Supplementary Methods

Mice All animal experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and performed in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*. Male 6- to 10-week old C57BL/6J mice, MMP9-/- mice, IL-10-/- mice and B6.SJL-Ptprca Pep3b/BoyJ mice (CD45.1 congenic mice) were purchased from The Jackson Laboratory. For $CD25^{+}$ cell depletion, 300μg anti-CD25 antibody (eBioscience) was diluted in PBS and injected ip 2d prior to MCAO. For granulocyte-specific depletion, 400μg functional rat anti-Gr1 antibody (eBioscience) was diluted in PBS and injected i.p. 24h prior to MCAO. Control mice received the same amount of IgG2a isotype antibody. See Supplementary material for the construction of bone marrow chimeric mice.

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Ischemia models

Cerebral focal ischemia was induced in mouse by intraluminal occlusion of the left MCA for 60min. Mice were anesthetized with 3% isoflurane in 67%:30% N2O/O2 (induction), until they were unresponsive to the tail pinch test and were then fitted with a nose cone blowing 1.5% isoflurane for anesthesia maintenance. A monofilament was introduced into the common carotid artery, advanced to the origin of the MCA, and left there for 60 min until reperfusion. The surgical procedure required approximately 10 minutes. The rectal temperature was controlled at 37.0 ± 0.5°C during surgery and MCA occlusion via a temperature-regulated heating pad. Mean arterial blood pressure was monitored during MCA occlusion through a tail cuff, and arterial blood gas was analyzed at 15 min after the onset of ischemia. Regional cerebral blood flow (CBF) was measured using laser-Doppler flowmetry. Animals that did not show a CBF reduction of at least 75% were excluded (less than 10% of stroke animals) from further experimentation. Animals that died after ischemia induction were also excluded from experimental groups. Sham-operated animals underwent the same anesthesia and surgical procedures except middle cerebral artery occlusion. Immediately after surgery, animals were randomly assigned to PBS, splenocyte or Tregs treatment groups. Treatment was done by an investigator blinded to treatment groups. Transient (120min) cerebral focal ischemia was induced in Sprague Dawley rats as described [1].

Deleted: Animals that did not show a CBF reduction of at least 75% were excluded (less than 10% of stroke animals) from further experimentation, as were animals that died after ischemia induction.

Two dimensional laser speckle imaging techniques

In selected experiment, cortical blood flow was monitored using the laser speckle technique. Briefly, a CCD camera (PeriCam PSI System; Perimed) was positioned above the head, and a laser diode (785 nm) illuminated the intact skull surface to allow penetration of the laser in a diffuse manner through the brain. Speckle contrast— defined as the ratio of the SD of pixel intensity to the mean pixel intensity—was used to measure CBF as it is derived from the speckle visibility relative to the velocity of the light-scattering particles (blood). This was then converted to correlation time values, which are inversely and linearly proportional to the mean blood velocity. Laser speckle perfusion images were obtained 10 min before MCAO and

continuing throughout the ischemic period until 5 min into the reperfusion. CBF was measured again in the same animals at 2h (right after Treg injection) and 24h after reperfusion. CBF changes were recorded over time and expressed as % of pre-MCAO baseline.

Measurement of infarct volume

For 2,3,5-triphenyltetrazolium chloride (TTC) staining: Brains were removed and sliced into 7 coronal sections, each 1 mm thick. Sections were immersed in prewarmed 2% TTC (Sigma) in saline for 10 min, and then fixed in 4% paraformaldehyde. Animals that developed massive hemorrhage were excluded from further evaluation (about 2% of stroke animals). For cresyl violet or MMP2 staining: Free floating sections were prepared from the fixed and dehydrated brains and stained with cresyl violet (Sigma) or MMP2 antibody (Santa Cruz Biotechnology). Infarct volume was determined with NIH Image J analysis by an observer blinded to the experimental group assignment. The actual infarct volume with correction of edema was calculated as the volume of the contralateral hemisphere minus the noninfarcted volume of the ipsilateral hemisphere.

Behavioral tests

Behavioral tests were performed by an individual blinded to experimental groups. The corner test was performed as described previously [2]. Corner test performance was expressed by the number of left turns out of 10 turn trials per day. For the vibrissae-induced forelimb placing test, the mice were held by their torsos with their forepaws hanging free and moved slowly toward the edge of a tabletop, touching the vibrissae to the edge of the tabletop. Each test was repeated for each paw 10 times. The number of completed placing responses out of the 10 times was recorded. The cylinder test was performed at 3, 5, 7, 10, 14, 21 and 28 d after stroke to assess forepaw use and rotation asymmetry. The mouse was placed in a cylinder 9 cm in diameter and 15 cm in height, and videotaped for 5 min. Videotapes were analyzed, and forepaw (left/right/both) use of the first contact against the cylinder wall after rearing and during lateral exploration was recorded. Non-impaired forepaw (right) preference is expressed as the relative proportion of right forepaw contacts, which was calculated as: (right - left) / (right + left + both) × 100.

