Alpha-synuclein-specific regulatory T cells ameliorate Parkinson’s disease progression in mice

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Research Article

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Abstract

Background

Parkinson's disease is a long-term neurodegenerative disease characterized by dopaminergic neuronal loss and the aggregation of alpha-synuclein in the brain. Cell therapy using regulatory T cells has therapeutic potential on Parkinson's progression in a 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine-induced mouse model; however, several challenges were associated with its applications. Polyclonal regulatory T cells can move to sites other than the disease loci and cause undesirable suppression. Moreover, the efficiency of regulatory T cells is reduced during the expansion process, accompanied by phenotypic changes. Here, we propose a strategy for regulatory T cell expansion using alpha-synuclein and bee venom phospholipase A2.

Methods

We presented alpha-synuclein to T cells via dendritic cells following bee venom phospholipase A2 treatment and analyzed their phenotype and mobility. These regulatory T cells were transferred to 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine-induced Parkinson's mouse model. First, we measured motor function using pole test. Next, we examined the expression of tyrosine hydroxylase, alpha-synuclein, ionized calcium binding adaptor molecule 1, and nitric oxide synthase 2 in the substantia nigra by immunohistostaining. The mRNA expression related to neuroinflammation was measured using real-time reverse transcription polymerase chain reaction.

Results

Our method increased the mobility of regulatory T cells towards the site of abundant alpha-synuclein in vitro and in vivo. Alpha-synuclein-specific bee venom phospholipase A2-treated regulatory T cells showed noteworthy neuroprotective effects against motor function deficits, dopaminergic neuronal loss, and alpha-synuclein accumulation in the Parkinson's mouse model. Furthermore, adoptive transfer of alpha-synuclein-specific bee venom phospholipase A2-treated regulatory T cells exerted immunosuppressive effects on activated microglia, especially pro-inflammatory microglia, in Parkinson's mice.

Conclusions

Our findings suggest that the combination of alpha-synuclein and bee venom phospholipase A2 may provide a significant improvement in neuroprotective activities of regulatory T cells and suggest the effective clinical application of T cell therapy in Parkinson's.

Background

Parkinson's disease (PD) is a progressive neurodegenerative disorder that leads to motor deficits, including tremors, rigidity, and postural instability (1). The current therapy for Parkinson's is aimed only at relieving the disease symptoms. None of the prescribed drugs, such as dopamine agonists and levodopa,
have been proven disease-modifying (2). Additionally, there are many side effects, such as drug-related motor fluctuations, on-off effects, and dyskinesia (3).

A characteristic feature of PD is the presence of soluble or insoluble aggregates of alpha-synuclein (α-syn) in the brain. Though α-syn is a synaptic protein present in the brain, it accompanies neurotoxicity and ultimately leads to neuronal dysfunction (4). Aggregated α-syn is phagocytosed by microglia and induces microglial activation with ROS production. Activated microglia are found in specific brain regions in patients with PD and are considered influential in the degeneration of dopaminergic neurons by amplifying neuronal inflammation (5, 6). The 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) model is the most widely used experimental model for studying PD in mice. MPTP causes α-syn accumulation in the substantia nigra (SN) and PD-like motor impairment (7, 8). In the MPTP model, as in patients with PD, microglial activation is induced before the dopaminergic neuronal loss and is implicated in PD pathology. Therefore, activated microglia are a promising therapeutic target for PD (9).

CD4+CD25+ regulatory T cells (Tregs) play a crucial role in controlling immune balance in the central nervous system (CNS). However, their suppressive activity is dysregulated during inflammation, aggravating the damage caused by autoreactive T effector cells (10, 11). Recently, Tregs have been considered an attractive therapeutic modality that induces neuroprotective activity (12). Adoptive transfer of Tregs has been attempted as a potential treatment for neurodegenerative diseases such as Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and even PD (13-15). However, these studies had some limitations, one being the off-target suppression by polyclonal Tregs. As a solution to this problem, the development of antigen-specific Tregs has been attempted. It has been reported that antigen-specific Tregs are more effective and safer than polyclonal Tregs in a mouse model of non-obese diabetes and graft-versus-host disease (GVHD) (16-19). Therefore, α-syn, a PD-specific antigen, could increase the effectiveness of Treg therapy targeting PD. Another obstacle is the difficulty of producing and maintaining suppressive phenotypes during Treg expansion. Previously, we reported that treatment of splenocyte cultures with bee venom phospholipase A2 (bvPLA2) maintained the expression of CD62L in CD4+CD25+ Tregs (20). The CD62Lhi Treg subset has been demonstrated to act as an optimal suppressor in vitro and restrains the donor T cells effectively in GVHD (21, 22). Therefore, we hypothesized that bvPLA2 could modulate the Treg phenotype during expansion and increase its therapeutic effects.

