

Inhibition of PAR1 attenuates meningeal macrophage activation and vascular inflammation in a rat model of thrombin-induced hydrocephalus

Xiaodi Hao

Zhejiang University <https://orcid.org/0000-0003-0584-9217>

Xianghua Ye

Zhejiang University

Dan Shen

Zhejiang University

Chensheng Le

Zhejiang University

Lusha Tong

Zhejiang University

Feng Gao (✉ 2202012@zju.edu.cn)

Zhejiang University

Research

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Abstract

Background and Purpose— Our previous studies demonstrated that intraventricular injection of thrombin could induce hydrocephalus. The inflammation of subarachnoid space plays a key role in hydrocephalus. As thrombin, inducing coagulation, could contribute to inflammation, its effects on subarachnoid space have not been well studied. Macrophagic dysfunction may contribute to this course. However, the mechanisms that how thrombin affects macrophage in subarachnoid space have not been illustrated. Our aim was to explore the possible role that macrophage played in thrombin-induced meningeal inflammation, and to furtherly understand its contribution during thrombin-induced hydrocephalus.

Methods— There were two parts in this study. Firstly, rats had an intraventricular injection of saline or thrombin. Secondly, rats received thrombin injection with vehicle or PAR1 antagonist treatment. Immunofluorescence staining was applied to observe the activation of meningeal macrophage and the expression of NeuN in the cortex. Meanwhile, the expression of intercellular adhesion molecule 1 (ICAM1) in meningeal vessels were tested to detect the vascular inflammation. Western blot was applied to measure the secretion of pro-inflammatory cytokines (IL-1 β and IFN γ).

Results— Our results demonstrated that intraventricular injection of thrombin caused significant activation of meningeal macrophages, vascular inflammation, and neuron loss. Inhibition of PAR1 pathway attenuated the M1 polarization of meningeal macrophage, reduced the inflammatory infiltrations and prevented the neuron loss, as well as hydrocephalus after thrombin injection.

Conclusions— Clinically available PAR1 antagonists may offer a novel therapeutic approach candidate for the prevention or the management of inflammation in hemorrhage-induced hydrocephalus.

Background

Hydrocephalus is an independent risk factor of hemorrhage-associated morbidity [1]. Our previous study found that thrombin played a critical role in hydrocephalus [2, 3]. Cerebrospinal fluid (CSF) is mainly produced in choroid plexus and absorbed in subarachnoid space, and the key point of hydrocephalus should be the changes in formation or absorption of CSF [4, 5]. Thrombin is produced immediately after hemorrhage stroke and plays an essential role in brain damage after intracerebral hemorrhage (ICH) [2, 3, 6, 7]. Thrombin could disrupt blood-cerebrospinal fluid (CSF) barrier and generate hydrocephalus via proteinase-activated receptor 1 (PAR1) pathway, which leads to vascular endothelial-cadherin (VE-cadherin) downregulation in choroid plexus [2, 3].

Inflammation in the ventricular system is strongly associated with the blood-CSF barrier and hydrocephalus formation [8–12]. Subarachnoid space plays a critical role in cerebrospinal fluid absorption, the dysfunction of which would contribute to hydrocephalus [5, 10, 13]. Acute inflammatory cell infiltration in subarachnoid space caused the transformation of pial cells into macrophages, promoted fibroblasts and extracellular matrix deposition leading to subarachnoid fibrosis and hydrocephalus [10, 13, 14]. The meningeal macrophage is one type of non-parenchymal macrophages

that mediate immune responses at brain boundaries [15, 16]. The relationship between thrombin and meningeal inflammation is still unknown. In this study, the thrombin-induced inflammation in subarachnoid space was investigated.

The high-affinity thrombin receptor, PAR1, has been implicated in mediating the interaction between coagulation and inflammation [17]. The role of inflammation and PAR1 in thrombin-induced hydrocephalus needs to be further elucidated. In this study, we aimed to explore the role of thrombin in brain injury and further illustrate the mechanism of hemorrhage-associated hydrocephalus.

Methods

Animals model

Animal protocols were approved by and conducted in accordance with the ethical guidelines of the Zhejiang University Animal Experimentation Committee and were in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. A total of 32 male Sprague–Dawley rats (3-month old, Zhejiang University Laboratories), at the weight of 280–320 g, were used in this study. Animals were anesthetized with pentobarbital (50 mg/kg intraperitoneally (IP)) and were positioned in a stereotaxic frame. A cranial burr hole (1 mm) was drilled and a 26-gauge needle was inserted stereotaxically into the right lateral ventricle (coordinates: 0.6 mm posterior, 4.5 mm ventral, and 1.6 mm lateral to the bregma). Saline or thrombin (3U in 50 μ l) was infused using a microinfusion pump (World Precision Instruments). The needle was removed after injection, the burr hole was filled with bone wax, and the skin incision was sutured.

Experimental groups

In brief, the rats were randomly divided into two parts. First, rats received an intracerebroventricular (ICV) injection of 3 units rat thrombin (Sigma-Aldrich, St Louis, MO, USA) in saline or saline alone (50 μ l) in 7 minutes and were euthanized for intracardiac perfusion and the brains were used for Western blot (n = 4 for each group), brain histology (n = 4 for each group) at 24 hours. Second, rats were treated with vehicle (1% dimethyl sulfoxide in saline) or PAR1 inhibitor (SCH79797, Abcam, USA, 25 μ g/kg) immediately while thrombin infusion. Rats were treated with SCH79797 at a dosage accordingly [3, 7]. The rats were euthanized at 24 hours after injection and the brains were used for western blot (n = 4 for each group) and brain histology (n = 4 for each group).

