Toll-Like Receptor 2 Deficiency Shifts PrP106-126-Induced Microglial Activation from a Neurotoxic to a Neuroprotective Phenotype

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Abstract Prion diseases are fatal neurodegenerative diseases characterized by spongiform change, neuronal loss, and gliosis involving microglial activation in the central nervous system. Microglial activation is thought to play a key role in the pathogenesis of prion disease; however, the molecular mechanisms underlying prion-induced microglial activation are not well understood. The present study underlines the importance of toll-like receptor (TLR)-2 in mediating PrP106-126-induced microglial activation. We found that PrP106-126 induced expression of proinflammatory molecules and TLR2 in microglial cells; however, functional blocking antibodies against TLR2 suppressed PrP106-126-induced expression of proinflammatory molecules. PrP106-126-induced expression of proinflammatory molecules was also reduced in microglial cells isolated from TLR2−/− mice compared to those isolated from wild-type mice. Consistent with the importance of nuclear factor kappa B (NF-κB) mediating TLR functions, NF-κB inhibition also inhibited PrP106-126-induced expression of proinflammatory molecules. To better understand the effect of TLR2 deficiency on active microglial cells, we studied the expression of Arg1 and Mrc1 and anti-inflammatory cytokines, which indicated that TLR2 deficiency in microglial cells results in a shift from neurotoxic to neuroprotective phenotype. Taken together, our results indicate that the TLR2 signaling pathway mediates PrP106-126-induced microglial activation and potentially reveal new therapeutic strategies for prion diseases that modulate TLR2 signaling.

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Abbreviations

GSS	Gerstmann-Sträussler syndrome
CJD	Creutzfeldt-Jakob disease
CNS	Central nervous system
PD	Parkinson's disease
MS	Multiple sclerosis
AD	Alzheimer's disease
TLR	Toll-like receptor
KO	Knockout
WT	Wild type
Aβ	Amyloid β

SCC	Strongly connected component
MAPK	Mitogen-activated protein kinase
ERK	Extracellular signal-regulated kinase
mFPR2	Formyl peptide receptor 2
miR	mRNA
RAGE	Receptor for advanced glycation end products
NF-κB	Nuclear factor kappa B
CCK-8	Cell counting Kit-8
DMEM	Dulbecco's modified Eagle's medium
cDNA	Complementary DNA
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
TNF	Tumor necrosis factor
IL	Interleukin
iNOS	Inducible isoform of nitric oxide synthase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
ECF	Chemifluorescence
ThT	Thioflavine-T
PDTC	Pyrrolidine dithiocarbamate
Arg	Arginase
Mrc	Macrophage mannose receptor
MyD88	Myeloid differentiation factor 88
TGF	Transforming growth factor
PAMPs	Pathogen-associated molecular pattern molecules
DAMPs	Damage-associated molecular pattern molecules
NTF	Neurotrophic factors
TIRAP	TIR domain-containing adaptor protein

Background

Prion diseases, also termed transmissible spongiform encephalopathy, are a group of untreatable and fatal neurodegenerative diseases. They include bovine spongiform encephalopathy in cows, scrapie in sheep, and chronic wasting disease in deer and elk, as well as Gerstmann-Sträussler syndrome (GSS), and Creutzfeldt-Jakob disease (CJD) in humans (Prusiner 1998). The major neuropathological features of prion disease are neuronal vacuolation, neuronal loss, and gliosis involving microglial activation in affected areas of the central nervous system (CNS) (Scott 1993). Prion diseases are enigmatic because PrP is a host-encoded protein that can promote immunotolerance to PrPSc, adaptive immunity against prion infection and therefore does not occur (Aguzzi and Polymenidou 2004), which makes innate immunity pivotal in the neuroinflammation of neurodegenerative disorders. A synthetic neurotoxic PrP peptide, PrP106-126, exhibits many biophysical properties similar to those of PrPSc, in which conversion of PrPC into the protease-resistant amyloid fibrils occurs in vitro. Therefore, PrP106-126 is a suitable model system to investigate PrPSc neurotoxicity (Gu et al. 2002; Singh et al. 2002; Ettaiche et al. 2000; Forloni et al. 1993).

Microglial cells, the resident macrophages in the CNS parenchyma, have multiple functions, including phagocytosis, growth factor and cytokine production, and antigen presentation; microglial cells are considered the first line of defense in the CNS, as they respond to infection, injury, or neurodegeneration and are critical for neuroinflammation. After activation, an innate immune response is initiated, characterized by neurotoxic factor and proinflammatory cytokine release and eventually phagocytosis (Colton and Wilcock 2010). Accumulating evidence suggests that microglial activation precedes or occurs simultaneously with neuronal and glial cell degeneration in neurodegenerative disorders, including prion disease, Parkinson's disease (PD), multiple sclerosis (MS), and Alzheimer's disease (AD), and it is thought to have both toxic and protective effects on neuronal survival (Perry et al. 2010; Gonzalez-Scarano and Baltuch 1999; Brown et al. 1996). Thus, controlling microglial activation is important for preventing neuronal injury.

Considerable evidence indicates that toll-like receptors (TLRs) mediate microglial activation. Because microglial activation is pivotal in prion disease, TLRs also may be involved in prion disease pathology (Ofodile 2011; Trudler et al. 2010). Several of TLRs were constitutively expressed in primary microglial cells in vitro, and TLR2 was the most highly expressed (Trudler et al. 2010). Substantial evidence indicates that TLR2 is crucial for microglial activation in various infectious and neurodegenerative diseases (reviewed in Oliveira-Nascimento et al. 2012; Hanke and Kielian 2011). Moreover, extensive proof indicates that TLR2 is critical in AD. Liu et al. (2012) showed that TLR2 is a primary Aβ receptor that triggers neuroinflammatory activation, and suggested TLR2 inhibition in microglial cells could be beneficial in the treatment of AD. TLR2 knockout (KO) reduced amyloid β (Aβ) deposition in brain before the age of 6 months, but increased the Aβ load thereafter in a mouse model of AD. TLR2 activation in microglia induced formyl peptide receptor 2 (mFPR2) expression through p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) 1/2-dependent pathways. Furthermore, TLR2 ligand-stimulated microglial cells showed a dramatically increased capacity to endocytose $A\beta_{42}$ via mFPR2 (Chen et al. 2006; Richard et al. 2008). In the brain of a RML scrapie model, the microglial surface receptor TLR2 is significantly overexpressed and therefore may be involved in microglial activation. In addition, overexpressed mRNA (miR)-146a induction may be secondary to the initial response triggered by TLR2 or TLR4 activation during prion infection (Saba

et al. 2012). Using a detailed network analysis, Crespo et al. (2012) identified a strongly connected component (SCC) that contained 16 members that included TLR2; this unique SCC may be central to prion pathogenesis. Moreover, receptor for advanced glycation end products (RAGE) and TLR family members may interact to coordinate and regulate immune and inflammatory responses (Ibrahim et al. 2013; Yang et al. 2010; Monden et al. 2013). We previously demonstrated that PrP106-126-induced nuclear factor kappa B (NF-κB) activation is RAGE-mediated (data unpublished).