The aim of the current study is to establish the effects of bvPLA2 and α-syn on ex vivo expansion of Tregs. Thus, we adoptively transferred expanded Tregs into an MPTP-induced mouse model of PD and assessed the disease pathology. The advantage of combination of α-syn and bvPLA2 showed the enhancement of the neuroprotective effects of Tregs on motor function, neuronal loss, and pro-inflammatory microglial activation. In conclusion, our findings provide insight into the clinical application of Treg therapy that has a great potential for PD.

**Materials And Methods**

**Animals**
Male C57BL/6 mice and Thy1.1 (B6.PL-Thy1<+>/CyJ) mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). The mice were maintained on a 12 h light/dark cycle and under temperature-controlled conditions, with food and water *ad libitum*. All experiments were performed in accordance with approved animal protocols and guidelines established by Kyung Hee University (KHUASP(SE)-22-148 and 20-240).

**Cell preparation**

To present the Parkinson's disease-specific antigen to Tregs via dendritic cells (DCs), α-syn (Prospec, EB, NJ, USA) was aggregated by incubation in a shaker at 37 °C for 7 days. To obtain bone marrow (BM)-DCs, BM-leukocytes were isolated from the femurs and tibiae of mice and resuspended in a medium containing 20 ng/mL Granulocyte-macrophage colony-stimulating factor R&D Systems, Minneapolis, MN, USA) (23). After 7 days, CD11c<+> DCs were isolated using CD11c MicroBeads (Miltenyi Biotec Inc., Auburn, CA, USA) and seeded at a density of 2 × 10^5/mL in 96-well U-bottom plates. For antigen presentation, half of the samples were treated with 2 μg/mL aggregated α-syn for 24 h. CD4 T cells were isolated from splenocytes using CD4 (L3T4) microbeads (Miltenyi Biotec). The cells were added to the DC at a ratio of 10:1 (CD4 T: DC). Half of the samples were treated with 0.4 μg/mL bvPLA2 (Sigma-Aldrich, St Louis, MO, USA). Four days after CD4 T cells-DC co-culture, CD4<+>CD25<+> T cells (Tregs) were isolated using magnetic-activated cell sorting, according to the manufacturer's protocol (CD4<+>CD25<+> Regulatory T Cell Isolation Kit; Miltenyi). CD4<+>CD25<+> T cells were stimulated using the Treg Expansion Kit (Miltenyi) for 14 days.

**Treg migration assay**

Treg migration was assayed using 24-well Transwell chambers with 5 μm pores. A total of 10^5 cells in 100 μL of media (RPMI 1640 with 0.5% Bovine Serum Albumin (BSA)) were placed in the upper chambers. Aggregated α-syn, diluted in 600 μL media, was placed in the lower wells, and the chambers were incubated at 37 °C. After 3 and 4 h, the migrated cells present in the bottom wells were counted using a LUNA-II automated cell counter (Logos Biosystems, Anyang, South Korea).

**Animal experiment**

For MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) intoxication, seven-week-old male mice received four i.p. injections of MPTP-HCl (12 mg/kg free base in saline; Sigma-Aldrich) at 2 h intervals as previously described (13). Twelve hours after the last MPTP injection, MPTP-intoxicated mice were randomly divided and received adoptive transfers of 5 × 10^5 Tregs.

** Trafficking**

For trafficking of adoptively transferred Tregs, four groups of Tregs were prepared from Thy 1.1-mice as described in the cell preparation section. MPTP-treated mice (Thy 1.2<+>) were divided randomly into four groups and received adoptive transfer of 1 × 10^6 Thy 1.1<+> Tregs. After 7 days, the mice were euthanized, and the inguinal lymph nodes, spleen, blood, lung, kidney, liver, and brain were harvested. T cells were
enriched by 30–70% Percoll (Cytiva, Marlborough, MA, USA) density gradient centrifugation and debris removal solution (Miltenyi) from the lung, kidney, liver, and brain.

