Supplemental Methods and Data

I. Supplemental Methods

1. Cerebrovascular anatomy

The circle of Willis and its branches were examined as previously described (Lambertsen, et al. 2009). PI3Kγ knockout (KO) and C57Bl6 wildtype (WT) were overdosed with pentobarbital and perfused with 10 ml of 0.9% saline, followed by 10 ml of a 50% solution of Quink carbon black ink (SolvX; Parker). Vessels were photographed under a dissecting microscope. The left and right posterior communicating (PCOM) arteries were scored (Lambertsen KL, 2009) as follows: 0, absent; 1, present, but poorly developed (hypoplastic); 2, well formed. A single PCOM score was obtained by averaging left and right scores. Results show that the circle of Willis was intact in both types of mice at the level of the anterior and middle cerebral arteries (Supple. Fig. 1). Most mice also had fully formed PCOM arteries; similar PCOM artery scores were obtained in WT and KO mice. These findings indicated that the reduced infarct volume observed in KO mice was not caused by differences in vascular anatomy between WT and KO mice.

2. Regional cerebral blood flow measurement

Regional cerebral blood flow (CBF) was monitored by laser Doppler flowmetry (MSP300XP; ADInstruments Inc). Probes were placed at the center (from bregma: 3.5 mm lateral, 1.5 mm caudal) and periphery (from bregma: 1.5 mm lateral, 1.5 mm caudal) of the ischemic territory as previously described (Jin, et al. 2009). After transient ischemia, CBF was restored by withdrawal of the nylon suture. Only animals that exhibited a reduction in CBF>85% during MCAO and a CBF recovery by >80% after 15 min of reperfusion were included in the study.

3. Western blot

Mice were killed at indicated times after tMCAO (n = 4-5 for per group per time point). Brains were stored at -80^{o}C until analysis. Western blot analysis was performed as described previously (Zhang, et al. 2007). Briefly, whole-cell protein was prepared from the ischemic cortices (between coronal levels +2 mm to −3 mm relative to bregma). Samples from sham-operated animals served as basal controls. Brain tissues were homogenized in 10× vol of cold protein extraction buffer (complete tablet; Roche), centrifuged at 13,000 rpm for 20 min at 4 °C, and the supernatant was used for analysis. After adding the 2× volume of sample buffer (Invitrogen) to the supernatant, equal amounts were loaded per lane. Antibodies used were as follows: rabbit anti-claudin-5 (1:500; Zymed Laboratories), goat anti-occludin (1:1000; Santa Cruz), and goat anti-collagen IV (1:2000; abcam). Protein samples (30 μg protein each lane) were separated by SDS-PAGE and proteins transferred to polyvinylidene difluoride membrane. Membranes were blocked [10% nonfat milk in 0.01% PBS-Tween20 (PBS-T)] and incubated in primary antibody (diluted in 1% bovine serum albumin in PBS-T) overnight at 4°C. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (in 5% milk in PBS-T). Immunopositive bands of horseradish peroxidase (HRP)-conjugated secondary antibodies were detected with an ECL system (GE Healthcare) and exposure to ECL Hyperfilm.

4. Cell Cultures

Primary mouse brain microvascular endothelial cells (BMECs) were prepared using the method as previously described (Song, et al. 2003; Wu, et al. 2003). Briefly, brain microvessels were harvested from the cerebral cortex of 6-8-week-old male wild-type mice (n=20) and PI3Kγ KO (n=20) mice, and BMECs were purified with anti-murine CD31 precoated M-450 Dynabeads (Dynal). Then cells were plated onto tissue culture dishes pre-coated with collagen I and cultured in complete growth medium (DMEM/F-12 containing 10% fetal bovine serum (FBS), 10% horse serum, 0.1 mg/ml endothelial cell growth supplement, 0.1 mg/ml heparin, and 100 units/ml

penicillin/streptomycin). The purity of BMECs was identified by the presence of factor VIII and von Willebrand factor was >95% of the cell population. All experiments were performed on confluent quiescent cells in primary cultures (passage 3-5) that were exposed overnight to 0.1%FBS/DMEM.

5. Neutrophil Firm Adhesion to Endothelial Cells

Neutrophil firm adhesion to endothelial cells was performed as previously described (Bhunia, et al. 1998; Li et al. 2008). BMECs, isolated from WT and PI3Kγ KO mice, were grown to confluence in 96-well gelatin-coated plates. Polymorphonuclear leukocytes (PMNs, or neutrophils) were isolated from peritoneal lavage of WT mice as previously described (Li et al. 2008). PMN purity was >95%, and the viability assessed by trypan blue exclusion was >98%. PMNs (1.5×10^5 in 100 µl of DMEM/0.1% BSA) labeled with calcein-AM (Molecular Probes) were added to BMECs pretreated with TNFα (10 ng/ml) for the indicated times at 37 °C in a 5% CO2 incubator. PMNs were incubated with BMECs for 1 h and nonadherent cells were removed by washing six times with DMEM/0.1%BSA. Neutrophil adhesion was assessed by a fluorescence microplate reader at 485-nm excitation and 530-nm emission. Data were expressed as the percentage of adherent cells in total cells by calculating the ratio of the fluorescence of calcein AM-loaded cells in each well before and after washing, respectively.

