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Original Article

CD206$^+$ dendritic cells might be associated with Heat-pattern and induced regulatory T cells after treatment with bee venom

Woo-Sang Jung[1], Seungwon Kwon[1*], Jung Yun Yang[1], Chul Jin[1], Seung-Yeon Cho[1], Seong-Uk Park[1], Sang-Kwan Moon[1], Jung-Mi Park[1], Chang-Nam Ko[1], Hyunsu Bae[2] and Ki-Ho Cho[1]

[1]Department of Cardiology and Neurology, College of Korean Medicine, Kyung Hee University
[2]Department of Physiology, College of Korean Medicine, Kyung Hee University

Objectives: Bee venom (BV) is a widely used therapy in Traditional East Asian Medicine (TEAM). We previously reported that BV was clinically effective for treating Parkinson's disease, that phospholipase A_2 (PLA_2) was the main component of BV, and that it induced regulatory T cells (Tregs) by binding CD206 on dendritic cells (DCs). Therefore, we aimed to reconfirm our findings in human blood samples and investigate the relationship between CD206$^+$ DCs and clinical syndrome differentiation in TEAM.
Methods: We surveyed 100 subjects with questionnaires on cold-heat patternization and obtained their blood samples. The obtained human peripheral blood monocytes (hPBMCs) were washed with phosphate-buffered saline (PBS). After resuspension with ex vivo media, numbers of cells were counted. Tregs were counted after culturing the samples in a 37℃ CO_2 incubator for 72 h.
Results: We divided the subjects into a relatively high CD206$^+$ group or a relatively low CD206$^+$ group. The heat factor scores of high CD206$^+$ group were significantly higher than that of low CD206$^+$ group (high vs low: 239.2 ± 54.1 vs 208.4 ± 55.1, p=0.023). After culturing with PLA_2, Tregs increased in the high CD206$^+$ group but decreased in the low CD206$^+$ group.
Conclusion: In this study, we reconfirm that CD206$^+$ DCs induced Treg differentiation by incubating human blood samples with PLA_2 and that they showed an association with syndrome differentiation, especially with heat patterns, in TEAM. A heat pattern in TEAM might be one indication for PLA_2 therapy because its score was elevated in the high CD206$^+$ group.

Key Words *: Bee venom; CD206, Pattern Identification, phospholipase A_2, Regulatory T cell*

Introduction

Parkinson's disease (PD) is a neurodegenerative disease with an increasing prevalence. Resting tremors, rigidity, masked face, and bradykinesia are representative symptoms caused by the lack of dopamine in the substantia nigra. Neuroinflammation plays an important role in its pathogenesis.[1,2] Bee venom (BV) is a representative therapy used in Traditional East Asian Medicine (TEAM) to treat autoimmune diseases such as rheumatoid arthritis or multiple sclerosis. Recent studies showed that BV might be beneficial for treating neurodegenerative diseases by suppressing immune activity.[3-6]

In our previous studies, we reported that BV had therapeutic effects in the 1-methyl-4-phenyl-1,2,3,6

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・Correspondence to : Seungwon Kwon
Department of Cardiology and Neurology, College of Korean Medicine, Kyung Hee University
23 KyunHeedae-ro, Dongdaemun-gu, Seoul 02447, Korea
Tel : +82-02-958-9127, Fax : +82-02-958-9132, E-mail : kkokkottung@hanmail.net

-tetrahydropyridine (MPTP)-induced mouse model of PD by promoting the survival of dopaminergic neurons and showed its clinical effectiveness in a randomized controlled trial.[7,8] We also found that, in mice, phospholipase A_2 (PLA_2) was the most important component in BV and that it had a close relationship with cluster of differentiation 206 (CD206) on dendritic cells (DCs) and regulatory T cells (Tregs).[9]

Therefore, we wanted to verify these findings and $CD206^+$ DCs association with clinical syndrome differentiation in TEAM using human blood samples and a validated questionnaire for cold-heat patternization, respectively.[10,11]

Methods

1. Subjects

Healthy subjects without any history of inflammation within the past month were recruited between March 1, 2018 and December 1, 2018 from the Departments of Cardiology and Neurology of Kyung Hee University Korean Medicine Hospital, Seoul, Korea. We surveyed the subjects using the questionnaire for cold-heat patternization (Appendix 1)[10,11] and collected 20cc blood samples from each participant. The Institutional Research Board of Kyung Hee University Korean Medicine Hospital approved this study in 2017 (KOMCIRB -170818-BR-033), and informed consent was obtained from the study subjects after providing a full explanation of this study. All procedure of this study has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

2. Isolation of Human Peripheral Blood Mononuclear cells (hPBMCs)

Samples were collected in BD Vacutainer CPT tubes (BD Bioscience, San Jose, CA, USA) and centrifuged at 2000 rpm for 20 min. The obtained hPBMC were washed with phosphate-buffered saline (PBS). The cells were resuspended with ex vivo media (Lonza, Walkersville, MD, USA), containing 10% human serum (Sigma, St. Louis, MO, USA) and 1% penicillin and streptomycin (Gibco, Gaithersburg, MD, USA) and counted. The samples were frozen and preserved by the addition of 10% DMSO (Sigma-Aldrich). When the samples were needed for future experiments, the cells were thawed in a 37℃ water bath and washed with sterile PBS before use.

