Carcinoembryonic Antigen–Related Cell Adhesion Molecule 1 Inhibits MMP-9–Mediated Blood–Brain–Barrier Breakdown in a Mouse Model for Ischemic Stroke

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Rationale: Blood–brain–barrier (BBB) breakdown and cerebral edema result from postischemic inflammation and contribute to mortality and morbidity after ischemic stroke. A functional role for the carcinoembryonic antigen–related cell adhesion molecule 1 (CEACAM1) in the regulation of reperfusion injury has not yet been demonstrated.

Objective: We sought to identify and characterize the relevance of CEACAM1-expressing inflammatory cells in BBB breakdown and outcome after ischemic stroke in Ceacam1−/− and wild-type mice.

Methods and Results: Focal ischemia was induced by temporary occlusion of the middle cerebral artery with a microfilament. Using MRI and Evans blue permeability assays, we observed increased stroke volumes, BBB breakdown and edema formation, reduction of cerebral perfusion, and brain atrophy in Ceacam1−/− mice. This translated into poor performance in neurological scoring and high poststroke-associated mortality. Elevated neutrophil influx, hyperproduction, and release of neutrophil-related matrix metalloproteinase-9 in Ceacam1−/− mice were confirmed by immune fluorescence, flow cytometry, zymography, and stimulation of neutrophils. Importantly, neutralization of matrix metalloproteinase-9 activity in Ceacam1−/− mice was sufficient to alleviate stroke sizes and improve survival to the level of CEACAM1-competent animals. Immune histochemistry of murine and human poststroke autopic brains congruently identified abundance of CEACAM1-matrix metalloproteinase-9* neutrophils in the ischemic hemispheres.

Conclusions: CEACAM1 controls matrix metalloproteinase-9 secretion by neutrophils in postischemic inflammation at the BBB after stroke. We propose CEACAM1 as an important inhibitory regulator of neutrophil-mediated tissue damage and BBB breakdown in focal cerebral ischemia. (Circ Res. 2013;113:1013-1022.)

Key Words: animal model of human disease remodeling • inflammation • ischemic stroke • neutrophils • vascular system injuries

Every year, ischemic stroke accounts for ≈10% of global deaths, leaving 5 million patients permanently disabled, not only in high-income, but also in less-developed countries.1 Besides the primary hypoxic damage and secondary processes, such as excitotoxicity, recruitment of inflammatory cells following restoration of cerebral perfusion contributes to ischemic brain injury.2,3 Especially, inflammatory events occurring at the blood–brain barrier (BBB) during cerebral ischemia are critical for the pathogenesis of tissue damage in ischemic stroke. Cell adhesion molecules (eg, intercellular adhesion molecule 1 and the selectins)4 are involved in the development of postischemic brain inflammation by promoting cell adhesion, migration, and activation of leukocytes. Although genetic deficiency or antibody blocking of cellular adhesion molecules, such as intercellular adhesion molecule 1 and platelet selectin, demonstrated attenuated cerebral damage in mice,5-7 links between early BBB breakdown and the innate immune response are still not fully understood. Neutrophils are an essential component of the innate immune system and among the first cells to adhere to the cerebral endothelium and infiltrate the ischemic brain.8 This postischemic inflammation leads to impairment of BBB function and neuronal damage by release of matrix metalloproteinas (MMPs), for example MMP-9 and proinflammatory cytokines, including interleukin (IL)-1ß, as well as reactive oxygen species.9

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Ceacam1−/− CEACAM1-deficient mice (8,17) Bederson’s test (18) was used to determine global neurological (tMCAO) with a silicone-coated microfilament for 60 minutes as described. Mice were randomized and experiments were performed in a blinded manner. Animals Old male mice by temporary occlusion of the middle cerebral artery (MCAO) to induce ischemic stroke in CEACAM1-competent and CEACAM1-null mouse lines to investigate the role of CEACAM1 to dampen the host inflammatory response.11,12 Congruently, lack of CEACAM1 expression results in hyperactivation of neutrophils and increased mortality in a model of Listeria monocytogenes infection as a result of increased neutrophil liver infiltration and elevation of inflammatory cytokine levels.13 Although the role of CEACAM1 in controlling neutrophil granulocyte function has been studied extensively in bacterial infections,13,14 it remains unclear whether CEACAM1 also exhibits a negative regulatory role in sterile inflammation. To address this question, we use the transient occlusion of the middle cerebral artery to induce ischemic stroke in CEACAM1-competent and CEACAM1-null mouse lines to investigate the role of CEACAM1 in postischemic reperfusion injury. Our data demonstrate that CEACAM1-expressing neutrophils are important regulators of reperfusion injury that dampen exacerbation of inflammation and breakdown of the BBB after ischemic stroke.

Methods

Animals
All animal experiments were approved by the local animal care committee (Behörde für Soziales, Familie, Gesundheit und Verbraucherschutz, Hamburg) and conducted after the recommendations for research in basic stroke studies.15 Cerebral ischemia was induced in 10- to 12-week-old male, C57BL/6 wild-type (WT), and CEACAM1-deficient mice (Ceacam1−/−-mice), described previously.16 Ceacam1−/− mice were backcrossed for least 10 generations.

Induction of Cerebral Ischemia and Neurological Scoring
Mice were randomized and experiments were performed in a blinded fashion. Focal cerebral ischemia was induced in 10- to 12-week-old male mice by temporary occlusion of the middle cerebral artery (MCAO) with a silicone-coated microfilament for 60 minutes as described.15 Bederson’s test (18) was used to determine global neurological function according to the following scoring system: 0, no deficit; 1, preferential turning; 2, circling; 3, longitudinal spinning; 4, no movement; and 5, death. The detailed experimental description can be found in Online Data Supplement methods section.

Assessment of BBB Breakdown
BBB breakdown was assessed as described.20 For quantification of extravasated Evans blue into the stroke tissue, mice were injected IV with 2% solution of Evans blue in normal saline (4 mL/kg of body weight) 4 h postsurgery. Evans blue was allowed to circulate for 4 h. Mice were euthanized and perfused with 50 mL of ice-cold phosphate-buffered saline. Brains were removed, cut into 1-mm slices, and stroke sizes were documented by 2% Evans blue and 50% trichloroacetic acid staining. Evans blue was fixed in 50% trichloroacetic acid (Sigma-Aldrich, St Louis, MO), and dye concentrations were quantified photometrically at 540 nm/600 nm.

Flow Cytometric Analyses
Flow cytometry was performed as described.8 Animals were euthanized and perfused with phosphate-buffered saline. After removal of the meninges and dissection into ipsilateral and contralateral hemispheres, brains were enzymatically digested followed by a Percoll gradient centrifugation step to remove myelin. The resulting single cell suspensions were incubated with the appropriate antibody cocktail followed by analyses with a LSR Fortessa flow cytometer (BD Pharmingen, Franklin Lakes, NJ) and FACS Diva software (version 6.1.3, BD Pharmingen, Franklin Lakes, NJ). The antibodies used in this study are listed in the Antibody reference list in the Online Data Supplement methods section.

Histological Analyses
Histological analyses were performed as described.17 Briefly, mice were deeply anesthetized with isoflurane inhalant and perfused through the left ventricle using 10 mL phosphate-buffered saline followed by 50 mL of cold 4% paraformaldehyde. Brains were then stained according to standard immunohistochemistry procedures with antibodies listed in the Antibody reference list in the Online Data Supplement methods section. For analysis of autopic human brain, tissue sections were selected from the files of the Institute of Neuropathology at the University Medical Center Hamburg-Eppendorf. Brains had been fixed in 4% paraformaldehyde for at least 3 weeks before paraffin-embedding. Brain sections (3 μm) were stained according to standard immunohistochemistry procedures.

Zymography
Zymography was performed according to the manufacturer’s instructions (Life Technologies Ltd, Darmstadt, Germany). Briefly, 30 μg of each brain lysate were loaded on a 10% gelatin gel; zymograms were renatured and developed with zinc-containing buffers. Gels were stained with Coomassie Brilliant Blue and areas of protease activity seemed as clear bands.

