

PYNOD reduces microglial inflammation and consequent neurotoxicity upon lipopolysaccharides stimulation

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Received May 1, 2017; Accepted March 28, 2018

DOI: 10.3892/etm.2018.6108

Abstract. PYNOD, a nod-like receptors (NLR)-like protein, was indicated to inhibit NF- κ B activation, caspase-1-mediated interleukin (IL)-1 β release and cell apoptosis in a dose-dependent manner. Exogenous addition of recombinant PYNOD to mixed glial cultures may suppress caspase-1 activation and IL-1 β secretion induced by A β . However, to the best of our knowledge, there no study has focused on the immunoregulatory effects of PYNOD specifically in microglia. The present study aimed to explore the roles of PYNOD involved in the lipopolysaccharides (LPS)-induced microglial inflammation and consequent neurotoxicity. Murine microglial BV-2 cells were transfected with pEGFP-C2-PYNOD (0-5.0 μ g/ml) for 24 h and incubated with or without LPS (1 μ g/ml) for a further 24 h. Cell viability was determined using MTT assay and the secretion of nitric oxide (NO), IL-1 β and caspase-1 was measured using the Griess method or ELISA. Protein expression levels of NF- κ B p65 and inducible nitric oxide synthase (iNOS) were detected by immunofluorescent staining and/or western blot analysis. Co-culture of BV-2 cells with human neuroblastoma cell line SK-N-SH was performed in Transwell plates and the cell viability and apoptosis (using flow cytometry) of SK-N-SH cells were determined. Results indicated that PYNOD overexpression inhibited NO secretion and iNOS protein expression induced by LPS in BV-2 cells, with no detectable cytotoxicity. PYNOD overexpression also reduced the secretion of IL-1 β and caspase-1 from BV-2 cells upon LPS stimulation. These effects were dose-dependent.

Additionally, PYNOD overexpression prevented LPS-induced nuclear translocation of NF- κ B p65 in BV-2 cells. The growth-inhibitory and apoptosis-promoting effects of BV-2 cells towards SK-N-SH cells were alleviated as a result of PYNOD overexpression. In conclusion, PYNOD may mitigate microglial inflammation and consequent neurotoxicity.

Introduction

Proteins with a nucleotide-binding oligomerization domain (NOD) and leucine-rich repeats (LRRs) are referred to as nod-like receptors (NLRs) and are involved in inflammation and apoptosis (1,2). Most NLRs have critical functions in sensing pathogenic organisms or other inducers of inflammation. Indeed, Nod1 and Nod2 promote NF- κ B activation by interacting with RICK/RIP2 (3). CARD12, NALP1, and PYPAF promote NF- κ B activation, caspase-1-associated interleukin (IL)-1 β synthesis, and/or apoptosis (4). On the other hand, PAN2 binds IKK α to reduce tumor necrosis factor (TNF)- α and IL-1 β -related NF- κ B activation (5). And PYPAF3 abrogates the secretion of IL-1 β by macrophages (6).

Recent years, several studies have reported a new NLR-like protein with anti-inflammatory effects (7), comprising pyrin domain (PYD) and NOD sequences but lacking LRRs; it was termed PYNOD (also named NLRP10 and NALP10) (8). Previous studies revealed that PYNOD binds to an adaptor protein, apoptosis-associated speck-like protein containing a CARD (ASC), and inhibits ASC-mediated NF- κ B activation and apoptosis; PYNOD also suppresses caspase-1-mediated IL-1 β maturation and secretion; and the effects are dose-dependent (8,9). PYNOD has been shown to be essential to the functions of immunocompetent cells such as dendritic cells (10-13), macrophages and neutrophils (8,9).

