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Blood vitronectin induces detrimental brain IL-6 and correlates with outcomes after stroke only in female mice

Cuihong Jia, Ph.D., **Hannah M. Malone, B.Sc.**, **Matthew P. Keasey, Ph.D.**, **Chiharu Lovins, M.Sc.**, **Jacob Elam, M.D.**, **Theo Hagg, M.D.,Ph.D.***

Department of Biomedical Sciences, Quillen College of Medicine, East Tennessee State University, Johnson City, Tennessee, USA, 37614

Abstract

Background and purpose: Women have worse stroke outcomes than men, especially after menopause. Few studies have focused on female-specific mechanisms, other than hormones. We investigated the role of the blood protein vitronectin after ischemic stroke in mice.

Methods: Adult male and female vitronectin knockout and wildtype littermates, and C57BL/6 mice received a middle cerebral artery occlusion and the injured brain tissue analyzed 24 hours to 3 weeks later for cell loss and inflammation, as well as neurological function. Blood vitronectin levels were measured before and after stroke.

Results: Intravenously injected vitronectin leaked extensively from bloodstream into brain infarct and penumbra by 24 hours after stroke. Strikingly, vitronectin was detrimental in female, but not male mice, as shown by reduced brain injury (26.2±2.6 vs. 13.4±3.8%, p=0.018, n=6,5) and forelimb dysfunction in female vitronectin knockout mice. Stroke increased plasma vitronectin 2- to 8-fold at 24 hours in females (36±4 vs. 145±24 μg/ml, p<0.0001, n=10,7), but not males (62±8 vs. 68±6, p>0.99, n=10,7), and returned to control levels by 7 days. Individually variable vitronectin levels at 24 hours correlated with stroke-induced brain injury at 7 days only in females. Vitronectin promoted stroke-induced microglia/macrophage activation and leukocyte infiltration in females. Pro-inflammatory interleukin-6 greatly increased in the striatum at 24 hours in wild type mice but was increased ~60% less in female (739±159 vs. 268±111, p=0.02, n=7,6), but not male (889±178 vs. 1179±295, p=0.73, n=10,11), knockout mice. In individual wildtype females, plasma vitronectin levels correlated with striatal interleukin-6 expression at 24 hours. The female-specific effect of vitronectin-induced interleukin-6 expression following stroke was not due to gonadal hormones, as shown by ovariectomy and castration. Lastly, intrastriatal injection of interleukin-6 in female mice immediately before stroke reversed the vitronectin knockout phenotypes of reduced brain injury and microglia/macrophage activation.

Conclusion: Vitronectin plays a novel sexually dimorphic detrimental pathophysiological role in females and might ultimately be a therapeutic target to improve stroke outcomes in women.

*Correspondence: Dr. Theo Hagg, PO Box 70582, East Tennessee State University, Johnson City, TN 37614, haggt1@etsu.edu, Phone: 423-439-6294.

Author contributions:

CJ, MPK and TH designed research, analyzed the data and wrote the paper. CJ, HM, MPK, CL and JE performed experiments.

Disclosures: None;

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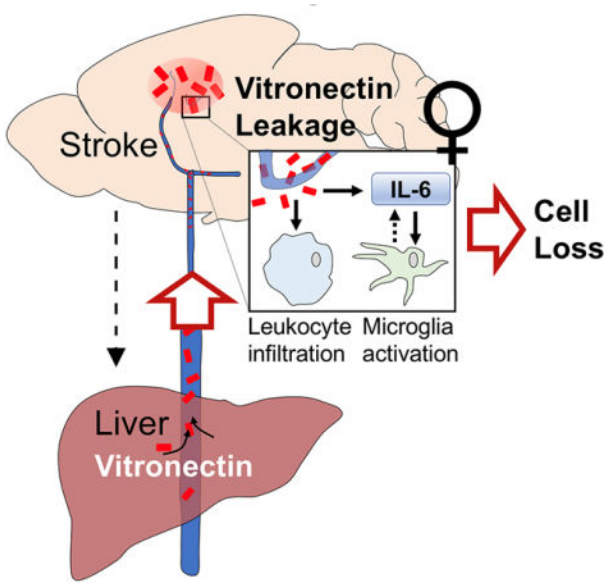
Graphical Abstract



Blood vitronectin, produced by the liver, leaks into injured brain regions after ischemic stroke in mice. Vitronectin worsens cell loss and neurological dysfunction only in females, through increased local IL-6 and inflammation. In females, stroke causes an increase in blood vitronectin levels at 24 h, which correlates with worse outcomes. Vitronectin might be a target to improve stroke outcomes in women.

Keywords

blood protein; cell loss; inflammation; ischemic stroke; prognostic; sex dimorphism

Subject Terms:

Stroke and Neuroscience

Introduction

Women have worse outcomes after ischemic stroke, particularly after menopause[1,2,3]. Early treatment with tissue plasminogen activator has more robust effects in women[4]. Despite sex differences in stroke patients, few preclinical and clinical studies focus on sex-specific mechanisms, other than hormones[2,3]. A better understanding of female-specific stroke mechanisms will help to develop novel strategies for stroke prevention, diagnosis and treatments in women.

Vitronectin (VTN) is a glycoprotein produced primarily by liver and released into the bloodstream, and affects hemostasis, immune responses, cell migration and attachment[5]. Blood VTN[6,7], fibrinogen[8] and fibronectin[9] do not normally enter into brain, but leak across regions of disrupted blood brain barrier under pathological conditions. VTN immunostaining

is present after ischemia-induced cerebral hemorrhagic transformation in primates[7]. VTN is one of the most abundant blood proteins, with a large variation in individual values in healthy humans and rodents[10]. Higher levels are associated with worse outcomes in coronary disease[11,12] and traumatic brain injury[13]. Blood fibrinogen levels are associated with stroke severity in humans[14] but it is not known whether VTN would be detrimental, or beneficial like leaked plasma fibronectin which reduces stroke injury in male mice[9].

The pathophysiological mechanisms and consequences of leaked VTN are not well-understood. VTN activates microglia in vitro and promotes matrix metallopeptidase-9 expression[15]. Microglia and macrophages contribute to detrimental inflammation after stroke in part by producing pro-inflammatory cytokines[16]. We previously found that VTN was unique among five tested ECM and plasma proteins, including fibronectin and fibrinogen, to induce interleukin-6 (IL-6) in vitro, and that VTN promotes IL-6 expression in the brains of naïve mice and after intracerebral hemorrhage[6,17]. IL-6 increases rapidly after stroke from almost non-existent levels[18,19] and induces cytotoxic cytokines such as TNFα[20,21]. IL-6 reportedly is beneficial after stroke but those studies used males[22,23]. The mechanism(s) that induce IL-6 after stroke may involve NMDA receptors but remains largely unresolved[24].

Here, we compared the role of VTN-induced IL-6 on stroke outcomes in male and female mice.

Materials and Methods

This manuscript follows ARRIVE guidelines and adheres to the AHA Journals' implementation of Transparency and Openness Promotion Guidelines. Additional methodological details are provided in the supplemental material. All data, analytic methods, and study materials are available from the corresponding author upon reasonable request. All analyses were done blinded to the genotype, sex or treatment.

