Blocking of α4 Integrin Does Not Protect From Acute Ischemic Stroke in Mice

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Background and Purpose—T lymphocytes have recently been identified as key mediators of tissue damage in ischemic stroke. The interaction between very late antigen-4 (VLA-4) and vascular adhesion molecule-1 is crucial for the transvascular egress of T lymphocytes, and inhibition of this interaction by specific antibodies is a powerful strategy to combat autoimmune neuroinflammation. However, whether pharmacological blocking of T-lymphocyte trafficking is also protective during brain ischemia is still unclear. We investigated the efficacy of a monoclonal antibody directed against VLA-4 in mouse models of ischemic stroke.

Methods—Transient and permanent middle cerebral artery occlusion was induced in male C57Bl/6 mice. Animals treated with a monoclonal anti-CD49d antibody (300 μg) 24 hours before or 3 hours after the onset of cerebral ischemia and stroke outcome, including infarct size, functional status, and mortality, were assessed between day 1 and day 7. The numbers of immune cells invading the ischemic brain were determined by immunocytochemistry and flow cytometry.

Results—Blocking of VLA-4 significantly reduced the invasion of T lymphocytes and neutrophils on day 5 after middle cerebral artery occlusion and inhibited the upregulation of vascular adhesion molecule-1. However, the anti-CD49d antibody failed to influence stroke outcome positively irrespective of the model or the time point investigated.

Conclusions—Pharmacological inhibition of the VLA-4/vascular adhesion molecule-1 axis in experimental stroke was ineffective in our hands. Our results cast doubt on the effectiveness of anti-CD49d as a stroke treatment. Further translational studies should be performed before testing anti-VLA-4 antibodies in patients with stroke. (Stroke. 2014;45:00-00.)

Key Words: infarction, middle cerebral artery • inflammation • natalizumab• vascular cell adhesion molecule-1 • very late antigen-4

The immune system is increasingly recognized as promising therapeutic target in acute ischemic stroke in which treatment options are still limited. Although the local and systemic immune response after cerebral ischemia comprises a complex network of cellular and soluble factors, T lymphocytes seem to be of particular relevance for stroke development and expansion. T cells start to enter the ischemic brain around day 1 with a maximum around day 5 both in patients with stroke and in rodents. The T-cell effector mechanisms that trigger tissue damage in ischemic stroke are still incompletely understood but might involve secretion of proinflammatory cytokines, such as interferon-γ or immediate cytotoxicity mediated for instance by perforins. In contrast, T-cell–driven adaptive immunity seems to be of minor pathophysiological importance at least during the acute stage of an ischemic insult. Interestingly, certain T-cell subsets exist that can exert more severe damage to the ischemic brain than others. Blocking of interleukin-17A producing γδ T cells, for instance, was protective after transient middle cerebral artery occlusion (tMCAO) in mice, and genetic depletion of regulatory T cells (Treg) at an early stroke stage likewise improved outcome under experimental conditions.

Transmigration across the activated blood–brain barrier is a mandatory step for T lymphocytes to cause harm to ischemic brain tissues. Cell trafficking from the vasculature into the brain parenchyma is a complex and tightly regulated process, which involves different adhesion receptors located on endothelial cells and immune cells. Invasion of T lymphocytes into...
the central nervous system critically depends on the interplay between leukocyte very late antigen-4 (VLA-4) with vascular cell adhesion molecule-1 (VCAM-1) expressed on endothelial cells. Structurally, VLA-4 represents an integrin heterodimer composed of a \( \alpha \) chain (integrin \( \alpha 4=CD49d \)) and a \( \beta \) chain (CD29). Blocking of VLA-4 using the monoclonal antibody natalizumab is highly effective in preventing relapses and disease progression in multiple sclerosis\(^{10} \) and Crohn’s disease.\(^{11} \)

However, contradictory reports exist on the efficacy of this approach in models of ischemic stroke. Here, VLA-4 inhibition might only act beneficially during distinct stages of infarction or in certain animal species.\(^{8,12,13} \) Albeit the preclinical package of a VLA-4 blockade in ischemic stroke is still preliminary and does not fulfill current consensus criteria that would recommend to move forward into clinical testing,\(^{14,15} \) there is at least 2 phase 2 trial being planned that aims to investigate the safety and efficacy of natalizumab in patients with stroke (Effect of Natalizumab on Infarct Volume in Acute Ischemic Stroke; ClinicalTrials.gov identifier: NCT01955707).

