



Knockdown of TLR4 Represses the Paraquat-Induced Neuroinflammation and Microglial M1 Polarization

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Abstract

Paraquat (PQ) is associated with multiple nervous system disorders including Parkinson's disease. Despite the evidence that PQ could induce inflammatory responses in the central nervous system and largely contribute to neurotoxicity, the mechanisms of PQ-induced neuroinflammation are not yet fully understood. Toll-like receptor 4 (TLR4) could recognize various pathogens and initiate inflammation processes. Therefore, we investigated the role of TLR4 in PQ-induced neuroinflammation by using murine microglial immortalized BV-2 cell line. Normal microglia and TLR4-knockdown microglia were treated with PQ to evaluate signal transduction molecular expression, inflammatory responses, and microglial functions. Compared with normal microglia, PQ-induced production of pro-inflammatory cytokines was significantly reduced in TLR4-knockdown microglia. Levels of M1 markers were decreased, while levels of M2 markers were increased upon PQ exposure, confirming that TLR4 depletion inhibited the microglial M1 polarization. Besides, the migration and phagocytosis capability reduced by PQ were to some extent recovered in TLR4-knockdown microglia. Taken together, our results suggested that TLR4 mediated the neuroinflammatory responses in microglia and the depletion of TLR4 protects against PQ neurotoxicity.

Keywords Paraquat · TLR4 · Neuroinflammatory responses · Microglia

Introduction

Paraquat (1, 1'-dimethyl-4, 4'-bipyridinium, PQ), a widely used highly efficient and nonselective herbicide, has been demonstrated to be associated with multiple nervous system disorders such as Parkinson's disease (Prasad et al. 2009; Lou et al. 2012). Neuroinflammation has been considered to be a crucial mediator in environmental neurotoxicant-induced progressive neural (Mittra et al. 2011; Costa et al. 2014). Microglia, the major resident immune cells in the central nervous system, plays as an active contributor to neuroinflammation (Rojo et al. 2014). Our previous study also revealed that PQ exposure could activate microglia into an inflammatory phenotype (Huang et al. 2019). However, the mechanisms of

PQ-induced neuroinflammation are not yet fully understood, and no effective therapeutic strategy has been found.

Toll-like receptors (TLRs), a panel of type I transmembrane proteins, could recognize pathogen-associated molecular patterns (PAMPs), thereby initiating innate immune responses and inflammation (Kuzmich et al. 2017). As a member of the TLRs family, TLR4 plays an important role in regulating cell signaling transduction and inflammatory responses (Kuzmich et al. 2017; Gioannini et al. 2005). It could be activated by stimuli such as bacterial lipopolysaccharide (LPS), subsequently recruits the central adapter protein MyD88, and then mediates the production of pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6 (Akira and Takeda 2004). This mechanism might be particularly relevant to microglial inflammatory responses as microglia was found to be the only TLR4-positive cellular population in the brain parenchyma of adult rats (Laflamme and Rivest 2001).

A variety of evidence showed that TLR4 could be affected by neurotoxicants. TLR4 expression was upregulated in the mouse model upon MPTP exposure and may affect α -synuclein clearance (Stefanova et al. 2011; Ros-Bernal et al. 2011). TLR4 deficiency could reduce the development of

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neuroinflammation and has a protective effect in the MPTP/probenecid mouse model of Parkinson's disease (Shao et al. 2019; Campolo et al. 2019). The deletion of MyD88 was found to be able to protect against MPTP neurotoxicity (Drouin-Ouellet et al. 2011). Li et al. reported that TLR4 is involved in triggering inflammatory responses in microglia up PQ exposure (Li et al. 2019). Our previous study also found that TLR4 signaling in microglia is activated upon PQ exposure (Huang et al. 2019). All the evidence suggested that TLR4 is a vital mediator in the development of neuroinflammation induced by neurotoxicants.

In this study, the murine microglial immortalized BV-2 cell line (Blasi et al. 1990) was applied to understand the role of TLR4 in PQ-induced microglia activation. We investigated the effects of TLR4 depletion on microglial inflammation responses and microglia activities such as migration and phagocytosis upon PQ exposure. Our results showed that TLR4 mediated the neuroinflammatory responses in microglia and depletion of TLR4 has a protective effect against the PQ neurotoxicity.

Materials and Methods

Chemicals and Reagents

Paraquat dichloride (molecular weight 257.16, analytical standard, PQ) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dosing solutions were prepared by dissolving the calculated amount of PQ in the cell culture medium, following the approved standard operating procedures for handling toxic agents. All other reagents were obtained from commercial sources and were of the highest available grade.

Cell Culture and Treatments

BV-2 mouse microglial cells were purchased from the Chinese Academy of Sciences Cell Bank in Kunming. Frozen cells were thawed and expanded in RPMI-1640 medium supplemented with 15% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were incubated in a humidified atmosphere with 5% CO₂ at 37 °C and then passaged by 0.25% trypsin at about 80% confluence. As for the PQ treatment, when reaching a confluence of 70–80%, cells were incubated in the complete culture media with the different final concentrations of PQ (0 or 0.06 µmol/l) for 24 h. After 24-h incubation, cells were washed with PBS once and then collected by 0.25% trypsin digestion.

TLR4-shRNA Design and Cell Transfection

shRNA targeting TLR4 mRNA (shTLR4) or negative control mRNA sequence (control shRNA) were designed and synthesized by using pGMLV-SC5 RNAi lentiviral vector. Synthesized oligonucleotides were annealed and ligated to the BamHI/EcoRI sites of pGMLV-eGFP to produce pGMLV-eGFP-shTLR4 or pGMLV-eGFP-control shRNA. The eGFP expression was used to detect the transfection of *Lentivirus*. BV-2 mouse microglial cells were plated in 6-well plates at a density of 3×10^4 cells/ml and incubated overnight. The other day, cells were cultured in complete culture medium (control group), complete culture medium containing shTLR4 diluent (TLR4 shRNA group), or complete culture medium containing control shRNA diluent (control shRNA group), respectively. After transfection for 24 h, the culture medium was replaced with complete culture medium; cells were incubated for 72 h and screened in media containing 4 µg/ml puromycin (#P8230, Beijing Solarbio Science & Technology Co., Ltd., China). Since pGMLV-SC5 RNAi lentiviral vector contains eGFP and anti-puromycin gene, the BV-2 cells transfected with a lentiviral vector can be observed with green fluorescent. Cells were harvested to determine the TLR4 protein level by western blotting assay.

Cell Viability Assay

Cell viability was determined by CCK8 assay. BV-2 cells growing at the exponential phase were seeded at a density of 7×10^4 cells/ml in 96-well plates. After different treatments, 10 µl CCK8 solution was added to each well, and absorbance was detected at 450 nm using a spectrophotometer. Each treatment group has five replicates. Cell viability was obtained as a percentage of the value of survival cells in the control groups.

