

M2 Microglia-Derived Migrasome-Enriched Extracellular Vesicles Restore Mitochondrial Homeostasis to Orchestrate Neurovascular Unit Recovery After Ischemic Stroke

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Supplementary Materials and methods

Cell culture

The murine BV2 microglial cell line and the murine C8-D1A astrocyte cell line were sourced from iCELL (Shanghai, China), while the rat PC12 pheochromocytoma cell line was procured from FuHeng Biology (Shanghai, China). The mouse brain microvascular endothelial cell line (bEnd.3) was acquired from Procell Life Science & Technology (Wuhan, China). The BV2, C8-D1A, PC12, and bEnd.3 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin.

Flow cytometry analysis

The polarization of BV2 cells was assessed through flow cytometry using antibodies against CD86 (Affinity, China) and CD206 (HUABIO, China). Apoptosis in PC12 cells was assessed utilizing Annexin V-FITC/PI double staining (Elabscience, China), and the percentages of apoptotic cells were determined.

Immunofluorescence

For cultured cells, samples were fixed, permeabilized when necessary, and blocked with 1% BSA. Primary antibodies were applied overnight at 4 °C, followed by appropriate fluorophore-conjugated secondary antibodies. Nuclei were counterstained with DAPI. For brain tissue sections, similar procedures were employed, incorporating antigen retrieval as required. Fluorescence images were acquired with a confocal laser scanning microscope (CLSM; LSM980, Zeiss, Germany) or a fluorescence microscope (BX53, Olympus, Japan), and quantified with ImageJ software. Detailed antibody information is provided in **Table S4**.

Western blotting

Protein lysates extracted from cultured cells or brain tissues were quantified. Western blotting was performed according to standard protocols. Comprehensive details regarding the antibodies are provided in **Table S4**.

Enzyme-linked immunosorbent assay (ELISA)

To detect inflammatory cytokines, brain tissues from the ipsilateral hemisphere and collected culture supernatants were collected and homogenized. Following centrifugation, the supernatants were extracted, and the concentrations of TNF- α , IL-1 β , IL-6, and IL-10 were

quantified using commercial ELISA kits in accordance with the manufacturers' protocols.

For the *in vitro* detection of cyclic adenosine monophosphate (cAMP), supernatants from cell cultures were collected, and cAMP levels were determined.

Measurement of nitric oxide production.

Nitric oxide (NO) production in BV2 microglial cells was determined using a commercial nitric oxide assay kit (Beyotime, China) according to the manufacturer's instructions. Briefly, cell culture supernatants were collected and reacted with the Griess reagent. The absorbance was measured at 540 nm using a microplate reader, and NO levels were calculated based on a sodium nitrite standard curve.

Uptake of migrasome-enriched extracellular vesicles (EVs) and inhibition assays

BV2 microglia were subjected to oxygen-glucose deprivation/reoxygenation (OGD/R) treatment prior to migrasome-enriched EVs incubation. Cells were then incubated with fluorescently labeled migrasome-enriched EVs for 6 h at 37°C unless otherwise specified. To assess the mechanisms underlying the internalization of migrasome-enriched EVs, cells were treated under the following conditions: (1) control, (2) low temperature (4°C) to inhibit energy-dependent uptake, (3) Dynasore (80 μ M, 30 min pretreatment) to inhibit dynamin-dependent endocytosis, and (4) EIPA (50 μ M, 30 min pretreatment) to inhibit macropinocytosis. After incubation, cells were washed with PBS to remove non-internalized migrasome-enriched EVs and fixed with 4% paraformaldehyde. Fluorescence signals were visualized using CLSM.

Intracellular trafficking analysis of migrasome-enriched EVs

For intracellular trafficking analysis, cells were incubated with labeled migrasome-enriched EVs for 6 h following OGD/R treatment. Cells were then immunostained with antibodies against EEA1 (early endosome marker) or TOMM20 (mitochondrial marker). Images were acquired using CLSM. Colocalization was assessed qualitatively based on the overlap of fluorescent signals.

