Overexpression of TGFβ1 in murine mesenchymal stem cells improve the lung inflammation via impacting Treg/Th17 balance in LPS-induced ARDS mice

CURRENT STATUS: UNDER REVIEW

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DOI:
10.21203/rs.3.rs-16570/v1

SUBJECT AREAS
Stem Cell & Developmental Cell Biology

KEYWORDS
Acute respiratory distress syndrome, Mesenchymal stem cells, TGFβ1, Th17/Treg
Abstract

Background: T helper 17 cells (Th17)/ regulatory T cells (Treg), as subtypes of CD4+T cells, played an important role in the inflammatory response of acute respiratory distress syndrome (ARDS). However, there is still a lack of effective methods to regulate the differentiation balance of Th17/Treg. It was proved that mesenchymal stem cells (MSCs) could regulate the differentiation of CD4+T cells, but the mechanism was still unclear. TGFβ1, as one of the paracrine cytokines of MSCs, could also regulate the differentiation of Th17/Treg but possess low expression in MSCs. Therefore, mouse MSCs (mMSCs) overexpressing TGFβ1 was constructed by lentivirus transfection and intratracheally transplanted into LPS-induced ARDS mice in our study. And the aim of which was to evaluate the therapeutic effects of mMSCs overexpressing TGFβ1 on inflammation and immunoregulation via impacting Th17/Treg balance in LPS-induced ARDS mice.

Methods: mMSCs with TGFβ1 overexpression were constructed using lentiviral vectors. Then mBM-MSCs and mBM-MSC-TGFβ1 (mMSCs overexpressing TGFβ1) were transplanted intratracheally into the ARDS mice induced by lipopolysaccharide. At 3d and 7d after transplantation, mice were sacrificed and the histopathology of lungs was assessed by hematoxylins and eosin staining and lung injury scoring. Homing of the mMSCs were assayed by ex vivo optical imaging. The relative number of Th17 and Treg in the lungs and spleens in mice were detected by FCM. IL-17A and IL-10 in the lungs of mice were analyzed by western blot. Permeability was evaluated by analysing the protein concentration of BALF using ELISA. Alveolar Lung fibrosis was assessed by Masson's trichrome staining and Ashcroft scoring. The mortality of ARDS mice was followed until 7 days after transplantation.

Results: The transduction efficiencies mediated by the lentiviral vectors were 82.3-88.6%. Overexpressing TGF-β1 inhibited the proliferation of mMSCs during day 5-7 (p<0.05), but made no effects on their differentiation or migration (p>0.05). Compared to the LPS+mBM-MSC-NC group, mMSCs overexpressing TGFβ1 engraftment led to improved histopathology of lung tissue in ARDS mice (p<0.05), much more differentiation of mMSCs into Th17 or Treg (p<0.05) and improved permeability of injured lungs (p<0.05). Moreover, IL-17A was also decreased while IL-10 increased in
the LPS+mBM-MSC-TGFβ1 group than in the LPS+mBM-MSC-NC group respectively (p<0.05). Finally, mMSCs overexpressing TGFβ1 did not aggravate the fibrosis of lungs in ARDS mice (p>0.05).

Conclusion: MSCs overexpressing TGFβ1 could regulate lung inflammation and attenuated lung injuries via modulating the imbalance of Th17/Treg in the lungs of ARDS mice.

Background
Acute respiratory distress syndrome (ARDS) which was initially defined 52 years ago as a multifactorial syndrome of severe lung injury\(^1\), is characterized by hypoxemia, loss of lung compliance, and pulmonary edema, that can in some instances progress to multiple organ failure\(^2\). ARDS can develop in response to multiple predisposing factors including pneumonia, systemic infection, and major surgery or multiple traumas\(^3\) and results in death in 30-45% of cases\(^4\).

In the past 50 years, considerable progress has been made in understanding the pathology of ARDS and the development of ARDS is strongly associated with a disordered immune response in the lung\(^5\). In previous studies, CD4\(^+\) T cells, as an important component of adaptive immune cells, were significantly activated in the early stage of ARDS, and differentiation of T helper 17 cells (Th17)/regulatory T cells (Treg) played an important role in the development of ARDS\(^5\). Th17 cells could release a lot of inflammatory cytokines which mediate the acute inflammatory response\(^6\). Treg, as an important immunosuppressive cell, could also activated in ARDS, and the transplantation of Treg into ARDS mice model may reduce the levels of proinflammatory cytokines in alveolus\(^7\) and inhibited neutrophils apoptosis and fibrocyte recruitments\(^8\). In addition, the differentiation balance of Th17/Treg is an independent predictor for 28-day mortality in patients with ARDS\(^9\). Thus, there is still a lack of effective methods to regulate the differentiation balance of Th17/Treg.

It was proved by our previous study that, mesenchymal stem cells (MSCs), with their properties of multi-potency and immunoregulation, could significantly improve the inflammation and repair lung injuries in ARDS mice\(^10\). The mechanism of their immunological regulation may be related to the modulation of T-cell expansion\(^11\). And it has also been confirmed in recently published study that
MSCs could regulate the imbalance of Th17/Treg, which is regulated by antigen-stimulated
costimulatory molecules, antigen-presenting cells, cytokines, and intracellular signals\textsuperscript{11}. TGF\textbeta\textsubscript{1}, as
one of the important cytokines to regulate the differentiation of Th17/Treg\textsuperscript{12}, possess low expression
in MSCs which is proved by our preliminary data. Thus, overexpression of TGF-\textbeta\textsubscript{1} is expected to
further optimize MSCs treatment for immunoregulation.

Therefore, the aim of this study is to evaluate the effect of mouse MSCs (mMSCs) overexpressing
TGF\textbeta\textsubscript{1} on inflammation and immunoregulation in LPS-induced ARDS mice.

