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Reduced Activated T Lymphocytes (CD4+CD25+) and Plasma Levels of Cytokines in Parkinson's Disease

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Abstract Parkinson's disease (PD) is the second most common neurodegenerative disease. The cause of neurodegeneration in PD is not completely understood, and evidence has shown that inflammatory/immune changes may be involved in PD pathophysiology. Herein, we aimed to determine the profile of the peripheral immune system in patients with PD in comparison with controls. Forty patients with PD and 25 age- and gender-matched controls were enrolled in this study. From these, 23 PD patients and 21 controls were included in the immunophenotyping analyses. Peripheral blood was drawn on the same day of the clinical assessment and submitted to plasma separation for enzyme-linked immunosorbent assay or cytometric bead array. Immunophenotyping analyses of the peripheral blood were performed by flow cytometry. We found that patients with PD presented peripheral immune changes evidenced by decreased percentage of T lymphocytes (CD3+ cells), especially activated T lymphocytes (CD4 +CD25+ cells), when compared with controls. In line with these results, we also found decreased plasma levels of the cytokines IL-4, IL-6, IL-10, TNF, IFN-γ, and IL-17A in the PD group. In vitro experiments demonstrated that the production of cytokines by peripheral blood mononuclear cells harvested from healthy young donors was reduced after exposure to the anti-parkinsonian drugs levodopa and pramipexole. Our data corroborate the hypothesis that immunological mechanisms are involved in PD. It is not clear whether the differences that we have found are due to adaptive mechanisms or to changes associated with PD, including pharmacological treatment, or even directly related to the disease pathophysiology. Future studies are needed in this regard.

Keywords Parkinson's disease · Immune system · Inflammation · Immunophenotyping · Leukocytes · Cytokines

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Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease. Pathologically, PD is defined by the progressive loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) and the presence of intraneuronal inclusions of α-synuclein (called Lewy bodies) in the remaining neurons. The neuronal death results in dopamine deficit in the basal ganglia, mainly striatum, and cortical brain regions, leading to the motor signs that characterize PD clinically: bradykinesia, resting tremor, rigidity, and postural instability [1]. In addition to the motor signs, patients with PD also experiment non-motor symptoms in any stage of the disease. Among these, cognitive impairment and mood changes are of special interest, since they are disabling and have a significant impact on quality of life, hospitalization, and healthcare costs [2].

The cause of neurodegeneration in PD is not completely understood. Although 5 to 10% of PD cases have a clear genetic origin [3], the mechanisms underlying neuronal death in PD are not known in the great majority of cases. Inflammation is one of the several factors proposed to contribute to PD pathophysiology. Inflammatory processes may participate in the cascade of events leading to neuronal degeneration. Genetic, epidemiological, and immunological studies in humans and animal models support this hypothesis [4]. For instance, studies have reported abnormalities in immune functions in patients with PD, including changes in lymphocyte subsets [5–10], poor response of peripheral blood mononuclear cells (PBMC) to mitogens [11], and impaired production of cytokine [12, 13]. These immunological changes do not seem to be the primary cause of neuronal cell death in PD, but they may exacerbate or hasten the progression of the disease.

Although there is evidence supporting the hypothesis of systemic immune dysfunction in PD, there is no consensus on its general pattern, providing a basis for further investigation. Therefore, this study aimed to determine the profile of the peripheral immune system in patients with PD in comparison with controls. Specifically, we investigated blood monocytes and lymphocyte subsets, and plasma levels of cytokines in patients with PD and matched controls. In addition, we investigated whether there was any association between immune parameters and clinical data, notably cognitive and depressive symptoms.

Methods

Subjects

This study included 40 patients with PD diagnosed according to the UK PD Brain Bank criteria [14] and a group of 25 controls matched by age, gender, body mass index (BMI), and educational level. From these, 23 patients with PD and 21 age- and gender-matched controls were included in the immunophenotyping analyses. Patients were recruited from the outpatient movement disorder clinic of the *Santa Casa de Belo Horizonte* Hospital, Belo Horizonte, Brazil. Controls were recruited from the local community. Participants were excluded if they had undergone previous neurosurgery or if they had any other neurological disorder and/or cognitive decline (i.e., delirium or dementia), significant sensory impairment, and infectious or autoimmune diseases in activity in the previous 4 weeks. In addition, individuals who had used corticosteroids, anti-inflammatories, or antibiotics in the 4 weeks prior to the study were excluded. All subjects provided written informed consent before admission to the study. The Research Ethics Committee of the *Universidade Federal de Minas Gerais*, Brazil, approved this study.

Clinical Evaluation

All patients were evaluated with the Unified Parkinson's Disease Rating Scale (UPDRS) [15] which assesses different signs and symptoms of PD. The UPDRS scores were obtained in the "on" state of the disease. The modified Hoehn and Yahr staging scale (HY) was used to establish the stage of PD [16]. The modified Schwab and England activities of daily living (ADL) scale (S&E) was used to assess daily routine of patients with PD [15].

