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Basic Study

Correlation between gut and lung microbiota homeostases and human umbilical cord mesenchymal stem cells in acute lung injury

Lv L *et al*. Role of HUC-MSCs on ALI

Abstract

BACKGROUND

Acute lung injury (ALI) and its final severe stage, acute respiratory distress syndrome, are associated with high morbidity and mortality rates in patients lacking effective specific treatments. Gut microbiota homeostasis, including that in ALI, is important for human health. Evidence suggests that the gut microbiota improves lung injury through the lung-gut axis. Human umbilical cord mesenchymal cells (HUC-MSCs) are attractive prospects for the ALI treatment. This study hypothesized that HUC-MSCs improve ALI *via* the lung-gut microflora.

AIM

To explore the effects of HUC-MSCs on lipopolysaccharide (LPS)-induced ALI in mice *via* the lung-gut axis.

METHODS

C57BL/6 mice were randomly divided into four groups (18 rats per group): Sham, sham + MSC, LPS, and LPS + MSC. ALI mice were established by intraperitoneal injections of LPS (10 mg/kg). After 6 h, mice were intervened with 0.5 mL phosphate buffered saline (PBS) containing 1 × 10^6 cells HUC-MSCs by intraperitoneal injections. For the negative control, 100 mL 0.9% NaCl and 0.5 mL PBS were used. Bronchoalveolar lavage fluid (BALF) was obtained from anesthetized mice, and their blood, lungs, ileum, and feces were obtained by an aseptic technique following CO_2 euthanasia. Wright's staining, ELISA, hematoxylin-eosin staining, Evans blue dye leakage assay, immunohistochemistry, fluorescence *in situ* hybridization, western blot, 16S rDNA sequencing, and non-targeted metabolomics were used to observe improvement the effect of HUC-MSCs on ALI mice *via* the lung–gut axis. One-way analysis of variance with post-hoc Tukey's test, independent-sample student's *t*-test, Wilcoxon rank-sum test, and Pearson correlation analysis were used for statistical analyses.

RESULTS

HUC-MSCs were observed to improve pulmonary edema and lung and ileal injury, and decreased mononuclear cell, neutrophil, and protein concentrations in BALF and inflammatory levels of serum, lung, and ileum in ALI mice. Especially, HUC-MSCs decreased Evans blue concentration and toll-like receptor 4, myeloid differentiation factor 88, p-nuclear factor kappa-B (NF-κB)/NF-κB, and p-inhibitor α of NF-κB (p-IκBα)/IκBα levels in the lung, and they raised the pulmonary vascular endothelial-cadherin, zonula occludens-1 (ZO-1), and occludin levels and ileum ZO-1, claudin-1, and occludin expression levels. HUC-MSCs improved gut and BALF microbial homeostasis, and the relationship between the gut and BALF microflora has been established. The number of pathogenic bacteria decreased in the BALF of ALI mice treated with HUC-MSCs. Concurrently, the abundances of *Oscillospira* and *Coprococcus* in the feces of HUS-MSC-treated ALI mice were significantly increased. In addition, *Lactobacillus*, *Bacteroides*, and *unidentified_Rikenellaceae* genera appeared in both feces and BALF. Moreover, this study performed metabolomic analysis on the lung tissue and indicated five upregulated metabolites and 11 downregulated metabolites in the LPS + MSC group compared to those in the LPS group, which were related to the purine metabolism and the taste transduction signaling pathways. Therefore, an intrinsic link between lung metabolite levels and BALF flora homeostasis was noted.

CONCLUSION

This study suggests that HUM-MSCs attenuated ALI by redefining gut and lung microbiota.

Key Words: Acute lung injury; Human umbilical cord mesenchymal cells; Lipopolysaccharide; Microflora; Untargeted metabolomics; Toll-like receptor 4/nuclear factor kappa-B signaling pathway

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Core Tip: This study provides a scientific basis for the biological mechanism and clinical application of human umbilical cord mesenchymal cells (HUC-MSCs), also provides new ideas for the development of therapeutic strategies for acute lung injury (ALI). Results suggest that HUC-MSCs inhibited the inflammatory cytokine expression levels in serum and the lung, which may be achieved by redefining gut and lung microbiota. This study provides a scientific basis for the pathophysiological mechanisms and clinical application of HUC-MSCs, also provides new ideas for the development of therapeutic strategies for ALI.

