Role of C1q in spleen-derived neutrophil infiltration and neuroinflammation after ICH in mice

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Research

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Abstract

Background: Neuroinflammation is a major detrimental role of secondary brain injury after spontaneously intracerebral hemorrhage (ICH). Neutrophil infiltration plays a key role in the pathophysiology of ICH, but the coming resource and mechanism is unknown. This study aims to investigate whether spleen-derived neutrophil infiltration accelerated neuroinflammation and the role of C1q classical pathway.

Methods: Male C57 mice were subjected to collagenase-induced ICH. If necessary, splenectomy was performed 2 weeks prior to ICH induction or anti-C1q neutralizing antibody (50mg/kg) was injected intravenously into the tail vein 15 minutes prior. Immunohistochemistry, Propidium Iodide staining, western blotting, ELISA and qRT-PCR were used to study the change of molecular proteins, neuronal cells and inflammatory factors. 7.0T animal MRI was used to assess hydrocephalus.

Results: At 0h, 6h, 12h, 24h and 48h post ICH induction, we found a significant increasing tendency of microglia activation and neutrophil infiltration around hematoma and that C1q upregulation was correlated with neuronal decrease, which peaked at 24h after ICH. Here, we demonstrated spleen atrophy and upregulation of neutrophil in spleen 24h after ICH. Splenectomy prior to ICH mice resulted in significant decrease of microglia and neutrophil infiltration compared with that in group of sham-splenectomy. Moreover, both anti-C1q antibody and splenectomy significantly attenuated neutrophil infiltration and neuron death, restored synapse VGAT, alleviated hydrocephalus and inflammatory factors, such as IL-1β, TNF-α and IL-6 after ICH.

Conclusion: The study demonstrated that spleen is a major source of brain neutrophil infiltration after ICH. C1q-targeted inhibition of classic complement pathway could prevent spleen-derived neutrophil infiltration and attenuate ICH induced neuroinflammation, which provides a novel therapeutic approach for hemorrhagical stroke.

Introduction

Spontaneous intracerebral hemorrhage (ICH) is the most risky subtype of stroke consisting approximately 10–20% of all strokes, which can cause severe morbidity and even mortality[1]. More and more evidence has shown that neuroinflammation is a major determination factor of early deterioration and poor long-term outcome after ICH. Previously, we successfully demonstrated that activated microglia played a key role in neuroinflammation induced by Germinal Matrix hemorrhage (GMH) [2, 3], a special type of ICH in newborn rats. Studies have been focused on microglia activation pathway and microglia polarization to search for therapeutic targets [3, 4]. Microglia plays important role in neuroinflammation of diseases such as multiple sclerosis (MS) and stroke. At the time of stroke, the blood brain barrier (BBB) is disrupted and peripheral immune cells gain access to the brain parenchyma, which accelerates neuroinflammation [5]. Neutrophil accumulates 24 h following microglia activation after ischemia stroke, and this has been a major target for therapeutic intervention [6]. Multiple studies have established that microglia activation and neutrophil infiltration play key roles in neuroinflammation after ICH [7, 8]. However, the source organ,

function and fate of neutrophil need to be better understood. Trials trying to block peripheral immune cells in stroke patients have been unsuccessful [9]. Identifying source organ of neutrophil and the potential molecular pathway regulating neutrophil infiltration would provide more therapeutic targets in anti-neuroinflammation after ICH.

Activation of complement system results in rapid brain injury by accelerating inflammation in the setting of ICH. Generally, complement cascade can be activated via three pathways- C1q pathway, mannose-binding lectin (MBL)/MAPS pathway and the alternative pathway [10]. Different initiating molecule activates different pathways, but they all converge on complement C3, and then different breakdown products lead to several different antibody-independent results: (1) immune-cell recruitment by the soluble activation products C3a and C5a, (2) direct immune-cell attack by activation products deposited on dead cell and debris (C1q itself as well as C4b and C3b), and (3) membrane damage caused by activation of the lytic membrane attack complex (C5b-9) [11]. C3 appears to play a central role in compliment and independent brain injury following ICH. Some studies suggested that activation of C5 may have beneficial effect after the onset of ICH [12]. Nakamura et al. found that C5-deficient animals demonstrated a significantly greater neurological deficit and brain water content at 3 days after ICH [13]. Nevertheless, it has been shown that selective C3a antagonist (C3aRA) can attenuate brain edema and reduce neurological deficits in mice 72 h after ICH [14]. In addition, MBL complement pathway was involved in ischemia and reperfusion damage [15]. C1q is the component of complement classic pathway first recognized, which can bind various ligands derived self or non-self [16],. C1q has the ability to recognize structures and ligands on apoptotic cells, debris and synapses, which results in microglia/neutrophil engulfment and synapse pruning. In the setting of ICH, C1q may be presented by damaged neurons and newly developed synapse, and C1q-induced microglia or neutrophil engulfment may accelerate brain injury.

Thanks to the growing understanding of post-stroke immunological responses, peripheral immune system attracted more research activity. A lot of studies have been focused on peripheral immune organs, such as bone marrow, blood and spleen, which are believed to contribute to neuroinflammation post-stroke[17, 18]. Among them, spleen is the largest immune organ in the body carrying lymphocytes, monocytes and neutrophils. Some studies with rodents and patients highlighted the key role of splenic atrophy and contraction after ischemic and hemorrhagic stroke, and traumatic brain injury (TBI) [19–21]. After ischemic stroke, spleen was activated and the brain-spleen cell cycling started. It is still controversial what plays the key role in the splenic response after stroke, the lymphocytes, monocytes, [22] neutrophil infiltration or the neutrophil-to-lymphocyte ratio (NLR)[23]. Moreover, it is worthwhile to elucidate the precise role of each cell type of spleen in secondary brain injury after stroke. It was reported that splenic volume was inversely related with neutrophil count in the blood, but not lymphocyte or monocyte [24]. Actually, splenic response after ICH is far less studied than that after ischemic stroke. The relationship between neutrophil infiltration and microglia induced neuroinflammation after ICH is still not clear. Recent work of Hooshmand demonstrated that neutrophils induced astroglia differentiation and migration of human Neural Stem Cells (NSCs) via C1q synthesis [25]. We hypothesize that C1q expressed in damaged

neurons and synapses is the key molecule inducing spleen-derived neutrophil infiltration, which accelerates neuroinflammation after ICH.

Materials And Methods

Ethics statement and mice

All animal studies were performed in accordance with the animal care guidelines approved by the Animal Ethics Committee of the Army Medical University, Chongqing, China. Eight-week-old CD-1 mouse (weight 35-40 g) were housed at room temperature and given free access to food and water. Male mice in good health were used in this study because sex and estrogen have been reported to be important factors mediating outcomes after stroke [26]. Splenectomy was performed two-weeks before ICH induction and anti-C1q antibody (Annexon Biosciences, CA, USA) was injected into the tail vein 15 minutes before ICH induction. 50mg/kg has been established as the maximal effective dose to attenuate complement cascade activation and deposition in mouse models of Guillain-Barré syndrome (GBS) [27].

Splenectomy

Splenectomy was performed according to the instruction of Chauhan et al [28] with modification. Generally, mice were gently anesthetized with 3 % isoflurane (in mixed air and oxygen) and positioned on a 37 °C heated plate. During the procedure, a longitudinal incision about 1 cm was made on the left dorsolateral side of the abdomen, caudal to the last rib. The spleen and splenic arteries were dissected gently in a good view. Then, the splenic arteries were cauterized before the spleen was dissected and no bleeding should be observed. Sham surgery was performed by dissecting the spleen and splenic arteries in view only. After surgery, the mice were allowed to recover for 2 weeks prior to induction of ICH. The harvested spleens were immediately weighted with an analytical microbalance with 1.0 mg precision.

ICH induction

Mice were intraperitoneally injected with 3% isoflurane (in mixed air and oxygen) and positioned in a stereotactic head frame (Kopf Instruments, Tujunga, CA, USA). The coordinates were on the right coronal suture, 2.2 mm lateral to the midline and 0.2 mm posterior to bregma. At the coordinates, a cranial burr hole (1 mm) was made and a 27-gauge 5-ml syringe (Hamilton Company, Reno, NV) with 0.3U clostridial collagenase VII-S (Sigma, St Louis, MO) was stereotaxically implanted in the right basal ganglia coordinates and 3.5 mm beneath the dura. Clostridial collagenase was injected through the syringe connected to a micro-infusion pump (Harvard Apparatus, Holliston, MA) at a rate of 0.25 ml/min. Mice in the sham-surgery group underwent needle insertion only. To prevent back-leakage of the collagenase, the syringe was maintained in that position for an additional 10 min. During the operative procedure, the mice were laid on a 37 °C heated plate, and they were allowed to recover on a 37 °C heated blanket until they were returned to their dams.

