

Original Article

S100- β aggravates spinal cord injury via activation of M1 macrophage phenotype

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Abstract

Objectives: S100- β has been identified as a sensitive biomarker in central nervous system injuries. However, the functions and mechanisms of S100- β are unknown in spinal cord injury. **Methods:** Spinal cord injury (SCI) mouse model was generated by surgical operation, microglia activation model was established by inducing BV-2 cells with LPS. The SCI model was evaluated by Basso-Beattie-Bresnahan (BBB) behavioral score, HE staining, and Nissl staining. The expression level of S100- β was detected by qRT-PCR, western blot, and immunofluorescence. qRT-PCR and western blot were used to detect the expression of iNOS and CD16. Pro-inflammatory cytokines TNF- α and IL-1 β levels were detected by qRT-PCR and ELISA. **Results:** The expression of IL-1 β , TNF- α , iNOS, and CD16 increased at 3rd day after SCI. In BV2 microglia, LPS treatment promoted the expression of S100- β , IL-1 β , TNF- α , iNOS, and CD16. Knockdown of S100- β reduced the expression of iNOS stimulated by LPS. Over-expression of S100- β increased IL-1 β and TNF- α , and S100- β inhibition suppressed IL-1 β and TNF- α . In SCI mice, knockdown of S100- β attenuated the spinal cord injury and inhibited the expression of iNOS, IL-1 β , and TNF- α . **Conclusions:** Down-regulation of S100- β could inhibit the pathogenesis of SCI and inhibit the activation of M1 macrophages. S100- β may be a useful diagnostic biomarker or therapeutic target for SCI.

Keywords: S100-B, Spinal Cord Injury, M1 Phenotype, Macrophage, Pro-Inflammatory Factors

Introduction

Spinal cord injury (SCI) is one common and serious neurological condition^{1,2}; the characteristics are high disability rate, irreversible motor and sensory deficits, which cause immense socioeconomic impact on affected individuals and the health care system^{3,4}. Currently, 2.5-4 million people around the globe suffer from pain due to SCI^{5,6}. However, there are no effective treatments for SCI^{7,8}. Therefore, understanding the pathogenesis is essential to develop effective therapeutic strategies for SCI.

The development of SCI is accompanied by a robust and persistent inflammatory response⁹. Microglia, the dominant immune cells of the central nervous system (CNS), had been reported as a key cellular component to protect neural tissues after SCI^{10,11}. Two macrophage subsets dominate sites of CNS injury, phenotype M1 (classical activation) and phenotype M2 (alternative activation)¹⁴. It is reported that SCI-related tissue damage is characterized by M1 phenotype activation and inflammation, promote microglia M2 polarization or inhibit microglia polarization to M1 phenotype could attenuate the inflammation after the SCI^{15,16}. For instance, tumor necrosis factor- α (TNF- α), activation factors of M1 phenotype, induced microglia polarization to M1 in the injured spinal cord and produce a variety of pro-inflammatory cytokines, including IL-1 β , TNF- α ¹⁷⁻¹⁹. Meanwhile, increased expression of markers of the M1 phenotype, include inducible nitric oxide (iNOS), and cell surface markers such as CD16, had been detected after SCI²⁰.

S100- β is a neuronal survival protein synthesized mainly by mature astroglia²¹. It is a member of S100 proteins

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family that is a part of the superfamily of calcium-modulated proteins²². S100 proteins are highly enriched in the nervous system, and many of them reside in the glia^{23,24}. As a marker for glial cells, the role of S100- β has been studied extensively in the past 30 years. Yasuda et al.²⁵ has been reported that higher concentration of S100- β in the extracellular space induces the production of iNOS in astrocytes, which contributes to infarct expansion that results in DNA damage or cell death. Additionally, recent research suggested that S100- β might be a valuable biomarker for silent brain injury in carotid revascularization²⁶. However, it is not clear that the functions and mechanisms of S100- β in SCI.

This study investigated the effects of S100- β on inflammation and activation of macrophages/microglia after SCI.

Materials and methods

Animals

To establish the SCI model, the C57B/6J mice (20-25 g, 8-week-old) were anesthetized by an intraperitoneal injection of xylazine (40 mg/kg) and ketamine (80 mg/kg). After skin and paravertebral muscle detachment, thoracic laminectomy was performed to expose the spinal cord on T9 and T10. The dorsal dural surface was then exposed to a commercial spinal cord contusion device (60 kdyn using Infinite Horizon impactor, Precision Systems & Instrumentation, Lexington, KY). The spinal cord injury mice (n=36) were randomly divided into six groups (6 mice per group), which were euthanized at 12 hours, 1 day, 3 days, 5 days, 7 days, 14 days after SCI. A group of 6 mice constituted the Sham group (surgical control) which had the same surgical procedures with SCI group but without contusion. The sham group was euthanized at 2 weeks. To prevent the infection and death of SCI mice caused by surgery, the mice placed in a constant temperature cage after surgery and treated with artificial bladder expression twice a day until recovery.

To reduce the expression of S100- β , 10 μ l of S100- β lentivirus (10^9 TU/mL) was injected into the injury site after SCI. The sham group and the SCI group were injected with the same dose of an empty lentiviral vector. More than 6 mice injected lentiviral in every group, and mice were euthanized at 3d after injection for further experiments.

This study was approved by the Committee on Animal Care and Use of the ethical committee of the Affiliated Hospital of Xi'an Jiaotong University.

Basso-Beattie-Bresnahan (BBB) behavioral score

BBB behavioral score was used to evaluation of hindlimb motor function. The stage score has been described previously²⁷. The mice were placed on a circular platform with a diameter of 2 m. The hindlimbs' walking and limb activity scores were observed and recorded at the 1, 3, 5, 7, 14 days after SCI and 1 day before surgery.

Tissue collection

At the predetermined time points, the mice were anesthetized, the diaphragm was cut, and the pericardium was opened. The connective tissue and meninges were stripped off, then the spinal cord above and below 5 mm of the injury site was carefully dissociated with scalpels and scissors. These tissues were placed into -80°C for further experiments. Some mice were perfused with PBS, and the right atrial appendage was cut at the same time. After the effluent liquid became clear, 4% PFA was perfused until the tissues became hard. The spinal cord about the injury site was removed and placed in 4% PFA overnight for histopathological staining and immunofluorescence.