**Material And Methods**

**Cell Culture**

Mouse MSCs (mMSCs) isolated from the bone marrow of C57BL/6mice (mBM-MSC) were purchased
from Cyagen Biosciences, Inc. (Guangzhou, China), and 293T cells were supplied by Zoonbio
Biotechnology Co., Ltd. (Nanjing, China). The mBM-MSC were identified by detecting cell surface
phenotypes and their multipotent potential for differentiation along the adipogenic, osteogenic, and
chondrogenic lineages as previously described\textsuperscript{13,14}.

Either mBM-MSC or 293T cells were cultured in a 1:1 mix of Dulbecco’s modified Eagle’s
medium/nutrient mixture F-12 (DMEM/F12) (Wisent, Inc., St-Bruno, Montreal, Quebec, Canada)
containing 10\% FBS (Wisent, Inc.) and 1\% antibiotic-antimycotic (streptomycin, penicillin and
amphotericin B; Wisent, Inc.), incubated at 37 °C in a humidified atmosphere of 5\% CO\textsubscript{2} and passaged
every 3–4 days by 0.25\% trypsin-ethylenediaminetetraacetic acid (EDTA; Gibco, Carlsbad, CA, USA)
when they reached about 80\% confluence. Passages between 5 and 10 were used for the
experimental protocols.

**Recombinant Lentivirus Vector Construction And Package**

The full-length coding sequences (CDS) of TGF\textbeta\textsubscript{1} was transferred into CMV promoter-dependent
lentivirus vector PDS159_pL6.3-CMV-GFPa1-IRES-MCS (Zoonbio Biotechnology Co., Ltd.).

Subsequently, the lentivectors CL721-pL6.3-CMV-GFPa1-IRES-mus-TGF-\textbeta (overexpressing TGF\textbeta\textsubscript{1})
which co-expressed the enhanced green fluorescent protein (eGFP) and TGF\textbeta\textsubscript{1} was obtained, and the
empty vector CL721-pL6.3-CMV-GFPa1-IRES was used as an empty vector control. Then, the recombinant plasmid, CL721-pL6.3-CMV-GFPa1-IRES-mus-TGF-β and CL721-pL6.3-CMV-GFPa1-IRES were separately co-transfected with packaging plasmids into 293T cells at the indicated concentrations using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer’s instructions, producing lentivirus LV402-pL6.3-CMV-GFPa1-IRES-mus-TGF-β and the negative control PDS019.

Lentiviral Vector Transduction And eGFP Reporter Gene Detection

The mBM-MSC (1 x 10^6/well seeded in six-well cell culture plates) were transduced with viral supernatant at a multiplicity of infection (MOI) value of 160:1 for 24 hours. Then the stable cell lines were harvested after selection using blasticidin (BSD; InvivoGen) at the minimal lethal concentration (6 µg/mL) as previously described 15 and cultured in normal culture media for 20 passages after transduction. Finally, the transduction efficiency of mBM-MSC and the percentage of eGFP positive cells were evaluated by fluorescence microscopy and flow cytometry analysis using a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

RNA Isolation And Quantitative Real-time Pcr (qRT-PCR)

Total RNA was isolated from the cells and tissues using TRIZol reagent (Invitrogen, Austin, TX, USA) according to the manufacturer’s protocol, and the purity of the RNA (260/280 nm absorbance ratio of 1.8–2.2) was assessed by a spectrophotometer (Tecan, Switzerland). Reverse transcription was completed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) with 1 mg of RNA according to the manufacturer’s instructions. The qRT-PCR reaction was performed by a CFX96™ Real-Time system (Bio-Rad). Relative changes in gene expression were normalized to the expression of actin and calculated by the 2(-ΔΔCt) method. The primer sequences used for PCR amplification in our study were designed based on the sequences of the genomic clones and are as follows:
| Gene     | Primer          | Primer sequence         | PCR amplified products (bp) |
|----------|-----------------|-------------------------|-----------------------------|
| Actin    | Forward         | 5’-AGAGGGAAATCGTGCGTGAC-3’ | 195                         |
|          | Reverse         | 5’-CCATACCCAAAGGAAGGCT-3’ |                             |
| GAPDH    | Forward         | 5’-TGTGTCGTCGTGGATCTGA-3’ | 150                         |
|          | Reverse         | 5’-TTGCTGTGGAAGTCGCAGGAG-3’ |                             |
| TGFβ1    | Forward         | 5’-GACTCTCCACCTGCAAGACC-3’ | 100                         |
|          | Reverse         | 5’-GGACTGGCGAGCCCTTAGTT-3’ |                             |
| Collagen I | Forward         | 5’-GTGTTTCTGTGCTACGT-3’ | 132                         |
|          | Reverse         | 5’-TCTTTCTCCTCCTGACCT-3’ |                             |
| α-SMA    | Forward         | 5’-CCTCGCCTCTACCTCTA-3’ | 120                         |
|          | Reverse         | 5’-ATTCCGCTTGCCTTTCCT-3’ |                             |

**Western Blot Analysis**

To evaluate the TGFβ1 concentration in mBM-MSC, the total cellular protein was extracted by RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) containing an antiprotease cocktail (1 mmol/L PMSF, 1 mmol/L NaF and 1 mmol/L Na3VO4; US Biological Inc., Swampscott, MA, USA) according to the manufacturer’s instructions, quantified by a BCA protein assay kit (Beyotime), separated by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) (10%), electro-transferred to PVDF membranes (Millipore, Bedford, MA, USA) and then incubated with primary antibodies against TGFβ1 (1:5000 dilution; Abcam Incorporated, Cambridge, MA) or β-actin (1:3000 dilution; Abcam Incorporated, Cambridge, MA) at 4°C overnight. The blots were washed three times with TBST and then incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (1:5000 dilution; Zoonbio Biotechnology) for 1 h at room temperature. Immunoreactive complexes were visualized by chemiluminescence reagents (Thermo Fisher Scientific Inc., Waltham, MA, USA) and immunoreactive bands were obtained using a chemiluminescence imaging system (BioshineChemiQ4800 mini; Ouxiang, Shanghai, China). Finally, intensity of those bands was analysed
Protein Concentration In Culture Medium

mBM-MSC, mBM-MSC-NC, and mBM-MSC-TGFβ1 were seeded in a 12-well plate at a density of $1 \times 10^5$ cells per well. After 12 h the culture medium was changed, and mBM-MSC were cultured in an incubator at 37 °C, 5% CO₂ for 24 h. The culture medium was then collected and TGFβ1 protein levels in the culture medium were quantified using an enzyme-linked immunosorbent assay (ELISA) kit (Abcam, USA) according to the manufacturer's instructions.

