Lack of collagen XV is protective after ischemic stroke in mice

Hiraman Dhungana, Mikko T Huuskonen, Taina Pihlajaniemi, Ritva Heljasvaara, Denis Vivien, Katja M Kanninen, Tarja Malm, Jari Koistinaho and Sighild Lemarchant

Collagens are key structural components of basement membranes, providing a scaffold for other components or adhering cells. Collagens and collagen-derived active fragments contribute to biological activities such as cell growth, differentiation and migration. Here, we report that collagen XV knock-out (ColXV KO) mice are resistant to experimental ischemic stroke. Interestingly, the infarcts of ColXV KO mice were as small as those of wild-type (WT) mice thrombolysed with recombinant tissue plasminogen activator (rtPA), the actual treatment for ischemic stroke. Importantly, there were no differences in the architecture of cerebrovascular anatomy between WT and ColXV KO mice. We found a twofold increase of the most potent pro-angiogenic factor, type A vascular growth endothelial factor (VEGF-A) in the ipsilateral cortex of rtPA-treated ischemic WT mice compared with untreated ischemic and sham-operated counterparts. A similar increase of VEGF-A was also found in both rtPA and untreated ischemic ColXV KO mice compared with sham ColXV KO mice. Finally, we evidenced that the levels of ColXV were increased in the plasma of WT mice treated with rtPA compared with untreated ischemic counterparts. Altogether, this study indicates that the lack of ColXV is protective after stroke and that the degradation of endothelial ColXV may contribute to the beneficial effect of rtPA after ischemic stroke. The neuroprotection observed in ColXV KO mice may be attributed to the increased VEGF-A production following stroke in the ischemic territory.

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Collagen XV and ColXVIII constitute the so called multiplexin family of non-fibrillar collagens, characterized by multiple triple helix interruptions and similar non-collagenous sequences. Nevertheless, they differ in their functional properties and expression patterns and for example, contrary to ColXVIII, ColXV predominantly carries chondroitin sulfate chains and was therefore classified as a chondroitin sulfate proteoglycan (CSPG). It is mainly produced by skeletal and cardiac muscle, and endothelial cells, occurring at the basement membranes adjacent to these cells. ColXV knock-out (KO) mice suffer from mild skeletal and cardiac myopathy and defective myelination of peripheral nerves.

In this study, we report for the first time the neuroprotective effect of ColXV deficiency in mice suffering from ischemic stroke. Accordingly, we found an increase of type A vascular endothelial growth factor (VEGF-A) in the ischemic cortex of ColXV KO mice. Additionally, we showed that rtPA increased the presence of unbound ColXV in the plasma of wild-type (WT) mice after stroke.

Results

Lack of collagen XV is protective after thromboembolic stroke. WT and ColXV KO mice were subjected to thromboembolic stroke provoked by a local injection of thrombin

1Department of Neurobiology, A. I. Virtanen Institute for Molecular Sciences, Biocenter Kuopio, University of Eastern Finland, Kuopio, Finland; 2Oulu Center for Cell-Matrix Research, Biocenter Oulu and Faculty of Biochemistry and Molecular Medicine, University of Oulu, Oulu, Finland; 3Centre for Cancer Biomarkers CCBIO, Department of Biomedicine, University of Bergen, Bergen, Norway; 4Normandie Univ, UNICAEN, INSERM U919, Serine Proteases and Pathophysiology of the neurovascular Unit, Cytocron, Caen, France and 5Department of Clinical Research, Caen University Hospital, Avenue de la côte de Nacre, Caen, France

*Corresponding author: S Lemarchant, Department of Neurobiology, A.I. Virtanen Institute for Molecular Sciences, University of Eastern Finland, PO Box 1627, Kuopio 70211, Finland. Tel: +35 620 675 740; Fax: +35 817 162 424; E-mail: sighild.lemarchant@uef.fi

