

Original Article

Allogeneic human umbilical cord-derived mesenchymal stem cells reduce lipopolysaccharide-induced inflammation and acute lung injury

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Received April 27, 2020; Accepted October 8, 2020; Epub October 15, 2020; Published October 30, 2020

Abstract: Acute lung injury (ALI) is the clinical disorder of acute hypoxemic respiratory deficiency and it is associated with a high mortality rate. Increased lung permeability, infiltration of inflammatory cells, secretion of inflammatory cytokines, and pulmonary edema are hallmarks of ALI. Currently, there is no effective pharmacological agent approved for ALI, and the treatment regimens available are mostly supportive. Mesenchymal stem cells (MSCs) are multipotent stromal cells with immunomodulating potential, which therefore hold great promise for the treatment of ALI. We established an LPS-induced ALI mouse model by intratracheal injection of lipopolysaccharide (LPS). Human umbilical cord-derived MSCs (hUC-MSCs) were delivered through the tail vein to assess the effects of MSCs on relieving LPS-induced ALI. Intratracheal injection of LPS increased the infiltration of neutrophils and enhanced the expression of pro-inflammatory cytokines, such as IL-6, IL-1 β and TNF- α . Administration of hUC-MSCs decreased pathological signs of inflammation, as well as reduced ALI scores. The levels of IL-6, IL-1 β and TNF- α were also dose-dependently inhibited in the bronchoalveolar lavage fluids from damaged lung tissues. Moreover, MPO and BAX levels were decreased by the hUC-MSC treatment, suggesting hUC-MSCs may play the role in inhibiting ROS production and apoptotic death in ALI repair. These results highlight the potential of hUC-MSCs to alleviate bacterial endotoxin-induced inflammation, and may represent an effective modality for the treatment of ALI in clinical settings.

Keywords: Umbilical cord-derived mesenchymal stem cells, inflammation, lipopolysaccharide, acute lung injury

Introduction

Acute lung injury (ALI) is a disorder of acute respiratory failure, which is characterized by increased permeability and edema in the lungs due to alveolar epithelial damage, severe acute inflammation and diminished gas exchange [1, 2]. Acute respiratory distress syndrome (ARDS) is a severe form of ALI, with a mortality rate reaching 30-40% [3]. ALI and ARDS are usually caused by sepsis, microbial-induced pneumonia, trauma, inhalation of toxic agents, and gastric aspiration [4]. Once an inflammatory response is triggered by pathogens or trauma, the inflammatory signals are induced as a protective machinery. However, prolonged inflammation in the lungs and dysregulated secretion of inflammatory cytokines destabilize the cadherin junctions and disrupt the micro-

vasculature barrier. Lung damage then leads to an increase in epithelial and endothelial permeability, which ultimately contributes to alveolar fluid accumulation and pulmonary edema. Increased alveolar fluid reduces pulmonary gas exchange, and hypoxemia may then occur [5]. In the injured lung, suppressing excessive inflammatory signals and promoting repair of the damaged pulmonary endothelium and epithelium are critical for recovery from ALI. Despite we have a better understanding of ALI, no curative pharmacological agent has yet been approved for its treatment. The demand for effective treatments, other than supportive fluid management and protective mechanical ventilation, has created substantial momentum for the development of novel therapies against ALI.

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Mesenchymal stem cells (MSCs) are multipotent precursor cells derived from different tissues, including peripheral blood, adipose tissue, bone marrow, the umbilical cord and placenta [6]. Considering their easy availability, relatively rare ethical considerations, their potential to differentiate into epithelial cells for pulmonary epithelium repair, and their ability to release anti-inflammatory cytokines, MSCs are considered a good source of cell therapy for ALI [4, 7-9]. Proof-of concept experiments have been performed in preclinical studies. In a paraquat-induced ALI model in rats, the administration of bone marrow-derived rat MSCs via the caudal vein reduced the expression of inflammatory cytokines, apoptosis, the lung injury score, and the wet-to-dry lung weight ratio [10]. Another report showed that in a lipopolysaccharide (LPS)-induced ALI mouse model, the infusion of bone marrow-derived MSCs by venipuncture alleviated the thickening of the alveolar wall, whereas the administration of lung fibroblasts did not [11]. Neutrophil infiltration, cytokine expression, and edema triggered by intraperitoneal injection of endotoxin, were also reduced in MSC-treated mice [11]. Gupta *et al.* injected bone marrow-derived MSCs via the intratracheal route in an LPS-induced ALI model and found that MSC treatment improved the survival rate of mice by up to 72 hours [12]. Diminished bronchoalveolar lavage (BAL) protein, an index for pulmonary endothelial and epithelial permeability, was observed in MSC-treated mice, along with a reduction in excess water in the lungs. The injection of MSCs also upregulated the expression of anti-inflammatory interleukin (IL)-10, while downregulating the expression of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)- α and macrophage inflammatory protein (MIP)-2 [12].