Cell Proliferation Assay

Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology, Haimen, China) assay was used to further investigate the effects of overexpressing TGFβ1 on mBM-MSC proliferation according to the manufacturer's instructions. Briefly, cells were seeded in 96-well plates at $2 \times 10^3$ cells per well in 100 µL of growth medium. After staining with CCK-8 (10 µL per well), the cells were incubated for 4 h at 37 °C. Absorbance was assessed at 450 nm with a microplate reader (Tecan, Switzerland).

Multi-differentiation Of mMSCs After Gene Transduction

For osteogenic differentiation, the cells were seeded in 6-well plates and cultured in 2 mL of DMEM/F12 supplemented with 10% FBS. When reached approximately 80–90% confluence, the cells were switched to C57BL/6 mouse MSC osteogenic differentiation medium (Cyagen Biosciences, Inc., Guangzhou, China) for 2–3 weeks. The calcium deposition was assessed by staining the cells with 40 mM Alizarin Red S solution at room temperature for 10 min.

For adipocytic differentiation, when reached confluence, the cells were treated with mouse MSC adipogenic differentiation basal medium A (Cyagen Biosciences, Inc., Guangzhou, China) for 3 days, followed by exchanged with mouse MSC adipogenic differentiation basal medium B (Cyagen Biosciences, Inc., Guangzhou, China) for 24 h and then switched back to basal medium A. After five to six cycles, the cells were cultured in basal medium B for 3 days until lipid vacuoles enlarged. To assess the accumulation of neutral lipid vacuoles, the cells were stained with filtered Oil red O solution for 10 min at room temperature, and the incorporated Oil red O was extracted by adding...
1 mL of isopropanol to each well at room temperature for 15 min.

For chondrogenic differentiation, \(2.5 \times 10^5\) cells were centrifuged in a 15 mL tube at 150 g for 5 min to form a pellet. Chondrogenic differentiation was processed by the three-dimensional culture method and C57BL/6 mouse MSC chondrogenic differentiation medium (Cyagen Biosciences, Inc., Guangzhou, China). After 28 days, the pellets were embedded in paraffin and then fixed in dimethylbenzene and ethanol. Five micrometer slides were cut and stained with Alcian Blue to determine polysaccharide amine combination.

In vitro scratch assay

The horizontal migration of cells was determined by the in vitro scratch assay. Cells were seeded in six-well culture plates. After reaching approximately 100% confluence, a scratch was made with a 10 µl sterile pipette tip. And then the cells were cultured in serum-free DMEM/F12 for another 12 h. The images of the wound area were recorded by a light microscope immediately after scratching and 12 h later. The horizontal migration ability of the cells was quantified by measuring the wound area in each group by Image J analysis software.

Transwell Migration Assay

The vertical migration of cells was determined by the Transwell migration assay. Transwell inserts (6.5 mm diameter and 8 mm pore size; Millipore) which were seeded with \(2 \times 10^4\) cells in 100µL of serum-free DMEM/F12 were loaded into lower chambers with 600 µL of DMEM/F12 supplemented with 10% FBS. After incubated for 12 h, the cells remaining on the upper surface of the inserts were removed with cotton swabs, and the cells that had migrated to the lower surface were stained with crystal violet (Beyotime Institute of Biotechnology, Haimen, China) for 20 min. The stained cells from four randomly chosen areas were measured under a light microscope.

Ethics Statement

All animal experiments in this study were performed in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Southeast University. Wild-type (WT) C57BL/6 mice aged 6–8 weeks were purchased from the
Laboratory Animal Centre (Shanghai, China). And mice were housed in individual microisolator cages under specific pathogen-free conditions, with free access to water and chow.

Murine Model Of Lipopolysaccharide-induced Acute Respiratory Distress Syndrome

After anaesthetised with an intra-peritoneal injection of pentobarbital at 50 mg/kg, mice were subjected to intratracheal (i.t.) administration of LPS (2 mg/kg, Escherichia coli serotype 0111: B4; Sigma-Aldrich, St Louis, MO, USA) dissolved in 20 µL sterile normal saline (NS). Sham operation was performed in a similar manner with same volume of only 0.9% NS instead of LPS. Then, the mice recovered until fully awake in a 100% oxygen chamber.

Experimental Protocol

The mice were randomly divided into five groups as follows: Control group, mice received phosphate-buffered saline (PBS) intratracheally 4 hours after i.t. administration of 0.9% NS; ARDS group, mice received PBS intratracheally 4 hours after LPS challenge; the LPS + mBM-MSC group received WT mBM-MSC (2 × 10^5 cells per mouse) intratracheally; the LPS + mBM-MSC-NC group received mBM-MSC-NC (normal control, 2 × 10^5 cells per mouse) intratracheally; and the LPS + mBM-MSC-TGFβ1 group received mBM-MSC-TGFβ1 (overexpressing TGFβ1, 2 × 10^5 cells per mouse) intratracheally. The mice were sacrificed at 1d, 3d or 7d after mBM-MSC injection, and the lung lobes were collected for further analysis.