Assessments of neurological deficit

Neurological deficit was assessed in a blinded fashion using a 5-point-scale neurological score in mice (0, no observable deficit; 1, torso flexion to right; 2, spontaneous circling to right; 3, leaning/falling to right; 4, no spontaneous movement; 5, death) and a 10-point-scale neurological score in rats (4 points if there is a reduction in the resistance to a contralateral push; 3 points if contralateral circling is evident; 2 points for the appearance of contralateral shoulder adduction; and 1 point for contralateral forelimb flexion, when suspended vertically by the tail).

Real-time PCR

Total RNA was isolated from ischemic brains at 24 h after stroke using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Five μg was used to synthesize the first strand of cDNA using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). PCR was performed on the Opticon 2 Real-Time PCR Detection System (Bio-Rad) using corresponding primers (Supplementary Table 1) and SYBR gene PCR Master Mix (Invitrogen). The cycle time values were normalized with GAPDH of the same sample; and then the expression levels of the mRNAs were calculated and expressed as fold changes versus sham control.

Cell preparation for flow cytometry

Spleen, lymph nodes, bone marrow, blood, liver, lung, kidney and brain were collected after MCAO and single cell suspensions were prepared for flow cytometric analysis. Briefly, liver, lung, kidney and brain were first flushed with PBS and chopped into fine particles in 4 ml of complete RPMI 1640 medium supplemented with 10% fetal calf serum. Tissues were then incubated in 10 ml of digestion buffer (2% FBS, 1 mg/ml collagenase II, 0.5 mg/ml of DNase I in RPMI 1640 medium) for 1h in 37°C water bath. The suspension was passed through a 70 μm cell strainer, resuspended in 40 ml of complete RPMI 1640 and pelleted at 2000g for 10 min at 4 °C. Cells were fractionated on a 30%–60% percoll gradient (GE Health) at 1000g for 25 min. The mononuclear cells in the interface were washed prior to staining. Bone marrow was prepared from femur and tibia bones. Peripheral blood was obtained from mice by cardiac puncture, and the RBCs were lysised by ACK lysis buffer (Sigma). Lymphocytes were isolated from spleens and lymph nodes by mechanical homogenization followed by lysis of RBCs using ACK lysis buffer**.** Isolated cells were resuspended at $1x10^6$/ml and stain with CD4, CD25, CD45.1 and Foxp3.

Flow cytometry

Cells were stained with anti-mouse CD3, CD4, CD8, CD25, B220, NK1.1, Gr-1, CD45.1 and the appropriate isotype controls following the manufacturer's instruction (eBioscience). For Foxp3 intracellular staining, cells were surface-stained with PB-conjugated anti-CD4 and PE-conjugated CD25, then permeabilized with the intracellular staining Kit (eBioscience) according to the manufacturer's protocol. Cells were then stained with APC-conjugated anti-Foxp3. Flow cytometric analysis was performed using a FACS flow cytometer (BD Biosciences).

Intravenous injection, quantification, and detection of tracers

Cadaverine conjugated to Alexa Fluor-555 (950Da, Invitrogen, 200μg/mouse) or bovine serum albumin conjugated to Alexa Fluor-555 (66kDa, Invitrogen, 2.5mg/mouse) was injected into the tail vein after reperfusion. For quantification of the tracers, animals were sacrificed at 24h after MCAO and perfused for 5min with HBSS. Brains were removed and homogenized

in 1% Triton X-100/PBS and centrifuged at 16,000 rpm for 20 minutes. The relative fluorescence of the supernatant was measured at ex/em 544/590 nm.

Immunohistochemistry

Free floating sections (25μm) or paraffin embedded sections (5μm) were used for immunohistochemical staining. The rabbit anti-Iba-1 (Wako), rat anti-CD31 (BD Pharmingen), rabbit anti-CD3 (Abcam), rat anti-F4/80 (BM8, Biolegend), and rabbit anti MPO (Abcam) were used to label microglia, endothelial cells, T cells, macrophages, and neutrophils, respectively. Other primary antibodies used include mouse anti-ZO-1 (Invitrogen), rabbit anti-Foxp3 (ebioscience), rabbit anti-MAP2 (Santa Cruz), mouse anti-NeuN (Millipore) and goat anti-MMP-9 (R&D System). Biotin conjugated anti-mouse IgG was used to detect extravascular IgG. Biotin was detected with fluorescence conjugated streptavidin (Jackson ImmunoResearch) or with ABC kit (Vector) followed by developing with NovaRED peroxidase substrate kit (Vector) according to the manufacturer's instructions.

Image processing and analysis

Cerebral T cell, macrophage, and neutrophil infiltration, and microglial activation were quantified in a blinded manner in both the cortex and striatum at two coronal levels (0.2 and −0.5 mm relative to bregma). All images were processed with Image J for cell-based counting of automatically recognized cells. The mean was calculated from the three fields in the cortex or striatum of each section and adjusted to express as mean number of cells per square millimeter.

The length of CD31 immunopositive blood vessels was measured using the Image J in both the cortex and striatum at the same coronal levels described above and adjusted to the total length per square millimeter. For quantification of the length of the MMP-9^{+} blood vessels, 15 μm maximum project z-stacks were reconstructed, measured, and adjusted to the total length per square millimeter using Image J.

