CCR6 (CC Chemokine Receptor 6) Is Essential for the Migration of Detrimental Natural Interleukin-17–Producing γδ T Cells in Stroke

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Background and Purpose—Immune-mediated tissue damage after stroke evolves within the first days, and lymphocytes contribute to the secondary injury. Our goal was to identify T-cell subpopulations, which trigger the immune response.

Methods—In a model of experimental stroke, we analyzed the immune phenotype of interleukin-17 (IL-17)–producing γδ T cells and explored the therapeutic potential of neutralizing anti-IL-17 antibodies in combination with mild therapeutic hypothermia.

Results—We show that brain-infiltrating IL-17–positive γδ T cells expressed the Vγ6 segment of the γδ T cells receptor and were largely positive for the chemokine receptor CCR6 (CC chemokine receptor 6), which is a characteristic for natural IL-17–producing γδ T cells. These innate lymphocytes are established as major initial IL-17 producers in acute infections. Genetic deficiency in Ccr6 was associated with diminished infiltration of natural IL-17–producing γδ T cells and a significantly improved neurological outcome. In the ischemic brain, IL-17–positive γδ T cells trigger the expression of CXC chemokines and neutrophil infiltration. Therapeutic targeting of synergistic IL-17 and tumor necrosis factor-α pathways by IL-17 neutralization and therapeutic hypothermia resulted in additional protective effects in comparison to an anti-IL-17 antibody treatment or therapeutic hypothermia alone.

Conclusions—Brain-infiltrating IL-17–producing γδ T cells belong to the subset of natural IL-17–producing γδ T cells. In stroke, these previously unrecognized innate lymphocytes trigger a highly conserved immune reaction, which is known from host responses toward pathogens. We demonstrate that therapeutic approaches targeting synergistic IL-17 and tumor necrosis factor-α pathways in parallel offer additional neuroprotection in stroke.

Visual Overview—An online visual overview is available for this article. (Stroke. 2017;48:1957-1965. DOI: 10.1161/STROKEAHA.117.016753.)

Key Words: brain ■ interleukin-17 ■ lymphocytes ■ neuroprotection ■ stroke

Stroke is worldwide ranked as the second most common cause of death and the third most common cause of disability-adjusted life years.1 Numerous experimental and clinical studies demonstrate that the activation of the systemic immune compartment and brain resident immune cells is a major element of the pathophysiology of stroke. Recently, clinical studies targeting T cells highlighted the therapeutic potential of immune modulatory treatment strategies.2 T-cell–related detrimental mechanisms in cerebral ischemia encompass perforin-mediated neurotoxicity and the production of proinflammatory cytokines interleukin-17 (IL-17) and IL-21.1,3 Whereas IL-21 and perforin are thought to be directly neurotoxic, IL-17 effects mainly depend on the induction of proinflammatory chemokines, which are leading to a rapid neutrophil infiltration into ischemic hemispheres. Major sources of IL-17 in ischemic brains are γδ T cells, which are producing IL-17 within 24 hours after the ischemic attack.5 The temporal dynamics of the IL-17 production in γδ T cells indicate that antigen-specific priming is not essential for their activation.5,6 In models of acute infection, similarly rapid IL-17 responses are mounted by γδ T cells before the antigen-specific activation of T cells and B cells.7 IL-17–producing γδ T cells can be divided into several subsets. So-called natural IL-17–producing γδ T cells (nTγδ17 cells) are producing IL-17 within 12 hours.8 The rapid antigen-independent activation of nTγδ17 cells can occur through...
the engagement of pathogen pattern recognition receptors and the cytokine receptors for IL-1β and IL-23, whereas the antigen receptor is not required. nTγδ17 cells express CCR6 (CC chemokine receptor 6) and can further be classified based on the expression of the variable (v) segment of the T-cell receptor. nTγδ17 cells express mostly Vγ4, or Vγ6, according to the classification proposed by Heilig and Tonegawa.

Although several studies suggest detrimental functions of IL-17–producing γδ T cells in stroke, their exact phenotype is unclear. In this study, we classified γδ T cells in detail, studied the role of the chemokine receptor CCR6 for their migration, and investigated their effects on stroke outcome. To further explore the therapeutic potential of an IL-17 neutralization, we combined the treatment with anti-IL-17 antibodies with mild therapeutic hypothermia (TH). TH is already established as a treatment for several central nervous system diseases and has a major effect on proinflammatory cytokines, that is, tumor necrosis factor-α (TNF-α) and IL-1β. In connection with IL-17, TH effects on TNF-α are potentially crucial because both cytokines have synergistic effects on the expression of neutrophil-attracting CXC chemokines.

