Original Contribution

FTY720 Ameliorates Acute Ischemic Stroke in Mice by Reducing Thrombo-Inflammation but Not by Direct Neuroprotection

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Background and Purpose—Lymphocytes are important players in the pathophysiology of acute ischemic stroke. The interaction of lymphocytes with endothelial cells and platelets, termed thrombo-inflammation, fosters microvascular dysfunction and secondary infarct growth. FTY720, a sphingosine-1-phosphate receptor modulator, blocks the egress of lymphocytes from lymphoid organs and has been shown to reduce ischemic neurodegeneration; however, the underlying mechanisms are unclear. We investigated the mode of FTY720 action in models of cerebral ischemia.

Methods—Transient middle cerebral artery occlusion (tMCAO) was induced in wild-type and lymphocyte-deficient Rag1−/− mice treated with FTY720 (1 mg/kg) or vehicle immediately before reperfusion. Stroke outcome was assessed 24 hours later. Immune cells in the blood and brain were counted by flow cytometry. The integrity of the blood–brain barrier was analyzed using Evans Blue dye. Thrombus formation was determined by immunohistochemistry and Western blot, and was correlated with cerebral perfusion.

Results—FTY720 significantly reduced stroke size and improved functional outcome in wild-type mice on day 1 and day 3 after transient middle cerebral artery occlusion. This protective effect was lost in lymphocyte-deficient Rag1−/− mice and in cultured neurons subjected to hypoxia. Less lymphocytes were present in the cerebral vasculature of FTY720-treated wild-type mice, which in turn reduced thrombosis and increased cerebral perfusion. In contrast, FTY720 was unable to prevent blood–brain barrier breakdown and transendothelial immune cell trafficking after transient middle cerebral artery occlusion.

Conclusions—Induction of lymphocytopenia and concomitant reduction of microvascular thrombosis are key modes of FTY720 action in stroke. In contrast, our findings in Rag1−/− mice and cultured neurons argue against direct neuroprotective effects of FTY720. (Stroke, 2013;44:00-00.)

Key Words: FTY720 ■ inflammation ■ ischemic stroke ■ MCAO ■ microvascular dysfunction ■ thrombo-inflammation

Ischemic stroke causes a profound local and systemic inflammatory response, which involves a variety of immune cells that migrate across the activated blood–brain barrier (BBB) to invade the brain parenchyma in a defined spatio-temporal sequence.1 Although previous research mainly focused on the role of innate immune cells,2 there is accumulating evidence suggesting that T lymphocytes, which belong to the adaptive immune system, are also critically involved in stroke development.1,3 T cells can be found in the postischemic brain as early as 24 hours after reperfusion,4 and it has been shown that lymphocyte-deficient Rag1−/− mice are profoundly protected from ischemic stroke.5–7 However, the pathomechanisms of T cell-mediated tissue damage in acute brain ischemia are only incompletely understood. We could recently show that lymphocytes foster ischemic neurodegeneration by inducing microvascular dysfunction.7 This microvascular dysfunction includes pathological interaction of T lymphocytes with endothelial cells and platelets, and eventually leads to increased thrombosis and...
impaired reperfusion of cerebral microvessels, a process commonly referred to as no reflow.6,7 Hence, there seems to be a tight interplay between thrombotic and inflammatory circuits in the pathophysiology of ischemic stroke, and this thrombo-inflammation might become a promising druggable target.8,9

FTY720 is a lipophilic immunomodulatory sphingosine-1-phosphate (SIP) analog and has recently been approved for the treatment of relapsing-remitting multiple sclerosis. After phosphorylation by the sphingosine-kinase isoform 2, FTY720 acts on 4 of the 5 known SIP receptor subtypes. SIP receptors are widely expressed in the body including the central nervous system (CNS) and therefore exert pleiotropic functions. The most prominent effect of FTY720 is the reduction of peripheral lymphocyte counts by blocking the egress of lymphocytes from lymphoid organs through agonist-induced receptor internalization.10 Given that T cells are obviously of major relevance for lesion development after cerebral ischemia,5,7 it is reasonable to analyze the efficacy of FTY720 on stroke outcome. Indeed, FTY720 has already been evaluated in different stroke models in rodents by several groups.11–17 Whereas most of these studies found a reduction of stroke volumes and improved neurological status after FTY720 treatment,11,13,14 the results on the modes of FTY720 action in the ischemic brain are conflicting.11,13,14,17 Putative mechanisms include (a) reduced brain inflammation,11,12,14 (b) attenuation of BBB disruption,14,17 or (c) direct neuroprotective effects.11,13,15

The present study assesses the relative contribution of these mechanisms in mouse models of brain ischemia/reperfusion injury and, for the first time, describes a direct modulation of thrombo-inflammation by FTY720.

