

S100A8 Promotes Inflammation via Toll-Like Receptor 4 After Experimental Traumatic Brain Injury

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Research

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Abstract

Background: S100A8 is involved in the pathological processes of a variety of central nervous system(CNS) diseases related to inflammation including traumatic brain injury (TBI). However, the underlying mechanism for the induction of inflammation in the brain by S100A8 after TBI remains unclear, which was investigated in the present study.

Methods: The weight-drop TBI model was used in this study. The mice were randomly assigned into 5 groups: the Sham, S100A8, S100A8 + TAK-242, TBI, and TBI + TAK-242 groups. In the S100A8 + TAK-242 and TBI + TAK-242 groups, mice were treated with TAK-242, an inhibitor of Toll-like receptor (TLR) 4, intraperitoneally at half an hour before TBI. In the S100A8 and S100A8 + TAK-242 groups, S100A8 recombinant protein was injected into the lateral ventricle of the brain. To explore the relationship between S100A8 and TLR4, Western Blot (WB), immunofluorescence, enzyme-linked immunosorbent assay (ELISA) and Nissl staining were employed. Neurological score and the brain water content were also assessed. Additionally, BV-2 microglial cells were stimulated with lipopolysaccharide (LPS) or S100A8 recombinant protein with/without TAK-242 *in vitro*. The expressions of the related proteins were subsequently detected with WB or ELISA.

Results: The levels of S100A8 protein and pro-inflammatory cytokines were significantly increased after TBI. After intracerebroventricular administration of S100A8, the neurological scores of non-TBI animals were decreased remarkably with severe brain edema. Furthermore, the levels of TLR4, p-p65 and myeloid differentiation factor 88(MyD88) were all increased after S100A8 administration or TBI, which could be restored by TAK-242. Meanwhile, p-p65 and MyD88 were upregulated after S100A8 or LPS stimulation *in vitro*, which also could be suppressed by TAK-242.

Conclusions: The present study demonstrated that TLR4-MyD88 pathway was activated by S100A8, which was essential to the development of inflammation in the brain after TBI.

Background

Traumatic brain injury (TBI) is a major cause of death and disability worldwide. The number of TBI patients is increasing and which is becoming a serious public health problem [1]. TBI might confer a long-term risk for cognitive impairment, dementia[2, 3], stroke[4, 5], parkinsonism[6–8], and is associated with an increased long-term mortality rate[9, 10]. With regard to the etiology of TBI, pathological changes such as glutamate excitotoxicity, oxidative stress, increased vascular permeability, disturbance of ionic homeostasis, and inflammation in the brain were triggered after the primary brain injury [11–13], leading to the secondary brain injury. Among them, inflammation plays a critical role in the brain damage after TBI and is an important contributor to neurological deficit [14].

S100 proteins, including S100A8, are the largest subset superfamily with the Ca²⁺-binding EF-hand. The EF-hand family results in a high Ca²⁺ binding affinity for the specific feature of helix–loop–helix motif with charged amino acid residues [15]. S100A8 has the potential to play multiple roles, depending on

posttranscriptional modifications, oligomeric forms, concentrations, as well as proximal microenvironments, for its unique structures [16]. S100A8 and S100A9 (also known as MRP8 and MRP14) serve as warning signals in the inflammatory process. This is a common feature of damage-associated molecular pattern (DAMP) molecules [17]. S100A8 participates in the pathology of various diseases and injuries of the CNS including TBI [18–20]. However, the signaling pathway downstream the S100A8 induced after TBI still remains unclear.

Increasing data showed that Toll-like receptors (TLRs) play an important role in innate immunity and inflammatory responses. TLR1-9 could be expressed in microglia, macrophage-like glial cells in the brain[21]. Among them, TLR4 could be activated by Lipopolysaccharide (LPS) and is extensively expressed in the brain. In endotoxin-induced shock, S100A8 could stimulate TLR4 as a previous study showed [22]. In addition, TLR4 was reported to be essential for the inflammatory response in the brain after TBI [23]. The adapter protein myeloid differentiation factor 88 (MyD88) mediates the TLR4 signaling pathway, and which subsequently activates transcription factor nuclear factor-kappa B (NF- κ B). As a result, plenty of pro-inflammatory cytokines are produced [24]. Nevertheless, the relationship between S100A8 and TLR4-MyD88 signaling pathway in response to TBI is unknown.

Hence, the present study was aimed to investigate whether S100A8 could promote the expressions of pro-inflammatory cytokines by activating the MyD88/NF- κ B signaling pathway via TLR4 in the brain after TBI.

Materials And Methods

1. Animal Preparation

All procedures relate to animals were approved by the Animal Care and Use Committee of Jinling Hospital. Adult male Institute of Cancer Research (ICR) mice (28–32 g) were purchased from the Animal Center of Qinglongshan (Nanjing, China). The mice were kept in cages with constant room temperature ($25 \pm 2^\circ\text{C}$) and air humidity ($50 \pm 10\%$). They could have free access for food and water and the light/dark cycle was 12 h.

2. Experimental designs

This study was divided into two parts: *in vivo* experiments and *in vitro* experiments.

2.1 *In vivo* experiments

First, the cortical expression of S100A8 after TBI was assessed. Twenty-one adult male ICR mice weighing 28 to 32 g were randomly divided into 7 groups: the Sham (n = 3/group) and TBI [3, 6, 9, 12, 24, 72 hours (h)] (n = 3/group) groups. The sham mice were euthanized at 24 h after surgery and the TBI

mice were euthanized at the indicated time-point after TBI. Brain samples were collected for Western Blot analysis. Six other mice were added into the Sham (n = 3) and TBI (n = 3) groups, which were euthanized at 24 h after TBI to collect the whole brains for immunofluorescence staining.

Then, one time point(24 h) was selected for further experiments according to the time-course of S100A8 expression. We randomly divided 105 male mice into 5 groups: (1) Sham group, (2) TBI group, (3) TBI + TAK-242 group, (4) S100A8 group, (5) S100A8 + TAK-242 group (n = 21 for each group). The sham mice were euthanized at 24 h after surgery. The mice in the TBI group were euthanized as mentioned later. TAK-242(MCE; Monmouth Junction, NJ, USA) was administered intraperitoneally to the mice in TBI + TAK-242 and S100A8 + TAK-242 groups at 0.5 h before TBI. In the S100A8 and S100A8 + TAK-242 groups, S100A8 recombinant protein was injected into the lateral ventricle of the brain according to the previous report [25]. Brain samples were collected for Western Blot (n = 3) and enzyme-linked immunosorbent assay (ELISA) (n = 3). Nissl staining (n = 3 for each group) was also performed. Besides, brain water content was measured using the wet/dry method (n = 6) as previously described[26] and neurological deficits (n = 6) were evaluated before they were euthanized.

2.2 *In vitro* experiments

BV-2 cells cultured in the six well plates were randomly divided into 5 groups: (1) Sham group, (2) S100A8 group, (3) S100A8 + TAK-242 group, (4) LPS group, (5) LPS + TAK-242 group. Cells were collected for Western Blot analysis (n = 3), and the cell-free supernatants were collected for ELISA (n = 3).

