Long noncoding RNA H19 promotes leukocyte inflammation in ischaemic stroke by targeting the miR-29b/C1QTNF6 axis

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Abstract

Background and Purpose Inflammatory processes induced by leukocytes are crucially involved in the pathophysiology of acute ischemic stroke. This study aimed to elucidate the inflammatory mechanism of long non-coding RNA (lncRNA) H19-mediated regulation of C1QTNF6 by sponging miR-29b in leukocytes during ischemic stroke. Experimental Approach H19 and miR-29b expression in leukocytes of patients with ischemic stroke and middle cerebral artery occlusion rats was measured through real-time PCR. Moreover, we performed in vivo and in vitro experiments to determine the impact of H19 and miR-29b on C1QTNF6 expression in leukocytes after ischemic injury. Key Results There was H19 and C1QTNF6 upregulation, as well as miR-29b downregulation, in neutrophils of patients with stroke. Moreover, miR-29b could bind C1QTNF6 mRNA to repress its expression while H19 could sponge miR-29b to maintain C1QTNF6 expression. Moreover, C1QTNF6 overexpression promoted the release of IL-1β and TNF-α in leukocytes and further exacerbates blood-brain barrier disruption. Conclusion and Implications Our findings confirm the inflammation-modulatory effect of the H19/miR-29b/C1QTNF6 axis on cerebral ischemic injury.

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Running title: The H19/miR-29b/C1QTNF6 axis in cerebral ischemic stroke

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Abbreviations

BBB, blood-brain barrier; C1QTNF, C1q and tumour necrosis factor; hCMEC/D3, Human brain capillary endothelial cells; IL-1β, Interleukin-1β· lncRNAs, long ncRNAs; miRs, MCAO, middle cerebral artery occlusion; MicroRNAs; NIHSS, National Institute of Health Stroke Scale; MMP-9, Matrix Metallopeptidase-9; ncRNA, non-coding RNA; OGD, Oxygen–glucose deprivation; TNF-α, tumour necrosis factor-α· TTC, 2,3,5-Triphenyl-2H-tetrazolium chloride; UTRs untranslated regions; ZO-1, Zonula occludens-1.

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Abstract

Background and Purpose

Inflammatory processes induced by leukocytes are crucially involved in the pathophysiology of acute ischemic stroke. This study aimed to elucidate the inflammatory mechanism of long non-coding RNA (lncRNA) H19-mediated regulation of C1QTNF6 by sponging miR-29b in leukocytes during ischemic stroke.

Experimental Approach

H19 and miR-29b expression in leukocytes of patients with ischemic stroke and middle cerebral artery occlusion rats was measured through real-time PCR. Moreover, we performed in vivo and in vitro experiments to determine the impact of H19 and miR-29b on C1QTNF6 expression in leukocytes after ischemic injury.

Key Results

There was H19 and C1QTNF6 upregulation, as well as miR-29b downregulation, in neutrophils of patients with stroke. Moreover, miR-29b could bind C1QTNF6 mRNA to repress its expression while H19 could

sponge miR-29b to maintain C1QTNF6 expression. Moreover, C1QTNF6 overexpression promoted the release of IL-1β and TNF-α in leukocytes and further exacerbates blood-brain barrier disruption.

Conclusion and Implications

Our findings confirm the inflammation-modulatory effect of the H19/miR-29b/C1QTNF6 axis on cerebral ischemic injury.

Key words: ischaemic stroke, leukocyte, C1QTNF6, inflammation

Bullet point summary:

What is already known:

miR-29b and H19 expression in leukocytes are significantly changed after ischemic injury.

C1QTNF6 directly modulate pro-inflammatory cytokine gene expression in leukocytes.

What this study adds:

C1QTNF6 promoted the release of IL-1β and TNF-α in leukocytes and further exacerbates blood-brain barrier disruption.

lncRNA H19 regulated the C1QTNF6 mRNA by sponging miR-29b in leukocytes during ischemic stroke.

Clinical significance:

Mechanism of H19/miR-29b/C1QTNF6 axis in leukocytes promotes BBB damage through IL-1β and TNF-α upregulation in cerebral ischemic injury.

INTRODUCTION

Stroke is a major mortality and morbidity cause worldwide (Ding et al., 2020; Ma, Jiang, Xu, Liu, & Guo, 2021). Ischemic stroke, which is caused by thrombosis or embolism in blood vessels, is the most common stroke subtype(Mendelson & Prabhakaran, 2021). Currently, there remains no specific treatment for improving the prognosis in patients with ischemic stroke given the complex pathogenesis. A series of post-stroke inflammatory responses are crucially involved in functional recovery, which compromises the ischemic stroke prognosis.

Peripheral leukocytes significantly affect the clinical outcome of stroke and could be a therapeutic target for treating and preventing ischemic stroke (G. Li, Ma, et al., 2018). There have been several studies on non-coding RNA (ncRNA) expression in peripheral leukocytes in patients with ischemic stroke. ncRNAs in leukocytes regulate the release of inflammatory factors and promote inflammatory responses after cerebral ischemic injury(Liu et al., 2020). ncRNAs can be classified as short ([?] 200 base pairs) or long ncRNAs (lncRNAs; $>$ 200 base pairs) (Palazzo & Koonin, 2020). MicroRNAs (miRNAs, miRs) are small ncRNAs with $<$ 22 nucleotides that modulate protein expression at the posttranscriptional level (G. Li, Morris-Blanco, et al., 2018). Mature miRNAs prevent mRNA translation or promote mRNA degradation by binding to the consensus seed sequences in the 3' untranslated regions (UTRs) of the target mRNAs(G. Li, Morris-Blanco, et al., 2018). Recent studies have demonstrated a mutual regulation between miRNAs and lncRNAs, including a direct interaction and an indirect role by binding to target genes in ischemic stroke(Ghafouri-Fard, Shoorei, & Taheri, 2020).

To identify leukocyte genes related to ischemic stroke, we previously detected mRNA expression in neutrophils using RNA-seq and compared it between patients with acute ischemic stroke and healthy controls. We found altered expression of C1q and tumour necrosis factor (C1QTNF)6 after ischemic stroke. C1QTNF6 was originally identified in the heart, brain, and peripheral inflammatory cells; further, it is involved in diverse processes, including apoptosis, metabolism, and release of inflammatory factors(Yan et al., 2021). C1QTNF6 can directly modulate pro-inflammatory cytokine gene expression in leukocytes(Lei et al., 2017). Our preliminary findings demonstrated that C1QTNF6 mRNA can be modulated by several miRNAs in

neutrophils, including miR-29b. miR-29b expression in leukocytes is significantly decreased after ischemic injury; moreover, changes in miR-29b are correlated with the prognosis of ischemic stroke(Y. Wang et al., 2015). miR-29b overexpression alleviates brain injury and attenuates blood-brain barrier (BBB) damage (Y. Wang et al., 2015). Moreover, we predicted potential targeting lncRNAs related to miR-29b and identified the binding site of miR-29b in H19 using Starbase (http://starbase.sysu.edu.cn/index.php). Blood H19 levels are increased after ischemic stroke in circulating blood, which promotes the release of inflammatory factors. Therefore, we hypothesised that C1QTNF6 in leukocytes may be regulated by H19 and miR-29b, which could be involved in the pathogenesis of ischemic stroke.

This study aimed to elucidate the inflammatory mechanism of lncRNA H19-mediated regulation of C1QTNF6 by sponging miR-29b in leukocytes during ischemic stroke.

MATERIALS AND METHODS

Participants

This study was registered with ClinicalTrials.gov (NCT03577093). We recruited 50 patients with acute ischemic stroke of the anterior circulation from the emergency department of Xuanwu Hospital between March 2016 and December 2017. Ischemic stroke was diagnosed by two neurologists based on the patient's history, laboratory examination, neurological deficits, and diffusion-weighted magnetic resonance imaging results. We included patients with first-time ischemic stroke with a National Institute of Health Stroke Scale (NIHSS) score < 25 points who were admitted within 6 hours after symptom onset. We excluded patients with a history of malignant tumour, acute infectious disease, haematologic disease, or renal or liver failure. Moreover, we included 42 age- and sex-matched healthy volunteers without central nervous system diseases from the medical examination centre as the control group.

Blood collection and separation of neutrophils

Venous blood samples (10 ml) obtained from the patients with ischemic stroke and healthy controls were collected in ethylene diamine tetraacetic acid anticoagulant vacuum tubes at admission. Blood samples were fractionated through centrifugation at 3000 × g for 10 min at 4°C. Next, the plasma layer was aliquoted and stored at -80°C for routine lab assays. Neutrophils were separated using a standard Ficoll-Paque Plus gradient method for RNA extraction and examination as previously described(G. Li, Ma, et al., 2018).

Animals

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Qingdao University. Male Sprague-Dawley rats weighing 260–280 g were purchased from Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). The animals were maintained in standard-housing open-top cages at a specific pathogen-free facility at 23 +- 1degC with 50%–60% humidity at Qingdao University. Before the procedure, all animals were fasted for 24 h with free access to tap water.

Middle cerebral artery occlusion

Rats underwent surgery for right middle cerebral artery occlusion (MCAO). After anesthetisation, a nylon filament with a silicon tip of a diameter of 0.37 mm was inserted into the right MCA to obstruct blood flow for 2 h. Subsequently, it was removed to allow reperfusion. Regional cerebral blood flow was observed using transcranial laser Doppler to confirm a decrease in blood flow by 20%–30% compared with the baseline value during MCAO. Sham-operated rats underwent the same anaesthesia surgical procedures without MCAO. After recovering from anaesthesia, the rats were returned to sterilised cages with regular access to food and water.

Rats were randomly divided into six groups (n = 12/group): sham group; MCAO + control group; MCAO + miR-29b antagomir group; MCAO + H19 siRNA group; MCAO + miR-29b antagomir group + H19 siRNA control group; MCAO + miR-29b antagomir + H19 siRNA group. miR-29b antagomir, H19 siRNA, and transfection reagent were purchased from GenePharma (Shanghai, China). Three days before MCAO, the rats were treated using a mixture of 100 μL miR-29b antagomir, H19 siRNA, or control +

32-μl transfection reagent through the tail vein. The sequences were 5'-CCACCGUAAUUCAUUUAGATT-3' (H19 siRNA), 5'-AACACUGAUUUCAAAUGGUGCUA-3' (miR-29b antagomir), and 5'-UUCUCC GAACGUGUCACGUTT-3' (H19 siRNA control).

