Supplemental Material

Ischemic stroke activates hematopoietic bone marrow stem cells

*Gabriel Courties PhD[1], *Fanny Herisson MD[2], Hendrik B. Sager MD[1], Timo Heidt MD[1], Yuxiang Ye PhD[1], Ying Wei MD[2], Yuan Sun MD PhD[1], Nicolas Severe PhD[3], Partha Dutta PhD[1], Jennifer Scharff[1], David Scadden MD[3], Ralph Weissleder MD PhD[1,4], Filip K. Swirski PhD[1], Michael A. Moskowitz MD[2], Matthias Nahrendorf MD PhD[1]

Corresponding author: Matthias Nahrendorf
Center for Systems Biology
185 Cambridge Street
Boston, MA 02114
Tel: (617) 643-0500
Fax: (617) 643-6133
mnahrendorf@mgh.harvard.edu

Detailed methods

Animals and stroke procedure

Adult C57BL/6 and FVB/N mice (10-12 weeks old) were obtained from Jackson Laboratories and repTOP™ mitoIRE mice were purchased from Charles River Laboratories. *Adrb3*$^{-/-}$ mice (gift from P. Frenette) and Nestin-GFP reporter mice (gift from G. Enikolopov) were bred in our facilities. Experimental stroke was induced by a transient occlusion of the middle cerebral artery (tMCAO). Mice were anesthetized with 1.5-2% isoflurane. Temperature was kept between 36-36.5°C using a temperature control system (Harvard Apparatus). After midline neck skin incision, the right carotid artery bifurcation was dissected, and the external carotid artery (ECA) was permanently ligated. A clip was then placed on the internal carotid artery, and a temporary ligation was tied onto the common carotid artery. An arteriotomy was then performed on the ECA. A commercial silicon coated filament (7019, Doccol corp, MA, USA) was then introduced and pushed from the ECA to the ICA after removal of the clamp, until blocked in the carotid termination. The filament was left in place for forty-five minutes and gently pulled out afterwards. A permanent knot on the ECA secured the arteriotomy from bleeding, and the temporary knot on the CCA was then removed to allow complete reperfusion. Painkillers were given at the completion of the surgical procedure (buprenorphine 0.1mg/kg subcutaneously) and mice recovered in a clean cage. In additional control experiments, sham surgery was performed using similar conditions as in tMCAO but without induction of brain ischemia. Specifically, the right carotid bifurcation was dissected and the external carotid artery permanently ligated. Afterwards, the incision was sutured and the mice allowed to wake up in a clean cage. No arteriotomy was performed. The Subcommittee on Research Animal Care at Massachusetts General Hospital approved procedures.

Flow cytometry

After bone marrow harvest, single cell suspensions were obtained and total number of cells per femur were determined using a hemocytometer and Trypan Blue staining method for cell viability (Cellgro, Mediatech, Inc, VA). All antibodies used in this study were purchased from eBioscience, BioLegend or BD Biosciences. For mature myeloid cells analysis, monoclonal antibodies including anti-CD11b (M1/70), Ly6G (1A8), CD115 (AFS98), Ly6C (AL-21) were used. Neutrophils were identified as CD11b^{+} Ly6G^{+}, Ly6C^{low} monocytes were identified as CD11b^{+} CD115^{+} Ly6C^{low} and Ly6C^{high} monocytes were defined as CD11b^{+} CD115^{+} Ly6C^{high}. For hematopoietic stem and progenitor cells analyses, cells were stained with biotin conjugated antibodies against lineage markers including B220 (RA3-6B2), CD4 (GK1.5), CD8α (53-6.7), NK1.1 (PK136), CD11b (M1/70), CD11c (N418), Gr-1 (RB6-8C5), Ter119 (TER-119) followed by streptavidin Pacific Orange™ or APC/Cy7 conjugates, and antibodies against c-Kit (2B8), Sca-1 (D7), IL7Rα (SB/199), CD93 (AA4.1), CD16/32 (2.4G2), CD34 (RAM34), CD135 (A2F10), CD48 (HM48-1) and CD150 (TC15-12F12.2). Hematopoietic stem cells (HSC) were identified as Lin^{-} c-Kit^{+} Sca-1^{+} CD48^{-} CD150^{+}, LKS were identified as Lin^{-} c-Kit^{+} Sca-1^{+}. Granulocyte-macrophage progenitors (GMP) were identified as Lin^{-} c-Kit^{+} Sca-1^{-} CD16/32^{+} CD34^{+} and macrophage and dendritic cell progenitors (MDP) were defined as Lin^{-} c-Kit^{+} Sca-1^{-} CD16/32^{+} CD34^{+} CD115^{+}. Common lymphoid progenitors (CLP) were identified as Lin^{-} IL7Rα^{+} c-Kit^{int} Sca-1^{int} and early immature B cells were defined as Lin^{-} B220^{int} CD93^{+}. Cell numbers per femur were calculated as total cells per femur sample multiplied by percentage of cells obtained from the appropriate FACS gates. For BrdU incorporation assays, 1 mg of BrdU was injected i.p. 24 hours before harvesting. BrdU staining was performed using the FITC BrdU flow kit (BD Biosciences) according to the manufacturer's protocol. For cell cycle analysis, cells were stained for LKS and HSC cell surface markers as described above, fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to manufacturer's instructions. Cells were then stained with anti-Ki67 antibody (SolA15, eBioscience) or isotype control. After washing, 1μl of DAPI (FxCycle™ Violet stain, Life Technologies) was subsequently added to samples. Data acquisition was performed using LSRII Flow Cytometer (BD). Data were analyzed using with FlowJo software (Tree Star).

