USP8 protects against lipopolysaccharide-induced cognitive and motor deficits by modulating microglia phenotypes through TLR4/MyD88/NF-κB signaling pathway in mice

JiaYi Zhao, Wei Bi, JiaWei Zhang, Shu Xiao, RuiYi Zhou, Chi Kwan Tsang, DaXiang Lu, Lihong Zhu

Department of Pathophysiology, School of Medicine, Jinan University, Guangzhou, Guangdong Province 510632, China
Department of Neurology, The First Affiliated Hospital, Jinan University, Guangzhou, Guangdong Province 510630, China
Clinical Neuroscience Institute, The First Affiliated Hospital of Jinan University, Guangzhou, Guangdong Province 510630, China

Abstract
Ubiquitin-specific protease 8 (USP8) regulates inflammation in vitro; however, the mechanisms by which USP8 inhibits neuroinflammation and its pathophysiological functions are not completely understood. In this study, we aimed to determine whether USP8 exerts neuroprotective effects in a mouse model of lipopolysaccharide (LPS)-induced cognitive and motor impairment. We commenced intracerebroventricular USP8 administration 7 days prior to i.p. injection of LPS (750 μg/kg). All treatments and behavioral experiments were performed once per day for 7 consecutive days. Behavioral tests and pathological/biochemical assays were performed to evaluate LPS-induced hippocampal damage. USP8 attenuated LPS-induced cognitive and motor impairments in mice. Moreover, USP8 downregulated several pro-inflammatory cytokines [nitric oxide (NO), tumor necrosis factor α (TNF-α), prostaglandin E2 (PGE2), and interleukin-1β (IL-1β)] in the serum and brain, and the relevant protein factors [inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2)] in the brain. Furthermore, USP8 upregulated the anti-inflammatory mediators interleukin (IL)-4 and IL-10 in the serum and brain, and promoted a shift from pro-inflammatory to anti-inflammatory microglial phenotypes. The LPS-induced microglial pro-inflammatory phenotype was abolished by TLR4 inhibitor and in TLR4−/− mice; these effects were similar to those of USP8 treatment. Mechanistically, we found that USP8 increased the expression of neuregulin receptor degradation protein-1 (Nrdp1), potently downregulated the expression of TLR4 and myeloid differentiation primary response protein 88 (MyD88) protein, and inhibited the phosphorylation of IκB kinase (IKK) β and kappa B-alpha (IkBα), thereby reducing nuclear translocation of p65 by inhibiting the activation of the nuclear factor-kappaB (NF-kB) signaling pathway in LPS-induced mice. Our results demonstrated that USP8 exerts protective effects against LPS-induced cognitive and motor deficits in mice by modulating microglial phenotypes via TLR4/MyD88/NF-kB signaling.

1. Introduction
Neuroinflammation is a major cause of neurodegenerative diseases (Allison and Ditor, 2014), of which many remain incurable. Current clinical therapeutic strategies for progressive neurodegenerative disorders are designed to control symptoms rather than address the underlying cause of neurodegeneration (Herrero and Morelli, 2017). Uncontrolled or chronically inflamed brains lead to neurotoxicity due to
production of neurotoxic mediators and inflammatory cytokines, which could aggravate the pathological symptoms of neurodegeneration (Nichols et al., 2019; Pimplikar, 2014). Mediators of neuroinflammation can directly affect cognition and memory, contributing to cognitive and motor impairment associated with several chronic neurological conditions (Jin et al., 2019). Strategies to prevent and/or ameliorate neuroinflammation are therefore essential to reduce brain damage and cognitive deficits (Verdile et al., 2015; Fan et al., 2014).

The neuroinflammatory response includes microglial activation resulting in a phagocytic phenotype, and subsequent release of inflammatory mediators (Cunningham, 2013). Microglial activation in the central nervous system is heterogeneous, and mainly results in the contrasting M1 and M2 phenotypes. M1 microglia are predominantly seen at injury sites at the end stage of disease, when the immune resolution and repair process of M2 microglia are dampened. (Tang and Le, 2016). The regulation of microglial polarization from the M1 to M2 phenotype could prove valuable in the development of therapeutic and preventive strategies against neurodegenerative diseases (Jin et al., 2019).

Microglial cells express TLR4 on their surface for actively monitoring the environment. TLR4 deficiency promotes a shift to the M2 microglial phenotype, which ameliorates neurological impairment (Yao et al., 2017). In response to lipopolysaccharide (LPS; a specific ligand for TLR4), microglia become hyper-activated, resulting in the production of cytotoxic factors such as NO, TNF-α, and PGE2 (Hoogland et al., 2015). Although microglia are nervous system-specific immune cells that influence brain development, response to injury, and tissue repair, excessive cytokine production and microglial activation can cause systemic inflammatory responses including neuroinflammation, which could impair cognitive and motor function (Rice et al., 2017). Subsequent microglial activation promotes the release of more inflammatory factors, thus initiating a cycle that often leads to neuronal death (Frank-Cannon et al., 2009).

LPS primarily activates the MyD88-dependent and independent pathways, which involve recognition of the lipid A-region of LPS by TLR4, suggesting that MyD88 pathways play important roles in inflammation and immune response (Rahimifard et al., 2017). Microglia are the main immune cells of the central nervous system that express TLR4, while LPS is a widely expressed bacterial TLR4 ligand that activates the innate immune response to infections (Döring et al., 2017). TLR4 could modulate the NF-kB inflammatory cascade, leading to neurodegeneration (Lysakova-Devine et al., 2010). Inhibiting microglial activation and the resulting neuroinflammation could serve as an adjunctive treatment for the progressive neurodegenerative disorders.

The ubiquitin proteasome system (UPS) is an efficient protein degradation pathway (Gallastegui and Groll, 2010), which regulates short half-life regulatory proteins in cells and structurally abnormal, misfolded, or damaged proteins. The UPS system includes ubiquitin and several other enzymes. USP8 is a member of DUBs, and regulates the stability of ubiquitin protein ligases (E3s) including Nrdp1, which is an E3 expressed primarily in the brain, heart, prostate, and skeletal muscle (Soares et al., 2004). Nrdp1, also known as FLRF or RBCC, is a newly E3 expressed primarily in the brain, heart, prostate, and skeletal muscle (Zhang et al., 2011). Nrdp1 inhibits the production of proinflammatory cytokines but increases interferon-β production in toll-like receptor-triggered macrophages by suppressing adaptor MyD88-dependent activation of NF-kB and activator protein-1 (AP-1) while promoting activation of the kinase TBK1 and transcription factor IRF3 (Wang et al., 2009). Nrdp1 is a specific target for USP8 deubiquitinating enzyme, and USP8 could augment Nrdp1 activity by mediating its stabilization (Wu et al., 2004). We previously reported a significant reduction in the degradation of Nrdp1 in BV2-immortalized murine microglial cells after transfection with USP8. USP8 overexpression also reduced the production of LPS-induced proinflammatory mediators. USP8 could therefore be a novel candidate for the treatment of neuroinflammatory disorders (Zhu et al., 2015).

