

# From SHP2 to DDR1-ERK: A critical signaling axis controlling microglial activation and neuropathic pain after spinal cord injury

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**Abstract.** This study explores the role of the src homology-2domain-containing protein tyrosine phosphatase-2 (SHP2)-discoid domain receptor 1 (DDR1)-extracellular signal-regulated kinase (ERK) signaling axis in neuropathic pain following spinal cord injury (SCI). Rats were transfected with LV-shSHP2 before SCI modeling. Hind limb function and paw withdrawal threshold were assessed using the Basso-Beattie-Bresnahan (BBB) scale, and Von Frey filaments, respectively. The effects of SHP2 knockdown on microglial activation and DDR1/SHP2/ERK pathway were examined by Western blotting and immunofluorescence. To further validate the regulatory mechanism of SHP2 in SCI, lipopolysaccharide (LPS)-activated BV-2 microglial cells were subjected to SHP2/DDR1 knockdown, or DDR1 overexpression. Results showed that SCI induced motor deficits, mechanical allodynia, and upregulation of spinal SHP2, DDR1, and p-ERK/ERK ratio, alongside microglial activation. These changes were reversed by LV-shSHP2 treatment. In LPS treated BV-2 cells, DDR1 overexpression promoted cell activation and ERK phosphorylation, while counteracting the effects of LV-shSHP2; however, DDR1 knockdown produced the opposite effects. A direct interaction between SHP2 and DDR1 was confirmed. In conclusion, SHP2 knockdown suppresses microglial hyperactivation and alleviates SCI-induced neuropathic pain by downregulating the DDR1-ERK signaling pathway, during which SHP2-DDR1 interaction has been confirmed as a key regulatory mechanism. Targeting this specific interaction axis may offer a novel therapeutic strategy for neuropathic pain management by selectively disrupting microglial activation.

**Key words:** Spinal cord injury — SHP2 — DDR1 — Extracellular signal-regulated kinase — Microglia

## Introduction

Neuropathic pain (NP), which occurs in 80% of patients with spinal cord injury (SCI), complicates clinical treatment (Lee et al. 2018). The study of post-SCI NP commonly adopts an animal model, with the low thoracic (T7-T10) contusion as the main modeling method (Kramer et al. 2017). Emerging evidence has demonstrated that mechanisms of SCI, including microglia activation, also contribute to the occurrence of NP (Shiao and Lee-Kubli 2018). However, strategies to relieve

NP by targeting microglia activation remain poorly developed. In this case, a deeper understanding on the underlying mechanisms of microglia activation is of great significance for the treatment of post-SCI NP. Microglial cells are resident macrophages of the central nervous system (CNS) (Cuadros et al. 2022). Recent studies have revealed the role of microglia cells in the alleviation and treatment of NP caused by SCI (Miranpuri et al. 2021). Besides, the inhibition of microglial activity reduces spinal cord inflammation and apoptosis injury after SCI (Wang et al. 2017). The stimulation of the spinal cord can promote microglial activation, resulting in compromised pain control (Shu et al. 2020). These findings underscored the inhibition of microglial activation as a potential therapeutic strategy, and corresponding methods are under development. Src homology-2 domain-containing

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protein tyrosine phosphatase-2 (SHP2) is a carcinogenic type of phosphatase (Liu et al. 2021). Previous studies found that the presence of SHP2 in the spinal dorsal horn augmented incisional pain (Ding et al. 2018).

SHP2 is also involved in the progression of NP (Peng et al. 2012), and more intriguingly, SHP2 inhibition plays a role in attenuation of microglial activation (Song et al. 2016). It has been reported that knockdown of spinal SHP2 suppresses BDNF-mediated GluN2B-NMDA receptor activation, spinal long-term potentiation induction and pain allodynia elicitation in intact rats, ultimately alleviating pain allodynia in neuropathic rats (Ding et al. 2015). SHP2 is also implicated in several allodynia-related signal transduction cascades (Hayano et al. 2016). SHP2 has several downstream targets including ERK signalling pathway (Vazhappilly et al. 2019), and acts as downstream activated gene of discoid domain receptor 1 (DDR1) signaling (Sogaard et al. 2019), suggesting its role in NP may involve the regulation of signaling pathways. DDR1, a non-integrin collagen receptor, is associated with the activation of microglia (Wang et al. 2019).

Reportedly, SHP2 binds to DDR1 in a collagen-dependent manner (Koo et al. 2006). DDR1 knockdown exerts an inhibitory effect on microglial activity induced by collagen (Hebron et al. 2017). Although both SHP2 and DDR1 have been confirmed to be associated with neuroinflammation and pain states, their direct mechanistic interaction in SCI and microglial function has not been established. In addition, DDR1 can trigger the extracellular signal-regulated kinase (ERK) signaling pathway (Zhao et al. 2021). The above insights implied the possibility that DDR1 may relieve activated microglia-induced NP via regulating the ERK pathway. ERK, a mitogen-activated protein kinase, has the ability to deliver extracellular information into intracellular responses (Xu et al. 2019). The ERK pathway has also been evidenced to play an essential role in the pathogenesis of NP and targeting inhibit ERK activation contributes to NP relief (Jia et al. 2018). The release of phosphorylate-ERK (p-ERK) in the spinal cord is related to microglial cells (Xu et al. 2019). Further, the activation of the ERK pathway has been also observed in activated microglia, hinting the potential of ERK pathway inhibition in activated microglia for pain relief (Lee et al. 2018). Accordingly, the present study conjectured that the loop of SHP2, ERK and DDR1 might contribute to microglial activation-induced NP after SCI. To verify this assumption, the present study explored the role and mechanism of SHP2 in SCI via DDR1/ERK signaling pathways.