In vivo staining of bone marrow vasculature and bone lining cells
To visualize bone structures, mice were administered intravenously with OsteoSense® 750EX, a fluorescent in vivo bisphosphonate imaging agent (4 nmol/mouse, PerkinElmer), 24 h before ex-vivo staining of sternal preparations. For in vivo endothelial cells labeling, mice were given i.v APC anti-mouse CD31 (MEC13.3), Alexa Fluor® 647 anti-mouse VE-Cadherin (BV13) and APC anti-mouse Sca-1 (D7, 2 µg/mouse in 100µl PBS), 30 min prior to organ harvest.

Whole mount immunofluorescence staining of the sternum
Sterna were harvested and processed as described in[1]. Briefly, sternal bones were transected with a surgical blade to obtain compartments of sternal BM. Each specimen was further sectioned longitudinally to expose the bone marrow. Sternal preparations were fixed with 4% paraformaldehyde in PBS in a 96-well plate at room temperature for 30 minutes. Whole mount tissues were blocked and permeabilized overnight with 20% goat serum and 0.5% Triton X-100 in PBS. After washing with 0.1% Triton X-100 in PBS (4 x 15 minutes), BM tissues were stained with rabbit anti-mouse Tyrosine Hydroxylase antibody (Millipore, 1:100) in 20% goat serum and 0.1%Triton X-100 in PBS, for 2 days at 4°C. After washing, samples were incubated for 2 hours at RT with Alexa Fluor 488 goat anti-rabbit secondary antibody (Life technologies, 1:200). After washing, whole-mount tissues were imaged using an Olympus IV100 microscope and z-stacks images acquired at 2-5µm steps were processed with ImageJ software (NIH).

Confocal microscopy
For serial intravital microscopy of the calvarium, SLAM HSCs (Lin^- c-kit^+ $Sca\text{-}1^+$ $CD48^-$ $CD150^+$) were FACS-sorted using a FACSAria II cell sorter (BD) from C57BL/6 mice and labeled ex-vivo with the Vybrant® DiD Cell-Labeling Solution (1,1′-dioctadecyl-3,3,3′,3′- tetramethylindodicarbocyanine perchlorate, Molecular Probes®) according to manufacturer's protocol. 20,000 labeled HSCs were injected i.v. into non-irradiated Nestin-GFP recipient mice. The GFP signal, while not specific for mesenchymal stem cells, was used as landmark to aid revisiting similar regions of interest in serial intravital imaging. To highlight bone architecture, OsteoSense® 750EX, a fluorescent bisphosphonate imaging agent, was administered i.v. 24 hours prior to imaging (4 nmol/mouse, PerkinElmer). To outline the vasculature, we used rhodamine-labelled Griffonia simplicifolia lectin (RL-1102, Vector Laboratories). Lectin was injected i.v. (50µl at 2 mg/ml) immediately prior imaging. In vivo imaging was performed on days 1 and 5 after the adoptive cell transfer using a confocal microscope (IV100 Olympus). Z-stacks images for each location were acquired at 2µm steps and post-processing was performed using Image J software (NIH). For visualization of LKS cells in sternal BM, Lin^- c-Kit^+ $Sca\text{-}1^+$ cells were FACS sorted and labeled ex-vivo with two different fluorescent dyes including CellTracker™ CM-Dil and SP-$DiOC_{18}$(3) (Molecular Probes) prior to adoptive transfer into recipient mice.