Behavioural tests

Behavioural tests were performed using 12 animals in each group. The rats underwent neurological evaluation using the Zea-Longa 5-point scoring system before and 24 h after surgery (Longa, Weinstein, Carlson, & Cummins, 1989).

2,3,5-Triphenyl-2H-tetrazolium chloride (TTC) staining

Rats were sacrificed 24 h after reperfusion. Brains were quickly removed and cut into 2-mm thick coronal slices for TTC staining. Infarct and oedema volume were detected using TTC staining. The infarct volume was calculated as follows: (contralateral hemisphere volume - undamaged ipsilateral hemisphere)/contralateral hemisphere volume × 100%. Volume calculation with oedema correction as follows: (ipsilateral hemisphere volume-contralateral hemisphere volume)/contralateral hemisphere volume × 100%.

Western blotting

The ipsilateral cerebral hemisphere, rat leukocytes, and cultured cells were homogenised in lysis buffer containing phosphatase and protease inhibitors. We used the following primary antibodies: anti-Interleukin (IL)-1β (Catalogue, AF-501-NA), anti-tumour necrosis factor (TNF)-α (Abcam, ab205587), anti-Matrix Metallopeptidase(MMP)-9 (Abcam, ab76003), anti-zonula occludens(ZO)-1 (arigo, ARG55738), anti-occludin (Abcam, ab216327), anti-caspase-3 (Abcam, ab13847; Affinity Biosciences, AF7022), anti-C1QTNF6 (Abcam, ab36900), and anti-β-actin (Abcam, ab20272). Signals were detected through incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) for 60 min at room temperature and using an enhanced chemiluminescence kit (Millipore, Darmstadt, Germany). Grey values of the protein bands were analysed using ALPHAEASE FC software (Alpha Innotech, San Leandro, CA, USA).

RT-PCR

Equivalent amounts of purified RNA obtained from neutrophils, leukocytes, and brain tissue were used as a template for cDNA synthesis using SYBR Green qPCR Master Mix (Fermentas, Burlington, Canada), oligo-d(T) primers, and SuperScript III/RNaseOUT Enzyme Mix (Invitrogen, Carlsbad, CA, USA). MiR-29b primers for human neutrophils were 5'-GGGTAGCACCATTTGAAATCA3' and 5'-GTGCGTGTCGTGGAGTCG-3'. MiR-29b primers for rat leukocytes were 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAG-3' and 5'-ACACTCCAGCTGGGTAGCACCATTTGAAATC-3'. H19 primers for human neutrophils were 5'-CTTCTTTAAGTCCGTCTCGTTC-3' and 5'-GAGGCAGGTAGTGTAGTGGTTC-3'. H19 primers for rat 5'- GGAATCGGCTCGAAGGTAAA-3' and 5'-GGGCCAGGCAGAGTTAGTTG-3'. C1QTNF6 primers for human neutrophils were 5'-TCAGTCCCTTCCACCAAA-3' and 5'-ACCTTGATAAAGCCTGGAGA-3'. C1QTNF6 primers for rat were 5'-GTTCGGGGTCTGTGAGTTGAG-3' and 5'-CTTTCAGGATGGTGATGTTGATGTA-3'. The relative gene expression was calculated using the 2^{-T} method, normalised, and expressed as fold change relative to U6.

NeuN/TUNEL staining and immunofluorescence analysis

The rats were killed through chloral hydrate administration and cardiac perfusion with physiological saline and 4% paraformaldehyde. Brains were harvested and fixed in 4% paraformaldehyde for 48 h, followed by dehydration in 30% sucrose. A cryostat vibratome was used to obtain 20-μm-thick brain coronal sections. The brain sections were blocked with 0.3% (w/v) BSA in PBS at room temperature for 1 h. Sections were incubated using NeuN antibodies (Millipore, 1:1000) overnight at 4°C. Slides were washed in TBST, incubated with Alexa Fluor 488 AffiniPure Donkey antibody, and counterstained with DAPI. The number of

NeuN/TUNEL-positive cells in the ischemic region was calculated using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

The brain coronal sections were incubated using primary antibodies against CD11b (NB100-65284) and C1QTNF6 (Abcam, ab36900) at 4°C overnight, followed by incubation with a fluorophore-labelled secondary antibody (Santa Cruz Biotechnology). Fluorescence images were acquired using an Olympus Fluoview FV1000 fluorescence microscope (Olympus, Japan).

ELISA

We collected plasma from patients with stroke and healthy volunteers as previously described(G. Li, Ma, et al., 2018). Approximately 200 μL of plasma was prepared in an ice-cold phosphate-buffered solution. Protein levels of TNF-α, IL-1β, and MMP-9 were quantified using a commercial ELISA kit (Abcam), following the manufacturer's instructions.

Cell culture, transfection, oxygen–glucose deprivation treatment, and co-culturing

The human acute myeloid leukaemia cell line, HL-60, was used as the neutrophil model. HL-60 cells were cultured in 1640 RPMI medium containing 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in an atmosphere of 5% CO_2. HL-60 cells were transfected with a mixture of H19 siRNA, miR-29b antagomir or control, and Lipofectamine RNAiMAX (GenePharma); subsequently, they were incubated for 24 h in a humidified incubator for further analyses.

Human brain capillary endothelial cells (hCMEC/D3) were grown in EBM-2 medium supplemented with vascular endothelial growth factor, insulin-like growth factor-1, epidermal growth factor, basic FGF, hydrocortisone, ascorbate, penicillin-streptomycin, and 2.5% FCS. Subsequently, they were maintained in an incubator at 37°C and 5% CO_2. Oxygen–glucose deprivation (OGD) and reoxygenation were used as in vitro models for replicating cerebral ischaemia. The hCMEC/D3 cells were kept in a glucose-free and hypoxic incubator chamber with a gas mixture of 94.5% N_2, 0.5% O_2, and 5% CO_2 at 37 °C for 2.5 hours, followed by co-culturing with HL-60 cells for 24 h. The hCMEC/D3 cells were divided into five groups: Sham + control-HL-60 group; OGD + control-HL-60 group; OGD + miR-29b antagomir-HL-60 group (20 nM); OGD + H19 siRNA-HL-60 group (20 nM); and the OGD + miR-29b antagomir (20 nM) and H19 siRNA (20 nM) groups.

In the co-culture experiments, 10 x 10^5 hCMEC/D3 cells/well were co-cultivated with HL-60 cells treated with H19 siRNAs, miR-29b, or controls under non-contact conditions. Further, 10 x 10^5 HL-60 cells/well were seeded on the apical side of Transwell membranes (Corning, 0.4 μm pore size) and cultivated with supplemented medium. Moreover, hCMEC/D3 cells were seeded to the basal side and further cultivated as aforementioned in the static monoculture model. At 24 h after co-culture, the cells were separately collected for further analysis.

Flow cytometry

The apoptosis ratio was estimated using the Dead Cell Apoptosis Kit with Annexin V Alexa Fluor 488 for flow cytometry, following the manufacturer's instructions (Invitrogen, Carlsbad, USA). Cells were collected, washed twice with cold PBS, and incubated with Annexin V-FITC mixed with propidium iodide for 10 min in the dark. Cellular fluorescence was assessed using flow cytometry (CytoFLEX S, Beckman, China).

Statistical analysis

Statistical analyses were performed using SPSS version 23.0. Numeric data are expressed as mean ± SD. Student's t-test was used for between-group comparisons. One-way analysis of variance with Tukey–Kramer post hoc test was used to compare several quantitative variables. Pearson's correlation test was used to assess between-variable correlations. Statistical significance was set at $P < 0.05$.

RESULTS

There is H19 and C1QTNF6 upregulation, as well as miR-29b downregulation, in neutrophils of patients with ischemic stroke

Neutrophils are the most abundant leukocytes; therefore, we detected the maximal changes in the neutrophil gene expression. We found a novel gene, C1QTNF6, which showed increased mRNA expression in the neutrophils of patients with acute ischemic stroke compared with healthy volunteers (Figure 1A, $P < 0.05$). Further analysis using TargetScan and StarBase databases revealed that C1QTNF6 is regulated by miR-29b and H19. Further, our analysis identified the putative miR-29b binding sites of H19 and 3' UTR of C1QTNF6 mRNA (Figure 1D). To confirm this, we conducted quantitative real-time PCR to evaluate the mRNA expression of miR-29b, H19, and C1QTNF6 in neutrophils. H19 expression in neutrophils was significantly increased in patients with stroke (Figure 1A, $P < 0.05$). Further, patients with stroke showed decreased miR-29b expression (Figure 1A,P <0.05), which significantly increased C1QTNF6 levels. Additionally, there was a negative linear correlation between miR-29b and C1QTNF6 levels in the neutrophils of patients with stroke (Figure 1B, r = -0.716, $P < 0.001$).

H19 and miR-29b levels are correlated with the National Institute of Health Stroke Score

To further elucidate the clinical significance of H19 and miR-29b in patients with stroke, we analysed the correlation of clinical parameters with H19 and miR-29b expression. H19 levels were positively correlated with the NIHSS score at admission (Figure 1C, r = 0.468, P= 0.002). However, miR-29b levels were negatively correlated with the NIHSS scores (Figure 1B; r = -0.491, $P < 0.001$). Furthermore, plasma IL-1β, TNF-α, and MMP-9 levels were significantly higher in patients with acute ischemic stroke than in healthy controls (Figure 1E, P <0.05). This further suggested that H19 and miR-29b in neutrophils are critically involved in ischemic stroke.

H19 expression is upregulated after MCAO injury while H19 inhibition alleviates rat MCAO injury

Given the increased H19 expression in patients with stroke, we further evaluated the role of H19 in cerebral ischemic injury through intravenous injection of H19 siRNA to rats at 3 days before MCAO. Quantitative PCR was performed to detect H19 expression and verify the efficacy of H19 siRNA transfection. First, there were increased H19 levels in the ischemic brain of rats at 24 h after MCAO (Figure 2A,$P < 0.001$). There were no significant differences in leukocyte H19 levels between MCAO rats and sham rats; however, there was an upregulation trend (Figure 2A, $P > 0.05$). There was decreased leukocyte H19 expression after treatment with H19 siRNA (Figure 2B, $P < 0.001$). Furthermore, TTC staining showed that H19 siRNA treatment decreased cerebral infarct volume and oedema volume compared with that in MCAO rats (Figure 2C,P <0.05). Moreover, H19 siRNA alleviated the MCAO-induced neurological deficits (Figure 2C, P <0.05).