Migration Assay

BV-2 cells with or without shTLR4 transfection were harvested and seeded (at a density of 5×10^4 cells/ml) on the upper chamber of a transwell filter with 8 µm pores (Costar), and the chamber was placed in completed cell culture media containing different doses of PQ (0 or 0.06 µmol/l). Following an incubation period of 24 h, cells were fixed with 4% paraformaldehyde in PBS for 25 min. Non-migrated cells on the upper side of the filter were removed with a cotton swab, and the cells on the underside of the filter were stained with 0.1% crystal violet at room temperature for 10 min. Images were captured using a microscope (OLYMPUS IX81). Cell migration in each group was determined by the number of migrated cells. For each experiment, the number of cells in five random fields on the underside of the filter was counted, and three independent filters were analyzed.

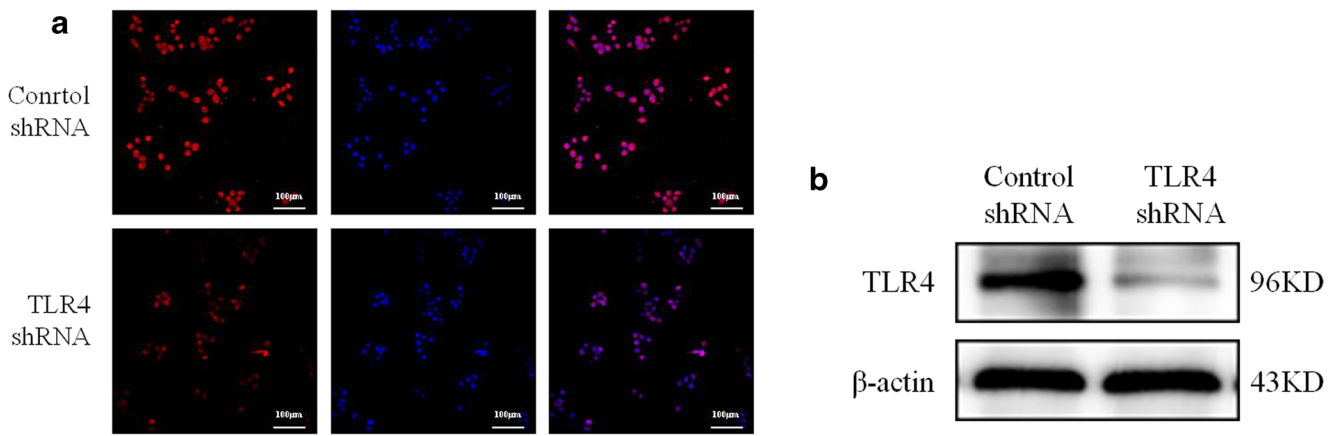


Fig. 1 Knockdown of TLR4 by *Lentivirus*-mediated shRNA in BV-2 microglia. Cells were transfected with TLR4 shRNA or control shRNA. **A** TLR4 protein was visualized by immunostaining with anti-TLR4 (red), and nuclei were visualized by staining with DAPI (blue). Scale bar = 100 μ m. **B** Total protein lysate was evaluated by western blot analysis for the expression level of TLR4 protein in each group. β -Actin was used as the internal control for normalization

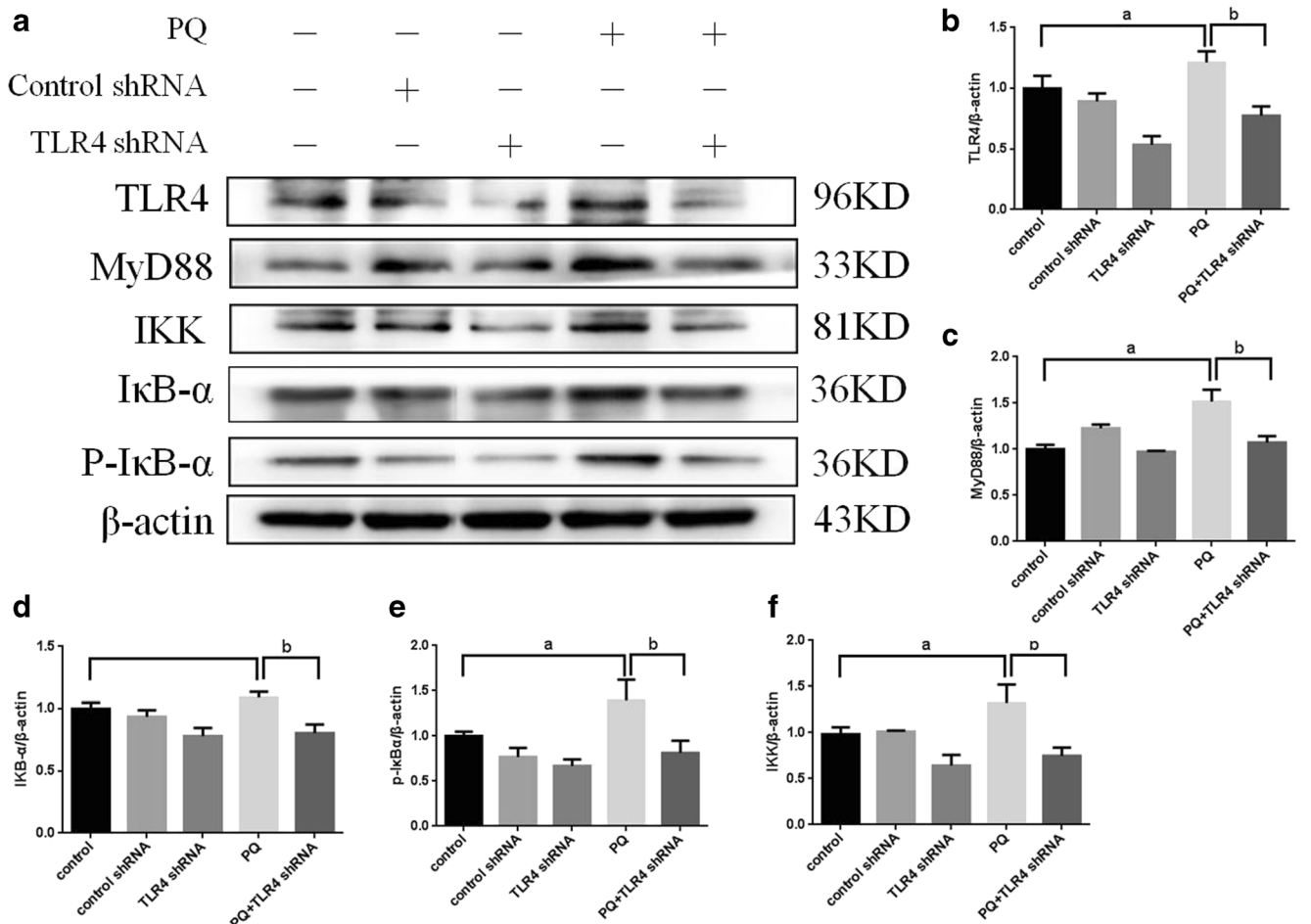


Fig. 2 Knockdown of TLR4 inhibited RAGE signaling pathway in PQ-treated BV-2 microglia. BV-2 microglia transfected with TLR4 shRNA or control shRNA were treated with 0.06 μ mol/l PQ for 24 h. **A** Total cell lysates were evaluated by western blot analysis for the expression level of TLR4, MyD88, IKK, I κ B α , and p-I κ B α protein. β -Actin was used as the internal control for normalization. **B–F** Histogram showed the quantitative evaluation of the protein band by densitometry. The data are presented as mean \pm SE. a: $p < 0.05$, compared with the vehicle control; b: $p < 0.05$, compared with the PQ treatment group

