**Online Data Supplement for**

**Mesenchymal stem cell-derived extracellular vesicles alleviate acute lung injury via transfer of miR-27a-3p**

Jiangmei Wang1; Ruoqiong Huang1; Qi Xu1; Guoping Zheng2; Guanguan Qiu2; Menghua Ge2; Qiang Shu1; Jianguo Xu2, 3 \*

**Supplementary Materials and Methods:**

**Isolation and culture of adipose-derived mesenchymal stem cells (MSCs)**

Subcutaneous adipose tissue was obtained from a healthy human donor (25 years old) during routine liposuction after informed consent. Protocol and procedures were previewed and preapproved by the ethical committee of Shaoxing Second Hospital. Adipose tissue was extensively washed with cold phosphate-buffered saline (PBS) until a clear solution was obtained. The adipose tissue sample was digested with 0.1% collagenase IA (Sigma, St. Louis, MO) in PBS at 37°C for 60 minutes and centrifuged at 300 x g for 5 minutes at room temperature. The cell pellets were cultured with expansion medium consisted of Dulbecco’s modified Eagle medium (DMEM, Thermo Fisher Scientific, Waltham, MA**)**) low glucose with 10% fetal bovine serum (FBS) plus epidermal growth factor (EGF) and fibroblast growth factor (FGF; R&D Systems, Minneapolis, MN, USA) and penicillin/streptomycin antibiotics (Thermo Fisher Scientific) in a humidified incubator at 37°C with 5% CO2 until approximately 80% confluence. Cells were then treated with 0.5 % trypsin-EDTA and re-plated at 1 x 104 cells/cm2 for more passages. The resulting MSCs in passage 4-6 were used for isolation of EVs.

**Isolation and treatment of mouse bone marrow-derived macrophages (BMDMs).**

For BMDMs, bone marrow cells were obtained from C57BL/6 mice (6-8 weeks old) by flushing the femurs and tibias with cold PBS. After red blood cells were removed by treating with 1x lysis buffer (10 mM KHCO3, 155 mM NH4Cl, 0.1 mM EDTA) for 3 minutes, cells were washed with PBS and resuspended in culture medium containing DMEM supplemented with 20 ng/ml GM-CSF (PeproTech, Rock Hill, NJ), 10% FBS (Biological Industries, Cromwell, CT), and 1% penicillin plus streptomycin at 37 oC with 5% CO2 overnight. Non-adherent bone marrow cells were collected and seeded on 6-well culture plates (2 x 106 cells/well). Fresh culture medium was changed every 3 days. Adherent BMDMs were dissociated with lidocaine/EDTA (0.4% lidocaine, 5 µM EDTA) after 7 days, counted, and reseeded onto cell culture plates for further experiments. BMDMs (3 x 105 cells) were cultured alone or cultured with MSC-EVs (100 µg/ml) in a standard 24-well plate and simultaneously stimulated with or without LPS for 24 h. Then, BMDMs were treated with Trizol and harvested for qRT-PCR analysis.

### Isolation of MSC-EVs

MSCs were cultured in DMEM low glucose supplemented with EV-depleted FBS obtained from ultracentrifugation (118,000 g for 16 h at 4 °C) until confluence. The culture medium was collected and centrifuged sequentially for 15 min at 1500 g and 30 min at 16,500 g, followed by centrifugation for 2 h at 118,000 g at 4°C in a swinging bucket rotor (Optima XPN- 80, SW 32 Ti rotor, Beckman Coulter). The resulting pellet was resuspended in 100-500 μl of PBS depending on the amount of starting volume of medium and stored at -80°C for further analysis. The protein concentration of EVs was quantified by a BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA).

**Transmission electron microscopy (TEM)**

Fresh EVs samples were isolated as mentioned above and resuspended in 100 µl of PBS. Samples were absorbed onto 200 mesh formvar copper grids.Then, the grids were negatively stained with 2% aqueous uranyl acetate for 30 sec and dried at room temperature. The grids were observed on a transmission electron microscope (FEI Tecnai Spirit G2) to view the EVs.

