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Recombinant Slit2 Attenuates Neuroinflammation After Surgical Brain Injury by Inhibiting Peripheral Immune Cell Infiltration via Robo1-srGAP1 Pathway in a Rat Model

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Abstract

Background and Purpose—Peripheral immune cell infiltration to the brain tissue at the perisurgical site can promote neuroinflammation after surgical brain injury (SBI). Slit2, an extracellular matrix protein, has been reported to reduce leukocyte migration. This study evaluated the effect of recombinant Slit2 and the role of its receptor roundabout1 (Robo1) and its downstream mediator Slit-Robo GTPase activating protein 1 (srGAP1)-Cdc42 on peripheral immune cell infiltration after SBI in a rat model.

Methods—One hundred and fifty-three adult male Sprague-Dawley rats (280–350 g) were used. Partial resection of right frontal lobe was performed to induce SBI. Slit2 siRNA was administered by intracerebroventricular injection 24 hours before SBI. Recombinant Slit2 was injected intraperitoneally 1 hour before SBI. Recombinant Robo1 used as a decoy receptor was co-administered with recombinant Slit2. srGAP1 siRNA was administered by intracerebroventricular injection 24 hours before SBI. Post-assessments included brain water content measurement, neurological tests, ELISA, western blot, immunohistochemistry, and Cdc42 activity assay.

Results—Endogenous Slit2 was increased after SBI. Robo1 was expressed by peripheral immune cells. Endogenous Slit2 knockdown worsened brain edema after SBI. Recombinant Slit2 administration reduced brain edema, neurological deficits, and pro-inflammatory cytokines after SBI. Recombinant Slit2 reduced peripheral immune cell markers cluster of differentiation 45 (CD45) and myeloperoxidase (MPO), as well as Cdc42 activity in the perisurgical brain tissue which was reversed by recombinant Robo1 co-administration and srGAP1 siRNA.

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Conflicts of Interest: None.

Conclusions—Recombinant Slit2 improved outcomes by reducing neuroinflammation after SBI, possibly by decreasing peripheral immune cell infiltration to the perisurgical site through Robo1-srGAP1 mediated inhibition of Cdc42 activity. These results suggest that Slit2 may be beneficial to reduce SBI-induced neuroinflammation.

Keywords

Surgical brain injury; Neuroinflammation; Brain edema; Peripheral Immune Cell Infiltration; Slit2; Robo1; srGAP1; Cdc42

INTRODUCTION

Surgical brain injury (SBI) is the inadvertent injury to brain tissue at the perisurgical site which occurs due to neurosurgical maneuvers such as incision, retraction, and electrocoagulation that can aggravate post-operative brain edema and neurological deficits (Jadhav et al., 2007a, Huang et al., 2014). Major complications are encountered in 13–27% patients after intracranial surgeries (Bruder, 2002). Measures to reduce neurosurgical complications are limited (Jadhav et al., 2007a). Furthermore, routine therapy used against SBI including hyperosmolar agents and steroids can have unwanted adverse effects (Li et al., 2014, Xu et al., 2014). Therapeutic strategies that augment endogenous protective mechanisms would be a safe approach to reduce post-operative complications in neurosurgical patients.

Neuroinflammation is a major pathophysiological consequence after SBI (Yamaguchi et al., 2007, Hyong et al., 2008) which contributes to brain edema that can worsen post-operative neurological function (Ayer et al., 2012). Following brain injury, resident immune cells get activated and release cytokines and chemokines that promote migration of peripheral immune cells to injury site in the brain (Wang and Dore, 2007, Rhodes, 2011, Ma et al., 2014). Immune cell infiltration and inflammatory mediators were increased in adjacent brain tissue at the perisurgical site following experimental resection (Jadhav et al., 2007a, Hyong et al., 2008). Infiltrated immune cells release inflammatory mediators that further promote neuroinflammation (Petty and Lo, 2002, Yilmaz et al., 2006, Lo, 2009).

Slit2 is a secreted extracellular matrix protein (Ballard and Hinck, 2012) expressed endogenously in the brain by neurons and astrocytes (Hagino et al., 2003, Prasad et al., 2007). Slit2 was recently identified as an inhibitor of leukocyte chemotaxis (Wu et al., 2001, Ballard and Hinck, 2012) and was shown to be protective in experimental models of systemic inflammation (Kanellis et al., 2004, Tole et al., 2009, London et al., 2010). Slit2 reduced migration of leukocytes to the cortical venules after global cerebral ischemia in mice (Altay et al., 2007). The anti-migratory function of Slit2 is mediated by binding to its receptor roundabout1 (Robo1) (Wong et al., 2002, Ballard and Hinck, 2012). Furthermore, it has been established that Slit-Robo GTPase Activating Protein 1 (srGAP1) a downstream effector of the receptor Robo1 (Wong et al., 2002) can inhibit Cdc42, which is a critical mediator for cell migration (Bishop and Hall, 2000, Yiin et al., 2009). Robo1 has been shown to be expressed on the surface of peripheral immune cells (Wu et al., 2001, Guan and Rao, 2003, Prasad et al., 2007), which we propose induces the signal transduction pathway

that mediates the anti-migratory effect of Slit2 and thereby reduces brain infiltration of peripheral immune cells.

The role of Slit2 after SBI is unknown, and the mechanism by which Slit2 elicits neuroprotection has not been evaluated. The objective of this study was to evaluate the effects of recombinant Slit2 as a novel therapeutic strategy to reduce neuroinflammation after SBI in a rat model.

MATERIALS and METHODS

Animals

All procedures were approved by the Institutional Animal Care and Use Committee at Loma Linda University and complied with NIH Guide for the Care and Use of Laboratory Animals. Adult male Sprague Dawley rats (280–350 g) were housed in humidity and temperature controlled environment with 12 hour light/dark cycle. One hundred and fifty-three rats were subjected to either Sham surgery (n=26) or SBI surgery (n=127) by inducing partial resection of the right frontal lobe.

Experimental Design

Experiment 1—The time course expression and localization of endogenous Slit2 and Robo1 was characterized at 24 hours, 72 hours, and day 7 after SBI. Rats (n=24) were divided 4 groups: Sham, SBI 24 hours, SBI 72 hours, and SBI day 7. Brain samples from the residual right frontal lobe were collected for enzyme linked immunosorbent assay (ELISA), western blot and for immunohistochemistry.

Experiment 2—The role of endogenous Slit2 after SBI was evaluated. Rats (n=37) were divided into 4 groups: Sham, SBI, SBI+Slit2 siRNA, SBI+Scramble siRNA. Endogenous Slit2 knockdown was performed by intracerebroventricular (ICV) injection of Slit2 siRNA (Life Technologies, Grand Island, NY, USA) 24 hours before SBI. Brain water content, neurological function and western blot was evaluated at 72 hours after surgery.

Experiment 3—The effect of exogenous recombinant Slit2 pretreatment for SBI was evaluated. Three doses of recombinant Slit2 (1 μg/Kg, 3 μg/Kg, 10 μg/Kg) (R and D Systems, Minneapolis, MN, USA) was tested. Rats (n=74) were divided into 5 groups: Sham, SBI+Vehicle, SBI+Slit2 (1 μg/Kg), SBI+Slit2 (3 μg/Kg), SBI+Slit2 (10 μg/Kg). The dose of recombinant Slit2 (3 μg/Kg) was chosen based on previous publication (Altay et al., 2007). Furthermore, since a dose response effect of Slit2 was previously reported in vitro studies (Prasad et al., 2007), we examined the effects of two additional doses to establish the optimal dose for SBI. Recombinant Slit2 or vehicle normal saline was injected intraperitoneally 1 hour before SBI. Brain water content and neurological function was evaluated at 24 and 72 hours after surgery. Brain samples from the residual right frontal lobe at the perisurgical site were collected at 24 hours for western blot and immunohistochemistry.

Experiment 4—The role of Robo1 and srGAP1 in recombinant Slit2 mediated protection after SBI was investigated. Rats were divided into 6 groups: Sham, SBI+Vehicle, SBI+Slit2

(10 μg/Kg), SBI+Slit2(10 μg/Kg)+Robo1(3 μg/Kg), SBI+Slit2(10 μg/Kg)+srGAP1 siRNA, SBI+Slit2(10 μg/Kg)+scramble siRNA. Rats (n=18) were added for the last 3 groups. Recombinant Robo1 (3 μg/Kg) (R and D Systems, Minneapolis, MN, USA) was co-administered with recombinant Slit2 by intraperitoneal injection 1 hour before SBI. srGAP1 or scramble siRNA (Life Technologies, Grand Island, NY, USA) was given by ICV injection 24 hours before SBI. Neurological function was evaluated at 24 hours after surgery and brain samples were collected at 24 hours for western blot and Cdc42 activity assay.

