Frontiers in Bioscience Landmark

Front. Biosci. (Landmark Ed) **2026**; 31(6): 47325
https://doi.org/10.31083/FBL47325

Original Research

Icariin Attenuates Neuroinflammation and Dopaminergic Degeneration in a Rodent Model of Parkinson's Disease by Promoting the Expansion of Regulatory T Cells

Sicong Xu[1,†], Li Jiang[1,†], Hao Zhang[1], Ping Zhang[1], Zhao Pan[1,*]

[1]Department of Neurology, The Affiliated Nanhua Hospital, Hengyang Medical School, University of South China, 421002 Hengyang, Hunan, China
*Correspondence: pzxnwh@163.com (Zhao Pan)
[†]These authors contributed equally.

Academic Editors: Stepan Dzhimak and Xudong Huang
Submitted: 13 October 2025 Revised: 5 May 2026 Accepted: 14 May 2026 Published: 26 June 2026

Abstract

Background: Icariin, a bioactive flavonoid, exhibits significant neuroprotective properties and has emerged as a promising candidate for preventing neurodegenerative diseases, including Parkinson's disease (PD). However, the mechanism underlying its action is not fully understood. Enhancing the function and frequency of peripheral regulatory T cells (Tregs) may mitigate dopaminergic degeneration. This study investigates the role of Tregs in icariin's neuroprotective effects in a rodent model of PD. **Methods**: PD was induced in mice via stereotactic injection of 6-hydroxydopamine (6-OHDA). Mice underwent pretreatment with saline, icariin, an androgen receptor inhibitor (ARI), or a combination of icariin and ARI. Motor function was assessed in each experimental group, and dopaminergic neuronal injury was evaluated using immunohistochemistry (IHC) staining for tyrosine-hydroxylase (TH) in the substantia nigra (SN) and striatum. IHC was used to quantify $CD4^+$ T-cell infiltration in the SN. Neuroinflammation was assessed through mRNA levels of the pro-inflammatory M1 phenotype of microglia, the anti-inflammatory M2 phenotype of microglia, and the levels of indicated pro-inflammatory cytokines in the SN. The frequency of Tregs among peripheral blood mononuclear cells (PBMCs) was analyzed by flow cytometry, and with Tregs were depleted using PC61 monoclonal antibodies. *In vitro* androgen receptor (AR) knockdown using shRNA in naïve $CD4^+$ T cells was performed to validate the AR-dependent mechanism. **Results**: The results revealed that icariin significantly alleviated dopaminergic degeneration. Mechanistically, icariin promotes the expansion of peripheral neuroprotective and immunosuppressive Tregs, thereby restricting $CD4^+$ T cell migration into the SN. This improvement in the inflammatory microenvironment reduced neuroinflammation and mitigated neurodegeneration. However, the neuroprotective and anti-inflammatory effects of icariin were lost when combined with ARI. Additionally, Treg depletion before 6-OHDA injection reversed the positive effects observed in the PD model. **Conclusions**: Icariin protects against neurodegeneration and neuroinflammation by boosting Tregs expansion in an androgen receptor-dependent manner.

Keywords: icariin; Parkinson's disease; androgen receptor; regulatory T cell

1. Introduction

Parkinson's disease (PD) is characterized by progressive loss of dopaminergic neurons within the nigrostriatal system [1]. The standard of care for PD is dopamine replacement therapy, which provides short-term relief but has limited long-term effectiveness. Side effects of dopamine treatment include gastrointestinal symptoms, symptom fluctuations, dyspraxia, and psychiatric symptoms, all of which can limit the patient's willingness to continue the medication [2]. This highlights the urgent need to find alternative therapeutic interventions for PD. Icariin, a bioactive isoprenylated flavonoid derived from plants in the genus Epimedium [3], exhibits a wide range of antioxidant, antibacterial, anti-inflammatory, and immunomodulatory properties [4]. Many studies have demonstrated that icariin has neuroprotective effects in various neurological conditions, including Alzheimer's [5], PD [6], cerebral infarction [7], and depression [8]. However, the mechanisms by which icariin confers these benefits in PD remain unclear.

Traditionally considered an immunologically privileged site, the brain is now recognized as a target of peripheral immune activity [9]. Notably, post-mortem analyses of PD brains frequently reveal $CD4^+$ T cell infiltrates, implicating adaptive immunity in disease pathogenesis [10,11]. $CD4^+$ T cells consist of neuroprotective regulatory (Treg) and neurodestructive effector (Teff) subsets. A shift in the Treg/Teff ratio towards a pro-inflammatory state is believed to facilitate T-cell migration into the brain parenchyma, thereby exacerbating neuroinflammation and dopaminergic degeneration [12]. Therapeutic strategies that enhance Treg function and increase their numbers can effectively alleviate neuroinflammation and mitigate neuronal degeneration [13]. Specifically in PD, a decrease in peripheral Treg number and function has been correlated with disease progression, while strategies to enhance Tregs have shown neuroprotective potential in preclinical models [12,13,14].

Icariin exhibits androgen-like properties, acting as a ligand for the androgen receptor (AR) [15]. AR signaling modulates immune responses by suppressing effector

T cell functions and promoting an immune-tolerant state, as seen in oncology research [16]. Specifically, within the $CD4^+$ T cell compartment, AR activation can skew differentiation away from pro-inflammatory Th1/Th2 lineages and towards the Treg phenotype [17]. Despite existing knowledge, the direct link between icariin's neuroprotection in PD and AR-driven immunomodulation, particularly through the expansion of Tregs, remains unexplored. Therefore, this study aimed to investigate whether icariin in a PD model is mediated through AR-dependent enhancement of Treg populations.

In this study, we investigated the therapeutic role of icariin in PD, and hypothesized that AR-mediated Tregs expansion might play a key role in this mechanism.

2. Materials and Methods

2.1 Animals and Treatment

This study was approved by the ethics committee of Affiliated Nanhua Hospital (Approval number: 2024-KY-024). C57BL/6JGpt mice (Strain NO. N000013) aged 8–10 weeks and weighing 25–30 g were utilized as experimental subjects. Animals with overt behavioral abnormalities, signs of infection, or significant weight loss (>20% of initial body weight) during the experiment were excluded from further analysis. The animals were purchased from GemPharmatech (Nanjing, China) and were housed in groups of five per cage under specific pathogen-free (SPF) conditions at Hunan Evidence Based Biotechnology Co., Ltd, Hengyang, China. All mice were housed and maintained under identical conditions. Cage positions were randomized on the rack, and the order of treatments and measurements was randomized to minimize potential confounders. Before the experimental procedures, mice were trained on the behavioral apparatus for 2 days, and those exhibiting insufficient equilibrium function were excluded from subsequent experiments. Sample size was determined based on the results of the preliminary experiment. For evaluating the therapeutic effects of icariin (ICA, HY-N0014, MedChemExpress, Monmouth Junction, NJ, USA) in the PD model, mice were randomly assigned to distinct treatment groups. The randomization sequence was generated using a computer-based random number generator. One group received saline (n = 20); another group received ICA (n = 20); a third group received an androgen receptor inhibitor (ARI, Enzalutamide, HY-70002, MedChemExpress, Monmouth Junction, NJ, USA) (n = 10); and a combined treatment group received both ICA and ARI (n = 10). ICA and enzalutamide were administered separately via oral gavage once daily. ICA was suspended in 0.5% carboxymethylcellulose (CMC-Na, 419273, Sigma-Aldrich, St. Louis, MO, USA) and given at a dose of 20 mg/kg. Enzalutamide was suspended in 1% CMC-Na with 0.1% Tween 80 (P1754, Sigma-Aldrich, St. Louis, MO, USA) and given at a dose of 10 mg/kg. To minimize potential drug interactions, the two drugs were administered sequentially with an interval of at least 8 hours. These interventions were conducted over a consecutive seven-day period. On the eighth day, ten mice were randomly selected from both the saline and ICA groups for the induction of PD through stereotactic injection of 6-hydroxydopamine (6-OHDA, HY-B1081, MedChemExpress, Monmouth Junction, NJ, USA) [18]. The mice were anesthetized via intraperitoneal injection of 1.25% avertin (MA0478-2, MeilunBio, Dalian, China) at a dose of 30 µL/g, with the anesthetic efficacy validated in prior pilot experiments. Following anesthesia, the mouse heads were securely fixed in a stereotaxic frame. After disinfection, the scalp was incised along the midline. The PD model was established by stereotaxic microinjection of 6-OHDA into the striatum. The injection was precisely guided relative to the bregma (coordinates: Posterior: 2.8 mm; Lateral: 1.3 mm; Ventral to the surface of the dura mater: 4.5 mm, 6 µg per mouse). Sham-operated mice served as controls. Following surgery, wounds were sterilized, sutured, and animals were returned to standard housing for postoperative care and continued feeding. Subsequent behavioral tests were performed on days 10 and 11, followed by the collection of specified biological samples (Fig. 1A). To evaluate the association between anti-inflammatory properties of Icariin and the expansion of Tregs, the mice were randomly assigned to: the saline group (n = 20), the ICA-treated group (n = 20), or the ICA + 6-OHDA-treated group (n = 20). After a seven-day pretreatment interval, the mice were intraperitoneally injected with PC61 (102002, BioLegend, San Diego, CA, USA) to deplete Tregs *in vivo*, with IgG1 (02-6100, Thermo Fisher Scientific, Waltham, MA, USA) administered as an isotype control. PC61 and IgG1 were administered at 150 µg/mouse once daily. All antibodies were diluted in sterile Phosphate-Buffered Saline (PBS, PB180327, Procell, Wuhan, China) to a final volume of 300 µL per mouse. On day 10, the mice underwent either stereotaxic injections or sham operations, followed by additional behavioral tests (Fig. 1B). After behavioral tests, mice were euthanized by cervical dislocation in accordance with American Veterinary Medical Association (AVMA) guidelines. The investigator conducting group allocation was aware of the assignments. All other investigators involved in animal handling, treatment administration, outcome assessment, and data analysis were blinded to group allocation.

