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Ageing and Longevity

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https://doi.org/10.3390/jal3010001

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David Goldeck [1,*], Claudia Schulte [2], Marcia Cristina Teixeira dos Santos [3], Dieter Scheller [4], Lilly Öttinger [5], Graham Pawelec [6,7], Christian Deuschle [2], Daniela Berg [2,8], Andre Nogueira da Costa [9] and Walter Maetzler [2,8]

1 Fairfax Centre, Kidlington OX5 2PB, UK
2 Department of Neurodegeneration, Hertie Institute for Clinical Brain Research, German Center for Neurodegenerative Diseases, University of Tübingen, 72076 Tübingen, Germany
3 Experimental Medicine and Diagnostics, Global Exploratory Development, UCB Biopharma SPRL, 1070 Brussels, Belgium
4 Consultancy Neuropharm, 41470 Neuss, Germany
5 Section for Dementia Research, Department of Psychiatry and Psychotherapy, University of Tübingen, 72076 Tübingen, Germany
6 Department of Immunology, University of Tübingen, 72076 Tübingen, Germany
7 Health Sciences North Research Institute, Sudbury, ON P3E 2H2, Canada
8 Department of Neurology, Kiel University, 24105 Kiel, Germany
9 Novartis Institutes for BioMedical Research, CH-4056 Basel, Switzerland
* Correspondence: dgoldeck@yahoo.de

Abstract: Immune cells are thought to be involved in a destructive cycle of sterile cerebral inflammatory responses in neurodegenerative diseases such as Parkinson's Disease (PD). Despite their peripheral origin, immune cells may enter the CNS due to impaired blood–brain barrier function and may potentially contribute to neuronal damage. Hence, specific characteristics of peripherally activated immune cells could help in understanding neurodegeneration in PD and could potentially serve as accessible disease markers. To investigate immune cell activation status, the expression of receptors for cell surface molecules CD161, NKG2A, NKG2C and NKG2D as well as chemokine receptors CCR6, CXCR2, CXCR3 and CCR5 associated with neurodegenerative diseases was investigated. The frequencies of peripheral CD8+ T-cells expressing the inhibitory and activating receptors NKG2A and NKG2C, and the activating receptor NKG2D were higher in PD patients than in healthy matched controls. The frequencies of NKG2C+CD8− cells were also higher, whereas the frequencies of CD161+ cells were not significantly different. Of the chemokine receptor-expressing cells, only the proportion of CD4−CD56+CCR5+ T-cells was higher in PD patients than in the controls. These observations support the hypothesis that an imbalance in the activation state of T-cells plays a role in the pathological processes of PD and suggest that peripheral blood immune cell phenotypes could be specific early markers for inflammation in PD.

Keywords: Parkinson's disease; chemokine receptors; NK-cell receptors

Citation: Goldeck, D.; Schulte, C.; Teixeira dos Santos, M.C.; Scheller, D.; Öttinger, L.; Pawelec, G.; Deuschle, C.; Berg, D.; Nogueira da Costa, A.; Maetzler, W. Higher Frequencies of T-Cells Expressing NK-Cell Markers and Chemokine Receptors in Parkinson's Disease. *J. Ageing Longev.* **2023**, *3*, 1–10. https://doi.org/10.3390/jal3010001

Received: 1 October 2022
Revised: 16 December 2022
Accepted: 19 December 2022
Published: 22 December 2022

1. Introduction

Ageing and neurodegeneration are both associated with low-grade chronic inflammation as measured by the levels of different serum molecules [1–5]. It has been reported that inflammatory factors secreted by activated microglia, astrocytes, neurons, T-cells and mast cells may all lead to neuroinflammation and, further, neurodegeneration and that, for microglia at least, secretion is greater in older adults [5]. These effects are exacerbated by a defective blood–brain barrier (BBB) in older adults [5], including PD patients [6]. The levels of cytokines such as IL-6 and TNF-alpha are reportedly associated with higher mortality as well as with functional decline in older adults [1–3]. Neuroinflammatory processes have also been suggested to participate in the pathogenesis of Parkinson's Disease (PD). Microglia in the substantia nigra have been shown to produce proinflammatory cytokines

such as IL-6, TNF-alpha and IL-1beta; the concentrations of these proinflammatory factors are also increased in the striatum [7] and in cerebrospinal fluid (CSF) in PD [8,9], and there are reports of elevated TNF-alpha and IL-6 in patients' plasma [10,11]. Microglia-mediated neuroinflammation is thought to play an important role in the pathogenesis of PD, but it is not clear whether the systemic inflammatory markers in patients represent a cause or a consequence of the ongoing neurodegenerative process.