INTRODUCTION

Acute lung injury (ALI), whose final severe stage was defined as acute respiratory distress syndrome (ARDS) is caused by various pathogenic factors, including acute pneumonia, sepsis, severe trauma, and acute pancreatitis[1]. It is primarily characterized by pulmonary edema and acute inflammation[2], and has high morbidity and mortality rates in patients lacking effective patient-specific treatments[3]. Therefore, ALI has received much attention from the research community, and significant developments have been made in understanding their pathophysiological mechanisms; however, clinically available treatments are still limited[4]. Lipopolysaccharide (LPS), is a common pathogenic factor associated with ALI, is the main constituent of gram-negative bacterial cell walls[5]. A previous study reported that LPS induced lung tissue damage and increased the expression of inflammatory factors in the bronchoalveolar lavage fluid (BALF)[6]. Animal model of LPS-induced lung injury are commonly used to study ALI[7]. Interestingly, LPS-induced ALI mice have a gut microbiota imbalance, and improvement in gut microbiota homeostasis can ameliorate lung inflammation of ALI mice and inhibit toll-like receptor 4 (TLR4)/nuclear factor kappa-B (NF-κB) signal

intensity in the lungs[8]. The above research suggests the possible involvement of the intestinal microbiota in ALI.

A prospective observational cohort study focused on the relationship between lung microbiota and ALI and found that BALF microbiota can predict clinical outcomes in critically ill patients, especially enrichment of the gut-associated bacteria in BALF[9]. There is increasing evidence that the effects of host-microorganism interactions extend well beyond the local environment and influence the responses of peripheral tissues[7]. Homeostasis of the gut microbiota is important for human health, including its modulatory effects on ALI[10]. A 16S rRNA amplicon and metagenomic sequencing study found that the composition of the gut microbiota has a significant impact on wasting and death in mice with ALI, suggesting the importance of the lung–gut microbiota crosstalk in lung injury[11]. Moreover, the depletion of gut microbes using an antibiotic cocktail improvs lung injury and decreases interleukin (IL)-6 levels in the BALF of ALI mice[12]. A clinical cohort study found that the gut microbiome composition in patients with coronavirus disease 2019 (COVID-19), a respiratory illness, was significantly altered compared with that in non-COVID-19 individuals, irrespective of whether patients had received medication, and that gut microbiome composition was correlated with disease severity[13]. Additionally, intestinal diseases alter the composition of the pulmonary microbiota and their metabolites[14]. Therefore, changes in the abundance and composition of the gut microbiota may be key to improving ALI *via* lung–gut microflora. Recently, the impact of gut microbiota homeostasis on lung diseases has come into focus.

Mesenchymal stem cells (MSCs) are multipotent cells that can differentiate into various specialized cell types, including osteoblasts, chondrocytes, and adipocytes[15]. It originates from the mesoderm and is widely derived from adult stem cells with multi-differentiation potential, and can be isolated from a variety of tissues, such as the bone marrow, umbilical cord, amniotic membrane, adipose tissue, and skeletal muscle[16]. In addition, they have been shown to have beneficial effects in ALI[17]. Human umbilical cord mesenchymal cells (HUC-MSCs) have gained popularity in stem cell research and

applications because of their specific advantages, including easily availability, abundance, lack tumorigenicity, and ethical compliance[18]. Ahn *et al*[19] found that HUC-MSCs improved chronic lung disease and bronchopulmonary dysplasia in premature infants, without any transplantation-related adverse outcomes. Additionally, HUC-MSCs improved lung injury and inhibited the pro-inflammatory cytokine levels in the lungs of LPS-induced ALI mice[20]. HUC-MSCs treatment ameliorated lung inflammation and fibrosis in bleomycin-induced pulmonary fibrosis model mice[21]. Furthermore, researchers have reported that intraperitoneal infusions of HUC-MSCs improve colitis by reshaping the diversification of the gut microbiota[22]. Additionally, the gut microbiota of pulmonary hypertension mice were reversed by MSCs treatment[23]. Studies on the HUC-MSC treatment of inflammation-related diseases are in developing rapidly; however, their mechanisms of action are poorly understood. Therefore, by analyzing the lung-gut microbiota and lung metabolomics, this study aimed to explore the underlying mechanisms of HUC-MSCs amelioration of ALI to provide a scientific basis for the clinical application of HUC-MSCs and a direction for the development of therapeutic strategies for ALI/ARDS.

MATERIALS AND METHODS

Basic information of animals and cells

Total 48 to 6-8 wk old male C57BL/6 mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and housed in a specific pathogen-free standard environment. Free-diet mice were isolated and allowed to adapt for one week. All procedures involving animals were approved and supervised by the Animal Experimentation Ethics Committee of Zhejiang Eyong Pharmaceutical Research and Development Center. The mycoplasma free HUC-MSCs (HUM-iCell-e009) were purchased from iCell Bioscience Inc. (Shanghai, China) and cultured in a specialized medium (PriMed-iCELL-012, iCell Bioscience, China) containing supplements at 37 °C in a 5% CO_2 incubator. Purity of HUC-MSCs was assessed by immunofluorescence and was typically greater than 90%. Cell identification conducted by iCell Bioscience Inc.