Microglia are immunocompetent cells of the brain and the first line effector cells responsible for immune reactions in the central nervous system (CNS) (14-16). Activation of microglia causes the secretion of multiple immune effectors, including nitric oxide (NO) via the inducible nitric oxide synthase (iNOS) and pro-inflammatory cytokines, e.g., IL-1 β , IL-6, and TNF- α , which restore CNS homeostasis by cell and debris removal (17-19). Microglia can activate the NF- κ B pathway during neuroinflammation (20-22). Taken together,

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Key words: PYNOD, microglial inflammation, neurotoxicity, nitric oxide release, caspase-1-mediated IL-1 β release, NF- κ B nuclear translocation

microglia activation results in chronic neuroinflammation and increased neuronal injury, and eventually neuronal death. The death of neurons usually follows microglia activation in multiple CNS diseases such as Alzheimer's disease (AD) (23), Parkinson's disease (PD) (24,25), and amyotrophic lateral sclerosis (ALS) (26). Microglia-related neurotoxicity represents an important mechanism for neurodegeneration and reduced neuron function. Therefore, suppressing microglia induction is considered a therapeutic tool in neurodegenerative diseases (27).

A previous study showed that the degradation of PYNOD in the cytoplasm of mixed glial cells allowed the formation of the inflammasome, while exogenous addition of recombinant PYNOD to the glial cultures could reduce A β -induced caspase-1 activation and IL-1 β release (28). So far, studies had only examined mixed glial cells and there is no study focusing on the immunoregulatory effects of PYNOD specifically in microglia. Whether PYNOD suppresses microglia activation under inflammatory conditions remains unknown.

Therefore, this study aimed to explore how PYNOD regulates the inflammatory signaling in microglia upon lipopolysaccharides (LPS) stimulation and how it affects microglia-induced neurotoxicity, using BV-2 cell line (raf/myc-immortalised murine neonatal microglia) as a substitute for primary microglia (29) and human neuroblastoma cell line SK-N-SH for neuronal cells (30).

Materials and methods

Cell culture and grouping. Murine microglial cell line BV-2 and human neuroblastoma cell line SK-N-SH were obtained from the Chinese Academy of Sciences (Beijing, China) and cultured in Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin (all from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), at 37°C in a humid environment containing 5% CO₂.

BV-2 cells cultured in 24-well plates (6x10⁴ cells/ml) or on glass coverslips (for immunofluorescent staining) were subjected to transfection of pEGFP-C2-PYNOD (0, 0.5, 2.5, and 5.0 μ g/ml; Takara Biotechnology Co., Ltd., Dalian, China) for 24 h, followed by incubation with or without LPS (1 μ g/ml; Sigma-Aldrich, St. Louis, MO, USA) for another 24 h; the only exception was that to detect the protein expression and distribution of NF- κ B p65, only 1 h was taken for LPS treatment). Then cell viability was determined; the levels of NO, IL-1 β and caspase-1 in supernatant of cell culture were measured; and the protein expressions of NF- κ B p65 and iNOS were detected by immunofluorescent staining and/or western blot analysis.

And a co-culture in transwell plates was performed to assess the effects of microglia on neuronal cells *in vitro*, according to a previous study (31). BV-2 cells were grown in culture inserts and were subjected to transfection of pEGFP-C2-PYNOD (0 or 5.0 μ g/ml) for 24 h, followed by incubation with or without LPS (1 μ g/ml) for another 24 h. SK-N-SH cells were then added into the wells. The co-culture lasted for 24 h. Then cell viability and apoptosis of SK-N-SH cells were determined after co-culture.

Cell viability. Cell viability was quantified using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium

bromide (MTT) assay. After removal of the medium, 0.5 mg/ml of MTT solution in medium was added for 3 h. Then, the supernatants were carefully aspirated, and formazan crystals were dissolved with DMSO; absorbance was measured at 540 nm.

Analysis of cell apoptosis using flow cytometry. Detection of apoptotic cells was performed using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay using an Annexin V-FITC/PI apoptosis detection kit (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Briefly, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide (PI) for 15 min at 4°C in the dark. Cell cycle distribution was quantified by flow cytometry.