Flow cytometry

For flow cytometry, cells were washed with BD FACS Stain buffer (BD Bioscience, San Jose, CA, USA) and stained with fluorescently labeled-antibodies for 30 min at 4 °C in the dark. The following antibodies were used: PE-Cy7-CD4 (Invitrogen, Carlsbad, CA, USA), APC-CD62L (Invitrogen), and APC-Cy7-CD25 (BD Pharmingen) for the Treg phenotype, PE-Cy5-CD4 (Invitrogen), and mouse Vβ TCR Screening Panel (BD Pharmingen, San Diego, CA, USA) for the T cell receptor (TCR) repertoire, and PE-cy5-CD4, BV421-Thy1.1 (BD OptiBuild, San Jose, CA, USA), and 7AAD (Invitrogen) for Treg trafficking. All data were acquired using FACSLyric™ (BD Biosciences) and analyzed using FACSuite software (BD Biosciences).

Pole test

Six days after the adoptive transfer of Tregs, a pole test was performed to determine forelimb and hindlimb motor coordination and balance. Briefly, the mice were placed on top of a gauze-banded wooden pole (50 cm in length and 0.8 cm in diameter) facing upward. Animals were allowed to climb down to the base of the pole. The time taken to turn completely downward and the total time taken for the mouse to reach the floor (time to down) were recorded. The maximum cut-off time to stop was 30 s.

Tissue processing and immunohistochemistry

Mice were anesthetized with isoflurane (Forane solution; ChoongWae Pharma, Seoul, South Korea) and transcardially perfused with Phosphate-buffered saline (PBS). Brains were dissected and divided in half. The brain halves were postfixed in 4% paraformaldehyde for 18 h at 4 °C, transferred to 30% sucrose solution, and subsequently frozen. Tissues were serially cut on a cryostat into 30-μm-thick coronal sections using a cryomicrotome (HM525 NX; Thermo Ficsher Scientific, Inc., Waltham, MA, USA).

To detect dopaminergic neurons, primary antibodies were directed against tyrosine hydroxylase (TH; 1:2000, Pel-Freez Clinical System, Rogers, AR, USA). The sections were washed with PBS, incubated with the appropriate biotinylated secondary antibody, and processed using an avidin-biotin complex kit (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA) for 1 h at 25 °C. The reaction product was visualized with 0.05% diaminobenzidine-HCl and 0.003% hydrogen peroxide in 0.1 M phosphate buffer. The labeled tissue sections were subsequently mounted and analyzed under a bright-field microscope (Nikon, Tokyo, Japan). An unbiased stereological estimation of the total number of TH-positive dopaminergic neurons in the SN was performed using the optical fractionator method on an Olympus computer-assisted stereological toolbox system version 2.1.4. (Olympus, Tokyo, Japan) as previously described (24). The sections used for counting covered the entire SN, from the rostral tip of the pars compacta to the caudal end of the pars reticulata.

For immunofluorescence, brain sections were incubated with 50% formic acid for 10 min at 25 °C and heated with 10 mM sodium citrate buffer (pH 6.0) for epitope retrieval. After washing with cold PBS,
nonspecific binding was reduced by blocking the sections with 5% BSA in 0.2% Triton X-100 in TBS for 30 min at 25 ºC. The sections were incubated with antibodies against ionized calcium binding adaptor molecule 1 (Iba1; 1:1000, Abcam, Cambridge, MA, USA), nitric oxide synthase 2 (NOS2; 1:500, Santa Cruz Biotechnology, Dallas, Texas, USA), and α-syn (1:100, Santa Cruz Biotechnology) O/N at 4 ºC. Brain sections were washed with TBSTr, incubated for 2 h at 25 ºC with Alexa 488- or 594-conjugated IgG secondary antibodies, and then counterstained with DAPI. The tissues were examined using an LSM 800 confocal laser-scanning microscope (Carl Zeiss, Oberkochen, Germany). The staining intensity was quantified by measuring the integral density of the region of interest from monochromatic images using the ImageJ software. The percentage of staining intensity was calculated relative to the MPTP group and multiplied by 100.

