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# IL-10 promotes development of acute respiratory distress syndrome via inhibiting differentiation of bone marrow stem cells to alveolar type 2 epithelial cells

Z. CHEN, Y. HU, T. XIONG, C. CHEN, X.-X. SU, Y. HUANG, L. ZHANG

Department of Pediatrics, West China Second University Hospital, Sichuan University, Chengdu, China

**Abstract. – OBJECTIVE: To explore whether interleukin-10 (IL-10) could promote the development of acute respiratory distress syndrome (ARDS) via inhibiting differentiation of bone marrow stem cells (BMSCs) to alveolar type 2 (AT II) epithelial cells.**

**PATIENTS AND METHODS: 25 ARDS (acute respiratory distress syndrome) patients admitted in our hospital from December 2015 to February 2018 were enrolled. Meanwhile, 25 healthy controls in the same period were selected as control group. Serum level of IL-10 in each subject was detected via ELISA (enzyme-linked immunosorbent assay). BMSCs were isolated and cultured, followed by identification of surface antigens and morphology observation using flow cytometry. For *in vitro* experiments, expression levels of AT II-related genes induced with or without IL-10 were detected by qRT-PCR (quantitative Real-time polymerase chain reaction) and Western blot, respectively. The culture medium of BMSCs induced with or without IL-10 was collected for detecting expression levels of interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) by ELISA.**

**RESULTS: IL-10 was overexpressed in ARDS patients than that of healthy controls. Primary BMSCs were elongated after culturing for 1-3 days. Negative-antigen CD34 (4.32%) and positive-antigen (99.87%) on the surface of BMSCs were identified by flow cytometry. Both mRNA and protein expressions of AT II-related genes increased in a time-dependent manner. ELISA results showed that IL-10 level in cell supernatant decreased with the prolongation of induction days. Moreover, IL-10 intervention downregulated the expressions of AT II-related genes.**

**CONCLUSIONS: IL-10 promotes ARDS development via inhibiting cell differentiation of BMSCs to AT II.**

*Key Words:*

Bone marrow mesenchymal stem cells, Exosomes, Apoptotic pathway, Osteoporosis, Osteoblasts, Proliferation.

## Introduction

Acute respiratory distress syndrome (ARDS) is a common acute and critical disease with a high mortality up to 30-50%[1]. Although the treatment methods have been progressed, supportive treatment is still the major treatment for ARDS. Therefore, it is urgent to explore effective treatment strategies to alleviate ARDS. ARDS is the lung parenchyma damage resulted from excessive inflammation, which seriously damages alveolar epithelial cells and pulmonary microvascular endothelial cells[2,3]. Hence, repairing alveolar epithelial cells may contribute to ARDS treatment. Stem cells possess potentials of self-renewal and multi-directional differentiation, which are valuable in ARDS treatment. Among them, bone marrow stem cells (BMSCs) are the most promising seed cells because of their easy isolation and range sources[4-6]. Researches on cell differentiation of BMSCs to type II alveolar epithelial cells (AT II) have become the hot topic in ARDS treatment. It is reported that inflammatory factors are involved in ARDS development. Interleukin-10 (IL-10) is a pro-inflammatory cytokine that is widely expressed in the early stage of ARDS. IL-10 is an essential mediator of pulmonary diseases, which is capable of inducing inflammatory cascades and nitric oxide synthase. Accumulation of nitric oxide further aggravates the disease condition of ARDS[7,8]. Therefore, it is of great significance to explore the underlying mechanism of BMSCs differentiation in ARDS development, so as to provide theoretical basis for improving clinical outcomes of ARDS patients.

## Patients and Materials

### Patients

A total of 25 ARDS patients admitted in West China Second University Hospital from

*Corresponding Author:* Li Zhang, MD; e-mail: zhanglihk@126.com

December 2015 to February 2018, were enrolled. These patients all underwent tracheal intubation or tracheotomy for mechanical ventilation. 25 healthy controls in the same period were selected as control group. Basic characteristics of ARDS patients and healthy controls were listed in Table I. This study was approved by the Ethics Committee of West China Second University Hospital. Signed written informed consents were obtained from all participants before the study.

