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ORIGINAL ARTICLE

Peripheral Lymphocyte Subsets as a Marker of Parkinson's Disease in a Chinese Population

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Abstract In this study, we conducted a clinical analysis of lymphocyte subtypes in 268 patients with Parkinson's disease (PD) to assess their clinical impact as a potential marker of advanced PD in Chinese patients. The participants comprised 268 sporadic PD patients and 268 healthy controls. The numbers of natural killer (NK) cells and CD3+, CD3+CD4+, CD3+CD8+, and CD19+ lymphocytes from peripheral blood were determined by immunostaining and flow cytometric analysis and the percentages of these CD+ T cells were calculated. The ratio of regulatory T (Treg)/helper T 17 (Th17) lymphocytes from 64 PD patients and 46 controls was determined by flow cytometric analysis. The results showed that the percentage of NK cells was higher in advanced PD patients than in controls (22.92% ± 10.08% *versus* 19.76% ± 10.09%, $P = 0.006$), while CD3+ T cells are decreased (62.93% ± 9.27% *versus* 65.75% ± 9.13%, $P = 0.005$). The percentage of CD19+ B cells in male patients was lower ($P = 0.021$) than in female patients, whereas NK cells were increased ($P < 0.0001$). The scores on the Unified Parkinson's Disease Rating Scale (UPDRS) and the Non-Motor Symptoms Scale in late-onset PD patients were significantly higher than those in early-onset patients ($P = 0.024$ and $P = 0.007$, respectively). The percentage of CD19+ B cells in patients with UPDRS scores >24 was lower than in those with scores <24 (10.17% ± 4.19% *versus* 12.22% ± 5.39%, $P = 0.009$). In addition, the Treg/Th17 ratio in female patients was higher than that in female controls (13.88 ± 6.32 *versus* 9.94 ± 4.06, $P = 0.042$). These results suggest that the percentages of NK cells, CD3+ T cells, and CD19+ B cells along with the Treg/Th17 ratio in peripheral blood may be used to predict the risk of PD in Chinese individuals and provide fresh avenues for novel diagnostic biomarkers and therapeutic designs.

Keywords Parkinson's disease · Biological marker · Lymphatic cell

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Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc) and decreased projections to the caudate and putamen [1]. PD is the second most common neurodegenerative disease after

Alzheimer's disease (AD) and afflicts ~1% of the population aged 60 and older worldwide [2]. It has been reported that the number of individuals suffering from PD will double from 2005 to 2030 [3], but its etiology is incompletely understood. Factors such as genetics, environmental elements, aging, abnormal immune responses, and a progressive inflammatory reaction are risks for PD pathogenesis [4]; inflammation and abnormal immune responses have been especially affirmed to play crucial roles in PD [5, 6]. Recently, inflammation both in the brain and in the peripheral immune system has been reported to lead to the onset and progression of neurodegenerative disorders such as PD, partly due to lymphocyte infiltration [6]. Abnormal immune responses have been found in the development of PD, including changes in lymphocyte subpopulations and increased immunoglobulin synthesis, cytokines, and acute-phase protein in both cerebrospinal fluid and peripheral blood [7].

