INTRODUCTION

Acute respiratory distress syndrome (ARDS) is a severe condition from acute lung injury (ALI) and is a respiratory failure disorder due to hypoxemia, with extremely high morbidity and mortality in critical care. It accounts for approximately 75,000 deaths annually in America. Pulmonary infection is the primary etiology of ARDS, in which, especially, lipopolysaccharide (LPS) in Gram-negative bacillus increases the permeability of the alveolar-capillary barrier, thereby leading to non-cardiogenic pulmonary edema. The inflammatory cascading reaction that is dominated by macrophages and neutrophils is the main mechanism in the pathological process of ARDS. Mass accumulation and infiltration of inflammatory cells in the lungs contribute to excessive inflammatory cytokine release and anti-inflammatory cytokine reduction with a consequence of inflammatory disequilibrium. Additionally, macrophages are considered indispensable mediators in ALI. In the lung microenvironment, the alveolar macrophages are polarized into two subpopulations, including M1 and M2 phenotype macrophages. M1-like polarization contributes to the pro-inflammatory reaction of host defense, whereas M2-like polarization exerts an important function in anti-inflammatory response and tissue remodeling. Therefore, alveolar macrophage polarization is critical for inflammatory equilibrium in the lungs.

Background: Acute respiratory distress syndrome (ARDS) is a severe disorder that is related to high mortality. Mesenchymal stem cells (MSCs) have shown strong effects in relieving lung injury. Aims: To determine the role of umbilical cord-derived MSCs (UC-MSCs) together with surfactant protein B (SP-B) in ARDS. Study Design: Animal experimentation. Methods: Immunophenotypic characteristics of UC-MSCs were identified. BALB/c mice were intratracheally administrated with lipopolysaccharide (LPS) and received UC-MSCs or UC-MSCs transfected with SP-B (UC-MSCs-SP-B). Pathological changes and lung injury degrees after transplantation were assessed by histological and biochemical analyses. Inflammatory chemokine and cytokine production in the bronchoalveolar lavage fluid (BALF) was measured using enzyme-linked immunoassay. Flow cytometry was used to examine macrophage phenotypes and differentiation of T-helper 17 (Th17) and T-regulatory (Treg) in the BALF. Results: Our results showed that isolated UC-MSCs possessed multilineage differentiation potential. SP-B transfection into UC-MSCs strengthened the effects of UC-MSCs on lung function repair in LPS-induced ARDS. UC-MSCs and UC-MSCs-SP-B attenuated cellular infiltration. Additionally, UC-MSCs and UC-MSCs-SP-B inhibited the inflammatory response by promoting M2-like polarization, as well as reduced Th17 differentiation and promoted Treg differentiation. Conclusion: UC-MSCs in combination with SP-B, alleviates inflammatory reaction in ARDS by regulating macrophage polarization.
higher gene transfection efficiency. Surfactant protein B (SP-B) is a key protein in pulmonary surfactant and functions in maintaining mammalian breathing and is required to maintain lung function, as genetic SP-B knockdown leads to respiratory failure in newborns and SP-B knockout mice. Animal-derived lung surfactants that contain native SP-B have been reported to improve outcomes in patients with RDS having surfactant deficiency.

We hypothesized that SP-B-transfected MSCs, which retain cell activity and various physiological effects, may yield stronger therapeutic effects on ARDS after transplantation into the lung. Therefore, this study aimed to transplant UC-MSCs transfected with SP-B into the lung of ARDS mice. By analyzing the pathological changes and inflammatory response of the lung tissue, the role of SP-B transfected UC-MSCs in ARDS was explored.

MATERIALS AND METHODS

Isolation of UC-MSCs

UC tissues (6–9 cm) were taken from pregnant women with full-term deliveries. This study was approved by the Ethics Committee of General Hospital of Central Theater Command of Chinese People’s Liberation Army (Hubei, China) (approval number: KY2019-023). Informed consent was signed by all participants. People’s Liberation Army (Hubei, China) (approval number: DWLL2019-011). Animal-derived lungs surfactants that contain native SP-B have been reported to improve outcomes in patients with RDS having surfactant deficiency.

The tissues of 1 mm³ were washed with phosphate-buffered saline (PBS), digested in 0.1% collagenase II (Gibco, USA) that contain 30 U/ml DNase I for 1.5 h, and treated with 0.125% trypsin (Gibco) for 1 h. After terminating the trypsin activity, the mixture was filtered through a 100-μm cell strainer. Cells were then washed and cultured in Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) (1:1) (Gibco) that contain 100 μg/ml of penicillin, 10% fetal bovine serum (FBS) (Gibco), and 10 μg/ml of streptomycin (Sigma–Aldrich) at 37°C with 5% CO₂. The adherent cells were incubated until confluence.

