Original Paper

Mesenchymal Stem Cell-Derived Exosomes Reduce A1 Astrocytes via Downregulation of Phosphorylated NFκB P65 Subunit in Spinal Cord Injury

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Key Words

Mesenchymal stem cell • Exosomes • Spinal cord injury • Astrocytes • Inflammation

Abstract

Background/Aims: Neurotoxic A1 astrocytes are induced by inflammation after spinal cord injury (SCI), and the inflammation-related Nuclear Factor Kappa B (NFκB) pathway may be related to A1-astrocyte activation. Mesenchymal stem cell (MSC) transplantation is a promising therapy for SCI, where transplanted MSCs exhibit anti-inflammatory effects by downregulating proinflammatory factors, such as Tumor Necrosis Factor (TNF)-α and NFκB. MSC-exosomes (MSC-exo) reportedly mimic the beneficial effects of MSCs. Therefore, in this study, we investigated whether MSCs and MSC-exo exert inhibitory effects on A1 astrocytes and are beneficial for recovery after SCI. **Methods:** The effects of MSC and MSC-exo on SCI-induced A1 astrocytes, and the potential mechanisms were investigated *in vitro* and *in vivo* using immunofluorescence and western blot. In addition, we assessed the histopathology, levels of proinflammatory cytokines and locomotor function to verify the effects of MSC and MSC-exo on SCI rats. **Results:** MSC or MSC-exo co-culture reduced the proportion of SCI-induced A1 astrocytes. Intravenously-injected MSC or MSC-exo after SCI significantly reduced the proportion of A1 astrocytes, the percentage of p65 positive nuclei in astrocytes, and the percentage of TUNEL-positive cells in the ventral horn. Additionally, we observed decreased lesion area and expression of TNFα, Interleukin (IL)-1α and IL-1β, elevated expression of Myelin Basic Protein (MBP), Synaptophysin (Syn) and Neuronal Nuclei (NeuN), and improved Basso, Beattie & Bresnahan (BBB) scores and inclined-plane-test angle. *In vitro* assay showed that MSC and MSC-exo reduced SCI-induced A1 astrocytes, probably via inhibiting the nuclear translocation of the NFκB p65. **Conclusion:** MSC and MSC-exo reduce SCI-induced

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A1 astrocytes, probably via inhibiting nuclear translocation of NFκB p65, and exert anti-inflammatory and neuroprotective effects following SCI, with the therapeutic effect of MSC-exo comparable with that of MSCs when applied intravenously.

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Introduction

Traumatic spinal cord injury (SCI) is a severe type of central nervous system (CNS) damage that frequently leads to deleterious functional loss below the level of the injury. The pathophysiology of SCI is complex with immediate primary mechanical injury followed by a cascade of secondary processes including neuroinflammation, ischemia, and excitotoxicity that exacerbate SCI damage [1].

Astrocytes play a crucial role in SCI secondary injury referred to as reactive astrogliosis, which can both hinder and support CNS recovery [2-5]. Liddelow et al. found that two types of reactive astrocytes, termed A1 and A2 astrocytes, are induced by neuroinflammation and ischemia, respectively [6]. A2 astrocytes exert protective effects by upregulating the expression of certain neurotrophic factors, whereas A1 astrocytes, which form rapidly after CNS injury, exert neurotoxic effects on the myelin sheath, synapses, and neurons. A recent study reported that neuroinflammatory A1 reactive astrocytes are induced by NFκB signaling [6]. Additionally, the marker of A1 astrocytes, complement component 3 (C3), is upregulated in an NFκB-dependent manner [7]. Moreover, blocking NFκB activity in astrocytes promotes oligodendrogenesis and axonal sparing through alteration of the inflammatory environment following SCI [8].

Numerous studies have demonstrated MSCs as ideal candidates for cell-based treatment of SCI. MSCs promote tissue repair mainly by inhibiting inflammation and activating endogenous repair mechanisms [9, 10]. Previous studies demonstrated that MSCs reduce inflammatory cytokine (TNFα, IL-1, IL-6) levels [11] and inhibit NFκB activation under inflammatory conditions [12]. Additionally, MSCs are capable of directing stimulated macrophages from a proinflammatory M1 phenotype to an anti-inflammatory M2 phenotype [13, 14]. Recent studies suggest that the therapeutic effects of MSCs might be mediated by exosomes [15-17]. Exosomes are small particles (40-100 nm in diameter) derived from multi-vesicular bodies and are secreted into extracellular fluid by most living cells. Exosomes carry complex cargo including proteins, lipids and nucleic acids, and transfer them to recipient cells to exert function. MSC-derived exosomes (MSC-exo) are reportedly capable of mimicking most biological functions of MSCs including anti-inflammatory and anti-apoptotic effects and can increase levels of anti-inflammatory cytokines, decrease levels of proinflammatory cytokines [18, 19], and inhibit activation of astrocytes [17] and macrophages [20]. Similar to MSCs, MSC-exo infusion can inhibit NFκB activation [21, 22]. Moreover the therapeutic effects of cell-free exosomes are comparable with those of intact MSCs after brain injury [16, 17, 23]. A previous study of applications of MSC-exo to an SCI model suggesting that systemic administration reduced levels of proinflammatory cytokines and improved function recovery [24]; however, the precise mechanisms associated with the beneficial effects on SCI remain unknown. Based on the reported anti-inflammatory effects, we hypothesized that both MSC and MSC-exo might play an inhibitory role on inflammation-induced A1 astrocytes.

In this study, we investigated the potential effect of MSCs and MSC-exo on A1 astrocytes after SCI. MSCs and MSC-exo derived from an equal number of MSCs were intravenously administered to SCI rats in order to evaluate their effect on A1 astrocytes in the ventral horn of the spinal cord, as well as the morphological reconstruction of the injured spinal cord and functional recovery. The astrocytes used in the experiments were purified from injured spinal-cord tissue and cultured *in vitro* to investigate the potential mechanism.

Materials and Methods

Animals

Adult male Sprague–Dawley rats, all specific pathogen-free, were provided by the Animal Institute of Zhengzhou University and housed individually in separate cages with free access to water and food, in a room with a 12-h light/dark cycle and controlled temperature (22–25°C) and humidity (40–60%).

Twelve SD rats (100–150 g) were used to isolate MSCs. For *in vivo* experiments, 100 rats (200–250 g) were randomly divided into four groups (25 rats/group): Sham, SCI+phosphate-buffered-saline (PBS), SCI+MSC, and SCI+MSC-exo. In each group, 10 rats were used for behavioral tests and morphological assessment, 3 rats were sacrificed to determine whether intravenously-infused MSCs or MSC-exo reached spinal cord tissue, 6 rats were used to perform western blot and enzyme linked immunosorbent assay (ELISA), and 6 rats were sacrificed to supply tissue for immunofluorescence assays and terminal deoxynucleotidyltransferase-mediated UTP end labelling (TUNEL) assay. For *in vitro* experiments, 3 rats from the Sham group and 18 from the SCI groups (200–250 g) were used to obtain astrocytes from spinal-cord tissue.