M1 polarization of BV2 microglia and isolation of M1 microglia-derived migrasome-enriched EVs

BV2 cells were seeded at an appropriate density and allowed to adhere overnight, followed by induction of M1 polarization through incubation with 100 ng/mL lipopolysaccharide (LPS;

L2630, Sigma, USA) and 20 ng/mL recombinant murine interferon- γ (IFN- γ ; 315-05-20, PeproTech, USA) for 24 h. M1 microglia-derived migrasome-enriched EVs were isolated using the same protocol as described above.

qPCR

Total RNA was extracted from cultured cells using an RNA isolation kit and quantified. RNA (1 μ g) was reverse transcribed into cDNA using HiScript III RT SuperMix. Quantitative real-time PCR (qPCR) was performed with SYBR Green Premix on a qPCR system. All reactions were performed in triplicate. The primers used are detailed in **Table S5**.

ROS detection

DCFH-DA (S0033S, Beyotime, China) and MitoSOX Red (HY-D1055, MCE, USA) kits were used to detect intracellular reactive oxygen species (ROS) and mitochondrial superoxide levels, respectively, following the manufacturers' protocols. Fluorescence images were captured using a CLSM.

CCK-8 assay

PC12 cell viability was determined with the Cell Counting Kit-8 (CCK-8; M0856, MIKX, China). Cells seeded in 96-well plates were subjected to indicated treatments and then incubated with CCK-8 reagent. Absorbance was measured at 450 nm using a microplate reader.

Live/dead staining

PC12 cells were treated with a staining solution comprising 1 μ M Calcein-AM and 1 μ M propidium iodide (PI) for 30 min in a dark environment. Live (green) and dead (red) cells were observed and quantified under a CLSM. ImageJ software was used for fluorescence intensity analysis.

EdU assay

Cell proliferation was evaluated utilizing the BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 594 (C0078S, Beyotime, China). EdU-positive cells were visualized through CLSM, and the proportion of EdU-positive cells was determined in relation to the total number of nuclei.

Transwell assay

Cell migration was examined using Transwell chambers with an 8 μ m pore size (Corning,

USA). Specifically, bEnd.3 cells (2×10^4 cells in 100 μ L of serum-free DMEM) were added to the upper chamber, and the lower chamber was filled with 500 μ L of DMEM that included 10% FBS. Following a 24-hour incubation period at 37 °C, the upper chamber was cleared of non-migratory cells. Crystal violet staining was performed on fixed cells attached to the lower surface.

Tube formation assay

Matrigel (Corning, USA) was placed into pre-chilled 96-well plates (50 μ L/well) and polymerized at 37 °C for 30 min. bEnd.3 cells were trypsinized, collected in DMEM containing 2% FBS, and plated onto Matrigel at 2×10^4 cells per well. After 4-6 h of incubation, tube formation was observed and photographed under a light microscope. Subsequently, the tubes were fixed and stained with 0.1% crystal violet for subsequent analysis. Total tube length and number of branch points were analyzed using ImageJ software with the Angiogenesis Analyzer plugin.

Experimental animals and grouping

All animal experiments received approval from the Animal Experiment Ethics Committee of Sun Yat-sen University (SYSU-IACUC-2025-002457) and were conducted in compliance with the ARRIVE guidelines. A cohort of 112 male C57BL/6J mice, aged 6-8 weeks and weighing between 20-25 grams, was obtained from Guangdong Yaokang Biotechnology Co., Ltd. (Guangzhou, China). The mice were maintained under specific pathogen-free (SPF) conditions, with a 12-hour light/dark cycle, a controlled temperature of 22 ± 2 °C, and a relative humidity of $50 \pm 10\%$. All animals had unrestricted access to standard rodent chow and water.

Brain stereotaxic injection

Mice underwent brain stereotaxic injection under anesthesia and were positioned in a stereotaxic apparatus (RWD, China). A volume of 1 μ L of migrasome-enriched EVs was administered into the left lateral ventricle using a microsyringe at a controlled rate of 1 μ L/min. The coordinates for the injection with respect to the bregma were anteroposterior (AP) -0.59 mm, mediolateral (ML) +1.25 mm, and dorsoventral (DV) -2.78 mm. The needle was maintained in position for an additional 2 minutes before being gradually withdrawn. To evaluate the therapeutic time window, migrasome-enriched EVs were administered at different

time points relative to reperfusion, including 30 min prior to reperfusion, at the onset of reperfusion (0 h), and 1 h after reperfusion, using the same dosage and injection protocol as described above.