Laboratory animal model of ALI

There were four groups, namely sham, sham + MSC, LPS, and LPS + MSC groups, with 18 mice in each group. The 36 randomly selected mice were intraperitoneal injected with 100 mL of LPS (10 mg/kg) to establish an ALI animal model[7], and sham mice were administered 100 mL 0.9% NaCl as the control. After 6 h, half of the ALI mice and half of the sham mice were given 0.5 mL phosphate buffered saline (PBS) containing HUC-MSCs (2×10^6 cells/mL) by intraperitoneal injections[24], and the other half of the ALI mice and sham mice were given 0.5 mL 0.9% NaCl.

Sample preparation

Three days after HUC-MSCs intervention in ALI mice, BALF was obtained after the mice were anesthetized with isoflurane as reported by Wu *et al*[24]. Briefly, the trachea was placed a 20-gauge catheter, flush five times with PBS, and all liquids were collected. Subsequently, all mice were euthanized with CO_2, and the blood, lungs, ileum, and feces were obtained using aseptic techniques. The lungs (one lung is evenly divided into four) from three mice in each group were taken and weighed, then, were baked in an oven at 80 °C for 48 h to determine the dry weight calculated by the ratio of wet lung weight to dry lung weight (weight ratio of W/D) as reported by Li *et al*[25]. In addition, a part of the remaining lung and ileum were made into paraffin block separately, and the other were stored at 80 °C for further analysis. The BALF was divided into two parts for inflammation index detection and 16S rDNA sequencing.

Detection of immune cells and inflammatory cytokines

The protein concentration in the BALF was measured using the BCA assay (Beyotime, China). Additionally, the cells in BALF were precipitated and resuspended, and mononuclear cells and neutrophils were counted using Wright's staining. The levels of tumor necrosis factor (TNF)-α (ml002095-1, Enzyme-linked, China), IL-1β (ml063132-1, Enzyme-linked, China), and IL-6 (ml002293-1, Enzyme-linked, China) in the serum,

lungs, and ileum were measured by ELISA according to the manufacturer's instructions.

Histopathological observation

Hematoxylin-eosin (H&E) staining was performed to observe the histopathology of the lungs and ileum. Briefly, the lungs and ileum were fixed in 10% formalin and removed. The tissue was then placed in paraffin, and then sections were stained with hematoxylin and eosin. The injury of lungs and ileum were scored in a blinded fashion as previously reported[26,27].

Measurement of alveolar-capillary permeability

Three mice were randomly selected from each group for the Evans blue dye leakage assay to explore the function of the lung tissue endothelial barrier as previously reported[28]. This assay was performed using 25 mg/kg of 0.5% blue dye (Sigma, United States) injected into mice *via* the tail vein 2 h before isoflurane anesthesia. After the heart of the anesthetized mice with exposed, their left ventricles were intubated and flushed with 4% normal saline. Subsequently, the mice were euthanized using CO_2. Lungs were homogenized in 2 mL PBS and treated with formamide (Sigma, United States) at 60 °C for 24 h. Finally, the concentration of Evans blue dye in the lung tissue was determined with a spectrophotometer at 620 nm by Microplate Reader (CMaxPlus, Molecular Devices, United States).

Immunohistochemistry for lung

Immunohistochemistry (IHC) was performed as described by Peng *et al*[29]. Paraffin blocks of lungs were deparaffinized, permeabilized, and blocked. The sections were then incubated at 4 °C over night with 1:100 dilutions of TLR4 antibody (Affinity, United States). After washing with PBS, the slides were incubated with goat anti-rabbit IgG HRP (Abcam, United States) at 37 °C for 30 min. The slides were washed again,

mounted with DAPI (Vector Laboratories, Burlingame, CA) and examined using an E100 fluorescence microscope (Nikon, Japan).

Fluorescence in situ hybridization

Fluorescence *in situ* hybridization (FISH) was performed on slides of ileum and lung as previously described[30], using a pan-bacteria FITC-labeled probe (EUB338) and an RNA FISH kit (Genepharma, China). After DAPI staining, the slides were visualized under an inverted fluorescence microscope (Ts2-FL; Nikon, Japan). Images were acquired using Micro-Manager and analyzed using ImageJ/FIJI software (National Institutes of Health, United States, version 1.53c).