Immunohistochemistry and HE staining

Rats were anesthetized with pentobarbital (100 mg/kg, IP) and perfused with 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (pH 7.4). The brains were removed and kept in 4% paraformaldehyde for 24 hours and then immersed in 30% sucrose for 2 to 3 days at 4°C. Brains were embedded in optimal

cutting temperature compound and 18- μ m thick slices cut using a cryostat (LEICA, CM3050S). Immunohistochemistry and HE staining were performed as previously described. The primary antibodies were goat anti-Iba1 (1:400 dilution; Abcam, Cambridge, MA, USA), mouse anti-CD68 (1:100 dilution; Abcam, Cambridge, MA, USA), mouse anti-NeuN (1:200 dilution; Abcam, Cambridge, MA, USA), rabbit anti-CD206 (1:200 dilution; Abcam, Cambridge, MA, USA).

Western blot analysis

Briefly, brain tissue was immersed in western sample buffer and lysed. The lysate was centrifuged at 12000 r/min for 30 min at 4°C and the supernatant was collected. Protein concentration was determined by the BCA protein assay kit (Pierce, Thermo Scientific, USA). Protein samples were separated using SDS-polyacrylamide gel electrophoresis and transferred to the nitrocellulose membrane. After blocking with 5% nonfat milk, the membranes were incubated with primary antibodies anti-IL-1 β (1:2000 dilution; Abcam, Cambridge, MA, USA), anti-IFN γ (1:2000 dilution; Abcam, Cambridge, MA, USA) and anti-GAPDH (1:3000; KangChen, Shanghai, China) at 4°C overnight. After repeated washes, the membranes were reacted with antibodies against rabbit IgG (1:3000; Multi Sciences, China) or mouse IgG (1:3000; Multi Sciences, China) for 2 h at room temperature. The relative densities of bands were analyzed with ImageJ software.

Statistical analysis

Values are given as means \pm SD. Student t-tests were used to compare the data between two groups. Differences were considered significant at $p < 0.05$.

Results

Thrombin promoted the activation of meningeal macrophage

We first observed the accumulation of inflammatory cells in subarachnoid space. Our findings suggested that inflammation is widespread throughout the subarachnoid space after intraventricular injection of thrombin. In this study, Iba1 was used to label meningeal macrophages, CD68 was used as a marker of M1 macrophages and CD206 was used as a marker of M2 phenotype. CD68 or CD206 positive inflammatory cells were counted and the results were expressed as positive cells per millimeter of meninges. In areas of subarachnoid space, there was abundant CD68 positive meningeal macrophages in the thrombin group compared with markedly lower numbers in the saline group (115.4 ± 28.41 versus 37.7 ± 17.19 positive cells/mm in the saline group, $p < 0.01$). It indicated that thrombin induced the M1 polarization of meningeal macrophage in subarachnoid space. While there was a potential increase tendency in the distribution of CD206 positive macrophages between thrombin and saline group (122.4 ± 60.76 versus 63.57 ± 14.72 positive cells/mm in the saline group, $p = 0.1088$). These results

demonstrated that thrombin remarkably promoted M1 polarized meningeal macrophage rather than M2 phenotypic macrophage.

Thrombin induced vascular inflammation in subarachnoid space

The adherence and migration of leukocytes through the endothelium of blood vessels in subarachnoid space is an important event which triggers the early inflammatory response [18]. In subarachnoid space, prominent ICAM1 staining of endothelial cells in the thrombin group was observed. A significant rise in the expression of ICAM1 in endothelial cells and meningeal macrophages after thrombin injection was observed. At 24 hours after thrombin injection, the higher levels of proinflammatory cytokines (IL-1 β , IFN γ) were also detected compared to the saline group.

Thrombin caused hydrocephalus and neuron loss

Ventricular volumes were measured by brain sections at 10 levels (50 μ m in thickness) and NeuN and Iba1 immunostaining were performed in primary somatosensory cortex. The results showed that thrombin injection caused significant hydrocephalus (22.28 \pm 6.062 versus 8.46 \pm 1.95 mm³ in saline group, p<0.01) and a significant reduction in numbers of neurons at 24 hours after intraventricular thrombin injections compared with the saline group.

Inhibition of PAR1 alleviated the meningeal macrophage activation in subarachnoid space

To examine the role of PAR1 in thrombin-induced inflammation, SCH79797 was used. In subarachnoid space, SCH79797 resulted in less inflammation infiltration. Additionally, fewer CD68 positive cells were observed in the SCH79797 group compared to the vehicle group (61.39 \pm 16.43 versus 126 \pm 36.6 positive cells/mm in the vehicle group, p<0.05). And there were less CD206 positive cells in subarachnoid space compared to vehicle group (77.59 \pm 29.74 versus 134.9 \pm 46.07 positive cells/mm in vehicle group, p = 0.0815).

SCH79797 reduced the infiltration and adhesion of inflammatory cells in subarachnoid space

Furthermore, thrombin-induced vascular inflammation was also suppressed by treatment with SCH79797, a specific PAR1 antagonist. Inhibition of PAR1 attenuated the expression of ICAM1 and proinflammatory cytokines (IL-1 β , IFN γ).

SCH79797 suppressed the thrombin-induced hydrocephalus and neuron loss

Treatment with SCH79797 protected the neuron loss and produced a negative effect on ventricle enlargement (11.45 ± 2.254 versus 20.79 ± 3.297 mm³ in the vehicle group, $p < 0.01$).