Tissue preparation and immunofluorescence

At different time points after the onset of ICH, mice were anesthetized with pentobarbital (100mg/kg) as previously described. Then, they were perfused intracardially with 100ml saline (0.9% Nacl) and with 100 ml 4% fixation solution (paraformaldehyde in 0.1M phosphate buffer). Brains were dissected immediately on ice, stored in 4% paraformaldehyde overnight and then transferred into 30% sucrose at 4℃ to dehydrate for 3 to 4 days. After that, the brain tissues were embedded in O.C.T (optimal cutting temperature compound, Sakura Finetek USA Inc., Torrance, CA). Generally, 18-μm slices were prepared using a cryostat microtome and then the immunofluorescence protocol was followed as previously described. The following primary antibodies were incubated with the sections: rabbit polyclonal Iba-1 antibody (diluted 1:1000; Wako Pure Chemical Industry, Japan), rat Anti-Neutrophil antibody (diluted 1:500; Abcam, MA, USA), rabbit polyclonal NeuN antibody (diluted 1:500; Abcam, MA, USA), chicken polyclonal MAP2 antibody (diluted 1:500; Abcam, MA, USA), rabbit polyclonal SLC32A1/VGAT antibody (diluted 1:500; Abcam, MA, USA) and mouse Anti-C1q antibody (diluted 1:500; Abcam, MA, USA). The sections were then washed with 0.01 M PBS (phosphorite buffer solution) and kept at room temperature for 45 min. Finally, the sections were incubated with DyLight 488-conjugated donkey anti-rat secondary antibody, DyLight 488-conjugated donkey anti-rabbit secondary antibody, DyLight 488-conjugated donkey anti-chicken secondary antibody and Cy3-conjugated donkey anti-rabbit secondary antibody for 1h at room temperature. All secondary antibodies were from Jackson ImmunoResearch Laboratories. DAPI was used to stain nuclei. At least four images around the hemorrhage were captured every ten slices using a 20 * 40 objective on a Zeiss confocal microscope (LSM780; Zeiss, Germany).

Administration of Propidium Iodide (PI) and PI staining

A total volume of no more than 100μl PI (0.4mg/kg, diluted in 0.9% Nacl, Sigma-Aldrich Corporation, St. Louis, MO, USA) was administered intraperitoneally 1h before decapitation. Mice were sacrificed 24h after ICH induction and the brains were frozen in nitrogen vapor. Cryostat brain sections of 12 μm were prepared from anterior to posterior at 100 to 150 xx (unit) intervals and then, the sections were placed on poly-L-Lysine slides and stored at -80℃ [29]. Brain sections were fixed in 100% ethanol for 10 minutes at room temperature for detection of PI positive cells. After being cover-slipped with Permount Mounting Medium, the sections were photographed using a Zeiss confocal microscope (LSM780; Zeiss, Germany) at 568/585 nm for PI. PI-positive cells were quantified in three fields of sections around hematoma every 10 slices. Six rat pups in each group were analyzed.

Western blot analysis

Western blot (WB) analysis was performed at 6h, 12h, 24h and 48h after ICH induction. Generally, mice were anesthetized by pentobarbital injected intraperitoneally and then perfused intracardially with 0.1M PBS. Total proteins were extracted from brain tissues by homogenization in RIPA buffer (Santa Cruz, USA). Homogenates were centrifuged at 14,000 ×g at 4℃ for 20 minutes to harvest proteins. Protein concentration was determined using BCA Protein Quantitation Kit (ab102536, Abcam, MA, USA). After SDS-Page protocol, proteins were separated on a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA) and blocked with 5% non-fat dry milk in TBS with 0.1% Tween20 (TBS-T). After being washed in

TBS-T, the membranes were incubated with antibodies, including rabbit Iba-1 antibody (diluted 1:1000; Wako Pure Chemical Industry, Japan), rat Anti-Neutrophil antibody (diluted 1:000; Abcam, MA, USA),rabbit Anti-C1q antibody (diluted 1:1000; Abcam, MA, USA) and rabbit VGAT antibody (diluted 1:500; Abcam, MA, USA) and appropriate secondary antibodies, including HRP-conjugated anti-rabbit or anti-rat (1:1000, Beyotime Biotechnology, China). Images were acquired with ChemiDOCTMxrs system (Bio-Rad) and processed with Image Lab (Microsoft).

Analysis of mRNA by qRT-PCR

Mice were anaesthetized with pentobarbital (100/kg,i.p.) 24h after ICH. Cortical tissues around hematoma were dissected on ice and stored at -80℃. Total RNAs were isolated with TRIzol reagent (Invintrogen) according to the manufacture's protocol. RNA quantity and quality were determined with NanoDrop spectrophotometer (ThermoScientific, USA) at A260/A280. Reverse transcription (RT) of RNA was performed as previously described[2]. HPRT, a housekeeping gene encoding Hypoxanthine Phosphoribosyltransferase, was used as internal control to evaluate mRNA expression. qPCR was performed as previously reported [30]. RT-PCR primers were presented below:

IL-1β: 5'-ACCTTCCAGGATGAGGACATGA-3' (forward)

5'-CTAATGGGAACGTCACACACCA-3'(reverse)

TNF-α:5'-GCCACCACGCTCTTCTGTCTAC-3' (forward)

5'-GGGTCTGGGCCATAGAACTGAT-3' (reverse)

IL-6: 5'-CACATGTTCTCTGGGAAATCG-3'(forward)

5'-TTGTATCTCTGGAAGTTTCAGATTGTT-3'(reverse);

HPRT: 5'-GTTAAGCAGTACAGCCCCAAA-3'(forward)

5'- AGGGCATATCCAACAACAAACTT-3' (reverse)

ELISA assay

Inflammatory factors in brain tissue were quantified using enzyme-linked immunosorbent assays (ELISA) kit (RayBiotech, Norcross, GA). Generally, tissues around hematoma were dissected and immediately immersed in liquid nitrogen for protein extraction. protease inhibitor cocktail in 0.01mol/L PBS (Roche, Indianapolis, IN) was used to dilute the tissue. The homogenates were centrifuged at 12,000 g at 4℃ for 20 minutes and the supernatants were used to determine the expression level of TNF-α, IL-6, and TGF-β with ELISA kit.

Magnetic Resonance Imaging and volume measurement

Images were captured at 12h, 24h and 48h after ICH for spleen volume determination and 28 days after ICH for ventricular volume determination by a 7.0 T/200 mm Varian MRI scanner (Bruker BioSpin, USA). Generally, mice were anesthetized with 2 % isoflurane/air mixture and a small animal physiological monitoring system (SA Instruments, Stony Brook, NY) was used to monitor status of anesthesia and animal physiology. The T_2 fast spin-echo sequence (TR/TE=3000/45 ms) was used for ventricular volume determination and T_1 was used for spleen volume determination. The lateral ventricular volume was calculated by summing areas of all lateral ventricles, and multiplied by the section thickness (0.7 mm). The spleen volume was calculated as reported by Laurence H. Jackson [31]. T_1-weighted spin-echo multi-slices sequence was used and the slice thickness was 1 mm. Image was reconstructed offline and an individual 2D slice were obtained. The spleen tissue was marked by drawing organ contours and subsequent organ volume detection was carried out.

Statistical analysis

Summary statistics are reported as mean ± standard derivation (STD). Two sample *t*-test was used for the comparison between XX and XX and one-way analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons was performed for comparisons involving more than 2 groups. A nonparametric Kruskal-Wallis test was used followed by a Nemenyi test if the data were not normally distributed. The results were considered significant at a level of $p< 0.05$. All statistical analyses were performed using GraphPad prism 6 and SPSS V13 (SPSS Inc., Chicago, IL, USA).

Results

Extensive neutrophil infiltration and microglia activation after ICH

It has been reported that microglia is activated and accumulates after ICH, but neutrophil infiltration around hematoma was not clear. In the study, we detected neutrophil and microglia by immunohistochemical staining and western blot analysis at 6h, 12h, 24h and 48h after ICH. As shown in Figure 1A, neutrophil (anti-neutrophil antibody, green) significantly accumulated at 12h (Figure 1C, $p<0.05$) and peaked at 24h ($p<0.01$) after ICH. Similarly, microglia was detected by anti-Iba-1 antibody at 12h (Figure 1B, $p<0.05$) after ICH, compared with that in sham group. At 24h (Figure 1B, $p<0.01$) after ICH, we detected significant accumulation of Iba-1 positive microglia around hematoma. In addition, we demonstrated that neutrophil protein increased in brain 12h after ICH, compared with that in sham group, and peaked at 24h (Figure 1D). Protein expression was upregulated earlier in microglia at 6h after ICH and also peaked at 24h after ICH (Figure 1E).

C1q in damaged neuronal cells and the relationship with immune cells

To test our hypothesis of expression of C1q protein in damaged neuronal cells and phagocytosis of immune cells. we labeled C1q protein in neurons, microglia and neutrophils at different time points after ICH and detected a significant increase of C1q positive cells and a significant decrease of NeuN positive neurons at different time points at early statge of brain injury (Figure 2A-2B). Moreover, cells with C1q and

NeuN protein expression increased significantly at 12h (Figure 2C, $p<0.05$) and peaked at 24h ($p<0.05$) after ICH, compared with that in sham group. Western Blot analysis demonstrated a significant increase of C1q protein at 6h, 12h, 24h and 48h after ICH and the expression peaked at 24h (Figure 2D). In addition, we demonstrated expression of C1q in neutrophils and microglia on brain slices of mice in sham group 24h after ICH (Figure 2E). Following C1q up-regulation, a significant infiltration of neutrophils and accumulation of activated microglia were observed. White arrows indicated that microglia was activated and its branches were shorten after ICH.