Currently, it is hypothesized that abnormal proinflammatory cytokine levels in the periphery (e.g., TNF-alpha and IL-6) reflect the presence of a cerebral inflammatory process. The roles of TNF-alpha and IL-6 are particularly well-established in acute inflammation and as mediators of lymphocyte differentiation, including B and T lymphocytes and NK-cells. In line with this, frequencies of NK-cells were reported to be higher in PD patients than controls [12,13]. NK-cells have also been found in human postmortem PD substantia nigra [14] and PD mouse model brains [15]. Potential actions of NK-cells in PD brains include the degradation of α-syn aggregates, support for repairing damaged neurons or glia, the modulation of inflammation by interacting with brain cells and by influencing other leukocytes infiltrating the CNS [16].

Proposed mechanisms responsible for PD pathology include altered expression of NK-cell receptor ligands on neurons and microglia influencing the activation status of the NK-cells [16]. The NK-cell surface receptors NKG2A, NKG2C and NKG2D can also be expressed on T-cells [17,18]. NKG2A expression is related to an effector phenotype and contributes to long-term maintenance of CD8+ T-cells in chronic disease [19]. T-cells activated via NKG2A suppress TNF production and cytotoxic function of CD8+ T-cells [20], whereas NKG2C, NKG2D and CD161 ligation results in increased cytokine production and higher levels of cytotoxic molecules, such as granzyme B and perforin [21]. CD161 expression characterizes T-cells that, after activation, produce greater quantities of proinflammatory cytokines, such as IFN-gamma and IL-17, than their CD161- counterparts [22,23]. The frequencies of NK-cells carrying NKG2A and NKG2D were reported to be different in PD patients relative to the controls in some studies [12,13], while others found lower NKG2A expression in PD and no difference in NKG2D+ NK-cells [12]. NKG2C+ cells and T-cells expressing CD161 have also been implicated in the pathogenesis of another neurodegenerative disease, multiple sclerosis [24]. Although PD and MS are very different diseases, this implies that, in general, NKG2C+ cells can affect the brain; hence, we were encouraged to investigate whether these cells are elevated in PD and possibly contribute to pathology also in this disease. Different T-cell immune signatures in PD have been reported, as, e.g., less late differentiated CD8+ T-cells in PD patients compared to the controls [25].

CD161+CD8+ IL-17-producing T-cells express chemokine receptors such as CCR6 and CCR2 [26]. This provides for further potential associations with diseases of the brain because CCR6 may be related to the homing of T-cells to the brain [27] and to the entry of IL-17-producing cells into the brain in autoimmune encephalomyelitis [28]. Along these lines, IL-17-producing CD4+ Th17 cells have been reported to be significantly increased in PD patients [29]. The increased expression of CCR6 on blood-derived brain cells in a murine model of inflammation in pre-Alzheimer's-like disease is also consistent with this notion [30]. CCR6 expression is also increased in PBMCs of Alzheimer's disease (AD) patients [30–32]. Another chemokine receptor, CCR5, plays a role in BBB transport [33,34]. Its deficiency results in the activation of astrocytes and Aβ deposition [35]. Therefore, it might represent a possible route through the BBB into the brain [35,36]. T-cells expressing the chemokine receptor CXCR3 have been reported to be decreased in PD patients [13]. CXCR3-positive cells have increased cytokine production capacity and can promote the development of cytotoxicity. Migration through endothelial cell layers, as part of the BBB, has been observed in this context [37], especially in an inflammatory environment where activated astrocytes secrete chemokines recruiting peripheral immune cells into the brain [38]. A study focusing on alterations in the levels of chemokines secreted by PBMCs from PD patients showed that these chemokines were more highly expressed relative to

controls, and correlated with the patients' UPDRS III score [39]. In early PD stages, the infiltration of cytotoxic CD8+ T-cells into the substantia nigra has also been reported [40].

