In Vivo Imaging of the Inflammatory Receptor CD40 After Cerebral Ischemia Using a Fluorescent Antibody

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Background and Purpose—Brain inflammation is a hallmark of stroke, where it has been implicated in tissue damage as well as in repair. Imaging technologies that specifically visualize these processes are highly desirable. In this study, we explored whether the inflammatory receptor CD40 can be noninvasively and specifically visualized in mice after cerebral ischemia using a fluorescent monoclonal antibody, which we labeled with the near-infrared fluorescence dye Cy5.5 (Cy5.5-CD40MAb).

Methods—Wild-type and CD40-deficient mice were subjected to transient middle cerebral artery occlusion. Mice were either intravenously injected with Cy5.5-CD40MAb or control Cy5.5-IgGMAb. Noninvasive and ex vivo near-infrared fluorescence imaging was performed after injection of the compounds. Probe distribution and specificity was further assessed with single-plane illumination microscopy, immunohistochemistry, and confocal microscopy.

Results—Significantly higher fluorescence intensities over the stroke-affected hemisphere, compared to the contralateral side, were only detected noninvasively in wild-type mice that received Cy5.5-CD40MAb, but not in CD40-deficient mice injected with Cy5.5-CD40MAb or in wild-type mice that were injected with Cy5.5-IgGMAb. Ex vivo near-infrared fluorescence showed an intense fluorescence within the ischemic territory only in wild-type mice injected with Cy5.5-CD40MAb. In the brains of these mice, single-plane illumination microscopy demonstrated vascular and parenchymal distribution, and confocal microscopy revealed a partial colocalization of parenchymal fluorescence from the injected Cy5.5-CD40MAb with activated microglia and blood-derived cells in the ischemic region.

Conclusions—The study demonstrates that a CD40-targeted fluorescent antibody enables specific noninvasive detection of the inflammatory receptor CD40 after cerebral ischemia using optical techniques. (Stroke. 2008;39:2845-2852.)

Key Words: focal ischemia • inflammation • near-infrared fluorescence • optical imaging

Focal cerebral ischemia initiates complex, multifaceted, and dynamic inflammatory processes that occur hours to days after the onset of ischemia and have been implicated in secondary damage as well as repair and recovery. To date, it is still controversial whether the beneficial effects of inflammation outweigh the detrimental effects.1,2 Imaging technologies that provide noninvasive visualization of inflammatory processes after ischemia using target-specific probes would provide powerful tools to further evaluate the role of these processes in stroke. Moreover, these technologies might be valuable in detecting and assessing the disease progression, stratifying patients for therapy, and monitoring the response to therapeutic intervention.

CD40 and its ligand CD 154 (CD40L) have been implicated in the regulation of inflammation and immune responses. The CD40 receptor is expressed on the surface of immune cells, including B cells, monocytes, and dendritic cells, as well as nonimmune cells such as endothelial cells, epithelial cells, diverse mesenchymal cells, platelets, and malignant tumor cells.3 In the central nervous system, CD40 is markedly upregulated after inflammatory stimulation on a variety of cells, including microglia, astrocytes, smooth muscle cells, and vascular endothelial cells.3,4 The interaction between CD40 and its ligand triggers a series of inflammatory cytokines and chemokines, and induces the expression of vascular adhesion molecules.5 In experimental studies, targeted deletion of the CD40 receptor or CD154 have led to a reduction in infarct size.6 In stroke patients, an increased expression of the CD40 receptor on monocytes as well as its ligand on platelets in the blood was observed.7 Because CD40 is considered proinflammatory, is thought to contribute to the expansion of the lesion, and is robustly upregulated on
various inflammatory cells in the brain, it might be a suitable molecular target for imaging.

In the recent years, an increasing number of target-specific probes have been developed for noninvasive visualization of physiological and pathophysiological processes using different imaging modalities, including near-infrared fluorescence (NIRF) imaging.8 NIRF imaging offers several advantages, including high sensitivity, the use of nonionizing radiation, as well as its demand for relatively simple and inexpensive instrumentation. We recently showed that NIRF dyes can be noninvasively detected with high sensitivity in the brain of living pigmented mice using a planar NIRF imaging system.9 Furthermore, microscopic techniques such as confocal microscopy or single-plane illumination microscopy (SPIM) offer the possibility to evaluate the distribution of injected probes in biological specimens on the cellular level with high spatial resolution. Together with staining methods, including immunohistochemistry, fluorescence imaging techniques allow the identification of binding sites and cell types.