Spleen shrinkage and neutrophil aggregation after ICH

To illustrate the role of spleen in neuroinflammation after ICH, abdominal MRI was carried out at 12h, 24h and 48h after ICH. Spleen volume contracted at 24h after ICH (Figure 3A). Spleen weight measurements presented similar result that ICH lead to a significant shrinkage of spleen at 24h (ICH 24h: 0.065±0.009 g vs. Sham: 0.090±0.016 g) and peaked at 48h after injury (Figure 3B, $p<0.05$). In addition, a significant increase of neutrophil in spleen was observed compared with that in sham group, even though spleen contracted after ICH (Figure 3C). A brief protocol schedule for further study was made, splenectomy was performed two weeks prior to ICH induction (Figure3 D).

Neutrophil infiltration and microglia activation decreased following splenectomy

Spleen is a major source of peripheral immune cells which could infiltrate into the brain after ischemic stroke and traumatic brain injury (TBI). Infiltration of T-cells and macrophages into the brain after ischemic stroke were well documented in the previous studies [28]. However, it is unclear whether the infiltrated neutrophils come from spleen after ICH. To parallel the spleen shrinkage study, we divided the mice into three groups, sham, ICH+ splenc. -sham (ICH mice with sham splenectomy) and ICH+ splenectomy. There were significant more infiltrated neutrophils in brain slice of ICH+ splenc. -sham mice (Figure 4B) compared with that of sham mice (Figure 4A) and C1q expression and neutrophil infiltration decreased in mice with splenectomy (Figure 4C). Co-expression of neutrophils and gC1qR were carried out immunohistochemically (Figure 4D). In addition, WB analysis presented the same result that neutrophil protein increased significantly after ICH in mice of sham-splenectomy group (Figure 4 E, $P<0.01$) and decreased in mice of splenectomy group ($p<0.05$, *vs.* group of ICH+ splenc. -sham).

In the same groups, we detected microglia activation after ICH and splenectomy. As a result, we found that Ibal-1 positive brain microglia in group of ICH+ splenc. -sham was activated significantly (Figure 5 B) with ameboid cell body, compared with that in sham group (Figure 5 A). C1q expression and activation of microglia decreased following splenectomy (Figure 5 C). Co-expression of microglia and gC1qR were carried out immunohistochemically (Figure 4 D). Moreover, we counted the number of neutrophil and Iba-1 positive cells in the brain slices. Neutrophils and microglia significantly increased in ICH+ splenc. -sham group (Figure 5E, $p<0.05$, $p<0.01$). number of neutrophils and microglia decreased following splenectomy ($p<0.05$).

C1q antibody and splenectomy reversed neutrophil infiltration and neuronal cell death after ICH

To illustrate the role of C1q in neutrophil infiltration and neuronal cell death, we administrated anti-C1q antibody intraventricularly to neutralize C1q domain. By immunohistochemical technique, we detected the expression of neutrophil and PI-labeled dead neuronal cells. As a result, a significant increase of neutrophil infiltration was detected in ICH mice in vehicle group. Neutrophils decreased following anti-C1q antibody injection and splenectomy (Figure 6 A). Accordingly, ICH induced neuronal cell death, and anti-C1q antibody and splenectomy reversed the damage (Figure 6 B). In addition, average cell count of three fields around the hematoma (without the field close to ventricle) gave the exact number of neutrophil infiltration and PI positive dead cells (Figure 6 C). Moreover, neutrophil protein was detected by WB analysis in each group and the usual result with immunohistochemistry was observed. Neutrophil protein upregulated by ICH decreased following anti-C1q antibody injection and splenectomy (Figure 6 D, $p<0.05$).

C1q antibody and splenectomy prevented synapse loss around hematoma

Synapse loss is a detrimental damage after ICH induced neuroinflammation. Microtubule-associated protein 2 (MAP2) is the major microtubule associated protein in brain tissue and exists in the cell bodies, dendrites and axons of mature neurons. In the study, we detected the expression of MAP2 and VGAT, an integral membrane protein involved in gamma-aminobutyric acid (GABA) and glycine uptake into synaptic vesicles. Co-expression of MAP2 and VGAT was demonstrated in Figure 7A. ICH induced a significant decrease of VGAT expression around MAP2 positive neurons and treatment with saline (vehicle) has no preventive effect. Sham-splenectomy prior to ICH could not prevent VGAT synapse loss either. VGAT expression increased following anti-C1q antibody injection and splenectomy prior to ICH. A brain slice of H&E staining was presented to demonstrate co-expression of MAP2 and VGAT (Figure 7B). The rest six mouse in each group were used for VGAT protein detection by WB analysis (Figure 7C). As a result, VGAT protein expression decreased significantly in group of intraventricular injection of saline (Vehicle, 2.7-folds, $p<0.05$) and in group of sham-splenectomy (2.1-folds, $p<0.05$). Interestingly, the level of VGAT protein increased significantly following anti-C1q antibody injection and splenectomy ($P<0.05$, versus the corresponding vehicle and sham-splenectomy group).

Effect of splenectomy and C1q antibody on ICH induced hydrocephalus

Volume of lateral ventricle and cortical thickness are typical indicators for hydrocephalus. In the study, 7.0 Tesla MRI on mice head was carried out 28 days after ICH in group of sham-ICH (sham), intraventricular injection of saline (Vehicle), sham-splenectomy (ICH+ splenc. -sham), intraventricular injection of anti-C1q neutralizing antibody (ICH+Anti-C1q) and splenectomy (ICH+ Splenc.). Ten mice met criteria for successful treatment and ICH model were brought into the experiment as the images shown in Figure 8A. By calculating volume of lateral ventricle, we found that ICH induced significant increase of ventricle volume and that intraventricular injection of saline and sham-splenectomy could not improve the damage (Figure 8B, $p<0.01$, versus the sham group). Fortunately, anti-C1q antibody improved ICH induced hydrocephalus by decreasing ventricle volume significantly (ICH+Anti-C1q: 14.25 ±2.43 mm^3 *vs.* Vehicle: 27.46± 4.32 mm^3, $p<0.05$). Splenectomy prior to ICH decreased the volume of ventricle enlarged

(ICH+ Splenc.: 13.46 ± 1.86 mm^3 vs. ICH+ splenc. -sham: 26.48 ± 2.68 mm^3, $^{\#}p<0.05$). ICH induced a significant decrease of cortical thickness compared with that in the group of sham (Figure 8C, $p<0.05$). No difference or protective effect were detected in mice injected intraventricularly with saline or suffered from sham-splenectomy. Splenectomy prior to ICH and anti-C1q antibody injection demonstrated protective effect by increasing cortical thickness ($P<0.05$).

Effect of splenectomy and C1q antibody on inflammatory factors after ICH

Pro-inflammatory cytokines play detrimental role in neuroinflammation induced by ICH. Thus, we measured protein level of IL-1β, TNF-α and IL-6 by ELISA 24 h after ICH. As a result, ICH induced a significant increase of protein level of IL-6, IL-1β, and TNF-α compared to mice in sham group (Figure 9A~C). Splenectomy prior to ICH and anti-C1q antibody injection showed protective effect by decreasing protein level of IL-6, IL-1β, and TNF-α compared with mice with saline injection and sham-splenectomy respectively. To confirm the results, we quantified the expression level of mRNA encoding these pro-inflammatory proteins by qPCR and similiar results were obtained (Figure 9 D~F). Expression of IL-6, IL-1β, and TNF-α protein up-regulated by ICH 24h after onset decreased following splenectomy and anti-C1q antibody injection prior to ICH.

Discussion

In this study, we presented three developments of neuroinflammation induced by central and peripheral immune system after ICH. Firstly, we demonstrated that microglia activation and neutrophil infiltration were two representative immune cell responses in central and peripheral immune system after ICH. Secondly, spleen was the major source of neutrophils infiltrated into the brain around hematoma after ICH. At last, we found that C1q expressed in damaged neuronal cells conducted spleen-derived neutrophil infiltration, which accelerated brain injury. Based on the results, we gave a potential sketch on the vital role of C1q connecting peripheral immune system and neuroinflammation after ICH (Fig. 10).

Secondary brain injury, such as blood-brain barrier (BBB) disruption, brain edema and hydrocephalus after ICH are directly caused by neuroinflammation, which involves the innate and adaptive immune system. Microglia activation has been extensively reported to initiate neuroinflammation and conducts CNS innate immune response. Previously, we demonstrated that microglia activation and accumulation around hematoma are the key immune response following germinal matrix hemorrhage (GMH) [2–4] and ICH in rats [32]. Accordingly, in this study we found a significant increase of microglia after ICH in mice and microglia activation peaked at 24 h after injury. Neutrophil- lymphocytes ratio (NLR) has been extensively reported as a risk factor associated with bad outcomes after ICH [23, 33]. Controversial opinions existed with regard to neutrophil counts infiltrated into brain after ICH. Xiurong Zhao et al reported that neutrophil depletion at 24 h after ICH in mice made the neurological deficits worse and concluded that neutrophil infiltrated into the brain at later stage following ICH could be beneficial [34]. In this study, we found similar increase of neutrophil counts with microglia from 6 h to 48 h after ICH in mice and demonstrated that spleen is the source of the infiltrated neutrophils. Studies have reported that

within hours of first wave of infiltration, Neutrophils continued to enter the ICH-affected site of brain for several days after ICH onset [35]. We found that splenectomy prior to ICH induction alleviated the neutrophils in injured brain and ameliorated the pro-inframammary factors, which is consistent with the finding that depletion of neutrophil prior to ICH could mitigate ICH-mediated injury [36].