HE staining

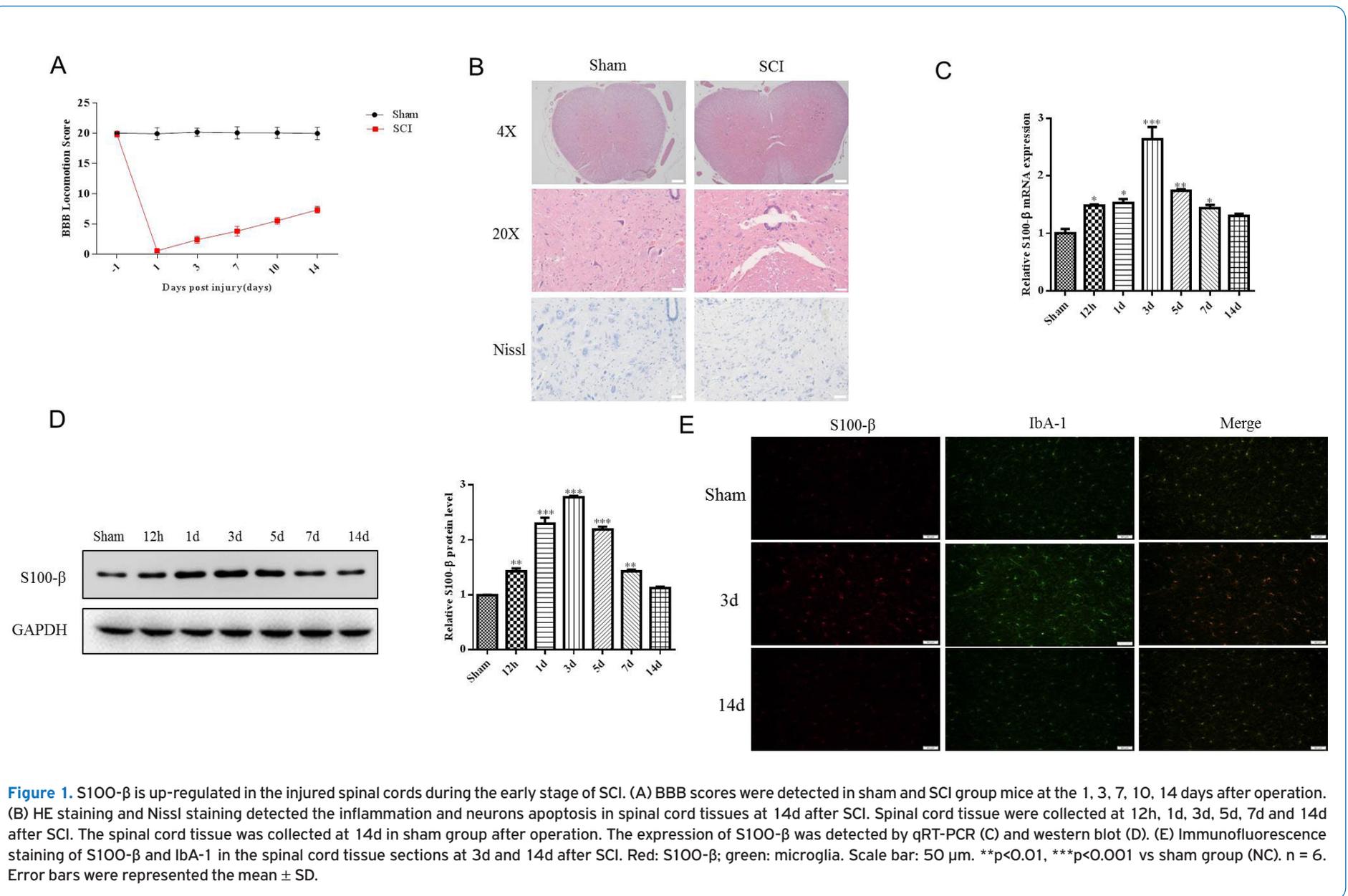
The tissues of 14d after SCI were dehydrated through xylene and gradient alcohol solutions. Then the tissues were embedded by paraffin and cut into 4 μ m sections. After dewaxing and hydration, the sections were stained with haematoxylin solution for 1 min and washed twice by distilled water. Following, the sections were differentiated in 1% hydrochloric acid and stained with eosin for 2 min after washed twice by distilled water. Finally, the sections were dehydrated with alcohol and vitrification by xylene. The stained sections were mounted by neutral balsam and observed through light microscope (Leica, Germany).

Nissl staining

The tissues of 14d after SCI were embedded by paraffin and cut 4 μ m sections. First, sections were deparaffinized with xylene and rehydrated with gradient alcohol. Then, sections were stained with Nissl staining solution for 5 min and mounted with neutral balsam. The apoptotic neurons were shrunken or contained vacuoles. The normal neurons had a relatively large and full soma, with round, large nuclei. The neurons were observed through microscope (Leica, Germany) randomly in five areas.

Immunofluorescence (IF)

The tissues of 3d and 14d after SCI were embedded by paraffin and cut 4 μ m sections. The sections were deparaffinized with xylene and rehydrated with gradient alcohol. Then, the antigen in sections was retrieval by citric acid. The sections were incubated with S100- β (ab52642, 1:100 dilution, Abcam, USA) and Iba-1 (ab48004, 1:100 dilution, Abcam, USA) antibody for overnight at 4°C. The sections were washed with PBS and incubated with appropriate fluorescently labeled secondary antibody for 1 hour at room temperature. The sections were mounted after wash three times by PBS. Fluorescence was detected using an Axio Imager optical sectioning microscope with ApoTome (Carl Zeiss).



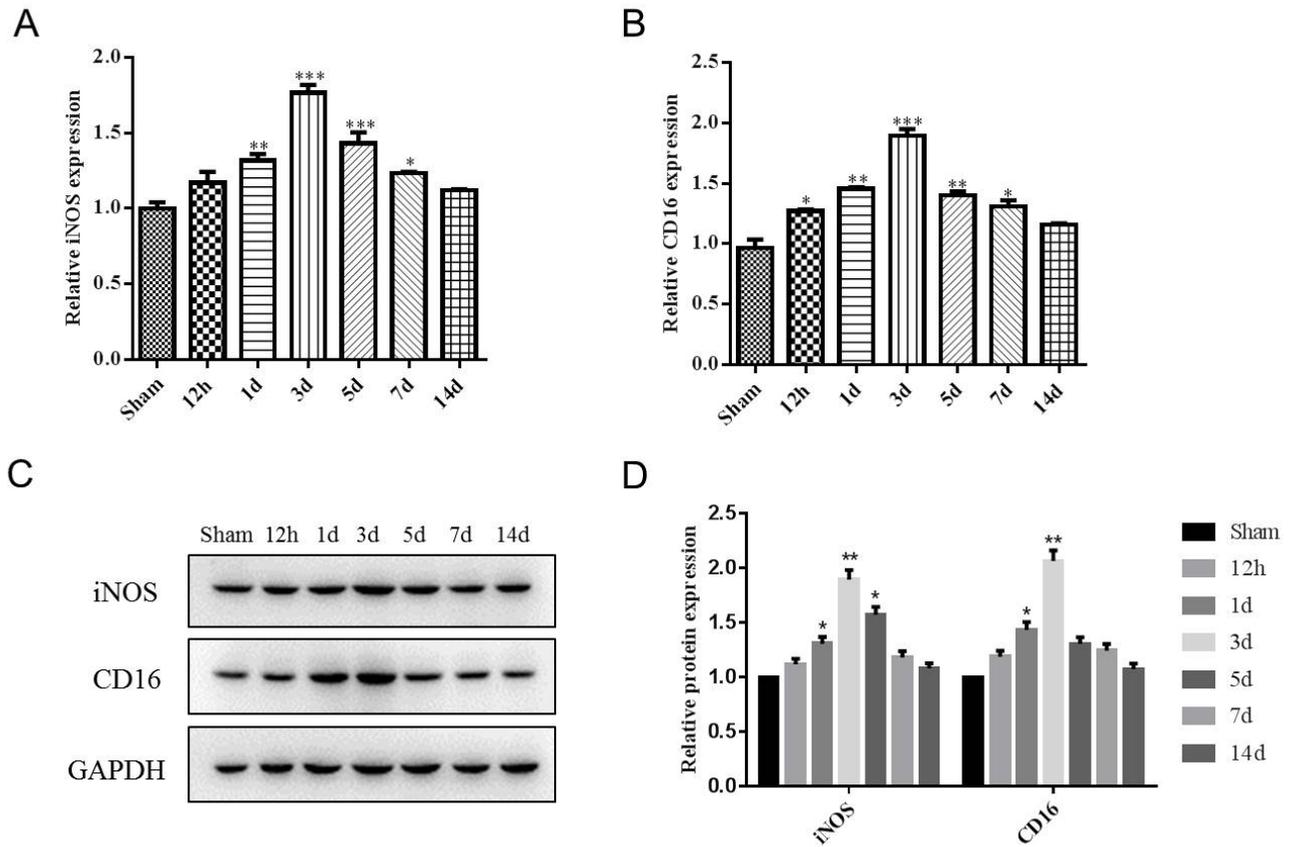


Figure 2. SCI promotes the activation of M1 macrophage. Determine the expression of M1 polarization markers in mice spinal cord tissue that 12h, 1d, 3d, 5d, 7d and 14d after SCI. qRT-PCR were performed to detect iNOS (A) and CD16 (B) expression in mRNA levels. (C) iNOS and CD16 protein expression were detected by western blot. (D) Quantitative analysis the western blot results. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs sham group (NC). Error bars were represented the mean \pm SD.