**Dynamic light scattering for EV size analysis**

Dynamic light scattering for EV size determination was performed per manufacturer’s instruction (Zetasizer Nano ZS90, Malvern Panalytical, Malvern, United Kingdom).

**Nanoparticle tracking analysis for particle analysis**

The particle number and concentration of EVs were measured using nanoparticle tracking analysis via ZetaView PMX 110 (Particle Metrix, Meerbush, Germany). Samples were diluted into appropriate concentrations using PBS and measured according to the operating instructions.

**Western blot analysis**

Whole-cell lysates and MSC-EVs were prepared in lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 0.5% Nonidet P (NP)-40, 1 mM Na3VO4, and 1 mM PMSF). The protein concentration was examined using a BCA protein assay kit. Equal amount of protein extracts (5 μg) was separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidenefluoride membranes (Millipore, Billerica, MA), and probed with the primary antibodies to CD63, CD81, CD105, CD44, CD40, GM130, calnexin, and NFKB1. After washing, membranes were incubated with secondary antibodies conjugated to horseradish peroxidase. The signals were detected via enzyme-linked chemiluminescence using the EZ-ECL kit (Biological Industries, Kibbutz Beit-Haemek, Israel).

**EV uptake assay in vitro and in vivo**

Isolated MSC-EVs were blocked with 5% BSA for 1 h, washed with PBS, and ultra-centrifuged at 118,000 g for 2 h at 4°C. Supernatant was discarded, and the EV pellet was resuspended with 100 µl of PBS. EVs were then incubated with or without mouse anti-CD63 antibody (Abcam, Cambridge, MA) overnight and washed for the second time. After that, EVs were incubated with cy3-conjugated donkey anti-mouse secondary antibody (MilliporeSigma, Burlington, MA) for 2 h and washed for the third time. For in vitro assay in THP-1 cells, THP-1 cells were seeded onto 6-well plate at 5 x 105 cells per well and incubated with CD63-labeled EVs for 24 h at 37 °C. Cells were stained with 1 μg/ml Hoechst 33342 (ImmunoChemistry Technologies, Bloomington, MN) to label the nucleus. Cells were imaged by confocal laser scanning microscopy (Carl Zeiss MicroImaging, Heidelberg, Germany) on confocal dishes. For in vitro assay in BMDMs, BMDMs were seeded onto 12-well plate at 8 x 105 cells per well and incubated with CD63-labelled EVs for 6 h at 37°C. Cells were collected and stained with PE-Cy7-conjugated anti-mouse F4/80 (Thermo Fisher Scientific). Cells were washed with PBS plus 2% BSA, 4 mM EDTA, and 0.01% NaN3. Data were collected on a BD LSRFortessa™ flow cytometer and analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

For in vivo assay, isolated MSC-EVs were labeled with Vybrant™ DiD cell-labeling solution for 10 min (5 μl/ml, Thermo Fisher Scientific). After labeling, EVs were washed with PBS and ultracentrifuged at 118,000 g for 2 h at 4°C. Supernatant was discarded, and the EV pellet was resuspended with 100 µl of PBS. Mice were treated with 5 mg/kg of LPS intratracheally (IT) and administered with labeled EVs (50 µg/0.05 ml) IT 30 minutes later. Bronchoalveolar lavage (BAL) was harvested 24 h later by flushing the lungs with 0.5 ml of cold PBS for 3 times. BAL was centrifuged at 400 x g for 5 min to collect total cells. Total cells were incubated with PE-Cy7-conjugated anti-mouse F4/80 antibody at 4°C for 30 min to label macrophages. Cells were washed with PBS plus 2% BSA, 4 mM EDTA, and 0.01% NaN3. Then, cells were analyzed via a BD LSRFortessa™ flow cytometer.

