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ORIGINAL PAPER

Enhanced Expression of PD-L1 on Microglia After Surgical Brain Injury Exerts Self-Protection from Inflammation and Promotes Neurological Repair

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Abstract

Neuroinflammation and brain edema are major complications in the pathophysiology of surgical brain injury (SBI). Programmed death-ligand 1 (PD-L1), an immune inhibitory receptor ligand, has been increasingly investigated for inhibition of T cell-mediated immunity and braking inflammatory response. However, the negative immunomodulatory capacity of PD-L1 and their possible mechanism in SBI is not yet clear. This study aimed to evaluate the expression and the role of PD-L1 in a mouse model of SBI induced inflammation and to further study the potential therapeutic effects of PD-L1 on SBI. Here we showed that PD-L1 expression was markedly elevated in the surrounding peri-resection brain tissue post-SBI in vivo. PD-L1 was up-regulated through ERK signal pathway in LPS-treated BV-2 cells in vitro. Furthermore, blockade of the PD-L1 checkpoint using PD-L1 antibody significantly enhanced brain edema, exacerbated apoptosis and increased neurodeficits post-SBI. Moreover, activated PD-1/PD-L1 with PD-L1 protein significantly attenuated the inflammation responses and brain edema post-SBI. These results suggest that enhanced expression of PD-L1 post-SBI exerts self-protection from inflammation and promotes neurological repair. PD-L1 signal may have therapeutic potential for neurodegenerative disorders.

Keywords Programmed death 1 · Programmed death ligand 1 · Surgical brain injury · Microglia · Astrocyte

Abbreviations

PD-L1	Programmed death-ligand 1
PD-1	Programmed death protein 1
SBI	Surgical brain injury
Akt	Ser/Thr kinase
mTOR	Mechanistic target of rapamycin
LPS	Lipopolysaccharide
ERK	Extracellular regulated protein kinases
PCR	Polymerase chain reaction
ELISA	Enzyme-linked immunosorbent assay
BBB	Blood–brain barrier
MRI	Nuclear magnetic imaging
EAE	Experimental autoimmune encephalomyelitis
ICH	Intracerebral hemorrhage
MCAO	Middle cerebral artery occlusion

Qian Chen, Lixia Xu and Tianjiao Du contributed equally to this work.

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Introduction

Surgical brain injury (SBI) is unavoidable concomitant injury caused by neurosurgical procedures and surgical instruments [1–3]. This inevitable brain injury can result in disruption of the blood–brain barrier (BBB), brain edema, nerve cell death, and aggravate neurological deficits. The methods of avoiding and alleviating SBI have become the focus of neurosurgeons [4].

Previous studies in our laboratory and other laboratories have clearly shown that regulating immune processes can be used in the treatment of SBI [5]. Of particular interest is how to induce immune tolerance. Programmed death 1 (PD-1) is a transmembrane glycoprotein belonging to the CD28/CTLA-4 immunoglobulin superfamily. There are two ligands for PD-1: Programmed Death Ligand 1 (PD-L1) and Programmed Death Ligand 2 (PD-L2) [6]. More and More works have focused on the impact of PD-1/PD-L1 pathway on T-cell tolerance [7–10]. The PD-1/PD-L1 signaling pathway has been reported to be involved in the regulation of the immune system in a variety of diseases, such as Alzheimer disease [11], multiple sclerosis [12, 13], rabies virus infection [14], lupus [15] and colitis [16]. PD-L1 is also known to induce regulatory T cells (Tregs) by inhibiting the Akt/mTOR signaling cascade [17]. Autoimmunity is accelerated by PD-1 deficiency on autoimmune-prone backgrounds, providing further evidence for a role for PD-L1/PD-1 in the induction and maintenance of tolerance [17]. Zhao and Han reported that PD-L1 improves the prognosis of ischemic stroke [18] and cerebral hemorrhage [19] induced brain injury. Taken together, manipulating PD-1/PD-L1 pathway may provide a novel approach for maintaining and inducing immune tolerance. This pathway may be a particularly attractive therapeutic target in immune-related diseases. In any case, the role of PD-1/PD-L1 plays in SBI and whether it can be applied to SBI treatment has not been reported.

The aim of our study was to assess the expression of PD-L1 on microglia in vitro and in vivo, further evaluate the consequences of PD-L1 blockade/PD-L1 protein in neurosurgical complications, and finally elucidate PD-L1-mediated possible protective mechanisms in a mouse SBI model.

Materials and Methods

Reagents

Antibodies against Iba-1, GFAP and PD-L1 were from Abcam (Cambridge, London, UK). Cleaved Caspase-3 and alexa fluor dyes conjugated secondary antibodies was obtained from Cell Signaling Technology (Danvers, MA, USA). FACS antibodies against CD3, CD11b, PD-1 and PD-L1 were from Biolegend (Cambridge, MA). LPS was provided by Sigma (Deisenhofen, Germany). ELISA kits were purchased from Hermes Criterion Biotechnology (Canada).

Animals

C57BL/6 mice (female; grade SPF; 6–7w) were purchased from Experimental Animal Center of Academy of Military Medical Sciences, China. They were housed in the animal care facility at Tianjin Huanhu Hospital (Tianjin, China). Experimental protocols were conducted in accordance with national legislation and associated guidelines and that the procedures were approved by the institutional animal care committee of Tianjin Key Laboratory of Cerebral Vascular and Neurodegenerative Diseases.

SBI Model

The mice were anesthetized with 20 µl/10 g of 10% chloral hydrate and placed prone in a stereotaxic frame with the head fixed. Skin and subcutaneous tissue were cut and the underlying periosteum was reflected to expose the right frontal skull. An operating square area (3 mm × 3 mm) was marked. A micro-drill was used to open the cranial window. 3 mm × 3 mm × 1 mm right frontal lobe brain tissue was removed. The mice were observed in the incubator for 2 h after the anesthesia was resuscitated [20].