The mechanism by which USP8 inhibits inflammation in vivo remains unclear. We hypothesized that USP8 could increase the expression of Nrdp1 and inhibit neuroinflammation by modulating the TLR4-mediated MyD88-dependent pathway. In this study, we used LPS (a specific ligand for TLR4) to induce neuroinflammation, VIPER (a specific TLR4 inhibitor), and TLR4-/- mice to elucidate the mechanism of action and potential targets of USP8-mediated effects on cognitive and motor impairments. Subsequently, we studied the effects of USP8 on LPS-induced animal behaviors, microglial morphology, release of inflammatory factors, protein expression, and activation of inflammatory pathways.

2. Materials and methods

2.1. Animals

C57BL/6J male mice (11 to 12 weeks old, Guangdong Medical Laboratory Animal Center) and transgenic TLR4-/- male mice (Model Animal Research Center of Nanjing University) were maintained and handled in accordance with the guidelines of the Animal Ethics Committee of Jinan University (SYXK 2017-0174). All mice were housed in a room with automatically controlled temperature (21–25 °C), relative humidity (45%–65%), and light-dark (12 h: 12 h) cycle.

2.2. USP8 treatment and LPS-induced cognitive and motor impairment

The mice were divided into the following groups: (1) Control, (2) Saline, (3) LPS, (4) USP8 + LPS, (5) USP8, and (6) NC + LPS. Intracerebroventricular (i.c.v.) USP8 (lentiviruses encoding mouse USP8 constructed and produced by Ohio Technology, Shanghai; 1 × 10⁸ TU/μL, 4 μL) administration was performed using a microsyringe with the stereotaxic coordinates −0.26 cm dorsal, −0.15 cm lateral, and −0.02 cm anterior from bregma as described by Haley and McCormick (1957), and was commenced 7 days prior to i.p. injection of LPS (750 μg/kg). The saline group received an equal volume of saline while the control group did not receive injections. The NC + LPS group received an equal volume of LV5 (EF-1a/GFP/Puro, purchased from Ohio Technology; 1 × 10⁸ TU/μL, 4 μL), starting 7 days prior to i.p. injection of LPS (750 μg/kg). Fig. 2A depicts the detailed experimental protocol.

We determined whether USP8 protected against LPS-induced cognitive and motor impairments by inhibiting TLR4, and compared the neuroprotective effects of USP8 with those of VIPER, a specific TLR4 inhibitor. Seven groups of animals were used for this experimental protocol: (1) Control group, (2) Saline group, (3) LPS group, (4) USP8 + LPS group, (5) VIPER + LPS group, (6) VIPER + USP8 + LPS group, and (7) VIPER group. Mice in the VIPER + LPS group were treated with VIPER (100 μg/kg, i.p.) 2 h before LPS injection (Fig. 5A).

To determine the role of TLR4 in USP8-mediated attenuation of cognitive impairment following neuroinflammation, animals were divided into: (1) Wild type (WT) control group; (2) WT LPS group; (3) TLR4-/- control group; and (4) TLR4-/- LPS group. A dose of LPS (750 μg/kg) was injected into mice daily for 7 days.

2.3. Neurocognitive behavior tests

To determine the therapeutic effects of USP8 in LPS-induced mice, we used the Morris water maze (MWM) test (Foster, 2012) and step-through passive avoidance test (PAT) (Shan et al., 2009) to assess learning and memory abilities.

The MWM was performed by Cheng Du Technology & Market Co., LTD. A circular pool (height: 35 cm, diameter: 120 cm) was filled with water made opaque with whole milk kept at 21–25 °C. An escape platform (height: 14 cm, diameter: 4-5 cm) was submerged 1–1.5 cm...
below the surface of the water at a specific position. The mice were trained with three trials per day for 7 days. After training, LPS or saline was administered 6 h prior to the beginning of the test every day, and the spatial probe test was conducted on the last day of testing. The tasks consisted of a place navigation test and spatial probe test.

The PAT, using a “step-through” apparatus (Cheng Du Technology & Market Co, LTD.) consisting of six reaction boxes, was used to test learning and memory ability. When mice entered the dark compartment, they received an electric shock (39 V, 3-s duration). Latency to enter the dark compartment was automatically recorded. Mice were placed in the illuminated compartment facing away from the dark compartment during the training trials for the first 3 days. Subsequently, LPS was injected 6 h before each daily test during the training phase (7 days).

2.4. Motor coordination tests

Motor behavior was evaluated using the pole test and traction test (Zhu et al., 2018). For the pole test, we conducted 5 days of training on a pole (diameter: 1 cm, length: 60 cm). On the sixth day, LPS was injected 6 h before testing. The time taken for mice to climb down was recorded (finishing the upper and lower halves of the pole and total length of the pole). The following standards were used for scoring: crossing the three parts within 3 s was scored as 3, within 6 s was scored as 2, and > 6 s was scored as 1. Results were expressed as total scores.

For the traction test, mice were placed on a horizontal wire by their front paws and scored as follows: 3 when mice grasped the wire with both hind paws, 2 when mice grasped the wire with one hind paw, and 1 when mice could not grasp the wire with either hind paw, including falling down.