These authors contributed equally to this work

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into the middle cerebral artery as previously described.13,14 Twenty minutes after clot formation, mice were treated with intravenous injections of saline or rtPA (10 mg kg$^{-1}$). Two days after stroke, magnetic resonance imaging (MRI) images revealed that the lesion volume was significantly smaller in ColXV KO mice compared with WT mice (Figures 1a and b: $24.2 \pm 2.5$ mm$^3$ for WT mice and $16.3 \pm 1.5$ mm$^3$ for ColXV KO mice; $P = 0.0179$). Interestingly, while early thrombolysis with rtPA was beneficial in WT mice (Figures 1a and b: $13.2 \pm 3.0$ mm$^3$ for WT mice injected with rtPA; $P = 0.0161$ compared with WT mice injected with saline), no additional benefit of rtPA to ColXV KO mice was observed (Figures 1a and b: $13.8 \pm 2.3$ mm$^3$ for ColXV KO mice injected with rtPA; $P > 0.05$ compared with ColXV KO mice injected with saline). Cerebral blood flow (CBF) was monitored by Doppler flowmetry during the surgery in WT and ColXV KO mice treated or not with rtPA. We evidenced a similar reduction of about 90% of CBF after thrombin injection (clot formation) in WT and ColXV KO mice (Figure 1c: $P > 0.05$ between groups after clot formation). While no changes of CBF were evidenced between clot formation and the end of saline injection in untreated WT and ColXV KO mice (Figure 1c: $P > 0.05$), thrombolysis with rtPA increased CBF in WT (Figure 1c: 10.8% of initial CBF at clot formation and 41.4% of initial CBF at the end of rtPA injection in WT mice; $P = 0.0352$) and ColXV KO mice (Figure 1c: 10.8% of initial CBF at clot formation and 39.5% of initial CBF at the end of rtPA injection in ColXV KO mice; $P = 0.0474$).

We then evaluated a set of features that could account for the protection observed in ColXV KO after ischemia. Importantly, we did not observe any difference between the architecture of cerebral vasculatures in the middle cerebral artery area of WT and ColXV KO healthy mice (Figure 2a). No differences in blood parameters, such as partial pressure O$_2$, partial pressure CO$_2$, pH or glucose, were found between WT and ColXV KO mice 20 min after stroke (Figure 2b). Brain edema has a central role in the pathophysiology of stroke. Therefore, we looked at brain swelling and aquaporin 4, the well-known glial water channel whose expression is increased after stroke and is extensively described for its contribution to brain edema formation and development.15 We did not observe any modification of the percentage of swelling between WT and ColXV KO mice treated or not with rtPA 2 days after stroke (Figure 3a: $P = 0.2444$). Similarly, no difference in aquaporin 4 immunoreactivity was found in the peri-ischemic area between WT and ColXV KO mice treated or not with rtPA 3 days after stroke (Figures 3b and c: $P = 0.6393$).

rtPA leads to an increase of collagen XV levels in the plasma of ischemic WT mice. We then measured the protein levels of ColXV in the plasma of sham-operated and ischemic WT mice treated or not with rtPA 3 days after stroke, by ELISA. Stroke did not influence plasma concentrations of ColXV (Figure 4: $P = 0.6242$ between sham-operated and untreated ischemic WT mice). However, rtPA tended to increase ColXV protein levels after ischemia in the plasma of WT mice (Figure 4: $+1.93 \mu g ml^{-1}$ in ischemic WT mice treated with rtPA compared with untreated ischemic WT mice, $P = 0.0526$).
To determine why ColXV KO mice are more resistant to ischemic stroke than WT mice, we then investigated whether the lack of ColXV could influence mechanisms mediating neuroinflammation or neuroprotection.

rtPA fails to reduce stroke-induced increases of interleukin-6 and chemokine ligand 2 protein levels in ischemic collagen XV KO mice. First, we did not observe any changes in astrogliosis or microgliosis between WT and ColXV KO mice treated or not with rtPA 3 days after stroke, measured respectively as GFAP (Figures 5a and b: \( P = 0.8330 \)) or Iba1 (Figures 5c and d: \( P = 0.6932 \)) reactivity in the peri-ischemic area.