Mice

A total of 317 mice were used. VTN breeders (B6.129S2(D2)-Vtn<tm1Dgi>/J, JAX 004371[25]; backcrossed 13 times to C57BL/6) and C57BL/6 mice (JAX 000664) were from Jackson Laboratory. Heterozygotes were bred to yield VTN+/+ (wildtype) and VTN−/− littermates. All animal work was approved by our University Committee on Animal Care and complied with the NIH Guide on Care and Use of Animals.

Surgical procedures

Mice were anesthetized with Avertin (i.p., 400 mg/kg 2,2,2-tribromoehtanol in 20 ml of 2% 2-methyl-2-butanol in saline, T48402, Sigma). A reliable unilateral 30 min MCAO or sham (without filament insertion) was performed in 12–16 week old mice. In some mice, 1 μl of PBS or recombinant human IL-6 (100 ng, 200–06, Peprotech) was stereotactically injected into the middle of ipsilateral striatum immediately before MCAO. In others, recombinant human VTN (rhVTN, 50 μg/100 μl saline, SPR3186, Sigma) was injected via the right jugular vein. This dose (50 μg/1.7 ml plasma for a 25g mouse equals 29.4 μg/ml) mimics normal plasma VTN levels (16–24 μg/ml[26], 29.5 μg/ml in C57BL/6 and 35.6 μg/ml in VTN

+/+ females, as described in the results). Cases of ovariectomy or castration were performed two weeks before MCAO.

Motor function tests

Forelimb motor functions were tested by a neurological deficit score system at 24 hours after MCAO and from videos taken during cylinder and grid walk tests at −1, 7, 14 and 21 days.

Histological analyses

Series of every sixth 30 μm thick sections through the injury were stained with antibodies (Supplementary Table I) and imaged in their entirety using mosaic stitching and 10x objectives. Injury size was measured by the GFAP-negative area surrounded by activated GFAP-positive astrocytes, or the area with cell loss (Cresyl Violet, NeuN staining), and normalized to the contralateral side (Image J software, NIH). Inflammation was quantified by the area and intensity/density of CD68 and CD45 staining.

Protein and mRNA measurements

Plasma VTN concentrations were measured by ELISA (MVNKT-TOT, Molecular Innovations) and in the injured striatum by western blot. Striatal IL-6, CNTF or LIF mRNA levels were quantified by Taqman probe RT-qPCR.

Statistical analyses

Data are presented as mean±SEM (or SD as indicated). Significance was set at $p<0.05$ and determined by t test (2 groups), one- or two-way ANOVA followed by Bonferroni post hoc tests (more than 2 groups), repeated measures ANOVA, or Pearson correlation analysis (GraphPad Prism 6.0).

Results

Blood VTN leaks into injured brain regions following stroke

To show that VTN leaks from the bloodstream into the brain, male and female VTN−/− mice were injected intravenously with rhVTN 24 hours after MCAO. Four hours later, extensive and intense VTN staining was seen in the injured striatum and cortex of both male and female mice, with no sex difference in the VTN-positive area and integrated density (Fig. 1A, B). VTN was present around microvessels and in the parenchyma among neurons (Fig. 1C). Peri-vascular VTN was also present in C57BL/6 mice 24 hours after MCAO (Supplementary Fig. IA), and increased after intravenous injection of rhVTN (Supplementary Fig. IB) despite reduction in local VTN mRNA (Supplementary Fig. IC). Thus, substantial amounts of VTN leak into the injured brain tissue over the first day following MCAO.

VTN increases stroke injury only in females

In female VTN−/− mice, the area of cell loss at 14 days after MCAO was decreased from 26% to ~17% of the contralateral hemisphere compared to VTN+/+ littermates, as measured in cresyl violet-stained brain sections and confirmed by GFAP staining (Fig. 2A). The

reduction in cortical blood flow during the MCAO was not different between VTN+/+ and VTN−/− female (Fig. 2B, left) or male mice (not shown) in any of the experiments. Neurological deficit scores for the affected forelimb at 24 hours were not different between VTN+/+ and VTN−/− females (Fig. 2B, right). Thus, the MCAO conditions and initial stroke injury was comparable between the two genotypes. However, motor function of the affected forelimb was improved in VTN−/− compared to VTN+/+ females at 7 days and beyond (Fig. 2B), as shown by the higher limb use index in a cylinder test and fewer placement errors in grid walking. This genotype difference remained after 7 days, suggesting that VTN plays a role during the first week following stroke. In these females, injury size was defined by the area of obvious loss of the neuronal nuclear maker NeuN (Fig. 2C, Supplementary Fig. IIB) because GFAP had appeared within the injured tissue (Supplementary Fig. IIA), making analyses difficult. These VTN−/− females had a smaller injury area (Fig. 2C). Injury size correlated to worse motor performance (Figure 2C). Remarkably, male VTN−/− and VTN+/+ mice showed no difference in injury size (Fig. 2D) or 24 hour IL-6 levels (see below). Therefore, we did not perform behavioral studies. The injury size was not different between VTN+/+ females and males (Fig. 2A,D, 26.2±2.3% vs. 20.6±1.8, p=0.095). Others found that female mice have smaller infarct sizes, as measured by TTC up to 3 days. Our results at 1–2 weeks, using histology noted to be good methods for chronic damage[27], are consistent with findings that sex differences are not maintained after 7 days[28].

Plasma VTN levels increase only in female mice after stroke and correlate with brain injury

Strikingly, plasma VTN was increased 4-fold at 24 hours after MCAO in females but remained the same in males (Fig. 3A, left). The concentrations before MCAO were not significantly different between females and males. In C57BL/6 mice, levels also increased only in females (Fig. 3A, middle vs. right). In another study, repeated measurements in C57BL/6 mice showed that VTN had returned to normal levels in females by 7 days after MCAO, without changes in males (Fig. 3B). In individual C57BL/6 females, the highly variable VTN concentrations at 24 hours after MCAO correlated positively to brain injury size at 7 days (Fig. 3C, left) but not in males (Fig. 3C, right). This again suggests that VTN plays a unique detrimental role in females. Lastly, as a control, we showed that plasma VTN in female C57BL/6 mice was increased at 24 hours after the MCAO compared to both naïve and sham operated mice (Fig. 3D).

VTN promotes cerebral inflammation following stroke in females

Activated microglia and macrophages exacerbate cell loss after stroke[19]. The area and density of CD68-positive activated microglia/macrophages (Fig. 4A) and infiltration of CD45-positive leukocytes (Fig. 4B) was reduced in female VTN−/− mice at 14 days after MCAO. Plasma VTN concentrations at 24 hours positively correlated with CD68 and CD45 density within the injured area at 7 days after MCAO in the same C57BL/6 females (Fig. 4C). In C57BL/6 males, no correlation was detected (Fig. 4D).

VTN promotes IL-6 expression acutely following stroke only in female mice

At 24 hours following MCAO, VTN+/+ female mice had a 738-fold increase in striatal IL-6 mRNA compared to sham-operated ones, but this extreme induction was markedly reduced

in VTN−/− females to 276-fold (Fig. 5A). The levels of IL-6 in sham females were not different between genotypes. The closely related IL-6 family members CNTF and LIF were induced to the same extent in both genotypes, suggesting a specificity of VTN in promoting IL-6 expression. In males, IL-6, CNTF and LIF induction was not significantly different between genotypes (Fig. 5B), suggesting that VTN does not regulate these cytokines in males after MCAO. Further, MCAO-induced IL-6 in female and male VTN+/+ mice was comparable and not significantly different. The female-specific VTN-induced IL-6 following MCAO is independent of gonadal hormones because the different levels of IL-6 between VTN+/+ and VTN−/− females after MCAO are not affected by ovariectomy, nor by castration in males (Supplementary Fig. III).