Infarct volume measurement and CBF measurement

Infarct volume was assessed by 2,3,5-triphenyltetrazolium chloride (TTC) staining. Coronal brain slices (1 mm thick) were prepared from rapidly extracted brains and incubated in 2% TTC solution (Sigma-Aldrich, USA) at 37 °C for 20 min. The stained sections were photographed, and infarct areas were quantified using ImageJ software.

CBF was assessed 24 hours post-reperfusion using a laser speckle contrast imaging system (PeriCam PSI, PERIMED AB, Sweden). During this procedure, anesthetized mice were subjected to scalp incision for skull exposure, and the imaging probe was positioned above the skull surface. Blood flow images were captured and analyzed utilizing the software provided by the manufacturer.

Magnetic resonance imaging (MRI) was conducted 24 hours after reperfusion using a 9.4 T small-animal MRI system (Aspect Imaging, Israel). For T2-weighted imaging, a turbo spin-echo sequence was applied with these parameters: repetition time (TR) = 2920 ms, echo time (TE) = 38 ms, echo train length = 7, slice thickness = 1 mm, matrix size = 256 × 256, and resolution = 0.148 × 0.148 mm².

H&E and Nissl staining

Brains were collected at 24 h post-MCAO following transcardial perfusion with 4% paraformaldehyde, then post-fixed, dehydrated, embedded, and sectioned. H&E staining was conducted using an H&E staining kit (Beyotime, China) following the manufacturer's guidelines.

For Nissl staining, frozen sections were fixed, stained with Nissl staining solution for 5 minutes, and differentiated in 0.1% glacial acetic acid. After dehydration and clearing, the sections were mounted with neutral gum. Images were subsequently captured and analyzed using a light microscope.

TUNEL staining

Neuronal apoptosis in brain sections was assessed using TUNEL staining. Frozen brain

sections were fixed with 4% paraformaldehyde and processed using a TUNEL BrightRed Apoptosis Detection Kit (A113, Vazyme, China) according to the manufacturer's instructions. Stained sections were examined under a confocal microscope.

Neurobehavioral assessments

The open field test was conducted on day 6 after MCAO to evaluate spontaneous locomotor and exploratory activity. Mice were placed in an open field arena and allowed to explore freely for 5 min. Total distance traveled, mean velocity, and immobility time were recorded and analyzed. The novel object recognition test was performed on day 7 after MCAO to evaluate recognition memory. During the training phase, mice were allowed to explore two identical objects. After a 6-h interval, one familiar object was replaced with a novel object during the testing phase. The percentage of exploration time spent with the novel object was calculated. The Y-maze test was conducted on day 8 after MCAO to evaluate spatial working memory and exploratory preference. During the test phase, mice were allowed to freely explore the three-arm maze for 5 min. The percentage of time spent in the novel arm was calculated and used for quantitative analysis.

Transmission electron microscopy (TEM) for mitochondrial morphology

BV2 cell pellets were subjected to fixation in 3% glutaraldehyde (4 °C, 24 h) followed by post-fixation in 2% osmium tetroxide (4 °C, 24 h). After dehydration through a graded ethanol series and propylene oxide, samples were embedded in Epon 812 resin at 60 °C for 48 h. Ultrathin sections were then placed on 200-mesh copper grids and examined by TEM.

Supplementary Tables and Figures

Table S1 Longa Score

Longa score	Neurological symptoms
0	no neurological deficit
1	an inability of the left forelimbs to fully extend
2	circling to the contralateral side with an inability to move in a straight line
3	difficulty in walking, accompanied by a slumped posture to the left
4	an inability to walk spontaneously, potentially accompanied by a loss of consciousness

Table S2 Modified Neurological Severity Score (mNSS)

Test	Neurological symptoms	Score
Motor tests		
lifting tail	Forelimb flexion	1
	Hindlimb flexion	1
	Head deviates by more than 10° within 30s	1
placing on the floor	Normal walk	0
	Inability to walk straight	1
	Circling toward the paretic side	2
	Fall down to the paretic side	3
Sensory test		
placing test	Visual tactile test	1
proprioceptive test	Deep sensation, pushing the paw against the table edge	1
beam balance test	Balance with steady posture	0
	Grasps side of beam	1
	Hugs the beam and one limb falls down from the beam	2
	Hugs the beam and two limbs fall down from the beam, or spins on beam (> 60s)	3
	Attempts to balance on the beam but falls off (> 40s)	4