2.2 Behavioral Test

2.2.1 Pole Test

The experimental protocol began with placing the mouse atop the vertical pole, with the head facing upwards. T-Turn was recorded as the time it took for the mouse to turn around at the top of the pole and begin descending headfirst. The time it took the mouse to climb from the top of the pole to the ground was recorded as T-Down [19].

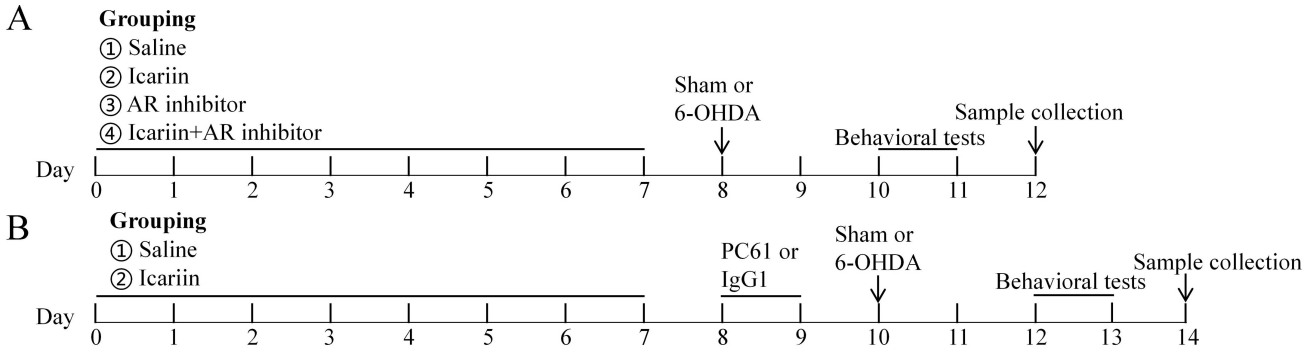

Fig. 1. Flow chart showing the experimental schedules. (A) The schedule used for evaluating the effects of icariin treatment on PD. (B) The schedule used to assess the effects of icariin treatment on PD following Treg depletion. AR, androgen receptor; 6-OHDA, 6-hydroxydopamine; PD, Parkinson's disease; Treg, regulatory T cells.

2.2.2 Rotarod Test

Mice were placed on a rotating rod with an incremental increase in rotational speed, ultimately reaching 40 revolutions per minute (rpm). The time elapsed until the mice fell off the rod was meticulously recorded, with a maximum threshold of 300 seconds assigned to those that remained on the rod for beyond this duration. The experiment was repeated three times, and the mean value for each mouse was recorded [19].

2.2.3 Apomorphine-Induced Rotation Test

To evaluate dopaminergic asymmetry and determine lesion severity, mice were injected with apomorphine (APO, 0.5 mg/kg, HY-12723, MedChemExpress, Monmouth Junction, NJ, USA) intraperitoneally to induce rotational behavior. Subsequently, the number of rotations toward the unlesioned side was recorded over a 30-minute observational period [18].

2.3 Immunohistochemistry

Following transcardial perfusion with saline and 10% paraformaldehyde (PFA), brains were extracted and fixed in 4% PFA. Tissue samples were then cryoprotected using a graded sucrose series (5%, 2.5%, and 1%, each for 48 hours). Coronal sections were cut and immunohistochemically stained. Briefly, sections were incubated overnight with primary antibodies targeting tyrosine hydroxylase (TH; 1:300, ab137869, Abcam, Cambridge, MA, USA) or CD4 (1:300, ab183685, Abcam, Cambridge, MA, USA). After washing, sections were treated with HRP-conjugated anti-rabbit IgG secondary antibody (1:50, A0210 Beyotime, Shanghai, China) and visualized under a light microscope.

2.4 Molecular Docking

The structural representations of the compound Icariin were retrieved from the PubChem database. The crystal structure of the AR target protein was acquired from the PDB database and subsequently processed using the Schrodinger Maestro software (Maestro 11.9 platform, San Diego, CA, USA). The protein was processed with Schrödinger's Protein Preparation Wizard to remove crystallization water, add missing hydrogen atoms, correct incomplete bond information, and restore missing peptide segments. After that, energy minimization and geometric optimization of the protein were carried out. Finally, we performed molecular docking and screening using the Standard Precision (SP) method to analyze the interaction between the compound and the target protein, particularly the specific contacts with key protein residues. This analysis allowed for an assessment of whether the screened compound exhibited a significant active effect based on its docking score.

2.5 Quantitative Reverse Transcription Polymerase Chain Reaction

Tissue was lysed using TRIzol reagent (Thermo Fisher, Waltham, MA, USA, catalog: 15596018CN), followed by the addition of chloroform to separate the aqueous and organic phases for RNA extraction. The extracted RNA was reverse-transcribed into cDNA using reverse transcriptase (Takara, Tokyo, Japan, catalog: RR047A). Real-time PCR was performed on the 7300 Plus real-time PCR System (Thermo Fisher, Waltham, MA, USA) using SYBR Premix Ex Taq (Takara, Tokyo, Japan, DRR041A). The reaction mixture consisted of 5 pmol of primer, 10 µL of 2× PCR master mix, 1 µL of cDNA template, and sufficient distilled water to achieve the final reaction volume. The following conditions were applied for thermocycling: Predenaturation at 95 ℃ for 15 s, followed by 40 cycles of denaturation at 95 ℃ for 10 s, and annealing at 60 ℃ for 30 s. The $2^{-\Delta\Delta Cq}$ method was used to calculate relative quantitative values. GAPDH was used as an internal reference. Primers used in this study are listed in Table 1.

2.6 Flow Cytometry

Peripheral blood mononuclear cells were harvested using a commercially available kit (Beyotime, Shanghai, China, Catalog: C0029S). Cells were fixed, permeabi-

Table 1. Primer used in this study.

Target gene	Forward primer sequence	Reverse primer sequence
iNOS	GGTGAAGGGACTGAGCTGTT	ACGTTCGTTCTCTTGCA
Arg-1	CACCTGAGCTTTGATGTCG	TGAAAGGAGCCCTGTCTTG
CD206	AAGGAAGGTTGGCATTTGT	CTTTCAGTCCTTTGCAAGC
GAPDH	GCCAAGGCTGTGGGCAAGGT	TCTCCAGGCGGCACGCAGA
TNF-α	GGAAAGCATGATCCGAGATG	CAGTAGACAGAAGAGCGTGGTG

lized, and then incubated with antibodies against CD4 (0.25 µg/test, eBioscience, San Diego, CA, USA, catalog: 11-0040-81), followed by CD25 (0.125 µg/test, eBioscience, San Diego, CA, USA, catalog: 17-0390-82) and FOXP3 (1 µg/test, eBioscience, San Diego, CA, USA, catalog: 12-5773-80). Data were acquired using a flow cytometer (CytoFLEX, Beckman Coulter, Miami, FL, USA). The gating strategy was as follows: lymphocytes were first gated based on forward scatter area (FSC-A) versus side scatter area (SSC-A) to exclude debris. Doublets were excluded by gating on FSC-width (FSC-W) versus FSC-H and SSC-W versus SSC-H to select single cells. Subsequently, $CD4^+$ T cells were gated from the single-cell population, and the percentages of $CD25^+FOXP3^+$ regulatory T cells (Tregs) were analyzed within the $CD4^+$ population.