6. Flow Cytometry

BMECs, isolated from wildtype and PI3Kγ KO mice, were used to determine the effect of PI3Kγ deficiency on TNFα-induced ICAM-1 cell surface expression. ICAM-1 expression is crucial for the integrin-mediated firm adhesion of leukocytes in endothelial cells. The confluent cell monolayers were serum-deprived for 24 h and then treated with 10 ng/ml TNF-α (Sigma) in DMEM supplemented with 0.1% BSA for the indicated times. The cells were then washed and fixed in 4% paraformaldehyde at 4^oC for 60 min. After extensive washing, cells were resuspended in PBS/0.1% BSA (2×10^5/ml in 200 ul volume) and were incubated with a 1:1000 dilution (in

PBS/azide) of FITC-labeled rat anti-mouse CD54/ICAM-1 antibody (eBioscience), or isotype control FITC-rat IgG2b,κ, for 30 minutes at room temperature in the dark. Samples were then fixed in 1% buffered paraformaldehyde and washed three times with PBS/0.5% BSA. After washing, cells were resuspended in 500 µl PBS and the level of ICAM-1 surface expression analyzed by FACScan flow cytometry (Becton-Dickinson) with CellQuest software as previously described (Ferran, et al 1998).

II. Supplemental Data

Suppl. Figure 1. A, Representative Photographs of the circle of Willis in mice. **B,** Quantitative measurement of PCOM artery score between WT and KO mice based on black ink labeling. MCA, middle cerebral artery; PCA, posterior cerebral artery; BA, basilar artery; SCA, superior cerebellar artery; PCOM, posterior communicating artery. Results are expressed as mean ± SD. P>0.05, n=5/group.

Suppl. Figure 2. Regional cerebral blood flow (rCBF) was measured using a laser Doppler flowmeter in the center of the ischemic focus (probe 1) and peripheral zones (probe 2) of the ischemic distribution. Ischemia led to the expected drops in rCBF. Reperfusion restored rCBF levels close to baseline. There were no significant differences between wildtype (WT) and PI3K knockout (KO) mice. R15: 15 min after reperfusion. n=12/group.

Suppl. Figure 3. Double immunostaining for claudin-5 (A) and phospho-MLC (B) with endothelial marker CD31 in the non-ischemic cortex in WT and PI3Kγ KO mice. Note that both claudin-5 and phospho-MLC were strongly co-localized with CD31, indicating endothelial origin. MLC: myosin light chain 2 (Ser19). Scale bars: 50 µm. Double immunostaining was described in "*Materials and Methods*".

Suppl. Figure 4. Western blot analysis of claudin-5 (A), occluding (B), and collagen IV (C) in the ischemic and contralateral (Ctrl) hemispheres of WT and PI3Kγ KO mice at indicated times after tMCAO. Each lane represents a sample from one brain. Note that claudin-5 was markedly reduced at 4 h, occludin was not changed at 4 and 24 h, collagen IV was reduced at 24 and 72 h after tMCAO in WT mice. Similar results were obtained in three separate experiments.

Suppl. Figure 5. Immunostaining for MMP-9 in two different microvessels within ischemic cortex in WT (A) and KO (B) mice at 24 and 72 h after tMCAO. Note that MMP-9 was strongly co-expressed in the endothelium of brain microvessels at 24 h time point in WT mice. Scale bars: 50 μm. Immunostaining was described in "*Materials and Methods*".

Suppl. Figure 6. PI3Kγ regulates TNFα-induced ICAM-1 cell surface expression and PMN firm adhesion in cultured mouse BMECs in vitro. A, TNFα induced ICAM-1 expression in BMECs isolated from wildtype mice, but this effect was blocked in BMECs isolated from PI3Kγ KO mice. Flow cytometry was performed as described in *Supplemental Materials and Methods*. Simialr results were obtained in three separate experiments. B, PMN firm adhesion to TNFα-activated BMECs was almost completely blocked in PI3Kγ-null BMECs. Neutrophil adhesion assay was described in *Supplemental Materials and Methods*. Data shown are mean ± SD from three separate experiments. ***P* < 0.05 versus respective WT controls.

Suppl. Figure 7. PI3Kγ deficiency blocks neutrophil adhesion in cerebral microvessels in vivo. Immunostaining of neutrophils shows the adhesion of neutrophils to the microvessels (arrows) either on the brain surface (panel a/c) or within the parenchyma (panel b/d) at 4 h after tMCAO in WT and PI3Kγ KO mice. Note that the early neutrophil adhesion was prominent in WT mice (panel a/b) but was absent in KO mice (panel c/d). Similar observations were noted in more than 10 mice for each group. Bar=100 um.

