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Human Umbilical Cord-Derived Mesenchymal Stem Cells Alleviate Acute Lung Injury Caused by Severe Burn via Secreting TSG-6 and Inhibiting Inflammatory Response

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Short title: TSG-6 from hUC-MSCs attenuates burn-induced ALI

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Abbreviation:

TNF-α: tumor necrosis factor α; TSG-6: TNF-a stimulated gene/protein 6; hUC-MSC: human umbilical cord-derived mesenchymal stem cell; ALI: acute lung injury; ELISA: enzyme linked immunosorbent assay; ARDS: acute respiratory distress syndrome; MSCM-sf: Mesenchymal Stem Cell Medium serum-free medium; GFP: green fluorescent protein; MPO: myeloperoxidase; CD68: cluster of differentiation 68; siRNA: small interference RNA; PMSF: Phenylmethanesulfonyl fluoride; IL: interleukin.
Abstract

Objectives To investigate whether hUC-MSCs attenuated severe burn-induced ALI and the effects were based on TSG-6 secreted from hUC-MSCs. Method Rat model was established and evaluated as follows: cytokine expression was measured by ELISA assay, and both inflammatory cell infiltration and lung injury were assessed by immunohistochemistry assay. Results In vitro, TSG-6 levels in serum from the burn group were significantly increased than that of the sham group. In vivo, TSG-6 levels of lung tissues and serum in the burn+ hUC-MSCs group were significantly increased than those of that in the burn group. Both in lung tissues and serum, increased levels of proinflammatory cytokines (TNF-α, IL-1β, IL-6) were remarkably decreased, but anti-inflammatory cytokine IL-10 increased after hUC-MSCs administration (p<0.05). These significant positive effects after hUC-MSCs transplantation did not occur in the Burn+siTSG-6 group. Conclusion Intra-tracheal implantation of hUC-MSCs has been an effective treatment for severe burn-induced ALI via promoting TSG-6 secretion and inhibiting inflammatory reaction in lung tissue.

Keywords hUC-MSCs, TSG-6, ALI, inflammation, burns
Introduction

Acute lung injury (ALI) and its severe manifestations acute respiratory distress syndrome (ARDS) are common forms of hypoxic respiratory failure in patients with a mortality rate of 25–40% [1]. ALI/ARDS also is a major complication and a common cause of mortality in severely burned patients [2]. Growing evidence from experimental and clinical studies have shown that the systemic inflammatory response plays critical roles in the development and mortality of ALI/ARDS [3-5]. Several studies have shown that transplantation of MSCs can reduce systemic inflammation, attenuate lung injury, as well as improve survival in models of ALI [6-8]. Unfortunately, there are no animal models of severe burn-induced ALI in these studies. The human umbilical cord MSCs (hUC-MSCs) emerged recently as an attractive source of MSCs for clinical application. The hUC-MSCs not only share the same features such as multilineage differentiation, paracrine functions, and immunomodulatory properties as well as of all MSCs, but also have some unique advantages, such as non-required for bone marrow matching, low immunogenicity, higher self-renewal capacities, and for accelerating injury tissue repair processes [9-10]. Our previous experiments had demonstrated that severe burns could bring a systemic inflammatory reaction. Meanwhile, hUC-MSCs could alleviate the systemic inflammatory reaction of severe burn through secreting a variety of bioactive factors [11]. However, little is known about hUC-MSCs in the prevention of severe burn-induced ALI. Multipotential anti-inflammatory protein tumor necrosis factor (TNF-a)–stimulated gene/protein 6 (TSG-6) has been found to be efficacious in a wide range of disease models, including respiratory disease models. Our previous study further testified the potential role and mechanism of hUC-MSCs was TSG-6, which secreted by hUC-MSCs attenuated severe burn-induced excessive inflammation via inhibiting activation of P38 and JNK signaling ways [11-12]. Above
discoveries prompted us to further investigate whether hUC-MSCs attenuate severe burn-induced ALI via paracrine secreting TSG-6, which inhibits inflammatory reaction in lung tissue. Within this study, we investigated the therapeutic potential and mechanism of hUC-MSCs on severe burn-induced ALI in a rat model.