Here, we tested the hypothesis that a NIRF-labeled monoclonal antibody can be used for the specific imaging of CD40, a receptor involved in brain inflammation after cerebral ischemia. To test the specificity of the imaging approach in vivo, we used a NIRF-labeled IgG antibody without binding affinity to CD40 and CD40-deficient mice as controls. In addition, we evaluated the distribution of the antibodies at the cellular level using microscopic techniques and immunohistochemistry.

**Materials and Methods**

**Animals**

All experimental procedures conformed to institutional guidelines and were approved by an official committee (G0229/05; Lagoso). Thirty-four male C57Bl6/N mice (wild-type; Bundesinstitut fuer Risikoforschung, Berlin, Germany) and 4 male B6.129P2-C57L/J mice (CD40-deficient, CD40−/−; Institute of Microbiology, ETH Zurich, Switzerland) weighing 18 to 26 grams were housed under standard conditions.

**Focal Cerebral Ischemia**

Middle cerebral artery occlusion (MCAO) was performed as described.10 Briefly, a monofilament was introduced into the common carotid artery under isoflurane anesthesia, advanced to the origin of the middle cerebral artery, and left there for 60 minutes, until reperfusion. Sham-operation involved surgical procedures, without occlusion of the middle cerebral artery.

**Lesion Determination**

Brain slices were incubated in a 2% triphenyltetrazolium chloride (TTC) solution (Sigma Aldrich) at 37°C for 30 minutes. An image of each slice was analyzed with NIH Image software. Lesion volumes were calculated as described.10

**Preparation of NIRF-Labeled Antibodies**

Rat anti-CD40 monoclonal antibodies (clone FGK45) were provided by A. Rolink (University of Basel, Switzerland). Rat IgG2A monoclonal antibodies (clone 1d10) were provided by R. Kroczek (Robert Koch Institute, Berlin, Germany). For Cy5.5 labeling, 1500 μL (10 mg/mL) of the antibodies in 0.1 mol/L borate buffer (pH 8.7) and 112 μL Cy5.5-N-hydroxysuccinimide ester (Cy5.5-NHS, PA 15601; Amersham Biosciences) were mixed in dimethylsulfoxide and allowed to react overnight at room temperature (calculated molar ratio, Cy5.5:antibody = 10:1). The Cy5.5 antibody conjugates were purified and transferred to 0.1 mol/L phosphate-buffered saline (PBS) by ultrafiltration (Vivaspin units, 10-kDa cut-off; Vivascience AG). The number of Cy5.5 molecules covalently bound to each antibody was calculated from protein measurements with bicinchoninic acid (BCA) Protein Assay (Pierce Biotechnology) and Cy5.5 concentrations, photometrically determined at 675 nm. The average Cy5.5-to-antibody ratio was 3:1.

**NIRF Imaging Protocol**

Eighty hours after reperfusion, 22 wild-type mice either received Cy5.5-CD40MAb (MCAO, n = 11; sham, n = 5) or Cy5.5-IgGMAb (MCAO, n = 7) as a control for unspecific distribution. Two MCAO animals (1 CD40 and 1 IgG mouse) died during the course of the experiments and were excluded from further analysis. Four CD40-deficient MCAO mice were injected with Cy5.5-CD40MAb. The fluorescent compounds were diluted in saline (equivalent to 1 mg Cy5.5/kg body weight) and injected into the tail vein as a bolus of 200 μL. Before our study we determined that this dose yields adequate contrast for in vivo NIRF imaging at data acquisition times of 1 second. Noninvasive NIRF imaging was performed 16 hours after injection of the compounds. Mice were anesthetized by intraperitoneal injection of a 5% chloral hydrate solution (200 mg/kg; Merck), and the skin overlying the head was depilated. For ex vivo imaging, brains were removed under deep anesthesia without previous perfusion of the animal, and coronal brain slices of 1-mm thickness were cut in a brain matrix using a razor blade.