Lung Histopathology Analysis

The right lung lobes (n = 3 for each group at each time-point) were collected and fixed in 4% paraformaldehyde, embedded in paraffin and sliced into 5 µm sagittal sections. After stained with a haematoxylin and eosin staining kit (Beyotime Institute of Biotechnology, Haimen, China), the slices were then viewed by a pathologist based on ten randomly selected high-power fields (400×) in each section according to oedema, alveolar and interstitial inflammation, alveolar and interstitial haemorrhage, atelectasis and necrosis, which was graded on a 0- to 4-point scale (0, no injury; 1, injury in 25% of the field; 2, injury in 50% of the field; 3, injury in 75% of the field; and 4, injury throughout the entire field). The total lung injury score was calculated as the sum of these scores,
which has been described previously\textsuperscript{14−16}.

Preparation Of Lung Tissue Lymphocytes And Flow Cytometry Analysis

After the mice were sacrificed, 5 mL PBS/0.6 mm EDTA was injected into the right ventricular cannula for lung perfusion. The lung was then isolated from the surrounding tissue, and added to the medium containing digestive enzymes (RPMI 1640, 20 mM HEPES, 10% FCS, 175 U/mL collagenase, 75 U/mL DNase I, 0.2 U/mL pancreatic elastase, 35 U/mL hyaluronidase, 100 IU/mL penicillin, and 100 mg/mL streptomycin) for incubation of 45 minutes at 37 °C. The resulting suspension was passed three times through a 19-gauge needle to break up the clumps and then through a 40 mm filter to remove debris. The leukocytes were enriched by discontinuous Percoll gradient centrifugation and recovered at the interface between 40% Percoll and 70% Percoll layers\textsuperscript{17}.

The following antibodies (Miltenyi, USA) were used for surface and nuclear staining: FITC-labeled anti-CD4, APC-labeled anti-CD25, PE-labeled anti-Foxp3, and PE-labeled anti-ROR\textgreek{g}. For the analysis of Tregs, cells were incubated with surface marker antibodies FITC-anti-CD4 and APC-anti-CD25, followed by fixation and permeabilization with Foxp3 staining buffer (Miltenyi) and intracellular staining with PE anti-Foxp3. To detect the phenotypes of Th17 in lungs, cells were incubated with surface marker antibodies FITC-anti-CD4 and APC-anti-CD25. After then, the cells were fixed and permeabilized using ROR\textgreek{g} staining buffer (Miltenyi), followed by intracellular staining with PE anti-ROR\textgreek{g}.

Protein Concentration In Lungs And Bronchoalveolar Lavage Fluid

To analyse the expression of IL-17A, IL-10 and occludin in the lungs after transplantation, total protein lysates were extracted by RIPA lysis buffer (Beyotime) from left lung lobes (n = 3 per group at each time-point) and measured by western blot as previously described. The PVDF membranes were incubated with primary antibodies against IL-17A (1:5000 dilution, Abcam), IL-10 (1:5000 dilution, Abcam), occludin (1:5000 dilution; Abcam) or \textbeta-actin (1:3000 dilution; Abcam Incorporated, Cambridge, MA).

Bronchoalveolar lavage fluid (BALF) was collected by flushing 1 mL ice-cold PBS back and forth three times through a tracheal cannula as previously described\textsuperscript{14,15}. After centrifugation at 800 × g for 10
minutes, total protein (TP) and albumin (ALB) concentrations in the BALF were measured by ELISA kits (Cusabio Biotech, Wuhan, China).

Labelling And Tracing Of Mesenchymal Stem Cells

WT mBM-MSC, mBM-MSC-NC and mBM-MSC-TGFβ1 were labelled with CellVue NIR815 dye (eBioscience Inc., San Diego, CA, USA) according to the manufacturer’s instructions. Then, NIR815-labelled cells (5 × 10^5 cells) were directly administered into the trachea of the mice in different groups according to the protocol. After 1d, 3d and 7d post-transplantation, three mice at each time-point were sacrificed, and ex vivo lungs were imaged using a Maestro in vivo optical imaging system (excitation = 786 nm, emission = 814 nm, and 4,000 ms exposure time; Caliper Life Sciences, MA, Boston, USA)\(^\text{15, 16}\). The autofluorescence spectra were then unmixed based on their spectral patterns using Maestro 2.4 software (Caliper Life Sciences). The fluorescence intensity of the lungs was measured by placing the regions of interest (ROIs) on the lungs, and the signals were analysed based on the total fluorescence counts of the ROIs.

Evaluation Of Lung Oedema

Lung oedema was evaluated using the ratio of lung wet weight to body weight (LWW/BW) that was measured as previously described\(^\text{16}\). Briefly, the whole lung was removed and cleared of all extrapulmonary tissues, and the LWW/BW was calculated based on the values of the lung wet weight and the body weight (mg/g).

Lung Fibrosis Analysis

The lung sections were stained sequentially with Weigert’s iron haematoxylin solution, Biebrich scarlet-acid fuchsina solution and aniline blue solution, and a blue signal indicated positive staining for collagen. The criteria of Ashcroft were used\(^\text{15, 16}\) to assess lung fibrosis, which was quantified based on the findings in ten randomly selected high-power fields (400×) for each slide by histopathologists blinded to the protocol. Collagen-I and α-SMA mRNA expression in lung tissues was measured by RT-PCR.

Statistical analysis
The data are presented as the means ± standard deviation (SD). Statistical analyses were performed using SPSS 26.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8 (GraphPad Software, La Jolla California USA). Comparisons among multiple groups were performed by one-way ANOVA followed by Bonferroni’s post hoc test if the data were normally distributed. Kaplan–Meier curves were used to describe the survival rate of mice in each group, and log-rank tests were performed to analyze the significance of differences. A p-value < 0.05 was considered statistically significant.

