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Mesenchymal stem cells protect the integrity of the alveolar epithelial barrier through extracellular vesicles by inhibiting MAPK-mediated necroptosis

Tao Ruan[1,2], Jiaming Han[2], Chengxu Xue[1], Fengyuan Wang[1] and Juntang Lin[2*]

Abstract

Background Alveolar–capillary barrier disruption is a hallmark of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). The contribution of necroptosis to the compromised alveolar-barrier in ALI remains unclear. Mesenchymal stem cells (MSCs) may contribute to tissue repair in ALI and ARDS. Here we evaluated the efficacy and explored the molecular mechanisms of menstrual blood-derived endometrial stem cells (MenSCs) and MenSC-derived extracellular vesicles (MenSC-EVs) in ALI-induced alveolar epithelial barrier dysfunction.

Methods Human lung epithelial cells were stimulated with endotoxin and treated with MenSCs or MenSC-EVs, and their barrier properties were evaluated. Lipopolysaccharide (LPS)-injured mice were treated with MenSCs or MSC-EVs, and the degree of lung injury and the alveolar epithelial barrier of the lung tissue were assessed.

Results We found that MenSCs reduced lung injury and restored alveolar-barrier integrity in lung tissue. In vitro, MenSCs reduced paracellular permeability and restored barrier integrity in human lung epithelial cells. MenSC-EVs replicated all these MenSC-mediated changes. Mechanistic research revealed that MenSCs inhibited MAPK signaling and necroptosis. JNK inhibition SP600125, and ERK inhibition U0126 or inhibition of necroptosis with Nec-1 or GSK872 diminished the beneficial anti-epithelial barrier dysfunction effects of MenSCs or MenSC-EVs.

Conclusions Our results suggest that human menstrual blood-derived endometrial stem cells mitigate lung injury and improve alveolar barrier properties by inhibiting MAPK-mediated necroptosis through extracellular vesicles, supporting the application of MenSCs or MenSC-derived extracellular vesicles to treat ALI or ARDS.

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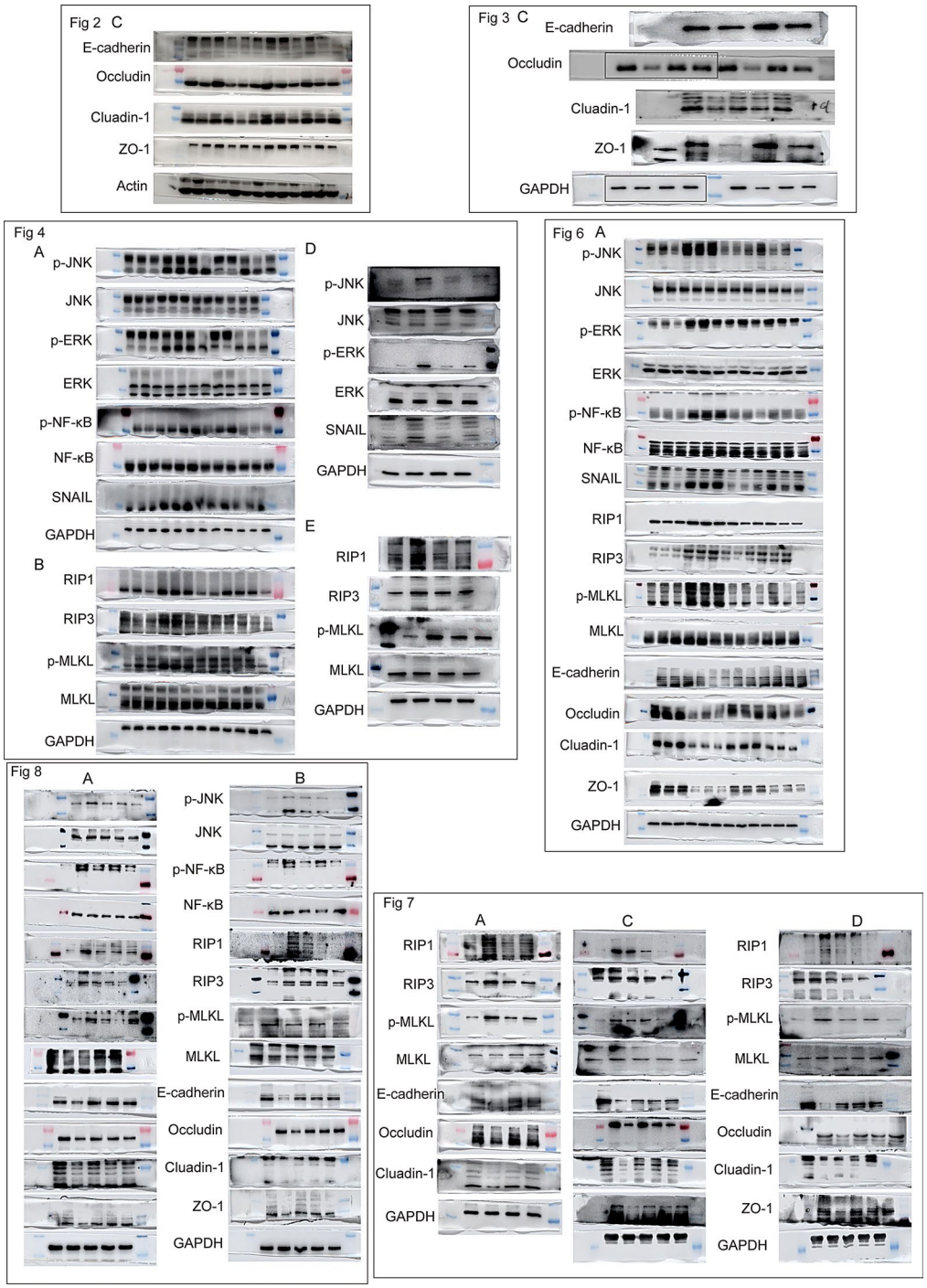
Graphical Abstract



Keywords Acute lung injury, Mesenchymal stem cells, Alveolar epithelial barrier, Tight junctions, Necroptosis

Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are a disease caused by various external pathogenic factors such as severe infection or trauma, and is characterized by decreased lung volume, decreased compliance, and an imbalance of in the ventilation/blood flow ratio [1, 2]. It is clinically characterized by progressive hypoxemia and respiratory distress, and pulmonary

imaging reveals heterogeneous exudative lesions. ALI/ARDS has a high clinical incidence rate, rapid progression and high mortality [3]. The pathogenesis of ALI/ARDS has not been fully elucidated, and although some progress has been made in airway management and protective mechanical ventilation strategies for ALI/ARDS treatment in recent years, effective drug treatment methods are still lacking. The exploration of key drug treatment targets in the pathogenesis of ALI/ARDS remains a hot research topic in respiratory and critical care medicine.

The airway and alveolar epithelial barrier are composed of tight junctions (TJs) between airway and alveolar epithelial cells and endothelial cells [4]. Tight junction related proteins, which are composed of peripheral cytoplasmic proteins such as membrane proteins and closed loop proteins, are important components involved in maintaining tight junction structure and function [4, 5]. Tight junction proteins, important components of the mechanical barrier between epithelial and endothelial cells, prevent solutes and water from freely passing through the intercellular spaces between epithelial or endothelial cells, playing crucial roles in maintaining the homeostasis of the intracellular environment [6–9]. In recent years, research has revealed that various lung diseases, including infection, pulmonary edema, fibrosis, and tumors, are closely related to abnormal expression of tight junction proteins and structural destruction of tight junction complexes.

Necroptosis was recently identified as a novel mechanism contributing to the inflammatory cascade response to ALI [10, 11]. Necroptosis occurs through the activity of the necroptosome, a complex of two essential proteins: receptor-interacting serine/threonine-protein kinase 3 (RIPK3) and mixed lineage kinase domain-like pseudokinase (MLKL) [12]. RIPK3 phosphorylates and recruits MLKL to the necroptosome. Then phosphorylated MLKL oligomerizes and translocates to the plasma membrane to create pores for osmotic rupture [13]. Necroptosis results in disruption of the cellular membrane and the release of components, including DAMPs and inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), which can cause self-amplified inflammation [14]. In macrophages, the human respiratory syncytial virus (RSV) induces necroptosis of macrophages, aggravating pneumonia [15]. Ripk3−/− mice are protected from RSV-induced weight loss and present with reduced viral loads in the lungs [11]. Nonetheless, the molecular mechanism that triggers necroptosis during ALI is not fully understood.