Western blot

The antibodies used for western blot (WB) are shown in Table 1. The lung and ileum tissues were homogenized in a lysis solution for the complete lysis of proteins. After centrifugation and supernatant collection, the samples were uniformly concentrated, added to the marker, and denatured. Briefly, sodium-dodecyl sulfate gel electrophoresis and membrane transfer was performed for 20 μg protein per group as reported by Li *et al*[3]. The membranes were incubated with primary antibodies overnight and then treated with secondary antibodies for 1 h. Finally, the membranes were subjected to chemiluminescence reactions and protein levels were measured by enhanced chemiluminescence. ImageJ/FIJI software was used to semiquantitative analysis.

The 16S rDNA sequencing for BALF and feces

High-throughput 16S rDNA sequencing was performed to analyze the gut and pulmonary microflora by BioDeep Co., Ltd (Suzhou, China). A sequencing library was prepared using the TruSeq Nano DNA LT Library Prep Kit of Illumina. A NovaSeq6000 (PE250, Illumina, United States) was used for sequencing as reported by Yi *et al*[31]. Then the QIIME2 DADA2 and OmicStudio platforms[32] were used to analyze the data and presentation.

Metabolomics for the lung tissue

The non-targeted metabolomics approach was performed by PANOMIX Biomedical Tech Co., Ltd. (Suzhou, China). The samples were treated with 75% methanol-chloroform (9:1) and 25% water for sonication and centrifugation to extract the metabolites. The 2 μL samples were analyzed by liquid chromatography-mass spectrum (MS) detection with the Vanquish UHPLC System (Thermo, United States). Additionally, an orbitrap Explorer (Thermo, United States) was used for mass spectrometry analysis. After data acquisition, the Proteowizard package (v3.0.8789) and the R xcms package were used to preprocess the data. Metabolites were identified on the public databases such like the Human Metabolome Database[33], MassBank (https://massbank.eu/MassBank/), Lipid Maps (https://Lipidmaps.org/), mzCloud (https://www.mzcloud.org/), Kyoto Encyclopedia of Genes and Genomes (KEGG)[34], and self-building repository, and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) analysis was performed to screen the differential metabolites.

Statistical analysis

Statistical analyses were conducted using SPSS (version 16.0; IBM, Armonk, NY, United States). Data from multiple *in vivo* experiments were analyzed using the one-way analysis of variance (ANOVA) with a post-hoc Tukey test. An independent-sample student's *t*-test was used for comparison between the two groups. The Wilcoxon rank-sum test was conducted to explore the microflora with a significant difference in abundance. Pearson's correlation analysis was used to explore the intrinsic associations. Additionally, the Variable Importance for the Projection (VIP) of OPLS-DA was used to screen for metabolites with biomarker potential (VIP > 1). Two-way Orthogonal Partial Least Squares (O2PLS) analysis was used to explore the links between BALF microbiota and lung metabolism. The threshold for significance was $P < 0.05$ for all tests.

RESULTS

HUC-MSCs treatment alleviated the lung injury and inflammation in ALI mice

This study used LPS to induce the ALI in a mouse model and collected the lungs and BALF to explore the ameliorating effect of HUC-MSCs on ALI mice by H&E staining and ELISA tests. ALI mice had a higher ratio of W/D lung weight and more mononuclear cells and neutrophils than sham mice ($P < 0.01$), whereas HUC-MSC treatment on ALI mice decreased the W/D weight ratio ($P < 0.05$), mononuclear cells, neutrophils count, and protein concentration ($P < 0.01$) (Figures 1A and B). Additionally, compared to sham mice, the lung of ALI mice had markedly thickened alveolar septa with significant inflammatory cell infiltration; however, HUC-MSCs treatment alleviated the degree of alveolar septal thickening and inflammatory cell infiltration in ALI mice (Figure 1C). Likewise, the H&E scores for lung injury in the LPS group were higher than those in the sham ($P < 0.01$) and the LPS + MSC groups ($P < 0.05$) (Figure 1D). Moreover, ALI mice had significantly increased levels of TNF-α, IL-1β, and IL-6 in their serum and lung tissues ($P < 0.01$) (Figures 1E and F). In particular, the above-mentioned inflammatory factor levels were decreased in the ALI mice treated with HUC-MSCs treatment ($P < 0.01$) (Figures 1E and F).