Results
The Efficiency Of Lentiviral Vector-mediated TGFβ1 Overexpression In mMSCs
The transduction efficiency, which was reflected by the eGFP-positive cell ratio in our study, mediated by the lentiviral vectors after 20 passages was detected by fluorescence microscopy and flow cytometry analysis. Transduction efficiencies of overexpressing TGFβ1 were 82.3–88.6% (Fig. 1A&B). TGFβ1 mRNA levels in mMSCs was detected by qRT-PCR. It was showed that TGFβ1 mRNA expression was significantly higher in the mBM-MSC-TGFβ1 group than in the mBM-MSC-NC group (p < 0.0001). However, there was no significant difference between the mBM-MSC and mBM-MSC-NC group (p > 0.05) (Fig. 1C). And secreted TGFβ1 in the culture media of mMSCs was significantly higher in the mBM-MSC-TGFβ1 group than that in the mBM-MSC-NC group (p < 0.0001, Fig. 1D). It also showed similar results for TGFβ1 protein expression in mMSCs by western blot analysis (Fig. 1E). Thus, lentivirus-mediated TGFβ1 transduction is stable and efficient.

In this study, calcium deposition examined by Alizarin Red S staining, lipid accumulation by Oil red O staining and polysaccharide amine combination by Alcian Blue staining experiment were used to evaluate the multi-differentiation of mMSCs after gene transduction. It was showed that TGFβ1 transfection did not change the multi-potential differentiation ability of mMSCs (Fig. 2).

Effects Of Overexpressing Tgfβ1 On The Proliferation Of mMSCs
CCK-8 assay was used to evaluate the effects of overexpressing TGFβ1 on cell proliferation. By comparing different growth curves, it was found that overexpression of TGFβ1 significantly decreased cell proliferation in the mBM-MSC-TGFβ1 group compared to the mBM-MSC-NC from days 5 to 7 (p < 0.05). There was no significant difference between the mBM-MSC and mBM-MSC-NC group (p > 0.05,
Effects Of Overexpressing TGFβ1 On The Migration Of mMSCs

The scratch assay and transwell assay were used to indicate the horizontal and vertical migration abilities of mMSCs. There were no significant differences of the wound areas in the scratch assay among mBM-MSC, mBM-MSC-NC and mBM-MSC-TGFβ1 groups after 12 h of incubation (p > 0.05, Fig. 4A). Similar results were also detected in the transwell assay. No significant differences among the groups were observed (p > 0.05, Fig. 4B).

Mesenchymal stem cells overexpressing TGFβ1 improved the pulmonary histopathology of lipopolysaccharide-induced ARDS mice

After LPS-induced lung injury, alveolar wall thickening, alveolar and interstitial inflammatory cell infiltration, haemorrhage, alveolar exudate and oedema were observed in the lung tissues of ARDS group mice (Fig. 5A), and the Smith score for quantifying lung injury was also increased significantly (p < 0.05, Fig. 5B) in the ARDS group. However, compared to the ARDS group, histopathologic characteristics and the Smith score were alleviated at 3 and 7 d in the LPS + mBM-MSC and LPS + mBM-MSC-NC groups (p < 0.05, Fig. 5A&B). The effects were greater in the LPS + mBM-MSC-TGFβ1 group than in the LPS + mBM-MSC-NC group (p < 0.05, Fig. 5A&B).

Mesenchymal stem cells overexpressing TGFβ1 inhibited lung inflammation of lipopolysaccharide-induced ARDS mice

The differentiation of Th17 and Treg cells in lungs of LPS-induced ARDS mice were detected by flow cytometry. At 3 days after mMSCs transplantation, the number of CD4^+ T cells in the lungs increased significantly in the LPS + mBM-MSC, LPS + mBM-MSC-NC and LPS + mBM-MSC-TGFβ1 groups compared with the ARDS group (p < 0.05, Fig. 6A). Meanwhile, the proportion of Th17 cells significantly decreased in the LPS + mBM-MSC and LPS + mBM-MSC-NC groups than that in the ARDS group (p < 0.05) while significantly increased in the LPS + mBM-MSC-TGFβ1 group compared to that in the LPS + mBM-MSC-NC (p < 0.05), but still lower than that in the ARDS group (p < 0.05, Fig. 6B). In addition, the proportion of Treg cells in the LPS + mBM-MSC group and LPS + mBM-MSC-NC groups were significantly lower than that in the ARDS group (p < 0.05), and which in the LPS + mBM-MSC-TGFβ1
group was significantly higher than that in the LPS + mBM-MSC-NC group (p < 0.05), even higher than that in the ARDS group (p < 0.05, Fig. 6C). Moreover, Th17/Treg ratio in the LPS + mBM-MSC and LPS + mBM-MSC-NC groups was significantly lower than that in the ARDS group (p < 0.05), and which in the LPS + mBM-MSC-TGFβ1 group was further lower than that in LPS + mBM-MSC-NC group (p < 0.05, Fig. 6D).

In addition, similar results were also observed at 7 days after mMSCs transplantation. Compared with ARDS group, the number of CD4⁺T cells increased significantly in the lungs of LPS + mBM-MSC, LPS + mBM-MSC-NC and LPS + mBM-MSC-TGFβ1 groups (p < 0.05, Fig. 7A). At the same time, the proportion of Th17 cells in the LPS + mBM-MSC and LPS + mBM-MSC-NC groups decreased significantly compared to ARDS group (p < 0.05), and which in the LPS + mBM-MSC-TGFβ1 group decreased further relative to that in the LPS + mBM-MSC-NC group (p < 0.05, Fig. 7B). Besides, the proportion of Treg cells in the LPS + mBM-MSC and LPS + mBM-MSC-NC groups was significantly lower than that in the ARDS group (p < 0.05), and which in the LPS + mBM-MSC-TGFβ1 group was slightly higher than that in LPS + mBM-MSC-NC group (p < 0.05), but still lower than that in ARDS group (p < 0.05, Fig. 7C). Th17/Treg ratio in the LPS + mBM-MSC-TGFβ1 group was significantly lower than that in LPS + mBM-MSC-NC group (p < 0.05, Fig. 7D). It was suggested that mMSCs overexpressing TGFβ1 could further regulate the differentiation of Th17 and Treg and improve the balance of Th17/Treg in the lungs of ARDS mice.

