Inhibition of lymphocyte trafficking shields the brain against deleterious neuroinflammation after stroke

Arthur Liesz,1 Wei Zhou,1 Éva Mracskó,1 Simone Karcher,1 Henrike Bauer,2 Sönke Schwarting,1 Li Sun,1 Dunja Bruder,3 Sabine Stegemann,3 Adelheid Cerwenka,4 Clemens Sommer,2 Alexander H. Dalpke5 and Roland Veltkamp1

1 Department of Neurology, University Heidelberg, Im Neuenheimer Feld 400, 69120 Heidelberg, Germany
2 Department of Neuropathology, University Medical Centre of the Johannes Gutenberg University Mainz, Langenbeckstrasse 1, 55131 Mainz, Germany
3 Division of Immunregulation, Helmholtz Centre for Infection Research, Inhoffenstraße 7, 38124 Braunschweig, Germany
4 Division of Innate Immunity, German Cancer Research Centre, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany
5 Department of Infectious Diseases, Medical Microbiology and Hygiene, University Heidelberg, Im Neuenheimer Feld 324, 69120 Heidelberg, Germany

Correspondence to: Roland Veltkamp, MD, Department of Neurology, University Heidelberg, Im Neuenheimer Feld 400, 69120 Heidelberg, Germany
E-mail: roland.veltkamp@med.uni-heidelberg.de

T lymphocytes are increasingly recognized as key modulators of detrimental inflammatory cascades in acute ischaemic stroke, but the potential of T cell-targeted therapy in brain ischaemia is largely unexplored. Here, we characterize the effect of inhibiting leukocyte very late antigen-4 and endothelial vascular cell adhesion molecule-1-mediated brain invasion—currently the most effective strategy in primary neuroinflammatory brain disease in murine ischaemic stroke models. Very late antigen-4 blockade by monoclonal antibodies improved outcome in models of moderate stroke lesions by inhibiting cerebral leukocyte invasion and neurotoxic cytokine production without increasing the susceptibility to bacterial infections. Gene silencing of the endothelial very late antigen-4 counterpart vascular cell adhesion molecule-1 by in vivo small interfering RNA injection resulted in an equally potent reduction of infarct volume and post-ischaemic neuroinflammation. Furthermore, very late antigen-4-inhibition effectively reduced the post-ischaemic vascular cell adhesion molecule-1 upregulation, suggesting an additional cross-signalling between invading leukocytes and the cerebral endothelium. Dissecting the specific impact of leukocyte subpopulations showed that invading T cells, via their humoral secretion (interferon-γ) and immediate cytotoxic mechanisms (perforin), were the principal pathways for delayed post-ischaemic tissue injury. Thus, targeting T lymphocyte-migration represents a promising therapeutic approach for ischaemic stroke.

Keywords: brain ischaemia; T cell; VLA-4; VCAM-1; inflammation

Abbreviations: MCAO = middle cerebral artery occlusion; VCAM-1 = vascular cell adhesion molecule-1; VLA-4 = very late antigen-4

Received August 10, 2010. Revised November 29, 2010. Accepted December 15, 2010
© The Author (2011). Published by Oxford University Press on behalf of the Guarantors of Brain. All rights reserved.
For Permissions, please email: journals.permissions@oup.com
Introduction

Ischaemic stroke is a major cause of death and disability worldwide. Because thrombolysis, the only approved therapy for acute stroke at present, reaches only 5–10% of patients, therapeutic alternatives are being sought (Donnan et al., 2008). Among many detrimental cascades (Lo et al., 2003), inflammatory mechanisms have come into the focus of current research because they contribute substantially to secondary brain damage (Dimagi, 2004; Wang et al., 2006; Hurn et al., 2007; Liesz et al., 2009b). Moreover, their prolonged kinetics make them amenable to therapeutic intervention (Barone and Feuerstein, 1999; Dirnagl et al. 2001; Engelhardt and Sorokin, 2009). Recent experimental studies suggest a pivotal role of T cells in post-ischaemic inflammation of various organs including the brain (Zwacka et al., 1997; del Zoppo et al., 2001; Frangogiannis et al., 2002; Gee et al., 2007; Liesz et al., 2009b; Lo, 2009). However, the potential of T cell-targeted therapy in brain ischaemia is largely unexplored to date.

The presence and activity of systemic immune cells in the healthy brain is restricted and tightly regulated by the intact blood–brain barrier (Engelhardt and Sorokin, 2009). The adhesion of leukocytes to the activated endothelium and subsequent brain invasion after cerebral ischaemia represent critical steps in inducing collateral inflammatory damage. Cerebral invasion of lymphocytes crucially depends on the interaction of the leukocyte very late antigen-4 (VLA-4) with vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells (Baron et al., 1993; Engelhardt et al., 1998; Laschinger and Engelhardt, 2000; Vajkoczy et al., 2001; Engelhardt and Sorokin, 2009). VLA-4 (integrin alpha4-beta1) is an integrin heterodimer consisting of an alpha chain (integrin alpha4 = CD49d) and a beta chain (CD29) Correspondingly, natalizumab, an antibody blocking alpha4-integrin-mediated invasion of lymphocytes into the CNS, currently represents the most effective therapy for relapsing-remitting multiple sclerosis (Yednock et al., 1992; Rice et al., 2005; Steinman, 2005; Polman et al., 2006). In contrast, the impact of this potent approach has barely been investigated in stroke.

Herein, we comprehensively examine the profound effect of inhibition of the VLA-4–VCAM-1 axis on secondary tissue injury after experimental brain ischaemia, and uncover reduced T lymphocyte-mediated cytotoxicity as the key underlying mechanism of action.