Although bone marrow is the main source of MSCs in isolation, its clinical application is limited due to the extremely invasive procedure required for sample collection. In addition, the differentiation capacity, life span and the number of MSCs derived from bone marrow markedly decrease with age [13]. Several reports have shown that human umbilical cord and placenta-derived MSCs are more primitive, proliferative, immunosuppressive and possess stronger anti-inflammatory properties [14-16]. Although human umbilical cord-derived MSCs

(hUC-MSCs) have been shown to have potential for preventing lung injury in pre-clinical murine models [17, 18], their therapeutic effects and potential functions in ALI models remain unclear. In the current study, the potential for hUC-MSCs to treat ALI was assessed in an LPS-induced ALI model in C57BL/6 mice. Treatment with hUC-MSCs significantly alleviated the pathological signs of pulmonary inflammation, inhibited the infiltration of mononuclear cells and neutrophils, reduced ALI histopathology scores, and suppressed the expression of pro-inflammatory cytokines in a dose-dependent manner. These results suggest that hUC-MSCs may serve as an efficacious option for the treatment of ALI and could confer many functional benefits.

Materials and methods

Mice

Male C57BL/6 mice (8~12 weeks old, 23-25 g) were purchased from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). All mice were kept in compliance with applicable regulations, with food and water accessible *ad libitum*. The animal experiment protocol (103-E01-001) was approved by the institutional animal care and use committee (IACUC) of Cardinal Tien Hospital (New Taipei, Taiwan).

Isolation and characterization of hUC-MSCs

hUC-MSCs were obtained from Meridigen Biotech Co., Ltd. (Taipei, Taiwan) and isolated as previously described [19] under an approved protocol by the Ethics Committee of the Cardinal Tien Hospital (IRB no. CTH-102-2-4-003). hUC-MSCs were passaged at approximately 80-90% confluence. For long-term storage, hUC-MSCs were suspended in CryoStor CS10 (STEMCELL Technologies, Vancouver, BC, Canada) and cryopreserved in a vapor phase liquid nitrogen tank. MSC characterization was based on the capability of tri-lineage differentiation (osteocytes, chondrocytes and adipocytes) and the expression of specific surface antigens on hUC-MSCs, as previously described [20].

ALI model

C57BL/6 mice were randomly grouped (n = 5 per group) and administered vehicle, 5 mg/kg

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or 10 mg/kg LPS from *Escherichia coli* O111:B4 (Sigma-Aldrich, St. Louis, MO, USA) through intraperitoneal or intratracheal injection as previously described [21]. For the intratracheal injections, the mice were randomly assigned to receive an injection of LPS or PBS. Briefly, after anesthesia with an intraperitoneal injection of 30 mg/kg pentobarbital sodium, the mice were fixed in a supine position at an angle of 50 degrees. 50 μ l PBS containing the indicated dose of LPS or the same volume of PBS was instilled into the trachea of the ALI mice and the sham mice, respectively using a safety catheter 22G needle. After instillation, the mouse was held in a vertical position and rotated for 1 minute to dispense the drip evenly into the mouse's lungs.

Treatment of ALI by hUC-MSC injection

At 24 h after LPS administration, the mice were intravenously injected with 1×10^5 , 5×10^5 , or 1×10^6 hUC-MSCs to treat the LPS-induced ALI. The mice were then sacrificed after 3 days of hUC-MSC treatment to collect the lungs and the BAL fluids for further analyses.

Immunohistochemistry and histopathology analyses

After sacrifice, the lung tissues were fixed in 10% formalin and then sent to the National Laboratory Animal Center (Taipei, Taiwan) for tissue processing and hematoxylin and eosin (H&E) staining. Images of the pulmonary tissues were acquired and evaluated by a veterinary pathologist at the Laboratory Animal Center, National Taiwan University College of Medicine (Taipei, Taiwan). The severity of lung injury was graded following the guidelines described [22]. Sections of paraffin-embedded tissue were subjected to immunohistochemical staining according to the instructions of the VisUCyte HRP Polymer-DAB Cell & Tissue Staining Kit (R&D Systems, Wiesbaden, Germany). The goat anti-human/mouse MPO antibody (AF3667, R&D Systems, Wiesbaden, Germany) was used at 1 μ g/mL, and the rabbit anti-human/mouse BAX (AF820, R&D Systems, Wiesbaden, Germany) was used at 2 μ g/mL.

Detection of IL-1 β , IL-6 and TNF- α by enzyme-linked immunosorbent assay (ELISA)

The mice were sacrificed under anesthesia by intraperitoneal injection with pentobarbital

sodium (45 mg/kg body weight, Sigma-Aldrich, St. Louis, MO, USA) and the BAL fluid samples were collected on ice. The concentrations of IL-6, IL-1 β and TNF- α BAL in fluid samples were assayed in duplicate with commercially available ELISA kits (Abcam, Cambridge, MA, USA) according to the manufacturer's protocols.