Supporting the idea that leaked blood VTN acutely induces striatal IL-6 mRNA at 24 hours after MCAO in females, we found a positive correlation between VTN and IL-6 in the same female VTN+/+ and C57BL/6 mice (Fig. 5C). CNTF and LIF mRNA expression levels also positively correlated to plasma VTN concentrations. IL-6 also correlated to CNTF (r=0.76, p=0.003) and LIF (r=0.85, p=0.0004) levels, consistent with our finding that intrastriatal IL-6 injection regulates their expression[21]. In contrast, in males, there were no correlations of plasma VTN with striatal IL-6, CNTF and LIF (Fig. 5D).

Acute IL-6 expression is a major contributor to the detrimental effects of VTN after stroke

To determine whether VTN is detrimental in female mice by inducing excessive IL-6, we performed a “rescue” experiment by injecting IL-6 into the striatum of VTN+/+ and VTN−/− females immediately before the MCAO. This mimics the peak IL-6 expression at 24 hours that returns to baseline levels by 48–72 hours after MCAO[18,29]. Because intrastriatal injection of IL-6 increased GFAP in injured regions making it difficult to define the injury area (Supplementary Fig. IV), we used NeuN. The IL-6 injection did not affect the injury size in VTN+/+ mice compared to PBS, as measured by the area absent of NeuN staining in the striatum (Fig. 6A,B). In VTN−/− mice, the reduced injury size (PBS) was reversed by the IL-6 injection. The IL-6 injection also reversed the reduced microglia/macrophage activation seen in VTN−/− mice (Fig. 6C). It did not affect leukocyte infiltration in VTN−/− mice (Fig. 6D).

Discussion

VTN is a novel female-specific detrimental protein after stroke

Our results suggest that blood VTN leakage into the brain was detrimental after ischemic stroke in a female-specific pathophysiological mechanism. Following MCAO, VTN entered male and female brains equally but only VTN−/− females had smaller injuries and plasma VTN levels correlated with injury in females only. VTN induced IL-6 in female brains after stroke, leading to increased inflammation and cell loss, and worse neurological recovery. It remains to be determined whether injection of VTN or overexpression in the liver would “rescue” the VTN−/− phenotype. This study identified blood VTN as a first-in-class detrimental female-specific soluble protein after ischemic stroke. Potential mechanisms underlying the sex differences remain to be identified but might include VTN isoforms and

VTN integrin receptor expression and downstream intracellular signaling pathways in the cells it binds to.

A number of mechanisms show sexual dimorphism after ischemic stroke[1,3]. Female rodents initially have smaller injuries and better functional outcomes due to the neuroprotective effect of estrogen and progesterone[1,30,31]. Our data suggest that gonadal sex hormones do not explain the effects of VTN on MCAO-induced harmful IL-6 in females, although we cannot exclude other sources. It remains to be confirmed that the hormones do not affect VTN-mediated MCAO infarct size. None of the more than 800 genes on the X chromosome appear to be connected to VTN or its signaling. Stroke-induced cell death is affected more by mitochondrial cytochrome-c release and caspase activation in females than in males[32]. Moreover, increased caspase activity by inhibition of X-link inhibitor of apoptosis enhances stroke-induced injury only in females[33]. Pannexin1, a gap-junction membrane channel, is also detrimental after ischemic stroke in females, but not males[34]. Besides the well-known protective role of estrogen and progesterone, the anti-inflammatory cytokine IL-4 is required for female-specific protection in mice[35]. Poly(ADP-ribose) polymerase-1 pathway is detrimental only in males[36,37]. TRPM2 channels are detrimental only in male mice[38], whereas hemeoxygenase-1/Wnt signaling protects males, but not females[39]. It remains to be determined whether or not VTN signaling intersects with any of these sex-specific pathophysiological mechanisms.

We cannot exclude the contribution of VTN produced by pericytes following MCAO[6]. The majority of VTN derives from the bloodstream, as shown by the leaked rhVTN injected into VTN−/− mice at 24 h, consistent with BBB breakdown[40], and reduced local VTN mRNA.

Variably induced plasma VTN levels positively correlate to brain injury outcomes after stroke

The VTN−/− data, showing reduced injury only in females, suggest that when VTN leaks into the brain, it is detrimental in females, but not in males, despite similar baseline plasma levels and leakage. Plasma VTN levels in females were increased by 24 hours after the MCAO and were reduced again to normal levels by 7 days, acting like other acute phase proteins made by the liver[41]. The substantially different individual levels of VTN in females after MCAO correlated with worse outcomes such as the injury size. Plasma VTN vary among presumed healthy people and also in patients[10,11,12], raising the possibility that prior higher plasma VTN levels caused by other diseases would impact stroke outcomes. High plasma VTN is associated with worse outcomes after cardiovascular disease[10,11,12,25]. It will be important to determine whether reducing VTN levels before or after, stroke would be beneficial. The acute nature of the increased VTN is consistent with the functional motor improvement in VTN−/− female mice by 7 days which is retained afterward. This suggests that treatments intervening with the acute VTN spike would result in lasting neuroprotection. It remains to be determined what regulates VTN in the liver but could include circulating IL-6, which also correlates to worse outcomes after human stroke[42] and can induce liver VTN in rats[43]. It will be important to test whether pharmacological drugs that reduce VTN production or integrin signaling would lack neuroprotective efficacy in VTN−/− females and might be specific treatments for females.

Female-specific harmful VTN-induced IL-6 and microglial activation

Ischemic stroke rapidly induces IL-6 expression in the brain that peaks around 24 hours and is reduced again after 2 days[18,24]. This peaking response paralleled MCAO-induced acute increase of plasma VTN in female mice and overlapped with the acute VTN leakage into the brain. Moreover, elevated plasma VTN correlated positively to IL-6 expression in the female striatum at 24 hours after MCAO. Indeed, VTN rapidly increases IL-6 expression in cultured cells and in naïve mice[17]. In female VTN−/− mice, the more than 700-fold MCAO-induced striatal IL-6 was reduced by ~60% compared to VTN+/+ females. Thus, VTN substantially, although not exclusively, regulates IL-6 expression in females. IL-6 is also much reduced but not completely blocked in VTN−/− mice in a hemorrhage model[17]. Thus, other mechanisms, including other blood-derived proteins probably play a role in inducing IL-6 as well, including the high concentrations of fibrinogen and fibronectin, which can induce inflammation in the brain[15]. In males, IL-6 increased to similar levels as in females, yet did not correlate with plasma VTN levels at 24 hours, which did not correlate with injury size at 7 days. This suggests that brain IL-6 is not detrimental in males, which would not be inconsistent with the apparent beneficial role of blood IL-6[22,23]. It remains to be determined which brain cells respond differently to IL-6, and which cells respond to VTN to produce IL-6 in females, but not in males. VTN can activate microglia[14] and promote macrophage adhesion[15,44]. Microglia play a role in BBB breakdown after stroke[45]. Thus, VTN leakage in females may exacerbate microglia activation and macrophage infiltration, which play a predominantly detrimental role[46] and produce IL-6 after stroke[47]. Indeed, the female VTN−/− mice had reduced microglia/macrophage activation and leukocyte infiltration after MCAO. Microglia have a sexually dimorphic response to ischemic stroke[48]. Astrocytes also produce IL-6 after stroke[24] and may play a sexually dimorphic role because IL-6 deletion in astrocytes reduces demyelination and inflammation in a multiple sclerosis mouse model only in females[49]. IL-6 reportedly is beneficial after ischemic stroke in male mice[22]. Here, female VTN−/− mice had reduced IL-6 expression and their smaller injuries after MCAO were reversed by intrastriatal IL-6 injection. This suggests that VTN-induced IL-6 after stroke in females is detrimental. The IL-6 injection did not affect leukocyte infiltration in either VTN+/+ and VTN−/− females, suggesting that leaked blood VTN activates local brain cells, including microglia.