Materials and Methods
A detailed description of the methods and supplementary figures are provided in the online-only Data Supplement.

Mice
A total of 235 C57Bl/6 (wild-type, WT) and 24 Rag1−/− mice were used in this study. Animal experiments were approved by legal state authorities (Regierung von Unterfranken and Regierungspräsidium Darmstadt) and performed according to the recommendations for research in experimental stroke studies11 and the current Animal Research: Reporting of In Vivo Experiments guidelines (http://www.nc3rs.org/ARRIVE).

Ischemia Model
Focal cerebral ischemia was induced in 6- to 8-week-old male mice (Harlan Winkelmann, Borchen, Germany) by 60 minutes or 90 minutes transient middle cerebral artery occlusion (tMCAO) as described.19,20 Mice were controlled for physiological parameters that could affect stroke outcome (Tables I and II; Figure 1 in the online-only Data Supplement). Edema-corrected infarct volumes were calculated from 2,3,5-triphenyltetrazolium chloride–stained brain slices. The Bederson score and the grip test score were used to monitor neurological function.4 Mice were randomly assigned to the operators by an independent person not involved in data analysis. We performed surgery and evaluation of all readout parameters while being blinded to the experimental groups.

FTY720 Treatment
FTY720 (1 mg/kg body weight dissolved in 0.9% sodium chloride, Cayman Chemical, 10006292) was applied immediately before reperfusion, that is, removal of the occluding filament, by an intraperitoneal injection. An equal volume of sodium chloride (0.9%) (vehicle) served as control.

Western Blot
Immunoreactivity against fibrinogen (anti-fibrinogen) pAb 1:500, antibody cross-reactive against fibrin and fibrinogen, Acris antibodies, occludin (anti-occludin mAb 1:1000, Abcam), and actin (antiactin mAb 1:75.000, Dianova) in the ischemic cortices and basal ganglia was detected by Western Blot.21

Determination of Blood–Brain Barrier Leakage
Blood–brain barrier leakage after tMCAO was quantified using the vascular tracer Evans Blue (Sigma Aldrich) and photometric measurements as described.22

Immunohistochemistry
Cryo-embedded brain slices were stained with antibodies against CD31 (mouse anti-mouse, abcam, ab9498, 1:100) or fibrinogen (rabbit anti-mouse, Acris, AP00766PU-N, 1:100).23 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) antibody in PBS containing 1% bovine serum albumine.

For calculation of the thrombosis index,24 the whole brain was sliced 24 hours after stroke, and hematoxylin and eosin staining was performed (slice thickness 10 μm). The number of occluded blood vessels within the ischemic hemispheres was counted in every 10th slice for FTY720-treated and untreated animals under a 40-fold magnification.

Immunofluorescence staining of hippocampal cultures was performed as described.22 The following antibodies were used: MAP2a/b (1:100, Abcam) and Alexa Fluor 488–coupled donkey antibody recognizing rabbit IgG (1:250, Serotec). For detection of apoptosis, the In Situ Cell Death Detection Kit (Roche) was used according to the manufacturer’s instructions.

Cell Separation and Flow Cytometry
For the isolation of brain-infiltrating mononuclear cells, a Percoll (GE Healthcare) density gradient (50%/30%) was used as described.7 Myeloid immune cells and lymphocytes were incubated in fluorescence-activated cell sorter buffer with monoclonal antibodies anti–CD45R/B220-PE (BD Bioscience, 553089), anti–CD11b-PE (BD-Pharmigen, 553052), anti–CD8a-PE (BD Bioscience, 553032), anti–Gr1-allophycocyanin (Ly-6G and Ly-6C, BD Bioscience, 557397), and anti–CD45R/B220-PE (BD Bioscience, 553089).