NO level measurement. The supernatant of BV-2 cell culture was obtained, and NO amounts were determined by the Griess method. The supernatants were added with equal amounts of Griess reagent (Promega Corporation, Madison, WI, USA) and kept at room temperature for 10 min. Optical density was measured at 540 nm. A standard curve was obtained with sodium nitrite at 10-100 M.

Enzyme-linked immunosorbent assay (ELISA). The supernatant of BV-2 cell culture was obtained, and IL-1 β (cat. no. PMLB00C; R&D Systems, Inc., Minneapolis, MN, USA) and caspase-1 (cat. no. K111-25; BioVision, Inc., Milpitas, CA, USA) amounts were measured by specific ELISA kits according to the manufacturer's instructions. Optical density was measured at 450 nm. According to the manufacturer, the IL-1 β kit had a sensitivity of 2.31 pg/ml, an intra-assay coefficient of variation (CV) of 3.0-7.5% and an inter-assay CV of 5.7-8.4%.

Immunofluorescent staining. BV-2 cells were fixed with 3.7% paraformaldehyde. After permeabilization (0.2% Triton X-100) and blocking (2% bovine serum albumin; Sigma-Aldrich), the samples were treated with rabbit polyclonal anti-NF- κ B p65 primary antibodies (1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and Cy5-labelled goat anti-rabbit secondary antibodies (1:5,000; Sigma-Aldrich). The co-staining of nucleus by Hoechst 33258 was performed. Analysis was carried out under a laser scanning confocal microscope (Carl Zeiss AG, Oberkochen, Germany).

Western blot analysis. Cytoplasmic and nuclear proteins were extracted with NE-PERTM nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific, Inc.). The BV-2 cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Total protein amounts were determined. Electrophoresis was performed with 25 μ g of proteins loaded in each lane, followed by electro-transfer onto polyvinylidene difluoride (PVDF) membranes. After blocking in 5% nonfat milk in Tris-buffered saline (TBS)/0.1% Tween-20, the samples were incubated with primary antibodies (rabbit anti-mouse iNOS, p65, and GAPDH polyclonal antibodies; all at 1:1,000) overnight at 4°C in blocking buffer. After thorough washing with phosphate buffered saline Tween-20

(PBST), horseradish peroxidase (HRP)-conjugated secondary goat anti-mouse antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA) were added for 1 h at room temperature. Immunoreaction was detected using an enhanced chemiluminescence detection system. The amounts of objective proteins were normalized to that of GAPDH.

Statistical analysis. All statistical analyses were performed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). The normal distribution of the data was tested using the Kolmogorov-Smirnov test and all data were found to be normally distributed. Data were presented as mean \pm standard deviation of three independent experiments. Statistical analysis was done using one-way analysis of variance followed by Tukey's post hoc test. $P < 0.05$ indicated statistically significant differences.

Results

PYNOD concentration-dependently decreases NO production without cytotoxicity in BV-2 microglial cells upon LPS stimulation. To explore how PYNOD affects the NO production in microglia under inflammatory conditions, BV-2 cells were overexpressed with pEGFP-C2-PYNOD plasmid, followed by LPS stimulation. PYNOD concentration-dependently reduced the NO secretion from BV-2 cells induced by LPS, with statistically significant differences (both $P < 0.05$; Fig. 1A). Furthermore, it was found that LPS stimulation increased the iNOS protein expression in BV-2 cells, which could be suppressed by PYNOD-overexpression, also in a dose-dependent manner (both $P < 0.05$; Fig. 2). Whether such inhibitory effect of PYNOD resulted from cytotoxicity was then examined. PYNOD-overexpression lead to no changes in the growth of BV-2 cells (Fig. 1B), suggesting that PYNOD reduced the NO production independently of cytotoxicity in BV-2 microglial cells upon LPS stimulation.

