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IMMU-KYN-PARK: EXPLORING INDOLEAMINE 2, 3-DIOXYGENASE 1 IN PERIPHERAL IMMUNE SYSTEM IN PARKINSON'S DISEASE

Tese no âmbito do Doutoramento em Biologia Experimental e Biomedicina, especialização em Neurociências e Doença, orientada pelo Professor Frederico Guilherme de Sousa da Costa Pereira, pela Professora Maria Cristina Januário dos Santos e pelo Professor Marco Cosentino e apresentada ao Instituto de
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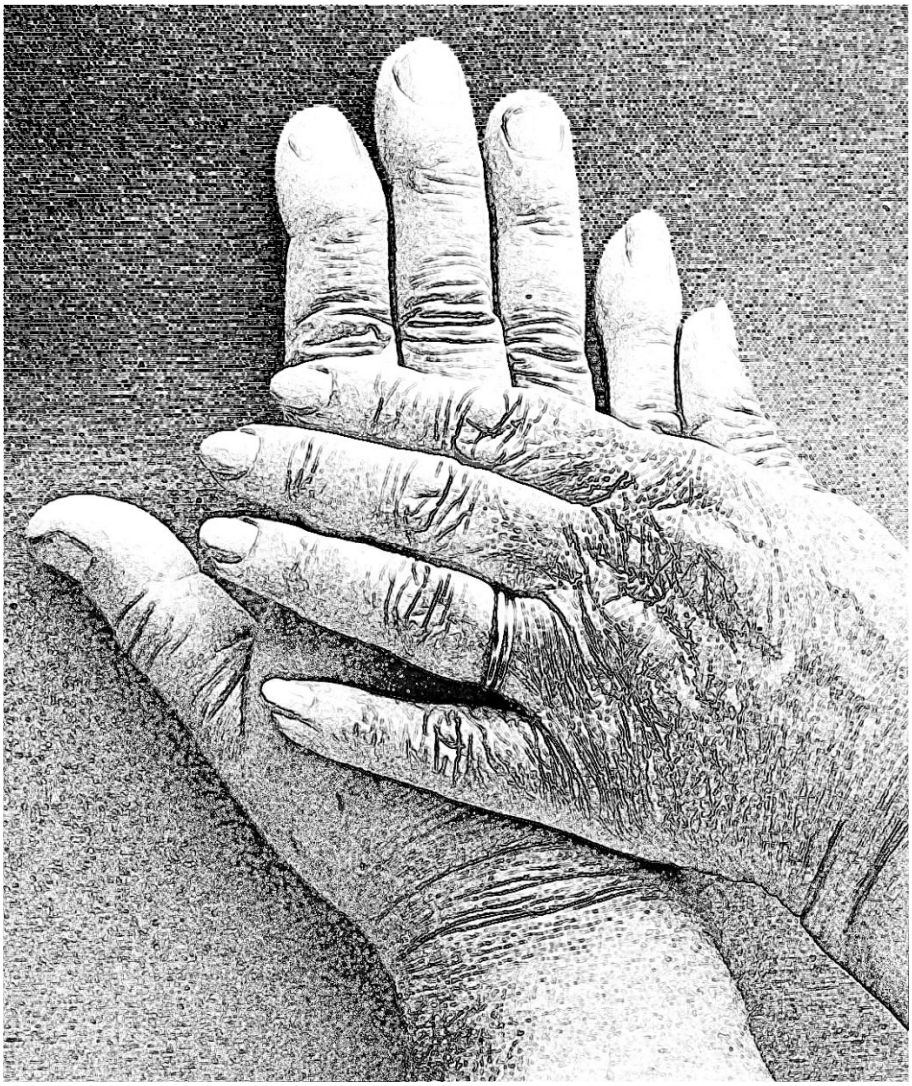

To my father, a Parkinson's Disease patient.

To my mother, a caregiver of a Parkinson's Disease patient.

To all the ones that live every single day with Parkinson's Disease.

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CHAPTER 1

Introduction and thesis outline

Introduction and thesis outline

Parkinson's Disease (PD) is the second most common neurodegenerative disease and the neurological disorder with the fastest growing globally. From 1990 to 2015, the number of people with PD more than doubled from 2.6 million to 6.3 million[1]. By 2040 the number will double again to at least 12.9 million[1]. Moreover, the poor diagnosis in developing countries led to underestimated numbers of PD prevalence[2]. The caregivers also represent a quite important fraction of people affected indirectly by the disease. Moreover, some of them get mental issues[3]. Unfortunately, we witness years after years of failed clinical trials of new drugs aiming to stop or modify the course of the disease[4]. The current dopaminergic therapies in PD include L-DOPA, which has been associated with late complications such as L-DOPA-induced dyskinesia (LIDs). This also affects patients' quality of life[5]. PD is a multifactorial disease with unknown etiology where environment and lifestyle play a strong role[6]. Multitarget strategies are now being described as a good approach to improving PD patients' quality of life. Medical doctors and scientists start to be aligned with a PACT (P=prevent, A=advocate; C=Care and T = Treat)[7]. This PACT emphasizes the need to take preventive measures (including cleaning the environment of hazardous chemicals and pesticides) to implement healthy lifestyle strategies including nutrition and physical exercise and find disease-modifying pharmacological therapeutics that would impact the quality of life of patients and consequently caregivers[7]. The immune system has acquired an important role in PD where immune changes have been pointed as initiators or exacerbators of neuroinflammation, thus being responsible for perpetuating neuroinflammation and neurodegeneration[8]. Moreover, it is hypothesized that gene-environment interaction intersected with immune aging contributes to the development and progression of PD[8]. Importantly, both peripheral and central immune cells contribute to the neuroinflammatory process in PD. However, less is known about the role of peripheral immune cells on disease pathology[8]. Considering that dendritic cells (DCs) and monocytes orchestrate the innate immune response, we propose to disclose the inflammatory profile of these circulating myeloid cells. Importantly, these cells induce the enzyme indoleamine 2, 3-dioxygenase 1 (IDO1), which may represent an immune regulator mechanism that has been proposed as a target in autoimmune and neurodegenerative diseases[9–12]. Another relevant issue covered by this thesis is the immunomodulatory role of catecholamines [13]. In fact, it is relevant to acknowledge that dopamine (DA), which is central in PD neuropathology, plays also a role in the neuro-immune cross-talk [13–15]. Moreover, DCs and monocytes, uptake (except DCs), synthesize, store, and/or release DA, besides expressing dopaminergic receptors[16].

Taking all this information into consideration the thesis will focus (1) on the role of IDO1 as an immune regulator mechanism; (2) on circulating myeloid cells (DCs and monocytes), which can simultaneously induce IDO1 and sense DA, and (3) on the effect of DA on immune regulation, including modulation of IDO1-induction by DCs and monocytes and cytokine production. [13]. We reviewed the state of the art of DCs and monocytes and the physiopathology of PD with particular emphasis on the induction of IDO1 by these cells (**Chapter 2**: Indoleamine 2, 3-dioxygenase – can DCs and monocytes expressing this moonlight enzyme change the phase of Parkinson's Disease?). In **Chapter 3** we developed an *in vitro* model that allowed us to explore IDO1 induction in peripheral blood mononuclear cells (PBMCs) under inflammatory stimuli. In particular, PBMCs proliferation was used as an inflammatory marker; IDO1 gene expression and kynurenine pathway (KP) metabolites (kynurenine and tryptophan; which were measured on cell culture supernatants) were used as markers of IDO1 induction/function (**Chapter 3:** A peripheral blood mononuclear cell-based *in vitro* model: a tool to explore indoleamine 2, 3-dioxygenase-1). We then performed an immune characterization of circulating DCs and monocytes from PD patients by immunophenotyping these cells and by measuring the level of inflammatory cytokines released in PD serum **(Chapter 4:** Circulating myeloid cells in Parkinson's Disease patients display a pro-inflammatory phenotype**)**. Finally, we studied the impact of DA on IDO1 induction and cytokines release under a lipopolysaccharide (LPS) stimulation condition (**Chapter 5:** The effect of salbutamol on IDO1 induction is impaired in monocytes from PD patients). **Chapter 6** is a general discussion that aims to integrate all generated data herein.

The ultimate goal of this thesis is two-fold: 1-to understand the extension of immune changes in PD and 2-to modulate $IDO1^{+}$DCs and $IDO1^{+}$monocytes by targeting their catecholaminergic system. Finally, $IDO1^{+}$DCs and $IDO1^{+}$monocytes may have diagnostics and therapeutic value in PD. And the knowledge of DA immune modulatory properties is instrumental to optimizing current therapeutic strategies.

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CHAPTER 2

Indoleamine 2, 3-dioxygenase – can DCs and monocytes expressing this moonlight enzyme change the phase of Parkinson's Disease?

Indoleamine 2, 3 - dioxygenase (IDO1) – can DCs and monocytes expressing this moonlight enzyme change the phase of Parkinson's Disease?

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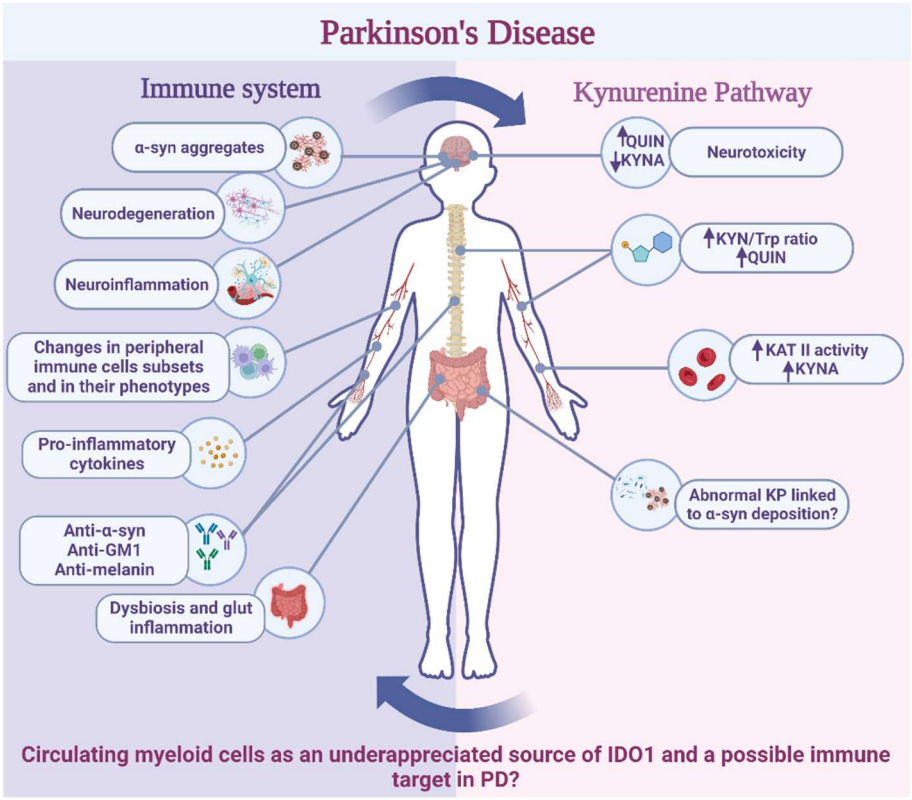

Abstract: Parkinson's Disease (PD) is the second most common neurodegenerative disease where central and peripheral immune dysfunctions have been pointed out as a critical component of susceptibility and progression of this disease. Dendritic cells (DCs) and monocytes are key players in promoting immune response regulation and can induce the enzyme indoleamine 2,3-dioxygenase 1 (IDO1) under pro-inflammatory environments. This enzyme with catalytic and signaling activity supports the axis IDO1-KYN-aryl hydrocarbon receptor (AhR), promoting disease-specific immunomodulatory effects. IDO1 is a rate-limiting enzyme of the kynurenine pathway (KP) that begins tryptophan (Trp) catabolism across this pathway. The immune functions of the pathway, which are extensively described in cancer, have been forgotten so far in neurodegeneration. However, dysfunctions of KP have been described in PD and associated with neurotoxic functions. With this review, we aim to focus on the immune properties of $IDO1^{+}$DCs and $IDO1^{+}$monocytes as a possible strategy to balance the pro-inflammatory profile described in PD. We also highlight the importance of exploring the role of dopaminergic therapeutics in IDO1 modulation to possibly optimize current PD therapeutic strategies.

Keywords: Parkinson's Disease (PD); indoleamine 2,3-dioxygenase (IDO1); dendritic cells (DCs); monocytes; Kynurenine pathway (KP); dopamine (DA).

1. Introduction

Parkinson's Disease (PD) is a progressive neurodegenerative disease clinically characterized by motor symptoms such as resting tremor, bradykinesia, rigidity, postural instability, and non-motor symptoms such as sensory abnormalities, sleep disturbance, depression, cognitive impairment, psychosis, gastrointestinal alterations, and sexual dysfunctions[1–3]. PD is now the fastest-growing neurological disorder worldwide with important socio-economic impacts[4,5]. Several factors appear to play a significant role in the course of the disease, including genetic inheritance, aging, and environmental factors[6]. Heritable forms of PD represent 5-10% of all cases, however, genetic alterations can be present in sporadic forms of the disease[7]. Increasing degeneration of the nigrostriatal dopaminergic pathway with consequent dopamine (DA) depletion in the striatum and the presence of α-synuclein (α-syn)-containing Lewy bodies are two essential neuropathological hallmarks of PD[8,9]. Several pathways appear to be chronically deregulated which contributes to neurodegeneration[10]. Alterations in α-syn proteostasis, mitochondrial dysfunction, oxidative stress, and neuroinflammation are some of the mechanisms that are present during the disease and contribute to its propagation[9,11,12]. Both peripheral and central innate and adaptive immunity are implicated in neuroinflammation, which seems to underlie neuronal dopaminergic loss in PD[16-18]. There is evidence of infiltration of peripheral immune cells into the brain parenchyma and activated glial cells in PD[13–15]. However, information about the peripheral-central neuroimmune crosstalk in PD is still lacking[16]. Importantly, evidence suggests that the immune system is the link between environmental factors and genetic susceptibilities in PD, inducing an autoimmune response [17–20]. Dendritic cells (DCs) and monocytes, which are key players in promoting immune response regulation, can induce the enzyme indoleamine 2,3-dioxygenase 1 (IDO1) [21–26]. IDO1, the rate-limiting enzyme of tryptophan (Trp) degradation into kynurenine (KYN) across the kynurenine pathway (KP, Figure 1), is increased in inflammatory environments and is considered an immune regulator with catalytic and signaling activity[27,28]. The catalytic activity supports the axis IDO1-KYN- AhR, promoting immunomodulatory effects: the activation of AhR by KYN sustains IDO1 expression and activity [29]. PD patients show dysfunctions in DCs, monocytes, and KP[30,31]. KP dysregulations in PD have been essentially associated with their neuromodulator properties and contribution to the neurotoxic environment in the central nervous system (CNS)[32]. Although metabolites and enzymes across the pathway are known by their immunomodulatory properties, both at the periphery and centrally, they remain poorly explored in the PD context[33]. This review aims to provide an overview of the role of IDO1 as an immune regulator mechanism in DCs and monocytes and explores this mechanism in the PD context. This knowledge can change the perspective on KP

dysregulations in PD and other neurodegenerative diseases, opening new possibilities for therapeutic strategies and biomarkers.

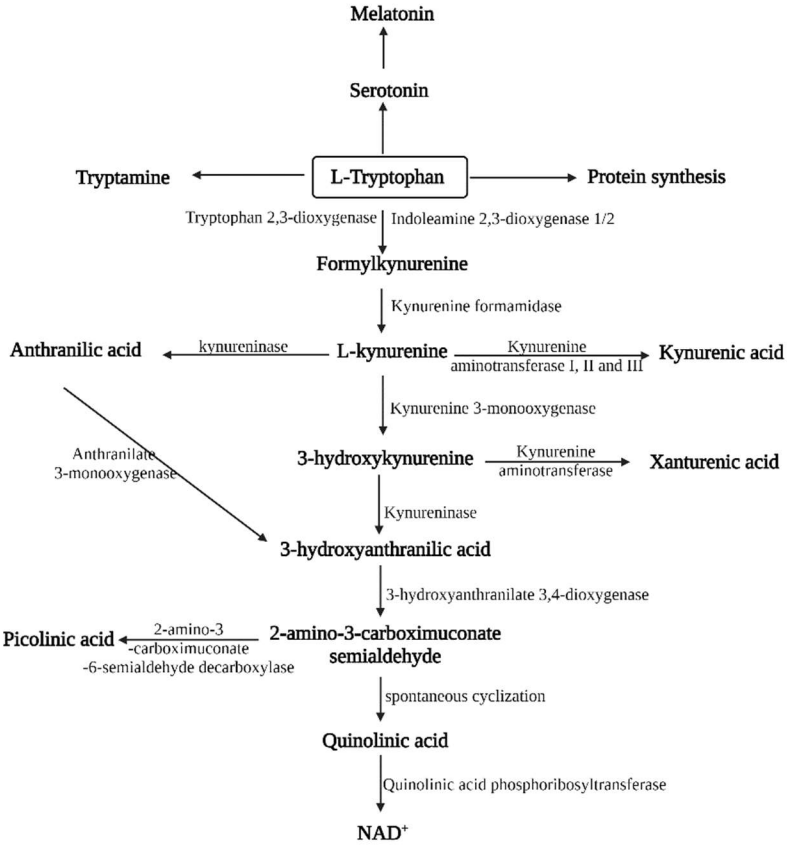

Figure 1. Degradation of tryptophan (Trp) across kynurenine pathway (KP). Trp also fuels the serotonin pathway and protein catabolism. The metabolites and the enzymes across KP are described.

Immune system and KP in PD

1.1. Immune dysfunctions and autoimmunity in PD

Several single-nucleotide polymorphisms (SNPs) were found in PD genes related to immune system functions [34,35]. These genetic changes associated with environmental risk factors can create conditions to initiate or accentuate the disease[36]. There is no consensus on whether inflammation is a cause or consequence of neurodegeneration[37], but it is accepted that neuroinflammation and dopaminergic neurodegeneration fuel each other entertaining a vicious cycle[38]. Interestingly, by using an α-syn overexpression mouse model it was shown that α-syn alone can activate microglia and start an adaptive immune response suggesting that neuroinflammation can start before neurodegeneration[39]. Furthermore, dopaminergic neurons in *substantia nigra* (SN) are particularly susceptible to cytokines and reactive oxygen species (ROS) released during the propagation of this cycle [40,41]. Moreover, SN

is one of the highest microglia density brain regions with high levels of tumor necrosis factor (TNF)-α receptor 1, which makes this region very susceptible to immune damage[42]. Some authors believe that microglia activation might precede neuronal death in PD[43,44] and strongly contributes to nigrostriatal pathway damage[45,46]. Moreover, activation of microglia led to the release of chemokines and cytokines that signal neurons and recruit peripheral immune cells to infiltrate the brain[46]. PD animal studies suggested that infiltrated $CD163^+$ and $CCR2^+$ monocytes and infiltrated $TCD4^+$ cells might mediate dopaminergic neurodegeneration [47–50]. In particular, $TCD4^+$ and $TCD8^+$ cells were found infiltrated in *post-mortem* brains of PD patients and PD animal models (eg. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-mouse model)[53-56]. Moreover, the lack of $TCD4^+$, but not $TCD8^+$, cells have been shown to strongly attenuate the dopaminergic neuronal death in MPTP animal model[50–53]. These infiltrated $TCD4^+$ cells can acquire a Th1 phenotype with the production of interferon (IFN)-γ and TNF-α or a Th17 phenotype with a production of IL-17 or IL-22 contributing to a chronic inflammatory process by perpetuating microglia activation[54]. Contrarily, T regulatory cells (Treg) increased protection against MPTP-induced DA cell death. However, it is not clear if their neuroprotective activity is due to the suppression of T effector cells (Teff) or to the switch of microglia from a proinflammatory to an anti-inflammatory phenotype[55,56]. Human studies also demonstrated changes in T cell subpopulations on PD[57]. However, the literature is not consensual describing these changes and different studies point to different outcomes, probably because of the use of different phenotyping methods[57]. These alterations can give us some important clues about the importance of T cell modifications but also highlight the importance of considering the functional changes of T cell subtypes on PD[57]. PD patients have impaired T regs which may be unable to suppress Teff cell proliferation and pro-inflammatory cytokines release[25,58]. Treg dysregulations seem to contribute to maintaining a pro-inflammatory profile both at the periphery and CNS[57]. Interestingly, Schwab *et al*., 2021 described that transforming the brain's immune microenvironment by targeting T cells is a novel therapeutic approach with neuroprotective functions[59]. Furthermore, more regulatory populations, such as B regulatory cells (Bregs) are also compromised in PD[60]. Moreover, autoantibodies against α-syn, DA, and melanin are present in sera and CSF of PD patients whereas α-syn autoantibodies have been correlated with disease severity[61–63]. Furthermore, it seems that there are reactive T cells to α-syn peptides and anti-α-syn, anti-melanin, and anti-ganglioside (GM1) antibodies can infiltrate the brain years before the disease onset, suggesting that autoimmunity plays a role in PD pathogenesis in the first phases of the disease[64–67]. Autoimmunity in PD is also demonstrated by mutations in the genes: *SNCA, LRRK2, PRKN, PINK1,* and *GBA,* which also encoded proteins related to immune functions and carcinogenesis (which has a strong immune dysfunction component)[68–71]. For example, SNCA-related alterations led to α-syn dysfunctions that target autoreactive T cells, increase cytokines levels,

and develop antibodies against α-syn promoting systemic inflammation and the pathogenesis of PD[67]. T cells of PD patients recognize α-syn peptides, and B cells produce deposits of IgG to surround dopaminergic neurons and coat the Lewy bodies, which suggests dopaminergic neurons are being targeted by these immunoglobulins[72]. LRRK2, which is also expressed in peripheral immune cells, increases under inflammatory environments, regulates immune responses, and is increased in sporadic PD[71]. Despite LRRK2 being increased in B cells of PD patients, LRRK2 knockout mice show B cell alterations[73]. PRKN and PINK1 mutations are essentially related to autophagy and mitochondria damage. Importantly, mitochondrial antigen presentation may be a mechanism linking PD to adaptive immunity[70]. On the other hand, autophagy plays an important role in the context of antigen-presenting cells (APC) and major histocompatibility complex (MHC) class II, in lymphocyte ontogenesis and differentiation and inflammasome regulation[74,75]. This means that PD patients with a mutation in PRKN and PINK1 might develop an auto-immune mechanism with the presence of cytotoxic T cells against mitochondria antigens[71]. Interestingly, autoantibodies from PD patients, but not healthy subjects provoke loss of dopaminergic neurons in rat SN[64]. The accumulation of α-syn aggregates is also related to defects in protein degradation systems such as lysosomal and autophagy referred to above[74,75]. Interestingly, infection triggers such viruses can induce α-syn pathology[18]. This is one of the mechanisms used to explain the gene-environment contribution to the etiology and disease progression[17]. The human leukocyte antigen (HLA) variants linked to antigen presentation which increases susceptibility to PD might be another mechanism to explain this interaction[57]. Accordingly, it has been established an association between HLA alleles and late-onset PD as well as a positive correlation between levels of major histocompatibility complex (MHC)-II expression and disease severity[57]. Importantly, genome-wide association (GWAS) studies are contributing to an increase in the complexity of PD genetics by identifying several genes or risk loci[34]. Moreover, several single nucleotide polymorphisms (SNPs) have been associated with an increased risk of developing PD. This also includes genetic risk variants that are shared by PD and autoimmune diseases. Moreover, patients with some auto-immune diseases have an increased risk of developing PD[26]. These similarities between PD and other autoimmune diseases might help us to find a pattern that allows us to understand the role of the autoimmune process in PD[26]. Similarly, natural killer (NK) cells are increased in the blood of PD patients, and they were also found close to the α-syn aggregates in PD brains[76]. Depletion of NK cells in a fibril α-syn–induced PD mouse model increased α-syn pathology because NK cells act as scavengers of α-syn species[76]. Changes in the peripheral immune system are not restricted to the blood: higher levels of inflammatory mediators have been found in the gut of PD patients and they are inversely correlated with the PD age onset. Moreover, immune changes have been associated with gut

dysbiosis suggesting a linkage between dysbiosis and inflammation that may contribute to the initiation of PD pathology[71,77,78].

