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PERIPHERAL IMMUNITY IN PARKINSON'S DISEASE

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ABSTRACT

Parkinson's disease (PD) is the second most common neurodegenerative disorder among elderly population, characterized by the progressive degeneration of dopaminergic neurons in the midbrain. Major hallmarks of PD are loss of dopaminergic neurons in substantia nigra pars compacta, occlusion of α -synuclein (α -syn)-rich intraneuronal inclusions (Lewy bodies, LB), and microglial activation.

To date, exact mechanism(s) of PD occurrence and pathogenesis remains unknown. Numerous evidences of the principal role of neuroinflammation in progression of PD have been accumulated in several past years. Neuroinflammation is characterized by presence of activated microglia in brain and elevated levels of pro-inflammatory cytokines in CNS. Recently, active participation of peripheral immune system (in a view of elevated levels of cytokines in blood, the presence of auto antibodies, and the infiltration of T cell in CNS) has been proposed. So far reported evidence suggests that CD4⁺ T lymphocytes contribute to neuroinflammation in PD. Moreover, infiltration and reactivation of pro-inflammatory CD4⁺ T cells in CNS could exacerbate neuroinflammation to greater levels since they are able to prime microglia into pro-inflammatory phenotype, which can trigger stronger response in CNS further perpetuating the on-going neurodegenerative process.

1. GENERAL INTRODUCTION

Parkinson's disease (PD) is second most common neurodegenerative disorder after Alzheimer's disease (AD) affecting 7-10 million people worldwide (Tysnes & Storstein, 2017) thus imposing an increasing social and economic burden on societies with increased aging of populations. Classical pathological hallmarks of PD are: loss of dopamine (DA)-producing neurons in the substantia nigra pars compacta (SN) of the midbrain (Samii et al., 2004; Gelb et al., 1999) and occurrence of intracellular inclusions called Lewy body (LB). LB are spherical, eosinophilic structures with a central, granular core surrounded by a fibrillary halo and positive for the protein α -synuclein (α -syn) (Jellinger, 2002).

PD is characterized by classic motor symptoms, including tremor at rest, bradykinesia, rigidity, and postural instability. In addition, dysfunction of the autonomic nervous system, depression and cognitive impairment occur as disease progresses (Boland & Stacy, 2012; Kowal et al., 2013; Johnson et al., 2013). It is generally accepted that both genetic and environmental factors contribute to the development of PD.

The potential contribution of peripheral immunity in neuroinflammation and neurodegeneration raised increasing interest only during the last decade (Mosley et al., 2012; Cappellano et al., 2013; González et al., 2014). In contrast to the wealth of studies on neuroinflammatory processes in the CNS of PD patients (reviewed in Hirsch & Hunot, 2009; Sanchez-Guajardo et al., 2013), the specific role of the peripheral immune system during PD is still weakly understood.

Neuroinflammation is nowadays recognized as one of the crucial mechanisms in the pathogenesis of several neurodegenerative disorders including PD, regardless of the initial triggering factor. It has been shown that both innate and adaptive immune response participate influencing the process of neuroinflammation to better or worse. More importantly it is now clear that participation of the immune system in those cases is not restricted to CNS only but involves changes also in periphery.

So far it has been shown that T cells indeed can be found in the SN of parkinsonian brains (Brochard et al., 2009). Both CD8⁺ and CD4⁺ T cells occur in postmortem brain specimens from PD patients as well as in the 1-methyl-4-phenyl-

1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD, and evidence from the mouse model indicates that CD4⁺ T cells determine T cell-mediated dopaminergic cell death (Brochard et al., 2009). Furthermore, beside the fact that T cells infiltrate CNS of PD patients, decreased numbers of CD3⁺ and CD4⁺ T lymphocytes have been consistently reported in peripheral blood of PD patients (Jiang et al., 2017). The most prominent change observed was reduced population of CD4⁺ T cells, specifically T naïve cells (Bas et al., 2001). In addition, lower ratio of CD4⁺ and CD8⁺ T cell and increased ratio of IFN- γ to IL-4-producing T cells in PD patients (Baba et al., 2005) have been reported, as well as decrease in the numbers of CD4⁺CD29⁺ cells and memory T cells (Stevens et al., 2012), decreased percentage of naïve and increased percentage of memory T cell in PD patients and increased percentages of Fas⁺ cells and decreased percentage of CD31⁺ cells (Hutter Saunders et al., 2012). All above mentioned data are clearly suggesting the presence of alterations of the adaptive immune system in peripheral blood of PD patients.

The possible involvement of the peripheral immune system in neurodegeneration and/or neuroprotection deserves much attention also in consideration of recent studies supporting the immunomodulating functions of dopamine, which, in addition to its role as central neurotransmitter, is an important molecule bridging the nervous and immune systems (Levite, 2012). It has the ability to affects both innate and adaptive immune cells, since virtually all immune cells produce DA, which may act as autocrine/paracrine mediator (Cosentino et al., 2000; Cosentino et al., 2002a; Cosentino et al., 2002b; Cosentino et al., 2005). The effects of DA are exerted through 5 different dopaminergic receptors (DR) grouped into the D1-like (D1 and D5) and the D2-like (D2, D3 and D4) (Beaulieu & Gainetdinov, 2011). CD4⁺ T cells express both D1-like and D2-like DR (Cosentino et al., 2007), with CD4⁺ naïve T cells expressing more D1-like DR while memory T cells express more D2-like DR (Kustrimovic et al., 2014).

No disease-modifying therapies are currently available for PD; symptomatic treatments rely mainly on the administration of dopamine precursor L-DOPA, dopaminergic agonists and other indirect dopaminergic agents. The emerging contribution of peripheral nervous system and central neuroinflammation are increasingly pointing to immunomodulation as a neuroprotective and therapeutic strategy for PD (Olson and Gendelman, 2015).

Having in mind all above mentioned the general aim of this thesis was to assess all the alterations (phenotypical and functional) of various subsets of CD4⁺ T cells in peripheral blood of PD patients and sex- and age-matched controls.

The program was developed firstly through the extensive reading and studying of available literature regarding observed alterations in peripheral immune response in PD pathology, with specific regard to the CD4⁺ T cells hence giving us the possibility to accurately plan the future experiments.

Peripheral venous blood samples were collected from patients with PD, that were drug-naïve, meaning that they have never received any antiparkinson drugs or on antiparkinson drug treatment, and from age- and sex-matched healthy subjects. Participants were recruited at the Movement Disorders Center of the University of Piemonte Orientale, Divisione di Neurologia, Ospedale Maggiore of Novara, at the Interdepartmental Research Center for Parkinson's Disease of the Neurological Institute "C. Mondino" of Pavia, and at the Centre for Parkinson's Disease and Movement Disorders of the Neurological Service at the Ospedale di Circolo of Varese, Italy. HS were spouses and caregivers of enrolled PD patients.

The Ethics Committees of Ospedale di Circolo of Varese and Neurological Institute "C. Mondino" of Pavia approved the protocol and all the participants signed a written informed consent before enrolment. The study was performed according to the Declaration of Helsinki and to the relevant ethical guidelines for research on humans. We have pursued two pathways in analysis of any potential change regarding CD4⁺ T cells. Firstly, we were focused on examination (both phenotypical and functional) of naïve/memory subset of CD4⁺ T cells and later on we continued with further research of changes in pool of effector T cells (T helper, Th1, Th2 and Th17) and regulatory cells. In order to successfully examine potential changes in function of various subsets of CD4⁺ T cells we have developed and validated several *in vitro* methods.

Obtained results of this thesis have been published in two peer review journals. In addition, in-depth overview of existing literature regarding the involvement of peripheral immune system in pathogenesis of PD have been submitted for publication in peer review journal.

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2. Peripheral Immunity, Immunoaging and Neuroinflammation in Parkinson's Disease

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This manuscript has been submitted to be considered for publication by an international peer reviewed journal and it is presently under evaluation.

2.1. OVERVIEW

In the present review was extensively discussed the fine interplay between neuroinflammation and the peripheral immune response in the pathobiology of PD. First of all, after brief introduction to the pathology of PD a summary of neuroinflammation and its main characteristics (such as regulation of microglial activation) in PD were specified and discussed. Afterwards, changes in peripheral immune systems (both innate and adaptive) that are so far reported were listed and discussed. In the end, some of proposed immunotherapies for treatment of PD were enumerated.

Neurodegenerative disorders are more and more present in the general population due to population ageing. On-going debate is whether an "aged" immune response is not able to handle with age-related stress, thus leading to occurrence of neurodegenerative diseases, or whether during ageing immune and inflammatory pathways become hyper-activated, hence promoting neurodegeneration.

2.2. MANUSCRIPT

Peripheral Immunity, Immunoaging and Neuroinflammation in Parkinson's Disease

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Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disorder among elderly population, and it is depicted by the degeneration of dopaminergic neurons in the midbrain which is progressive. Up to the present time, the precise cause remains ill-defined and the mechanism of neurons death indeterminate. It is typically considered as a disease of central nervous system (CNS). Nevertheless, numerous evidences have been accumulated in several past years testifying undoubtedly about the principal role of neuroinflammation in progression of PD.

Neuroinflammation is associated with presence of activated microglia in brain and elevated levels of cytokine levels in CNS.

Nevertheless, active participation of immune system as well has been noted, such as, elevated levels of cytokines in peripheral blood, as well as, the presence of auto antibodies. In addition, the infiltration of T cell in CNS has been reported. Moreover, infiltration and reactivation of those T cells could exacerbate neuroinflammation to greater neurotoxic levels. Hence, peripheral inflammation is able to prime microglia into pro-inflammatory phenotype, which can trigger stronger response in CNS further perpetuating the on-going neurodegenerative process.

In the present review, the interplay between neuroinflammation and the peripheral immune response in the pathobiology of PD will be discussed.

First of all, an overview of regulation of microglial activation and neuroinflammation is summarized and discussed. Afterwards, we try to collectively analyze changes that occurs in peripheral immune system of PD patients and trying to link them to the potential exacerbation of the neuroinflammation and hence the symptoms of the disease. In the end, we summarize some of proposed immunotherapies for treatment of PD.

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Key words (6-8): Parkinson's disease, neuroinflammation, peripheral immunity, alpha-synuclein, immunoaging, immunomodulation.

1. INTRODUCTION

Neuroinflammation is increasingly considered a pivotal mechanism in the pathogenesis of several neurodegenerative disorders including Alzheimer's disease (AD) [1], Parkinson's disease (PD) [2], amyotrophic lateral sclerosis (ALS) [3] and multiple sclerosis (MS) [4].

In all these disorders, no matter what the initial causing factor may be both, the innate and adaptive immune systems contribute, leading to neuronal death. Participation of the immune system in neurodegenerative diseases is not limited to the certain brain sites but also includes changes also in peripheral immune system. Nonetheless, it is still unclear if changes occurring in the peripheral immune system are consequences to the process of cell degeneration in the central nervous system (CNS), or if they play a causal role in neurodegeneration. Due to population ageing, neurodegenerative disorders which increase with age are becoming more frequent [5]. Ageing is also associated with changes in peripheral immunity, and it is still a matter of debate whether an "aged" immune response may fail to cope with age-related stress, thus contributing to development of neurodegenerative diseases, or whether during ageing immune and inflammatory pathways become hyper-activated, hence promoting neurodegeneration. So far, what is common for various neurodegenerative disease is the deficiency of successful therapies that are focused on the cause of the disease rather on the symptoms. In that matter the development of innovative approaches is now mandatory. The immune system might represent a potential target for novel therapeutics, provided that its contribution to neuroinflammation and neurodegeneration will be clarified.

2. PARKINSON'S DISEASE

PD is second most common neurodegenerative disorder after AD affecting 7-10 million people worldwide [6]. Classical pathological hallmarks of PD are: loss of dopamine (DA)-producing neurons in the substantia nigra pars compacta (SN) of the midbrain [7,8,9] and occurrence of intracellular inclusions called Lewy body (LB). LB are sphere-shaped structures rich with the protein α -synuclein (α -syn) [9]. PD is characterized by classic motor symptoms, including tremor at rest, bradykinesia, rigidity, and postural instability [9]. In addition, dysfunction of the autonomic nervous system, depression and cognitive impairment occur as disease progresses [10]. The etiology of sporadic PD is difficult to determine, since when clinical manifestation appear most of dopaminergic neurons have already been lost, usually more than 70% [11].

So far has been confirmed that genetic and environmental factors contribute to the development of PD. Treatments currently available for PD remain predominantly symptomatic, and the search continues for therapeutics that target disease mechanism, thus resulting in PD prevention, slowing of disease progression, and even rescue of damaged neurons. At present, the mainstay of PD treatment is based on DA replacement, by using L-3,4- dihydroxyphenylalanine (L-DOPA), the direct precursor of DA, some dopaminergic agonists as well as other indirect dopaminergic agents [12].

NEUROINFLAMMATION IN PARKINSON'S DISEASE

The typical features of neuroinflammation are activated microglia, reactive astrocytes in CNS parenchyma and the release of inflammatory mediators such as cytokines, chemokines, complement cascade proteins, prostaglandins, nitric oxide (NO), reactive oxygen species (ROS) and reactive nitrogen species (RNS). All of these factors can disrupt the integrity of the blood brain barrier (BBB) thus allowing the entry of adaptive immune cells into the CNS [13]. One of the main features of neuroinflammatory processes in PD is a significant increase in the levels of pro-inflammatory cytokines in the striatum and cerebrospinal fluid (CSF) of PD patients in comparison to healthy subjects (reviewed in [14]), including tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6, IL-2, and several growth factors (e.g., epidermal growth factor (EGF), tumor growth factor (TGF)- β , basic fibroblast growth factor (bFGF), tumor growth factor (TGF)- β 1). Increased levels of cytokines were observed only in

nigrostriatal pathway and not in cortical regions, proposing that cytokine production may be restrained to specific sites of the brain [15, 16]. McGeer and colleagues [17] were among the first detecting human leukocyte antigen DR (HLA-DR)-positive (activated) microglia, particularly in the close proximity of the dying neurons in post-mortem examination of SN obtained from PD patients [17], and since then activated microglia has been consistently observed in PD patients. In addition, increased level of $\beta 2$ -microglobulin, the light chain of major histocompatibility complex (MHC) class I, has been found in the striatum of PD patients [18].

Activated microglia produces various pro-inflammatory mediators such as cytokines, chemokines, NO, RNS and ROS. Long lasting or disproportionate microglial activation may result in pathological forms of inflammation that are capable of exacerbation of the neurodegenerative diseases [19]. On the other hand, microglia secrete neurotrophic factors as well, such as insulin-like growth factor 1 (IGF1), brain-derived neurotrophic factor (BDNF), transforming growth factor- β (TGF- β) and nerve growth factor (NGF) [20]. Microglia mediates the immune response triggered by pathogen-associated molecular patterns (PAMPs) by modulating Toll like receptors (TLRs), scavenger receptors, phagocytosis and complement-mediated responses. Microglial cells express all nine TLRs [21], and such expression is dynamically regulated [22]. Activation of TLR1 induces IL-6 and IL-1 secretion, TLR2 is highly expressed on activated microglia, while activation of TLR3 is considered as the strongest activation stimulus leading to TNF- α production. Expression of TLR4 is low and can be increased by some stimuli such as MPTP or LPS. TLR4 stimulates inducible nitric oxide synthase (iNOS), IL-1, IL-6, IL-12 and IFN- α and β production [23]. The exact role of TLRs in PD still awaits its clarification and confirmation, but the present data propose potential influence in α -syn clearance [24].

In addition, microglia can be activated by: (i) complement [24, 25] (ii) monocyte chemoattractant protein-1 (CCL2), secreted by dopaminergic neurons which interacts with its receptor CCR2 expressed on microglia [26]; (iii) C-reactive protein; (iv) chromogranin A [27]; (v) matrix metalloproteinase-3 and (vi) aggregated α -syn, which is liberated from stressed and/or dying neurons [28]. Additionally, overexpression of α -syn or presence abnormal forms of α -syn can activate microglial cells. Abnormal posttranslational modifications of α -syn, such as nitration, oxidation and phosphorylation, can be found in LB inclusions present in the brains of PD patients

[29] and cause the protein to aggregate [30] and activates microglia [28]. Activated microglia produces NO and iNOS, which will cause increased nitration of α -syn, perpetuating the ongoing neuroinflammation [31]. Proinflammatory and neurotoxic cytokines and chemokines produced by activated microglia may also result in disruption of the BBB and the attraction of lymphocytes to the site of neuronal injury. Taken together, these data support the hypothesis that activation of microglial cells, directly contribute to the pathobiology of PD. All so far published evidences of neuroinflammatory processes in the brains of PD patients are listed in **Table 1**.

Table 1. Evidences of neuroinflammatory process in the brains of PD patients.

			REFERENCES
Post-mortem studies	Substantia nigra	Expression of HLA-DR, HLA-DP, HLA-DQ and CD68 on microglia CD68	[17, 32, 33]
		Augmented levels of CD23, IL-1 β , IFN- γ , TNF- α	[15, 34, 35]
		Increased expression of iNOS, COX-2	[35, 36]
	Straitum	Increased levels of IL-1 β , IL-2, IL-6, IFN- γ , EGF, TNF- α , TGF- α , TGF- β 1,	[18, 37, 38, 39]
In vivo studies	Serum	Increase concentrations of IL-2, TNF- α and IL-6, RANTES	[40, 41, 42]
		Presence of antibodies against DA-oxidized proteins	[43]
	CSF	Increase in IL-1 β , IL-6 and TNF- α	[37, 44]

3.1. Pathways involved in neuroinflammation and neuronal death

Major source of the oxidative stress, that is believed to be principally responsible for the death of DA neurons, superoxide radicals produced by activated microglia

Hirsch & Hunot [45] provided one of the most plausible hypotheses about the connections between neuroinflammation and oxidative stress involving several enzymatic systems that results in creating deadly oxidative environment (**Figure 1**). In the normal CNS, expression of iNOS is not detectable and NADPH-oxidase is dormant, but in patients with PD, both iNOS and NADPH-oxidase are noticeably expressed and active in microglia leading to high production of NO free radicals [46, 47]. Confirmation of pathogenic role in PD of two inflammatory enzymes, iNOS and NADPH-oxidase, came from animal studies involving mutant mice, lacking those enzymes, where the loss of dopaminergic neurons after MPTP administration was reduced [46, 47]. For sure one of the most important mediators of neuroinflammation in PD are pro-inflammatory cytokines like IL-1 β , IFN- γ and TNF- α .

So far two mechanisms have been proposed for their involvement in neurotoxicity. The first one is a direct mechanism through receptor binding expressed on dopaminergic neurons, resulting in activation of death pathways, while the second one is an indirect mechanism involving microglial activation (**Figure 2**). Direct mechanism of cytokine-induced death of dopaminergic neurons are the consequence of activation of cytokine receptors, such as the TNF- α receptors (TNF- R), expressed by neurons and coupled to intracellular death-related signaling pathways (e.g. JNK, NF κ B, and p38 MAP kinase pathways). Findings like ability of microglia to produces TNF- α , expression of TNF-R like p55 TNF-RI and p75 TNF-RII on the surface of nigral dopaminergic neurons [48], and increased expression of TNF- RI in PD patients [49] just add to the confirmation of neurotoxicity of this pro- inflammatory cytokine. TNF- α released from activated microglia binds to the TNF-RI hence leading to the activation of a cascade of cysteine proteases (caspases) which are key players in initiating the apoptosis process in mammalian cells [50]. In addition, Fas ligand, a cell-surface molecule expressed on activated T cells, can be involved in cell death. Namely, Fas ligand can activate Fas receptor expressed on astrocytes resulting in secretion of several pro-inflammatory cytokines and chemokines, including IL-6, IL-8, and

CCL2/MCP1 [51], or it can activate Fas receptor expressed on microglial cells thus inducing secretion of pro-inflammatory cytokines, IL-1 β , IFN- γ and TNF- α [45]. Most importantly Fas ligand can induce apoptosis of the cells that are expressing Fas receptor, such as the case of the dopaminergic neurons [45]. The harmful consequence of Fas–Fas ligand pathway activation in PD has only recently been recognized and still awaits clarification since it is unclear whether this pathway is per se toxic for dopaminergic neurons or leads to indirect toxicity by stimulation of the production of glial-derived inflammatory factors.

In the addition, pro-inflammatory cytokines are strong regulators of the respiratory burst apparatus, since it has been confirmed that IFN- γ and TNF- α are strong inducers of iNOS and NADPH-oxidase in macrophages and microglial cells of rodents. Interestingly, induction of iNOS expression in human microglial cells using pro-inflammatory cytokines like IL-1 β , IFN- γ and TNF- α per se was not successful [52], indicating existence of another mechanisms of activation of iNOS in humans. It has been proposed that this alternative mechanism involves cytokine-stimulated expression of the low-affinity IgE receptor CD23. Activation of CD23 leads to induced expression of iNOS and subsequent high release of NO [45]. Increase expression of CD23 was observed in glial cells in the SN of PD patients, and not in control subjects. CD23-dependent iNOS expression in human glial cells is strongly potentiated by the combined action with the pro-inflammatory cytokines [45].

The use of non-steroidal anti-inflammatory drugs (NSAIDs) decrease the risk of developing PD as shown by some epidemiological studies have [53]. Exactly those studies have provided a key motivation to the concept that inflammation in PD influence the neurodegenerative process, stimulating the progression and spread of the disease.

Nowadays, neuroinflammation has been recognized as an essential feature of PD. However, the origin neuroinflammatory process still awaits clarification. Notwithstanding the advancement made so far, remains the question: Is neuroinflammation a meek consequence of neuronal alterations and death of neurons or neuro-inflammatory processes can be a main reason of the disease.

4. PERIPHERAL IMMUNE SYSTEM IN PARKINSON'S DISEASE

The potential contribution of peripheral immunity in neuroinflammation and neurodegeneration raised increasing interest only during the last decade [54, 55, 56]. In contrast to the abundance of studies available on neuroinflammatory processes that occur in the CNS during the progression of PD (reviewed [45, 57]), the specific role of the peripheral immune system during PD is still weakly understood. Indeed, studying the role of peripheral immunity in PD is difficult, further complication arising from the complex interrelation among PD, aging and immunity. Aging has a major influence on peripheral immunity, and the terms "immunoaging" or "immunosenescence" have been created to describe the complex changes occurring with age in the innate and adaptive immune response [58]. When studying peripheral immunity on PD specific attention must be exerted in dissecting the immune changes attributable to the disease from those associated with aging. On the other side, it cannot be excluded that immunoaging itself can play a role contributing to the pathogenesis and/or development of PD. Actually, the mutual influence between PD, aging and immunity is still an unresolved issue.

4.1. Peripheral innate immunity in PD

4.1.1. Dendritic cells

In PD, very few studies of DC frequency are available. Ciaramella et al., [59] examined the frequency of circulating myeloid DC (mDC) and plasmacytoid DC (pDC) in PD patients. pDC differentiate into typical DC and produce type I interferon, while mDC migrate in inflamed tissues and in lymph nodes, and recognize blood borne pathogens [60]. It has been found mild decrease of percentage of pDC, while the number and the percentage of mDC were more profoundly reduced in PD patients [59]. More recently, the increased frequency of CD16+ DC, which produce pro-inflammatory cytokines, were found in PD patients that were previously infected with cytomegalovirus (CMV) [61]. In addition, the frequency of CD16-, CD141+ and CD1c+ mDCs were found decreased in PD patients compared to controls, (only in CMV-seropositive people) [62]. Recently was proposed that in PD a certain percentage of peripheral DC may reach the brain, at the levels of choroid plexus or meninges, where they may encounter various antigens and contribute to the neuroinflammatory process [63]. Additional studies are needed in order to clarify whether such DC subserve

antigen presentation locally, to CD4+ T cells that have already entered the brain, or they can also migrate back to peripheral tissues reaching nearby draining lymph nodes (such as the cervical lymph nodes, CLN) and present brain antigens to peripheral T cells [64].

4.1.2. Monocytes

Neuromelanin and α -syn are examples of DAMP that strongly activate these cells by engaging TLR [65] hence these molecules can be strong activators of peripheral monocytes and macrophages as well, especially α -syn, which occurs also in peripheral tissues and may be increased in PD patients [66]. Recently, it has been demonstrated that α -syn is able to induce activation of TLR2 expressed on human monocytes, resulting in increased production of IL-1 β [67]. Grozdanov et al., [68] showed that monocytes obtained from peripheral blood of PD patients have a pro-inflammatory profile. The augmented number of classical monocytes and decreased number of the non-classical monocytes in the blood of PD can be result of an increased differentiation of monocytes or increased migration from the bone marrow. Indeed, elevated level of CCL2 have been found in the serum of PD patients, which can be a signal for recruitment of bone marrow monocytes [69]. In addition, upregulation of expression of CCL2 receptor on classical monocytes isolated from PD patients was confirmed [70]. Recent study on monocytes isolated from peripheral blood cells in PD patients showed that lower production of cytokines in response to TLR2/Pam 3 Cys (IL-1 β , IL-6, IL-10 and TNF- α ,) and TLR7/8/R848 (IL-6, TNF- α ,) activation, and no modification in the percentage of resident or inflammatory monocytes and their TLR2 and TLR4 expression in PD patients [71].

4.1.3. Macrophages

Studies that are dealing with potential alterations in number and/ or function of macrophages in peripheral blood of PD patients are almost non-existent which is difficult to believe especially having in mind novel strategies of potential treatment of PD based on cell-mediated transfer of catalase nanoparticles from macrophages to brain [72] and macrophage-mediated delivery of neurotrophic factors [73, 74]. Nowadays, potentially effective approach includes cell-carriers. Those cell-carriers would be genetically modified in order to produce therapeutically active molecules,

such as neurturin (NTN), neurotrophic factors, GDNF, or vascular endothelial growth factor (VEGF) [73, 75]. It has been shown that delivery of GDNF by macrophages-led to dramatic reduction of inflammation and neuronal degeneration in SN [73].

4.1.4. Natural killer cells

So far has been confirmed increased percentage of NK cells in PD patients with no changes in activity of NK cells in PD patients and healthy controls [76, 77]. In addition, increase in NK cell number correlated with the severity of disease. In addition, it has been confirmed increased expression of receptor NKG2A in PD patients [76, 77]. Conversely, depending on the study, no modification [106] or an increased [77] expression of NKG2D on NK cells was found. Furthermore, NKCA was positively correlated with PD severity [76].

4.1.5. Granulocytes

In the studies performed three decades ago circulating neutrophils were used for the examination of the function of NO in PD [78]. It was confirmed that production of NO by activated peripheral neutrophils was about 60% higher in PD patients both newly diagnosed and treated patients with PD [79]. In the neutrophils obtained from PD patients an overexpression of the nNOS gene was recorded, [80, 81]. Although obtained results are regarding the periphery authors however speculate that they can partially reflect the events occurring in the CNS. Furthermore, Gatto et al., [81] have found augmented phorbol-12-myristate-13-acetate (PMA) activation of the H₂O₂ release of neutrophils in drug treated PD patients. In addition, plasma samples that were obtained from drug naïve or drug treated PD patients increased the release of NO by PMA-stimulated neutrophils of control subjects but did not affect the production of H₂O₂. Furthermore, impairment in the phagocytic capacity of latex particles of neutrophils in PD patients, both treated and untreated, was demonstrated [82].

Major changes that occurs in peripheral innate immune response in patients with PD are listed in the **Table 2**.

Table 2. Changes in peripheral innate immunity during PD.

	CHANGES IN PD	REFERENCE
Granulocytes	- Increased production of NO by activated peripheral neutrophils - Augmented H₂O₂ release after PMA activation of the neutrophils	[79]
	Decreased capacity of phagocytosis of latex particles	[82]
Monocytes and macrophages	Hyperactive in response to LPS stimulation, correlated with disease severity	[68]
	Upregulation of CCL2 receptor (CCR2) expression on classical monocytes	[70]
	Decreased production of cytokines like IL-1β, IL-6, IL-10 and TNF-α, in response to the activation of TLR2, TLR7 and TLR8.	[71]
	No change of CD14⁺CD16⁺ or CD14⁺CD16⁻ percentages and expression of TLR2 and TLR4	
NK cells	- Increased activity with disease progression - Elevated percentage of CD3⁺CD56⁺ - Increased expression of the inhibitory receptor NKG2A	[76]
	Increased expression of the inhibitory receptor NKG2D	[77]
Dendritic cells	Decreased percentage and absolute count of myeloid DC	
	Decreased percentage, but not the absolute count of plasmacytoid DC	[59]
	Inverse correlation of frequency of both blood DC with motor functions decline	
	Higher frequency of CD16⁺ mDCs in CMV+ PD patients	[62]

-
- Decreased frequency of CD16⁻ cells, CD141⁺ and CD1c⁺ mDCs in CMV⁺ PD patients
-

4.2. Adaptive immunity in PD

4.2.1. B cells

B lymphocytes are the antibody-secreting cells and key mediators of humoral immunity. The numbers of B cells were reported as reduced in PD patients using both CD19+ [83, 84] and CD20+ activation marker [77], but these findings still need to be explained and put in correct context since for example infiltration of B cells into CNS of PD patients has not been confirmed and in addition significant reduction of total C19+ B cells in the periphery occurs in elderly population [85]. It has been observed the down-regulation of numerous genes responsible for encoding the CD surface molecules such as B cell specific immunoglobulins, immunoglobulin heavy constant mu and immunoglobulin heavy constant delta as well as regulators of differentiation and activation of B cells. All of that can contribute to the observed decrease in the B cell number. Reduced mRNA levels of CD19 were confirmed by quantitative real-time PCR analyses as well as by microarray analysis [86]. It is important to emphasize that observed changes were not correlated to the on-going treatment of PD patients but rather to the disease itself. Additionally, major down-regulation of 16 B cell related genes (membrane-bound molecules that are involved in B cell signal transduction) in peripheral blood of PD patients have been recorded [87].

Having in mind available data and findings it is obvious that B cells might be actively involved in PD, but further studies are required.

4.2.2. CD4+ and CD8+ T cells

The early studies on alterations of peripheral adaptive immune response in PD have found diminished numbers of lymphocytes in PD patients, mainly due to the reduction in population of CD4+ T cells [83, 88, 89]. The most prominent change was observed in the pool of naive T cells (CD45RA+) in a way of dramatically reduced percentage [83]. The ratio of CD4+ and CD8+ T cell was significantly lower in PD patients. One of the most interesting finding was an increased ratio of IFN- γ to IL-4-producing T cells in PD patients, indicating the shift toward the Th1-type immune response [89]. Furthermore, it has been found that memory (CD45RO+) T cells from PD patients have elevated co-expression CD25 (α -chain of the IL-2 receptor), TNF- α receptors, and reduced expression of IFN- γ receptors which all is implying that those cells are activated [90]. In the studies performed recently was confirmed a reduction

in CD4⁺ T cells in peripheral blood of PD patients while the proportions of CD8⁺ T cells remained unchanged [84, 91, 92]. In addition, it was reported more profound reduction in the numbers of CD4⁺CD29⁺ cells and CD4⁺CD45RO⁺ memory T cells [84], reduced percentage of CD45RA⁺ naïve and an increased percentage of memory T cell subset from CD4⁺ T cells in PD patients [91, 92]. Interestingly it has been confirmed elevated presence of CD4⁺ and CD8⁺ T cells that express $\gamma\delta$ TCR in peripheral blood of PD patients. So far has been found that those cells have pathological relevance for PD, namely these cells are recognized as active player and regulators of CNS inflammation [93]. More interestingly it has been found in CSF of PD patients have been found augmented percentage of $\gamma\delta$ TCR expressing cells with high expression of CD25 meaning that preferentially this type of cells is activated in PD [94]. Furthermore, Hutter-Saunders et al., [91] have shown increased percentages of Fas⁺ cells and decreased percentage of CD31⁺ cells. As previously mentioned, in some studies have been described decreased number of lymphocytes in peripheral blood of PD patient that could be caused by Fas-mediated apoptosis [83, 95]. Nevertheless, increases in apoptosis may not be due to Fas alone since it was shown decreased expression of CD31 (platelet endothelial cell adhesion molecule-1 (PECAM-1)), which was related with disease severity. Under normal conditions, CD31 is responsible for controlling the amplitude of clonal expansion, T cell tolerance and establishment of regulatory functions [96] and some recent studies performed on CD31 deficient mice showed that loss of this marker increases T cell susceptibility to apoptosis [97]. So, observed decrease in the expression of CD31 on CD4⁺ T cells may contribute to the reduction in total CD4⁺ T cell counts by increasing the process of apoptosis. Summarizing, in peripheral blood of PD patients are increased memory and decreased naïve CD4⁺ T cells and increased Fas⁺ and decreased CD31⁺ CD4⁺ T cells, which are characteristics of activated or proliferating T cells [98, 99].

All above mentioned data are implying that T effector memory phenotype is favored in PD as confirmed by recently published results from our laboratory. Namely, we have reported that in peripheral blood of PD patients are present augmented numbers of effector memory CD4⁺ T cells and their increase correlate with increased motor dysfunction. Further, we have recorded reduction of absolute count of CD4⁺ T naïve cells, and unchanged T central memory (TCM) cells in PD patients in comparison to healthy controls. Specifically, our flow cytometric strategy [100] permitted us to characterize two different population of memory cells, TCM and TEM.

T central memory cells are responsible for reactive memory, they migrate to T cell areas of secondary lymphoid organs. On the other hand, T effector memory cells, provide protective memory, they are able to migrate directly to the inflamed peripheral tissues and displaying immediate effector function [101]. Further, we have recorded positive correlation between reduction of CD4⁺ T naive cells with increasing age in controls but not in PD patients. In literature it is well described the decrease of T naive cells in senior population and is believed to be the consequence of thymic involution as well as differentiation of T naive cells into antigen- experienced cells [102]. Obtained above mentioned results have led us to the formulation of the hypothesis that PD is associated with increased peripheral immune exposure to antigens.

Interestingly, a significant reduction in $\alpha 4\beta 7^{+}$ CD4⁺ T cells and a slight increase in $\alpha 4\beta 1^{+}$ CD4⁺ T cells in PD patients has been recorded [91]. Interaction between $\alpha 4\beta 7$ and mucosal addressin cell adhesion molecule 1 (MAdCAM-1) allows entry of T cells into the gut [103], while interaction of $\alpha 4\beta 1$ and vascular cell adhesion molecule 1 (VCAM-1) that is expressed on endothelial cells allows entrance of T cells into the CNS [104]. These findings are even more important in the light of dual hit hypothesis of PD. Namely, it has been observed increase in inflammatory responses in the gut by the presence of pro-inflammatory immune mediators [96, 105] and by increased expression of MAdCAM-1 on endothelial cells in inflamed gut tissue [106], and by increased intestinal permeability, which correlated with E. coli bacteria, nitro tyrosine and α -syn staining [105]. So, increased expression of MAdCAM-1 will augments homing of $\alpha 4\beta 7^{+}$ T cell, which eventually will lead to decreased frequency of those cells in the peripheral blood.

In the context of presented findings of chronic inflammation in the periphery a “dual hit” hypothesis is suggesting that the etiology of PD may be infection by a pathogen that gains entry to the CNS through the periphery (e.g., nasal and gut tissues) [107].

4.2.3. T regulatory cells

When it comes to the potential differences of this specific cell subset in patients with PD so far has been done very few studies. It has been shown that the percentages of peripheral CD4⁺CD25⁺ Treg and CD4⁺CD25^{high} Treg cells are lower in patients with PD [83, 89] and that the ability of Treg to suppress proliferation of Teff is impaired with lower Treg:Teff ratios [91]. In addition, it was found that CD31 expression on Treg

cells is negatively correlated with disease severity with the reduction in function of Treg at low Treg:Teff ratios [91]. In the MPTP mouse model of PD was shown that Treg are able to reduce ROS production and NFkB activation (via mechanisms that modulate redox enzymes, cell migration, and phagocytosis) hence reducing the activation of microglia [108, 109, 110].

Furthermore, it has been shown that the adoptive transfer of Treg protected dopaminergic neurons from dying by 90%, while adoptive transfer of pro-inflammatory Th1 or Th17 cells exacerbated neurodegeneration caused by MPTP intoxication [110]. Taken together these data imply the potential use of Treg as suppressors of immune responses in PD pathogenesis.

4.2.4. Cytokine/Chemokine production

Stypula et al., [40] reported augmented levels of IL-2 in the serum of PD patients, while Kluter et al., [111] reported a significantly reduced secretion of IL-2 by mitogen stimulated PBMC obtained from PD patients. Reports regarding the levels of cytokine production are often contradictory like increased PBMC basal and LPS stimulated production of IL-1 β , IL-6 and TNF- α , LPS- induced in PD patients was reported [112] and IL-1 β , IL-8, IFN- γ , MCP-1, MIP-1 α , and TNF- α in the patients with PD [113], and on the other hand lower production of IL-1 α , IL-1 β , IL-6 and TNF- α , by unstimulated PBMC or stimulated with LPS or Con A [114]. Interestingly, a positive correlation has been observed between increased production of cyto/chemokines with increasing UPDRS III score [113]. In addition, augmented levels of circulating IL-10, IL-15 and RANTES were found in PD patients [115].

All major changes that occurs in peripheral adaptive immune response in patients with PD are listed in the **Table 3**.

Table 3. List of most important currently available studies regarding changes in adaptive immune response in PD, with briefly summarized results.

	CHANGES IN PD PATIENTS	REFERENCE
B cells	Decreased number and percentage	[83, 84]
	- Downregulation of genes encoding IGHM and IGHD - Decreased mRNA levels of CD19	[86]
	Downregulation of 16 B cell related genes	[87]
T cells	Higher number of $\gamma\delta^+$ T cells	[94]
	- Unchanged number of CD8⁺ T cells - Diminished number of CD4⁺ T cells - Higher ratio of CD4^{bright+}CD8^{dull+}	[88]
	- Increased number of CD8⁺ T cells - Diminished number of CD4⁺ T cells - Reduced percentage of CD4⁺ T naïve cells - Increased percentage of activated CD4⁺CD25⁺ T cells	[83]
	- Diminished number of total lymphocytes - Diminished number of CD4⁺ T cells - Increased number of CD8⁺ T cells - Increased ratio of IFN-γ- to IL-4-producing T cells - Higher ratio of CD4^{bright+}CD8^{dull+} - Lower percentage of CD4⁺CD25⁺ and CD4⁺CD25^{high} T cells	[89]
	Increased percentage of FAS⁺CD4⁺ T cells	[95]
	- Diminished number of CD4⁺ T cells - Increased numbers of memory T cells	[84]
	- Diminished number of CD4⁺ T cells - Increased numbers of memory T cells - Increased percentage of Fas⁺CD4⁺ T cells - Decreased percentage of CD31⁺ cells - Decreased percentage of $\alpha 4\beta 7^+$ and increased percentage of $\alpha 4\beta 1^+$ CD4⁺ T cells	[91]

	• Impaired function of Treg cells (inhibition of proliferation)	
	• Diminished number of CD4⁺ T cells • Reduced percentage of CD4⁺ T naïve cells • Increased numbers of effector memory T cells • No difference in immune profile of drug treated PD patients and drug naïve patients • More pronounced response to common recall antigen tetanus toxoid • Increased percentage of T central memory cells after stimulation with fibrillar α-syn	[92]
Cytokines	• Increased levels of IL-2 in the serum • Decreased levels of secretion of IL-2 by mitogen stimulated PBMC • Elevated secretion of IL-1β, IL-6 and TNF-α • Lower levels of secretion of IL-1α, IL-1β and IL-6 and TNF-α by unstimulated or ConA stimulated PBMC • Lower levels of IFN-γ secretion by LPS stimulated PBMC • Elevated levels of circulating IL-10, IL-15 and RANTES • Increased PBMC basal and LPS stimulated production of IL-1β, IL-8, IFN-γ, MCP-1, MIP-1α, and TNF-α	[40] [111] [112] [114] [115] [113]

In advanced age immune system undergoes various changes that are briefly listed in **Table 4**.

Table 4. List of most important changes that affects immune system cells during ageing observed so far, with briefly summarized results.

