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Original article

Stem-leaf saponins of *Panax notoginseng* attenuate experimental Parkinson's disease progression in mice by inhibiting microglia-mediated neuroinflammation *via* P2Y2R/PI3K/AKT/NFκB signaling pathway

Hui Wu, Chenyang Ni, Yu Zhang, Yingying Song, Longchan Liu, Fei Huang*, Hailian Shi, Zhengtao Wang, Xiaojun Wu*

Shanghai Key Laboratory of Compound Chinese Medicines, The Ministry of Education (MOE) Key Laboratory for Standardization of Chinese Medicines, Institute of Chinese Materia Medica, The MOE Innovation Centre for Basic Medicine Research on Qi-Blood TCM Theories, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China

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ABSTRACT

Stem-leaf saponins from *Panax notoginseng* (SLSP) comprise numerous PPD-type saponins with diverse pharmacological properties; however, their role in Parkinson's disease (PD), characterized by microglia-mediated neuroinflammation, remains unclear. This study evaluated the effects of SLSP on suppressing microglia-driven neuroinflammation in experimental PD models, including the 1-methyl-4-phenylpyridinium (MPTP)-induced mouse model and lipopolysaccharide (LPS)-stimulated BV-2 microglia. Our findings revealed that SLSP mitigated behavioral impairments and excessive microglial activation in models of PD, including MPTP-treated mice. Additionally, SLSP inhibited the upregulation of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX2) and attenuated the phosphorylation of PI3K, protein kinase B (AKT), nuclear factor-κB (NFκB), and inhibitor of NFκB protein α (IκBα) both *in vivo* and *in vitro*. Moreover, SLSP suppressed the production of inflammatory markers such as interleukin (IL)-1β, IL-6, and tumor necrosis factor alpha (TNF-α) in LPS-stimulated BV-2 cells. Notably, the P2Y2R agonist partially reversed the inhibitory effects of SLSP in LPS-treated BV-2 cells. These results suggest that SLSP inhibit microglia-mediated neuroinflammation in experimental PD models, likely through the P2Y2R/PI3K/AKT/NFκB signaling pathway. These novel findings indicate that SLSP may offer therapeutic potential for PD by attenuating microglia-mediated neuroinflammation.

1. Introduction

Parkinson's disease (PD) is an age-related neurodegenerative disorder characterized by the degeneration and loss of dopaminergic neurons in the substantia nigra pars (SNpc) [1]. Despite its discovery several decades ago, curative therapy remains elusive, and its pathogenesis is not fully understood. The pathophysiology of PD involves various pathways and mechanisms, including neuroinflammation, oxidative stress, glial cell activation, and mitochondrial dysfunction [2]. As our understanding of PD's pathological mechanisms deepens, the role of the inflammatory response in this disease has gained significant attention [3]. Neurotoxins and inflammatory factors secreted by activated microglia are identified as key contributors to the dysfunction of dopamine secretion by dopaminergic neurons in PD, thus promoting disease progression [4-7].

Microglia serve as the primary immune cells in the central nervous system, playing a crucial role in immune surveillance. Their functions encompass phagocytosis, proinflammatory cytokine release, and dead cell clearance. However, their response to injury or infection can trigger neuroinflammation, potentially leading to various neurological disorders. In their resting state, microglia exhibit small cell bodies with highly branched processes [8]. Upon activation, these cells undergo morphological changes, including enlarged cell bodies and shortened, thickened protrusions, gradually transforming into amoeboid-like activated macrophages without protrusions [9]. Activated microglia release inflammatory mediators and peroxidative neurotoxic substances, such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, IL-1β, and nitric oxide (NO), which induce nervous tissue necrosis [10]. In response to inflammatory stimuli, cyclooxygenase-2 (COX2) and induced NO-synthase (iNOS) are upregulated in microglia-mediated neuroinflammation [11]. Gene knockout studies blocking the expression of inflammatory-related proteins like COX2 [12, 13] or iNOS [14-16] have demonstrated potential neuroprotective effects on dopaminergic neurons in PD models. Moreover, research indicates that inhibiting microglia-mediated neuroinflammation can improve the environment for dopaminergic neurons, thereby producing protective effects and mitigating PD symptoms [17-19]. Consequently, suppressing neuroinflammation associated with microglial activation may represent an effective strategy for treating Parkinson's disease.

In addition to exogenous factors triggering microglia-mediated inflammation, nucleotides such as UTP and ATP released by

* Corresponding author.
E-mail addresses: Fei_H@hotmail.com (F. Huang); xiaojunwu320@126.com (X. Wu)

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apoptotic neurons and activated glia can induce inflammation through purine receptors [20, 21]. The P2Y2 receptor (P2Y2R) has been implicated in multiple inflammatory reactions. ATP and UTP stimulate P2Y2Rs found in astrocytes and microglia, leading to the initiation of Rho and Rac-dependent integrin activation, promoting glial cell migration. Moreover, P2Y2 receptor (P2Y2R) activation enhances microglial responses associated with neuroinflammation [22]. An elevated level of inflammatory cytokine IL-1β in Alzheimer's disease has been observed to coincide with the upregulation of P2Y2R [23]. These findings suggest a potential role for P2Y2R in the pathogenesis of neuroinflammatory disorders mediated by microglia. However, further studies are necessary to validate this conclusion.

Panax notoginseng (Burk) F. H. Chen is a Chinese medicinal herb highly valued for its therapeutic properties. The roots of *P. notoginseng* are widely utilized as a herbal remedy in Asian countries for treating cardiovascular and cerebrovascular disorders. Saponins constitute the principal active components of the *P. notoginseng* rhizome and are extensively employed in the management of stroke, cerebral ischemia, and cardiovascular pathologies [24-27]. The compositions and contents of the saponins from stem and leaves of *Panax notoginseng* (SLSP) differ from those of the underground parts. SLSP primarily comprise PPD-type saponins such as ginsenosides Rb3, Rb1, Rc and notoginsenoside Fc [28]. Research indicates that SLSP may be effective in treating depression and anxiety disorders [29]. Additionally, SLSP treatment has been proposed as a method to stimulate neuronal plasticity following localized cerebral ischemic events [30]. Our previous research demonstrated that SLSP protected hippocampal neurons and exerted cardiac protection in mice subjected to sleep deprivation by suppressing abnormal autophagy [31, 32]. However, the effects of SLSP on PD require further investigation. This study aimed to examine the impact of SLSP on the models of PD, including 1-methyl-4-phenylpyridinium (MPTP)-induced mouse model of PD and neuroinflammation in lipopolysaccharide (LPS)-induced BV-2 microglia cells. Furthermore, we explored the molecular mechanisms underlying the anti-inflammatory effects of SLSP.

2. Materials and methods

2.1. Reagents

SLSP were obtained from Qidan Co., Ltd. (purity > 95%, Wenshan, China). The primary saponins in SLSP included notoginsenoside IX (7.72%), ginsenoside Rd (3.04%), notoginsenoside Fa (4.13%), notoginsenoside FP2 (5.59%), ginsenoside Rb3 (17.4%), notoginsenoside Fc (11.8%), ginsenoside Rc (11.1%), and ginsenoside Rb1 (4.86%) as reported in our previous publication [32]. MPTP (purity > 98%, Cat#: ST1020) was supplied by Beyotime Biotech., Inc. (Shanghai, China). Minocycline (Mino, Cat#: MB1477) and Dexamethasone (Dex, Cat#: MB1434) were procured from Dalian Meilun Biotech Co., Ltd. (Dalian, China). LPS (Cat#: L4391) was acquired from Sigma-Aldrich Company (MO, USA). Antibodies against COX2 (Cat#:1282), tyrosine hydroxylase (TH, Cat#: 2791), p-AKT (Cat#: 9271), p-NFκB (Cat#: 3033), phosphoinositol 3-kinase (PI3K) (Cat#: 4257), p-PI3K (Cat#: 4228), IκB (Cat#:4812) and p-IκB (Cat#: 2859) were purchased from Cell Signaling Technology (MA, USA). NFκB (Cat#: A2547), AKT (Cat#: A22770), and GAPDH (Cat#: AC002) were obtained from ABclonal Technology (Wuhan, China). Antibodies against iNOS (Cat#: ab15323) and Iba-1 (Cat#: ab178846) were sourced from Abcam Branch (Shanghai, China). Primers for IL-1β, IL-6, TNF-α, iNOS, COX2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were provided by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). The agonist of P2Y2R (Diquafosol (Diq) tetrasodium, $C_{18}H_{22}N_4Na_4O_{23}P_4$, purity > 99%, CAS: 211427-08-6) was acquired from MedChemExpress Co., Ltd. (Shanghai, China).