Mesenchymal stem cells (MSCs) are multipotent progenitors with differentiation capacities, that can be isolated from different tissues, such as adipose, umbilical cord, and bone marrow [16, 17]. MSCs can be induced to differentiate into fat, bone, cartilage and other types of cells in vitro. To date, MSCs have shown good application prospects in the treatment of various diseases due to their ability to secrete cytokines and mediate immune regulation [18]. Because of their advantages, such as noninvasive sample collection, multiple collections from the same donor, access to a large number of seed cells with the same genetic background, and autotransplantation, menstrual blood derived endometrial stem cells (MenSCs) have gradually become important cell sources for stem cell therapy [19]. However, whether MenSCs can play a critical role in alveolar epithelial barrier maintenance is still unknown. The mechanism by which MenSCs alleviate ALI is not completely understood. An increasing number of studies suggest that the therapeutic effects of MSCs in other diseases are related to their paracrine action [20, 21]. EVs secreted by MSCs contain multiple bioactive molecules, which could mediate the therapeutic effects of MSCs [20]. In this study, we explored the contribution of necroptosis to alveolar epithelial barrier injury in ALI and the ability of MenSCs or MenSC-derived EVs to modulate alveolar-barrier functions.

Materials and methods

Reagents

Lipopolysaccharide (LPS) was purchased from Sigma (St. Louis, MO). FITC-conjugated dextran (MW 4000) was purchased from Sigma (St. Louis, MO) and dissolved in PBS. Antibodies against ZO-1 (13663 S), JNK (9252 S), phospho-JNK (Thr183/Tyr185) (4668 S), ERK (4695 S), phospho-ERK (Thr202/Tyr204) (4370 S), RIP (3493 S), RIP3 (13526 S), MLKL (14993 S), phospho-MLKL (Ser358) (91689 S), NF-κB p65 (8242 S), phospho-NF-κB p65 (Ser536) (3033 S) were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against E-cadherin (sc-8426), Occludin (sc-133256), Claudin-1 (sc-166338), SNAI1 (sc-393172), GAPDH (sc-47724) and Actin(sc-8432) were obtained from Santa Cruz Biotechnology Inc. (San Diego, CA). Alexa Fluor® 488-conjugated anti-rabbit IgG and Alexa Fluor® 488-conjugated anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA).

Cell culture

A549, and BEAS-2B cells were purchased from the American Type Culture Collection (Manassas, VA). The cells were maintained in complete Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin–streptomycin (Corning, USA) at 37 °C in a humidified 5% CO_2 incubator.

MenSC culture and MenSC-EV isolation

MenSCs were obtained from healthy young female individuals (aged 25–35 years) and provided by the Zhongyuan Stem Cell Research Institute of the Xinxiang High Tech Zone. The third to fifth passages of MenSCs were cultured for subsequent experiments. MenSCs were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin–streptomycin (Corning) at 37 °C in a humidified 5% CO_2 incubator. The culture medium was changed every 3 days until the cells reached 80% confluency. MenSC-EVs were isolated from MenSC-conditioned medium (CM) by ultracentrifugation according to Lian et al. [22], and characterized according to the International Society for Extracellular Vesicles guidelines [23]. The isolated MenSC-EVs were characterized by TEM (HT-7700, Hitachi, Japan) and nanoparticle flow cytometry (N30E, NanoFCM, China), and surface markers of EVs, including CD9 (20597-1-AP, Proteintech, China), CD63 (25682-1-AP, Proteintech, China), CD81 (27855-1-AP, Proteintech, China), and Alix (2171 S, Cell Signaling Technology, USA), were identified by western blot, as shown Supplementary Material.

Animals

C57BL/6 male mice (6–8 weeks of age) were provided by Vital River Laboratories [Beijing, China; license no. SCXK (Beijing) 2012-0001] and maintained in SPF facilities at Xinxiang Medical University. The use of animals was approved by the Institutional Animal Care and Use Committee of Xinxiang Medical University and was in accordance with the Guide for the Care and Use of Laboratory Animals by the National Research Council. A total of 40 male C57BL/6 mice were randomly distributed into four groups. Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital administration (50 mg/kg body weight) and then the ALI model was induced by intratracheal inhalation of 5 mg/kg LPS (Sigma, Beijing, China), after 4 h, followed by intratracheal administration of 50 μL of MenSCs (1×10^6 cells) (Fig. 1A). For MenSC-derived extracellular Vesicles (MenSC-EVs) 200 μg was administered via intratracheal administration. The mice in the PBS treatment group received an equal amount of PBS. At the end of the experiment (Day 4), the mice were euthanized by an intraperitoneal injection of (100 mg/kg body weight) pentobarbital, and their sera, and lungs were harvested for further examination. All procedures were performed in agreement with the ARRIVE guidelines 2.0.

Histopathology

Lung tissue Sect. (5 mm) were stained with hematoxylin and eosin (H&E). Pathologic lesions were graded using a semiquantitative histology scoring system in a blinded fashion as previously described [4, 24]. The severity of lesions was graded on a scale of 0 to 4 (0 for none or

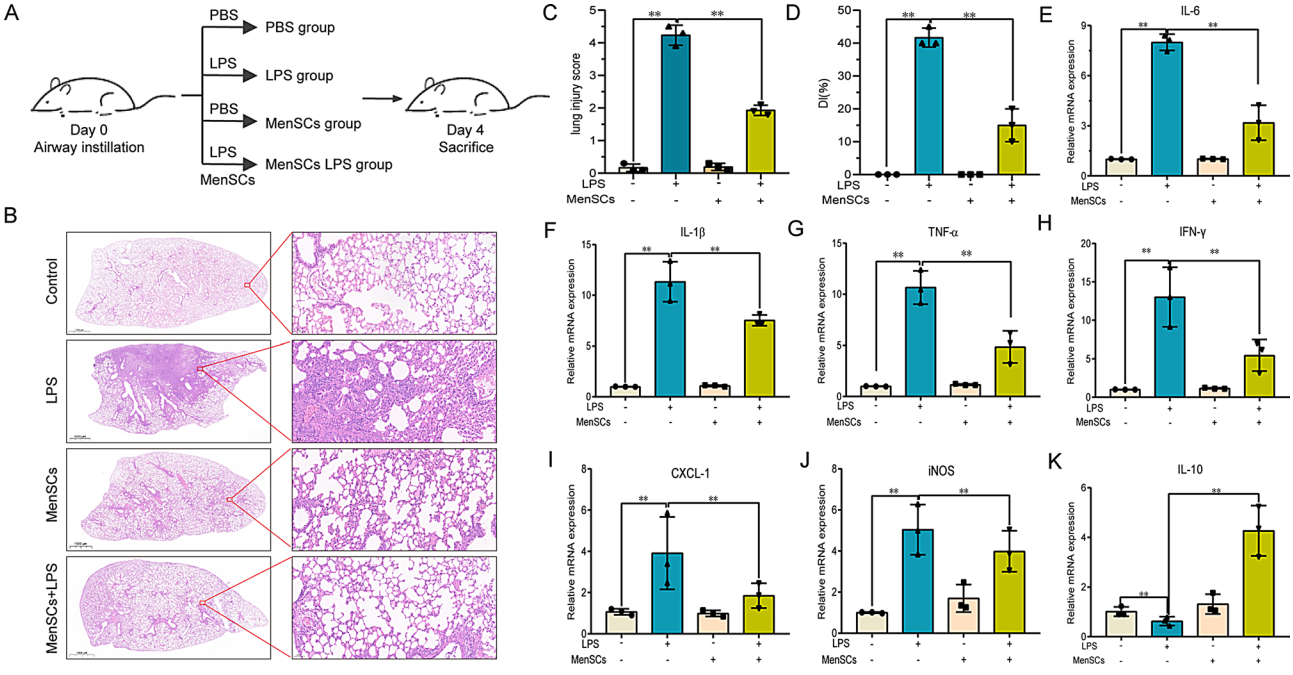


Fig. 1 Intratracheal MenSC transplantation alleviates LPS-induced ALI in vivo. (**A**) Schematic representation of the mouse model established in this study. PBS, LPS (4 mg/kg), and MenSCs (1×10^6 cells) were intratracheally administered to the mice. (**B**) Hematoxylin and eosin (H&E)-stained images of the lungs. (**C**) Lung injury score. (**D**) Destructive index (DI). (**E**-**K**) Relative mRNA expression of proinflammatory factors in the lungs of mice that were administered MenSCs in the presence of LPS (4 mg/kg) was detected via qRT–PCR. PBS was used as a negative control

very minor, 1 for mild, 2 for intermediate, 3 for moderately severe, and 4 for severe and widespread) based on the infiltration of immune cells such as macrophages and neutrophils. The numbers of infiltrates in 5 randomly selected fields (40×) in different areas of the lung (top, bottom, left, right, and central regions) were counted. The infiltration was scored as follows: 1, 0–10 cells/field; 2, 11–30 cells/field; 3, 31–60 cells/field; and 4, > 61 cells/field. The mean scores of the control and MenSC or MenSC-EV-treated groups (10 mice per group) were calculated, statistically analyzed using an unpaired Student's t test and plotted in a bar graph.

