Original paper

Folia Neuropathologica

Bone marrow stromal cells reverse the microglia type from pro-inflammatory tumour necrosis factor α microglia to anti-inflammatory CD206 microglia of middle cerebral artery occlusion rats through triggering secretion of CX3CL1

Xiu-Juan Bai[1], Lei Hao[2], Yan-E Guo[1], Xiao-Bing Shi[1], Wei-Ping Wu[1]

[1]Geriatric Neurological Department of the Second Medical Center & National Clinical Research Center for Geriatric Diseases, Chinese People's Liberation Army General Hospital, Beijing, China, [2]Department of Neurology, Ren Ji Hospital University of Chinese Academy of Sciences, Chongqing, China

Folia Neuropathol 2021; 59 (1): 20-31 *DOI: https://doi.org/10.5114/fn.2021.105129*

Abstract

The middle cerebral artery occlusion (MCAO) model has been extensively applied to study ischaemic stroke. This study attempted to clarify effect of bone marrow stromal cells (BMSCs) on infarct injury of MCAO rats. BMSCs were isolated and identified by staining CD29/CD44 and CD31/CD45. CX3CL1 silencing vector (pLVX-shRNA-CX3CL1) was generated and infected to BMSCs. pLVX-shRNA-CX3CL1 infected BMSCs were transplanted into brain tissue of MCAO rats. Real-time PCR was used to determine CX3CL1 expression. Infarct areas were stained with TTC to evaluate infarct size. Double-staining immunofluorescence was conducted to determine anti-inflammatory type CD206 and pro-inflammatory type tumour necrosis factor α (TNF-α) microglia. Isolated BMSCs were positively presented for CD29/CD44, and negatively for CD31/CD45. CX3CL1 was significantly lower in the BMSC + pLVX-shRNA2-CX3-CL1 group compared to the BMSCs + pLVX group ($p < 0.05$). According to TTC and neurological scores, MCAO rats were successfully generated. BMSCs transplantation significantly increased CD206 microglia and decreased TNF-α microglia. However, shRNA-CX3CL1-infected BMSCs remarkably reduced CD206 microglia and enhanced TNF-α microglia compared to the MCAO + BMSCs group. In conclusion, BMSCs reverse microglia from pro-inflammatory type TNF-α microglia to anti-inflammatory type CD206 microglia in the infarct region of MCAO rats (3rd to 7th days post BMSC transplantation), through triggering of CX3CL1 secretion. Therefore, the potential effects of CX3CL1 secreted by BMSCs would provide an insight for stem cell-dependent therapeutic strategies in treating ischaemic stroke-associated disorders.

Key words: *middle cerebral artery occlusion, CX3CL1, BMSC transplantation, ischaemic stroke.*

Introduction

In the recent years, stroke has been become a common cerebrovascular disorder and has led to a higher proportion of disability and death throughout the world [20]. It is estimated that the incidence of stroke reached to 70/100,000 individuals in 2005 [9], and it is predicted to reach as much as 7.8 million patients by 2030 [16]. There are also 1.5 million deaths from stroke each year in China, making it the third most common reason for death after cardiovas-

Communicating author:
Prof. Wei-Ping Wu, Geriatric Neurological Department of the Second Medical Center & National Clinical Research Center for Geriatric Diseases, Chinese People's Liberation Army General Hospital, Beijing, China, e-mail: zucccyang@sina.com

cular disorders and cancer in developing countries [7]. Among all stroke patients, ischaemic strokes account for about 85% of cases; patients demonstrate severe dysfunctions of neurons because of the remarkable decrease of the cerebral blood flow in the brain [10]. Ischaemic stroke is mainly characterized by strong inflammations and thromboembolic vascular occlusion, therefore demonstrating an incidence rate about 400/100,000 individuals every year worldwide [8]. At present, the only available strategies for treating ischaemic stroke are thrombolytic treatments. However, there are also a few complications for thrombolytic treatment; therefore, the application of this is limited [13]. Therefore, it is necessary and critical to develop a method for treating ischaemic stroke with effective outcome and higher safety.

Cerebral ischaemia usually triggers the release and activation of chemokines and cytokines [15]. After brain ischaemia, the neurons can release soluble fractalkine (CX3CL1), which may inhibit microglial activation through binding the CX3CR1 in the brain tissues [22]. Meanwhile, CX3CL1 can also decrease the toxicity of microglia and alleviate the damage of neurons in animal models with neuropathology and inflammation [2,4]. Meanwhile, CX3CL1 reduces glutamate-induced neural death and has a neuroprotective effect on neurons [11]. Therefore, we hypothesised that neuron-expressing CX3CL1 might be involved in ischaemia-induced injury. In order to verify this hypothesis, we structured the shRNA-CX3CL1-expressing lentivirus vector and infected to bone marrow stromal cells (BMSCs), then the CX3CL1-expressed BMSCs were transplanted into middle cerebral artery occlusion (MCAO) rat models. Finally, the anti-inflammatory type CD206 microglia and pro-inflammatory type tumour necrosis factor α (TNF-α) microglia in infarct regions of MCAO rats were determined. The findings of this study reveal the target against ischaemic injury, which provides promising insight for improving the therapeutic strategy of stroke.

Material and methods

Animals

In this study, SD rats (3 weeks old) were purchased from the Experimental Animal Centre of Chongqing Medical University (Chongqing, China). The rats were given free access to food and water and were kept at room temperature in a light/dark cycle of 12 h/12 h.

The animal experiments were conducted in accordance with guide for Care and Use of Laboratory Animals of the NIH. All experimental procedures were approved by the Ethical Committee of The People's Liberation Army General Hospital, Beijing, China.

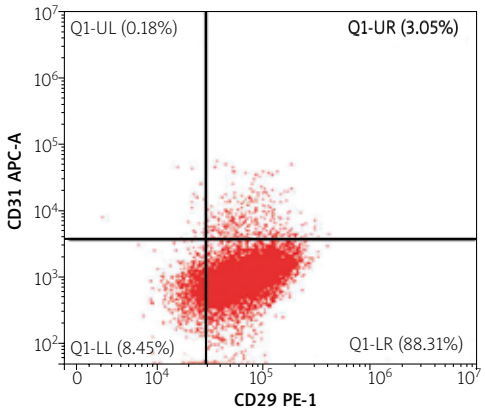
Isolation and identification of BMSCs

The isolation of BMSCs was conducted according to the instructed protocol of a form study [19]. In brief, the rats were killed by dislocating the cervical vertebrae and were then incubated with 75% alcohol for 15 min. The humeri and femurs of rats were isolated, placed on a culture plate containing 75% alcohol, and treated for 10 min. Then, the bone marrow canals in the humeri and femurs were washed using a DMEM-F12 (Gibco BRL. Co. Ltd., Grand Island, New York, USA)-filled sterile syringe repeatedly. The washed cells in bone marrow canals were cultured under condition of 5% CO_2 at 37°C for 24 h. After several passages, the adherent cells were harvested and subjected to identification.