CHANGES DURING AGEING		REFERENCE
Granulocytes	Decreased capability of oxidative burst, phagocytic capacity, bactericidal activity and chemotaxis	[116, 117, 118]
Monocytes and macrophages	Increased chemotaxis and phagocytic capacity, production of PGE2 and synthesis of pro-inflammatory cytokines IL-6 and TNF-α and chemokines: IL-8, MIP-1α and RANTES	[119, 120]
	Decreased TLR1/2-induced levels of intracellular cytokines TNF-α and IL-6, chemotaxis and phagocytic capacity and CD14 expression	[121, 122]
	Increase in release of PGE2.	[122]
NK cells	Reduced cytotoxicity and proliferative response to IL-2	[123, 124]
Dendritic cells	Decreased number	[125, 126, 127]
	Decline in capacity to present antigens to T cells, lymph node homing, ability to secrete pro-inflammatory cytokines or to elicit T cell proliferation	
T cells	- Increased number of memory and effector cells, increased numbers of effector cells clone - Augmented production of pro-inflammatory cytokines - Reduced number of naïve T cells, diversity of T cells repertoire and expression of co-stimulatory molecules, CD27, CD28 and CD40	[128, 129]

- Reduced ability of naïve CD4⁺ T cells to respond to IL-4 and increased ability to respond to IL-1, IL-6, TGF- β , and IL-23 in order to generate Th17 cells [130, 131]
 - Increased number of CD4⁺ Treg cells in peripheral blood with higher suppressive activity [132, 133, 134]
-

Some of listed changes during immunosenescence of peripheral immune system resemble strongly to the changes so far recorded in peripheral blood of PD patients, so one might easily ascribe observed changes to the process of ageing and not to the disease itself. Nevertheless, all studies previously discussed were performed in comparison with age-matched group of healthy subjects and in all the studies those recorded changes were significantly different. So, it seems that recorded changes are consequence of PD pathology rather than disease.

What remains to be clarified is whether changes in immune system that we see in advanced age can be one of the reason for developing neurodegenerative disease? And if yes, more important question remains open: what can be the “defying factor” that determine why some individuals in elderly develop PD and others no?

5. RELATIONSHIP BETWEEN PERIPHERAL IMMUNE SYSTEM AND NEUROINFLAMMATION IN PARKINSON'S DISEASE

BBB disruption and breakdown and inflammation appear to be one of the major players in the pathology of various neurodegenerative diseases. The event of BBB integrity alterations compromises the vascular component of the brain and can lead to leukocyte migration within the brain [135]. BBB disturbance has been documented in PD patients by the analysis of CSF and positron- emission tomography imaging. In PD disruption of BBB integrity has been confirmed histologically by the presence of the CD4+ and CD8+ lymphocytes [136]. In the recent study authors Gray & Woulfe [137] use various methods to demonstrates the damaged BBB integrity in the striatum of PD patients. Some of the methods included examination of leakage of serum proteins outside vessel walls that were stained and erythrocyte extravasation etc. Alterations of BBB function during the course of PD can led to engagement of the peripheral immune response by increased efflux of antigen from the CNS that will further lead to the increased infiltration of peripheral immune cells and hence influence the neuroinflammation for better or worse.

In normal circumstances, activated T and B cells are able to patrol within the CNS in very low numbers, while naive lymphocytes are nearly excluded [138, 139]. Nevertheless, during infections and/ or inflammations of the CNS, peripheral immune cells have somewhat unrestrained entrance to the CNS [140].

Indeed, different immune cell populations were found in the CNS of a PD patients [136, 141]. As mentioned, increased permeability and disruption of the BBB of the PD patients may stimulate an efflux of antigen from the CNS that can reach periphery where adaptive immunity can be engaged [142, 143]. Since it has been confirmed that in some instance CNS is the immune privilege site, it can be logical to speculate that adaptive immune responses against CNS neo-antigens are initiated in the periphery. Recently, it has been shown that encephalitogenic CD4+ T cells can be activated during neurodegenerative diseases and are able to express TCRs that are specific to neo-antigens originating from CNS. Initially, these neo-antigens are presented by APCs, that reside inside the CNS. APCs after capturing these antigens migrate into the cervical lymph nodes and present them to peripheral naive CD4+ T cells. Subsequently, after antigen presentation naïve T cells will differentiate into

memory and effector CD4⁺ T cells that will migrate towards the site of inflammation and could be re-stimulated in situ by APCs residing in the brain parenchyma or in the CSF [140, 143, 144]. Microglia express both MHC I and MHC II class molecules providing thus the opportunity to directly interact with cells of the adaptive immune system, even acting as an antigen presenting cells.

In vitro studies in mice have shown the ability of microglia to present antigens through MHC II molecules and activate CD4⁺ T cells [145]. The re-stimulation of infiltrated auto-reactive CD4⁺ T cells in the CNS site with on-going inflammatory process would result in production of pro-inflammatory helper T cells such as Th1 and/or Th17 that are characterized by production of inflammatory cytokines, which are able to act directly or indirectly on microglial cells. Pro-inflammatory Th1 cells would produce IFN- γ and TNF- α , hence forcing microglial differentiation towards pro-inflammatory M1-like phenotype [2], while Th17 cells would mainly participate in disrupting BBB integrity by producing IL-22 and IL-17 cytokines [146]. On the other hand, T regulatory cells have anti-inflammatory functions since they are able to provoke the phenotypic switch from the neurotoxic M1-like phenotype of microglial cells to the neurotrophic M2-like phenotype through the downregulation of iNOS and upregulation of arginase 1 in microglia [54].

In the proposed hypothesis of CNS neo-antigen activation of the peripheral adaptive immune system it is believed that infiltrated activated T cells might subsequently acquire Th1 or Th17 pro-inflammatory phenotypes of cells and migrate to the CNS where pro-inflammatory cytokines secretion might provide further support to the activation of M1 microglia and increased destabilization of the BBB, resulting in a feed-back loop amplification of neuroinflammation and neurodegeneration (**Figure 3**). It is still unclear whether the activation of the peripheral immune response is an initial or consequential process in the PD and the answer to this question is very hard to give since it is extremely difficult to identify the persons with early PD.

In the meantime, we can rely on the evidences provided by studying the various animal models (with all limitations given). For example, in the MPTP mouse model, after the MPTP intoxication following changes have been observed: activation of microglia (via elevated expression of MHC I and MHC II molecules), augmented expression of adhesion molecules on endothelial cells and infiltration of T lymphocytes (CD4⁺ and CD8⁺ T cells) in the SN [147, 136]. Convincing reduction of dopaminergic neurodegeneration have been observed in animal studies with MPTP intoxication and

use of specific animals such as recombination-activating-gen-1 (RAG1) knockout mice, TCR- β -chain-deficient mice and SCID mice [136, 148]. Those data are strongly suggesting the importance of infiltrating T cells in the pathogenesis of PD. Furthermore, another set of evidences from animal studies strongly supports the idea of importance of α -syn. Namely in these studies have been confirmed that abnormal species of α -syn are closely connected to the activation of microglia, oxidative stress occurrence and hence loss of dopaminergic neurons [28, 147, 149] and that α -syn-related neo-antigens provide a major contribution to activation of the peripheral adaptive immune response. Those studies strongly emphasize the importance of modulation of the adaptive immune response that can lead towards different outcomes of neurodegenerative diseases. Both native and altered α -syn species occur not only in the CNS but in peripheral tissues as well [150]. So far it has been confirmed the presence of nitrated form of α -syn in cervical lymph nodes of MPTP-treated mice [148]. Interestingly, some modified forms of α -syn were also detected in the gut of PD patients [105, 151]. The occurrence of modified forms of α -syn in lymph nodes and gut supports the speculation that they might play a role as neo-antigens, leading to initiation of the peripheral adaptive immune response. In MPTP mouse model of PD have been shown that nitrated α -syn can drain from the CNS into CLN [148]. In this study was shown that immunization of the mice with the C-terminus of nitrated α -syn elicits strong adaptive immune responses and exacerbate dopaminergic neuronal loss. Different study showed that effector CD4⁺ T cells (Teff cells) isolated from mice immunized with nitrated α -syn secreted mostly IL-17 and TNF- α after ex vivo re-stimulation [110]. Furthermore, mice immunized with nitrated α -syn were used as a source of Teff cells that were in vitro polarized into various subsets and then adoptively transferred into wild type (wt) mice that was MPTP intoxicated. Results have shown that transfer of Th17-polarized Teff cells exacerbated MPTP-induced neurodegeneration. This was not the case with the transfer of Th1 or Th2 polarized cells. Finally, adoptive transfer of Treg cells resulted in weakened neurodegeneration [110] by suppressing ROS production and NF- κ B activation via mechanisms that modulate cell migration, phagocytosis and redox enzymes [108, 109, 110].

In addition, it is noteworthy to mention the study done by Kustrimovic et al., in which has been shown the ability of α -syn, wild type and fibrillated form, to influence the percentual distribution of peripheral T naïve, TEM and TCM cells in PD patients.

Intriguingly, authors have shown that both forms of α -syn induced a similar response as one evoked by common recall antigen, tetanus toxoid, meaning reduced occurrence of T naive cells and increased percentage of TCM and TEM cells [92]. Presented results do not immediately imply that the recorded responses to α -syn are due to recognition of α -syn as the neo-antigen by T memory cells, even so the response pattern is very much similar to the one induced by TTd. In addition, the ability of the peripheral immune system to recognize α -syn as an antigen is reinforced by the recorded presence of specific antibodies in the serum of the patients with PD [152, 153]. Recent study by Sulzer et al., [154] addressed the important question whether PD is linked with T cell recognition of α -syn epitopes presented by precise MHC alleles. Results have shown that peptides that are originating from two regions of α -syn are able to provoke immune responses in PD patients.

If in the propagation of PD is indeed involved the response of the adaptive immune system in a sense of recognition of specific antigens by CD4⁺ T cells, then it involves the antigen presenting cells as well. Indeed, it has been shown in mice deficient in MHC class II molecules, increase resistance to the activation of microglial cells and neurodegeneration [155]. It has been recently proposed that certain antigens could synergize with a defined MHC class II genome and hence potentially increase the risk for PD development. In humans, the MHC region comprises more than 200 HLA loci, which are located in proximity on chromosome 6. Proteins encoded by the MHC class I genes are three and are named as HLA A, HLA B and HLA C. On the other hand, MHC class II genes are responsible for encoding six main proteins as follows: HLA DPA1, HLA DPB1, HLA DQA1, HLA DQB1, HLA-DRA and HLA DRB1 [156]. Diverse human leukocyte antigen (HLA) alleles comprise different peptide-binding affinities and thus has the ability to activate different T cell clones and provoke various Th response whether they are pro-inflammatory or anti-inflammatory T-cells [157]. MHC class I alleles HLA-A*2, HLA-B*17, HLA-B*18 and HLA-A*28 have been associated to higher risk of developing PD [158, 159]. Furthermore, it has been confirmed that MHC class II alleles HLA-DQB1*06 and HLA-DRB1*0301 are significantly more frequent in PD patients [158, 159]. Interestingly, allele HLA-DRB1*0301 also contributes to the predisposition of some typical autoimmune diseases like diabetes [160] and MS [161] leading to potential hypothesis of the presence of autoimmune component in PD [162, 163, 164]. Another relevant issue is the potential role of the gastro intestinal tract (GIT) as site for inflammation and

initiation of PD. Nowadays is known existence of bidirectional communication between the CNS and the GIT, so called the brain-gut axis [165, 166]. GIT has perpetual interaction with the environment, the widespread neuronal system and unique immunological repertoire so it has been proposed the idea of its potential role in the development of PD (reviewed in [167]).

GIT is characterized by the presence of specific gut microbiota that if changed can lead to major changes in the function of gut barrier and increased intestinal permeability. Changes in gut barrier and increased permeability will activated the immune system and change the function of enteric nervous system (ENS) [168] altogether leading to the change of the pro- and anti-inflammatory responses [169]. It has been reported recently that at very early stage of PD can be spotted lesions in the enteric nervous system (ENS), much before the lesions in the CNS [96, 151]. All of these data have led to the development of what is now known as “Braak’s hypothesis”. Braak hypothesis states that α -syn pathology initiate in the submucosal plexus of the ENS and retrogradely propagates towards the CNS [170]. In 2007 was presented dual hypothesis which further have reinforced Braak’s theory. Namely in dual hit hypothesis was stated that α -syn can be transmitted to midbrain via two different pathways. First one is nasal (from the olfactory bulb to the temporal lobe) and the second one is gastric (through the ENS) [171].

Nevertheless, even if extremely interesting and tempting for speculation the causal rapports between GIT and PD still awaits the full clarification to be given since the better understanding of this relationship could permit earlier diagnosis of PD as well as development of novel therapeutics to be used in treatment of PD.

6. IMMUNOMODULATORY THERAPIES IN PARKINSON'S DISEASE

The role of dopaminergic pathways in PD are essentials, nevertheless, only recently has begun research on dopaminergic pathways that are able to significantly modulate the immune responses. Results that have accumulated showed substantial connection between DA and peripheral immune system. So far it has been confirmed that immune cells can encounter DA not only in brain but also in blood, lymphoid organs and in some peripheral tissues, like kidney and the hepatic vasculature [172]. In addition, DA can be produced by various immune cells hence acting as autocrine/paracrine manner hence influencing the function of themselves and the neighboring cells as well [173, 174, 175, 176, 177]. Furthermore, a large body of evidence have revealed expression of dopaminergic receptors (DR) on various cells of immune system (reviewed in detail in [178]). DA can affect T and B lymphocytes, DC, neutrophils and NK cells (reviewed in [178]). The effect that DA can exert on immune cells will depend not only on type of the cells but also on activation status of immune cells, expression of DR, DA concentration. Relationship between DA and immune system so far has been established in several human diseases such as multiple sclerosis [172, 179, 180], rheumatoid arthritis [181, 182]; and Parkinson's disease [183].

Having in mind above said, the implication of published results of the role of DA in immune system regulation in PD is substantial for correct usage of traditional therapeutic approaches in treatment of PD. In addition, clarifying direct effects of DA on peripheral immune activity could eventually pave a way to a potential drug repurposing. The growing evidence indicating a role for neuroinflammation, supported by peripheral immune activation, in the pathogenesis of PD fostered intensive interest in the development of novel therapeutics targeting the immune response. To date a number of different approaches has been tested with some success in animal models of PD (**Table 5**).

Table 5. Preclinical studies of immunotherapeutic approaches in animal models of PD.

Adapted from Sanchez-Guajardo et al., 2013 [57]

	Model; species	Target	Effect	Reference(s)
Intervention				
dnTNF	6-OHDA; rat	Soluble TNF	Reduced nigral degeneration by 50%	[184, 185]
GDNF (striatal)	MPTP / 6-OHDA; rodents	Neurons	Induced neuroprotection of DA neurons	[186]
GDNF (nigral)	AAV- α syn overexpression; rat	Neurons	No effect	[187, 188]
Chemical intervention				
Celecoxib	Striatal 6-OHDA; rat	COX2	- Decreased microglia activation - Prevented neurodegeneration	[189]
Hydrogen Sulfide L-DOPA	human monocyte THP-1 cell line; human astrocytoma U373 cell lines; human microglia; human SH-SY5Y neuroblastoma cells	Microglia and astrocytes	- Increased levels of DA and glutathione in brain; - Decreased production of IL-6/ TNF-α and NO by microglia	[190]
Minocycline	MPTP; mice	Microglia	Attenuated MPTP-induced neurodegeneration in SN (increased number of TH+ neurons)	[191]

			• Decreased production of nitrotyrosine (participate in NO-related oxidative damage) • Inhibited microglial activation (reduced expression of Mac-1 marker) • Inhibited production of IL-1β, ROS and NO	
Minocycline	Primary spinal cord rat cell line; primary mixed glial and microglial cell cultures	Neurons	• Prevents excitotoxic cell death • Inhibits microglial proliferation • Inhibits activation of microglia by preventing activation of p38 MAPK in microglia	[192]
Minocycline	MPTP model; mice	Microglia	• Augment MPTP-induced damage to DA neurons by alerting the VMAT2 function	[193]
Naloxone	LPS induced parkinsonism; rat	Microglia	• Reduced superoxide production from microglia • Reduced release of TNF-α from microglia • prevented DA cell loss	[194]
Naloxone	LPS treatment; mesencephalic neuron-glia cultures; rat	Microglia	• Protection against LPS-Induced reduction in high-affinity DA uptake of cell cultures • Protection of TH+ and MAP-2+ neurons • Inhibits activation of microglia and secretion of pro-inflammatory factors	[195]

			• Reduced levels of generation of ROS in cell cultures •	
Dexamethasone	Intranigral LPS; rats	Microglia	• Prevented degeneration of DA neurons induced by LPS administration • Reduced microglial activation • Prevented loss of catecholamine content and TH activity	[196]
Peripheral immune system modification				
Glatiramer acetate (GA)	MPTP model with cell transfer from GA immunized animals; mice	T cells	• Conferred dopaminergic neuroprotection • Reduced microglial reaction • Increased GDNF expression in ventral midbrain • Neuroprotection is dependent on T cells	[197]
Glatiramer acetate (GA)	MPTP model; with cell transfer from GA immunized animals; mice	T cells	• Lymphoid cells from GA immunized mice protected SN neurons from MPTP toxicity • Attenuation of SN cell loss by transferred T cells was dose dependent • Neuroprotection was accomplished by transferred CD4+ T cells from GA immunized mice	[198]

			GA specific antibodies are not neuroprotective	
Myelin oligodendrocyte glycoprotein (MOG) with CFA or CFA alone	MPTP model; mice	Peripheral immune system	- immunization with MOG prior to MPTP treatment, led to a reduction in DA depletion - Partial rescue of DA neurons;	[199]
Systemic CFA	6-OHDA; rats	Peripheral immune system	- Reduced neuronal loss in SN - Increases GDNF in striatum; - Increased survival of TH+ cells in SN; - Decreases activation of microglia (decreased CD11b expression)	[200]
Live Bacill Calmett-Guerin (BCG); TH in CFA; Copaxone in CFA; CFA alone	MPTP model; mice	Peripheral immune system	- BCG reduced microgliosis - Partial DA neuron rescue	[201]
Granulocyte-monocyte-colony stimulating factor (GM-CSF)	MPTP model; mice	T regulatory cells	- Demonstrated link between neuroprotective effects of peripheral GM-CSF administration and neuroprotection - Increased neuroprotection - Decreased microglial activation - GM-CSF activated T regulatory cells in dose-dependent manner and those cells when administered to MPTP-	[202]

			treated mice elicited robust neuroprotective response	
Activated regulatory cells	MPTP model; mice adoptive transfer of Treg cells	T regulatory cells	• Nigrostriatal protection significantly increased (by 90%) • Neuroprotective effect was dose-dependent • Increased levels of GDNF and TGF-β in midbrain • Decreased levels of iNOS and TNF-α in midbrain • Increased TH + neuron survival	[108]
Vasoactive intestinal peptide (VIP) or nitrated α -syn	MPTP model; mice T cell transfer from VIP or nitrated α -syn immunized animals	T regulatory cells	• Nitrated α-syn induce increase in number of Th17 pro-inflammatory T cells and inhibit Treg function; • VIP induced increased expression of Foxp3 and IL-10 and reduced expression of Mac1 on microglia • Adoptive transfer of Treg (natural and VIP-induced), devoid of DC, attenuated both neuroinflammatory and neurodegenerative activities in MPTP-treated animals	[110]
Peptide sensitized dendritic cells (DC)	transgenic mice expressing human A53T variant of α -syn	Peripheral immune response	• Development of specific anti-α-syn antibodies • Reduced markers of neuroinflammation in the CNS • Reduced levels of brain IL-1 α cytokine	[203]

α-syn aimed therapies				
Active immunization using full-length human α -syn	α -syn overexpressing mice under the PDGF- β promoter	Peripheral immune response	- Intracellular deposition of antibody in neurons - Induced antibody-mediated lysosomal degradation of α-syn	[204]
Passive immunization with α -syn antibody	α -syn overexpressing mice under the PDGF- β promoter	Peripheral immune response	- Reduced activation of astroglia - Increased clearance of pathological α-syn aggregates	[205]
α -syn in CFA/iFA	AAV- α syn SN expressing rats	Peripheral immune response	- Increased levels of GDNF in striatum - Accumulation of Treg in SN - Reduction of α-syn aggregates	[24]
pVAX1-IL-4/SYN-B	MPTP model; mice	Peripheral immune response	- High anti-α-syn antibody response - Improvement in motoric function - Diminished death of TH neurons in the SN - Overexpression of GDNF - Increased levels of IL-4 in the serum of the animals	[206]
AFFITOPE ^{VR} -KLH conjugates (AFF1)	PDGF- α -syn transgenic mice mThy1.2 α -syn transgenic mice	Peripheral immune response	- AFF 1 immunization reduced accumulation of α-syn (oligomers only, not monomers) - Ameliorated neurodegeneration - Promoted microglial clearance of α-syn - Increased production of anti-inflammatory cytokines	[207]

Vaccination with three different epitope vaccines: α -syn ₈₅₋₉₉ – P30-MAP; α -syn ₁₀₉₋₁₂₆ – P30-MAP and α -syn ₁₂₆₋₁₄₀ – P30-MAP	Mouse, Wt-C57Bl/6	Peripheral immune response	All 3 conjugates induce human α-syn –specific Abs that bounds to Lewis bodies	[208]
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Notes: **6-OHDA**, 6-hydroxi-dopamine; **α -syn**, α -synuclein; **AAV**, adeno associated virus; **CFA**, complete Freund's adjuvant; **DA**, dopamine; **DC**, dendritic cells; **dnTNF**, dominant negative Tumor Necrosis Factor; **GDNF**, glia cell line derived neurotrophic factor; **iNOS**, inducible nitric oxide synthase; **LB**, Lewy bodies; **L-DOPA**, L-3,4-dihydroxyphenylalanine; **LPS**, lipopolysaccharide; **MHC II**, Major Histocompatibility Complex II; **MPTP**, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; **NADPH**, nicotinamide adenine dinucleotide phosphate-oxidase; **NO**, nitric oxide; **N- α -syn**, Nitrated- α -synuclein; **PDGF**, platelet-derived growth factor; **PDGF- β** , platelet derived growth factor β ; **SN**, substantia nigra; **TH**, Tyrosine Hydroxylase; **Th**, T helper CD4 + cell; **TGF β** , transforming growth factor β ; **Treg**, regulatory CD4 + T cell; **WT**, wild-type;

Some of the approaches are focused at the inflammatory response and include use of anti-inflammatory gene therapy. Overexpression of a dominant negative TNF molecule in SN that is used to block endogenous TNF signaling can be taken as an illustration. This approach has proven effective in 6-OHDA rat model of PD reducing the DA neuronal loss [184, 185].

Interesting proposition for sure represent use of delivery of specific neurotrophic factor, GDNF, that is able to suppress degeneration of dopaminergic neurons in long-terms. Transplantation of GDNF-secreting cells that are encapsulated is an interesting ex vivo cell-based gene delivery model since it enables the removal of the cells if unwanted effects occur (reviewed in [186]). Nevertheless, this approach did not appear to be effective in rAAV- α -syn-overexpression rat animal models [187].

As previously mentioned, in various studies has been confirmed the beneficial role of nonsteroidal anti-inflammatory drugs (NSAID) and reduced risk of PD development since it has been confirmed by several epidemiological studies that those drugs have a sparing effect on PD [209, 210]. In addition, usage of celecoxib, a selective inhibitor of COX-2, has been proven useful in 6-OHDA rat model of PD, since it has reduced significantly DA cell loss [189]. Further, compounds, such as dexamethasone, naloxone and minocycline, all anti-inflammatory compounds, have shown the ability to reduce activation of microglia, hence reducing neurodegeneration in various animal models of PD [189, 190, 191, 192]. More specifically, application of minocycline led to the inhibition of activation of microglia and microglial-T cells interaction in human cells, by decreasing the expression of CD40 ligand on T cells and disabling them to interact with microglia, thus diminishing production of pro-inflammatory factors [211]. Alternatively, hydrogen sulphide-releasing L-DOPA, exerting antioxidant and anti-inflammatory characteristics, has been proven neuroprotective properties through reduction of IL-6, TNF- α and NO release from activated human microglial cells [190].

Copolymer-1 (Cop-1; Copaxone®, glatiramer acetate, GA) is an immunomodulatory agent. This is a polymer comprised of four amino acids (glutamic acid, lysine, alanine, and tyrosine). GA is found in myelin basic protein and it is able to induces a shift in the T cell responses towards an anti-inflammatory phenotype [212]. In multiple sclerosis, GA treatment results in increased Treg frequency and

restored Treg suppressive properties hence reducing the level of pro-inflammatory cytokines [213]. Evidence for GA efficacy in PD has been provided by showing that adoptive transfer of splenocytes isolated from GA -treated animals to MPTP- treated receivers led to the reduction in CD4+ T cell infiltration in the SN, as well as reduction in activation of microglia. Furthermore, this method led to increased neurotrophic factors and neuronal survival [198]. Recorded neuroprotective functions are linked to the production of anti-inflammatory cytokines such as IL-4, IL-10, TGF- β and neurotrophins like BDNF and GDNF. Up to day GA has been approved for treatment of relapsing-remitting multiple sclerosis with therapeutic responses in 30% to 80% of patients [214]. Nevertheless, the mechanisms by which GA mediates neuroprotection still remains inconclusive, but several potential points have been addressed such as its ability to modulate function of innate response, ability to block presentation of antigen and induction of Treg cells [215, 216]. In addition, neuroprotective effects of substance fingolimod (FTY720), a sphingosine-1-phosphate receptor (S1PR) agonist, has been tested on 2 different mouse models of PD, 6-OHDA or rotenone models. It has been found that sfingosine-1 is able to reduce the deficit of motor function, diminished the loss of TH-positive neurons in the SN and slow down the decrease of striatal DA in mice [217].

With increasing knowledge about the pivotal role of α -syn in PD pathogenesis, several attempts were made to develop α -syn-targeted vaccines. Masliah et al. [204] used transgenic mice overexpressing human α -syn, which were immunized with recombinant human α -syn. Immunization resulted in increased anti- α -syn antibody titers, leading to increased α -syn clearance and reduced levels of α -syn in the CNS, leading to the ameliorations of the symptoms of the pathology, but interestingly did not exert effect on microglial activation [204, 205]. To date, only a few studies targeting α -syn reached the clinical stage. The Michael J. Fox Foundation funded a preclinical study testing of the vaccine PD01, developed in 2010 by AFFIRiS (Vienna, Austria). AFFiRiS used short peptides, (AFFITOPEVR) that mimic a specific region of the α -syn molecule [218], with two identified candidates for vaccine PD01A and PD03A. PD01A (Affiris, study code PD01A –clinicaltrials.gov/ct2/show/NCT02216188) represents the first in class PD vaccine that is constructed to target aggregates of α -syn which have been confirmed as crucial players in PD [219]. The vaccine was tested in advanced PD patients by administration of two doses throughout a 12 months period. Available reports state that one half of the vaccinated patients developed anti- α -syn antibodies

in serum and in some patients the presence of anti- α -syn antibodies was recorded even in CSF. Overall was reported that the vaccine was well tolerated and safe. In addition, a phase II trial is ongoing (clinicaltrials.gov/ct2/show/NCT01885494). A second study that includes different group of peptides (Afiris, PD03 - clinicaltrials.gov/ct2/show/NCT02267434) is under valuation in a randomized placebo-controlled trial that convolves patients with early onset of PD [220].

Several researchers actively pursue passive immunotherapy as a strategy for treatment of PD at present. Clinical phase of testing of a monoclonal antibody against α -syn (PRX002) has begun. Up to day it has been observed that the treatment with PRX002 led to a quick dose-dependent reduction of levels of α -syn in serum (clinicaltrials.gov/ct2/show/NCT021557714) [221]. Recently several researchers have tried a novel approach in animal models. Namely, it has been proposed the use of the small molecules with the ability to modulate or block aggregation of a α -syn. Up to date two compounds are proposed for clinical testing: NPT200-11 [222] and ANLE138b [223]. It has been confirmed for both substances are able to pass the BBB and to modify and reduce the aggregation of α -syn in vitro as well as in animal models [220].

On the other hand, very promising approach in immunotherapy of PD represent the route that is directed in modifying the adaptive immune response and hence reduce the neuroinflammation in CNS. These approaches include adoptive transfer of lymphocytes from an immune donor to a naive recipient or stimulation of specific subsets of T cells in order to enhance their anti-inflammatory properties. Immunization strategy is thought to act on two levels; first clearance of aberrant forms of α -syn, and second, in controlling the neuroinflammation by boosting the Treg cell. T cells may be polarized into inducible Treg by IL-2, IL-10, TGF- β and all-trans retinoic acid [224], as well as by vasoactive intestinal peptide (VIP) [225]. Besides suppressing the activity of effector T cells, Treg may also induce increased production of BDNF and GDNF by astrocytes [108, 197] It has been shown for the first time that adoptive transfer of CD3-activated Treg to MPTP intoxicated mice reduces microgliosis, increases neurotrophic factors expression and provides significant protection to dopaminergic neurons [108]. In addition, Tregs can modulate the phenotype from neurotoxic to neurotrophic of microglia and control microglial responses to nitrated form of α -syn, LPS and TNF- α [108]. Furthermore, co-adoptive transfer of splenocytes isolated from VIP-treated mice together with the splenocytes obtained from the animals immunized with specific

epitopes of nitrated α -syn, lead to a decrease in activation of microglia and an increase in survival of dopaminergic neurons [108]. In addition, if CD4⁺ T cells were polarized before transfer into pro-inflammatory Th1 and Th17 cells neurodegeneration induced by MPTP was worsen, while transfer of Tregs increased survival of dopaminergic neurons by nearly 96% [110] leading to the important conclusion of significant utility of Treg cells in reducing neurotoxic activity of microglia and ameliorating the survival of dopaminergic neurons.

One more interesting approach to increase peripheral Treg cells is by using granulocyte macrophage colony stimulating factor (GM-CSF). Pretreatment with GM-CSF in the MPTP rodent model of PD induced increase in number of CD4⁺CD25⁺FoxP3⁺ Treg in a dose- dependent manner, decreased microglial activation and prevented neurodegeneration [226]. This study has paved the way towards a clinical trial using sargramostim, (Sanofi US, Bridgewater, NJ) [227]. So far has been published data from double-blind phase 1 clinical trial. In the PD patients treated with sargramostim, UPDRS, Part III scores was slightly improved after 6 and 8 weeks of treatment when compared with placebo together with cortical motor activities. In addition, numbers and function of Treg cells was increased [227]. Nevertheless, the study included only 22 PD patients so further confirmation of obtained results that could lead to the firm conclusions requires higher number of enrolled patients and additional clinical research.

Nonetheless, development of immunotherapeutic approaches for PD require thorough knowledge of the targeted underlying pathogenic mechanisms, and of the potential effects of their modifications. Remarkably, just a few years ago an innovative immunomodulatory therapy for AD dreadfully went wrong, due to an unexpected immune side effect of the tested vaccine AN1792 based on the human synthetic peptide A β 42. Preclinical studies were conducted on transgenic mice for human amyloid precursor protein (APP) driven by the human PDGF- β promoter (PDAPP). Animals were actively immunized with A β 42 prior the beginning of neuropathology, resulting in reduced plaque formation and gliosis [228]. This effect was associated with the increase of the antibody titers against A β 42 in the serum, diminished active microglia and augmented levels of in the anti-inflammatory cytokines IL-4, IL-10 and TGF- β [229]. Several other studies confirmed the beneficial effects vaccination, including reduced A β deposition in the CNS and reduced A β plasma levels [230, 231, 232]. Phase I clinical trial confirmed safety of the vaccine and permitted a Phase II

clinical trial, which was soon dismissed. Namely, in 6% of the patients enrolled in the trial have developed autoimmune meningoencephalitis [233, 234]. Subsequent investigation revealed the occurrence of a Th1-type shift of the immune response [233]. This case has influenced scientific community greatly and stressed out once again the importance of controlled immune response in any kind of immunotherapy approach.

7. CONCLUSION

Parkinson's disease is multifactorial neurodegenerative disorder. Up to the present time, only treatments that are focused on ameliorating symptoms are available including DA replacement therapy or DA agonists. In past few years activation of peripheral immune system and neuroinflammation has taken places as very important, if not key players in pathogenesis of PD. Even if numerous data from preclinical studies based on various animal models suggests a detrimental role of immune-associated mechanisms in PD, the fundamental goal, which is efficient therapy that is directed towards the cause remains distant. What can be concluded so far is that an effective treatment for PD should attack the disease from several angles. Undoubtedly the therapy should also involve substitution of damaged neurons by implementation of stem cell therapy and reversal of any present damage on neurons by application of neurotrophic and/or growth factors or by engaging the immune system to repair the damage. At the end, remains open the difficult question what can be the best approach in controlling the production of aggregated, misfolded or otherwise changed proteins, such as α -syn that can be neurotoxic. The abovementioned clinical studies are targeting the clearance of α -syn by activating the humoral immune response, but nevertheless the effects on neuronal tissues and/or immune system of those proteins still remains ill-defined provoking significant concern regarding proposed therapies. Almost certainly a successful result in creating efficient therapy will require a combination of various strategies rather than just focusing to one specific factor or a process.

CONFLICT OF INTEREST

The authors declare that they have no competing financial interests.

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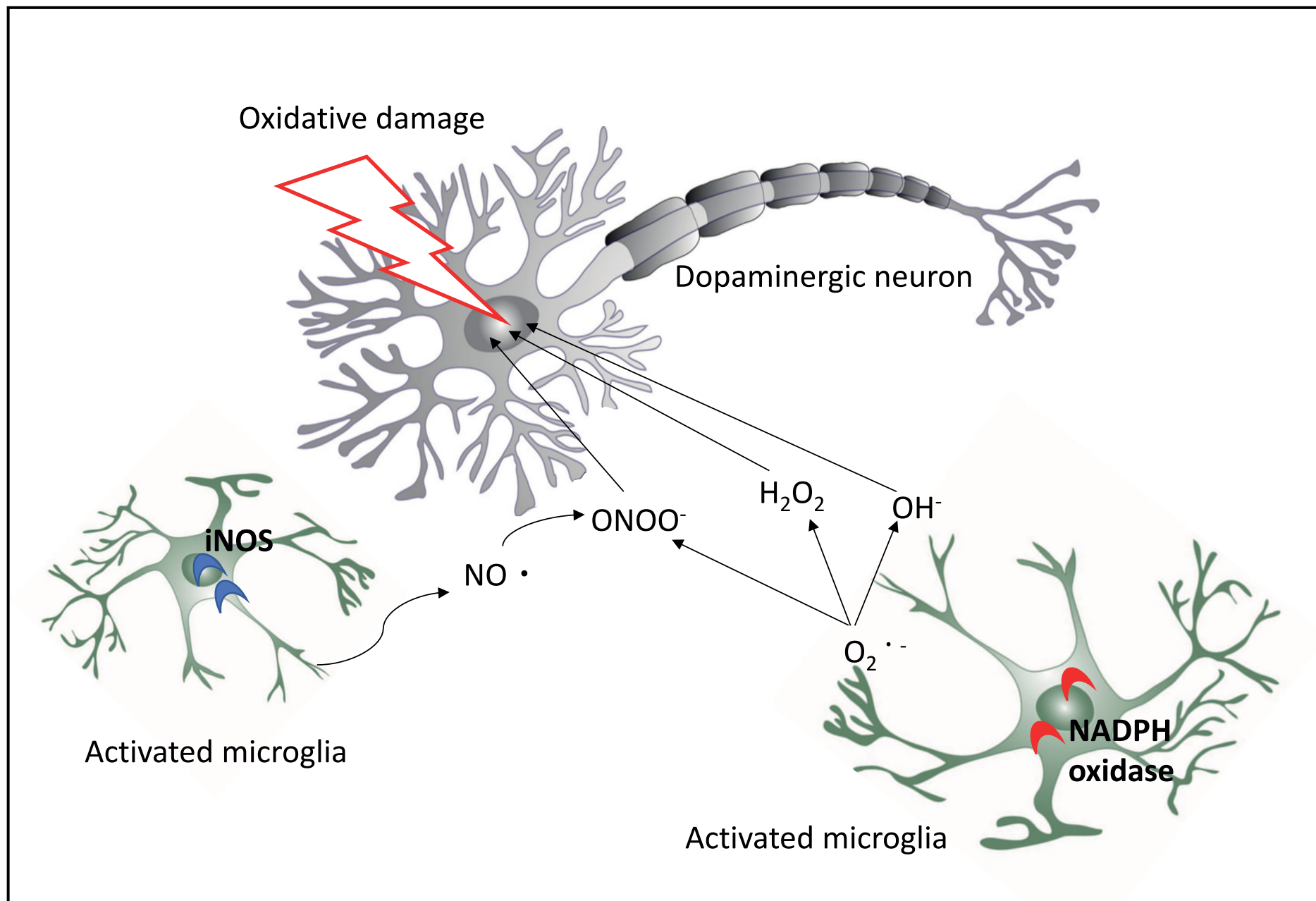


Figure 1. Molecular pathways of oxidative damage and potential link with neuroinflammation and death of dopaminergic neurons in Parkinson's disease. Under pathological conditions, activated microglia express iNOS and produce high levels of NO free radicals. In addition, upregulated expression and activation of NADPH oxidase augments, leading to the production of high amounts of O_2^- free radicals. Newly formed O_2^- and NO free radicals react and generate $ONOO^-$, which further cause oxidative damage to proteins within dopaminergic neurons. Conversely, O_2^- radicals might also interact with H_2O_2 , resulting in formation of OH radical, further causing oxidative damage to the neuronal cell. iNOS=inducible nitric oxide synthase.

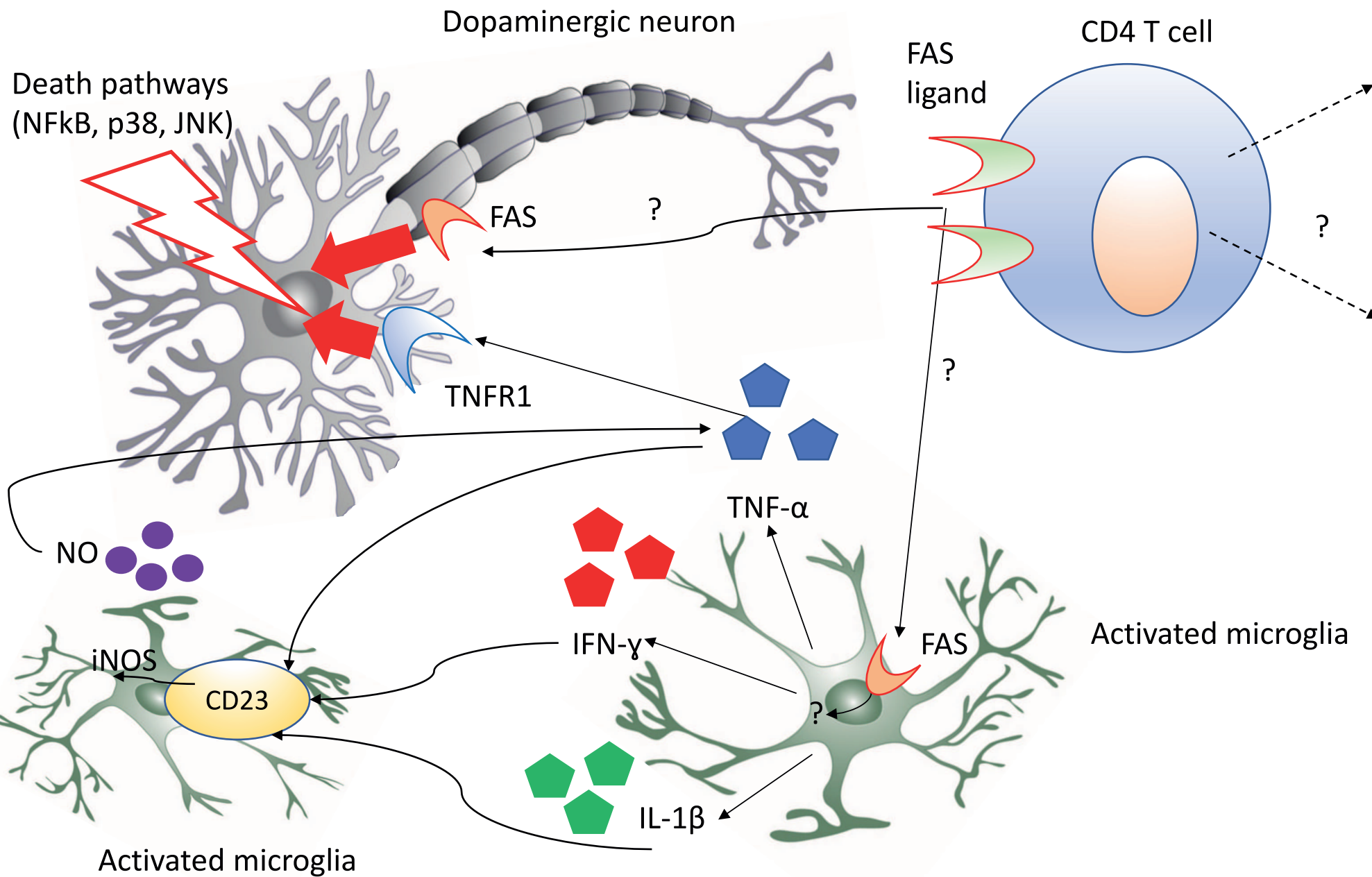


Figure 2. Possible mechanisms involved in propagation of neuroinflammation and its deleterious mechanisms in PD.