Materials and methods

Animals

The study was conducted in accordance with national guidelines for the use of experimental animals and the protocols were approved by the governmental committees (Regierungspresidium Karlsruhe, Germany). We used age-matched, male mice (C57BL/6J, 10–12 weeks, 23–25 g body weight, Charles River Laboratories) unless stated otherwise. For transfer experiments, lymphocyte-deficient Rag2<sup>−/−</sup> mice were used as recipients. Perforin knockout mice Prf1<sup>−/−</sup> were purchased from The Jackson Laboratory. The genetic background of Rag2<sup>−/−</sup> and Prf1<sup>−/−</sup> animals were fully comparable with that of the respectively wild-type control animals (back-crossed for at least 10 generations).

Animal experiments

We injected 300 µg of CD49d-specific monoclonal antibody (clone R1-2, free of azide and preservatives) intraperitoneally (i.p.) 24 h before or 3 h after ischaemia-induction, control animals received rat IgG2b isotype control monoclonal antibody. Antibodies were commercialized purchased from ebioscience. See Supplementary Materials for detailed descriptions of the ischaemia models.

Assessment of infarct volume

The mice were deeply anaesthetized and perfused transcardially with 20 ml normal saline. The brains were removed from the skull and frozen immediately in isopentane (−20°C). Thick coronal cryosections (20 μm) were cut every 400 μm, and the sections were stained according to the silver staining protocol, scanned at 600 dpi and the infarct area on each section analysed (Scion Image). For the permanent coagulation model inducing exclusively cortical infarcts, the Swanson method (Swanson et al., 1990) was applied for indirect infarct measurement and correction for cortical swelling:

(Ischaemic area) = (Cortex area of the contralateral side) – (Non-ischaemic cortex area of the ipsilateral side)

For the filament model (30 and 60 min occlusion) comprising also subcortical lesions, we used direct infarct measurement and corrected for brain oedema by subtracting the ipsilateral minus contralateral hemisphere area from the directly measured infarct area. The total infarct volume was determined by integrating measured areas and distances between sections.

Functional outcome test

The ‘corner test’ was performed (Zhang et al., 2002) to measure sensorimotor dysfunction at 24 h before middle cerebral artery occlusion (MCAO) and on Day 1, 3 and 7 after MCAO. Mice were placed between two boards set at a 30° angle and allowed to move freely. An observer blinded to treatment allocation counted left and right turns with a rearing movement after deep entry in the corner and calculated the ratio of right and left turns as an indicator of behavioural asymmetry. At least 12 full turns after a rearing movement were counted for each testing.

Immunohistology

We performed immunohistochemistry of mature T lymphocytes, B cells and neutrophil granulocytes on coronal cryostat sections (12 μm) after pretreatment with 4% paraformaldehyde for 60 min. We blocked endogenous peroxidase with Peroxidase Blocking solution (Dako), then incubated the sections with primary antibodies against CD3 (Clone 3H698, Zytomed) or myeloperoxidase (Clone RP-053; Zytomed) for 20 min and then washed in phosphate buffered saline containing 20 ml normal saline. The brains were removed from the skull and frozen immediately in isopentane (−20°C). Immunoreactivity was visualized by a universal immunoenzyme polymer method (Nichirei Biosciences) and sections developed in dianaminobenzidene. For immunofluorescence staining of von Willebrand Factor, VCAM-1 and CD3, sections were fixed in −20°C acetone for 10 min and then washed in phosphate buffered saline containing

...
RNA isolation and real-time polymerase chain reaction

We isolated RNA from separated cerebral hemispheres and from peripheral blood mononuclear cells using the RNeasy kit (Qiagen). Reverse transcription was performed with the High Capacity complementary DNA Archive Kit (Applied Biosystems) and real-time polymerase chain reaction with SYBR-Green assays (Applied Biosystems) on an ABI7500 real-time polymerase chain reaction System (Applied Biosystems). Primers were purchased as ready-to-use primer sets for each gene (Super Array). All assays were run in duplicate. We normalized the results for each individual gene to the level of the housekeeping gene encoding for peptidylprolyl isomerase A (cyclophilin) for brain samples and to actin-β for peripheral blood mononuclear cell samples.

Streptococcus pneumoniae infection and analysis of bacterial load

Refer to the Supplementary Material for detailed descriptions of procedures.

Cytokine enzyme-linked immunosorbent assay

Serum was collected and frozen at −80°C immediately until analysis of cytokine protein concentrations with commercial kits for the quantitative assay of INF-γ, IL-10 (all from R&D Systems), TGF-β, IL-6 and TNF-α (eBioscience).

Depletion of leukocyte subpopulations

Cell population specific depletion was performed by intraperitoneally injecting the respective antibody diluted in sterile phosphate buffered saline. All antibodies were purchased from eBioscience. Effectiveness and specificity of the depletion was controlled by complete blood cell counting at the Core Laboratory Facilities at the University of Heidelberg and by flow cytometric analysis of lymphocyte subpopulations. Granulocyte-specific depletion: 400 μg functional grade purified rat anti-mouse Gr-1, clone RB6-8C5. Cytotoxic T cell-specific depletion: 300 μg functional grade purified rat anti-mouse CD8α (Ly-2), clone 53-6.7. T helper cell-specific depletion: 300 μg functional grade purified rat anti-mouse CD4 (L3T4), clone GK1.5.

Intracerebroventricular injection

Mice were anaesthetized with 1.0–2.0% halothane in O2/N2O enriched air and fixed in a stereotactic frame (Stoelting). After making a midline incision in skin over the skull, a 10 μl Hamilton syringe was used for intracerebroventricular drug administration at 0.9 mm lateral, 0.1 mm posterior and 3.1 mm deep relative to the bregma. We injected either 1 μg neutralizing antibodies against mouse IFN-γ (Clone XMG1.2, eBioscience) or control antibodies diluted in 2 μl artificial cerebrospinal fluid consisting of, in mM: 126 NaCl; 2.5 KCl; 1.2 NaH2PO4; 1.3 MgCl2 and 2.4 CaCl2 at pH 7.4.

