# CD69 plays a beneficial role in ischemic stroke by dampening endothelial activation

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## ABSTRACT

**Rationale:** CD69 is an immunomodulatory molecule induced during lymphocyte activation. Following stroke, T lymphocytes upregulate CD69 but its function is unknown.

**Objective:** We investigated whether CD69 was involved in brain damage following ischemic stroke.

**Methods and Results:** We used adult male mice on the C57BL/6 or BALB/c backgrounds, including wild type mice and $CD69^{-/-}$ mice, and $CD69^{+/+}$ and $CD69^{-/-}$ lymphocyte-deficient $Rag2^{-/-}$ mice, and generated chimeric mice. We induced ischemia by transient or permanent middle cerebral artery occlusion. We measured infarct volume, assessed neurological function, and studied CD69 expression, as well as platelet function, fibrin(ogen) deposition, and von Willebrand factor (vWF) expression in brain vessels and vWF content and activity in plasma, and performed the tail-vein bleeding test and the carotid artery $FeCl_3$-induced thrombosis model. We also carried out primary glial cell cultures, and sorted brain $CD45^-CD11b^-CD31^+$ endothelial cells for mRNA expression studies. We blocked vWF by i.v. administration of anti-vWF antibodies. $CD69^{-/-}$ mice showed larger infarct volumes and/or worse neurological deficits than the wild type mice after ischemia. This worsening effect was not attributable to lymphocytes or other hematopoietic cells. CD69 deficiency lowered the time to thrombosis in the carotid artery despite platelet function not being affected. Ischemia upregulated *Cd69* mRNA expression in brain endothelial cells. CD69-deficiency increased fibrin(ogen) accumulation in the ischemic tissue, and plasma vWF content and activity, and vWF expression in brain vessels. Blocking vWF reduced infarct volume and reverted the detrimental effect of $CD69^{-/-}$ deficiency.

**Conclusion:** CD69 deficiency promotes a pro-thrombotic phenotype characterized by increased vWF and worse brain damage after ischemic stroke. The results suggest that CD69 acts as a down-regulator of endothelial activation.

**Keywords:** Brain ischemia, CD69, endothelium, arterial thrombosis, von Willebrand factor

### Non-standard abbreviations and acronyms

MCA: middle cerebral artery
MCAo: middle cerebral artery occlusion
tMCAo: transient MCAo
pMCAo: permanent MCAo
TTC: 2,3,5-Triphenyl-tetrazolium chloride
CBF: cerebral blood flow
vWF: von Willebrand factor

## INTRODUCTION

CD69 is a type II transmembrane protein of the C-type lectin family.[1] CD69 expression increases in all bone marrow-derived leukocytes following cell stimulation.[1-3] CD69 is regarded as an immunomodulatory molecule.[4,5] In a previous study in lymphoid tissue of stroke patients and controls we detected enhanced CD69 expression in T cells after stroke,[6] but the functional meaning of this finding is unknown. CD69-deficient mice exhibit very few immune abnormalities under control conditions, suggesting that the function of CD69 is not essential or that it may be replaced by other molecules.[7] However, CD69-deficient mice showed enhanced inflammatory responses in models of allergic asthma, skin contact hypersensitivity,[8] collagen-induced arthritis,[9] NK-sensitive tumors,[10] and experimental autoimmune myocarditis.[11] In contrast, CD69-deficient mice were found to have less inflammation in models of dextran sulfate sodium-induced acute and chronic colitis,[12] cigarette smoke-induced pulmonary inflammation,[13] bleomycin-induced lung inflammation and fibrosis,[14] another study of collagen-induced arthritis,[15] and allergic airway inflammation,[16] and they were also protected from vaccinia virus infection.[17] These results suggest that the role of CD69 depends on the type of disease and the cell types involved, and it may also depend on the strain of CD69-deficient mice used.[5]

The first aim of this study was to investigate whether CD69 was involved in stroke outcome, and we examined this in both transient and permanent cerebral ischemia. In our study, CD69 deficiency increased brain damage after ischemic stroke, and worsened functional outcome. Furthermore, CD69 deficiency in immunodeficient mice ($Rag2^{-/-}$) also worsened the brain injury in relation to $CD69^{+/+}Rag2^{-/-}$ mice, thus illustrating a beneficial role of CD69 in ischemic stroke not related to lymphocytes. The results show that CD69 deficiency promotes a pro-thrombotic phenotype and suggests that CD69 acts as a negative regulator of endothelial activation following the ischemic insult.

## METHODS

An extended version of the Methods is provided in the Online Supplement.

*Animals*

We used $CD69^{+/+}$ wild type (wt) mice and $CD69^{-/-}$ mice on the C57BL/6 background.[7] A group of male $CD69^{+/+}$ and $CD69^{-/-}$ immunocompetent and lymphocyte deficient $Rag2^{-/-}$ mice were on the BALB/c background.[18] $CD69^{-/-}$ mice had been backcrossed on the C57BL/6 and the BALB/c backgrounds at least nine times.[17] Unless otherwise stated, the studies were performed in mice on the C57BL/6 background. Animal work was conducted with approval of the ethical committee of the University of Barcelona (CEEA) in compliance with the local regulations, the Spanish legislation (Real Decreto 53/2013), and the European Community Directives, and it is reported following the ARRIVE guidelines.

*Brain ischemia*

Transient occlusion of the right middle cerebral artery (tMCAo) was carried out in adult (3-4 month old) male mice. The MCA was occluded under isoflurane anesthesia using a monofilament, as reported.[19,20] Cerebral blood flow (CBF) was monitored with laser Doppler flowmetry (Perimed, AB, Järfälla, Sweden). Reperfusion was induced after 45, 60 or 85 min of arterial occlusion. For permanent ischemia (pMCAo), the right MCA was cauterized under isoflurane anesthesia, as reported.[21]

*Chimeric mice*

We generated chimeric mice by chemical ablation of the bone marrow of 2-month old male wt mice followed by transplantation of bone marrow from CD69$^{-/-}$ mice or wt mice, as described.[24] 45-min MCAo was carried out eight weeks after grafting.

*Infarct volume and neurological deficit*

Infarct volume was measured with MRI (T2 relaxometry maps) (7T, BioSpec, Bruker) or the 2,3,5-triphenyltetrazolium chloride (TTC) technique, as reported.[20] We assessed behaviour with different tests including the adhesion/removal tape test,[21] and the hanging wire test.[25] Various neurological score tests were used for the different experimental models, with higher scores indicating worse deficits.[19,20] The animals that died did not receive a score but mortality was compared between groups.

*Tail bleeding test*

Tail bleeding time measurements were performed in adult (3-4 month old) wt mice and CD69$^{-/-}$ mice, as described earlier.[26]

*Ferric chloride ($FeCl_3$)-induced murine model of carotid artery injury*

These studies were performed in adult (3-4 month old) male wt mice and CD69$^{-/-}$ mice, according to standardized protocols.[27,28] A neurological score[19] was performed 24 hours after surgery and then, mice were euthanized by cervical dislocation and the brain and carotid artery were removed and processed for histology.

*Brain cell isolation and flow cytometry*

Cell isolation from brain tissue was performed as described.[29] Isolated cells were incubated at 4 ºC for 10 min with FcBlock (1:200; Clone 2.4G2; BD Pharmingen) followed by primary antibodies (BD Pharmingen): rat anti-mouse CD11b (clone M1/70, APC-Cy7), CD45 (clone 30-F11, FITC), CD3 (clone 17A2, PE), CD45R (clone RA3-6B2, V450), and Ly6G (clone 1A8, PE-Cy7). Data was acquired on a BD FacsLSRII cytometer using the FacsDiva software (BD Biosciences). Data analysis was performed with FlowJo software (version 7.6.5, TreeStar Inc., Ashland, OR, USA).

