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M2 microglia-derived migrasome-enriched extracellular vesicles restore mitochondrial homeostasis to orchestrate neurovascular unit recovery after ischemic stroke

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Abstract

Ischemic stroke is a major cause of disability with few treatment options available. Microglia-driven neuroinflammation contributes significantly to stroke pathology, and promoting anti-inflammatory microglial phenotypes represents a promising strategy. Migrasomes are newly discovered organelles mediating intercellular communication, yet their role in ischemic stroke remains unexplored. This study demonstrates that M2 microglia-derived migrasome-enriched extracellular vesicles (EVs) exert potent neuroprotection in both OGD/R cell models and MCAO mice. These migrasome-enriched EVs were efficiently internalized by microglia, astrocytes, neurons, and microvascular endothelial cells, promoting microglial M2 polarization, suppressing astrocytic aberrant activation, reducing neuronal apoptosis, and enhancing angiogenesis. Intracerebral administration of M2 microglia-derived migrasome-enriched EVs significantly reduced infarct volume, ameliorated cerebral edema, improved cerebral blood flow, and accelerated neurological and cognitive recovery without detectable toxicity. Mechanistically, migrasome-enriched EVs activated the cAMP/EPAC1/Rap1 signaling pathway in microglia, leading to restored mitochondrial homeostasis. Collectively, these findings identify M2 microglia-derived migrasome-enriched EVs as novel intercellular messengers that orchestrate neurovascular unit recovery after ischemic stroke, positioning migrasome-enriched EVs as promising candidates for stroke therapy.

Keywords Migrasome-enriched extracellular vesicles, Ischemic stroke, Microglia polarization, Mitochondrial homeostasis, Neurovascular unit recovery

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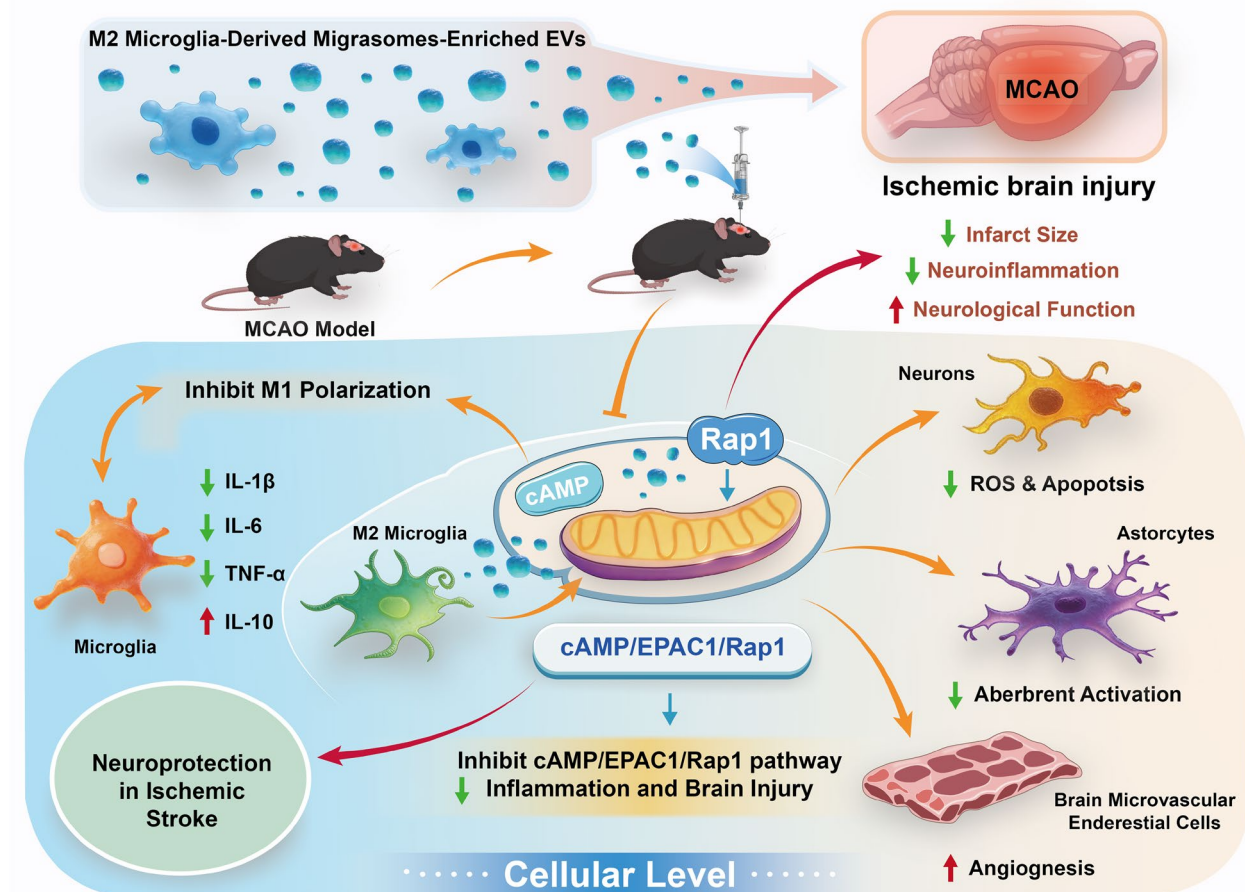
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Graphical Abstract



Introduction

Ischemic stroke significantly contributes to fatalities and lasting disabilities, imposing substantial burdens on healthcare systems globally [1]. As a pressing global health challenge necessitating immediate intervention, research into its therapeutic strategies has been prioritized in the healthcare agendas of numerous countries [2–4]. The homeostasis of microglia after cerebral ischemic injury is a key factor influencing treatment prognosis [5–8]. Therefore, the development of novel intervention strategies to restore microglial homeostasis is a current research focus.

It is well-established that the extent of neuroinflammation following cerebral ischemia-reperfusion serves as a critical target influencing the therapeutic time window, with its regulatory pathways being closely linked to the immune system within brain tissue [9]. As the primary executors of innate immunity in brain tissue, glial cells are the initial responders. Microglia, functioning as first responders, are rapidly activated after ischemic stroke, transitioning from a resting state (M0) to various

functional phenotypes [10]. M1 microglia release mediators that exacerbate blood-brain barrier (BBB) disruption and promote neuronal programmed death [11]. In contrast, M2 microglia attenuate pathological damage and promote functional recovery by releasing reparative mediators [12]. Consequently, modulating the functional state of microglia within the ischemic penumbra to favor a repair-supportive phenotype has become a research priority in many recent studies. Nonetheless, the mechanisms aimed at inhibiting M1 subtype polarization or facilitating the conversion from M1 to M2 subtypes are frequently hindered by operational complexities and ethical considerations [13], thereby impeding their clinical translation. This has resulted in an accumulation of basic research findings, while clinical treatment options remain scarce. In response, research on the neuroprotective effects of endogenous M2-type microglia transplantation post-stroke has progressively advanced [14]. However, the proportion of endogenous M2-type microglia significantly diminishes during the acute ischemic phase, and the cost associated with cell transplantation poses a

challenge to its therapeutic viability [15]. Thus, the critical question arises as to whether exogenous approaches can replicate or deliver the protective signals of M2 cells to achieve comparable or superior therapeutic outcomes without the need for cell transplantation. Addressing this question is of paramount importance.

The primary scientific challenge in achieving the translational breakthrough previously mentioned involves the precise and efficient delivery of the beneficial effects of anti-inflammatory microglia to adjacent cell populations within neurovascular units. M2 microglia, as pivotal coordinators of the post-ischemic brain micro-environment, may exert protective functions remotely by releasing specific signaling molecules. In this context, migrasomes have recently garnered significant research attention as newly identified organelles. These membrane-encapsulated vesicles are formed at the tips or branch points of retraction fibers in migrating cells [16]. These structures contain smaller intracellular vesicles and bioactive molecules, including proteins and mRNA [17]. Since their discovery, migrasomes have shown considerable potential as functional carriers. Migrasomes can also be conceptualized as a class of bio-derived nanoscale vesicular carriers that mediate intercellular delivery of bioactive cargos, thereby aligning with emerging principles in nanobiotechnology. They have been confirmed to exhibit high migratory productivity in immune cells, including macrophages, microglia, monocytes, and neutrophils [18], and facilitate communication and signal exchange among microglia within the central nervous system [19]. Notably, recent findings link migrasomes to cellular mitochondrial homeostasis. Migrasomes not only carry mitochondria-related components but can also directly regulate the energy metabolism and redox balance of recipient cells by transferring healthy mitochondria or mitochondrial DNA, thereby supporting the preservation of mitochondrial homeostasis of target cells [20–22]. The intrinsic anti-inflammatory and tissue-repairing properties of M2 microglia suggest that their derived migrasome-enriched extracellular vesicles (EVs) may function as a preconfigured protective signaling module, particularly through the mechanism of regulating mitochondrial homeostasis. Upon uptake by neighboring cells, they may trigger a cascade of repair responses. Therefore, in the context of ischemic stroke, intervention using migrasome-enriched EVs derived from M2 microglia holds significant therapeutic potential.

In conclusion, this study systematically elucidates the neuroprotective impacts and the fundamental molecular processes of M2 microglia-derived migrasome-enriched EVs in the context of ischemic stroke. Utilizing middle cerebral artery occlusion (MCAO) mouse models and oxygen-glucose deprivation/ reoxygenation (OGD/R) cell

models, the research demonstrates that M2 microglia-derived migrasome-enriched EVs therapy significantly reduces cerebral infarct volume, enhances neurological function, and modulates microglial polarization towards the M2 phenotype. Additionally, it inhibits aberrant astrocyte activation, promotes neuronal survival, and facilitates endothelial cell angiogenesis. Through comprehensive transcriptomic screening and functional validation, the study reveals that M2 microglia-derived migrasome-enriched EVs preferentially activate the cAMP/EPAC1/Rap1 signaling pathway within microglia, thereby restoring mitochondrial homeostasis and initiating a cascade of neurovascular unit repair. This study provides the first systematic evidence that M2 microglia-derived migrasome-enriched EVs function as multi-cellular regulatory units to restore neurovascular homeostasis after ischemic stroke. These findings support further investigation of EV-based neuroprotective strategies involving migrasome-enriched vesicle populations in ischemic stroke and broader neuroinflammatory conditions.

Materials and methods

M2 polarization and identification of BV2 microglia

BV2 cells were seeded at an appropriate density and allowed to adhere overnight, followed by induction of M2 polarization through 48 h incubation with 20 ng/mL recombinant murine interleukin-4 (IL-4; RP01161, Abclonal, China) [23, 24]. Flow cytometry, Western blotting, and immunofluorescence staining confirmed the efficiency of M2 polarization in IL-4-treated BV2 cells.