### Reagents and Instruments

Glutamine, α-MEM and penicillin were obtained from Gibco (Grand Island, NY, USA); fetal bovine serum (FBS), 0.25% trypsin, type I collagenase, bovine pituitary extract, recombinant human epidermal growth factor, recombinant human insulin, hydrocortisone, adrenaline, triiodothyronine, iron binding globulin, retinoic acid and fatty acid free-bovine serum albumin were obtained from Sigma-Aldrich (St. Louis, MO, USA); inverted phase contrast microscopy camera system was obtained from Nikon (Tokyo, Japan); scanning electron microscope was obtained from Hitachi (Tokyo, Japan); flow cytometry was obtained from Bio-Tek (Winooski, Vermont, USA).

### Isolation and Culture of BMSCs

4-week-old Sprague-Dawley (SD) rats (Model Animal Research Center of Nanjing University, Nanjing, China) were sacrificed with dislocation of cervical vertebra. Rat femur and tibia were collected under aseptic condition. The marrow cavity was washed with L-DMEM (L-Dulbecco's Modified Eagle Medium) (Gibco, Grand Island, NY, USA). After centrifugation at 1000 r/min for 5 min, BMSCs were re-suspended in L-DMEM containing 10% FBS. BMSCs were then seeded in 6-well plates at a density of $1.0\times10^6$/L for cell culture.

### BMSCs Differentiation to AT II

Primary BMSCs were maintained in DMEM/F12 containing 20% FBS. Cell passage was performed when the confluence was up to 100%. Fourth-passage BMSCs were seeded into 6-well plates at a density of $2\times10^5$/mL. 2 mL of epithelial cell medium-basal-phenol red free was replaced until 80% of confluence. Culture medium was replaced every 2 days. 4 μL of bovine pituitary extract, 10 ng/mL recombinant human epidermal growth factor, 5 μg/mL recombinant human insulin, 0.5 μg/mL hydrocortisone, 0.5 μg/mL adrenaline, 6.7 ng/mL triiodothyronine, 10 μg/mL iron binding globulin, 0.1 ng/mL retinoic acid and 2.5 mg/mL fatty acid free-BSA (bovine serum albumin) were added into the culture medium from the first medium replacement.

### Cell Transfection

$2\times10^5$/mL BMSCs were seeded into 6-well plates. In brief, siRNA-IL-10 or siRNA control was transfected according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), respectively.

**Table I.** Basic characteristics of study population.

| | ARDS group (n = 25) | Control group (n = 17) | *p* |
|---|---|---|---|
| Age (days) | 18 (6-27) | 17.7 (4-26) | 0.637 |
| Weight (Kg) | 1.7 (1.3-3.8) | 2.6 (1.4-4.3) | 0.825 |
| Male sex | 12 (48.0%) | 7 (47.1%) | 0.364 |
| PRISM-III 24 | 15.2 (8.4-22.3) | 2.5 (2-4.4) | < 0.001 |
| Modified Murray's score | 4.9 (4.3-5.7) | 0.62 (0.4-1.3) | < 0.001 |
| $PaO_2/FiO_2$ | 125 (83-164) | 473 (363-659) | < 0.001 |
| OI | 13.2 (9.4-18.2) | 1.6 (1.1-2.5) | 0.041 |
| Crs (mL/cm $H_2O$/kg) | 0.44 (0.32-0.49) | 0.78 (0.7-0.9) | < 0.001 |
| Deaths | 4 (16.0%) | 1 (6.9%) | |
| Co-morbidities | Severe sepsis (4)<br>RSV infection (5)<br>H1N1 flu (3)<br>Aspiration (5)<br>Trauma (2)<br>Late onset GBS infection (1)<br>Malignancy (5) | Metabolic disease (3)<br>SAH (4)<br>Status epilepticus (3)<br>General anesthesia (7) | |

IQR, interquartile range; HCT, hematopoietic cellular transplantation; ARDS, acute respiratory distress syndrome; PRISM-3, Pediatric Risk of Mortality III score.