2.5. Immunofluorescence staining

Samples were transferred to 30% sucrose solution, and 10-μm-thick sequential sections were prepared on a microtome (Leica CM 1850; Leica Microsystems, Seoul, Korea) and incubated at 37°C overnight. After three washes in phosphate buffer saline (PBS) (pH 7.4) for 5 min each, brain sections were permeabilized with Triton X-100 (0.3% in TBST) at room temperature for 10 min followed by three washes in 0.025% TBST for 5 min each. Then, sections were blocked for 1 h in 1% bovine serum albumin (BSA) solution and incubated overnight at 4°C with rabbit polyclonal antibody against microtubule-associated protein 2 (MAP2; Millipore Corp, Billerica, MA, USA) or anti-chitinase 3 like protein 3 (YM-1; Abcam, Inc., Cambridge, MA, USA), mouse monoclonal antibody against USP8 (1:50; Sigma Chemical, St Louis, MO, USA) or anti-TNF-α (Abcam, Inc., Cambridge, MA, USA), or goat polyclonal antibody against ionized calcium-binding adapter protein 1 (IBA1; Millipore Corp., Billerica, MA, USA). The day after incubation with primary antibodies, the sections were washed three times in PBS (pH 7.4) for 10 min each. After washing, brain sections were incubated for 1 h at room temperature with Alexa-Fluor 488- or 647-conjugated donkey anti-goat secondary antibody (Invitrogen-Molecular Probes, Carlsbad, CA), Alexa-Fluor 488- or 555-conjugated donkey anti-rabbit secondary antibody (Invitrogen-Molecular Probes, Carlsbad, CA), and Alexa-Fluor 546-conjugated donkey anti-mouse secondary antibody (Invitrogen-Molecular Probes, Carlsbad, CA). Sections were then washed in PBS (pH 7.4) three times for 10 min each. Images were obtained using fluorescence microscopy (Leica Microsystems, Wetzlar, Germany).

2.6. Nitrite and ELISA assay

NO production was determined by measuring nitrite levels in the serum. Nitrite is a stable oxidative product of NO and is assessed by the Griess reaction. For the ELISA assay, serum was collected after treatment. IL-1β and TNF-α were measured using an ELISA kit from eBioscience (Vienna, Austria), PGE2 was measured using an ELISA kit from R&D Systems (Minneapolis, MN), and IL-4 and IL-10 were measured with an ELISA kit from USCN (Wuhan, China) according to manufacturers’ instructions.

2.7. Western blot analysis

Brain tissue was lysed in ice-cold RIPA lysis buffer (Beyotime, Nantong, Jiangsu, China) containing 1 mM phenylmethylsulfonyl fluoride. For full cleavage, the tissue was lysed using an ultrasonic instrument and centrifuged for 15 min at 12,000 rpm and 4°C. Supernatant was collected for protein analysis. Cytoplasmic and nuclear p65 detection was performed using a NE-PER® kit according to manufacturer's instructions (Thermo Scientific, Rockford, IL, USA). Equal amounts of protein (10–50 μg) were resolved on 8–10% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA), blocked with 5% non-fat milk, and washed with TBST. The membranes were incubated overnight at 4°C with primary antibodies. To detect target proteins, specific antibodies against COX-2, iNOS, IKK, Phospho-IKK-α/β (p-IKK α/β) inhibitory IκBα, phospho-IκBα (p-IκBα), NF-κB, MyD88, Tir domain-containing adaptor inducing interferon-beta (TRIF), TLR4, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Tublin and Lamin-B1 (Cell Signaling Technology Inc, MA, USA) were used. Antibodies against USP 8 and Nrdp1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The membranes were then incubated with the corresponding conjugated goat anti-mouse or goat anti-rabbit IgG-horseradish peroxidase (HRP) secondary antibodies (Cell Signaling Technology Inc, MA, USA) for 1 h at room temperature and subsequently developed with enhanced chemiluminescence detection reagent. The results were quantified using scanning densitometry.

2.8. Statistical analysis

Data are presented as the mean ± SEM derived from three experiments. Comparisons between two groups were made using Student’s t-test. Comparisons among multiple groups were made using one-way ANOVA followed by post hoc pairwise comparisons. The level of statistical significance was set at p < 0.05.

3. Results

3.1. USP8 expression in the brain after intraperitoneal injection of LPS

To determine the expression of endogenous USP8 in the brains of LPS-induced mice, we injected mice with LPS intraperitoneally for 1, 3, 5, 7, and 9 days. USP8 protein levels in the brain decreased with time and the decrease was significant after 3 days of LPS injection (control: 0.86 ± 0.044, LPS (3 d): 0.21 ± 0.054, LPS (5 d): 0.19 ± 0.012, LPS (7 d): 0.20 ± 0.010, LPS (9 d): 0.18 ± 0.0114; Fig. 1A). LPS injection induced a time-dependent decrease in endogenous USP8 levels. In order to increase intracellular USP8 protein levels, we performed intracerebroventricular (i.c.v.) USP8 injection in the mice, which resulted in higher USP8 protein levels (1.21 ± 0.04; Fig. 1B) in the brain tissues compared to those in control and saline groups (control, 1.04 ± 0.04; saline, 1.12 ± 0.06).

To determine whether USP8 is expressed in microglial cells in the brain, we detected the expression of USP8 and IBA-1 by immunofluorescence. USP8 was mainly co-localized with IBA1-positive cells (Fig. 1C), indicating that USP8 is expressed in microglia. Consistently, we observed that IBA1 expression was significantly reduced after LPS administration, and icv injection of USP8 restored microglial USP8 levels (Fig. 1B, C).
3.2. Inhibitory effects of USP8 on LPS-induced cognitive and motor dysfunction

To elucidate the effects of LPS and USP8 on hippocampus-dependent learning and memory, we conducted MWM and PAT. Control mice exhibited short escape latency to reach the platform. LPS-induced mice took a longer time to reach the platform location than did control mice (control, 9.31 ± 1.10 s; saline, 9.13 ± 1.05 s; LPS, 17.64 ± 0.92 s; \( P < 0.01 \); Fig. 2B), suggesting that LPS treatment caused memory deficits. Mice that received i.c.v injections of USP8 displayed significantly reduced escape latency after LPS treatment (11.50 ± 1.50 s; \( P < 0.01 \), Fig. 2B). In the spatial probe test, the mean incidences of crossing the removed platform and time in target section were increased in USP8-pretreated mice (\( P < 0.01 \), Fig. 2C), suggesting that USP8 treatment alleviates LPS-induced memory deficits.

Fig. 1. USP8 expression in microglia after i.p. injection of LPS. (A) USP8 protein level in mouse brain was significantly decreased at different time points in mice received i.p. injection of LPS. USP8 protein level was detected by western blot using anti-USP8 antibody. GAPDH was used as a loading control. Lower panel, quantification of USP8 protein levels, \( n = 6 \). (B) Intracerebroventricular injection of USP8 enhanced USP8 protein level in the brain. Lower panel, quantification of USP8 protein levels in mouse brains (C) Double immunofluorescence and confocal imaging confirmed the colocalization of USP8 (red) and IBA1 (green) in hippocampus. DAPI indicates nuclear staining in blue (Scale bar, 25 \( \mu \)m.). Data are presented as mean ± SEM. * \( P < 0.05 \), ** \( P < 0.01 \) compared to control group and saline group; # \( P < 0.05 \), ## \( P < 0.01 \) compared to USP8 + LPS group and USP8 group, analyzed by one-way ANOVA. Error bars indicate SEM.
We used the pole and traction tests to determine whether USP8 could improve LPS-induced impairments in locomotion. As shown in Fig. 2D and E, test scores in USP8-pretreated mice were significantly higher than those in USP8-untreated mice (P < 0.01), indicating that USP8 treatment improves locomotive performance.

