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Research Article

IMPACT OF LRRK2G2019S IN PRO-INFLAMMATORY CYTOKINES IN PARKINSON'S DISEASE PATIENTS

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Abstract:

PD is a neurodegenerative disorder, where the accumulation of α -SN aggregates is a central hallmark of the disease pathogenesis. However, the precise pathophysiological processes remain largely unidentified yet. Some of these genes seem to be particular relevant for the disease, including for example α -SN, glucocerebrosidase (GBA), parkin (PARK2), Pten-induced kinase 1 (PINK1), microtubule-associated protein tau (MAPT) and LRRK2. The LRRK2 is only gene discovered so far with familial and sporadic form of the PD. The clinically indistinguishable symptoms of the LRRK2 from the idiopathic form of the disease points towards the same pathogenic process. The prior studies focused on the role of monocytes involvement in the neuroinflammatory process in the PD patients. However this study is first to study pro-inflammatory cytokines productions by classical and non-classical monocytes in idiopathic (PDI) and LRRK2(PDL) mutated groups of the PD. The trends towards increased production of pro-inflammatory cytokines in the LRRK2 mutated PD patients points towards a significant disease process in a PD patients. Our result concludes the increased production of pro-inflammatory cytokines in PDL patients in both classical and non-classical monocytes. This in consistence with the prior studies that showed increased cytokines production in the PD patients. Thus non-classical and classical monocytes could be useful biomarkers for detecting PD at early stage. Importantly presentation of the processed antigen by the macrophages activates the T-lymphocytes of the adaptive immunity that release cytokines to activate B-cells to generate immunoglobulin against the antigen. The antibodies produce by the Bcells can crossed the BBB and exert their influence via Fc receptors present in the microglia. Since blocking the Fc receptors present on the monocytes could halt the ongoing inflammatory process needs further research. In addition the influence of LRRK2 on the maturation B-lymphocytes requires further studies as B-cells has high expression of LRRK2 after macrophages.

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INTRODUCTION:

PD is a neurodegenerative disorder, where the accumulation of α -SN aggregates is a central hallmark of the disease pathogenesis. However, the precise pathophysiological processes remain largely unidentified yet. [1] It is one of the most prevalent neurodegenerative disorders worldwide, affecting about 2%-3% of community older than age 65. Until now 20 genes have been associated to the familial forms of the PD, whereas more than 20 genetic loci linked to increased susceptibility for the development of the PD were identified from genome-wide association studies (GWAS). Some of these genes seem to be particular relevant for the disease, including for example α -SN, glucocerebrosidase (GBA), parkin (PARK2), Pten-induced kinase 1 (PINK1), microtubule-associated protein tau (MAPT) and LRRK2. [2] The LRRK2 is only gene discovered so far with familial and sporadic form of the PD. The clinically indistinguishable symptoms of the LRRK2 from the idiopathic form of the disease points towards the same pathogenic process. LRRK2 has established role in inducing mitochondrial functioning, inflammatory responses, apoptosis, deregulating the immune system and endocytosis. [3] The role of the LRRK2 in immune system has been established too. It is well documented that LRRK2 expressed on the immune cells. The highest expression found in the macrophages followed by the B-cells and the dendritic cells. [4]

The present study focused on sub-population of the monocytes i.e classical and inflammatory monocytes in idiopathic and LRRK2G2019S mutation groups of patients. CD14-CD16+ monocytes are considered to represent a inflammatory monocyte population that is characteristically more pro-inflammatory and typically display increased cytokine secretion and greater antigen presentation compared to the classical monocyte (CD14+CD16--) sub-population. [5] Remarkably, elevated levels of serum cytokines (IL-2, IL-4, IL-6, IL-10, TNF α) in PD patients point to an involvement of the peripheral immune system in the pathogenesis of PD [6]. Since PD is the clinical diagnosis therefore there is urgent need for the biomarkers that detect PD at earlier stage when the non-motor symptoms (NMS) predominate. This is important as 80% of dopamenergic neurons already damaged when the motor symptoms dominate. At this stage only symptomatic treatment could slow the process but there is no cure to halt the process of

neurodegeneration. Prior results suggest increased peripheral inflammation in the early stage of PD, but the role of inflammation in motor and non-motor symptoms is unclear[7]. Here, we study the contribution of LRRK2 to the dysregulation of monocytes in Parkinson's disease. We found an increase number of "classical and inflammatory" monocytes subpopulation in the peripheral blood of PD patients which points towards the dysregulation of inflammatory pathways.

Methods and Materials

Healthy and the diseased subjects participating in the study:

Total 41 Parkinson's disease (PDT) patients older than 30 years were included in this study. All the recruited patients were attending the Neurology out-Patient clinic of the Services Hospital Lahore (SHL). The patients were diagnosed following the United Kingdom Parkinson's Disease Society Brain Bank Criteria[8]. Patients were evaluated using several tests, in brief: 1). a standard clinical evaluation; 2). Screening for the dementia was carried out using the Mini Mental Status Examination (MMSE)[9]. 3). Screening for several cognitive domains was carried out using Montreal Cognitive Assessment (MoCA)[10]. The patients were divided in two groups i.e idiopathic(n=20)(PDI) and LRRK2 G2019S mutation(n=21)(PDL). The presence of the LRRK2 G2019S mutation was confirmed in a first and second round of the PCR amplification of the exon41. Demographic data are summarized in Table2. The control group(CNT)(n=40) included the cognitively healthy adults belonging to the local community (recruited among the patients' spouses, hospital or university staff, or their relatives), that were age and education matched to the patients. They had no history of the neurological or the psychiatric relevant condition, including abuse of alcohol or drugs or the head trauma, neither significant motor, visual or auditory deficits which could influence the neuropsychological performance. Moreover, to be eligible for this particular study, we considered the exclusion of the patients or the controls with the diagnosis of the diabetes, chronic inflammatory or the neoplastic diseases, or the medications susceptible of influencing the study variables. All subjects or their legal guardians signed an informed written consent and the study was approved by the CHUC ethics committee according to the guidelines of the Helsinki declaration on studies with the human subjects.

	Controls(CNT)	LRRK2 G201S mutant PD patients(PDL)	Idiopathic PD patients(PDI)
Male:Female (n)	20:20	10:11	10:10
Age(years)	61.87±2.12	67.3±2.7	62.7±3.2
Disease duration(years)		14.9±1.9	10.15±2.9

Table 2: Demographic data of the study population. Data are expressed as mean \pm SEM, except when indicated otherwise.