Neutrophil Isolation and Stimulation With N-Formylmethionyl-Leucyl-Phenylalanine
Neutrophils were isolated as described.21 Briefly, neutrophils were enriched from bone marrow following negative selection with an antibody cocktail–depleting lymphocytes and natural killer cells (see Antibody reference list in the Online Data Supplement methods section). Purity of isolated neutrophils was by light microscopy and by flow cytometry (data not shown, purity>90%). After isolation, 1×10^6 neutrophils were stimulated with 1 μmol/L N-formylmethionyl-leucyl-phenylalanine (Sigma-Aldrich, St Louis, MO).
St Louis, MO). MMP-9 levels were measured in the supernatant with a total MMP-9 ELISA according to the manufacturer (Mouse Total MMP-9 DuoSet, R&D Systems, Minneapolis, MN).

**Inhibition of MMP-9 With SB-3CT**

MMP-9 inhibition was performed as previously described. Briefly, 2-[(4-phenoxyphenyl)sulfonyl]methyl]-thiirane (SB-3CT; 25 mg/kg body weight, Enzo Life Sciences GmbH, Lörrach, Germany) was injected IP as a suspension in a vehicle solution (10% dimethyl sulf oxide in normal saline). SB-3CT was administered 2 h and 5 h after stroke induction. Control groups received vehicle solution instead.

**Statistical Analyses**

For statistical analysis, Prism Graph version 4.0 software (GraphPad Software, La Jolla, CA) was used. After testing for Gaussian distribution with the D’Agostino and Pearson omnibus normality test, data were either analyzed using the unpaired 2-tailed Student t test or non-parametric tests (Mann–Whitney U test). More than 2 groups were analyzed by 1-way ANOVA with post hoc Bonferroni adjustment for P values. Bederson scores were analyzed with the Wilcoxon rank-sum test. P<0.05 were considered statistically significant.

**Results**

**CEACAM1+ Cells Infiltrate the Ischemic Brain Lesion During Postischemic Inflammation**

Ischemia and reperfusion alter the expression of adhesion molecules on microvessels and leukocytes. To document whether the cellular adhesion molecule, CEACAM1, displays changes in its expression pattern after ischemic stroke, we analyzed basic vascular anatomy and CEACAM1 expression in Ceacam1−/− and WT mice before and after ischemic brain injury. In vascular corrosion casts and laser Doppler flowmetry, we confirmed that no anatomic deviations in the cerebral vascular trees were produced by deletion of the Ceacam1 gene in unchallenged Ceacam1−/− and WT mice (Online Figure I). In the uninjured brain, CEACAM1 was only expressed on endothelial cells (EC; Online Figure II). This expression pattern changed during the first 24 h of postischemic inflammation. In whole brain lysates, we found increased CEACAM1 expression in the ipsilateral compared with the contralateral hemisphere after induction of the ischemic stroke, whereas CEACAM1 was only expressed on basal levels in sham-operated mice (Figure 1A). After stroke, CEACAM1 was predominantly expressed on CD11b+ cells, but not microglia (Figure 1B; inlay; asterisk symbol), as demonstrated by histological analysis of ischemic brain sections (Figure 1B). Further differentiation of these cells by flow cytometry revealed that only neutrophils, macrophages, and dendritic cells expressed high levels of CEACAM1 (Figure 1C), whereas microglia (Figure 1C) and lymphocytes (data not shown) expressed CEACAM1 on very low levels. Additionally, immunohistochemical staining of postmortem adult human brain sections confirmed these findings. We found no CEACAM1 expression in the uninjured brain but prominent influx of CEACAM1+ cells 24 h after stroke, which were also identified as neutrophils (Figure 1D). These data show that CEACAM1+ neutrophils are among the earliest inflammatory leukocyte population to infiltrate the ischemic brain.

**CEACAM1 Improves Outcome After Ischemic Stroke**

Accumulation of neutrophils in infarcted cerebral tissue correlates with severity of secondary tissue damage and poor neurological outcome. We, therefore, hypothesized that absence of the inhibitory function of CEACAM1 could negatively influence BBB function and stroke outcome.

**Figure 1.** Carcinoembryonic antigen–related cell adhesion molecule 1 (CEACAM1) is up-regulated during postischemic inflammation. Twenty-four hours after stroke, CEACAM1 expression was analyzed by Western blotting (A), immunofluorescence (B), flow cytometry (C), and in human brain tissue (D). Increased levels of CEACAM1 were detected in ipsilateral brain lysates (A). In histological sections, an influx of CD11b+CEACAM1+ cells was detected (B). CD11b-labeling is shown in green, CEACAM1 expression is shown in red, nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI), blue; scale bar=50 µm (B). Flow cytometry analyses identified granulocytes, macrophages, and dendritic cells (DCs) as CEACAM1-expressing cells (C). In human brain sections, an influx of CEACAM1-expressing cells was also observed (D; shown after peroxidase-assisted detection of CEACAM1). The infiltrating CEACAM1-positive cells (red) showed positive staining for myeloperoxidase (MPO, green) but not CD68 (green), indicating that in human stroke, only neutrophils express CEACAM1 but not macrophages (D, lower row); scale bar=50 µm. H&E indicates hematoxylin and eosin; and WT, wild type.
Following this idea, CEACAM1-competent and Ceacam1−/− mice were subjected to tMCAO to analyze infarct volumes (Figures 2 and 3). Twenty-four hours after stroke, we found that Ceacam1−/− mice had ≈30% larger infarcts compared with WT mice (Figure 2A; 34.79±4.06 mm³ versus 43.72±4.44 mm³; P<0.001). Observing increased midline shift in Ceacam1−/−, edema was measured with a noninvasive method, which highly correlates with absolute brain water content.19 This analysis revealed that Ceacam1−/− mice developed more pronounced cerebral edema (Figure 2B; 9.53±3.36% versus 14.17±3.88% increase of the ipsilateral hemisphere volume; P=0.0018), resulting in lower striatal and cortical perfusion rates compared with the contralateral hemispheres (Figure 2C: striatal: 0.87±1.03% versus −2.40±3.59%; P=0.0083; cortical: 6.42±2.62% versus 2.41±7.35%; P=0.0051). We investigated whether this larger tissue damage in Ceacam1−/− mice was also functionally relevant by scoring the mice according to Bederson test, which rates poststroke neuronal function and morbidity.18 In these scores, Ceacam1−/− mice performed significantly worse compared with WT mice, which became evident by significant higher frequencies in circling (Bederson score 2), longitudinal spinning (Bederson score 3), and poststroke mortality (Bederson score 5) in the Ceacam1-negative genotype (Figure 3A; P=0.0364 on d1; P=0.031 on d2; P<0.0001). The increased poststroke morbidity in Ceacam1−/− mice was also associated with a remarkably delay in body weight gain as a consequence of reduced motility and neurological performance (Figure 3B; at d14, 99.47±5.26% versus 90.76±4.44% of the initial weight; P=0.0005). The overall poststroke survival of mice was significantly lower compared with WT animals (Figure 3C; 57% versus 100% in WT mice). After 2
Ceacam1−/− mice displayed significantly larger brain atrophy compared with CEACAM1-competent mice (Figure 3D; 2.35±3.91 mm³ versus 10.38±9.95 mm³; P=0.0189), whereas the degree of vascular remodeling and angiogenesis did not differ significantly between the mouse lines (Online Figure III).

Taken together, we found increased stroke volumes and cerebral edema, associated with decreased cerebral perfusion, and overall worse outcome in the absence of CEACAM1 suggesting that CEACAM1 is involved in early pathophysiological cascades after ischemic stroke.

**Increased BBB Breakdown in Ceacam1−/− Mice**

Increase in BBB permeability during ischemic stroke lead to influx of plasma proteins and subsequently to formation of the cerebral edema.25 Because we observed increased cerebral edema in Ceacam1−/− mice, we suspected that impaired BBB function was related to the worse outcome in Ceacam1−/− mice after stroke. Therefore, we investigated stability of the BBB during reperfusion after intravenous injection of Evans blue (Figure 4) and with MRI (Online Figure IV). We observed increased perivascular staining with Evans blue, indicative of a more severe BBB breakdown in the CEACAM1-negative genotype (Figure 4A; 1.9±0.7 versus 3.1±0.8-fold increase of Evans blue intensity; P<0.0001). In both cortical and striatal strokes, enhanced Evans blue extravasation was observed in Ceacam1−/− mice. Normalization of extravasated Evans blue to the stroke volume demonstrated that BBB breakdown was independent of the stroke size in Ceacam1−/− mice (Figure 4B; 5.24±1.20 ng/mm³ in WT versus 12.41±1.54 ng/mm³ in Ceacam1−/− mice), whereas sham mice showed similar basal Evans blue leakage in both genotypes (Figure 4C; 6.377±1.839 µg/g in WT versus 5.086±0.8558 µg/g in Ceacam1−/− mice). No differences were observed in serum levels of Evans blue that served as an internal standard to document comparable abundance of intravascular dye (Figure 4D; 0.0985±0.0155 ng/µL in WT versus 0.1070±0.0105 ng/µL in Ceacam1−/− mice).