In a parallel cohort of animals, we investigated the expression of cytokines present in protein extracts from the contralateral and ipsilateral cortices of WT and ColXV KO mice treated or not with rtPA 3 days after stroke. Cytometric bead assay revealed a significant increase of interleukin-6 (IL-6) or chemokine ligand 2 (CCL2) protein levels in the ipsilateral cortex of mice suffering from stroke compared with corresponding contralateral cortex or to corresponding sham-operated mice (Figures 6a and b). We observed that thrombolysis with rtPA led to a twofold decrease of stroke-induced IL-6 (Figure 6a: −58.7 pg ml\(^{-1}\) in ischemic WT mice treated with rtPA compared with untreated ischemic WT mice, \( P = 0.0143 \)) and CCL2 (Figure 6b: −288.7 pg ml\(^{-1}\) in ischemic WT mice treated with rtPA compared with untreated ischemic WT mice, \( P = 0.0500 \)) increases in the ipsilateral cortex of WT mice. Interestingly, rtPA failed to reduce stroke-induced IL-6 (Figure 6a: \( P = 0.6242 \)) and CCL2 (Figure 6b: \( P = 0.9999 \)) increases in the ipsilateral cortex of ColXV KO mice. There were no differences of IL-6 (Figure 6c: \( P = 0.2506 \)) or CCL2 (Figure 6d: \( P = 0.6015 \)) protein levels in the ipsilateral cortex of WT and ColXV KO mice. No modifications of tumor necrosis factor \( \alpha \) (TNF-\( \alpha \)) and interferon \( \gamma \) (IFN-\( \gamma \)) protein levels were found between the groups (Figures 6c and d: \( P = 0.3841 \) for TNF-\( \alpha \), \( P = 0.3570 \) for IFN-\( \gamma \)). IL-10 and IL-12p70 were not detected in the samples.

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**Figure 2** No differences of blood parameters and cerebral vasculatures between WT and ColXV KO mice. (a) Measurements of blood parameters in WT (\( N = 4 \)) and ColXV KO (\( N = 3 \)) mice 20 min after thromboembolic stroke. No significant difference in any of these parameters was noted between WT and ColXV KO mice. (b) Cerebral vasculature of WT and ColXV KO mice. Black circles indicate the middle cerebral artery bifurcation.

**Figure 3** Lack of ColXV does not influence brain edema. (a) Brain edema was measured as a % of swelling by using the formula ((V ipsilateral hemisphere − V contralateral hemisphere)/V contralateral hemisphere)\(^*100\). Values plotted are mean ± S.E.M. Kruskall–Wallis test: \( P > 0.05 \). (b–c) Photomicrographs of aquaporin 4 (b) and corresponding immunoreactivity quantifications (c) in the peri-ischemic area of injured WT and ColXV KO mice thrombolysed or not with rtPA 20 min after thromboembolic (TE) stroke. Values plotted are mean ± S.E.M. Kruskall–Wallis test: \( P > 0.05 \); \( N = 4–5 \) per group. PI, peri-ischemic area.
VEGF-A expression is increased in the ischemic cortex of collagen XV KO mice after thromboembolic stroke. We then studied the expression of VEGF-A, a neuroprotective molecule in the CNS\textsuperscript{16} including after ischemic stroke.\textsuperscript{17}

We observed a twofold increase of VEGF-A in the ipsilateral cortex of ischemic WT mice treated with rtPA compared with untreated ischemic WT mice or sham-operated WT counterparts (Figures 7a and b: +110.7\% in ischemic WT mice treated with rtPA compared with untreated ischemic WT mice, \(P=0.0209\)). Interestingly, we observed a similar increase of VEGF-A in ischemic ColXV KO mice treated or not with rtPA compared with sham-operated ColXV KO counterparts (Figures 7c and d: +131.9\% in ischemic ColXV KO mice compared with sham-operated ColXV KO counterparts, \(P=0.0209\); \(P=0.3865\) between ischemic ColXV KO mice treated or not with rtPA).

**Discussion**

Collagens, laminins, nidogens and perlecan are major structural proteins of basement membranes, self-assembled with other extracellular matrix (ECM) components, which altogether represent a complex network providing a crucial molecular and physical scaffold for cells.\textsuperscript{18} Here, we investigated for the first time the role of ColXV in the CNS after acute ischemic stroke in mice. Interestingly, we demonstrated that ColXV-deficient mice are more resistant to thromboembolic stroke than WT siblings. Indeed, we observed a 33\% decrease

**Figure 4** rtPA leads to an increase of ColXV levels in the plasma of WT mice. ELISA for mouse ColXV in the plasma of sham-operated and ischemic WT mice thrombolysed or not with rtPA 20 min after thromboembolic (TE) stroke, at 3 days post injury. Values plotted are mean ± S.E.M. Mann–Whitney U-tests: \(P=0.0526\); \(N=3–5\) per group.