**NIRF Imaging System**

The NIRF imaging system was recently described in detail.9 For excitation of Cy5.5, light from a Thermo Electric Cooling (TEC)-cooled laser diode emitting at 682 nm (Roithner Lasertechnik) was kept constant at a power of 40 mW. The light was directed into a dark chamber by optical fiber bundles (diameter, 0.5 mm; Loptek) and coupled into 2 fibers. The head of the mice were illuminated from above by 2 overlapping light cones with a circle diameter of ≈4 cm at an object distance of ≈10 cm. The fluorescence emission was detected by using a back-illuminated nitrogen cooled charge-coupled device (CCD) camera (Vers Array 512, 512×512; Roper Scientific Inc) fitted above the head of the animal with an vertical interval of 15 cm. The CCD camera was equipped with a focusing lens system (Nikkor macro lens f = 50 mm, f/1.2; Nikon). Two 710-nm interference filters (FWHM 20 nm; Andover Corp) were used for the detection of the fluorescence and blocking the excitation light. To correct for illumination inhomogeneities and to provide an anatomic reference for the fluorescence images, images where acquired without the filters blocking the excitation light. To prevent saturation of the camera, an optical density filter (OD4; Melles Griot GmbH) was used. Data acquisitions times were 1 second.

**Data Processing and Analysis of the NIRF Images**

Data were processed and analyzed as described previously, including normalization and correction for illumination inhomogeneities. Region of interest (ROI) analysis was performed by a person blinded to the experimental groups. Rectangular ROIs were selected over the right and left hemisphere using normalized noninvasive NIRF images (Figure 1). The average fluorescence intensity of all pixels within the ROI was calculated. Target-to-background ratios (TBRs) were calculated by dividing ROI values from the left hemisphere by ROI values from the right hemisphere. Data are presented as means ± SD.

**Statistical Analysis**

Comparisons between TBR were made using a 1-way ANOVA test followed by Bonferroni posttest. TBRs were plotted against lesion volumes, followed by a linear regression analysis to calculate R² and to determine the regression equation.

**Cell Tracking With 6-Carboxyfluorescein Diacetate**

Intraperitoneal injection of the fluorescent cell tracker 6-carboxyfluorescein diacetate (CFDA) was performed as described.11 Under
Figure 1. Representative noninvasive NIRF images (A, upper row) and ex vivo NIRF images of brains removed from the skull (A, bottom row) 16 hours after intravenous injection of the fluorescent compounds. The images have the same scaling given by the color bar. The rectangles give an example of the ROIs selected over the right and left hemisphere. TBR calculated from ROI analyses (B).
isoflurane anesthesia, the left lateral abdomen was opened and 100 μL of a 2% CFDA solution (Invitrogen) in 0.1 mol/L PBS was slowly injected into the spleen of 2 wild-type MCAO and 2 wild-type sham-operated mice that were injected with Cy5.5-CD40MAb, 80 hours after reperfusion. As a control for unspecific extravasation, 2 wild-type MCAO mice were intravenously injected with CFDA. To obtain a control for changes in the autofluorescence of brain tissue after MCAO, 2 wild-type mice received an intrasplenic injection of PBS. Brains were taken 16 hours after injection of the CFDA.

**Immunohistochemistry**

Immunohistochemistry was performed 16 hours after injection of the labeled antibodies (ie, 96 hours after reperfusion). Coronal 20-μm sections were cut with a cryostat and fixed in 4% paraformaldehyde in 0.1 mol/L PBS for 30 minutes before staining with FITC-conjugated antirat antibodies (Dianova) at a concentration of 1:250 (rabbit; Wako Chemicals) at a concentration of 1:500. TRITC-conjugated secondary antibodies (antirabbit) were used at a concentration of 1:250 (Dianova).

**Confocal Microscopy**

Sections were placed under the 40× objective of a confocal microscope with spectroscopic detection (TCS SP2; Leica). For separate inspection of the 3 dyes, appropriate lasers (excitation at 488 nm, 543 nm, and 633 nm) and filter blocks (triple dichroic FITC-TRITC-Cy5) were used. Image acquisition times were 9 seconds. The brains of 6 wild-type MCAO mice injected with Cy5.5-CD40MAb or Cy5.5-IgGMAb (n=3 each) and all CFDA-injected mice were examined. From each brain, the cortex, stratum, and peri-ischemic territory of 3 coronal sections taken between 1.6 to −1.3 from bregma were inspected. To estimate the number of Iba1 and CFDA-positive cells that are positive for fluorescence from injected NIRF compound, 50 Iba1 and 50 CFDA-positive cells in each section in randomly selected high-power fields were examined respectively.