TTC staining
Brains were removed and sliced on a brain matrix every millimeter. Fresh tissue sections were then soaked in a solution of 2% TTC (2,3,5-Triphenyltetrazolium chloride, Sigma, St. Louis, MO) for 15 minutes protected from light. Stroke volume was measured using ImageJ software (imagej.nih.gov) by planimetry. Indirect stroke volume was given subtracting the volume of the non-injured parenchyma of the stroke hemisphere from the volume of the contralateral hemisphere.

In vivo bioluminescence imaging
Transgenic repTOP™ mitoIRE mice were anesthetized and injected i.p. with 150 mg/kg body weight with D-Luciferin (RR Labs Inc, San Diego, CA). Five minutes later, mice were moved to the imaging chamber of the IVIS™ 100 imaging system in a supine position. A region of interest (ROI) was drawn to encompass the entire mouse and light emission was recorded every 5 min until the signal intensity passed its peak value. Signal intensities were measured in photons per second using Living Image® software (Caliper Life Sciences).

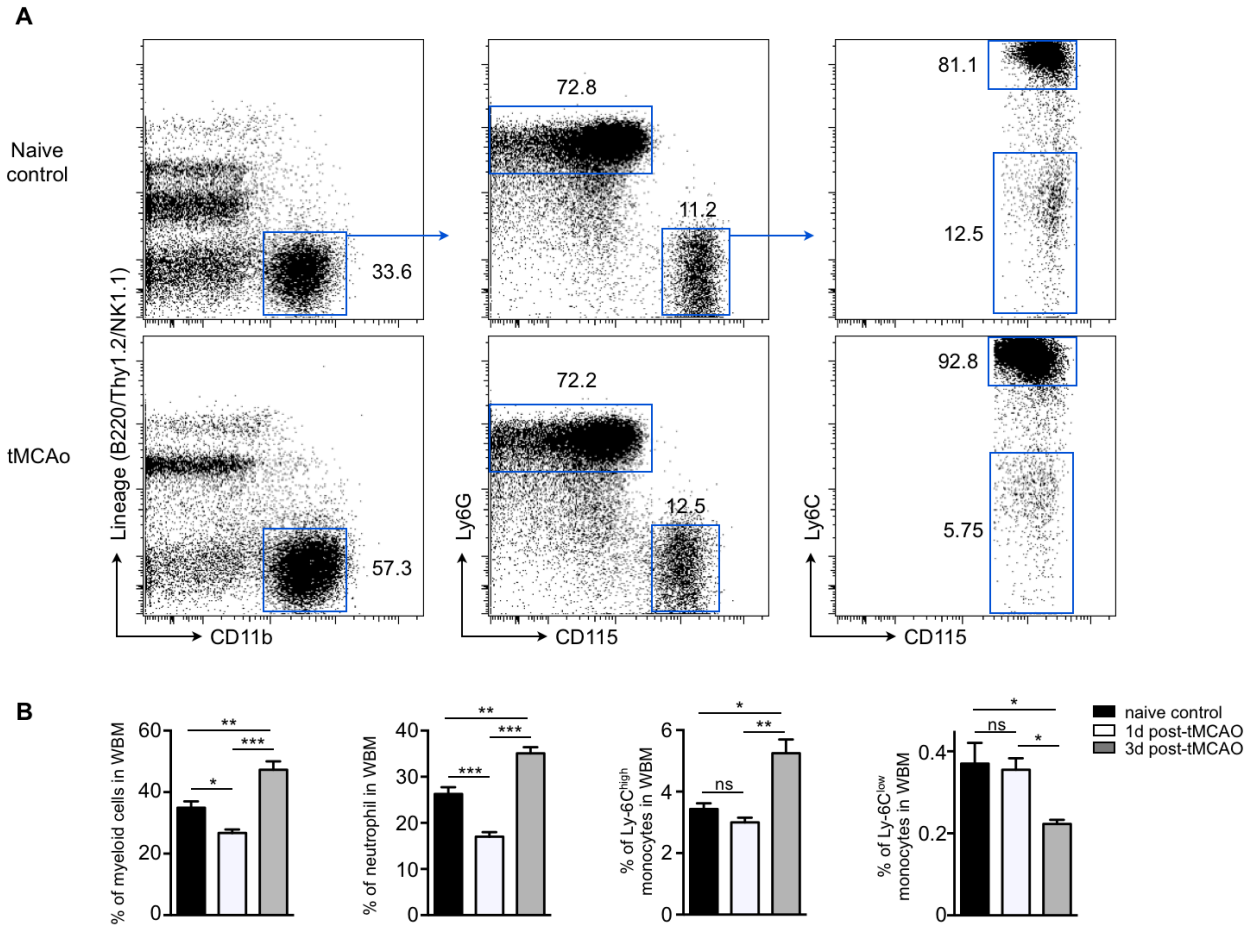
Colony-Forming cell assay
CFC assays were performed using a semi-solid cell culture medium (MethoCult® GF M3434, STEMCELL Technologies) following the manufacturer's instructions. Bones were flushed with Iscove's Modified Dulbecco's Medium (Lonza) supplemented with 2% FCS and 2×10^4 whole bone marrow cells were cultured in 35 mm dishes in duplicates and incubated at 37°C, 5% CO_2 in a humidified incubator. Colonies were counted after 7 days using a low magnification inverted microscope (Nikon Eclipse TE2000-S).