**RNA extraction and quantitative Real-Time PCR (qRT-PCR)**

Total RNA was isolated from Tregs or unfixed brain halves using the easy-BLUE RNA extraction kit (iNtRON Biotechnology, Seongnam, South Korea), and cDNA was synthesized using Cyclescript reverse transcriptase (Bioneer, Daejeon, South Korea). Samples were prepared for real-time PCR using the SensiFAST SYBR no-Rox kit (Bioline, Randolph, MA, USA). The cycling conditions were: 1 cycle at 95 ºC for 30 s, 49 cycles at 95 ºC for 10 s, 55 ºC for 30 s, followed by a melting curve at 95 ºC for 10 s, 50 ºC for 5 s, and then a gradual increase until 95 ºC was reached. The base sequences of the primers are shown in Table 1.

**Table 1. The base sequence of primers for rtPCR**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forwad primer sequence (5′–3′)</th>
<th>Reverse primer sequence (5′–3′)</th>
</tr>
</thead>
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<tr>
<td>β-actin</td>
<td>GTG CTA TGT TGC TCT AGA CTT CG</td>
<td>ATG CCA CAG GAT TCC ATA CC</td>
</tr>
<tr>
<td>FoxP3</td>
<td>CTG CTC CTC CTA TTC CCG TAA C</td>
<td>AGC TAG AGG CTT TGC CTT CG</td>
</tr>
<tr>
<td>GATA3</td>
<td>GAA GGC ATC CAG ACC CGA AAC</td>
<td>ACC CAT GGC GGT GAC CAT GC</td>
</tr>
<tr>
<td>IL-10</td>
<td>CAG CCG GGA AGA CAA TAA CTG</td>
<td>CCG CAG CTC TAG GAG CAT GT</td>
</tr>
<tr>
<td>Areg</td>
<td>ACT GTG CAT GCC ATT GCC TA</td>
<td>ACT GGG CAT CTG GAA CCA TC</td>
</tr>
<tr>
<td>NOS2</td>
<td>AGG ACA TCC TGC GGC AGC</td>
<td>GCT TTA ACC CCT CCT GTA</td>
</tr>
<tr>
<td>TNF-α</td>
<td>GGC AGG TTC TGT CCC TTT CAC</td>
<td>TTT TGT GCT CAT GGT GTC TTT TCT</td>
</tr>
<tr>
<td>IL-1β</td>
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<td>TGA GGC CCA AGG CCA CAG G</td>
</tr>
<tr>
<td>IL-6</td>
<td>TTC CAT CCA GTT GCC TTC TTG</td>
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<td>AGG AGC TGT CAT TAG GGA CAT C</td>
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<tr>
<td>IL-4</td>
<td>ATC CTG CTC TTC TTT CTC GAA TGT</td>
<td>GCC GAT GAT CTC TCT CAA GTG ATT</td>
</tr>
</tbody>
</table>
Statistical analysis

All data were analyzed using GraphPad Prism 5.01 (GraphPad Software Inc., San Diego, CA, USA). The data are presented as the mean and standard error of the mean (SEM), where indicated. The statistical significance of each variable was evaluated by one-way analysis of variance (ANOVA), followed by Tukey’s multiple comparison test for multiple comparisons. Multiple comparisons within groups were analyzed using a two-way ANOVA, followed by Bonferroni post-hoc tests. All experiments were performed in a blinded manner and were repeated independently under identical conditions. Statistical significance was set at P < 0.05.

Results

Treatment of bvPLA2 maintains the suppressive phenotype during ex vivo expansion of Tregs

To prepare antigen-specific suppressive Tregs, CD4+ T cells were co-cultured with poly- or α-syn-presenting DCs for four days with or without bvPLA2. After 4 days, CD4+CD25+ Tregs were isolated and expanded for 14 days (Fig. 1A). Thereafter, Tregs were divided into four groups: untreated (None), bvPLA2 treated (bvPLA2), α-syn-specific (α-syn), and α-syn-specific bvPLA2 treated (α-syn+bvPLA2) Tregs. The expression of CD62L in Tregs were analyzed using flow cytometry (Fig. 1B). At the start (day 0), there were no differences among the four groups. However, on day 14 higher expression of CD62L was observed in bvPLA2 and α-syn+bvPLA2 Tregs (Fig. 1C). After expansion, mRNAs were extracted from Tregs, and the expression of Treg markers, such as forkhead box P3 (FoxP3), GATA3, Interleukin (IL)-10, and Amphiregulin (Areg), was analyzed (Fig. 1D). Treatment with bvPLA2 increased the expression of these markers, regardless of antigen presentation. These results suggest that bvPLA2 maintains the immunosuppressive Treg phenotype during ex vivo expansion.