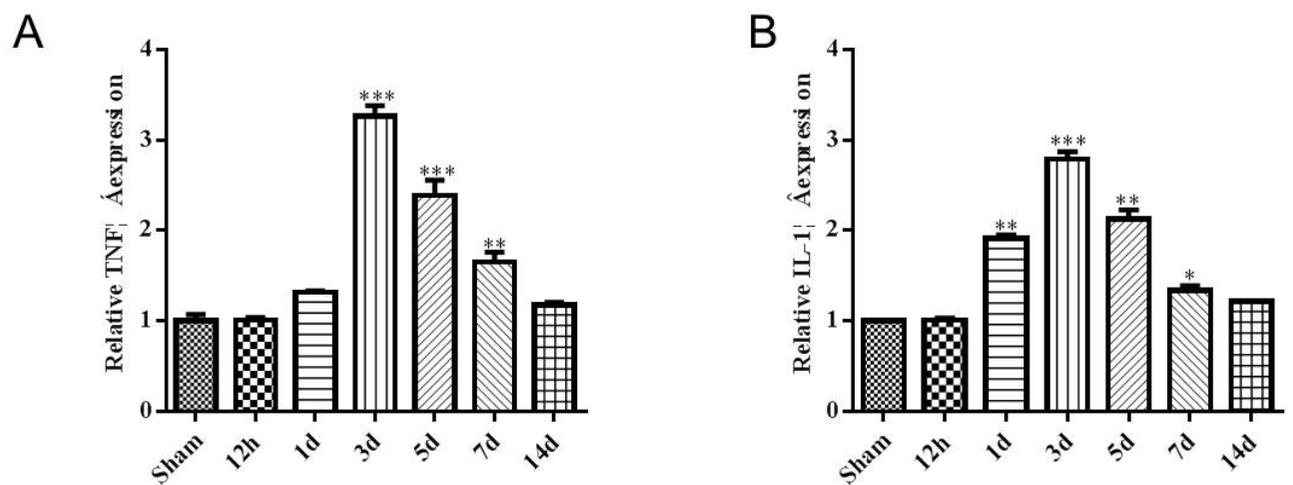
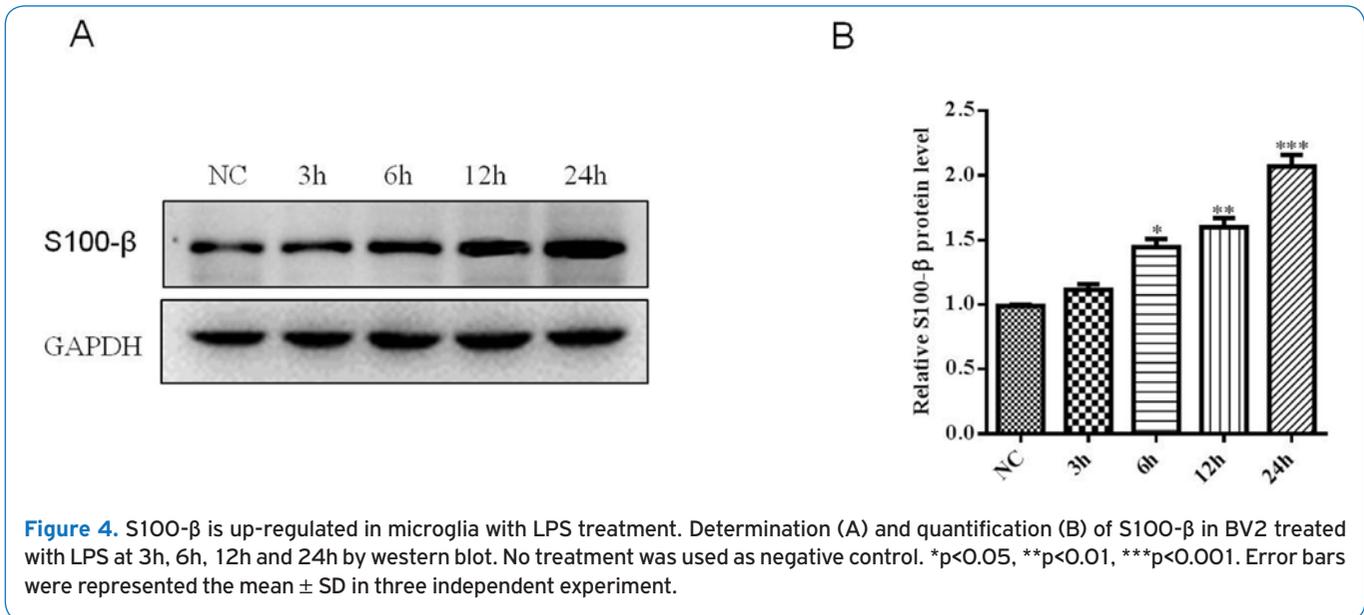


Figure 3. SCI promotes the release of pro-inflammatory factors. qRT-PCR analysis of pro-inflammatory factors after 12h, 1d, 3d, 5d, 7d and 14d post-SCI in spinal cord tissue of mice. (A) TNF- α ; (B) IL-1 β ; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs sham group (NC). Error bars were represented the mean \pm SD.



Cell culture and cell transfect

BV2 cells were purchased from Procell Life Science&Technology Co.,Ltd (Wuhan, Hubei, China), and cultured in DMEM (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS (Gibco, Carlsbad, CA, USA), 50 g/ml streptomycin (Invitrogen, Carlsbad, CA, USA), and 50 U/ml penicillin in a humidified atmosphere of 95% air and 5% CO₂. BV2 cells were grown for 4 days, and medium was replaced by DMEM (Dulbecco's Modified Eagle's Medium) solution containing LPS (lipopolysaccharides, 1 ug/ml). BV2 cells were collected for further experiments cultured with LPS for 3h, 6h, 12h, 24h.

