Drug Design, Development and Therapy

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ORIGINAL RESEARCH

Paeoniflorin Combined with Neural Stem Cell Transplantation for Parkinson's Disease: Dual Mechanism of Cell Therapy and Inflammation Regulation

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Introduction: Parkinson's disease (PD) is a neurodegenerative disorder lacking therapies to replace lost dopaminergic neurons. Neural stem cell (NSC) transplantation faces survival and differentiation challenges. This study investigated feasibility and efficacy of paeoniflorin (PF) combined with NSC transplantation for PD treatment.

Methods: NSCs were isolated from E14 SD rat embryos. Differentiation medium induced dopaminergic progenitors and mature midbrain dopaminergic (mDA) neurons. Immunofluorescence identified NSCs and mDA neurons. CCK-8 and Calcein-AM/PI staining evaluated PF's effect on cell viability. Primary microglia were co-cultured with mDA neurons under PF treatment, with LPS-induced inflammation modeling. ELISA measured inflammatory cytokines, and Western blot analyzed TLR4 pathway and NLRP3 inflammasome proteins. In vivo, a PD rat model was established by injecting 6-hydroxydopamine into the substantia nigra, and apomorphine-induced rotational behavior validated the model. mDA cells, alone or with PF, were transplanted into the striatum. Tyrosine hydroxylase staining evaluated mDA differentiation and survival, and immunohistochemistry and Western blot verified inflammatory protein changes.

Results: NSCs formed neurospheres with high Nestin+ purity. Successful differentiation into dopaminergic lineages observed. PF had no significant cytotoxicity to NSCs or microglia, reduced inflammatory damage to mDA neurons, and enhanced maturation when microglia were pre-treated. In PD rats, apomorphine induced >7 rotations per minute, and TH staining confirmed dopaminergic neuron loss, validating the model. PF combined with mDA transplantation improved dopaminergic neuron differentiation and survival in the striatum. Mechanistically, PF suppressed the TLR4/MYD88/NF-κB signaling pathway and NLRP3 inflammasome, reducing inflammation, and stabilizing the neural microenvironment.

Conclusion: Paeoniflorin lessens inflammatory damage to transplanted cells, promotes survival and differentiation, and outperforms mDA-only transplantation for neuronal survival and functional recovery. By regulating inflammation, PF optimizes the neural microenvironment, offering new perspectives for combined cell transplantation therapy in PD.

Keywords: Parkinson's disease, paeoniflorin, neural stem cells, cell transplantation, inflammatory response

Introduction

Parkinson's disease (PD) is a common chronic progressive neurodegenerative disorder, characterized primarily by motor symptoms such as tremor, rigidity, bradykinesia, and postural instability.[1] The characteristic pathological changes of PD are the loss of dopaminergic neurons in the dense part of the substantia nigra and the formation of Lewy bodies from misfolded alpha-synuclein aggregates within the cells.[2] Currently, the treatment of PD mainly relies on pharmacological and surgical interventions. Pharmacotherapy primarily targets symptom relief, but its long-term use is complicated by motor fluctuations and dyskinesia.[3] Deep brain stimulation offers an alternative for advanced-stage patients, yet its

Received: 10 March 2025
Accepted: 26 August 2025
Published: 4 September 2025

invasive nature carries the risk of complications.[4] Although these approaches can improve symptoms in the short term, they cannot replace the lost neurons and halt the progression of the disease.

In recent years, Neural Stem Cells (NSCs) transplantation in the treatment of PD has received extensive attention. With self-renewal ability and multi-lineage potential, NSCs can theoretically replace the lost dopaminergic neurons, differentiate NSCs into dopaminergic neurons, and integrate them into the brain of patients, so that they can re-innervate the striatum.[5,6] However, how to ensure that the transplanted NSCs survive in the harsh microenvironment of the brains of PD patients and differentiate into dopaminergic nerve cells remains a key problem to be solved urgently. Neuroinflammatory response not only plays a crucial role in the pathogenesis of PD, but also has a direct impact on the effect of NSCs transplantation.[7] The latest research results show that penetrating trauma during cell transplantation can directly induce a rapid inflammatory response, which poses a serious threat to the survival of transplanted cells in the short term.[8] Moreover, interventions targeting inflammatory pathways, such as the TNF-NF-kappaB-p53 axis, can increase the survival rate of transplanted dopaminergic neurons and improve motor deficits in PD models.[9] Therefore, mitigating transplantation-related inflammation is key to enhancing the success of cell transplantation therapy for PD.

Paeoniflorin (PF), a primary bioactive compound extracted from *Paeonia lactiflora*, has garnered considerable attention due to its neuroprotective effects.[10] It exhibits a range of pharmacological actions in the central nervous system, including anti-inflammatory, antioxidant, and anti-apoptotic effects.[11,12] For PD, PF may protect dopaminergic neurons by regulating inflammatory responses and antioxidant pathways, thereby slowing the progression of the disease to some extent.[13] Although the anti-inflammatory and neuroprotective effects of PF have been reported in a large number of studies, the feasibility, and effectiveness of PF combined with NSCs transplantation in the treatment of PD are still relatively scarce.

The purpose of this study was to investigate the feasibility and effectiveness of PF combined with NSCs transplantation in the treatment of PD. We will systematically evaluate the effects of PF on the survival and differentiation of transplanted cells and its potential to improve motor symptoms in animal models of PD. In addition, we will comprehensively explore the regulatory effect of PF on the inflammatory environment in the brain after cell transplantation, and the effect of this regulatory effect on the transplantation effect of NSCs. It is expected that this study will not only improve the therapeutic effect, but also ensure the safety of treatment, and open up a new strategy for the treatment of PD.

Methods

Isolation, Culture, and Identification of NSCs

Brain tissue was obtained from the embryos of SD rats at embryonic day 14 (E14). Under a microscope, the hippocampal region was dissected and placed in D-Hank's Balanced Salt Solution. The tissue was thoroughly minced with ophthalmic scissors and then filtered through a 70-μm mesh filter. Gentle mechanical dissociation was performed to separate NSCs from the brain tissue. The cells were cultured in a mixed medium consisting of DMEM/F12 basal medium, 2% B27 supplement (Gibco), 20 ng/mL epidermal growth factor (EGF, Gibco), 20 ng/mL basic fibroblast growth factor (bFGF, Gibco), and penicillin/streptomycin (100 U/mL, Invitrogen) at 37°C with 5% CO2, with medium changes every other day. After approximately 3 days, the cells exhibited neurosphere-like growth. Passaging was performed using Accutase (Invitrogen, Carlsbad, CA). After 7 days, the purity of NSCs was identified by immunocytochemical detection of the stem cell marker Nestin and neural cell markers. Neurospheres from the third passage were used for subsequent differentiation experiments.

For the isolation of microglia, the whole brain of neonatal pups was placed in a sterile Petri dish containing HBSS on ice after removing the meninges. The tissue was minced and digested with trypsin for 15 minutes, then cultured in DMEM basal medium supplemented with 10% FBS at 37°C with 5% CO2. After 24 hours, when the cells were fully adhered, the medium was changed. After 7 days, when the cells showed a clear stratification, the medium was changed and the culture was shaken at 200 r/min for 30 minutes at 37°C to collect detached cells. The cells were then seeded onto culture bottles coated with poly-L-lysine. After 1 hour, the medium was changed to remove non-adherent cells. The purity of the cells was identified by immunofluorescence staining.

Directed Induced Differentiation of Dopaminergic Neurons

Neurospheres were dissociated using Accutase and plated at a density of 1.5×10^4 cells/cm^2 on culture dishes pre-coated with laminin. The midbrain dopaminergic neurons (mDA) differentiation medium consisted of DMEM/F12, Neurobasal medium, non-essential amino acids, 200 mM GlutaMAX, B27 supplement, 200 ng/mL SHH, and 100 ng/mL FGF8, with the medium changed every other day for a period of 10 days. On day 10, the cells were again dissociated with Accutase and replated at a density of 2×10^5 cells/cm^2 on laminin-coated culture dishes. The dopaminergic maturation medium was composed of Neurobasal medium, GlutaMAX, B27 supplement, 200 μM ascorbic acid, 0.5 mM cAMP, 10 μM DAPT, 1 ng/mL TGF-βIII, 20 ng/mL BDNF, and 20 ng/mL GDNF, and the cells were further cultured for 2 weeks. Immunocytochemical fluorescence detection of dopaminergic neurons was performed on days 0, 12, and 20. We selected LMX1A, TUJ1, and TH as stage-specific markers representing mDA progenitors, immature neurons, and mature dopaminergic neurons, respectively. Pax6, although commonly used to mark early neural progenitors, is primarily associated with forebrain identity and is downregulated during midbrain floor plate specification. Thus, it was not used in this context to avoid misrepresenting regional lineage commitment.

