Article

J. Biol. Regul. Homeost. Agents. 2024; 38(5): 4141–4152
https://doi.org/10.23812/j.biol.regul.homeost.agents.20243805.329

IL-10R Modulates Microglial Polarization and Attenuates Neuroinflammation Damage in Mouse Brain via TLR4/NF-κB Pathway

Bin Zheng[1], Tao Wu[1], Yun Qian[1], Lu Teng[2,*], Changyou Yin[2,*]

[1]Department of Neurosurgery, The Second Affiliated Hospital of Shandong First Medical University, 271000 Taian, Shandong, China
[2]Department of Neurosurgery, Yantai Yuhuangding Hospital, 264000 Yantai, Shandong, China
*Correspondence: yhdnstl@163.com (Lu Teng); yinchy99@163.com (Changyou Yin)
Published: 1 May 2024

Background: Intracerebral hemorrhage (ICH) stands as a crucial cerebrovascular pathology, wherein subsequent neuroinflammatory reactions augment neuronal detriment. Microglia, the principal immune effectors within the central nervous system, significantly influence the inflammatory cascade following ICH. Interleukin (IL)-10 is an anti-inflammatory cytokine that functions via the IL-10 receptor (IL-10R). It has been demonstrated to facilitate M2 polarization of microglial cells, thereby aiding in the resolution of inflammation and the repair of neural tissue. However, the specific mechanism of IL-10R in regulating microglial polarization remains unclear. Therefore, this study aimed to elucidate the role of IL-10R in facilitating microglia differentiation into the M2 phenotype, specifically focusing on its modulation of the toll-like receptor 4 (TLR4)/nuclear factor κB (NF-κB) signaling pathway.

Methods: We established a mouse model of intracerebral hemorrhage (ICH) to investigate the impact of neural stem cell (NSC) transplantation. IL-10R knockdown was achieved through cell transfection. The expression levels of inducible nitric oxide synthase (iNOS) and the mannose receptor (CD206) were evaluated using Western blot analysis to assess microglial polarization. Furthermore, we quantified inflammation levels by assessing the expressions of IL-10R, IL-1β, and tumor necrosis factor-alpha (TNF-α) using enzyme-linked immunosorbent assay (ELISA). Assessment of motor function was conducted using the modified neurological severity score (mNSS) and the rotarod performance test. Neuronal injury and mortality were determined by measuring brain water content, conducting Nissl staining, and performing the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Additionally, Western blot analysis was employed to evaluate the activity of the toll-like receptor 4 (TLR4)/nuclear factor κB (NF-κB) pathway, with its activation being suppressed by the specific inhibitor Resatorvid (TAK-242).

Results: We found that NSC transplantation significantly decreased microglial M1 polarization, inflammatory response, brain water content, neuronal cell damage, and apoptosis while increasing microglial M2 polarization and mice motor function following ICH. Inhibition of IL-10R reversed the effect of NSC transplantation, indicating its crucial role in NSCs-mediated neuroprotection. Furthermore, the activity of the TLR4/NF-κB inflammatory pathway was enhanced after ICH, which was suppressed by NSCs. Following NSC treatment, the IL-10R inhibition activated the TLR4/NF-κB pathway, which was reversed by TLR4/NF-κB pathway inhibitor TAK-242. Additionally, IL-10R inhibition counteracted the effect of NSC transplantation, which was disrupted when the TLR4/NF-κB pathway was inhibited.

Conclusion: This study suggests that IL-10R plays a crucial role in NSC transplantation for treating ICH. Inhibiting the TLR4/NF-κB pathway facilitates M2 polarization of microglial cells, leading to a decrease in neuroinflammatory response. This finding provides a new molecular target for treating ICH and other neuroinflammatory diseases. It emphasizes the potential therapeutic value of NSC transplantation in conjunction with the modulation of IL-10R signaling.

Keywords: IL-10R; TLR4/NF-κB pathway; microglial cells; M2 polarization; neuroinflammation

Introduction

Neuroinflammation following brain injury is a critical factor leading to neuronal death and neurological dysfunction [1–3]. Microglia, the principal immune cells within the central nervous system, are essential in controlling the inflammatory response post-brain trauma [4,5]. The interaction between pro-inflammatory M1-type and anti-inflammatory M2-type microglial cells is pivotal in regulating tissue repair and functional recovery after injury [6]. However, effectively modulating microglial polarization to enhance therapeutic outcomes post-brain injury remains a significant scientific challenge.

Interleukin (IL)-10 functions as a crucial anti-inflammatory cytokine, exerting its effects through the IL-10 receptor (IL-10R) [7–9]. Recent researches indi-

Journal of
BIOLOGICAL REGULATORS
and Homeostatic Agents

cate that IL-10 not only suppresses the production of pro-inflammatory cytokines but also promotes the transition of microglial cells into the M2 phenotype [10–12]. However, the precise molecular mechanisms underlying IL-10R's regulation of microglial polarization remain partially elucidated.

The toll-like receptor 4 (TLR4) and nuclear factor κB (NF-κB) signaling pathways play crucial roles in regulating the inflammatory response and are significant contributors to the activation and polarization of microglia [13,14]. Studies have suggested that overactivation of the TLR4/NF-κB signaling pathway is closely associated with neuroinflammatory responses subsequent to brain trauma [15,16]. Therefore, inhibiting this pathway could potentially alleviate the inflammatory response and promote tissue repair [17]. Despite extensive research on the roles of IL-10, IL-10R, and the TLR4/NF-κB pathway in inflammation, there remains a gap in understanding the interactions and specific mechanisms in regulating microglial polarization. Particularly, the dynamic alterations and control mechanisms governing their interaction in a model of brain injury remain incompletely explored.

This study aims to elucidate how IL-10R facilitates the differentiation of microglia into the M2 phenotype, specifically focusing on its modulation of the TLR4/NF-κB signaling pathway. By conducting IL-10R siRNA interference experiments in a mouse brain injury model, we evaluated the impact of IL-10R on the TLR4/NF-κB pathway and its role in regulating microglial polarization and reducing neuroinflammation. Our findings not only reveal a novel mechanism by which IL-10R regulates microglial function following brain injury but also provide scientific evidence for developing novel therapeutic strategies. These insights potentially hold significant clinical implications for improving the prognosis of brain injury.

Methods

Mice Preparation

We obtained 180 male C57BL/6 mice, aged 8 weeks and weighing 20–30 g, from Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, with specific pathogen-free (SPF) status. Mice were housed in a controlled environment with temperatures between 26 and 28 °C and a 12-hour light-dark cycle. They were provided ad libitum access to food and water. All experimental procedures adhered to the ethical standards for the care and use of animals and received approval from the Ethics Committee of Yantai Yuhuangding Hospital (approval No. 2024-108). After experimental procedures, the mice were humanely euthanized using compressed CO_2 gas, followed by cervical dislocation. This method of euthanasia was selected for its efficacy and compliance with the guidelines recommended by the American Veterinary Medical Association (AVMA), aiming to minimize animal suffering.