Nevertheless, there is no direct evidence that indicates whether TLR2 is critical to prion disease pathology. In the present study, we investigated the exact role of TLR2 in PrP106-126-induced murine microglial cell activation. We found that TLR2 mediates proinflammatory cytokine expression through the NF-κB pathway and therefore plays a role in PrP106-126-induced microglial activation. Similarly, TLR2 ablation shifted microglial activation from a neurotoxic to neuroprotective phenotype.

Methods

Animals and Reagents

TLR2−/− mice used in this study were obtained from Model Animal Research Center of Nanjing University. Dulbecco's modified Eagle's medium (DMEM) was purchased from Hyclone (Logan, UT, USA). Inactivated fetal bovine serum (FBS), streptomycin, and penicillin were obtained from Life Technologies Corporation (Gibco, Grand Island, NY, USA). Reagents and apparatus used in Western blot were purchased from Bio-Rad (Hercules, CA, USA). Rabbit anti-mouse TLR2 antibody was acquired from Bioss (Biosynthesis Biotechnology, Beijing, China). Rabbit NF-κB antibody was from Cell Signaling Technology (Cambridge, MA, USA). Function blocking antibodies to TLR2 (T2.5) were purchased from eBioscience. Cell Counting Kit-8 (CCK-8) was purchased from Beyotime Biotechnology (Wuhan, Hubei, China). Rabbit anti-mouse max and rabbit anti-mouse β-actin antibody were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Microglial Cells Isolation and Culture

The murine microglial BV2 cell line (Xiehe Medical University, Yuqin Liu, Cell Culture Center, Beijing, China) was cultured in DMEM (Hyclone, Logan, UT, USA) containing 10 % heat-inactivated FBS (Gibco, Grand Island, NY, USA), 100 μg/mL streptomycin, 100 U/mL penicillin (Gibco), and 2 mmol/L glutamine in a 95 % air/5 % CO$_2$-humidified incubator.

Primary microglial cells were obtained according to a previously described procedure (Garcao et al. 2006). Briefly, brains from neonatal mice (0–3 days old) were sterilized with 75 % ethanol and dissected. The meninges were carefully and the brains were digested with 0.25 % trypsin at 37 °C. After repeated agitation, the cells were passed through a 200-μm mesh, separated by centrifugation at 200g for 10 min, and resuspended in DMEM with 10 % FCS, and then cultured for approximately 18–20 days. Following culture, microglial cells were separated by gentle shaking on an orbital shaker at 37 °C and then the medium was harvested into a new flask. After incubating for 2 h at 37 °C, nonadherent cells were removed.

Flow Cytometry

Isolated microglial cells stained with anti-CD11b (BD Biosciences) were analyzed by flow cytometry to determine cell purity. We examined 10,000 cells in each experiment.

PrP106-126 Peptides Preparation

PrP106-126 (sequence KTNMKHMAGAAAAGA VVGGLG) and scrambled PrP106-126 (ScrPrP106-126) with sequence of AVGMHAGKGLANTAKAGAMVG were purchased from Sangon Bio-Tech (China). The purity was >95 %. Peptide aggregation was performed by agitation at 37 °C for 12 h in 0.1 mol/L phosphate-buffered saline (PBS) at a concentration of 1 mmol/L. The final concentration used in our study was at 100 μM.

Thioflavine-T Fluorometric Assay

A thioflavine-T (ThT) fluorometric assay was used to measure fibril formation of PrP106-126 according to previously described methods (Pan et al. 2014).

Cell Viability Assays

Cell viability was measured by CCK-8 assay; 100 μl of cell suspension with a total of 5000 cells per well were plated in a 96-well plate, and 10 μl of pyrrolidine dithiocarbamate (PDTC) with different concentrations was added and incubated for 24 h. Then, 10 μl of CCK-8 reagent was added to each well and cells were incubated for 2 h at 37 °C and 5 % CO$_2$. The absorbance of the samples was measured at 450 nm using a microplate reader with a background blank as the control. The cell survival ratio was expressed as the percentage of the control.

Enzyme-Linked Immunosorbent Assay

The levels of tumor necrosis factor (TNF)-α and NO were determined by enzyme-linked immunosorbent

assay (ELISA) assay using commercial ELISA kit (Wuhan Boster Biotech) in accordance with the manufacturer's instructions.

Western Blotting

After treatment, cytoplasmic and nuclear proteins were prepared using a commercial protein extraction kit (Cytoplasmic and Nuclear Protein Extraction Kit; Wuhan Boster Biotech) following the manufacturers' instructions. After protein separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12 % gels and nitrocellulose membranes transfer, membranes were blocked with 5 % fat-free dried milk in tris-buffered saline (TBS-T) and then incubated overnight at 4 °C with primary antibodies: [rabbit anti-NF-κB p65 (1:1000), anti-TLR2 (1:500)]. They were then washed and incubated with the secondary antibody (1:5000). The signal was visualized using a commercial chemifluorescence (ECF) system (Versadoc; Bio-Rad).

Quantitative PCR

After treatment, total RNA was extracted from microglial cells using the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the instructions. RNA was used for cDNA synthesis by a commercial kit (cDNA Synthesis Kit; Fermentas, Glen Burnie, MD, USA) and quantitative polymerase chain reaction (qPCR) was carried out with a commercial mix (SYBR Green Master Mix; Bio-Rad) and a thermal cycler (DNA Engine Opticon™ 2 system; MJ Research, Waltham, MA, USA). The primers used in our study are listed in Table 1. The sequences and amplification efficiency of these primers were provided upon request. The total volume for qPCR and PCR amplification procedure was as previously described (Shi et al. 2012; Kouadir et al. 2012). The relative mRNA expression levels were calculated using the comparative CT method ($2^{-\Delta\Delta CT}$).