## Materials and Methods

### Ethics statement

All animal experiments were approved by the Institutional Animal Care and Use Committee, Zhejiang Baiyue

Biology Technology Co. Ltd. (Approval No. ZJBYLA-IACUC-20240814) and were performed in compliance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (McGrath et al. 2010).

### SCI model

Sprague Dawley adult rats (male, 250–300 g, Vital River Laboratories, Beijing, China) were housed under a 12-hour light/dark cycle with 40–60% humidity at 22°C with free access to food and water. Rats were randomly assigned to the sham group, SCI group, lentivirus-short hairpin RNA against SHP2 (LV-shSHP2) group and LV-negative control (LV-NC) group. LV-shSHP2 and its negative control were constructed by GenePharma (Shanghai, China). Briefly, shSHP2 and its control were cloned into lentiviral vector GV118 (Genechem, Shanghai, China) and transfected into the human embryonic kidney (HEK) 293T cells with lentivirus packaging vector Helper vector-I (GenePharma, China). The final titer of LV-shSHP2 was  $1.0 \times 10^9$  transducing units (TU)/ml (Ding et al. 2015). For the LV-shSHP2 group, rats were intrathecally injected with 10  $\mu$ l LV-shSHP2 at  $1.0 \times 10^9$  TU/ml (Ding et al. 2015). For the LV-NC group, rats were intrathecally injected with LV-NC in the same volume. After 5 days, rats in both LV-shSHP2 and LV-NC groups were subjected to SCI surgery. Prior to operation, intraperitoneal anaesthesia was initially performed with sodium pentobarbital (50 mg/kg). Rats in the SCI group, LV-NC group and LV-shSHP2 group received the laminectomy at the thoracic 10 (T10) level, followed by a moderate contusion injury on the spinal cord (10 g $\times$ 25 mm) by a New York University (NYU) impactor. Rats in the sham group underwent laminectomy only (Lee et al. 2018). The whole experiment lasted for 28 days after SCI modeling.

### Basso-Beattie-Bresnahan (BBB) test

The 22-point BBB locomotor rating scale (score range of 0–21) was adopted to evaluate the recovery of hindlimb function of rats after SCI. Rats in each group were placed in the open field, and hind limb movements, including joint and paw movement, limb coordination, the control of tail and trunk as well as stepping ability, were evaluated. Scores of each group were assessed before the SCI operation (D0), and on the 1<sup>st</sup>, 7<sup>th</sup>, 14<sup>th</sup>, 21<sup>th</sup> and 28<sup>th</sup> days after SCI by two observers who were blinded to the condition of each group.

### Paw withdrawal threshold (PWT) test

Mechanical allodynia was assessed by the von Frey filaments method using PWT. Rats were placed in wire cages (20 $\times$ 20 $\times$ 30 cm) with a mesh bottom (5 $\times$ 5 mm mesh) and

acclimatized for 30 min. The electronic von Frey filament (76-0976, Harvard Apparatus, Holliston, MA, USA) was applied against the central edge of each hind paw to exert stimulation. The test was carried out before the SCI surgery (D0), and on the 7<sup>th</sup>, 14<sup>th</sup>, 21<sup>th</sup> and 28<sup>th</sup> days after SCI, and was repeated three times on each rat before the mean PWT was evaluated.

### Cell culture

The mouse microglial BV-2 cell line was purchased from the National Collection of Authenticated Cell Cultures (GNM45, Shanghai, China). Cells were cultivated in RPMI 1640 medium (R0083, Sigma-Aldrich, Deisenhofen, Germany) with 10% fetal bovine serum (FBS, 12103C, Sigma-Aldrich, Germany) at 37°C in a 5% CO<sub>2</sub> incubator (Z742533, Sigma-Aldrich, Germany).

### Cell transfection and treatment

DDR1 (reference sequence: NM\_001198833.1) overexpression vector was synthesized by GenePharma (China) using the pcDNA 3.1 control vector (V87020, Invitrogen, New York, NY, USA). Si-DDR1 (siB160909022350-1-5, RiboBio, Guangzhou, China), Si-negative control (Si-NC, siN0000004-4-10, RiboBio, China), DDR1 overexpression and control vector were separately transfected into separately cultured BV-2 cells by Lipofectamine 3000 transfection reagent (L3000015, Invitrogen, USA). Before LV-NC or LV-shSHP2 infection, BV-2 cells received 4-h starved culture. Then, 2 µg/ml puromycin (ab141453, Abcam, Cambridge, UK) was used to obtain stable BV-2 cells. To induce the activation of microglia, 1 µM lipopolysaccharide (LPS, 00-4976-93, Invitrogen, USA) was added to the culture medium of BV-2 cells for 24 h at 37 °C (Wang et al. 2018).

### Quantitative real-time polymerase chain reaction (qRT-PCR)

After transfection, total RNAs were extracted from BV-2 cells using a Universal RNA extraction kit (9767, TaKaRa, Tokyo, Japan) before cDNA synthesis with PrimeScript RT kit (RR037Q, TaKaRa, Japan). Subsequently, qPCR was carried out using a qPCR kit (QR0100, Merck, Germany) on a Thermal Cycler Dice PCR System (TP950, TaKaRa, Japan). Cycling parameters were set as follows: pre-denaturation at 95 °C for 3 min 40 cycles of denaturation at 95°C for 30 seconds (s), annealing at 60°C for 10 s and extension at 60°C for 30 s. Relative expressions were presented using the 2<sup>-ΔΔCT</sup> method. Primers involved in the assay were as follows: SHP2: 5'-TATCCTCTGAACTGTGCAGATCC-3'(forward), 5'-TCTGGCTCTCTCGTACAAGAAAA-3'(reverse); DDR1: 5'-GCGTCTGTCTGCGGGTAGAG-3'(forward), 5'-ACGGCCTCAGATAAATACATTGTCT-3'

(reverse); GAPDH: 5'-TCCCTCAAG ATTGTCAGCAA-3'(forward), 5'-AGATCCACA ACGGATACATT-3'(reverse).