**Increased Levels of MMP-9 Exacerbate BBB Breakdown and Tissue Damage in Ceacam1−/− Mice**

Based on these findings, we went on to further define the underlying cause for more severe BBB breakdown in Ceacam1−/− mice. Inverse cerebrovascular permeability after stroke is caused by MMP-9, which is produced by different cell types, such as myeloid cells, glial cells, and EC.26–28 We analyzed the infiltrating inflammatory cells to identify the cell population that expresses MMP-9 and is causally involved in BBB breakdown in our model. Because we showed that CEACAM1 is prominently expressed in CD11b+ cells, namely neutrophils, and neutrophils were identified as the principal cell population present in the infarcted areas (Figure 1B and 1C), we characterized their spatial distribution and secretion products in our model after 8 h (Online Figure V) and 24 h (Figure 5). We observed increased accumulation of CD11b+MMP-9+ cells in Ceacam1−/− ischemic hemispheres compared with WT mice (Figure 5A; Online Figure VB and VC). Furthermore, only neutrophils expressed MMP-9 in significant amounts among myeloid cells during the first phase of the postischemic inflammation (Figure 5B). To date, a link between CEACAM1 expression on neutrophils and the regulation of MMP-9 production has not been reported. Consistent with our histological observations, flow cytometry analyses of the ischemic brains also revealed significantly higher absolute numbers of neutrophils in the ischemic hemispheres of Ceacam1−/− mice (Figure 5C; 20.90±2.90% versus 40.12±3.70% infiltrating neutrophils; P<0.0001; n=6 per

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**Figure 4. Blood–brain–barrier (BBB) breakdown is increased in Ceacam1−/− mice.** Stability of the BBB during reperfusion (A and B, n=10 per group, representative microscopic images) and in sham mice (C, n=4 per group) was analyzed after IV injection of Evans blue. Semi-quantitative immunohistochemical analysis demonstrated higher BBB breakdown (A). This effect was independent of stroke size as shown in B. No differences of Evans blue leakiness was observed in sham-operated mice. Serum levels of Evans blue were measured as an internal control (D). A. Representative microscopic images of brain sections after injection of Evans blue (red), scale bar: 2 mm. The stroke area was identified with anti–microtubule-associated protein 2 (MAP-2) antibodies (white; the stroke area did not stain positive for MAP-2, black areas, marked with white dots). B. Representative macroscopic images of Evans blue leakage (blue) combined with 2,3,5-tetrazolium chloride–life/dead staining (the infarcted area is shown in white) of cortical (B, upper row) and striatal strokes (B, lower row). Data are presented as mean with 95% confidence intervals; data were evaluated with the Mann–Whitney U test; each white and black symbol represents data from 1 WT or Ceacam1−/− brain section. tMCAO indicates temporary occlusion of the middle cerebral artery.
group). Normalization of the infiltrating cell numbers relative to resident microglia proved a solely higher influx of neutrophils into the ischemic hemispheres of Ceacam1−/− mice (Figure 5D and 5E). Macrophage numbers were comparable 24 h postinfarction between the different genotypes (numbers of infiltrating neutrophils were normalized to microglia: 0.0784±0.0471 in WT versus 0.2914±0.0996 in Ceacam1−/− mice; and neutrophil numbers were correlated to macrophages: 0.1789±0.1187 in WT versus 0.2876±0.1560 in Ceacam1−/− mice [Figure 5D and 5E]).

In addition, we evaluated total MMP-9 activity in brain homogenisates from sham controls and mice 8 h and 24 h after tMCAO. High MMP-9 levels and activity were confirmed in ipsilateral hemispheres from Ceacam1−/− mice (Figure 6A and 6B; (Online Figure VE and VF). Expectedly, MMP-9 levels and activity in the contralateral hemispheres were comparable between both genotypes (Online Figure VI), indicating that higher MMP-9 levels in ischemic hemispheres of Ceacam1−/− mice resulted from postischemic inflammation. Moreover, we identified 200-kDa MMP-9 dimers in zymography that identify neutrophils as a major source of MMP-9 in our model, because these dimers are specifically secreted by neutrophils.29 Flow cytometric analyses of MMP-9 expression in neutrophils also revealed elevation of MMP-9 levels in CEACAM1-negative neutrophils (Figure 6C; MMP-9 levels are expressed as differences in ipsilateral hemispheres from Ceacam1−/− mice (Figure 6A and 6B; (Online Figure VE and VF). Expectedly, MMP-9 levels and activity in the contralateral hemispheres were comparable between both genotypes (Online Figure VI), indicating that higher MMP-9 levels in ischemic hemispheres of Ceacam1−/− mice resulted from postischemic inflammation. Moreover, we identified 200-kDa MMP-9 dimers in zymography that identify neutrophils as a major source of MMP-9 in our model, because these dimers are specifically secreted by neutrophils.29 Flow cytometric analyses of MMP-9 expression in neutrophils also revealed elevation of MMP-9 levels in CEACAM1-negative neutrophils (Figure 6C; MMP-9 levels are expressed as differences
in mean fluorescence intensity after normalization to a MMP-9-negative cell population (microglia); 29.32±8.99 in WT versus 42.07±11.48 in Ceacam1−/− cells; \( P=0.0281 \). To prove that CEACAM1 participates in the regulation of MMP-9 levels in neutrophils, we performed in vitro stimulation of untouched neutrophils from CEACAM1-competent and Ceacam1−/− mice with N-formylmethionyl-leucyl-phenylalanine. We demonstrate that exposure of primary neutrophils to N-formylmethionyl-leucyl-phenylalanine induces secretion of significantly higher levels of MMP-9 over time in Ceacam1−/− mice compared with WT, indicating a CEACAM1-dependent regulation of MMP-9 production (Figure 6D).

**Inhibition of MMP-9 With SB-3CT Produces a Phenocopy of WT Infarction Sizes in Ceacam1−/− Mice**

Because we show a causative role for CEACAM1 in controlling neutrophilic MMP-9 secretion, we hypothesized whether inhibition of MMP-9 activity was sufficient to reduce postischemic inflammation in the CEACAM1 null mice after tMCAO and to improve their poststroke outcome. Hence, we repeated our experiments in the presence of an MMP-9 inhibitor, SB-3CT in vivo. 24 h after tMCAO, we observed that SB-3CT application ameliorated the exacerbation of ischemic brain damage in Ceacam1−/− mice (Figure 7A: stroke volumes: 28.25±4.46 mm³, WT with SB-3CT, \( n=8 \), versus 32.15±3.12, Ceacam1−/− mice with SB-3CT, \( n=8 \), versus 38.26±1.51 mm³, WT animals with vehicle control, \( n=4 \), versus 45.84±3.98 mm³, Ceacam1−/− animals with vehicle control, \( n=5 \); Figure 7B: edema: 8.57±2.38%, WT with SB-3CT, versus 8.69±1.93%, Ceacam1−/− mice with SB-3CT, versus 12.38±1.25% WT animals with vehicle control, versus 17.56±2.36%, Ceacam1−/− animals with vehicle control; Figure 7C: perfusion: striatal: 4.51±2.69%, cortical: 16.56±6.57%, WT with SB-3CT; striatal: 4.32±2.34%, cortical: 15.10±7.79% Ceacam1−/− mice with SB-3CT; striatal: −0.03±1.68%, cortical: 12.93±6.94% WT animals with vehicle control; striatal: −5.15±2.31%, cortical: −2.37±4.78%, Ceacam1−/− animals with vehicle control).