Among the subpopulations of T lymphatic cells, regulatory T cells (Tregs) and helper T 17 cells (Th17) are the two subtypes of T lymphocytes. CD4+CD25+ Tregs mainly play a positive role in neurodegenerative diseases [8] by suppressing immune activation, maintaining immune homeostasis and tolerance, and mediating neural protection by suppressing microglial responses to α-synuclein aggregation [9, 10]. Accumulation of Tregs decreases the cytotoxic activity of CD8+ T cells and natural killer (NK) cells and the production of interleukin (IL)-2 [11]. Several studies have shown that Tregs improve disease outcomes in animal models of neurodegenerative disorders, especially PD and Alzheimer's disease [8, 12]. In contrast, Th17 cells, a lineage of CD4+ T cells defined by IL-17A and IL-17F, contribute to the progression of neuroinflammation mediated by the pro-inflammatory cytokines IL-1, IL-6, IL-17, tumor necrosis factor alpha, and interferon gamma [13]. It is worth noting that the proportion of CD4+CD25+ Tregs in the peripheral blood changes with aging [14]. In the case of the inflammatory response in PD, Tregs can be transformed into Th17 cells in peripheral blood and lymphoid tissue [15]. However, Treg/Th17 cell transformation in peripheral blood has not been investigated in PD patients, especially at an advanced stage. In this study, we conducted a classification analysis of lymphocyte subtypes to investigate the clinical impact of NK cells and other major lymphocyte subpopulations in 268 PD patients, to explore the potential of the Treg/Th17 ratio as a biological marker for assessing PD progression.

Participants and Methods

Participants

Two hundred sixty-eight sporadic PD patients and 268 age- and environment-matched controls were recruited from the outpatient clinic in the Neurology Department of the First Affiliated Hospital of Sun Yat-sen University between January 2014 and December 2015 (many had migrated from other regions of China). In addition, another set of 64 PD patients and 46 controls were recruited for analysis of the Treg/Th17 ratio. PD patients were diagnosed using the UK Brain Bank clinical criteria. The procedures were approved by the Ethics Committee of Sun Yat-sen University and all patients and controls provided written informed consent. Age- and gender-matched controls were identified during routine health examinations. Patients and controls with a history of an autoimmune or inflammatory disorder and those receiving chronic immunosuppressive therapy were excluded. A brief screening was conducted to exclude controls with symptoms likely to represent PD. Patient information on demographics, PD features, diagnostic features, family history, environmental risk, Unified Parkinson's Disease Rating Scale (UPDRS), Non-Motor Symptoms Scale for Parkinson's disease (NMSS), and Hoehn-Yahr (H&Y) stage was collected.

Blood Sample Collection

Blood samples were collected aseptically by venipuncture from PD patients and controls in the morning after 8 h of fasting. Four milliliter of whole blood was collected into an acid citrate dextrose-coated tube, stored at room temperature, and processed within 6 h. The samples were assessed by flow cytometric analysis of peripheral blood mononuclear cells (PBMCs).

Preparation of Peripheral Blood Mononuclear Cells

PBMCs were collected by density gradient centrifugation using lymphocyte separation medium according to the manufacturer's instructions (MP Biomedicals, Santa Ana, CA) and used for flow cytometry. Briefly, the same volume of phosphate-buffered solution (PBS) was mixed with the blood sample on the clean bench. Then, the diluted sample was carefully layered on the lymphocyte separation medium. After that, the mixture was centrifuged at 2000 rpm for 20 min. The mononuclear cell layer was carefully transferred into another centrifuge tube. Finally, the lymphocytes were washed with PBS and re-suspended in RPMI-1640 medium. The collected PBMCs were used for flow cytometry within 48 h.

Cell Staining and Flow Cytometric Analysis of T Cell Subtypes

We prepared T cell subtypes with fluorochrome-conjugated monoclonal antibodies. For staining of Treg cells (CD4+CD25+FoxP3+ T cells), the PBMCs were re-