Isolation and characterization of migrasome-enriched vesicle fractions

Migrasome-enriched vesicle fractions were isolated from M2-polarized BV2 microglia using differential centrifugation and density gradient ultracentrifugation, following established protocols [16]. Initially, BV2 cells were plated on 150 mm culture dishes that had been pre-coated with fibronectin (Sigma-Aldrich, USA) to facilitate cell migration and migrasome formation. Following a 12-hour incubation period, both cells and associated migrasome-enriched EVs were collected by gentle scraping in ice-cold phosphate buffered saline (PBS) supplemented with a protease inhibitor cocktail. The resultant suspension was subjected to a series of centrifugation steps at 4 °C: initially at 1,000 × g for 10 min to remove cellular debris, followed by 4,000 × g for 20 min to eliminate larger fragments. The supernatant was then centrifuged at 20,000 × g for 30 min to isolate crude migrasome-enriched vesicle fractions. This pellet was then resuspended in PBS and applied to a discontinuous Optiprep™ density gradient (Axis-Shield, Norway; comprising layers of 5%, 10%, 15%, 20% [sample], 25%, 30%, 35%, and 40%), followed by

ultracentrifugation at $150,000 \times g$ for 4 h at 4 °C. Fractions enriched in migrasome-like vesicles were collected, diluted with PBS, and subjected to a final centrifugation at $20,000 \times g$ for 30 min at 4 °C. The final vesicle pellet (migrasome-enriched fraction) was subsequently resuspended in PBS.

The integrity and enrichment of migrasome-associated structures in the isolated vesicle fractions were verified through Western blot analysis, assessing enrichment of migrasome-associated markers and evaluating the relative depletion of classical exosomal markers. To assess migrasomes morphology, BV2 cells cultured on fibronectin-coated confocal dishes were stained for 1 h with Alexa Fluor™ 647-conjugated wheat germ agglutinin (WGA; Solarbio, China) and visualized using a confocal laser scanning microscope (CLSM; LSM980, Zeiss, Germany). For ultrastructural examination, vesicle fractions enriched in migrasome-like structures were subjected to negative staining with 2% uranyl acetate and analyzed via transmission electron microscopy (TEM). Scanning electron microscopy (SEM) was used to examine migrasomes on the cell surface.

For ultrastructural and size characterization, isolated migrasome-enriched vesicle fractions were subjected to negative staining with 2% uranyl acetate and analyzed by TEM. In addition, nanoparticle tracking analysis (NTA) (NanoSight, Malvern Instruments, UK) was performed to determine the size distribution of the isolated vesicle fractions according to the manufacturer's instructions.

For cellular uptake assays, migrasome-enriched EVs were labeled with 1 µg/mL WGA-Alexa Fluor™ 647, washed, and resuspended in PBS at 5×10^{10} particles/mL. The BV2 microglia, C8-D1A astrocytes, PC12 neuronal cells, and bEnd.3 endothelial cells were seeded onto confocal dishes. These cells were then incubated with the labeled migrasome-enriched EVs for durations of 6, 12, and 24 h. Post-incubation, the cells underwent washing and fixation with 4% paraformaldehyde. Uptake of migrasome-enriched EVs was assessed through CLSM.

OGD/R model

To replicate the ischemic microenvironment characteristic of ischemic stroke in vitro, an OGD/R model was implemented based on previously established methodologies. Cells were seeded in suitable culture plates and allowed to adhere overnight. The culture medium was then exchanged for glucose- and fetal bovine serum (FBS) -free Dulbecco's Modified Eagle Medium (DMEM), followed by exposure to hypoxic conditions (1% O₂, 5% CO₂, 94% N₂) for 4 h. After the OGD treatment, cells were returned to normal culture medium containing glucose and 10% FBS and maintained under normoxic conditions (21% O₂, 5% CO₂) for 24 h. The following three groups were used in this study: (1) Ctrl group, (2) OGD/R

group, and (3) MIGs group. In the MIGs group, M2 microglia-derived migrasome-enriched EVs were introduced at an optimized concentration of 50 µg/mL at the initiation of reperfusion. Control cells in the Ctrl group were maintained under normoxic conditions in glucose-containing DMEM for the duration of the experiment.

Experimental animals and grouping

A cohort of 112 male C57BL/6J mice, aged 6–8 weeks and weighing between 20 and 25 g, was obtained from Guangdong Yaokang Biotechnology Co., Ltd. (Guangzhou, China). Three groups were established by random assignment: (1) Sham group, (2) MCAO group (subjected to middle cerebral artery occlusion/reperfusion), and (3) MIGs group (MCAO mice treated with M2 microglia-derived migrasome-enriched EVs via stereotaxic injection 30 min before reperfusion). Detailed surgical procedures and postoperative care are described below.

In vivo fluorescence imaging

The migrasome-enriched EVs, freshly isolated, were labeled with WGA-Alexa Fluor™ 647. Following labeling, the migrasome-enriched EVs were washed and resuspended in PBS, and stereotactically injected into the left lateral ventricle as previously described. Mice were imaged with an in vivo imaging system (AniView Phoenix, BLT Photon Technology) post-injection.

Biosafety evaluation

Healthy mice were divided into control and migrasome-enriched EV-treated groups ($n=6$ per group) to assess the biosafety of migrasome-enriched EVs administration. Seven days after stereotaxic injection, blood samples were obtained to perform hematological and biochemical analyses. Serum concentrations of alanine aminotransferase liver and kidney function markers were quantified using an automated biochemical analyzer. To assess humoral immune responses, serum immunoglobulin G (IgG) and immunoglobulin M (IgM) were measured by enzyme-linked immunosorbent assay (ELISA) as per the manufacturer's protocol. Major organs were excised and subjected to H&E staining for histological evaluation.

MCAO model

Induction of focal cerebral ischemia was achieved through transient MCAO [25]. Following anesthesia, the left common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were carefully isolated. A 6 – 0 silicone-coated monofilament (602256PK5Re, Doccol, USA) was inserted into the CCA and gently advanced into the ICA until it blocked the origin of the middle cerebral artery (MCA). After 60 min of blockage, the filament was taken out to reestablish cerebral blood flow (CBF). In the MIGs group, mice received

a single stereotaxic injection of migrasome-enriched EVs precisely 30 min prior to reperfusion. Mice in the Sham group underwent an identical surgical procedure without the insertion of the filament. Throughout the surgical process, body temperature was maintained at 37 ± 0.5 °C using a heating pad.

Neurobehavioral and cognitive tests

Behavioral tests were conducted by two blinded investigators. Neurological deficits were evaluated 24 h post-reperfusion using the Longa scoring system (Table S1). Mice with scores of 2 or higher were considered to have been successfully modeled.

Neurological function was evaluated on days 1, 4, and 7 following MCAO utilizing the modified Neurological Severity Score (mNSS) and Garcia tests. The mNSS evaluates motor, sensory, reflex, and balance functions, with higher scores correlating with greater impairment severity (Table S2). The Garcia test consists of six subscales, each ranging from 0 to 3, where higher scores denote better function (Table S3).

Bulk RNA sequencing

Total RNA was isolated from the ipsilateral brain tissues of mice in both the MCAO and MIGs groups ($n=3$ per group). RNA sequencing was conducted using the Illumina NovaSeq 6000 platform. Differentially expressed genes (DEGs) were identified based on a fold change greater than 2 and an adjusted p -value less than 0.05. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were conducted to functionally annotate the DEGs.

Mitochondrial morphology and membrane potential assay

Mitochondrial morphology in BV2 cells was assessed using MitoTracker Red (C1049B, Beyotime, China) as per the manufacturer's protocol. Mitochondrial membrane potential ($\Delta\Psi_m$) was determined by JC-1 staining (C2003S, Beyotime, China), followed by nuclear counterstaining with Hoechst 33,342. Images were acquired via CLSM and analyzed using ImageJ software.

GGTI298 administration

For Rap1 inhibition experiments, mice received an intraperitoneal injection of GGTI298 (25 μ M/kg; HY-100876, MCE, USA) 30 min prior to reperfusion.

Statistical analysis

All data are expressed as mean $\pm$ standard deviation (SD). Statistical analyses were conducted utilizing GraphPad Prism 9. Comparisons between two groups were performed using Student's t -test, while comparisons among multiple groups were assessed via one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test

for normally distributed data. Data that did not meet normality assumptions were analyzed using the Kruskal-Wallis test. Statistical significance was determined at $p < 0.05$.

Results

Characterization of M2-polarized microglia and the isolation of migrasome-enriched vesicle fractions derived from M2 microglia

To obtain functional migrasome-enriched EVs from a specific cellular source, BV2 microglia were polarized into the M2 phenotype through IL-4 stimulation (Fig. 1A). Flow cytometry analysis confirmed a significant shift towards the M2 phenotype post-IL-4 treatment, as indicated by an increased population of CD206-positive cells compared to controls (Fig. 1B). This phenotypic conversion was further corroborated. Western blot analysis revealed that IL-4 stimulation significantly down-regulated the M1 marker inducible nitric oxide synthase (iNOS) while upregulating M2 marker arginase 1 (ARG1) (Fig. 1C, D). Immunofluorescence staining consistently demonstrated an increased fluorescence intensity of M2-associated markers, including CD206, ARG1, and TREM2 (Fig. 1E, F). Collectively, these findings validate the successful transformation of BV2 microglia into the M2 phenotype.

Following the establishment of the cellular source, migrasome-enriched vesicle fractions were isolated from the culture supernatant of M2-polarized BV2 cells. Fluorescence microscopy identified numerous spherical structures (indicated by white arrows) along retraction fibers (indicated by green arrows), a hallmark feature of migrasomes (Fig. 1G). These structures were further validated by SEM and TEM, which confirmed the presence of membrane-bound vesicular structures associated with retraction fibers (Fig. 1H, I).

To further characterize the isolated vesicle fractions and address concerns regarding vesicle identity and heterogeneity, multiple complementary approaches were employed. TEM of the isolated fraction revealed heterogeneous membrane-bound vesicles, including large vesicular structures consistent with migrasome-like vesicles, along with smaller vesicles (Fig. 1J), suggesting the presence of mixed EV populations, which is commonly observed in vesicle preparations. Consistently, NTA demonstrated a heterogeneous size distribution with a mean particle diameter of approximately 401 nm and a predominant peak at around 365 nm, with the majority of particles ranging from 200 to 600 nm (Fig. 1K), which is consistent with previously reported size distributions of migrasomes and migrasome-like vesicular structures.