	Attempts to balance on the beam but falls off (> 20s)	5
	Falls off: no attempt to balance or hang on the beam (< 20s)	6
Reflexes absent and abnormal movements		
auricle reflex	No head shaking or slow response	1
corneal reflex	No blinking or slow response	1
startle reflex	Motor response to a brief noise	1
seizures, myoclonus or myodystony	If present	1
	Maximum scores	18

Table S3 Garcia score

Projects	Judging by	Score
Spontaneous activity	Moves around, explores cage	3
	Moves around reluctantly, reaches at least one side of cage	2
	Barely moves in cage, does not rise to any side of cage	1
	Does not move at all	0
Symmetry in the movement of four limbs	All four limbs extend symmetrically	3
	Limbs on contralateral side extend less than those on ipsilateral side	2
	Limbs on contralateral side exhibit minimal movement	1
	Forelimb on contralateral side does not move at all	0
Forepaw outstretching	Forelimbs outstretched, walking symmetrically on forepaws	3
	Left side outstretched less than right, forepaw walking impaired	2
	Left forelimb has limited movement	1
	Left forelimb does not move at all	0
Climbing	Climbs, grips tightly with both forepaws	3
	Left side impaired, does not grip as tightly and releases	2

	before right	
	Does not climb	1
Body proprioception	Reacts by turning head, equally startled by stimulus on both sides	3
	Reacts slowly to stimulus on left side	2
	Does not react to stimulus on left side	1
Vibrissae touch	Reacts by turning head, equally startled by stimulus on both sides	3
	Reacts slowly to stimulus on left side	2
	Does not react to stimulus on left side	1
Maximum scores		18

Table S4 Antibodies and reagents used for immunofluorescence and Western blotting.

Antibodies/Reagents	Source	Identifier
anti-CD206	HUABIO	HA720214F
anti-ARG1	CST	93668T
anti-TREM2	CST	55739S
anti-TSPAN4	Bioss	bs-9413R
anti-NDST1	Proteintech	26203-1-AP
anti-CPQ	Abclonal	A12062
anti-TSG101	Affinity Biosciences	DF8427
anti-CD63	Affinity Biosciences	AF5117
anti-GM130	Affinity Biosciences	DF75567
anti-Histone H3	Signalway Antibody	32667
anti-CD86	HUABIO	HA750109
anti-EEA1	HUABIO	HA722147
anti-TOMM20	HUABIO	HA750166
anti-GFAP	Proteintech	16825-1-AP
anti-BAX	Abcam	ab32503

anti-Bcl-2	Abcam	ab194583
anti-Iba1	Oasis Biofarm	OB-PGP049
anti-iNOS	CST	20609T
Anti-CD68	Abcam	ab283654
anti-C3	Proteintech	21337-1-AP
anti-S100A10	Proteintech	11250-1-AP
anti-NeuN	Proteintech	26975-1-AP
anti-CD31	Abcam	ab182981
anti-EPAC1	Proteintech	12572-1-AP
anti-RAP1	Abclonal	A9725

Table S5 Primer sequences for qRT-PCR.

Genes	Primer sequences (5' to 3')
<i>Tnf</i>	Forward: CAGCCGATTTGCCACTTCATA Reverse: TCCTTAGGGCAAGGGCTCTT
<i>Il1b</i>	Forward: AGGCTTCCTTGTGCAAGTGT Reverse: TGTCGAGATGCTGCTGTGAG
<i>Il6</i>	Forward: CCCAACTTCCAATGCTCTCCTAATG Reverse: GCACACTAGGTTTGCCGAGTAGACC
<i>Il10</i>	Forward: TGAGCTTCTCTGTGAACGATTTA Reverse: GTCACCCTATGGAAACAGCTTA