To determine whether H19 siRNA preserves neurons from ischemic injury, we examined the number of NeuN/TUNEL-positive cells in rats after MCAO. MCAO injury increased the number of cortical TUNEL-positive cells compared with sham-operated rats, which was abrogated by H19 siRNA treatment (Figure 2D, E $P < 0.05$). These results demonstrate that H19 inhibition can attenuate cerebral ischemic injury in the cortex.

MiR-29b expression is downregulated after MCAO injury while miR-29b inhibition exacerbates cerebral injury

We investigated the effect of intravenous injection of miR-29b antagomir by measuring the infarct volume and neurological deficits after MCAO injury. First, we found decreased miR-29b levels in the ischemic brain and leukocytes at 24 h in MCAO rats (Figure 2A, $P < 0.05$). Second, there was downregulated miR-29b expression in leukocytes after miR-29b antagomir injection (Figure 2B, $P < 0.05$). Third, TTC staining showed that miR-29b antagomir treatment increased the cerebral infarct volume and oedema volume compared with that in MCAO rats (Figure 2C, $P < 0.05$). MiR-29b antagomir significantly promoted MCAO-induced neurological deficiency (Figure 2C, $P < 0.05$). Fourth, miR-29b antagomir increased the

number of cortical TUNEL-positive cells in MCAO rats (Figure 2D, $P < 0.05$). Fifth, H19 siRNA promoted miR-29b expression, which indicated that H19 interacts with miR-29b in the leukocytes of MCAO rats (Figure 2B, $P < 0.05$).

H19 ανδ μιP-29β ρεγυλατες MΆO-ινδυςεδ ΄1XTNΦ6, IΛ-1β, ανδ TNΦ-α εξπρεσσιον

To determine whether miR-29b is involved in the pathophysiology of cerebral ischemic stroke, MCAO rats were intravenously injected with miR-29b antagomir to decrease miR-29b expression in leukocytes. First, miR-29b antagomir treatment significantly increased C1QTNF6 mRNA levels in leukocytes of MCAO rats (Figure 3A, $P < 0.05$). Moreover, miR-29b antagomir treatment increased C1QTNF6 protein levels in the brain (Figure 3B,$P < 0.05$). Additionally, miR-29b antagomir treatment increased IL-1β and TNF-α levels in MCAO rats (Figure 3A, B, $P< 0.05$).

Additionally, we analysed IL-1β and TNF-α expression in MCAO rats treated with H19 siRNA. Treated with H19 siRNA significantly increased brain and leukocyte levels of IL-1β and TNF-α (Figure 3A, B, $P< 0.05$). Additionally, C1QTNF6 mRNA and protein expression in leukocytes and the brain, respectively, were significantly downregulated in MCAO rats treated with H19 siRNA (Figure 3A, B, $P < 0.05$). Taken together, our findings suggest that H19 and miR-29b regulate C1QTNF6 levels; moreover, they affect IL-1β and TNF-α expression in leukocytes and the brains of MCAO rats.

H19 inhibition reversed MCAO injury worsened by miR-29b antagomir

To further elucidate the relationships among H19, miR-29b, and C1QTNF6 in cerebral ischemic injury, we intravenously injected H19 siRNA in MCAO rats with miR-29b antagomir and analysed the infarct volume and brain oedema at 24 h after MCAO. H19 siRNA reduced cerebral infarct volume and brain oedema compared with that in MCAO rats treated with miR-29b antagomir (Figure 4A,$P < 0.05$). Additionally, H19 siRNA significantly improved neurological deficits in MCAO rats with miR-29b antagomir treatment (Figure 4A, $P < 0.05$).

C1QTNF6 mRNA and proteins in leukocytes and the brain, respectively, were significantly higher in MCAO rats treated with miR-29b antagomir than in MCAO rats. Moreover, this increase was downregulated by H19 siRNA treatment (Figure 4C, D, $P < 0.05$). Additionally, cerebral and leukocyte TNF-α levels, as well as IL-1β levels, were decreased in rats treated with H19 siRNA than in MCAO rats treated with miR-29b antagomir (Figure 4C, D, $P < 0.05$). These findings suggest that H19 promotes TNF-α and IL-1β secretion by targeting the miR-29b/C1QTNF6 axis in leukocytes.

H19 and miR-29b in HL-60 cells regulate hCMEC/D3

apoptosis

To explore whether C1QTNF6 in leukocytes affects hypoxia-induced BBB disruption through an inflammatory response, we established an in vitro model in which OGD-exposed hCMEC/D3 cells were co-cultured with HL-60 cells. HL-60 cells in the different groups were treated as aforementioned. Flow cytometry analysis revealed a decreased apoptosis ratio in hCMEC/D3 cells in the OGD+H19 siRNA-HL-60 group than in the OGD+control-HL-60 group (Figure 6A, B, $P < 0.05$). However, there was an increased apoptosis ratio in hCMEC/D3 cells in the OGD+miR-29b antagomir-HL-60 group than in the OGD+control-HL-60 group (Figure 6A, B, $P < 0.05$). However, the increased hCMEC/D3 cell apoptosis induced by OGD+miR-29b antagomir-HL-60 was reversed by H19 siRNA treatment (Figure 6A, B, $P < 0.05$).

Additionally, we measured C1QTNF6, IL-1β, and TNF-α expression in HL-60 cells and ZO-1 and occludin expression in hCMEC/D3 cells. Treatment with miR-29b antagomir significantly increased C1QTNF6, IL-1β, and TNF-α expression in HL-60 cells (Figure 6C, $P < 0.05$). There was a significant decrease in C1QTNF6, IL-1β, and TNF-α expression in HL-60 cells treated with H19 siRNA than in OGD HL-60 cells (Figure 6C,$P < 0.05$). Furthermore, we observed changes in ZO-1 and occludin levels in hCMEC/D3 cells, as well changes in C1QTNF6, IL-1β, and TNF-α expression in HL-60 cells. (Figure 6C). These findings suggest that H19 in leukocytes aggravates hypoxia-induced hCMEC/D3 apoptosis by targeting miR-29b/C1QTNF6.

DISCUSSION

Our findings confirmed H19 upregulation in neutrophils in patients with acute ischemic stroke, which is positively associated with the severity of neurological deficits. Moreover, we observed miR-29b downregulation in neutrophils in patients with acute ischemic stroke, which was negatively associated with H19 levels. Additionally, we demonstrated that H19 regulates C1QTNF6 expression by sponging miR-29b, which facilitates IL-1β and TNF-α release by leukocytes and BBB disruption during cerebral ischemic injury. Our findings may provide new insights into the targeted treatment of ischemic stroke.

Emerging evidence regarding peripheral immune cells has provided insights into novel inflammatory mechanisms contributing to neuronal cell death in cerebral ischemic injury(Huang, Hussain, & Chang, 2021). Leukocytes are a sensitive indicator of inflammatory stimuli in patients with ischemic stroke(L. Wang et al., 2020). In patients with ischemic stroke, there is altered leukocyte expression of multiple ncRNAs, including H19, which is crucially involved in the inflammatory response of ischemic stroke(J. Wang et al., 2017). We previously reported that H19 increases the immune response by increasing plasma TNF-α and IL-1β levels, as well as decreasing plasma IL-10 levels, after ischemic stroke; moreover, it affects the subsequent pathological outcome(J. Wang et al., 2017). Wan et al. found that H19 upregulates MCAO-induced IL-1β and IL-18 expression by triggering caspase-1 signals; further, H19 excision exerted anti-inflammatory effects by inhibiting the production of these cytokines(Wan et al., 2020). Additionally, H19 functions as a competitive endogenous RNA for regulating neuronal apoptosis by sponging different miRNAs. Cheng et al. reported H19 upregulation in patients with diabetes mellitus, which directly suppresses miR-29b levels (Cheng et al., 2019). A recent study reported that H19 inhibition ameliorated OGD-induced hippocampal neuronal apoptosis and increased inflammatory cytokine levels by directly targeting miR-29b(Xu, Wang, Meng, & Xu, 2021). We observed significant H19 upregulation in the leukocytes of patients with acute ischemic stroke and MCAO rats; moreover, H19 excision suppressed IL-1β and TNF-α secretion by directly targeting miR-29b. Moreover, we identified the binding site of miR-29b in H19 using the Starbase database. Our findings suggest that H19 and miR-29b interactions are crucially involved in inflammatory signalling during cerebral ischemic injury.

Multiple basic studies have demonstrated that miR-29b is a key mediator of the ischemic cascade in regulating inflammation and neuronal apoptosis (Jia, Hao, Wang, & Qu, 2015). There is decreased miR-29b expression in the ischemia region and in OGD-activated microglia, which contributes to injury in focally ischemic brains (H. Wang, Li, Gao, & Liao, 2019). Additionally, miR-29b can inhibit neuronal apoptosis by suppressing TNF-α and IL-1β release in OGD-activated microglia(H. Wang et al., 2019). There are significantly decreased blood miR-29b levels in patients with ischemic stroke, as well as decreased blood and brain levels of miR-29b in patients with cerebral ischemic injury(Y. Wang et al., 2015). This suggests that miR-29b in the brain and circulating blood could be involved in regulating the post-stroke immune response, which is consistent with our findings. We found that miR-29b excision promoted neuronal apoptosis and upregulated TNF-α and IL-1β expression by targeting C1QTNF6. Similarly, Wang et al. reported that miR-29b promoted IL-1β, IL-6, and IL-8 expression by inhibiting C1QTNF6 in human bronchial epithelial cells (J. Wang et al., 2020).