Post-stroke immunological response has been extensively studied, especially in ischemia stroke [19]. Peripheral immune system plays a detrimental role in the progression of brain injury following stroke. Several organs have been reported to be involved in immune response after stroke, such as bone marrow, blood and spleen [17]. Among them, spleen is a lymphoid organ that plays a key role in both adaptive and innate immune response. The detrimental role of spleen contraction and immune cells infiltration have been reported in several CNS disease such as ischemia[24] ICH[37] and traumatic brain injury (TBI) [38]. Compared with ischemia stroke, studies on the role of spleen are badly needed to illustrate the participation of peripheral immune system in ICH. A recent study with158 patients with ischemia and intracerebral hemorrhage demonstrated that 40% of stroke patients experienced substantial post-stroke reduction in splenic volume and that spleen contraction was significantly associated with upregulation of cytokine IL-6 in plasma [20]. Another clinical research found that hematoma size can predict the extent of spleen contraction in patient suffering from ICH and demonstrated that perihematomal edema was smaller in patients with severe spleen shrinkage compared with that in patients with lesser spleen shrinkage 3d after ICH [39]. The study suggested protective effect of spleen contraction, which is in accordance with a previous study reporting that neutrophil infiltration at a later stage after ICH could be beneficial [34]. The positive effects of spleen shrinkage after ICH indicated the activation of self-protection system, but the detrimental role of infiltration of spleen-derived immune cells and subsequent secretion of inflammatory cytokines should not be ignored. In the present study, we found significant shrinkage of spleen after collagenase induced ICH in mice at 24 h and 48 h after injury. At 24 h after injury, a significant increase of neutrophil counts was detected compared with that in spleen of sham-operated mice, which is in accordance with the result reported in study concerning mice with ischemia stroke [40]. In this study, we concerned the detrimental role of spleen contraction at an early stage of 48 h after ICH. We demonstrated that splenectomy two weeks prior to ICH mitigated hydrocephalus and synapse loss induced by hematoma, and reduced inflammatory cytokine such as TNF-α, IL-1β and IL-6 in brain tissue.

Since splenectomy is not a potential treatment option after ICH, we can alleviate the infiltration of neutrophils derived from spleen by inhibiting the molecular pathway leading to neutrophil infiltration. In the study, we found significant increase of C1q protein at 12 h, 24 h and 48 h after ICH. Especially, number of neurons (NeuN+) expressing C1q protein was found at peak around hematoma at 24 h after ICH. Accordingly, a study showed that C1q was not expressed in cultured PC12 neurons before hypoxia [41] and studies on autopsy brains demonstrated a significant increase of C1q mRNA/protein in neurons of AD brain compared with controls [42]. These studies suggested that neurons under stress or in neuropathological environment were activated to express C1q. The important role of C1q after neuropathology cannot be ignored because it can recognize various structures and ligands on microbial surfaces or apoptotic cells. C1q triggers a protease cascade, leading to the deposition of the downstream

complement protein C3, which coats dead cells, pathogens, or debris [43]. C1q interacts directly with damaged neurons and unwanted synapse in an antibody independent way, leading to inflammation and synaptic removal by macrophages and microglia [44]. Inhibition of C1q prevents activation of all complement components downstream of C1q but leaves the full lectin and alternative pathways in place [11]. In our study, we used ant-C1q antibody as an inhibitor of C1q by intraventricular injection. As a result, ventricular volume in mice treated with anti-C1q antibody significantly decreased compared with that in ICH mice treated with saline. Moreover, Protein and mRNA levels of tissue inflammatory cytokines, such as TNF-α, IL-1β and IL-6 were reduced by anti-C1q antibody after ICH. Accordingly, C1q-deficiency is reported to be neuroprotective against hypoxic-ischemic brain injury in premature mice [45] .

C1q has long been considered as a typical innate immune molecule with a range of ligands and functions. Complement activation by classical way, alternative and lectin way was defined separately, which work in a complex system and interacted with each other. In this study, we focused on the effects of C1q classical way of complement activation after ICH and maintained the normal working way of alternative and lectin way. It has been reported that the roles of C1q in the CNS pathophysiology and development are some of the highlights of complement research in last decade [16]. Much more studies focusing on complement pathways after ICH are needed to provide potential therapeutic targets.

Conclusions

This study demonstrated that C1q activation after ICH attracted the infiltration of spleen-derived neutrophils, which aggravated neuroinflammation. Both splenectomy prior to ICH onset and anti-C1q antibody injection prevented synapse loss, alleviated hydrocephalus and decreased secretion of inflammatory cytokines after ICH. It provided proof-of-concept for monoclonal antibody inhibition of C1q classical complement pathway as a potential therapeutic strategy for ICH.

Declarations

Funding

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Not applicable

Availability of Data and Materials

The authors do not wish to publicly share the data and materials, please contact author for data requested.

Authors' contributions

ZC, JT, GZ and HF conceived and designed the experiments; TTL, JJG, BZ and YN performed the experiments; JLSJ, lw and TTL analysed the data; and JT and ZC wrote and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experimental procedures and animal care procedures were approved by the Ethics Committee of Southwest Hospital (Army medical university), performed in accordance with the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and reported following the ARRIVE guidelines (Animal Research: Reporting in Vivo Experiments, https://www.nc3rs.org.uk/ arrive-guidelines).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

Abbreviations

ICH: Intracerebral hemorrhage; CNS: Central nervous system; ELISA: Enzyme linked immunosorbent assay; TNF-α: Tumor necrosis factor alpha; IL-1β: Interleukin-1 beta; IL-6: Interleukin-6; TBI: traumatic brain injury; NLR: Neutrophil- lymphocytes ratio; GMH: Germinal matrix hemorrhage; BBB: Blood-brain barrier; GABA: Gamma-aminobutyric acid; MS: Multiple sclerosis

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Figures

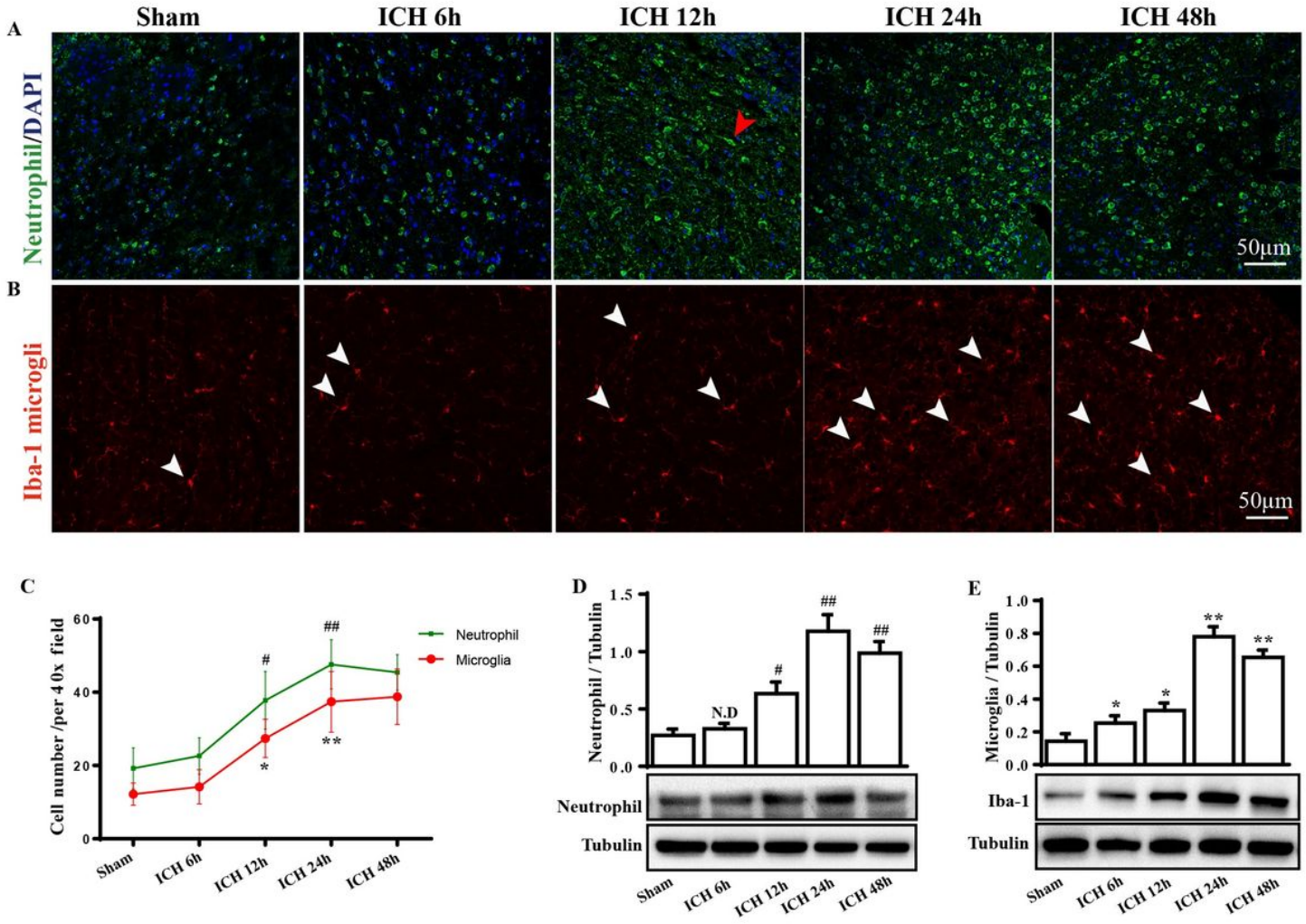

Figure 1

Immunohistochemical staining and western blotting analysis of neutrophil and microglia in mice at different time points after ICH. (A) Neutrophil (green) expression increased at 12h (red arrow) after ICH and peaked at 24h. (B) Microglia (red) activated (round cell bodies with low processes, white arrow) at 12h, 24h and 48h after ICH. (C) Cell counts of neutrophils and microglia per 40×field of every brain slice around hematoma at 6h, 12h, 24h and 48h after ICH (#<0.05, ##<0.01 for neutrophil vs. sham; *<0.05, ** <0.01 for microglia vs. sham). (D) Western blotting analysis of neutrophil protein by rabbit anti-neutrophil antibody and (E) microglia by rabbit anti-Iba-1 antibody at 6h, 12h, 24h and 48h after ICH (#<0.05, ## <0.01 for neutrophil vs. sham; *<0.05, **<0.01 for microglia vs. sham). One-way-ANOVA analysis was performed and summary statistics were reported as mean ± STD. Bar= 50μm.