HUC-MSCs treatment improved the endothelial barrier function and integrity of lung in ALI mice

In addition, this study observed the endothelial barrier function and integrity in the lungs using Evans blue, WB, and IHC assays. As shown in Figure 2A, ALI mice had a higher concentration of Evans blue dye in the lungs than did the sham mice, and HUC-MSC treatment reduced Evans blue concentration in the lungs of ALI mice ($P < 0.01$). In particular, the levels of endothelial barrier-associated proteins, such as vascular endothelial (VE)-cadherin, zonula occludens-1 (ZO-1), and occludin, were markedly decreased in ALI mice ($P < 0.05$, $P < 0.01$); however, HUC-MSC treatment reversed these expression levels ($P < 0.01$) (Figures 2B-D). In addition, this study explored the signal intensity of the TLR4/myeloid differentiation factor 88 (Myd88)/NF-κB signaling

pathway. The TLR4, Myd88, p-NF-κB/NF-κB, and p-inhibitor α of NF-κB (p-IκBα)/IκBα levels of the lung were all increased in ALI mice compared to the sham mice ($P < 0.01$); however, in ALI mice with HUC-MSCs treatment, these levels were decreased ($P < 0.05$ or $P < 0.01$) (Figures 2E-I). Similarly, TLR4 detected by IHC had a strong positive expression in the lungs of ALI mice, whereas HUC-MSCs treatment attenuated the positive expression of TLR4 ($P < 0.05$ or $P < 0.01$) (Figures 2J and K).

HUC-MSCs treatment improved injury, endothelial barrier integrity, and bacterial translocation of the ileum in ALI mice

Studies have reported not only lung function injury but also intestinal dysfunction in ALI[17]. Therefore, the ileum was examined using H&E staining, ELISA, WB, and FISH. The ileal tissue in ALI mice had shorter and ruptured villi with significant inflammatory cell infiltration compared to sham mice, whereas these ileal injuries in ALI mice treated with HUC-MSCs were improved ($P < 0.05$, $P < 0.01$) (Figure 3A). In addition, LPS treatment on mice markedly increased the ileal TNF-α, IL-1β, and IL-6 levels ($P < 0.01$), whereas HUC-MSC treatment inhibited them ($P < 0.01$) (Figure 3B). The suppressed expression of endothelial barrier-associated proteins, including claudin, ZO-1, and occludin expression by LPS was enhanced by HUC-MSC treatment in ALI mice ($P < 0.05$) (Figures 3C and D). Furthermore, the EUB338 counts of the ileum epithelium and lungs in ALI mice were measured to observe the lung translocation of gut bacterial, and this study found that they were increased in ALI mice, whereas HUC-MSCs treatment reduced them ($P < 0.01$) (Figures 3E-H).

HUC-MSCs treatment attenuated ALI via regulating lung-gut microbiota homeostasis

The 16S rDNA sequencing was used to explore the effect of the lung and gut microbiota on the HUC-MSCs-mediated amelioration of ALI. BALF and fecal samples were collected for 16S rDNA sequencing. After homogenizing the sequencing depth of each group, 48 BALF and fecal samples from the four groups were identified at the genus level, with an average of 48.25 units in each group. Figures 4A and B showed the top 20

flora with the highest average abundance at the genus level. Additionally, the Shannon and Simpson indices of alpha diversity reflected richness and community evenness. According to the Kruskal-Wallis rank-sum test, the Shannon index of alpha diversity showed no significant differences (P = 0.056) (Figure 4C). Additionally, the Bray-Curtis distance of beta diversity reflected microbial diversity between groups and was analyzed by principal coordinates analysis, which revealed that the projection distance of the LPS + MSC group on the coordinate axis was closer to that of the negative control group than that of the LPS group (Figure 4C). Similarly, the gut microflora Simpson index for alpha diversity showed no significant differences (P = 0.058), and the projection distance of gut microflora was closer to that of negative control group than to that of the LPS group (Figure 4D).

Subsequently, the Wilcoxon rank-sum test was conducted to explore the microflora with a significant difference in abundance (marked microflorae) (Figures 4E and F). In particular, there were 21 microflora with upregulated abundance and 12 microflora with downregulated abundance in the BALF of the LPS + MSC group compared to the LPS group (P < 0.05) (figure 4E), and 17 microflora with upregulated abundance and 3 microflora with downregulated abundance in feces (P < 0.05) (Figure 4F). The 33 marked microflorae with the largest upregulation or downregulation of OTU in the BALF of ALI mice with/without HUC-MSCs are shown in Figure 5A, and 20 marked microflorae in feces are shown in Figure 5B. *Rhizobiales* had the largest log2 fold change (FC) in the BALF of the LPS + MSC group compared to the LPS group [log2(FC) = 9.3264, P = 0.0284], and *Elizabethkingia* had the lowest log2FC in the BALF of the LPS + MSC group compared to that of the LPS group [log2(FC) = -5.1799, P = 0.028] (Figure 5A). In fecal samples, the log2FC of *unclassified_Bacteroidales* was the highest in the marked microflorae of the LPS + MSC group compared to that of the LPS group [log2(FC) = 4.7549, P = 0.027], and the unidentified_F16 was the lowest [log2(FC) = -4.6328, P = 0.012] (Figure 5B).