Herein, we demonstrate that blocking of the VLA-4-VCAM-1 axis using a monoclonal CD49d-specific antibody does not improve stroke outcome in mice irrespective of the stage or type of cerebral ischemia.

Materials and Methods

A detailed description of the Methods, the Surgical Procedures, and the Stroke Study Population is provided in the online-only Data Supplement.

Ischemia Model

A total of 153 mice (Charles River) were included in the study, which was conducted in accordance with institutional guidelines for the use of experimental animals (University of Würzburg, Germany and University Hospital Hamburg-Eppendorf, Hamburg, Germany), and the protocols were approved by governmental authorities (Regierung von Unterfranken, Würzburg, Germany and Behörde für Lebensmittelsicherheit und Veterinärwesen, Hamburg, Germany). If not otherwise mentioned, we performed 30 minutes of tMCAO in 6- to 8-week-old male C57Bl/6 mice weighing 20 to 25 g, as described.\(^{16} \) For permanent MCAO (pMCAO), the coagulation model according to Tamura et al\(^{17} \) was used.

We injected 300 \( \mu \)g of CD49d-specific monoclonal antibody (clone R1-2; eBioscience) intraperitoneally 24 hours before or 3 hours after the induction of ischemia based on the study of Liesz et al.\(^{16} \) Control animals were injected with 300-\( \mu \)g rat IgG2b isotype control antibodies (eBioscience).

Mice were controlled for several physiological parameters that can critically affect stroke outcome (blood pressure, heart rate, and arterial blood gases; Figure I in the online-only Data Supplement; Table I in the online-only Data Supplement).

We calculated edema-corrected infarct volumes from coronal brain slices stained with 2,3,5-triphenyltetrazolium chloride (TTC). All results were expressed as mean±SEM except for ordinal functional outcome scales that were 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 \( \geq 0.2 \) after 30 minutes of tMCAO (IgG control mice versus anti–CD49d-treated mice) were determined via a priori sample size calculation with the following assumptions: \( \alpha=0.05, \beta=0.2, \) mean, 20% SD of the mean (GraphPad Stat Mate 2.0; GraphPad Software). For statistical analysis, the GraphPad Prism 5.0 software package (GraphPad Software) was used. Data were tested for Gaussian distribution with the D’Agostino and Pearson omnibus normality test and then analyzed by 1-way ANOVA; in case of measuring the effects of 2 factors simultaneously, 2-way ANOVA with post hoc Bonferroni correction for multivariate analyses was used. If only 2 groups were compared, unpaired, 2-tailed Student t test was applied. Nonparametric functional outcome scores were compared by Kruskal–Wallis test with post hoc Dunn multiple comparison test. For comparison of survival curves, the log-rank test was used. \( P \) values <0.05 were considered statistically significant.

Results

CD49d Inhibition Does Not Improve Outcome After Experimental Stroke

We first analyzed whether a monoclonal antibody directed against CD49d would improve outcome after mild brain ischemia–reperfusion injury in mice. To this end, 300 \( \mu \)g of the antibody was administered intraperitoneally 24 hours before 30 minutes of tMCAO, and stroke size was assessed on day 1. Stroke volumes did not differ between the groups at this early time point (30.1±7.9 \( \text{mm}^3 \) for control mice versus 32.4±7.7 \( \text{mm}^3 \) for anti–CD49d-treated mice; \( P=0.83; \) Figure 1A). To test whether blocking of CD49d exerts beneficial effects at more advanced stages of infarct development, we also determined stroke volumes in antibody-treated mice and controls on day 7, but again did not observe any relevant differences (20.7±4.3 \( \text{mm}^3 \) for control mice versus 19.2±5.4 \( \text{mm}^3 \) for anti–CD49d-treated mice; \( P=0.83; \) Figure 1A). In human stroke studies, functional outcome rather than stroke size is regarded as the most relevant function, motor function, and coordination in mice. The corner test\(^{20} \) and mortality rates were assessed on a daily basis until day 7 after stroke (Methods in the online-only Data Supplement).