Intracerebral Hemorrhage (ICH) Model

The method for modeling ICH is consistent with previous studies [18,19]. Briefly, to establish intracerebral hemorrhage models, mice were anesthetized through intraperitoneal administration of 3% sodium pentobarbital (50 mg/kg sodium pentobarbital) (Cat. No. P3761, Sigma-Aldrich, Saint Louis, MO, USA). Following stabilization, cranial fur was meticulously removed, and a precise 1-cm incision at the scalp's midpoint was developed. After skull exposure, a 1-mm borehole was created 0.2 mm anterior to bregma and 2.0 mm to the right of the midline. A 26-gauge needle mounted on a stereotaxic frame was inserted to a depth of 3.7 mm into the right striatum. Using a syringe pump (Legato 130, KD Scientific Inc., Holliston, MA, USA), Collagenase VII-S (Catalog No. C2399, Sigma-Aldrich, Saint Louis, MO, USA) was precisely delivered into the brain tissue. The administered dose of Collagenase VII-S consisted of 0.075 U dissolved in 0.5 μL of isotonic saline solution (0.9%; Catalog No. ST341, Beyotime, Shanghai, China) at a rate of 2 μL/min. After injection, the needle remained *in situ* for an additional 10 minutes before being retracted at a velocity of 1 mm/min. The Sham group underwent identical surgical procedures, excluding the administration of collagenase. Subsequently, the incision was sutured, and the mice were returned to their housing for recovery. The success of the ICH models was verified by magnetic resonance imaging (MRI). All animal ICH model was successful; thus, no animal was excluded from analysis.

Intracerebroventricular Injection of Small Interfering (si)RNA and TLR4 Inhibitor

After anesthetizing the mice and exposing the skull as described above, a 1-mm burr hole was drilled on the left front side of the head. For the targeted suppression of IL-10R in mouse brain tissue, siRNA specific to IL-10R (si-*IL-10R*; sequence: 5′-GAGCCTAGAATTCATTGCATACGAA-3′) and a corresponding negative control (si-*NC*; sequence: 5′-GAGGATTAAACTGTTTACCACCGAA-3′) were synthesized by GenePharma Co., Ltd. (Shanghai, China). The siRNAs were dissolved in sterile, RNase-free aqueous solution according to the manufacturer's instructions. Subsequently, a 26-pin stereotactic needle was inserted to a depth of 2.3 mm, and the siRNA solution was delivered into the brain tissue at a controlled rate of 0.67 μL/min using a SYRINGE PUMP (legato 130, KD Scientific Inc., Holliston, MA, USA). Following the injection, the needle was left for an additional 5 minutes before being removed at a speed of 1 mm/min. After this, the burr hole was sealed, and the scalp was sutured. To inhibit the TLR4/NF-κB pathway, the specific TLR4 inhibitor Resatorvid (TAK-242) (3 mg/kg; S7455, Selleck, Houston, TX, USA)

was added in the siRNA solution and co-administered into the brain. This intracerebroventricular (i.c.v.) injection was performed 1 day before establishing ICH.

Preparation and Transplantation of Neural Stem Cells (NSCs)

The neural stem cells (NSCs; C17.2; iCell-m012) were obtained from iCell Bioscience Inc. (Shanghai, China) and cultured in a specialized medium designated for C17.2 cells (iCell-m012-001b, iCell Bioscience Inc., Shanghai, China). Culture conditions were maintained at 37 °C with 5% CO_2 and 70–80% humidity. For transplantation, a suspension of 1×10^5 cells in 3 µL of NSC-specific medium was injected into the ICH core at a rate of 1 µL/min, 1 hour after ICH induction. The cells used in this study have undergone mycoplasma detection, and the results indicate the absence of mycoplasma contamination.

Furthermore, sixty mice were randomly divided into 6 groups, including the Sham, ICH, ICH+NSCs, ICH+NSCs+si-NC, ICH+NSCs+si-IL-10R, and ICH+NSCs+si-IL-10R+TAK-242 groups, each comprising 10 mice. The detailed procedure of treatment was as described above.

Modified Neurological Severity Score (mNSS) Assessment and Rotarod Test

Seventy-two hours after ICH surgery, mice underwent mNSS scoring to evaluate motor and sensory function, balance beam performance, reflexes, and abnormal movements. The mNSS scoring system ranged from 0 to 18 points, with 1–6 points indicating mild injury, 7–12 points indicating moderate injury, and 13–18 points indicating severe injury.

After evaluating the mNSS, the motor coordination and balance were further assessed through the rotarod test, which determines the ability of mice to maintain their position on a rotating rod. Initially, mice underwent an adaptability assessment on a rotating rod set at 4 rotations per minute (rpm). Subsequently, four additional trials were conducted with the speed progressively increasing from 4 rpm to 40 rpm over 5 minutes. The average time of mice falling from the rotating rod during the experiment was recorded.

Collection of Brain Tissue Samples and Their Water Content Measurement

After completing the neurobehavioral assessment, the brain water content (BWC) was determined to evaluate brain edema. Following the previously described procedures [20,21], mice were euthanized via cervical dislocation, followed by the extraction of brain tissue. In each group, brain tissue from 5 mice was used for assessing BWC, while the remaining 5 brain tissues were used for obtaining coronal sections. To determine BWC, brain specimens were initially recorded for their wet weight using an electronic balance (Model 02202101, Fisher Scientific, Shanghai, China). Subsequently, these specimens were desiccated in an oven at 100 °C for 14 hours to ascertain the dry weight. Moreover, BWC was calculated using the formula: [(wet weight – dry weight) / wet weight] × 100%. For tissue sectioning, 2-mm coronal slices from the hemisphere ipsilateral to the ICH were either immediately analyzed or paraffin-embedded and preserved at –80 °C for subsequent investigations.

Western Blot Analysis

Tissue samples were immersed in ice-chilled RIPA lysis buffer (Catalog No. P0013B, Beyotime, Shanghai, China) added with phenylmethylsulfonyl fluoride (PMSF; Catalog No. ST505, Beyotime, Shanghai, China) in a 100:1 ratio. Following a 30-minute incubation, samples were homogenized using a homogenizer (Model CN-41056-98, Cole-Parmer Instrument Company, LLC., Shanghai, China). The homogenate was centrifuged at 12,000 rpm for 20 minutes at 4 °C, and the supernatant was retrieved for total protein isolation. After this, the proteins were separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Catalog No. P0012A, Beyotime, Shanghai, China) and subsequently transferred onto a polyvinylidene difluoride (PVDF) membrane (Catalog No. IPVH00010, Millipore, Billerica, MA, USA). The membrane was then blocked with 5% skim milk for 2 hours and incubated overnight with primary antibodies, including anti-inducible nitric oxide synthase (iNOS) (1:1000; Catalog No. ab178945, Abcam, Cambridge, MA, USA), anti-CD206 (1:1000; Catalog No. ab64693, Abcam, Cambridge, MA, USA), anti-TLR4 (1:1000; Catalog No. ab22048, Abcam, Cambridge, MA, USA), anti-NF-κB (1:1000; Catalog No. 51-0500, Invitrogen, Carlsbad, CA, USA), anti-p-NF-κB (1:1000; Catalog No. MA5-15160, Invitrogen, Carlsbad, CA, USA), and anti-Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) (1:2000; Catalog No. ab8245, Abcam, Cambridge, MA, USA) at 4 °C. The next day, the membrane was treated with HRP-conjugated secondary antibody (1:2000; Catalog No. ab7090, Abcam, Cambridge, MA, USA) at room temperature for 2 hours. The protein bands were visualized using an enhanced chemiluminescence system (ECL; Catalog No. 1622301, Millipore, Billerica, MA, USA) and were quantitatively analyzed utilizing ImageJ software (version 1.48, National Institutes of Health, Rockville, Maryland, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of IL-10R (ml037888, MLbio, Shanghai, China), IL-1β (EK201B, MultiSciences, Hangzhou, China), and tumor necrosis factor-alpha (TNF-α; Catalog No. ab100747, Abcam, Cambridge, MA, USA) within the injured brain tissue sections were conducted using commercially available ELISA kits, following the instructions provided by the manufacturer. Moreover, the quantitative results were expressed in picograms per milliliter (pg/mL).

