IL-4-polarized BV2 microglia cells promote angiogenesis by secreting exosomes

Yuan Tian^{1,2,B-D}, Pei Zhu^{1,B}, Shanshan Liu^{1,B}, Zheng Jin^{1,B}, Dong Li^{1,E}, Heng Zhao^{3,A},
Xun Zhu^{1,A}, Chang Shu^{4,C}, Dongmei Yan^{1,A,D-F}, Zehua Dong^{5,A,F}

¹ Department of Immunology, College of Basic Medical Sciences, Jilin University, Changchun, China

² Key Laboratory of Molecular Enzymology and Engineering under the Ministry of Education, College of Life Sciences, Jilin University, Changchun, China

³ Department of Neurosurgery, Stanford University, USA

⁴ Department of Obstetrics, First Hospital of Jilin University, Changchun, China

⁵ Intensive Care Unit, the Affiliated Hospital of Qingdao University, China

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;

D – writing the article; E – critical revision of the article; F – final approval of the article

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Address for correspondence

Dongmei Yan
E-mail: dmyan@jlu.edu.cn

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Conflict of interest

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Abstract

Background. The microglia cell transfer has been shown to play a protective role in ischemic stroke. Microglia cells may play this nerve-protective role via the promotion of angiogenesis. However, the underlying mechanisms are largely unknown and need further investigation.

Objectives. The aim of this study was to investigate the pro-angiogenesis effects of unpolarized, interleukin 4 (IL-4)-polarized or lipopolysaccharide (LPS)-polarized BV2 microglia cells both in vivo and in vitro. We also investigated the potential mechanisms of these pro-angiogenesis effects.

Material and methods. BV2 cells were polarized using phosphate-buffered saline (PBS), LPS or IL-4, respectively. The gene expression pattern was analyzed with reverse transcription polymerase chain reaction (RT-PCR). The transfer of polarized BV2 cells was performed with an intravenous injection into mice 45 min after the middle cerebral artery (MCA) occlusion. Angiogenin expression was assessed with immunofluorescence. We also examined the angiogenesis effect of polarized BV2 cells and their exosomes through 3-dimensional co-cultures in vitro. Finally, the microRNA (miRNA) profiles of exosomes released by BV2 cells under different polarization conditions were examined using miRNA microarray.

Results. The IL-4-polarized BV2 transplantation promoted angiogenin expression in the ischemic brain. Interleukin 4-polarized microglia increased the tube formation of endothelial cells by secreting exosomes. The miRNA profiles of exosomes released by BV2 cells under different polarization conditions were different. Exosomes from IL-4-polarized BV2 cells contained higher amounts of miRNA-26a compared to those from the LPS-polarized and unpolarized BV2 cells.

Conclusions. Interleukin 4-polarized microglia cells might ameliorate the damage caused by ischemic stroke by promoting angiogenesis through the secretion of exosomes containing miRNA-26a.

Key words: angiogenesis, exosomes, microglia, interleukin 4

Introduction

Stroke is the second leading cause of death worldwide. It is most often caused by a thrombus or an embolus in the middle (MCA) or anterior cerebral artery (ACA), where an infarct develops after a few minutes of ischemia. Microglia are a type of neuroglia (glial cells) located throughout the central nervous system; they account for 10–15% of all cells within the brain and spinal cord. Microglia are also a type of resident macrophage cells, and are the first responding cells during the development of a stroke.^{1,2} There have been reports indicating that transplanting unstimulated HMO6 human microglial cells could protect animals from neural damage in a stroke by reducing neuronal cell death.³

Microglia/macrophages are known to have distinct phenotypes with various and even opposing functions. It has recently been reported that microglia with the M2-like phenotype (interleukin 4 (IL-4)-polarized microglia) are initially recruited at the injury site after the middle cerebral artery occlusion (MCAO), but they polarize to the M1-like phenotype (lipopolysaccharide (LPS)-polarized microglia) at a later stage.^{4,5} The M2-like microglia which are induced by type II cytokines like IL-4 and IL-13 could secrete cytokines that promote regenerative processes and neurogenesis while suppressing type I immunity; thus, the M2-like microglia could be beneficial in spinal cord injury and in stroke.⁶ Moreover, IL-4 has been proven to be essential for the treatment of ischemic brain damage, as well as for polarizing microglia/macrophages to a M2-like subset.⁷ Therefore, manipulating the polarization of microglia/macrophages might be a better treatment method not only for experimental stroke but in clinical treatment as well.^{8,9} A direct injection of M2-type bone marrow-derived macrophages (BMDM) after ischemia did not lead to a significant improvement,¹⁰ but this may be due to different causes of stroke, and different micro-environments may drive BMDM to different phenotypes with opposing functions under hypoxia conditions.¹¹ In short, the therapeutic effect of a direct M2 microglia/macrophages transplantation has not yet been proven. Polarizing resident microglia/macrophages in vivo may be a more suitable approach to the treatment of stroke.