RNA isolation and gene expression
Total RNA was isolated using RNeasy mini kit (Qiagen) according to the manufacturer's protocol. For qRT-PCR, RNA was treated with DNase I and reverse-transcribed using the high capacity RNA to cDNA kit (Applied Biosystems). Runs were performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems) using Taqman primers and reagents (Applied Biosystems). Threshold cycle values from target genes were normalized to housekeeping genes expression including Actinb (*Actb*) or glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) using the $2^{-(Ct(\text{target gene}) - Ct(\text{housekeeping gene}))}$ method.

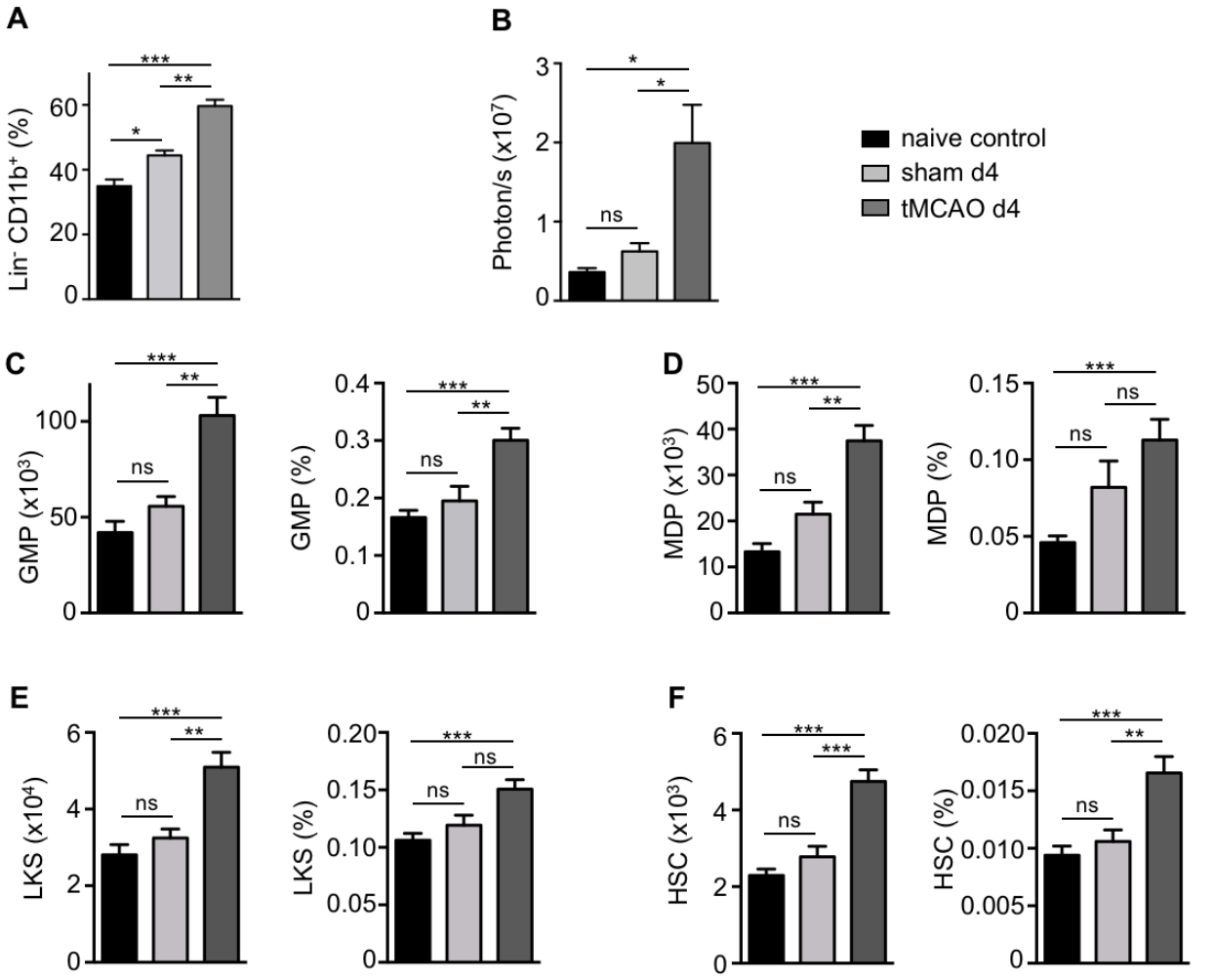
ELISA
Quantitative measurement of norepinephrine in the bone marrow was determined using a noradrenaline sensitive ELISA Assay Kit according to manufacturer's protocol (DLD Diagnostika, Eagle Biosciences). Femurs were harvested and immediately snap-frozen and homogenized in a catecholamine stabilizing solution (4mM sodium metabisulfite, 1mM EDTA and 0.01N hydrochloric acid, pH=7.5) prior to the procedure.