All individuals were subjected to cognitive examination which included the Mini-Mental State Examination (MMSE) [17] adapted for the Brazilian elderly population. MMSE is a brief test for cognitive screening, comprising items from different domains such as orientation, attention, memory, and language. Since impairment in executive functioning is the most common cognitive deficit in PD, the Frontal Assessment Battery (FAB) was also applied [18]. FAB is a brief assessment tool that evaluates executive functioning and consists of six subtests exploring cognitive processes related to the frontal lobes: conceptualization mental flexibility, motor programming, sensitivity to interference, inhibitory control, and environmental autonomy. In each subtest, scores range from 0 (worst) to 3 (best). The total FAB score is calculated by the sum of the scores from each of the six subtests. In addition, all participants were evaluated using the Beck Depression Inventory (BDI), a self-rating instrument for depressive symptoms comprising 21 items, each one ranging from 0 to 3 according to the severity of symptoms [19]. BDI has been validated as a tool for depression screening and diagnosis in PD.

Blood Samples

Ten milliliters of blood was drawn by venipuncture in vacuum tubes containing heparin or ethylenediamine tetraacetic acid (EDTA) (Vacuplast, Huangyan, China) on the same day of the clinical assessment. In order to rule out any confounding factors caused by circadian rhythm, all samples were collected at the same time of the day (between 14 and 16 h).

Immunophenotyping

Blood samples collected in EDTA-containing tubes were kept at room temperature and used within 16 h for immunophenotyping analyses. Erythrocytes were briefly lysed using an ammonium chloride-based solution. After washing twice with phosphate-buffered saline (PBS), cells were stained for 30 min with combinations of the following monoclonal anti-human antibodies: anti-CD3 FITC, anti-CD4 PECy5, anti-CD8 PECy5, anti-CD25 FITC, anti-CD19 FITC, anti-CD56 PE, anti-CD16 PECy5, anti-CD14 FITC (all from BD Biosciences, San Jose, CA, USA). After staining, cells were washed and fixed with 2% formaldehyde in PBS solution. For intracellular staining for FoxP3, the fixed cells were permeabilized with 0.1% saponine for 10 min. After washing twice with PBS, cells were stained using anti-FoxP3 PE monoclonal antibodies (BD Biosciences, San Jose, CA, USA) for 30 min. Immediately after staining, cells were washed, resuspended, and analyzed by flow cytometry. FITC- and PE-labeled immunoglobulin control antibodies and a control of non-stained cells were included in all experiments. Preparations were acquired using a FACSCanto II cytometer (BD Biosciences, San Jose, CA, USA). A minimum of 50,000 gated events on lymphocytes and monocyte population identified by size (FSC) and granularity (SSC) were acquired for analysis. The instrument has been checked for sensitivity and overall performance with Cytometer Setup and Tracking beads (BD Biosciences, San Jose, CA, USA) prior to data acquisition. Data were analyzed using FlowJo 7.6.5 software (Tree Star, Ashlamb, OR, USA). A representative example of analysis strategy performed using FlowJo 7.6.5 software is given in Fig. 1.

Plasmatic Cytokine Assessment

Blood samples collected in heparinized tubes were used for plasma obtained within 2 h of the blood draw. These samples were centrifuged at 3000g for 10 min, 4 °C, twice. The plasma was collected and stored at −70 °C until assayed. The samples were thawed, and cytokines were measured as routinely performed in our laboratory. Multiple cytokines [interleukin (IL)-2, IL-4, IL-6, IL-10, tumor necrosis factor (TNF), interferon (IFN)-γ, and IL-17] were simultaneously measured by flow cytometry using the Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine Kit (BD Biosciences, San Jose, CA, USA). Acquisition was performed using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). The instrument has been checked for sensitivity and overall performance with Cytometer Setup and Tracking beads (BD Biosciences) prior to data acquisition. Quantitative results were generated using FCAP Array v1.0.1 software (Soft Flow Inc., Pecs, Hungary). Plasma levels of IL-1β were measured by high-sensitivity enzyme-linked immunosorbent assay (ELISA) according to the procedures supplied by the manufacturer (R&D Systems, Minneapolis, MN, USA). Concentrations are expressed as picograms per milliliter.

## Effects of Dopaminergic Drugs on Cytokine Production

We aimed to evaluate cytokine production after exposition of PBMC to the anti-parkinsonian drugs levodopa (dopamine precursor) and pramipexole (dopaminergic agonist). Peripheral blood samples were harvested from five healthy donors (two male, three female; 32.2 ± 3.8 years) in heparin-containing tubes. Fresh blood samples were diluted in PBS (1:1), gently layered on Ficoll solution (Ficoll-PaquePlus, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) at room temperature, and centrifuged at 405g for 40 min. The PBMC-containing layer was collected and washed twice in PBS at 4 °C. The viable cells, as determined by trypan blue exclusion, were resuspended at the concentration of 1×10^7 cells/mL in a medium composed of RPMI-1640 (Roswell Park Memorial Institute-1640) with L-glutamine (Cultilab, Campinas, Brazil), 40 IU/mL penicillin (Ariston, São Paulo, Brazil), and 40 μg/mL gentamicin (Nova Farma, Anápolis, Brazil), supplemented with 10% heat-inactivated human serum (Sigma-Aldrich, St. Louis, MO, USA).