Administration of PD-L1 Protein and PD-L1 Antibody

Flow chart showed the experimental design for analyzing the expression of PD-L1 post-SBI at different time, and the effects of PD-L1 mAb/PD-L1 protein on brain injury in vivo (Fig. S1). Mice were given 50 µg of either monoclonal anti-PD-L1 antibody (BP0101, clone 10F.9G2, BioXcell, NH, West Lebanon, USA) or an isotype matched control (anti-Keyhole Limpet Hemocyanin-KLH, clone LTF-2, BioXcell, West Lebanon, NH, USA) intraperitoneally injection in 100 µl PBS, 1 h following SBI [19], respectively, as the anti–PD-L1 mAb and Isotype mAb groups. In PD-L1 treat SBI part, mice were given 50 µg of PD-L1 protein (ACRO Biosystems) dissolved in 100 µl PBS or 100 µl PBS only, 1 h following SBI, and labeled with SBI&PD-L1 group, SBI group, respectively.

BV-2 Microglia

The BV2 microglia was purchased from China Center for Type Culture Collection, Wuhan University, cell ID: 3142C0001000000337. BV2 cells were normally cultured in completely medium containing Dulbecco's modified Eagle medium (DMEM) (Gibco, Los Angeles, CA, USA) supplemented with 10% fetal bovine serum (Gibco) and 100 U/ml penicillin/streptomycin, maintained in a humidified atmosphere at 37 °C containing 5% CO_2. The ERK blockade was purchased from Cell Signaling Technology (Cat.No: PD98059#9900, China), concentration 10 µM/ml was used to blocked ERK signaling pathway.

Q-PCR

Total RNAs from the harvested BV-2 cells with TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. RNAs were reverse transcribed using the RT-PCR kit (Takara Bio, Beijing, China). Real-time PCR reactions were performed using SYBR Premix DimerEraser system (Takara Bio, Beijing, China). The PCR primers were designed as follows:

mouse PD-L1 forward, 5′-GCCACCTTCACCTGCAGC TTGT′ and reverse, 5′-AAACCGGCCTTCTGGTTTGGGC-3′; and mouse IL-6 forward, 5′-ACGCTTCTGGGCCTG TTGTT-3′ and reverse, 5′-CCTGCTGCTGGTGATTCTCT′-3′. PCR cycles were carried out by initial denaturation at 95 °C for 5 min, then running 40 cycles of 95 °C for 10 s and 60 °C for 1 min. Duplicate experiments were conducted to calculate the mean ΔΔCt, mean RQ (fold-change) and standard deviation on Lightcycler 480II (Roche, Switzerland).

Flow Cytometry

For each sample, 1×10^6 cells were harvested, washed with FACS buffer (1% BSA and 0.05% sodium azide in PBS). The cells were resuspended in 100 µL of FACS buffer containing 1 µg of the indicated antibody, incubated on ice for 30 min, resuspended in 500 µL of FACS buffer, and then analyzed within 1 h. A Coulter FACS equipment and FlowJo analysis software were used.

Immunofluorescence

The mice were anesthetized, sacrificed, and fixed with 4% paraformaldehyde, brain tissue was taken for paraffin sectioning. The mouse brain sections were then dewaxed three times in xylene for 15 min each. Then the brain sections were immersed in 100% ethanol for 5 min, 95% ethanol for 3 min, 80% ethanol for 3 min, and 75% ethanol for 1 min. Fluorescent staining was performed after high pressure repair of antigen with 1 × EDTA. The primary antibody was incubated at 4 °C for 12 h and the secondary antibody was incubated at 37 °C for 4 h. Finally, brain sections were observed and photographed with a fluorescence microscope.

Enzyme Linked Immunosorbent Assay

The level of cytokines, TGF-β, IL-10, IL-6 and iNOS, were determined with ELISA kits. Firstly, 100 µl of each standards and specimens were added into microtiter plate, and 50 µl Enzyme Conjugate was added into wells. Plate was incubated at 37 °C for 60 min. Then remove the incubation, rinse and empty the microtiter wells 5 times with 1 × washing buffer. Then dispense 50 µl of Color A and Color B regent into each wells, gently mix for 5 s. The colorimetric reaction was stopped after 15 mins of incubation at 37 °C by adding 50 µl of stop solution to each wells. The absorbance at 450 nm was immediately measured using a microtiter reader (Biosciences Pharmingen, USA).

Neurological Function Evaluation

Neurological function was assessed with the modified Garcia test which was applied on mice at baseline, 1 day, 3 days, 7 days and 14 days after SBI [21]. The modified Garcia test involves a 21-point sensorimotor assessment that includes seven tests. Each test has a score ranging from 0 to 3, with a maximum score of 21. The tests evaluate spontaneous activity, side stroking, vibrissae touch, limb symmetry, climbing, lateral turning, and forelimb walking.

Evaluation of Cerebral Edema

Fresh brains were removed at 0 (sham group), 1, 3, 7and 14 days after SBI. A 30-mg section of peri-resection region was obtained from each extracted brain to evaluate cerebral edema [22]. These sections were immediately weighed and then dried at 100 °C for 24 h. The dry weight was then determined. The brain water content was determined using the following equation: Water content (%) = (wet weight − dry weight)/wet weight × 100%.

In Vivo MRI Brain Imaging

Mice were scanned by a clinical 3.0 T MR clinical scanner (Magnetom Trio with Tim, Siemens, Germany). T2-weighted MR images were acquired under the following parameters: TE = 72 ms and TR = 2500 ms with the same other parameters.

Statistical Analysis

The quantitative data are presented as the mean ± standard deviation (SD) values. Each experiment was executed three times. Repeated measure analysis of variance was used to comprehensively analyze the effects of treatment and timepoints on SBI. Other data that did not include multi-timepoints were analyzed using one-way ANOVA analysis. $p < 0.05$ was defined significant. All data was collected and analyzed by at least two investigators who were blind to the group assignment.

Results

PD-L1 is Highly Induced Post-SBI In Vivo

PD-L1 expression and microglia activation were measured using double immunofluorescence staining (PD-L1 & Iba-1)

in the surrounding peri-resection brain samples collected at 1d, 3d, 7d, 14d post-SBI. Immunofluorescence staining showed that numbers of Iba-1 positive cells (microglia activation) were significantly higher at 1d, 3d, 7d but subsequently down-regulated at days 14 (Fig. 1). Meanwhile, PD-L1 protein expression in post-SBI 1d, 3d and 7d also increased. This enhanced expression of PD-L1 protein decreased in the post-SBI 14d subgroup, which was similar as the Iba-1 expression level (Fig. 1b). The trend of PD-L1 expression was consistent with that of microglia activation. The expression of PD-1/PD-L1 in T cells and monocytes in peripheral blood were also increased (Fig. S2). These results showed that PD-L1 expression is markedly elevated and correlated with microglia activation post-SBI in vivo.