2.7 In Vitro Treg Differentiation and AR Knockdown

Naïve $CD4^+$ T cells were isolated from spleens of C57BL/6J mice using a naïve $CD4^+$ T cell isolation kit (Vazyme, Nanjing, China, catalog: CS105-01). The purity (>95% $CD4^+$, >90% $CD62L^+$, <2% $CD25^+$) was confirmed by flow cytometry. Purified cells were transduced with lentivirus expressing AR shRNA (Lv-AR) or negative control scramble shRNA (Lv-NC). The AR shRNA target sequence was 5′-CCGCCGACATTAAAGACATTT-3′ (Gene ID: 11835, mouse); the scramble shRNA sequence was 5′-TTCTCCGAACGTGTCACGT-3′. After transduction, cells were resuspended in RPMI-1640 medium (11875093, Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, A5669801, Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (A5873601, Gibco, Grand Island, NY, USA), and cultured at 37°C in a humidified atmosphere containing 5% CO_2. Cells were then divided into four groups: (1) Lv-NC (negative control lentivirus); (2) Lv-NC + Icariin; (3) Lv-AR (lentivirus expressing AR shRNA); (4) Lv-AR + Icariin. Treg polarization was induced with anti-CD3 (2 µg/mL), anti-CD28 (2 µg/mL), TGF-β (2 ng/mL) and IL-2 (100 U/mL). Icariin (Sigma-Aldrich, St. Louis, MO, USA, catalog: I1286-1G) was added at the beginning of culture and replenished every 2 days. After 5 days, cells were harvested for flow cytometry analysis. For surface staining, cells were incubated with CD4 (0.25 µg/test, eBioscience, San Diego, CA, USA, catalog: 11-0040-81) and CD25 (0.125 µg/test, eBioscience, San Diego, CA, USA, catalog: 17-0390-82) for 30 minutes at room temperature in the dark. After washing, cells were fixed with Intracellular Fixation Buffer for 30 minutes at room temperature, then permeabilized with 1× Permeabilization Buffer. For intracellular staining, cells were incubated with Foxp3 (1 µg/test, eBioscience, USA, catalog: 12-5773-80) for 30 minutes at room temperature in the dark. Stained cells were analyzed using a flow cytometer (Beckman CytoFLEX, Miami, FL, USA). The percentage of $CD4^+CD25^+Foxp3^+$ cells among $CD4^+$ T cells was recorded for each group. Statistical analysis for the *in vitro* experiment was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. All data are presented as mean ± standard error of the mean (SEM). A $p$value < 0.05 was considered statistically significant.

2.8 Enzyme-Linked Immunosorbent Assay

Levels of pro-inflammatory cytokines (IL-6, IL-1β, TNF-α) in midbrain tissue block containing the SN were quantified using commercial ELISA kits (Abcam, ab222503, ab197742, ab208348) according to the manufacturer's protocols.

2.9 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Peripheral blood was obtained via orbital sinus puncture, and red blood cells were lysed by treatment with a commercial lysis buffer (Beyotime, China) for 15 minutes. The cell suspension was centrifuged, and the pellet was resuspended and subjected to density gradient centrifugation with lymphocyte separation liquid (Solarbio, China; P8620) at 2000 rpm for 20 minutes. Cells from the interface layer were carefully aspirated and transferred into a 15 mL tube. Finally, PBMCs collected from the interface layer were harvested for further use.

2.10 Statistics

Statistical analyses were performed using IBM SPSS Statistics 25.0 (IBM Corp., Armonk, NY, USA). The data are presented as mean ± SEM. The normality of data distribution was assessed using the Shapiro-Wilk test, while the Levene's test was used to confirm homogeneity of variances. For comparisons involving three or more experimental groups, one-way analysis of variance (ANOVA) was performed. When a significant main effect was detected (p < 0.05), planned pairwise comparisons between groups of

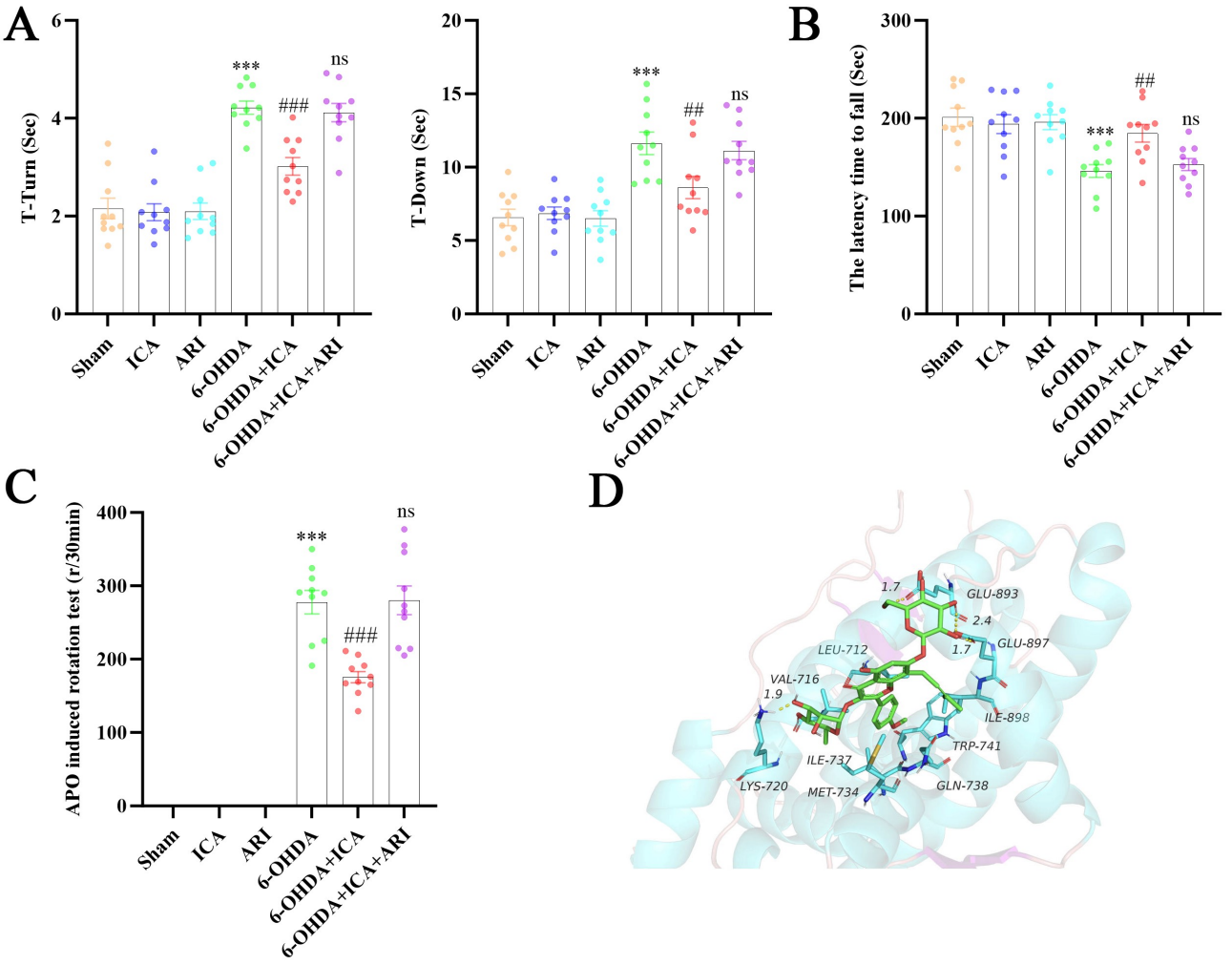

Fig. 2. Icariin induces functional improvement in the PD model. (A) Pole test in each group (n = 10). (B) Rotarod test in each group (n = 10). (C) Apomorphine-induced rotation test (n = 10). (D) Molecular docking analysis. ***$p < 0.001$ compared to the sham group; $^{###}p < 0.001$, $^{##}p < 0.01$ compared to the 6-OHDA group. ns: not significant, compared to the 6-OHDA group. ICA, icariin; ARI, androgen receptor inhibitor.

specific interest were conducted using Fisher's Least Significant Difference (LSD) post hoc test. This approach was chosen to concentrate on specific, pre-defined contrasts rather than all possible group pairings. The 95% confidence intervals (CI) were calculated and reported where applicable, with a significance threshold set at $p < 0.05$.