PBMC were transferred to a u-bottom 96-well cell culture cluster (Costar, New York, NY, USA) at 1×10^6 cells/mL final concentration. Cells were then treated with levodopa (LD) or pramipexole (PX) at three different concentrations, as follows: (i) the peak plasma concentration (LD 6 μg/mL; PX 2 ng/mL); (ii) 0.1× the peak plasma concentration (LD 0.6 μg/mL; PX 0.2 ng/mL), and (iii) 100× the peak plasma concentration (LD 600 μg/mL; PX 200 ng/mL). Phytohemagglutinin (PHA) at 1% (Sigma-Aldrich, St. Louis, MO, USA) served as non-specific stimulus for PBMC (positive control). Non-treatment (i.e., absence of any drug or stimulus, referred to as baseline) was used as control. Cells were incubated at 37 °C in a 5% CO_2 atmosphere. The supernatants were collected 24 h after treatment and stored at −70 °C until assayed. The CBA Human Inflammatory Cytokine Kit (BD Biosciences, San Jose, CA, USA) was used to quantitatively measure IL-1β, IL-6, IL-8, IL-10, TNF, and IL-12p70 in the supernatant samples. Procedures followed the manufacturer's recommendations as described above, as well as acquisition and analysis.

Statistical Analysis

Association between dichotomous variables was assessed with the Fisher exact test. All variables were tested for Gaussian distribution by the Kolmogorov-Smirnov normality test. Two groups (patients vs. controls) were compared by Mann-Whitney or Student's t tests when non-normally or normally distributed, respectively. Spearman's correlation analyses were performed to examine the

Fig. 1 Representative example of the analysis strategy performed using FlowJo 7.6.5 software. The immunophenotyping of leukocytes was verified by flow cytometry assays. Whole-blood samples from Parkinson's disease (PD) patients and controls were stained with surface markers and intracellular FoxP3 after erythrocytes lyse. Total lymphocytes were gated (a) and fluorescent dot-plots were selected, for example for T lymphocytes (CD3+), as demonstrated in (b). Then, the lymphocyte subset, for instance T helper cells (c), was gated based on T lymphocyte (b) or total lymphocyte (a) gates. In some cases, double-positive gates (for example, CD4+CD25+, (d)) were necessary. Intracellular staining for FoxP3 was analyzed through histogram (e *red/left*: isotype control, *blue/right*: positive cells for FoxP3) based on the CD4 +CD25+ gate (d)
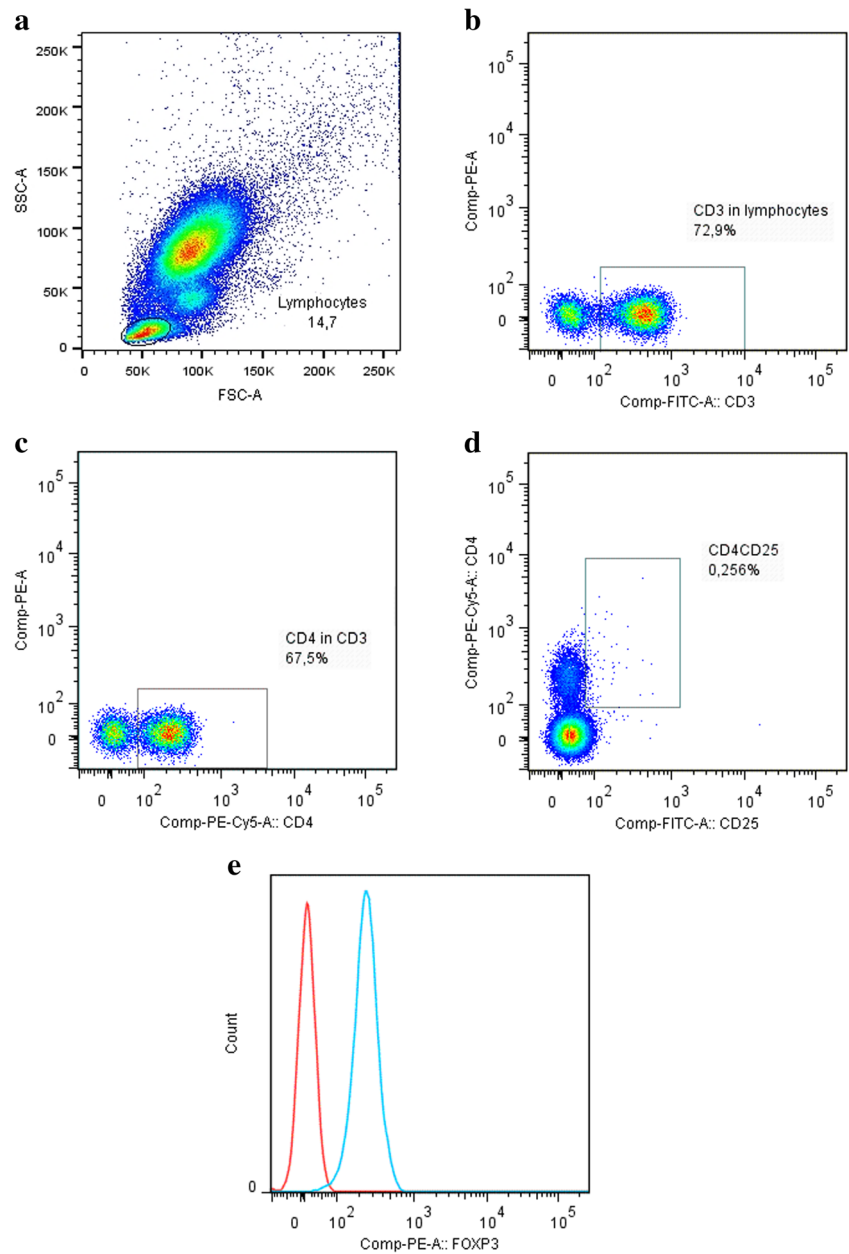
relationship between clinical variables and percentage of cell subset or plasma levels of cytokines.