The flow cytometry assay was conducted to verify the BMSCs as described in the previous study [6] with a few modifications. Briefly, the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, Missouri, USA) for 15 min and blocked with 5% BSA at 4°C for 60 min. Then, the cells were incubated using R-CD29 PE (CD44) and R-CD31 eFluor660 (CD45) antibodies (eBioscience, Santiago, CA, USA). Finally, the fluorescence signals were examined with a flow cytometer (Model: FC500 MPL, BD Biosciences, San Jose, CA, USA) at 488 nm.

shRNA-CX3CL1 synthesis

The targeting oligonucleotides for shRNA-CX3CL1 were synthesised by Western Biotech Co. Ltd. (Chongqing, China) as instructed by the sequence illustrated in Figure 1. Then, the above oligonucleotides were sub-cloned into the pLVX-shRNA2 vector to generate the pLVX-shRNA2-CX3CL1 plasmid. The pLVX-shRNA2-CX3CL1 plasmid was identified with both the gene sequencing method and enzyme digestion approach. Then, pLVX-shRNA2-CX3CL1 plasmid and the packing-necessary elements (including psPAX2, pMD2.G, and RNAi-mate) were mixed and co-cultured in 293T cells, as described in a previous study [21]. Moreover, the infecting efficacy of pLVX-shRNA2-CX3CL1 lentivirus was evaluated by examining the green fluorescence under a fluores-



Population	Percentage
Q1-UR: CD29+CD31+	2.84%
Q1-UL: CD31+	0.20%
Q1-LR: CD29+	89.43%
Q1-LL: Not labelled	7.54%

Fig. 1. Identification for the BMSCs by examining the CD29/CD44 and CD31/CD45 molecule with flow cytometry assay.

cence inverted microscope (model: TE2000U, Nikon, Tokyo, Japan).

Real-time PCR (RT-PCR)

The total RNAs in BMSCs were extracted with Trizol reagent (Beyotime Biotech., Shanghai, China) as instructed elsewhere. The complementary DNAs (cDNAs) were then generated using cDNA Synthesis reagent (Western Biotech., Chongqing, China) as instructed in the manufacturer's protocol. The mRNA transcription of CX3CL1 gene and β-actin gene was determined using SYBR Green I PCR kit (Western Biotech.). The specific primers for the gene amplification are illustrated in Table I. Eventually, the expression of CX3CL1 was analysed by normalizing to β-actin and calculated using the $2^{-\Delta\Delta ct}$ formula as described in a previous study [12].

MCAO rat model

The MCAO rat models were generated using the approach described by Longa *et al*. [16], with some modifications. In brief, the SD rats aged from 6 to 8 weeks (250-280 g) were anaesthetized by intraperitoneally injecting with 10% chloral hydrate (0.5 ml/100 g). The common carotid (right), external carotid (right), and the internal carotid arteries were then separated and exposed. About 18-22 mm of the nylon surgical thread was inserted into the right internal carotid artery via the external carotid artery blocking the origin to right middle cerebral artery. One hour after occlusion, the thread was removed slowly to made complete blood reperfusion for ischaemic regions. During the whole surgery process, the rats were maintained with a heating blanket to keep the body temperature at 37°C. The generated MCAO rats were divided into the MCAO model group (n = 9), the MCAO + BMSCs group (n = 9), and the MCAO + BMSCs + pLVX-shRNA2-CX3CL1 group (n = 9). Another 3 rats without any treatment were assigned as the control group. Also, the above MCAO group, MCAO + BMSCs group, and MCAO + BMSCs + pLVX-shRNA2-CX3CL1 group were sub-divided into the following groups: 1 day (n = 3), 3 days (n = 3), and 7 days (n = 3).

Table I. Primer sequences for the PCR assay

Genes		Sequences (5'-3')	Length
CX3CL1	Forward	ATGGTGGCAAGTTTGAGAAGC	145 bp
	Reverse	TCCTGGGAAATAGCAGTCGG	
β-*actin*	Forward	CCCATCTATGAGGGTTACGC	150 bp
	Reverse	TTTAATGTCACGCACGATTTC	

Transplantation of BMSCs + pLVX-shRNA2-CX3CL1 into the lateral ventricle

About 24 h after the blood reperfusion (MCAO rats), the rats were weighed, anaesthetized, and transplanted with BMSCs or BMSCs plus pLVX-sh-

Table II. Neurological functional scoring system evaluated 24 h post-operatively with Longa's method

Neurological score	Neurological status
0 score	No neurological symptoms
1 score	Inability to completely extend the right front paw
2 scores	Rotating while crawling and falling to the right side
3 scores	Inability to walk without assistance
4 scores	Unconscious

The rats with neurological scores ranging from 1 to 3 were included for experiment, and those scoring of 0 or 4 were excluded.

RNA2-CX3CL1 into the lateral ventricle. Briefly, the head was fixed on a brain stereotaxic instrument after skin preparation. The scalp was cut along the midline, the fascia was peeled off, and the anterior fontanelle was marked. The high-speed skull was drilled right above the transplantation site of the right ventricle and the skull was pierced. A micro-injector was inserted and the BMSCs + pLVX-shRNA2-CX3CL1 was injected slowly (assigned as the BMSCs + pLVX-shRNA2-CX3 CL1 group). The injection speed was 0.5 µl/min, and the injection volume was 5 µl/rat (including 10^5 cells). Meanwhile, the MCAO rats underwent the above processes, and transplanted with only BMSCs was included in the MCAO group (as the control group).

Evaluation for neurological scores

About 7 days, 24 h post infection of BMSCs + pLVX-shRNA2-CX3CL1 lentivirus into the MCAO rats, neurological scores were assessed by employing the scoring system reported by Longa *et al*. [14]. The scoring system was graded into 5 items, including scores of 0, 1, 2, 3, and 4, all of which are illustrated in Table II.

2,3,5-triphenyltetrazolium chloride (TTC) staining and measurement of infarct size

After evaluating for neurological functions, the rats were euthanised for infarct size analysis. Whole brain tissues were quickly isolated from rats and frozen at –20°C for 30 min. Cerebral infarct of brain tissues were stained with 2% TTC at 37°C for 10 min without light and then sliced into sections (2 mm thickness). Subsequently, sections were fixed with 4% paraformaldehyde at 37°C for 30 min. Eventually, the cerebral infarct size was analysed with image analysis software by integrating the infarct regions. The infarct size was calculated using the equation reported in a previous study [17].