Flow Cytometry

Flow cytometry was applied to determine the presence of specific surface antigens of MSCs. Briefly, 1 x 10⁴ UC-MSCs that are suspended in 500 μl staining buffer with 1% FBS were centrifuged at 1000 rpm for 10 min, washed with PBS, digested in 0.1% collagenase II (Gibco) that contain 100 μg/ml of penicillin, 10% fetal bovine serum (FBS) (Gibco), and 10 μg/ml of streptomycin (Sigma–Aldrich) at 37°C with 5% CO₂. The adherent cells were incubated until confluence.

Western Blotting

Radioimmunoprecipitation assay buffer (Invitrogen) was employed to extract the protein from the transfected UC-MSCs. Protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes. The membranes were incubated with primary antibodies SP-B (ab271345) and glyceraldehyde 3-phosphate dehydrogenase (ab181602) overnight at 4°C after blocking with 5% skim milk. Appropriate secondary antibodies were then added for 1 h of incubation at 4°C. Blot bands were then visualized with enhanced chemiluminescence reagent (Amersham, USA) and analyzed with Image J software.

Experimental Animal

Male BALB/c mice (8-week-old; Vital River Co. Ltd, Beijing, China) were raised under controlled conditions (22 °C–26 °C; 12 h light/dark cycle). Animal experiments were approved by the Animal Ethics Committee of General Hospital of Central Theater Command of Chinese People’s Liberation Army (Hubei, China) (approval number: DWLL2019-011).

Forty-two mice were evenly divided into six groups as follows: sham (intratracheally received normal saline); UC-MSCs (intratracheally received normal saline plus UC-MSCs); UC-MSCs-SP-B (intratracheally received normal saline plus UC-MSCs transfected with pRES2-EGFP-SP-B); LPS (intratracheally received LPS); LPS + UC-MSCs-vector (intratracheally received LPS plus UC-MSCs transfected with empty plasmid), and LPS + UC-MSCs-SP-B (intratracheally received LPS plus UC-MSCs transfected with pRES2-EGFP-SP-B).

Experimental Model

BALB/c mice were administrated with LPS. Animals were anesthetized via an intraperitoneal injection of pentobarbital sodium (60 mg/kg) before trachea exposure. After the anesthesia,
mice were intratracheally injected with LPS (O55:B5; 4 mg/kg; Sigma) in 100 μl of PBS or normal saline (0.1 ml/mouse) as control through a 20-gage catheter. After 6 h, 1 × 10^6 UC-MSCs or UC-MSCs transfected with SP-B in 100 μl of PBS were intratracheally injected into the mice. After 72 h, the mice were euthanized by cervical dislocation under isoflurane. Lung tissues and bronchoalveolar lavage fluids (BALFs) were collected.

**Histology Analysis**

Lung tissues from each group were excised 72 h post-transplantation and fixed in 10% formalin under a pressure of 15 cm H2O. After fixation for 24 h, tissues were embedded in paraffin. Next, 5-μm-thick specimens were deparaffinized and then stained with hematoxylin and eosin. Lung injury degree was observed with a microscope and semi-quantified according to a previously published lung injury scoring system.

**Wet/dry (W/D) Ratio**

Lung tissues were stored in a dry plastic plate, and the wet weight was immediately weighed with an electronic scale. The tissues were dried in an oven for 48 h at 60 °C and the dry weight was weighed. The W/D ratio indicated the ratio of the wet weight to the dry weight.

**Arterial Blood Gas**

As documented, mice were anesthetized with pentobarbital sodium 72 h post-transplantation. All mice maintained spontaneous breathing at the time of the experiment. Blood was taken from the celiac artery. An ABL90 FLEX analyzer (Radiometer, Denmark) was used to analyze oxygen partial pressure (pO2) and saturation. The pO2/fraction of inspired oxygen (FiO2) ratio was calculated.

**Measurement of BALF**

Lung tissues were washed with 0.35 ml PBS for BALF sample collections, and BALF samples were centrifuged at 300 g for 10 min at 4 °C. Inflammatory cytokines were measured in the supernatants. Total protein concentration was assessed with the bicinchoninic acid assays (Sigma). The precipitated cells were used to analyze oxygen partial pressure (pO2) and saturation. The pO2/fraction of inspired oxygen (FiO2) ratio was calculated.

**Statistical Analysis**

Statistical analysis was performed using the Statistical Package for the Social Sciences version 21.0 (Chicago, IL, USA). Values are expressed as the means ± standard deviation. One-way analysis of variance followed by Tukey’s post-comparison test or t-test was applied to analyze the differences between groups. The normal distribution of data was tested using the Shapiro–Wilk test. The Mann–Whitney U test was used for non-normally distributed data. A p-value of <0.05 was statistically significant. A sample size of seven cases for each group was required to detect at least 50% difference between the control and test groups, with a power of 85% at the 1.25% significance level.