Real-time quantitative PCR

Total RNA was extracted from human alveolar epithelial cells and lung tissues using an RNAeasy™ Kit (R0026; Beyotime Biotechnology). cDNA was synthesized from 500 ng of total RNA with the HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, R211–01). Quantitative real-time PCR (qPCR) for E-cadherin, Occludin, Claudin-1, ZO-1, GAPDH and inflammatory cytokines was performed with AceQ qPCR SYBR Green Master Mix (Vazyme, Q111-02) on a Real-Time System (Bio-Rad, USA) according to the manufacturer's instructions. Primers for E-cadherin, Occludin, Claudin-1, ZO-1, GAPDH and inflammatory cytokines were obtained from GENEWIZ (Suzhou, China). The relative amount of each gene was normalized to that of GAPDH. The fold change was calculated by the ΔΔCT method. The percent expression was calculated as the ratio of the normalized value of each sample to that of the corresponding untreated control sample. All real-time RT–PCR analyses were performed in triplicate. The primer sequences used in the qPCR analysis are shown in Supplementary Material Tables S1 and S1.

Western blot analysis

The cells were grown in 6-well plates, then harvested and lysed in RIPA lysis buffer (P0013B, Beyotime). After being incubated on ice for 20 min, the cell lysates were centrifuged at 4 °C, and 15,000 rpm for 15 min. Lung tissues were lysed and homogenized in RIPA lysis buffer containing phosphatase and protease inhibitors (P0013B, Beyotime) and then centrifuged at 15,000 rpm for 15 min at 4 °C. Proteins were separated via SDS–PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore), blocked in NcmBlot blocking buffer (NCM Biotech) for 10 min, and incubated overnight with the appropriate primary antibody at 4 °C. The primary antibodies included E-cadherin, Occludin, ZO-1, JNK, Phospho-JNK (Thr183/Tyr185), ERK, Phospho-ERK (Thr202/Tyr204), RIP, RIP3, MLKL, Phospho-MLKL (Ser358), NF-κB p65, Phospho-NF-κB p65 (Ser536), SNAI1, GAPDH and Actin. After being washed three times with Tris-buffered saline containing Tween-20, the membranes were incubated with an appropriate HRP-conjugated secondary antibody at room temperature for 1 h and with SuperSignal Western Pico enhanced chemiluminescence substrate (Pierce Chemical Co., Rockford, IL). The density of the bands was analyzed using ImageJ software (NIH, Bethesda, MD, USA) (https://imagej.nih.gov/ij/) and normalized to the corresponding total protein or β-actin as indicated. The quantified results are presented as the means ± standard deviation (SD) from three experiments in bar graphs.

Immunofluorescence staining

A549 cells were seeded on coverslips in 24-well flat-bottom plates and treated with LPS for 24 h. The cells were fixed with 100% methanol at -20°C for 20 min. The cells were permeabilized with 0.25% Triton X-100 in PBS for 20 min and washed three times with PBS. Lung tissues were sectioned into 4-μm-thick sections, deparaffinized with xylene, and then dehydrated with an ethanol gradient. After thermal repair of the antigens, the samples were incubated with 5% bovine serum albumin (BSA) in PBS for 30 min at room temperature, and then with anti-Ecadherin, Occludin, Claudin-1, and anti-ZO-1 antibodies (1:100) in PBS overnight at 4°C. After incubation, the cells were washed in blocking buffer and stained with Alexa488-conjugated anti-mouse and anti-rabbit IgG (1:100), for 1 h at room temperature. Finally, the cells were stained with 10 μM DAPI (4',6-diamidino-2-phenylindole) for 5 min. After the cells were washed in PBS, fluorescence images were captured under a Nikon fluorescence microscope.

Statistical analysis

The differences in the mRNA levels and Western blot band densities in the cells exposed to LPS in the absence or presence of various treatments were statistically analyzed using unpaired Student's t tests. The data are presented as the means ± SD of three independent experiments. A p value of < 0.05 was considered statistically significant. All the statistical analyses were performed with GraphPad Prism (GraphPad software 8.0; (https://www.graphpad.com/scientific-software/prism).

Results

Intratracheal transplantation of menscs alleviates LPS-induced ALI

MSCs have promising application prospects in the treatment of various diseases through secretion of cytokines and immune regulatory effects. To define the regulatory role of MenSCs in pulmonary inflammatory injury, the mice were divided into four groups: PBS, LPS, MenSCs and LPS/MenSCs (Fig. 1A). After MenSC or PBS administration, hematoxylin and eosin (H&E) revealed showed extensive morphological damage, such as hemorrhaging,

congestion, thickening of the alveolar walls, interstitial infiltration, and infiltration of inflammatory cells, especially neutrophils, in LPS-exposed lungs; in contrast, assessment of lung pathology revealed markedly more intact alveolar walls and decreased inflammation after MenSC treatment (Fig. 1B). Additionally, LPS-induced the lung-injury score, alveolar wall destruction was greatly counteracted by MenSCs administration (Fig. 1C, D). Moreover, we further measured the expression of inflammatory factors in the lungs of the mice and found that the expression of inflammatory factors, including IL-6, IL-1β, TNF-a, IFN-γ, CXCL1, iNOS, and was notably attenuated, and that the anti-inflammatory cytokine IL-10 was increased by MenSC treatment (Fig. 1E-K). These results indicat that MenSC inhibited cytokine and chemokine expression and affected lung inflammation and injury.

MenSCs ameliorate alveolar epithelial barrier dysfunction in ALI

Tight junctions are the most important connections between alveolar epithelial cells. Pathogens or exogenous substances destroy tight junctions, increase the permeability of epithelial cells and endothelial cells, impair lung barrier function, and ultimately lead to lung injury. We next investigated the role of MenSCs in the repair of the alveolar epithelial barrier. The concentration of FITC-dextran in the serum was quantified to determine alveolar epithelial barrier permeability after LPS administration. The results demonstrated that permeability was significantly greater in the LPS group than in the LPS/MenSC group (Fig. 2A). MenSCs effectively restored the E-cadherin, Occludin, Claudin-1, and ZO-1 mRNA levels in ALI mice (Fig. 2B). Similarly, the Western blot results showed significantly greater protein expression of E-cadherin, Occludin, Claudin-1, and ZO-1 in the LPS/MenSC mice than in the LPS mice (Fig. 2C). Immunofluorescence staining revealed that the expression of the tight junction-related proteins Occludin and Claudin-1 was significantly decreased in the LPS group compared with the that in control group. MenSCs partially restored the expression of Occludin and Claudin-1 in lung tissue of ALI/ MenSC mice (Fig. 2D). IF intensity analysis revealed that LPS significantly decreased the Occludin and Claudin-1 signals located in the intercellular junctions, which was largely reversed by MenSCs (Fig. 2E, F). These results indicate that MenSCs can repair the alveolar epithelial barrier by increasing the expression of tight junction proteins in lung tissue.

MenSCs alleviate LPS-induced structural damage to tight junctions

Using transwell inserts, a non-contact coculture system was established in vitro between MenSCs and A549 cells. The concentration of FITC-dextran in the culture supernatant was quantified to determine alveolar epithelial barrier permeability after LPS administration. The results demonstrated that permeability was significantly greater in the LPS group than in the LPS/MenSCs group (Fig. 3A). The mRNA and protein levels of tight junction genes were evaluated by qPCR and Western blot. As shown in (Fig. 3B, C), the qPCR and Western blot results revealed significantly greater protein expression of E-cadherin, ZO-1, Occludin, and Claudin-1 in the LPS/MenSC group than in the LPS group. Immunofluorescence staining revealed that MenSCs restored E-cadherin, Occludin, Claudin-1, and ZO-1 expression on the cell membrane of LPS-stimulated A549 cells (Fig. 3D). These results indicate that MenSCs increased the expression of tight junction proteins in alveolar epithelial cells downregulated by LPS, restored alveolar epithelial barrier function.

MenSCs inhibit MAPK signaling and necroptosis in LPS-induced ALI

Accumulating evidence suggests that the MAPK signaling pathway mainly includes p38, ERK and JNK, which jointly participate in regulating tight junction barrier function [25]. Here, we first determined whether the MAP kinase pathway is involved in regulating necroptosis-induced expression of epithelial junction proteins. In ALI mice, the phosphorylation of JNK, ERK and NF-KB p65 was greater than that in ALI/ MenSC mice, and was decreased by MenSC injection (Fig. 4A). Moreover, the levels of RIP1, and RIP3 and the level of phosphorylated MLKL were significantly higher in ALI mice than in ALI/ MenSC mice, but were obviously decreased by MenSC intervention (Fig. 4B). In addition, immunofluorescence staining of RIP1, pMLKL revealed that MenSC injection decreased the proportion of RIP1, pMLKL positive lung, which indicated that MenSCs inhibited the level of necroptosis in the lung (Fig. 4C). To further characterize the direct cell-autonomous effect of MenSCs on the lung, we used A549 cells, a human lung adenocarcinoma alveolar epithelial cell line. A549 cells were treated with 50 μg/mL LPS to induce alveolar epithelial cell dysfunction. The Western blot results revealed that LPS treatment robustly promoted the expression of phosphorylated of JNK, ERK and SNAIL, whereas MenSC treatment abrogated the LPS-induced increase in the phosphorylation of JNK, ERK and SNAIL expression (Fig. 4D). Moreover, LPS increased the expression of RIP1, and RIP3 and the phosphorylation of MLKL. MenSC treatment significantly decreased the expression of RIP1, and RIP3 and the phosphorylation of MLKL (Fig. 4E). These results suggest that MenSCs inhibit MAPK signaling and necroptosis in LPS-induced ALI.