The levels of IL-17A and IL-10 were measured in lung homogenates at 3 day and 7 days after mMSCs transplantation by western blot. Compared with the ARDS group, IL-17A was significantly decreased in the LPS + mBM-MSC and LPS + mBM-MSC-NC groups (p < 0.05). mMSCs overexpressing TGFβ1 could further reduce the concentration of IL-17A in the lungs compared with that in the LPS + mBM-MSC-NC group (p < 0.05, Fig. 8A). Meanwhile, compared with the ARDS group, IL-10 was significantly elevated in the LPS + mBM-MSC and LPS + mBM-MSC-NC groups (p < 0.05). mMSCs overexpressing TGFβ1 could further increase the concentration of IL-10 in the lungs compared with that in the LPS + mBM-MSC-NC group (p < 0.05, Fig. 8B).

It was suggested that mMSCs overexpressing TGFβ1 could inhibit the expression of IL-17A while increase IL-10 level in the lungs of ARDS mice.
Effects of overexpressing TGFβ1 on the graft retention of mMSCs in the lung after lipopolysaccharide challenge

To track intrapulmonary mMSCs, ex vivo NIR imaging was performed on the lungs at 1d, 3d and 7d after mMSCs administration. There were no significant differences of fluorescent counts of ROIs among the groups (p > 0.05, Fig. 9A&B).

Overexpression of TGFβ1 in mesenchymal stem cells improved the permeability of ARDS

The LWW/BW was calculated to evaluate lung oedema. The LWW/BW was significantly reduced in the LPS + mBM-MSC, LPS + mBM-MSC-NC and LPS + mBM-MSC-TGFβ1 groups compared with that in the ARDS group at 3d and 7 d (p < 0.05, Fig. 10A).

To evaluate whether mMSCs overexpressing TGFβ1 could make a difference in the permeability of the lung, total protein and albumin concentrations in the BALF were measured by mouse-specific ELISAs. The total protein and albumin concentrations were significantly reduced in the LPS + mBM-MSC and LPS + mBM-MSC-NC groups compared with those in the ARDS group at both 3d and 7 d (p < 0.05).

Significant decreases in the total protein and albumin concentrations were also observed in the LPS + mBM-MSC-TGFβ1 group compared with the LPS + mBM-MSC-NC group at 3d and 7d (p < 0.05, Fig. 10B&C).

Additionally, occludin protein expression level increased significantly in the LPS + mBM-MSC and LPS + mBM-MSC-NC groups compared with that in the ARDS group (p < 0.05). A significant increase was also observed in the LPS + mBM-MSC-TGFβ1 groups compared to the ARDS group (p < 0.05), and the increase was much greater than that in the LPS + mBM-MSC-NC group (p < 0.05, Fig. 10D).

Effects of mMSCs overexpressing TGFβ1 on the lung fibrosis in ARDS mice

To assess lung fibrosis, collagen deposition (which was stained as blue) in lung tissue at 7 d after LPS challenge was analysed by Masson’s trichrome staining and was markedly increased in the ARDS group compared with that in the control group (p < 0.0001). The lung fibrosis score decreased significantly in the LPS + mBM-MSC, LPS + mBM-MSC-NC and LPS + mBM-MSC-TGFβ1 groups compared to that in the ARDS group (p < 0.0001). Reduced collagen I and α-SMA mRNA was also observed after intervention with mBM-MSC, mBM-MSC-NC or mBM-MSC-TGFβ1 compared with the ARDS group (p <
0.0001), but the increased α-SMA mRNA was found in the LPS + mBM-MSC-TGFβ1 group than in the LPS + mBM-MSC-NC group (p < 0.0001, Fig. 11A-D).

Effects of mMSCs overexpressing TGFβ1 on the survival of ARDS mice
Mice that received LPS challenge statistically decreased the survival rate at 7d than the Control group (p < 0.05). Although there was no significant difference of the survival rate in the LPS + mBM-MSC, LPS + mBM-MSC-NC and LPS + mBM-MSC-TGFβ1 groups compared to ARDS group, an upward trend was still found after mMSCs treatment (p > 0.05, Fig. 12).

Discussion
Immunotherapy, as one of the important treatments for ARDS, has not made any breakthrough in the past two decades. A large number of studies have shown that MSCs, as multi-functional stem cells with low immunogenicity, could significantly improve the immunity state of ARDS in animal models. And the mechanism of which was mainly due to the regulation of cell function and state by paracrine a variety of cytokines. TGFβ1, as one of the main paracrine cytokines of MSCs, could regulate the systematic immunity state by modulating the differentiation of T cells in ARDS. In our study, we firstly constructed mMSCs with overexpression of TGFβ1, and the main findings were as follows: (1) Overexpressing TGF-β1 inhibited the proliferation of mMSCs during day 5–7, but made no effects on their differentiation or migration. (2) mMSCs overexpressing TGFβ1 improved histopathology of lung tissue, decreased permeability and inflammatory cytokines in ARDS mice. (3) mMSCs overexpressing TGFβ1 could significantly modulate the differentiation of T cells into Th17 and Treg while inhibiting the ratio of Th17 and Treg. And IL-17A was also decreased while IL-10 was increased in the lungs of ARDS mice after mMSCs overexpressing TGFβ1 treatment. (4) mMSCs overexpressing TGFβ1 did not aggravate the fibrosis of lungs in ARDS mice.