Statistics
Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, Inc.). Results are reported as mean ± standard error of mean. For a two-group comparison, a Mann-Whitney test for nonparametric data was used. For a comparison of more than two groups, an ANOVA test, followed by a Bonferroni test for multiple comparisons, was applied.

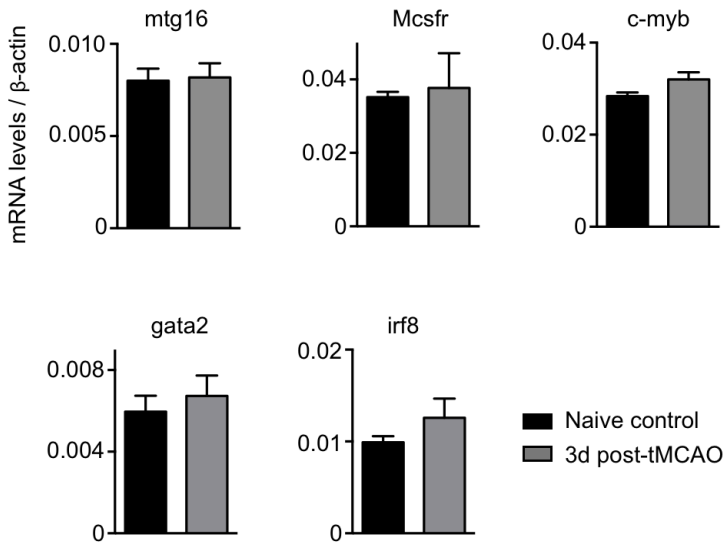
References
1. Takaku T, Malide D, Chen J, Calado RT, Kajigaya S, Young NS. Hematopoiesis in 3 dimensions: human and murine bone marrow architecture visualized by confocal microscopy. *Blood.* 2010;116:e41–e55.