Double-staining immunofluorescence assay for determining anti-inflammatory CD206 and pro-inflammatory TNF-α microglia

The tissues of infarct regions (the adjacent tissues of necrotic tissues or non-obvious necrotic areas) of MCAO rats were isolated and fixed with 4% paraformaldehyde. Then, the tissues were embedded, cut into sections with thickness of 5 µm (at least 3 sections for each group), and incubated with 5% non-fat milk at room temperature for 1 h. The sections were incubated using mouse anti-rat Iba antibody (first antibody) at 4°C overnight and washed with phosphate-buffered saline (PBS) 3 times. Subsequently, the sections were incubated with goat anti-mouse immunoglobulin G (IgG) (FITC labeled) at room temperature for 2 h. In the case of positively expressed antigens under a fluorescence microscope, the sections were incubated with rabbit anti-rat CD206 or anti-rat TNF-α at 4°C overnight and washed with PBS 3 times. Then, the sections were treated using goat anti-rabbit IgG (Cy5 labelled), washed with PBS 3 times, and observed under a fluorescence microscope. Approximately 45 visual fields of the infarct regions of 3 rats (3 sections for each rat and 5 fields for each section) in one group (including the 1-day, 3-day, and 7-day groups) were selected to count the Iba-positive microglia, CD206/TNF-α positive staining microglia, and double-staining microglia. The CD206 or TNF-α positive cells were represented as cells/mm^2.

Statistical analysis

The data were represented as mean ± standard deviation (SD) and analysed using SPSS software (version: 18.0, SPSS Inc., Chicago, IL., USA). Differences of data among groups were analysed by utilizing the ANOVA test validated by Tukey's post hoc test. $P < 0.05$ was assigned as a significant difference.

Results

Identification for BMSCs from rats

The surface biomarkers for the passaged BMSCs were determined using flow cytometry. The results showed that the immunophenotypes for BMSCs were positively presented for CD29 and CD44, but negative for CD31 and CD45 (Fig. 1). These results suggest that the BMSCs were successfully generated in this study.

pLVX-shRNA2-CX3CL1 lentivirus was generated

By using the targeting oligonucleotides for CX3CL1 gene (Fig. 2A), we synthesized the pLVX-shRNA-CX3CL1 plasmid. The pLVX-shRNA-CX3CL1 plasmid was identified using the enzyme digestion approach (Fig. 2B) and gene sequencing method (Fig. 2C); the results of both methods showed that pLVX-shRNA2-CX3CL1 plasmid was successfully generated. Meanwhile, the green fluorescence results also indicated that the infecting efficacy of pLVX-shRNA2-CX3CL1 lentivirus was high enough for the following experiments (Fig. 2D).

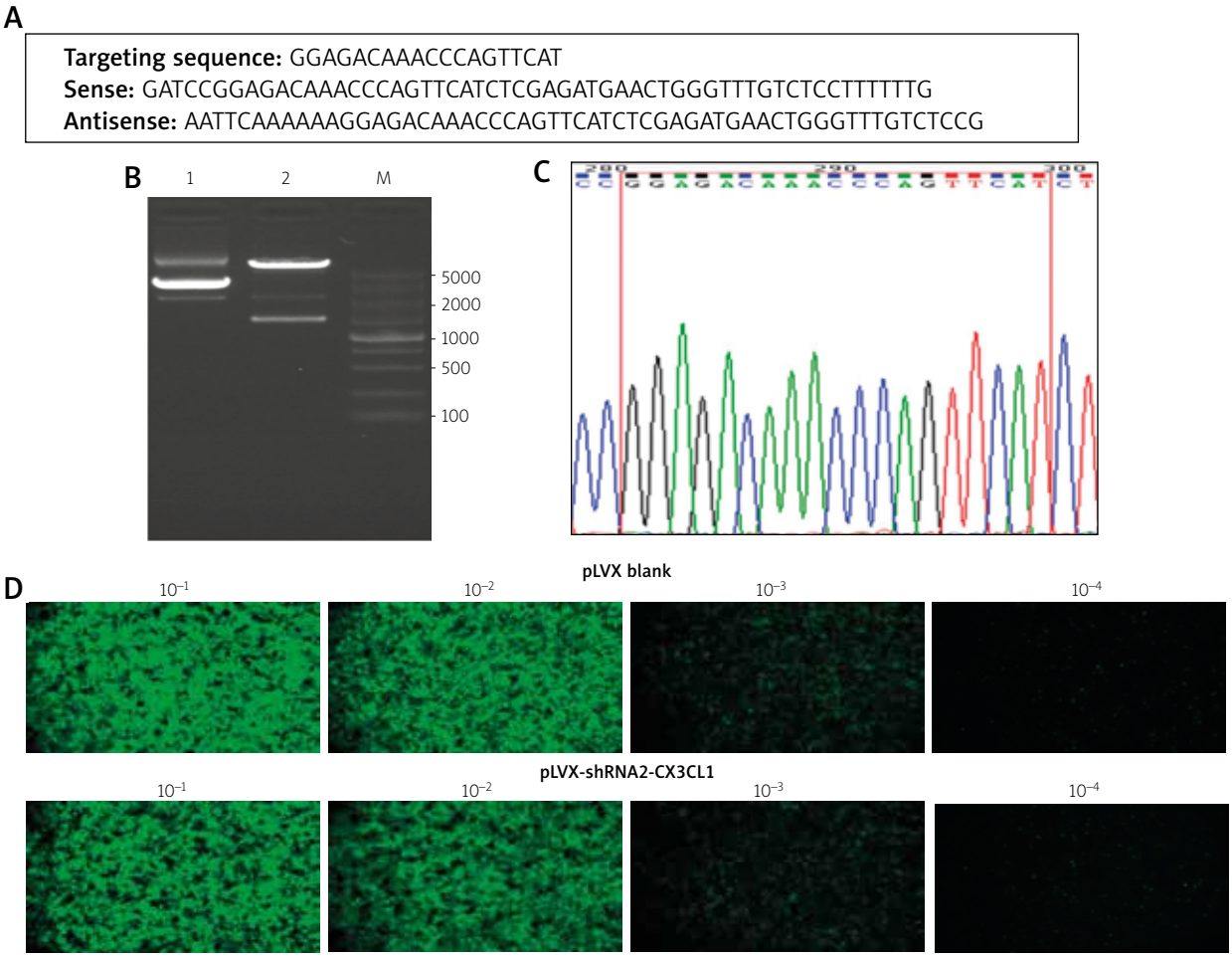


Fig. 2. Identification and infecting efficacy determination for generated pLVX-shRNA-CX3CL1 lentivirus. **A**) Targeting oligonucleotides for *CX3CL1* gene. **B**) pLVX-shRNA-CX3CL1 plasmid identification using enzyme digestion. **C**) pLVX-shRNA-CX3CL1 plasmid identification using gene sequencing method. **D**) Determination for infecting efficacy of pLVX-shRNA2-CX3CL1 lentivirus.