The flow cytometric analysis of the peripheral blood monocytes of the patient's blood samples:

To determine the frequency and MFI of the pro-inflammatory cytokines(TNF α and IFN γ) and the receptor CD68 ,peripheral blood was collected from patients with the PD and the healthy controls (HC)by the veno-puncture.The peripheral blood monocytes were stained in a standard method using fluorochrome-conjugated mouse anti-human monoclonal antibodies against:CD11b, CD14 and CD16 (Biolegend and eBioscience).Intracellular staining was done after saponin permeabilization using fluorochrome-conjugated mouse anti-human monoclonal antibodies against: TNF α ,IFN γ and CD 68.Irrelevant, directly conjugated, murine IgG1 or IgG2 (Biolegend) were used to ascertain background staining. Samples were run on a FACSCanto II (Becton Dickinson) and data were analyzed with the FlowJo 7.6.4 software (Tree Star).This protocol was repeated three times independently.

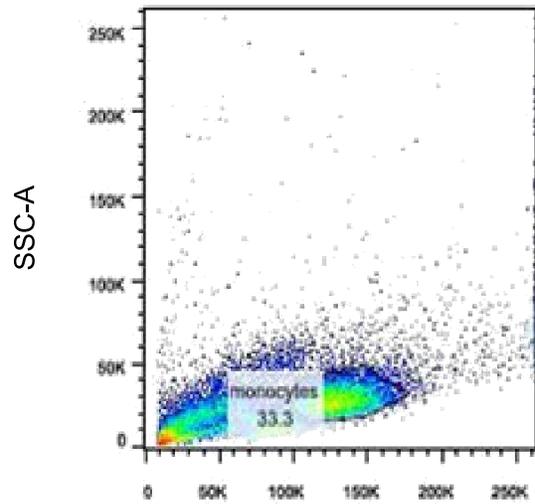
Statistical Analysis

The one-way analysis of variance (ANOVA) evaluations were used to compare the mean of pro-inflammatory cytokines and chemokines. Unpaired Student's t-test(two-tailed) was applied for assessing

the differences between the two groups Differences between the groups were considered statistically significant when $p < 0.05$.Statistical analysis was performed using the GraphPad Prism version 5.00 for Windows, GraphPad Software (San Diego California USA, www.graphpad.com)

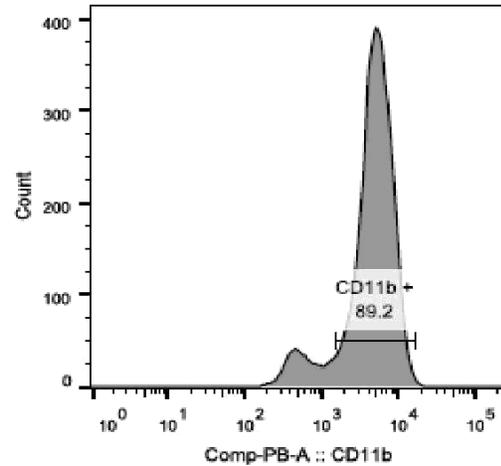
RESULTS:

To evaluate the fraction of the classical(CD14+/CD16-), non classical(CD14+/CD16+) and intermediate (CD14/CD16+)monocytes, FACS analysis was performed using the CD14 and CD16 specific antibodies. The analysis of the proinflammatory cytokines(TNF α and IFN γ) and the CD68 receptor in peripheral blood of the classical monocytes from the PDT,PDL,PDI patients and the HC subjects, were based on this subset division. Gates were set on the live cells by the FSC and SSC(Fig.1a).Gates were further set on the CD11b+ cells to exclude the B and T cells from the analysis(Fig.1b) and then the frequency and production of the pro-inflammatory cytokines(TNF α and IFN γ) and the CD68 was determined in the gated classical(CD14+/CD16-) monocytes(Fig.1c).

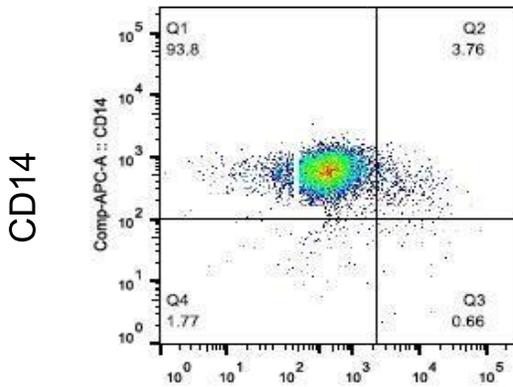


FSC -A

(a)



(b)



CD16 (c)

Figure.1: The FACS analysis of the peripheral blood monocytes.(a) Representative dot plot of the monocytes from peripheral mononuclear cells by gating in the forward scatter(FSC) and side scatter(SSC) channels. (b) Dot plot histogram showing gating of the CD11b⁺ monocytes. (c) The gated classical monocytes from the peripheral blood in the upper left quadrant.

The CD68 receptor on the classical monocytes from the PD patients' blood:

The CD68 selective expression on the surface of monocytes/macrophages is recognized and utilized for their recognition [11].To assess the number of the monocytes, the frequency and the MFI of CD68⁺ on the surface of the classical monocytes in the controls and PDT patients were analysed using flow cytometry. There are increase number of the CD68⁺ receptors in

the PDT than the CNT (Fig.2a and b). Between PDI and PDL however increase numbers of CD68⁺ receptors were found in the PDI than the PDL. The frequency of the CD68⁺ cells was 1.3 times higher in the PDI than the PDL and MFI of the CD68⁺ cells was 4.4 times higher in the PDI than the PDL higher in the PDI which denotes that PDL has low levels of the classical monocytes than the PDI group (Fig.3a and b).