Cell Viability Assay

NSCs were dissociated into single suspended NSCs using Accutase and plated on coverslips pre-coated with poly-L-ornithine and laminin. After directed differentiation into dopaminergic progenitor cells using differentiation medium, cell viability was assessed using the Cell Counting Kit-8 (CCK8). Cells were seeded at a density of 2×10^3 cells/well in 96-well plates and treated with different concentration gradients of PF. After incubation at 37°C and 5% CO2 for 24 hours, 10 μL of CCK8 (Sigma) was added to each well and incubated for an additional hour. Cell viability was then measured using a microplate reader at an absorbance of 450 nm. The Calcein-AM/PI double staining method was employed to evaluate cell survival status. After washing with PBS, the staining solution (Calcein-AM 1–2 μM, PI 1–5 μg/mL) was added and thoroughly mixed, followed by incubation at 37°C in the dark for 20–30 minutes. The live/dead ratio of cells was detected using a fluorescence microscope, with green fluorescence indicating live cells and red fluorescence indicating dead cells, to assess cell survival.

Cell Co-Culture and Animal Ethics

Primary microglia, extracted and undifferentiated, can be induced into an inflammatory state after stimulation with lipopolysaccharide (LPS) at a concentration of 1 μg/mL for 24 hours. These microglia were co-cultured with mDA differentiated from NSCs on day 10 in a 0.4 μm chamber. The microglia were cultured in serum-free medium at the bottom layer, while the top layer contained the mature culture medium for the differentiated mDA. 100 μM PF was added to continue the co-culture of mDA cells and microglia 12 hours before LPS stimulation of microglia. After 24 hours of LPS stimulation, the upper layer was tested for inflammatory factors and related proteins.

Sprague Dawley (SD) rats were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd. All animals were housed in a sterile environment with a 12-hour light/dark cycle and ample food and water. All animal breeding and experimental procedures were conducted in accordance with National and International Animal Ethics Guidelines. This study was approved by the Ethics Committee of Peking University People's Hospital and adhered to the Declaration of Helsinki.

Model Construction and Cell Transplantation

Sprague-Dawley rats of 8 weeks of age were selected to ensure that their body weight was in the range of 220–250 grams. After general anesthesia with isoflurane, the rats were firmly fixed in the stereotaxic frame. According to the coordinates of the substantia nigra in the rat brain atlas (A/P = 5.2 mm, -L = 2.2 mm, V = −8.0 mm), 10 μL of 6-hydroxydopamine (6-OHDA) at a concentration of 2 mg/mL was injected. The 6-OHDA was dissolved in 0.9% saline containing 0.2 mg/mL ascorbic acid. Four weeks post-surgery, the PD animal model rotation test was conducted by intraperitoneal injection of apomorphine (0.2 mg/mL; Sigma). A rotation rate of ≥7 times per minute on the same side indicated successful model construction. PD rats with stable rotation counts of over 7 times after apomorphine injection

at 4- and 8-weeks post-surgery were selected for further cell transplantation studies. One week before transplantation, PF was administered intraperitoneally (100 mg/kg). The cell transplantation site was the striatum on the same side as the lesion damage (A/P = 1.0 mm, -L = 2.5 mm, V = −6.8 mm). A Hamilton syringe was used to inject 5×10^6 cells into the striatum at a rate of 1 μL/min. After the injection was completed, the syringe was maintained in place for 10 minutes before being slowly withdrawn. After the injection procedure is completed, the surgical wound is carefully sutured. Tissue samples were collected at 4 weeks after cell transplantation for sectional staining in order to systematically assess dopaminergic neuron survival and related mechanisms.

Immunohistochemical and Cell Staining

Cells were fixed with 4% paraformaldehyde, and brain tissue was perfused with 4% paraformaldehyde through the heart, removed, and placed in a 30% sucrose solution for dehydration at 4°C until submerged. Cells were permeabilized with 0.1% Triton™ X-100 (Sigma-Aldrich) and blocked with 10% normal goat serum at room temperature. The following primary antibodies were used for overnight incubation at 4°C: rabbit anti-Nestin (1:500; Sigma-Aldrich), rabbit anti-microtubule-associated protein-2 (MAP-2) (1:200; Millipore), rabbit anti-glial fibrillary acidic protein (GFAP) (1:1000; Abcam, UK), rabbit anti-CNPase (1:100; Cell Signaling Technology), rabbit anti-Tyrosine Hydroxylase (TH) (1:1000; Abcam, UK), and rabbit anti-LMX1A (10 μg/mL; Abcam, UK). After incubation, cells were washed three times with PBS for 5 minutes each and incubated for 1 hour at room temperature with the following secondary antibody: Alexa Fluor 488/594-conjugated goat anti-rabbit (1:500; Invitrogen). After staining the nucleus with DAPI, the sample was observed under a fluorescence microscope, and the expression and distribution of the target protein were analyzed according to the localization of different fluorescent antibodies.

WB and ELISA

The cells were placed in a lysate containing a Protease Inhibitor Cocktail (Thermo Fisher Scientific), incubated on ice for 30 minutes to achieve full lysation, and the brain tissue samples were shaken and shredded. Then the protein was quantified by BCA method (Sigma-Aldrich), and the quantified protein was separated by SDS-PAGE and transferred to 0.45 μm PVDF (Millipore) membrane. The membrane was closed with 3% BSA at room temperature, incubated with the diluted primary antibody at 4°C overnight, washed three times with TBST for 5 minutes each time, and incubated with the second antibody of the same species at room temperature for 1 hour, then developed with ECL, the protein band image was obtained by the imaging system, and the relative expression of the semi-quantitative target protein with gray value was analyzed by software. At the same time, ELISA kit was used to quantitatively detect inflammatory factors, the cell supernatant was added to the ELISA plate hole coated with corresponding antibodies, so that inflammatory factors and antibodies were specifically bound, after washing, HRP was added to label the second antibody to form a complex, after washing again, enzyme substrate was added, HRP catalyzed the substrate to glow, and specific wavelength absorbance or light intensity was measured by photometer. The concentration of inflammatory factors was calculated according to the standard curve.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism version 8.0 (GraphPad Software, La Jolla, CA, USA). Data are expressed as the mean±standard deviation of six independent biological replicates. For pairwise comparisons between two groups, Student's *t*-test was applied when both normality assumptions and homogeneity of variances were satisfied. For comparisons across three or more groups under parametric conditions, one-way analysis of variance (ANOVA) was performed, followed by Tukey's post-hoc testing. In the case of non-normally distributed data, the Kruskal–Wallis test was used, with subsequent Dunn's post-hoc test for pairwise comparisons. Normality was assessed using the Shapiro–Wilk test (with $p > 0.05$ considered indicative of normal distribution), and results were visually confirmed with Q–Q plots (Supplementary Figure S1). Homogeneity of variance was tested using Levene's test. Where the assumptions of normality or equal variance were not met, non-parametric tests (Mann–Whitney U) were employed. Statistical significance was defined as $P < 0.05$, with $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and ns: not significant.