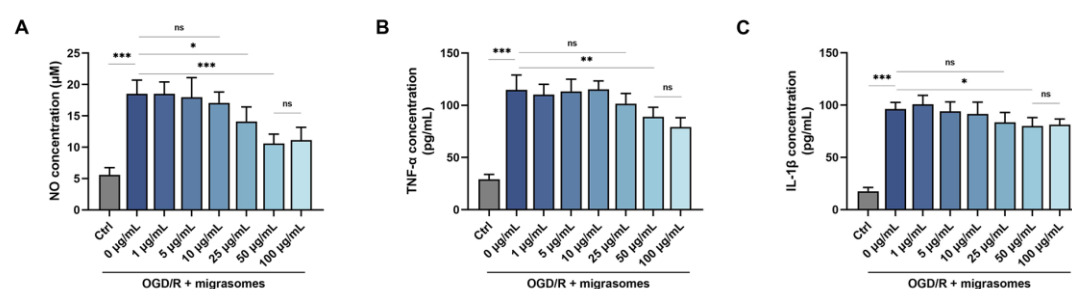


Figure S1. Dose-response analysis of M2 microglia-derived migrasome-enriched EVs in BV2 microglia. BV2 cells were treated with increasing concentrations of M2 microglia-derived

migrasome-enriched EVs (0, 1, 5, 20, 50, and 100 $\mu\text{g/mL}$). (A) Nitric oxide (NO) production was measured using the Griess assay. (B-C) Levels of pro-inflammatory cytokines TNF- α and IL-1 β were quantified by ELISA.

Low concentrations (≤ 10 $\mu\text{g/mL}$) showed minimal effects, while 50 $\mu\text{g/mL}$ significantly reduced inflammatory responses. No further significant improvement was observed at 100 $\mu\text{g/mL}$, indicating a plateau effect. Data are presented as mean $\pm$ SD ($n = 6$ per group). ns: no significance, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