Database analyses

The raw dataset of GSE66890 and GSE40180 were downloaded from the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>). GSE66890 includes the expression profiles of whole blood RNAs from 29 sepsis-related ARDS and 28 sepsis alone patients. Gene Set Enrichment Analysis (GSEA) [23, 24] was also used to analyse the enriched pathways from these differentially expressed genes between the septic ARDS group and the sepsis alone group. The Molecular Signatures Database (MSigDB) used in this study was "h.all.v7.1.symbols.gmt". The gene sets with $P < 0.05$ and a false discovery ratio (FDR) q -value < 0.25 were considered significant. The RNA expression data from lungs in sham-operated, cecal ligation and puncture (CLP)-operated, and CLP-operated followed by MSC treatment C57Bl/6J mice was extracted from GSE40180 and each scatter dot plot was obtained for the expression pattern analysis.

Statistical analyses

Statistical analyses were analyzed using GraphPad Prism Software (San Diego, CA, USA). Fisher's exact test was used to compare the ALI scores between the hUC-MSC treated and the sham mice. The Wilcoxon two-sample rank sum test was used to compare the cytokine levels in the BAL fluids between the ALI and sham mice. The unpaired two-tailed t -test were used to compare between two groups in the database analyses. All results were expressed as the mean \pm standard deviation (SD). P value < 0.05 was considered for statistical significance.

Results

LSP administration through intratracheal injection causes ALI

To establish the ALI mouse model, 5 or 10 mg/kg LPS was administrated to C57Bl/6 mice

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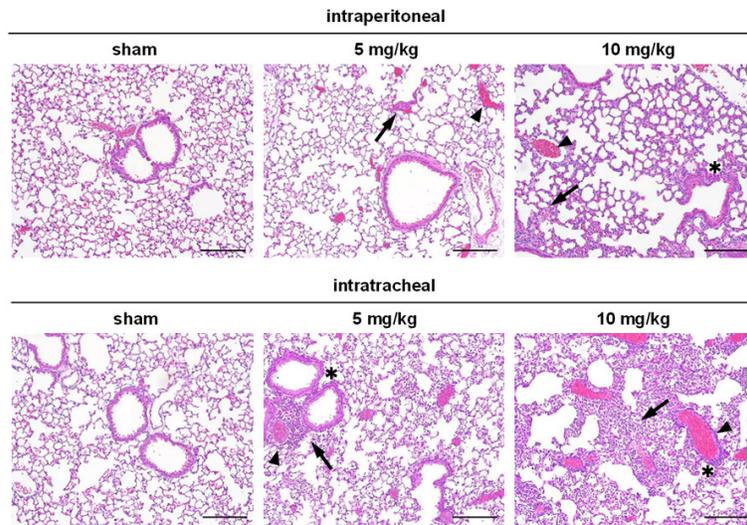


Figure 1. The representative lung histological images of LPS-induced ALI. ALI was induced in mice after intraperitoneal or intratracheal delivery of vehicle (sham) or LPS (5 and 10 mg/kg) for 24 h. Neutrophil infiltration in the alveolar spaces (arrow), vascular congestion (arrow head), and thickening of the alveolar walls (star) were significantly increased in 10 mg/kg LPS injected lungs of mice. Intratracheal injection of LPS consistently caused stronger ALI signs than intraperitoneal injection. 200 \times , H&E. Scale bar, 200 μ m.

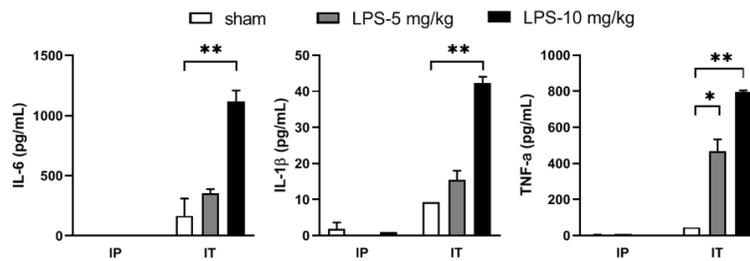


Figure 2. Change of inflammatory cytokines in the BAL fluids of mice after LPS injection. The BAL fluids were collected 24 hours after delivery of vehicle (sham) or LPS (5 and 10 mg/kg) via intraperitoneal or intratracheal injection. The concentrations of the pro-inflammatory cytokines, IL-6, IL-1 β and TNF- α in the BAL fluids collected from mice were detected by ELISA. Data are presented as the mean \pm SD. Wilcoxon two-sample rank sum test, * P <0.05, ** P <0.01. IP: intraperitoneal; IT: intratracheal.