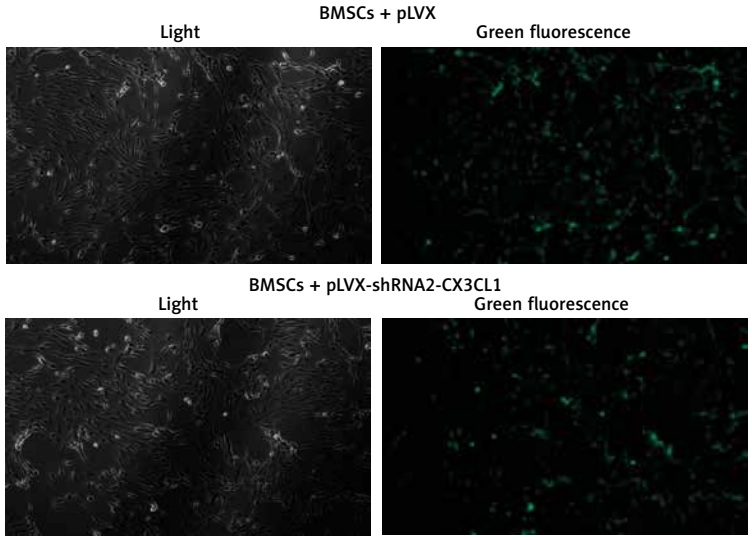


Fig. 3. Cell morphology and infecting efficacy of pLVX-shRNA-CX3CL1 lentivirus in BMSCs. Magnification, 400×.

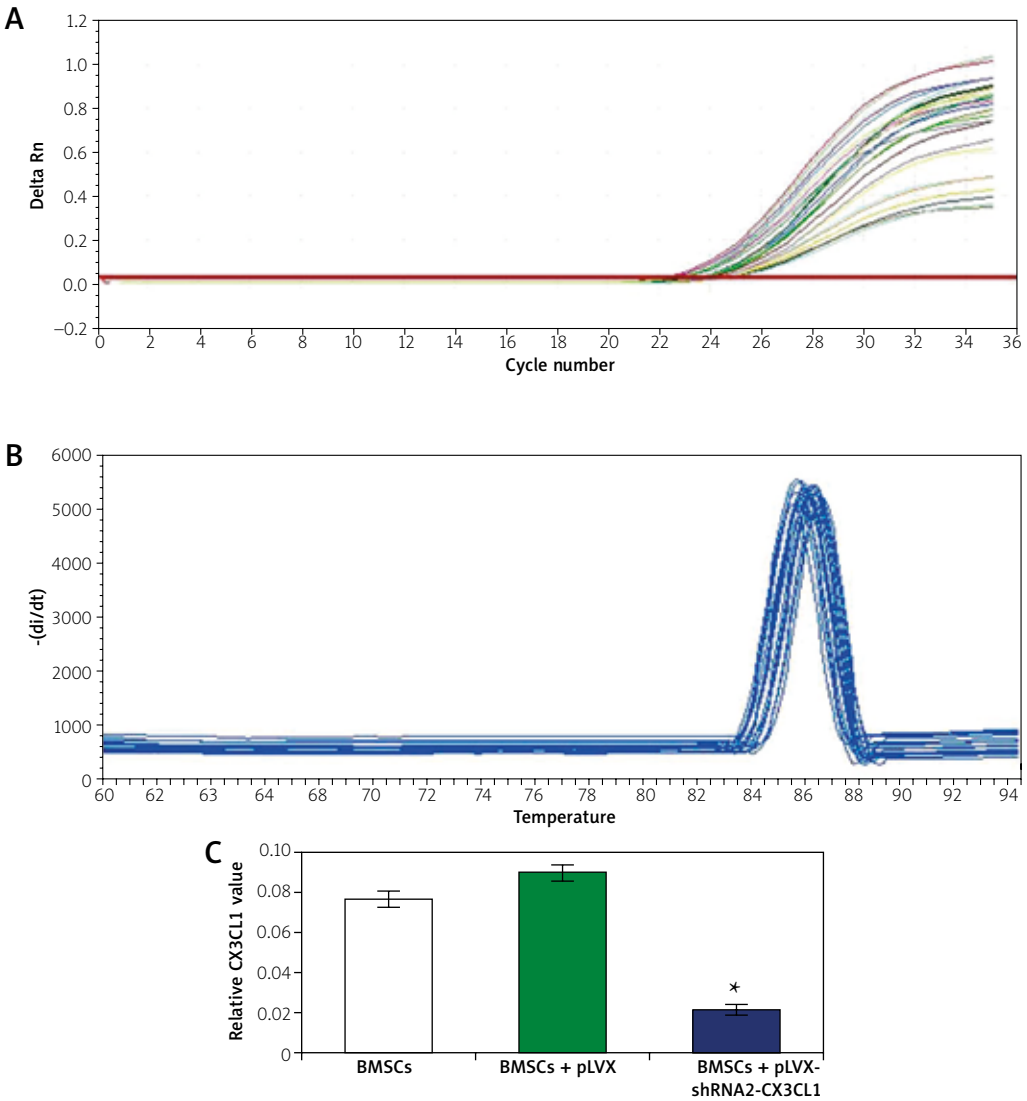


Fig. 4. Effects of pLVX-shRNA2-CX3CL1 lentivirus infection on CX3CL1 expression in BMSCs. **A**) Amplification curve of *CX3CL1* gene. **B**) Dissolution curve of *CX3CL1* gene. **C**) pLVX-shRNA2-CX3CL1 lentivirus infection reduced *CX3CL1* gene expression in BMSCs. $^{*}p < 0.05$ vs. BMSCs + pLVX group.

Moreover, pLVX-shRNA-CX3CL1 lentivirus infecting BMSCs kept the normal cell morphology with higher infecting efficacy (Fig. 3).

pLVX-shRNA2-CX3CL1 infection efficiently reduced CX3CL1 expression

Real-time PCR findings showed that the CX3CL1 expression was significantly lower in the BMSC + pLVX- shRNA2-CX3CL1 group compared to that in the BMSCs + pLVX group (Fig. 4, $p < 0.05$). However, no significant difference was found between the BMSCs group and the BMSCs + pLVX group (Fig. 4, $p > 0.05$). These results hint that pLVX-shRNA2-CX3CL1 lentivirus infection significantly suppressed the CX3CL1 expression in BMSCs.