### 3.3. USP8 suppressed neuroinflammation in LPS-induced mice

Microglial activation and cytokines are essential for LPS-induced neuroinflammation. We therefore monitored the levels of select pro-inflammatory cytokines (TNF-α, IL-1β, PGE₂, and NO) in serum and brain homogenates, and inflammation-related protein factors (iNOS and COX-2) in the brain. ELISA and Griess assay revealed that the levels
Fig. 3. Inhibitory effects of USP8 on LPS-induced neuroinflammation. (A) USP8 treatment decreased LPS-induced pro-inflammatory cytokines in serum and brain. The indicated pro-inflammatory cytokines were detected by ELISA and Griess assay. n = 5. (B) USP8 treatment increased LPS-induced anti-inflammatory cytokine production in the serum and brain. The indicated anti-inflammatory cytokines were detected by ELISA. n = 5. (C) Effect of USP8 treatment on iNOS and COX-2 levels in the brains after LPS-induced neuroinflammation. USP8 treatment alleviated the expression of iNOS and COX-2 protein. n = 6. Lower panel, quantification of the western blot result. (D) USP8 treatment alleviated neuronal loss induced by LPS-mediated neuroinflammation. Neurons and microglia were stained by MAP2 antibody (red) and IBA1 antibody (green) in the hippocampus after treatment. Images were acquired by double immunofluorescence with confocal microscopy. DAPI indicates nuclear staining (blue). n = 5 (Scale bar, 100 μm.). Data are presented as mean ± SEM. *P < 0.05, **P < 0.01 compared to control group and saline group; #P < 0.05, ##P < 0.01 compared to USP8 + LPS group and USP8 group, analyzed by one-way ANOVA. Error bars indicate SEM.
Microglia exhibit several phenotypes, including the classical pro-inflammatory (M1) and alternative anti-inflammatory (M2) phenotypes. In the presence of LPS, microglial cells (IBA1-positive cells) in the hippocampus were predominantly M1-phenotypes (IBA1-positive cells) (Wang et al., 2009). We subsequently used immunofluorescence to detect neuronal cells (MAP2-positive cells) and microglial cells (IBA1-positive cells) in the hippocampus. As expected, LPS induced prominent loss of cholinergic neurons (labeled by MAP2) and activation of microglia (labeled by IBA1; Fig. 3D). Treatment with USP8 significantly reduced neuronal loss and microglial activation (Fig. 3D).

3.4. USP8 increased anti-inflammatory factor levels in LPS-induced mice

We performed ELISA to detect the levels of anti-inflammatory markers (IL-4 and IL-10) in serum and brain homogenates to assess the anti-inflammatory effect of USP8 treatment. As shown in Fig. 3B, USP8 treatment significantly increased IL-4 and IL-10 levels in the serum and brain after LPS injection (P < 0.01, Fig. 3B). This result suggests that USP8 could facilitate the clearance of inflammation.

3.5. Inhibitory effects of USP8 on LPS-induced MyD88-dependent signaling pathway activation

We then studied the molecular mechanisms underlying the therapeutic effects of USP8 on neuroinflammation-associated cognitive impairment. LPS is a direct ligand of TLR4 (Laird et al., 2009). We confirmed that the expression of TLR4 was elevated in LPS-induced mice (0.68 ± 0.09) compared to control and saline groups, whereas USP8 pre-treatment significantly reduced TLR4 expression (control, 0 ± 0; saline, 0.02 ± 0.01; USP8 + LPS, 0.07 ± 0.06; P < 0.01). This result is consistent with those of other studies showing that increased inflammation in the brain is at least partly due to TLR4 activation in LPS-induced mice (Pardon, 2015). Importantly, USP8 treatment could inhibit TLR4 expression.

To determine whether USP8 could inhibit the TLR4 signaling pathway, nuclear extracts from mouse hippocampi were prepared and assayed for TLR4 signaling pathway activity. TLR4 interacts with the adapter protein MyD88 or/and TRIF to activate NF-κB, which regulates the expression of inflammatory mediators (Lin et al., 2012). In mice injected with LPS, we found no significant differences in TRIF protein expression compared with control, saline, and USP8-treated groups (Fig. 4A and B); however, P65 protein was translocated to the nucleus, and IkBα was phosphorylated (control, 0 ± 0; saline, 0 ± 0; LPS, 0.87 ± 0.06; USP8 + LPS, 0 ± 0; P < 0.01). USP8 treatment inhibited LPS-induced translocation of P65 to the nucleus and IkBα phosphorylation (P < 0.01, Fig. 4A and B). Thus, USP8 suppresses the signaling activity of the MyD88-dependent pathway by inhibiting the expression of its signaling components.

3.6. USP8 treatment induced an M1 to M2 switch in microglial phenotype in the hippocampi of LPS-induced mice

TLR4 activation by LPS can trigger neuroinflammation by activating inflammatory cells such as microglia (Laird et al., 2009; Pardon, 2015). Microglia exhibit several phenotypes, including the classical pro-inflammatory (M1) and alternative anti-inflammatory (M2) phenotypes (Song and Suk, 2017). TNF-α is expressed by the M1 phenotype, and YM-1 is expressed by the M2 phenotype (Wu et al., 2015; Pan et al., 2015). After LPS injection, TNF-α positive cells increased (control, 18.37% ± 3.81%; saline, 14.23% ± 9.25%; LPS, 93.71% ± 1.17%; USP8 + LPS, 9.59% ± 3.92%; P < 0.01), and almost no YM-1-positive cells were observed, indicating that microglia were predominantly in the M1 phenotype. Conversely, USP8 treatment caused a dramatic increase in the M2 microglial phenotype (Fig. 4C); YM-1-positive cell count (control, 81.63% ± 3.81%; saline, 85.77% ± 9.28%; LPS, 6.29% ± 1.17%; USP8 + LPS, 90.41% ± 3.92%; P < 0.01) and the expression of IL-4 and IL-10 increased (Fig. 3B). Thus, LPS increased MyD88-dependent signaling pathway activation, which could be inhibited by USP8 treatment, indicating that USP8 may act on microglia by inhibiting TLR4 to attenuate LPS-induced neuroinflammation in the hippocampus and the consequent memory deficits.

