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RESEARCH ARTICLE

Capturing Microglia-Derived Extracellular Vesicles in Acute Ischemic Stroke: A Bead-Based Flow Cytometry Approach

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Objective: Recent findings suggest that microglia, the resident immune cells of the central nervous system (CNS), respond to stimuli in the internal environment by dynamically changing their phenotype and releasing extracellular vesicles (EVs) that contribute to neurorepair and neuroprotection after stroke. We investigated the isolation of EVs secreted by microglia (MDEVs) from plasma samples of acute ischemic stroke (IS) patients.

Methods: EVs were isolated from patients' plasma at three key time points—24 hours, 7 days, and one month following symptom onset—using the ExoQuick® ULTRA EV precipitation kit. Subpopulations of MDEVs were purified based on common EV protein markers (CD81, CD63, and CD9 tetraspanins), as well as the transmembrane protein 119 (TMEM119), which specifically indicates microglia, through the Basic Exo-Flow Capture kit. The obtained antibody-coupled bead-associated particles were then labeled with Exo-FITC and analyzed using the BD FACSAria™ III flow cytometer.

Results: Flow cytometry analysis confirmed a pure and highly enriched MDEV suspension appropriate for a variety of downstream applications in future research. Fluorescein isothiocyanate (FITC) median fluorescence intensities (MFI) remained consistent across all evaluated post-stroke time points, indicating that similar amounts of EVs were recovered from each patient with uniform capture efficiency. However, compared to controls, FITC-MFIs were significantly higher in IS patients.

Conclusions: Studying EV populations is challenging due to their heterogeneity. This MDEV purification protocol may provide a basis for developing new, noninvasive methods for CNS monitoring, and could support future biomarker discovery for the diagnosis, prognosis, and treatment of IS.

Keywords: microglia-derived extracellular vesicles, flow cytometry, TMEM119, ischemic stroke

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Introduction

Despite advances in prevention and treatment, stroke remains a leading cause of death and disability globally [1,2]. Post-stroke pathophysiological changes, such as energy failure, oxidative stress, excitotoxicity, inflammation, hemostatic activation, and blood-brain barrier (BBB) disruption, lead to dysfunction or death of the affected neurons, resulting in secondary injury to more distant cells [3]. These notable changes are reflected in the neurovascular unit [4], a multicellular entity that maintains the homeostasis of the cerebral microenvironment through complex interactions among neurons, glial cells, and blood vessels [5].

Microglia, along with oligodendrocytes and astrocytes, form the three glial cell lines of the central nervous system (CNS) [6]. As the brain's resident immune cells, they are essential for responding to acute neurological injuries [7]. Following an ischemic insult, microglia rapidly proliferate and, even with peripheral macrophages infiltrating, they remain the primary cellular population within the lesion site [8]. Upon activation, microglia adopt an amoeboid phenotype, marked by the secretion of various inflammatory signals and neurotrophic factors, as well as phagocytosis of cellular debris [7,9]. Activated microglia have been considered detrimental during ischemic stroke (IS), as inhibiting their activation can reduce brain injury caused by ischemia. These cells can also contribute to disrupting the BBB and promote neuronal cell death. However, this process is complex and may also exert beneficial effects by enhancing neurogenesis, reducing neuronal apoptosis, and improving functional recovery following IS [10]. The adaptive nature of microglia allows them to shift between different phenotypes, with M1 mediating pro-inflammatory and neurotoxic responses, and M2 supporting anti-inflammatory and neuroprotective actions [11]. These different polarization states likely explain their biphasic role and function [10].

Researchers have proposed using circulating microglia-derived extracellular vesicles (MDEVs) as indicators of mi-

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croglial phenotypes [12]. Extracellular vesicles (EVs) are nano-sized, lipid-bilayer structures released by all cells into the extracellular space, serving as key mediators of intercellular communication [13]. These vesicles can be detected in various biofluids, are relatively stable, and can express surface markers and carry cargo, such as proteins, nucleic acids (e.g., mRNA, miRNA), and lipids, reflecting their cell of origin [14–17]. Moreover, they can cross the BBB, allowing the detection of EVs released by various brain cells into the peripheral circulation [18]. These unique qualities make EVs attractive candidate biomarkers in several conditions.