To verify the identity and enrichment of migrasome-associated structures in the isolated vesicle fractions,

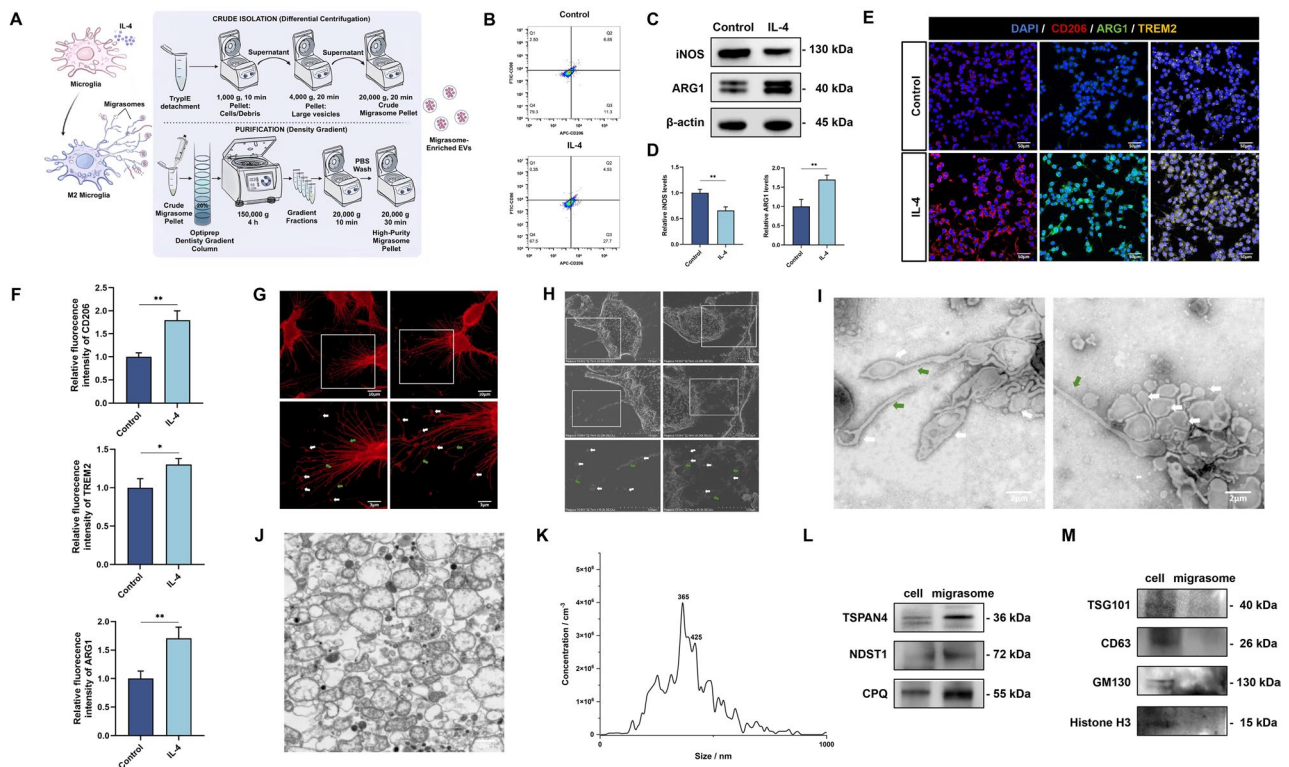


Fig. 1 Induction of M2 phenotype in BV2 microglia and characterization of their derived migrasome-enriched vesicle fractions. **(A)** Schematic diagram illustrating the procedure for preparing migrasome-enriched EVs from M2-polarized microglia. **(B)** Representative flow cytometry plots and quantification analyzing M1/M2 surface markers in BV2 cells. **(C, D)** Western blot analysis of iNOS and ARG1 in control and IL-4-treated BV2 cells. **(E, F)** Representative immunofluorescence images and quantitative analysis showing the expression and co-localization of CD206, ARG1 and TREM2 in BV2 cells. **(G)** Fluorescent image of a BV2 cell showing migrasomes (white arrows) formed along retraction fibers (green arrows). **(H)** SEM image confirming the presence of migrasomes (white arrow) on retraction fibers (green arrow). **(I)** TEM image showing migrasomes (white arrows) associated with BV2 cells, highlighting their ultrastructural features in situ. **(J)** TEM image of isolated migrasome-enriched vesicle fractions showing membrane-bound vesicular structures with heterogeneous sizes and internal vesicles. **(K)** NTA showing the size distribution of isolated vesicle fractions. **(L)** Western blot analysis of migrasome-associated markers in the isolated migrasome-enriched vesicle fraction and whole cell lysates. **(M)** Western blot analysis of EV-related and intracellular markers in the isolated migrasome-enriched vesicle and whole cell lysates. Data are presented as mean $\pm$ SD from at least three independent experiments. * $p < 0.05$, ** $p < 0.01$

Western blot analysis was conducted. Migrasome-associated proteins (TSPAN4, NDST1, and CPQ) were enriched in the migrasome-enriched vesicle fraction compared with cell lysates (Fig. 1L), supporting the enrichment of migrasome-associated vesicular components. In contrast, classical EV markers such as TSG101 and CD63 were markedly reduced, and intracellular organelle markers including GM130 and Histone H3 were largely absent (Fig. 1M), indicating minimal contamination from intracellular components.

Collectively, these results indicate that the isolated vesicles are enriched in migrasome-like structures with limited contamination from other cellular components. Although a degree of heterogeneity typical of EV preparations remains, the enrichment of migrasome-associated markers together with the depletion of classical exosomal and intracellular markers suggests that the observed biological effects are largely driven by migrasome-enriched fractions.

M2 microglia-derived migrasome-enriched EVs modulate the phenotypic polarization of microglia and astrocytes in vitro

The uptake and functional impact of M2 microglia-derived migrasome-enriched EVs on BV2 microglia exposed to OGD/R were examined to assess their modulatory role in glial responses under ischemic conditions. Fluorescence labeling experiments demonstrated a time-dependent internalization of migrasome-enriched EVs by BV2 microglia (Fig. 2A). The concentration of M2 microglia-derived migrasome-enriched EVs (50 μ g/mL) used in the following experiments was determined based on preliminary dose-response analyses, which demonstrated near-maximal anti-inflammatory effects without additional benefit at higher concentrations (Figure S1A–C). To investigate the mechanisms underlying internalization and intracellular trafficking of migrasome-enriched EVs, BV2 microglia subjected to OGD/R treatment were incubated with labeled migrasome-enriched EVs

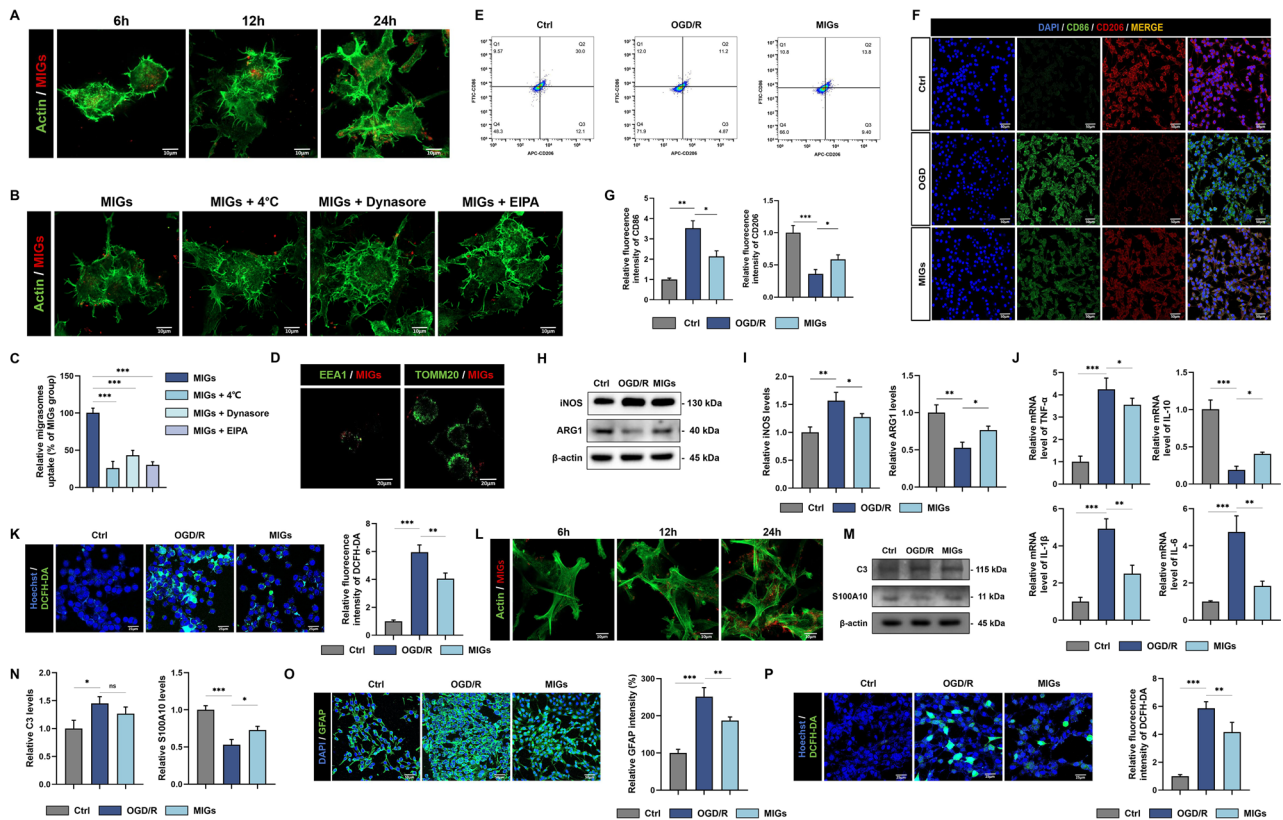


Fig. 2 M2 microglia-derived migrasome-enriched EVs promote protective phenotypes in microglia and astrocytes under OGD/R. **(A)** Representative fluorescence images showing the time-dependent uptake of labeled migrasome-enriched EVs (red) by BV2 microglia. **(B)** Representative fluorescence images showing uptake of labeled migrasome-enriched EVs (red) by BV2 microglia under different conditions, including MIGs, low temperature (4 °C), Dynasore, and EIPA treatment. **(C)** Quantitative analysis of uptake of migrasome-enriched EVs (%). **(D)** Representative confocal images showing the intracellular localization of labeled migrasome-enriched EVs (red) with EEA1 or TOMM20 (green) in BV2 microglia. **(E)** Representative flow cytometry plots analyzing the surface expression of CD86 and CD206 on BV2 cells. **(F, G)** Representative immunofluorescence images and quantitative analysis of CD86 and CD206 expression in BV2 cells. **(H, I)** Western blot analysis and quantification of iNOS and ARG1 protein levels in BV2 cells. **(J)** qPCR analysis of cytokine mRNA levels in BV2 cells. **(K)** Measurement of intracellular ROS levels in BV2 cells using DCFH-DA fluorescence. **(L)** Representative images showing the uptake of labeled migrasome-enriched EVs (red) by C8-D1A astrocytes. **(M, N)** Western blot analysis and quantification of C3 and S100A10. **(O)** Representative immunofluorescence images and quantification of GFAP in C8-D1A astrocytes. **(P)** Measurement of intracellular ROS levels in C8-D1A astrocytes. Data are presented as mean ± SD from at least three independent experiments. ns: no significance, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

and analyzed at an early 6-hour time point, which was selected to capture initial uptake and trafficking events. Confocal imaging revealed robust intracellular accumulation of migrasome-enriched EVs in the MIGs group. In contrast, uptake of migrasome-enriched EVs was markedly reduced under low-temperature conditions (4 °C), indicating that the internalization process is energy-dependent. Furthermore, treatment with Dynasore, an inhibitor of dynamin-dependent endocytosis, significantly decreased uptake of migrasome-enriched EVs. Similarly, inhibition of macropinocytosis using EIPA also resulted in a substantial reduction in intracellular migrasome-enriched EVs. Quantitative analysis further confirmed these findings, with the most pronounced inhibition observed at 4 °C (Fig. 2B, C). These findings suggest that migrasome-enriched EVs internalization is an energy-dependent process involving multiple endocytic pathways.

To further examine intracellular trafficking, colocalization analysis was performed using the early endosomal marker EEA1 and the mitochondrial marker TOMM20. Migrasome-enriched EVs exhibited partial and regionally enriched co-localization with EEA1, indicating that a subset of internalized migrasome-enriched EVs is trafficked through the endosomal pathway. In contrast, minimal co-localization with TOMM20 was observed, suggesting that migrasome-enriched EVs do not directly localize to mitochondria (Fig. 2D). These findings support that migrasome-enriched EVs are internalized via active endocytic pathways and primarily undergo endosomal trafficking, rather than directly targeting mitochondria.