1.2. Inflammation and KP dysfunctions feed each other in PD

IDO1 is expressed in various immune cell types including fibroblasts, macrophages, microglia, monocytes, DCs, and astrocytes, and is the rate-limiting enzyme of the KP that uses Trp as a precursor [79 80,81]. IDO1 is a heme protein, and the active-site heme is key for IDO1 dioxygenase activity[38]. This enzyme needs an environment abundant in compounds with high redox power to regenerate constantly Fe^{2+}, which facilitates the binding of O_2 and Trp to form the active ternary complex[82]. IDO1 dioxygenase activity increases levels of KYN, which is further processed in the KP. KP pathway also includes kynurenine aminotransferase (KAT) I and II and 3-hydroxy anthranilic acid oxygenase, the enzymes responsible for producing kynurenic acid (KYNA) and quinolinic acid (QUIN), respectively[83]. QUIN has a neurotoxic activity by activating n-methyl D-aspartate (NMDA) receptors and KYNA acts as a neuroprotective agent by antagonizing both NMDA and nicotinic acetylcholine receptors (nAChRs). Under basal conditions, IDO1 expression is very low, however proinflammatory cytokines, such as IFN-γ, TNF-α, transforming growth factor beta (TGF)-β, and pathogen-associated molecular pattern (PAMP), including lipopolysaccharide (LPS), induce IDO1 expression in monocytes and DCs (Figure 2)[84,85]. Therefore, immune dysfunction might be linked with KP dysregulations in PD. Indeed, Heilman *et al*., 2020, found an association between acute-phase proteins and KYN/Trp ratio and quinolinic acid (QUIN), a neurotoxic KP metabolite[86] in serum from PD patients. These authors also showed that serum and CSF high levels of neurotoxic KP metabolites are correlated with more severe symptoms[86]. Moreover, high levels of the ratio KYN/Trp were found in the serum and CSF of PD patients and correlated with neopterin and disease severity, reflecting an immune activation[87]. Overall, both, peripheral and central inflammation might contribute to the imbalance between the neurotoxic QUIN and the neuroprotector KYNA in PD brains[88]. A shift toward neurotoxic QUIN synthesis and away from neuroprotective KYNA production in PD is suggested based on the following findings: lower KYNA/KYN ratio, higher QUIN level, and QUIN/KYN ratio observed in PD patients compared to healthy subjects (HS); PD patients at an advanced stage (Hoehn-Yahr stage >2) showed lower KYNA and KYNA/KYN ratio, as well as higher QUIN and QUIN/KYNA ratio compared to PD patients at the early stage (Hoehn-Yahrstage≤2) and HS [89]. An increase in QUIN or a decrease in KYNA production may result in excessive activation of NMDA receptors [90]. Dopaminergic neurons seem to be protected by KYNA which antagonizes QUIN-mediated excitotoxicity[91]. NMDA receptors have an important, but not entirely understood, role in L-DOPA-induced dyskinesia (LID),

and antagonists of this receptor are shown to reduce the severity of PD symptoms as well as reduce LID in patients with advanced PD[92] [93]. Altered levels of KYN metabolites can affect glutamatergic transmission and may play a role in the development of LID. Plasma and CSF of PD patients with LID showed increased 3-hydroxykynurenine (3-HK)/KYNA ratios and decreased anthranilic acid (AA) levels [94]. Interestingly, although LID mechanisms are not known, it is accepted that neuroinflammation and systemic inflammation exacerbate LID[95]. In both,

Moreover, KAT and 3-hydroxy anthranilic acid oxygenase, have been found in astrocytes surrounding

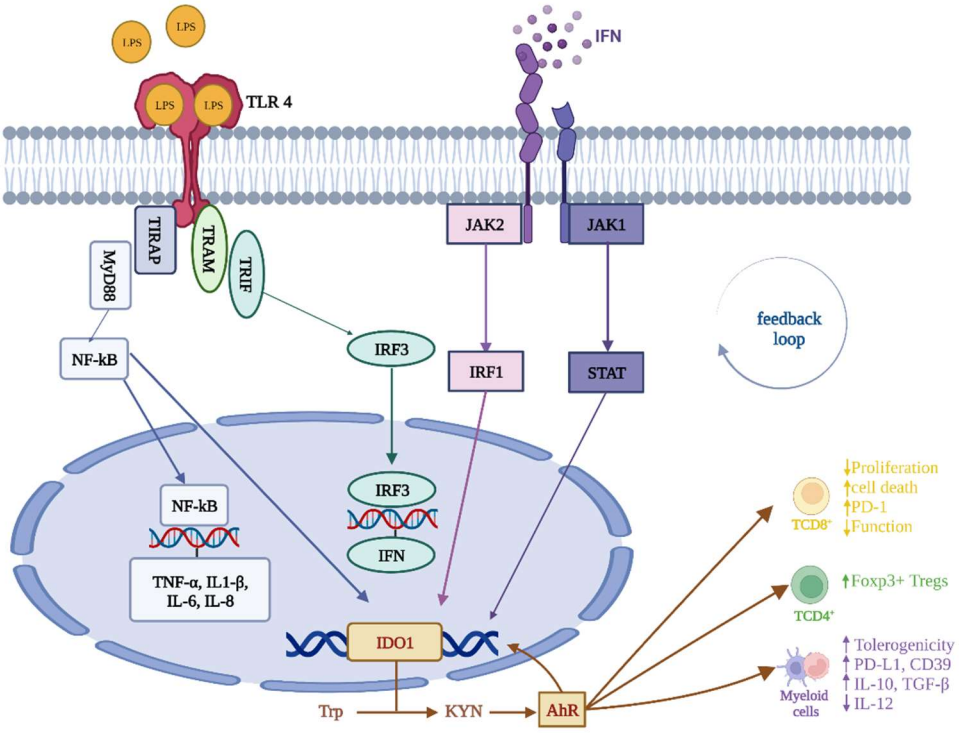

Figure 2. Schematic representation of indoleamine 2,3-dioxygenase 1 (IDO1) induction by lipopolysaccharide (LPS) and interferon (IFN)-γ in dendritic cells and monocytes. IDO1 can be induced under inflammatory conditions, including in the presence of LPS through a pathogen-associated molecular pattern (PAMP) triggering the NF-kB pathway or in the presence of INF-γ that triggers JAK/STAT pathway. Both culminate with increased IDO1 expression that converts tryptophan (Trp) into kynurenine (KYN) through the kynurenine pathway (KP). KP supports the axis IDO1-KYN-AhR which modulates immune response. Additionally, AhR enhances their activity through an IDO1-AhR-IDO1 positive feedback loop prolonging activation induced by PAMPS and cytokines, in dendritic cells and monocytes. Legend: [AhR] – Aryl hydrocarbon receptor; [IL] – interleukin; [IRF3] - interferon regulatory factor 3; [MyD88] - myeloid differentiation primary response gene 88; [NF-kB] - nuclear factor-kB; [PD-L1] - Programmed death-ligand 1; [TCD8+]- T effector cell; [TCD4+] - T helper cell; [TIRAP] - TIR domain-containing adaptor protein; [TRAM] - TRIF-related adaptor molecule; [TRIF] - TIR domain-containing adaptor inducing IFN-β.Created in Biorender.com.

glutamatergic afferents and DA neurons in the SNc of rats[83]. 3-HK levels have also been found to be

increased in the putamen, prefrontal cortex, and pars compacta of the SNc from PD patients[98]. The decreased levels of endogenous KYNA might be ineffective in blocking the NMDA receptor and preventing neurotoxicity induced by 3-HK[99]. *Post-mortem* samples of PD patients have shown an accumulation and deposition of QUIN[100]. Intrastriatal inoculation of mice with QUIN results in increased levels of phosphorylated α-syn and neurodegeneration suggesting the pathological role of QUIN accumulation in protein aggregation[100]. On the other hand, elevated levels of KYNA correlated with an increase in KAT II activity in red blood cells of PD patients which indicates dysregulation of the pathway at the periphery[101]. The KP pathway has also a bioenergetic role, given by the generation of the nicotinamide adenine nucleotide (NAD^+)[102]. Interestingly, neuronal bioenergetics failure has been linked to PD pathogenesis[103]. Collectively, this evidence suggests that KP metabolite imbalance might contribute to PD progression by disturbing several pathways: the immune response, cytotoxic effects, and bioenergetic impairments. These also suggest that the peripheral pro-inflammatory profile described in PD patients might contribute to sustaining this KP imbalance peripherally and centrally (Figure 3)[88]. Finally, more studies that explore the immunomodulatory profile of KP are mandatory in the PD context. For example, KYN is also an agonist of AhR. The AhR is a ligand-activated transcription factor that integrates environmental, dietary, microbial, and metabolic cues to control complex transcriptional programs in the immune system in a ligand-specific, cell-type-specific, and context-specific manner[104]. KYN specifically promotes the differentiation of Tregs and increases IDO1 in DCs (please see section 3). KYNA also has an anti-inflammatory action and plays a chemokine-like role by modulating leukocyte-endothelial interactions and leukocyte recruitment. [89]. $IDO1^+$DCs and $IDO1^+$monocytes can modulate T cell responses by impairing Teff cells and enhancing the activity of Tregs[105,82]

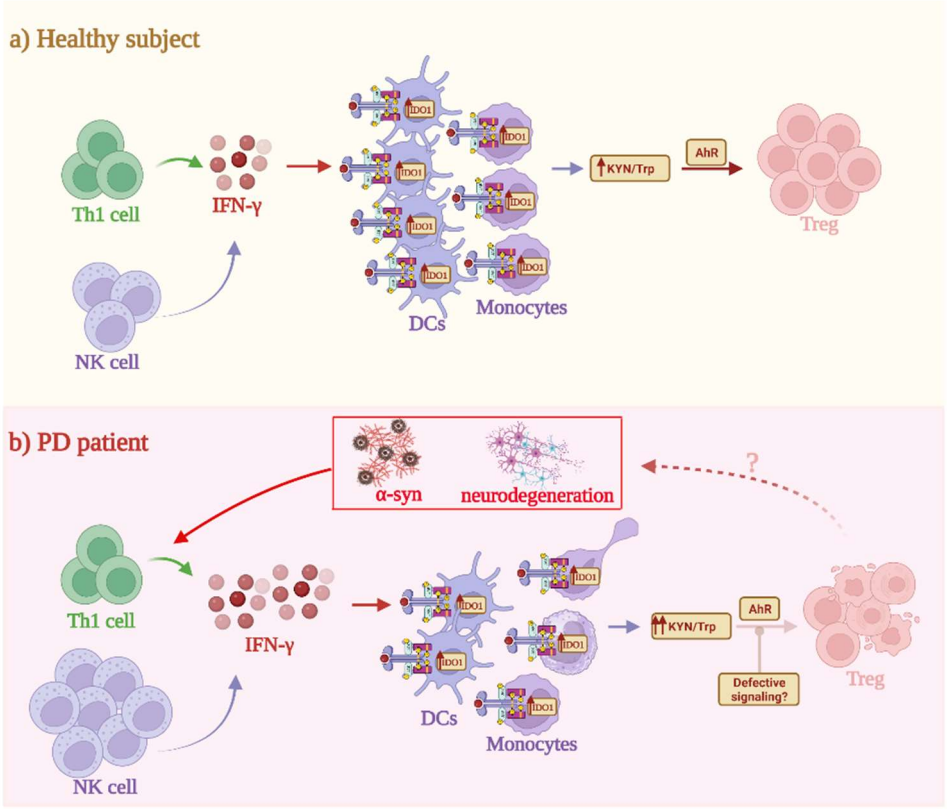

Figure 3 – Schematic representation of indoleamine 2,3-dioxygenase 1 (IDO1) dysregulation on PD. IDO1 can be induced under inflammatory conditions. Immune response in a healthy subject (a) Th1 cells and NK cells produce INF-γ that triggers the JAK/STAT pathway in circulating myeloid cells, including DCs and monocytes. The activation of these cells includes increased IDO1 expression that degrades tryptophan (Trp) into kynurenine (KYN) across the kynurenine pathway (KP). The activation of KP increases the KYN/Trp ratio which supports the axis IDO1-KYN-AhR promoting T regulatory (Treg) cells activation with the resolution of inflammation. Parkinson's Disease (PD) patients (b) show α-synuclein (α-syn) aggregates and neurodegeneration with dopaminergic neuron loss which contributes to a chronic low-grade inflammatory profile able to increase the proportion of IFN-γ^+Th1 and IFN-γ^+NK positive cells. Moreover, these patients also show increased levels of natural killer (NK) cells which also are potent IFN-γ producers. PD patients show lower levels of circulating DCs and changes in monocyte cell subsets. Even in the presence of a decreased number of DCs and monocyte profile changes PD patients show increased serum KYN/Trp ratios when compared with healthy subjects indicating KP dysregulations. Tregs of these patients are also compromised which might also indicate dysfunction in the KP-immune system communication. For example, AhR downregulation or AhR signaling disruption is associated with the differentiation of Foxp3$^+$Tregs. Created in Biorender.com.

2. IDO1: so much more than an enzyme!

The complexity of IDO1 led some authors to call it a 'moonlight' protein[106,107]. IDO1 is just not an enzyme: it is an immune regulator, and it has been implicated in several physiologic and pathological processes, including pregnancy, autoimmune diseases, chronic inflammation, and tumor immunity[81,102]. Moreover, IDO1 is not exclusively expressed in the immune system since it seems to be present in neurons and other cells that are present in the inflammation area, including tumoral cells[108]. Additionally, IDO1 can acquire distinct functions according to the environment and cell needs[109]. This might explain the different IDO1 phenotypes described in the literature: IDO1$^+$DCs that don't break down Trp; IDO1$^+$DCs able to degrade Trp without suppressing T cell responses; and

IDO1^{+}DCs with the ability to breakdown Trp and suppress T-cell responses[108]. IDO1 is also a catalyst of physiological peroxidase reactions. Importantly, hydrogen peroxide (H_2O_2) activates the peroxidase function of IDO1 to induce protein oxidation and inhibit dioxygenase activity[82]. The following are more particularities of IDO1: (i) IDO1 is endowed with signaling activity when phosphorylated in critical tyrosine residues; (ii) IDO1 exists also in an apo form (heme-unbound) with unknown functions and (iii) IDO1 shows distinct intra and extracellular topology[106]. Moreover, different functions of IDO1 have been associated with different conformations creating an enzyme with high conformational plasticity[106]. On the other hand, the non-enzymatic function of IDO1 is due to the presence of ITIMs (functional immunoreceptor tyrosine-based inhibitory motifs; Y111, Y249) in small non-catalytic domains responsible for the activation of the noncanonical NF-kB pathway that induces the expression of the *ido1* and *tgfb1* genes, which establishes a positive feedback loop that confers a long-term immunoregulatory phenotype on DCs[80,106]. Interestingly, accordingly, the phosphorylation of the two ITIMs motifs can reduce or maintain IDO1 expression[110]. Additionally, IDO1^{+}DCs and IDO1^{+}monocytes can modulate T cell responses by impairing Teff cells and enhancing the activity of Tregs[105,82]. IDO1 plasticity is particularly important in the disease context[102]. For example, cancer cells constitutively express IDO1, and IDO1 inhibition has been discussed as a potential therapeutic approach[82]. Oppositely, the rescuing of the IDO1 function might be a target in autoimmunity. Mondaneli *et al*., 2019, applied this strategy in multiple sclerosis (MS) by using N-acetylserotonin (NAS; an IDO1 positive allosteric modulator (PAM)), in an animal model of MS [experimental autoimmune encephalomyelitis (EAE)] which can activate the IDO1-KYN-AhR in DCs and in peripheral blood mononuclear cells (PBMCs) of patients with relapsing-remitting MS. NAS activated the IDO1-KYN-AhR in DCs (animal model) and increased KYN levels released by cells isolated from patients[111]. Importantly, IDO1 SNPs seem to influence the age onset of PD[112]. Finally, whether IDO1 is a driving force, or a compensatory mechanism might be disease-specific and should be investigated in PD[102].

3. DCs and monocytes as an underappreciated source of peripheral IDO1

3.1. DCs and monocytes

Circulating myeloid cells as DCs and monocytes play important immune functions such an internalization and antigen presentation in the MHC context to B and T cells[113]. These cells present

pattern recognition receptors (PRR) that ensure that they recognize danger-associated molecular patterns (DAMPs)[113]. This is the first step to distinguishing the self from the non-self and signaling different molecules and pathways that will lead to antigen presentation, co-stimulation, and migration. DCs also promote the balance between exacerbated immune responses and immune tolerance[113]. The different subsets of DCs and monocytes drive different immune responses. Single-cell studies using flow cytometry, mass spectrometry, and single-cell RNA sequencing are showing a complex heterogeneity of these cells and call for a redefinition of the classical classification of DCs and monocytes, which would consider not only the surface markers but also the functional and transcriptional signatures of these cells[114,115]. At this point, DCs are classified according to surface markers as follows: conventional DCs (cDCs; HLA-DR$^+$, CD11c$^+$, CD123$^{-/dim}$) and plasmacytoid DCs (pDCs; HLA-DR$^+$, CD11c$^-$, CD123$^+$). Moreover, cDCs can be divided into cDC1s (if expressing CD141) and cDC2s (if expressing CD1c). The different cell subsets are associated with different functions: (i) pDCs can produce high amounts of INF-γ in response viruses; (ii) cDC1s perform cross-presentation of exogenous antigens via MHC-I and induce cytotoxic TCD8$^+$ cells, being important in responses against virus and cancer; and (iii) cDC2s are highly efficient in phagocytosis and MHC-II antigen presentation inducing TCD4$^+$[115]. Similarly, monocytes are classified into three main populations based on their lineage expression markers and different functions: classical monocytes (CD14hiCD16-), intermediate monocytes (CD14hiCD16low), and non-classical monocytes (CD14lowCD16hi)[116]. Interestingly, the expression of adhesion molecules (CD62L, CD43, CD49d), chemokine receptors (CCR2, CCR5, and CXCR1), and surface markers (HLA-DR, CD86, and CD80), was used to identify a new human monocyte population[117]. Importantly, these adhesion molecules were related to different phenotypes suggesting the utility of the use of multiparameter analysis to redefine immune populations[117]. Similarly, 6-sulfo LacNac (SLAN) is suggested to be a marker of non-classical monocytes and suggested as a biomarker in disease staging in leukemia[118,119]. In homeostatic conditions, monocytes maintain peripheral reservoirs and resident macrophages, and under pathological conditions, monocytes acquire inflammatory and regulatory functions[120]. In particular, classical monocytes under inflammatory environments show phagocytic activity, antigen-presentation function, tissue-repairing properties, and both, pro and anti-inflammatory activities[120]. Interestingly, monocytes can present antigens *in vivo* however, it is not known if this contributes in a significant way to prime T cells when compared with DCs[120]. Intermediate monocytes can secrete massive amounts of pro-inflammatory cytokines including TNF-α, and IL-1β when stimulated with LPS [a toll-like receptor 4 (TLR4) agonist][121], thus promoting TCD4$^+$ cell proliferation[116,122,82]. The non-classical monocytes actively patrol the endothelium[122]. However, the heterogeneity of these cells has been underappreciated and it is dependent on inflammatory environments[114]. Considering the

plasticity of these cells as well as the plasticity of the moonlight IDO1, we can argue that studying the function of IDO1$^+$circulating myeloid cells seems to be a complex task. There is a limited number of studies aiming to characterize IDO1 expression across the DCs and monocyte population. For example, a recent study showed that cDC1 presented an IDO1-dependent regulatory function that controlled the ability of cDC2 to acquire tolerogenic competence also mediated by IDO1[123]. Specifically, both cDC1 and cDC2 expressed IDO1 when stimulated together with LPS. However, when isolated, cDC1 but not cDC2, expressed IDO1. The release of KYN by cDC1 seems to be an important mechanism to spread tolerogenic activity from cDC1 to cDC2 and this mechanism seems to occur in both, mice and human DCs under LPS stimulation[123]. Moreover, cDC2 stimulated by LPS seems to release more IL-6 which targets IDO1 proteasomal degradation[123]. Accordingly, another study showed that IFN-γ and LPS induced IDO1 expression in cDC1 and cDC2 but not in pDCs[124]. Other studies showed that both, human and mouse pDCs can express IDO1 in specific environments, such as tumor environments or infections[125]. IDO1 expression in monocytes is described as the upregulation of IDO1 in total, classical, and intermediate monocytes but not in non-classical after IFN-γ stimulation of human monocytes[126]. On the other hand, IDO$^+$ non-classical monocytes were identified in dengue infection of B cell lymphoma[127,128]. These IDO1$^+$circulating myeloid cells also might acquire different functions according to the inflammatory environment. [102]. Overall, the crosstalk inter and intrapopulation, like antigen-presenting cells (APCs) and T cells, seems to be important in tolerogenic immunocompetence acquirement[129,130]. Considering the importance of cell crosstalk and the specificity of the inflammatory environment on IDO1 modulation, studying IDO1$^+$circulating myeloid cells in a PD context seems to offer a possibility to understand the impaired immune mechanism and potentiate the opportunity to develop therapeutic strategies.

3.2. Are IDO1$^+$DCs and IDO1$^+$monocytes dysfunctional in PD?

PD patients show fewer levels of circulating DCs which is associated with increased impairment of motor functions [31,131]. The migration of DCs to the inflammation brain is one possibility suggested to explain the decreased DCs numbers in PD[31]. DCs are key elements of brain immune surveillance, however, the identification of infiltrated myeloid subsets is hard in neurodegeneration, due to the restricted number of cells, morphological heterogeneity, and lack of specific markers[41,132]. Importantly, α-syn can activate macrophages and DCs, which implicates an immune response orchestration with an adaptive immune response, including anti- α-syn antibody production [133–135]. Similarly, human DCs exposed to neuromelanin (NM) can phagocyte and become mature, thus triggering T cell maturation

and B cell antibody production[136]. Interestingly, LRRK2 inhibitors blocked TNF-α release by cultured human DCs[137]. Tolerogenic DCs of PD patients demonstrated a decrease in the expression of programmed death-ligand 1 (PD-L1), which plays an important role in the development and maintenance of Tregs[60]. DCs of patients also correlated positively with activation molecules such as CD80, CD86, and CD40, indicating activation of DCs in PD[60]. Additionally, PD patients positive for cytomegalovirus (CMV) showed increased levels of pro-inflammatory DCs ($CD16^{+}$ILT2 high). Moreover, it was shown that CMV impaired IDO1 activity in human fibroblasts [86,138]. Patients with elevated H&Y scores exhibited increased expression of toll-like receptor 2 (TLR2) in DCs, which might suggest an increase in DCs susceptibility to α-syn[48,139]. Interestingly, granulocyte-macrophage colony-stimulating factor (GM-CSF) promotes DCs tolerogenic phenotypes and Treg responses, thus conferring neuroprotection in an MPTP animal model [140]. However, IDO1 expression and function in DCs have been overlooked in PD. The important immunoregulatory function of these cells and their potential as a therapeutic target warrant a better characterization of DCs in PD. A PD sex-based characterization in cell frequency, phenotype, and function (including IDO1 expression and function) can be important to disclose impaired mechanisms implied in disease progression or pathology. Regarding blood monocytes, while some studies reported increased classical monocytes and decreased intermediate and non-classical frequencies, others failed to find differences in monocytes[48,141]. On the other hand, CSF of PD patients showed increased levels of non-classical monocytes[142]. Moreover, monocytes of PD patients seem to show altered phenotypes according to disease stage and, possibly, there are also sex-based differences in the activation of monocytes[143,144]. A characteristic gene expression profile was described in PD's early stages [145]. Furthermore, monocytes increased the levels of surface markers CCR2 and CD11b at early stages, which were correlated with worse cognition, and CD163, which correlated with phagocytic activity[143].Additionally, the number of monocytes expressing HLA-DR increased in the late stages[143]. Moreover, TLR2 expression in monocytes, which can be induced by α-syn[146], is related to worse motor symptoms[143]. Interestingly, monocytes might participate actively in spreading α-syn from the periphery to the brain [147]. $CD163^{+}$ cells, which seem to be useful in identifying infiltrated monocytes, were present in SN of PD patients. In addition, both, $CD163^{+}$ cells and $CCR2^{+}$monocytes were found in the brain of PD mouse models[49,148,149]. Moreover, soluble CD163 has been proposed as a disease biomarker of cognitive deficits in PD[131]. Interestingly, the number of monocytes expressing TLR4 in rapid-eye-movement (REM) sleep behavior disorder (iRBD), which identifies a prodromal state of PD, is positively related to immune activation and negatively correlated with dopaminergic transmission[150]. However, these patients seem to show a correlation between the expression of the CD163 receptor and lower inflammation, showing a protective role of CD163[150]. LRRK2 was increased in monocytes of PD patients and contributes to

monocyte dysregulation in PD[151,152]. The increased LRRK2 expression occurs in a pro-inflammatory environment or due to the presence of pathologic α-syn, which can induce monocyte recruitment to the brain[153,154]. Glucocerebrosidase (GBA) also seems to be implicated in monocyte dysregulation in PD: (i) monocytes of PD patients with GBA mutation showed a different transcriptome from the ones without mutation; (ii) total and classic PD monocytes showed GBA reduced activity; (iii) GCase activity in classical monocytes was inversely correlated to motor severity and (iv) reduced monocyte GBA activity in sporadic forms is followed by increased plasmatic levels of CXCL8, CCL2, and CCL3 [152,155,156]. The functional monocyte studies in PD patients show incongruent data in response to LPS and or α-syn, similarly to monocyte frequencies data, as follows: (i) PD monocytes showed decreased sensitivity to LPS and fibrillary α-syn; (ii) PD monocytes showed increased reactivity to pathological α-syn and (iii) PD monocytes showed no differences in response to LPS when compared with HS[48,141,157]. Importantly, techniques, cohorts, and population subsets might be the origin of the differences seen between studies[48,141]. The phagocytic activity might also decrease across disease stages. More severe PD patients show less ability to uptake α-syn, which might be age-related [48,158]. Impaired cytokine production by PD monocytes has been also reported[152]. It is noteworthy that human monocytes from healthy donors stimulated with INF-γ up-regulated the enzymes IDO1, KMO, and quinolinate phosphoribosyltransferase (QPRT) and this up-regulation was followed by increased KYN/Trp ratio, QUIN levels, and neopterin (which is a pro-inflammatory immune response marker in the culture medium)[159]. The use of monocytes as a PD biomarker has been suggested: (i) as an early biomarker to define sargramostim (GM-CSF) treatment outcomes for PD (please see section 5.2); (ii) to distinguish between PD phenotypes (with dementia or depression)[160–162]. Overall, the described alterations increase the probability of IDO1 impairments in these cells. This may have implications in terms of peripheral and central immune response regulation in PD, considering that monocytes can infiltrate the PD brain. Finally, more studies are necessary to address the immune changes (frequency, phenotype, and function) of monocytes in PD, including IDO1^{+}monocytes.