Increased PD-L1 Expression in Activated Microglia

To further confirm the correlation of PD-L1 up-regulation and microglia activation after SBI, we analyzed the expression level of PD-L1 in vitro neuroinflammatory process. We exposed BV-2 microglia to lipopolysaccharide (LPS, 500 ng/ml) for 24 h to induce inflammatory response and analyzed the expression level of PD-L1. Double immunofluorescence staining showed that LPS significantly enhanced the expression of PD-L1, in the meantime, the expression of Iba-1 protein was also increased by LPS stimulation (Fig. 2a). In support, FACS results showed that LPS significantly up-regulated the expression of PD-L1 (Fig. 2b). To confirm these findings, we also analyzed mRNA level of PD-L1. BV-2 cells were stimulated with different concentrations of LPS (250, 500, 1000 ng/ml) for 6 h. Q-PCR results showed that LPS enhanced IL-6 mRNA expression, and the expression of PD-L1 mRNA was also significantly increased, simultaneously (Fig. 2c). Taken together, our data clearly demonstrate that PD-L1 is highly induced in LPS-stimulated BV-2 cells.

LPS Up-Regulates PD-L1 Expression Via ERK Signaling Pathway

ERK signal provides an important role for PD-L1 expression in tumor cells [23–25]. To find out whether this signaling pathway is involved in LPS-stimulated PD-L1 expression in BV-2 cells, we treated the cells with PD98059 (10 μM/ml), a known ERK inhibitor. Immunofluorescence staining (Fig. 3a, b) and Q-PCR (Fig. 3c) showed that PD98059 treatment led to an apparent reduction in LPS-induced PD-L1 upregulation at both mRNA and protein levels, meanwhile,

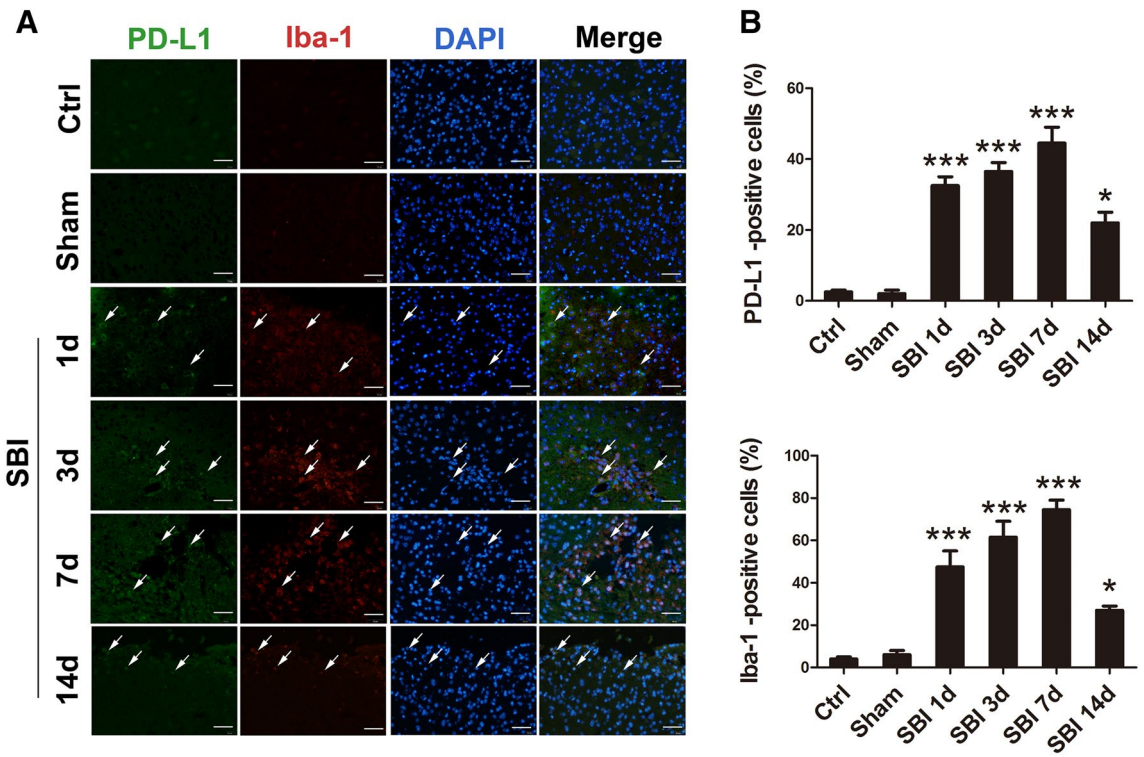

Fig. 1 PD-L1 expression is markedly elevated and correlated with microglia activation post-SBI. **a** Expression of PD-L1 and Iba-1 in the surrounding peri-resection brain samples at different time post-SBI (green, PD-L1; red, Iba-1; blue, DAPI, scale bar, 50 μm); **b** percentage of PD-L1 and Iba-1 positive cells. Data are mean ± SD. $^{*}p < 0.05$, $^{***}p < 0.001$, n = 8 animals per group (Color figure online)