Under OGD/R conditions, flow cytometry analysis revealed a lower percentage of CD86⁺ (M1) cells and a higher percentage of CD206⁺ (M2) cells following migrasome-enriched EVs treatment, compared to the OGD/R

group (Fig. 2E). This phenotypic shift was further validated through immunofluorescence staining and quantification of the M1 markers (CD86 and iNOS) and M2 markers (CD206 and ARG1) (Fig. 2F, G; Figure S2A-C). The molecular level revealed that migrasome-enriched EVs treatment downregulated iNOS and upregulated ARG1 expression (Fig. 2H, I). To further determine whether these effects are specific to M2 microglia-derived migrasome-enriched EVs, a comparative analysis was performed using migrasome-enriched EVs derived from M1-polarized microglia. Under OGD/R conditions, M1 microglia-derived migrasome-enriched EVs exhibited an opposite regulatory effect, characterized by increased expression of M1 markers (CD86) and reduced expression of M2 markers (CD206), as assessed by immunofluorescence staining. In contrast, M2-derived migrasome-enriched EVs promoted a shift toward an anti-inflammatory phenotype. These findings indicate that the observed immunomodulatory effects are not a general property of microglia-derived migrasome-enriched EVs but are dependent on the polarization state of the donor cells (Figure S3A-C).

Additionally, suppression of pro-inflammatory TNF- α , IL-1 β , and IL-6 transcription, alongside enhanced anti-inflammatory IL-10 expression, was observed in MIGs group by quantitative real-time PCR (qPCR) analysis (Fig. 2J). Additionally, treatment with migrasome-enriched EVs significantly reduced OGD/R-induced intracellular reactive oxygen species (ROS) overproduction in BV2 cells (Fig. 2K). To assess microglial phagocytosis and lysosomal status, uptake of fluorescently labeled latex beads and CD68 expression analysis were performed. OGD/R significantly increased phagocytic uptake versus control, an effect attenuated by the M2 inducer (Figure S2D, E), suggesting a functional shift away from non-specific phagocytosis upon M2 polarization. Interestingly, despite reduced phagocytosis, CD68 protein levels remained comparable between OGD/R and M2 inducer-treated groups (Figure S2F, G), with no significant differences in fluorescence intensity.

Subsequently, the effects of migrasome-enriched EVs on astrocytes were investigated under identical ischemic conditions. Similar to their uptake by microglia, C8-D1A astrocytes efficiently internalized fluorescently labeled migrasome-enriched EVs (Fig. 2L). Western blot analysis of A1/A2 astrocyte phenotypic markers revealed that migrasome-enriched EVs markedly decreased the expression of the harmful A1 marker C3 while enhancing the expression of the neuroprotective A2 marker S100A10 (Fig. 2M, N). Consistent with this phenotypic shift, OGD/R injury led to astrocyte activation, as indicated by an increase in GFAP immunofluorescence intensity, which was significantly attenuated in MIGs group (Fig. 2O). Furthermore, migrasome-enriched EVs

effectively suppressed the production of pro-inflammatory cytokines and attenuated OGD/R-induced ROS generation in astrocytes (Fig. 2P; Figure S4A-D). Collectively, these in vitro results indicate that migrasome-enriched EVs derived from M2 microglia directly facilitate the promotion of protective phenotypes in both microglia and astrocytes, while mitigating oxidative stress under ischemic-like conditions.

M2 microglia-derived migrasome-enriched EVs protect neuronal cells and enhance angiogenic functions of endothelial cells under OGD/R

To elucidate whether M2 microglia-derived migrasome-enriched EVs confer direct neuroprotection under ischemic conditions, their effects were initially evaluated using PC12 neuron-like cells. The PC12 cells exhibited time-dependent internalization of fluorescently labeled migrasome-enriched EVs (Fig. 3A). Under conditions of OGD/R, cell viability, as measured by the CCK-8 assay, was significantly diminished; however, this reduction was notably ameliorated following treatment with migrasome-enriched EVs (Fig. 3B). Consistent with these findings, live/dead cell staining revealed that the percentage of dead cells was markedly reduced in the MIGs group (Fig. 3C, D). Flow cytometry analysis employing Annexin V-FITC/PI double staining further corroborated that migrasome-enriched EVs significantly reduced the apoptotic rate of PC12 cells exposed to OGD/R (Fig. 3E). At the molecular level, migrasome-enriched EVs treatment downregulated the pro-apoptotic protein Bax and upregulated the anti-apoptotic protein Bcl-2, as revealed by Western blot analysis, consequently reducing the Bax/Bcl-2 ratio (Fig. 3F, G). Additionally, migrasome-enriched EVs effectively mitigated the OGD/R-induced increase in intracellular ROS levels (Fig. 3H, I). Finally, EdU incorporation assay demonstrated that while OGD/R injury suppressed PC12 cell proliferation, migrasome-enriched EVs treatment effectively restored their proliferative capacity (Fig. 3J).

Following the establishment of the direct neuroprotective effects, the subsequent investigation focused on the impact of migrasome-enriched EVs on brain endothelial function. Consistent with observations in other cell types, bEnd.3 endothelial cells demonstrated efficient uptake of labeled migrasome-enriched EVs over time (Fig. 3L). In a Transwell migration assay, the migratory capacity of bEnd.3 cells, which was compromised by OGD/R injury, was significantly restored upon treatment with migrasome-enriched EVs (Fig. 3M, N). Notably, in an in vitro tube formation assay using Matrigel, treatment with migrasome-enriched EVs markedly ameliorated the OGD/R-induced impairment of angiogenic function. bEnd.3 cells treated with migrasome-enriched EVs exhibited the formation of capillary-like structures

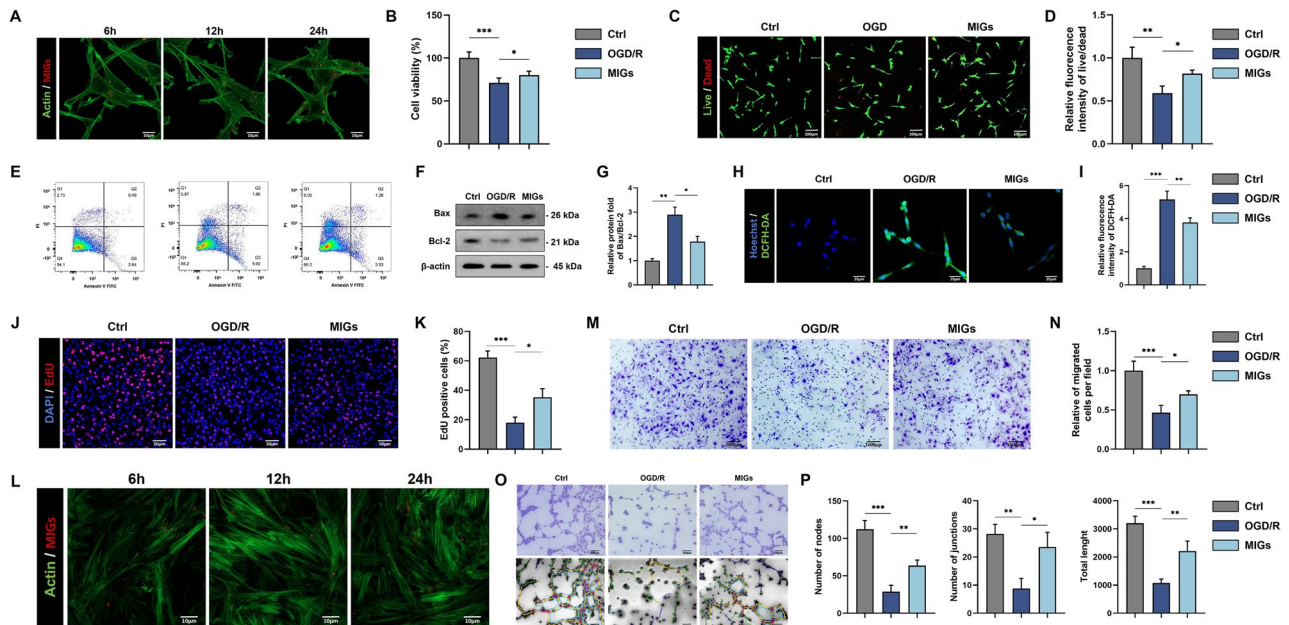


Fig. 3 Neuroprotective and pro-angiogenic effects of M2 microglia-derived migrasome-enriched EVs on PC12 and bEnd.3 cells under OGD/R. **(A)** Representative fluorescent images showing the time-dependent uptake of labeled migrasome-enriched EVs (red) by PC12 neuron-like cells. **(B)** PC12 cell viability assessed by CCK-8 assay. **(C, D)** Representative fluorescent images and quantification of live/dead staining in PC12 cells. **(E)** Representative flow cytometry of apoptosis in PC12 cells detected by Annexin V-FITC and PI double staining. **(F, G)** Western blot analysis and quantitative ratio of Bax and Bcl-2 protein expression in PC12 cells. **(H, I)** Representative fluorescent images and quantification of intracellular ROS levels in PC12 cells detected by DCFH-DA probe. **(J, K)** Quantification of PC12 cell proliferation assessed by EdU incorporation assay. **(L)** Representative fluorescent images showing the time-dependent uptake of labeled migrasome-enriched EVs (red) by bEnd.3 endothelial cells. **(M, N)** Representative images and quantitative analysis of bEnd.3 endothelial cells migration assessed by Transwell assay. Migrated cells were stained with crystal violet. **(O, P)** Representative images and quantitative analysis of number of nodes, number of junctions and the total tube length in the bEnd.3 endothelial cells in vitro tube formation assay on Matrigel. Data are presented as mean $\pm$ SD from at least three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

with significantly greater total tube length and number of junctions compared to the OGD/R group (Fig. 3O, P). Collectively, these findings indicate that migrasome-enriched EVs derived from M2 microglia exert direct protective effects on neuronal cells by enhancing cell viability, suppressing apoptosis and oxidative stress, and restoring proliferation. Moreover, migrasome-enriched EVs augment critical reparative functions of brain endothelial cells by promoting migration and angiogenic tube-forming capacity under ischemic conditions.

In vivo tracking and systemic biosafety evaluation of intracerebrally injected M2 microglia-derived migrasome-enriched EVs

To verify the intracranial retention and assess the systemic safety of migrasome-enriched EVs administration, stereotactic injection of labeled migrasome-enriched EVs was conducted into the left cerebral hemisphere of mice. In vivo fluorescence imaging demonstrated successful localization and retention of migrasome-enriched EVs at the injection site within the brain over a 24-hour period (Fig. 4A).

To evaluate potential systemic toxicity following migrasome-enriched EVs administration, a comprehensive

biosafety assessment was performed seven days post-injection. Red blood cell (RBC) count, platelet (PLT) count, total white blood cell (WBC) count, and the proportions of neutrophils, lymphocytes, and monocytes were measured as key hematological parameters; none showed significant changes after migrasome-enriched EVs treatment (Figure S5A, B). Biochemical analysis of serum markers revealed that liver function (alanine aminotransferase, ALT; aspartate aminotransferase, AST) and renal function (urea nitrogen, UREA; creatinine, CREA) remained within normal ranges, unaffected by migrasome-enriched EVs injection (Figure S5C, D). Additionally, the levels of immunoglobulins (IgG, IgM) remained stable, which indicates no humoral immune response (Figure S5E).