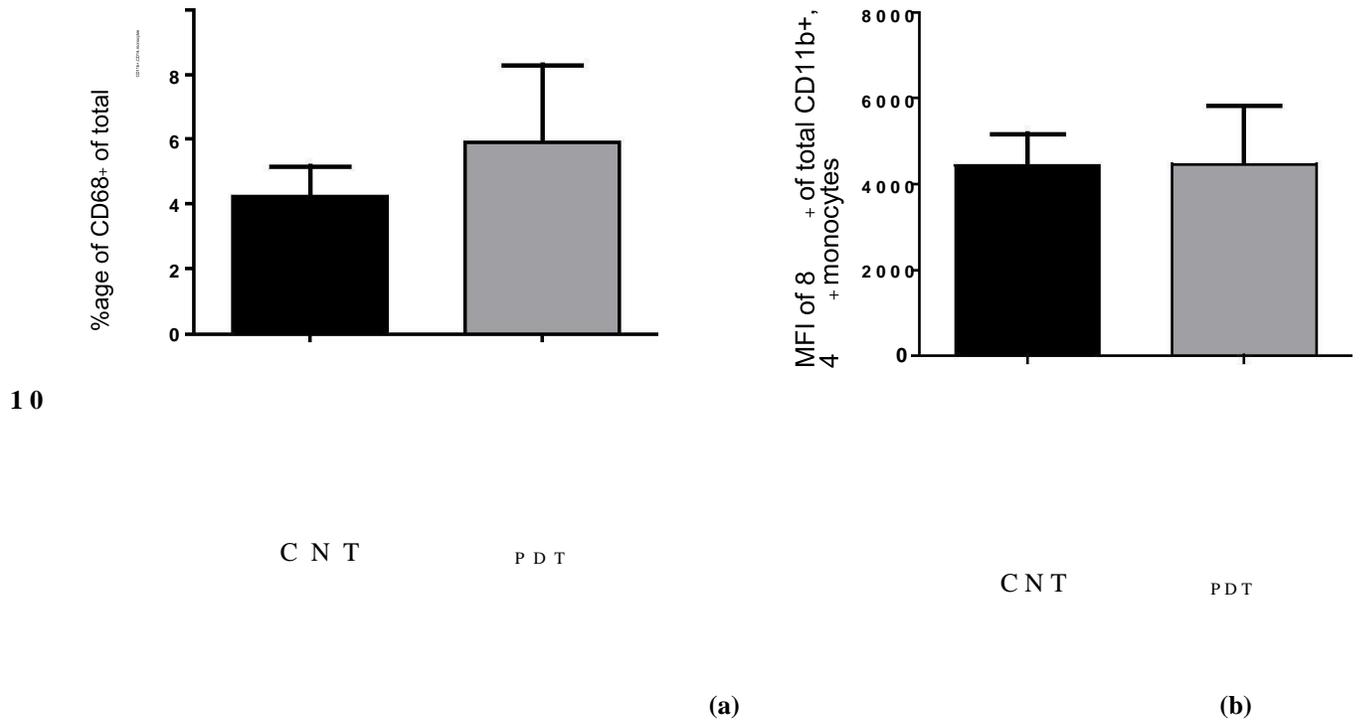


Figure.2: Flow cytometric analysis of the CD68⁺ classical monocytes in the HC individuals and the PDT.(a) The CD14⁺ monocytes were stained with CD68⁺ antibody and sorted through flow cytometer.(b) The MFI of CD68⁺ cells in the classical monocytes.

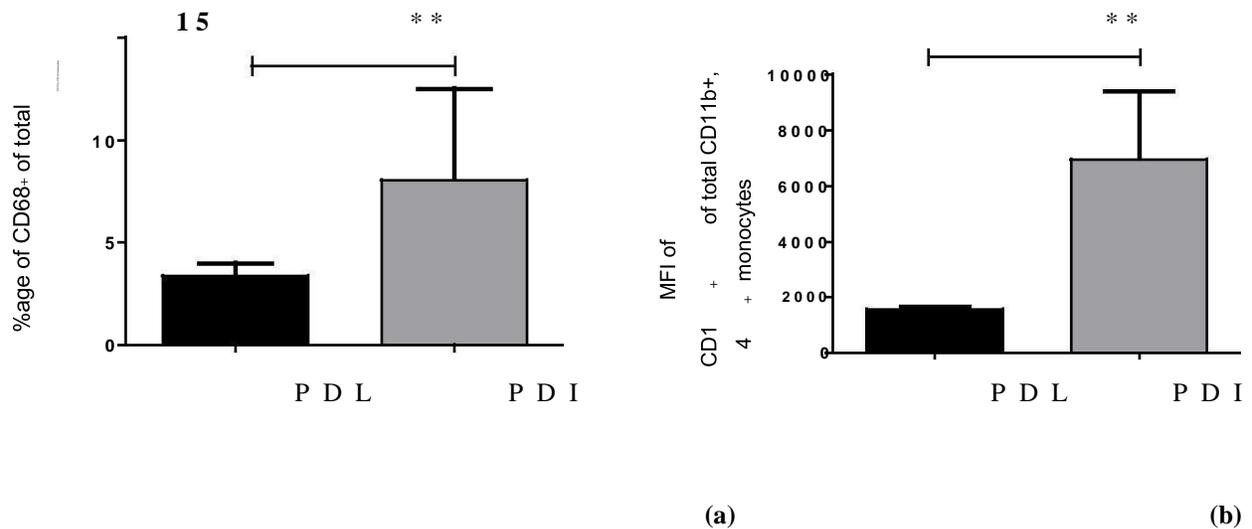


Figure.3:Flow cytometric analysis of the CD68⁺ classical monocytes in the PDL patients and the PDI patients.(a) The CD14⁺ monocytes were stained with CD68⁺ antibody and sorted through flow cytometer.(b) The MFI of CD68⁺ cells in the classical monocytes.(*p<0.05, **p<0.01, ***p<0.001).

The pro-inflammatory TNF α production in the classical monocytes:

Production of the pro-inflammatory TNF α was evaluated in the classical monocytes isolated from the peripheral blood. Considerable difference in the frequency and MFI of the TNF α in the HC and the PDT was observed when analyzed through the flow cytometry. The frequency of the TNF α was 2.4 times higher in the PDT than the CNT and MFI was 2.9 folds elevated in the PDT than the CNT group (Fig.3a and b) when statistical analysis was performed. Further

checking the assembly of the TNF α in the sub-groups of the PDT (PDL and PDI) via the same parameters showed increased levels of the TNF α remains in the PDL than the PDI (Fig.4a and b) when statistical analysis was done.

Overall the results illustrated increased levels of the pro-inflammatory TNF α in the PDT and PDL groups of patients when compared HC and PDI groups respectively.