Surgical Brain Injury Model

Rats were subjected to surgical brain injury as previously described (Jadhav et al., 2007a, Yamaguchi et al., 2007). Briefly, anesthesia was induced by 4% isoflurane in an induction chamber and maintained at 2.5% using a nasal mask. The skin was incised and periosteum was reflected to expose the bregma and frontal bone. A square craniotomy 5×5 mm was made on the right frontal bone with left lower corner towards bregma by using a micro drill. The dura was incised and a partial resection of the right frontal lobe was performed with margins of resection at 2 mm lateral to sagittal suture and 1 mm proximal to coronal suture, with the depth extending to base of skull. Normal saline irrigation and intraoperative packing was done to ensure complete hemostasis after which skin incision was sutured. Sham animals were subjected to right frontal craniotomy but without dural incision or frontal lobe resection. Buprenorphine 0.03 mg/Kg was given by subcutaneous injection for post-operative analgesia. Post-operatively rats were closely observed for complete recovery from anesthesia.

Intracerebroventricular Injection

Rats were anesthetized with isoflurane and placed prone on a stereotactic frame. A 10 μL Hamilton syringe (Hamilton Co, Reno, NV, USA) was inserted through a burr hole on the skull into right lateral ventricle using the following coordinates relative to bregma: 1.0 mm lateral, 1.5 mm posterior and 3.2 mm below the horizontal plane of bregma as previously described (Suzuki et al., 2010, Chen et al., 2013). Slit2 siRNA 500 pmol or srGAP1 siRNA 500 pmol (Life Technologies, Grand Island, NY, USA) each in 2 μL sterile saline was injected at a rate 0.5 μL/min as previously described (Suzuki et al., 2010, Chen et al., 2013). The same volume of scramble siRNA (Life Technologies, Grand Island, NY, USA) was injected as a negative control. The needle was left in situ for additional 10 mins and then slowly withdrawn over 5 mins to prevent leakage. The burr hole was sealed with bone wax and skin incision was sutured. Rats were returned to recovery cages and closely observed for complete recovery from anesthesia.

Brain Water Content Measurement

Brain edema was evaluated at 24 and 72 hours after surgery by wet weight/dry weight method as previously described (Jadhav et al., 2007b, Yamaguchi et al., 2007). The brains were quickly removed and dissected into six parts: right frontal, left frontal, right parietal, left parietal, cerebellum and brainstem. The samples were weighed immediately (wet weight) and then placed in an oven at 100°C for 48 hours and weighed again (dry weight). The percent of brain water content in each region was calculated using the formula [(wet weight – dry weight)÷wet weight]×100 (Yamaguchi et al., 2007).

Assessment of Neurological Function

Neurobehavioral deficits were evaluated by an examiner blinded to the groups by using modified Garcia test and beam balance test at 24 and 72 hours after SBI as previously described (Garcia et al., 1995, Yamaguchi et al., 2007). Briefly, Garcia test assessed sensorimotor deficits and included seven tests: spontaneous activity, body proprioception, vibrissae touch, limb symmetry, lateral turning, forepaw outstretching, and climbing. Each test was scored from 0 to 3 with a maximum score of 21. In beam balance test, the rats were allowed to walk on a 90 cm × 2.25 cm beam for 1 min during which distance traveled and time taken to travel the distance was recorded. The score ranged from 0 to 5. Higher scores indicated better function for both the tests.

Enzyme Linked Immunosorbent Assay (ELISA)

Slit2 concentration in brain samples were measured using a Slit2-HRP conjugated Elisa kit (MyBiosource, San Diego, CA, USA) as previously described (Doyle et al., 2008, Hu et al., 2014). Sample supernatants from the residual right frontal lobe were incubated in a microtiter plate pre-coated with biotin conjugated antibody specific for rat Slit2. Next, the samples were incubated with avidin conjugated HRP after which TMB substrate was added. The absorbance was measured spectrophotometrically at wavelength of 450 nm using a microplate reader (Biorad, Irvine, CA, USA).

Western Blotting

Western blot was performed as previously described (Hasegawa et al., 2011, Chen et al., 2013). Briefly, brain samples were collected after rats were transcardially perfused with phosphate buffered saline (PBS). Samples were homogenized in Ripa lysis buffer (Santa Cruz Biotechnology, Dallas, TX, USA) for protein extraction (Hasegawa et al., 2011) Supernatants from the residual right frontal lobe samples were collected after centrifugation at 14,000 g at 4°C for 30 mins and protein concentration was determined using a detergent compatibility assay (Biorad, Irvine, CA, USA). Equal amounts of protein (50 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes after which the membranes were incubated overnight with the respective primary antibodies: anti-Slit2 (1:200), anti-Robo1 (1:200), anti-myeloperoxidase (MPO) (1:500) (all from Santa Cruz Biotechnology, Dallas, TX, USA), anti-cluster of differentiation 45 (CD45) (1:500), anti-IL-1β (1:1000) and anti-INF-γ (1:1000) (all from Abcam, Cambridge, MA, USA). The same membranes were probed with anti-β Actin (1:2000) (Santa Cruz Biotechnology, Dallas, TX, USA) as loading controls. The membranes were incubated with appropriate secondary antibodies (1:4000) (Santa Cruz Biotechnology, Dallas, TX, USA) for 1 hour at room temperature. Bands were visualized using ECL Plus Chemiluminescence kit (Amersham Biosciences, Arlington Heights, IL, USA) and quantified using the software Image J (National Institutes of Health, Bethesda, MD, USA). Results are expressed as relative density to β-actin and then normalized to average value of the sham group as previously described (Hasegawa et al., 2011).

Immunohistochemistry

Briefly, after PBS perfusion and post-fixation in formalin, brain samples were sectioned into 10-μm-thick slices using a cryostat (CM3050S; Leica Microsystems, Bannockburn, IL, USA). Immunofluorescence staining was performed as previously described (Hasegawa et al., 2011, Altay et al., 2012). The sections were incubated overnight at 4°C with the following primary antibodies: anti-Slit2 (1:200), anti-Robo1 (1:200), anti-MPO (1:200) (all from Santa Cruz Biotechnology, Dallas, TX, USA), anti-neuronal nuclei (NeuN) (1:500), anti-glial fibrillary acidic protein (GFAP) (1:100) and anti-CD45 (1:150) (all from Abcam, Cambridge, MA, USA). Sections were then incubated with FITC- and Texas Red-conjugated appropriate secondary antibodies (1:100) (Jackson Immuno Research, West Grove, PA, USA) for 2 hours at room temperature and visualized with a fluorescence microscope (Olympus BX51).

Cdc42 Activity Assay

A pull down assay was performed using Cdc42 activity assay kit (Cell Biolabs, San Diego, CA, USA) as previously described (Yiin et al., 2009). Briefly, samples were mixed with the p21-activated protein kinase (PAK1)-p21-binding domain (PBD) agarose beads and incubated at 4°C for 1 hour. The beads were then re-suspended in sample buffer and separated by 10% polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. The membrane was probed with anti-Cdc42 specific antibody provided in the kit to detect the GTP-bound Cdc42.

Statistical Analysis

Statistical analysis was performed using the Sigma Plot 10.0 and Sigma Stat version 3.5 (Systat Software, San Jose, CA, USA). Data were presented as mean ± SEM. Statistical differences between groups were analyzed using one-way ANOVA for multiple comparisons followed by Tukey or Student-Newman-Kuels post hoc tests. P values less than 0.05 was considered statistically significant.

RESULTS

All sham-operated rats survived. Nine rats died of the 127 SBI rats. The overall mortality in the SBI group was 7.09%.

Temporal expression and localization of endogenous Slit2 after SBI

The expression of Slit2 at the perisurgical site in the brain was measured at 24 hours, 72 hours and 7 days after SBI. The level of endogenous Slit2 was increased at 24 hours following SBI and reached a peak at 72 hours compared to sham ($p<0.05$ compared to sham, Fig. 1A). The increase in the level of Slit2 was sustained to 7 days after SBI ($p<0.05$ compared to sham). Double immunofluorescence staining showed that Slit2 was expressed by the neurons and astrocytes (Fig. 1C).

Temporal expression and localization of Robo1 after SBI

The expression of Robo1 in the brain was measured at 24 hours, 72 hours and 7 days after SBI. Robo1 expression increased at all-time points with a peak at 24 hours after SBI ($p<0.05$ compared to sham, Fig. 2A). Double immunofluorescence staining of brain samples showed that Robo1 co-localized with the marker for peripheral immune cells, cluster of differentiation 45 (CD45) and with the marker for neutrophils, myeloperoxidase (MPO) at the perisurgical site after SBI (Fig. 2B).