Antigen presentation increases the mobility of Tregs toward disease-site

To determine whether antigen presentation affected TCR Vβ usage, the TCR repertoire was assessed using the Mouse Vβ TCR Screening Panel (Fig. 2A). The usage of some Vβ, particularly Vβ 8.1/2 and 13, increased slightly after antigen presentation.

It is presumed that antigen-specific Tregs are more efficient than poly-Tregs because they migrate towards the specific area of the brain where the antigen exists (25). Therefore, we performed an in vitro migration assay to assess the mobility of Tregs towards α-syn (Fig. 2B). After 4 h of seeding Tregs, approximately 50% of α-syn and α-syn+bvPLA2 Tregs moved to the bottom where α-syn existed, whereas less than 10% of None and bvPLA2 Tregs moved. To determine whether these in vitro results were reproduced in vivo, we produced None, bvPLA2, α-syn, and α-syn+bvPLA2 Tregs expressing Thy1.1 from Thy1.1+ mice (Fig. 2C). These Tregs were adoptively transferred to Thy1.2+ MPTP-intoxicated mice. Their distributions were detected by Thy1.1 expression in CD4+7AAD- cells. The results showed that α-syn, and α-syn+bvPLA2 Tregs accounted for a higher proportion in the brain, while there were no differences among the groups in other tissues such as lung, spleen, kidney and lymph node. These results support
that α-syn presentation improves the mobility of Tregs to α-syn-rich environments \textit{in vitro} as well as \textit{in vivo}.

**α-syn-specific bvPLA2 treated Tregs improve motor function in MPTP-induced Parkinson's disease mice**

To investigate the effect of Tregs on MPTP-induced neurodegeneration, $5 \times 10^5$ Tregs were adoptively transferred into MPTP-treated mice (12 mg/kg) (Fig. 3A). A pole test was carried out to examine PD-related motor deficits on day 6, and mice were sacrificed on day 7. MPTP treatment causes deficits in motor function resembling those observed in human PD (26). As expected, MPTP treatment significantly extended the time taken to turn downward compared to the wild type (WT) (Fig. 3B). This time was significantly shortened in all Tregs, except for None Tregs. In particular, α-syn+bvPLA2 Tregs significantly improved motor function compared with None Tregs in both indices. These results suggest that α-syn+bvPLA2 Tregs significantly improve motor function.

**α-syn-specific bvPLA2 treated Tregs reduce Parkinson's disease pathology in MPTP-induced Parkinson's disease mice**

PD is characterized by the loss of dopaminergic neurons in the SN (27). To confirm the loss of dopaminergic neurons, TH was assessed in the SN (Fig. 4A). MPTP treatment significantly reduced the number of TH-positive dopaminergic neurons compared with the control. Transfer of bvPLA2, α-syn, and α-syn+bvPLA2 Tregs increased the number of TH-positive neurons in the SN of MPTP intoxicated mice. Interestingly, α-syn+bvPLA2 Tregs significantly inhibited the loss of dopaminergic neurons compared with N, bvPLA2, and α-syn Tregs.

To investigate the effects of Tregs on the upregulation of α-syn in the MPTP-induced mouse model of PD, SNs were stained with α-syn (Fig. 4B). The α-syn intensity in the SN was increased in MPTP-intoxicated mice, and the adoptive transfer of all Tregs showed a significant reduction. Notably, α-syn+bvPLA2 Tregs decreased the accumulation of α-syn in the SN compared to MPTP and None Tregs. These results suggest that a combination of antigen presentation and bvPLA2 treatment improves the effects of Tregs on PD pathology.