3. Results

3.1 Icariin Improves the Motor Performance in the PD Model

Motor function was assessed using the pole, rotarod, and apomorphine-induced rotation tests. As expected, 6-OHDA-lesioned mice exhibited significant motor deficits compared to sham controls (pole test: 95% CI: 3.91–4.52, T-turn: $p < 0.001$; 95% CI: 9.90–13.36, T-down: $p < 0.001$; rotarod test: 95% CI: 131.49–160.97, $p < 0.001$; rotation: 95% CI: 241.70–313.90, $p < 0.001$; Fig. 2A–C). Icariin pretreatment substantially ameliorated these deficits, improving performance in the pole (95% CI: 2.61–3.43; T-turn: $p < 0.001$; 95% CI: 6.89–10.32, T-down: $p = 0.0012$; Fig. 2A) and rotarod (95% CI: 164.01–205.21, $p = 0.002$, Fig. 2B) tests. Importantly, this beneficial effect was abolished upon co-administration of an androgen receptor (AR) inhibitor (Fig. 2A,B). Molecular docking further confirmed a stable interaction between icariin and AR, with a binding energy of –7.04 kcal/mol (Fig. 2D), supporting a direct AR-engagement mechanism. In addition, the binding energy between ICA and AR was –7.04 kcal/mol, indicating a strong interaction (a binding energy less than –6 kcal/mol) and a high degree of structural compatibility. These data suggest that icariin improves motor performance in the PD model through an AR-dependent mechanism.

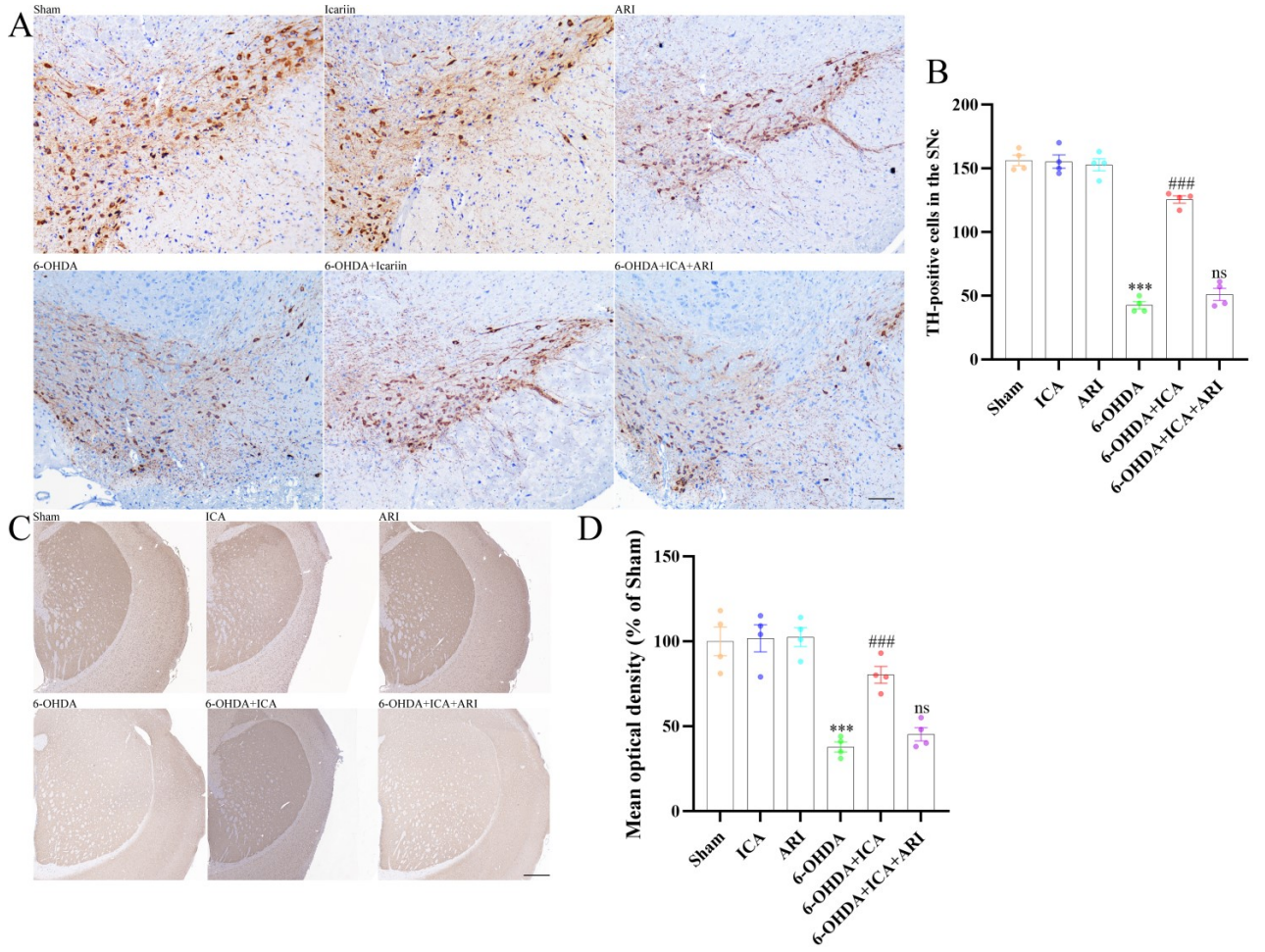

Fig. 3. Icariin protects against dopaminergic neuronal degeneration in the PD model. (A) Representative IHC for TH staining in the SN. Scale bar = 100 μm. (B) Bar graph showing TH-positive cells in the SNc. n = 4 per group. (C) Representative IHC for TH staining in the striatum. Scale bar = 200 μm. (D) Bar graph showing the mean optical density of TH in the striatum. n = 4 per group. ***$p < 0.001$ compared to the sham group; $^{###}p < 0.001$ compared to the 6-OHDA group. ns: not significant, compared to the 6-OHDA group. ICA, icariin; ARI, androgen receptor inhibitor; IHC, immunohistochemistry; TH, tyrosine-hydroxylase; SN, substantia nigra.

3.2 Icariin Protects Against Neuronal Degeneration in the PD Model

Having established that ICA improves motor function, we next asked whether it also confers neuroprotection at the cellular level. We conducted immunohistochemistry (IHC) for tyrosine-hydroxylase (TH) in the substantia nigra (SN) (Fig. 3A) and striatum (Fig. 3C) to investigate the potential neuroprotective actions in the PD model. Icariin supplementation remarkably attenuated 6-OHDA-induced dopaminergic neuronal injury, as evidenced by the increase in TH-positive cells in the SNc (95% CI: 116.27–134.73, $p < 0.001$, Fig. 3B) and mean intensity of TH in the striatum (95% CI: 64.59–95.92, $p < 0.001$, Fig. 3D). No significant differences were found between the 6-OHDA-treated group and the group that received the combination treatment with 6-OHDA+ICA+ARI. These findings suggest that icariin protects against neurodegeneration in the PD model in an AR-dependent manner.

3.3 Icariin Restrains Neuroinflammation in the PD Model

Icariin administration markedly altered the microglial activation profile in PD mice, characterized by a downregulation of pro-inflammatory M1 markers (95% CI: 176.57–268.43, Inducible Nitric Oxide Synthase, iNOS: $p < 0.001$; 95% CI: 212.86–355.64, TNF-α: $p < 0.001$; Fig. 4A) coupled with an upregulation of anti-inflammatory M2 markers (95% CI: 43.07–70.93, CD206: $p < 0.001$; 95% CI: 57.90–82.60, Arg-1: $p < 0.001$; Fig. 4B). Concordantly, icariin treatment suppressed the elevated levels of pro-inflammatory cytokines (95% CI: 26.28–50.72, TNF-α: $p < 0.001$; 95% CI: 39.77–52.89, IL-1β: $p < 0.001$; 95% CI: 30.50–45.51, IL-6: $p < 0.001$; Fig. 4C). All these anti-inflammatory effects were dependent on AR signaling, as they were not observed in the group co-treated with the AR inhibitor.