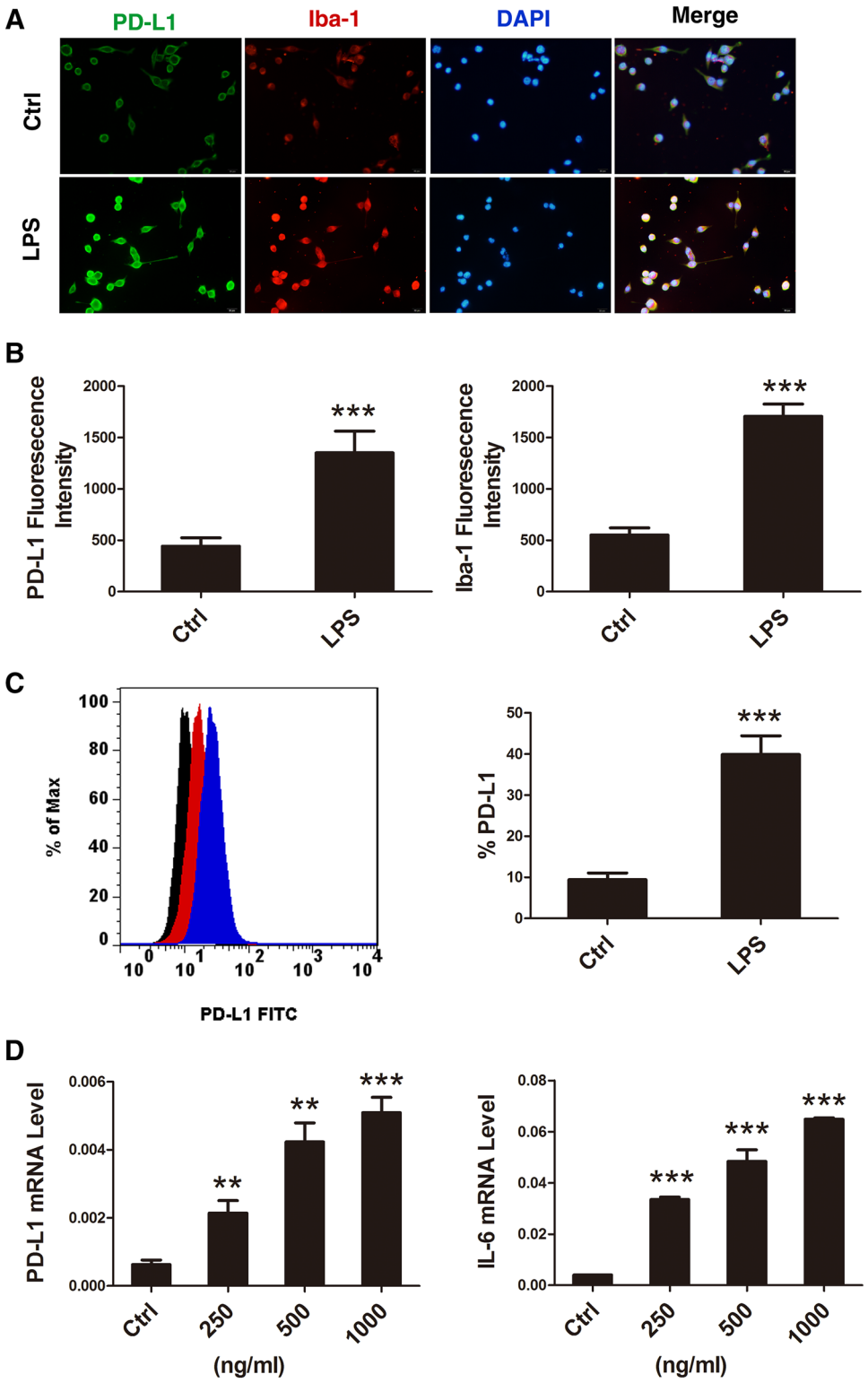

Fig. 2 Increased PD-L1 expression in activated microglia. **a** Immunofluorescence staining images of BV-2 cells treated with LPS for 24 h. Green, PD-L1; red, Iba-1; blue, DAPI; the below histogram showed the quantitative analysis. **b** FACS analysis of cultured BV-2 cells treated with LPS for 24 h. Black, IgG; red, Ctrl; blue, LPS. The right histogram showed the quantitative analysis; **c** Q-PCR analysis of mRNA levels of PD-L1 and IL-6 in cultured BV-2 treated with LPS (250, 500, 1000 ng/ml) for 6 h. Data are mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$ (Color figure online)

Iba-1 and IL-6 expression were also blocked. These findings indicate that ERK signaling is responsible for mediating LPS-mediated upregulation of PD-L1 expression in BV-2 microglia.

PD-L1 Blockade Enhances Cerebral Edema and Inflammation Post-SBI In Vivo

To determine the role of the excessive expression of PD-L1 post-SBI, mice were intraperitoneally injected with