In light of the above findings, to investigate the specific activation status of T-cells in a well-characterized and homogeneous cross-sectional cohort focusing on early PD patients, four receptors (NKG2A, NKG2C, NKG2D and CD161) were selected, and to characterize a possible role of chemotaxis, three receptors (CCR5, CCR6 and CXCR3) that have been proposed to play critical roles in PD were chosen. Additionally, the proportion of NK-cells was compared between PD patients and controls according to their maturation stages (CD56highCD16low marking immature NK cells, CD56lowCD16high marking mature, and CD56lowCD16highCD57+ late-stage or terminal).

2. Materials and Methods

2.1. Cohorts

Eighteen PD patients and twenty-four controls were recruited from the outpatient clinic at the Department of Neurodegenerative Diseases of the University of Tübingen, Germany, and clinical data were collated (Table S1). The study was approved by the Ethics Committee of the Medical Faculty of the University of Tübingen (480/2015BO2). All participants provided written informed consent. PD was diagnosed according to the United Kingdom Brain Bank Society Criteria. Control individuals were assessed as having no neurological disease or inflammatory disease and were chosen to be age and sex-matched to the PD patients. PD patients were chosen to represent a homogeneous cohort with very early disease state (blood withdrawal 0–3 years after disease onset, mean 1.89 years) and to have the akinetic-rigid subtype of PD. Plasma was obtained and CMV serostatus determined via an anti-CMV IgG ELISA (Genesis Diagnostics CMV IgG ELISA kit GD84, Omega Diagnostics, Reinbeck, Germany).

2.2. Surface Marker Staining of PBMCs

PBMCs were isolated from EDTA-whole blood by Ficoll-Hypaque (Linaris, Mannheim, Germany) gradient centrifugation. PBMCs were frozen at −80 °C in 10% DMSO (Serva, Heidelberg, Germany) and 20% FBS (Sigma, Pittsburgh, PA, USA) and subsequently stored in liquid nitrogen. After thawing, non-specific antibody binding was blocked with 1% Gamunex (human IgG from Talecris, Clayton, NC, USA). Cells were then stained with ethidium monoazide bromide (Biotium, Fremont, CA, Canada) for live-dead discrimination. Surface marker staining of 2×10^6 PBMCs in PBS (PAA, Pasching, Austria) with 2% (*v*/*v*) FBS (Sigm, Pittsburgh, PA, USA), 2 mM EDTA (Serva, Heidelberg, Germany) and 0.01% (*w*/*v*) NaN3 (Sigma, Pittsburgh, PA, USA) was performed using the antibodies shown in Table S2. Markers were chosen to identify T-cells (CD3+), NK-cells (CD16+CD56+), their activation and differentiation status (CD161, NKG2A, NKG2C, NKG2D, CD57) and their expression of selected chemokine receptors (CCR5, CCR6, CXCR3). Measurements were performed with a BD LSRII flow cytometer and DIVA 6.1 software. To ensure performance quality, control BD CS&T tracking beads and BD 8 peak rainbow beads were applied, and for each measurement run, cells from the same batch of a control donor were included as a standard biological control. To determine frequencies of immune cells and their expression of markers, FlowJo software version 7.2.5 (Treestar) was used with the gating strategy shown in Figures S1–S8.

2.3. Statistics

Statistical analysis was performed using SPSS software. Age and length of PBMC storage were compared between PD patients and controls using the *t*-Test, gender and CMV serostatus using Pearson Chi Square Test. The analysis included the cell phenotypes CD8+/CD8−, CD4+/CD4− and CD56+/CD56− T-cells and CD56highCD16low, CD56lowCD16high and CD56lowCD16highCD57+ NK-cells. Age, gender, length of PBMC storage and CMV serostatus were not significantly different between PD and control samples (Table S1) and were thus not included as covariates. The proportions of cells expressing

the receptors of interest were compared between the groups of PD patients and controls using Mann–Whitney U Tests. For NKG2D, expression was determined as median fluorescence intensity of staining with the conjugated antibody and used for comparison between PD patients and controls.