suspended in staining buffer and plated at 1×10^6 cells/mL medium. Cells were then incubated with anti-human CD4 (FITC, Biolegend, San Diego, CA) at 4 °C in the dark for 30 min, then incubated with anti-human CD25 (APC, Biolegend) for 30 min. After that, fixation/permeabilization was performed with the Foxp3/Transcription Factor Staining Buffer Set Kit according to the manufacturer's instructions (eBioscience, San Diego, CA). Finally, the mixture was incubated with anti-human Foxp3 (PE, eBioscience). For staining Th17 cells, The PBMCs were re-suspended at 1×10^6 cells/mL in RPMI 1640 medium. Leukocyte Activation Cocktail (2 μL) was added to 1 mL medium and left for 4 h–6 h. Cells were incubated with anti-human CD3 (FITC, Biolegend) in 4 °C in the dark for 30 min, and then incubated with anti-human CD8a (PerCP, Biolegend) for another 30 min. After incubation, fixation/permeabilization procedure was performed as above. Finally, PBMCs were incubated with anti-human IL-17A (PE, Biolegend). Isotype-matched mouse or rat monoclonal antibodies were used as the negative control. We performed multicolor flow cytometric analysis using a FACS Calibur flow cytometer (BD Bioscience). Using the same methods and different combinations of specific antibodies as above, we calculated the percentages of CD3+, CD3+CD4+, CD3+CD8+ T cells in the PBMCs, and anti-human CD19 (Sigma-Aldrich, St. Louis, MO) and CD56 (Sigma-Aldrich) were used in marking CD19+ T cell and NK cell (CD3+CD56+), respectively.

Statistical Analysis

Data with a normal distribution are expressed as mean ± SD and were analyzed with the independent sample test. Data with a skewed distribution are expressed as the median and were analyzed with the Mann-Whitney test. The categorical variable (gender) is shown as a percentage. The variables tested were age, PD duration, UPDRS, UPDRS III, NMSS, H&Y stage, percentages of Tregs and Th17, the Treg/Th17 ratio, and percentage of CD3+, CD3+CD4+, CD3+CD8+, and CD19+ lymphocytes and NK cells. SPSS 16.0 software (Chicago, IL) was used for the statistical analyses. $P < 0.05$ was regarded as statistically significant.

Results

Patient Characteristics

In this study, 268 PD patients (mean age 60.59 ± 11.11 years, 58% male, 42% female) and 268 controls (mean age 59.41 ± 11.11 years, 63% male, 37% female) were enrolled from the First Affiliated Hospital of Sun Yat-sen University between January 2014 and December 2015 (Table 1). There was no significant difference of age and gender between PD group and control group.

Peripheral Lymphocyte Subsets in PD Patients and Controls

The percentage of NK cells in peripheral blood from PD patients was higher than that from the control group ($P = 0.006$, Table 2), while the percentage of CD3+ T cells in the PD group was lower than that in the control group ($P = 0.005$, Table 2). In addition, no significant difference was found for CD3+CD4+ T cells, CD3+CD8+ T cells, and CD19+ B cells between the two groups.

Peripheral Lymphocyte Subsets in PD Patients Stratified by Gender

The percentage of NK cells in male patients was higher than that in female patients ($P < 0.0001$, Table 2). The percentage of CD19+ B cells in male patients was lower than that in female patients ($P = 0.021$, Table 2). However, there was no significant difference in CD3+ T cells, CD3+CD4+ T cells, and CD3+CD8+ T cells between the genders.

Clinical Indices and Peripheral Lymphocyte Subsets in PD Patients Stratified by Age at Disease Onset

Next, we divided PD patients into two groups by age at disease onset. The patients were diagnosed as the early-onset if the onset age was <45 years, and the rest were defined as the late-onset. Although the disease duration and UPDRS III score did not significantly differ between the two groups, the H & Y stage, average UPDRS and NMSS scores in late-onset PD patients were higher than those in the early-onset patients ($P = 0.001$, $P = 0.024$, $P = 0.007$, Table 3), while other clinical indices and lymphocyte subsets in peripheral blood did not differ between the two groups (Table 4).

Relationship Between Peripheral Immune Indices and PD Severity

We also stratified the PD patients by UPDRS score and found that the percentage of CD19+ B cells in those with a score >24 was lower than in those with a score <24 [10.17% ± 4.19% *versus* 12.22% ± 5.39%, $P = 0.009$]. When the patients were stratified by NMSS score (cut-off, 15), no significant difference in lymphocyte subsets was found between the two groups.