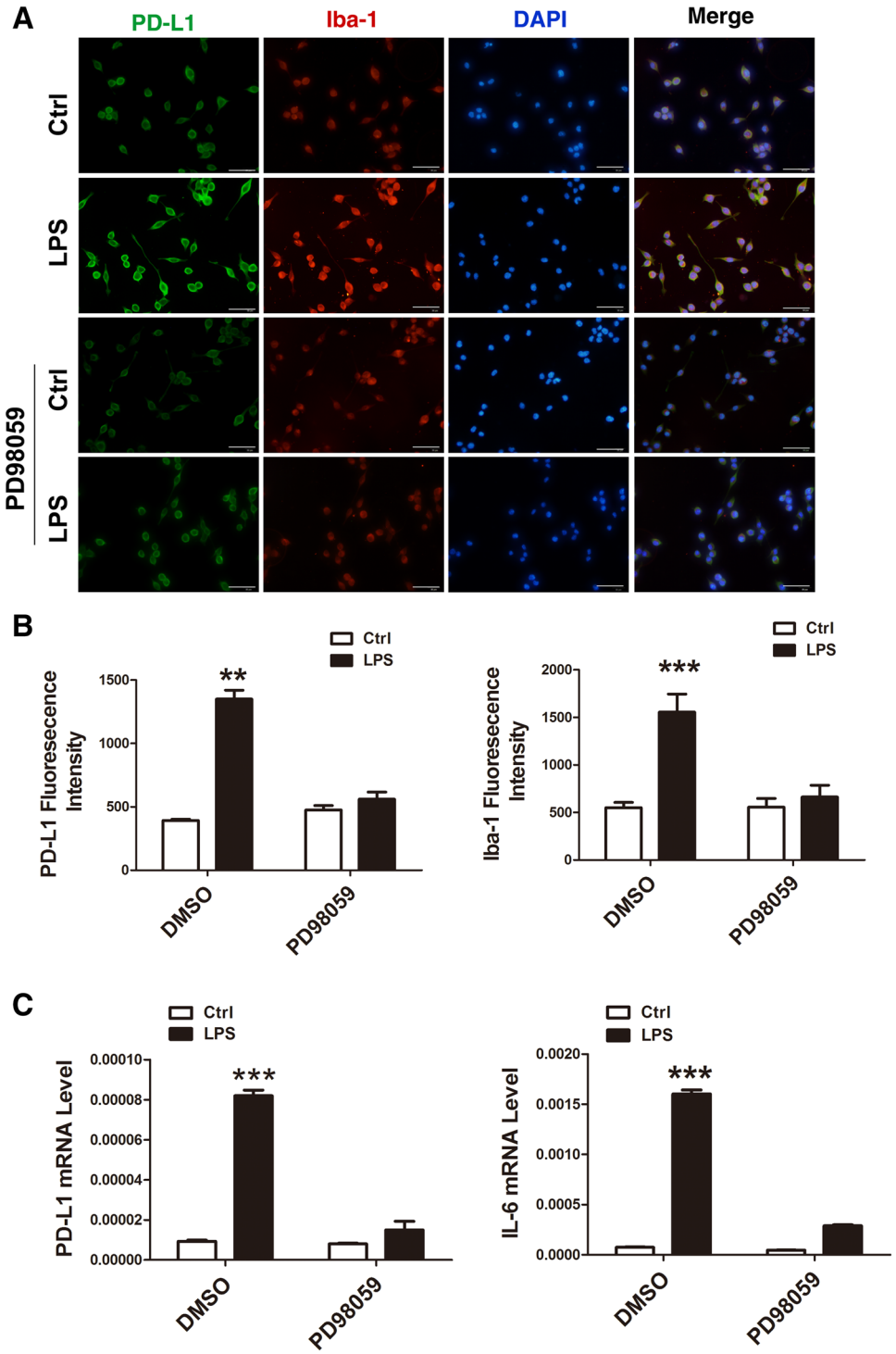

Fig. 3 LPS up-regulates PD-L1 expression via ERK signaling pathway. **a** Immunofluorescence staining of LPS- treated BV-2 cells in the presence of PD98059 or not for 24 h. **b** Quantitative analysis of PD-L1 and Iba-1 level. **c** Q-PCR analysis of LPS- treated BV-2 cells in the presence of PD98059 or not for 6 h. Data are mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$

a single dose of anti-PD-L1 mAb (50 μg) 1 h post-SBI and evaluated for infarct volumn, inflammatory cytokines expression levels and microglia activation. Neurological function scoring demonstrated that PD-L1 mAb group performed worse than that in Isotype mAb group (F = 102.99, $p < 0.001$), the difference in timepoints was also significant (F = 44.98, $p < 0.001$). The neurological function scoring of the 3d, 7d, and 14d post-SBI was statistically significant compared with the 1d ($p < 0.05$) (Fig. 4a). The difference in brain water content between different treatment groups (PD-L1 antibody and Isotype) was statistically significant (F = 9.35, P = 0.012), and the difference in different time points was also statistically significant (F = 34.55, $p < 0.001$). The brain water content of the 3d,7d and

14d was statistically significant compared with the 1d ($P < 0.05$). MRI results showed that cerebral edema in PD-L1 mAb group was significantly more severe than that in the isotype mAb group (Fig. 4c). Further, we detected the concentrations of the pro-inflammatory cytokine (IL-6 & iNOS) and anti-inflammatory cytokine (IL-10 & TGF-β) in the peripheral blood. ELISA results showed that the expression levels of IL-6 and iNOS (Fig. 4d) were higher in the PD-L1 mAb group than that in the Isotype group ($F = 22.79$, $P < 0.001$; $F = 16.03$, $P = 0.0025$; respectively), The IL-6 expression of the 3rd and 7th days was statistically significant compared with the 1st day ($P < 0.05$), iNOS was the same. The difference between PD-L1 mAb group and Isotype group in IL-10 expression is significant ($F = 33.02$, $P < 0.001$). The IL-10 expression of the 3, 7, 14d was significantly increased compared with 1d ($P < 0.05$). Although the difference in different timepoints on TGF-β expression is significant ($F = 58.38$, $P < 0.001$), there showed no significant difference between PD-L1 mAb group and Isotype group ($F = 0.78$, $P = 0.39$). When compared with 1d, the expression of TGF-β on 3d and 7d was increased significantly. These findings indicate that PD-L1 blockade enhances infarct volume and inflammation post-SBI in vivo.

PD-L1 Blockade Promotes Microglia and Astrocytes Activation Post-SBI In Vivo

Microglia and astrocytes are considered key players in initiating an inflammatory response after injury. To determine the role of the excessive expression of PD-L1 post-SBI, we observed the effects of PD-L1 mAb on microglia and astrocytes activation in vivo. Immunofluorescence staining showed that Iba-1 (Fig. 5a; Fig. S3) and GFAP (Fig. 5b) expression in PD-L1 mAb treated group and Isotype group was significantly different ($F = 135.74$, $P < 0.001$; $F = 102.11$, $P < 0.001$ respectively). The difference on each timepoints was also significant ($F = 523.51$, $P < 0.001$; $F = 55.07$, $P < 0.001$ respectively). The difference of Iba-1 and GFAP expression of the 3d, 7d,14d was statistically significant compared with the 1d ($P < 0.05$). Taken together, these results indicated that PD-L1 blockade promotes microglia and astrocytes activation post-SBI in vivo.

PD-L1 Antibody Exacerbates Cell Death Post-SBI In Vivo

We next analyzed the role of PD-L1 mAb on cell apoptosis post-SBI. Extent of cell apoptosis as determined by counting