*Immunofluorescence*

Mice were perfused through the heart with heparinized saline followed by 4 % PFA and the brain was cryoprotected, frozen, cut in a cryostat and processed for immunofluorescence with a sheep polyclonal antibody against von Willebrand Factor (vWF) (#ab11713, Abcam) and rabbit polyclonal antibodies against ionized calcium-binding adapter molecule-1 (Iba-1) (#016-20001, Wako Chemicals, Neuss, Germany), both diluted 1:100, or fibrin(ogen) (#A0080, DAKO) diluted 1:2,000, followed by secondary antibodies (Alexa Fluor®; Molecular Probes; Life Technologies). Images were

obtained in a confocal microscope (TCS SPE-II, Leica Microsystems) with LAS software (Leica).

*Treatments*
Mice received i.v. administration of 125 μg anti-vWF antibody (#A0082, DAKO)[22,23] or corresponding immunoglobulins (#A903, DAKO) 10 min after pMCAo. Treatment was randomly allocated and administration and outcome measures were carried out in a blinded fashion.

*Histology*
Mice were perfused through the heart with heparinized saline and 4% PFA. Carotid arteries were embedded in paraffin and 5-μm thick coronal sections were cut on a microtome and processed with the Carstairs' method for fibrin and platelet staining.

*Platelet activation and flow cytometry*
Platelets were isolated from the blood of the vena cava collected in citrate dextrose from anesthetized wt and $CD69^{-/-}$ mice under basal conditions and 6 and 24 hours after tMCAo. The blood was then diluted 1:1 in Hepes-Tyrode's Buffer (HTB). The platelet rich plasma (PRP) was processed as reported.[30] PRP was stimulated with either 0.02 or 0.05 IU/mL of thrombin (Chrono-Long) and incubated with the following primary antibodies: rat anti-mouse CD62P (clone Wug.E9, FITC; Emfret Analytics), CD42b (clone Xia.G5, PE; Emfret Analytics), CD41 (clone MWreg30, PE-Cy7; Biolegend), and integrin αIIbβ3 (clone JON/A, PE; Emfret Analytics), and armenian hamster anti-mouse CD61 (clone 2C9.G2, Alexa Fluor 647; Biolegend). Data was acquired on a BD FacsLSRII cytometer using the FacsDiva software (BD Biosciences) and was analyzed with FlowJo software as above.

*Quantification of von Willebrand factor (vWF):antigen (Ag)*
Determination of vWF:Ag was carried out by capture ELISA with a rabbit polyclonal IgG to vWF (Dako North America Inc, Carpinteria, CA, USA) diluted in 0.05 mol/L carbonate buffer pH 9.6. Calibration curves were constructed with decreasing dilutions of standard plasma (Control Plasma N, Siemens Corporation, Washington D.C., USA). Samples were incubated with a peroxidase-conjugated rabbit anti-human vWF IgG (Dako North America Inc, Carpinteria, CA, USA) (162 ng/well) and the reaction was developed with 3,3',5,5'-tetramethylbenzidine (TMB, 1-step Ultra TMB-ELISA, Thermo Scientific, Rockford, IL, USA) during 20 min. The reaction was stopped by adding 100 μL of 2M $H_2SO_4$ to each well, and was read at 450 nm wavelength.

*Determination of vWF collagen binding activity (vWF:CBA).* Measurements of vWF:CBA were performed following a reported technique.[31]

*Quantitative vWF multimer analysis.*
Multimeric structure was studied by discontinuous SDS-agarose gel electrophoresis as previously reported.[32]

*Cell cultures*
Primary microglial and astroglial cell cultures were obtained from newborn mice as reported.[33]

*Fluorescence activated cell sorting (FACS) of endothelial cells and gene expression*
$CD45^-CD11b^-CD31^+$ brain endothelial cells were sorted using Aria II cell sorter (BD Biosciences) with the following antibodies: CD31 (clone 390, PE-CY7, eBiosciences), and CD45 and CD11b (as above). Purity of the sorted endothelial cells was checked by flow cytometry (95±2%, mean±SD, n=4) (Supplementary Fig. S1). In independent experiments, sorted cells were collected in RNAse-free PBS, and the total RNA was extracted with the PureLink™ RNA Micro Scale Kit (Invitrogen) for qRT-PCR analysis.

*Statistical analyses*
Two-group comparisons were carried out using a two-tailed Mann–Whitney test or *t*-test, as required after testing for normality. Multiple groups were compared with a one-way ANOVA or Kruskal-Wallis test, or a two-way ANOVA followed by post-hoc analysis. The specific tests used in each experiment are stated in the Figure Legends.

## RESULTS

### CD69 deficiency worsens stroke outcome

$CD69^{-/-}$ mice showed larger lesion volumes following transient ischemia compared to wt mice, as assessed by T2*w* MRI at several time points after occlusion of the MCA for different periods of time (Fig. 1A,B, Supplementary Fig. S2). Accordingly, $CD69^{-/-}$ mice showed more weight loss and had worse neurological deficits (Fig. 1A,B, Supplementary Fig. S2A,B). These effects were not accompanied with greater blood-brain barrier (BBB) breakdown at reperfusion in $CD69^{-/-}$ mice as assessed by Evans blue extravasation at different time points after tMCAo (Supplementary Fig. S2C). We did not find differences in mortality between groups after tMCAo (11% in the wt group and 9% in the $CD69^{-/-}$ group). We then tested the effect of CD69 deficiency after permanent ischemia (pMCAo) by carrying out longitudinal T2*w* MRI studies at days 1, 4 and 7 post-ischemia. Group differences in lesion volume did not reach statistical significance but the $CD69^{-/-}$ mice showed a worse performance in the adhesion/removal tape test at day 7 (Fig. 1C). pMCAo in mice on the BALB/c background showed slightly larger lesion volumes in the $CD69^{-/-}$ group (Fig. 1D). There were no differences between genotypes in systolic blood pressure measured by tail-cuff plethysmography (mean±SD, 100.0±3.3 mmHg n=6 wt mice, and 103.7±5.4 mmHg n=5 $CD69^{-/-}$ mice, Mann-Whitney test p=0.329). Likewise, there were no significant differences in basal cerebral blood flow determined by arterial spin labelling MRI (Supplementary Fig. S3). Collectively, these findings suggested that CD69 exerts beneficial effects in brain ischemia.

### Despite increased CD69 expression in lymphocytes after ischemia, the exacerbated brain damage in CD69 deficiency is not attributable to lymphocytes

tMCAo (85-min MCAo) reduced the numbers of lymphocytes in the blood at 4 days (Fig. 2A). $CD69^{-/-}$ mice had a slightly higher proportion of T cells and lower proportion of B cells in the spleen and CLN under basal conditions, but the differences were maintained after ischemia (Fig. 2A). In the brain tissue, the $CD69^{-/-}$ mice tended to show higher infiltration of T cells and myeloid cells 4 days after tMCAo compared to the wt mice, but

differences were not statistically significant. However, CD69 deficiency caused a significant increase in the numbers of $Ly6G^+$ neutrophils while other myeloid cell populations remained unaffected (Fig. 2B). In the pMCAo model, the proportion of T cells in the CLN of wt mice 7 days after MCAo and controls was inversely correlated with the size of the brain lesion at 24 hours, as assessed by MRI (Fig. 2C). The proportion of $CD69^+$ T cells in the CLN increased 7 days after pMCAo versus control, suggesting that ischemia increased the percentage of activated T cells despite reducing the T cell population (Fig. 2C). To test the role of CD69 expression in lymphocytes, we induced pMCAo in lymphocyte deficient $Rag2^{-/-}$ BALB/c mice that were either $CD69^{+/+}$ or $CD69^{-/-}$. In these mice, lesion volume was larger in the absence of CD69 (Fig. 2D), suggesting that CD69 deficiency exerted detrimental effects in the ischemic brain independently of lymphocytes.