3.7. USP8 increased Nrdp1 levels in LPS-induced mice

To provide insight into the potential molecular mechanism underlying the therapeutic effects of USP8, we examined its effect on Nrdp1 expression because it has been reported that USP8 augments Nrdp1 activity by mediating its stabilization (Wu et al., 2004), and that Nrdp1 is known to inhibit the production of proinflammatory cytokines in toll-like receptor-activated macrophages by suppressing its specific adaptor MyD88 (Wang et al., 2009). As shown in Fig. 4D, Nrdp1 expression in the brain of USP8-LPS-treated mice was significantly higher than that in LPS-treated mice (P < 0.01, Fig. 4D). This result suggests that USP8 increases the expression of Nrdp1 which is associated with the potent downregulation of TLR4 signaling activity in LPS-induced mice.

3.8. USP8 and VIPER had similar effects on cognitive impairment and motor dysfunction

To compare the effect of USP8 on cognitive and motor function, we administered a TLR4 inhibitor (VIPER) in LPS-induced mice. Memory function and motor coordination were assessed using the MWM, PAT, pole test, and traction test. In the MWM, VIPER-treated mice reached the platform faster than VIPER-untreated controls (control, 6.50 ± 0.57; saline, 6.19 ± 0.77; LPS, 18.14 ± 2.40; USP8 + LPS, 7.97 ± 0.77; VIPER + LPS, 6.63 ± 0.67; USP8 + VIPER + LPS, 6.78 ± 0.48; VIPER, 5.30 ± 0.44; P < 0.01, Fig. 5B). In addition, the VIPER-treated mice spent more time in the target quadrant (control, 38.78 ± 2.89; saline, 40.25 ± 2.87; LPS, 18.67 ± 2.51; USP8 + LPS, 33.33 ± 2.76; VIPER + LPS, 37.75 ± 3.46; USP8 + VIPER + LPS, 33.50 ± 1.71; VIPER, 37.00 ± 4.22; P < 0.01), and showed more platform crossings (control, 9.44 ± 0.99; saline, 10.25 ± 1.03; LPS, 3.33 ± 0.78; USP8 + LPS, 8.89 ± 0.89; VIPER + LPS, 9.38 ± 0.63; USP8 + VIPER + LPS, 9.30 ± 0.68; VIPER, 8.67 ± 0.88; P < 0.01; Fig. 5B) compared with the VIPER-untreated control mice. These data indicate that TLR4 is involved in learning and memory deficits in LPS-induced mice. USP8 had similar effects to those of VIPER on learning and memory deficits in LPS-injected mice; there was no significant difference between the USP8 + LPS and VIPER + LPS groups (Fig. 5B). In the PAT, compared with untreated mice, USP8-treated and VIPER-treated mice spent more time in the illuminated chamber after LPS injection, indicating that USP8 and VIPER treatment alleviated LPS-induced memory deficit (Fig. 5C). In the pole test, motor coordination scores in USP8-treated mice were significantly higher than those in the untreated group (P < 0.05, Fig. 5D). In the traction test, the motor function of VIPER-treated mice was similar to that of USP8-treated mice (Fig. 5E).

3.9. USP8 and VIPER attenuated neuroinflammation in the serum and brain tissues of LPS-induced mice

To confirm that the behavioral changes observed were caused by neuroinflammation, we analyzed the expression of pro-inflammatory and anti-inflammatory markers. The levels the LPS-induced pro-inflammatory markers TNF-α (control, 7.49 ± 1.16; saline, 7.70 ± 0.70; LPS, 82.03 ± 4.20; USP8 + LPS, 11.92 ± 2.99; VIPER + LPS, 10.44 ± 1.50; USP8 + VIPER + LPS, 7.28 ± 1.09; VIPER, 9.18 ± 3.30 in serum, and control, 3.92 ± 1.61; saline, 2.02 ± 1.29; LPS, 26.23 ± 1.48; USP8 + LPS, 1.81 ± 0.90; VIPER + LPS, 4.54 ± 2.06; USP8 + VIPER + LPS, 3.70 ± 1.31; VIPER, 4.97 ± 1.28 in brain; P < 0.01), IL-1β (control, 6.50 ± 1.49;
USP8 treatment shifted microglial polarization from pro-inflammatory phenotypes toward anti-inflammatory phenotypes after LPS-induced neuroinflammation. (A) Signaling components of MyD88-dependent signaling pathway were analyzed in the hippocampus using western blot with the indicated antibodies. (B) Quantification of the immunoblots in A, n = 6. (C) Percentage of microglia with M1 phenotype in the hippocampus was determined by the ratio of TNF-α (red) to IBA1 (purple) positive cells by confocal immunofluorescence microscopy. M2 phenotype was determined by the ratio of YM-1 (green) to IBA1 (purple) positive cells. DAPI was used for nuclear staining, blue, n = 5 (Scale bar, 75 μm.). Lower panel, quantification of the percent M1 and M2 phenotypes after different treatments as described in C. (D) The therapeutic effects of USP8 on the expression of Nrdp1 after LPS stimulation. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01 compared to control group and saline group; †P < 0.05, ‡P < 0.01 compared to USP8 + LPS group and USP8 group, analyzed by one-way ANOVA. Error bars indicate SEM.
saline, 10.21 ± 1.79; LPS, 69.86 ± 2.57; USP8 + LPS, 9.79 ± 2.32; VIPER + LPS, 7.74 ± 3.84; USP8 + VIPER + LPS, 11.64 ± 1.61; saline, 3.42 ± 1.44; LPS, 1042.82 ± 23.32; USP8 + LPS, 497.99 ± 26.21; VIPER + LPS, 494.58 ± 17.89; VIPER, 543.34 ± 32.37 in brain; P < 0.01), analyzed by one-way ANOVA. Error bars indicate SEM.