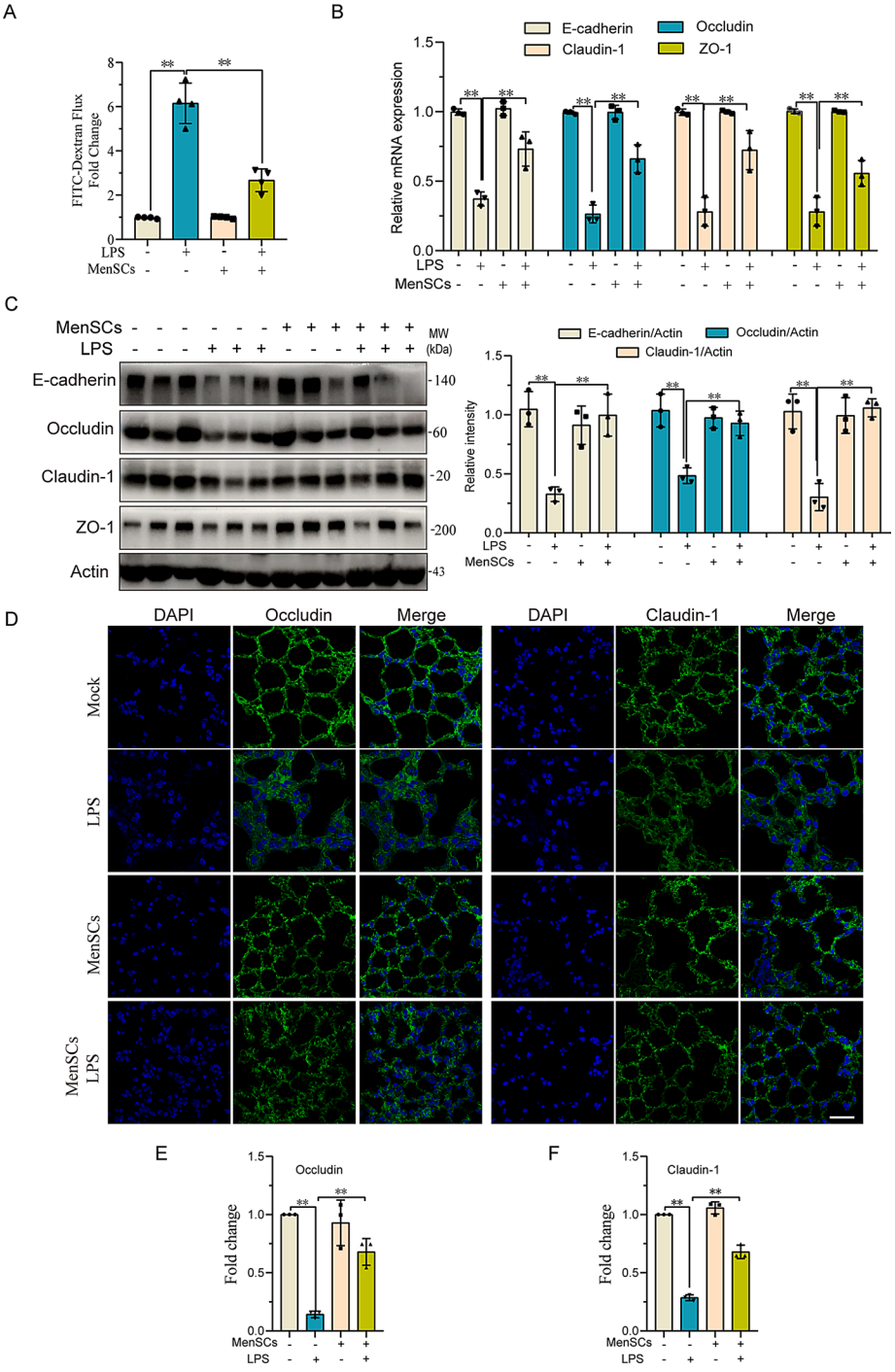


Fig. 2 MenSCs ameliorate alveolar epithelial barrier dysfunction in ALI. (**A**) LPS-induced increases in lung permeability were markedly attenuated after MenSC treatment. (**B**) Relative mRNA expression of tight junction proteins in the lungs of mice administered MenSCs in the presence of LPS was detected via qRT–PCR. (**C**) Western blot analysis of the expression levels of the tight junction markers E-cadherin, Occludin, and Claudin-1 in lung tissue. (**D**) MenSCs ameliorate the LPS induced deterioration of the intercellular junction structure. Lung tissue were immunostained with antibodies against occludin and claudin-1 and visualized under a confocal microscope. (**E**, **F**) Quantiication of fluorescence signals. The monolayers immunostained for occludin and claudin-1 in D were analyzed for immunofluorescence intensity by using Image J software

MenSC-EVs alleviate LPS-induced ALI and inhibit the MAPK/necroptosis signaling

Accumulating evidence suggests that MSC therapy is effective due to its paracrine action rather than its differentiation mechanism [21, 26]. As important paracrine factors, extracellular vesicles contain exosomes and microvesicles, are released by all cell types and are considered promising therapies [27]. Therefore, we

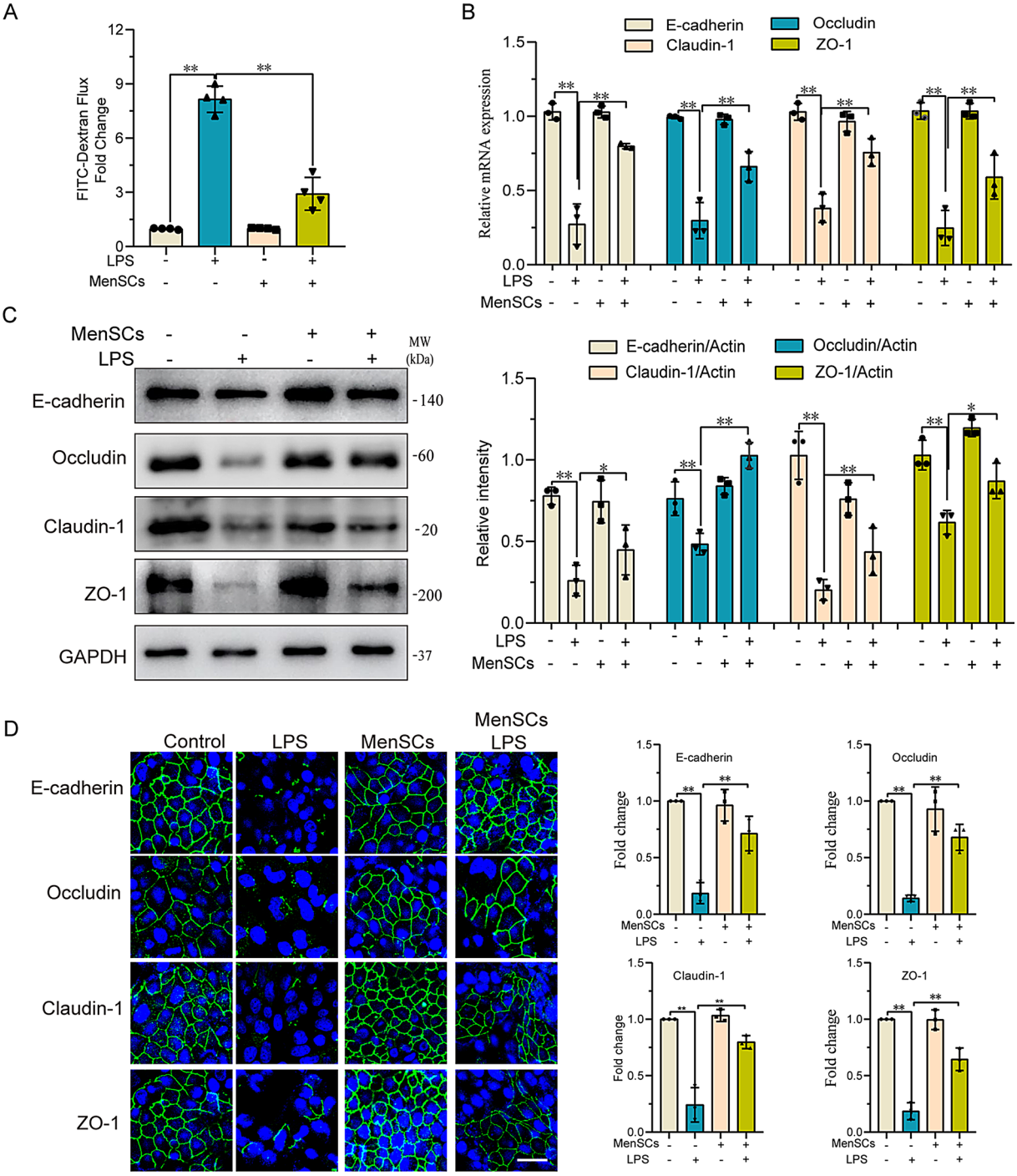


Fig. 3 MenSCs alleviate LPS-induced alveolar epithelial cell dysfunction in vitro. A non-contact co-culture system was established in vitro between MenSCs and A549 cells. (**A**) Alveolar epithelial cell permeability was determined in the FD4 supernatant level after MenSC treatment. (**B**) Relative mRNA expression of tight junction proteins in A549 cells treated with MenSCs in the presence of LPS was detected via qRT–PCR. (**C**) Western blot analysis of the expression levels of the tight junction markers E-cadherin, Occludin, and Claudin-1 in A549 cells. (**D**) The localization of E-cadherin, Occludin, Claudin-1, and ZO-1 in A549 cells was determined by immunofluorescence staining. Scale bar = 100 μm

