Stroke

BRIEF REPORT

Postischemic Neuroprotection Associated With Anti-Inflammatory Effects by Mesenchymal Stromal Cell-Derived Small Extracellular Vesicles in Aged Mice

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BACKGROUND AND PURPOSE: Small extracellular vesicles (sEVs) obtained from mesenchymal stromal cells (MSCs) were shown to induce ischemic neuroprotection in mice by modulating the brain infiltration of leukocytes and, specifically polymorphonuclear neutrophils. So far, effects of MSC-sEVs were only studied in young ischemic rodents. We herein examined the effects of MSC-sEVs in aged mice.

METHODS: Male and female C57Bl6/j mice (8–10 weeks or 15–24 months) were exposed to transient intraluminal middle cerebral artery occlusion. Vehicle or sEVs (equivalent of 2×10^6 MSCs) were intravenously administered. Neurological deficits, ischemic injury, blood-brain barrier integrity, brain leukocyte infiltration, and blood leukocyte responses were evaluated over up to 7 days.

RESULTS: MSC-sEV delivery reduced neurological deficits, infarct volume, brain edema, and neuronal injury in young and aged mice of both sexes, when delivered immediately postreperfusion or with 6 hours delay. MSC-sEVs decreased leukocyte and specifically polymorphonuclear neutrophil, monocyte, and macrophage infiltrates in ischemic brains of aged mice. In peripheral blood, the number of monocytes and activated T cells was significantly reduced by MSC-sEVs.

CONCLUSIONS: MSC-sEVs induce postischemic neuroprotection and anti-inflammation in aged mice.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: exosomes ■ inflammation ■ ischemic stroke ■ macrophages ■ neutrophils

Small extracellular vesicles (sEVs) including exosomes (70–150 nm) mediate complex signaling between cells.[1] Derived from the right cell type, sEVs can promote neurological recovery, neuronal survival, and brain remodeling.[2–5] We have shown recently that mesenchymal stromal cell (MSC)-derived sEVs induce postischemic neuroprotection by modulating brain leukocyte and, specifically, polymorphonuclear neutrophil (PMN) infiltrates in mice.[6] So far, studies using MSC-sEVs have been performed in young rodents. Ischemic stroke mostly affects old individuals. The efficacy of MSC-sEVs in aged rodents was not explored. In a head-to-head comparison, we compared the effects of MSC-sEVs in young (8–10-week-old) and aged (15–24-month-old) mice exposed to intraluminal middle cerebral artery occlusion (MCAO).

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Nonstandard Abbreviations and Acronyms

ICAM-1	intercellular cell adhesion molecule-1
MCAO	middle cerebral artery occlusion
MSC	mesenchymal stromal cell
NeuN	neuronal nuclear antigen
PMN	polymorphonuclear neutrophil
sEV	small extracellular vesicle

METHODS

Detailed data that support the findings of this study are available from the corresponding author upon reasonable request. Experiments were performed with local government approval in accordance to E.U. directive 2010/63/EU and local institutional guidelines. Experimental details, including sample size calculation, randomization, blinding, inclusion, and exclusion criteria, and dropouts are reported in the Materials and Methods in the Supplemental Material.

MSCs were raised from healthy human bone marrow.[6] sEVs were harvested from conditioned media using polyethylene glycol-6000 precipitation followed by ultracentrifugation.[6] MSC-sEVs were characterized according to International Society of Extracellular Vesicles recommendations.[7] The sEV particle concentration, size, protein content, and the presence of exosome markers (CD9, CD63, CD81) were determined as previously described.[6] MSC and MSC-sEV characteristics are presented in Figures S1 and S2 and Table S1 and S2.

Thirty minutes MCAO was induced in young (8–10 weeks) or aged (15–24 months) male or female C57BL6/j mice, as outlined in Figure S3.[6] Laser Doppler flow was recorded