Statistical Analysis

Data are presented as the mean±standard deviation (SD). Parametric data were compared by one-way ANOVA or Student's t test followed by Tukey's post hoc test. Graphics were generated with Graph Pad Prism (Graph Pad Prism 4.0, San Diego, CA). SPSS software (version 13.0: SPSS Inc., Chicago, IL, USA) was used for statistical analyses. A probability value (p)<0.05 were considered statistically significant.

Table 1 Primers used for quantitative PCR

Name	Sequence (5′-3′)
β-actin	GCTTCTTTGCAGCTCCTTCG
	CCTTCTGACCCATTCCCACC
TLR2	GTTTGCTATGATGCCTTTG
	TTCGTACTTGCACCACTCG
TNF-α	CCCTTCCTCCGATGGCTAC
	CGCCTCCTTCTTGTTCTGG
IL-1β	CAGGCTCCGAGATGAACAA
	CCCAAGGCCACAGGTATTT
iNOS	TGTGTCAGCCCTCAGAGTAC
	CACTGACACTTCGCACAA
TGF-β	CACTGATACGCCTGAGTG
	GTGAGCGCTGAATCGAAA
IL-10	GCTCTTACTGACTGGCATGAG
	CGCAGCTCTAGGAGCATGTG
Arg1	GCATATCTGCCAAAGACATCG
	CCATCACCTTGCCAATCCC
Mrc1	GCAGTGGGCTGGAGGAA
	TGCTGAGGGAATGATAAATGG

Results

TLR2 Expression Is Increased in Microglial Cells with PrP106-126 Treatment

To investigate whether TLR2 participated in PrP106-126-induced microglial activation, we used qPCR to examine whether PrP106-126 stimulation increased TLR2 mRNA expression. BV2 cells and primary microglial cells were incubated with 100 μM of neurotoxic PrP106-126 for 6, 12, or 24 h. The purity of the isolated cells was >94 %, as determined by expression of the marker CD11b using flow cytometry (Fig. 1a, b). PrP106-126 treatment upregulated TLR2 mRNA expression in BV2 cell lines (Fig. 1d) and in primary microglial cells (Fig. 1e) at all time points. Similarly, zymosan A, a well-characterized TLR2 agonist, induced significant overexpression of TLR2. We also performed Western blotting to confirm TLR2 protein expression at 6, 12, and 24 h. As expected, PrP106-126 induced TLR2 protein expression in primary microglial cells (Fig. 1f, g). Together, these findings suggest that TLR2 is involved in PrP106-126-induced microglial activation. ThT fluorometric assay revealed amyloid aggregates in PrP106-126 preparations with BV2 and primary microglial cells treatment (Fig. 1c).

Effect of PrP106-126 on Different Proinflammatory Molecules in Microglial Cells

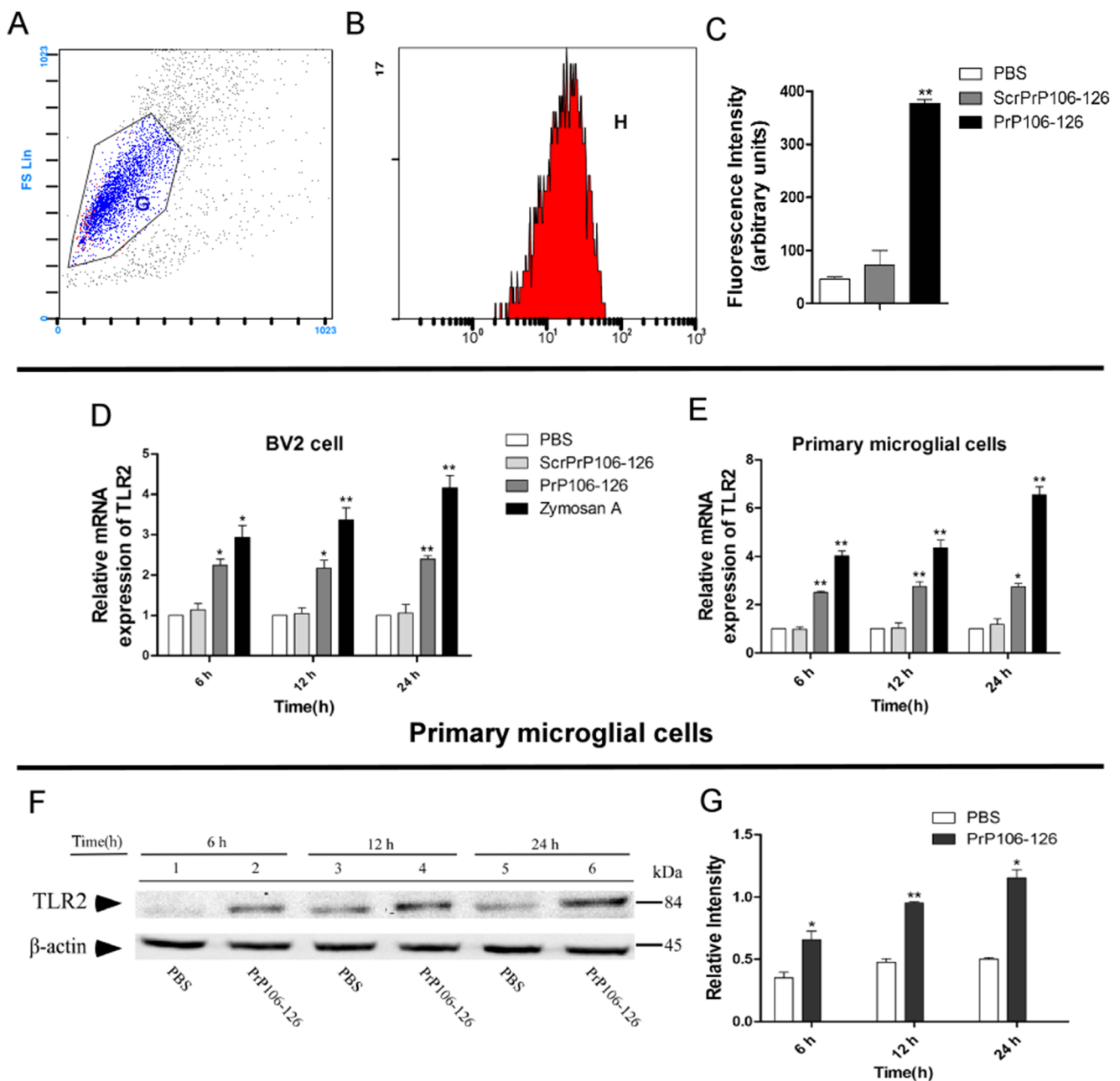To investigate whether interactions between PrP106-126 and microglial cells could induce proinflammatory molecule