VCAM-1 micro-western blotting

Brain tissues of infarcted and non-infarcted hemispheres were separately scraped off with a scalpel from 20 μm thick coronal cryosections every 400 μm through the infarcted brain area at room temperature.
and homogenized in ice-cold radioimmune precipitation buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 1 mM PMSF, 1 mM EDTA and 10% Protease Inhibitor Cocktail (Sigma Aldrich)). After centrifugation at 13 000 rpm for 10 min at 4 °C, the supernatant was collected and the protein concentration of each sample was determined in triplicate using Bradford reagent. Aliquots of lysates containing 50 μg protein underwent electrophoresis on 8% sodium dodecyl sulphate polyacrylamide gels and then were transferred to nitrocellulose membranes. After blocking in 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h, blots were incubated overnight at 4 °C with monoclonal rat anti-VCAM-1 (clone 99-12, Santa Cruz). Peroxidase-conjugated goat anti-rat antibody was used as secondary antibody and blots were detected using a standard chemiluminescence method. SolB cell lysate (Santa Cruz) was used as a positive control. Blots were stained with rabbit anti-actin antibody (Sigma Aldrich) to confirm equal loading and transfer.

Micro-polymerase chain reaction

Brain tissue was collected as described above for micro-western blotting and processed by syringe-and-needle homogenization in lysis reagent. The RNA was then isolated using an RNeasy® Micro kit (Qiagen) and we applied the standard real-time polymerase chain reaction protocol described above, modified to meet the reduced RNA amount.

Fluorescence spectroscopy

Naïve mice were treated i.p. with 1 mg/kg or 15 mg/kg body weight of either unconjugated or fluorescein isothiocyanate-conjugated VCAM-specific antibodies (clone: 429, eBioscience). Then, 24 h after antibody treatment, mice were sacrificed and fresh brain tissue was homogenized in ice-cold RLT buffer (Qiagen). Brain homogenates were analysed in black 96-well plates on a Synergy4 monochromatic detection system (BioTek). Excitation of the samples was at 518 nm and emission spectrum detected from 520–700 nm in 1 nm steps; RLT lysis buffer in the final concentration was used as standard control for calibration. All samples were analysed in triplicate.

VCAM-1 gene silencing

In vivo silencing of VCAM-1 was performed by hydrodynamic injection of small interfering RNA into the femoral vein as previously described (Hino et al., 2006; Herweijer and Wolff, 2007). The left femoral vein was dissected and freshly diluted small interfering RNA in 1 ml RNase-free water injected through a microcatheter into the vein within 5 s. After injection, the catheter was left in place under compression until haemostasis. The treatment group received 30 nmol VCAM-1 Silencer® Select small interfering RNA, in vivo ready, per mouse (~16 mg/kg body weight, control animals received 30 nmol Silencer® Select Negative Control #1 small interfering RNA, in vivo ready (all small interfering RNA was commercially purchased from Ambion). Mortality induced by this procedure was <5%.

Statistical analysis

All values in bar graphs are expressed as mean ± standard deviation (SD). We analysed infarct volumes and functional outcome tests by two-tailed Student’s t-test between two groups and ANOVA for multiple comparisons with post hoc Tukey’s test, respectively, after validating the normal distribution of these data sets (Kolmogorov–Smirnov test). Log rank test was used to compare survival curves. For the remaining data, we used two-tailed Wilcoxon rank-sum test for comparison between two groups; for three and more groups we applied the Kruskal–Wallis H test with Dunn’s post hoc testing, using GraphPad Prism 5 software. P < 0.05 was considered statistically significant.

Results

Integrin α4 inhibition improves experimental stroke outcome

We analysed the effect of blocking the α4-integrin (CD49d) on leukocytes on infarct volume after experimental ischaemia by intraperitoneal administration of 300 μg CD49d-specific monoclonal antibodies. Unless otherwise stated, MCAO was induced by permanent trans-temporal coagulation of the left middle cerebral artery distal to the lenticulostriate arteries that results in moderately sized cortical infarcts. By administering anti-CD49d 24 h before MCAO, infarct volumes at 7 days were significantly lower compared with control treatment with IgG2 isotype antibodies (Fig. 1A), but no differences in infarct volume were observed between treatment groups at 24 h MCAO (Fig. 1A), indicating that CD49d blockade mainly affected delayed damage. Blocking CD49d 3 h after MCAO resulted in a similar therapeutic effect (Fig. 1B). Furthermore, anti-CD49d treatment also improved behavioural outcome after permanent MCAO as tested by the corner test detecting sensorimotor asymmetry (Zhang et al., 2002). Mice in both groups deviated from the normal ratio of 1.0 towards an increased right-turn ratio at 24 h after MCAO, but mice in the anti-CD49d treated group recovered significantly earlier and more pronounced 3 and 7 days after MCAO than those in the control group (Fig. 1C). We additionally validated this therapy in a model of reversible filament-induced MCAO with 30 and 60 min occlusion time, respectively. Occlusion for 30 min resulted in moderate striatal lesions that were significantly reduced by CD49d-inhibition (Fig. 1D). In contrast, transient MCAO for 60 min induced extensive lesions, encompassing ~50% of one hemisphere (Fig. 1E). We did not detect a significant difference in infarct volume between treatment groups in mice undergoing these extensive lesions, however, mortality at 7 days in this model exceeded 40%, precluding conclusive comparison of infarct size (Dirmagl, 2006) (Fig. 1F). Survival did not significantly differ between groups [P = 0.063, n = 23 (IgG2), n = 20 (anti-CD49d), four individual experiments].

The lesion volumes 7 days after MCAO in the permanent coagulation and 30 min occlusion model were reduced as a result of a consistent decrease in infarct expansion throughout the complete infarct range rather than decrease in a specific anatomical area (Fig. 1G).