Materials and Methods

**hUC-MSCs Preparation**

All experimental procedures were in accordance with the international guidelines for biomedical research in experimental animals issued by the Committee of International Organizations of Medical scientific (CIOMS). We approved the certification of the institutional animal care and used committee of the Fourth Medical Center of PLA General Hospital and obtained the informed consent of all subjects. The primary human umbilical cord mesenchymal stem cells (hUC-MSCs) were purchased from ScienCell Research Laboratories (ScienCell, San Diego, CA). hUCMSCs were examined using flow cytometry and are positive for CD105, CD90,CD44, CD29, and HLA-I and negative for CD34, CD45, HLA-DR, CD31, and vWF as described in our previous publication [12]. They have the potential to develop into mature cells that produce fat, cartilage, bone, tendons, and muscle. These properties, in combination with their developmental plasticity, have generated tremendous interest because of the potential use of MSC in regenerative medicine to replace damaged tissues.

**Ex vivo evaluation of TSG-6 releases from hUC-MSCs in serum of burn animals**

The hUC-MSCs were plated into 6-well plates with a cell density of 5000 cells/cm² and incubated with 3 ml mesenchymal stem cell medium serum free (MSCM-sf) (ScienCell Research Laboratories, San Diego, CA) for 1 day, which were subsequently cultured continuously with 3 mL of MSCM-sf containing 10% sham serum or burn serum
at 24 hours post severe burn. Currently, the levels of inflammatory mediators, oxidative stress factors, and other factors were very high. TSG-6 secretion from hUC-MSCs after 10% burn serum continuous culture, and the supernatant of the cells was assayed by ELISA kit.

**hUC-MSCs were transfected with siRNA for TSG-6**

We selected the lenti-viral expression vector containing the TSG-6 siRNA sequence (GeneChem, Shanghai, China) for specific targeting TSG-6, including Lenti-TSG-6-siRNA, and its negative control vector, Lenti-GFP-siRNA. TSG-6 siRNA lenti-virus vectors were generated by ligating the vector PGC-LV. TSG-6 siRNA oligonucleotide sequences are:

Forward, 5'-'CCGGTTTCTCCGAACGTGCAGTTTTCAAGAGAAGCTGCAAACGTGCAGTTTCGGAGAATTTTTG-3';
Reverse, 5'-'AATTCAAAAAATCTCCGAACGTGCAGTTTTCTCTGAAACGTGACACGTTCGGAGA-3'.

The sequences of control siRNA are:

Forward, 5'-'CCGGGCAAGCTGACCCTGAAGTTCATCTCGAGATGAACTTCAGGGGTCACGTTGCTTTTTG-3';
Reverse, 5'-'AATTCAAAAAAGCAAGCTGACCCTGAAGTTCATCTCGAGATGAACTTCAGGGGTCACGTTGC-3'.

The recombinant lenti-virus was produced by cotransfecting HEK293T cells with pGC-LV-GFP-siRNA or pGC-LV-TSG-6-siRNA, pHelper 1.0, and pHelper 2.0 plasmid using Lipofectamine 2000 (GeneChem, Shanghai, China). hUC-MSCs were transfected with the prepared lenti-virus (Lenti-TSG-6-siRNA or Lenti-GFP-siRNA). The hUC-MSCs were plated into either 6-well plates or T-75 culture bottles with a cell density of 5000 cells/cm² and incubated with 3 ml or 10 ml MSCM-sf without antibiotics for 1 day. And then, these cells were transfected in 1 ml medium of Lenti-TSG-6-siRNA or Lenti-GFP-siRNA according to the protocol provided by the manufacturer. After
12 hours, the medium was changed, and these cells were incubated with MSCM-sf without antibiotics for 3-4 days sequentially. The efficiency of TSG-6 gene knockdown in hUC-MSCs were evaluated using GFP-fluorescence observation and Western blot. Then hUC-MSCs in T-75 culture bottles were harvested with 0.25% trypsin and re-suspended at $5 \times 10^6$ cells in 1 mL sterile PBS for subsequent implantation.

