Influenza Virus Infection Aggravates Stroke Outcome

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Background and Purpose—Stroke is triggered by several risk factors, including influenza and other respiratory tract infections. However, it is unknown how and in which way influenza infection affects stroke outcome.

Methods—We infected mice intranasally with human influenza A (H1N1) virus and occluded the middle cerebral artery to induce ischemic strokes. Infarct volume and intracerebral hemorrhage were determined by histology. To evaluate the integrity of the blood–brain barrier and inflammation, we measured various cytokines in vivo and in vitro and performed immunohistochemistry of leukocyte markers, collagen IV, immunoglobulins, and matrix metalloproteinase-9.

Results—Influenza virus infection increased infarct size. Whereas changes in cardiovascular parameters did not explain this effect, we found evidence for an inflammatory mechanism. In influenza virus infection, the respiratory tract released cytokines into the blood, such as RANTES that induced macrophage inflammatory protein-2 and other inflammatory mediators in the ischemic brain. In infected mice, there was an increased number of neutrophils expressing the matrix metalloproteinase-9 in the ischemic brain. This was accompanied by severe disruption of the blood–brain barrier and an increased rate of intracerebral hemorrhages after tissue plasminogen activator treatment. To investigate the role of cytokines, we blocked cytokine release by using GTS-21, a selective agonist of the α7 nicotinic acetylcholine receptor. GTS-21 ameliorated ischemic brain damage and improved survival.

Conclusions—Influenza virus infection triggers a cytokine cascade that aggravates ischemic brain damage and increases the risk of intracerebral hemorrhage after tissue plasminogen activator treatment. Blockade of cytokine production by α7 nicotinic acetylcholine receptor agonists is a novel therapeutic option to treat stroke in a proinflammatory context. (Stroke. 2011;42:783-791.)

Key Words: α7 nicotinic acetylcholine receptor ■ cytokines ■ influenza ■ RANTES ■ stroke
Methods

Influenza Virus and Infection

Human influenza virus A/Puerto Rico/8/34 (H1N1; PR8) was propagated as described earlier. Male C57BL/6 mice (Friedrich-Loeffler Institute) at an age of 3 to 4 months if not indicated otherwise were anesthetized by intraperitoneal injection of xylazine (50 μL per 10 g body weight, 0.1%) and ketamine (50 μL per 10 g body weight, 0.5%). We administered the H1N1 virus (1×10⁵ plaque-forming units) or the control inoculum (phosphate-buffered saline) in a final volume of 50 μL (25 μL in each nasal opening).

The infection led to disease as shown by ruffled fur, reduced locomotor activity, unnatural posture, and a fast breathing rate indicative of the respiratory pathology. Successful infection was controlled by virus titration of lungs. To investigate survival, we used C57BL/6 mice at an age of 7 to 9 weeks because the LD₅₀ of PR8 (2×10⁴ plaque-forming units) was evaluated in this age group of C57BL/6 mice. All animal experiments were approved by the local animal welfare committee.

Middle Cerebral Artery Occlusion

In all experiments, the animals were randomized and investigators were blinded to the treatment. For permanent middle cerebral artery occlusion (MCAO), male mice were anesthetized at the age of 3 to 4 months by intraperitoneal injection of 2.5% tribromoethanol (150 μL per 10 g body weight). The experimental procedure has been described previously. To evaluate the effects of hypoxemia, mice were exposed to normobaric hypoxia at 16% oxygen during surgery and for a period of 48 hours after surgery. For transient MCAO, male mice were anesthetized at the age of 3 to 4 months by intraperitoneal injection of xylazine (50 μL per 10 g body weight, 0.1%) and ketamine (50 μL per 10 g body weight, 0.5%). A median neck incision was performed and the left common carotid artery was exposed. A silicon rubber-coated 7-0 monofilament of 20-mm length (Doccol Corporation, Catalog No. 7019PK5Re) was inserted into the common carotid artery on the left side. The filament was advanced into the internal carotid artery approximately 11 mm from the bifurcation until the coated part of the filament was no longer visible. The filament was reversibly fixed during the whole time of ischemia. After 45 minutes of occlusion, the filament was removed to establish reperfusion. During the time of occlusion, the femoral vein was exposed and cannulated with a 20-cm long catheter (polyethylene-10). Human tissue plasminogen activator (tPA, 10 mg/kg in a volume of 300 μL; Boehringer Ingelheim) was administered starting 30 minutes after MCAO with a bolus (1 mg/kg). The remaining dose (9 mg/kg) was infused over 20 minutes with a pump (PHD2000 Programmable; Harvard Apparatus). At 24 hours after transient MCAO, the animals were reanesthetized and perfused intracardially with 4% paraformaldehyde.

