Selectin-Mediated Recruitment of Bone Marrow Stromal Cells in the Postischemic Cerebral Microvasculature

Gokhan Yilmaz, MD, PhD; Shantel Vital, MS; Cigdem Erkuran Yilmaz, MD, PhD; Karen Y. Stokes, PhD; J. Steven Alexander, PhD; D. Neil Granger, PhD

Background and Purpose—The therapeutic potential of bone marrow stromal cells (BMSCs) has been demonstrated in different models of stroke. Although it is well established that BMSCs selectively migrate to the site of brain injury, the mechanisms underlying this process are poorly understood. This study addresses the hypothesis that selectins mediate the recruitment of BMSCs into the postischemic cerebral microvasculature.

Methods—Focal ischemic stroke was induced by middle cerebral artery occlusion and reperfusion. Cell recruitment was monitored using either fluorescent- or radiolabeled BMSCs detected by intravital microscopy or tissue radioactivity. Mice were treated with either a blocking antibody against P- or E-selectin or with the nonselective selectin antagonist, fucoidin. The role of CD44 in cell recruitment was evaluated using BMSCs from CD44 knockout mice.

Results—Middle cerebral artery occlusion and reperfusion was associated with a significantly increased adhesion of BMSCs in cerebral venules compared with sham mice. Immunoneutralization of either E- or P-selectin blocked the middle cerebral artery occlusion and reperfusion-induced recruitment of adherent BMSCs. An attenuated recruitment response in the postischemic hemisphere was also noted after fucoidin treatment or administration of CD44-deficient BMSCs.

Conclusions—Cerebral vascular endothelium assume a proadhesive phenotype after ischemic stroke that favors the recruitment of BMSCs, which use both P- and E-selectin to home into the infarct site. CD44 may serve as the critical ligand for selectin-mediated BMSC recruitment. *(Stroke. 2011;42:806-811.)*

Key Words: acute stroke ■ adhesion molecules ■ focal ischemia ■ inflammation ■ stem cells

Stroke, the second most common cause of death worldwide and a major cause of disability, is expected to realize a 30% increase in incidence over the next decade. Despite the severity and growing incidence of this disease, few therapeutic options are available to medical professionals who care for patients with stroke. The only effective therapeutics available at present are directed toward rapid vessel recanalization or symptomatic relief. Although most drugs that have reduced tissue injury and enhanced functional recovery in animal models of ischemic stroke have not yielded similar therapeutic benefit in the clinical setting, the search for agents or interventions that confer protection against ischemic stroke continues to gain momentum. In this regard, one of the most promising therapeutic strategies involves the local or systemic administration of stem cells derived from either the hematopoietic or central nervous systems.

The therapeutic potential of exogenously administered bone marrow stromal cells (BMSCs) has been demonstrated in several experimental models of stroke,1,2 experimental autoimmune encephalitis,3 and in patients with an ischemic stroke.4 Studies in mice, rats, and primates have shown that intravenously administered BMSCs are able to home into different tissues (eg, bone marrow,5 lungs,6,7 liver and spleen8). Furthermore, there is evidence that BMSCs will selectively home and migrate into injured tissues, including the brain.8,9 These observations suggest that BMSCs may engage specific adhesion receptors that allow for the selective recruitment and infiltration of the regenerative cells into injured tissue.

Although the adhesion receptors that mediate BMSC recruitment into the brain after ischemic stroke remain unknown, there are several lines of evidence that suggest a potential role for the selectins. Under shear flow, BMSCs have been shown to display P-selectin-dependent rolling and adhesion on endothelial cells in vitro as well as P-selectin-dependent rolling and adhesion into murine skin venules in vivo.10 A similar mechanism was suggested for the homing of

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CD44 expressing human BMSCs into specialized bone marrow microvessels that constitutively express E-selectin on endothelial cells.11 Studies of the cerebral microvascular responses to ischemic stroke have also implicated the selectins. The postischemic cerebral microvasculature expresses high levels of E- and P-selectin12–14 and recruits large numbers of leukocytes and platelets.15 P-selectin has been shown to mediate the enhanced rolling and firm adhesion of both leukocytes and platelets in postischemic cerebral venules and to contribute to the tissue injury associated with focal ischemia–reperfusion.16 E-selectin has also been implicated in the inflammation and tissue injury associated with ischemic stroke.17

BMSCs derived from H-2Kb-tsA58 transgenic mice18 are conditionally immortalized and are an attractive alternative source to BMSCs isolated from wild-type mice without the limitation of passage number, low yield, and spontaneous differentiation.19,20 In our study, large-T-carrying immortal cells were used as a ubiquitous source of BMSCs to compare their adhesive properties in ischemic stroke with the responses produced by BMSCs derived from wild-type mice.