Endogenous Slit2 knockdown worsened outcomes after SBI

Western blot showed that Slit2 siRNA effectively suppressed the expression of Slit2 ($p<0.05$ compared to SBI and scramble siRNA groups, Fig. 3A). SBI rats that received Slit2 siRNA had significantly higher brain water content in the residual right frontal lobe ($p<0.05$ compared to SBI and scramble siRNA groups, Fig. 3B). Garcia test showed worsened neurological function in the Slit2 siRNA group compared to scramble siRNA ($p<0.05$ compared to scramble siRNA, Fig. 3C), but there was no significant difference between the groups in beam balance test (Fig. 3D).

Recombinant Slit2 administration reduced brain edema and improved neurological function 24 and 72 hours after SBI

Brain water content was significantly increased at the perisurgical site in the residual right frontal lobe at 24 and 72 hours after SBI ($p<0.05$ compared to sham, Fig. 4A and 4B, respectively). Recombinant Slit2 3 μg/Kg and 10 μg/Kg significantly reduced brain water content at both time points ($p<0.05$ compared to vehicle). Neurological function evaluated by Garcia test was significantly worse at 24 and 72 hours after SBI ($p<0.05$ compared to sham, Fig. 4C and 4D, respectively). All three doses of recombinant Slit2 significantly improved Garcia scores at 24 hours ($p<0.05$ compared to vehicle). However, only recombinant Slit2 10 μg/Kg improved performance in the Garcia test at 72 hours ($p<0.05$ compared to vehicle). Beam balance test showed significantly worse neurological function after SBI at 24 and 72 hours ($p<0.05$ compared to sham, Fig. 4E and 4F, respectively). Slit2 10 μg/Kg showed a tendency to improve beam balance scores at 72 hours but was not significant.

Recombinant Slit2 reduced neuroinflammation and peripheral immune cell infiltration at the perisurgical site 24 hours after SBI

The expression of pro-inflammatory cytokines IL-1β and INF-γ in the residual right frontal lobe was quantified by western blot, which showed significant increase after SBI compared to sham ($P<0.05$ compared to sham, Fig. 5A and 5B, respectively). Recombinant Slit2 10 μg/Kg significantly reduced the expression of both IL-1β and INF-γ ($p<0.05$ compared to vehicle). Immunofluorescence staining showed increased CD45 and MPO positive cells at the perisurgical site after SBI compared to sham (Fig. 5C and 5D, respectively). Recombinant Slit2 10 μg/Kg treated group had fewer cells positively stained for CD45 and MPO compared to vehicle group.

Recombinant Slit2 reduced peripheral immune cell infiltration to the perisurgical site dependent on Robo1 receptor and its downstream mediator srGAP1

Western blot showed that CD45 and MPO expression was significantly increased after SBI ($p<0.05$ compared to sham, Fig. 6A and 6B, respectively). Recombinant Slit2 10 μg/Kg significantly reduced the expression of both markers ($p<0.05$ compared to vehicle). Recombinant Robo1 administration abolished the effects of recombinant Slit2 by increasing expression of CD45 and MPO ($p<0.05$ compared to Slit2) and worsened neurological deficits which was evaluated using Garcia test ($p<0.05$ compared to Slit2, Fig. 6C). Likewise, srGAP1 siRNA abolished the effects of recombinant Slit2 by increasing the expression of CD45 and MPO ($p<0.05$ compared to Slit2, Fig 7A and 7B, respectively) and worsened neurological deficits which was evaluated using Garcia test ($p<0.05$ compared to Slit2, Fig 7C).

Recombinant Slit2 reduced Cdc42 activity after SBI by Robo1-srGAP1 pathway

Cdc42 activity was significantly increased 24 hours after SBI ($p<0.05$ compared to sham, Fig. 8A), which was reduced with recombinant Slit2 10 μg/Kg administration ($p<0.05$ compared to vehicle). Recombinant Robo1 3 μg/Kg partially reversed the reduction in Cdc42 activity by recombinant Slit2 ($p=0.097$ compared to SBI+Slit2 10 μg/Kg, Fig. 8A), whereas srGAP1 siRNA reversed the reduction in Cdc42 activity by recombinant Slit2 ($p<0.05$ compared to Slit2, Fig. 8B).

DISCUSSION

In this study we focused on the anti-inflammatory function of recombinant Slit2 after SBI, particularly in terms of reducing peripheral immune cell migration to the brain after injury. We observed that endogenous Slit2 was upregulated after SBI, and knockdown of endogenous Slit2 worsened SBI induced brain edema. Robo1, the receptor for Slit2 was expressed by brain infiltrated peripheral immune cells. Administration of recombinant Slit2 prior to inducing SBI reduced brain edema, neurological deficits, neuroinflammation, and peripheral immune cell infiltration at the perisurgical site. Recombinant Slit2 reduced immune cell infiltration and Cdc42 activity after SBI was reversed by recombinant Robo1 co-administration as well as by inhibition of the downstream effector srGAP1. These results suggest that Slit2 may have protective function after SBI, and that recombinant Slit2 may be a beneficial candidate to reduce neuroinflammation.

Among members of the Slit family, Slit2 has important function in the brain especially in regulating migration of neurons and axons in the CNS during development (Marillat et al., 2002). However, its function in the adult brain is still being explored (Hagino et al., 2003). In addition, there are limited studies on the effect of Slit2 on neuroinflammation after brain injuries (Altay et al., 2007). Slit2 was detected at the peri-lesional site 2 days after traumatic brain injury and peaked at 7 days followed by a subsequent decline by day 14 after the injury in mice (Hagino et al., 2003). Consistent with these studies, our results showed that endogenous Slit2 increased at 24 hours, 72 hours and 7 days after SBI with a peak at 72 hours after injury. Slit2 was localized in neurons and astrocytes, which we speculate is the source for increase in endogenous Slit2 after SBI. This observation complements previous

studies that have reported Slit2 was expressed in the neurons and astrocytes (Hagino et al., 2003, Mertsch et al., 2008).

Roundabout 1 (Robo1), the receptor for Slit2 has been shown to mediate the anti-migratory function of Slit2 (Prasad et al., 2007, Yiin et al., 2009). Studies show that Slit2 exerts its repellent function on various cell types by binding to its receptor Robo1 (Guan and Rao, 2003). Robo1 is highly expressed by neutrophils (Tole et al., 2009) and lymphocytes (Wu et al., 2001) and moderately expressed by monocytes (Prasad et al., 2007). We observed that Robo1 expression increased at the perisurgical site after SBI and peaked at 24 hours and started decreasing 72 hours after injury. Robo1 was co-localized with the brain infiltrated peripheral immune cells.

To elucidate the role of endogenous Slit2 after SBI, we performed siRNA knockdown of endogenous Slit2 and evaluated brain edema and neurological function at 72 hours after SBI since endogenous Slit2 levels peaked at this time point. Slit2 knockdown worsened brain edema after SBI. However, there was no significant difference in neurological function after Slit2 siRNA knockdown compared to the SBI group. Given that Garcia test and beam balance test examine the overall gross sensorimotor deficits, it is possible that these tests were not sensitive enough to detect fine sensorimotor deficits which may result from the perisurgical site edema (Krafft et al., 2014, McBride et al., 2015). Additionally, these tests include parameters that evaluate function of the bilateral side and it is likely that the rats compensated for deficits using the uninjured side which may have reduced sensitivity of these tests (Krafft et al., 2014). Nevertheless, our observations suggest that Slit2 is upregulated in response to brain injury possibly as an endogenous protective mechanism after SBI. Endogenous Slit2 may be protective after SBI in several ways. First, Slit2 is a secreted protein (Marillat et al., 2002) that may be secreted into the CSF and thence into blood stream where it may directly bind to Robo1 receptors on peripheral immune cells and thereby eventually slow down migration of these cells to the brain parenchyma. Interestingly, we observed that Robo1 expression started decreasing as the levels of endogenous Slit2 temporally increased after SBI. We speculate that Robo1 expression possibly decreased at 72 hours after SBI as a result of reduced immune cell infiltration by endogenous Slit2. Endogenous Slit2 may be elevated as a potential compensatory mechanism after injury. Second, it is possible that endogenous Slit2 may be increased to compensate for the endothelial barrier breakdown after injury. Slit2 was shown to reduce endothelial barrier permeability through binding to the endothelial specific receptor Robo4 (Jones et al., 2008, Jones et al., 2009). We speculate that Slit2 may be a compensatory mechanism to prevent further breakdown of the blood brain barrier, which could also reduce peripheral immune cell infiltration to the brain parenchyma after SBI. This could likely explain why endogenous Slit2 knockdown worsened brain edema. Lastly, Slit2 may exert direct anti-inflammatory effects by interacting with the Robo4 receptor. Previous study showed that Slit2 reduced pro-inflammatory cytokine and chemokine expression by directly interacting with the endothelial specific Robo4 receptor (Zhao et al., 2014). Although we did not investigate the protective mechanism of endogenous Slit2 after SBI, our results suggest that Slit2 has a protective function after SBI. Further investigation is needed to explore protective mechanism of endogenous Slit2 and the role of endothelial Robo4 receptor in Slit2 mediated protection.