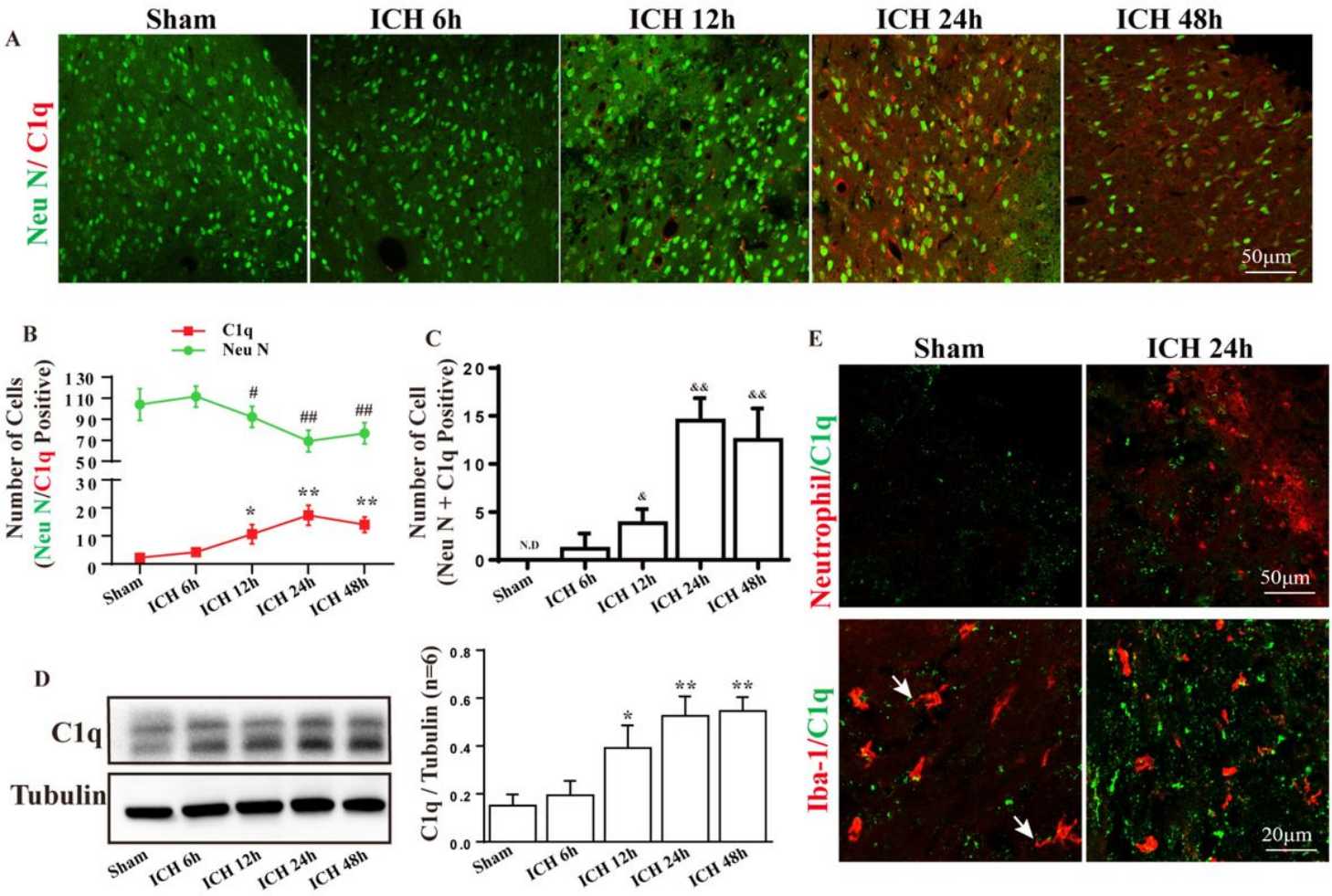

Figure 2

C1q protein expression in neurons, microglia and infiltrated neutrophils. (A) Double staining of neurons (Neu N+, green) and C1q (red) in brain slice of sham surgery mice and mice at 6h, 12h, 24h and 48h after ICH. (B) Cell counts of Neu N positive (green line) and C1q positive (red line) cells. (C) Cell counts of Neu N+C1q+ cells per 40×field of brain slice (vs. Sham, N.D: not detected, &<0.05, &&<0.01). (D) Western blotting analysis of C1q protein by rabbit anti-C1q antibody (vs. sham, *<0.05, **<0.01, One-way-ANOVA). We detected a significant increase of C1q protein (green) with neutrophil infiltration (red) and microglia activation (red, white arrow). Bar scale (A, E-neutrophil) 50μm, (E-Iba-1) 20μm. Summary statistics was reported as mean ± STD.

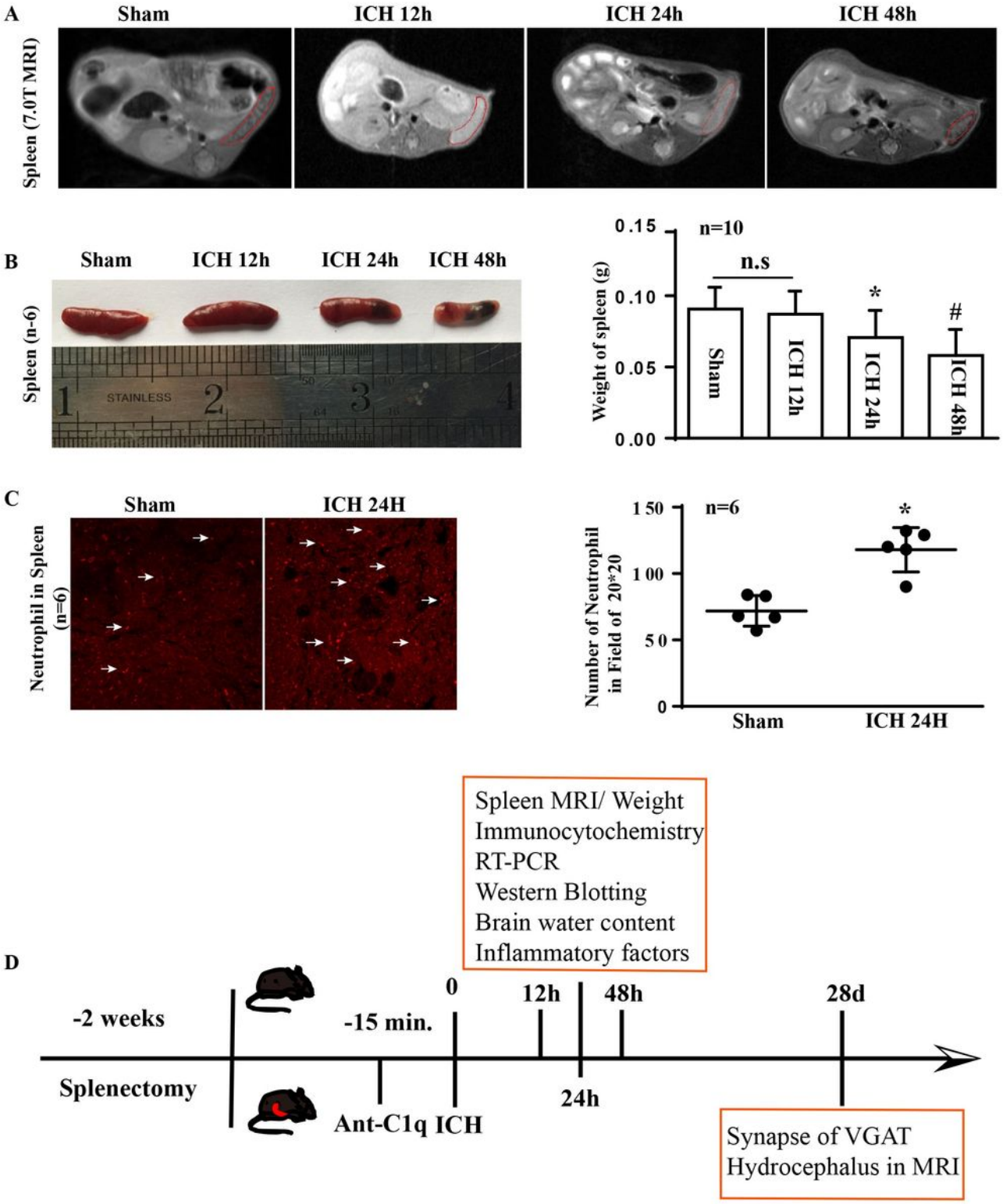

Figure 3

Spleen contracted at early stage following ICH and expression of neutrophils in spleen. (A) 7.0T MRI for spleen of mice in sham group and ICH group at12h, 24h and 48h after surgery. (B) General photograph of spleen and weight of spleen in sham group and ICH group at 12h, 24h and 48h after surgery (n.s: not significant, *<0.05 vs. sham and #<0.05 vs. ICH 24h). (C) Immunohistochemical staining and cell counts

of neutrophil in spleen of mice in sham group and 24h after ICH (bar=50μm, *<0.05 vs. sham). (D) A brief protocol schedule for further study was made. Summary statistics was reported as mean ± STD.