4. IDO1 as a therapeutic target in PD

4.1. IDO1 inhibition

IDO1 inhibitors have been extensively studied in the cancer context despite the difficulties associated with the plasticity of IDO1[163]. IDO1 inhibitors [eg. 1-methyl-D-tryptophan (1-MT), epacadostat,

navoximod, BMS-986205, and PF-06840003] have shown immunosuppressive activity in tumor microenvironments containing tumor progression in preclinical models of several cancers[105]. For example, the competitive inhibitor IDO1 epacadostat showed powerful antitumoral effects by increasing natural killer (NK) and T cells in preclinical studies[163]. This molecule was tested in clinical trials in monotherapy and combined with the immune checkpoint inhibitors (anti-CTLA-4 inhibitor and anti-PD-L1 inhibitor). However, its benefit in different cancers was not confirmed in the final phases of the trials[163]. This failure might be explained by insufficient inhibition of IDO1, wrong selection of the patients (not based on IDO1 expression), the existence of TDO2, failure to block the KP downstream enzymes, and failure to block AhR activation (interleukin-4-induced was discovered as another AhR activator)[163]. There is still an ongoing phase 3 trial using the IDO1 inhibitor (BMS-986205) alone or combined with nivolumab (which is an anti-PD1) [164]. Interestingly, IDO1 modulators (eg. indoximod), beyond IDO1 inhibitors, also showed some benefits. However, the mechanism explaining their action is not completely understood[164]. Overall, the possible reasons to explain the failure of IDO1 inhibitors in cancer seem to be all related to an inefficient inhibition of the Trp metabolism[163]. This may hint at the importance of Trp metabolism in immune system modulation and stress the difficulties regarding KP modulation. Interestingly, IDO1 inhibition or deletion has also been tested as a strategy to target neurodegeneration. For example, Ido -/- mice injected with intra-striatal QUIN are less sensitive to QUIN neurotoxicity and showed discrete impairments in KP metabolism[165]. Moreover, the increased levels of KP metabolites in the periphery and brain achieved after 24h following LPS injection are abolished in both Ido-/- and Tdo-/- mice[166]. The inhibition of IDO1 by 1-MT (2.5, 5 and 10 mg/kg, ip during 15 days, starting 15 days post-intrastriatal injection) in a 6-hydroxydopamine (6-OHDA) murine model of PD induced the following in a dose-dependent way: (i) improved motor function; (ii) reduced oxidative stress, mitochondrial dysfunction, and neuronal apoptosis, and (iii) increased neurotransmission (as gauged by DA and homovalinic acid values)[167]. Moreover, studies on Alzheimer's Disease (AD) demonstrated that oral administration of the IDO inhibitor coptisine (50mg/kg, once daily for a month) to APP/PS1 transgenic mice decreased the activation of microglia and astrocytes and consequently prevented neuron loss, reduced amyloid plaque formation, and ameliorated impaired cognition. In the same study, this inhibition increased cell viability by reducing IDO activity[168]. In an experimental MS mouse model (EAE), IDO1 inhibition by 1-MT (100mg/kg i.p during 7 days in different disease stages defined accordingly with the EAE score) increased FoxP3 cells and amelioration of EAE[169]. Overall, these experiments addressed the neuropathological aspects related to KP in animal models of neurodegenerative disease. However, the contribution of IDO1 inhibition, if any, to resolving the pro-inflammatory profile described in PD patients, thus halting the neurodegenerative course, remains to be known[111].

4.2. Can IDO1$^+$ tolerogenic DCs be recruited to fight PD?

DCs are important immune modulators, which may be useful in PD[132]. The treatment of PD patients with sargramostim (recombinant GM-CSF), which stimulates the innate immune response by activating macrophages and DCs, may suggest the benefits of DCs in PD[170]. Subcutaneous administration of this drug for 56 days increased the activity and numbers of their Tregs and improved the Unified Parkinson's Disease Rating Scale (UPDRS) III scores[171]. Interestingly, Treg data was linked with increased levels of KP metabolites, such as KYN and QUIN[171]. Moreover, the administration of GM-CSF to a MPTP PD mouse model induced tolerogenic DCs derived from its bone marrow that increased Tregs numbers, which in turn attenuated neuroinflammation and neurodegeneration[140]. Additionally, the inoculation of Tregs isolated from mice treated with GM-CSF to a MPTP mouse model showed anti-inflammatory and neuroprotective abilities[140]. Furthermore, the induction of Foxp3$^+$ regulatory T cells via prostaglandin E_2 receptor signaling, by targeting DCs with venom phospholipase A_2 modulated the neuroinflammatory responses in the MPTP mouse model[172]. Importantly, dendritic cell-based vaccines have shown to be an effective treatment for synucleinopathies in animal models[173]. Specifically, intravenous administration of bone marrow-derived DCs sensitized with α-syn to a transgenic mouse expressing the human A53T variant of α-syn induced the production of α-syn antibodies that reduced the α-syn aggregates and improved the locomotor function[173,174]. In another study, three out of ten anti-α-syn antibodies isolated from memory B-cells of PD patients inhibited intracellular synuclein aggregates and recognized Lewy bodies from post-mortem PD brain tissues[72]. Similarly, administration of human recombinant α-syn in α-syn transgenic mice decreased α-syn aggregates in neuronal cells and improved neurodegeneration[175]. Also, recombinant α-syn was preventively administrated (before the lesion) to a group of rats that were inoculated unilaterally with recombinant adeno-associated viral vector aiming to overexpress human α-syn in their SN and to induce a progressive neuropathologic process. This vaccination strategy led to the activation of microglia and increased Tregs levels in this model[64,176]. This activation of microglia by immunization is also supported by a study where stereotaxic administration of antibodies in an α-syn transgenic mouse reduced the accumulation of α-syn in microglia and improved neurodegeneration[175 65,177]. These studies strongly suggest that DCs-based vaccination is a promising strategy aiming to balance Treg/Teff and/or induce antibody production, thus controlling the immune response[71,175]. The ability of IDO1$^+$DCs to induce Treg and decrease Teff cells might improve the efficacy of this strategy[140]. Excitingly, proteomic analysis of *in vitro* inoculated human DCs with cholera toxin B subunit revealed a strong up-regulation of IDO1, which means that using DCs-based vaccination in PD might already be taking advantage of IDO1 as an immunoregulatory mechanism[178].

The authors also suggested the importance of disclosing the underlying mechanism of IDO1 modulation to facilitate vaccine efficacy and safety[178].

5. Do current PD therapeutics affect IDO1?

L-DOPA represents the most effective symptomatic treatment for PD aiming to replace DA in the striatum. Other dopaminergic therapies that include dopaminergic agonists, monoamine oxidase B (MAOB), and catechol-ortho-methyltransferase (COMT) inhibitors and COMT are also useful therapies[179,180]. DA agonists and MAO-B inhibitors are less effective than L-DOPA but have lower dyskinesia risk and DA agonists are associated with a higher overall risk of adverse events[181]. COMT inhibitors are useful as add-on therapies to reduce L-DOPA-induced wearing-off [181]. Individuals experiencing L-DOPA-induced complications, such as worsening symptoms and functional impairment when a medication dose wears off ("off periods"), medication-resistant tremor, and dyskinesia, benefit from advanced treatments such as therapy with levodopa-carbidopa enteral suspension, or deep brain stimulation[181]. Subcutaneous apomorphine (D1/D2 agonist) injections and inhaled levodopa can be used to achieve a faster medication response in individuals with severe off periods and delayed onset with subsequent dosing[180]. Non-DAergic drugs may also be used in PD and include anticholinergics, amantadine (ionotropic glutamate receptor antagonist) istradefylline (adenosine receptor antagonist), and clozapine[181]. Nonmotor symptoms require no dopaminergic approaches (eg, selective serotonin reuptake inhibitors and clozapine for psychiatric symptoms, cholinesterase inhibitors for cognition)[182,183]. Since PD's current therapies are symptomatic and focused on improvement in motor and non-motor symptoms, the emergence of disease-modifying therapies that would delay disease progression and influence the pathological process is an unmet need[179.] Interestingly, DA was called a "NeuroImmunotransmitter" due to its role as an immune modulator[184]. It is noteworthy that many immune cells, including monocytes and DCs, express DA receptors [D1- and D2-like receptors, which are members of the monoaminergic G protein-coupled receptor (GPCR) family] and other dopamine-related proteins, enabling them to respond to DA[185]. Interestingly, examination of ventral striatum to ventromedial prefrontal cortex resting-state functional connectivity in responses to L-DOPA and placebo across a range of plasma C-reactive protein (CRP) concentrations (0-1, >1-2, >2-3. And >3 mg/L) showed that only patients with CRP > 2 mg/L had a positive response to L-DOPA in subjects with major depressive disorder[186]. This work further suggests that DA is endowed with inflammation-modulatory properties. In this context, understanding the putative interaction between DA and IDO1 is mandatory. Importantly, increasing evidence suggests that L-DOPA, d-amphetamine (DA releaser), and apomorphine decrease KP metabolites, including

the striatal KYN and KYNA in mice and rats[101,187,188,189]. PD patients taking L-DOPA presented significantly reduced concentrations of KYN and KYNA in the frontal cortex in post-mortem examinations[190]. Moreover, LIDs were correlated with altered KP metabolism in plasma and CSF in PD[191]. This L-DOPA-KP metabolites relationship was also explored in MPTT monkeys. In particular, the administration of Ro 61-8048 (10, 30, and 100 mg/kg; an inhibitor of KMO) was used as a strategy to increase, dose-dependently, KYNA levels in serum and CSF[192]. Importantly, co-administration of Ro-61-8048 with L-DOPA produced a moderate but significant reduction in the severity of dyskinesia while maintaining the motor benefit[192]. The same strategy was applied in dyskinesia MPTT monkeys[193-195]. Daily administration of Ro 61-8048 (50mg/kg) before L-DOPA administration induced KYNA serum levels and reduced the development of LIDs[193-195]. In a mouse model injected unilaterally in the striatum with a NMDA or quinolinate, the administration of DR1 (SHC23390, 1mg/kg, 15 minutes before the lesion) and DR2 (raclopride, 2mg/kg, 15 minutes before the injection) antagonists prevented the reduction in brain KYNA and abolished NMDA neurotoxicity [188]. Another study in mice aimed to evaluate whether the DA antagonist's haloperidol (1.5mg/kg i.p., and 0.025mg/mL dissolved in water), clozapine (20mg/kg i.p., 0.33mg/mL dissolved in water) and raclopride (10mg/kg, i.p., 0.17mg/mL dissolved in water) administration contributed to increase KYNA levels[194]. These authors showed that chronic but not acute administration of these drugs for one month (administration in water) reduced KYNA levels in the striatum, hippocampus, and frontal cortex [196]. Interestingly, these strategies already explore the communication of DA-IDO1 a possible therapeutic strategy to target excitotoxicity-driven LIDs. However, the neuroinflammation-driven LIDs were left out of the equation. Therefore, the communication between the dopaminergic system and KP may be an important axis to mediate immune function in PD, thus being, possibly, useful in the management of L-DOPA-induced motor complications[195]. Overall, this DA-IDO1 axis needs further investigation and may represent a potential therapeutic target in PD[197]. Moreover, these studies have essentially explored the central but not peripheral DA-KP axis. Furthermore, carbidopa, an inhibitor of DOPA decarboxylase which is used in combination with L-DOPA in PD, showed an inhibitory effect of the IDO1 that is increased in cancer cells and led the authors to re-purpose carbidopa in cancer [198,199]. Interestingly, it was shown that this carbidopa-mediated IDO1 suppression in pancreatic ductal adenocarcinoma by at least two mechanisms: carbidopa is an AhR agonist and interacts with JAK1/STAT1 signaling (which means interaction with activation of IFN-γ-IDO1 mediated pathway)[195]. Despite this data concerning cancer cells, this information is particularly important to alert us that IDO1-AhR might also decrease IDO1 expression under certain circumstances, which makes this interaction complex and possibly cell and environment-dependent.

Interestingly, PD patients show a lower incidence of most cancers, except melanoma. This is not due to L-DOPA and may be attributable to carbidopa[196].

6. Conclusions

With this review, we aim to shed light on the use of IDO1 as an immunomodulatory strategy, beyond targeting the neurotoxic branch of the KP pathway, as currently being done with beneficial effects on neurodegeneration. Nonetheless, it is first necessary to explore the expression and activity of $IDO1^+$DCs and $IDO1^+$monocytes in neurodegenerative diseases, including PD. The changes verified in DCs and monocytes in PD patients increase the probability of IDO1 dysfunctions on these cells that contribute to an impairment of anti-inflammatory strategies and low-grade chronic inflammation in PD. Thus, targeting $IDO1^+$DCs and $IDO1^+$monocytes seem to be an attractive strategy to balance the pro-inflammatory profile described in PD. Considering that these cells infiltrate the PD brain, makes this case stronger. Importantly, tolerogenic DCs are also being explored as a therapeutic strategy, and disclosing the IDO1 role in these cells might help to improve the efficacy of tolerogenic DCs. The study of $IDO1^+$DCs and $IDO1^+$monocytes in different disease stages can also be explored as potential biomarkers. Furthermore, looking at the described effect of carbidopa as an AhR agonist and considering the DA immunomodulatory effects, disclosing the interaction between PD therapeutics and IDO1 (especially, the DA-IDO1 axis) can further be important to optimize PD current therapeutic strategies. In the end, we propose to re-look at IDO1 role in PD by considering both its immune and neurotoxic function.

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Disclosers

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Author Contributions

Milene Gonçalves: conceptualization, writing original draft, writing-review, and editing. Paulo Rodrigues Santos: writing-review and editing. Cristina Januário: writing-review and editing. Marco Cosentino: writing-review and editing. Frederico C. Pereira: conceptualization, writing-review, and editing.

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CHAPTER 3

A peripheral blood mononuclear cell-based *in vitro* model: a tool to explore indoleamine 2,3-dioxygenase-1

(Submitted to the European Journal of Pharmacology-under review)

A peripheral blood mononuclear cell-based *in vitro* model: a tool to explore indoleamine 2, 3-dioxygenase-1 (IDO1)

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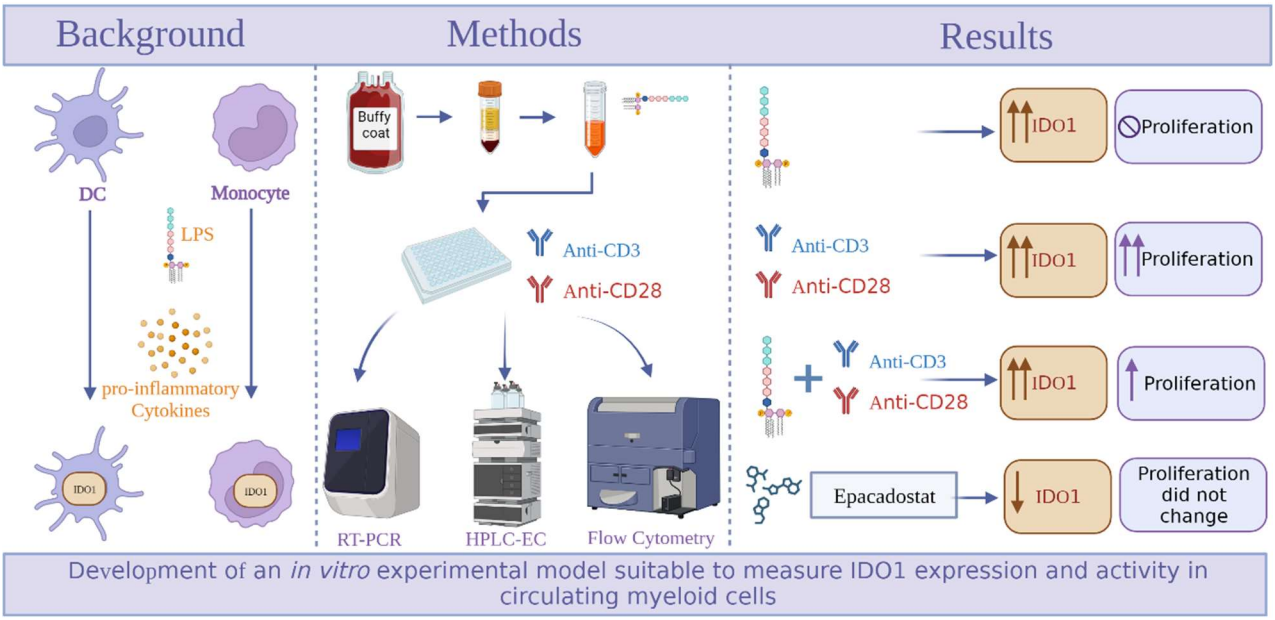

Abstract

Background

Proinflammatory cytokines powerfully induce the rate-limiting enzyme indoleamine 2, 3-dioxygenase-1 (IDO-1) in dendritic cells (DCs) and monocytes, it converts tryptophan (Trp) into L-kynurenine (KYN), along the kynurenine pathway (KP). This mechanism represents a crucial innate immunity regulator that is able to modulate T cells. This work explores the role of IDO1 in lymphocyte proliferation within a specific pro-inflammatory milieu.

Methods

PBMCs were isolated from buffy coats taken from healthy blood donors and exposed to a pro-inflammatory milieu triggered by a double-hit stimulus: lipopolysaccharide (LPS) plus anti-CD3/CD28. The IDO1 mRNA levels in the PBMCs were measured by RT-PCR; the IDO1 activity was analysed using the KYN/Trp ratio, measured by HPLC-EC; and lymphocyte proliferation was measured by flow cytometry. Trp and epacadostat (EP) were used as an IDO1 substrate and inhibitor, respectively. KYN, which is known to modulate Teffs, was tested as a positive control in lymphocyte proliferation.

Results

IDO1 expression and activity in PBMCs increased in an *in vitro* pro-inflammatory milieu. The lymphoid stimulus increased IDO1 expression and activity, which supports the interaction between the activated lymphocytes and the circulating myeloid IDO1-expressing cells. The addition of Trp decreased proliferation but EP, which abrogated the IDO1 function, had no impact on proliferation. Additionally, incubation with KYN seemed to decrease the lymphocyte proliferation.

Conclusion

IDO1 inhibition did not change T lymphocyte proliferation. We present herein an *in vitro* experimental model suitable to measure IDO1 expression and activity in circulating myeloid cells.

Keywords: inflammation; indoleamine 2,3-dioxygenase 1, Tryptophan, Kynurenine, myeloid cells, T lymphocyte proliferation.

1. Introduction

The Kynurenine pathway (KP) is the main route (about 90-95%) of degradation of the essential amino acid L-Tryptophan (Trp)[1]. Other Trp routes include: the serotonin pathway, protein synthesis, decarboxylation (to tryptamine), and transamination (to indol-3-yl pyruvic acid)[2]. The degradation of Trp by the KP occurs over several steps and generates several metabolites with biological functions including immunomodulatory activity [3,4]. KP dysregulations have been linked to several inflammation-driven pathologies including: neurodegenerative (eg. Parkinson´s Disease), oncological, and cardiovascular diseases[5,6]. Importantly, the rate-limiting enzyme indoleamine 2,3-dioxygenase-1 (IDO-1), which converts Trp into L-kynurenine (KYN) is powerfully induced by proinflammatory cytokines, such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-6 and LPS[7,8]. KYN feeds the two main branches of KP leading to the formation of two relevant metabolites: quinolinic acid (QUIN) and kynurenic acid (KYNA) with neuroactive and immunomodulatory properties[9]. A brain imbalance between the neuroprotective KYNA and the neurotoxic QUIN is well established in neurodegenerative diseases; however, the role of periphery KP metabolites in these diseases has been almost completely ignored[10–12]. IDO1, which can be upregulated in dendritic cells (DCs) and monocytes (0.1 to 1% and 1 to 6% of leukocytes in the human peripheral blood)[13,14], is a crucial innate immunity regulator that seems to play a role in suppressing effector T-cell (Teffs) cells and activating regulatory immune responses in inflammatory microenvironments[14–16]. IDO1 presents immunomodulatory functions related to Trp starvation and increased KYN levels. For example, the starvation of Trp leads to cell cycle arrest and T cell apoptosis whereas KYN activates the transcription factor aryl hydrocarbon receptor (AhR) which induces the differentiation of $CD4^+$ T cells into Tregs[17–19]. IDO1 modulation has been indicated as an object of study in autoimmune and neuroinflammatory diseases because of its anti-inflammatory and immunoregulatory effects, both at the periphery (peripheral blood mononuclear cells - PBMCs) and the central nervous system (CNS)[20,21] Therefore, targeting IDO1 might be an important strategy to control the Teff-mediated pro-inflammatory process in the neuroimmune interface and to manage neurodegenerative diseases[22,23]. We explored, therefore, the impact of IDO1 on lymphocyte proliferation within a specific pro-inflammatory milieu. To reach this goal, PBMCs were subjected sequentially to lipopolysaccharide (LPS, myeloid stimulus), which can induce IDO1, and to anti-CD3/CD28 (lymphoid stimuli), which increase T cell proliferation[4-8].

2. Material and Methods

2.1. Subjects

PBMCs were isolated from the buffy coats (BC) of healthy donors and collected in the Immunohematology and Transfusion Medicine service of *Ospedale di Circolo e Fondazione Macchi di Varese*, Italy. There is no information concerning the gender or age of the individuals.

2.2. 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 obtained from Pharmacia Biotech (Uppsala, Sweden, GEH1714403). The phosphate buffer saline (PBS (g/L) NaCl 8.0, KCl 1.5, Na_2HPO_4 1.44, KH_2PO_4 0.24, pH 7.4) used for the isolation and cell culture of PBMCs, as well as the lysis buffer ((g/L) NH_4Cl 8.248, $KHCO_3$ 1.0, EDTA 0.0368), also used for the isolation of PBMCs, were prepared with reagents from Sigma-Aldrich (Saint Louis, MO, USA). Trypan Blue solution 0.5% (ECM0990D-100mL) was purchased from Euroclone, Italy. Purified mouse ab anti-human CD3 (555330, clone UCHT1, Mouse IgG1, κ) and purified mouse ab anti-human CD28 (clone CD28.2, Mouse C3H x BALB/c, IgG1, κ) were obtained from Becton Dickinson, Italy. The cell Proliferation Dye, eFluor 670 (CPD) was obtained from eBioscience-Prodotti Gianni, Italy (65-0840). Lipopolysaccharide (LPS, Standard lipopolysaccharide from E. coli 0111:B4 strain; TLR4 ligand, tlrl-eblps) was obtained from Invivogen). Tryptophan (Trp, 73-22-3) and kynurenine (KYN, 2922-83-0) were from Sigma-Aldrich (Saint Louis, MO, USA). Epacadostat (EP, 1204669-58-8) was obtained from Cayman Chemical Company. BD Cytofix/Cytoperm (554714, Becton Dickinson, San Diego, CA, USA).