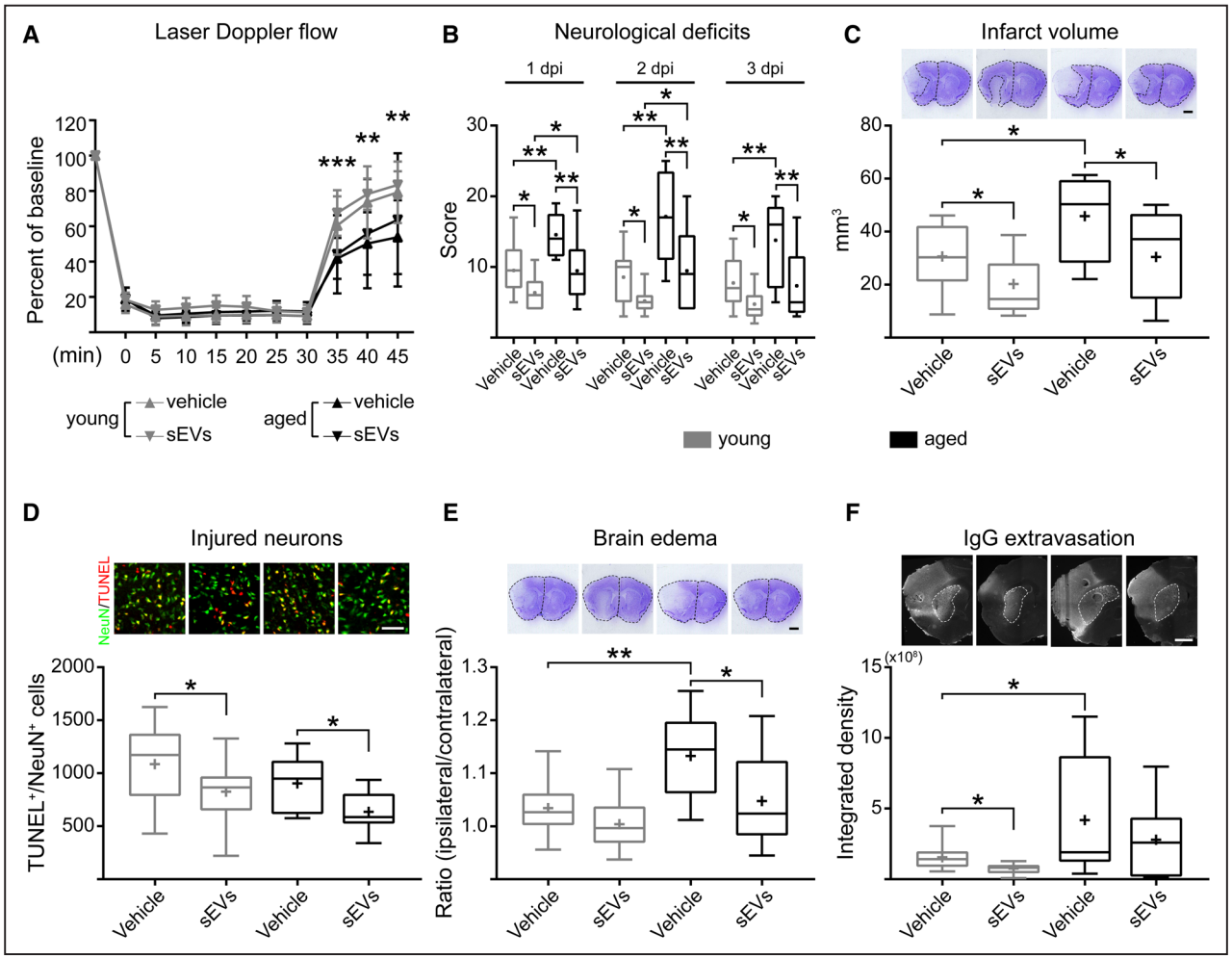

Figure 1. Mesenchymal stromal cell (MSC)-small extracellular vesicles (sEVs) reduce postischemic neurological deficits and induce neuroprotection in young and aged mice.

A, Laser Doppler flow (LDF), (**B**) neurological deficits, (**C**) infarct volume evaluated by cresyl violet staining, (**D**) neuronal injury in the ischemic striatum assessed by TUNEL/NeuN histochemistry, (**E**) brain edema examined by cresyl violet staining, and (**F**) blood-brain barrier breakdown determined by IgG extravasation in young and aged male mice exposed to 30 min intraluminal middle cerebral artery occlusion (MCAO). Vehicle or MSC-sEVs (2×10^6 cell equivalents) were intravenously administered immediately after reperfusion. Animals were sacrificed at 72 h post-MCAO. Representative brain sections are shown. Note the more severe brain injury associated with reduced reperfusion and exacerbated blood-brain barrier breakdown in aged compared with young mice. Data are means±SD (in **A**) or box blots with medians (lines inside boxes)/means (crosses inside boxes)±interquartile ranges with minimum and maximum values as whiskers (in **B**–**F**). *P<0.05/**P<0.01/***P<0.001 (n=9–11 animals/group). Scale bars: 1 mm (in **C**, **E**, and **F**)/50 µm (in **D**).