Results

Cell Extraction and Identification

After isolating the hippocampal region of embryonic rat brain tissue, cells were selected and cultured using an NSC-specific medium. The primary NSCs grew as individual floating cells (Figure 1A). As the culture time extended, individual NSCs could further expand by proliferation and aggregation into neurosphere-like clusters (Figure 1B). Immunofluorescence detection using the NSC marker (Nestin) revealed that the aggregated neurospheres were of high purity for NSCs (Figure 1C). After digestion with Accutase, the cells were directly induced to differentiate into specific neural types. Microglial cells obtained from the brain tissue of newborn rat pups grew adherently, exhibiting a uniform round or oval transparent appearance (Figure 1D). Under the induction and stimulation of LPS, the cells showed changes in protrusions and became morphologically irregular (Figure 1E). Immunofluorescence identification using the microglial cell marker (IBA1) indicated that the extracted microglial cells had a high degree of purity and could be directly stimulated for inflammatory induction for subsequent experiments (Figure 1F).

NSC Differentiation into mDA Neurons

NSCs extracted from the hippocampal region of rat embryos possess the capacity for self-renewal and multipotent differentiation. Under various induction conditions, they differentiate through glial progenitor cells and neural progenitor cells into astrocytes (GFAP), oligodendrocytes (CNPase), and neurons (TUJ1) (Figure 2A). After the aggregated neurospheres are digested with Accutase, the dissociated individual NSCs differentiate and mature into dopaminergic neurons under the induction of mDA culture medium. The process of cell proliferation and differentiation of NSCs into mDA can be observed at days 0, 2, 4, 6, 8, and 10 (Figure 2B). Cell immunofluorescence staining results on days 12 and 20 show that mDA progenitor cells (LMX1A) are the predominant cell type, characterized by immature neuronal markers (TUJ1) and mature neuronal markers (MAP2) (Figure 2C). The sequential expression of LMX1A, TUJ1, MAP2, and TH defines stage-specific milestones in mDA neuronal maturation. At Day 12, LMX1A marks committed midbrain progenitors, with concurrent TUJ1 expression confirming neuronal lineage entry. By Day 20, MAP2 replaces TUJ1 as the dominant cytoskeletal marker, reflecting dendritic maturation and structural competence. Terminal differentiation is evidenced by TH expression in MAP2+ neurons, validating functional dopaminergic identity. This hierarchy—where LMX1A initiates

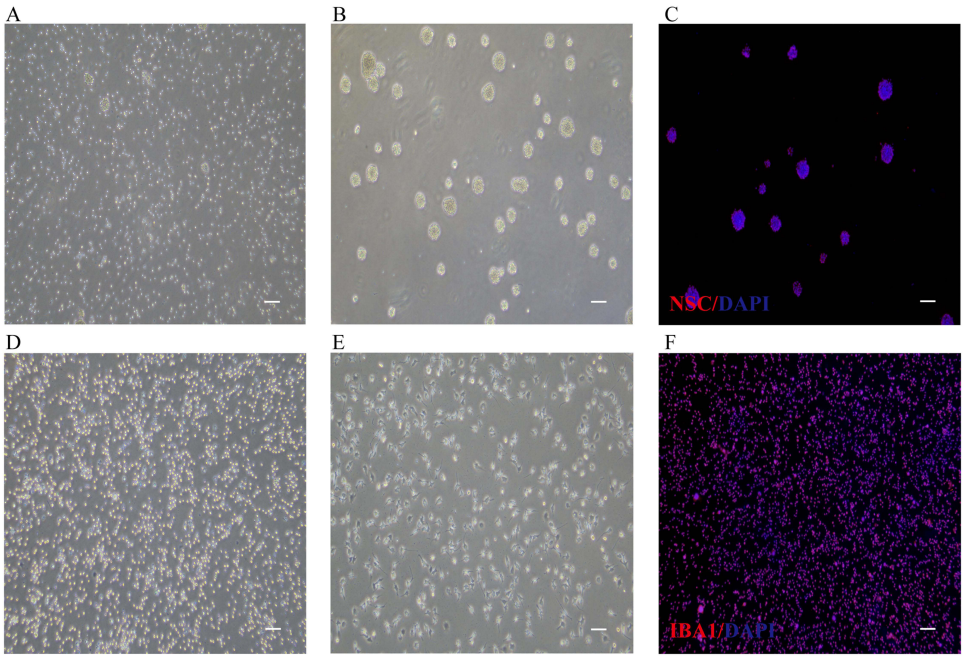

Figure 1 Isolation, culture, and identification of neural stem cells and microglia. (**A**) Individual neural stem cells in suspension culture; (**B**) Neurospheres formed by the proliferation of neural stem cells; (**C**) Nestin immunostaining to assess the purity of neurospheres; (**D**) The translucent morphology of undifferentiated microglia; (**E**) Microglia differentiated after LPS stimulation; (**F**) IBA1 immunolabeling for the identification of microglia. Scale bar: 100 μm.

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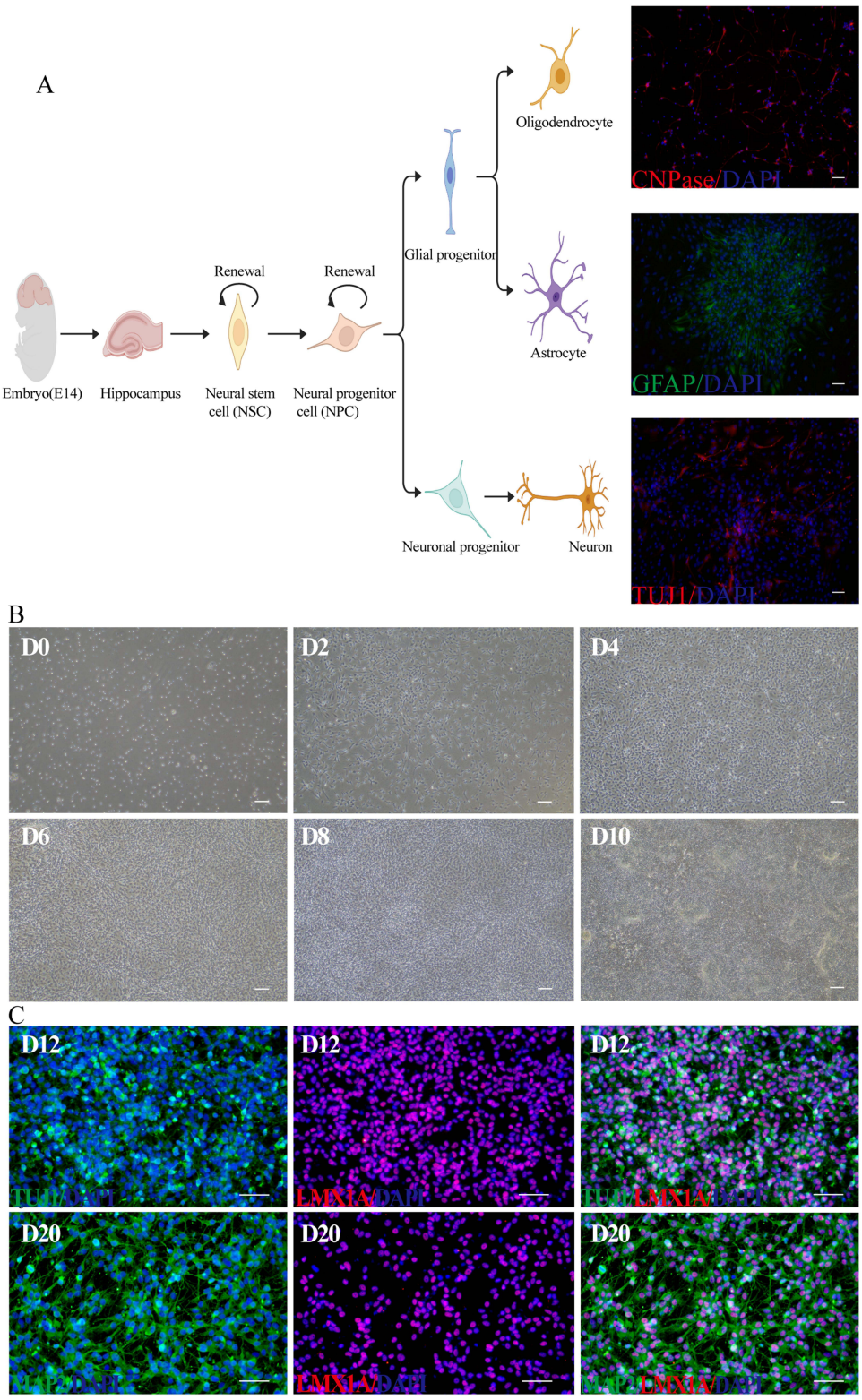

Figure 2 Differentiation of neural stem cells into dopaminergic (DA) neurons. (**A**) The self-renewal and multilineage differentiation potential of neural stem cells; (**B**) Morphological changes of cells at various differentiation time points; (**C**) Immunostaining identification of neuronal markers on days 12 and 20. Scale bar: 50 μm.

midbrain specification, TUJ1 marks neuronal birth, MAP2 enables synaptic integration, and TH confers neurotransmitter phenotype—recapitulates endogenous nigrostriatal development and ensures transplanted cells are functionally competent.