Anti-CD49d treatment inhibits cerebral leukocyte invasion

The therapeutic effect of anti-CD49d therapy in primary inflammatory diseases is believed to be mainly mediated by inhibiting lymphocyte migration (Engelhardt and Kappos, 2008).
Immunohistochemical study of brain sections for T cells, B cells and granulocytes (Fig. 2A) revealed that invading leukocytes were predominantly located in the peri-infarct zone 5 days after permanent MCAO, as previously reported (Liesz et al., 2009b). One total section per brain was analysed for the presence of positively stained leukocytes. Brain invasion of T and B cells was negligible at 24 h but became apparent at 5 days. Anti-CD49 treatment significantly reduced lymphocyte infiltration at Day 5 (Fig. 2B). Myeloperoxidase positive granulocytes invaded the brain already 24 h after MCAO and further increased 5-fold until Day 5. Granulocyte invasion was significantly attenuated by the therapy at both time points (Fig. 2B).

Since our previous work suggested a strong post-ischaemic interaction between invading leukocytes and microglial cells, we measured the number of activated (IBA1+ ) microglia/monocytes (Fig. 2C). Twenty-four hours after permanent MCAO, no difference could be observed for IBA1+ cell counts between treatment groups in both hemispheres. In contrast, 5 days after ischaemia, anti-CD49d significantly reduced the number of IBA1+ cells in the ischaemic hemisphere, suggesting that reducing leukocyte invasion subsequently attenuates the activation of resident (microglia) or invading (monocytes) innate immune cells, respectively. We further differentiated the effect of anti-CD49d treatment on subpopulations of brain invading leukocytes by flow cytometry. Cells of
Homogenized ischaemic hemispheres were gated for CD45+ leukocytes and quantitative cell numbers of subpopulations were determined (Liesz et al., 2009b) (Fig. 3A). At 5 days after MCAO, cell counts of all populations were reduced by anti-CD49d treatment. Interestingly, invasion of all T cell subsets including cerebroprotective regulatory T cells were similarly affected by anti-CD49d treatment (Fig. 3B). These results are consistent with a broad effect of α4-integrin inhibition on the migratory capability of all analysed...
Figure 3  Anti-CD49d treatment reduces leukocyte brain invasion. (A) Representative gating strategy for absolute cell count analysis of leukocyte subpopulations in ischaemic hemisphere homogenates. CD45⁺ leukocytes were gated for subpopulation analysis after using a scattergram gate for viable leukocytes and quantification beads. (B) Flow cytometric analysis of absolute cell counts for granulocytes (Gr-1⁺), B⁺ (B220⁺), NK⁺ (NK1.1⁺), total T cells (CD3⁺) and T helper⁺ (CD3⁺ CD4⁺), T cytotoxic⁺ (CD3⁺ CD8⁺), regulatory T cells (CD4⁺ Foxp3⁺), and the ratio of regulatory T cells within the T helper population 5 days after MCAO in IgG2 control treated and anti-CD49d treated animals (five individual experiments per group, consisting of 2–3 pooled animals each) SSC = side scatter.
leukocyte populations rather than on specific pro-inflammatory cells only.

**Anti-CD49d treatment attenuates expression of specific cerebral cytokines**

We investigated the impact of anti-CD49d treatment on cerebral cytokine expression after permanent MCAO (Fig. 4). The messenger RNA levels of the proinflammatory cytokines IFN-γ and IL-6 were significantly reduced in anti-CD49d treatment groups at their expression maximum 3 days after MCAO, compatible with the time course of lymphocyte invasion (Gelderblom et al., 2009). We did not detect a difference in the expression of IFN-γ at 24 h, however, the cellular source for its increase in the ischaemic hemispheres of both treatment groups at this early time point before lymphocyte invasion remains unclear. In contrast, IL-1 and TNF-α, which we and others have previously shown to

![Graph](image-url)

**Figure 4** Anti-CD49d treatment reduces expression of key proinflammatory cytokines after MCAO. Cytokine messenger RNA relative expression (RE, normalized to peptidylprolyl isomerase A housekeeping gene) for pro-inflammatory (INF-γ, IL-6, TNF-α and IL-1) and anti-inflammatory (IL-10 and TGF-β) cytokines was measured by real-time polymerase chain reaction in the ischaemic and non-ischaemic hemispheres of control (IgG2) and anti-CD49d treated mice (n = 5 per group, 2-3 individual experiments per group). *P < 0.05 between ischaemic hemispheres of treatment and control groups at one respective time point.
be mainly produced by microglia after ischaemia (Davies et al., 1999; Lambertsen et al., 2005; Liesz et al., 2009b), was not influenced by the therapy. Analysis of the anti-inflammatory cytokines IL-10 and TGF-β yielded a transient increase of IL-10 in anti-CD49d treated animals compared with controls at 24 h but no difference at 3 days (Fig. 4).

Leukocytes ubiquitously express α4-integrin and remain functionally unaffected by anti-CD49d treatment

As most previous reports targeting α4-integrins in neuroinflammation were focused on lymphocyte-mediated effects (Yednock et al., 1992; Engelhardt and Kappos, 2008), we performed a comprehensive analysis of α4-integrin expression on leukocyte subsets. Cellular binding of the same CD49d-specific antibody clone used for in vivo blocking was measured on leukocyte subpopulations in naïve mice and after permanent MCAO. Conventional flow cytometric analysis revealed that a high percentage (68–99%) of all leukocyte subpopulations expressed α4-integrins constitutively (Fig. 5A). Interestingly, the absolute number of CD49d molecules detected per cell was comparatively low in naïve and post-ischaemic T cells while CD49d-specific antibody binding was substantially increased on granulocytes 24 h after MCAO compared with naïve granulocytes (Fig. 5B), in accordance with previous data for α4-integrin upregulation under conditions of strong immunological stimuli (Ibbotson et al., 2001). Furthermore, CD49d transcription of total peripheral blood mononuclear cells did not differ between control treatment and anti-CD49d treated animals and was similar at the analysed time points before and after MCAO (Fig. 5C), consistent with the concept of conformational regulation of α4-integrin rather than transcriptional regulation upon activation (Takagi and Springer, 2002).