**SPIM**

SPIM was described previously. Brains of 6 MCAO mice (Cy5.5-CD40MAb and Cy5.5-IgGMAb, n=3 each) were removed 16 hours after injection of the labeled compounds (ie, 96 hours after reperfusion) and cut through the stroke area into 2 coronal pieces. The frontal parts of the brains were examined with SPIM immediately after brain removal without processing the tissue. The dorsal parts of the brains were first dehydrated in methanol (25%, 50%, 75%, 100% methanol, 2 hours each), and then left in a clearing medium (Murray clear, 1:1 mixture of benzyl benzoate and benzyl alcohol) for 3 hours. All specimens were inspected with a 2.5× objective (Fluar 2.5×/0.12; Zeiss). For the excitation of Cy5.5, a 633-nm laser was used. As a detector, a CCD camera preceded by a 633-nm-long pass interference filter (RazorEdge 633; Semrock) to block the excitation light was used. Image acquisition times were 8 minutes for a stack of ~1000 slices. Pixel spacing was 2.6 μm. Binning (2×) was used for data presentation.

**Results**

**Noninvasive NIRF Imaging and Ex Vivo NIRF Imaging of the Brains Removed From the Skull**

No differences between the hemispheres were seen in the 3 control groups, which were sham-operated wild-type mice that received Cy5.5-CD40MAb, wild-type MCAO animals that received Cy5.5-IgGMAb, and CD40−/− MCAO mice that received Cy5.5-CD40MAb (Figure 1A). Higher fluorescence intensities over the ischemic hemisphere compared with the contralateral side were detected noninvasively only in wild-type MCAO mice injected with Cy5.5-CD40MAb. The corresponding ex vivo NIRF images showed equally low fluorescence intensities over both hemispheres in brains of sham-operated animals that were injected with Cy5.5-CD40MAb. A low signal over the ischemic hemisphere was seen in wild-type MCAO animals injected with Cy5.5-IgGMAb and in CD40−/− mice that received Cy5.5-CD40MAb. Intense fluorescence was only seen over the ischemic hemisphere of wild-type MCAO mice that had received Cy5.5-CD40MAb. The supplementary material provides additional noninvasive and ex vivo NIRF images (supplemental Figure I, available online at http://stroke.ahajournals.org).

In Figure 1B, TBRs calculated from ROI analyses of noninvasive NIRF images are depicted. TBRs of all experimental groups passed the Kolmogorov-Smirnov goodness-of-test for normality (P>0.2). Sham-operated wild-type mice that received Cy5.5-CD40MAb, wild-type MCAO animals that received Cy5.5-IgGMAb, and CD40−/− MCAO mice that received Cy5.5-CD40MAb showed statistically significant lower TBRs compared to wild-type MCAO mice that received Cy5.5-CD40MAb (0.94±0.08, 1.0±0.14, and 0.95±0.08 vs 1.47±0.16, respectively; P<0.001).

In Figure 1C, the TBRs were plotted against the lesion volumes estimated from TTC-stained brain slices. Linear regression analysis demonstrated no linear correlation between the calculated TBRs and the lesion volume with r²=0.159.

**Ex Vivo NIRF Imaging of Brain Slices and TTC Staining**

A lesion was clearly delineated by TTC staining in all MCAO animals 96 hours after reperfusion (Figure 2). CD40−/− and wild-type MCAO mice showed equal lesion size.

In sham-operated animals receiving Cy5.5-CD40MAb, weak fluorescence was evenly distributed over the brain slice. Areas of low fluorescence intensities, corresponding to the pallor in TTC staining, were seen in wild-type MCAO animals injected with Cy5.5-IgGMAb and in the CD40−/− mice that received Cy5.5-CD40MAb. Areas with high fluorescence intensities were observed over the ischemic region in wild-type MCAO mice that had received Cy5.5-CD40MAb. The supplementary material provides additional NIRF images of serial brain slices (supplemental Figure II, available online at http://stroke.ahajournals.org).