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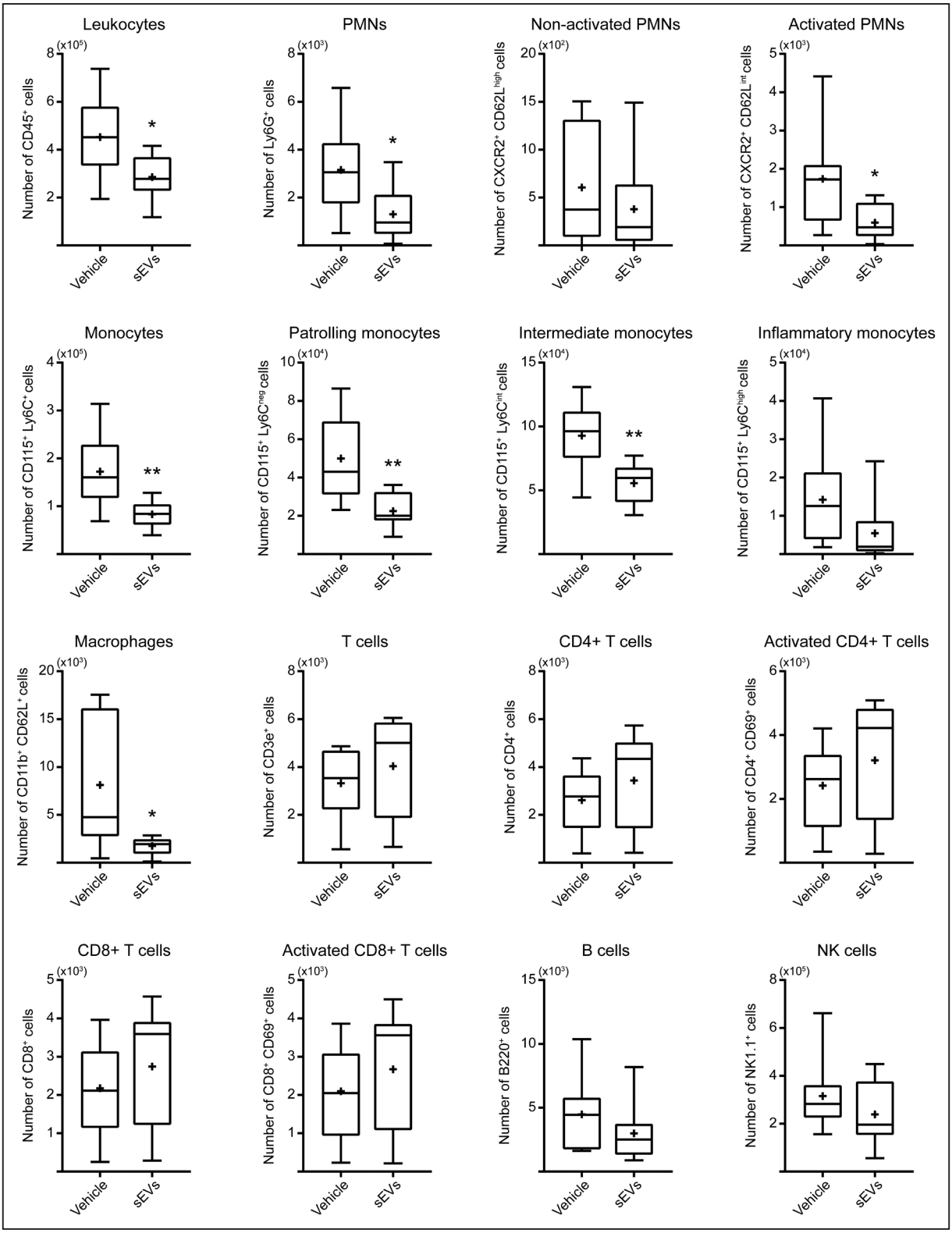

Figure 2. Mesenchymal stromal cell (MSC)-small extracellular vesicles (sEVs) reduce leukocyte and specifically polymorphonuclear neutrophil (PMN), monocyte, and macrophage infiltrates in the ischemic brain of aged mice.
Total counts of leukocytes and leukocyte subsets in the brains of aged male mice exposed to intraluminal middle cerebral artery occlusion (MCAO) evaluated by flow cytometry. Vehicle or sEVs (2×10^6 cell equivalents) were intravenously applied immediately after reperfusion. Animals were sacrificed at 72 h post-MCAO. Data are box blots with medians (lines inside boxes)/means (crosses inside boxes)±interquartile ranges with minimum and maximum values as whiskers. **P*<0.05/***P*<0.01 (n=8–9 animals/group).

above the middle cerebral artery territory.[6] Immediately after reperfusion or with 6 hours delay, 200 µL vehicle (normal saline) or MSC-sEVs (equivalent of 2×10^6 MSCs, in normal saline) were intravenously administered.[6] Neurological deficits were evaluated using the Clark score.[6] Blood samples were obtained by cardiac puncture. Mice were euthanized after 72 hours or 7 days.