H&E staining of major organs demonstrated an absence of structural damage, inflammation, or pathological changes in animals exposed to migrasome-enriched EVs (Fig. 4B). These findings collectively indicate that the intracerebral administration of M2 microglia-derived migrasome-enriched EVs is a viable approach, demonstrating a favorable biosafety profile without detectable systemic hematological, biochemical, immunological, or organ-level toxicity.

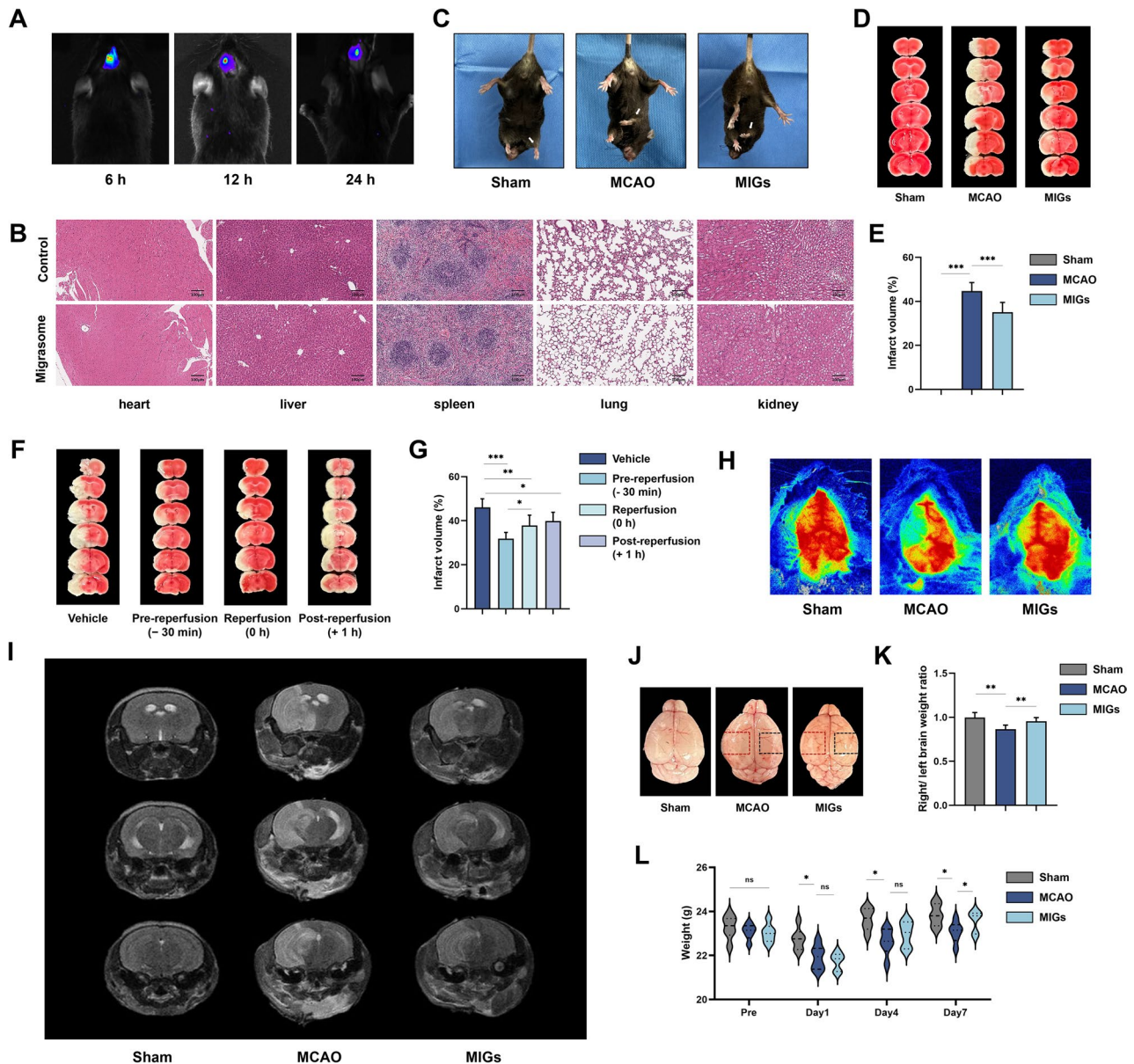


Fig. 4 Therapeutic effects of M2 microglia-derived migrasome-enriched EVs on ischemic brain injury and functional recovery after MCAO. **(A)** In vivo fluorescence imaging of mouse brains following intracerebral administration of labeled migrasome-enriched EVs (red pseudocolor), shown at 6, 12, and 24 h. **(B)** Representative images of major organ sections stained with H&E from mice with or without migrasome-enriched EVs administration. **(C)** Representative photographs of mice post-MCAO. The arrow indicates the failure to extend the left forelimb, a sign of contralateral neurological deficit. **(D, E)** Representative TTC-stained brain sections and quantitative analysis of infarct volume from Sham, MCAO, and MIGs groups at 24 h post-reperfusion. The pale area indicates the infarct region. **(F, G)** Representative TTC-stained brain sections and quantitative analysis from mice treated at different time points relative to reperfusion, including vehicle (MCAO), 30 min prior to reperfusion (–30 min), at the onset of reperfusion (0 h), and 1 h after reperfusion (+1 h). **(H)** Representative laser speckle contrast images in the ipsilateral hemisphere. **(I)** Representative T2-weighted MRI of mouse brains 24 h after MCAO. Hyperintense signals indicate brain edema and injury. **(J)** Representative photographs of brain coronal sections. The black and red dashed lines outline the contralateral and ipsilateral hemispheres, respectively, highlighting the asymmetry due to edema. **(K)** Quantification of brain edema presented as the ratio of the weight of the contralateral (right) hemisphere to the ipsilateral (left) hemisphere. **(L)** Body weight changes of mice before surgery (Pre) and on days 1, 4, and 7 after MCAO. Data are presented as mean $\pm$ SD ($n=6-8$ mice per group). ns: no significance, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

M2 microglia-derived migrasome-enriched EVs attenuate brain injury and promote functional recovery after MCAO

The therapeutic efficacy of M2 microglia-derived migrasome-enriched EVs was evaluated in a clinically relevant stroke model using transient MCAO in mice (Figure

S6A). Both MCAO and MIGs groups exhibited significantly elevated Longa neurological scores at 24 h post-reperfusion compared to the Sham group, confirming comparable severity of initial injury upon model establishment (Figure S6B). Successful induction of cerebral

ischemia was confirmed by the presence of marked neurological deficits, including contralateral forelimb paralysis (Fig. 4C) and circling behavior toward the paretic side.

The neuroprotective effects of migrasome-enriched EVs were further validated by a reduction in cerebral infarction. According to 2,3,5-triphenyltetrazolium chloride (TTC) staining, the MCAO group had a large, pale infarct area, which was considerably smaller in the MIGs group (Fig. 4D, E). To evaluate the therapeutic time window of migrasome-enriched EVs, additional experiments were performed in which migrasome-enriched EVs were administered at different time points relative to reperfusion, including 30 min prior to reperfusion, at the onset of reperfusion, and 1 h after reperfusion. Notably, migrasome-enriched EVs treatment at all three time points significantly reduced infarct volume compared with the Vehicle group. Among these, administration prior to reperfusion exhibited the strongest protective effect, followed by treatment at reperfusion, whereas administration 1 h post-reperfusion still conferred significant but comparatively reduced protection (Fig. 4F, G). These results indicate that migrasome-enriched EVs retain therapeutic efficacy within a clinically relevant time window, with earlier intervention yielding greater benefit. Consistent with these findings, evaluation of cerebral perfusion 24 h after reperfusion, conducted using laser speckle contrast imaging, indicated that migrasome-enriched EVs treatment partially restored CBF in the ischemic hemisphere (Fig. 4H). This observation was supported by T2-weighted magnetic resonance imaging (MRI), which revealed a smaller hyperintense lesion area, indicative of reduced edema and injury, in the MIGs group (Fig. 4I).

Additionally, migrasome-enriched EVs treatment mitigated post-ischemic cerebral edema. Gross examination of brain coronal sections showed pronounced swelling of the ischemic hemisphere (area within the red dashed line) post-MCAO, which was markedly attenuated in MIGs group (Fig. 4J). This reduction was quantitatively confirmed by a significantly lower right/left hemisphere weight ratio (Fig. 4K).

Finally, monitoring of body weight as a general indicator of recovery revealed that MCAO induced significant and sustained weight loss. Mice treated with migrasome-enriched EVs demonstrated a more rapid and significant recovery of body weight towards pre-operative levels by day 7 post-stroke (Fig. 4L). Collectively, these findings indicate that intracerebral administration of M2 microglia-derived migrasome-enriched EVs effectively mitigates acute ischemic brain damage, reduces infarct size and edema, enhances cerebral perfusion, and accelerates functional recovery in mice.

M2 microglia-derived migrasome-enriched EVs ameliorate neuroinflammation, protect neurons, modulate glial responses, and promote angiogenesis in the ischemic brain.

To understand how migrasome-enriched EVs contribute to neuroprotection in the ischemic brain, ELISA analysis was performed on ipsilateral brain homogenates. The results showed a surge in TNF- α , IL-1 β , and IL-6 following MCAO, which was substantially attenuated by migrasome-enriched EVs treatment. The MIGs group exhibited elevated levels of the anti-inflammatory cytokine IL-10 (Fig. 5A).

Histopathological analysis using H&E staining revealed substantial damage following MCAO. The neuronal layers exhibited loosening and disorganization, accompanied by pronounced vacuolization and a marked reduction in neuronal density, indicative of severe injury. Treatment with migrasome-enriched EVs led to a partial yet noticeable amelioration; the tissue architecture appeared more organized, neuronal layers were more distinct, and the degree of vacuolization and rarefaction was intermediate between the other two groups (Fig. 5B). Similarly, Nissl staining indicated a considerable decline in Nissl bodies post-MCAO, with this loss being considerably mitigated in the MIGs group (Fig. 5C).

To evaluate microglial activation and polarization *in situ*, immunofluorescence staining was conducted. MCAO elicited a substantial increase in IBA1⁺ microglia, with a high proportion co-expressing the M1 marker iNOS. Migrasome-enriched EVs treatment significantly decreased the total number of IBA1⁺ cells and altered their phenotype, reducing iNOS co-localization while enhancing co-localization with the M2 marker ARG1 (Fig. 5D-F). Concurrently, migrasome-enriched EVs treatment modulated reactive astrogliosis. An increase in GFAP⁺ astrocytes, many expressing the detrimental A1 marker C3, was observed after MCAO. However, treatment with migrasome-enriched EVs facilitated a shift towards a protective A2 phenotype, as indicated by an increased co-expression of GFAP with the A2 marker S100A10 (Fig. 5G-I).