through intraperitoneal or intratracheal injection. Compared to intraperitoneal injection, intratracheal injection of LPS consistently led to stronger ALI signs after 24 h (Figure 1). Pronounced infiltration or aggregation of mononuclear cells and neutrophils in the alveolar space or in the vessel wall, thickness of the alveolar wall, and vascular congestion were significantly observed in the mice given 10 mg/kg LPS via the intratracheal route. Elevated expression of IL-6, IL-1 β and TNF- α were detected in the BAL fluids from the lung tissues of intratracheally injected mice, compared with

the intraperitoneally injected group (Figure 2). The induction of IL-6, IL-1 β and TNF- α production by LPS stimulation was dose-dependent. Hence, LPS administration with 10 mg/kg for 24 h, through intratracheal injection, was selected as the model used to investigate the effects of hUC-MSCs on repairing ALI.

hUC-MSC administration reduces LPS-induced lung injury in terminal bronchioles and alveoli

To explore the potential of hUC-MSCs on ALI repair, three different doses of hUC-MSCs (1×10^5 , 5×10^5 , 1×10^6 cells per animal, reflecting 4 to 40×10^6 cells per kg body weight) were used to treat LPS-induced ALI mice through tail vein injection. As shown in Figure 3B, histological examination confirmed that LPS administration resulted in a robust inflammatory response compared to the sham group. Treatment with hUC-MSCs in the LPS-induced ALI mice reduced the infiltration of inflammatory cells in the alveolar space or the vessel wall, and partially rescued the alveolar and vascular structure. The ALI scores were assessed based on alveolar capillary congestion, haemorrhage, infiltration or aggregation of neutrophils in

the alveolar space or the vessel wall, and the thickness of the alveolar wall (Table 1). While there was no significant difference in the scores between the mice treated with hUC-MSCs alone and the sham mice, the LPS group had a significantly higher ALI score compared with the sham group. When hUC-MSCs were administered following LPS instillation, the ALI score was significantly reduced compared with that of the LPS group alone. The best therapeutic efficacy was seen in the high-dose treatment group (1×10^6 cells). These results suggest that hUC-MSCs might migrate to the lungs via