MSC isolation and characterization

Fetal bovine serum (FBS; Gibco, Gaithersburg, MD, USA) was ultra-centrifuged (120,000g for 18 h) to remove exosomes in serum. Bone marrow was obtained by flushing the femurs of rats with Dulbecco's modified Eagle medium (DMEM; GIBCO) supplemented with 10% exosome-depleted FBS. Cells were cultured in DMEM culture medium supplemented with 10% exosome-depleted FBS plus 1% penicillin-streptomycin (GIBCO) and digested with 0.25% trypsin plus 0.02% EDTA.

The expression of MSC surface markers was detected by flow cytometry. Briefly, passage 4 (P4) MSCs were digested with 0.125% trypsin and suspended in PBS containing 2% bovine serum albumin (BSA). Subsequently, a suspension of 1×10^5 cells was incubated with fluorescein isothiocyanate- or phycoerythrin-conjugated monoclonal antibodies, including those against CD45 (B168471; Biolegend, San Diego, CA, USA), CD90 (B178360; Biolegend), and CD29 (B203041; Biolegend), for 30 min in the dark. Controls were incubated with IgG1 isotype-control antibodies. Thereafter, all cells were washed and re-suspended in PBS, and the samples were analyzed by flow cytometry (Accuri C6; BD Biosciences, Franklin Lakes, NJ, USA). Data were processed using FlowJo software 8.7.

MSC labeling

Bone marrow mesenchymal stem cells (BMSCs) at passage 4 were labeled with PKH-26 Red fluorescent dye (Sigma-Aldrich, St. Louis, MO, USA). Briefly, cells were trypsinized with 0.25% trypsin-EDTA solutions, washed, and resuspended in 1 mL of Diluent C (Sigma-Aldrich). An equal volume of the PKH26 solution (4 nM) was then mixed with the cell suspension, and the mixture was incubated at 25°C for 4 min, after which an equal volume of FBS was added to the mixture to stop the reaction. Cell pellets were washed with PBS and seeded in culture dishes. PKH26-positive BMSCs were observed with a fluorescence microscope at 24 h post labeling.

Cell proliferation assay

The proliferation of PKH26-labeled MSCs was determined using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Miyagi, Japan) according to manufacturer's instructions. Unlabeled-MSC were used as controls. Unlabeled-MSCs and PKH26-labeled MSCs were plated in 96-well plates at 5×10^3 cells/well, respectively, and allowed to proliferate for 24 h or 48 h, after which 10 μ L of CCK-8 solution was added to each well, followed by incubation for 3 h. The absorbance at 450 nm was measured using a microplate reader (Biotek, Winooski, VT, USA).

MSC-exo isolation and characterization

Exosomes were isolated from the supernatants of passage 4 MSC by differential centrifugation (300 g for 10 min; 2,000 g for 10 min; and 10,000 g for 30 min), followed by ultracentrifugation (110,000 g for 70 min), and washing of the exosomes with PBS (110,000 g for 70 min). All centrifugation steps were performed at 4°C. Subsequently, the supernatant was removed and the exosome pellet was resuspended in PBS.

Exosome morphology was observed by transmission electron microscopy. 10 μ L of the suspension was loaded onto formvar/carbon-coated grids at 25° C for 10 min. After removing excess fluid with filter paper, exosomes were negatively stained with 3% aqueous phosphotungstic acid for 5 min, and the exosome-containing grids were observed using a transmission electron microscope (HT7700, HITACHI, Tokyo, Japan) operating at 100 kV.

Expression of the exosomal markers was detected through western blot. Exosomes were lysed with ice-cold radioimmunoprecipitation assay buffer (Solarbio, Beijing, China) supplemented with a protease- and phosphatase-inhibitor cocktail (Abcam, Cambridge, MA, USA), and the protein concentration was estimated using a BCA protein assay kit (Solarbio). Forty micrograms of protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes, which were blocked in blocking buffer, followed by incubation with primary antibodies against CD63 (1:500; 25682-1-AP; Proteintech, Wuhan, China), CD9 (1:1000; ab92726; Abcam) and CD81 (1:1000; ab109201; Abcam) at 4°C overnight. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody and bands were developed using enhanced chemiluminescence.

Analysis of size distribution was performed using tunable resistive pulse sensing (TRPS) on a qNano system (Izon, Christchurch, New Zealand). Briefly, samples or calibration particles were placed in the NP100A nanopore and measured with a voltage of 0.74 V.

MSC-exo labeling

MSC-exo were labeled with PKH26 dye (Sigma-Aldrich). Briefly, 2 μ L of PKH26 was added to 1 mL of Diluent C containing 25 μ g of exosomes, followed by incubation at 25°C for 20 min and the subsequent addition of 1 mL of 5% BSA to stop the staining reaction. Eight microliters of PBS was added to the mixture, followed by an ultra-centrifugation (4°C, 110,000 g for 70 min). The pelleted exosomes were resuspended in 10 mL of PBS and ultra-centrifuged again (4°C, 110,000 g for 70 min). The supernatant was removed and the exosomes were resuspended in PBS.

SCI models

For all rats, anesthesia was induced using 4% isoflurane in oxygen (1 L/min) and maintained with 2% isoflurane in oxygen (1 L/min). Laminectomy of a single vertebra at the level of T10 was performed to expose the spinal cord. Following this, a contusive injury (200 kilodyne) was applied to the exposed dura mater, using a spinal cord impactor (IH Impactor; Precision Systems and Instrumentation, Lexington, KY). The SCI models met the following inclusion criteria: spinal cord hemorrhage and edema around the wound, flicking of bilateral hind limbs, swaying reflex of the tail. The Sham group underwent only a laminectomy.

MSC or MSC-exo Injection

Rats were randomly divided into four groups: Sham, SCI rats receiving PBS (SCI+PBS), SCI rats receiving passage 4 MSCs (SCI+MSC), and SCI rats receiving MSC-exo (SCI+MSC-exo). For the SCI+MSC group, 200 μ L PBS containing 1×10^6 MSCs was infused into the tail vein (200 μ L/min) of each rat at 30-min post-SCI. At 1 dpi, 200 μ L of PBS containing 1×10^6 MSCs was injected in the same manner. For the SCI+MSC-exo group, 200 μ L of exosomes (200 μ g/mL, derived from $\sim 1 \times 10^6$ MSCs) was injected into the tail vein (200 μ L/min) of each rat at 30-min post-SCI. Subsequently, 200 μ L of exosomes (200 μ g/mL) was injected in the same manner at 1 dpi. For the SCI+PBS group, 200 μ L PBS was injected into the tail vein at the same time points as described for the SCI+MSC-exo group.

BBB locomotor rating scale

BBB scores were determined before surgery and at 1, 3, 7, 14, 21, and 28 days after SCI by two independent observers who were blinded to animal grouping. Rats were placed in an open field and allowed to move freely in the field for 5 min. The scores ranged from 0 (no limb movement or weight support) to 21 (normal locomotion). The average locomotor scores were calculated and recorded.

Inclined plane test

This test was performed on a board that was raised progressively on an inclined plane system, using a stepping motor that changed the angle of the inclined plane (0-90°). The inclined plane angle was recorded as the maximum angle at which rats were able to maintain their position for 5 s without sliding.