Immunohistology

Staining protocols for T lymphocytes and neutrophils are described in the Methods in the online-only Data Supplement.

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.\(^{8} \) Myeloid immune cells and lymphocytes were incubated in fluorescence-activated cell sorter buffer with monoclonal antibodies anti–CD3-Alexa Fluor 488 (clone 17A2; Biolegend), anti–CD11b-PE (clone M1/70; Biolegend), and anti–Gr-1-allophycocyanin (clone RB6-8C5; BD Bioscience). Cells were counted on a fluorescence-activated cell sorter Calibur (Becton Dickinson; Methods in the online-only Data Supplement).

Western Blot Analysis

Immunoreactivity for VCAM-1 (anti–VCAM-1 mAb 1:2000; Abcam) was detected by Western blot analysis and quantified by densitometry. Actin was served as loading control for all Western blot experiments (see online-only Data Supplement).

Statistics

All results were expressed as mean±SEM except for ordinal functional outcome scales that were 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 \( \geq 0.2 \) after 30 minutes of tMCAO (IgG control mice versus anti–CD49d-treated mice) were determined via a priori sample size calculation with the following assumptions: \( \alpha=0.05, \beta=0.2, \) mean, 20% SD of the mean (GraphPad Stat Mate 2.0; GraphPad Software). For statistical analysis, the GraphPad Prism 5.0 software package (GraphPad Software) was used. Data were tested for Gaussian distribution with the D’Agostino and Pearson omnibus normality test and then analyzed by 1-way ANOVA; in case of measuring the effects of 2 factors simultaneously, 2-way ANOVA with post hoc Bonferroni correction for multivariate analyses was used. If only 2 groups were compared, unpaired, 2-tailed Student t test was applied. Nonparametric functional outcome scores were compared by Kruskal–Wallis test with post hoc Dunn multiple comparison test. For comparison of survival curves, the log-rank test was used. \( P \) values <0.05 were considered statistically significant.
end point. Therefore, we applied different test batteries to evaluate the neurological status in both mouse groups. Blocking of VLA-4 failed to influence motor function and coordination as assessed by the Bederson score (day 1, 2.0 [1.75, 2.0] for control mice versus 2.0 [2.0, 3.0] for anti–CD49d-treated mice; \( P \geq 0.05 \) and day 7, 1.5 [1.0, 2.0] for control mice versus 1.5 [1.0, 2.0] for anti–CD49d-treated mice; \( P \geq 0.05 \)) and the grip test (day 1, 4.0 [3.75, 4.0] for control mice versus 3.0 [0.0, 4.0] for anti–CD49d-treated mice; \( P \geq 0.05 \) and day 7, 4.0 [4.0, 5.0] for control mice versus 4.5 [4.0, 5.0] for anti–CD49d-treated mice; \( P \geq 0.05 \)) on day 1 and day 7 after tMCAO (Figure 1B).

Furthermore, the results from the corner test revealed that anti–CD49d treatment did not significantly improve sensorimotor asymmetry over time (\( P = 0.08 \); Figure 1C). Finally, VLA-4 inhibition had no positive effect on overall survival (Figure 1D).