2.3. Isolation of PBMCs

BC were diluted (1:1) in PBS ((g/L) NaCl 8.0, KCl 1.5, Na_2HPO_4 1.44, KH_2PO_4 0.24, pH 7.4) and then the PBMCs were isolated by Ficoll-Paque Plus density gradient centrifugation. The cells were resuspended, and any residual contaminating erythrocytes were lysed by the addition of 5 mL of the lysis buffer ((g/L) NH_4Cl 8.248, $KHCO_3$ 1.0, EDTA 0.0368), followed by immediate centrifugation at 100 g for 10 min at room temperature (RT). The cells were washed in PBS/FBS 2% and centrifuged at 300 g for 10 min at RT and resuspended at the final concentration of 10×10^6 cells in 10 ml of RPMI for subsequent culture. Cell viability was assessed by trypan blue, and purity was assessed by turk

solution [(v/v): gentian violet 0.02%+acetic acid 3% prepared in water]. The viability of the cultured cells was ≥ 95%. The PBMC suspension obtained by this method contained at least 90% of lymphocytes and a few contaminant polymorphonuclear (PMN) cells (≤ 5%), as confirmed by flow cytometry.

2.4. Cell culture

2.4.1. LPS stimulus

The PBMCs were resuspended at the concentration of 1 × 10^6/ mL in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and 100 U/mL penicillin/streptomycin and activated with LPS (100 ng/mL) in a cell culture flask, for 6h at 37 °C in a moist atmosphere of 5% CO_2. These cells were then transferred to the 96-well plates coated with anti-CD3. This time-point was chosen based on a time-course evaluating IDO1 induction in monocytes and dendritic cells across 24h-stimulation of total blood (Supplementary Figure S1a).

2.4.2. Anti-CD3/CD28 stimuli

First, the 96-well flat bottom plate was coated with 100 µL of anti-CD3 antibody (2 µg/mL in sterile PBS 1X). The lid-covered plate was incubated for 2h at 37°C with 5% of CO_2. Then the unbound antibody was washed twice with 200 µL of sterile 1X PBS. 250 µL of resuspended PBMCs (1 × 10^6/ mL in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and 100 U/mL penicillin/streptomycin) pre-stimulated or not with LPS (100 ng/mL for 6h) were added to each well and stimulated with soluble anti-CD28 (2 µg/mL) for 24 or 120h at 37 °C in a moist atmosphere of 5% CO_2.

2.4.3. IDO1 activation by Trp supplementation

We adjusted the Trp concentration present in RPMI (25 µM) to human physiologic values according to Geisler *et al*., 2015 (67.4±10.2 µM)[24] for the assays with Trp supplementation. Trp was added conjointly with LPS. The final concentration of Trp (73.00±10.70 µM) in the culture supernatants was determined after 126h of cell culture at 37ºC with 5% of CO_2 by HPLC-EC.

2.4.4. IDO1 inhibition by Epacadostat

Epacadostat (EP) is an IDO1 inhibitor that acts as a Trp-competitive inhibitor of the catabolic activity of human IDO1 in cell-based assays (IC_{50}=12 nmol/L) with >100-fold selectivity exhibited against IDO2 and TDO2[25]. The EP was dissolved in DMSO and used at a concentration of 1.0 µM, which mimics the serum concentration observed in patients receiving 300 mg BID, where there was > 90% inhibition of IDO1[26]. The EP was added conjointly with LPS and Trp.

2.4.5. KYN incubation

KYN was directly added to the cell culture after 6h of incubation with LPS (100 ng/mL) and immediately before the anti-CD3/CD28 stimuli at a concentration of 50 μM[27]. The final concentration of KYN (47.69±4.32μM) in the culture supernatants was determined after 120h of cell culture at 37ºC with 5% of CO_2 by HPLC-EC and may reflect the exogenous addition plus the KYN released by the cells into the medium in the pro-inflammatory environment.

2.5. IDO1 expression by Real Time-PCR (RT-PCR)

Cells were collected after 24 or 120h of cell culture (counting from the last stimuli – anti-CD3/CD28) at 37 °C with 5% CO_2 for IDO1 expression analysis. RT-PCR of IDO1 was performed according to a previously reported method but with modifications[28]. Briefly, *circa* 50,000 PBMCs were resuspended in PerfectPure RNA lysis buffer (5 Prime Gmbh, Hamburg, Germany) to isolate the RNA. Total RNA was extracted by PerfectPure RNA Cell KitTM (5 Prime Gmbh), and the amount of RNA extracted was estimated by spectrophotometry at $\lambda = 260$ nm. The total mRNA obtained from the PBMCs was reverse-transcribed using a random primer, high-capacity cDNA RT kit (Applied Biosystems). The cDNA was amplified with the SsoAdvanced™ Universal SYBR® Green Supermix (BIORAD) for analysis and then assayed on the StepOne® System (Applied Biosystems). The linearity of the real-time PCR assays was tested by constructing standard curves using serial 10-fold dilutions of a standard calibrator cDNA for the gene, and the regression coefficients (r2) were always > 0.999; a melting curve was also performed to check for the specificity of IDO1. The gene expression level in any given sample was represented as $2^{-\Delta Ct}$ where Δ Ct = [Ct (sample) – Ct (housekeeping gene)]. The relative expression was determined by normalization to 18 S cDNA. Analysis of the data was performed by StepOne software™ 2.2.2- Applied Biosystems.

2.6. Proliferation Assay

T lymphocyte proliferation was measured after 120h of cell culture after the last stimuli at 37 °C with 5% CO2, using standard staining with the proliferation dye CPD and flow cytometric analysis [BD FACSCelesta flow cytometer, Becton Dickinson, Milan, Italy with BD FACSDiva software (version 8.0.1.1)]. Resting and activated lymphocytes were identified according to their typical morphological parameters in the Forward and Side Scatter dot- plot (FSC vs. SSC plot) and a sample of a minimum of 20,000 cells was acquired at the gate (Supplementary Table S1 and Supplementary Figure 3S). CPD is a red fluorescent dye that binds to any cellular protein containing primary amines and as cells divide

the dye is distributed equally between daughter cells resulting in a successive halving of the fluorescence intensity during cell division. The lymphocyte proliferation can be therefore calculated as a percentage (%) of the CPD^{low} cells collected at the gate. The necessary amount of PBMCs, including the cells to be stained with CPD (CPD^{+}) and the cells that were not stained with CPD (CPD^{-}), were counted by using a cellometer (cellometer Auto T4, Nexcelom Bioscience, Euroclone). The cells were then washed with PBS/FBS 0.1% to a volume of 5 mL and centrifuged at 1,200 g for 5 min at RT. The supernatants were carefully removed and CPD^{-} cells were resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and 100 U/mL penicillin/streptomycin at a concentration of $1x10^6$ cells/mL. These cells were incubated at 37 °C with 5%CO_2 until CPD staining of the remaining cells was completed. CPD (2.5µM) was added to the remaining cells that were resuspended in 1 mL of PBS/FBS 0.1% (for up to $10x10^6$ cells), the suspension was immediately mixed using vortex and then incubated at RT for 8 min, protected from light. The reaction was stopped by the addition of an equal volume of pre-warmed (37°C) FBS (1mL) and incubated at 37°C for 10 min. Next, PBS/FBS 10% was added to a volume of 10 mL. The suspension was centrifuged at 1,200 g, for 5 min at RT and washed twice with 10 mL PBS/FBS 10% at 1,200 g for 5 min. The cells were resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and 100 U/mL penicillin/streptomycin at a concentration of $1x10^6$ cells/mL to continue for cell culture.

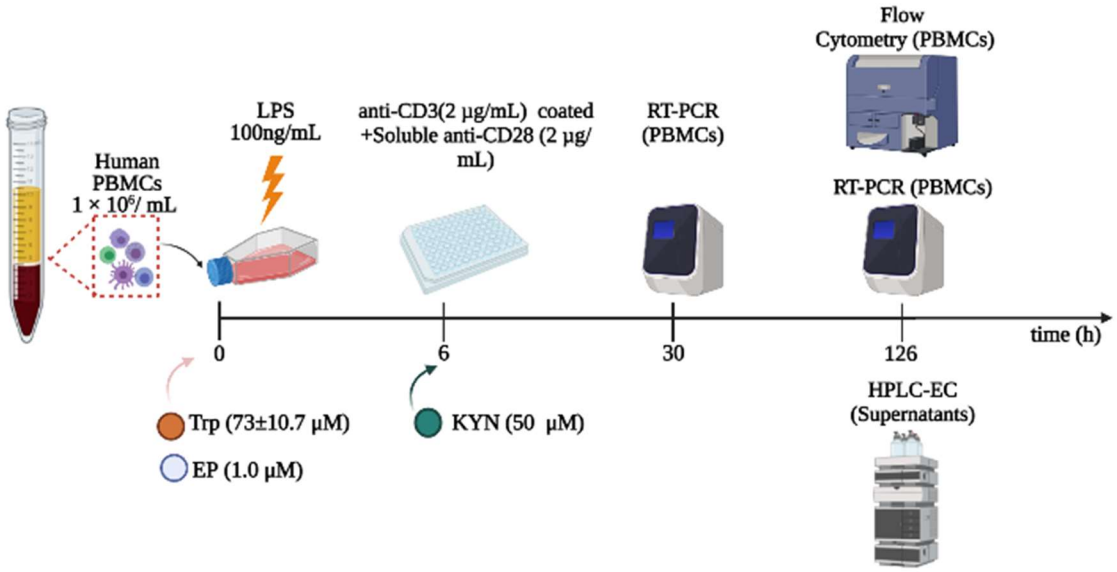

Figure 1. Schematic representation of the work timeline created in BioRender.com. Isolated peripheral blood mononuclear cells (PBMCs) were incubated with lipopolysaccharide (LPS) during 6h at 37ºC, 5%CO_2 in the presence or absence of tryptophan (Trp) and/or Epacadostat (EP). Control cells were unstimulated. The unstimulated PBMCs and the ones pre-incubated with LPS were then transferred to a 96 well plate coated with anti-CD3 (2µg/mL) whereas soluble anti-CD28 (2µg/mL) was added simultaneously in the presence or absence of Kynurenine (KYN). Real-time PCR (RT-PCR) was used to analyse IDO1 mRNA levels of PBMCs 24 or 120h after incubation with anti-CD32/CD28 at 37ºC, 5%CO_2.

After 120h of incubation at 37ºC, 5%CO_2, cells were collected to analyse lymphocytes proliferation by flow cytometry and supernatants were collected to measure Trp and KYN metabolites in PBMCs supernatants (HPLC-EC).

2.7. High-Performance Liquid Chromatography -Electrochemical (HPLC-EC) measurements of Trp and KYN

Supernatants were collected after 120h of cell culture with the last stimuli at 37 °C with 5% CO_2 and diluted (1:1) with perchloric acid ($HClO_4$) 0.2 N. Then, the solution was centrifuged at 10,000g and 4ºC. The supernatants were recovered and stored at -80ºC until quantification. The supernatants were then thawed and centrifuged at 10,000g at 4ºC immediately before the analysis. These KP metabolites (Table S3, Supplementary files) were assayed by HPLC with electrochemical detection, using a previously described method, with modifications[29]. Briefly, the HPLC system consisted of a pump (model LC10ADVp, Shimadzu, Kyoto, Japan), a Waters X-Bridge Shield C18 RP (150*4,6mm, 3,5 µm, 80A) column, an autosampler (model SIL9A, Shimadzu), and an electrochemical detector (ESA Couloarray 5800A, ESA, Bedford, MA, USA) with an analytical coulometric cell with 4 electrodes (model 6210, ESA). The chromatograms were collected, stored, and processed with a computerized integrator (ESA Coularray version 3.1, ESA, Bedford, MA, USA). The optimal composition of the mobile phase was ultrapure water/acetonitrile (90.7:9.3 v/v), 0.6264 mM octansulfonic acid, 0.349 mM Na_2EDTA and 0.049 mM potassium phosphate monobasic with an adjusted (using H_3PO_4) pH of 3.2. The freshly prepared mobile phase was filtered (GSWP 04700, 0.45 mm, Millipore, Bedford, MA) and degassed in a vacuum for 10 min. The flow rate was 0.9 ml/min. The volume of the sample injected was 30 µl. The system was running continuously with the mobile phase being recycled back into the reservoir. Trp was detected in channel 1 (600mV) and KYN was detected in channel 2 (500mV) simultaneously. Linearity and sensitivity of the method (detection limit: 0.1µM, for both metabolites) were tested by constructing standard curves using serial dilutions of the standards (Trp and KYN) and the regression coefficients (r^2) were > 0.98 (Table S2, Supplementary files). Trp and KYN were reconstituted according to the manufacturer's specifications.

2.8. Statistical analysis

Data are reported as means ± SD of the indicated number of experiments. Data came from independent experiments. Statistical analysis and graphic illustrations were performed using GraphPad Prism 8.0.1 software (San Diego, CA). Paired two-tailed Student's t-tests, ANOVA with posthoc analysis: Tukey test and Friedman test with posthoc analysis and the Dunn test were used to calculate p values. A p-value of less than 0.05 was considered significant.

3. Results

The pro-inflammatory milieu increased IDO1 expression and activity. Different stimuli were applied to the isolated PBMCs from healthy blood donors to study the impact of a pro-inflammatory environment on the IDO1 expression and activity *in vitro*, they were LPS alone, anti-CD3/CD28, and LPS combined with anti-CD3/CD28 (LPS+anti-CD3/CD28). IDO1 expression was increased at 126h in the LPS condition (2.8-fold vs. unstimulated; Figure 2D). However, the KYN and Trp levels (Figures 2 A and B) and KYN/Trp (Figure 2C) were not altered, at the same timepoint in the culture medium in the LPS condition, when compared to the unstimulated condition. On the other hand, the anti-CD3/CD28 stimuli increased the KYN levels (1.37±0.84µM vs. 4.93±1.51µM, p=0.0017; Figure 2A), decreased the Trp levels (29.5±3.28µM vs. 10.40±2.82µM, p=0.0003; Figure 2B) and increased the IDO1 activity (0.05±0.03 vs. 0.48±0.24, p=0.0858; Figure 2C) and IDO1 expression (3.1-fold; Figure 2D), when compared to the unstimulated condition. The combination of both stimuli (LPS and anti-CD3/CD28) also increased KYN levels (1.37±0.84µM vs. 6.64±2.18µM, p=0.0039; Figure 2A), decreased Trp levels (29.50±3.28µM vs. 15.80±5.93µM, p=0.0033; Figure 2B) and increased IDO1 activity (0.05±0.03 vs. 0.66±0.54, p=0.0197; Figure 2C) and IDO1 expression (3.1-fold, Figure 2D) when compared to the unstimulated condition. Moreover, KYN levels in the LPS+anti-CD3/CD28 condition were significantly higher when compared to anti-CD3/CD28 (6.64±2.18µM vs. 4.93±1.51µM, p=0.0109, t-student; Figure 2A, inset).

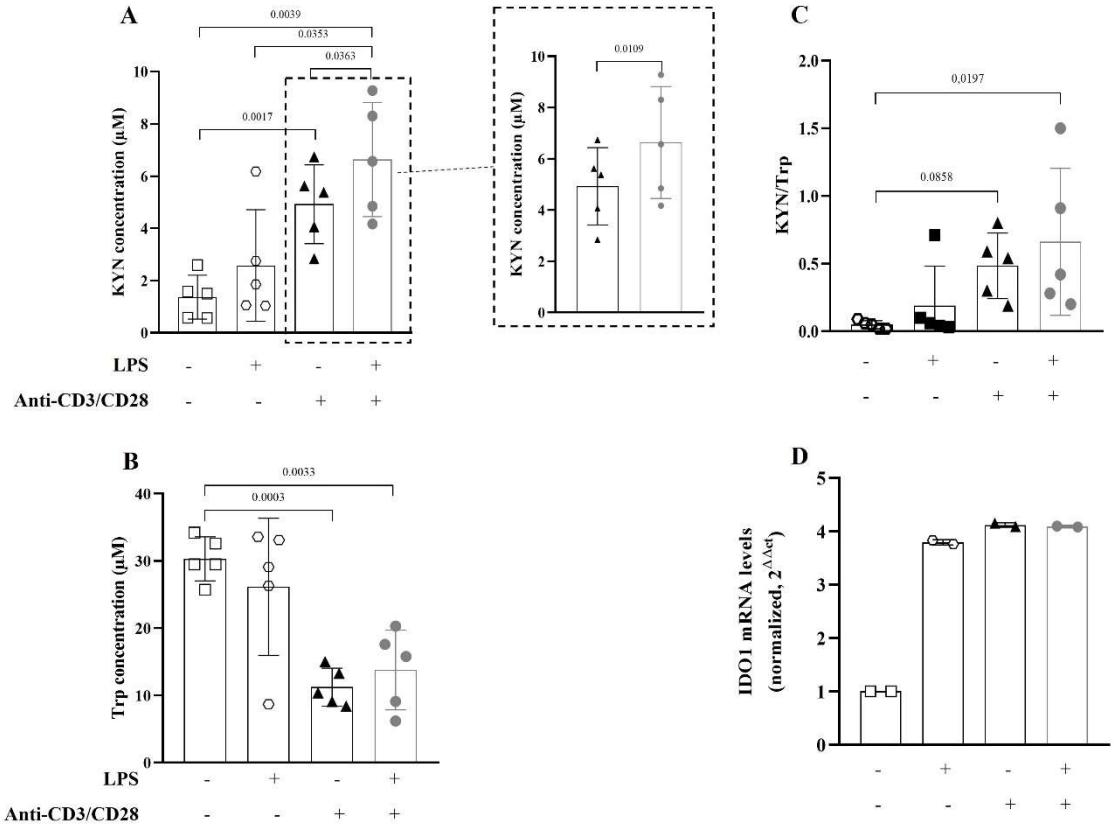

Figure 2. A pro-inflammatory milieu increased IDO1 expression and activity. Different pro-inflammatory stimuli changed IDO1 mRNA levels and activity. LPS (100 ng/mL) was applied 6h before the anti-CD3/CD28 (2µg/mL) stimuli. (A) *In vitro* kynurenine (KYN) and (B) tryptophan (Trp) concentration (µM) measured in peripheral blood mononuclear cells (PBMCs) culture supernatants (HPLC-EC). Statistics: one-way ANOVA, post-hoc analysis: Tukey test, n=5. Inset – the effect of LPS in KYN levels achieved by the anti-CD3/CD28 stimuli. Statistical analysis: t-student, n=5. (C) IDO1 activity given by kynurenine (KYN)/Tryptophan (Trp) ratio measured in supernatants of PBMCs by HPLC-EC. Statistics: Friedman test, post-hoc analysis - Dunn test, n=5. (D) normalized IDO1 mRNA levels were measured in PBMCs lysates by RT-PCR. No statistical analysis, n=2. All measurements were performed after 120h incubation of PBMCs in the presence or absence of anti-CD3/CD28 following a pre-exposure with LPS (6h) at 37ºC, 5%CO_2. IDO1 mRNA absolute values ($2^{\Delta ct}$) resting condition were $3.69x10^{-6} \pm 1.63x10^{-7}$, represented as mean±SD.

LPS attenuated anti-CD3/CD28-induced lymphocyte proliferation.

Anti-CD3/CD28, but not LPS, increased lymphocyte proliferation (51.96±10.05% vs. 1.30±0.84%, p=0.0011; Figure 3). Interestingly, pre-incubation with LPS attenuated anti-CD3/CD28-induced lymphocyte proliferation (37.46±11.20 vs. 51.96±10.05%, p=0.0327, t-student; Figure 3, inset)

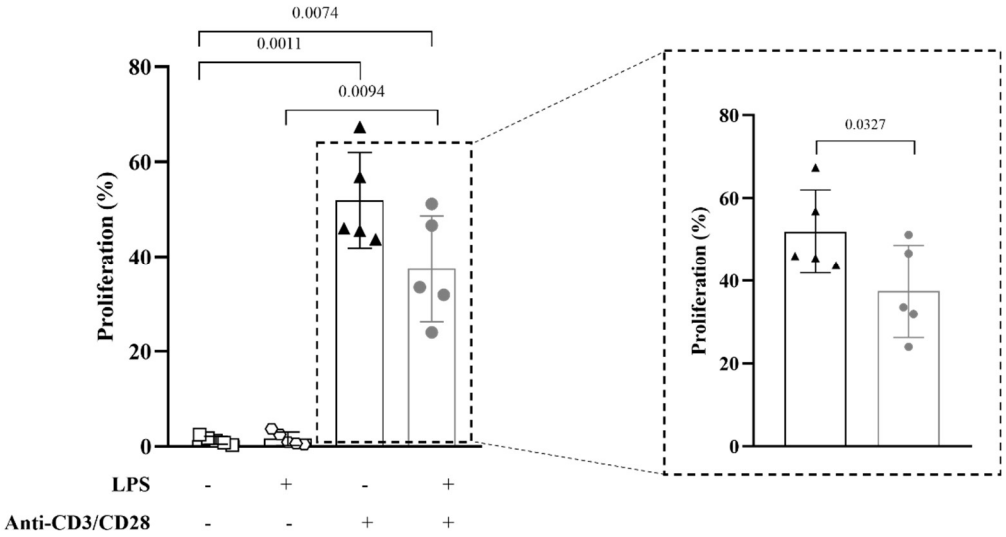

Figure 3. LPS attenuated anti-CD3/CD28-induced lymphocyte proliferation. Lymphocytes proliferation was measured by Flow cytometry after 120h incubation of PBMCs with anti-CD3/CD28 (2µg/mL) following a pre-exposure to LPS (100 ng/mL, 6h) at 37ºC, 5%CO_2. Statistical analysis: one-way ANOVA, post-hoc analysis - Tukey test; n=5. Inset – the effect of LPS in lymphocyte proliferation triggered by the anti-CD3/CD28 stimuli. Statistical analysis: t-student, n=5.

IDO1 inhibition did not change lymphocyte proliferation. We supplemented the culture medium with Trp (IDO1 substrate) reaching physiological values of 73.00±10.70 µM to increase IDO1 activity, thus feeding KP. Trp addition further attenuated lymphocyte proliferation (37.46±11.20 vs. 16.76±7.33%, p=0.0630; Figure 4D) without reaching a statistical significance. However, Trp showed

a tendency to decrease Kyn levels (Figure 4C) without IDO1 mRNA alterations (Figure 4A). Further, we used EP, which is an IDO1 catalytic inhibitor, to restore lymphocyte proliferation. In fact, EP reduced IDO1 mRNA levels (3.3-fold; Figure 4B) and KYN levels (3.80±2.49 vs. 0.68±0.11µM, p=0.0498; Figure 4C). However, the IDO1 inhibitor did not restore lymphocyte proliferation (19.94±18.56 vs. 21.08±15.42%, p=0.5942; Figure 4D).

