Regulatory T Cells Are Important Cerebroprotective Immunomodulators in Acute Experimental Stroke

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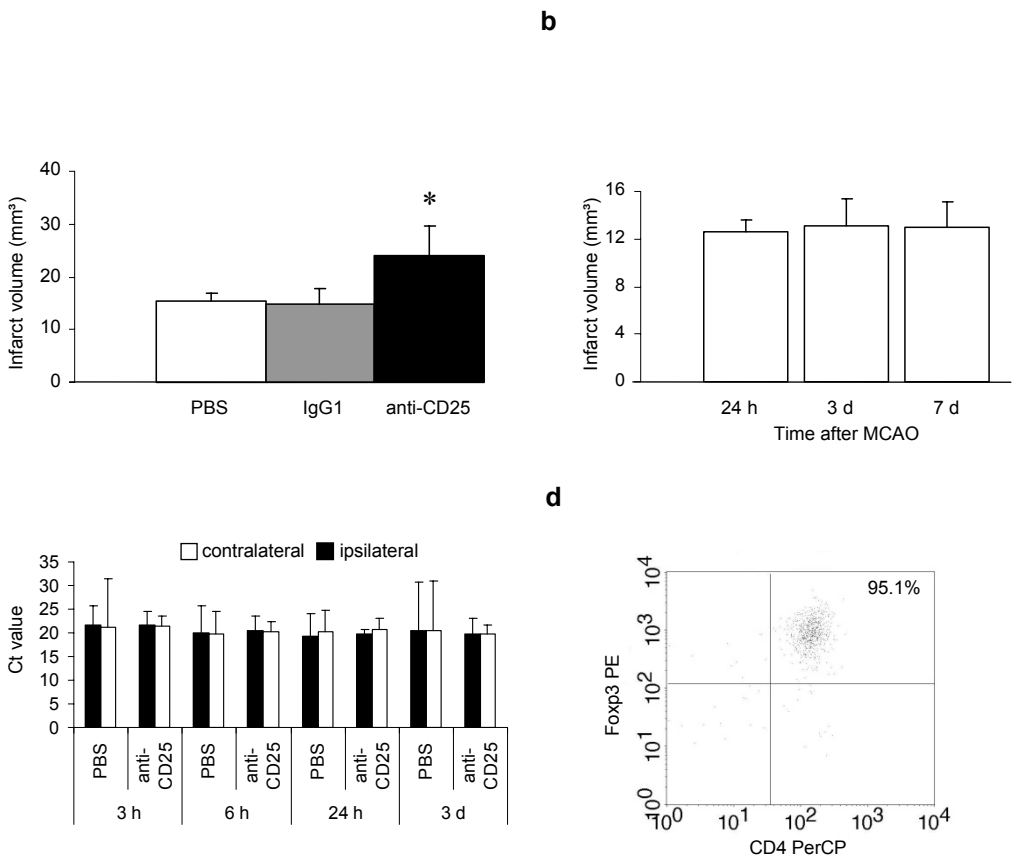
Nature Medicine: doi:10.1038/nm.1927



Supplementary Figure 1
Control experiments

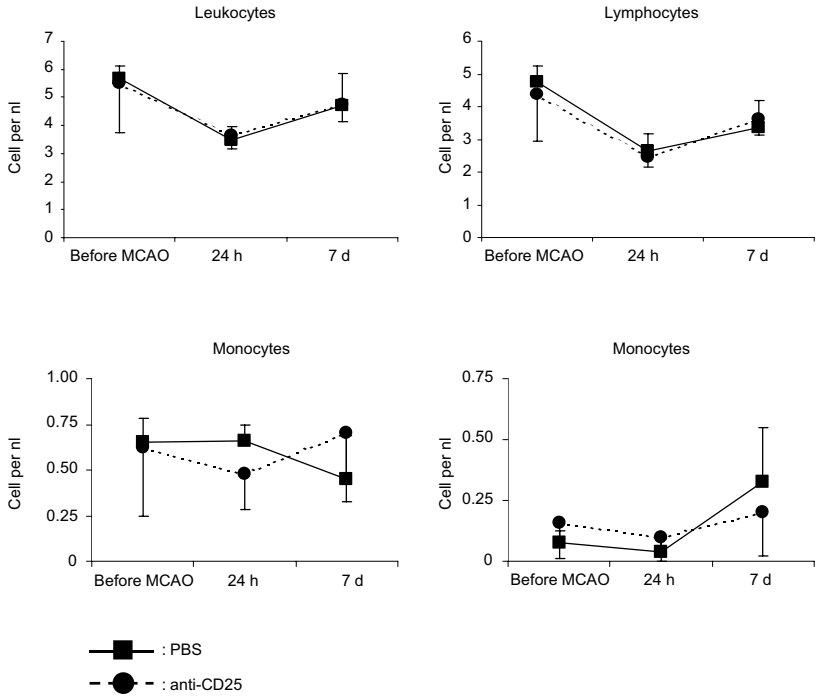
(**a**) Pretreatment with PBS and IgG1 isotype control 48 h before MCAO resulted in almost identical infarct volumes at day 7 after MCAO, while anti-CD25 injection significantly increased the lesion volume (n=10, p <0.001).