Journal of
BIOLOGICAL REGULATORS
and Homeostatic Agents

Nissl Staining

Nissl staining was utilized to evaluate neuronal survival in brain specimens. The prepared slices were coronal sections of the brain. These prepared slices were treated with Nissl staining reagent (Catalog No. C0117, Beyotime, Shanghai, China) at 40 °C for 10 minutes. Subsequent rinsing of the sections was carried out using 95% and 70% ethanol solutions (Catalog No. 10009128, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). Visualization of Nissl-stained sections was accomplished using light microscopy (Model BX51, Olympus, Tokyo, Japan), with quantification of Nissl-positive neurons using ImageJ software (version 1.48, National Institutes of Health, Rockville, MD, USA).

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL)

Fresh tissue sections were fixed in 4% paraformaldehyde (Catalog No. P0099, Beyotime, Shanghai, China) and permeabilized utilizing Proteinase K (Catalog No. P9460, Solarbio, Beijing, China). After rinsing with phosphate-buffered saline (PBS; Catalog No. C0221A, Beyotime, Shanghai, China), the sections underwent labeling with a TUNEL assay kit (Catalog No. C1082, Beyotime, Shanghai, China) and were subsequently stained with 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI; Catalog No. C1002, Beyotime, Shanghai, China). Apoptotic cells were enumerated in four arbitrarily selected visual fields using a fluorescence microscope (Model CKX53, OLYMPUS, Tokyo, Japan). In cases where sections were paraffin-embedded, initial deparaffinization in xylene was required (Catalog No. 10023418, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), followed by rehydration through a series of graded ethanol (Catalog No. 100091192, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China).

Statistical Analysis

Statistical analyses were performed using SPSS version 22.0 (IBM SPSS, Armonk, NY, USA). Data were expressed as mean ± standard deviation. Multiple group comparisons were conducted using one-way ANOVA followed by Bonferroni correction. A p-value < 0.05 was considered statistically significant.

Results

NSC Transplantation Attenuates Microglial Activation and Neuroinflammation Post-Brain Hemorrhage

Utilizing a rodent model of ICH, we explored the effects of NSC transplantation on microglial activation, focusing on inducible nitric oxide synthase (iNOS) expression, a marker of the pro-inflammatory M1 microglial phenotype. We observed a significant increase in iNOS expression in the ICH group compared to sham controls, highlighting the impact of NSC transplantation on microglial activation dynamics post-ICH (Fig. 1A, $p < 0.001$). However, this effect was reversed by the NSCs transplantation in the ICH+NSCs group ($p < 0.001$). These findings indicated that NSC transplantation could reduce the M1 activation state of microglial cells post-brain hemorrhage. Conversely, CD206, a marker of M2-type microglial cells, was significantly increased in the ICH group (Fig. 1A, $p < 0.001$), which was further enhanced following NSC transplantation ($p < 0.001$). These observations indicate that NSC transplantation can promote the polarization of microglial cells towards the M2 type, contributing to the alleviation of neuroinflammatory responses.

Furthermore, IL-1β (Fig. 1B) and TNF-α (Fig. 1C) were significantly increased in the ICH group compared to the Sham group ($p < 0.001$). However, these pro-inflammatory factors were significantly decreased in the ICH+NSCs group ($p < 0.001$), indicating that NSC transplantation can mitigate neuroinflammatory reactions following cerebral hemorrhage. The expression of IL-10R, a marker of anti-inflammatory activity, was found to be elevated in the ICH group relative to the Sham group (Fig. 1D, $p < 0.001$), which was further increased in the ICH+NSCs group ($p < 0.001$). These findings demonstrate that NSC transplantation can increase the expression of the anti-inflammatory factor IL-10R, contributing to the alleviation of neuroinflammatory responses.

Inhibition of IL-10R Diminishes the Efficacy of NSC Transplantation

As shown in Fig. 2A, the level of IL-10R was substantially reduced in the ICH+NSCs+si-IL-10R group compared to the ICH+NSCs+si-NC group ($p < 0.001$), indicating successful downregulation of IL-10R in NSC-treated ICH mouse models post-transfection. Moreover, the inhibition of IL-10R led to the increase of IL-1β (Fig. 2B, $p < 0.001$) and TNF-α (Fig. 2C, $p < 0.001$) levels, indicating enhanced neuroinflammatory responses. Additionally, following treatment with si-NC and si-IL-10R, the expression levels of iNOS and CD206 were assessed using Western blot analysis. As depicted in Fig. 2D, the ICH+NSCs+si-IL-10R group exhibited significantly elevated iNOS level ($p < 0.001$) and reduced CD206 level ($p < 0.001$) than the ICH+NSCs+si-NC group. This indicates that the inhibition of IL-10R attenuates the therapeutic effect of NSC transplantation, leading to microglial cells being more inclined towards the M1 activation state rather than M2. ELISA analysis demonstrated that in the si-IL-10R treated ICH group, brain tissue levels of IL-1β and TNF-α were substantially higher compared to the si-NC treated ICH group ($p < 0.001$). These observations suggested that the inhibition of IL-10R enhanced neuroinflammatory responses and shifted microglial towards the M1 activation state, thereby attenuating the therapeutic effect of NSC transplantation.