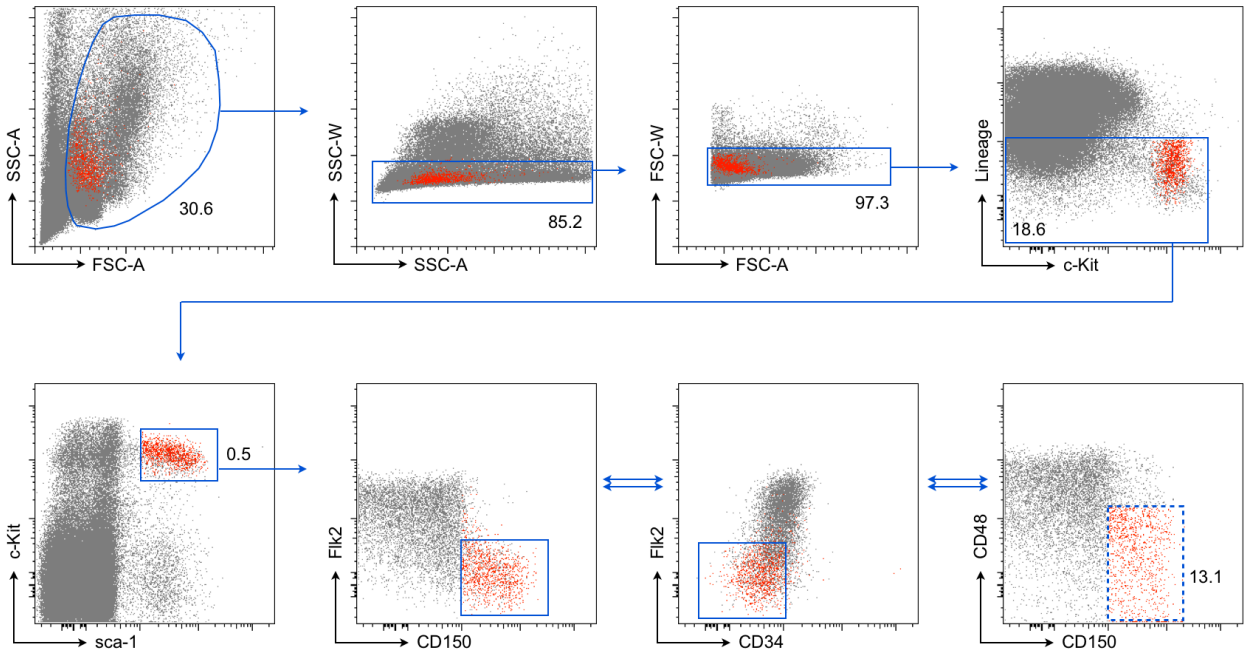
Online Figure I: Bone marrow myeloid cell content after stroke
A, FACS gating strategy and representative dot plots for identification of myeloid cell subsets in the bone marrow in non ischemic controls (upper panels) and on day 3 after stroke (lower panels) induced by tMCAO in C57BL/6 mice. **B,** Percentages of myeloid cells (Lin$^-$ CD11b$^+$), neutrophils (Lin$^-$ CD11b$^+$ Ly6G$^+$ CD115$^-$), Ly6C^{high} monocytes (Lin$^-$ CD11b$^+$ Ly6G$^-$ CD115$^+$ Ly6C^{high}) and Ly6C^{low} monocytes (Lin$^-$ CD11b$^+$ Ly6G$^-$ CD115$^+$ Ly6C^{low}) in whole bone marrow at indicated time points following stroke. n= 4-6 mice per group, one-way ANOVA. Mean ± s.e.m., *P < 0.05, **P < 0.01, ***P < 0.001.



Online Figure II: Sham surgery mildly alters myeloid content in the bone marrow.
Bone marrow cell suspensions from naive controls, sham-operated controls and mice with tMCAO (day 4) were stained for stem and progenitor cells. FACS enumeration per femur and percentages of mature myeloid cells (A), GMP (C), MDP (D), LKS (E), HSC (F) in whole bone marrow (n= 4-6 mice per group, one-way ANOVA). B, Quantification of luciferase activity in MITO-Luc mice on day 4 following sham operation and tMCAO (n= 4 mice per group, one-way ANOVA). Mean ± s.e.m., *P < 0.05, **P < 0.01, ***P < 0.001.



Online Figure III: Unchanged gene expression in LKS after stroke
Experimental stroke was induced or not in C57BL/6 mice by tMCAO and LKS from the bone marrow were sorted by FACS three days later. Gene expression levels of mtg16, Mcsfr, c-myb, gata2 and irf8 in LKS were not altered after ischemic injury of the brain as assessed by RT-qPCR. n = 4-5 mice per group.



Online Figure IV: FACS gating strategy for identification of hematopoietic stem cells in the bone marrow.
SLAM HSC (red population) are identified as lineage negative ($B220^-$ $CD4^-$ $CD8^-$ $NK1.1^-$ $CD11b^-$ $CD11c^-$ $Gr\text{-}1^-$ $Ter119^-$ $IL7R\alpha^-$) $c\text{-}kit^+$ $Sca\text{-}1^+$ $CD135^-$ (Flk2), $CD34^-$ $CD48^-$ $CD150^+$.