According to current quality guidelines in experimental stroke research, the safety and efficacy of any novel therapeutic approach should be evaluated in both models of transient and permanent stroke.\(^4\) Therefore, we also induced pMCAO by transtemporal coagulation of the right MCA distal to the lenticostriate arteries.\(^17\) This procedure leads to circumscribed cortical infarcts, which are highly reproducible in size and location. In line with the findings after tMCAO, prophylactic application of the anti-CD49d antibody was also unable to modify stroke volumes on day 7 (18.5±2.5 mm\(^3\) for control mice versus 20.8±2.4 mm\(^3\) for anti–CD49d-treated mice; \( P = 0.51 \)) after permanent ischemia (Figure 2A). Accordingly, we did not observe differences in the corner test between treated and untreated mice under these conditions (\( P = 0.13 \); Figure 2B).

Figure 1. Prestroke CD49d inhibition does not improve stroke outcome in a transient middle cerebral artery occlusion (tMCAO) model. A, Left, Representative 2,3,5-triphenyltetrazolium chloride stains of 3 corresponding coronal brain sections of an IgG-treated C57Bl/6 mouse (IgG control) and a C57Bl/6 mouse treated with a specific antibody against CD49d (300 \( \mu \)g IP) 24 hours before 30 minutes of tMCAO. Animals were euthanized on day 1 or day 7 after tMCAO. Infarct expansion (white arrows) seems to be similar between the 2 treatment groups at either time point and this was confirmed by infarct volumetry (right; \( n = 10 \) per group), unpaired, 2-tailed Student t test. B and C, Blocking of CD49d does not improve functional outcome on day 1 and day 7 after tMCAO as assessed by the Bederson score (left), the grip test (right), and the corner test (C; \( n = 10 \) per group); Kruskal–Wallis test with post hoc Dunn multiple comparison test. D, Kaplan–Meier curve reveals that blocking of CD49d does not influence long-term survival after tMCAO (\( n = 10 \) per group), log-rank test.
To exclude inappropriate timing as the reason underlying the inefficacy of VLA-4 inhibition in our stroke models, we also applied the anti-CD49d antibody in a therapeutic approach (ie, 3 hours after the onset of transient brain ischemia). This therapeutic regimen likewise did not affect stroke outcome on day 1 or day 7 (infarct volume; day 7, 26.9±5.3 mm³ for control mice versus 22.5±2.1 mm³ for anti-CD49d-treated mice; P≥0.05; Bederson score: day 1, 2.0 [1.5, 3.0] for control mice versus 2.0 [1.0, 2.5] for anti–CD49d-treated mice; P≥0.05; Bederson score: day 7, 0.5 [0.0, 2.0] for control mice versus 1.0 [1.0, 1.75] for anti–CD49d-treated mice; P≥0.05; grip test: day 1, 3.0 [3.0, 4.0] for control mice versus 4.0 [3.0, 5.0] for anti–CD49d-treated mice; P≥0.05; grip test: day 7, 5.0 [4.25, 5.0] for control mice versus 5.0 [5.0, 5.0] for anti–CD49d-treated mice; P≥0.05; corner test, P=0.92; Figure 3A–3C).

Anti-CD49d Treatment Reduces Leukocyte Numbers in the Ischemic Brain
Suppression of lymphocyte invasion into the central nervous system is considered the key mode of natalizumab action in multiple sclerosis.10 Thus, we next analyzed whether blocking of VLA-4 also prevents immune cell infiltration after ischemic stroke. Indeed, anti-CD49d treatment significantly reduced the numbers of brain-infiltrating T lymphocytes (14.8±1.9 cells per slice in the ischemic hemisphere for control mice versus 9.3±1.3 cells for anti–CD49d-treated mice; P<0.05) and granulocytes (23.5±5.7 cells per slice in the ischemic hemisphere for control mice versus 12.2±1.9 cells for anti–CD49d-treated mice; P<0.05) on day 5 after tMCAO, as assessed by immunocytochemistry (Figure 4A and 4B) and flow cytometry (CD3+ T lymphocytes, 4760±500 cells for control mice versus 3463±239 cells for anti–CD49d-treated mice; P=0.03; Gr-1+ neutrophils, 8620±921 cells for control mice versus 3996±477 cells for anti–CD49d-treated mice; P=0.016; CD11b+ [a marker for activated microglia/macrophages] cells, 1702±1653 cells for control mice versus 5978±685 cells for anti–CD49d-treated mice; P=0.016; Figure 4C). Moreover, the anti-CD49d antibody significantly inhibited the upregulation of VCAM-1,11 the main endothelial ligand of VLA-4, in the ischemic brains on day 5 after stroke (Figure 5).