2.2. Animals and treatment

Male C57BL/6 mice, aged 11 weeks, were obtained from the Animal Research Center of Shanghai University of Traditional Chinese Medicine (Shanghai, China). The animals were allowed a one-week acclimation period before the commencement of experiments. All mice were housed under controlled environmental conditions, with a temperature maintained at 23 °C and a 12-h light/dark cycle. Food and water were provided *ad libitum*. All animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care, as approved by the Experimental Animal Ethical Committee of Shanghai University of Traditional Chinese Medicine (Approval No: PZSHUTCM-201016011).

The mice were allocated into six groups: control, MPTP, minocycline (50 mg·kg^{-1}), and SLSP (25, 50, and 100 mg·kg^{-1}). The SLSP dosage and administration duration for this study were slightly modified from previous research [32]. The control and MPTP groups received daily saline treatment, while the SLSP groups were administered SLSP *via* gavage for 15 consecutive days. The minocycline group, serving as a positive control, received intraperitoneal injections of minocycline (50 mg·kg^{-1}) for 15 consecutive days. From days 11 to 15, the MPTP, SLSP, and minocycline groups received daily intraperitoneal injections of MPTP at 30 mg·kg^{-1}. The control group was administered an equivalent volume of normal saline solution intraperitoneally once daily.

2.3. Pole test

The pole test is a widely employed experimental method for assessing coordination and muscle function in mice. It is particularly utilized in investigating PD models and evaluating the effects of neurological impairment on motor coordination. Initially, we constructed a vertical pole suitable for mice to ascend. The mice were allowed an acclimatization period to improve their coordination before formal testing commenced. On the 15th day, following intraperitoneal administration of MPTP to each group, the mice underwent behavioral testing on the pole. The time required for mice to complete a full downward movement was recorded to evaluate the extent of bradykinesia in the subjects.

2.4. Immunohistochemistry

The mice were anesthetized, and the chest cavity skin was incised to expose the heart and liver. Initially, the mice were perfused with PBS, followed by 4% paraformaldehyde (PFA). The extracted mouse brains were placed in a 24-well plate containing 4% perfused with 4% PFA and then transferred to 4 °C for overnight fixation. Subsequently, the brains were dehydrated in a 15% sucrose solution for 24 h, followed by a 30% sucrose solution for an additional 24 h. The brains were then embedded in OCT (Yeasen Biotechnology, 36309ES61) and sectioned to 20 μm thickness. The sections were permeabilized with 0.3% Triton-X-100 and blocked with 5% donkey serum for 1 h. The brain sections were then incubated with primary antibodies against TH or Iba-1 at 4 °C overnight. Subsequently, the sections were incubated with secondary antibodies conjugated with Alexa fluorophore for 1 h in dark conditions. Finally, fluorescence images were acquired using an Olympus VS120 Virtual Slide Scanner (Olympus, Tokyo, Japan).

2.5. Cell culture and treatments

The BV-2 microglia cell line, derived from a mouse brain, was

obtained from the ATCC and maintained in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 incubator. Upon reaching appropriate density, the cells were transferred to a medium containing SLSP (50, 100 and 200 μg·mL^{-1}), Dex (10 μmol·L^{-1}), or Diq (10 μmol·L^{-1}) and cultured for 2 h. Subsequently, LPS (200 ng·mL^{-1}) was added for an additional 3 or 22 h treatment.

2.6. Immunocytochemistry

Initially, a 14 mm circle cover glass pre-coated with rat tail collagen was placed into a 24-well plate. BV-2 cells were seeded in the plates and treated with SLSP (50, 100, and 200 μg·mL^{-1}) and Dex (10 μmol·L^{-1}) for 2 h, followed by stimulation with LPS for 6 h. The cells were washed once with incubated PBS, fixed with 4% paraformaldehyde for 20 min, and penetrated with 0.3% Triton-X-100 for 20 min. After blocking with 5% donkey serum for 1 h, the cells were incubated in a solution containing NFκB (1∶200) primary antibody overnight at 4 °C. The following day, the cells were transferred to a solution containing the secondary antibody conjugated with Alexa fluorophore. They were incubated for 1 h before placing the coverslip containing the cells on the slide with DAPI. Finally, the cover glasses were scanned using an Olympus VS120 Virtual Slide Scanner to acquire fluorescence images.

2.7. Measurement of NO release

Upon reaching appropriate cell density, the culture medium was supplemented with SLSP (50, 100, and 200 μg·mL^{-1}), Dex (10 μmol·L^{-1}), or Diq (10 μmol·L^{-1}) for a 2-h treatment period. Following this, LPS (200 ng·mL^{-1}) was introduced to the cell culture and maintained for an additional 22 h. Subsequently, the NO released into the medium was quantified using the methodology described in our previous publication [33].

2.8. Transient transfection of siRNA or plasmid

Upon reaching 70%–80% confluence, BV-2 cells underwent transient transfection with mP2Y2R-PCMV plasmids (0.5 μg/well) using Tenfect DNA transfection reagent, in accordance with the manufacturer's protocol. Alternatively, cells were transfected with P2Y2R siRNA (RiboBio, China) using riboFECT CP transfection reagent as per the manufacturer's instructions. Following a 6 h transfection period, the transfection reagent-containing medium was replaced with a fresh DMEM, and cells were cultured for an additional 18 h. Subsequently, BV-2 cells were treated with LPS (200 ng·mL^{-1}) for 24 h. Post-treatment, the culture medium was collected for the Griess assay, and cells were lysed for Western blotting analysis, as described in the following sections.

2.9. Western blotting analysis

An appropriate volume of RIPA Lysis Buffer containing inhibitor cocktails was applied to the harvested BV-2 cells or mouse SN tissue samples. The specimens were maintained on ice and subsequently lysed *via* ultrasonic disruption for 1 min. The lysed samples underwent centrifugation in a cryo-centrifuge (15 min, 4 °C, 12 000 r·min^{-1}). Protein concentration was determined using the BCA quantification method. The resulting supernatant was utilized for western blotting analysis following established protocols [34]. Immunoreactive bands were visualized using Chemiluminescence Solution, and band images were quantified using FluorChem FC3 software.

2.10. Quantitative PCR

The treated BV-2 cells were collected, and 500 μL of Trizol reagent was added to each sample. Total RNA was extracted using Trizol following the manufacturer's guidelines (Life Technologies, USA). Initially, RNA was transcribed into cDNA using a reverse transcription kit (Cat#: R223, Vazyme Biotech, China). Gene expressions were quantified utilizing a real-time PCR kit (Cat#: Q511, Vazyme Biotech, China). Subsequently, normalization was performed relative to the GAPDH measurement in the same sample. The primer sequences employed are detailed in Table S1.

2.11. Statistical analysis

All data were presented as mean ± standard error of the mean (SEM). Comparisons among groups were determined by one-way analysis of variance (ANOVA) with Dunnett's post-hoc test. Comparison between the two groups was conducted using an unpaired Student's *t*-test. A P value < 0.05 was considered statistically significant.