Effects of PF on Cell Viability

Microglial cells extracted were subjected to a 24-hour culture under varying concentrations of PF, followed by CCK8 viability assays. The results indicated that PF had no significant impact on the viability of microglial cells (Figure 3A). However, excessively high concentrations of PF could affect the activity of these cells. The effects of PF on the activity of mDA derived from NSCs were detected at different time points, such as 12 hours, 24 hours, 48 hours, and 72 hours. Compared with the control group that was not stimulated by PF, the activity of cells stimulated by 100μM PF was decreased after 48 hours of culture. However, with the further extension of culture time, mDA cells continued to expand, and there was no significant difference in the effect of different concentrations of PF on its activity (P>0.05) (Figure 3B). In addition, Calcein/PI cell activity and cytotoxicity assay were used to evaluate the cell status after 72 hours of different concentrations of PF. The results showed that there was no significant difference in the proportion of dead cells to live

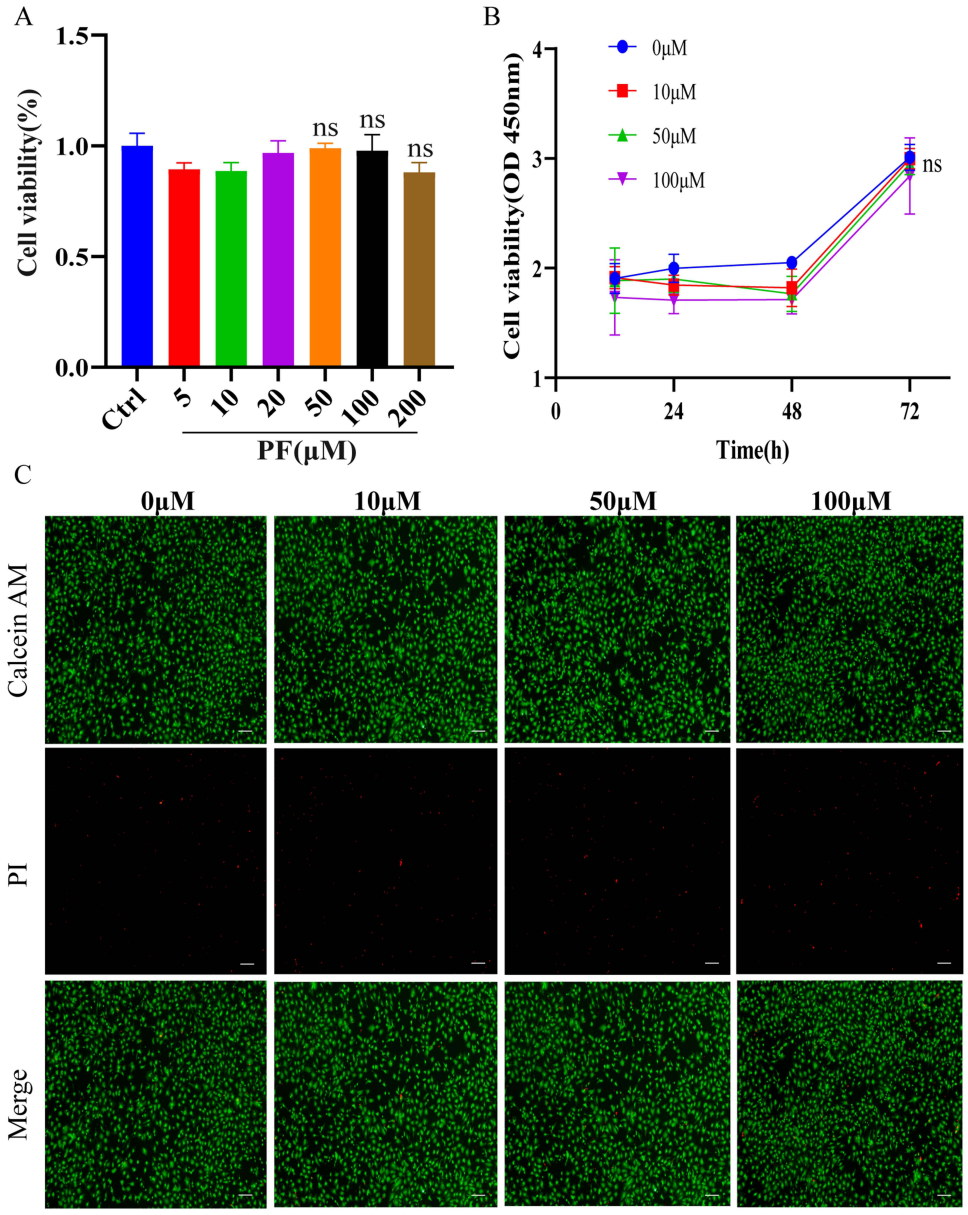

Figure 3 The effect of PF on cell viability. (**A**) The impact of varying concentrations of PF on microglial viability (n = 6 per group); (**B**) The effect of PF at different times and concentrations on the viability of dopaminergic (DA) cells; (**C**) Calcein/PI double staining to assess the activity and toxicity of dopaminergic (DA) cells after 72 hours of PF exposure at different concentrations. Scale bar: 50 μm. ns. indicates no statistical significance.

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cells in each experimental group (Figure 3C), which further confirmed that PF had a weak effect on the activity of mDA cells within the range of conventional concentration under long-term treatment.

Influence of Inflammation on mDA Cells

Microglia and mDA cells were co-cultured in a 0.4um transwell system. The inflammatory response induced by LPS stimulation of microglia can affect the activity of the overlaying mDA cells (Figure 4A). After 24 hours of LPS stimulation of microglia, compared to the unstimulated group, the LPS-induced inflammatory response significantly reduced the activity of the overlaying mDA cells ($P < 0.05$). Moreover, the activity of mDA cells stimulated by LPS was notably improved after pre-treatment with 100μM PF, indicating that PF has the effect of inhibiting inflammatory damage to mDA cells (Figure 4B). Additionally, in the LPS-stimulated group, there were more dead cells observed in the upper chamber, with a decrease in the number of viable mDA cells and a significant reduction in cell viability. However, in the group pre-treated with PF for 24 hours, there was no significant decrease in cell viability, and the ratio of viable to dead cells showed no significant difference (Figure 4C). Under the influence of inflammatory factors, there is a significant death of mDA cells, with a marked reduction in the number of immature neurons and dopaminergic progenitor cells ($P < 0.05$). The number of cells in the LPS-stimulated group pre-treated with PF was significantly higher than that in the group stimulated with LPS alone, indicating that pre-treatment with PF can prevent the damage to neural cells caused by inflammatory factors (Figure 4D). In the mature stage of dopaminergic neurons, PF can promote the expression of TH, a marker of dopaminergic neurons. LPS stimulation significantly reduced the number of dopaminergic neurons, whereas PF pretreatment significantly increased the number of dopaminergic neurons ($P < 0.05$), and the proportion of dopaminergic neurons among total neurons also increased significantly (Figure 4E). PF not only shows significant anti-inflammatory properties, but also promotes the maturation of dopaminergic neurons, and plays an important role in regulating the inflammatory response of microglia and maintaining the function of mDA cells.