Immunofluorescence-Labeled Phagocytosis Assay

BV-2 cells with or without shTLR4 transfection were plated in 24-well plates overnight. The cells were then incubated with different doses of PQ (0 or 0.06 $\mu\text{mol/l}$) for 24 h. The fluorescent microspheres, as a marker of fluid phase phagocytosis, were added to the treated cells for 1-h incubation (0.4 μwell). Cells were then fixed with 4% paraformaldehyde for 15 min, blocked with 5% BSA for 2 h, incubated with rabbit anti-Iba-1 antibody (1:100) at 4 °C overnight, and then incubated with goat anti-rabbit FITC-IgG for 2 h. Finally, coverslips were then incubated with DAPI (1:1000) for double staining and then mounted on glass slides. Three random fields of cells were selected under an inverted fluorescent microscope. The number of cells containing microspheres and the total number of cells were counted, respectively. Phagocytic efficiency (%) = the number of cells containing microspheres / the total number of cells \times 100%.

Phagocytosis Function Measured by Flow Cytometry

BV-2 cells with or without shTLR4 transfection were collected, and 10^5 cells were plated in 6-well plates overnight. The cells were then incubated with different doses of PQ (0 or 0.06 $\mu\text{mol/l}$) for 24 h. The fluorescent microspheres, as a marker of fluid phase phagocytosis, were added to the treated cells for 1 h incubation (1.67 μwell). Cells were then disassociated by using 0.25% EDTA trypsin after being washed three times by PBS with 1% BSA. Pellet cells were resuspended in PBS, which is ready for flow cytometry measurement. Cells containing microspheres were detected by flow cytometry. Phagocytic efficiency (%) = the number of cells containing microspheres / the total number of cells \times 100%.

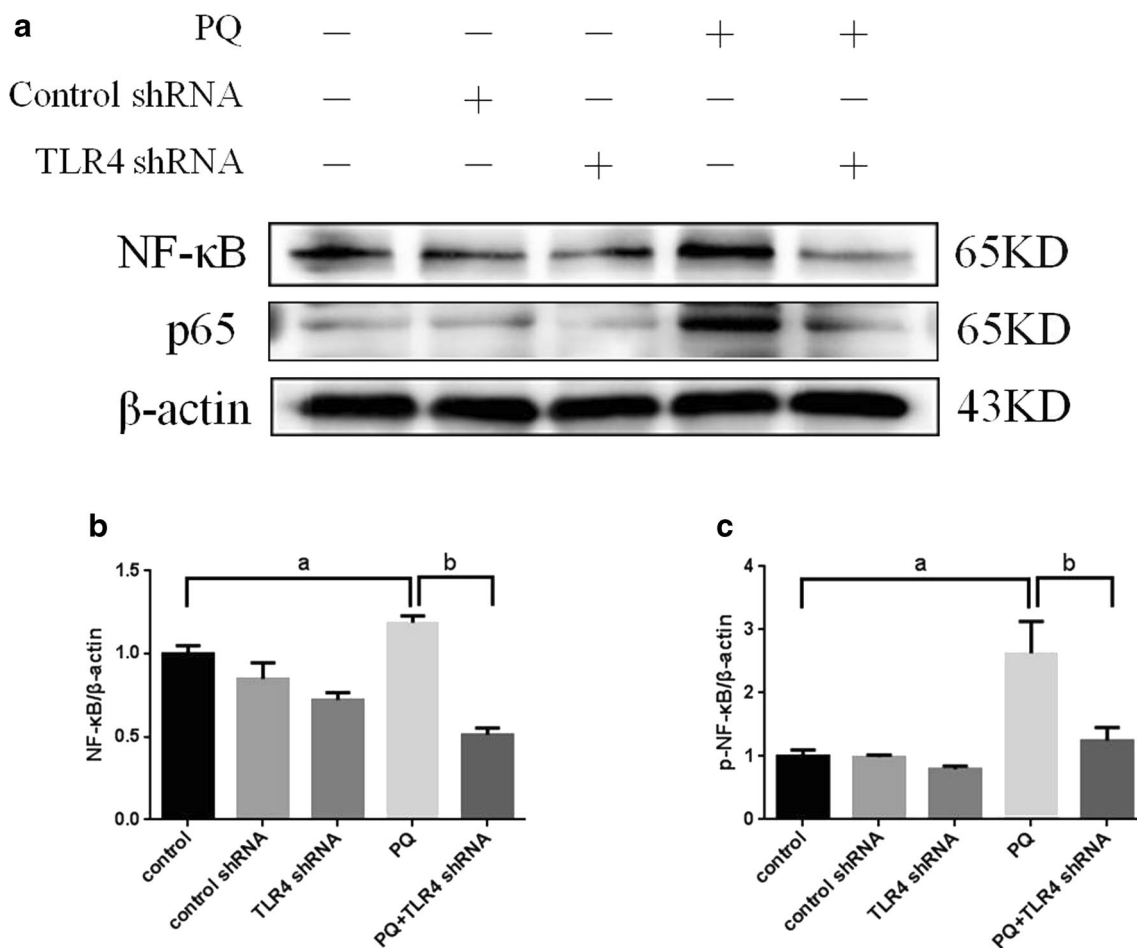


Fig. 3 Knockdown of TLR4 inhibited NF- κ B in PQ-treated BV-2 microglia. BV-2 microglia transfected with TLR4 shRNA or control shRNA were treated with 0.06 $\mu\text{mol/l}$ PQ for 24 h. **A** Total cell lysates were evaluated by western blot analysis for the expression level of NF- κ B p65 and total NF- κ B protein. β -Actin was used as the internal control

for normalization. **B–C** Histogram showed the quantitative evaluation of the protein band by densitometry. The data are presented as mean \pm SE. a: $p < 0.05$, compared with the vehicle control; b: $p < 0.05$, compared with the PQ treatment group