In the present study, an established murine model of transient focal cerebral ischemia was used to address the following objectives: (1) to determine whether BMSCs adhere in cerebral microvessels after ischemia/reperfusion (I/R); and (2) to assess the contributions of E- and P-selectin to the I/R-induced recruitment of BMSCs in the cerebral microvasculature. Our findings implicate both endothelial cell selectins in the I/R-induced stem cell recruitment and suggest that CD44 may serve as the ligand on stem cells that adheres in cerebral microvessels after ischemia–reperfusion.17 Although the difference between the responses to E- and P-selectin on the BMSC recruitment response elicited by cerebral I/R. The postischemic cerebral microvasculature expresses high levels of E- and P-selectin12–14 and recruits large numbers of leukocytes and platelets.15 P-selectin has been shown to mediate the enhanced rolling and firm adhesion of both leukocytes and platelets in postischemic cerebral venules and to contribute to the tissue injury associated with focal ischemia–reperfusion.16 E-selectin has also been implicated in the inflammation and tissue injury associated with ischemic stroke.17

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**Methods**

**Animals**

All in vivo experiments were performed on C57Bl/6J male mice (WT; 6 to 8 weeks old; Jackson Laboratories, Bar Harbor, ME). BMSCs were isolated from H-2Kb-tsA58 mice expressing the temperature-sensitive SV40 large-T antigen (large T; CBA/ca X C57Bl/10 hybrid; Charles River Laboratories, Wilmington, MA), CD44 knockout mice (B6.Cg-Cd44tm1Hbg/J, Jackson Laboratories), or from WT mice. The experimental procedures were approved by the Louisiana State University Institutional Animal Care and Use Committee and were in compliance with the guidelines of the National Institutes of Health.

**Middle Cerebral Artery Occlusion and Reperfusion**

Transient (60 minutes) focal cerebral ischemia was induced by occlusion of the left middle cerebral artery using an intraluminal filament method.21 At the end of experiments, the production of an infarct was confirmed by 2,3,5-triphenyltetrazolium chloride.

**Intravitral Videomicroscopy**

Mice were randomly allocated into experimental groups for intravitral videomicroscopic evaluation of BMSC adhesion in postischemic cerebral venules. Sham and middle cerebral artery occlusion and reperfusion mice receiving (5 minutes before administration of BMSCs) neutralizing antibodies against either E-selectin or P-selectin or isotype-matched control were anesthetized after a 24-hour reperfusion period. BMSCs (8×10⁶) were infused intravenously. The interaction of fluorescently labeled cells with cerebral microcirculation was observed through a cranial window.21 The intravitral microscopy experiments were not conducted in a blinded fashion. BMSC adhesion (stationary for ≥30 seconds) was expressed as the number of cells per 1 mm² venular surface, calculated from the diameter and length, assuming a cylindrical geometry.

**BMSC Isolation and Labeling**

BMSCs were cultured from WT, large-T, or CD44 knockout mice as previously described.5,20 BMSCs were labeled by carboxyfluorescein-diacetate-succinimidyl-ester. A total of 8×10⁶ viable BMSCs in 150 µL of phosphate-buffered saline (phosphate-buffered saline in controls) were injected into the mice 10 minutes before obtaining estimates of cell adhesion through videomicroscopy. Flow cytometry was used to detect the expression of P-, E-, and L-selectins; P-selectin glycoprotein ligand-1; CD24; CD43; and CD44 on large-T BMSCs in their naïve state or after incubation with ischemic brain extracts for 15 minutes.

**51Cr-Labeling of BMSCs**

BMSCs were radioactively labeled by Na⁵¹CrO₄. In WT mice, 2×10⁶ ⁵¹Cr-labeled BMSCs were injected intravenously 24 hours after either sham surgery or middle cerebral artery occlusion and reperfusion with or without treatment with the pan-selectin blocker fucoidin (30 mg/kg intravenously once a day for 3 days). After 72 hours, brain ⁵¹Cr activity per gram of tissue was determined by a gamma counter.