C1QTNF6 is an important member of the C1QTNF family. It was originally found to exert inflammatory-regulating effects in several diseases(Kirketerp-Moller, Bayarri-Olmos, Krogfelt, & Garred, 2020). C1QTNF6 is crucially involved in alleviating OGD-induced inflammatory molecules, including TNF-α, IL-1β, IL-6, and IL-10, in PC12 cells. (Y. Li, Sun, Gu, & Gao, 2020). CIQTNF6 overexpression protects against OGD-induced inflammation and cell apoptosis by activating PI3K/Akt signalling(Y. Li et al., 2020). CIQTNF6 induces IL-10 expression in macrophages, which may represent a novel target for pharmacological therapy in inflammatory diseases(Kim, Lee, Park, & Park, 2010). Consistent with previous findings, we found that CIQTNF6 is among the target genes of miR-29b and is significantly downregulated in leukocytes of patients with stroke and MCAO rats. However, C1QTNF6 overexpression with miR-29b inhibition promoted MCAO-induced expression of pro-inflammatory cytokines in leukocytes, including TNF-α and IL-1β. Peripheral inflammation is a common contributing factor to ischemic stroke deterioration(Meng et al., 2019). Ischemic stroke involves BBB damage; moreover, peripheral inflammatory factors, including IL-1β and TNF-α, are

more likely to aggravate BBB damage and even worsen stroke outcomes(Yuan, Liu, & Qi, 2020). IL-1β and TNF-α could reduce tight junction expression or false adjacent blood vessels and further destroy BBB integrity and normal function (Rodrigues & Granger, 2015). Neutrophils produce various pro-inflammatory cytokines that affect BBB function, including IL-1β and TNF-α, which can further promote neutrophil recruitment to the brain parenchyma and exacerbate inflammation(Koh et al., 2015Koh et al., 2015). We observed increased IL-1β and TNF-α secretion after C1QTNF6 overexpression in the peripheral leukocytes of MCAO rats. Additionally, increased IL-1β and TNF-α levels in the brain promote leukocyte migration into the brain parenchyma and increase BBB permeability, which forms a vicious cycle. Consistent with our in vivo study, C1QTNF6 overexpression promoted IL-1β and TNF-α expression in HL-60 cells. IL-1β and TNF-α upregulation can directly cause apoptosis of endothelial cells, which is an important BBB component. Taken together, our findings support the hypothesis that C1QTNF6 induces BBB damage by promoting IL-1β and TNF-α secretion; moreover, C1QTNF6 suppression exerts neuroprotective effects.

In summary, this is the first study to demonstrate H19 and C1QTNF6 upregulation, as well as miR-29b downregulation, in the leukocytes of patients with ischemic stroke and MCAO rats. H19 promotes C1QTNF6 expression by directly targeting miR-29b in the leukocytes of MCAO rats. Furthermore, C1QTNF6 upregulation in leukocytes aggravates cerebral ischemic injury and promotes BBB damage through IL-1β and TNF-α upregulation. Our findings demonstrate the inflammation-modulatory effect of the H19/miR-29b/C1QTNF6 axis in the process of cerebral ischemia injury.

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AUTHOR CONTRIBUTIONS

G.L. and X.M. conducted study design, animal experiments, histology experiment, and manuscript preparation. H.Z., J.F. and T.L. designed clinical experiment. Y.L. and Y.G. designed and managed the research.

CONFLICT OF INTEREST

None.

REFERENCES

Cheng, X. W., Chen, Z. F., Wan, Y. F., Zhou, Q., Wang, H., & Zhu, H. Q. (2019). Long Non-coding RNA H19 Suppression Protects the Endothelium Against Hyperglycemic-Induced Inflammation via Inhibiting Expression of miR-29b Target Gene Vascular Endothelial Growth Factor a Through Activation of the Protein Kinase B/Endothelial Nitric Oxide Synthase Pathway. *Front Cell Dev Biol, 7* , 263. doi:10.3389/fcell.2019.00263

Ding, R., Hase, Y., Ameen-Ali, K. E., Ndung'u, M., Stevenson, W., Barsby, J., ... Kalaria, R. N. (2020). Loss of capillary pericytes and the blood-brain barrier in white matter in poststroke and vascular dementias and Alzheimer's disease.*Brain Pathol, 30* (6), 1087-1101. doi:10.1111/bpa.12888

Ghafouri-Fard, S., Shoorei, H., & Taheri, M. (2020). Non-coding RNAs participate in the ischemia-reperfusion injury. *Biomed Pharmacother, 129* , 110419. doi:10.1016/j.biopha.2020.110419

Huang, X., Hussain, B., & Chang, J. (2021). Peripheral inflammation and blood-brain barrier disruption: effects and mechanisms. *CNS Neurosci Ther, 27* (1), 36-47. doi:10.1111/cns.13569

Jia, L., Hao, F., Wang, W., & Qu, Y. (2015). Circulating miR-145 is associated with plasma high-sensitivity C-reactive protein in acute ischemic stroke patients. *Cell Biochem Funct, 33* (5), 314-319. doi:10.1002/cbf.3116

Kim, M. J., Lee, W., Park, E. J., & Park, S. Y. (2010). C1qTNF-related protein-6 increases the expression of interleukin-10 in macrophages. *Mol Cells, 30* (1), 59-64. doi:10.1007/s10059-010-0088-x

Kirketerp-Moller, N., Bayarri-Olmos, R., Krogfelt, K. A., & Garred, P. (2020). C1q/TNF-Related Protein 6 Is a Pattern Recognition Molecule That Recruits Collectin-11 from the Complement System to Ligands. *J Immunol, 204* (6), 1598-1606. doi:10.4049/jimmunol.1901316

Koh, H. S., Chang, C. Y., Jeon, S. B., Yoon, H. J., Ahn, Y. H., Kim, H. S., ... Park, E. J. (2015). The HIF-1/glial TIM-3 axis controls inflammation-associated brain damage under hypoxia. *Nat Commun, 6* , 6340. doi:10.1038/ncomms7340

Lei, X., Seldin, M. M., Little, H. C., Choy, N., Klonisch, T., & Wong, G. W. (2017). C1q/TNF-related protein 6 (CTRP6) links obesity to adipose tissue inflammation and insulin resistance. *J Biol Chem, 292* (36), 14836-14850. doi:10.1074/jbc.M116.766808

Li, G., Ma, Q., Wang, R., Fan, Z., Tao, Z., Liu, P., ... Luo, Y. (2018). Diagnostic and Immunosuppressive Potential of Elevated Mir-424 Levels in Circulating Immune Cells of Ischemic Stroke Patients. *Aging Dis, 9* (2), 172-181. doi:10.14336/AD.2017.0602

Li, G., Morris-Blanco, K. C., Lopez, M. S., Yang, T., Zhao, H., Vemuganti, R., & Luo, Y. (2018). Impact of microRNAs on ischemic stroke: From pre- to post-disease. *Prog Neurobiol, 163-164* , 59-78. doi:10.1016/j.pneurobio.2017.08.002

Li, Y., Sun, J., Gu, L., & Gao, X. (2020). Protective effect of CTRP6 on cerebral ischemia/reperfusion injury by attenuating inflammation, oxidative stress and apoptosis in PC12 cells. *Mol Med Rep, 22* (1), 344-352. doi:10.3892/mmr.2020.11108

Liu, W., Wang, Z., Liu, L., Yang, Z., Liu, S., Ma, Z., ... Cao, X. (2020). LncRNA Malat1 inhibition of TDP43 cleavage suppresses IRF3-initiated antiviral innate immunity.*Proc Natl Acad Sci U S A, 117* (38), 23695-23706. doi:10.1073/pnas.2003932117

Longa, E. Z., Weinstein, P. R., Carlson, S., & Cummins, R. (1989). Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke, 20* (1), 84-91. doi:10.1161/01.str.20.1.84

Ma, H., Jiang, Z., Xu, J., Liu, J., & Guo, Z. N. (2021). Targeted nano-delivery strategies for facilitating thrombolysis treatment in ischemic stroke. *Drug Deliv, 28* (1), 357-371. doi:10.1080/10717544.2021.1879315

Mendelson, S. J., & Prabhakaran, S. (2021). Diagnosis and Management of Transient Ischemic Attack and Acute Ischemic Stroke: A Review. *JAMA, 325* (11), 1088-1098. doi:10.1001/jama.2020.26867

Meng, H., Zhao, H., Cao, X., Hao, J., Zhang, H., Liu, Y., ... Xu, Y. (2019). Double-negative T cells remarkably promote neuroinflammation after ischemic stroke. *Proc Natl Acad Sci U S A, 116* (12), 5558-5563. doi:10.1073/pnas.1814394116

Palazzo, A. F., & Koonin, E. V. (2020). Functional Long Non-coding RNAs Evolve from Junk Transcripts.*Cell, 183* (5), 1151-1161. doi:10.1016/j.cell.2020.09.047

Rodrigues, S. F., & Granger, D. N. (2015). Blood cells and endothelial barrier function. *Tissue Barriers, 3* (1-2), e978720. doi:10.4161/21688370.2014.978720

Wan, P., Su, W., Zhang, Y., Li, Z., Deng, C., Li, J., ... Zhuo, Y. (2020). LncRNA H19 initiates microglial pyroptosis and neuronal death in retinal ischemia/reperfusion injury. *Cell Death Differ, 27* (1), 176-191. doi:10.1038/s41418-019-0351-4

Wang, H., Li, Z., Gao, J., & Liao, Q. (2019). Circular RNA circPTK2 regulates oxygen-glucose deprivation-activated microglia-induced hippocampal neuronal apoptosis via miR-29b-SOCS-1-JAK2/STAT3-IL-1beta signaling. *Int J Biol Macromol, 129* , 488-496. doi:10.1016/j.ijbiomac.2019.02.041

Wang, J., Zhao, H., Fan, Z., Li, G., Ma, Q., Tao, Z., ... Luo, Y. (2017). Long Noncoding RNA H19 Promotes Neuroinflammation in Ischemic Stroke by Driving Histone Deacetylase 1-Dependent M1 Microglial Polarization. *Stroke, 48* (8), 2211-2221. doi:10.1161/STROKEAHA.117.017387

Wang, J., Zhu, M., Ye, L., Chen, C., She, J., & Song, Y. (2020). MiR-29b-3p promotes particulate matter-induced inflammatory responses by regulating the C1QTNF6/AMPK pathway. *Aging (Albany NY), 12* (2), 1141-1158. doi:10.18632/aging.102672

Wang, L., Cheng, Q., Peng, M., Lv, D., Zi, W., Xu, G., & Liu, X. (2020). The relationship between the platelet to leukocyte ratio and mechanical thrombectomy outcomes in acute ischemic stroke patients. *Neurol Res, 42* (10), 890-896. doi:10.1080/01616412.2020.1790868

Wang, Y., Huang, J., Ma, Y., Tang, G., Liu, Y., Chen, X., ... Yang, G. Y. (2015). MicroRNA-29b is a therapeutic target in cerebral ischemia associated with aquaporin 4.*J Cereb Blood Flow Metab, 35* (12), 1977-1984. doi:10.1038/jcbfm.2015.156

Xu, J., Wang, C., Meng, F., & Xu, P. (2021). Long noncoding RNA H19 inhibition ameliorates oxygenglucose deprivationinduced cell apoptosis and inflammatory cytokine expression by regulating the microRNA29b/SIRT1/PGC1alpha axis. *Mol Med Rep, 23* (2)doi:10.3892/mmr.2020.11770

Yan, S., Ding, J., Zhang, Y., Wang, J., Zhang, S., Yin, T., & Yang, J. (2021). C1QTNF6 participates in the pathogenesis of PCOS by affecting the inflammatory response of granulosa cells. *Biol Reprod* doi:10.1093/biolre/ioab094

Yuan, S., Liu, K. J., & Qi, Z. (2020). Occludin regulation of blood-brain barrier and potential therapeutic target in ischemic stroke. *Brain Circ, 6* (3), 152-162. doi:10.4103/bc.bc_29_20

Figure legends

Figure 1. H19, miR-29b, and C1QTNF6 expression in patients with acute stroke. **A** . Quantitative PCR of H19, miR-29b, and C1QTNF6 levels in neutrophils of patients with acute stroke. Acute ischemic stroke group (N = 50) and control group (N = 42). **B** . Correlation between miR-29b and C1QTNF6 expression at admission (N = 50). **C** . Correlation of H19 and miR-29b expression with the NIHSS score at admission (N = 50). **D** . The predicted binding sites between miR-29b and C1QTNF6 and between miR-29b and H19.**E.** Plasma TNF-α, IL-1β, and MMP-9 levels in patients with acute ischemic stroke (N = 50). *P < 0.05, ***P < 0.001 vs. control group.