Table S1. Physiological Variables in Wild-type and PI3Kγ KO Mice (mean ± SD)

Variables	WT-Sham	WT-tMCAO	KO-Sham	KO-tMCAO
Body weight (g)	23.8 ± 0.7	23.5 ± 0.8	23.3 ±0.6	23.1 ±0.7
BP (mm Hg)	101± 9.3	95 ± 10	99.5 ± 8.4	91 ± 9
pH	7.35 ± 0.1	7.28 ± 0.08	7.4 ± 0.09	7.26 ± 0.09
pCO2 (mm Hg)	37.4 ± 4.3	42.5 ± 3.9	37.2 ± 4.1	41.7 ± 5.2
pO2 (mm Hg)	115 ± 21	107 ± 29	109 ± 36	105 ± 34

During these experiments, blood pressure (BP) was monitored through a femoral artery and blood gas was analyzed at the end (i.e. 24 h after tMCAO or sham procedure). Each data in the Table represents the mean ± s.d. from eight mice. No significant differences were noted between the two mouse strains.

Table S2. Stroke study population

Mice	Procedure	Randomized, n	Drop-outs, n (reason)	Analyzed, n(%)	Read-out (number of mice)
C57BL/6	tMCAO Sham	177	6 (BS=0) 7 (SAH) 7 (died during or after surgery	157/177 (87.7)	Infarct volumes (12), including ∗Functional scores (12) ∗Regional cerebral blood flow (12); Cerebral vasculature (5); Physiological parameters (8); Evans blue leakage (15); Brain water content (8); Immunohistochemistry (20); DHE staining (10); ELISA assay (10); Gelatin Zymography (10); Western blot (16); RT-PCR (28); Sham controls (15).
PI3Kγ KO	tMCAO Sham	170	8 (BS=0) 2 (SAH) 3 (died during or after surgery	157/170 (92.3%)	Infarct volumes (12), including ∗Functional scores (12) ∗Regional cerebral blood flow (12); Cerebral vasculature (5); Physiological parameters (8); Evans blue leakage (15); Brain water content (8); Immunohistochemistry (20); DHE staining (10); ELISA assay (10); Gelatin Zymography (10); Western blot (16); RT-PCR (28); Sham controls (15).
C57BL/6	Cell culture	20	0	20/20 (100%)	Brain microvascualr endothelial cell culture
PI3Kγ KO	Cell culture	20	0	20/20 (100%)	Brain microvascualr endothelial cell culture
Total		387	33/387 (8.5%)	354/387 (91.4)	

Note BS, Bederson score; BS=0 indicating no deficits; SAH, subarachnoid hemorrhage; tMCAO, transient middle cerebral artery occlusion; Sham, sham-operated.

Only male mice (8-10 weeks old) were used in this study. The number of animals used for each experimental group are also provided in the figure legends.

Table S3. Mouse real-time RT-PCR primers

Gene name	Forward primers 5' – 3'	Reverse primer 5' – 3'
Nox1	CATCCAGTCTCCAAACATGACA	GCTACAGTGGCAATCACTCCAG
Nox2	CCAACTGGGATAACGAGTTCA	GAGAGTTTCAGCCAAGGCTTC
Nox4	TGAACTACAGTGAAGATTTCCTTGAAC	GACACCCGTCAGACCAGGAA
E-selectin	CTCACTCCTGACATCGTCCTC	ACGTTGTAAGAAGGCACATGG
P-selectin	ATGCCTGGCTACTGGACACT	CTTCATCGCACATGAACTGG
ICAM-1	GCCTTGGTAGAGGTGACTGAG	GACCGGAGCTGAAAAGTTGTA
HPRT	TGTTGGATACAGGCCAGAC	CGTGATTCAAATCCCTGAAGT

References

Bhunia AK, Arai T, Bulkley G, and Chatterjee S. Lactosylceramide mediates tumor necrosis factor-α-induced intercellular adhesion molecule-1 expression and the adhesion of neutrophils in human umbilical vein endothelial cells. J Biol Chem 273: 34349–34357, 1998

Bensaad K, Cheung EC, Vousden KH. Modulation of intracellular ROS levels by TIGAR controls autophagy. EMBO J. 2009;28:3015-3026.

Ferran C, Stroka DM, Badrichani AZ, et al. A20 inhibits NF-κB activation in endothelial cells without sensitizing to TNF-mediated apoptosis. *Blood.* 1998; 91: 2249–2258.

Jin G, Tsuji K, Xing C, Yang YG, Wang X, Lo EH. CD47 gene knockout protects against transient focal cerebral ischemia in mice. Exp Neurol. 2009: 217:165-170.

Lambertsen KL, Clausen BH, Babcock AA, Gregersen R, Fenger C, Nielsen HH, Haugaard LS, Wirenfeldt M, Nielsen M, Dagnaes-Hansen F, Bluethmann H, Faergeman NJ, Meldgaard M, Deierborg T, Finsen B. Microglia protect neurons against ischemia by synthesis of tumor necrosis factor. J Neurosci. 2009;29(5):1319-30.