For the knockdown or overexpression of S100-β, BV2 cells cultured in normal DMEM medium or LPS contained DMEM medium for 24h and were transfected with S100-β siRNA (30 nM) or S100-β plasmid by using Lipofectamine 3000 (Thermo-Scientific, USA) and cultured for 48h. The negative control (NC) siRNA and S100-β siRNA sequences were purchased from GenePharma (Shanghai, China). S100-β siRNA, sense: AAUCCAAGUUACAUUUAAAUA, anti-sense: UUUAAAUGUAACUUGGAUUUAU. NC siRNA, sense: UUCUCCGAACGUGUCACGUTT, anti-sense: ACGUGACACGUUCGGAGAATT.

qRT-PCR

According to the manufacturer's protocols, total RNA was extracted and reverse transcribed using a PrimeScript RT reagent kit with random primers (Invitrogen, San Diego, CA, USA). The qRT-PCR was performed using 2×SYBR Green PCR Master Mix (Takara, Dalian, China). The qRT-PCR reaction system contained 1 μl of cDNA, 0.5 μl of forward primer (10 μM), 0.5 μl of reverse primer (10 μM), 5 μl of 2×SYBR Green PCR Master Mix and 3 μl of double-distilled water. The qRT-PCR reaction was conducted at 95°C for 30

s, followed by 40 cycles of 95°C for 10 s, and 60°C for 30 s. The results were normalized to GAPDH and then calculated using the 2^{-ΔΔCq} method.

The primers sequence is as follows: S100-β (forward: GATGTCCGAGCTGGAGAAGG, reverse: CCTGCTCCTTGATT CCTCA); iNOS (forward: GTTCTCAGCCCAACAATACAAGA, reverse: GTTCTCAGCCCAACAATACAAGAC); CD16 (forward: CTGCTGCTGTTTGCTTTTGC, reverse: TTCGCACATCAGT GTCACCA); TNF-α (forward: GATCGGTCCCCAAAGGGATG, reverse: CCACTTGGTGGTTTGTGAGTG); IL-1β (forward: TGCCACCTTTTGACAGTGATG, reverse: TGATGTGCTGCTGCC AGATT).

Western blot

Total protein was extracted in a RIPA buffer (Beyotime, China), and subsequently, the BCA assay (Thermo Scientific, USA) was used to analysis concentration. The dissolved protein samples were separated by SDS-polyacrylamide gel electrophoresis, which was then transferred to nitrocellulose membranes. Subsequently, membranes were incubated with 5% skim milk in TBST (25 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.5) at 1h to block reactions. Membranes were incubated with primary antibodies against S100-β (1:1000, Abcam, USA), iNOS (1:1000, Abcam, USA), CD16 (1:1000, Abcam, USA) and GAPDH (1:1000, Abcam, USA) overnight at 4°C, follow incubated by goat anti-rabbit HRP-conjugated secondary antibody (1:5000, Abcam, USA) at room temperature for 1h, and section colors were developed with ECL (Synthgene, China).

Enzyme-linked immunosorbent assay (ELISA)

Inflammatory factors of TNF-α and IL-1β in the injured spinal cord were detected by ELISA kit (Beyotime

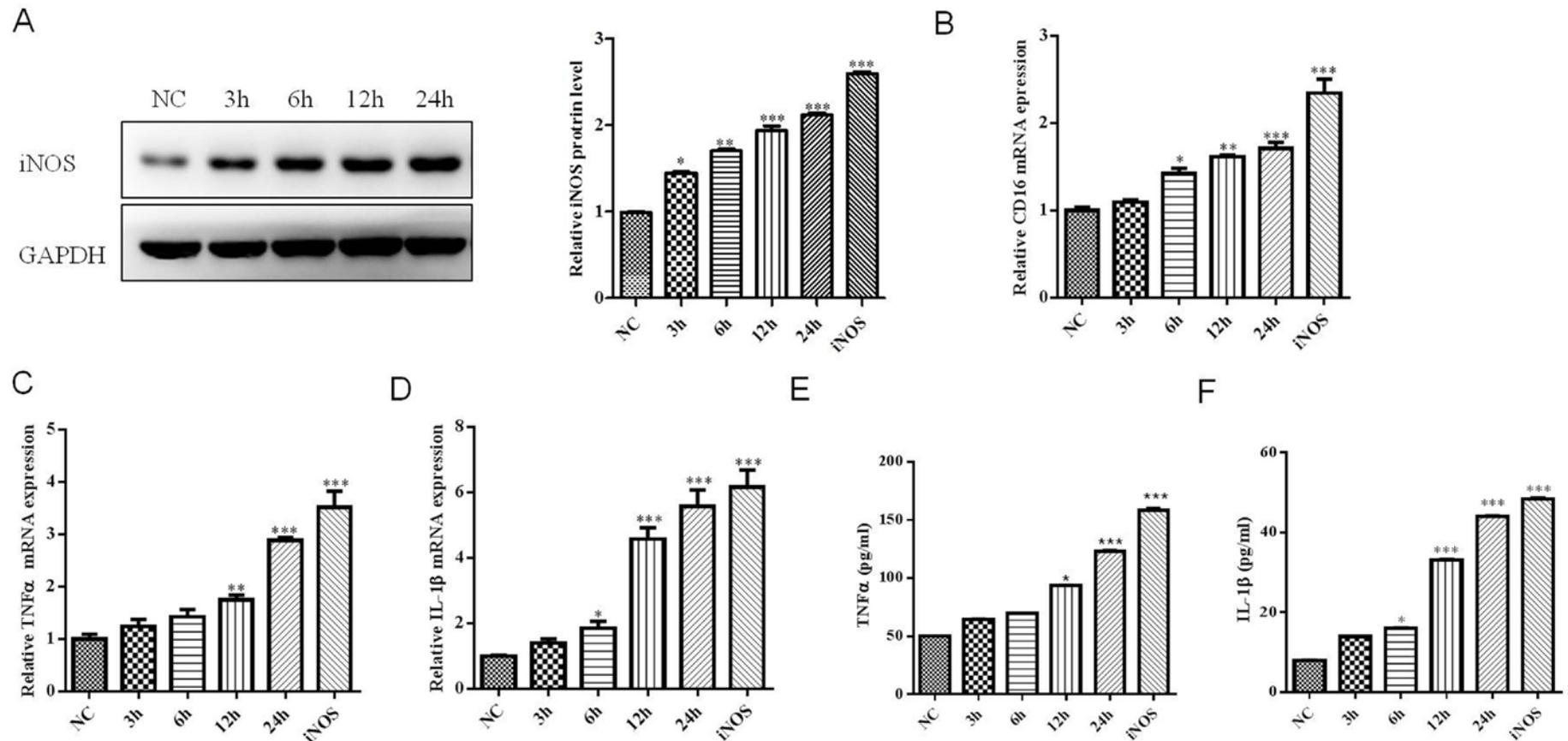


Figure 5. LPS treatment induces the activation of M1 phenotype and inflammation. Determination of the expression of M1 polarization markers and pro-inflammatory cytokines in BV2 treated with LPS at 3h, 6h, 12h and 24h. (A) Western blot detected and quantitative analysis iNOS protein expression. (B) qRT-PCR analysis CD16 expression in mRNA levels. qRT-PCR analysis inflammatory cytokines TNF- α (C) and IL-1 β (D) expression in mRNA levels. TNF- α (E) and IL-1 β (F) protein expression was detected by ELISA assay. No treatment was used as negative control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Error bars were represented the mean \pm SD in three independent experiment.

Statistical analysis

Biotechnology, China). The absorbance value at 450 nm was measured by Multiplex Microplate Reader (Thermo-Fisher, USA). The measured OD values were converted into concentration value.

