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Original Article

The clinical significance of the imbalance of Th17 and Treg cells and their related cytokines in peripheral blood of Parkinson's disease patients

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Abstract: This study aims to detect the proportion of Th17 and Treg cells and the levels of their related cytokines in peripheral blood of Parkinson's Disease (PD) patients. Flow cytometry was employed to detect peripheral ($CD3^+CD8^-CD17^+$) Th17 and ($CD3^+CD4^+CD25^+Foxp3^+$) Treg cells. ELISA was used to detect the cytokine levels in serum. Real-time PCR was used to detect the expression of nuclear transcription factors RORγt and Foxp3. Th17 cell proportion was increased and the proportion of Treg cells was significantly decreased in peripheral blood of PD patients' ($P<0.05$). The serum level of IL-10 and TGF-β in PD patients was remarkably reduced. In contrast, serum level of IL-6 and IL-17 was increased in PD patients. In addition, PD patients had significantly lower level of Foxp3 mRNA and slightly higher RORγt mRNA in mononuclear cells of peripheral blood ($P<0.05$). In conclusion, in the peripheral blood of PD patients, the proportions of Th17 cells and the related cytokines markedly increased, but the proportion of Treg cells decreased. Therefore, the immunological imbalance of Th17, Treg cells and related cytokines might play a role in the development of PD.

Keywords: Parkinson's disease (PD), Th17 lymphocytes, Treg lymphocytes, cytokines

Introduction

Parkinson's disease (PD) is a chronic degenerative disorder of the central nervous system which is characteristic of the degeneration of substantia nigra striatum pathway. Its pathologic features are the dopamine neuronal degeneration in the pars compacta region of substantia nigra with the formation of Lewy body, that is, cytoplasmic eosinophilic inclusion bodies, which causes the damage of substantia nigra striatum pathway and the decrease of dopamine in caudate nucleus and putamen. The typical clinical symptoms of PD are resting tremor, rigidity, slowness of movement, and damaged postural reflex. The pathogenesis is still poorly understood. The present researches show that genetic, environmental factors and immunological imbalance are possibly associated with the pathogenesis [1]. In recent years, immunological imbalance has become the focus in studying PD pathogenesis and development, and the immunological imbalance of Th17 and Treg cells is a new focus in the study of PD pathogenesis. As is reported, Th17 cells can combine with the receptor expressed in neuron via its secretion IL-12, and thus induce the apoptosis or death of neurons [2]. Treg cells have the neuroprotective effect by inhibiting the reaction of microglia cells to stimulus like nitrated α- synaptic nuclear protein [3]. In addition, Reynolds et al [4] found that regulatory T (Treg) cells can effectively reduce the degeneration of dopamine neuronal degeneration in the pars compacta region of substantia nigra induced by Th17 cells. Preliminary researches have manifested that in animal models, Th17 and Treg cells have some effects in the pathogenesis of PD, but no report on the possible effects of Th17 and Treg cells in PD patients is available. In this research, the proportions of Th17 cells to Treg cells in the peripheral blood of PD patients and healthy controls were detected, as well as the expression of related cytokines and nuclear

transcription factors, so as to investigate the changes and their clinical significances in PD pathogenesis.

Subjects and methods

Subjects

40 cases of primary PD patients visiting our outpatients of the hospital were selected, and they conformed to the PD diagnosis criteria formulated by Neurology branch of Chinese Medical Association [5], including 22 males and 18 females, aging (60.4±5.8) on average. None of the cases underwent any treatment. A total of 40 healthy people were selected as control who visited the physical examination center of our hospital, including 24 males and 16 females, aging (58.6±6.2) on average. The persons who had recent infection, tumors, immunologic diseases, diseases of the blood system, chronic infectious disease or history of smoking, alcoholism, or the ones who had taken antibiotics, non-steroidal anti-inflammatory agents and immunosuppressive agents were excluded. All the subjects had signed informed consent. No statistical difference in age and gender exists between the healthy control group and PD group. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. Written informed consent was obtained from all participants.

Specimen collection

Venous blood was taken from all subjects and divided into three tubes, 2 mL/tube. Tube A was conducted anticoagulation with heparin lithium for detecting the proportion of Th17 to Treg cells; tube B was naturally coagulated and centrifugated and then serum was separated for detecting related cytokines; tube C was conducted anticoagulation with heparin lithium to extract RNA for RT-PCR.