3. Regulatory T-cell expansion

To count Tregs, the blood samples were diluted to a concentration of 1×10^6 cells/ml. Next, 100 μl (1×10^5 cells) hPBMCs, 20 μl of CD3/CD28 MACSiBead, 100 μl of the media, and 500 U of rhIL-2 (PeproTech, Rocky Hill, NJ, USA) were added to every well of a U-bottom 96 well plate. Each sample was treated with 0.4 ug/ml of BV PLA_2 ($bvPLA_2$, Sigma-Aldrich, St. Louis, MO, USA). Tregs were counted after the samples were cultured for 72 h in a 37℃ CO_2 incubator.

4. Flow cytometric analysis

To analyze monocytes or lymphocytes in the samples, hPBMCs were stained with either BV510-CD11c and PE-CD206 or with FITC-CD4 and APC-Cy7-CD3, respectively. To stain the samples, resuspended cells were incubated at 4℃ for 30 min and then washed with stain buffer.

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FITC-CD4, BV421-CD25, PE-CD161, PE-Cy7 -CD45RA, and BB700-CD127 surface antibodies were used to detect Tregs. To detect forkhead box P3 (FOXP3), samples were mixed with a 1:3 ratio of Foxp3/Transcription factor fixation/permeabilization concentrate and diluent (eBioscience, San Diego, CA, USA). After a 30-min incubation at 4℃, an equal volume of permeabilization buffer (1X, eBioscience) was added to the samples. After centrifugation for 5 min at 400 g, the supernatants were removed, and a diluent containing AF647 -FOXP3 antibody was added to the samples. Finally, after an incubation at 4℃ for 30 min, the samples were washed with permeabilization buffer.

5. Statistical analysis

The subjects were divided into two groups, the top 40% and the bottom 40%, based on the proportion of $CD206^+$ DCs in the total DC population. The chi-square test for categorical variables and the independent t-test for continuous variables were used to compare the two groups. The analysis was performed using SPSS for Windows, version 10.0 (SPSS Inc., Chicago, IL, USA).

Results

1. Baseline Characteristics

One hundred subjects were included in the study. In baseline assessments, the average age was higher and the heat factor scores were lower in the low $CD206^+$ group than in the high $CD206^+$ group. These differences were statistically significant (Table 1).

2. Changes in regulatory T cells after culture with PLA_2

After culture with PLA_2, Tregs significantly increased in the high $CD206^+$ group and significantly decreased in the low $CD206^+$ group. The decrease in resting Tregs was greater in the low $CD206^+$ group than in the high $CD206^+$ group (Table 2).

Discussion

Previously, we reported the clinical effectiveness of BV pharmaco-acupuncture for the treatment of idiopathic PD. After eight weeks, BV-treated participants showed a significant improvement in

Table 1. Baseline Characteristics

	Low $CD206^+$ (N=40)	High $CD206^+$ (N=40)	P-values
Age, *yr*	37.9 ± 12.7	32.0 ± 12.1	0.044
Sex, *m (%)*	11 (27.5)	11 (27.5)	N.S.
BMI, kg/m^2	22.6 ± 3.0	21.2 ± 2.4	N.S.
Heat factor scores	208.4 ± 55.1	239.2 ±54.1	0.023
Cold factor scores	222.8 ± 57.7	235.3 ± 67.3	N.S.
Helper T cells, *n*	15.2 ± 16.2	14.3 ± 17.4	N.S.
Regulatory T cells, *n*	118.2 ± 151.9	127.1 ± 156.5	N.S.