**CD69 expression in hematopoetic cells does not explain the worsening effect of CD69 deficiency in brain ischemia**

The vast majority of research performed on CD69 has focused on its effects on the immune system. However, CD69 is also constitutively expressed in different cells, including platelets.[2,7,34] To test whether CD69 deficiency might affect platelet function, we isolated platelets from wt and $CD69^{-/-}$ mice under basal conditions and 6 and 24 hours following tMCAo and examined platelet activation induced by thrombin, as assessed by measuring the expression of P-selectin (CD62P) and $\alpha$IIb$\beta$3 (JON/A) by flow cytometry. In these *ex vivo* experiments, we could not find differences in the response to thrombin between genotypes in any of the experimental conditions (Fig. 3A and Supplementary Fig. S4). We then examined *in vivo* whether CD69 deficiency affected the bleeding time using the tail-bleeding test. Total bleeding time tended to be lower in the $CD69^{-/-}$ mice, and these mice showed significantly less re-bleedings than the wt mice (Fig. 3B).

To further elucidate the role of hematopoietic versus non-hematopoietic CD69 expression in ischemic brain damage, we generated chimeric mice by transplanting bone marrow of either $CD69^{-/-}$ mice or wt mice to recipient wt mice. CD69 deficiency restricted to hematopoietic cells did not worsen the lesion volume, the neurological score, or the loss of body weight 24h after tMCAo (Fig. 3C). Therefore, CD69 deficiency in blood cells did not explain the worsening effects of total CD69 deficiency in ischemic brain damage, suggesting that non-hematopoietic CD69 expression may play a role.

**The worse stroke outcome under $CD69^{-/-}$ deficiency is not attributable to pro-inflammatory mediators**

Ischemia induced the expression of CD69 mRNA in brain tissue (Fig. 4A). To find out whether CD69 is induced in glial cells, we carried out glial cell cultures and exposed them to LPS or IL-4. LPS upregulated the expression of CD69 in microglia and, to a lesser extent, astrocytes (Fig. 4A). CD69 expression was also upregulated after exposure of glial cells to IL-4 (Fig. 4A), suggesting that CD69 is a global marker of microglial cell activation. Microglial cell cultures obtained from $CD69^{-/-}$ mice showed an enhanced production of pro-inflammatory mediators after exposure to LPS (Fig. 4B) indicating that CD69 exerted some negative regulation of inflammation. However, this effect was not

observed after exposing microglial cells to a transient episode of oxygen deprivation, which induced a milder inflammatory response compared with LPS (Fig. 4C). Furthermore, the $CD69^{-/-}$ mice did not over-express pro-inflammatory mediators in relation to the wt mice following tMCAo, at least at the time points that we examined (Fig. 4D). These findings suggested that enhanced brain inflammation was not the main determinant of the detrimental effect of CD69 deficiency following brain ischemia.

**CD69-deficiency promotes arterial thrombus formation**

CD69 may play a role in the activated endothelium since CD69 expression is upregulated in endothelial cells exposed to thrombin.[35] We used the carotid artery ferric chloride-induced thrombosis model, an *in vivo* assay based on redox-induced endothelial damage that recapitulates vascular injury and platelet activation and aggregation. CD69 deficient mice formed thrombi faster and showed a higher total time in occlusion than the wt mice (Fig. 5A). Moreover, $FeCl_3$-induced carotid artery thrombosis had worse neurological consequences in $CD69^{-/-}$ mice than in the wt mice, as assessed with a neurological score 24 hours after $FeCl_3$ application (Fig. 5A), even though we did not detect signs of neuronal death at this time point (Supplementary Fig. S5). We assessed possible differences in endothelial function in $CD69^{-/-}$ mice by studying carotid artery reactivity by myography. Contractile responses to KCl, and relaxation responses to acetylcholine and NO-donor sodium nitroprusside were similar in wild type and $CD69^{-/-}$ mice (Supplementary Fig. S6), indicating that CD69-deficiency did not alter smooth muscle contraction or endothelium-dependent and -independent relaxation.

Together these results show that CD69-deficiency causes the mice to be prone to arterial thrombosis. Given that we were unable to find alterations in thrombin-induced platelet activation in prior experiments, it is possible that the endothelium of $CD69^{-/-}$ mice was involved in their exacerbated response to brain ischemia. Ischemia induced fibrin(ogen)$^+$ blood vessels and fibrin(ogen) extravasation to the brain parenchyma (Fig. 5B). Notably, brain areas showing fibrin(ogen) extravasation were similar in wt and $CD69^{-/-}$ mice, which also showed similar levels of albumin in the brain tissue (Fig. 5C) indicating that CD69-deficiency did not promote further BBB breakdown. However, $CD69^{-/-}$ mice had more fibrin(ogen)$^+$ vessels (Fig. 5B) resulting in increased tissue fibrin(ogen) (Fig. 5C) and fibrin (Suppl. Fig. S7) deposition, and higher plasma fibrinogen levels (Fig. 5E). We then isolated $CD45^-CD11b^-CD31^+$ endothelial cells from the brain of wt mice and $CD69^{-/-}$ mice by fluorescence activated cell sorting (FACS) and obtained mRNA from the sorted cells for gene expression studies. Wt endothelial cells showed low *Cd69* mRNA expression that significantly increased 3 hours post-ischemia, before mRNA induction of inflammatory molecules, like *Vcam1* (Fig. 5F). This result suggested a possible role of CD69 in the vasculature.

**Detrimental effects of vWF in CD69 deficient mice**

Thrombus formation involves the interaction of platelets with the damaged endothelium through exposure of vWF.[36] Notably, we found that the plasma of $CD69^{-/-}$ mice contained more vWF antigen and showed higher binding of vWF to collagen under control

conditions and after pMCAo (Fig. 6A). However, the ratio of vWF antigen/collagen binding only showed a trend to increase in the CD69 deficient mice versus the wt mice (Fig. 6A, Supplementary Fig. S8A-C) and the formation of plasma vWF multimeric complexes (Fig. 6B) and the liver expression of the vWF degrading enzyme ADAMST13 (Supplementary Fig. S8D) were not significantly affected, showing that there was not a systemic defect in vWF processing. The main producer of plasma vWF is the endothelium.[37,38] We found a higher expression of *vWF* mRNA in $CD31^+$ endothelial cells FACS-sorted from the brain of $CD69^{-/-}$ mice than wt mice (Fig. 6C). Accordingly, there was more vWF protein in the cerebral blood vessels of $CD69^{-/-}$ mice than in the wt mice (Fig. 6D). To underscore whether vWF was involved in the exacerbated ischemic brain damage in $CD69^{-/-}$ mice, we administered anti-vWF blocking antibody or control antibody i.v. 10 min after pMCAo to wild type and $CD69^{-/-}$ mice. Anti-vWF antibody reduced lesion volume and prevented the detrimental effect of CD69 deficiency, since the groups of mice that received the anti-vWF antibody showed a similar mean lesion size regardless of the genotype (Fig. 6E). Therefore, our results suggest that CD69 might exert some negative regulatory function on endothelial cell activation and vWF production.