MCAO rat model generation

According to the TTC staining results, there were no infarcts in brain tissues of the Normal-group rats (Fig. 5A). However, the infarct size of the MCAO rats was signifi-

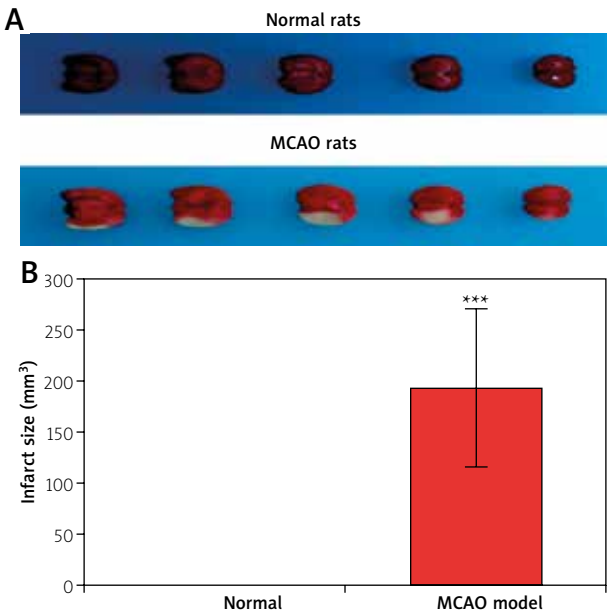


Fig. 5. Comparison for the infarct sizes of MCAO rats between normal rats and MCAO rats, determined by TTC staining. **A**) Infarct images of normal rats and MCAO rats. **B**) MCAO rats illustrated obvious infarct size. $^{***}p < 0.001$ vs. normal group.

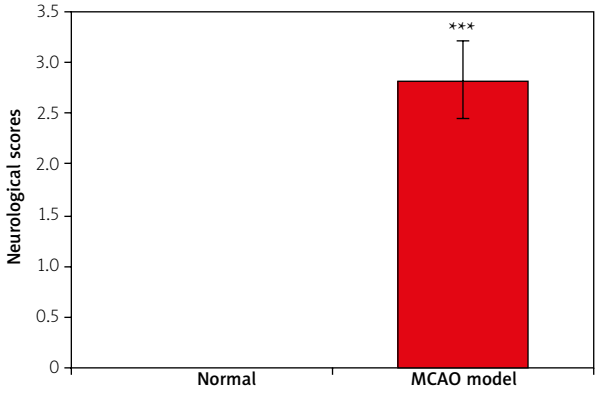


Fig. 6. Comparison for neurological scores of rats in normal group and MCAO rats. $^{****}p < 0.001$ vs. MCAO model group.

cantly increased in the MCAO model group compared to that in rats of the normal group (Fig. 5B, $p < 0.05$).

The MCAO rat model demonstrated significantly reduced neurological scores

In order to confirm established MCAO rat model, neurological function was evaluated. The findings showed that all of the MCAO rat models demonstrated neurological scores from 1 to 3, which suggests that the MCAO rat model was successfully established (Fig. 6).

BMSCs transplantation increased anti-inflammatory type CD206 microglia through triggering secretion CX3CL1 molecule

The results showed that BMSC transplantation (MCAO + BMSCs group) clearly increased amounts of anti-inflammatory type CD206 microglia in the infarct region of MCAO rats compared to that in the MCAO group at 1 day (Fig. 7A), at 3 days (Fig. 8A), and 7 days (Fig. 9A) post transplantation of BMSCs (all $p < 0.05$). However, the BMSC transplantation-induced increased amounts of anti-inflammatory type CD206 microglia in the infarct region of MCAO rats were significantly decreased by the interfering CX3CL1 administration (MCAO + BMSCs + shRNA-CX3CL1 group), when compared with that of the MCAO + BMSCs group at 1 day (Fig. 7A), 3 days (Fig. 8A), and 7 days (Fig. 9A) post treatment (all $p < 0.05$). These results suggest that BMSC transplantation might reverse MCAO-induced decreased amounts of anti-inflammatory type CD206 microglia by secreting the CX3CL1 molecule.

BMSC transplantation decreased pro-inflammatory type TNF-α microglia *via* triggering secretion of the CX3CL1 molecule

The double-staining results of Iba and TNF-α demonstrated that BMSC transplantation (MCAO + BMSCs group) clearly decreased amounts of pro-inflammatory type TNF-α microglia in the infarct region of MCAO rats compared to that in the MCAO group at 3 days (Fig. 8B) and 7 days (Fig. 9B) post transplantation of BMSCs (all $p < 0.05$). Meanwhile, there were no obvious pro-inflammatory type TNF-α microglia in the infarct region of MCAO rats at 1 day post BMSCs transplantation (Fig. 7B). Moreover, when compared with the MCAO + BMSCs group at 3 days (Fig. 8B) and 7 days (Fig. 9B) post treatment, the reduced amounts of pro-inflammatory type TNF-α microglia in the infarct region of MCAO rats were remarkably increased by interfering CX3CL1 administration (MCAO + BMSCs + shRNA-CX3CL1 group) (all $p < 0.05$). However, there were even no effects of interfering CX3CL1 administration on the amounts of TNF-α microglia in the infarct region of MCAO rats (Fig. 7B). These findings hint that BMSC transplantation could reverse MCAO-induced