Statistical significance of difference was examined by the unpaired two-tailed Student's t-test. Differences were considered significant for $P < 0.05$. Statistical

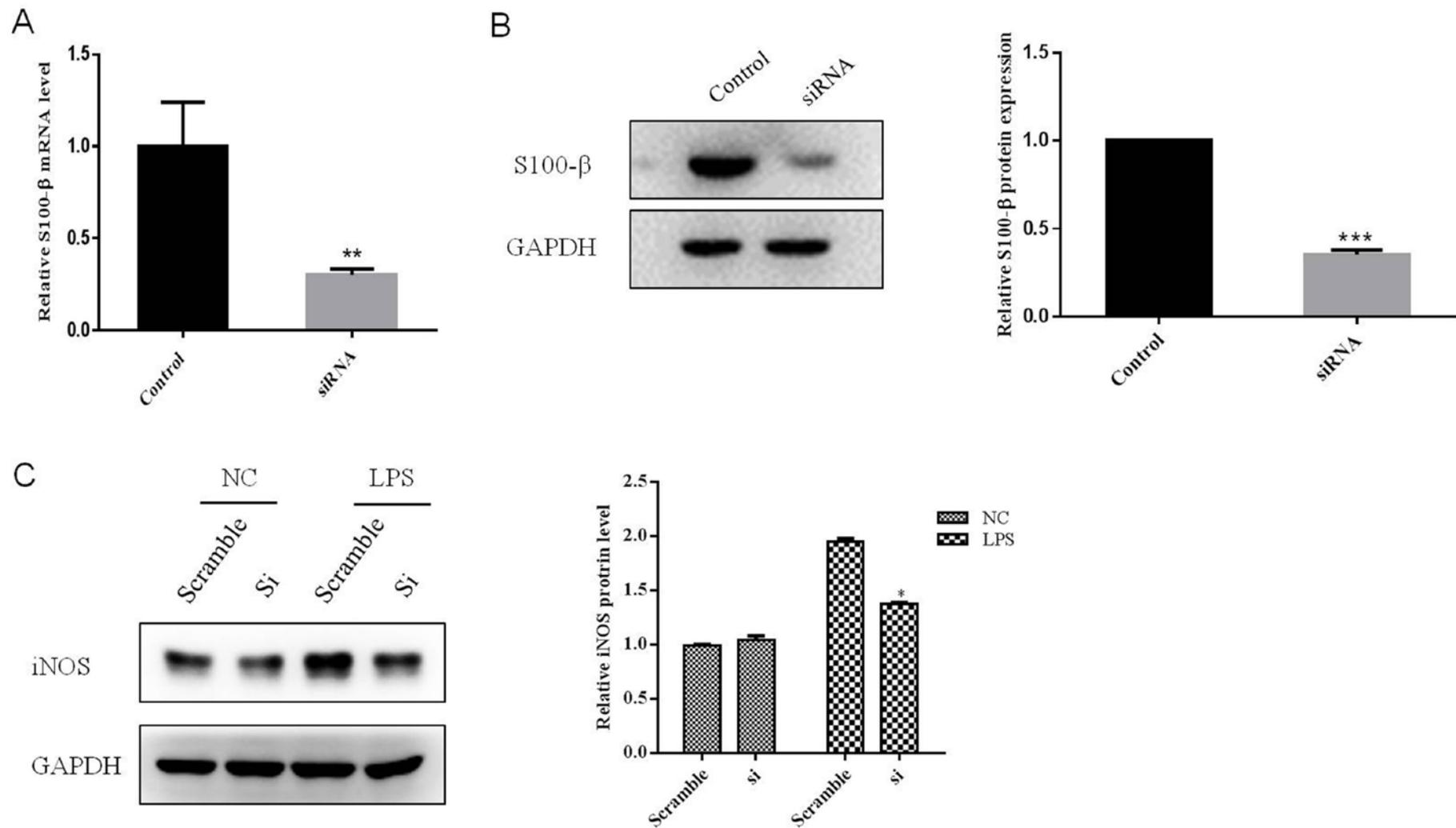


Figure 6. S100- β silencing decreases the overexpression of iNOS induced by LPS in microglia. (A) S100- β mRNA expression detected by qRT-PCR in transfected si-S100- β and LPS-stimulated BV2 cells. (B) Determination and quantification the protein expression of S100- β in LPS-stimulated and transfected with si-S100- β BV2 cells. (C) BV2 cells cultured 24h with control medium or LPS stimulated medium and then transfected with si-S100- β or si-NC. Western blot was used to detect iNOS protein expression. * $p < 0.05$ vs scramble. Error bars were represented the mean \pm SD in triplication experiments.

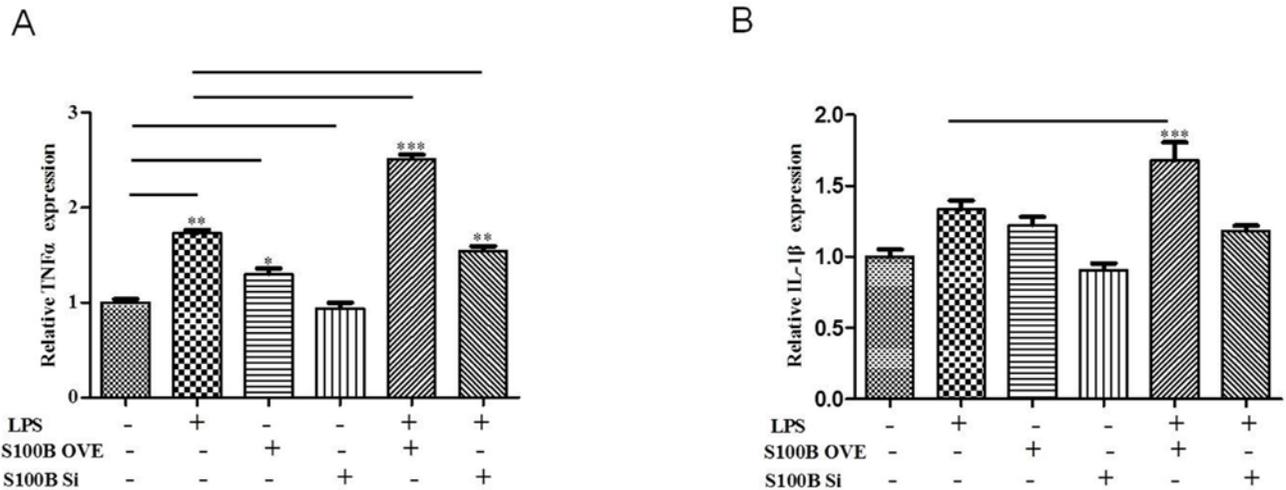


Figure 7. Effects of S100-β on the expression of inflammation-associated genes in BV2 treated with LPS or without. BV2 cells cultured 24h with control medium or LPS contained medium. Then the BV2 cells transfected with si-S100-β, S100-β vector or corresponding control, separately. The mRNA expression of pro-inflammatory factors TNF-α (A) and IL-1β (B) was detected by qRT-PCR. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Error bars were represented the mean \pm SD in triplication experiments.

analysis was performed using GraphPad Prism 7.0. Data were expressed as mean \pm SD.