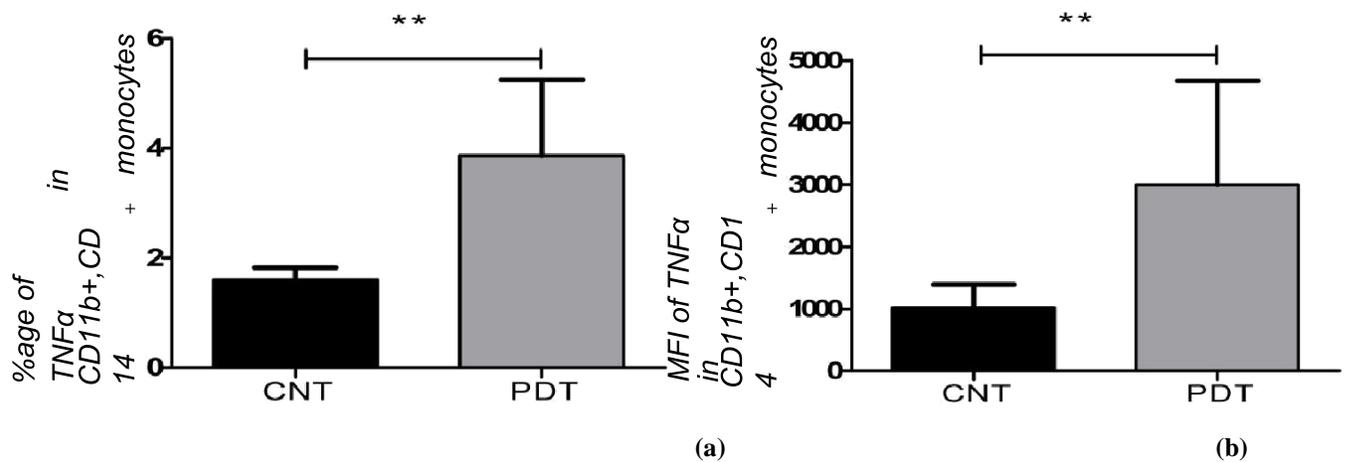
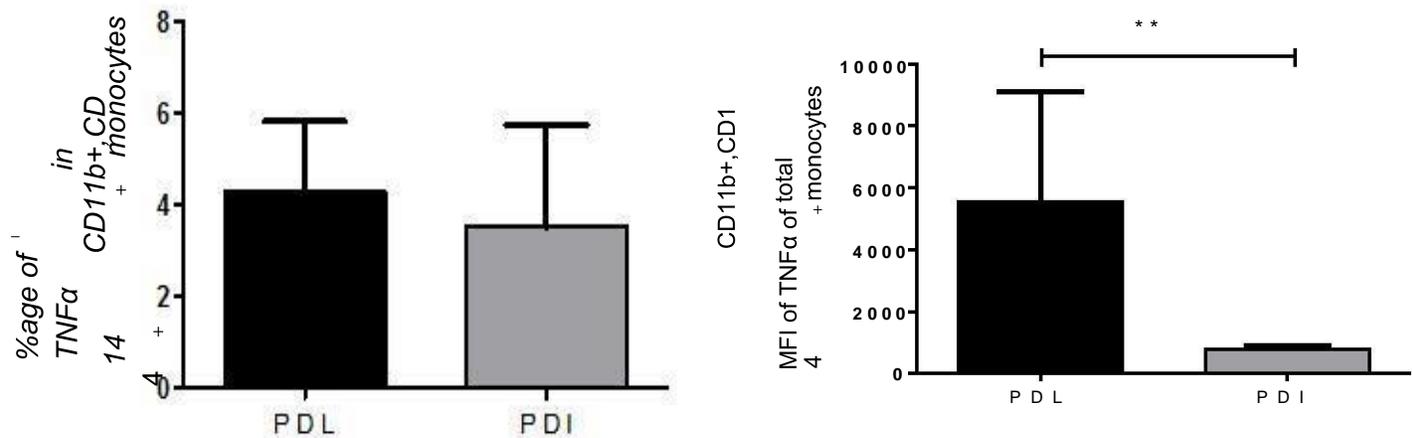


Figure.4: Flow cytometric analysis of the TNF α in the classical monocytes in the HC and the PDT.(a) The TNF α in the classical monocytes were stained and sorted based on the TNF α production through the flow cytometer.(b) The MFI of the TNF α in the classical monocytes. (*p<0.05, **p<0.01, ***p<0.001).



(a)

(b)

Figure.5:The flow cytometric analysis of the TNF α in the classical monocytes of the PDL patients and the PDI patients.(a) The CD14⁺ monocytes were stained and sorted based on the TNF α production through the flow cytometer.(b) The MFI of the TNF α in the classical monocytes.(*p<0.05, **p<0.01, ***p<0.001).

The pro-inflammatory IFN γ production in the classical monocytes.

The IFN γ levels was assessed in the classical monocytes isolated from the peripheral blood. The results showed increased levels of the IFN γ in the PDT than the CNT (Fig.6a and b). The flow-cytometry

analysis showed high occurrence of the IFN γ in the PDL than the PDI (Fig.7a and b).In general the results suggests increased production of IFN γ by classical monocytes in the PDT group of patients and PDL groups patients.