tested the effects of MenSC-derived extracellular vesicles (MenSC-EVs) on ALI mice. MenSC-EVs (200 μg dissolved in 200 μL of PBS) was administered via intratracheal administration for 4 days; 200 μL per mouse PBS served as the control (Fig. 5A). Hematoxylin and eosin (H&E) staining showed striking histological improvements in LPS-triggered pulmonary inflammatory injury after MenSC-EV administration, as shown by

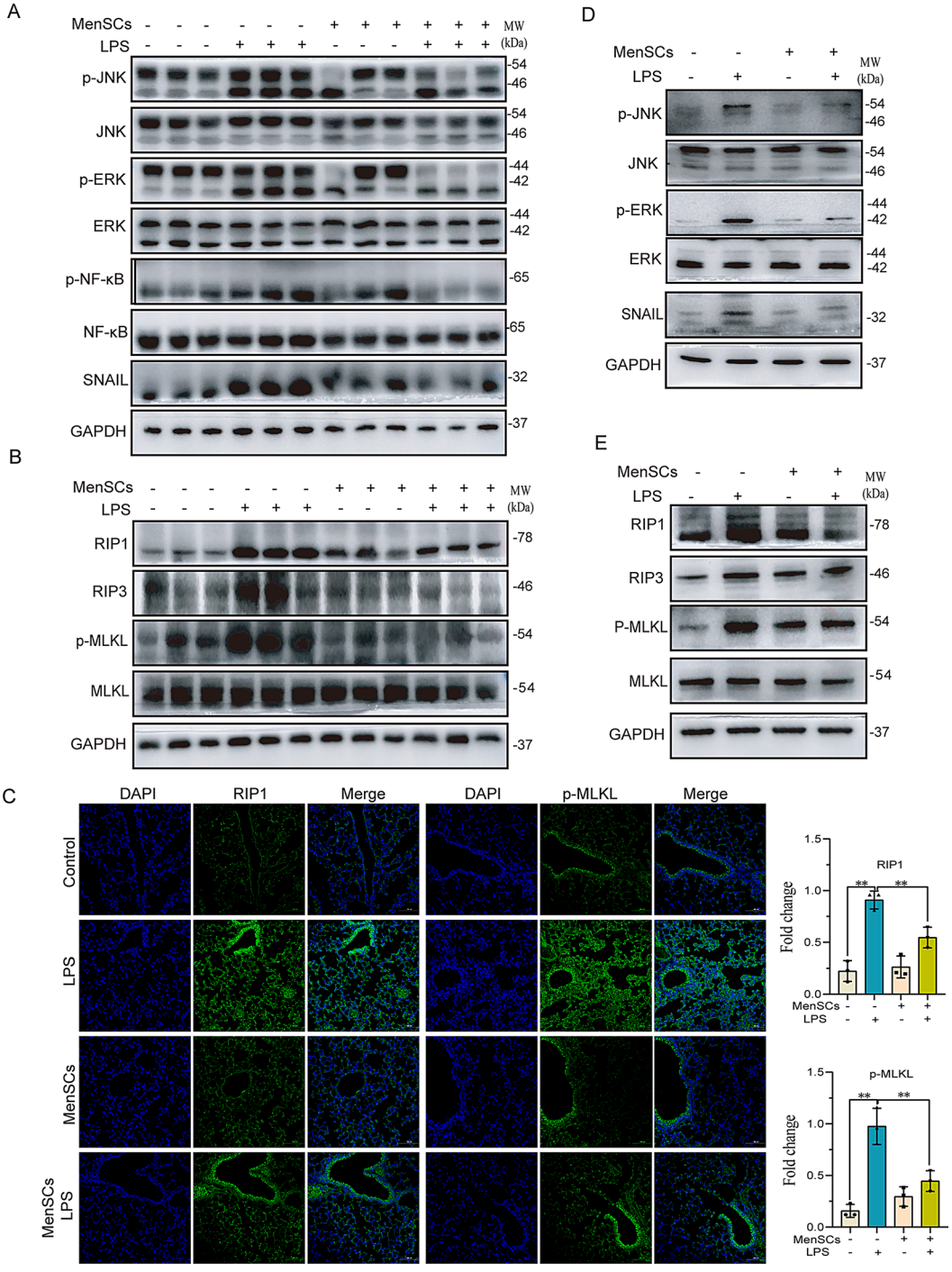


Fig. 4 MenSCs inhibit MAPK signaling and necroptosis in LPS-induced ALI. (**A**,**B**) Western blot analysis of the phosphorylation of JNK, ERK, NF-κB p65, RIP1, and RIP3 and the phosphorylation of MLKL in the lung tissue of ALI mice. (**C**) The localization of RIP1, pMLKL in the mouse lung was determined on the last day of the experiment via immunofluorescence staining. Scale bar = 100 μm. (**D**,**E**) Western blot analysis of the phosphorylation of JNK, ERK, RIP1, and RIP3 and the phosphorylation of MLKL in A549 cells treated with LPS and MenSCs

alleviated alveolar congestion and thickening, alveolar septa, and edema (Fig. 5B). Additionally, LPS-induced the lung-injury score, alveolar wall destruction were greatly counteracted by MenSC-EV administration (Fig. 5C, D). Moreover, the qPCR results revealed that MenSC-EVs suppressed the expression of inflammatory factors CXCL1, IL-1β, IFN-γ, TNF-a, CXCL2, iNOS, and increased the expression of anti-inflammatory cytokine IL-10 (Fig. 5E-K).

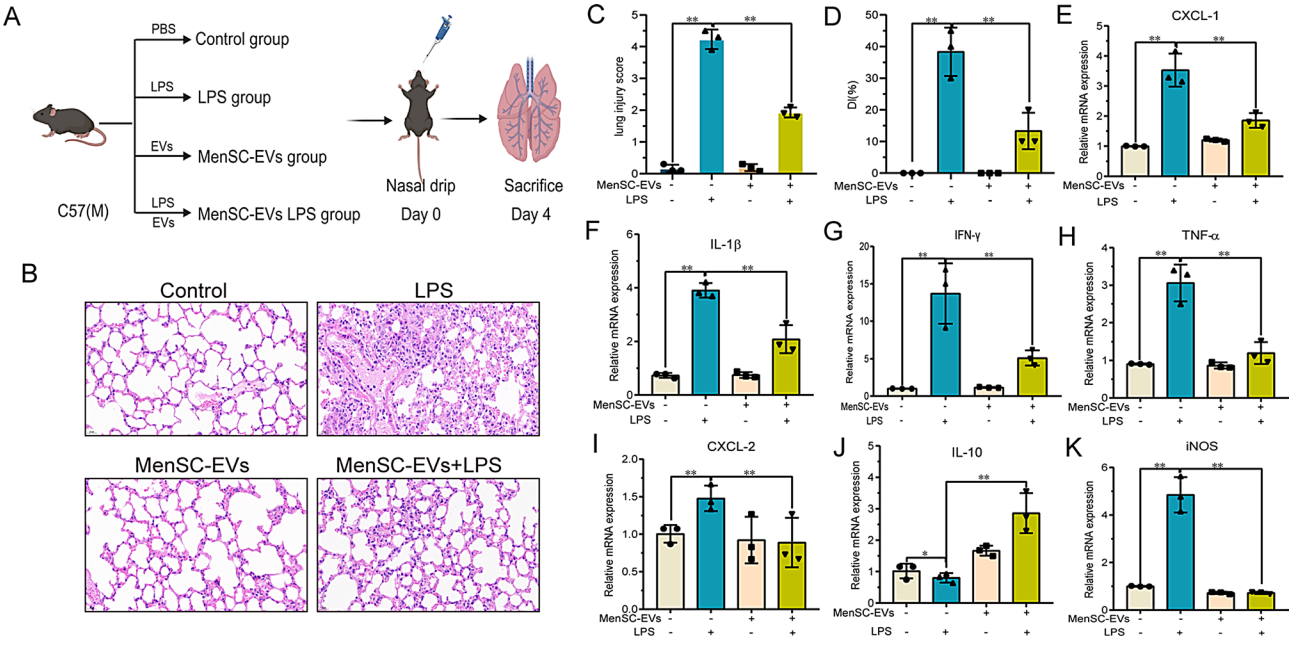


Fig. 5 MenSC-EVs alleviate LPS-induced ALI and inhibit the MAPK/necroptosis signaling. (**A**) Schematic representation of the mouse model established in this study. PBS, LPS (4 mg/kg), and MenSC-EV (200 μg) were intratracheally administered to the mice. (**B**) Hematoxylin and eosin (H&E)-stained images of lungs. (**C**) Lung injury score. (**D**) Destructive index (DI). (**E**-**K**) Relative mRNA expression of inflammatory factors in the lungs of mice administered MenSC-EVs in the presence of LPS (4 mg/kg) was detected via qRT–PCR