Methods

Animals

All animal experiments were approved by the local animal care committee (Behörde für Lebensmittelsicherheit und Veterinärwesen Hamburg). We conducted the experiments according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 83-123, revised 1996) and performed all procedures in accordance with the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments; http://www.nc3rs.org/ARRIVE) between January 2015 and April 2017. Ccr6–/– homozygous mice (C57BL/6 background; strain B6.129P2-Ccr6<sup>–/–</sup>) were described before. Transgenic mice were back-crossed at least 10 generations to the C57BL/6 background. Age-matched male wild-type littermates served as controls. We randomized all mice and conducted transient middle cerebral artery occlusion for 45 minutes as previously described using the intraluminal filament method (6-0 nylon) in a blinded fashion. The detailed experimental description can be found in the online-only Data Supplement. Experimental groups and animal numbers are shown in Table I in the online-only Data Supplement.

Antibody Treatment

Animals were treated with 500 µg of mouse monoclonal anti–murine IL-17A antibody (Clone MM17F3; 16.6 mg/kg of bodyweight) or with 500 µg of isotype control antibody (IgG1). Mice were injected intravenously once at 90 minutes after onset of ischemia.

Analysis of Infarct Size by 2,3,5-Triphenyl-2-Hydroxy-Tetrazolium Chloride Staining

We analyzed infarct size by harvesting brains and cutting them into 1 mm slices (Braintree Scientific, 1 mm) followed by vital staining using 2% (wt/vol) 2,3,5-triphenyl-2-hydroxy-tetrazolium chloride in phosphate buffer. We determined infarct volumes in a blinded fashion using NIH ImageJ software.

Stroke Assessment by Magnetic Resonance Imaging

Magnetic resonance imaging was performed in normal mice on a dedicated 7-T magnetic resonance small animal imaging system (ClimScan, Bruker). The image protocol comprised T2-weighted imaging, diffusion-weighted imaging, and 3-dimensional time-of-flight angiography.

Temperature Modulation Methodology

Core body temperature was maintained at 37°C throughout surgery for normothermia and 33 to 34°C for mild hypothermia as described before by using a feedback-controlled heating device (DC temperature controller, FHC Apparatus). The detailed experimental description can be found in the online-only Data Supplement.

Antibodies and Flow Cytometry

We performed flow cytometry for the analysis of cell types as previously described. The detailed experimental description can be found in the online-only Data Supplement.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction

We obtained real-time PCR primers from Applied Biosystems. The detailed experimental description can be found in the online-only Data Supplement.

Statistical Analysis

Data are reported as mean±SD. Statistical analyses were performed using the appropriate test indicated in the figure legends. Briefly, Student t test was used to compare infarct volumes and quantitative polymerase chain reaction data; Mann–Whitney U test for the comparison of clinical scores and 1-way ANOVA for multiple comparisons with Bonferroni post hoc test, after validating the normal distribution of these data sets (Kolmogorov–Smirnov test). P values <0.05 were considered statistically significant.

Results

Vγ6<sup>+</sup>/CCR6<sup>+</sup> γδ T Cells Are the Major Producers of IL-17 in the Ischemic Brain

Others and we have shown that γδ T cells are detrimental in stroke, but the exact phenotype of these innate-like T lymphocytes is unknown.

When we measured the infiltration of γδ T cells on day 3 after experimental stroke, we found 16±64±387.6% γδ T cells per ischemic hemisphere (Figure 1A). Analysis of the cellular infiltrate and intracellular cytokines levels at early time points revealed that γδ T cells were already present at 12 hours, and that 41±3% of the infiltrating γδ T cells were positive for IL-17. The IL-17 levels persisted on similarly elevated levels until day 3 (45±12%). In comparison to IL-17, interferon-γ levels were significantly lower with 10±12% (12 hours) and 15±9% (3 days), respectively. We did not observe γδ T cells double positive for IL-17 and interferon-γ. Seventy-nine ±15% of infiltrating γδ T cells were positive for TNF-α on day 3.

To differentiate infiltrating γδ T-cell subsets in our stroke model, we analyzed the expression of the variable (v) segments Vγ1, Vγ4, and Vγ6 of the T-cell receptor, according to the classification proposed by Heilig and Tonegawa.

Staining for Vγ6 was performed as described before, after pretreatment with anti-Cδ. We found that 18±2% were positive for Vγ1, 19±2% for Vγ4, 40±5% for Vγ6, and 24±9% were stained...
by none of the antibodies against Vγ1/4/6 (Figure 1B). In the next step, we measured IL-17 levels in the different subsets and found that the vast majority of IL-17+ γδ T cells expressed Vγ6 (95±5%; Figure 1B). In contrast, we did not observe Vγ1+ IL-17+ γδ T cells. Only few IL-17+ γδ T cells were positive for Vγ4 (6±2%) or negative for antibodies against Vγ1/4/6 (1±1%; Figure 1B). It is known from other studies that subsets of IL-17–producing γδ T cells express the chemokine receptor CCR6. We observed that 59±7% of the infiltrating γδ T cells were positive for CCR6 (Figure 1C). When we further gated
on Vγ6+ γδ T cells, we determined that CCR6 was expressed on 80±6% of Vγ6+ γδ T cells and on 47±8% of IL-17+/Vγ6+ γδ T cells, whereas Vγ1+ and Vγ4+ γδ T cells were negative for CCR6 (Figure 1D).