3. Results

3.1. T-Cell Activation Status

The frequencies of CD8+ T-cells expressing the inhibitory receptor NKG2A in relation to the total % of CD3+ T-cells were significantly higher in PD patients (0.58%) compared to controls (0.35%; $p = 0.021$; Figure 1a), as were the frequencies of those cells expressing the activating receptor NKG2C in relation to % CD3+ cells (0.30%) compared to controls (0.18%, $p = 0.047$; Figure 1b). The latter was also the case for CD8−T-cells (PD patients 0.35%; controls 0.28%; $p = 0.036$; Figure 1c). The density of expression of the activating receptor NKG2D on CD8+ T-cells was higher in patients than in controls as analyzed by median fluorescence intensity (2103-vs.-1894, $p = 0.04$; Figure 1d). The other investigated activating receptor, CD161, showed no statistically significant difference between PD patients and controls when analyzing the median frequencies of positive cells.

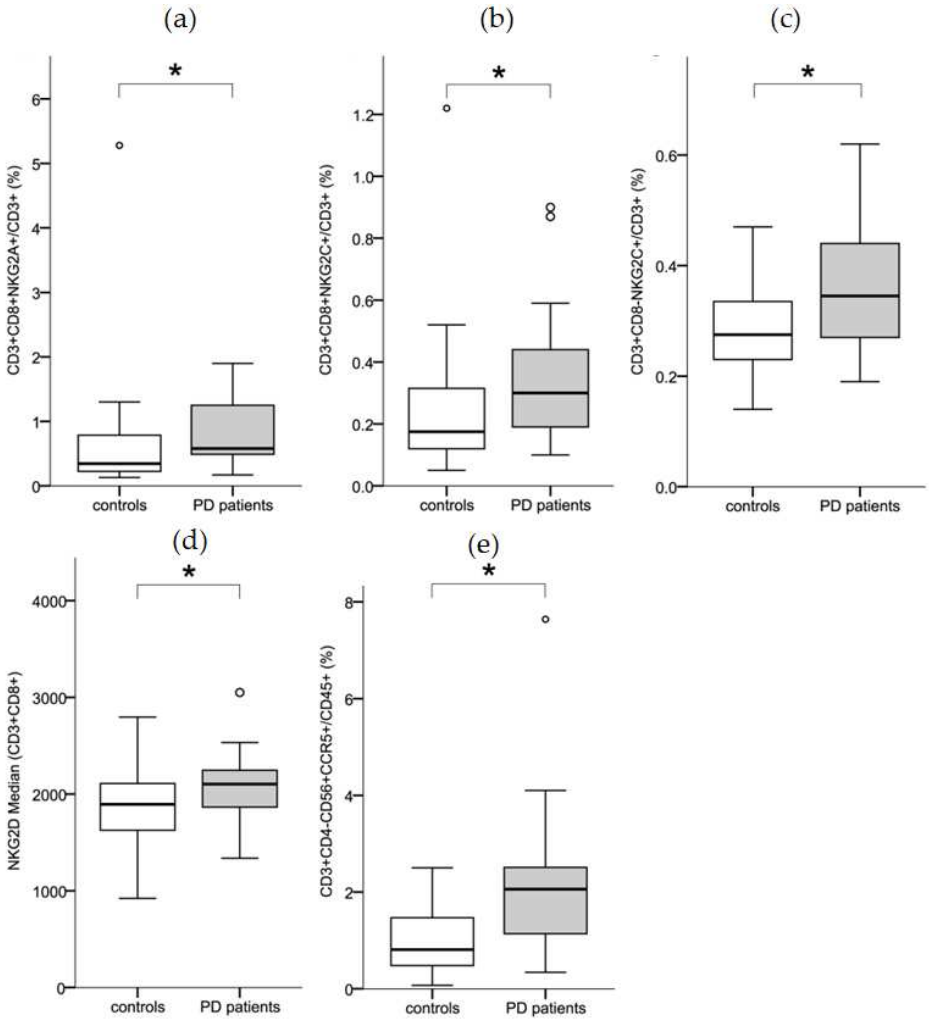

Figure 1. CD8+/− T-cells. Frequencies of CD8+ T-cells expressing the receptors NKG2A (**a**) and NKG2C (**b**) and frequencies of CD8− T-cells expressing the receptor NKG2C (**c**) relative to CD3+ cells. Median fluorescence intensities of receptor NKG2D on CD8+ T-cells (**d**). Frequencies of CD4-CD56+ NKT-cells expressing CCR5 (**e**). *p*-values were calculated using Mann–Whitney U Tests comparing PD patients to controls. Outliers are presented as circles. * $p < 0.05$.

3.2. Chemokine Receptor Expression

Of the three investigated chemokine receptors, the frequencies of T-cells and NKT-cells expressing CCR6 or CXCR3 were not significantly different between PD patients and controls. Only the proportion of CD4-CD56+ NKT-cells expressing CCR5 was found to be higher in PD patients (2.06% in PD patients and 0.81% in controls; $p = 0.013$; Figure 