For other methods, see supplementary material (available at http://stroke.ahajournals.org).

Results

Influenza Aggravates Concomitant Stroke

Mice were infected intranasally with the human H1N1 influenza A virus PR8. To induce an ischemic stroke, we permanently occluded the middle cerebral artery (MCAO) 2 to 5 days after infection. This time window was chosen because symptoms of respiratory tract infections often precede stroke by 1 to 7 days. Influenza virus infection increased the infarct size if permanent MCAO was performed on Day 3 to 5 but not on Day 2 (Figure 1A). It cannot be excluded that at earlier time points influenza infection may affect the infarct size, but this is unlikely because infected mice did not show any signs of infection on Day 1. Infarcts were mostly limited to the cortex in...
the permanent MCAO stroke model (Figure 1B). Influenza virus infection enlarged the infarct size in the full rostrocaudal extension of the lesion (Figure 1C). However, the infection had no effect on the mortality of mice after stroke (permanent MCAO; Supplemental Table I).

Neural cells express the influenza receptors Sia\(\alpha2\)-3gal and Sia\(\alpha2\)-6gal (Figure 2A, left panel). Therefore, we investigated whether infection of ischemic brain tissue could underlie the increased infarct size. At 5 days after infection, we detected the viral nucleoprotein, a marker of ongoing viral replication, in lung tissue but not in ischemic brain tissue (Figure 2A, left panel). In addition, we could recover PR8 virus from lung tissue but not from blood or the brain (Figure 2A, right panel). These data argue against a role of direct brain infection in the aggravation of stroke.

Influenza virus pneumonia may lead to hypoxemia. Although oxygen saturation in awake mice was not reduced 3, 4, and 5 days after influenza virus infection (Figure 2B, left panel), the arterial pO\(_2\) measured in anesthetized mice dropped after influenza virus infection (Figure 2B, right panel). A similar drop of pO\(_2\) levels was measured in uninfected mice if the O\(_2\) concentration of the air was reduced to 16% corresponding to an altitude of approximately 2400 m (Figure 2C, left panel). Exposure of mice to 16% O\(_2\) during and after permanent MCAO had no effect on the infarct size (Figure 2C, right panel). This indicates that the enlarged infarct size after influenza virus infection is not explained by the mild hypoxemia. Furthermore, we confirmed that body temperature decreases in mice after influenza virus infection in contrast to other species. Body temperature did not differ between the sham and stroke group (Figure 2D). Because hypothermia is neuroprotective, the lower body temperature in influenza virus-infected mice would clearly not enlarge the infarct size. Other physiological parameters that are known to influence the infarct size were not altered after influenza infection (Supplemental Table II).

**Respiratory Influenza Virus Infection Augments Neuroinflammation in Stroke**

Influenza virus infection leads to a profound inflammatory response in the lung that may have distant effects on the ischemic brain. In accordance with previous reports, we
found elevated concentrations of MIP-1α, interleukin (IL)-6, IL-1β, monocyte chemotactrant protein-1, RANTES, and MIP-2 in lung tissue of influenza virus-infected mice (Figure 3A, left panel). In plasma, RANTES levels were higher after influenza virus infection both in sham and stroke mice (Figure 3A, middle panel). In contrast, plasma levels of other cytokines were not affected by influenza infection in stroke mice (Figure 3A, middle panel; Supplemental Figure IA–D).
left panel). In the brain, influenza virus infection alone had no effect on cytokine expression. However, it significantly increased the expression of IL-1β, monocyte chemoattractant protein-1, and MIP-2 in concomitant stroke (Figure 3A, right panel). Also, granulocyte colony-stimulating factor, MIP-1β, and tumor necrosis factor were increased (Supplemental Figure 1A, C, D right panel). However, IL-17 (Supplemental Figure 1B, right panel), granulocyte/macrophage colony-stimulating factor, interferon-γ, and HMGB1 were not affected (data not shown).