Table 1 Characteristics and clinical indices of PD patients and controls.

Group	*n*	Age (years) Mean ± SD	Gender Male/Female	Duration (years) Mean ± SD	UPDRS Mean ± SD	UPDRS III Mean ± SD	H&Y stage Mean ± SD	NMSS Mean ± SD
PD	268	60.59 ± 11.112	156/112	4.88 ± 4.00	27.14 ± 15.69	12.77 ± 7.703	1.903 ± 0.865	9.716 ± 6.673
Control	268	59.41 ± 11.113	168/100	NA	NA	NA	NA	NA
P-value		0.931	0.289					

UPDRS, Unified Parkinson's Disease Rating Scale; H&Y stage, Hoehn–Yahr stage; NMSS, Non-motor Symptoms Scale for Parkinson's disease; NA, not applicable; PD, Parkinson's disease; SD, standard deviation; UPDRS III, motor function evaluation.

Table 2 Subset analysis of lymphocytes from PD patients stratified by gender.

Group	*n*	CD3+ T (62%–76%)	CD3+CD4+ T (32%–46%)	CD3+CD8+ T (18%–32%)	CD19+ B (7%–18%)	NK (7%–18%)
PD	268	62.93 ± 9.27	33.54 ± 7.59	23.68 ± 8.51	11.14 ± 4.89	22.92 ± 10.08
Control	268	65.75 ± 9.13	34.61 ± 7.61	24.86 ± 7.87	11.93 ± 5.94	19.76 ± 10.09
P-value		0.005	0.217	0.207	0.241	0.006
Male PD	156	62.13 ± 9.66	33.07 ± 8.09	23.38 ± 9.71	10.31 ± 5.21	25.16 ± 10.36
Female PD	112	63.96 ± 8.68	34.14 ± 6.91	24.07 ± 6.65	12.14 ± 4.31	19.90 ± 8.89
P-value		0.15	0.34	0.58	0.021	<0.0001
Male control	168	64.56 ± 8.24	32.97 ± 6.18	22.46 ± 7.05	11.06 ± 4.58	20.78 ± 9.87
P-value (*vs* Male PD)		0.03	0.46	0.61	0.09	0.008
Female control	100	66.15 ± 10.44	33.67 ± 7.28	25.27 ± 8.09	12.69 ± 5.97	18.42 ± 9.06
P-value (*vs* Female PD)		0.001	0.25	0.36	0.32	0.09

NK, natural killer; PD, Parkinson's disease; SD, standard deviation. Mean ± SD.

Table 3 Clinical indices in early-onset (<45 years) and late-onset PD patients (≥45 years).

Group	Duration (years)	UPDRS	UPDRS III	H&Y stage	NMSS
Early-onset	3.94 ± 2.32	21.52 ± 10.31	11.00 ± 4.63	1.47 ± 0.51	6.68 ± 4.89
Late-onset	4.95 ± 4.10	27.71 ± 16.11	12.89 ± 7.88	1.93 ± 0.88	10.31 ± 8.51
P-value	0.102	0.024	0.128	0.001	0.007

Mean ± SD.

Table 4 Subset analysis of lymphocytes from PD patients stratified by disease onset (45 years as the cut-off).

Group	CD3+ T (62%–76%)	CD3+CD4+ T (32%–46%)	CD3+CD8+ T (18%–32%)	CD19+ B (7%–18%)	NK (7%–18%)
Early-onset	64.74 ± 5.93	33.68 ± 4.06	23.64 ± 5.13	12.21 ± 6.04	20.11 ± 7.32
Late-onset	62.82 ± 9.44	33.53 ± 7.78	23.68 ± 8.70	11.08 ± 4.83	23.11 ± 10.22
P-value	0.063	0.023	0.161	0.417	0.067

UPDRS, unified Parkinson's disease rating scale; H&Y stage, Hoehn–Yahr stage; NMSS, Non-motor Symptoms Scale for Parkinson's disease; SD, standard deviation; UPDRS III, motor function evaluation. Mean ± SD.