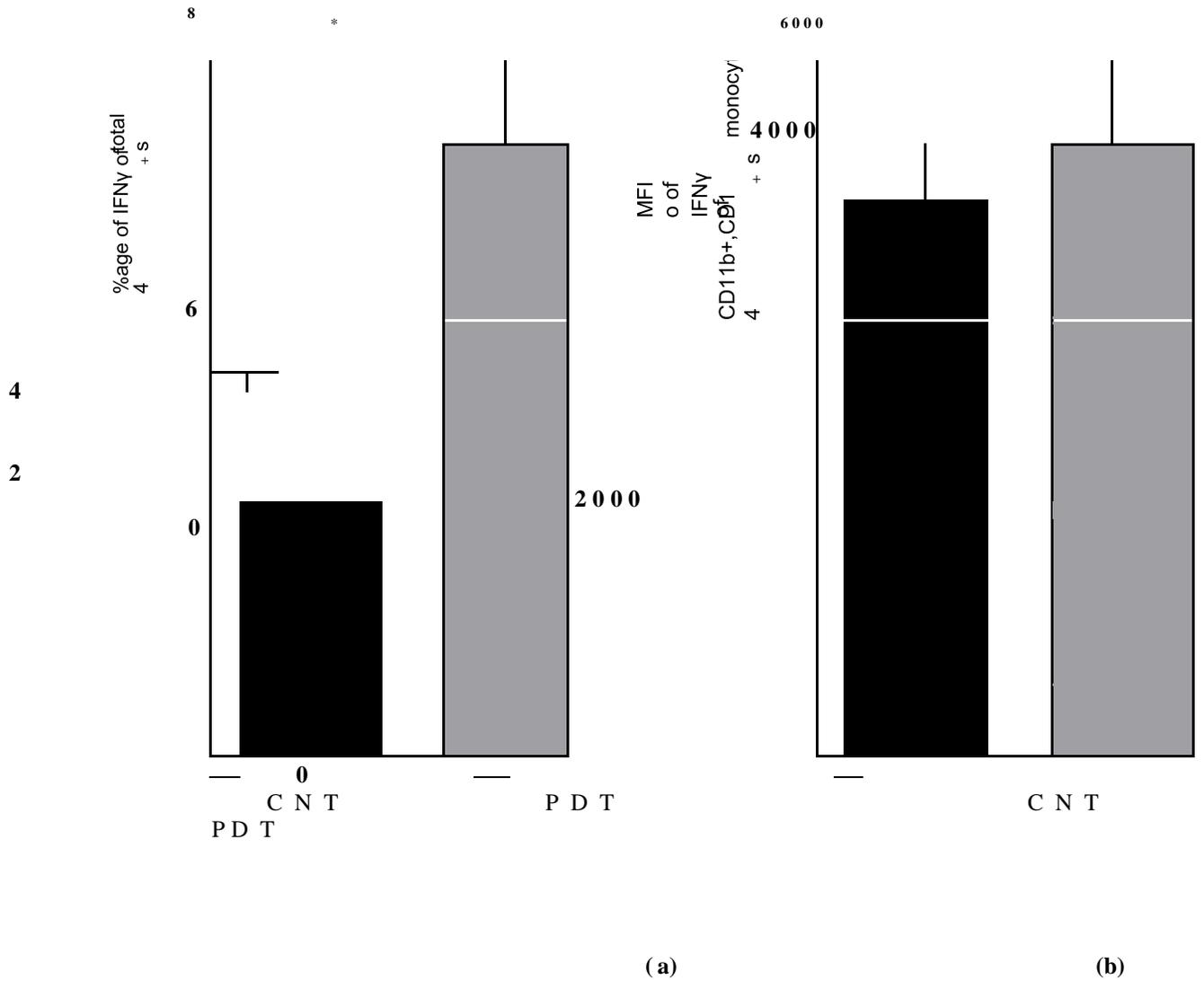


Figure.6: Flow cytometric analysis of the IFN γ in the classical monocytes in the HC and the PDT.(a) The IFN γ in the classical monocytes were stained and sorted based on the IFN γ production through the flow cytometer.(b) The MFI of the IFN γ in the classical monocytes. (*p<0.05, **p<0.01, ***p<0.001).

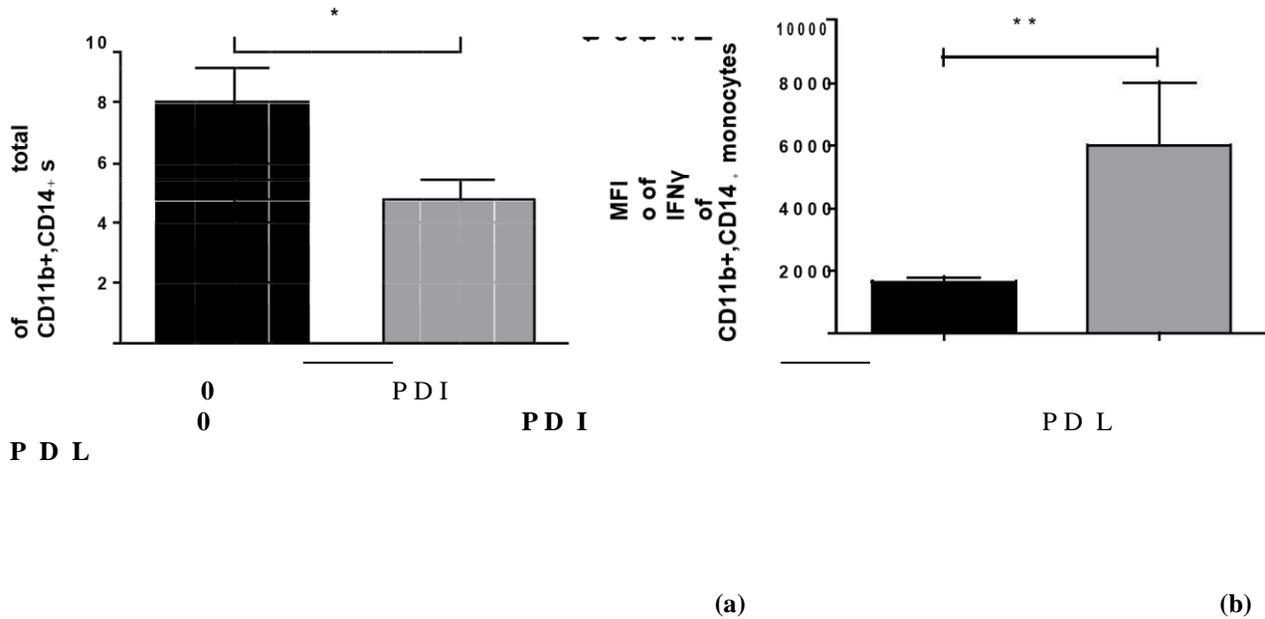


Figure 7: Flow cytometric analysis of the IFN γ in the classical monocytes of the PDL patients and the PDI patients.(a) The CD14 $^{+}$ monocytes were stained and sorted based on the IFN γ production through the flow cytometer.(b) The MFI of the IFN γ in the classical monocytes. (*p<0.05, **p<0.01, ***p<0.001).

Isolation of the non-classical monocytes from the patients' blood samples:

For identification of the non classical(CD14 $^{-}$ /CD16 $^{+}$) monocytes the same gating strategy was used as described in the section 3.1(Fig.9) using flowjo on the isolated peripheral blood monocytes from patients samples. The variations of the CD68 $^{+}$ cells and the levels of the pro-inflammatory cytokines (TNF α and IFN γ) were evaluated between the HC subjects and the PDT and within the diseased groups (PDL and PDI) of the PDT in the non-classical (CD14 $^{-}$ /CD16 $^{+}$) monocytes. The non classical monocytes have high phagocytic activity and are coupled with the release of cytokines and also well-known as the 'inflammatory' monocytes [12].