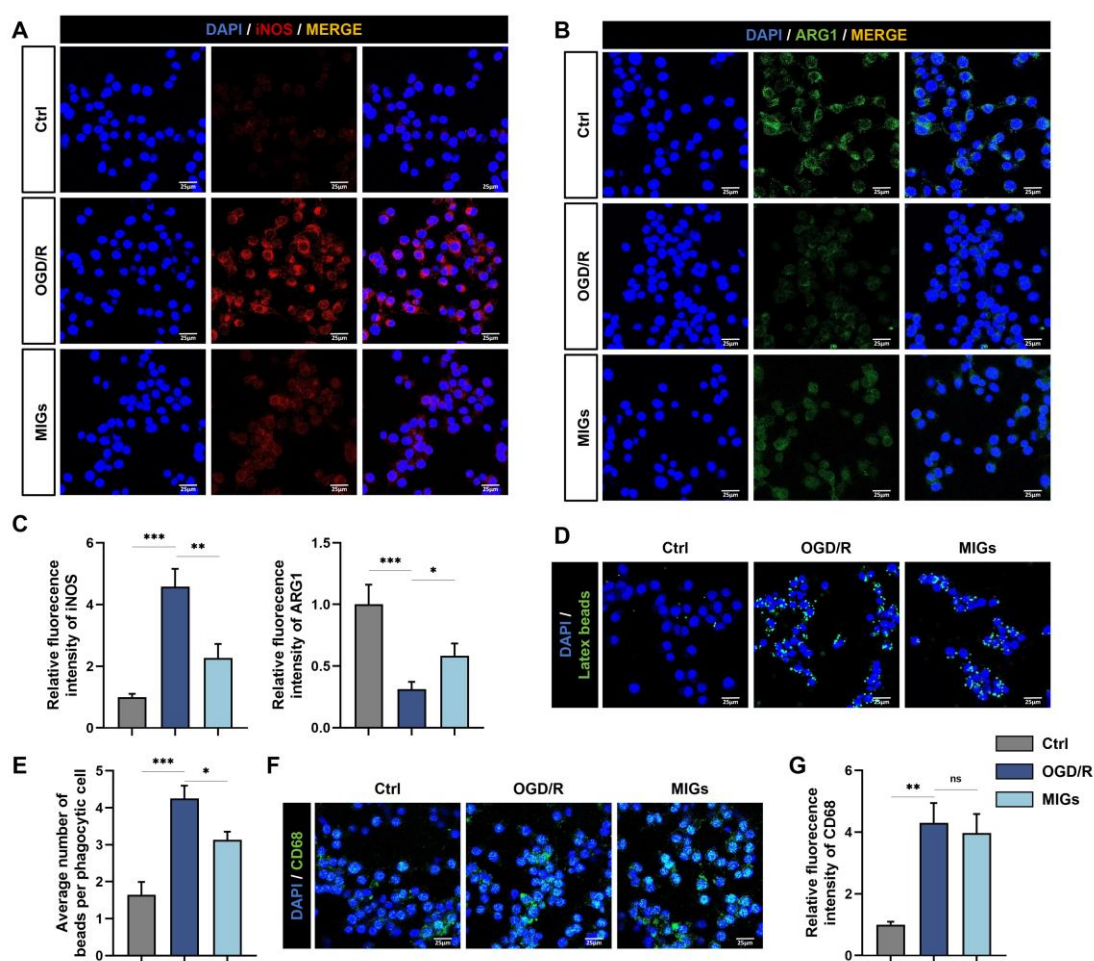


Figure S2. M2 microglia-derived migrasome-enriched EVs modulate microglial phenotype and phagocytic function under OGD/R conditions. (A, B) Representative immunofluorescence images showing the expression of the M1 marker iNOS and the M2 marker ARG1 in BV2 cells. (C) Quantitative analysis of iNOS and ARG1 fluorescence intensity. (D) Representative images of BV2 cells phagocytosing fluorescently labeled latex beads

(green). (E) Quantitative analysis of phagocytic activity. (F) Representative immunofluorescence images of CD68 expression in BV2 cells. (G) Quantitative analysis of CD68 fluorescence intensity. Data are presented as mean $\pm$ SD from at least three independent experiments. ns: no significance, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

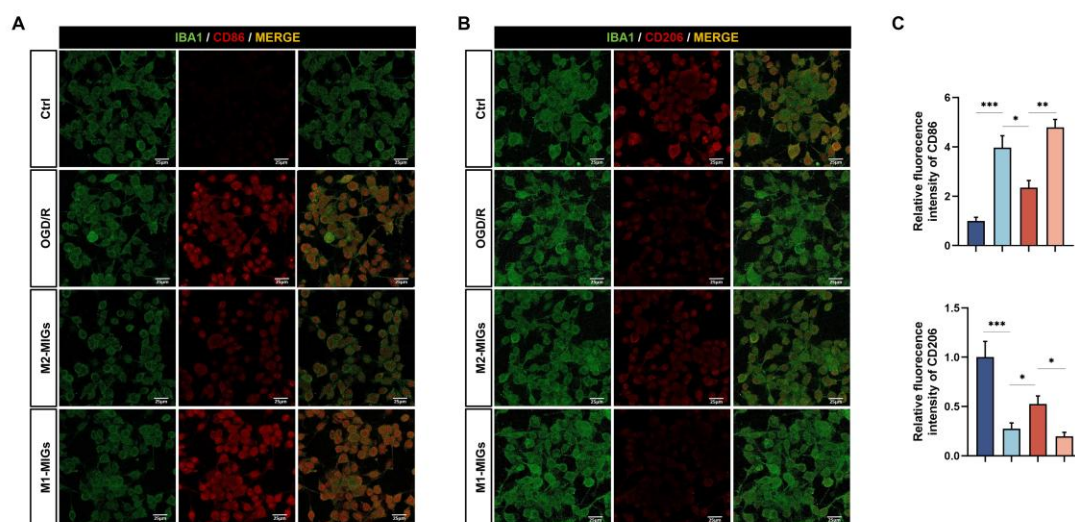


Figure S3. M2 microglia-derived migrasome-enriched EVs promote anti-inflammatory polarization of microglia compared with M1 microglia-derived migrasome-enriched EVs under OGD/R conditions. (A) Representative immunofluorescence images of microglia stained for IBA1 (green) and the pro-inflammatory marker CD86 (red) under different conditions, including Control (Ctrl), oxygen-glucose deprivation/reoxygenation (OGD/R), OGD/R treated with M2 microglia-derived migrasome-enriched EVs (M2-MIGs), and OGD/R treated with M1 microglia-derived migrasome-enriched EVs (M1-MIGs). (B) Representative immunofluorescence images of microglia stained for IBA1 (green) and the anti-inflammatory marker CD206 (red) under the same experimental conditions. (C) Quantitative analysis of relative fluorescence intensity of CD86 and CD206. Data are presented as mean $\pm$ SD from at least three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