Conclusions

This study identified blood VTN as a first-in-class detrimental female-specific blood protein after ischemic stroke that acted by inducing excessive IL-6 expression in the brain. It points to opportunities to investigate whether VTN blood levels are prognostic for stroke outcomes and tailoring neuroprotective treatments that target VTN in women.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Kim T, Chelluboina B, Chokkalla AK, Vemuganti R. Age and sex differences in the pathophysiology of acute cns injury. Neurochem Int. 2019;127:22–28 [PubMed: 30654116]
2. Ahnstedt H, McCullough LD, Cipolla MJ. The importance of considering sex differences in translational stroke research. Transl Stroke Res. 2016;7:261–273 [PubMed: 26830778]
3. Bushnell CD, Chaturvedi S, Gage KR, Herson PS, Hurn PD, Jimenez MC, et al. Sex differences in stroke: Challenges and opportunities. J Cereb Blood Flow Metab. 2018;38:2179–2191 [PubMed: 30114967]
4. Sohrabji F, Park MJ, Mahnke AH. Sex differences in stroke therapies. J Neurosci Res. 2017;95:681–691 [PubMed: 27870437]
5. Preissner KT, Reuning U. Vitronectin in vascular context: Facets of a multitalented matricellular protein. Semin Thromb Hemost. 2011;37:408–424 [PubMed: 21805447]
6. Jia C, Keasey MP, Malone HM, Lovins C, Sante RR, Razskazovskiy V, et al. Vitronectin from brain pericytes promotes adult forebrain neurogenesis by stimulating cntf. Exp Neurol. 2019;312:20–32 [PubMed: 30408465]
7. del Zoppo GJ, Frankowski H, Gu YH, Osada T, Kanazawa M, Milner R, et al. Microglial cell activation is a source of metalloproteinase generation during hemorrhagic transformation. J Cereb Blood Flow Metab. 2012;32:919–932 [PubMed: 22354151]
8. Petersen MA, Ryu JK, Akassoglou K. Fibrinogen in neurological diseases: Mechanisms, imaging and therapeutics. Nat Rev Neurosci. 2018;19:283–301 [PubMed: 29618808]
9. Sakai T, Johnson KJ, Murozono M, Sakai K, Magnuson MA, Wieloch T, et al. Plasma fibronectin supports neuronal survival and reduces brain injury following transient focal cerebral ischemia but is not essential for skin-wound healing and hemostasis. Nat Med. 2001;7:324–330 [PubMed: 11231631]
10. Conlan MG, Tomasini BR, Schultz RL, Mosher DF. Plasma vitronectin polymorphism in normal subjects and patients with disseminated intravascular coagulation. Blood. 1988;72:185–190 [PubMed: 2455567]
11. Derer W, Barnathan ES, Safak E, Agarwal P, Heidecke H, Mockel M, et al. Vitronectin concentrations predict risk in patients undergoing coronary stenting. Circ Cardiovasc Interv. 2009;2:14–19 [PubMed: 20031688]
12. Yaghoubi A, Ghojazadeh M, Abolhasani S, Alikhah H, Khaki-Khatibi F. Correlation of serum levels of vitronectin, malondialdehyde and hs- crp with disease severity in coronary artery disease. J Cardiovasc Thorac Res. 2015;7:113–117 [PubMed: 26430499]
13. Anada RP, Wong KT, Jayapalan JJ, Hashim OH, Ganesan D. Panel of serum protein biomarkers to grade the severity of traumatic brain injury. Electrophoresis. 2018;39:2308–2315 [PubMed: 29570807]
14. del Zoppo GJ, Levy DE, Wasiewski WW, Pancioli AM, Demchuk AM, Trammel J, et al. Hyperfibrinogenemia and functional outcome from acute ischemic stroke. Stroke. 2009;40:1687–1691 [PubMed: 19299642]
15. Milner R, Crocker SJ, Hung S, Wang X, Frausto RF, del Zoppo GJ. Fibronectin- and vitronectin-induced microglial activation and matrix metalloproteinase-9 expression is mediated by integrins alpha5beta1 and alphavbeta5. J Immunol. 2007;178:8158–8167 [PubMed: 17548654]
16. del Zoppo GJ, Milner R, Mabuchi T, Hung S, Wang X, Berg GI, et al. Microglial activation and matrix protease generation during focal cerebral ischemia. Stroke. 2007;38:646–651 [PubMed: 17261708]
17. Keasey MP, Jia C, Pimentel LF, Sante RR, Lovins C, Hagg T. Blood vitronectin is a major activator of lif and il-6 in the brain through integrin-fak and upar signaling. J Cell Sci. 2018;131
18. Kang SS, Keasey MP, Arnold SA, Reid R, Geralds J, Hagg T. Endogenous cntf mediates stroke-induced adult cns neurogenesis in mice. Neurobiol Dis. 2013;49:68–78 [PubMed: 22960105]