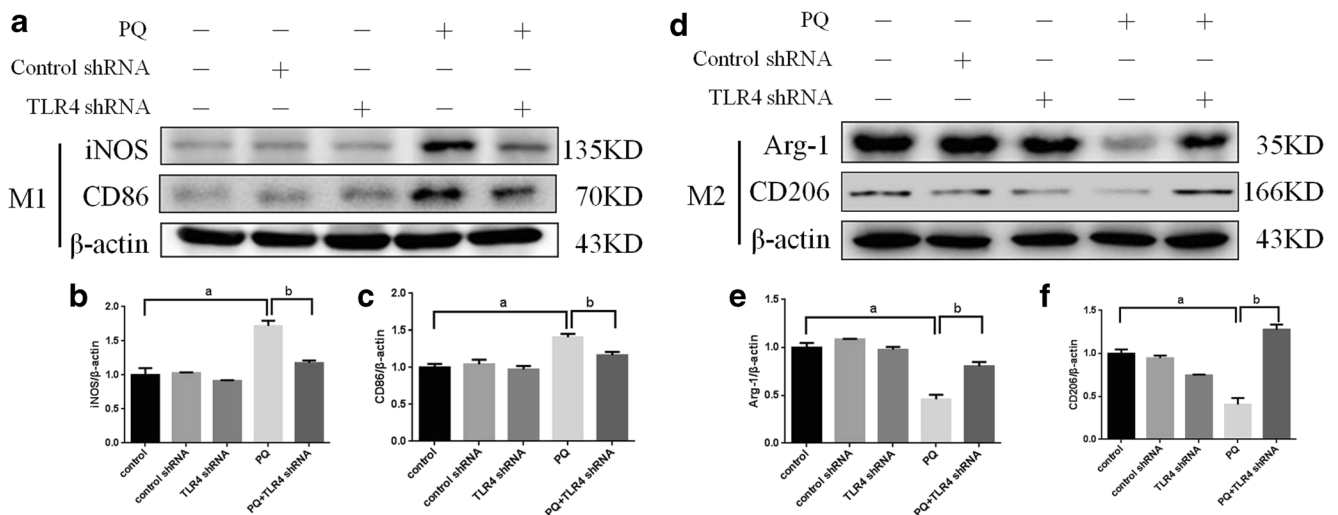


Fig. 4 The knockdown of TLR4 inhibits PQ-induced polarization of BV-2 microglia cells. BV-2 microglia transfected with TLR4 shRNA or control shRNA were treated with 0.06 $\mu\text{mol/l}$ PQ for 24 h. **A** Total protein lysates were evaluated by western blot analysis for the expression of M1 markers (iNOS, CD86). β -Actin was used as the internal control for normalization. **B–C** Histogram showed the quantitative evaluation of the protein band by densitometry. **D** Total protein lysates were evaluated

by western blot analysis for the expression of M2 markers (Arg-1, CD206). β -Actin was used as the internal control for normalization. **E–F** Histogram showed the quantitative evaluation of the protein band by densitometry. The data are presented as mean \pm SE. a: $p < 0.05$, compared with the vehicle control; b: $p < 0.05$, compared with the PQ treatment group

Western Blotting Assay

BV-2 cells with or without shTLR4 transfection were plated in 100-mm dishes at a density of 10^6 cells/ml and treated with different doses of PQ (0 or 0.06 $\mu\text{mol/l}$) for 24 h. Cells were washed with ice-cold PBS and then were incubated with lysis buffer on ice for 10 min. The cell lysates were centrifuged at $12000 \times g$ for 5 min at 4 $^{\circ}\text{C}$. Protein concentrations were determined by the BCA method. Nuclear and cytoplasmic extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. To measure the expression level of the TLR4 signaling pathway, the membranes were probed with TLR4 (1:1000), MyD88 (1:1000), $\text{p-I}\kappa\text{B}\alpha$ (1:1000), $\text{I}\kappa\text{B}\alpha$ (1:1000), IKK (1:1000), and $\text{NF-}\kappa\text{B/p65}$ (1:1000) antibodies. To measure the level of pro-inflammatory cytokines, the membranes were probed with $\text{TNF-}\alpha$ (1:1000), IL-6 (1:1000), and IL-1 β (1:1000) antibodies. To measure the expression level of M1 markers, the membranes were probed with iNOS (1:1000) and CD86 (1:1000) antibodies. To measure the expression level of M2 markers, the membranes were probed with Arg-1 (1:1000) and CD206 (1:1000) antibodies. β -Actin (1:10000) antibodies were used as the internal control for normalization. Membranes were incubated with different antibodies at 4 $^{\circ}\text{C}$ overnight. After washing with PBST for 1 h, membranes were incubated with HRP-conjugated secondary antibody and then imaged using ThermoFisher iBright Imaging System. Quantification of the band density was determined by densitometric analysis.

Statistical Analysis

Data were shown by the means \pm SE of at least three independent experiments. For each independent experiment, we have included at least three replicates in each treatment group. Statistical differences between experimental groups were determined by ANOVA followed by the Tukey post hoc test. The significance level was set at $p < 0.05$.

Results

Lentivirus-Mediated shRNA-Downregulated TLR4 in BV-2 Microglial Cells

Our previous study has demonstrated that TLR4 is involved in the inflammatory responses of BV-2 microglia upon PQ exposure; we further investigated the role of TLR4 by knockdown of the *TLR4* gene. BV-2 cells were transfected with TLR4 shRNA to achieve knockdown with the scrambled shRNA (control shRNA) as a negative control. No significant difference in cell viability was observed in cells transfected with TLR4 shRNA or control shRNA when compared with normal BV-2 microglia. Both immunofluorescence assay and western blotting results showed that the expression of TLR4 protein was significantly suppressed in BV-2 microglia transfected with the TLR4 shRNA group when compared with the BV-2 microglia transfected with control shRNA (Fig. 1). In contrast, the TLR4 protein level in the NC shRNA group exhibited no significant difference when compared with the