Given that Slit2 has a protective function after SBI, next we examined whether exogenous recombinant Slit2 administration would have beneficial effects after SBI. Recombinant Slit2 was shown to protect against various systemic inflammatory conditions including renal, peritoneal and lung inflammation in rodents (Kanellis et al., 2004, Tole et al., 2009, Ye et al., 2010). Since neurosurgical procedures are planned events, it allows for pretreatment before injury is inflicted. Three doses of recombinant Slit2 were tested by evaluating outcomes at 24 and 72 hours after injury to establish the most effective dose to reduce SBI. Recombinant Slit2 10 μg/Kg reduced brain edema and improved performance in the Garcia test at both 24 and 72 hours after SBI. We therefore used this dose to complete subsequent experiments.

Inflammatory markers were shown to be elevated at the perisurgical site 24 and 72 hours after SBI (Yamaguchi et al., 2007, Hyong et al., 2008). Resident immune cells get activated due to the primary injury and release chemokines and pro-inflammatory cytokines that induce endothelial cells to upregulate adhesion molecules which promotes peripheral leukocyte trafficking to the injury site (Hickey, 1999, Ransohoff et al., 2003). A number of studies show that leukocytes accumulate in the CNS in response to an injury (Ransohoff et al., 2003, Wang and Dore, 2007, Ma et al., 2014). Infiltrated immune cells and activated resident microglia release inflammatory mediators such as INF-γ and IL-1β that increases tissue injury and potentiates neuroinflammation (Yilmaz et al., 2006). Slit2 has been shown to have anti-migratory function including inhibition of leukocyte migration (Wu et al., 2001, Tole et al., 2009, London et al., 2010). We observed that recombinant Slit2 reduced expression of proinflammatory cytokines and peripheral immune cell markers at the perisurgical site. These results suggest that recombinant Slit2 mediates protection at least in part through reduction of neuroinflammation by decreasing brain infiltration of peripheral immune cells.

To elucidate the anti-migratory mechanism of recombinant Slit2, we explored the role of its receptor Robo1 (Guan and Rao, 2003) which we observed was expressed by the peripheral immune cells. The second LRR domain of Slit2 can directly bind with the first extracellular immunogloblulin (Ig) domain of Robo1 (Wong et al., 2002, Guan and Rao, 2003, Ballard and Hinck, 2012). Robo1 knockdown neutralized the inhibitory effect of Slit2 for cellular chemotaxis in vitro (Prasad et al., 2007, Mertsch et al., 2008). Altay et al. previously showed that recombinant Robo1 acts as a decoy receptor by binding to recombinant Slit2 which thereby makes less Slit2 available to bind to the Robo1 receptors on immune cells to exert its anti-migratory function (Altay et al., 2007). We therefore co-administered recombinant Robo1 along with recombinant Slit2 as a decoy receptor to neutralize the effects of recombinant Slit2. Our results complement the findings of Altay et al. and showed that recombinant Robo1 reversed the protective effect of recombinant Slit2 by increasing brain infiltrated immune cell markers. This suggests that Slit2 regulates the migration of peripheral immune cells to the brain dependent on Robo1.

Slit-Robo GTPase Activating Protein 1 (srGAP1) is a downstream effector of Robo1 receptor (Wong et al., 2002). The SH3 domain of srGAP1 directly binds to the intracellular CC3 motif of Robo1 receptor (Wong et al., 2001, Guan and Rao, 2003). In the presence of Slit2, there is increased recruitment of srGAP1 to Robo1 which induces the downstream

signal transduction pathways. srGAP1 inactivates Cdc42, a small GTPase protein, by converting the active GTP-bound form of Cdc42 to inactive GDP-bound form of Cdc42 (Wong et al., 2001, Yiin et al., 2009). Cdc42 is a critical mediator for cell migration (Bishop and Hall, 2000, Yiin et al., 2009). Activation of Cdc42 leads to filopodial extension at leading edge of migrating cells which enables cells to move forward (Bishop and Hall, 2000, Kumar et al., 2012). Our results showed that srGAP1 siRNA reversed the protective effects of recombinant Slit2 against SBI induced immune cell infiltration. Additionally, we observed that Cdc42 activity was reduced by recombinant Slit2 and this reduction was partially neutralized by recombinant Robo1 co-administration and reversed by srGAP1 siRNA. This suggests that Slit2 activation of the Robo1-srGAP1 pathway inhibits Cdc42 activity which possibly reduced peripheral immune cell infiltration and neuroinflammation after SBI.

In conclusion, our findings indicate that Slit2 contributes to protection against neuroinflammation after SBI. Administration of recombinant Slit2 reduced neuroinflammation after SBI by inhibiting peripheral immune cell infiltration possibly through Robo1-srGAP1 pathway mediated Cdc42 inactivation. These observations suggest that Slit2 may be a potential therapeutic option to reduce neurosurgical injury. Modulation of neuroinflammation by targeting peripheral immune cell infiltration may be beneficial to improve post-operative outcomes in neurosurgical patients.

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ABBREVIATIONS

SBI	Surgical brain injury
Robo1	Roundabout1
CD45	Cluster of differentiation 45
MPO	Myeloperoxidase
srGAP1	Slit-Robo GTPase activating protein 1
ELISA	Enzyme linked immunosorbent assay
ICV	Intracerebroventricular
PBS	Phosphate buffered saline
NeuN	Neuronal nuclei
GFAP	Glial fibrillary acidic protein
PAK	p21 activated kinase
PBD	p21 binding domain

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HIGHLIGHTS

- Peripheral immune cells infiltration increased at the perisurgical site after SBI
- Endogenous Slit2 was increased as a protective mechanism after SBI
- Recombinant Slit2 attenuated brain edema by reducing neuroinflammation
- Recombinant Slit2 reduced neuroinflammation by decreasing immune cell infiltration
- Recombinant Slit2 inhibited immune cell migration dependent on Robo1-SrGAP1 pathway