(**b**) Infarct volumes of *Rag2*$^{-/-}$ mice were similar at 24 h, 3 d and 7 d after MCAO.

(**c**) Treg depletion (anti-CD25) did not alter the expression of the housekeeping gene cyclophilin A (*Ppia*) at any measured time point after MCAO (Ct : cycle threshold for *Ppia*).

(**d**) Representative original FACS plot of MACS enriched regulatory T cells. Purity of regulatory T cells used for adoptive cell transfer experiments was >90% CD4^{+}Foxp3^{+} cells.

a

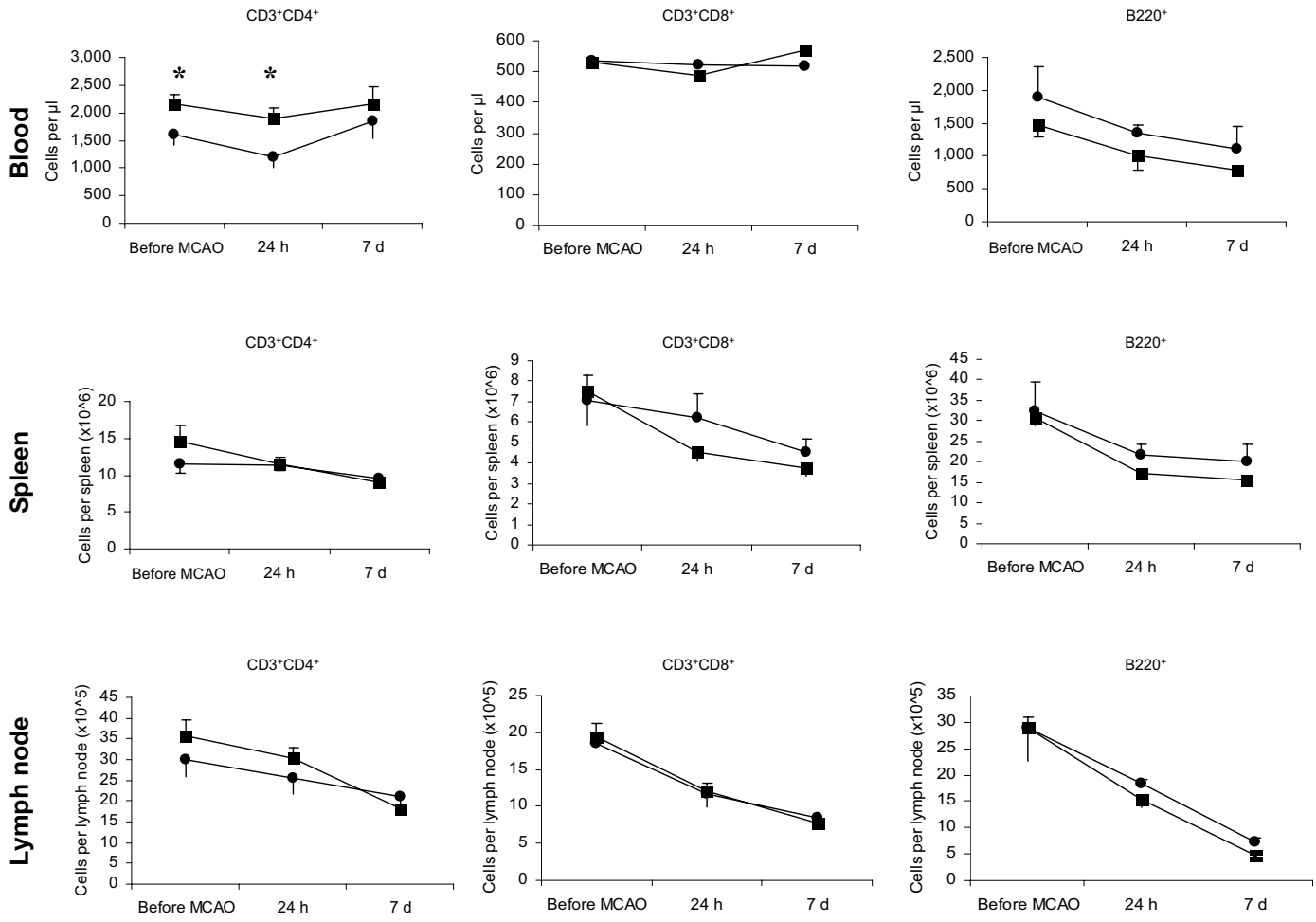


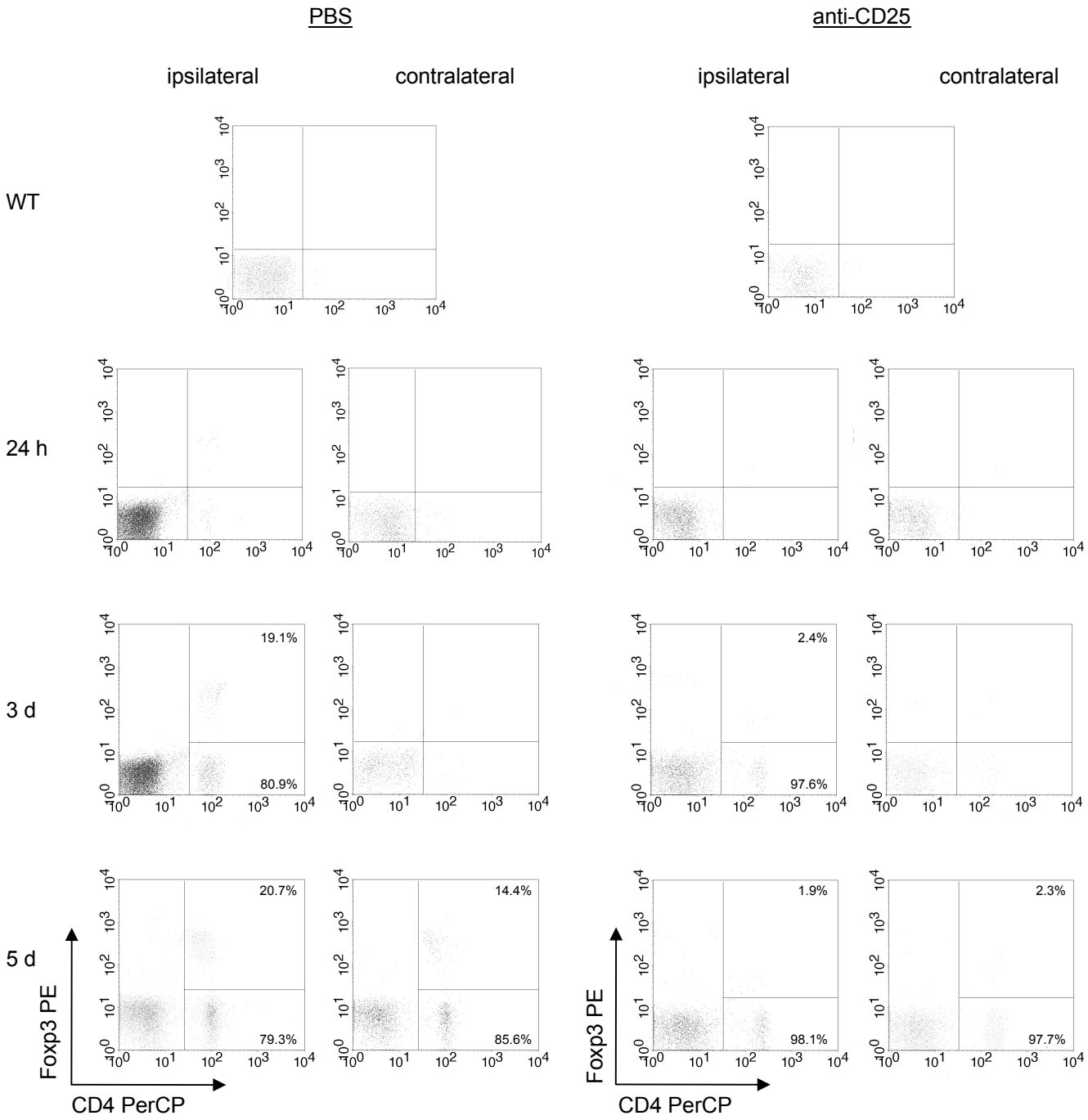
Supplementary Figure 2
The impact of anti-CD25 treatment on leukocyte subpopulations

(**a**) Treg depletion (anti-CD25) does not alter the number of blood circulating leukocytes and their subsets compared to control animals (PBS) before MCAO and 24 h and 7 d after stroke induction.