**Animals**

All studies adhered to procedures consistent with the International Guiding Principles for Biomedical Research Involving Animals issued by the Council for the International Organizations of Medical Sciences (CIOMS), and all experimental protocols were approved by the Institutional Animal Care and Use Committee at the Fourth Medical Center of PLA General Hospital and the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition. Six-week-old male Wistar rats (180–220g) were obtained from the local animal facility, housed at the Institute of Animal Experiments of the Forth Medical Center of PLA General Hospital in stables with a temperature of 22° C, a relative humidity of 55% and a day/night cycle of 12/12 hours, with food and water ad libitum throughout the experiment.

**Experimental Groups**

Wistar rats were randomly divided into 5 groups: 1). Sham; 2). Burn; 3). Burn + hUC-MSCs (burn and implantation with hUC-MSCs); 4). Burn +Vehicle (burn and implantation with hUC-MSCs transfected with Lenti-GFP-siRNA); 5). Burn + siTSG-6 (burn and implantation with hUC-MSCs transfected with Lenti-TSG-6-siRNA). Rats in each group were divided equally into three subgroups of 6 rats according to the indicated time points of d1, d3, and d7 for the samples collected.
Animal Treatment

Rats were anesthetized with Avertin (20 mg/ml, 300 mg/kg) (2, 2, 2-tribromoethanol, Sigma, USA) via intra-peritoneal injection. The dorsal and abdominal hair was completely shaved. Rat models with 50% TBSA full-thickness burn injury were established via placing the backside and abdomen into hot water (94°C) for 12 s and 6s, respectively [11,13,14]. Intra-peritoneal injections of balanced salt solution (40 ml/kg) were immediately administered to prevent shock in all groups. We adopted 20-gauge central catheter as a tracheal catheter, while rats were restrained on the 45°inclined operating board with their head facing the operator. The upper incisors of the rat were fixed with a cotton thread to the nails at one end of the operating board, so the head of the rat was raised, and we inserted the laryngoscope into its mouth. The glottis of the rat can be seen in the light of the laryngoscope. The tracheal catheter was quickly inserted into the airway when the glottis was opened [15-16]. The rats in the Burn + hUC-MSCs group immediately received intra-tracheal implantation of \(5 \times 10^6\) (1 ml) hUC-MSCs after burn. The rats in the Burn +Vehicle and Burn+siTSG-6 groups received intra-tracheal implantation of \(5 \times 10^6\) (1 ml) hUC-MSCs transfected with Lenti-GFP siRNA or Lenti-TSG-6-siRNA at the same time. The rats in the sham and burn groups received intra-tracheal implantation of 1 mL PBS. All rats in each group were clinically evaluated. The burn wound and rats in the sham group were treated as previously described [11]. Blood and lung tissue samples were collected at each indicated time points. After anesthesia with the above anesthetics, rats’ blood was extracted from the inferior vena cava. And then, rats were euthanized with an overdose intravenous of pentobarbital sodium.

At each indicated time point, blood samples from the inferior vena cava were extracted, and lung tissue samples were collected. And then, rats were euthanized with an overdose intravenous of pentobarbital sodium.
**Specimen collection and detection**

Both the lung tissue and blood samples in all groups were taken from the aortaventralis at d1, d3, and d7 after injury. Subsequently, one piece of lung specimen was stored in liquid nitrogen for TSG-6 detection using ELISA kit, as well as another piece was fixed using 4% paraformaldehyde for immunohistochemical analysis and H.E. staining. The detection range of the kit is from 0.312 ng/ml to 20 ng/ml. 100mg lung tissue was rinsed with PBS, homogenized in 1 ml of PBS, and stored overnight at -20°C. After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 μg, 2 - 8°C. The supernate was removed and assayed immediately. Standard: Pipette 250 μl of Sample Diluent into each tube. Use the stock solution to produce a 2-fold dilution series. Mix each tube thoroughly before the next transfer. The undiluted Standard serves as the high standard (20 ng/ml). Sample Diluent serves as the standard zero.