Anti-Inflammatory Effects of PF

Following 24 hours of co-culture stimulation with LPS, the expression levels of inflammatory cytokines IL-1β, IL-6, and TNF-α in the supernatant significantly increased (Figure 5A–C), indicating that the inflammatory response induced by LPS in microglia cells at the bottom layer spread to the supernatant in the upper layer through co-culturing. After pretreatment with PF, the expression levels of inflammatory cytokines IL-1β, IL-6, and TNF-α induced by LPS stimulation were significantly reduced, with statistically significant differences compared to the LPS-only stimulated group ($P < 0.05$), suggesting that PF pretreatment effectively inhibits the expression of LPS-induced inflammatory cytokines. In microglia cells not stimulated by LPS, the expression levels of NF-κB and p-NF-κB were at basal levels, with NF-κB primarily existing in an inactive form in the cytoplasm, bound to IκB. After LPS stimulation, the expression levels of inflammation-related proteins TLR4, MYD88, and NF-κB significantly increased, and the expression level of p-NF-κB (the phosphorylated form of NF-κB) also significantly increased, indicating that NF-κB was activated and translocated to the nucleus, promoting the transcription of inflammatory cytokines (Figure 5D). However, after PF pretreatment, the increase in the expression levels of inflammation-related proteins induced by LPS stimulation was not significant, with statistically significant differences compared to the LPS-stimulated group ($P < 0.05$), and the expression level of p-NF-κB induced by LPS stimulation was significantly reduced, indicating that PF pretreatment effectively inhibits the activation of NF-κB induced by LPS. Additionally, the expression of NLRP3 inflammasome-related proteins NLRP3, caspase-1, and cleaved-caspase-1 also significantly increased after LPS stimulation (Figure 5E). After PF pretreatment, the expression level of cleaved-caspase-1 induced by LPS stimulation was significantly reduced, with statistically significant differences compared to the LPS-stimulated group ($P < 0.05$), suggesting that PF pretreatment effectively inhibits the activation of caspase-1 induced by LPS. PF pretreatment effectively alleviated LPS-induced inflammation by inhibiting LPS-stimulated NF-kappa B activation and NLRP3 body formation.

Construction and Verification of PD Model

We constructed a rat animal model of PD by stereotactic injection of 6-hydroxydopa, as shown in Figure 6A. To evaluate the behavioral alterations, we induced rotational behavior by intraperitoneal injection of apomorphine and recorded its

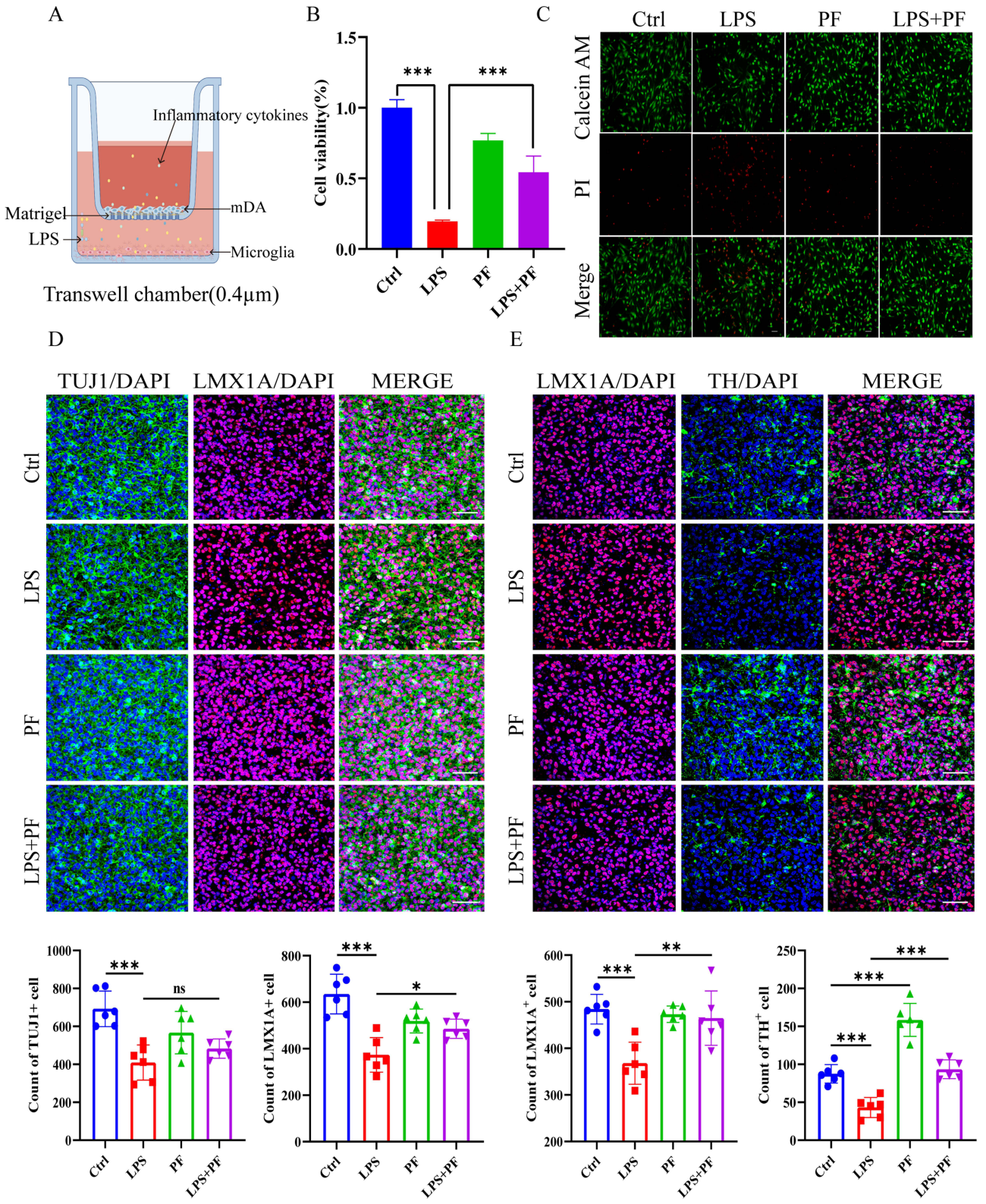

Figure 4 The influence of inflammation on dopaminergic cells. (**A**) Interactions between microglia and dopaminergic cells in a Transwell co-culture system; (**B**) The impact of LPS stimulation on the activity of dopaminergic cells under co-culture conditions; (**C**) Calcein/PI double staining for quantitative analysis of cell viability; (**D**)Changes in the number of dopaminergic cells under inflammatory conditions; (**E**) PF promotes the differentiation of dopaminergic neurons (n = 6 per group). Scale bar: 50 μm. *p < 0.05; **p < 0.01; ***p < 0.001; ns. indicates no statistical significance.