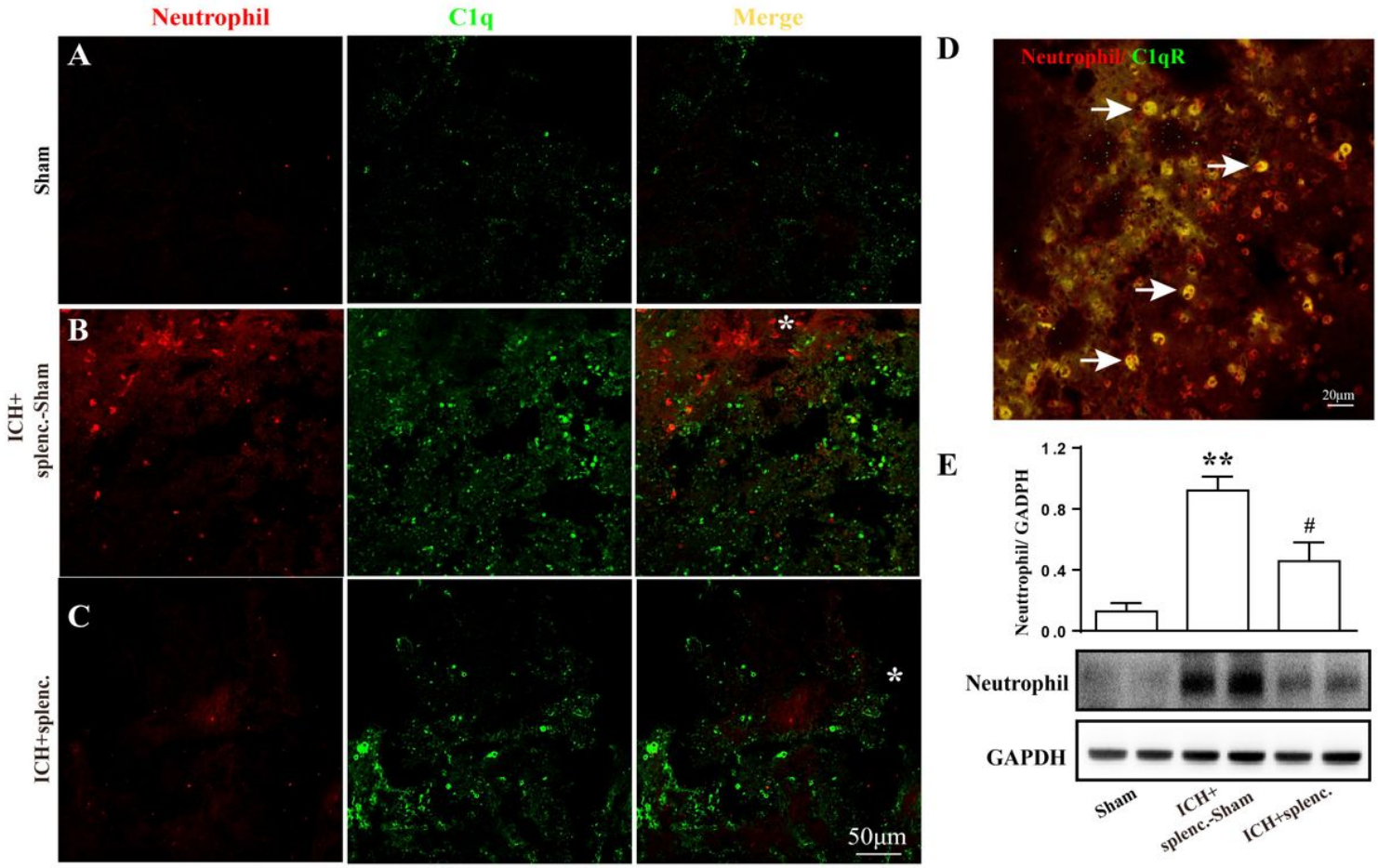

Figure 4

Splenectomy decreased the expression of C1q protein after ICH. (A) Double staining of neutrophil (red) and C1q (green) in brain slice of mice in sham group. (B) ICH induced over-expression of C1q protein surrounding infiltrated neutrophils. (C) Splenectomy downregulated C1q protein expression (bar=50μm, * indicated hematoma). (D) Co-expression of neutrophil (red) and C1qR (green) in ICH mice treated with vehicle (bar=20μm, white arrow indicated neutrophil+C1qR+ cells). (E) Western blotting analysis of C1q protein in group of sham (no ICH), ICH+ splenc. -sham (sham-splenectomy) and ICH+ splenc. (splenectomy prior to ICH). **<0.01 vs. sham, #<0.05 vs. ICH+ splenc. -sham.

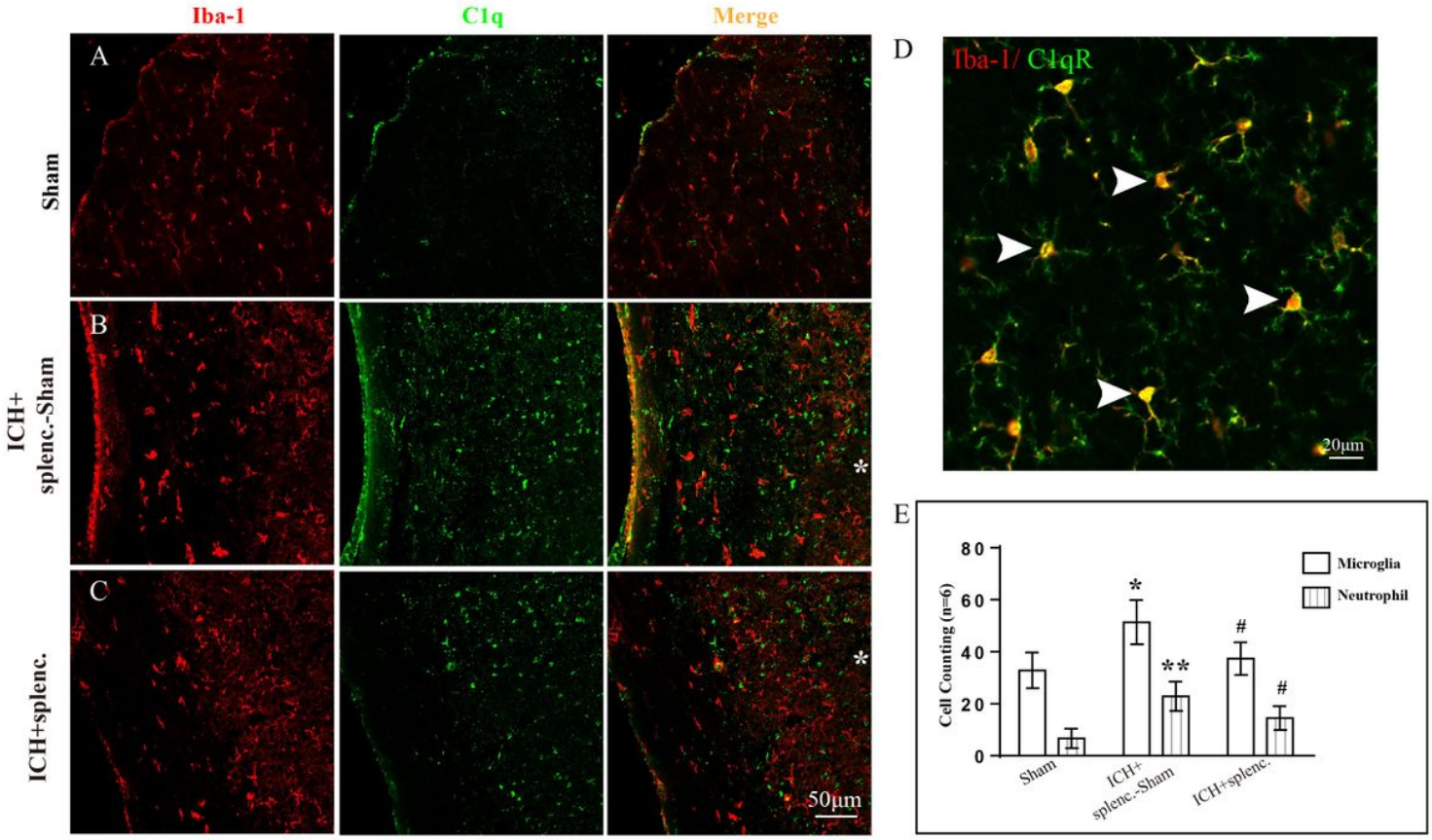

Figure 5

Immunohistochemical staining of microglia and C1q/C1qR, and cell counts of microglia and infiltrated neutrophils. (A~C) Double staining of microglia (red) and C1q (green) in brain slice of mice in group of sham (no ICH), ICH+ splenc. -sham (sham-splenectomy) and ICH+ splenc. (splenectomy prior to ICH), (bar=50μm, * indicated hematoma). (D) Co-expression of microglia (red) and C1qR (green) in ICH mice treated with vehicle (bar=20μm, white arrow indicated microglia+C1qR+ cells). (E) Cell counts of microglia and neutrophils per 40×field of brain slice (*<0.05, **<0.01 vs. sham, #<0.05 vs. ICH+ splenc. -sham). Summary statistics was reported as mean ± STD.