Exosomes are small vesicles originating from the fusion of multivesicular bodies with a plasma membrane. The contents of exosomes, including proteins, mRNAs and microRNAs (miRNAs), play a crucial role in the activation, polarization or inhibition of immune cells. Recent findings indicate that exosomes derived from mesenchymal stromal cells significantly improved functional recovery by promoting angiogenesis in stroke rats.¹² Angiogenesis stimulates other downstream events, including neurogenesis, synaptogenesis, and neuronal and synaptic plasticity, which are all involved in the long-term repair and restoration process of the brain after an ischemic event. Particularly, recent studies have shown that the conditioned medium from metformin-treated BV2 cells can promote

angiogenesis in vitro.¹³ Whether IL-4-polarized microglia may promote angiogenesis by secreting exosomes needs further investigation.

In this work, we transferred polarized microglia to the brain in a mouse stroke model to determine the protective effect of microglia in vivo; we also investigated the underlying mechanisms in vitro by co-culturing endothelial cells with polarized microglia cells or exosomes secreted by those cells. Our results showed that M2-like microglia could protect the central nervous system, probably through the secretion of pro-angiogenesis exosomes.

Material and methods

BV2 cell culture and polarization

Mouse microglial cell line BV2 cells were cultured in a complete medium (Roswell Park Memorial Institute (RPMI) 1640 with L-glutamine, supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin) (North China Pharmaceutical Group Corp., Shijiazhuang, China). In order to eliminate exosomes, FBS was ultracentrifuged at 120,000 g for 90 min using a Ti70 rotor (Optima™ LE-80K Ultracentrifuge; Beckman Coulter Life Sciences, Indianapolis, USA) before being added to the medium. The cells were maintained at 37°C in a 5% CO₂ incubator. BV2 cells were seeded at 1×10^6 per well in a 25 cm² plate for 24 h before being stimulated with LPS (1 µg/mL) or murine IL-4 (25 ng/mL) for 48 h to generate classically activated macrophages (M1) or alternatively activated macrophages (M2), respectively.

Gene expression of polarized BV2 cells by reverse transcription polymerase chain reaction

For the reverse transcription polymerase chain reaction (RT-PCR) assay, BV2 cells were seeded in a 25 cm² flask and polarized with LPS (1 µg/mL) and IL-4 (25 ng/mL) to generate M1 and M2, respectively. After polarization, total RNA was extracted with TRIzol™ Reagent (Invitrogen, Carlsbad, USA), following the manufacturer's protocol, and then reverse-transcribed into complementary DNA (cDNA) using the SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen). The gene primers are shown in Table 1. The parameters for RT-PCR were as follows: denaturing at 94°C for 3 min, followed by 25 cycles of 94°C for 30 s, 51°C for 1 min and 72°C for 1 min, and then extension at 72°C for 2 min.

Middle cerebral artery occlusion model and transplantation of microglia

The animal procedures were approved by the Stanford Institutional Animal Care and Use Committee (Stanford University, USA) and were in accordance with the National