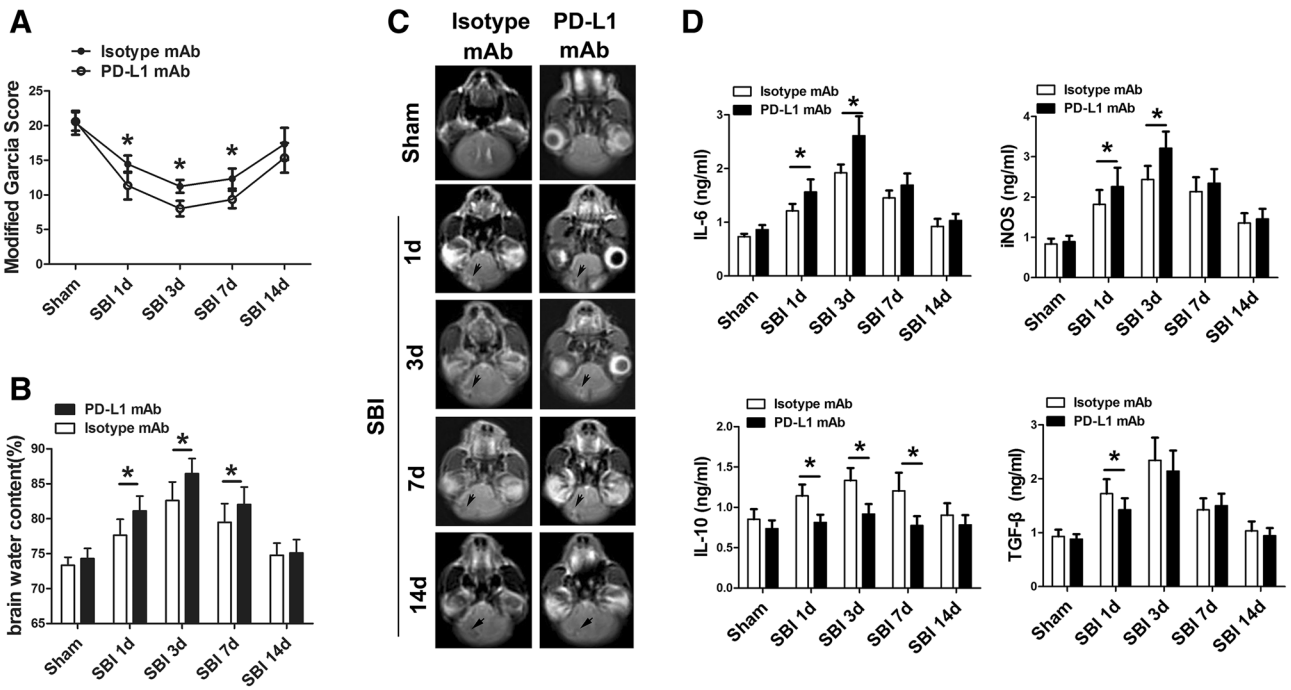

Fig. 4 Treatment with anti-PD-L1 mAb 1 h after SBI enhances infarct volume and inflammation. **a** Neurological function scoring histogram. **b** Brain water content (%) post-SBI 1d, 3d, 7d and 14d treated with isotype mAb or PD-L1 mAb. **c** MRI scanning post-SBI 1d, 3d, 7d and 14d treated with isotype mAb or PD-L1 mAb. The bright area shown by the arrow is edema area. **d** ELISA analysis of pro-inflammatory cytokine (IL-6, iNOS) and anti-inflammatory cytokine (IL-10, TGFβ) in serum post-SBI 1d, 3d, 7d and 14d treated with isotype mAb or PD-L1 mAb. Data are mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, n = 8 animals per group (Color figure online)