3. TBI model in mice

The weight-drop model of TBI in this study was followed Flierl et al. [27]. In brief, after the mouse was anesthetized, it was placed onto the platform under the weight-drop device. To expose the skull, a 1.5 cm midline longitudinal scalp incision in the mouse's head was made. The target area, 1.5 mm lateral to the midline on the mid-coronal plane, was impact by an object which is 200 g weight. We used 0.9% normal saline solution to perfuse the mouse transcardially after it was deeply anesthetized via inhalation of isoflurane. At the end, the brain was collected for subsequent analyses.

4. Neurological scoring

The modified Garcia's method [28] was used to calculate neurological score at 24 h after TBI by two investigators blinded to the grouping. The modified Garcia method consists of 6 tests, which were Spontaneous activity (in cage for 5 min), symmetry in the movement of four limbs, Symmetry of forelimbs (outstretching while held by tail), Climbing wall of wire cage, Reaction to touch on either side of trunk, and Response to vibrissae stimulation and its scores range from 3 to 18 (Table 1).

Table 1
Neurological Evaluation in Sham, S100A8, S100A8 + TAK-242, TBI and TBI + TAK-242 groups

Test	Score			
	0	1	2	3
Spontaneous activity (in cage for 5 min)	No movement	Barely moves	Moves but does not approach at least three sides of cage	Moves and approaches at least three sides of cage
Symmetry in the movement of four limbs	Left side: no movement	Left side: slight movement	Left side: moves slowly	Both sides: move symmetrically
Symmetry of forelimbs (outstretching while held by tail)	Left side: no movement, no outreaching	Left side: slight movement to outreach	Left side: moves and outreaches less than right side	Symmetrical outreach
Climbing wall of wire cage	...	Fails to climb	Left side is weak	Normal climbing
Reaction to touch on either side of trunk	...	No response on left side c	Weak response on left side	Symmetrical response
Response to vibrissae touch	...	No response on left side	Weak response on left side	Symmetrical response

5. Brain water content

The brain water content was measured by the dry-wet weight method [26]. The anesthetized mice's brain was removed. After removed the right cerebral hemispheres, brain stem and cerebellum, the remaining left cerebral hemispheres were collected and obtained the wet weight (WW). After that, we obtained the dry weight (DW) after the left hemispheres were dried at 72 °C for 72 h. The results of brain water content were calculated as: $(WW - DW)/WW \times 100\%$.

6. BV-2 microglial cell culture

BV-2 microglial cells were purchased from Cobioer BioScience Co., Ltd (Nanjing, China) and were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) which contains 5% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (pen/strep; Gibco, Grand Island, NY, USA); TAK-242 was added to the cells at the concentration of 10 nM just before the stimulation of S100A8 protein or LPS. Cells were treated with the S100A8 recombinant protein at final concentrations of 0.5 μ l/ml; LPS (Lipopolysaccharide, Sigma-Aldrich, St. Louis, MO, USA) was added to stimulate the BV-2 cell line at the final concentration of 0.1 μ g/mL according to the previous study [30].

7. Western Blot analysis

In vivo study, the brain tissue located over the injure site was collected. The Total Protein Extraction Kit (Beyotime, Nantong, China) was used to extract protein according to the manufacture's instruction. The BCA Protein Assay Kit (Beyotime, Nantong, China) was adopted to determine the protein concentrations. Equal amounts of protein were subjected to 10–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by being transferred to polyvinylidene-difluoride (PVDF) membranes. The membranes were blocked with 5% freshly prepared skim milk-1 for 2 h and then incubated overnight at 4 °C with following primary antibodies: S100A8 (1:1000; Abcam, Cambridge, MA, USA), TLR4 (1:200; Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA), MyD88 (1:500; Abcam, Cambridge, MA USA), p-p65 (1:200; Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA), β -actin (1:5000, Bioworld Technology, Bloomington, MN, USA). At the end, we use the horseradish peroxidase (HRP)-linked secondary antibodies to incubate the membranes for 1 h. The blotted protein bands were visualized by enhanced chemiluminatescence (ECL) kit (EMD Millipore, Billerica, MA, USA) and were recorded by Tanon 5500 Chemiluminescence Imaging system (Tanon Science and Technology Co., Ltd., Shanghai, China). Band density was quantified using Image J Software (NIH, Bethesda, MA, USA), with normalization to β -actin.

Similarly, Western Blot analysis was done in the cultured BV2 cells. Cells were lysed in lysis buffer containing Phosphatase Inhibitor Cocktail 2(Sigma-Aldrich, St. Louis, MO, USA). Protein concentrations were quantified by a BCA protein kit. Samples (50 μ g protein per lane) were loaded on 12% SDS-PAGE gels. The following steps were consistent with the experiments *in vivo*.

8. ELISA

For *in vivo* study, to quantify the release of cytokines from the brain after TBI (n = 3/group), mice were sacrificed at 24 h after TBI, brain homogenates were centrifuged at 5000 g for 5 min at 4°C. The cytokines interleukin (IL)-1 β and tumor necrosis factor(TNF)- α were quantified in the supernatant used commercial ELISA kits (both purchased from Multisciences, Hangzhou, China). All procedures are performed according to manufacturers' protocols.

For *in vitro* study, cell-free supernatants were collected by centrifugation at 1000 g for 20 min and assayed by IL- 1 β and TNF- α ELISA kits.

9. Immunofluorescence staining

Immunofluorescence staining was performed as previously described[31]. Briefly, the brain sections were incubated overnight at 4°C with the antibody against S100A8 (1:200; Abcam, Cambridge, MA, USA). After washed with phosphate buffered saline (PBS), Iba-1 (1:200; EMD Millipore, Billerica, MA, USA) was added and incubated overnight. The goat anti-rabbit IgG (diluted 1:200) was used to incubate the brain sections at room temperature for 10 min after washed with PBS. Then, we incubated the sections at room

temperature for 5 min with the DAPI dye solution (Kaiji Biological, Nanjing, China). At the end, anti-fluorescence quenching was used to slightly dry and seal the sections. A fluorescence microscope was used to observe, then the images were collected.

10. Nissl Staining and Cell Counting

To assess neuronal cell death, Nissl staining was performed as previously described [32]. The fixed brain samples were dehydrated, paraffin-embedded, and sliced into 10- μ m thick, followed by dewaxing 3 times in xylene for 5 min each time and then placing them in anhydrous ethanol for 5 min, 90% ethanol for 2 min, 70% ethanol for 2 min, and distilled water for 2 min. Specimens underwent Nissl staining for 10 min and then were rinsed twice with distilled water for a few seconds each time. They were then dehydrated twice in 95% ethanol for 2 min each time and made transparent by treatment with xylene twice for 5 min each time, followed by sealing with neutral gum. Specimens were viewed under a light microscope. Neurons have big cell body and rich cytoplasm under normal conditions. In contrast, shrunken cell body and many empty vesicles could be detected in damaged neurons. Cell counting was calculated within the brain cortex. Six random high power fields (x400) were randomly chosen to calculate the mean number of surviving neurons in each coronary section. Every third coronary section, starting from 3.0 mm posterior to the optic chiasma, was selected and we collected 4 sections in each animal for quantification. At the end, the average number of the 12 sections from three individual mice's brain was considered as the data for each group. We presented the data as the number of surviving neurons per high-power field.