Because RANTES plasma levels were elevated after influenza virus infection, we wondered whether RANTES would induce cytokine expression in neural cells. In primary glial cells, RANTES stimulated MIP-2 release into the medium (Figure 3B). Likewise, oxygen glucose deprivation (OGD), an in vitro model of cerebral ischemia, raised MIP-2 release but the combination of RANTES and OGD had no further effect on MIP-2 secretion (Figure 3B). In brain endothelial bEnd.3 cells, RANTES and OGD alone had no effect on MIP-2 release, but the combination increased MIP-2 release (Figure 3C). The stimulation of MIP-2 release by the combination of OGD and RANTES may explain the synergistic effect of stroke and influenza on MIP-2 levels in the brain (Figure 3A, right panel).

The cytokines MIP-2, IL-1β, and monocyte chemoattractant protein-1, which were upregulated in the ischemic brain after influenza virus infection, are involved in the recruitment of neutrophils. Indeed, we found an elevated number of neutrophils in the brain of influenza infected mice 48 hours after permanent MCAO (Figure 4A) but no change in the number of CD11b-positive macrophages and microglia (data not shown). At the same time, levels of matrix metalloproteinase-9 (MMP-9), a neutrophilic enzyme, increased in the ischemic brain of influenza virus-infected mice as compared with uninfected controls (Figure 4B). Double staining confirmed that MMP-9 was expressed by neutrophils.
but also revealed a vascular staining pattern for MMP-9 (Figure 4C). This supports the notion that MIP-2 attracted neutrophils expressing MMP-9 in the ischemic brain after influenza virus infection.

**Disruption of the BBB Increases Hemorrhagic Risk in Thrombolysis**

MMP-9 is known to degrade the extracellular matrix of the BBB. Therefore, we investigated protein levels of collagen IV, a component of the basal membrane, in the ischemic brain by immunohistochemistry. After influenza virus infection, we found reduced collagen IV staining in the ischemic area (Figure 4D, left panel). The extravasation of IgG was increased in stroke mice after influenza virus infection as compared with uninfected animals (Figure 4D, right panel), indicating that a concomitant influenza virus infection aggravates the disruption of the BBB in cerebral ischemia.

Disruption of the BBB is an important cause of hemorrhage in thrombolytic treatment. To evaluate the consequences of influenza virus infection for thrombolysis, we used a stroke model (transient MCAO) that has been used previously to investigate complications of tPA treatment.18 We administered tPA already 30 minutes after occlusion to minimize intracerebral hemorrhages in uninfected mice (Figure 5A–B). However, after influenza virus infection, tPA treatment significantly increased the hemorrhage volume that was assessed by the hemoglobin concentration in the brain (Figure 5A). A similar result was obtained when the number of macroscopic and microscopic hemorrhages on the side of the lesion was scored (Figure 5B). In parallel, plasma levels of MMP-9 were elevated in influenza virus-infected mice suggesting a possible cause for the increased rate of hemorrhages (data not shown). However, the effect of MMP-9 may be dampened by a similar increase in the MMP-9 inhibitor TIMP-1 (data not shown). Surprisingly, in the transient MCAO stroke model, the infarct size was not larger in influenza virus-infected mice (Figure 5C). Still, mortality increased significantly after influenza virus infection in transient MCAO (Supplemental Table I). Because only surviving mice were included in the analysis of infarct size, it is possible that death due to a large infarct concealed an effect of influenza on infarct size.

**Activation of α7 Nicotinic Acetylcholine Receptor to Combat Neuroinflammation in Influenza-Associated Stroke**

If inflammatory mediators are the link between influenza virus infection of the respiratory tract and aggravated ischemic brain damage, anti-inflammatory treatment should be beneficial. In influenza virus infection, lung epithelial cells and macrophages produce cytokines.19 Both cell types express the α7 nicotinic acetylcholine receptor (α7nAchR) that limits cytokine release in the reflex control of immunity.20,21 Therefore, we investigated whether the α7nAchR agonist GTS-21 interferes with the effects of influenza virus infection. GTS-21 increased the viability of A549 lung epithelial cells infected by influenza virus (Figure 6A) but had no effect on viral replication (data not shown). Interestingly, GTS-21 blocked the release of RANTES from A549 cells after influenza virus infection (Figure 6B). To exploit this mechanism, we administered GTS-21 to mice (10 mg/kg intraperitoneally, before and 6 hours after surgery). Also in vivo, GTS-21 lowered RANTES plasma concentrations in infected mice (Figure 6C, upper left panel). In contrast, GTS-21 had...
no effect on plasma levels of MIP-1α and IL-6 (Supplemental Figure II). In the ischemic brain, GTS-21 reduced levels of IL-1β, monocyte chemotactic protein-1, and MIP-2 that were elevated by influenza virus infection (Figure 6C). In contrast, GTS-21 had no effect on the expression of MIP-1α or RANTES in the ischemic brain (Supplemental Figure II). Importantly, GTS-21 significantly reduced the infarct size of influenza-infected mice but had no effect on the infarct size in noninfected animals (Figure 6D, left panel), suggesting that blockade of cytokine release by GTS-21 specifically interferes with influenza-triggered aggravation of ischemic brain death.