Fluorescence intensity of IgG extravasation was determined by the gray value of both the cortex and striatum part from the full view image of coronal sections, and was showed using the surface plot of the image J.

Zymography

Brain and blood samples were collected at 24 h after stroke for gel zymography. Anti-coagulated blood samples were immediately centrifuged at 4000 r.p.m for 15 min and plasma was collected and frozen at -80 ^{o}C until use. Brain samples were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM CaCl2, 0.05% BRIJ-35, 0.02% NaN3, 1% Triton X-100), and then centrifuged to obtain supernatants. Total protein concentrations were determined (Biorad). Forty mg of total protein, which was corrected for protein concentration, 5 μl plasma, and 1 μg of MMP-9 human standards (Millipore) were diluted in equal volume of 2x nonreducing sample buffer (0.4 M Tris, pH 6.8, 5% SDS, 20%

glycerol, 0.05% bromophenol blue). Samples were loaded and separated by a 10% Tris-glycine gel with 0.1% gelatin, and then washed with renaturing buffer (Invitrogen) for 90 min and further incubated with developing buffer (Invitrogen) at 37 ^{o}C for 30 h. The gels were then stained with 0.5% Coomassie blue R-250 (Biorad) for 1 h and destained with destain buffer (Biorad). Band intensities of pro- and active-MMP-9 were quantified using gel analysis of Image J and expressed as a fold change over sham group.

Transmission electron microscopy

Sample for transmission electron microscopy was collected at 48h after ischemia from the injured cortex and was fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) for 12h and 1% osmium tetroxide for 1h. After dehydration in an alcohol series, the tissues were embedded in epoxy resin. The ultrathin sections were prepared using a Reichert ultramicrotome, contrasted with uranyl acetate and lead citrate, and examined under a CM120 electron microscope at 80kv.

Analysis of neutrophil-derived MMP9 in vivo

Blood neutrophils from MCAO mice with or without Treg treatment were isolated as described above. After cell counting using a haemocytometer, the isolated neutrophils were lysed in lysis buffer (Cell Signaling), and cell lysates were subjected to MMP9 ELISA (R&D System).

Cytokine enzyme-linked immunosorbent assay.

Blood plasma, neutrophil lysate, and cell culture media were collected as described above. Protein concentrations were measured with commercial ELISA quantification kits for TNF-α, IL-10, IL-6, pro-MMP9 (R&D Systems) and neutrophil elastase (Biotang Inc.) according to the manufacturer's instructions. Pro- and active MMP9 activities were measured by a commercial kit (GE Health) according to the manufacturer's instructions.

References:

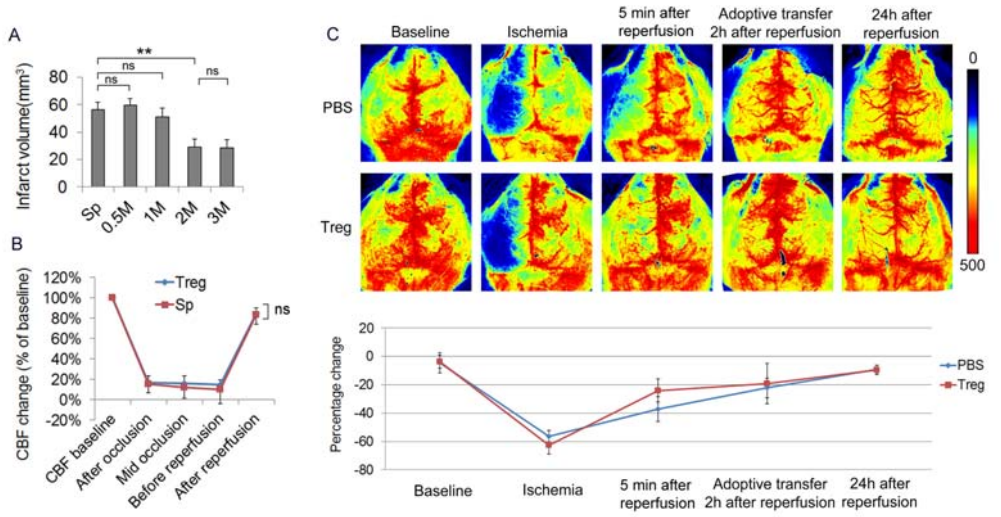
1. Zhang R, Xue YY, Lu SD, et al. Bcl-2 enhances neurogenesis and inhibits apoptosis of newborn neurons in adult rat brain following a transient middle cerebral artery occlusion. Neurobiology of disease. 2006 Nov;24(2):345-56.

2. Stetler RA, Cao G, Gao Y, et al. Hsp27 protects against ischemic brain injury via attenuation of a novel stress-response cascade upstream of mitochondrial cell death signaling. J Neurosci. 2008 Dec 3;28(49):13038-55.