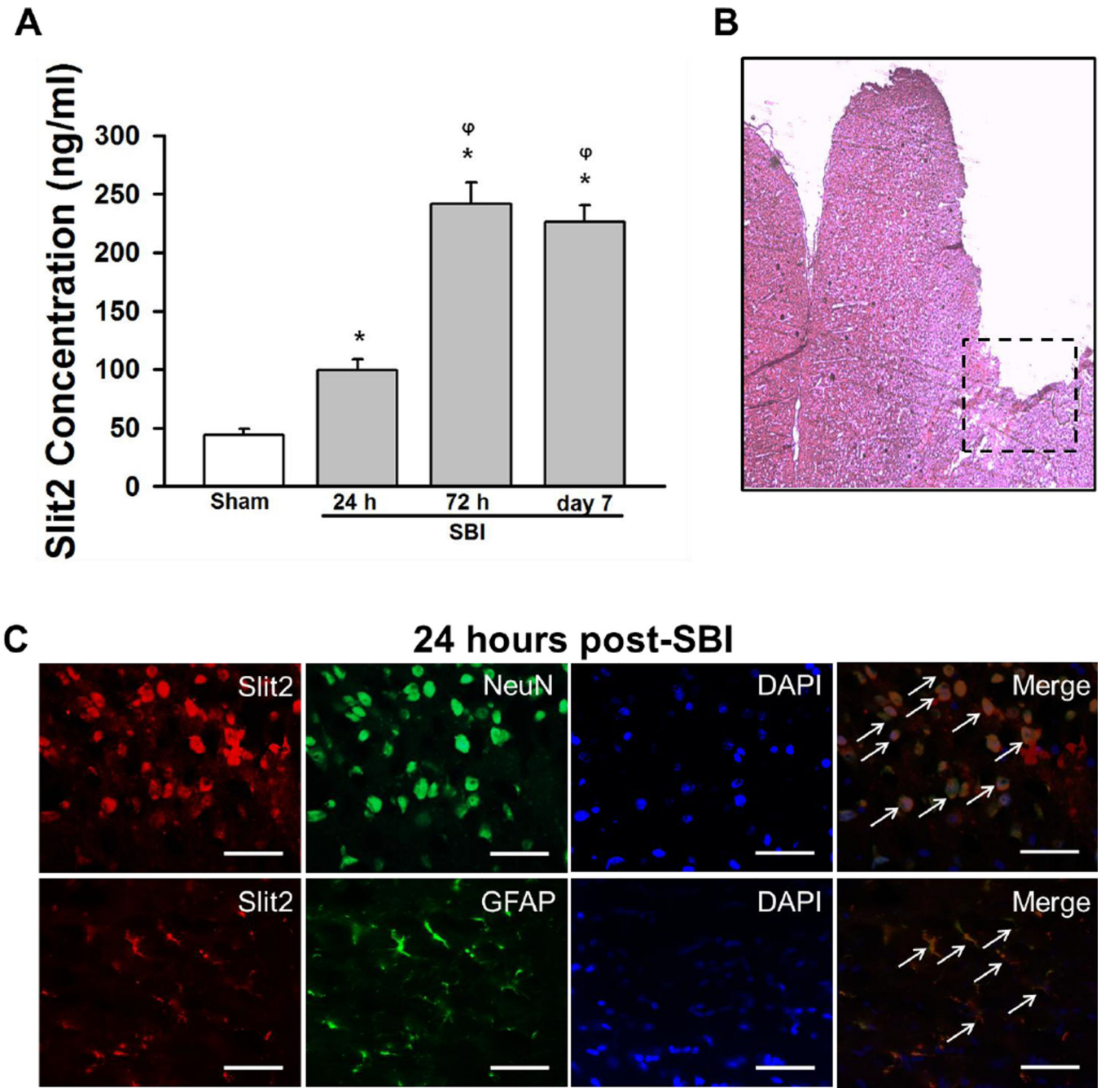

Figure 1.

Temporal expression and localization of endogenous Slit2 in the brain after SBI. (**A**) ELISA showed that Slit2 concentration increased at 24 hours, 72 hours and 7 days after SBI with a peak at 72 hours. Data are expressed as mean ± SEM. N=4 to 6 per group. ANOVA, SNK. *p<0.05 compared to Sham, ᵠp<0.05 compared to 24 hours SBI. (**B**) Histology section showing perisurgical site in the inset used for immunofluorescence staining. (**C**) Representative microphotographs of immunofluorescence staining showing co-localization of Slit2 (Texas Red/red), with neuronal nuclei (NeuN) or glial fibrillary acidic protein

(GFAP) (FITC/green) and DAPI at 24 hours after SBI. Arrows indicate merged cells. Scale bar=50 μm.

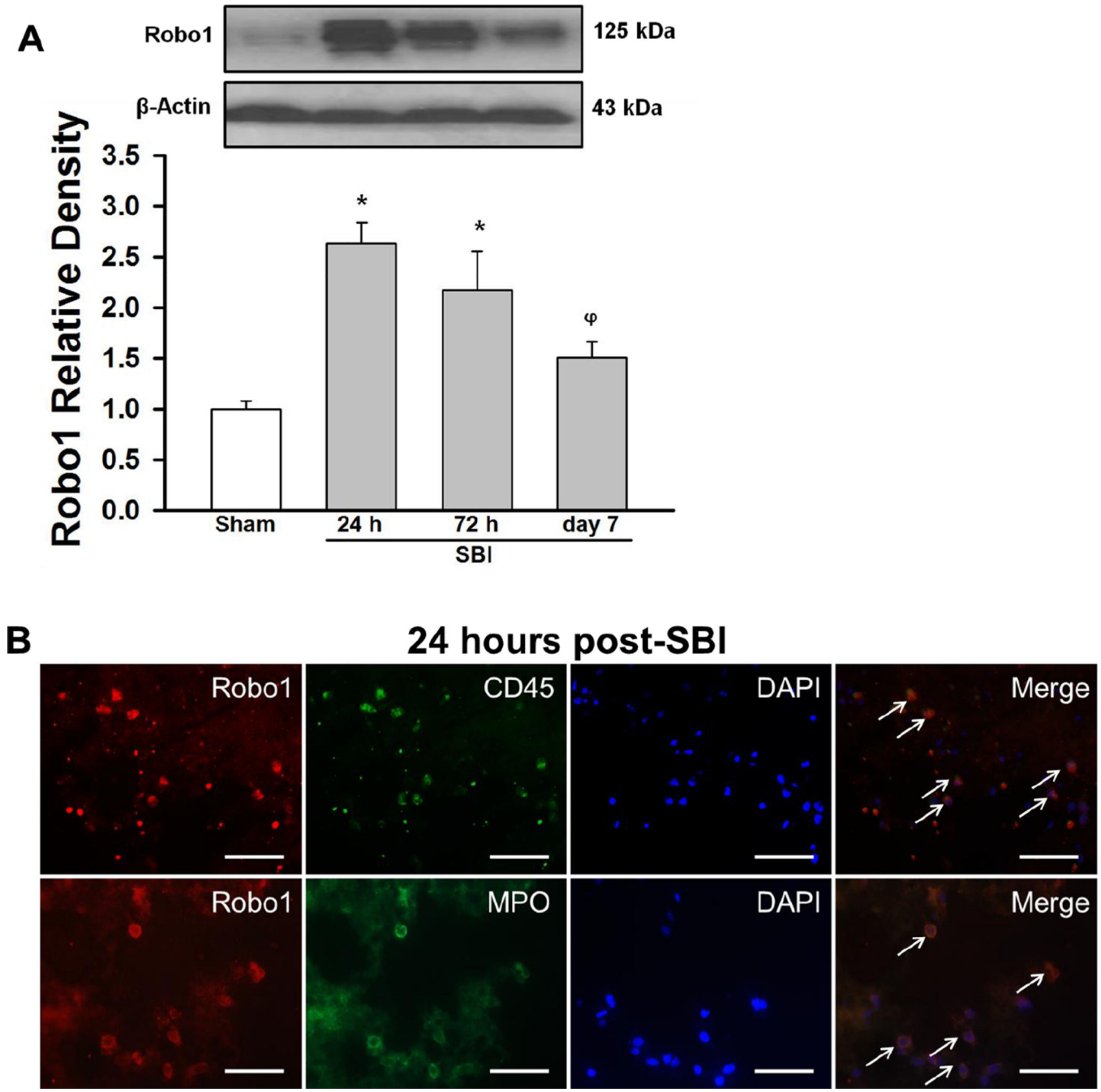

Figure 2.

Temporal expression and localization of Robo1 in the brain after SBI. (**A**) Representative image of western blot and quantitative analysis showed that Robo1 expression increased at 24 hours, 72 hours and 7 days after SBI with a peak increase at 24 hours. Data are expressed as mean ± SEM. N=4/group. ANOVA, SNK. *$p<0.05$ compared to Sham, $^{\varphi}p<0.05$ compared to 24 hours SBI. (**B**) Representative microphotographs of immunofluorescence staining showing co-localization of Robo1 (Texas Red/red) with immune cell markers

cluster of differentiation 45 (CD45) or neutrophil marker myeloperoxidase (MPO) (FITC/green) and DAPI at 24 hours after SBI. Arrows indicate merged cells. Scale bar=50 μm.