**α-syn-specific bvPLA2-treated Tregs modulate microglial polarization in MPTP-induced Parkinson's disease mice**

Activated microglia secrete M1-associated pro-inflammatory cytokines that aggravate neurodegeneration in the PD brain, while M2 microglia participate in tissue repair by producing anti-inflammatory cytokines (28). To assess the activation of pro-inflammatory M1 microglia, SNs were immunostained with Iba1 as a marker of activated microglia and NOS2 as a marker of pro-inflammatory microglia (Fig. 5A). The increased intensities of NOS2 and Iba1 in MPTP-treated mice were significantly decreased by the adoptive transfer of α-syn and α-syn+bvPLA2 Tregs. In particular, the intensities of Iba1 and NOS2 in the α-syn+bvPLA2 Treg group were significantly different from those in the None Treg group. To further confirm the effects of Tregs on microglial polarization, the mRNA expression of M1 and M2 microglial
markers was evaluated by quantitative real-time PCR (Fig. 5B, C). The relative expression of NOS2, an M1 phenotypic marker, was significantly reduced by the α-syn and α-syn+bvPLA2 Treg transfer. Interestingly, the expression levels of the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), IL-1β, and IL-6 were significantly decreased with any other types of Treg. However, the adoptive transfer of Tregs has no significant effect on the relative mRNA level of arginase 1 (Arg1) and IL-4, an M2 phenotypic marker. Additionally, the mRNA levels of IL-10 and FoxP3 were significantly increased in the α-syn+bvPLA2 Treg-transferred mice brain (Fig. 5D). Collectively, these data show that Tregs, which combine with bvPLA2 treatment and α-syn presentation, have the best therapeutic effects on modifying microglial imbalances in the MPTP-intoxicated mice brains.

Discussion

Our present study proposes bvPLA2 treatment and α-syn presentation as methods for enhancing the therapeutic effects of Tregs in PD. To prepare Tregs, bvPLA2 was treated to CD4+ T cells during α-syn presentation via DCs. CD4+CD25+ Tregs were isolated and expanded ex vivo. The α-syn-specific bvPLA2 treated Tregs showed the following characteristics. First, the expression of suppressive markers was steadily maintained following bvPLA2 treatment. Second, these Tregs moved more frequently towards α-syn both in vitro and in vivo. Finally, Tregs showed better neuroprotective effects on the PD pathology with improved motor deficits, dopaminergic neuronal loss, and α-syn accumulation.

Tregs tightly regulate immune balance in the CNS as immune suppressors. In neurodegenerative diseases, Treg dysfunction leads to neuroinflammation. Many studies have focused on the importance of Tregs in the pathology of neurodegenerative diseases (10, 29). Tregs are significantly reduced or show poor suppressive activity in mild AD, ALS, and multiple sclerosis (MS) (30-32). In line with this, Treg therapy has been attempted, and its neuroprotective effects have been demonstrated in neurodegenerative diseases, including AD, ALS, and PD (13-15). In patients and mouse models with PD, CD4+ and CD8+ T cells were demonstrated to infiltrate the brain (33, 34). Other studies have reported that CD3+ T cells were accumulated in the brains of AD and MS patients (35, 36). These studies suggest the possibility of infiltration of adoptively transferred cells into the brain with neurodegenerative diseases and its potential as a therapeutic strategy for these diseases targeting adaptive immunity.

For efficient Treg therapy, it is important to diminish the number of Tregs to be administrated. Previously, Ashley et al. demonstrated that adoptively transfer of higher than 3.5 × 10^6 Tregs was necessary to elicit sufficient neuroprotective effects in behavior test and TH immunoreactivity MPTP-mice model (13). Similarly, in AD and ALS models, the neuroprotective effects of Tregs have been showed with transferring 1 × 10^5 or more Tregs which cultured with stimulation for 4 days (14, 15). To obtain such amount of Tregs in human, ex vivo expansion is essential for patients and many protocols for ex vivo expansion of Tregs have been developed (18, 37, 38). In this study, we adoptively transferred only 5 × 10^5 ex vivo expanded Tregs to MPTP-intoxicated mice and demonstrated that they could show sufficient neuroprotective effects by bvPLA2 and α-syn. Moreover, we established the improved efficacy of Tregs as
much as eight-fold compared with conventional expansion methods. However, since the Tregs should be isolated from peripheral blood mononuclear cells (PBMC) in human for clinical Treg therapy, there is a need for adequate modifications with antigen presentation and expansion.