Results

S100-β is up-regulated in the injured spinal cord during the early stage of SCI

The BBB scores were assessed to evaluate the locomotion recovery after SCI²⁸. The BBB score of sham group mice was unaltered after the operation. At the same time, the BBB score of SCI group mice was severely impaired immediately after the SCI and gradually recovered within 14d after operation (Figure 1A). The HE staining of paraffin-embedded showed that the infiltration of inflammation cells was increased in SCI mice at 14d after operation. The Nissl staining results showed that the apoptosis ratios of neurons were increased in SCI mice at 14d after operation, compared with the sham group mice (Figure 1B). To explore the effects of S100-β in SCI, we calculated whether the injury affected the expression of S100-β. qRT-PCR and western blot were performed to detect the expression of S100-β in mRNA and protein levels, respectively (Figure 1C & D). We found that S100-β was generally up-regulated after SCI and reached the peak on the third day. Similarly, IF staining exhibited that S100-β were higher in the tissue sections at 3rd day post-SCI than sham group while decreasing at 14th day (Figure 1E). These results demonstrated that S100-β was up-regulated during the early stage of SCI.

SCI promotes the activation of M1 phenotype and inflammation

We next detected the expression of M1 polarization markers, iNOS and CD16, and the pro-inflammation factors, TNF-α and IL-1β, after SCI. The results indicated that iNOS and CD16 were significant increased from day 1 to 3 after SCI, and decreased from day 3 to 14 after SCI (Figure 2). Meanwhile, the pro-inflammation factors, TNF-α and IL-1β, were induced and peaked at day 3 (Figure 3). These results suggested SCI promoted M1 phenotype activation and inflammation.

S100-β is up-regulated in microglia with LPS treatment

The effects of S100-β were further investigated in BV2 microglia treated with LPS. As shown in Figure 4, S100-β was highly increased in LPS-stimulated BV2. Moreover, LPS treatment induced over-expression of M1 polarization markers and inflammatory cytokines, including TNF-α and IL-1β (Figure 5).

S100-β-knockdown reduces the overexpression of iNOS induced by LPS in microglia

To investigate whether S100-β could regulate M1 phenotype activation of microglia, the expression level of iNOS was determined in BV2 that cultured in LPS conditions or transfected S100-β siRNA, separately or simultaneously. S100-β knock down was detected by qRT-PCR and western blot (Figure 6A-B). Western blot analysis indicated that LPS-stimulated over-expression of iNOS was partly eliminated by S100-β-knockdown, while no significant difference

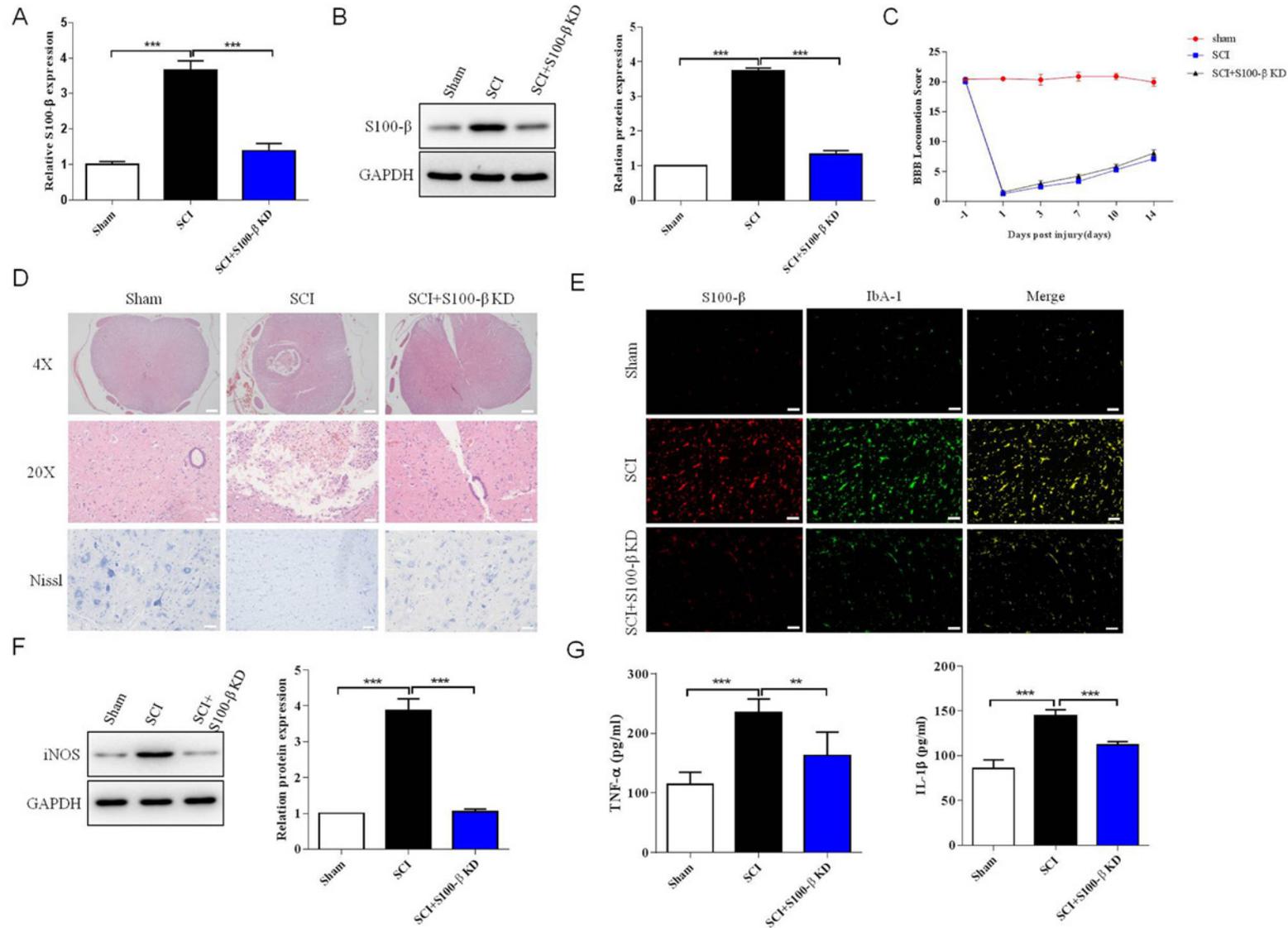


Figure 8. S100-β-knockdown inhibits the activation of M1 phenotype and the expression of pro-inflammation factor *in vivo*. S100-β siRNA lentivirus were injected in the injury sites after SCI. Control lentivirus were injected in the SCI mice and sham mice after operation. At the 3d after operation, the spinal cord tissues about the injury sites were collected. The expression of S100-β was detected by qRT-PCR (A) and western blot (B). (C) The BBB scores were detected at the 1, 3, 7, 10, 14 days after operation. (D) HE staining and Nissl staining detected the inflammation and neurons apoptosis. (E) Immunofluorescence staining of S100-β and IbA-1. (F) Western blot detected the expression of iNOS at the protein level. (G) The level of TNF-α and IL-1β was detected by ELISA. ** $p < 0.01$, *** $p < 0.001$. $n = 6$. Error bars were represented the mean \pm SD.

was observed in BV2 without LPS (Figure 6C). The result indicated that S100- β silence could inhibit M1 polarization in an inflammatory environment, while no apparent functions at normal conditions.

Expression of pro-inflammatory factors in S100- β -treated microglia

In order to study whether or not S100- β influences inflammatory responses, we detected the expression of IL-1 β and TNF- α in BV2 that use LPS stimulated or transfect S100- β plasmid and siRNA, respectively or simultaneously. As shown in Figure 7, both IL-1 β and TNF- α were significantly higher in S100- β -treatment BV2, whether LPS-stimulated or not. Notably, the expression of TNF- α regulated by S100- β is more pronounced than IL-1 β . These findings demonstrated that high expression of S100- β aggravates inflammation, and S100- β -knockdown inhibits the release of pro-inflammatory factors.