Importantly, the cerebral influx of neutrophils into the ischemic hemispheres of Ceacam1−/− mice was diminished to that WT levels after treatment with SB-3CT and led to improved survival of the Ceacam1−/− mice (Figure 7D and 7E; cerebral influx of neutrophils: 20.33±5.17%, WT treated with SB-3CT, \( n=4 \), versus 13.28±6.72, Ceacam1−/− mice treated with SB-3CT, \( n=5 \); survival rates: 83.3% Ceacam1−/− mice/+SB-3CT versus 60% Ceacam1−/− mice without SB-3CT).

**Figure 7. Inhibition of matrix metalloproteinase-9 (MMP-9) in Ceacam1−/− mice decreases stroke size to wild-type (WT) levels.** After inhibition of MMP-9, stroke sizes (A, representative T2 images), cerebral edema (B), arterial spin-labeled perfusion (C, representative ASL perfusion maps) were measured with MRI 24 h after temporary occlusion of the middle cerebral artery. Infiltrating neutrophils were examined by flow cytometry (D), and survival was recorded over a time course of 3 days (E). Neutralization of MMP-9 improved outcome of Ceacam1−/− mice to WT levels. Histological analysis showed that MMP-9 is not only coexpressed by murine carcinoembryonic antigen–related cell adhesion molecule 1 positive (CEACAM1+) cells (F, left), but also by human cells (F, right) in the ischemic hemispheres (CEACAM1 expression is shown in red, MMP-9 in green, 4,6-diamidino-2-phenylindole [DAPI] in blue, scale bar=50 μm). Data are presented as mean with 95% confidence intervals; \( P<0.05 \), \( P<0.001 \), \( **P<0.0001 \); data from stroke volume, edema, perfusion measurements, and quantification of infiltrating cells were analyzed with a Bonferroni-corrected ANOVA; each white and black square represents data from 1 WT or Ceacam1−/− animal; each white and black symbol represents data from 1 WT or Ceacam1−/− animal.
These data demonstrate that CEACAM1+ neutrophils are a critical determinant in the regulation of BBB breakdown; because CEACAM1 is a functionally and structurally conserved molecule, we were prompted to analyze whether human CEACAM1+ neutrophils could also potentially attribute to MMP-9–mediated BBB breakdown during cerebral ischemic insult. As shown in Figure 7F, MMP-9+CEACAM1+ neutrophils are present both in murine and human ipsilateral hemispheres. Hence, it is tempting to assume that CEACAM1 may also contribute to reperfusion injury after ischemic stroke in humans.

**Discussion**

In this study, we report on the function of CEACAM1 in inflammation after ischemic stroke. We demonstrate that lack of CEACAM1 expression on neutrophils leads to exacerbation of BBB breakdown and increased neutrophil infiltration into the ischemic hemispheres. As a consequence, extensive cerebral edema, reduced cerebral perfusion, and worse overall outcome were observed in Ceacam1−/− mice. Cerebral edema after BBB breakdown contributes to severe morbidity and mortality after ischemic stroke and is a major therapeutical challenge. Although decompressive hemicranieotomy after malignant MCA territory infarction has reduced stroke-associated mortality rates from 71% to 22%, still 31% of these patients are left severely disabled (modified Rankin scale score 4). Therefore, early therapeutic intervention and reestablishment of perfusion are the immediate goals of poststroke treatment, albeit that restoration of cerebral blood flow causes reperfusion injury with exacerbated inflammation–induced tissue damage.

Inflammation after cerebral ischemia, proinflammatory cytokines, IL-1β, IL-6, tumor necrosis factor-α, monocyte chemotactic protein-1, and reactive oxygen species are released by necrotic and injured tissue. Consequently, astrocytes, microglia, and EC are activated, which initiates the posts ischemic inflammatory response. During this phase, induction of leukocyte adhesion molecules on cerebral microvessels and circulating leukocytes triggers leukocyte adhesion and extravasation and cytokine-induced remodeling of EC–EC tight junctions. In parallel, disruption of EC–EC junctions and enzymatic degradation of the perivascular extracellular matrix (mainly by MMP-9) destabilize the vasculature and facilitate plasma protein and leukocyte extravasation leading to formation of the vasogenic edema. The vasogenic edema is a result of biphasic BBB breakdown caused by activated MMP-2 and the cytokine-inducible MMPs stromelysin (MMP-3) and MMP-9. The breakdown of the BBB is initially reversible. It is largely catalyzed by MMP-2, but subsequent release of IL-1β, tumor necrosis factor-α, and the influx of neutrophils induce MMP-3 and MMP-9 activity producing irreversible breakdown of the BBB. Therefore, it is important to identify novel proteins or specific cell types involved in regulation of MMP-9 production in ischemic stroke. Recent reports highlighted the immunomodulatory role of CEACAM1 on myeloid and ECs at the inflammatory endothelial interface. We and others described CEACAM1 as a regulator of vascular homeostasis and integrity decreasing basal and acute vascular permeability. Under hypoxic conditions, CEACAM1 is up-regulated after hypoxic preconditioning in myocardial ischemia on myocytes and endothelia and was suspected to elicit cardioprotective effects. Furthermore, CEACAM1-expressing myeloid cells catalyze collateral formation and improve tissue perfusion after permanent femoral artery occlusion in mouse model for hindlimb ischemia. We demonstrated up-regulation of CEACAM1 in the ischemic hemisphere after stroke due to an influx of CEACAM1+ neutrophils. CEACAM1 is an immunoreceptor tyrosine-based inhibition motif-containing pathogen receptor inhibits the inflammatory response elicited by toll-like receptor 4–CEACAM1 complex formation on neutrophils in response to pathogen challenge, leading to reduction of proinflammatory cytokine production. Ischemia and reperfusion injury share many phenotypic parallels with bacterial infections, such as signaling through pattern-recognition molecules (toll-like receptors), leukocyte adhesion molecule–dependent recruitment and activation of immune cells of the innate and adaptive immune system and endothelial activation. Interestingly, reperfusion injury is reduced in mouse lines with systemic deletion of leukocyte or endothelial adhesion molecules, eg, intercellular adhesion molecule 1, platelet selectin, or toll-like receptor 4.

To date, it has not been investigated whether CEACAM1 had any inhibitory effects on sterile inflammation in cerebral reperfusion injury. Hence, one could argue that CEACAM1 exerts neuroprotective effects by enhancing EC–cell contacts, and thus, limiting vascular permeability. As presented here, systemic deletion of the Ceacam1 gene did not increase basal cerebral endothelial permeability. Contradicting these results, vascular hyperpermeability in Ceacam1−/− mice has been reported in endothelium of the lung, in tumors, and after topical application of mustard oil or injection of vascular endothelial growth factor. However, we reported earlier that endothelial CEACAM1 is of a lesser vasoprotective relevance during vascular remodeling, because transfer of CEACAM1+ myeloid cells into CEACAM1-deficient hosts could remedy defects in collateral formation and wound healing. On the contrary, depletion of CEACAM1+CD11b+ granulocyte antigen 1+ cells compromised this beneficial effect. This suggests that BBB breakdown and cerebral endothelial permeability are not solely influenced by endothelial CEACAM1 expression, but rather involve CEACAM1-expressing inflammatory cells. In addition, the cerebral vasculature consists of a specialized endothelium that is devoid of fenestrations and is highly impermeable. Therefore, absence of vascular endothelial CEACAM1 may be of minor impact on cerebral vessel stability.