**SPIM**

SPIM revealed the distribution of fluorescent antibody in the unprocessed frontal part of the brain of wild-type MCAO mice that received Cy5.5-CD40MAb (Figure 3A). In both hemispheres the signal from Cy5.5 was associated with vessels. In contrast to the nonischemic cortex and the ischemic cortex after injection of Cy5.5-IgGMAb (data not shown), additional signal was detected in the ischemic cortex in the form of dots of cellular size, which appeared to be located within or adjacent to the vessels and in the brain parenchyma. We did not detect fluorescence signals from structures deeper than 50 μm with SPIM in unprocessed brain tissue. After tissue processing, we were able to visualize the distribution of the compounds in the dorsal part of the brains. In Figure 3B, projections from a 3-dimensional reconstruction demonstrate the distribution of Cy5.5-CD40MAb and Cy5.5-CD40MAb in volumes of 2×2×2 mm. Mice injected with Cy5.5-CD40MAb showed significantly higher fluorescence.
cence intensities in the ischemic territory compared to mice injected with Cy5.5-IgGMAb. The signal in both animal groups was found to be associated with vessels. In mice that received Cy5.5-CD40MAb, additional signal in the form of dots of cellular size was detected, whereas no dots were visible in mice injected with Cy5.5-IgGMAb. In the parenchyma of those mice, a scarce and diffusely distributed fluorescence was observed.

Figure 2. Representative ex vivo NIRF images of coronal brain slices (A, upper row) having the same scaling given by the color bar and images of the same slices after TTC staining to delineate the lesion area (bottom row) 16 hours after intravenous injection of the fluorescent compounds. Plot of TBR against the lesion volumes estimated from TTC-stained brain slices (B).
Confocal Microscopy and Immunohistochemistry
In the ischemic cortex of mice that had received Cy5.5-CD40MAb, greater abundance of Cy5.5 fluorescence was detected compared to mice that were injected with Cy5.5-IgGMAb (Figure 4). In MCAO mice that either received Cy5.5-CD40MAb or Cy5.5-IgGMAb, the fluorescence from injected compounds and the antibody staining against the injected compounds revealed a similar fluorescence pattern. In the ischemic cortex of animals that had either received Cy5.5-CD40MAb or Cy5.5-IgGMAb, a large number of Iba1-positive cells were detected and had similar densities in the inspected areas in both groups of animals. Mice that received Cy5.5-CD40MAb showed a partial colocalization of fluorescence from the injected compound with the Iba1 staining. Confocal analysis revealed colocalization with CD40MAb in approximately half of the Iba1-positive cells. By contrast, fluorescence and immunoreactivity from control IgG were detected as a diffuse signal, without cellular localization.

Figure 3. SPIM 16 hours after intravenous injection of the fluorescent antibodies (ie, 96 hours after reperfusion). Z-projections of unprocessed tissue showing the ischemic (A, ipsilateral) and the nonischemic (A, contralateral) cortex surface of a MCAO mouse injected with Cy5.5-CD40MAb. Projections (0°, 45°, and 90°) from a 3-dimensional reconstruction of processed brain tissue showing ischemic cortex and striatum of a MCAO mice injected with Cy5.5-CD40MAb (B, upper row) and Cy5.5-IgGMAb as a control (B, bottom row).

Figure 4. Confocal images of brain sections showing the ipsilateral cortex of MCAO animals 16 hours after intravenous injection of Cy5.5-CD40MAb (upper row) and Cy5.5-IgGMAb (bottom row). The blue (NIRF channel) shows the distribution of the injected Cy5.5-labeled compounds, the green shows the staining with FITC-labeled antibodies against CD40MAb or IgGMAb, and the red (rhodamine) shows the staining against Iba1. The images on the last line show an overlay of the NIRF channel and the Iba1 stain.
parenchyma and associated with vessels. In contrast, the highly fluorescent dots of cellular size were detected in the tissue of wild-type mice that received the CD40 antibody, CD40MAb and control Cy5.5-IgGMAb. In the ischemic area, revealed marked differences in the distribution of Cy5.5-invasive NIRF. Inspection of tissue samples with SPIM beneath the detection limit and were not detected with noninvasive fluorescent compounds in the ischemic tissue were clearly higher compared to nonischemic tissue. The amounts of fluorescence intensities in the ischemic tissue were slightly lower compared to nonischemic tissue. The signal was rather diffuse and scarcely visible in mice that received fluorescent IgG. Taken together, this suggests that the signal after injection of fluorescent-labeled CD40 antibody is largely attributable to specific binding or uptake of the compound rather than being attributable to leakage.

The CD40 receptor is highly upregulated on activated immune cells residing within or infiltrating the central nervous system under inflammatory conditions and thereby provides numerous sites for binding a CD40-directed imaging probe.3,4 Activated monocytes and microglia are likely candidates for binding or uptake of the fluorescent CD40MAb in the ischemic area. In a first experiment, we stained tissue sections for the presence of Iba1 cells. Fluorescence from the injected CD40 antibody was found to be largely associated with Iba1 staining (≈50%). The fluorescence not associated with Iba1 staining might represent cross-sectioned capillaries filled with circulating compound, because mice were not perfused to prevent wash-out of the probe, or other cell types that express CD40, such as activated endothelial cells or B cells.