### Immunofluorescence

On the postoperative day 28, rats were anaesthetized with diethyl ether (613479, Sigma-Aldrich, Germany) and perfused transcardially with PBS and 4% paraformaldehyde (P0099, Beyotime, China). Afterwards, rats were sacrificed by cervical dislocation. T10 spinal cord tissues (5 µm) from rats after SCI were fixed in 4% paraformaldehyde (P0099, Beyotime, Shanghai, China) for 24 h, dehydrated in a gradient concentration of ethanol, and embedded in paraffin (1.07150, Sigma-Aldrich, Germany). For immunofluorescence, paraffin sections were deparaffined and hydrated. LPS-induced BV-2 cells after transfection/infection were fixed in 4% paraformaldehyde for 20 min. After washing with phosphate buffered saline (PBS, C0221A, Beyotime, China), tissues and BV-2 cells were treated with 0.3% Triton X-100 (ST677, Beyotime, China) and primary antibodies. Tissues were probed with anti-cell-type-specific Abs for microglia (anti-Iba-1) (ab178846, 1:200, Abcam, UK), anti-DDR1 (sc-390268, 1:100, Santa Cruz Biotechnology, Dallas, TX, USA) and anti-p-ERK (#4370, 1:200, Cell Signaling Technology, USA), while BV-2 cells were treated with anti-CD68 (ab237968, 1 µg/ml, Abcam, UK) at 4°C overnight. Then, tissues and cells were incubated with Alexa Fluor® 488-conjugated Goat Anti-Rabbit IgG H&L (green, ab150077, 1:200, Abcam, UK), Alexa Fluor® 488-conjugated Goat Anti-Rat IgG H&L (green, ab150165, 1:200, Abcam, UK) and Alexa Fluor® 647 conjugated-Goat Anti-Mouse H&L (red, ab150115, 1:200, Abcam, UK) for 30 min at room temperature (RT), protected from the light. The nuclei were counterstained with DAPI for 15 min at RT (blue, ab104139, 1:100, Abcam, UK). Tissues labelled by DDR1 and p-ERK were double stained by Iba-1. BV-2 cells were labelled by CD68. Fluorescence images were detected under a BX63 fluorescence microscope (OLYMPUS, Tokyo, Japan, magnification ×100 and ×200, scale bar = 100 µm) and analyzed by the ImageJ software (US National Institutes of Health, Bethesda, MD, USA) (Xu et al. 2019).

### Western blot

The spinal cord of T10 after SCI and LPS-induced BV-2 cells after transfection/infection were collected and homogenized to extract proteins using a protein extraction kit (P0027, Beyotime, USA). The total proteins were then quantified by a CBQCA Protein Quantitation kit (C6667, ThermoFisher, Waltham, MA, USA). After being separated by Pierce SDS-PAGE (89888, ThermoFisher, USA), proteins were transferred to PVDF membranes (03010040001, Merck, Germany) and cultivated in blocking reagent (11921673001, Merck, Germany) for 1 h at RT. Later, membranes were subjected to

