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ORIGINAL RESEARCH

Tenascin-C Facilitates Microglial Polarization via TLR4/MyD88/NF-κB Pathway Following Subarachnoid Hemorrhage

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Purpose: This study primarily aims to elucidate the underlying mechanism of Tenascin-C in neuroinflammation and microglia polarization in a mouse model of subarachnoid hemorrhage (SAH).

Methods: The subarachnoid hemorrhage model was constructed by injecting blood into the anterior chiasmatic cistern and stimulating primary microglia with hemoglobin in vitro. Then, Imatinib mesylate was used to inhibit Tenascin-C. Through neurological function scoring, brain edema, primary cell extraction, immunofluorescence staining, CCK8, Tunel staining, Elisa, Western blot and other methods, the potential mechanism of Tenascin-C induced microglia cell polarization was explored.

Results: The results of this study observed that the expression of Tenascin-C was up-regulated after subarachnoid hemorrhage. Inhibiting the increase of Tenascin-C by imatinib could significantly ameliorate neuroinflammation, neuronal apoptosis, blood brain barrier disruption and brain edema. When the level of Tenascin-C decreased, the numbers of TLR4 positive, MyD88 positive and NF-κB positive microglial cells decreased accordingly. Moreover, after subarachnoid hemorrhage, the number of microglial cells positive for M1-type markers increased significantly. After imatinib inhibited Tenascin-C, the number of M1-type microglial cells decreased and the number of M2-type microglial cells increased significantly.

Conclusion: In summary, the elevated level of Tenascin-C after subarachnoid hemorrhage induces the activation of microglia, releasing a large number of inflammatory factors and aggravating early brain injury.

Keywords: SAH, microglia, Tenascin-C, polarization

Introduction

Subarachnoid hemorrhage (SAH) is a cerebrovascular disease with high mortality and disability rates. In recent years, the early brain injury stage has been attracting the attention of researchers constantly.[1] Early brain injury is the trigger point of many pathophysiological responses, including cerebral edema, destruction of the blood-brain barrier, neuroinflammation, elevation of intracranial pressure, etc.[2,3] Therefore, alleviating early brain injury may be helpful to improve the prognosis of patients with subarachnoid hemorrhage. Among them, neuroinflammation is regarded as an important factor for early brain injury, cerebral vasospasm and delayed brain injury after subarachnoid hemorrhage.[4–6] As the resident immune cells in brain tissue, microglia are also considered as potential participants in early brain injury.[7,8] Activated microglia can differentiate into cells with different functions. M1-type microglia mainly release inflammatory factors such as IL-1β and TNF-α. While M2-type microglia mainly plays an anti-inflammatory role. And under a specific internal environment, M1-type can be converted to M2-type.[9–11]

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Some scholars have found that the expression level of Tenascin-C in the cerebrospinal fluid of patients with subarachnoid hemorrhage will increase,[12] and Tenascin-C is an endogenous activator of TLR4.[13,14] In recent years, studies have reported that Tenascin-C participates in the inflammatory reaction through TLR4 in rheumatoid arthritis and osteoarthritis,[15,16] and Tenascin-C regulates the phagocytic activity of microglia and the production of inflammatory factors through TLR4 in the central nervous system.[17] When TLR4 is activated, its downstream signaling pathway factor MyD88 will be activated, further activating the NF-κB signaling pathway and causing a cascade reaction of neuroinflammation, promoting the release of inflammatory factors such as IL-1β and TNF-α and aggravating early brain injury.[18] For this reason, we wonder whether Tenascin-C can induce the activation of microglia through TLR4/MyD88/NF-κB, thereby affecting the early brain injury of subarachnoid hemorrhage, and whether inhibiting the expression level of Tenascin-C can promote the transformation of M1-type microglia to M2-type microglia to alleviate brain injury after subarachnoid hemorrhage.

Therefore, research on the underlying mechanism between the increased expression level of Tenascin-C and microglia polarization after subarachnoid hemorrhage is helpful to find potential targets for new drug research and prognosis improvement in subarachnoid hemorrhage. In previous years, some scholars have proved that imatinib can effectively reduce the expression level of Tenascin-C.[19] In this study, imatinib will be used as an effective inhibitor of Tenascin-C.

Methods and Materials

Animals and SAH Models

The male C57BL/6J mice (weighing about 25g, aged 6–7 weeks) were provided by the Animal Center of Yijishan Hospital of Wannan Medical College. All animal study protocols were approved by the Animal Experiment Ethics Committee of Yijishan Hospital of Wannan Medical College and strictly adhere to the "Guide for the Care and Use of Laboratory Animals" issued by the National Institutes of Health in the United States. All experimental mice were kept at a temperature of about 25°C and a humidity controlled between 40%-70%, with free access to food and water. The subarachnoid hemorrhage (SAH) model was induced using the anterior optic chiasm cistern. After inhaled isoflurane (2% oxygen, 300 mL/min) anesthesia, the mice were fixed on a stereotactic device. After disinfection, a scalp incision of approximately 1.0 cm was made in the sagittal midline, and a hole with a diameter of about 1.0 mm was drilled at the midline of the skull, 4.5 mm from the suture. One mouse was euthanized as an arterial blood donor, and about 50 μL of arterial blood was collected by puncture at the exposed apex and injected into the anterior optic chiasm cistern through the prepared hole, with the needle kept in place for at least 2 minutes to prevent blood reflux or cerebrospinal fluid (CSF) leakage. The drilling point was sealed with bone wax and the incision was sutured and disinfected. Then, the animals were allowed to recover for 45 minutes after surgery, returned to the cage and maintained at a temperature of 25°C. The sham operation group of mice underwent the same procedure of puncturing into the brain, but no arterial blood was injected. Twenty-four hours later, the neurological function was assessed using the modified Garcia score, and mice with a score of <6 were excluded.

Experimental Program in Figure 1

Experiment 1: The aim of this experiment is to assess the time-dependent expression changes of Tenascin-C in the early brain injury stage after SAH and to determine the optimal dosage of imatinib. Animals were randomly divided into six groups (Sham, 6h, 12h, 24h, 48h, 72h, with 15 mice per group). At specified time points (6h, 12h, 24h, 48h, 72h after SAH), mice were euthanized. Six mice from each group were randomly selected for Western Blot to determine the time point with the highest Tenascin-C expression. Then, different concentration gradients of imatinib (#HY-50946 MCE Nanjing China) were set at this time point. Mice were randomly divided into six groups (sham, SAH, SAH+Vehicle, SAH+80mg/kg imatinib, SAH+40mg/kg imatinib, SAH+20mg/kg imatinib, with 15 mice per group). Six mice from each group were randomly selected for Western Blot, and the remaining mice had their brain tissues removed for frozen sections and subsequent immunofluorescence staining analysis.

Experiment 2: The aim of this experiment is to evaluate the effect of Tenascin-C on neurological function after SAH in mice. Mice were randomly divided into four groups (Sham, SAH, SAH+vehicle, SAH+imatinib, with 15 mice per