Discussion
The present study failed to confirm a positive effect when blocking the VLA-4–VCAM-1 axis in ischemic stroke in mice.6 Prophylactic or therapeutic application of a monoclonal CD49d-specific antibody did not affect stroke size or neurological deficits in models of transient and permanent brain ischemia. Moreover, we did not find evidence that the efficacy of VLA-4 inhibition in stroke is time dependent because the infarctions after anti-CD49d treatment were unaltered both during the acute and during the chronic stage.

There are currently 3 reports that investigated the efficacy of specific antibodies against VLA-4 in rodent models of ischemic stroke.6,12,13. Becker et al12 and Relton et al13 described reduced infarct volumes and improved functional deficits after transient focal cerebral ischemia in rats receiving anti-VLA-4 treatment but only focused on the acute phase after the ischemic challenge (24 and 48 hours, respectively). In contrast, the study by Liesz et al6 used C57Bl/6 mice, which were subjected to both transient and pMCAO. Importantly, later time points (until day 7) were taken into account in this study as well. Contrary to the findings in rats,12,13 Liesz et al6 failed to confirm that blocking of VLA-4 with a monoclonal CD49d-specific antibody improves stroke outcome on day 1 after focal brain ischemia in mice, which is in accordance with the observations from our study. Surprisingly, however, the same antibody obviously prevented secondary infarct growth because stroke volumes were significantly smaller and neurological deficits were less pronounced on day 7 after MCAO in the study by Liesz et al.6

Although the discrepancies in stroke outcomes between the rat12,13 and the mouse studies6 can be reasonably explained by species differences, as well as differing antibody formulations and application routes, the reasons why we were unable to reproduce the findings made by Liesz et al6 at the late stage of infarction (day 7) remain unclear. We used identical procedures (transient filament occlusion or permanent electrocoagulation of the MCA) to induce focal cerebral ischemia in male C57Bl/6 mice of comparable age and outcome assessment on the time points after MCAO or the neurological test batteries was largely the same. Moreover, the
same anti-CD49d antibody clone (R1-2) was purchased from the same commercial provider (eBioscience). In addition, we strictly followed the time points and application routes as indicated for the anti-CD49d antibody by Liesz et al.6 Importantly, the location (only the basal ganglia or only cortex, respectively) and variability of infarctions induced by 30-minute tMCAO or pMCAO in the present study well matched with those reported by Liesz et al.6 although total infarct volumes on day 7 after tMCAO were moderately larger in our hands (≈10 versus ≈20 mm3, respectively). Different methods for the assessment of infarct volumes (TTC staining versus silver staining) probably accounted for these variations. Also, both studies were obviously of high quality6,15 because masking of raters was ensured, and a sufficient number of animals was included and tested in different stroke models to allow for valid statistical conclusions; although in contrast to our study, Liesz et al.6 did not declare dropout rates and did not provide prespecified sample size calculations, limitations which are known to be potential sources of study bias.14,15 However, several other reasons are also conceivable. For example, the housing conditions and subsequently the microbiota are substantially different between animal facilities, which in turn will influence most inflammatory reactions.22 Small differences in the handling of the animals or different antibody batches could also be of relevance. Stroke size seems to determine the postischemic immune response critically.23,24 Therefore, we cannot exclude that the anti-CD49d antibody would have worked in case of longer periods of brain ischemia (eg, 60 or 90 minutes); although in the study by Liesz et al.6 the stroke protective effect seen on day 7 after 30 minutes of tMCAO was lost after 60 minutes of tMCAO. Taken together, these divergent results underline the complexity of studying the role of the immune system in brain ischemia and once more spell out the difficulty in reproducing key findings from experimental stroke studies between different laboratories.25