Fig. 5. Effect of VIPER on ameliorating LPS-induced cognitive and motor impairments was similar to that of USP8 treatment. (A) A scheme for VIPER treatment and assessment of cognitive and motor functions in mice. Arrow heads represent days on which acquisition tests were conducted. Mice were injected intracerebroventricularly with USP8 7 days prior to intraperitoneal injection with LPS, or injected intraperitoneally with VIPER 2 h prior to LPS injections. The Morris water maze (MWM) and passive avoidance test (PAT) were performed as described in the Methods section. (B) Results of the MWM test for USP8- and VIPER-administered mice treated with LPS, n = 15. (C) Results of the PAT test of USP8- and VIPER-administered mice treated with LPS, n = 15. (D) Motor coordination scores in USP8- and VIPER-administered mice treated with LPS, n = 15. (E) The traction test in USP8- and VIPER-administered mice treated with LPS, n = 15. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01 compared to control group and saline group; #P < 0.05, ##P < 0.01 compared to USP8 + LPS group, VIPER + LPS group, and USP8 + VIPER + LPS group, analyzed by one-way ANOVA. Error bars indicate SEM.
2.73 ± 0.36; LPS, 15.31 ± 0.78; USP8 + LPS, 2.84 ± 0.18; VIPER + LPS, 2.68 ± 0.65; USP8 + VIPER + LPS, 2.68 ± 0.65; VIPER, 4.27 ± 0.34 in brain; P < 0.01) were decreased after USP8- or VIPER treatment (P < 0.01, Fig. 6A). In contrast, the levels of IL-4 (control, 55.21 ± 1.28; saline, 52.12 ± 1.17; LPS, 1.91 ± 1.01; USP8 + LPS, 60.77 ± 1.97; VIPER + LPS, 53.46 ± 1.64; USP8 + VIPER + LPS, 52.18 ± 2.62; VIPER, 53.46 ± 2.52 in serum, and control, 50.84 ± 1.03; saline, 46.48 ± 0.71; LPS, 27.26 ± 0.82; USP8 + LPS, 51.31 ± 1.08; VIPER + LPS, 49.30 ± 1.16; USP8 + VIPER + LPS, 50.37 ± 0.66; VIPER, 50.31 ± 0.87 in brain; P < 0.01) and IL-10 (control, 274.71 ± 3.43; saline, 282.37 ± 4.01; LPS, 86.64 ± 5.48; USP8 + LPS, 239.35 ± 5.79; VIPER + LPS, 267.33 ± 0.28; USP8 + VIPER + LPS, 266.22 ± 1.11; VIPER, 266.78 ± 3.43 in serum, and control, 167.11 ± 7.69; saline, 174.21 ± 5.37; LPS, 90.53 ± 3.55; USP8 + LPS, 160.56 ± 1.58; VIPER + LPS, 164.04 ± 1.47; USP8 + VIPER + LPS, 176.01 ± 3.83; VIPER, 170.99 ± 4.12 in brain; P < 0.01) were higher in the serum and brain homogenates of USP8- or VIPER-treated groups compared to those in the untreated groups after LPS injection (Fig. 6B).

We used immunofluorescent staining of microglia and neurons to...
verify that VIPER inhibited microglial activation and neuronal damage. The number of IBA1-positive cells was lower and the number of MAP2-positive cells higher in VIPER treated animals (Fig. 6C) than in animals not treated with VIPER.

Furthermore, VIPER + LPS-treated animals exhibited significantly lower TLR4 expression in the brain than the LPS group (Fig. 7A, B). In addition, VIPER abolished the effects of LPS on microglial M1 polarization under neuroinflammation (Fig. 7C).

These data indicated the involvement of the MyD88-dependent signaling pathway in activating inflammation, resulting in the activation of microglia and subsequent cognitive impairment. Notably, USP8 treatment inhibited TLR4-mediated neuroinflammation.

3.10. USP8 attenuated LPS-mediated cognitive deficits, motor impairment, and inflammation via TLR4

We determined whether TLR4 deficiency affects cognitive impairment after LPS-induced neuroinflammation by injecting TLR4 knockout mice (TLR4−/−) with LPS and evaluating memory and motor function. TLR4−/− (10.87 ± 1.52 s) mice reached the platform faster than did WT mice (17.64 ± 0.92 s; P < 0.01, Fig. 8A), and spent more time in the platform quadrant and on the platform. In the PAT, TLR4−/− mice spent less time in the dark compartment. The number of errors in the passive avoidance test was approximately reduced by half in TLR4−/− mice (0.38 ± 0.18) compared to that in WT mice.
Fig. 8. Knocking out TLR4 in mice protects against LPS-induced neuroinflammation and cognitive and motor impairments. (A) USP8 targeted TLR4 to ameliorate LPS-induced neuroinflammation. Morris water maze (MWM), passive avoidance test (PAT), pole test, and traction test were performed as described in the Methods section to test the memory ability and motor coordination in mice received the indicated treatments, n = 10. (B) The expression of pro-inflammatory cytokines, TNF-α, IL-1β, and PGE₂ in mouse serum and brain were investigated using ELISA kits, n = 5. (C) The expression levels of the signaling components of TLR4-pathway in mouse hippocampus were investigated using western blot with the indicated antibodies, n = 6. Lower panel shows that quantification of the western blot result. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01 compared to control group; #P < 0.05, ##P < 0.01 compared to LPS (TLR4−/−) group, analyzed by one-way ANOVA. Error bars indicate SEM.
(0.78 ± 0.19; P < 0.01, Fig. 8A). Motor coordination scores of TLR4−/− mice were significantly higher than those of WT mice (P < 0.05, Fig. 8A). We then studied pro-inflammatory cytokine expression in TLR4−/− mice after i.p. injection of LPS. TLR4−/− mice exhibited significantly lower levels of TNF-α, IL-1β, and PGE2 in hippocampal tissue 7 days after LPS injection (P < 0.01, Fig. 8B). To elucidate whether the MyD88-dependent signaling pathway was involved in cognitive impairment, we assessed hippocampal iKBα, p65, MyD88, and TLR4 protein expression in TLR4−/− and WT mice. WT mice had significantly higher iKBα, P65, MyD88, and TLR4 protein expression than did TLR4−/− mice (P < 0.01, Fig. 8C).

We then examined the effects of USP8 treatment on LPS-induced neuroinflammation and cognitive and motor impairment. USP8-treated mice exhibited recovery of cognitive and motor impairment similar to that in TLR4−/− mice (Figs. 2, 5, 8A). To verify the relationship between TLR4 and USP8 in cognitive and motor impairment following LPS-induced neuroinflammation, we measured brain USP8 protein expression in TLR4−/− mice. The expression of USP8 was reduced after LPS injection in WT mice; however, TLR4 knockout abolished LPS-induced USP8 repression (Fig. 8C). These results were similar to those observed (behavior, pro-inflammatory cytokines, and signaling pathway activity) in VIPER-treated mice (Figs. 5–7). Thus, USP8-treated mice exhibited similar protective effect to that in TLR4−/− mice and VIPER-treated mice.