(**b**) Flow cytometric analysis of lymphocyte subpopulations in blood, spleen and lymph node revealed a significant reduction of cell counts in Treg depleted mice compared to PBS treatment only for T helper cells ($CD3^+CD4^+$) in the blood before and 24 h after MCAO (* = $p<0.05$, n=5 per group), compatible with the proportion of depleted Treg.

b

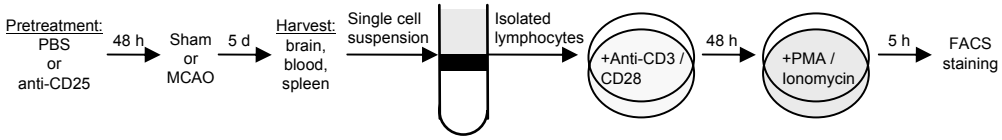




Supplementary Figure 3 The brain invasion of Treg after MCAO

The presence of $CD4^+Foxp3^+$ regulatory T cells was analysed by flow cytometry in brains of unaffected mice (WT) and in the ipsilateral (ischemic) and contralateral (non-ischemic) hemispheres at 24 h, 3 d and 5 d after MCAO in control (PBS) and Treg depleted (anti-CD25) mice. Values in the plots indicate the percentage of Foxp3 positive / negative cells in the $CD4^+$ population, if detectable, respectively. Representative plots of 3 independent experiments.

a



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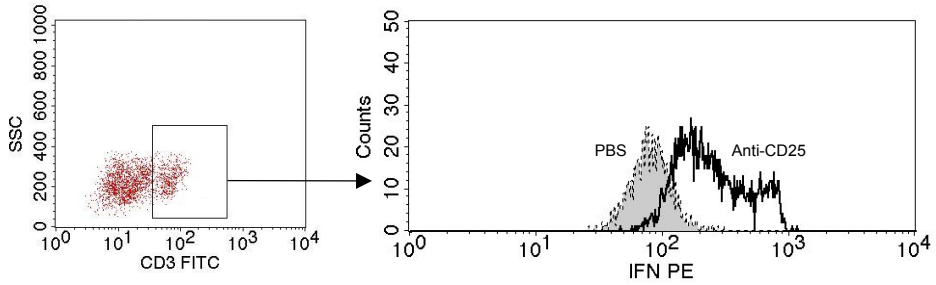

Supplementary Figure 4
Experimental protocol and plots of intracellular cytokine FACS

(**a**) Pretreatment protocol for cells used for flow cytometric analysis of intracellular cytokine production. Lymphocytes were isolated from the ischemic hemisphere (as well as from blood and spleen, respectively) of anti-CD25 and PBS treated mice 5 d after MCAO. Brain invading lymphocytes were isolated from homogenized brain hemispheres using dicontinuous Percoll gradients. The cells were primed for 48 h with anti-CD3 / anti-CD28 and restimulated for 5 h (PMA / Ionomycin) before respective intracellular cytokine FACS analysis.

(**b**) In $CD3^+$ T cells isolated from brain, spleen and blood the quantity of intracellular IFN-γ molecules per cell was measured by quantitative FACS. Two representative original plots of 7 individual experiments are shown. The dot blot on the left indicates gating of $CD3^+$ T cells and the histogram on the right shows IFN-γ-PE fluorescence intensity of cells isolated from ischemic brains 5 d after MCAO from Treg depleted (anti-CD25) and control mice (PBS).

Supplementary methods

Coagulation model. We used this model for all experiments, except for data depicted in Fig. 1d. Mice were anesthetized with 1.0–2.0% halothane in O_2/N_2O. After a 1 cm skin incision between left eye and ear, we drilled a burr hole through the temporal skull. We removed the dura mater and occluded the middle cerebral artery (MCA) permanently using a bipolar electrocoagulation forceps (ERBOTOM, Erbe). For laser doppler measurements, we placed the probe (P403, Perimed) 3 mm lateral and 6 mm posterior to the bregma and obtained relative perfusion units (Periflux 4001, Perimed). During the operation, body temperature was kept at 37 °C using a feedback controlled heating pad. We stitched the skin lesion and placed the mouse in a cage under an infrared heating lamp until recovery from anaesthesia. Sham treatment was given according to the same surgical protocol as for MCAO but without coagulation of the MCA. The overall mortality in the coagulation model was less than 5% for a 7 d observation period.