GTS-21 treatment did not affect viral titers in the lung 4 days after infection (vehicle-treated group, 5.53 ± 0.05 log_{10} plaque-forming units/mL; GTS-21-treated group, 5.61 ± 0.12 log_{10} plaque-forming units/mL; n = 3). Furthermore, the effect of GTS-21 treatment on survival after influenza infection and stroke was investigated. GTS-21 treatment prolonged survival for 1 day (Figure 6D, right panel). These data exclude an aggravation of the influenza infection due to GTS-21 treatment.

**Discussion**

Epidemiological data establish influenza as a stroke trigger.2– 7 We show in a mouse model that influenza aggravates stroke pathophysiology. The detrimental effect could not be explained by an infection of the brain, fever, or hypoxemia, but rather, cytokines likely mediate the effect of influenza virus infection on stroke pathophysiology.

Hypercytokinemia has been linked to highly virulent forms of influenza viruses.22–24 However, elevated cytokine levels have also been observed with less virulent viral strains.25 Our data suggest a mechanism through which cytokines contribute to ischemic brain damage. Influenza virus infection of epithelial cells and alveolar macrophages in the respiratory tract elicits the release of cytokines into the blood such as RANTES. Circulating RANTES induces the expression of MIP-2 and possibly other cytokines in cells of the BBB. Consequently, MIP-2 recruits neutrophils to the ischemic brain of influenza virus-infected mice. This mechanism is likely to contribute to the aggravation of stroke by influenza, because neutrophils are known to mediate ischemic brain damage.8,26–28 In addition, neutrophils release MMP-9, a protease that degrades collagen IV and other components of the extracellular matrix of the BBB.29 In line with this notion, we found evidence that influenza virus infection elevates MMP-9 levels in the brain and enhances the ischemia-induced degradation of collagen IV and the disruption of the BBB, although the brain is not a direct target of the virus. Disruption of the BBB by MMP-9 has been implicated in tPA-induced brain hemorrhages.30–32 Indeed, a concomitant influenza virus infection increased brain hemorrhage after tPA treatment in our mouse model. Because tPA treatment does not lead to reperfusion in the transient intraluminal MCAO model we have used, we cannot judge whether influenza infection may interfere with the potential therapeutic benefit of tPA treatment. However, our study provides evidence that standard stroke treatment may be risky in the case of concomitant influenza. Our data suggest neutralization of RANTES as a strategy to interfere with the pathogenic cascade that worsens ischemic brain damage and disrupts the BBB. However, selective antagonism of RANTES has been reported to weaken antiviral defense and to prolong infection.

**Figure 6.** The α7nAChR agonist GTS-21 reduced cytokine expression and stroke volume after influenza virus infection. A, GTS-21 improved cell viability in A549 cells after influenza infection. Analysis of variance, F(3/44) = 35.15, P < 0.0001. **P < 0.0001** (Newman-Keuls post hoc test). B, GTS-21 blocked the RANTES release from influenza virus-infected lung epithelial A549 cells. Analysis of variance, F(3/16) = 1354, P < 0.0001. **P < 0.0001** compared with other influenza virus infected groups (Newman-Keuls post hoc test). C, Cytokine levels in plasma and brain after GTS-21 treatment (10 mg/kg intraperitoneally, immediately before and 6 hours after surgery). At 3 days after influenza virus infection, stroke was induced (permanent MCAO [pMCAO]). Cytokine levels were measured 15 hours after pMCAO. Cytokine levels are expressed relative to the value measured in vehicle-treated groups with the same infection and stroke status. **P < 0.05** (t test with Bonferroni correction for multiple testing). D, left panel, Infarct volume in GTS-21-treated mice. GTS-21 (10 mg/kg) was administered before surgery and 6 hours, 24 hours, and 36 hours after surgery. The infarct size was determined 48 hours after surgery. Chi square 15.18, P < 0.0001 (log-rank test, 10 mice per group).
of the lung.\textsuperscript{33} Furthermore, other cytokines probably contribute to the interaction between influenza and stroke. In this situation, activation of the cholinergic anti-inflammatory pathway seems to be a better choice.