Figure 2. Changes in H19 and miR-29b levels, as well as subsequent effects on cerebral injury and neurological deficits in MCAO rats. **A** . H19 and miR-29b expression in leukocytes and ischemic brain tissue after MCAO injury (N = 9). **B** . Quantitative PCR was performed to confirm the efficacy of H19 siRNA and miR-29b antagomir transfection (N = 9). **C** . Effects of intravenous injections of H19 siRNA and miR-29b antagomir on cerebral injury and neurological function deficits at 24 h after stroke (N = 12). **D** and**E** . Neuronal apoptosis in the ipsilateral cortex as detected by NeuN/TUNEL immunofluorescence double staining (N = 6). *P < 0.01, ***P < 0.001 vs. sham group. #P < 0.05, ###P < 0.001 vs. MCAO group. Scale bar = 20 μm.

Figure 3. H19 and miR-29b regulated C1QTNF6 expression in the leukocytes of MCAO rats **A.** C1QTNF6, IL-1β, and TNF-α protein levels in leukocytes were assessed by western blotting (N = 6).**B.** C1QTNF6, IL-1β, and TNF-α protein levels in the ipsilateral brain tissue were assessed by western blotting (N = 6). *P < 0.05 vs. sham group; #P < 0.05, MCAO group.

Figure 4. H19 siRNA reversed miR-29b antagomir-aggravated acute cerebral ischemic injury. **A.** Cerebral infarct volume and brain oedema evaluated by TTC staining of coronal brain sections (N = 12).**B.** Neuronal apoptosis in the ipsilateral cortex as detected by NeuN/TUNEL immunofluorescence double staining (N = 6). **C.**C1QTNF6 mRNA, IL-1β, and TNF-α protein levels in the leukocytes were assessed by qRT-PCR and western blotting (N = 6). **D.** C1QTNF6, IL-1β, and TNF-α protein levels in the ipsilateral brain tissue were assessed by western blotting (N = 6). &P < 0.05, &&&P < 0.001 vs. MCAO + miR-29b antagomir + H19 siRNA control group. Scale bar = 20 μm.

Figure 5. MiR-29b antagomir and H19 siRNA regulated C1QTNF6 levels in leukocytes. **A.** Immunofluorescence detection of C1QTNF6 in leukocytes (CD11b+) at 24 hours after MCAO (N = 6).**B.** Leukocytes

(CD11b+) levels in brain tissue (N = 6).**C.** C1QTNF6 expression levels in leukocytes were assessed by immunofluorescence (N = 6). ***P < 0.001 vs. Sham group; #P < 0.05 vs. MCAO group. &P < 0.05 vs. MCAO+miR-29b antagomir + H19 siRNA control group. Scale bar = 20 μm.

Figure 6. H19 and miR-29b regulated ZO-1 and occludin expression in hCMEC/D3 cells **A and B.** hCMEC/D3 cell apoptosis induced by H19 siRNA and miR-29b antagomir treatment (N = 6).**C.** C1QTNF6, IL-1β, and TNF-α protein levels in HL-60 cells were assessed by western blotting (N = 6). **D.** ZO-1 and occludin protein levels in hCMEC/D3 cells were assessed by western blotting (N = 6). * P < 0.05 vs. Sham group; *** P < 0.001 vs. Sham group; # P < 0.05 vs. OGD group; ## P < 0.01 vs. OGD group; &P < 0.05 vs. OGD+miR-29b antagomir group; &&&P < 0.001 vs. OGD+miR-29b antagomir group.

Figure 7. Schematic diagram depicting the possible involvement of the H19/miR-29b/C1QTNF6 axis in cerebral ischemic stroke. The ischemia-dependent activation of the H19/miR-29b/C1QTNF6 axis enhances IL-1β and TNF-α expression in leukocytes; moreover, it causes further BBB disruption to aggravate neuron injury.

Fig 1

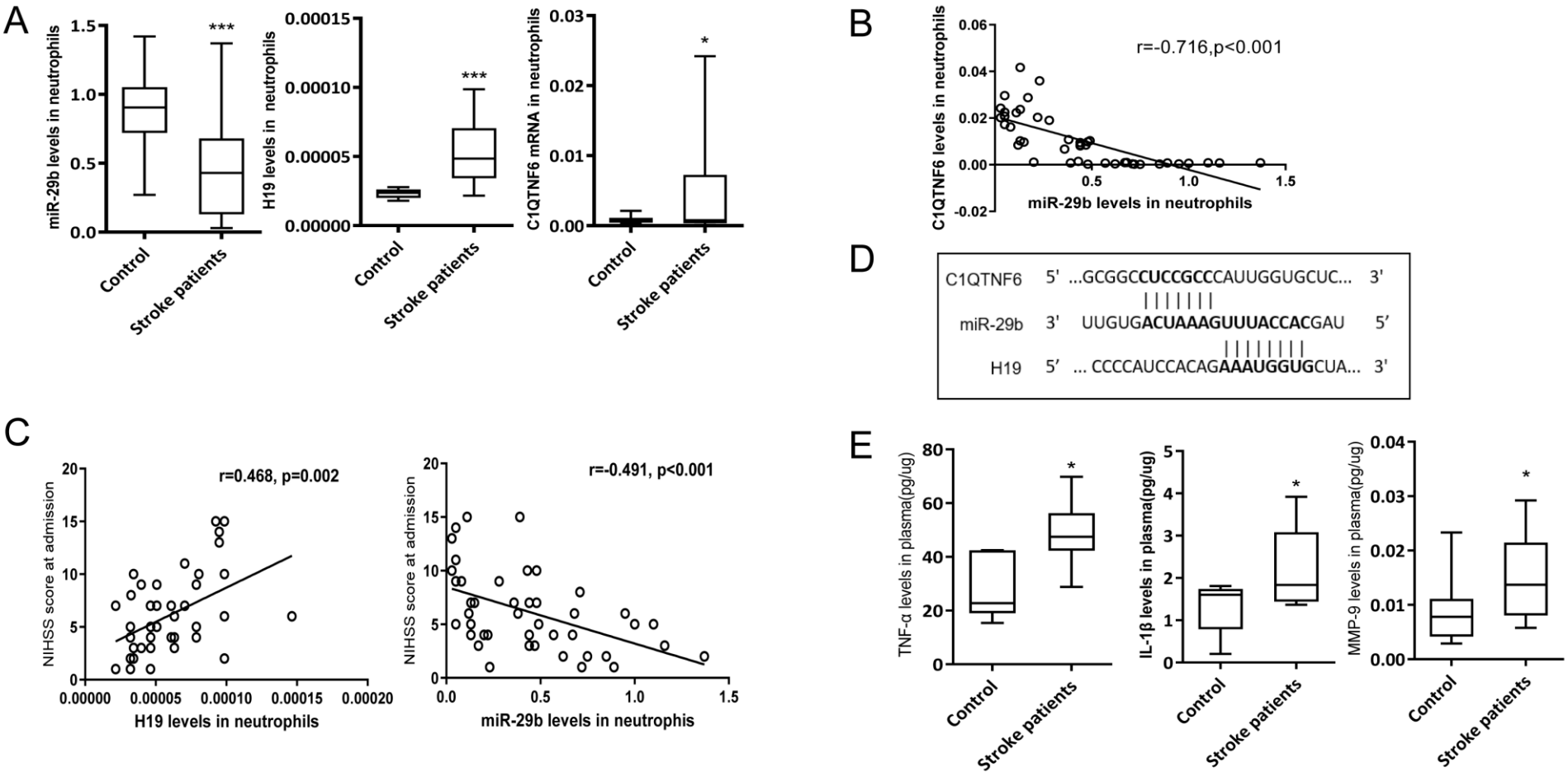
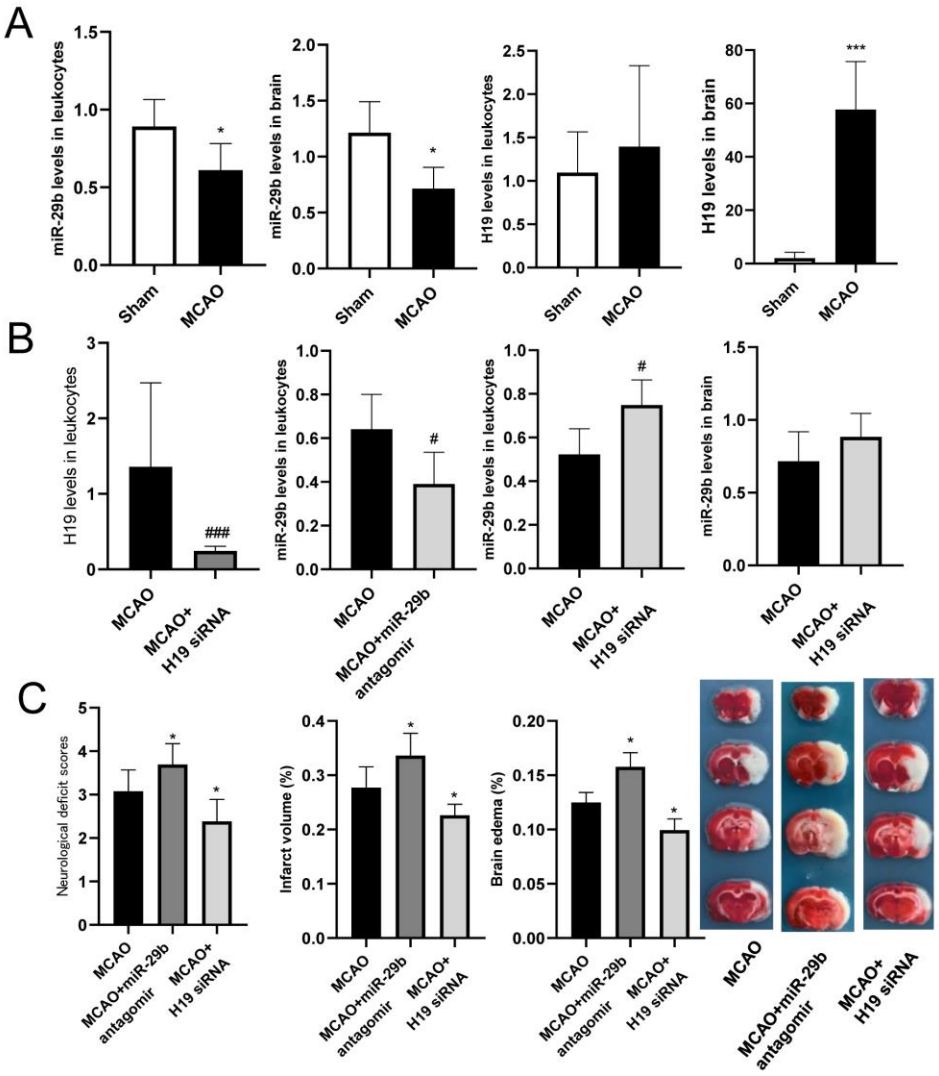
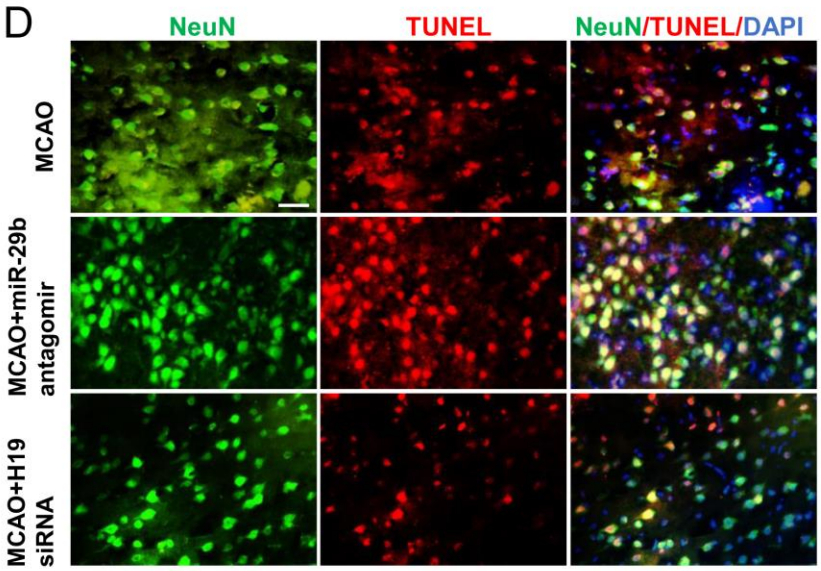

