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Saikosaponin A mitigates the progression of Parkinson's disease *via* attenuating microglial neuroinflammation through TLR4/MyD88/NF-κB pathway

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Abstract. – OBJECTIVE: Neuroinflammation caused by excessive microglial cell activation and the subsequent death of dopaminergic neurons plays a role in the pathogenesis of Parkinson's disease (PD). Saikosaponin A (Ssa), a triterpene saponin derived from Radix Bupleuri, has anti-inflammatory and antioxidant functions. This research aimed to investigate whether Ssa has a therapeutic effect on PD.

MATERIALS AND METHODS: BV2 microglia- and SH-SY5Y cells were treated with a neurotoxin N-methyl-4- phenylpyridinium (MPP+) and Ssa. Cell viability, apoptosis, inflammatory reactions, and expression levels of oxidative stress mediators were assessed. A PD rat model was created by intraperitoneal injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), followed by the Ssa treatment. Hematoxylin-eosin (H&E) staining, Nissl staining, and immunohistochemistry were used to detect neuronal apoptosis and microglial activation. Open-field test (OFT) was performed to evaluate the locomotion of the rats. The underlying mechanism of Ssa effect in PD was explored using network pharmacology analysis and verified experimentally.

RESULTS: Ssa dampened neuronal apoptosis and had anti-inflammatory and anti-oxidative stress proprieties in MPP+-treated SH-SY5Y cells and BV2 microglia. As shown in *in-vivo* experiments, Ssa reduced MPTP-mediated neuronal apoptosis and motor dysfunction and lowered the expression of inflammatory factors and oxidative stressors in the substantia nigra (SN) of the PD rat. Additionally, Ssa inactivated the TLR4/MyD88/NF-κB pathway.

CONCLUSIONS: This study provides the first evidence that Ssa prevents dopaminergic neurodegeneration caused by microglia activation by modulating the TLR4/MyD88/NF-κB axis.

Key Words:
Parkinson's disease, Neuroinflammation, Saikosaponin A, Microglia, Neuroprotection.

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease involving a reduction in the neurotransmitter dopamine. The main symptoms of PD include resting tremor, rigidity, bradykinesia/dysmotility, and postural instability[1]. Neuroinflammation and microglial activation are considered the hallmarks of PD neuropathology[2,3]. Microglia are the resident macrophages of the central nervous system that are rapidly activated in almost all types of neurological diseases[4]. Activated microglia can secrete multiple factors (including cytokines, chemokines, prostaglandins, reactive oxygen and nitrogen species, and growth factors) that increase oxidative stress and initiate an apoptotic cascade of neuronal responses[5]. Hence, inhibition of microglia activation may contribute to the treatment and prevention of PD.

Saikosaponin A (Ssa), the active ingredient extracted from the root of Saikosaponin, is a natural compound with diversified pharmacological activities that may be used for anti-tumor applications[6,7]. Studies[8-11] show that Ssa inhibits inflammation and oxidative stress in acute lung injury[8], allergic rhinitis[9,] and acute liver injury[10] by blocking Nuclear factor-κB (NF-κB) activation and NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome expression. Furthermore, in neuroinflammatory diseases, Ssa pretreatment lowers high-mobility group box 1 (HMGB1) levels in the serum of middle cerebral artery occlusion (MCAO) rats and decreases the levels of inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), and interleukin 6 (IL-6) in the brain tissue of MCAO rats[11]. Besides, Ssa administration re-

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markably improves depression-like behaviors and curbs neuronal apoptosis after ischemic brain injury in rats[12]. However, the effect of Ssa on PD is still unclear.

Toll-like receptors (TLRs) are essential proteins that act as a bridge between non-specific and specific immunity[13]. TLR4, a member of the TLRs, is activated by lipopolysaccharide (LPS), a component of Gram-negative bacteria that triggers the generation of pro-inflammatory mediators[14]. MyD88 is a canonical adapter of the downstream inflammatory pathway for members of the TLR- and interleukin-1 (IL-1) receptor families[15]. NF-κB comprises a family of transcription factors that modulate immune responses and inflammation[16]. Yang et al[17] showed that increased TLR4 expression is closely associated with the PD onset, progression, effectiveness of drug therapy, disease stage, and duration, which are significant for PD diagnosis and treatment. In addition, blocking TLR4/MyD88/NF-κB activation reduces LPS-induced neuroinflammation in microglia in a rat PD model[18,19]. These findings demonstrated that the inactivation of the TLR4/MyD88/NF-κB axis suppressed neuroinflammation in PD.

The main goal of this study is to investigate whether Ssa could mitigate the -methyl-4- phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neuroinflammatory response and to further characterize the specific role of Ssa and the TLR4/MyD88/NF-κB axis in neuroinflammation. Our results revealed that Ssa reduced microglia activation and the release of inflammatory factors and delayed the progression of PD *in vivo* and *in vitro* by blocking the TLR4/MyD88/NF-κB pathway.

Materials and Methods

Animals

Forty male Sprague-Dawley (SD) rats (10-12 weeks, 250±20 g) were acquired from the Animal Center of Wuhan University. All rats were housed in a light/dark cycle animal room for 12 hours at a fixed temperature of 23±1°C and 60% humidity and allowed to eat and drink freely. All animal experiments adhere to the Basel Declaration and the International Council for Laboratory Animal Science (ICLAS) ethical guidelines. The current research protocol was approved by the Animal Research Committee of Wuhan University.

Animals and Drug Therapy

The rats were randomly divided into the sham-operated group (Sham) (n=10), the Ssa group (5 mg/kg, n=10), the MPTP group (n=10) and the MPTP+Ssa group (5 mg/kg, n=10) as described previously[20]. Rats in the MPTP group were anesthetized with sodium pentobarbital (45 mg/kg) (Sigma-Aldrich, St-Louis, MO, USA), and the PD model was constructed by intraperitoneal (i.p) injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, 20 mg/kg, Sigma-Aldrich, St-Louis, MO, USA). MPTP (20 mg/kg) was dissolved in saline and injected intraperitoneally (i.p) into rats at 12-hour intervals for two days. The total dose of Ssa (Sigma-Aldrich, St-Louis, MO, USA) was 80 mg/kg per rat. Following injection, PD rats exhibited a wobbly gait, reduced activity, and slow movements. Sham rats were given an equal volume of sterile saline solution (0.9%). In parallel, rats in the Ssa and the MPTP+SSa groups were administered Ssa (5 mg/kg, dissolved in equal amounts of DMSO) by intraperitoneal injection twice daily for two weeks, three days prior to the construction of the PD model. Rats in the Sham group did not receive any treatment[21,22].

Hematoxylin-eosin (H&E) Staining

On the second week after the MPTP injection, substantia nigra (SN) tissues from each group of rats were stripped and fixed using 100 mL/L formalin for one day. After paraffin embedding, the tissues were sectioned (4 μM thickness) and baked in a constant temperature oven at 65°C for six hours. After routine dewaxing and hydration, the sections were stained with H&E, sealed with neutral gum, and reviewed under a microscope (Olympus, Shinjuku, Tokyo, Japan).

Nissl Staining

Brain sections were cleaned three times with phosphate buffer saline (PBS) (15 minutes each time) and then dyed with 0.5% cresyl violet solution for 10 minutes. The sections were then flushed with distilled water and dehydrated with graded alcohol (70%, 95%, and 100%). SN tissue sections were cleaned in xylene for five minutes and sealed with a neutral resin (non-fluorescent). Next, they were observed under a microscope (Olympus, Shinjuku, Tokyo, Japan) and photographed. Quantitative analysis of the number of normal neurons was carried out with the aid of the Image-Pro Plus 6 software[18] (National Institutes of Health, Bethesda, MD, USA).

Immunohistochemistry (IHC)

Brain tissues from each group were routinely paraffin-sectioned and examined by IHC. Sections were incubated with anti-tyrosine hydroxylase (TH) (1:1000; ab137869, Abcam, CA, USA) and anti-ionized calcium-binding bridging submolecule 1 (IBA-1) (1: 1000; ab178846, Abcam, CA, USA) primary antibodies overnight at 4°C. After washing, a drop of Goat anti-Rabbit IgG (H+L) biotin-labeled secondary antibody (1:500, Cat. No. 65-6140, Thermo Fisher Scientific, Waltham, MA, USA) was added to the sections and incubated for 10 minutes at room temperature (RT). Next, the sections were washed and incubated with Streptomyces anti-biotin-peroxidase solution for 10 minutes at RT. The sections were then subjected to washing, DAB color development at RT, hematoxylin re-staining, blocking, and microscopic examination. Five high magnification (×400) fields were picked at random, and the number of positive cells in each section was calculated separately with a microscope (Olympus, Shinjuku, Tokyo, Japan). TH- and IBA-1-positive cells were independently counted by three investigators, and the mean values were taken.

Open-Field Test (OFT)

As previously described[25], the volume of the open field device is 100×100×40 cm. The bottom of the box consists of a 20×20 cm black grid. OFT was conducted two weeks following MPTP injection to determine the impact of Ssa on motor function in rats. The rats were tested in a quiet and dimly lit environment. The rats were put in the same position against the wall and allowed to explore the apparatus freely for five minutes. After the OFT test, the bottom of the box was cleaned with a 5% water-ethanol solution to avoid the influence of previous rats.

Western Blot (WB)

After SN tissues and cells were processed, the medium was discarded, and the total proteins were extracted using Radioimmunoprecipitation assay (RIPA) lysis buffer (Roche Applied Science, Penzberg, Germany). The total proteins (30 μg) were separated on 10% SDS polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with 5% skimmed milk powder for one hour at RT, the membranes were rinsed three times with TBST for 10 minutes each. Subsequently, membranes were incubated overnight at 4°C with the following primary antibodies: TLR4 (ab13556, 1:1000, Abcam, CA, USA), MyD88 (ab133739, 1:1000), TH (ab137869, 1:1000), NF-κB (ab32536, 1:1000), p-NF-κB (ab76302, 1:1000), Bax (ab32503, 1:1000), C-caspase-3 (ab32351, 1:1000), Bcl-2 (ab182858, 1:1000), and GAPDH (ab9485, 1:1000). Membranes were then washed with TBST, and incubated with horseradish peroxidase (HRP)-labeled anti-rabbit secondary antibody (ab6721, 1:3000) for one hour at RT. All primary antibodies and secondary antibodies were purchased from Abcam. The membranes were then washed three times with TBST (10 minutes each). Finally, the proteins were imaged using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Invitrogen, Waltham, MA, USA), and each protein's grey value was determined using Image-Pro Plus 6 software[18] (National Institutes of Health, Bethesda, MD, USA).

Cell Culture and Treatment

BV2 microglia and SH-SY5Y cells were acquired from the American Typical Culture Collection (ATCC, Manassas, VA, USA). Cells were grown at 37°C in a humidified incubator (incubator with 5% CO_2) in Dulbecco's modified Eagle medium (DMEM, Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (Gibco, Rockville, MD, USA) and 1% penicillin/streptomycin (Gibco, Rockville, MD, USA).

BV2 microglia and SH-SY5Y cells were grown in 6-well plates at the density of 2×10^5 cells/well, and incubated with varying concentrations of Ssa (2 μmol/L, 4 μmol/L) for four hours, followed by the treatment with N-methyl-4- phenylpyridinium (MPP^+) (5 mM, Sigma-Aldrich, St. Louis, MO, USA) for 14 hours[22]. In the inhibitor group, cells were preincubated with 20 μg/mL TAK-242 (TLR4 inhibitor) (Sigma-Aldrich, St-Louis, MO, USA) for three hours before incubation with Ssa (2 μmol/L, 4 μmol/L) and 5 mM of MPP^+ for 14 hours.

Cell Viability Assay

The impact of Ssa on the viability of SH-SY5Y and BV2 cells was examined using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St-Louis, MO, USA) assay. Briefly, SH-SY5Y or BV2 cells were grown in 96-well plates at the density of 1×10^4 cells/well and kept overnight at 37°C in a 5% CO_2 atmosphere. Cells were then incubated with the different concentrations of Ssa (2 μmol/L, 4 μmol/L) for 24 hours. MTT (5 mg/mL) was then added to each well. After incubating for four hours at 37°C, DMSO (100 μL) was added into each well to dissolve the crystals, and

the absorbance was measured at 570 nm. Three replicate experiments were carried out for both concentrations of Ssa.

TdT-Mediated dUTP Nick end Labeling (TUNEL) Staining

After an appropriate treatment as described before, cells were washed once in PBS, fixed in 4% Polyformaldehyde (Cat. No. P0099, Beyotime, Shanghai, China) for 30 minutes, and washed with PBS. The cells were then penetrated with the 0.3% Triton X-100 dissolved in PBS at room temperature for five minutes. TUNEL solution (50 µl, Cat. No. C1089, Beyotime, Shanghai, China) was added to the sample and maintained at 37°C for 60 minutes in the dark, followed by three washes with PBS. After sealing the slides with an anti-fluorescence quenching blocking solution, the cells were reviewed under a fluorescent microscope (Olympus, Shinjuku, Tokyo, Japan) with excitation light at 450-500 nm and emission light at 515-565 nm (green fluorescence). Five fields of view were randomly picked for each sample, and the apoptotic rate was measured as follows: Apoptosis rate = apoptotic cell number/total cell number×100%.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total cellular RNA was separated with the TRIzol reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's protocol. RNA concentration and purity were measured with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the PrimeScript-RT Kit (Madison, WI, USA). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was done using SYBR® Premix-Ex-Taq™ (Takara, Dalian, Shengyang, China) and the ABI7300 system (Applied Biosystems, Waltham, MA, USA). The total volume of the PCR system was 30 µL, and each sample contained 300 ng of cDNA. The amplification procedure consisted of an initial denaturation at 95°C for 10 minutes, followed by 45 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and 85°C for 20 seconds. We converted all fluorescence data to relative quantification, with GAPDH as an endogenous control for the assay. The qRT-PCR reactions were repeated three times. The specific primers used for qPCR are as follows:

CD86, forward, 5'-TGTTTCCGTGGAGACGCAAG-3' and reverse, 5'-TTGAGCCTTTGTAAATGGGCA-3'. IL-1β, forward, 5'-TGCCACCTTTTGACAGTGATG-3' reverse, 5'-AAGGTCCACGGGAAAGACAC-3'. IL-6, forward, 5'-AAAGAGTTGTGCAATGGCAATTCT-3' and reverse, 5'-AAGTGCATCATCGTTGTTCATACA-3'. TNF-α, forward, 5'-CATCTTCTCAAAATTCGAGTGACAA-3' and reverse, 5'-TGGGAGTAGACAAGGTACAACCC-3'. IL-8, forward, 5'-TTGCCAAGGAGTGCTAAAGAA-3' and reverse, 5'-GCCCTCTTCAAAAACTTCTCC-3'. CD206, forward, 5'-CGTTTCGGT GGACTGTGGA-3' and reverse, 5'-GTTGTGGGCTCTGGTGGG-3'. IL-10, forward, 5'-ACCTGGTAGAAAGTG ATGCCC-3' and reverse, 5'-ACACCTTGGTCTTGGAGCTT-3'. Arg1, forward, 5'-CTCCAAGCCAAAGTCCTTA GAG-3' and reverse, 5'-AGGAGCTGTCATTAGGGACATC-3'. IL-4, forward, 5'-GCCTCACATTGTCACTGCAA-3' and reverse, 5'-GTGTTCTTGGAGGCAGCAAA-3'. IL-13, forward, 5'-GGATAAGGGGCGTTGACTCA-3' and reverse, 5'-GATGCTCCATACCATGCTGC-3'. GAPDH, forward, 5'-CGGAGTCAACGGATTTGG TCGTAT-3' and reverse, 5'-AGCCTTCTCCATGGTGGTGAA GAG-3'[25].

Enzyme-Linked Immunosorbent Assay (ELISA)

Concentrations of IL-1β, IL-6 and TNF-α in brain tissues and cell supernatants were assessed by ELISA kits as described by the manufacturer (Abcam, Shanghai, China).

Detection of Oxidative Stress

SOD and GSH-Px activities and MDA levels of the rat brain and cell supernatants were assayed with the colorimetric assay using a microplate reader (BECKMAN-Coulter Co., Miami, FL, USA) following the kit instructions (Nanjing Jiancheng Institute of Biological Engineering, Nanjing, Jiangsu, China). The experiment was repeated three times.

Statistical Analysis

All experiments were done in triplicates, and the data obtained were presented as mean± stand error. The results obtained from the experiment were statistically analyzed using the SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). One-way analyses of variance (ANOVA) were conducted to compare means between groups for data that met normal distribution and Chi-squared. A difference of $p<0.05$ was considered statistically significant. If the variance was not uniform or did not conform to a normal distribution, the analysis was carried out using the rank sum test.

Results

Network Pharmacology Prediction

To investigate the mechanism of action of Ssa in PD, a network pharmacology analysis was performed, and the analysis flow is shown in Figure 1A. Disease targets for PD were obtained from the GeneCards (https://www.genecards.org/) and OMIM (https://www.omim.org/) databases, and drug targets for Ssa were acquired from the SwissTarget (http://www.swisstargetprediction.ch/), Pharmmapper (https://www.lilab-ecust.cn/pharmmapper/), and PubChem (https://pubchem.ncbi.nlm.nih.gov/) databases. Targets were uploaded onto the Venn diagram online tool, and 70 overlapping targets were obtained (Figure 1B), which can be considered potential targets for the neuroprotective role of Ssa in PD. We introduced the common targets of drugs and diseases from the analysis into Cytoscape software to visualize the drug target network (Figure 1C). Next, we analyzed the 70 target genes for GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment using the DAVID (https://david.ncifcrf.gov/) database (Figure 1 D-E). GO analysis includes biological processes (BP), cellular components (CC), and molecular functions (MF), which contain neuronal apoptotic processes that are involved in the pathological evolution of PD. KEGG enrichment analysis showed that TLR4/MyD88/NF-κB was enriched in the Toll-like receptor pathway in the top 30 signaling pathways. Together with previous observations that TLR4/MyD88/NF-κB pathway mediates neuroinflammation in PD, and that inhibition of TLR4/MyD88/NF-κB hinders the LPS-induced

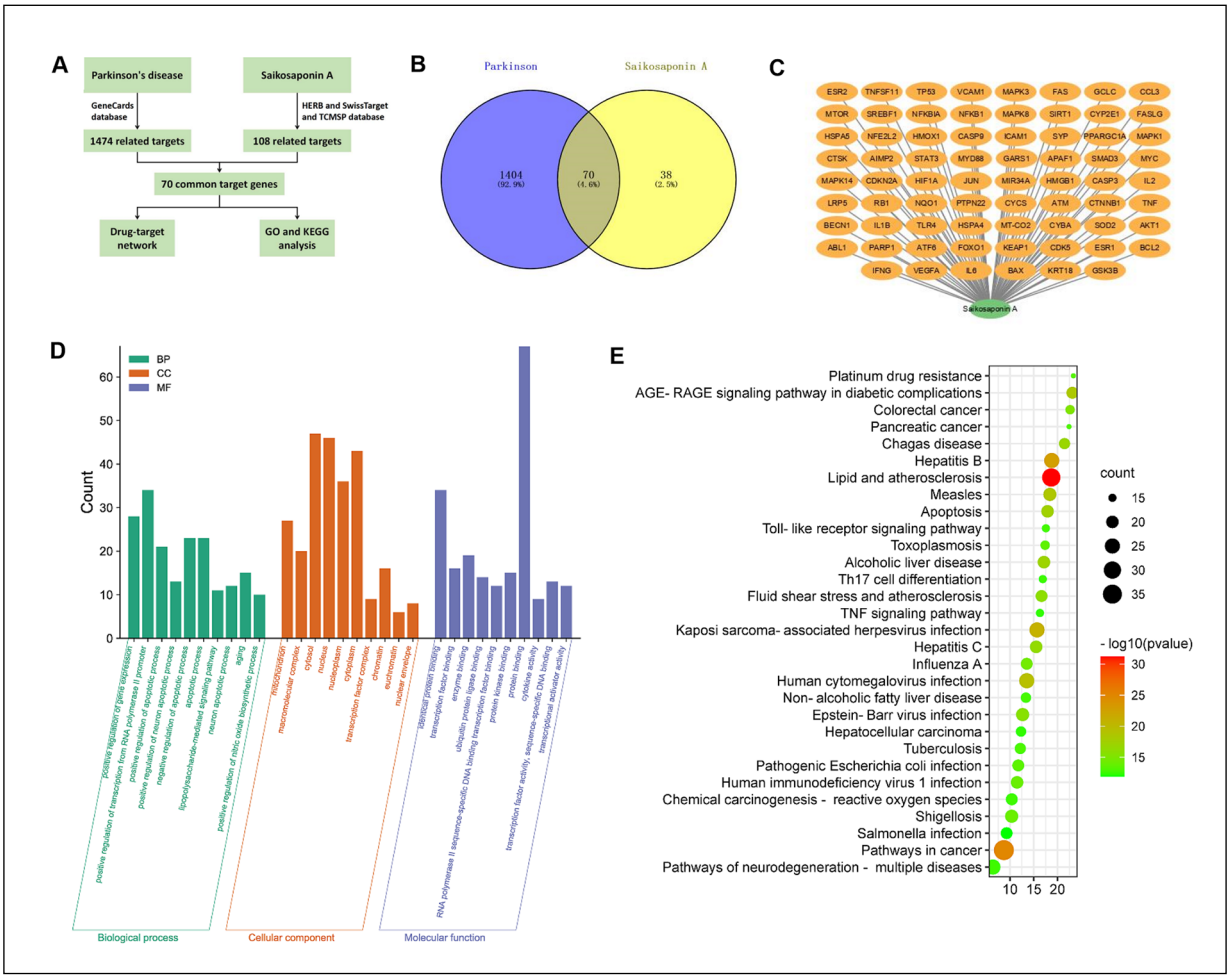

Figure 1. Network Pharmacology Prediction. **A,** Flow chart of pharmacological analysis. **B,** Venn diagram analysis of the intersection of disease targets of PD and Ssa-related targets. **C,** Common targets of drugs and diseases are imported into Cytoscape software to visualize drug-target networks. **D-E,** DAVID (https://david.ncifcrf.gov/home.jsp) was used for analyzing the enriched KEGG and GO functions of Ssa.

neuroinflammatory response in the brain tissue of PD rats, our results suggest that Ssa contributed to PD by modulating the TLR4/MyD88/NF-κB pathway.

Saikosaponin A Mitigated MPP+-Induced Damage in SH-SY5Y Cells

To test the impact of Ssa on neurons, we pretreated SH-SY5Y cells with Ssa (2 μM, 4 μM) for four hours and then treated them with 5 mM MPP + for 14 hours. The survival rate and apoptosis of SH-SY5Y cells were tested by MTT and TUNEL staining. The survival rate of MPP+-induced SH-SY5Y cells was dramatically reduced, and apoptosis rate was significantly increased compared to the Control group. Ssa-pretreated cells exhibited a notable rise in viability and a decline in apoptosis (Figure 2 A-B). As shown by the Western Blot analysis, levels of Bax and C-caspase3 increased, and the level of Bcl-2 was significantly decreased in MPP+-induced SH-SY5Y cells compared to the Control group. The expression of Bax and C-caspase3 was lower, and the Bcl-2 level was significantly higher in the Ssa-pretreated cells (Figure 2C). The influence of Ssa on MDA expression and GSH-Px and SOD activities in MPP+-induced SH-SY5Y cells was examined with a commercial kit. After Ssa treatment, inflammatory cytokine, and MDA levels were reduced in SH-SY5Y, while GSH-Px and SOD activities were markedly increased (Figure 2D). Thus, Ssa suppressed MPP+-induced SH-SY5Y cell damage.

Saikosaponin A Abated MPP+-Induced Inflammatory Responses in BV2 Microglia and Inactivated the TLR4/MyD88/NF-κB Axis

Next, we assayed the impact of Ssa on MPP+-induced inflammation in BV2 microglia. The effect of Ssa on the viability of BV2 microglia was examined with MTT. The results displayed that MPP+ had no major effect on the survival rate of BV2 microglia as compared to the Control group, and the viability of MPP+-induced BV2 microglia was not markedly altered after Ssa treatment (Figure 3A). The expression of inflammatory factors in BV2 cells was tested by qRT-PCR. Ssa substantially suppressed the expression of pro-inflammatory factors IL-1β, TNF-α, IL-6, IL-8, and CD86 and upregulated the expression of anti-inflammatory factors CD206, Arg1, IL-4, IL-13, and IL-10 in MPP+-induced BV2 microglia *vs.* the MPP+ group (Figure 3B). The release of IL-1β, TNF-α, and IL-6 in MPP+-induced BV2

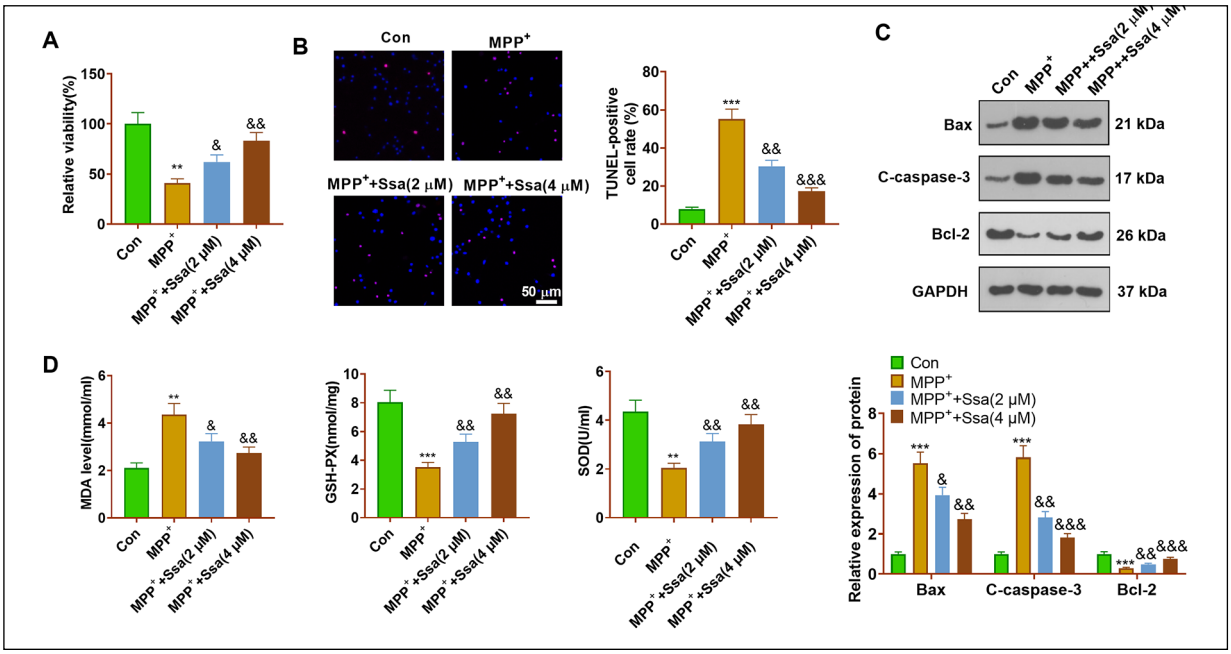

Figure 2. Saikosaponin A eased MPP+-induced damage in SH-SY5Y cells. SH-SY5Y cells were pretreated with Ssa (2 μM,4 μM) for four hours and then treated with 5 mM MPP + for 14 hours. **A**, MTT cell viability assay. **B**, TUNEL staining. Scale bar=50 μm. **C**, WB analysis of the expression levels of apoptosis-related proteins Bax, C-caspase3 and Bcl-2 in cells. **D**, The MDA expression and GSH-Px and SOD activities in cells are measured by commercial kits. Data are expressed as mean ± SEM, N=3. ***p<0.001 (*vs.* Con group), &p<0.05, &&p<0.01, &&&p<0.001 (*vs.* MPP+ group).

microglia was determined by ELISA. Ssa substantially inhibited the release of these inflammatory factors in MPP+-induced BV2 microglia *vs.* the MPP+ group (Figure 3C). These findings corroborated the anti-inflammatory effects of Ssa in MPP+-induced BV2 microglia. A previous study[19] supports the contribution of neuroinflammation to PD. As shown by the Western Blot analysis, the expression of TLR4 and MyD88 was elevated in MPP+-induced microglia, and phosphorylation of NF-κB was enhanced. In contrast, Ssa lowered the levels of TLR4 and MyD88 and the phosphorylation of NF-κB (Figure 4A). Thus, Ssa alleviated MPP+-induced inflammatory responses in BV2 microglia *in vitro*, possibly by attenuating the LR4/MyD88/NF-κB pathway activation.

Inhibition of TLR4/MyD88/NF-κB Enhanced the Anti-Apoptotic and Anti-Inflammatory Effects of Ssa

Cells were pre-cultured with 20 μg/mL TAK-242 (TLR4 inhibitor) for three hours, followed by incubation with Ssa (4 μM) and 5 mM of MPP+ for 14 hours. As shown in Figure 5A, TAK-242 down-regulated TLR4 and MyD88 expression and reduced NF-κB phosphorylation in BV2 cells. Next, we assessed the influence of TAK-242 on SH-SY5Y cell viability and apoptosis. TAK-242 potentiated the effects of Ssa in MPP+-induced SH-SY5Y cells, including further increasing SH-SY5Y cell viability and inhibiting cell apoptosis (Figure 5B, C). Additionally, adding TAK-242 further reduced MDA expression and enhanced

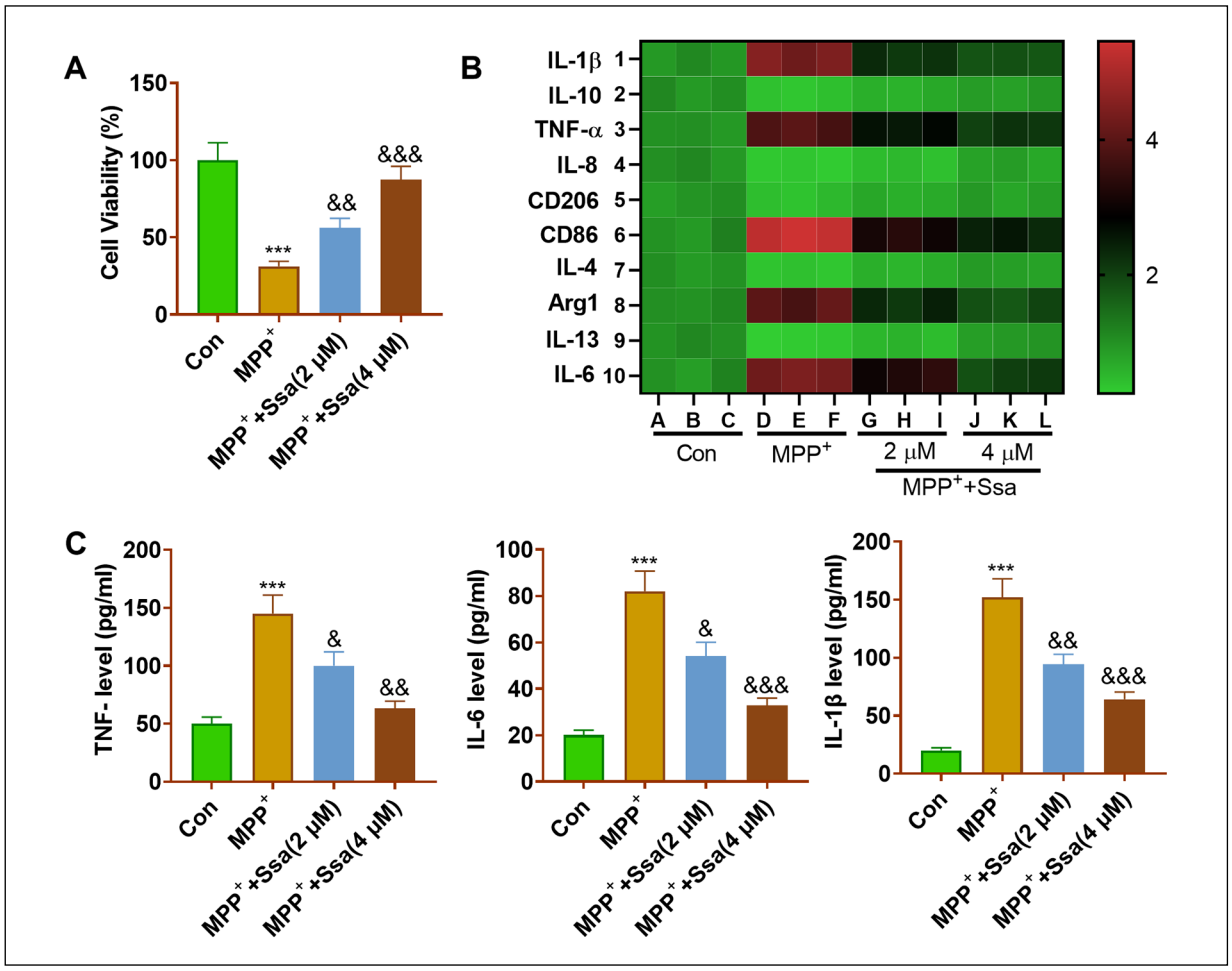

Figure 3. Saikosaponin A alleviated MPP+-induced inflammatory response in BV2 microglia and BV2 microglia were pretreated with Ssa (2 μM, 4 μM) for four hours, followed by treatment with 5 mM MPP + for 14 hours. **A**, MTT was utilized to verify cell viability. **B**, qRT-PCR measurement of the levels of pro-inflammatory factors IL-1β, TNF-α, IL-6, IL-8 and CD86 and anti-inflammatory factors CD206, Arg1, IL-4, IL-13 and IL-10 in cells. **C**, Expression of IL-1β, TNF-α and IL-6 in BV2 microglia detected by ELISA. Data are expressed as mean ± SEM, N=3. ***$p<0.001$ (*vs.* Con group) &$p<0.05$, &&$p<0.01$, &&&$p<0.001$ (*vs.* MPP+ group).

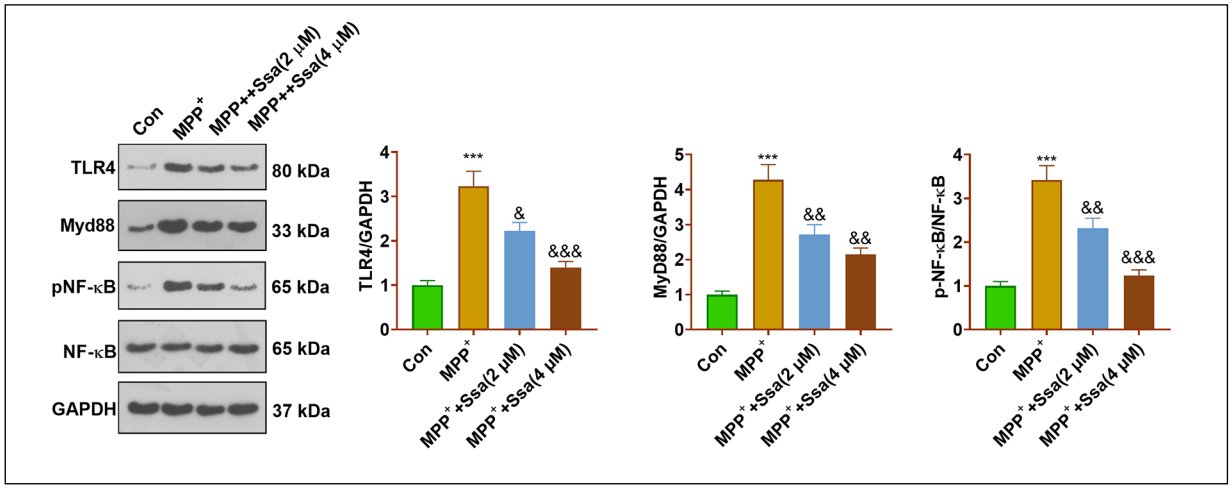

Figure 4. Saikosaponin A attenuated the TLR4/MyD88/NF-κB pathway activation, WB examined TLR4 and MyD88 expression and NF-κB phosphorylation in BV2 microglia. Data are expressed as mean ± SEM, N=3. ***p<0.001 (*vs.* Con group), &p<0.05, &&p<0.01, &&&p<0.001 (*vs.* MPP$^+$ group).

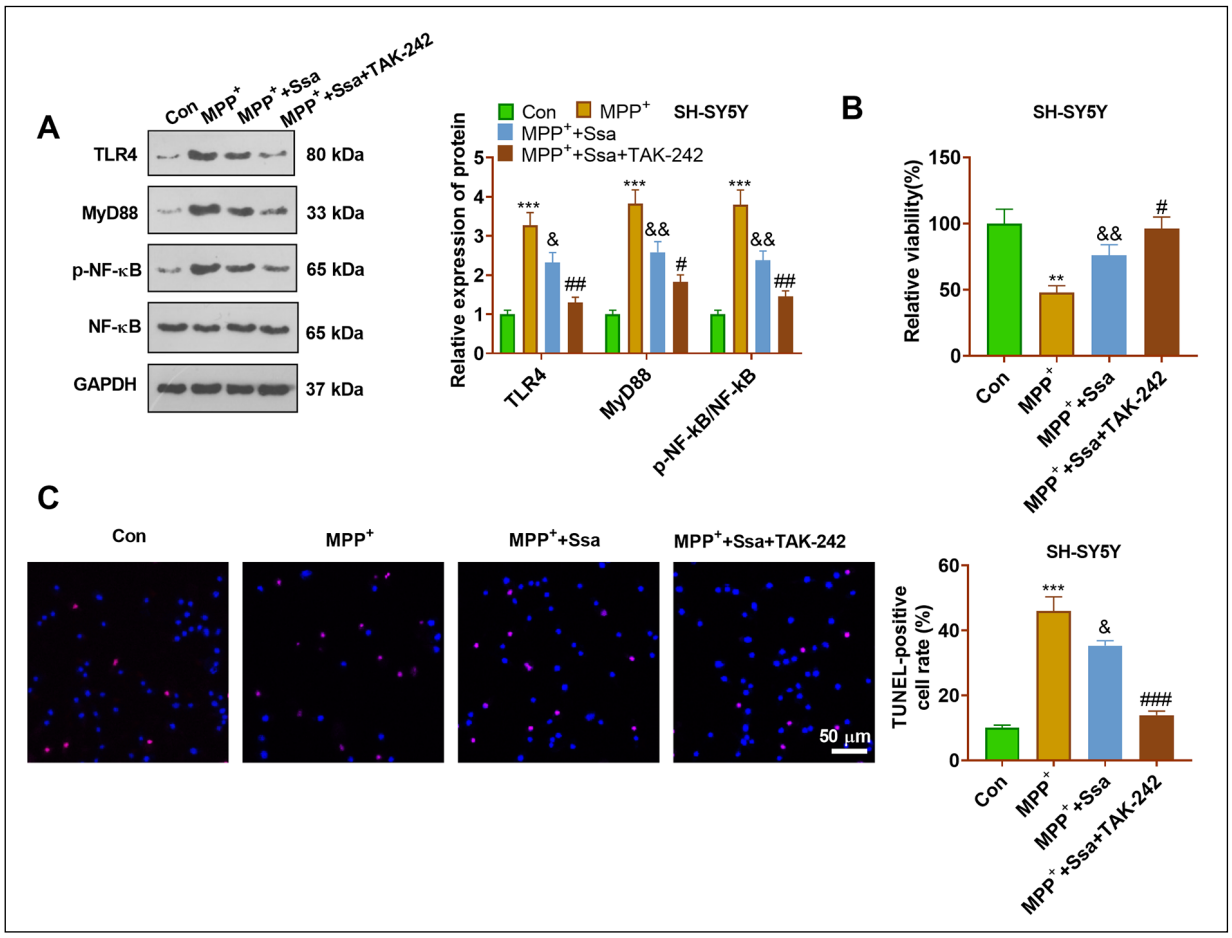

Figure 5. Inhibiting the TLR4/MyD88/NF-κB pathway enhanced the anti-apoptotic. SH-SY5Y cells and BV2 microglia were pre-cultured with 20 μg/ ml TAK-242 (TLR4 inhibitor) for three hours, followed by incubation with Ssa (4 μM) and 5 mM MPP$^+$ for 14 hours, respectively. **A**, WB assessed TLR4 and MyD88 expression and NF-κB phosphorylation in BV2 microglia. **B**, SH-SY5Y cell viability was detected by MTT. **C**, SH-SY5Y cell apoptosis was checked by TUNEL staining. Scale bar = 50 μm. Data are expressed as mean ± SEM, N=3. **p<0.01, ***p<0.001 (*vs.* Con group), &p< 0.05, &&p<0.01 (*vs.* MPP$^+$ group). #p<0.05, ##p<0.01, ###p<0.001 (*vs.* MPP++Ssa group).

GSH-Px and SOD activities in SH-SY5Y cells compared to the MPP$^+$+Ssa group (Figure 6A-C). ELISA showed that adding TAK-242 further reduced the expression of inflammatory factors in BV2 cells *vs*. the MPP$^+$+Ssa group (Figure 6D-E). In summary, Ssa exerted its anti-inflammatory effects by modulating the MPP$^+$-induced TLR4/MyD88/NF-κB pathway in BV2 microglia.

Saikosaponin A Reduced MPTP-Mediated Neuronal Apoptosis and Ameliorated MPTP-Mediated Motor Dysfunction in Rats

The PD rat model was set up *via* intraperitoneal (i.p) injection of MPTP (20 mg/kg) twice daily, with a total of 80 mg of MPTP per rat. Ssa (5 mg/kg dissolved in an equal amount of DMSO) was administered twice daily for two weeks by intraperitoneal injection three days prior to the modeling. The experimental design is presented in Figure 7A. At week two after the MPTP injection, SN tissues from PD rats were isolated and stained with H&E and Nissl to observe the effect of Ssa on the MPTP-induced morphological changes of hippocampal neurons. As shown in Figure 7B, neurons in the Sham and Ssa groups were neatly arranged and clearly visible, whereas most neurons in the MPTP group were disordered, with substantial neuronal loss, contracted, and stained darkly. In contrast, pre-administration of Ssa lessened the changes in hippocampal neurons in the brain tissue of PD rats, with hippocampal neurons having clear bounds and being more organized compared to the MPTP group. In Figure 7C, Nissl staining showed a significant amount of Nissl body degeneration in the MPTP group. Pretreating with Ssa substantially decreased neuronal degeneration in the SN region of PD rats. Tyrosine hydroxylase (TH) is the rate-limiting enzyme involved in catecholamine and dopamine syn-

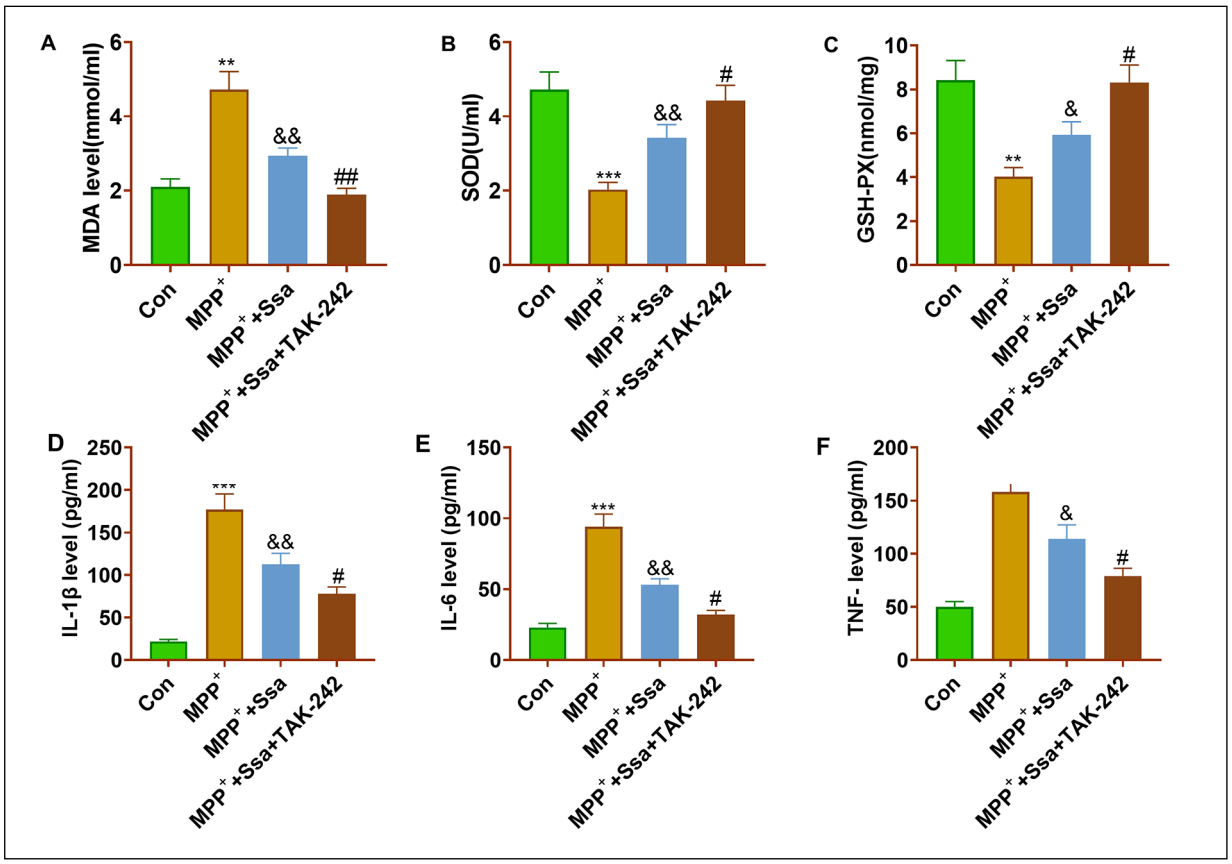

Figure 6. Inhibiting the TLR4/MyD88/NF-κB pathway enhanced the anti-inflammatory and antioxidative effects of Ssa. SH-SY5Y cells and BV2 microglia were pre-cultured with 20 μg/ ml TAK-242 (TLR4 inhibitor) for three hours, followed by incubation with Ssa (4 μM) and 5 mM MPP$^+$ for 14 hours, respectively. **A-C**, Commercial kits were utilized to measure MDA expression and GSH-Px and SOD activities in SH-SY5Y. **D-F**, The expressions of inflammatory cytokines IL-1β, TNF-α and IL-6 in BV2 microglia were compared by ELISA. Data are expressed as mean ± SEM, N=3. **p<0.01, ***p<0.001 (*vs*. Con group). &p< 0.05, &&p<0.01, (*vs*. MPP$^+$ group). #p<0.05, ##p<0.01 (*vs*. MPP++Ssa group).

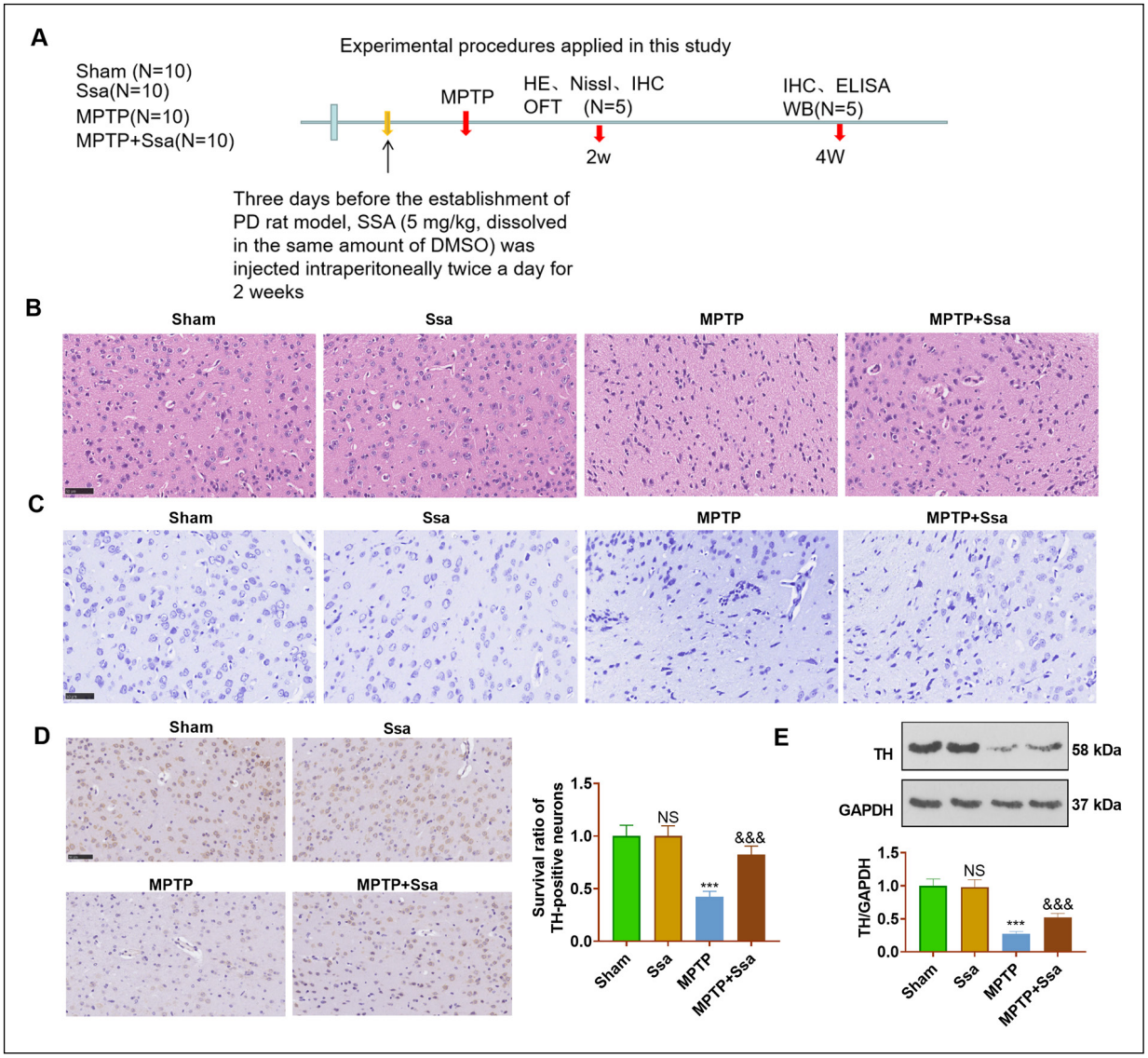

Figure 7. Saikosaponin A reduced MPTP-mediated TH-neuronal loss in rats. MPTP (20 mg/kg) was administered intraperitoneally (i.p) to rats to construct a PD model, twice daily, for a total of 80 mg of MPTP per rat. Ssa (5 mg/kg dissolved in an equal amount of DMSO) was administered intraperitoneally to rats three days prior to the construction of the PD model, twice daily for two weeks. **A**, Schematic diagram of experimental design. **B**, H&E staining in rat SN. Scale bar =50 µm. **C**, Nissl staining in rat SN. Scale bar =50 µm. **D**, Immunohistochemical staining of TH-positive cells at a scale of 50 µm and quantification of TH-positive cells. Data are expressed as mean ± SEM, N=5. NS $p>0.05$, *** $p<0.001$ (*vs*. Sham group), &&&$p<0.001$ (*vs*. MPTP group). **E-F**, WB determined the expression of TH proteins and apoptosis-related proteins in rat SN tissues.

thesis. Brainstem dopamine and norepinephrine deficiency due to neurodegeneration of dopamine and norepinephrine neurons are mainly associated with non-motor and motor symptoms of PD[26]. Therefore, we measured the expression of TH protein in MPTP-induced rat SN using immunohistochemistry and Western blot (WB). The number of TH-positive neurons and TH protein levels were significantly lower in the SN of the MPTP-treated rats compared to the Sham group. Notably, Ssa significantly increased the number of TH-positive cells and TH protein expression in the MPTP-induced rat SN (Figure 7 D-E). On the other hand, MPTP treatment increased the expression of Bax and C-caspase-3 and lowered the expression of Bcl-2 compared to the Sham group. Ssa treatment inhibited the expression of Bax and C-caspase-3 and increased the expression of Bcl-2 (Figure 8A). Behavioral examinations of PD rats two weeks after MPTP administration showed that rats in the MPTP group had significantly fewer center entries and spent less time in the center area compared

to the Sham group. Ssa treatment substantially improved the impairment of motor movements, as demonstrated by the increased number of center entries and prolonged time spent in the center area in PD rats (Figure 8B, C). There was no statistical difference between the Sham and Ssa groups. Our results show that Ssa diminishes MPTP-mediated neuronal apoptosis and alleviates MPTP-mediated motor dysfunction in the rat brain.

Saikosaponin A Blocked MPTP-Induced Activation of Microglia in Rat Hippocampal Tissues

Microglia are usually activated in the presence of neuroinflammation. Four weeks after the MPTP injection, SN was isolated from the brains of PD rats, and the expression levels of the microglia activation marker IBA1 were detected using immunohistochemical staining. There was a significantly higher level of IBA1-positive staining in the SN of MPTP-induced rats compared to the Sham group. Ssa pretreatment reduced IBA1 cell number in the hippocampal cortical area of rats (Figure 9A). In addition, MPTP induced an increase in IL-1β, TNF-α, IL-6, and MDA contents and a decrease in SOD and GSH-Px activities in the SN of rats compared to the Sham group. Ssa down-regulated the expression of these inflammatory markers and oxidative stressors in the SN of LPS-treated rats (Figure 9 B-G).

Saikosaponin A Impeded TLR4/MyD88/NF-κB Expression in the Rat Hippocampus

The TLR-4/MyD88 pathway is key to the induction of inflammation[27]. At week 4 after MPTP injection, TLR4/MyD88/NF-κB expression in the SN of MPTP-induced rats was measured using WB. Elevated levels of TLR4 and MyD88 and increased NF-κB phosphorylation were found in the hippocampus of MPTP-treated rats compared to the Sham group. Ssa significantly reversed the MPTP-induced overexpression of TLR4 and MyD88 and reduced NF-κB phosphorylation (Figure 10A).

Discussion

The results of the current study show that Ssa inhibited microglia activation and diminished oxidative stress and inflammatory factor expression both *in vivo* and *in vitro*, and improved neuronal apoptosis and motor function in PD rats.

Parkinson's disease, a progressive neurodegenerative disorder manifested as tremors and bradykinesia, is the most common severe movement disorder, affecting approximately 1% of 60% of adults globally[28,29]. The main feature of PD is the degeneration of dopaminergic neurons in the substantia nigra (SN) through activation of programmed neuronal death[30]. A recent study[31] has shown that microglia activation is triggered earlier than other degenerative events in PD, suggesting that hyperactivation (including different states of polarization) of microglia may induce loss of dopaminergic neurons, thus triggering an endless cycle of inflammation/ degeneration[31]. Therefore, inhibition of microglia activation may be an effective approach to treating PD. MPTP-induced PD rat models[32,33] and MPP^+-treated SH-SY5Y cells[34] have been widely used to study inflammatory processes in PD pathogenesis and anti-inflammatory therapies for

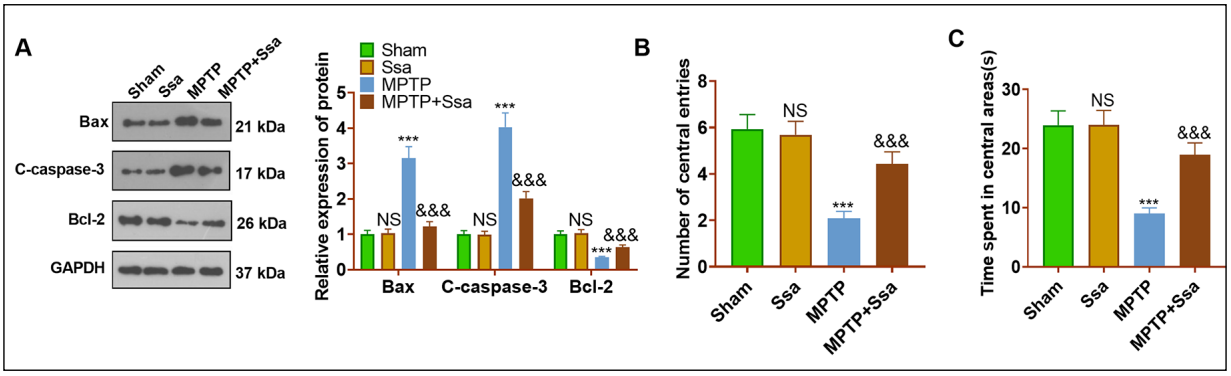

Figure 8. Saikosaponin A abated MPTP-mediated neuronal apoptosis and improved MPTP-mediated motor dysfunction in rats. **A**, WB determined the expression of TH proteins and apoptosis-related proteins in rat SN tissues. **B** and **C**, Number of center entries and duration in the center region in rats in the OFT two weeks following LPS injection. Data are expressed as mean ± SEM, N=5. NS $p>0.05$, ***$p<0.001$ (*vs.* Sham group), &&&$p<0.001$ (*vs.* MPTP group).

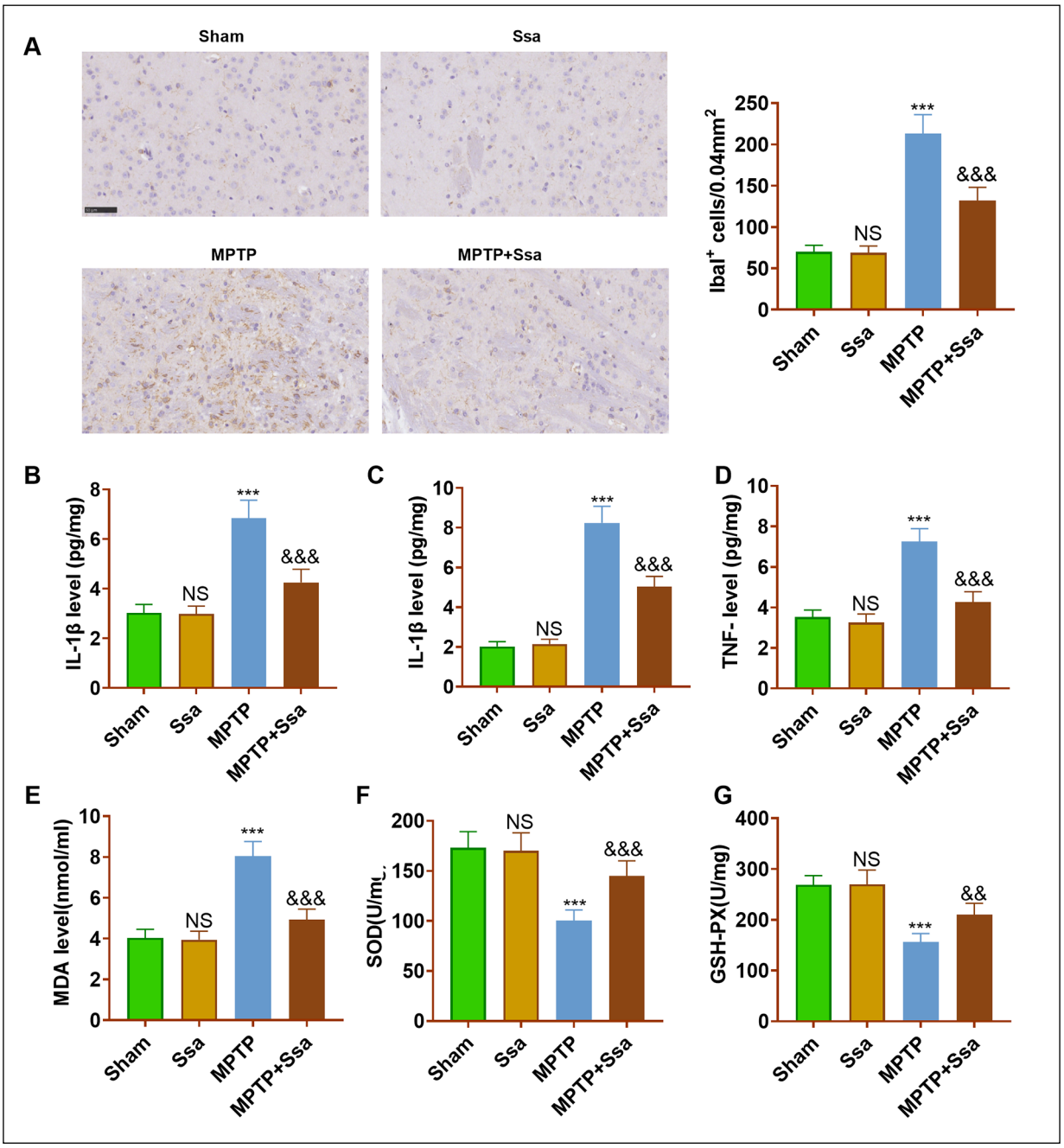

Figure 9. Saikosaponin A blocked MPTP-induced microglia activation in hippocampal tissues of rats. Four weeks following MPTP injection, SN was separated from PD rats' brains. **A**, Morphological changes of SN microglia were observed by IBA-1 immunohistochemical staining. Representative micrographs of the SN region are exhibited, and the number of IBA-1-positive cells was counted. **B-D**, ELISA measured the production of pro-inflammatory mediators (TNF-α, IL-1β, and IL-6). **E-G**, Commercial kits were utilized to measure the production of oxidative stressors (MDA, SOD, and GSH-Px). Data are expressed as mean ± SEM, N=5. NS $p>0.05$, ***$p<0.001$ (*vs*. Sham group), && $p<0.01$, &&&$p<0.001$ (*vs*. MPTP group).

PD treatment. We constructed *in-vivo* and *in-vitro* PD models using MPTP and MPP^+ in rat and SH-SY5Y cells, respectively.

Radix Bupleurum (RB) has been applied extensively in Chinese medicine for over 2000 years. In the last few decades, about 74 compounds have been isolated from RB, including essential oils, triterpenoid saponins, polyacetylenes, flavonoids, lignans, fatty acids, and sterols[35]. Saikosaponin (Sss, especially Ssa, Ssc

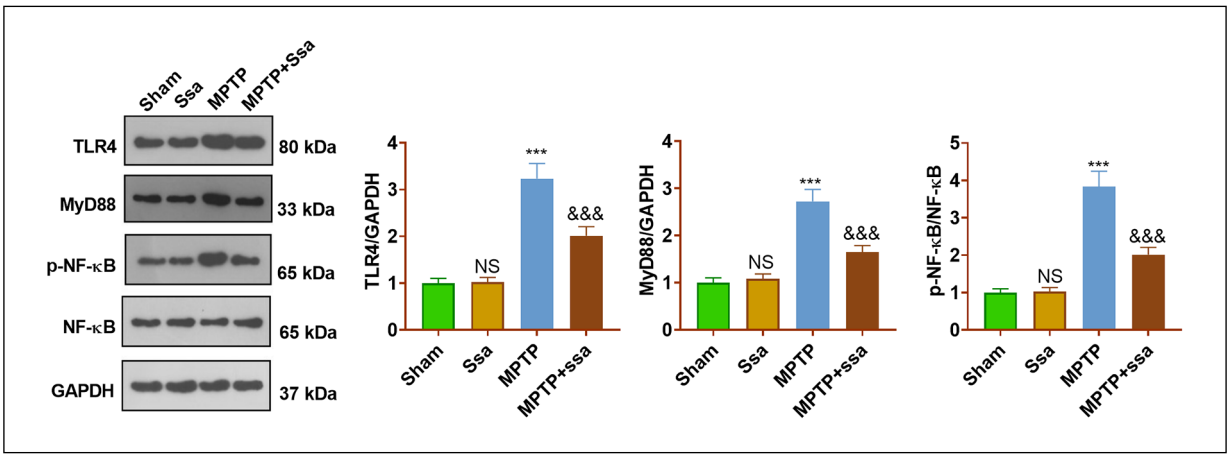

Figure 10. Saikosaponin A hindered TLR4/MyD88/NF-κB expression in the rat hippocampus. Expression of TLR4/MyD88/NF-κB in MPTP-induced rat SN was determined using WB at week four after MPTP injection. Data are expressed as mean ± SEM, N=5. ***$p<0.001$ (*vs*. Sham group), NS $p>0.05$, &&&$p<0.001$ (*vs*. MPTP group).

and Ssd), the main bioactive compounds in RB, have anti-inflammatory, anti-tumor, antioxidant, anti-viral, and hepatoprotective properties[36]. Zhou et al[37] showed that Ssa can effectively reduce neuropathic pain in chronic constriction injury (CCI) rats by inactivating the p38 MAPK and NF-κB pathways in the spinal cord[37]. In a rat model of controlled cortical shock (CCI)-induced traumatic brain injury (TBI), Ssa eases the inflammatory response and improves neurological function and cognition in traumatic brain injury (TBI) rats and diminishes brain edema and blood-brain barrier permeability by blocking the MAPK signaling pathway[38]. It is thus clear that Ssa suppresses the inflammatory response in neuroinflammatory diseases. Nevertheless, there is little research on the effect of Ssa in neurodegenerative diseases, although it has been well-studied as one of the main active components of RB. Zhou et al[39] demonstrated that Ssd treatment slowed the activation of microglia and astrocytes, reduced neuronal apoptosis, and abated amyloid-β deposition in the hippocampus of mice with Alzheimer's disease (AD) by blocking the NF-κB pathway, with a neuroprotective effect against progressive neurodegeneration. Ssd prevents SH-SY5Y cells from glutamate-induced oxidative cytotoxicity by inducing endogenous antioxidant enzyme activity and HO-1 expression through activation of PI3K and subsequent Nrf2 nuclear translocation[40]. Additionally, Ssd lowers H_2O_2-induced reactive oxygen species (ROS) accumulation and H_2O_2-induced PC12 cell apoptosis by inhibiting the MAPK signaling pathway[41]. These findings further strengthen the theory that Ssd may be a promising candidate for the prevention and treatment of AD and other oxidation-related diseases. We researched the role and the mechanism of action of Ssa using *ex-vivo* and *in-vivo* models of MPTP and MPP^+-induced PD and showed that SSa was able to reverse elevated expression of inflammatory factors and oxidative stressors and increased neuronal apoptosis in *in-vivo* PD model. Our results imply that Ssa may have a neuroprotective effect.

TLR4/MyD88/NF-κB is a classical inflammatory signaling pathway that mediates neuroinflammation. Several studies have demonstrated that inhibition of the TLR4/MyD88/NF-κB pathway lowers the production of pro-inflammatory mediators in activated microglia[18,42-46], thereby having a neuroprotective effect in I/R[47,48], subarachnoid hemorrhage[49], traumatic brain injury[50], encephalitis[51], and neurodegenerative diseases[52]. Using *in vitro* model of MPP^+-induced PD, Yu et al[53] showed that Curcumin depressed activation of MPP+-induced primary astrocytes and reduced the levels of inflammatory factors and oxidative stressors in astrocytes by lowering the levels of MPP^+-induced TLR4 and its downstream effectors, including NF-κB, IRF3, MyD88 and TIRF[53]. These studies[49-53] suggested that inhibition of TLR4/MyD88/NF-κB may play a beneficial role in the pathophysiology of PD with respect to neuroinflammation. Based on the above studies, we explored the association between Ssa and TLR4/MyD88/NF-κB. In both *in-vivo* and *in-vitro* PD models, TLR4 and MyD88 were up-regulated, and NF-κB phos-

phorylation was enhanced, although Ssa treatment inhibited TLR4/MyD88/NF-κB activation. Inhibition of the TLR4/MyD88/NF-κB pathway amplified the inflammatory inhibitory effect of Ssa on MPP^+-induced BV2 microglia.

Conclusions

To our knowledge, this is the first study to describe that Ssa lessens neurotoxicity and neuroinflammation in *in-vivo* and *ex-vivo* PD models by blocking the TLR4/MyD88/NF-κB signaling axis. Our data suggest that Ssa may be a potential drug for the treatment of inflammatory pathology in PD. Nevertheless, there are some limitations to our study. We have mainly studied the effects of Ssa on oxidative stress and neuroinflammation in PD and its potential mechanisms. Comprehensive and detailed studies on Ssa in terms of pharmacodynamics and pharmacokinetics are still needed to develop its bioactive compounds as effective drugs.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Ethics Approval

The Ethics Committee of Hebei North University approved this study, No. HBNV20220310223042, Date: March 13th, 2022.

Informed Consent

Not applicable.

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Data Availability

The data that support the findings of this study are available on request from the corresponding author.

Authors' Contribution

X.-L. Liu and L. Fan conceived and designed the analysis; X.-L. Liu, B.-Ho. Yue and Z. Lou collected the data; X.-L. Liu contributed data or analysis tools; L. Fan, B.-Ho. Yue, and Z. Lou performed the analysis; X.-L. Liu wrote the paper..

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