**Statistical Analysis**

All results are expressed as mean±SEM. Statistical comparisons were made with a one-way analysis of variance followed by a Tukey-Kramer post hoc analysis. Statistical significance was assessed at P<0.05. See http://stroke.ahajournals.org for detailed methods.

**Results**

**BMSCs Adhere to Cerebral Microvessels After Focal Cerebral I/R Through Both E- and P-Selectins**

BMSCs isolated from large-T mice that were administered (intravenously) 24 hours after ischemic stroke were observed to firmly adhere in cerebral venules (Figure 1C; 144±17.9 cells/mm², n=8) In contrast, BMSCs adhered in venules of sham-operated mice in very low numbers (Figure 1A–B; 15±7.4 cells/mm², n=8). Selectin immunoblockade, using antibodies directed against either E- or P-selectin administered before injection of BMSCs, significantly decreased the number of adherent BMSCs in cerebral microvessels (46±6.3 cells/mm², n=7 and 32±4.5 cells/mm², n=6, respectively; Figure 2). Isotype matched (control) antibodies had no impact on the BMSC recruitment response elicited by cerebral I/R. Although the difference between the responses to E- and P-selectin blockade were not statistically significant, blocking P-selectin decreased the number of adherent BMSCs more dramatically than E-selectin blockade (32±4.5 versus 46±6.2 cells/mm²). In a separate series of experiments (Figure 3), focal cerebral I/R elicited a much larger accumulation of radioabeled BMSCs in the ischemic (left hemisphere) brain (1174.9±114.3 cells/g tissue, n=3) compared with sham controls (189.3±84.9 cells/g tissue, n=3). The I/R-induced recruitment of radioabeled BMSCs was largely prevented in mice treated (for 3 days) with the pan-selectin blocker fucoidin (392.7±117.9 cells/g tissue), providing additional support for a role for selectins in mediating the stem cell recruitment response.
Large-T BMSCs Exhibit Similar Properties to WT BMSCs After Focal Cerebral I/R

A comparison of the large-T and WT BMSC recruitment responses in cerebral venules of sham mice and mice exposed to focal I/R (Figure 1A) revealed no differences in the numbers of adherent WT cells or large-T cells under both sham (21 ± 21.2 cells/mm², n = 3 versus 15 ± 7.5, n = 4, respectively) and postischemic conditions (122 ± 21.2 cells/mm², n = 4 versus 144 ± 17.9 cells/mm², n = 8, respectively). In addition, neither WT cells nor large-T cells reduced infarct size in stroke mice (29.8% ± 6.2%, n = 6 stroke mice versus 27.6% ± 3.74%, n = 5 WT BMSC treatment and 31.5% ± 3.84%, n = 6 large-T treatment). See http://stroke.ahajournals.org for supplemental data.

CD44 Expressed on BMSCS Contributes to the Recruitment of These Cells in the Postischemic Brain

Because BMSCs appear to use both E- and P-selectins for adhesion in cerebral venules subjected to focal I/R, we used
Surface expression of selectin ligands CD24 and CD44 on BMSCs under basal conditions and after incubation with ischemic brain extract (I/R brain). Values represent duplicate measurements from the same sample.

Figure 4. Surface expression of selectin ligands CD24 and CD44 on BMSCs under basal conditions and after incubation with ischemic brain extract (I/R brain). Values represent duplicate measurements from the same sample.

flow cytometry to identify potential selectin ligands that are expressed on BMSCs. The surface expression of P-selectin, L-selectin, P-selectin glycoprotein ligand-1, CD24, CD43, and CD44 was examined on BMSCs. Under basal conditions, BMSCs did not express CD43, P-selectin, L-selectin, or P-selectin glycoprotein ligand-1 (see http://stroke.ahajournals.org for supplemental information). However, expression of CD24 (5.1%) and CD44 (11.8%) were detected. Incubation of BMSCs with ischemic brain extract for 15 minutes resulted in increased expression of both CD24 (6.9%) and CD44 (20.4%; Figure 4). Because the flow cytometric analysis suggests that CD44 may represent a quantitatively important ligand on BMSCs for endothelial selectins, we studied the effects of CD44 deficiency on BMSC recruitment after focal cerebral I/R using radiolabeled BMSCs. As shown in Figure 5, the I/R-induced BMSC recruitment into the postischemic (left) hemisphere (2534±1190 cells/g tissue, n=3) was not observed for BMSCs derived from CD44 knockout mice (106±27 cells/g tissue, n=5), whereas a robust response was noted using the same focal I/R protocol for BMSCs derived from WT mice.