Tissue preparation for immunofluorescence

At 3 dpi, rats (n = 6) were anesthetized and perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde in phosphate buffer (4°C; pH 7.4). A 1-cm long spinal cord segment, centered on the injury site was dissected, placed in the same fixative within 4 h to 6 h, and cryoprotected in sucrose solution (a graduated series of 10%, 20%, and 30%) at 4°C. These segments were cryosectioned into 20-μm-thick sections.

Lesion size analysis

At 28 dpi, the spinal cord specimens from each group were harvested (n = 6) and the 4% paraformaldehyde-fixed specimens were embedded into paraffin for production of 5-μm-thick transverse sections. Every 40th section of the lesion-site sample was subjected to hematoxylin-eosin (HE) and Nissl staining. The middle three HE-stained sections (centered in the epicenter of the lesion site, with an interval of 200 μm) of each sample were used for lesion area analysis performed using ImageJ. Lesion percentage was calculated as the lesion area divided by the whole transverse area of the spinal cord.

TEM

Ultrathin sections of the injury site were observed by a transmission electron microscope (HITACHI) at 28 dpi (n = 4). The sections were collected on copper grids and counterstained with 2% uranyl acetate for 20 min and 0.04% lead citrate for 10 min.

ELISA

Spinal cord tissues were collected and homogenized as described for western blot. Concentrations of proinflammatory cytokines, including TNF-α, IL-1α and IL-1β, were measured using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to manufacturer's protocol. Assays were performed in duplicates, and data are expressed as pg/mg protein.

TUNEL assay

At 3 dpi, cell apoptosis on injury site was assessed using an in situ cell death detection kit (Roche, Mannheim, Germany) according to manufacturer's instructions. Tissue sections were dewaxed, rehydrated, and washed with PBS, followed by pre-treatment of the sections with proteinase-K for 30 min and incubation with TUNEL reaction mixture for 1 h at 37°C. The converter POD was then added to the sections. After incubation for 30 min at 37°C, the sections were washed with PBS and incubated with diaminobenzidine for 10 min. Images were captured using a microscope at 400× magnification.

Astrocytes isolation

Astrocytes were isolated according to previous methods [25, 26]. At 3d post SCI, rats from the Sham and SCI groups (n = 6) were sacrificed, and the T9-T11 segment of the ventral spinal cord was dissected from Sham rats while a 2-cm long ventral segment that included the rostral and caudal areas to the injury epicenter was removed from SCI rats. The spinal cord tissues were minced by mechanical shearing and then digested in PBS containing 0.125% trypsin and 0.01% papain for 30 min at 37°C. After centrifugation at 1000 g for 5 min, cells were re-suspended in DMEM/F12 supplemented with 10% exosome-depleted FBS and penicillin-streptomycin (Gibco). Cells were cultured for 7 days at 37°C under 5% CO₂, with changes to the medium 3-days post plating. After 7 days, microglia and oligodendrocytes were removed by shaking the culture flasks at 210 rpm for 6 h at 37 °C. The astroglia phenotype of Glial Fibrillary Acidic Protein (GFAP) was characterized by immunofluorescence.

Astrocyte coculture with MSCs or MSC-exo

Seven groups were used for *in vitro* experiment: astrocytes isolated from Sham rats (Sh-AST); astrocytes isolated from SCI rats (SCI-AST); astrocytes isolated from SCI rats and supplemented with Pyrrolidine Dithiocarbamate (PDTC), an inhibitor of NFκB which inhibits the nuclear translocation of NFκB p65 [27] by suppressing the degradation of IκB (SCI-AST+PDTC); astrocytes isolated from SCI rats and co-cultured with MSC (SCI-AST+MSC); astrocytes isolated from SCI rats, co-cultured with MSC and supplemented with TNF-α, an activator of NFκB (SCI-AST+MSC+TNFα); astrocytes isolated from SCI rats and co-cultured with MSC-exo (SCI-AST+MSC-exo); astrocytes isolated from SCI rats, co-cultured with MSC-exo, and supplemented with

TNF- α (SCI-AST+MSC-exo+TNF α). Cocultures were established using a 6-well Transwell cell culture system with a 0.4- μ m membrane that cells cannot get through. For the SCI-AST+PDTC group, 5×10^4 astrocytes were seeded on the upper chamber and supplemented with 10 μ M PDTC [27-29]. For the SCI-AST+MSC and SCI-AST+MSC+TNF α groups, 5×10^4 astrocytes were seeded on the upper chamber in the absence and presence of TNF- α (50 ng/mL; R&D Systems), respectively [30-34], with 5×10^4 MSCs seeded on the lower chamber. Cells were cultured in DMEM supplemented with 10% exosome-depleted FBS and co-cultured for 48h. For the SCI-AST+MSC-exo and SCI-AST+MSC-exo+TNF α groups, MSCs were first seeded in 6-well plates (5×10^4 /well) and cultured for 48h, followed by collection of the supernatants to isolate exosomes, which were placed in the lower chamber and co-cultured with 5×10^4 astrocytes for 48h. Astrocytes co-cultured with MSCs or MSC-exo were then harvested for immunofluorescence and western blot analyses.

Immunofluorescence

The 20- μ m cryo-sections (200- μ m rostral to the injury epicenter; n = 6) and the astrocytes cultured in Transwells (n = 6) were washed in PBS and incubated with 5% BSA to block nonspecific antibody binding. The sections were first incubated with the following primary antibodies overnight at 4°C: rabbit anti-C3 (1:100) and mouse anti-GFAP (1:200; [2A5]ab4648; Abcam); rabbit anti-p65 (1:100; 10745-1-AP; Proteintech) and mouse anti-GFAP (1:200; [2A5]ab4648; Abcam); mouse anti-NeuN (1:200; [1B7]ab104224; Abcam); rabbit anti-Syn antibody (1:100, 17785-1-AP; Proteintech); rabbit anti-MBP (1:100; 10458-1-AP; Proteintech). The sections were rinsed in PBS and incubated with respective secondary antibodies, including Red cyanine-3-conjugated goat anti-rabbit IgG (1:800; 111-165-003; Jackson, West Grove, PA, USA) and Green 488-conjugated goat anti-mouse IgG (1:800; 129586; Jackson) for 2 h at 37°C. The sections were then washed in PBS and mounted with 4',6-diamidino-2-phenylindole (DAPI; #033M4064V; Sigma-Aldrich). Sections were observed at 400 $\times$ magnification with a fluorescence microscope (BX53; Olympus, Tokyo, Japan).

Image analysis

Quantifications were performed using ImageJ software. Three sections from each rat were examined (n = 6), and the area proportion of double-label-positive astrocytes was analyzed in four randomly chosen fields of the ventral horn within each section. The percentage of Syn positive area and the percentage of MBP positive area was analyzed across the entire section, respectively. The number of NeuN positive cells and the percentage of TUNEL-positive cells were calculated in four randomly selected fields of the ventral horn within each section.