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For determining the number of CD4+ and CD8+ T lymphocytes within the intracranial vascular compartment, brains were harvested from non-perfused and perfused mice and analyzed by flow cytometry. The difference in T lymphocyte counts between nonperfused and perfused brains reflects the amount of T lymphocytes exclusively within the intracranial vasculature.

For flow cytometry analysis of peripheral immune cells, 200 μl of blood was harvested transcardially in heparin-coated tubes, and red blood cells were lysed using red blood cell lysis buffer (Biolegend) following the manufacturer description.

Thrombosis Assays
Platelet adhesion assays under flow conditions and intravital microscopy of thrombus formation in FeCl3-injured mesenteric arterioles were performed as described.24
Hippocampal Neuronal Cell Cultures

Neuronal cell cultures were obtained from C57Bl/6 mice embryos (E18) as described previously. Neuronal cultures were incubated at 37°C and 5% CO₂ and maintained in culture for up to 5 to 7 days before performing the experiments. Cell viability was assessed by the In Situ Cell Death Detection Kit (TMR red, Roche) according to the manufacturer's instructions. For ischemic conditions, O₂ was restricted per incubation at 37°C in a humidified incubator with 5% CO₂, 5% O₂, and 90% N₂. FTY720-phosphate (FTY-P, Santa Cruz) or vehicle control was added to the cell cultures.

Statistics

All data are given as mean±SEM except for the ordinal Bederson score and the grip test score that are depicted as scatter plots including median with the 25% percentile and the 75% percentile given in brackets in the text. Numbers of animals (n=10) necessary to detect a standardized effect size on infarct volumes ≥ 0.25 (FTY720-treated versus untreated mice) were calculated via a priori sample size analysis with the following assumptions: α=0.05, β=0.2 (power 80%), mean, standard deviation 20% of the mean (StatMate 2.0, GraphPad Software). For statistical analysis, PrismGraph 5.0 software package (GraphPad Software) was used. Data were tested for Gaussian distribution with the Kolmogorov–Smirnov test and then analyzed by 1-way ANOVA, or in case of nonparametric data (Bederson score, grip test), Kruskal–Wallis test with post hoc Dunns correction. If only 2 groups were compared, unpaired, 2-tailed Student t test or Mann–Whitney Test (Bederson score, grip test); *P<0.05, **P<0.001 (power 80%).

Results

FTY720 Reduces Thrombo-Inflammation in Stroke

Next, we assessed whether FTY720 protects from acute ischemic stroke in a clinically relevant scenario, that is when applied after the onset of stroke. C57Bl/6 WT mice were subjected to 60 minutes of tMCAO and received 1 mg/kg FTY720 immediately before reperfusion (Figure 1A). Stroke volumes on day 1 were significantly reduced, by ~30%, in FTY720-treated mice compared with vehicle-treated controls (108.1±33.6 versus 78.2±29.4 mm³, P=0.048; Figure 1A). Importantly, reduced stroke size also translated into improved functional outcomes as assessed by the Bederson score, reflecting the global neurological status (median 3.0 [3.0, 3.0] [vehicle] versus 3.0 [2.0, 3.0] [FTY720], P=0.03) and the grip test reflecting motor function and coordination (median 2.5 [0.5, 4.0] [vehicle] versus 4.0 [3.5, 4.0] [FTY720], P=0.02; Figure 1B).

To exclude the possibility that FTY720 simply delays ischemic brain damage rather than preventing it, we also investigated stroke volumes on day 3 after MCAO (Figure 1A). Again, FTY720 treatment led to significantly smaller infarctions compared with controls mice also at that later time point, and we did not observe any relevant changes in stroke volumes between day 1 and day 3.

FTY720 Does Not Alter the Cellular Infiltrate in the Ischemic Brain

It is well established that the local postischemic inflammatory response that evolves after stroke modulates lesion development. Therefore, we evaluated whether FTY720 alters the composition of the cellular infiltrate within the ischemic brain. Although FTY720 led to a rapid and significant reduction of leukocytes and lymphocytes in the peripheral blood (Figure