The meningeal macrophage activation was associated with the neuron loss and hydrocephalus

To better evaluate the association between meningeal inflammation and thrombin-induced hydrocephalus, a detailed quantitative analysis of ventricular volume and CD68 positive cell numbers was carried out in all cases with and without the thrombin injection. The density of CD68+ meningeal macrophages reflected the severity of hydrocephalus and neuron loss in the early phase of thrombin-induced brain injury.

Discussions

The major findings of this study are as follows: (1) thrombin promoted M1 polarization of meningeal macrophage; (2) thrombin induced vascular inflammation in subarachnoid space; (3) inhibition of PAR1 attenuated the inflammatory response in subarachnoid space after thrombin injection; (4) meningeal inflammation was associated with severity of thrombin-induced hydrocephalus and neuron loss.

Thrombin is an important component of the coagulation cascade and plays an essential role in hydrocephalus [2, 3, 6]. Our previous study found that thrombin disrupted the blood-CSF barrier via VE-cadherin downregulation and led to hydrocephalus [3]. And the inflammation response was previously proved to be closely associated with the formation of hydrocephalus [9, 10, 19, 20]. Systemic inflammation is a therapeutic target in acute ischemic stroke [21, 22]. Also, it was indicated that anti-inflammation treatment could alleviate spontaneous hydrocephalus [23]. In the current study, the role of inflammation in thrombin-induced hydrocephalus was investigated.

Non-parenchymal brain macrophages are mononuclear phagocytes that are increasingly recognized to be critical players in the diseases of the central nervous system [23–26]. There are three types of central nervous system macrophages between parenchyma and circulation- namely, meningeal, perivascular and choroid plexus macrophages [15, 16, 26]. Meningeal inflammation has been identified as a key feature of hydrocephalus [10, 13, 19] and may contribute to the extensive cortical pathology that accompanies progressive disease [27–30]. Meningeal cells are involved in cortical development, fibrotic scar formation, brain inflammation, and neurodegenerative disorders such as Multiple Sclerosis (MS) and Alzheimer's disease (AD) and other brain disorders [29–31]. Here, our study aimed to explore whether meningeal macrophages were involved in the inflammation of subarachnoid space and the formation of hydrocephalus [10, 13]. Two macrophage populations were found in the central nervous system, corresponding to the proinflammatory M1 and "alternatively activated" anti-inflammatory M2 classes [16,

25]. The M1 macrophages are characterized by the high expressions of oxygen intermediates and proinflammatory cytokines which are closely associated with brain injury [23, 32]. While the M2 phenotype is considered to be involved in the promotion of tissue remodeling [16, 32]. In our study, a significant increase in M1 polarization was observed in meningeal macrophages and many of them are CD68 positive after thrombin injection. Interestingly, there was no significant difference in M2 macrophages which were CD206 positive between saline and thrombin group.

One of the most important features of proinflammatory macrophage is the secretion of pro-inflammatory cytokines [32]. In this study, activated endothelial cells and inflammatory infiltrations were observed after thrombin injection, pro-inflammatory cytokines were also significantly increased. Cytokines (like IL-1 β , IFN γ) were shown to decrease VE-cadherin protein levels and induce endothelial barrier dysfunction [33, 34]. Intercellular adhesion molecule 1 (ICAM1), induced by IL-1 β , IFN γ [35], is best known for its role in leukocyte adhesion and typically expressed on endothelial cells and cells of the immune system [18, 36]. Additionally, ICAM1 could mediate Src activation [37, 38], which is involved in the regulation of VE-cadherin [3, 7]. In this study, the increased expression of ICAM1, IL-1 β , and IFN γ after thrombin injection were observed, which highlighted the role of the inflammatory response in thrombin-induced hydrocephalus. Furthermore, a growing body of evidence demonstrated that leukocyte-endothelial adhesion interaction could lead to posthemorrhagic vasospasm which also could exacerbate the neuron loss [39–41].

To investigate the relationship between inflammation and thrombin-induced brain injury, the ventricular volume and the neuron loss in the cortex were analyzed. Interestingly, the level of infiltrating CD68 positive macrophages in meninges, correlated modestly with the severity of hydrocephalus and neuron loss. Rats with increased meningeal inflammation had a more severe brain injury. These results suggested that diffuse meningeal inflammation may contribute to the pathological mechanisms driving the progression of hydrocephalus and cortical neuronal pathology. These findings are consistent with other observations in meningeal inflammation [27–30].

The cellular signaling effects of thrombin are mediated by protease-activated receptors (PARs) [2, 3, 42]. PAR1 plays a critical role in the detrimental thrombin signaling [7, 17, 43]. The activation of PAR1 disrupts vascular integrity[3, 7], results in neurotoxicity[44, 45] and increases secondary brain injury after stroke[2, 6, 43]. In our study, inhibition of PAR1 reduced the meningeal inflammation, neuron loss, and hydrocephalus. It is possible that anti-inflammation will provide novel therapeutic targets for hydrocephalus management in the future.

Conclusion

These results demonstrated that meningeal macrophages were activated by intraventricular thrombin injection and it also exaggerated neuron loss and peri-vascular inflammation in the subarachnoid space. The clinically available PAR1 antagonists could reverse the meningeal injury and inflammation, thus

might offer a new therapeutic approach for management in ventricular hemorrhage-induced hydrocephalus.