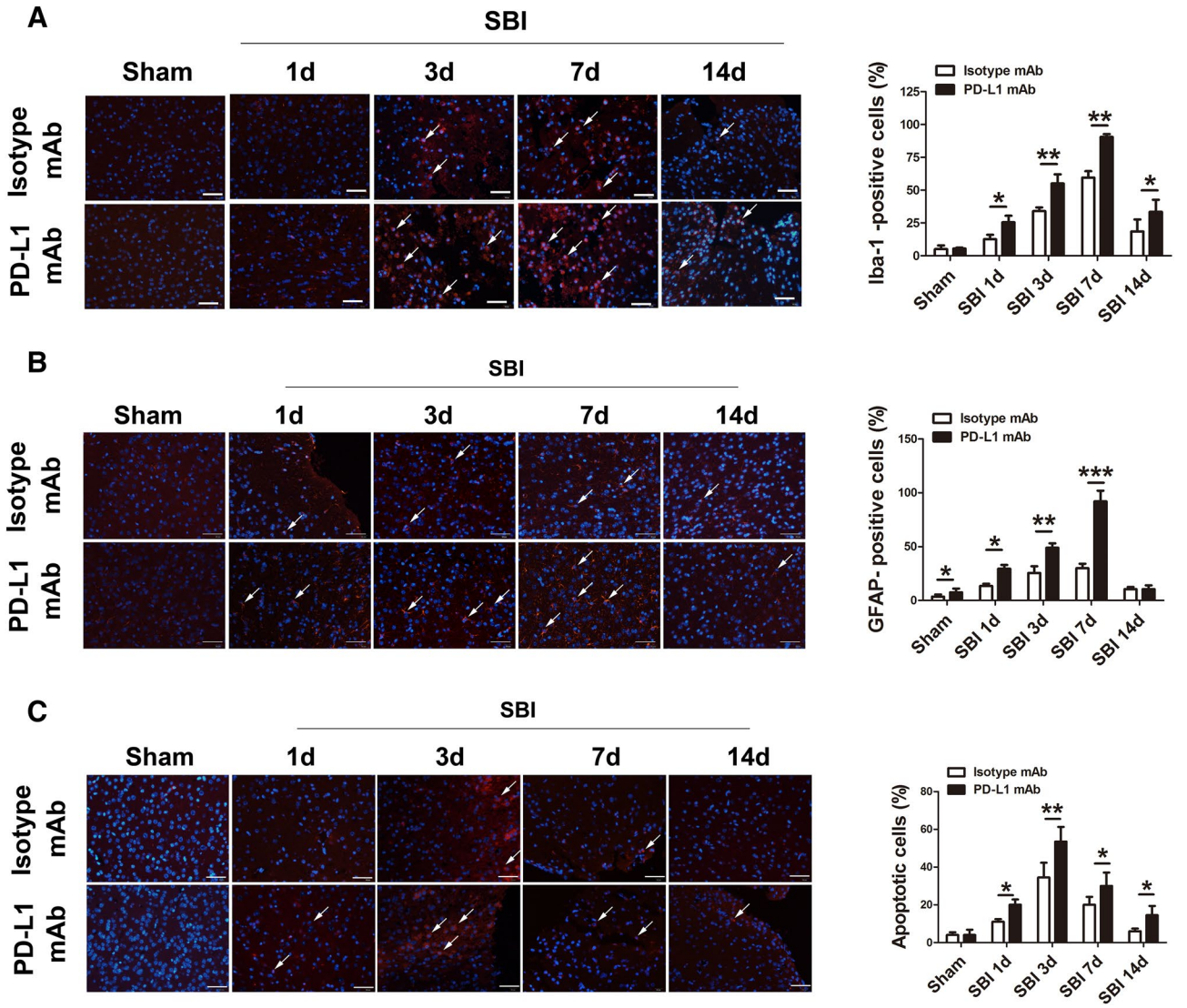

Fig. 5 PD-L1 blockade increases the activation of microglia and astrocytes post-SBI in vivo. **a** Immunofluorescence staining images of microglia at different time points post-SBI treated with isotype mAb or PD-L1 mAb (red, Iba-1; blue, DAPI, scale bar, 50 μm); the right histogram showed the quantitative analysis; **b** Immunofluorescence staining images of astrocytes at different time post-SBI treated with isotype mAb or PD-L1 mAb (red, GFAP; blue, DAPI, scale bar, 50 μm); **c** Immunofluorescence staining images of Caspase-3 at different time post-SBI treated with isotype mAb or PD-L1 mAb (red, Caspase-3; blue, DAPI, scale bar, 100 μm). The right histogram showed the quantitative analysis. Data are mean ± SD. $*p<0.05$, $**p<0.01$, $***p<0.001$, n = 8 animals per group (Color figure online)

the number of cleaved Caspase-3 positive cells in the surrounding peri-resection brain samples at different time post-SBI treated with isotype mAb or PD-L1 mAb. The injured brains were quickly collected and analyzed using immunofluorescence staining analysis. The data showed that there was a significant increase in the expression of cleaved Caspase-3 in the PD-L1 mAb group post-SBI when compared to Isotype group ($F = 23.18$, $P < 0.001$) (Fig. 5c). The difference of Caspase-3 expression of the 3d, 7d, 14d was statistically significant compared with the 1st day ($P < 0.05$). These findings indicate that PD-L1 blockade exacerbates cell death post-SBI in vivo.

PD-L1 Protein Attenuates the Inflammatory Responses and brain Edema Post-SBI

To further confirm the potential therapeutic effects of PD-L1 protein on SBI, we detected the inflammation responses and brain edema on the 3rd day post-SBI (the most serious time point of cerebral edema after SBI). Although the increase of neurological function score in SBI mice treated with PD-L1

protein was not significant (P = 0.27) (Fig. 6a), the water content of brain tissue in PD-L1 treated group decreased significantly compared with the SBI group (P = 0.03) (Fig. 6b). ELISA results showed that the expression levels of IL-6 (Fig. 6c) were lower in the PD-L1 treated group than that in the SBI group, whereas the expression of IL-10 was the opposite. Immunofluorescence staining showed that the expression of Iba-1 in microglia and GFAP in astrocytes in PD-L1-treated SBI mice were significantly decreased (Iba-1: P = 0.039; GFAP: P = 0.033) (Fig. 6d). These results above indicate that PD-L1 protein has potential therapeutic effects on SBI.