10. Statistical analysis

All data in this study were expressed as mean \pm SD. Statistical analysis was performed by the one-way ANOVA according to Tukey's post hoc tests. $P < 0.05$ was considered statistically significant.

Results

1. Time-course expression and location of S100A8 after experimental TBI

Until now, there are little data about the changes of S100A8 in the brain following TBI. Therefore, Western Blot was performed for the brain samples from the area adjacent to the contusion at different time points after TBI (Fig. 1a). The protein levels of S100A8 were not significantly increased until 24 h after TBI (Fig. 1b). And, we noticed that protein level of S100A8 is higher at 24 h than that at 3 d. Therefore, the subsequent experiments in the present study were performed at the time point of 24 h after TBI.

Immunofluorescence staining also showed the high expression of S100A8 in the TBI group. It was detected that S100A8 was co-localized with microglia marker Iba-1 after TBI (Fig. 1c). Thus, we

performed the *in vitro* study in the cultured microglia BV-2.

2. TLR4-MyD88 dependent signaling was activated by S100A8 or TBI

A number of studies revealed that TLR4 plays a significant role in initiating the inflammatory response after stroke or TBI [33, 34]. We hypothesized that S100A8 could induce the inflammation in the brain after TBI through the key inflammatory signaling pathway TLR4-MyD88 pathway. Thus, the expression of TLR4 and MyD88 in the brain were detected in the TBI and S100A8 groups by Western Blot, respectively. Our results showed that intracerebroventricular injection of S100A8 protein remarkably increased the protein levels of TLR4 and MyD88 in the brain (both $p < 0.05$ vs the Sham group), which were both lessened by TAK-242, an inhibitor of TLR4 (Fig. 2a, c and d). TLR4 and MyD88 levels in the TBI group were both higher than that in the Sham group (both $p < 0.05$) (Fig. 2b, e and f). The increased TLR4 and MyD88 expressions after TBI were suppressed partly by TAK-242 (Fig. 2b, e and f).

3. NF- κ B acts as the downstream effector of TLR4-MyD88 dependent pathway after TBI

Previous studies have demonstrated that TLRs could trigger inflammation through activation of NF- κ B, and subsequent upregulation of pro-inflammatory cytokine expression.[35] It was revealed that NF- κ B was involved in the development of inflammation in S100A8/A9-stimulated BV-2 cells [17]. We hypothesized that NF- κ B could act as the downstream node of TLR4-MyD88 dependent pathway after TBI. Thus, it was attempted to investigate the expression of phosphorylated-p65, the main subunit of NF- κ B, at 24 h in the TBI and S100A8 groups by Western Blot analysis. The results showed that intracerebroventricular injection of S100A8 protein or TBI markedly increased the level of p-p65 compared with the Sham group ($p < 0.01$ for each). In contrast, compared with the S100A8 group or TBI group, p-65 were significantly reduced in both TAK-242 treatment groups (both $p < 0.05$) (Fig. 3).

4. Pro-inflammatory cytokine production after intracerebroventricular injection of S100A8 protein or TBI

In the present study, the inflammatory response in the brain of the mice of TBI or intracerebroventricular injection of S100A8 protein was evaluated by the levels of pro-inflammatory cytokines IL-1 β and TNF- α via ELISA. Compared with the Sham group, the concentrations of IL-1 β and TNF- α were significantly increased in the S100A8 group ($p < 0.01$ and $p < 0.001$, respectively) (Fig. 4a and b). In contrast, the concentrations of pro-inflammatory cytokines were reduced at the S100A8 + TAK-242 group compared with S100A8 group ($p < 0.05$ and $p < 0.001$, respectively). And the result in the TBI and TBI + TAK-242

groups were turned out to be the same as S100A8 and S100A8 + TAK-242 groups (Fig. 4c and d), suggesting that S100A8 is involved in the pathology of inflammatory response after TBI.

5. The expressions of p-p65, MyD88, as well as pro-inflammatory cytokines in BV-2 microglial cells after the stimulation of S100A8 protein or LPS

To further confirm that S100A8 promotes the production of pro-inflammatory cytokines in microglia via TLR4/MyD88/NF- κ B signaling pathway, we investigated the expressions of MyD88 and p-p65 at the S100A8, S100A8 + TAK-242, LPS and LPS + TAK-242 groups *in vitro*. The results showed that S100A8 treatment caused noticeably increased expressions of MyD88 and p-p65 compared with the Sham group (Fig. 5a, c and d) ($p < 0.01$). Instead, the expressions of MyD88 and p-p65 in the TAK-242 + S100A8 groups were reduced compared with those in the S100A8 group (Fig. 5a, c and d) ($p < 0.05$). The effects of LPS incubation were similar to that of S100A8 (Fig. 5b, e and f) .

To assess the inflammatory response in BV-2 cells stimulated by S100A8 and LPS, we detected the expressions of pro-inflammatory cytokines by ELISA. As a positive control, IL-1 and TNF- α were significantly increased in the LPS group when compared with the Sham group ($p < 0.05$, $p < 0.0001$, respectively) (Fig. 5i and j). The productions of IL-1 β and TNF- α were also dramatically increased in cultured microglia after the stimulation of S100A8 ($p < 0.001$, $p < 0.01$, respectively) (Fig. 5g and h). At the same time, the expressions of inflammatory cytokines were reduced in the S100A8 + TAK-242 and LPS + TAK-242 group compared with S100A8 or LPS + TAK-242 group, respectively.

Therefore, our data suggested that TLR4/MyD88/NF- κ B signaling pathway was activated in BV-2 microglial cells incubated with S100A8 and subsequently promoted the productions of the pro-inflammatory cytokines such as IL-1 β and TNF- α .

6. S100A8 and TBI promoted neuronal cell death in mice

Figure 6a exhibited Nissl staining of the area adjacent to the contusion brain tissue in different groups. Obvious damage could be seen in the S100A8 and TBI groups with decreases in cell number, sparse cell arrangements and loss of integrity. However, TAK-242 treatment improved the morphological damage induced by S100A8 or TBI (Fig. 6a). The cell numbers were also increased after TAK-242 treatment.. Quantitative results of cell count were shown in Fig. 6b and c.

7. TBI and S100A8 exaggerated neurological deficits and brain edema, which were alleviated by TAK-242 treatment.

To assess the neurological deficits after TBI and intracerebroventricular injection of S100A8, the modified Garcia method was used in the present study. The scores were almost full marks in the Sham group, but there were marked differences between the TBI and Sham groups as well as between the S100A8 and Sham groups ($P < 0.001$ for each). Besides, the mice in the TBI + TAK-242 or S100A8 + TAK-242 group had significant higher neurological scores than those in the TBI group or S100A8 group ($p < 0.01$ and $p < 0.001$ respectively) (Fig. 7a and b), suggesting that S100A8 is harmful to the brain via TLR4 signaling mostly.