Flow cytometry detection

RPMI-l640 buffer solution 1:1 diluted peripheral blood; Ficoll separating medium density gradient centrifugation method was used to collect peripheral blood mononuclear cells (PBMCs). RPMI-l640 was employed to regulate the cell concentration to be $2^{*}10^{6}$/mL; PMA (eBioscience, SanDiego, CA, USA) (1 μg/ml) 2.5 μL, Ionomycin (eBioscience, SanDiego, CA, USA) (50 μg/ml) 2 μL and Brefeldin-A (eBioscience, SanDiego, CA, USA) 2 μL was added into the reaction system every 100 μL, and cultured in incubator at 37°C, 5% CO_2 for 6 h. When the cells were taken out from the incubator, they were washed with washing liquor once and the supernatant was removed, and then the cells were suspended with 100 μL staining buffer, while 1.25 μL APC-CD3 (eBioscience, SanDiego, CA, USA), 1 μL FITC-CD8 antibody (eBioscience, SanDiego, CA, USA) was added for 30 min of incubation at 4°C away from light. Later, they were washed by washing liquor and the supernatant was removed, and 1 mL fresh Fixation/Permeabilization fluid (eBioscience, SanDiego, CA, USA) was added to be vortex mixed, and then incubated for 1 h at 4°C away from light. It was washed with 2 mL 1× Permeabilization Buffer, and the supernatant was removed; 100 μL 1× Permeabilization Buffer was used to suspend cells, and 1.5 μL PE-IL-17 (eBioscience, SanDiego, CA, USA) was added; 5 μL PE-IgG1 (eBioscience, SanDiego, CA, USA) was added into the flow tube of the same type as control, and incubated for 30 min at 4°C away from light. 2 mL 1× Permeabilization Buffer was used for washing the cells twice, and the supernatant was removed; 500 μL Staining buffer was taken to suspend cells, and Th17 cell proportion was detected with flow cytometry (FACSCalibur; BD Biosciences, New Jersey, USA).

100 μL cell suspension was taken and Fcblock incubated for 10 min at 4°C away from light; 0.5 μL Percp-CD3e (eBioscience, SanDiego, CA, USA), 0.3 μL FITC-CD4 (eBioscience, SanDiego, CA, USA) and 0.3 μL APC-CD25 (eBioscience, SanDiego, CA, USA) was added for surface dyeing, and then 30 min of incubation was conducted at 4°C away from light. After being washed with liquor twice, 1 mL Fixation/Permeabilization liquid was added, mixed and incubated for 30 min at 4°C away from light. Washing liquor was used twice; 2.5 μL PE-Foxp3 (eBioscience, SanDiego, CA, USA) was added for nuclear staining; 5 μL PE-IgG1 (eBioscience, SanDiego, CA, USA) was added into the flow tube of the same type as control for 30 min of incubation at 4°C away from light. After washing twice, 500 μL Staining buffer suspended cells, and flow cytometry was used to detect the proportion of Treg cells.

Table 1. The primer sequences used in this study

Gene		Primer sequence (5'→3')
β-actin	Forward primer	CCTCTATGCCAACACAGTGC
	Reverse primer	GTACTCCTGCTTGCTGATCC
RORγt	Forward primer	AGTAGGCCACATTACACTGCT
	Reverse primer	GACCCACACCTCACAAATTGA
Foxp3	Forward primer	TACACCCAGGAAAGACAGCAACCT
	Reverse primer	TCTGCTTGGCAGTGCTTGAGAA

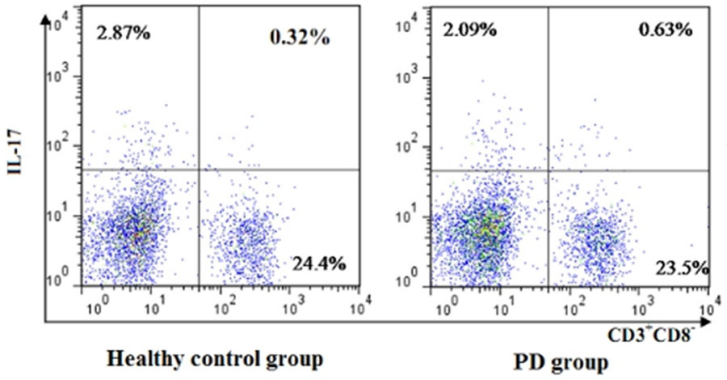

Figure 1. Th17 ($CD3^+CD8^-IL\text{-}17^+$) flow cytometry detection.