Table 1. Forward and reverse gene primers sequences

Gene	Primers
iNOS (forward)	ATGGCAACATCAGGTCGG
iNOS (reverse)	GCACAACCTGGGTGAACTCC
TNF- α (forward)	ACTGAACTTCGGGGTGATCG
TNF- α (reverse)	CCACTTGGTGGTTTGCTACG
Arg1 (forward)	CAGTCTGGCAGTTGGAAGC
Arg1 (reverse)	GGTGTGCAGGGGAGTGTG
TGF- β (forward)	GGACTACTACGCCAAGAAG
TGF- β (reverse)	TCAAAAGACAGCCACTCAGG
GAPDH (forward)	GACTTCAACAGCAACTCCCACTC
GAPDH (reverse)	TAGCCGTATTCTTGTCTATACCAG
VEGF (forward)	CTGCTGTAAACGATGAAGCCCTG
VEGF (reverse)	GCTGTAGGAAGCTCATCTCTCC
HGF (forward)	CTCCTGAAGGCTCAGACTTGGT
HGF (reverse)	CCAGAAGTAAATACTGCAAGTGG
FGF (forward)	AAGCGGCYCYACYGCAAGAACG
FGF (reverse)	CCTTGATAGACACAACCTCTCTC
EGF (forward)	ACTGGTGTGACACCAAGAGGTC
EGF (reverse)	CCACAGGTGATCCTCAAAACACG
PGF (forward)	TGCTGTGGTGATGAAGGTCTGC
PGF (reverse)	GCATTACAGAGCACATCCTGAG
PDGF (forward)	GTGGTCCTTACCGTCATCTCTC
PDGF (reverse)	GTGGAGTCGTAAGGCAACTGCA
MMP2 (forward)	CAAGGATGGACTCCTGGCACAT
MMP2 (reverse)	TACTCGCCATCAAGCGTTCCCAT
MMP9 (forward)	GCTGACTACGATAAGGACGGCA
MMP9 (reverse)	TAGTGGTGCAGGCAGAGTAGGA

Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

Male C57BL/J (20 $\pm$ 2 g) mice were purchased from The Jackson Laboratory (Bar Harbor, USA). Transient MCAO was induced using the method of intraluminal vascular occlusion described in our previous studies.¹⁴ Briefly, the mice were initially anesthetized with 5% isoflurane, and maintained at 1–2% during the surgery in a 70% N₂O and 30% O₂ mixture using a face mask. In order to maintain the body core temperature of the mice at 37 $\pm$ 0.5°C, a surface heating pad was used and the temperature was monitored by a rectal probe during the entire procedure. Then, the left common carotid artery, external carotid artery and internal carotid artery were surgically exposed by a ventral midline neck incision. The mice were subjected to MCAO with a 6-0 nylon monofilament suture (Doccol Corp., Sharon, USA), coated with silicone. At 45 min after MCAO, the occluded animals were re-anesthetized, the nylon monofilament suture was removed and the end of the external carotid artery was tied. The mice were allowed to wake up from anesthesia and returned to the cages.

The mice were randomly divided into 4 groups as follows (n = 5–7 mice per group): 1. vehicle treatment; 2. treatment with unpolarized microglia (the control

group); 3. treatment with LPS-polarized microglia; and 4. treatment with IL-4-polarized microglia. One million microglia cells in 0.1 mL of phosphate-buffered saline (PBS) were administered to each mouse with vein infusion immediately after the transient MCAO; the vehicle-treated mice were injected with an equal volume of PBS.

Immunofluorescence staining

At 2 days after MCAO, 3 groups of 5 experimental animals each were sacrificed and their organs were fixed by transcardial perfusion with PBS. The brain tissues were immersed in 4% paraformaldehyde (PFA) in 0.1 mol/L PBS (pH 7.4) for 48 h. The brain tissues were cut into equal samples (thickness: 50 μ m).

The slides were fixed with methyl alcohol and acetone (1:1) for 10 min, and then washed 3 times with PBS. After washing, the slides were blocked with 5% bovine serum albumin (BSA) and incubated overnight with anti-angiogenin Ab (diluted 1:200; Abcam, Cambridge, USA). The slides were washed 3 times in a wash buffer (PBS with 0.05% Tween[®] 20 (Institute of Chemical Technology, Beijing, China) for 15 min each time. After washing, the secondary antibody coupled with fluorescence was added and incubated for 2 h at room temperature. Then, 4',6-diamidino-2-phenylindole (DAPI) was added for staining for 5 min, followed by washing. The slides were sealed with Fluoromount[™] (Sigma-Aldrich, St. Louis, USA) and observed with confocal laser scanning microscopy using an Axio Vert inverted scanning microscope (Carl Zeiss AG, Oberkochen, Germany).