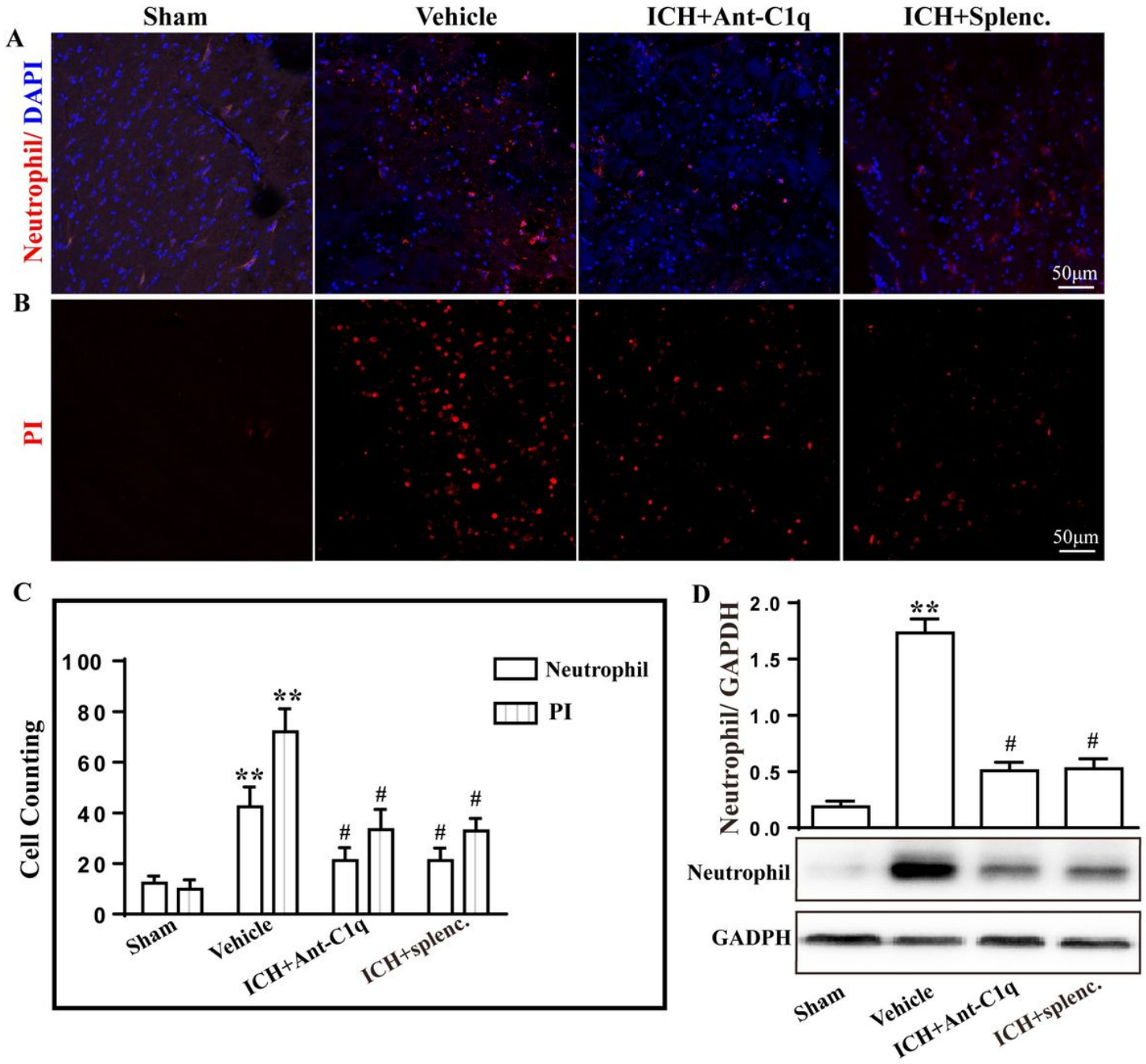

Figure 6

Splenectomy and anti-C1q antibody decreased neutrophil infiltration and PI positive dead cells following ICH. (A) Immunohistochemical staining of neutrophil (red) and DAPI (blue) in mice of sham (no ICH), vehicle (injected with saline), ICH+Anti-C1q (injected with Anti-C1q antibody prior to ICH) and ICH+ splenc. (splenectomy prior to ICH), bar= 50μm) group. (B) Immunohistochemical staining of PI (red) and DAPI (blue) in mice of sham, vehicle, ICH+Anti-C1q and ICH+ splenc. (bar= 50μm) group. (C) Cell counts of neutrophils and PI positive cells per 40×field of brain slice (**<0.01 vs. sham, #<0.05 vs. vehicle). (D) Western blot analysis of neutrophil protein in group of sham, vehicle, ICH+Anti-C1q and ICH+ splenc. (** <0.01 vs. sham, #<0.05 vs. vehicle) mice.

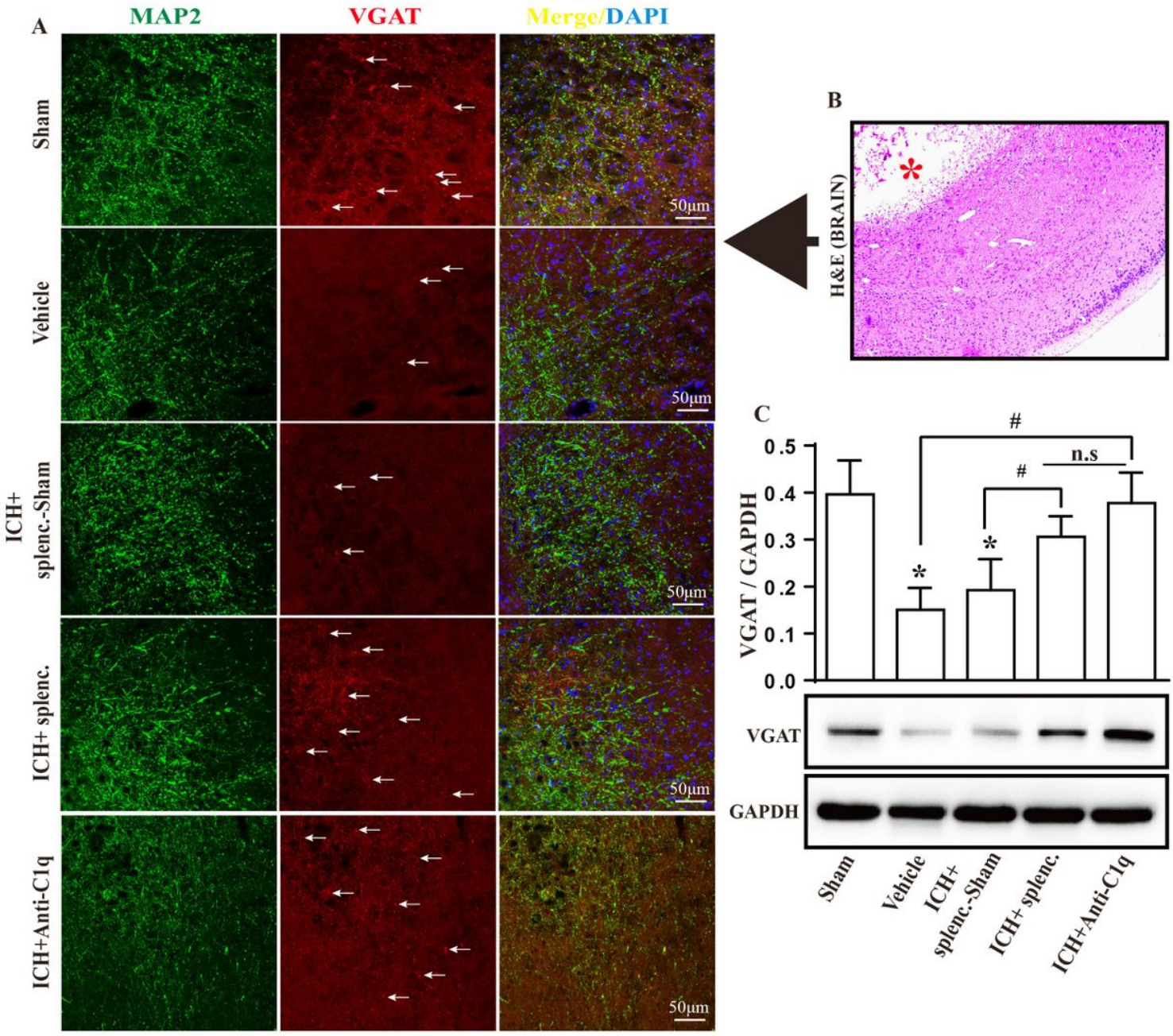

Figure 7

Splenectomy and anti-C1q antibody prevented synapse loss induced by ICH. (A) Double-labelling immunofluorescent staining of MAP2 (green) and VGAT (red), an integral membrane protein involved in gamma-aminobutyric acid (GABA) and glycine up-taking into synaptic vesicles, in group of sham, vehicle, ICH+ splenc. -sham, ICH+Anti-C1q and ICH+ splenc. (bar= 50μm, white arrow indicates positive VGAT) mice. (B) A brain slice of hematoxylin-eosin (H&E) staining indicated where the double-labelling immunofluorescence was observed. (C) Western blot analysis of VGAT protein in group of sham, vehicle, ICH+ splenc. -sham, ICH+Anti-C1q and ICH+ splenc. mice (*<0. 05 vs. sham, #<0.05 and n.s indicates not significant).