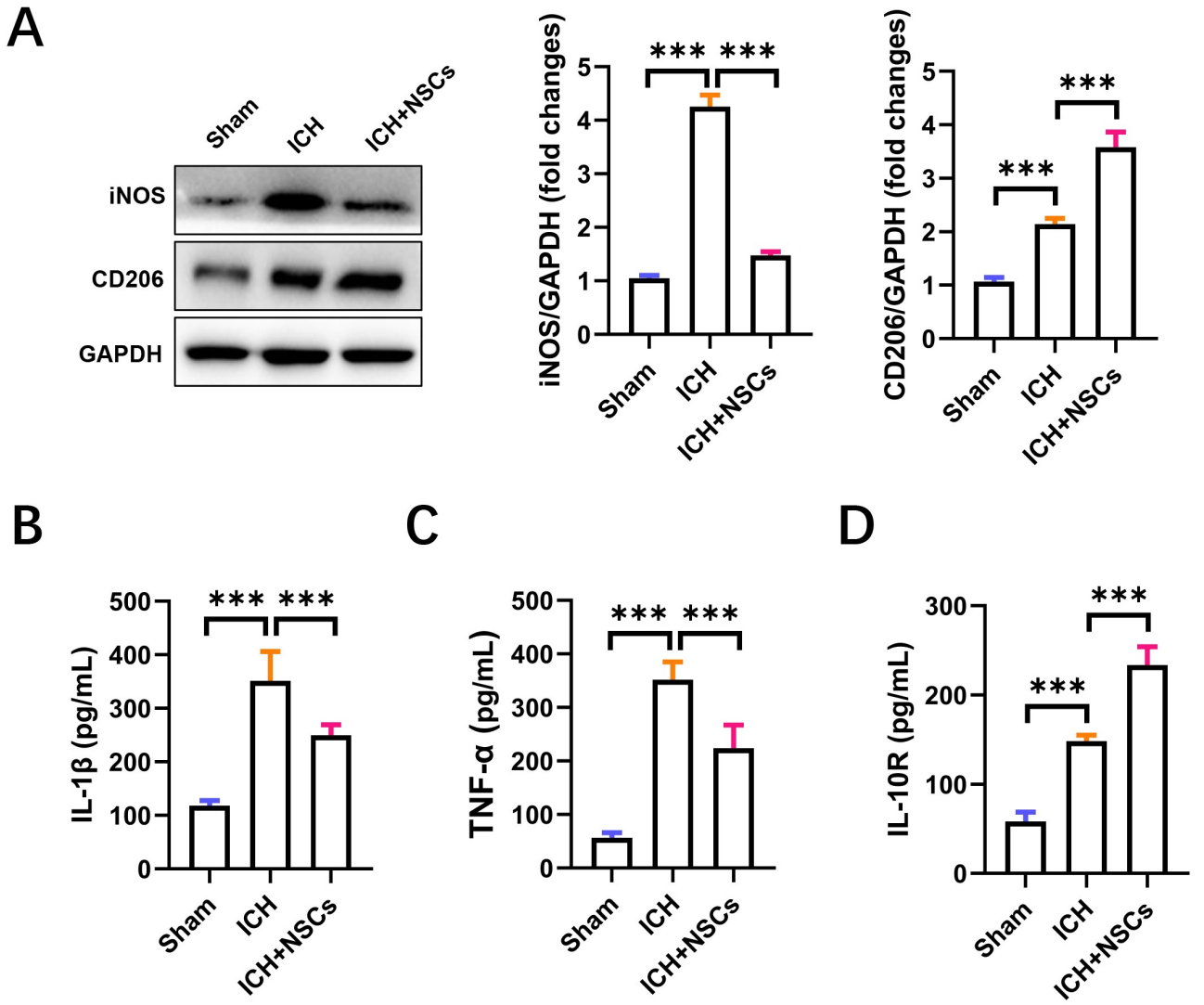

Fig. 1. Neural stem cell (NSC) transplantation alleviates microglial cell activation and inflammatory response following intracerebral hemorrhage. (A) The expression levels of inducible nitric oxide synthase (iNOS) and CD206 were assessed using Western blot analysis. (B–D) The expression levels of interleukin (IL)-1β, tumor necrosis factor-alpha (TNF-α), and IL-10 receptor (IL-10R) in the cerebral tissues of mice were quantified utilizing enzyme-linked immunosorbent assay (ELISA) techniques. N = 10. ***$p < 0.001$. ICH, intracerebral hemorrhage; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase.

Inhibiting IL-10R Reverses the Neuroprotective Benefits of NSC Transplantation

The mNSS and rotarod tests are commonly used to evaluate mouse neurological function. We observed that mNSS score (Fig. 3A) was significantly decreased in the ICH group ($p < 0.001$), which was subsequently restored following NSC transplantation ($p < 0.05$). In contrast, the improved mNSS score was decreased by downregulating IL-10R ($p < 0.05$). Similarly, the time of mice staying on the rotating rod (Fig. 3B) was decreased in the ICH group ($p < 0.001$), which was effectively increased by NSCs ($p < 0.001$). Conversely, the increased time was decreased by downregulating IL-10R ($p < 0.001$). Our findings suggest that NSCs enhance the motor function of ICH mice, but this enhancement is disrupted by inhibiting IL-10R. Measurement of brain water content indicates the degree of brain tissue edema. As shown in Fig. 3C, we observed a significant increase in brain water content within the ICH group ($p < 0.001$), which was subsequently alleviated by NSCs ($p < 0.001$). In contrast, the decreased brain water was increased again by inhibiting IL-10R ($p < 0.05$). These findings indicate that the NSCs could reduce the level of brain edema, which was disrupted by IL-10R inhibition. Furthermore, Nissl staining was used to observe the cellular status around the hemorrhagic site, with a reduction in the number of positive cells (Nissl bodies) possibly indicating cell damage. As shown in Fig. 3D, a significant decrease in Nissl bodies was found in the ICH group ($p < 0.001$), which was subsequently restored by NSCs ($p < 0.001$), indicating a neuroprotective effect of NSCs in ICH models. However, in the

Journal of
BIOLOGICAL REGULATORS
and Homeostatic Agents

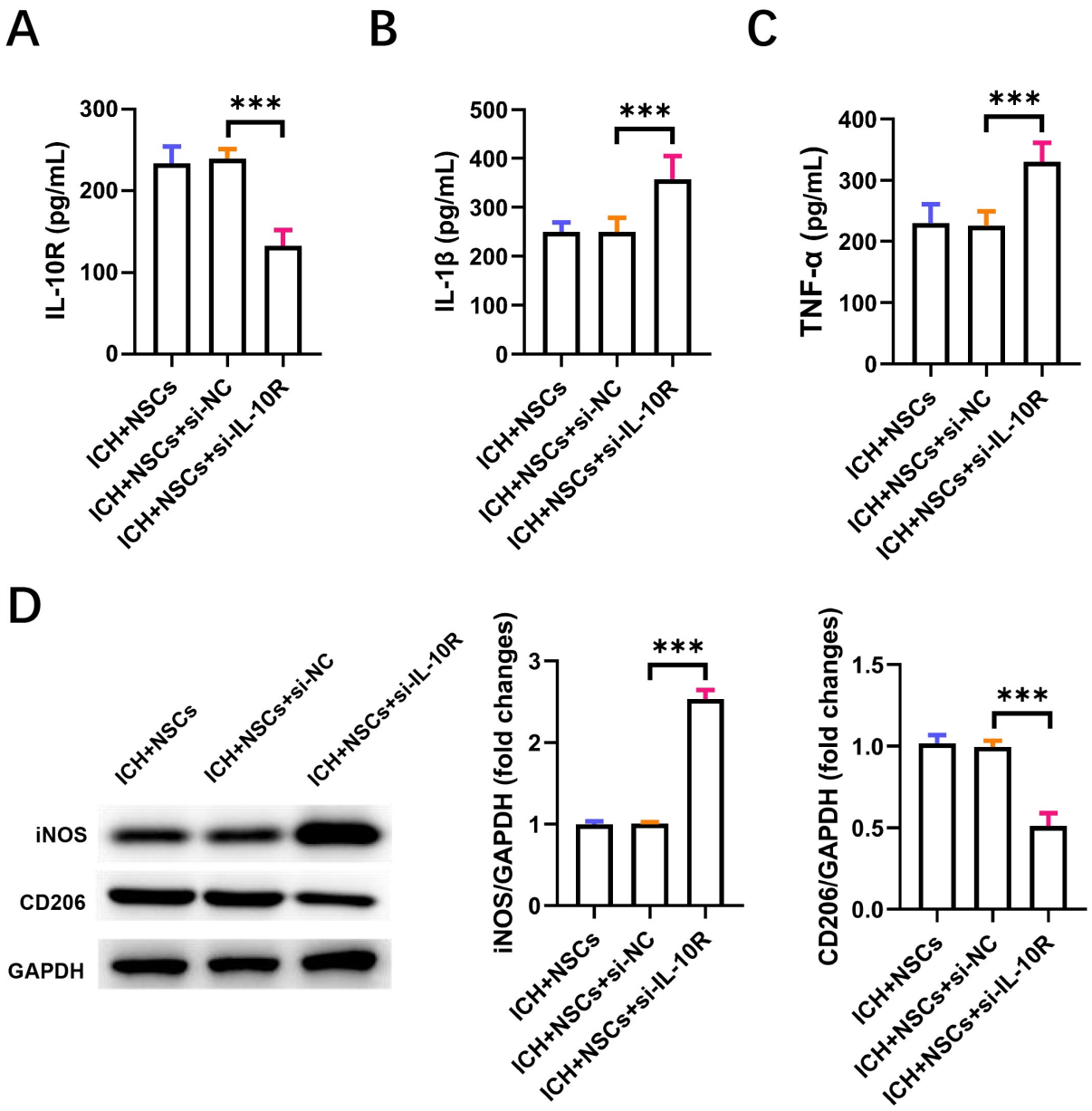

Fig. 2. Blocking IL-10R diminishes the therapeutic impact of NSC transplantation. (A–C) The levels of IL-10R (A), IL-1β (B), and TNF-α (C) in the brain tissue of ICH mice were determined using ELISA. (D) Protein expression of iNOS and CD206. N = 10. ***$p <$ 0.001.