Li G, Sanders JM, Bevard MH, Sun Z, Chumley JW, Galkina EV, Ley K, Sarembock IJ. CD40 ligand promotes Mac-1 expression, leukocyte recruitment, and neointima formation after vascular injury. Am J Pathol. 2008;172:1141-1152.

Sardina JL, López-Ruano G, Sánchez-Abarca LI, Pérez-Simón JA, Gaztelumendi A, Trigueros C, Llanillo M, Sánchez-Yagüe J, Hernández-Hernández A. p22phox-dependent NADPH oxidase activity is required for megakaryocytic differentiation. Cell Death Differ. 2010;17:1842-1854.

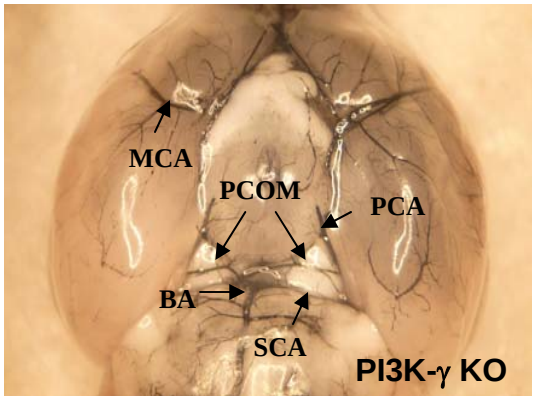
Song L , Pachter JS. Culture of murine brain microvascular endothelial cells that maintain expression and cytoskeletal association of tight junction-associated proteins. In Vitro Cell Dev Biol Anim 2003; 39:313–320

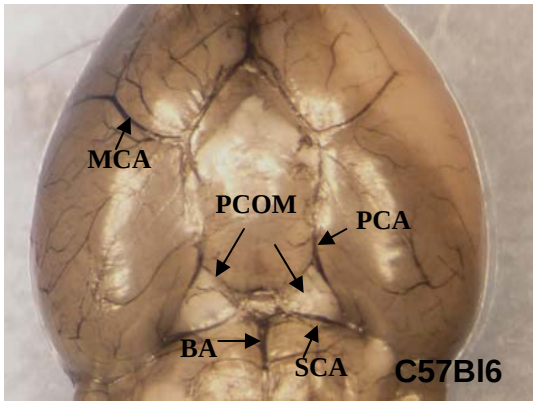
Takada Y, Mukhopadhyay A, Kundu GC, Mahabeleshwar GH, Singh S, Aggarwal BB. Hydrogen peroxide activates NF-kappa B through tyrosine phosphorylation of I kappa B alpha and serine phosphorylation of p65: evidence for the involvement of I kappa B alpha kinase and Syk protein-tyrosine kinase. J Biol Chem. 2003; 278(26):24233-41.

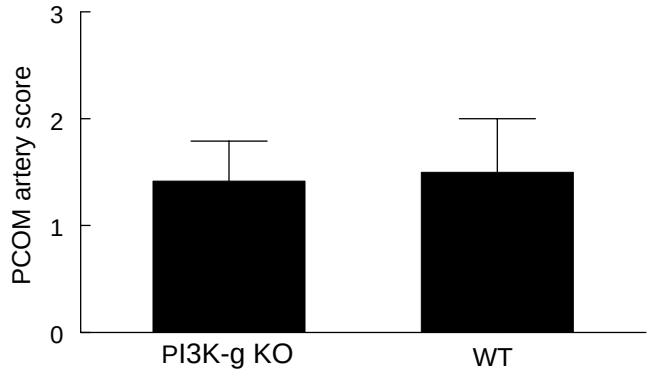
Wu Z, Hofman FM, Zlokovic BV. A simple method for isolation and characterization of mouse brain microvascular endothelial cells. J Neurosci Methods 2003;130:53–63.

Zhang X, Polavarapu R, She H, Mao Z, Yepes M. Tissue-type plasminogen activator and the low-density lipoprotein receptor-related protein mediate cerebral ischemia-induced nuclear factor-kappaB pathway activation. Am J Pathol. 2007; 171:1281-1290.