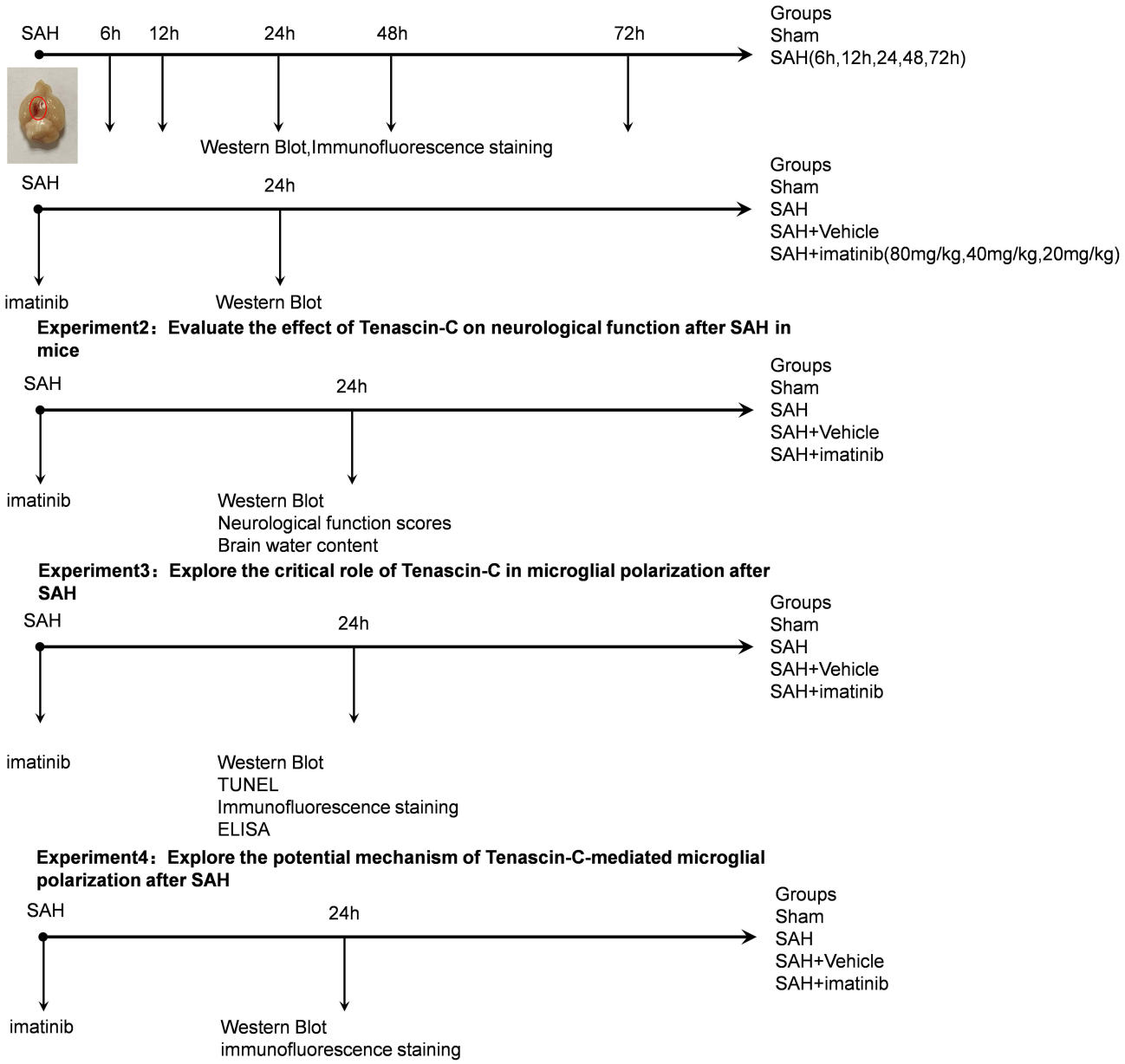

Figure 1 Animal experimental design and groups.

group). Neurological function scores, balance beam tests, and brain edema detection were performed 24 hours after SAH. Western Blot was used to detect the expression of tight junction proteins in the blood-brain barrier.

Experiment 3: The aim of this experiment is to explore the critical role of Tenascin-C in microglial polarization after SAH. After induction of SAH, mice were immediately given intraperitoneal injection of imatinib. Twenty-four hours later, mice were randomly divided into four groups (sham, SAH, SAH+vehicle, SAH+imatinib, with 15 mice per group). Western Blot, immunofluorescence staining, TUNEL staining, and ELISA were used to verify the correlation between Tenascin-C and microglial polarization.

Experiment 4: The aim of this experiment is to explore the potential mechanism of Tenascin-C-mediated microglial polarization after SAH. Mice were randomly divided into four groups (Sham, SAH, SAH+vehicle, SAH+imatinib, with 15 mice per group). The experimental methods included Western Blot and immunofluorescence staining.

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Cellular and in vitro Model Culture

To culture primary microglia, the brain of neonatal mice must be collected within 24 hours after birth. The animal procedure has been approved by the Animal Experiment Ethics Review Committee of Yijishan Hospital of Wuhu Medical College. Under a microscope, the meninges are removed to preserve the cortex, and the brain tissue is carefully cut and placed in a petri dish on ice. It is then cut into small pieces with a microscissors and the cortical tissue is placed in preheated TrypLE (#12563029, Gibco, Suzhou, China). The brain tissue is digested for 10 minutes in a 37-degree Celsius incubator. Then, 2 mL of FBS is added to stop the digestion, and the digested tissue is transferred to a culture flask. Primary microglia are cultured in high-glucose medium (DMEM, #C11995500BT, Gibco, Suzhou) supplemented with 10% fetal bovine serum (FBS, #10099141C, Gibco, Australia) and 1% penicillin-streptomycin (#10378016, Gibco, Suzhou). The medium is changed on days 3 and 7. On days 10 and 13, the culture flask is gently shaken to collect the suspended microglia, which are then transferred to experimental wells. To simulate an in vitro SAH model, primary microglia are typically stimulated with 25 μmol/L hemoglobin. The primary microglia are divided into three groups: control group, OxyHB group, and imatinib group. The levels of inflammatory factors are detected by ELISA and immunofluorescence is performed.

Brain Edema Measurement

The degree of brain edema in mice was assessed by the wet-dry method; in brief, the mice were sacrificed 24 hours after modeling, and the wet weight of their brain tissue was measured. Then, the samples were dried in an oven at 37 degrees Celsius for 72 hours, after which the dry weight was measured. The brain water content was calculated as [(wet weight - dry weight)/wet weight] × 100%.

Behavioral Assessment

Twenty-four hours after subarachnoid hemorrhage, short-term neurological function was assessed using the modified Garcia score and the balance beam test. The modified Garcia score evaluated six domains: spontaneous movement, posture symmetry, forelimb extension, climbing ability, bilateral body tactile response, and bilateral whisker tactile response. Each domain was scored from 0 to 3 points, with a total score ranging from 3 to 18, where a higher score indicated less severe neurological damage. The balance beam test measured motor coordination and balance ability. Each mouse was tested three times, and the average score was recorded. The scoring criteria were as follows: 0 points, the mouse could not grab or immediately fell; 1 point, the mouse could grab but did not move and remained for 1 minute; 2 points, the mouse could move but fell within 1 minute; 3 points, the mouse could walk from one end of the beam to the other but easily fell; 4 points, the mouse moved freely on the beam.

Immunofluorescence Staining (IF)

After modeling, the mice were perfused with PBS (#KGL2206-500, KGI, China) and then with 4% paraformaldehyde. After perfusion, the intact brain tissue was removed and fixed in 4% paraformaldehyde (#P0099, Beyotime, New Jersey) for 24 hours. Then, the tissue was dehydrated using 15% and 30% sucrose solutions. After dehydration, the tissue was dried with dust-free paper towels, and 12 μm thick frozen sections were cut. The frozen sections were placed in a 37°C oven for 2 hours, and a hydrophobic pen was used to mark around the tissue. The sections were washed three times with PBS, permeabilized with TritonX-100 (X100, Sigma, USA) for 30 minutes, and blocked with immunofluorescence blocking solution (#P0102, Beyotime, China) at room temperature. Then, the sections were incubated with primary antibodies overnight at 4°C. After washing three times with PBS, the sections were incubated with corresponding fluorescent secondary antibodies for 2 hours at room temperature, followed by three washes with PBS. Finally, the sections were mounted with DAPI (#D21490, Thermo Fisher Scientific, USA) containing fluorescence quencher. Immunofluorescence staining of microglia involved seeding cells into culture plates, followed by fixation, permeabilization, blocking, and incubation with corresponding antibodies, and finally mounting. Fluorescent images were captured using a Leica Thunder fluorescence microscope system.

Cell Viability Analysis

The viability of cultured primary microglia was assessed using a Cell Counting Kit-8 (CCK-8) kit (#C0038, Beyotime, China) according to the manufacturer's instructions. This was done to investigate the optimal effective dose of imatinib.