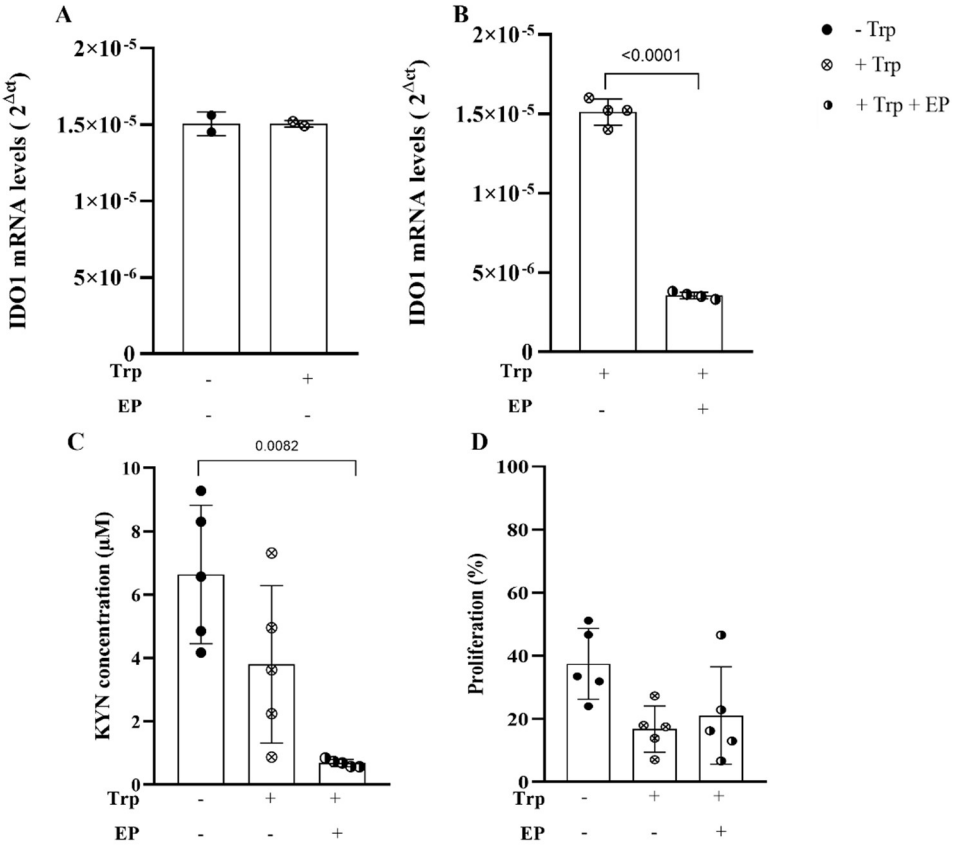

Figure 4. IDO1 inhibition did not change T lymphocyte proliferation. The pro-inflammatory milieu used was the following: pre-incubation with LPS (100 ng/mL, 6h) in combination with anti-CD3/CD28 (2µg/mL, during 120h) 37ºC, 5%CO_2. The impacts of tryptophan (Trp; 73.00±10.70 µM) and epacadostat (EP, 1µM; IDO1 inhibitor) addition are shown. (A) IDO1 mRNA levels were measured in lysates from PBMCs cultured in media Trp supplementation for 126h (RT-PCR). Data are expressed as 2Δct. No statistical analysis, n=2. (B) IDO1 mRNA levels were measured in lysates from PBMCs cultured in medium supplemented with Trp in the presence or absence of epacadostat for 30h (RT-PCR). Data are expressed as 2Δct. Statistical analysis: t-student, n=4. (C) *In vitro* kynurenine (KYN) levels (concentration, µM) were measured in PBMCs supernatants (HPLC-EC). Statistical analysis: one-way ANOVA, n=5. (D) Lymphocytes proliferation was measured by Flow Cytometry. Statistical analysis: one-way ANOVA, n=5

The addition of a higher concentration of KYN decreased lymphocyte proliferation without reaching significance. As a positive control, a higher concentration of KYN (48.95±2.51µM) was added to the cell culture in our specific pro-inflammatory milieu LPS+anti-CD3/CD28. KYN was able

to decrease the proliferation of lymphocytes further, reaching levels that were less than half of the values obtained with LPS+anti-CD3/CD28. However, statistical significance was not reached (37.46±11.20 vs. 15.21±17.31%, p=0.1250; Figure 5).

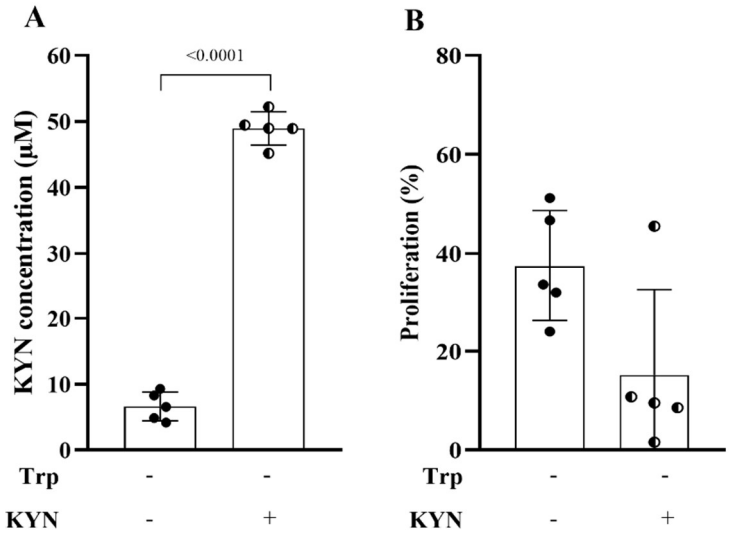

Figure 5. Addition of exogenous kynurenine (KYN) seemed to decrease lymphocyte proliferation. The pro-inflammatory milieu was set by using a pre-incubation with LPS (100 ng/mL, 6h) in combination with anti-CD3/CD28 (2µg/mL, during 120h). (A) *In vitro* KYN levels (concentration, µM) were measured in peripheral blood mononuclear cells (PBMCs) culture supernatants, after the addition of 50 µM of KYN to the culture medium (HPLC-EC); t-student, n=5. (B) the impact of the addition of 50 µM of KYN in PBMCs proliferation was measured by Flow Cytometry, t-student, n=5. Both measurements were made after 120h incubation of PBMCs with anti-CD3/CD28 following a pre-incubation with LPS (6h) at 37ºC, 5%$CO_{2.}$

4. Discussion

First we need to emphasize that the aim of the *in vitro* model was to reproduce the pro-inflammatory milieu seen in pathological contexts and which enabled us to simultaneously determine IDO1 expression (mRNA levels by RT-PCR) and activity (KYN/Trp ratio by HPLC-EC), as well as lymphocyte proliferation by flow cytometry[30].

IDO1 modulation by different pro-inflammatory stimuli. The KP imbalance that occurs in several pathologies has been associated with inflammation [31]. PBMCs, namely circulating myeloid cells, can use KP to orchestrate peripherally and central (CNS) immune responses[20]. Thus, KP has inflammatory modulation properties[32]. It has been suggested that tryptophan-derived catabolites, including KYN, are responsible for the inhibition of T-cell proliferation induced by IDO1[50]. Therefore, we used a pro-inflammatory milieu to induce IDO1 in PBMCs and to study the impact of IDO1 manipulation on

lymphocyte proliferation. Importantly, we show herein that this model is suitable to measure IDO1 expression and activity. We used double-hit stimuli that combined two pro-inflammatory stimuli: LPS (DCs and monocytes) and anti-CD3/CD28 (T cells). Innate cells, including monocytes and DCs, can be activated through pattern recognition receptors (PRRs) with pathogen-associated molecular patterns (PAMPs) such as LPS[33,34]. Furthermore, LPS is known to induce IDO1 in DCs and monocytes[34,35]. This is consistent with our flow cytometry data showing that LPS (100 ng/ml) induced IDO1 in DCs and monocytes in a time-dependent fashion, in total blood samples (Supplementary Figure S1b). We specifically showed that 34% of DCs and 43% of monocytes were $IDO1^+$ at 6h. Moreover, DCs and monocytes showed their maximum IDO1 induction at 12h (55.4 and 95%, respectively). This level of induction lasted remained up to 24h (Supplementary Figure S1b). Accordingly, our gene expression analysis showed that LPS increased IDO1 in PBMCs lysates. However, LPS failed to significantly increase KYN levels and KYN/Trp (this ratio has been validated as an *in vitro* measure of IDO1 activity for several studies)[30]. On the other hand, the activation of T cells with anti-CD3/CD28, both in the presence or absence of LPS, increased all the IDO1 parameters measured: gene expression, KYN levels, and KYN/Trp. Moreover, all experimental conditions increased IDO1 mRNA levels similarly. Importantly, our flow-cytometry data also showed that LPS, as well as anti-CD3/CD28 and the combination of both stimuli are unable to induce IDO1 in B, T, and NK cells (Supplementary Figure S2b). This clearly shows that circulating myeloid cells are the main source of IDO1 in PBMCs cultures. Therefore, this suggests that myeloid cells are the chief contributors to IDO1 data in this study. Additionally, our data suggest that T-cell activation is necessary to sustain IDO1 activity in the circulating myeloid cells in our model. This is consistent with what was reported in cell cultures with high myeloid/T cell ratios, that mimic tumor microenvironments: interferon-gamma (IFN-γ) released by activated T cells triggers the monocytes to express IDO1[36]. Indeed, we are working with PBMCs that comprise both myeloid cells and T cells. T cell activation with the eventual production of IFN-γ might interact with IDO1-expressing myeloid cells in our cultures, thus driving IDO1 induction and activity. This cell-cell interaction was also explored by Lawlor *et al*., 2020, in a holistic study of circulating immune cell responses where the authors observed that anti-CD3/CD28 activated all classes of lymphocytes either directly (T cell subset) or indirectly (B and NK cell subsets[37]). One should not forget that NK cells also release IFN-γ, thus also contributing to IDO1 modulation[38].

LPS attenuated lymphocyte proliferation. The ability of T cells to proliferate in response to antigens has been used as an indicator of the presence of antigen-specific T cells. Thus, measuring T cell functions in terms of lymphocyte proliferation is used as an immunological marker in the present work[39]. we noted that LPS attenuated lymphocyte proliferation while there were increased levels of KYN in supernatants of LPS+anti-CD3/CD28 when compared with anti-CD3/CD28 alone. The

mechanisms whereby LPS attenuated proliferation herein may rely on monocyte priming prior to the lymphoid stimulation. This may include: a reduction in the production of pro-inflammatory cytokines, an increased expression of anti-inflammatory cytokines, a decrease in the antigen-presenting capacity partly due to a reduced HLA-DR (major histocompatibility complex cell surface receptor) expression and the upregulation of numerous mechanisms that negatively regulate toll-like receptor (TLR)-associated signaling pathways[40]. Overall, this altered monocyte function can, in part, contribute to a weaker activation of the T cell response with a consequent reduction in lymphocyte proliferation.

IDO1 inhibition does not affect lymphocyte proliferation. Since the double-hit stimuli both increased IDO1 expression and changed the lymphocyte proliferation, we investigated the role of IDO1 in these proliferation changes. We decided first to feed KP by supplementing the culture medium with Trp to reach physiological values[24]. Interestingly, Trp supplementation further aggravated the effects of LPS on lymphocyte proliferation without changing IDO1 mRNA. Moreover, TRP also seemed to decrease KYN levels, without a reaching statistical significance. There are important points that need to be considered: 1) mRNA levels do not necessarily reflect IDO1 protein levels because IDO1 is also controlled by post-transcriptional mechanisms [41,42]; 2) KYN/Trp, which is a proxy for IDO1 activity, could not be compared between conditions with and without Trp supplementation, given that the initial amount of Trp is different between conditions; 3) IDO1 may be inhibited by an excess of Trp[43]. In fact, some authors have stated that Trp concentrations higher than 50 μM might inhibit the KYN/Trp ratio and IDO1 activity[30]. On the one hand, we used a Trp concentration which is *circa* 5x TRP KM (~15 μM)[44], on the other hand normal Trp plasma levels are between *circa* 46-83μM. However, others have shown that supplementing the culture medium with 100μM of Trp triggered an increase in extracellular levels of KYN in an *in vitro* increment approach[45,46], and 3) Trp could be feeding other immune modulator pathways[20,24,30,47]. For example, Trp could be feeding the serotonin pathway, and this indolamine has immunoregulatory properties by acting on serotonin receptors that are present in peripheral immune cells[48]. Nonetheless, information about the immunomodulator mechanisms of serotonin in peripheral immune cells is controversial and still scarce[49] . Finally, there is an alternative KP route that includes the enzyme interleukin 4-induced 1 (IL4I1) that degrades Trp into an indole metabolite (indole-3-pyruvic acid) which is converted into KYNA and other derivatives that activate AhR, thus exerting immune modulatory action and anti-inflammatory activity[50]. We next used IDO1 inhibition as a strategy to further understand the impact of IDO1 on lymphocyte proliferation. The EP concentration used herein corresponds to the plasmatic EP concentration seen in melanoma patients treated with EP 300–400 mg BID (metastatic melanoma trial). One should point out that this trial failed since only one third of the patients achieved 90% inhibition of IDO1 at these dose levels. It was argued that this failure was due to low levels of EP[51].

Nonetheless, we showed that EP triggered a robust decrease in IDO1 mRNA (at 24h) and KYN levels (at 120 h). EP is, usually, described as a Trp-competitive inhibitor of the catabolic activity of human IDO1[52,53]. However, we show that it also reduced IDO1 gene expression. This may have strongly contributed to the decreased levels of KYN. In addition to catalytic activity, IDO1 is also endowed with nonenzymatic functions that contribute to reprograming the immunoregulatory phenotype of immune cells[54]. We have shown that both catalytic and non-enzymatic activities were nearly abolished by EP. However, this did not have an impact on lymphocyte proliferation. On the contrary, others have suggested that EP, at concentrations lower than the one we used, have actually increased human lymphocyte proliferation[39,40]. The apparent contradictions regarding EP as well as Trp data may stem from cell culture conditions, incubation times, and stimuli which are different across studies[55,56]. Overall, our study clearly shows that IDO1 inhibition did not change lymphocyte proliferation in this *in vitro* experimental model.

Inhibition of lymphocyte proliferation is dependent on KYN concentration. Finally, we added KYN (50μM) directly to the PBMCs culture, as a positive control. KYN reached a final concentration of 47.69±4.32μM in the culture supernatants. This may reflect the exogenous addition plus the KYN released by the cells to the medium. KYN supplementation reduced lymphocyte proliferation by more than half of the values obtained with LPS+anti-CD3/CD28. However, this reduction did not reach statistical significance (all but one sample showed a robust proliferation reduction). Nonetheless, our data seems to be consistent with others showing: 1) that T cell proliferation is dose-dependently inhibited by KYN and 2) that KYN inhibits the proliferation of PBMCs at lower concentrations in the absence of Trp[27,57,58]. Others showed that KYN used at 50μM induced primary human CD8 T-cell death[22]. Therefore, one can argue that KYN can reduce T cell proliferation in a concentration-dependent manner and the KYN concentrations triggered by our double-hit protocol were not sufficient to inhibit lymphocyte proliferation.

Study limitations and future directions. It has been suggested that both gender and age (immunoaging) have an impact on the responses of the immune system in acute and chronic inflammatory diseases[59–62]. Furthermore, monocytes, one of the cell types expressing IDO1 in our experimental model, showed a sex-dependent response to LPS[63]. Generally, there is a lack of information and consensus about the role of gender in IDO1 expression. A study using in vitro PBMCs showed increased levels of basal IDO1 expression in males when compared with females[64]. On the other hand, IDO1 expression on colorectal cancer and solid tumors in humans may not be related to gender[65,66]. Interestingly, aging seems to foster the activation of the IDO1-KYN-AhR pathway which might be related to the suppression of effector immune cells and immunosenescence[67]. All together, we can conclude that sex and age might affect both the immune profile of cells and IDO1 expression.

Based on this, we consider that the lack of availability of information concerning biological sex and age regarding BC is a strong limitation of this study. We consider that, in the future, this model must also address gender and age differences, including the phase of the menstrual cycle and the use of oral contraceptives, when used in the future. Additionally, we also consider it important to explore this model in the context of different diseases, such as neurodegenerative diseases in order to explore the role of IDO1 with regard to these patients' PBMCs. Therefore, we suggest that this model is also suitable to explore other pharmacological agents with an immunomodulatory function.

5. Conclusion

The undoubted importance of KP regarding cancer, associated with the discovery the immune properties of KP metabolites has led to a growing interest in exploring KP in other diseases with inflammatory components. Several disruptions in metabolite balance have been associated with specific diseases, namely neurological ones. However, as referred to by Joisten *et al*., 2021, it has been hard to understand the physiopathological role of IDO1 immunoregulatory mechanisms: do they drive pathological states or are they compensatory mechanisms? We were able to create a pro-inflammatory environment where IDO1 is increased and could be manipulated. We observed that the changes in the lymphocyte proliferation parameter seem to be IDO1-independent. Moreover, our study also highlights the importance of further investigation of the Trp immunomodulatory IDO1-independent properties. Importantly, the suggestion that KYN impacts on proliferation in a concentration- and environment-dependent manner increases the importance of carefully analyzing KP imbalances for disease staging (diagnostics) and therapeutic purposes in the context of neurodegenerative diseases.

Data Availability Statement

All datasets generated for this study will be disclosed in response to a reasonable request.

Ethics Statement

All blood samples were collected from healthy individuals after they signed an informed consent to participate in the present study approved by the Ethical Committee of the Coimbra Hospital and University Centre (Portugal; CHUC-131-19).

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Disclosers

No conflicts of interest, financial or otherwise, are declared by the authors.

Author Contributions

Milene Gonçalves: conceptualization, methodology, validation, investigation, formal analysis, and writing of the original draft. **Alessia Furgiuele**: methodology. **Emanuela Rasini**: methodology, formal analysis. **Massimiliano Legnaro**: methodology, formal analysis. **Marco Ferrari**: methodology, formal analysis. **Paulo Rodrigues Santos**: methodology, resources, funding acquisition. **Francisco Caramelo**: formal analysis. **Franca Marino:** Writing-review and editing. **Frederico C. Pereira**: conceptualization, resources, writing-review and editing, supervision, funding acquisition. **Marco Cosentino**: resources, writing-review and editing, supervision, funding acquisition.

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1. Supplementary Files

1.1. Supplementary Methods

Two experiments were made aiming to: first - choose a time point where cells were $IDO1^+$ (Supplementary Figures S1a and S1b) and second - to prove that circulating myeloid cells are the only cells expressing IDO1 (Supplementary Figures S2a and S2b). The methods related to the first experiment are described above (1.1.1 and 1.1.2). Regarding the second experiment: PBMCs were isolated from healthy donors BC provided by the Portuguese Blood and Transplantation Institute. Isolation of PBMCs was made as described in the original paper (methods, section 2.3), LPS stimulation was performed according to the section methods (2.4.1 and 2.4.2) and immunophenotyping was performed as defined in this file (1.1.2).

1.1.1. LPS stimulation of total blood

Blood was collected from five healthy donors (age mean=55 years, 60% females) in the Institute of Immunology of the Faculty of Medicine, Coimbra University (Ethical Committee of the Coimbra Hospital and University Centre (Portugal CHUC-131-19). Total blood was incubated with RPMI 1640 medium (1:1, 500µL of total blood + 500µL of RPMI) supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and 100 U/mL penicillin/streptomycin) and stimulated with LPS (100 ng/ml) at 37 °C in a moist atmosphere of 5% CO_2 (Supplementary Figure S1a). To analyze the relative frequency of $IDO1^+$cells (DCs and monocytes) across time, we collected extracellular media at times 0, 1h, 3h, 6h, 12h, 18h, and 24h (Supplementary Figure S1a and Figure S1b) and we immunophenotyped cells as described above by flow cytometry.

1.1.2. IDO1 analysis by flow cytometry

100 uL of the well content (extracellular media) were added to 12 × 75 mm tubes and stained with the extracellular antibodies (Table S4). After 15 min of incubation in the dark at room temperature (RT), suspensions were treated with BD Cytofix/Cytoderms (Becton Dickinson, San Diego, CA, USA). First with 500 µL of permeabilization/fixation solution for 20 minutes. Second and after a spin of 5 minutes, 1500rpm, the cells were washed by adding 2mL of BD perm/wash buffer and incubated for 10 minutes at RT in the dark. After a spin of 5 minutes, 1500 rpm, we aspirated the supernatant and resuspended the cell pellets in 100 µL of BD perm/wash buffer containing the intracellular antibody IDO1 followed by an incubation of 30 minutes at RT in the dark. Cells were then washed in 2 mL of BD perm/wash buffer followed by a 5-minute spin at 1500 rpm for 5 minutes. In the end, cell suspensions were resuspended in 1 × PBS and acquired in FACS Canto II with DIVA software (Becton Dickinson, San Jose, CA, USA).

1.2. Supplementary Figures

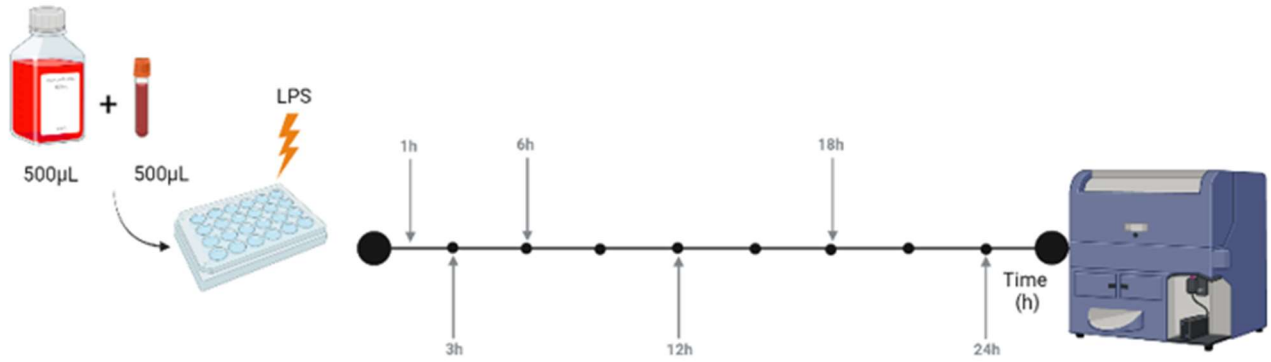

Figure S1a. Schematic representation of a time-course of peripheral blood mononuclear cells (PBMCs) exposure to LPS. Created in Biorender.com. Total blood was incubated with culture medium (1:1) and lipopolysaccharide (LPS) in a concentration of 100ng/mL across 24h at 37ºC, 5% CO_2. $IDO1^+$ cells were immunophenotyped by flow cytometry in the following time points: 0, 1, 3, 6,12,18, and 24h (used antibodies are described in supplementary table S1).

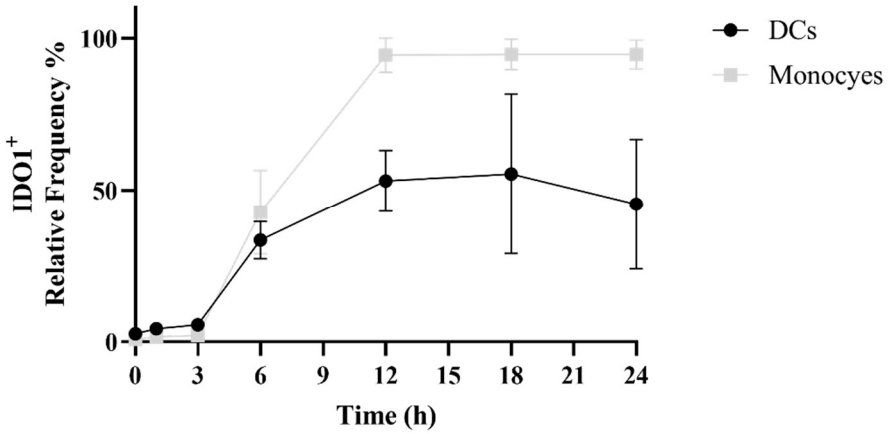

Figure S1b. $IDO1^+$ circulating myeloid cells (DCs and monocytes) relative frequency in percentage (%) across 24h. The total blood of five healthy individuals (age mean=55 years, 60% females) was incubated with culture medium (1:1) and lipopolysaccharide (LPS) in a concentration of 100ng/mL for 24h at 37ºC, 5%CO_2. IDO1+ cells were analysed by flow cytometry at the following time points: 0, 1, 3, 6,12,18, and 24h.

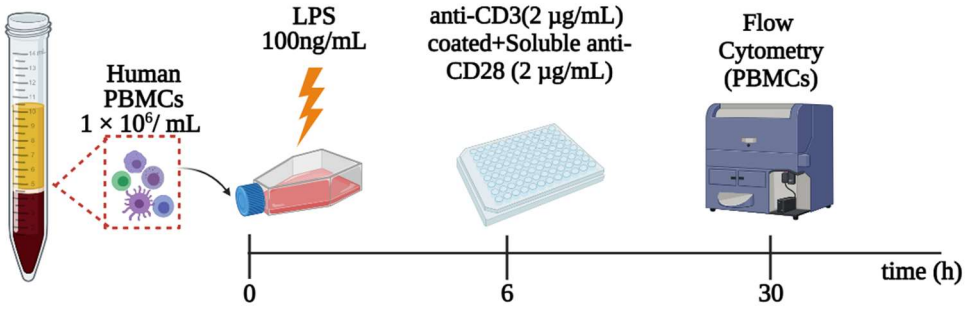

Figure S2a. Schematic representation of the timeline of the experiment 2. Created in BioRender.com**.** Isolated peripheral blood mononuclear cells PBMCs were incubated in the presence or absence of lipopolysaccharide (LPS) for 6h at 37ºC, 5%CO_2. Control cells were unstimulated. The unstimulated PBMCs and the ones pre-incubated with LPS were then transferred to a 96-well plate coated with anti-CD3 (2µg/mL) whereas soluble anti-CD28 (2µg/mL) was added simultaneously. Immunophenotyping was used to analyse IDO1 by flow cytometry 24h after the lymphoid stimulus-incubation at 37ºC, 5%CO_2.