**LPS-induced lung injury model and harvesting alveolar macrophages from BAL**

C57BL/6 mice (6-8 weeks old) were purchased from Shanghai Laboratory Animal Center (Shanghai, China) and used in all experiments. All mice were housed in a temperature-controlled room at the Zhejiang University Laboratory Animal Center. Protocols for animal research were preapproved by the Institutional Animal Care and Use Committee of Zhejiang University School of Medicine. Approximately 265 mice in total were used in the study, of which 30 were lost due to death or loss of instilled solutions. Mice within each cage were randomly selected and allocated into 5 treatment groups in random order: control, LPS, LPS + MSCs intravenously (IV), LPS + EVs IV, and LPS + EVs intratracheally (IT). LPS from Escherichia coli O111:B4 was acquired from Sigma (St. Louis, MO) and diluted in 2 mg/ml of PBS. Mice were anaesthetized with 4% trichloroacetaldehyde and instilled with 5 mg/kg of LPS or PBS IT (approximately 50 µL). Based on our experience with C57BL/6 mice, the optimal dose of LPS to induce significant acute lung injury without high death rate within 48 h was 5 mg/kg. Thirty minutes after PBS or LPS treatment, 1 × 106 cells, 50 µg of EVs or PBS (0.2 mL) were administered via the tail veins of the mice. In another group, 50 µg (50 µL) of EVs was administered via IT. Animals were sacrificed at 48 h after LPS or PBS treatment. Lungs and BAL samples were collected for analysis. To separate alveolar macrophages, BAL was centrifuged at 400 x g for 5 minutes at 4 oC. The cell pellet was resuspended with RPMI-1640 at 2.5 x 105 cells per ml and plated 2 ml into each 6-well culture dish. The cells were then allowed to adhere to the bottom of the wells by incubating in a 37 oC humidified incubator with 5% CO2. Non-adherent cells and debris were removed by changing the culture medium. Cells were further incubated for 24 h prior harvest. Approximately 90% of the adherent cells were positive for macrophage markers.

**Inﬂammatory cell counts, protein, and cytokines in BAL**

To obtain BAL cells from mice, lungs were lavaged three times with 0.5 ml of cold PBS. Total cell counts in the BAL were determined using a hemocytometer. BAL was labeled with FITC-conjugated anti-mouse Ly-6G (Gr-1) antibody (Thermo Fisher Scientific) and analyzed for percentage of neutrophil via flow cytometry. Neutrophil counts were calculated by multiplying the percentage of neutrophil by the total number of white blood cells in the BAL. BAL was then centrifuged at 400g for 5 minutes to collect supernatant for analysis of total protein and cytokine levels. Protein concentration in BAL, which is an index of lung injury and capillary leakage, was determined using a BCA Protein Assay Kit. IL-1β, IL-6 and TNF-α levels in the BAL were analyzed by multiplex cytokine array (Millipore, Billerica, MA). Assay was performed using a Qiagen LiquiChip 200 machine and analyzed via LiquiChip Analyzer Software (Qiagen, Venlo, Netherlands).

#### **Transducing anti-miR-27a-3p into MSCs and BMDMs**

Anti-mouse-miR-27a-3p and control anti-miR lentivirus were purchased from Genechem (Shanghai, China). The control anti-miR had no homologous sequence in the mouse genome. Primary BMDMs and human MSCs were infected with a mixture containing polybrene (5 μg/ml, Genechem) and lentiviruses (multiplicity of infection of 60). Four days after the transduction, the transduction efficiency was determined from the proportion of GFP-positive MSCs and BMDMs using flow cytometry. BMDMs were cocultured with MSC-EVs (100 µg/ml) and simultaneously stimulated with LPS (100 ng/mL) for 24 hours. Then, BMDMs were harvested for qRT-PCR analysis. MSC-EVs from transduced MSCs were prepared as described earlier and used for in vivo experiments.