1e), although significance was reduced to a trend when %CD3+CD56− T-cells were used as the reference population instead of %CD45+ cells. Median frequencies of CCR5-expressing CD4+ T-cells did not reveal a difference between PD patients and controls, in neither the activated (CD56+) nor not activated states (CD56−) (Figure S9). Regarding NK-cells, median frequencies of CCR5-expressing mature NK-cells did not differ significantly between PD patients and controls (Table 1).

Table 1. Frequencies of NK-cells in PD and expression of CCR5 and CXCR2.

Markers	Cells	PD Patients % (Median (Range))	Control % (Median (Range))	*p* Values [1]
CD3-CD56+ [2]	Total NK-cells	5.27 (0.51–11.52)	5.42 (2.25–14.54)	0.77
CD3-CD56highCD16low [2]	Immature NK-cells	0.30 (0.06–0.70)	0.24 (0.09–0.98)	0.52
CD3-CD56lowCD16high [2]	Mature NK-cells	4.72 (0.33–10.68)	4.93 (1.55–13.92)	0.67
CD3-CD56lowCD16high CD57+ [2]	Late stage mature NK-cells	2.44 (0.27–5.77)	3.00 (0.71–11.15)	0.20
CD3-CD56lowCD16high CD161+ [2]	CD161+ mature NK-cells	3.89 (0.28–9.01)	4.53 (1.19–12.51)	0.57
CD3-CD56lowCD16high CCR5+ [3]	CCR5 expressing mature NK-cells	0.10 (0.04–1.00)	0.08 (0.02–0.58)	0.44
CD3-CD56lowCD16high CCR5 median [4]	CCR5 expressing mature NK-cells	899 (232–543)	347 (202–541)	0.37
CD3-CD56lowCD16high CXCR2 median [4]	CXCR2 expressing mature NK-cells	1008 (717–1585)	382 (538–1998)	0.15

[1] *p* values were calculated using Mann–Whitney U Tests; [2] analysis based on % of CD45+, [3] analysis based on % of CD3+, [4] analysis based on median fluorescence intensities.

3.3. NK-Cell Differentiation States

No significant differences between PD patients and controls were found for frequencies of either immature NK-cells (CD3-CD56highCD16low), mature NK-cells (CD3-CD56lowCD16high) or late-stage NK-cells (CD3-CD56lowCD16highCD57+) (Table 1).