PYNOD concentration-dependently reduces the secretion of IL-1 β and caspase-1 from BV-2 microglial cells upon LPS stimulation. IL-1 β secretion from BV-2 microglial cells was significantly higher (551.5 pg/ml) after LPS stimulation ($P < 0.05$, Fig. 3A); however, the increase of IL-1 β secretion was inhibited by PYNOD overexpression (both $P < 0.05$, Fig. 3A), declining to 340.6 pg/ml (2.5 μ g/ml pEGFP-C2-PYNOD transfection) or 250.3 pg/ml (5.0 μ g/ml pEGFP-C2-PYNOD transfection). Caspase-1 amount in the supernatant of BV-2 cell culture was 7.62-fold higher after LPS stimulation ($P < 0.05$; Fig. 3B), while it was significantly suppressed by PYNOD overexpression to lower level, about 5.47-fold or 3.32-fold of the un-stimulated control (2.5 or 5.0 μ g/ml pEGFP-C2-PYNOD transfection) (both $P < 0.05$; Fig. 3B).

PYNOD prevent the nuclear translocation of NF- κ B in BV-2 microglial cells upon LPS stimulation. The protein amount of NF- κ B p65 in nucleus of BV-2 cells was high after LPS stimulation (Fig. 4A), which was significantly decreased by PYNOD overexpression ($P < 0.05$; Fig. 4A). And immunofluorescent staining confirmed that the intracellular distribution of NF- κ B p65 was restricted in nuclear or perinuclear regions (Fig. 4B); however, PYNOD overexpression resulted in cytoplasmic distribution of NF- κ B p65. The results suggested that PYNOD

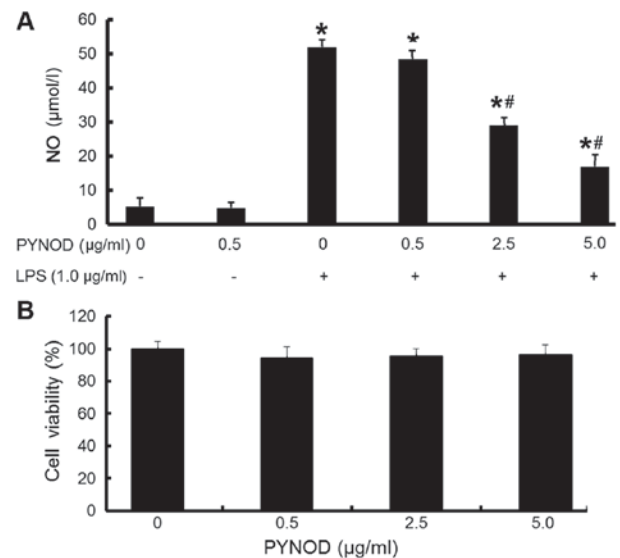


Figure 1. PYNOD decreases NO secretion in LPS-stimulated BV-2 microglial cells in a concentration-dependent manner. (A) Murine microglial cell line BV-2 were transfected with pEGFP-C2-PYNOD (0, 0.5, 2.5 and 5.0 μ g/ml) for 24 h and then subjected to LPS treatment (1.0 μ g/ml) for another 24 h. NO level in the supernatant of cell culture was measured using the Griess method. (B) BV-2 cells were transfected with pEGFP-C2-PYNOD (0, 0.5, 2.5 and 5.0 μ g/ml) for 24 h. Cell viability was determined using MTT assay. The results were expressed as mean \pm standard deviation of three independent experiments. * $P < 0.05$, compared with the un-stimulated control group; # $P < 0.05$, compared with the LPS treatment plus no transfection group. NO, nitric oxide; LPS, lipopolysaccharides.