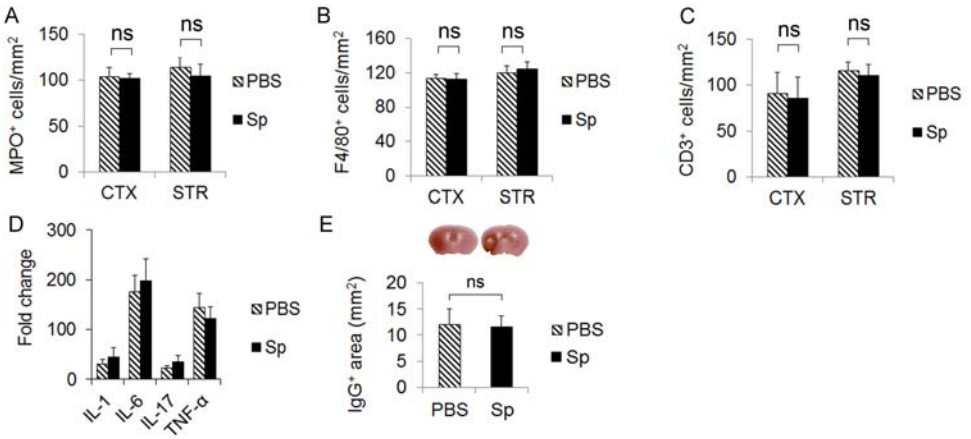
Supplementary Table 1. Primers for RT-PCR

Gene	Primer	Product size
IL-6	sens: agttgccttcttgggactga revs: tccacgatttcccagagaac	159 bp
IL1β	sens: gcccatcctctgtgactcat revs: agctcatatgggtccgacag	129 bp
TNFα	sens:agaagttcccaaatggcctc revs: ccacttggtggtttgctacg	120bp
IL-17	sens: tccctctgtgatctgggaag revs: ctcgaccctgaaagtgaagg	154bp
IL-10	sens: ccaagccttatcggaaatga revs: ttttcacaggggagaaatcg	162bp
TGFβ	sens: tgcgcttgcagagattaaaa revs: cgtcaaaagacagccactca	135bp

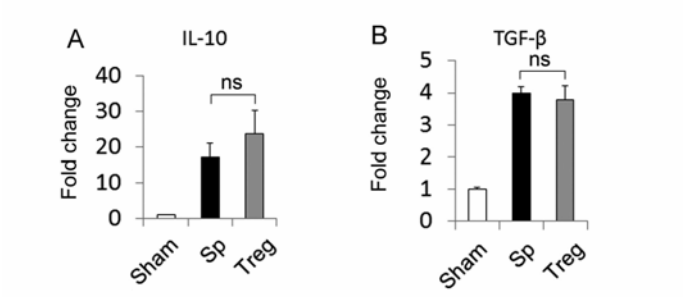
Supplementary Figures



Supplementary Figure 1. Delayed Tregs treatment improves neurological deficits after cerebral ischemia. Tregs were iv injected 2h after reperfusion. (A) Dose-effect of Treg treatment. M: 1×10^6 cells. (B) Regional cerebral blood flow (CBF) was measured before, during and after MCAO. Animals that showed CBF reduction of equal to or greater than 75% of pre-MCAO levels were randomly assigned to Tregs, splenocyte, or PBS treatment groups. Retrospectively, we found no statistical difference in CBF reduction during and after MCAO among the various experimental groups. (C) CBF monitored using 2-D laser speckle imaging techniques before MCAO, during MCAO and at 5 min, 2h, and 24h after reperfusion. Results were expressed as percent change from baseline (pre-MCAO). Treg-treated mice showed similar CBF reduction as compared to PBS-treated mice. n=3-4 per group. Data are mean ± SE. *P<0.05, ** P<0.01.



Supplementary Figure 2. Splenocyte-treated and PBS-treated MCAO mice developed same extent of cerebral inflammation and BBB leakage. (A-C) Quantification of the infiltration of MPO^+ neutrophilic granulocytes (A), $F4/80^+$ macrophages (B) and $CD3^+$ T cells (C) in the ischemic brains of PBS-treated and splenocyte-treated MCAO mice (n=4/group). (D) Quantitative real-time PCR for mRNA expression of IL-1β, IL-6, IL-17 and TNF-α in the ischemic hemispheres from animals with 60 min MCAO and 24h reperfusion. (n=4/group). (E) Quantification of IgG leakage determined by positive area of mouse IgG immunohistochemical staining. (n=4/group). There is no significant difference in the inflammatory cell infiltration or brain inflammatory cytokine levels between PBS-treated and splenocyte-treated groups. Data are mean ± SE.



Supplementary Figure 3. Tregs modulate post-ischemic inflammation without changing anti-inflammatory cytokines. Quantitative real-time PCR for mRNA expression of IL-10 (A) and TGF-β (B) in the ischemic hemispheres from animals with 60 min MCAO and 24h reperfusion. There is no significant change in the IL-10 and TGF-β mRNA levels in the brains between different treatment groups. n=6/group. Data are mean ± SE.