ICH+NSCs+si-IL-10R group, there were fewer Nissl bodies compared to the ICH+NSCs+si-NC group ($p < 0.001$), suggesting that inhibiting IL-10R attenuated the protective effect of NSCs on mouse neurons. Additionally, TUNEL staining (Fig. 3E) revealed an increase in cell apoptosis in the ICH group ($p < 0.001$), which was mitigated following NSC transplantation in the ICH+NSCs group ($p < 0.01$). Conversely, inhibiting IL-10R resulted in an elevated number of apoptotic cells following NSC transplantation ($p <$ 0.01). These results suggest that IL-10R inhibition reverses the neuroprotective effect of NSC transplantation in ICH, thereby leading to neurological impairment, brain edema, cell damage, and cell apoptosis.

NSCs Suppress the Activation of the TLR4/NF-κB Inflammatory Pathway Post-ICH by Upregulating IL-10R

TLR4 is a cell surface receptor whose activation can initiate the signaling pathway of inflammatory reactions. p-NF-κB, the phosphorylated form of NF-κB, indicates the activation status of NF-κB, which mediates inflammatory responses. We observed significantly elevated TLR4 levels and the ratio of p-NF-κB to NF-κB ($p < 0.001$) compared to the Sham group, indicating activation of the TLR4/NF-κB inflammatory pathway after ICH (Fig. 4). Conversely, in the ICH+NSCs group, TLR4 levels and the p-NF-κB/NF-κB ratio were significantly lower

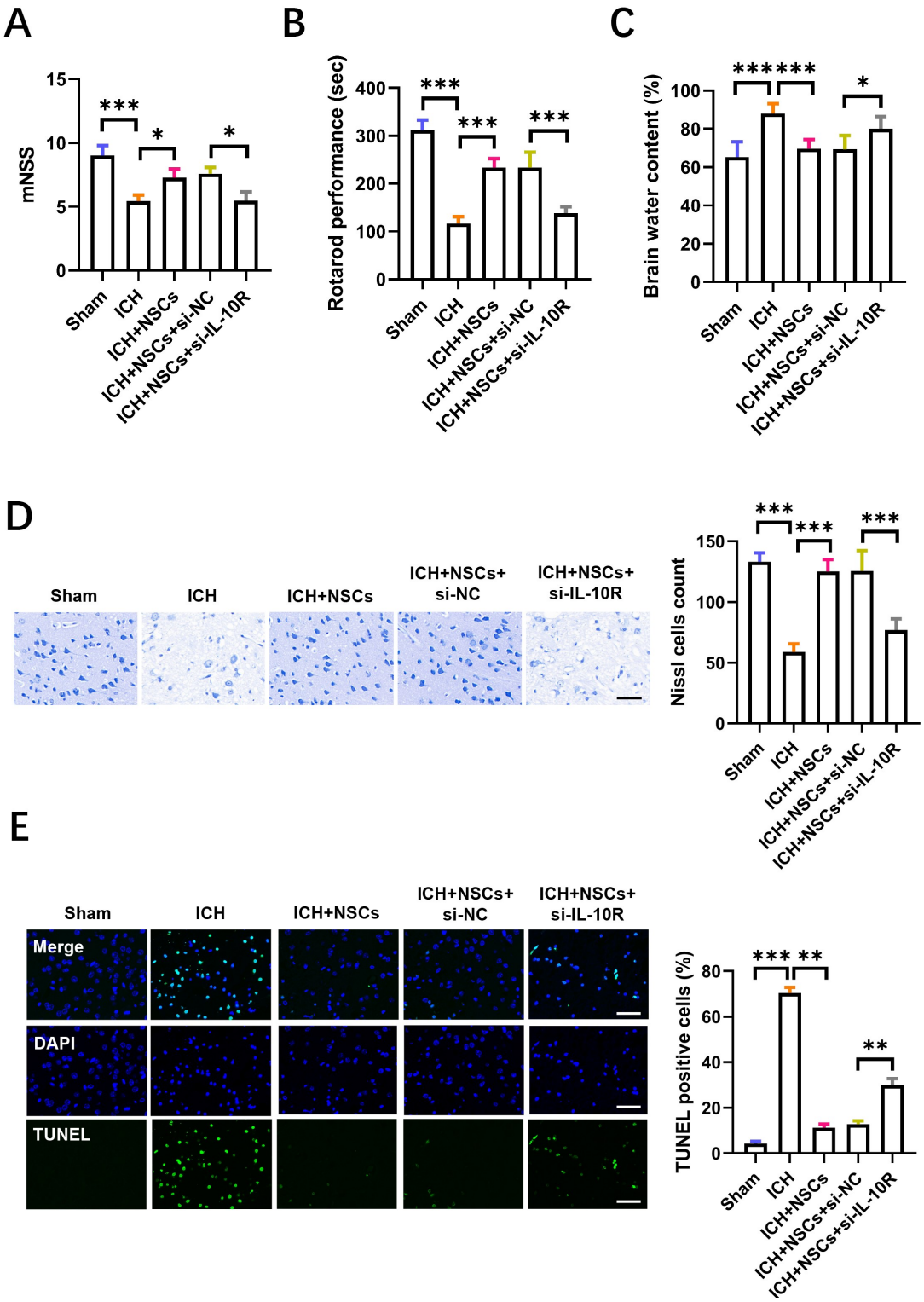

Fig. 3. Inhibiting IL-10R reverses the neuroprotective effect of neural stem cell (NSC) transplantation. (A,B) The modified neurological severity score (mNSS) and Rotarod tests were performed 72 hours post-ICH induction to evaluate neurological behavior. (C) Brain water content was quantified. (D) Nissl staining was performed to observe cellular status around the ICH site (scale: 50 μm). (E) Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was utilized to observe cellular apoptosis near the ICH site. (scale: 50 μm). N = 10. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. DAPI, 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride.

Journal of
BIOLOGICAL REGULATORS
and Homeostatic Agents

compared to the ICH group ($p < 0.001$) (Fig. 4), suggesting the anti-inflammatory effects of NSCs. Furthermore, the levels of TLR4 and p-NF-κB/NF-κB were substantially higher in the ICH+NSCs+si-IL-10R group compared to the ICH+NSCs+si-NC group ($p < 0.001$) (Fig. 4). This indicates that the NSCs suppress the TLR4/NF-κB inflammatory pathway by promoting IL-10R. When IL-10R is knocked down, the suppression of inflammation is disrupted (Fig. 4). Moreover, the levels of TLR4 and p-NF-κB/NF-κB were reduced in the ICH+NSCs+si-IL-10R+TAK-242 group compared to the ICH+NSCs+si-IL-10R group ($p < 0.001$), indicating that the TAK-242 suppresses the TLR4/NF-κB inflammatory pathway. Accordingly, NSCs exhibit an anti-inflammatory effect on ICH, but IL-10R inhibition reduces this effect, thereby failing to reduce the activity of the inflammatory pathway.

IL-10R Inhibition Promotes Inflammation and Decreases M2 Polarization of Microglial Cells by Activating the TLR4/NF-κB Pathway

As depicted in Fig. 5A, si-IL-10R significantly decreased IL-10R level ($p < 0.001$) in NSCs treated ICH mice, while the TLR4/NF-κB pathway inhibitor TAK-242 did not affect the IL-10R level. The IL-10R inhibition increased TNF-α (Fig. 5B, $p < 0.001$) and IL-1β (Fig. 5C, $p < 0.001$) levels, which were reversed by suppressing TLR4/NF-κB pathway ($p < 0.001$). IL-10R inhibition enhanced the inflammatory response by activating the TLR4/NF-κB pathway. Western blot analysis was employed to assess the expression of iNOS and CD206. The findings (Fig. 5D) revealed that IL-10R inhibition led to an increase in iNOS expression and a decrease in CD206 expression ($p < 0.001$), both of which were reversed by suppressing the TLR4/NF-κB pathway ($p < 0.001$). These findings suggest that IL-10R inhibition promotes M1 polarization and suppresses M2 polarization by activating the TLR4/NF-κB pathway.