We next further explored the role of MenSC-EVs in repairing the alveolar epithelial barrier. The results demonstrated that MenSC-EVs suppressed the phosphorylation of JNK, ERK and NF-κB p65, and reduced the levels of SNAIL, RIP1, and RIP3 and decreased the level of p-MLKL; these effects significantly restored the protein levels of E-cadherin, Occludin, Claudin-1, and ZO-1 (Fig. 6A). Immunofluorescence staining revealed that MenSC-EVs increased the expression of the tight junction-related proteins E-cadherin, Occludin, Claudin-1, and ZO-1 (Fig. 6B). These results suggest that MenSC-EV injection protects against LPS-induced ALI, which is consistent with the roles of MenSCs.

MenSC-EVs alleviate LPS-induced alveolar epithelial cell dysfunction by inhibiting MAPK-mediated necroptosis

In A549 cells stimulated with or without LPS, we treated A549 cells with 25 μg/mL MenSC-EVs for 24 h. The Western blot results revealed that EV treatment significantly decreased the expression of RIP1, and RIP3 and the phosphorylation of MLKL, and increased the expression of the tight junction-related proteins E-cadherin, Occludin, and Claudin-1 (Fig. 7A). Immunofluorescence staining revealed that MenSC-EVs increased the expression of the tight junction-related proteins Occludin, and ZO-1 (Fig. 7B). The qPCR results revealed that MenSC-EVs suppressed the expression of inflammatory factors IL-6, TNF-a, CXCL1, and iNOS in virto study (Supplementary Material Fig. S2). To further address the cell-autonomous mechanism of extracellular vesicles, we treated A549 cells with 25 μg/mL MenSC-EVs or PBS as the control for 24 h in the presence or absence of Nec-1 or GSK872 targeting RIP1 or RIP3. The dosage of MenSC-EVs was based on previous literature, which demonstrated that this dosage did not affect cell viability. Nec-1 or GSK872 inhibited LPS-induced RIP1 or RIP3, and phosphorylation of MLKL upregulation, and inhibited LPS-induced tight junction-related proteins E-cadherin, Occludin, Claudin-1, and ZO-1 downregulation (Fig. 7C, D).

The JNK inhibitor SP600125 suppressed the phosphorylation of JNK, and mimicked the ability of Nec-1 to diminish MenSC-EV effects on p-JNK/p-ERK, RIP1, and RIP3 and the phosphorylation of MLKL, and tight junction-related proteins (Fig. 8A). Similarly, ERK inhibitor U0126 replicated all these SP600125-mediated changes(Fig. 8B). Similar observations were made with BEAS-2B cells, a normal human lung epithelial cell line that has been widely used for studying ALI (Supplementary Material Fig.S3). These results indicate that MenSC-EVs mitigate alveolar epithelial cell dysfunction by inhibiting MAPK-mediated necroptosis(Fig. 8C).

Discussion

ALI causes immune dysfunction, promotes the release of proinflammatory factors, increases the number of white blood cells, and can progress to ARDS. Although treatment methods are constantly improving, the mortality

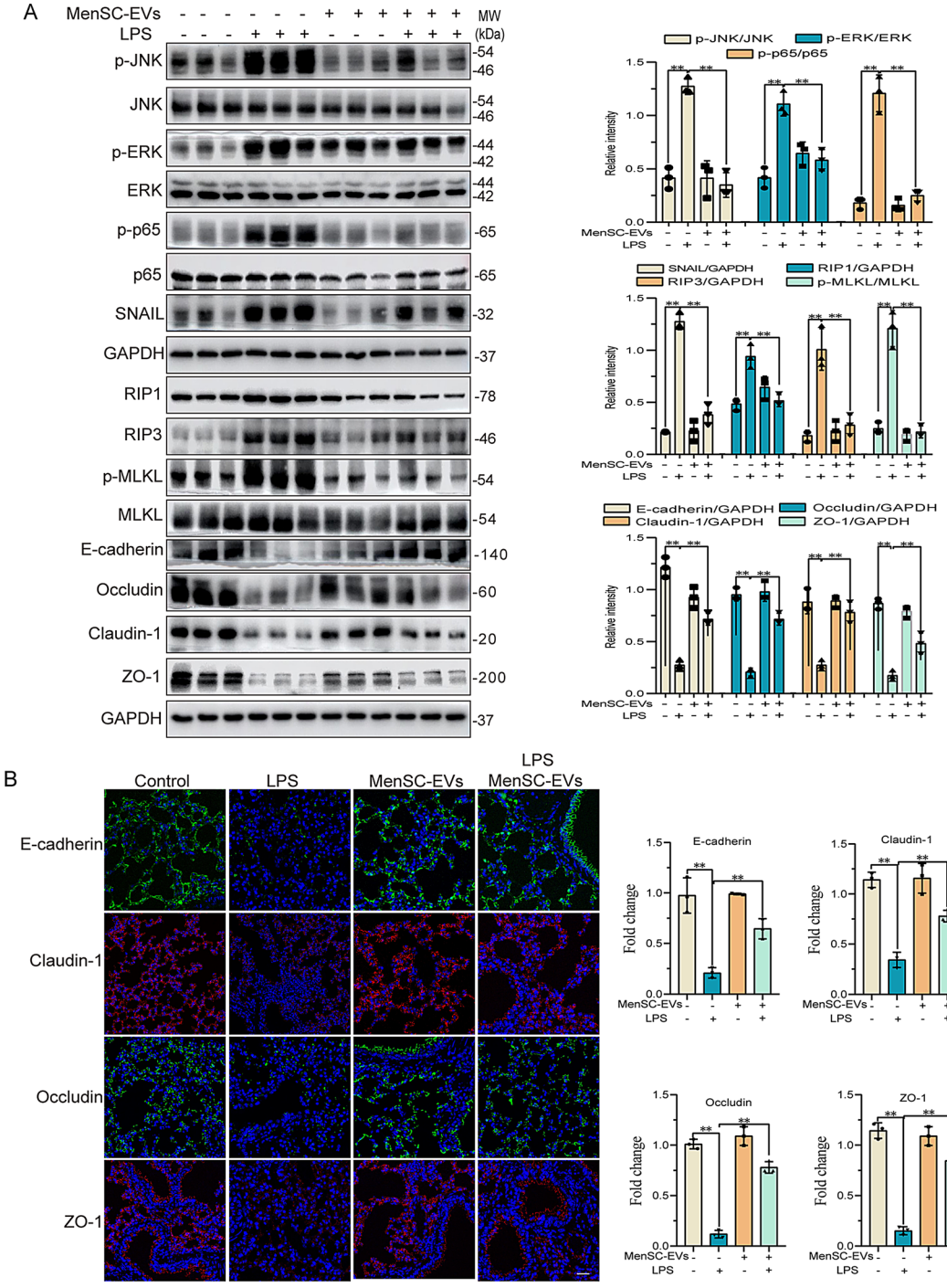


Fig. 6 MenSC-EVs ameliorate alveolar epithelial barrier dysfunction in ALI. (**A**) Western blot analysis of the phosphorylation of JNK, ERK NF-KB p65, MLKL, RIP1, RIP3 and the tight junction markers E-cadherin, Occludin, Claudin-1, and ZO-1 in the lung tissue of ALI mice treated with LPS and MenSC-EVs. (**B**) Immunofluorescence staining analysis of the localization of E-cadherin, Occludin, Claudin-1, and ZO-1 in the mouse lung

rate is still high. In animal models and human patients with acute lung inflammation and injury, MSC administration has been shown to have the potential to become an effective therapeutic strategy for treating ALI [28–32]. However, a complete understanding of the mechanisms of the therapeutic potential of MSCs in acute lung inflammation and injury is lacking. MSCs have potential as a therapeutic approach for pneumonia because of their immunosuppressive activity. The lower inflammatory response and longer survival rates observed in ALI patients treated with MSCs support this conclusion. Our study provides first-hand evidence of the potential