Surface proteins, including the microglia transmembrane protein 119 (TMEM119), CD11b, CD14, and CD68 [12,19] are expressed on MDEVs, enabling their detection in plasma using advanced, high-throughput techniques like nanoimaging or nanoflow cytometry. A study has shown that EVs released from pro-inflammatory microglia can be identified using these methods, and reported an increase in their circulating plasma levels in a preclinical model of stroke [20].

Our exploratory study investigated the isolation of the TMEM119-positive EV subpopulation, presumed MDEVs, from plasma samples of acute IS patients during the first month after symptom onset, using a combination of precipitation and bead-based flow cytometry. Although MDEVs are increasingly studied in preclinical stroke models, few clinical studies have explored their presence in patient populations. Detecting these vesicles in plasma could be an initial step toward developing new, non-invasive methods to monitor microglial activity in vivo.

Methods

Study Population

The study protocol, prepared in accordance with the principles outlined in the Declaration of Helsinki, was approved and annually renewed by the Ethics Committee for Scientific Research of the George Emil Palade University of Medicine, Pharmacy, Science, and Technology of Targu Mures (approval no. 1361/10.05.2021, 1721/28.04.2022, 2226/22.03.2023). Written informed consent was obtained from all patients or their family members prior to their participation. Participants were assigned codes, and their data were kept and managed anonymously.

A total of eighteen patients with middle cerebral artery stroke, including nine who received thrombolysis, were enrolled in this study in the acute phase, within one day of symptom onset. Participants were selected from the 1st and 2nd Neurology Clinics at the County Emergency Clinical Hospital of Targu Mures between December 2021 and May 2023. Exclusion criteria included hemorrhagic transformation of IS; intracranial hemorrhage; a history of stroke within 12 months prior to admission; stroke-mimic pathologies; patients undergoing mechanical thrombectomy; acute infections; and other severe health issues, such as malignancies. Stroke severity was determined using the National Institutes of Health Stroke Scale (NIHSS), a validated and reliable instrument for evaluating neurological impairment post-stroke [21]. Nine control subjects with no brain lesions, neurological disorders, or infectious or inflammatory diseases, and who had similar demographics and cardiovascular risk factors to the patient group, were also included. Peripheral blood sampling was performed at three intervals: 24 hours (D1), seven days (D7), and one month (M1) in patients, and once in control subjects. Data recorded for each patient included demographics, risk factors, clinical details, and computed tomography scans on D1 and D7 to exclude a hemorrhagic transformation.

Isolation and Characterization of EVs from Plasma

Whole blood samples were collected from participants in 10-ml K2-EDTA vacutainer tubes (#367525, Becton Dickinson) and processed within 2 hours of collection to obtain plasma. Centrifugation was performed at 4°C in a two-step procedure: an initial spin at 300 × g for 10 minutes, followed by a second spin at 2000 × g for 20 minutes to remove platelets, apoptotic bodies, and cell debris. The plasma was then frozen and stored at –80°C until downstream analyses were performed. After thawing, EVs were extracted from plasma samples using the ExoQuick® ULTRA (#EQULTRA-20A-1, System Biosciences) EV precipitation kit in accordance with the manufacturer's protocols [22]. Following the initial precipitation and resuspension of the EV pellet, a second purification step was performed using columns to reduce background interference and minimize contamination by proteins such as IgG and albumin.

Then, to evaluate the presence of EVs in plasma samples obtained from stroke patients and control subjects, we assessed the expression of tetraspanin surface markers (CD81, CD63, and CD9) using bead-based flow cytometry, a widely used approach for profiling EV surface proteins [23]. Tetraspanins are transmembrane proteins commonly used to identify EV populations regardless of their cellular origin [24]. We used the Basic Exo-Flow Capture kit (#CSFLOWBASICA-1, System Biosciences) to immunocapture tetraspanins on EVs following the manufacturer's protocols [25]. This method allows for the selective isolation of CD81+, CD63+, and CD9+ EVs on streptavidin-coated large magnetic beads conjugated with biotinylated antibodies that target these tetraspanins: anti-human CD81 (#130-122-217, Miltenyi Biotec); anti-human CD63 (#130-100-169, Miltenyi Biotec), and anti-human CD9 (#130-103-954, Miltenyi Biotec). After capturing, bead-associated particles were labeled with Exo-FITC, which comprises fluorescein isothiocyanate (FITC) conjugated to a protein that recognizes post-translational modifications (carbohydrate additions, glycosylation) on EV surface proteins. Beads with detection antibody alone, without captured EVs, served as negative controls. Sample acquisitions were conducted using the BD FACSAria III

