Mice, stroke model and FTY720 treatment
A total of 235 C57Bl/6 and 24 Rag1−/− mice were used in this study. Animal experiments were approved by legal state authorities (Regierung von Unterfranken and Regierungspräsidium Darmstadt) and conducted according to the recommendations for research in experimental stroke studies1 and the current ARRIVE guidelines (http://www.nc3rs.org/ARRIVE). Focal cerebral ischemia was induced in 6-8-weeks old male mice (Harlan Winkelmann) by 60 min or 90 min transient middle cerebral artery occlusion (tMCAO) as previously described.2,3 Mice were anesthetized with 2.5% isoflurane (Abbott). Following a midline skin incision in the neck, the proximal common carotid artery and the external carotid artery were ligated and a standardized silicon rubber-coated 6.0 nylon monofilament (60SPPK10; Doccol Corp.) was inserted and advanced via the right internal carotid artery to occlude the origin of the right MCA. The operators were blinded to the treatment groups and the maximum operation time per animal did not exceed 15 minutes. Immediately before the removal of the filament FTY720 (1 mg/kg bodyweight, Cayman Chemical, 10006292) or vehicle (0.9% sodium chloride) was applied by a single intraperitoneal (i.p.) injection. After that, cerebral reperfusion was allowed by removing the filament.

The following conditions excluded mice from end-point analyses (exclusion criteria):
1. Death within 24h after MCAO
2. Subarachnoidal hemorrhage (SAH) or bleeding into the brain parenchyma (as macroscopically assessed during brain sampling)
3. Bederson score = 0 (immediately after reperfusion)
4. Operation time > 15 minutes

Determination of stroke size
Animals were sacrificed 24h after MCAO. Brains were removed and cut into three 2mm thick coronal slices using a mouse brain slice matrix (Harvard Apparatus). The sections were stained with 2,3,5-triphenyltetrazoliumchloride (TTC) for 20 min at 37°C and edema-corrected infarct volumes were quantified by planimetry (ImageJ software, National institutes of health) according to the following equation:

\[ V_{\text{indirect}}(\text{mm}^3) = V_{\text{infarct}} \times (1-(VI – VC)/VC), \]

with the term (VI – VC) representing the volume difference between the ischemic hemisphere and the control hemisphere and (VI – VC)/VC expressing this difference as a percentage of the control hemisphere.

Assessment of functional outcome
24h after tMCAO the modified Bederson score4 was used to determine global neurological function according to the following scoring system: 0, no deficit; 1, forelimb flexion; 2, decreased resistance to lateral push; 3, unidirectional circling; 4, longitudinal spinning; 5, no movement. Motor function and coordination were evaluated by the grip test.5 For this test, the mouse was placed midway on a string between two supports and rated as follows: 0, falls off; 1, hangs onto string by one or both forepaws; 2, as for 1, and attempts to climb onto string; 3, hangs onto string by one or both forepaws plus one or both hindpaws; 4, hangs onto string by fore- and hindpaws plus tail wrapped around string; 5, escape (to the supports). Neurological
scores were assessed by an independent investigator blinded to the experimental conditions of the mice.

**Laser-Doppler flowmetry**
Laser-Doppler flowmetry (Moore Instruments) was performed in FTY720- and vehicle treated wild-type mice before (baseline), during (ischemia) and immediately after tMCAO (reperfusion) as well as after 12h and 24h. The regional cerebral blood flow (CBF) was measured in the territory of the right middle cerebral artery (MCA) (6 mm lateral and 2 mm posterior from bregma).6

**Invasive hemodynamics**
For the assessment of blood pressure and heart rate, FTY720- and vehicle treated wild-type mice were anesthetized with 2.0% isoflurane and catheterized via the right carotid artery with a high-fidelity 1.4 F Millar microtip catheter (Milar Instruments) as described.7

**Blood gas analysis**
100µl of arterial blood was drawn from the left cardiac ventricle of anesthetized mice by a heparinized syringe. We determined PaO₂, PaCO₂ and pH in FTY720- and vehicle-treated mice using an ABL 77 automated blood gas analyzer (Radiometer).

**Counting of platelets and erythrocytes**
50 µl of blood were drawn from the retroorbital plexus of anesthetized mice by siliconized microcapillaries and collected in a 1.5 ml tube containing 300 µl of heparin in TBS (20 U/ml, pH 7.3). We determined platelet counts and size as well as erythrocytes using a Sysmex KX-21N automated haematology analyzer (Sysmex).