**Histological analyses**

After fixation with 4% paraformaldehyde for 1 week at room temperature, the specimens were embedded in paraffin and sectioned into five micrometer-thick sections. Then, the slices were deparaffined with dimethyl benzene, rehydrated, and then stained with H&E in accordance with standard procedures. Infiltrations of neutrophils and macrophages were detected via incubating with specific antibodies (MPO, CD68; from R&D Systems, Minneapolis, MN), the corresponding secondary antibody and the PAP (peroxidase–anti-peroxidase) complex in turn, and finally stained with DAB (3,3’-diaminobenzidine). Myeloperoxidase (MPO) exists in the cytoplasm of neutrophils and can be used as an indicator of neutrophils in lung tissue. The positive reaction of neutrophils is brown-yellow colorization. CD68 is a macrophage specific antigen which is direct evidence for the identification of macrophages.
The positive reaction of macrophages is brown colorization. Randomly, five different visual fields under a light microscope (400x) of each respective section were chosen and CD86 and MPO positive cells were counted by an experienced scientist in a blinded manner.

**Western blotting**

Protein from hUC-MSCs was isolated using RIPA buffer (Sigma Life Science), containing PMFS (Sigma Life Science) and complete Mini Protease inhibitor cocktail (Roche). Cells were centrifuged at 3000g for 10 min to collect the nuclei, and then the supernatants were further centrifuged at 17,000g for 20 min to separate the mitochondrion. Cytoplasm was diluted in Laemmli buffer and denaturized at 95°C for 10 min. Protein was quantified using a commercial bicinechonic acid (BCA) kit (BCA Protein Assay Kit; Pierce Biotechnology Inc., Rockford, IL, USA). Equal amounts of protein were subjected to SDS-PAGE gel as previously described [17], and anti-TSG-6 antibody (1:1,000) and anti-β-actin antibody (1:20,000) (R&D Systems, Minneapolis, MN) were used for protein expression assay.

**Enzyme-linked immunosorbent assay (ELISA)**

100mg lung tissue was rinsed and homogenized, then stored overnight at -20°C. The samples were performed to break the cell membranes; the homogenates were centrifuged for 5 minutes at 5000 µg, 2 - 8°C. The supernatant was removed. The double-antibody sandwich ELISA kits (eBioscience, USA) were used to detect the levels of tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), IL-6, IL-10, MPO and TSG-6 in lung tissues and serum according to the manufacturer’s protocols.

**Statistical analysis**
All data were expressed as the mean ± SD and analyzed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). ANOVA followed by Tukey Cicchetti test for multiple comparisons or nonparametric Kruskal-Wallis test, as appropriate was used for statistical analysis. Statistical significance level was set at $p<0.05$.

Results

**TSG-6 levels in supernatant/lung tissues/serum and TSG-6 knockdown efficiency**

To investigate whether TSG-6 secreted from hUC-MSCs influenced its therapeutic effect or not, we firstly examined the levels of TSG-6 secretion from hUC-MSCs after treatment with serum of sham or burn. Compared with the sham group, TSG-6 levels in the supernatant of burn serum were significantly increased in vitro at 24 hours post burn (Figure 1a). hUC-MSCs cultured in vitro were transfected with Lenti-GFP siRNA or Lenti-TSG-6-siRNA. As showing in Figure 1b, representative photographs demonstrated successful transfection with Lenti-GFP siRNA or Lenti-TSG-6-siRNA. Data from Western blotting showed that TSG-6 expression was significantly decreased after Lenti-TSG-6-siRNA transfection than that of the vehicle group (Figure 1c).