cytometer (BD Biosciences), processed with BD FACSDiva™ v8.0 Software, and exported as ".csv" files. For each sample, data acquisition included at least 10,000 single-bead events. The FITC median fluorescence intensity (MFI) of each sample, normalized to the negative control, was used for statistical analysis. Our protocol has been described in detail previously [26,27].

Morphological characterization of EVs was performed using electron microscopy (EM), a technique capable of detecting and visualizing EVs regardless of their size [23]. EV suspensions were fixed with glutaraldehyde at a 1:1 ratio, resulting in a final concentration of 2.5%. A 5 µL aliquot of the fixed preparation was applied to Formvar-carbon-coated grids, followed by observation and imaging under a Hitachi HD-2700 scanning transmission electron microscope (Hitachi STEM High-Technologies Corporation, Japan) operated at 200 kV. Images were captured at magnifications of 50,000x, 200,000x, and 400,000x, without contrast enhancement.

Purification of TMEM119+ EVs

The specific capture of EVs expressing TMEM119, a marker for microglia, was conducted according to the previously established purification protocol using the Basic Exo-Flow Capture kit. Exo-Flow beads were incubated with biotinylated anti-TMEM119 antibody (#FAB10313B, Novus Biologicals) to isolate the presumed MDEV subpopulation. Sample acquisitions were conducted using the BD FACSAria III cytometer (BD Biosciences), and data were analyzed with the BD FACSDiva™ v8.0 Software. After flow sorting, bead–antibody–EVs–FITC complexes were treated with Exosome Elution Buffer to eliminate the fluorescent label, yielding a supernatant enriched with eluted, intact TMEM119+ EVs.

Statistical Analyses

Data were analyzed using GraphPad® Prism Software version 10.3.0. Descriptive data are presented as mean ± standard deviation (SD) or median with interquartile range (IQR, Q1-Q3). Differences between patients and controls were assessed with the nonparametric Mann–Whitney U test. To evaluate whether the number of EVs captured on beads (FITC-MFIs) varied among patients during follow-up, the nonparametric Friedman's ANOVA test was used. A p-value of < 0.05 was considered statistically significant.

Results

Study Population

The study included 18 patients with acute IS and 9 control individuals. The mean age of patients was 66 ± 7.5 years, and 65 ± 7 years for control subjects at the time of study inclusion. Eight patients and four controls were females. All patients had hypertension; nine had diabetes mellitus; four had hyperlipidemia; three had atrial fibrillation, and three were smokers. The median stroke severity was 7.5 at D1, 5.5 at D7, and 2 at M1, according to the NIHSS. Fourteen strokes were caused by large artery atherosclerosis, four were cardioembolic, and none resulted from small artery occlusion or other etiologies according to the TOAST classification. Eleven patients had left-sided strokes. Nine patients received intravenous thrombolysis. A total of twelve samples were collected at one month, as six patients could not attend the one-month follow-up due to reasons such as SARS-CoV-2 infection, stroke reinfarction, or transfer to other rehabilitation facilities.

Bead-based flow cytometry revealed the expression of tetraspanins on EV surfaces

To assess the efficiency of the EV isolation protocol, we analyzed the surface expression of CD81, CD63, and CD9 on purified EVs using flow cytometry. Tetraspanin+ EVs exhibited higher FITC-MFIs during acquisitions. Forward scatter (FSC) vs. FITC intensity plots indicated that the no-EVs buffer control displayed only a minimal presence of FITC-positive complexes, while nearly all particles in the EV samples from patients and controls were FITC-positive. The differences between the buffer-only control and the study population samples arise from statistical variability in fluorescence measurements, which amplifies as more tetraspanin+ EVs are captured on magnetic beads and labeled with Exo-FITC. The histograms show the level of separation between the negative and positive bead–EV complexes in FITC fluorescence intensities (Figure 1).