Regarding the analyses on the effects of anti-parkinsonian drugs on cytokine production, we used one-way analysis of variance (ANOVA) to test group differences. Dunnett's multiple comparison test was used to compare each treatment with a single control (i.e., baseline condition).

All statistical tests were two tailed and were performed using a significance level of $\alpha = 0.05$. Statistical analyses were performed using SPSS software version 22.0 (SPSS Inc., Chicago, IL, USA), as well as GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Socio-demographic and Clinical Results

Demographic and clinical features of both groups are shown in Table 1. Patients with PD and controls did not differ regarding age, gender, body mass index, and educational level. Patients with PD presented a worse performance in the MMSE in comparison with controls ($Z = -3325$; $p = 0.001$). There was no difference between PD and control individuals regarding total FAB performance. Nonetheless, the analysis of the subtests demonstrated that the PD group presented a lower

Table 1 Demographic and clinical features of Parkinson's disease (PD) patients and control subjects

	PD patients (n = 40)	Control subjects (n = 25)	p value
Gender (female/male)	13/27	6/19	0.58[a]
Age in years (mean ± SD)	68.71 ± 10.07	65.23 ± 8.75	0.20[b]
Body mass index in kg/m² (mean ± SD)	26.02 ± 3.73	27.64 ± 3.71	0.09[c]
Educational level in years (mean ± SD)	4.72 ± 2.87	6.72 ± 5.37	0.16[b]
MMSE [mean ± SD (median)]	*24.00 ± 3.99 (25)*	*27.00 ± 3.57 (29)*	*0.001*[b]
FAB [mean ± SD (median)]	11.49 ± 2.99 (12)	12.32 ± 3.67 (13)	0.32[c]
Conceptualization	1.23 ± 1.01 (1)	1.64 ± 1.11 (2)	0.12[b]
Mental flexibility	1.82 ± 1.10 (2)	2.08 ± 1.04 (2)	0.34[b]
Programming	*1.74 ± 0.91 (2)*	*2.24 ± 0.83 (2)*	*0.04*[b]
Sensitivity to interference	2.26 ± 0.94 (3)	1.84 ± 1.25 (2)	0.21[b]
Inhibitory control	1.41 ± 0.88 (1)	1.52 ± 1.09 (1)	0.73[b]
Environmental autonomy	3.00 ± 0.00 (3)	3.00 ± 0.00 (3)	1.00[b]
BDI [mean ± SD (median)]	*8.64 ± 7.58 (6)*	*2.76 ± 3.35 (1)*	*<0.001*[b]
Medication in use (frequency in %)			
Anti-hypertensive (%)	55.00	48.00	0.62[a]
Anti-diabetic (%)	10.00	20.00	0.29[a]
Hypolipidemic (%)	10.00	24.00	0.17[a]
Levothyroxine (%)	10.00	4.00	0.64[a]
Anti-depressants	20.00	12.00	0.51[a]
Levodopa	37 (92.50)	–	–
Pramipexole	20 (50.00)	–	–
Entacapone	7 (17.50)	–	–
Amantadine	11 (27.50)	–	–
Length of illness in years [mean ± SD (range)]	5.45 ± 4.13 (0.4–18)	–	–
UPDRS [mean ± SD (range)]	51.82 ± 25.27 (11–105)	–	–
UPDRS I [mean ± SD (range)]	3.36 ± 2.96 (0–11)	–	–
UPDRS II [mean ± SD (range)]	14.08 ± 7.14 (2–31)	–	–
UPDRS III [mean ± SD (range)]	34.56 ± 18.43 (8–69)	–	–
HY [mean ± SD (range)]	2.44 ± 0.69 (1–4)	–	–
S&E in % [mean ± SD (range)]	77.95 ± 11.96 (50–100)	–	–

BDI Beck Depression Inventory, *FAB* Frontal Assessment Battery, *HY* Hoehn and Yahr staging scale, *MMSE* Mini-Mental State Examination, *PD* Parkinson's disease, *SD* standard deviation, *S&E* Schwab and England activities of daily living scale; *UPDRS* Unified Parkinson's Disease Rating Scale

[a] Fisher's exact test

[b] Mann-Whitney test

[c] Student's *t* test Statistically significant values are *italicized*.

score in programming ($Z = -2107$; $p = 0.04$). In addition, BDI score was higher in patients with PD in comparison with controls ($Z = -3528$; $p < 0.001$).

Immunophenotyping

Monocytes and lymphocyte subpopulations were evaluated by the expression of the membrane-bound molecules CD14, CD19, CD56, CD3, CD4, and CD8 and by the activation marker CD25. In addition, FoxP3 expression was assessed in order to investigate regulatory T lymphocyte (Treg) population. Results about immunophenotyping are shown in Table 2.