19. Lambertsen KL, Biber K, Finsen B. Inflammatory cytokines in experimental and human stroke. J Cereb Blood Flow Metab. 2012;32:1677–1698 [PubMed: 22739623]
20. Barone FC, Arvin B, White RF, Miller A, Webb CL, Willette RN, et al. Tumor necrosis factor-alpha. A mediator of focal ischemic brain injury. Stroke. 1997;28:1233–1244 [PubMed: 9183357]
21. Kang SS, Keasey MP, Hagg T. P2×7 receptor inhibition increases cntf in the subventricular zone, but not neurogenesis or neuroprotection after stroke in adult mice. Transl Stroke Res. 2013;4:533–545 [PubMed: 24312160]
22. Yamashita T, Sawamoto K, Suzuki S, Suzuki N, Adachi K, Kawase T, et al. Blockade of interleukin-6 signaling aggravates ischemic cerebral damage in mice: Possible involvement of stat3 activation in the protection of neurons. J Neurochem. 2005;94:459–468 [PubMed: 15998296]
23. Gronhoj MH, Clausen BH, Fenger CD, Lambertsen KL, Finsen B. Beneficial potential of intravenously administered il-6 in improving outcome after murine experimental stroke. Brain Behav Immun. 2017;65:296–311 [PubMed: 28587928]
24. Suzuki S, Tanaka K, Suzuki N. Ambivalent aspects of interleukin-6 in cerebral ischemia: Inflammatory versus neurotrophic aspects. J Cereb Blood Flow Metab. 2009;29:464–479 [PubMed: 19018268]
25. Zheng X, Saunders TL, Camper SA, Samuelson LC, Ginsburg D. Vitronectin is not essential for normal mammalian development and fertility. Proc Natl Acad Sci U S A. 1995;92:12426–12430 [PubMed: 8618914]
26. Courey AJ, Horowitz JC, Kim KK, Koh TJ, Novak ML, Subbotina N, et al. The vitronectin-binding function of pai-1 exacerbates lung fibrosis in mice. Blood. 2011;118:2313–2321 [PubMed: 21734232]
27. Liu F, McCullough LD. Middle cerebral artery occlusion model in rodents: Methods and potential pitfalls. J Biomed Biotechnol. 2011;2011:464701 [PubMed: 21331357]
28. Craft TK, Glasper ER, McCullough L, Zhang N, Sugo N, Otsuka T, et al. Social interaction improves experimental stroke outcome. Stroke. 2005;36:2006–2011 [PubMed: 16100018]
29. Berti R, Williams AJ, Moffett JR, Hale SL, Velarde LC, Elliott PJ, et al. Quantitative real-time rt-pcr analysis of inflammatory gene expression associated with ischemia-reperfusion brain injury. J Cereb Blood Flow Metab. 2002;22:1068–1079 [PubMed: 12218412]
30. Gibson CL, Coomber B, Murphy SP. Progesterone is neuroprotective following cerebral ischaemia in reproductively ageing female mice. Brain. 2011;134:2125–2133 [PubMed: 21705427]
31. McCullough LD, Mirza MA, Xu Y, Bentivegna K, Steffens EB, Ritzel R, et al. Stroke sensitivity in the aged: Sex chromosome complement vs. Gonadal hormones. Aging (Albany NY). 2016;8:1432–1441 [PubMed: 27405096]
32. Liu F, Li Z, Li J, Siegel C, Yuan R, McCullough LD. Sex differences in caspase activation after stroke. Stroke. 2009;40:1842–1848 [PubMed: 19265047]
33. Siegel C, Li J, Liu F, Benashski SE, McCullough LD. Mir-23a regulation of x-linked inhibitor of apoptosis (xiap) contributes to sex differences in the response to cerebral ischemia. Proc Natl Acad Sci U S A. 2011;108:11662–11667 [PubMed: 21709246]
34. Freitas-Andrade M, Bechberger JF, MacVicar BA, Viau V, Naus CC. Pannexin1 knockout and blockade reduces ischemic stroke injury in female, but not in male mice. Oncotarget. 2017;8:36973–36983 [PubMed: 28445139]
35. Xiong X, Xu L, Wei L, White RE, Ouyang YB, Giffard RG. Il-4 is required for sex differences in vulnerability to focal ischemia in mice. Stroke. 2015;46:2271–2276 [PubMed: 26130091]
36. Liu F, Lang J, Li J, Benashski SE, Siegel M, Xu Y, et al. Sex differences in the response to poly(adp-ribose) polymerase-1 deletion and caspase inhibition after stroke. Stroke. 2011;42:1090–1096 [PubMed: 21311064]
37. Li J, McCullough LD. Sex differences in minocycline-induced neuroprotection after experimental stroke. J Cereb Blood Flow Metab. 2009;29:670–674 [PubMed: 19190654]
38. Jia J, Verma S, Nakayama S, Quillinan N, Grafe MR, Hurn PD, et al. Sex differences in neuroprotection provided by inhibition of trpm2 channels following experimental stroke. J Cereb Blood Flow Metab. 2011;31:2160–2168 [PubMed: 21587268]
39. Tulsulkar J, Ward A, Shah ZA. Ho1 and wnt expression is independently regulated in female mice brains following permanent ischemic brain injury. Brain Res. 2017;1662:1–6 [PubMed: 28219651]

40. Krueger M, Bechmann I, Immig K, Reichenbach A, Hartig W, Michalski D. Blood-brain barrier breakdown involves four distinct stages of vascular damage in various models of experimental focal cerebral ischemia. J Cereb Blood Flow Metab. 2015;35:292–303 [PubMed: 25425076]

41. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. N Engl J Med. 1999;340:448–454 [PubMed: 9971870]

42. Spalletta G, Cravello L, Imperiale F, Salani F, Bossu P, Picchetto L, et al. Neuropsychiatric symptoms and interleukin-6 serum levels in acute stroke. J Neuropsychiatry Clin Neurosci. 2013;25:255–263 [PubMed: 24247852]

43. Seiffert D, Geisterfer M, Gauldie J, Young E, Podor TJ. Il-6 stimulates vitronectin gene expression in vivo. J Immunol. 1995;155:3180–3185 [PubMed: 7545714]

44. Kanse SM, Matz RL, Preissner KT, Peter K. Promotion of leukocyte adhesion by a novel interaction between vitronectin and the beta2 integrin mac-1 (alphambeta2, cd11b/cd18). Arterioscler Thromb Vasc Biol. 2004;24:2251–2256 [PubMed: 15458976]

45. Su EJ, Cao C, Fredriksson L, Nilsson I, Stefanitsch C, Stevenson TK, et al. Microglial-mediated pdgf-cc activation increases cerebrovascular permeability during ischemic stroke. Acta Neuropathol. 2017;134:585–604 [PubMed: 28725968]

46. Iadecola C, Anrather J. The immunology of stroke: From mechanisms to translation. Nat Med. 2011;17:796–808 [PubMed: 21738161]

47. Taylor RA, Sansing LH. Microglial responses after ischemic stroke and intracerebral hemorrhage. Clin Dev Immunol. 2013;2013:746068 [PubMed: 24223607]

48. Rahimian R, Cordeau P Jr, Kriz J. Brain response to injuries: When microglia go sexist. Neuroscience. 2019;405:14–23 [PubMed: 29526689]

49. Erta M, Giralt M, Jimenez S, Molinero A, Comes G, Hidalgo J. Astrocytic il-6 influences the clinical symptoms of eae in mice. Brain Sci. 2016;6