Taken together, we show that the vast majority of the infiltrating IL-17+ γδ T cells display a T-cell receptor that comprises Vγ6+. It is this Vγ6+ subset of γδ T cells, which is positive for the chemokine receptor CCR6. Thus, Vγ6+/CCR6+ γδ T cells are the major producers of IL-17 in the ischemic brain.

Ccr6−/− Mice Are Protected From Experimental Stroke

To investigate whether CCR6 has effects on the recovery of animals, we analyzed the neurological outcome in Ccr6−/− mice and littermates in our middle cerebral artery occlusion model. On day 3, Ccr6−/− mice showed significantly reduced infarct sizes and milder disability compared with littermate controls (Figure 2A and 2B). Mortality, physiological parameters, vessel occlusion during transient middle cerebral artery occlusion, and vasculature were not altered between genotypes (Figure IA and Table II in the online-only Data Supplement; Figure 2C).

CCR6 Is Required for the Infiltration of IL-17+ γδ T Cells in Experimental Stroke

To determine whether the expression of CCR6 is required for the migration of γδ T cells, we analyzed the cellular infiltrate in Ccr6−/− mice and littermates by flow cytometry. On day 3, we detected a significant reduction in absolute numbers of γδ T cells in Ccr6−/− mice in comparison to littermate controls (Figure 3A), whereas CD4 and CD8 T cells (Figure 1B in the online-only Data Supplement) were unchanged. When we measured intracellular cytokine levels in infiltrating γδ T cells, we determined reduced IL-17 levels in Ccr6−/− mice, whereas interferon-γ and TNF-α levels were not affected (Figure 3A). In contrast, cytokine levels in infiltrating αβ T cells were not altered (Figure IC and ID in the online-only Data Supplement). To analyze whether Ccr6−/− mice exhibit an altered repertoire of γδ T cells in the peripheral immune system, we measured γδ T cell subsets and cytokine levels in γδ T cells in cervical and mesenteric lymph nodes without detecting significant changes between genotypes (Figure IIA and IIB in the online-only Data Supplement). The analysis of Ccl20 RNA levels in whole brain RNA of ischemic hemisphere revealed a robust upregulation of the CCR6 ligand at 12 and 24 hours (Figure 3B). We next analyzed the expression of Vγ1, Vγ4, and Vγ6 on infiltrating γδ T cells in Ccr6−/− mice and observed a significant reduction in absolute numbers selectively for the Vγ6+ γδ T cell subset (Figure 3C). This was paralleled by a dramatic decrease of IL-17 levels in the Vγ6+ subset of Ccr6−/− mice (Figure 3D).

Taken together, we show that the expression of the chemokine receptor CCR6 is required for the infiltration of IL-17+ Vγ6+ γδ T cells in stroke.

Deficiency in CCR6 Affects Neutrophil Infiltration

Others and we have previously reported that γδ T cells are detrimental in stroke via an IL-17–mediated induction of the neutrophil-attracting chemokine CXCL1.5 To assess whether diminished IL-17 levels in Ccr6−/− mice are associated with decreased neutrophil levels, we analyzed cellular infiltrates by flow cytometry and immunohistochemistry and observed a selective significant reduction in neutrophil numbers on day 3 in Ccr6−/− mice (Figure 4A). Cell numbers and intracellular TNF-α levels of microglia, monocytes/macrophages, and dendritic cells were similar between genotypes (Figure 4B and 4C). When we analyzed levels of the Cxcl1 transcript in whole brain RNA, we found reduced levels in Ccr6−/− mice (Figure 4D). These findings link the CCR6/IL-17+ γδ T cells with the IL-17–dependent induction of CXC chemokines and the subsequent infiltration of neutrophils.