In vivo, TSG-6 levels of burn+ hUC-MSCs group in lung tissues were significantly increased compared with the burn group at d1, d3, and d7 post-severe burn (Figure 1d). Meanwhile, TSG-6 levels of burn+ hUC-MSCs group in serum were significantly increased compared with the burn group in vivo (Figure 1e). TSG-6 levels of lung tissues and serum reached the peak at d3 in the burn+ hUC-MSCs group.
Figure 1 Secretion and knockdown efficiency of the TSG-6

The effect of hUC-MSCs and TSG-6 knockdown on structural damage and total inflammatory cell infiltration in lung tissues after severe burn

We evaluated the therapeutic influence of hUC-MSCs and TSG-6 knockdown on the structural changes and infiltrated degree of total inflammatory cells in the lungs at d1, d3 and d7 post-severe burn using H&E staining. As showing in Figure 2, compared with the sham group, the structural damages, including alveoli fusion, interstitial hemorrhage, alveoli exudation, and infiltrated degree of total inflammatory cells in the lung tissues of burn group were remarkably increased at d1. Meanwhile, these changes were less severe in the burn +hUC-MSCs group at d1.
Destroyed structure and inflammatory cell infiltration after burn, such as neutrophil infiltration, interstitial edema, and pulmonary hemorrhage, were all improved gradually at d3 and d7. However, we noted that burn+hUC-MSCs recovered faster than other groups. Moreover, we found that the therapeutic effect of hUC-MSCs on the structural damage and total inflammation was sharply decreased after TSG-6 knockdown (Figure 2). Respectively, we calculated the neutrophil and macrophage infiltration of lung tissues induced by severe burns to evaluate the therapeutic influence of hUC-MSCs and TSG-6 knockdown on lung tissues at d1, d3 and d7 after severe burns using immunohistochemical staining. As shown in Figure 3, compared with the sham group, the neutrophil infiltration degrees of lung tissues in the burn group were remarkably increased, and they were markedly decreased by hUC-MSCs administration ($p<0.05$). Furthermore, the data showed that the therapeutic effect of hUC-MSCs on neutrophil infiltration in the lung was sharply decreased after TSG-6 knockdown (Figure 3a). The quantitative analysis was reported in the corresponding histogram ($p<0.05$) (Figure 3b). As well as showing in Figure 4, in comparison with that in the sham group, macrophage infiltration degrees of lung tissues in the burn group were significantly increased, which were dramatically decreased after hUC-MSCs implantation ($p<0.05$). Furthermore, the data showed that the therapeutic effect of hUC-MSCs on macrophage infiltration in the lung was also sharply decreased after TSG-6 knockdown (Figure 4a). The quantitative analysis was reported in the corresponding histogram ($p<0.05$) (Figure 4b). It suggested that the therapeutic effect of hUC-MSCs on attenuating burn-induced neutrophil and macrophage infiltration was dependent on the level of TSG-6 expression. Down-regulation of TSG-6 expression in hUC-MSCs significantly reduced its therapeutic function on improving structural damages and total inflammatory cells infiltrations of the lung tissues (Figures 2, 3 and 4).
Figure 2 Effect of TSG-6 on the structural damages and total inflammatory cells infiltrations of the lung tissues induced by severe burn.

Figure 3 Effect of TSG-6 secretion and knockdown on neutrophil infiltrations of the lung tissues induced by severe burn.
The effect of hUC-MSCs and TSG-6 knockdown on inflammatory cytokines in lung tissues after severe burn

We also tested the levels of the proinflammatory cytokines of TNF-α, IL-1β, IL-6, MPO, and anti-inflammatory cytokine IL-10 in lung tissues at d1, d3 and d7 after severe burn using ELISA. As shown in Figure 5, compared with the sham group, the levels of proinflammatory cytokines, such as TNF-α (Figure 5a), IL-1β (Figure 5b), IL-6 (Figure 5c), and MPO (Figure 5d) in the lung tissues of the burn group were remarkably increased, and they were all markedly decreased after hUC-MSCs administration ($p<0.05$). It’s worth noting that the level of anti-inflammatory cytokine IL-10 (Figure 5e) in burn+hUC-MSCs group was significantly higher than that in the burn group and sham group, and that in the burn group was also higher than that in the sham group ($p<0.05$).