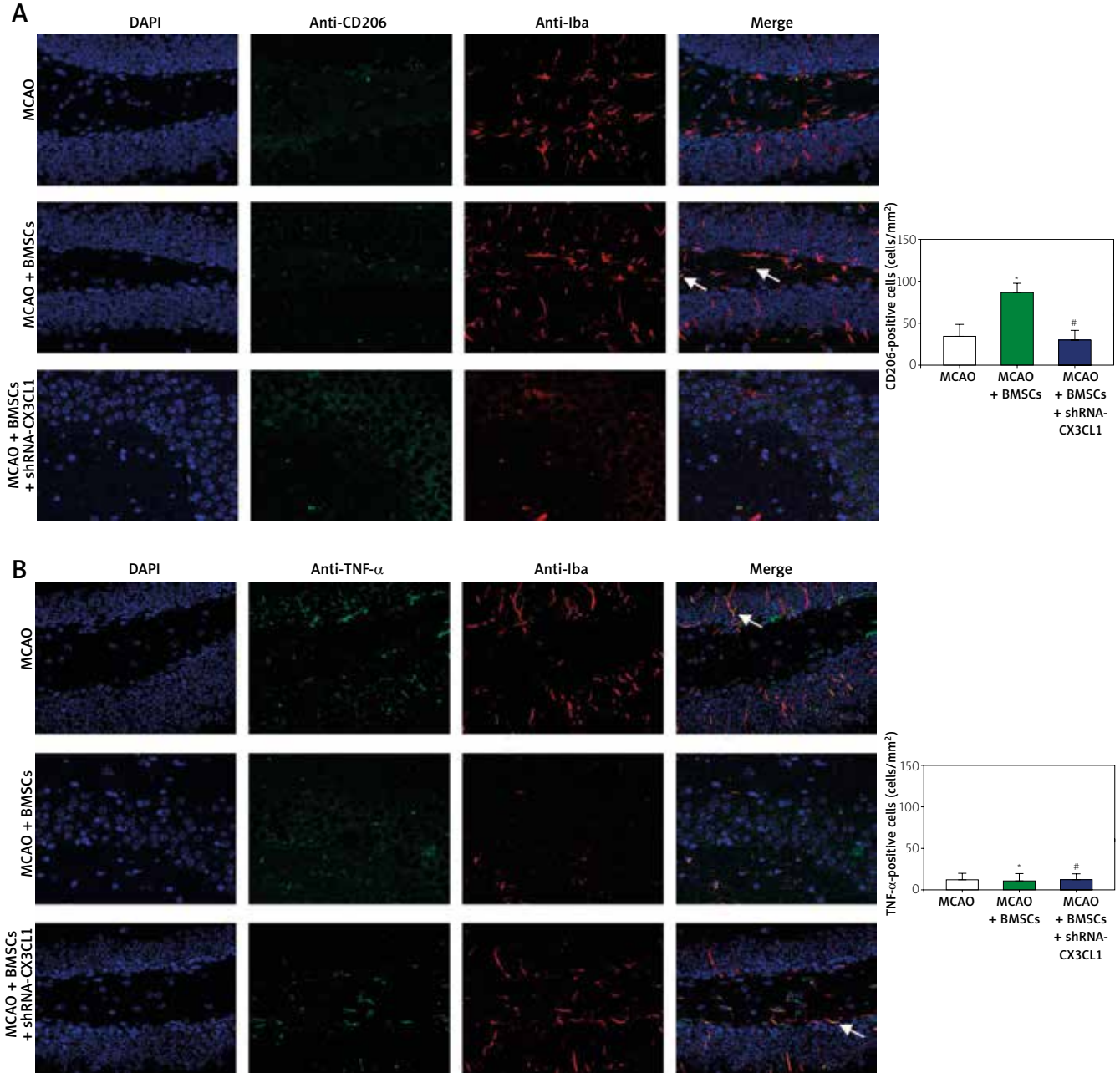


Fig. 7. BMSCs transplantation increased anti-inflammatory type CD206 microglia and decreased pro-inflammatory TNF-α microglia in MCAO rats at 1 day post administration. **A**) Double-staining images for the Iba and CD206 in microglia in MCAO, MCAO + BMSCs, and MCAO + BMSCs + shRNA-CX3CL1 group. **B**) Double-staining images for the Iba and CD206 in TNF-α microglia in MCAO, MCAO + BMSCs, and MCAO + BMSCs + shRNA-CX3CL1 group. The white arrows represented the double-stained microglia. *$p < 0.05$ vs. MCAO model group; #$p < 0.05$ vs. MCAO + BMSCs group. Magnification, 400×.

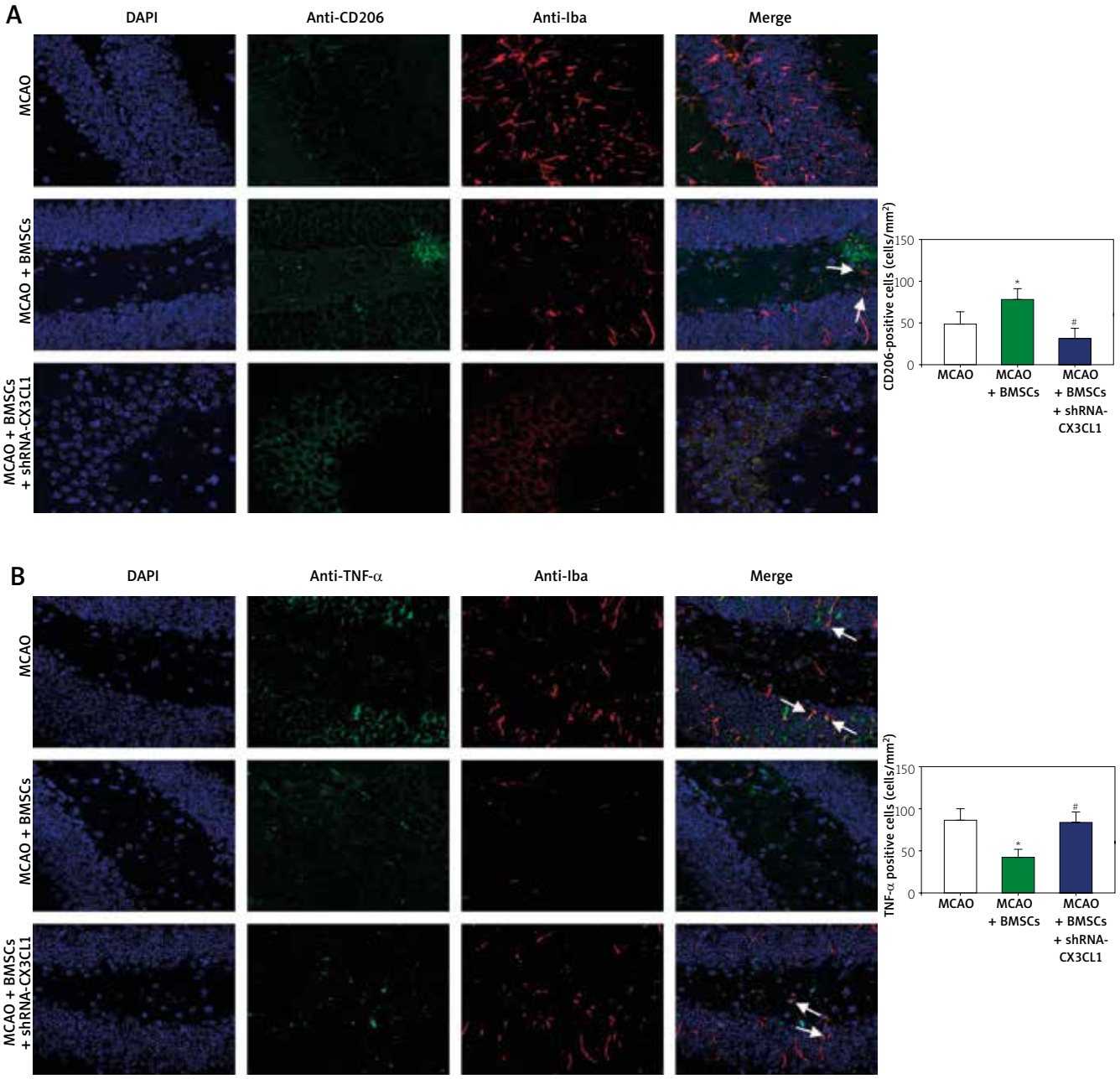


Fig. 8. BMSC transplantation increased anti-inflammatory type CD206 microglia and decreased pro-inflammatory TNF-α microglia in MCAO rats at 3 days post administration. **A**) Double-staining images for the Iba and CD206 in microglia in MCAO, MCAO + BMSCs, and MCAO + BMSCs + shRNA-CX3CL1 group. **B**) Double-staining images for the Iba and CD206 in TNF-α microglia in MCAO, MCAO + BMSCs, and MCAO + BMSCs + shRNA-CX3CL1 group. The white arrows represented the double-stained microglia. *$p < 0.05$ vs. MCAO model group; #$p < 0.05$ vs. MCAO + BMSCs group. Magnification, 400×.