Anti-CD49d antibody treatment significantly reduced the cerebral invasion of T lymphocytes and granulocytes on day 5 after experimental brain ischemia and inhibited the upregulation of VCAM-1, which is in line with the supposed mechanisms of drug action and similar to previous studies.6,12,13,21 These observations prove that the anti-CD49d antibody clone
applied in our study was biologically active thereby excluding a spoilt antibody preparation as an explanation for the discrepant study results although we cannot definitely exclude that it was slightly less functional than the one used by Liesz et al.\textsuperscript{6}

Moreover, invading T cells and granulocytes are obviously of minor relevance for mediating tissue damage in the ischemic mouse brain during the later stages of infarction. However, we cannot rule out from our studies that distinct T-cell subsets (and potentially other immune cells) were unaffected by the anti-CD49d antibody and, therefore, might have mediated most of the inflammatory damage.\textsuperscript{26} Controversial effects on stroke outcome have also been described for other immunomodulatory therapies, such as FTY720\textsuperscript{27,28} or antineutrophil agents.\textsuperscript{29,30} Furthermore, even the long claimed characteristic of neutrophils as the leading stroke enhancers has recently been called into question.\textsuperscript{31}

Taken together, immune-mediated tissue damage after stroke is a complex process, which is only incompletely understood.
Although there is little doubt that certain immune cell subsets are critically involved in secondary infarct expansion, pharmacological strategies to modulate the inflammatory response on brain ischemia produced controversial results. The VLA-4-VCAM-1 axis, for example, had no effect on several critical outcome parameters of experimental stroke in our hands although a prominent role was described by others. Because ≥ 1 phase 2 clinical trial is currently underway that aims to test the safety and efficacy of a monoclonal antibody (natalizumab) in patients with acute ischemic stroke (Effect of Natalizumab on Infarct Volume in Acute Ischemic Stroke; ClinicalTrials.gov Identifier: NCT01955707), we think that our findings are critically involved in secondary infarct expansion, phosphochromically induced ischemia of the rat cortex. J Cereb Blood Flow Metab. 1995;15:42–51.


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Disclosures
None.

References


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SUPPLEMENTAL MATERIAL

Supplemental Methods
Induction of cerebral ischemia in mice
Focal cerebral ischemia was induced by 30 min of transient middle cerebral artery occlusion (tMCAO) as described.1,2 Mice were anesthetized with 2.5% isoflurane (Abbott) in a 70% N2O/30% O2 mixture. Core body temperature was maintained at 37°C throughout surgery by using a feedback-controlled heating device. 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 (6021; Doccol) was inserted and advanced via the right internal carotid artery to occlude the origin of the right MCA. The intraluminal suture was left in situ for 30 minutes. Then animals were re-anesthetized and the occluding monofilament was withdrawn to allow for reperfusion. Sham-operated mice underwent the same surgical procedure except for the insertion of the filament.
Permanent MCA occlusion was performed according to Tamura et al.3 Briefly, the skin between the right eye and the right ear was incised and the temporal muscle was retracted. To expose the MCA, a small craniotomy was performed and the dura mater was excised. The MCA was occluded permanently using bipolar electrocoagulation forceps (Codman, 0.25 mm Tip Diameter) and the incision wound was sutured afterwards.
We injected 300 µg of a CD49d-specific monoclonal antibody (clone R1-2, eBioscience) intraperitoneally 24 h before or 3 h after the induction of ischemia.4 Control animals were injected with 300 µg rat IgG2b isotype control antibodies (eBioscience) 24 h before or 3 h after induction of ischemia.
Assessment of functional outcome
Neurological deficits were scored and quantified according to Bederson5 immediately after reperfusion, i.e. 30 min after ischemia (tMCAO group only), and again on day 1 and day 7 after MCAO: 0, no deficit; 1, forelimb flexion; 2, as for 1, plus decreased resistance to lateral push; 3, unidirectional circling; 4, longitudinal spinning; 5, no movement. For the grip test,6 the mouse was placed midway on a string between two supports and rated as follows: 0, falls off; 1, hangs on to string by one or both fore paws; 2, as for 1, and attempts to climb on to string; 3, hangs on to string by one or both fore paws plus one or both hind paws; 4, hangs on to string by fore and hind paws plus tail wrapped around string; 5, escape (to the supports). The corner test was used according to Zhang et al.7 Mice were placed in a corner (30° angle) and the chosen sides to leave the corner were counted. Each mouse was tested before and after surgery. We calculated the ratio of right and left turns as an indicator of behavioural asymmetry. At least 10 full turns were counted for each testing.
Stroke study design
All mice were randomly assigned to the operators by an independent person not involved in data acquisition and analysis. We performed surgery and evaluation of all read-out parameters while blinded to the experimental groups. The following conditions excluded animals from end-point analyses (exclusion criteria): 1. Death within 24 h after MCAO 2. Subarachnoidal hemorrhage (SAH) or intracerebral hemorrhage present within the brain parenchyma (ICH) (as macroscopically assessed during brain sampling) 3. Bederson score = 0 (60 min after tMCAO)
Of the 146 mice subjected to 30 minutes tMCAO, 13 mice (8.9%) met at least one exclusion criterion after randomization and were withdrawn from the study resulting in 133 mice with tMCAO finally included in the study. From the 20 mice subjected to electrocoagulation all mice survived until the endpoint of the study at day 7. Drop-out rates were evenly distributed between the groups (six mice in the IgG control treated vs seven for CD49d treated mice group, P > 0.05).