Filament model. We used this model exclusively for experiments shown in Fig. 1d. Mice were anesthetized with 1.0–2.0% halothane in O_2/N_2O enriched air. We placed the laser doppler probe over the cortical area supplied by the MCA (3 mm lateral and 6 mm posterior to the bregma). Baseline CBF was measured as relative perfusion units and defined as 100% flow. After neck dissection, we made an incision into the external carotid artery (ECA) between two ligations and advanced a silicon-covered 8-0 nylon monofilament through the Internal Carotid Artery to occlude the MCA. MCA occlusion was documented as a decrease of relative perfusion values by laser doppler to less than 20% of primary flow. We fixed the filament in this position by ligation, closed the neck, removed the doppler probe and placed the mouse to its cage. 30 min or 90 min, respectively, after filament insertion the mouse was re-anesthetized and the filment removed. After we closed the surgical wound, the mice were transferred to their cages with free access to water and food. During the operations body temperature was kept at 37 °C with a feedback controlled heating pad. We maintained normal body temperature of the mice between operations and until recovery after the procedure by an infrared heating lamp. Sham operation was performed identically as described above, including re-anesthesia 30 min or 90 min after Sham surgery, except for only brief introduction of the filament into the ECA. The overall mortality in the 30 min filament model was less than 5%, while the mortality was 25% after 90 min filament occlusion for a 7 d observation period.

Functional outcome tests. For the “forelimb use asymmetry test”; we placed the mice in a transparent glass cylinder and videotaped them for 3–10 min, depending on degree of movement. The independent use of the forelimb was analyzed by a video player with slow motion and frame-by-frame capabilities (VLC Media Player). To assess independent use of left or right forelimb we scored (1) contact of the cylinder wall with one forelimb during a full rear and (2) landing with only one forelimb at the cylinder bottom after a full rear. At least 20 independent contacts were counted for one forelimb and each experiment was performed twice, with a 1 h break for the mouse between testing. Forelimb use asymmetry was expressed as a ratio of right- and left-sided, independent forelimb use.
For the “corner test”, we placed the mice between two boards set at a 30 ° angle and allowed to move freely. We counted left and right turns with a rearing movement after deep entry in the corner and calculated the ratio of right and left turns as an indicator

of behavioural asymmetry. At least 12 full turns after a rearing movement were counted for each testing.

Intracerebroventricular injections. Mice were anesthetized with 1.0–2.0% halothane in O_2/N_2O enriched air and fixed in a stereotactic frame (Stoelting). After a midline incision of the skin, we used a 10 µl Hamilton syringe for i.c.v. drug administration and injected control vehicle [Bovine serum albumin (BSA) in 2 µl artificial cerebrospinal fluid (aCSF) consisting of mM: 126 NaCl; 2.5 KCl; 1.2 NaH_2PO_4; 1.3 $MgCl_2$ and 2.4 $CaCl_2$ at pH 7.4], or 100 ng IL-10 [recombinant mouse protein (rmIL-10, LabGEN) in 2 µl aCSF] immediately after MCAO 0.9 mm lateral, 0.1 mm posterior, and 3.1 mm deep relative to the bregma. We achieved i.c.v. cytokine antagonization by a single injection of 1 µg neutralizing mouse TNF-α-specific antibodies (Clone MP6-XT22, eBioscience) or 1 µg neutralizing antibodies against mouse IFN-γ (Clone XMG1.2, eBioscience) diluted in 2 µl aCSF.

Assessment of infarct volume. We deeply anesthetized the mice with tribromoethanol and perfused them transcardially with 20 ml normal saline. We removed the brains from the skull and froze them immediately in isopentane (-20 °C). We cut 20 µm thick coronal cryosections every 400 µm, stained the sections by the silver staining protocol, scanned them at 600 dpi, and measured the infarct area using a public domain image analysis program (Scion Image). The total infarct volume was determined by integrating measured areas and distances between sections. We applied correction for brain edema by subtracting the ipsilateral minus contralateral hemisphere volume from the directly measured infarct volume.