### Western Blot

Total protein was extracted from treated cells by radioimmunoprecipitation assay (RIPA) solution (Beyotime, Shanghai, China). Protein sample was separated by electrophoresis on 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and then transferred to PVDF (polyvinylidene difluoride) membrane (Millipore, Billerica, MA, USA). After membranes were blocked with skimmed milk, they were incubated with primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The membranes were then washed with TBST (Tris-buffered Saline with Tween 20) (Beyotime, Shanghai, China) and followed by the incubation of secondary antibody. The protein blot on the membrane was exposed by chemiluminescence (Thermo Fisher Scientific, Waltham, MA, USA).

### RNA Extraction and qRT-PCR (Quantitative Real-Time Polymerase Chain Reaction)

RNA was extracted by TRIzol method (Invitrogen, Carlsbad, CA, USA) and reversely transcribed to cDNAs using Easy Script First-Strand complementary Deoxyribose Nucleic Acid (cDNA) Synthesis Super Mix (TaKaRa, Otsu, Shiga, Japan). QRT-PCR was performed according to the instructions of Trans Start-TM SYBR Green PCR Supermix (Invitrogen, Carlsbad, CA, USA). The relative expression of target gene was calculated using $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were as follows: Occludin, F: 5'-TGGCATACTCTTCCAATGGC-3', R: 5'-GTCATCCACAGGCGAAGTTA-3'; KGF, F: 5'- CATGGATCCATGAGCTATGATTACATGGAAG -3', R: 5'-GTCGAATTCTTAAGTGATTGCCATAGGCAG-3'; CK18, F: 5'-AAGAAAACCCGAAGAGG-3', R: 5'-CTGACTCAAGGTGCAGC-3'; SpA, F: 5'-GGATAGTAGCCATGTCACTGTGTTC-3', R: 5'-TGATACCAGCGACAACAGTCAAG-3'; SpB, F: 5'-CTCACAAAGATGACCAAGGAAGA-3', R: 5'-GGGCAGGTAGACATCAAGCACT-3'; SpC, F: 5'-GCATCCCTAGTCTTGAGGCTTTG-3', R: 5'-GAATCAGAATCGGATCCAGCAC-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

### ELISA (Enzyme-Linked Immunosorbent Assay)

According to the instructions of ELISA kit (EBiosciences, San Diego, CA, USA), corresponding antibodies were diluted in the coating buffer at a density of 1-10 μg/mL. Briefly, cells were blocked in 5% FBS for 40 min. After washing with phosphate-buffered saline (PBS) for three times, each sample and enzyme-labeled antibody were added to each well. Substrate solution was used to terminate the reaction and ELISA results were determined within 20 min. Absorbance at the wavelength of 450 nm was detected using an ELISA detector.

### Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was introduced for statistical analysis. The quantitative data were represented as mean ± standard deviation ($\bar{x} \pm s$). The *t*-test was used for comparing differences between the two groups. $p < 0.05$ was considered statistically significant.

## Results

### IL-10 Expressions in ARDS Patients and Healthy Controls

Basic characteristics of 25 ARDS patients and 25 healthy controls were listed in Table I. ELISA assay results elucidated that IL-10 expression was higher in ARDS patients than that of healthy controls (Figure 1), indicating that IL-10 is overexpressed in serum of ARDS patients.

### Phenotype Identification of BMSCs

Primary BMSCs were observed in adherent growth after isolation and cell culture for 1 day. BMSCs were in typical morphology after culturing for 3 days (Figure 2A). We then identified specific surface antigens by flow cytometry. Negative-antigen CD34 (4.32%) and positive-antigen (99.87%) on cell surface indicated that BMSCs have been successfully isolated *in vitro*.

### Cell Differentiation of BMSCs to AT II

The mRNA levels of AT II-related genes, including Occludin, KGF, CK18, SpA, SpB and SpC increased with the prolongation of cell differentiation (Figure 3A-3F). Western blot results also elucidated that protein levels of Occludin and CK18 increased in a time-dependent manner (Figure 3G). However, IL-10 expression in culture medium during cell differentiation gradually decreased detected by ELISA (Figure 3H). The above results indicated that BMSCs could be differentiated to AT II.