Because we observed an apparent increase of neutrophil influx into the ischemic hemispheres of Ceacam1−/− mice, we focused on these inflammatory cells to identify a novel CEACAM1-dependent mechanism attributing to the integrity of the BBB. Excessive neutrophil infiltration into tissues correlates with increase in MMP-9 production. Furthermore, the induction of irreversible cerebrovascular permeability after stroke is substantially caused by MMP-9. Therefore, we analyzed presence and distribution of MMP-9 in our model. Both in vivo and in vitro analyses showed increased MMP-9 induction in CEACAM1-deficient neutrophils, suggestive of a disturbed regulation of MMP-9 synthesis and activation. The significant accumulation of neutrophils in ipsilateral hemispheres of Ceacam1−/− mice may be both, cause or consequence of elevated MMP-9 activity, because MMP-9 degrades the basal...
lamina and impairs vessel stabilization, but simultaneously also stimulates neutrophil migration. Various cytokines, including IL-1β, can induce MMP-9. Recently, Lu et al. identified CEACAM1 as a down-regulator of inflammatory cascade and IL-1β production in neutrophils. Hence, we also assumed that exaggerated inflammatory cytokine release contributed to enhanced MMP-9 induction in CEACAM1-negative backgrounds. The source of MMP-9 after ischemic stroke, however, is still discussed controversially: although endothelial-adherent and infiltrating neutrophils were identified as the main source of MMP-9 that is responsible for the early loss of BBB integrity in the first 24 h, it was also suggested that neutrophils do not contribute to MMP-9 activity in insulted tissue. Still, our analysis of the composition and activities of gelatinase-type MPPs revealed high levels of neutrophil-specific dimeric MMP-9 isoforms in brain lysates from Ceacam1−/− mice. Flow cytometric and histological analyses, our data add further proof that MMP-9 was indeed secreted by neutrophils. Moreover, it is of note that high MMP-9 levels correlate with infarction formation, poor neurological outcome, increased risk of hemorrhagic transformation, and intracranial hemorrhage. Inhibition experiments, we demonstrate that reduction of MMP-9 activity alone can compensate for loss of CEACAM1 expression, in that BBB breakdown, diminished secondary tissue damage, and improved neurological outcome and survival after ischemic stroke. Moreover, lower MMP-9 levels decreased BBB breakdown in reperfusion injury by controlling MMP-9 levels in neutrophils. This assumption is profoundly supported by the herein presented immunohistological labelings of human poststroke autopic brains, where we identified cerebral influx of human neutrophilic granulocytes as the principal source of MMP-9.

In summary, we described the contribution of CEACAM1 to the pathophysiology of reperfusion injury, BBB breakdown, and cerebral edema in a mouse model for ischemic stroke. We demonstrated for the first time that CEACAM1 dampened inflammation in reperfusion injury by controlling MMP-9 levels in neutrophils. As a consequence, lower MMP-9 levels decreased BBB breakdown, diminished secondary tissue damage, and improved neurological outcome and survival after ischemic stroke. Moreover, our analysis of human autopic brain tissue showed that the CEACAM1+ neutrophils might be involved in the pathophysiology of ischemic stroke in humans. Future studies will need to be conducted to address a putative analogous function of human and murine CEACAM1 in ischemic stroke and reperfusion injury.

Acknowledgments

We thank the FACS Sorting Core Unit at the University Medical Center Hamburg-Eppendorf (UKE) and the Core Facility for Mouse Pathology (UKE) for their support and Christa Reinhold and Krimhild Scheike for expert technical assistance.

Sources of Funding

This project was funded by the Deutsche Forschungsgemeinschaft (Bonn, Germany) to Drs Horst and Magnus (HO-3312-1) and the ERA-NET grant (NanoStroke) to Dr Magnus.

Disclosures

None.

References

Neutrophilic MMP-9 levels are regulated by CEACAM1 at the ischemic brain.


CEACAM1 deficiency can be functionally compensated by MMP-9 inhibition. Thus, stroke outcome is determined by the CEACAM1-dependent regulation of neutrophilic MMP-9 production. The mechanisms underlying edema formation after stroke are poorly defined. In this study, we demonstrate a link between the cellular adhesion molecule, CEACAM1, and the extent of BBB breakdown. We found that CEACAM1 is a negative regulator of MMP-9 production by neutrophils, and therefore, protects from metalloproteinase-induced BBB damage. The loss of CEACAM1 results in an increase in cerebral vascular permeability producing larger infarction sizes, increased tissue damage, poor neurological outcome, and higher mortalities. These changes can be reversed by MMP-9 inhibition. Altogether, these findings reveal for the first time a negative regulator for cerebral edema formation after stroke. Hence, the modulation of CEACAM1-mediated effects might offer a new venue for understanding and future interventions in cerebral edema formation after stroke.

What New Information Does The Article Contribute?

- Levels of BBB-disrupting matrix metalloproteinase-9 (MMP-9) in the infarcted areas are elevated by leukocyte influx and activation of endothelial and brain-resident leukocyte populations.
- The carcinoembryonic antigen–related cell adhesion molecule 1 (CEACAM1) is an essential regulator of vascular integrity (vascular remodeling) and leukocyte activity in infections and in the resolution of inflammation.
- Loss of CEACAM1 enhances BBB breakdown, subsequent increase of stroke sizes, and exacerbates poststroke inflammation.
- Neutrophilic MMP-9 levels are regulated by CEACAM1 at the ischemic BBB.

What Is Known?

- CEACAM1 can be functionally compensated by MMP-9 inhibition. Thus, stroke outcome is determined by the CEACAM1-dependent regulation of neutrophilic MMP-9 production.
Carcinoembryonic Antigen−Related Cell Adhesion Molecule 1 Inhibits MMP-9−Mediated Blood−Brain−Barrier Breakdown in a Mouse Model for Ischemic Stroke

Peter Ludewig, Jan Sedlacik, Mathias Gelderblom, Christian Bernreuther, Yücel Korkusuz, Christoph Wagener, Christian Gerloff, Jens Fiehler, Tim Magnus and Andrea Kristina Horst

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http://circres.ahajournals.org/content/suppl/2013/06/18/CIRCRESAHA.113.301207.DC1
Supplemental Material

Ludewig et al. CEACAM1 inhibits MMP-9-mediated blood-brain-barrier breakdown in a mouse model for ischemic stroke

Supplemental Methods

Induction of cerebral ischemia
Mice were kept under a 12h light/dark cycle, constant temperature (22±2°C), and with food and water ad libitum. Focal cerebral ischemia was induced in 10-12 week-old male mice by transient middle cerebral artery occlusion (tMCAO) for 60 min, as described 1,2. Briefly, Mice were anesthetized with 1.5% isoflurane (Abbott, Wiesbaden, Germany) in 100% O₂, and intraperitoneal injection of 0.05 mg/kg bodyweight buprenorphine in saline. After a midline skin incision in the neck, the proximal common carotid artery, and the external carotid artery were ligated, and a standardized silicone-coated 6.0 nylon filament (6023; Doccol Corp., Redlands, CA, USA) was inserted in the left internal carotid artery to occlude the origin of the left middle cerebral artery (MCA). The occluding monofilament was withdrawn after 60 min, and the ligature of the common carotid artery was opened to allow reperfusion. Sham operated mice were treated identically except that the filament was removed immediately after insertion. Mice were blinded to the genotype and operation time per animal did not exceed 15 min. In the first three days after tMCAO, daily, 1 ml of saline was injected intraperitoneally to prevent dehydration.

Laser-Doppler flowmetry
Laser-Doppler flowmetry (Moor Instruments, Axminster, U.K.) was used in all animals to monitor regional cerebral blood flow (rCBF) in the MCA territory (6 mm lateral and 2 mm posterior from Supplemental Figure II, G,H).

Assessment of the cerebral vasculature
For assessment of the cerebral vasculature WT and Ceacam1— mice (n=3/group) were deeply anesthetized with CO₂. Post mortem, mice were injected with an acrylic-based resin (Acryfix, Evonik, Dortmund, Germany), for preparation of anatomical corrosion castings. After hardening, tissues were corroded away with 25% KOH, and removed carefully with forceps. The plastinated vasculature was exposed and examined under a binocular.

Stroke assessment by magnetic resonance imaging
Magnetic resonance imaging (MRI) was performed repeatedly at 24h and 14 days after stroke on a dedicated 7T MR small animal imaging system (ClinScan, Bruker, Ettlingen, Germany) with a 4 element phased array mouse brain receiver coil and a circular polarized whole body transmit coil. The image protocol comprised T2 weighted sequence to assess location and extend of infarction. Pulsed arterial spin labeling (PASL) perfusion imaging was acquired to assess the extent of perfusion deficit. 3D time-of-flight (TOF) angiography was performed in oblique coronal-axial slice orientation to allow sufficient inflow of fresh spins. T2w TSE sequence parameters were: TE = 57 ms, TR = 4650 ms, BW = 100 Hz/pixel, turbo factor 7, matrix = 256x192, FOV = 20x15 mm2, 28 slices, and 0.4 mm slice thickness with 0.1 mm gap. PASL sequence parameter were: inversion time = 1800 ms, labeling time = 700 ms and saturation stop time = 1600 ms, TE = 14 ms, TR = 2400 ms, BW = 1985 Hz/pixel, EPI factor 72 with PA acceleration factor 2, matrix = 72x72, FOV = 20x20 mm2, 9 slices, 0.5 mm slice thickness with 0.1 mm gap. TOF image parameters were set as follows: TR/TE = 18/3.7 ms, flip angle (FA) = 25°, BW = 150 Hz/pixel, matrix size = 192 x 192 and FOV = 20 x 20 mm2. Five image slabs containing 24 slices each (slice thickness 0.12 mm and 25% overlap) were acquired.