Tunel

As mentioned previously for immunofluorescence staining, a one-step TUNEL staining kit (#C1089, Beyotime, China) was used to perform dual immunostaining for NeuN and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) according to the manufacturer's instructions. The frozen tissue sections were incubated with the TUNEL detection solution at room temperature for 1 hour, protected from light, and then mounted with a fluorescence quencher containing DAPI. Subsequently, the TUNEL staining results were observed under a fluorescence microscope using a wavelength of 550 nm.

Western Blot (WB)

Brain tissue and cell samples were lysed using RIPA buffer (#P10013B, Beyotime, Shanghai) containing phosphatase inhibitor (#GRF102, Epizyme, Shanghai) and protease inhibitor (#GRF101, Epizyme, Shanghai). After lysis, the extracted protein suspension was quantified using a BCA protein assay kit (#P0012S, Beyotime, Nanjing). For Western Blot, equal amounts of protein samples were separated by polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene fluoride membrane (PVDF, #1620177, #1620264, Millipore). The membrane was blocked with 5% skimmed milk at room temperature for 2 hours, washed three times with Tris-buffered saline containing Tween 20 (TBST, #PS103, Yase, Shanghai), and then incubated with the corresponding primary antibody overnight at 4°C. The next day, the PVDF membrane was washed three times with TBST and incubated with the corresponding secondary antibody at room temperature for 2 hours. Protein bands were incubated with enhanced chemiluminescence solution (#BMU102-CN, Abbkine, China) and analyzed using a Biorad WB imager. All antibodies used in this experimental procedure are listed in Table 1.

Table 1 Antibodies Used in the Article

Protein	Product Code	Application	Dilution Ratio	Company	Affiliating Area
Tenascin-C	67710-1-Ig	WB	1:2000	Proteintech	Wuhan, China
		IF	1:200		
TLR4	P60712RIS	WB	1:2000	Abmart	ShangHai, China
		IF	1:200		
MyD88	A21905	WB	1:2000	Abclonal	NanJing, China
		IF	1:200		
NF-κB	10745-1-AP	WB	1:2000	Proteintech	Wuhan, China
p-NF-κB	82335-1-RR	WB	1:2000	Proteintech	Wuhan, China
		IF	1:200		
iNOS	18985-1-AP	WB	1:2000	Proteintech	Wuhan, China
		IF	1:200		
CD206	81525-1-RR	WB	1:2000	Proteintech	Wuhan, China
		IF	1:200		
Arg-1	16001-1-AP	WB	1:2000	Proteintech	Wuhan, China
		IF	1:200		
CD86	TD6332S	WB	1:2000	Abmart	ShangHai, China
		IF	1:200		
ZO-1	21773-1-AP	WB	1:2000	Proteintech	Wuhan, China
Occludin	ab216327	WB	1:2000	Abcam	Shanghai, China
β-tubulin	80713-1-RR	WB	1:5000	Proteintech	Wuhu, China
GAPDH	60004-1-Ig	WB	1:5000	Proteintech	Wuhan, China

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Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of IL-1β and TNF-α in the mouse brain tissue homogenates collected 24 hours after subarachnoid hemorrhage were detected using a Mouse IL-1β ELISA Kit (#RK00006, Abclonal, China) and a Mouse TNF-α ELISA Kit (#RK00027, Abclonal, China). All assays were performed strictly according to the manufacturer's instructions.

Statistical Analysis

All data were analyzed using the mean ± standard error (SEM). For data that met the assumption of normality, one-way ANOVA was used for inter-group comparisons as appropriate. For non-normally distributed data, inter-group comparisons were performed using the Kruskal–Wallis test or Mann–Whitney *U*-test. A p-value < 0.05 was considered statistically significant. All statistical analyses were performed using Prism 10 (GraphPad Software, USA).

Results

Mortality in the SAH Model

No significant differences were observed in physiological parameters, including mean arterial pressure, arterial blood pH, arterial oxygen partial pressure, arterial carbon dioxide partial pressure, and blood glucose levels among all groups during the surgery (data not shown). No mortality was observed in the sham-operated group in any of the experimental groups. In Experiment 1, the mortality rate after SAH surgery was 30.67% (23/75) at the detection time gradient. In the optimal dose detection group of imatinib, the mortality rates were as follows: SAH group, 33.33% (5/15); SAH+Vehicle group, 40.00% (6/15); SAH+80mg/kg imatinib group, 20.00% (3/15); SAH+40mg/kg imatinib group, 26.67% (4/15); SAH+20mg/kg imatinib group, 40.00% (6/15). In Experiment 2, the mortality rates were as follows: SAH group, 40.00% (6/15); SAH+Vehicle group, 40.00% (6/15); SAH+imatinib group, 20.00% (3/15). In Experiment 3, the mortality rates were as follows: SAH group, 33.33% (5/15); SAH+Vehicle group, 33.33% (5/15); SAH+imatinib group, 20.00% (3/15). In Experiment 4, the mortality rates were as follows: SAH group, 33.33% (5/15); SAH+Vehicle group, 40.00% (6/15); SAH+imatinib group, 26.67% (4/15). At the same time, after we obtained the optimal action concentration of imatinib, we counted the overall mortality rates in Experiment 2, Experiment 3 and Experiment 4. In the SAH group, it was 35.56% (16/45). In the SAH + Vehicle group, it was 37.78% (17/45). And in the SAH + imatinib group, it was 22.22% (10/45). Mice were randomly selected for the experiments.

Trend of Tenascin-C Expression

To evaluate the expression trend of Tenascin-C after SAH, we used Western blotting to detect changes in Tenascin-C expression in the ipsilateral cortex of mice. The expression of Tenascin-C began to increase at 6 h after SAH and reached a peak at 24 h (Figure 2A). To simulate an in vitro SAH model, we treated primary microglia with OxyHB for 24 h. Immunofluorescent double staining of mouse brain tissue sections and primary microglia revealed colocalization of microglia and Tenascin-C (Figure 2B and C).

Tenascin-C Inhibitor Imatinib Can Reverse Neurological Impairment, Brain Edema and Blood-Brain Barrier Damage After SAH

To uncover the potential mechanism of Tenascin-C in SAH, we used the Tenascin-C inhibitor imatinib. Before starting the administration, Western blotting was used to detect the optimal dose of imatinib. Compared with SAH+Vehicle and SAH, 80 mg/kg imatinib and 40 mg/kg imatinib could reduce Tenascin-C expression to varying degrees, but 80 mg/kg had the most significant inhibitory effect (Figure 3A). Therefore, subsequent experiments used 80 mg/kg imatinib for mouse administration. In vivo experiments, the levels of tight junction proteins ZO-1 and Occludin were detected by Western blotting to reflect the degree of blood-brain barrier injury. The expression of tight junction proteins ZO-1 and Occludin decreased in the SAH group and SAH+vehicle group compared to the sham group, while the imatinib group significantly reversed this result (Figure 3B and C). We used the modified Garcia score and balance beam test to evaluate the neurological dysfunction of mice, and it was obvious that the imatinib group improved the neurological function of mice (Figure 3D and E). In addition, we also used the detection of water content in mouse brain tissue to reflect brain edema, and it was clear that