The autonomic nervous system regulates immunity.\textsuperscript{21} In influenza or stroke, the sympathetic nervous system influences the immune response and outcome.\textsuperscript{34,35} but the role of the parasympathetic cholinergic nervous system has not been studied in detail. Cholinergic control of immunity converges on the $\alpha_7$-nAchR.\textsuperscript{31} This receptor is expressed by alveolar macrophages and epithelial cells in the respiratory tract, which are the main sources of cytokine production in influenza.\textsuperscript{20,21} Our study demonstrates that activation of $\alpha_7$-nAchR by the selective agonist GTS-21 inhibited the release of RANTES from influenza virus-infected lung epithelial cells. On GTS-21 treatment, lower levels of circulating RANTES were associated with decreased levels of inflammatory mediators in the brain and smaller infarct size. In addition, GTS-21 may activate $\alpha_7$-nAchR in glial and endothelial cells,\textsuperscript{36,37} and thereby inhibit cytokine expression in the ischemic brain. However, it is noteworthy that GTS-21 only reduced the infarct size in influenza virus-infected mice suggesting that GTS-21 specifically targets the aggravation of ischemic brain damage by influenza. $\alpha_7$-nAchR activation is known to reduce cytokine release in sepsis models.\textsuperscript{38} Our data show that this strategy may also be successful to dampen the hypercytokinemia in influenza without any direct effect on viral replication. This effect may help to reduce influenza-associated mortality, which is largely due to cardiovascular diseases in both the common seasonal form of influenza and during pandemics.\textsuperscript{39} Notably, GTS-21 was still effective when administered 3 days after infection, suggesting a wider therapeutic window than for antiviral treatment. Basal RANTES levels in GTS-21-treated mice probably prevented the increased susceptibility to the viral infection that was observed in RANTES-deficient mice.\textsuperscript{33}

In stroke research, preclinical studies have had a notoriously low predictive value for the clinical efficacy of compounds. An important reason for the disparity between preclinical and clinical data is probably the fact that preclinical studies relied largely on healthy young rodents, whereas clinical stroke often occurs in the context of pre-existing diseases.\textsuperscript{40} Many of the disorders that predispose to stroke such as atherosclerosis, obesity, and infection are associated with systemic inflammation.\textsuperscript{41} Our study demonstrates that concomitant inflammatory disease has an important impact on the safety of thrombolysis, the only approved stroke treatment. An increased bleeding risk of thrombolysis in influenza virus-infected patients has been indirectly suggested by clinical data showing that MMP-9 blood levels, which correlate with an increased risk of hemorrhage after tPA, rise in influenza.\textsuperscript{42–44} A recent study reporting an increased rate of hemorrhagic transformation of infaracts in obese mice suggests that the chronic low-grade inflammation of obesity also weakens the BBB.\textsuperscript{45} However, the impact of this finding for thrombolysis is still unclear. Whereas the risk of thrombolysis in influenza may be greater, an $\alpha_7$-nAchR agonist is only efficacious in association with an inflamma-
tory condition. Thus, our study suggests that stroke treatment should be stratified according to concomitant diseases.

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**Disclosures**

YAA: ownership interest and patents.

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SUPPLEMENTAL MATERIAL

Supplementary Methods

Measurement of physiological parameters

To determine physiological parameters that could affect stroke pathophysiology, the femoral artery was cannulated after anesthesia with Rompun and Ketamine (see above) in a separate cohort of animals. Arterial blood gases and glucose were measured 10 min before and 10 min into pMCAO in a blood sample of 100 µl. For laser Doppler measurements, the probe (P415-205; Perimed, Järfälla, Sweden) was placed 3 mm lateral and 6 mm posterior to the bregma. Relative perfusion units were determined (Periflux 4001; Perimed). For the measurement of body temperature after infection we used a telemetry monitoring system (VitalView®, Minimiter, USA) as described previously. 1 Oxygen saturation was measured in awake mice using the MouseOx® (Starr Life Sciences Corp).