**Figure 2.** CCR6 (CC chemokine receptor 6)-deficient mice are protected from experimental stroke. A, Triphenyltetrazolium chloride (TTC) staining was used to quantify (representative TTC staining) infarct volume at d 3 after middle cerebral artery occlusion (MCAO, left) in littermate controls and Ccr6−/− mice. Data are presented as means±SD of 13 littermate controls and 15 Ccr6−/− animals. B, Neurological scores (Bederson score; Corner test) were performed 3 d after MCAO. C, In all mice subjected to MCAO, regional cerebral blood flow (rCBF) was measured with Laser Doppler. The Circle of Willis was visualized by time-of-flight angiography in Ccr6−/− mice and littermate controls. Student t test was used to assess statistical significance for stroke size and Mann–Whitney U test for neurological scores. *P<0.05, **P<0.01. WT indicates wild type.
Combined Disruption of IL-17 and TNF-α Pathways Results in an Additional Decrease of Infiltrating Neutrophil

Both mild hypothermia and neutralization of IL-17 are neuroprotective in experimental stroke. Protective effects of mild hypothermia include a robust downregulation of TNF-α. Others and we have previously shown that TNF-α and IL-17 synergistically induce CXC chemokines. The synergistic function of both cytokines let us speculate that a combined treatment—hypothermia and neutralization of IL-17—might exert additive beneficial effects on stroke outcome. When we investigated effects of mild hypothermia, we found significantly diminished TNF-α levels in microglia and monocytes/macrophages under TH (Figure 5A). We next observed that the deficiency in CCR6 in combination with TH led to a significant reduction of infiltrating γδ T cells and IL-17 levels when compared with hypothermic wild-type animals, indicating that γδ T cells were still functional under TH (Figure 5B). Finally, we found that TH in Ccr6−/− mice significantly further diminished Cxcl1 levels and numbers of infiltrating neutrophil in comparison to hypothermic littermate controls (Figure 5C and 5D).

Taken together, we show that the combined reduction of TNF-α and IL-17 levels results in a significant decrease of neutrophil levels in ischemic brains in comparison to approaches, which are targeting either TNF-α or IL-17 pathways alone.

Neutralization of IL-17 Provides Additional Protective Effects Under Conditions of Mild Hypothermia

We next analyzed the neurological outcome after middle cerebral artery occlusion in hypothermic mice with and without disruption of the CCR6/IL-17-axis. Three days after the ischemic attack, hypothermic Ccr6−/− mice showed a significant reduction in infarct size and improved neurological outcome in comparison to hypothermic littermate controls (Figure 6A). In addition, and much more interesting for therapy, administration of a single dose of anti-IL-17A (500 µg) antibody 90 minutes after stroke induction led to a significant reduction in infarct sizes and improved clinical outcomes in hypothermic wild-type animals compared with the injection of an isotype control antibody (Figure 6B). To demonstrate that the detrimental effects of CCR6+ γδ T cells were IL-17 dependent, we
finally analyzed the effect of the anti-IL-17A neutralization in Ccr6−/− mice and found that the anti-IL-17A antibody did not exert additional protective effects.

Discussion

Our study in a model of experimental stroke clarifies the mechanism how detrimental IL-17+ γδ T cells are recruited to the site of ischemic tissue damage. We show that the IL-17+ γδ T cells can be classified as so-called nTγδ17 cells and that the migration of these harmful nTγδ17 cells into ischemic hemispheres is CCR6 dependent.

Although a proinflammatory role of IL-17–positive γδ T cells in stroke was suggested by several studies, recruitment mechanisms and the phenotype of these innate lymphocytes were unclear.5,10 The observation that brain-infiltrating IL-17+ γδ T cells display the phenotype of CCR6+Vγ6+ nTγδ17 cells now explains why these innate-like lymphocytes are capable to exert their detrimental function already in the first days. The physiological role of nTγδ17 cells is to immediately produce IL-17 in response to pathogens,4 which can be triggered through IL-1, IL-23, and toll-like receptor agonists without explicit involvement of the T-cell receptor.17,18 nTγδ17 cells are the major innate producers of IL-17, which is the central to induce host defenses.19 IL-17 immediately induces chemokines, which are orchestrating the neutrophils influx to the site of injury. In mice, nTγδ17 can be characterized by the expression of CCR6 and either Vγ1, Vγ4, or Vγ6.5 We now show that equally to bacterial infections, sterile inflammation after stroke triggers similarly conserved pathways: (1) production of IL-17 by Vγ6+/CCR6+ nTγδ17 cells; (2) induction of CXC chemokines; and (3) infiltration of neutrophils. Notably, consequences for affected tissues differ. Whereas the neutrophil influx is protective in infections, the infiltration into the highly vulnerable ischemic brain leads to additional collateral damage. Accordingly, blocking of neutrophils responses reduces pathology in ischemia reperfusion injury.5,10,20