Fig. 1 Effect of PrP106-126 peptides on TLR2 expression in mouse BV2 and primary microglial cells. The purity of isolated WT and TLR2−/− microglial cells determined by CD11b marker expression using flow cytometry was >95 and 94 %, respectively (**a, b**), and the presence of PrP106-126 amyloid aggregates was examined using thioflavine-T (ThT) fluorometric assay (**c**). Mouse BV2 microglial cells (**d**) and primary microglial cells (**e**) were stimulated with 100 μM PrP106-126, ScrPrP106-126, or zymosan A for 6, 12, or 24 h. The mRNA level of TLR2 was determined by qPCR. **f, g** Primary microglial cells were stimulated with PBS or PrP106-126 (100 μM) and TLR2 expression was determined by Western blot assays using anti-TLR2 antibody. *Bars* showed the relative change of TLR2 level compared with β-actin. Data are expressed as the mean±SD of triplicate experiments (vs. PBS; *$p<0.05$; **$p<0.001$)

expression in our cultures, we examined mRNA expression at different time points after treatment with PrP106-126. Cells were also stimulated with zymosan A as a positive control. PrP106-126 induced overexpression of inflammatory molecules, including inducible isoform of nitric oxide synthase (iNOS), TNF-α, and IL-1β, in BV2 cell line as well as in primary microglial cells. Proinflammatory molecule

expression peaked after 12 h and remained stable for 24 h (Fig. 2a–f). In addition, PrP106-126 treatment significantly increased iNOS-generated nitric oxide production and TNF-α levels in primary microglial cells assayed by ELISA (Fig. 2g, h). Consistent with previous reports, our results suggest that PrP106-126 can increase proinflammatory molecule expression and release in microglial cells.
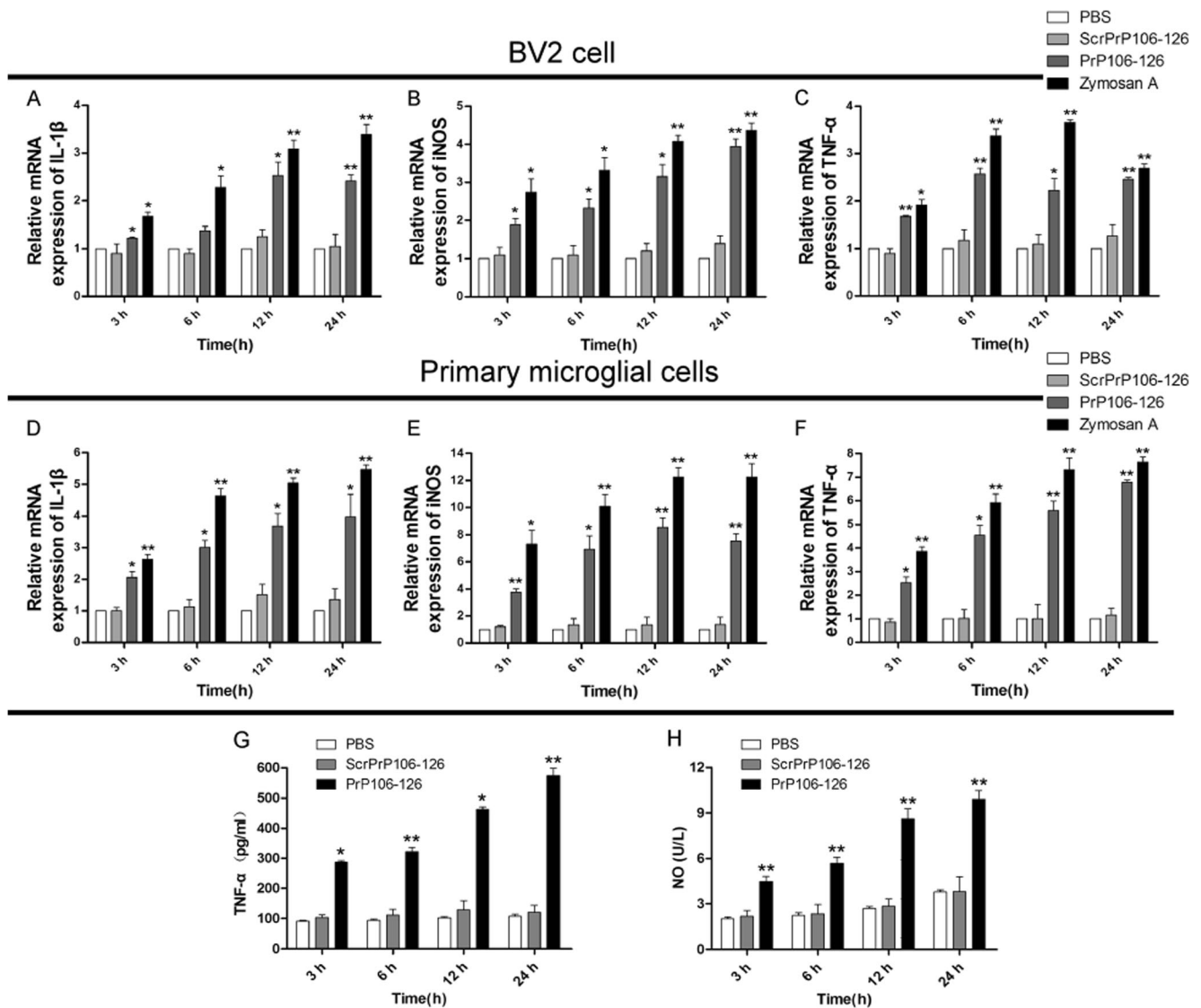
Fig. 2 Expression of proinflammatory cytokines in BV2 and primary microglial cells treated with PrP106-126. Mouse BV2 microglial cells (**a**–**c**) and primary microglial cells (**d**, **e**) were treated for 3, 6, 12, or 24 h with PrP106-126 (100 μM), ScrPrP106-126 (100 μM), or zymosan A (100 μM). The mRNA expression of proinflammatory cytokines were determined by qPCR. All the levels of mRNA expression are expressed as the fold increase over PBS-treated cells. The level of released TNF-α (**g**) and NO (**h**) was measured by ELISA following instructions. Data are expressed as the mean±SD of triplicate experiments and are representative of an experimental $n=3$ or 4 (vs. PBS; *$p<0.05$; **$p<0.001$)