3. Results

3.1. SLSP protect against MPTP-induced behavioral impairment and dopaminergic neuronal loss in mice

The pole test was conducted to evaluate motor impairments and bradykinesia in mice. A significant increase in total locomotor activity time was observed following MPTP treatment (Fig. 1A, P < 0.001). Conversely, mice pretreated with all three doses of SLSP and the positive control minocycline (P < 0.001) exhibited considerably shorter durations to reach the platform compared to the MPTP group, indicating a reduction in bradykinesia. To further assess the impact of SLSP on dopamine neuron survival in MPTP-induced mice, we examined the expression of TH, a key enzyme in dopamine synthesis, in the striatum and SN. The expression of TH protein in the SN (Figs. 1B and 1C, P < 0.001) and striatum (Figs. 1D and 1E, P < 0.001) of mice in the MPTP group showed a significant reduction compared to the control group, consistent with our prior findings [33, 35]. However, SLSP treatment reversed the decrease in TH protein expression in the SN (Figs. 1B and 1C, P < 0.05 or P < 0.01) and the striatum (Figs. 1D and 1E, P < 0.05 or P < 0.001) of MPTP-induced mice. Similarly, MPTP induced a reduction in TH-positive cells in the SNpc region of mice (Figs. 1F and 1G, P < 0.001) and a significant decrease in TH-immunoreactive neurofilaments in the striatum (Figs. 1H and 1I, P < 0.001). SLSP treatment increased the TH-positive cells in SNpc (Figs. 1F and 1G, P < 0.05 or P < 0.001) and TH-immunoreactive neurofilaments in the striatum (Figs.1H and 1I, P < 0.05 or P < 0.001). Likewise, minocycline reversed the decrease in TH protein expression in the SN (Figs. 1B and 1C, P < 0.05) and striatum (Figs. 1D and 1E, P < 0.001); it also reversed the decrease in TH-positive cells in the SNpc (Figs. 1F and 1G, P < 0.001) and increased TH-immunoreactive neurofilaments in the striatum (Figs. 1H and 1I, P < 0.001).

3.2. SLSP inhibit microglia over-activation in SN of MPTP-treated mice

To assess SLSP's potential in reducing microglia over-activation in PD mice, we examined the expression of Iba-1, a molecular marker for microglia. As illustrated in Figs. 2A and 2B, the MPTP group mice exhibited significantly increased fluorescence intensity of Iba-1-positive cells compared to the control group (P < 0.001). In contrast, the SLSP groups demonstrated markedly lower fluorescence intensity of Iba-1-positive cells than the MPTP group (P < 0.01 or P < 0.001). Furthermore, SLSP treatment mitigated the increase in Iba-1 protein expression in SN (Figs. 2C and 2D, P < 0.01). These results indicate that SLSP significantly inhib-

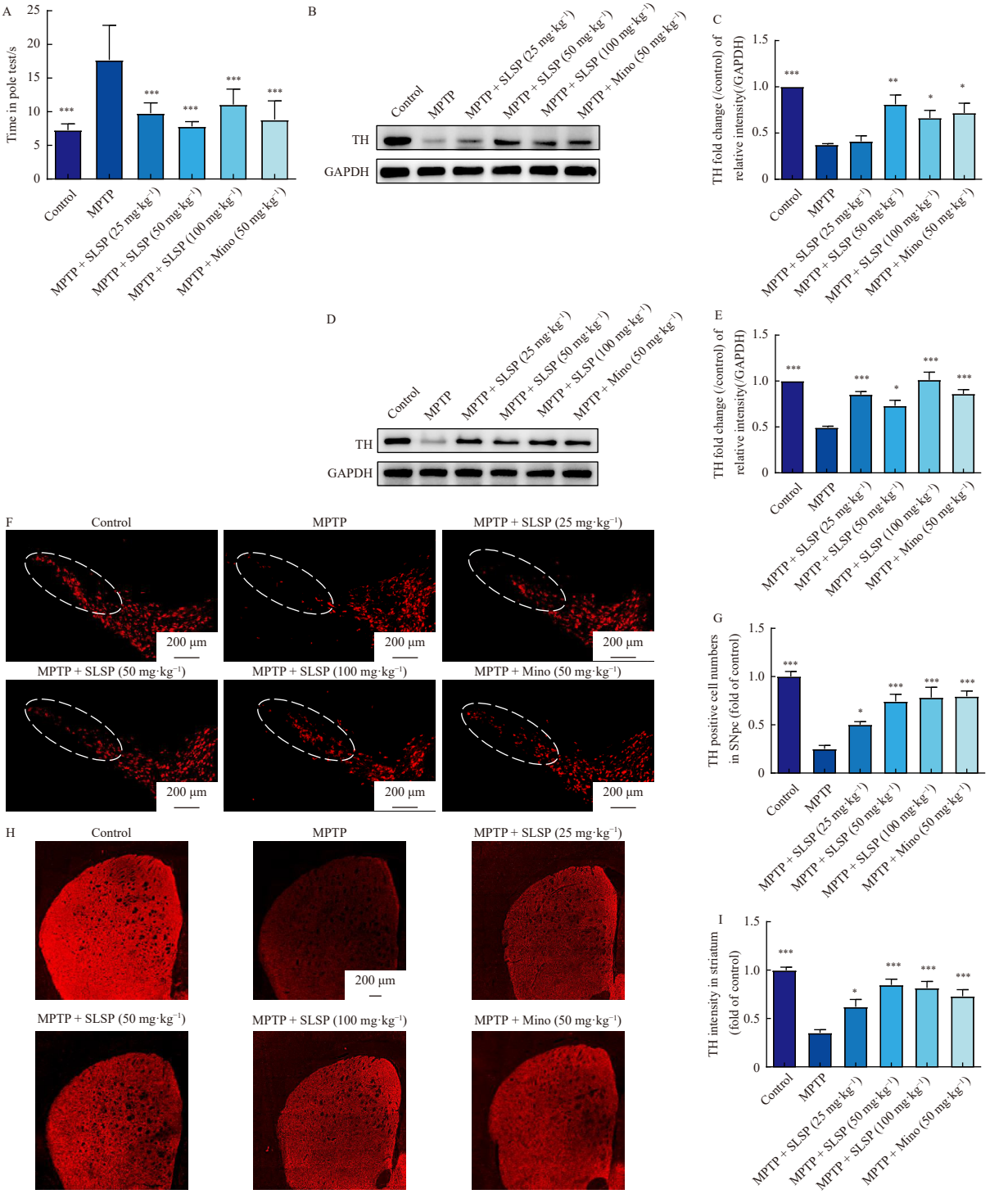

Fig. 1 SLSP protect against MPTP-induced behavioral impairment and dopaminergic neuronal loss in mice. (A) Time duration required for the mice to reach the platform in the pole test (n = 8/group). (B, C) SLSP regulated TH protein expression in SN (n = 4/group). (D, E) SLSP regulated TH protein expression in the striatum (n = 4/group). (F, G) Immunostaining and fluorescent intensity analysis of TH in SNpc (scale bar is 200 μm, n = 4/group). (H, I) Immunostaining and fluorescent intensity analysis of TH in the striatum (scale bar is 200 μm, n = 4/group). Data were presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* MPTP group.

ited microglia overactivation in the SN of MPTP-treated mice. Similarly, minocycline attenuated microglia overactivation in the SN of MPTP-treated mice.