A
miR-29b levels in neutrophils
H19 levels in neutrophils
C1QTNF6 mRNA in neutrophils
Control
Stroke patients
***
***
*
B
C1QTNF6 levels in neutrophils
r=-0.716,p<0.001
miR-29b levels in neutrophils
D
C1QTNF6 5' ...GCGGCCUCCGCCCAUUGGUGCUC... 3'
miR-29b 3' UUGUGACUAAAGUUUACCACGAU 5'
H19 5' ... CCCCAUCCACAGAAAUGGUGCUA... 3'
C
NIHSS score at admission
r=0.468, p=0.002
H19 levels in neutrophils
r=-0.491, p<0.001
miR-29b levels in neutrophis
E
TNF-α levels in plasma(pg/ug)
IL-1β levels in plasma(pg/ug)
MMP-9 levels in plasma(pg/ug)
Control
Stroke patients

Fig 2


A
miR-29b levels in leukocytes
miR-29b levels in brain
H19 levels in leukocytes
H19 levels in brain
Sham
MCAO
B
H19 levels in leukocytes
miR-29b levels in leukocytes
miR-29b levels in brain
MCAO
MCAO+ H19 siRNA
MCAO+miR-29b antagomir
C
Neurological deficit scores
Infarct volume (%)
Brain edema (%)
MCAO
MCAO+miR-29b antagomir
MCAO+ H19 siRNA


D
NeuN
TUNEL
NeuN/TUNEL/DAPI
MCAO
MCAO+miR-29b antagomir
MCAO+H19 siRNA

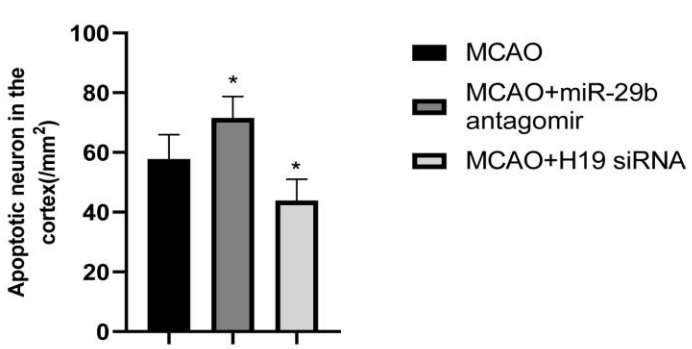

E
Apoptotic neuron in the cortex(/mm2)
MCAO
MCAO+miR-29b antagomir
MCAO+H19 siRNA

Fig 3

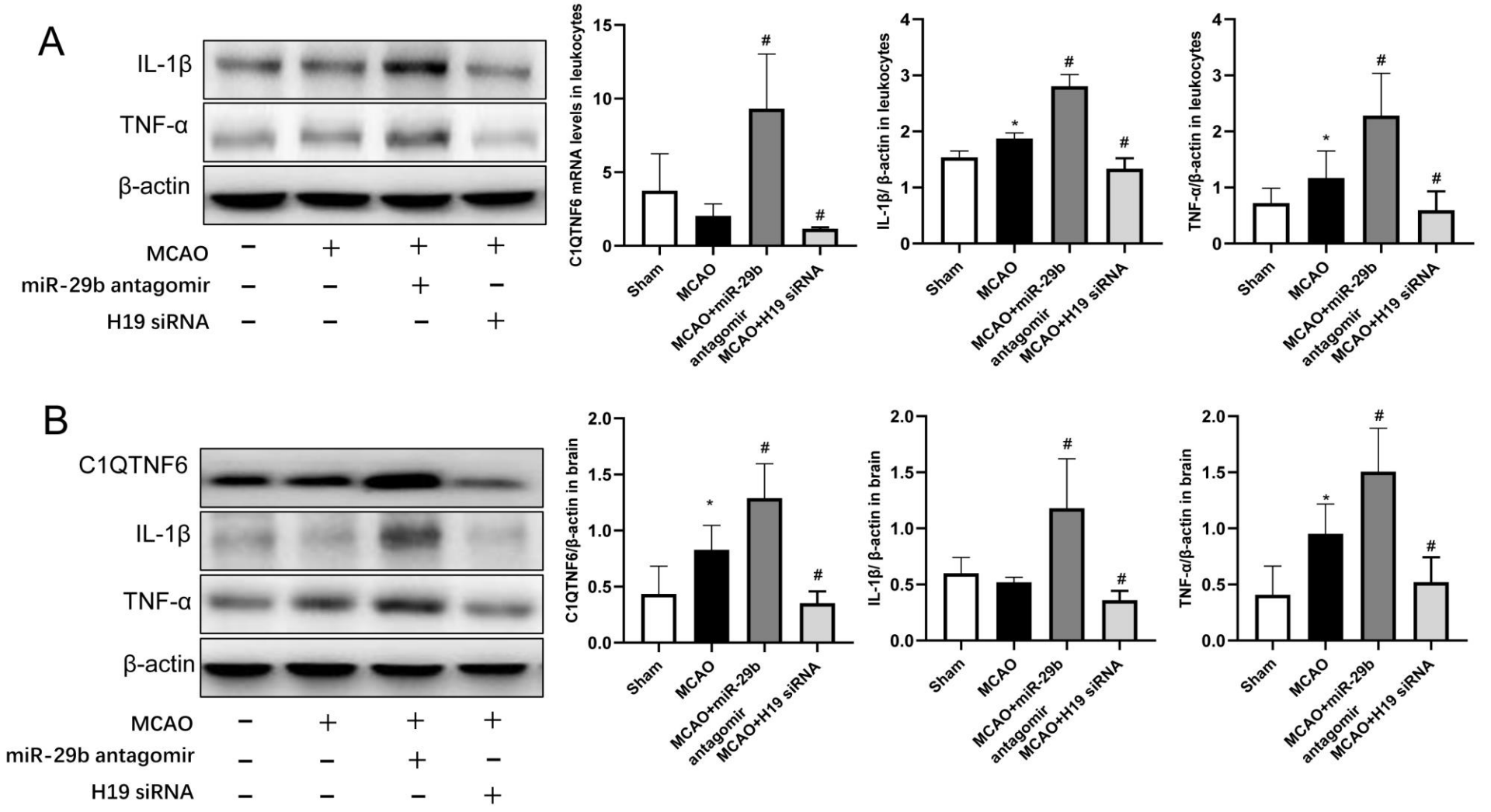

A
IL-1β
TNF-α
β-actin
MCAO − + + +
miR-29b antagomir − − + −
H19 siRNA − − − +
C1QTNF6 mRNA levels in leukocytes
IL-1β/ β-actin in leukocytes
TNF-α/β-actin in leukocytes
Sham
MCAO
MCAO+miR-29b antagomir
MCAO+H19 siRNA
B
C1QTNF6
IL-1β
TNF-α
β-actin
MCAO − + + +
miR-29b antagomir − − + −
H19 siRNA − − − +
C1QTNF6/β-actin in brain
IL-1β/ β-actin in brain
TNF-α/β-actin in brain
Sham
MCAO
MCAO+miR-29b antagomir
MCAO+H19 siRNA