Abbreviations

CNS: Central nervous system; CSF: Cerebrospinal fluid; ICAM1: intercellular adhesion molecule 1; ICH: intracerebral hemorrhage; VE-cadherin: vascular endothelial cadherin; PAR1: proteinase-activated receptor 1

Declarations

Ethics approval and consent to participate

Human volunteers did not participate in the study. Animal experiments were conducted after the approval from the Zhejiang University Animal Experimentation Committee.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

XH, XY, DS, and CL performed the experiments, analyzed the data, prepared the figures and wrote the manuscript. LT and FG designed the experiments, interpreted the data, and edited the manuscript.

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Figures

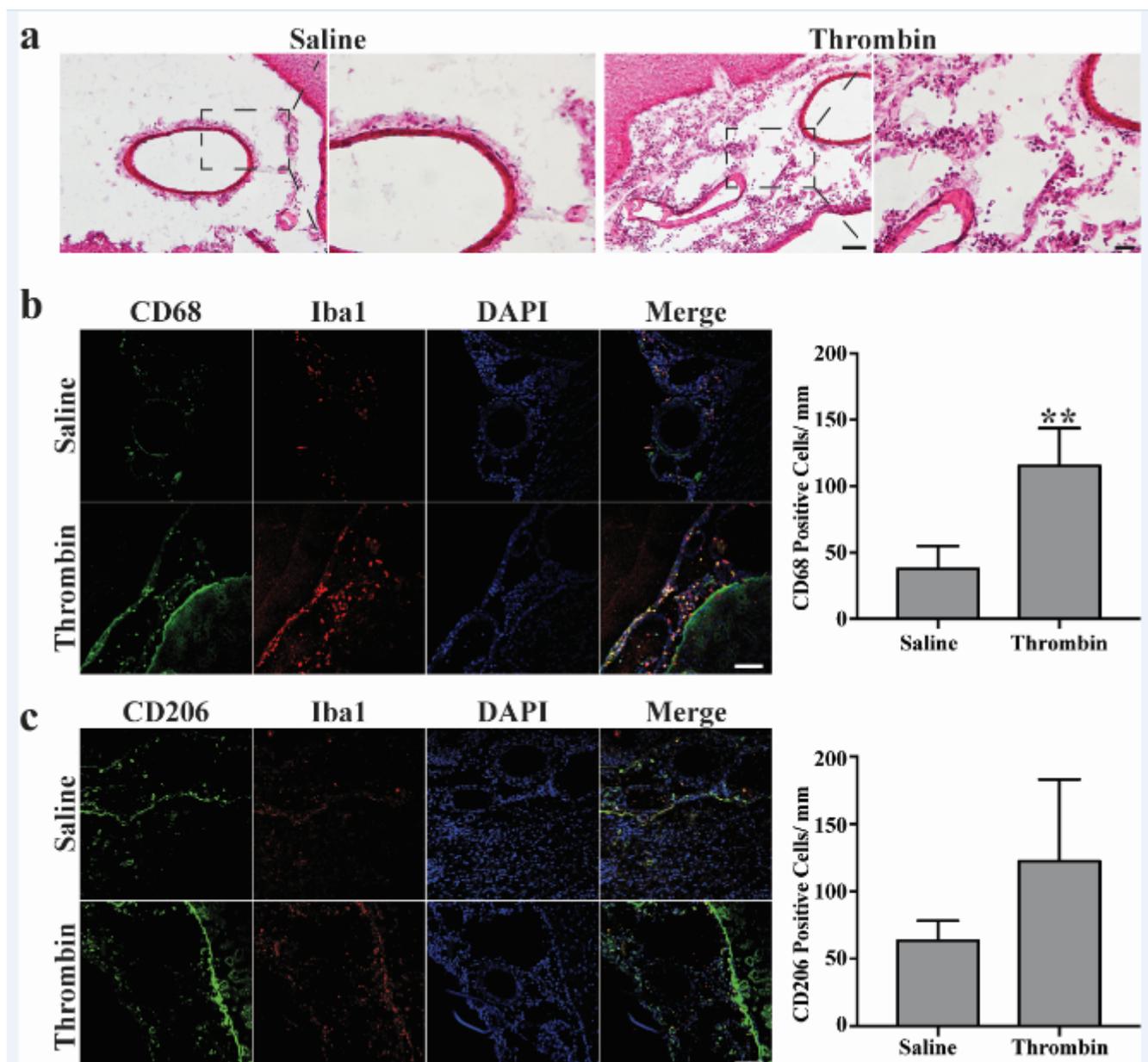


Figure 1

Distribution of inflammatory cells in the subarachnoid space in the saline or thrombin group. a H & E staining showed more inflammation infiltration in the thrombin group. Scale bar=20 μm (high magnification) and 50 μm (low magnification). b CD68 and Iba1 immunohistochemistry in rats injected with saline or thrombin. Scale bar=200 μm . Values are means \pm SD, $n = 4$, $**P < 0.01$ vs saline group. c CD206 and Iba1 immunohistochemistry in saline or thrombin group. Scale bar=200 μm .