The brain water content was also examined in the present study. In accordance with previous studies[36], the brain water content was significantly increased in the TBI group compared with the Sham group ($p < 0.001$) (Fig. 7d). However, they were dramatically reduced by administration of TAK-242 ($p < 0.05$) (Fig. 7d). In the S100A8 group, the brain water content was also increased when compared with the Sham group ($p < 0.001$) (Fig. 7c), which was suppressed after TAK-242 treatment ($p < 0.05$) (Fig. 7c).

Discussion

The present study demonstrates the findings as following: i) the expression of S100A8 in the brain was increased at 24 h after TBI in mice; ii) exogenous S100A8 could induce the activation of inflammatory signaling pathway TLR4/MyD88/NF- κ B, which is similar as TBI; iii) TAK-242, an inhibitor of TLR4, could suppress the activation of TLR4/MyD88/NF- κ B and the production of downstream inflammatory cytokines by S100A8 or TBI.

Previous studies showed that S100A8 is involved in the pathophysiology of diverse diseases in the CNS, such as TBI and stroke [18, 19]. Over-expression of S100A8 was demonstrated to play a critical role in modulating the inflammatory response through the ways of inducing cytokine secretion and stimulating leukocyte recruitment [16]. In contrast, Goyette J and colleagues showed that S100A8 induces the anti-inflammatory properties in macrophages [37]. Further studies revealed that S100A8 may play different roles in different diseases, which depend on proximal microenvironments, oligomeric forms, post-transcriptional modifications and concentrations [16]. Thus, one main aim of the present study is to elucidate the expression and role of S100A8 in TBI. Our data showed that the expression of S100A8 was increased at 24 after TBI and which could induce inflammatory cytokine production by activation of TLR4/MyD88/NF- κ B signaling pathway.

Microglia and blood-derived macrophages are recruited rapidly to the injury site, and are activated by CNS damage or infection in pathophysiological conditions as the previous study showed [14]. In response to a variety of ligands, TLR4, mainly expressed in microglia in CNS, promotes microglial activation and the expression of pro-inflammatory mediators[38]. TLR4 signaling results the activation of NF- κ B and then drives the transcription abundance of pro-inflammatory cytokines that may produce tissue destruction and activate the innate immune system [39–41]. During septic shock, S100A8, as an endogenous ligand of TLR4, results in elevated expression of TNF- α in phagocytes for inducing the translocation of MyD88 and activating of NF- κ B [22]. Similarly, our data demonstrated that the protein of S100A8 may cause inflammation after TBI via TLR4/MyD88 dependent pathway.

The protein of S100A8 had a strong positive effect on inflammatory signaling pathway (i.e. NF- κ B) as our results showed. According to the findings in previous studies, NF- κ B is also a central regulator of microglial responses to activating stimuli [42]. In the present study, S100A8 treatment significantly enhanced the levels of p-p65 *in vitro* and *in vivo*. Hence, the involvement of NF- κ B in the induction of inflammatory response by S100A8 after TBI was confirmed.

Our data showed that S100A8 represents a molecular system participated in the pathogenesis of inflammatory response upstream of TNF α and IL-1 β induction. Because of the high abundance of S100A8 and its involvement in inflammatory diseases, a potential therapy inhibiting uncontrolled inflammatory processes by targeting it is feasible.

Conclusion

In summary, our data indicate that S100A8 promoted the production of pro-inflammatory cytokines via activation of TLR4/MyD88/NF- κ B signaling pathway after TBI. Thus, S100A8 could be a potential biomarker for inflammation in the brain after TBI. Furthermore, inhibition of S100A8 may partly alleviate adverse consequence after TBI and which could be a target for treatment of TBI.

Abbreviations

CNS: central nervous system; DAMP: damage-associated molecular pattern; DMEM: Dulbecco's Modified Eagle Medium; DW: dry weight; ECL: enhanced chemiluminatescence; ELISA: enzyme-linked immunosorbent assay; FBS: fetal bovine serum; HRP: horseradish peroxidase; IL: interleukin; ICR: Institute of Cancer Research; LPS: Lipopolysaccharide; MyD88: myeloid differentiation factor 88; NF- κ B: nuclear factor-kappa B; PBS: phosphate buffered saline; PVDF: polyvinylidene-difluoride; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBI: Traumatic brain injury; TLRs: Toll-like receptors; TBST: Tris Buffered Saline with Tween20; WB: Western Blot; WW: wet weight.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

GYH contributed in the design and conception and writing of the manuscript. MLZ, YLH, and DGW participated in several experiments and carried out the analysis, acquisition, and interpretation of the data. HC and LAS made the statistical analysis and revision of the manuscript. GFD and XCJ supported in the technical support, such as conception, design and the funding, and revision of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval

The experimental protocols in the present study including all the surgical procedures and animal usages were approved by the Animal Care and Use Committee of Jinling Hospital.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