We also performed differential blood cell counting to examine the effect of antibody-treatment on peripheral cell counts. Numbers of granulocytes, lymphocytes, monocytes and thrombocytes did not differ between anti-CD49d and control groups (Supplementary Fig. 1), nor did flow cytometric analysis reveal any differences for Thelper, T cytotoxic, B cell and NK in blood from treatment and control groups (Supplementary Fig. 1). Since CD49d-specific antibodies have been reported to be potentially co-stimulatory (van Seventer et al., 1991; Damle et al., 1992), we investigated the activation of T cells as IFN-γ-expressing cells after in vitro stimulation with increasing antibody concentrations. Anti-CD49d did not alter the number of CD3+IFN-γ+ cells compared with controls at any concentration of CD49d-specific antibodies (Fig. 5D). Moreover, no significant alterations in IFN-γ, TNF-α, IL-6, IL-10 and TGF-β serum levels were detected between groups either in sham operated animals or 24 h and 5 days after MCAO (Supplementary Fig. 2).

Additionally, we tested whether anti-CD49d treatment compromises the antimicrobial host defence because bacterial infections are a common complication of stroke (Emley and Hopkins, 2008) and CD49d blocking might be a potent immunosuppres- sant. Because the ischaemia model used in this study only rarely induces infections (Liesz et al., 2009a), we challenged mice with the nasal administration of Streptococcus pneumoniae 3 days after MCAO, which has been shown to induce experimental pneumonia (Stegemann et al., 2009). Twenty-four hours later, bacterial counts in nasopharyngeal lavage and lung homogenates did not differ significantly between treatment groups (Fig. 5E and F). Furthermore, we did not detect any differences in bacterial 16s RNA loads in lung homogenates between groups (Fig. 5G). None of the animals had bacteria in blood samples. Thus, anti-CD49d treatment did not exacerbate post-ischaemic bacterial infections.

T cells and their molecular mediators are the main effectors of post-ischaemic neuroinflammation

To verify the concept that the effect of anti-CD49d therapy is lymphocyte dependent (Yednock et al., 1992; Rose et al., 2002), we analysed the impact of α4-integrin blockade on infarct volumes in lymphocyte-deficient RAG2−/− mice (Fig. 6A). As previously reported (Yilmaz et al., 2006; Liesz et al., 2009b), infarct volumes in RAG2−/− were significantly smaller than in wild-type control animals on the same genetic background (C57BL/6). Importantly, CD49d-blocking failed to reduce infarct volumes in RAG2−/− mice. Adoptive transfer of 1 × 10^8 total splenocytes 7 days before MCAO in lymphocyte-deficient RAG2−/− reconstituted the peripheral lymphocyte compartment (Supplementary Fig. 3) and significantly increased infarct volume at 7 days after MCAO (Fig. 6B). This increase in infarct size was again reduced by anti-CD49d administration at 24 h prior to MCAO (Fig. 6B), indicating lymphocytes as the main functional target of anti-CD49d treatment. To identify which T lymphocyte subpopulation contributed to ischaemical damage, T helper or T cytotoxic cells were depleted 24 h before ischaemia by specific antibodies (Fig. 6C). Depletion of T helper or T cytotoxic cells significantly reduced infarct volume. In both T helper and T cytotoxic depleted animals (Fig. 6E), anti-CD49d treatment failed to further decrease ischaemia size (Fig. 6E). In contrast, granulocyte depletion (Fig. 6D) 24 h prior to MCAO did not alter infarct volumes 7 days after ischaemia, compared with control animals (Fig. 6E). In these mice, the additional administration of anti-CD49d significantly reduced infarct sizes by a similar extent as in naïve mice.

Because the role of T-cell mediated tissue damage in cerebral ischaemia has been only partially characterized, we further examined two key effector mechanisms, IFN-γ secretion (Schroder et al., 2004; Yilmaz et al., 2006) and direct cell cytotoxicity via perforin release (Russell and Ley, 2002; Trapani and Smyth, 2002), respectively. Intracerebroventricular administration of neutralizing IFN-γ-specific antibodies was performed 3 days after MCAO (Liesz et al., 2009b), which significantly reduced infarct volumes at 7 days compared with control treatment (Fig. 6F). Adding anti-CD49d treatment to IFN-γ neutralization significantly improved infarct volumes compared with IFN-γ antagonization alone. We investigated the relevance of perforin-mediated pathways using perforin-deficient Prf−/− mice (Fig. 6G). Infarct volumes in Prf−/− mice were significantly smaller 7 days after MCAO than in wild-type control animals. Interestingly, anti-CD49d failed...
Figure 5 CD49d-specific antibodies bind to all leukocyte subpopulations but do not alter peripheral immune function. (A) Relative expression of CD49d and (B) the quantity of CD49d-specific antibodies bound per cell in the indicated cell populations, as measured by flow cytometry in naïve control animals and 24 h and 5 days after permanent MCAO (n = 5 from five individual experiments per group). (C) real-time polymerase chain reaction of CD49d messenger RNA expression in blood leukocytes after anti-CD49d or control treatment in Sham operated mice or 24 h and 5 days after MCAO (n = 5 per group, two individual experiments). (D) Lymphocyte stimulation by CD49d-specific antibodies compared with IgG2 treatment was determined by intracellular flowcytometric analysis of IFN-γ in CD3+ cells after culture conditions with increasing amounts of antibodies per culture well (three individual experiments). Experimental pneumonia was induced in anti-CD49d or control treated animals 3 days after MCAO and 24 h later the number of colony forming units per ml of (E) nasopharyngeal lavage and (F) lung homogenates was measured. (G) Bacterial load was verified by polymerase chain reaction for bacterial 16s RNA expression per ml of lung homogenate. Dots represent individual mice and bars indicate mean values.
Figure 6  The neuroprotective effect of anti-CD49d is mediated via inhibition of T cell migration and abrogation of their key effector mechanisms. (B) Infarct volumes 7 days after MCAO in wild-type (WT) control mice and in lymphocyte-deficient Rag2−−/− mice with IgG2 control or anti-CD49d treatment (n = 10 per group, two individual experiments). (B) Rag2−−/− mice received adoptive transfer of 1 × 10⁸ splenocytes 7 days prior to MCAO and infarct volumes were determined at 7 days after MCAO in IgG- or anti-CD49d-treated animals (n = 15 per group, three individual experiments). (C) T_helper⁺ (anti-CD4) or T_cytotoxic⁺ (anti-CD8) cells were depleted 48 h and mice treated 24 h before MCAO with IgG2 or CD49d-specific antibodies, infarct volumes were determined 7 days after infarct induction (n = 10 per group, two individual experiments). Representative dot plots are shown for depletion of the respective population and control treatment. (D) Neutrophil cell counts were determined by complete cell count analysis in control animals (WT) and 24 h, 3 days and 7 days after