**Determination of infarct size**

After sacrificing the mice the brains were quickly removed and cut in three 2-mm thick coronal sections using a mouse brain slice matrix (Harvard Apparatus). The slices were stained for 20 min at 37°C with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich) in PBS to visualize the infarctions.\(^8\)

Indirect, i.e. corrected for brain edema, infarct volumes were calculated by volumetry (ImageJ software, National Institutes of Health) according to the following equation:

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

whereas the term \( (V_{\text{ih}} - V_{\text{ch}}) \) represents the volume difference between the ischemic hemisphere and the control hemisphere and \( (V_{\text{ih}} - V_{\text{ch}})/V_{\text{ch}} \) expresses this difference as a percentage of the control hemisphere.

**Invasive hemodynamics**

For the assessment of blood pressure and heart rate, IgG treated C57Bl/6 mouse (IgG control) and C57Bl/6 mouse treated with a specific antibody against CD49d (300 µg i.p.) 24 h before measurement, were anesthetized with 2.0% isoflurane and catheterized via the right carotid artery with a high-fidelity 1.4 F Millar microtip catheter (Millar Instruments) as described.\(^9\) Hemodynamic data were digitized via a MacLab system (AD Instruments) connected to an Apple G4 PowerPC computer and analyzed.

**Blood gas analysis**

100 µl of arterial blood was drawn from the left cardiac ventricle of anesthetized mice by a heparinized syringe. We determined PaO2, PaCO2 and pH in anti-CD49d treated mice and IgG treated control mice using an ABL 77 automated blood gas analyzer (Radiometer).

**Histology and immunohistochemistry**

Cryo-embedded brains were cut into 10 µm thick slices and fixed in acetone. Blocking of epitopes was achieved by pre-treatment with 5% bovine serum albumin (BSA) in PBS for 45 min to prevent unspecific binding. For staining of neutrophils rat anti-mouse Ly-6B.2 alloantigen (MCA771G, AbD Serotec) at a dilution of 1:500 in PBS containing 1% BSA was added overnight at 4°C. Afterwards, slides were incubated with a biotinylated anti-rat IgG (BA-4001, Vector Laboratories) diluted 1:100 in PBS containing 1% BSA for 45 min at room temperature. Following treatment with Avidin/Biotin blocking solution (Avidin/Biotin Blocking Kit, Sp-2001, Vector Laboratories) to inhibit endogenous peroxidase activity, the secondary antibody was linked via streptavidin to a biotinylated peroxidase (POD) according to the manufacturer’s instructions (Vectorstain ABC Kit, Peroxidase Standard PK-4000, Vector Laboratories). Antigens were visualized via POD using the chromogen 3,3’-Diaminobenzidin (DAB) (Kem-En-Tec Diagnostics).