Measurement of infarct volume

At the indicated time points after MCAO, mice were deeply reanesthetized with tribromoethanol and perfused intracardially with 4% paraformaldehyde (PFA). The brains were kept in 4% PFA for 3–4 h and then transferred to 30% sucrose solution for 24 h. Finally, the brains were frozen on dry ice. Coronal cryosections of the brains (20 µm in thickness) were cut every 400 µm and stained with the Nissl technique. Infarct volumes were corrected for brain edema as previously described. 2

Influenza virus titration

To assess the number of infectious particles (plaque titers) in lung, blood, brain, and cell culture supernatants a plaque assay using Avicel® was performed in 96-well plates as described previously. 3 Organs were homogenized in saline buffer. A 10% homogenate was titrated and used for infection of MDCK target cells. After a 48-h incubation period the virus-infected MDCK cells were immunostained by incubating for 1 h with a monoclonal antibody specific for the influenza A virus nucleoprotein (Serotec) followed by 30-min incubation with peroxidase-labeled anti-mouse antibody (DIANOVA) and 10-min incubation with True Blue™ peroxidase substrate (KPL). Stained plates were scanned on a flat bed scanner and the data were acquired by Microsoft® Paint software. The virus titer is given as the logarithm to the base 10 of the mean value. The detection limit for this test was <1.7 log 10 PFU/ml.

Immunohistochemistry

For fluorescent immunohistochemistry, cryosections of 20-µm thickness were fixed in 4% PFA for 30 min. Sections were then permeabilized with 0.25% Triton X-100 in PBS for 5 min and blocked in 5% normal horse serum or 5% bovine serum albumin for 90 min except for collagen staining, for which sections were permeabilized by 0.25% Triton X-100 for 2.5 h. The following primary antibodies were used: rabbit anti-mouse collagen IV (1:500; Abcam, Cambridge, UK; Cat. No. ab19808), rabbit anti-mouse polymorphonuclear leukocyte (1:100; Accurate Chemical & Scientific, Westbury, NY, USA; Cat. No. AIAG31140), goat anti-mouse MMP-9 (1:50; R&D-Systems, Wiesbaden-Nordenstadt, Germany; Cat. No. AF909), and rat anti-mouse CD11b (1:100 AbD, Serotec, Düsseldorf, Germany Cat. No. MCA 711G). The primary antibodies were applied overnight at room temperature with the exception of the anti-PMN antibody, which was applied for 2 h. After washing, the following secondary antibodies were added: Cy3-conjugated donkey anti-goat (1:200; Dianova, Hamburg, Germany; Cat. No. 705-165-147), Cy3-conjugated donkey anti-rabbit (1:200; Dianova; Cat. No. 711-165-152), Alexa-Fluor-488 donkey anti-goat (1:200; Invitrogen, Karlsruhe, Germany; Cat. No.
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To stain the viral nucleoprotein and sialic acid-containing influenza receptors, cryosections were used. The sections were fixed in 4% PFA for 30 min. Endogenous peroxidase activity was then blocked by incubation in PBS containing 3.0% H₂O₂ for 10 min. After incubation with 2.5% normal horse serum or 3% bovine albumin serum for 20 min, goat anti-nucleoprotein (AbD Serotec; 1:4000), biotinylated Sambucus nigra lectin (α 2,6; Vector Laboratories, Burlingame, CA; 10 µg/ml), or biotinylated Maackia amurensis II lectin (α 2,3; Vector laboratories; 10 µg/ml) were applied overnight. After adding ImmPRESS REAGENT (anti-goat IgG; Vector Laboratories Cat. No. MP-7405) or ABC reagent for 30 min, sections were stained with 3,3'-diaminobenzidine (Vector laboratories) according to the manufacturer's protocol. Finally, the sections were counterstained with hematoxylin, dehydrated with ascending alcohol row and incubated in Roti Histoclear solution for 10 min. The sections were covered in ENTellan (Merck) and dried for 30 min.

MMP-9 and collagen IV staining were quantified in predefined areas (Figure 3C) on coronal sections containing the anterior commissure without knowledge of treatment using Leica DM4000B (Leica Microsystems) and PictureFrame Application 2.3 (Camera Type: MICROFIRE_C). Neutrophils were counted in the infarct area on a section containing the anterior commissure. For quantification of integrated density we used Image J (Wayne Rasband, National Institute of Mental Health, Maryland, USA). For confocal microscopy a Nikon A1R 4 laser line confocal microscope with hybrid scanner and 32 channel spectral detector was used.