Furthermore, the Pearson's correlation analysis was used to analyze the correlation between marked microflorae of the gut and lungs, and most of the bacteria in the BALF

and feces had a strong or extremely strong correlation (Figure 6). The *Desulfovibrio* genus of feces is positively correlated with *Stenotrophomonas* in BALF ($P < 0.05$) (Supplemental Table 1).

HUC-MSCs treatment on ALI mice regulated metabolic profiling of lung tissue

The base peak chromatograms in the positive and negative modes of the four groups showed similar trends, suggesting good repeatability and reliable results (Figure 7A). Additionally, Partial Least Squares-Discriminant Analysis (PLS-DA) was used to distinguish metabolite differences between groups in the positive/negative mode. In particular, all blue Q2 positions in the permutation test chart were lower than the original points on the far right, suggesting that the PLS-DA models were valid (Figures 7B and C). In the positive mode, MS analysis identified a total of 1206 common biomarkers between the sham and LPS + MSC groups, 362 common biomarkers between the LPS and sham + MSC groups, 347 common biomarkers between the LPS and sham groups, 343 common biomarkers between LPS + MSC and LPS groups, and 181 common biomarkers between the sham + MSC and sham groups (Figure 7D). In the negative mode, this study identified 905 common biomarkers between the sham and LPS + MSC groups, 476 common biomarkers between the LPS and sham + MSC groups, 225 common biomarkers between the LPS and sham groups, 112 common biomarkers between the LPS + MSC and LPS groups, and 139 common biomarkers between the sham + MSC and sham groups (Figure 7E).

Subsequently, precise screening of the metabolic profiles by MS/MS was performed to eliminate false positives. This study identified four upregulated metabolites (3-succinoylpyridine, nicotinamide ribotide, aldosterone, and phenylacetic acid) and one downregulated metabolite (lithocholic acid) in the sham + MSC group compared to those in the sham group. In addition, KEGG enrichment analysis suggested that these differential metabolites might participate in nicotinate and nicotinamide metabolism, aldosterone-regulated sodium reabsorption, and aldosterone synthesis and secretion pathways (Figures 8A-C). Additionally, 12 markedly up-regulation metabolites

(hydroxyindole, xanthine, and N-acetyl-L-aspartic acid, *etc.*) and 20 markedly downregulated metabolites (prostaglandin E2, argininosuccinic acid, and guanine, *etc.*) were identified in the LPS group compared to the sham group, these metabolites were predicted to be potentially involved in the alanine, aspartate and glutamate metabolism, the oxidative phosphorylation, the cAMP signaling pathway, and so on (Figures 8A, D and E). In particular, five upregulated metabolites (anabasine, IMP, lidocaine, salicylic acid, and propionylcarnitine) and 11 downregulated metabolites (N-acetylleucine, guanosine, and guanine, *etc.*) were identified in the LPS + MSC group compared to those in LPS group (Figures 8A and F). They were related to the Purine metabolism and the taste transduction signaling pathways ($P < 0.001$) (Figure 8G).

Significant correlations between microbes in BALF and metabolites in lung tissues

To explore the role of HUC-MSCs in regulating the microflora of the lung-gut axis to improve ALI, we performed an O2PLS correlation analysis on the expression of microflora in BALF and the expression of metabolites in the lungs to determine the microflora involved in the improvement of MSC and their impact on metabolism. In Figure 9A, the top 25 bacteria and top 25 metabolites are shown with a large absolute value of the joint loading values, suggesting that they have a large weight in the improvement of ALI following HUC-MSCs treatment. Subsequently, these flora and metabolites were analyzed using correlation analysis (Supplemental Table 1). Significantly related bacteria and metabolites were screened based on $P < 0.05$. The number of metabolites that significantly correlated with BALF microbes is shown in Figure 9B. Additionally, metabolites with a large role in the improvement of ALI by HUC-MSC treatment were subjected to KEGG enrichment analysis and were found to be mainly involved in the signaling pathways of drug metabolism-other enzymes, tyrosine metabolism, autophagy-animal, and endocytosis (Figure 9C).

DISCUSSION

MSCs have emerged as a promising therapeutic strategy for inflammatory diseases, owing to their low immunogenicity, ability to stabilize immunity, and ability to ameliorate inflammatory responses[35]. Moreover, HUC-MSCs not only ameliorate acute and chronic pneumonia, but also enteritis[36,37]. This study found that HUC-MSCs improved pulmonary edema, alleviated pathological damage to the lungs and ileum, and inhibited the levels of inflammatory cells in the BALF and factors in the serum, BALF, lungs, and ileum. Furthermore, HUC-MSCs treatment of ALI mice improved endothelial barrier integrity in the lungs and ileum. Endothelial permeability is regulated by intercellular junctions including adherens junctions and tight junctions, which are composed of cell junction proteins including occludin, claudin 1, and VE-cadherin, *etc*.[38]. Claudin-1 in the ileum and VE-cadherin, ZO-1, and occludin in the lung and ileum play key roles in maintaining vascular integrity. This decrease indicated endothelial barrier disruption in lungs and ileum. In this study, HUC-MSCs treatment of ALI mice advanced lung VE-cadherin, ZO-1, and occludin expression signal intensity and upregulated claudin-1, ZO-1, and occludin expression levels in the ileum. In summary, treatment of ALI mice with HUC-MSCs ameliorated lung and ileal barrier integrity.