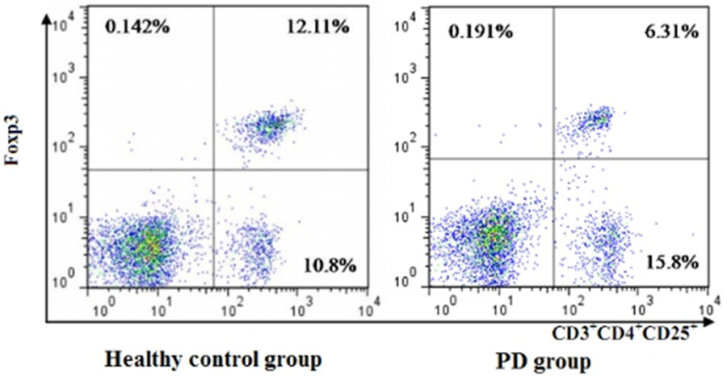

Figure 2. Regulatory Treg ($CD3^+CD4^+CD25^+Foxp3^+$) flow cytometry detection.

Cytokine detection

Enzyme linked immunosorbent assay (ELISA) was adopted to detect the levels of the Th17 and Treg cell- related cytokines like IL-6, IL-10, IL-17, IL-23 and TGF-β in serum between PD group and control group, and the operation followed the instruction of ELISA kit (R&D Systems Inc, Minneapolis, MN, USA). 8 samples were randomly detected for each indicator. The microplate reader was Austria CliniBio128C automatic microplate reader.

RORγt/Foxp3 mRNA level in peripheral blood

Mononuclear cells were separated and 1 mL Trizol (TaKaRa, Dalian, China) was added for fully homogenate. The total RNA was extracted and dissolved in 25 mL DEPC solution. The total RNA was conducted quantification and purity detection with ultraviolet spectrophotometer, and reverse-transcripted into cDNA as the instruction of reverse-transcription kit (TaKaRa, Dalian, China). Samples were added into RT-PCR reaction system as the instruction of the kit. 2× Power SYBR® Green PCR Master Mix (TaKaRa, Dalian, China) 10 μL, upstream primer (20 pmol/μL) 0.4 μL, downstream primer (20 pmol/μL) 0.4 μL, cDNA template 2 μL; sterile ddH_2O was supplemented until the total system was 20 μL. PCR procedures: 50°C incubation 2 min, 95°C Taq enzyme activation 10 min, 95°C 15 s, 60°C 1 min, a total of 40 cycles. PCR primer sequences were listed in **Table 1**. The PCR instrument was PRISM® 7500 Real-time PCR Systems (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

All data were analyzed with SPSS-19.0 statistical software. The comparison between two groups was conducted with Student's *t* test. $P<0.05$ indicates that the difference is statistically significant.

Results

Comparison of Th17 and Treg cell percentage in peripheral blood in PD group and control group

Compared with the control group, the PD group had significantly higher Th17 cell proportion in peripheral blood, and the difference is statistically significant ($P<0.05$) (**Figure 1**), while the regulatory Treg cell proportion is remarkably lower, and the difference is statically significant

Table 2. Comparison of Th17 and Treg cell proportions in peripheral blood

Group	Cases	Th17 cells (%)	Treg cells (%)	Treg/Th17
Healthy control group	40	0.34±0.03	10.39±2.33	30.9±1.67
PD group	40	0.69±0.11	5.55±0.62	8.25±1.2
T, P		19.415, 0.000	12.696, 0.000	69.660, 0.000

Table 3. The levels of IL-6, IL-10, IL-17, IL-23 and TGF-β in serum (n=8)

Group	IL-6 (pg/ml)	IL-10 (pg/ml)	IL-17 (pg/ml)	IL-23(pg/ml)	TGF-β (pg/ml)
Healthy control group	91.3±5.1	72.2±15.7	38.6±4.9	179.2±14.7	7850±250
PD group	176.8±7.3	40.3±8.4	58.8±6.2	192.4±14.1	5940±210
T, P	27.157, 0.000	5.067, 0.000	7.230, 0.000	1.833, 0.088	16.546, 0.000