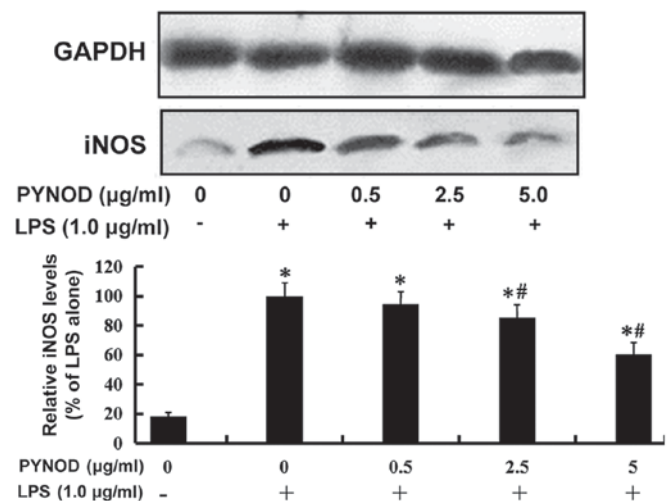


Figure 2. PYNOD decreases the protein expression of iNOS in LPS-stimulated BV-2 microglial cells in a concentration-dependent manner. BV-2 cells were transfected with pEGFP-C2-PYNOD (0, 0.5, 2.5 and 5.0 μ g/ml) for 24 h and then subjected to LPS treatment (1.0 μ g/ml) for another 24 h. 25 μ g total protein extracted from the cells was subjected to western blot analysis. Relative protein expression of iNOS in each group was quantified with GAPDH as the internal control. The results were expressed as mean \pm standard deviation of three independent experiments, and normalized to the LPS treatment plus no transfection group. * $P < 0.05$, compared with the un-stimulated control group; # $P < 0.05$, compared with the LPS treatment plus no transfection group. iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharides.

might prevent the nuclear translocation of NF- κ B p65 in BV-2 cells upon LPS stimulation, thus inhibiting the generation of inflammatory singling.

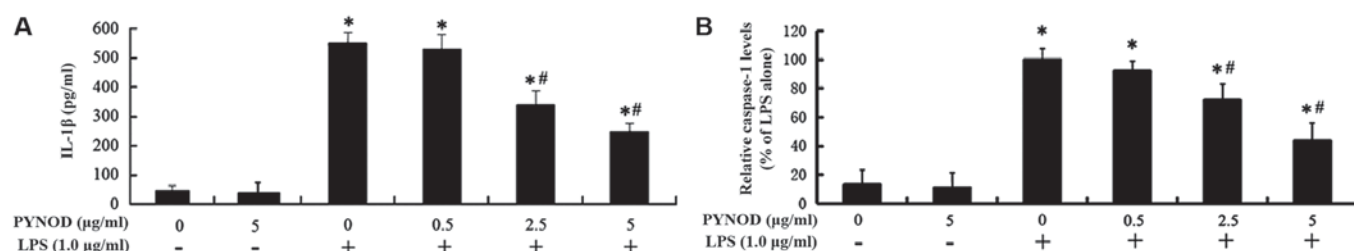


Figure 3. PYNOD reduces the secretion of IL-1 β and caspase-1 in LPS-stimulated BV-2 microglial cells in a concentration-dependent manner. BV-2 cells were transfected with pEGFP-C2-PYNOD (0, 0.5, 2.5 and 5.0 μ g/ml) for 24 h and then subjected to LPS treatment (1.0 μ g/ml) for another 24 h. The supernatant of cell culture was assessed for (A) IL-1 β and (B) caspase-1 amounts. The results were expressed as mean \pm standard deviation of three independent experiments, and the results in (B) were normalized to the LPS treatment plus no transfection group. * P <0.05, compared with the un-stimulated control group; # P <0.05, compared with the LPS treatment plus no transfection group. IL, interleukin; LPS, lipopolysaccharides.