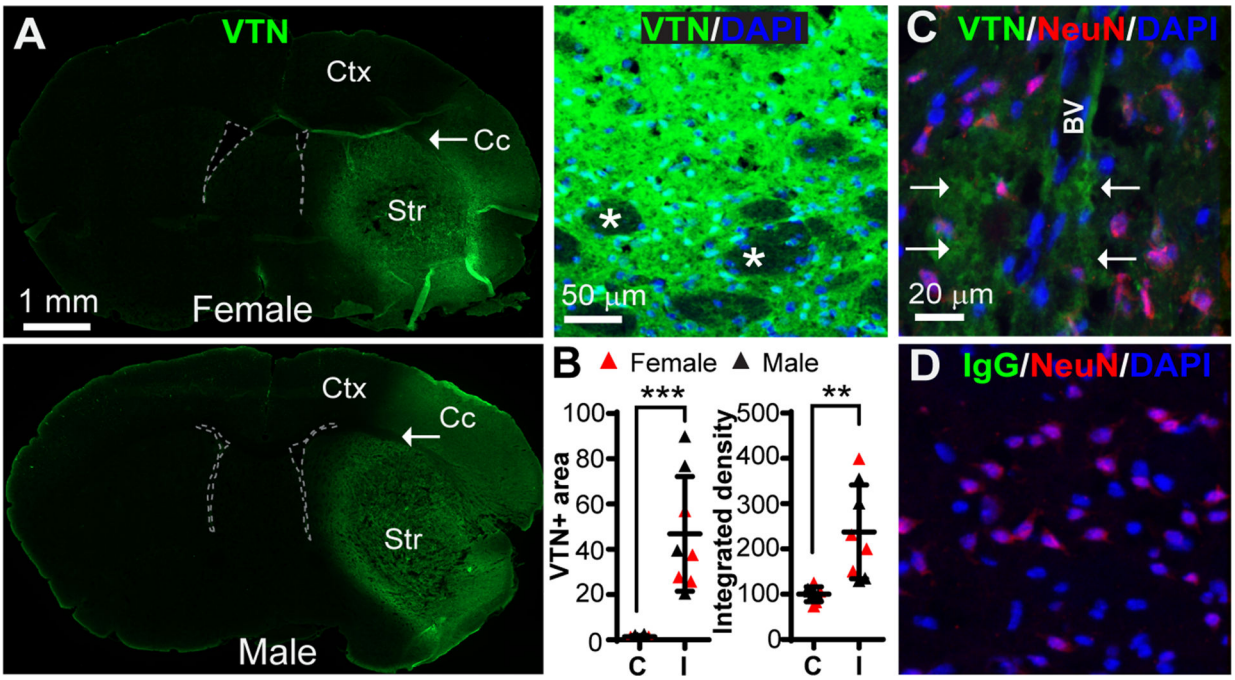

Figure 1. Blood vitronectin (VTN) leaks into brain following ischemic stroke.

A) VTN injected i.v. into VTN−/− mice at 24 hours after a unilateral MCAO leaks into the injured brain regions, as shown by VTN immunostaining 4 h later. Cc, corpus callosum, Ctx, cortex, Str, striatum. Dashed lines delineate the lateral ventricles. A higher magnification shows intense VTN staining in the striatum among DAPI-stained nuclei. * indicate white matter tracts. **B**) The VTN-positive area and VTN integrated density was not different between females and males (N=4,4). **C**) Leaked VTN was present around blood vessels (BV) and in the parenchyma intermixed with NeuN-positive neurons shown in a 0.67 μm thick confocal image. Arrows indicate an area of substantial VTN leakage. **D**) No staining was present using purified IgG as primary antibody, confirming immunostaining specificity.

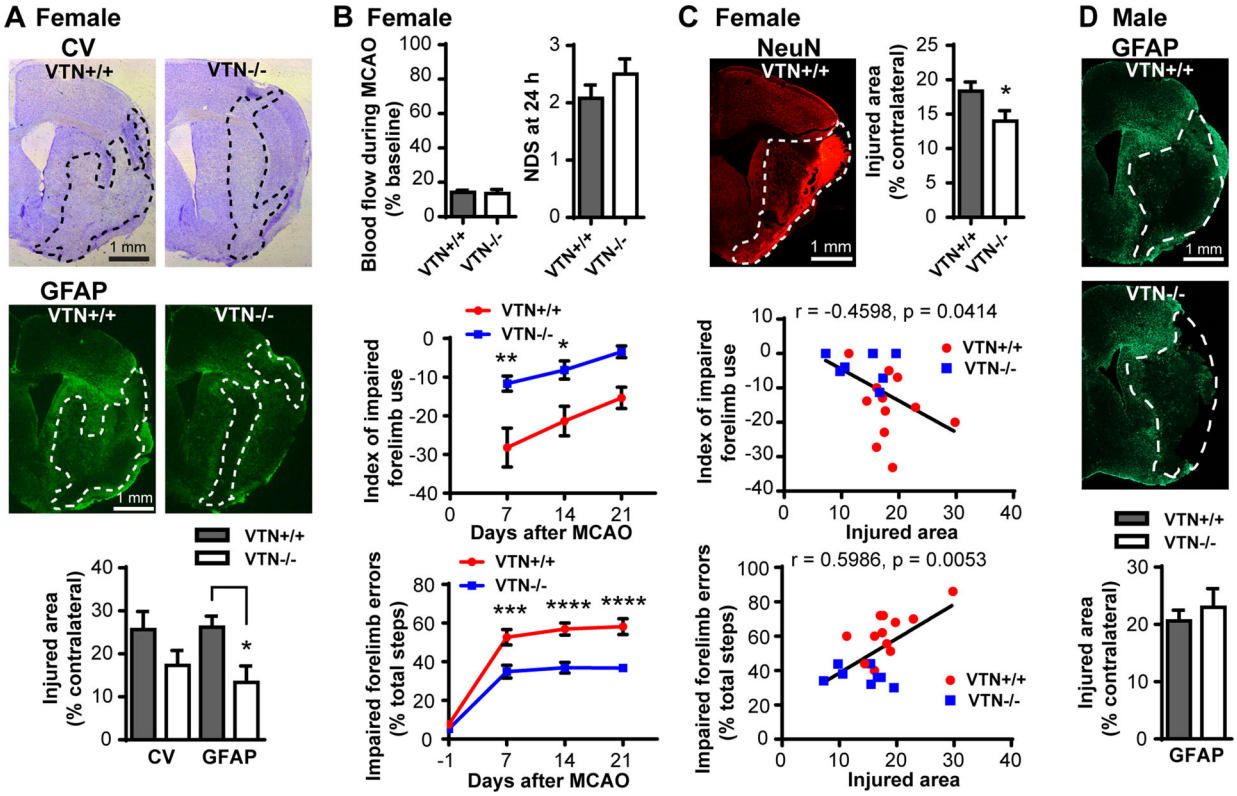

Figure 2. VTN increases stroke injury only in female mice.

A) At 14 days after MCAO, VTN−/− females had smaller injuries (dashed lines) than VTN +/+ females as shown by Cresyl Violet (CV) and GFAP staining and quantification of the area of cell loss (N=6,5, confirmed in Fig. 3 by inflammatory markers). **B**) Reduced cortical blood flow during MCAO and neurological deficit scores (NDS) at 24 hours were not different between genotypes (N=12,8). These VTN−/− females had a higher (better) index of impaired forelimb use in a cylinder test (N=12,8), and made fewer stepping errors with the impaired forelimb in a grid walk test (N=8,8, 4 VTN+/+ excluded for technical video issues). **C**) The injury was identifiable by a NeuN-negative area (dashed lines) at 21 days after MCAO (Supplementary Fig. IIB). The bright area in the injury area is a staining artifact. VTN−/− females had smaller injuries, accounting for substantial ipsilateral hemisphere shrinkage (Supplemental materials) that did not differ between genotypes (79.0% vs. 81.1% of contralateral, p=0.70). The injury size negatively correlated to the impaired forelimb index (lower is worse) and positively to placement errors (higher is worse) at 21 days. **D**) MCAO-induced injured size measured in GFAP-stained sections was similar in VTN+/+ and VTN−/− males at 14 days (N=9,6). * p<0.05, ** p<0.01, *** p<0.001.