To demonstrate the pivotal role of the IL-17 axis and the CCR6 expression on nTγδ17, we further analyzed effects of an IL-17 neutralization and genetic depletion of CCR6. We found that both the neutralization of IL-17 and the inhibition of the nTγδ17 cell migration into ischemic brains in Ccr6−/− mice had beneficial effects on the neurological outcome. These results are underlining the essential function of CCR6 for the migration of nTγδ17 in stroke and are in correspondence with previous reports, demonstrating neuroprotective effects for the intracerebroventricular neutralization of CCL20,21 which is the main ligand for CCR6.22 In principle, reduction of γδ T cells in ischemic brain of Ccr6−/− mice can also be attributed to smaller infarct volumes. However, the
Figure 5. Interleukin-17 (IL-17) and tumor necrosis factor-α (TNF-α) pathways synergistically induce expression of CXCL1 and infiltration of neutrophils. A, Immunohistochemical analysis of Iba1 immunoreactive brain microglia/macrophages in ischemic cores and flow cytometric analysis of absolute cell numbers of macrophages (CD45<sup>hi</sup>, Ly6G<sup>−</sup>, CD11c<sup>−</sup>, CD11b<sup>+</sup>) in ischemic hemispheres analyzed 3 d after middle cerebral artery occlusion (MCAO) in CCR6<sup>−/−</sup> mice and littermate controls under normothermic and hypothermic conditions. Frequencies of TNF-α-positive microglia and macrophages were analyzed by flow cytometry. B, Absolute numbers of γδ T cells and IL-17A production by γδ T cells analyzed by flow cytometry in CCR6<sup>−/−</sup> mice and littermate controls under normothermic and hypothermic conditions 3 d after MCAO. True count beads were used to quantify absolute cell numbers of macrophages and γδ T cells. C, Relative gene expression of Cxcl1 in stroked brains of Ccr6<sup>−/−</sup> mice and littermate controls under normothermic and hypothermic conditions. RNA was obtained from the affected hemisphere 24 h after tMCAO (n=5–6). D, Flow cytometric analysis of absolute neutrophil numbers (CD45<sup>hi</sup>, Ly6G<sup>+</sup>, CD11b<sup>+</sup>) in Ccr6<sup>−/−</sup> mice and littermate controls under normothermic and hypothermic conditions. Statistical significance analyzed by (A–D) 2-way ANOVA with Bonferroni post hoc test. *P<0.05, **P<0.01. A, Scale bar 50 μm. Iba1 indicates ionized calcium binding adaptor molecule 1; Ly6G, lymphocyte antigen 6 complex, locus G; and WT, wild type.
finding of a selective reduction of Vγ6⁺γδ T cells and IL-17⁺/Vγ6⁺γδ T cells in Ccr6−/− mice argues for specific effects of the CCR6 deficiency on the IL-17⁺ subset of γδ T cells.
pathways in parallel might, therefore, offer new therapeutic options. A prototypical pair of cytokines, which are synergistically enhancing the gene expression of proinflammatory CXC chemokines, are IL-17 and TNF-α.5,24 Similar to IL-17, expression levels of TNF-α are rapidly upregulated in ischemic brains, and anti-TNF therapy proved to be neuroprotective in experimental stroke.25 Besides antibody-mediated neutralization, mild TH diminishes TNF-α levels in ischemic brains.12 Because TH is already established as a treatment for several central nervous system diseases, that is, cerebral hypoxia, we performed a proof of principle study, combining IL-17 neutralization and TH. Supporting the concept that the inhibition of synergistic immunologic pathways can provide additional neuroprotection, we found that the neutralization of IL-17 in combination with TH led to an additional decrease of CXC chemokine and neutrophil levels and improved neurological outcome in comparison to anti-IL-17 antibody treatment or TH alone.

Taken together, our study shows that the initiation of the sterile immune response in stroke follows highly conserved patterns. Here, we describe for the first time IL-17–producing γδT cells as the main source of IL-17, which seem to play a similar role in ischemic stroke as previously described for infectious diseases.

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Disclosures
None.

References
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Cover title: CCR6 in stroke

CCR6 is essential for the migration of detrimental natural IL-17 producing γδ T cells in stroke

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Supplemental methods:

In vivo stroke model:
We conducted transient middle cerebral artery occlusion (tMCAO) for 45 min using the intraluminal filament method (6-0 nylon) as described before\textsuperscript{1}. Mice for all animal experiments were randomized and coded by an independent researcher, so experiments were carried out blindly. Sample size calculation was performed (stroke size from pilot experiments, significance level 0.05, power 90\%) and resulted in 8 animals per group to see a difference of 23\% in stroke size. We monitored mice for heart rate, respiratory rate, oxygen saturation, and rectal body temperature. To exclude an insufficient occlusion of the middle cerebral artery in our stroke model in littermate controls, and $Ccr6^{-/-}$ we performed laser doppler and time of flight angiography. Exclusion criteria were defined in a dropout score, which included weight loss, general condition, spontaneous behavior, and impairment of wound healing. We anesthetized all mice (20 to 25 g, 12 weeks; TVH, University Medical Center Hamburg-Eppendorf) using isoflurane 1\% to 2\% v/v oxygen and we injected buprenorphine 0.03 mg/kgBW intraperitoneally (i.p.) every 12 hours for 24 hours as analgesia. After stroke induction, we repeatedly scored every mouse on a scale from 0–5 immediately after reawakening and every day until sacrifice. Bederson Score: 0 no deficit, 1 preferential turning, 2 circling, 3 longitudinal rolling, 4 no movement, 5 death. For “corner test”\textsuperscript{2}, we placed the mice between two boards set at a 30° angle and mice were placed to enter the board freely. We counted left and right turns with a rearing movement after deep entry in the corner at least 10 full turns were counted for each testing. For “Cylinder test”\textsuperscript{3}, mice were placed in a glass cylinder. Behavior was quantified by determining the Limb use Asymmetry Score as published previously. Detailed neurological-scoring was adapted from\textsuperscript{4}. Blinded, trained investigator performed the experiments. 11 different scores that were rated on two neurologic functions (general deficit and focal deficit) were performed at 2 hours and 72 hours after MCAO. The general deficit scale includes evaluation of hair (0-2), eyes (0-2), hearing ability of ears (0-2), spontaneous activity (0-3), posture (0-3), where as focal deficit scale includes gait (0-4), body symmetry (0-3), climbing in an inclined plane (0-4), forelimb symmetry (0-4), whisker response to a light touch (0-4), gripping (0-3). Mice were sacrificed three days after reperfusion using isoflurane and decapitation. Only mice with a Bederson score greater than or equal to one after reawakening, and an sufficient occlusion of the middle cerebral artery during MCAO as measured by laser doppler were included for stroke size analysis.

Temperature modulation methodology:
Mild hypothermia (33-34°C) was induced 15 minutes before ischemia onset by spraying 100\% alcohol on the mice body as described before\textsuperscript{5,6}. Body temperature was controlled by a heating pad underneath the mice and monitored every 15 min. Body temperature was maintained at 33-34°C for 45 minutes during the surgery and 3 hours after the surgery by keeping the mice under ventilation and repeated spraying of alcohol. Following mild hypothermia the animals were rewarmed using heating mats placed under the cages and by providing blankets. Time to target temperature was less than 10 min. In the normothermic groups, rectal temperature was maintained at 37°C. Both groups were kept under anesthesia for 4h.

Antibodies and flow cytometry:
We performed flow cytometry for the analysis of cell types as previously described\textsuperscript{1}. Mouse antibodies were as follows: CD3 (145-2C11), CD11b (M170), CD11c (N418),
CD45 (30-F11), IFN-γ (XMG1.2), TNF-α (MP6-XT22), Secondary anti-rat IgM-PE (HIS40) (eBioscience); B220 (RA3-6B2), CD4 (RM4-5), CD8a (53-6.7), NK1.1 (PK136), TCR-γδ (GL-3), CCR6 (29-2L17), IL-17A (TC11-18H10.1), Vy1.1 (2.11), Vy4 (UC3-10A6), Ly6C (HK1.4) (Biolegend); Ly6G (1A8) (BD Biosciences); Vy6 (17D1; IgM) kindly provided by Immo Prinz. For flow cytometry analysis animals were euthanized and perfused with phosphate-buffered saline. Only ipsilesional hemispheres, cervical lymph nodes or mesenteric lymph nodes were dissected, digested for 30 min at 37°C (1 mg/ml collagenase (Roche), 0.1 mg/ml DNAse I (Roche) in DMEM), and pressed through a cell strainer. Cells were incubated with standard erythrocyte lysis buffer on ice and separated from myelin and debris by Percoll gradient (GE Healthcare) centrifugation. For absolute quantification, TrueCount tubes (Becton Dickinson) containing fluorescence beads were used according to the manufacturer’s protocol and 10% of the sample volume was counted. For intracellular cytokine staining T cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (100ng/ml; Sigma) and ionomycin (1µg/ml; Sigma) in the presence of brefeldin A (3µg/ml; eBioscience) for 4 hours. After staining of surface markers, cells were fixed, permeabilized using (IC) Fixation Buffer in conjunction with Permeabilization Buffer (eBioscience) and stained with anti-cytokine antibodies and the corresponding isotype controls. For intracellular staining of monocytes, neutrophils and microglia, intracellular transport was blocked with brefeldin A (3µg/ml; eBioscience) for 3 hours. After staining of surface markers cells were fixed, permeabilized and stained for intracellular cytokines using (IC) Fixation Buffer in conjunction with Permeabilization Buffer (eBioscience). Data were acquired with a Fortessa FACS system (BD Biosciences) and analyzed with FlowJo (TreeStar). Doublets were excluded with FSC-A and FSC-H linearity. Samples were randomized and coded by an independent researcher, so experiments were carried out blindly.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction:
We isolated RNA from brain tissue by killing the mice with isoflurane at indicated time points (24 hours) after stroke induction. Hemispheres were separated and homogenized in TRIzol Reagent (1ml per 100 mg tissue), chlorophorm was added, samples were centrifuged at 12000 g for 15 min at 4 °C and the upper aqueous phase was collected. RNA was precipitated by addition of isopropyl alcohol, washed and dissolved in TE-Buffer. We obtained real-time PCR primers from Applied Biosystems (Carlsbad, CA): Ccl20 (Mm00444228_m1); Cxcl1 (Mm00433859_m1); Mmp3 (Mm00440295_m1); Tnf (Mm00443258_m1). We purchased probe mixtures from Fermentas (Waltham, MA). The relative gene expression was calculated using ΔΔCt method, and the samples were normalized to control population and to the expression of Sdha. Untreated samples were used as a calibrator. Samples were randomized and coded by an independent researcher, so experiments were carried out blindly.