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**Figure 1.** FTY720 improves stroke outcome. A, Top, Representative 2,3,5-triphenyltetrazolium chloride stains of 3 corresponding coronal brain sections of a vehicle-treated wild-type (WT) mouse and an FTY720-treated WT mouse euthanized on day 1 or 3 after transient middle cerebral artery occlusion. FTY720 was applied immediately before reperfusion, that is 1 h after stroke onset, at a dosage of 1 mg/kg. Infarcts seemed to be smaller in the groups receiving FTY720, and this could be confirmed by infarct volumetry (n=9–11/group; bottom). Note that there was no further change in infarct size in FTY720-treated animals between day 1 and day 3. B, Reduced stroke volumes after FTY720 treatment also translated into a better functional outcome on day 1 as assessed by the Bederson score (top) and the grip test (bottom), n=9 to 11/group; unpaired, 2-tailed Student’s t test (infarct volumes) or Mann–Whitney Test (Bederson score, grip test); *P<0.05, **P<0.001.
Stroke-Protective Effect of FTY720 Depends on the Induction of Lymphocytopenia but Not Direct Neuroprotection

We next sought to clarify the mechanisms underlying these stroke-protective properties of FTY720. *Rag1*−/− mice lack T cells and B cells and are profoundly protected from ischemic neurodegeneration in the tMCAO model.5–7 Therefore, *Rag1*−/− mice are a useful tool to test whether FTY720 modulates stroke outcome via mechanisms that are independent of lymphocytopenia, for instance by mediating direct neuroprotection. In this experimental setup, vehicle-treated WT mice or FTY720-treated WT mice and *Rag1*−/− mice were subjected to 90 minutes of tMCAO. This prolonged period of ischemia was chosen because 60 minutes of tMCAO only induces small infarctions in untreated *Rag1*−/− mice on day 1,5–7 making the detection of any additional infarct lowering effect of FTY720 difficult. Again, injection of FTY720 into WT mice immediately before reperfusion produced significantly smaller strokes (121.7±26.6 versus 75.9±29.7 mm³, *P*<0.001) and better functional outcomes (Bederson score: median 3.0 [3.0, 4.0] [vehicle] versus 3.0 [2.0, 3.0] [FTY720], *P*=0.03; Grip test: median 2.0 [2.0, 3.0] [vehicle] versus 3.0 [3.0, 4.0] [FTY720], *P*>0.05) on day 1 compared with vehicle-treated WT mice (Figure 3A and 3B). In line with previous studies,5–7 untreated *Rag1*−/− mice displayed a stroke-protective phenotype in the tMCAO model compared with untreated WT mice (infarct volumetry: 121.7±26.6 [WT] versus 86.4±24.4 mm³)

Figure 2. FTY720 does not alter the local inflammatory response in the ischemic brain. The total number of viable cells per ipsilesional hemisphere and distinct immune cell subsets (CD4+ and CD8+ T cells, CD11b+ macrophages/microglia, Gr-1+ neutrophils) were quantified by flow cytometry 24 h after transient middle cerebral artery occlusion in vehicle-treated wild-type (WT) mice or FTY720-treated WT mice (n=8–10/group). No significant differences were observed for any cell type or treatment group. n.s.=not significant, 1-way ANOVA followed by Bonferroni multiple comparison test.

Figure 3. FTY720 improves stroke outcome independent of direct neuroprotective effects. Infarct volumes (A) and functional outcomes (B) on day 1 after 90 minutes of transient middle cerebral artery occlusion in wild-type (WT) mice and *Rag1*−/− mice with or without FTY720 treatment (1 mg/kg immediately before reperfusion). Although FTY720 significantly improved stroke outcome in WT mice, it was unable to further reduce stroke volumes or to improve neurological status in T lymphocyte–deficient *Rag1*−/− mice, which are protected from stroke already under naïve conditions. n=11/group, *P*<0.05, **P*<0.001, n.s.=not significant, 1-way ANOVA followed by Bonferroni multiple comparison test (infarct volumes) or Kruskal–Wallis test followed by Dunns multiple comparison test (Bederson score, grip test). C. Bar graph presentation of the percentage of dead neurons under different experimental conditions (normoxia versus hypoxia (5% CO₂, 5% O₂, 90% N₂); 0 versus 10 versus 100 nmol/L FTY720-P). Hypoxia significantly increased the number of apoptotic neurons (*P*<0.0001), and application of FTY720-P could not prevent neuronal loss both under normoxic or hypoxic conditions. n=7 to 13/group, n.s.=non significant, 1-way ANOVA followed by Bonferroni multiple comparison test.
(Rag1−/−), P<0.05; Figure 3A and 3B). Importantly, however, FTY720 was unable to further reduce stroke size or improve functional status in Rag1−/− mice (infarct volumetry: 86.5±36.4 mm³, P>0.05; Bederson score: median 2.0 [2.0, 4.0], P>0.05; Grip test: median 4.0 [1.0, 4.0], P>0.05; Figure 3A and 3B). This indicates that FTY720 acts beneficially in acute stroke mainly by affecting lymphocyte counts, whereas other potential modes of FTY720 action such as direct neuroprotection are obviously of minor importance. However, one cannot definitely exclude from these experiments that the protection achieved in Rag1−/− mice at a certain point reached a ceiling effect that did not allow observing small benefits of FTY720 that were not mediated by T lymphocytes.