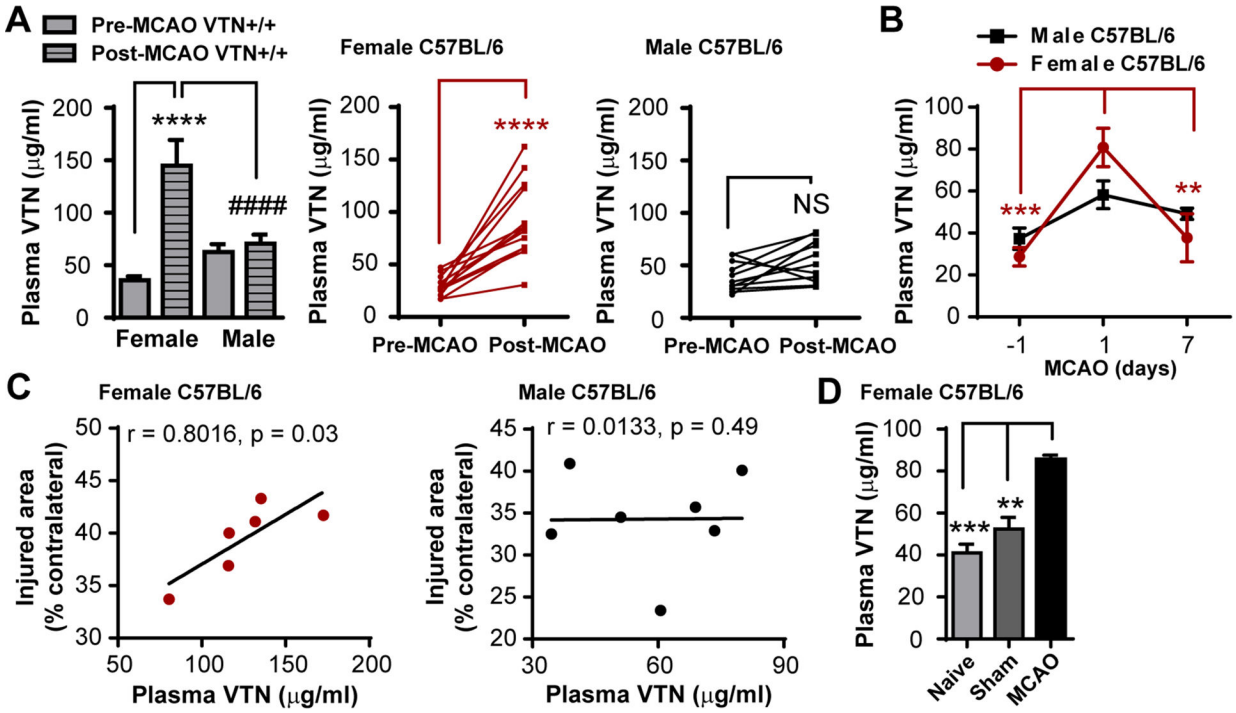

Figure 3. Plasma VTN increases only in female mice after stroke and are prognostic of brain injury.

A) Plasma VTN concentrations were increased 24 hours after compared to 24 hours before MCAO in female, but not male, VTN+/+ (N=10,7,10,12) and C57BL/6 mice (N=13,11). **B**) At 7 days, plasma VTN returned to baseline levels in females, but MCAO did not change plasma VTN in males (N= 6,7, repeated measures). **C**) Plasma VTN concentrations in female, but not male, C57BL/6 mice at 24 hours after MCAO correlated to injury size at 7 days, measured in GFAP-stained sections (N=6,7). **D**) MCAO increased plasma VTN at 24 hours compared to both naïve and sham operated C57BL/6 female mice (N=6,7,4). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** or #### $p<0.0001$

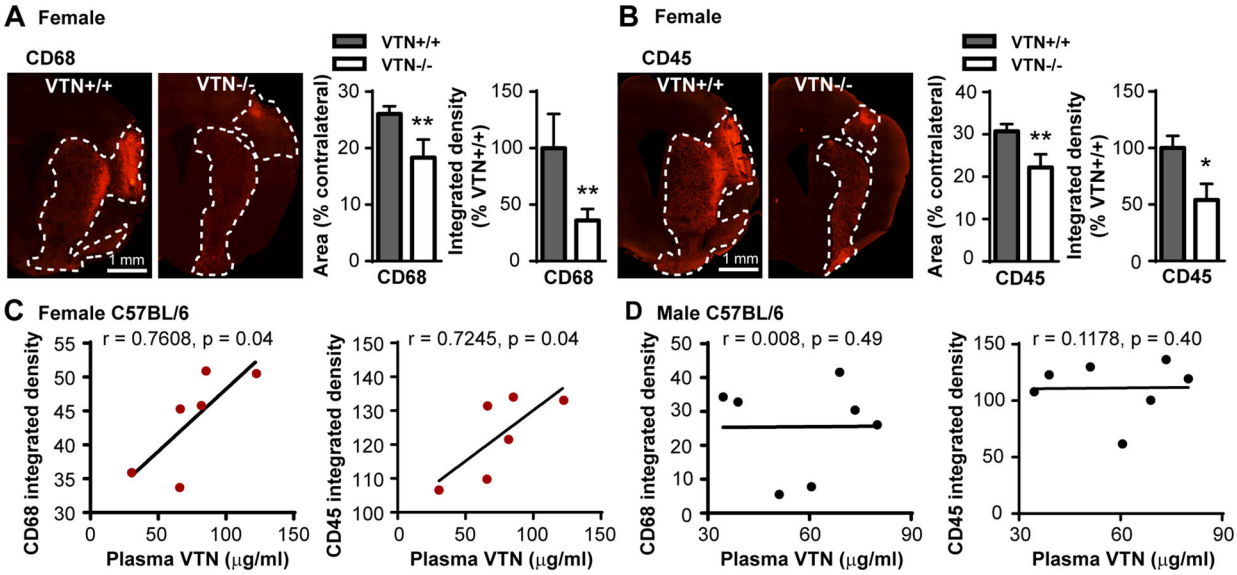

Figure 4. VTN promotes microglia/macrophage activation and leukocyte infiltration in females following stroke.

At 14 days after MCAO, VTN−/− female mice showed less **A**) CD68-positive activated microglia/macrophages and **B**) CD45-positive infiltrated leukocytes than VTN+/+ females, as shown by area and density measurements (N=6,5; * $p<0.05$, ** $p<0.01$). Dashed lines delineate injured areas with dense immunostained cells. **C**) Plasma VTN concentrations in female C57BL/6 mice at 24 hours correlated to CD68 and CD45 density at 7 days after MCAO (N=6). **D**) No correlation was present in males (N=7).