The subsequent neuronal outcomes were evaluated using NeuN and TUNEL double staining. MCAO led to a notable reduction in NeuN⁺ neurons alongside a considerable increase in TUNEL⁺ apoptotic cells within the peri-infarct cortex. In contrast, migrasome-enriched EVs treatment effectively preserved NeuN⁺ neurons and substantially reduced the number of TUNEL⁺ cells (Fig. 5J, K). Additionally, the impact on cerebral vasculature was assessed by staining for the endothelial marker CD31. MCAO injury decreased CD31⁺ microvessel density in the ischemic border zone versus the Sham group. Migrasome-enriched EVs treatment partially, yet significantly,

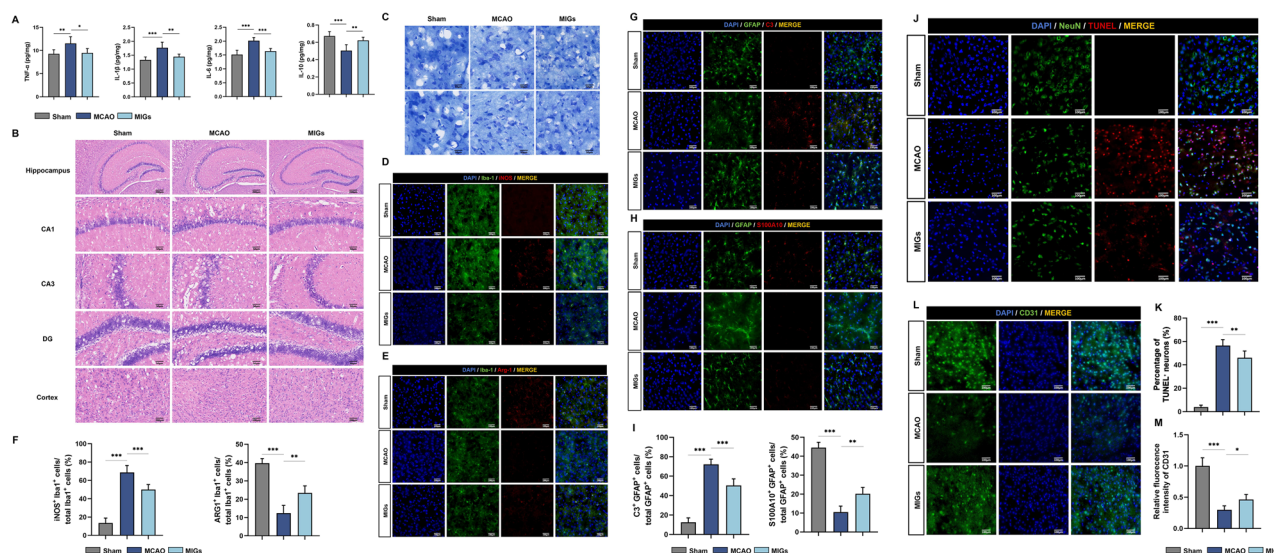


Fig. 5 M2 microglia-derived migrasome-enriched EVs mitigate neuroinflammation, cell death, and promote a protective microenvironment after MCAO. **(A)** Levels of inflammatory cytokines in the ipsilateral hemisphere brain homogenates measured by ELISA. **(B)** Representative H&E-stained images of the hippocampus (including CA1, CA3, and DG subregions) and the ischemic cerebral cortex. **(C)** Representative Nissl-stained images in the cortical peri-infarct area. **(D-F)** Representative immunofluorescence images and quantitative analysis of IBA1⁺ microglia and their phenotypic characterization in the peri-infarct cortex. **(G-I)** Representative immunofluorescence images and quantitative analysis of GFAP⁺ astrocytes and their phenotypic characterization in the peri-infarct cortex. **(J, K)** Representative immunofluorescence images and quantitative analysis of neuronal survival and apoptosis in the peri-infarct cortex. **(L, M)** Representative immunofluorescence images and quantitative analysis of CD31⁺ microvessels in the ischemic border zone. Data are presented as mean $\pm$ SD ($n = 6$ mice per group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

EV-treated mice traveled a significantly greater total distance and exhibited a higher mean velocity, while the prolonged immobility time observed in MCAO mice was reduced (Fig. 6E). The data suggest that migrasome-enriched EVs alleviate stroke-induced hypolocomotion and restore spontaneous exploratory activity during the subacute phase.

To evaluate cognitive function, specifically recognition memory, the novel object recognition test was administered (Fig. 6F). During the training phase, both mice in MCAO and MIGs group exhibited similar exploration behavior with two identical objects. However, during the testing phase, migrasome-enriched EV-treated mice showed a significantly stronger preference for the novel object relative to the MCAO group (Fig. 6G, H), indicating that migrasome-enriched EV-treatment partly preserved the ability to discriminate and remember novel stimuli.

Spatial working memory was examined using the Y-maze test (Fig. 6I). The exploration pattern in the novel arm (Arm 3) was more pronounced in migrasome-enriched EV-treated mice, as evidenced by their trajectories and heat maps (Fig. 6J). The quantification of the time duration in the novel arm demonstrated an increase in the MIGs group following MCAO, indicating enhanced spatial curiosity and short-term memory (Fig. 6K).

Collectively, the administration of M2 microglia-derived migrasome-enriched EVs not only alleviated

To assess general locomotor and exploratory activity, the open field test was conducted (Fig. 6C). Representative movement trajectories and heat maps visually illustrated reduced exploration in the MCAO group, which was ameliorated in the MIGs group (Fig. 6D). Quantitative analysis demonstrated that migrasome-enriched

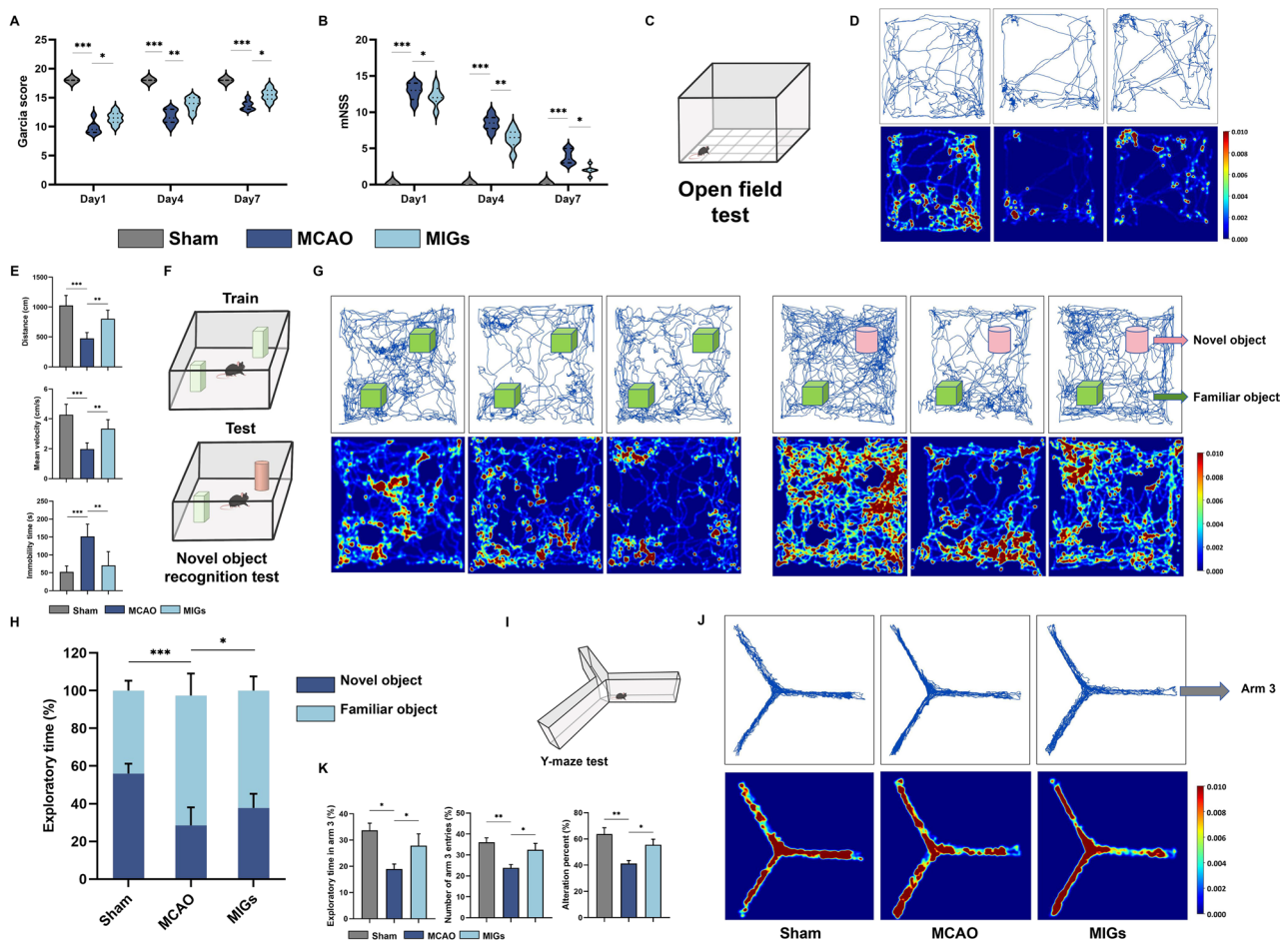


Fig. 6 M2 microglia-derived migrasome-enriched EVs treatment improves subacute neurological outcomes after MCAO. (A, B) Sequential evaluation of neurological function using the Garcia score and the mNSS on days 1, 4, and 7 after reperfusion. (C) Schematic of the open field test apparatus. (D) Representative movement trajectories and heat maps of mice in the open field test. (E) Quantitative analysis of locomotor activity in the open field test, including total distance traveled, mean velocity, and immobility time. (F) Schematic of the novel object recognition test procedure. (G) Representative movement trajectories and heat maps from the testing phase of the novel object recognition test. (H) Quantification of cognitive performance in the novel object recognition test, presented as the percentage of time spent exploring the novel object. (I) Schematic of the Y-maze test apparatus. (J) Representative movement trajectories and heat maps from the Y-maze test. (K) Quantification of exploratory behavior in the Y-maze, presented as the percentage of time spent in the novel arm (Arm 3). Data are presented as mean $\pm$ SD ($n = 8-10$ mice per group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

acute injury but also promoted comprehensive functional improvements during the subacute phase following ischemic stroke, including improvements in neurological deficits, locomotor activity, and cognitive functions during the subacute phase following ischemic stroke.

M2 microglia-derived migrasome-enriched EVs exert neuroprotection by activating the cAMP/EPAC1/Rap1 signaling axis and improving mitochondrial function in microglia

To elucidate the signaling pathways underlying migrasome-enriched EV-mediated neuroprotection, transcriptomic analysis was conducted on ipsilateral brain tissues. Unsupervised clustering effectively differentiated the transcriptomic profiles of the MCAO and MIGs groups (Fig. 7A). Volcano plot analysis identified numerous

DEGs (Fig. 7B), and KEGG pathway enrichment analysis identified significant enrichment in pathways associated with the “cAMP signaling pathway” and “Rap1 signaling pathway” (Fig. 7C). These findings suggest that the cAMP/Rap1 signaling cascade is dysregulated following MCAO and that migrasome-enriched EV-treatment may modulate this pathway.