Quantification of hemorrhagic transformations

To quantify hemorrhagic transformation, mice were perfused 24 h after tMCAO as described above. We scored hemorrhages at 20-21 levels throughout the brain without knowledge of the treatment as described previously. Briefly, microscopic hemorrhage (evident to the eye aided by a 10 x magnifying glass) was scored 2 and macroscopic hemorrhage (evident to the unaided eye) was scored 3, 4, or 5 according to its size. To visualize the hemorrhage (Figure 5D) we stained the sections with hematoxylin and eosin and diaminobenzidine (DAB). DAB is known to react with peroxidases in the red blood cells, facilitating precise identification of intracranial hemorrhage.

Hemoglobin assay

To measure the hemoglobin content in the brain we performed a spectrophotometric assay using Drabkin’s solution. Briefly, the animals were perfused transcardially 24 h after tMCAO with PFA (4%, 20 ml with a perfusion speed of 1.5 ml/min). To prepare the standards, fresh blood was injected into the cortex immediately before perfusion. The brains were homogenized by sonication in 1 ml PBS per hemisphere. After centrifugation 120 µl of Drabkin’s solution containing Drabkin’s reagent (Sigma, Cat. No.: D5941) and Brij 35 solution (0.015%, Sigma, Cat. No. B 4184) was added to 30 µl of supernatant and the absorption was measured at 540 nm (Spectra MAX 250, Molecular Devices Corp).

Measurement of cytokines

On day 3 after influenza virus infection male C57BL/6 mice were subjected to pMCAO as described above. At 15 h after occlusion mice were deeply reanesthetized with tribromoethanol. Blood was drawn from the caval vein and plasma was stored at -20°C until analysis by Bioplex.
Influenza and stroke

Cell culture

Glial cells were prepared from the brains of neonatal (postnatal day 2) mice as has been described. Cells were cultured in DMEM (Invitrogen) containing glucose (4.5 g/l), L-glutamine (0.5 mM), fetal calf serum (FCS, 10%, Invitrogen), penicillin (100 IU/ml), and streptomycin (100 µg/ml). The mouse brain endothelial cell line bEnd.3 was obtained from American Type Culture Collection (Manass, VA, USA) and grown in DMEM containing glucose (4.5 g/l), FCS (10%), penicillin (100 IU/ml), streptomycin (100 µg/ml), and L-glutamine (2 mM). Oxygen glucose deprivation (OGD) was used as an in vitro model of ischemia. At 1 h before oxygen deprivation, recombinant mouse RANTES (100 ng/ml, Peprotech, Hamburg, Germany) and 2-deoxy-D-glucose (5 mM) were added to medium. Cells were placed in an anaerobic chamber flushed with 5% CO₂ in 95% N₂ for 15 min before the chamber was sealed and incubated for 3 h at 37°C. Control cells were kept under normoxic conditions without 2-deoxy-D-glucose for 3 h. The medium was then replaced and the cells were allowed to recover for 24 h under normal conditions. MIP-2 release in the medium was measured using the mouse MIP-2 Quantikine ELISA (R&D).

The human lung epithelial cell line A549 was grown in minimal-essential medium (MEM) supplemented with 10% heat-inactivated FCS, penicillin (100 U/ml), and streptomycin (100 µg/ml). Cells were infected with PR8 (MOI = 1) and treated with GTS-21 for 48 h after infection. RANTES was measured in the medium with the human RANTES direct ELISA kit (Invitrogen).

For the cell viability assay, A549 cells were infected with PR8 virus at MOI of 1. At 48 h after addition of GTS-21, cells were fixed and viable cells were stained with crystal violet (Sigma-Aldrich, Steinheim, Germany). After extraction of crystal violet from viable cells with 100% methanol (Carl Roth, Karlsruhe, Germany), the extinction was measured by 450 nm with a microplate absorbance reader (Sunrise, Tecan).

Statistics

Student’s t-test was used to compare two groups and one-way ANOVA (analysis of variance) to compare more than two groups, followed by the Newman-Keuls multiple comparison test. Data are expressed as means ± sem.
Legends to supplementary figures

Supplementary Figure 1. Levels of G-CSF, IL-17, MIP-1β, and TNF in plasma, and brain after influenza virus infection and stroke. At 3 days after influenza virus infection pMCAO or sham surgery were performed and cytokine levels were determined 15 h later. *p<0.05 (Newman-Keuls post-hoc test, if ANOVA showed a significant difference between groups). Only significant differences between influenza virus infected and uninfected mice were indicated.