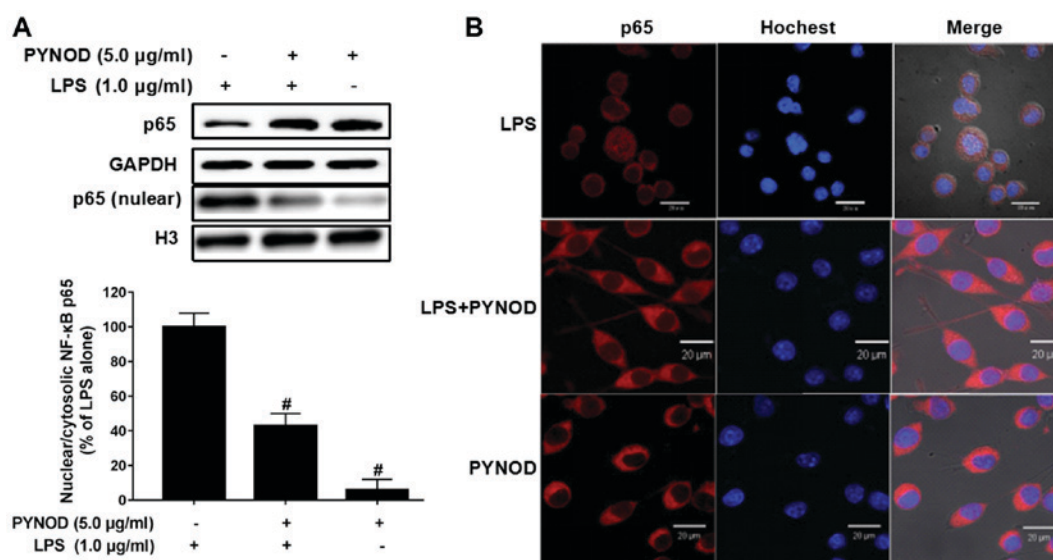


Figure 4. PYNOD prevents the nuclear translocation of NF- κ B in LPS-stimulated BV-2 microglial cells. BV-2 cells were transfected with pEGFP-C2-PYNOD (0, 0.5, 2.5 and 5.0 μ g/ml) for 24 h and then subjected to LPS treatment (1.0 μ g/ml) for another 1 h. (A) Nuclear protein and cytoplasmic protein were respectively extracted from the cells, and assessed by western blot for NF- κ B p65 detection; GAPDH was used as an internal control. (B) Immunofluorescent staining was performed to detect the cellular distribution of NF- κ B p65 (red). Hoechst 33258 (blue) was used to stain the nuclei. Scale bar, 20 μ m. The results were expressed as mean \pm standard deviation of three independent experiments, and the results in (A) were normalized to the LPS treatment plus no transfection group. # P <0.05, compared with the LPS treatment plus no transfection group. LPS, lipopolysaccharides.

PYNOD alleviated the neurotoxic effects of microglia upon LPS stimulation. There was a decrease of cell viability (P <0.05; Fig. 5A) and an increase of apoptosis (P <0.05; Fig. 5B) in human neuroblastoma SK-N-SH cells when the co-cultured BV-2 cells had been subjected to LPS stimulation. However, these changes in SK-N-SH cells were significantly alleviated (both P <0.05; Fig. 5A and B) if the co-cultured BV-2 cells had been overexpressed with PYNOD before LPS stimulation. Notably, PYNOD overexpression in BV-2 cells without LPS stimulation would never affect the cell viability or cell apoptosis of co-cultured SK-N-SH cells.

Discussion

This study aimed to explore the roles of PYNOD involved in the LPS-induced microglial inflammation and consequent neurotoxicity. The results showed that PYNOD overexpression inhibited NO release and iNOS protein expression induced by LPS in BV-2 cells, independently of cytotoxicity. It also reduced the secretion of IL-1 β and caspase-1 from BV-2 cells upon

LPS stimulation. The effects were dose-dependent. PYNOD overexpression prevented LPS-induced nuclear translocation of NF- κ B p65 in BV-2 cells. The growth-inhibitory and apoptosis-promoting effects of BV-2 cells towards SK-N-SH cells were alleviated as a result of PYNOD overexpression.