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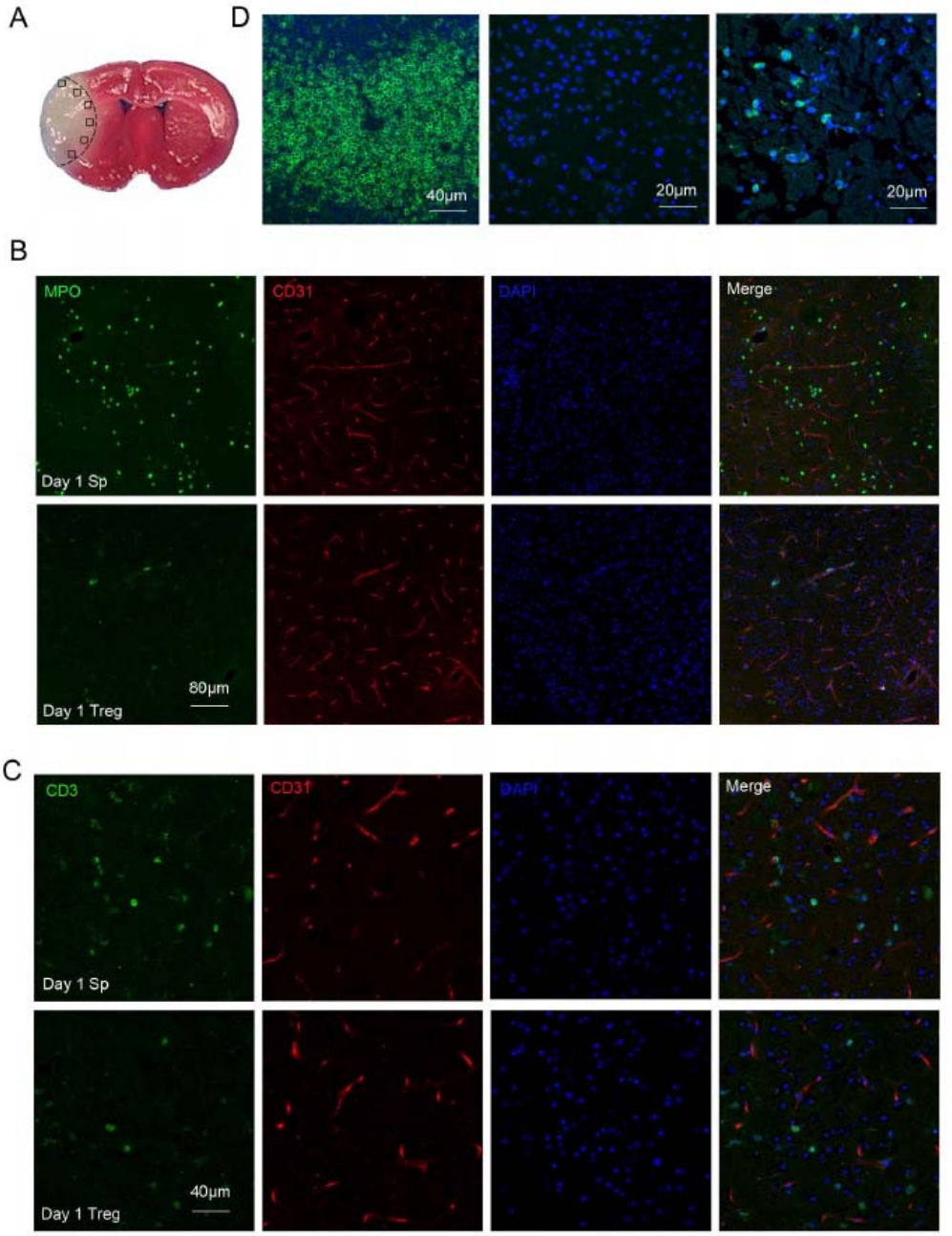

Supplementary Figure 4. Treg treatment inhibited the infiltration of neutrophils and T cells into the brain as early as 1 day after stroke. (A) Topographic map shows that MPO, CD3, F4/80 (BM8), and Iba1 staining was imaged in 6 regions (black boxes) at inner boundary of infarct. (B) The infiltration of MPO+ neutrophils in the brain was significantly decreased in the Treg-treated mice at 1 day after stroke. (C) The infiltration of CD3+ T cells in