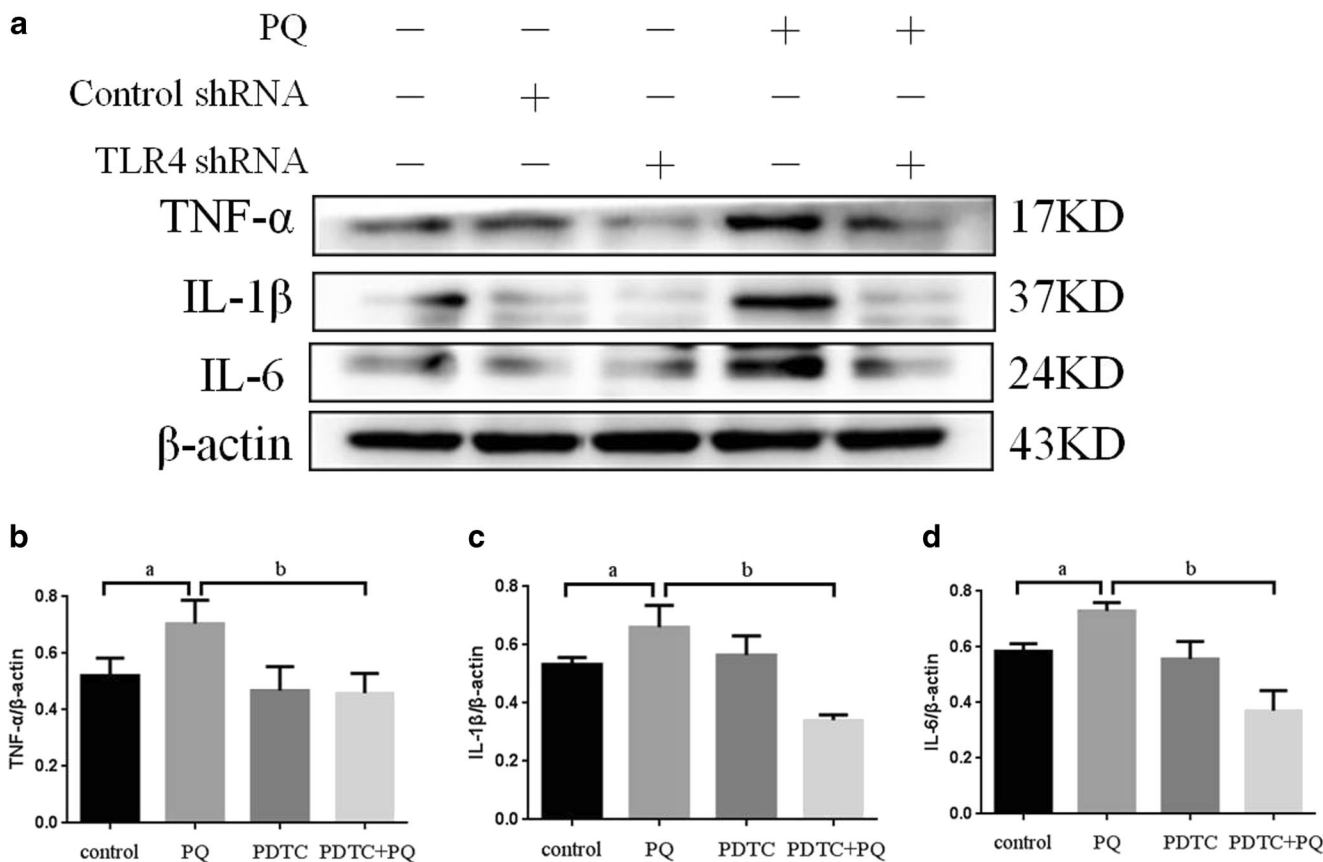


Fig. 5 The knockdown of TLR4 alleviated inflammatory responses in PQ-treated BV-2 microglia. BV-2 microglia transfected with TLR4 shRNA or control shRNA were treated with 0.06 μ mol/l PQ for 24 h. **A** Total cell lysates were evaluated by Western blot analysis for the expression level of TNF- α , IL-1 β , and IL-6 protein. β -Actin was used

as the internal control for normalization. **B–D** Histogram showed the quantitative evaluation of the protein band by densitometry. The data are presented as mean \pm SE. a: $p < 0.05$, compared with the vehicle control; b: $p < 0.05$, compared with the PQ treatment group

control group, confirming the role of scrambled shRNA as a negative control (Fig. 1).

Knockdown of TLR4 Inhibited TLR4 Signaling Pathway in PQ-Treated BV-2 Microglia

To clarify whether knockdown of TLR4 reverses the PQ-activated TLR4 signaling pathway, we treated BV-2 microglia (with or without TLR4 knockdown) with 0.06 μ mol/l PQ for 24 h. We first confirmed that there is no obvious interaction between PQ and control shRNA, whereas TLR4 shRNA dramatically attenuated the increase of TLR4 protein induced by PQ exposure (Fig. 2A–B). Subsequently, the expression of PQ-activated key molecules along the TLR4 signaling pathway such as MyD88, I κ B α , and p-I κ B α was inhibited by TLR4 knockdown. Also, the increased expression of I κ B kinase enzyme complex (IKK), which could phosphorylate the I κ B α protein into p-I κ B α , was also attenuated by TLR4 knockdown (Fig. 2A and C–F).

Since p-I κ B α could dissociate itself from NF- κ B, thereby releasing the p65 subunit of NF- κ B, we next measure the effects

of TLR4 knockdown on the abundance of NF- κ B p65 and total NF- κ B in BV-2 microglia treated with PQ. The results showed that although PQ exposure significantly increased the abundance of NF- κ B p65, in the absence of TLR4, the increment was significantly alleviated (Fig. 3). These results confirmed that knockdown of TLR4 effectively reverses the activation of TLR4 signaling pathway upon PQ exposure.

Knockdown of TLR4 Inhibited the PQ-Induced M1 Polarization of BV-2 Microglia

To further confirm whether microglial M1/M2 phenotype polarization affected by PQ could be reversed by TLR4 knockdown, we detected the expressions of M1/M2 specific markers in BV-2 microglia by western blotting assay. We observed that the expression levels of M1 markers (iNOS and CD86), increased upon PQ treatment, were decreased after TLR4 knockdown. On the other hand, the expression levels of M2 markers (Arg-1 and CD206), decreased upon PQ treatment, were increased after TLR4 knockdown (Fig. 4). These results indicated that knockdown of TLR4