Patients with PD and controls presented similar percentage of monocytes (CD14+), B lymphocytes (CD19+), and NK cells (CD56+). The percentage of T lymphocytes (CD3+) was decreased in the peripheral blood of patients with PD in comparison with controls. We did not find any difference between patients with PD and controls regarding the percentage of neither CD4+ nor CD8+ lymphocytes, as well as the CD4/CD8 ratio. Interestingly, patients with PD presented a lower percentage of activated T lymphocytes (CD4+CD25+ lymphocytes). In addition, patients with PD and controls did not differ with respect to the percentage of Treg cells (FoxP3+ in CD4+CD25+ lymphocytes).

Table 2 Immunophenotyping of monocytes and lymphocyte subsets

Marker	Cell type	Controls	PD patients	p value
% CD14+ monocytes	Monocytes	82.75 ± 10.36	77.28 ± 10.36	0.11[a]
% CD19+ lymphocytes	B lymphocytes	4.46 ± 2.67	5.05 ± 2.97	0.41[b]
% CD3+ lymphocytes	T lymphocytes	*79.37 ± 9.53*	*62.07 ± 15.71*	*0.01*[a]
% CD4+ in CD3+ lymphocytes	T helper lymphocytes	49.95 ± 9.00	53.90 ± 19.89	0.29[b]
% CD8+ in CD3+ lymphocytes	T cytotoxic lymphocytes	24.53 ± 5.61	31.14 ± 13.95	0.06[a]
% CD4+CD25+ in total lymphocytes	Activated T lymphocytes	*5.31 ± 2.25*	*0.87 ± 1.32*	*0.001*[b]
% FoxP3+ in CD4+CD25+ lymphocytes	Regulatory T lymphocytes	70.82 ± 13.13	43.50 ± 33.83	0.14[b]
% CD56+ lymphocytes	NK cells	18.21 ± 14.62	12.50 ± 6.00	0.86[b]
CD4:CD8 ratio	–	2.20 ± 0.85	2.35 ± 1.40	0.62[b]

PD Parkinson's disease, *Th* T helper cell, *Tc* T cytotoxic cell

[a] Student's *t* test

[b] Mann-Whitney test Statistically significant values are *italicized*.

Plasma Levels of Cytokines

Th1/Th2/Th17 cytokines (IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ, and IL-17A) were assessed in plasma by CBA. In addition, plasma levels of IL-1β were evaluated by ELISA. As shown in Table 3, there was no significant difference between PD patients and controls regarding plasma levels of IL-2 and IL-1β. Patients with PD presented lower plasma levels of IL-4, IL-6, IL-10, TNF, IFN-γ, and IL-17A than controls.

To further investigate the cytokine profile in PD, we also compared cytokine ratios (Fig. 2). Patients with PD showed higher IFN-γ/IL-4 ($p < 0.01$, Student's *t* test), IL-2/IL-4 ($p < 0.001$, Student's *t* test), and IFN-γ/IL-17A ratios ($p = 0.03$, Student's *t* test) in comparison with controls, suggesting a bias towards a Th1 profile. There was no statistical difference regarding IFN-γ/IL-10 ratio ($p > 0.05$, Student's *t* test). The PD group also presented lower IL-6/IL-10 ($p < 0.001$, Mann-Whitney test) and TNF/IL-10 ($p < 0.001$, Mann-Whitney test) ratios compared to controls, showing an anti-inflammatory-weighted imbalance in PD.

Table 3 Plasma levels of cytokines in PD patients and controls

Cytokine (pg/mL)	Controls	PD patients	p value
IL-2 (mean ± SD)	1.65 ± 0.23	1.56 ± 0.10	0.08[a]
IL-4 (mean ± SD)	*0.59 ± 0.15*	*0.49 ± 0.06*	*<0.001*[a]
IL-6 (mean ± SD)	*3.82 ± 3.48*	*1.85 ± 0.87*	*<0.001*[a]
IL-10 (mean ± SD)	*1.53 ± 0.27*	*1.36 ± 0.12*	*<0.001*[a]
TNF-α (mean ± SD)	*1.11 ± 1.00*	*0.33 ± 0.13*	*<0.001*[a]
IFN-γ (mean ± SD)	*1.25 ± 0.12*	*1.19 ± 0.08*	*0.01*[a]
IL-17A (mean ± SD)	*3.35 ± 2.15*	*2.57 ± 2.48*	*0.01*[a]
IL-1β (mean ± SD)	3.02 ± 1.73	2.81 ± 2.35	0.14[a]

IFN interferon, *IL* interleukin, *PD* Parkinson's disease, *SD* standard deviation, *TNF* tumor necrosis factor

[a] Mann-Whitney test Statistically significant values are *italicized*.

Among patients with PD, higher TNF/IL-10 ratios were associated with worse cognitive performance, since TNF/IL-10 correlated with MMSE ($\rho = -0.451$, $p < 0.01$) and the domains of FAB mental flexibility ($\rho = -0.400$, $p = 0.01$) and sensitivity of interference ($\rho = -0.365$, $p = 0.027$).