Fig 4

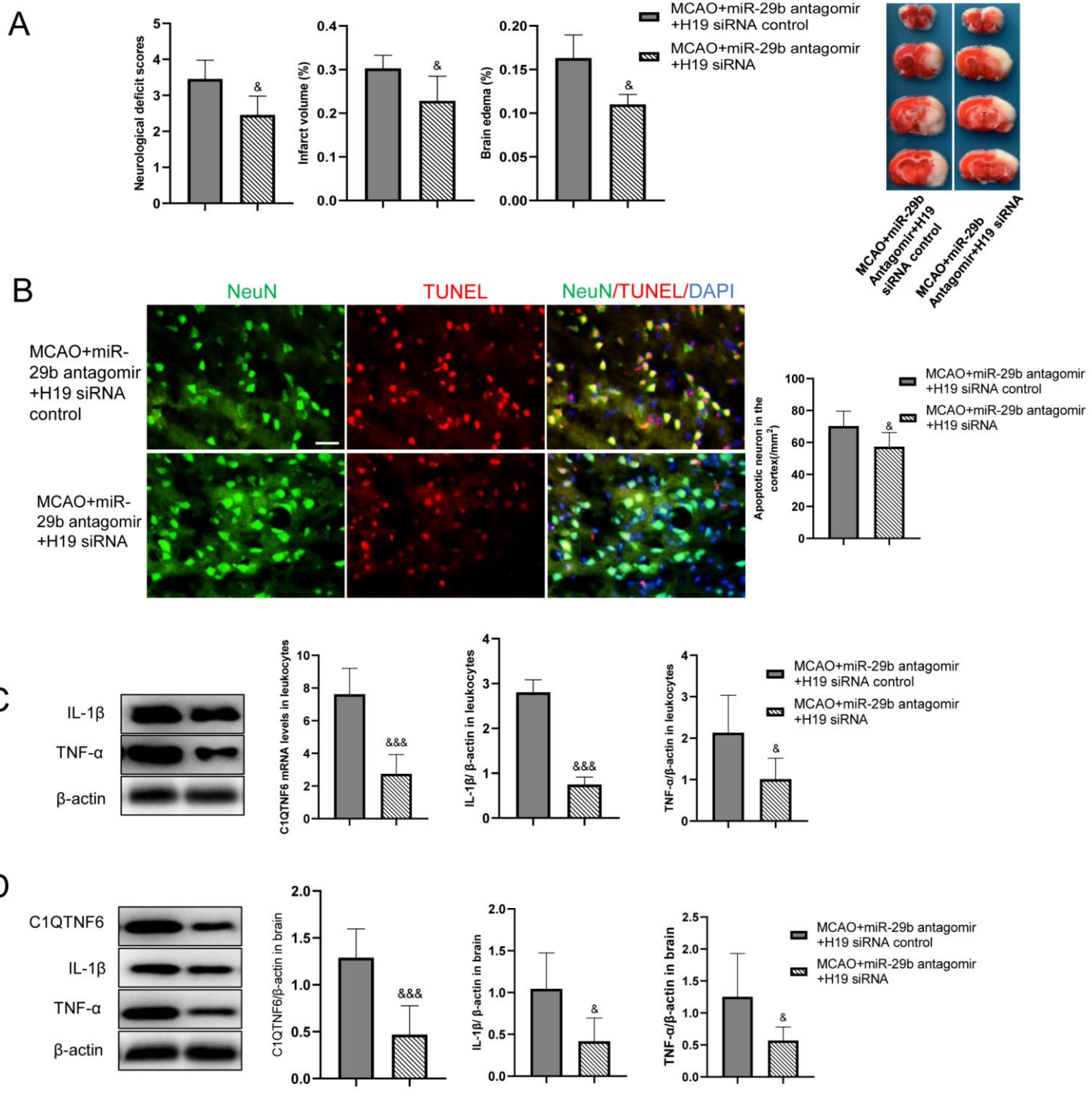
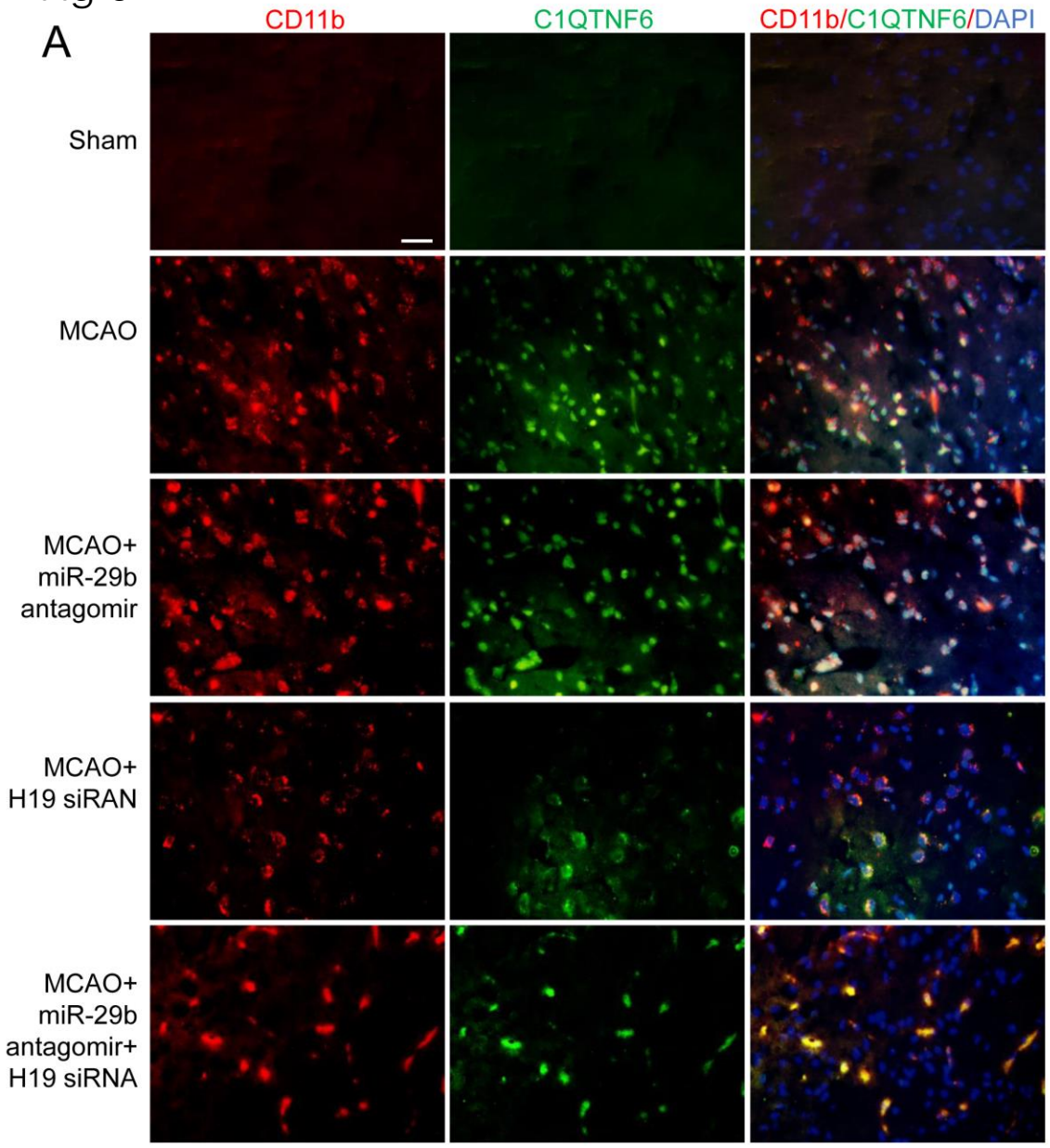
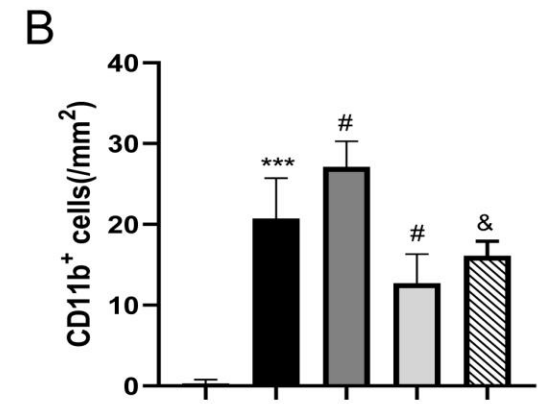

A
Neurological deficit scores
Infarct volume (%)
Brain edema (%)
MCAO+miR-29b antagomir +H19 siRNA control
MCAO+miR-29b antagomir +H19 siRNA
MCAO+miR-29b Antagomir+H19 siRNA control
MCAO+miR-29b Antagomir+H19 siRNA
B
NeuN
TUNEL
NeuN/TUNEL/DAPI
MCAO+miR-29b antagomir +H19 siRNA control
MCAO+miR-29b antagomir +H19 siRNA
Apoptotic neuron in the cortex(/mm²)
C
IL-1β
TNF-α
β-actin
C1QTNF6 mRNA levels in leukocytes
IL-1β/ β-actin in leukocytes
TNF-α/β-actin in leukocytes
D
C1QTNF6
IL-1β
TNF-α
β-actin
C1QTNF6/β-actin in brain
IL-1β/ β-actin in brain
TNF-α/β-actin in brain

Fig 5


A
CD11b
C1QTNF6
CD11b/C1QTNF6/DAPI
Sham
MCAO
MCAO+ miR-29b antagomir
MCAO+ H19 siRAN
MCAO+ miR-29b antagomir+ H19 siRNA