Electron microscopy revealed EV morphology and integrity

The EM enabled nanometer-scale visualization of the isolated EVs, providing detailed insights into their size and morphology. The observed particles were round, ovoid, or cup-shaped, with diameters ranging between 30 and 1000 nm, which corresponds to EV sizes. These vesicles had a well-defined outer boundary, although differences in internal contents and membrane thickness led to variations in contrast (Figure 2).

Bead-based flow cytometry detected the MDEV subpopulation

To obtain EVs derived from microglia, the surface expression of the microglial marker TMEM119 was analyzed on the previously isolated EVs using bead-based flow cytometry. TMEM119+ EVs exhibited an increase in FITC-MFIs during analysis. Plots of FSC vs. FITC intensity revealed very few FITC-positive complexes in the no EVs buffer control, whereas almost all particles in EV aliquots from both patients and controls were FITC-positive. The differences between the study samples and buffer control can be attributed to variability in fluorescence signal intensity, which tends to increase proportionally with the number of bead-bound, stained TMEM119+ EVs. The histograms demonstrate clear separation between negative and positive complexes based on their FITC fluorescence intensi-

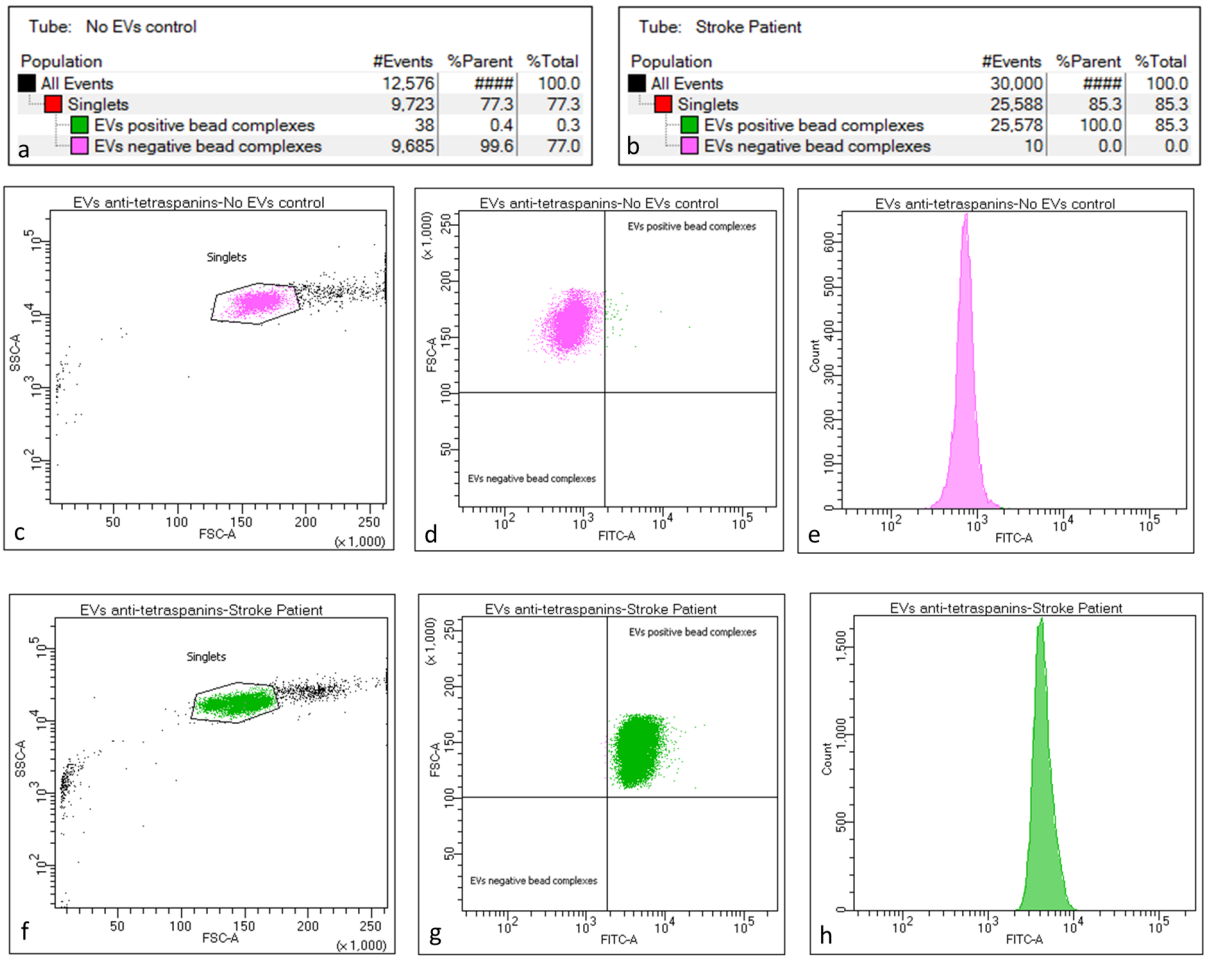
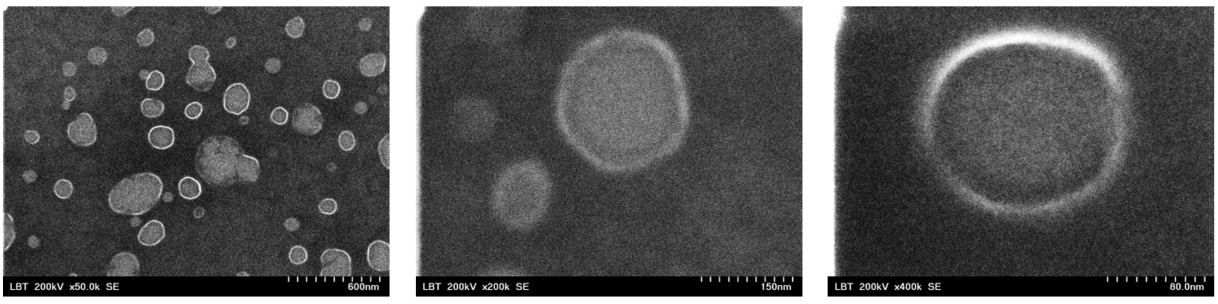