4. Discussion

USP8 is a cysteine protease of the USP/UBP subfamily (Nijman et al., 2005), and is a growth-regulated enzyme essential for cell proliferation and survival. Conditional knock-out of murine USP8 promotes a dramatic loss in expression of receptor tyrosine kinases including EGFR, ErbB3, and c-Met (Niendorf et al., 2017); USP8 inactivation causes enhanced ubiquitination of ligand-activated EGFR (Alwan and Leeuwen, 2007). In conjunction with components of the ESCRT-0 complex, USP8 plays an integral role in the early endosomal sorting machinery that protects EGFR from lysosomal degradation (Berlin et al., 2010). In addition to regulating growth-related proteins, USP8 regulates the stability of its effector protein Nrdp1 under different stimuli (Wu et al., 2004). Moreover, Nrdp1 and USP8 may reciprocally regulate each other (Ceuninck et al., 2013). Nrdp1 regulates TLR signaling via inhibition of NF-κB and AP-1 and activation of TBK1 and IRF3, leading to attenuated production of pro-inflammatory cytokines (Wang et al., 2009). In the present study, we showed that USP8 treatment ameliorates microglia-mediated cognitive and motor impairments following neuroinflammation. The detailed molecular mechanisms involved merit detailed study.

The hippocampus plays an important role in learning and memory consolidation (Betto et al., 2017). LPS treatment caused learning and memory impairment as well as hippocampal microglial activation (labelled by IBA-1) and neuronal cell loss (labelled by MAP-2) in mice (Zhao et al., 2019). We observed that LPS-induced systemic inflammation caused cognitive impairment, which was ameliorated by USP8 pretreatment (Fig. 2B and C). Further, LPS-treated mice obtained lower scores on the pole and traction tests, and these effects were reduced by USP8 treatment (Fig. 2D and E). Thus, USP8 exerts neuroprotective effects that manifest as cognitive and motor improvements in mice. The neuroprotective mechanism of USP8 therefore merits further study.

Like peripheral macrophages, microglia includes heterogeneous populations of cells that display functional variability due to different polarization statuses (Kettenmann et al., 2011). “Classically activated” M1 phenotypes are characterized by the ability to release pro-inflammatory cytokines (Satjio and Glass, 2011), and “alternatively activated” M2 phenotypes are characterized by the ability to produce anti-inflammatory and immunosuppressive factors (including Arg-1 and YM-1), and upregulate anti-inflammatory cytokines (Row et al., 2006). The classical M1 state is neurotoxic and contributes to secondary neuronal damage and cell death, thereby leading to neurodegeneration (Crain et al., 2013). In this study, we showed that USP8 suppresses cognitive and motor impairments after neuroinflammation by significantly decreasing M1 phenotype-associated pro-inflammatory cytokines (TNF-α, IL-1β, PGE2, and NO), and increases anti-inflammatory cytokine (IL-4 and IL-10) expression (Fig. 3A–C). Moreover, our immunofluorescence data suggested that USP8 may inhibit activated microglia and attenuate loss of neurons (Fig. 3D).

After LPS stimulation, TLR4 binds to MyD88 at the IL-1 receptor cytoplasmic domain, leading to the recruitment of IL-1 receptor-associated kinase IRAK4 (Akira and Takeda, 2004). The phosphorylation of TAK-1 induces the activation of IKK, which in turn phosphorylates the IκB protein. This leads to their proteosome-mediated degradation, and the phosphorylated form of IκB dissociates from the NF-κB p50/p65 dimer, after which it enters the nucleus (Simon et al., 2015). NF-κB subsequently activates proinflammatory mediators, which activate iNOS and inducible COX-2 (Saha and Pahan, 2006). We found no significant differences in TRIF protein expression between the control, saline, and USP8-treated groups (Fig. 4A and B). Nrdp1 is a specific target of USP8 deubiquitinating enzyme (Wu et al., 2004), and regulates TLR signaling, which leads to downregulation of pro-inflammatory cytokines (Wang et al., 2009). Since we found that USP8 treatment increased the Nrdp1 levels in LPS-induced mice (Fig. 4D), we speculate that this may downregulate the expression of TLR4 and MyD88 protein, and subsequently inhibit the phosphorylation of IκKβ and IκBα, leading to reduction of nuclear translocation of p65 by inhibiting the activation of the NF-κB signaling pathway in LPS-induced mice. The detailed underlying mechanism will require further characterization and validation in future work.

Activation of M1 microglia results in increased iNOS expression. Ablation of iNOS in APP/PS1 mice can protect the mice from plaque formation and premature mortality (Tichauer and Von Bernhardi, 2012). Intracerebral injection of anti-inflammatory cytokines, such as IL-4 and IL-13, reduced Aβ plaque load in APP23 mice, which was accompanied by improved cognition and upregulation of Arg1 and YM1-positive M2 cells (Kawahara et al., 2012). Our results clearly showed that microglia in USP8-treated mice expressed the alternative phenotype. In USP8-treated mice, the number of microglia co-labeled with YM1-positive cells increased. In contrast, in LPS-treated mice, there were almost no microglial cells co-labeled with YM1-positive cells, and the number of microglial cells co-labeled with TNF-α-positive cells was higher (Fig. 4C). These results were consistent with our ELISA and Griess results showing pro-inflammatory (TNF-α, IL-1β, PGE2, and NO) and anti-inflammatory (IL-4 and IL-10) cytokine production.

We used VIPER and TLR4−/− mice to compare the protective effects of USP8. The results from VIPER administration and the KO mouse model indicated that, similar to USP8 treatment, blocking TLR4 attenuated cognitive and motor impairments. Moreover, NF-κB activated by LPS triggered neuroinflammation in neuronal cells by activation of microglial cells via a series of inflammatory cytokines; however, this phenomenon was suppressed in VIPER-treated mice or TLR4−/− mouse brains (Figs. 5–8). These findings provide strong evidence that USP8-treated mice exhibited similar protective effect to that in TLR4−/− mice and VIPER-treated mice.