To further corroborate our hypothesis that FTY720 does not protect from ischemic stroke by shielding neurons directly (neuroprotection), we performed additional experiments in neuronal cell cultures under normoxic and hypoxic conditions (Figure 3C). In line with our in vivo findings, FTY720 was unable to reduce neuronal cell death in vitro even when different concentrations of FTY720 were used.

**FTY720 Reduces Microvascular Dysfunction and Thrombus Formation**

We could recently show that in the tMCAO model in mice T lymphocytes interact with endothelial cells and platelets to induce microvascular dysfunction and capillary thrombosis. Therefore, we speculated that FTY720 would be able to mitigate these processes by reducing the numbers of circulating lymphocytes and subsequently the event rate of harmful lymphocyte–endothelium–platelet interactions in the brain vasculature. Indeed, FTY720 treatment not only reduced the number of circulating T lymphocytes in the peripheral blood (Figure II A and II B in the online-only Data Supplement) but also in the intracranial vascular compartment on day 1 after tMCAO (1245±786 [vehicle] versus 447±676 [FTY720], P<0.05; Figure III in the online-only Data Supplement). Here, the number of T lymphocytes present specifically in the brain vasculature was determined by counting cells from nonperfused and perfused brains of untreated mice and FTY720-treated mice and calculating the difference afterward.

In line with our hypothesis, the decline in T lymphocytes within the cerebral vasculature after FTY720 treatment resulted in less thrombus formation. Western blot analysis revealed that the amount of fibrin(ogen) on day 1 after tMCAO in the ischemic cortices of FTY720-treated mice was significantly lower compared with untreated controls (optical density in ipsilesional cortices 1.6±0.3 versus 4.1±0.9, P=0.002; Figure 4A), whereas no significant differences were found in the basal ganglia (optical density in ipsilesional basal ganglia 1.8±0.6 versus 2.3±0.3, P=0.17; Figure 4A).

**Figure 4. FTY720 reduces microvascular thrombosis and improves cerebral reperfusion after transient middle cerebral artery occlusion (tMCAO).** A. Deposition of fibrin(ogen) in the ipsilateral (ipsi) or contralateral (contra) cortex or basal ganglia (BG) of vehicle-treated wild-type (WT) mice or FTY720-treated (1 mg/kg) WT mice as determined by immunoblot and densitometric quantification of the bands. Actin was used as loading control. FTY720 was applied immediately before reperfusion, and brains were analyzed on day 1 after tMCAO. n=4/group, *P<0.05, n.s.=not significant, 2-way ANOVA followed by Bonferroni multiple comparison test. AU=arbitrary units. B. Immunohistochemical localization of fibrin(ogen) in the lumina of brain microvessels (stained with the endothelial marker CD31) 24 h after transient middle cerebral artery occlusion (tMCAO) in the ipsilateral (ipsi) or contralateral (contra) cortex of vehicle-treated WT mice or FTY720-treated WT mice. One representative panel per group of 3 independent experiments is shown. Scale bar represents 100 μm. C. Left. Representative hematoxylin and eosin stains from the infarcted basal ganglia of vehicle-treated WT mice or FTY720-treated WT mice with (1 mg/kg immediately before reperfusion) on day 1 after tMCAO. Thrombotic vessels were abundant in control animals (arrows), whereas the microvascular patency was significantly increased in the mouse group receiving FTY720 (arrowheads), and this was confirmed by calculation of the thrombosis index (right). n=4/group, *P<0.05, unpaired, 2-tailed Student t test. Scale bar represents 100 μm. D. Reduction of intracerebral thrombosis in FTY720-treated mice improved cerebral blood flow (CBF) in the territory of the right middle cerebral artery 12 and 24 h after reperfusion compared with control mice as determined by serial laser Doppler flow measurements. No differences in CBF were detectable at baseline (before ischemia), immediately after insertion of the filament (ischemia), or immediately after removal of the filament (reperfusion). n=8/group and time point, ***P<0.0001, n.s.=not significant, 2-way ANOVA followed by Bonferroni multiple comparison test.
Immunohistochemistry consistently demonstrated intravascular fibrinogen deposits that occluded brain vessels in untreated mice and markedly reduced fibrinogen deposits in mice treated with FTY720 (Figure 4B). Accordingly, histological sections of infarcted brain tissue from untreated mice showed numerous occlusions of vessel lumina (Figure 4C). In comparison, the microvascular patency was significantly increased in mice receiving FTY720 (thrombosis index: 16.0±1.4 versus 9.5±1.7, P=0.01).