**Figure 5** Lack of ColXV does not influence gliosis. Photomicrographs of GFAP (a), Iba1 (c) and corresponding immunoreactivity quantifications (b,d) in the peri-ischemic area of injured WT and ColXV KO mice thrombolysed or not with rtPA 20 min after thromboembolic (TE) stroke. Values plotted are mean ± S.E.M. Kruskall–Wallis test: \(P>0.05\); \(N=4–5\) per group. IC, ischemic core; PI, peri-ischemic area.
Figure 6  rtPA fails to reduce stroke-induced increases of IL-6 and CCL2 protein levels in the ischemic cortex of ColXV KO mice. IL-6 (a), CCL2 (b), TNF-α (c) and IFN-γ (d) protein levels at 3 days post injury in the contralateral (CL, black bars) and ipsilateral (IL, white bars) cortices of sham-operated and injured WT and ColXV KO mice thrombolysed or not with rtPA 20 min after TE stroke. Values plotted are mean ± S.E.M. Kruskal–Wallis: $P<0.05$ for TNF-α and IFN-γ, $P<0.0001$ for IL-6 and CCL2. Mann–Whitney U-tests: *$P<0.05$, **$P<0.01$ compared to the contralateral hemisphere, *$P<0.05$ compared to the ipsilateral cortex of saline TE WT; $N = 4-5$ per group

Figure 7  Lack of ColXV increases VEGF-A expression in the ischemic cortex. Immunoblots for VEGF-A (a and c) and corresponding quantifications (b and d) in the ipsilateral cortex of sham-operated and injured WT (a and b) and ColXV KO (c and d) mice thrombolysed or not with rtPA 20 min after thromboembolic (TE) stroke, at 3 days post injury. Values plotted are mean ± S.E.M. Mann–Whitney U-tests: *$P<0.05$; $N = 4$
The collagen matrix is continuously remodeled by protease-dependent collagen degradation and production of new collagens. In this regard, our results suggest the possible cleavage of endothelial Col15 by rtPA, possibly participating to the rtPA beneficial effects in ischemic WT mice. This hypothesis is confirmed by previous reports showing the ability of rtPA to cleave/ degrade CSPGs. Proteolytic cleavages of ColIV, Col15 and Col15II produce fragments with anti-angiogenic activities: arrestin, canstatin or tumstatin for ColIV, resin for Col15 and endostatin for Col15II. Increasing post-stroke repair constitutes one direction of interest to establish new effective therapies. In this regard, VEGF-A is of particular interest for its involvement in mechanisms mediating neuroprotection, angiogenesis, neuregogenesis, neuronal migration and survival and axon guidance. Nevertheless, previous studies caution the use of VEGF for stroke therapy at appropriate dose and time post-stroke. Here, we have focused on short-term time points: MRI was conducted at 2 days post injury, and the mice were sacrificed one day later. Considering the anti-angiogenic effect of resin and the pro-angiogenic effect of VEGF-A, further investigations are now needed to study a long-term time point, to establish whether the increased VEGF-A production observed early after stroke is not transient but promotes long-lasting angiogenesis in Col15 KO mice after ischemic stroke. In line with our study, compelling evidence shows that CSPGs and heparan sulfate proteoglycans (HSPGs) have critical roles in mechanisms mediating neuroprotection and angiogenesis after stroke. Deglycosylation of CSPGs by the bacterial enzyme chondroitinase ABC reduced glial scar formation, promoted axonal regeneration and collateral sprouting, and improved functional outcome after experimental stroke in rodents. Additionally, chondroitinase ABC and glycinin increased the production of neurotrophic factors in primary cultures of cortical neurons (brain-derived neurotrophic factor for chondroitinase ABC and fibroblast-growth factor 2 for glycinin). The C-terminal protein fragment domain V of the HSPG perlcan reduced the infarct size and enhanced angiogenesis in a VEGF-dependent manner in the peri-ischemic area thereby promoting functional recovery in several rodent models of ischemic stroke.

This study indicates that the lack of Col15 is protective for ischemic stroke. The neuroprotection observed in Col15-deficient mice may be attributed to the acutely increased production of a neuroprotective molecule, VEGF-A, following stroke in the ischemic territory. Further studies are now warranted to decipher the mechanism(s) underlying VEGF-A production in Col15 KO mice during stroke, to determine the cellular origin of VEGF-A (endothelial cells? neurons? glia?), and finally, to investigate whether the increased production of VEGF-A in Col15-deficient mice may increase angiogenesis during the chronic phase of ischemic stroke. This study also highlights that Col15 may represent a substrate for rtPA, the actual treatment of ischemic stroke.
Materials and Methods

E thics. Animal experiments were conducted according to the national regulation of the usage and welfare of laboratory animals, approved by the National Animal Experiment Board of Finland and followed the Council of Europe legislation and regulation for animal protection.