While the data also indicated that the therapeutic effect of hUC-MSCs knockdown TSG-6 surely increased the levels of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, MPO and decreased anti-inflammatory cytokine IL-10 in lung tissues ($p<0.05$) (Figure 5).
Figure 5 Effect of TSG-6 secretion and knockdown on inflammatory cytokines of the lung tissues induced by severe burn

Discussion

During the last few years, human MSCs as a cellular therapy for ALI/ARDS has ignited much interest of researchers and the therapeutic effects have been demonstrated in different animal models [18-20]. Studies have shown that MSCs have therapeutic effects in various preclinical models of lung diseases via direct differentiation and paracrine action [21-23]. Indeed, there were a few of MSCs survive for more than one week after systemic administration, suggesting that the main effects of MSCs are probably mediated by paracrine mechanisms [24].

Patients with ALI caused only by severe burns or scald were not uncommon in the clinic, especially in children. In the present study, we established a severe burn-induced ALI in a rat model to investigate the therapeutic effects of
hUC-MSCs and its possible mechanism. We found that intra-tracheal implantation of hUC-MSCs increased the survival rates and significantly attenuated severe burn-induced ALI via inhibiting pulmonary inflammation, and the therapeutic effects of hUC-MSCs were strongly reduced when the expression of TSG-6 were inhibited via RNA interference. It indicated that the therapeutic effects of hUC-MSCs in severe burn-induced ALI rats were associated with the soluble factor TSG-6 that secreted by hUC-MSCs itself.

We created a rat model with a classical pulmonary inflammatory state via severe burn [13-14, 25]. The severity of the lung injury was characterized by several pathological parameters, just as described in the diagram (Fig.2-4). All these pathological changes are closely tied to the human ALI, which indicates that our model successfully duplicated human ALI. These parameters were all improved after intra-tracheal implantation of hUC-MSCs, which indicated the therapeutic benefits of hUC-MSCs implantation on severe burn-induced ALI. These findings proved that the intra-tracheal injection of hUC-MSCs attenuated lung injury in severe burn-induced ALI rats. Our results were consistent with some other previous studies, in which the administration of hUC-MSCs or BM-MSCs reduced systemic inflammation and attenuated LPS-induced ALI in rats [26].

ALI is an uncontrollable pulmonary inflammatory disease characterized by the release of cytokines and neutrophil accumulation. Neutrophils are the primary cells being convened to the site of inflammation and releasing inflammatory cytokines. Macrophages are another predominant cell existing in either a pro-inflammatory (M1) or anti-inflammatory (M2) state, acting as a trigger of the inflammatory response, and contributing to both the initiation and resolution of inflammation. MSCs treatment could regulate macrophages with anti-inflammatory phenotype and simultaneously enhance the phagocytic activity of macrophages [27]. In our study, the infiltration of neutrophils and
macrophages in lung tissue in the burn group were remarkably increased and surely reduced after treated with hUC-MSCs. This alleviative lung injury of hUC-MSCs was once confirmed in Canine radiation-induced ALI model via reducing oxidative stress, inflammatory reactions, and TGF-β-Smad 2/3 pathway activation [28].