Pro-inflammatory cytokines contribute to the dopaminergic cell death as well. TNF- α might have a direct effect on dopaminergic neurons by activating an intracellular death pathway coupled with TNF receptor 1 (TNFR1) expressed on the cell surface of these cells. Additionally, pro-inflammatory cytokines might also stimulate the expression of iNOS within microglia (and possibly astrocytes) through the expression and activation of the low-affinity receptor of immunoglobulin E (CD23), thus leading to the production of toxic amounts of NO free radicals, that in turn, potentiate the expression and release of TNF- α by neighbor microglia. Furthermore, recruitment of activated CD4⁺ T cells into CNS could enhance the microglial-dependent inflammatory reaction, by secreting pro-inflammatory cytokines such as, such as TNF- α , IFN- γ . Fas ligand-derived CD4⁺ T cells might have a deleterious effect on dopaminergic neurons directly (by activating an intracellular death pathway coupled with Fas receptor expressed on the cell surface of dopaminergic neurons) or indirectly (by activating Fas receptor expressed on activated microglial cells), thereby stimulating their activation and additional release of pro-inflammatory factors. IL=interleukin. iNOS=inducible nitric oxide synthase. NF κ B=nuclear factor kappa B. NO=nitric oxide. TNF=tumor necrosis factor. TNFR1=tumor necrosis factor receptor 1.

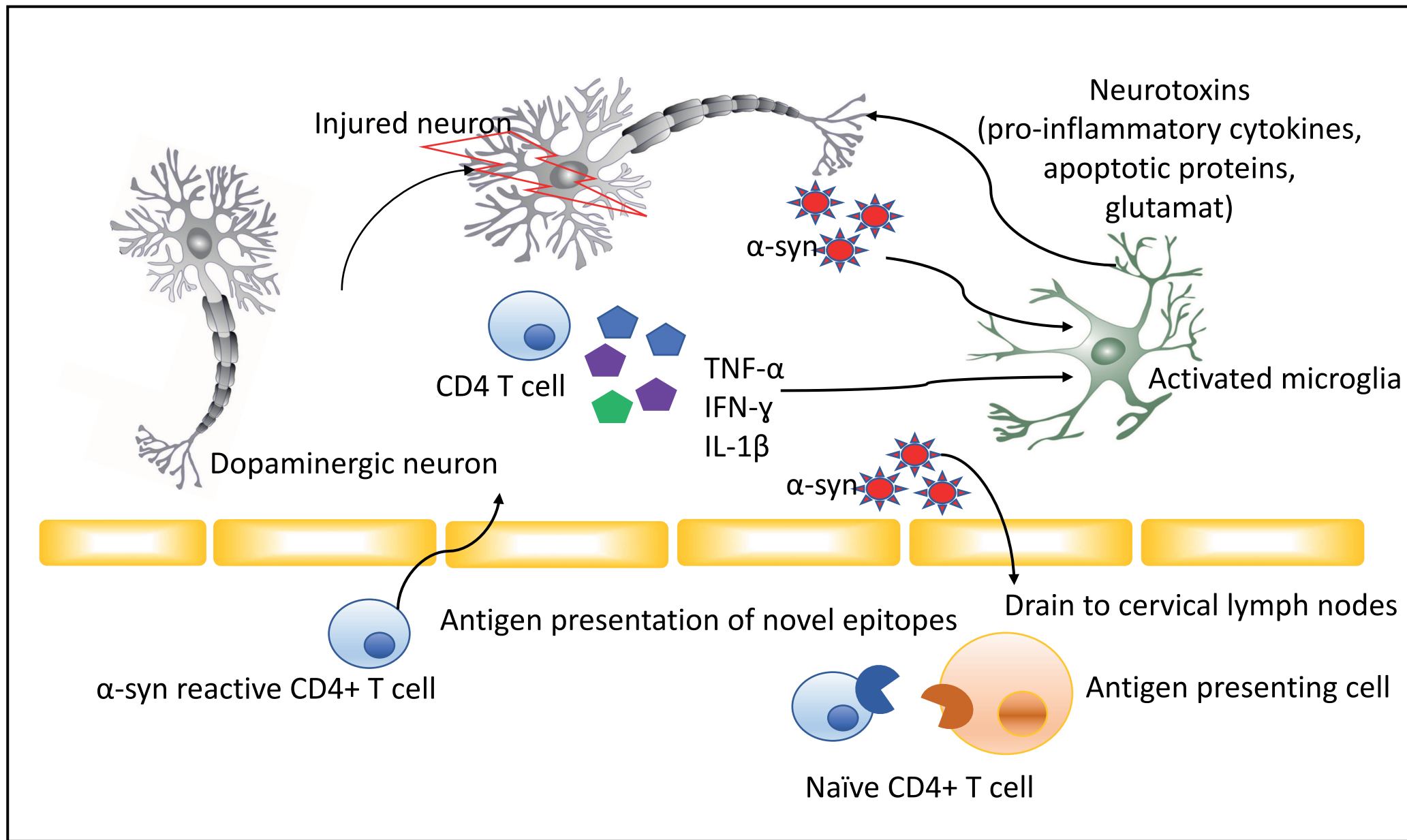


Figure 3: Contribution of peripheral immune system to the PD pathogenesis.

Activated microglia is a conspicuous feature of PD and it is very sensitive to changes in microenvironment, such as factors released from damaged/dying neurons like modified and aggregated proteins (α -synuclein, α -syn). Activated microglia may respond to the presence of these factors by secreting pro-inflammatory cytokines and ROS/RNS that will further perpetuate death of dopaminergic neurons. Furthermore, α -syn may drain to the peripheral lymph nodes where it will be recognized as an antigen and, that will differentiate into effector T cells and re-enter the CNS. Increased numbers of lymphocytes are found in the SN of PD patients' brains, but their exact role is still to be clarified. Nonetheless, experimental evidence suggests that effector T cell responses contribute to microglia activation and accelerate neurodegeneration.

3. Dopaminergic Receptors on CD4+ T Naïve and Memory Lymphocytes Correlate with Motor Impairment in Patients with Parkinson's Disease

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3.1. OVERVIEW

The present study was designed to examine alterations of CD4+ T cells and their expression of DR, with specific regard to naive and memory subsets, in peripheral blood of PD patients and in healthy subjects. Correlations of CD4+ T cell and cell subsets, as well as their respective expression of DR, were investigated with demographic and clinical features of the subjects. In addition, in preliminary experiments the *in vitro* effects of α -syn were evaluated on naive and memory CD4+ T cells. α -syn is the main component of LB and it has been confirmed that contributes to the pathogenesis of PD through different mechanisms, such as direct activation of microglial cells. In addition, it has been proposed that α -syn can act as an antigen itself leading to the activation of the adaptive immune response in the periphery (Harms et al., 2013; Reynolds et al., 2009; Sanchez-Guajardo et al., 2015). For these reasons, the effects of α -syn on CD4+ T cells were compared to effects that of a common recall antigen tetanus toxoid (TTd).

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3.2. *MANUSCRIPT*

**DOPAMINERGIC RECEPTORS ON CD4+ T NAIVE AND MEMORY LYMPHOCYTES
CORRELATE WITH MOTOR IMPAIRMENT IN PATIENTS WITH PARKINSON'S
DISEASE**

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27 **Abstract**

28 Parkinson's disease (PD) is characterized by loss of dopaminergic neurons in substantia nigra pars
29 compacta, α -synuclein (α -syn)-rich intraneuronal inclusions (Lewy bodies), and microglial
30 activation. Emerging evidence suggests that CD4⁺ T lymphocytes contribute to neuroinflammation
31 in PD. Since the mainstay of PD treatment is dopaminergic substitution therapy and dopamine is an
32 established transmitter connecting nervous and immune systems, we examined CD4⁺ T naive and
33 memory lymphocytes in PD patients and in healthy subjects (HS), with specific regard to
34 dopaminergic receptor (DR) expression. In addition, the *in vitro* effects of α -syn were assessed on
35 CD4⁺ T naive and memory cells. Results showed extensive association between DR expression in
36 T lymphocytes and motor dysfunction, as assessed by UPDRS Part III score. In total and CD4⁺ T
37 naive cells expression of D₁-like DR decrease, while in T memory cells D₂-like DR increase with
38 increasing score. In vitro, α -syn increased CD4⁺ T memory cells, possibly to a different extent in
39 PD patients and in HS and affected DR expression with cell subset-specific patterns. The present
40 results support the involvement of peripheral adaptive immunity in PD, and may contribute to
41 develop novel immunotherapies for PD, as well as to better use of current dopaminergic
42 antiparkinson drugs.

43

44 Parkinson's disease (PD) is the second most common neurodegenerative disorder, affecting an
45 estimated 7 to 10 million people worldwide and resulting in both motor and cognitive
46 disturbances^{1,2,3}. The main neuropathological features of PD are the progressive loss of
47 dopaminergic neurons in the substantia nigra pars compacta, the appearance of intraneuronal
48 inclusions called Lewy bodies, and the occurrence of microglial activation. Microglial cells in
49 particular are key players in neuroinflammation and neurodegeneration, and peripheral adaptive
50 immunity has been recently proposed as a major determinant in the regulation of microglial
51 function during neurodegenerative disease^{4,5,6,7}.

52 Over the last 15 years, several studies described the occurrence of peculiar modifications of
53 peripheral immunity in PD, such as decreased CD4⁺/CD8⁺ T-cell ratios, fewer CD4⁺CD25⁺ T
54 cells and increased ratios of interferon (IFN)- γ -producing to interleukin (IL)-4-producing T cells⁸,
55 as well as decreased CD4⁺ T lymphocytes and CD19⁺ B cells^{9,10}. Both CD8⁺ and CD4⁺ T cells
56 (but not B cells) were identified in the brain in both postmortem human PD specimens and in the 1-
57 methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD, and evidence obtained in
58 the animal model pointed to CD4⁺ T cells as main determinants of T cell-mediated dopaminergic
59 cell death¹¹. Remarkably, Saunders et al.¹² recently reported that PD patients have increased
60 effector/memory CD4⁺ T cells and decreased CD31⁺ and α 4 β 7⁺ CD4⁺ T cells, which are
61 associated with progressive motor dysfunction, suggesting a direct relationship between chronic
62 immune stimulation and PD neuropathology and disease severity, as well as strengthening the idea
63 that in PD the lead actors among adaptive immune system cells are CD4⁺ T lymphocytes.

64 No therapies are currently available for the neurodegenerative processes underlying PD, and
65 symptomatic treatments rely on the dopamine (DA) precursor L-DOPA as well on dopaminergic
66 agonists and on other indirect dopaminergic agents. Dopaminergic drugs relieve to some extent the
67 loss of brain dopaminergic neurons occurring in PD, although, as disease progresses, both nonmotor
68 and motor symptoms emerge that are resistant to dopaminergic medications¹³. Interestingly DA,
69 besides its role as brain neurotransmitter, is also an established transmitter connecting the nervous

70 and the immune system, as well as immune cells and peripheral tissues^{14,15,16}. DA affects both
71 innate and adaptive immune system cells, and immune cells produce DA, which may act as
72 autocrine/paracrine mediator on immune cells themselves and on neighboring cells^{17,18,19,20,21,22}.
73 Among T lymphocytes, CD4⁺ T cells may represent a major target for DA. DA subserve an
74 (auto)inhibitory loop in human CD4⁺CD25^{high} regulatory T lymphocytes, a specialized T cell subset
75 playing a key role in the control of immune homeostasis²³, and dendritic cells-derived DA affects
76 the differentiation of naive CD4⁺ T cells²⁴. The effects of DA are exerted through 5 different
77 dopaminergic receptors (DR) grouped into the D₁-like (D₁ and D₅) and the D₂-like (D₂, D₃ and
78 D₄)^{25,26}. Immune cells express all DR and in particular CD4⁺ T cells express both D₁-like and D₂-
79 like DR^{23,27}, with CD4⁺ naive T cells expressing more D₁-like than D₂-like DR, which on the
80 contrary are increased in memory T cells²⁸. Despite extensive evidence supporting the involvement
81 of CD4⁺ T cells (and in particular of memory T cells) in PD pathogenesis and progression^{8,10,11,12},
82 no information exists on DR expression on CD4⁺ T cells in PD patients. Nonetheless, a recent
83 investigation in the MPTP mouse model of PD suggested that DR D₃ expressed on CD4⁺ T cells
84 are critical for T cell-dependent microglial activation, which finally results in neurodegeneration²⁹.
85 The present study examined CD4⁺ T cells, as well as naive and memory CD4⁺ T cell subsets, in
86 PD patients and in healthy subjects, with specific regard to DR expression. Correlations of CD4⁺ T
87 cell and cell subsets, as well as their respective expression of DR, were investigated with
88 demographic and clinical features of the subjects. Patients on dopaminergic agents were compared
89 with recently diagnosed patients who never received dopaminergic medications. In addition, in
90 preliminary experiments the *in vitro* effects of α -synuclein (α -syn) were assessed on CD4⁺ T naive
91 and memory cells. α -syn is a protein expressed in brain and in peripheral tissues. It is the main
92 component of Lewy bodies and it may contribute to the pathogenesis of PD through different
93 concurrent mechanisms, including direct activation of microglial cells as well as possibly by acting
94 as an antigen itself, triggering the adaptive immune response in the periphery^{30,31,32}. For these

95 reasons, the effects of α -syn on CD4⁺ T naive and memory cells were also compared with those of
96 a common recall antigen like tetanus toxoid.

97

Results

Subjects. We enrolled 53 PD patients and 28 HS (Table 1). Patients comprised 16 subjects who had been never treated with antiparkinson drugs before enrollment and were therefore drug naive (PD-dn). In comparison to patients on antiparkinson drugs (PD-dt), PD-dn were younger and had on average lower H&Y stage, as well as UPDRS Part III score (Table 2). Plasma dopamine was 3.2 ± 5.7 nM in HS, 2.7 ± 3.3 nM in PD-dn patients ($P = 0.779$ vs. HS), and 8.0 ± 9.8 nM in PD-dt patients ($P = 0.003$ vs. HS).

Complete blood counts of PD patients and HS were all within normal limits (Table 3), however PD patients had less total lymphocytes, both in terms of absolute number (on average, about 17% less) and as percentage of white blood cells (-3.5%).

Complete blood count did not differ between PD-dn and PD-dt patients (Table 4), with the only exception of percentage of basophils, which were slightly lower in PD-dt.

T lymphocytes and CD4⁺ T naive and memory subsets. Reduction of lymphocytes in PD patients was accounted for essentially by T cells (Table 3). In particular, CD4⁺ T cells were about 21% less in PD patients in comparison to HS, even if the CD4⁺/CD8⁺ ratio did not change in patients, likely due the overall reduction of T lymphocytes. T lymphocyte subsets did not differ in PD-dn and PD-dt patients in terms of absolute counts, even if in PD-dn patients CD3⁺ T cells as percentage of total lymphocytes were less (-5.4%) and CD4⁺ T cells as percentage of CD3⁺ T cells were more (+6.4%) (Table 4).

To identify CD4⁺ T cell subsets, the gating strategy included the use of a biparametric dot plot CD45RA vs. CCR7, that allowed the identification of T naive (CD3⁺CD4⁺CD45RA⁺CCR7⁺), T_{CM} (CD3⁺CD4⁺CD45RA⁻CCR7⁺), and T_{EM} (CD3⁺CD4⁺CD45RA⁻CCR7⁻)²⁸. Among CD4⁺ T cells, PD patients showed decreased number of T naive cells (Fig. 1a). CD4⁺ T_{CM} and T_{EM} absolute numbers were not different in PD patients and in HS, however T_{EM} as percentage of total CD4⁺ T

123 cells were increased in PD patients (+3.3% on average) (Fig. 1c). PD-dn and PD-dt patients did not
124 differ in either absolute number or percentage of T naive, T_{CM} and T_{EM} (Fig. 1b,d).

125

126 **DR expression on CD4+ T cells.** DR expression was assessed at both mRNA level (in total
127 CD4+ T cells) and membrane protein level (in total CD4+ T cells and in T naive and memory
128 subsets). In comparison to cells from HS, CD4+ T cells from PD patients had lower mRNA levels
129 of the D₁-like DR D₅ and of the D₂-like DR D₃ and D₄, and higher mRNA levels of the D₂-like DR
130 D₃, while D₁-like DR D₁ mRNA levels were not different between cells from PD patients and HS
131 (Fig. 2a). Interestingly, both the D₁-like DR D₅ and the D₂-like DR D₂ mRNA levels were lower in
132 cells from PD-dn patients compared to cells from PD-dt patients (Fig. 2b).

133 Flow cytometric analysis of DR expression on CD4+ T cells revealed that in PD patients DR D₅+
134 CD4+ T cells were decreased in terms of both absolute number and percentage of total CD4+ T
135 cells (Fig. 2c,e), and that PD-dn patients had lower number and percentage of DR D₁+ and D₃+
136 CD4+T cells in comparison to PD-dt subjects (Fig. 2d,f).

137

138 **DR expression on CD4+ T naive and memory cells.** In comparison to HS, PD patients had
139 less D₁-like DR D₁+ and D₅+, as well as less D₂-like DR D₂+ and D₃+ T naive cells, both in terms
140 of absolute numbers and, for DR D₃+ cells, also of percentage of total CD4+ T cells (Fig. 3a,c). PD-
141 dn patients had less DR D₁+ T naive cells in comparison to PD-dt patients, in terms of both absolute
142 numbers and percentage of total CD4+ T cells (Fig. 3b,d).

143 No difference was found in DR expression in T_{CM} and T_{EM} between PD patients and HS, except for
144 DR D₃+ T_{CM} which were higher in PD patients in terms of percentage of total CD4+ T cells, and for
145 DR D₄+ T_{EM} which were higher in PD patients in terms of both absolute numbers and percentage of
146 total CD4+ T cells (see Supplementary Fig. S1 and S2 online). DR expression on T_{CM} or T_{EM} did
147 not differ between PD-dn and PD-dt patients (see Supplementary Fig. S1 and S2 online).

148

149 **Correlations between CD4+ T cells and demographic and clinical features of HS and PD**

150 **patients.** Age exerts major effects on lymphocyte function³³, and in particular T naive cells
151 may be reduced in elderly subjects³⁴. In agreement with these findings, in HS both the absolute
152 number as well as the percentage of CD4+ T naive cells negatively correlated with age (-0.395 (-
153 0.026 to -0.670), $P = 0.037$, and -0.472 (-0.120 to -0.718), $P = 0.011$). No correlation on the
154 contrary was found between age and the immune profile in PD patients, either as a whole or in PD-
155 dn and PD-dt patients.

156 The relationship between disease severity and CD4+ T cells was assessed by dividing PD patients
157 into 3 groups according either to the UPDRS Part III score or the H&Y stage (Table 1), thereafter
158 comparing each group with HS and analyzing the linear trend throughout the groups by means of
159 ANOVA. No relationship was found between CD4+ T naive or memory cells and the UPDRS Part
160 III score or the H&Y stage, with the only exception of a positive linear trend in the percentage of
161 T_{CM} cells and UPDRS Part III (see Supplementary Fig. S3 online).

162 In PD-dt patients, no relationship was found between either CD4+ T cells as a whole or CD4+ T
163 naive or memory cells and disease duration or LED (data not shown).

164

165 **Correlations between DR expression on CD4+ T cells and demographic and clinical features**

166 **of HS and PD patients.** DR mRNA levels in CD4+ T cells showed extensive correlations with
167 the UPDRS Part III score (Fig. 4a). The D₁-like DR D₅ mRNA levels decreased with increasing
168 UPDRS Part III scores. In comparison to CD4+ T cells from HS, in cells from PD patients DR D₅
169 mRNA levels were lower in the >20 UPDRS Part III score group. The D₁-like DR D₁ and the D₂-
170 like DR D₂, D₃ and D₄ mRNA levels did not show linear trends over the UPDRS Part III score,
171 however DR D₃ mRNA levels were higher than those in HS in the 1-10 and 11-20 UPDRS Part III
172 score groups, while DR D₄ mRNA levels were lower in all the UPDRS Part III score groups (Fig.
173 4a). Some correlations were also observed between DR mRNA levels and H&Y stage, as DR D₃

mRNA levels were higher than those in HS in the H&Y 1 and 2 stage groups, while DR D₄ was lower in H&Y stage 2 (see Supplementary Fig. S4 online).

Similar correlations with the UPDRS Part III score were observed for DR expression on CD4⁺ T cell membranes, in the case of DR D₅ (Fig. 4b,c). Correlations with the H&Y stage included DR D₅ expression lower than that in HS in the H&Y 2 and 3 stage groups, and DR D₃ expression lower than that in HS in the H&Y 3 stage groups (see Supplementary Fig. S5 online).

D₁-like DR D₁ and D₅ expression on cell membranes negatively correlated with the UPDRS Part III score in CD4⁺ T naive cells, while D₂-like DR D₂, D₃ and D₄ didn't show any major change (Fig. 5). On the contrary, D₂-like DR D₂ and D₄ increased with the UPDRS Part III score in both CD4⁺ T_{CM} and T_{EM} cells, while D₁-like DR did not change either in T_{CM} or T_{EM} (Fig. 5 and Supplementary Fig. S6 online). Only minor correlations were observed between DR expression and H&Y stage: D₁-like DR D₁ and D₅ and D₂-like DR D₃ were reduced in T naive from PD patients in comparison to cells from HS, and D₂-like DR D₂ were increased in T_{CM} (see Supplementary Fig. S7-S9 online).

No correlations were observed between DR mRNA levels and protein expression on CD4⁺ T cells or DR protein expression in CD4⁺ T naive and memory cells and age of HS or PD patients, or LED in PD-dt patients. However, in comparison to PD-dt patients treated with L-DOPA and dopamine agonists, those treated with L-DOPA alone had lower mRNA levels of DR D₁ ($6.0 \pm 4.1 \times 10^{-8}$ vs. $10.8 \pm 5.4 \times 10^{-8}$, $P = 0.035$), D₅ ($9.7 \pm 6.9 \times 10^{-8}$ vs. $20.6 \pm 7.5 \times 10^{-8}$, $P = 0.003$), and D₂ ($6.0 \pm 4.4 \times 10^{-8}$ vs. $9.4 \pm 3.3 \times 10^{-8}$, $P = 0.050$), as well as less percentage of CD4⁺ T cells which were DR D₁⁺ ($7.2 \pm 2.5\%$ vs. $10.3 \pm 3.5\%$, $P = 0.025$) or DR D₃⁺ ($4.5 \pm 1.5\%$ vs. $6.4 \pm 1.9\%$, $P = 0.017$), and of CD4⁺ T naive cells which were DR D₃⁺ ($3.5 \pm 1.2\%$ vs. $7.9 \pm 4.6\%$, $P = 0.009$). Patients treated with L-DOPA alone had also higher UPDRS Part III score (19.0 ± 4.3 vs. 14.8 ± 5.0 , $P = 0.028$) but similar H&Y stage (1.8 ± 0.7 vs. 1.8 ± 0.5 , $P = 0.933$).

***In vitro* responses of CD4⁺ T naive and memory cells to TTd and to α -syn.** The effect of α -syn on the frequency of CD4⁺ T naive and memory cells was tested on PBMC obtained from a group of 8 HS (F/M = 4/4, age = 58.1 $\pm$ 14.5 years) and 6 PD patients (F/M = 2/4, age = 76.7 $\pm$ 7.0 years, UPDRS Part III = 20.5 $\pm$ 3.1, H&Y = 1.8 $\pm$ 0.8) all treated with L-DOPA without (n = 4) and with DA agents (n = 2, in both cases rasagiline, in one case also ropinirole), with LED = 551.7 $\pm$ 140.1 mg/day.

Incubation of PBMC for 48 h with TTd resulted in reduced CD4⁺ T naive and increased T_{CM} and T_{EM} in both HS and PD patients, however the increase in T_{CM} and T_{EM} was higher in PD patients (Fig. 6a, left). Incubation of PBMC for 48 h with either monomeric or fibrillar α -syn resulted in reduced CD4⁺ T naive cells and increased T_{EM} cells in both HS and PD, however in PD patients fibrillar α -syn also increased T_{CM} and induced a more pronounced reduction of T naive cells than in HS (Fig. 6a).

Both monomeric and fibrillar α -syn induced several changes in the expression of DR on CD4⁺ T naive and memory cells (Fig. 6b). In particular, monomeric α -syn increased DR D₅ and D₂ in T naive cells, and DR D₂ in T_{CM}, while fibrillar α -syn increased DR D₁, D₂ and D₄ in T_{CM} and DR D₁ and D₄ in T_{EM}.

Co-incubation of PBMC with either DA, the D₁-like DR agonist SKF-38393, or the D₂-like DR agonists 7-OH-DPAT and PD-168,077 did not affect the frequency of CD4⁺ T naive and memory cells (see Supplementary Table S3, online). SKF-38393 (1 μ M) or the D₂-like DR agonist pramipexole (1 μ M) did not modify the effects of monomeric and fibrillar α -syn in PBMC of either HS and of PD patients (data not shown).

221

222 **Discussion**

223 The main result of our study is the evidence supporting an extensive association between DR
224 expression in T lymphocytes and motor dysfunction, as assessed by the UPDRS Part III score,
225 which is commonly used to measure disease severity in the clinical setting³⁵. Specifically, in total
226 CD4⁺ T cells as well as in CD4⁺ T naive cells the expression of D₁-like DR D₁ and D₅ decrease
227 with increasing UPDRS Part III score. On the contrary, D₂-like DR show changes only at the
228 mRNA level in total CD4⁺ T cells, do not exhibit major changes in CD4⁺ T naive cells, but show a
229 clear trend to increased expression with increasing UPDRS Part III score in T_{CM} and in T_{EM}. This is
230 the first study showing a connection between PD severity and DR expression on CD4⁺ T cells,
231 suggesting that dopaminergic pathways in peripheral immune cells are actively involved in PD. In
232 addition, we provided preliminary evidence that α -syn might affect CD4⁺ T memory cells, possibly
233 to a different extent in PD patients in comparison to HS.

234 Our results are in line with previous studies showing decreased CD4⁺ T lymphocytes in PD
235 patients^{9,10}, and in particular with Saunders et al.¹², who recently reported that in PD patients
236 increased effector/memory CD4⁺ T cells correlated with increased motor dysfunction. In our study
237 PD patients had decreased absolute count of CD4⁺ T naive cells, increased percentage of T_{EM} cells,
238 and T_{CM} not different from those in HS. Indeed, our flow cytometric strategy²⁸ allowed to
239 distinguish between T_{CM}, which mediate reactive memory by homing to T cell areas of secondary
240 lymphoid organs, and T_{EM}, which afford protective memory, by migrating to inflamed peripheral
241 tissues and displaying immediate effector function^{36,37}. At apparent variance with the study by
242 Saunders et al.¹², who reported that in PD patients effector/memory CD4⁺ T cells increased with the
243 UPDRS Part III score, we did not identify any clear correlation between T naive/memory cells and
244 the UPDRS Part III score. Saunders et al.¹² however identified T memory cells by using CD45RO
245 expression, and found increased CD4⁺ T memory cells only in PD patients with UPDRS Part III
246 score ≥ 31 , while in our study we enrolled only 7 patients with a score above 20, the highest score

247 being 24, and nonetheless we identified increased T_{EM} in PD patients, possibly also thanks to the
 248 specific flow cytometry staining strategy which included the expression of CD45RA and CCR7,
 249 and allowed to distinguish between T_{EM} and T_{CM}²⁸. It remains however to be established whether
 250 the enhanced peripheral T memory function occurring in PD patients is mainly T_{EM}, in line with the
 251 possibility that peripheral immune activation in PD has at least in part a protective role.
 252 Concerning the general peripheral immune profile, we also observed reduced CD4⁺ T naive cells
 253 with increasing age in HS but not in PD patients. Reduction of T naive cells in elderly subjects is
 254 well described and is believed to result from thymic involution in combination with ongoing
 255 differentiation of T naive cells into antigen-experienced memory/effector cells³⁴. In PD patients, the
 256 absence of correlations between T naive cell count and age, together with the reduced number of T
 257 naive cells in comparison to HS, is indeed in agreement with the hypothesis that PD is associated
 258 with increased peripheral immune exposure to antigens. A contribution by dysregulated thymic T
 259 cells development cannot be discarded, however, also in view of the lack of studies on thymic
 260 function during PD.
 261 Although many immune cell subsets are dysregulated in PD, the key role of CD4⁺ T cells in the
 262 pathogenesis of the disease is supported by their presence, together with CD8⁺ T cells, in the brain
 263 in both postmortem human PD specimens and in the MPTP mouse model of PD, and evidence
 264 obtained in the animal model indicate that CD4⁺ T cells are determinants of T cell-mediated
 265 dopaminergic cell death¹¹. Moreover, a recent study in MPTP-treated mice showed that CD4⁺ T
 266 cells are necessary for MPTP-induced neurodegeneration and that D₂-like DR D₃ expressed on T
 267 cells favor their activation and acquisition of the Th1 inflammatory phenotype²⁹.
 268 Our results show that mRNA expression of several DR are dysregulated in CD4⁺ T cells from PD
 269 patients: in particular, in comparison to cells from HS, in cells from PD patients mRNA for the D₁-
 270 like DR D₅ and for the D₂-like DR D₃ and D₄ are decreased, and mRNA for the D₂-like DR D₃ is
 271 increased (Fig. 2a,b). Flow cytometry analysis of DR expression on CD4⁺ T cell membranes
 272 provides however a more homogeneous picture (Fig. 2c,d,e,f), with D₁-like DR D₅ clearly reduced

273 by 30-49% in cells from PD patients. Interestingly, reduction of D₁-like DR was evident for both
274 DR D₁ and D₅ in CD4⁺ T naive cells (Fig. 3), while no difference occurred in T_{CM} or T_{EM} cells (see
275 Supplementary Fig. S1 and S2 online).

276 Little information is available on the physiopharmacology of D₁-like DR-operated pathways in T
277 cells. D₁-like DR D₅ likely mediate the inhibitory effects of dopamine on proliferation and
278 cytotoxicity of human CD4⁺ and CD8⁺ T cells⁴², however they also play a role in the inhibition of
279 human CD4⁺CD25^{high} regulatory T cells, thus resulting in a “suppression of the suppressors”²³.

280 Interestingly, *in vitro* in human naive CD4⁺ T cells, dopamine via D₁-like DR shifted T-cell
281 differentiation towards Th2, in response to stimulation with anti-CD3 and anti-CD28 mAb²⁴.

282 Reduced D₁-like DR on CD4⁺ T cells in PD patients might thus lead to several effects, such as
283 increased CD4⁺CD25^{high} regulatory T cell function and increased Th1/Th2 balance. Saunders et
284 al.¹² however, reported impaired function of CD4⁺CD25^{high} regulatory T cells from PD patients,
285 thus suggesting that reduced D₁-like DR may have no direct effects on this specialized cell subset.

286 Indeed, our preliminary unpublished data from another protocol, included in this same research
287 program and aimed at investigating DR expression on CD4⁺ T helper subsets, likely suggest that
288 PD patients have a Th1-biased peripheral immune profile. This observation is in agreement with the
289 previously reported increased ratios of IFN- γ -producing to IL-4-producing T cells in PD patients⁸,
290 as well as with the role of D₁-like DR on human CD4⁺ T naive cells which, according to Nakano et
291 al.²⁴, shift T-cell differentiation towards Th2. It can thus be suggested that reduced D₁-like DR on
292 CD4⁺ T naive cells in PD patients impair their ability to differentiate towards Th2, promoting a
293 Th1-biased proinflammatory profile.

294 D₁-like DR on CD4⁺ T lymphocytes, which are generally reduced in PD, also display a close
295 correlation with PD patients motor dysfunction, as assessed by the UPDRS Part III score. Indeed,
296 DR D₅ expression diminishes with increased UPDRS Part III score, both at the mRNA level as well
297 as in terms of percentage of CD4⁺ T cells which express the specific receptors (Fig. 4), a behavior
298 which is evident also in CD4⁺ T naive cells, for both DR D₁ and D₅, but not in T_{CM} or T_{EM} (Fig. 5).

299 By contrast, CD4⁺ T_{CM} and T_{EM} cells generally display a linear trend towards increased D₂-like DR
300 (Fig. 5 and Supplementary Fig. S6). Such close association with the UPDRS Part III score was not
301 always parallel with the H&Y stage. In particular, in CD4⁺ T naive cells DR D₁ and D₅ decreased,
302 and in T_{CM} and T_{EM} DR D₂ and D₄ increased with increasing UPDRS Part III score (Fig. 5 and
303 Supplementary Fig. S6) but not with increasing H&Y scale stage (Supplementary Fig. S7-9). A
304 likely explanation is that only one of the 7 subjects with UPDRS Part III >20 is included among the
305 4 subjects with H&Y 2.5-3.0 (Table 2). Whether UPDRS Part III scale profiles the underlying
306 immune dysfunction occurring in PD patients better than the H&Y scale remains to be established.
307 Unfortunately, the only other study correlating CD4⁺ T cells and motor dysfunction in PD
308 considered just the UPDRS Part III score.¹²

309 PD patients with more severe motor dysfunction (score >20) have T_{CM} cells expressing 131-134%
310 more DR D₂ and 112-126% more DR D₄ than cells from HS, and 64-100% more DR D₂ 64-105%
311 more DR D₄ in comparison to cells from PD patients with score 1-10. The picture is similar with
312 T_{EM}, as PD patients with score >20 have T_{EM} cells expressing 93-112% more DR D₂ and 48-71%
313 more DR D₄ than cells from HS, and 71-111% more DR D₂ and 22-38% more DR D₄ in
314 comparison to cells from PD patients with score 1-10. As a whole, it appears therefore that, with
315 increasing motor dysfunction, D₁-like DR decrease on CD4⁺ T lymphocytes and in particular on
316 CD4⁺ T naive cells, while D₂-like DR, increase specifically on CD4⁺ T_{CM} and also on T_{EM} cells.
317 As discussed above, reduced D₁-like DR on CD4⁺ T naive cells may promote a Th1-biased
318 proinflammatory profile, and the present results suggest that such trend increases with increasing
319 motor dysfunction. There is on the contrary paucity of data regarding the role of D₂-like DR on T
320 lymphocytes, even if Levite et al.⁴³ showed that activation of either DR D₂ or D₃ might induce T
321 cell proliferation and adhesion. Of potential relevance for the present results, it was recently
322 reported that, in the MPTP mouse model of PD, D₂-like DR D₃ expressed on CD4⁺ T cells are
323 critical for T cell-dependent microglial activation²⁹. If the same applied to PD patients and to D₂-
324 like DR-operated pathways as whole (as in PD patients DR D₂ and D₄, but not DR D₃, correlated

with motor impairment), increased D₂-like DR in the more advanced stages of the disease might imply increased activation of the peripheral immune system, in turn triggering central neuroinflammation leading to neurodegeneration and disease progression. Nonetheless, such findings should be interpreted cautiously since studies also exist showing that at least stimulation of the D₂-like DR D₄ may result in quiescence of human T cells³⁰. It is therefore necessary to clarify the role of individual DR in the modulation of memory T lymphocytes and in their relationship with microglia in PD. In addition, the eventual role of antiparkinson treatments on DR expression on T lymphocytes needs careful consideration, as discussed hereafter.

Comparison between PD-dn and PD-dt patients did not reveal any major differences in the peripheral immune profile. In particular, absolute numbers of CD3+ and CD4+ T cells were not different, although percentage CD3+T cells were slightly higher in PD-dt patients while percentage CD4+T cells were slightly higher in PD-dn patients (Table 4), and T naive, T_{CM} and T_{EM} were similar in the two patient populations (Figure 1b,d). A remarkable difference was however found in D₁-like DR D₁ expression in CD4+ T cells and in particular in T naive cells (Figures 2 and 3), as PD-dt patients had higher expression of DR D₁ in comparison to PD-dn. This difference might be of interest as PD-dn patients have on their T naive cells only 22-35% DR D₁ in comparison to HS T naive cells, while PD-dt patients have on their T naive cells 41-63% DR D₁ in comparison to HS T naive cells. Whether this is an effect of dopaminergic antiparkinson treatments cannot be established on the basis of the present results, also taking into account that no relationship was found between PD duration and/or LED and the immune profile or DR expression in CD4+ T cells and cell subsets. The hypothesis should be nonetheless taken into account, since - as above discussed - D₁-like DR on human CD4+ T naive cells may shift T-cell differentiation towards Th2²⁴. Provided that this role of D₁-like DR on T naive cells has any clinical relevance, it might be predicted that in the study which we are presently performing, aimed at investigating DR expression on CD4+ T helper subsets in PD patients, we will find less Th1 cells in PD-dt patients in comparison to PD-dn patients.

351 α -Syn is the major component of Lewy bodies and a key factor in PD pathogenesis. Pathological α -
352 syn released by degenerating neurons activates microglia to a proinflammatory profile³², and directs
353 cell migration⁴⁴. Efflux of α -syn from the brain to peripheral blood has been reported in mice and
354 possibly in PD patients⁴⁵, and it has been hypothesized that it might prime T cells that, in turn,
355 would enter the brain and sustain microglia activation and neurodegeneration⁴⁶. It has also been
356 suggested that the presence of aberrant forms of α -syn in the periphery may represent a possible
357 means for exposure as a neoantigen and subsequent activation of the adaptive immune system⁴⁷.
358 In our study, we performed preliminary experiments aimed at assessing the effects of different
359 forms of α -syn on CD4⁺ T naive and memory cells, in comparison to a well established recall
360 antigen like TTd. We tested both monomeric and fibrillar α -syn since accelerated fibril formation
361 by certain variants of α -syn are associated to PD pathogenesis^{48,49}. As expected, TTd reduced the
362 frequency of T naive cells while increasing T_{CM} and T_{EM}. The effect on T_{CM} and T_{EM} was however
363 more pronounced in cells from PD patients, possibly in line with the activated profile of peripheral
364 immune system in PD. Interestingly, both monomeric and fibrillar α -syn induced a response which
365 was qualitatively similar to the one evoked by TTd. Both PD patients and HS responded to the same
366 extent to α -syn, with the only exception of fibrillar α -syn, which increased T_{CM} cells in PD patients
367 but not in HS.

368 Available evidence of course does not allow to conclude that the responses to α -syn are actually due
369 to recognition of the protein by T memory cells, although the response pattern is similar to that
370 induced by TTd, and the ability of the peripheral immune system to recognize α -syn is also
371 supported by the occurrence of specific antibodies in the serum of PD patients and HS^{50,51,52}.
372 Nonetheless, the ability of fibrillar α -syn to increase T_{CM} is suggestive, as these cells mediate
373 reactive memory, by homing to T cell areas of secondary lymphoid organs and readily proliferating
374 and differentiating to effector cells upon antigenic stimulation^{36,37}.

375 It is also remarkable that incubation with α -syn affected DR expression on CD4⁺ T cells, and that in
376 particular fibrillar α -syn induced increased expression of DR D4 in both T_{CM} and T_{EM}, a finding

377 which resembles increased D₂-like DR in T_{CM} and T_{EM} of PD patients with more severe motor
378 dysfunction. Whether increased expression of DR corresponds to increased responsiveness, and
379 which consequences might be implied for the pro/antiinflammatory balance of peripheral (and
380 possibly also central) immunity need to be carefully considered.