Effects of Dopaminergic Drugs on Cytokine Production

The highest doses of both levodopa and pramipexole decreased the production of cytokines by PBMC (Fig. 3). Specifically, the levels of IL-1β, IL-6, IL-8, IL-10, and TNF were significantly lower in the supernatants from PBMC exposed to levodopa (600 µg/mL). In addition, pramipexole (200 ng/mL) significantly decreased the production of IL-6, IL-8, and TNF by PBMC.

Discussion

In the current report, we demonstrated that patients with PD presented peripheral immune changes evidenced by a decreased percentage of T lymphocytes (CD3+ cells), especially activated T lymphocytes (CD4+CD25+ cells), when compared with age-related controls. In line with these results, we also found decreased plasma levels of the cytokines IL-4, IL-6, IL-10, TNF, IFN-γ, and IL-17A in patients with PD.

Corroborating our data, several studies have shown a lower percentage of total lymphocytes [5, 6, 9] in addition to CD3+ [7, 8], CD4+ [8, 10], and CD19+ cells [10] in PD patients as compared with controls. One study has also found a lower percentage of CD4+CD25+ T cells in patients with PD [6]. The reduced percentage of T lymphocytes might be due to enhanced apoptosis process [20]. Also similar to our results, some studies have found no changes in the percentage of B lymphocytes (CD19+) and NK cells (CD56+), as well as CD4+ and CD8+ lymphocytes and CD4/CD8 ratio in PD [7, 10, 11, 20].
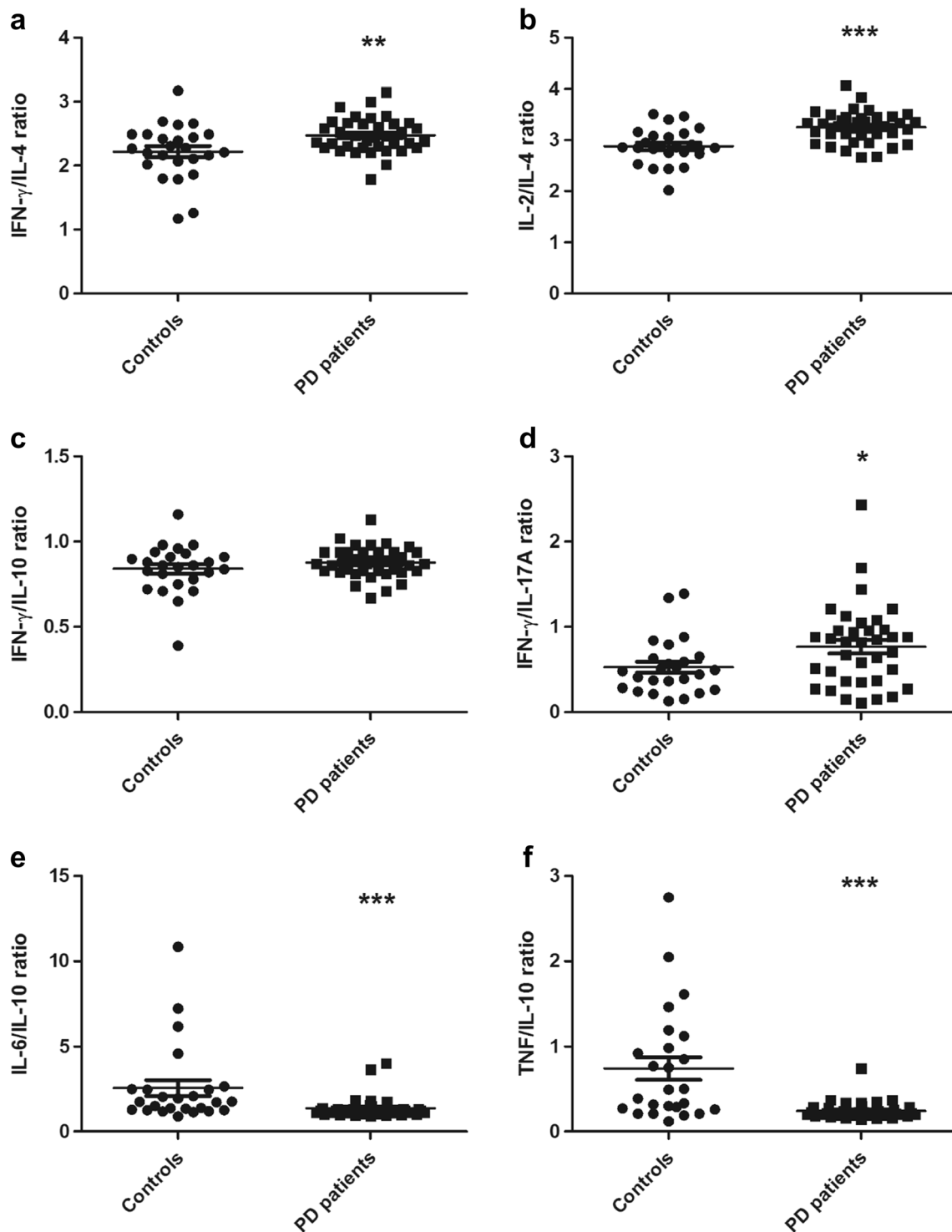