For application of Treg therapy to actual patients, it is also desirable to increase their suppressive potentials. Several studies suggest the importance of the CD62L expression in Tregs for clinical manipulation in GVHD (21, 22). Highly expressed CD62L is rapidly lost after antigen experience (39); however, bvPLA2 treatment led to the maintenance of the CD62L expression regardless of antigen presentation in this study. Notably, bvPLA2 was treated for only 4 days before expansion, but its effects on CD62L expression were maintained for 14 days after expansion. Finally, we also examined the expressions of Treg specific genes. Areg is a critical factor for Treg suppressive function in vivo and in vitro (40). Tregs produce Areg and IL-10 for tissue repair in damaged tissues, regulated by GATA3 (41). The increased expression of these markers supports the hypothesis that bvPLA2 enhances the suppressive function of Tregs. Additionally, the viability and persistence of infused cells are important factors to increase therapeutic potential for adoptive transfer. There is an emerging need for strategies to improve the viability of ex vivo expanded cells (42, 43). In Treg trafficking, we found that bvPLA2 treated Tregs were more frequently detected in most tissues, including the liver, after transferred 7 days. This result demonstrated the potential of bvPLA2 for enhancing the efficiency of Treg therapy.

Microglia, the primary immune cells in the CNS, play crucial roles in orchestrating brain inflammation (44). Microglia may perform pathogenic or neuroprotective functions, depending on their phenotype. M1 microglia express NOS2 as a phenotypic marker and secrete pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α. M2 microglia, which express Arg1, are associated with tissue repair by producing IL-4 and IL-10 (28). Activated microglia are involved in the pathogenesis of certain neurological diseases such as PD (45). In the brains of patients with PD, increased level of activated microglia is representative, and it causes neurodegeneration. In animal models, activation of microglia and secretion of M1-associated pro-inflammatory cytokines are induced by MPTP (28). Additionally, α-syn acts as a stimulus that activates M1 microglia in the PD brain (46). Therefore, targeting the balance of impaired pro- and anti-inflammatory microglia can be a strategy for disease modification (47). Tregs ameliorate neuroinflammatory injury by regulating microglial polarization. In addition, loss of Tregs induced pro-inflammatory microglial activation in ICH and stroke models (48, 49). Indeed, studies attempting Treg therapy in neurodegenerative diseases have commonly observed the inhibition of microglial inflammation after Treg transfer (13-15). Our findings are consistent with those of previous studies showing that Tregs inhibit neuroinflammation by modulating microglial polarization.

The current data showed that α-syn presentation and bvPLA2 treatment of Tregs improved the neuroprotective effects in a PD mouse model by ameliorating neurodegeneration, α-syn accumulation, and microglial inflammation. While this study effectively demonstrated the potential of incorporating bvPLA2 and antigen presentation into Tregs, there are certain limitations. Further studies are required for a better understanding of the mechanisms of Treg therapy. Resolving this issue will enable the
development of a more efficient protocol for Treg expansion. Ultimately, our strategy may provide a promising avenue for immunotherapy with clinical relevance and cost-effectiveness for treating PD.

**Conclusions**

The implication is clear that treatment with bvPLA2 and α-syn improved Treg suppressive potential and mobility towards the disease site in a mouse model of PD. These improvements increase the therapeutic effects of Tregs on amelioration of PD progression by the modulating microglial polarization (Fig. 6).

**Abbreviations**

AD: Alzheimer's disease; ALS: amyotrophic lateral sclerosis; ANOVA: analysis of variance; Areg: Amphiregulin; Arg1: arginase 1; α-syn: alpha-synuclein; BM: bone marrow; BSA: bovine serum albumin; bvPLA2: bee venom phospholipase A2; CNS: central nervous system; DCs: dendritic cells; DMSO: dimethyl sulfoxide; FoxP3: forkhead box P3; GVHD: graft-versus-host disease; Iba1: ionized calcium binding adaptor molecule 1; IL: interleukin; MPTP: 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine; MS: multiple sclerosis; NOS2: nitric oxide synthase 2; PBS: phosphate-buffered saline; PD: Parkinson's disease; SEM: standard error of the mean; SN: substantia nigra; TH: tyrosine hydroxylase; TCR: T cell receptor; TNF-α: tumor necrosis factor-α. Treg: regulatory T cells; WT: wild type.

**Declarations**

**Ethics approval and consent to participate**

All animal experiments were performed in accordance with the approved animal protocols and guidelines established by Kyung Hee University (KHUAP(SE) 22-148 and 20-240).

**Consent for publication**

Not applicable.