**Determination of blood-brain barrier leakage**
To determine blood-brain-barrier leakage 100 µl Evans Blue (2% diluted in 0.9% NaCl) (Sigma) were injected i.v. 1h after the induction of tMCAO.8 After 24h, mice were sacrificed and brains were quickly removed and cut into 2 mm thick coronal sections using a mouse brain slice matrix (Harvard Apparatus). Afterwards, brain slices were fixed in 4% PFA at 4°C for 2h in the dark. Then, brain slices were cut into small pieces and transferred into Eppendorf tubes. 500 µl formamide were added to each tube and incubated for 24h at 50°C in the dark. Tubes were centrifuged for 20 min at 16.000 g and 50 µl of the supernatant were transferred to a 96 well plate. Fluorescence intensity was determined in duplicates by a microplate fluorescence reader (Fluoroskan Ascent, Thermo Scientific) with an excitation at 620 nm and emission at 680 nm. The concentration of EB in the brain tissue was calculated for each sample from a standard curve using linear regression analysis.

**Protein extraction and Western blot analysis**
After TTC staining, cortices or basal ganglia were dissected from formalin-fixed brain slices and homogenized in RIPA buffer (25mM Tris pH 7.4, 150mM NaCl, 1% NP40) containing 0.1% SDS and 0.25% proteinase inhibitor. The samples were sonicated for 10 sec. After that, tissue lysates were centrifuged at 15.000 x g for 30 min at 4°C and supernatants were used for BCA protein assay and subsequent Western blot analysis.

The total lysates were treated with 4x SDS-PAGE loading buffer (final concentration: 62.5 mM Tris pH 6.8, 3% beta-mercaptoethanol, 8% SDS, 15% glycerol) at 95°C for 5 min. 20 µg of total protein was electrophoresed and transferred to a PVDF
membrane. After blocking for 30 min with blocking buffer (5% nonfat dry milk, 50 mM Tris-HCl pH 7.5, 0.05% Tween-20), membranes were incubated with the primary antibody at 4°C overnight at the following dilutions: anti-fibrinogen pAb 1:500 (cross reactive for fibrin and fibrinogen, Acris Antibodies), anti-occludin pAb 1:1000 (Abcam), and anti-actin mAb 1:75,000 (Dianova). After a washing step with TBST (50 mM Tris-HCl pH 7.5, 0.05% Tween-20), membranes were incubated for 1h with HRP-conjugated donkey anti-rabbit IgG (for fibrinogen and occludin) (Dianova) or donkey anti-mouse IgG (for actin) (Dianova) at a dilution of 1:5000 and were finally developed using ECLplus (GE Healthcare) and a Kodak X-Omat 5000 RA developer (Kodak). Bands were quantified by densitometric analysis using ImageJ software (National Institutes of Health) and normalized to the actin band, which served as loading control.9,10

Immunohistochemistry and histology
Cryo-embedded brains were cut into 10-µm thick sections on day 1 after MCAO. We pretreated the sections with 10% BSA and 1% goat serum with 0.2% Triton-X 100 for 30 min to prevent unspecific binding. For specific staining, the following antibodies were added overnight at 4°C: CD31 (mouse anti-mouse, abcam, ab9498, 1:100) and fibrinogen (rabbit anti-mouse, acri, AP00766PU-N, 1:100). Subsequently, slices were incubated with Dylight488-coupled goat anti-mouse (abcam, 96871, 1:100), AlexaFluor594 (invitrogen, A11012, 1:100) and biotinylated horse anti-mouse (vector, BA-2001, 1:100) in PBS containing 1% BSA. Sections were analyzed under a microscope (Nikon Eclipse 50i) equipped with a CCD camera. Negative controls included omission of primary or secondary antibody and gave no signals (not shown). For calculation of the thrombosis index, the whole brain was sliced 24h after tMCAO (slice thickness 10 µm). H&E staining was performed according to standard procedures. For quantification, stainings were examined in a blinded fashion and the number of occluded blood vessels within the ischemic hemispheres was counted in every tenth slice for FTY720-treated and untreated animals under a 40-fold magnification.

Cell separation
For the isolation of brain infiltrating mononuclear cells 24h after tMCAO, mice were transcardially perfused with 1x PBS supplemented with 1% heparin. Ischemic brain hemispheres were collected in 1x PBS, mechanically homogenized and transferred into a Percoll (GE Healthcare) density gradient (50%/30%) and centrifuged at 600 x g for 30 min without break. Mononuclear cells were collected from the interface of the Percoll gradient, washed and resuspended in 1 x PBS for further analysis. For flow cytometry analysis of peripheral immune cells, 150-200 µl blood was harvested transcardially in heparin-coated tubes and red blood cells were lysed using RBC lysis buffer (Biolegend) following the manufacturer's description. The amount of T lymphocytes within the brain vasculature was calculated form the difference (delta) between cell numbers from the brains of non-perfused mice and the brains of perfused mice.