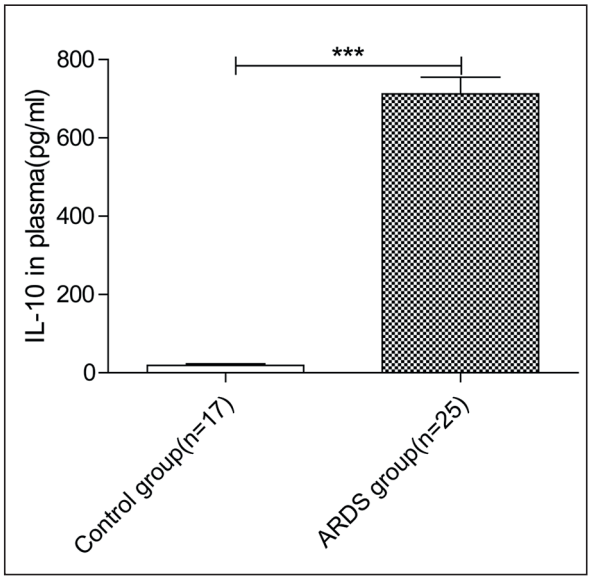


**Figure 1.** IL-10 expressions in ARDS patients and healthy controls. ELISA results elucidated that IL-10 expression was higher in ARDS patients than that of healthy newborns.

### *IL-10 Promoted ARDS Development via Inhibiting BMSCs Differentiation*

In the process of cell differentiation, IL-10 overexpression downregulated mRNA levels of Occludin, KGF, CK18, SpA, SpB and SpC detected by qRT-PCR (Figure 4A-4F). Similarly, protein expressions of Occludin, CK18 and KGF also decreased after IL-10 intervention (Figure 4G). Increased levels of interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were observed in culture medium after IL-10 treatment (Figure 4H-4J). Our results indicated that IL-10 promotes ARDS development *via* inhibiting BMSCs differentiation.

## Discussion

ARDS is one of the most serious diseases characterized by respiratory distress and hypoxemia predominate. Severe ARDS without time-