As an important part of adaptive immunity, T cell immunity is involved in the development of ARDS. Several studies have shown that specific regulation of T cell differentiation could improve the immunity and inflammation state of ARDS in animal models, resulting in improvement of their prognosis. It was also found in our study that the regulation of T cell differentiation in patients
with community-acquired pneumonia was directly related to their prognosis. Th17 and Treg, as paired CD4+ T cell subsets, are directly contributed with the prognosis of patients with ARDS. At present, the regulation of Th17 and Treg differentiation mainly depends on antigen-presenting cells, cytokines, and intracellular signaling pathways. However, TGFβ1, as an important factor in regulating T cell differentiation, was with very low expression in MSCs. In our study, overexpression of TGFβ1 in mMSCs by lentivirus transfection could significantly increase its mRNA and protein levels in the cells, as well as in the supernatant of culture media. But there was no significant effect on the differentiation and migration of MSCs after transduction. As for the inhibited migration of mMSCs overexpressing TGFβ1, it may be related to the involvement of TGFβ1 in the differentiation of MSCs via cell cycle regulation, which still needs further studies.

In was found in this study that the therapeutic effect of MSC after TGFβ1 transfection was significantly improved, which mainly due to the better regulation of T cell differentiation in ARDS mice on day 3 and day 7 and reduced Th17/Treg ratio, thus balancing inflammatory cytokines in vivo. In our previous studies, specify regulation of inflammatory cytokines such as ACE2 or PGE2 in MSCs could also improve their therapeutic effects for ARDS animals, which was in parallel with the results of this study. In addition, a recent study also indicated that MSCs could prevent the differentiation of naive CD4+T cells into Th17 cells, inhibit the production of inflammatory cytokines by Th17 cells, and induce the Treg phenotype in vitro. However, in our study, the effect of MSCs overexpressing TGFβ1 on CD4+T cells was mainly to increase the amount of Treg without reducing the number of Th17, which may be related to the animal status and detection methods. How to regulate Th17 and Treg differentiation specifically still needs further studies in vitro.

TGFβ1 is also an important index of fibrosis and inflammation. Despite this, pulmonary fibrosis of ARDS mice was also detected after MSCs transplantation. According to Masson’s staining or specific mRNA expression, it was suggested that MSCs overexpressing TGFβ1 did not significantly increase pulmonary fibrosis in ARDS mice, indicating that there might be no side effects related to MSCs.
transplantation.

This study still has several limitations. First, we induced ARDS by LPS in mice. The murine model of ARDS induced by LPS injection intratracheally just focused on inflammation in the lungs and cannot fully reflect the complexity of systematic inflammation as ARDS patients. Second, MSCs were only administered once intratracheally and the mice were then sacrificed at 3 and 7 days later after that, which may also not fully reflect the clinical application of such therapeutics and still needs further studies focusing on different routes and doses for MSCs treatment.

Conclusion

MSCs overexpressing TGFβ1 could regulate lung inflammation and attenuated lung injuries via modulating the imbalance of Th17/Treg in the lungs of ARDS mice.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All the authors have given final approval of the version to be published and have agreed to be accountable for all aspects of this work.

Availability of data and material

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the National Natural Science Foundations of China (No. 81571874, 81930058), the Postgraduate Research & Practice Innovation Program of Jiangsu Province (KYCX17_0168), Jiangsu Provincial Special Program of Medical Science (BE2019749, BE2018743), and National Science and Technology Major Project for Control and Prevention of Major Infectious Diseases of China (2017ZX10103004).

Consent for publication
Not applicable.

Authors’ contributions

CJX participated in the study design, performed the laboratory work and statistical analysis, prepared the drafts of the manuscript and revised the manuscript according to advice from the other authors. ZWX and XM participated in the laboratory work, performed the statistical analysis and drafted the manuscript. XJF participated in the study design and assisted in the statistical analysis. LL and YY participated in the study design and helped to revise the manuscript. QHB were responsible for the study design and revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

Acknowledgements

None declared.

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Figures
Figure 1

Measurement of TGFβ1 expression in mMSCs after lentiviral vector transfection. A. The mBM-MSC, mBM-MSC-NC, and mBM-MSC-TGFβ1 were cultured for 20 passages and observed with light microscopy (top) and fluorescence microscopy with green fluorescent protein (bottom), 200×. B. The percentage of GFP-positive cells was analyzed by flow cytometry at passage 20 after transduction. C. Evaluation of TGFβ1 mRNA expression in mMSCs after transfection (n=3; *p<0.0001 vs. mBM-MSC-NC). D. Evaluation of secreted TGFβ1 in the culture media of mMSCs after transfection by ELISA (n=3; *p<0.0001 vs. mBM-MSC-NC). E. Detection of TGFβ1 protein levels in mMSCs after transfection by Western blot analysis (n=3; *p<0.0001 vs. mBM-MSC-NC). mMSCs, mouse mesenchymal stem cells; NC,
normal control.