To further address whether reduced clot formation in the FTY720 group also translates into better cerebral (re)perfusion after tMCAO, we measured the cerebral blood flow (CBF) over time in the territory of the right middle cerebral artery by Laser Doppler flowmetry. No differences in baseline CBF (before ischemia), CBF after insertion of the occluding filament (ischemia), or CBF after removal of the occluding filament (reperfusion) were observed between FTY720-treated mice and vehicle-treated mice (P>0.05), indicating comparable procedural conditions in both groups (Figure 4D). However, 12 hours (percentage of baseline CBF: 56.4±8.7% [vehicle treated] versus 89.3±11.5% [FTY720 treated], P<0.001) and 24 hours (percentage of baseline CBF: 52.9±10.0% [vehicle treated] versus 89.1±17.4% [FTY720 treated], P<0.001) after reperfusion, CBF in the brains of FTY720-treated mice was significantly higher compared with controls (Figure 4D).

To exclude that FTY720 causes a general defect in platelet function that could account for the profound protection observed in ischemic stroke, we analyzed the ability of FTY720-treated mice to form stable thrombi in standardized thrombus formation assays. First, we studied platelet adhesion and aggregate formation on a collagen-coated surface in an ex vivo whole-blood perfusion system under high shear conditions (1000 s⁻¹).24 In this setting, platelets from naïve mice and mice treated with FTY720 adhered to collagen fibers and formed aggregates within 2 minutes that consistently grew into large thrombi (Figure 5A). By the end of the perfusion period, the surface area covered by platelets did not significantly differ between the groups (P>0.05; Figure 5A). We also studied the effects of FTY720 on occlusive thrombus formation in vivo after FeCl₃-induced injury of mesenteric arterioles.7 No significant differences in the time to first appearance of thrombi >10 μm (not shown) or median occlusion time of damaged arterioles were observed between FTY720-treated mice and controls (P>0.05; Figure 5B). These findings demonstrate that FTY720 application does not impair the general ability of platelets to form stable thrombi, at least in the setting of severe artificial vessel wall injury leading to the exposure of tissue factor and prothrombotic subendothelial matrix proteins. In line with these findings, no differences in platelet counts and volumes were observed between FTY720-treated mice and untreated controls (Table II in the online-only Data Supplement).

**FTY720 Does Not Stabilize the Blood–Brain Barrier After Stroke**

BBB disruption and subsequent edema formation represent frequent causes of secondary infarct growth, and S1P receptors have been shown to be expressed at the BB barrier.28–31 Thus, we analyzed the consequences of FTY720 on BBB structure and function after brain ischemia. The extent of edema formation as assessed by the concentration of the vascular tracer Evans blue leaking into the brain parenchyma was similar between FTY-treated mice and vehicle-treated controls on day 1 after tMCAO (control: 23.2±5.0 ng/mg brain tissue, FTY720: 29.9±6.9 ng/mg brain tissue, P>0.05; Figure 6A). In line with these findings, the expression of the tight junction protein occludin was unchanged in the cortices and basal ganglia of FTY720-treated compared with vehicle-treated mice (P>0.05; Figure 6B).