**Availability of data and materials**

Not applicable.

**Competing Interests**

The authors declare that there were no commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors’ contributions

S.-Y.P. performed the experiment and wrote the manuscript. H.Y., H.G., and H.K. performed the experiment and discussed the data and manuscript. H.B. was designed this study. All authors read and approved the final manuscript.

Acknowledgements

Not applicable

References


**Figures**
Figure 1

**Effects of bvPLA2 on Treg phenotype during *ex vivo* expansion**

A, B Schematic diagram of Treg preparation and expansion. C On days 0 and 14 after Treg isolation, the expression of CD62L was analyzed using flow cytometry. D The relative mRNA expression of FoxP3, GATA3, IL-10, and Areg in expanded Tregs was analyzed. Data are presented as the mean ± SEM. Statistical analyses were conducted using one-way analysis of variance (ANOVA); *p < 0.05, **p < 0.01. n = 3-4.
Figure 2

Effects of α-syn on Treg mobility toward disease area

A TCR repertoire assessed using the mouse Vβ TCR screening panel. B Tregs and α-syn were seeded in the upper and lower chambers of the transwell, respectively. Migration of Tregs was assessed after 3 and 4 h. C Thy1.1+ Tregs were isolated from the splenocytes of Thy1.1+ mice. Thy1.1+ Tregs were adoptively transferred to MPTP-intoxicated (Thy1.2+) mice and detected in the lymph nodes, spleen, blood, lung, kidney, liver, and brain after 7 days. Data are presented as the mean ± SEM. Statistical analyses were conducted using two-way ANOVA; ***p<0.001. n=3-4.
Figure 3

Tregs moderate motor dysfunction of MPTP-induced mice model of PD.

A Schematic diagram of Treg transfer in MPTP-induced PD mice. B Time taken to turn downward and descend the pole was measured by the pole test on day 6. Data are presented as the mean ± SEM. Statistical analyses were conducted with one-way ANOVA; *p < 0.05, **p < 0.01 vs. WT, #p < 0.05, ##p < 0.01, vs. MPTP, $p < 0.05 vs None Tregs. n=8-10.
**Figure 4**

**Immunohistochemical staining of TH and α-syn in the SN of MPTP-induced mice model of PD.**

**A** Immunohistochemistry was performed for TH, a dopaminergic neuron marker, expression in the SN of the MPTP-induced PD mouse model. **B** To measure α-syn accumulation, the brain sections were stained with α-syn. The intensity was calculated and normalized with MPTP group. Data are presented as the mean ± SEM. Statistical analyses were conducted with One-way ANOVA; *p < 0.05, **p < 0.01, and ***< 0.001 vs. WT, #p < 0.05, ##p< 0.01, and ###p< 0.001 vs MPTP;

\[ p < 0.01 \text{ and} \]

$p<0.001$ vs. None Treg, $^\wedge^\wedge<0.01$ vs. bvPLA2 Treg, $^\wedge^\wedge^\wedge<0.01$ vs. a-syn Treg. n=4-6.
Figure 5

Tregs modulate microglial polarization.

A The expression of NOS2 and Iba1 was detected in the SN. B-D The relative mRNA expression of pro-inflammatory markers (NOS2, TNF-α, IL-1β, and IL-6), anti-inflammatory markers (Arg1 and IL-4), and Treg markers (IL-10 and FoxP3) were analyzed in the brain. Data are presented as the mean ± SEM. Statistical analyses were conducted using one-way ANOVA; *p < 0.05, **p < 0.01, and *** p < 0.001 vs. WT, #p < 0.05, ##p< 0.01, and ###p< 0.001 vs. MPTP, $$$p<0.01 vs. None Treg, ^<0.05 vs. bvPLA2 Treg. n=4-6. Scale bars: 50 μm.
Figure 6

Adoptive transfer of α-syn-specific Tregs alleviated PD in MPTP-intoxicated mice

DCs and CD4+ T cells were isolated from the bone marrow and spleen, respectively. CD4+CD25+ Tregs were isolated and expanded ex vivo after treatment of bvPLA2 and α-syn presentation. Adoptive transfer of α-syn specific Tregs inhibited pro-inflammatory M1 microglial activation and alleviated PD pathologies such as motor dysfunction and dopaminergic neuronal loss in MPTP-induced PD mice.