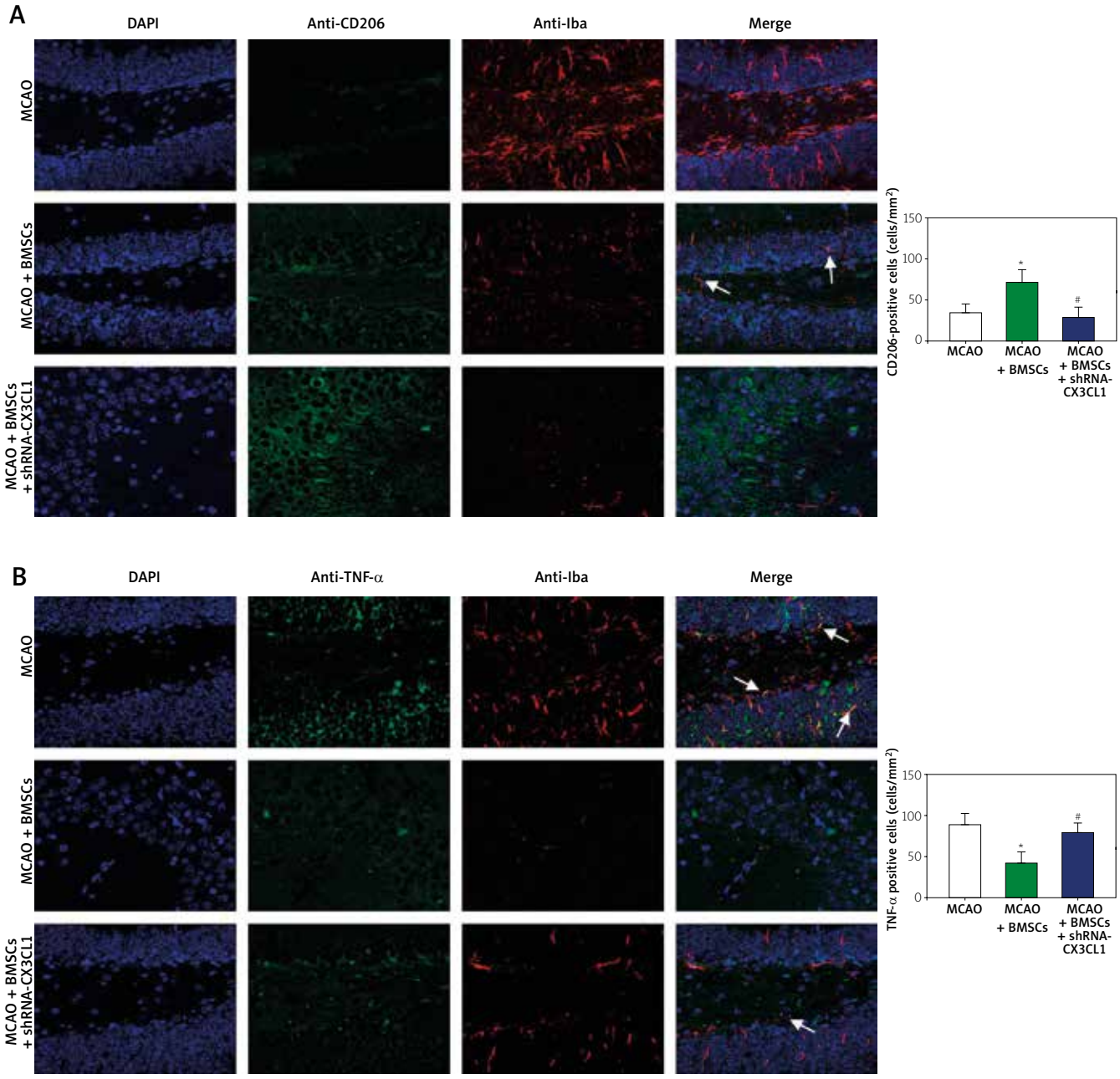


Fig. 9. BMSC transplantation increased anti-inflammatory type CD206 microglia and decreased pro-inflammatory TNF-α microglia in MCAO rats at 7 days post administration. **A**) Double-staining images for the Iba and CD206 in microglia in MCAO, MCAO + BMSCs, and MCAO + BMSCs + shRNA-CX3CL1 group. **B**) Double-staining images for the Iba and CD206 in TNF-α microglia in MCAO, MCAO + BMSCs, and MCAO + BMSCs + shRNA-CX3CL1 group. The white arrows represented the double-stained microglia. $^{*}p < 0.05$ vs. MCAO model group; $^{\#}p < 0.05$ vs. MCAO + BMSCs group. Magnification, 400×.

increased amounts of pro-inflammatory type TNF-α microglia through secretion of the CX3CL1 molecule.

Discussion

The results of the present study expand the application of BMSCs in treating ischaemic stroke. In our study, we proved that BMSC transplantation could decrease the BMSCs transplantation increased anti-inflammatory type CD206 microglia and decrease pro-inflammatory type TNF-α microglia *via* triggering secretion of CX3CL1 molecule. We speculated that both of the BMSCs-triggered increased CD206 microglia and decreased TNF-α microglia might be attributed to effects of BMSCs on improving the neurological functions of MCAO rats.

Previous studies [1,18] reported that BMSCs could significantly alleviate the symptoms of cardiovascular disorders, protect the normal functions of organs, and repair the damaged tissues. Moreover, BMSCs also play neuroprotective roles in retinal ganglion cells [5]. Therefore, in the present research, we generated the MCAO rat models and transplanted with BMSCs to observe the inflammatory response in the infarct regions of MCAO rats. The BMSCs were isolated and identified due to the positively expressed CD29/CD44 and negatively expressed CD31/CD45 molecule. Based on the commonly applied method for generating the MCAO model [14], MCAO rats were generated, illustrating the neurological dysfunction (with score from 1-3) and obvious infarcts in the brain. According to a previous study [3], the generation of an MCAO animal model induces brain/neurological dysfunctions, which is consistent with our findings. Until now, the MCAO rat model was established successfully, which would be used for the following experiments.

In order to clarify the effects of BMSCs on nerve regeneration and nerve repair, the BMSCs were directly transplanted to infarct regions of MCAO rats. We found that BMSC transplantation obviously increased amounts of anti-inflammatory type CD206 microglia and decreased amounts of pro-inflammatory type TNF-α microglia, which suggest that BMSCs play a role in protecting against the inflammatory response of microglia.