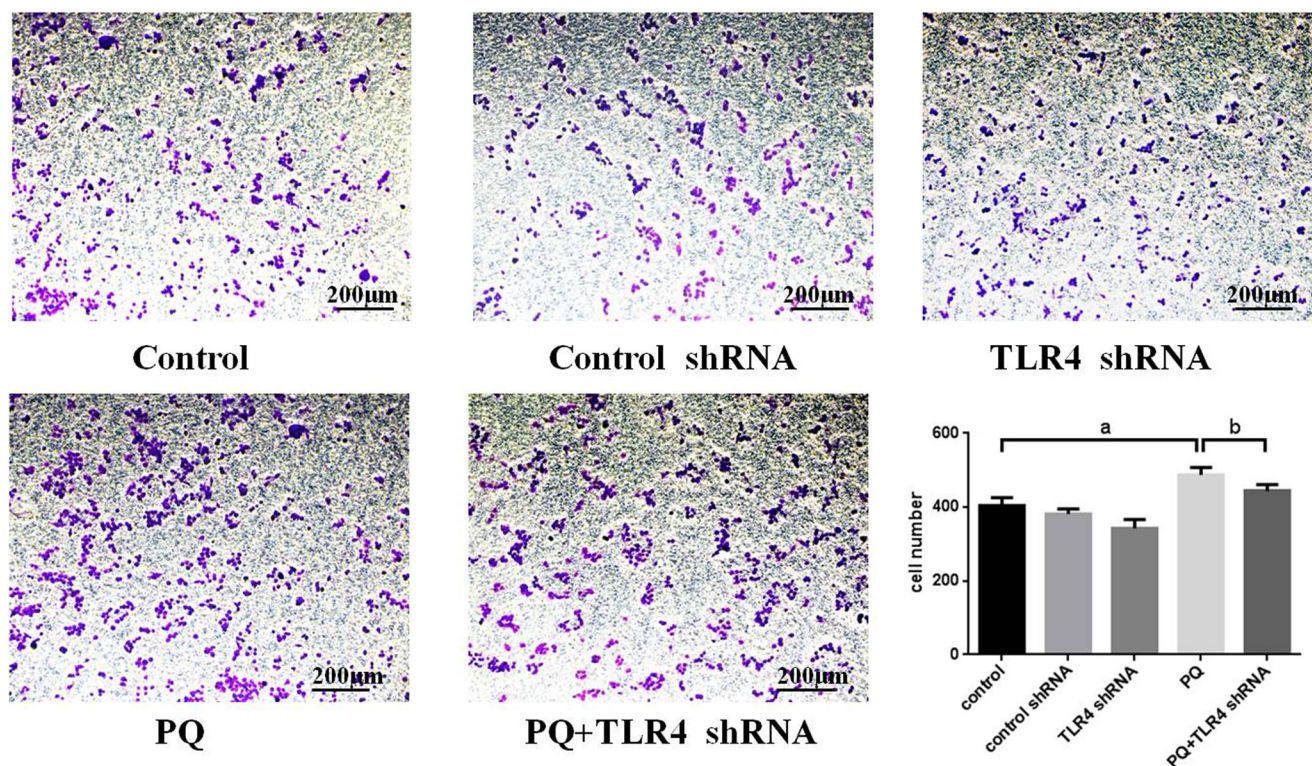


Fig. 6 The effects of TLR4 knockdown on the migration capability of BV-2 microglia. BV-2 microglia transfected with TLR4 shRNA or control shRNA were treated with 0.06 $\mu\text{mol/l}$ PQ for 24 h. Motility of cells was evaluated by the transwell migration assay. Cells were plated in the upper chamber of the filters and incubated for 24 h. Scale bar = 200 μm .

Cell migration in each group was determined by the number of migrated cells. Data are presented as mean \pm SE from at least three independent experiments. a: $p < 0.05$, compared with the vehicle control; b: $p < 0.05$, compared with the PQ treatment group

could reverse the polarization towards M1 phenotype induced by PQ exposure.

Knockdown of TLR4 Alleviates Inflammatory Responses in PQ-Treated BV-2 Microglia

To further illustrate the role of TLR4 signaling in PQ-induced inflammatory responses, validation of promotion or inhibition of pro-inflammatory cytokines release after PQ treatment in BV-2 microglia (both wild type and TLR4 knockdown) was performed by using western blotting. We found that the protein expressions of TNF- α , IL-1 β , and IL-6, which were significantly increased upon PQ exposure, were decreased significantly in the presence of TLR4 shRNA (Fig. 5). These results suggested that knockdown of TLR4 could alleviate the inflammatory responses induced by PQ in BV-2 microglia.

Knockdown of TLR4 Attenuates the PQ-Enhanced Migration Capability of BV-2 Microglia

To investigate whether inhibiting TLR4 signaling affects the migration capability of BV-2 microglia, transwell migration assay was performed on BV-2 microglia (both wild type and

TLR4 knockdown) upon different treatment. Cells were plated in the upper chamber of the filters and were allowed to migrate for 24 h. The number of cells transfected with control shRNA that had migrated to the underside of the filters was similar to that of the control group, whereas cells transfected with TLR4 shRNA showed a decreased motility when compared with the vehicle control group. While PQ exposure significantly increased the motility of BV-2 cells, knockdown of TLR4 to some extent attenuated this enhancement (Fig. 6). These results indicated that the migration capability of BV-2 microglia was affected by PQ exposure through TLR4 signaling pathway.

Knockdown of TLR4 Attenuates the PQ-Enhanced Phagocytic Activity of BV-2 Microglia

We further explore whether inhibiting TLR4 signaling affects the phagocytic function of BV-2 microglia by both immunofluorescence method and flow cytometry. BV-2 microglial cells (transfected with TLR4 shRNA or control shRNA) upon different treatments were incubated with microspheres for 1 h, and phagocytosis was monitored by the ingestion of fluorescent microspheres. Both immunofluorescence (Fig. 7A) and flow cytometry (Fig. 7B) results revealed that PQ exposure