3.3. SLSP reduce the expressions of inflammation-related proteins and modulate the P2Y2R/PI3K/AKT/NFκB signaling pathway in SN of MPTP-induced PD mice

The administration of MPTP resulted in elevated protein levels of iNOS and COX2 in the SN of mice, compared to the control group (Figs. 3A and 3B, $P < 0.05$ or $P < 0.001$). However, SLSP or minocycline significantly inhibited the expression of iNOS and COX2 in the SN of PD mice (Figs. 3A and 3B, $P < 0.05$, $P < 0.01$ or $P < 0.001$). The effects of SLSP on the P2Y2R/PI3K/AKT/NFκB signaling pathway were also examined. As previously reported, P2Y2R activation can induce the phosphorylation of PI3K, AKT, NFκB, and IκB [36-39]. Compared with the control group, the phosphorylation levels of PI3K, AKT, NFκB, and IκB in the SN of mice in the MPTP group were significantly increased (Figs. 3C and 3D, $P < 0.05$, $P < 0.01$ or $P < 0.001$). In contrast, the disturbed phos-

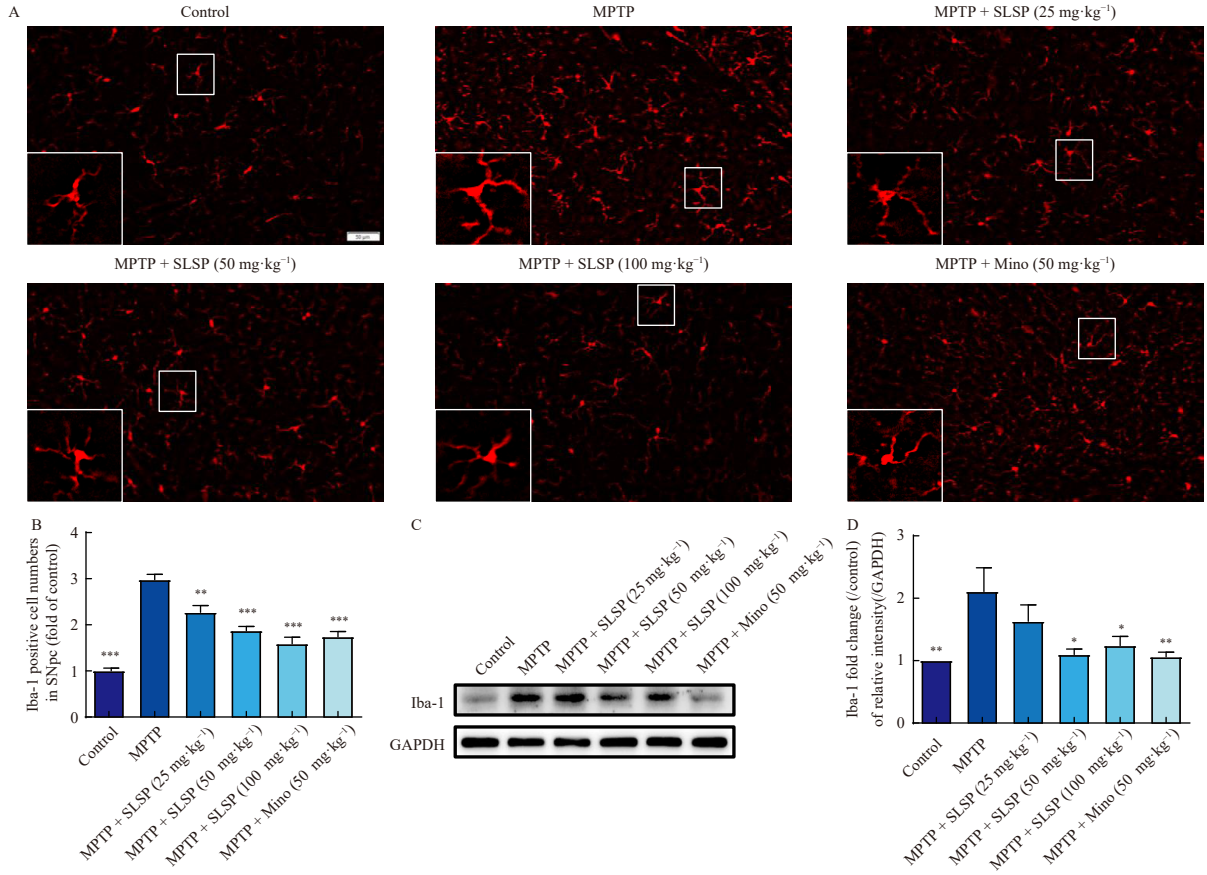

Fig. 2 SLSP inhibit microglia overactivation in SN of MPTP-treated mice. (A, B) Immunostaining and fluorescent intensity analysis of Iba-1 in SNpc (scale bar is 50 μm, n = 4/group). (C, D) SLSP regulated Iba-1 protein expression in SN. Data were presented as mean ± SEM (n = 4/group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* MPTP group.

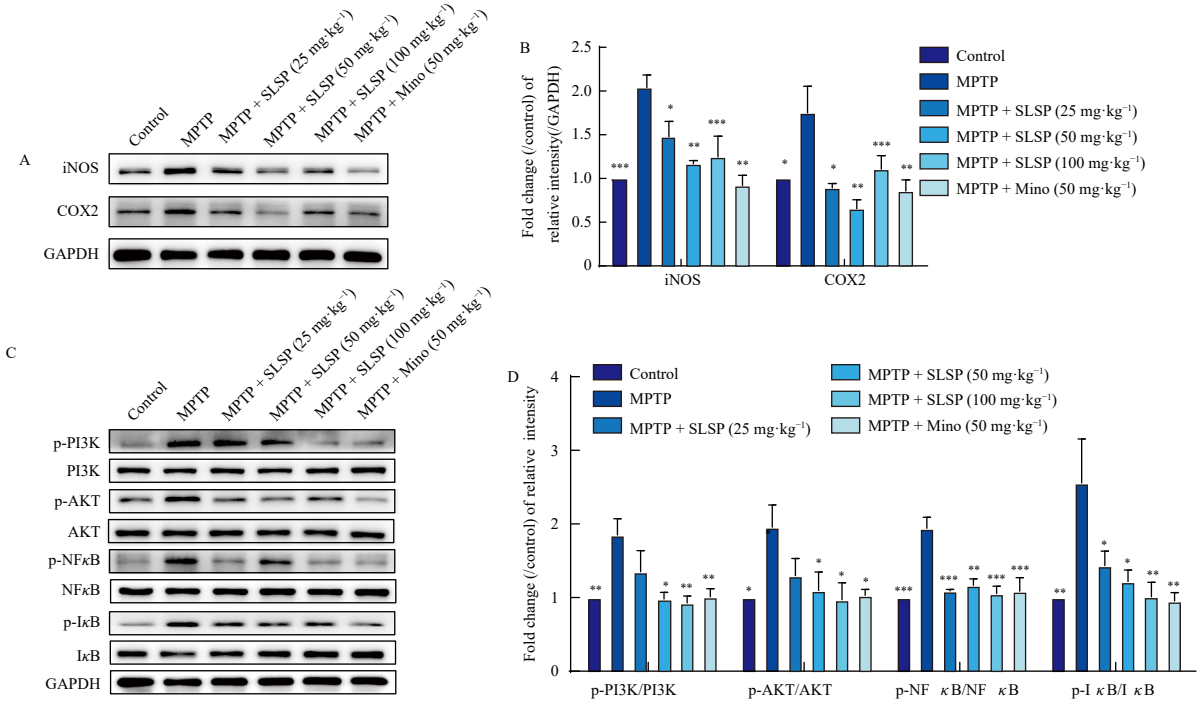

Fig. 3 SLSP reduce the expressions of inflammation-related proteins and modulate the P2Y2R/PI3K/AKT/NFκB signaling pathway in SN of MPTP-induced PD mice. (A, B) SLSP regulated iNOS and COX2 protein expression in SN. (C, D) SLSP regulated p-PI3K, p-AKT, p-NFκB, and p-IκBα protein expression in SN. Data were presented as mean ± SEM (n = 4/group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* MPTP group.

phorylation levels of these proteins in the SLSP and minocycline groups were significantly restored (Figs. 3C and 3D, $P < 0.05$, $P < 0.01$ or $P < 0.001$). These findings suggest that SLSP may regulate the production of proinflammatory factors *via* the P2Y2R-medi-

ated signaling pathway.