Immunofluorescence:
Tissue samples were fixed by perfusing animals through with 4% Paraformaldehyde (PFA). Cryostat brain sections (10 µm) were stained with antibodies against Ly-6G (1:200; 1A8; Biolegend) GFAP (1:400; 4A11; BD Biosciences), Iba-1 clone (1:100; A1F1; Proteintech). Sections were then incubated with the appropriate secondary antibodies (Alexa Fluor 488 goat anti-mouse; Alexa Fluor 546 goat anti-rat IgG; Invitrogen) at room temperature for 1h, and counterstained with DAPI (1:1000; Invitrogen). Negative control sections from each animal were obtained by omitting primary antibodies. To perform quantification of neutrophils in ischemic hemispheres
we cut 20µm free floating sections. We analyzed three sections per animal (n=3) at the bregma, and the stroke area was detected with Isolectin (GS-IB4 from Griffonia simplicifolia, Alexa Fluor® 488 Conjugate). Neutrophils were counted within the stroke area. Supplemental Figure II C shows the Isolectin staining. Sections were examined with a Leica DM5000B microscope with Leica software. Samples were randomized and coded by an independent researcher, so experiments were carried out blindly.
### Table I

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<th></th>
<th>C57Bl/6 Normothermia (incl./omitted)</th>
<th>Ccr6-/- Normothermia (incl./omitted)</th>
<th>C57Bl/6 Hypothermia (incl./omitted)</th>
<th>Ccr6-/- Hypothermia (incl./omitted)</th>
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<td><strong>Infarct size:</strong></td>
<td>13/0</td>
<td>16/1</td>
<td>9/1</td>
<td>11/0</td>
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<tr>
<td>anti IL-17 mAb</td>
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<td>0/0</td>
<td>18/1</td>
<td>9/2</td>
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<tr>
<td>IgG1 Isotype</td>
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<td>0/0</td>
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<td>0/0</td>
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<td><strong>Immunohistochemistry (tMCAO Model d3):</strong></td>
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<td>Ly6G</td>
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<td>4/1</td>
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<td>GFAP &amp; IBA1</td>
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<td>6/2</td>
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<tr>
<td>MRI</td>
<td>2/1</td>
<td>3/2</td>
<td>0/0</td>
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<td><strong>RNA-Analysis:</strong></td>
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<tr>
<td>Sham</td>
<td>8/2</td>
<td>9/3</td>
<td>8/3</td>
<td>6/2</td>
</tr>
<tr>
<td>12 hrs</td>
<td>7/1</td>
<td>8/2</td>
<td>7/2</td>
<td>7/2</td>
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<tr>
<td>24 hrs</td>
<td>8/2</td>
<td>6/1</td>
<td>7/2</td>
<td>9/4</td>
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<td><strong>FACS-Analysis:</strong></td>
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<tr>
<td>12h; 3d</td>
<td>45/3</td>
<td>38/1</td>
<td>24/1</td>
<td>22/3</td>
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<td><strong>Cylinder Test:</strong></td>
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<td>0/0</td>
<td>0/0</td>
<td>8/1</td>
<td>9/0</td>
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<table>
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<tr>
<th></th>
<th>C57Bl/6</th>
<th>Ccr6-/-</th>
<th>Total</th>
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<tbody>
<tr>
<td></td>
<td>232</td>
<td>208</td>
<td>440</td>
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*Table I: Experimental groups.*
<table>
<thead>
<tr>
<th></th>
<th>WT (n=3)</th>
<th>Ccr6−/− (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Before MCAO</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate beats/min</td>
<td>409.7 ± 46.77</td>
<td>361.3 ± 25.30</td>
</tr>
<tr>
<td>SpO2, %</td>
<td>89.00 ± 3.512</td>
<td>86.67 ± 1.202</td>
</tr>
<tr>
<td>Temperature</td>
<td>37.31 ± 0.5</td>
<td>37.92 ± 0.2</td>
</tr>
<tr>
<td><strong>During MCAO</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate beats/min</td>
<td>422.7 ± 15.38</td>
<td>429.7 ± 6.642</td>
</tr>
<tr>
<td>SpO2, %</td>
<td>96.33 ± 0.6667</td>
<td>92.00 ± 3.606</td>
</tr>
<tr>
<td>Temperature</td>
<td>36.88 ± 0.48</td>
<td>37.02 ± 0.12</td>
</tr>
<tr>
<td><strong>After MCAO</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate beats/min</td>
<td>509.7 ± 2.963</td>
<td>441.3 ± 32.84</td>
</tr>
<tr>
<td>SpO2, %</td>
<td>89.00 ± 3.786</td>
<td>91.00 ± 1.528</td>
</tr>
<tr>
<td>Temperature</td>
<td>36.56 ± 0.6</td>
<td>36.13 ± 0.5</td>
</tr>
</tbody>
</table>