Endothelial cell co-culture with polarized BV2 cells and tube formation assay

In order to analyze the mechanism of the therapeutic effect of IL-4-polarized microglia and to investigate whether these cells can promote angiogenesis, we introduced a co-culture of endothelial cells and microglia. For all the in vitro experiments, the human umbilical vein endothelial cells (HUVEC) were used. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 μ g/mL streptomycin. Then, 48-well plates were filled with 150 μ L Matrigel[®] (BD Biosciences, Franklin Lakes, USA) and allowed to solidify at 37°C for 30 min. Subsequently, BV2 microglia subsets (4 $\times$ 10³ cells/well) were co-incubated with the HUVEC (4 $\times$ 10⁴ cells/well). After 24 h, network structures were analyzed at $\times$ 40 magnification using the AxioVision Microscopy software (Carl Zeiss AG) and photographed with a digital camera (ECLIPSE TS 100-F; Nikon, Tokyo, Japan).^{15,16} The number of tubes per picture was counted using the ImageJ program (National Institutes of Health, Bethesda, USA).

Assessment of vascular endothelial growth factor secreted by polarized BV2 cells

The supernatants of the polarized BV2 cells were collected after 48 h of LPS and IL-4 stimulation. Then, the concentration of vascular endothelial growth factor (VEGF) secreted by the polarized BV2 cells was detected using the SMMV00 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Inc., Minneapolis, USA). The detection of the concentration of VEGF in the control, M1 and M2 supernatants was repeated 3 times.

Isolation and identification of microglia exosomes

The microglia exosome isolation procedures were performed at 4°C as described in the literature, using an exosome extraction kit (System Biosciences, Palo Alto, USA). Briefly, the cell supernatants were centrifuged at 3000 g for 15 min to remove cells and cell debris; the supernatants were added to an exosome extraction reagent and mixed gently. After 12 h at 4°C, the mixture was centrifuged at 10,000 g for 30 min; the pellets were collected and resuspended in 50–100 µL of PBS, and used for the analysis of the exosome-enriched fraction. For the transmission electron microscopy (TEM) morphology investigation, the pellets obtained by this process were subjected to uranyl acetate negative staining on the formvar/carbon-coated 400-mesh copper electron microscopy grids (FCF400-Cu; Electron Microscopy Sciences, Hatfield, USA). Twenty microliters of the sample were applied to the grid and incubated for 1 min at room temperature, and then the excess solution on the grid was wicked off and dried for 30 min with filter papers. An equal part of 10% uranyl acetate was added to the grid for 1 min for negative staining. The preparations obtained were examined at 70 kV with a Philips 208 electron microscope (Philips Healthcare, Bothell, USA) with the DigitalMicrograph™ (Gatan, Inc., Pleasanton, USA). Western blot was used to identify TSG101, CD81 and CD63 (primary antibody, 1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, USA), the specific exosomal protein markers. The protein concentrations of the exosome preparations were determined using the micro bicinchoninic acid protein assay (Thermo Fisher Scientific, Lafayette, USA).

Tube formation by microglia exosomes

Exosomes from other cells, such as multiple myeloma cells and mesenchymal stem cells, can promote angiogenesis. The effect of exosomes from IL-4-polarized microglia on angiogenesis has not been clear. The HUVEC were cultured as described above, then 48-well plates were filled with 150 µL Matrigel (BD Biosciences) and allowed

to solidify at 37°C for 30 min. The exosomes were separated by the method described above. Briefly, the exosomes from 5×10^6 polarized cells were co-incubated with the HUVEC. After 12 h, network structures were analyzed at $\times 40$ magnification using the AxioVision Microscopy software (Carl Zeiss AG) and photographed with a digital camera (Nikon). The number of tubes per image was counted using ImageJ (NIH).

RNA extraction and microRNA array

RNA was extracted with TRIzol (Invitrogen) according to the manufacturer's protocol, and the Nanodrop™ Spectrophotometer (Thermo Fisher Scientific, Waltham, USA) was used to assess the RNA present. The samples extracted from M0, M1 and M2 type microglia exosomes were then delivered to GMINIX Co. (Shanghai, China). Commercial mouse miRNA microarrays, containing 1,908 mouse mature microRNAs from the Sanger mirBase database v. 20.0 (2 probes for each miRNA on each chip) were used to analyze the expression of miRNA in different types of BV2 cells. The tagged miRNAs were purified and hybridized with the GMINIX microRNA Microarray-Single according to the manufacturer's instructions. After the hybridization, the chips were subjected to a stringent wash and fluorescence data were collected using the GeneChip Scanner 3000 (Thermo Fisher Scientific, Waltham, USA); the chips were scanned at a pixel size of 10 µm with Cyanine 3 (Cy3) Gain at 460 nm and Cyanine 5 (Cy5) Gain at 470 nm scanning. Equal RNA from 6 individual cell samples with the same treatment was mixed and each mixture sample was repeated twice.