S100- β -knockdown inhibits the activation of M1 phenotype and the expression of pro-inflammation factor in vivo

The S100- β siRNA lentivirus was injected in injury site of SCI mice after the operation. The control lentivirus was injected in the same sites of SCI mice and sham mice after the operation. To evaluate the knockdown of S100- β , qRT-PCR and western blot were performed, and the results showed that S100- β expression was decreased in SCI and S100- β siRNA injected group than SCI group after the 3d of operation (Figure 8A, 8B). According to BBB score, we found that the mice were severely impaired immediately after the SCI and gradually recovered within 14d after operation. The knockdown of S100- β induced better recovery of motor function in SCI mice (Figure 8C). Then, HE staining and Nissl staining were used to detect the inflammation and neurons apoptosis in 3d after operation. The HE staining results showed that decreased the expression of S100- β inhibited the infiltration of inflammatory cells and promoted the recovery of SCI. Nissl staining results showed that knockdown of S100- β decreased the apoptosis ratios of neurons (Figure 8D).

Similarly, IF staining results displayed that S100- β were higher in the tissue sections at 3 day post-SCI compared to sham group. Meanwhile, the expression of S100- β was decreased in SCI, and S100- β siRNA injected group than SCI group at 3 day post-SCI (Figure 8E). To confirm S100- β affects the M1 macrophage and pro-inflammatory factor, we detected the expression of iNOS through western blot and detected TNF- α , IL-1 β levels through ELISA. The results showed that SCI induced the increase of iNOS, TNF- α , and IL-1 β levels. However, knockdown of S100- β inhibited the effect of SCI on these genes expression (Figure 8F & 8G). These results suggested that knockdown of S100- β inhibited the activation of M1 phenotype and the expression of pro-inflammation factor *in vivo*.

Discussion

The pathophysiology of SCI is divided into two stages: the primary stage is caused by the serious mechanical injury, and the following stage comprises a cascade of degenerative events to complicate further and enlarge the mechanical damage^{29,30}. Multiple molecular mechanisms are involved in the progressive spread of secondary injury, and inflammation is the most critical pathological process. The present study demonstrated the molecular mechanism of S100- β after SCI, emphasizing macrophages activation and inflammatory response.

First of all, our data indicated that S100- β was over-expressed in the injured spinal cord tissues in SCI mice. Further, with the up-regulation of S100- β , M1 polarization markers, iNOS and CD16, and pro-inflammatory factors, TNF- α and IL-1 β , were increased significantly. It is reported that microglia and macrophages rapidly accumulated at the injury site within the first hours after mechanical damage and remain there until the end of the first week^{31,32}. Meanwhile, activated macrophages release various inflammation cytokines, including TNF- α and IL-1 β ^{33,34}. Notably, in our study, the expression of S100- β was continually increased from 12 h to day 3 post-SCI and then dropped off. In addition, M1 activation markers and pro-inflammatory factors exhibited similar expression patterns to S100- β . These results suggested that S100- β might play critical roles in both M1 activation and inflammation, which trigger an acute and rapid response to aggravate SCI.

In the injury environment, microglia and macrophages, because of heterogeneity of these cells' responses to injury, present their unique functional roles, including but not limited to activation of distinct immune phenotypes, production of reactive oxygen species, and inflammation^{16,35-37}. At the primary stage of SCI, the mechanical forces applied to the tissue destroys the plasma membranes of neuron and endothelial cells³². The deterioration induces the release of inflammatory factors, and secondary damage occurs³⁸. The pro-inflammatory cytokines, such as TNF- α and IL-1 β are secreted mainly by microglia and activate other macrophage cells. Activated macrophage cells extensively secrete pro-inflammatory cytokines, consequently accelerating the inflammation and SCI^{39,40}. In our study, the expression level of S100- β was successfully induced in LPS-stimulated BV2 cells. LPS treatment promoted the expression of pro-inflammatory regulators, including TNF- α and IL-1 β , which were dramatically down-regulated by S100- β -knockdown in BV2 cells. Additionally, knockdown of S100- β in SCI mice down-regulated the expression of pro-inflammatory. These results suggested S100- β promoted SCI via regulating the expression of the pro-inflammatory factors.

On the other hand, activated macrophages are divided into two main subtypes: the classically activated M1 macrophages and the alternatively activated M2 macrophages^{14,16}. The phenotypic responses and function are strongly regulated by cytokines in the microenvironment^{16,41}. M1 macrophages

participate in neuro-inflammation and lesion expansion by releasing pro-inflammatory cytokines and free radicals, while M2 macrophages release anti-inflammatory cytokines to reduce inflammation⁴². In our study, M1 phenotype marker, iNOS was highly induced by LPS in BV2 cells while being reversed by S100- β -silence. The results consistent with the *in vivo* experiments results. These results suggested that S100- β inhibition suppressed the activation of M1 phenotype.

In summary, S100- β was increased in the injured spinal cord tissues in SCI mice and LPS-treated BV2 cells. SCI and LPS promoted inflammatory response and activation of M1 macrophages. Over-expression of S100- β induced TNF- α and IL-1 β , while S100- β -silence suppressed the inflammation and polarization of M1 macrophages. These findings indicated that inhibition of S100- β might reduce secondary damage, which has great therapeutic potential to improve SCI.