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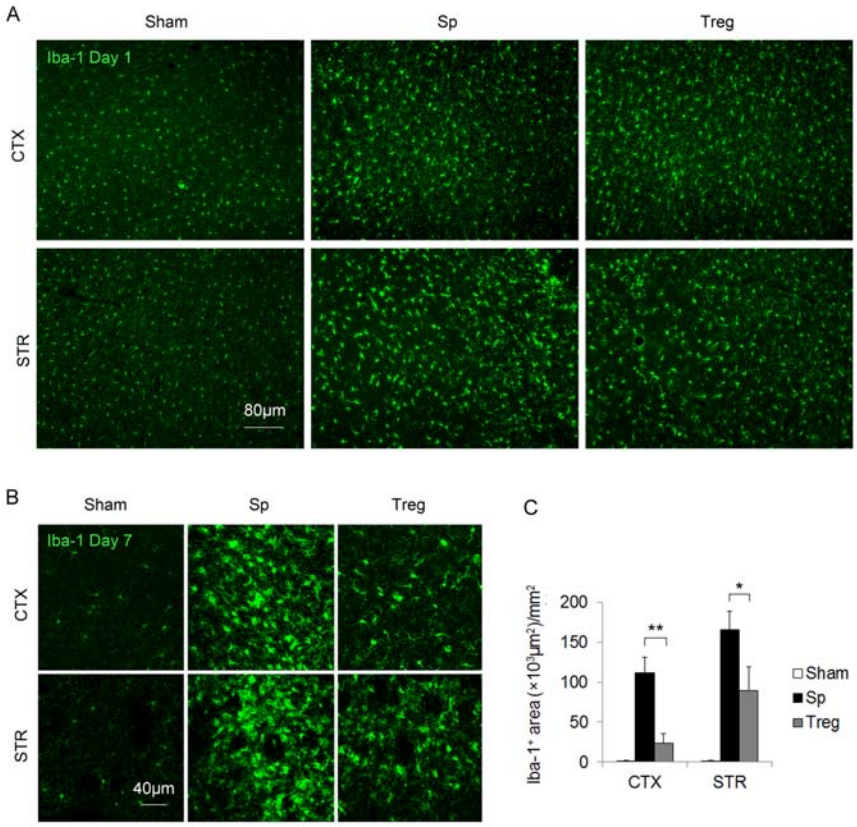
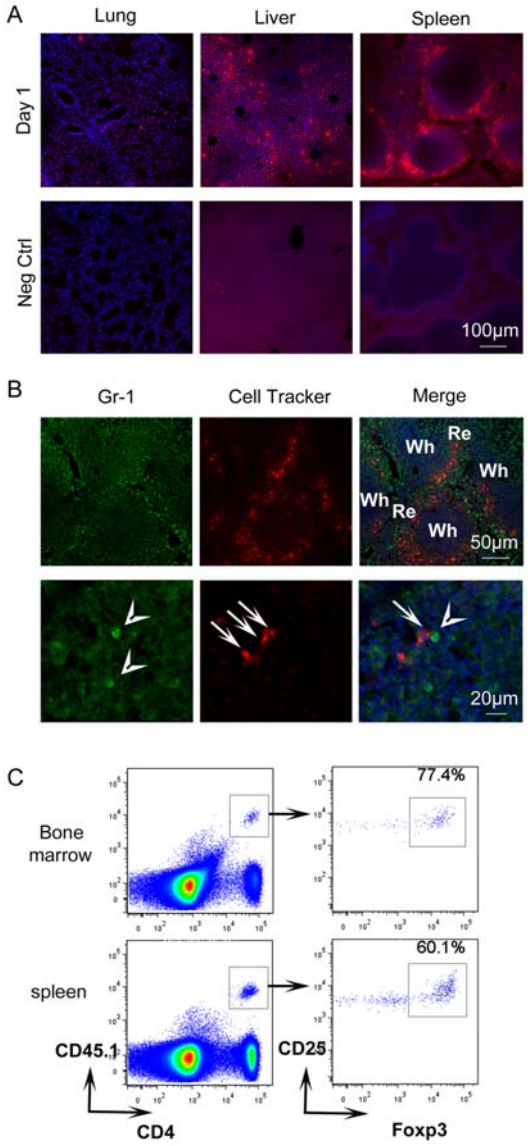
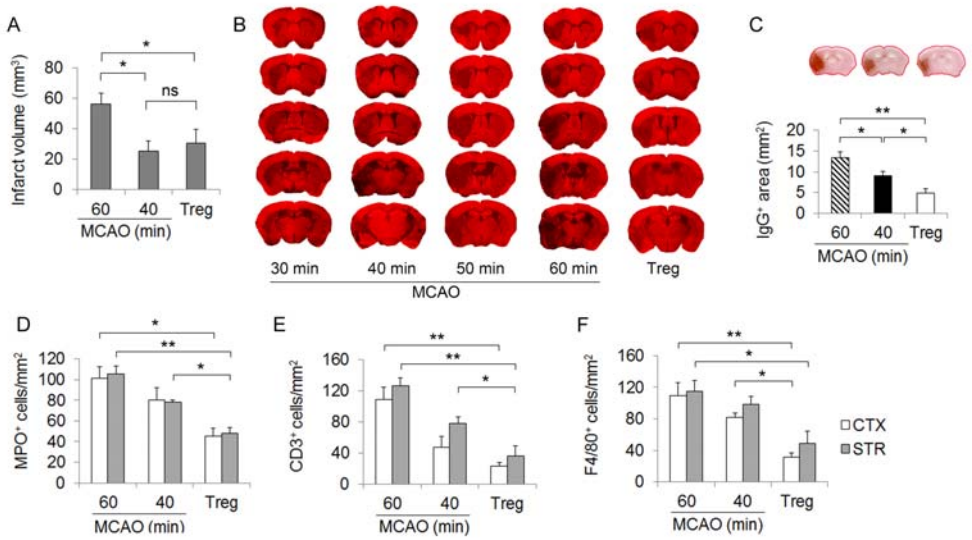
the brain was also decreased in the Treg-treated mice 1 day after stroke. Images are representatives of sections from six animals per group. (D) CD3 antibody specificity was confirmed using sham brain slice as negative control and spleen slice as positive control.