Discussion

Brain injury is a common and severe neurological disorder, with its pathogenesis involving complex inflammatory processes [22,23]. Overactivation of the neuroinflammatory response following brain injury often exacerbates neuronal damage and functional impairment, significantly impacting patient recovery and quality of life [24]. Therefore, finding effective therapeutic strategies to modulate neuroinflammatory response and promote injury recovery holds paramount clinical significance [25]. Microglial cells, the primary immune cells within the central nervous system, play a pivotal role in modulating the inflammatory response after brain injury [26]. The activation state of microglial cells significantly impacts the severity of neuroinflammation and the effectiveness of the injury repair process. Studies have shown that microglial cells undergo polarization into pro-inflammatory M1 and anti-inflammatory M2 phenotypes, with M2 microglia being particularly important in promoting injury repair and restoring neurological function [27–29]. This study aimed to investigate the influence of interleukin-10 receptor (IL-10R) on microglial cell polarization towards the M2 phenotype by modulating the TLR4/NF-κB signaling pathway, and its effect on mitigating neuroinflammation in mice following brain injury. Our findings indicate that activating IL-10R effectively promotes the polarization of microglial cells towards the M2 type, thereby reducing the inflammatory response following brain injury. These observations provide a novel strategy for treating brain injury.

In this study, we found a correlation between IL-10R and the increase in M2-type markers in the brain injury model, suggesting a potentially beneficial role for IL-10R in microglial cell polarization. Utilizing IL-10R interference RNA experiments, we validated the enhancing effect of IL-10R on M2-type polarization. Additionally, we explored the regulatory role of the TLR4/NF-κB pathway in microglial polarization, indicating that IL-10R activation could suppress the TLR4/NF-κB pathway, a pivotal regulator of the inflammatory response. These findings suggest a potential mechanism by which IL-10R promotes M2-type polarization, thus exerting an anti-inflammatory effect.

Furthermore, our experimental results demonstrate that IL-10R activation significantly decreases the expression of neuroinflammatory biomarkers, including TNF-α and IL-1β, post-brain injury. Additionally, mice activated by IL-10R showed improved neurological function recovery, indicating that M2-type polarization could play a critical role in inhibiting neuroinflammatory responses and promoting neurological repair. The exacerbation of the neuroinflammatory response following brain injury leads to neuronal damage, impacting the recovery and repair processes. Therefore, by modulating IL-10R and the TLR4/NF-κB pathway, the severity of the neuroinflammatory response can be effectively reduced, thereby enhancing neuroprotection and providing new insights and strategies for treating brain injury.

The findings of our study align with established medical literature, indicating that IL-10R exerts an inhibitory effect on the TLR4/NF-κB pathway. IL-10R can inhibit TLR4-mediated signaling, thereby suppressing the production of inflammatory factors [30]. Additionally, our findings validate the role of IL-10R in promoting M2 polarization of microglial cells [31,32]. IL-10 through its receptor can promote M2 polarization of macrophages. Overall, numerous studies have highlighted the critical role of IL-10R in regulating immune responses and promoting tissue repair [33–35].

Furthermore, our study revealed the significant role of NSC transplantation in modulating neuroinflammatory responses. As a potential therapy, NSCs can regulate microglial cell activation, decrease the release of inflammatory factors, and enhance neuroprotection. Therefore, NSC

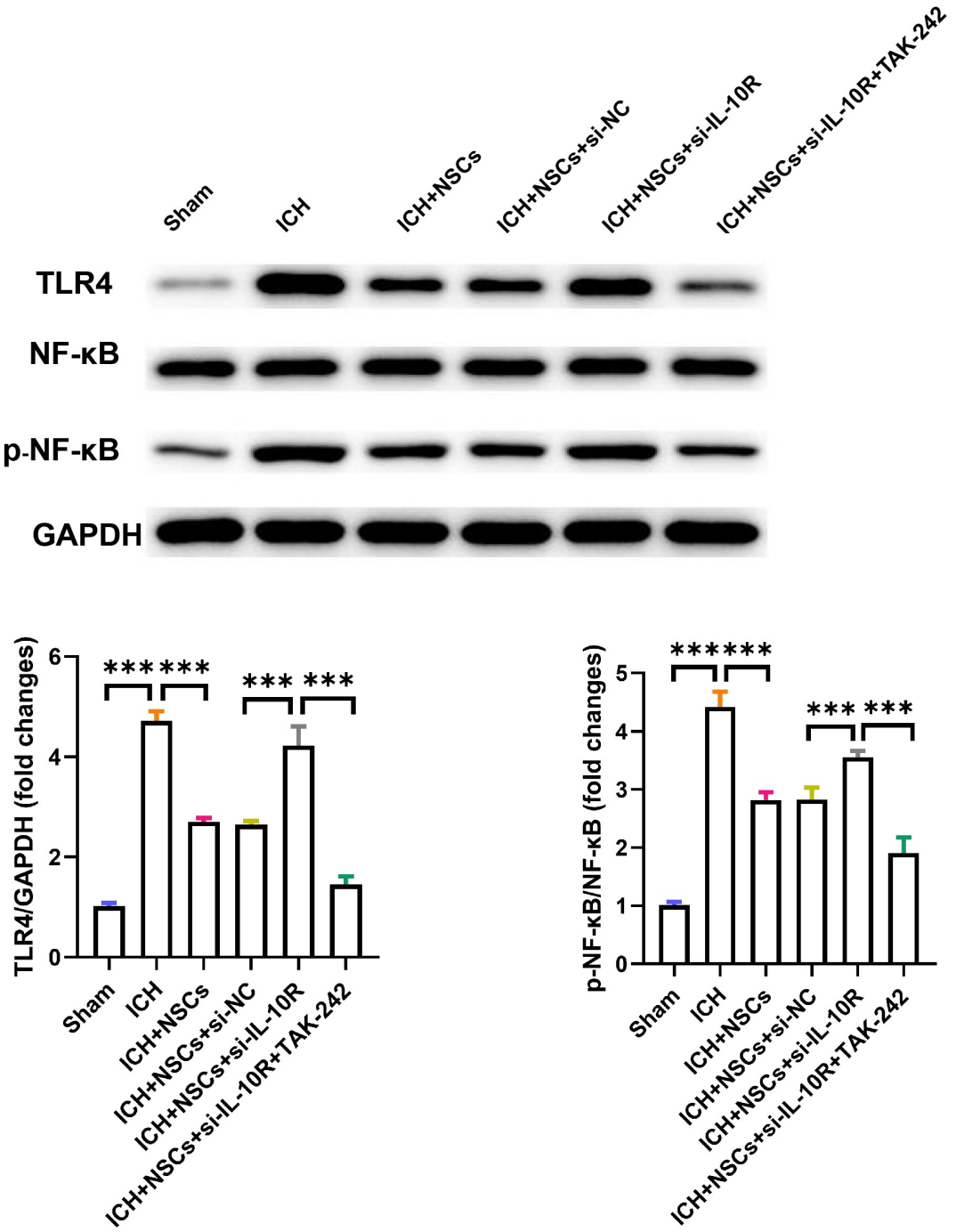

Fig. 4. Toll-like receptor 4 (TLR4)/nuclear factor κB (NF-κB) inflammatory pathway is activated after ICH and is suppressed by NSCs through increasing IL-10R. N = 10. ***$p < 0.001$. TAK-242, Resatorvid.

transplantation holds promise as an effective strategy for treating brain injuries. We analyzed the long-term effects of IL-10R activation on microglial cell function and its potential clinical applicability. While our study provides promising prospective insights, we recognize the limitations of the research, including the need for further preclinical studies to validate these findings and a deeper investigation of the role of IL-10R in various brain injury models.