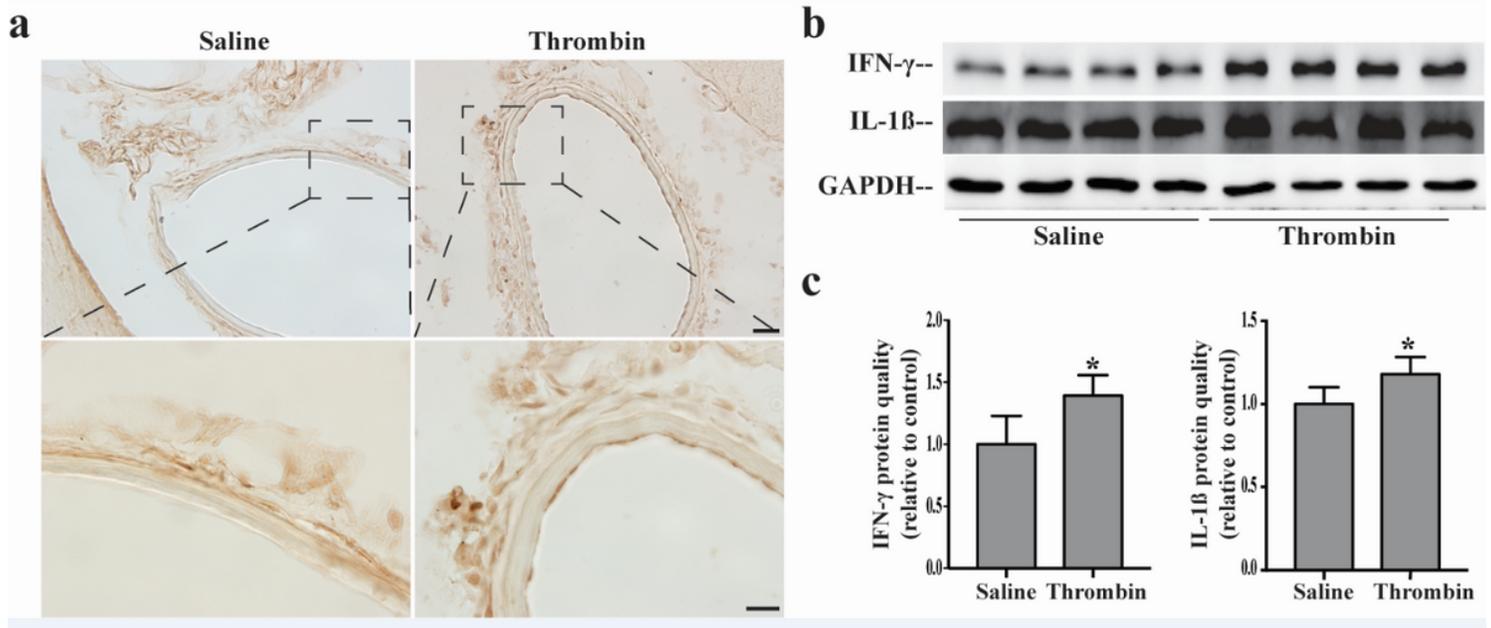


Figure 2

a Examples of ICAM1 staining in subarachnoid space. Scale bar=10 μm (high magnification) and 20 μm (low magnification). b The level of inflammatory cytokines was assessed by Western blot. c Quantitative analysis of inflammatory cytokines. Values are means \pm SD, $n = 4$, $*P < 0.05$ vs saline group