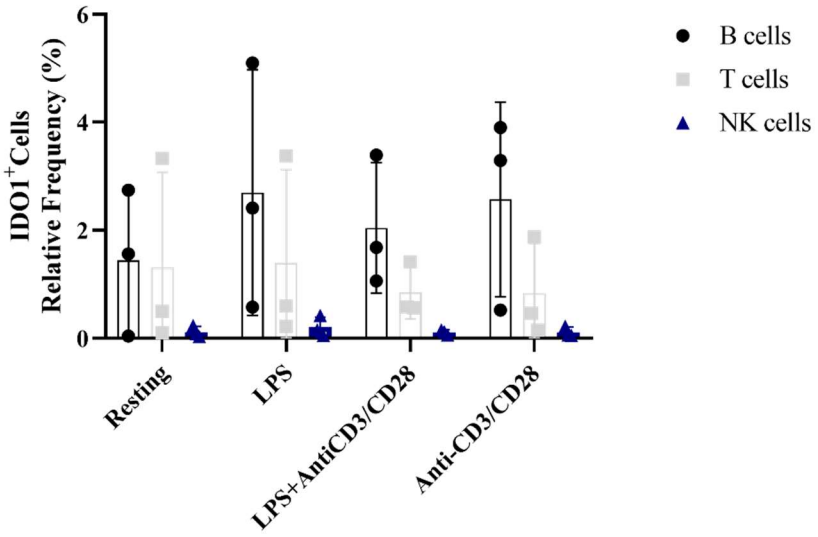

Figure S2b. Lymphocytes and NK cells do not induce IDO1. This figure shows $IDO1^+$ B cells, $IDO1^+$ T cells, and $IDO1^+$ Natural Killer (NK) cells analysed by flow cytometry in peripheral blood mononuclear cultures (PMBCs) after incubation, at 37ºC, 5%CO_2, with the following stimuli: LPS (100 ng/mL), anti-CD3/CD28 (2µg/mL) and LPS (100 ng/mL) applied 6h before the anti-CD3/CD28 (2µg/mL) stimuli. The PBMCs were isolated from 3 buffy coats of healthy donors.

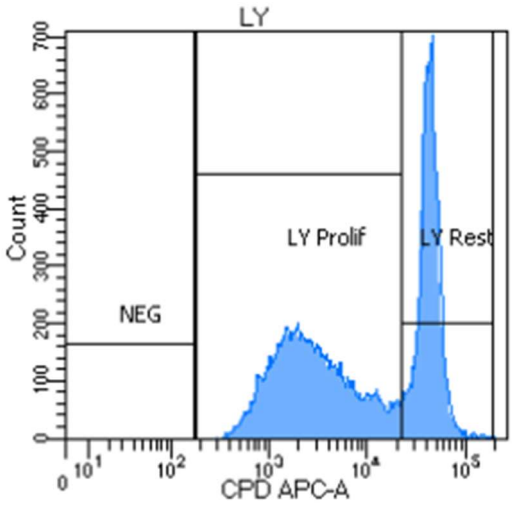

Figure S3. Lymphocyte proliferation assay. Representative figure of proliferation assay where one can see the lymphocytes on proliferation (Ly prolif) and on resting stage (Ly rest) analysed by flow cytometry.

1.3. Supplementary Tables

Table S1. Percentage of cells in flow cytometry – Percentage of cells in the gate, in resting state, and proliferation state by flow cytometry. [BC] – buffy coat

	Unstimulated			LPS			Anti-CD3/CD28			LPS+Anti-CD3/CD28		
Sample	**Cells in the gate (%)**	**Resting cells (%)**	**Proliferating cells (%)**	**Cells in the gate (%)**	**Resting cells (%)**	**Proliferating cells (%)**	**Cells in the gate (%)**	**Resting cells (%)**	**Proliferating cells (%)**	**Cells in the gate (%)**	**Resting cells (%)**	**Proliferating cells (%)**
BC1	47.8	98.1	0.3	37.4	95.6	0.4	35.7	51.7	46.1	38.6	50.6	46.7
BC2	60.7	98.3	1.2	49.2	97.9	0.9	61	31.3	67.4	52.2	46.6	51.2
BC3	55.1	97.4	1.7	51.4	98.2	0.6	55.5	55.5	43.8	50.5	65.8	33.5
BC4	72.6	98.5	0.8	69.8	96.7	2.5	68.8	54.1	45.6	60.1	75.6	24
BC5	52.2	96.9	2.5	53.7	95.5	3.7	59.7	42.8	56.9	48.7	67.6	31.9
Culture medium supplemented with Trp												
BC1	73.3	98.8	0.8	71.4	99.2	0.5	68.7	56.4	43.2	65.2	72.4	27.3
BC2	69.7	99	0.6	69.4	98.7	0.6	62.6	77.7	21.8	59.6	92.4	7.1
BC3	66	99.2	0.2	65	99.1	0.2	59.6	49.7	49.2	57.8	80.7	18
BC4	58.5	99.2	0.5	58.7	98.9	0.6	48.8	48.2	51.4	40.4	82.1	17.5
BC5	57.3	96.6	2.1	53.7	97.1	1.9	48.1	53.2	46	38.1	84.7	13.9
In the presence of Epacadostat												
BC1	56.1	99.4	0.3	49	99.4	0.1	52.4	46.1	53.1	47.5	86.3	13
BC2	72.6	99.5	0.4	75.1	99.3	0.5	71.8	63.4	36	71.6	76.5	22.9
BC3	69.5	98.8	1	71.1	98.2	1.6	63	52.3	46.8	58.9	83.2	16.2
BC4	74.2	98.4	1.3	73.9	98.5	1.2	61.4	90.4	9	66.4	92.8	6.7
BC5	66.8	96.2	3.2	72.9	95.1	4.4	67.2	39.4	59.5	65.3	52.4	46.6
Culture medium supplemented with KYN												
BC1	52	99.5	0.2	50.4	99.5	0.1	47.2	65.6	33.4	41.1	34.4	64.9
BC2	65.9	99.1	0.8	76.3	99.4	0.5	71.3	62.3	37.1	63.7	67.1	32.2
BC3	69.2	98.6	1	68.8	98.2	1.5	56.5	45.4	53.6	62.6	47.4	51.8
BC4	75.1	98.4	1.2	69.7	97.7	1.9	56.8	94.8	4.1	60.2	85.1	13.4

BC5	64.2	93.4	5.7	69	93	6.3	66.3	29.8	70	69.5	28.3	71.4

Table S2. Kynurenine Pathway metabolites HPLC-EC. [KYN – kynurenine; Trp – Tryptophan; LOD – Limit of detection; LOQ - Limit of quantification].

KP metabolite	Retention time (min)	Regression equation	Linear range (µM)	R^2	LOD (µM)	LOQ (µM)
KYN	4.97	Y = 63886902*X + 47,80	0.5-100	0.9984	0.06	0.18
Trp	10.15	Y = 146661960*X + 458,2	0.1-150	0.9778	0.25	0.75

Table S3. Kynurenine metabolites were measured by HPLC-EC under different stimuli. The table shows (1) the peak height in the HPLC-EC chromatogram calculated as the mean of two replicates; (2) the correspondent concentration (µM) calculated from the peak height; (3) the ration KYN/Trp that corresponds to IDO1 activity. [BC – buffy coat; KYN – kynurenine; Trp – Tryptophan; LPS – lipopolysaccharide].

	Unstimulated			**LPS**			**Anti-CD3/CD28**			**LPS+Anti-CD3/CD28**		
						KYN						
Sample	**Peak height**	**µM**	**Ratio (KYN/Trp)**	**Peak height**	**µM**	**Ratio (KYN/Trp)**	**Peak height**	**µM**	**Ratio (KYN/Trp)**	**Peak height**	**µM**	**Ratio (KYN/Trp)**
BC1	83.75	2.6		60.05	1.86		217.5	6.74		299.5	9.28	
BC2	18.75	0.58		33.55	1.04		131	4.06		156.5	4.85	
BC3	18.35	0.57		34.1	1.06		91.6	2.84		134.5	4.17	
BC4	51.1	1.58		199.5	6.18		181.5	5.62		268	8.3	
BC5	48.5	1.5		88.75	2.75		173.5	5.38		212	6.57	
						Trp						
BC1	2348.5	29.5	0.088	2632	33.06	0.056	667	8.38	0.8	493	6.19	1.5
BC2	2720	34.16	0.017	2673	33.57	0.031	1061	13.33	0.3	1403	17.62	0.28
BC3	2597	32.62	0.017	2314	29.06	0.036	1197.5	15.04	0.19	1619	20.33	0.2
BC4	2042.5	25.65	0.062	691.5	8.69	0.712	829	10.41	0.54	727.5	9.14	0.91

BC5	2349	29.5	0.051	2093	26.29	0.105	724	9.09	0.59	1258.5	15.81	0.42
KYN - culture medium supplemented with Trp												
BC1	ND			17.3	0.54		503	15.59		236	7.31	
BC2	ND			12.7	0.39		71	2.2		28.1	0.87	
BC3	ND			14	0.43		199.5	6.18		160	4.96	
BC4	ND			27.6	0.86		88.25	2.73		72.25	2.24	
BC5	ND			50	1.55		136.5	4.23		117	3.63	
Trp - culture medium supplemented with Trp												
BC1	9723.5	122.13	NA	9093	114.21	NA	7016.5	88.13	NA	7420	93.19	NA
BC2	9491.5	119.21	NA	8924.5	112.09	NA	7873.5	98.9	NA	7974	100.15	NA
BC3	7680.5	96.47	NA	7225.5	90.75	NA	5634.5	70.78	NA	5823.5	73.14	NA
BC4	7548	94.8	NA	6986.5	87.75	NA	5902	74.13	NA	5816.5	73.06	NA
BC5	7360	92.44	NA	6599.5	82.89	NA	5526	69.41	NA	5869	73.71	NA
KYN - addition of epacadostat												
BC 1	11.28	0.35		12.78	0.4		18.5	0.57		18.4	0.57	
BC 2	11.69	0.36		11.25	0.35		20.65	0.64		18.6	0.58	
BC 3	13.71	0.42		19.1	0.59		20.3	0.63		22.3	0.69	
BC 4	13.07	0.41		21.65	0.67		22.75	0.7		23.75	0.74	
BC 5	14.35	0.44		19.8	0.61		23.8	0.74		27.2	0.84	
Trp - addition of epacadostat												
BC 1	6336	79.58	0.004	6427	80.72	0.005	5573.5	70	0.01	5953.5	74.78	0.01
BC 2	5951.5	74.75	0.005	6335	79.57	0.004	5434.5	68.26	0.01	5949.5	74.73	0.01
BC 3	7132.5	89.58	0.005	6624	83.2	0.007	6033	75.77	0.01	6439.5	80.88	0.01
BC 4	6868.5	86.27	0.005	6684.5	83.96	0.008	5949	74.72	0.01	6402	80.41	0.01
BC 5	6741	84.67	0.005	6672	83.8	0.007	5854	73.53	0.01	6354.5	79.81	0.01
KYN - addition of KYN												
BC 1	1416	43.88	NA	1586	49.15	NA	1673	51.84	NA	1457.5	45.16	NA
BC 2	1315	40.75	NA	1563.5	48.45	NA	1644	50.94	NA	1684.5	52.2	NA
BC 3	1218	37.74	NA	1295.5	40.14	NA	1342	41.59	NA	1580	48.96	NA

BC 4	1177	36.47	NA	1366.5	42.35	NA	1585.5	49.13	NA	1579.5	48.95	NA
BC 5	1332	41.28	NA	1560.5	48.36	NA	1451	44.96	NA	1596	49.46	NA
Trp - addition of KYN												
BC 1	1994	25.04	NA	3000	37.68	NA	1617.5	20.32	NA	2156	27.08	NA
BC 2	1340	16.83	NA	853	10.71	NA	147	1.85	NA	271.5	3.41	NA
BC 3	2267.5	28.48	NA	2270.5	28.52	NA	184.5	2.32	NA	294	3.69	NA
BC 4	1821	22.87	NA	1592.5	20	NA	806	10.12	NA	1896.5	23.82	NA
BC 5	1826	22.93	NA	1484	18.64	NA	1007.5	12.65	NA	1744.5	21.91	NA

Table S4. Monoclonal antibodies used on the flow cytometry studies**.**

Specificity	Fluorochrome	Clone	Isotype	Brand
CD3	APC-H7	SK7	IgG_1	BD
CD3	V450	UCHT1	IgG_1	BD
CD4	PerCp-Cy5.5	RPA-T4	IgG_1	BD
CD8a	APC-H7	SKI	IgG_1	BD
CD11c	PE-Cy7	BLY6	IgG_1	BD
CD14	V450	MOP9	IgG_2	BD
CD16	FITC	B73.1	IgG_1	BD
CD19	FITC	HIB19	IgG_1	BD
CD19	APC-H7	SJ2501	IgG_1	BD
CD20	APC-H7	2H7	IgG_1	BD
CD56	PE	MY31	IgG_1	BD
CD123	PerCp-Cy5.5	7G3	IgG_2	BD
IDO1	AF647	V50-1886	IgG_1	BD
HLA-DR	V500	G46-6	IgG_2	BD

CHAPTER 4

Circulating myeloid cells in Parkinson's Disease patients display a pro-inflammatory phenotype

Circulating myeloid cells in Parkinson's Disease patients display a pro-inflammatory phenotype

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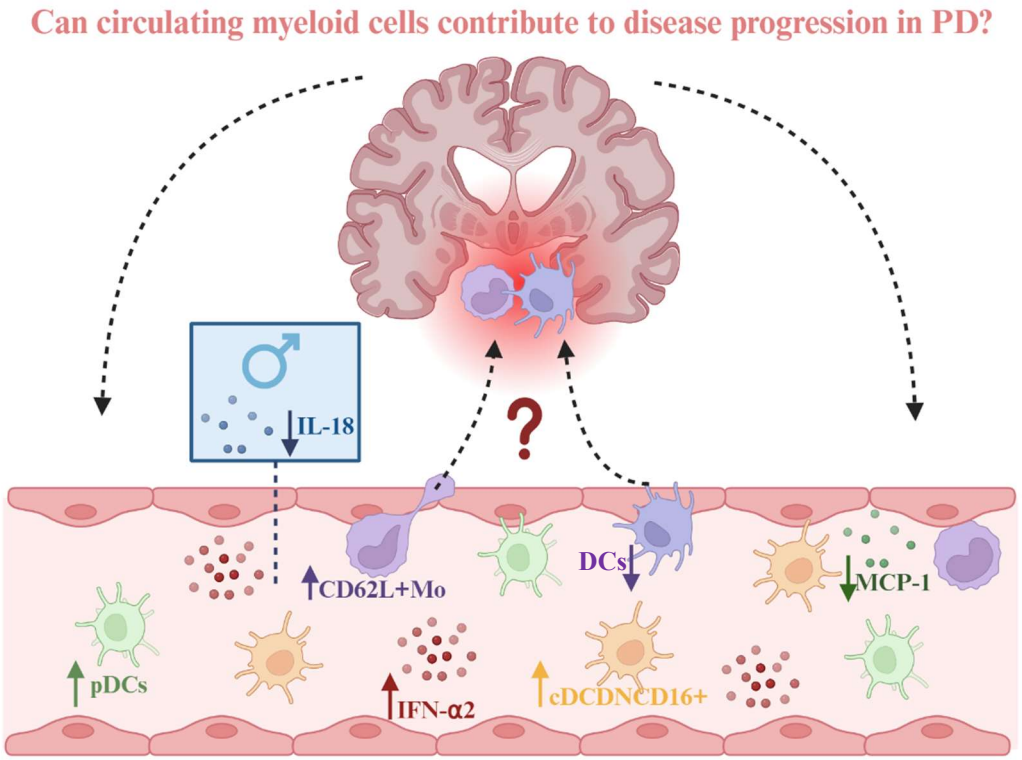

Abstract

Background: Dysfunctions in the immune system are being pointed out as a critical component of susceptibility and progression of Parkinson's disease (PD). Circulating myeloid cells such as dendritic cells (DCs) and monocytes are key players in promoting immune responses and maintaining the balance between tolerance and immunity. Immunosuppression mechanisms, including indoleamine 2, 3-dioxygenase 1 (IDO1) expression on those cells might be a target in neuroinflammatory diseases. This study aims to immunophenotype DCs and monocytes of PD patients and healthy subjects (HS), including their ability to induce IDO1.

Methods: Peripheral blood was collected from PD patients and healthy matched subjects and DCs and monocytes were immunophenotyped by flow cytometry. A bead-based immunoassay allowed the measurement of 13 cytokines simultaneously (IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL12p70, IL-17A, IL-18, IL-23, IL-33) in serum from both study groups.

Results: The population of cDCs that are negative to CD1c and CD141 (DN) and express CD16 is significantly increased in PD patients. Monocytes expressing the adhesion molecule CD62L are significantly increased in patients, and biological sex analysis showed that PD males presented higher frequencies of these CD62L-expressing monocytes when compared with healthy age-matched males. These monocytes are also moderately correlated with age in the PD group but not in the HS group. Additionally, PD patients showed significant changes in IFN-α2, MCP-1, and IL-18 (males). Furthermore, there is a moderate negative correlation between IL-8 and UPDRS III. However, $IDO1^{+}$DCs and $IDO1^{+}$monocytes from PD patients remained at low levels.

Conclusion: Collectively, the circulating myeloid cells and cytokine profile suggest that PD patients have a pro-inflammatory state that might contribute to disease progression and non- and motor symptomatology.

Keywords: Parkinson's Disease; inflammation; indoleamine 2,3-dioxygenase 1, Dendritic cells, monocytes.

1. Introduction

Parkinson's Disease (PD) is the second most common neurodegenerative disease and the neurological disorder with the fastest growing globally with profound consequences on caregivers and a huge economic burden to society[1,2]. Dopamine (DA), which is the gold standard therapeutic strategy in PD, only relieves PD symptomatology[1]. Dysfunctions in the immune system (central and peripheral) are being pointed out as a critical component of susceptibility and progression of the disease[3,4]. The neuroinflammation, which accounts for infiltrated peripheral immune cells, may create a self-sustained loop with neurodegeneration that sustains each other[5,6]. Moreover, α-synuclein (α-syn), which is dysfunctional in PD, is associated with both innate and adaptive immune responses by interacting with microglia and T cells[6]. PD is a multifactorial disease where age (and immune aging), genetic alterations, and risk factors are identified as modulators of immune function in PD[7]. For example, viral or bacterial exposure, pesticides, and alterations in gut microbiota have been implicated in the disease pathogenesis which shows us the increased need to study immune system changes in PD[8,9]. Immune system alterations seem to be present from the prodromal and early PD stages to the late stages of the disease[4]. Neurophysiological and neuroimaging studies showing activated microglia and neuroinflammation signals as well as changes in inflammation markers and immune cell populations in peripheral blood and cerebrospinal fluid (CFS) support this idea[4,10]. Circulating myeloid cells such as dendritic cells (DCs) and monocytes are key players in promoting immune responses and maintaining the balance between tolerance and immunity[11](see Chapter 2, Gonçalves *et al*., 2023a, in preparation). The expression of the enzyme indoleamine 2,3-dioxygenase 1 (IDO1) is a key mechanism involved in immunosuppression mediated by circulating myeloid cells[12] (see Chapter 3, Gonçalves *et al.,* 2023b, submitted). IDO1 is the rate-limiting enzyme of tryptophan (Trp) degradation into kynurenine (KYN) in the kynurenine pathway (KP) and its expression increases in the presence of pro-inflammatory cytokines like interferon (IFN)-γ[13]. Importantly, KP dysfunction has been associated with PD, and higher levels of neurotoxic KP metabolites in plasma and CSF (which correlated with more severe symptoms) were proposed as PD biomarkers[14]. Furthermore, dopaminergic agents (mainstay therapy) seem to modulate KP in PD[14]. Havelund *et al*., 2017 also established a correlation between L-DOPA-induced dyskinesia (LIDs) and altered KP metabolism in plasma and CSF in PD[15]. Excitingly, IDO1 has been further pointed out as a target in autoimmune and neuroinflammatory diseases[16]. Due to the multifactorial origin of PD and the presence of cofounder factors, we aim to characterize DCs and monocytes of PD patients and HS taking into consideration the following t: i) different stages of the disease might show different immune alterations[17]; ii) DA shows immunomodulatory properties which means that dopaminergic replacement therapies might

have an impact on immune function of PD patients[17]; iii) age is the major risk factor to neurodegenerative diseases and simultaneously, age modifies immune system in a phenomenon called immunoaging[4]; iv) gender has a role in immune system and concomitantly, PD is more common on men[18]. To achieve our goal, we immunophenotyped DCs and monocytes of PD patients and healthy-matched subjects, we measured inflammatory cytokines in the serum of these two groups and we correlated this information with clinical demographic and clinical information such as gender, age, Unified Parkinson's Disease Rate Scale (UPDRS)III, non-motor symptoms questionnaire (NMSQ)[19] score and L-DOPA equivalent daily dose (LEDD).

2. Material and Methods

2.1. Subjects

Peripheral blood samples were collected from PD patients and healthy age-matched subjects after signed informed consent, which was approved by the Ethical Committee of the Coimbra Hospital and University Centre (Portugal; CHUC-131-19). Patients were selected from the Movement Unit of the same hospital. Sample characterization with demographic and clinical information is described in Table 1. NMSQ was filled by all the participants of the study and is based on a validated European Portuguese version of the 'non-motor symptoms questionnaire[19] (appendix 1, Supplementary files)

Table 1 – Sample characterization. The table includes number of individuals and percentage. Parkinson's Disease (PD) patient information is described as Unified Parkinson's Disease rating scale (UPDRS) III, Non-motor symptoms questionnaire (NMSQ) score (appendix 1, supplementary files), L-DOPA equivalent daily dose (LEDD), gender and risk factors. Healthy subjects (HS) information included gender, NMQS, and risk factors.

	HS		**PD patients**	
	Number	%	Number	%
Subjects enrolled	40	100	62	100
Age, years				
Mean	62		66	
Range	47-83		43-87	
<50 years	2	5	14	23
>50 years	38	95	48	77
Gender				
Female	21	52	28	45
Male	19	48	34	55
UPDRSIII score				
Mean			19	
SD			8	
NMSQ score				

Mean	5		13	
SD	3		4	
LEDD				
Mean			493	
SD			214	
Risk factors				
Diabetes	6	15	14	23
Low Literary abilities	25	63	38	62
Smoker	6	15	9	15
Urban Environment	9	23	20	32
Exposure risks (toxins and metals)	14	35	32	52
Brain injury	3	8	4	6

2.2. Reagents

Phosphate buffer saline (PBS) was purchased by Invitrogen (Carlsbad, CA, USA). Fix and Perm A solution, 7-amino actinomycin D (7-AAD), and BD Lysing solution were from BD (Becton Dickinson, San Diego, CA, USA). Human Inflammation Panel 1(13-plex) with V-bottom Plate, LEGENDplex™ Muli-Analyte Flow Assay Kit came from Biolegend (San Diego, CA, USA). Reagent C of NK test™ was from Glycotope Biotechnology (Heidelberg, Germany). Antibodies description is on Table S1, Supplementary files.

2.3. Immunophenotyping

We collected 100 µL of total blood or up to 1×10^6 cells that were added into 12 × 75 mm tubes and stained them with the extracellular antibodies that will help to immunophenotype monocytes and DCs (Table S1, Supplementary files). After 15 min of incubation with the antibodies in the dark at room temperature (RT), suspensions were treated with Fix and Perm A solution for 10 min, also in the dark and at RT. The suspensions were then washed with 2 mL of 1 × PBS and centrifuged at 1500 rpm for 5 min. Next, cells were incubated with Fix and Perm B solution and the intracellular antibody IDO1 (Table S1, Supplementary files) for 20 min, in the dark at RT. Cell suspensions were washed twice with 2mL of 1 × PBS in the same conditions. Finally, cell suspensions were resuspended in 200µL of 1 × PBS and acquired in FACS Canto II with DIVA software (Becton Dickinson, San Jose, CA, USA).

2.4. Data analysis and representation

The FCS files from the immunophenotyping assay were analysed with FlowJo™ v10.8.1 (BD, Becton Dickinson, San Jose, CA, USA). The identification of the DCs and monocyte populations as well as IDO1^{+}cells was made by gating strategy (Supplementary Figure S1). Cells negative to the immunological markers CD3, CD19, CD20, CD14, and CD56, and positive to the HLA-DR were identified as total DCs. Inside total DCs the cells positive to CD11c were classified as myeloid DCs (mDCs) or classical DCs (cDCs) and cells positive to CD123 were considered plasmacytoid DCs (pDCs). cDCs were then divided into type 1 and type 2 cDCs (cDC1 and cDC2) according to the expression of CD141 (identify the cDC1 subpopulation) or CD1c (identify the cDC2 population). The subpopulation of cDCs negative to CD141 and CD1c was called double negative (cDCDN). This DN subpopulation was also divided into cDCDNCD16^{+} and cDCDNCD16^{-}, according to the expression of CD16. IDO1 might be identified in all these DC subsets. On the other hand, monocytes were classified according to the expression of CD14 and CD16. We identified 3 types of monocytes: classical (C-Mo, CD14^{++}CD16^{-}), intermediate (I-Mo, CD14^{+}CD16^{+}), and non-classical (NC-Mo, CD14$^{-/+}$CD16^{++}). We also analysed the expression of adhesion molecules (CD43, CD62L, CD49) and 6-sulfo LacNAc (SLAN) on total monocytes and their subpopulations. The flow cytometry standard (FCS) files acquired in cytokine assay were analysed using BioLegend's LEGENDplex™ data analysis software (biolegend.com/en-us/legendplex) online. Curve information about each cytokine is available in Table S2 (Supplementary files).