**RESULTS**

**Identification of MSC Characteristics**

First, the antigenic profile of isolated UC-MSCs was determined according to minimal criteria for defining pluripotent mesenchymal stromal/stem cells. The population of UC-MSCs highly expressed the MSCs markers, CD44 and CD29, rather than the hematopoietic markers, CD34 and CD45 (Figure 1A-1D). Next, UC-MSCs were induced to differentiate into osteoblasts and adipocytes in a selective culture medium. As shown in Figure 1E, Alizarin Red Staining of UC-MSCs showed large calcium phosphate deposits in the osteogenic induction medium. Additionally, UC-MSCs showed intracellular lipid droplets after adipogenic differentiation induction. These findings suggested that isolated UC-MSCs possess multilineage differentiation potential. We then transfected pIRES2-EGFP-SP-B into UC-MSCs and verified SP-B expression upregulation after transfection (p = 0.003) (Figure 1F).
The therapeutic effects of UC-MSCs were examined. Histological changes in the lung were observed by hematoxylin and eosin staining. No significant difference was found in the histologic change among sham, UC-MSCs, and UC-MSCs-SP-B groups (Figure 2A). Normal alveolar and interstitial tissue structures were destroyed, and the inflammatory infiltration was serious in the LPS group, whereas these changes were markedly attenuated after UC-MSCs transplantation together with SP-B transfection. LPS increased lung injury score in mice (vs. sham group: $p = 0.000$). UC-MSCs-SP (vs. LPS group, $p = 0.009$) and UC-MSCs-SP-B (vs. LPS + UC-MSCs-vector group, $p = 0.017$) markedly reduced lung injury score (Figure 2B). The effect of UC-MSCs with SP-B transfection on pulmonary function was evaluated by measuring the W/D ratio and arterial blood gases. Data showed that the mice in LPS + UC-MSCs (vs. LPS group: $p = 0.018$) and LPS + UC-MSCs-SP-B (group vs. LPS + UC-MSCs-vector group: $p = 0.003$) groups had lower W/D ratio of 6.0 and 4.2, respectively, than 8.0 in LPS (Figure 2C), which suggest that UC-MSCs either alone or combined with SP-B attenuates the degree of LPS-induced lung edema. LPS also reduced the $pO_2/FiO_2$ ratio (vs. sham group: $p = 0.000$), whereas UC-MSCs (vs. LPS group: $p = 0.008$) and UC-MSCs-SP-B (vs. LPS + UC-MSCs-vector group: $p = 0.017$) markedly restored the $pO_2/FiO_2$ ratio (Figure 2D), which demonstrated lung function improvement. Overall, SP-B transfection into UC-MSCs strengthens the protective effects of UC-MSCs against LPS-stimulated ARDS.

**SP-B Strengthens the Protection of UC-MSCs Against LPS-stimulated ARDS**

UC-MSCs and UC-MSCs-SP-B Decreases the Degree of BALF Changes

Cell counting and protein level in BALF were measured for further analysis of lung damage and inflammation. Total protein level was elevated in LPS group (vs. sham group: $p = 0.000$) but reduced in LPS + UC-MSCs-vector (vs. LPS group: $p = 0.012$) and LPS +
concentrations of pro-inflammatory cytokines (IL-1β and TNFα) and anti-inflammatory chemokines (IL-10, IL-13, TGF-β and PGE2) in BALF to analyze inflammatory regulation mediated by UC-MSCs. The increased levels of LPS-induced IL-1β and TNFα were significantly reversed by UC-MSCs (vs. LPS group: \( p < 0.006 \) and \( p = 0.006 \), respectively) or UC-MSCs-SP-B (vs. LPS + UC-MSCs-vector group: \( p = 0.001 \) and \( p = 0.003 \), respectively) (Figure 4E, 4F). Additionally, in parallel, the levels of IL-10, IL-13, TGF-β, and PGE2 were increased in LPS (vs. sham group: \( p = 0.004 \), \( p = 0.001 \), \( p = 0.001 \), and \( p = 0.001 \), respectively), LPS + UC-MSCs-vector (vs. LPS group: \( p = 0.010 \), \( p = 0.001 \), \( p = 0.001 \), and \( p = 0.001 \), respectively), and LPS + UC-MSCs-SP-B groups (vs. LPS + UC-MSCs-vector group: \( p = 0.013 \), \( p = 0.015 \), \( p = 0.010 \), and \( p = 0.008 \), respectively) (Figure 4G-4I). Overall, UC-MSCs and UC-MSCs-SP-B inhibits inflammation by promoting M2-like polarization in ARDS.