hUC-MSCs reduce LPS-induced lung injury

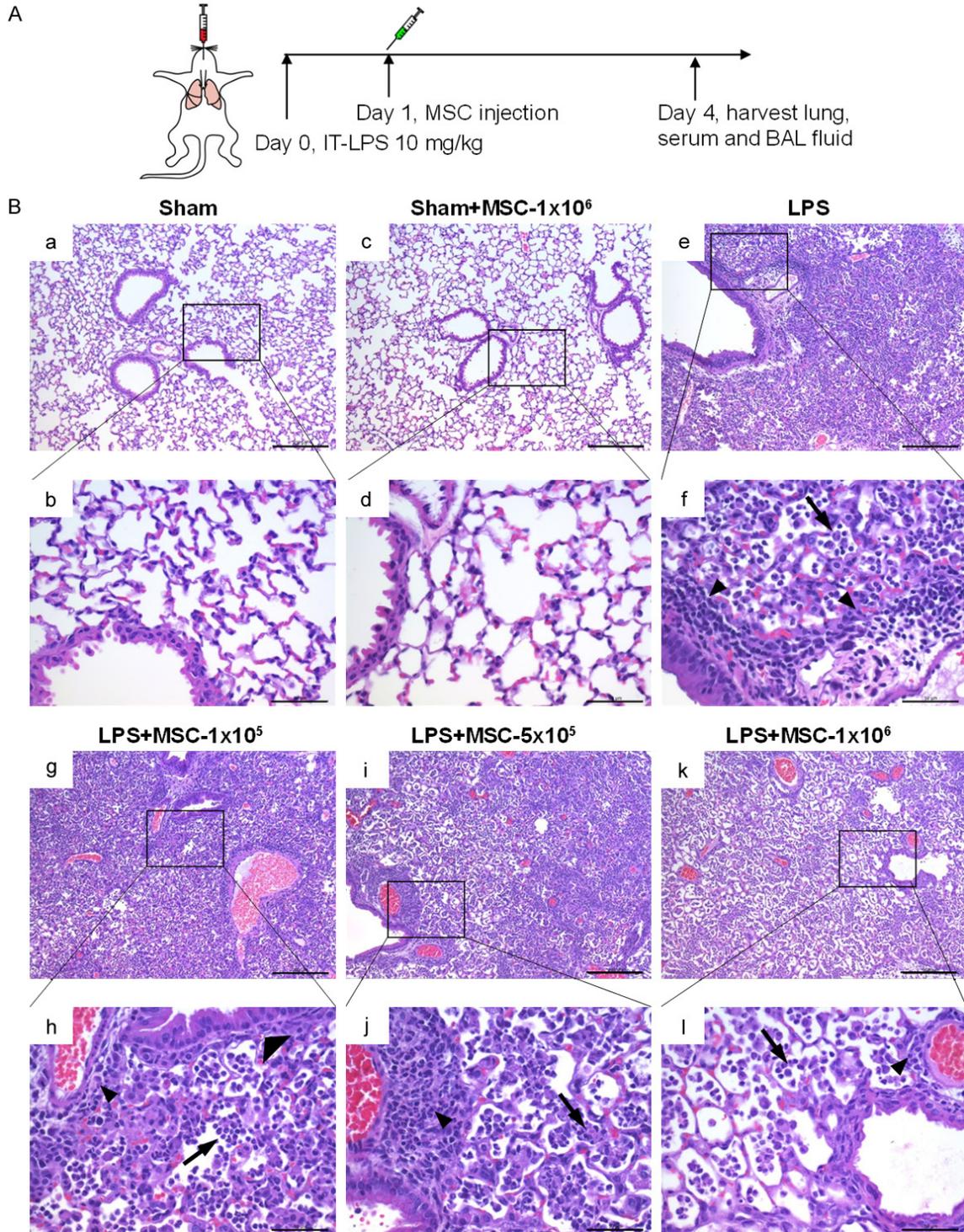


Figure 3. Administration of hUC-MSCs rescued the pathological signs of LPS-induced ALI in mice. (A) The experimental scheme for the treatment of LPS-induced ALI in mice via intravenous injection of hUC-MSCs. Vehicle (sham) or 10 mg/kg LPS was delivered by intratracheal injection to induce mouse ALI. Different doses of hUC-MSCs were intravenously administered 24 hours after LPS instillation. The lungs, serum, and BAL fluids were harvested for analyses on day 4. (B) Effects of hUC-MSCs on LPS-Induced ALI. Representative lung histological examinations revealed severe/high infiltration of neutrophils in the peribronchiolar/perivascular areas (arrow head) and alveoli (arrow) in the lung tissues after LPS-administration (e, f) compared with the lung tissues in the sham group (a, b). hUC-MSCs reduced the number of infiltrative neutrophils and injured areas in the LPS group (g-l), while hUC-MSCs have no effect in the sham group (c, d). Scale bars measure 200 μ m and 50 μ m at 200 \times and 400 \times , respectively.

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Table 1. The administration of hUC-MSCs significantly reduced the ALI score in mice

Group	ALI Incidence	ALI score
Sham	0/8 (0%)	NA
Sham-1×10 ⁶	1/8 (12.5%)	0.30±0.70
LPS	10/10 (100%)	4.40±0.70
LPS-1×10 ⁵	8/8 (100%)	4.50±0.53
LPS-5×10 ⁵	7/8 (87.5%)	4.29±0.76
LPS-1×10 ⁶	9/9 (100%)	3.89±0.33*

The degree of ALI was scored on alveolar capillary congestion, haemorrhage, infiltration or aggregation of neutrophils in the airspace or the vessel wall, and thickness of the alveolar wall. Five grades of lung injury were assigned for the histopathological evaluation: Grade 1 represents minimal (<1%); Grade 2 represents slight (1-25%); Grade 3 represents moderate (26-50%); Grade 4 represents moderately severe (51-75%); and Grade 5 represents severe/high (76-100%). NA = no significant lesions. *P value of the ALI scores between the LPS group and the LPS-1×10⁶ group was 0.0325 (by Fisher's exact test).

the blood circulation and promote the repair of damaged lung tissues.

hUC MSCs suppress the expression of pro-inflammatory cytokines

IL-6, IL-1 β and TNF- α are well-characterized pro-inflammatory cytokines due to their increased expression in a number of inflammatory diseases, including ALI. To explore the potential molecular machinery involved in hUC-MSC-mediated ALI repair, the secretion levels of these cytokines were examined by ELISA assay. Assessment of the cytokine expression profile in the BAL fluids revealed that administration of hUC-MSCs repressed the expression of pro-inflammatory cytokines IL-6, IL-1 β and TNF- α in a dose-dependent manner, while LPS exposure boosted their expression (Figure 4). Significant reductions in IL-6 and TNF- α levels were observed in mice injected with a high-dose of hUC-MSCs, suggesting that hUC-MSCs contribute to ALI repair via suppressing inflammation.

hUC-MSCs may play the role in inhibiting ROS production and apoptosis in ALI repair

In addition to immunomodulatory capacity in anti-inflammation, MSCs are self-renewing multipotent cells harbouring great potential in tissue regeneration and differentiation. It has been known that excess ROS produced by damaged endothelium/epithelium and leukocytes

recruited by pro-inflammatory play a major role in the process of ARDS and lung injury [25]. To further explore the potential mechanism of hUC-MSC in ALI repair, we conducted a gene set enrichment analysis (GSEA) in the publicly available expression profiles of sepsis patients (GSE66890). The results revealed that hypoxia, reactive oxygen species (ROS), apoptosis and G2/M checkpoint pathways were upregulated in severe sepsis patients with ARDS compared to sepsis patients (Figure 5A). To identify the expression changes associated with MSCs in promoting ALI repair, we then analysed the expression of genes involved in ROS pathway (i.e., *Mpo*, *Srxn1*, *Gsr*), cell proliferation (i.e., *Ki67*, *Pcna*, *Mcm2*) and apoptosis (i.e., *Bcl2l11*, *Bax*, *Casp4*) in sepsis mice treated with hUC-MSCs (GSE40180). The results showed that MSC treatment reduced the ROS and apoptosis pathways in septic lungs (Figure 5B), while no significant effect on proliferation was observed. To verify this finding, we examined the expression of MPO and BAX in lung tissues of sham-operated mice and LPS-injected mice without or with hUC-MSC treatment. As shown in Figure 6, MPO and BAX staining intensities were decreased in hUC-MSC-treated, LPS-injected mouse lungs compared with LPS-injected mouse lungs. The result suggested that hUC-MSCs inhibited ROS production and apoptosis in LPS-induced ALI.