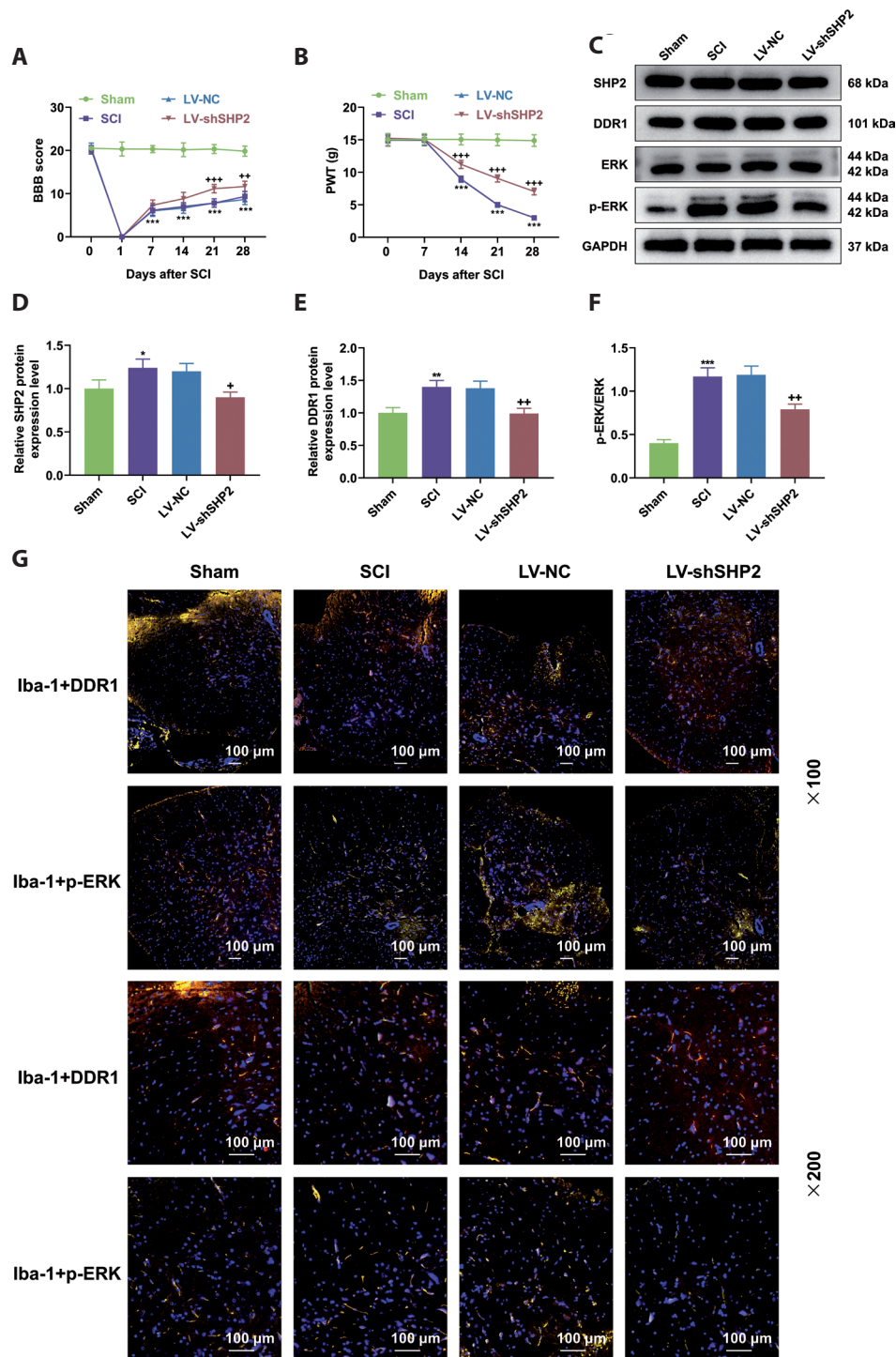


**Figure 1.** LV-shSHP2 relieved neuropathic pain-related behaviour after SCI in T10 spinal cord tissues. **A.** BBB scores were applied to assess the behavioural changes of rats after SCI. **B.** PWT was utilized to evaluate the responses of rats to pain after SCI. **C–F.** Western blot was performed to measure SHP2 and DDR1 protein levels and p-ERK/ERK signaling pathway in the T10 spinal cord of rats, GAPDH as an internal reference gene. **G.** Immunolabeling of Iba-1 with DDR1 and p-ERK in T10 spinal cord tissues (magnification ×100 and ×200, scale bar = 100 μm). n = 6. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 vs. Sham, +*p* < 0.05, ++*p* < 0.01, +++*p* < 0.001 vs. LV-NC. SHP2 (src homology-2 domain-containing protein tyrosine phosphatase-2); LV-shSHP2, lentivirus-short hairpin SHP2; LV-NC, LV-negative control; SCI, spinal cord injury; BBB, Basso-Beattie-Bresnahan; PWT, paw withdrawal threshold; Iba-1, cell-type-specific Abs for microglia; DDR1, discoid domain receptor 1; ERK, extracellular signal-regulated kinase; p-ERK, phosphorylate-ERK; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase. For more abbreviations see Figure 1.

incubation with primary antibodies anti-SHP2 (ab187040, 1:5000, 68kDa, Abcam, UK), anti-DDR1(ab193550, 1:500, 101kDa, Abcam, UK), anti-ERK (ab184699, 1:10000, 42, 44kDa, Abcam, UK), anti p-ERK (ab201015, 1:1000, 42, 44kDa, Abcam, UK) and anti-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, reference gene, ab181602, 1:500, 37kDa, Abcam, UK) overnight at 4°C, followed by 2-h culture with secondary antibody Goat Anti-Rabbit IgG H&L (1:5000) at RT. Lastly, the proteins in the membrane were detected by an enhanced chemiluminescence kit (ECL, ab133406, Abcam, UK) and further analyzed with Image Lab software (BIO-RAD, Hercules, CA, USA).

#### *Pull-down assay*

Pierce Magnetic RNA-Protein Pull-Down Kit (20164, ThermoFisher, USA) was used to perform the pull-down assay. DDR1 or SHP2 RNAs were first labelled with T4 RNA Ligase (EL0021, ThermoFisher, USA) and captured to magnetic beads, so that RNA could be oriented for protein binding. Then, RNA-bound beads were equilibrated in binding buffer, followed by the addition of protein lysate. After washing, samples were eluted with SDS-PAGE. Bound proteins were later detected by Western blot using anti-DDR1 or anti-SHP2 and anti-IgG (ab133470, 1:1000, Abcam, UK). GAPDH was used as the internal reference protein in this assay.

#### *Statistical analysis*

All data were analyzed by GraphPad 8.0 software (GraphPad, USA) and showed as the mean  $\pm$  standard deviation (SD). For the data in Figure 1A,B the repeated-measures analysis of variance (ANOVA) was performed, followed by Tukey post-hoc test for multiple comparisons. For the comparisons among other groups, one-way ANOVA and the Tukey test were used. Statistical significance was established at  $p < 0.05$ .