Increased amounts of NO produced by iNOS in induced microglia are considered to be cytotoxic in CNS inflammation, and related to neurodegenerative disease etiology (32). Indeed, agents inhibiting iNOS expression carry potential benefits in the treatment of high-NO-related inflammatory disorders (33). Pro-inflammatory cytokines, like IL-1 β , are produced in large amounts by microglia after stimulation by molecules such as IFN- γ and LPS during the inflammatory process, constituting potential factors causing neurological diseases (34). Caspase-1 is known to cleave pro-IL-1 β for activation, a process critical to IL-1 β secretion. A previous study showed that treating mixed glial cells with recombinant PYNOD led to reduced caspase-1 activation and IL-1 β secretion (28). The present study was carried out using microglial cells (which are included among the mixed glial cells) and

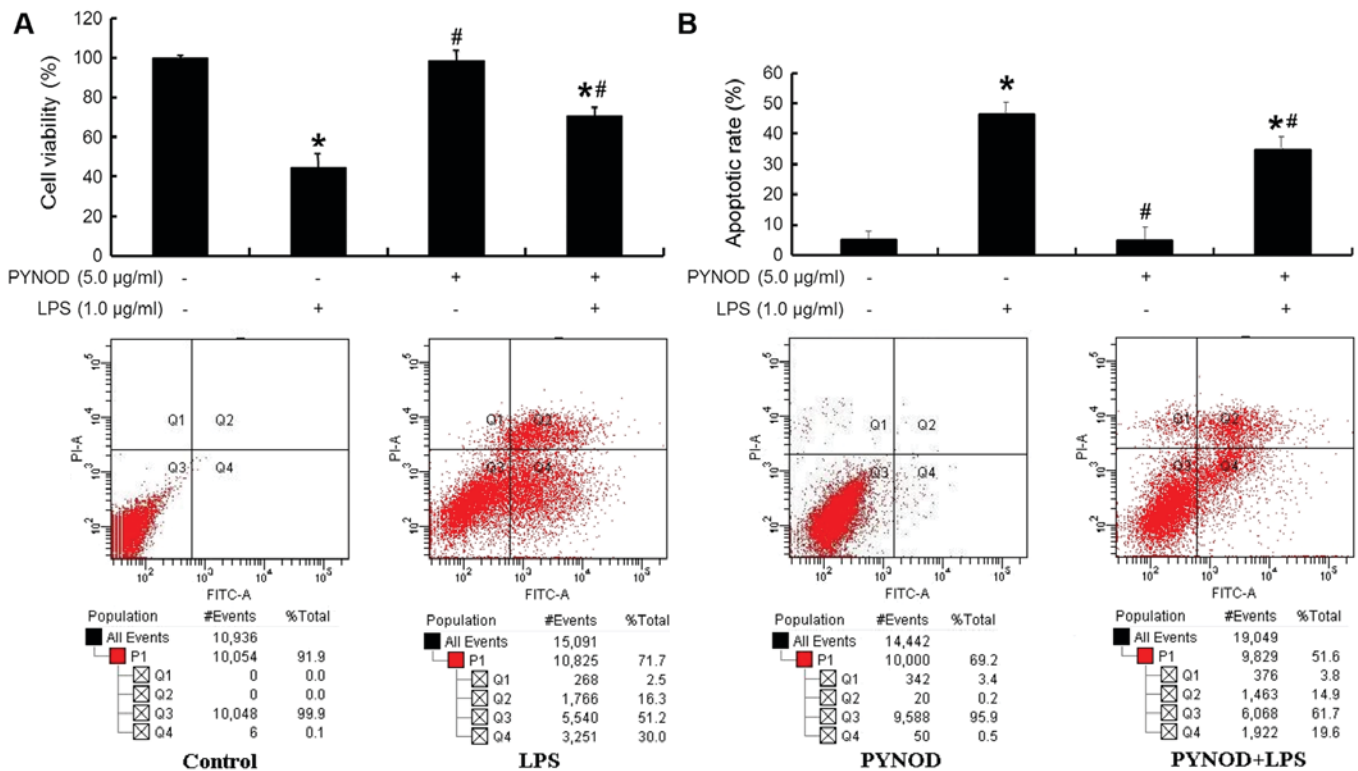


Figure 5. PYNOD alleviated the neurotoxic effects of microglia upon LPS stimulation. A co-culture assay in Transwell plates was performed: BV-2 cells were subjected to transfection of pEGFP-C2-PYNOD (0 or 5.0 $\mu\text{g/ml}$) for 24 h, followed by incubation with or without LPS (1 $\mu\text{g/ml}$) for another 24 h. Then the human neuroblastoma cell line SK-N-SH was added for 24 h co-culture. (A) Viability of SK-N-SH cells was determined by the MTT assay. (B) Apoptosis level of SK-N-SH cells was determined by flow cytometry. The results were expressed as mean \pm standard deviation of three independent experiments. * $P < 0.05$, compared with the un-stimulated control group; # $P < 0.05$, compared with the LPS treatment plus no transfection group. LPS, lipopolysaccharides.

showed that PYNOD reduced LPS-induced production of IL-1 β and caspase-1 in a dose-dependent manner. The present study implied that PYNOD modulates the secretion of the pro-inflammatory cytokine IL-1 β after microglia activation through caspase-1 inhibition.

NF- κ B plays key roles in multiple pathologies and represents a main drug target for various diseases (35,36). The promoter region of the mouse gene encoding iNOS harbors an NF- κ B binding site, and NF- κ B induction is key in NO and iNOS production in LPS-stimulated microglia (37). As shown above, PYNOD prevented the nuclear translocation of LPS-stimulated p65 in LPS-treated BV-2 microglial cells, revealing a potential role for NF- κ B in PYNOD's suppressive effects on inflammatory effectors such as NO and IL-1 β .