The data was shown as mean $\pm$ standard deviation (SD). The p-values were calculated using Student's t-test and post hoc test for multiple comparisons.

Results

Interleukin 4-polarized BV2 cells upregulated the expression of angiogenin in the ischemic brain

Angiogenin is a potent angiogenic growth factor that degrades the basement membrane, thereby facilitating cell invasion and migration. To check whether BV2 cells could induce angiogenesis by producing angiogenin, we polarized BV2 cells with either LPS or IL-4. The gene expression pattern was analyzed by RT-PCR (Fig. 1). We also detected the expression of angiogenin in the ischemic brain after IL-4-polarized BV2 treatment using immunofluorescence staining. The results showed that IL-4-polarized microglia significantly increased the expression of angiogenin and vascular density after 14 days compared with the control mice (Fig. 2).

Fig. 1. Gene expression of polarized BV2 cells

A. Reverse transcription polymerase chain reaction (RT-PCR) results showing that BV2 express M1 markers (*TNF-α* and *iNOS*) or M2 markers (*Arg-1* and *TGF-β*)

B. Quantitative data for the expression of *TNF-α*, *iNOS*, *Arg-1*, and *TGF-β*

Data is presented as mean ± standard deviation (SD) from triplicates; *Arg-1* – arginase 1; *GAPDH* – glyceraldehyde 3-phosphate dehydrogenase; *IL-4* – interleukin 4; *iNOS* – inducible nitric oxide synthase; IOD – integrated optical density; LPS – lipopolysaccharide; *TGF-β* – transforming growth factor β; *TNF-α* – tumor necrosis factor α; M1 – classically activated macrophages; M2 – alternatively activated macrophages.

Fig. 2. Expression of angiogenin in ischemic mice increased 14 days after the M2 microglia transplantation

A. Immunostaining of angiogenin in the ischemic brain on day 2 and day 14 after control and interleukin 4 (IL-4)-polarized BV2 treatment (4',6-diamidino-2-phenylindole (DAPI): blue; angiogenin: red; n = 3 per group; the scale bar represents 20 μm)

B. Total fluorescence of angiogenin in the ischemic brain on day 2 and day 14

M0 – control; M2 – IL-4-polarized BV2 treatment; ** p < 0.01 compared to the M0 group.

Fig. 3. Interleukin 4-polarized microglia promoted angiogenesis in vitro. BV2 microglia subsets (4×10^3 cells/well) were co-incubated with human umbilical vein endothelial cells (HUVEC) (4×10^4 cells/well) for 24 h

A. Network structures were analyzed at $\times 40$ magnification and photographed with a digital camera (n = 3 per group; the scale bar represents 200 μm)

B. The number of tubes per picture was counted using ImageJ software

M1 – LPS-polarized BV2 treatment; * p < 0.05 compared to the HUVEC group; NS – statistically nonsignificant.

Fig. 4. Angiogenesis-related gene expression of polarized BV2 cells. The expression of angiogenesis-related genes was detected with RT-PCR and the release of VEGF was detected in the culture supernatants of polarized BV2 cells using enzyme-linked immunosorbent assay (ELISA)

A. Expression of genes related to angiogenesis in M0, M1 and M2 BV2 cells

B. Concentrations of VEGF in the culture supernatants of M0, M1 and M2 BV2 cells; there were no significant differences between these cells ($p > 0.05$)

EGF – epidermal growth factor; FGF – fibroblast growth factor; HGF – hepatocyte growth factor; MMP2 – matrix metalloproteinase 2; MMP9 – matrix metalloproteinase 9; PDGF – platelet-derived growth factor; PGF – placental growth factor; VEGF – vascular endothelial growth factor.

Fig. 5. Characteristics of exosomes

A. Transmission electron micrographs of the exosomes derived from BV2 cells stimulated by IL-4 (the scale bar represents 200 nm)

B. Western blotting analysis for exosome markers CD63, CD81 and TSG101

cont – positive control.