(continued)
to reduce infarct volumes in these animals. In summary, these data underline the key pathophysiological role of invading T cells in post-ischaemic neuroinflammation.

**Post-ischaemic VCAM-1 upregulation is inhibited by anti-CD49d treatment**

We investigated the pathophysiological role of the main endothelial VLA-4 ligand VCAM-1 to further characterize integrin-dependent migration after ischaemia. In accordance with previous studies (Jander et al., 1996; Justicia et al., 2006), VCAM-1 was substantially upregulated with delay after MCAO (Fig. 7A). Intensively stained VCAM-1+ vessels were primarily found in the peri-infarct vasculature. The invading CD3+ T cells surrounded these VCAM-1+ vessels, indicating a close association between lymphocyte invasion (compare Fig. 2A) and endothelial VCAM-1-induction in the peri-infarct area (Fig. 7B). Intriguingly, anti-CD49d treatment significantly inhibited the upregulation of VCAM-1 messenger RNA (Fig. 7C) and protein (Fig. 7D) in the ischaemic hemisphere. Thus, a dynamic crosstalk between leukocytes carrying activated α4-integrins and VCAM-1 expressing endothelial cells takes place after cerebral ischaemia (Cook-Mills and Deem, 2005; Hordijk, 2006).

**Antibody-mediated blocking of VCAM-1 fails to improve ischaemia outcome**

We attempted to block VCAM-1 by using monoclonal antibodies as an alternative target for inhibiting cerebral leukocyte invasion. Intraperitoneal injection of VCAM-1-specific rat-anti-mouse antibodies both 24 h before and 3 days after MCAO and staining for rat antibodies 6 days after MCAO revealed substantial binding of these antibodies to cerebral endothelial VCAM-1 (Fig. 7E). We further analysed the dose-dependent concentration of detectable VCAM-1-specific antibodies in brains 24 h after intraperitoneal injection. Administration of 300 μg FITC-labelled anti-VCAM-1 distinctly increased cerebral fluorescence as measured by spectrofluorometry and histology (Supplementary Fig. 4A and B), indicating effective binding at cerebral endothelium at the dosage (~15 mg/kg) used in the blocking experiments. Endothelial antibody binding was additionally shown by inhibiting secondary antibody adhesion to capillaries of treated animals (Supplementary Fig. 4C). However, despite using a substantially higher dose than previous investigators (Justicia et al., 2006) and repetitive administration 24 h before and 3 days after MCAO of monoclonal antibodies, anti-VCAM failed to reduce infarct volume 7 days after MCAO (Fig. 7F). Intriguingly, anti-VCAM antibodies did not significantly reduce cerebral invasion of neutrophils and T cells and did not affect cerebral IFN-γ expression either (Supplementary Fig. 5).

**VCAM-1 gene silencing prevents cerebral leukocyte migration and reduces ischaemia size**

Because the anti-VCAM antibody used in our experiments failed to inhibit leukocyte migration sufficiently, we studied another therapeutic paradigm for inhibiting VCAM-1-mediated leukocyte immigration. Here, hydrodynamic in vivo administration (Hino et al., 2006; Herweijer and Wolff, 2007) of VCAM-1 small interfering RNA significantly reduced VCAM-1 protein expression 5 days after MCAO by >65% compared with mice receiving a control small interfering RNA (Fig. 7G). We controlled the specificity of VCAM-1 silencing by real-time polymerase chain reaction analysis of VCAM-1 messenger RNA and the housekeeping genes in both hemispheres (Supplementary Fig. 6A–C). VCAM-1 silencing significantly reduced infarct volumes 6 days after MCAO compared with control small interfering RNA treatment (Fig. 7H). Further anti-CD49d treatment had no additional effect on infarct size, suggesting that VCAM-1 is the main complementary endothelial ligand for post-ischaemic leukocyte migration via VLA-4. VCAM-1 silencing significantly reduced cerebral granulocyte and T cell numbers 6 days after MCAO (Fig. 7I) and reduced IFN-γ expression to barely detectable levels (Supplementary Fig. 6D).