Steady state susceptibility contrast-enhanced magnetic resonance imaging
We thank Guerbet, Roissy, France, for the generous gift of Endorem®.
$\Delta R_2$, $\Delta R_2^*$, vascular density, and size were evaluated by steady state susceptibility contrast-enhanced (ssCE) MRI. To determine $\Delta R_2$ and $\Delta R_2^*$, multi echo turbo spin echo (mTSE) and multi echo gradient echo (mGRE) pulse sequences were performed before and after an injection of iron oxide (Endorem, Guerbet GmbH, Sulzbach, Germany) at a dose of 300 µmol Fe/kg. Endorem was selected because it is an intravascular contrast agent with a long plasma half-life. The postcontrast image acquisition was delayed by 2 mins to ensure a steady-state distribution of contrast agent in the vessels. mTSE and mGRE images were obtained in the same location with a field of view (FOV) of 2cm x 1.5 cm. mTSE sequence parameters were: TR = 4480 ms, TEs = 14, 56, 97 ms, turbo factor = 4, NEX = 2, matrix = 128x96 and 24 slices of 0.4 thickness and 0.1 mm gap. mGRE sequence parameters were: TR = 60 ms, TEs = 1.9, 3.8, 5.7, 7.7, 9.6, 11.5 and 13.5 ms, flip angle =30°, NEX = 1, 3D matrix = 192x144x48 and 3D slab thickness of 12 mm. Transverse relaxation rates were calculated by pixelwise non-linear least square fitting of the signal decay measured at the different echo times which yields image maps of the pre- and post-contrast transverse relaxation rates: $R_2^{\text{pre}}$, $R_2^{\text{post}}$ and $R_2^*^{\text{pre}}$, $R_2^*^{\text{post}}$, respectively. Changes of the relaxation rates were given by $\Delta R_2 = R_2^{\text{post}} - R_2^{\text{pre}}$ and $\Delta R_2^* = R_2^*^{\text{post}} - R_2^*^{\text{pre}}$. Vessel density and size imaging were derived, pixel by pixel, using Matlab (MathWorks, Ismaning, Germany), according to the ratio of relaxation rate shift $\Delta R_2/\Delta R_2^{*2/3}$ (or Q) and $\Delta R_2^*/\Delta R_2$.

Analysis of Lesion evolution (ADC, T2W) in WT and Ceacam1+/- mice

Magnetic resonance imaging (MRI) was performed repeatedly at 90min, 270min, 330min, 450min, 510min and 24h after stroke on a dedicated 7T MR small animal imaging system (ClinScan, Bruker, Ettlingen, Germany) with a 4 element phased array mouse brain receiver coil and a circular polarized whole body transmit coil. The image protocol comprised of axial diffusion weighted (DWI) and T2 weighted sequence to assess location and extent of infarction. From the DWI images, pixel-by-pixel maps of the apparent diffusion coefficient (ADC) of water were calculated. DWI sequence parameter were: b-values 0, 500 and 1000 s/mm², TE = 31 ms, TR = 8000 ms, BW = 2790 Hz/pixel, echo planar imaging (EPI) factor 128 with parallel acquisition (PA) acceleration factor 2, matrix = 128x96, FOV = 20x15 mm², 14 slices, 0.8 mm slice thickness with 0.2 mm gap. T2w TSE sequence parameter were: TE = 57 ms, TR = 4650 ms, BW = 100 Hz/pixel, turbo factor 7, matrix = 256x192, FOV = 20x15 mm², 28 slices, 0.4 mm slice thickness with 0.1 mm gap.

Data were analyzed as described. Briefly, for monitoring of the changes in ADC values and T2w signal intensities, regions of interest (ROI) were defined on T2w images obtained after 24h, where the maximum extent of the lesion was identified by hyperintense T2w signals. The ischemic damaged region was defined by using the threshold T2w signal of mean +/-2 standard deviations from the averaged T2w signal measured in the contralateral hemispheres. The ROIs completely covered the hyperintense regions containing the T2 maxima. ROIs were then transferred to the corresponding T2w images and ADC maps obtained during reperfusion at 90min, 270min, 330min, 450min, 510min. Control values were measured from an ROI in the non-ischemic tissue contralaterally (same anatomic structures as ipsilaterally), and all values of signal intensities are hemispheric ratios (ratio of ipsilateral to contralateral signal intensities). ADC lesion volumes were identified and measured on ADC maps using a relative ADC threshold of 80% of the mean contralateral hemisphere.

Histological analyses

Histological analysis was performed, as described. Briefly, mice were anesthetized with isoflurane inhalant and perfused through the left ventricle using 10ml PBS followed by 50ml of cold 4% PFA. The brains were post-fixed in 4% PFA overnight at 4°C and cryoprotected in 30% sucrose (w/v) in PBS until the brains sank to the bottom of the solution. After snap freezing of the Tissue-Tek® OCT™ Compound (Sakura Finetek Europe B.V, Flemingweg, Netherlands)-embedded brains in isopentane precooled with liquid nitrogen, brains were cut into 6µm frontal sections using a Leica CM3050 cryotome. Sections were then exposed to PBS containing 0.3% Triton X-100 and 10% normal goat serum to block nonspecific antibody binding for a minimum of 60 min, followed by incubation with the primary antibodies at 4°C.
overnight. Sections were then incubated with the appropriate secondary antibody at room temperature for 1h. Control sections from each animal were prepared for immunohistochemical staining in an identical manner except that the primary antibodies were omitted. Stained sections were then examined under a Leica DM5000B microscope. For quantitative analysis, 3 mice per group and 3 sections at bregma area per mouse were analyzed.

Human brain sections were deparaffinized in Histo Clear (National Diagnostics, Atlanta, Georgia) and rehydrated through graded alcohol washes. For antigen retrieval, the slides were heated in citrate buffer (10 mM, pH 6.0) for 20 minutes. After antigen retrieval, endogenous peroxidase was quenched with 3% H2O2 in methanol for 10 minutes followed by a blocking step with 5% normal goat serum in TBS containing 0.1% Tween20 for 30min. Sections were incubated with the primary antibodies at 4 °C overnight. Sections were then incubated with the appropriate secondary antibody at room temperature for 1h. Sections for horseradish peroxidase staining were incubated with biotin-labeled anti-mouse immunoglobulins for 60 minutes, followed by incubation in the avidin-peroxidase complex for 60 minutes. Finally, the immunoreaction products were visualized with a 3,3-diaminobenzidine (DAB) substrate solution (Dako, Glostrup, Denmark). To verify the specificity of the stainings, some control sections were incubated with the appropriate isotype control. Stained sections were then examined under a Leica DM5000B microscope.

Protein extraction from brain tissue and cerebral microvessel isolation

Mouse tissues were dounced in freshly prepared kinase lysis buffer (KLB, 25 mM Tris/HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA (pH 8.0), 10% glycerol (v/v), 1% Triton X-100 (v/v), 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mM β-glycerolphosphate, protease inhibitors: 0.1 M PMSF, aprotinin, 50 mM sodium pervanadate solution, 1 M NaF). After centrifugation (20000 rcf, 10 min, 4 °C), the supernatant was transferred to fresh tubes, and the total protein concentration was determined with a NanoDrop spectrophotometer at 280nm (Thermo Scientific, Wilmington, USA). Cerebral microvessels were isolated, as described 13. Briefly, after centrifugation of the brain homogenisates in 30% dextran to remove myelin, myelin-free tissue was poured through a 70µm cell strainer (BD Pharmingen, Franklin Lakes, NJ, USA). Microvessels did not pass the mesh and could be washed off the filter. Purity of the isolated microvessels was confirmed under a microscope (Leica DM5000B). Subsequently, the microvessels were subjected to protein extraction and analysis by Western Blotting.