Interleukin 4-polarized BV2 cells increased the tube formation in vitro

To study the role of macrophage subsets in angiogenesis, we performed in vitro tube formation assays using a co-culture of endothelial cells and macrophages on a Matrigel base. Culturing endothelial cells in these settings led to the formation of tubular structures after 24 h. Adding IL-4-polarized BV2 cells to endothelial cells increased the number of tubes as compared to the control situation (Fig. 3).

Interleukin 4-polarized BV2 cells promote the tube formation by secreting exosomes

In order to ascertain the mechanism by which IL-4-polarized BV2 cells promote angiogenesis, we first detected

the gene expression of secreted growth factors related to angiogenesis, such as *VEGF*, hepatocyte growth factor (*HGF*), fibroblast growth factor (*FGF*), epidermal growth factor (*EGF*), placental growth factor (*PGF*), platelet-derived growth factor (*PDGF*), matrix metalloproteinase 2 (*MMP2*), and matrix metalloproteinase 9 (*MMP9*). Although the *VEGF* gene expression increased in the IL-4-polarized BV2 group compared with the control group, its level was lower than in the LPS-polarized group (Fig. 4A). There were no significant differences in the ELISA results for VEGF between the control culture, LPS-polarized BV2 cells and IL-4-polarized BV2 cells (Fig. 4B). The expression of other genes showed no significant increase in the IL-4-polarized BV2 group compared with the LPS-polarized group (Fig. 4A). These results showed that the tube formation of endothelial cells

Fig. 6. IL-4-polarized microglia exosomes promoted angiogenesis in vitro. The exosomes from 5×10^6 polarized cells were co-incubated with HUVEC cells for 12 h

A. Network structures were analyzed at $\times 40$ magnification and photographed with a digital camera ($n = 3$ per group; the scale bar represents 200 μm)
 B. The number of tubes per picture was counted using ImageJ software

exo – exosomes; * $p < 0.05$ compared to the HUVEC group; NS – statistically nonsignificant.

Fig. 7. Heat map of microRNA expression array of M0, M1 and M2 microglia-derived exosomes. An intensity key is given next to the heat map. Each sample is represented as an average of duplicates

promoted by IL-4-polarized macrophages was not related to the secretion of VEGF, HGF, EGF, PGF, PDGF, MMP2, and MMP9.

We next investigated the exosomes secreted by polarized BV2 cells in terms of size, ultrastructures and quantity, and then analyzed their activities promoting angiogenesis. Transmission electron microscopy revealed that the size of the exosomes was about 50–100 nm and each vesicle showed the classic cup-shaped appearance (Fig. 5A). Western blotting results showed the expression of common exosome markers like CD63, CD81 and TSG101 (Fig. 5B). Then, a tube formation assay was performed with exosomes from different microglia subsets. In comparison with the controls, the exosomes from IL-4-polarized BV2 cells stimulated the tube formation: the quantities of tubes

increased significantly (Fig. 6). There was no difference between the LPS-polarized group and the controls. This showed that the exosomes from IL-4-polarized BV2 microglia had pro-angiogenic properties.

The promotion of angiogenesis by the M2-type BV2 cells may be due to the secretion of miRNA-26a. Exosomes have been shown to play an important role in the regulation of cell activities. Recent studies have also shown that the content of exosomes, like miRNA, could enter a recipient cell once the exosome membrane fuses with the cell membrane. In this work, we analyzed the different miRNA profiles of unpolarized, LPS-polarized and IL-4-polarized BV2 cells (Fig. 7). We found that miRNA-26a, which has been shown to have angiogenic properties, was selectively upregulated by IL-4 polarization (Table 2).¹⁷

Table 2. MicroRNAs of exosomes released by M0-, M1- and M2-type microglia

Transcript ID	Mean signal of group M0	Mean signal of group M1	Mean signal of group M2
miR-466b-3p	5.521937	4.806909	2.125578
miR-466c-3p	5.521937	4.806909	2.125578
miR-466p-3p	5.521937	4.806909	2.125578
miR-8117	9.017336	8.827255	7.226154
miR-151-5p	8.958132	8.5831889	6.680118
miR-3620-5p	11.610691	11.805563	13.094678
miR-7221-3p	12.137256	12.323571	13.408188
miR-677-3p	9.540374	9.610824	11.439959
miR-1956	7.252635	7.637256	7.637256
miR-6360	4.987463	4.509745	2.048182
miR-6970-5p	11.378213	11.735456	12.896675
miR-346-3p	12.529241	12.37427	13.65795
miR-26a-5p	9.300976	8.937378	7.642079
miR-7033-5p	10.858716	10.955668	12.375583
miR-702-5p	9.151745	9.149582	8.055301
miR-15b-5p	9.812329	9.292808	7.689586
miR-7235-5p	11.251865	11.324551	12.264028
miR-1940	7.170623	7.549589	5.388706
miR-7049-5p	7.799654	7.473127	6.029101
miR-466a-5p	5.572361	5.641108	2.280584