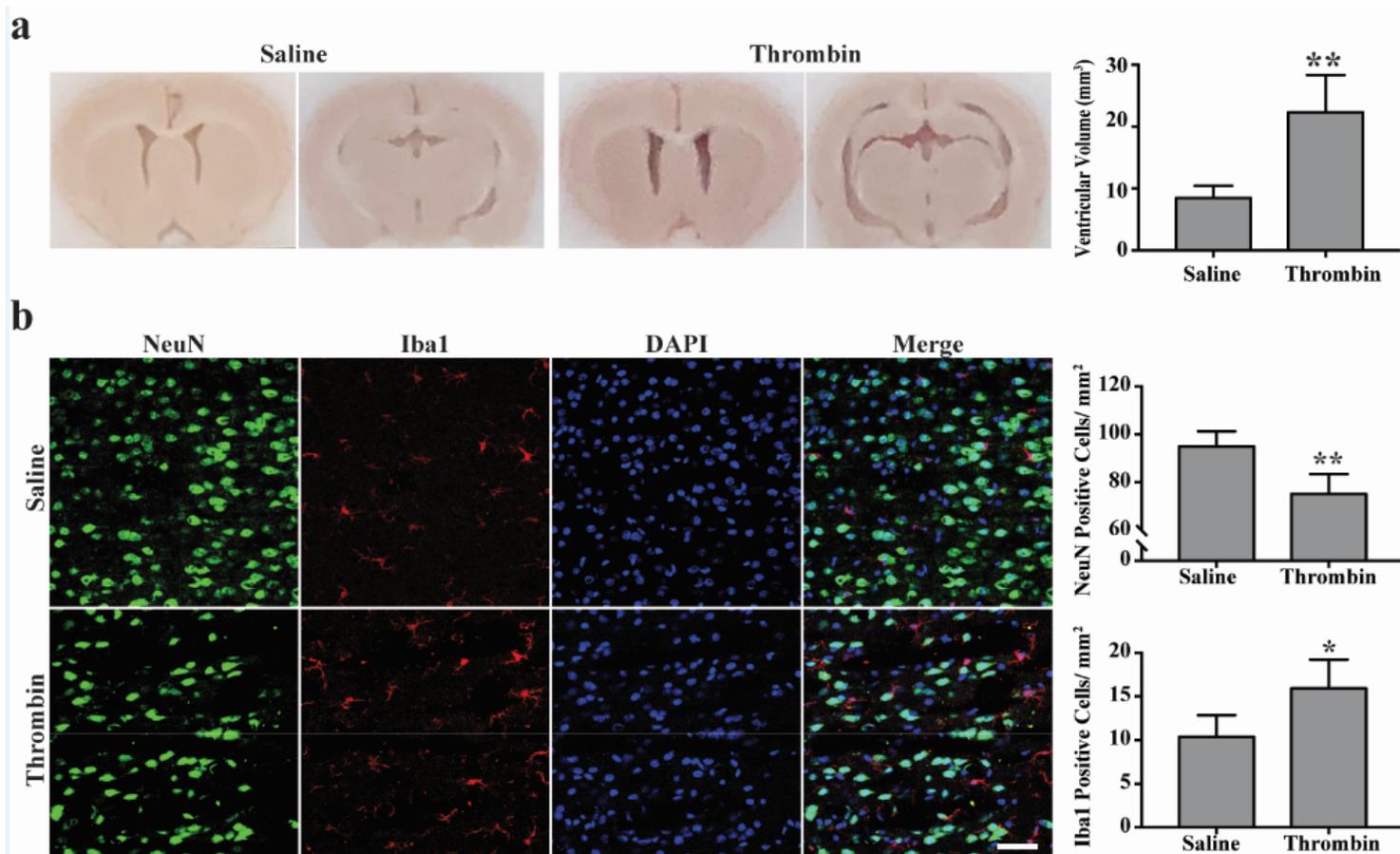


Figure 3

a Ventricles volumes were confirmed by examining coronal sections of frozen brains. $n = 4$, $**P < 0.01$ vs saline group. b NeuN and Iba1 positive cells in the cortex. Scale bar=50 μm . Values are means \pm SD, $n = 4$, $*P < 0.05$ and $**P < 0.01$ vs saline group.

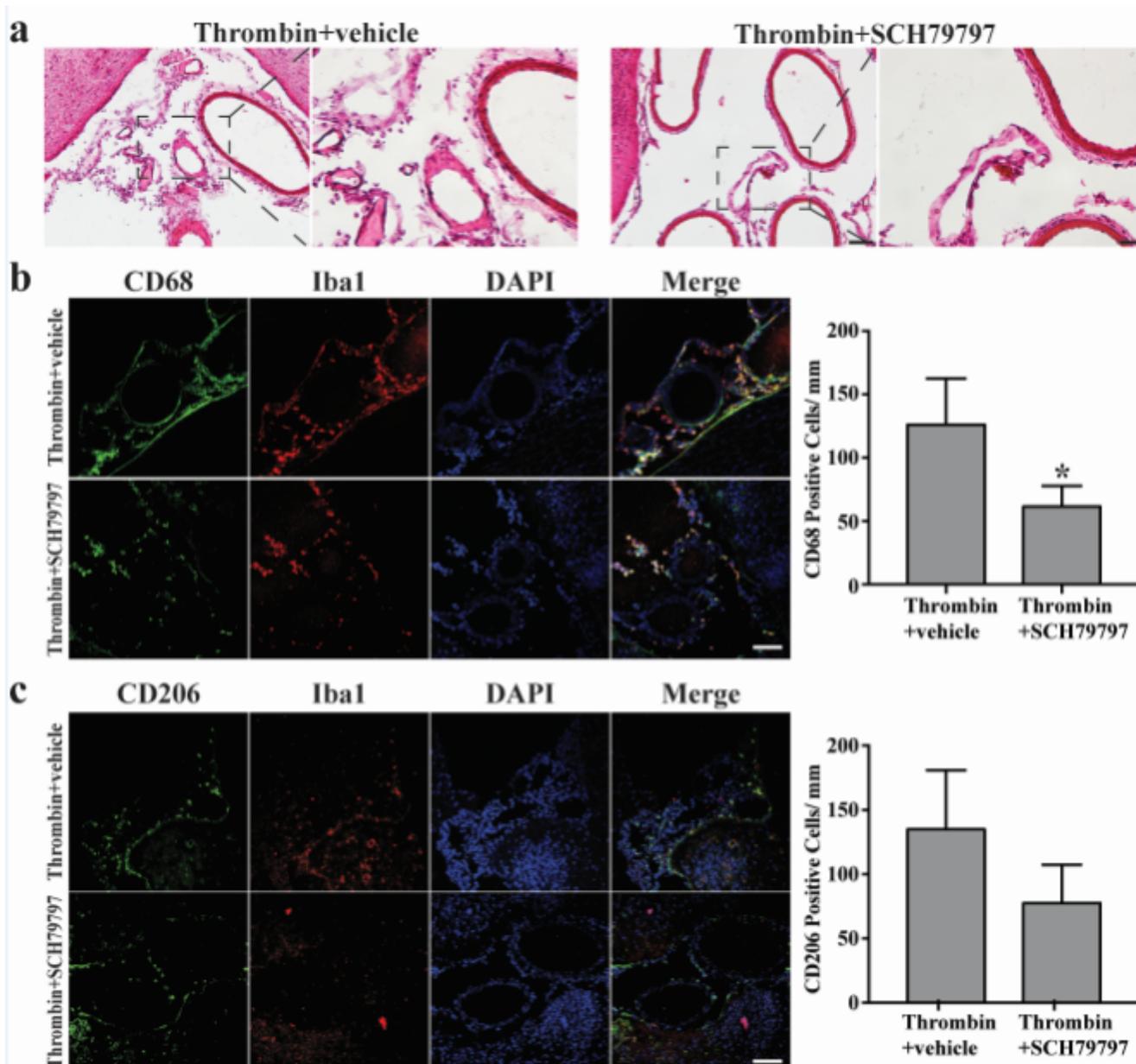


Figure 4

Distribution of inflammatory cells in the subarachnoid space in rats treated with vehicle or SCH79797. a H & E staining showed less inflammation infiltration in the SCH79797 group. Scale bar=20 μ m (high magnification) and 50 μ m (low magnification). b CD68 and Iba1 positive cells in subarachnoid space. Scale bar=200 μ m. Values are means \pm SD, n = 4, *P <0.05 vs vehicle group. c CD206 and Iba1 immunohistochemistry in thrombin + vehicle or SCH79797 group. Scale bar=200 μ m. Values are means \pm SD, n = 4.