TLR2 Is Involved in Proinflammatory and Neurotoxic Cytokine Expression in Microglial Cells After PrP106-126 Treatment

Since the expression of proinflammatory cytokines and TLR2 was upregulated in murine microglial cells with PrP106-126 stimulation, we hypothesized that PrP106-126 increases proinflammatory molecule expression in microglial cells by stimulating TLR2. To test this hypothesis, we examined whether anti-TLR2-neutralizing antibody affected PrP106-126-induced expression of proinflammatory molecules in primary microglial cells. Primary microglial cells were pretreated with or without 30 μM anti-TLR2-blocking antibody (T2.5) for 1 h and

then stimulated with PrP106-126 for 3, 6, 12, and 24 h. Figures 3a–c showed that PrP106-126-induced mRNA expression of proinflammatory cytokines in microglial cells was significantly inhibited by anti-TLR2-blocking antibodies treatment.

To confirm TLR2 participation in PrP106-126-induced microglial activation, primary microglial cells were isolated from wild type (WT) and TLR2 KO mice. As shown in Fig. 3d, e, PrP106-126-induced mRNA expression of IL-1β, iNOS, and TNF-α was significantly reduced in microglial cells isolated from TLR2−/− mice compared to those from WT mice. Similarly, TLR2 deficiency significantly abrogated zymosan A-induced upregulation of these proinflammatory molecules in microglial cells. These results suggested that
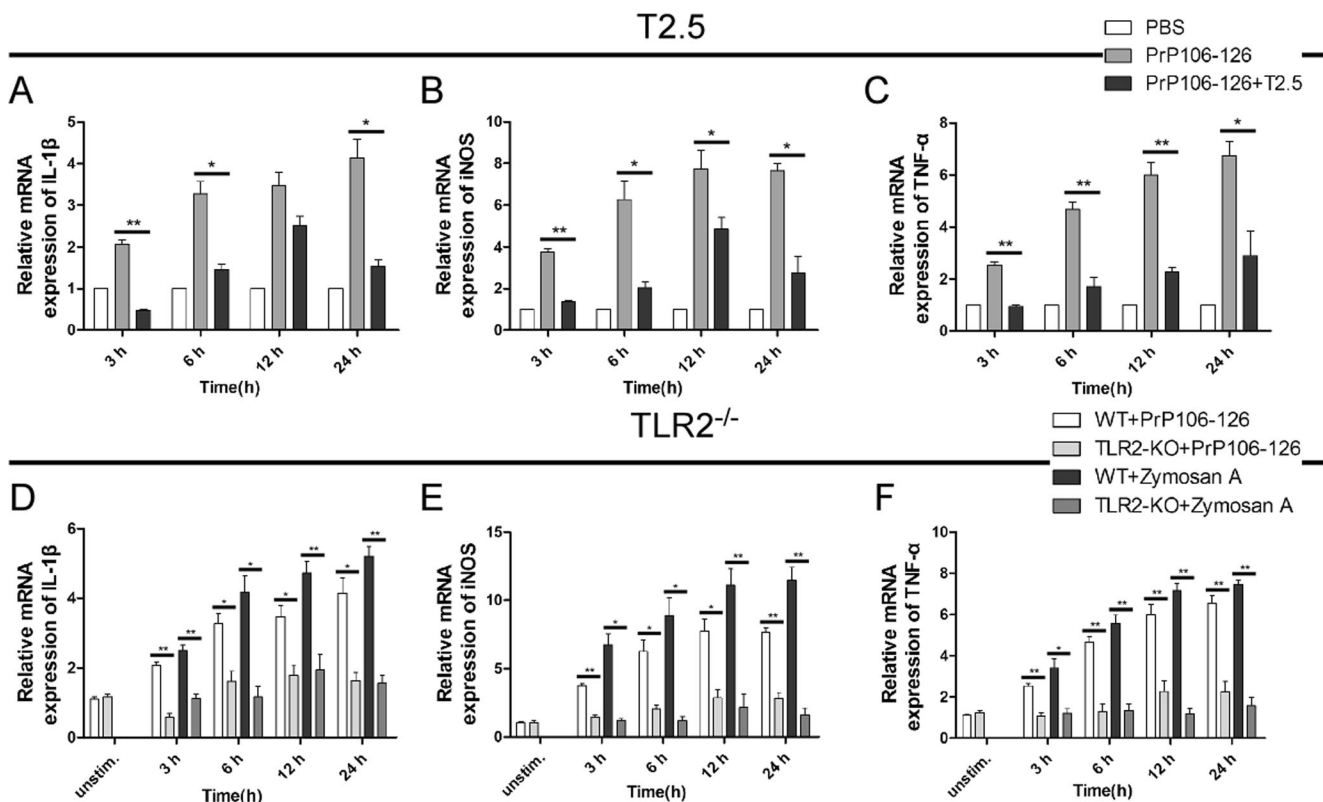
Fig. 3 Effect of TLR2 deficiency on PrP106-126-induced expression of proinflammatory molecules in microglial cells. **a–c** Primary microglial cells were preincubated with or without functional blocking antibodies T2.5 (30 μg/mL) against TLR2 for 1 h and then incubated with PrP106-126 or zymosan A for 3, 6, 12, or 24 h, and then analyzed for IL-1β (**a**), iNOS (**b**), and TNF-α (**c**) mRNAs using qPCR. **d, e** Microglial cells obtained from neonatal WT and TLR2−/− mice were treated with PrP106-126 (100 μM). After treatment, qPCR was performed to measure the mRNA expression of IL-1β (**d**), iNOS (**e**), and TNF-α (**f**). Data are expressed as the mean±SD of triplicate experiments and are representative of an experimental $n=3$ (vs. PBS; *$p<0.05$; **$p<0.001$)

TLR2 was involved in the expression of IL-1β, iNOS, and TNF-α in PrP106-126-stimulated microglial cells.