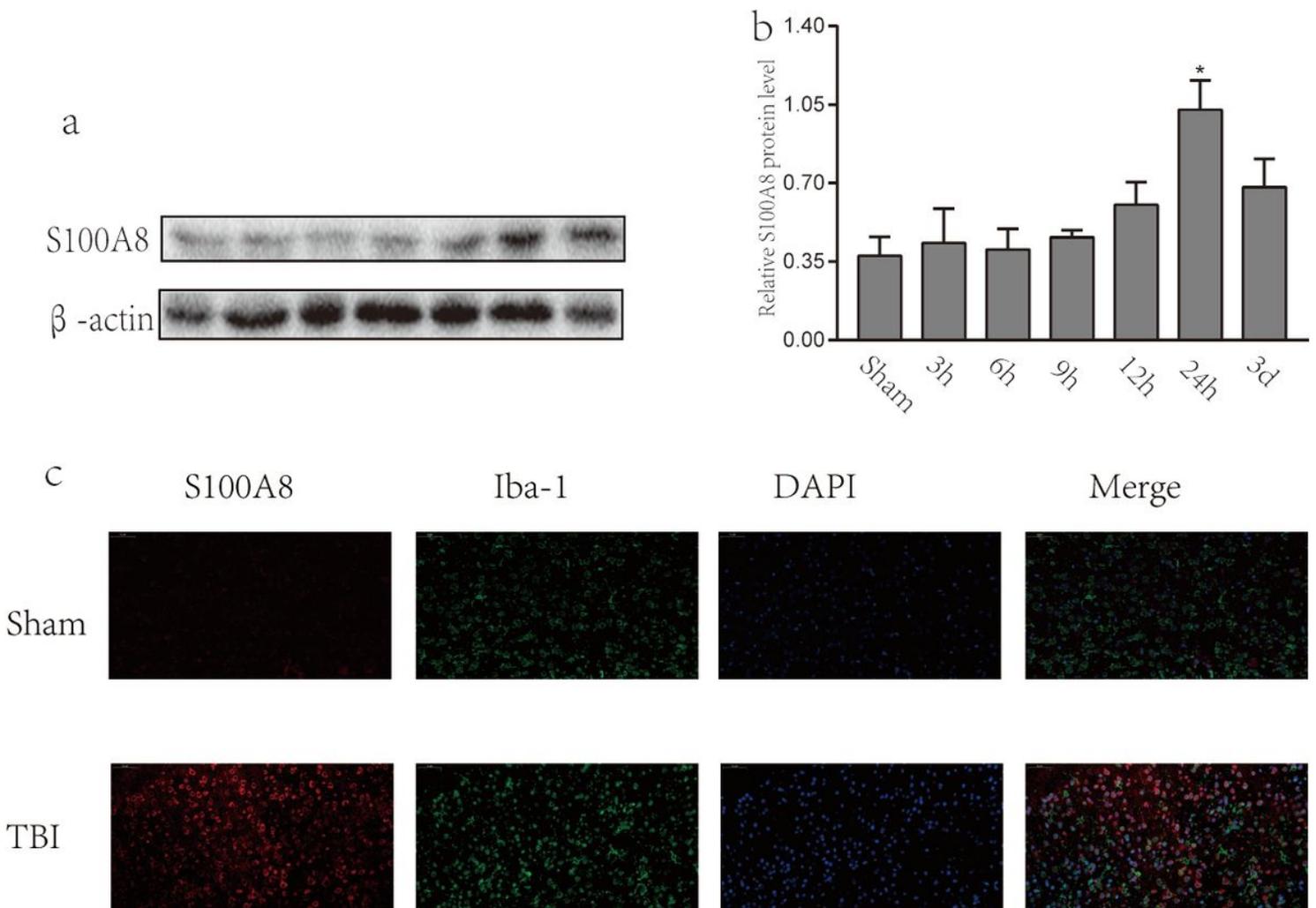


Figure 1

Time-course expression and Location of S100A8 after Experimental TBI. (a and b) Time-course expression and semiquantitative analysis of S100A8 after experimental TBI in mice; (c) Representative

immunofluorescence images show that S100A8 co-localized with microglia in vivo. Data are represented as mean \pm SD (n=3, per group). *p<0.05 compared with the Sham group.

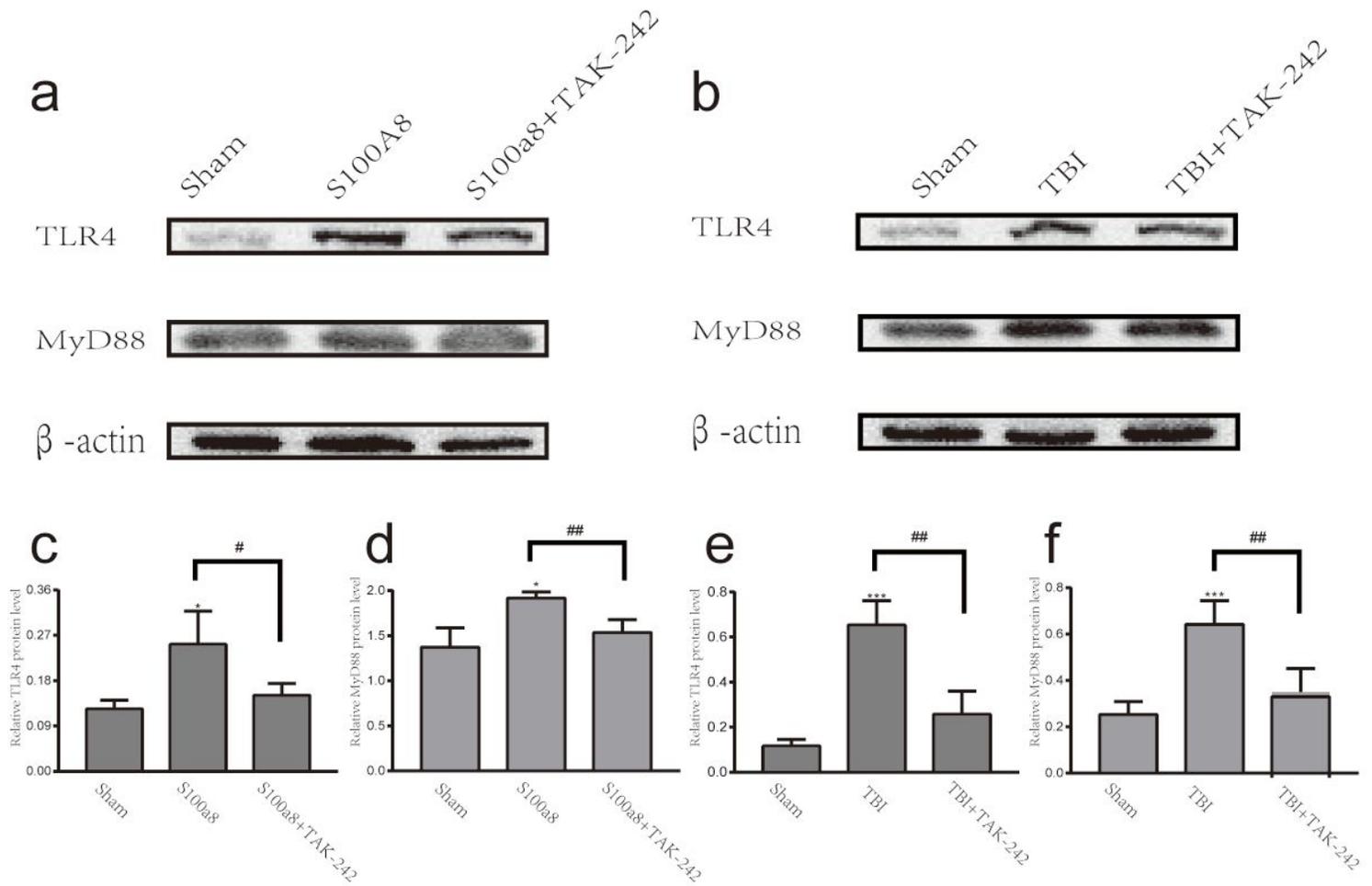


Figure 2

Evidence for Toll-like receptor 4 (TLR4)/MyD88 dependent signaling pathway was involved in TBI; (a, c and d) Representative Western Blot bands and semiquantitative analysis of TLR4 and MyD88 expression in different groups after intracerebroventricular injection of S100A8 protein; (b, e and f) Representative Western Blot bands and statistical analysis of TLR4 and MyD88 expression in different groups after TBI in mice. Data are represented as mean \pm SD (n=3, per group). *p<0.05 and ***p<0.001 compared with the Sham group; #p<0.05, ##p<0.01 compared with the S100A8 or TBI group.