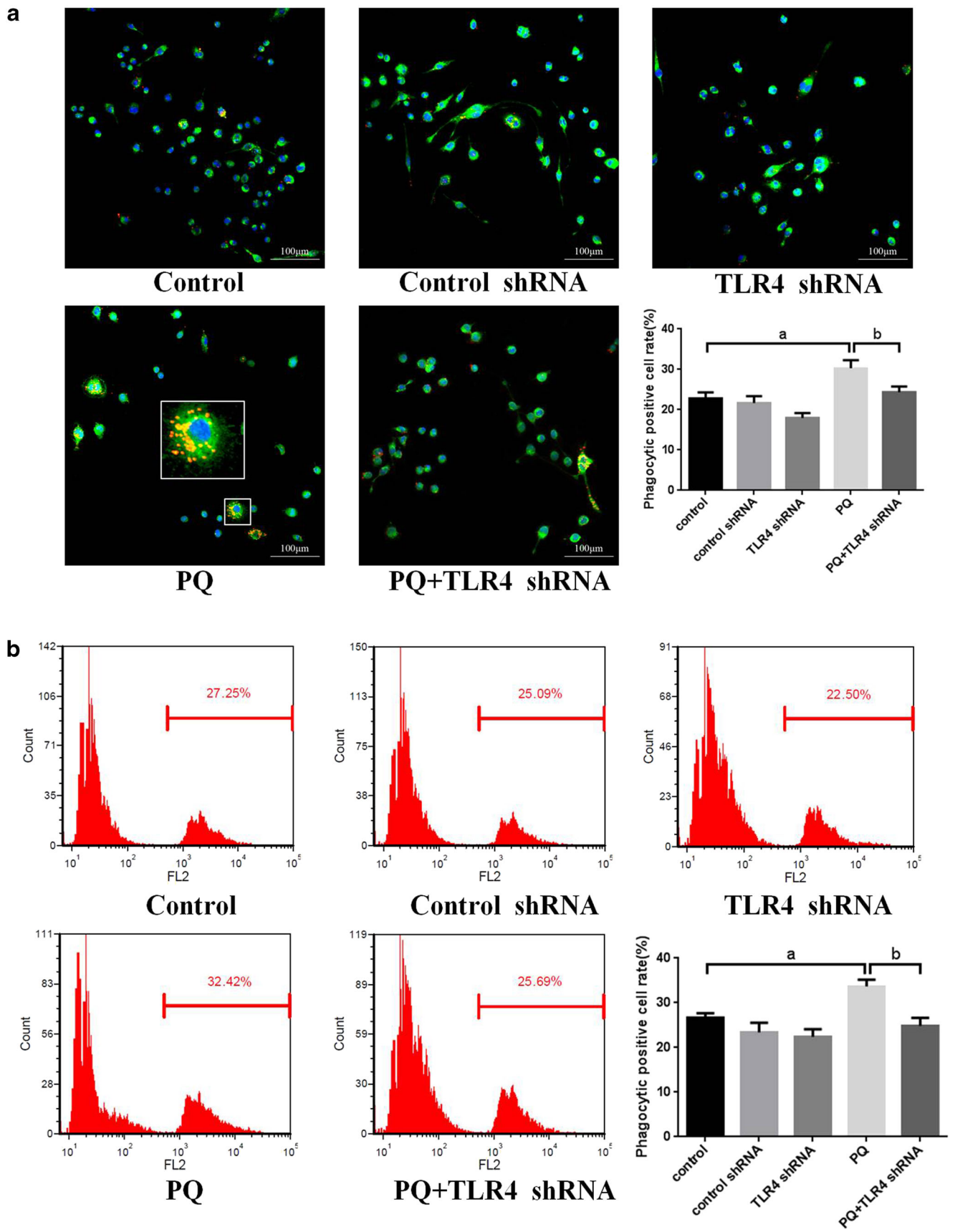


Fig. 7 The effects of TLR4 knockdown on microglial phagocytosis. BV-2 microglia transfected with TLR4 shRNA or control shRNA were treated with 0.06 $\mu\text{mol/l}$ PQ for 24 h. Fluorescent microspheres (red) were then added for 1 h. **A** Fluorescent images of the distinct microglial phagocytosis by immunofluorescent assay. Cells were fixed with 4% PFA and labeled with Iba-1 antibody and stained with FITC for visualization (green). The nucleus was visualized with DAPI (blue). Scale bar = 100 μm . **B** Flow cytometry peak images of BV-2 cells in each treatment group. Data are presented as mean \pm SE from at least three independent experiments. a: $p < 0.05$, compared with the vehicle control; b: $p < 0.05$, compared with the PQ treatment group

increases the phagocytic activity of BV-2 microglia, which could be reduced by TLR4 knockdown, suggesting that the phagocytic function of BV-2 microglia was affected by PQ exposure through TLR4 signaling pathway.

Discussion

In light of the evidence that PQ could induce inflammatory responses in the central nervous system thereby largely contributing to its neurotoxicity (Mitra et al. 2011; Costa et al. 2014; Purisai et al. 2007), targeting specific signaling pathways for neuroinflammation may provide a novel therapeutic strategy for protecting against PQ neurotoxicity.

TLR4, a member of the toll-like receptors family, plays a major role in regulating innate immunity in response to both exogenous and endogenous molecular patterns (Kuzmich et al. 2017; Gioannini et al. 2005). TLR4 is primarily known for initiating inflammatory responses in various organs including the central nervous system. Results obtained from both in vitro and in vivo models of environmentally neurotoxicants strongly supported that microglia plays as an active contributor to neuroinflammation with TLR4 activation involved (Laflamme and Rivest 2001; Stefanova et al. 2011; Ros-Bernal et al. 2011; Shao et al. 2019; Campolo et al. 2019). Thus, it is reasonable to hypothesize that TLR4 is the mediator in microglial inflammation contributing to the neurotoxicity of PQ. In this study, we investigated whether and how TLR4 affects the microglial inflammatory responses and microglial functions upon PQ exposure.

The murine microglial immortalized BV-2 cell line, a well-established microglia cell line originally derived by Dr. Elisabetta Blasi, was selected as our in vitro model (Blasi et al. 1990). Its similar phenotypic and functional properties to reactive microglia make it suitable as an alternative model for primary microglia culture or animal experiments to examine neuroinflammation (Henn et al. 2009). Since our previous study has found that TLR4 signaling in microglia is activated upon PQ exposure (Huang et al. 2019), herein we knockdown TLR4 by RNA interference and measure the subsequent effects. Based on our previous findings that BV-2 microglia was modulated to inflammatory phenotype (M1) with upregulated

TLR4 expression upon treatment with PQ at low doses of PQ (0.03 $\mu\text{mol/l}$, 0.06 $\mu\text{mol/l}$, and 0.12 $\mu\text{mol/l}$), 0.06 $\mu\text{mol/l}$ PQ was selected as the dosage in this study.

Upon PQ treatment, activated TLR4 recruits the central adapter protein MyD88, which then activates I κ B kinase (IKK) to phosphorylate I κ B α . This phosphorylation results in the dissociation of I κ B α from NF- κ B, thereby leading to the production of pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6 (Kawasaki and Kawai 2014; Lawrence 2009). In this study, the increased expressions of MyD88, IKK, p-I κ B α , and NF- κ B p65 protein upon PQ exposure were dramatically attenuated by the knockdown of TLR4, indicating that the knockdown of TLR4 directly leads to the repression of TLR4 signaling pathway. The levels of TNF- α , IL-1 β , and IL-6 were also inhibited after TLR4 knockdown, supporting our hypothesis that TLR4 signaling pathway is involved in PQ-induced inflammatory responses. Consistent with our findings, Zhang group reported that inhibiting TLR4 significantly reduces the intensity of inflammation in PQ-activated microglia (Li et al. 2019).

The activated microglia has demonstrated to be able to migrate to and phagocytosis infectious agents and senescent cells, as we previously reported that PQ exposure enhances the mobility and phagocytic activity of microglia (Huang et al. 2019; Mosher and Wyss-Coray 2014). Although not in the nervous system, TLR4 has been demonstrated to be associated with migration and phagocytosis in multiple systems. LPS, the classical activator of TLR4 signaling, increases the migration of melanoma cells, and this effect was substantially inhibited by TLR4 or MyD88 knockdown (Takazawa et al. 2014). TLR4 activation could increase the phagocytosis capability of macrophages (Anand et al. 2007). Here in the PQ-activated microglia, we observed that both microglial mobility and phagocytic activity were significantly reduced after inhibiting TLR4.