3.4. SLSP attenuate LPS-induced inflammatory response in BV-2 microglia

To determine the potential cytotoxicity of SLSP on BV-2 cells, we initially evaluated BV-2 cell viability following SLSP treatment using the CCK-8 assay. As illustrated in Fig. 4A, BV-2 cell viability remained unaffected by SLSP treatment at concentrations of 50, 100, and 200 μg·mL^{-1}. Subsequently, we investigated the anti-inflammatory effects of SLSP on LPS-induced BV-2 cells. Our results indicated that LPS significantly stimulated NO release (Fig. 4B, $P < 0.001$) and enhanced the protein expressions of COX2 and iNOS (Figs. 4C and 4D, $P < 0.001$). Treatment with SLSP or Dex, the positive control drug, inhibited NO production and the protein expressions of COX2 and iNOS (Figs. 4B–4D, $P < 0.001$). To examine the effect of SLSP on proinflammatory mediator mRNA expression, we performed qPCR analysis. As depicted in Figs. 4E–4I, LPS significantly increased the mRNA expression levels of *TNF-α*, *IL-6*, *IL-1β*, *iNOS*, and *COX2* ($P < 0.001$). However, SLSP treatment significantly attenuated this upregulation (Figs. 4E–4I, $P < 0.05$, $P < 0.01$ or $P < 0.001$). These findings suggest that SLSP possess the potential to suppress inflammatory reactions in microglial cells.

3.5. P2Y2R actively involves in the inflammatory responses in LPS-induced BV-2 cells

The P2Y2R expression was concurrently detected at both mRNA and protein levels in BV-2 cells induced with LPS. As illustrated in Figs. 5A and 5B, LPS significantly upregulated the mRNA and protein levels of P2Y2R ($P < 0.001$). Subsequently, P2Y2R was overexpressed in BV-2 cells, and inflammatory mediators were assessed upon LPS stimulation. In comparison to the vector group (LPS treated), the P2Y2R-OE group exhibited markedly increased protein levels of iNOS and NO release (Figs. 5C–5F; $P < 0.05$ or $P < 0.001$). Conversely, P2Y2R knockdown attenuated the protein levels of iNOS and NO release in response to LPS stimulation (Figs. 5G–5J; $P < 0.001$). These findings indicate that P2Y2R plays a role in the elevated levels of inflammatory mediators in LPS-stimulated BV-2 cells.

3.6. SLSP modulate the P2Y2R/PI3K/AKT/NFκB signaling pathway in LPS-induced BV-2 cells

The NFκB transcription factor plays a crucial role in microglial activation as a mediator of the inflammatory response. As

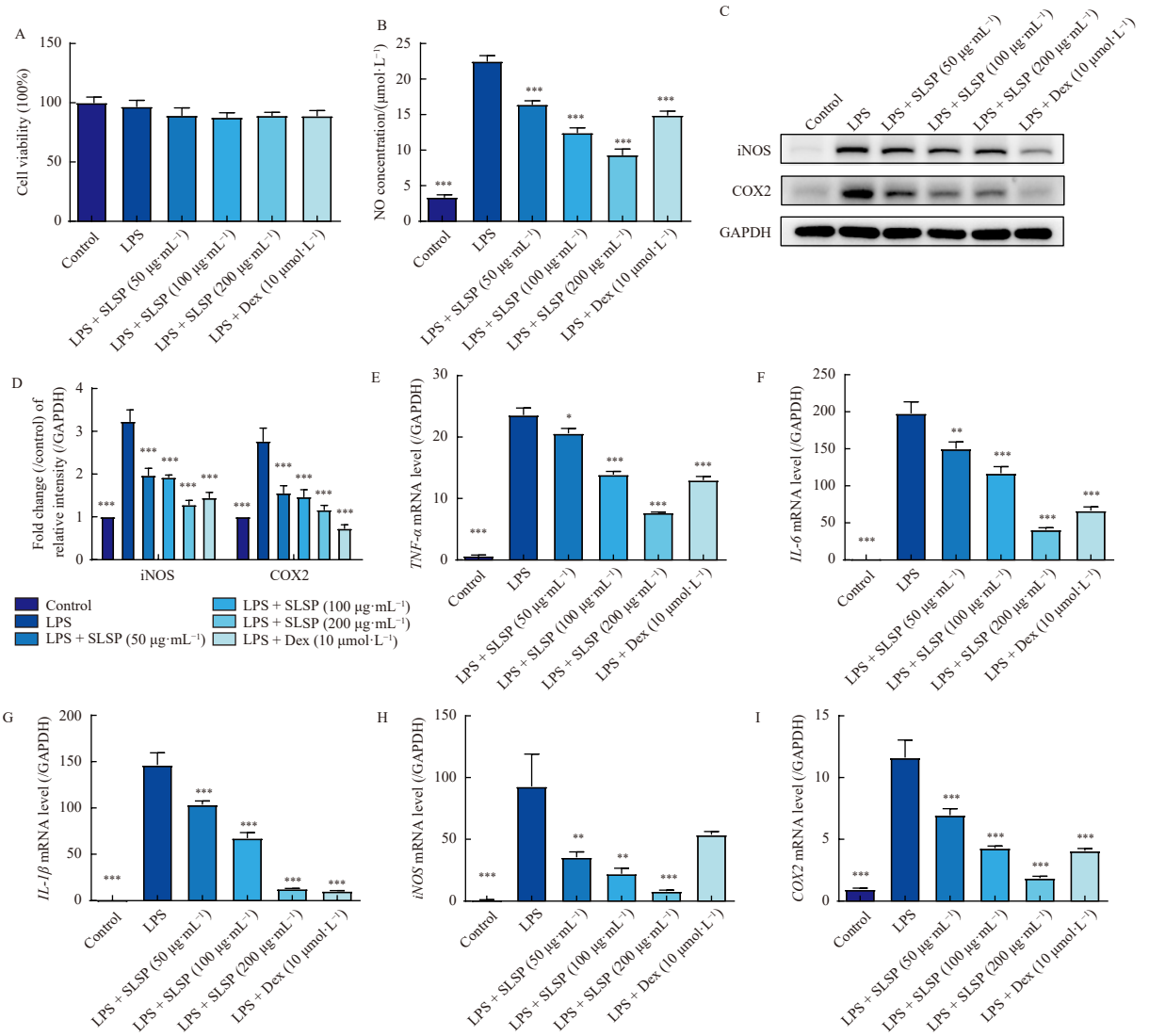

Fig. 4 SLSP attenuate LPS-induced inflammatory response in BV-2 microglia. (A) Effect of SLSP on cell viability. (B) Effect of SLSP on NO release. (C, D) SLSP regulated iNOS and COX2 protein expression in BV-2 cells. (E–I) SLSP regulated mRNA expressions of *TNF-α*, *IL-6*, *IL-1β*, *iNOS*, and *COX2* in BV-2 cells. Data were presented as mean ± SEM (n = 4/group). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ *vs* LPS group.