The academic achievement and contribution of this study lie in elucidating the regulatory mechanism of IL-10R in neuroinflammatory responses following brain injury, providing a new perspective on the regulation of microglial polarization. Our findings not only validate IL-10R's role in suppressing the TLR4/NF-κB pathway but also elucidate its impact on promoting M2 polarization of microglial cells. This discovery offers the potential for developing new treatment strategies, particularly for brain in-

Journal of
BIOLOGICAL REGULATORS
and Homeostatic Agents

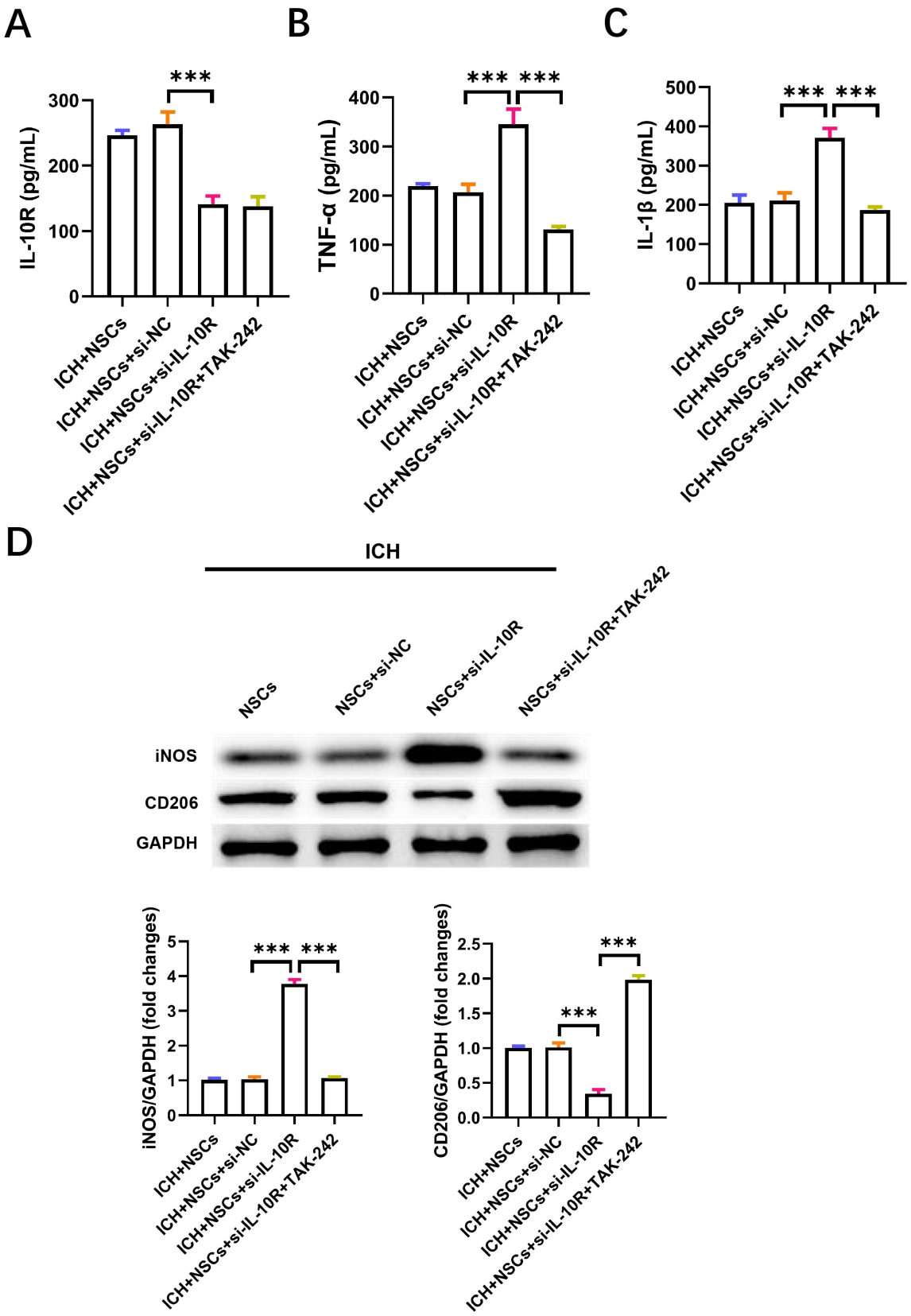

Fig. 5. IL-10R inhibition triggers inflammation and reduces M2 polarization in microglial cells by activating TLR4/NF-κB pathway. (A–C) The expression levels of IL-10R (A), TNF-α (B), and IL-1β (C) in the brain tissue of ICH mice were assessed using ELISA. (D) The expression levels of iNOS and CD206 were evaluated utilizing western blot. N = 10. ***$p < 0.001$.

juries induced by neuroinflammation, by potentially mitigating the inflammatory response and promoting the repair and functional recovery of neural tissue through IL-10R.

Conclusion

Our study underscores IL-10R's pivotal role in modulating microglial M2 polarization and reducing neuroinflammation post-brain injury. It also elucidates the regulatory mechanism of the TLR4/NF-κB pathway. These findings not only provide new insights into the regulation of inflammation following brain injury but also offer a scientific basis for the development of new treatment strategies.

Availability of Data and Materials

Data are available from the corresponding authors upon reasonable request.

Author Contributions

BZ and CY designed the experiments. BZ, TW and YQ carried out animal, cell experiments and sample analysis; LT and CY analyzed data; BZ wrote the manuscript, and CY revised the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All experimental procedures adhered to the ethical standards for the care and use of animals and received approval from the Ethics Committee of Yantai Yuhuangding Hospital (approval No. 2024-108).

Acknowledgment

Not applicable.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Dinet V, Petry KG, Badaut J. Brain-Immune Interactions and Neuroinflammation After Traumatic Brain Injury. Frontiers in Neuroscience. 2019; 13: 1178.

[2] Morganti-Kossmann MC, Semple BD, Hellewell SC, Bye N, Ziebell JM. The complexity of neuroinflammation consequent to traumatic brain injury: from research evidence to potential treatments. Acta Neuropathologica. 2019; 137: 731–755.

[3] van Vliet EA, Ndode-Ekane XE, Lehto LJ, Gorter JA, Andrade P, Aronica E, *et al*. Long-lasting blood-brain barrier dysfunction and neuroinflammation after traumatic brain injury. Neurobiology of Disease. 2020; 145: 105080.

[4] Olakowska E, Rodak P, Pacwa A, Machowicz J, Machna B, Lewin KJ, *et al*. Surgical Menopause Impairs Retinal Conductivity and Worsens Prognosis in an Acute Model of Rat Optic Neuropathy. Cells. 2022; 11: 3062.

[5] Han B, Jiang W, Cui P, Zheng K, Dang C, Wang J, *et al*. Microglial PGC-1α protects against ischemic brain injury by suppressing neuroinflammation. Genome Medicine. 2021; 13: 47.

[6] Chong SA, Balosso S, Vandenplas C, Szczesny G, Hanon E, Claes K, *et al*. Intrinsic Inflammation Is a Potential Anti-Epileptogenic Target in the Organotypic Hippocampal Slice Model. Neurotherapeutics. 2018; 15: 470–488.

[7] Wabnitz GH, Kirchgessner H, Jahraus B, Umansky L, Shenolikar S, Samstag Y. Protein Phosphatase 1α and Cofilin Regulate Nuclear Translocation of NF-κB and Promote Expression of the Anti-Inflammatory Cytokine Interleukin-10 by T Cells. Molecular and Cellular Biology. 2018; 38: e00041–18.

[8] Shields PG, Song MA, Freudenheim JL, Brasky TM, McElroy JP, Reisinger SA, *et al*. Lipid laden macrophages and electronic cigarettes in healthy adults. EBioMedicine. 2020; 60: 102982.