TLR2-NF-κB Pathway Is Involved in PrP106-126-Induced Expression of Proinflammatory Molecules in Microglial Cells

We then examined the role of the TLR2-NF-κB pathway in PrP106-126-induced proinflammatory molecule expression. Using Western blot analysis, we showed that nuclear translocation of p65 was induced in primary microglial cells isolated from WT, but not TLR2−/− mice after treatment with PrP106-126 (Fig. 4a). This suggests that the TLR2-NF-κB pathway may play a role in PrP106-126-induced expression of proinflammatory factors.

To determine whether NF-κB contributed to PrP106-126-stimulated microglial activation, we determined whether inhibition of NF-κB by pyrrolidine dithiocarbamate (PDTC, a selective chemical inhibitor of NF-κB) affected the proinflammatory or neurotoxic cytokine mRNA expression. NF-κB inhibition significantly attenuated, but did not inhibit, PrP106-126-induced upregulation of TNF-α, and iNOS mRNA expression in all the time points examined; interestingly, the mRNA of IL-1β was reduced at 3, 6, and 12 h but

not at 24 h, suggesting the existence of other signaling pathways responsible for IL-1β expression, which may rescue the inhibitory effect of TLR2 deficiency on IL-1β expression (Fig. 4c–e). This finding suggests that TLR2-mediated NF-κB activation at least partly contributed to proinflammatory and neurotoxic cytokine transcription.

PDTC treatment, however, could detrimentally affect cell survival and thus could have affected the present findings. To rule out this possibility, we used CCK-8 assay to determine whether PDTC treatment affected cell viability. We found that treatment with 30 μM PDTC did not significantly affect cell viability (Fig. 4b), suggesting that PDTC exerted its effects on PrP106-126-stimulated cells by inhibiting NF-κB activation. Together, our findings suggest that transcription of proinflammatory cytokines in microglial cells induced by PrP106-126 is partly dependent on the TLR2-NF-κB pathway.

TLR2 Deficiency Enhanced the Expression of Mrc1, Arg1, IL-10, and TGF-β in Primary Mouse Microglial Activation with PrP106-126 Treatment

Our previous findings indicated that interactions between microglial cells and amyloidogenic peptides
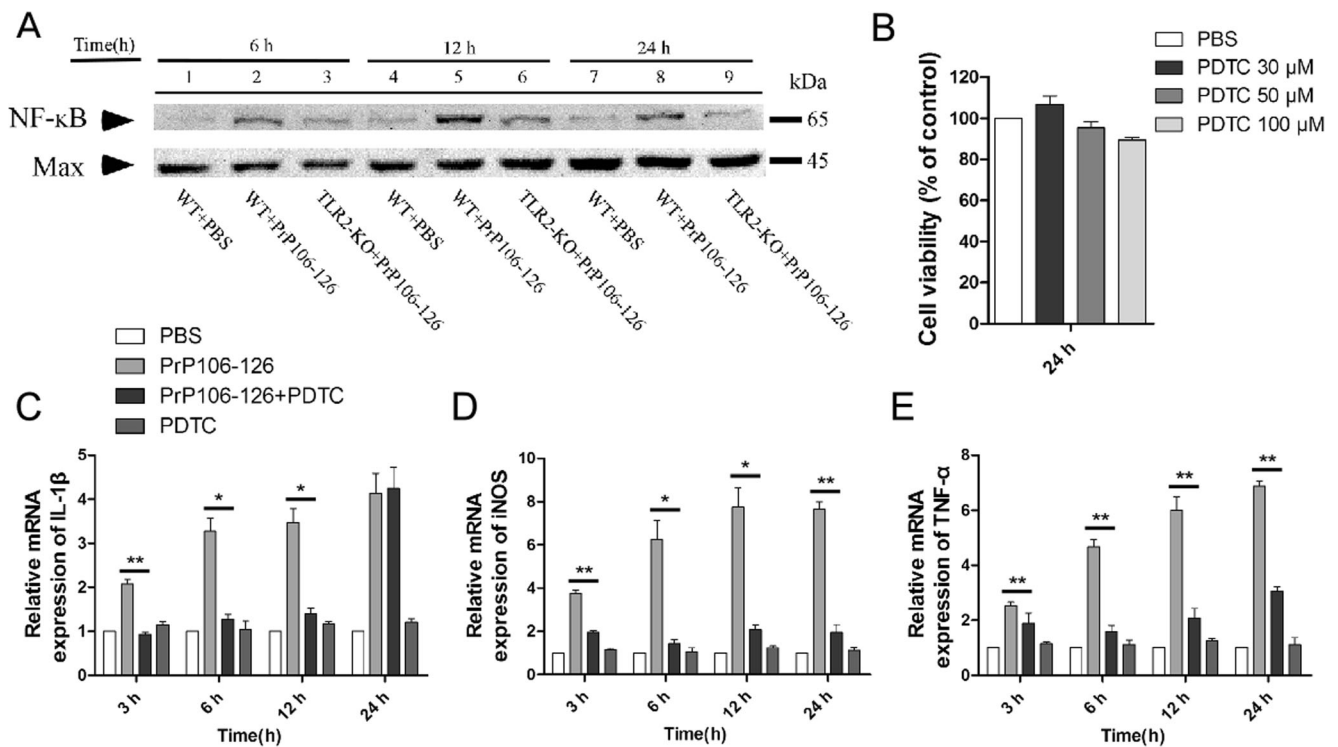
Fig. 4 PrP106-126-induced proinflammatory cytokine expression is mediated by a NF-κB-dependent pathway. **a** Western blot assays of NF-κB p65 expression in wild-type (WT) primary microglial cells treated with PBS (1, 4, 7) or PrP106-126 (2, 5, 8), and TLR2−/− primary microglial cells treated with PrP106-126 (3, 6, 9) was measured by Western blot assays. **b** CCK-8 assay showed microglial cells viability after incubated with different concentrations of PDTC, a selective chemical inhibitor of NF-κB, for 24 h. **c–e** Primary microglial cells preincubated with PDTC for 1 h were stimulated with PrP106-126 for 3, 6, 12, or 24 h, and then analyzed for IL-1β (**c**), iNOS (**d**), and TNF-α (**e**) mRNAs using qPCR. Data are expressed as the mean±SD of triplicate experiments and are representative of an experimental n=3 or 4 (*p<0.05; **p<0.001)