Western blot

The T10 segment of the spinal cord was rapidly collected at 3 dpi (n = 6), and the ventral section was dissected for western blot analysis. Astrocytes were harvested at 48 h after co-culture (n = 6), and proteins from cytoplasm and nuclear fraction were separately extracted using a nuclear protein extraction kit (R0050; Solarbio). Forty micrograms of proteins was separated and transferred onto polyvinylidene fluoride membranes, which were blocked in blocking buffer for 2 h and then incubated with primary antibodies against p65 (1:1000; #8242; Cell Signaling Technology, Danvers, MA, USA), phosphorylated-p65 (1:1000; #3033; Cell Signaling Technology), p-IKB α (1:1000; #2859; Cell Signaling Technology), IKB α (1:1000; #4812; Cell Signaling Technology), GFAP (1:500; SAB4300647; Sigma), C3 (1:100; ab11887; Abcam), β -actin (1:1000; 20536-1-AP; Proteintech), and Histone H3 (1:1000; #9715; Cell Signaling Technology) at 4°C overnight. After washing, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody, for 2 h at 25°C. Bands were developed using enhanced chemiluminescence and band intensity was quantified using ImageJ.

Statistical analysis

Statistical analyses were performed using Graph Pad Prism 5.0 (San Diego, CA, USA). All data are presented as mean $\pm$ standard error of the mean (SEM). The data analysis of BBB scores and inclined plane test was conducted using two-way analysis of variance with repeated measures. Other data were analyzed using one-way analysis of variance and a Bonferroni post-test. P<0.05 was considered statistically significant.

Results

Identification and labeling of MSCs and MSC-exo

MSCs were obtained from the bone marrow of rats and cultured in dishes (Fig. 1A). Passage 4 MSC adhered to the bottom of the culture dishes and grew with a fibroblast-like morphology (Fig. 1B). The expression of mesenchymal markers CD90 (96.4%), CD29 (99.9%), and CD45 (0.11%) was measured by flow cytometry (Fig. 1C), and MSCs stained red with PKH26 were observed by fluorescence microscopy (Fig. 1D). The CCK8 assay revealed no significant differences in cell proliferation between MSCs and PKH26-labeled MSCs within 72 h ($P > 0.05$, Fig. 1E).

Fig. 1. Isolation, characterization and labeling of BMSCs. (A) Schematic representation of the isolation of BMSC from femurs of SD rats. (B) The observation of the passage 4 BMSCs by a light microscope. Scale bar = 200 μm. (C) Flow cytometric analysis showing the expression levels of CD90, CD29 and CD45 in the passage 4 of BMSC. (D) Immunofluorescence image showing the PKH26-stained BMSC (red). Scale bar = 200 μm. (E) The CCK8 assay of PKH26-stained BMSCs and BMSCs without staining at different time points. ^{ns} $P > 0.05$.

Exosomes were isolated from passage-4 MSC-culture supernatant by ultracentrifugation (Fig. 2A), and TEM observation of MSC-exo samples showed the presence of numerous saucer-shaped vesicles (Fig. 2B). MSC-exo stained red with PKH26 dye were observed by fluorescence microscopy (Fig. 2C), with subsequent TRPS analysis showed a diameter distribution profile ranging from 30 nm to 150 nm (Fig. 2D). We verified high levels of expression of the exosome markers, CD63, CD9 and CD81 in the MSC-exo samples (Fig. 2E).

MSCs and MSC-exo reduce the proportion of A1 astrocytes *in vitro*

To assess the effects of MSCs and MSC-exo on A1-astrocytes *in vitro*, we isolated astrocytes from spinal cord tissue of the sham and SCI groups (Fig. 3A). Astrocytes derived from SCI rats were co-cultured respectively with MSCs and MSC-exo in a Transwell system (Fig. 3A). Astrocytes were observed at a magnification of $\times 100$ (Fig. 3B). At 6 and 12 h after co-culture with MSC-exo, astrocytes were harvested to determine the extent of MSC-exo internalization. We observed PKH26-labeled MSC-exo present in the cytoplasm of astrocytes, especially at 12 h after co-culture (Fig. 3C). At 48 h after co-culture, immunofluorescence assays were performed (Fig. 3D), and the proportion of A1 astrocytes was evaluated in each group by calculating C3+GFAP⁺/GFAP⁺ area. The ratio of C3+GFAP⁺ area was 0.139 ± 0.033 in the SCI-AST as compared with 0.003 ± 0.001 in the Sh-AST ($P < 0.001$, Fig. 3E). Co-culture with MSCs (SCI-AST+MSC) or MSC-exo (SCI-AST+MSC-exo) reduced the proportion of A1-astrocytes as compared with that in the SCI-AST ($P < 0.001$; $P < 0.01$). There was no significant difference in A1 proportion between the SCI-AST+MSC (0.019 ± 0.011) and SCI-AST+MSC-exo (0.023 ± 0.012) groups.

Fig. 2. Isolation, characterization and labeling of exosomes. (A) Schematic representation of the isolation of exosomes from culture supernatant of BMSC using ultracentrifugation. (B) Morphologic observation of the exosomes by transmission electron microscopy. Scale bar = 100 nm. (C) Immunofluorescence image showing the PKH26-stained exosomes (red). Scale bar = 20 μ m. (D) Representative size distribution of the exosomes derived from BMSCs. (E) Expression of exosome-markers (CD9, CD63, CD81) was assessed using western blot.

Fig. 3. MSCs or MSC-exo co-culture reduce the proportion of A1 astrocytes. (A) Schematic representation of the astroglia isolation from spinal cord tissue and co-culture with MSC or MSC-exo in the transwell system. (B) Observation of the passage 1 astrocytes by a light microscope. Scale bar = 50 μ m. (C) Immunofluorescence images showing the internalization of PKH26-labeled exosomes (red) by astrocytes (green) at 0, 6, and 12 h after co-culture. Scale bar = 20 μ m. (D) Immunofluorescence images of the C3⁺GFAP⁺ astrocytes (yellow) of different groups. Scale bar = 20 μ m. (E) Ratio of C3⁺GFAP⁺ area to GFAP⁺ area in different groups (n = 6). Error bars are standard errors. **P<0.01; ***P<0.001; ^{ns}P>0.05.

MSCs and MSC-exo reduce the proportion of A1 astrocytes in ventral horn of spinal cord after SCI

To investigate the effect of intravenously-administered MSCs or MSC-exo on A1 astrocytes of SCI rats, the rats were randomly divided into four groups (Fig. 4A), and we assessed whether intravenously-injected MSCs or MSC-exo arrive at the lesion site. The injured spinal cord was removed at 1 dpi and prepared for observation by fluorescence microscopy. PKH26-labeled MSC-exo were observed at the injury site, whereas PKH26-labeled MSCs were not detected (Fig. 4B). Analysis of the proportion of C3⁺GFAP⁺ astrocytes on the ventral horn of the spinal cord in different groups (Fig. 4C) revealed increases in SCI+PBS group (0.330 ± 0.078) as compared with the Sham group ($P < 0.001$, Fig. 4D). Furthermore, both MSCs and MSC-exo treatment decreased SCI-induced activation of A1 astrocytes ($P < 0.01$ and $P < 0.05$, respectively), with the proportion of A1 astrocytes at 0.090 ± 0.025 in the SCI+MSC group and 0.111 ± 0.041 in the SCI+MSC-exo group.