Inflammatory factors and cells promote ALI injury. A study reported that TNF-α could promote M1 macrophage activation which has pro-inflammatory effects in sepsis-related ALI[39]. Neutrophil overactivation promotes the development of inflammation and injury in ALI[40]. Microvascular endothelial barrier dysfunction, the main pathophysiological feature of ARDS/ALI, induces capillary leakage and edema, which further intensifies inflammatory injury, thus causing high morbidity and mortality[41]. Botros and colleagues reported that stabilizing the endothelial barrier during inflammation alleviated inflammatory responses, edema, and lung injury in mice with ALI[42]. Interestingly, the inhibition of the TLR4 signaling pathways is related to the integrity of the pulmonary endothelial barrier. One study found that the TLR4 knockdown decreased the sensitivity to particulate matter-induced pulmonary edema in ALI mice and increased the signal expression intensity of VE-cadherin[43].

Furthermore, MSC treatment in paraquat-induced ALI rats downregulated the TLR4 and NF-κB protein levels in the lungs[44]. Besides, LPS facilitates TLR4 activation to recruit MyD88, thereby activates NF-κB to promote the production of pro-inflammatory factors such as TNF-α and IL-6[45]. Similarly, this study reported the inhibition effect of HUC-MSCs on TLR4, Myd88, and NF-κB in lung of LPS-induced ALI mice, which suggests that HUC-MSCs may mitigate microvascular endothelial barrier dysfunction and inflammation in ALI *via* the TLR4/Myd88/NF-κB signaling pathway.

There is a correlation between microbiota levels and lung diseases[46]. By analyzing the 16S rDNA microflora in BALF, we observed a decrease in some pathogenic bacteria. For example, the *Stenotrophomonas* genus was decreased in the BALF of the LPS + MSC group. One study has reported that *Stenotrophomonas* maltophilia is commonly found in respiratory tract infections[47]. Additionally, the *Comamonas* genus is an important opportunistic pathogen in human[48]. The genus *Elizabethkingia* has recently emerged as the cause of life-threatening infections in humans, most commonly causing meningitis[49]. *Acinetobacter Baumannii* infections have also been linked to ventilator-associated pneumonia[50]. This suggests that HUC-MSCs ameliorate ALI by inhibiting the abundance of pathogenic bacteria. Additionally, by analyzing the microflora in the feces, *Oscillospira* and *Coprococcus* genus abundance in the LPS + MSC group was increased. *Oscillospira*, a common genus of gut bacteria, positively correlates with gut microbiota diversity[51]. Moreover, butyrate, a product of the *Coprococcus* genus, is thought to be able to participate in anti-inflammatory[52]. Thus, *Oscillospira* and *Coprococcus* may be involved in the restorative effects of HUC-MSCs treatment on gut microflora homeostasis. Additionally, some bacteria have contradictory roles in various diseases. The *Mucispirillum* genus is associated with Crohn's disease-like colitis in immunodeficient mice and is linked to health promotion in immunocompetent hosts[53]. In this study, the improvements in ALI may have been correlated with the abundance of microflora.

Correlation analysis of the gut and lung microflora noticed *Desulfovibrio* genus is positively correlated with *Stenotrophomonas*. The *Desulfovibrio* genus is a candidate

microbe that induces weight of mice with ALI and is directly related to survival[11]. This study not only noted bacteria that strongly correlated with the downregulation of pathogenic bacterial abundance in BALF but also those that were present in both the gut and lungs. *Lactobacillus*, *Bacteroides* and *unidentified_Rikenellaceae* genera appeared in the feces and BALF. Moreover, the *Bacteroides* in feces significantly related to *Lactobacillus* in the BALF. Significant changes in their abundance may be associated with the mechanism of lung-gut axis. Although the *Lactobacillus* genus is a beneficial flora most of the time in colitis[54], *Lactobacillus* rhamnosus GG treatment in patients with severe pneumonia does not improve the clinical outcomes[55], suggesting that changes in the abundance of *Lactobacillus* may be a consequence of the improvement of HUM-MSCs in ALI and could be used as biomarkers. *Lactobacillus*, *Bacteroides*, and *unidentified_Rikenellaceae* genera are potential biomarkers for evaluating the treatment efficacy of HUC-MSCs.