381 **Concluding remarks and perspectives**

382 It is noteworthy that in the present study we were unable to find any association between
383 dopaminergic substitution treatments and the peripheral immune profile. Possible explanations
384 include that L-DOPA may undergo conversion to dopamine only in the brain, and that dopaminergic
385 receptor agonists are usually D₂-like DR selective (pramipexole, ropinirole). Rotigotine is the only
386 dopaminergic agonist currently used in PD that has comparable affinity for D₂-like DR and at least
387 for the D₁-like D₅, however our study enrolled only four subjects on rotigotine (out of a total of 45).
388 Our study included also a group of newly diagnosed PD patients who never received dopaminergic
389 treatments. The main difference between newly diagnosed and antiparkinson-treated subjects
390 consisted in an even lower expression of D₁-like DR D₁ on total CD4⁺ T cells as well as in T naive
391 cells in newly diagnosed patients, however it remains to be established whether the increased D₁-
392 like DR D₁ expression in antiparkinson-treated patients is actually due to antiparkinson drugs and/or
393 to other factors. In order to clarify this issue, we have already started a longitudinal study on a
394 larger sample of drug naïve PD patients, who will be tested before and after pharmacological
395 treatment.

396 Anyway, from a general point of view it is possible to conclude that dopaminergic substitution
397 treatments have only minor, if any, impact on the peripheral immune system of PD patients, which
398 on the other side shows profound differences in comparison to that of HS. In particular, specific
399 differences related to dopaminergic pathways in immune cells definitely support the notion of a
400 chronic peripheral immune activation in PD patients, which may affect disease severity.

401 Immunotherapy is being increasingly regarded as an attractive strategy even in PD⁴⁷, and it is
402 therefore a priority to unravel the peripheral immune dysregulation occurring in PD patients, to plan

403 adequate immunotherapeutic interventions. In addition, since antiparkinson therapy still lies mainly
404 (if not only) on dopaminergic substitution therapy, detailed understanding of the role of
405 dopaminergic pathways in the immune system might possibly allow a more appropriate use of
406 available drugs, simply by better exploitation of their immunomodulating potential^{14,15,16,53}.
407

408 **Materials and Methods**

409 **Subjects.** Peripheral venous blood samples were collected from patients with idiopathic PD⁵⁴,
410 either drug naive (PD-dn, i.e. PD patients who never received L-DOPA, DA agonists and/or other
411 antiparkinson drugs) or on antiparkinson drug treatment (PD-dt), and from age- and sex-matched
412 healthy subjects (HS). PD was diagnosed according to the United Kingdom Parkinson's Disease
413 Society Brain Bank Criteria. Patients and controls with a history of autoimmune or inflammatory
414 disorders and those receiving chronic immunosuppressive treatment were excluded.

415 Participants were recruited through the Centre for Parkinson's Disease and Movement Disorders of
416 the Neurological Service at the Ospedale di Circolo of Varese, the Interdepartmental Research
417 Center for Parkinson's Disease of the Neurological Institute "C. Mondino" of Pavia, and the
418 Movement Disorders Center of the University of Piemonte Orientale, Divisione di Neurologia,
419 Ospedale Maggiore of Novara, Italy. Healthy subjects were spouses and caregivers of enrolled PD
420 patients. The Ethics Committees of Ospedale di Circolo of Varese and Neurological Institute "C.
421 Mondino" of Pavia approved the protocol and all the participants signed a written informed consent
422 before enrollment. The study was performed according to the Declaration of Helsinki and to the
423 relevant ethical guidelines for research on humans.

424 After enrollment, subjects were submitted to a complete examination. PD patients were staged
425 according to the criteria of Hoehn and Yahr (H&Y)⁵⁵ and evaluated by means of the Unified
426 Parkinson's Disease Rating Scale (UPDRS) Part III⁵⁶. UPDRS Part II score was also assessed
427 whenever possible. Data on patients and healthy controls were collected using standard data forms,
428 which included demographics, diagnostic features, family history, primary diagnosis, PD features,
429 UPDRS Part III score, and Hoehn and Yahr (H&Y) stage. Antiparkinson drug doses were recorded
430 at the time of enrollment and L-DOPA equivalent doses (LED) were calculated according to
431 established guidelines⁵⁷.

432 Withdrawal of 30 ml venous blood was performed after a fasting night, between 8:00 a.m. and
433 10:00 a.m., in EDTA-coated tubes (BD Vacutainer). Tubes were subsequently coded and stored at

room temperature until processing, which occurred within 24 hours after collection. Complete blood cell count with differential analysis was conducted on separate blood samples collected in EDTA-coated tubes (BD Vacutainer). Serum levels of dopamine were assayed by high-performance liquid chromatography with multielectrode electrochemical detection (HPLC-ED) according to a previously described method²³.

Reagents. Bovine serum albumin (BSA) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma, Italy. RPMI 1640, heat-inactivated fetal bovine serum (FBS), glutamine, and penicillin/streptomycin were obtained from Euroclone, Italy. Ficoll-Paque Plus was from Pharmacia Biotech (Uppsala, Sweden). Purified mouse ab anti-human CD3 (code 555330, clone UCHT1, Mouse IgG1, κ) and purified mouse ab anti-human CD28 (code 555726, clone CD28.2, Mouse IgG1, κ) were obtained from Becton Dickinson, Italy. ($\pm$)SKF-38,393 hydrochloride (cod. D047), R(+)-7-OH-DPAT hydrobromide (code H168), PD-168,077 maleate (code P233), pramipexol dihydrochloride (code A1237), and dopamine hydrochloride (code H8502) were all from Sigma, Italy. Human recombinant α -synuclein and its fibrillar form were a kind gift from Dr. Lars Kjær and Dr. Daniel Otzen (iNANO - Interdisciplinary Nanoscience Center, Aarhus University, Aarhus, Denmark), and were prepared as published before⁵⁸.

Flow cytometric analysis of naive and memory subsets of CD4⁺ T cells and of DR expression in whole blood. Analysis of CD4⁺ T naive and memory subsets and of DR expression was performed according to previously established method²⁸. Briefly, 100 μ l aliquots of whole blood were prepared, and erythrocytes were removed by means of a lysis buffer ((g/L) NH₄Cl 8.248, KHCO₃ 1.0, EDTA 0.0368). Samples were then centrifuged, supernatants were removed and cells were washed in PBS (pH 7.4) supplemented with 1% BSA (PBS/BSA) and resuspended in PBS/BSA. Total leukocytes were counted by means of a hemocytometer and cell viability, determined by the Trypan blue exclusion test, was always >99%.

460 From each subject 7 aliquots of 100 μ L were prepared: 5 were used for DR staining, 1 was used as
461 control for the secondary PE-goat anti-rabbit (PEGAR) ab, and 1 was used as negative control (no
462 ab). The staining protocol consisted of two steps. During the first step each aliquot was stained for
463 one of the five DR by an indirect labeling procedure (primary ab + secondary ab labeled with PE).
464 During the second step all the aliquots were incubated with a cocktail of anti-human CD3, CD4,
465 CD45RA and CCR7 ab for the identification of T lymphocytes, CD4⁺ T lymphocytes and the
466 following CD4⁺ T lymphocyte subsets: naive (CD3+CD4+CD45RA+CCR7⁺), central memory
467 (T_{CM}, CD3+CD4+CD45RA-CCR7⁺), and effector memory (T_{EM}, CD3+CD4+CD45RA-CCR7⁻).
468 The complete list of ab used in the study is shown in Supplementary Table S1, online.
469 Acquisition was then performed on a BD FACSCanto II flow cytometer (Becton Dickinson, Milan,
470 Italy) with BD FACSDiva software (version 6.1.3). Lymphocytes were identified by means of their
471 classical forward scatter (FSC) and side scatter (SSC) signals and a minimum of 20,000
472 lymphocytes from each sample was collected in the gate. Data were analyzed with the FlowJo
473 software (version 8.3.2). The results were finally expressed as absolute numbers (10⁶/ml) as well as
474 percentage of positive cells (%).

475

476 **Isolation of peripheral blood mononuclear cells (PBMC).** PBMC were isolated from whole
477 blood by using Ficoll-Paque Plus density gradient centrifugation. Cells were resuspended and, if
478 necessary, any residual contaminating erythrocytes were lysed by addition of 5 mL of lysis buffer,
479 followed by incubation for 5 min, during which samples were gently vortexed, and centrifugation at
480 100 g for 10 min at RT. Cells were washed twice in PBS by addition of 15 ml of PBS and
481 centrifugation at 300 g and 10 min at RT, and resuspended at the final concentration of 10x10⁶ cells
482 in 10 ml of RPMI/10% FBS for subsequent culture. Typical PBMC preparations contained at least
483 80% lymphocytes, as assessed by flow cytometry. Cell viability assessed by the Trypan blue
484 exclusion test was always >99%.

485

Real-time PCR assay of DR mRNA in CD4+ T cells. CD4+ T cells were isolated from PBMC
 by immunomagnetic sorting using Dynalbeads CD4 Positive Isolation kit (Life Technologies, code
 11145D). Real-time PCR of DR mRNA was performed according to a previously reported method
 with modifications⁵⁹. Briefly, to isolate RNA, at least 50000 CD4+ T cells were resuspended in
PerfectPure RNA lysis buffer (5 Prime GmbH, Hamburg, Germany), total RNA was extracted by
PerfectPure RNA Cell KitTM (5 Prime GmbH), and the amount of extracted RNA was estimated by
 spectrophotometry at $\lambda = 260$ nm. Total mRNA obtained from CD4+ T cells was reverse-
 transcribed using a random primer, high-capacity cDNA RT kit (Applied Biosystems). cDNA was
 then amplified with *SsoAdvancedTM Universal Probes Supermix* (BIORAD) for the analysis of DR
 D₂, DR D₃, and DR D₅ gene expression, and with *SsoAdvancedTM Universal SYBR[®] Green
 Supermix* (BIORAD) for analysis of DR D₁, and DR D₄ gene expression. cDNA was assayed on
 StepOne[®] System (Applied Biosystems). Real-time PCR conditions are shown in Supplementary
 Table S2, online.

Linearity of real-time PCR assays were tested by constructing standard curves by use of serial 10-
 fold dilutions of a standard calibrator cDNA for each gene, and regression coefficients (r^2) were
 always >0.999; a melting curve was also performed to check for specificity of DR D₁ (melting
 temperature = 83.5°C) and DR D₄ (melting temperature = 90°C). Gene expression level in a given
 sample was represented as $2^{-\Delta Ct}$ where $\Delta Ct = [Ct(\text{sample}) - Ct(\text{housekeeping gene})]$. Relative
 expression was determined by normalization to 18S cDNA. Analysis of the data were performed by
 StepOne softwareTM 2.2.2 - Applied Biosystems).

Frequency of CD4+ T naive and memory subsets in cultured PBMC. Isolated PBMC
 were cultured in RPMI/10% FBS for 48 h at 37°C in a moist atmosphere of 5% CO₂, without or
 with anti-CD3/anti-CD28 ab (0.1 µg/ml). Tetanus toxoid (TTd, 3 µg/ml), monomeric or fibrillar α -
 syn (both 500 nM) were added at the beginning of cell culture. Cells were finally harvested and

511 stained for flow cytometric analysis of naive and memory subsets of CD4⁺ T cells, as described in
512 section regarding flow cytometric analysis of naive and memory subsets of CD4⁺ T cells.
513

514 **Statistical analysis.** Distribution of the values was assessed by the D'Agostino & Pearson
515 normality test. Statistical significance of the differences between HS and PD patients and between
516 PD-dn and PD-dt patients was then analyzed by means of two-tailed Student's *t* test or by the Mann-
517 Whitney test, as appropriate, for continuous variables, and by the Fisher's exact test for categorical
518 variables. Correlations among continuous variables were assessed by Pearson or Spearman
519 correlation analysis. Differences between HS and PD patients categorized for UPDRS Part III score
520 or H&Y stage were analyzed by ordinary one-way ANOVA or by the Kruskal-Wallis test, with
521 either Holm-Sidak's or Dunn's adjustments for multiple comparisons, and trend analysis in PD
522 patients was performed by ANOVA post test for linear trend. Calculations were performed using
523 commercial software (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego
524 California USA, www.graphpad.com).
525

526

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548

549 **Author contribution**

550 **Study conception and design:** MC, FM, FB

551 **Acquisition of data:** NK, ER, ML, RB, IA, BM, GR

552 **Analysis and interpretation of data:** NK, MC, FB, CC, MM, VSG, FM

553 All authors were involved in drafting the article or revising it critically for important intellectual
554 content, and all authors approved the final version to be published. All authors agree to be
555 accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity
556 of any part of the work are appropriately investigated and resolved and declare to have confidence
557 in the integrity of the contributions of their co-authors.

558

559 **Competing financial interests**

560 The authors declare that they have no competing financial interests.

561

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697

698

a

1500



HS



PR

1000

699

700 **Figure 1. CD4+ T naive and memory cells in HS and PD patients.** Cells are shown as absolute
701 numbers (panels a and b) and as percentage of total CD4+ T cells (c and d). Data are shown as
702 medians with 25°-75° percentiles (boxes) and min-max values (whiskers). Comparisons are shown
703 between HS and PD patients as a whole (a and c) and between drug naive (PD-dn) and drug treated
704 (PD-dt) patients (b and d). Differences were analyzed by means of two-tailed Student's *t* test or by
705 Mann-Whitney test, as appropriate. P values less than 0.05 are indicated in the graphs.

706

a

**Δ levels
(-Ct)**

10^{-4}



HS



PD

10^{-5}

10^{-6}

707

708 **Figure 2. DR expression on CD4+ T cells from HS and from PD patients.** DR expression is
709 shown as mRNA levels (panels a and b) and as protein expression on the membranes of CD4+ T
710 cells, expressed as absolute numbers of DR+ cells (c and d) and as percentage of total CD4+ T cells
711 (e and f). Comparisons are shown between HS and PD patients as a whole (a, c, and e) and between
712 drug naive (PD-dn) and drug treated (PD-dt) patients (b, d, and f). Data are shown as medians with
713 25°-75° percentiles (boxes) and min-max values (whiskers). Differences were analyzed by means of
714 two-tailed Student's *t* test or by Mann-Whitney test, as appropriate. P values less than 0.05 are
715 indicated in the graphs.

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a

500



P



P

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718 **Figure 3. DR expression on CD4+ T naive cells from HS and from PD patients.** DR+ cells are

719 shown as absolute numbers (panels a and b) and as percentage of total CD4+ cells (c and d). Data

720 are shown as medians with 25°-75° percentiles (boxes) and min-max values (whiskers).

721 Comparisons are shown between HS and PD patients as a whole (a and c) and between drug naive

722 (PD-dn) and drug treated (PD-dt) patients (b and d). Differences were analyzed by means of two-

723 tailed Student's *t* test or by Mann-Whitney test, as appropriate. P values less than 0.05 are indicated

724 in the graphs.

725

D₁-like

726

727 **Figure 4. DR expression on CD4+ T cells and UPDRS-III score.** DR expression is shown as
728 mRNA levels (panel a) and as protein expression on the membranes of CD4+ T cells, expressed as
729 absolute numbers of DR+ cells (b) and as percentage of total CD4+ T cells (c). Data are medians
730 with 25°-75° percentiles (boxes) and min-max values (whiskers). Differences in DR expression
731 between HS and PD patients were analyzed by parametric ANOVA or Kruskal-Wallis
732 nonparametric ANOVA, as appropriate, with either Holm-Sidak's or Dunn's adjustments for
733 multiple comparisons, where * = $P < 0.05$ and ** = $P < 0.01$. Trend analysis in PD patients was
734 performed by ANOVA post test for linear trend.

735

D₁-like

DI

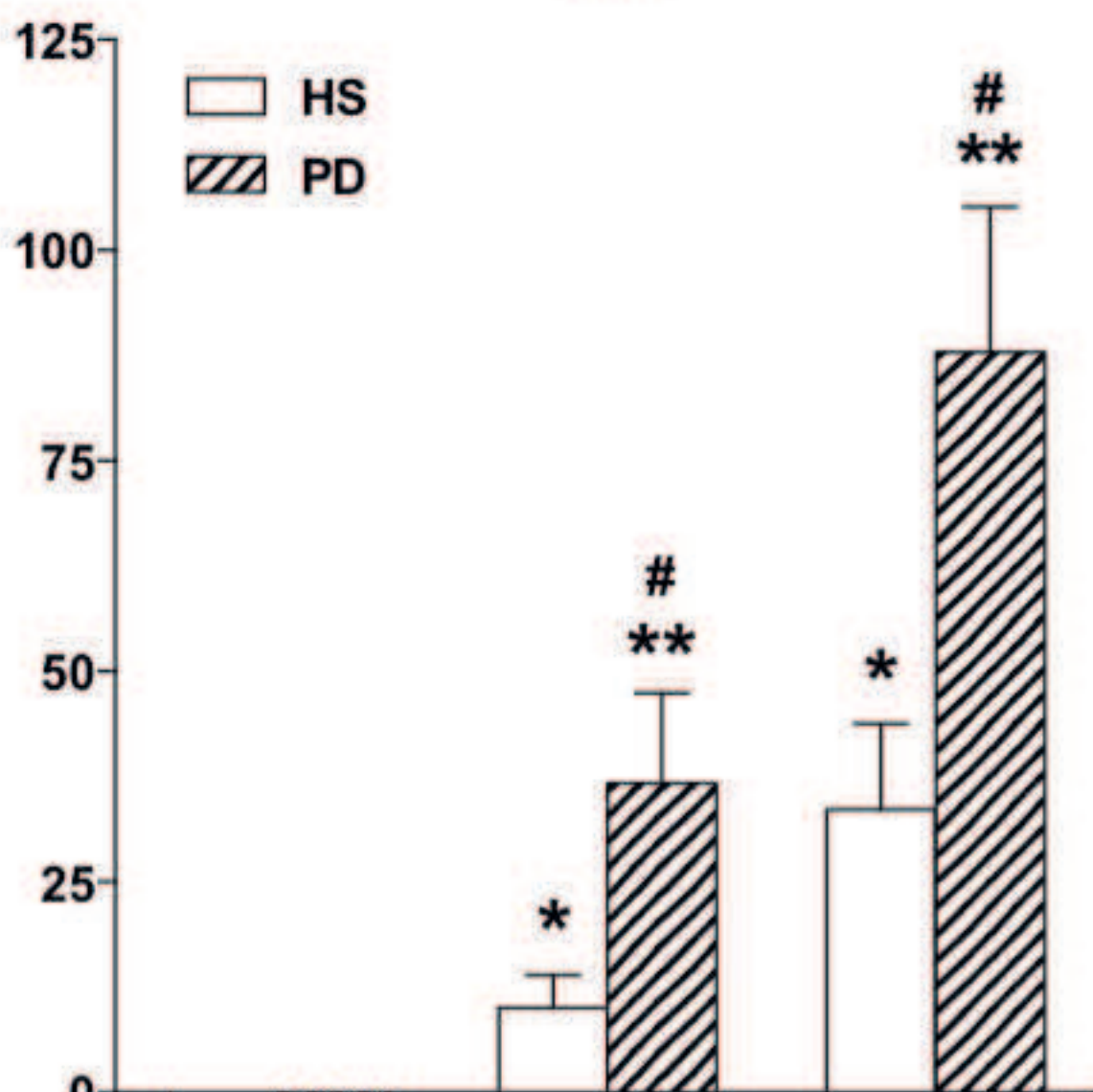
736

737 **Figure 5. DR expression in CD4+ T naïve, T_{CM} and T_{EM} and UPDRS-III score.** DR expression
738 is shown as protein expression on the membranes of CD4+ T naïve (left), T_{CM} (center) and T_{EM}
739 (right) cells, expressed as absolute numbers of DR+ cells. Data are medians with 25°-75°
740 percentiles (boxes) and min-max values (whiskers). Differences between DR levels in HS and in
741 PD patients were analyzed by parametric ANOVA or Kruskal-Wallis nonparametric ANOVA, with
742 either Holm-Sidak's or Dunn's adjustments for multiple comparisons, where * = P<0.05 and ** =
743 P<0.01. Trend analysis in PD patients was performed by ANOVA post test for linear trend.

744

a

TTd



745

746 **Figure 6. Effect of TTd, monomeric, and fibrillar α -syn on the frequency of CD4+ T naive and**
747 **memory subsets.** Panel a: Effects of TTd (left), monomeric (middle), and fibrillar α -syn (right) in
748 cells from HS (open columns) and PD patients (hatched columns). Data are expressed as percentage
749 variation with respect to control conditions (without TTd or α -syn), and are means $\pm$ SEM of n = 6-8
750 separate experiments each performed in duplicate. * = P<0.05 and ** = P<0.01 vs. control
751 conditions, and # = P<0.01 vs. HS. Panel b: monomeric (hatched columns), and fibrillar α -syn
752 (shaded columns) on DR expression in T naive (left), T_{CM} (center) and T_{EM} cells (right) from 5 HS.
753 Data are means $\pm$ SEM. * = P<0.05 and ** = P<0.01 vs control (open columns); # = P<0.01 vs
754 monomeric α -syn.

755

Supplementary Table S1. List of antibodies used in flow cytometric assays.

Target	Supplier	Species	Type	Conjugation	Dilution	Reference
human DR D ₁	Calbiochem-Inalco	rabbit	IgG, polyclonal	n/a	1:100	324390
human DR D ₅	Calbiochem-Inalco	rabbit	IgG, polyclonal	n/a	1:100	324408
human DR D ₂	LifeSpan-Space Import Exp	rabbit	IgG, polyclonal	n/a	1:100	LS-C22924
human DR D ₃	Calbiochem-Inalco	rabbit	IgG, polyclonal	n/a	1:100	324402
human DR D ₄	LifeSpan-Space Import Exp	rabbit	IgG, polyclonal	n/a	1:100	LS-C22938
anti-rabbit IgG	R&D System, Space Import Exp	goat	IgG, polyclonal	phycoerythrin	undiluted	F0110
human CD3	Biolegend–Campoverde	mouse	IgG2a κ, clone OKT3	PerCPCy5.5	1:1	317336
human CD4	Becton Dickinson	mouse	IgG1 κ, clone RPA-T4	APC-Cy7	1:1	557871
human CD8	Becton Dickinson	mouse	IgG1 κ, clone RPA-T8	PE-Cy7	1:1	557746
human CD45RA	Biolegend–Campoverde	mouse	IgG2b κ, clone HI100	FITC	1:1	304106
human CCR7 (CD197)	Becton Dickinson	rat	IgG2a κ, clone 3D12	Alexa Fluor 647	undiluted	557734

Supplementary Table S2. Real-Time PCR conditions.

Gene	UniGene ID	Interrogated sequence <i>RefSeq/GenBank mRNA</i>	Detected coding transcripts	Amplicon context sequence	Chromosome location	Amplicon length	Annealing temperature (°C)	Efficiency (%)
<i>DRD1</i>	Hs.2624	NC_000005.9 NG_011802.1 NT_023133.13	ENST00000329144 ENST00000393752	GACAGGAAACAGGCAGTGAGGATACGAA CAGAGAAGTCCCTTCCACCACCAGCCCAG TCCCGTCCATGGCAGAGGTGTTTCAGAGTC CTCATCTTCCTAAGAGAAAGCACATCA	5:174870011-174870124	84	60	100
<i>DRD2</i>	Hs.73893	NC_000011.9 NG_008841.1 NT_033899.8	ENST00000346454 ENST00000362072 ENST00000544518 ENST00000542968 ENST00000538967 ENST00000355319	AGCAGGGTGACAATGAAGGGCACGTAGA AGGAGACGATGGAGGAGTAGACCACGAA GGCCGGGTTGGCAATGATGCACTCGTTCT GGTCTGCGTTATTGAGTCCGAAGAGGAG	11:113286246-113287609	83	60	103

<i>DRD3</i>	Hs.121478	NC_000003.11 NG_008842.1 NT_005612.16	ENST00000460779 ENST00000467632 ENST00000295881 ENST00000383673 ENST00000281274	TTAAGGATGCTGGCTGTACACATCATGAC ATCCAGGGTGACAAAAACATCACAGCAA ATGCGGCTGAAATTCCAGACTCCACCTGT CACCTCCAGGTATACCACCCAGGGCATCA CCAAGGTGGCCACCAGCAAGTCTGCCACA GCCAGGCTCACTA CTAAGTAGT	3:113878626- 113890646	136	60	100
<i>DRD4</i>	Hs.99922	NC_000011.9 NG_021241.1 NT_009237.18	ENST00000176183	GCTGTGCTGGACGCCCTTCTTCGTGGTGCA CATCACGCAGGCGCTGTGTCCTGCCTGCT CCGTGCCCCCGCGGCTGGTCAGCGCCGTC ACCTGGCTGGGCTACGTCAACAGCGCCCT CAACCCCGTCATCTACACTGTCTTCAACGC CGAGTTCCGCAACGTCTTCCGCA	11:640411- 640580	140	60	98
<i>DRD5</i>	Hs.380681	NC_000004.11 NG_012024.1 NT_006316.16	ENST00000304374	TTTTAAACAGCAGGTTGTGTGTGTGTGCA GTGATGTGGTGGGAGCACAGCTTTCCTGG GTCTGGATTCCCGTGGCTTTGTGCTTATGT CATTTCTTCTCTCTGTGCTGGTGGGGGCCT CTTTACCATAGCTTAAG	4:9785451- 9785585	105	60	97

RPS18	Hs.627414		ENST00000454021	GTGGAACGTGTGATCACCATTATGCAGAA TCCACGCCAGTACAAGATCCCAGACTGGT TCTTGAACAGACAGAAGGATGTAAAGGAT GGAAAATACA	6:33243742- 33243838	67	60	98
			ENST00000486781					
		NC_000006.11	ENST00000484321					
		NT_007592.15	ENST00000211372					
		NT_113891.2	ENST00000477055					
		NT_167245.1	ENST00000476288					
		NT_167247.1	ENST00000439602					
		NT_167248.1	ENST00000474973					
		NT_167249.1	ENST00000457341					
			ENST00000494232					
			ENST00000434122					

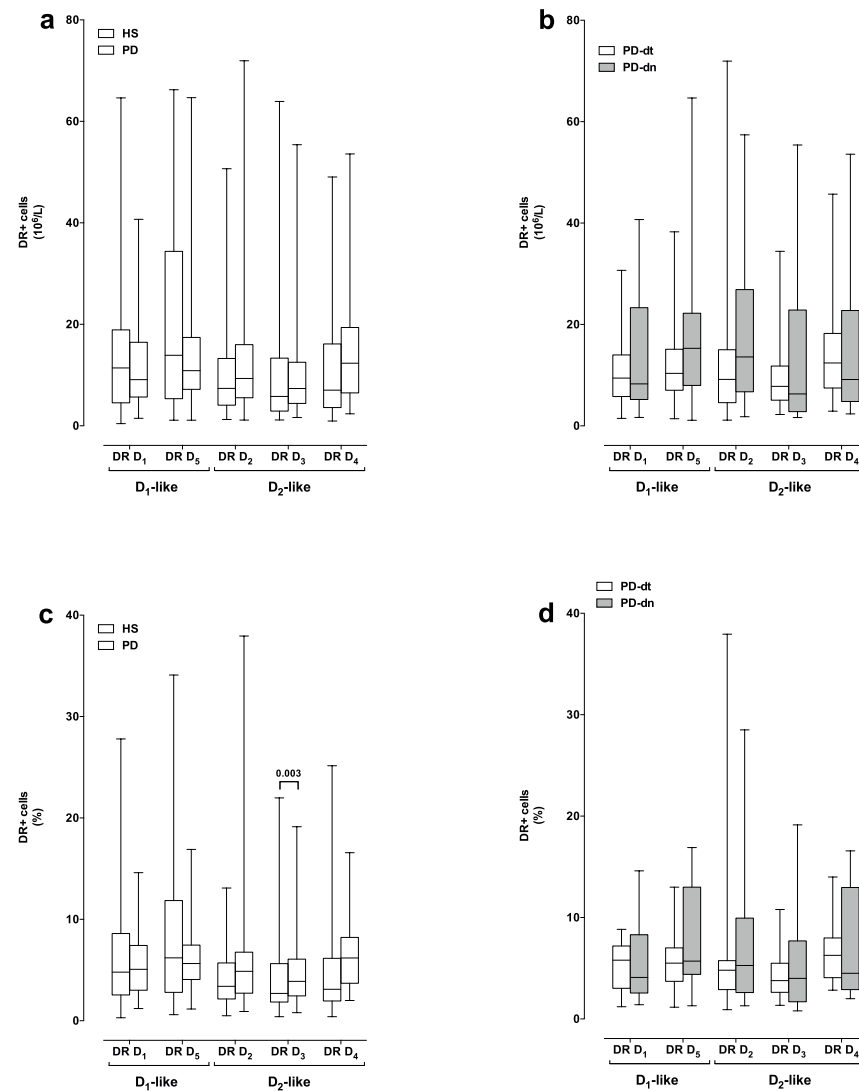
Supplemental Table S3. Effect of dopamine (a) and of dopaminergic agonists (b) on the frequency of CD4+ T naive and memory cells in cultured human PBMC. Data are means $\pm$ SEM of 3 separate experiments. Frequency is expressed as percentage of total CD4+ T cells. P always >0.05 vs. respective controls.

a.

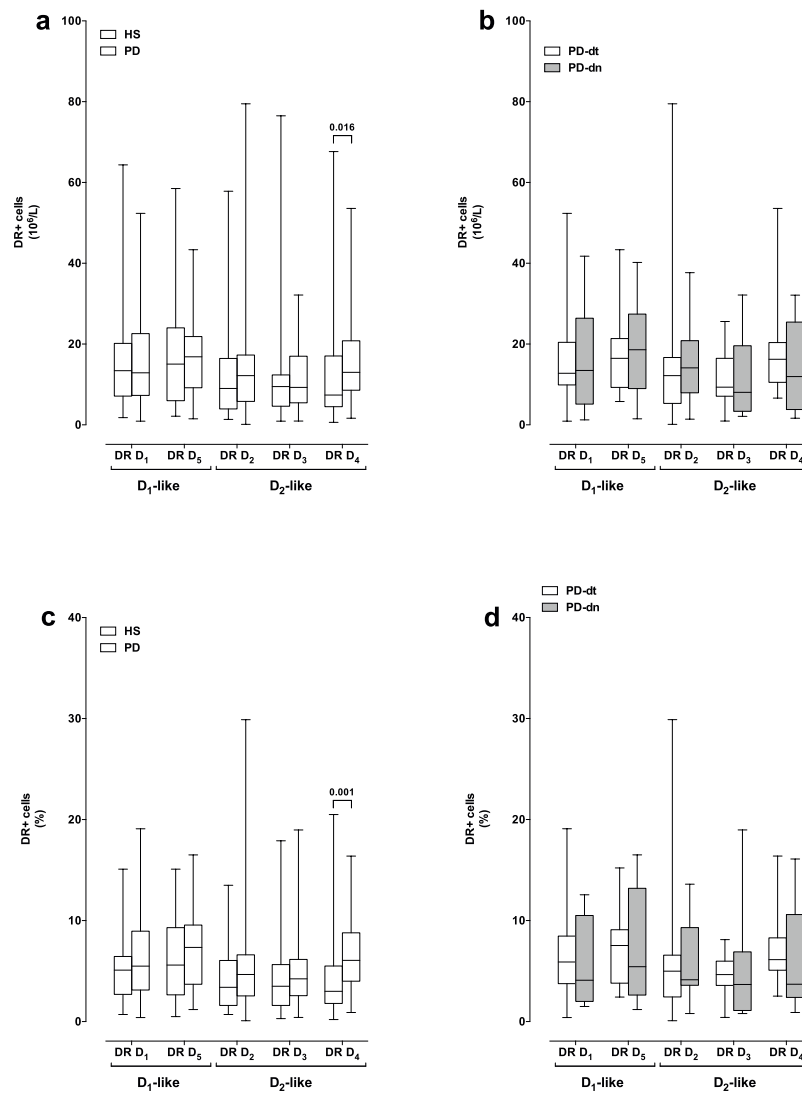
	T naive	T_{CM}	T_{EM}
Control	50.9 $\pm$ 5.2	25.0 $\pm$ 5.5	19.3 $\pm$ 3.5
Dopamine			
1 pM	52.0 $\pm$ 5.5	26.9 $\pm$ 5.3	17.0 $\pm$ 2.2
100 pM	51.8 $\pm$ 4.5	25.1 $\pm$ 4.8	18.3 $\pm$ 1.5
10 nM	52.8 $\pm$ 5.6	26.5 $\pm$ 4.2	16.8 $\pm$ 3.5
1 μ M	52.7 $\pm$ 5.1	27.9 $\pm$ 7.3	15.3 $\pm$ 2.2
100 μ M	49.7 $\pm$ 5.6	20.5 $\pm$ 4.1	23.5 $\pm$ 2.8

b.

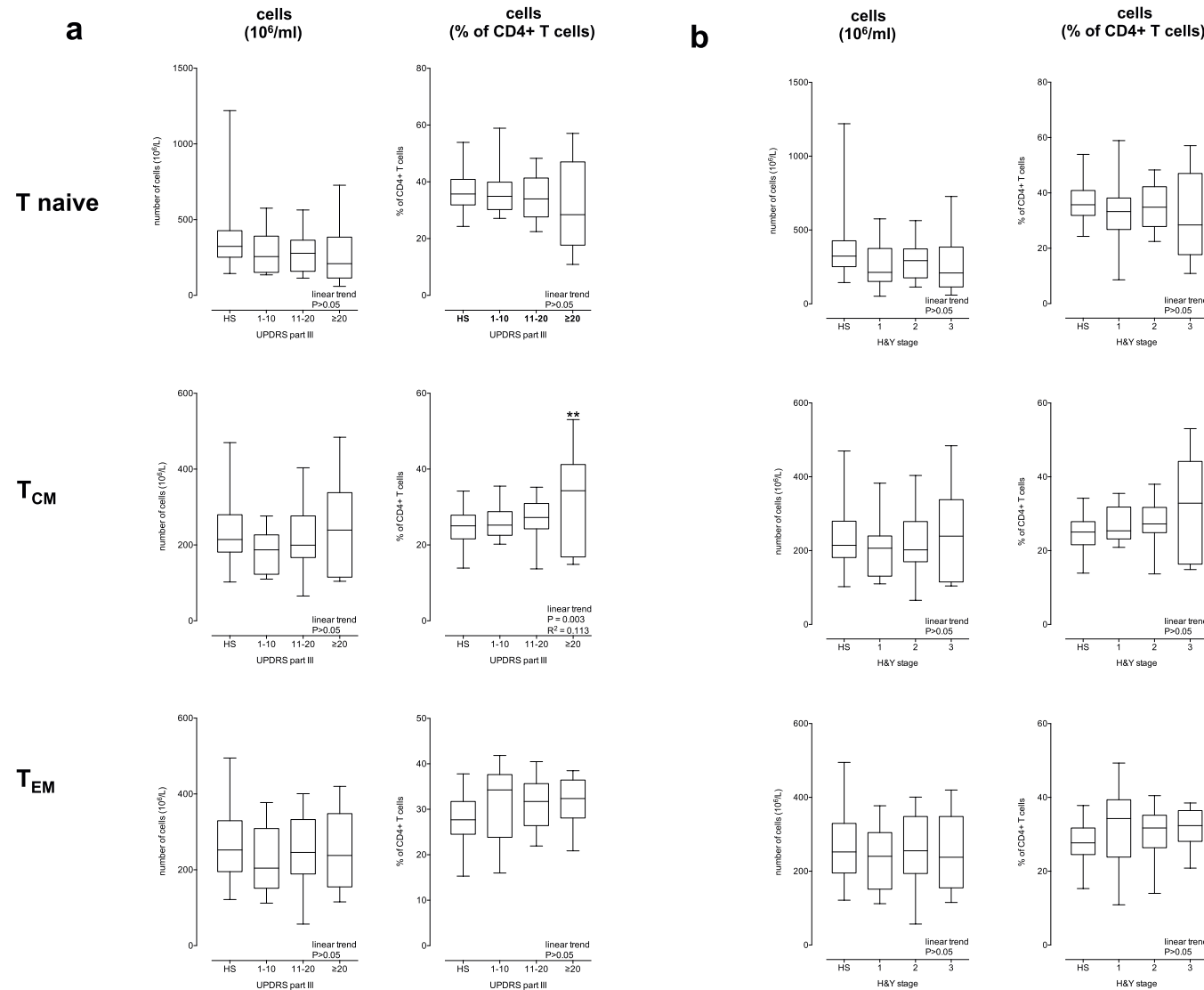
	T naive	T_{CM}	T_{EM}
Control	40.6 $\pm$ 6.3	28.3 $\pm$ 4.3	26.7 $\pm$ 1.8
SKF-38,393			
100 nM	40.8 $\pm$ 5.8	30.4 $\pm$ 5.6	24.3 $\pm$ 0.8
1 μ M	41.7 $\pm$ 5.9	29.8 $\pm$ 5.3	23.9 $\pm$ 1.1
10 μ M	40.5 $\pm$ 5.8	27.7 $\pm$ 4.8	27.2 $\pm$ 2.1
7-OH-DPAT			
10 nM	39.9 $\pm$ 5.9	30.9 $\pm$ 5.6	24.5 $\pm$ 0.8
100 nM	40.5 $\pm$ 6.6	27.4 $\pm$ 4.6	27.1 $\pm$ 2.5
1 μ M	41.5 $\pm$ 6.2	32.3 $\pm$ 6.9	21.9 $\pm$ 4.3
PD-168,077			
1 nM	40.7 $\pm$ 6.2	29.0 $\pm$ 5.3	25.6 $\pm$ 2.5
10 nM	40.6 $\pm$ 6.1	31.4 $\pm$ 5.6	23.7 $\pm$ 1.6
100 nM	41.1 $\pm$ 6.2	31.7 $\pm$ 4.5	23.2 $\pm$ 2.1



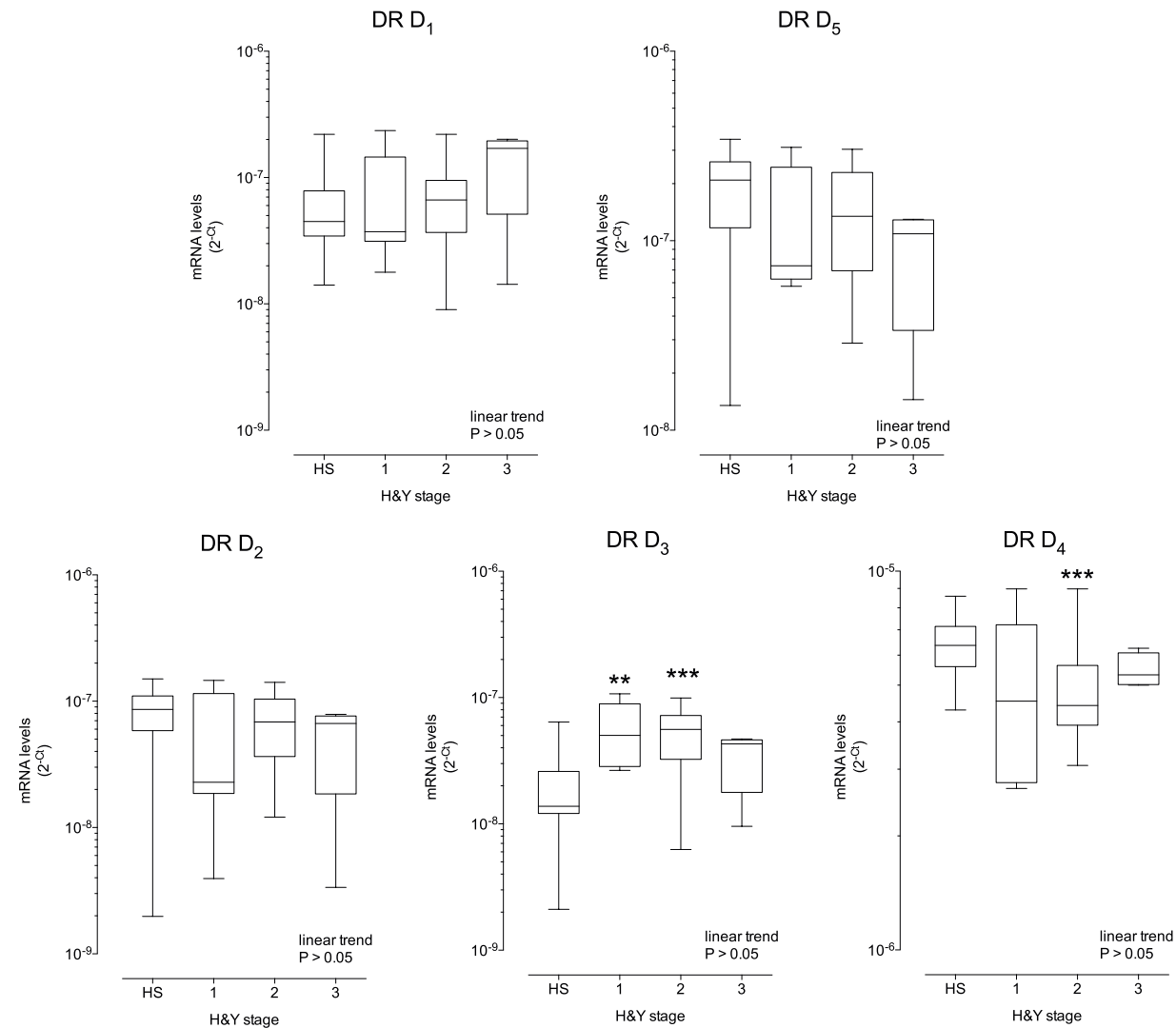
Supplementary Fig. S1 DR expression in CD4⁺ TCM cells from HS and from PD patients. DR⁺ cells are shown as absolute numbers (panels a and b) and as percentage of total CD4⁺ cells (c and d). Data are shown as medians with 25°-75° percentiles (boxes) and min-max values (whiskers). Comparisons are shown between HS and PD patients as a whole (a and c) and between drug naive (PD-dn) and drug treated (PD-dt) patients (B and D). Differences were analyzed by means of two-tailed Student's *t* test or by Mann-Whitney test, as appropriate. P values less than 0.05 are indicated in the graphs.