Treg and Th17 Cell Frequencies in PD Patients and Controls

To define changes in the percentage and phenotype of Treg and Th17 cells, we conducted flow cytometric analyses of PBMCs from 64 PD patients and 46 controls. CD4+ T cell populations were identified by high expression of CD4 and low side-scatter, and Tregs were identified within the CD4+ T cell population as CD25+FoxP3+ (Fig. 1). The Th17 cell population was identified as CD3+CD8–IL-

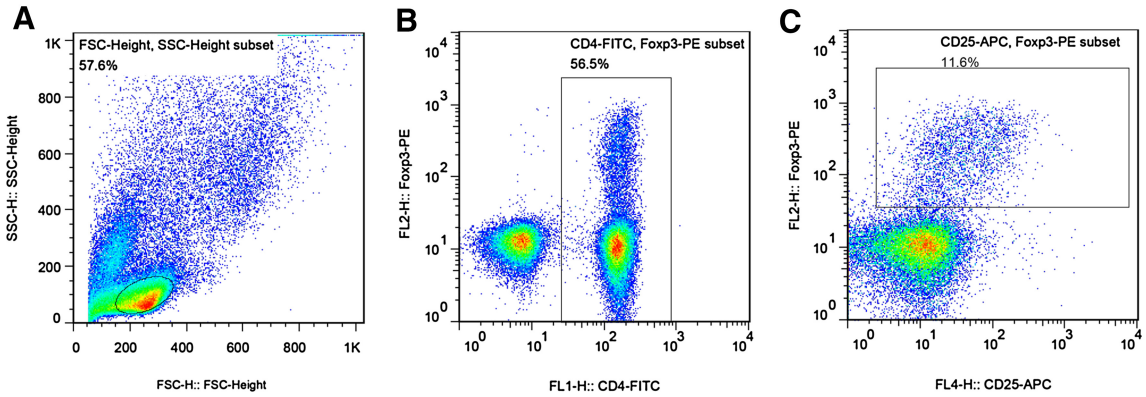

Fig. 1 Gating strategy for flow cytometric analysis of PBMCs and Treg cells frequency. **A** Representative flow cytometric scatter plots used for data collection. **B** CD4+ T cell population identified with high expression of CD4 and high expression of FoxP3. **C** Treg cells were identified within the CD4+FoxP3+ T cell population as CD25+.

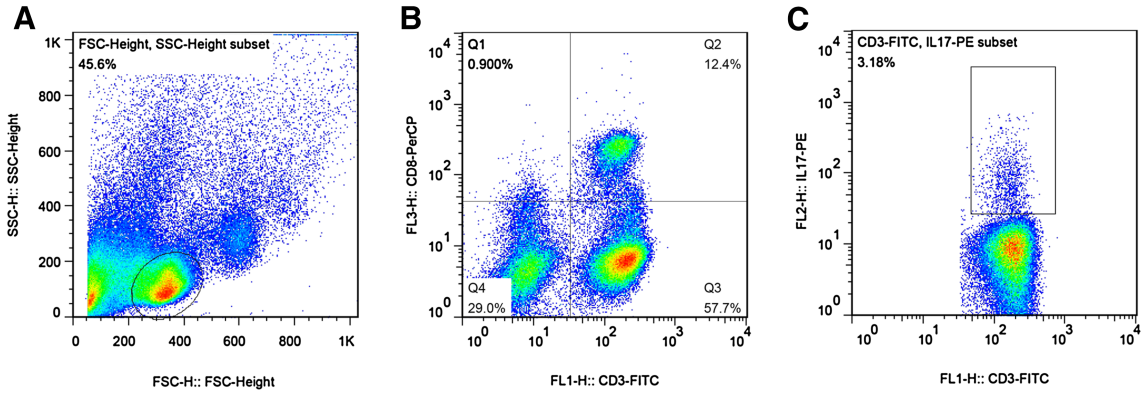

Fig. 2 Gating strategy for flow cytometric analysis of PBMC and Th17 cell frequency. **A** Representative flow cytometric scatter plots used for data collection. **B** The gated T cell population was identified by high expression of CD3 and negative expression of CD8. **C** Th17 was identified within the CD3+CD8−T cell population as IL-17A+.