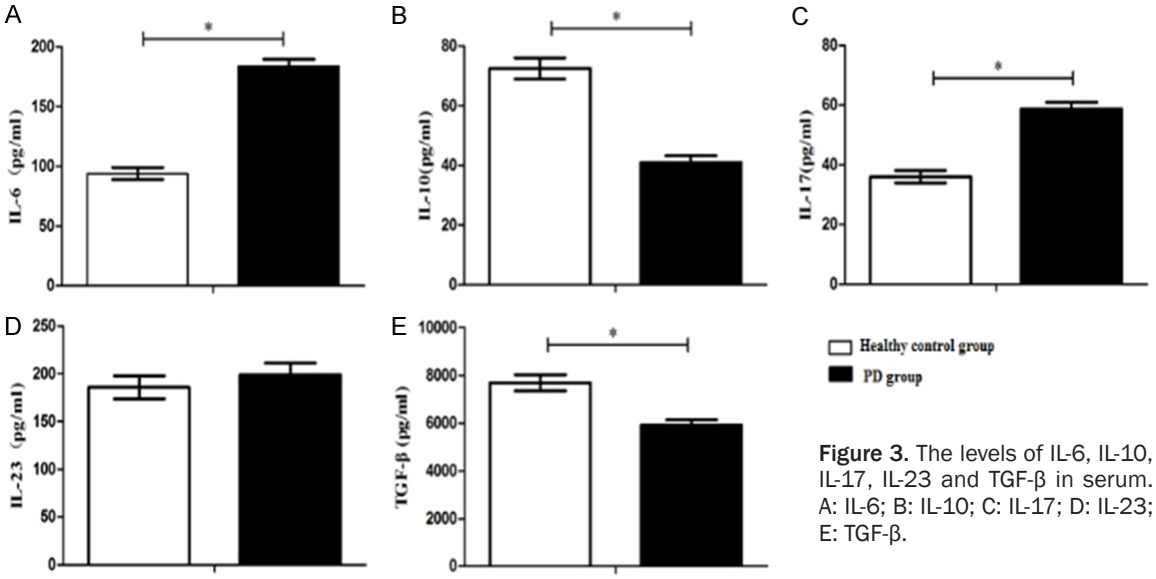

Figure 3. The levels of IL-6, IL-10, IL-17, IL-23 and TGF-β in serum. A: IL-6; B: IL-10; C: IL-17; D: IL-23; E: TGF-β.

Table 4. Comparison of RORγt and Foxp3 mRNA levels in peripheral blood (n=8)

Group	RORγt mRNA	FoxP3 mRNA
Healthy control group	0.51±0.05	1.08±0.10
PD group	0.56±0.15	0.51±0.07
T, P	0.894, 0.394	13.208, 0.000

(P<0.05) (**Figure 2**). The ratio of TReg/Th17 in the peripheral blood of PD patients is smaller than that of the control group, and the difference is of statistical significance (P<0.05) (**Table 2**).

The expression levels of Th17 and Treg cells related cytokines in serum between PD group and control group

ELISA detection shows that IL-6 and IL-17 levels in serum of PD patients significantly increased (P<0.05), while the levels of IL-10 and TGF-β were markedly lower than those of the control (P<0.05), and the expression of IL-23 is slightly higher than that of the control, but the difference is of no statistical significance (P>0.05) (**Table 3**; **Figure 3**).

Comparison of RORγt and Foxp3 mRNA levels in the peripheral blood of PD group and control group

Fluorescence quantitative PCR detected the expression of RORγt and Foxp3 mRNA levels in the peripheral blood of PD group and the control group, and the results indicate that the expression of RORγt and Foxp3 mRNA levels in the peripheral blood of PD patients was (0.56±0.39, 0.52±0.26) separately. Compared with the their expression of the control group (0.52±0.15, 1.08±0.32), PD group had signifi-