**Microarray analysis**

THP-1 is an immortalized monocyte-like cell line derived from the peripheral blood of a case of acute monocytic leukemia. THP-1 cells were cultured alone (LPS) or cocultured with MSCs (LPS + MSCs) via Transwell and treated with LPS (100 ng/ml) for 24 h. THP-1 cells in both groups were harvested and preserved with Trizol. Total RNA was extracted using the mirVana miRNA Isolation Kit (Ambion, Austin, TX) per the manufacturer’s protocol. Microarray experiments were performed using the miRCURY LNA Array platform (Exiqon, Vedbaek, Denmark). Data were analyzed via GenePix Pro 6.0 software (Molecular Devices, San Jose, CA). After normalization, significant differentially expressed miRNAs between two groups were identified through fold change and p-value. The threshold value to define an upregulation of miRNAs was a fold change of 1.5 with *p* < 0.1. Finally, heat map was performed to show distinguishable miRNA expression profiling among samples.

**Histopathology**

BAL collection and lung tissue collection were performed on separate mice. To harvest the lungs for immunohistochemistry, the tracheas were cannulated with a needle and the lungs fixed with 0.6 ml of chilled 4% paraformaldehyde. After overnight fixation, lung tissue was embedded in paraffin wax and sliced at 5 μm in thickness. Tissue sections were then stained with Hematoxylin and eosin (H&E) to examine the morphology and inflammatory infiltrate. Images were taken with an Olympus BX53 microscope (Shinjuku, Tokyo, Japan).

**Quantitative real-time PCR (qRT-PCR)**

Total RNA was extracted from the cells using Trizol reagent (Thermo Fisher Scientific). The RNA concentration was evaluated by Nanodrop Spectrophotometer (ND-2000; Thermo Fisher Scientific). Mature miR-27a-3p and pri-miR-27a were determined using Bulge-LoopTM miRNA qRT-PCR Starter Kit (Ribobio Co.Ltd, Guangdong, China) followed by real-time PCR using MicroRNA assay kit (Ribobio Co.Ltd, Guangdong, China). mRNA was assayed by the Superscript III First-strand kit (Thermo Fisher Scientific) followed by Power SYBR Green RT-PCR reagent kit (Takara Bio, Kusatsu, Japan). Values were normalized by the expression levels of β-actin for mRNA or U6 for miRNA and expressed as relative expression compared with control samples. miR-27a-3p primer was ordered from GeneCopoeia (Rockville, MD). The following primer sequences were used: U6 snRNA 5′TCGTGAAGCGTTCCATATTTTTAA3′; IL-1β forward 5'GAAATGCCACCTTTTGACAGTG3', reverse 5'TGGATGCTCTCATCAGGACAG3'; TNF-α forward 5'CAGGCGGTGCCTATGTCTC3', reverse 5'CGATCACCCCGAAGTTCAGTAG3'; iNOS forward 5'CAGGCTGGAAGCTGTAACAAAG3', reverse 5'GAAGTCATGTTTGCCGTCACTC3'; YM-1 forward 5'CAGGTCTGGCAATTCTTCTGAA3', reverse 5'GTCTTGCTCATGTGTGTAAGTGA3'; CD206 forward, 5'CTCTGTTCAGCTATTGGACGC3', reverse 5'TGGCACTCCCAAACATAATTTGA3'; arginase-1 (ARG-1) forward 5'CTCCAAGCCAAAGTCCTTAGAG3', reverse 5'GGAGCTGTCATTAGGGACATCA3'; IL-10 forward 5'CTTACTGACTGGCATGAGGATCA3', reverse 5'GCAGCTCTAGGAGCATGTGG3'; FIZZ 1 forward 5'AGGTCAAGGAACTTCTTGCCAATCC3', reverse 5'AAGCACACCCAGTAGCAGTCATCCC3'; β-actin forward 5'CGTTGACATCCGTAAAGACC3', reverse 5'AACAGTCCGCCTAGAAGCAC3'; pri-miR-27a forward 5'CTATCATGACAACTGGCCTGAG3', reverse 5'GACTTTGCTGTGGACCTTGC3'; NFKB1 forward 5'ATGGCAGACGATGATCCCTAC3', reverse 5'TGTTGACAGTGGTATTTCTGGTG3'.