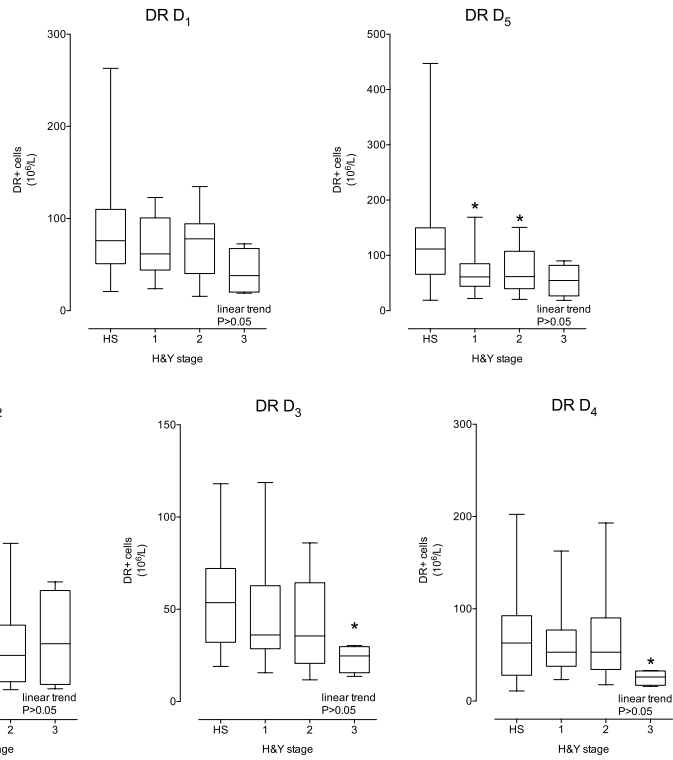
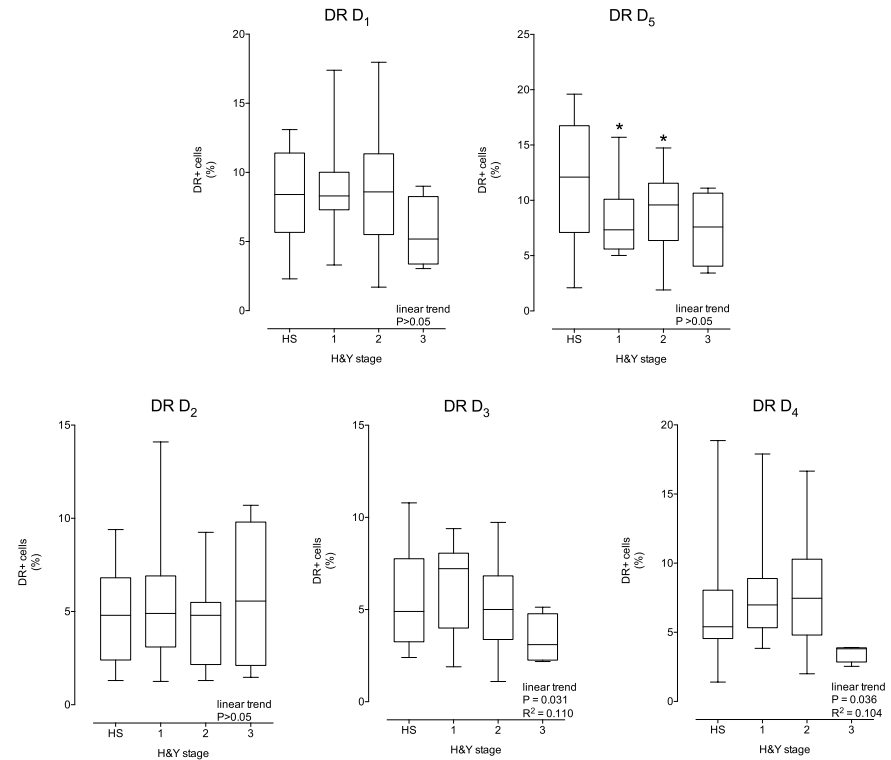
Supplementary Fig. S2 DR expression in CD4+ TEM cells from HS and from PD patients. DR+ cells are shown as absolute numbers (panels a and b) and as percentage of total CD4+ cells (c and d). Data are shown as medians with 25°-75° percentiles (boxes) and min-max values (whiskers). Comparisons are shown between HS and PD patients as a whole (a and c) and between drug naive (PD-dn) and drug treated (PD-dt) patients (b and d). Differences were analyzed by means of two-tailed Student's *t* test or by Mann-Whitney test, as appropriate.



Supplementary Fig. S3 CD4⁺ T naive and memory cells and UPDRS-III score (a) and H&Y stage (b). Cells are shown as absolute numbers (left) and as percentage of total CD4⁺ T cells (right). Data are shown as medians with 25°-75° percentiles (boxes) and min-max values (whiskers). Differences between HS and PD patients were analyzed by parametric ANOVA or Kruskal-Wallis nonparametric ANOVA, with either Holm-Sidak's or Dunn's adjustments for multiple comparisons, where * = P<0.05. Trend analysis in PD patients was performed by ANOVA post test for linear trend.

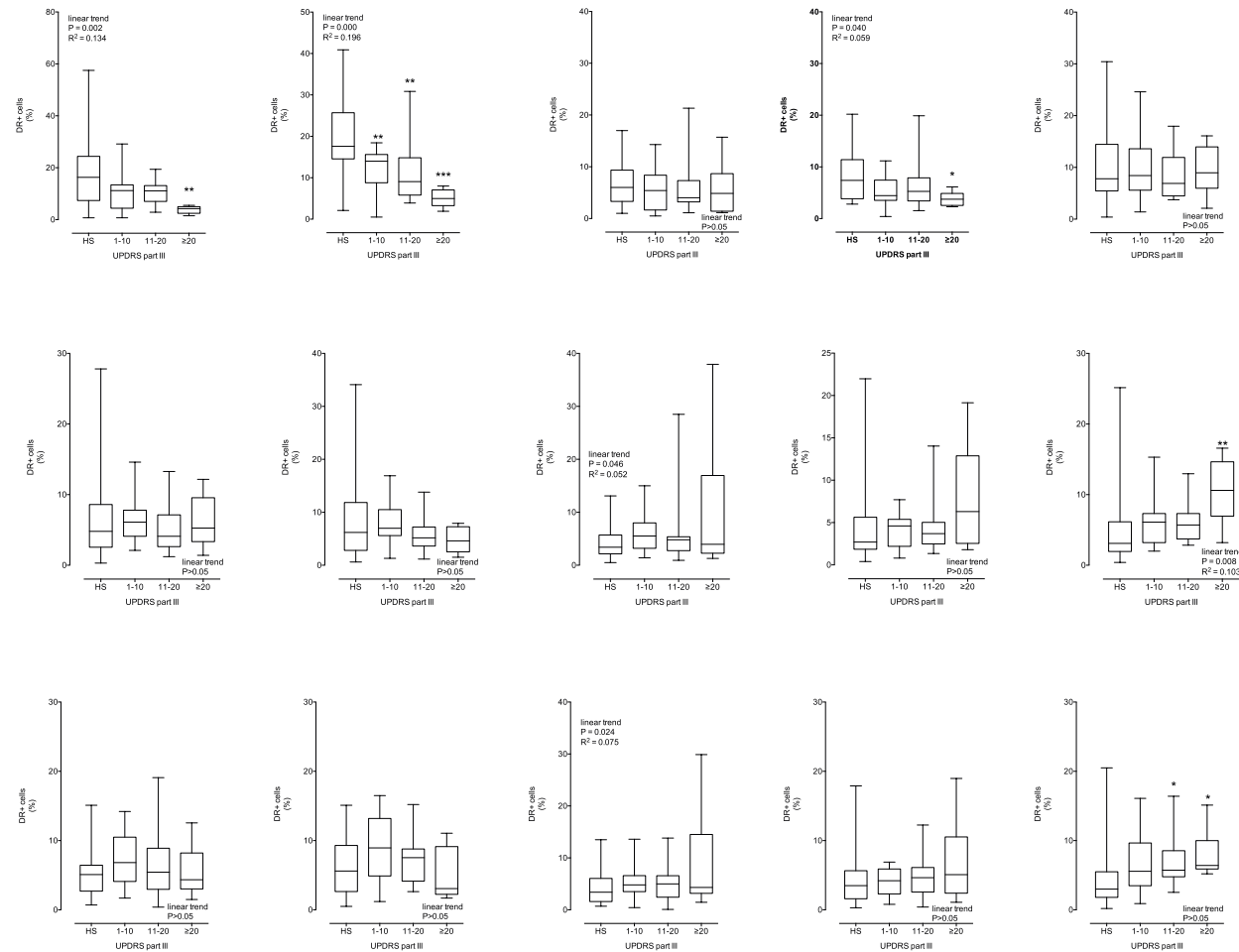


Supplementary Fig. S4 DR mRNA expression in CD4+ T cells and H&Y stage. Differences between DR mRNA levels in HS and in PD patients were analyzed by parametric ANOVA or Kruskal-Wallis nonparametric ANOVA, as appropriate, with either Holm-Sidak's or Dunn's adjustments for multiple comparisons, where * = $P < 0.05$ and *** = $P < 0.001$. Trend analysis in PD patients was performed by ANOVA post test for linear trend.

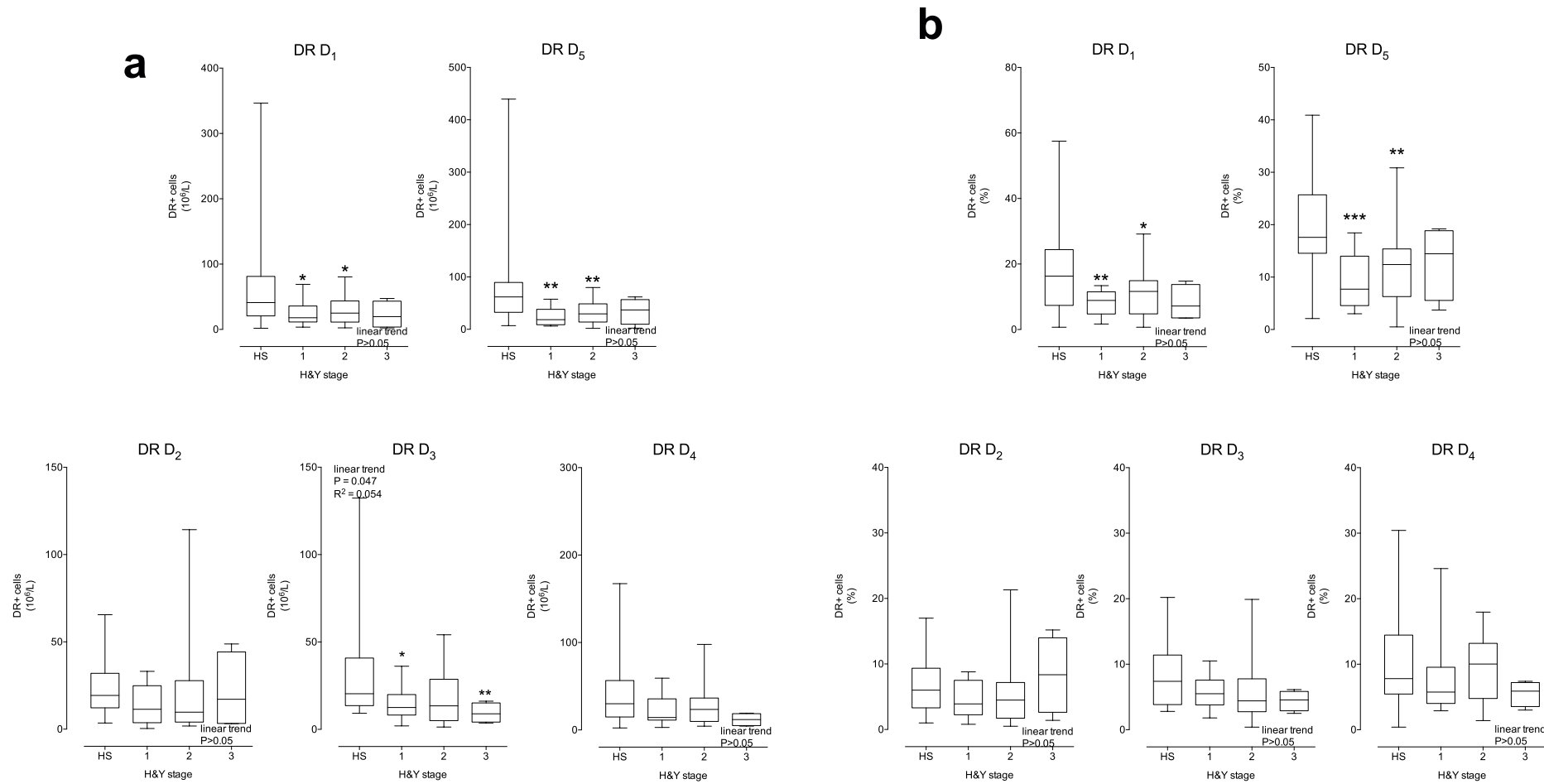
a**b**

Supplementary Fig. S5 DR expression in CD4⁺ T cells and H&Y stage. DR⁺ cells are shown as absolute numbers (a) and as percentage of total CD4⁺ cells (b). Data are medians with 25th-75th percentiles (boxes) and min-max values (whiskers). Differences between DR levels in HS and in PD patients were analyzed by parametric ANOVA or Kruskal-Wallis nonparametric ANOVA, with either Holm-Sidak's or Dunn's adjustments for multiple comparisons, where * = P < 0.05. Trend analysis in PD patients was performed by ANOVA post test for linear trend.

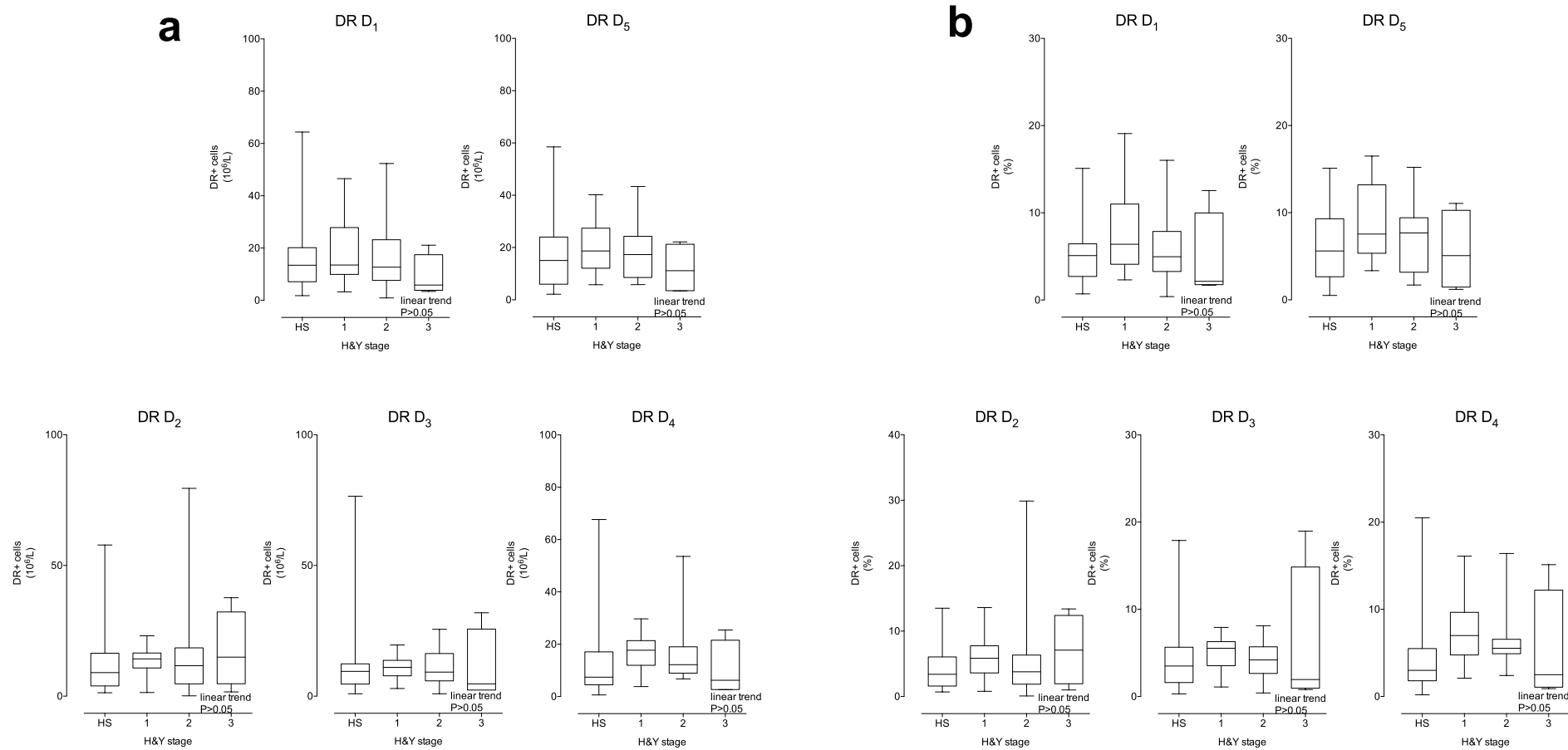
D₂-like

 T_{EM} 

Supplementary Fig. S6 DR expression in CD4+ T naive (upper), T_{CM} (middle) and T_{EM} (lower) and UPDRS-III score. DR expression is shown as protein expression on the membranes of CD4+ T cells, expressed as percentage of CD4+ cells. Data are medians with 25°-75° percentiles (boxes) and min-max values (whiskers). Differences between DR levels in HS and in PD patients were analysed by parametric ANOVA or Kruskal-Wallis nonparametric ANOVA, with either Holm-Sidak's or Dunn's adjustments for multiple comparisons, where * = P<0.05, ** = P<0.01 and *** = P<0.001. Trend analysis in PD patients was performed by ANOVA post test for linear trend.



Supplementary Fig. S7 DR expression in CD4⁺ T naive cells and H&Y stage. DR⁺ cells are expressed as absolute numbers (A) and as percentage of total CD4⁺ T cells (B). Data are shown as medians with 25th-75th percentiles (boxes) and min-max values (whiskers). Differences between DR levels in HS and in PD patients were analyzed by parametric ANOVA or Kruskal-Wallis nonparametric ANOVA, with either Holm-Sidak's or Dunn's adjustments for multiple comparisons, where * = P<0.05, ** = P<0.01 and *** = P<0.001 . Trend analysis in PD patients was performed by ANOVA post test for linear trend.



Supplementary Fig. S9 DR expression in CD4⁺ T_{EM} cells and H&Y stage. DR⁺ cells are expressed as absolute numbers (A) and as percentage of total CD4⁺ T cells (B). Data are shown as medians with 25°-75° percentiles (boxes) and min-max values (whiskers). Differences between DR levels in HC and in PD patients were analyzed by parametric ANOVA or Kruskal-Wallis nonparametric ANOVA, with either Holm-Sidak's or Dunn's adjustments for multiple comparisons. Trend analysis in PD patients was performed by ANOVA post test for linear trend.

756 **Table 1. Comparison between HS and PD patients.** Data are means±SD unless otherwise
757 indicated.

	HS	PD	P
n	28	53	
Gender (F/M)	11/17	17/36	0.625
Age (years)	68.8±8.1	69.7±9.5	0.545 ^a
UPDRS Part II (score)^b		7.2±3.5	
1-10 (n)		35	
11-20 (n)		6	
UPDRS Part III (score)^c		14.0±6.0	
1-10 (n)		18	
11-20 (n)		27	
>20 (n)		7	
H&Y scale (stage)		1.6±0.6	
1.0 (n)		21	
1.5-2.0 (n)		25	
2.5-3.0 (n)		4	

758
759 **Notes:** a = by Mann-Whitney *U* test; b = data missing for 1 PD-dn and 11 PD-dt; c = data missing
760 for 1 PD-dn patient.

761

762

763 **Table 2. Comparison between PD-dn and PD-dt.** Data are means±SD unless otherwise indicated.

	PD-dn	PD-dt	P
n	16	37	
Gender (F/M)	7/9	10/27	0.337
Age (years)	65.6±10.8	71.4±8.4	0.041^a
UPDRS Part II (score)	6.9±4.7 ^b	7.5±2.6 ^b	0.606 ^d
UPDRS Part III (score)	9.9±5.9 ^d	15.6±5.3	0.002^a
H&Y scale (stage)	1.2±0.4	1.8±0.5	<0.001^a
LED (mg/day)		459.4±247.4	
Drugs			
L-DOPA (n)		27 ^e	
DA agonists (n)		27 ^f	
pramipexole (n)		19	
ropinirole (n)		4	
rotigotine (n)		4	
Rasagiline (n)		19	

764

765 **Notes:** a = by Student's *t* test; b = data missing for 1 PD-dn and 11 PD-dt; c = data missing for 1

766 PD-dn patient; d = by Mann–Whitney *U* test; e = 8 taking L-DOPA alone, and 19 taking L-DOPA +

767 DA agents; f = 10 taking DA agonists alone (4) or with rasagiline (6), and 17 taking DA agonists +

768 L-DOPA, without (6) or with rasagiline (11).

Table 3. Complete blood count, comparison between HS and PD patients. Data are means±SD

unless otherwise indicated.

	units	range	HS	PD	P
RBC	10 ¹² /L	4.50-6.00	4.9±0.4	4.7±0.4	0.100 ^a
hemoglobin	g/dL	13.0-17.5	14.4±1.1	14.2±1.2	0.472 ^a
hematocrit	%	42.0-54.0	43.4±3.6	42.6±3.3	0.439 ^a
MCH	pg	27.0-32.0	29.8±1.8	30.1±2.3	0.253 ^a
MCHC	g/dL	32.0-36.0	33.7±2.8	33.4±1.7	0.780 ^a
Platelets	10 ⁹ /L	150-450	238.2±50.1	242.8±69.1	0.853 ^a
WBC	10 ⁹ /L	4.30-11.00	6.9±1.6	6.6±1.7	0.310 ^a
lymphocytes	10 ⁹ /L	1.50-5.50	2.12±0.73	1.76±0.48	0.027^b
	%	10.0-45.0	30.8±7.4	27.3±6.8	0.050^a
monocytes	10 ⁹ /L	0.2-1.1	0.5±0.1	0.5±0.2	0.620 ^b
	%	2.0-12.0	7.5±1.9	7.5±2.2	0.991 ^b
neutrophils	10 ⁹ /L	1.50-5.50	4.1±1.1	4.1±1.4	0.781 ^b
	%	40.0-80.0	58.9±8.0	62.4±7.2	0.120 ^a
eosinophils	10 ⁹ /L	0.0-0.8	0.2±0.3	0.2±0.1	0.679 ^a
	%	0.0-7.0	2.3±1.6	2.3±1.6	0.838 ^a
basophils	10 ⁹ /L	0.0-0.2	0.0±0.0	0.0±0.0	0.793 ^a
	%	0.0-1.6	0.5±0.2	0.5±0.4	0.469 ^a
lymphocyte subsets					
CD3+	10 ⁶ /L		1515.0±651.7	1241.0±366.2	0.109 ^a
	% of total lymph		70.8±9.4	69.6±8.7	0.549 ^a
CD4+	10 ⁶ /L		1012.0±439.1	797.4±263.3	0.035^a
	% of CD3+		67.3±10.8	64.7±10.8	0.419 ^a
CD8+	10 ⁶ /L		334.0±162.8	274.9±151.5	0.134 ^b
	% of CD3+		23.4±9.3	22.0±9.7	0.562 ^b
CD4+/CD8+	ratio		3.8±2.9	4.0±3.4	0.979 ^a

Notes: Abbreviations: RBC, red blood cells; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; WBC, white blood cells. a = by Mann–Whitney *U* test; b = by Student's *t* test.

Table 4. Complete blood count, comparison between PD-dn and PD-dt. Data are means±SD

unless otherwise indicated.

	units	range	PD-dn	PD-dt	P
RBC	10 ¹² /L	4.50-6.00	4.8±0.4	4.7±0.4	0.250 ^a
hemoglobin	g/dL	13.0-17.5	14.4±0.9	14.1±1.3	0.456 ^a
hematocrit	%	42.0-54.0	43.3±2.9	42.2±3.4	0.324 ^a
MCH	pg	27.0-32.0	29.9±1.7	30.2±2.5	0.317 ^a
MCHC	g/dL	32.0-36.0	33.3±1.6	33.5±1.8	0.847 ^a
Platelets	10 ⁹ /L	150-450	239.3±48.8	244.4±76.8	0.859 ^a
WBC	10 ⁹ /L	4.30-11.00	6.2±1.6	6.7±1.7	0.400 ^a
<i>lymphocytes</i>	10 ⁹ /L	1.50-5.50	1.63±0.41	1.81±0.50	0.222 ^b
	%	10.0-45.0	27.1±6.6	27.5±6.9	0.841 ^b
<i>monocytes</i>	10 ⁹ /L	0.2-1.1	0.5±0.2	0.5±0.1	0.750 ^a
	%	2.0-12.0	8.0±2.6	7.2±2.0	0.221 ^b
<i>neutrophils</i>	10 ⁹ /L	1.50-5.50	3.9±1.4	4.2±1.4	0.315 ^a
	%	40.0-80.0	61.6±7.7	62.7±7.1	0.532 ^a
<i>eosinophils</i>	10 ⁹ /L	0.0-0.8	0.2±0.1	0.2±0.1	0.668 ^a
	%	0.0-7.0	2.6±2.0	2.2±1.5	0.581 ^a
<i>basophils</i>	10 ⁹ /L	0.0-0.2	0.0±0.0	0.0±0.0	0.378 ^a
	%	0.0-1.6	0.7±0.4	0.4±0.3	0.019^a
<i>lymphocyte subsets</i>					
CD3+	10 ⁶ /L		1115.9±302.0	1295.0±381.8	0.143 ^a
	% of total lymph		65.9±9.5	71.3±7.9	0.037^b
CD4+	10 ⁶ /L		757.3±208.3	814.8±284.7	0.672 ^a
	% of CD3+		69.2±12.2	62.8±9.6	0.046^b
CD8+	10 ⁶ /L		220.7±134.6	299.7±154.1	0.071 ^a
	% of CD3+		19.5±9.4	23.1±9.8	0.220 ^b
CD4+/CD8+	ratio		5.3±5.3	3.4±1.8	0.053 ^b

Notes: Abbreviations: RBC, red blood cells; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; WBC, white blood cells. a = by Mann–Whitney *U* test; b = by Student's *t* test.

4. Parkinson's disease patients have a complex phenotypic and functional Th1 bias: cross-sectional studies of CD4+ Th1/Th2/T17 and Treg in drug-naïve and -treated patients

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4.1. OVERVIEW

The aim of present study was to investigate the phenotypical and functional profile of Th1, Th2, Th17 and Treg in the peripheral blood of PD patients (drug-naïve or drug-treated) and age- and sex-matched healthy subjects. Two clinical studies were conducted, with the first study dealing with the T helper (Th1, Th2 and Th17) profile and function in peripheral blood, while in the second protocol dealt with Treg frequency and function.

4.2. MANUSCRIPT

1 **Parkinson's disease patients have a complex phenotypic and functional Th1 bias: cross-**
2 **sectional studies of CD4+ Th1/Th2/T17 and Treg in drug-naïve and -treated patients**

3
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32 **Abstract**

33 ***Background***

34 Parkinson's disease (PD) affects an estimated 7 to 10 million people worldwide, and only
35 symptomatic treatments are presently available to relieve the consequences of brain dopaminergic
36 neurons loss. Neuronal degeneration in PD is the consequence of neuroinflammation in turn
37 influenced by peripheral adaptive immunity, with CD4⁺ T lymphocytes playing a key role. CD4⁺ T
38 cells may however acquire proinflammatory phenotypes, such as T helper (Th) 1 and Th17, as well
39 as antiinflammatory phenotypes, such as Th2 and the T regulatory (Treg) one, and to what extent
40 the different CD4⁺ T cell subsets are imbalanced and their functions dysregulated in PD remains
41 largely an unresolved issue.

42 ***Methods***

43 We performed two cross-sectional studies in antiparkinson drug-treated and -naïve PD patients, and
44 in age- and sex-matched healthy subjects. In the first one we examined circulating Th1, Th2, Th17,
45 and in the second one circulating Treg. Number and frequency of CD4⁺ T cell subsets in peripheral
46 blood were assessed by flow cytometry and their functions were studied in *ex vivo* assays. In both
47 studies, complete clinical assessment, blood count and lineage-specific transcription factors mRNA
48 levels in CD4⁺ T cells were independently assessed and thereafter compared for their consistency.

49 ***Results***

50 PD patients have reduced circulating CD4⁺ T lymphocytes, due to reduced Th2, Th17 and Treg.
51 Naïve CD4⁺ T cells from peripheral blood of PD patients preferentially differentiate towards the
52 Th1 lineage. Production of interferon- γ and tumor necrosis factor- α by CD4⁺ T cells from PD
53 patients is increased and maintained in the presence of homologous Treg. This Th1-biased
54 immune signature occurs in both drug-naïve patients and in patients on dopaminergic drugs,
55 suggesting that current antiparkinson drugs do not affect peripheral adaptive immunity.

56 ***Conclusions***

57 The complex phenotypic and functional profile of CD4⁺ T cell subsets in PD patients strengthen
58 the evidence that peripheral adaptive immunity is involved in PD, and represents a target for the
59 preclinical and clinical assessment of novel immunomodulating therapeutics.

60
61 **Key words:** Parkinson's disease; CD4⁺ T lymphocytes; Th1; Th2; Th17; Treg.

62

63

Background

Parkinson's disease (PD) is among the most common neurological diseases, affecting 1-2 per 1000 in the general population with increasing prevalence with age, and up to 1 in 100 individuals above 60 years of age, resulting in an estimated 7 to 10 million people with PD worldwide [1, 2]. People with PD suffer from motor symptoms, including bradykinesia, rigidity, resting tremor, and postural instability, as well as from non-motor symptoms, such as autonomic disturbances, depression and cognitive impairment, resulting in dramatically poor quality of life and rising economic burden for patients, caregivers and the healthcare systems [3-5]. Available treatments for PD are only symptomatic, and aim at relieving the loss of brain dopaminergic neurons by using the dopamine (DA) precursor L-DOPA, some dopaminergic agonists as well as other indirect dopaminergic agents [6]. Dopaminergic replacement improves patients' quality of life, although as disease progresses non-motor and motor symptoms emerge that may be resistant to dopaminergic medications [7, 8]. No treatments currently exist to prevent PD or delay its progression, mainly due to the still limited comprehension of the events ultimately leading to neurodegeneration. PD is characterized by the progressive loss of dopaminergic neurons in the *substantia nigra pars compacta* and by the appearance of Lewy bodies, which are intracellular inclusions of aggregated α -synuclein [9-12]. Despite extensive knowledge about the mechanisms leading to neuronal death, which include mitochondrial dysfunction, oxidative and proteolytic stress, and neuroinflammation, understanding the causes of neurodegeneration in PD remains so far an elusive goal. In this regard, novel clues are possibly coming from evidence concerning the role of peripheral adaptive immunity in the regulation of neuroinflammation [13-16]. T cells indeed can be found in the *substantia nigra* of parkinsonian brains [17, 18]. Both CD8⁺ and CD4⁺ T cells (but not B cells) occur in postmortem brain specimens from PD patients as well as in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD, and evidence from the mouse model indicates that CD4⁺ T cells determine T cell-mediated dopaminergic cell death [18]. Although T lymphocytes infiltrate parkinsonian brains, decreased numbers of CD3⁺ and CD4⁺ T lymphocytes have been consistently reported in peripheral blood of PD patients [19]. CD4⁺ T lymphocytes play a crucial role in the orchestration of an effective immune response during host defense as well as in the pathogenesis of inflammatory disease. To this end, CD4⁺ T cells may acquire proinflammatory phenotypes, such as T helper (Th) 1 and Th17, as well as antiinflammatory phenotypes, such as Th2 and the T regulatory (Treg) one [20, 21], and evidence from animal models of PD suggests that Th1 and Th17 may be detrimental while Th2 and Treg may be protective (reviewed in 22). Whether CD4⁺ T cell subsets are imbalanced and their functions are dysregulated in PD patients remains however largely an unresolved issue.

98 The aim of this study was to investigate the profile of Th1, Th2, Th17 and Treg in the peripheral
99 blood of PD patients, either drug-naïve or on dopaminergic replacement therapy, and to examine
100 their function. Since we previously found that in PD patients CD4⁺ T naïve cells D₁-like
101 dopaminergic receptors (DR) decrease, while in T memory cells D₂-like DR increase, with
102 increasing motor disability [23], we also assayed DR on all CD4⁺ T cells subsets. To this end, we
103 conducted two clinical studies, recruiting PD patients and age- and sex-matched healthy subjects. In
104 the first study, Th1, Th2 and Th17 count and function were examined, while in the second study
105 Treg frequency and function were assessed. In both protocols, complete clinical assessment, blood
106 count and lineage-specific transcription factors mRNA levels in CD4⁺ T cells were independently
107 assessed and thereafter compared for their consistency. Results provide for the first time a detailed
108 phenotypic and functional profile of the CD4⁺ T cell compartment in peripheral blood in both drug-
109 naïve PD patients and in patients on dopaminergic drugs, which extends available knowledge about
110 the involvement of CD4⁺ T cell subsets in PD and offers an array of biomarkers for the assessment
111 of novel unconventional therapeutics targeting peripheral immunity.
112
113

114

115 **Methods**

116 **Subjects.** Peripheral venous blood samples were collected from patients with idiopathic PD [24],
117 either drug-naïve (PD-dn, i.e. PD patients who never received any antiparkinson drugs) or on
118 antiparkinson drug treatment (PD-dt), and from age- and sex-matched healthy subjects (HS). PD
119 was diagnosed according to the United Kingdom Parkinson's Disease Society Brain Bank Criteria.
120 Exclusion criteria were history of autoimmune or inflammatory disorders and chronic
121 immunosuppressive treatment.

122 Participants were recruited at the Movement Disorders Center of the University of Piemonte
123 Orientale, Divisione di Neurologia, Ospedale Maggiore of Novara, at the Interdepartmental
124 Research Center for Parkinson's Disease of the Neurological Institute "C. Mondino" of Pavia, and
125 at the Centre for Parkinson's Disease and Movement Disorders of the Neurological Service at the
126 Ospedale di Circolo of Varese, Italy. HS were spouses and caregivers of enrolled PD patients.
127 The Ethics Committees of Ospedale di Circolo of Varese and Neurological Institute "C. Mondino"
128 of Pavia approved the protocol and all the participants signed a written informed consent before
129 enrollment. The study was performed according to the Declaration of Helsinki and to the relevant
130 ethical guidelines for research on humans.

131 After enrollment, subjects were submitted to a complete examination. PD patients were staged
132 according to the criteria of Hoehn and Yahr (H&Y) [25] and evaluated by means of the Unified
133 Parkinson's Disease Rating Scale (UPDRS) Part III [26]. Antiparkinson drug doses were recorded
134 at the time of enrollment and L-DOPA equivalent doses (LED) were calculated according to
135 established guidelines [27].

136 Withdrawal of venous blood was performed after a fasting night, between 8:00 a.m. and 10:00 a.m.,
137 in EDTA-coated tubes (BD Vacutainer). Tubes were subsequently coded and stored at room
138 temperature until processing, which occurred 24 hours after collection, to ensure homogeneous
139 treatment of all the samples. Complete blood cell count with differential analysis was conducted on
140 separate blood samples collected in EDTA-coated tubes (BD Vacutainer).

141

142 **Reagents.** Bovine serum albumin (BSA) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
143 (HEPES) were purchased from Sigma, Italy. RPMI 1640, heat-inactivated fetal bovine serum
144 (FBS), glutamine, and penicillin/streptomycin were obtained from Euroclone, Italy. Ficoll-Paque
145 Plus was from Pharmacia Biotech (Uppsala, Sweden, code GEH1714403). Purified mouse ab anti-
146 human CD3 (code 555330, clone UCHT1, Mouse IgG1, κ) and purified mouse ab anti-human
147 CD28 (code 555726, clone CD28.2, Mouse IgG1, κ) were obtained from Becton Dickinson, Italy.

148 Phytohaemagglutinin (PHA, code L8902), recombinant interleukin (IL)-2 (code 0208AF12),
 149 dopamine hydrochloride (code H8502), phorbol 12-myristate 13-acetate (PMA, code P813), and
 150 ionomycin (code I3909) were all from Sigma Aldrich (Saint Louis, MO, USA).
 151 Human ab anti-INF- γ (code 130-095-743) and anti-IL-4 (code 130-095-753), IL-1 β (code 130-093-
 152 895), IL-4 (code 130-095-373), IL-6 (code 130-095-365), IL-12 (code 130-096-704) and TGF- β
 153 (code 130-095-067) were from Miltenyi Biotec, Bergisch Gladbach, Germany.
 154 ELISA kits for human interferon (IFN)- γ (code EHIFNG), tumor necrosis factor (TNF)- α (code
 155 EH3THFA), IL-4 (code EHIL4), IL-10 (code EHIL10) and IL-17A (code EHIL17A) were all from
 156 Thermo Scientific, Rockford, USA.

157
 158 **Flow cytometric analysis of CD4⁺ T helper cells in whole blood.** Analysis was performed in two
 159 steps. In the first step, 100 μ l aliquots of whole blood were prepared from each subject (5 were
 160 used for DR staining, 1 as control for the secondary PE-goat anti-rabbit (PEGAR) ab, and 1 as
 161 negative control, i.e. without any ab). All the aliquots were incubated with a cocktail of anti-human
 162 CD4, CXCR3 (CD183), CCR4 (CD194) and CCR6 (CD196) ab for the identification of CD4⁺ T
 163 lymphocytes and of the following CD4⁺ T helper subsets: Th1 cells (CD4⁺CXCR3⁺CCR4⁻CCR6⁻),
 164 Th2 cells (CD4⁺CXCR3⁻CCR4⁺CCR6⁻), Th17 cells (CD4⁺CXCR3⁻CCR4⁺CCR6⁺) and Th1/17
 165 cells (CD4⁺CXCR3⁺CCR4⁺CCR6⁺). After 20 min in the dark, erythrocytes were removed by
 166 means of a lysis buffer ((g/L) NH₄Cl 8.248, KHCO₃ 1.0, EDTA 0.0368). Samples were then
 167 centrifuged, supernatants were removed and cells were washed in PBS ((g/L) NaCl 8.0, KCl 0.2,
 168 Na₂HPO₄ 1.42, KH₂PO₄ 0.24, pH 7.4) supplemented with 1% BSA (PBS/BSA), and resuspended in
 169 PBS/BSA. Total leukocytes were counted by means of a hemocytometer and cell viability,
 170 determined by the Trypan blue exclusion test, was always >99%. During the second step, each
 171 aliquot was stained for one of the five DR by an indirect labeling procedure. Briefly, samples were
 172 stained with primary ab and incubated for 30 min on ice. After washing, samples were incubated
 173 with PEGAR ab for 30 min on ice in the dark. Samples were then washed and resuspended in 350
 174 μ l PBS and left on ice until acquisition. Ab used in the study are listed in Additional Table S1, and
 175 the gating strategy is shown in Additional Figure S1. Acquisition was then performed on a BD
 176 FACSCanto II flow cytometer (Becton Dickinson, Milan, Italy) with BD FACSDiva software
 177 (version 6.1.3). Lymphocytes were identified by means of their classical forward scatter (FSC) and
 178 side scatter (SSC) signals and a minimum of 20,000 lymphocytes from each sample was collected
 179 in the gate. Data were analyzed with the FlowJo software (version 8.3.2). The results were finally
 180 expressed as absolute numbers (10⁶/mL) as well as percentage of positive cells (%).