## DISCUSSION

CD69 is a marker of lymphocyte activation and has immunomodulatory functions, but various cell types can express this molecule. We show here that CD69 deficiency increases acute stroke brain damage by promoting a pro-thrombotic phenotype mediated by the increased production of vWF by endothelial cells. In our study, CD69 deficiency worsened the outcome of ischemic stroke. The detrimental effect of CD69 deficiency was not mediated by lymphocytes since CD69 deficiency increased lesion size in lymphopenic $Rag2^{-/-}$ mice compared to $CD69^{+/+}$ $Rag2^{-/-}$ mice. In addition to the important functions of CD69 in lymphocytes, various cells express CD69, including other leukocytes, platelets,[2,34] and activated endothelial cells.[35] CD69 deficiency reduced re-bleeding and the time to thrombosis in the carotid artery $FeCl_3$-induced thrombosis model. Ischemic stroke and reperfusion induces platelet activation and adherence to the cerebral endothelium, which promotes thrombus formation.[39] Because of the expression of CD69 in platelets, we first argued that CD69 deficiency in platelets might alter platelet function. However, we could not detect changes in platelet activation in the absence of CD69. Furthermore, experiments with chimeric mice reconstituted with either wt or $CD69^{-/-}$ bone marrow showed that the absence of CD69 in blood cells could not reproduce the enhanced ischemic damage observed in the $CD69^{-/-}$ mice.

Thrombus formation requires the interaction of platelets with the damaged endothelium. Endothelial activation induces up-regulation of CD69 expression,[35] but the function of CD69 under these conditions is currently unknown. We report that cerebral ischemia increased *Cd69* mRNA expression in brain endothelial cells. Furthermore, CD69 deficiency promoted fibrin(ogen) deposition in the ischemic brain vessels. These results suggested that lack of CD69 favored microvascular thrombosis. After severe ischemic conditions, recanalization of the occluded vessels does not ensure complete reperfusion since microthrombi can impair microvascular reperfusion.[40] However, CD69-deficient

mice did not display more BBB leakage, possibly because microthrombi sealed the affected vessels.

CD69 deficiency also increased the plasma levels of vWF antigen and vWF binding to collagen. vWF is synthesized in endothelial cells and megakaryocytes and is stored in the Weibel-Palade bodies and α-granules, respectively. It is secreted constitutively from endothelial cells, and upon activation of endothelial cells and platelets.[38,41] Endothelial damage exposes vWF on the endothelial surface that mediates platelet adhesion promoting platelet activation, leukocyte adhesion and thrombosis. Interestingly, plasma vWF is almost completely derived from endothelial cells,[37] and its levels increase following endothelial damage, so plasma vWF is considered a marker of endothelial damage or dysfunction.[42] In the present study, higher vWF expression was found in the brain vessels of the ischemic CD69$^{-/-}$ mice than the wt mice, suggesting that CD69 acts as a negative regulator of endothelial activation. Hypoxia induces the release of vWF by promoting exocytosis of endothelial cell Weibel-Palade bodies facilitating neutrophil recruitment.[43] Accordingly, in our study we found increased neutrophil infiltration in the ischemic tissue of CD69-deficient mice. vWF KO mice have been reported to have reduced infarct volume and increased functional outcome after cerebral ischemia/reperfusion. The reconstitution of the plasma with vWF returned infarct and outcome to baseline levels confirming that plasma vWF is important in ischemic brain damage.[44-46]

vWF is a highly glycosylated protein and its carbohydrate structures are involved in vWF binding to platelets.[47] The interaction between vWF and GPIbα is crucial in the initial stages of platelet adhesion to the endothelium under high-shear flow[48] as well as playing a role in platelet-platelet adhesion.[49] When vWF KO mice were supplemented with vWF protein deficient in binding to either collagen or GPIbα, infarct size and functional outcome remained the same as in the KO mice, suggesting that the interactions between vWF and both collagen and GPIbα are crucial in the pathogenesis of focal ischemic stroke.[46] In agreement with these findings, complete blockade of the vWF binding site of GPIbα before ischemia lead to a significant reduction in ischemia lesion volume and an improvement in functional outcome 24 hours after tMCAo, and this was still effective when treatment was provided 1 hour after MCAo.[50] Furthermore, blockade of the GPIbα-vWF interaction caused reperfusion of a photochemically-induced MCAo model and reduced brain infarct size in guinea pigs,[51] and restored vessel patency after occlusive thrombosis in a MCAo model in mice.[52] In humans, a positive association between high vWF levels and ischemic stroke has been found.[53-55] Furthermore, various vWF polymorphisms have been identified that are associated with an increased risk of stroke,[56,57] and some platelet glycoprotein Ibα (GPIbα) polymorphisms that may increase its interaction with vWF, increase the risk of stroke.[58,59] Therefore, vWF is regarded as a potential target for stroke therapy.[60] In our study, blocking vWF reverted the detrimental effect of CD69-deficiency demonstrating the participation of vWF in the exacerbated ischemic brain damage found in CD69-deficient mice. Carbohydrate structures on the vWF are recognized by the S-type lectins, galectins,[61] which have been reported to impair vWF-platelet string formation.[62] Of note, galectin-1 has been identified as a ligand of CD69.[63] These findings suggest a potential molecular link between CD69 and vWF

mediated by galectin-1. However, demonstration of the mechanistic pathway connecting CD69 signalling with specific prothrombotic pathways will require further investigation.

Limitations of our study include that we measured endothelial function in the carotid artery rather than in brain vessels, so we cannot readily extrapolate on brain microvascular endothelial function based on the results obtained in a different vascular bed. Also, results will require validation in female mice due to sex differences in the response to stroke,[64] and studies of CD69 in aged animals with risk factors would stress the potential relevance of our findings in a more realistic stroke context. The vasculature suffers alterations with aging and co-morbidities, such as hypertension and diabetes, involving endothelial dysfunction and vascular remodeling, amongst other changes.[65] Further investigation is required to find out whether CD69 might be a druggable target with a relevant therapeutic window for the treatment of acute ischemic stroke or for primary/secondary prevention of thromboembolic events.

Altogether, our findings show that CD69 deficiency worsens stroke outcome by enhancing the endothelial release of vWF and favouring arterial thrombosis that may secondarily exacerbate ischemic brain damage. The results suggest that CD69 acts as a negative regulator of endothelial cell activation after ischemic stroke.