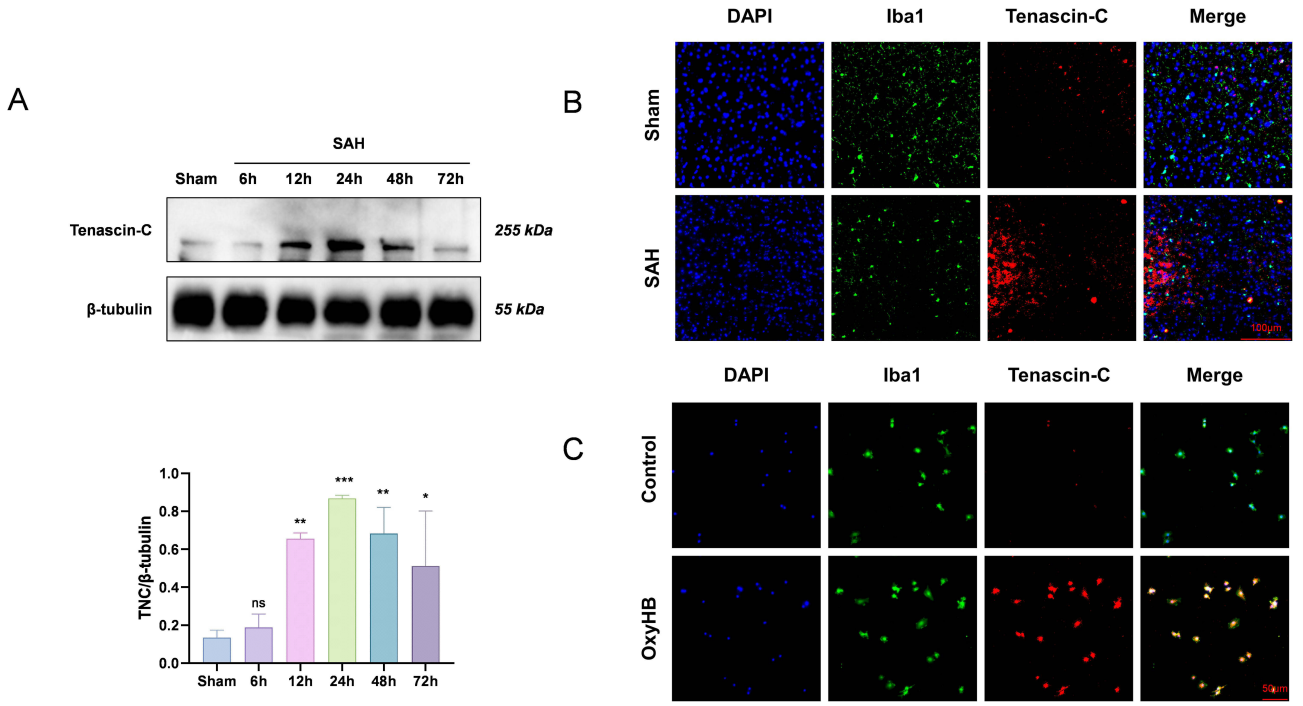

Figure 2 Changes in Tenascin-C levels in in vivo and in vitro subarachnoid hemorrhage models. (**A**)The relative expression levels of Tenascin-C in the in vivo SAH model at 6h, 12h, 24h, 48h, and 72h. (**B** and **C**) Immunofluorescent co-staining of Iba1 (green) and Tenascin-C (red) in microglia in vivo and in vitro after 24h SAH. Data are expressed as mean ± SD, ns indicates not significant,*P < 0.05, **P < 0.01, ***P < 0.001.

the imatinib group improved brain edema compared to the SAH+vehicle group (Figure 3F). The above studies suggest that the Tenascin-C inhibitor imatinib significantly Reduce brain injury after subarachnoid hemorrhage.

Imatinib Reduced the Inflammatory Response After SAH

Previous studies have found that Tenascin-C is considered an important inducer of the neuroinflammatory cascade. We used ELISA to detect the expression levels of IL-1β and TNF-α in mouse cortical brain tissue and primary microglia culture medium. According to statistical analysis, the expression levels of IL-1β and TNF-α were significantly increased after SAH in vivo, but the imatinib group significantly reduced the levels of inflammatory factors compared to the SAH +vehicle group (Figure 4D and E). For the in vitro model, we set different concentration gradients of imatinib, and detected the lowest concentration of the drug that affected primary microglia cells by CCK8 and compared it with the OxyHB group. 10μM imatinib and 5μM imatinib had the smallest difference from the OxyHB group (Figure 4A). The results of the in vivo experiment were also verified in the in vitro model. We used OxyHB to stimulate primary microglia cells, and the inflammatory factor levels in the culture medium of the imatinib+OxyHB group decreased (Figure 4B and C), suggesting that inhibiting Tenascin-C expression can alleviate the inflammatory response after SAH.

Inhibition of Tenascin-C Expression Can Promote Microglia M1 to M2 Phenotype Transition and Reduce Neuronal Apoptosis

After SAH occurs, microglia that were originally in a resting state are immediately activated to the M1 type. M1 microglia promote the development of inflammation and exacerbate early brain injury, while M2 microglia release anti-inflammatory factors that can alleviate early brain injury. According to our research findings, Western blotting detected that the expression levels of CD86 and iNOS were significantly increased in the SAH group compared to the sham group, while the expression levels of CD86 and iNOS in the imatinib group were significantly reduced compared to the SAH +Vehicle group (Figure 5A and B). By detecting the markers of M2 microglia, we surprisingly found that the expression levels of CD206 and Arg-1 in the imatinib group were significantly increased (Figure 6A and D). In

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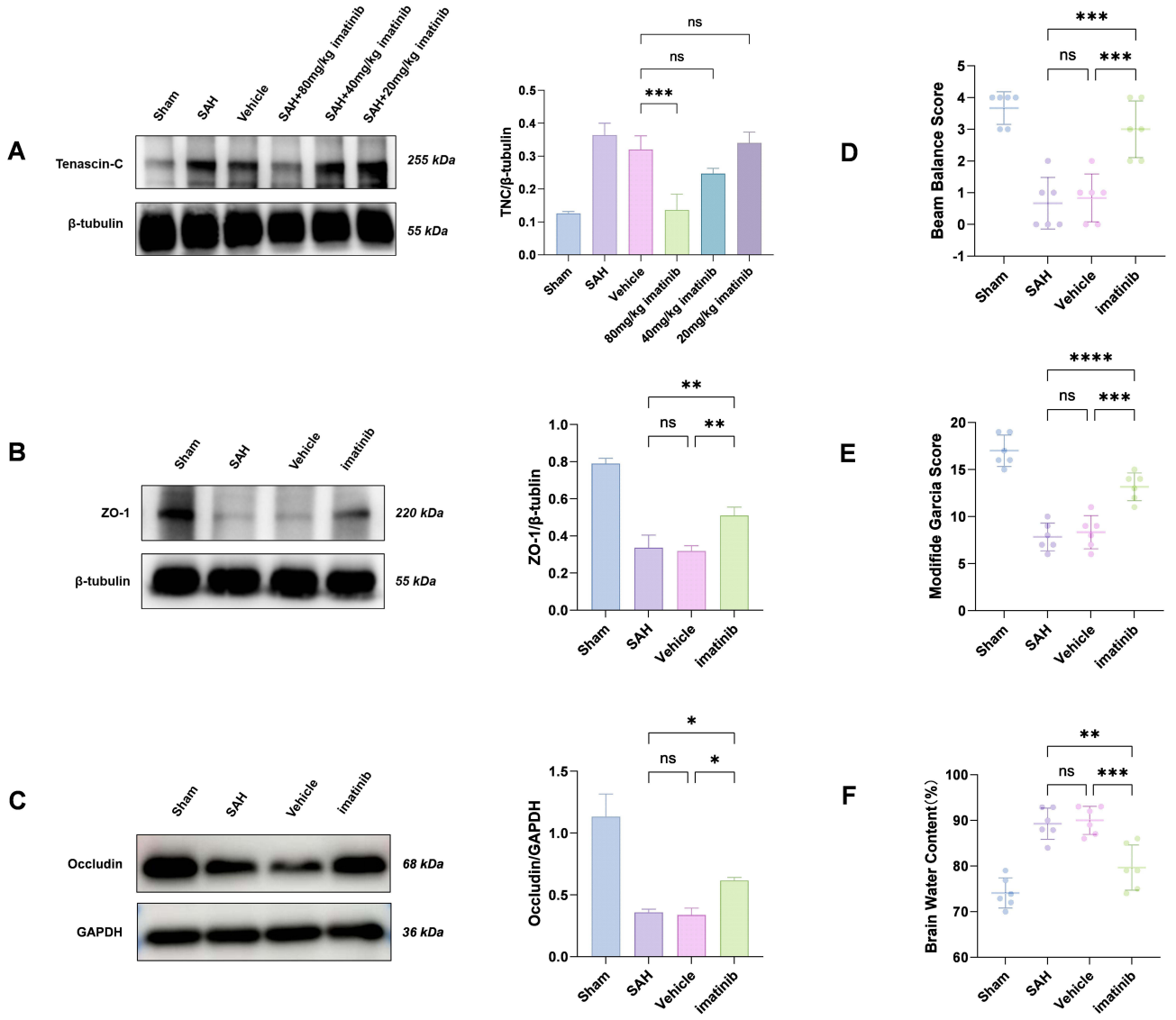