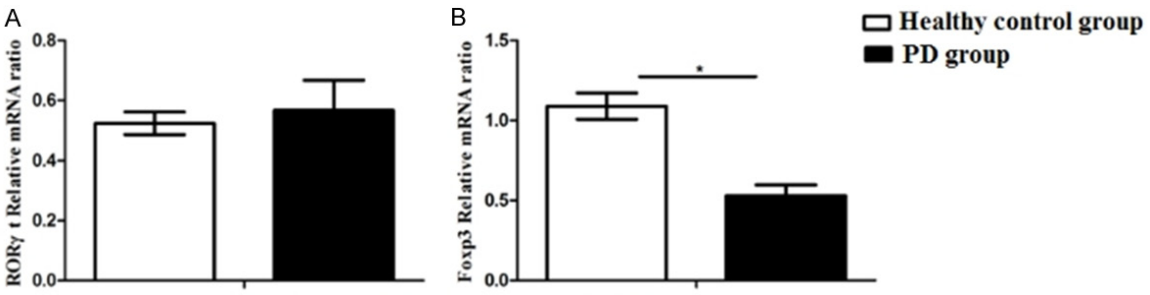

Figure 4. Comparison of RORγt and Foxp3 mRNA levels in peripheral blood. A: RORγt; B: Foxp3.

cantly lower Foxp3 mRNA, and the difference is statistically significant (P<0.05), while RORγt mRNA of PD group was higher than that of the control, but the difference was of no statistical significance (**Table 4**; **Figure 4**).

Discussion

PD is a common neurodegenerative disease and characteristic of the denaturation loss of dopamine (DA) energy neuron in substantia nigra and corpus striatum caudate putamen nerve fibers, clinically performing to be resting tremor, rigidity, slowness of movement, and abnormal posture and walking [6]. In recent years, the study of the correlation between the inflammation reaction of cell-mediated immunity, degeneration of central nervous system and the damage of substantia nigra striatum receives increasing focus [3, 7, 8].

Auxiliary Th17 cells and regulatory T cells (Treg) are the focus in the study of CD4 subpopulation cells. Treg cells can function immunologic regulation and immunosuppression, while Th17 cells can greatly induce inflammation. The balance of Th17 and Treg cells is involved in the immune response and immune tolerance of organism. The imbalance of Th17 and Treg cells plays a vital role in the inflammation reaction, graft versus host reaction, autoimmune diseases and tumors [9-13]. As is reported, Th17 cells can combine with receptors expressed in neurons via its secretion IL-21, and thus induce the apoptosis or death of neurons [2]. Treg cells can protect nerves by inhibiting the reaction of microglia to stimulus like nitrated α-synaptic nuclear protein [14]. Researches show that Treg cells are closely linked with neurous system diseases.

Reynolds et al. [4] also found that in the animal models in which MPTP induced PD, the degeneration and substantia nigra striatum induced by neuronin nitrated α-synaptic nuclear protein is greatly mediated by Th17 cells, which is accompanied with the malfunction of Treg cells. In contrast, normal Treg cells can reverse the degeneration and substantia nigra caused by N-α-syn T cells. The study indicates that Treg cells can reduce the neurodegenerative changes of substantia nigra striatum dopamine mediated by Th17 cells. The research results demonstrate that the proportion of Th17 cells in peripheral blood significantly increases, while Treg cell proportion and Foxp3 mRNA levels are markedly lower than those of the control, and Treg/Th17 ratio obviously changed as compared with that of the control group, which suggests that the imbalance of Th17 and Treg cells has some effects on the occurrence and development of PD.

Previous studies found that subtle regulation exists in the induction and differentiation of Th17 cells and Treg cells. TGF-β is an essential cytokine which induces Treg and generate immunological tolerance and also promotes the differentiation of Th17 [15, 16]. The changes of organisms and local microenvironment and the maintenance of immune balance require different Th17/Treg regulation. IL-17 content in the serum of AD rats induced by $A\beta_{1-42}$ significantly increases, which indicates that Th17 cells possibly cooperatively promote PD nerve inflammation and neural degeneration [17]. Treg cells protect nerves by secreting cytokines TGF-β and IL-10, as the protection of TGF-β to dopaminergic neurons has been reported [18]. Currently, the high expression of IL-17 has been detected in the serum and tissues of the patients with autoimmune diseases [19, 20]. IL-17 causes the inflammatory injury of tissues and organs by inducing inflammatory mediates and chemotaxis factor. In this

research, the levels of Treg-related cytokines IL-10 and TGF-β in the serum of PD patients were lower than those of the control, and the expression of IL-17 significantly grew, which indicates that Th17/Treg related cytokines are also correlated with the development of PD.

In summary, the immunological imbalance of Th17, Treg cells and their related cytokines has some effects on the occurrence and development of PD, which provides a new idea to investigating the function of immunological mechanism in PD pathogenesis.

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Disclosure of conflict of interest

None.

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