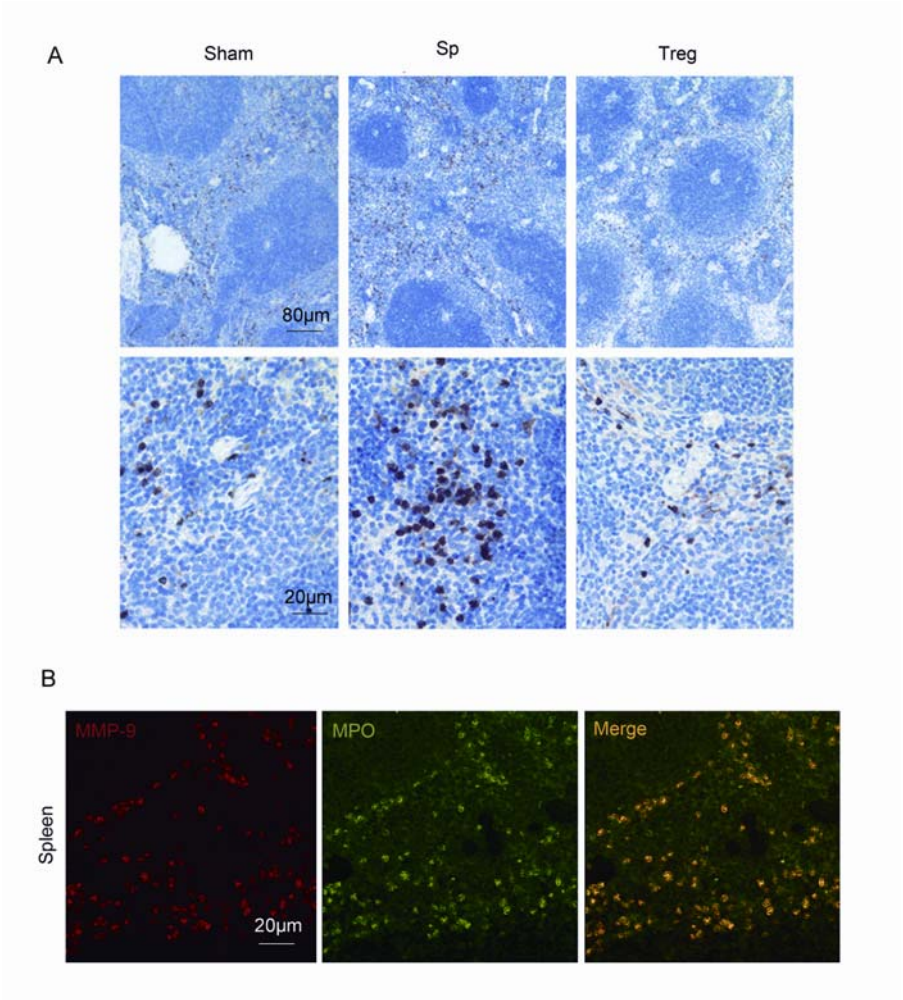
Supplementary Figure 5. Tregs treatment did not ameliorate microglia activation until 7 days after stroke. Microglia were labeled with Iba-1 on brain sections. (A) Iba-1 staining on brain sections obtained at 1 day after MCAO. (B) Iba-1 staining on brain sections obtained at 7 days after MCAO. Images are representatives of sections from six animals per group. (C) Quantification of Iba-1+ area/mm2 in the inner bounder of infarct zone at 7 days after MCAO (n=6/group). The activation of microglia did not differ between the Treg- or splenocyte-treated groups at 1 day after MCAO. At 7 days after stroke, Treg treatment significantly reduced the amount of Iba-1+ microglia compared to splenocyte-treated mice. Data are mean ± SE. *P<0.05, **P<0.01.



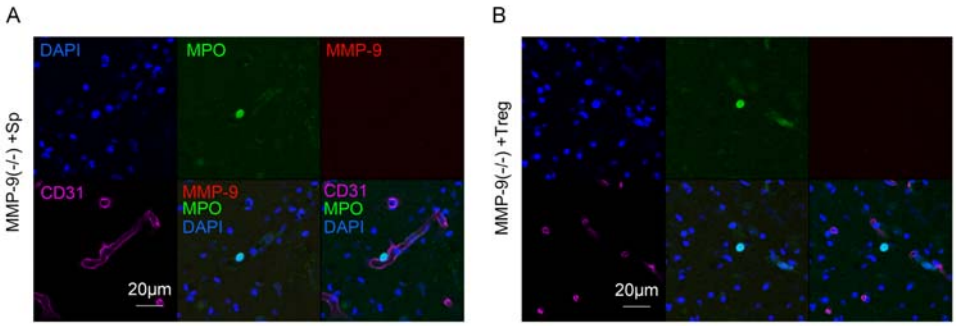
Supplementary Figure 6. Cell-tracker labeled Tregs traffic to the liver, lung and spleen at 24h after MCAO. Cell-tracker labeled Tregs were injected into the mice through tail vein at 2h after MCAO. Animals were sacrificed at 24h after MCAO. (A) Cell tracker-positive Tregs could be observed in the lung, liver and spleen. Red: cell tracker; blue: Dapi. (B) MPO staining in the spleen showing the contact between $Gr1^+$ neutrophil (green) and cell-$tracker^+$ Tregs (red). Upper panel: low power. Lower panel: high power. Arrow: cell-$tracker^+$ Tregs; arrow head: $Gr1^+$ neutrophils. Re: red pulp. Wh: white pulp. (C) Flow cytometry analysis showing adoptively transferred $CD45.1^+CD4^+$ cells present in the spleen and bone marrow at 1d after MCAO. Majority of these cells are positive for Treg markers CD25 and Foxp3.