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**Disclosures**

None

**Data, Materials, and Code Disclosure Statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## Figures

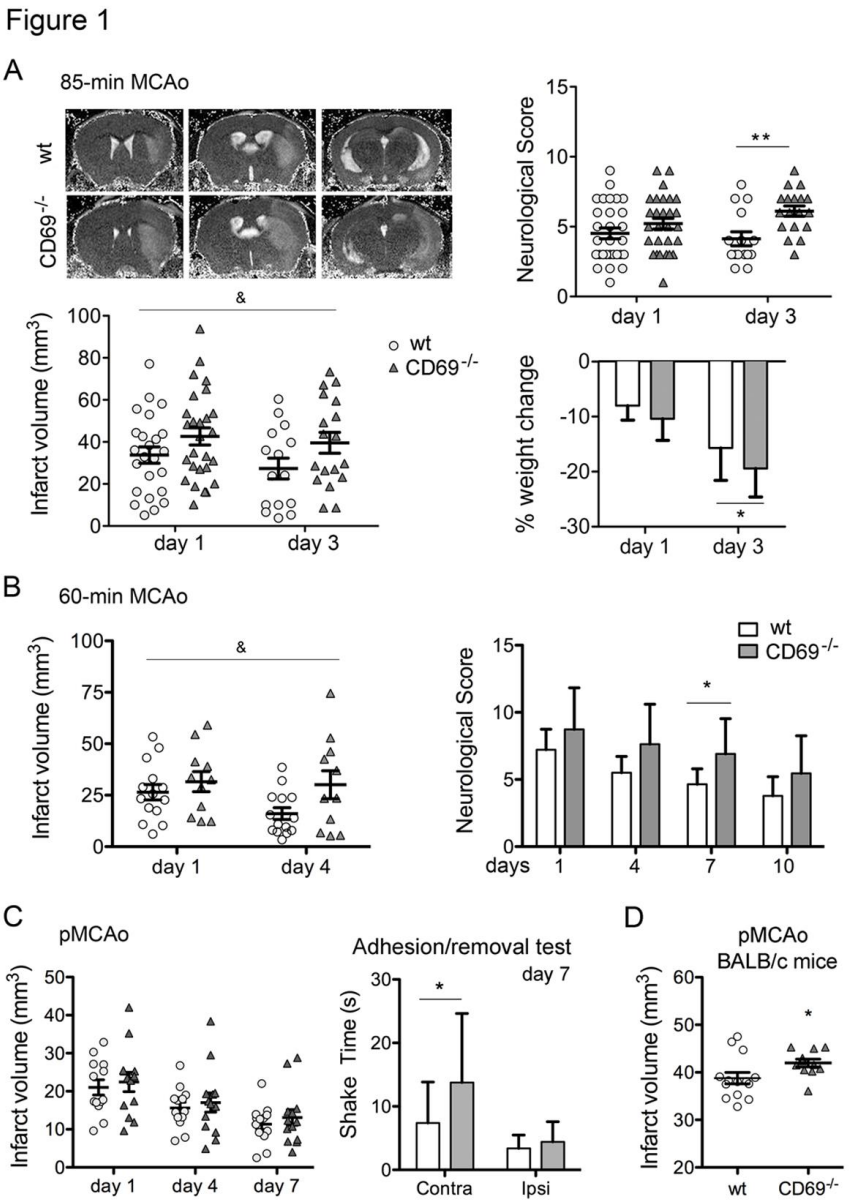


**Figure 1. CD69 deficiency worsens brain damage after ischemia.** Outcome of transient ischemia (tMCAo) (A-B) and permanent ischemia (pMCAo) (C) in wild type mice (wt) and CD69$^{-/-}$ mice. Brain lesion volume (T2*w* MRI). A,B) Transient ischemia was induced by either 85-min MCAo (A) or 60-min MCAo (B). A) Representative brain MRI of wt mice (n=24) and CD69$^{-/-}$ mice (n=27) 1 day post-ischemia. A subgroup of wt mice (n=15) and CD69$^{-/-}$ mice (n=18) was studied again by MRI at day 3. Infarct volume was larger in the CD69$^{-/-}$ group ($^{\&}$ genotype effect $p<0.05$). The neurological score was worse in CD69$^{-/-}$ mice (genotype effect $p<0.005$), and the change in body weight was more pronounced in CD69$^{-/-}$ mice (genotype effect $p<0.002$). B) An independent group of wt mice (n=14) and CD69$^{-/-}$ mice (n=11) received 60-min MCAo and was subjected to MRI and neurological testing at several time points post-ischemia. CD69-deficiency increased lesion volume ($^{\&}$ genotype effect $p<0.05$) and worsened the neurological score (genotype effect $p<0.03$). C) Lesion volume after pMCAo in wt mice (n=13) and CD69$^{-/-}$ mice (n=13) showed no statistically significant differences, but the behavioral performance in the adhesion/removal tape test showed worse time to shake in the contralateral (Contra), but not the ipsilateral (Ipsi), paw of the CD69$^{-/-}$ group at day 7. D) In BALB/c mice, lesion volume 1 day after pMCAo was slightly

larger in the CD69$^{-/-}$ (n=11) than the wt (n=14) group (two-tailed *t*-test, * $p<0.05$). Multiple group comparisons were conducted with a two-way ANOVA, by genotype and either time point or body side. Specific significant differences between groups according to post-hoc analysis are indicated by *$p<0.05$ and **$p<0.01$. Bar graphs show the group mean ± SD.