Figure 1. EVs negative and positive capture bead complexes from the buffer-only control (a, c–e) and from a stroke patient (b, f–h), coupled with anti-CD81, anti-CD63, and anti-CD9 antibodies.



a b c

Figure 2. Representative scanning EM (SE) overview of EV suspensions obtained at magnifications 50,000x (a); 200,000x (b); 400,000x (c).

ties. The analysis revealed a pure, abundant suspension of TMEM119+ EVs, indicating successful isolation of the presumed MDEV subpopulation and suitability for further cargo analysis (Figure 3).

We also measured the amount of MDEVs captured on magnetic beads by analyzing FITC-MFIs in the flow cytometer. The same plasma volume was used from each subject to isolate EVs. Under these conditions, similar FITC-MFIs were observed at different time points in stroke patients (n = 12; p = 0.916), but significantly higher ones compared to controls [median (IQR) D1, n = 18: 3915.5 (2879–6129) vs. 949 (0–1623), p = 0.003; D7, n = 18: 2812 (1790–5978) vs. 949 (0–1623), p = 0.001; M1, n = 12: 2474 (1734.5–7713.5) vs. 949 (0–1623), p = 0.0009].

Discussions

In this study, we identified presumed MDEVs in plasma samples from eighteen stroke patients and nine control subjects, demonstrating the feasibility of detecting TMEM119-positive EVs after stroke.