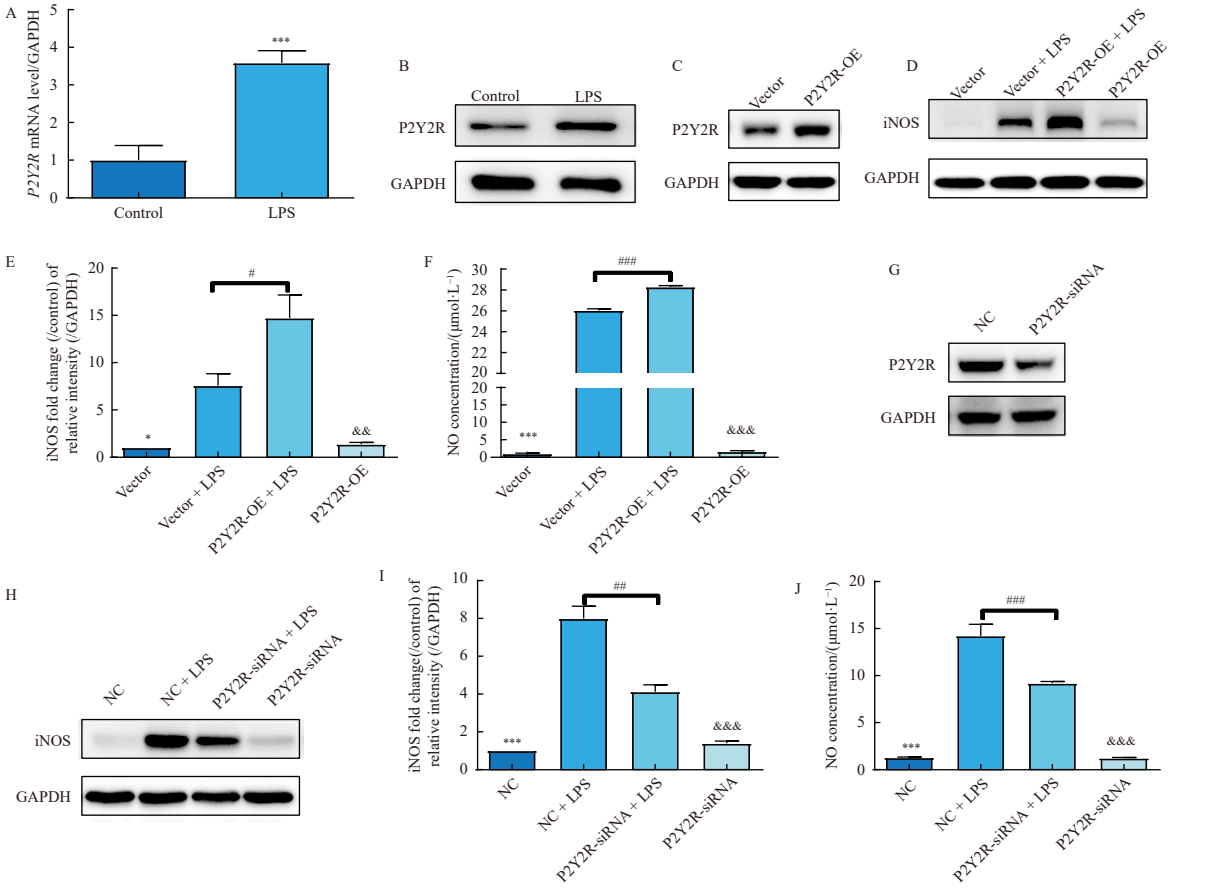

Fig. 5 P2Y2R actively participates in the inflammatory responses in LPS-induced BV-2 cells. (A) Effect of LPS on mRNA expression of *P2Y2R* ($^{***}P < 0.001$ *vs* Control). (B) Effect of LPS on the protein expression of P2Y2R. (C–E) Western blotting analysis of P2Y2R and iNOS in BV-2 cells with P2Y2R overexpression. (F) NO release was measured in the presence of P2Y2R overexpression ($^{*}P < 0.05$, $^{***}P < 0.001$ *vs* Vector + LPS; $^{\&\&}P < 0.01$, $^{\&\&\&}P < 0.001$ *vs* P2Y2R-OE + LPS; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ *vs* Vector + LPS). (G–I) Western blotting analysis of P2Y2R and iNOS in the presence of P2Y2R knockdown. (J) NO release was measured in case of P2Y2R knockdown ($^{***}P < 0.001$ *vs* NC + LPS; $^{\&\&\&}P < 0.001$ *vs* P2Y2R-siRNA + LPS; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* NC + LPS). Data were presented as mean ± SEM ($n = 4$/group).

demonstrated in Fig. 6A, compared to the control group, the majority of cells in the LPS group exhibited NFκB translocation into the nucleus. However, SLSP and Dex significantly inhibited this NFκB translocation. To investigate the effects of SLSP on the P2Y2R/PI3K/AKT/NFκB signaling pathway in BV-2 cells upon LPS stimulation, we initially examined P2Y2R protein expression. As illustrated in Figs. 6B and 6C, LPS induced a significant increase in P2Y2R in BV-2 cells ($P < 0.001$). SLSP treatment did not alter P2Y2R protein expression. Nevertheless, SLSP influenced the P2Y2R/PI3K/AKT/NFκB signaling pathway. As shown in Figs. 6D and 6E, SLSP treatment suppressed the LPS-induced increased phosphorylation of PI3K, AKT, NFκB, and IκB in BV-2 cells ($P < 0.01$ or $P < 0.001$), indicating that SLSP could modulate P2Y2R activity.

3.7. Activation of P2Y2R counteracts the inhibition of SLSP on the P2Y2R/PI3K/AKT/NFκB signaling pathway of LPS-induced BV-2 cells

To further elucidate the role of SLSP in the P2Y2R/PI3K/AKT/NFκB signaling pathway, we employed the P2Y2R agonist diquafosol. Compared to the LPS + SLSP group, the LPS + SLSP + Diq cells exhibited a significant increase in NO release (Fig. 7A, $P < 0.001$). Additionally, diquafosol partially counteracted SLSP's inhibitory effect on iNOS and COX2 protein expression (Figs.7B and 7C, $P < 0.05$ or $P < 0.001$). Moreover, it partially reversed SLSP's inhibition of PI3K, AKT, NFκB, and IκB phosphorylation (Figs. 7D and 7E; $P < 0.05$, $P < 0.01$ or $P < 0.001$). These results suggest that SLSP mitigate the production of excessive proinflammatory mediators by targeting the P2Y2R/PI3K/AKT/NFκB signaling pathway.

4. Discussion

An effective treatment for PD remains a significant, yet elusive, goal. Mounting experimental evidence and post-mortem analyses indicate that inflammation plays a crucial role in driving PD progression [40-42]. Consequently, inhibiting neuroinflammation, primarily mediated through microglial activation, presents a logical and promising approach for mitigating dopamine neuron degeneration in PD. This study demonstrates that SLSP improved behavioral deficits in PD mice while attenuating microglia-mediated neuroinflammation. Additionally, SLSP significantly inhibited the inflammatory mediator response in LPS-induced BV-2 cells. These findings suggest that SLSP inhibit excessive microglial activation in PD models by reducing microglia-mediated inflammation.

To date, researchers have utilized the classical *in vitro* model of LPS-stimulated BV-2 microglia to investigate inflammation-mediated mechanisms and evaluate potential anti-inflammatory agents [43]. In the central nervous system, LPS can directly affect microglial cells and contribute to significant oligodendrocyte death due to its neurotoxic properties [44]. Likewise, MPTP induces severe and uncontrollable Parkinson's-like behavioral disorders in experimental animals [45, 46]. Furthermore, studies have reported increased immune molecules and early microglial activation following MPTP intoxication [47]. Consequently, the activation of microglial cells by LPS *in vitro* and the MPTP-induced mouse model of PD *in vivo* are widely recognized as reliable methods for examining agents with the potential to inhibit neuroinflamma-