Flow cytometry
Myeloid immune cells and lymphocytes were incubated in FACS buffer (1x PBS, 0.1% BSA, 0.1% sodium azide) with monoclonal antibodies (anti-CD4-PerCP (BD Bioscience, 553052), anti-CD8a-PE (BD Bioscience, 553032)) at 4°C for 30 min. Isotype controls were: anti-rat IgG2b PerCP (BD Bioscience, 550764) and anti-rat IgG2b PE (BD Bioscience, 556925). Subsequently, cells were washed and
resuspended in FACS buffer and subjected to flow cytometry using a FACSCalibur (Becton Dickinson). FlowJo software (Tree Star) was used for analysis.

**Platelet adhesion under flow conditions**
Rectangular coverslips (24 x 60 mm) were coated with 0.2 mg/ml fibrillar type I collagen (Nycomed) for 1h at 37°C and blocked with 1% bovine serum albumin (BSA). Heparinized whole blood was perfused as previously described. Image analysis was performed offline using Metavue software (Visitron). Thrombus formation was expressed as the mean percentage of total area covered by thrombi.

**Intravital microscopy of thrombus formation in FeCl₃-injured mesenteric arterioles**
Mice were anesthetized, and the mesentery was exteriorized through a midline abdominal incision. Arterioles measuring 35 to 60 µm in diameter were visualized at 10-fold magnification with an inverted microscope (Axiovert 200; Carl Zeiss) equipped with a 100-WHBO fluorescent lamp source and a camera (CoolSNAP-EZ; Visitron). Digital images were recorded and analyzed offline using Metavue software. Injury was induced by topical application of a 3-mm² filter paper saturated with 20% FeCl₃ for 10 seconds. Adhesion and aggregation of fluorescently labeled platelets (DyLight 488–conjugated anti-GPIX Ig derivative, Emfret Analytics) in arterioles was monitored for 40 minutes or until complete occlusion occurred (blood flow stopped for more than 1 minute).
Supplemental references


<table>
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<tr>
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<td>3</td>
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**Supplemental Table I** Blood gas analysis in vehicle- or FTY720-treated wild-type mice. No significant differences were observed between the groups. n.s.=not significant, unpaired, two-tailed Student’s t-test.
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<thead>
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<th>Vehicle</th>
<th>FTY720 (1 mg/kg bodyweight)</th>
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<td>n</td>
<td>5</td>
<td>5</td>
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<tr>
<td>Platelet count (x10^3/µl)</td>
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<td>Platelet volume (fl)</td>
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<td>5.6 ± 0.29</td>
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<tr>
<td>Red blood cell count (x10^3/µl)</td>
<td>6.6 ± 0.31</td>
<td>6.2 ± 0.27</td>
<td>n.s.</td>
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**Supplemental Table II** Platelet counts, platelet volumes, and red blood cell counts in vehicle- and FTY720-treated C57Bl/6 wild-type mice. No significant differences were observed between the groups. n.s.=not significant, unpaired, two-tailed Student’s t-test.
Supplemental Figure I  Mean arterial blood pressure (RR) and heart rate are unaltered in vehicle- and FTY720-treated wild-type (WT) mice as assessed by invasive hemodynamics. n=5/group, n.s.=not significant, unpaired, two-tailed Student's t-test.
Supplemental Figure II FTY720 induces rapid lymphocytopenia in the peripheral blood. (A) Total blood leukocyte counts in C57Bl/6 wild-type mice before, 3h and 24h after administration of FTY720 (1 mg/kg) as assessed by flow cytometry. n=4/group, ***p<0.0001, 1-way ANOVA followed by Bonferroni multiple comparison test. (B) Fraction of CD4+ and CD8+ T cells calculated from the total number of viable cells before, 3h and 24h after administration of FTY720. n=4/group, ***p<0.0001, 2-way ANOVA followed by Bonferroni multiple comparison test.
Supplemental Figure III FTY720 induces rapid lymphocytopenia also in the cerebral vasculature. Number of T cells (CD4+ plus CD8+ lymphocytes) in the cerebral vasculature of vehicle-treated wild-type (WT) mice or FTY720-treated WT mice on day 1 after tMCAO as assessed by flow cytometry. In both groups, lymphocytes were harvested from the ischemic brains of non-perfused and perfused mice. The difference (delta) in cell counts between non-perfused and perfused brains represents the amount of T lymphocytes exclusively in the cerebral vasculature. Note, that FTY720 treatment also led to a significant reduction in T cell numbers within the brain vasculature. n=10/group, *p<0.05, unpaired, two-tailed Student’s t-test.