Moreover, lung tissue metabolomics was performed in this study, and the composition of metabolites was different in sham mice and ALI mice; HUM-MSC treatment in ALI mice changed the lung metabolite composition. The results of the metabolomic analysis suggest that HUM-MSC treatment alters the bile secretion pathway. Bile and its nuclear receptor farnesoid X are involved in inflammatory liver and bowel diseases[56]. In addition, this study verified the correlation between the BALF microflora and lung tissue metabolites using the O2PLS method. *Haemophilus* took a pivotal role in improving HUC-MSCs treatment in ALI mice. One study reported that non-typeable *Haemophilus influenzae* induces neutrophilic inflammation in severe asthma[57]. Moreover, Yue *et al*[58] found that autophagy can combat the inflammation caused by *Haemophilus parasuis* acting as a cellular defense mechanism. Similarly, KEGG enrichment results of the high-impact metabolites predicted by O2PLS suggested that autophagy-related pathways may play a critical role in HUC-MSCs treatment in ALI mice. The exploration of lung metabolites contributes to the biological mechanism and biomarker discovery of HUM-MSCs treatment in ALI.

Naturally, this study only examined the correlation between microarray and metabolomics in the lung and gut and does not have conclusive evidence to confirm that the lung-gut axis microbiota is a crucial factor behind the ability of HUC-MSCs to enhance ALI. Animal and clinical studies are necessary to validate the role of gut and lung microorganisms in the cellular improvement of HUC-MSCs in ALI.

CONCLUSION

This study shown that HUM-MSC treatment of ALI improved the edema, tissue injury and endothelial barrier function of the lung; upregulated the VE-cadherin, ZO-1, and occludin levels in the lung; and inhibited the inflammatory cytokine expression levels in the serum and lung. Moreover, HUM-MSC treatment on ALI attenuated the expression of the TLR4/Myd88/NF-κB signaling pathway in the lung tissue. In addition, HUM-MSCs treatment of ALI mice improved ileal histopathological damage, reduced the levels of inflammatory factors, promoted ZO-1, claudin-1, and occludin protein expression, and decreased EUB338 counts in the lung and ileum. In particular, this study found that the gut and lung microflora and metabolites were significantly different between ALI and HUM-MSCs-treated mice. There was a correlation between the abundances of the gut and lung microflora. *Lactobacillus*, *Bacteroides*, and *unidentified_Rikenellaceae* genera are potential biomarkers for evaluating the treatment efficacy of HUC-MSCs. Additionally, this study contributes to the biological mechanism and biomarker discovery of HUM-MSC treatment of ALI by the combined analysis of lung tissue metabolomics and microbiota. This study provides a scientific basis for the biological mechanism and clinical application of HUC-MSCs and new ideas for the development of therapeutic strategies for ALI.

ARTICLE HIGHLIGHTS

Research background

Acute lung injury (ALI) has high morbidity and mortality rates and needs effective treatment. Research found gut microbiota improves lung injury through the lung-gut axis. Human umbilical cord mesenchymal cells (HUC-MSCs) can improve ALI.

Research motivation

Although HUC-MSCs can improve ALI, their biological mechanism of action is not yet clear.

Research objectives

To explore changes in the microbiota in the lung-gut axis and the relationships with HUC-MSC treatment.

Research methods

C57BL/6 mice were used to establish the ALI animal model by intraperitoneal injections of lipopolysaccharide. Wright's staining, ELISA, hematoxylin-eosin staining, Evans blue dye leakage assay, immunohistochemistry, fluorescence *in situ* hybridization, and western blot were used to observe improvement the effect of HUC-MSCs on ALI mice. The high-throughput 16S rDNA sequencing was used to observe the microbiota homeostases in the lung-gut axis. The non-targeted metabolomics was used to explore changes in lung tissue metabolites.

Research results

HUC-MSCs ameliorate histopathological damage in the lung and ileum of ALI mice. HUC-MSCs treatment improved inflammation, endothelial barrier integrity, and bacterial translocation in the lungs and ileum of ALI mice. HUC-MSCs regulated lung-gut microbiota homeostasis. HUC-MSCs treatment on ALI mice regulated metabolic profiling of lung and ileum.

Research conclusions

This study shows the improvement of HUC-MSCs in the lung and ileum of ALI mice. This study suggests a correlation between HUM-MSCs improved ALI and gut and lung microbiota homeostases.

Research perspectives

This study observes the biological mechanism of HUC-MSCs improving ALI from the perspective of the correlation between the microbiota in the lung-gut axis and lung tissue metabolites, providing a research basis for HUC-MSC treatment.

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