Microglial activation is deleterious to neurons, causing apoptosis (19). Neurotoxic microglial-neuronal interactions are considered to be important in the development of neurological diseases (38). Our results showed that neurons co-cultured with LPS-treated BV-2 microglial cells showed enhanced cell death and apoptosis. Nevertheless, transfection with PYNOD in LPS-stimulated co-cultures resulted in higher cell viability and less apoptotic cells. These results suggested a beneficial effect of PYNOD on microglia neurotoxicity in co-cultures. Thus, neuroprotection by PYNOD might result from its effects on microglia. Nevertheless, other cells in the brain may also be affected by PYNOD. Indeed, PYNOD is expressed in neurons and its expression is increased in injured neurons (39). NLRs (including PYNOD) play important roles in the cerebral endothelial cells and the maintenance

of the brain-blood barrier under immune and inflammatory processes (40). NLRs (again including PYNOD) also play roles in the non-canonical inflammasome activation in pericytes (41). Taken together, these results strongly suggest that PYNOD is involved in the neuroinflammatory process of many cell types in the CNS. Additional studies are necessary to determine its exact roles in the brain and how these different cell types interact together in neuroinflammation. However this protein was recently discovered and none of the inhibitors against it, so activators/inhibitors of downstream signalling molecules (such as IL-1 β , NF- κ B) will need to be demonstrate the specific effects of PYNOD. In addition, knock-out models and PYNOD-transgenic mice will be necessary to delineate the exact roles of PYNOD.

Taken together, the present study revealed that PYNOD could mitigate microglial inflammation and consequent neurotoxicity. Therefore, PYNOD might be therapeutically used as an anti-inflammatory drug target suppressing excessive microglia activation associated with exacerbated neuronal cell death under brain injury conditions.

Acknowledgements

Not applicable.

Funding

The present study was supported by grants from the National Natural Science Foundation of China (grant

no. 81441046), Science Foundation of Jiangxi Province (grant no. 20142BAB215055), and Science and Technology Commission of Jiangxi Province Health Department (grant no. 20143124).

Availability of data and materials

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

Authors' contributions

QZ contributed to the study design, data acquisition and analysis and drafted the manuscript; CH was involved in data acquisition and revision of the manuscript; RQ was involved in data acquisition and analysis; DL worked on aspects of the study design, data acquisition and analysis and revised the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflict of interests

The authors declare that they have no competing interests.

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