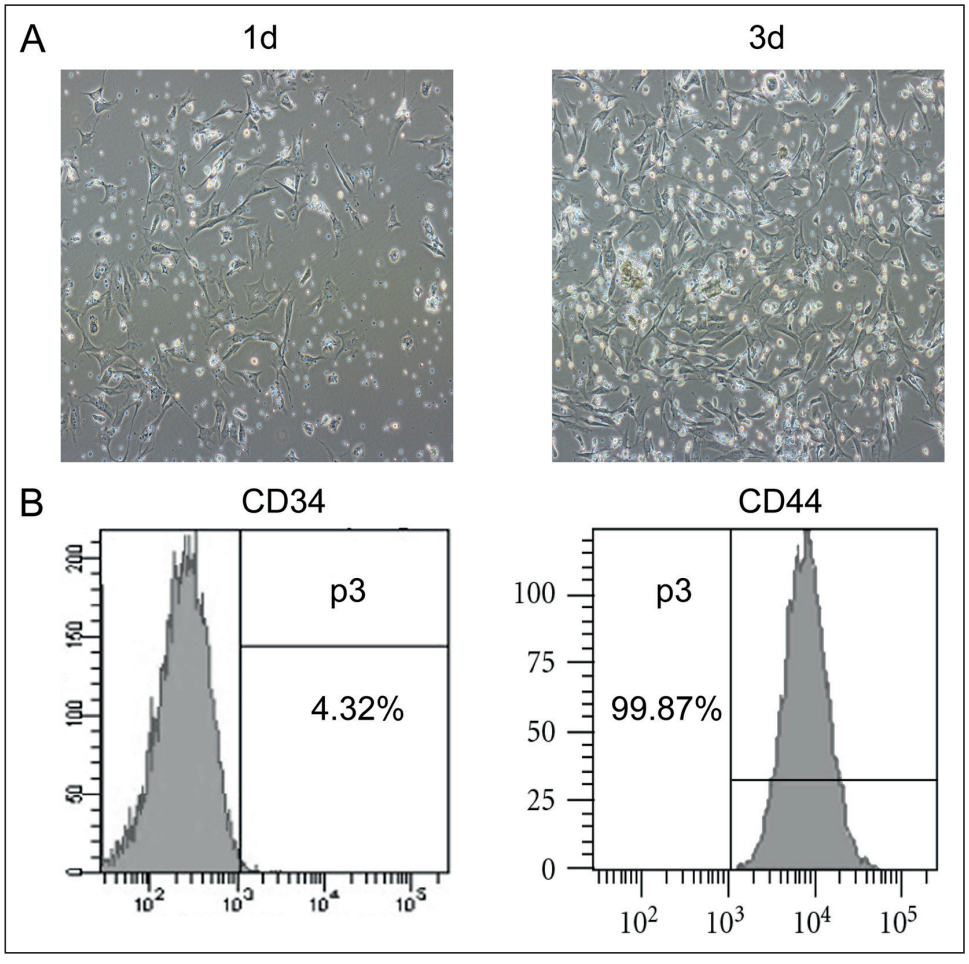


**Figure 2.** Phenotype identification of BMSCs. ***A,*** Primary BMSCs were observed in adherent growth after isolation and cell culture for 1 day. BMSCs were in typical morphology after culturing for 3 days. ***B,*** Negative-antigen CD34 (4.32%) and positive-antigen (99.87%) detected by flow cytometry.