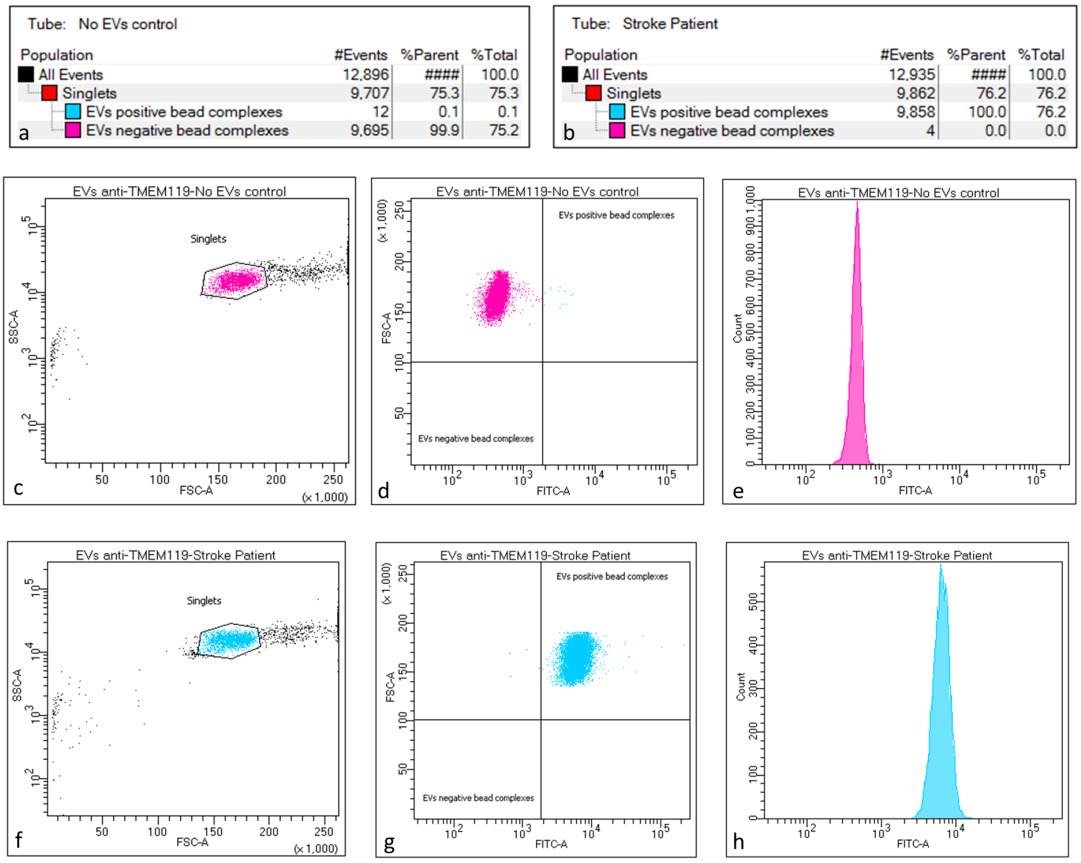


Figure 3. EVs negative and positive capture bead complexes from the buffer-only control (a, c–e) and from a stroke patient (b, f–h), coupled with anti-TMEM119 antibody.

The small size and heterogeneity of EVs present significant challenges for their characterization and subsequent analyses [28]. Several techniques have been developed to isolate EVs from various samples using different principles. Traditional methods include commonly used ultracentrifugation or density gradient ultracentrifugation, and its alternatives: size exclusion liquid chromatography, polymer-based precipitation, immune-affinity capture, and filtration [29]. Recently, many novel isolation techniques have emerged due to advancements in nanomaterials and microfluidic chips. These include microfluidic technologies such as deterministic lateral displacement, asymmetric-flow field-flow fractionation, dielectrophoresis, non-contact microfluidics, and acoustic fractionation, along with other approaches, such as capture by centrifugal microfluidic disc system (Exo-CMDS), ultra-fast exosome isolation system (EXODUS), and 3D ZnO nanoarrays. Another group includes immunological methods such as lipid microarrays and immunomagnetic bead capture using synthetic peptide Vn96 or hedgehog particles, Exosome Separation and Detection (ExoSD) chip, and 3D paper-based sEV isolation system. Additionally, EVs can be captured by covalent chemistry and other strategies [30]. Despite the variety of available methods, a standardized approach remains elusive. We used the ExoQuick® ULTRA, a commercially available polymer-based precipitation method, to purify EVs regardless of their cellular origin. This technique offers a high particle recovery rate, requiring minimal sample volume [31] and includes a second purification step using columns to reduce background signals and decrease IgG and albumin content in the resuspended EV pellet, enabling a biochemically cleaner evaluation of these nanovesicles in subsequent steps.