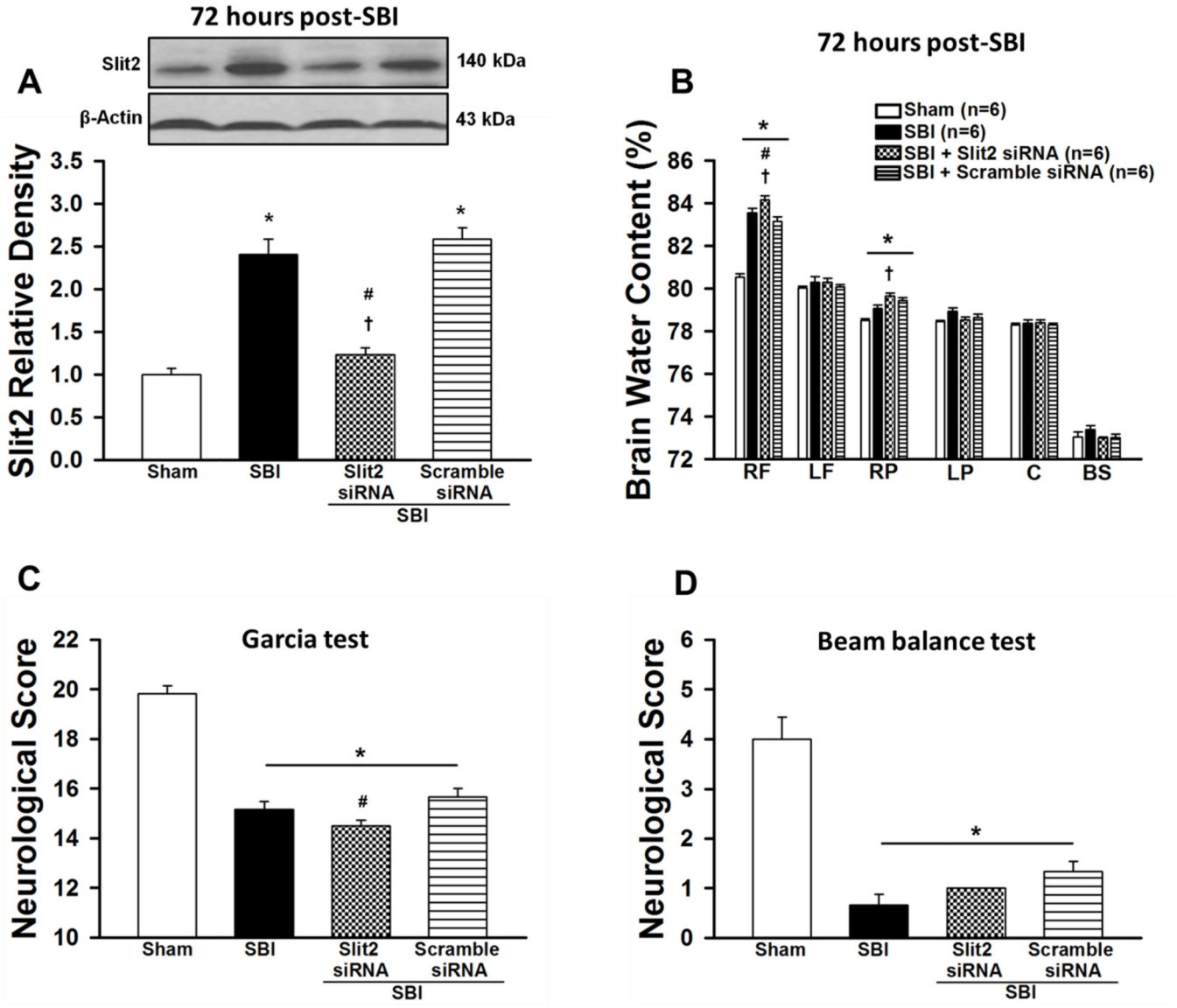

Figure 3.

Effect of endogenous Slit2 knockdown on Slit2 expression, brain water content and neurological function 72 hours after SBI. (**A**) Representative image of western blot and quantitative analysis of Slit2 expression after siRNA knockdown showed that Slit2 siRNA but not scramble siRNA significantly reduced the expression of endogenous Slit2 after SBI. (**B**) Brain water content was significantly increased at the residual right frontal lobe 72 hours after SBI. Slit2 siRNA worsened brain edema compared to SBI and scramble siRNA. RF: right frontal, LF: left frontal, RP: right parietal, LP: left parietal, C: cerebellum, BS: brainstem. (**C**) Garcia test and (**D**) Beam balance test showed significantly worse neurological function 72 hours after SBI compared to sham. Slit2 siRNA group performed worse than scramble siRNA group in the Garcia test. Data are expressed as mean ± SEM. N=6/group. ANOVA, SNK. *p<0.05 compared to sham, †p<0.05 compared to SBI, $^{\#}$p<0.05 compared to SBI+scramble siRNA.

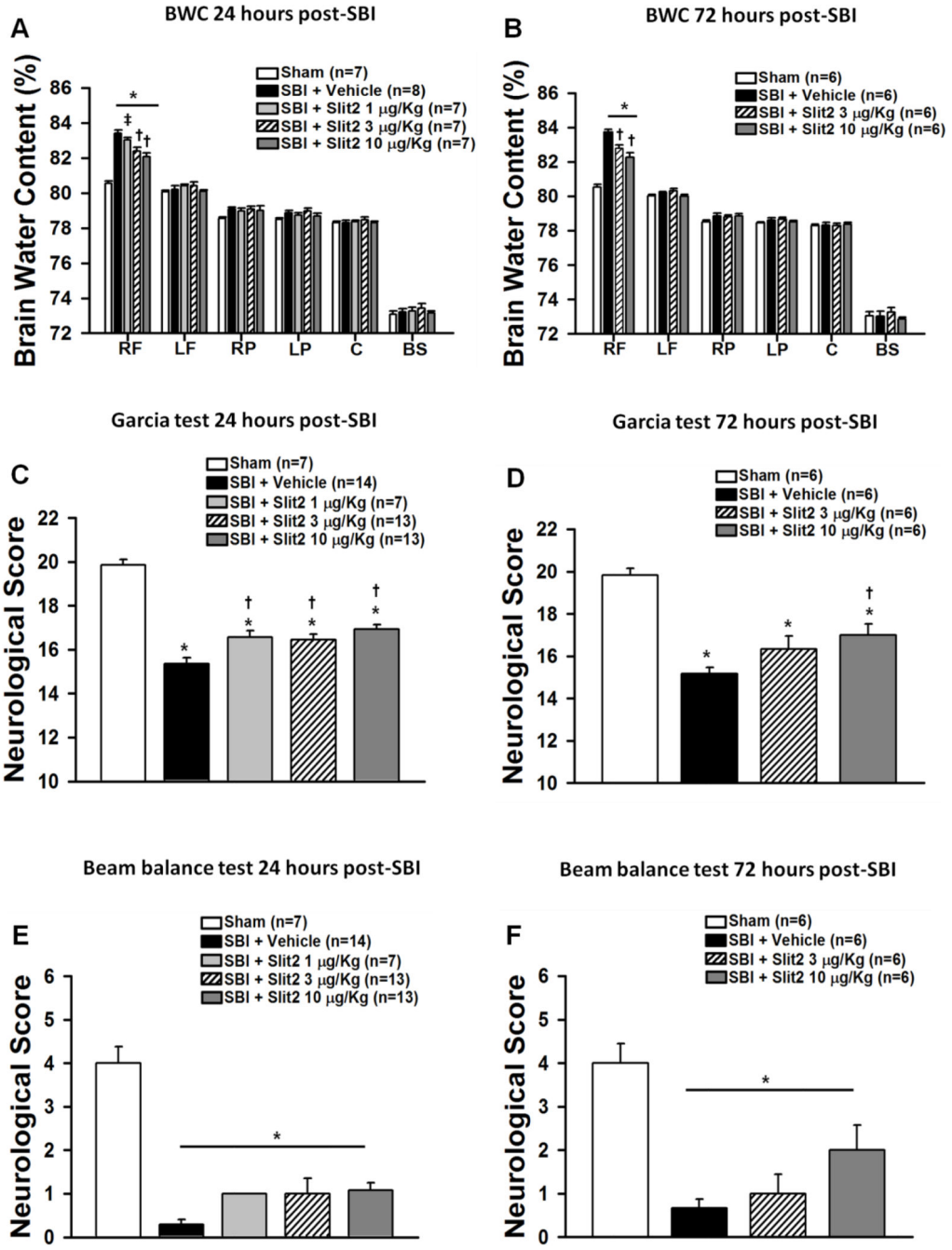

Figure 4.

Effect of recombinant Slit2 on brain water content and neurological function at 24 and 72 hours after SBI. Brain water content was significantly increased in the residual right frontal lobe at 24 hours (**A**) and 72 hours (**B**) after SBI. Recombinant Slit2 3 μg/Kg and 10 μg/Kg significantly reduced brain edema at both time points. RF: right frontal, LF: left frontal, RP: right parietal, LP: left parietal, C: cerebellum, BS: brainstem. Garcia test (**C** and **D**) and Beam balance test (**E** and **F**) showed significantly worse neurological function at 24 and 72 hours after SBI, respectively. Slit2 10 μg/Kg significantly improved performance in the

Garcia test 24 and 72 hours after SBI. Data are expressed as mean ± SEM. ANOVA, Tukey. *p<0.05 compared to Sham, †p<0.05 compared to SBI+Vehicle, ‡p<0.05 compared to SBI +Slit2 10 μg/Kg.