4. Discussion

4.1. NK-Cell Receptor-Expressing T-Cells in PD Patients vs. Controls

Here, we report a higher proportion of T-cells expressing the activating receptor NKG2C and a higher density of expression of the activating receptor NKG2D on circulating lymphocytes from PD patients relative to age- and sex-matched controls. However, a higher frequency of T-cells expressing the inhibitory receptor NKG2A was also present. These observations suggest that an imbalance in the activation states has occurred. NKG2A expression is enhanced by anti-inflammatory cytokines [41] possibly secreted to restore inflammatory equilibrium after viral infection. In PD, NKG2A-expressing T-cells might play a similar role, i.e., to dampen inflammation. NKG2C was previously described as an alternative activating receptor promoting proliferation and effector functions of cytotoxic T-cells [42]. Its expression (as well as that of other NKG2 receptors) has been reported to be elevated in conditions of CMV infection, of ageing or of chronic immune stimulation during disease [43]. Once they infiltrate the brain, cytotoxic NKG2C+ CD4+ T-cells can contribute to tissue damage, as shown by the example of oligodendrocyte damage observed in studies on multiple sclerosis [21]. These cells co-expressed CD56 and NKG2D receptors and produced more cytotoxic molecules (FasL, granzyme B and perforin) than NKG2C-negative T-cells [21]. In a mouse PD model, cytotoxic CD4+ T-cells contributed to neurodegeneration via the Fas/FasL pathway [44]. The dysregulation of NKG2D was proposed to lead to autoreactive T cell stimulation and to promote a self-perpetuating pathology in autoimmune disease [45]. Both NKG2C and NKG2D are linked to a cytotoxic pathway, and our study indicates that they might be markers of an inflammatory state in PD pathology that originates from the periphery.

4.2. Chemokine Receptor Expression on T-Cells

Inflammation at the BBB, e.g., induced by proinflammatory cytokines secreted by microglia [39], can result in structural changes at cell–cell junctions and the upregulated expression of adhesion molecules. This leads to increased leukocyte transmigration [46]. Chemokines are important for crossing the BBB—one such is CCL5 (also known as RANTES). It is one of the many inflammatory mediators secreted by activated microglia, astrocytes, neurons, T-cells and mast cells in the context of neurodegenerative diseases [47]. CCL5 was found at higher levels in PD patients compared to a control group [48] and was even higher in PD patients treated with levodopa [49]. In addition, serum CCL5 levels correlated with the UPDRS III score [48], the Hoehn–Yahr score and disease duration [50]. Reale et al. postulated that the "systemic presence of CCL5 in PD may reflect both a central and/or peripheral source of activation" [51]. The corresponding receptor CCR5 is a major driver of brain infiltration by T-cells via the BBB. It is expressed by T-cells, which were demonstrated to cross an in vitro BBB model [34]. Furthermore, these CCR5-expressing cells have been found in MS lesions [34], and CCR5 and CCL5 expression was increased upon stimulation with Aβ, a marker for Alzheimer pathology [51,52]. Similarly, in PD, alpha-synuclein might be able to induce CCR5 expression, but this remains speculative. A recent study found CCR5 enrichment among transcriptomic signatures previously associated with PD [53]. In addition, work on a mouse model of PD revealed that stimulation of TH17 cells with alpha-synuclein could cause neuronal cell death in the substantia nigra (SN) [54]. More research would be needed to determine whether CD4+ TH17 cells exhibit increased chemokine receptor expression. As a possible indication of increased migration of peripheral cells, we found higher frequencies of T-cells expressing the chemokine receptor CCR5 (CD56+CD4-CCR5+ T-cells) in PD patients than in controls. Brain-infiltrating T-cells may induce excessive microglial inflammation and oxidative stress, promoting further neurodegeneration in PD [55]. Additionally, CCR5 expression correlates with inflammation mediated by type 1 T helper cells (TH1) [56,57], which mainly produce proinflammatory cytokines and support macrophages and cytotoxic T-cells, whereas Type 2 (TH2) cells primarily produce anti-inflammatory cytokines and induce humoral responses. In chronic inflammation, the fine balance of the TH1 and TH2 system may be shifted towards the TH1 pathway, and NK-cells and T-cells may be activated. This shift towards the TH1 pathway might be a general finding in PD. Interestingly, an available CCR5 antagonist has already been proposed as a possible drug for neuroinflammatory diseases, with a suggested mechanism of action focusing on hindering the migration of CCR5+ cells into the CNS [58]. In this context, limiting the impact of the intervention to CCR5 on peripheral cells would need to be considered because of the possibility that bidirectional interactions between neurons and glial cells via CCR5 in the brain may be beneficial for dopaminergic neuron survival [59]. These findings parallel the observation of elevated frequencies and surface levels of migration markers CCR2 and CD11b on classical and intermediate monocytes in early Parkinson's disease, which correlate with worse cognition [60]. Thus, the current study provides evidence that peripheral activation of inflammatory processes could potentially cause cerebral cell death via transendothelial activation of cerebral inflammatory responses. As our data show the imbalance in the activation of T-cells in the periphery, further data are needed to confirm their direct intracerebral effects.