in vitro stimulate classical microglial activation but suppress alternative activation (Shi et al. 2013). Based on these findings, we concluded that PrP106-126 stimulates classical activation of microglial cells. TLR2 deficiency significantly reduced PrP106-126-stimulated overexpression of proinflammatory molecules in primary microglial cells (Fig. 3); however, to better understand how TLR2 deficiency affects microglial activation after PrP106-126 treatment, we examined the difference in gene expression profiles over time. PrP106-126 treatment reduced expression of arginase (Arg) 1 and macrophage mannose receptor (Mrc) 1, which are associated with resolution of inflammation, and tissue remodeling. However, TLR2 deficiency enhanced PrP106-126-induced transcription of Arg1 and Mrc1 in microglial cells (Fig. 5a, b). We also examined anti-inflammatory cytokine expression in PrP106-126-challenged WT and TLR2−/− microglial cells to determine whether the TLR2 deficiency affected the inflammatory response. We observed that PrP106-126 stimulation induced weak upregulation of IL-10 and transforming growth factor (TGF)-β. Interestingly, TGF-β and IL-10 expression were higher in TLR2−/− microglial cells than that in WT microglial cells with treatment of PrP106-126

(Fig. 5c, d). Thus, the microglial phenotype could be polarized to an anti-inflammatory neuroprotective status.

Discussion

Although microglial cells constitutively expresses several TLRs, TLRs 1–4 are the most prevalent, with TLR2 being the most highly expressed (Trudler et al. 2010). Previous studies using DNA microarray analyses found that TLR2 gene expression was significantly upregulated in the prion-infected mouse (Saba et al. 2012). Microglial activation increases production of neurotoxic factors and proinflammatory cytokines, whereas PrP106-126 activates microglial cells (Shi et al. 2012). Thus, in this work, we investigated whether TLR2 participated in PrP106-126-induced microglial activation. We found that PrP106-126 induced expression of proinflammatory molecules and TLR2 in microglial cells; however, functional blocking antibodies against TLR2 suppressed PrP106-126-induced expression of proinflammatory molecules. PrP106-126-induced expression of proinflammatory molecules was also reduced in microglial cells isolated from TLR2−/− mice compared to that in wild type mice.

Fig. 5 TLR2 deficiency enhanced the expression of Mrc1, Arg1, IL-10, and TGF-β in primary microglial cells. Microglial cells obtained from neonatal WT and TLR2−/− mice were treated with PrP106-126 for 3, 6, 12, or 24 h and qPCR was performed to analyze the mRNA expression of Mrc1 (**a**), Arg1 (**b**), IL-10 (**c**), and TGF-β (**d**). Data are expressed as the mean±SD of triplicate experiments and are representative of an experimental *n*=3 or 4 (vs. PBS; *$p<0.05$; **$p<0.001$). Significant differences between untreated versus PrP106-126-treated WTs are denoted with *asterisks* (*$p<0.05$; **$p<0.001$); significant differences between PrP106-126-treated WTs and TLR2 KO microglial cells are also indicated ($^{\#}p<0.05$; $^{\#\#}p<0.001$)
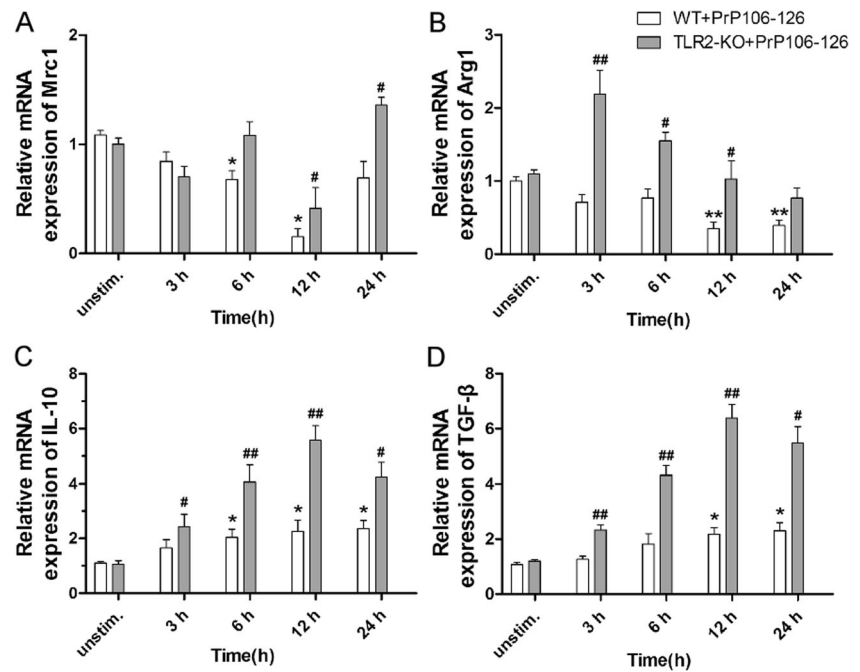
Upon activation, multiple proinflammatory factors released by microglial cells were involved in mediating the inflammatory response via various signaling pathways including transcription factor NF-κB pathway. Our previous work demonstrated that NF-κB participated in PrP106-126-induced Ana-1 activation (Lu et al. 2012). Furthermore, many studies have confirmed the involvement of NF-κB activation by PrP106-126 stimulation in microglial cells (Shi et al. 2012; Lu et al. 2012; Yang et al. 2008; Julius et al. 2008). However, the upstream mediator of NF-κB activation induced by PrP106-126 remains unknown. In the present study, we showed that PrP106-126 activated microglial cells via the TLR2-NF-κB pathway. However, inhibition of TLR2 or NF-κB reduced, but did not completely halt, inflammatory cytokine transcription. Therefore, other pathways responsible for inflammatory cytokine expression, such as the NLRP3 inflammasome, may exist (Shi et al. 2012). It has been demonstrated that myeloid differentiation factor 88 (MyD88) signal deficiency did not markedly change the prion disease neuropathogenesis during prion infection, which indicates that NF-κB activation resulting from MyD88-mediated TLR signaling does not contribute to prion pathogenesis (Prinz et al. 2003). However, since the inflammatory response in prion disease in vivo is much more complex and microglial activation is a double-edged sword, responsible both for the clearance of protein amyloids and neurotoxic mediators, these findings may explain the discrepancy between the results of MyD88 and TLR2 deficiency. On the other hand, identification of the signaling pathways contributing to microglial activation that are independent of MyD88 should be investigated further.