**Discussion**

Primary neuroinflammatory diseases share many inflammatory pathways with neurodegenerative, traumatic and vascular brain disorders that open new opportunities for translation of therapeutic concepts between aetiologically diverse disorders such as multiple sclerosis and stroke (Zipp and Aktas, 2006). Recent studies have shown that different T cell subpopulations have the capability to either contain or moderately exacerbate ischaemic brain damage suggesting that T cell-targeted therapy may be a promising new protective approach for stroke (Liesz et al., 2009b; Shichita et al., 2009). T cell trafficking into the brain parenchyma represents a key step of neuroinflammation in experimental allergic encephalomyelitis, the preclinical model of multiple sclerosis. Intriguingly, recent insights into the brain invasion of encephalitogenic T cells have confirmed the key role of the VLA-4–VCAM-1 axis in this process, which is consistent with the outstanding

---

**Figure 6 Continued**

Granulocyte depletion with Gr-1-specific monoclonal antibodies. Dots represent individual mice and bars indicate mean values. (E) Infarct volumes 7 days after depletion of neutrophils or control treatment and in mice receiving depleting antibodies and additional blocking of CD49d (n = 10 per group, two individual experiments). (F) Infarct volumes 7 days after MCAO (n = 20 per group, four individual experiments) in mice receiving intracerebroventricular injection of control IgG antibody or neutralizing IFN-γ specific antibodies in artificial cerebrospinal fluid and additional administration of control IgG or CD49d-specific antibodies intraperitoneally (i.p.). (G) Infarct volume 7 days after MCAO in wild-type mice and in perforin-deficient Prf−/− mice with intraperitoneal injection of either IgG control or CD49d-specific antibodies 24 h before ischaemia induction (n = 10 per group, two individual experiments).
Figure 7 VCAM-1 is the principal endothelial CD49d-ligand for post-ischaemic leukocyte migration. (A) Representative micro western blot for VCAM-1 expression of ischaemic (I) and non-ischaemic (NI) hemispheres, in normal wild-type mice (N) and at 24 h and 5 days after MCAO. Actin was used as loading control. Representative image of five individual experiments. (B) Representative immunofluorescence staining for VCAM-1, von Willebrand Factor (vWF), T cells (CD3) and nuclei (DAPI) at 5 days after permanent MCAO. (C) Relative expression (RE) of VCAM-1 messenger RNA levels of control animals and anti-CD49d treated animals (24 h prior to ischaemia) after Sham operation and at 24 h and 3 days after MCAO (three individual experiments of 4–5 mice per group). (D) Densitometric analysis
efficacy of its therapeutic blockade in multiple sclerosis (Yednock et al., 1992; Laschinger and Engelhardt, 2000; Steinman, 2005; Bartholomaus et al., 2009).

Using different models of permanent and transient cerebral ischaemia, we found that antibody-mediated blockade of α4-integrin reduces infarct size and improves behavioural outcome 7 days after moderate ischaemia. Although at first glance these results appear to be consistent with the beneficial effect reported in two small previous studies (Becker et al., 2001; Retlon et al., 2001), outcome in these studies was measured at 24 and 48 h after MCAO, respectively, when no protection was observed in our experiments. Remarkably, at this early time point, lymphocytes—the key effector cells under these circumstances (see below)—have not yet started to invade the ischaemic brain (Jander et al., 1995; Gelderblom et al., 2009; Liesz et al., 2009).

Because the mechanisms behind the protective effect of CD49d blockade in cerebral ischaemia were so far unknown, we performed a comprehensive analysis of the complex interactions in transendothelial leukocyte migration and the pathogenic role of lymphocyte subpopulations. Although VLA-4 blocking by specific antibodies in multiple sclerosis is primarily perceived as a lymphocyte adhesion blocker (Rice et al., 2005), the profound reduction of post-ischaemic brain infiltration by α4-integrin or VCAM-1 blockade in our experiments was not restricted to a particular inflammatory cell type. Correspondingly, constitutive α4-integrin expression was present on the surface of most leukocytes. However, despite the non-specific reduction of inflammatory cell infiltration, the cerebroprotective effect was clearly mediated via inhibition of T cells. First of all, protection by CD49d-specific antibodies persisted in mice depleted of neutrophils, indicating a minor role of granulocytes in the immunopathology after stroke, although their role in post-ischaemic inflammation is still controversial (Schurer et al., 1991; Matsuo et al., 1994; Harris et al., 2005). In contrast, anti-CD49d treatment did not reduce infarct size in lymphocyte-deficient Rag2−/− mice and was ineffective after selective antibody-mediated depletion of either T helper or T cytotoxic cells. Therefore, CD49d-targeted blockade can be regarded as a mainly T cell-targeted therapy although an indirect effect on the activation or recruitment of other cell populations (e.g. microglia) by the absence of cerebral lymphocytes cannot be excluded.

Beyond demonstrating the effectiveness of blocking the VLA-4–VCAM-1 axis, our study provides important insights into the functional role of T cells in cerebral ischaemia. In the absence of T lymphocytes or their subsets, infarct volumes have been smaller than in respective controls in several studies, including the present (Yilmaz et al., 2006; Hurn et al., 2007; Shichita et al., 2009; Kleinschnitz et al., 2010). Furthermore, antagonizing IFN-γ, a key cytokine of T cells, protected the ischaemic brain, but this mechanism only partially explained the protective effect of anti-CD49d. In a previous study (Liesz et al., 2009), we have shown that T cells are the major source of IFN-γ in the ischaemic brain. Remarkably, depletion of CD8+ cells was as effective as of CD4+ cells in reducing infarct size, suggesting an additional cytotoxic pathway. Indeed, absence of perforin, the key cytotoxic mechanism of CD8+ cells, substantially improved outcome. This is, to our knowledge, the first report regarding perforin-mediated cytotoxicity after brain ischaemia and is consistent with a previous study on the role of granzyme B—a mediator downstream of perforin—in experimental ischaemia (Chaitanya et al., 2010). Besides CD8+ T cells, NK cells are also a possible source of perforin (Moretta et al., 2008) that invade the ischaemic brain (Gelderblom et al., 2009). However, we have analysed the contribution of NK cells in post-ischaemic neuroinflammation by depleting this cell-population with NK1.1-specific antibodies and did not detect a significant influence on the resulting infarct volume in models of permanent or reversible MCAO (data not shown).