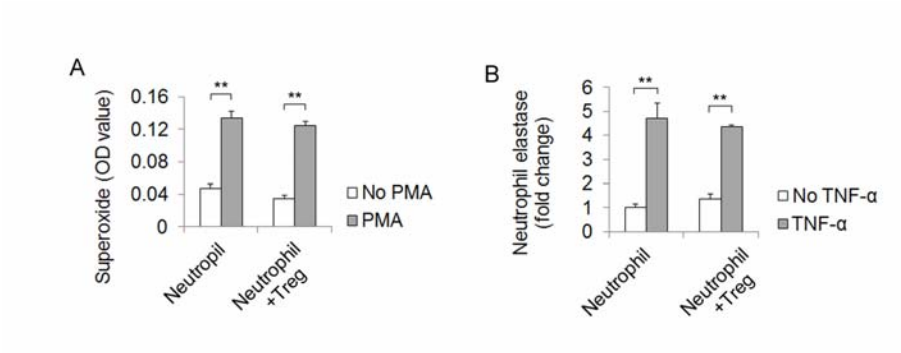
Supplementary Figure 7. BBB protection and anti-inflammation effect observed in Treg-treated animals are not the consequences of the reduced infarct volume. (A) Mice subjected to 40 minutes MCAO without treatment and mice subjected to 60 minutes MCAO with Treg treatment developed equivalent size of infarct. (B) Representative images of MAP2 staining showing cerebral lesion volumes in MCAO mice with different ischemia durations. Images are representatives of brain sections from four mice in each group. (C) Quantification of endogenous IgG positive area determined by immunohistochemical staining of mouse IgG. (D-F) Quantification for the infiltration of MPO^+ neutrophilic granulocytes (D), $CD3^+$ T cells (E), and $F4/80^+$ macrophages (F) in the ischemic brains. Treg-treated animals shown significantly reduced IgG extravasation and inflammatory cell infiltration as compared to untreated animals even when the cerebral lesion volumes were matched between the groups. N=3-4/group. Data are mean ± SE. *P<0.05, **P<0.01.



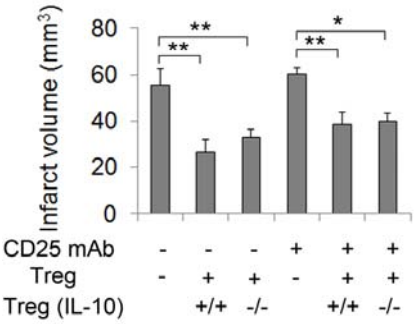
Supplementary Figure 8. Treg treatment inhibited the increase of MMP-9 expression in the spleen. (A) Immunohistochemical staining of MMP-9 of spleen sections from mice sacrificed 1 day after stroke. The number of MMP-9+ cells was significantly increased after stroke in the spleens of splenocyte-treated mice. Treg treatment effectively inhibited the increase of MMP-9 expression. (B) Double staining the MMP-9 and MPO on the spleen section confirmed the presence of MMP-9+/MPO+ neutrophils 1 day after MCAO. Images are representatives of sections from four animals per group.



Supplementary Figure 9. Treg-conferred suppression on immune cell infiltration was abolished in MMP-9 deficient mice. Triple staining of MMP-9, MPO and CD31 on brain sections from MMP-9(-/-) mice subjected to 60 min of MCAO and 3 days of reperfusion. The infiltration of MPO+ neutrophils after MCAO was significantly decreased compared to the wide type counterparts (shown in Figure 6I). Treg treatment did not further decrease the neutrophil extravasation on MMP-9(-/-) mice compared to splenocyte-treated mice. Images are representatives of sections from six animals per group.



Supplementary Figure 10. Tregs do not inhibit the production of superoxide and neutrophil elastase from neutrophils upon stimulation. (A) Tregs did not inhibit superoxide production from cultured neutrophils (n=4/group). Neutrophils isolated from blood and bone marrow were cultured with or without CD3/CD28 antibodies-primed Tregs, and subjected to PMA (100 ng/ml) for 10 min. The production of superoxide was measured by a commercial kit (National Diagnostics). (B) Tregs did not inhibit TNF-α-induced neutrophil elastase production from cultured neutrophils (n=6/group). TNF-α (100 ng/ml, 4h)-treated neutrophils were cultured with or without CD3/CD28 antibodies-primed Tregs for 24 hrs. The release of neutrophil elastase in the culture medium was measured. Neutrophil elastase level was expressed as fold change over non-treated neutrophil control. Data are mean ± SE. *P<0.05, **P<0.01.



Supplementary Figure 11. IL-10 is not a direct protective mediator for exogenous Treg-afforded early neuroprotection. Quantification of TTC-stained brain slices showing that transplantation of IL-10 deficient Tregs, either in the presence or absence of endogenous Tregs, protect the brain against ischemia at 3d after MCAO.