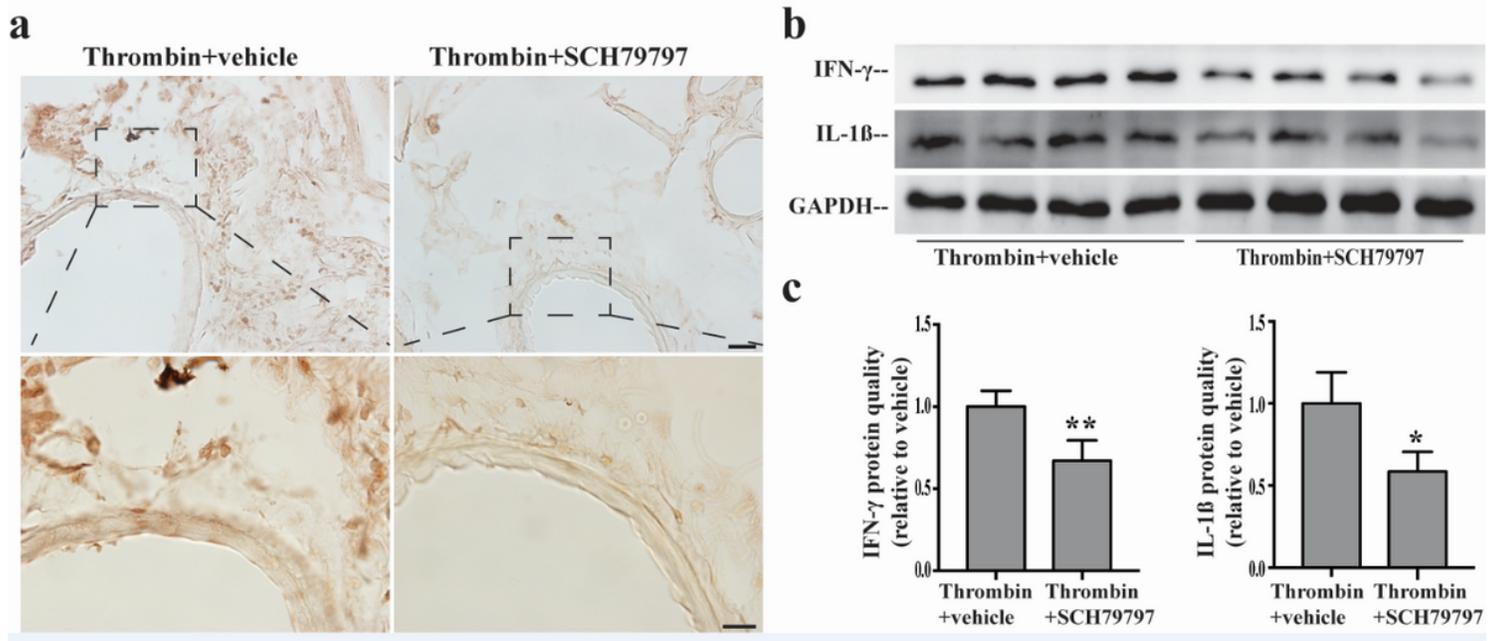


Figure 5

a ICAM1 staining in rats treated with vehicle or SCH79797. Scale bar=10 μ m (high magnification) and 20 μ m (low magnification). b Inflammatory cytokines levels determined by Western blot, and c quantitative analysis of inflammatory cytokines. Values are means \pm SD, n = 4, *P < 0.05 and **P < 0.01 vs vehicle group