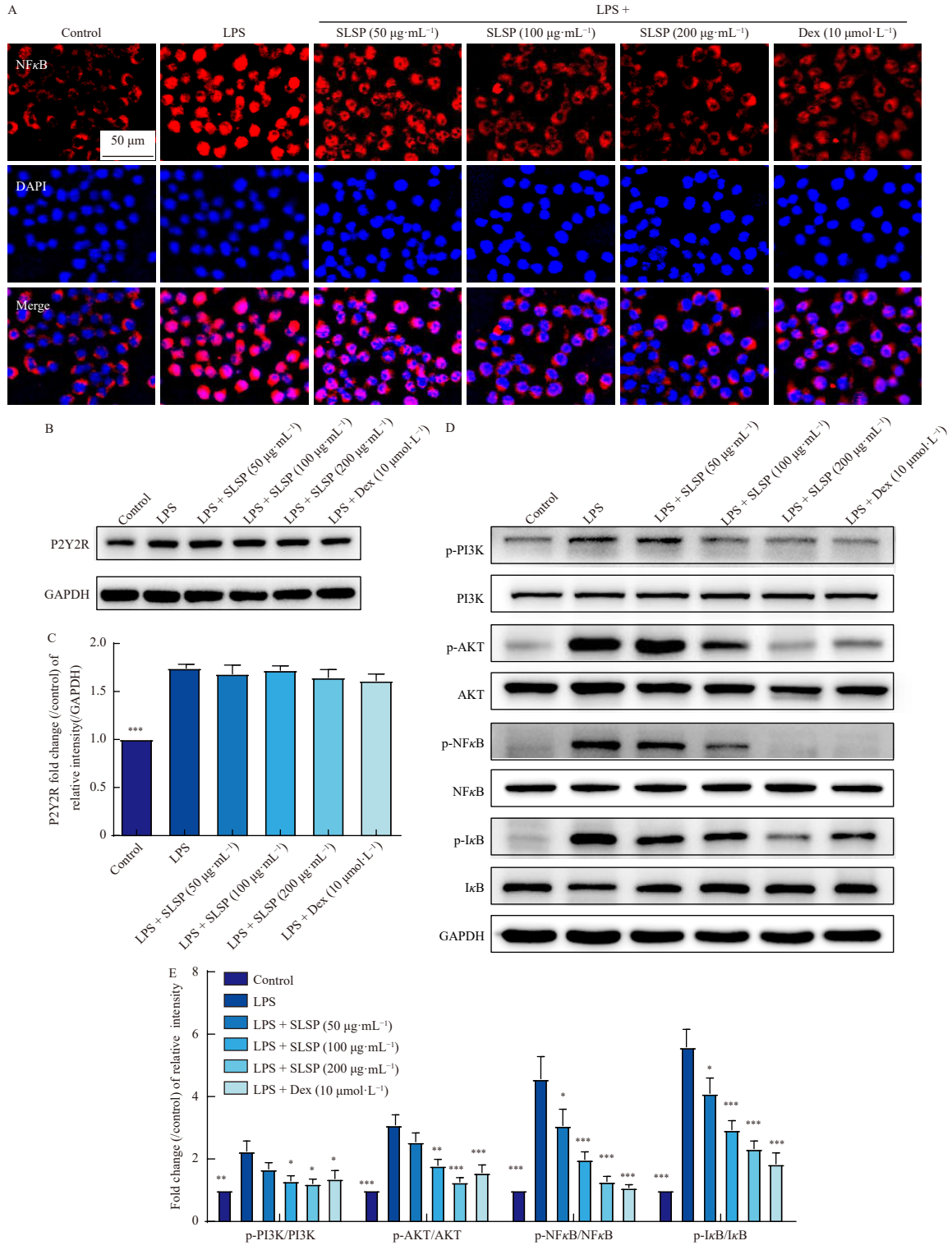

Fig. 6 SLSP modulate the P2Y2R/PI3K/AKT/NFκB signaling pathway in LPS-induced BV-2 cells. (A) SLSP inhibited the translocation of NFκB into the nucleus (scale bar is 50 μm). (B, C) Effect of SLSP on protein expression of P2Y2R. (D, E) SLSP regulated p-PI3K, p-AKT, p-NFκB, and p-IκBα expressions in BV-2 cells. Data were presented as mean ± SEM (n = 4/group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* LPS group.

tion [48-50]. In this study, these two models were employed to evaluate the efficacy and underlying mechanisms of SLSP.

Research has demonstrated that MPTP toxicity induces behavioral impairments in mice [45, 46]. MPTP causes the destruction of dopaminergic neurons in the SNpc and subsequent loss of their dopamine-secreting function [35, 49, 50]. Additionally, it is characterized by activated microglial cells in the striatum and SNpc. Neuroinflammation in PD is typically identified by the reactive morphology of glial cells and the presence of inflammatory mediators in the tissue [51]. Our findings revealed that SLSP mitigated behavioral impairment and significantly prevented MPTP-induced loss of TH protein in the SNpc and striatum of PD mice. SLSP also inhibited microglial activation in MPTP-induced PD mice by reducing the number of activated microglia and suppressing neuroinflammation. In conclusion, this study demonstrated that SLSP played a role in preventing neurological damage by modulating microglia-mediated neuroinflammation, ultimately alleviating behavioral impairment in a PD model.

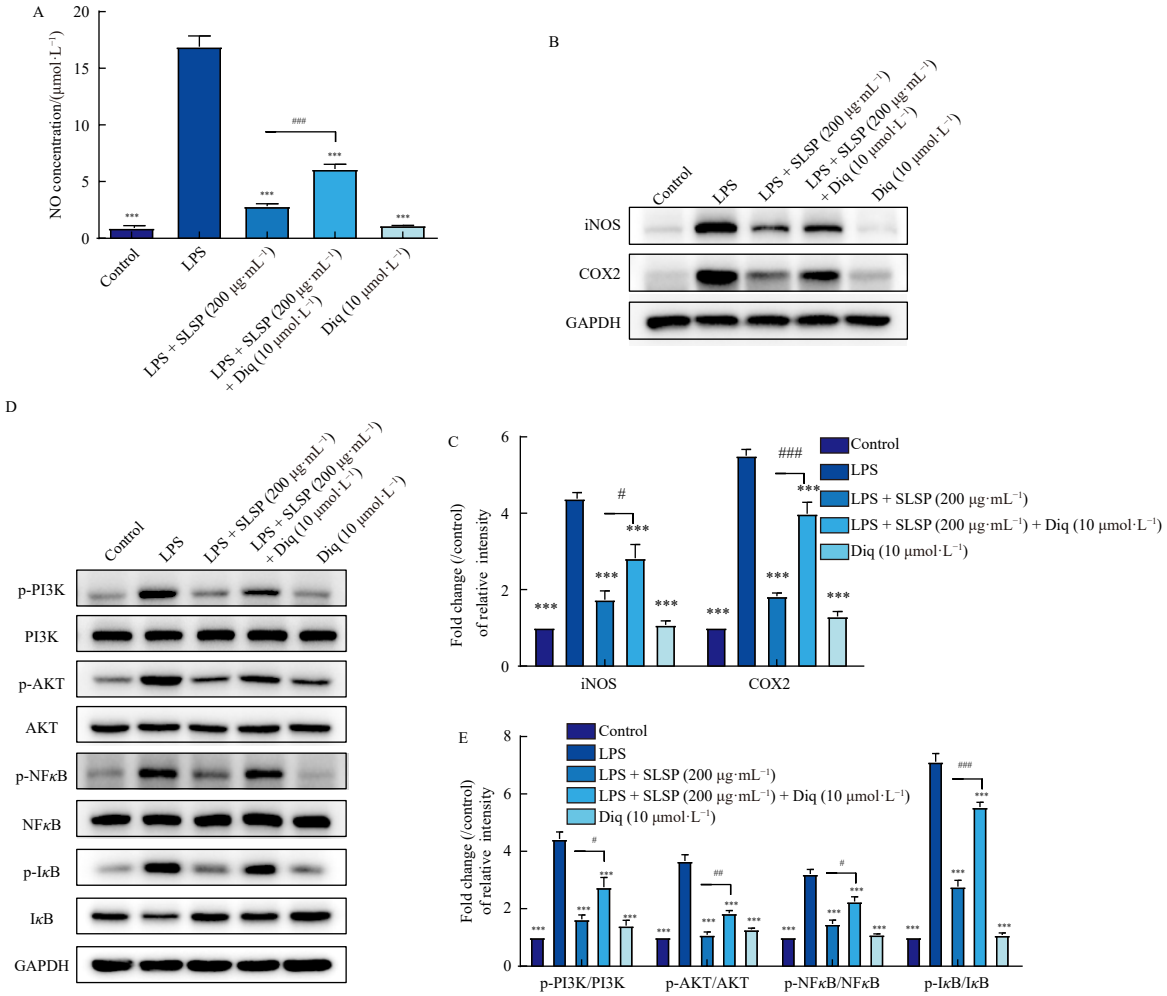

Fig. 7 Activation of P2Y2R counteracts the inhibitory effect of SLSP on the P2Y2R/PI3K/AKT/NFκB signaling pathway in LPS-induced BV-2 cells. (A) Effect of SLSP on NO release in the presence of diquafosol. (B, C) Representative Western blots of iNOS and COX2 expression with diquafosol in BV-2 cells. (D, E) Representative Western blots of p-PI3K, p-AKT, p-NFκB, and p-IκBα expressions in the presence of diquafosol in BV-2 cells. Data were presented as mean ± SEM (n = 4/group). $^{***}P < 0.001$ *vs* LPS group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* LPS + SLSP group.