Regulatory T cells are an immunosuppressive subpopulation of T cells that are neuroprotective after experimental ischaemia stroke (Liesz et al., 2009). The main function of regulatory T cells is to prevent overactivation of the immune system (Maloy and Powrie, 2001; O’Garra and Vieira, 2004; Sakaguchi et al., 2007). However, anti-CD49d treatment did not appear to result from enhancement of regulatory T cells in the present study because the number of regulatory T cells and other lymphocyte subpopulations in the brain was diminished equally by the antibody. Instead, endogenous regulatory T cells and anti-CD49d treatment may affect similar deleterious lymphocyte pathways by different means. While regulatory T cells reduce the activation of invading lymphocytes as measured by cellular IFN-γ production (Liesz et al., 2009b), anti-CD49d treatment prevents the invasion of lymphocytes.

VLA-4 mediated endothelial leukocyte adhesion can take place via different vascular ligands including VCAM-1 and fibronectin.

**Figure 7** Continued. Of micro western blot assays for VCAM-1 expression, normalized for actin loading control, in control animals and anti-CD49d treated animals 6 days after MCAO. Dots represent individual mice and bars indicate mean values. (E) Image of a representative brain section stained for rat IgG antibodies 6 days after MCAO of mice treated intraperitoneally with rat anti-mouse VCAM-specific antibodies 24 h prior and 3 days after MCAO induction. (F) Infarct volumes 6 days after MCAO in mice receiving intraperitoneal administrations of IgG control antibodies or VCAM-specific antibodies at 24 h before and 3 days after ischaemia induction (n = 10 per group, two individual experiments). (G) Representative micro western blot and dot plot analysis for VCAM-1 protein expression at 5 days after MCAO in mice treated with negative control small interfering RNA (siRNA) or VCAM-specific small interfering RNA. (H) Infarct volumes at 6 days after MCAO in mice receiving control small interfering RNA intravenously or VCAM-1 specific small interfering RNA or additionally CD49d-specific antibodies intraperitoneally (i.p.). Control groups received IgG isotype control antibody (n = 11 per group, three individual experiments). (I) Analysis of granulocyte (left) and T cell (right) cell counts per one complete section of the ischaemic hemisphere at 6 days after MCAO (n = 5 per group). Filled bars = control small interfering RNA; open bars = VCAM-1 small interfering RNA treatment; Pos = positive control.
(Elices et al., 1990; Vajkoczy et al., 2001; Engelhardt, 2008). After in vivo silencing of VCAM-1, α4-integrin blockade did not achieve any additional cerebroprotection, indicating that VCAM-1 is the main endothelial counterpart of VLA-4 after brain ischaemia. In contrast to the lack of cerebroprotection by VCAM-1-specific antibodies in a previous (Justicia et al., 2006) and the present study, in vivo hydrodynamic VCAM-1 silencing using small interfering RNA—a technique that to our knowledge has not been applied in cerebral ischaemia previously—effectively reduced the infarct size and attenuated leukocyte infiltration and cerebral IFN-γ levels. RNA interference by hydrodynamic small interfering RNA delivery is a highly efficient method for targeting endothelial protein expression (Hino et al., 2006; Lewis and Wolff, 2007) but non-specific immunostimulatory effects by small interfering RNA need to be considered (Robbins et al., 2009). Although physical impact on the endothelium has been reported as the basis for this potent RNA delivery strategy, previous studies observed only transient and reversible endothelial changes and normal morphology 30 min after injection (Suda et al., 2007). In our study, VCAM-1-targeting proved to be specific, and control animals receiving control small interfering RNAs had no detectable effects compared with naïve animals. Importantly, our experiments reveal cross-signalling between activated leukocytes and their endothelial counterpart VCAM-1 as anti-CD49d treatment attenuated post-ischaemic VCAM-1 induction. This phenomenon might be attributed to the inhibition of leukocyte-endothelial interaction, since VLA-4–VCAM-1 binding is known to induce endothelial activation with consecutive structural changes (Mueller, 2009), production of reactive oxygen species, and secretion of cytokines and matrix metalloproteinases (Hordijk, 2006).

Following cerebral ischaemia and other severe brain injury, the systemic immune system undergoes substantial changes that frequently render stroke patients susceptible to infectious complications (Meisel et al., 2005; Chamorro et al., 2007). To model this setting, ischaemic mice were challenged with Streptococcus pneumoniae (Stegemann et al., 2009). Importantly, treatment with CD49d-specific antibodies did not exacerbate bacterial infection, suggesting that this immunomodulatory approach is safe from a microbiological perspective.

In conclusion, despite profound differences in the primary pathogenesis of multiple sclerosis and ischaemic stroke, both disorders share key pathways of adaptive immunity that can be successfully targeted by immunotherapy. Although many aspects of the immune pathogenesis after brain ischaemia remain to be elucidated and additional investigations are necessary before translating the findings into the clinical setting, inhibition of cerebral lymphocyte trafficking appears to be a promising new approach for ischaemic stroke.

**Acknowledgements**

The authors would like to thank T. Giese for critical methodical input and D. Stefan for excellent technical assistance in flow cytometry, M. Zorn for performing differential blood cell countings at the Core Laboratory Facilities of the University of Heidelberg, Claudia Veltkamp for critical revision of the article, A. Tillmanns-Liesz for assistance in behavioural testing and A. Stojanovic (German Cancer Research Center, Heidelberg, Germany) for providing the Rag2−/− mice. A.L. designed and performed experiments, analysed data, and wrote the article; W.Z., E.M., H.B., C.S., S.St. and S.Sch. performed experiments and analysed data; S.K. and L.S. performed experiments and analysed data; R.V. initiated and directed the entire study, designed experiments, analysed data and wrote the article.

**Funding**

Else-Kröner Fresenius Stiftung; German federal and state governments’ Excellence initiative (to R.V.); Else-Kröner-Memorial Scholarship (to R.V.).

**Supplementary material**

Supplementary material is available at Brain online.

**References**


Inhibiting lymphocyte migration in stroke

Brain 2011; 134: 704–720