Cerebrovascular anatomy

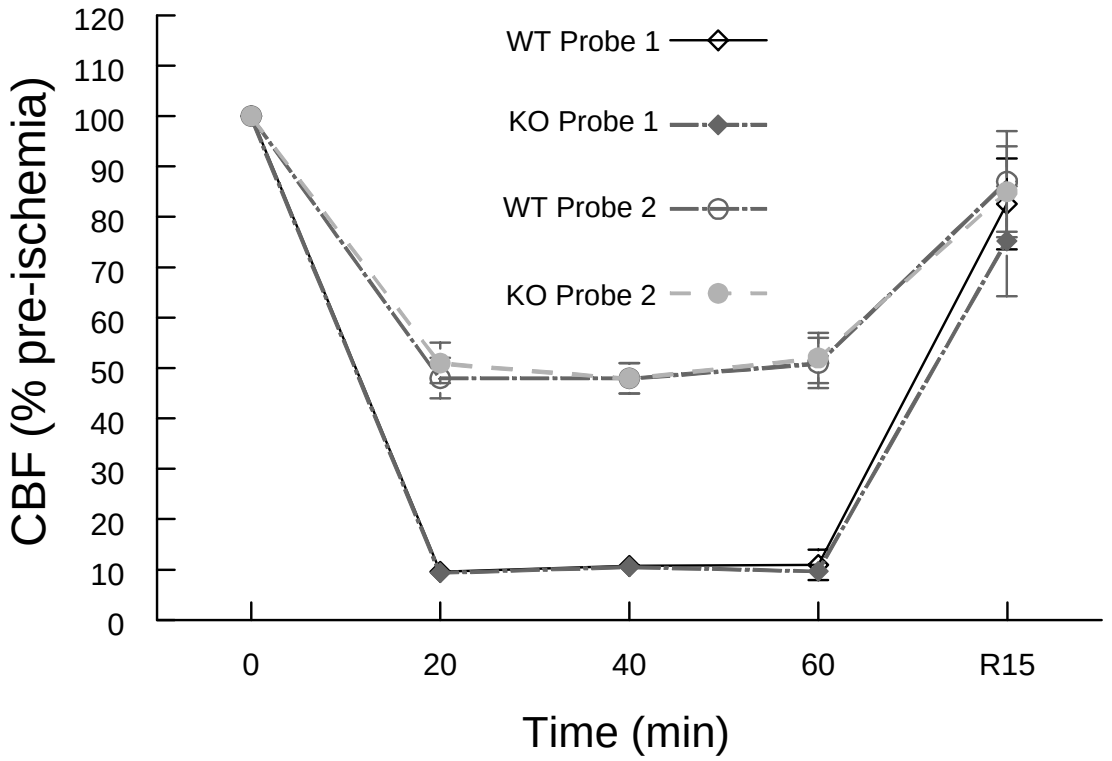






Suppl.Fig.1

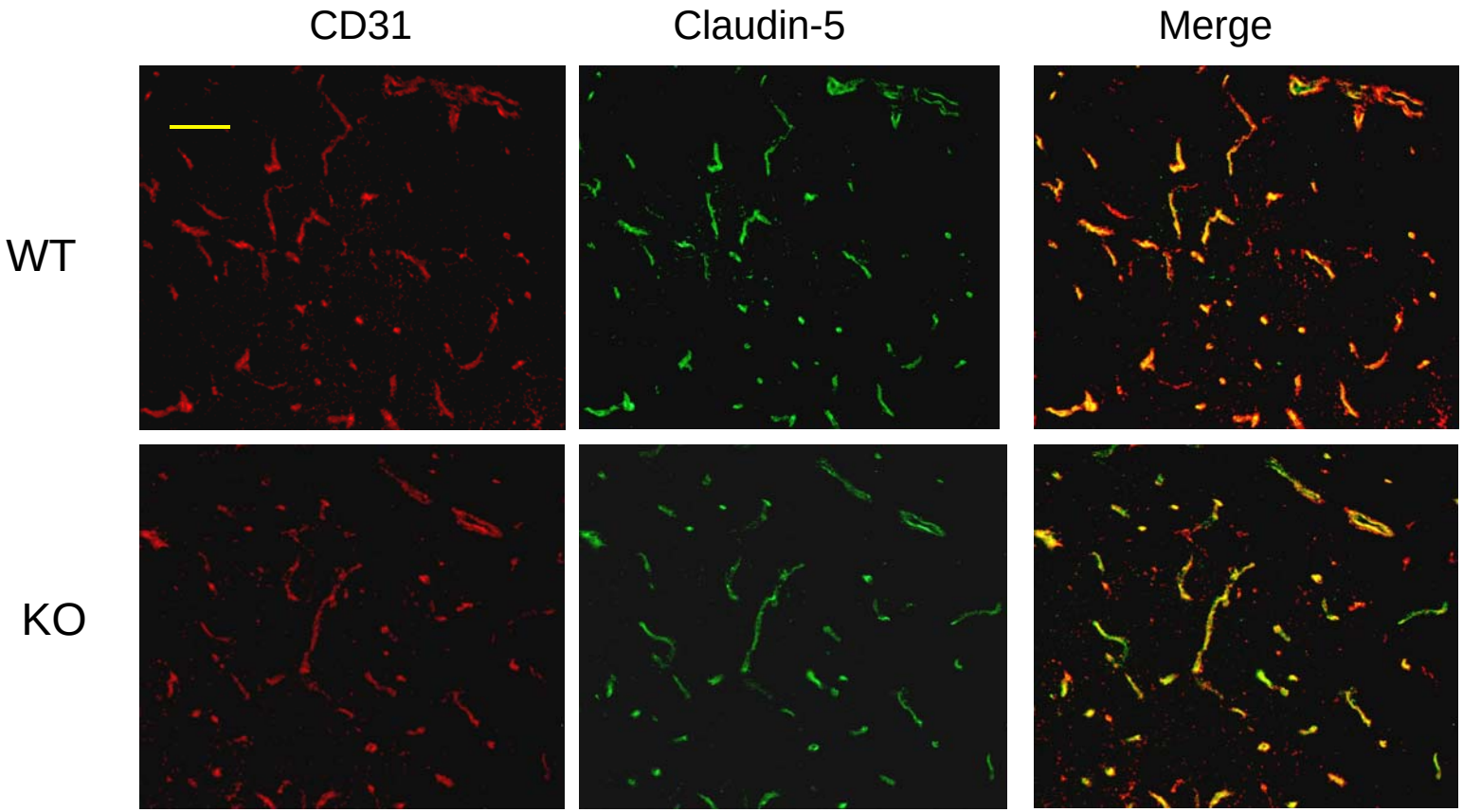
Regional cerebral blood flow measurement