Figure 2

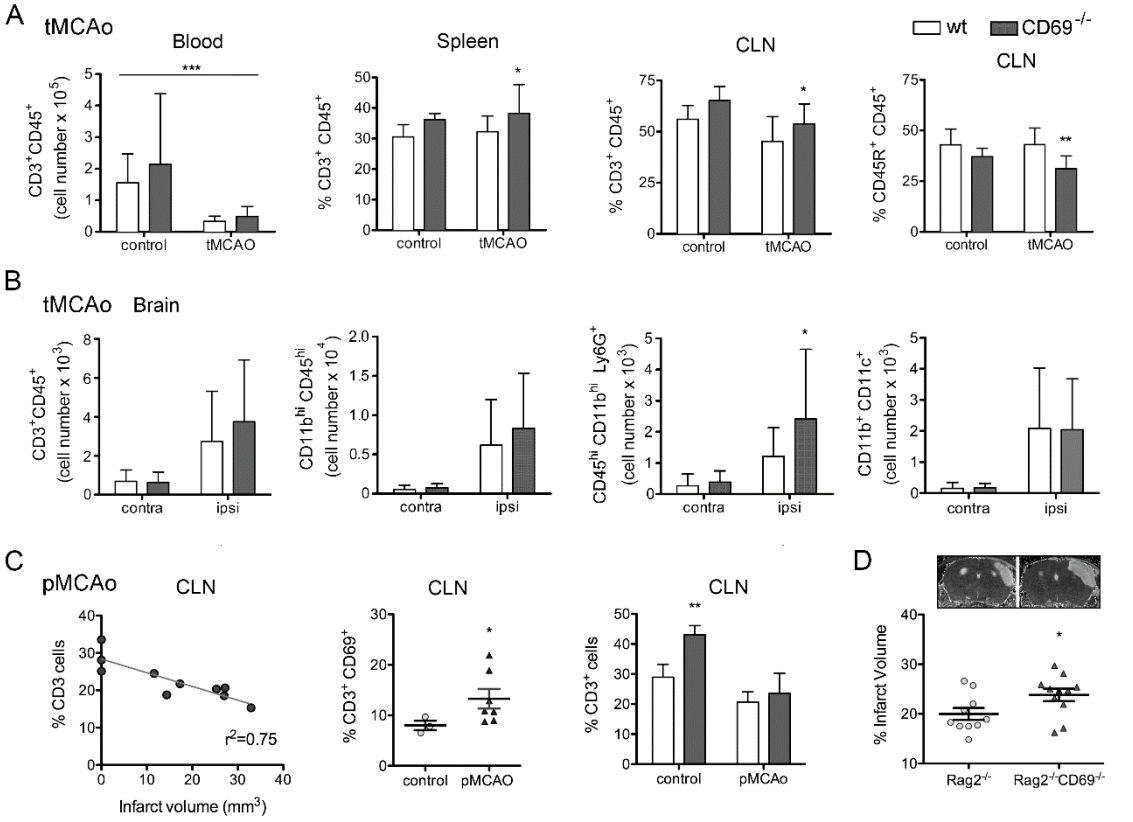


**Figure 2. The worsening effect of CD69-deficiency is not attributable to lymphocytes.** A) Flow cytometry analysis of peripheral lymphocytes in control wild type (wt) mice (n=7-8) and CD69$^{-/-}$ mice (n=7), and 4 days after 85-min ischemia (tMCAo) in wt mice (n=10-13) and CD69$^{-/-}$ mice (n=11-17). Ischemia causes a similar degree of lymphopenia ($p<0.001$) in both genotypes. CD69$^{-/-}$ mice show a higher proportion of CD3$^{+}$ T cells in the spleen (genotype effect $p<0.01$) and cervical lymph nodes (CLN) (genotype effect $p<0.01$), and a lower percentage of CD45R$^{+}$ B cells in the CLN (genotype effect $p<0.05$) (Two-way ANOVA by genotype and condition (control vs. tMCAo) followed by the Bonferroni test that highlights specific group differences indicated by * $p<0.05$ and ** $p<0.01$). B) Flow cytometry analysis of immune cells in the contralateral and ipsilateral brain hemispheres 4 days after 85-min MCAo in wt mice (n=13) and CD69$^{-/-}$ mice (n=15). Ischemia increases all the studied cell populations in the ipsilateral ischemic (Ipsi) hemisphere versus the contralateral (Contra) hemisphere of mice of both genotypes ($p<0.001$), but the CD69$^{-/-}$ mice show more infiltrating Ly6G$^{+}$ neutrophils in the ipsilateral hemisphere than the wt mice (*$p<0.05$) (Two-way ANOVA by genotype and brain hemisphere followed by the Bonferroni test). C) The extent of T cell reduction in the CLN at day 7 after pMCAo correlated with the volume of the brain lesion at 24h (T2*w* MRI). T cells in the CLN of naïve mice (n=3) and 7 days after pMCAO (n=7) were studied by flow cytometry. $r^2$ indicates the goodness of the fit in linear regression analysis. pMCAo in wt mice increases the proportion of CLN CD69$^{+}$ T cells vs. control naïve mice (Mann-Whitney test $p<0.05$) at day 7. CD69$^{-/-}$ mice show a higher proportion of T cells in the CLN vs. wt mice (n=3 per group) (*$p<0.01$), but pMCAo (n=9-15) reduces the percentage of T cells in both genotypes (Two-way ANOVA by genotype and condition). D) CD69-deficiency worsens the brain lesion independently of lymphocytes since pMCAo in lymphocyte-deficient Rag2$^{-/-}$ mice on the BALB/c background that were either CD69$^{+/+}$ (n=10) or CD69$^{-/-}$ (n=11) induced larger lesions (T2*w* MRI at 24h) in the latter group (Mann-Whitney test, $p<0.05$). Bar graphs show the group mean±SD.

Figure 3

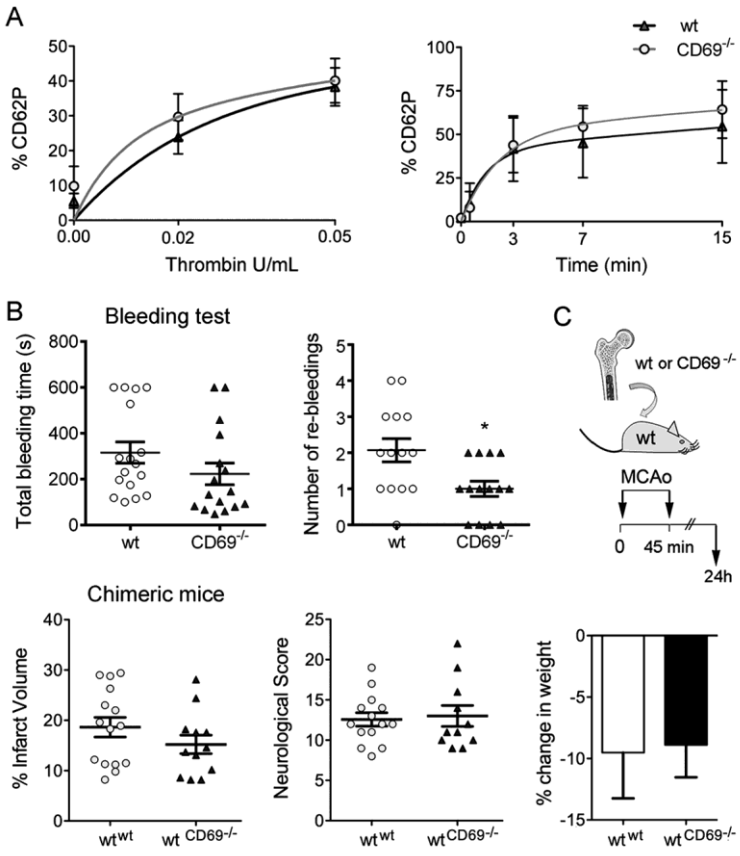


**Figure 3. The worsening effect of CD69-deficiency is not attributable to hematopoietic cells**. A) Platelets isolated from blood of wt mice (n=6) and CD69$^{-/-}$ mice (n=6) 6h post-ischemia (45-min MCAo) were stimulated *ex vivo* with several concentrations of thrombin for 15 min and at different time points after stimulation with 0.02 U/mL thrombin. Platelet activation was assessed by measuring the % of P-selectin (CD62P)$^{+}$ platelets by flow cytometry. There were no significant differences between wt and CD69-deficient platelets. B) For an *in vivo* functional assay, we carried out the tail vein bleeding test in wt and CD69$^{-/-}$ mice (n=16-17 mice per group). The total bleeding time tended to be lower (p=0.07) and the number of re-bleedings was significantly inferior (p<0.05) in the CD69$^{-/-}$ mice than in the wt mice (Mann-Whitney test). C) Chimeric mice were generated by transplanting bone marrow of CD69$^{-/-}$ donor mice to wt recipient mice (n=12). Controls were recipient wt mice that received bone marrow of different wt donor mice (n=15). After 45-min MCAo, lesion volume, neurological score, and body weight (mean±SD) at 24h showed no significant differences between these chimeric groups.