Cipriani *et al*. [4] reported that CX3CL1 demonstrates long-term neuroprotective effects on the cerebral ischaemia in rodents. Therefore, to explore the specific mechanism that triggers the protective effects of BMSCs on inflammation in microglia, we sub-cloned the shRNA-CX3CL1 gene into pLVX lentivirus to form pLVX-shRNA2-CX3CL1 lentivirus, which could efficiently inhibit expression of CX3CL1. Then, pLVX-shRNA2-CX3CL1 lentivirus-infected BMSCs (BMSCs + shRNA-CX3CL1) were transplanted to the infarct regions of MCAO rats. The findings showed that BMSC transplantation-induced increased amounts of anti-inflammatory type CD206 microglia in infarct regions of MCAO rats were significantly decreased by the interference of shRNA-CX3CL1. Meanwhile, the reduced amounts of pro-inflammatory type TNF-α microglia in the infarct region of MCAO rats were also increased by interfering with shRAN-CX3CL1. The above results suggest that the number of anti-inflammatory microglia increased and the number of pro-inflammatory microglia decreased in the MCAO model group after transplantation of BMSCs. Therefore, BMSC transplantation might reverse the distribution of anti-inflammatory and pro-inflammatory type (from pro-inflammatory type TNF-α microglia to anti-inflammatory type CD206 microglia), so as to inhibit the inflammatory responses in the infarct regions of MCAO rats. This reversal of anti-inflammatory microglia and pro-inflammatory microglia might be related to the secretion of CX3CL1 through BMSCs. After mild MCAO, the infarct always includes striatum and neocortex; however, the morphological features of necrosis (or cortical neuron damages) were not discovered in our MCAO rats (this might be associated with isolation of tissues from non-obvious necrotic areas), which should be considered as a limitation and clarified in a following study.

In conclusion, BMSC transplantation could reverse microglia from pro-inflammatory type TNF-α microglia to anti-inflammatory type CD206 microglia in the infarct region of MCAO rats (especially from 3 days to 7 days post BMSC transplantation), through triggering of the secretion of CX3CL1. Therefore, the potential effects of CX3CL1 secreted by BMSCs would provide an insight for stem cell-dependent therapeutic strategies in ischaemic stroke-associated disorders.

Disclosure

The authors report no conflict of interest.

References

1. Asakura H, Asamura R, Ontachi Y, Hayashi T, Yamazaki M, Morishita E, Miyamoto KI, Nakao S. Selective inducible oxide synthase inhibition attenuates organ dysfunction and elevated endothelin levels in LPS-induced DIC model rats. J Thromb Haemost 2005; 3: 1050-1055.
2. Catalano M, Lauro C, Cipriani R, Chece G, Ponzetta A, Di Angelantonio S, Ragozzine D, Limatola C. CX3CL1 protects neurons against excitotoxicity enchancing GLT-1 activity on astrocytes. J Neuroimmunol 2013; 263: 75-82.
3. Chen W, Guo Y, Yang W. Protective effect of ginsenoside Rb1 on integrity of blood-brain barrier following cerebral ischemia. Exp Brain Res 2015; 233: 2823-2831.
4. Cipriani R, Villa P, Chesse G, Lauro C, Paladini A, Micotti E, Perego C, De Simoni MG, Fredholm BB, Eusebi F, Limatola C. CX3CL1 is neuroprotective in permanent focal cerebral ischemic in rodents. J Neurosci 2011; 31: 16327-16335.
5. Cui Y, Xu N, Xu W, Xu G. Mesenchymal stem cells hydrogen peroxide-induced oxidative stress and enhance neuroprotective effects in retinal ganglion cells. In Vitro Cell Dev Biol Anim 2017; 53: 328-335.
6. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop DJ, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The international Society of Cellular Therapy position statement. Cytotherapy 2006; 8: 315-317.
7. Duan X, Wang W, Liu X, Yan H, Dai R, Lin Q. Neuroprotective effect of ethyl acetate extract from gastrodia elata against ttansient focal cerebral ischemia in rats induced by middle cerebral artery occlusion. J Tradit Chin Med 2015; 35: 671-678.
8. Favate AS, Younger DS. Epidemiology of ischemic stroke. Neurol Clin 2016; 34: 967-980.
9. Feigin V, Lawes CM, Bennett DA, Barker-Collo SL, Parag V. Worldwide stroke incidence and early case fatality reported in 56 population-based studies, a systematic review. Lancet Neurol 2009; 8: 355-369.
10. Hossmann KA. Pathophysiology and therapy of experimental stroke. Cell Mol Neurobiol 2006; 26: 1057-1083.
11. Limatola C, Lauro C, Catalano M, Ciotti MT, Bertollini C, Di Angelantonio S, Ragozzino D, Eusebi F. Chemokine CX3CL1 protects rat hippocampal neurons against glutamate-mediated excitotoxicity. J Neuroimmunol 2005; 166: 19-28.
12. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta ct}$ method. Methods 2001; 25: 402-408.
13. Logan JK, Pantle H, Huiras P, Bessman E, Bright L. Evidence-based diagnosis and thrombolytic treatment of cardiac arrest of periarrest due to suspected pulmonary embolism. Am J Emerg Med 2014; 32: 789-796.
14. Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without ccraniectomy in rats. Stroke 1989; 20: 84-91.
15. Pluta R, Ulamek-Koziol M, Januszewski S, Czuczwar S. Amyloid pathology in the brain after ischemia. Folia Neuropathol 2019; 57: 220-226.
16. Strong K, Mathers C, Bonita R. Preventing stroke: saving living lives around the world. Lancet Neurol 2007; 6: 182-187.
17. Sui R, Zang L, Bai Y. Administration of troxerutin and cerebroprotein hydrolysate injection alleviates cerebral ischemia/reperfusion injury by down-regulating caspase molecules. Neuropsychiatr Dis Treat 2019; 15: 2345-2352.
18. Wang B, Wu S, Ma Z, Wang T, Yang C. BMSCs pre-treatment ameliorates inflammation-related tissue destruction in LPS-induced rat DIC model. Cell Death Dis 2018; 9: 1024.
19. Wang B, Wu SM, Wang T, Liu K, Zhang G, Zhang XQ, Yu JH, Liu CZ, Fang CC. Pre-treatment with bone marrow-derived mesenchymal stem cells inhibits systemic intravascular coagulation and attenuates organ dysfunction in lipopolysaccharide-induced disseminated intravascular coagulation rat model. Chin Med J (Engl) 2012; 125: 1753-1759.
20. Xu B, He X, Sui Y, Wang X, Wang X, Ren L, Zhai YX. Ginkgetin aglycone attenuates neuroinflammation and neuronal injury in the rats with ischemic stroke by modulating STAT3/JAK2/SIRT1. Folia Neuropathol 2019; 57: 16-23.
21. Zhang J, Liu Y, Zang M, Zhu S, Chen B, Li S, Xue B, Yan L. Lentivirus-mediated CDglyTK gene modified free flaps by intra-artery perfusion show targeted therapeutic efficacy in rat model of breast cancer. BMC Cancer 2019; 19: 921.
22. Zhu J, Zhou Z, Liu Y, Zheng J. Fractalkine and CXCR1 are involved in the migration of intravenously grafted human bone marrow stromal cells toward ischemic brain lesion in rats. Brain Res 2009; 1287: 173-183.