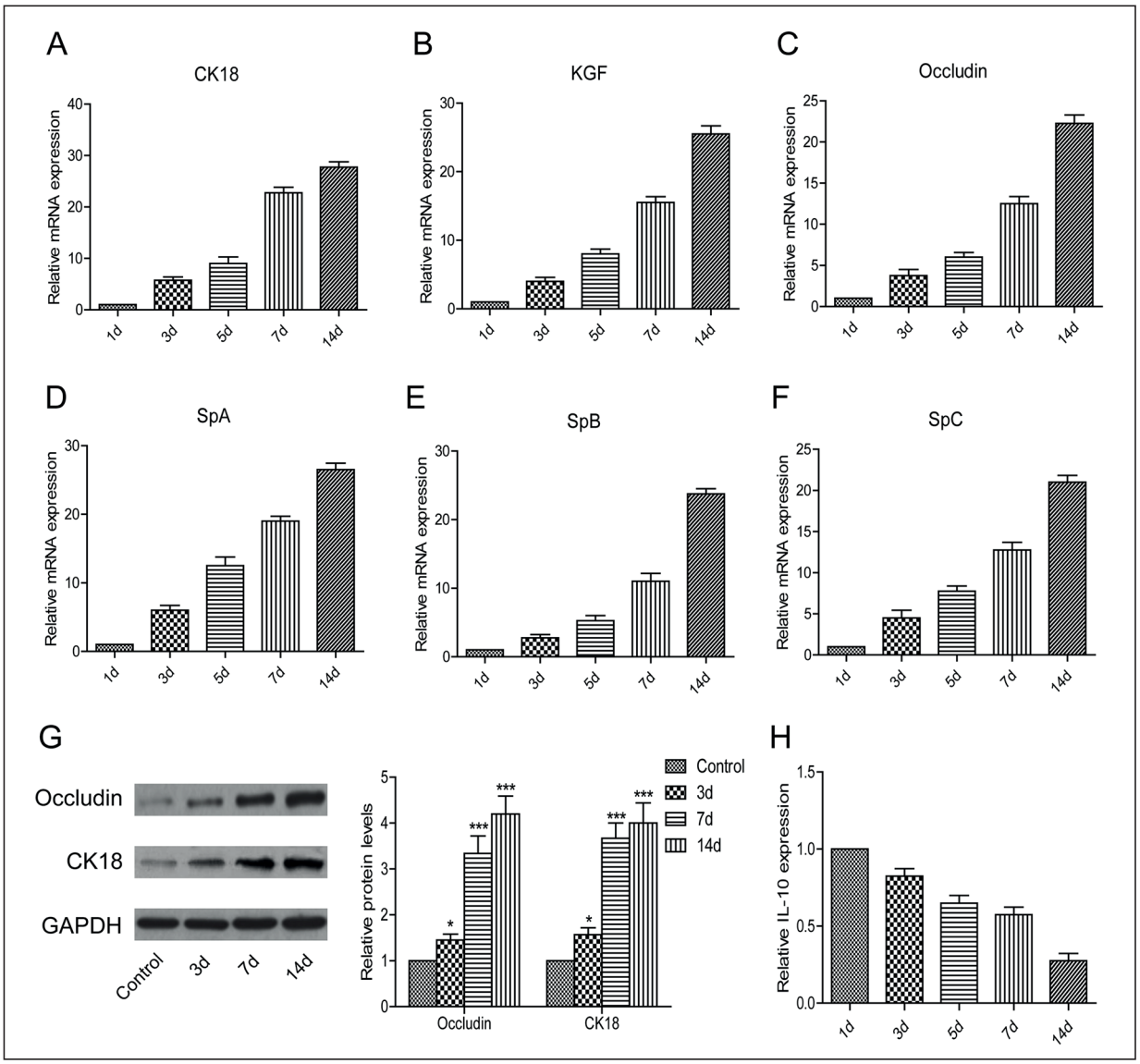


**Figure 3.** Cell differentiation of BMSCs to AT II. ***A-F,*** The mRNA levels of AT II-related genes, including Occludin, KGF, CK18, SpA, SpB and SpC. ***G,*** Protein levels of Occludin and CK18 increased with the prolongation of induction days. ***H,*** ELISA results showed decreased IL-10 expression in culture medium.

ly treatment would easily cause multiple organ failure and even death[9-11]. So far, the specific pathogenesis of ARDS has not been fully elucidated. Infection, trauma, shock, and toxic gases are considered to be the risks of ARDS[12-14]. In recent years, tissue engineering has been greatly advanced, especially stem cell technology, which has been applied in the treatment of lung injuries. Certain stem cells could be activated under damage, contributing to repair and regeneration of the impaired sites[15]. In particular, BMSCs belong to special adult cells. BMSCs possess the advantages of easy collection, large amplification and good biocompatibility[16]. Therefore, clinical application of BMSCs may be an ideal treatment for ARDS patients.

Recent studies[17] have shown that BMSCs are beneficial to scavenge pulmonary edema, reduce inflammation and prevent bacterial infection. Phase 1 clinical studies have suggested the safety of BMSCs for the treatment of human ARDS. Phase 2 clinical trial (NCT02575774) of BMSCs therapy for human ARDS is ongoing[4,6,17]. BMSCs application in ARDS treatment shows a promising prospect in the clinical practice. Gas exchange between alveoli and blood depends