* P-values were calculated using independent t-tests for the continuous variables and by chi-square tests for the categorical variables.
BMI, Body Mass Index.

the Unified Parkinson's Disease Rating Scale compared to the control group.[8] In addition, we reported that the modulation of peripheral immune tolerance by Tregs may contribute to the neuroprotective effects of BV in the MPTP -induced mouse model of PD.[7] Tregs, identified by CD4, FOXP3, and CD25 expression, are a subpopulation of T cells that play an important role in immunosuppression.[12] Tregs have recently been reported to have neuroprotective effects by suppressing the secretion of inflammatory cytokines, and thus, they represent a potential therapeutic approach for neuroinflammation -mediated disorders, including PD.[13-15] In a follow-up study, we found that bvPLA$_2$ directly bound to CD206 on DCs and consequently promoted the secretion of PGE$_2$. PGE$_2$ secretion from DCs resulted in Treg differentiation via PGE$_2$ (EP2) receptor signaling in FOXP3(-)CD4(+) T cells. CD206 is a mannose receptor that is mainly present on the surface of macrophages and DCs.[16,17] It is associated with clathrin-mediated endocytosis,[18] phagocytosis of pathogens, antigen presentation, and production of inflammatory cytokines.[19,20] These observations suggest that PLA$_2$-CD206-PGE$_2$-EP2 signaling promotes immune tolerance through Tregs in PD.[9]

In this study, we wanted to confirm our previous findings in human blood samples by comparing the proportion of CD206$^+$ DCs in two groups, the high CD206$^+$ group and the low CD206$^+$ group. To confirm the differences between the two groups, we excluded the middle population (median ± 5%) that had moderate levels of CD206. After PLA$_2$ treatment, Tregs increased in the high CD206$^+$ group and decreased in the low CD206$^+$ group. Activated Tregs, known as effector Tregs, increased in both groups, while resting Tregs decreased to a greater extent in the low CD206$^+$ group than in the high CD206$^+$ group. These findings are in accordance with our previous observations.

Traditional East Asian Medicine (TEAM) is a comprehensive medical system with its own theories and approaches for treatment. Syndrome differentiation is a unique diagnostic system that comprehensively analyzes patients' symptoms. The benefit of TEAM is that it can magnify the effectiveness of treatment and minimize adverse effects by leading doctors to consider each

Table 2. Changes in regulatory T cells after PLA$_2$ treatment

	Low CD206$^+$ (N=40)	High CD206$^+$ (N=40)	P-values
PBS-Tregs, *n*	118.2 ± 151.9	127.1 ± 156.5	N.S.
PLA$_2$-Tregs, *n*	51.9 ± 47.0	155.3 ± 241.2	0.017
△ Tregs, *n*	-74.6 ± 125.9	10.8 ± 152.0	0.011
△ Resting Tregs, %	-7.9 ± 13.4	-0.3 ± 17.0	0.036
△ Activated Tregs, %	7.9 ± 13.4	0.3 ± 17.0	0.036

* Phosphate-buffered saline is a buffer solution commonly used in biological research. It is a water-based salt solution that contains ions and has the same osmolarity as the human body (isotonic).
†△: The difference between before and after experiments.
‡P-values were calculated by independent t-tests.
PBS, Phosphate-buffered saline; PLA$_2$, Phospholipase A$_2$; Tregs, Regulatory T cells.

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patient's specific situation.[21] In Korea, several questionnaires on syndrome differentiation have been recently developed.[10,11,22,23] One of these questionnaires evaluated heat-cold patternization and has been validated with reasonable statistical methods.[10,11] We examined heat-cold patternization in our study because, according to TEAM theory, symptoms related to heat and cold such as fever have a close relationship with immunity.[24] Interestingly, the average heat factor scores were elevated in the high $CD206^+$ group. Heat factor scores were calculated from items in the questionnaire, such as thirst, chest discomfort, reddish-yellow urine, constipation, indigestion, bleeding, aversion to heat, and a strong desire to drink cold water, which are commonly associated with inflammation or accelerated immune activity. Therefore, the heat pattern in TEAM may reflect hyperactivity of the immune system, and patients with high heat patterns could be more sensitive to PLA_2 therapy because they have a higher $CD206^+$ concentration. Based on this finding, we suggest that PLA_2 treatment could have clinical implications in the near future.

In this study, we reconfirmed that $CD206^+$ DCs induced Treg differentiation in human blood samples after incubation with PLA_2, and that there was an association between Treg differentiation and syndrome differentiation, especially heat patterns, in TEAM.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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ORCID

Woo-Sang Jung	http://orcid.org/0000-0001-7355-7684
Seungwon Kwon	http://orcid.org/000-0002-1857-3515
Jung Yun Yang	http://orcid.org/0000-0001-5867-6546
Chul Jin	http://orcid.org/0000-0002-4646-3495
Seung-Yeon Cho	http://orcid.org/0000-0003-3149-9759
Seong-Uk Park	http://orcid.org/0000-0002-4617-2719
Sang-Kwan Moon	http://orcid.org/0000-0003-0497-3080
Jung-Mi Park	http://orcid.org/0000-0002-0851-4431
Chang-Nam Ko	http://orcid.org/0000-0002-4749-7961
Hyunsu Bae	http://orcid.org/0000-0002-0299-3582
Ki-Ho Cho	http://orcid.org/0000-0001-5824-9114