The levels of CD68 $^{+}$ cells from the Parkinson's patients peripheral blood samples in the inflammatory monocytes:

The analysis of the CD68 $^{+}$ cells in the inflammatory (CD14 $^{-}$ /CD16 $^{+}$) monocytes in the PDT patients using flow cytometry illustrates high frequency of the CD68 $^{+}$ cells in the PDT than HC. The frequency of the CD68 $^{+}$ cells was 4.7 times high in PDT than the HC (Fig.8a and b).Further observation of the variation of CD68 $^{+}$ cells in the two diseased groups(PDL and PDI) through flow cytometry demonstrate noteworthy decreased levels in the PDL than the PDI group (Fig.9a and b).

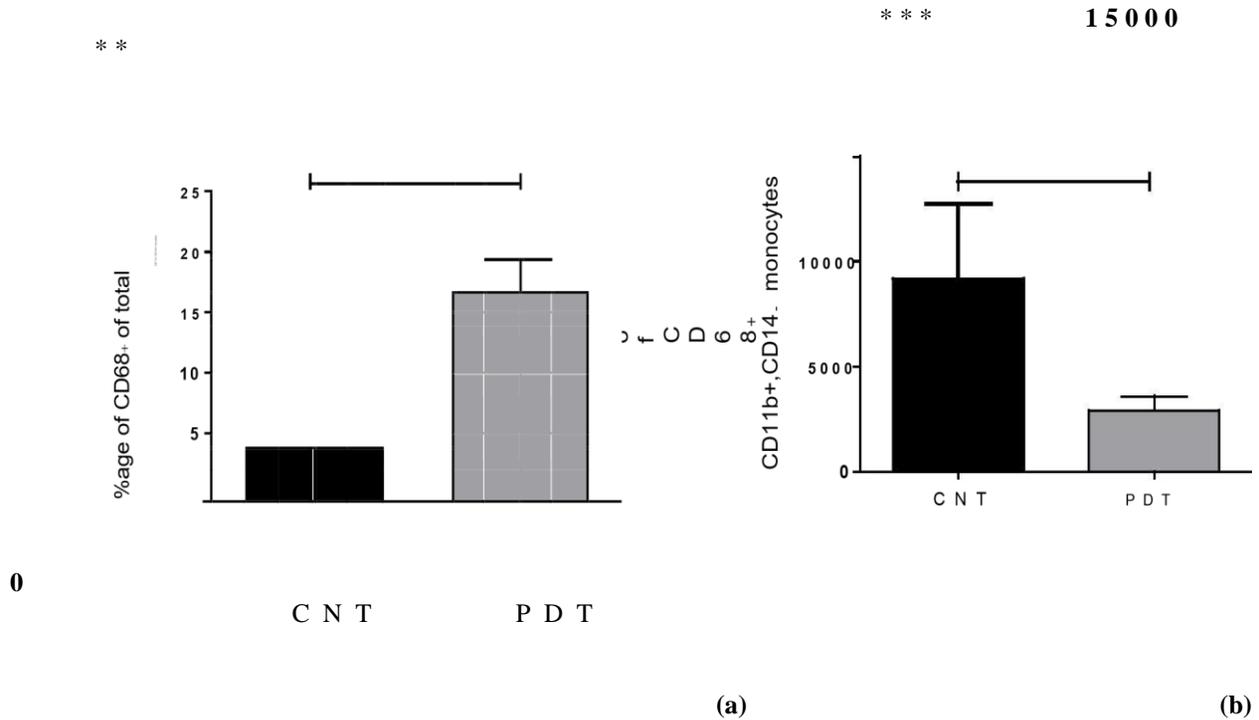


Figure.8:Flow cytometric analysis of the CD68⁺ cells in the inflammatory monocytes within the CNT and the PDT patients.(a) The CD14⁺ monocytes were stained and sorted based on the CD68⁺ cells through the flow cytometer.(b) MFI of the CD68⁺ cells in the inflammatory monocytes.(*p<0.05, **p<0.01, ***p<0.001).

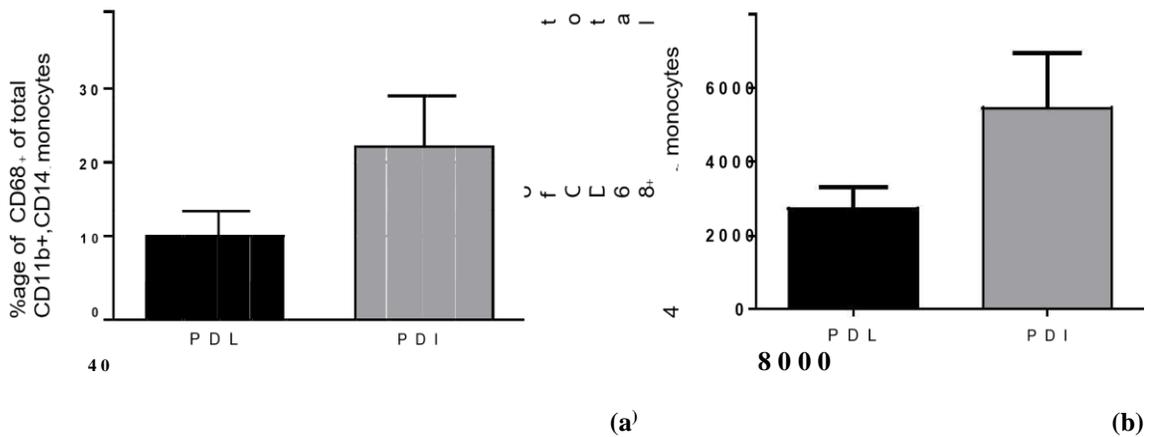


Figure.9:Flow cytometric analysis of the CD68⁺ cells within the inflammatory monocytes in the PDL and the PDI patients.(a) The inflammatory monocytes were stained and sorted based on the CD68⁺ cells through the flow cytometer.(b) The MFI of CD68⁺ cells in the inflammatory monocytes.

The pro-inflammatory TNF α production in the inflammatory monocytes:

The inflammatory monocytes were assessed for the production of the TNF α in the isolated peripheral blood monocytes. The samples were run on the FACS and analyzed through the flowjo. The rate of recurrence and the MFI of the cells showed an increase

tends towards production of TNF α in the PDT than the HC (Fig10a and b).Flow cytometric analysis of the assembly of the TNF α involving PDL and PDI in the inflammatory monocytes was also performed. The fraction of the cells between the PDL and the PDI illustrated significant trends of production of TNF α in the PDL than the PDI (Fig.11a and b).