## **Results**

#### *LV-shSHP2 relieved NP-related behaviours after SCI in T10 spinal cord tissues*

Compared with the Sham group, the BBB score significantly decreased from day 7 to day 28 in SCI group (Fig. 1A,  $p < 0.001$ ). Compared with the LV-NC group, rats in the LV-shSHP2 group presented a higher BBB score from day 21 to day 28 (Fig. 1A,  $p < 0.01$ ). In contrast to the Sham group, the SCI group experienced a sharp decrease in PWT on the 14<sup>th</sup> day after SCI (Fig. 1B,  $p < 0.001$ ). The drop of PWT in the LV-shSHP2 group was lower than that in the LV-NC group (Fig. 1B,  $p < 0.001$ ). Western blot analysis of T10 spinal cord tissues revealed that different from the Sham

group, an increase in SHP2, DDR1 and p-ERK/ERK protein levels was observed in the SCI group (Fig. 1C–F,  $p < 0.05$ ). The treatment with LV-shSHP2 diminished the protein levels of SHP2, DDR1 and p-ERK/ERK in T10 spinal cord tissues, relative to LV-NC control (Fig. 1C–F,  $p < 0.05$ ). The levels of Iba-1, DDR1 and p-ERK in T10 spinal cord tissues were higher in the SCI group than in the Sham group (Fig. 1G); conversely, they were lower in the LV-shSHP2 group than in the LV-NC group (Fig. 1G).

#### *DDR1 overexpression stimulated microglial activation in LPS-induced BV-2 cells while Si-DDR1 and LV-shSHP2 inhibited microglial activity in LPS-induced BV-2 cells*

Relative to the LV-NC group, LV-shSHP2 led to a decline in SHP2 mRNA expression level in BV-2 cells (Fig. 2A,  $p < 0.001$ ). Compared with the vector group, DDR1 overexpression significantly boosted DDR1 mRNA expression in BV-2 cells (Fig. 2B,  $p < 0.001$ ). The expression of DDR1 in BV-2 cells transfected with Si-DDR1 was lower than that in cells transfected with Si-NC (Fig. 2B,  $p < 0.001$ ). Compared with the control group, BV-2 cell activation was promoted by LPS (Fig. 2C). LPS-induced BV-2 cell activation was dampened by LV-shSHP2 and Si-DDR1, when comparison was made with LV-NC and Si-NC (Fig. 2C). DDR1 overexpression further stimulated the activation of LPS-induced BV-2 cells, compared to the Vector group (Fig. 2C).

#### *Si-DDR1/LV-shSHP2 inhibited p-ERK/ERK protein expression in LPS-induced BV-2 cells while DDR1 overexpression reversed the impacts of LV-shSHP2 on LPS-induced microglial activation in BV-2 cells*

Compared with the control group, LPS elevated SHP2, DDR1 and p-ERK/ERK protein levels in BV-2 cells, which were reversed by LV-shSHP2 (Fig. 3A–D,  $p < 0.01$ ). After the transfection of Si-DDR1, DDR1 and p-ERK/ERK protein levels in LPS-induced BV-2 cells were lower than that in Si-NC-transfected BV-2 cells, while no significant change was observed in SHP2 protein levels (Fig. 3A–D,  $p < 0.001$ ). As opposed to the vector group, DDR1 overexpression elevated DDR1 and p-ERK/ERK protein levels in LPS-induced BV-2 cells, whereas no statistical differences were found in SHP2 protein levels (Fig. 3A–D,  $p < 0.05$ ). The immunofluorescence assay data demonstrated that DDR1 overexpression reversed the inhibitory effect of LV-shSHP2 on LPS-induced BV-2 cell activation (Fig. 3E).

#### *DDR1 overexpression offset the effects of LV-shSHP2 on DDR1 and ERK signaling pathways in LPS-induced BV-2 cells*

Compared with the LV-shSHP2+vector group, LV-shSHP2 +DDR1 group presented promoted DDR1 and p-ERK/ERK

protein levels in LPS-induced BV-2 cells (Fig. 4A–D,  $p < 0.05$ ). The pull-down assay results confirmed the interaction between DDR1 and SHP2 (Fig. 4E). Anti-DDR1 treatment resulted in enriched SHP2 protein and anti-SHP2 treatment was found to enrich DDR1 protein (Fig. 4E).

## Discussion

Post-SCI NP has an over 50 % prevalence rate (Burke et al. 2017), which also remains tricky for existing treatments and has a poor prognosis (Burke et al. 2017), necessitating deeper investigation of NP's mechanisms and novel approaches. As contusion-based SCI rat models are commonly adopted in NP-related studies (Finnerup et al. 2021), the present study established an SCI rat model to investigate the functions of SHP2 on post-SCI NP. The principal finding of this study was the molecular interaction

between SHP2 and DDR1 in microglia as a key regulatory mechanism controlling NP after SCI. While prior studies have established the independent roles of SHP2 and DDR1 in pain and neuroinflammation (Ding et al. 2015; Hebron et al. 2017), our data provided the first evidence that they functioned as a coordinated, upstream signaling complex of ERK to drive microglial hyperactivation.

The study found that NP-related behaviours following SCI could be mitigated by inhibiting SHP2. BBB scoring has been used in the standardization of SCI progression (All et al. 2020). According to our results, intrathecal injection of shSHP2 promoted the recovery of motor ability after SCI. Besides, the PWT assay data showed pain hypersensitivity was also attenuated by shSHP2, indicating that SHP2 contributed to NP-associated behaviours after SCI. The positive impacts of shSHP2 on mechanical allodynia and pain hypersensitivity in neuropathic rats discovered in previous studies (Ding et al. 2015) were consistent with the findings of the