To ascertain whether migrasome-enriched EVs activate the cAMP/EPAC1/Rap1 signaling axis in microglia, BV2 cells were subjected to OGD/R conditions. ELISA results revealed that migrasome-enriched EV-treatment significantly elevated cAMP levels (Fig. 7D). Further analyses through immunofluorescence and Western blotting revealed that migrasome-enriched EVs substantially upregulated EPAC1, a key mediator of cAMP signaling that activates Rap1, as well as Rap1 itself (Fig. 7E-I).

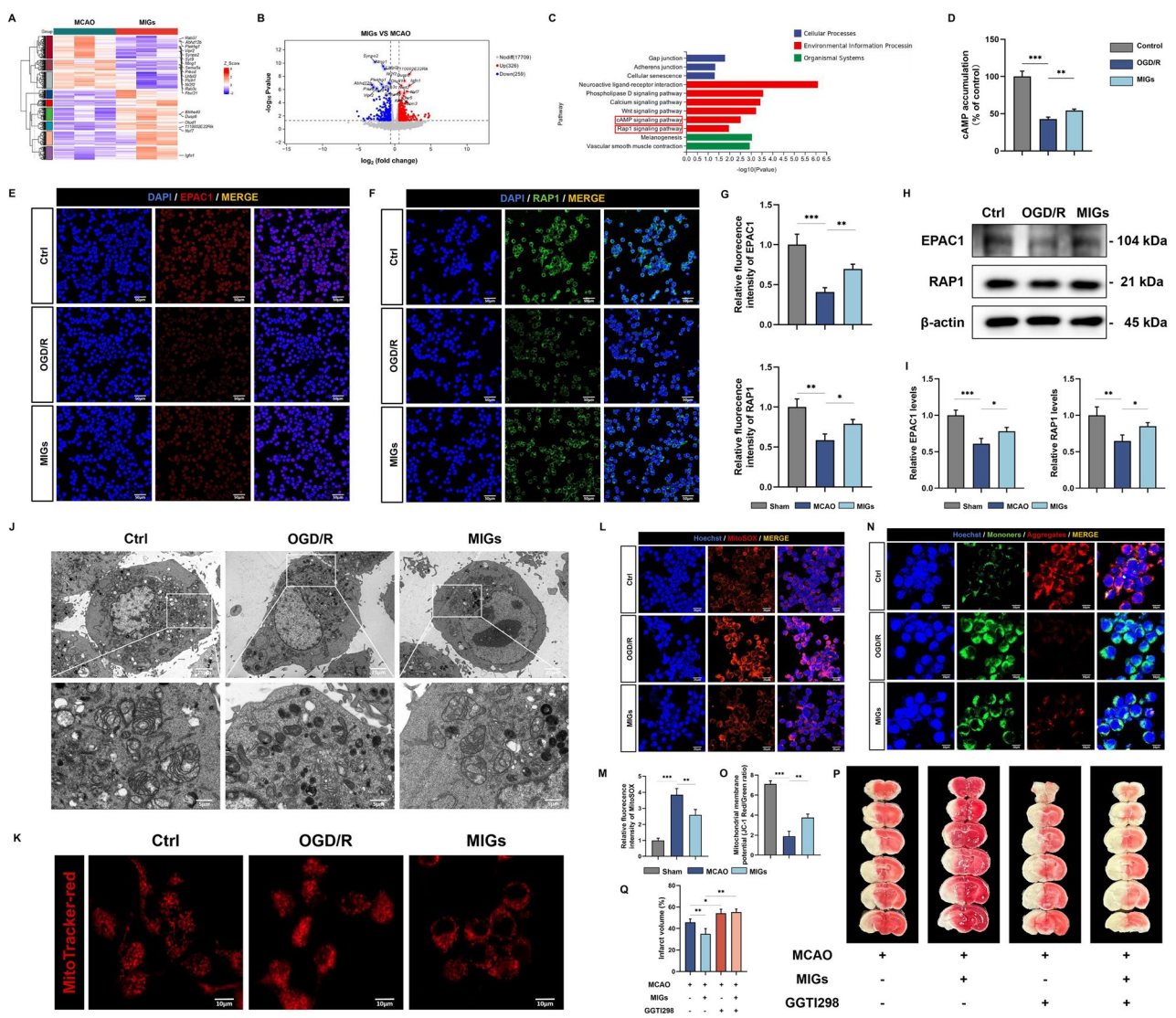


Fig. 7 M2 microglia-derived migrasome-enriched EVs activate the cAMP/EPAC1/Rap1 pathway to improve mitochondrial function in microglia. **(A)** Heatmap of differentially expressed genes in ipsilateral brain tissue between the MCAO and MIGs groups. **(B)** Volcano plot of DEGs between the MCAO and MIGs groups. **(C)** KEGG pathway enrichment analysis. The top enriched pathways are shown, with the cAMP signaling pathway and Rap1 signaling pathway highlighted. **(D)** cAMP levels in BV2 cells. **(E-G)** Representative immunofluorescence image and quantitative analysis of EPAC1 and Rap1 in BV2 cells. **(H, I)** Western blot analysis and quantification of EPAC1 and Rap1 protein expression in BV2 cells. **(J)** Representative TEM images of mitochondria in BV2 cells. **(K)** Representative images and quantification of mitochondrial mass. **(L, M)** Representative images and quantification of mitochondrial superoxide levels. **(N, O)** Representative images and quantification of mitochondrial membrane potential ($\Delta\Psi_m$) assessed by JC-1 staining (red: J-aggregates, high $\Delta\Psi_m$; green: monomers, low $\Delta\Psi_m$). **(P)** Representative TTC-stained brain sections from MCAO mice treated with MIGs in the presence or absence of the Rap1 inhibitor GGTI298. **(Q)** Quantitative analysis of infarct volume. Data are presented as mean $\pm$ SD from at least three independent experiments (cells) or $n=6$ mice per group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

These findings corroborate the induction of the cAMP/EPAC1/Rap1 signaling cascade by migrasome-enriched EVs in microglia.

Considering the well-documented role of cAMP in metabolic coupling and mitochondrial ATP production [26], the relationship between pathway activation and mitochondrial function was subsequently explored. TEM demonstrated that OGD/R conditions induced significant mitochondrial swelling and cristae disruption in BV2 cells, which were substantially mitigated by

migrasome-enriched EV-treatment (Fig. 7J). Concordantly, migrasome-enriched EV-treatment resulted in an increase in mitochondrial mass, as evidenced by MitoTracker Red staining (Fig. 7K), and significantly reduced the overproduction of mitochondrial superoxide induced by OGD/R (Fig. 7L, M). In addition, JC-1 staining demonstrated that migrasome-enriched EVs repaired the mitochondrial membrane potential ($\Delta\Psi_m$) affected by OGD/R (Fig. 7N, O).

To clarify the cause-and-effect relationship between Rap1 activation and the observed neuroprotection, a rescue experiment was performed utilizing the Rap1 inhibitor GGTI298. In vivo administration of GGTI298 substantially diminished the protective effect of migrasome-enriched EVs, as indicated by a significant reversal in the reduction of cerebral infarct volume, which was assessed via TTC staining (Fig. 7P, Q). Collectively, these findings indicate that migrasome-enriched EVs derived from M2 microglia modulate the cAMP/EPAC1/Rap1 signaling pathway in microglia, thereby enhancing mitochondrial integrity and function. The inhibition of Rap1 negates the neuroprotective effect of migrasome-enriched EVs, thereby establishing this axis as a critical mechanistic pathway for migrasome-mediated therapy in ischemic stroke.

Discussion

These findings highlight a previously unrecognized mechanism by which migrasome-enriched EVs orchestrate coordinated repair across multiple cell types within the neurovascular unit. The pathological essence of ischemic stroke is the holistic collapse of the neurovascular unit, making it difficult to achieve functional recovery through single-target interventions [27–29]. The findings of this study indicate that M2 microglia-derived migrasome-enriched EVs enhance the neurofunctional outcomes of ischemic stroke by concurrently modulating the functional trajectories of four critical cell types. Specifically, these cells facilitate the microglia shift towards the M2 phenotype, switch astrocytes to neuroprotective phenotypes, mitigate neuronal apoptosis and oxidative stress, and restore the migratory and angiogenic capabilities of cerebral microvascular endothelial cells. These effects are not isolated or parallel; rather, they converge through the M2 phenotype polarization of microglia, which acts as an upstream hub. This process is amplified through intercellular cascades, ultimately integrating into a synergistic benefit for neuronal survival and vascular repair. The multi-target, synergistic protective mechanism of these migrasome-enriched EVs offers a plausible explanation for their superior neuroprotective efficacy observed in animal models, setting them apart from previous stroke treatment strategies that primarily focus on single pathways. This represents an innovative therapeutic approach with significant translational potential.

Importantly, the isolated vesicle preparations in this study are referred to as M2 microglia-derived migrasome-enriched EVs, rather than homogeneous or purified migrasomes. This terminology reflects the enrichment of TSPAN4-positive migrasome-like structures and migrasome-associated proteins, while acknowledging the heterogeneous nature of the isolated fractions and the possible coexistence of other extracellular vesicle

subtypes. Therefore, the biological effects observed in this study should be interpreted as effects of migrasome-enriched EV populations, rather than effects attributable exclusively to pure migrasomes.

In recent years, migrasomes have emerged as novel intercellular communication carriers, noted for their contributions to tissue healing and disease regulation. These vesicular structures are formed and released during the retraction of the fibrous ends of migrating cells and are rich in intraluminal vesicles capable of transporting proteins, mRNA, and even intact organelles [30]. Recipient cells can internalize migrasomes after they dissociate from the parent cell, which can modify their functions [19]. An increasing body of evidence indicates that migrasomes are implicated in various pathological conditions within the central nervous system [31]. Emerging evidence highlights migrasomes as vital components in cell-cell communication and immune regulation. Their capability to package and transport signaling molecules facilitates precise intercellular targeting in the brain, particularly within neuroinflammatory microenvironments [32]. However, the therapeutic potential and underlying mechanisms of migrasome-enriched EVs in the context of ischemic stroke remain unexplored. Microglia represent a pivotal focus in ischemia/reperfusion neuroprotection research [33]. As cerebral ischemia commences, microglia are swiftly activated, releasing inflammatory agents and undergoing phenotypic changes [34]. In this investigation, M2 microglia were chosen as the source of migrasome-enriched EVs due to their intrinsic anti-inflammatory and tissue-repairing attributes. The protective functions of M2 microglia may be partly conveyed by their migrasome-enriched EV populations, which contain bioactive cargos capable of modulating recipient-cell responses in the post-stroke microenvironment. This approach may offer a more direct and efficient alternative to relying solely on endogenous phenotypic transformation.

Notably, this strategy is conceptually related to previous therapeutic approaches based on M2 microglia and their derived EVs, particularly exosomes. Prior studies have demonstrated that transplantation of M2-polarized microglia or administration of M2 microglia-derived exosomes can exert neuroprotective effects in ischemic stroke by modulating neuroinflammation and promoting tissue repair [35–37]. In this context, M2 microglia-derived migrasome-enriched EVs may be considered an EV-based extension of these strategies. Compared with direct cell transplantation, vesicle-based approaches may avoid limitations including low cell survival and high cost, while enabling the delivery of bioactive cargos. The present findings suggest that M2 microglia-derived migrasome-enriched EV populations can influence several neurovascular unit-associated cell types, including

microglia, astrocytes, neuronal cells, and endothelial cells. This broad cellular activity may be related to the vesicular nature of the preparations and the enrichment of migrasome-associated structures and cargos, as suggested by previous studies on migrasome biology and EV-mediated intercellular communication [18, 38]. However, given the heterogeneous nature of the isolated fractions, these effects should not be attributed exclusively to pure migrasomes. Further studies are required to define the relative contribution of migrasome-like vesicles and other EV subtypes within these preparations.