**Discussion**

We identify herein lymphocytopenia as the key mechanism responsible for the stroke-protective properties of FTY720 in mice. The reduction of lymphocytes in the brain vasculature attenuated microvascular thrombus formation and increased CBF during the reperfusion phase after tMCAO. In contrast,
FTY720 does not seem to shield neurons directly under conditions of acute ischemia because FTY720 could not further improve stroke outcome in lymphocyte-deficient Rag1−/− mice. Finally, we did not find evidence for a BBB stabilizing or anti-inflammatory effect of S1P receptor modulation in the acute phase of stroke.

By taking advantage of transgenic mouse models, we could recently show that depletion of T cells6 or specialized T cell subsets, such as regulatory T cells (Treg),7 dramatically protects from acute ischemic brain damage. Mechanistic studies revealed that the stroke-promoting properties of T cells and Treg do not depend on their classical immune function. Rather, T cells interact functionally with activated cerebral endothelial cells and platelets for instance via the lymphocyte function-associated antigen-1/intercellular adhesion molecule-1 pathway. This interaction, which already occurs within a few hours after the onset of ischemia, causes microvascular dysfunction, leading to increased thrombus formation and subsequently impaired cerebral reperfusion after tMCAO (no reflow).6,7

The present study for the first time confirms this novel concept of T cell–induced thrombo-inflammation in stroke6,8 by using a pharmacological approach. FTY720 traps T lymphocytes within lymphoid organs, thereby inducing rapid and profound lymphocytopenia in the peripheral blood.8 Importantly, FTY720 also decreased the number of T lymphocytes within cerebral blood vessels after tMCAO (Figure III in the online-only Data Supplement), thereby reducing the probability of detrimental lymphocyte–endothelial cell–platelet interactions. As a consequence, less thrombi were formed in the brain, and CBF improved in the mouse group receiving FTY720. In full support of our hypothesis, a recent study reported fewer intercellular adhesion molecule-1 positive brain vessels in FTY720-treated mice after tMCAO.14 Whether platelets carry S1P receptors or undergo activation after S1P binding is controversial15,16 and that is why we cannot definitely exclude direct (antithrombotic) effects of FTY720 on platelet function. However, our observation of regular clot formation and clot stability after severe artificial vessel wall injury in the presence of FTY720 argues against a direct impact of FTY720 on platelet aggregation and thrombus formation but rather suggests indirect mechanisms operating for instance through the reduction of peripheral lymphocyte counts.

FTY720 acts on the S1P receptor subtypes 1, 3, 4, and 5. Neurons express mainly S1P1 and S1P3, and S1P1 shows a widespread expression in the brain.6,7 This, together with the fact that FTY720 can readily cross the BBB,6,7 points towards a potential neuroprotective effect of FTY720.6,7 Indeed, FTY720 restored synaptic transmission in cortico-striatal brain slice preparations from myelin oligodendrocyte glycoprotein-immunized experimental autoimmune encephalomyelitis mice via a direct influence on glutamatergic signaling. Notably, this effect was accompanied by structural protection of dendritic spines.6,7 In line with these in vitro findings, mice that are deficient of the S1P1 receptor only in neurons and astrocytes are largely protected from experimental autoimmune encephalomyelitis.38 Although pharmacological S1P receptor modulation might mediate direct neuroprotection in models of autoimmune central nervous system inflammation, spinal cord injury,49 or certain neurodevelopmental disorders such as Rett Syndrome,50 the situation in ischemic stroke seems to be different. FTY720 did not protect primary neurons against glutamate excitotoxicity14 or hypoxia (this study). The fact that FTY720 does not affect stroke outcome after permanent MCAO56 and the inability of FTY720 to further reduce lesion size in lymphocyte-deficient Rag1−/− mice after tMCAO (this study) likewise argues against a genuine neuroprotective mode of action.

FTY720 was unable to maintain BBB structure and function on stroke in our hands, although S1P receptors have been shown to be induced at the BBB in an ischemic environment.30,31 Moreover, a similar amount of immune cells including T lymphocytes invaded the ischemic brain tissue of FTY720-treated mice or control mice on day 1. Hence, the beneficial effect of FTY720 cannot be ascribed to a relief of BBB breakdown or local inflammation at least during the acute phase after an ischemic insult. Our findings are in agreement with the recent findings of Cremer et al.8,9}