Figure 2

The effects of overexpressing TGFβ1 on multi-abilities of mMSCs. A. The effects of overexpressing TGFβ1 on the proliferation of mMSCs. The growth curves of the cells after transduction for 7 days were evaluated by CCK-8 assay (n=3; *p<0.05 vs. mBM-MSC-NC). B. The effects of overexpressing TGFβ1 on the multi potential differentiation of mMSCs. The cells were stained with Alizarin Red S at day 21 to detect calcium deposition (top), with Oil red O after 28 days of adipogenic differentiation to detect the accumulation of neutral lipid vacuoles (middle), and with Alcian Blue after 28 days of chondrogenic differentiation with three-dimensional culture method to detect polysaccharide amine combination (bottom), 200×. C. The effects of overexpressing TGFβ1 on the horizontal migration of mMSCs, which was examined by in vitro scratch assay. The wound areas were photographed at 0 and 12 h.
and quantified by measuring the wound area in each group, 100× (n=3). D. The effects of overexpressing TGFβ1 on the vertical migration of mMSCs, which was examined by Transwell assay. The migrated cells on the lower surface of Transwell inserts were stained with crystal violet and measured under a light microscope form four randomly chosen areas, 100×(n=3). CCK-8, cell counting kit-8; mMSCs, mouse mesenchymal stem cells; NC, normal control.
The effect of overexpressing TGFβ1 on the graft retention of mMSCs in the lungs after LPS challenge. A. Typical ex vivo NIR images of injured lungs from mice at 1d, 3d, and 7d after mMSCs administration. B. Average signal from ex vivo NIR imaging of injured lungs from mice at 1d, 3d, and 7d after mMSCs administration (n = 3). mMSCs, mouse mesenchymal stem cells; NIR, near-infrared fluorescent.
Figure 4

The effect of mMSCs overexpressing TGFβ1 on the differentiation of CD4+ T cells in lungs of ARDS mice at 3d after mMSCs transplantation. A-D. The differentiation of Th17 and Treg cells in lungs of LPS-induced ARDS mice were detected by flow cytometry (n = 3, *p<0.05 compared with the Control group; #p<0.05 compared with the ARDS group; δp<0.05 compared with the LPS+mBM-MSC-NC group). E. Representative flow cytometry analysis were shown for each treatment group. ARDS, acute respiratory distress syndrome; mMSCs, mouse mesenchymal stem cells; NC, normal control.
Figure 5

The effect of mMSCs overexpressing TGFβ1 on the differentiation of CD4+ T cells in lungs of ARDS mice at 7d after mMSCs transplantation. A-D. The differentiation of Th17 and Treg cells in lungs of LPS-induced ARDS mice were detected by flow cytometry (n = 3, *p<0.05 compared with the Control group; #p<0.05 compared with the ARDS group; δp<0.05 compared with the LPS+mBM-MSC-NC group). E. Representative flow cytometry analysis were shown for each treatment group. ARDS, acute respiratory distress syndrome; mMSCs, mouse mesenchymal stem cells; NC, normal control.
The effect of mMSCs overexpressing TGFβ1 on the inflammatory factors in lungs of ARDS mice. A. Expression of the IL-17A and B. IL-10 protein in the lungs of all the experimental groups at 3 and 7d after mMSCs administration was evaluated by western blot analysis (n = 3, *p<0.05 compared with the Control group; #p<0.05 compared with the ARDS group; δp<0.05 compared with the LPS+mBM-MSC-NC group). ARDS, acute respiratory distress syndrome; mMSCs, mouse mesenchymal stem cells; NC, normal control.
The effect of mMSCs overexpressing TGFβ1 on the permeability of LPS-induced lungs in mice. A. Lung oedema was analysed by LWW/BW. The results are shown for samples of all the experimental groups at 3 and 7d after LPS exposure (n = 3, *p<0.05 compared with the Control group; #p<0.05 compared with the ARDS group). B. Total protein and C. albumin concentrations in bronchoalveolar lavage fluid were analysed by a mouse-specific ELISA kit to evaluate the permeability of the lung (n = 3, *p<0.05 compared with the Control group; #p<0.05 compared with the ARDS group; δp<0.05 compared with the LPS+mBM-MSC-NC group). D. Expression of the occludin protein in the lungs of all the experimental groups at 3 and 7d after mMSCs administration was evaluated by western blot analysis (n = 3, *p<0.05 compared with the Control group; #p<0.05 compared with the ARDS group; δp<0.05 compared with the LPS+mBM-MSC-NC group). ALB, albumin; ARDS, acute respiratory distress syndrome; ELISA, enzyme-linked immunosorbent assay; LWW/BW, lung wet weight/body weight; mMSCs, mouse mesenchymal stem cells; NC, normal control; TP, total protein.
The effect of mMSCs overexpressing TGFβ1 on the histopathology of LPS-induced lung injury in mice. A. Typical histopathological images of the lung tissues from mice of all the experimental groups at 3d and 7d after LPS exposure (HE staining, 50×). B. Lung injury score (Smith score) of the lung tissues from mice of all the experimental groups at 3d and 7d after LPS challenge (n = 3 at each time-point for each group; *p<0.05 compared with the Control group; #p<0.05 compared with the ARDS group; δp<0.05 compared with the LPS+mBM-MSC-NC group). ARDS, acute respiratory distress syndrome; mMSCs, mouse mesenchymal stem cells; NC, normal control.
The effect of mMSCs overexpressing TGFβ1 on lung fibrosis in ARDS mice. A. Lung fibrosis was evaluated by Masson’s trichrome staining at 7d after LPS exposure (200×). A blue signal indicated positive staining for collagen and a red signal represented muscle fibrosis and cellulose. B. The quantification of lung fibrosis is shown as an Ashcroft score (n = 10, *p<0.05 compared with the Control group; #p<0.05 compared with the ARDS group). C. Collagen I and D. α-SMA mRNA in lungs were quantified by qRT-PCR (n = 3 at each time-point for each group; *p<0.05 compared with the Control group; #p<0.05 compared with the ARDS group; δp<0.05 compared with the LPS+mBM-MSC-NC group). ARDS, acute respiratory distress syndrome; α-SMA, alpha smooth muscle actin; mMSCs, mouse mesenchymal stem cells; NC, normal control.
Kaplan-Meier survival curves. Mice that received LPS challenge in each group were followed 7d after mMSCs administration (n = 18 for each group, *p<0.05 compared with the Control group).

Figure 11 not included with this version.

Figure 12 not included with this version.