4.3. Observations on NK-Cells

We also investigated the NK-cell frequencies, but we did not find any significant differences between PD patients and controls. Our results stand in contrast to three previous studies [12,13,61] that found elevated frequencies of NK-cells in PD patients. These studies found a positive correlation between disease duration and NK-cell frequency or activity. Compared to these cohorts of PD patients, our cohort was in a very early disease stage, since they were recruited only up to 3 years after disease onset (mean 1.89 years), which is in line with the hypothesis that relevant immune changes occur early on, are possibly causative, but are less pronounced at later stages [62]. Hence, if the frequency of NK-cells is

similar in PD patients and controls in early disease stage and increases, and then decreases again with disease progression, the potential of this parameter in serving as a dynamic marker of progression could be of value in future studies. Consistent with this notion, a different study found a significant elevation of NK-cell frequencies in late-onset PD patients compared to healthy controls, but this did not reach significance when comparing early onset PD patients to controls [63].

5. Conclusions

The specific pattern of peripherally activated T-cells in the blood of early PD patients, as detected in this study, suggests a mechanism contributing to neuronal degeneration in PD that could potentially be included in a panel of markers for early disease diagnosis. The present data confirm the complexity of inflammatory processes in PD. Further studies on longitudinal samples might aid in better deciphering these processes. Parallel with disease severity, further studies should also consider the influence of potential confounders such as ethnic variations, as discussed recently [63].

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/jal3010001/s1, Figure S1. Gating strategy for the populations of panel 1 part 1. Figure S2. Gating strategy for the populations of panel 1 part 2. Figure S3. Gating strategy for the populations of panel 1 controls. Figure S4. Gating strategy for the populations of panel 1 controls. Figure S5. Gating strategy for the populations of panel 2 part 1. Figure S6. Gating strategy for the populations of panel 2 part 2. Figure S7. Gating strategy for the populations of panel 2 part 3. Figure S8. Gating strategy for the populations of panel 2 part 4. Figure S9. Frequencies of chemokine receptor-expressing CD4+ T-cells (a) and CD4+ NKT-cells (b) among CD45+ cells displayed as Tukey plots Mann–Whitney U Tests comparing PD patients (PD) and controls (C) did not reveal any significant differences. Table S1. Cohort Summary. Table S2. Monoclonal antibodies employed.

Author Contributions: Conceptualization, D.G., G.P., A.N.d.C., D.S. and W.M.; methodology, L.Ö., D.G. and M.C.T.d.S.; formal analysis, L.Ö. and C.S.; investigation, L.Ö., C.D. and D.G.; resources, C.D. and W.M.; data curation, L.Ö., D.G., C.S., C.D. and W.M.; writing—original draft preparation, C.S. and D.G.; writing—review and editing, M.C.T.d.S., G.P., D.S., W.M., D.B. and A.N.d.C.; visualization, C.S.; supervision, G.P. and W.M.; project administration, D.B., A.N.d.C., D.S. and W.M.; funding acquisition, G.P., A.N.d.C. and W.M. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by grants from the German Federal Ministry for Education and Research (BMBF) within the BioPharma initiative Neuroallianz, project D13 B (grants 16GW0066K and 16GW0067) and by UCB Pharma GmbH (Monheim, Germany). We acknowledge support by Deutsche Forschungsgemeinschaft.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Medical Faculty of the University of Tübingen (480/2015BO2).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available from the corresponding author upon request.

Acknowledgments: Samples were obtained from the Neuro-Biobank of the University of Tübingen, Germany. This biobank is supported by the local University, the Hertie Institute and the DZNE.

Conflicts of Interest: The authors declare no conflict of interest.

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