Some hypotheses have been proposed to interpret pathogenesis of prion disease, which include PrPSc gain-of-toxic function, PrPSc loss-of-function, chronic inflammation, activation of apoptotic or autophagy death pathways, and endoplasmic reticulum stress, induced by proteins misfolding and neuronal damage (Soto 2003). The findings from a gene regulatory network analysis performed by Crespo et al. (2012) support the idea that inflammation is the key to the neurodegeneration process in prion disease. Indeed, uncontrolled and overactivated microglial cells can trigger neurotoxicity by releasing neurotoxic factors. Recent studies have reported that phagocytosis activity of microglia cells is the main reason of neuronal loss and synaptic degeneration induced by Aβ, whereas neuronal loss was restraint by inhibition of phagocytosis (Neniskyte et al. 2011). Therefore, controlling microglial activation and inflammation is important for treating neurodegenerative diseases.

After exposure to pathogen-associated molecular pattern molecules (PAMPs) and damage-associated molecular pattern molecules (DAMPs), microglial cells can be activated and play a specialized immune surveillance function in the CNS. Activated microglial cells can release several factors, including cytokines, neurotrophic factors (NTF), and reactive oxygen and nitrogen species (ROS), to modulate the innate immune response (Colton and Wilcock 2010; Hanke and Kielian 2011; Nimmerjahn et al. 2005). Among these factors, some are anti-inflammatory and neuroprotective and contribute to tissue repair. However, others have proinflammatory activities, exhibit detrimental effects, and are toxic to neurons. Thus, it is critical to balance the pro- and anti-inflammatory

responses, thereby inhibiting the potentially harmful effects of dysregulated inflammatory responses on vulnerable neurons.

There is functional heterogeneity in the activation states of microglial cells, with distinct expression profiles, morphologies, and effector function (Martinez et al. 2009; Ransohoff and Perry 2009). Therefore, it is critical to determine the exact role that microglial activation, as well as each phenotype, plays in neurodegenerative disorders. In the present study, we observed that PrP106-126 induced microglial activation with overexpression of proinflammatory cytokines, including IL-1β, iNOS, and TNF-α, which are known to be neurotoxic. However, TLR2 deficiency reduced expression of proinflammatory cytokines, but enhanced the transcription of IL-10 and TGF-β in microglial cells induced by PrP106-126, as well as transcription of Arg1 and Mrc1, indicating that the microglial phenotype are polarized to an anti-inflammatory, neuroprotective status. By expressing and releasing neurotrophic proteins such as arginase 1 and IL-10 (Kuo et al. 2011; Ma et al. 2010), this microglial phenotype facilitates neurotoxic inflammatory resolution (Martinez et al. 2009) and promotes neuronal protection and regeneration. Considering previous and present findings, it is likely that TLR2−/− microglial cells activated by PrP106-126 have neuroprotective functions.

The mechanisms through which PrP106-126 engages microglial TLR2 are unknown. Aβ1-42 can bind to TLR2 on the surface of microglial cells (Liu et al. 2012). Considering the similarities between PrP106-126 and Aβ1-42 aggregates, PrP106-126 may directly bind to TLR2. Conversely, our previous findings indicated that PrP106-126 is associated or interacts with some cell surface receptors, such as α5β1-integrin (Chang et al. 2012), the scavenger type B receptor CD36 (Kouadir et al. 2012), which suggests that a cell surface receptor complex mediates interactions between microglial cells and neurotoxic PrPs. More importantly, disrupting interactions between PrP106-126 and any component of the receptor complex will inhibit PrP106-126 actions. Therefore, TLR2 may be part of the receptor complex that participates in PrP106-126-induced microglial activation.

Previous findings demonstrate that RAGE and TLRs share several common ligands, including HMGB1, the bacterial cell wall component LPS, β-sheet fibrils including serum amyloid A, and amyloid β. RAGE also appears to interact with TIR domain-containing adaptor protein (TIRAP) and MyD88, both of which are intracellular adaptor proteins that TLRs use to activate downstream signaling pathways (Ibrahim et al. 2013). We demonstrated that RAGE mediates PrP106-126-induced NF-κB activation (data unpublished). Thus, it is possible thar PrP106-126 binds to RAGE, which may associate or interact with TLR2. However, the current understanding of the mechanisms underlying RAGE-TLR cross talk at the receptor level is limited and important questions remain to be addressed.

CD14 can form clusters with TLR2-TLR6 heterodimers (Ofodile. 2011; Trudler et al. 2010; Oliveira-Nascimento et al. 2012; Hanke and Kielian 2011). Sakai et al. (2013) showed that increased microglial activation in prion-infected CD14−/− occurs concomitantly with increased anti-inflammatory cytokine expression, including IL-10. Thus, TLR2 might transduce cellular signaling through CD14, thereby contributing to microglial adhesion and recognition of PrP106-126. Our groups are working to further delineate such mechanisms.

In conclusion, we showed that PrP106-126 induced microglial activation via the TLR2/NF-κB pathway, and inhibiting TLR2 signaling shifted microglial activation from a neurotoxic to a neuroprotective state. Nevertheless, these preliminary findings need to be confirmed by further in vitro and in vitro investigation. Our results indicate a previously unidentified role for the TLR2 signaling pathway in modulating PrP106-126-induced microglial activation and provide new perspectives for therapeutic strategies that target TLR2-mediated signaling pathways.

Conflict of interest The authors declare no financial or commercial conflicts of interest.

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