**Knockdown of miR-27a-3p in mice**

Lenti-anti-miR-27a-3p virus (1 x 107 infectious units/mouse in 50 µl PBS), or an identical amount of anti-miR control was intratracheally instilled into anesthetized C57BL/6 mice 5 days before LPS treatment. GFP positive macrophages in BAL were examined via fluorescence microscopy 5 days later. Then, mice were subjected to LPS-induced lung injury delineated above.

**Flow cytometry for alveolar macrophages from in vivo studies**

For determination of M2 macrophages in BAL, cells were labelled with surface markers of PE anti-mouse F4/80 (BioLegend, San Diego, CA) and APC anti-mouse CD206 (Thermo Fisher Scientific). Cells were then washed and resuspended with PBS for flow cytometry analysis. All data were collected on a flow cytometer (BD LSRFortessa™) and analyzed using FlowJo vX software.

**Phagocytosis assay in vitro**

Cultured BMDMs were incubated with DMEM with 2% FBS for 2 h. After that, BMDMs were cultured alone, with LPS (100 ng/ml), or with MSC-EVs (100 µg/ml) plus LPS (100 ng/mL). BMDMs were then cultured with fresh DMEM along with ﬂuorescently labeled heat-inactivated *E coli* (K-12 strain) BioParticles™, Alexa Fluor™ 594 conjugate (Thermo Fisher Scientific), for 30 minutes per the manufacturer’s instructions. Cells were harvested and examined for Alexa Fluor 594 fluorescence intensity via flow cytometry. To determine the role of miR-27a-3p on phagocytosis, BMDMs were transduced with anti-miR-27a-3p or control anti-miR lentivirus. Four days later, BMDMs were cocultured with MSC-EVs (100 µg/ml) and simultaneously stimulated with LPS (100 ng/mL) for 24 hours. The same assay protocol was followed to assess the phagocytotic activity. Fluorescence intensity was measured as image-integrated pixel intensity per cell using ImageJ software.

**Dual luciferase assay**

For dual luciferase assay, a 206 bp 3′UTR segment and a mutant segment of NFKB1 corresponding to the putative binding site of miR-27a-3p were synthesized and inserted into the pGL3 vector (Promega, Madison, WI). pcDNA-miR-27a-3p was constructed by inserting miR-27a-3p into pcDNA3.1(+) vector (Invitrogen). HEK293 cells were co-transfected in 24-well plates with either 0.1 μg of pGL3-NFKB1 3’UTR or 0.1 μg of mutant pGL3-NFKB1 3’UTR along with 0.4 μg of pcDNA-miR-27a-3p or empty pcDNA vector. Firefly and Renilla luciferase activities in cell lysates were measured 48 h after transfection using a dual luciferase assay kit (Promega).

**Statistical methods**

Data are presented as mean ± standard deviation of the mean (SD). Student’s t test or one-way analysis of variance (ANOVA) with Bonferroni’s post hoc analysis was performed for parametric data with n ≥ 8. The Mann-Whitney test or Kruskal-Wallis test with Dunn’s post hoc analysis was performed for small sample size (n < 8) and non-parametric data with n ≥ 8. Data were tested for normality by using the D’Agostino-Pearson Omnibus normality test, the Shapiro-Wilk normality test, and Kolmogorov-Smirnov test with Dallal-Wilkinson-Lilliefor corrected P value. Statistical analysis was carried out using the GraphPad Prism 7. Results were considered significant if p < 0.05.