Based on transcriptomic profiling and subsequent experimental validation, this study reveals that the protective role of migrasome-enriched EVs is connected to the enhanced mitochondrial function induced by modulating cAMP/EPAC1/Rap1 signaling pathway in microglia. As the primary target cells involved in the regulation of neuroinflammation within the brain [39–41], microglia represent a critical cellular context for understanding this signaling cascade. Notably, research within the domain of EV has yielded considerable evidence indicating that vesicles originating from specific cell types may exhibit preferential interactions with their parental or related cell populations [42, 43]. Although the targeting patterns of migrasome-enriched EVs have not been formally documented, it is hypothesized, based on their partial homology with exosomes in terms of biogenesis and membrane composition, that they may engage in cell-type-associated interactions. In the present study, efficient uptake of migrasome-enriched EVs by microglia was observed, suggesting a functional interaction between migrasomes and microglia in the post-ischemic microenvironment, although whether this interaction reflects preferential or homologous targeting remains to be fully clarified. More significantly, the M2 polarization of microglia acts as an upstream event that coordinates the repair of neurovascular units by migrasomes [44–46]. Consequently, elucidating the activation mechanism of the cAMP/EPAC1/Rap1 pathway in microglia is pivotal, as it serves as a critical link between transcriptomic findings in brain tissue and the overall protective effects observed in the animal model.

cAMP is a pivotal intracellular messenger molecule extensively implicated in both cellular physiology and pathological processes [47, 48]. It exerts numerous biological effects during the pathological progression of ischemic stroke. While cAMP is involved in extracellular metabolism, its principal function resides in intracellular signaling. Increased cAMP levels facilitate the production of factors that support neuronal survival and function [49]. Recent evidence suggests that increasing cAMP levels following ischemic stroke can significantly ameliorate neuroinflammatory responses, pointing to the vital role of the cAMP signaling pathway in this context

[50, 51]. Augmenting cAMP signaling can synergistically regulate multiple cellular functions within neurovascular units by inhibiting microglial migration and pro-inflammatory activation, thereby preserving neuronal morphology and functional integrity and reducing central nervous system damage [52]. However, the upstream regulatory mechanisms linking cAMP activation to migrasome-enriched EVs signaling remain incompletely understood.

The upstream mechanism by which M2 microglia-derived migrasome-enriched EVs induce cAMP elevation in recipient microglia remains to be fully elucidated, despite the observed increase in intracellular cAMP levels following migrasome-enriched EVs treatment. In the context of ischemic stroke, EV-mediated intercellular communication has been shown to modulate intracellular signaling pathways, including cAMP-related responses [53, 54]. Migrasomes, as a distinct class of vesicular structures enriched in bioactive cargos, may similarly influence intracellular signaling through the delivery of functional molecules to recipient microglia. One plausible explanation is that migrasome-delivered bioactive cargos may modulate intracellular signaling pathways in recipient microglia, potentially through receptor-mediated activation or metabolic regulation, thereby contributing to the observed elevation of cAMP levels. Nevertheless, the specific components responsible for cAMP elevation remain to be identified, which represents a limitation of the current study.

The Epac1/Rap1 signaling axis represents a crucial downstream signaling cascade that operates independently of the classical cAMP pathway [55, 56]. Epac1, a guanosine nucleotide exchange factor, promotes Rap1 activation by catalyzing GDP-GTP exchange [57]. The Epac1/Rap1 pathway is integral to essential pathophysiological processes, including vascular barrier maintenance, oxidative balance, and immune-inflammatory cascades [58–61]. In ischemic injury models, Epac1 activation has been shown to prevent neuronal cell death, mitigate BBB damage, and influence immune system reactions [62–64], aligning closely with the protective effects of migrasome-enriched EVs observed in this study. Rap1, a member of the Ras family of small GTPases, was initially recognized as an immunomodulator involved in immunity and has demonstrated significant neuroprotective potential [65, 66]. This study further elucidates the causal necessity of Rap1 in mediating this protective effect through *in vivo* rescue experiments. The application of the Rap1-specific inhibitor GGTI298 completely negated the migrasome-enriched EV-mediated reduction in cerebral infarct volume (Fig. 7P, Q). This finding indirectly corroborates the notion that Rap1 activation is an essential and indispensable node for M2 microglia-derived migrasome-enriched EVs to exert its neuroprotective effects.

The key question pertains to the mechanisms by which Rap1 activation specifically facilitates the neuroprotective effects of migrasome-enriched EVs. Subsequent investigations concentrated on mitochondria, which serve as pivotal centers for energy metabolism and cellular survival decisions, and are recognized as established downstream effectors of Rap1 signaling [67–69]. Experimental validation substantiated that migrasome-enriched EVs preserve mitochondrial structure and function under OGD/R conditions (Fig. 7J–O), thereby functionally linking upstream signaling pathways to the protection of downstream organelles. These findings suggest that migrasome-enriched EVs are internalized through active endocytic pathways and primarily trafficked via endosomal compartments, supporting the notion that their effects on mitochondrial function are mediated indirectly through intracellular signaling cascades rather than direct organelle targeting. Importantly, mitochondrial homeostasis supports not only the survival of microglia but also their metabolic reprogramming towards an anti-inflammatory phenotype [70]. Consequently, M2 microglia-derived migrasome-enriched EV-mediated mitochondrial protection may constitute the metabolic foundation for both the phenotypic transition of microglia and subsequent multicellular reparative processes.

The present findings demonstrate that M2 microglia-derived migrasome-enriched EVs orchestrate neurovascular unit recovery after ischemic stroke by restoring mitochondrial homeostasis. These migrasome-enriched EVs are effectively internalized by microglia via modulating the cAMP/EPAC1/Rap1 signaling pathway and subsequently restoring mitochondrial structure and function. This process not only drives microglial polarization toward the M2 phenotype but also serves as an upstream initiating signal, coordinating a multicellular cascade involving astrocytes, neurons, and microvascular endothelial cells to achieve synergistic neurovascular repair. Importantly, migrasome-enriched EVs retained protective efficacy when administered at the onset of reperfusion, suggesting that their therapeutic effects are not limited to a preconditioning paradigm. Further studies will be required to define the therapeutic window for migrasome-based therapy.

It should be emphasized that the current study is not intended to establish a clinically applicable delivery route, but rather to validate the intrinsic therapeutic potential of M2 microglia-derived migrasome-enriched EVs in the brain. In the present study, migrasome-enriched EVs were administered via intracerebral injection to establish proof-of-concept efficacy within the brain. Although this route is not readily applicable in current clinical practice, it is widely adopted in early-stage preclinical studies to evaluate the intrinsic therapeutic potential of novel agents while minimizing confounding factors such as

systemic biodistribution and BBB limitations. The BBB represents a major obstacle for the delivery of vesicular therapeutics to the brain, particularly in ischemic stroke, where both barrier dysfunction and delivery inefficiency complicate systemic administration [71, 72]. Moreover, emerging engineering strategies may facilitate the delivery of vesicular therapeutics across the BBB, including surface modification, ligand-mediated targeting, and biomimetic engineering approaches [73–76], which provide potential avenues to enhance the translational applicability of migrasome-based therapies. Therefore, this approach serves as a necessary first step to directly assess the neuroprotective effects of M2 microglia-derived migrasome-enriched EVs. Future studies will focus on developing clinically translatable and less invasive delivery strategies, including intravenous administration, along with the application of engineering approaches to enhance BBB penetration and targeting efficiency.

The improvement in cerebral perfusion observed in this study further suggests that migrasome-enriched EVs may contribute to the restoration of neurovascular function following ischemic injury. This improvement is more likely associated with enhanced neurovascular unit integrity and preservation of endothelial function. Consistent with this interpretation, the *in vitro* findings demonstrate that migrasome-enriched EVs promote endothelial cell migration and tube formation. These observations raise the possibility that migrasome-enriched EVs may act as multi-functional regulators within the neurovascular unit, coordinating interactions between microglia, endothelial cells, and other cellular components in response to ischemic stress. However, whether migrasome-enriched EVs directly induce angiogenesis, vascular remodeling, or other forms of vascular adaptation *in vivo* remains to be further investigated. A more comprehensive understanding of their vascular effects, including potential roles in perfusion regulation and microvascular stability, will be important for fully elucidating their therapeutic potential.

Notwithstanding these findings, several limitations should be acknowledged. The *in vivo* distribution and safety profile of migrasome-enriched EVs require further systematic investigation. In addition, behavioral assessments were conducted only up to 7 days after MCAO; therefore, the current findings primarily reflect beneficial effects during the acute and early subacute phases. Furthermore, future studies will be required to explore less invasive and clinically relevant delivery strategies, such as intravenous administration. It is also important to note that only young male mice were used in this study, which may limit the generalizability of the findings. Accordingly, future studies incorporating female and aged animals will be necessary to fully evaluate the therapeutic potential of migrasome-enriched EVs across different

biological conditions. Taken together, despite these limitations, the present findings provide a foundation for further investigation into migrasome-based therapeutic strategies in ischemic stroke.

Conclusion

This investigation provides initial evidence that M2 microglia-derived migrasome-enriched EVs restore mitochondrial homeostasis in microglia via modulating cAMP/EPAC1/Rap1 signaling pathway. Acting as an upstream initiating event, this restoration of mitochondrial function subsequently orchestrates a multicellular cascade that promotes anti-inflammatory polarization of astrocytes, supports neuronal survival, and facilitates endothelial angiogenesis, thereby driving the synergistic repair of the neurovascular unit after ischemic stroke. These findings extend the research scope of migrasome-enriched EVs to the context of ischemic stroke and reveal a novel mechanism of intercellular protection. As naturally derived, multi-targeted, and acellular therapeutic vehicles, migrasome-enriched EVs hold significant translational potential for ischemic stroke and broader neuro-inflammatory diseases.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12951-026-04643-4>.

Supplementary Material 1.

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Author contributions

Yuanjia Zhang: Writing—original draft, Methodology, Investigation, Formal analysis, Conceptualization. Jie Wu: Writing—original draft, Visualization, Methodology, Investigation. Leyi Liu: Investigation, Formal analysis, Methodology, Conceptualization. Yiqian Zhu: Visualization, Methodology. Jierui Zhang: Software, Data curation. Jinghong Xu: Visualization. Xuefeng Shi: Methodology. Yu Yu: Methodology. Chao Wang: Conceptualization. Jiajing Xiao: Methodology. Xuanchenye Zhang: Formal analysis. Zhaorong Li: Data curation. Wenqi Huang: Supervision, Funding acquisition. Wenzhi Li: Supervision, Conceptualization. Zhongxing Wang: Supervision, Project administration, Funding acquisition. Tingting Li: Supervision, Investigation, Project administration, Funding acquisition, Conceptualization.

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Data availability

Data are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Animal Experiment Ethics Committee of Sun Yat-sen University (SYSU-IACUC-2025-002457) in accordance with the National Institutes of Health guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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