Supplementary Materials & Methods

Cell preparation and fluorescence flow cytometry analysis of CD4+Foxp3+ cells

Fluorescence flow cytometry analyses were performed to determine percentage of Foxp3+ cells in CD4+ cells. This method is a modification of an inflammatory cell isolation protocol developed previously (Campanella et al. 2002; Kurnick et al. 1979). Four days after sham-operation, or 2, 4, and 7 days after pMCAO surgery, 5 mL peripheral blood (PB) was obtained from femoral artery under anesthesia. The animals were then euthanized under deep anesthesia and transcardiac perfusion with 50 mL ice-cold PBS was carried out to remove the retained blood from brain vasculature. Spleen, superficial or deep cervical lymph nodes (SCLNs or DCLNs), and ipsilateral forebrains were isolated, and passed through a 40-μm nylon cell strainer (BD Biosciences, San Jose, CA, USA) to prepare a single-cell suspension. The red blood cells in PB and spleen were lysed using ACK lysing buffer (Quality Biological, Inc, Gaithersburg, MD, USA), and incubating for 3 min at room temperature. After washing with RPMI (Invitrogen, Carlsbad, CA, USA), the suspension was centrifuged at 1700 *rpm* for 7 min at room temperature; the pellet was resuspended in 2 mL of PBS with 0.1% BSA (FACS buffer) and overlaid on the top of a gradient containing 3.0 mL of

20% or 37% (brain sample) and 3.0 mL of 70% Percoll solution (Sigma-Aldrich, St. Louis, MO, USA) at room temperature (Campanella et al. 2002; Kurnick et al. 1979). Percoll was prepared by dilution in Hanks' balanced salt solution (HBSS) (Bio-Whittaker, Walkersville, MD, USA). The gradient was centrifuged at 1500 *rpm* for 20 min without braking at room temperature; cells were collected from the 20% or 37% (brain sample) to 70% interface and washed once with FACS buffer.

After Percoll separation, $1x10^5$ cells per tube were washed with FACS buffer, and incubated 30 min at 4°C with 1 µg PE-conjugated mouse IgG_{2a} anti-rat CD4 (clone: OX-35, BD Biosciences). Subsequently, the cells were washed twice with 1 mL of FACS buffer, fixed with 4% paraformaldehyde (PFA, pH 7.3) for 20 min at room temperature, and washed twice. They were then washed in 2 mL 1X Permeabilization buffer (eBioscience, San Diego, CA, USA) containing 0.1% saponin, and incubated with 1 µg AlexaFluor 488-conjugated rat IgG_{2a} anti-mouse/rat Foxp3 (clone: FJK-16s, eBioscience) in 1mL 1X Permeabilization Buffer overnight at 4°C.

After washing twice with FACS buffer, the CD4+ cells or Foxp3+ cells were discriminated from cell debris using DAPI staining. The cells were analyzed using the FACSVantage SE flow cytometer (BD Biosciences) equipped with Argon ion laser tuned to 488 nm and UV laser and standard FITC/PE/DAPI filter sets to detect emission.

CellQuest Acquisition and Analysis Software (BD Biosciences) was used to quantify approximately $1x10^5$ cells per sample. For each experiment, cells were stained with appropriate isotype control antibodies to establish background staining and to set quadrants before calculating the percentage of positive cells. The percentage of Foxp3+ cells in CD4+ cells was calculated using simple ratios.

Cell Quantification

To estimate the total number of BrdU+ or PCNA+ cells in the ipsilateral SVZ (the area that lines the lateral walls of the lateral ventricle and is a predominant source of neuronal progenitors), 10 serial sections, spaced 300 μm apart, through the anterior to posterior SVZ (bregma level 1.70 to −1.30 mm) were quantified with the unbiased optical fractionator approach (StereoInvestigator, MicroBrightField, Colchester, VT, USA). The analysis was performed using a Leica DM 4000B microscope with a motorized specimen stage controlling movements in the x-y axis. We outlined a contour of the ipsilateral SVZ at low magnification (4x Leica HCX N Plan, N.A. 0.12). The optical disector probes were performed using 63x oil-immersion lens (Leica PL APO, N.A. 1.40). A 3-dimensional sampling box (counting frame area 50 x 50 μm; sampling box height 15 μm; guard area 5 μm at the top and bottom of the section) was focused

through the section. BrdU+ or PCNA+ cells were marked when they were in focus within the counting frame. We marked at least 100 cells per region in each brain. In all cases, the Schmitz-Hof coefficient of error (CE) was less than 0.10, reflecting the benefits of the high-precision design-based stereology approach used here.

Statistics

Statistical analysis was conducted with Stat View version 5.0 (SAS Institute, Cary, NC) or SAS (SAS Institute). Two-way factorial analysis of variance (ANOVA) or one factor ANOVA with post hoc Bonferroni/Dunn test was used for the comparison among pMCAO and sham-operated group with or without E-selectin tolerization in the quantification of the percentage of Foxp3+ cells in total CD4+ cells examined with flow cytometry, Dcx protein levels with immunoblotting, and BrdU+ or PCNA+ cells in the ipsilateral SVZ. The unpaired Student's t test was used for the comparison among the E-selectin tolerized and the PBS treated pMCAO group in the quantification of total infarction volume and the average number of Foxp3+, BrdU+/Dcx+, BrdU+/NeuN+, and vWF+/TNF+ cells, and Dcx+ cells attached to vWF+/TNF- or vWF+/TNF+ blood vessels. For the comparison among the E-selectin tolerized and the PBS tolerized pMCAO groups in the quantification of the right-biased body swing rate and the

removal time in behavioral tests, the results were analyzed by repeated-measures ANOVA with independent variables of treatment group and day of testing, followed by the Bonferroni post hoc test for multiple comparisons between groups. All values are presented as means ± SD (standard deviation). $P < 0.05$ was considered to be statistically significant.

References

Campanella M, Sciorati C, Tarozzo G, Beltramo M. (2002) Flow cytometric analysis of inflammatory cells in ischemic rat brain. *Stroke* 33:586-592

Kurnick JT, Ostberg L, Stegagno M, Kimura AK, Orn A, Sjoberg O. (1979) A rapid method for the separation of functional lymphoid cell populations of human and animal origin on PVP-silica (Percoll) density gradients. *Scand J Immunol* 10:563-573