181

182 **Flow cytometric analysis of CD4⁺ Treg subsets in whole blood.** Analysis of CD4⁺ Treg was
183 performed according to Miyara et al. [28]. Briefly, 100 μ L aliquots of whole blood were prepared as
184 above described, and 7 aliquots of 100 μ L were prepared from each subject (5 for DR staining, 1 as
185 control for the secondary PEGAR ab, and 1 as negative control). The staining protocol consisted of
186 two steps. During the first step, each aliquot was stained for one of the five DR by an indirect
187 labeling procedure (primary ab + secondary ab labeled with PE). During the second step, all the
188 aliquots were incubated with a cocktail of anti-human CD4, CD25, CD127, and CD45RA ab for the
189 identification of CD4⁺ T lymphocytes, of total Treg (cTreg, CD4⁺CD25^{high}CD127^{low}), and of naïve
190 Treg (nTreg, CD4⁺CD25^{high}CD127^{low}CD45RA⁺) and activated Treg (aTreg,
191 CD4⁺CD25^{high}CD127^{low}CD45RA⁻). Ab used in the study are listed in additional Table S1, and the
192 gating strategy is shown in Additional Figure S2. Acquisition, lymphocyte identification, data
193 analysis and final expression of results were performed as described above.

194

195 **Isolation of peripheral blood mononuclear cells (PBMC).** PBMC were isolated from whole
196 blood by using Ficoll-Paque Plus density gradient centrifugation. Cells were resuspended and, if
197 necessary, any residual contaminating erythrocytes were lysed by addition of 5 mL of lysis buffer
198 ((g/L) NH₄Cl 8.248, KHCO₃ 1.0, EDTA 0.0368), followed by incubation for 5 min, during which
199 samples were gently vortexed, and centrifugation at 100 g for 10 min at room temperature (RT).
200 Cells were washed twice in PBS by addition of 15 mL of PBS and centrifugation at 300 g and 10
201 min at RT, and resuspended at the final concentration of 1 x 10⁶ cells/mL of RPMI/10% FBS for
202 subsequent culture. Typical PBMC preparations contained at least 80% lymphocytes, as assessed by
203 flow cytometry. Cell viability, assessed by the trypan blue exclusion test was always >99%.

204

205 **Real-time PCR assays of CD4⁺ T cells.** CD4⁺ T cells were isolated from PBMC by means of
206 *Dynabeads CD4 Positive Isolation kit* (Life Technologies, code 11145D). At least 50000 CD4⁺ T
207 cells were thereafter resuspended in *PerfectPure RNA lysis buffer* (5 Prime GmbH, Hamburg,
208 Germany), and total RNA was extracted by *PerfectPure RNA Cell KitTM* (5 Prime GmbH, code
209 2302340). The amount of extracted RNA was estimated by spectrophotometry at λ = 260 nm. Total
210 mRNA was then reverse-transcribed using a random primer and a high-capacity cDNA RT kit
211 (Applied Biosystems, code 4368813), and the resulting amount of cDNA was estimated by
212 spectrophotometry at λ = 260 nm. Real-Time PCR reactions were then started with 1 μ M cDNA.
213 Amplification of cDNA was performed by means of the *SsoAdvancedTM Universal Probes*
214 *Supermix* (BIORAD, code 1725282) for the analysis of mRNA levels of the transcription factor
215 genes *TBX21*, *STAT1*, *STAT3*, *STAT4*, *STAT6*, *RORC*, *GATA3*, *FOXP3*, and *NR4A2*. cDNA was

assayed on StepOne® System (Applied Biosystems). Real-time PCR conditions are shown in Additional Table S2.

Linearity of assays was tested by constructing standard curves using serial 10-fold dilutions of a standard calibrator cDNA for each gene. Regression coefficients (r^2) were always >0.999 . Assays were performed in triplicate for each sample, and levels of mRNA were finally expressed as $2^{-\Delta Ct}$ where $\Delta Ct = [Ct \text{ (sample)} - Ct \text{ (housekeeping gene)}]$. Relative expression was determined by normalization to expression of *RPS18*, which is the gene for 18S cDNA. Data analysis was performed by StepOne software™ 2.2.2 (Applied Biosystems).

Separation of naïve CD4⁺ T cells and polarization assay. The isolation of naïve CD4⁺ T cells from PBMC was performed using the human CD4⁺ Naïve T cell Isolation Kit (Miltenyi Biotec, code 130-094-131), and polarization was assayed by the Human Th1/Th2/Th17 Phenotyping Kit (BD, code 560751), according to the manufacturers' instructions. Purity of separated naïve CD4⁺ T lymphocytes was always more than 95%, as confirmed by flow cytometric analysis.

The polarization assay was developed according to published methods [29-31], with modifications. Briefly, naïve CD4⁺ T cells were cultured in U-bottomed 96-well plates primed with anti-CD3/CD28 ab, in standard conditions (Th0), or under the different following polarizing conditions: IL-12 (10 ng/mL) and anti-IL-4 ab (10 µg/mL) for Th1; IL-4 (10 ng/mL) and anti-IFN-γ ab (10 µg/mL) for Th2; IL-1β (10 ng/mL), IL-6 (50 ng/mL), TGF-β (5 ng/mL), anti-IL-4 ab (10 µg/mL) and anti-IFN-γ ab (10 µg/mL) for Th17. Cells were incubated for 4 days, at 37 °C in a moist atmosphere of 5% CO₂. Cells were thereafter observed under light microscopy to confirm formation of clusters indicating cell activation. Wells were then replenished with 500 µL fresh medium containing human recombinant IL-2 (10 ng/mL for Th1 and Th2, and 2 ng/mL for Th17), and left in the incubator for another 3 days. Cells were subsequently collected, centrifuged at 600 x g for 5 min, resuspended in 1 mL RPMI with 10% FBS, adjusted to the final concentration of 1×10^6 cells/mL for each sample, and stimulated with PMA (50 ng/mL), ionomycin (1µg/mL), and GolgiStop™ Protein Transport Inhibitor (included in the Phenotyping Kit) for 5 h at 37°C in 5% CO₂. Cells were finally harvested, stained with the Human Th1/Th2/Th17 Phenotyping Kit, and analyzed by flow cytometry according to the manufacturer's protocol.

Purification and functional assays of CD4⁺ T regulatory (Treg) and T effector (Teff) cells. Treg and Teff were purified from PBMC by means of the human CD4⁺CD25⁺ Regulatory T cell Isolation Kit (Miltenyi Biotec, code 130-091-301), according to the manufacturer's instructions. Both Treg and Teff viability were more than 99% as assessed by the trypan blue exclusion test.

250 Flow cytometric analysis showed that CD4⁺CD25^{high}CD127^{low} Treg cells were 7.1±0.2% in the
251 Teff fraction and 76.1±3.2% in the Treg fraction (mean±SD, n = 3). Treg and Teff cells were placed
252 in U-bottomed 96-well plates at the concentration of 1 x 10⁶/mL in RPMI 1640 medium
253 supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and 100 U/mL
254 penicillin/streptomycin, at 37°C in a moist atmosphere of 5% CO₂. Treg and Teff were cultured
255 alone or in co-culture in different Teff:Treg ratios (1:1, 1:0.5, 1:0.25, and 1:0.125), in resting
256 conditions or added with PHA (5 µg/mL) and IL-2 (40 ng/mL). Measurement of cell proliferation
257 was performed after 5 days, by means of standard staining with Cell Proliferation Dye eFluor 670
258 (eBioscience-Prodotti Gianni, Italy, code 65-0840) and flow cytometric analysis. Production of
259 IFN-γ, TNF-α, IL-4, IL17A, IL-10 and TGF-β was assessed in supernatants, which were collected
260 after 48 h and frozen at -80°C until analysis, performed by means of standard ELISA assays.

261

262 **Statistical analysis.** Distribution of the values was assessed by the D'Agostino & Pearson normality
263 test. Statistical significance of the differences between HS and PD patients and between PD-dn and
264 PD-dt patients was then analyzed by means of two-tailed Student's *t* test or by the Mann-Whitney
265 test, as appropriate, for continuous variables, and by the Fisher's exact test for categorical variables.
266 Correlations among continuous variables were assessed by Pearson or Spearman correlation
267 analysis. Differences between HS and PD patients categorized for UPDRS Part III score or H&Y
268 stage were analyzed by ordinary one-way ANOVA or by the Kruskal-Wallis test, with either Holm-
269 Sidak's or Dunn's adjustments for multiple comparisons, and trend analysis in PD patients was
270 performed by ANOVA post test for linear trend. Calculations were performed using commercial
271 software (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California
272 USA, www.graphpad.com).

273

274

275

276 **Results**

277 ***Study #1 - CD4⁺ T cells and Th subsets in HS and PD patients.***

278 **Subjects.** The study included 47 HS and 82 PD patients (Table 1). Patients comprised 26 subjects
279 who had been never treated with antiparkinson drugs before enrollment (drug-naïve PD patients,
280 PD-dn), and 56 patients on antiparkinson drugs (drug-treated PD patients, PD-dt). In comparison to
281 PD-dn patients, a larger fraction of PD-dt patients had UPDRS Part III score higher than 10 and
282 H&Y stage 1.5 or more. Disease duration of PD-dt patients was 5.5 ± 4.7 years (range: 0.5-22 years).
283 Complete blood counts of HS and PD patients were all within normal limits (Additional Table
284 S3A), however in comparison to HS both PD-dn and PD-dt patients had less total lymphocytes (on
285 average, about -19% in PD-dn and -16% in PD-dt patients). Complete blood count did not differ
286 between PD-dn and PD-dt patients (Additional Table S3A).

287 **Circulating CD4⁺ T cells and Th subsets.** In both PD-dn and PD-dt patients, CD4⁺ T cells were
288 less than in HS, both as absolute counts and as percentage of total lymphocytes. There was on the
289 contrary no difference between PD-dn and PD-dt patients (Figure 1). Th1 cells did not differ
290 between HS and PD patients in terms of absolute counts but their frequency among CD4⁺ T cells
291 was higher in PD patients ($18.1 \pm 9.0\%$ vs $14.6 \pm 6.1\%$, $P < 0.042$), mainly due to increased proportion
292 in PD-dt patients (Figure 2). Th2 cells were less in PD patients in comparison to HS ($54.9 \pm 35.2 \times$
293 $10^6/L$ vs $78.7 \pm 63.7 \times 10^6/L$, $P < 0.017$), due to decreased count in PD-dt patients, and their frequency
294 was lower in PD-dt in comparison to PD-dn patients (Figure 2). Absolute counts (but not
295 frequencies) of Th17 and Th1/17 cells were less in both PD-dn and PD-dt patients in comparison to
296 HS (Figure 2). In comparison to HS, PD patients as a whole showed similar Th1/Th2 ratio
297 (2.94 ± 2.15 vs 2.48 ± 1.70 in HS, $P = 0.292$) but higher Th1/Th17 ratio (2.69 ± 1.85 vs 2.00 ± 1.40 in
298 HS, $P = 0.040$). In comparison to PD-dn patients, PD-dt patients had higher Th1/Th2 ratio and
299 similar Th1/Th17 ratio (Figure 2). Finally, there were no major differences in DR expression on any
300 Th subsets among HS, PD-dn and PD-dt patients (Additional Figures S3-6).

301 **Transcription factors mRNA levels in CD4⁺ T cells.** In comparison to HS, both PD-dn and PD-dt
302 patients had lower levels of *TBX21*, *STAT3*, *STAT4*, and *NR4A2*, and higher levels of *STAT6*,
303 *GATA3*, and *FOXP3* (Figure 3). *RORC* was also lower in PD-dn and PD-dt patients in comparison
304 to HS, however the difference reached the statistical significance in PD-dt patients only. There was
305 no difference in *STAT1* expression among HS, PD-dn or PD-dt patients (Figure 3).

306 **Polarization of naïve CD4⁺ T cells.** Under standard conditions, the proportion of IFN- γ -positive
307 Th0 cells was nearly twofold in PD patients in comparison to HS (on average, +85% in PD-dn and
308 +87% in PD-dt patients), while there was no difference in either IL-4- or IL17A-positive cells

309 (Figure 4). Th1 polarising conditions resulted in increased IFN- γ -positive cells in both HS and PD
310 patients, however the final proportion of IFN- γ -positive cells was higher in PD patients in
311 comparison to HS (on average, +39% in both PD-dn and PD-dt patients). Th2 polarising conditions
312 resulted in increased IL-4-positive cells in HS and PD-dn patients only, and as a consequence the
313 proportion of IL-4-positive cells in PD-dt patients was less than in HS (on average, -52%) (Figure
314 4). Th17 polarising conditions resulted in increased IL17-A-positive cells in HS only, thus the final
315 proportion of IL17-A-positive cells in PD patients was less than in HS (on average, -39% in PD-dn
316 and -41% in PD-dt patients) (Figure 4).

317

318 ***Study #2 - CD4⁺ Treg in HS and PD patients.***

319 **Subjects.** The study included 33 HS and 66 PD patients (Table 2), 30 PD-dn and 36 PD-dt. In
320 comparison to PD-dn patients, a larger fraction of PD-dt patients had H&Y stage 1.5 or more.
321 Disease duration of PD-dt patients was 6.0 ± 4.3 years (range: 1-16 years). Complete blood counts of
322 HS and PD patients were all within normal limits (Additional Table S3B). Compared to HS
323 however PD-dn patients had less total lymphocytes (on average, about -18%). PD-dt patients had
324 total lymphocyte count lower than HS but higher than PD-dn patients, and the differences with both
325 groups didn't reach the statistical significance.

326 **Circulating Treg.** In agreement with study #1, in both PD-dn and PD-dt patients CD4⁺ T cells
327 were less than in HS, both as absolute counts and percentage of total lymphocytes, without any
328 difference between PD-dn and PD-dt patients (Additional Figure S7). Both total circulating Treg as
329 well as total nTreg and aTreg were less in PD-dn and PD-dt patients than in HS, while on the
330 contrary their frequency was the same in HS and PD patients, due to reduced total CD4⁺ T cells.
331 There was no difference between PD-dn and PD-dt patients (Figure 5). In total Treg, the absolute
332 count of D₂-like DR⁺ cells was lower in both PD-dn and PD-dt patients in comparison to HS, while
333 for D₁-like DR⁺ cells the difference with HS was significant for PD-dn patients only, even if there
334 was no difference between PD-dn and PD-dt patients. Frequency of total DR⁺ Treg among CD4⁺ T
335 cells was on the contrary the same in HS and PD patients (Additional Figure S8). A similar pattern
336 of differences in DR⁺ cells between HS and PD patients was observed in nTreg, while for aTreg the
337 differences were only minor (Additional Figures S9 and S10).

338 **Transcription factors mRNA levels in CD4⁺ T cells.** The pattern of differences between HS and
339 PD patients was the same as in study #1 (Additional Figure S11), with lower levels of *TBX21*,
340 *STAT3*, *STAT4*, and *NR4A2*, and higher levels of *STAT6*, *GATA3*, and *FOXP3* in cells from PD
341 patients. In study #2, *RORC* was lower not only in cells from PD-dt but also from PD-dn patients.
342 *STAT1* expression was not different among HS, PD-dn or PD-dt patients (Additional Figure S11).

343 **Proliferation of Teff and inhibition by Treg.** Teff proliferation in response to PHA was not
344 different in HS, PD-dn and PD-dt patients (Figure 6, panel A), and Treg effectively inhibited Teff
345 proliferation to a similar extent in both HS and PD patients at all the Teff:Treg ratios tested (Figure
346 6, panel B). The inhibitory effect of Treg however was reduced by DA 1 μ M only in HS and PD-dn
347 but not in PD-dt patients (Figure 6, panel C).

348 **Cytokine production by Teff and Treg.** Production of IFN- γ and TNF- α was stimulated by PHA
349 in Teff from HS, PD-dn and PD-dt patients. PHA-induced increase of IFN- γ and TNF- α production
350 was however higher in cells from PD-dn and PD-dt patients compared to HS (on average, +126%
351 and +179% in PD-dn, and +82% and +236% in PD-dt patients). There was no difference in IFN- γ
352 or TNF- α production between cells from PD-dn and PD-dt patients (Figure 7). Production of IL-4
353 and IL17A was not different in Teff from HS, PD-dn and PD-dt patients, and was not affected by
354 PHA (Figure 7). Production of IL-10 was increased by PHA in cells from HS, but not from PD-dn
355 and PD-dt patients (Figure 7).

356 Coincubation of Teff with Treg 1:1 reduced PHA-induced production of IFN- γ on average by 81%
357 in cells from HS but only by 24% and 22% in cells from PD-dn and PD-dt patients. A similar
358 pattern was observed with TNF- α (-87% in cells from HS, -16% and -36% in cells from PD-dn and
359 PD-dt patients) (Figure 8). Production of IL-10 was not different in Treg from HS, PD-dn and PD-
360 dt patients (21.1 $\pm$ 1.49, 14.6 $\pm$ 7.2 and 19.4 $\pm$ 6.2 pg/mL, respectively).

361

362 **No correlations between circulating CD4⁺ T cells, DR expression, transcription factors**
363 **mRNA levels, demographic and clinical features of HS and PD patients.** Circulating Th and
364 Treg absolute counts or frequency, DR expression, and transcription factors mRNA levels in CD4⁺
365 T cells did not correlate with age in HS or with age, UPDRS Part III score, and H&Y stage in PD-
366 dn and PD-dt patients, or with disease duration and LED in PD-dt patients (data not shown).

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370 **Discussion**

371 Peripheral adaptive immunity in PD has been the subject of increasing interest over the last two
372 decades, since in animal models of neurodegeneration T lymphocytes provide a major contribution
373 to neuroinflammation and neuronal death, and targeting the peripheral immune system, e.g. by
374 increasing Treg activity, may result in effective neuroprotection [32, 33]. Various studies indeed
375 suggest the occurrence of peripheral immune changes in PD patients, including decreased
376 CD4⁺/CD8⁺ T-cell ratios, fewer CD4⁺CD25⁺ T cells, increased ratios of IFN- γ -producing to IL-4-
377 producing T cells [34], and decreased CD4⁺ T lymphocytes and CD19⁺ B cells [35, 36]. A recent
378 metaanalysis identified decreased numbers of CD3⁺ and CD4⁺ T lymphocytes as a consistent
379 finding across 21 studies including 943 cases of PD [19]. These observations, together with the
380 occurrence of T lymphocytes in postmortem brain specimens from PD patients [17, 18] as well as in
381 a mouse model of PD [18], and with the markedly reduced dopaminergic neuronal death in mice
382 lacking CD4⁺ T cells [18], strengthened the hypothesis that CD4⁺ T cells are crucial for
383 neurodegeneration during PD, possibly through Th1-dependent mechanisms. We now provide
384 detailed evidence about CD4⁺ T cell phenotypes count and frequency in the peripheral circulation
385 of PD patients as well as about their functional profile. Of particular importance, we included in our
386 studies both patients on dopaminergic substitution treatments as well as drug-naïve patients, who
387 never received any dopaminergic drugs, thus allowing to assess any differences associated with
388 current antiparkinson therapies. Main results can be summarized as follows: (i) PD patients, either
389 drug-naïve or on dopaminergic drugs, have reduced circulating CD4⁺ T lymphocytes; (ii) CD4⁺ T
390 cells reduction is accounted for mainly by reduced circulating Th2, Th17, Th1/17 and Treg,
391 resulting in increased Th1/Th2 and Th1/Th17 ratio; (iii) lineage-specific transcription factors
392 mRNA levels in CD4⁺ T cells from patients exhibit distinctive patterns; (iv) naïve CD4⁺ T cells
393 isolated from blood of patients preferentially differentiate towards the Th1 lineage, and production
394 of IFN- γ and of TNF- α by CD4⁺ T cells from patients is increased and maintained even in the
395 presence of homologous Treg. Remarkably, only minor differences occurred between drug-naïve
396 patients and patients on dopaminergic drugs, namely in the latter group Treg insensitivity to DA.

397 ***Decreased circulating CD4⁺ T cells***

398 Decreased numbers of circulating CD4⁺ T lymphocytes in PD patients has been consistently
399 described by several studies [19]. We previously reported that PD patients have on average $214.5 \times$
400 $10^6/L$ less lymphocytes in comparison to HS [23], a difference which is confirmed in the present
401 investigation by both study #1 ($-244.0 \times 10^6/L$) and study #2 ($-268.9 \times 10^6/L$). Additional Table S4
402 summarizes the differences observed between PD patients and HS in our previous study [23] and in

the present investigation. According to the whole picture, CD4⁺ T cells reduction in PD patients is accounted for by reduced Th2 (on average, $-23.7 \times 10^6/L$), Th17 ($-31.1 \times 10^6/L$), Th1/17 ($-33.8 \times 10^6/L$), Treg ($-30.4 \times 10^6/L$), and T naïve cells ($-117.2 \times 10^6/L$), which altogether make a total of $236.2 \times 10^6/L$ less cells, an amount well matching with the global differences above reported for total CD4⁺ T cells. In this scenario, Th1 cell count is not different between PD patients and HS, however in patients the contextual reduction of the other phenotypes leads to their relative increase (on average, +3.5% of total CD4⁺ T cells).

The Th1 bias

Reduced circulating Th2, Th17, Th1/17 and Treg in PD patients leads to a relative increase of Th1 cells, which constitutes the basis for a possible Th1 bias, as also suggested by the increased Th1/Th2 and Th1/Th17 ratios in PD patients. Such bias is however made clear by results of *in vitro* functional tests, showing first of all a preferential differentiation of naïve CD4⁺ T cells from both drug-naïve patients and patients on dopaminergic drugs towards the Th1 lineage. Indeed, IFN- γ -positive Th0 cells were nearly twofold in PD patients in comparison to HS, and Th1 polarising conditions resulted in increased IFN- γ -positive cells in all PD patients, while Th2 polarising conditions resulted in less IL-4-positive cells, at least in patients on dopaminergic drugs. Finally, Th17 polarising conditions resulted in increased IL17-A-positive cells in HS, but not in PD patients either drug-naïve or on dopaminergic drugs. Preferential differentiation of naïve CD4⁺ T cells from PD patients towards the Th1 lineage suggests the involvement of homeostatic mechanisms driving naïve T cell differentiation [37]. Increased production of the Th1 cytokines IFN- γ and TNF- α by CD4⁺ effector T cells from PD patients also contributes to the Th1 bias. In comparison to cells from HS, IFN- γ and TNF- α production in cells from either drug-naïve PD patients or from patients on dopaminergic drugs was indeed between two- and nearly three-fold higher. Finally, support to such Th1 bias in PD patients likely derives also from the lack of reduction of IFN- γ and TNF- α production by CD4⁺ effector T cells in the presence of Treg, as well as by the decreased production of IL-10 by CD4⁺ effector T cells themselves [38]. Figure 9 summarizes the differences occurring in circulating CD4⁺ T cells between HS and PD patients.

The occurrence of imbalances in the differentiation process towards the different CD4⁺ T cell lineages/phenotypes is indirectly supported also by the profound modifications of transcription factor genes expression in CD4⁺ T cells from PD patients in comparison to those from HS. In PD patients, CD4⁺ T cells expressed lower levels of *TBX21* and *STAT4*, which together with *STAT1* drive Th1 differentiation [39], as well as of *RORC* and *STAT3*, which regulate the differentiation towards the Th17 lineage [40], and higher levels of *STAT6* and *GATA3*, which are master regulators of Th2 development [41, 42]. *FOXP3*, which is centrally involved in the development and

437 maintenance of the Treg phenotype [43], was upregulated, while *NR4A2*, encoding the orphan
438 nuclear receptor Nurr1 which affects Treg cell development through activation of *FOXP3* [44], was
439 downregulated. Transcription factor genes were assessed by means of mRNA levels, which
440 however are not necessarily a homogeneous measure of gene transcript activity. Indeed,
441 increased/decreased mRNA levels might mirror either increased/decreased transcription or
442 decreased/increased translation (or any combination of either). Modification of transcription factors
443 mRNA levels must be taken therefore as an indicator of their involvement in the disease, and not as
444 a quantitative measure of transcription factors activation. For instance, modification of *TBX21* and
445 *STAT4*, which mediate primarily IL-12-induced Th1 differentiation, but not of STAT1, which is
446 involved in IFN- γ -dependent reinforcement of the Th1 phenotype [39], might therefore indicate a
447 preferential involvement of IL-12-dependent pathways and a minor, if any, role of IFN- γ signalling
448 in the increased Th1 differentiation and function of CD4⁺ T cells occurring in PD patients.
449 Remarkably, such modifications occurred to the same extent in both drug-naïve patients as well as
450 in patients on dopaminergic substitution treatments (Figure 3 and Additional Figure S11),
451 suggesting that modifications of the transcription factors network in CD4⁺ T cells occurs early in
452 PD. The similar pattern and extent of modifications in all the patients and the absence of
453 correlations between any of the transcription factors and patients characteristics, including age, age
454 at onset, disease duration, LED, and UPDRS Part III score, suggests that modification of CD4⁺ T
455 cell differentiation mechanisms is independent from PD progression and severity, and insensitive to
456 drug treatments.

457 ***No relationship with dopaminergic substitution therapy or with PD patients' characteristics***

458 Our investigation provides phenotypic and functional evidence supporting a Th1 bias of circulating
459 CD4⁺ T cells in PD patients, thus giving clinical relevance to preclinical observations in animal
460 models of PD supporting the contribution of Th1-related mechanisms to neuroinflammation and
461 neurodegeneration [45, 46]. Our results also reveal a dysfunction of the Treg compartment, since
462 Treg from PD patients inhibit the proliferation of effector CD4⁺ T cells to the same extent as those
463 from HS, however they completely fail to reduce production of the Th1 cytokines IFN- γ and TNF-
464 α . Dysfunctional Treg from PD patients were recently reported also by Saunders et al. (39), who
465 however did not investigate the ability of these cells to modulate cytokine production. On the other
466 side, reduced circulating Th17 and Th1/17 cells in PD patients, as well as no evidence for increased
467 production of IL-17 by CD4⁺ T cells of patients, suggests a marginal if any role for this cell lineage
468 in PD, despite evidence from animal models [46, 48]. Such peripheral Th1 bias, eventually
469 amplified by dysfunctional Treg, occurs in both drug-naïve patients and in patients on dopaminergic
470 drugs, suggesting that dopaminergic substitution treatments do not exert major effects on peripheral

471 CD4+ T cells. Actually, in patients on dopaminergic drugs results show also insensitivity of Treg to
472 dopaminergic functional inhibition. DA exerts a physiological inhibition of Treg suppressive
473 activity [49], and resistance to DA in PD patients on dopaminergic drugs might possibly arise from
474 desensitization of DA-operated pathways in these cells. The lack of major differences in DR
475 expression on Treg between drug-naïve patients and patients on dopaminergic drugs however points
476 to intracellular mechanisms. In any case, Treg resistance to DA might also imply that dopaminergic
477 drugs would not affect Treg suppressive activity, a condition which at least in principle could be
478 beneficial in PD.

479 ***Possible origin and implications of the Th1 bias***

480 The major question posed by this finding is whether such immune profile represents a preexisting
481 milieu favouring subsequent neuroinflammation, or whether it arises as a consequence of peripheral
482 leakage of CNS-derived neoantigens, such as modified α -synuclein within Lewy bodies released
483 from dying or dead dopaminergic neurons, as suggested by animal models of neurodegeneration
484 [50]. In this regard, careful consideration might be deserved by emerging evidence about the role of
485 the immune system as key regulator of the influence on brain development and homeostasis exerted
486 by the gut microbiome [51]. A recent study compared the fecal microbiomes of PD patients and
487 controls, showing in feces of PD patients nearly 80% decreased abundance of *Prevotellaceae*, and
488 increased abundance of other families including *Lactobacillaceae* [52]. Although how the intestinal
489 microbiome contributes to development, homeostasis and activation of the immune system is still
490 largely unknown, it is remarkable that *Prevotellaceae* abundance may be associated with increased
491 Th17 -mediated mucosal inflammation, in line with their ability to drive Th17 immune responses *in*
492 *vitro* [53]. On the other side, several lines of evidence indicate that *Lactobacillaceae* may induce
493 Th1-type immune responses [54]. Taking together available evidence, it might be speculated that
494 modifications of the intestinal microbiome in PD is a major driver for the development of immune
495 traits such as reduced Th17 cells and Th1-biased immunity. Indeed, clinical and pathological
496 evidence support the hypothesis that PD starts in the gut, where α -synuclein-related
497 neurodegeneration of the enteric nervous system is a frequent and early manifestation of PD
498 (reviewed in 55). Presentation of α -synuclein to lymphocytes homing into the gut-associated
499 lymphoid tissue, in the context of a disbiotic intestinal milieu, might well promote α -synuclein
500 antigenic potential, driving detrimental T cell responses in PD patients [56]. The peripheral immune
501 signature of PD might thus arise in the gut from a complex interaction among intestinal
502 microbioma, the enteric nervous system and the immune system, with the possible influence of
503 environmental factors eventually affecting any of the three actors.

504 Defining the time sequence of peripheral immune activation with respect to central
505 neuroinflammation would be indeed of key importance to establish the potential of peripheral
506 immunity as target for novel therapeutic interventions aimed at preventing neurodegeneration. A
507 major obstacle in this regard is however the lack of solid biomarkers for the early identification of
508 very initial preclinical conditions which will over the years progress to clinically established PD.
509 Interestingly, constipation is a very common occurrence in PD and evidence suggests that it may
510 even represent a major risk factor for subsequent PD development even several years later (see e.g.
511 57). Indeed, circumstantial evidence suggests that chronic constipation might be associated with
512 immune activation [58]. Would it be possible that among subjects with severe constipation those
513 subsequently developing PD would be preferentially those with the most prominent
514 proinflammatory immune profile? A prospective study including an adequate sample of subjects
515 with severe chronic constipation periodically assessed for their peripheral immune profile in
516 comparison to their neurological conditions might possibly contribute to answer these questions.

517 ***Limits of the investigation***

518 The main limitations of our studies are the cross-sectional design and the inability to perform all the
519 assays in all the subjects. A longitudinal design would allow to study the same subjects over a
520 period of time, detecting changes at both group and individual level, thus allowing to establish a
521 sequence of events. Our project, aimed at investigating peripheral adaptive immunity on PD, started
522 however with a set of cross-sectional studies as this kind of studies can be done more quickly than
523 longitudinal studies, and allowed us to establish main differences and similarities between PD
524 patients and HS, as well as between drug-naïve patients and patients on dopaminergic drugs. Based
525 on results, we already started a longitudinal protocol including all the drug-naïve patients so far
526 recruited, as well as additional ones that we are presently recruiting, to assess any changes in the
527 phenotypic and functional CD4⁺ T cells profile over several years. Planned evaluations include at
528 present both 2- and 4-year follow-up.

529 Inability to perform all the assays in all the subjects and the subsequent need to include in the
530 present investigation two study protocols, the first focussing on Th1/Th2/Th17 and the second one
531 on Treg, was due mainly to the time sequence of validation of the different flow cytometric panels
532 and of the *in vitro* models for the functional assessment of CD4⁺ T cell subsets. Requirement of
533 significant amounts of blood for *in vitro* models was another factor which limited the possibility to
534 assess all the cellular phenotypes and functions in all the subjects. Nonetheless, performing two
535 separate studies also allowed us to check the reproducibility of many results. Indeed, total leukocyte
536 and specific CD4⁺ T cell counts, as well as transcription factors expression in CD4⁺ T cells were
537 performed in all the subjects and provided consistent results across the studies, as shown in

538 Additional Table S3A and B for total leukocytes, in Figure 1 and in Additional Figure S7 for CD4+
539 T cell counts, and in Figure 3 and in Additional Figure S11 for transcription factors. An additional
540 limitation is the assessment of dopaminergic substitution treatment only at the time of enrollement.
541 Regarding dopaminergic drugs, although we used LED [27] to obtain a homogenous measurement
542 of treatment intensity, we were unable to assess treatment duration and changes in time. Such
543 limitation will be overcome only using a longitudinal study design.

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Conclusions

The results of our investigation provide for the first time a comprehensive and detailed account of a complex phenotypic and functional Th1-biased adaptive immune response occurring in peripheral blood of PD patients. Reduction of CD4⁺ T cells as also reported in previous studies might apparently suggest impairment of peripheral immunity. The present results however, including detailed analysis of CD4⁺ T cell subsets coupled to their functional characterization, allow now detailed understanding of many subtle and concurrent changes affecting several components of the CD4⁺ T cell compartment, leading as a whole to a preminence of Th1 immunity. In particular, reported functional responses specific to the different CD4⁺ T cell subsets represent suitable biomarkers for both the *ex vivo/in vitro* assessment of candidate peripheral immune system-targeted treatments for PD, as well as for the assessment of their effects in clinical trials. Recently, β_2 -adrenoceptors (β_2 -AR) were suggested as brand novel therapeutic targets in PD, based on their ability to modulate α -synuclein gene expression in human cells and to abrogate neurodegeneration in the MPTP mouse model of PD, and considering the association in the Norwegian population between use of the β_2 -AR agonist salbutamol and of the β_2 -AR antagonist propranolol with, respectively, reduced and increased risk of PD [59]. Remarkably, β_2 -AR are the main interface between sympathoadrenergic nerves and immune cells [60-62], and stimulation of β_2 -AR on CD4⁺ T lymphocytes inhibits Th1 and stimulates Th2 responses [60, 61], and possibly enhances Treg suppressive functions [63]. β_2 -AR agonists represent therefore obvious candidates to be tested on CD4⁺ T cells from PD patients for their ability to correct dysfunctional responses such as Th1-biased differentiation of CD4⁺ naïve T cells and increased production of Th1 cytokines insensitive to Treg inhibition. Any drugs which would result effective in such *in vitro* testings might well be candidate for therapeutic trials in PD patients as add-ons to conventional dopaminergic substitution treatments.

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Declarations

Ethics approval and consent to participate

The Ethics Committees of Ospedale di Circolo of Varese (I) and Neurological Institute “C. Mondino” of Pavia (I) approved the protocol and all the participants signed a written informed consent before enrollment.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Study conception and design: FB, GB, FM, MC

Recruitment of healthy subjects and PD patients: CC, LM, BM, GR, AS, MM, GB

Acquisition of data: NK, CC, ER, ML, RB, IA, BM, GR

Analysis and interpretation of data: NK, CC, ER, ML, FB, GB, FM, MC

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity

606 of any part of the work are appropriately investigated and resolved, and declare to have confidence
607 in the integrity of the contributions of their co-authors.

608

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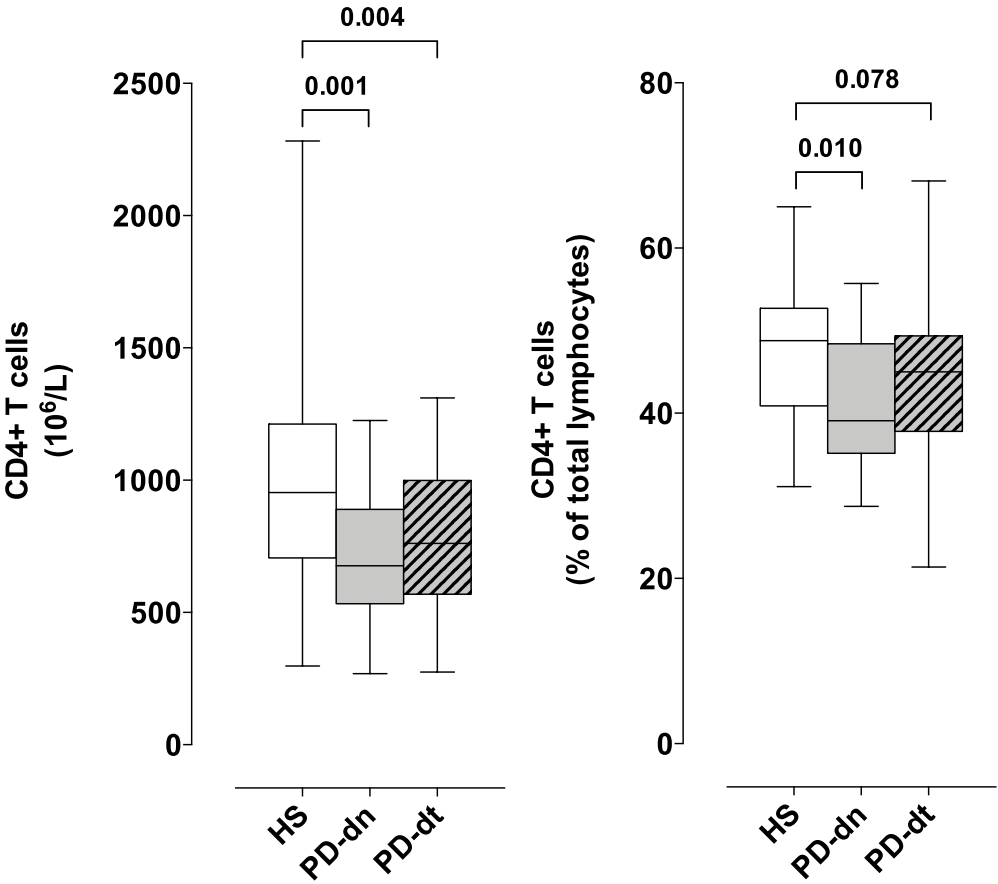
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Figure 1

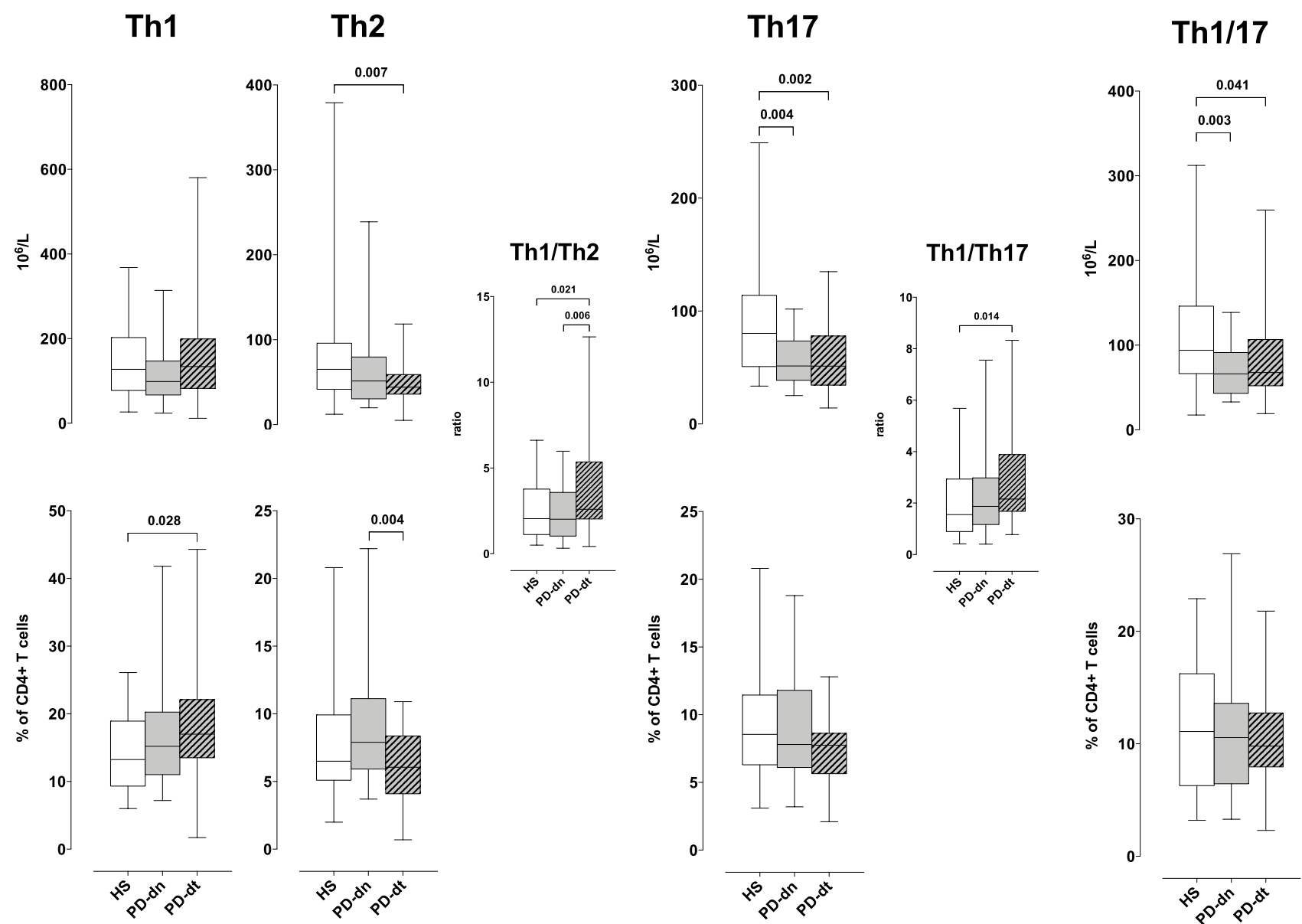


793

794 **Figure 1. CD4+ T cells in HS and PD patients.** Cells are shown as absolute numbers (left panel)
795 and as percentage of total lymphocytes (right panel). Data are medians with 25°-75° percentiles
796 (boxes) and min-max values (whiskers).