Researchers have previously isolated CNS-derived EVs, detecting unique surface proteins of specific cells using immunoassays [32]. This discovery has generated considerable interest in evaluating brain-derived EVs from peripheral blood to gain insights into the brain's pathophysiological changes, which are otherwise inaccessible in living humans. EVs can freely pass the BBB, enabling them to deliver their cargo into peripheral circulation [33]. We opted for immunocapture of MDEVs using the Basic Exo-Flow Capture kit, targeting TMEM119, a specific marker for microglia. According to previous literature, TMEM119 is expressed on all resting microglia, making it a distinct and consistent marker for distinguishing resident microglia from infiltrating peripheral macrophages in the human brain. During microglial activation, TMEM119 gene expres-

sion is reduced, although the protein expression remains preserved on the cell surface [34,35]. Roseborough et al. reported plasma-based detection of dual-labeled activated MDEVs in an experimental stroke model. TMEM119+/CD14+ and TMEM119+/MHC-II+ vesicle populations showed increased levels at 28 days, whereas no significant changes were observed at 7 days post-stroke [20]. In addition, MDEVs carrying fibrinogen are also detectable in peripheral circulation, with elevated TMEM119+/fibrinogen+ EVs at 28 days post-stroke [36].

We analyzed the FITC-MFIs to quantify the amount of EVs captured on magnetic Exo-Flow beads. Since signals derive from multiple particles bound to individual beads, variations in signal intensity may indicate relative abundances of EV subsets, or differences in particle concentration, diameter distributions, or epitope densities [23]. We observed comparable FITC-MFIs at different time points in patients, indicating that the beads have reached their saturation capacity for binding. This suggests that the FITC-MFIs are determined by the beads' capturing efficiency rather than the plasma EV concentration, as excess EVs are washed out during the protocol. As a result, we could capture an equal amount of EVs from each patient with consistent capture capabilities indispensable for downstream analyses. In addition, IS patients showed higher FITC-MFIs compared to controls. However, TMEM119 has also been reported to be mainly degraded or cleaved during microglial activation [37] or to be reduced in ramified microglia proximal to the ischemic injury site, 24 hours following the insult [38]. Using TMEM119 in combination with other relevant microglial surface markers may facilitate the identification of microglial subpopulations in flow cytometry analysis [37].

We acknowledge that this study has certain limitations due to its methodological focus. We identified presumed MDEVs in a relatively small sample, with fewer available at 1 month, and did not conduct correlation analyses with clinical parameters (e.g., stroke severity, imaging results, or outcome), so the clinical significance remains preliminary. TMEM119 distinguishes microglia from infiltrating peripheral macrophages and monocytes. Prioritizing methodological feasibility, we used it as a single marker for the initial capture of MDEVs. In the setting of stroke, the cellular milieu is markedly altered by increased infiltration of peripheral myeloid cells expressing canonical markers (e.g., CD68, Iba1) [39]. Although TMEM119 retains comparatively high specificity for microglia, its expression may be modulated under inflammatory conditions. Further integration of multiple markers alongside characterization of MDEV cargo would more accurately define microglial subpopulations, particularly in the acute phase of stroke.

Conclusion

Analyzing EV populations is challenging due to their heterogeneity. This MDEV purification protocol could serve as an initial step toward exploring a noninvasive approach, offering perspectives on directly and peripherally monitoring CNS signals in vivo and opening new possibilities for applying liquid biopsy techniques in stroke patient care. Future studies incorporating larger cohorts, multimarker strategies, and integration with clinical data are needed to strengthen the biological interpretation and translational significance of MDEVs in IS.

Abbreviations

BBB – blood-brain barrier
CNS – central nervous system
EM – electron microscopy
EVs – extracellular vesicles
FCS – forward scatter
FITC – fluorescein isothiocyanate
IQR – interquartile range
IS – ischemic stroke
MDEVs – microglia-derived extracellular vesicles
MFI – median fluorescence intensity
NIHSS – National Institutes of Health Stroke Scale
SD – standard deviation
TMEM119 – transmembrane protein 119

Authors' contributions

TF (Conceptualization; Formal analysis; Funding acquisition; Investigation; Methodology; Resources; Visualization; Writing – original draft; Writing – review & editing)

DRM (Data Curation; Investigation; Methodology; Resources; Validation; Writing – review & editing)

RB (Conceptualization; Supervision; Validation; Writing – review & editing)

Conflict of interest

None to declare.

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