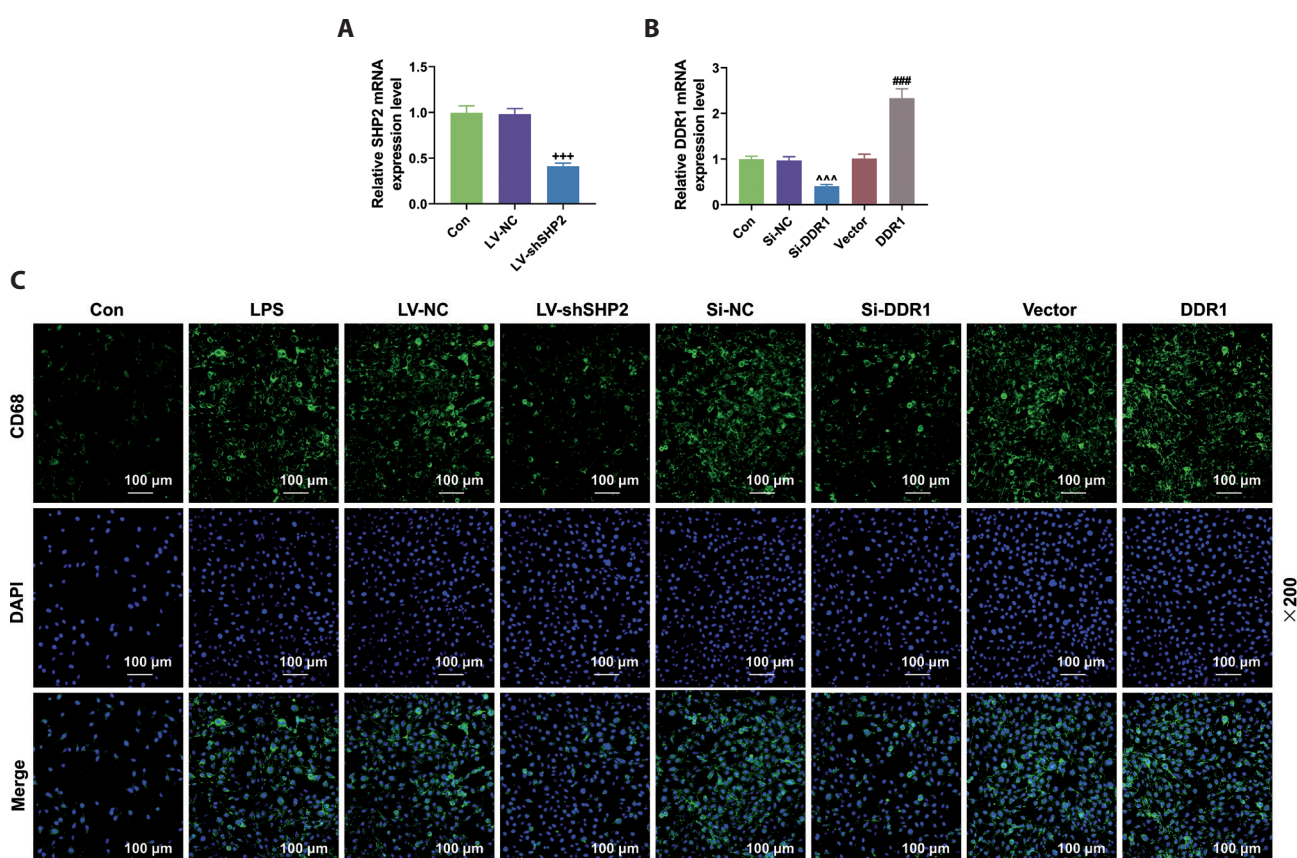


**Figure 2.** DDR1 overexpression stimulated microglial activation in LPS-induced BV-2 cells while Si-DDR1 and LV-shSHP2 inhibited microglial activity in LPS-induced BV-2 cells. **A–B.** SHP2 and DDR1 mRNA expression levels in BV-2 cells after transfection/infection detected by qPCR. **C.** Immunolabeling of CD68 with DAPI for assessing the activation of BV-2 cells treated with/without LPS and infected/transfected with LV-NC/LV-shSHP2/Si-DDR1/Vector/DDR1 overexpression (magnification  $\times 200$ , scale bar = 100  $\mu\text{m}$ ).  $n = 3$ . <sup>^^^</sup> $p < 0.001$  vs. Si-NC, <sup>+++</sup> $p < 0.001$  vs. LV-NC, <sup>###</sup> $p < 0.001$  vs. Vector. qRT-PCR, quantitative real-time polymerase chain reaction; DAPI, 4',6-diamidino-2-phenylindole. For more abbreviations see Figure 1.