Figure 3 Suppressing the level of Tenascin-C can improve neurological function, blood brain barrier injury and cerebral edema after subarachnoid hemorrhage (SAH). (**A**) Detect the optimal concentration of imatinib in mice by Western Blot. (**B** and **C**) Detect the tight junction proteins ZO-1 and Occludin in the blood brain barrier to reflect the damage degree of the blood brain barrier. (**D** and **E**) Evaluate the neurobehavioral ability of mice through the balance beam test and Modified Garcia Score. (**F**) Evaluate the cerebral edema status of mice. Data are expressed as mean ± SD, ns indicates not significant.*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

immunofluorescence, the number of CD86 and iNOS-positive microglia in the SAH+vehicle group significantly increased (Figure 5C and D), while the number of CD206 and Arg-1-positive microglia in the imatinib group significantly increased (Figure 6B and E). The same research results also appeared in the in vitro model. We stimulated primary microglia with OxyHB and found by double immunofluorescence staining that the proportion of CD86 and iNOS-positive microglia in the OxyHB group increased (Figure 5E and F), while the proportion of CD206 and Arg-1-positive microglia in the drug group increased (Figure 6C and F). Additionally, through Tunel staining, it was found that neuronal apoptosis in the imatinib group was significantly reduced compared to the SAH+Vehicle group (Figure 6G).

Tenascin-C Aggravate Brain Injury After SAH Through TLR4/MyD88/NF-κB Pathway

Tenascin-C is an endogenous activator of TLR4. When TLR4 is stimulated, it regulates the NF-κB signaling pathway through MyD88, triggering a cascade of inflammatory reactions. In our study, Western blotting revealed that the expression level of TLR4 in the SAH group was significantly higher than that in the sham group, and the expression level of MyD88 was also increased. Similarly, the expression level of p-NF-κB increased. Through statistical analysis of

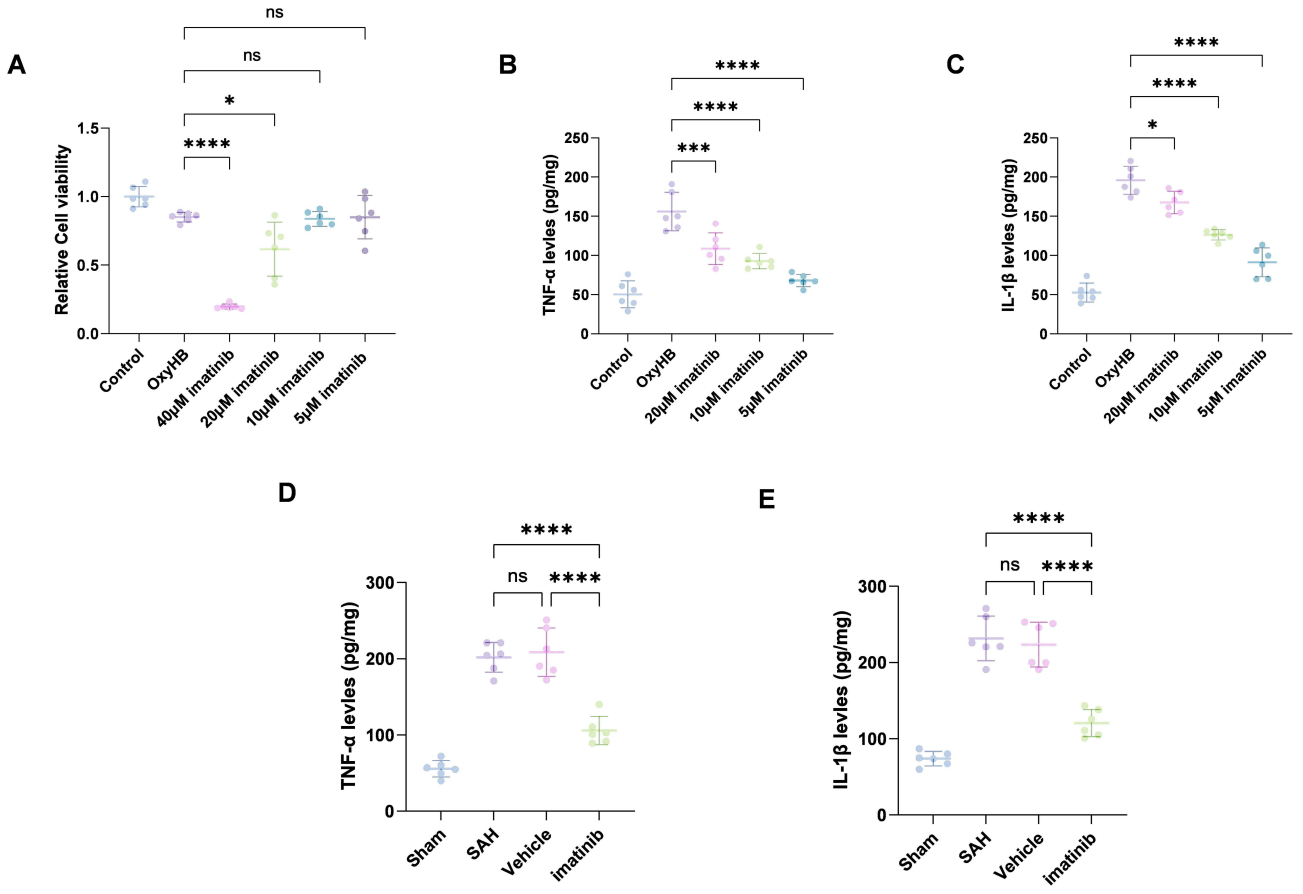

Figure 4 Inhibition of Tenascin-C levels in vivo and in vitro after subarachnoid hemorrhage can reduce the inflammatory factors TNF-α and IL-1β. (**A**)Detect the optimal concentration range of imatinib on primary microglial cells by CCK8. (**B** and **C**) Study the levels of inflammatory factors TNF-α and IL-1β in the control group, OxyHB group and imatinib added group of the in vitro SAH model by ELISA. (**D** and **E**) Detect the levels of inflammatory factors TNF-α and IL-1β in the sham group, SAH group, vehicle group and imatinib group of the in vitro SAH model by ELISA. Data are expressed as mean ± SD, ns indicates not significant,*P < 0.05,***P < 0.001, ****P < 0.0001.

the SAH+Vehicle group and the imatinib group, we found that after inhibiting the expression of Tenascin-C, the expression levels of the above pathway markers all decreased significantly (Figure 7A–C). Immunofluorescence co-staining of microglia and pathway markers also showed the same result, with a significant decrease in the number of TLR4, MyD88, and NF-κB-positive microglia after inhibiting Tenascin-C (Figure 7D–F). Interestingly, in the in vitro model, we used OxyHB to stimulate primary microglia and found the same trend as the in vivo model through immunofluorescence staining. The proportion of TLR4, MyD88, and p-NF-κB-positive microglia in the OxyHB group increased significantly, and adding the inhibitor imatinib successfully reversed this result (Figure 7G–I). In summary, we have reasons to believe that the elevated Tenascin-C level after SAH could induce the activation of microglia via the TLR4/MyD88/NF-κB signaling pathway.