Animals. Adult WT and transgenic female mice lacking in the α1 chain of collagen XV (ColXV KO) by site-specific Cre-loxP-mediated deletion in embryonic stem cells from C57BL/6J background were used in this study.11 Transgenic genotypes were identified by PCR amplification of ear DNA a few days after birth and after death to confirm the results of the first genotyping. The mice were housed under controlled temperature, humidity and light conditions (12 h light and dark cycles) with free access to food and water. Animals were housed in groups of up to 5 in cages.

Thromboembolic stroke model. Nine month-old WT and ColXV KO female mice were anesthetized by 5% isoflurane in 30% O2/70% N2O and the surgical anesthesia was maintained by 2% isoflurane. Temperature was maintained at 37 ± 1 °C by thermostatically controlled heating blanket (Harvard apparatus; PanLab, Cornellia, Spain). In a parallel group, a femoral artery was catheterized for monitoring of blood gases (pH, pO2, pCO2) 20 min after ischemia. Mice were placed in a stereotactic frame, the skin between the right eye and the right ear was incised, and the temporal muscle was retracted. A small craniotomy was performed, the dura was excised, and the middle cerebral artery (MCA) was exposed. One μL containing 1 IU purified murine α-thrombin (Enzyme Research Laboratories, South Bend, IN, USA) was injected into the MCA using a micropipette to induce the formation of a stable clot in situ.12 Cerebral blood velocity in the MCA territory was followed during the surgery by laser Doppler (Moor Instruments, Axminster, UK) to confirm the drop of the blood flow after the clot formation. Thrombolysis was induced after 30 min of occlusion by injecting 200 μL of rtPA (10 mg/Kg; Actilyse, Boehringer Ingelheim, Germany) intravenously to the tail vein (10% bolus, 90% perfusion for 40 min). Control mice received 200 μL of saline under the same conditions. Mice were randomized into six groups using GraphPad Quickcalcs (PFA; as described in the immunohistochemistry section) for staining purposes 15 min at 2000 × g until analysis. After washing with PB, PBS and PBS–tween (0.05% Tween-20), sections were treated with antibodies (1/500, Sigma-Aldrich) and unspecific bindings were blocked with 1×-h incubation in 10% normal goat serum (NGS, Merck Millipore, Billerica, MA, USA). Incubation with primary antibodies was conducted overnight at RT with dilutions as follows: rabbit anti-NeuN (glial fibrillary acidic protein, 1/200; Dako), rabbit anti-Iba1 (ionized calcium-binding adapter molecule-1, 1/250; Wako Pure Chemical Industries, Tokyo, Japan) or rabbit anti-aquaporin 4 (1/300; Merck Millipore). After washing with PBS–tween, sections were incubated with corresponding fluorescent Alexa fluor 488 or 568-conjugated secondary antibodies (1/200; Life Technologies) for 2 h at RT, then washed again and finally mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA).

Magnetic resonance imaging. MRI was performed in vivo at 2 days post injury in anesthetized mice to determine the lesion volume using a horizontal 9.4 T Oxford NMR 400 magnet (Oxford instrument PLC, Abington, UK) as previously described.70 Middle cerebral artery territory. To evaluate the cerebral vasculature anatomy of WT and ColXV KO mice, anesthetized mice were perfused with saline as described in the IHC section, then with a diluted India blue ink dye solution and finally with PFA. The brains were dissected out and the images were taken using a digital camera (Color View 12, soft Imaging System, Muenster, Germany) using Soft Imaging software. Immunoreactivity were quantified using Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA) at a pre-defined range, measured as the relative immunoreactive area for GFAP..rtP A were measured by using the cytometric bead assay Th1/Th2/Th17 kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer’s instructions. Data were analyzed using FACS Calibur (BD Biosciences, San Jose, CA, USA) and analyzed by FCAP Array software (Soft Flow, St. Louis Park, MN, USA).

Magnetic resonance imaging. MRI was performed in vivo at 2 days post injury in anesthetized mice to determine the lesion volume using a horizontal 9.4 T Oxford NMR 400 magnet (Oxford instrument PLC, Abington, UK) as previously described.40 Multi-slice T2-weighted images were acquired: echo time/repetition time of 40 ms/3000 ms, matrix size 128 × 256, field of view 19.2 × 19.2 mm², slice thickness 0.8 mm and number of slices 12. Images were then analyzed using Aedes software (Kuopio, Finland) under Matlab program (Math-woks, Natick, USA).