Isolation of brain invading lymphocytes. We perfused the mice transcardially with 20 ml normal saline and removed the brains immediately from the skull. We split the hemispheres, mechanically homogenized the tissue after incubation in dissociation buffer (10 mL RPMI-1640, 180 units collagenase IV, 250 units Dnase), and overlaid the cell suspension on Percoll gradients of 1.03 and 1.088 g mL^{-1} density. The collected mononuclear cells from the interphase were processed for flow cytometry. For intracellular cytokine staining, we plated single cell suspensions of 300,000 blood mononuclear cells on 96-well plates (RPMI-1640, + 10% FCS, + Glutamine, + HEPES, + Penicillin/Streptomycin, + Mercaptoethanol). Each sample was processed in triplicate. We primed the cells for 48 h with plate-bound anti-mouse CD3e (Clone145-2C11, BD Biosciences) and 2 µg ml^{-1} soluble anti-mouse CD28 (Clone 37,51, BD Biosciences), followed by restimulation with 500 ng Calcimycin (Sigma-Aldrich), 5 ng ml^{-1} PMA (Sigma-Aldrich), and protein transport inhibitor (BD Golgi Plug, BD Biosciences) for 5 h.

FACS Analysis. We stained the respective single cell suspensions for anti-mouse CD3 (Clone 17A2), CD4 (Clone RM 4-5), CD25 (Clone 7D4), Foxp3 (Clone FJK-16s), IFN-γ (Clone XMG1.2), IL-4 (Clone 11B11), CD11b (Clone M1/70), and the appropriate isotype control by following the manufacturer's protocols (eBioscience). We performed flow cytometry on a Becton Dickinson FACS Calibur and analyzed the data by CellQuest Pro software. Gates were set according to unstained samples and isotype control; compensation was adjusted using BD CaliBRITE Beads (BD Bioscience). We used quantitative calibration with the BD QuantiBRITE PE Beads (BD Bioscience) for flow cytometric quantification of intracellular cytokines per cell and QuantiQuest software to standardize the quantification in terms of antibodies bound per cell following the manufacturer's protocol.

Immunohistology. We used 4 µm thick paraffin-embedded coronal brain sections for intracellular staining of Foxp3 using Retrievagen A and the anti-rat Ig HRP Detection Kit (both from BD Pharmingen) and DAB (Dako) with rat anti-mouse primary Foxp3-specific antibodies (Clone FJK-16s, eBioscience).
We performed immunohistochemistry to determine the regional distribution of mature T lymphocytes and neutrophilic granulocytes on coronal cryostat sections (12 µm). After pretreatment with 4% PFA for 60 min, we blocked endogenous peroxidase by Peroxidase Blocking solution (Dako), then incubated the sections with primary antibodies against CD3 (Clone 3H698, Zytomed) or myeloperoxidase (Clone RP-053; Zytomed) for 60 min at 21 °C. Immunoreactivity was visualized by a universal immunoenzyme polymer method (Nichirei Biosciences) and sections developed in diaminobenzidine.
We investigated the distribution of microglia by immunohistochemical staining of coronal cryostat brain sections (12 µm) for IBA1 (Wako). Immunoreactivity was visualized by the avidin-biotin complex method and sections developed in diaminobenzidine.
For analysis of cell number in the peri-infarct area and the corresponding contralateral area, we captured the images on a Zeiss Axiovert 200M microscope. Blinded samples were analyzed by a technician not informed about treatment groups.

Combination of immunocytochemistry with *in situ* hybridization. We deeply anesthetized mice and rapidly perfused them transcardially 24h after MCAO with 0.9% saline, followed by 4% paraformaldehyde in 100 mM borax buffer (pH 9.5, 4 °C). We removed the brains from the skull, cut them in 25 µm coronal sections, and mounted every 12th section on Colorfrost/Plus. *In situ* hybridization for the proinflammatory cytokine TNF-α was performed by the following protocol: We dehydrated slides, dried them, and incubated the slides overnight with hybridization mixture. After exposure to an x-ray film (Kodak) for 17 h at 4 °C, we defatted the slides in xylene, dipped them in NTB2 nuclear emulsion (Kodak), and exposed them for 21 d. We developed the slides in D19 developer and fixed them in rapid fixer (Kodak). The anti-mouse IBA1-specific antibody was used to stain microglial cells by using the avidin-biotin bridge method with peroxidase as a substrate and developed in PBS with DAB (0.05%) and 0.003% H_20_2. We acquired images using a digital camera (DC500, Leica) on a Leica microscope (DM-R HC).