2.5. Cytokine assay

Standard and reagent preparation was made according to the manufacturer's instructions (human Inflammation Panel 1(13-plex) with V-bottom Plate, LEGENDplex™ Muli-Analyte Flow Assay Kit). This kit allows the measurement of 13 cytokines simultaneously: IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL12p70, IL-17A, IL-18, IL-23, IL-33 (Table S2). Serum was obtained after centrifugation of dry tubes at 2500 rpm for 10 minutes and if necessary diluted with the assay buffer. Protocol was performed exactly as described in protocol instructions for the V-bottom plate. Briefly, 25µL of assay buffer and 25µL of previously diluted samples or prepared calibrator were placed in the respective wells simultaneously with mixed beads and incubated in a shaker for 2h, protected from light. The plate was then centrifuged at 1050 rpm for 5 minutes and the supernatant was discarded without disturbing the pellet. Wells were washed twice with a 1x wash buffer. 25µL of detection antibodies was added to each well followed by an incubation of 1 hour at room temperature protected from light. 25 µL of SA-PE was added directly to each well without previous wash and the plate was

incubated in the same conditions for 30 minutes. A second wash was performed twice with a 1x wash buffer. Final pellets containing beads were resuspended in 150 µL of 1x wash buffer by pipetting and acquiring in FACS Canto II with DIVA software (Becton Dickinson, San Jose, CA, USA).

2.6. Statistical analysis

Data are reported as means ± standard deviation (SD) of the indicated number of experiments. The data acquired by flow cytometry is presented as relative frequency (RF, in percentage). For correlation, Spearman's rank (or Pearson) correlation coefficients were calculated by analyzing the association between immune parameters (cell populations or cytokine levels) and UPDRSIII, NMS, and LEDD. For correlation coefficients, the values 0–0.29 were indicative of weak correlation, the values 0.30–0.59 were indicative of moderate association, and the values 0.60–1.00 were considered as high association[20]. Statistical analysis and graphic illustrations were performed using GraphPad Prism 8.0.1 software (San Diego, CA). Unpaired Student's t-tests, Mann-Whitney test, One-way ANOVA with the post hoc analysis -Tukey test - and Friedman test with the post hoc analysis -Dunn test - and two-way ANOVA – with the posthoc analysis Tukey's multiple comparisons test - were used to calculate p values. p-values were corrected with the Bejamine Hochberg (q*) test. $IDO1^+$DCs and $IDO1^+$monocytes represent the DCs or monocytes expressing IDO1 (represented as RF). Table S3 (Supplementary files) shows means of both groups±SD in cell percentage or cytokine concentration, p-values, corrected p-values (q*) and delta differences (%) between PD and HS means. Images were created with Biorender.com.

3. Results

PD patients showed increased cDCDNCD16$^+$ frequencies without significant changes on IDO1. PD patients (2.34±0.92%) showed a decrease in DCs which did not reach statistical significance (12%, p=0.0255>q*) (Figure 1) when compared to HS (2.67±0.78%). We further analysed DCs subpopulations by a gating strategy (Supplementary Figure 1). We did not find differences in the RF of cDCs (PD=76.39±8.55% vs HS =79.55±5.65%, Figure 1). However, pDCs showed increased RF in PD patients when compared with HS which did not reach statistical significance (PD=6.34±3.46% vs HS =5.04±2.57%, 26%, p=0.0267>q*; Figure 1). No significant differences were found in cDCs subpopulations, including cDC1, cDC2, and cDCDN cells (Figure 1A). However, we analysed the RF of DN cells expressing CD16 (PD=78.84±21.09% vs HS =60.78±28%, Figure 1) cells and we verified that PD patients showed statistically significant higher levels of these cells expressing CD16

(cDCDNCD16^{+}; PD= vs HS, p<q*, Figure 1). We also compared IDO1-positive DC populations between groups. Although PD patients showed higher percentages of IDO1^{+}DCs (*circa* 27% increase, p=0.119>q*) this increase did not reach statistical significance in any of the DCs subpopulations (Figure 1B), IDO1^{+}cDC1 were not analysed due to the lower number of cDC1 population. Finally, IDO1^{+}pDCs were not detected in our work.

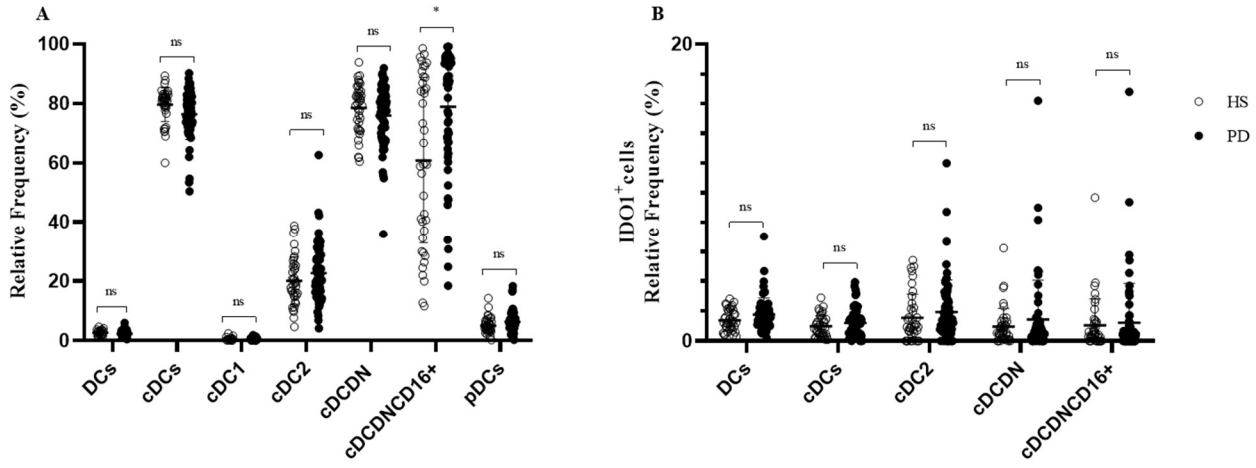

Figure 1. Relative frequency (RF) of (A) dendritic cells (DCs) and (B) indoleamine 2,3-dioxygenase 1 (IDO1)$^{+}$DCs in the blood of Parkinson's disease patients (PD, bar with black dots) and healthy subjects (HS, bar with white dots). Classical DCs (cDCs); plasmacytoid DCs (pDCs); cDCs type 1 (cDC1cDCs type 2 (cDCs2); double negative (DN) cDCs. PD with n=62 and HS with n=40. Mann-Whitney test was performed between both groups and statistical differences was accepted with corrected Bejamini Hochberg (q*). Significant results are indicated in the figure (p<q* and q*=0.0025).

PD patients showed no significant changes in monocyte populations. PD patients did not show significant changes in RF of total monocytes and monocyte subpopulations when compared with HS (Figure 2A). We also analysed the expression of molecules related to monocyte activation (CD43, CD49, CD62L, HLA-DR, SLAN) on total monocytes and monocytes subpopulations. In this analysis, we observed that PD patients had higher levels of total monocytes expressing CD62L when compared with the group HS (PD=77.21±27.37% vs HS=57.97±33.30%, p=0.005>q*; Figure 2B), which is essentially produced by C-Mo (PD=71.77±33.54% vs HS=58.93±29.14%, p=0.0115>*q; Figure 2B). The other activation-related molecules were not significantly different in any of the monocyte subpopulations between groups (Supplementary Table S3). We also aimed to compare IDO1^{+}monocyte populations between groups. There was no statistically significant difference in IDO1^{+}monocytes and their subpopulations (Figure 2C) between groups.

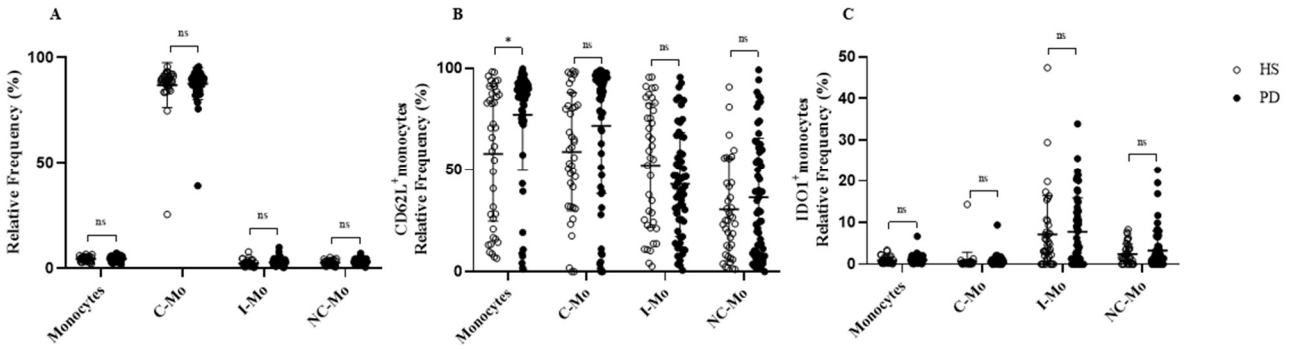

Figure 2. Relative frequency (RF) of (A) monocytes (Mo) and Mo subpopulations, (B) CD62L^{+}monocytes and (C) IDO1^{+}monocytes in the blood of Parkinson's disease patients (PD, bar with black dots) and healthy subjects (HS, bar with white dots). Total Mo – monocytes; classical Mo (C-Mo); intermediate Mo (I-Mo); non-classical Mo (NC-Mo). PD with n=62 and HS with n=40. Mann-Whitney test was performed between both groups and statistical differences was accepted with corrected Bejamini Hochberg (q*). Significant results are indicated in the figure (p<q* and q*0.0025).

PD patients showed different levels of cytokines. PD patients showed higher levels of IFN-α2 (PD=5.16±8.34 vs HS=3.28±3.93 pg/mL, p<q, 57%, Supplementary Table S3, Figure 3A) and lower levels of MCP-1 (PD=411.6±256.70 vs HS=572.3±326.50 pg/mL, q*<0.005, 28%, Supplementary Table S3, Figure 3B) in serum when compared with the HS. Additionally, IL12p70 and IL-33 showed increased levels (with high delta – from 36% to 54%, p=0.0548>q* and 0.0231>q*, respectively; Supplementary Table S3) in PD patients when compared with HS without reaching statistical significance. IL-1β, TNF-α, and IL-23 were not analysed because they were below LOD (Supplementary Table S2). Other measured cytokines also did not show statistical differences between groups (Supplementary Table S3).

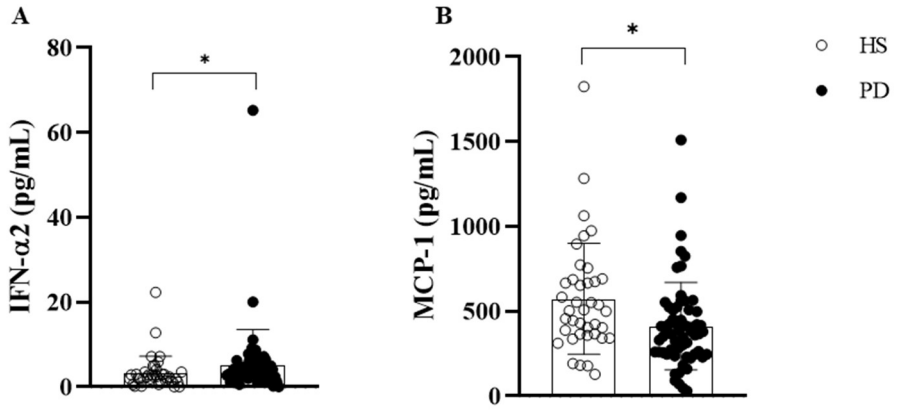

Figure 3. Circulating cytokines in Parkinson's disease patients (PD, bar with black dots) and healthy subjects (HS, bar with white dots). (A) Serum levels of interferon (IFN) alfa (α) 2 (pg/mL) and (B) monocyte chemotactic protein 1 (MCP-1). Mann-Whitney test was performed between both groups and statistical differences was accepted with corrected Bejamini Hochberg (q*). Significant results are indicated in the figure (p<q* and q*0.005).

The impact of gender on circulating myeloid profile of PD patients and age-matched HS. To analyse the impact of gender on the DCs and the monocyte profile of PD patients, we divided both groups by gender and performed a two-way ANOVA analysis. We verified that gender did not significantly impact on DCs and monocyte profile (total and subpopulations) of PD patients when compared to HS. Moreover, when analyzing all the molecules produced by these populations, including IDO1, only the RF of total monocytes expressing CD62L showed significant differences with higher levels of $CD62L^{+}$Mo in males with PD (81.56±24.48%) when compared with male HS (57.89±33.38%, p=0.0331, Figure 4A). These two groups also showed differences in IL-18, with PD males (383.4±197.1%) producing lower levels of this cytokine when compared with males from the HS group (601.8±471.7%, p=0.0360; Figure 4B).

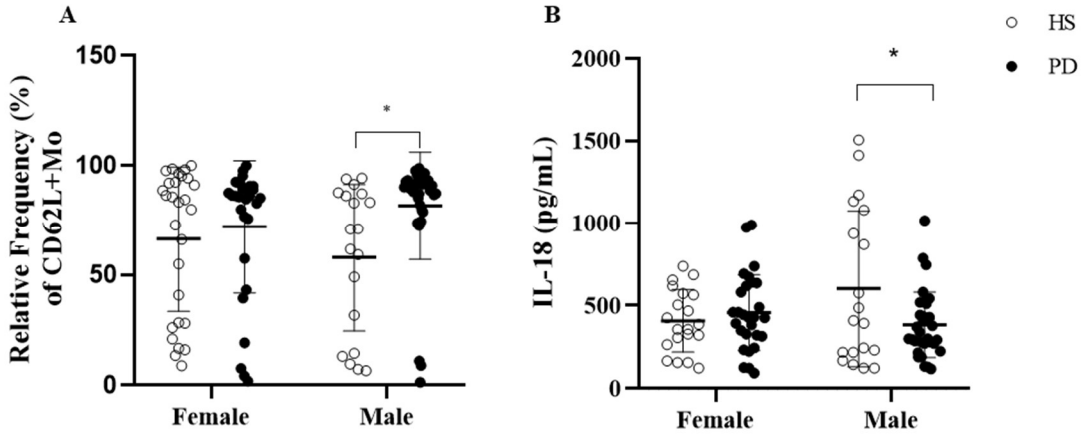

Figure 4. Impact of gender on (A) total monocytes (Mo) expressing CD62L ($CD62L^{+}$Mo) molecule and interleukin 18 (IL-18) in Parkinson's disease (PD, bar with black dots) and healthy subjects (HS bar with white dots). n=62, 28 females and 34 males and HS with n=40, 21 females and 19 males. A two-way ANOVA test was performed between groups and statistical differences were accepted with $p<0.05$.

The impact of age on circulating myeloid profile of PD patients and age-matched HS. To analyse the impact of age on DCs and monocyte profiles, we performed two analyses. First, we divided the PD group into younger individuals (before 50 years) and older individuals (more than 50 years) and second, we correlated DCs and monocyte populations with age in both groups of PD and HS. We did not divide the HS by age because we just had two HS under 50 years of age. There was no difference between the younger and older patients on DCs profile and serum cytokine levels, however, total monocytes expressing CD62L (Figure 5A), $CD62L^{+}$C-Mo (Figure 5B), and $CD62L^{+}$I-Mo (Figure 5C) were higher in PD late-onset patients ($CD62L^{+}$Mo=83.9±17.9%; $CD62L^{+}$C-Mo=79.7±26.3%; $CD62L^{+}$I-Mo=54.7±30.4%) when compared with both young onset PD ($CD62L^{+}$Mo=54.4±40.5%;

C-Mo^{+}CD62L^{+}=44.7±42.0%; I-MoCD62L^{+}C-Mo=29.3±36.5%) and HS (CD62L^{+}Mo=58.0±33.3%; CD62L^{+}C-Mo =58.9±29.1%; CD62L^{+}I-Mo= 28.7±22.9%). UPDRS is a widely applied index of PD severity. We classified disease stages (mild, moderate, severe) based on the UPDRSIII score using the criteria defined by Martinez-Martim *et al.,* 2014. Herein, the majority of patients are in the mild stage (below 33 scores) and just 7 patients are moderate (above 33 scores) and 1 patient is in the severe stage (above 58 scores). There is a positive, yet weak, correlation between age and UPDRS III (Spearman r=0.286, p=0.0283, Figure 5D). On the other hand, we established a positive, moderate, correlation between age and total monocytes expressing CD62L (Spearman r=0.333, p=0.0081, Figure 5E). Additionally, we could not find a correlation between age and CD62L^{+}monocytes in the HS.

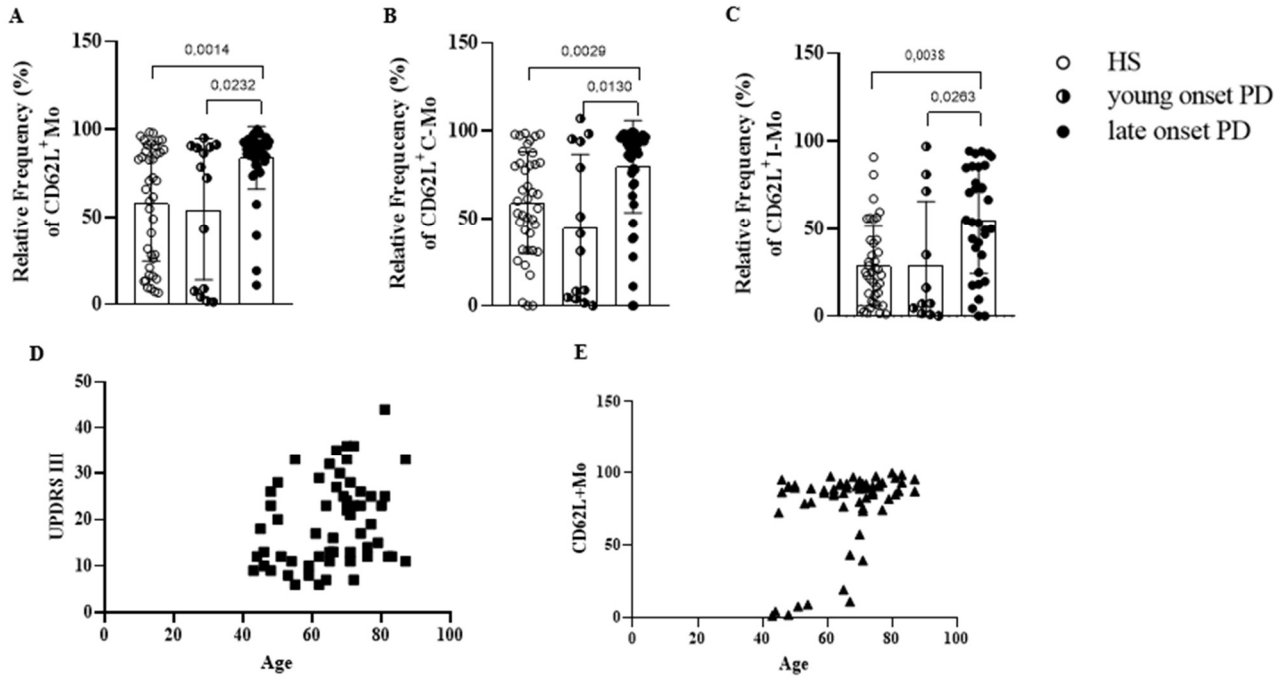

Figure 5. The impact of age on monocytes (Mo) expressing CD62L (CD62L^{+}) and UPDRSIII. (A) Relative frequency (RF) of total monocytes expressing CD62L molecule (CD62L^{+}Mo); (B) RF of Classical monocytes expressing CD62L (CD62L^{+}C-Mo); (C) RF of intermediate monocytes expressing CD62L (CD62L^{+}I-Mo); (D) Correlation of CD62L^{+}Mo with age (Kruskal-Wallis test and Spearman r (0.333, p=0.0081) and (E) Correlation of Unified Parkinson's Disease Rate Scale (UPDRS)III with age (Statistical test and Spearman r (0.286, p=0.0283). PD patients; N=62, 14 individuals <50 years (young onset PD, half black dots) and 48 individuals >50 years (late-onset PD, black dots) and HS (white dots) with n=40, 2 individuals <50 years, and 38 individuals>50 years. Statistical differences were accepted when p<0.05.

L-DOPA equivalent daily dose (LEDD) and IL-8, but not the immune profile of circulating myeloid cells, are correlated with UPDRS III in PD. Aiming to understand if the inflammatory profile of DCs in PD patients was related to motor symptoms (UPDRS III) we performed correlations between immune cells and UPDRSIII. No significant correlations were found between the cell immune profile of DCs and monocytes and UPDRSIII. We only found a moderate correlation between UPDRSIII and LEDD (Pearson r: 0.333, p=0.0108, Figure 6A), and IL-8 (Spearmen r: -0.4309, p=0.0007, Figure 6B), and age (as referred above, Figure 5D). Due to the immunomodulator role that has been given to DA, we wanted to verify if medication taken by patients could be related to immune cell profile, serum cytokine levels, and motor symptoms. With this goal, we performed different analyses: (1) we divided PD patients according to LEDD dose (< to 500 mg and >500 mg) and performed a correlation between LEDD with all cell populations and interleukins; (2) we divided PD patients according to the type of medication: (i) without medication (naïve, n=2), ii) taking levodopa/carbidopa alone (n=34), iii) taking levodopa/carbidopa plus agonists (n=15), iv) taking levodopa/benserazide alone (n=2), v) levodopa/benserazide plus agonists (n=2), and vi) taking the agonist alone (n=3). We then tried to establish a correlation between the dopaminergic medication and immune cell profile and serum cytokine levels. Since we excluded small groups (n≤3) we only considered the following groups: levodopa/carbidopa and levodopa/carbidopa plus agonists. We did not find any significant difference in this analysis.

NMSQ score is weakly correlated with different interleukins in PD. We used NMSQ based on a validated European Portuguese version of the 'non-motor symptoms questionnaire[19] (appendix 1, Supplementary files) to evaluate the NMS of PD patients. We then wanted to understand whether the DCs and monocytes' immune changes were in turn related to this NMS. We performed a correlation analysis between NMSQ score circulating myeloid cell populations and cytokine levels in PD patients. The NMSQ was significantly correlated only with cytokine profile in serum and not with cell populations. Particularly, we were able to establish a negative, yet weak, correlation between the NMSQ score and IL-6 (Spearman r=-0.254, p=0.0479, Figure 6C) in the PD group.

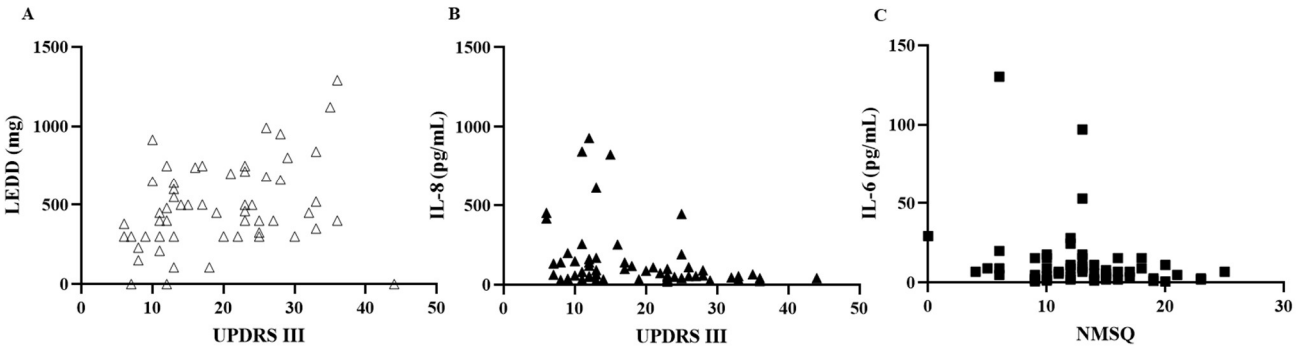

Figure 6. Unified Parkinson's Disease Rate Scale (UPDRS)III (A, B) and Non-motor Symptoms Questionnaire (NMSQ) score (C, D) correlations in Parkinson's Disease. (A) Correlation of UPDRSIII with L-DOPA equivalent daily dose (LEDD, white triangle, Pearson r=0.333, p=0.0108) and (B) with IL-8 (black triangle, Spearman r=-0.4309, p=0.0007); (C) Correlation of NMSQ with Interleukine-6 (dark square, Pearson r=-0.254, p=0.0479) in Parkinson's disease (PD) group. PD with n=62. HS with n=40. Statistical differences were accepted with $p<0.05$.