Discussion

Subarachnoid hemorrhage (SAH) is a serious threat to the lives and health of patients and brings a huge economic burden to their families. This study is the first to reveal the role of Tenascin-C in microglial polarization during the early brain injury stage, aiming to provide a potential direction for the treatment of SAH. In our current study, we found that the expression level of Tenascin-C increased stepwise during the early brain injury stage of SAH and reached its highest point at 24 hours. Imatinib mesylate has been shown to effectively reduce the expression level of Tenascin-C and alleviate the cerebral vasospasm caused by SAH.[20]

In our study, imatinib did indeed reduce the expression level of Tenascin-C during the early brain injury stage, and the results showed that inhibiting the expression level of Tenascin-C effectively weakened the activation of M1 microglia. It

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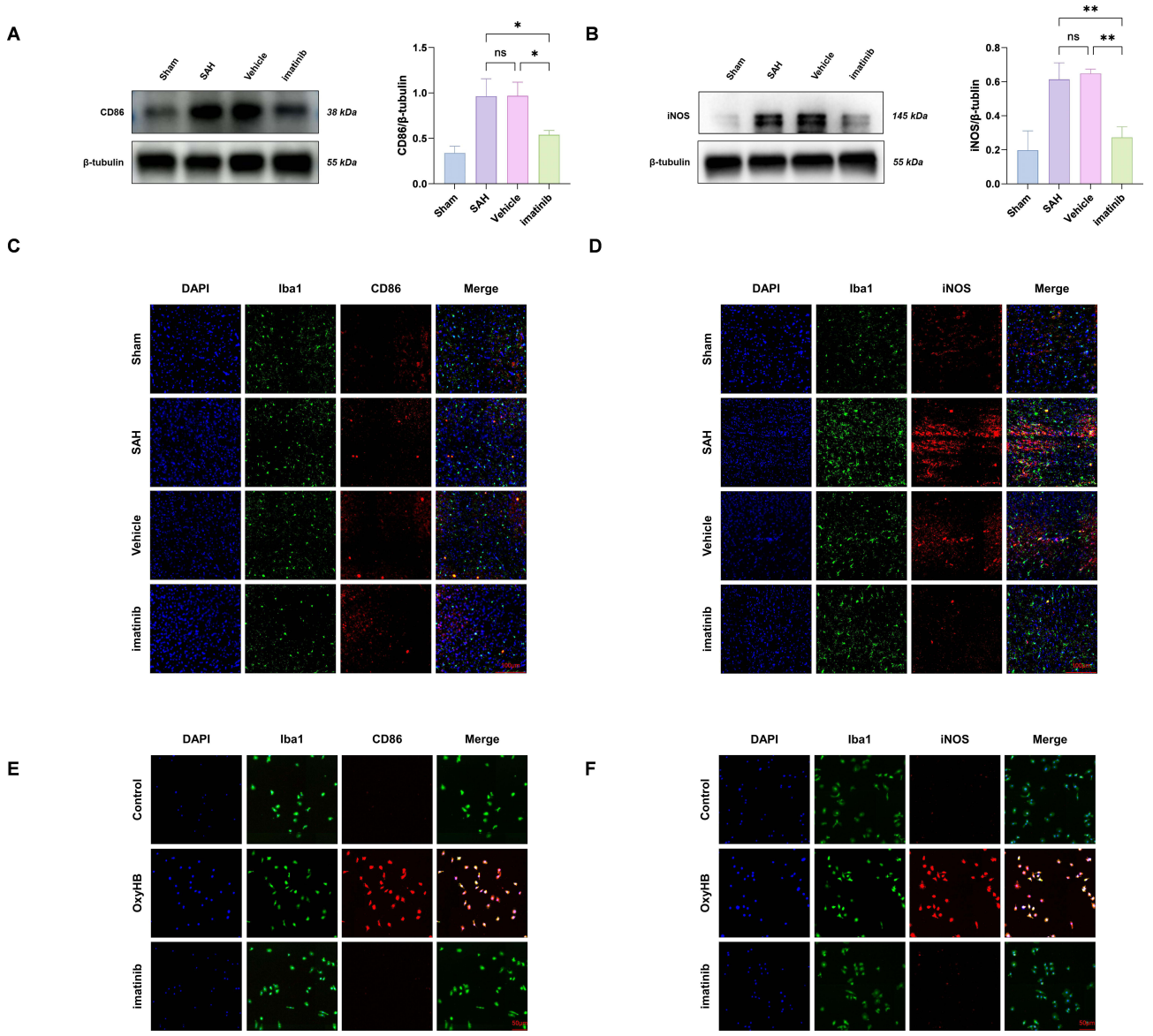

Figure 5 Inhibiting the levels of Tenascin-C can reduce M1 microglia. (**A** and **B**) Analyzed the levels of M1 microglial markers CD86 and iNOS in vivo using Western blot in the Sham group, SAH group, Vehicle group, and imatinib group of the SAH model. (**C**–**F**) Additionally, detected the M1 microglial markers (CD86 and iNOS, red) and microglial marker Iba1 (green) in vivo in the SAH model Sham group, SAH group, Vehicle group, and imatinib group, as well as in vitro in the Control group, OxyHB group, and imatinib group. Data are expressed as mean ± SD, ns indicates not significant,*P < 0.05, **P < 0.01.

also reversed the brain edema, neurological function, and the expression level of inflammatory factors after SAH. Toll-like receptors (TLRs) belong to the pattern recognition receptor family and play a crucial role in the inflammatory response. Especially, TLR4 is extremely important in this family and is abundantly expressed on microglia.[21–23] TLR4 is activated by many endogenous substances, such as heme, fibrinogen, and heat shock proteins. Activated TLR4 interacts with two different adapter proteins (MyD88 and Toll receptor-associated interferon activator (TRIF)) and activates two parallel signaling pathways to promote the release of inflammatory factors. In addition, the TRIF-dependent pathway induces late activation of NF-κB, while the MyD88-dependent pathway is an early activation of NF-κB.[24–26] We found that after inhibiting Tenascin-C with imatinib, the levels of markers of the TLR4/MyD88/NF-κB pathway were also inhibited. According to our immunofluorescence results, the number of TLR4/MyD88/NF-κB-positive microglia in the imatinib group of mice was significantly reduced, and this result was also confirmed in primary microglia in vitro. These results demonstrate that Tenascin-C promotes the activation of TLR4 receptors, which further activates the NF-κB