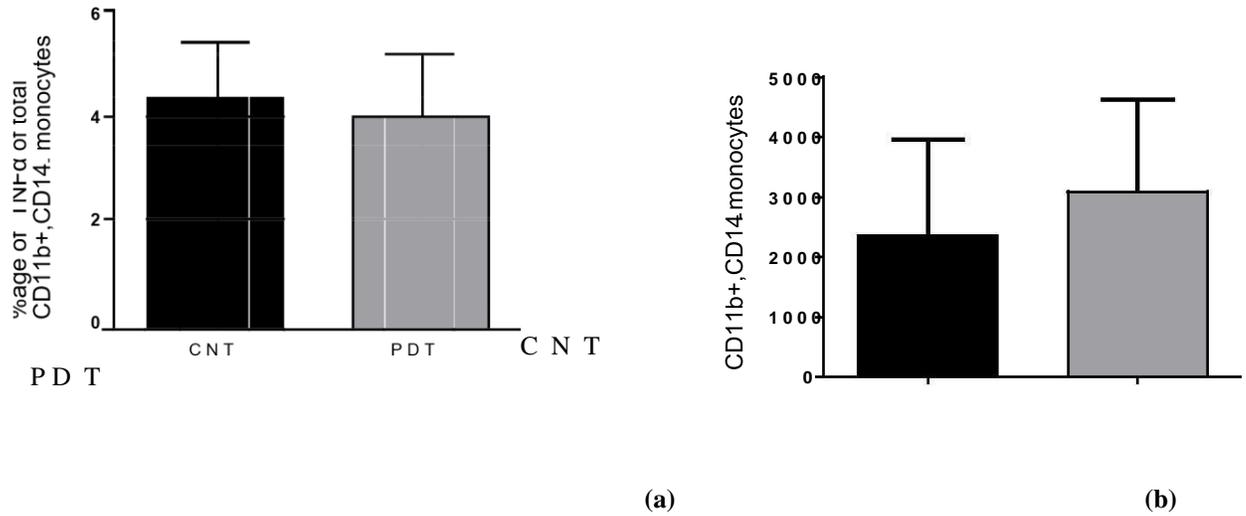


Figure.10: Flow cytometric analysis of the TNFα in the inflammatory monocytes between the control and the PDT patients.(a) The CD14⁻ inflammatory monocytes were stained and sorted based on the TNFα production through the flow cytometer.(b) The MFI of TNFα in the inflammatory monocytes.

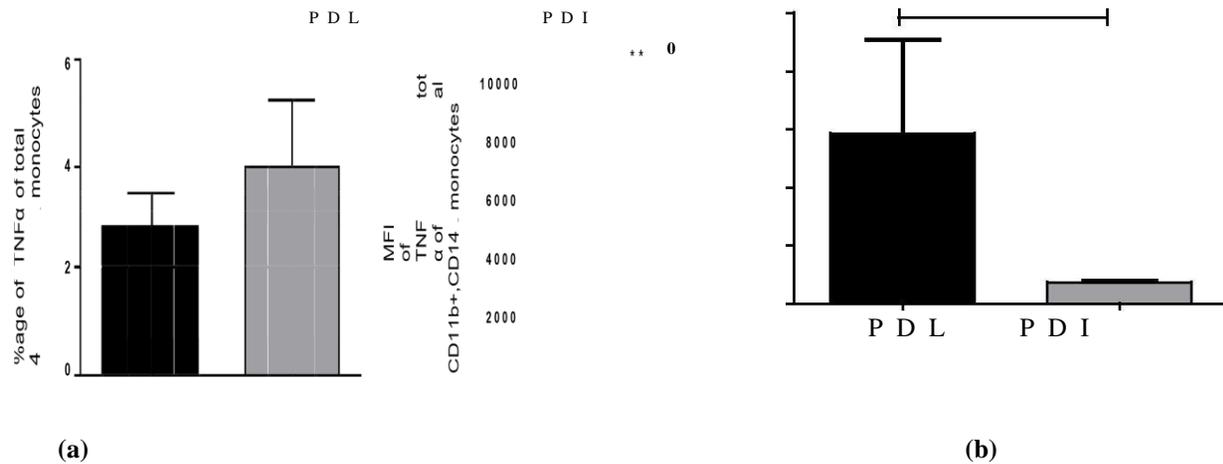


Figure.11:Flow cytometric analysis of the TNFα in the inflammatory monocytes within the PDL and the PDI patients.(a) The CD14⁻ inflammatory monocytes were stained and sorted based on the TNFα assembly through the flow cytometer.(b) The MFI of the TNFα in the inflammatory monocytes. (*p<0.05, **p<0.01, ***p<0.001).

The IFNγ production in the inflammatory monocytes

The rate of cells producing the IFNγ was high in the PDT than the HC (Fig.12a and b).Flow cytometric analysis of the production of the IFNγ in the PDL and the PDI within the inflammatory monocytes was in addition made. The proportion of cells with IFNγ did

not give an idea about considerable variation in the two diseased groups of PD(PDL and PDI).However there are trends towards increased production of IFNγ in the PDL group of patients. Nonetheless the MFI of the cells in the PDI was higher than the PDL (Fig.13a and b).

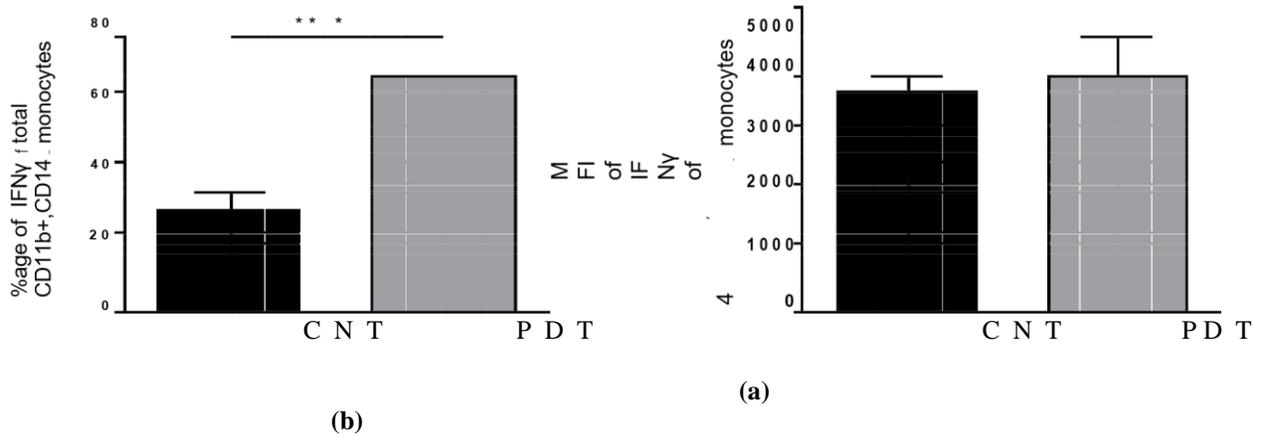


Figure.12:Flow cytometric analysis of the IFN γ in the inflammatory monocytes in CNT and PDT patients.(a) The inflammatory monocytes were stained and sorted based on the IFN γ production through the flow cytometer.(b) The MFI of the IFN γ in the inflammatory monocytes. (*p<0.05, **p<0.01, ***p<0.001)