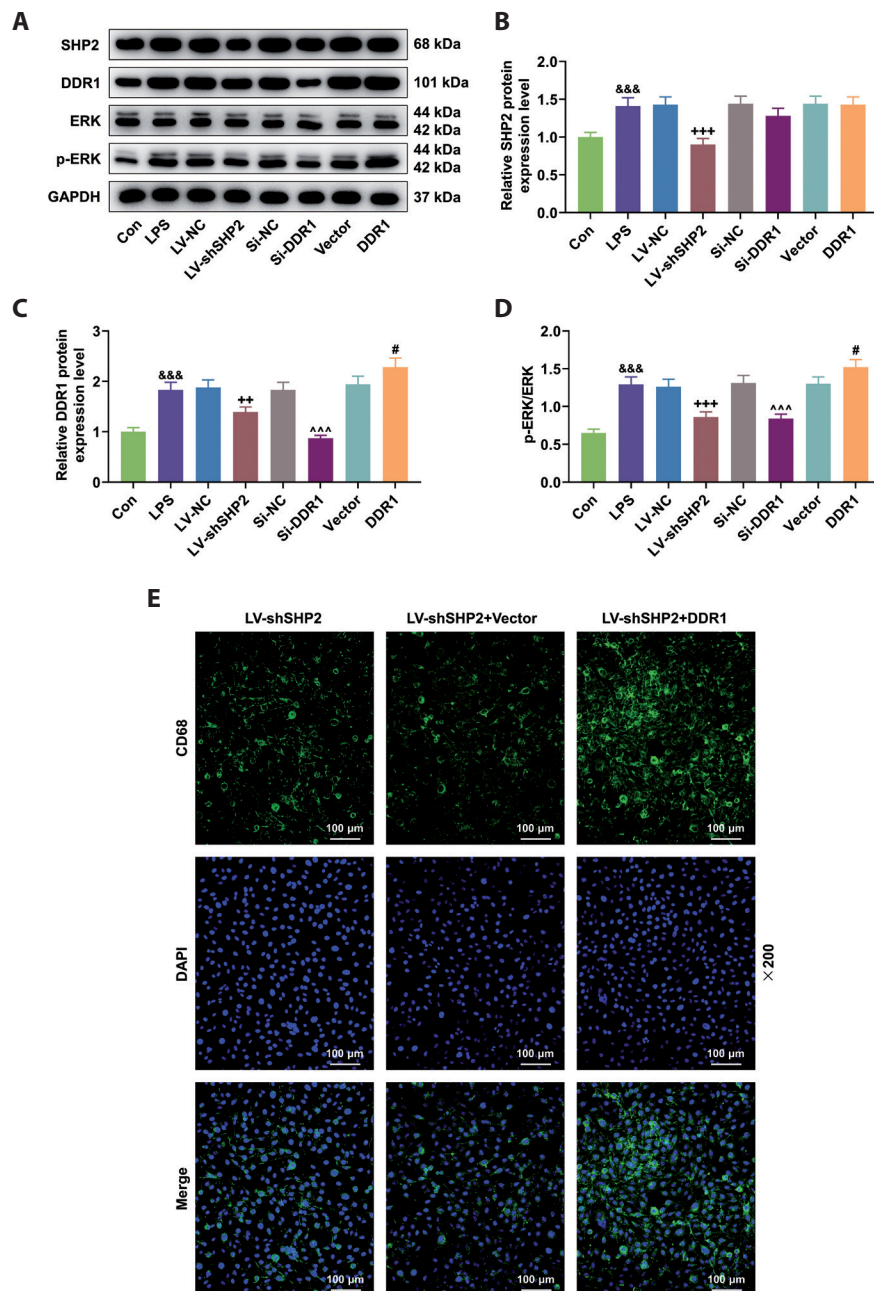


**Figure 3.** Si-DDR1/LV-shSHP2 inhibited p-ERK/ERK protein expression in LPS-induced BV-2 cells while DDR1 overexpression reversed the impacts of LV-shSHP2 on LPS-induced BV-2 cell activation. **A–D.** Western blot was conducted for analyzing SHP2, DDR1 and p-ERK/ERK protein levels in BV-2 cells treated with/without LPS and infected/transfected with LV-NC/LV-shSHP2/Si-DDR1/Vector/DDR1 overexpression. GAPDH was used as an internal reference. **E.** Microglial activation in LPS-induced BV-2 cells infected by LV-shSHP2 and transfected with DDR1 overexpression/Vector was examined by immunolabeling of CD68 with DAPI (magnification  $\times 200$ , scale bar = 100  $\mu\text{m}$ ).  $n = 3$ . &&  $p < 0.01$ , &&&  $p < 0.001$  vs. Con, ++  $p < 0.01$ , +++  $p < 0.001$  vs. LV-NC, ^^^  $p < 0.001$  vs. Si-NC, #  $p < 0.05$  vs. Vector. Si-DDR1, silencing-DDR1; Si-NC, silencing-negative control. For more abbreviations see Figure 1.

present study. Furthermore, the mitigation of post-SCI NP by shSHP2 was achieved *via* inhibiting microglial activation. SHP2 overexpression has been detected in microglia and plays a crucial role in neuroinflammation (Yue et al. 2020),

while shSHP2 weakens the accumulation of Iba-1 microglia (Han et al. 2022). Immunofluorescence assay data demonstrated that shSHP2 caused the deactivation of microglia, echoing with previous findings. Reportedly, microglia play