Table 5 Phenotypic analysis of T cell subsets from female PD patients.

Group	*n*	Age (years)	Treg (%)	Th17 (%)			Treg/Th17
		Mean ± SD	Mean ± SD	Median	Min	Max	Mean ± SD
Female PD	20	59.75 ± 8.55	7.67 ± 1.49	0.53	0.36	3.31	13.88 ± 6.32
Female control	28	53.94 ± 14.25	7.83 ± 2.04	0.86	0.45	1.64	9.94 ± 4.06
P-value		0.174	0.835	0.074			0.042

PD, Parkinson's disease; SD, standard deviation.

17A+ (Fig. 2). No significant difference in measures of Treg (%), Th17 (%), or the Treg/Th17 ratio was found between PD patients and controls. When the PD patients and controls were divided into two groups by gender, the Treg/Th17 ratio in female PD patients was higher than in female controls (13.88 ± 6.32 *versus* 9.94 ± 4.06, $P = 0.042$, Table 5). But in the male PD patients and controls, no significant difference was found (Table 5).

Discussion

It has been widely acknowledged that neuroinflammation contributes to the pathophysiology of PD [5–7, 16, 17]. Triggering factors may include pathogens like bacteria or viruses, the dysregulation of inflammatory pathways (immune changes associated with aging or autoimmune disorders), protein aggregates (amyloid β peptides and α-

synuclein), and primary inflammatory responses in neurons [17]. In PD, a prolonged neurodegenerative period leads to the death of cells in the central nervous system (CNS) and presentation of their antigens to the immune system with activation of T and B lymphocytes. B cells or specific autoantibodies might enter the CNS across a dysfunctional blood-brain barrier, produce cytokines which in turn activate microglia, and release autoantibodies, leading to further inflammation and subsequent cell death [18]. Brochard [19] reported that CD8+ and CD4+ T cells but not B cells infiltrate into the brain tissues of the post-mortem specimens from PD patients and the MPTP mouse model of PD, mainly in the area surrounding the SNc. But T cell-mediated DA toxicity is almost exclusively arbitrated by CD4+ T cells. It has been noted that there is a remarkable change of immune function in the CNS in PD [19]. The disturbances in the function of CNS immunocytes activate microglia, worsen the injury of neurons in the SNc, and consequently result in the occurrence and development of PD. Recent studies have indicated that the suppressed inflammatory function of Tregs from peripheral blood is impaired in PD patients, and notably the effector memory T cell activation and Treg dysfunction may be linked to PD pathobiology and disease severity, especially motor dysfunction [20]. Stevens [21] reported that CD4+ T cell and CD19+ B cell in peripheral blood change with the severity of PD. CD4+ T cells are negatively correlated with H&Y stage and UPDRS score, and CD19+ B cells are negatively correlated with H&Y stage. NK cells, non-specific cytotoxic lymphocytes, are the third major lymphocyte for lymphoid-progenitors of B and T lymphocytes [22]. It has been noted that both the number of NK cells and their activity are elevated in PD patients [23]. Moreover, in another clinical investigation, Niwa and colleagues [24] demonstrated that the percentage of NK cells increases, while Th cells, particularly Th1 cells, decrease in the peripheral blood of PD patients. Both cells have been positively correlated with UPDRS scores, but no significant predominance was reported for Th1/Th2 or Treg/Th17 cells in that study. Accordingly, in the present study, we found that the percentage of NK cells increased in our PD patients, while the percentage of CD3+ cells decreased, in accordance with previous studies. To investigate the relationship between peripheral immune indices and the gender of PD patients, we divided the PD patients and controls into two subgroups by gender, and found that the percentage of CD19+ B cells in female patients was higher than that in male patients. Then we analyzed male PD *versus* male control, and female PD *versus* female control, and found the percentage of NK cells increased in male cases, but did not significantly differ between female patients and female controls. For CD19+ B cells, no significant difference was found between the two groups, so an increase of CD19+ B cells was detected in all female participants including patients and controls. In addition, after stratified analysis by UPDRS score, the percentage of CD19+ B cells in PD patients whose UPDRS score was >24 was markedly lower than in those with scores <24, indicating that CD19+ B cells are negatively correlated with PD severity. Thus, we suggest that the percentage of CD19+ B cells may be used to predict the progression of PD.