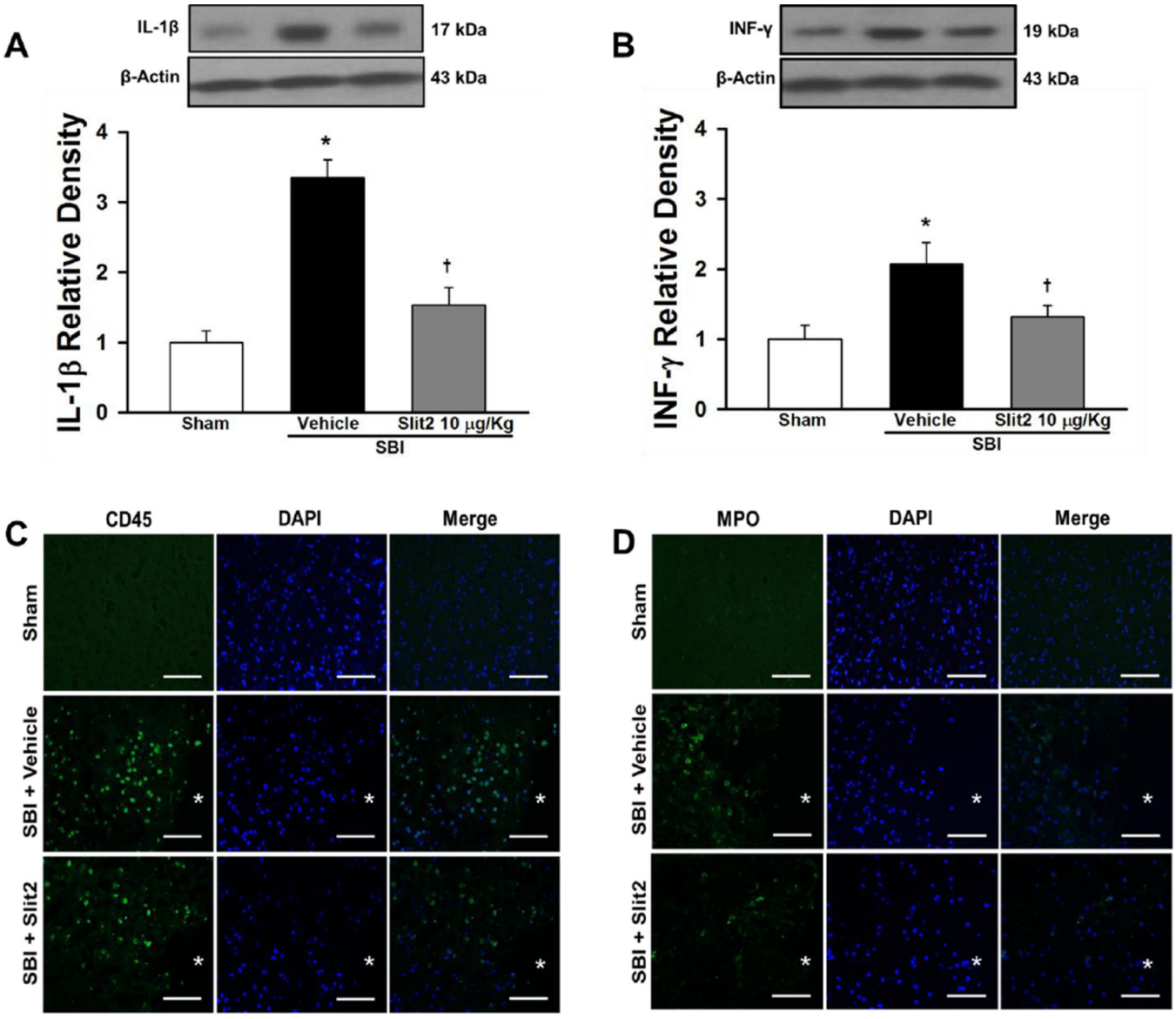

Figure 5.

Effect of recombinant Slit2 on neuroinflammation and peripheral immune cell infiltration at 24 hours after SBI. Representative western blot image and quantitative analysis showed that IL-1β (**A**) and INF-γ (**B**) expression was significantly increased after SBI. Slit2 10 μg/Kg significantly reduced the expression of both pro-inflammatory cytokines. Data are expressed as mean ± SEM. N=6/group. ANOVA, SNK. *p<0.05 compared to Sham, †p<0.05 compared to SBI+Vehicle. (**C**) Representative microphotographs of immunofluorescence staining showing co-localization of peripheral immune cell marker cluster of differentiation 45 (CD45)-FITC/green with DAPI at the right frontal perisurgical site. Recombinant Slit2 10 μg/Kg group had fewer CD45 positively stained cells. (**D**) Representative microphotographs of immunofluorescence staining showing co-localization of neutrophil marker myeloperoxidase (MPO)-FITC/green with DAPI at the perisurgical site. Fewer MPO

positive cells were visualized in the recombinant Slit2 10 μg/Kg group. Asterix denotes site of injury and scale bar=100 μm for panels C and D.

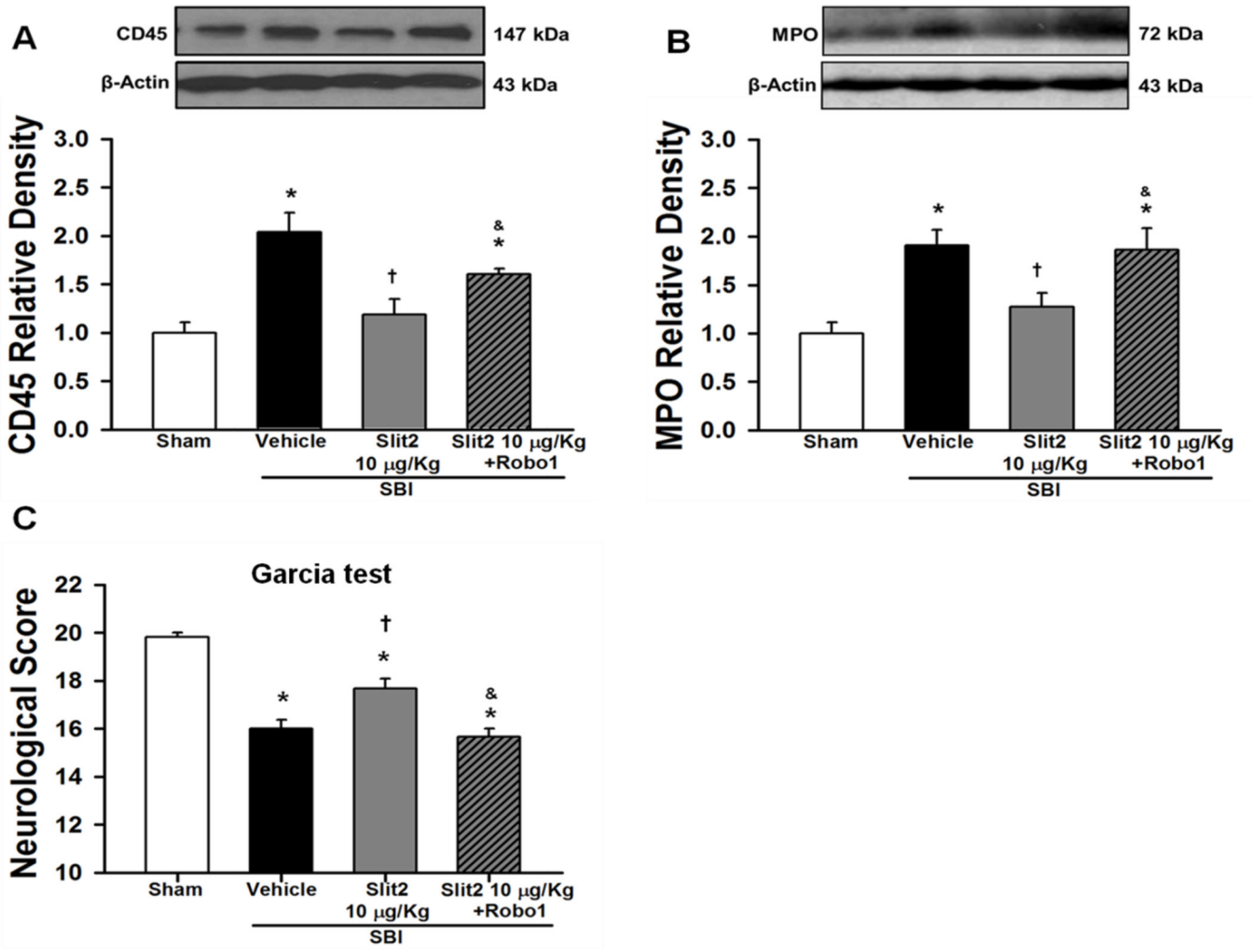

Figure 6.

Role of Robo1 in Slit2 mediated decrease in peripheral immune cell infiltration. (**A**) Representative western blot image and quantitative analysis showing peripheral immune cell marker cluster of differentiation (CD45) expression was increased in the right frontal perisurgical site at 24 hours after SBI compared to sham. Slit2 10 μg/Kg significantly reduced the expression of CD45 which was reversed with recombinant Robo1 co-administration. (**B**) Representative western blot image and quantitative analysis showing increased expression of neutrophil marker myeloperoxidase (MPO) at the perisurgical site after SBI. Slit2 10 μg/Kg significantly reduced the expression of MPO which was reversed with recombinant Robo1 co-administration. (**C**) Slit2 10 μg/Kg significantly improved neurological function evaluated using Garcia test 24 hours after SBI which was reversed with recombinant Robo1 co-administration. Data are expressed as mean ± SEM, N=6/group. ANOVA, SNK. *p<0.05 compared to Sham, †p<0.05 compared to SBI+Vehicle, &p<0.05 compared to SBI+Slit2 10 μg/Kg.