[9] Tao W, Zhang G, Liu C, Jin L, Li X, Yang S. Low-dose LPS alleviates early brain injury after SAH by modulating microglial M1/M2 polarization via USP19/FOXO1/IL-10/IL-10R1 signaling. Redox Biology. 2023; 66: 102863.

[10] Chen M, Chen Q, Tao T. Tanshinone IIA Promotes M2 Microglia by ERβ/IL-10 Pathway and Attenuates Neuronal Loss in Mouse TBI Model. Neuropsychiatric Disease and Treatment. 2020; 16: 3239–3250.

[11] Han R, Lan X, Han Z, Ren H, Aafreen S, Wang W, *et al*. Improving outcomes in intracerebral hemorrhage through microglia/macrophage-targeted IL-10 delivery with phosphatidylserine liposomes. Biomaterials. 2023; 301: 122277.

[12] Wen RX, Shen H, Huang SX, Wang LP, Li ZW, Peng P, *et al*. P2Y6 receptor inhibition aggravates ischemic brain injury by reducing microglial phagocytosis. CNS Neuroscience & Therapeutics. 2020; 26: 416–429.

[13] Wolfson B, Lo PK, Yao Y, Li L, Wang H, Zhou Q. Impact of miR-140 Deficiency on Non-Alcoholic Fatty Liver Disease. Molecular Nutrition & Food Research. 2018; 62: e1800189.

[14] Ran Y, Qie S, Gao F, Ding Z, Yang S, Tian G, *et al*. Baicalein ameliorates ischemic brain damage through suppressing proinflammatory microglia polarization via inhibiting the TLR4/NF-κB and STAT1 pathway. Brain Research. 2021; 1770: 147626.

[15] Wang P, Li C, Liao G, Huang Y, Lv X, Liu X, *et al*. Vanillin Attenuates Proinflammatory Factors in a tMCAO Mouse Model via Inhibition of TLR4/NF-kB Signaling Pathway. Neuroscience. 2022; 491: 65–74.

[16] Hu J, Wang X, Chen X, Fang Y, Chen K, Peng W, *et al*. Hydroxychloroquine attenuates neuroinflammation following traumatic brain injury by regulating the TLR4/NF-κB signaling pathway. Journal of Neuroinflammation. 2022; 19: 71.

[17] Wang H, Song X, Li M, Wang X, Tao Y, Xiya X, *et al*. The role of TLR4/NF-κB signaling pathway in activated microglia of rats with chronic high intraocular pressure and vitro scratch injury-induced microglia. International Immunopharmacology. 2020; 83: 106395.

[18] Song Y, Liu X, Yuan J, Sha Z, Jiang W, Liu M, *et al*. Atorvastatin combined with low-dose dexamethasone improves the neuroinflammation and survival in mice with intracerebral hemorrhage. Frontiers in Neuroscience. 2022; 16: 967297.

[19] Zhang Y, Chen Y, Wu J, Manaenko A, Yang P, Tang J, *et al*. Activation of Dopamine D2 Receptor Suppresses Neuroinflammation Through αB-Crystalline by Inhibition of NF-κB Nuclear

Journal of
BIOLOGICAL REGULATORS
and Homeostatic Agents

Translocation in Experimental ICH Mice Model. Stroke. 2015; 46: 2637–2646.

[20] Choi KH, Park MS, Kim HS, Kim KT, Kim HS, Kim JT, *et al*. Alpha-lipoic acid treatment is neurorestorative and promotes functional recovery after stroke in rats. Molecular Brain. 2015; 8: 9.

[21] Sun Z, Wu K, Gu L, Huang L, Zhuge Q, Yang S, *et al*. IGF-1R stimulation alters microglial polarization via TLR4/NF-κB pathway after cerebral hemorrhage in mice. Brain Research Bulletin. 2020; 164: 221–234.

[22] Yang T, Zang S, Wang Y, Zhu Y, Jiang L, Chen X, *et al*. Methamphetamine induced neuroinflammation in mouse brain and microglial cell line BV2: Roles of the TLR4/TRIF/Peli1 signaling axis. Toxicology Letters. 2020; 333: 150–158.

[23] Karimy JK, Reeves BC, Kahle KT. Targeting TLR4-dependent inflammation in post-hemorrhagic brain injury. Expert Opinion on Therapeutic Targets. 2020; 24: 525–533.

[24] Gong P, Jia HY, Li R, Ma Z, Si M, Qian C, *et al*. Downregulation of Nogo-B ameliorates cerebral ischemia/reperfusion injury in mice through regulating microglia polarization via TLR4/NF-kappaB pathway. Neurochemistry International. 2023; 167: 105553.

[25] Liu L, Li X, Wang Y, Cao F, Zhang S, Zhan Z, *et al*. Impacts of mild hypothermia on LPS-mediated TLR4/NF-κB signaling pathway in microglia. Journal of Biosciences and Medicines. 2019; 7: 86–97.

[26] Liu Y, Zhao C, Meng J, Li N, Xu Z, Liu X, *et al*. Galectin-3 regulates microglial activation and promotes inflammation through TLR4/MyD88/NF-kB in experimental autoimmune uveitis. Clinical Immunology (Orlando, Fla.). 2022; 236: 108939.

[27] Vargas AM, Rivera-Rodriguez DE, Martinez LR. Methamphetamine alters the TLR4 signaling pathway, NF-κB activation, and pro-inflammatory cytokine production in LPS-challenged NR-9460 microglia-like cells. Molecular Immunology. 2020; 121: 159–166.

[28] Lv J, Zhu J, Wang P, Liu T, Yuan J, Yin H, *et al*. Artemisinin exerts a protective effect in the MPTP mouse model of Parkinson's disease by inhibiting microglial activation via the TLR4/Myd88/NF-KB pathway. CNS Neuroscience & Therapeutics. 2023; 29: 1012–1023.

[29] Wang L, Geng G, Zhu T, Chen W, Li X, Gu J, *et al*. Progress in Research on TLR4-Mediated Inflammatory Response Mechanisms in Brain Injury after Subarachnoid Hemorrhage. Cells. 2022; 11: 3781.

[30] Shi J, Shi S, Xie W, Zhao M, Li Y, Zhang J, *et al*. IL-10 alleviates lipopolysaccharide-induced skin scarring via IL-10R/STAT3 axis regulating TLR4/NF-κB pathway in dermal fibroblasts. Journal of Cellular and Molecular Medicine. 2021; 25: 1554–1567.

[31] Miki S, Suzuki JI, Takashima M, Ishida M, Kokubo H, Yoshizumi M. S-1-Propenylcysteine promotes IL-10-induced M2c macrophage polarization through prolonged activation of IL-10R/STAT3 signaling. Scientific Reports. 2021; 11: 22469.

[32] Cianciulli A, Dragone T, Calvello R, Porro C, Trotta T, Lofrumento DD, *et al*. IL-10 plays a pivotal role in anti-inflammatory effects of resveratrol in activated microglia cells. International Immunopharmacology. 2015; 24: 369–376.

[33] Glocker EO, Kotlarz D, Klein C, Shah N, Grimbacher B. IL-10 and IL-10 receptor defects in humans. Annals of the New York Academy of Sciences. 2011; 1246: 102–107.

[34] Ding Y, Qin L, Zamarin D, Kotenko SV, Pestka S, Moore KW, *et al*. Differential IL-10R1 expression plays a critical role in IL-10-mediated immune regulation. Journal of Immunology (Baltimore, Md.: 1950). 2001; 167: 6884–6892.

[35] Shouval DS, Biswas A, Goettel JA, McCann K, Conaway E, Redhu NS, *et al*. Interleukin-10 receptor signaling in innate immune cells regulates mucosal immune tolerance and anti-inflammatory macrophage function. Immunity. 2014; 40: 706–719.