Although the exact mechanism remains unclear, studies have hypothesized that neuroinflammation and the immune response are involved in the pathophysiological process of PD [5, 6, 16]. The changes of peripheral lymphocyte subsets in PD patients in our study precisely reflected the relationship between the immune response and the development of PD. In order to identify representative lymphocyte subsets as potential biomarkers of PD progression, researchers have continued to focus on the CD4+ lymphocyte subsets, including Treg and Th cells. In this study, for the first time, we investigated the percentage of Treg and Th17 cells in the peripheral blood of the same individual to determine whether there is a significant difference in the Treg/Th17 ratio between PD patients and healthy controls. While no significant difference in the measures of Tregs (%), Th17 cells (%), or the Treg/Th17 ratio was detected between the PD and control groups, our data revealed that the Treg/Th17 ratio in female PD patients was significantly higher than in female controls, but there was no difference between the male patients and controls, indicating that the Treg/Th17 ratio varies between gender.

Previous studies have indicated that both the prevalence and incidence of PD are significantly higher in men than in women [25–28]. Epidemiological studies suggest that symptom onset may be later in women [29]. This difference may be due to the neuroprotective effects of estrogen [30, 31]. Estrogens regulate immune responses at multiple levels, including cell development, proliferation, cytokine or antibody production, and apoptosis in infection, autoimmunity, and other inflammatory diseases [32–34]. Estrogen also enhances the number and function of CD4+CD25+ Tregs in multiple sclerosis [35], suggesting a potential interaction between this hormone and the immunoregulatory mechanism [36]. It is possible that once the innate and adaptive immune system is activated in the early stage of PD, Treg cells start to increase and play a neuroprotective role enhanced by the functional regulation of Tregs by estrogen, although the mechanism is still unclear.

In conclusion, we not only investigated the percentage of NK cells, lymphocyte subsets, and other peripheral immune indices in a Chinese PD population, but also analyzed the clinical indices associated with disease

severity. We found that the percentage of NK cells increased in PD patients, while CD3+ T cells decreased. We also found that some peripheral lymphocyte subsets changed with PD severity and gender. In mild PD patients evaluated by UPDRS, the percentage of CD19+ B cells decreased. Furthermore, the percentage of NK cells increased, while CD19+ B cells decreased, in male compared with female PD patients. In addition, the Treg/Th17 ratio in female PD patients was higher than that in female controls. Taken together, we speculate that NK cells and CD3+ T cells in peripheral blood may be worth consideration as potential biomarkers for the progression of PD, and the Treg/Th17 ratio may be a special biomarker useful for female PD patients. CD19+ B cells in peripheral blood may be a potential predictor of PD progression according to its negative correlation with PD severity. Further studies are needed to expand the patient samples to explore more immune indices for PD prediction and understanding their biological characteristics in the pathogenesis as well as clinical values for the assessment of disease severity.

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Compliance with Ethical Standards

Conflict of interest The authors declare that no conflicts of interest exist.

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