Discussion

BMSCs hold great potential for treatment of ischemic tissue diseases, including stroke22 and myocardial infarction.23 Although adequate delivery of BMSCs to a site of tissue injury is critical for therapeutic efficacy, only a limited number of studies have evaluated the adhesive behavior of BMSCs in postischemic tissues. For example, it has been shown that BMSCs, administered intra-arterially, accumulate in renal glomeruli after renal I/R.24 Although intravenous BMSC has been used as a therapeutic agent in animal models of ischemic stroke25 and in humans subjects with stroke,7 the present study represents the first effort to assess BMSC recruitment in the microcirculation of the brain after focal I/R. Using 2 sources of BMSC, that is, from WT and H-2Kb-tsA58 transgenic mice, we have demonstrated a 7- to 9-fold increase in the number of adherent BMSCs that accumulate in cerebral venules after focal I/R. The selective accumulation of BMSCs in the ischemic region of the brain was verified by a comparison of the recruitment of radiolabeled BMSCs in the left (ischemic) and right brain hemispheres after focal I/R. Our findings are consistent with the view that BMSCs will selectively home and migrate into injured tissues, including the brain.5,9

Another major objective of our study was to determine whether the selectins act as the specific endothelial cell adhesion receptors to which BMSCs engage in the postischemic cerebral microvasculature, allowing for their selective recruitment and infiltration into areas of ischemia-induced brain injury. We focused on the endothelial selectins (P- and E-selectins) because previous in vitro and in vivo studies indicate that endothelial cells in culture10 and venules of the mouse ear10 and bone11 can sustain selectin-mediated BMSC adhesion as well as evidence that the postischemic cerebral microvasculature expresses elevated levels of E- and P-selectin.12–14 In this study, BMSCs were administered 24 hours after the induction of focal cerebral ischemia based on the previous reports showing effective functional neurological recovery after experimental stroke using the same dose and time administration as our study.25,26 Furthermore, E-selectin immunoreactivity,14 mRNA for E-selectin17,27 and E-selectin protein17 are reported to be high 24 hours after focal cerebral ischemia. Although the maximum level for E-selectin expression appears to occur at 6 to 12 hours after stroke, the studies mentioned report E-selectin expression levels of approximately 80% of maximum 24 hours after stroke induction. Although not as dramatic (35% of maximum expression observed 24 hours after stroke), previous reports also indicate sustained elevations in P-selectin mRNA28 and immunoreactivity13,14 in the ischemic hemisphere 24 hours after the onset of cerebral ischemia. Our results provide 2 lines of evidence that implicate both P- and E-selectin as major homing molecules for BMSCs in postischemic brain tissue: (1) immune neutralization of P- or
E-selectin with adhesion molecule-specific antibodies dramatically blunts the BMSC recruitment responses elicited by focal cerebral I/R; and (2) the pan-selectin blocker fucoidin produced an equally profound attenuation of the recruitment of radiolabeled BMSCs into postischemic brain tissue. It has been proposed that BMSCs may accumulate in some vascular beds due to simple entrapment related to steric hindrance in capillaries, however, this appears unlikely in our study because fucoidin was so effective in preventing the I/R-induced BMSC recruitment response. Although our findings strongly support a role for the endothelial selectins as homing receptors for BMSCs in the postischemic brain, a role for other adhesion molecules cannot be discounted. Indeed, in vitro model systems suggest that P-selectin as well as vascular cell adhesion molecule-1 contribute to the adhesive interactions between BMSCs and endothelial cells.