Fig. 2 Th1/Th2 and Th17 cytokines were evaluated in the plasma of Parkinson's disease (PD) patients and controls by cytometric bead array (CBA). PD patients showed higher IFN-γ/IL-4 (**a**), IL-2/IL-4 (**b**), and IFN-γ/IL-17A (**d**) ratios in comparison with controls, suggesting a bias towards a Th1 profile. There was no statistical difference regarding IFN-γ/IL-10 ratio (**c**). In addition, PD patients presented lower IL-6/IL-10 (**e**) and TNF/IL-10 (**f**) ratios compared to controls, showing an anti-inflammatory-weighted imbalance in PD. **a**–**d** = Student's *t* test. **e**, **f** = Mann-Whitney test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Changes in peripheral lymphocyte subsets in PD may result in changes in cytokine levels. We found a reduction in the percentage of activated T lymphocytes in PD, and, as a consequence, PD patients presented lower plasma levels of cytokines IL-4, IL-6, IL-10, IL-17A, TNF, and IFN-γ. Corroborating our data, the production of IL-6 and TNF-α by PBMC and TNF-α by
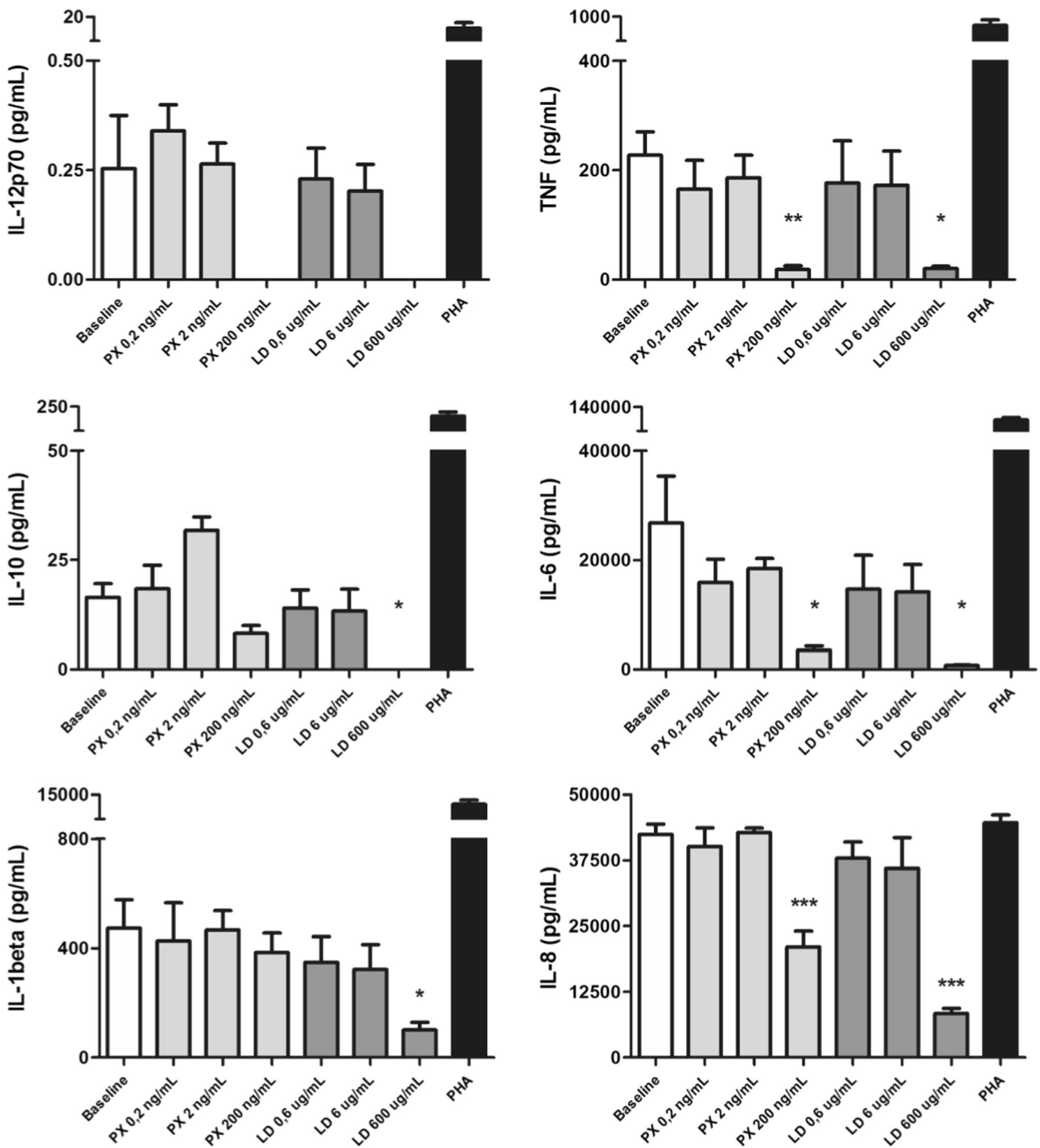
Fig. 3 In vitro exposure of peripheral blood mononuclear cells (PBMC) to high doses of the anti-parkinsonian drugs pramipexole (PX) and levodopa (LD) resulted in decreased production of cytokines. PBMC were harvested from healthy young donors. Phytohemagglutinin (PHA) was used as positive control. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was used to compare each treatment with the baseline condition. *$p < 0.05$, ***$p < 0.001$