B
CD11b+ cells(/mm2)
40
30
20
10
0
***
#
#
&

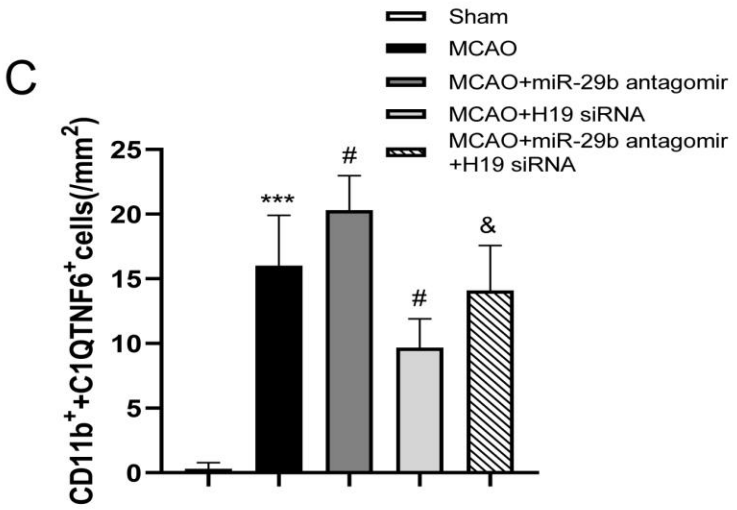

C
CD11b+C1QTNF6+cells(/mm2)
25
20
15
10
5
0
***
#
#
&
Sham
MCAO
MCAO+miR-29b antagomir
MCAO+H19 siRNA
MCAO+miR-29b antagomir +H19 siRNA

Fig 6

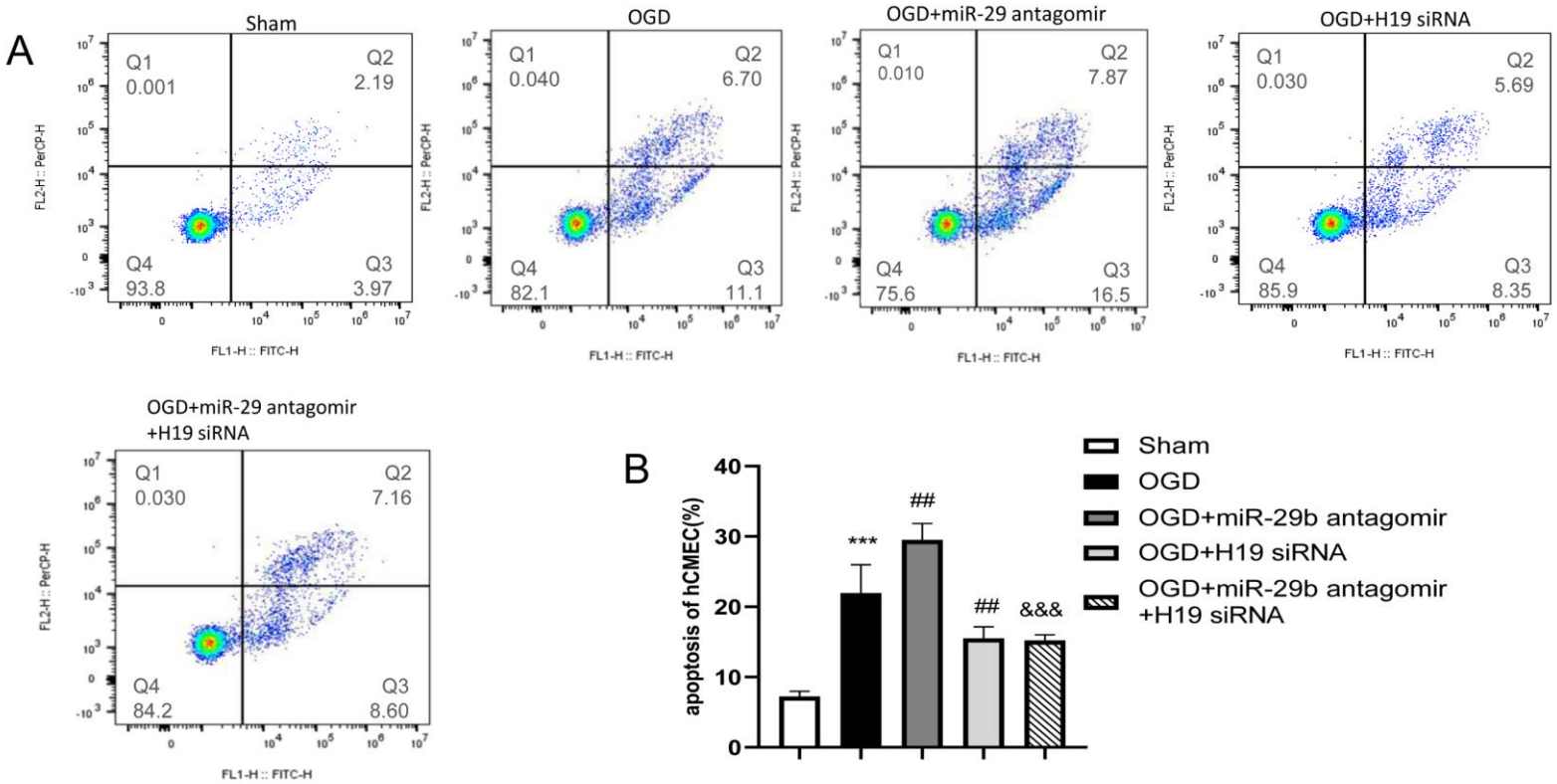
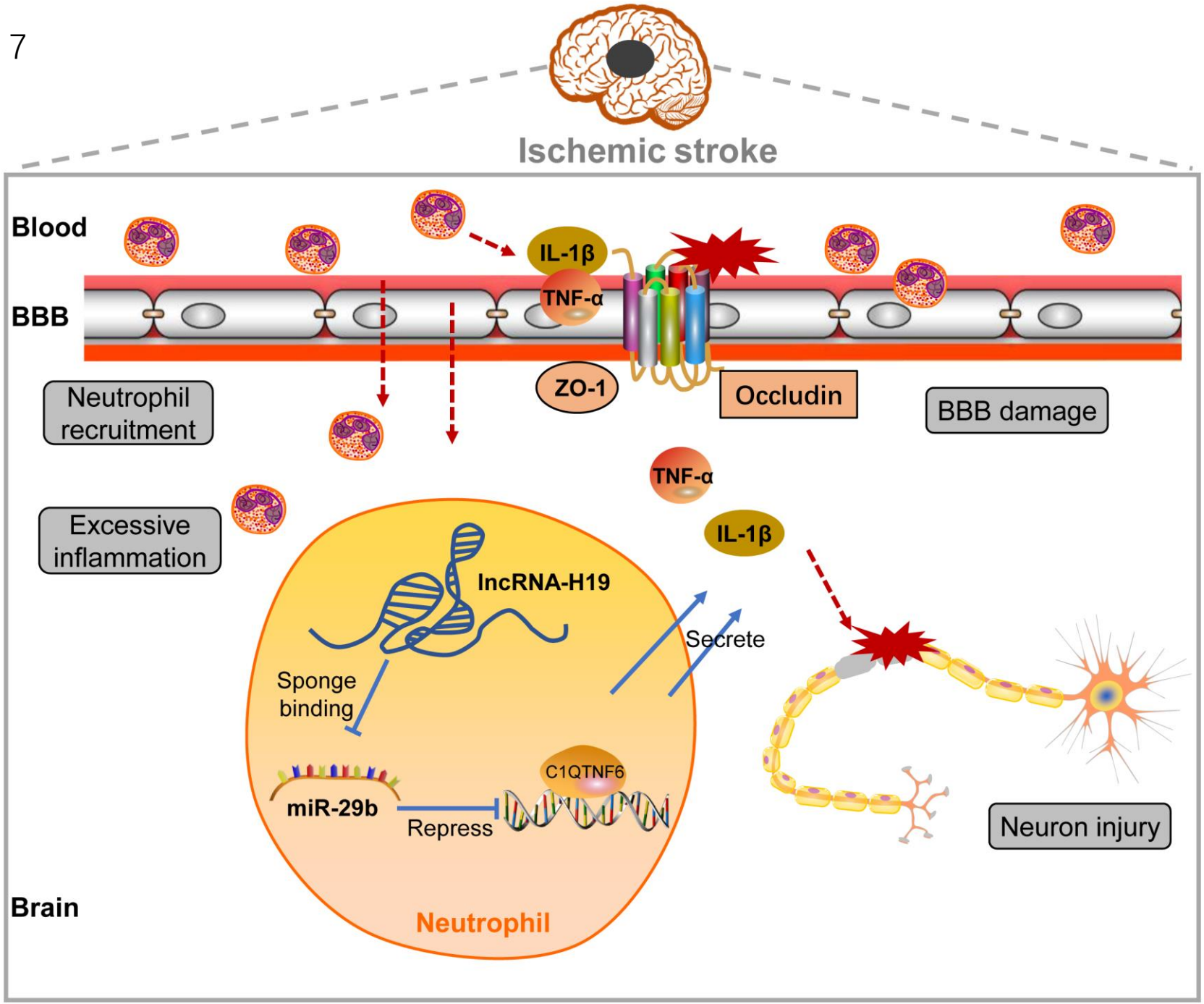

A
Sham
Q1 0.001
Q2 2.19
Q4 93.8
Q3 3.97
OGD
Q1 0.040
Q2 6.70
Q4 82.1
Q3 11.1
OGD+miR-29 antagomir
Q1 0.010
Q2 7.87
Q4 75.6
Q3 16.5
OGD+H19 siRNA
Q1 0.030
Q2 5.69
Q4 85.9
Q3 8.35
OGD+miR-29 antagomir +H19 siRNA
Q1 0.030
Q2 7.16
Q4 84.2
Q3 8.60
FL2-H :: PerCP-H
FL1-H :: FITC-H
B
apoptosis of hCMEC(%)
***
##
##
&&&
Sham
OGD
OGD+miR-29b antagomir
OGD+H19 siRNA
OGD+miR-29b antagomir +H19 siRNA
C
C1QTNF6
IL-1β
TNF-α
miR-29b antagomir
H19 siRNA
C1QTNF6/β-actin in HL-60 cells
IL-1β/β-actin in HL-60 cells
TNF-α/β-actin in HL-60 cells
D
Occludin
ZO-1
β-actin
OGD
ZO-1/β-actin in hCMEC/D3 cells
Occludin/β-actin in hCMEC/D3 cells
Sham
OGD
OGD+miR-29b antagomir
OGD+H19 siRNA
OGD+miR-29b antagomir +H19 siRNA

Fig 7


Ischemic stroke
Blood
BBB
IL-1β
TNF-α
ZO-1
Occludin
BBB damage
Neutrophil recruitment
Excessive inflammation
TNF-α
IL-1β
lncRNA-H19
Secrete
Sponge binding
C1QTNF6
miR-29b
Repress
Neuron injury
Brain
Neutrophil