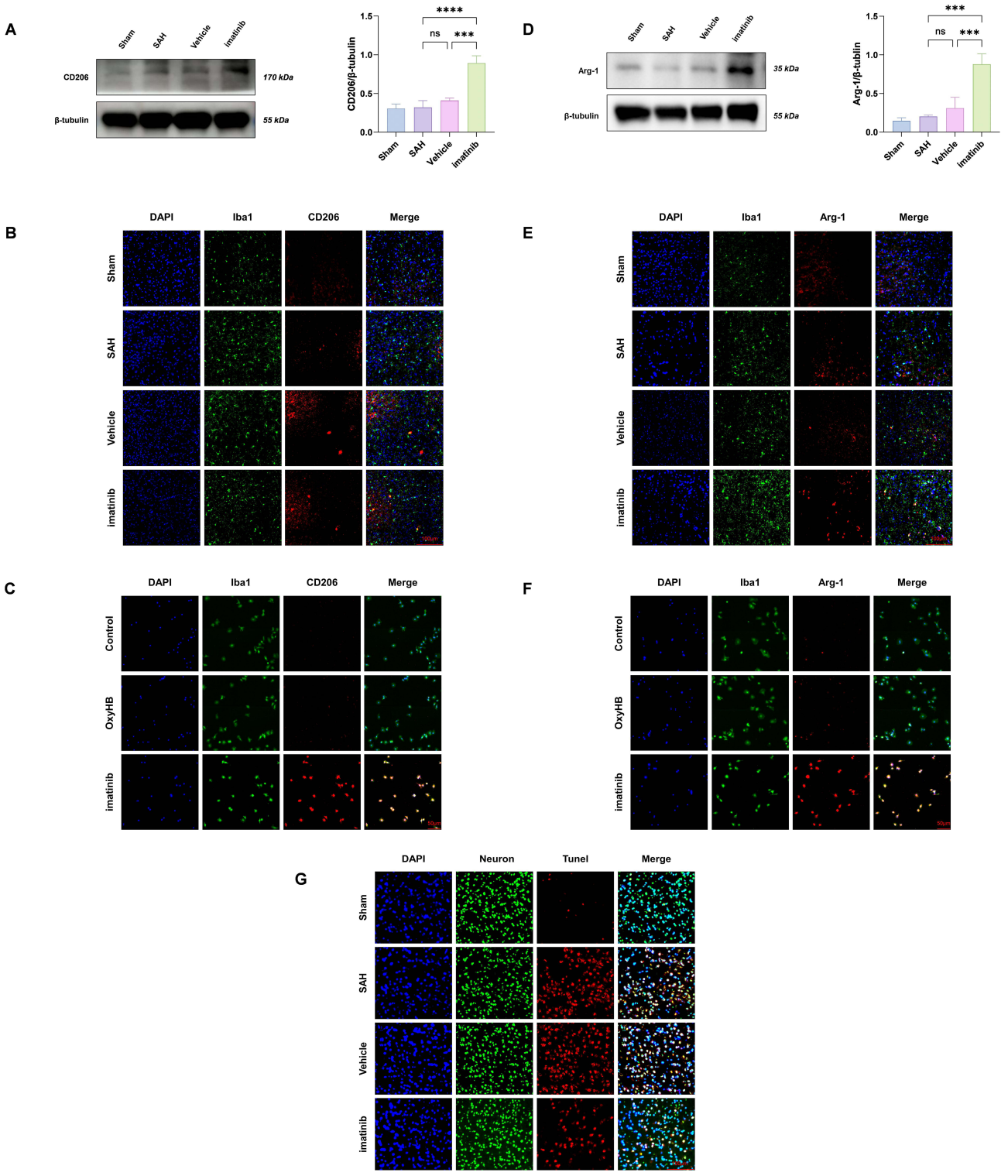

Figure 6 Inhibiting the levels of Tenascin-C can promote the transformation of microglia to the M2 phenotype And reduce neuronal apoptosis. (**A** and **D**) Analyze the levels of M2 microglial cell markers CD206 and Arg - 1 in the Sham group, SAH group, Vehicle group and imatinib group in the SAH in vivo model by Western blot. (**B, C, E** and **F**) And detect M2 microglial cell markers (CD206 and Arg - 1, red) and microglial cell Iba1 (green) in the Sham group, SAH group, Vehicle group, imatinib group of the in vivo SAH model and the Control group, OxyHB group, imatinib group of the in vitro SAH model by immunofluorescence staining.(**G**) Evaluate the apoptosis of neurons by Tunel staining. Data are expressed as mean ± SD, ns indicates not significant, ***P < 0.001, ****P < 0.0001.

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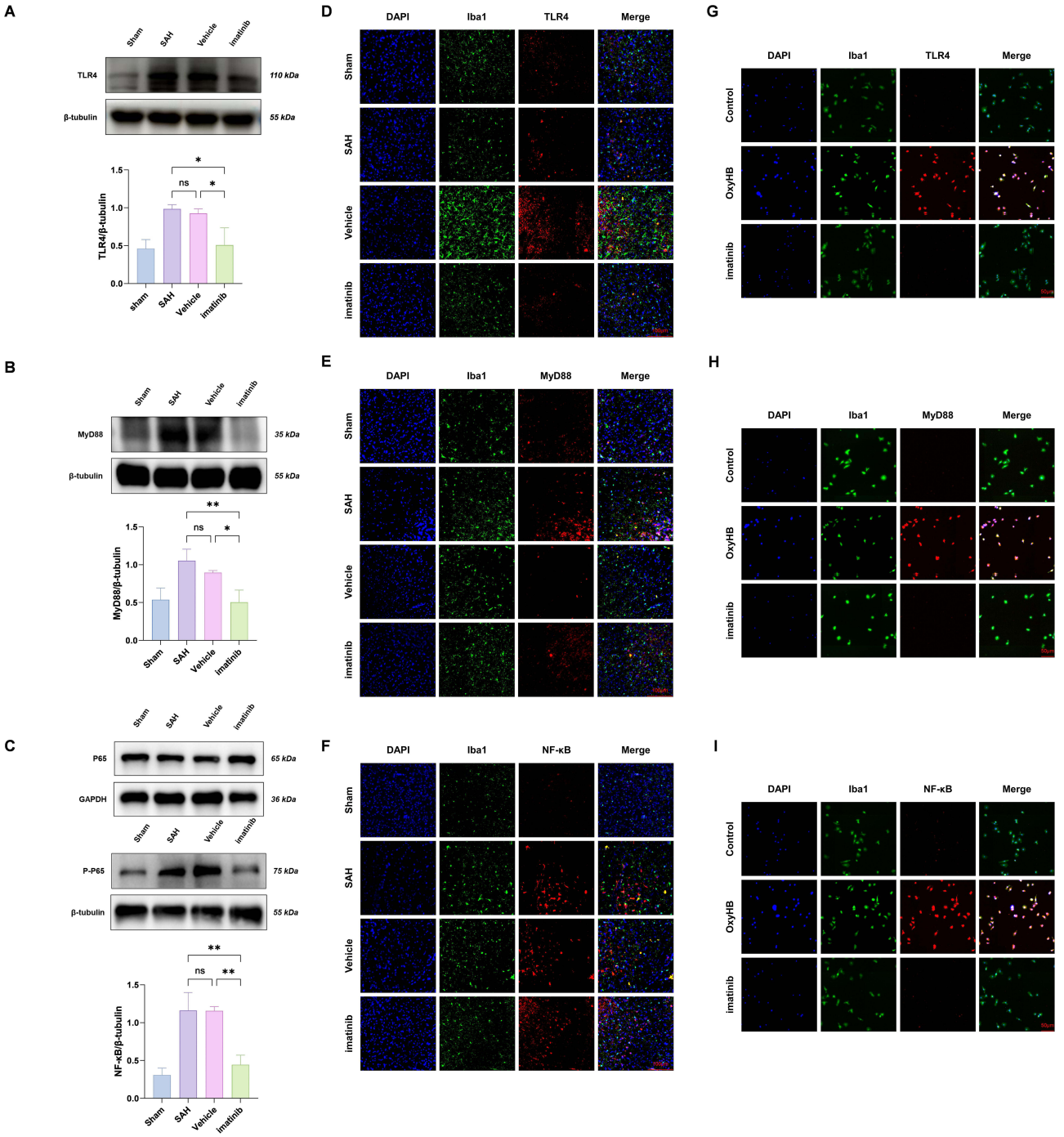

Figure 7 Tenascin-C induces microglial polarization through the TLR4/MyD88/NF-κB pathway. (**A**–**C**) Quantitatively analyze the activation levels of the TLR4/MyD88/NF-κB related signaling pathways in the in vivo SAH model by Western blot. Four groups are set up in the in vivo SAH model: Sham group, SAH group, Vehicle group, and imatinib group. Three groups are set up in the in vitro SAH model: Control group, OxyHB group, and imatinib group.(**D**–**I**) Analyze the activation levels of TLR4/MyD88/NF-κB related molecular signals (red) by immunofluorescence co-staining of microglia (green). Data are expressed as mean ± SD, ns indicates not significant,*P < 0.05, **P < 0.01.

signaling pathway through the MyD88-dependent pathway, leading to the activation of microglia and the release of a large amount of inflammatory factors, thereby exacerbating early brain injury. The integrity of the blood-brain barrier is also a key factor in inducing early brain injury. The integrity of the blood-brain barrier depends on tight junction proteins. After SAH, tight junction proteins are degraded, and the permeability of the blood-brain barrier increases. The infiltration of peripheral inflammatory factors further exacerbates neuroinflammation and brain edema.[27–29] In our study, imatinib-treated mice reduced the degradation of tight junction proteins (ZO-1, Occludin). Therefore, the increase in the

expression level of Tenascin-C during the early brain injury stage also leads to the degradation of tight junction proteins and aggravates the damage to the blood-brain barrier. By detecting the markers of M1 microglia (iNOS, CD86)[30,31] and M2 microglia (Arg-1, CD206),[32] we found that when Tenascin-C was inhibited, the immunofluorescence results showed that the number of M1 pro-inflammatory microglia decreased, while the number of M2 anti-inflammatory microglia increased, both in vivo and in vitro experiments. The levels of the inflammatory factors IL-1β and TNF-α further confirmed this experimental result. And through the TUNEL staining results, the neuronal apoptosis was indeed improved after inhibiting Tenascin-C. Inhibiting M1 microglia to drive M2 microglia has an extremely important significance in the treatment of early brain injury. Provide a potential treatment strategy for SAH. The study showed that Tenascin-C plays an important role in the activation mechanism of microglia after SAH. It activates M1 microglia through the TLR4/MyD88/NF-κB molecular signaling pathway. Imatinib can inhibit the expression level of Tenascin-C during early brain injury and promote the activation of M2 microglia, thus reducing the neuroinflammation, blood-brain barrier damage, and brain edema during early brain injury.