In summary, our in vivo study provided evidence that USP8 may act on the TLR4-mediated MyD88-dependent pathway to direct microglia into the M2 phenotype, ultimately reducing hippocampal inflammation and the consequent cognitive and motor impairments. USP8 could be a novel target to develop strategies to alleviate neuroinflammation-associated cognitive and motor impairments. Detailed studies on the signaling mechanisms underlying the interactions of USP8, Nrdp1, and TLR4 could lead to the identification of novel drug targets.
Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors thank JYZ and BW designed the experiment, carried out the MWM, ELISA and Western blotting measurement and drafted the manuscript. RY performed the immunohistochemistry measurement. D.L. and T.C.K contributed to perform the statistical analysis and manuscript editing. SX prepared the animals models and contributed to date the analysis. J.W.Z helped to perform the tissue sampling, biochemical analysis and interpretation. L.H.Z conceived of the study, participated in its design and coordination, secured funding for the project, helped to draft the manuscript and took over all responsibility for the work. All authors read and approved the final manuscript. This study was supported by grants from the National Science Foundation of China (Nos. 81371442), the Training program for outstanding young teachers in higher education institutions of Guangdong Province (Nos. YQ2015024), the Major special foundation for Science and Technology Planning Project of Guangzhou (Nos. 201506010095), and the Fundamental Research Funds for the Central Universities (Nos. 21615419 and Nos. 21615474).

References

Akira, K., Takeba, S., 2004. TLR signaling pathways. Semin. Immunol. 16, 3–9 [PubMed: 14751757].
Allison, D.J., Ditor, D.S., 2014. The common inflammatory etiology of depression and cognitive impairment: a therapeutic target. J. Neuroinflamm. 11, 151 [PubMed: 25178630].
Alper, H.A., Leruven, J.E., 2007. UBPY-mediated epidermal growth factor receptor (EGFR) de-ubiquitination promotes EGFR degradation. J. Biol. Chem. 33, 1568–1569 [PubMed: 17121848].
Berlin, I., Schwartz, H., Nash, P.D., 2010. Regulation of epidermal growth factor receptor ubiquitination and trafficking by the USP8-STAT3 complex. J. Biol. Chem. 45, 34099–34321 [PubMed: 20736164].
Bettio, L.E.B., Rajendran, L., Gil-Mohapel, J., 2017. The e...

b signaling pathway, CNS Neurosci. Ther. 25, 575–590 [PubMed: 30676698].

Kawahara, K., Suenobu, M., Yoshida, A., Koga, K., Hyodo, A., Ohtsuka, H., Kuniyasu, A., Tamakami, N., Sugimoto, Y., Nakayama, H., 2012. Intracerebral microinjection of interleukin-2/interleukin-13 reduces beta-amyloid accumulation in the ipsilateral side, improves cognitive deficits in young amyloid precursor protein mouse 3 mice. Neurobiology 207, 243–260 [PubMed: 22342431].

Kennethmann, H., Hansich, U.K., Noda, M., Verkhratsky, A., 2011. Physiology of microglia. Physiol. Rev. 91, 461–553 [PubMed: 21527311].

Laird, M.H., Laird, S.H., Bee, D.J., Perkins, A.E., Medvedev, W., Piao, M.J., Vogel, S.N., 2009. TLR4/MyD88/PI3K interactions regulate TLR4 signaling. J. Leukoc. Biol. 6, 966–977 [PubMed: 19289601].

Lin, S., Yin, Q., Zhong, Q., Li, F., Zhou, Z., Yang, Y., Zhang, D.F., Xu, J., Jiang, H.Y., Du, K., Qian, P., Yao, W.F., Gao, H., Wei, M.J., 2015. Systemic in...

Sohrabi, M., Neher, J.J., Tremblay, M.E., 2017. Combs CK. Inflammatory mechanisms in neurodegeneration. J. Neurochem. 149, 562–581 [PubMed: 30702751].

Niendorf, S., Okule, A., Kisser, A., Lohler, J., Frisbee, M., Schorf, S., Lewitzy, M., Horak, I., Knebel, K.P., 2017. Essential role of ubiquitin-specific protease 8 for receptor tyrosine kinase stability and endocytosis trafficking in vivo. Mol. Cell. Biol. 13, 5029–5039 [PubMed: 17452457].

Nijman, S.M., Luna-Vargas, M.P., Velds, A., Brummelkamp, T.R., Dine, A.M., Simka, T.K., Berns, W., 2006. A genome-wide functional inventory of deubiquitinating enzymes. Mol Cell. 5, 773–786 [PubMed: 16325527].

Pan, J., Jin, J.L., Ge, H.M., Yin, K.L., Chen, X., Han, L.J., Chen, Y., Qian, L., Li, X.X., Yu, Y., 2015. Malbital4 regulates microglia M1/M2 polarization in experimental stroke. J. Neuroinflamm. 12, 51 [PubMed: 25889516].

Pardou, M.C., 2015. Lipopolysaccharide hyporesponsiveness: protective or damaging role to the brain? J. Morph. Embryol. 3, 903–913 [PubMed: 26662122].

Printses, I., Yen, L., Sweeney, C., Caraway, C., 2014. Oligomerization of the Nrdp1 E3 ubiquitin ligase is necessary for efficient autoubiquitination but not E63 ubiquitin-...

J. Zhao, et al.

Brain, Behavior, and Immunity 88 (2020) 582–598

595
signal ablation attenuated neurological deficits by regulating microglial M1/M2 phenotype after traumatic brain injury in mice. J. Neuroimmunol. 310, 38–45 [PubMed: 28778443].
Zhang, Y., Kang, Y.M., Tian, C., Zeng, Y., Jia, L.X., Ma, X., Du, J., Li, H.H., 2011. Overexpression of Nrdp1 in the heart exacerbates doxorubicin-induced cardiac dysfunction in mice. PLoS ONE 6, e21104 [PubMed: 21738612].
Zhao, J., Bi, W., Xiao, S., Lan, X., Cheng, X., Zhang, J., Lu, D., Wei, W., Wang, Y., Li, H., Fu, Y., Zhu, L., 2019. Neuroinflammation induced by lipopolysaccharide causes cognitive impairment in mice. Sci. Rep. 9, 5790 [PubMed: 30962497].
Zhu, L., Bi, W., Lu, D., Zhang, C., Shu, X., Wang, H., Qi, R., Shi, Q., Lu, D., 2015. Regulation of ubiquitin-specific processing protease 8 suppresses neuroinflammation. Mol. Cell. Neurosci. 64, 74–83 [PubMed: 24861766].
Zhao, Y.L., Sun, M.F., Jia, X.B., Zhang, P.H., Xu, Y.D., Zhou, Z.L., Xu, Z.H., Cui, C., Chen, X., Yang, X.S., Shen, Y.Q., 2018. Aucubin alleviates glial cell activation and preserves dopaminergic neurons in 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine-induced parkinsonian mice. NeuroReport 29, 1075–1083 [PubMed: 29985188].