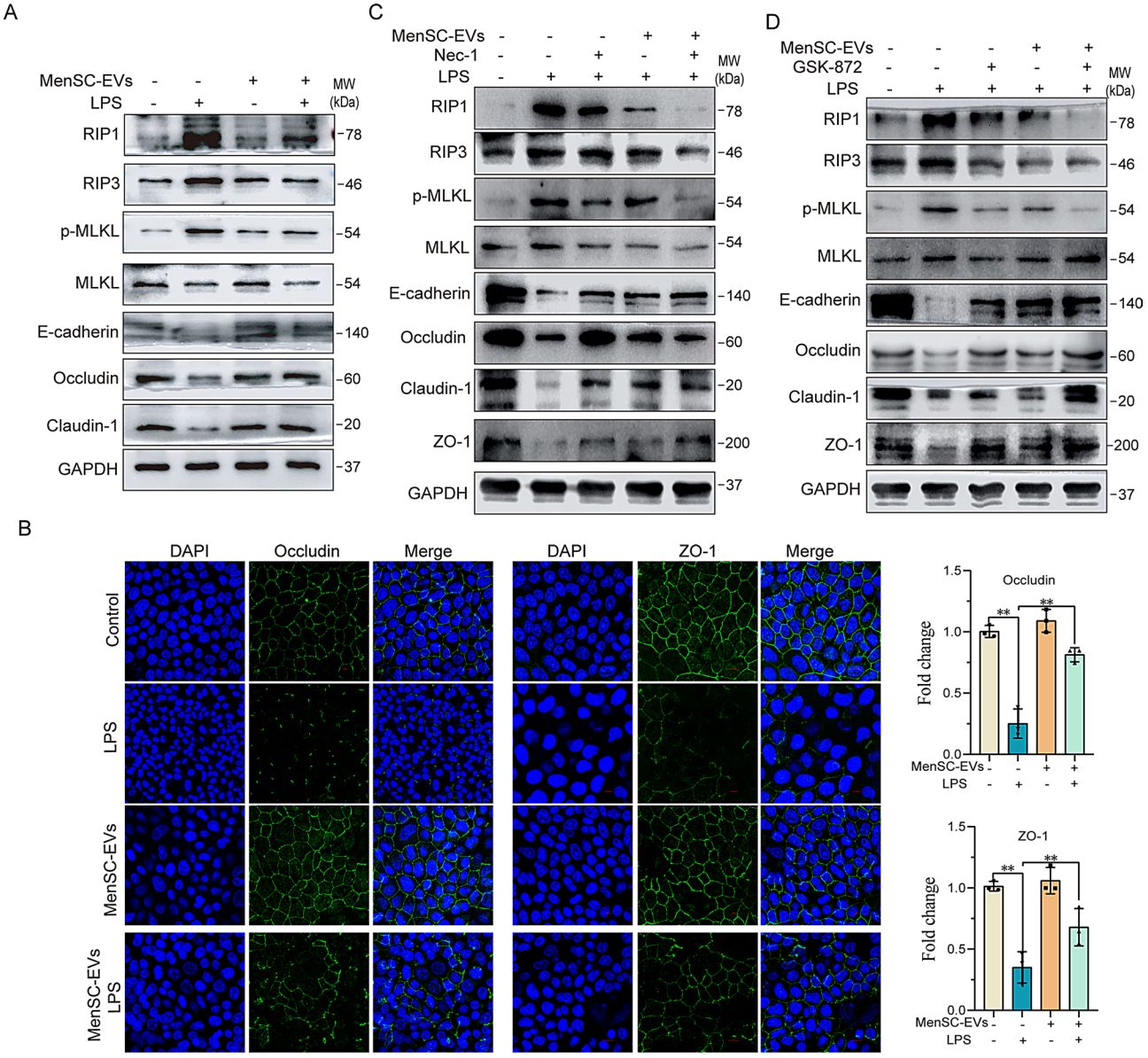


Fig. 7 MenSC-EVs alleviate LPS-induced alveolar epithelial cell dysfunction by inhibiting necroptosis. (**A**) The expression of p-MLKL, RIP1, RIP3, E-cadherin, Claudin-1, Occludin and ZO-1 was detected via Western blotting. (**B**) Immunofluorescence staining analysis of the localization of Occludin, and ZO-1 in A549 cell. (**C**) Western blot analysis of p-MLKL, RIP1, RIP3 and the tight junction markers E-cadherin, Occludin, Claudin-1, and ZO-1 in A549 cells treated with MenSC-EVs and the necroptosis inhibitor Nec-1. (**D**) Western blot analysis of p-MLKL, RIP1, RIP3 and the tight junction markers E-cadherin, Occludin, Claudin-1, and ZO-1 in A549 cells treated with MenSC-EVs and the necroptosis inhibitor GSK872

molecular mechanism by which MenSC-EVs improve acute lung injury and alveolar barrier properties by inhibiting MAPK-mediated necroptosis.

Accumulating literatures have reported that MSCs have the potential to relieve the pathology of ALI. Gupta et al. [33] demonstrated that intravenous infusion of bone marrow-derived MSCs significantly improved lung function and reduced inflammation in a rat model of ALI induced by lipopolysaccharide (LPS). Zhu et al. [34] found that intratracheal administration of human umbilical cord-mesenchymal stem cells (UC-MSCs) significantly improved survival and attenuated the lung inflammation in lipopolysaccharide (LPS)-induced ALI mice. Compared to other sources of MSCs, MenSCs have gradually become an important cell source in stem cell therapy because of their advantages such as periodicity, non-invasively, and without trauma [35, 36]. In this study, we strongly demonstrated that MenSC transplantation alleviated lung pathology and decreased the levels of the proinflammatory cytokines, repair damaged alveolar barrier through MAPK-mediated necroptosis.

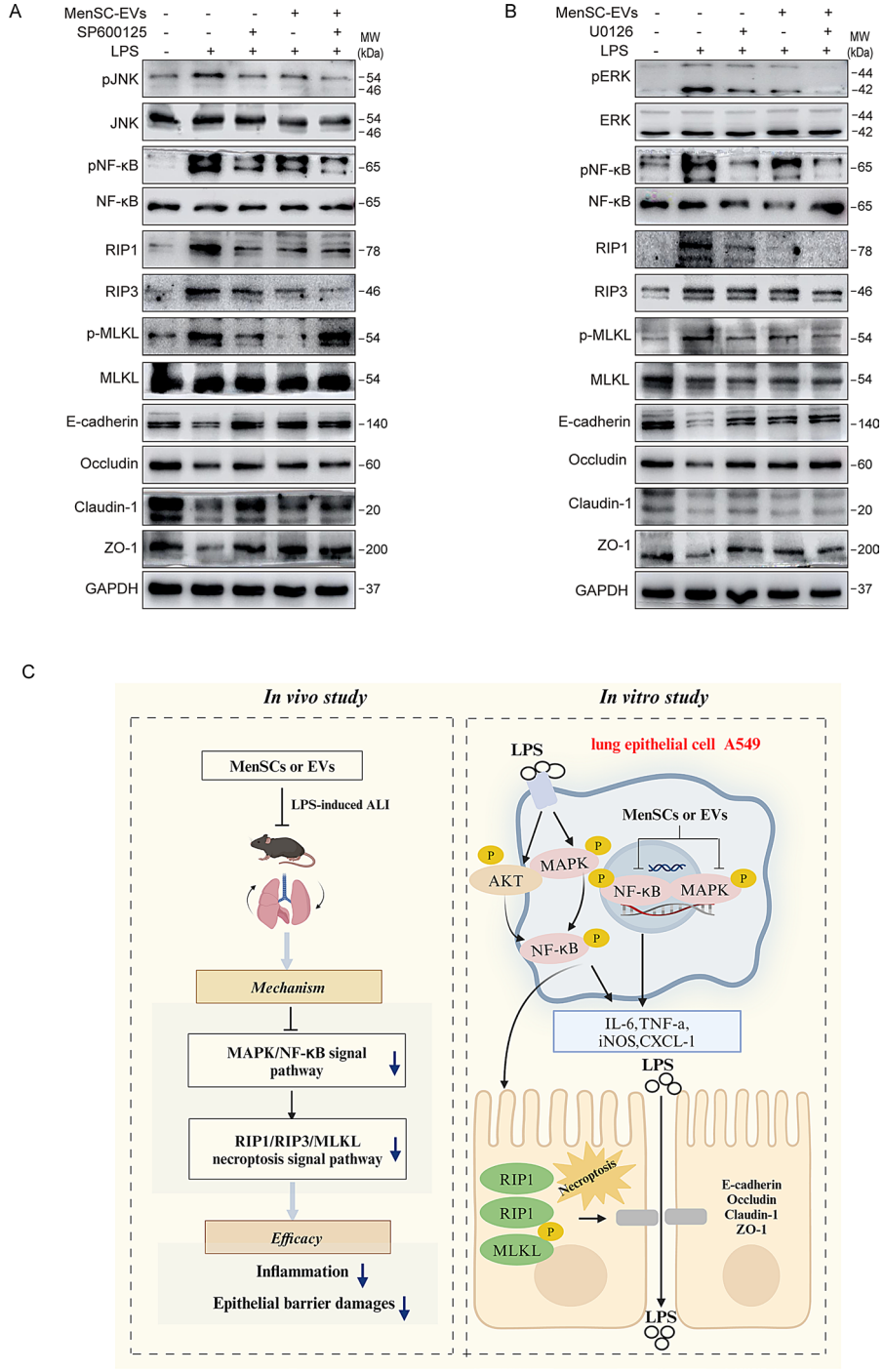


Fig. 8 MenSC-EVs alleviate LPS-induced alveolar epithelial cell dysfunction by inhibiting MAPK-mediated necroptosis. (**A**) Western blot analysis of p-JNK, p-MLKL, RIP1, RIP3 and the tight junction markers E-cadherin, Occludin, Claudin-1, and ZO-1 in A549 cell treated with MenSC-EVs and the JNK inhibitor SP600125. (**B**) Western blot analysis of p-ERK, p-MLKL, RIP1, RIP3 and the tight junction markers E-cadherin, Occludin, Claudin-1, and ZO-1 in A549 cell treated with MenSC-EVs and the ERK inhibitor U0126. (**C**) Schematic model of the efficacy and mechanism of MenSC-EVs on the lung inflammation and epithelial barrier damages in the treatment of ALI