Figure 4

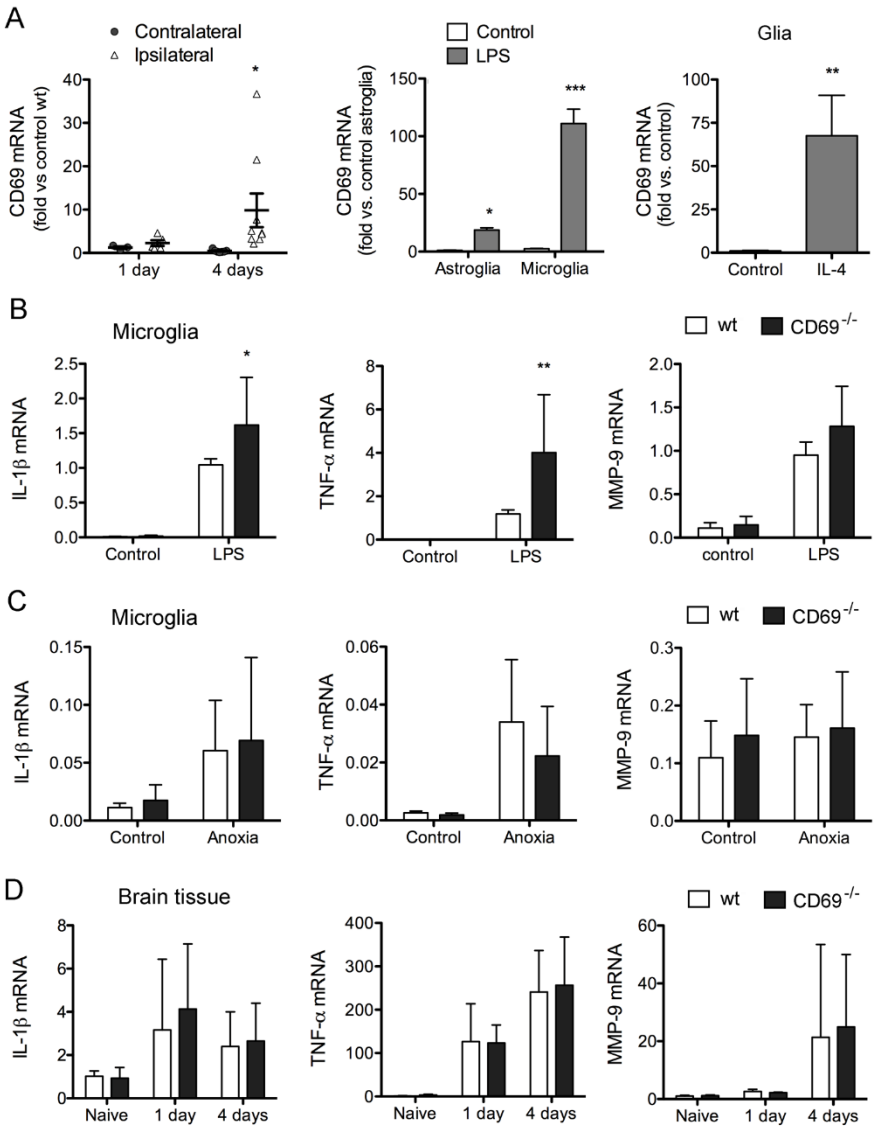


**Figure 4. CD69 mRNA and cytokine mRNA expression in the brain after ischemia and in vitro in glial cells.** A) Ischemia (45-min MCAo) up-regulates the cerebral expression of CD69 mRNA in the ipsilateral versus the contralateral hemisphere at 4 days (two-way ANOVA by region and time point, n=5-9). Cultured astroglial and microglial cells up-regulate the expression of CD69 mRNA after exposure to LPS (10 ng/mL) for 4 hours (n=3 replicates in a representative example of three independent experiments; two-way ANOVA by cell type and condition). Exposure to IL-4 (50 ng/mL) for 4 hours also induced the expression of CD69 mRNA in mixed glial cells (mean of two independent experiments with n=3 replicates each; Mann-Whitney test). B,C) Cultures of $CD69^{-/-}$ microglia (three cultures with n=2-3 replicates each) and wt microglia (two cultures with n=3-4 replicates each) exposed either to LPS (10 ng/mL) for 6 hours (B) or to three-hour oxygen deprivation followed by 3 hours of normoxia (C) showed higher mRNA induction of IL-1β, TNF-α, and MMP-9 versus the control condition. Compared to wt microglia, $CD69^{-/-}$ microglia showed higher induction of IL-1β mRNA and TNF-α mRNA after LPS but not after anoxia (two-way ANOVA by genotype and condition). D) Cerebral mRNA expression of IL-1β, TNF-α, and MMP-9 increased after ischemia (45-min MCAo) in the ipsilateral versus the contralateral hemisphere but we could not detect differences between genotypes at the indicated time points (n=5-9 per group, two-way ANOVA by genotype and time point). *p<0.05, **p<0.01. Bar graphs show the group mean±SD.

Figure 5

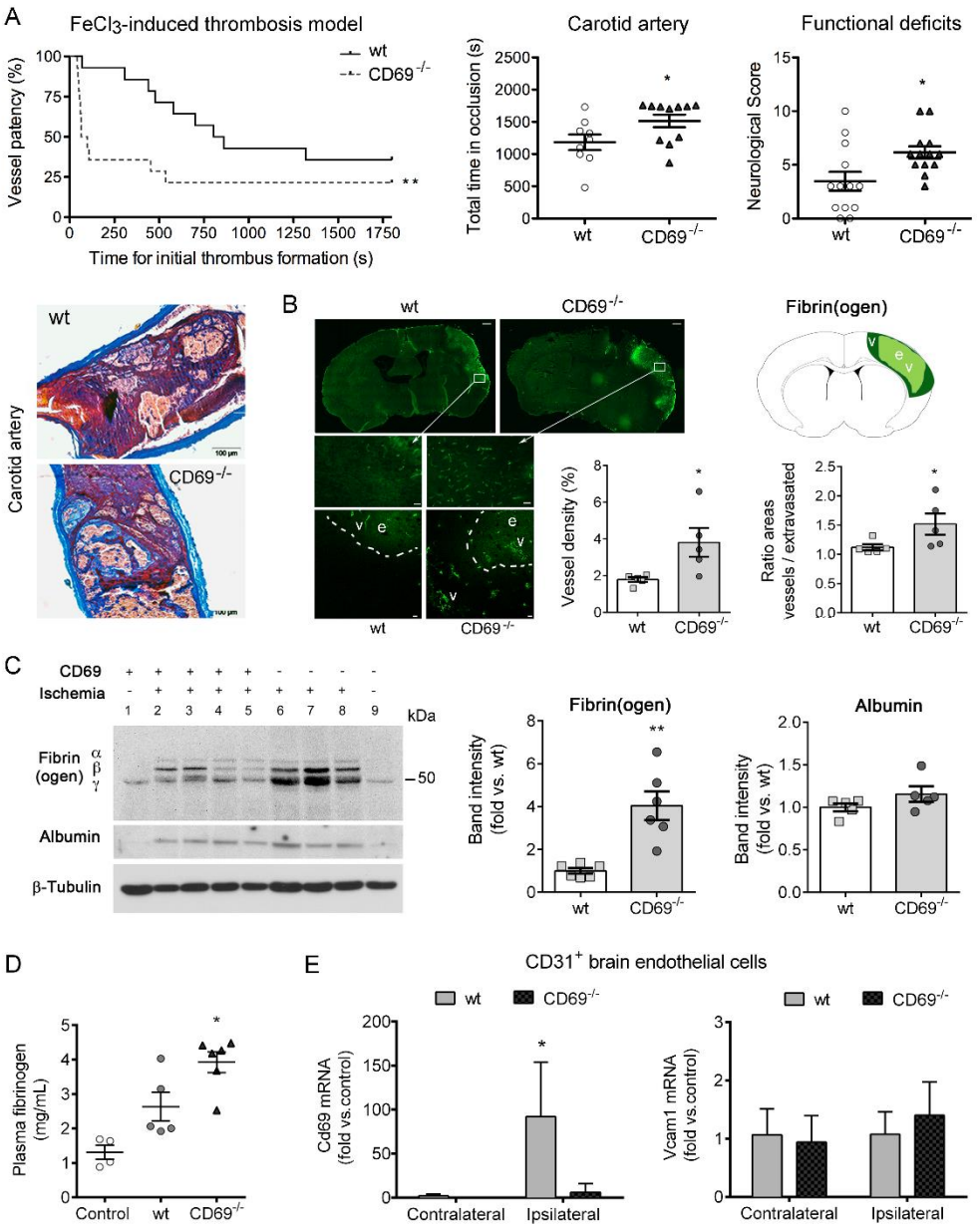


**Figure 5. CD69 deficiency facilitated thrombosis and increased fibrin(ogen) in the ischemic brain tissue.** A) Carotid artery thrombosis was induced by the $FeCl_3$ model in wild type (wt) mice and CD69$^{-/-}$ mice (n=14 per group). CD69 deficiency significantly reduced the time for initial thrombus formation (Gehan-Breslow-Wilcoxon test). Occlusions occurred in n=9 wt mice and n=11 CD69$^{-/-}$ mice, and in these mice the total time in occlusion was higher in CD69$^{-/-}$ mice than in the wt mice (Mann-Whitney test). The neurological score 24h after induction of thrombosis was worse in CD69$^{-/-}$ mice than in wt mice (Mann-Whitney test). Carstair's thrombus staining showed the presence of fibrin (red) and platelets (gray-blue to navy blue) in both genotypes. Scale bar: 100 µm. B) Fibrin(ogen) immunoreactivity in brain sections obtained 6h after pMCAo (BALB/c mice) showing one entire coronal section per genotype (upper images), corresponding magnifications of the zones indicated by squares to illustrate fibrin(ogen)$^+$ vessels (middle images), and images of the border of the lesion (lower images), representative of n=5 mice per genotype. Density of fibrin(ogen)$^+$ vessels (v) was higher in CD69$^{-/-}$ mice (Mann-Whitney test *p=0.016). The area with diffuse fluorescence corresponding to plasma fibrinogen extravasation (e, highlighted by a surrounding white dotted line) was similar in both genotypes (0.200±0.093