Suppl.Fig.2

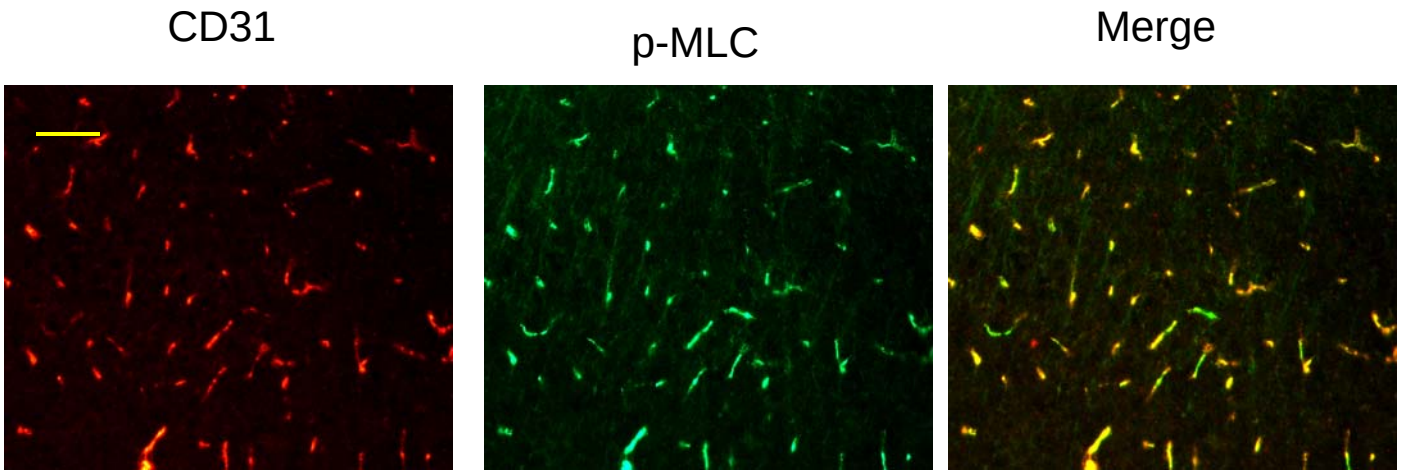
A.

Double staining showing the co-localization of claudin-5 with CD31 in non-ischemic cortex



Suppl.Fig.3

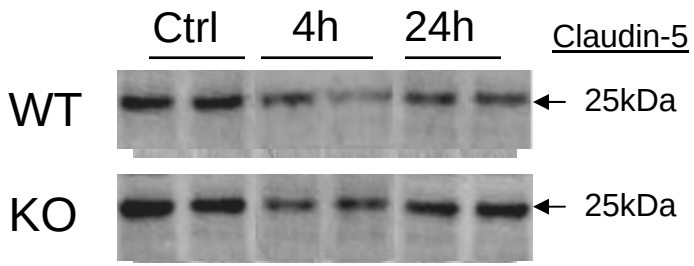
B. Double staining showing the co-localization of the MLC (Ser19) with CD31 in ischemic cortex of WT mice 4 h after tMCAO



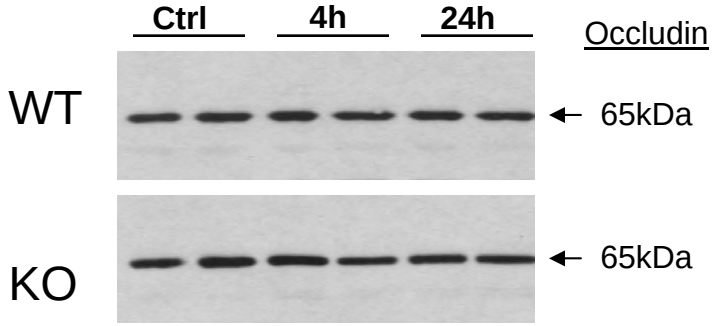
Suppl.Fig.3

Western blot

A.