Additionally, C3 and GFAP expression in the ventral spinal cord was evaluated by western blot (Fig. 5A) showing levels in the SCI+PBS group higher than those in the Sham group ($P < 0.001$ and $P < 0.001$, respectively; Fig. 5B). At 3-days post-administration of MSCs, we observed attenuated C3 and GFAP expression relative to that in SCI+PBS rats ($P < 0.01$ and $P < 0.01$, respectively). MSC-exo treatment also resulted in decreased levels of C3 and GFAP ($P < 0.01$ and $P < 0.05$, respectively). Importantly, no significant differences were found in levels of C3 and GFAP between the SCI+MSC and SCI+MSC-exo groups ($P > 0.05$).

Fig. 4. Intravenously-injected MSCs or MSC-exo reduce the proportion of A1 astrocytes in the ventral horn after SCI. (A) Schematic diagram of the SCI model of different groups and experiments performed at different time points. (B) Observation of the transverse sections of spinal cord of different groups with a fluorescence microscope to trace the PKH26-labeled MSC or MSC-exo (red) in the spinal cord. Scale bar = 20 μ m. (C) Immunofluorescence images of the C3*GFAP* astrocytes (yellow) in ventral horn of spinal cord of different groups. Scale bar = 20 μ m. (D) The ratio of C3*GFAP* area to GFAP* area in different groups (n = 6). Error bars are standard errors. ** P<0.01; *** P<0.001; ns P>0.05.

MSCs and MSC-exo attenuate proinflammatory cytokine expression in ventral spinal cord after SCI

Previous studies showed that A1 astrocytes can be induced by proinflammatory factors, such as TNF and IL-1 [6, 35]. Therefore, we performed the ELISA assays at 3 dpi to assess levels of TNF- α , IL-1 α , and IL-1 β in the ventral spinal cord of different groups. TNF- α was 160.665 $\pm$ 17.095 pg/mg in the SCI+PBS group, which was significantly higher than that in the Sham group (58.304 $\pm$ 4.083 pg/mg; P<0.001; Fig. 5C). TNF- α in the SCI+MSC and SCI+MSC-exo groups were lower than that in the SCI+PBS group (107.724 $\pm$ 12.059 and 102.671 $\pm$ 13.713 pg/mg, respectively), with no significant difference between MSC and MSC-exo treatment groups (P>0.05). IL-1 α and IL-1 β levels in the SCI+PBS group were 24.605 $\pm$ 4.378 and

Fig. 5. Expression of C3, GFAP and proinflammatory cytokines (TNF- α , IL-1 α and IL-1 β) in ventral spinal cord at 3 dpi. (A) Protein expression of C3 and GFAP in the ventral spinal cord of different groups was analyzed via western blot. (B) Quantification of the relative expression of C3 and GFAP in all four groups ($n = 6$). Error bars are standard errors. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns $P > 0.05$. (C) Expression levels of TNF- α , IL-1 α , and IL-1 β in ventral spinal cord at 3 dpi detected by ELISA assays ($n = 6$). Error bars are standard errors. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns $P > 0.05$.

76.140 $\pm$ 9.763 pg/mg, respectively, as compared with 7.967 $\pm$ 1.332 and 22.394 $\pm$ 2.918 pg/mg, respectively, in the Sham group. In the SCI+MSC group, IL-1 α and IL-1 β levels were 11.880 $\pm$ 2.411 and 32.480 $\pm$ 5.799 pg/mg, respectively, and in the SCI+MSC-exo group, IL-1 α and IL-1 β levels were 12.100 $\pm$ 1.997 and 39.841 $\pm$ 8.707 pg/mg, respectively. These results revealed substantial increases in TNF- α , IL-1 α and IL-1 β levels in the SCI+PBS group ($P < 0.001$, $P < 0.01$, and $P < 0.001$, respectively); however, MSC or MSC-exo treatment reduced these levels relative to that observed in the SCI+PBS group, with no significant difference in cytokine levels observed between MSC and MSC-exo treatment groups ($P > 0.05$).

MSCs and MSC-exo attenuate lesion size and improved functional recovery in SCI rats

Ten rats from each group were sacrificed at 28 dpi following behavioral analyses, and histopathological evaluation was performed in the spinal cord of those rats (6 rats for HE and Nissl, 4 rats for TEM; Fig. 6A). The lesion percentages in the SCI+PBS, SCI+MSC, and SCI+MSC-exo group were 34.933 $\pm$ 2.182%, 12.471 $\pm$ 1.853%, and 10.034 $\pm$ 2.105%, respectively, with significant changes in the treatment groups relative to the percentage measured in the SCI+PBS group ($P < 0.001$ and $P < 0.001$, respectively).

Fig. 6. Morphological evaluation of the injured spinal cord and locomotor functional assessment. (A) Images of the transverse sections of spinal cord of different groups using HE, nissl staining ($n = 6$) and transmission electron microscopy ($n = 4$). Scale bar = 200 μm , 50 μm , 20 μm , and 2 μm . (B) Quantification of the lesion area percentage in injury group and treatment groups ($n = 6$). *** $P < 0.001$; $^{ns}P > 0.05$. (C) Representative BBB scores of rats from all four groups at different time points ($n = 10$). *** $^{(green)}$ SCI+MSC vs SCI+PBS group $P < 0.001$; *** $^{(blue)}$ SCI+MSC-exo vs SCI+PBS group $P < 0.001$; $^{ns}^{(blue)}$ SCI+MSC-exo vs SCI+MSC group $P > 0.05$; $^{ns}^{(black)}$ $P > 0.05$ among the SCI+PBS, SCI+MSC, and SCI+MSC-exo group. (D) Representative inclined-plane-test angles of rats from all four groups at different time points ($n = 10$). *** $^{(green)}$ SCI+MSC vs SCI+PBS group $P < 0.01$; *** $^{(green)}$ SCI+MSC vs SCI+PBS group $P < 0.001$; *** $^{(blue)}$ SCI+MSC-exo vs SCI+PBS group $P < 0.001$; $^{ns}^{(blue)}$ SCI+MSC-exo vs SCI+MSC group $P > 0.05$; $^{ns}^{(black)}$ $P > 0.05$ among the SCI+PBS, SCI+MSC, and SCI+MSC-exo group.

Fig. 7. Expression of Syn, MBP, and NeuN, and TUNEL assay in the lesion at 3 dpi. (A) Immunofluorescence staining for Syn (red), MBP (red) in the transverse sections and NeuN (green) in the ventral horn of the transverse spinal cord sections from different groups. Scale bar = 20 μ m. (B) TUNEL⁺ cells (brown) in the ventral horn of the transverse spinal cord sections from different groups. Scale bar = 20 μ m. (C) Representative of the Syn⁺ area percentage, the MBP⁺ area percentage in the whole transverse sections, and the number of NeuN⁺ cells per high-power field in the ventral horn of the transverse spinal cord sections from different groups (n = 6). Error bars are standard errors. *P<0.05; **P<0.01; ***P<0.001; nsP>0.05. (D) The percentage of TUNEL⁺ cells (cell apoptosis) in the ventral horn of the transverse spinal cord sections (n = 6). Error bars are standard errors. **P<0.01; ***P<0.001; nsP>0.05.

The ultrastructure of the lesion tissue from all groups was observed using TEM (Fig. 6A). In the Sham group, the myelin sheath and axons displayed intact structures, whereas in the SCI+PBS group, we observed loose, onion-like demyelination and degenerated axons. However, in the SCI+MSC and SCI+MSC-exo groups, these morphological phenomena were alleviated.