monocytes/macrophages was found to be significantly lower in patients with PD as compared with control groups composed of age-related healthy donors and patients with cerebrovascular diseases [21]. Klüter and colleagues (1995) evaluated cytokine production in the supernatants of mitogen-stimulated whole-blood cultures, finding reduced production of IL-2 in PD but no differences regarding sIL-2R, IL-6, IFN-α2, and IFN-γ levels. Interestingly, the mean dose of levodopa was

negatively associated with IL-2 production [11]. IL-2 production by PBMC from patients with PD was found to be significantly lower than that from controls, while the production of IL-6 and IL-1β did not differ between groups [12]. The production of IL-2 and IFN-γ by whole-blood cultures of PD patients was also markedly decreased in PD compared with healthy controls and major depression patients. After amantadine treatment, IL-2 secretion was comparable to controls [13].

Specific drugs used for PD treatment may influence lymphocyte subsets, activation, and, therefore, cytokine levels. Long-term treatment with anti-parkinsonian drugs may influence the immune system through direct effects on lymphocytes or in antigen-presenting cells. For example, treatment with amantadine, originally established as an anti-viral drug but also with a weak anti-NMDA effect, has been demonstrated to alter lymphocyte subsets. Short-term treatment with amantadine was associated with an increase of the CD4/CD8 ratio [22]. Levodopa therapy is also capable of inducing changes in T lymphocyte proteome [23]. After treatment with levodopa, both the CD95/CD3 ratio and the number of lymphocytes dead due to apoptotic processes were found to be decreased. These data indicate that levodopa treatment in PD has an impact on apoptotic processes, and this should be taken into consideration as a positive event in the pathomechanism precipitated by this treatment [20].

The main limitation of our study comprises the fact that all patients were medicated and the observed findings might be influenced by patients' ongoing treatment. In order to investigate the putative effect of anti-parkinsonian drugs on the immune system, we evaluated cytokine production after exposition of PBMC to levodopa and pramipexole. We found that both levodopa and pramipexole were able to decrease the production of cytokines by PBMC, suggesting that anti-parkinsonian treatment is responsible, at least in part, for the observed findings. The observed immune changes in PD, however, might also result from other reasons, such as immune alterations related to aging process and/or peripheral immune alterations due to neuronal death.

We observed that PD patients showed increased ratios of IFN-γ/IL-4, IL-2/IL-4, and IFN-γ/IL-17A in comparison with controls, suggesting a bias towards a Th1 profile in PD. Increased ratios of IFN-γ/IL-4-producing T cells have also been described in PD patients in comparison with controls [6]. When activated, T helper (Th) lymphocytes can differentiate into three different types of effective Th cells, named Th1, Th2, and Th17. Th1 cells act mainly in macrophage and T cytotoxic cell activation, Th2 cells contribute to B lymphocyte activation, while Th17 cells are responsible for tissue inflammation induction and protection against extracellular pathogens [24]. Th1 cells are responsible for cell-mediated immunity raised by intracellular pathogens, being also involved in autoimmunity. The bias towards a Th1 profile seen in PD reinforces the hypothesis that PD is associated with immunological imbalance or dysfunction. A shift to a Th1-type immune response has already been described in the peripheral immune system in PD patients [6]. In addition, we found that higher TNF/IL-10 ratios were associated with worse cognitive performance in PD patients. This result is in line with the idea that a shift to a pro-inflammatory status is directly associated with worse cognitive performance. A growing body of evidence suggests the existence of a link between inflammatory responses mainly mediated by pro-inflammatory cytokines and cognitive and mood alterations (for a review, see [25]).

We identified both humoral and cellular changes in PD. To the best of our knowledge, this is the first report to describe reduced T lymphocyte activation and decreased plasma levels of inflammatory cytokines in a sample of PD patients in comparison with age-related controls. Despite the fact that all patients with PD were medicated, the strict exclusion criteria, the selection of controls with comparable age and gender, and the analysis of clinical and inflammatory parameters together can be regarded as strengths of the current study. In addition, we showed that the production of cytokines by PBMC in vitro is reduced with the exposure to the anti-parkinsonian drugs levodopa and pramipexole. More studies are needed in order to elucidate the role of anti-parkinsonian drugs in the immune system.

Conclusions

Our data together with previous studies corroborate the hypothesis that immunological mechanisms are involved in PD. It is clear that there is a peripheral immune alteration in PD, which is more than simply a decrease in immune responses. Immune changes observed in PD partially resemble those seen in normal aging, though being exaggerated in PD. PD is clearly an age-related disease, and it is possible that the immune alterations observed during aging are more pronounced in individuals who suffer from PD. Use of anti-parkinsonian drugs might also result in peripheral immune changes. It remains a challenge to fully understand how the immune system is linked to PD; therefore, future studies into this subject are needed to confirm the current findings.

Authors' Contributions NPR, ALT, and HJR worked on the conception and organization of the research project. NPR, FA, ELMV, PLS, IGB, MSS, and PPC worked on the execution of the research project. NPR and ALT designed and executed the statistical analyses and wrote the first draft of the manuscript. HJR and ALT reviewed the statistical analyses and the manuscript. All authors read and approved the final manuscript.

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