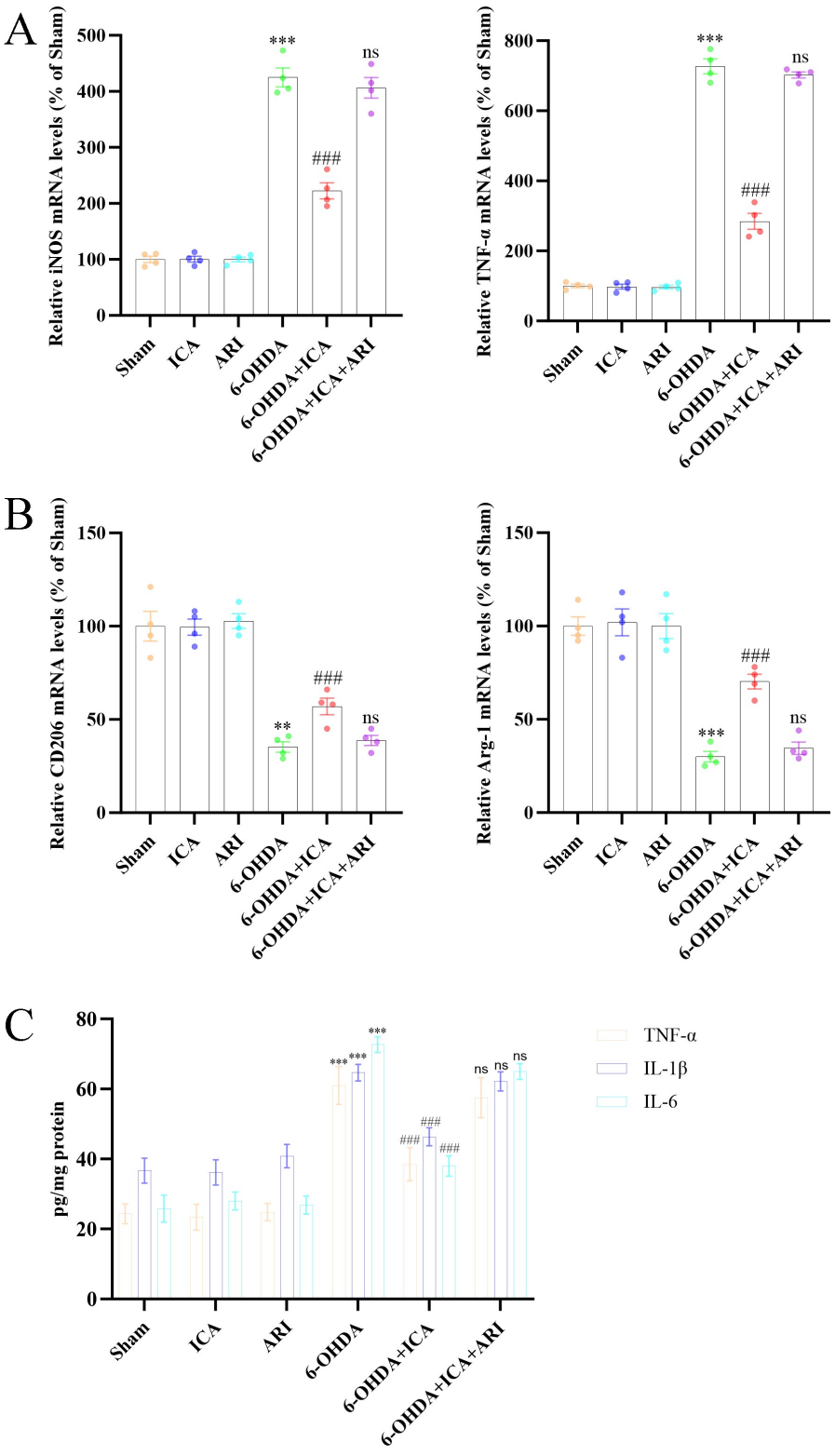

Fig. 4. Icariin restrains neuroinflammation in the PD model. (A) The mRNA abundance of the indicated microglial M1 phenotype markers in each group (n = 4). (B) The mRNA abundance of indicated microglial M2 phenotype markers in each group (n = 4). (C) The levels of indicated pro-inflammatory cytokines were quantified using ELISA (n = 6). $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the sham group; $^{\#\#\#}p < 0.001$ compared to the 6-OHDA group. ns: not significant, compared to the 6-OHDA group. ICA, icariin; ARI, androgen receptor inhibitor.

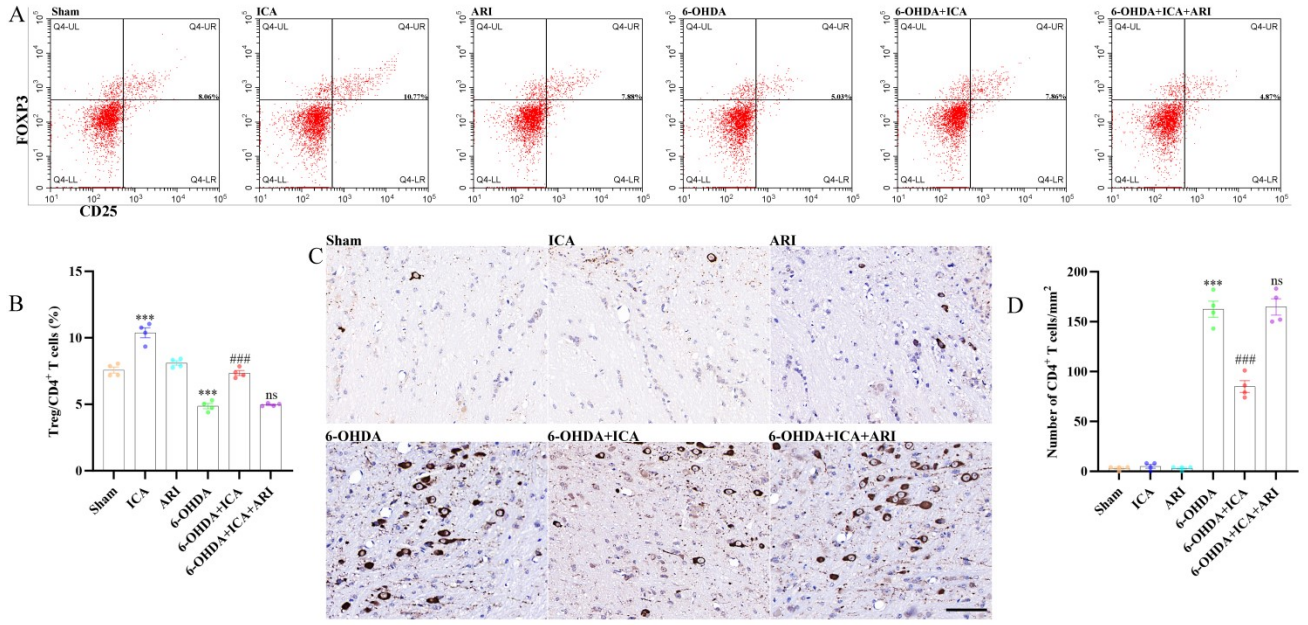

Fig. 5. Icariin promotes Tregs expansion and limits CD4$^+$ T cells migration into the SN. (A) Representative scatter plot showing the number of Treg in the PBMC. (B) Bar graph showing the frequency of Treg in each experimental group (n = 4). (C) Representative IHC for CD4 in the SN. (D) Bar graph showing the number of infiltrating CD4 T cells in the SN (n = 4). Scale bar = 100 µm. ***$p < 0.001$ compared to the sham group; $^{###}p < 0.001$ compared to the 6-OHDA group. ns: not significant, compared to the 6-OHDA group. ICA, icariin; ARI, androgen receptor inhibitor; PBMC, peripheral blood mononuclear cell.