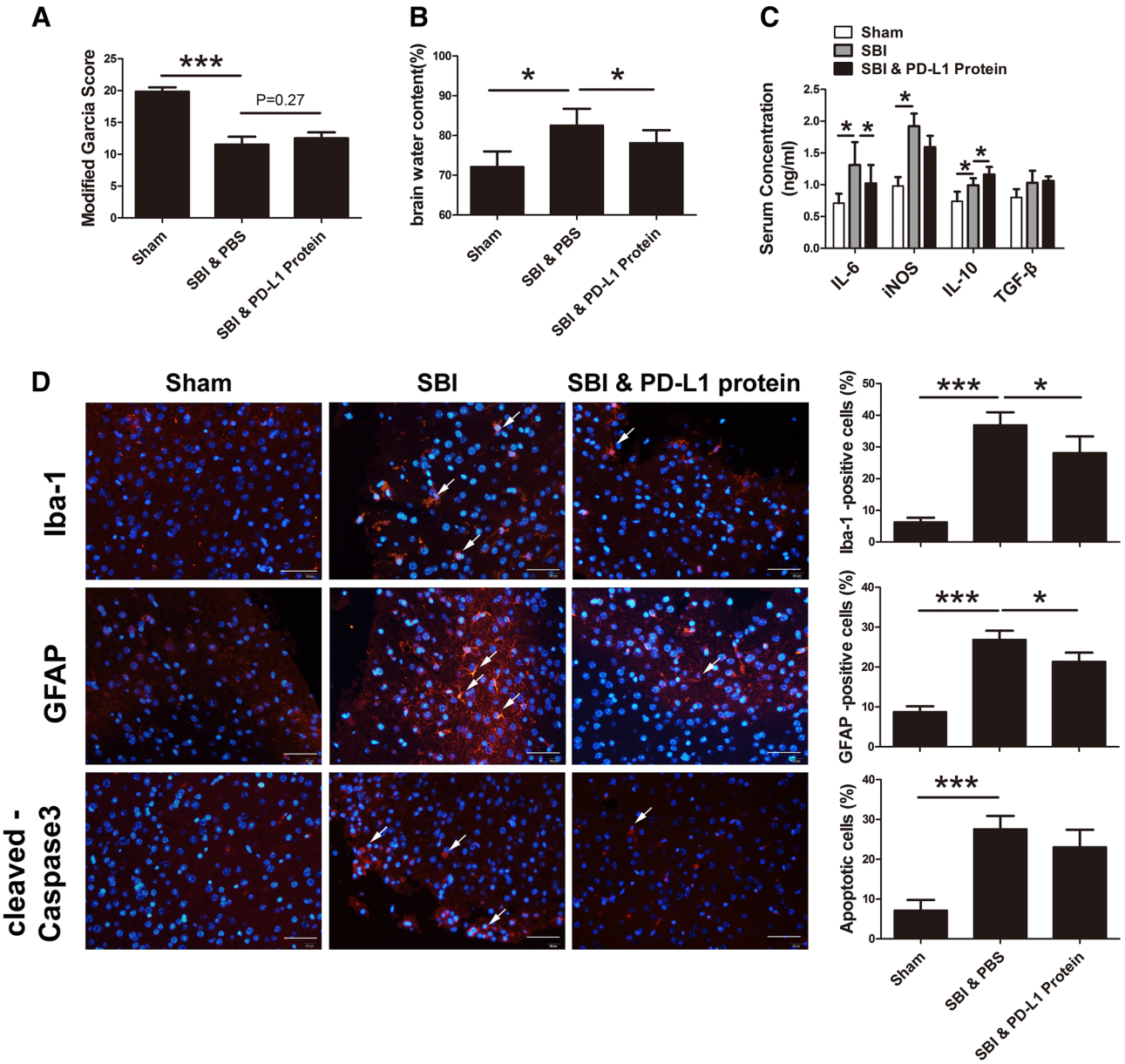

Fig. 6 PD-L1 protein attenuates the inflammatory responses and brain edema post-SBI. **a** Neurological function scoring histogram at 3d post-SBI treated with PBS or PD-L1 protein. **b** Brain water content (%) at 3d post-SBI treated with PBS or PD-L1 protein. **c** ELISA analysis of pro-inflammatory cytokine (IL-6, iNOS) and anti-inflammatory cytokine (IL-10, TGF-β) in serum 3d post-SBI treated with PBS or PD-L1 protein. **d** Immunofluorescence staining images of Iba-1 in microglia, GFAP in astrocytes and Caspase-3 (red, Iba-1, GFAP, Caspase-3 from top to bottom respectively; blue, DAPI, scale bar, 50 μm), the right histogram showed the quantitative analysis. Data are mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, n = 8 animals per group (Color figure online)

Discussion

In this experiment, we constructed a mouse SBI model to clarify the role of PD-1/PD-L1 in SBI. We found that after SBI, the mouse body was in an active state of inflammation, and microglia in the surrounding peri-resection brain samples of SBI mice was significantly activated. However, it is surprising that the expression of PD-L1 in SBI mice is also significantly increased while the body's inflammatory response is very active. It is well known that activation of the PD-1/PD-L1 signaling pathway inhibits the activation of immune cells and allows the body to enter an immune resting state. Why do these seemingly contradictory results appear on SBI mice at the same time?

Based on the experimental results, we speculated that after SBI, the body is in a state of stress inflammation, which leads to secondary brain damage. In response to this state, the body initiates a "self-protection mechanism"-activating the PD-1/PD-L1 pathway to suppress an excessively strong inflammatory response and avoiding unnecessary secondary damage. To verify our inference, we blocked the body's "self-protection mechanism" (PD-1/PD-L1 pathway) to see what changes will happen to the body. The results showed that the brain edema of the SBI mice in the PD-L1 antibody group was exacerbated, the activated microglia in the brain was significantly increased, and the expression of inflammatory factors in the blood was also significantly increased. Furthermore, it is interesting that the expression change of PD-L1 and Iba-1 in microglia showed a synchronous trend. In the 1d, 3d and 7d after SBI, the PD-L1 and Iba-1 expression increased significantly and decreased on 14d. The possible explanation may be the expression of PD-L1 changed with the expression of Iba-1 in microglia. On the 14th day after injury, the body has almost recovered by self-healing, the neuroinflammation has almost recovered and the number of activated microglia has returned to normal levels, so the PD-L1 level has also decreased simultaneously. Intervention of PD-1/PD-L1 pathway may be able to treat SBI by affecting microglia activity. To further confirmed the potential therapeutic effects of PD-L1, SBI mice were administrated with PD-L1 protein. The results indicated that the PD-L1 protein significantly attenuated the inflammation responses both in brain and blood.

This self-protection mechanism may be achieved by affecting T cells through the PD-1/PD-L1 signaling pathway. Yang et al. reported that PD-L1 can inhibit the production of effector T cells [26]. In addition, administration of PD-L1 antibody significantly increased the $CD4^+$T cells and ameliorating DSS-induced colitis [16]. PD-L1 also reduces the immunopathological damage in experimental autoimmune encephalomyelitis (EAE) by ameliorates T cell activation [27]. Pittet et al. also reported that blocking PD-L1 significantly increases $CD4^+$ and $CD8^+$T cell transmigration in a multiple sclerosis model [28]. Tian and Ding et al. confirmed that when the PD-1/PD-L1 pathway is activated, it can upregulate the expression of Treg cells and inhibit the ability of T cells to respond to inflammation [29, 30]. Francisco reported that PD-L1 can inhibit T cell responses by promoting both the induction and maintenance of regulating induced T reg (iT reg) through Akt and mTOR signal [17].

PD-1/PD-L1 pathway also influences the activity of microglia. The role of microglia in the brain injury is a double-edged sword. After brain injury, the microglia will differentiate into two opposite directions, which can differentiate into pro-inflammatory state (M1) or anti-inflammatory state (M2) [31, 32]. Current research showed that PD-L1 can promote the differentiation of microglia into anti-inflammatory state (M2) and reduce secondary brain damage caused by intracerebral hemorrhage (ICH) [33]. Our data showed that activation of microglia is always accompanied by increased expression of PD-L1, and this process is completed through ERK pathway. We also presented that blocking PD-1/PD-L1 pathway increased the percentage of active microglia, contribute to more severe brain edema and neurological deficit.

However, our experiments also have some limits. We have not presented a more exhaustive process which SBI causes the increase in PD-L1 expression. Moreover, some researchers reported results that are contrary to us. Bodhankar reported that PD-L1 knocked mice showed a better prognosis than the wild type mice in middle cerebral artery occlusion (MACO) models [34]. Li's explanation for this discrepancies is that the functions of PD-L1 in the ischemic brain are cell-specific. However, our results failed to explain this phenomenon better [35].

In conclusion, our data indicated that PD-1/PD-L1 serve as a "self-protection mechanism" in SBI. When the body's immune response is too strong, the PD-1/PD-L1 signaling pathway is activated to protect the body from excessive immune responses.

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Authors Contributions HY and QW designed the study. QC and LX accomplished the SBI model, specimen acquisition, FACS assay and immunofluorescence staining. TD, WF and YH carried out ELISA and PCR. QC, LX and SL performed data analysis. QC, LX and TD wrote the manuscript. QC, LX and TD contributed equally to this study. All authors read the final version and approved the submission and publication of the manuscript.

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Compliance with Ethical Standards

Conflict of interest All authors claim that there are no conflicts of interest.

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