Supplementary Figure 2. Cytokine levels in plasma and brain after GTS-21 treatment (10 mg/kg i.p., immediately before and 6 h after surgery). At 3 days after influenza virus infection stroke was induced (pMCAO). Cytokine levels were measured 15 h after pMCAO. Cytokine levels are expressed relative to the value measured in vehicle-treated groups with the same infection and stroke status.

References

Supplementary Table 1. Survival of mice after permanent MCAO (pMCAO) and transient MCAO (tMCAO). *p<0.005 in comparison to uninfected mice (Fisher’s exact test).

Survival after pMCAO (48 h)

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<tr>
<th>Groups</th>
<th>Number of mice [n]</th>
<th>Surviving mice [% (n)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected – pMCAO</td>
<td>40</td>
<td>87.5% (35)</td>
</tr>
<tr>
<td>Infected – pMCAO</td>
<td>50</td>
<td>78.0% (39)</td>
</tr>
<tr>
<td>Uninfected – GTS-21 – pMCAO</td>
<td>10</td>
<td>90.0% (9)</td>
</tr>
<tr>
<td>Infected – GTS-21 – pMCAO</td>
<td>10</td>
<td>90.0% (9)</td>
</tr>
</tbody>
</table>

Survival after pMCAO (15 h)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of mice [n]</th>
<th>Surviving mice [% (n)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected - Sham</td>
<td>23</td>
<td>100.0% (23)</td>
</tr>
<tr>
<td>Infected - Sham</td>
<td>23</td>
<td>95.6% (22)</td>
</tr>
<tr>
<td>Uninfected - pMCAO</td>
<td>25</td>
<td>100.0% (25)</td>
</tr>
<tr>
<td>Infected - pMCAO</td>
<td>25</td>
<td>100.0% (25)</td>
</tr>
<tr>
<td>Uninfected – GTS-21 - Sham</td>
<td>6</td>
<td>100.0% (6)</td>
</tr>
<tr>
<td>Infected – GTS-21 - Sham</td>
<td>6</td>
<td>100.0% (6)</td>
</tr>
<tr>
<td>Uninfected – GTS-21 - pMCAO</td>
<td>6</td>
<td>100.0% (6)</td>
</tr>
<tr>
<td>Infected – GTS-21 - pMCAO</td>
<td>6</td>
<td>100.0% (6)</td>
</tr>
</tbody>
</table>

Survival after tMCAO (24 h)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of mice [n]</th>
<th>Surviving mice [% (n)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected - tMCAO</td>
<td>20</td>
<td>80.0% (16)</td>
</tr>
<tr>
<td>- Vehicle</td>
<td>- 10</td>
<td>- 80% (8)</td>
</tr>
<tr>
<td>- tPA</td>
<td>- 10</td>
<td>- 80% (8)</td>
</tr>
<tr>
<td>Infected - tMCAO</td>
<td>41</td>
<td>39.0%* (16)</td>
</tr>
<tr>
<td>- Vehicle</td>
<td>- 18</td>
<td>- 44.4% (8)</td>
</tr>
<tr>
<td>- tPA</td>
<td>- 23</td>
<td>- 34.8% (8)</td>
</tr>
</tbody>
</table>
**Supplementary Table 2.** Physiological parameters in influenza-infected mice and controls. There were no statistically significant differences between infected and control mice (n=7-8).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control mice</th>
<th>Influenza infected mice (day 3 post infection)</th>
<th>Influenza infected mice (day 4 post infection)</th>
<th>Influenza infected mice (day 5 post infection)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before pMCAO</td>
<td>After pMCAO</td>
<td>Before pMCAO</td>
<td>Before pMCAO</td>
</tr>
<tr>
<td>MABP (mm Hg)</td>
<td>75.9 ± 2.2</td>
<td>65.1 ± 2.6</td>
<td>76.5 ± 1.7</td>
<td>63.1 ± 2.1</td>
</tr>
<tr>
<td>pCO₂ (mm Hg)</td>
<td>58.1 ± 2.7</td>
<td>59.1 ± 2.5</td>
<td>57.9 ± 2.6</td>
<td>64.5 ± 2.2</td>
</tr>
<tr>
<td>pH</td>
<td>7.20 ± 0.00</td>
<td>7.16 ± 0.01</td>
<td>7.25 ± 0.02</td>
<td>7.19 ± 0.02</td>
</tr>
<tr>
<td>Drop in Doppler signal (%)</td>
<td>72.9 ± 1.7</td>
<td>74.4 ± 2.8</td>
<td>72.8 ± 4.8</td>
<td></td>
</tr>
</tbody>
</table>