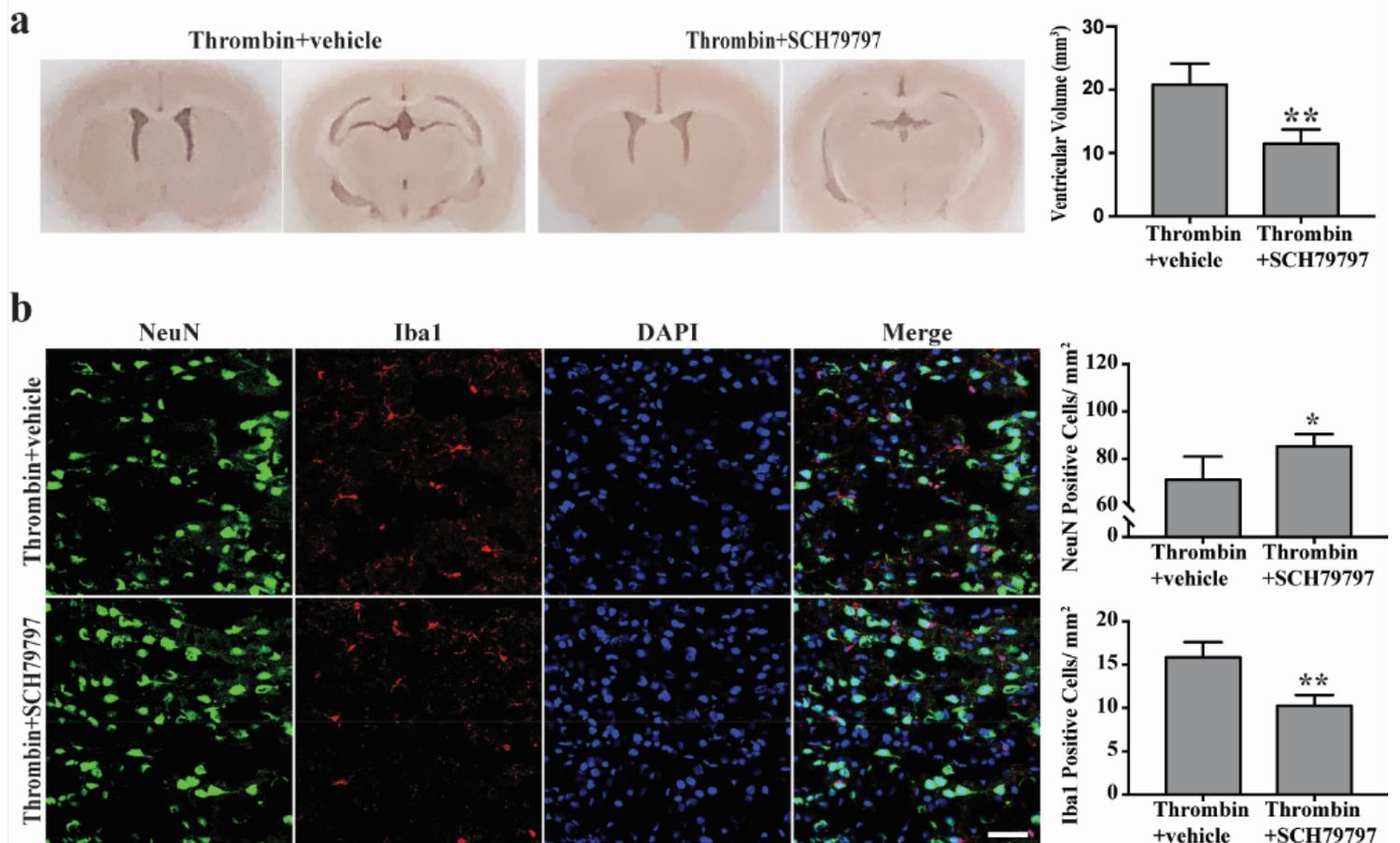


Figure 6

a Representative frozen sections from thrombin + vehicle or SCH79797 group. n = 4, **P <0.01 vs vehicle group. b Immunofluorescence staining of NeuN and Iba1 in the cortex. Scale bar=50 μ m. Values are means \pm SD, n = 4, *P <0.05 and **P <0.01 vs thrombin + vehicle group.

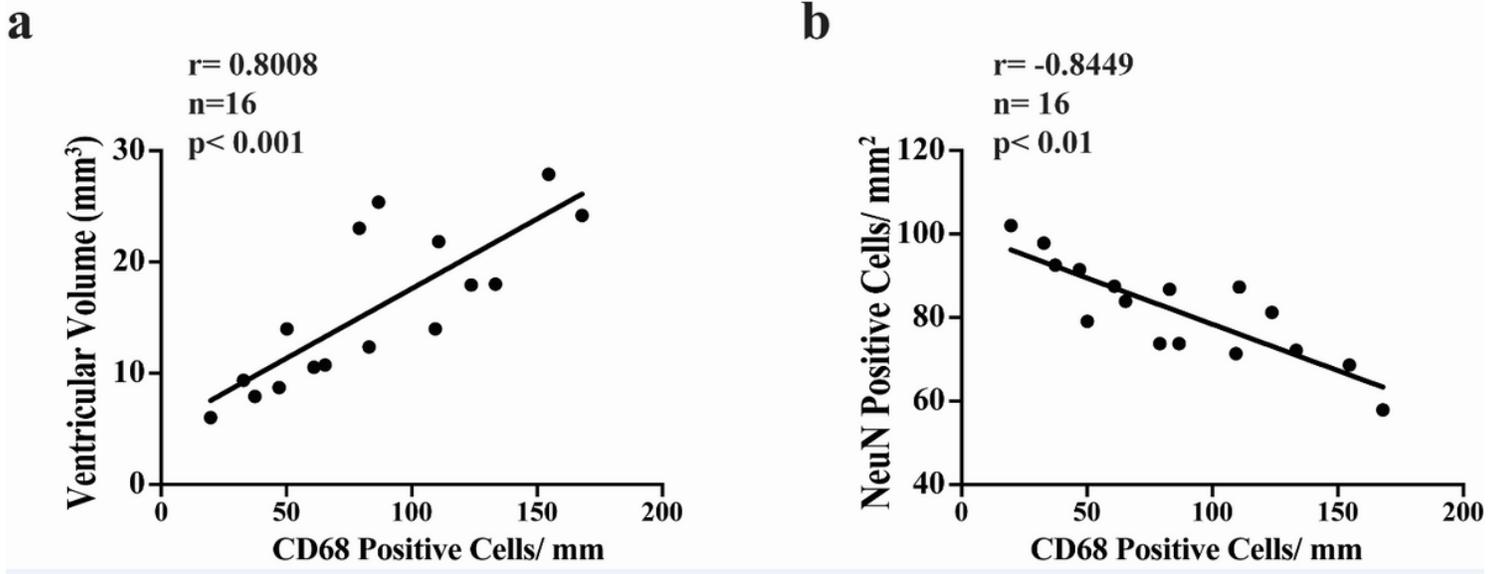


Figure 7

Pearson's correlation analysis between CD68 positive cells and ventricular volume a or NeuN positive cells b.