**Figure 6.** FTY720 does not stabilize the blood–brain barrier after ischemic stroke. A, Top, Representative coronal brain sections from a vehicle-treated wild-type (WT) mouse and a WT mouse treated with FTY720 (1 mg/kg immediately before reperfusion) on day 1 after transient middle cerebral artery occlusion (tMCAO) and injection of the vascular tracer Evans blue. Bottom, No significant difference in the concentration of Evans Blue leaking into the brain parenchyma was observed between the 2 groups; n=5 to 6/group, n.s.—not significant, 2-way ANOVA followed by Bonferroni multiple comparison test. AU=arbitrary units.
part discrepant to previous studies describing BBB stabilization and reduced transendothelial trafficking of immune cells mediated by FTY720 in cerebral ischemia and intracranial hemorrhage.14,16,17,41 The exact reasons for these contrary findings are unclear at present, but differences in the stroke models and animal species used might play a role. In particular, other doses of FTY720 than the one used here might produce different results in terms of BBB stabilization and immune cell transmigration.13,14 Moreover, our study only investigated the consequences of FTY720 treatment on immune cell invasion on day 1. At this early stage of infarction, the number of blood-borne immune cells present in the brain parenchyma is still low,4 and we cannot exclude FTY720 effects on the composition of the cellular infiltrate at later time points.13,16

FTY720 has proven to mitigate ischemia-induced tissue damage in a clinically relevant setting also in other organs such as the liver42 and the kidney.43 This, together with the considerable number of positive studies on FTY720 in experimental stroke,11-15,17 suggests that pharmacological S1P receptor modulation is effective in ischemia/reperfusion injury in general. Although it is tempting to speculate that short-term treatment with FTY720 is beneficial in patients with stroke as well, one has to be cautious when transferring the findings from animal models to the human situation, in particular in the field of experimental stroke research.44 Also, our proof-of-principle study was not designed to address translational aspects of FTY720 in stroke, that is, to mimic the clinical situation exactly, but rather wanted to clarify the consequences of FTY720 treatment on immune cell invasion and reduced transendothelial trafficking of immune cells.13,14 Moreover, our proof-of-principle study was not designed to address translational aspects of FTY720 in stroke, that is, to mimic the clinical situation exactly, but rather wanted to clarify the to date unknown mode of FTY720 action in acute cerebral ischemia. Certainly, different FTY720 treatment intervals or dosing regimens or the use of older and comorbid animals might have produced different results. Moreover, specific side effects of FTY720 affecting, for instance, the cardiovascular system (induction of cardiac arrhythmias and increase in blood pressure) would probably hamper its use in clinical stroke. Finally, lymphocytopenia induced by FTY720 could further enhance stroke-related immunodepression that has recently been identified as a major cause of poststroke infection and mortality.5,56

In conclusion, lymphocytopenia and concomitant reduction of intravascular thrombo-inflammation, but not direct neuroprotection, are critical for the stroke-protective action of FTY720. Transient inhibition or short-term depletion of lymphocytes might become a novel approach to combat ischemic stroke in the future.

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References


FTY720 Ameliorates Acute Ischemic Stroke in Mice by Reducing Thrombo-Inflammation but Not by Direct Neuroprotection

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SUPPLEMENTAL MATERIAL

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 described²,³ 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-fibrin(ogen) 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 fibrin(ogen) 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 fibrin(ogen) (rabbit anti-mouse, acris, 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
**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.

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>FTY720 (1 mg/kg bodyweight)</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>3</td>
<td>3</td>
<td>n.s.</td>
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<tr>
<td>PaO₂ (mmHg)</td>
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<td>69.2 ± 7.0</td>
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<tr>
<td>PaCO₂ (mmHg)</td>
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<td>40.1 ± 1.1</td>
<td>n.s.</td>
</tr>
<tr>
<td>pH</td>
<td>7.36 ± 0.04</td>
<td>7.42 ± 0.08</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Vehicle</td>
<td>FTY720 (1 mg/kg bodyweight)</td>
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<tr>
<td>n</td>
<td>5</td>
<td>5</td>
<td>n.s.</td>
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<tr>
<td>Platelet count (x10^3/µl)</td>
<td>608 ± 133</td>
<td>621 ± 22</td>
<td>n.s.</td>
</tr>
<tr>
<td>Platelet volume (fl)</td>
<td>5.8 ± 0.53</td>
<td>5.6 ± 0.29</td>
<td>n.s.</td>
</tr>
<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.