797

Figure 2

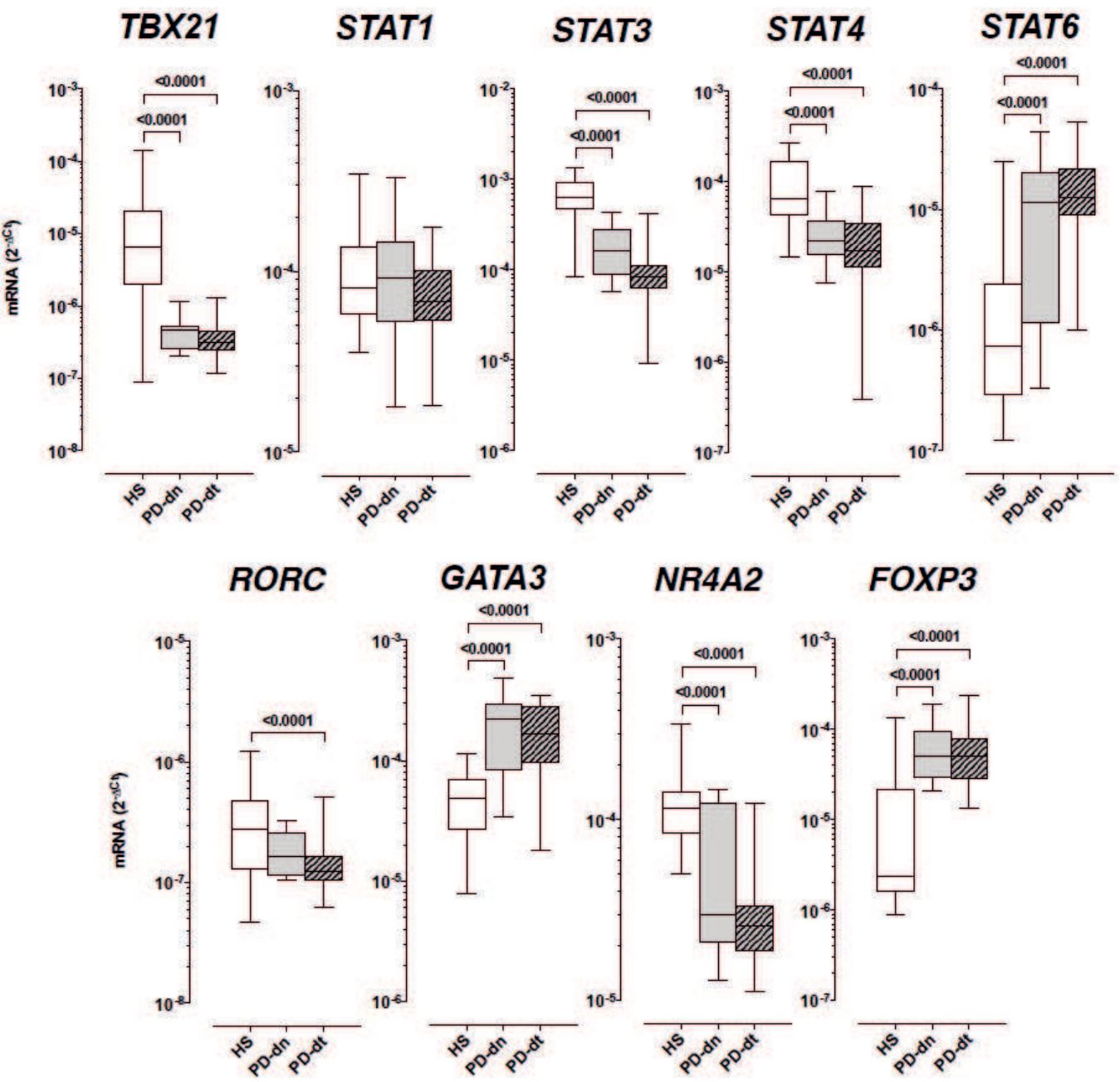


798

799 **Figure 2. Th subsets in HS and PD patients.** Cells are shown as absolute numbers (upper panels)
800 and as percentage of total lymphocytes (lower panels). Th1/Th2 ratio and Th1/Th17 ratio are also
801 shown (middle panels). Data are medians with 25°-75° percentiles (boxes) and min-max values
802 (whiskers).

803

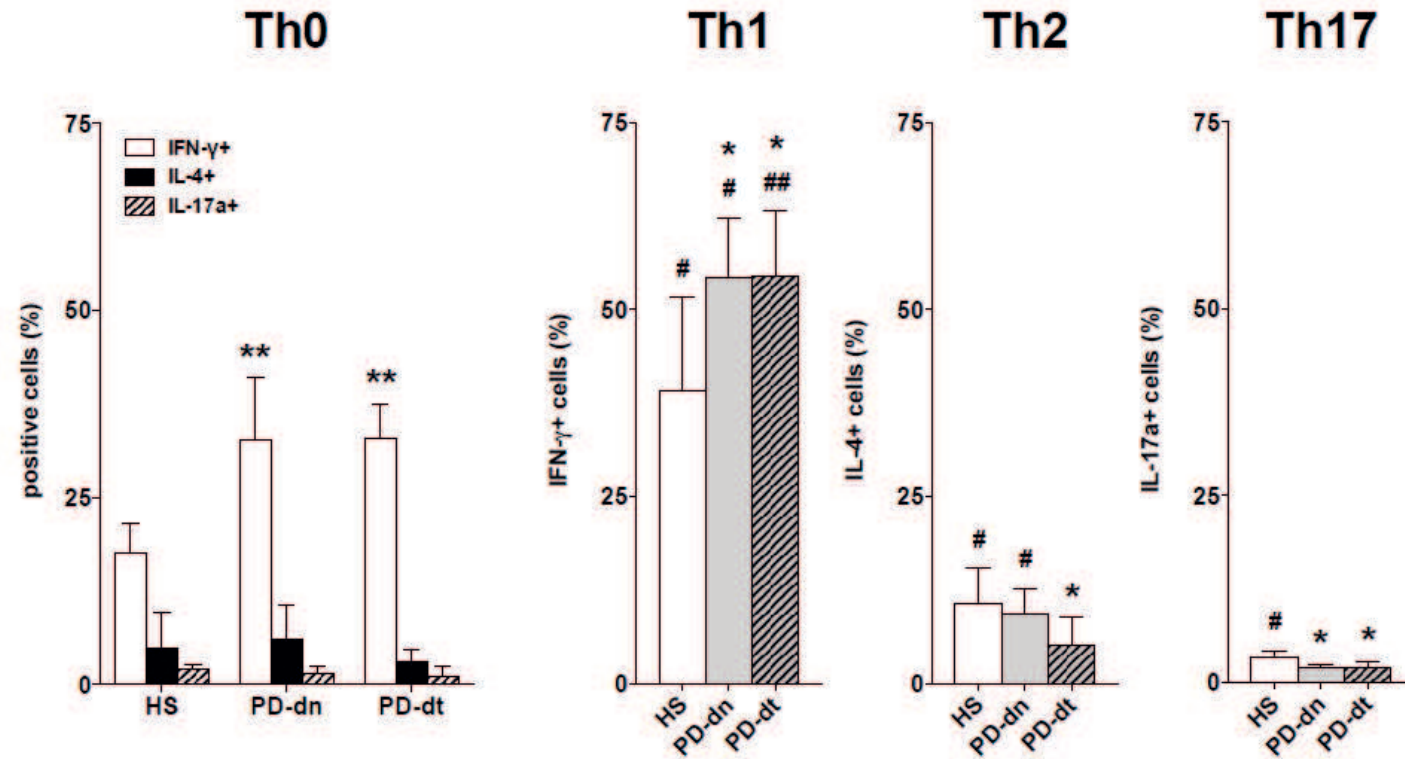
Figure 3



804

805 **Figure 3. Transcription factors mRNA levels in CD4+ T cells of HS and PD patients.** Data are
806 medians with 25°-75° percentiles (boxes) and min-max values (whiskers).

807



808

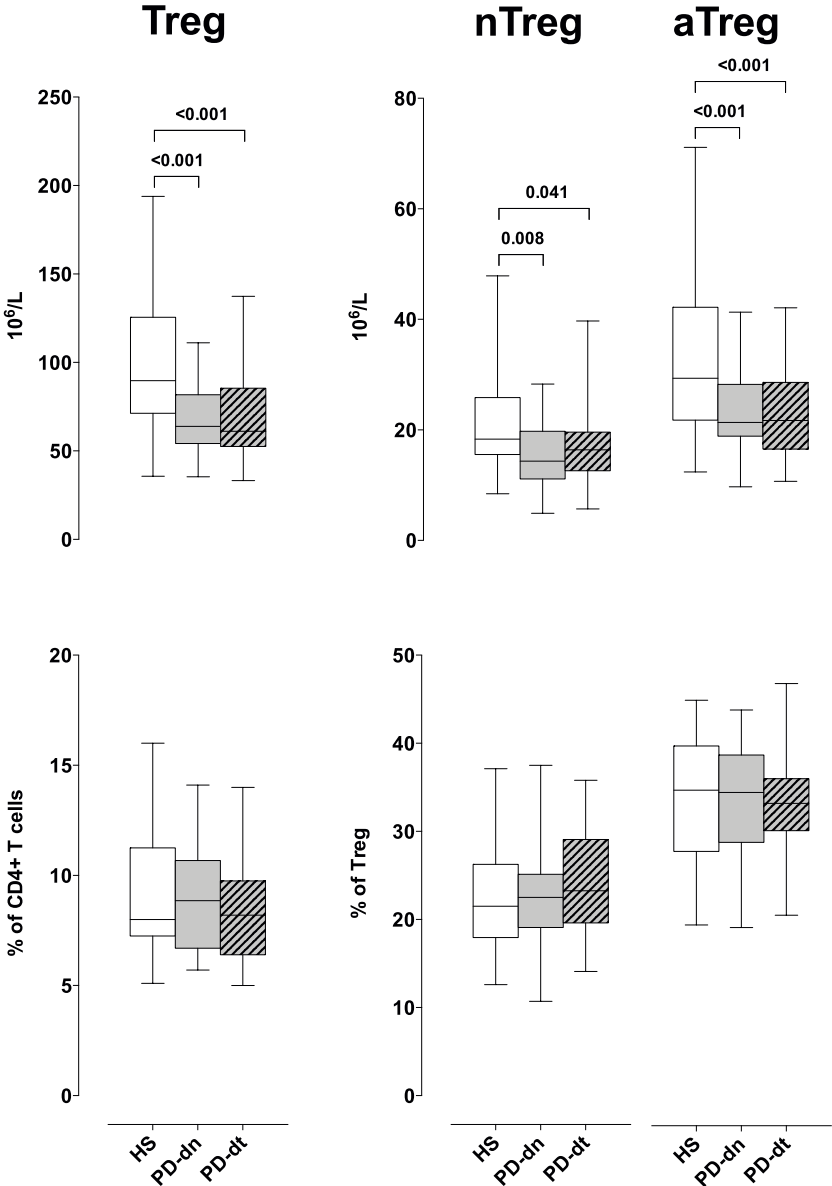
809 **Figure 4. Polarisation of naïve CD4⁺ T cells towards Th1, Th2 and Th17 in HS and PD**

810 **patients.** Data are means±SD of n = 5-6 subjects tested in triplicate. * = P<0.05 and ** = P<0.01 vs

811 HS; # = P<0.05 and ## = P<0.01 vs standard conditions (Th0).

812

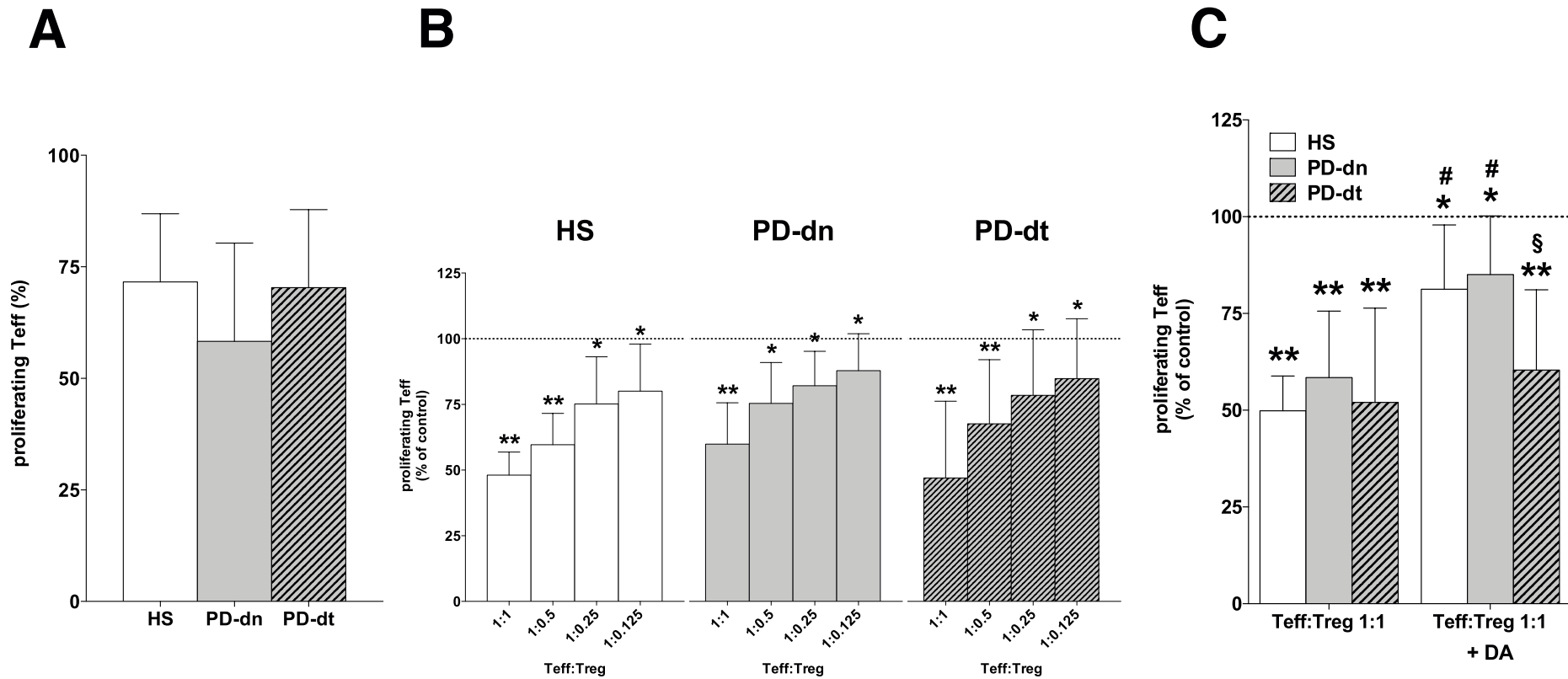
Figure 5



813

814 **Figure 5. Treg cells in HS and PD patients.** Cells are shown as absolute numbers (upper panels)
815 and as percentage of total CD4+ T cells and of total Treg, respectively (lower panels). Data are
816 medians with 25°-75° percentiles (boxes) and min-max values (whiskers).

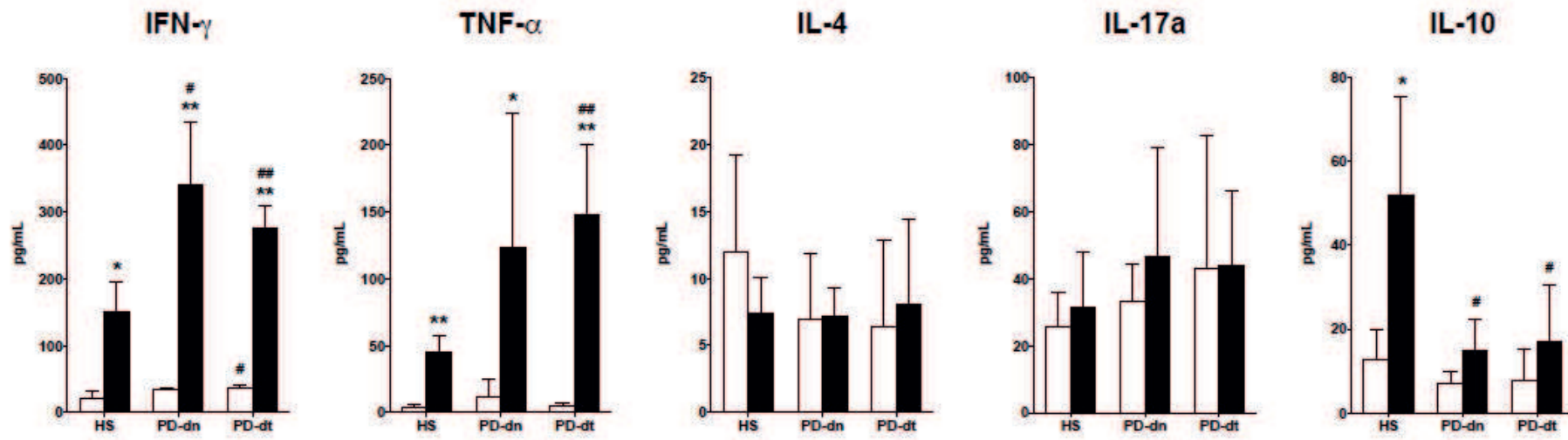
817



818

819 **Figure 6. Treg-induced inhibition of Teff proliferation in HS and PD patients.** Teff from HS
820 and PD patients proliferated to a similar extent in the presence of PHA (A), and were concentration-
821 dependently inhibited by Treg (B). Treg inhibition of Teff proliferation was reduced by DA 1 μ M in
822 cells from HS and PD-dn patients but not from PD-dt patients (C). Data are means $\pm$ SD of n = 9-17
823 subjects. * = P<0.05 and ** = P<0.01 vs respective Teff alone; # = P<0.05 vs respective Teff:Treg
824 1:1; § = P<0.05 vs both HS and PD-dn.

825

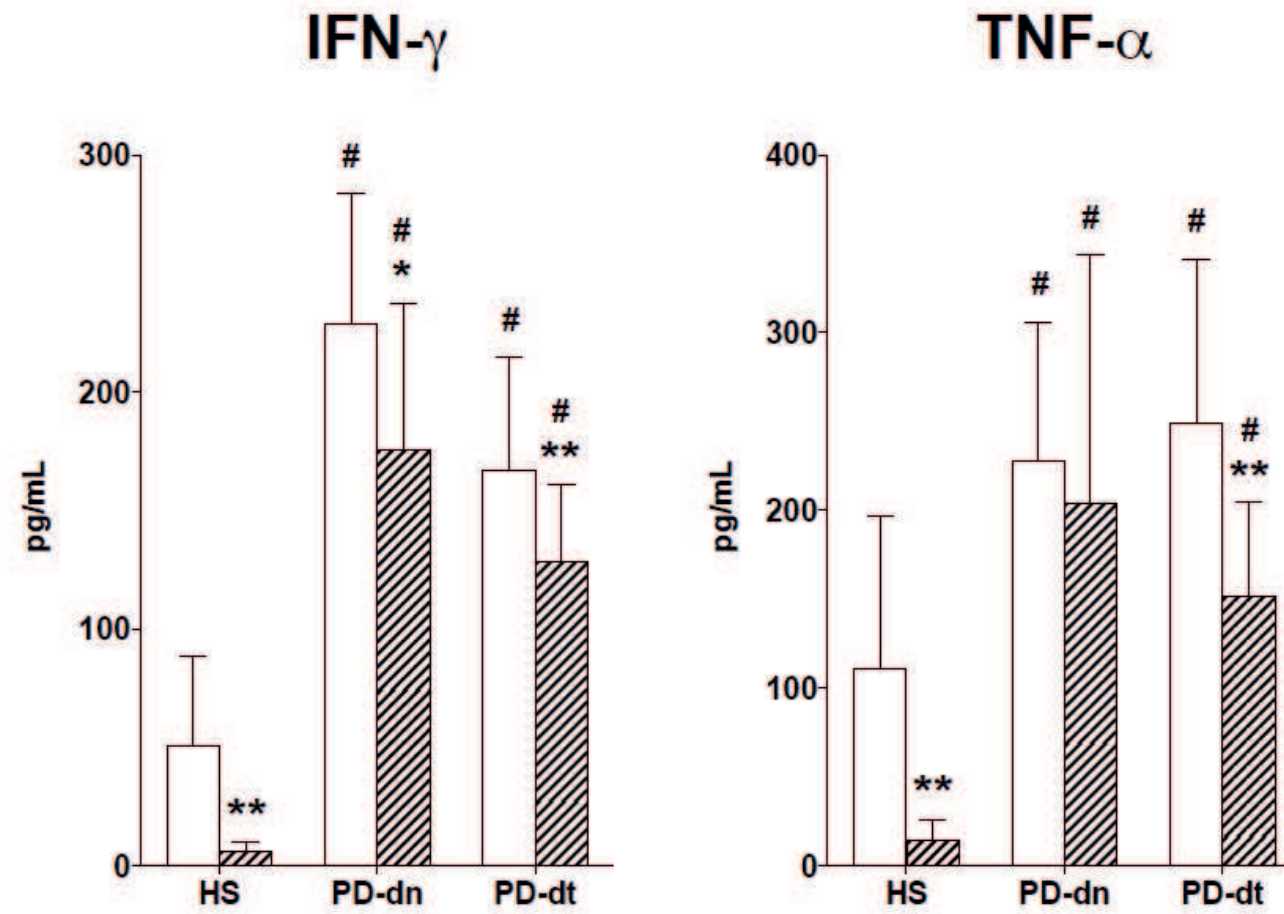


826

827 **Figure 7. Cytokine production by Teff cells in HS and PD patients.** Cytokines were measured in
828 supernatants of resting cells (white columns) and of cells stimulated with PHA (black columns).

829 Data are means $\pm$ SD of n = 4-9 subjects. * = P<0.05 and ** = P<0.01 vs resting cells; # = P<0.05
830 and ## = P<0.01 vs HS.

831



832

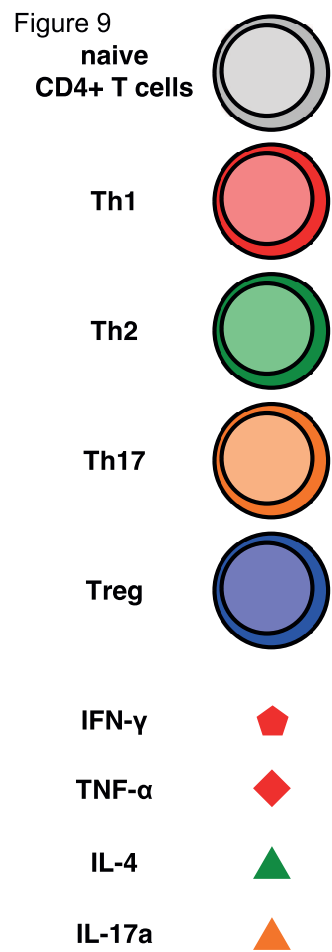
833 **Figure 8. Teff production of IFN- γ and TNF- α and inhibition by Treg in HS and PD patients.**

834 Teff were stimulated with PHA alone (white columns) or in the presence of Treg 1:1 (hatched

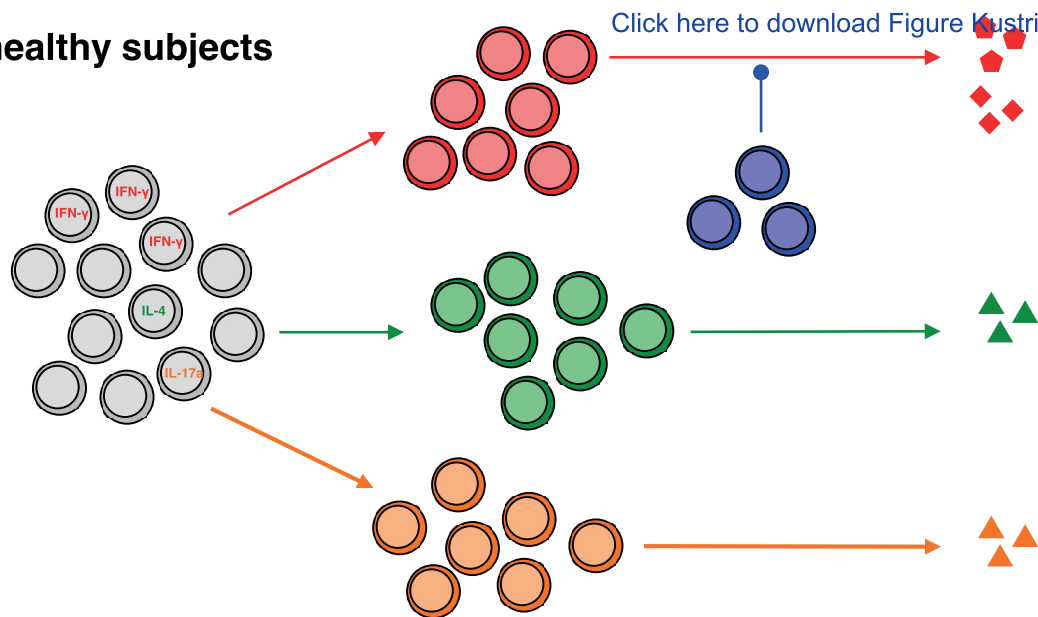
835 columns). Data are means $\pm$ SD of n = 6-10 subjects. * = P<0.05 and ** = P<0.01 vs Teff alone; # =

836 P<0.01 vs HS.

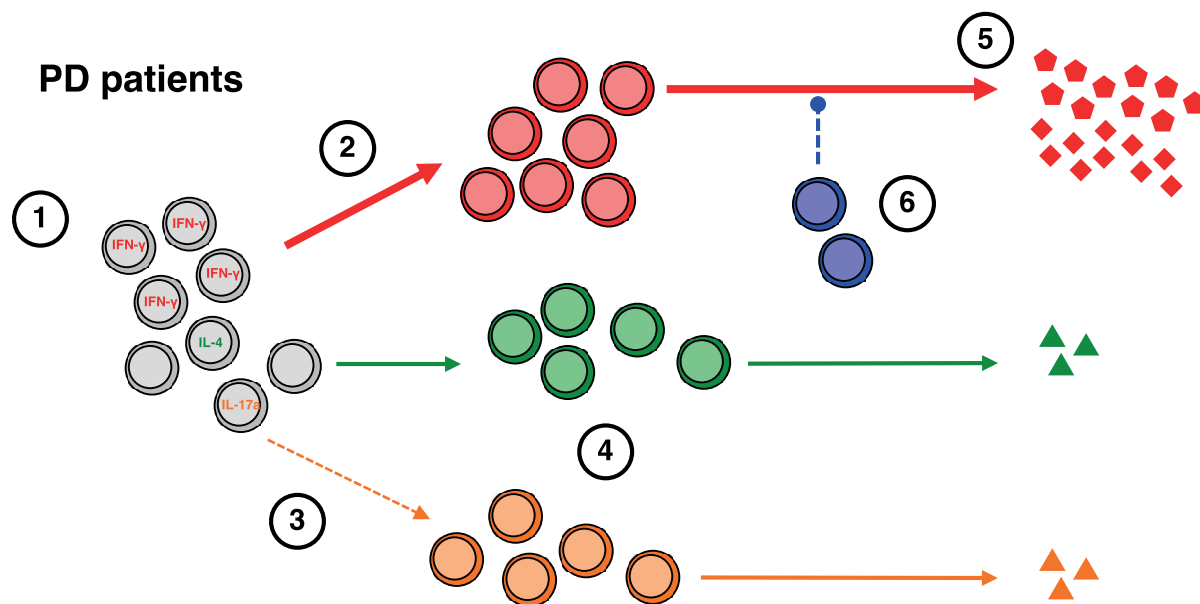
837



healthy subjects



PD patients



838

839 **Figure 9. The Th1 bias in PD.** Circulating CD4⁺ T naive cells in PD patients are reduced by about
840 30%, however the proportion of IFN- γ -positive cells is increased (1). Differentiation towards the
841 Th1 lineage is increased (2), while differentiation towards Th17 is impaired (3), and both Th2 and
842 Th17 cells in blood are reduced on average by 20-30% (4). Production of IFN- γ and TNF- α by Th1
843 cells is strongly increased (5) and not impaired in the presence Treg, which are also reduced by
844 30% in the circulation (6). Reduced production of IL-10 by CD4⁺ effector T cells, which likely
845 contributes to amplify the Th1 bias, is not represented.

846

847

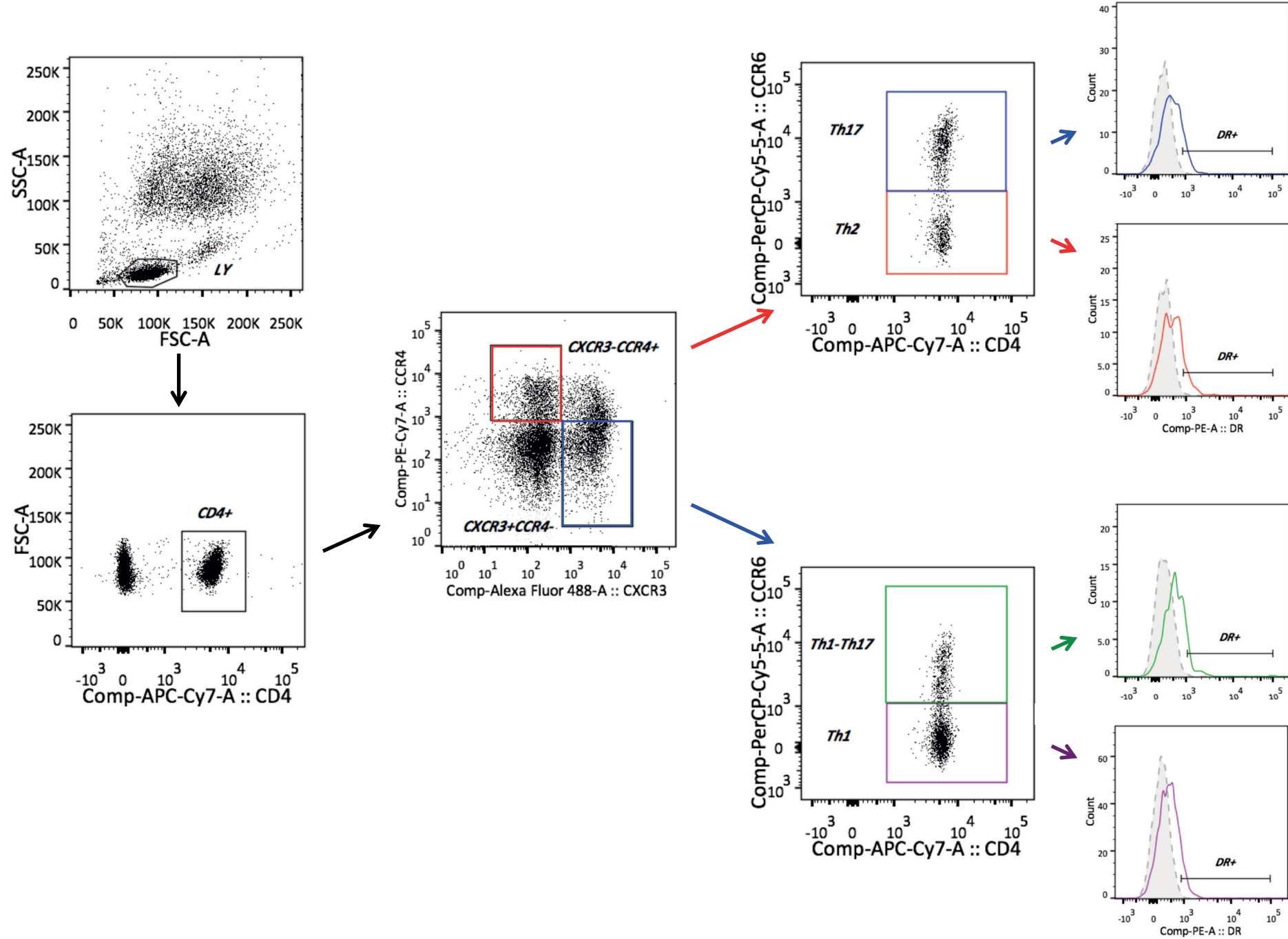


Figure S1

848

849 **Additonal Figure S1. CD4+ Th cells and DR expression in whole blood.** Gating strategy used to
850 identify DR+CD4+ T lymphocytes. Total lymphocytes were identified on a biparametric dot plot
851 SSC vs FSC (left, upper panel [LY]), to exclude monocytes, granulocytes and debris. From the cells
852 in LY, CD4+ T cells were selected by using the FSC vs CD4 dot plot (left, lower panel [CD4+]). Th
853 subsets were thereafter identified according to the expression of CXCR3, CCR4 and CCR6. Finally,
854 DR expression was assessed by a single-parameter histogram (right panels), where the solid lines
855 represent DR+ cells and the dashed lines represents the negative control (cells stained with the
856 secondary PEGAR ab only). DR D5 Staining is shown in the example.

857

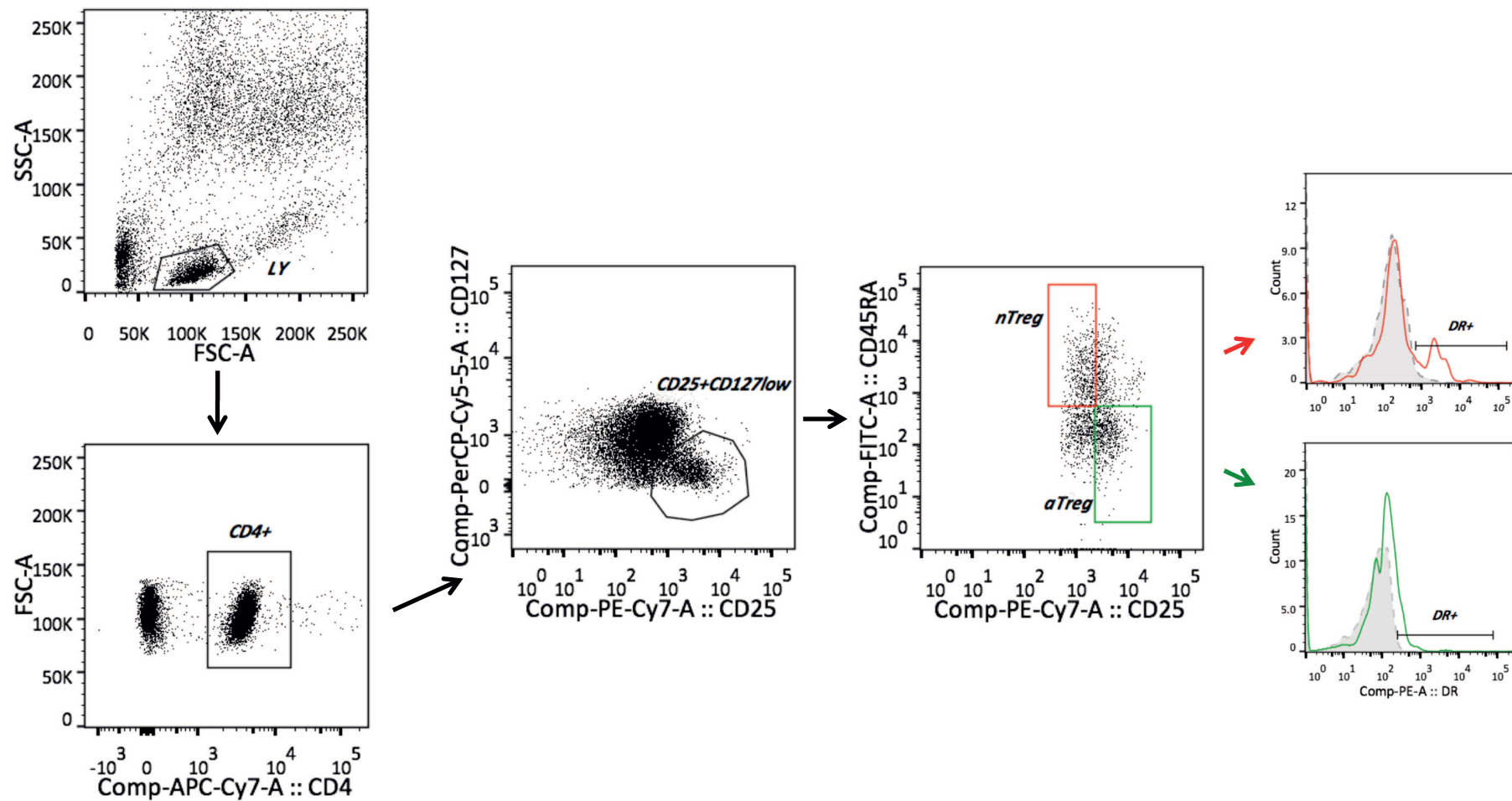


Figure S2

858

859 **Additional Figure S2. CD4+ Treg cells and DR expression in whole blood.** Gating strategy used
860 to identify DR+CD4+ Treg cells. Total lymphocytes were identified on a biparametric dot plot SSC
861 vs FSC (left, upper panel [LY]), to exclude monocytes, granulocytes and debris. From the cells in
862 LY, CD4+ T cells were selected by using the FSC vs CD4 dot plot (left, lower panel [CD4+]). Treg
863 were thereafter identified according to the expression of CD25 and CD127, and nTreg and aTreg
864 were separated by means of CD45RA. Finally, DR expression was assessed by a single-parameter
865 histogram (right panels), where the solid lines represent DR+ cells and the dashed lines represents
866 the negative control (cells stained with the secondary PEGAR ab only). DR D5 Staining is shown in
867 the example.