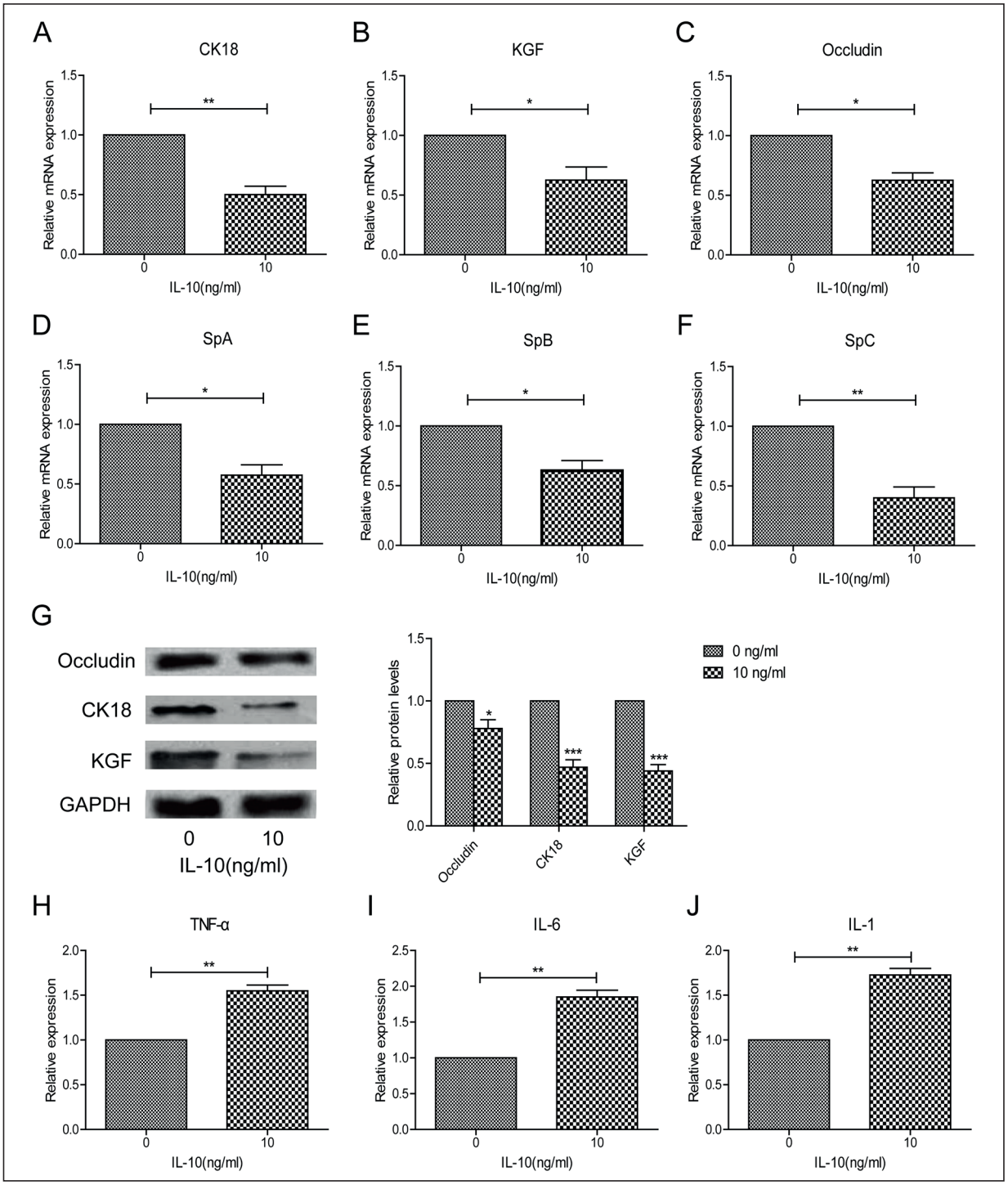


**Figure 4.** IL-10 promoted ARDS development *via* inhibiting BMSCs. ***A-F,*** IL-10 overexpression led to decreased expressions of Occludin, KGF, CK18, SpA, SpB and SpC detected by qRT-PCR. ***G,*** Protein expressions of Occludin, CK18 and KGF decreased after IL-10 intervention. ***H-J,*** Increased levels of IL-1, IL-6 and TNF-α were observed in culture medium after IL-10 induction.

on the complete structure of blood gas barrier. Although the pathogenesis of ARDS has not been fully elucidated, current researches mainly believed that inflammation-induced diffuse damage to the blood gas barrier is the major manifestation of ARDS[4,18]. Inflammatory factors have been proved to participate in ARDS development. In our study, IL-10 levels were overex-

pressed in ARDS patients than those of healthy controls. ARDS is the result of interaction of multiple inflammatory mediators. Dynamic balance between proinflammatory cytokines and anti-inflammatory cytokines is an important step affecting the prognosis of ARDS. It is reported that inflammation stimulates and induces the release of BMSCs to promote the early recovery of body defection. BMSCs, as adult stem cells, have strong proliferation ability, multi-directional differentiation potential, and low immunogenicity, which is an ideal seed cell line for repairing tissue damage[10,19,20]. In the present study, expression levels of AT II-specific genes remarkably increased during the process of BMSCs differentiation, whereas IL-10 level decreased. On the contrary, IL-10 induction remarkably decreased expressions of AT II-related genes. To sum up, BMSCs extracted from bone marrow could be served as effective treatment for ARDS. BMSCs reduced release of inflammatory factors to directly repair the damaged pulmonary epithelium. Besides, IL-10 was closely related to ARDS development. It inhibited the cell differentiation of BMSCs to AT II, so as to promote the disease condition of ARDS. Monitoring IL-10 expression in early stage of ARDS contributes to evaluate the disease condition and improve prognosis of ARDS patients.

## Conclusions

We showed that IL-10 promotes ARDS development via inhibiting cell differentiation of BMSCs to AT II.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

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