B.



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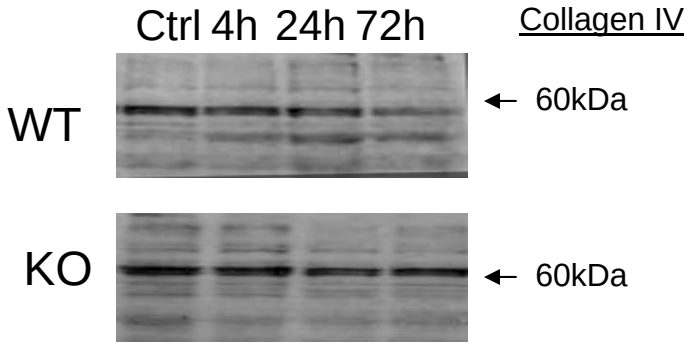
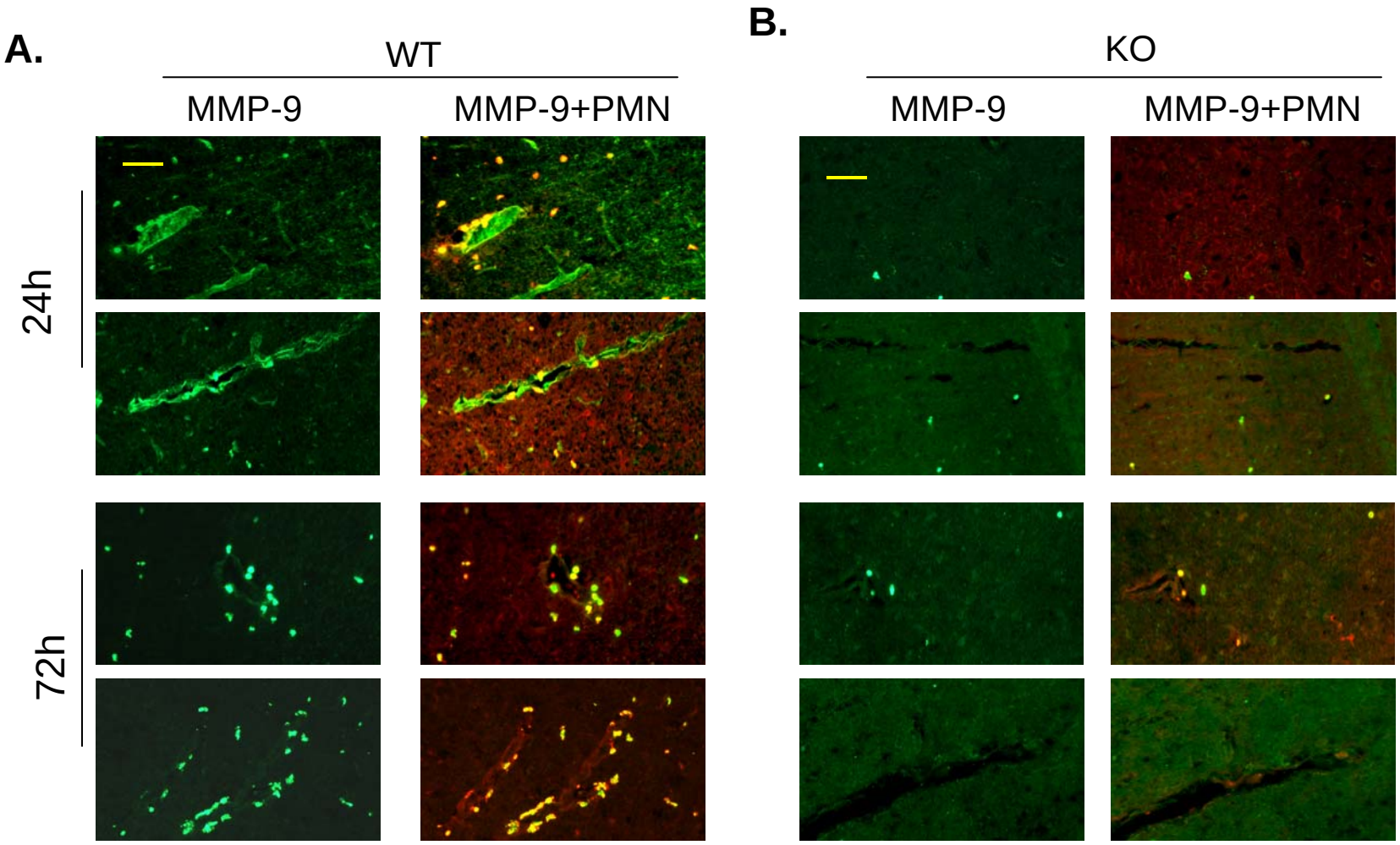