Protein extraction. Contralateral and ipsilateral cortices were collected and stored at −70 °C for protein purposes (N=4–5 in each group). For the second study, mice were sacrificed 3 days after stroke by terminal perfusion with heparinized saline, brains were dissected and the contralateral and ipsilateral cortices were collected and stored at −70 °C for protein purposes (N=4–5 in each group).

Western blot. Proteins (5 μg) were resolved on 14% polyacrylamide gel under denaturing conditions and transferred onto a polyvinylidene difluoride membrane. Membranes were blocked with phosphate-buffered saline (PBS)–tween (0.2%; Tween-20; Sigma-Aldrich) and 5% of milk. Blots were incubated overnight at 4 °C with the mouse anti-VEGF-A (1/500; Santa Cruz Biotechnology, Dallas, TX, USA) primary antibody diluted in PBS–tween containing 5% of milk. After a 2-h incubation at room temperature (RT) with the peroxidase-conjugated anti-mouse secondary antibody (1/5000; GE Healthcare Life Sciences, Uppsala, Sweden), proteins were revealed with an enhanced chemiluminescence ECL-Plus kit (Amersham Pharma Biotech) as loading control and visualized by Alexa fluor 647-conjugated anti-secondary antibody (1/1000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

Immunohistochemistry. Anesthetized mice were perfused with cold heparinized saline, followed by a perfusion with 4% PFA in 0.1 M phosphate buffer (PB) pH 7.4. Brains were collected and rinsed in a PB containing 20% sucrose for cryoprotection for 24 h and then embedded and frozen in OCT (Optimal Cutting Temperature; Sakura Finetek, Tokyo, Japan). Six 20-μm coronal sections 200 μm apart of each brain were cut on a cryostat (Leica Microsystems, Wetzlar, Germany), collected on poly-lysine glasses (Thermo Scientific, Leicestershire, UK), and stored at −70 °C until analysis. After washing with PB, PBS and PBS–tween (0.05% Tween-20), sections were treated with 1 μg/mL of PFA (Triton X-100) (0.4%, Sigma-Aldrich) and unspecific bindings were blocked by 1×-h incubation in 10% normal goat serum (NGS, Merck Millipore, Billerica, MA, USA). Incubation with primary antibodies was conducted overnight at RT with dilutions as follows: rabbit anti-NeuN (1/200; Dako), rabbit anti-Iba1 (1/250; Wako Pure Chemical Industries, Tokyo, Japan) or rabbit anti-aquaporin 4 (1/300; Merck Millipore). After washing with PBS–tween, sections were incubated with corresponding fluorescent Alexa fluor – 488 or – 568-conjugated secondary antibodies (1/200; Life Technologies) for 2 h at RT, then washed again and finally mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA). Negative controls for unspecific binding of the secondary antibodies were conducted in parallel sections following the same procedures described above, except the incubation in primary antibodies. For the analyses, the peri-ischemic area was imaged using 10x magnification on an AX70 microscope (Olympus corporation, Tokyo, Japan) coupled to a digital camera (Color View 12, soft Imaging System, Muenster, Germany) using Soft Imaging software. Immunoreactivity were quantified using Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA) at a pre-defined range, measured as the relative immunoreactive area for GFAP. for IL-6, CCL2, TNF-α, IFN-γ, IL-10 and IL-12p70 proteins contained in protein lysates from the contralateral and ipsilateral cortices of sham-operated and ischemic WT and ColXV KO mice treated or not with tPA were measured by using the cytometric bead assay (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer’s instructions. Data were acquired using FACS Calibur (BD Biosciences, San Jose, CA, USA) and analyzed by FCAP Array software (Soft Flow, St. Louis Park, MN, USA).

Cytokine protein expression. IL-6, CCL2, TNF-α, IFN-γ, IL-10 and IL-12p70 proteins contained in protein lysates from the contralateral and ipsilateral cortices of sham-operated and ischemic WT and ColXV KO mice treated or not with tPA were measured by using the cytometric bead assay (BD Biosciences, Franklin Lakes, NJ, USA). Statistical analyses were performed with the Statview software package (v5.0). A Wallis test was used for intergroup multiple comparisons. In significant cases, Mann–Whitney U-test was applied as post hoc test.

Conflict of Interest

The authors declare no conflict of interest.
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Author contributions

JK and SL designed research, analyzed and/or interpreted the data. HD, MH, KK, TM and SL performed experiments. TP, RH and DV provided mice and materials. SL wrote the article, and JK, TP, DV and KK gave critical comments on the draft of the manuscript. All authors read and approved the final version of the manuscript.

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