A variety of counterreceptors for the endothelial selectins have been identified and detected on circulating cells, including P-selectin glycoprotein ligand-1, L-selectin, sialyl Lewis x, CD43, and CD44. We evaluated the possible expression of these ligands as well as P- and E-selectin on BMSCs and found that CD44 was the only possible selectin counterreceptor that exhibited a significant level of expression on unstimulated murine BMSCs, and its expression was elevated when exposed to ischemic brain extract. CD44, a hyaluronan receptor expressed on hematopoietic stem cells, BMSCs, and activated T lymphocyte, mediates the adhesion of these cells to endothelium and into the extracellular matrix. CD44 expression can be induced by products of tissue injury such as platelet-derived growth factor. Our finding that BMSCs isolated from CD44 knockout mice were not able to home into the postischemic tissue suggests that CD44 may recognize homing ligands other than selectins. A likely candidate molecule is hyaluronic acid, a major CD44 ligand, which is highly expressed in the postischemic brain. Because CD44 signaling is antiapoptotic and stimulates proliferation of the cells carrying the ligand, the expression of CD44 may also serve to enhance the survival and differentiation of BMSCs that infiltrate injured brain tissue.

Another novel feature of the present study was the use of large-T antigen-carrying BMSCs derived from H-2Kb-tsA58 transgenic mice. Large-T antigen-carrying cells are conditionally immortalized and exhibit differentiation when cultured at 37°C. These cells retain their stem cell characteristics up to 1 year, and we have shown that large-T BMSCs can confer protection against the neurological deficits induced by focal I/R that is very similar to that observed after treatment with WT BMSC. Furthermore, the I/R-induced BMSC adhesion response observed for the large-T cells was virtually identical to that seen for WT BMSCs. The similar responses of WT and large-T BMSCs suggest that the latter cells may represent a useful model for detailed characterization of stem cell recruitment and differentiation in postischemic brain tissue.

In conclusion, our findings indicate that cerebral endothelium assume a proadhesive phenotype after focal ischemic stroke that favors the recruitment of BMSCs into the infarct area. BMSCs appear to use E- and P-selectins that are expressed on postischemic endothelium to home into the infarct site. The expression of CD44 on BMSCs appears to be critical for the selectin-mediated recruitment of stem cells into the region of postischemic tissue. The adhesive interface between the blood and endothelial cell surfaces may represent an important therapeutic target for enhancement of stem cell delivery to sites of ischemic infarction after stroke.

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Disclosures

None.

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Supplemental Methods

**Middle cerebral artery (MCA) occlusion and reperfusion (MCAo/R):** The mice were anesthetized by intraperitoneal injection of ketamine (50mg/kg) and xylazine (2.5mg/kg). Transient (60 minutes) focal cerebral ischemia was induced by occlusion of the left middle cerebral artery (MCAo) using a modification of intraluminal filament method. The blunted tip of a 6-0 nylon monofilament was advanced to the level of the carotid bifurcation via the internal carotid artery until light resistance was felt. The distance from the nylon thread tip to the internal carotid artery–pterygopalatine artery bifurcation was slightly >6 mm, and the distance to the bifurcation of the internal and external carotid arteries was slightly <9 mm. The monofilament was removed after 60 minutes of occlusion. In the sham group, these arteries were visualized but not disturbed. Ischemia and reperfusion (I/R) were verified using a LASER Doppler flow probe (MSP300XP, ADInstruments Inc.) attached to the left parietal cranium. At the end of experiments, mice were euthanized with a lethal dose of pentobarbital (150 mg/kg, i.p.). The brains were immediately removed, and then stained with 2% 2,3,5-triphenyltetrazolium chloride to confirm the production of an infarct. The total areas of each brain section and the infarcted region were quantified on digitized images with the public domain NIH image. Infarct volume was corrected for edema Swanson formula.  

**Intravital videomicroscopy:** Mice were randomly allocated into different experimental groups for intravital videomicroscopic evaluation of BMSC adhesion in postischemic cerebral venules. Sham mice and MCAo/R mice receiving (i.v., 5 min before administration of BMSC) neutralizing antibodies (2.5 mg/kg i.v.) directed against either E-selectin (rat anti-mouse CD62E, BD Pharmingen) or P-selectin (rat anti-mouse CD62P, BD Pharmingen), or an isotype-matched control antibody (rat IgG2a κ isotype control, rat IgG1, λ isotype control, BD Pharmingen) were anesthetized with ketamine & xylazine after a 24 h reperfusion period. The right femoral vein was cannulated for infusion (5 min) of BMSC (8 x 10^6), which were allowed to circulate for 15 min before visualization. The femoral artery was cannulated to monitor mean arterial blood pressure and to sample arterial blood for blood gases. All mice were tracheotomized and mechanically ventilated with room air during observation by intravital microscopy.