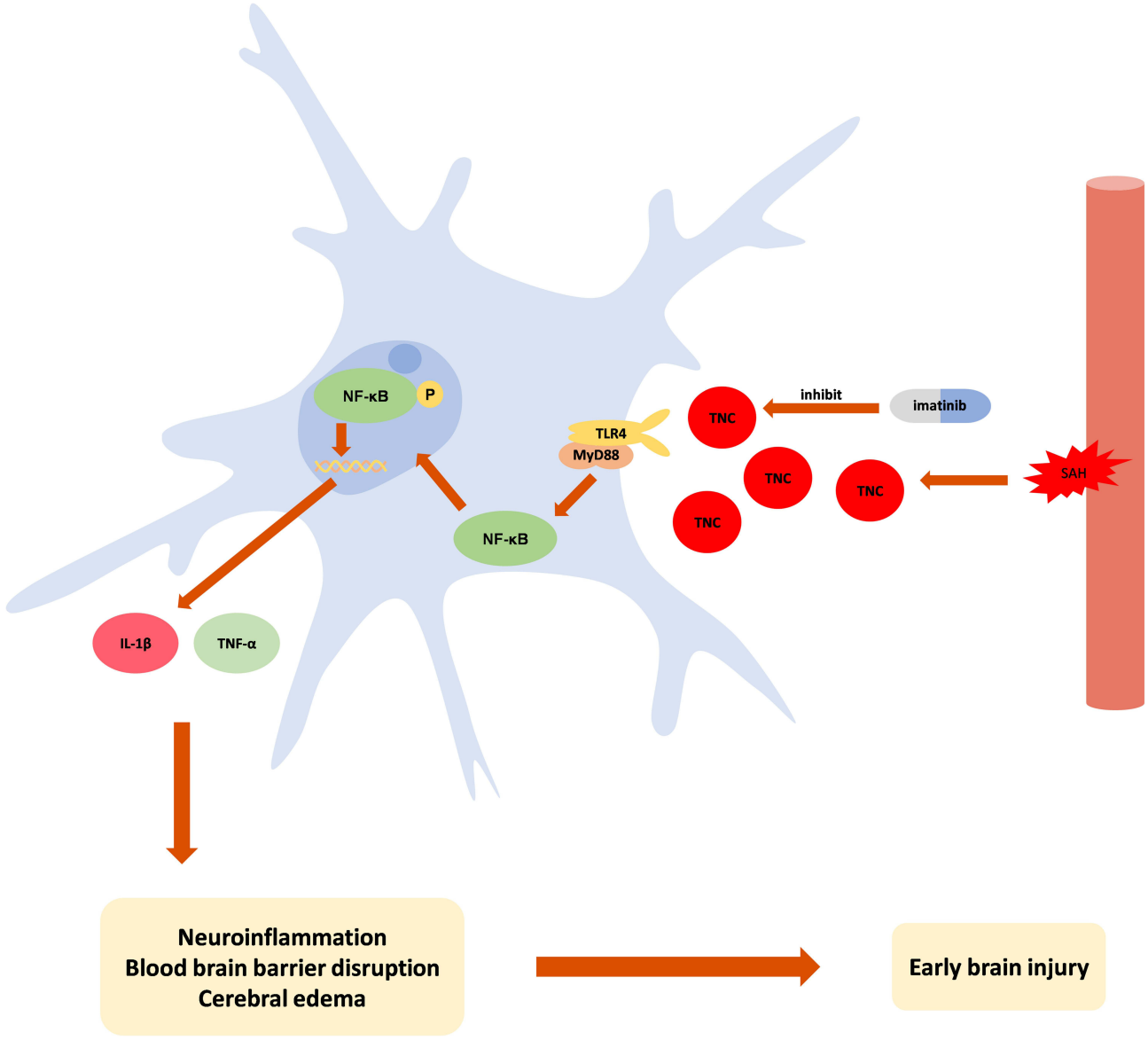

Figure 8 Tenascin-C induces microglial polarization through the TLR4/MyD88/NF-κB pathway. After subarachnoid hemorrhage, the expression level of Tenascin-C increases. It induces the activation of microglia through the TLR4/MyD88/NF-κB signaling pathway, releases a large number of inflammatory factors TNF-α and IL-1β, promotes neuron damage, blood - brain barrier disruption, etc. Imatinib can effectively reduce Tenascin-C induced early brain injury.

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Based on the above research findings, we have proposed new targets for the treatment of subarachnoid hemorrhage (SAH). Our research has found that in the early brain injury stage of SAH, Tenascin-C plays an important role in the activation process of microglia. In the early brain injury stage of SAH, the level of Tenascin-C rises, which activates microglia into pro - inflammatory cells through the TLR4 receptor on the surface of microglia, releasing a large amount of inflammatory factors and exacerbating the early brain injury. However, after inhibiting Tenascin-C with imatinib, the number of anti-inflammatory microglia begins to increase, alleviating the inflammatory response in the early brain injury stage. Consequently, it improves the symptoms after SAH. Tenascin-C affects the classification of microglia through the TLR4 receptor. Therefore, research on Tenascin-C is helpful for us to open up new avenues for the treatment of SAH.

At the same time, we also admit that our experiment has many limitations. First, imatinib is not a specific inhibitor of Tenascin-C, and secondly, whether Tenascin-C acts on microglia through other molecular signaling pathways, and whether Tenascin-C affects other subtypes of M2 microglia, such as M2a, M2b, M2c subtypes,[11] and whether Tenascin-C affects other cells in the nervous system, such as neurons and astrocytes. These are what we need to explore in detail in future experiments. However, we have provided new insights into the effect of Tenascin-C on microglial polarization and provided a new theoretical basis for the research and treatment strategy of new drugs for SAH. In the future, our research should knock down the Tenascin-C-related genes through adenovirus. Further explore the correlation between Tenascin-C and various cells in the nervous system after SAH and the underlying molecular signaling mechanisms. Formulate more detailed experimental procedures to reveal the molecular signaling mechanisms related to Tenascin-C and early brain injury, aiming to find new treatment directions for SAH and improve the quality of life of patients.

Conclusion

After the occurrence of subarachnoid hemorrhage, the expression level of Tenascin - C in the circulatory system increases. This study has for the first time confirmed that Tenascin - C induces the activation of microglia through the TLR4/MyD88/NF-κB molecular signaling pathway, aggravating early brain injury (Figure 8).

Abbreviations

SAH, Subarachnoid hemorrhage; TUNEL, Terminaldeoxynucleotidyl transferase dUTP nick end labeling; TLRs, Toll-like receptors; MyD88, Myeloid differentiation primary response 88; NF-κB, Nuclear factor kappa B; IL-1β, Interleukin-1β; TNF-α, Tumor necrosis factor-α.

Ethics Approval and Informed Consent

This study has been approved by the Laboratory Animal Welfare and Ethics Committee of Wannan Medical College (Approval No: WNMC-AWE-2024224), and all individuals participating in this study or their legal representatives have signed the informed consent form. The research protocols for all animal experiments have been approved by the Laboratory Animal Welfare and Ethics Committee of Wannan Medical College.

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Author Contributions

Zheng-qing Hu completed the experimental design and wrote the first draft of the manuscript. Ruijie Ma carried out the experiments on primary microglia cells. Jia-qing Sun guided the writing of the manuscript and conducted data analysis. Min Peng was responsible for the illustration layout of the article as well as Western Blot (WB). Jinlong Yuan and Niansheng Lai respectively carried out ELISA and immunofluorescence staining. Dayong Xia provided constructive guidance on the design of the entire manuscript and experimental process. All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and

interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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