At 1 and 3 dpi, neither the BBB scores nor the inclined-plane angles among the SCI+PBS, SCI+MSC, and SCI+MSC-exo groups showed any significant difference ($P > 0.05$; $n = 10$; Fig. 6C, D). However, the SCI+MSC and SCI+MSC-exo groups showed significantly higher BBB scores than the SCI+PBS group from 7 dpi (7 dpi: $P < 0.001$ and $P < 0.001$; 14 dpi: $P < 0.001$ and $P < 0.001$; 21 dpi: $P < 0.001$ and $P < 0.001$; 28 dpi: $P < 0.001$ and $P < 0.001$). At 28 dpi, the BBB scores in the MSC and MSC-exo treatment groups were 13.800 ± 0.396 and 14.450 ± 0.411 , respectively, differences between which were not significant ($P > 0.05$). The inclined-plane angles at 28 dpi in the MSC and MSC-exo treatment groups were $55.250 \pm 0.786^\circ$ and $58.750 \pm 1.070^\circ$, respectively.

These results suggested that MSC and MSC-exo treatment attenuated lesion size and improved functional recovery after SCI. Additionally, the effects of MSCs and MSC-exo on functional recovery were equivalent.

MSCs and MSC-exo exert neuroprotective and anti-apoptotic effects on SCI rats

A1 astrocytes are toxic to synapses, myelin sheath, and neurons in the CNS. As a specific marker for presynaptic terminals, Syn is often used to detect the synapse density and distribution. MBP is the major protein of the myelin sheath in the CNS and maintains myelin sheath stability. NeuN is a neuron-specific nuclear protein that serves as an excellent marker for neurons in the central and peripheral nervous systems. To assess the loss of synapses, myelin sheath and neurons in injured spinal cord from different groups, Syn, MBP, and NeuN expression was detected, respectively, by immunofluorescence (Fig. 7A). The percentages of Syn⁺ area in the Sham, SCI+PBS, SCI+MSC, and SCI+MSC-exo groups were $30.213 \pm 1.529\%$, $4.250 \pm 1.814\%$, $13.565 \pm 2.088\%$, and $16.067 \pm 2.671\%$, respectively (Fig. 7C). The percentages of MBP⁺ area in these groups were $41.678 \pm 1.291\%$, $10.008 \pm 2.298\%$, $25.573 \pm 3.256\%$, and $28.448 \pm 2.948\%$, respectively, and the number of NeuN⁺ cells per high-power field (400 $\times$) on the ventral horn of the spinal cords from each group was 30.167 ± 2.892 , 7.167 ± 2.120 , 22.833 ± 5.180 , and 21.667 ± 2.801 , respectively. These results showed that MSC and MSC-exo treatment remarkably attenuated the loss of Syn ($P < 0.05$ and $P < 0.01$, respectively), MBP ($P < 0.01$ and $P < 0.001$, respectively), and NeuN ($P < 0.05$ and $P < 0.05$, respectively).

To assess apoptosis, a TUNEL assay was performed in tissue from the ventral horn of spinal cords from all groups ($n = 6$). As shown in Fig. 7B, TUNEL-positive cells were rarely observed in the Sham group, whereas the percentage of TUNEL-positive cells was significantly higher in the SCI+PBS group ($50.357 \pm 5.292\%$; Fig. 7D) than in the Sham group ($4.522 \pm 0.662\%$; $P < 0.001$). Both the SCI+MSC and SCI+MSC-exo groups showed lower percentages of TUNEL-positive cells ($25.638 \pm 4.771\%$ and $21.085 \pm 5.211\%$, respectively) relative to the SCI+PBS group ($P < 0.01$ and $P < 0.001$, respectively).

MSCs and MSC-exo inhibit SCI-induced nuclear translocation of the NF κ B p65 subunit in astrocytes

Previous reports demonstrated that C3 is an astroglial target of NF κ B, and that A1 astrocytes are induced by NF κ B activation [6, 7]. Expression and distribution of the NF κ B p65 subunit in the spinal cord was detected by immunofluorescence and western blot (Fig. 8A, C). The percentage of p65⁺ nuclei in GFAP⁺ cells counted on the ventral horn was elevated in the SCI+PBS group ($23.373 \pm 3.469\%$) as compared with that in the Sham group ($4.745 \pm 0.846\%$; $P < 0.001$; Fig. 8B). Treatment with MSCs or MSC-exo reduced the percentage of p65⁺ nuclei in GFAP⁺ cells ($9.208 \pm 2.649\%$ and $10.318 \pm 2.286\%$, respectively) as compared with that in the SCI+PBS group ($P < 0.01$ and $P < 0.01$, respectively), with no significant difference observed between the MSC and MSC-exo treatment groups ($P > 0.05$). These results suggested that MSC and MSC-exo inhibited the astroglial p65 nuclear translocation induced by SCI in the ventral horn of the spinal cord.

The expression levels of p-IK β (NF-kappa-B inhibitor alpha) and nuclear p65 (p-p65) in the ventral spinal cord were significantly elevated in the SCI+PBS group as compared with those in the Sham group ($P < 0.001$ and $P < 0.001$, respectively; Fig. 8D). Additionally, in the SCI+PBS group, the expression of cytoplasmic IK β in ventral spinal cord decreased as

Fig. 8. Expression and distribution of the NFκB p65 in the ventral spinal cord of different groups. (A) Immunofluorescence images of the ventral horn that were double-stained by p65 (red) and GFAP (green). The p65-translocated nuclei (purple) are labeled with p65 and DAPI. Scale bar = 20 μm. (B) Quantification of the percentage of p65+ nuclei in GFAP+ cells in the ventral horn of spinal cord sections from different groups (n = 6). Error bars are standard errors. **P<0.01; ***P<0.001; nsP>0.05. (C) Western blot analysis of the expression of the cytoplasmic p-IKBα, IKBα, and p65, and the nuclear p-p65 in the ventral spinal cord of all groups. (D) Quantification of the relative expression of cytoplasmic p-IKBα, IKBα, and p65, and the nuclear p-p65 in the ventral spinal cord of all groups (n = 6). Error bars are standard errors. *P<0.05; **P<0.01; ***P<0.001; nsP>0.05.

compared with that in the Sham group (P<0.001). When compared with the SCI+PBS group, MSC and MSC-exo treatment showed reduced levels of p-IKBα and nuclear p65, and elevated level of cytoplasmic IKBα, with no significant difference observed in the levels of p-IKBα, cytoplasmic IKBα, and nuclear p65 between the MSC and MSC-exo treatment groups (P>0.05). Moreover, the level of cytoplasmic p65 did not differ among the four groups (P>0.05). These results indicated that MSCs and MSC-exo displayed equivalent effects on inhibition the SCI-induced phosphorylation and degradation of IKB, and nuclear translocation of NFκB p65 in the ventral spinal cord.