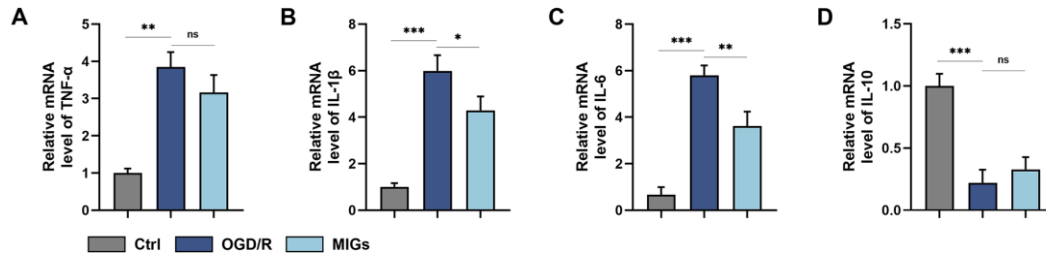


Figure S4. M2 microglia-derived migrasome-enriched EVs alter C8-D1A astrocytic inflammatory cytokine expression under OGD/R conditions. The mRNA levels of pro-inflammatory cytokines TNF- α (A), IL-1 β (B), IL-6 (C), and the anti-inflammatory cytokine IL-10 (D) were measured by qPCR. Data are presented as mean $\pm$ SD from at least three independent experiments. ns: no significance, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

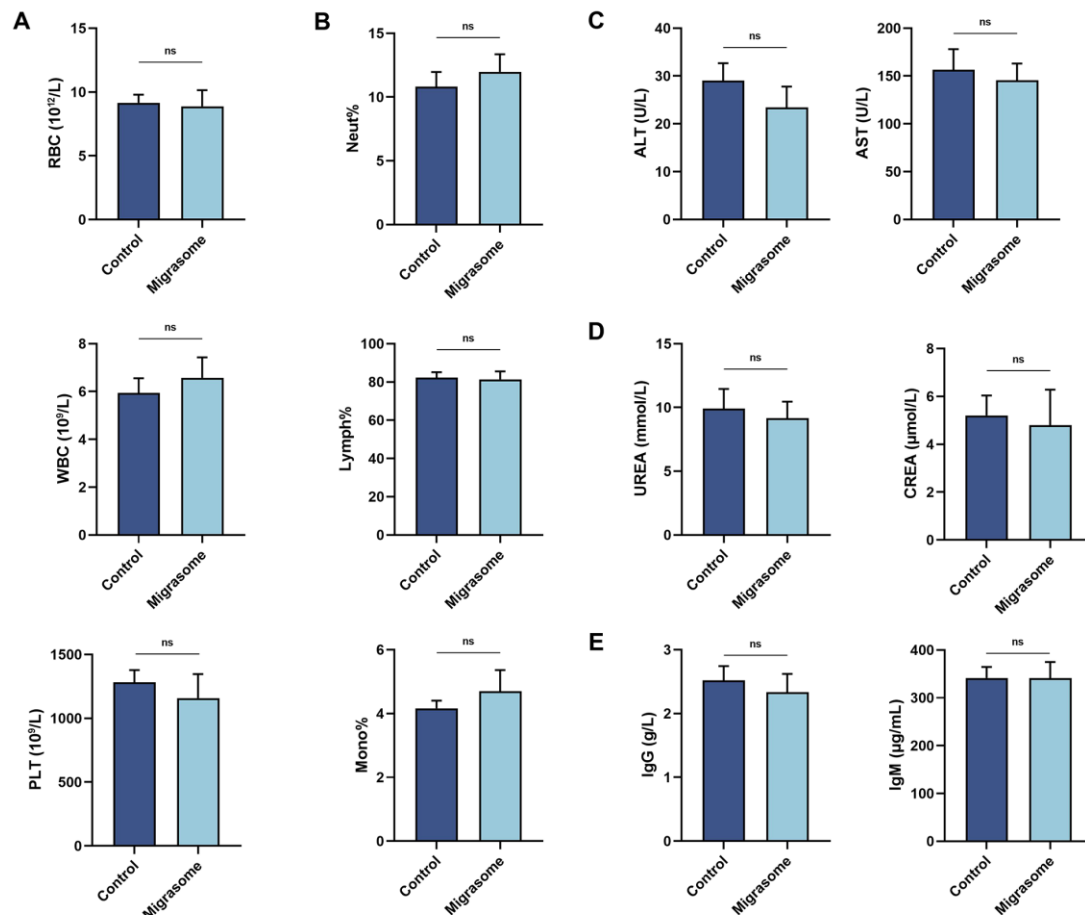


Figure S5. Systemic biosafety of stereotactically injected M2 microglia-derived migrasome-enriched EVs. (A, B) Hematological analysis of peripheral blood collected 7 days post-injection. Parameters include red blood cell (RBC) count, platelet (PLT) count, total white