Microglia serve a crucial function in neuronal protection, acting as the primary defense mechanism against potential chemical or physical brain damage. Their contribution to the brain's immune system is an essential safeguard against potential harm [9]. In pathological conditions, microglial cells can produce free radicals, chemokines, and proinflammatory cytokines that stimulate neuroinflammation, potentially leading to neuronal toxicity and neurodegeneration [3, 40]. In this study, SLSP demonstrated potent inhibition of microglia-mediated inflammation. SLSP suppressed both the mRNA and protein expression of iNOS, resulting in the inhibition of NO release. Microglial activation leads to increased COX2 expression, a contributing factor in the progression of PD [12, 13]. This research showed that SLSP effectively inhibited the increase of both COX2 mRNA and protein expression in BV-2 cells induced by LPS. NFκB is a key transcription factor for microglial activation. The translocation of NFκB into the nucleus is crucial for inducing the expression of various inflammatory genes during microglial activation, such as TNF-α, IL-1β, and IL-6 [52]. Moreover, studies have demonstrated that elevated levels of proinflammatory cytokines TNF-α, IL-6, and IL-1β play a significant role in neuron damage caused by inflammation. In this study, SLSP significantly inhibited the translocation of NFκB into the nucleus and the production of proinflammatory mediators induced by LPS. These results suggest a suppressive effect of SLSP on microglial activation.

Purinergic signaling plays a crucial role in triggering both local and systemic inflammatory responses [53]. Under normal physiological conditions, nucleotide release is strictly regulated; however, inflammatory states lead to increased nucleotide release [54]. P2Y2R, a significant member of the P2Y receptor family, has been implicated in various inflammatory responses. P2Y2R inhibitors can suppress neuronal inflammation by reducing the expression of proinflammatory cytokines, including TNF-α, IL-1β, and IL-6, ultimately exerting a protective effect on spinal cord injury [55]. Our research demonstrated that LPS triggered an increase in P2Y2R expression in BV-2 cells. Overexpression or knockdown of P2Y2R in BV-2 cells affected the expression of iNOS protein and NO release following LPS induction. These studies suggest that P2Y2R may significantly impact microglia-induced inflammation. Previous research indicates that P2Y2R activation can enhance the phosphorylation levels of PI3K, AKT, NFκB, and IκB [36-39]. Diquafosol is a potent, selective agonist for P2Y2R with an average EC_{50} of 0.1 μmol·L^{-1} in human 1321N astrocytoma cells that stably express P2Y2R [56]. As a P2Y2R agonist, diquafosol activates P2Y2R [57], leading to Src phosphorylation, which further activates PI3K/AKT/NFκB [37-39]. In this study, SLSP demonstrated the ability to reduce the phosphorylation of PI3K, AKT, NFκB, and IκB both *in vivo* and *in vitro*. These findings strongly suggest that SLSP suppressed microglia-mediated neuroinflammation in PD models through the P2Y2R/PI3K/AKT/NFκB signaling pathway. To confirm this conclusion, the P2Y2R agonist diquafosol was employed. Our experimental results revealed that diquafosol partially antagonized SLSP's inhibitory effect on inflammatory mediator production and P2Y2R/

PI3K/AKT/NFκB signaling pathway activation. These findings demonstrate that SLSP inhibited microglial activation by repressing the P2Y2R signaling pathway. Although diquafosol was used in *in vitro* cell experiments to treat BV-2 cells and verify that SLSP inhibited microglia activation through P2Y2R, appropriate P2Y2R agonists still need to be used in *in vivo* animal experiments to confirm that SLSP improves PD-like behavior in mice through P2Y2R. Simultaneously, a study investigating P2Y2R's role in MPTP-induced PD mice by creating mice with microglia-specific overexpression of P2Y2R is also needed. This will further confirm that SLSP improves PD mice symptoms by inhibiting microglia through P2Y2R.

In conclusion, SLSP mitigated experimental PD progression in mice by inhibiting microglia-mediated neuroinflammation, likely *via* the P2Y2R/PI3K/AKT/NFκB signaling pathway (Fig. 8). This novel study suggests that SLSP may serve as a potential therapeutic intervention for both the progression and onset of PD, particularly in cases where microglia-driven neuroinflammation is a prominent pathological feature.

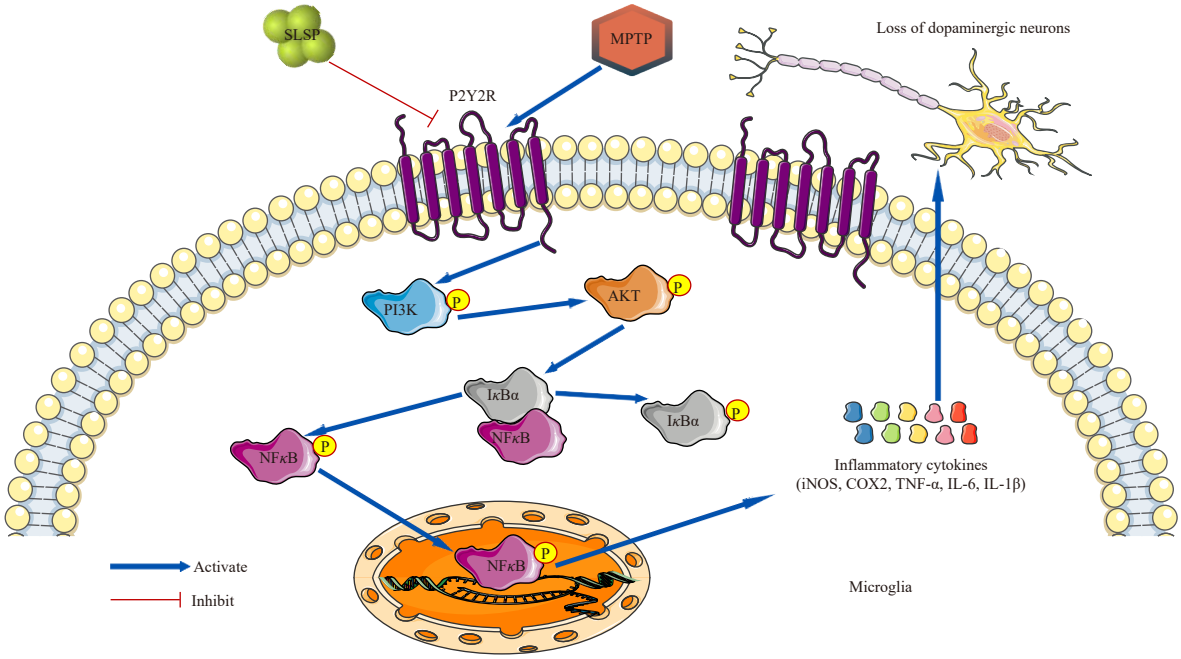

Fig. 8 Schematic illustration: SLSP mitigate the development of PD in mice by inhibiting microglia activation through the P2Y2R/PI3K/AKT/NFκB signaling pathway.

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Declaration of competing interest

These authors have no conflict of interest to declare.

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