Fig. 9. The percentage of the p65-translocated nuclei and the proportion of A1 astrocytes in Sh-AST and SCI-AST with different treatment. (A) Schematic representation of the different groups of Sh-AST, SCI-AST, SCI-AST treated with PDTC, SCI-AST cocultured with MSC, SCI-AST cocultured with MSC and treated with TNF α , SCI-AST cocultured with MSC-exo, SCI-AST cocultured with MSC-exo and treated with TNF α . (B) Immunofluorescence images of the astrocytes double-stained with p65 (red) and GFAP (green) in different groups. The p65-translocated nuclei (purple) are labeled with p65 and DAPI. Scale bar = 20 μ m. (C) Immunofluorescence images of the astrocytes double-stained with C3 (red) and GFAP (green) in different groups. A1 astrocytes are double-labeled with C3 and GFAP. Scale bar = 20 μ m. (D) Quantification of the percentage of p65-positive nuclei in GFAP-positive cells in the seven groups (n = 6). Error bars are standard errors. * P<0.05; ** P<0.01; *** P<0.001; ns P>0.05. (E) Quantification of the ratio of C3/GFAP-double-positive area to GFAP positive area in the seven groups (n = 6). Error bars are standard errors. ** P<0.01; *** P<0.001; ns P>0.05.

MSCs and MSC-exo reduce A1 astrocytes via inhibiting the nuclear translocation of NF κ B p65

To verify whether A1 astrocyte reductions following MSC and MSC-exo treatment were mediated by NF κ B, we measured the percentage of p65⁺ nuclei in GFAP⁺ cells, as well as the proportion of A1 astrocytes in the seven groups (Fig. 9A). Immunofluorescence images showed the p65/GFAP/DAPI and C3/GFAP/DAPI-staining of different groups, respectively (Fig. 9B, C).

The percentage of p65⁺ nuclei and the proportion of A1 astrocytes in the SCI-AST group were $16.750 \pm 2.071\%$ and 0.139 ± 0.033 , respectively, which were higher than those observed in the Sh-AST group (P<0.001 and P<0.001, respectively; Fig. 9D, E). Compared with the

Fig. 10. Expression of C3, GFAP, p-IKBα, IKBα, p65, and nuclear p-p65 in different groups. (A) Western blot analyses of C3, GFAP, p-IKBα, IKBα, p65, and nuclear p-p65 levels in astrocytes from different groups. (B) Quantification of the relative expression of C3, GFAP, p-IKBα, IKBα, p65, and nuclear p-p65 in astrocytes from different groups (n = 6). Error bars are standard errors. *P<0.05; **P<0.01; ***P<0.001; nsP>0.05.

untreated SCI-AST, PDTC treatment reduced the percentage of p65⁺ nuclei (3.295±0.803%) and the proportion of A1 astrocytes (0.030±0.010). In the SCI-AST+MSC group, the percentage of p65⁺ nuclei and the proportion of A1 astrocytes were 3.760±0.884% and 0.019±0.011, respectively, and in the SCI-AST+MSC-exo group, these values were 4.072±0.869% and 0.023±0.012, respectively, with no significant difference observed in the values among the SCI-AST+PDTC, SCI-AST+MSC, and SCI-AST+MSC-exo groups. However, upon treatment of astrocytes with excess TNF-α, the reducing effects of MSCs and MSC-exo on p65⁺ nuclei and A1 astrocytes were attenuated.

Additionally, we evaluated the expression levels of A1 markers (C3 and GFAP) and NFκB-related proteins (p-IKBα, IKBα, p65 and p-p65) in different groups by western blot (Fig 10A). The results revealed increased levels of C3, GFAP, p-IKBα, and nuclear p-p65, and decreased expression of IKBα in the SCI-AST group as compared with that in the Sh-AST group ($p < 0.01$, $p < 0.01$, $p < 0.01$, $p < 0.001$, and $p < 0.001$, respectively; Fig. 10B). Treatment with PDTC, MSC, or MSC-exo reduced the levels of C3, GFAP, p-IKBα, and nuclear p-p65, and increased the expression of IKBα in astrocytes (Fig. 10B), with no significant difference observed in these protein levels between the MSC and MSC-exo treatment groups ($p > 0.05$). Moreover, the effects of MSC-exo treatment on C3, GFAP, p-IKBα, IKBα, and nuclear p-p65 levels and those of MSC treatment on the expression of C3, IKBα, and p-p65 were attenuated

by excess TNF α , whereas GFAP and p-IKB α levels showed no significant difference between the SCI-AST+MSC and SCI-AST+MSC+TNF α groups ($p > 0.05$).

In summary, these results implied that MSCs and MSC-exo may reduce A1 astrocytes via inhibiting the nuclear translocation of p65.

Discussion

In this study, we provided the first evidence showing that MSCs and MSC-exo significantly reduced A1 astrocyte proportion by inhibiting NF κ B activation, which resulted in their comparable beneficial effects on an SCI rat model. The intravenously applied MSCs and MSC-exo contributed to an improved locomotor function, reductions in proinflammatory cytokine levels, and the neuroprotective effect on residual neurons, synapses, and myelin sheath.

Following primary mechanical injury, limiting the development of secondary injury is key to treating SCI. Reactive astrocytes play a significant role in secondary injury in SCI, with “reactive astrocyte” first described during the 1970s after discovery of GFAP [36], which is currently used as a routine identifier of astrocytes in the healthy CNS. Elevated GFAP level is considered a marker of astrocyte reactivity [37]. Numerous studies report that reactive astrocytes rather than a single type, are highly heterogeneous, and that neuroinflammation and ischemia can induce two different types of reactive astrocytes, “A1” and “A2,” respectively. This terminology is similar to the nomenclature of microglia in the CNS, termed “M1” and “M2” [38, 39]. Additionally, previous studies demonstrated that A1 astrocytes might be activated via NF- κ B signaling [6]. A1 astrocytes lose most of their original astrocyte function and exhibit new functions where they secrete toxins that induce apoptosis of neurons and are toxic to synapses and the myelin sheath. C3 is a marker for A1 astrocytes but not expressed in A2 astrocytes [6, 35, 37, 40]. In this study, C3/GFAP was used as a double-marker for labeling A1 reactive astrocytes in the spinal cord.

MSC have been shown to promote anatomical and functional recovery in SCI models [41, 42]. Early intravenous delivery of MSC attenuates injury by regulating inflammation in SCI [43] and traumatic brain injury (TBI) [44-46]. Additionally, MSC transplantation can reduce the levels of proinflammatory cytokines, such as TNF- α and IL-1 [11, 47], and inhibit NF κ B signaling [12, 48, 49]. Moreover, MSCs are capable of switching microglia from a proinflammatory M1 phenotype towards an anti-inflammatory M2 phenotype *in vitro* [13] and in SCI models [50]. Furthermore, MSCs inhibit reactive astrogliosis in inflammation-induced preterm brain injury [51], attenuate GFAP overexpression in astrocytes in stroke [52], and modulate astrocytic end-feet in lipopolysaccharide-treated rats [53]. However, little is known about the effect of MSCs on inflammatory A1 astrocytes after SCI.