3.4 Icariin Boosts Tregs Expansion and Restrains CD4$^+$ T Cells Migration

Strategies for enhancing Tregs expansion show potent anti-inflammatory and neuroprotective effects in neurodegeneration, partially by limiting CD4 T cell migration. We performed flow cytometry on PBMCs to evaluate the frequency of peripheral Tregs (Fig 5A). 6-OHDA treatment decreased the frequency of Tregs (95% CI: 4.24–5.50, $p <$ 0.001, Fig. 5B). Mice pretreated with icariin showed increased Treg frequency compared to the sham group (95% CI: 9.20–11.57, $p < 0.001$, Fig. 5B). In addition, mice pretreated with icariin before 6-OHDA injection showed elevated Tregs frequency compared to the 6-OHDA-only group (95% CI: 6.76–7.95, $p < 0.001$, Fig. 5B). IHC for CD4$^+$ T cells (Fig. 5C) indicated that icariin significantly reduced 6-OHDA-induced CD4$^+$ T cells migration into the SN (95% CI: 66.31–103.69, $p < 0.001$, Fig. 5D). No differences were observed when comparing 6-OHDA and 6-OHDA+ICA+ARI treatment groups. These data indicate that icariin shows robust potential for promoting Tregs and limiting CD4 T cell infiltration into the brain.

3.5 The Anti-Inflammatory Action of Icariin in the PD Model Is Associated With Tregs Expansion

To evaluate whether the anti-inflammatory effect of icariin in PD relies on Tregs, mice were administered PC61 monoclonal antibodies for three consecutive days to deplete Tregs *in vivo*, as shown in Fig. 1B. In this experiment, IgG1 served as an isotype control. Following PC61 treatment, a depletion exceeding 80% of Foxp3$^+$ cells within the CD4-gated PBMCs population (95% CI: 0.79–1.02, $p < 0.001$, Fig. 6A). Behavioral tests revealed, no significant differences between the 6-OHDA and 6-OHDA+icariin treatment groups following Tregs depletion (Fig. 6B). Additionally, the anti-inflammatory effect of icariin in PD was counteracted upon Tregs depletion. No significant differences in pro-inflammatory cytokine levels were observed between the 6-OHDA and 6-OHDA+ICA groups (Fig. 6C). Similarly, following Tregs depletion, the infiltrating CD4$^+$ T cells were not significantly altered between the 6-OHDA and 6-OHDA+ICA groups (Fig. 6D,E). These findings suggest that icariin alleviates neuroinflammation in PD, at least in part, by promoting the expansion of regulatory T cells.

3.6 AR Knockdown Abrogates Icariin-Induced Increase of CD4$^+$CD25$^+$Foxp3$^+$ Cells In Vitro

To directly test whether Icariin promotes Treg expansion in an AR-dependent manner, we performed lentivirus-mediated AR shRNA knockdown in primary mouse naïve CD4$^+$ T cells. After transduction, cells were cultured under Treg-polarizing conditions for 5 days, and the frequency of CD4$^+$CD25$^+$Foxp3$^+$ cells was analyzed by flow cytometry (Fig. 7). Cells were divided into four groups: Lv-NC (negative control lentivirus); Lv-NC + Icariin; Lv-AR (lentivirus expressing AR shRNA); Lv-AR + Icariin. In Lv-NC transduced cells, Icariin treatment significantly increased the percentage of CD4$^+$CD25$^+$Foxp3$^+$ cells (Lv-NC + Icariin) compared with the Lv-NC control (95% CI:

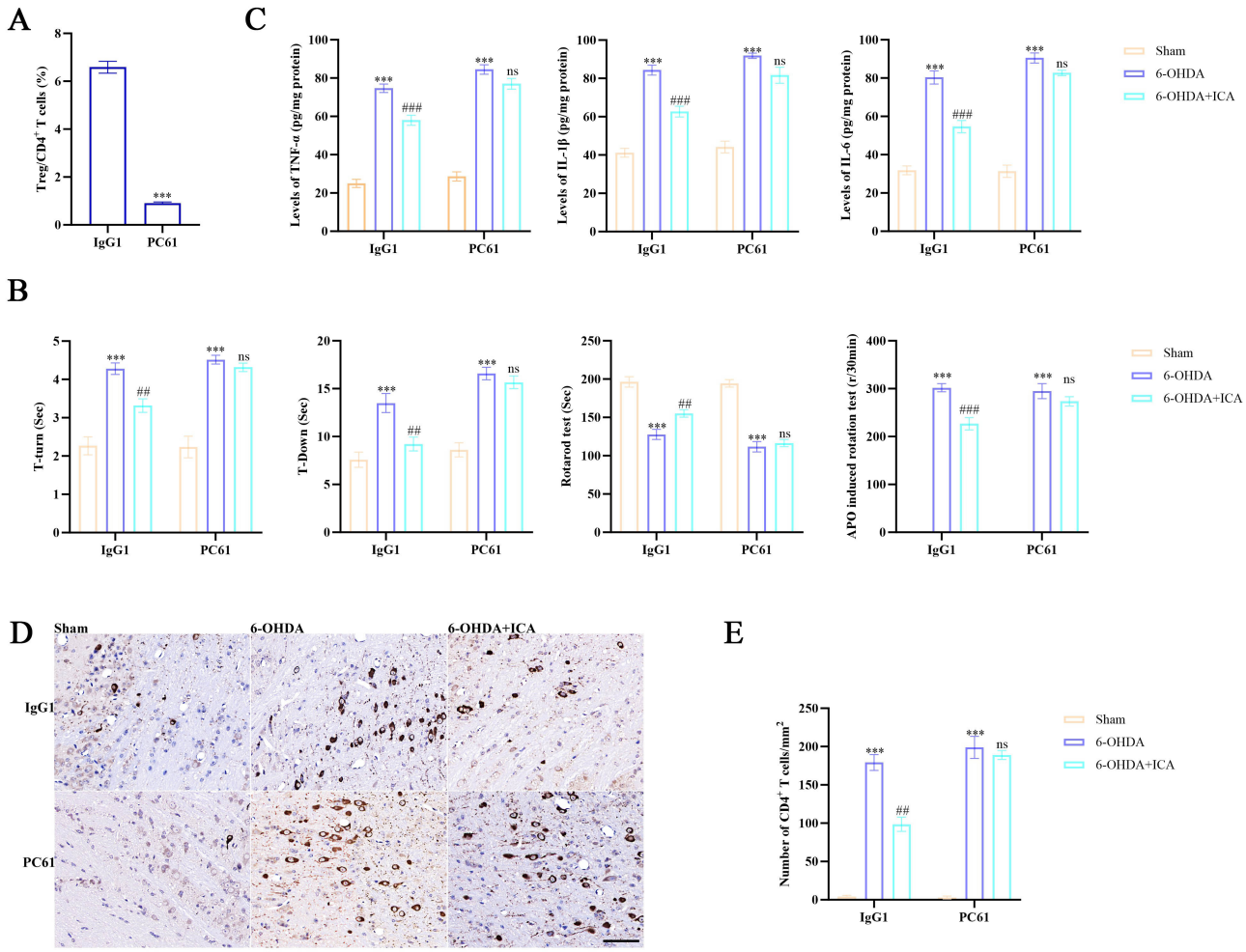

Fig. 6. The anti-inflammatory effects of icariin are counteracted upon Tregs depletion. (A) Tregs frequency following PC61/IgG1 treatment (n = 10). (B) Pole test and Rotation test in each group. n = 10 per group. (C) The abundance of pro-inflammatory cytokines was measured using ELISA (n = 6). (D) Representative IHC for $CD4^+$ in the SN. (E) Bar graph showing the number of $CD4^+$ cells in the SN (n = 4). Scale bar = 100 μm. ***$p < 0.001$, compared to the sham group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared to the 6-OHDA group. ns: not significant, compared to the 6-OHDA group. ICA, icariin; ARI, androgen receptor inhibitor.

2.0450–3.0117, $p < 0.001$, Fig. 7B). Lv-AR transduction alone did not affect the baseline frequency of these cells relative to the Lv-NC control. Notably, when Lv-AR transduced cells were treated with Icariin (Lv-AR + Icariin), the percentage of $CD4^+CD25^+Foxp3^+$ cells was not significantly different from that of the Lv-AR group, and was markedly lower than that of the Lv-NC + Icariin group (95% CI: –3.1917 to –2.2250, $p < 0.001$, Fig. 7B). These results demonstrate that AR is essential for Icariin-induced expansion of $CD4^+CD25^+Foxp3^+$ cells, providing direct genetic evidence for an AR-dependent mechanism.

4. Discussion

In this study, we observed that icariin supplementation significantly improved function, blocked dopaminergic neuronal degeneration, and reduced neuroinflammation in a rodent model of PD in an androgen receptor-dependent manner. By specifically targeting the androgen receptor, icariin markedly promoted the expansion of peripheral Tregs, which in turn restricted $CD4^+$ migration into the SN. This improvement in the pro-inflammatory environment effectively curbed dopaminergic degeneration (Fig. 8). Notably, we identified excessive infiltration of $CD4^+$ T cells; however, pretreatment with icariin dramatically reduced this infiltration.

Our findings align with the growing consensus that infiltrating $CD4^+$ T cells play a crucial role in dopaminergic neurodegeneration. In PD, a compromised blood-brain barrier facilitates the entry of these peripheral immune cells into the CNS, where they can exacerbate underlying pathologies such as mitochondrial dysfunction [20] and oxidative stress [21]. Preclinical studies, including those using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) models, demonstrate that depletion of $CD4^+$ T cells protects

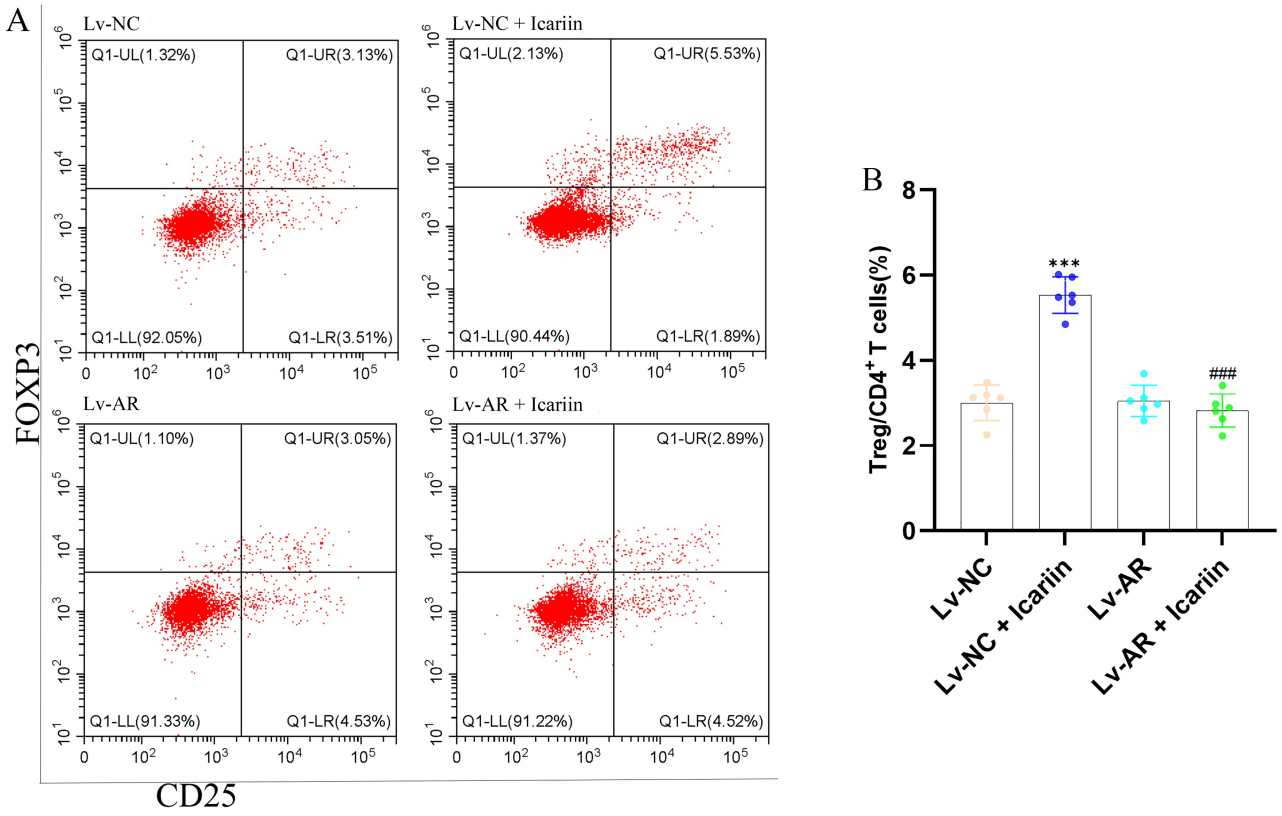

Fig. 7. AR shRNA knockdown abolishes icariin-induced Treg expansion *in vitro*. Cells were transduced with Lv-NC (negative control lentivirus) or Lv-AR (lentivirus expressing AR shRNA) and cultured under Treg-polarizing conditions with or without Icariin (10 μM) for 5 days. (A) Representative flow cytometry histograms showing Foxp3 expression in $CD25^+$ cells under indicated conditions. (B) Quantitative summary of the percentage of $CD4^+CD25^+Foxp3^+$ cells. Data are presented as mean ± SEM from six independent experiments (n = 6). $^{***}p < 0.001$ compared to the Lv-NC group; $^{\#\#\#}p < 0.001$ compared to the Lv-NC + Icariin group.

dopaminergic neurons, underscoring their toxic effects, often mediated by cytokines like IFN-γ [14]. In our model, we observed that icariin's reduction of $CD4^+$ T cell infiltration correlated with diminished microglial activation. This finding aligns with the established concept of deleterious microglia-T cell crosstalk: activated microglia recruit T cells, which in turn release pro-inflammatory factors such as IFN-γ that further polarize microglia, creating a self-perpetuating cycle of neuroinflammation [22,23].

Modulating the immunophenotype has emerged as a viable treatment strategy for neurodegenerative diseases. Beyond PD, evidence from other disorders underscores the broad relevance of regulatory T cells (Tregs) in neuroprotection. In Alzheimer's disease (AD), deficits in Treg function are linked to worsened pathology. Critically, adoptive transfer of Aβ-specific regulatory T cells has been shown to directly suppress neuroinflammation, reduce Aβ and tau pathology, and rescue cognitive deficits in AD mouse models [24]. Similarly, in amyotrophic lateral sclerosis (ALS), a high frequency of activated regulatory T cells in patients is associated with better survival and slower disease progression [25]. Tregs are a specialized subset of $CD4^+$ T cells that maintain immune homeostasis. They achieve this by regulating immune activation and preventing the development of pathological immune responses. A principal mechanism by which Tregs exert immunomodulatory effects and sustain immune tolerance is the secretion of cytokines with inhibitory properties, such as TGF-β and IL-10 [26]. Some Treg subsets can bind sufficient levels of IL-2, thereby limiting its availability for Teffs and preventing their clonal expansion. This mechanism may enable Tregs to mitigate neurotoxicity associated with Teffs' activity [27]. While our PC61 depletion experiments demonstrate that Tregs are required for icariin's neuroprotective effects, the precise site of Treg action—whether directly within the CNS or indirectly through peripheral immune modulation—remains to be determined. This study demonstrated that the increase in Treg levels induced by icariin is contingent on AR activation. The role of AR in Tregs represents a significant area of research. Recent studies suggest that the expression and functionality of androgen receptors in Tregs exhibit sex-specific characteristics, which influence the quantity and phenotype of Tregs, ultimately modulating the immune response [28]. Studies have estab-

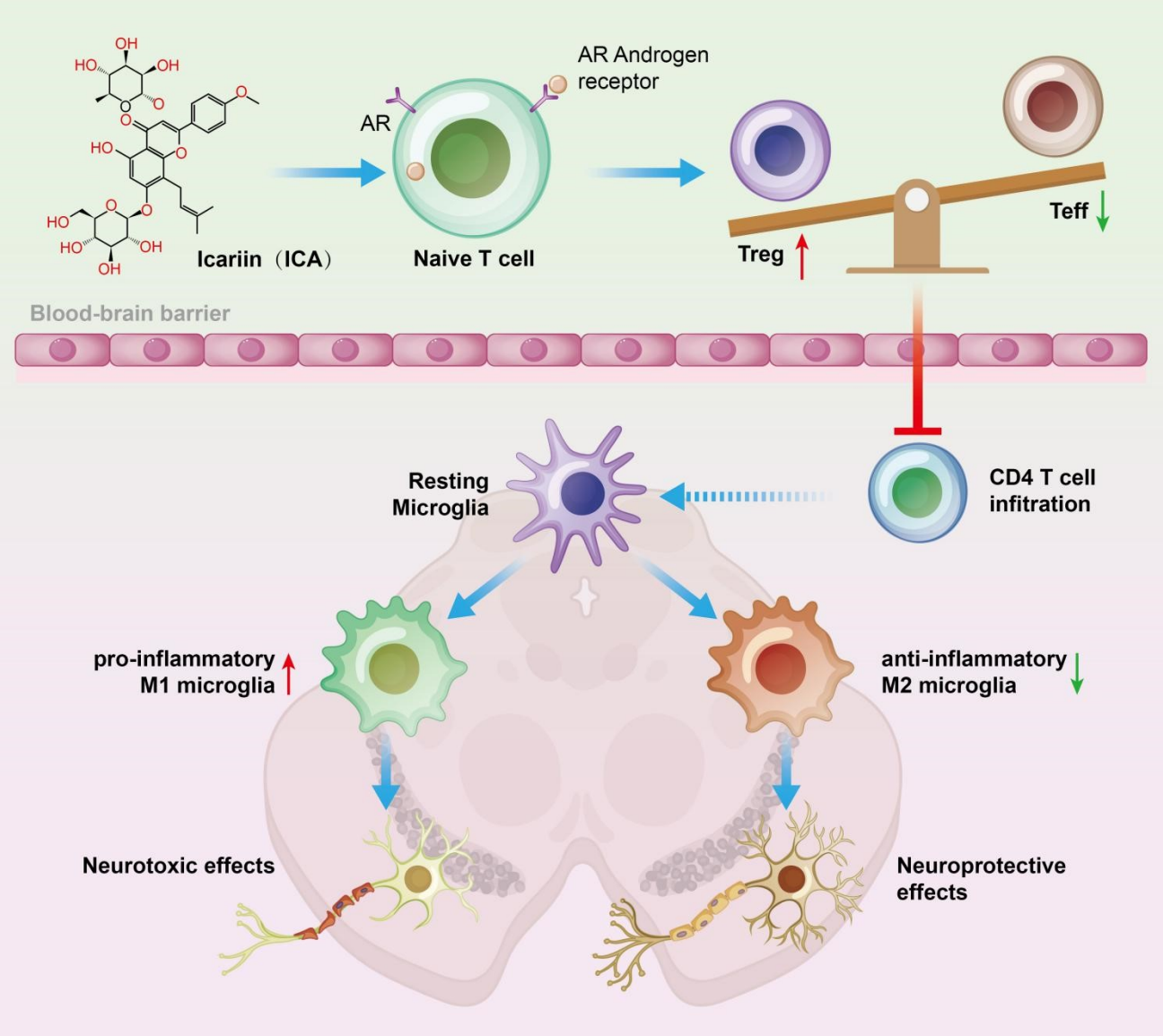

Fig. 8. The sketch elucidates the exact mechanism underlying the protective effects of icariin on PD. The red upward arrow (↑) indicates cell expansion, while the green downward arrow (↓) indicates a decrease in cell number.

lished a critical link between AR signaling and Treg biology in males, demonstrating that genetic ablation of the AR in male mice results in a marked decrease in Treg numbers and a consequent phenotype resembling that of females [29]. Androgen signaling critically shapes the local niche around Tregs, thereby influencing their recruitment and functional state [16]. For instance, androgens facilitate the development of stromal cells that produce IL-33, a cytokine essential for the expansion and functional activity of Tregs [30]. This androgen-mediated production of IL-33 limits the Treg population in male adipose tissue, potentially influencing male-specific immune responses and metabolic conditions [29]. These findings open new therapeutic avenues for addressing male-centric diseases, such as prostate cancer [31]. Evidence suggests that modulating the number and function of Tregs, either with antagonists or with agonists targeting androgen receptors, may enhance the efficacy of prostate cancer immunotherapy. This presents an innovative therapeutic strategy that leverages androgen receptor modulation to enhance anti-tumor immune responses [32].

To directly test whether AR is cell-intrinsically required for Icariin-induced Treg expansion, we performed AR shRNA knockdown in primary naïve $CD4^+$ T cells. *In vitro* Treg polarization assays revealed that Icariin significantly increased the percentage of $CD4^+CD25^+Foxp3^+$ cells in scramble shRNA-transfected cells, confirming its pro-Treg activity. AR knockdown alone did not affect baseline Treg differentiation, excluding nonspecific effects of shRNA transfection. Notably, AR knockdown completely abrogated Icariin-induced Treg expansion, as the percentage of $CD4^+CD25^+Foxp3^+$ cells in the AR shRNA + Icariin group was comparable to that of the AR shRNA group and

significantly lower than that of the scramble + Icariin group (Fig. 7). These results provide direct genetic evidence that Icariin promotes Treg differentiation in an AR-dependent manner, addressing the limitation of our previous pharmacological inhibition approach.

Based on our molecular docking evidence demonstrating high-affinity binding of icariin to AR, together with pharmacological blockade results showing that an AR inhibitor completely reversed all icariin-mediated effects, we propose a plausible mechanistic framework for the AR-dependent action of icariin. As a ligand-activated transcription factor, AR can directly regulate key genes involved in Treg differentiation and function in $CD4^+$ T cells. For instance, AR signaling has been shown to promote Foxp3 expression, the master transcription factor for Treg lineage commitment [17]. Concurrently, AR signaling can suppress the differentiation of pro-inflammatory T helper subsets such as Th1 and Th17, thereby indirectly favoring an immunosuppressive microenvironment [16]. Moreover, AR may shape the immune niche by modulating cytokine production—for example, by influencing stromal cell-derived IL-33, which supports Treg expansion and survival [27,28]. We therefore hypothesize that icariin, via AR activation, likely enhances peripheral Treg pool expansion through dual, synergistic pathways: direct upregulation of Treg-critical molecules and indirect remodeling of the immunomodulatory microenvironment. These expanded Tregs may then migrate into the central nervous system or exert systemic immunomodulatory effects, ultimately suppressing neuroinflammation and protecting dopaminergic neurons.

This study has several limitations. Firstly, the experiments were conducted exclusively in male murine models; given the established sexual dimorphism in androgen-immune interactions, evaluating icariin in female models is an essential future step to assess its broader therapeutic relevance. Secondly, while the complete reversal of icariin's effects by a specific AR inhibitor strongly supports AR-dependence, the use of a single inhibitor remains a limitation. Future studies employing additional AR antagonists or conditional AR-knockout models would further confirm the pathway's specificity. Finally, the precise molecular mechanisms by which icariin promotes Treg cell expansion require further investigation, and the translational potential of these findings necessitates future clinical evaluation in patients with Parkinson's disease.

As an adjunct to established pharmacological therapies (such as levodopa), icariin may help mitigate adverse effects, enhance therapeutic efficacy, and potentially slow disease progression. For individuals in the prodromal stage of the disease, it may serve as a preventive intervention to delay the onset of clinical manifestations.

5. Conclusions

In conclusion, the findings demonstrate that icariin protects against neuroinflammation and neurodegeneration in a rodent model of PD via an AR-dependent mechanism. It was further found that Tregs play a critical role in mediating this effect. These results provide direct evidence of icariin's neuroprotective effects in Parkinson's disease and lay the foundation for further investigation and potential clinical translation.

Availability of Data and Materials

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

SCX, ZP, PZ and LJ designed the research study. SCX, ZP, LJ, HZ and PZ performed the research. HZ and SCX contributed to behavioral tests; SCX contributed to Molecular docking; ZP, SCX and JL conducted experiments. ZP, PZ and HZ analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The animal study protocol was approved by the Institutional Review Board of The Affiliated Nanhua Hospital (Approval number: 2024-KY-024). The study was carried out in accordance with the ARRIVE guidelines. All animal experiments were conducted in accordance with the Chinese national guidelines for laboratory animal welfare and ethics.

Acknowledgment

We thank EJEAR's English editing service for assisting in the preparation of this manuscript.

Funding

This study was supported by the Clinical Medical Technology Innovation Guide Project of Hunan Province, China (grant number 2021SK51901), the Project of Hunan Provincial Department of Education (grant number 21B0412), and The general project of the Health Commission of Hunan Province (grant number 202203072781, 202203074169).

Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL47325.

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