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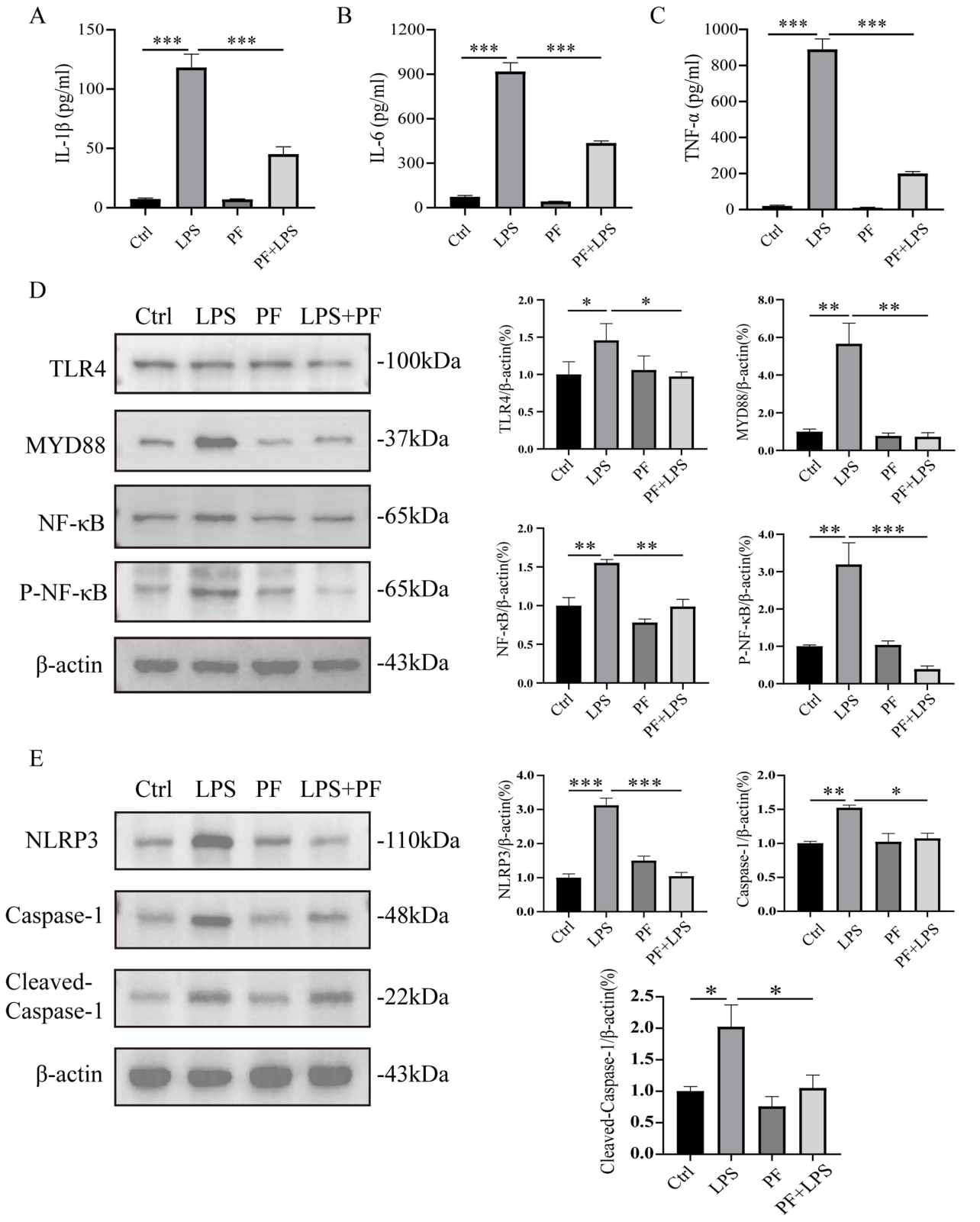

Figure 5 The anti-inflammatory mechanism of PF. (**A–C**) Changes in the expression of inflammatory cytokines IL-1β, IL-6, and TNF-α; (**D**) Activation of the TLR4/MYD88/NF-κB signaling pathway; (**E**) Formation of the NLRP3 inflammasome (n = 6 per group). *p < 0.05; **p < 0.01; ***p < 0.001.

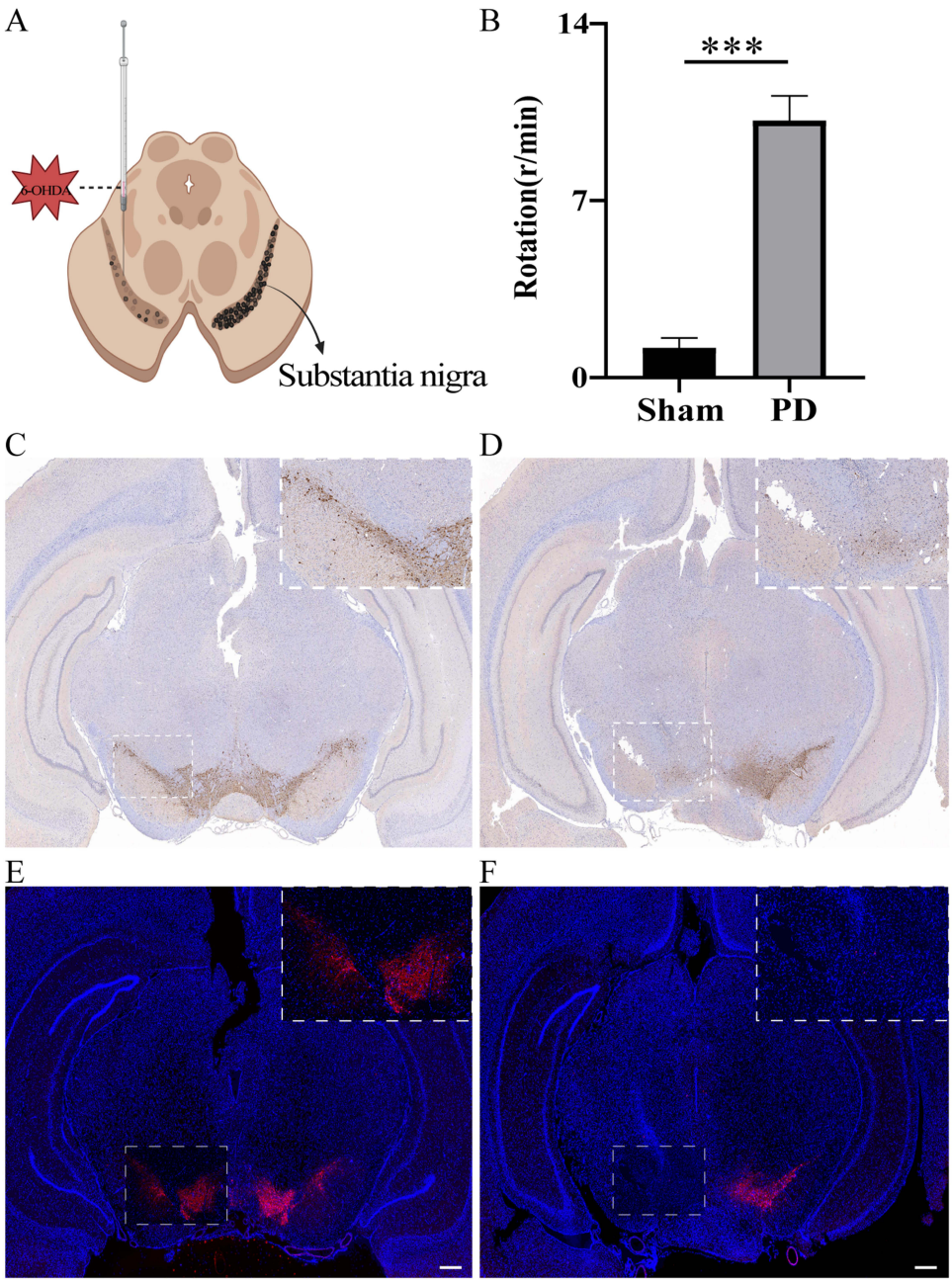

Figure 6 Experimental procedures and results for the construction and evaluation of a Parkinson's disease (PD) rat model. (**A**) Stereotactic injection of 6-hydroxydopamine (6-OHDA) into the substantia nigra to induce PD pathological features; (**B**) Assessment of behavioral responses in the PD model using apomorphine-induced rotational behavior tests (n=6); (**C** and **D**) Immunohistochemical staining showing differences in dopaminergic neurons in the substantia nigra coronal section between sham surgery (**C**) and PD model (**D**) groups; (**E** and **F**) Immunofluorescence staining further reveals the contrast of dopaminergic neurons in the substantia nigra coronal section between sham surgery (**E**) and PD model (**F**) groups. Scale bar: 500 μm. ***$p < 0.001$.

frequency. The results showed that the number of rotations in the PD model group was all more than 7 per minute, which was significantly higher than that in the sham-operated group (Figure 6B). This result indicates the successful construction of the PD animal model. Further, we performed cardiac perfusion treatment on the rat brain tissue and performed histoimmunochemical staining from the largest section of the substantia nigra. Compared with the sham-operated group (Figure 6C), the PD model group showed significant dopaminergic nerve cell loss in the unilateral substantia nigra region (Figure 6D). In addition, histochemical fluorescence staining confirmed significant loss of dopaminergic nerve cells on

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the PD modeling side under the action of 6-OHDA compared to the sham-operated group without 6-OHDA injection (Figure 6E and F).