Initially cell replacement potential was considered as the mechanism through which MSCs exert therapeutic function in injury repair [54]; however, little evidence exists supporting transplanted-MSCs differentiation into functional cells *in vivo* [55]. Instead, paracrine capacity is a more important factor in the therapeutic effects provided by MSCs. The systemic administration of conditioned medium or the secretome from MSCs promotes recovery after SCI [56] and improves the outcomes of TBI in rats [57]. Recent studies showed that extracellular vesicles (EVs)/exosomes alone contributed to the therapeutic effects of MSCs in multiple animal models, such as those for ischemic stroke [16, 58, 59], acute kidney injury [60] and traumatic brain injury [17]. Exosomes comprise one of the main subclasses of EVs and are considered the most promising therapeutic mediator of MSC function by altering the activity of target cells via horizontal transfer of mRNAs, microRNAs, and proteins [61]. Compared with factors in the absence of membrane packaging, exosomes are more stable and capable of targeting inflammatory sites due to their enhanced permeability and retention effects [62, 63]. MSC-exo have been confirmed to exert beneficial effects on functional recovery and suppress the activation of A1 neurotoxic reactive astrocytes after SCI [24, 64]; However, it remains unclear whether these effects are comparable to those of live MSCs when applied systemically to SCI rats. In addition, the underlying mechanisms of

the A1-inhibitory effects exerted by MSC-exo have not been investigated before. This is the first study to compare the efficacy of MSCs and cell-free exosomes derived from MSCs on SCI models. MSCs and exosomes secreted by an equal number of MSCs were applied to SCI rats via tail vein. Given that the permeability of the blood-spinal cord-barrier (BSCB) increased significantly at the lesion site at 1 and 3 days post-SCI, fell greatly by 1 week post-SCI, and remained stable at uninjured sites [65], the ability of MSCs or exosomes to pass through the BSCB may be higher at injection time points within 1-week-post-SCI. Additionally, the inflammatory response develops within hours after SCI [66]; therefore, in this study, the first time point of intravenous-injection was chosen at 30 min post SCI to ensure that MSCs or MSC-exo exerted their functions at the beginning of the inflammatory cascade in spinal cord. Then the second injection was performed at 1 dpi to strengthen the effects.

Our findings suggested that both MSCs and MSC-exo displayed suppressive effects on A1 astrocytes, and showed an equal neuroprotective effect following SCI. Consistent with previous studies showing that MSC application decreased GFAP expression [67, 68], we found the MSCs and MSC-exo not only downregulated the general reactivity of astrocytes (GFAP) but also reduced the A1 proportion in the whole astrocytes.

Notably, PKH26-labeled exosomes were present at lesion sites, whereas PKH26-labeled MSCs were not detected at lesion sites. This finding has been previously and consistently observed: intravenously administered MSCs were not detected at lesion sites of SCI, whereas they showed beneficial effects on functional recovery [42, 65]. However, in immunosuppressed rats, intravenously applied MSCs migrated to the lesion sites of SCI [69] or cerebral ischemia injury [70], although no engrafted MSCs were detected at lesion sites 3 weeks post administration [71]. These results suggest that the differentiation ability and direct cell-to-cell contact are not the primary mechanisms that mediate the therapeutic functions of MSCs in injury repair. Although in the SCI models we work with, the efficacy of purified exosomes is equivalent to that of live MSCs, it is possible that MSCs exert other important functions undetected in this study. There are other components such as microvesicles (>500 nm in diameter) and tunneling tubes that might be involved in MSC functions [72-75]. However, in the present study, our results indicated that exosomes secreted by MSCs played a major role in the therapeutic function of intravenously administered MSCs in the SCI model.

NFκB signaling is widely activated after SCI [76] by multiple pro-inflammatory agents, such as cytokines (TNF-α and IL-1), stress, free radicals, and reactive oxygen species [77, 78]. Moreover, secondary inflammation in SCI is regulated by NFκB pathway [79], and inhibition of astroglial NFκB improves functional recovery after SCI [76]. In unstimulated cells, NFκB exists in the cytoplasm in the form of a complex with IκB, an NFκB inhibitor. When stimulated by proinflammatory cytokines, such as TNF-α [32] and IL-1, NFκB is activated. The activation of NFκB involves the phosphorylation and degradation of IκB, followed by the nuclear translocation of the NFκB p65 subunit. The nuclear-translocated NFκB units exert the function as a transcription factor that positively regulate the transcription of target genes by binding to their promoter regions [80, 81]. As a target gene of NFκB, the C3 promoter contains two κB binding sites [82, 83]. Additionally, C3 is a direct NFκB target in astroglia [84], and C3⁺ A1 astrocytes are induced through NFκB signaling. Therefore, we hypothesized that reductions in A1-astrocyte proportion following MSC or MSC-exo administration might be mediated by NFκB. Treatment with MSCs or MSC-exo attenuated the nuclear translocation of p65 accompanied by reductions in the proportion of the A1 subtype in cultured astrocytes. However, these effects were attenuated by excess TNF-α (an activator of NFκB). Although the direct interaction of p65 with C3 was not evaluated in the present study, we verified that MSC and MSC-exo reduced the A1 astrocyte proportion, at least partially through inhibition of NFκB p65 translocation/activation. Furthermore, we used a co-culture system that precluded direct MSC contact with astrocytes to ensure that the effect of the MSCs was mediated by their paracrine function. The exosomes secreted by an equal amount of MSCs exerted comparable effects on NFκB activation and reductions in the A1 subtype to live MSCs, indicating that among the paracrine components of MSCs, exosomes might play a central role in inhibiting NFκB p65 activation and reducing A1 astrocytes.

Previous researches have showed that MSC-exo may have multiple cell targets in the CNS. Nakano et al. reported that intraventricularly infused PKH26 labeled MSC-exo localized mainly to astrocytes in diabetic mouse brains [85]. Likewise, in a paper by Xiong et al., intravenously injected CD63 GFP-tagged MSC-exo were found taken up by neurons and astrocytes in traumatic brain injury rat brains [86]. However, a recent study reported that intravenously delivered DiR-labeled MSC-exo were detected predominantly within M2 macrophages, but only a small amount of them localized within astrocytic process endings in contused spinal cord [87]. It shall be noted that in this study, DiR-labeled MSC-exo were infused at one week post-contusion, whereas in our study, PKH26-labeled MSC-exo were infused at 30-min and 1-day post-injury. Considering the dynamic pathophysiological process after SCI, the different infusion time points may lead to diverse cellular localization for infused MSC-exo.

Here we focused on the effects of MSCs and MSC-exo on A1 astrocytes which are induced by SCI. However, we did not investigate other types of cells which may be involved in the activation of A1 astrocytes, such as microglia/macrophages and neutrophil. Activated microglia/macrophages and immune cells at the lesion site after SCI can produce a series of proinflammatory cytokines, including TNF- α , IL-1 and IL-6 [88, 89] that may lead to the activation of astroglial NF κ B. Given that both MSCs and MSC-exo have been confirmed to inhibit proinflammatory microglia in SCI [50], it is possible that MSCs and MSC-exo exert effects on A1 astrocytes not only in a direct way but also mediated by a variety of inflammatory cells.

Conclusion

In conclusion, the present study may provide a useful roadmap for future work on the mechanistic research about cell-free therapy for CNS injury.

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All animal procedures were approved by the Laboratory Animal Care Center of Zhengzhou University and were conducted in accordance with the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Zhengzhou University, China.

Disclosure Statement

No conflict of interests exists.

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