Discussion

A growing body of evidence from preclinical studies of lung injury, sepsis, and other lung diseases indicates that MSCs possess potent immunomodulatory properties and have regenerative potential, thus making them an attractive option for treating lung diseases. Due to their great self-renewal capacity, and their ease of acquirement, hUC-MSCs are a great choice for cell therapy. Herein, the current study determined that allogenic hUC-MSCs reduced disease severity and inflammatory responses in LPS-induced ALI in C57BL/6 mice.

MSCs are considered to be hypoimmunogenic and more tolerated than other types of stem cells by the host immune system [26], making them a great cell source for transplantation to treat ALI. In preclinical models, MSCs could be administered by different routes. For example, MSCs can reach target organs by the blood cir-

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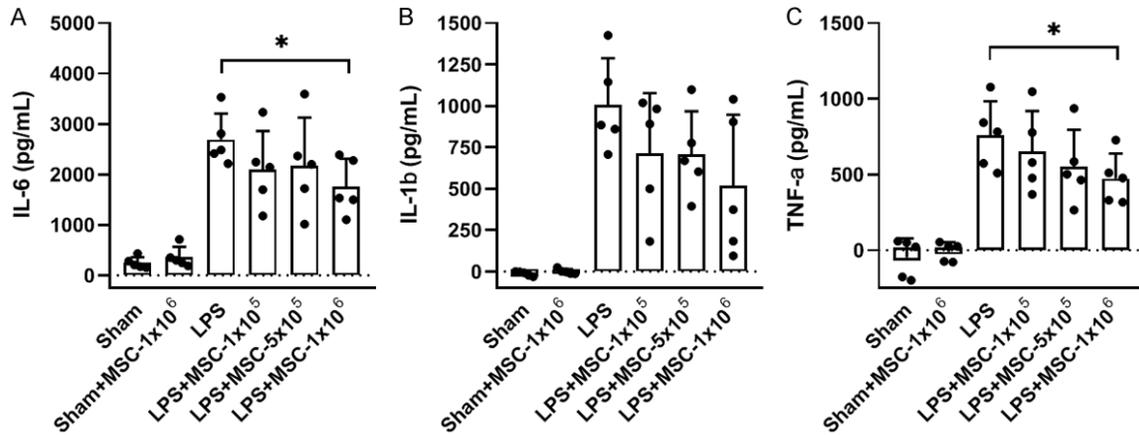


Figure 4. Reduction of inflammatory cytokines in the BAL fluids after hUC-MSC treatment in the LPS-induced ALI model. The concentrations of IL-6, IL-1 β and TNF- α in the BAL fluids of different groups, as described in **Figure 3**, were detected by ELISA. Wilcoxon two-sample rank sum test, * $P < 0.05$.

cultivation via intravenous injection. MSCs can also be delivered directly from the trachea to the lung tissues. It has been reported that delivery of MSCs via intravenous injection may be as effective as via interalveolar administration [9]. Considering all parameters, including the ease of injection, and the fact that MSCs delivered through the systemic circulation may still reach other injured tissues (such as kidneys in ALI patients), administration of MSCs through intravenous injection was the method of choice for the current study. The results revealed that hUC-MSCs given via intravenous injection not only decreased the infiltration of mononuclear cells and neutrophils (**Figure 3**), and reduced ALI scores (**Table 1**), but they also inhibited the expression of inflammatory cytokines (**Figure 4**). These results confirmed that intravenous injection of hUC-MSCs can lead to a significant histological improvement in the extent of lung injury through the circulation. Although only partial ALI repair was observed in the current study, this may be because the specimen was taken after only three days of treatment with hUC-MSCs. It may be that by extending the observation period after treatment with MSCs, administering repeated MSC treatments, or increasing the number of MSCs given as a single treatment, could improve their therapeutic effects on ALI repair, and warrants further investigation.