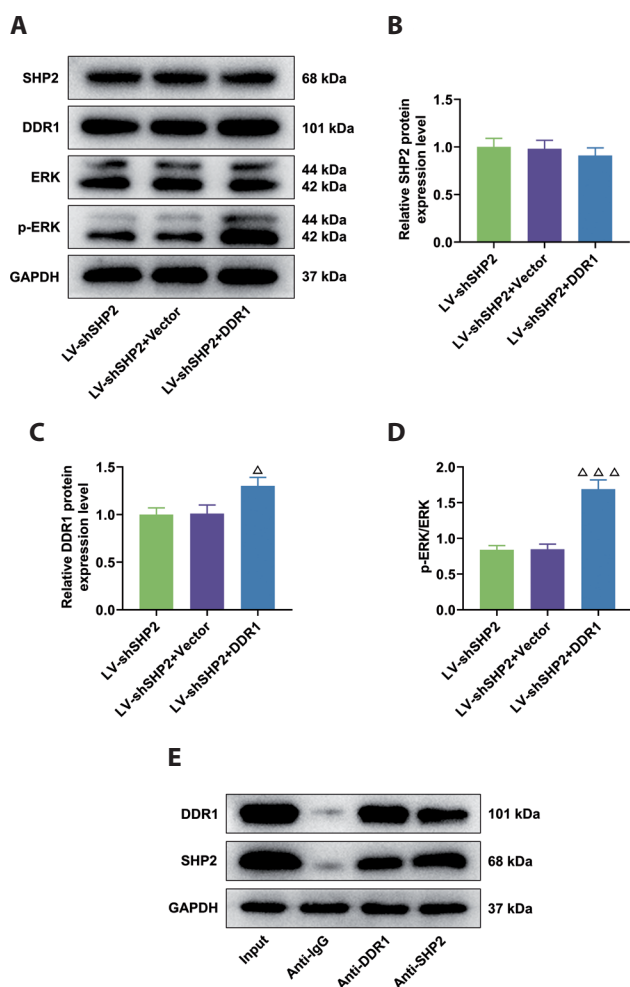


**Figure 4.** DDR1 overexpression reversed the effects of LV-shSHP2 on DDR1 and ERK signaling pathway in LPS-induced BV-2 cells. **A–D.** Western blot analysis was carried out to test the effects of DDR1 overexpression on DDR1 and p-ERK/ERK protein levels in LPS-induced BV-2 cells infected by LV-shSHP2 and transfected with DDR1 overexpression/Vector. GAPDH served as the internal control. **E.** The Pull-down assay was applied to verify the interaction between SHP2 and DDR1 in BV-2 cells with GAPDH as the reference gene.  $n = 3$ .  $\Delta p < 0.05$ ,  $\Delta\Delta\Delta p < 0.001$  vs. LV-shSHP2+Vector. For more abbreviations see Figure 1.

an important role in the development of NP (Ji and Xu 2021). Microglia activate as a result of hazardous factor stimulation, and accordingly release inflammatory mediators targeting NP (Zhong et al. 2018). Hence, microglia-related signaling pathways are promising targets for NP therapy.

The present study revealed that DDR1 could promote the activation of microglia. DDR1 has been targeted for the treatment of neurodegenerative diseases (Fowler et al. 2020) and DDR1 inhibition is implicated in suppressing microglial activity (Hebron et al. 2017). Through Western blot and

immunofluorescence assay, we indicated that DDR1 was upregulated after SCI. Meanwhile, DDR1 overexpression augmented microglial activity while DDR1 inhibition repressed the activation of microglia. These findings suggested the role of DDR1 downregulation in relieving NP triggered by activated microglia.

Moreover, DDR1 has been reported to regulate the ERK signaling pathway (Zhao et al. 2021). ERK is highly expressed in neuronal cells, and ERK signaling contributes to injury-induced pain hypersensitivity (Xu et al. 2008). ERK also amplifies pain signals and promotes the progression of NP through its phosphorylation (Lin et al. 2018). The inhibition of ERK attenuates the activation of microglia and further soothes radicular pain behaviours (Zhong et al. 2018). Therefore, the effects of shSHP2 on NP alleviation might be related to the DDR1-ERK signaling pathway. Our study further analyzed SHP2 and DDR1 targeting the ERK pathway, and found that shSHP2 inhibited the ERK signaling pathway and DDR1. DDR1 overexpression and Si-DDR1 were found to possess pro- and anti-microglia activation abilities, respectively, suggesting the regulation of DDR1 to ERK signaling pathway. Importantly, the modulation of SHP2 to the ERK pathway has been identified in previous studies (Ahmed et al. 2019). By stimulating the ERK pathway, SHP2 plays a carcinogenic role in many diseases (Song et al. 2022).

Ample evidence has demonstrated that SHP2 could be activated by DDR1 (Yeh et al. 2019). DDR1-SHP2 signaling complex is also implicated in cell migration (Mehta et al. 2021). The present study confirmed the interaction between SHP2 and DDR1 *via* the pull-down assay and the role of the SHP2-DDR1 pathway in microglial activity. DDR1 overexpression reversed the inhibition of microglial activation and p-ERK signaling pathway by shSHP2, which positioned DDR1 as a mediator in SHP2 suppressing ERK-stimulated microglial activation.

Beyond the mechanistic insights, our findings possessed notable clinical implications. The most immediate translational question is the feasibility of targeting the SHP2-DDR1 axis for treating NP. Encouragingly, several allosteric inhibitors of SHP2 have been developed and clinical trials for cancer are currently underway, which provides a valuable foundation for potential drug repurposing. Assessing the efficacy of the existing SHP2 inhibitors in preclinical models of NP could significantly accelerate translational progress. While the development of specific DDR1 inhibitors is less advanced, our study highlighted DDR1, and particularly its interaction with SHP2, as a compelling and novel target for future therapeutic development. The finding that SHP2 inhibition exerts its analgesic effect primarily by down-regulating DDR1-ERK signaling in microglia suggests that a strategy focused on this specific axis could achieve desired efficacy while potentially avoiding the broader biological consequences of complete SHP2 or DDR1 knockout. Future

work should focus on evaluating established SHP2 inhibitors in our model and exploring strategies to directly disrupt the SHP2-DDR1 interaction.

To sum up, the study suggests that the SHP2-DDR1 interaction in microglia as a critical axis promotes NP after SCI by activating the ERK pathway, and inhibition of SHP2 can attenuate activated microglia-induced NP after SCI by downregulating the DDR1/ERK signaling pathway. The findings of the present study support the efficacy of targeting microglial activity in post-SCI NP treatment, and further provide a novel insight into the modulation of microglia activation. However, the study has several limitations that should be addressed in future work. First, the experiments were conducted exclusively in male rats. Involvement of female subjects is necessary to determine the generalizability and potential sexual dimorphism of this signaling mechanism. Second, the behavioral assessment was limited to mechanical hypersensitivity. Evaluating other relevant pain modalities, such as thermal hyperalgesia and ongoing spontaneous pain, will provide a more comprehensive understanding of SHP2's role in NP. Finally, while we established DDR1 as a key mediator, other downstream pathways of SHP2 that might contribute to microglial activation and pain processing have not been explored, which warrants further investigation.

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