$mm^2$ wt mice; 0.199±0.102 $mm^2$ $CD69^{-/-}$ mice, values are the mean±SD). The area containing fibrin(ogen)$^+$ vessels coincided with the area of fibrinogen extravasation in wt mice whereas it was larger in $CD69^{-/-}$ mice, as shown by the higher ratio between both areas (Mann-Whitney test *p=0.032). Scale bar: 100µm (upper images); 10µm (middle and lower images). C) Western blotting of fibrin(ogen) and albumin in the brain tissue of $CD69^{-/-}$ mice and wt mice 6h after pMCAo as above. Ischemia (+) refers to tissue from the ipsilateral (ischemic) hemisphere, and ischemia (-) indicates tissue from the contralateral (unaffected) hemisphere. CD69-deficient mice (-) show more fibrinogen accumulation (Mann-Whitney test, **p=0.002, n=6 per group), but not albumin, in the ischemic tissue than wt mice (+). D) Plasma fibrinogen is significantly higher in $CD69^{-/-}$ mice (n=6) than wt mice (n=5) 6h postischemia as assessed by ELISA (Mann-Whitney test, *p=0.030). Controls indicate samples from non-ischemic wt mice (n=4). E) $CD45^-CD11b^-CD31^+$endothelial cells were FACS sorted from the ipsilateral (ischemic) and contralateral hemisphere of wt mice (n=4) and $CD69^{-/-}$ mice (n=3) (BALB/c background) 3 h after pMCAo. Two-way ANOVA by genotype and hemisphere shows a significant increase of *Cd69* mRNA expression in the ischemic tissue of wt mice (genotype effect, p=0.026, Bonferroni test, *p<0.05). In contrast, the expression of *Vcam1* mRNA does not change (two-way ANOVA, genotype effect p=0.679).

Figure 6

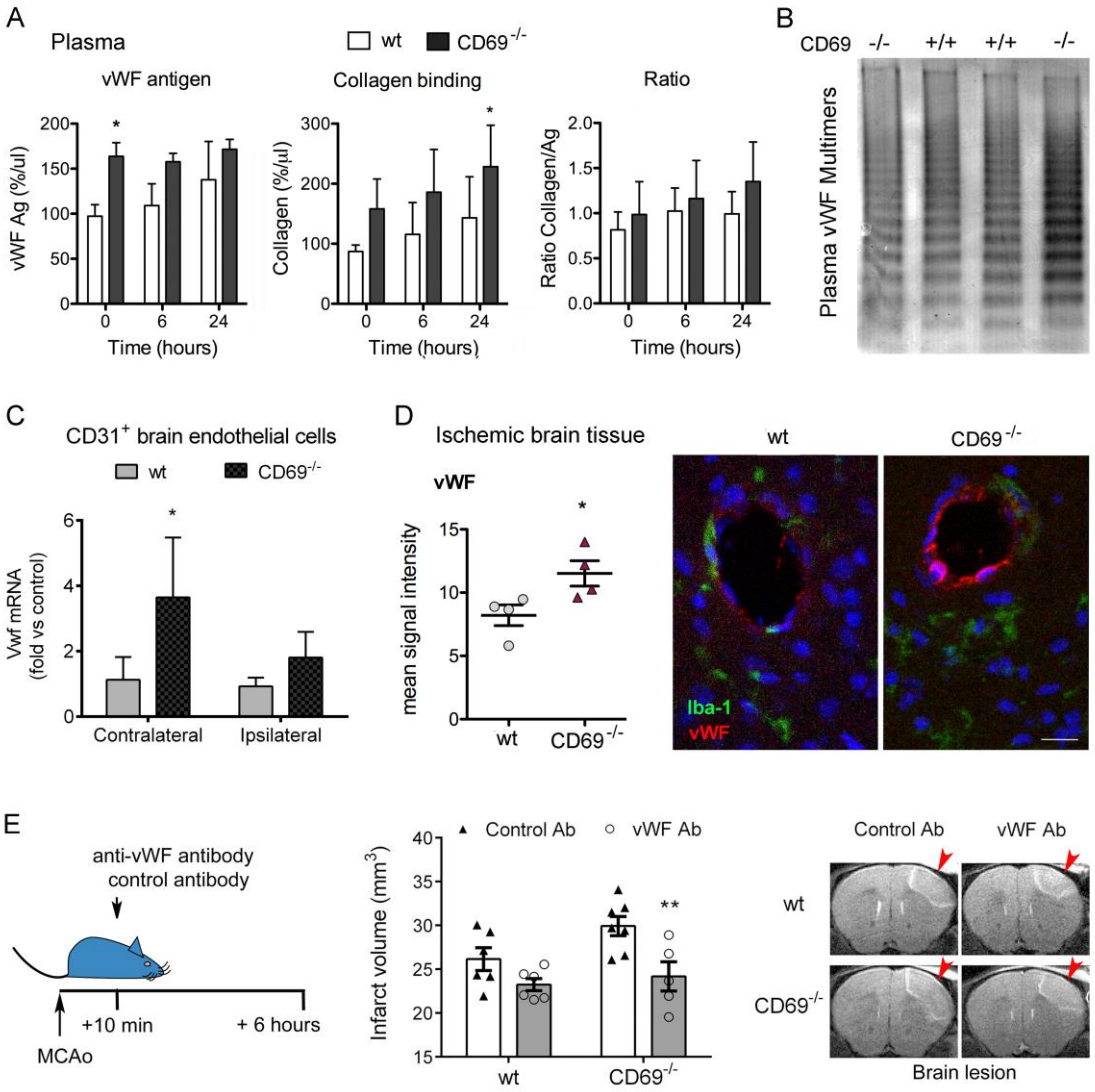


**Figure 6. Involvement of vWF in the exacerbated ischemic brain damage of CD69$^{-/-}$ mice.** A) Plasma vWF antigen (Ag) and collagen binding in control mice (time 0) and at 6h and 24h after pMCAo were higher in CD69$^{-/-}$ mice (n=11) than wt mice (n=22) (BALB/c background) (two-way ANOVA, genotype effect $p<0.001$, graphs show the group mean±SD). The ratio of collagen binding/Ag showed a trend to increase in the plasma of CD69$^{-/-}$ mice (two-way ANOVA, genotype effect $p=0.06$). B) vWF multimer formation in plasma was similar in both groups. C) Expression of *vWF* mRNA is higher in CD31$^{+}$ endothelial cells FACS-sorted from the ipsilateral (ischemic) and contralateral (unaffected) hemispheres of CD69$^{-/-}$ mice than wt mice (BALB/c) after 3h pMCAo (n=4 per genotype and hemisphere). Two-way ANOVA by genotype and hemisphere shows a significant genotype effect for vWF ($p=0.007$) that is more marked in the contralateral hemisphere (Bonferroni test, *$p<0.05$). D) vWF immunostaining in brain sections of wt and CD69$^{-/-}$ mice (n=4 per group) 24h after 45-min MCAo showed higher signal intensity in the latter (Mann-Whitney test). *$p<0.05$, **$p<0.01$. Scale bar: 25 µm. E) Blockade of vWF eliminates differences in brain lesion volume between wt and CD69$^{-/-}$ mice (BALB/c). Mice received anti-vWF antibody (vWF Ab) (n=6 wt mice and n=5 CD69$^{-/-}$ mice) or control antibody (Control Ab) (n=6 wt mice, n=7 CD69$^{-/-}$ mice), 10 min after pMCAo. Infarct volume (T2*w* MRI) was measured after 6 hours. Anti-vWF antibody reduced the lesion size in both genotypes (Two-way ANOVA, treatment effect $p=0.002$), with a marked effect in CD69$^{-/-}$ mice (Bonferroni test **$p<0.01$). Representative T2*w* MRI images illustrate the brain lesion in each group (arrowheads).