The head of each mouse was fixed in a plastic frame in the sphinx position. The left parietal bone was exposed by a midline skin incision, followed by a craniectomy at 1 mm posterior from the bregma and 4 mm lateral from the midline. The dura mater was not cut because the fluorescently labeled blood cells were easily observed and intracranial pressure was well maintained in the absence of this procedure. The observation area largely represented the region of infarcted tissue produced by MCAo/R. A 12-mm glass coverslip was placed over the craniectomy, and the space between the glass and dura mater was filled with artificial cerebrospinal fluid. An upright Nikon microscope equipped with a SIT camera (C2400-08; Hamamatsu Photonics K.K., Shizuoka, Japan) and a mercury lamp was used to observe the cerebral microcirculation. Venular segments (100 μm length, 20-80 μm diameter) were observed and the number of adherent cells (stationary for ≥ 30 sec) determined. BMSC adhesion was expressed as the number of cells per 1 mm² venular surface, calculated from the diameter and length, assuming a cylindrical geometry.

**BMSC isolation and labeling:** Primary cultures of BMSC were obtained from WT, large-T or CD44ko mice as previously described. Briefly, fresh complete bone marrow was harvested aseptically from tibias and femurs and cultured in Iscove's Modified Dulbecco's medium supplemented with fetal bovine serum (10%). After 3 days of incubation, non adherent cells were removed and cells tightly adherent to plastic were isolated and resuspended in fresh
Isocove's Modified Dulbecco's medium for further growth in new flasks. Consistent with previous reports, by passage 3, there was less than 1% CD11b and CD45 expressing cells as assessed by flow cytometry and isolated cells were Sca-1 positive (70%).

BMSC were harvested using non-enzymatic dissociation solution (Sigma Chemicals). The cells were labeled by incubation with 90 µmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE) for 10 min. Labeled cells were washed two times with PBS. Cells were then centrifuged and filtered through a 70 μm cell strainer (BD, Falcon) and resuspended in PBS (pH 7.4). The cells were counted and 8 x 10^6 viable BMSC in 150 µl of PBS (or 150 µl of PBS in controls) were injected into the mice 10 min. prior to obtaining estimates of cell adhesion via videomicroscopy. Flow cytometry was used to detect the cell surface expression of P-, E-, and L-selectins, PSGL-1, CD24, CD43, and CD44 on large-T BMSC in their naïve state or after incubation with ischemic brain extracts for 15 min. Ischemic brains were isolated from WT mice that were subjected to MCAO/r surgery. Brains were homogenized in PBS containing antiproteinase cocktail and were centrifuged for 10 min at 10000 rpm. Supernatants were collected and used as ischemic brain extracts.

51Cr-labelling of BMSC: 51Cr-labelling of BMSC is derived from a previously used method. Bone marrow stromal cells (3 x 10^6 cells/ml) were suspended in PBS and incubated with 150 μCi Na^{51}CrO_4 at 37°C for 60 min. The cells were washed twice with PBS at 250 x g for 8 min to remove unincorporated radioactivity and then were resuspended in HBSS containing 1% FBS. In wild type mice, 2 x 10^6 51Cr-labeled BMSC were injected (via the femoral vein) 24 hours after either sham surgery or MCAo/R, with or without treatment with the pan-selectin blocker fucoidin (30 mg/kg i.v. once a day for 3 days). After 72 hrs, the mice were anesthetized and transcardially perfused with PBS (100 mmHg) for 5 minutes. The brain was then removed, weighed and each hemisphere was placed in a 14800 Wizard 3 gamma counter (Wallac), with automatic correction for background activity and spillover, for determination of tissue 51Cr activity per mass of tissue.

Supplemental Figures
S1. Flow cytometric analysis of L-selectin, CD62, and P-selectin expression on BMSC.

S2. Infarct size in stroke mice 6 days after treatment with saline (I/R, n=6), 2x10⁶ wild type BMSC (I/R+WT, n=6) or 2x10⁶ large T BMSC (I/R+Large T, n=5). No difference was found in infarct size among the groups (One-way Analysis of Variance P= 0.9648).

Supplemental References