868

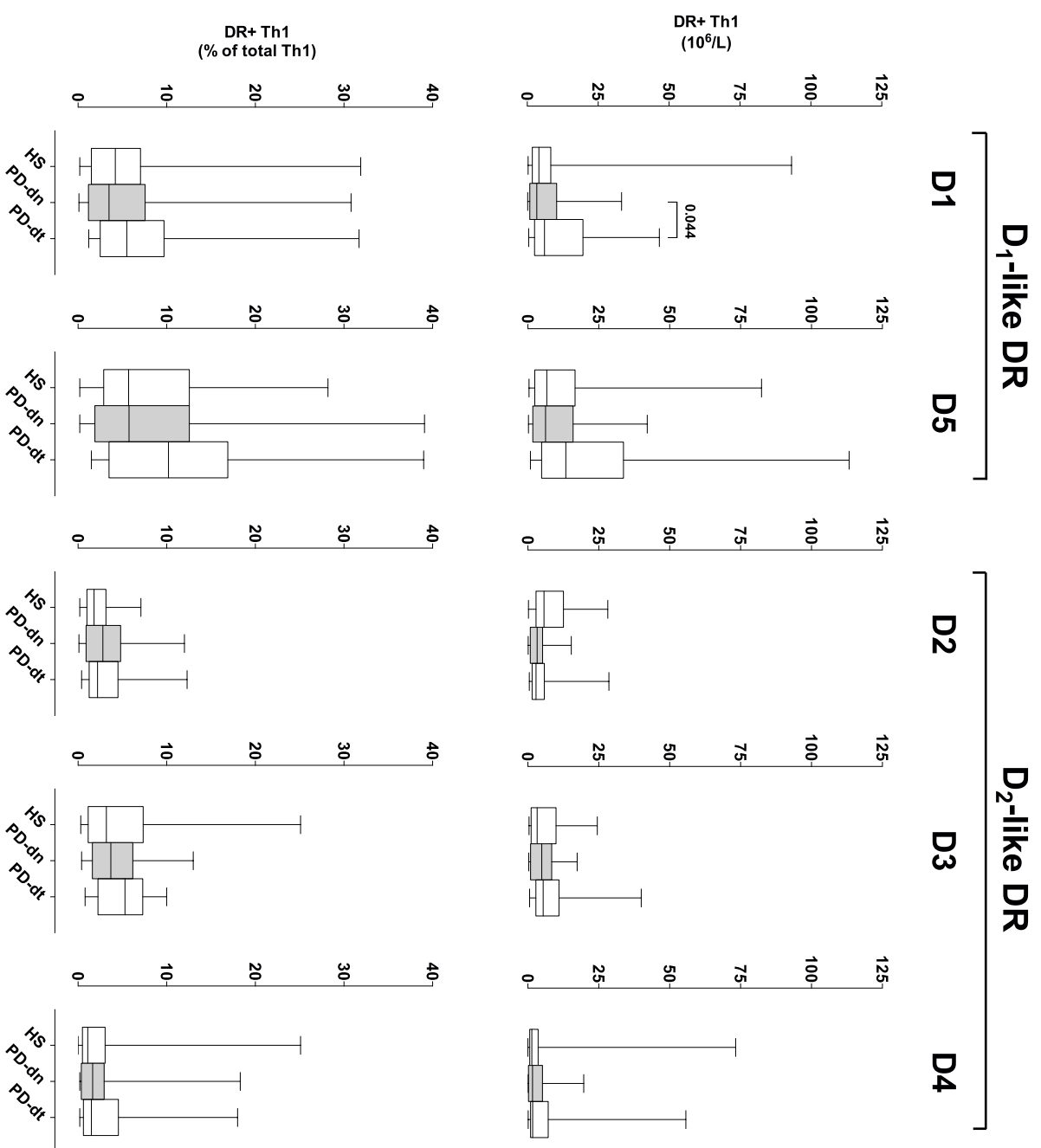


Figure S3

869

870 **Additional Figure S3. DR expression on Th1 cells in HS and PD patients.** Cells are shown as
871 absolute numbers (upper panels) and as percentage of total Th1 cells (lower panels). Data are
872 medians with 25°-75° percentiles (boxes) and min-max values (whiskers). Differences were
873 analyzed by means of two-tailed Student's *t* test or by Mann-Whitney test, as appropriate.
874

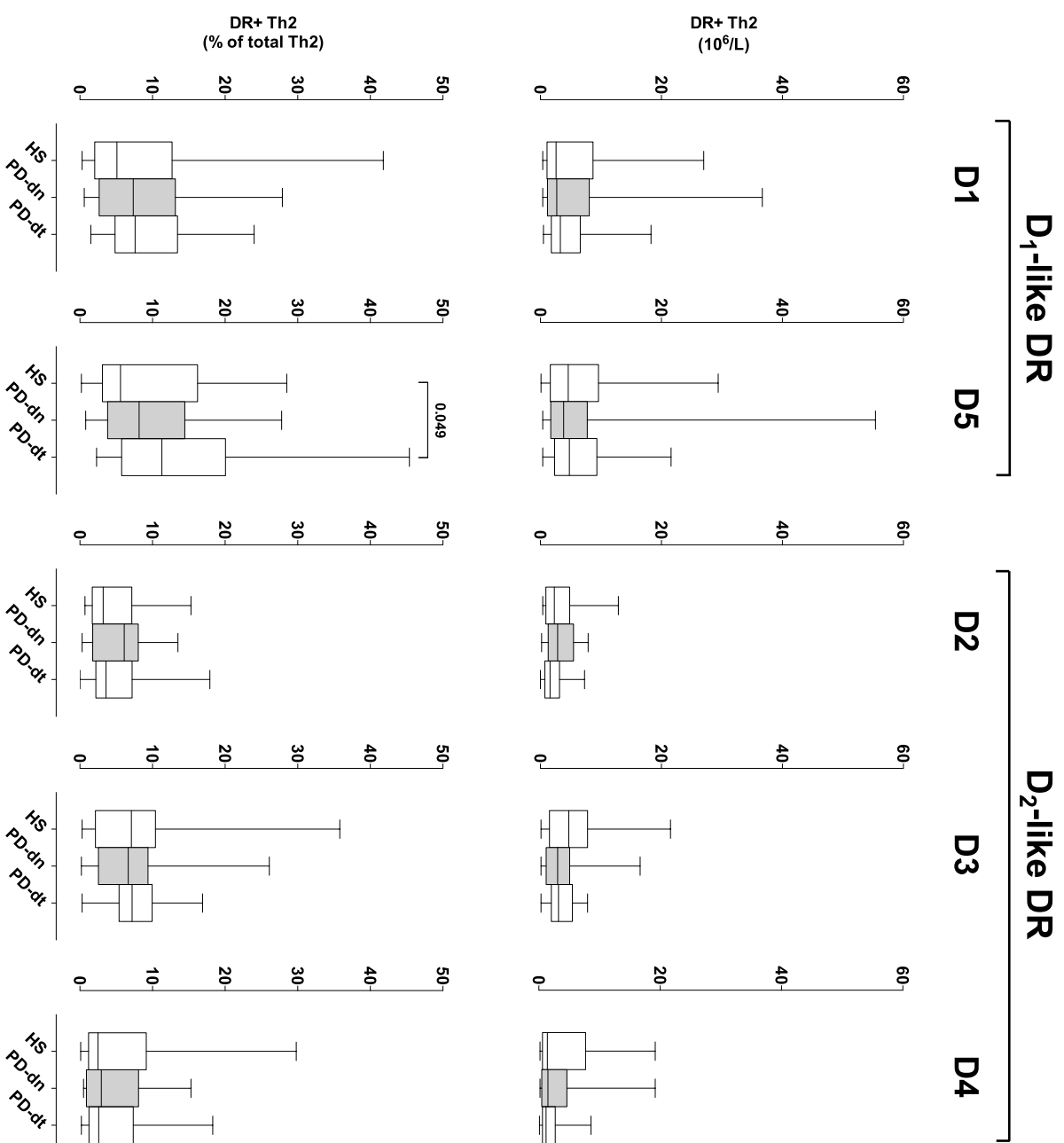


Figure S4

875

876 **Additional Figure S4. DR expression on Th2 cells in HS and PD patients.** Cells are shown as
877 absolute numbers (upper panels) and as percentage of total Th2 cells (lower panels). Data are
878 medians with 25°-75° percentiles (boxes) and min-max values (whiskers). Differences were
879 analyzed by means of two-tailed Student's *t* test or by Mann-Whitney test, as appropriate.
880

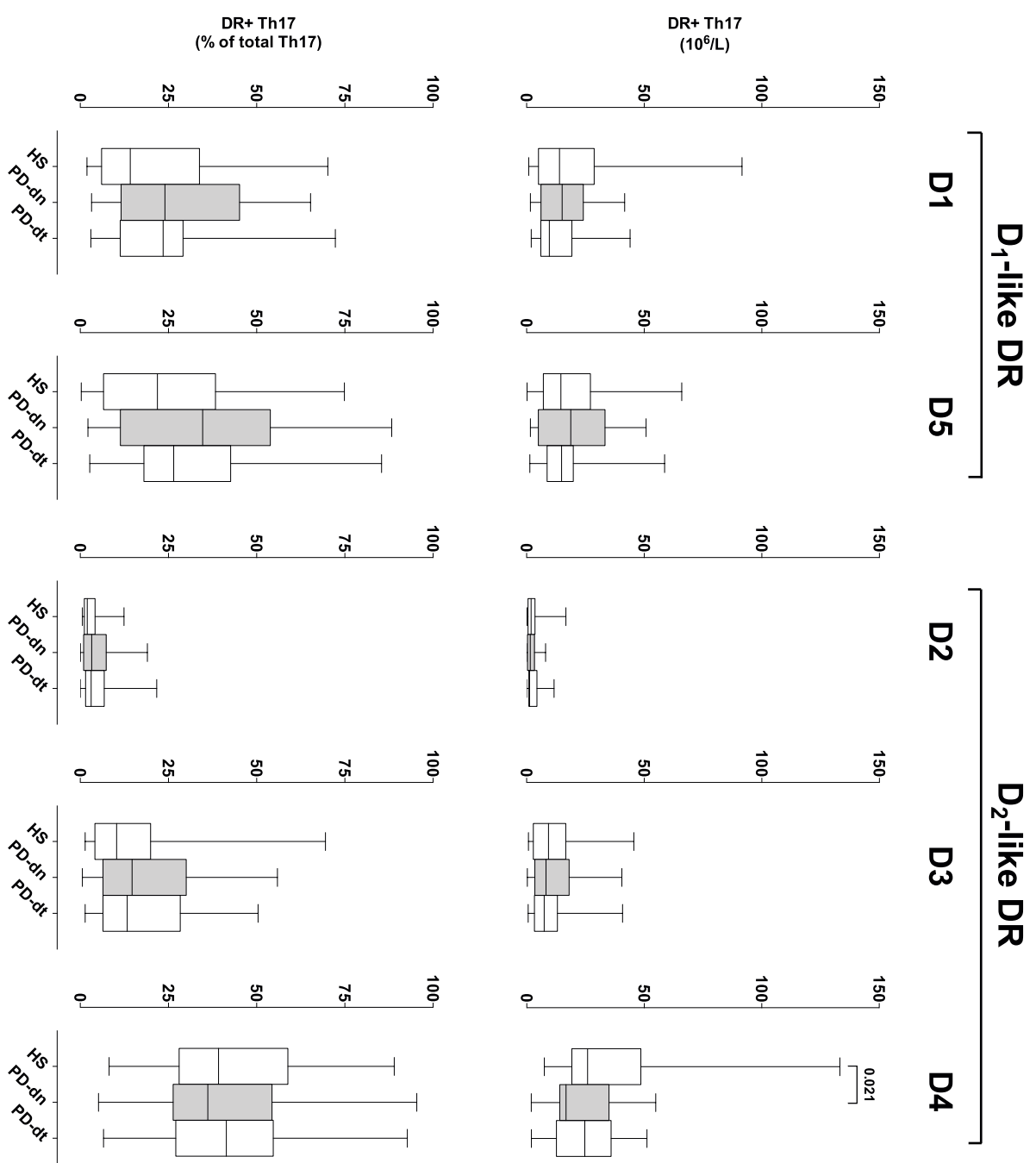


Figure S5

881

882 **Additional Figure S5. DR expression on Th17 cells in HS and PD patients.** Cells are shown as
883 absolute numbers (upper panels) and as percentage of total Th17 cells (lower panels). Data are
884 medians with 25°-75° percentiles (boxes) and min-max values (whiskers). Differences were
885 analyzed by means of two-tailed Student's *t* test or by Mann-Whitney test, as appropriate.
886

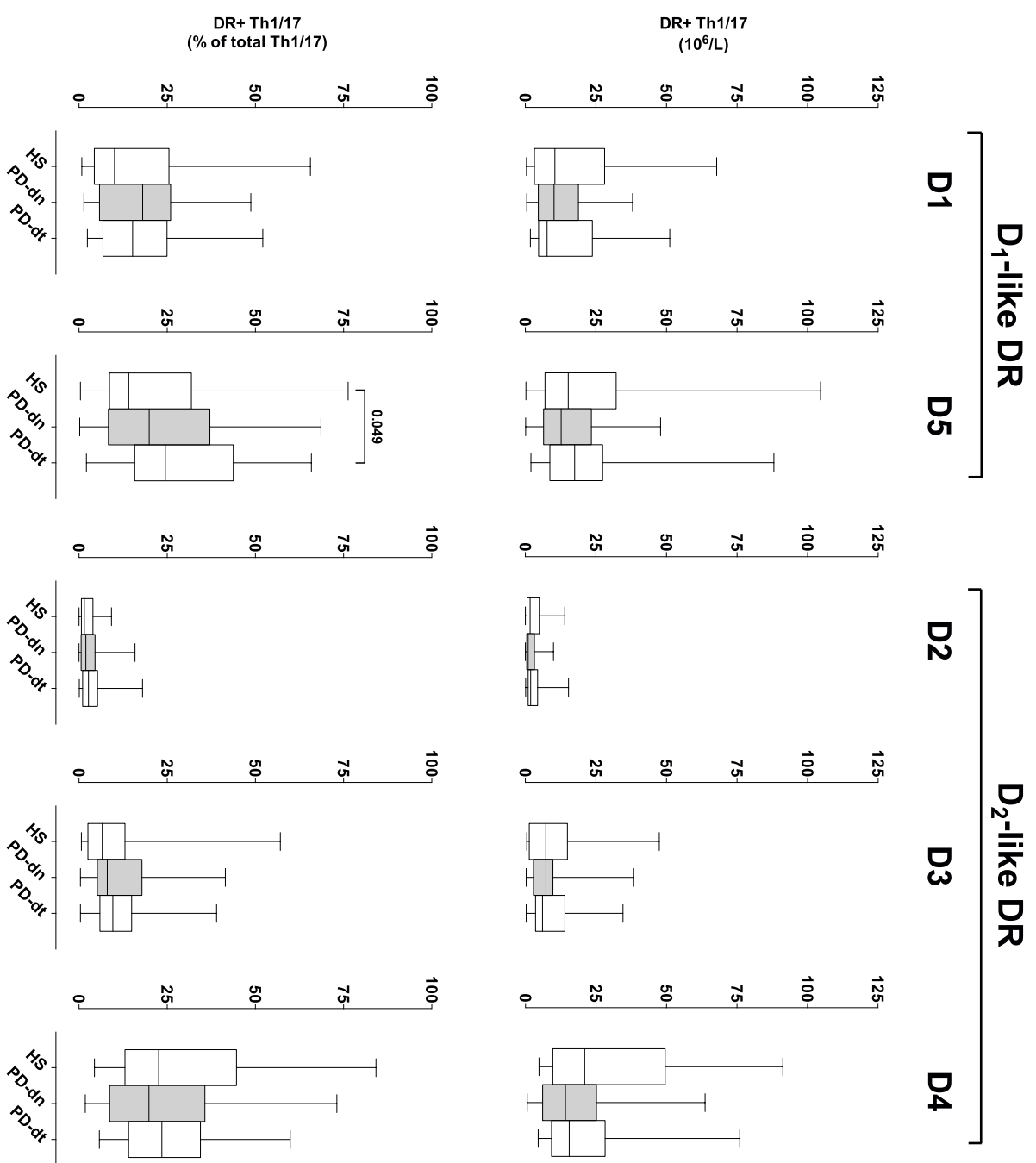


Figure S6

887

888 **Additional Figure S6. DR expression on Th1/17 cells in HS and PD patients.** Cells are shown as
889 absolute numbers (upper panels) and as percentage of total Th1/17 cells (lower panels). Data are
890 medians with 25°-75° percentiles (boxes) and min-max values (whiskers). Differences were
891 analyzed by means of two-tailed Student's *t* test or by Mann-Whitney test, as appropriate.
892

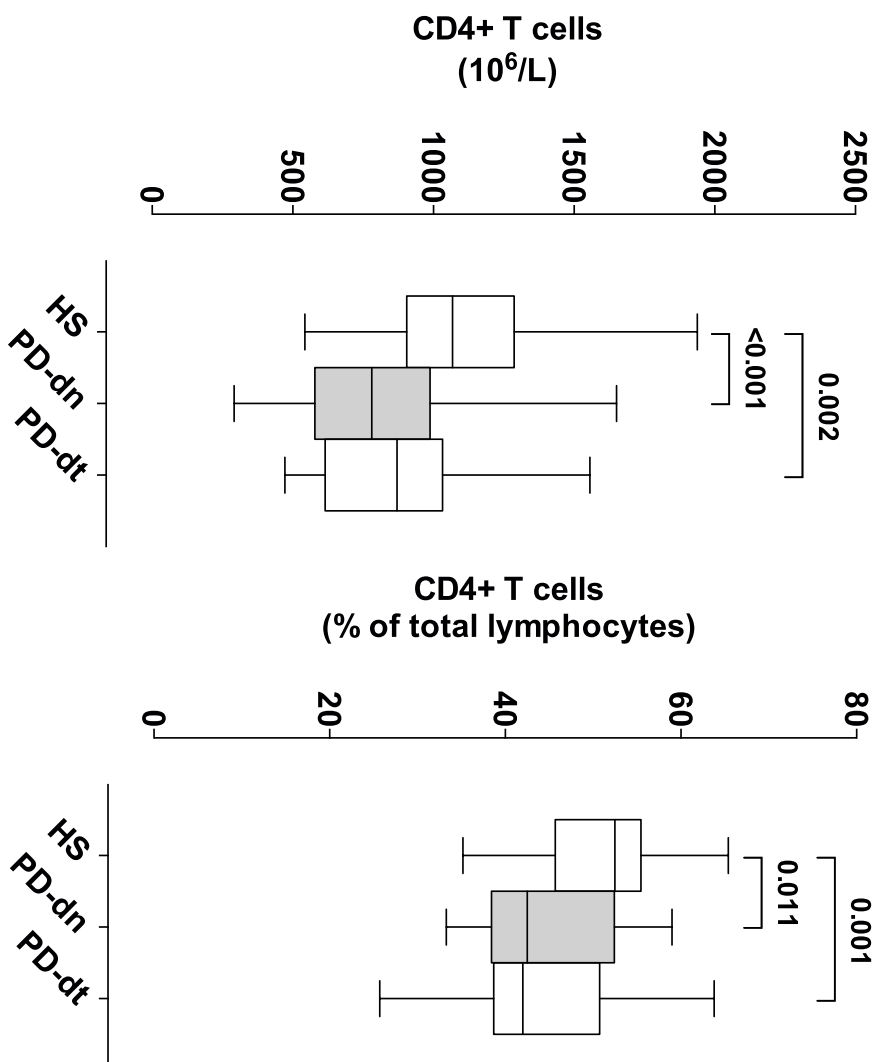


Figure S7

893

894 **Additional Figure S7. CD4+ T cells in HS and PD patients enrolled in study #2.** Cells are
895 shown as absolute numbers (left panel) and as percentage of total lymphocytes (right panel). Data
896 are medians with 25°-75° percentiles (boxes) and min-max values (whiskers). Differences were
897 analyzed by means of two-tailed Student's *t* test or by Mann-Whitney test, as appropriate.
898

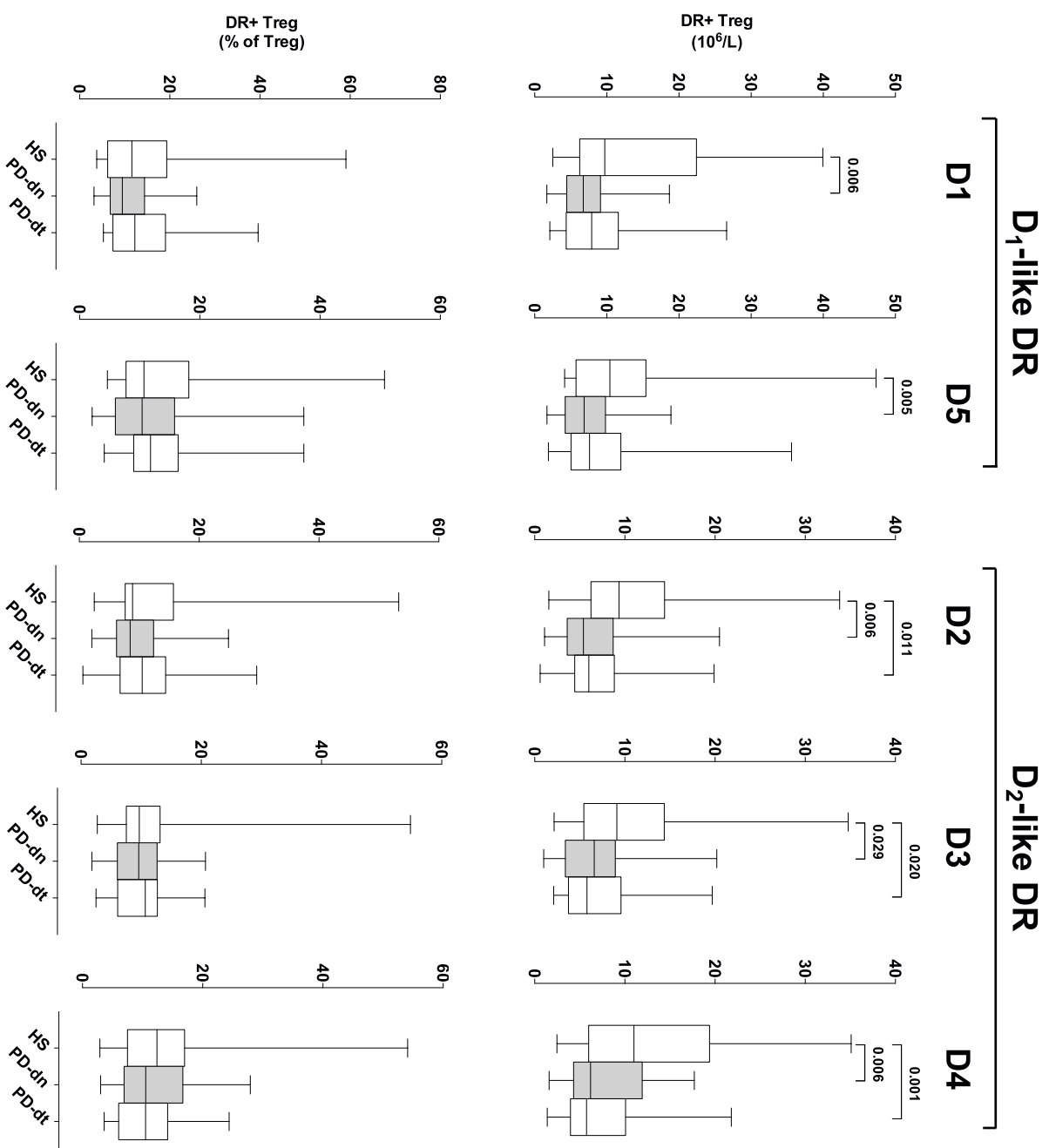


Figure S8

899

900 **Additional Figure S8. DR expression on Treg cells in HS and PD patients.** Cells are shown as
901 absolute numbers (upper panels) and as percentage of total Treg cells (lower panels). Data are
902 medians with 25°-75° percentiles (boxes) and min-max values (whiskers).

903

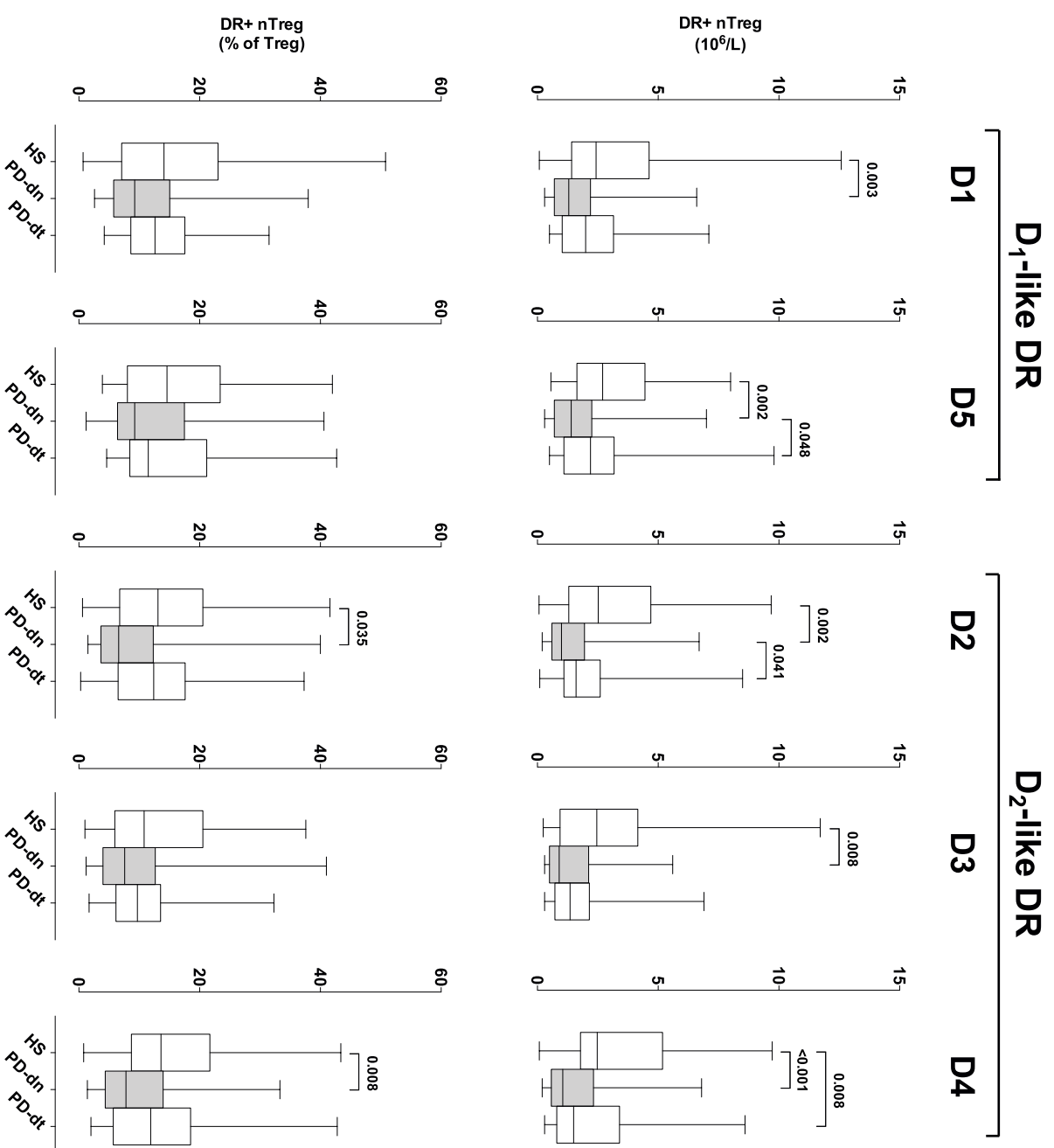


Figure S9

904

905 **Additional Figure S9. DR expression on nTreg cells in HS and PD patients.** Cells are shown as
906 absolute numbers (upper panels) and as percentage of total nTreg cells (lower panels). Data are
907 medians with 25°-75° percentiles (boxes) and min-max values (whiskers).

908

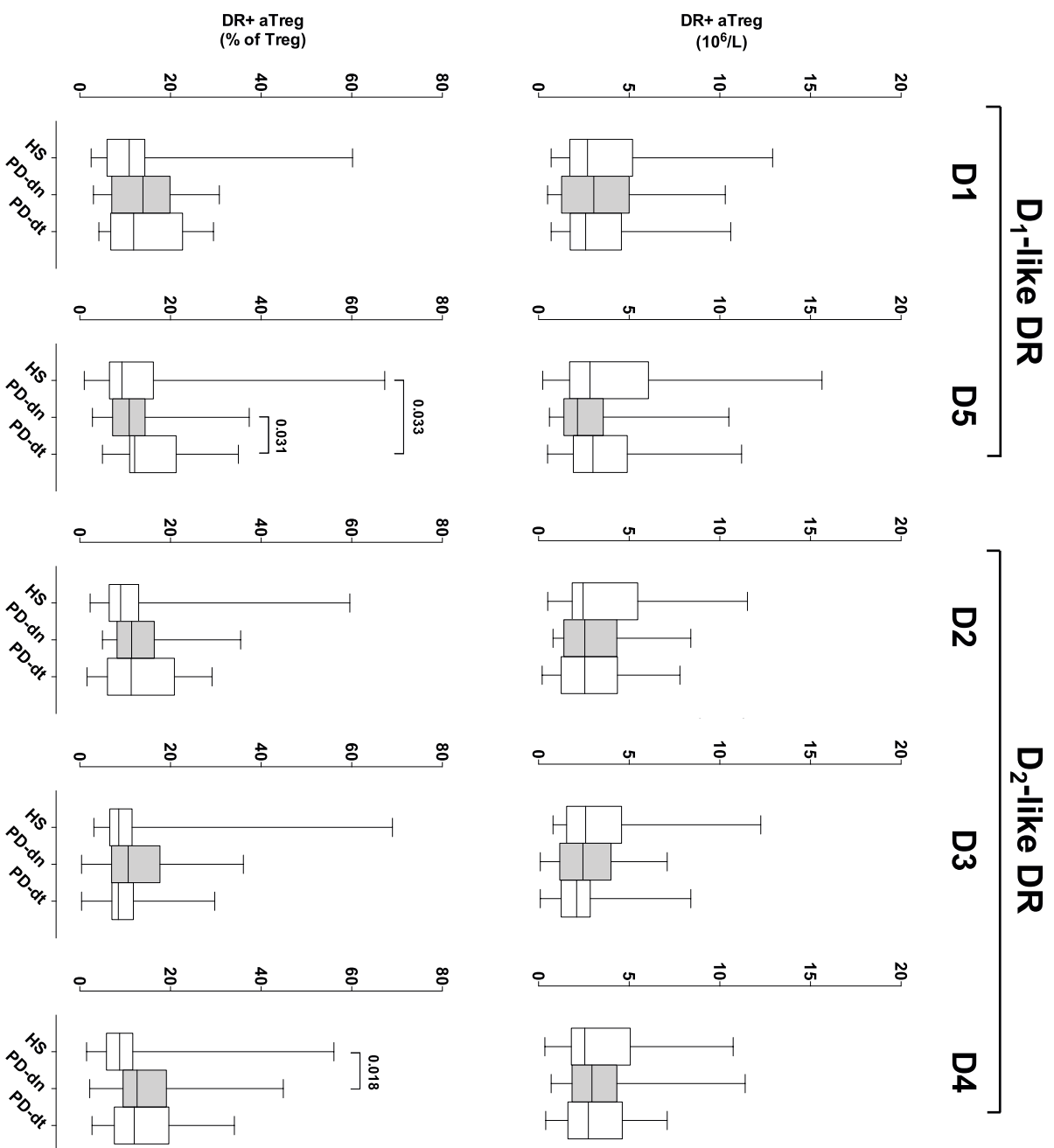


Figure S10

909

910 **Additional Figure S10. DR expression on aTreg cells in HS and PD patients.** Cells are shown as
911 absolute numbers (upper panels) and as percentage of total aTreg cells (lower panels). Data are
912 medians with 25°-75° percentiles (boxes) and min-max values (whiskers).

913

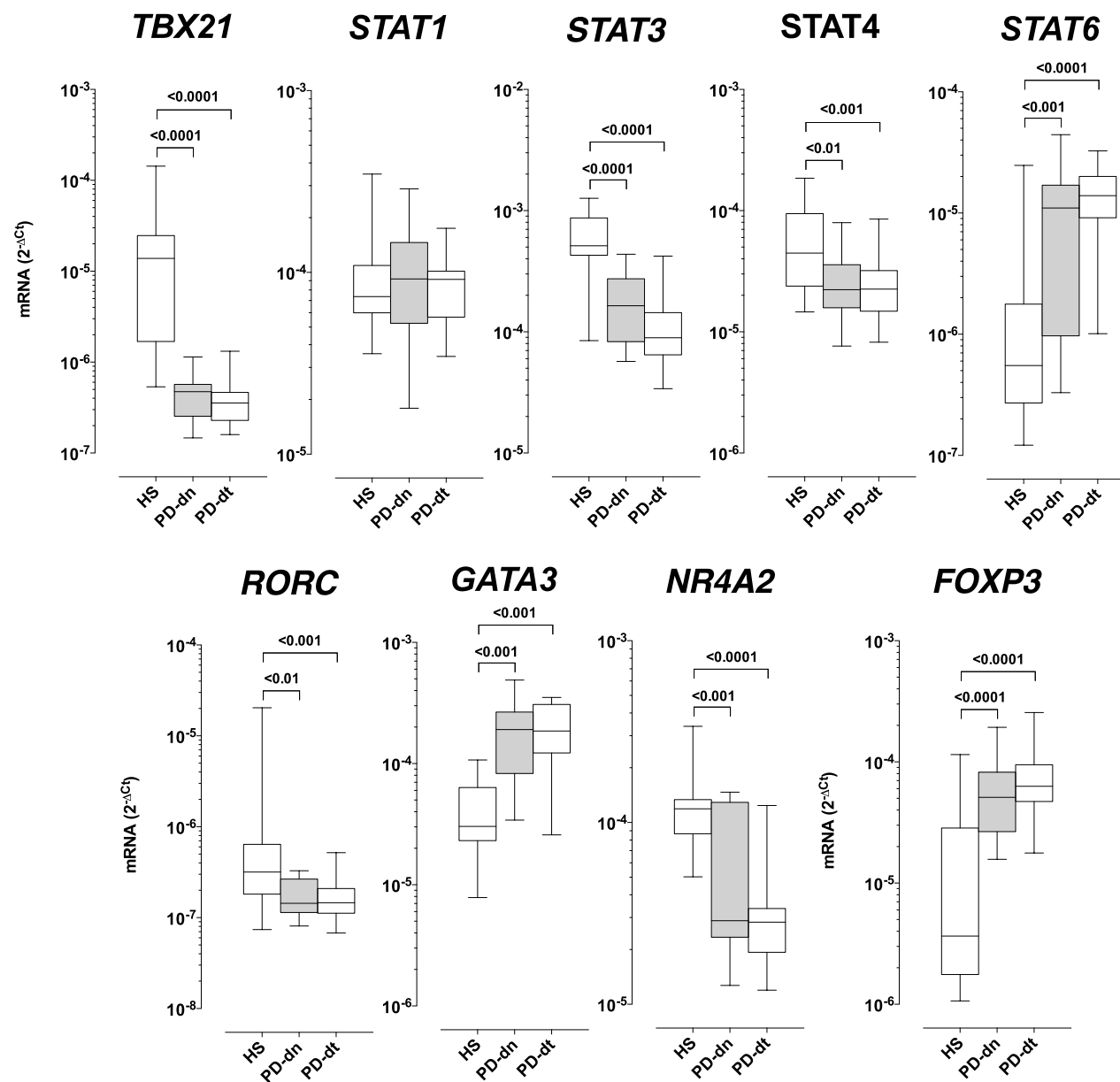


Figure S11

914

915 **Additional Figure S11. Transcription factors mRNA levels in CD4+ T cells of HS and PD**
916 **patients enrolled in study #2.** Data are medians with 25°-75° percentiles (boxes) and min-max
917 values (whiskers).

918

919

920 **Table 1. Characteristics of HS and PD patients enrolled in study #1.** Data are means±SD unless
921 otherwise indicated.

922

	HS	PD-dn	PD-dt	P		
				HS vs PD-dn	HS vs PD-dt	PD-dn vs PD-dt
n	47	26	56			
Gender (M/F)	25/22	15/11	34/22	0.808	0.549	0.813
Age (years)	66.8±10.3	68.0±9.3	70.4±8.5	0.632	0.057	0.254
UPDRS Part III						
(score)		13.6±7.2	15.3±6.6			0.101
1-10 (n)		14	13			0.010
11-20 (n)		7	34			
>20 (n)		5	9			
H&Y scale (stage)		1.3±0.5	1.9±0.7			0.001
1.0 (n)		16	14			0.004
1.5-2.0 (n)		9	31			
>2.5 (n)		1	11			
LED (mg/day)			533.2±360.1			
Drugs						
L-DOPA (n)			45 ^a			
DA agonists (n)			36 ^b			
pramipexole (n)			23			
ropinirole (n)			5			
rotigotine (n)			8			
Rasagiline (n)			21			

923 **Notes:** a = 11 taking L-DOPA alone, and 34 taking L-DOPA + DA agents; b = 9 taking DA agonists
924 alone (4) or with rasagiline (5), and 34 taking DA agonists + L-DOPA, without (19) or with
925 rasagiline (15).

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928 **Table 2. Characteristics of HS and PD patients enrolled in study 2.** Data are means±SD unless
929 otherwise indicated.

930

	HS	PD-dn	PD-dt	P		
				HS vs PD-dn	HS vs PD-dt	PD-dn vs PD-dt
n	33	30	36			
Gender (M/F)	19/14	16/14	20/16	0.803	0.799	1.000
Age (years)	65.1±11.4	68.2±9.1	70.0±8.1	0.241	0.133	0.698
UPDRS Part III						
(score)		14.2±7.0	17.3±8.4			0.076
1-10 (n)		14	7			0.051
11-20 (n)		9	19			
>20 (n)		7	10			
H&Y scale (stage)		1.4±0.5	1.9±0.8			0.002
1.0 (n)		17	10			0.014
1.5-2.0 (n)		12	17			
>2.5 (n)		1	9			
LED (mg/day)			522.3±356.5			
Drugs						
L-DOPA (n)			31 ^a			
DA agonists (n)			17 ^b			
pramipexole (n)			8			
ropinirole (n)			6			
rotigotine (n)			3			
Rasagiline (n)			19			

931 **Notes:** a = 15 taking L-DOPA alone, and 16 taking L-DOPA + DA agents; b = 4 taking DA agonists
932 alone (2) or with rasagiline (2), and 13 taking DA agonists + L-DOPA, without (10) or with
933 rasagiline (3).

934

5. CONCLUSIONS

Parkinson's disease is multifactorial neurodegenerative disorder resulting in part, as a consequence of progressive nigrostriatal degeneration. To date, there are only symptomatic treatments available with DA replacement therapy or DA agonists. As already mentioned, in past few years activation of peripheral immune system and neuroinflammation has taken places as very important, if not key players in pathogenesis of PD.

Nevertheless, in order to successfully apply the approaches of immunotherapy we first need to address several important questions regarding exact involvement of immune system in pathogenesis of PD, or at least since PD therapy still lies on dopaminergic substitution therapy, detailed understanding of the role of dopaminergic pathways in the immune system might allow a more appropriate use of available drugs.

In both of our studies we have recorded profound alterations in the pool of peripheral CD4⁺ T cells in the peripheral blood of PD patients when compared to healthy controls. Observed changes include decreased overall count of CD4⁺ cells, decreased absolute count of CD4⁺ T naive cells, increased percentage of T effector memory (T_{EM}) cells, and unchanged T central memory (T_{CM}) cells; reduction of Th2, Th17, Th1/17 and Treg, which results in increased Th1/Th2 and Th1/Th17 ratio. Furthermore, we have seen that the CD4⁺ cells isolated from peripheral blood of PD patients have different functional characteristics. Preliminary experiments aimed at assessing the effects of different forms of α -syn (monomeric and fibrillar) on naive and memory CD4⁺ T cells, showed reduced frequency of T naive cells while increasing T_{CM} and T_{EM}. Interestingly this response was very similar to the one induced by TTd. These results of course do not imply immediately that the α -syn is acting as a recall antigen and further research is needed. In addition, naïve CD4⁺ T cells isolated from blood of patients preferentially differentiate towards the Th1 lineage. Furthermore, production of pro-inflammatory cytokines IFN- γ and of TNF- α by CD4⁺ T cells from patients is increased and maintained even in the presence of homologous Treg.

At the end we have also examined expression of DR on specific subsets of CD4⁺ T cells and we have performed in-depth correlation analysis regarding potential influence of dopaminergic substitution therapy and observed changes. Dopaminergic substitution treatments have only minor, if any, impact on the peripheral immune

system of PD patients. Specific differences related to dopaminergic pathways in immune cells definitely support the notion of a chronic peripheral immune activation in PD patients, which may affect disease severity.

The present results reported functional responses specific to the different CD4+ T cell subsets that can represent appropriate biomarkers for both the *ex vivo* and/or *in vitro* assessment of candidate peripheral immune system-targeted treatments for PD, as well as for the assessment of their effects in clinical trials. Any drug that exhibit the ability to correct dysfunctional responses such as Th1-biased differentiation of naïve CD4+ T cells and/or reduce increased production of Th1 cytokines might be taken in consideration for therapeutic trials in PD patients as attachments to conventional PD treatment.

In past few years activation and changes of peripheral immune system have been given right place in pathogenesis of PD. Nonetheless, remains a question if all observed alterations in periphery are the main cause or simply contribute to the progressive loss of dopaminergic neurons or they are the consequence of present pathology? Unfortunately, the ultimate goal which is the efficient therapy of PD, still remains distant. One thing is sure, an effective treatment for PD should be multifunctional and include correction of any present damage on neurons and by engaging the immune system to repair the damage by natural means. A successful outcome will require a combination of various immunomodulatory strategies proposed so far.

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