Funding

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References

- Adams CM, Stacy R, Rangaswamy N, Bigelow C, Grosskreutz CL, Prasanna G. Glaucoma - Next Generation Therapeutics: Impossible to Possible. *Pharm Res* 2018;36(2):25.
- Wang J, Ishfaq M, Xu L, Xia C, Chen C, Li J. METTL3/m6A/miRNA-873-5p Attenuated Oxidative Stress and Apoptosis in Colistin-Induced Kidney Injury by Modulating Keap1/Nrf2 Pathway. *Front Pharmacol* 2019;10:517.
- Cui M, Ma X, Sun J, He J, Shen L, Li F. Effects of STAT3 inhibitors on neural functional recovery after spinal cord injury in rats. *Biosci Trends* 2017;10(6):460-466.
- Dietz V, Fouad K. Restoration of sensorimotor functions after spinal cord injury. *Brain* 2014;137(Pt 3):654-67.
- Oyinbo CA. Secondary injury mechanisms in traumatic spinal cord injury: a nugget of this multiply cascade. *Acta Neurobiol Exp (Wars)* 2011;71(2):281-99.
- van den Berg ME, Castellote JM, de Pedro-Cuesta J, Mahillo-Fernandez I. Survival after spinal cord injury: a systematic review. *J Neurotrauma* 2010;27(8):1517-28.
- Xu S, Zhu W, Shao M, Zhang F, Guo J, Xu H, Jiang J, Ma X, Xia X, Zhi X, Zhou P, Lu F. Ecto-5'-nucleotidase (CD73) attenuates inflammation after spinal cord injury by promoting macrophages/microglia M2 polarization in mice. *J Neuroinflammation* 2018;15(1):155.
- Rabchevsky AG, Patel SP, Springer JE. Pharmacological interventions for spinal cord injury: where do we stand? How might we step forward? *Pharmacol Ther* 2011;132(1):15-29.
- Song G, Yang R, Zhang Q, Chen L, Huang D, Zeng J, Yang C, Zhang T. TGF- β Secretion by M2 Macrophages Induces Glial Scar Formation by Activating Astrocytes *In Vitro*. *J Mol Neurosci* 2019;69(2):324-332.
- Karavelioglu Y, Cagli K, Yetim M, Bekar L, Golbasi Z. Missed unroofed coronary sinus. *Echocardiography* 2019;36(3):613-614.
- Kettenmann H, Hanisch UK, Noda M, Verkhratsky A. Physiology of microglia. *Physiol Rev* 2011;91(2):461-553.
- Hains BC, Waxman SG. Activated microglia contribute to the maintenance of chronic pain after spinal cord injury. *J Neurosci* 2006;26(16):4308-17.
- Schomberg D, Olson JK. Immune responses of microglia in the spinal cord: contribution to pain states. *Exp Neurol* 2012;234(2):262-70.
- Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective. *Annu Rev Immunol* 2009;27:451-83.
- Kroner A, Rosas Almanza J. Role of microglia in spinal cord injury. *Neurosci Lett* 2019;709:134370.
- Ghosh M, Xu Y, Pearse DD. Cyclic AMP is a key regulator of M1 to M2a phenotypic conversion of microglia in the presence of Th2 cytokines. *J Neuroinflammation* 2016;13:9.
- Kroner A, Greenhalgh AD, Zarruk JG, Passos Dos Santos R, Gaestel M, David S. TNF and increased intracellular iron alter macrophage polarization to a detrimental M1 phenotype in the injured spinal cord. *Neuron* 2014;83(5):1098-116.
- Streit WJ, Graeber MB, Kreutzberg GW. Functional plasticity of microglia: a review. *Glia* 1988;1(5):301-7.
- Benoit M, Desnues B, Mege JL. Macrophage polarization in bacterial infections. *J Immunol* 2008;181(6):3733-9.
- Chhor V, Le Charpentier T, Lebon S, Oré MV, Celador IL, Jossierand J, Degos V, Jacotot E, Hagberg H, Sävman K, Mallard C, Gressens P, Fleiss B. Characterization of phenotype markers and neuronotoxic potential of polarised primary microglia *in vitro*. *Brain Behav Immun* 2013;32:70-85.
- Çevik S, Özgenç MM, Güneyk A, Evran Ş, Akkaya E, Çalış F, Katar S, Soyalt C, Hanımoğlu H, Kaynar MY. NRG1, S100B and GFAP levels are significantly increased in patients with structural lesions resulting from mild traumatic brain injuries. *Clin Neurol Neurosurg* 2019;183:105380.
- Marshall DR. S100 beta as a neurotrophic factor. *Prog Brain Res* 1990;86:169-81.
- Bock E. Nervous system specific proteins. *J Neurochem* 1978;30(1):7-14.
- Umekawa H, Naka M, Inagaki M, Onishi H, Wakabayashi T, Hidaka H. Conformational studies of myosin phosphorylated by protein kinase C. *J Biol Chem* 1985;260(17):9833-7.
- Yasuda Y, Tateishi N, Shimoda T, Satoh S, Ogitani E, Fujita S. Relationship between S100beta and GFAP expression in astrocytes during infarction and glial scar formation after mild transient ischemia. *Brain Res*

- 2004;1021(1):20-31.
26. Alserr AH, Elwan H, Antonopoulos CN, Abdelreheem A, Elmahdy H, Sayed A, Taha A, Maratou E, Brountzos E, Khairy H, Liapis CD. Using serum s100- β protein as a biomarker for comparing silent brain injury in carotid endarterectomy and carotid artery stenting. *Int Angiol* 2019;38(2):136-142.
 27. Zeng H, Liu N, Yang YY, Xing HY, Liu XX, Li F, La GY, Huang MJ, Zhou MW. Lentivirus-mediated downregulation of α -synuclein reduces neuroinflammation and promotes functional recovery in rats with spinal cord injury. *J Neuroinflammation* 2019;16(1):283.
 28. Li HT, Zhao XZ, Zhang XR, Li G, Jia ZQ, Sun P, Wang JQ, Fan ZK, Lv G. Exendin-4 Enhances Motor Function Recovery via Promotion of Autophagy and Inhibition of Neuronal Apoptosis After Spinal Cord Injury in Rats. *Mol Neurobiol* 2016;53(6):4073-4082.
 29. Gensel JC, Kigerl KA, Mandrekar-Colucci SS, Gaudet AD, Popovich PG. Achieving CNS axon regeneration by manipulating convergent neuro-immune signaling. *Cell Tissue Res* 2012;349(1):201-13.
 30. Anwar MA, Al Shehabi TS, Eid AH. Inflammogenesis of Secondary Spinal Cord Injury. *Front Cell Neurosci* 2016;10:98.
 31. Beck KD, Nguyen HX, Galvan MD, Salazar DL, Woodruff TM, Anderson AJ. Quantitative analysis of cellular inflammation after traumatic spinal cord injury: evidence for a multiphasic inflammatory response in the acute to chronic environment. *Brain* 2010;133(Pt 2):433-47.
 32. Şaker D, Sencar L, Yılmaz DM, Polat S. Relationships between microRNA-20a and microRNA-125b expression and apoptosis and inflammation in experimental spinal cord injury. *Neurol Res* 2019;41(11):991-1000.
 33. Faden AI, Wu J, Stoica BA, Loane DJ. Progressive inflammation-mediated neurodegeneration after traumatic brain or spinal cord injury. *Br J Pharmacol* 2016;173(4):681-91.
 34. Kumar H, Ropper AE, Lee SH, Han I. Propitious Therapeutic Modulators to Prevent Blood-Spinal Cord Barrier Disruption in Spinal Cord Injury. *Mol Neurobiol* 2017;54(5):3578-3590.
 35. Kigerl KA, Gensel JC, Ankeny DP, Alexander JK, Donnelly DJ, Popovich PG. Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord. *J Neurosci* 2009;29(43):13435-44.
 36. London A, Cohen M, Schwartz M. Microglia and monocyte-derived macrophages: functionally distinct populations that act in concert in CNS plasticity and repair. *Front Cell Neurosci* 2013;7:34.
 37. Rowland JW, Hawryluk GW, Kwon B, Fehlings MG. Current status of acute spinal cord injury pathophysiology and emerging therapies: promise on the horizon. *Neurosurg Focus* 2008;25(5):E2.
 38. Beattie MS, Hermann GE, Rogers RC, Bresnahan JC. Cell death in models of spinal cord injury. *Prog Brain Res* 2002;137:37-47.
 39. Goldshmit Y, Kanner S, Zacs M, Frisca F, Pinto AR, Currie PD, Pinkas-Kramarski R. Rapamycin increases neuronal survival, reduces inflammation and astrocyte proliferation after spinal cord injury. *Mol Cell Neurosci* 2015;68:82-91.
 40. Li JL, Liang YL, Wang YJ. Knockout of ALOX12 protects against spinal cord injury-mediated nerve injury by inhibition of inflammation and apoptosis. *Biochem Biophys Res Commun* 2019;516(3):991-998.
 41. Wang X, Cao K, Sun X, Chen Y, Duan Z, Sun L, Guo L, Bai P, Sun D, Fan J, He X, Young W, Ren Y. Macrophages in spinal cord injury: phenotypic and functional change from exposure to myelin debris. *Glia* 2015; 63(4):635-51.
 42. Haan N, Zhu B, Wang J, Wei X, Song B. Crosstalk between macrophages and astrocytes affects proliferation, reactive phenotype and inflammatory response, suggesting a role during reactive gliosis following spinal cord injury. *J Neuroinflammation* 2015;12:109.