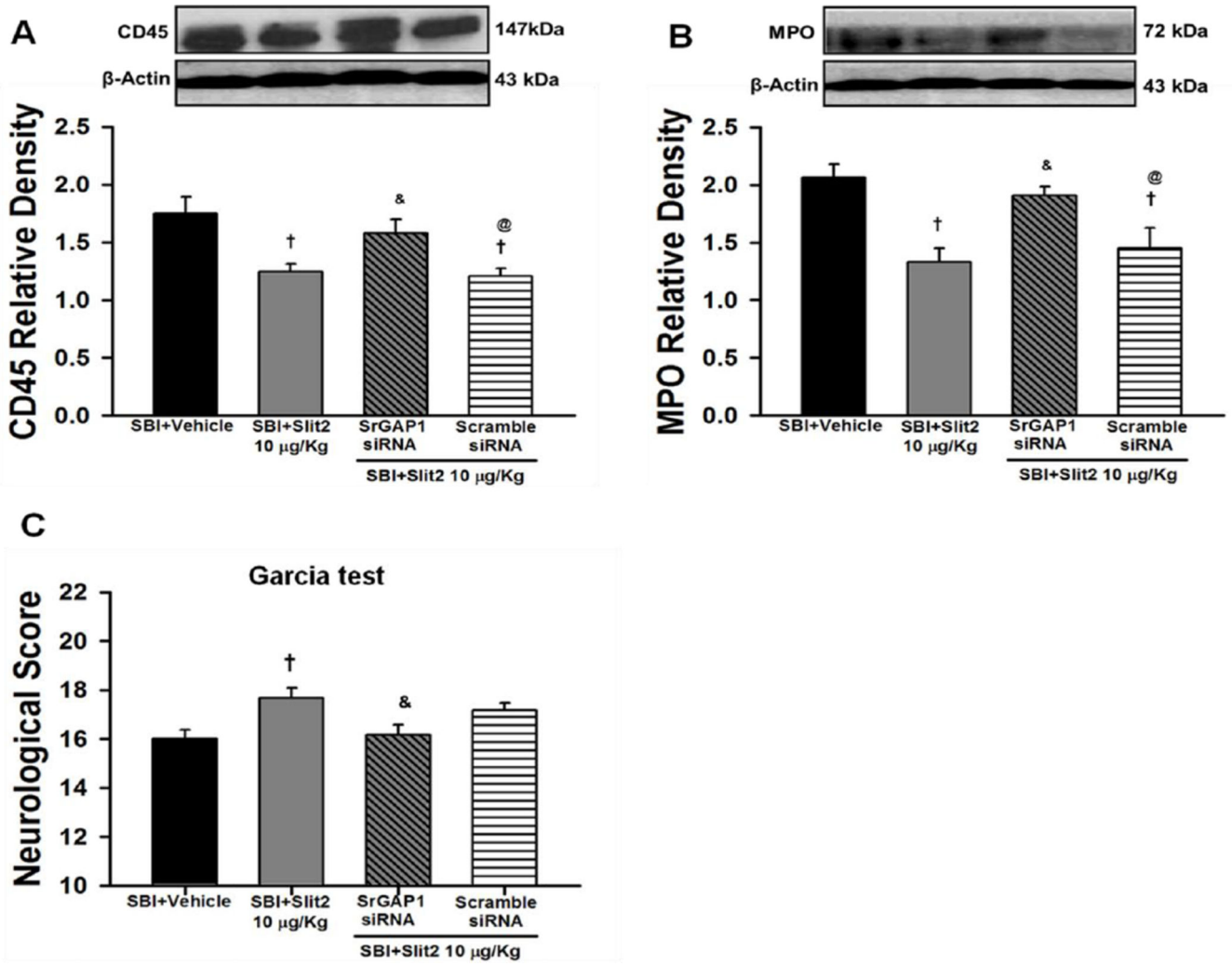

Figure 7.

Role of srGAP1 in Slit2 mediated decrease in peripheral immune cell infiltration. (**A**) Representative western blot image and quantification showing Slit2 10 μg/Kg significantly reduced the expression of peripheral immune cell marker cluster of differentiation 45 (CD45) which was reversed with srGAP1 siRNA but not scramble siRNA. (**B**) Slit2 10 μg/Kg significantly reduced the expression of neutrophil marker myeloperoxidase (MPO) which was reversed with srGAP1 siRNA but not scramble siRNA. (**C**) Slit2 10 μg/Kg significantly improved neurological function evaluated using Garcia test 24 hours after SBI which was reversed with srGAP1 siRNA. Data are expressed as mean ± SEM, N=6/group. ANOVA, SNK. †$p<0.05$ compared to SBI+Vehicle, &$p<0.05$ compared to SBI+Slit2 10 μg/Kg, @$p<0.05$ compared to SBI+Slit2+srGAP1 siRNA.

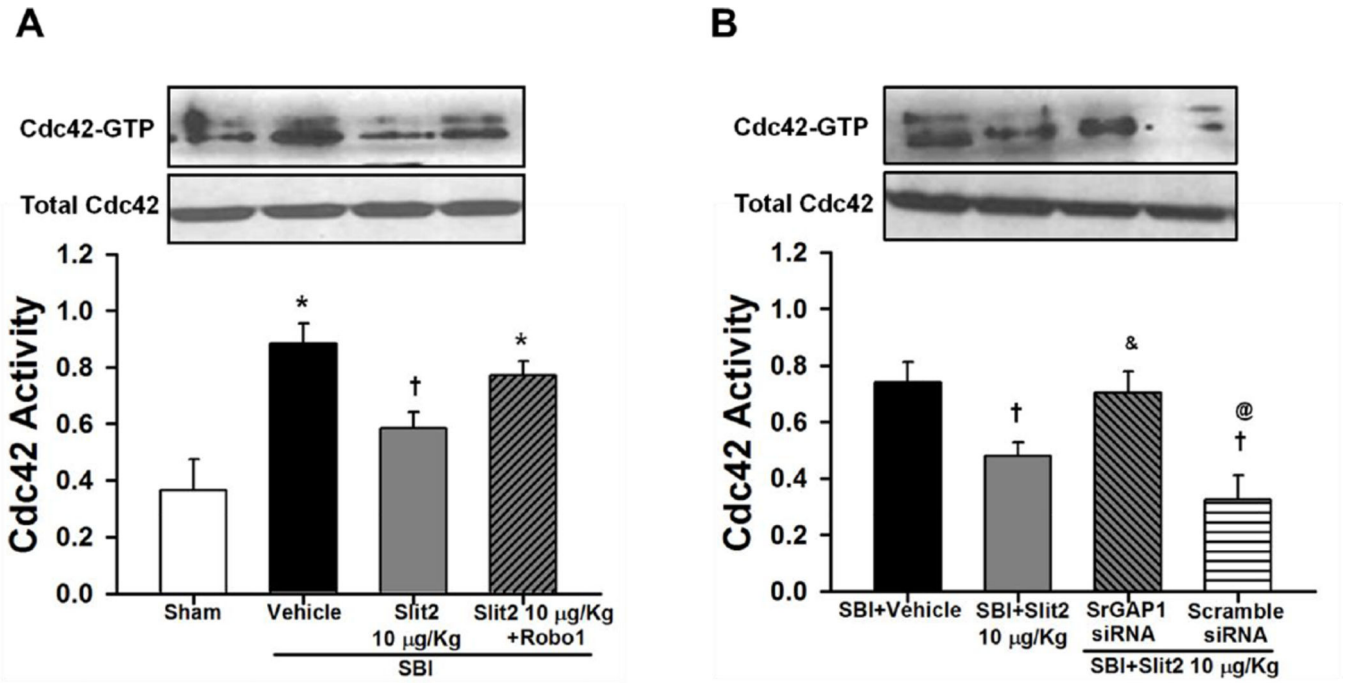

Figure 8.

Cdc42 activity assay in the right frontal perisurgical site at 24 hours after SBI. (**A**) Cdc42 activity was increased 24 hours after SBI which was significantly reduced by recombinant Slit2 10 μg/Kg. Robo1 co-administration partially reversed the effect of Slit2 on Cdc42 activity (p=0.097 compared to SBI+Slit2 10 μg/Kg). (**B**) srGAP1 siRNA but not scramble siRNA reversed the effect of Slit2 on Cdc42 activity. Data are expressed as mean ± SEM. N=5/group. ANOVA, SNK. *p<0.05 compared to Sham, †p<0.05 compared to SBI +Vehicle, &p<0.05 compared to SBI+Slit2 10 μg/Kg, @p<0.05 compared to SBI +Slit2+srGAP1 siRNA.