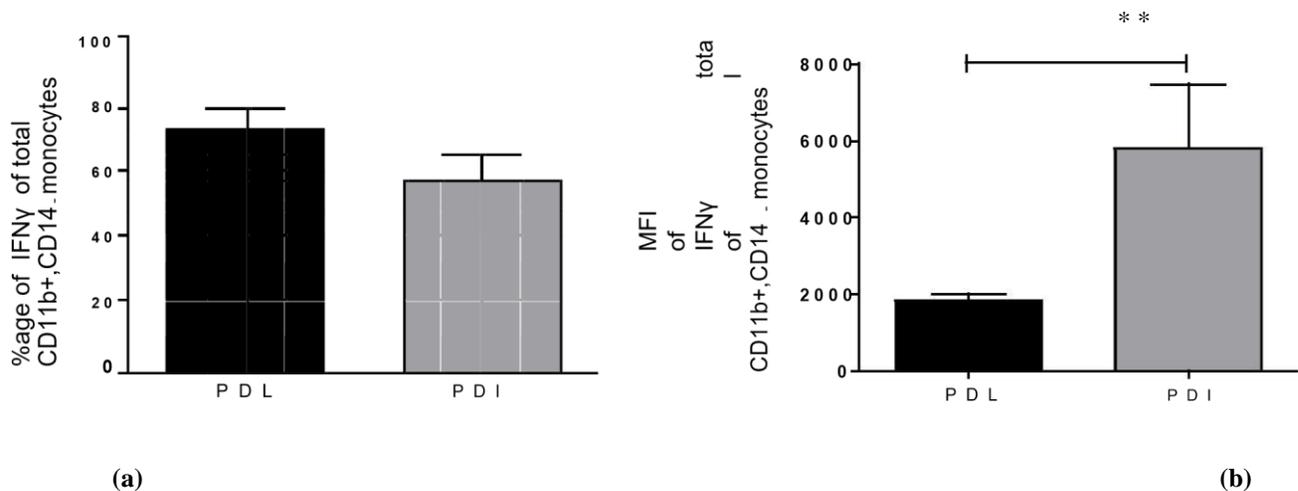


Figure.13:Flow cytometric analysis of the IFN γ in the inflammatory monocytes in the PDL and the PDI patients.(a) The inflammatory monocytes were stained and sorted based on the IFN γ production through the flow cytometer.(b) The MFI of the IFN γ in the inflammatory monocytes. (*p<0.05, **p<0.01, ***p<0.001).

Discussion and future directions:

The role of the LRRK2 in immune system has been well established. The highest expression of the LRRK2 found in the macrophages followed by the B-cells and the dendritic cells [5]. The macrophages and DCs are the antigen presenting cells (APCs) of the innate immune system that are professional in presenting cells to the adaptive immune system. The function of microglia in the CNS is the same as that is performed by the macrophages in the peripheral immune system [6]. Thus, the macrophages in the

peripheral blood of the PD patients are good target to study the LRRK2 physiology and pathology. Since blood brain barrier (BBB) becomes leaky with the passage of age and crosstalk between the CNS and peripheral immune system become more fluid [11]. Therefore, studying monocytes and its subset provides better understand of the ongoing neuroinflammatory process in the PD patients. Prior studies showed alterations in the pro-inflammatory cytokines in the classical and inflammatory monocytes in the PD patients [5, 6]. However in contrast to previous studies

our study the described alterations in the pro-inflammatory cytokines (TNF α and IFN γ) in the total PD patients as a whole in comparison to the healthy controls as well as between the idiopathic and LRRK2G2019S mutated group of PD patients. Thus this provides a better understanding of the peripheral inflammation process in the PD patients. The classical and the non-classical monocytes were identified through the flow cytometer. The CD68 signifies a typical and extensively used immunohistologic indicator molecule for cells of the monocyte/macrophage and dendritic cell(DC) system[10]. The production of pro-inflammatory TNF α and IFN γ was higher in PDT which authenticates previous studies of immune dysregulation in the PD [5, 6].In the diseased groups(PDL and PDI), the pro-inflammatory cytokines were higher in the PDL. This suggests a well increase in the concentration of pro-inflammatory cytokines inside the brain which may have a venerable, collective consequence on neuronal toxicity. Moreover the PDL group in this study has more duration of illness and age than the PDI group. It has been revealed that the BBB becomes injured during age and leukocyte extravasation has been observed in autoptic SNpc from PD patients [13, 14].Furthermore the LRRK2 may possibly function as a transcription controller of immune linked pathways by adjusting the function of transcription factors such as NFAT1 and NF- κ B. [15].

This study is of first type in terms of studying the pro-inflammatory cytokines productions between idiopathic and LRRK2 mutated groups of patients. The statistics shows increased trends towards production of pro-inflammatory cytokines (TNF α and IFN γ) by non-classical monocytes in LRRK2 mutated PD patients. Thus non-classical monocytes could be useful biomarkers for detecting PD at early stage. The non-classical monocytes releases large amount of cytokines upon exposure to the antigens (5). Previous data also suggest that LRRK2 could be regulating the antigen presentation function of human monocytes, and that such instruction is distorted in PD patients [5]. Presentation of the processed antigen by the macrophages activates the Tlymphocytes of the adaptive immunity that release cytokines to activate B-cells to generate immunoglobulin against the antigen. Importantly the antibodies produce by the B-cells can crossed the BBB and exert their influence via Fc receptors present in the microglia [16]. Since the Fc receptors present on the monocytes could halt the ongoing inflammatory process needs further research. In addition the influence of LRRK2 on the maturation B-lymphocytes requires further studies as B-cells are

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