Effect and Mechanism Verification of mDA and PF Transplantation

After PD model was stable for 4 weeks, it was used for cell transplantation. mDA cells were transplanted into the striatum of the side of the model, and samples were collected at the transplant site 4 weeks after surgery to detect the transplanted cell markers. The process of animal model construction and cell transplantation is shown in Figure 7A. The results of immunofluorescence staining for TH clearly showed significant differences among different treatment groups (Figure 7B). In the control group, the number of TH positive cells was very rare, and the distribution was very scattered, which fully reflected the large loss of dopaminergic neurons. The number of TH positive cells in the single mDA transplantation group was limited, and the cell morphology and distribution had not reached the ideal state. In contrast,

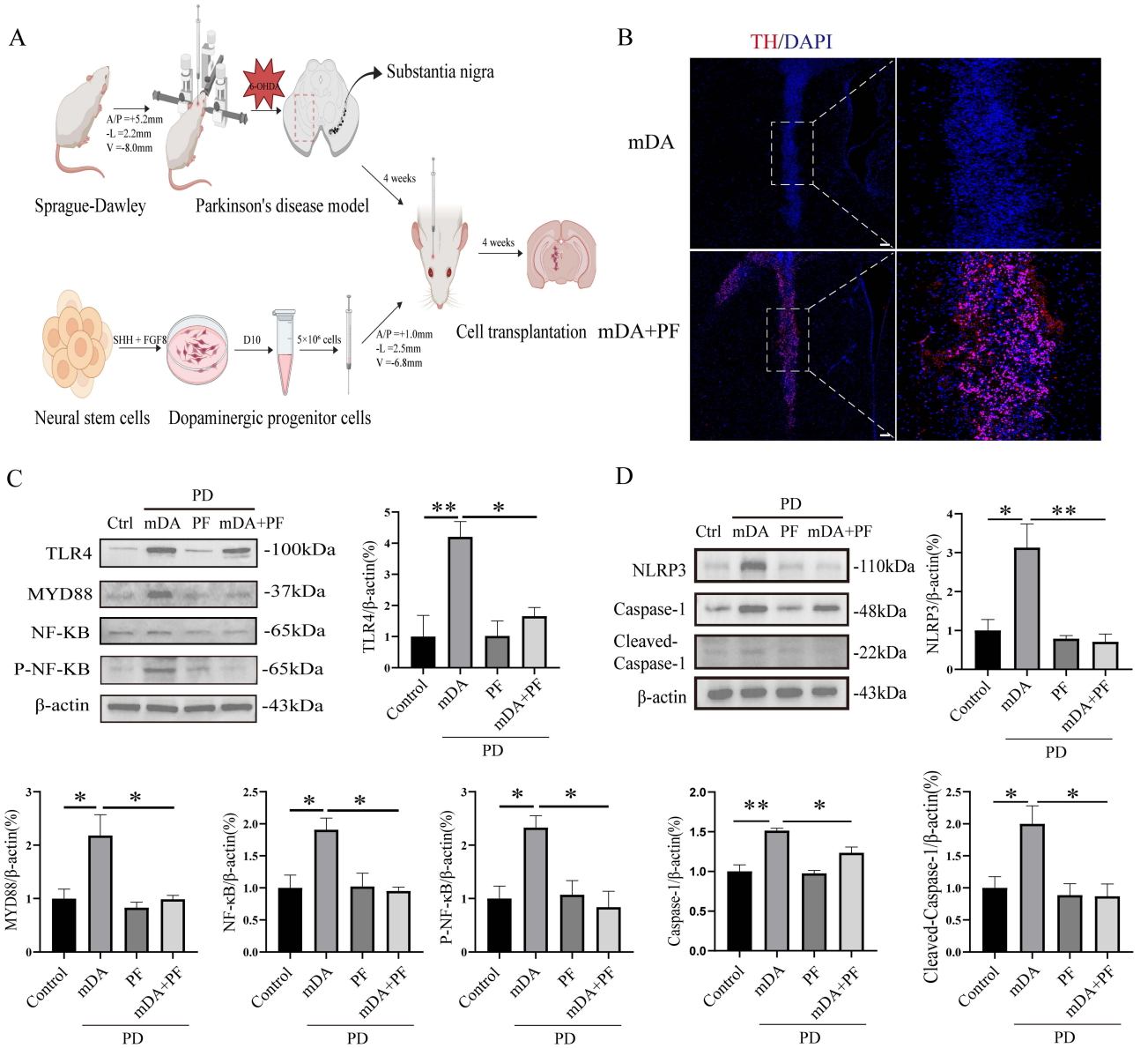

Figure 7 Experimental illustration of mesencephalic midbrain dopaminergic neurons (mDA) combined with paeoniflorin (PF) in the treatment of Parkinson's disease rats. (**A**) Construction of PD rat model and schematic diagram of cell transplantation; (**B**) Tyrosine hydroxylase (TH) immunofluorescence staining results show the survival of dopaminergic nerve cells transplanted with mDA alone and mDA combined with PF; (**C** and **D**) are the results of TLR4-MYD88-NF-kappa B signaling pathway and the expression of NLRP3 inflammatory body-related proteins (n=6). Scale bar: 200 μm. *p < 0.05; **p < 0.01.

the number of TH-positive cells in mDA + PF group increased significantly, and the distribution of TH-positive cells in the striatum was more extensive and uniform. These results strongly suggest that PF can significantly promote the differentiation and survival of dopaminergic neurons. In order to verify the anti-inflammatory mechanism of PF, in the detection of TLR4/MYD88/NF-kappa B signaling pathway-related proteins (Figure 7C), compared with the control group, the expression levels of TLR4, MYD88, NF-kappa B, and P-NF-kappa B in the mDA group were significantly increased ($P < 0.05$), which suggested that the mDA transplantation process may be activated by factors such as puncture injury, which may lead to inflammatory response. In the mDA + PF group, the expression level of the above proteins was significantly decreased compared with that in the mDA group ($P < 0.05$), which clearly indicated that PF could effectively inhibit the activation of this signaling pathway, thereby alleviating the inflammatory response. Significant differences were also observed in NLRP3 inflammator-associated proteins (Figure 7D). Compared with the control group, expression levels of NLRP3, Caspase-1, and activated Cleaved Caspase-1 were significantly increased in the mDA group ($P < 0.05$), indicating that mDA transplantation activated the NLRP3 inflammasome-related pathway and intensified the inflammatory response. The expression levels of these proteins in mDA + PF group were significantly lower than those in the mDA group ($P < 0.05$), further confirming that PF can inhibit the inflammatory response caused by transplantation puncture and maintain the stability of neural microenvironment.

Discussion

Parkinson's disease is a neurodegenerative disease dominated by movement disorders, and cell replacement therapy aims to improve movement symptoms in patients by replenishing missing dopaminergic neurons. Recent studies have demonstrated that dopaminergic neurons derived from human NSCs can effectively improve motor symptoms.[5] However, the low cell survival rate due to the traumatic inflammatory response associated with transplantation and the inflammatory microenvironment at the transplantation site leads to inconspicuous long-term symptom improvement.[8] In this study, we confirmed that PF can effectively inhibit the damage caused by inflammatory response to transplanted cells, and significantly promote cell survival and differentiation. In a rat model of PD, PF combined with mDA transplantation showed better cell survival and a higher level of differentiation than cell transplantation alone. Further studies showed that PF could significantly improve the neural microenvironment by regulating the inflammatory response, providing more favorable conditions for the survival and function of transplanted cells. This discovery provides a new idea and method for improving the efficacy of NSC transplantation in the treatment of PD and is expected to bring new breakthroughs in clinical treatment.

Cell replacement therapy using NSCs for PD has made significant progress in recent years, particularly in the treatment with dopaminergic precursor progenitor cells derived from NSCs. This therapeutic strategy involves differentiating NSCs into dopaminergic precursor progenitor cells to replace the loss of substantia nigra dopaminergic neurons caused by PD, thereby restoring dopamine levels in the brain and improving motor symptoms.[5] However, the acute inflammatory reaction caused by transplantation will have a direct and strong impact on the transplanted cells in a short time, seriously affecting their initial survival state.[8] Long-term chronic neuroinflammation at the transplant site continuously interferes with the survival microenvironment of cells;[14] In addition, the immune response of the host immune system to exogenous transplanted cells may also cause the transplanted cells to suffer damage, loss of function, and even rejection by the body.[15,16] In-depth research on the mechanism of transplant-related inflammation and the formulation of effective control measures have become the key to improve the effectiveness of cell replacement therapy, which is of vital significance to promote the clinical transformation of PD treatment.

