AMSC-EVs Attenuate Acute Kidney Injury Through TXNIP-IKKα/NFκB Signaling-mediated Renal CX3CR1^{+} Macrophage Polarization

Wei-juan Deng[1,2]*, Rui Tang[1]*, Meng-qing Ma[3]*, Hao Zhang[3], Chang-chun Cao[1]

1. Department of Nephrology, Sir Run Run hospital, Nanjing Medical University, Nanjing, China

2. Department of Nephrology, Jiujiang No.1 People's Hospital, Jiujiang, China

3. Department of Nephrology, Nanjing First Hospital, Nanjing Medical University, Nanjing, China

Corresponding author:

Sir Run Run Hospital, Nanjing Medical University, No. 109 Longmian Road, Nanjing 211112, China (Chang-chun Cao). E-mail: caochangchun@njmu.edu.cn (Chang-Chun Cao)

*These authors contributed equally to this work.

SUPPLEMENTARY FIGURE LEGEND

Figure S1. Isolation and characterization of AMSC. (A) AMSC-specific surface markers such as CD34, CD45, CD90, CD73, CD105 and HLR-DR measured by flow cytometry. (B) AMSC in vitro exhibited the typical morphology of shuttle-forming fibroblasts. (C) Positive Alcian Blue staining of AMSC assessed after osteogenic differentiation induction for 3 weeks. (D) Positive Oil-Red-O staining of AMSC was assessed after adipogenic differentiation induction for 2 weeks.

Figure S2. Depletion of CX3CR1^{+} macrophages. (A) Schematic diagram illustrating the experimental procedure in this section. (B) Representative contour plots of flow cytometric analysis of renal tissue macrophages in each group. Untreated refers to the group not injected with DT, littermate mice represent the non-depleted control group from the same litter, CX3CR1-DTR indicates the double

heterozygous CX3CR1^{+} macrophage depletion group. (C) Bar graph showing the proportion of macrophages (F4/80^{+}CD11b^{+}) to total leukocytes (CD45^{+} cells) in each group, n=3. The information is displayed as mean ± SEM, ****p < 0.0001.

Figure S3. Flow cytometry analysis of TXNIP expression in CX3CR1^{+} macrophages. (A) Effect of AMSC-EVs on TXNIP expression in CX3CR1^{+} macrophages of kidney tissue from cisplatin-induced AKI mice (n=3). (B) Effect of AMSC-EVs on TXNIP expression in THP-1 cells (n=3). Data are presented as mean ± SEM, *P < 0.05, **P < 0.01.

Figure S4. Cisplatin alters thioredoxin-system components in vivo and in THP-1 Macrophages. (A) RT-qPCR analysis of Trx1, Txnrd1, Trx2, and Txnrd2 mRNA in cisplatin-treated mice (n=4–6). (B) Western blot analysis of Trx1 and Txnrd1 protein in cisplatin-treated mice (n=3). (C) RT-qPCR analysis of Trx1and Txnrd1 mRNA in cisplatin-treated THP-1 cells (n=6). (D) Western blot analysis of Trx1 and Txnrd1 protein in cisplatin-treated THP-1 cells (n=3). Data are presented as mean ± SEM, ns, not significant; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

Figure S5. Flow cytometry analysis of macrophage polarization in THP-1 cells after treatment with AMSC-EVs and TXNIP overexpression. (A) Representative plots of CD86 expression in THP-1 cells: control, cisplatin-treated, AMSC-EVs-treated, and AMSC-EVs + TXNIP overexpression groups. (B) Representative plots of CD206 expression across the same experimental groups. (C) Quantification of CD68^{+}CD86^{+}macrophages in each group (n=3). (D) Quantification of CD68^{+}CD206^{+}macrophages in each group (n=3). Data are presented as mean ± SEM, *p < 0.05, ***p < 0.001, ****p < 0.0001.

SUPPLEMENTAL METHODS

1. AMSC isolation and characterization

For surface marker analysis, third-passage AMSC were collected and washed with PBS. Monoclonal antibodies against PE-CD34 (343605, BioLegend, USA), FITC-CD45 (304005, BioLegend, USA), APC-HLA-DR (307609, BioLegend, USA), PE-CD73 (344003, BioLegend, USA), PE-Cy7-CD90 (328123, BioLegend, USA) and APC-CD105 (800507, BioLegend, USA) were used, along with corresponding isotype controls. They were then mixed thoroughly and incubated at 4°C for 30 minutes before being subjected to flow cytometry for analysis.

To assess whether AMSC could differentiate into osteogenic and adipogenic lineages, third-passage AMSC were cultured in adipogenic differentiation induction medium for 2 weeks and stained with Oil Red O (OILR-10001, OriCell, Guangzhou, China), or in osteogenic differentiation induction medium for 3 weeks and stained with Alizarin Red (ALIR-10001, OriCell, Guangzhou, China) separately. Images of stained cells were captured using microscope (Olympus, Tokyo, Japan).

2. Flow cytometric assay

For kidney tissues, a single-cell suspension was first prepared. Approximately 10^6 cells per sample were suspended in 100 μl PBS, followed by the addition of 50 μl Brilliant Stain Buffer (563794, BD Biosciences, USA). The cells were incubated with fluorescently labeled antibodies on ice for 30 minutes. The unstained group, single-antibody stained group, and fluorescence-minus-one controls were used as references. Dye eFluor™ 780 (2594685, BD Biosciences, USA) was used to distinguish live from dead cells. The antibodies used were as follows: CD45 (BD 564279, Biosciences, USA), CD11b (101263, BioLegend, USA), F4/80 (123116, BioLegend, USA), CX3CR1 (149020, BioLegend, USA), TXNIP (A01409-1, Boster, USA). To assess the polarization of THP-1 cell-derived macrophages under different treatments, cells from the three groups were stained with CD68 (333805, BioLegend, USA), CD86 (374208, BioLegend, USA), and CD206 (321121, BioLegend, USA) at

25°C in the dark for 30 minutes. Flow cytometry was performed using the Cytek NL-CLC system, and sorting was completed with a BD FACSAria Fusion flow cytometer and analyzed with FacsDiva 8 software (BD). Data analysis was performed using FlowJo version 10 software.