Flow cytometric analyses

Flow cytometry was performed, as described [3]. Animals were euthanized and perfused with phosphate-buffered saline. After removal of the meninges, brains were mechanically dissected, cerebella were removed, and hemispheres divided into left ischemic and right non-ischemic. Each hemisphere was incubated for 30 min at 37°C (1 mg/mL collagenase, 0.1 mg/mL DNase I in DMEM), and passed through a cell strainer (40 µm; BD Pharmingen, Franklin Lakes, NJ, USA). Next, cells were incubated with standard erythrocyte lysis buffer on ice, followed by myelin removal with a Percoll gradient (1095 g/mL and 1030 g/mL; GE Healthcare, Buckinghamshire, UK) centrifugation. Purified cells were incubated with appropriate antibody cocktails (30 min, room temperature; see antibody reference) in flow cytometry buffer (0.5% bovine serum albumin, 0.02% sodium azide in phosphate-buffered saline). Cells were analyzed using a LSR Fortessa flow cytometer (BD Pharmingen, Franklin Lakes, NJ, USA) and FACS Diva software (BD Pharmingen, Franklin Lakes, NJ, USA). Approximately 2 000 000 events per tube were recorded. The antibodies used in this study are listed below in the Antibody reference list.

Assessment of BBB breakdown

BBB breakdown analysis was performed, as described14. A 2% solution of Evans blue (Sigma- Aldrich, Saint Louis, MI, USA) in normal saline (4mL/kg of body weight) was injected intravenously 4h after stroke. Evans blue was allowed to circulate for 4h. Mice were then
perfused with 50ml of ice-cold PBS followed by 4% PFA. Brains were processed as described under "Histological analyses". Three 50µm sections at bregma region were photographed with a Leica DM5000B microscope followed by staining for MAP2 to reveal the stroke area. Mean fluorescence intensity was measured in the stroke area and compared the correspondent contralateral region. Sham operated mice were injected with 2% solution of Evans blue in normal saline (4 mL/kg of body weight) intravenously 4h after operation. Evans blue was allowed to circulate for 4h. Mice were then perfused with 50ml of ice-cold PBS followed by homogenization in 1 mL of 50% trichloroacetic acid (Sigma-Aldrich, Saint Louis, MI, USA) and centrifugation (20 min, 20000 rcf, 4°C). Evans blue stain was measured at 620 nm and quantified according to a standard curve. The results are presented as µg of Evans blue stain/g of tissue. For normalization of extravasated Evans blue to the stroke tissue, operated mice were injected with 2% solution of Evans blue in normal saline (4 mL/kg of body weight) intravenously 4h after operation. Evans blue was allowed to circulate for 4h. Mice were then perfused with 50ml of ice-cold PBS, followed by TTC staining for assessment of the stroke size. The sections were then homogenized in 1 mL of 50% trichloroacetic acid (Sigma-Aldrich, Saint Louis, MI, USA) and centrifuged (20 min, 20000 rcf, 4°C). Since TTC has an absorbance that overlaps with the absorbance of Evans blue, fluorescence intensities of Evans blue were measured (excitation wavelength 540nm, emission wavelength 600nm) and quantified according to a standard curve. Extravasated Evans blue was normalized to the contralateral hemisphere and to stroke volume. The results are presented as ng of Evans blue /mm³ of stroke tissue.

**Identification of infracted areas after stroke by TTC staining**

Mice were anesthetized with 5% isoflurane and euthanized by cervical dislocation. Brains were removed and cut coronally into 1 mm slices with a brain matrix (Braintree Scientific, Braintree, MA, USA) on ice. Brain sections were incubated in 2% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma- Aldrich, Saint Louis, MI, USA) in phosphate buffered saline for 20 min at room temperature to identify the stroke area. Pictures of the brains sections were documented with a scanner (Canon, Europark Fichtenhain, Germany).
## Antibodies reference list

### Antibodies used in flow cytometry:

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### Antibodies used for Western blot analyses:

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### Secondary antibodies for flow cytometry or immunohistochemistry, and Western blotting:

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<td>isotype</td>
<td>concentration catalog number supplier</td>
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<td>ProteinA horseradish-peroxidase labeled</td>
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<td>1:20000</td>
<td>NA9120-1ml GE Healthcare, Buckinghamshire, UK</td>
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<td>Anti-mouse AlexaFluor488 (used for hCEACAM1, hCD68)</td>
<td>Goat</td>
<td>IgG (H+L)</td>
<td>1:200 A11001 Life Technologies Ltd Darmstadt, Germany</td>
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<td>DAKO Liquid DAB+ Substrate</td>
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<td>K3467</td>
<td>Dako, Glostrup, Denmark</td>
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<td>eBioscience, San Diego, USA</td>
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<td>005-00-121</td>
<td>Dianova, Hamburg, Germany</td>
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**Antibodies used for neutrophil isolation:**

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<th>Concentration catalog number supplier</th>
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<td>F4/80</td>
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<td>isotype</td>
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<td>Streptavidin magnetic beads</td>
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Supplemental Figure legends

Supplemental Figure I. Characterization of the cerebral vasculature in Ceacam1<sup>−/−</sup> and WT mice. Anatomic organization of the cerebral vasculature was assessed macroscopically (A, B), by MRI (C, D), in immune fluorescence (E, F) and with laser Doppler (G, H). Plastinates of the cerebral vasculature of WT (blue) and Ceacam1<sup>−/−</sup> mice (red; (A, B)); an intact Circle of Willis was identified in all animals (n=3 per group), and the distribution of the MCA trunk and branch appeared to be anatomically unaltered in the different genotypes (asterisks; (A-D)). In (C, D) representative results from time-of-flight MR angiography (horizontal plane), are shown. Additionally, histological analyses showed similar cortical mean vessel density (E, F). Vessels were stained with anti-CD31-antibodies shown in green; nuclei were stained with DAPI; scale bar 50µm. Binding of the primary antibodies was visualized after incubation with the appropriate fluorescently labeled secondary antibodies; please refer to the Antibody reference list. Microvessel densities were 541±49.35 vessels/mm<sup>2</sup> vs. 534.5 vessels/mm<sup>2</sup> in WT and Ceacam1<sup>−/−</sup> mice, respectively. In all mice subjected to tMCAO, regional cerebral blood flow (rCBF) was measured with Laser Doppler (G, H). The decrease in rCBF was similar between Ceacam1<sup>−/−</sup> and WT mice, indicating effective occlusion of the MCA origin. Ten minutes after reperfusion rCBF was reconstituted to at least 60% of baseline levels and was unaltered between WT and Ceacam1<sup>−/−</sup> animals. Laser Doppler analyses during tMCAO produced the following relative perfusion rates after occlusion: 12.1 ± 2.4% of baseline level in WT mice vs. 10.2 ± 6.2% of baseline level in Ceacam1<sup>−/−</sup> mice; P>0.05; reperfusion: 74.8 ± 8.3 % of baseline level in WT mice and 77.3 ± 5.4% of baseline level in Ceacam1<sup>−/−</sup> mice; P>0.05. Data are shown as mean with 95% confidence intervals.

Supplemental Figure II. In the uninjured brain, CEACAM1 is mainly expressed on endothelial cells. CEACAM1 expression was analyzed by Western Blotting (A), and immunofluorescence (B-D). In sham operated mice, CEACAM1 expression was detectable in brain lysates, as well as on brain endothelial cells (BECs), as demonstrated by Western Blotting (A). Endothelial cells were purified from brains and analyzed by Western Blotting, antibody used: P1 (see antibody references). Histological analysis confirmed these results and showed CEACAM1 expression mainly on endothelial cells (B). In representative brain cross sections from Ceacam1<sup>−/−</sup> mice, no CEACAM1-expression is detected (C). In panel (D), lack of CEACAM1 expression on infiltrating CD11b positive cells is demonstrated in the ischemic hemisphere of a Ceacam1<sup>−/−</sup> mouse. Endothelia are stained with anti-CEACAM1-antibodies (shown in red) and anti-CD31 antibodies, (shown in green; (B,C)). Coexpression of CEACAM1 and CD31 is shown in yellow (B). CD11b expressing cells are labeled with anti-CD11b antibodies (shown in green; (D)). Binding of the primary antibodies was visualized after incubation with the appropriate fluorescently labeled secondary antibodies; see Antibody reference list. Nuclei are stained with DAPI. Scale bars: 50µm.