MPO is an enzyme stored in neutrophils and macrophages and released into the extracellular fluid in the setting of the inflammatory process. Since MPO is an important enzyme in the inflammatory process, there is an ongoing interest in the use of MPO as a biomarker for assessing the extent of inflammatory response in our present study. After a severe burn, a remarkable increase of MPO in lung tissues was detected in the burn group, and the peak level was shown at d1. As we expected, treatment with hUC-MSCs clearly reduced the MPO activity at d1, d3, and d7. Previously, Li et al. found that intravenously infused with hUC-MSCs significantly inhibited LPS-stimulated MPO activity in rat lung tissues [26]. Reduced MPO activity indicates an improvement of lung injury and confirmed the therapeutic effects of hUC-MSCs on burn-induced lung injury through promoting anti-inflammatory homeostasis.

The pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 are the primary mediators of the acute phase response. Previous studies showed that decreased neutrophil recruitment into the lung tissue and suppressed expression of TNF-α, IL-1β, and IL-6 can improve the outcomes of ALI [29]. Several other studies have also shown that MSCs could decrease the plasma level of TNF-α and IL-1β through paracrine secretion [30]. Consistent with previous studies, increased levels of these inflammatory cytokines were found in our model of severe burn-induced ALI. After severe burns, the concentration of TNF-α, IL-1β, and IL-6 significantly increased. The difference is simply that the peak level occurs at different times. For TNF-α and IL-1β, the peak level appeared on d1, while the peak level of IL-6 appeared on d3. When hUC-MSCs were intra-tracheal implanted after burn, the concentration of
pro-inflammatory cytokines in lung tissues decreased significantly, which was mostly in agreement with previous studies demonstrating that BM-MSCs can reduce TNF-α and IL-6 secretion by lung macrophages via paracrine pathway or direct contact with host cells [30]. Our results also confirmed that hUC-MSCs were able to protect the lung from injury through reducing the inflammatory response.

Interleukin-10 (IL-10), one of the most important anti-inflammatory cytokines, is known to reduce the synthesis of pro-inflammatory cytokines. IL-10 treatment is supposed to be a promising therapeutic strategy to reduce lung injury [30-32]. Some argued that the progression of ALI is associated with decreased expression and secretion of IL-10 [33]. However, in our study, the IL-10 level rose in lung tissues after burn injury, and it were further markedly elevated after hUC-MSCs administration. Thus, the protective effect of IL-10 in lung inflammation response, which had been well-described in previous studies, might partially explain the mechanism through which hUC-MSCs exerted their therapeutic effects on severe burn-induced ALI.

According to the above findings, it can be inferred that hUC-MSCs administration improved ALI by balancing the homeostasis of the cytokine network. Furthermore, we would like to know more about how hUC-MSCs did work. In our previous study, we found the levels of TSG-6 levels were significantly elevated in the systemic inflammatory response in burn rats. TSG-6 expression is induced as a result of an inflammatory response. Our data showed that severe burn-induced ALI could unregulated the expression of TSG-6, which was in agreement with our previous study [11]. The even higher level of TSG-6 in the burn+ hUC-MSCs group might relate to the TSG-6 secreted by hUC-MSCs in response to inflammatory signals. To test our hypothesis and investigate the role of TSG-6, TSG-6 knockdown was achieved via RNA interference by transfection with siRNA for TSG-6 in our ALI rat model. In the
Burn+siTSG-6 group, the level of TSG-6 was significantly decreased. Meanwhile, pathological changes and inflammatory response were detected in burn+siTSG-6 group and Burn +vehicle group. These results showed that the efficacies of hUC-MSCs implantation, including improvement of lung function and pulmonary metabolism function, structure protection of lung tissues, reduction of inflammatory cell infiltration, suppression of pro-inflammatory cytokines, and promoting expression of anti-inflammatory, diminished by TSG-6 knockdown. Our data inferred that the anti-inflammatory properties of hUC-MSCs in severe burn-induced ALI are explained, at least in part, by the activation of hUC-MSCs to secrete TSG-6. These research further proved that TSG-6 is an inflammation-associated secreted protein that has been implicated as having important and diverse tissue protective and anti-inflammatory properties [34]. Our results showed the observed beneficial effects of hUC-MSCs in animal models of ALI and suggest that the anti-inflammatory properties of hUC-MSCs in the lung are explained, at least in part, by the activation of hUC-MSCs to secrete TSG-6. TNFα-stimulated gene-6 (TSG-6), a 30-kDa protein generated by activated macrophages, modulates inflammation; however, its mechanism of action and role in the activation of macrophages were not fully understood. From our earlier report [11] and some literatures [35], we could find that TSG-6 inhibited the association of TLR4 with MyD88, thereby suppressing NF-κB activation. TSG6 also prevented the expression of pro-inflammatory proteins (iNOS, IL-6, TNFα, IL-1β, and CXCL1) while increasing the expression of anti-inflammatory proteins (CD206, Chi3l3, IL-4, and IL-10) in macrophages. This shift was associated with suppressed activation of pro-inflammatory transcription factors STAT1 and STAT3. Thus, TSG-6 functions by converting macrophages from a pro-inflammatory to an anti-inflammatory phenotype secondary to suppression of TLR4/NF-κB signaling and STAT1 and STAT3 activation.
It was well known that delivering locally has the advantage of reaching the target organ directly with relatively small dosage, faster and stronger effectiveness, and slighter systemic adverse reactions. In clinical, physicians prefer to use medicine through intra-tracheal administration or aerosol inhalation for patients with lung diseases, especially for severe cases. Thus, we chose the intra-tracheal implantation as the route of medication. While increasing the dose of drugs might increase the efficacy of the treatment, we couldn’t exclude systemic adverse reactions and more waste of resources caused by larger doses. Taking these factors into consideration, we selected the optimal dosage of hUC-MSCs (1×10^6) as suggested in our previous study [36].