Edema-corrected infarct volume and brain edema were determined on 20-µm-thick coronal brain sections. Sections from the bregma level were immunolabeled for NeuN (neuronal nuclear antigen), extravasated serum IgG, the adhesion molecule ICAM-1 (intercellular cell adhesion molecule-1), the endothelial marker CD31 (cluster of differentiation-31), the pan-leukocyte marker CD45, the PMN marker Ly6G, and the T cell marker CD3 (see the Supplemental Material for details). NeuN stainings were processed for terminal transferase-mediated dUTP-nick end labeling (TUNEL). Brains and blood samples were analyzed by flow cytometry. The antibody cocktails and gating strategy are summarized in Table S3 and Figure S4.

Data were analyzed by 2-way repeated measurement (longitudinal analyses) or 2-way (cross-sectional analyses) ANOVA followed by 2-tailed *t* tests. For statistical analysis, SPSS22.0 (IBM, Armonk, NY) was used. $P<0.05$ were considered significant.

RESULTS

Laser Doppler flow decreased to ≈10% of baseline during MCAO in all groups, followed by laser Doppler flow recovery post-MCAO to 81.1±13.7% and 58.5±29.6%, respectively, of baseline in young and aged male vehicle-treated mice (Figure 1A). Laser Doppler flow was not influenced by MSC-sEVs in both sexes (Figure 1A; Figure S5A). MCAO induced reproducible neurological deficits and brain infarcts, which were more severe in aged than young male mice (Figure 1B through 1D). Neurological deficits, infarct volume, and the number of DNA-fragmented (ie, irreversibly injured) $TUNEL^+/NeuN^+$ neurons and $TUNEL^+$ cells in the ischemic striatum were reduced by MSC-sEVs in young and aged male and female mice, both when sEVs were administered immediately after reperfusion (Figure 1B through 1D; Figures S5B, S5C, and S6A) or with 6 hours delay (Figure SVIIB and SVIIC). Hence, MSC-sEVs decreased infarct volume at 3 days poststroke by 34.0%, 33.6%, and 36.1% in young male, aged male, and aged female mice, respectively, when sEVs were administered immediately after reperfusion. MSC-sEVs decreased brain edema and ICAM-1 abundance, but not IgG extravasation on ischemic microvessels of aged male mice, which was not significant in young male and aged female mice (Figure 1E and 1F; Figure S5D and S6B). Total leukocytes, PMNs (including activated PMNs), monocytes (both patrolling and intermediate monocytes), and macrophages were reduced by MSC-sEVs in ischemic brains of aged mice (Figure 2; Figure S8). Peripheral blood leukocytes were higher in young than aged vehicle-treated mice, and blood $Ly6G^+$ PMNs higher in aged than young mice (Figure S9). MSC-sEVs reduced blood $CD45^+$ leukocytes, which was significant in young mice, and decreased blood monocytes and activated T cells in aged mice.

DISCUSSION

We show that MSC-sEVs very similarly induce postischemic neuroprotection and functional neurological improvements in young and aged male and female mice, when administered immediately or 6 hours post-MCAO. Brain leukocyte, including PMN, monocyte, and macrophage infiltrates were reduced by MSC-sEVs in aged mice. Ischemic injury was more severe in aged than young mice. Until now, MSC-sEV effects on stroke outcome in rodents have been studied in middle-aged (12-month-old) mice.[8] Perhaps due to differences in experimental protocols or MSC properties (MSCs were raised from embyonic stem cells), no neuroprotective effects were noted.[8] In good agreement with the present and our previous[3,6] studies, MSC-EVs were found to modulate brain immune responses and to enhance fine motor recovery in aged (16–26-year-old) Rhesus monkeys exposed to cortical cold injury.[9] The combined evidence of this previous[9] and the present rodent study encourages further proof-of-concept studies evaluating the efficacy of MSC-sEVs in clinic-relevant stroke settings.

ARTICLE INFORMATION

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Disclosures

Drs Hermann, Giebel, and Doeppner hold a patent on extracellular vesicles for the treatment of inflammatory conditions (US9877989B2). Dr Giebel is advisory board member of Mursla Ltd and Innovex Therapeutics SL and founding director of Exosla. The other authors report no conflicts.

Supplemental Material

Supplemental Materials and Methods
Figures S1–S9
Table S1–S5
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