Supplemental Figure III. Postischemic angiogenesis and vascular remodeling are comparable in the different genotypes after tMCAO. Over a period of 14 days after tMCAO, vascular remodeling in WT and Ceacam1<sup>−/−</sup> mice was assessed with immunofluorescence (A-C) and steady-state susceptibility contrast enhanced MRI (ssCE-MRI) at day 14 (D-K). Vasculature was stained with anti-CD31 antibodies and AlexaFluor488-labelled anti-rat antibodies (A), representative histological stainings, CD31 expression is shown green; dashed lines define cortex and striatum, as indicated; scale bars: 50µm). Vascular density and vascular size were calculated for the cortex and striatum (A-C); n=3 per group). In agreement with the literature<sup>11</sup>, we observed an initial decrease in vascular density at days 1, 3 and 7 (A-B) and an increase in vessel perimeter (A,C). Beginning on day 7, highly angiogenic areas in the ipsilateral cortex were observed, with an increase in vascular density, whereas subcortical vascular density remained low (A,B). Interestingly, no differences were observed between WT and Ceacam1<sup>−/−</sup> mice. The different vessel densities are as follows: Sham striatal vessel density: 470±14.18 vessels/mm<sup>2</sup> vs 499±8.767 vessels/mm<sup>2</sup>; sham striatal average vessel perimeter 31.75±4.226 µm vs...
32.72±2.589 µm; sham cortical 499.7±9.812 vessels/mm² vs 501.3±16.37 vessels/mm²; sham cortical average vessel perimeter 31.18±4.240 µm vs 34.06±3.186 µm. **D1 striatal vessel density:** 477±19.57 vessels/mm² vs 479±19.91 vessels/mm²; D1 striatal average vessel perimeter 57.80±5.113 µm vs 60.90±1.798 µm; D1 cortical 380.3±59.09 vessels/mm² vs 398.5±59.99 vessels/mm²; D1 cortical average vessel perimeter 48.67±12.34 µm vs 54.77±8.001 µm. **D7 striatal vessel density:** 260±80.56 vessels/mm² vs 306.5±19.82 vessels/mm²; D7 striatal average vessel perimeter 57.41±6.834 µm vs 58.64±9.067 µm; D7 cortical 411.0±19.25 vessels/mm² vs 438.1±19.83 vessels/mm²; D7 cortical average vessel perimeter 45.12±10.24 µm vs 42.35±11.47 µm. **D14 striatal vessel density:** 278.5±37.54 vessels/mm² vs 274.7±11.42 vessels/mm²; D14 striatal average vessel perimeter 52.24±4.026 µm vs 48.35±4.024 µm; D14 cortical 514.0±16.39 vessels/mm² vs 529.4±27.18 vessels/mm²; D14 cortical average vessel perimeter 37.33±0.88 µm vs 41.06±4.087 µm.

To confirm these results, the vasculature was analyzed with ssCE-MRI at day 14 (n=5 per group). (D-G) display colored maps of ssCE-MRI (black coloring represents low values, red coloring represents high values; ∆R₂, ∆R₂* correspond to units sec⁻¹, Q represents units sec⁻¹/³). Calculations of each parameter are shown as ratios of [ipsilateral/contralateral] hemispheres (H-K). The ∆R₂ value expresses vessels with small diameter (e.g., capillaries), whereas the ∆R₂* value expresses vessels with a larger lumen. The ratio of changes in R₂ and R₂* directly relates to microvessel morphology. Findings of ssCE-MRI were consistent with our histological findings. Note that in both settings, increased cortical and decreased subcortical vessel densities are found. The ∆R₂* values (E, I) were increased in the lesion compared to the contralateral side, suggestive of hyperperfusion caused by improvement of collateral circulation and increase in vessel diameter. The ipsilateral cortical region revealed an increased ∆R₂ (D, H) and a high Q value (F, J) suggestive of enhanced microvascular blood flow and microvascular density, as found in histology. The ipsilateral subcortical region showed lower ∆R₂ and Q but high ∆R₂*/∆R₂ (G, K) values, suggesting decreased microvascular density but relatively high average vessel size.

**Supplemental Figure IV. Development of ADC and T2 lesions in WT and Ceacam1⁻/⁻ mice after 90, 270, 330, 450, 510 minutes and 24 hours.** Panel A illustrates the MRI patterns observed during reperfusion in two representative animals during the time course of 24h. Time points for each measurement are indicated. Panels B, C depict the changes in relative signal intensities in ADC and T2 values and Panels D,E the changes in lesion volumes on diffusion- and T2- weighted images after reperfusion (n=5 per group). Panels F, G show the analyses of diffusion- and T2- weighted images after 24 hours. In the first 270min, average ADC values (B) and signal intensities of T2 weighted images of the ischemic region (C) and lesion volumes (D, E) did not differ between WT and Ceacam1⁻/⁻ mice. After 5 hours of reperfusion, the appearance of the ischemic lesion changed substantially and differed between WT and Ceacam1⁻/⁻ mice with significant higher lesion volumes and signal intensities of T2 weighted images in Ceacam1⁻/⁻ mice, suggestive of increased vasogenic edema and BBB breakdown. Additionally, we reanalyzed the data from main Figure 2 (F,G) and found similar results with increased T2w-intensities in the stroke lesion of Ceacam1⁻/⁻ mice but similar ADC-values. Data are presented as mean with 95% confidence intervals; *P<0.05, **P<0.001, ***P<0.0001; data were evaluated with the Mann-Whitney-U test; each white and black symbol represent data from one WT or Ceacam1⁻/⁻ animal.

**Supplemental Figure V. Increased numbers of MMP-9⁺ neutrophils and MMP-9 levels in ischemic hemispheres of Ceacam1⁻/⁻ mice after 8h of reperfusion.** Neutrophils, identified by Ly-6G, and expression of the neuronal marker NeuN were examined in brain sections (A-C). Presence and distribution of neutrophilic granulocytes in the ischemic hemispheres was...
determined in immune fluorescence (B,C), and in peripheral blood by flow cytometry (D). MMP-9 contents were analyzed in protein lysates of the ischemic hemispheres 8h (E) and 24h (F) after induction of the ischemic stroke in zymography and Western Blotting. The stroke area was identified with NeuN staining (A, white/grey staining; scale bar 1mm). Absence or low intensity of NeuN staining represents areas with neuronal death. In these areas, MMP-9 positive neutrophils were identified by co-expression of MMP9 and Ly-6G ((B), MMP9 (red), Ly-6G (green), NeuN (grey), DAPI (blue); scale bars: 100µm) and quantified. Ceacam1⁻/⁻ mice showed increased neutrophil counts in the ischemic hemisphere compared to WT mice 8 h after stroke induction (C). As a control, neutrophil numbers were analyzed in peripheral blood (D) 8h after stroke but did not show differences between the genotypes. MMP-9 activity and protein levels were compared in protein lysates by zymography and Western blots (E) 8h after stroke. Data are presented as mean with 95% confidence intervals; *P<0.05, **P<0.001, ***P<0.0001; data were evaluated with the Mann-Whitney-U test; each white and black symbol represent data from one WT or Ceacam1⁻/⁻ animal.

Supplemental Figure VI. MMP-9 expression is not increased in sham operated mice and contralateral hemispheres of Ceacam1⁻/⁻ compared to WT mice. Zymography (A) and Western Blotting (B) of uninjured brain tissue (30µg) showed similar levels of MMP-9 in Ceacam1⁻/⁻ mice compared to the WT controls.
Supplemental references


encoded by the biliary glycoprotein gene, a member of the carcinoembryonic antigen gene family. *J Immunol.* 1993;150:4978-4984
A  CEACAM1 expression in sham operated mice

150 kD

100 kD

CEACAM1

actin

B

SHAM

CEACAM1

CD31

DAPI

WT

C

SHAM

CEACAM1

CD31

DAPI

Ceacam1

D  Negative control CEACAM1 expression 24h

CEACAM1

CD11b

DAPI

Ceacam1+/−

Ludewig et al., Supplemental Figure II
**A**

SHAM WT

WT D1 contralateral

SHAM Ceacam1<sup>−/−</sup>

Ceacam1<sup>−/−</sup> D1 contralateral

200 kDa

100 kDa

72 kDa

MMP-9 dimers

MMP-9

MMP-2

**B**

MMP-9-expression in contralateral brain lysates

WT

Ceacam1<sup>−/−</sup>

100kD

MMP-9

actin

Ludewig et al., Supplemental Figure VI