In our rat model, full-thickness burn injury was established via placing the backside and abdomen into hot water, but not the chest, so ALI is caused by severe burn-induced excessive inflammation and serious metabolic disturbances rather than full thickness escharotic at the back and abdomen.

Although the 50% TBSA burn was severe, we managed to avoid the death of the rat model. In the early period of this study, we performed consistent treatments as follows: keep warmth and then intra-peritoneal injections of balanced salt solution (40 ml/kg) were immediately administered to prevent shock. And the burn wound was then treated with a 1% tincture of iodine and kept dry to prevent infection.

To the best of our knowledge, this is the first report about the therapeutic evaluation of hUC-MSCs in severe burn-induced ALI. TSG-6 secreted from hUC-MSCs played a key role in severe burn-induced ALI via inhibiting the inflammatory reaction in lung tissue. It suggests that intra-tracheal implantation of hUC-MSCs is an effective treatment for severe burn-induced ALI rat model. Meanwhile, knockdown of TSG-6 mRNA expression in hUC-MSCs did not completely abrogate the anti-inflammatory effects suggesting some other unknown mechanisms.
were involved. It means additional experiments are required to determine the relative contribution of these factors to the beneficial effects of hUC-MSCs in lung injury.
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Statement

I acknowledged that an identical manuscript was presented as a preprint version according to the following link: https://www.researchsquare.com/article/rs-127492/v1 due to the absence of peer reviews and official publication.
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37. Preprint link: https://www.researchsquare.com/article/rs-127492/v1
Declarations

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**Conflicts of interest/Competing interests:**

The authors declare that they have no competing interests.

**Availability of data and material**

All authors are sure that all data and materials as well as software application or custom code support our published claims and comply with field standards. All material in the manuscript including figures, tables, or text passages is originated from the authors.

**Authors' contributions**

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Lingying Liu, Yu Wang, Yanan Liu and Zhongyuan Li. The first draft of the manuscript was written by Xiaohong Hu, and Yu Wang. The manuscript revised by Xiaohong Hu and Yonghui Yu. All authors read and approved the final manuscript.

**Ethics approval**

The present study was approved by the Fourth Medical Center of PLA General Hospital Ethics Committees. All experimental procedures were in accordance with the international guidelines for biomedical research in
experimental animals issued by CIOMS. After samples collection, rats were euthanized following the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition.