

ORIGINAL ARTICLE

Bee venom phospholipase A2 ameliorates motor dysfunction and modulates microglia activation in Parkinson's disease alpha-synuclein transgenic mice

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α -Synuclein (α -Syn) has a critical role in microglia-mediated neuroinflammation, which leads to the development of Parkinson's disease (PD). Recent studies have shown that bee venom (BV) has beneficial effects on PD symptoms in human patients or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxin-induced PD mice. This study investigated whether treatment with BV-derived phospholipase A2 (bvPLA2) would improve the motor dysfunction and pathological features of PD in human A53T α -Syn mutant transgenic (A53T Tg) mice. The motor dysfunction of A53T Tg mice was assessed using the pole test. The levels of α -Syn, microglia and the M1/M2 phenotype in the spinal cord were evaluated by immunofluorescence. bvPLA2 treatment significantly ameliorated motor dysfunction in A53T Tg mice. In addition, bvPLA2 significantly reduced the expression of α -Syn, the activation and numbers of microglia, and the ratio of M1/M2 in A53T Tg mice. These results suggest that bvPLA2 could be a promising treatment option for PD.

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INTRODUCTION

Parkinson's disease (PD) is the most extensively perceived synucleinopathy because it is the most general movement disorder, with a prevalence of ~1% at 65 years of age, increasing to 4–5% by 85 years of age.¹ Increasing evidence shows that neuroinflammation has a significant role in the pathogenesis of PD.² Activated microglia and recruited peripheral macrophages release neurotoxic species, such as pro-inflammatory cytokines, and promote neurodegeneration.^{3–6} Activated microglia/macrophages are classified into M1 or M2 phenotypes. When they are polarized to release neurotoxic factors, such as inflammatory cytokines, reactive oxygen species (ROS), nitric oxide synthase (NOS) and glutamate, they are defined by the M1 phenotype. However, when they release diverse neuroprotective molecules, such as anti-inflammatory cytokines, neurotrophins and membrane receptors, they are defined by the M2 phenotype.⁷ An increased ratio of M1 over M2 microglia/macrophages has been reported in various neurodegenerative pathologies, such as Alzheimer's disease, and in the elderly brain; modulation of microglia polarization

has been suggested as a neuroprotective therapy.^{8,9} In the PD brain, microglia proliferate early in the disease process, remaining comparatively static and unrelated to the extent of striatal degeneration or clinical severity.¹⁰ However, microglia polarization to proinflammatory M1 or anti-inflammatory M2 phenotypes has been poorly studied in PD; the cytokine levels from autopsy studies have suggested that both M1 and M2 microglia may coexist in a brain with Parkinson's disease.¹¹ In addition, studies conducted in a transgenic α -synuclein (Syn) A53T model have provided crucial insight into the dichotomous response, which microglia can activate.

Bee venom (BV) has been used in traditional medicine to cure some immune-related diseases, such as rheumatoid arthritis, in humans and animals.^{12,13} Recently, several clinical trials have shown that BV might be beneficial in the treatment of neurodegenerative diseases, including PD.^{14,15} Our recent study demonstrated that the modulation of peripheral immune tolerance by regulatory T cells (Tregs) may contribute to the neuroprotective effect of BV in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD.¹⁶ Bee venom

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phospholipase A2 (bvPLA2) is an enzyme that breaks down membrane phospholipids to produce free fatty acids and lysophospholipids.^{17,18} PLA2 can be classified into three broad classes based on its cellular distribution: secreted or extracellular type (sPLA2), cytosolic or intracellular type (cPLA2) and Ca²⁺-independent PLA2. bvPLA2 is of the sPLA2 type; sPLA2 is generally present in the venom of snakes, bees, cnidarians and scorpions. sPLA2 has a central role in various cellular responses, including phospholipid metabolism, signal transduction and the initiation and regulation of inflammatory and immune responses.^{19–22} bvPLA2 is a typical Group III sPLA2 member and comprises ~12% of the dry weight of venom in the European honeybee, *Apis mellifera*.^{17,23} bvPLA2 has a wide variety of pharmacological properties, including anti-HIV activity, myotoxicity and neurite outgrowth induction.^{24,25} However, the efficacy and the mechanism of the action of bvPLA2 that is involved in PD remains largely unknown.

In this study, we evaluated for the first time the motor dysfunction of A53T human α -Syn Tg mice and age-matched non-transgenic (nTg) littermates using a modified pole test. In addition, α -Syn aggregation and M1/M2 polarization were analyzed in the spinal cord.

MATERIALS AND METHODS

Animals and treatment

Animal procedures were approved by the University of Kyung Hee Institutional Animal Care and Usage Committee and were in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Human A53T α -Syn transgenic (A53T Tg) mice (B6;C3-Tg(Prnp-SNCA**A53T*)83Vle/J) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All animals were kept in a pathogen-free environment on a 12-h light/dark cycle and had access to food and water *ad libitum*. The A53T Tg mice were randomized into five groups. The mice received repeated intraperitoneal treatment of bvPLA2 (0.2 or 1 mg kg⁻¹) or phosphate-buffered saline (PBS). Starting at 3 months of age, the mice received PBS and bvPLA2 once a week for 3 months until they were 6 months old.

Cell culture and treatment

The murine BV-2 microglial cell line was maintained in RPMI 1640 medium (Welgene, Gyeongsan, Korea) supplemented with 10% fetal bovine serum (Welgene, Gyeongsan, Korea), 100 U ml⁻¹ penicillin and 100 μ g ml⁻¹ streptomycin (Invitrogen Life Technologies, Rockville, MD, USA). The cells were cultured every 2–3 days until the cells became 80% confluent. The cells were incubated at 37 °C with 95% humidity and 5% CO₂ for all experiments. For polarization, the cells were seeded in six-well plates at a density of 5 × 10⁵ cells per ml and treated the following day. Immediately before treatment, the cells were washed twice with serum-free RPMI medium and supplemented with 2 ml of warm serum-free RPMI medium containing experimental treatments. The cells were pretreated with 0.1, 1 or 10 μ g ml⁻¹ bvPLA2 for 30 min, then 1 μ g ml⁻¹ lipopolysaccharides (LPS) (Sigma-Aldrich, St Louis, MO, USA) or 20 ng ml⁻¹ murine recombinant interleukin (IL)-4 (R&D Systems, Minneapolis, MN, USA) were added to each well. After 24 h of treatment, the cells were rinsed twice with PBS and collected for RNA extraction.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from BV-2 cells using an Easy-BLUE RNA extraction kit (iNtRON Biotechnology, Inc., Seongnam, Korea). The RNA quality and concentration were determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Inc., ND-1000; Wilmington, DE, USA) and standardized to the lowest concentration with RNase free water. The RNA was reverse transcribed to cDNA using CycleScript Reverse Transcriptase and Random Oligonucleotide Primers (Bioneer, Deajeon, Korea) following the manufacturer's instructions. Quantitative real-time PCR was performed using the SensiFAST SYBR No-ROX Kit (Bioline, Taunton, MA, USA) and analyzed with the LightCycler 480 system (Roche Ltd, Basel, Switzerland). The PCR reactions were subjected to 55 cycles of denaturation at 95 °C for 10 s, annealing at 72 °C for 10 s and extension at 60 °C for 10 s, with fluorescence measured at the end of each cycle. The data are expressed as 2^{- $\Delta\Delta$ CT} for the experimental gene normalized to GAPDH, and presented as the fold change relative to the saline-treated control. The following primers were used: TNF- α for: 5'-TTCTGTCTACTGAACCTCGGGGTGATCGGTCC-3'; TNF- α rev: 5'-GTATGAGATAGCAAATCGGCTGACGGTGTGGG-3'; iNOS for: 5'-GGCAGCCTGTGAGACCTTTG-3'; iNOS rev: 5'-CATTGGAA GTGAAGCGTTTCG-3'; Arg1 for: 5'-AGACAGCAGAGGAGGTG AAGAG-3'; Arg1 rev: 5'-CGAAGCAAGCCAAGGTTAAAGC-3'; MMR for: 5'-AGTGGCAGGTGGCTTATG-3'; MMR rev: 5'-GGT TCAGGAGTTGTTGTG-3'; GAPDH for: 5'-ACCCAGAAGACTGT GGATGG-3'; GAPDH rev: 5'-CACATTGGGGGTAGGAACAC-3'

Measurement of motor activity

The pole test has been used to measure motor coordination and balance in mouse models of PD.²⁶ The pole test was performed 12 weeks after starting the experiment. In the test, the mice were placed on top of a rough-surfaced iron pole (50 cm in length and 0.8 cm in diameter) and could climb down to the base of the pole. The number of times that it took for the mouse to turn completely downward (time to turn; *T*-turn) and then to reach the floor (locomotion activity time; *T*-LA) were measured, with a cutoff limit of 30 s. The average of the three measurements was used as the result.

Tissue preparation

Transcardial perfusion was performed with a saline solution containing 0.5% sodium nitrate and heparin (10 U ml⁻¹); then, fixation was performed with 4% paraformaldehyde in 0.1 M phosphate buffer. Each brain was dissected, post-fixed overnight in buffered 4% paraformaldehyde at 4 °C and stored in a 30% sucrose solution at 4 °C until it sank. Freeze sectioning was performed on a sliding microtome to generate 30- μ m-thick coronal sections.

Immunofluorescence staining and quantification

For immunofluorescence staining, A53T Tg and non-transgenic littermate wildtype (WT) mice were transcardially perfused with PBS followed by 4% paraformaldehyde. The brain and spinal cord were removed and post-fixed in buffered 4% paraformaldehyde for 6 h. After immersion in a 30% sucrose solution, the frozen spinal cord was cut into 30- μ m-thick coronal sections. The slices were washed with PBS, incubated for 24 h at room temperature with human anti- α -synuclein (Santa Cruz Biotechnology, Dallas, TX, USA), and diluted 1:200 with 0.5% bovine serum albumin in PBS. The sections were then incubated with AlexaFluor 594 goat anti-rabbit (1:200, Vector Laboratories, Burlingame, CA, USA) for 2 h in the dark. The sections were washed thoroughly and mounted with fluorescent mounting

medium (Vector Laboratories). The number of cells labeled in the dorsal column was counted using a confocal microscope equipped with argon and HeNe1 lasers (LSM-5; Zeiss, Jena, Germany). Fluorescent images were sequentially acquired for the red and green channels to prevent the crossover of signals from the green to red or from the red to green channels. The images were viewed as z-stacks, both as a series of optical sections and as a projection of merged images.

Statistical analysis

All of the values are expressed as the mean \pm s.e.m. The statistical significance ($P < 0.05$ for all analyses) was assessed by one-way analysis of variance followed by Tukey's *post hoc* test for multiple comparisons using Prism 5.01 software (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Inhibitory effects of bvPLA2 on motor impairment in A53T Tg mice

The pole test is a useful method for evaluating the motor dysfunction caused by PD. Prolonged *T*-turn and *T*-LA are known characteristic motor dysfunctions of PD in mice.

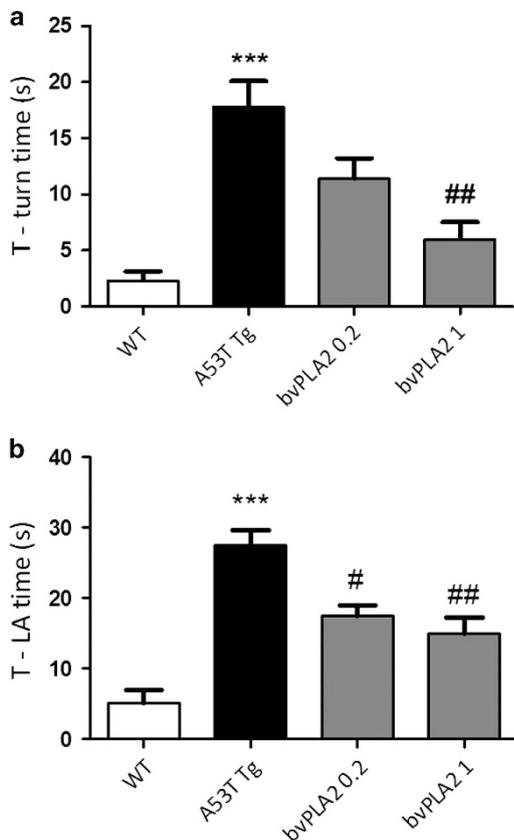


Figure 1 Inhibitory effects of bvPLA2 on motor impairment (*T*-turn and *T*-LA) in A53T Tg mice. A53T Tg mice were pretreated with 0.2 or 1 mg kg⁻¹ bvPLA2 for 3 months. (a) The amount of time for the mice to turn completely downward (*T*-turn) was recorded. (b) The amount of time for the mice to reach the floor (*T*-LA) was recorded. The values indicate the mean \pm s.e.m. *** $P < 0.001$, compared with the WT groups; # $P < 0.05$, ## $P < 0.01$, compared with the A53T Tg group. Tg, transgenic; WT, wild type

To confirm the effect of bvPLA2 on motor dysfunction in an *in vivo* PD model, we treated mice with bvPLA2 or PBS and carried out the pole test. The *T*-turn and *T*-LA of the A53T Tg group were markedly prolonged, at 17.8 ± 2.3 s and 27.5 ± 2.15 s, respectively, compared with the WT group (*T*-turn: 2.3 ± 0.84 s; *T*-LA: 5.1 ± 1.87 s; Figure 1). However, the *T*-turn time for the bvPLA2 1 mg kg⁻¹ per day treated group was shortened to 6 ± 1.54 s, and the *T*-LA time of the bvPLA2 0.2 and 1 mg kg⁻¹ per day treated groups were decreased to 17.5 ± 1.46 s and 15 ± 2.21 s, respectively (Figure 1).

Inhibitory effects of bvPLA2 on α -syn pathology in A53T Tg mice

Because α -Syn is known to contribute to PD pathogenesis in various ways,²⁷ it is the gold standard in the evaluation of PD. To confirm the effect of bvPLA2 on the α -Syn pathology, α -Syn immunostaining was performed using spinal cord tissue sections from 6-month-old A53T Tg mice and age-matched WT mice. The intensity of α -Syn in the dorsal column region of the spinal cord was significantly increased in A53T Tg mice (640 ± 89) compared with the WT group (220 ± 54 , $P < 0.01$; Figure 2). bvPLA2 treatment effectively inhibited α -Syn pathology (1 mg kg⁻¹, 326 ± 45 , $P < 0.05$; Figure 2) compared with A53T Tg mice.

Treatment with bvPLA2 induces microglial deactivation in the spinal cord

The activation of microglial cells is also one of the characteristics of PD. To determine whether the neuro-protective effect of bvPLA2 resulted from the inhibition of microglial activation in the spinal cord, we performed immunohistochemistry with anti-CD11b to detect microglia/macrophages in the spinal cord sections (Figure 3). In WT mice, few CD11b⁺ microglia/macrophages, with resting morphologies characterized by small cell bodies and thin processes, were observed in the spinal cords. In contrast, numerous CD11b⁺ microglia/macrophages, with activated morphologies characterized by larger cell bodies and thick processes, were obvious in the spinal cords of A53T Tg mice. In A53T Tg mice treated with bvPLA2, the number of activated microglia/macrophages decreased in the spinal cord.

Treatment with bvPLA2 normalizes the ratio of M1/M2 microglial phenotypes

While M1 microglia produce toxic substances to neurons, such as proinflammatory cytokines and reactive oxygen species, M2 microglia produce anti-inflammatory and tissue repair factors to promote survival and repair. We tested the effects of bvPLA on the M1/M2 microglial phenotypes using immunofluorescence double-labeling and confocal microscopy. Specifically, we documented the distribution and magnitude of microglia/macrophages expressing M1 (CD86) and M2 (Arginase 1) phenotypic markers (Figure 4). The M1:M2 ratio decreased markedly upon bvPLA2 treatment, mostly because of the increase of labeling for M2 phenotypic markers (Figure 4).

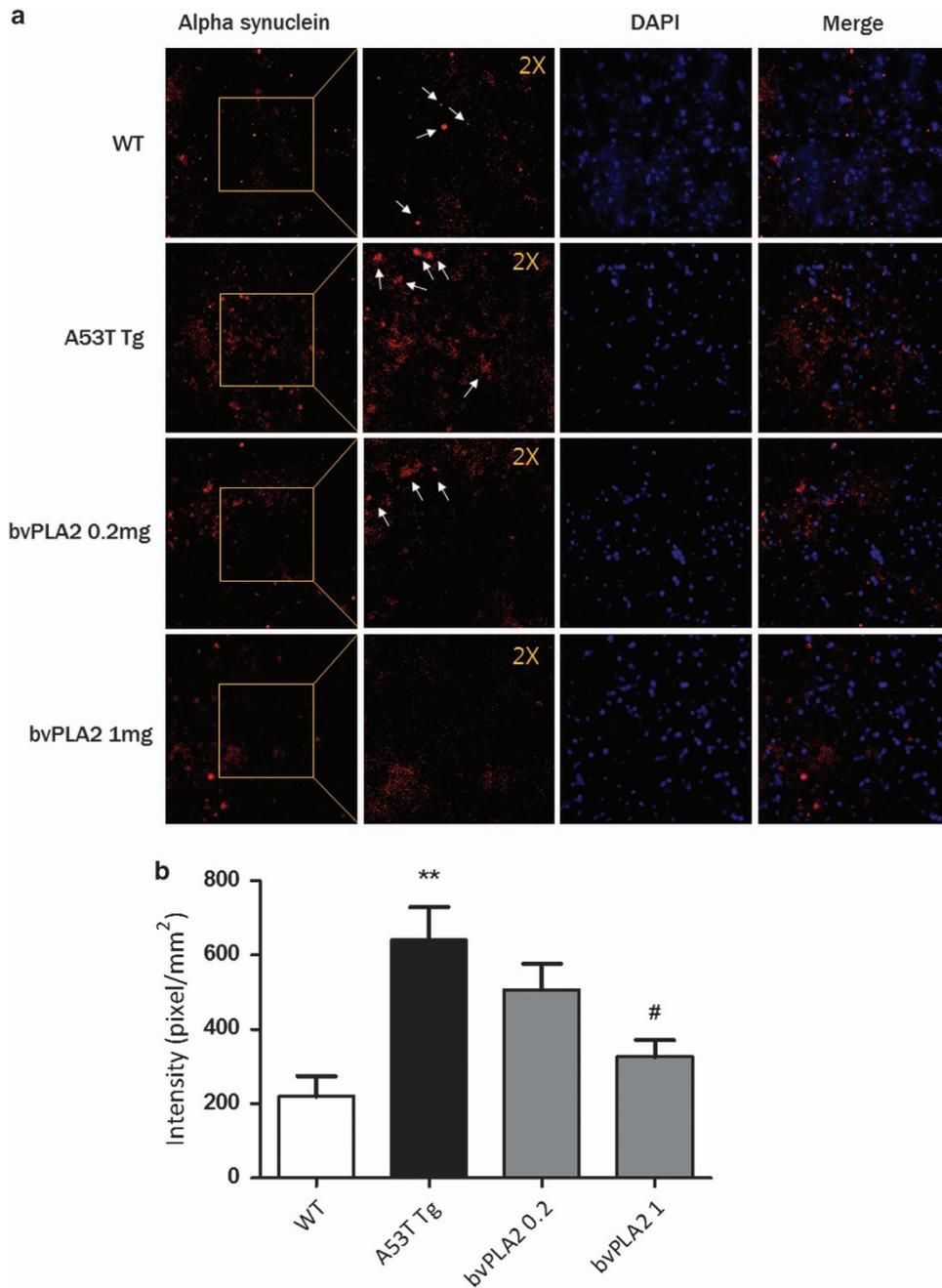


Figure 2 bvPLA2 inhibits α -Syn in the spinal cord in A53Tg mice. **(a)** Confocal microscopic imaging of α -Syn fluorescence (red) in the spinal cord. The nuclei were counterstained with DAPI (blue). The insets are a $\times 2$ magnification of the boxed area. The representative immunostained α -Syn inclusions are indicated as arrows. **(b)** The confocal image shown in **a** was analyzed. The values indicate the mean \pm s.e.m. ** $P < 0.01$ compared with the WT group, # $P < 0.05$ compared with the A53T Tg group. α -Syn, α -Synuclein; WT, wild type.

bvPLA2 influences the polarization of BV-2 microglial cells to the M2 phenotype

We tested the effects of bvPLA2 on the M1/M2 polarization of microglial cells by quantifying the gene expression upon exposure to M1-inducing conditions (LPS) or M2-inducing conditions (IL-4). Tumor necrosis factor alpha (TNF- α) and inducible nitric oxide synthase (iNOS) served as M1 phenotypic markers, whereas macrophage mannose receptor (MMR; CD206) and

arginase-1 (Arg1) served as M2 markers. The messenger RNA (mRNA) levels of TNF- α and iNOS were significantly increased compared with the vehicle control when the cells were exposed to LPS (Figure 5). bvPLA2 effectively blocked the polarization of BV-2 cells to the M1 phenotype in a dose-dependent manner during LPS exposure. No changes were observed in M1 markers during IL-4 exposure. MMR was slightly upregulated and Arg1 was significantly increased by IL-4 compared with the vehicle

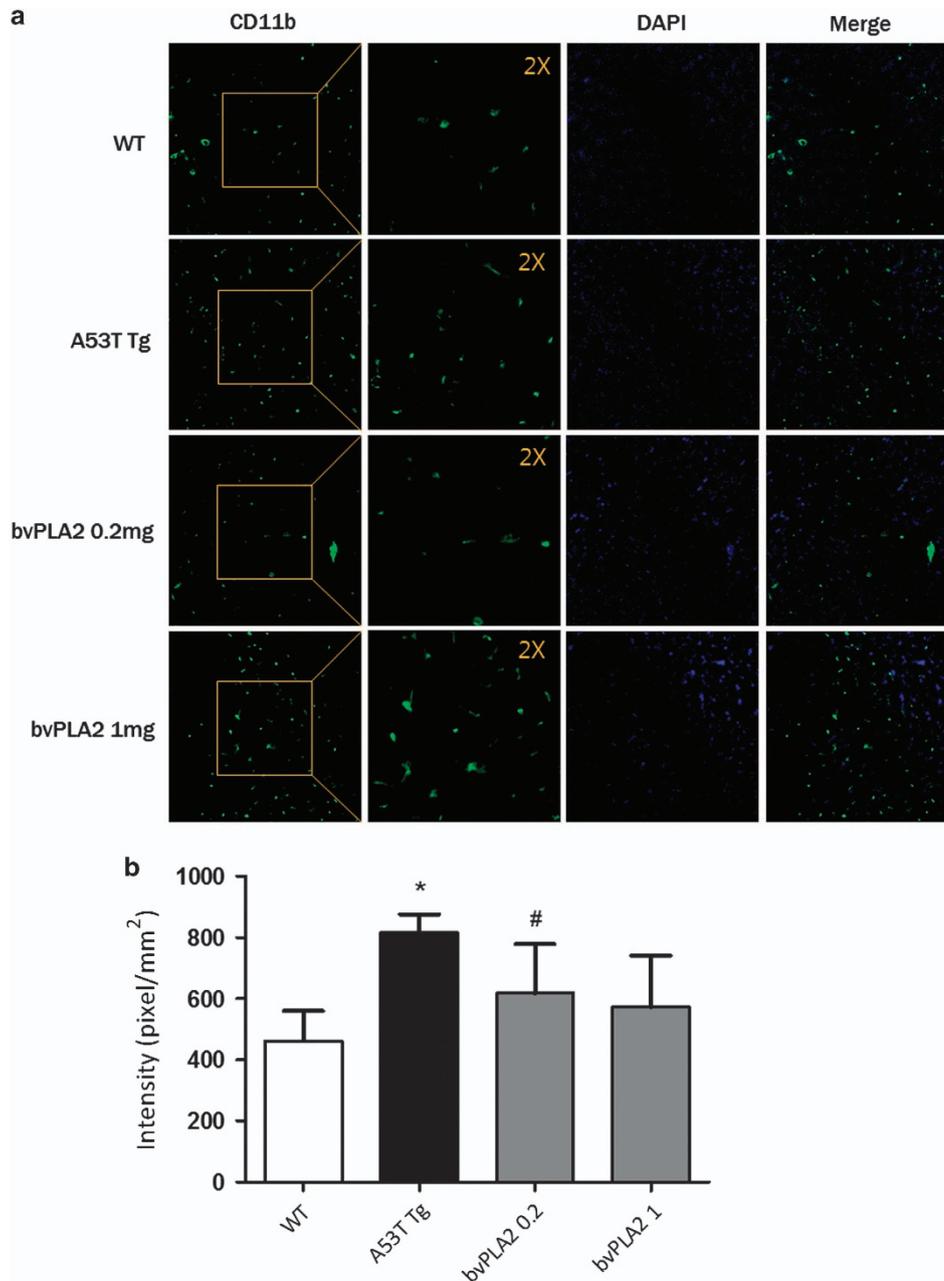


Figure 3 Treatment with bvPLA2 induces microglial deactivation in the spinal cord. **(a)** A section was selected and processed for CD11b immunofluorescence. Confocal microscopic observation revealed microglia fluorescence (green) in the spinal cord. The nuclei were counterstained with DAPI (blue). The insets are $\times 2$ magnification of the boxed area. The representative immunostained α -Syn inclusions are indicated as arrows. **(b)** The confocal image shown in **(a)** was analyzed. The values indicate the mean \pm s.e.m. * $P < 0.05$ compared with the WT group, # $P < 0.05$ compared with the A53T Tg group. α -Syn, α -Synuclein; Tg, transgenic; WT, wild type.

control. The mRNA levels of both were significantly increased upon bvPLA2 treatment when the cells were exposed to IL-4. There were no significant differences in M2 markers during LPS exposure. Taken together, these results demonstrate that bvPLA2 promotes M2 microglia polarization

DISCUSSION

We report that progressive degeneration in A53T Tg mice and the improvements resulting from bvPLA2 were associated with

α -Syn accumulation in the spinal cord. At the behavioral level, these alterations were accompanied by significantly reduced *T*-turn and *T*-locomotor times in a modified pole test. The behavior effects of bvPLA2 were accompanied by differential changes in the expression of inflammatory mediators in the spinal cords of A53T Tg mice. Specifically, treatment with bvPLA2 reduced the expression of the microglial activation marker CD11b and the M1 marker CD86 in the spinal cords of A53T Tg mice. In contrast, bvPLA2 treatment increased the

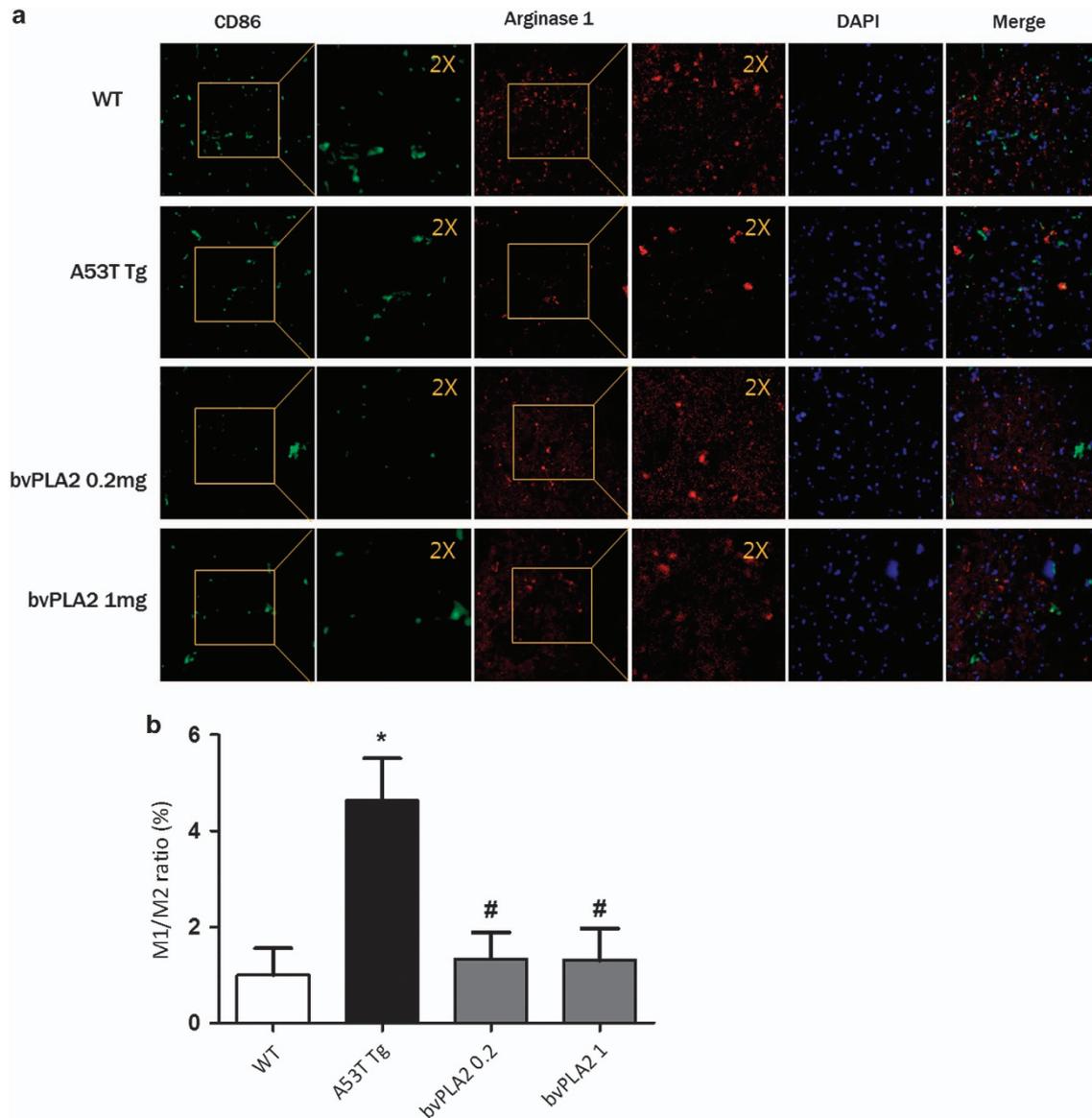


Figure 4 bvPLA2 changes the M1/M2 ratio in the spinal cords of A53T Tg mice. **(a)** Confocal microscopic imaging of CD86⁺M1 macrophage fluorescence (red) and Arginase I fluorescence (green) M2 macrophages in the spinal cord. The nuclei were counterstained with DAPI (blue). The insets are a $\times 2$ magnification of the boxed area. The representative immunostained α -Syn inclusions are indicated as arrows. **(b)** The confocal image shown in **a** was analyzed. The values indicate the mean \pm s.e.m. * $P < 0.05$ compared with the WT group, # $P < 0.05$ compared with the A53T Tg group. Tg, transgenic; WT, wild type.

expression of the M2 marker arginase 1 in the spinal cords of A53T Tg mice. These *in vivo* results were confirmed by *in vitro* experiments using BV-2 microglia. Although bvPLA2 treatment promoted M2 differentiation, M1 differentiation was inhibited by bvPLA2 treatment.

In recent years, several strains of genetically modified mice have been generated as models for studying PD, including the BAC-Tg3 mice reported by Hilton *et al.* and the A30P+A53T transgenic mice reported by Ikeda *et al.*²⁸ In the present study, we used A53T Tg mice, a model expressing mutant α -Syn under the control of the mouse prion protein promoter. The A53T mutant human α -Syn homozygous mice showed severe motor disturbance, with accumulation of α -Syn in the

brain, similar to patients with the A53T mutation.¹ A53T Tg mice have many similarities with human neuronal α -synucleinopathies (especially familial PD) because of the A53T α -Syn mutation. Specifically, the PrP-driven expression of human A53T α -Syn results in a mid- to late-onset neurodegenerative disorder that coincides with the accumulation of filamentous α -Syn cytoplasmic inclusions throughout the neuraxis, similar to patients with the A53T mutation.²⁹ No α -Syn pathology was detected in young animals expressing A53T human α -Syn, and these animals did not present an overt neurological impairment.

These results indicate that the pathological features that mimic PD in A53T mice remain stable. Previous studies have

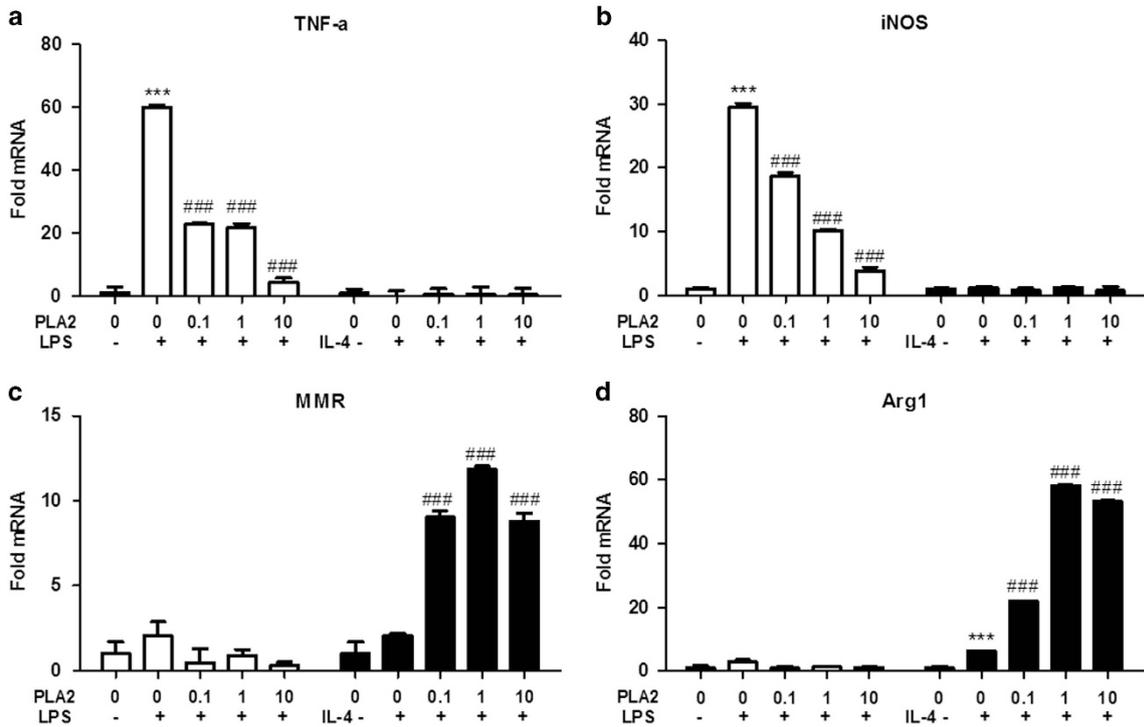


Figure 5 bvPLA2 directly induces M2-like polarization of BV-2 microglial cells. Cells were pretreated with bvPLA2 for 30 min and stimulated with LPS or IL-4 for 24 h. The gene expression profile of BV-2 microglial cells was determined by quantitative real-time PCR. (a, b): M1-phenotypic marker (A, TNF- α ; B, iNOS) and (c, d): M2-phenotypic marker (C, MMR; D, Arg1) mRNA expression in BV-2 microglial cells. The fold increase in each gene was normalized against the level of the unstimulated (vehicle-treated) control. The values indicate the mean \pm s.e.m., with each experiment performed in triplicate. *** $P < 0.0001$ compared with the control group, ### $P < 0.0001$ compared with the LPS or IL-4 only treated groups. BV, bee venom; bvPLA2, BV-derived phospholipase A2; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharides; MMR, macrophage mannose receptor; mRNA, messenger RNA; TNF, tumor necrosis factor.

reported the pharmacological efficacy of bvPLA2 in various PD models. bvPLA2 was shown to have an important role in MPTP-induced neurotoxicity and oxidative stress in the nigrostriatal system.³⁰ Our study demonstrated that treatment with bvPLA2 resulted in a significant restoration of motor dysfunction in PD mice. These results suggest that bvPLA2 treatment may be effective at ameliorating locomotive impairment caused by PD. α -Syn is a presynaptic neuronal protein that is linked genetically and neuropathologically to PD^{31–33} and multiple system atrophy.³⁴ α -Syn is not only a biomarker for diagnosing PD but is also an important target for PD therapy. We demonstrated that treatment with bvPLA2 is a feasible and effective way to decrease α -Syn in A53T Tg mice. However, the detailed mechanism by which bvPLA2 reduces α -Syn remains unclear and requires further exploration.

Microglia are the resident macrophages of the brain; they have crucial roles in the development and preservation of the neural environment.³⁵ However, in the presence of adverse stimuli, microglia can induce chronic, damaging inflammation that ultimately leads to neuronal cell death.³⁶ Microglia are readily activated during most neuropathological conditions, such as PD and Alzheimer's disease.³⁷ Our results show that bvPLA2 significantly decreases microglia activation in the spinal cord, suggesting that a decrease in microglia activation may contribute to the therapeutic effect of bvPLA2. Multiple

reports have suggested that the phenotype of activated microglia (M1/M2) controls the repair and regeneration response following nerve injury.^{38,39} When we tested the effects of bvPLA2 on the M1/M2 polarization of microglial cells, bvPLA2 treatment promoted M2 polarization and suppressed M1 polarization in BV-2 microglia. These observations strongly support a dual role for activated microglia in human brains with ongoing synucleinopathies. M1 microglia are thought to be proinflammatory (IL-1 β , TNF- α) and result in deleterious neuroinflammation, whereas M2 microglia (IL-10, neurotrophins) are considered to promote neuroprotection.^{39–42} Therefore, some researchers suggest that future immunomodulatory therapies for PD should focus on promoting the M2 profile over the M1 profile to promote regeneration and neuroprotection.⁹ Our results show that bvPLA2 significantly promotes the M2 profile over the M1 profile in A53T Tg mice and BV-2 microglia. We cannot rule out the possibility of the involvement of other immune cells in addition to macrophages, such as Tregs, in the effects of bvPLA2.

Previously, we reported that bee venom ameliorated MPTP-induced PD via Treg induction.¹⁶ We also revealed that bvPLA2 induced a Treg population via CD206 on DCs.⁴³ Recently, we revealed that bvPLA2 protects dopaminergic neurons by inhibiting neuroinflammatory responses in an MPTP-induced PD mouse model.⁴⁴ In the study, bvPLA2

had no effect on mice depleted of Tregs by anti-CD25 Abs, and we also demonstrated that bvPLA2-CD206-PGE2-EP2 signaling promotes Treg differentiation. Thus, it is reasonable to predict that the increase in Tregs mediated by bvPLA2 treatment could reduce neuroinflammation in A53T Tg mice.

These findings suggest that treatment with bvPLA2 ameliorates motor deficits and reduces α -Syn in A53T Tg mice. These effects are associated with a decrease in the M1/M2 ratio and the activation of microglia in A53T Tg mice. These results provide strong evidence for the use of bvPLA2 as a novel strategy for PD treatment.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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