Table II: Physiological measurements before, during and after MCAO. Values shown are in mean ± SD. Statistical analyses were performed by Student’s t-test. P-values ≤ 0.05 were considered significant. Before MCAO: 15 mins before MCAO; during MCAO: at 30 mins of MCAO; after MCAO: 1 hour following reperfusion.
Figure I: Comparison of survival and flow cytometric analysis of cellular infiltrates in ischemic hemispheres of Ccr6−/− mice and littermate controls.

(A) Survival was recorded over a time course of 3 days in normothermic and hypothermic WT and Ccr6−/− mice. (B) Absolute numbers of lymphocytes, T-lymphocytes, CD4+ T cells and CD8+ T cells did not differ between genotypes. The graphs show means ± SD of 8-10 animals per group analysed three days after MCAO in 4-5 independent experiments. (C-D) Statistical analyses were performed by Student’s t-test. P-values < 0.05 were considered significant.
Figure II: Flow cytometric analysis of γδ T cells from cervical (cLN) and mesenteric (mLN) lymph nodes of WT and Ccr6⁻/⁻ mice.

(A) Flow cytometric analysis of relative γδ T cell numbers and Vy1, Vy4 and Vy6 expression in cervical (cLN) and mesenteric (mLN) lymph nodes of WT and Ccr6⁻/⁻ mice. Lymphocytes were stained for B220, CD45, CD3, CD4, CD8, NK1.1, γδ TCR, Vy1, Vy4 and Vy6. (B) Flow cytometric analysis of IL-17A and IFN-γ levels in γδ T cells isolated from mesenteric lymph nodes (mLN) and cervical lymph (cLN) nodes at day 3 after sham operation. (C) For the analysis of the neutrophil infiltration the stroke area was detected with Isoclectin (GS-IB4 from Griffonia simplicifolia, Alexa Fluor® 488 Conjugate). (A, B) The graphs shows means ± SD of 3 animals per group analysed three days after sham operation in 3 independent experiments. Statistical analyses were performed by Student’s t-test. P-values < 0.05 were considered significant.
References:


# Table III

## Stroke Online Supplement

### Checklist of Methodological and Reporting Aspects for Articles Submitted to *Stroke* Involving Preclinical Experimentation

<table>
<thead>
<tr>
<th>Methodological and Reporting Aspects</th>
<th>Description of Procedures</th>
</tr>
</thead>
</table>
| **Experimental groups and study timeline**                   | ☑️ The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study.  
|                                                                  | ☐ An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated.  
|                                                                  | ☑️ An overall study timeline is provided.                                                                                                                                                                                                                                                                                                                       |
| **Inclusion and exclusion criteria**                          | ☐ A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article.                                                                                                                                                                                                                                             |
| **Randomization**                                             | ☑️ Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided.  
|                                                                  | ☐ Type and methods of randomization have been described.  
|                                                                  | ☐ Methods used for allocation concealment have been reported.                                                                                                                                                                                                                                                                                                 |
| **Blinding**                                                  | ☑️ Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible.  
|                                                                  | ☐ Blinding procedures have been described with regard to masking of group assignment during outcome assessment.                                                                                                                                                                                                                                             |
| **Sample size and power calculations**                        | ☑️ Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided.                                                                                                                                                                 |
| **Data reporting and statistical methods**                    | ☑️ Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups.  
|                                                                  | ☐ Baseline data on assessed outcome(s) for all experimental groups have been reported.  
|                                                                  | ☐ Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms.  
|                                                                  | ☐ Statistical methods used have been reported.  
|                                                                  | ☐ Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures.                                                                                                                                                                                                 |
| **Experimental details, ethics, and funding statements**      | ☑️ Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described.  
|                                                                  | ☑️ Different sex animals have been used. If not, the reason/justification is provided.  
|                                                                  | ☐ Statements on approval by ethics boards and ethical conduct of studies have been provided.  
|                                                                  | ☐ Statements on funding and conflicts of interests have been provided.                                                                                                                                                                                                                                                                 |