MSCs are adult progenitor cells with the capability to differentiate into multiple cell types, such as epithelial cells, adipocytes, fibroblasts,

myoblasts, and chondroblasts [6, 27]. It has also been reported that MSCs can be directed toward endothelial cells *in vitro* [28]. However, there is still limited evidence for effective engraftment of MSCs and subsequent differentiation to relieve ALI. In fact, it has been published that no donor MSC is observed in the lung 14 days after transplantation [11]. In some lung injury studies, cell engraftment rates were as low as <1% [29, 30]. On the other hand, the therapeutic potential of MSCs for ALI repair may come from their ability to secrete trophic factors involved in tissue repair, and restore permeability and immune system regulation [9, 31]. The secretion of angiopoietin-1 by MSCs has been suggested to be involved in restoring epithelial permeability [31]. Administration of MSCs enhanced the expression of anti-inflammatory IL-10 [12], while it suppressed the expression of pro-inflammatory molecules [10, 12, 32, 33]. Once the inflammation subsides, the impaired alveoli may be repaired and the accumulated water on the lungs may decrease, leading to the resolution of ALI. It has been known that alveolar macrophages can be polarized to M1 macrophages by LPS stimulation and then secrete a large number of cytokines, including IL-6, IL-1 β and TNF- α [34]. In the present study, hUC-MSCs significantly inhibited the secretion of IL-6 and TNF- α in BAL fluids from the lung tissues of LPS-induced ALI mice. Whether hUC-MSCs contribute to inhibiting the activation of alveolar macrophages remains to be clarified. Nevertheless, the reduction of IL-6 and TNF- α

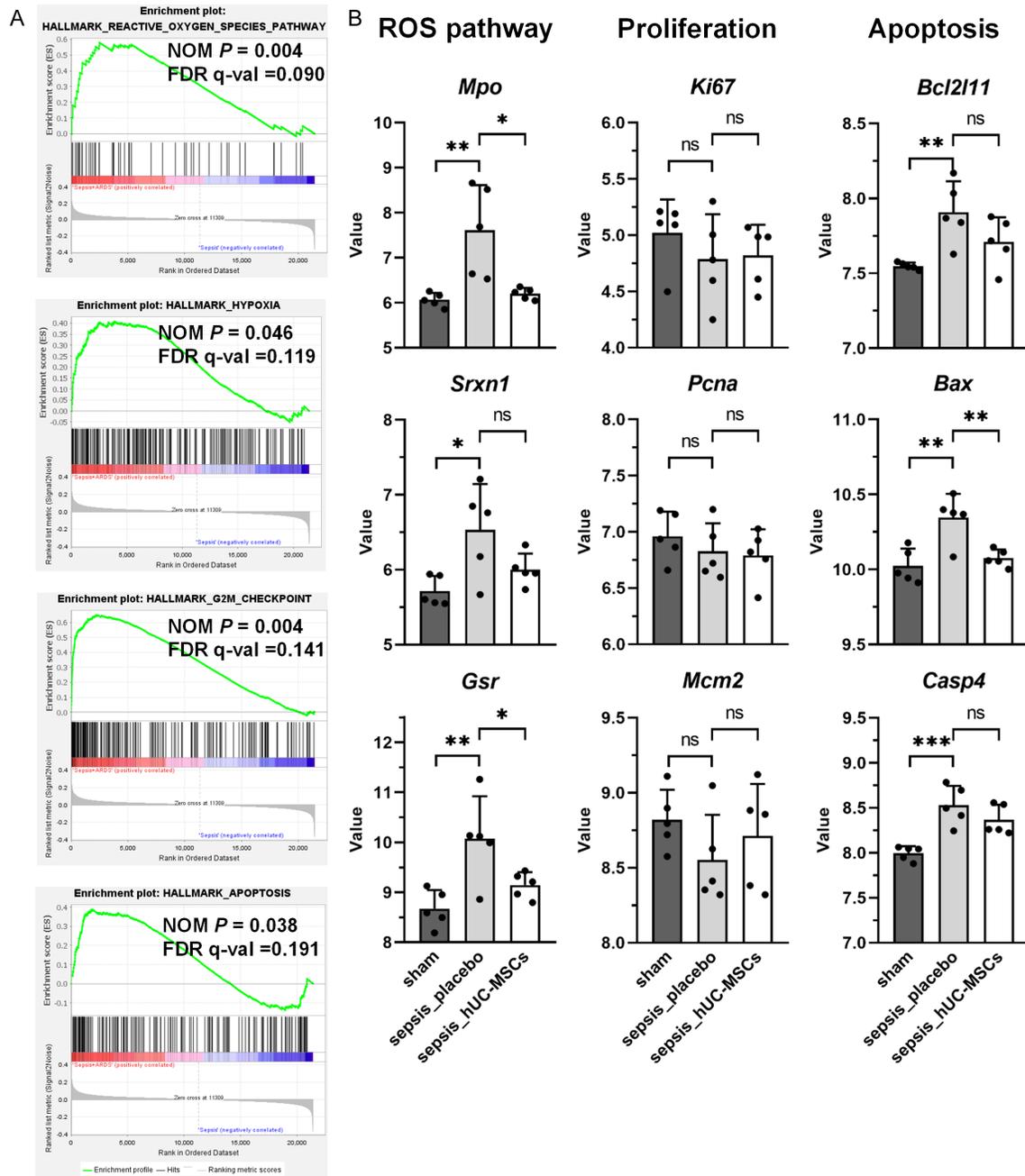


Figure 5. The potential functions of MSCs in inhibiting ROS production and apoptosis in ALI. A. GSEA plots showed that hypoxia, ROS, apoptosis, and G2/M checkpoint are the most upregulated pathways in severe sepsis patients with ARDS (GSE66890). B. The expression of genes involved in ROS pathway, cell proliferation and apoptosis in the lungs of sham, septic mice, and septic mice treated with MSCs (GSE40180). Unpaired two-tailed t-test, *P<0.05, **P<0.01.