One of the features of ALI is damage to the alveolar epithelial barrier. Damage to the barrier causes the alveolar cavity to be rich in protein edema fluid, and pulmonary edema. Protection of the integrity of alveolar epithelial cells and tight junction proteins is key for reducing pulmonary edema during influenza virus infection [37]. An alveolar epithelial screen in LPS-induced ARDS mice increases barrier permeability, which is caused mainly

by the breakdown of adhesion and tight juntion proteins [38]. Therefore, repair of the alveolar epithelial barrier plays an important role in the treatment of ALI/ARDS. The permeability and functional stability of the alveolar epithelial barrier are closely related to the integrity of alveolar epithelial cells and tight junction proteins, and the breakdown of the barrier will cause pulmonary edema. Recently, MSCs have been shown to be biologically active and able to exert therapeutic effects on various organ injury models [39]. In vitro studies have demonstrated that co-culture of endothelial cells with MSCs protects against inflammatory disruption of barrier function by modulating vascular endothelial cadherin/β-catenin signaling [40]. Furthermore, in vivo studies have demonstrated that MSCs are able to mobilize adherens and tight junction proteins and reduce the binding of inflammatory cells to the endothelium, resulting in preservation of vascular endothelial integrity [40, 41]. MSC MV significantly reduces pulmonary edema in models of lipopolysaccharide and the *E. coli* pneumonia in mice [42, 43] as well as in an ex vivo perfused human lung model of ischemia/reperfusion [44]. In this study, we established an ALI model in vitro and in vivo through LPS and MenSC treatment. Our results revealed that MenSCs increased the expression of the tight junction proteins E-cadherin, Occludin, Claudin-1 and ZO-1, reduced alveolar epithelial permeability and inhibited the levels of proinflammatory cytokines, suggesting that MenSCs can restore epithelial barrier function.

The mechanism by which MenSCs repair epithelial barrier function remains unclear. MSCs are thought to directly differentiate into epithelial cells and replace damaged cells. However, previous studies did not observe differentiation of MSCs into epithelial cells in LPS-induced ALI [45, 46]. Although MSCs can differentiate into multiple cell types [47], there is controversy over the localization, survival, and persistence of MSCs in vivo after administration. An increasing number of studies suggest that MSCs have the capacity to maintain the growth and viability of certain cell types by secreting trophic factors. MSCs can secrete various trophic factors, such as growth factors, cytokines, and chemokines, which can promote the proliferation, migration, and differentiation of epithelial cells [48]. The extracellular vesicles secreted by MSCs contain multiple bioactive molecules, which are considered promising therapies [22, 49]. In the present study, MenSC-EVs alleviated lung pathology injury, increased tight junction protein expression, reduced alveolar epithelial permeability and inhibited the levels of proinflammatory cytokines, which coincides with the effects of MenSCs. Moreover, MenSC-EVs could be easily taken up by A549 and BEAS-2B cells in vitro and ameliorate LPS-induced alveolar epithelial cell dysfunction. Our results demonstrate that MenSCs ameliorate ALI through extracellular vesicles, although the molecules in the extracellular vesicles underlying these effects remain to be identified.

Necroptosis is critical for maintaining the homeostasis of the alveolar epithelial barrier under physiological and pathological conditions [50]. Some microorganisms or proinflammatory mediators mediate the necroptosis of immune cells, including macrophages and lung epithelial cells with significantly elevated RIPK3/MLKL. The MLKL-induced membrane ruptures with the release of DAMPs, causing an excessive inflammatory response and aggravating lung tissue damage in mice [51–53]. RIPK1 inhibition by Nec-1 has been reported to play a protective role, decreasing lung injury and increasing survival [54]. In the present study, we also obtained similar results. The inhibited necroptosis in lung or alveolar epithelial cells treated with MenSCs or MenSC-EVs in our study suggests that necroptosis improves lung pathology Injury or alveolar epithelial barrier dysfunction.

The MAPK pathway is commonly activated by a variety of extracellular stimuli, including growth factors, cytokines, oxidative stress, and the Toll-like receptor [55]. Accumulating evidence suggests that the MAPK pathway plays an important role in regulating the expression of genes encoding tight junction proteins [25]. BDE-209 induces intestinal oxidative stress, induces necrosis and inflammation through the MAPK/NF-κB signaling pathway, which triggers intestinal barrier damage as well as intestinal flora disorders [56]. LPS exposure can cause severe lung injury and histological damage as indicated by the high expression of the necroptosis related genes (RIPK1, RIPK3 and MLKL) [57]. Coleman, Fadie T. demonstrated that pneumonia can be prevented by inhibiting necroptosis [58]. Here, we observed that MAPK is involved in the amelioration of necroptosis by MenSCs and MenSC-EVs, which provides a new scientific basis and intervention target for MSCs or extracellular vesicles to treat ALI, similar to the above studies. Obviously, this study is only a preliminary observation and exploration of the mechanism by which MenSCs and MenSC-EVs alleviate lung pathology injury. However, there are still obstacles to applying MenSC-EVs to ALI therapy in clinical state: there is an urgent need for scalable and cost-effective methods for the production and purification of extracellular vesicles, and further research is needed on the homing and immunomodulatory effects of MenSCs and MenSC-EVs.

Conclusions

In conclusion, our results suggest that by secreting extracellular vesicles, MenSCs inhibit MAPK-mediated necroptosis, which restores epithelial barrier function, and alleviates lung pathology injury. These findings

demonstrate the therapeutic potential of MenSCs and extracellular vesicles in treating ALI.

Abbreviations

ALI	Acute lung injury
ARDS	Acute respiratory distress syndrome
MSC	Mesenchymal stem cells
MenSCs	Menstrual blood-derived stem cells
EVs	Extracellular vesicles; LPS: Lipopolysaccharide
TJs	Tight junctions
RIPK3	Receptor-interacting serine/threonine-protein kinase 3
MLKL	Mixed lineage kinase domain-like pseudokinase
TNF-α	Tumor necrosis factor-alpha
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
H&E	Hematoxylin and eosin
qPCR	Quantitative real-time PCR
PVDF	Polyvinylidene fluoride
DAPI	4',6-diamidino-2-phenylindo
IL-1β	Interleukin 1 beta
TNF-a	Tumor necrosis factor alpha
IFN-γ	Interferon gamma
Inos	Inducible nitric oxide synthase
CXCL1	Chemokine 1

Supplementary Information

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Supplementary Material 1

Acknowledgements
The authors declare that they have not use AI-generated work in this manuscript in this section.

Author contributions
Tao Ruan and Jiaming Han designed and conducted the experiments and performed the data analysis. Jiaming Han and Fengyuan Wang administered the project. Tao Ruan wrote the manuscript. Juntang Lin was responsible for overall supervision. All the authors read and approved the Final manuscript.

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Data availability
The datasets in this study are available from the corresponding authors upon request. All data generated or analysed during this study are included in this published article and in its supplementary information files.

Declarations

Ethics approval and consent to participate
All animal experiments were approved by the Ethics Committee of Xinxiang Medical University (The approved title is role of TGF-β-activated kinase 1 (TAK1) activation in H5N1 virus induced lung injury by regulating E3 ubiquitin ligase Itch to mediate tight junction degradation. Approval number is XYLL-20220141. Date of approval is March 9, 2022). This study is reported in accordance with ARRIVE guidelines for animal experiments. Written informed consent was obtained from all menstrual blood donors involved in this study. The human-derived A549, and BEAS-2B cells were obtained from American Type Culture Collection (Manassas, VA, USA). American Type Culture Collection Company has confirmed that there was initial ethical approval for collection of human cells, and that the donors had signed informed consent. The human menstrual blood-derived endometrial stem cells used in this study were provided by the Zhongyuan Stem Cell Research Institute of the Xinxiang High Tech Zone. According to the source provider's confirmation, the original source has confirmed that there was initial ethical approval for collection of human cells.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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