Moreover, by looking into the microglial phenotypic markers, we proved that the knockdown of TLR4 reverses the PQ-induced microglial polarization towards the M1 phenotype. A similar phenomenon has been reported in microglial inflammation induced by other stimuli. For example, the absence of TLR4 induces microglial polarization towards the M2 phenotype and alleviates the development of neuroinflammation after traumatic brain injury (Yao et al. 2017). Curcumin could alleviate LPS-induced neuroinflammation by promoting microglial M2 polarization via inhibiting TLR4 pathways (Zhang et al. 2019).

Taken together, we can conclude that TLR4 plays a key role in the neuroinflammation induced by PQ and the knockdown of TLR4 attenuates the inflammatory responses. Therefore, our findings provide an insight that TLR4 can be considered a potential therapeutic target for protecting against PQ neurotoxicity.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Akira S, Takeda K (2004) Toll-like receptor signalling. *Nat Rev Immunol* 4(7):499–511
- Anand RJ, Kohler JW, Cavallo JA, Li J, Dubowski T, Hackam DJ (2007) Toll-like receptor 4 plays a role in macrophage phagocytosis during peritoneal sepsis. *J Pediatr Surg* 42(6):927–932 **discussion 933**
- Blasi E, Barluzzi R, Bocchini V, Mazzolla R, Bistoni F (1990) Immortalization of murine microglial cells by a v-raf/v-myc carrying retrovirus. *J Neuroimmunol* 27(2–3):229–237
- Campolo M, Paterniti I, Siracusa R, Filippone A, Esposito E, Cuzzocrea S (2019) TLR4 absence reduces neuroinflammation and inflammasome activation in Parkinson's diseases in vivo model. *Brain Behav Immun* 76:236–247
- Costa KM, Maciel IS, Kist LW, Campos MM, Bogo MR (2014) Pharmacological inhibition of CXCR2 chemokine receptors modulates paraquat-induced intoxication in rats. *PLoS One* 9(8):e105740
- Drouin-Ouellet J, Gibrat C, Bousquet M, Calon F, Kriz J, Cicchetti F (2011) The role of the MYD88-dependent pathway in MPTP-induced brain dopaminergic degeneration. *J Neuroinflammation* 8: 137
- Gioannini TL, Teghanemt A, Zhang D, Levis EN, Weiss JP (2005) Monomeric endotoxin: protein complexes are essential for TLR4-dependent cell activation. *J Endotoxin Res* 11(2):117–123
- Henn A, Lund S, Hedtjam M, Schrattenholz A, Porzgen P et al (2009) The suitability of BV2 cells as alternative model system for primary microglia cultures or for animal experiments examining brain inflammation. *ALTEX* 26(2):83–94
- Huang M, Li Y, Wu K, Yan W, Tian T, Wang Y, Yang H (2019) Paraquat modulates microglia M1/M2 polarization via activation of TLR4-mediated NF-kappaB signaling pathway. *Chem Biol Interact* 310:108743
- Kawasaki T, Kawai T (2014) Toll-like receptor signaling pathways. *Front Immunol* 5:461
- Kuzmich NN, Sivak KV, Chubarev VN, Porozov YB, Savateeva-Lyubimova TN et al (2017) *TLR4 signaling pathway modulators as potential therapeutics in inflammation and sepsis*. *Vaccines (Basel)* 5(4)
- Laflamme N, Rivest S (2001) Toll-like receptor 4: the missing link of the cerebral innate immune response triggered by circulating gram-negative bacterial cell wall components. *FASEB J* 15(1):155–163
- Lawrence T (2009) The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb Perspect Biol* 1(6):a001651
- Li XL, Wang YL, Zheng J, Zhang Y, Zhang XF (2019) Inhibiting expression of HSP60 and TLR4 attenuates paraquat-induced microglial inflammation. *Chem Biol Interact* 299:179–185
- Lou D, Chang X, Li W, Zhao Q, Wang Y, Zhou Z (2012) Paraquat affects the homeostasis of dopaminergic system in PC12 cells. *Pestic Biochem Physiol* 103(2):81–86
- Mitra S, Chakrabarti N, Bhattacharyya A (2011) Differential regional expression patterns of alpha-synuclein, TNF-alpha, and IL-1beta; and variable status of dopaminergic neurotoxicity in mouse brain after paraquat treatment. *J Neuroinflammation* 8:163
- Mosher KI, Wyss-Coray T (2014) Microglial dysfunction in brain aging and Alzheimer's disease. *Biochem Pharmacol* 88(4):594–604
- Prasad K, Tarasewicz E, Mathew J, Strickland PA, Buckley B et al (2009) Toxicokinetics and toxicodynamics of paraquat accumulation in mouse brain. *Exp Neurol* 215(2):358–367
- Purisai MG, McCormack AL, Cumine S, Li J, Isla MZ, di Monte DA (2007) Microglial activation as a priming event leading to paraquat-induced dopaminergic cell degeneration. *Neurobiol Dis* 25(2):392–400
- Rajo AI, McBean G, Cindric M, Egea J, Lopez MG et al (2014) Redox control of microglial function: molecular mechanisms and functional significance. *Antioxid Redox Signal* 21(12):1766–1801
- Ros-Bernal F, Hunot S, Herrero MT, Pamadeau S, Corvol JC, Lu L, Alvarez-Fischer D, Carrillo-de Sauvage MA, Saurini F, Coussieu C, Kinugawa K, Prigent A, Hoglinger G, Hamon M, Tronche F, Hirsch EC, Vyas S (2011) Microglial glucocorticoid receptors play a pivotal role in regulating dopaminergic neurodegeneration in parkinsonism. *Proc Natl Acad Sci U S A* 108(16):6632–6637
- Shao QH, Chen Y, Li FF, Wang S, Zhang XL, Yuan YH, Chen NH (2019) TLR4 deficiency has a protective effect in the MPTP/probenecid mouse model of Parkinson's disease. *Acta Pharmacol Sin* 40(12):1503–1512
- Stefanova N, Fellner L, Reindl M, Maslah E, Poewe W, Wenning GK (2011) Toll-like receptor 4 promotes alpha-synuclein clearance and survival of nigral dopaminergic neurons. *Am J Pathol* 179(2):954–963
- Takazawa Y, Kiniwa Y, Ogawa E, Uchiyama A, Ashida A, Uhara H, Goto Y, Okuyama R (2014) Toll-like receptor 4 signaling promotes the migration of human melanoma cells. *Tohoku J Exp Med* 234(1): 57–65
- Yao X, Liu S, Ding W, Yue P, Jiang Q, Zhao M, Hu F, Zhang H (2017) TLR4 signal ablation attenuated neurological deficits by regulating microglial M1/M2 phenotype after traumatic brain injury in mice. *J Neuroimmunol* 310:38–45
- Zhang J, Zheng Y, Luo Y, Du Y, Zhang X et al (2019) Curcumin inhibits LPS-induced neuroinflammation by promoting microglial M2 polarization via TREM2/TLR4/NF-kappaB pathways in BV2 cells. *Mol Immunol* 116:29–37

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