The inflammatory response in the treatment of PD by cell transplantation is caused by many factors, including the immunogenicity of the transplanted cells, the surgical trauma during transplantation, and the change of the microenvironment at the transplant site. Exogenous or allogeneic surface antigens of transplanted cells are recognized by host antigen-presenting cells, activating T and B cells, and producing specific immune responses.[17–19] Even autologous cell transplantation can activate the innate immune system by recognizing injury-associated molecular patterns (DAMPs) such as high mobility group protein B1 (HMGB1), heat shock proteins (HSPs), or exogenous molecules expressed by the transplanted cells are recognized by Toll-like receptors (TLRs) and NOD-like receptors (NLRs), resulting in an inflammatory response.[14,20,21] Moreover, the release of inflammatory mediators can activate the NLRP3 inflammatory

corpuscles, enable immune cells to migrate to the graft site, further strengthen the local neuroinflammatory response, and have a toxic effect on the transplanted cells, affecting the transplantation effective.[22,23] Tissue injury and cell destruction release DAMPs, a large number of pro-inflammatory mediators, including pro-inflammatory cytokines (IL-1β, IL-6, TNF-α), chemokines (MCP-1, IL-8), and complement components (C3a, C5a), which promote inflammation and trigger a cascade of reactions through recruitment and activation of immune cells.[24–26] Our study focused on the acute pro-inflammatory cascade induced by LPS, which primarily activates M1 polarization and upregulates IL-1β, IL-6, TNF-α, and the TLR4/NF-κB/NLRP3 pathway. While Arg1 and IL-10 are important markers of anti-inflammatory and M2-like microglial responses, Arg1 and IL-10 expression levels under these conditions are typically low unless M2-polarizing stimuli are present. Therefore, we prioritized evaluating PF's inhibitory effects on the dominant pro-inflammatory axis. In addition, surgical trauma may disrupt the blood–brain barrier, making it easier for peripheral immune cells to enter the central nervous system, participating in the local inflammatory response together with the microglia of central nervous cells.[27,28] Surgical trauma can also lead to the release of a large number of ROS and oxygen-free radicals, which damage cell membranes, proteins, and DNA, and further exacerbate the inflammatory response.[29,30] The long-term chronic inflammatory microenvironment of PD may also exacerbate the immune response after cell transplantation. Persistent microinflammatory states inhibit the survival, differentiation, and functional integration of transplanted cells, thus affecting the transplant efficacy.[31,32]

Anti-inflammatory drugs and immunomodulators were used to improve the microenvironment, regulate microglia to reduce inflammatory response, and optimize the microenvironment of transplanted cells to promote the survival and integration of transplanted cells.[9,33,34] Nonsteroidal anti-inflammatory drugs can reduce the production of pro-inflammatory prostaglandins by inhibiting cyclooxygenase.[35] Immunomodulators (cyclosporine, tacrolimus) inhibit the activation and proliferation of T cells and reduce immune-mediated rejection.[36,37] Improve the microenvironment of transplantation to reduce inflammation and promote cell integration by modulating the microenvironment of the transplanted area. Neurotrophic factors such as BDNF and GDNF, promote the survival and regeneration of nerve cells and regulate the inflammatory response.[38,39] Antioxidants such as N-acetylcysteine, vitamin E, protect transplanted cells from damage by ROS by reducing oxidative stress levels.[40] In this study, the mechanism of action of PF in the treatment of PD cell transplantation, mainly through its anti-inflammatory properties to improve the survival rate and functional recovery of transplanted cells. In our Transwell co-culture system, LPS-stimulated microglia in the lower chamber release pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) that diffuse across the porous membrane to the upper chamber, triggering secondary inflammatory responses in midbrain dopaminergic neurons. Our results confirm this inflammatory cascade: elevated cytokine levels, neuronal damage, and upregulation of TLR4, MyD88, and p-NF-κB indicate activation of the TLR4/NF-κB signaling pathway. Furthermore, the suppression of these responses by paeoniflorin highlights its anti-inflammatory potential through modulation of this signaling axis. During cell transplantation, damaged tissues and cells cause an acute inflammatory response. PF effectively inhibits the acute inflammatory response in the transplanted area by inhibiting the activation of microglia and reducing the release of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6). Toll-like receptors, especially TLR4, play an important role in the initiation of neuroinflammation.[41] PF can inhibit the expression of TLR4 and its downstream signaling pathway, thereby reducing the pro-inflammatory response. The NLRP3 inflammasome plays an important role in neuroinflammation and the pathology of PD, and its activation leads to further release of inflammatory factors such as IL-1β and IL-18.[42,43] In the long term after transplantation, chronic inflammation persists at the transplantation site. PF can effectively reduce chronic inflammation and maintain functional stability of transplanted cells by inhibiting the activation of NF-κB and NLRP3 inflammasome, thus providing a more favorable survival environment for transplanted cells.

This study acknowledges several methodological constraints. Although previous studies have demonstrated that paeoniflorin can cross the blood–brain barrier and exert central effects in rodent models, direct pharmacokinetic measurements were not performed in our study. Given that BBB permeability may be altered in pathological conditions such as Parkinson's disease or neuroinflammation, it is plausible that PF reached the brain parenchyma at therapeutically relevant concentrations. Nevertheless, we acknowledge the absence of direct CNS distribution data as a limitation, and future work will incorporate tissue-level quantification and tracer studies to verify BBB penetration under our dosing regimen. Behavioral assessments relied exclusively on apomorphine-induced rotation tests, which validated lesion

severity and motor asymmetry but omitted nuanced evaluations (eg, cylinder, stepping tests) critical for quantifying fine motor control and postural stability, thereby restricting comprehensive functional interpretation. Inflammatory modeling faced dual limitations: the LPS-stimulated microglial system effectively mimicked acute TLR4-driven inflammation but neglected sustained astrocyte reactivity, α-synuclein pathology, and adaptive immune crosstalk inherent to chronic PD neuroinflammation; similarly, the 6-OHDA model induced acute dopaminergic lesions but failed to recapitulate progressive α-synucleinopathy and complex chronic inflammation. While our observation period confirmed short-term efficacy, extended chronic PD models are needed to assess PF's sustained therapeutic benefits. Mechanistic analyses prioritized TLR4/NF-κB and NLRP3 pathways but overlooked PF's potential synergies with mitochondrial protection or autophagy—pathways central to α-synuclein clearance. Finally, the Transwell co-culture system clarified microglia-neuron interactions but excluded astrocytes and peripheral immune cells, oversimplifying the in vivo neuroinflammatory niche and leaving PF's immunomodulatory effects on astrocyte reactivity and T-cell infiltration unexplored. Addressing these gaps through multicellular systems, chronic models, and pathway interaction studies will enhance translational relevance and mechanistic depth.

Conclusion

Paeoniflorin, as a neuroprotective and anti-inflammatory agent, can effectively inhibit the damage of inflammatory response to transplanted cells, significantly promote cell survival and differentiation, and improve the efficacy of cell transplantation in the treatment of PD, especially in improving cell survival, promoting differentiation, and regulating inflammatory response. This study provides a new theoretical basis for PF combined cell transplantation in the treatment of PD, and provides a new idea and method for improving the efficacy of NSC-based cell transplantation in the treatment of PD, and is expected to promote a breakthrough in clinical treatment.

Data Sharing Statement

The datasets used during the study are available from the corresponding author on reasonable request.

Ethics Approval

The present study was approved by the Ethics Committee of Peking University People's Hospital (No.2021PHE042).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This work was supported by funding from the National key research and development program of China (2019YFE0117100), the National Natural Science Foundation of China (82104603, 82074174), Peking University People's Hospital Research and Development Fund (RS2021-12 and RDX2021-08).

Disclosure

The authors declare that they have no competing interests.

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