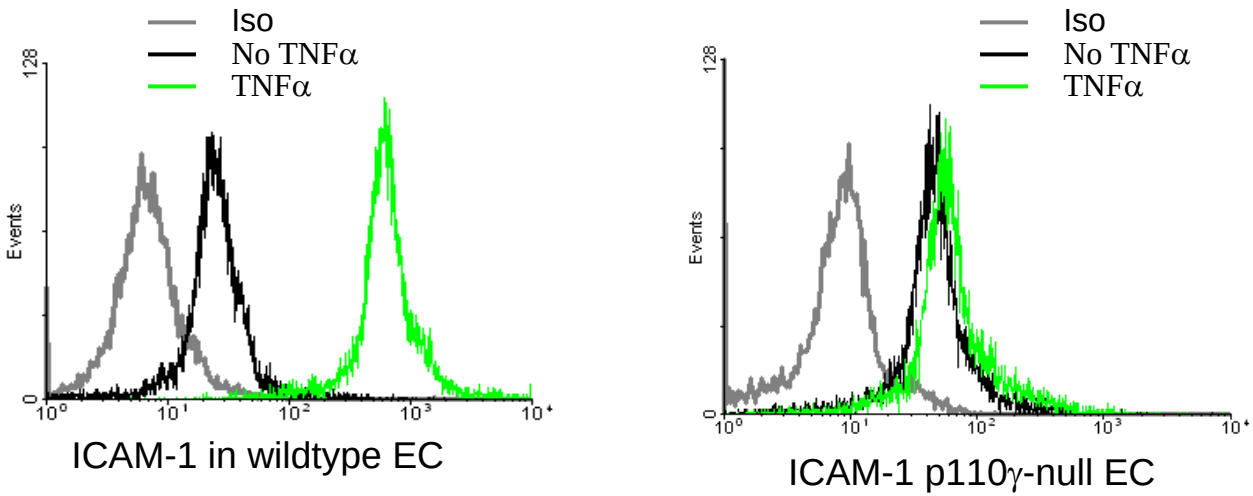
Suppl.Fig. 4

Immunostaining show MMP-9 present in the endothelium of brain microvessels



Suppl. Figure 5

A.



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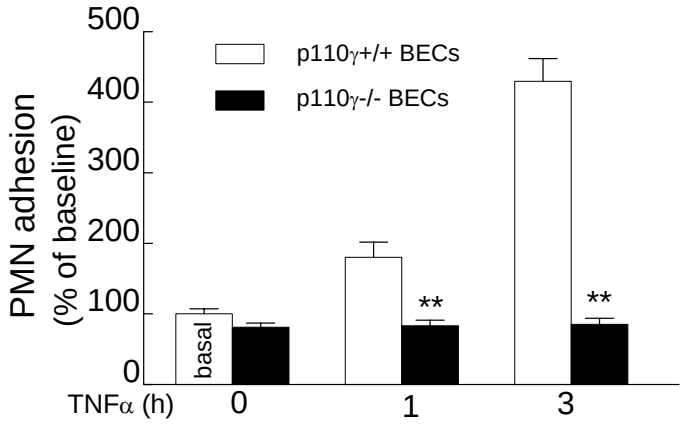
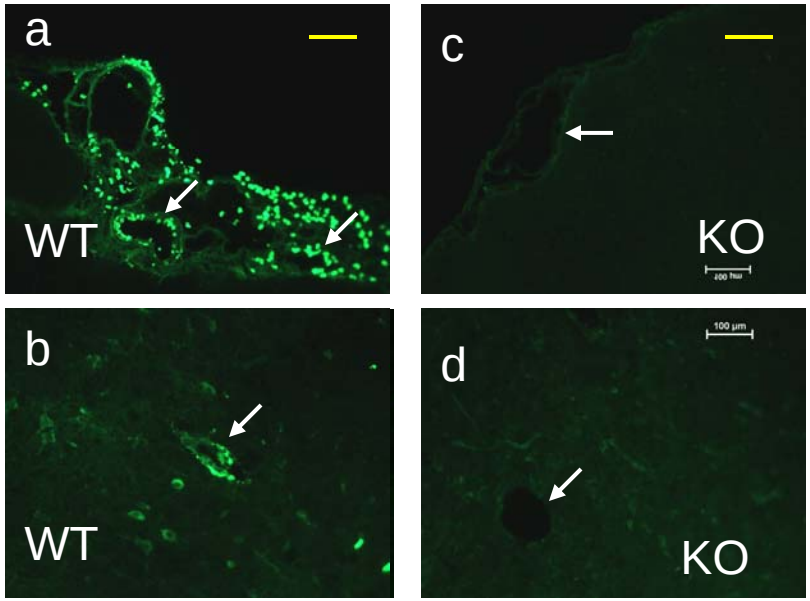


Suppl. Figure 6

PI3Kγ deficiency blocks neutrophil adhesion in cerebral microvessels in vivo



Suppl. Figure 7