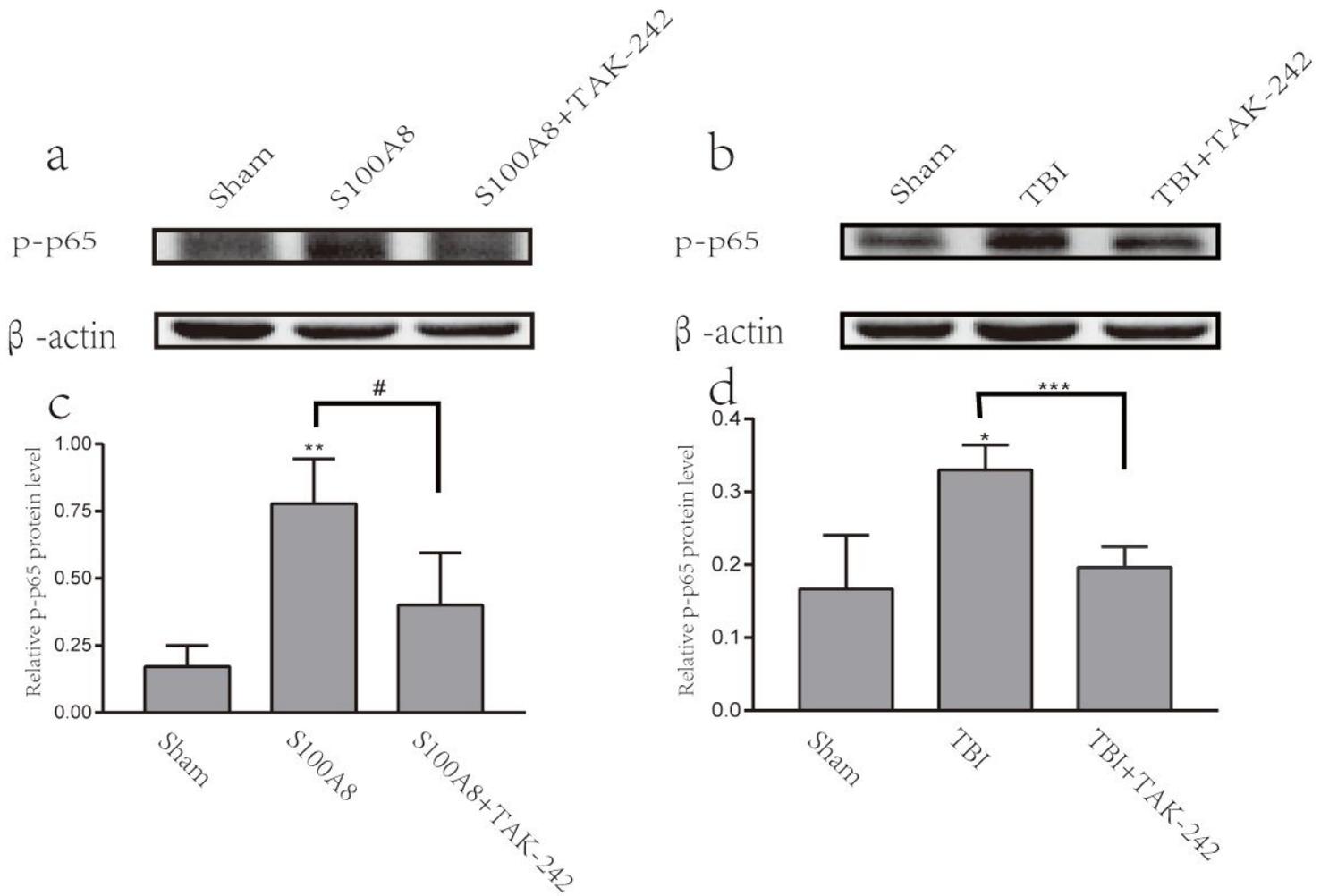


Figure 3

Involvement of NF- κ B activation after S100A8-stimulated and TBI. (a and c) Representative western blot bands and statistical analysis of p-p65 expression in different groups after intracerebroventricular injection of S100A8 protein; (b and d) Representative Western Blot bands and semiquantitative analysis of p-p65 expression in different groups after TBI in mice. Data are represented as mean \pm SD (n=3, per group); *p<0.05 and **p<0.01 compared with the Sham group; #p<0.05 and ###p<0.001 compared with the S100A8 or TBI group.

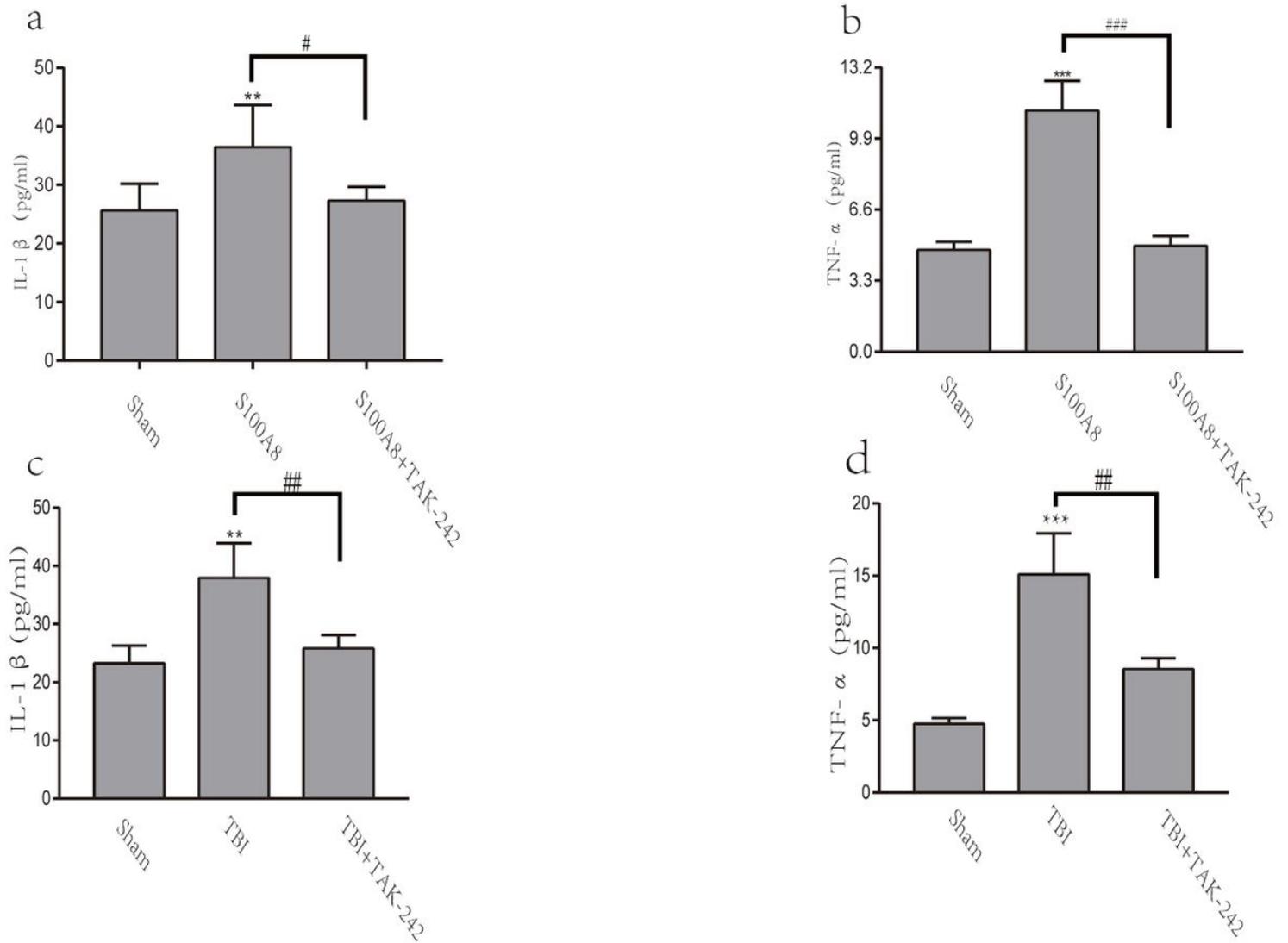


Figure 4

Detection of inflammatory cytokines by ELISA in vivo. (a and b) TNF- α and IL-1 β levels were determined by ELISA after intracerebroventricular injection of S100A8 protein. (c and d) Pro-inflammatory cytokine production in the Sham, 24 h post-TBI and TAK-242 treatment TBI mice. Data are represented as mean \pm SD (n=3, per group). **p<0.01 and ***p<0.001 compared with the Sham group; #p<0.05, ##p<0.01 and ###p<0.001 compared with the S100A8 or TBI group.