in ALI mice suggests that lung injury was repaired after treatment with hUC-MSCs. Furthermore, ROS are known to significantly contribute to bacterial clearance, but they also aggravate lung dysfunction and tissue damage during sepsis [35]. Treatment of hUC-MSCs in

LPS-induced ALI mice can effectively reduce ROS generator MPO, which has a positive correlation with the reduction of pro-apoptotic protein BAX. This indicates that hUC-MSC can reduce the damage of ROS to lung cells during sepsis.

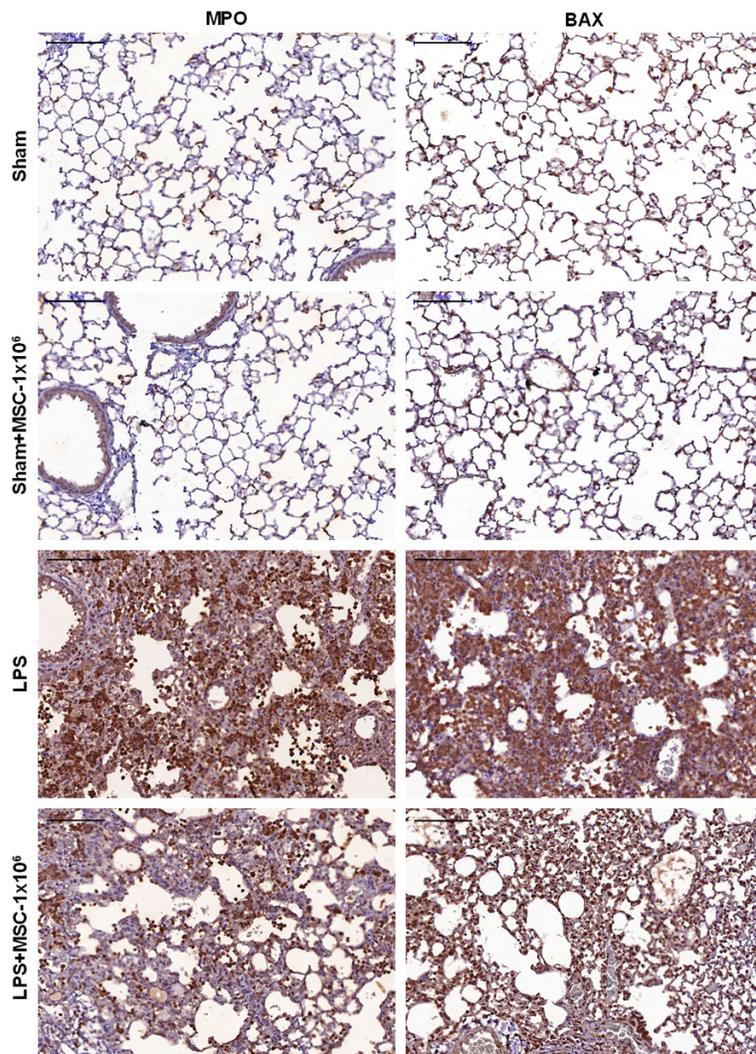


Figure 6. Effects of hUC-MSC treatment on MPO and BAX levels in LPS-induced lung injury. Representative DAB-colored images of anti-MPO (left panel) and anti-BAX (right panel) immunostaining. Paraffin-embedded lung tissues from sham-operated and LPS-injected mice without or with hUC-MSC treatment (1×10^6 cells) as indicated in **Figure 3** were subjected to immunostaining with anti-MPO and anti-BAX antibody. The scale bar indicates 100 μm .

In conclusion, the results reported herein highlight the potential of hUC-MSCs as another treatment regime for ALI, which currently lacks an effective treatment option. This study harnesses a cell-based therapy approach for the treatment of experimental acute lung injury model, and demonstrates the potential application of hUC-MSCs in acute lung injury, and potentially, acute respiratory distress syndrome associated complications. This study also paves the way for further clinical investigation into using hUC-MSCs to treat ALI.

Acknowledgements

This study was supported by grants from Cardinal Tien Hospital (CTH105B-225, CTH-109A-2209, and CTH109A-NDMC-2232) and Meridigen Biotech Co., Ltd., Taipei, Taiwan.

Disclosure of conflict of interest

The authors declare no conflict of interest. We wish to disclose that Dr. Willie Lin is an employee of Meridigen Biotech Co., Ltd., Taipei, Taiwan.

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