blood cell (WBC) count, neutrophil percentage, lymphocyte percentage and monocyte percentage. (C, D) Serum biochemistry analysis assessing liver function (ALT, alanine aminotransferase; AST, aspartate aminotransferase) and renal function (blood urea nitrogen, UREA; creatinine, CREA). (E) Measurement of serum immunoglobulin levels (IgG, IgM). Data are presented as mean $\pm$ SD (n = 6 mice per group). ns: no significance.

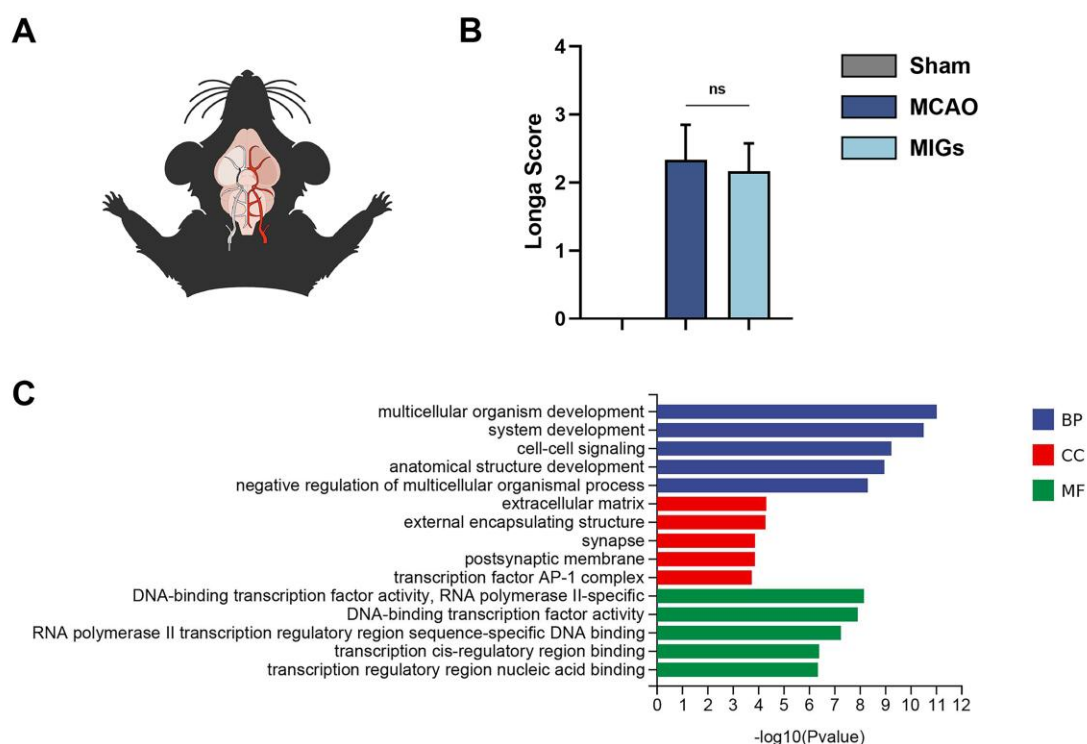


Figure S6. MCAO model validation and transcriptomic analysis of migrasome-enriched EV-treated brains. (A) Schematic illustration of the transient middle cerebral artery occlusion (MCAO) model in mice. (B) Longa neurological scores assessed at 24 h post-reperfusion in Sham, MCAO, and MIGs groups. Data are presented as mean $\pm$ SD (n = 6 mice per group). ns: no significance. (C) Gene Ontology (GO) enrichment analysis of differentially expressed genes from ipsilateral brain tissues of mice in MCAO and MIGs groups. Selected biological processes (BP), cellular components (CC), and molecular functions (MF) are shown.