In a second experiment, we injected CD40MAb into the spleen to give an approximation on the overall contribution of blood-derived cells to the CD40–NIRF signal. CFDA-positive cells were found in the ischemic territory of MCAO mice and showed partial colocalization with the fluorescence from injected Cy5.5-CD40MAb. Cells infiltrating the ischemic brain tissue either could have carried the fluorescently labeled CD40 antibody inside the brain or could have bound or taken-up the label within the brain parenchyma.

In summary, the CD40-targeted antibody is mainly found attached to cell populations involved in stroke-induced brain inflammation, with a substantial contribution of blood-derived cells. We have chosen 96 hours after reperfusion as an experimental paradigm for the subacute stage of inflammation, which recently received increasing attention.1 Ishikawa et al6 demonstrated that targeted deletions of CD40 receptor or CD154 lead to a significant reduction of infarct sizes 24 hours after MCAO. In our study we did not find differences between lesion size of CD40–/– and wild-type mice. In addition, no correlation between lesion volume and TBR was observed in wild-type MCAO mice injected with Cy5.5-CD40MAb. We observed numerous cells that have bound or incorporated the injected antibody directed against CD40 in the ischemic region. This gives evidence that the receptor is involved in the inflammatory processes after cerebral ischemia at a later stage of the disease and that correlation with the detection of a marker of inflammation with lesion size is likely to be time-dependent.

Although several optical imaging studies in animal models of disease have been published,8 only few studies so far have reported noninvasive fluorescence imaging of targets within the brain.14–16 We have previously shown that NIRF fluorochromes can be visualized with high sensitivity using noninvasive planar NIRF imaging in the brains of a pigmented mouse strain widely used in stroke research.9 The technique has several limitations. Because of scattering of light in tissue and a fluorescent source deeply located in the brain beneath the skull, the spatial resolution of planar noninvasive NIRF images is only a few millimeters. Planar NIRF does not allow for absolute quantitation. However, TBRs can be used to make comparisons between mice. In this study, we demon-
strated using a CD40 receptor targeted fluorescent probe in a mouse model of cerebral ischemia that noninvasive NIFR imaging specifically detects an inflammatory molecule involved in the pathophysiology of the disease.

Several fluorescence-based imaging technologies are in clinical use or undergoing investigation for a clinical setting.\textsuperscript{17,18} We recently described that depth-resolved in vivo NIFR measurements after intravenous injection of indocyanine green can be performed in the brains of human adults.\textsuperscript{19} Moreover, we showed blood perfusion imaging with indocyanine green in the cortex of stroke patients.\textsuperscript{20} However, these approaches are limited by the ultimate penetration depth of the excitation and emission light, which was found to be \( \approx 1.5 \) mm into the head, and thus only a few millimeters into the brain.\textsuperscript{21}

In conclusion, NIFR imaging using a CD40-targeted antibody may be helpful as experimental tool to understand disease progression in preclinical research and drug development. The specificity of approaches using targeted probes for image stroke pathophysiology can be tested using optical techniques. However, for full-head clinical imaging, labeling of the targeted antibody for MRI or nuclear imaging should be considered.

**Acknowledgments**

The authors gratefully acknowledge Jana Goldmann (Center for Anatomy Charité, Berlin) and Ingo Bechmann (Institute for Clinical Neuroanatomy, Goethe-University, Frankfurt) for help with CFDA. The authors also thank Annette Oxenius (Institute of Microbiology, ETH, Zürich) for providing the CD40-deficient mice, and Thomas Christian Weber (RCHCI, ETH, Zürich) for organizing the transport of the mice.

**Sources of Funding**

This study was supported by grants from the Europäischer Fond für regionale Entwicklung (J.K.), the Bundesministerium für Bildung und Forschung (Center for Stroke Research Berlin and Berlin NeuroImaging Centre), the VolkswagenStiftung (M.E.), the Deutsche Forschungsgemeinschaft (M.E., U.D.), the Herman and Lilly Schilling Stiftung (U.D.), the European Union (M.E., U.D.).

**Disclosures**

None.

**References**


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Stroke. 2008;39:2845-2852; originally published online July 17, 2008; doi: 10.1161/STROKEAHA.107.509844

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