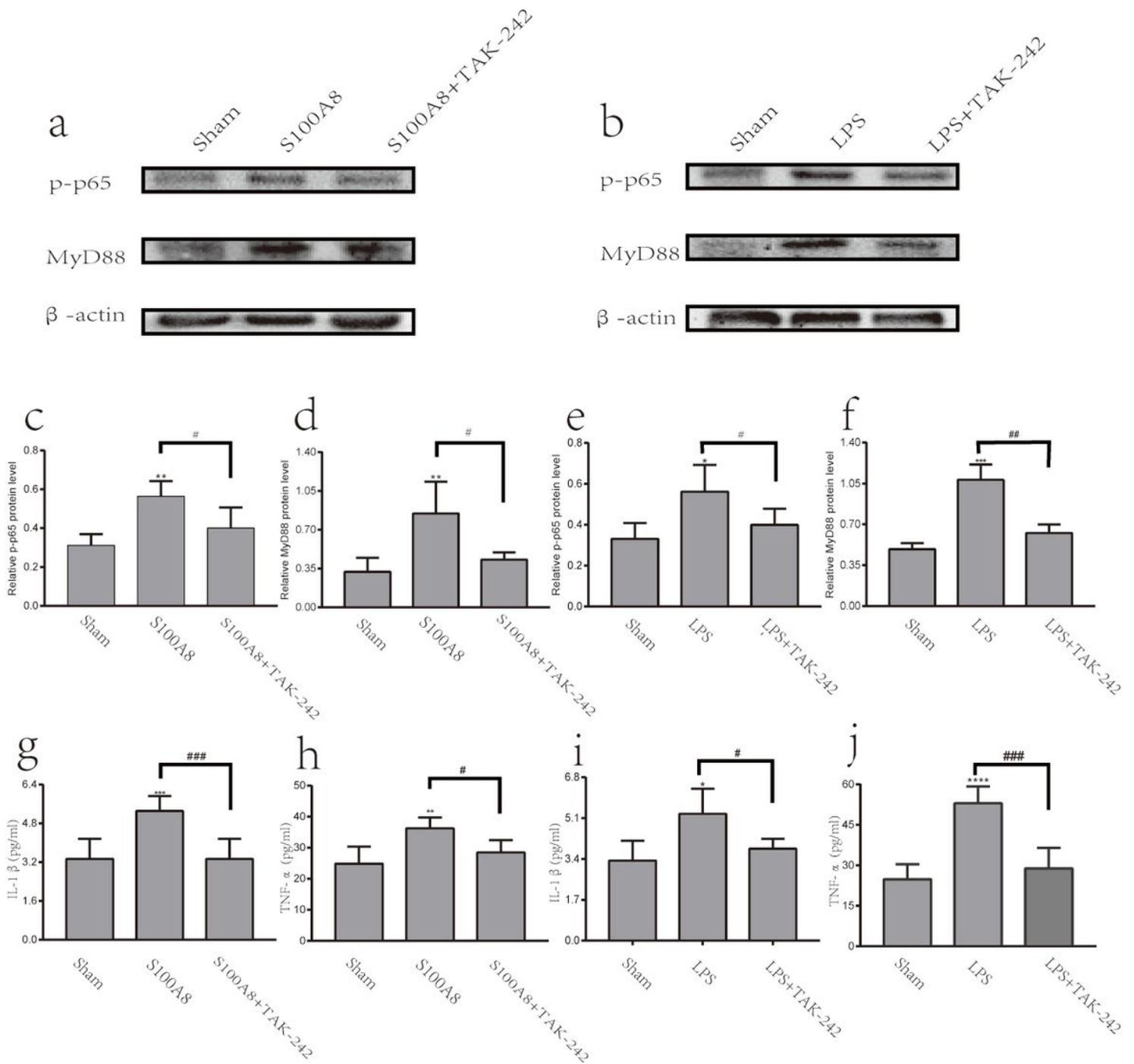


Figure 5

TLR4/MyD88/NF-κB signaling pathway was activated in the stimulation of S100A8 or LPS in vitro; (a, c and d) Representative Western Blot bands and quantitative data for MyD88 and p-p65 in the stimulation of S100A8 protein. (b, e and f) Representative Western Blot bands and quantitative data for MyD88 and p-p65 in the stimulation of LPS; (g, h, i and j) Pro-inflammatory cytokine production in BV-2 microglial cells after S100A8 or LPS stimulation. Each value represents the mean ± SD (n=3, per group) of three

independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ compared with the Sham group; # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ compared with the S100A8 or LPS group.