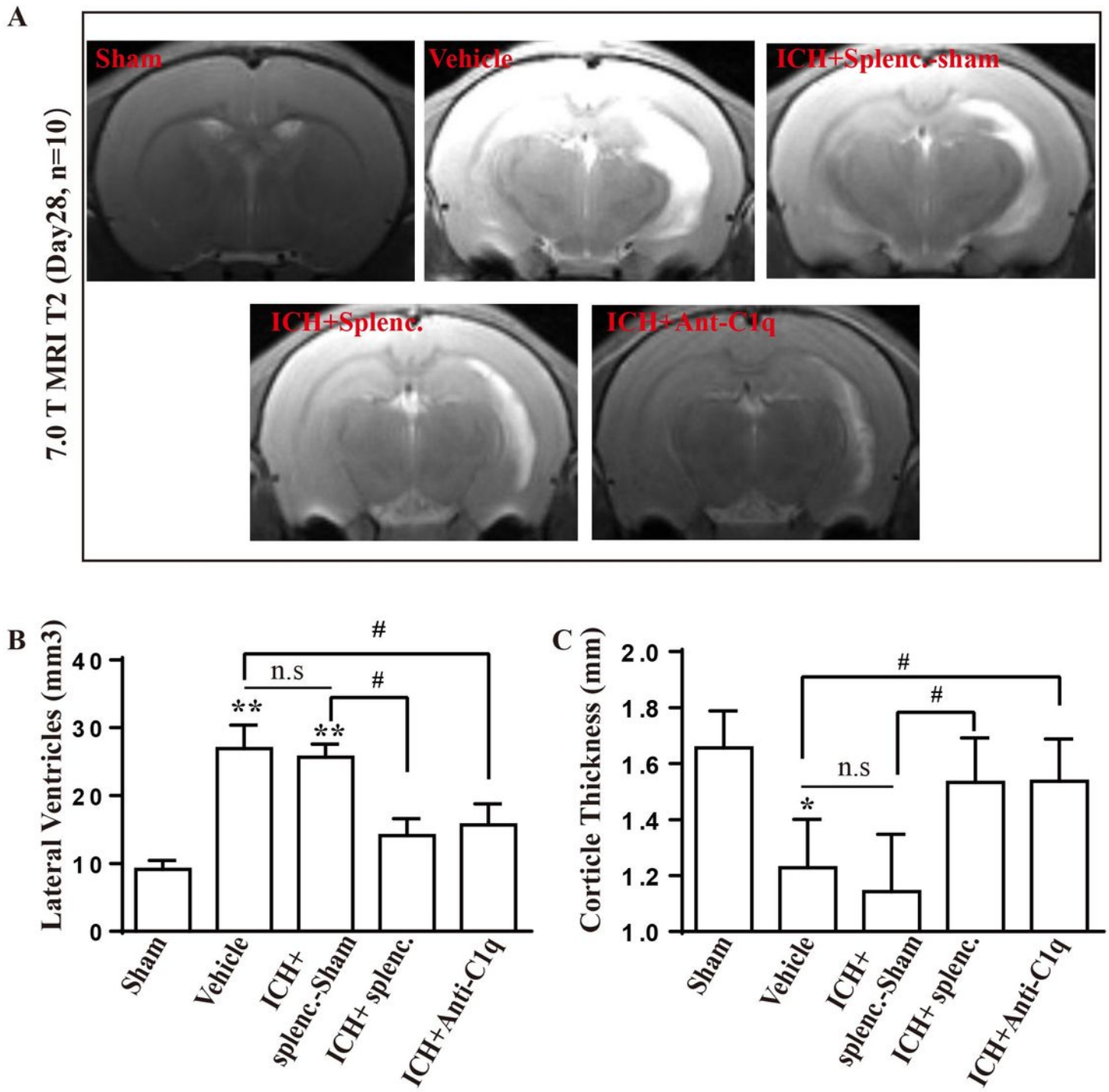

Figure 8

Hydrocephalus after ICH was improved by splenectomy and anti-C1q antibody in long term. (A) Representative images of mice brain containing ventricle by 7.0 Tesla animal MRI (T2) in group of sham, vehicle, ICH+ splenc. -sham, ICH+Anti-C1q and ICH+ splenc. mice 28 days after surgery (n=10). (B) Mean volume (mm3) of ventricles and (D) mean thickness (mm) of cortex in mice of each group. Summary statistics was reported as mean ± STD.

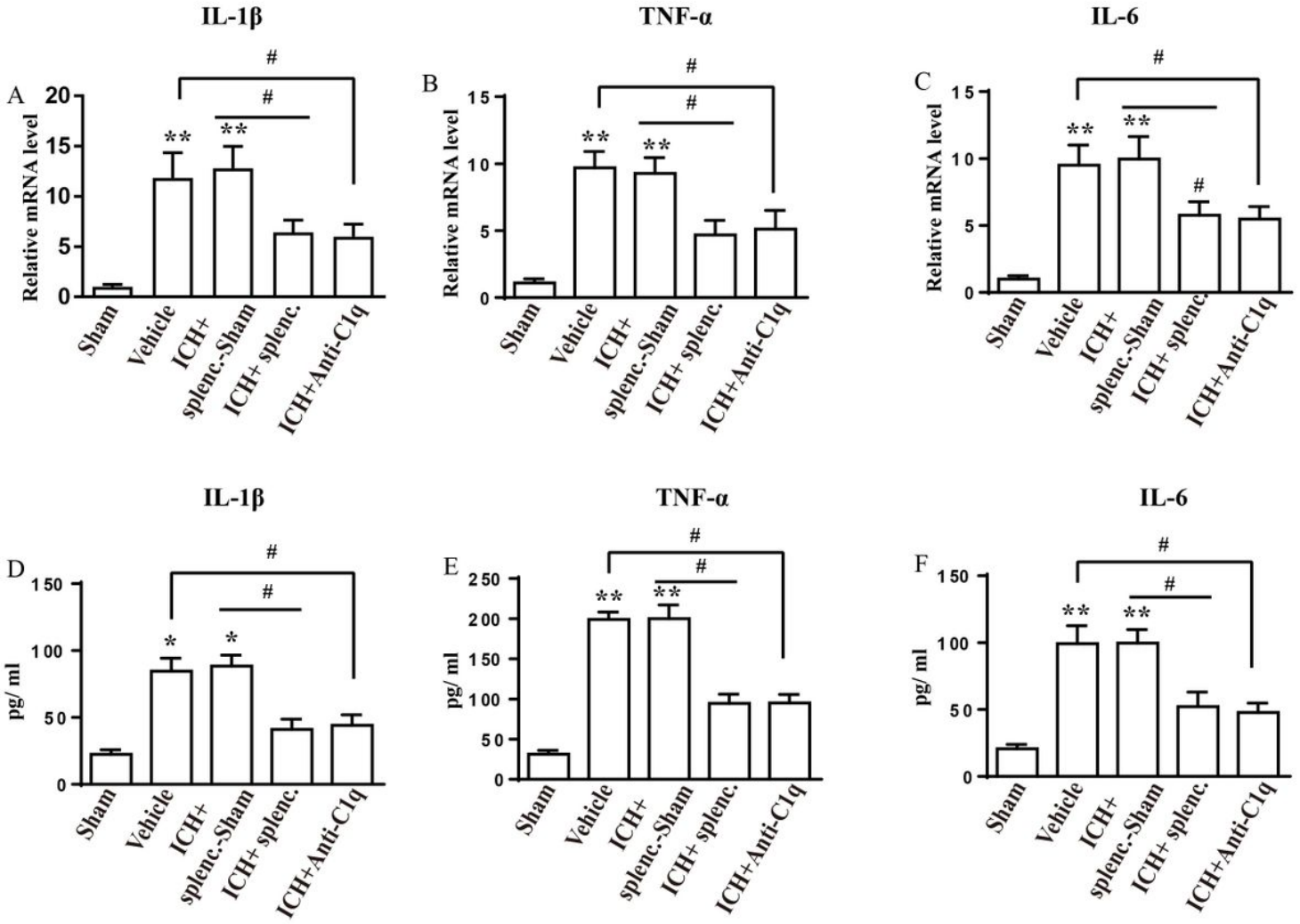

Figure 9

qPCR analysis of mRNA and ELISA analysis of protein of brain tissue isolated from perilesional ipsilateral cortex. ICH mice treated with vehicle injection or sham-splenectomy showed significant upregulation of pro-inflammatory cytokines (A) IL-1 β, (B) TNF-α and (C) IL-6 compared with that in sham-ICH mice. Relative mRNA and protein level coding the pro-inflammatory cytokines decreased following splenectomy and anti-C1q antibody. (D) IL-1 β, (E) TNF-α and (F) IL-6 24h after ICH. (*<0. 05, **<0.01 vs. sham, #<0.05).