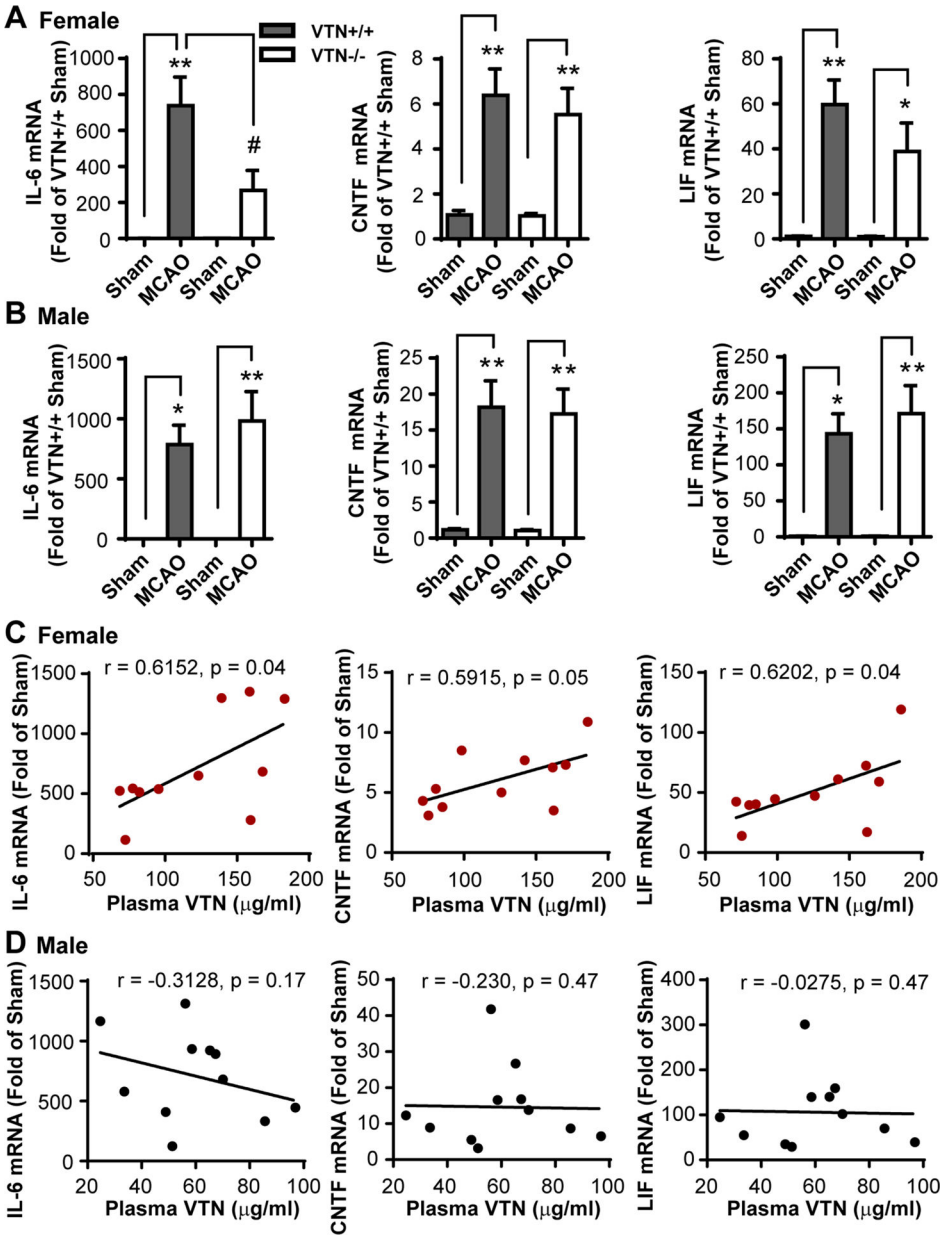

Figure 5. VTN promotes IL-6 expression acutely following MCAO only in female mice.

A) In VTN−/− females, MCAO-induced IL-6 in the injured striatum at 24 hours after MCAO was much less compared to VTN+/+ females. There were no genotype differences in MCAO-induced ciliary neurotrophic factor (CNTF) or leukemia inhibitory factor (LIF). N=5,7,6,6, * or # p<0.05, ** p<0.01. **B**) Males did not show genotype differences in MCAO-induced IL-6, CNTF or LIF (N=6,10,8,11). Plasma VTN concentrations correlated with IL-6, CNTF and LIF expression in females **C**), but not males **D**), N=11/group.

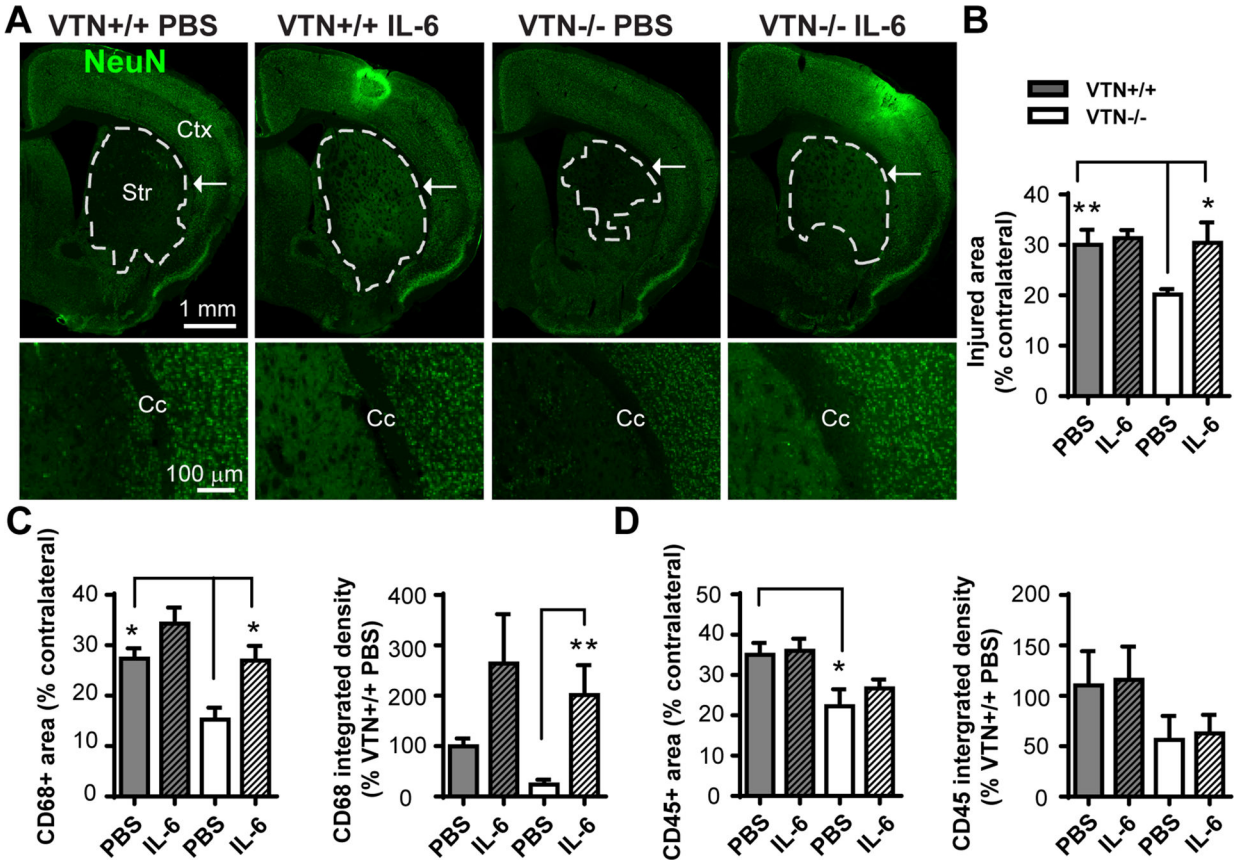

Figure 6. IL-6 is a major contributor to the detrimental effects of VTN after stroke.

A) Loss of NeuN-positive neurons (dashed lines) was reduced in VTN−/− females when injected with PBS into the striatum just before MCAO. IL-6 injection reversed this neuroprotective phenotype in VTN−/− females (right panels). High magnification images of areas indicated by arrows are shown below. Cc, corpus callosum, Ctx, cortex, Str, striatum. **B**) Quantification of the area of neuronal loss. **C**) IL-6 injection also reversed the VTN−/− phenotype of reduced microglia/macrophage activation (CD68 area and density), but **D**) did not affect genotype differences in CD45-positive leukocyte infiltration (N=8,6,5,5). * $p<0.05$, ** $p<0.01$.