**UC-MSCs-SP-B Reduces Th17 Differentiation and Promotes Treg Differentiation**

The effects of UC-MSCs-SP-B on the regulation of T cell and Th17 were further examined. As expected, a markedly increased number of IL-17+/CD4+ cells and reduced Foxp3+/CD25+ after the addition of LPS was found (vs. sham group: \( p = 0.001 \) and \( p = 0.001 \), respectively). Notably, UC-MSCs (vs. LPS group: \( p = 0.013 \) and \( p = 0.001 \), respectively) or UC-MSCs-SP-B (vs. LPS + UC-MSCs-vector group: \( p = 0.029 \) and \( p = 0.017 \), respectively) inhibited Th17 differentiation, whereas promoted Treg differentiation (Figure 5A, 5B). Therefore, UC-MSCs-SP-B displays a key role in modulating the inflammatory differentiation of T cells.

**DISCUSSION**

ARDS is related to diffuse epithelial injury that is induced by an impaired alveolar-capillary barrier with consequently raised permeability, pulmonary edema, and neutrophil accumulation. During this process, persistent inflammation and plasma protein loss contribute to lung dysfunction. Insufficient effective treatment for ARDS is associated with the multifactorial etiology of this disorder.29,30 MSCs could be a promising candidate for cell therapy in inflammatory pulmonary diseases. Evidence has shown beneficial effects from the transplant of bone marrow or umbilical cord-derived MSCs in lung injury treatment.17 The effects of UC-MSCs in combination with SP-B in ARDS were preliminarily explored.
have suggested that UC-MSCs infusion effectively decreases lung inflammation, prevents pulmonary fibrosis, increases blood counts, recovers hematopoiesis, and improves survival in shrews with acute radiation injury. 15 Moreover, UC-MSC infusion was an effective therapeutic tool for patients with ARDS. 16 This study revealed that intratracheal infusion of UC-MSCs in combination with SP-B attenuated inflammation and improved lung function in experimental ARDS models.

Resident inflammatory cells-alveolar macrophages are activated after stimulation and produce numerous cytokines, which promote the chemotaxis of neutrophils, monocytes, and lymphocytes, thereby aggravating the inflammatory lung responses. 17 M1 macrophages potentiate the local inflammatory response by secreting inflammatory mediators, whereas M2 macrophages can inhibit inflammatory damage by releasing anti-inflammatory mediators. Once the pulmonary microenvironment is stimulated, alveolar macrophages are immediately transformed into the M1 phenotype. 18, 38 Our results showed that the M1 phenotype was dominant after LPS stimulation compared to the M2 phenotype. MSCs promote the differentiation of macrophages into M2 and exert an anti-inflammatory effect in neonatal lung injury. 19 MSCs treatment alleviates bronchopulmonary dysplasia, improves lung function, and ameliorates pulmonary hypertension by pulmonary macrophage phenotype regulation. 20 According to previous research, the ratio of pulmonary M1/M2 macrophages is increased accompanied by reduced SP-B expression, which indicates lung injury initiation. 21 Here, after LPS treatment, intratracheal transplantation of either UC-MSCs or UC-MSCs-SP-B suppressed the M1-like polarization and augmented the M2-like polarization in the BALF. This suggested that UC-MSCs transplanted with SP-B regulate inflammation in ARDS through macrophage polarization modulation. Moreover, UC-MSCs-SP-B reduced Th17 differentiation and promoted Treg differentiation after ARDS.

SP-B preferentially binds to anionic surfactant lipids that potentiate the capacity of SP-B of facilitating lipid adsorption to the air-water interface. 42 The SP-B dimer is oligomerized in a circular structure, which mediates the connection between surfactant membranes. 33, 44 SP-B knockdown contributes to lung inflammation, thereby supporting the models that surface force disruption activates pro-inflammatory signaling. 45 After SP-B is lost, L-selectin is rapidly produced. L-selectin can mediate lung inflammation through increased leukocyte mobility into the lungs. 46 STAT-3 activates many genes that are involved in lung protection. After doxycycline withdrawal, STAT-3 is phosphorylated and upregulated, which may indicate a compensatory response to SP-B deficiency. 47 Additionally, phosphorylation of STAT-3 was reported crucial for repolarization of M1 to M2 type. 48 Therefore, M1/M2 polarization mechanisms regulated by SP-B may involve multiple molecules and signals, which need further investigations in the future.

Overall, our findings showed that transplantation of UC-MSCs transfected with SP-B could potentiate M2 macrophage polarization and further relieve LPS-stimulated lung injury. This demonstrated that the combination of UC-MSCs with SP-B yields significant alleviative effects on ARDS, which may be a promising strategy for ARDS treatment.

REFERENCES

Xu et al. Role of UC-derived MSCs Together with SP-B in ARDS


37. Fan EKY, Fan J. Regulation of alveolar macrophage death in acute lung inflammation. Respir Res. 2018;19:30. [CrossRef]


Balkan Med J,