Discussion

Our study offers a few points on the therapeutic effect of IL-4-polarized microglia in the acute and chronic phases of ischemic stroke. We showed that IL-4-polarized BV2 cells may promote the tube formation *in vitro* and angiogenesis *in vivo* through the secretion of exosomes containing miRNA-26a. Moreover, exosomes released by IL-4-polarized microglia may have a potential therapeutic value in the treatment of stroke.

Current stroke therapies include regulatory T cells,¹⁸ BMDM,¹⁹ human mesenchymal stem cells,²⁰ and neural stem cells,²¹ all of which might have the potential to shift the inflammatory environment and restore the neurological function after a stroke. However, as a kind of resident macrophages, microglia could be polarized or stimulated directly by the changes in the microenvironment in the brain during all stages of a stroke. Angiogenesis is essential in physiological processes, such as embryonic development and wound-healing tissue repair.²² New capillaries are formed from pre-existing blood vessels, allowing the recovery of the supply of anti-inflammatory factors and neuron growth factors. Microglia/macrophages are not only key players in inflammatory diseases, but also in promoting angiogenesis.²³ In this work, our results showed that microglia could promote angiogenesis not by conventional pathways (i.e., direct cell–cell contact or VEGF signaling), but by secreting exosomes, which concurs with previous reports.^{24–26}

The proteomic analysis of exosomes has revealed the presence of specific proteins involved in cell motility, angiogenesis, inflammatory regulation, or neuromodulation,

suggesting that glial cells use unconventional pathways for protein secretion.²⁷ Some reports have shown that the overexpression of CXCR4 in exosomes secreted from mesenchymal stem cells (ExoCR4) also promotes the recovery of cardiac functions after myocardial infarction (MI).^{25,28} Other reports have also shown that the exosome miRNAs from breast cancer (let-7a, miR-23b, miR-27a/b, miR-21, let-7, and miR-320b) are known to present anti-angiogenic activity.²⁹ In our study, the expression of miRNA-26a in M0, M1 and M2 microglia was quite different, and there have been numerous reports showing that miRNA-26a is closely related to angiogenesis. Qian et al. and other authors found that miRNA-26a promoted tumor growth and angiogenesis in glioma by directly targeting prohibitin.¹⁷ On the other hand, miRNA-26a suppresses the epithelial mesenchymal transition in human hepatocellular carcinoma; miRNA-26a is also a key factor in angiogenesis in diabetic wound healing.^{30,31} These results indicate that different exosomes from various cell types have different effects on angiogenesis under special circumstances.³²

The hypoxic state is a situation in which exosome-mediated signaling promotes angiogenesis in some solid tumors and ischemic infarction.^{32–34} In the neural system, the hypoxia-inducible factor (HIF) pathway is involved in angiogenesis in an exosome-dependent manner.³⁵ The study of exosomes provided a platform for the diagnosis and monitoring of neurodegenerative progression.³⁶ In our experiment, the M2-like microglia could secrete specific exosomes to promote neovascularization, and then carry more Th2/M2-type cells into the ischemic region, which would be beneficial in the recovery from ischemic stroke.

We injected the M1-type microglia into mice along with the M2-type microglia, but the mice died rapidly, perhaps due to strong inflammatory responses. The HIF pathway may be involved in the pro-angiogenic activity of the exosomes secreted from the M2 microglia.

Taken together, our data demonstrates that IL-4-polarized microglia could ameliorate the damage caused by ischemic stroke by promoting angiogenesis through the secretion of exosomes. Still, the exact mechanisms of how IL-4-polarized microglia secrete these exosomes or how these exosomes help in recovery still need further investigation.³⁷ The exact contents of these exosomes and the potential effects of this substance need to be elucidated as well. Nevertheless, our current data suggests that using exosomes derived from IL-4-polarized microglia may be considered a novel therapeutic method for the treatment of ischemic stroke.

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