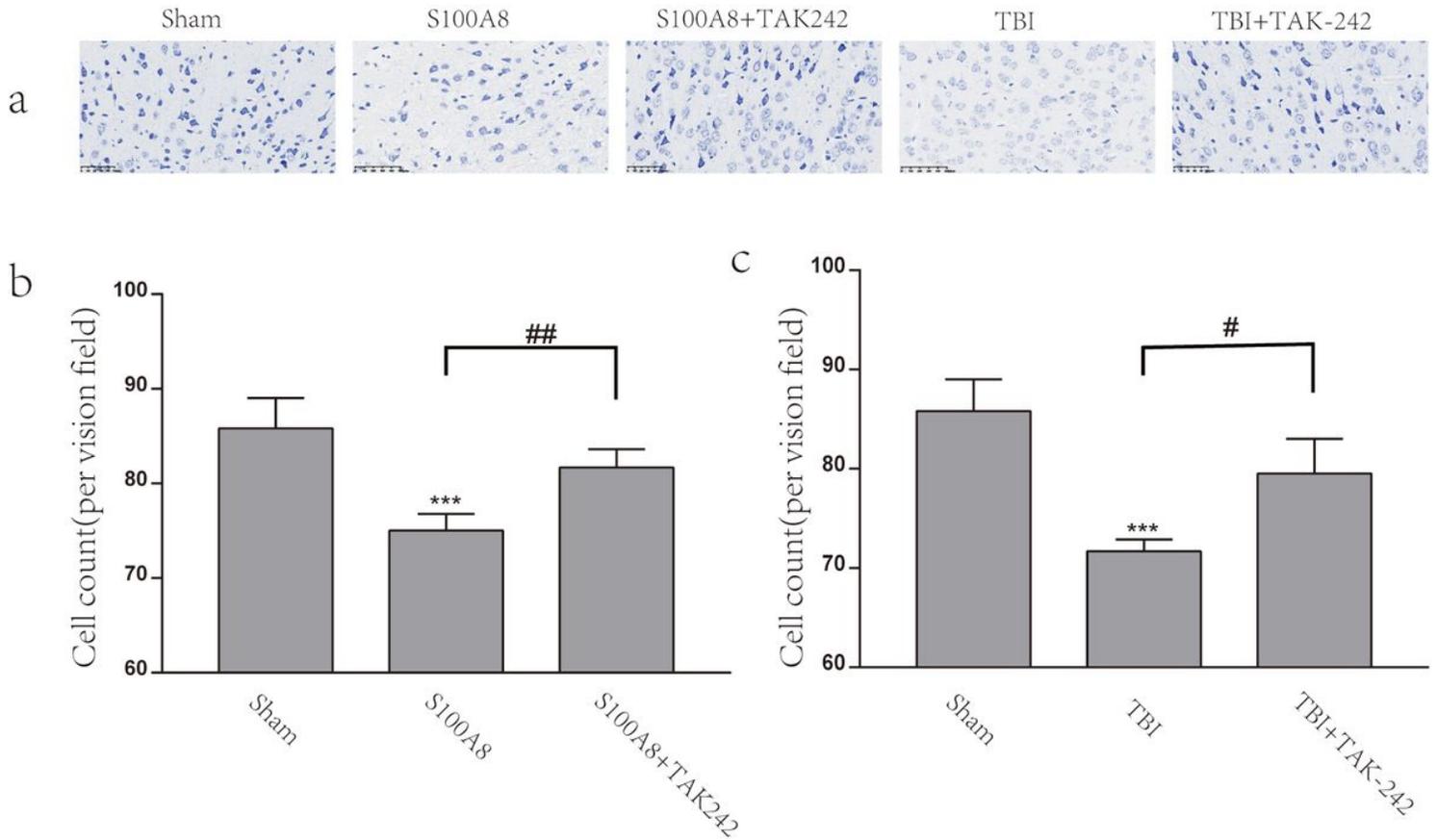


Figure 6

S100A8 and TBI promoted neuronal cell death in mice. (a) Representative nissl staining images of the mice' brain in different groups. (b and c) Statistical analysis of cell counts of survival neurons in different groups ($n=3$, *** $p < 0.001$ compared with the Sham group, # $p < 0.05$ and ## $p < 0.01$ compared with the S100A8 or TBI group).

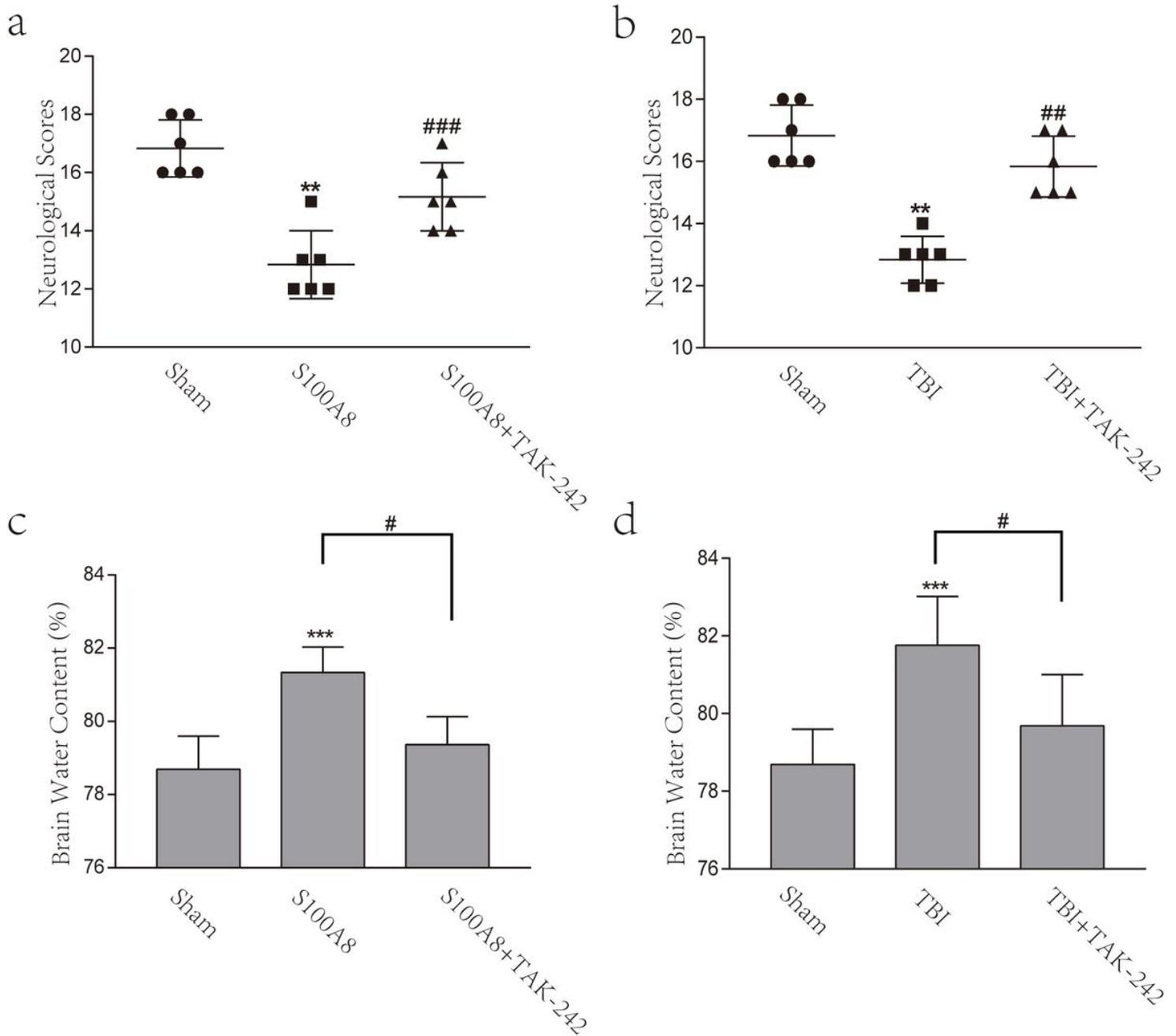


Figure 7

Effects of S100A8 treatment on neurological deficits and brain edema. (a and b) In S100A8 and TBI groups, the neurological deficits are lower than those in the S100A8+TAK-242 and TBI+TAK-242 groups, respectively. The neurological scores in the Sham group was higher than that in any other group; (c and d) Brain edema was exacerbated in TBI and S100A8 groups. Data are represented as mean ± SD (n=6, per group). **p<0.01 and ***p<0.001 compared with the Sham group; #p<0.05, ##p<0.01 and ###p<0.001 compared with the S100A8 or TBI group.