For immunofluorescence staining against CD3, cryo-embedded brain slices were fixed in 4% PFA in PBS for 15 minutes and blocked for 1 h at room temperature in 5% BSA with 0.2% Triton in PBS to prevent unspecific binding. A rat anti-mouse CD3
antibody conjugated to Alexa Fluor 488 (Clone 17A2, Biolegend, dilution 1:50) was applied for 1h at room temperature in PBS containing 1% BSA. For staining of DNA a fluorescent Hoechst dye (Hoechst 33342, Sigma-Aldrich) was added for 10 min at a concentration of 2 ng/ml. Sections were embedded in Mowiol 4-88 (Sigma) and analyzed under a microscope (Nikon Eclipse 50i).

For quantification of neutrophils and CD3+ T lymphocytes identical brain sections (thickness 10 µm) at the level of the basal ganglia (0.5 mm anterior from bregma) were selected and cell counting was performed from 5 subsequent slices (distance 100 µm) from 5 (neutrophils) or 7 (CD3+ T lymphocytes) different animals under a Nikon microscope Eclipse 50i (Nikon).10,11

For all histological experiments negative controls included omission of primary or secondary antibody and gave no signals (not shown).

Cell separation and flow cytometry
For the isolation of brain infiltrating mononuclear cells 5 days after tMCAO, mice were transcardially perfused with 1x PBS. Ischemic brains 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.12

Mononuclear cells were collected from the interface of the Percoll gradient, washed and resuspended in 1 x PBS for further analysis. Myeloid immune cells and lymphocytes were stained with monoclonal antibodies anti–CD3-Alexa Fluor® 488 (Clone 17A2, Biolegend), anti–CD11b-PE (Clone M1/70, Biolegend) and anti Gr-1-allophycocyanin (Clone RB6-8C5, BD Bioscience) following standard protocols. To determine absolute cell numbers by flow-assisted cell sorting Calibrite beads (BD Biosciences) were added.

Western blot
Cortices or basal ganglia were dissected from the mouse brains and homogenized in RIPA buffer (25 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40) containing 0.1% SDS and 4% proteinase inhibitor (complete protease inhibitor cocktail, Roche). Samples were sonified for 10 sec. Afterwards tissue lysates were centrifuged at 15.000×g for 30 min at 4°C and supernatants were used for bicinchoninic acid (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. 10 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 anti-VCAM-1 (ab 134047, Abcam, 1:2000) and anti-actin (Dianova, 1:75.000) antibodies at 4°C overnight. 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 VCAM-1) (Dianova) or donkey anti-mouse IgG (for actin) (Dianova) at a dilution of 1:5000 and were finally developed using ECLplus (GE Healthcare).10,11
Supplemental Tables

Table I
Results of blood gas analysis in IgG control-treated C57Bl/6 mice and mice receiving anti-CD49d antibodies, respectively. No significant differences were observed between the groups. (Mann Whitney Test)

<table>
<thead>
<tr>
<th></th>
<th>IgG control</th>
<th>anti-CD49d</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>PaO₂ (mmHg)</td>
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<td>78.8 ± 10.0</td>
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<td>pH</td>
<td>7.34 ± 0.05</td>
<td>7.43 ± 0.03</td>
<td>0.2683</td>
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</tbody>
</table>

Supplemental Figures

Figure I
Treatment with anti-CD49d does not alter mean arterial blood pressure (P = 0.25) or heart rate (P = 0.73) compared with isotype control-treated mice (IgG control) as assessed by invasive hemodynamics (n=5/group), unpaired, two-tailed Student’s t-test.
Supplemental References