4. Discussion

The immune profile of circulating myeloid cells is changed in PD patients. Myeloid cell profiling in PD is instrumental in characterizing peripheral and maybe central immune changes and the inflammatory status in PD[21]. With this work, we characterized circulating myeloid cell profiles, including their ability to induce IDO1, a powerful immune regulator mechanism in these myeloid cells. Firstly, we disclosed that the RF of DCs in PD showed a tendency to decrease when compared with HS. These observations are aligned with Ciaramella *et al*., 2013 which showed a decreased blood DCs frequency (mainly cDCs) that was associated with motor symptoms in PD[22]. Migration to the brain as a local of the inflammation is one of the hypotheses for this blood frequency reduction[22]. Interestingly, we also found reduced levels of MCP-1, which is a chemokine that attracts DCs and monocytes. A study that analysed the MCP-1 in CSF of PD patients showed that MCP-1 is correlated with PD progression, but it did not allow to distinguish between PD and HS[23]. Furthermore, higher expression of the CCR2 and its ligand MCP-1 has been associated with PD, and polymorphisms in MCP-1 and CCR2 genes were studied as risk factors for PD with inconsistent data[24]. The kinetics of seric MCP1 may be complex and may reflect different PD stages. Our PD patients seem to be in a mild stage and at this point, MCP-1 may be lower than the HS before starting to increase. Importantly, our study adds information about cDC1 and cDC2 in PD, and no differences in these cells were seen when compared to HS. However, and accordingly with Rhodes *et al.*, 2019, we identified a population of DCs with a lack of expression of cD1c and CD141 and positive to CD16 (cDCDNCD16^{+})[25] and we found this population to be significantly increased in PD patients when compared to HS. This population seems to display a pro-inflammatory role and produce large amounts of pro-inflammatory cytokines[25]. However, the same authors also argued that there is the possibility that these cells could be a subset of CD16^{+}NC-Mo[25]. Moreover, the RF of monocytes (and C-Mo) expressing the adhesion molecule CD62L seemed to be increased in PD patients when compared to HS. CD62L or L-selectin is an important regulator of adhesion, migration, and signaling that can be produced by monocytes, especially C-Mo[26]. Importantly, L-selectine may mediate the recruitment of monocytes to endothelial monolayers, and monocytes expressing this molecule have a more invasive phenotype which means they could be recruited to infiltrate brain parenchyma[26]. From our knowledge, changes in the

expression of CD62L in monocytes were not described previously in PD patients. However, changes in PD monocytes have been already reported including high frequencies of migration (CCR2, CD11b), and phagocytic (CD163) markers[26], hyperreactivity to LPS[27], higher proliferative capacity[24], increased number of classical and non-classical monocytes in CSF[28]. On the other hand, and contrary to Ciaramella *et al*., 2013, we found a tendency towards an increase in RF of pDCs. pDCs are potent inducers of IFNs that are involved in viral responses and tumor environments[29]. Moreover, pDCs can infiltrate tumor environments which might suggest that pDCs can also migrate to the brain as the local of major infection in PD or be responsible for chronic T cell activation[29]. We did not find increased levels of IFN-γ in PD patients' serum when compared with healthy subjects, but we found increased levels of IFN-α2. We did not find a significant increase of $IDO1^+$circulating myeloid cells RF, thus $IDO1^+$DCs and $IDO1^+$monocytes remained at low levels in PD patients. Nonetheless, other authors showed changed KP metabolite levels and increased IDO1 activity by showing increased KYN/Trp ratio in PD patients[30,31]. The lack of information about KP metabolites (useful to calculate IDO1 activity) in our samples constitutes a limitation of our study. Importantly, most of the patients included in this study are in PD mild stage, and the inflammatory profile seen in our study might not be sufficient to trigger IDO1 changes in PD patients. Therefore, more severe stages might come with significant differences in IDO1 parameters. Moreover, it is also important to consider that immune changes might precede PD symptoms for several years, and changes in myeloid cells might contribute to a low chronic inflammatory state, impaired α-syn homeostasis, T cell activation, and T regulatory cell dysregulation[29].

Immune profile correlations. We then verified whether these immune changes correlated with biological factors such as gender and age, as well as motor (UPDRSIII) and non-motor (NMSQ score) symptoms. We did not find immune cells or cytokines differences between males and females. We only found that PD males showed increased monocytes expressing CD62L and decreased IL-18 when compared with male HS. This means that probably, the increased monocytes expressing CD62L that we found in our study are more accentuated in men. Oppositely, men showed decreased levels of IL-18. IL-18 is a pleiotropic pro-inflammatory cytokine that seems to be a key player in neuro-inflammation and -degeneration[32]. Pre-clinical studies showed that abolishing the IL-18 levels in mice might be neuroprotective[32]. One cannot exclude the hypothesis whereby this decrease in IL-18 might be a protective strategy. However, levels of serum IL-18 may not necessarily reflect what is happening in the PD brain. This needs to be further explored by comparing serum and CSF in PD patients. Differences between males and females were expected because of two important aspects: i) gender affects immune system functions at both levels of innate and adaptive immune responses mainly due to differential regulation of sex chromosome genes and sex hormones[23] and ii) gender differences are

found in nigrostriatal dopaminergic pathway whereas female gonadal hormones provide resilience to DA loss contrarily to male gonadal hormones; PD is more prevalent in men, which also have earlier age of onset; PD therapeutics might benefit from addressing gender differences[33]. Similarly, we expected to have an age impact in our analysis because of two main aspects: i) immune aging – it is well known that there is an age-related susceptibility to infection and impaired immune response[34], and ii) age is the major risk factor to neurodegenerative diseases[35]. In our study we found increased RF of monocytes expressing CD62L in patients with more than 50 years. Moreover, we established a positive moderate correlation between these cells and age in PD patients but not in healthy age-matched subjects. However, we saw a weak correlation between age and motor symptoms (UPDRS III). Older patients usually have more years of diagnosis, which means they have more time for disease progression, thus showing a worsening of motor symptoms. Again, future studies should consider including more patients at more elevated disease stages to help us to clarify these points. On the other hand, PD patients usually take higher LEDD, which may explain the positive, yet moderate, correlation between UPDRSIII and LEDD that we also found in this study. In turn, UPDRSIII is negatively and moderately correlated with IL-8. Considering IL-8 as a pro-inflammatory cytokine responsible for activating immune cells, we could expect a positive correlation between UPDRS III levels and this cytokine. IL-8 was also analysed in the serum of PD patients by other groups which also did not find IL-8 changes or correlations with motor abnormalites[36,37]. Interestingly, low levels of IL-8 were found in dementia with Lewy bodies a, and an IL-8 gene polymorphism (-251T>A) was associated with contributing to AD susceptibility[38,39]. Cognitive impairment in PD might be a very heterogeneous non-motor symptom from subtle changes to mild cognitive impairment[40]. All these together suggest that stratification of patients with more cognitive impairment symptoms should be done and correlated with IL-8 serum levels in future studies. On the other hand, NMSQ was negatively and weakly correlated with IL-6 in PD patients. Changes in IL-6 levels in PD patients' serum have been seen on PD and positively correlated with motor scores[36,41]. Nonetheless, a study aiming to assess whether increased levels of cytokines may indicate cognitive impairment progression in PD patients, showed that patients with increased levels of IL-6 at the baseline showed worse depression scores after 2 years of disease progression[42]. Although our study does not seem to unveil a strong contribution of cytokines to non-motor as well as motor symptoms, additional studies are warranted to further study the relevance of cytokines to these PD symptoms. Moreover, the characterization of a specific cytokine profile instead of showing discrete changes in cytokines should be more informative about a pathological state or a correlation. Although without reaching statistical significance, PD patients in general showed changed deltas (Supplementary Table S3) of these cytokines supporting a chronic inflammatory environment. Importantly, immune parameters as cytokines and even immune cell

populations have showed contradictory outcomes, which might be due to technical issues, the different immune states across disease progression, the lack of stratification of motor and non-motor symptoms and reduced numbers of individuals on the studies. Start to uniformed immune techniques might be an important point aiming to at least eliminate technical issues as a source of variability. Finally, and due to the immunomodulatory role of DA, we analysed the impact of LEDD and the type of dopaminergic medication on immune changes[43]. PD therapeutics are based on dopaminergic replacement therapies, including levodopa/benserazide, levodopa/carbidopa, and DA agonists[44]. DA agonists act on both type (1 and 2) dopamine receptors (DR) and DR are present in immune cells and its expression might change under inflammatory conditions. Moreover, carbidopa has also demonstrated to have immune properties including being an AhR agonist, thus increasing IDO1 levels[45,46]. We divided PD patients into several groups as described previously. There were two important limitations to this analysis: i) the number of individuals in each group – actually, only three groups allowed us to perform statistical analysis and ii) the lack of a robust naïve PD group constituted by patients without taking medication which are very hard to find. Some final remarks need to be pointed out. Regarding point i) the lack of differences seen in this analysis may be justified by the presence of L-DOPA in both groups and regarding point ii) usually the patients that arrive for the first time to our movement disorder appointment are taking L-DOPA which was prescribed by a private neurologist. Therefore, functional *in vitro* studies to characterize DR signaling in the circulating myeloid cells of PD patients might be more informative and are warranted in additional studies.

5. Conclusion

Our data clearly shows that circulating myeloid cells of PD patients show a pro-inflammatory phenotype. Decreased RF of DCs with increased frequencies of pDCs and DNCD16^{+} suggest a dysregulated DC profile that may impact antigen presentation and T cell response dysfunction leading to chronic inflammation. Moreover, increased frequencies of monocytes expressing CD62L were present in PD patients and might also contribute to an inflammatory status. Despite these cells not presenting significant changes in IDO1 expression, IDO1 *in vitro* functional studies will provide more information about this immune regulator mechanism. Discrete cytokine changes are also observed in PD patients when compared with healthy subjects, however, focusing on particular cytokines might be a fragile strategy in a multifactorial disease such as PD. We propose to use profiles as biomarkers, instead of discrete cytokines or cell types. Moreover, herein we are focusing on the mild PD stage. However, it is mandatory to establish immune profile in each disease stage, including more severe ones. Nonetheless, monocytes expressing CD62L were correlated with age and were higher in male

PD patients when compared with healthy age-matched males. More studies should be addressed to understand if CD62L-expressing monocytes could be a suitable marker of disease progression. Moreover, motor symptoms are positively and moderately correlated with LEDD, and understanding the role of dopaminergic agents in the immune modulation of PD immune cells could be important to optimize therapeutic strategies. Overall, an altered landscape of circulating myeloid cells is seemingly present and may contribute to deregulated immune responses and a chronic inflammatory state. This may play a role in disease progression. Anti-inflammatory strategies, including IDO1 modulation, might constitute important therapeutic strategies to control inflammation at the periphery and centrally.

Data Availability Statement

All datasets generated for this study will be disclosed in response to a reasonable request.

Ethics Statement

Total blood samples were collected from healthy individuals after signed informed consent to participate in the present study approved by the Ethical Committee of the Coimbra Hospital and University Centre (Portugal; CHUC-131-19).

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Disclosers

No conflicts of interest, financial or otherwise, are declared by the authors.

Author Contributions

Milene Gonçalves: conceptualization, methodology, validation, investigation, formal analysis, writing the original draft. **Ana Morgadinho**: selection and characterization of PD patients. **Paulo Rodrigues Santos**: methodology, resources, funding acquisition. **Jani Almeida**: methodology. **Vera Alves**: methodology. **Ivo Cruz:** methodology. **Isabel Costa**: research nurse, blood collection PD patients, and healthy subjects. **Cristina Januário**: clinical selection of PD patients, writing-review and editing. **Marco Cosentino**: writing-review and editing. **Frederico C. Pereira**: conceptualization, resources, writing-review and editing, supervision, funding acquisition.

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1. Supplementary Files

1.1. **Appendix 1:** Validated European Portuguese version of the "Non-Motor Symptoms Questionnaire. Copyright© [2021] International Parkinson and Movement Disorder Society (MDS). All rights reserved. The provisional translation was used with the permission of MDS. Silva A, *et al*. Portuguese version of the NMS-Quest for Parkinson's disease, Acta Med Port 2021 Jan;34(1):6-11

SINTOMAS NÃO-MOTORES NA DOENÇA DE PARKINSON

Os sintomas motores da Doença de Parkinson são bem conhecidos. Contudo, outros problemas ocorrem como parte da doença ou do seu tratamento. É importante que o médico tenha conhecimento sobre estes, particularmente se são incómodos para si. Abaixo encontra enumerada uma série de problemas. Por favor, preencha com uma cruz a caixa "Sim" se teve ou sentiu algum destes durante o último mês. O seu médico ou enfermeiro podem fazer-lhe algumas questões para o ajudar a decidir. Caso não tenha sentido o problema no último mês preencha uma cruz a caixa "Não". Deverá escolher "Não" se já tiver tido o problema no passado, mas não no último mês.

		Sim	Não
1.	Babar-se durante o dia	☐	☐
2.	Perda ou alteração da capacidade de saborear ou cheirar	☐	☐
3.	Dificuldade em engolir comida, beber líquidos ou engasgar-se	☐	☐
4.	Vómitos ou sensação de mal-estar (náuseas)	☐	☐
5.	Obstipação (menos de 3 dejeções numa semana) ou maior esforço para defecar (fezes)	☐	☐
6.	Incontinência intestinal (perda de fezes)	☐	☐
7.	Sensação de esvaziamento intestinal incompleto, depois de ir à casa de banho	☐	☐
8.	Sensação de urgência para urinar, que o obriga a correr até à casa de banho	☐	☐
9.	Levantar se várias vezes de noite para urinar	☐	☐
10.	Dores sem explicação (que não sejam causadas por uma doença já conhecida, como por exemplo artrite)	☐	☐
11.	Mudança no peso sem explicação (sem ter alterado a alimentação)	☐	☐
12.	Dificuldade em lembrar-se de acontecimentos recentes ou esquecer-se de fazer coisas	☐	☐
13.	Perda de interesse no que se passa à sua volta ou em fazer as coisas	☐	☐
14.	Ver ou ouvir coisas que sabe não serem reais	☐	☐
15.	Dificuldade em concentrar-se ou manter-se focado	☐	☐
16.	Sentir-se triste, em baixo ou deprimido	☐	☐
17.	Sentir-se ansioso, assustado ou em pânico	☐	☐
18.	Sentir-se menos ou mais interessado a nível sexual	☐	☐
19.	Dificuldade em ter relações sexuais quando tenta	☐	☐
20.	Sentir a cabeça vazia, tonta ou fraca, quando se põe em pé depois de estar sentado ou deitado	☐	☐
21.	Cair	☐	☐
22.	Ter dificuldade em permanecer acordado durante atividades como trabalhar, conduzir ou comer	☐	☐
23.	Ter dificuldade em dormir à noite ou em manter o sono durante a noite	☐	☐
24.	Ter sonhos muito reais, intensos ou assustadores	☐	☐
25.	Falar ou mexer-se durante o sono, como se estivesse a viver o sonho	☐	☐
26.	Sensações desagradáveis nas pernas à noite ou em repouso, com a necessidade de se mexer	☐	☐
27.	Pernas inchadas	☐	☐
28.	Suor excessivo	☐	☐
29.	Ver a dobrar	☐	☐
30.	Acreditar em coisas que estão a acontecer consigo, mas que as outras pessoas dizem não ser verdade	☐	☐

1.2. Supplementary Figures

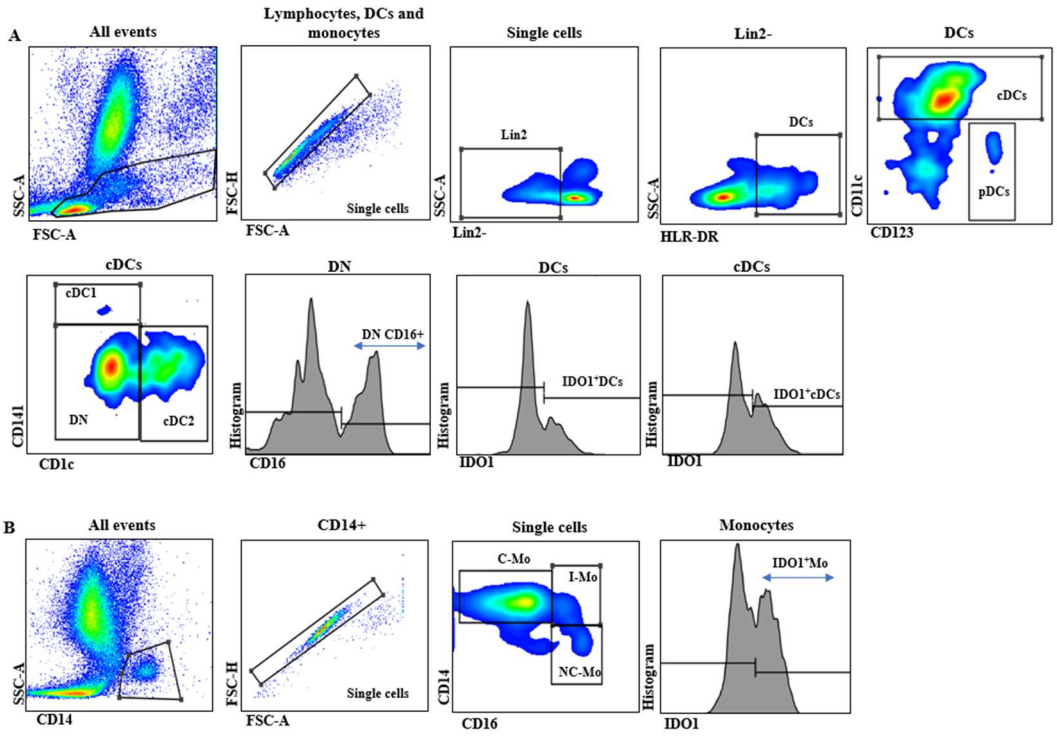

Figure S1 – Gate strategy. Schematic representation of (A) dendritic cells (DCs) and (B) monocytes (Mo) identification by gate strategy using the software FlowJo™ v10.8.1. Gates are made based on cell surface markers. Cell populations are identified based on these markers and subpopulations are gated inside total cell populations. SSC-A - Side Scatter parameter; FSC-A – Forward Scatter parameter; cDCs – clasical DCs; cDC1 – cDC type 1; cDC2 – cDC type 2; pDCs – plasmacytoid DCs; DN – Double negative; DNCD16^{+} - DN population Lin2 – Lineage 2 cocktail (CD3, CD14, CD19, CD20, CD56); HLA - human leukocyte antigen; IDO1 – Indoleamine 2,3 dioxygenase.

1.3. Supplementary Tables

Table S1. Monoclonal antibodies used on the flow cytometry studies.

Specificity	Fluorochrome	Clone	Isotype	Brand
CD1c	BV421	F10721A3	IgG_1	BD
CD3	APC-H7	SK7	IgG_1	BD
CD11c	PE-Cy7	BLY6	IgG_1	BD
CD14	V450	MOP9	IgG_2	BD
CD16	FITC	B73.1	IgG_1	BD
CD19	FITC	HIB19	IgG_1	BD
CD19	APC-H7	SJ2501	IgG_1	BD
CD20	APC-H7	2H7	IgG_1	BD
CD43	PE	EBlo84-3C1	IgG_1	eBioscience
CD49d	PerCp-Cy5.5	9F10	IgG_1	BD
CD56	PE	MY31	IgG_1	BD
CD62L	PE-Cy7	DREG-56	IgG_1	Biolegend

CD123	PerCp-Cy5.5	7G3	IgG_1	BD
CD141	PE	M80	IgG_1	BD
IDO1	AF647	V50-1886	IgG_1	BD
HLA-DR	V500	G46-6	IgG_2	BD
SLAN	FITC	DD-1	IgG_1	Milteryl
LIN2	FITC	CD3 – SK7, CD19 SJ25C1, CD20 – L27, CD14 MoP9, CD56 NCAM 16.2	IgG_1	BD

Table S2. Curve details of cytokine assay. LOD – Limit of detection (pg/mL); LOQ – limit of quantification (pg/mL); 5PL- five parameter logistic; IFN - interferon

TARGET	FIT	IC50	R^2	LOD	LOQ
IL-1B	5PL	7.9	0.999	7.342	30.787
IFN-A2	5PL	480.4	0.998	0.634	3.169
IFN-Γ	5PL	579.4	0.994	3.771	125.048
TNF-A	5PL	5.3	0.994	60.297	420.891
MCP-1	5PL	4.6	0.997	33.949	114.304
IL-6	5PL	39.8	0.998	1.203	8.243
IL-8	5PL	5.6	0.995	50.32	475.209
IL-10	5PL	2.7	0.998	3.986	27.526
IL-12P70	5PL	2.9	0.998	3.12	15.827
IL-17A	5PL	1.4	0.999	0.413	1.491
IL-18	5PL	26.1	0.998	8.447	59.44
IL-23	5PL	48.9	0.998	12.332	97.895
IL-33	5PL	158.6	0.998	77.712	368.645

Table S3. Statistics of circulating myeloid cells and cytokines.

	HS		PD		PD vs HS		
	Mean%	**SD**	**Mean %**	**SD**	**p-value**	**q***	**Delta (%)**
Cells							
DCs	2.67	0.78	2.34	0.92	0.0255	0.0100	-12.38
cDC2	20.06	8	22.62	10.33	0.2719	0.0250	12.76
DNCD16$^+$	60.78	28	78.84	21.09	0.0004	0.0025	29.71
pDCs	5.04	2.57	6.34	3.46	0.0267	0.0125	25.65
IDO1$^+$DCs	1.39	0.7	1.77	1.11	0.1109	0.0200	27.23
IDO1$^+$cDCs	0.99	0.7	1.22	0.91	0.2274	0.0225	23.04
IDO1$^+$cDC2	1.56	1.58	1.94	2.15	0.4097	0.0375	24.25
IDO1$^+$DN	0.97	1.23	1.43	2.64	0.9523	0.0500	47.45
IDO1$^+$DNCD16$^+$	1.05	1.75	1.22	2.65	0.4084	0.0350	15.56
NC-Mo	2.59	1.13	2.92	1.25	0.326	0.0325	12.81

I-Mo	2.28	1.44	2.85	1.87	0.0832	0.0175	25.08
CD62L^{+}Mo	57.97	33.3	77.21	27.37	0.005	0.0050	33.19
IDO1^{+}Mo	0.89	0.79	1.05	1.01	0.3243	0.0300	18.07
SLAN^{+}Mo	4.46	7.15	3.95	13.12	0.9289	0.0475	-11.33
CD62L-C-Mo	58.93	29.14	71.77	33.54	0.0115	0.0075	21.79
CD62L-NC-Mo	30.54	25.36	36.41	29.26	0.4174	0.0400	19.22
IDO1-NC-Mo	2.44	2.4	3.28	4.73	0.9053	0.0450	34.21
SLAN-NC-Mo	28.2	22.27	32.39	22.55	0.2763	0.0275	14.86
CD62L-I-Mo	54.69	30.42	29.3	36.52	0.0355	0.0150	-46.43
SLAN-I-Mo	36.07	28.71	32.03	25.81	0.5502	0.0425	-11.2
			Cytokines				
IFN-α2	3.284	3.93	5.156	8.34	0.0043	0.005	57
IFN-γ	6.202	8.6	9.498	24.47	0.3847	0.005	53.14
MCP-1	572.3	326.5	411.6	256.7	0.0032	0.005	-28.08
IL-6	7.759	11.06	10.91	17.76	0.1126	0.005	40.61
IL-8	1078	2289	146.9	199.5	0.6554	0.005	-86.37
IL-10	12.37	19.77	23.3	61.66	0.286	0.005	88.36
IL-12p70	4.752	7.75	6.503	11.01	0.0548	0.005	36.85
IL-17A	0.781	0.72	1.199	1.62	0.116	0.005	53.52
IL-18	502.1	364.5	416.6	212.2	0.6655	0.005	-17.03
IL-33	102.3	185.4	157.8	296.3	0.0231	0.005	54.25

CHAPTER 5

The effect of salbutamol on Indoleamine 2, 3-dioxygenase 1 induction is impaired in monocytes from Parkinson's Disease patients

The effect of salbutamol on Indoleamine 2, 3-dioxygenase 1 induction is impaired in monocytes from Parkinson's Disease patients

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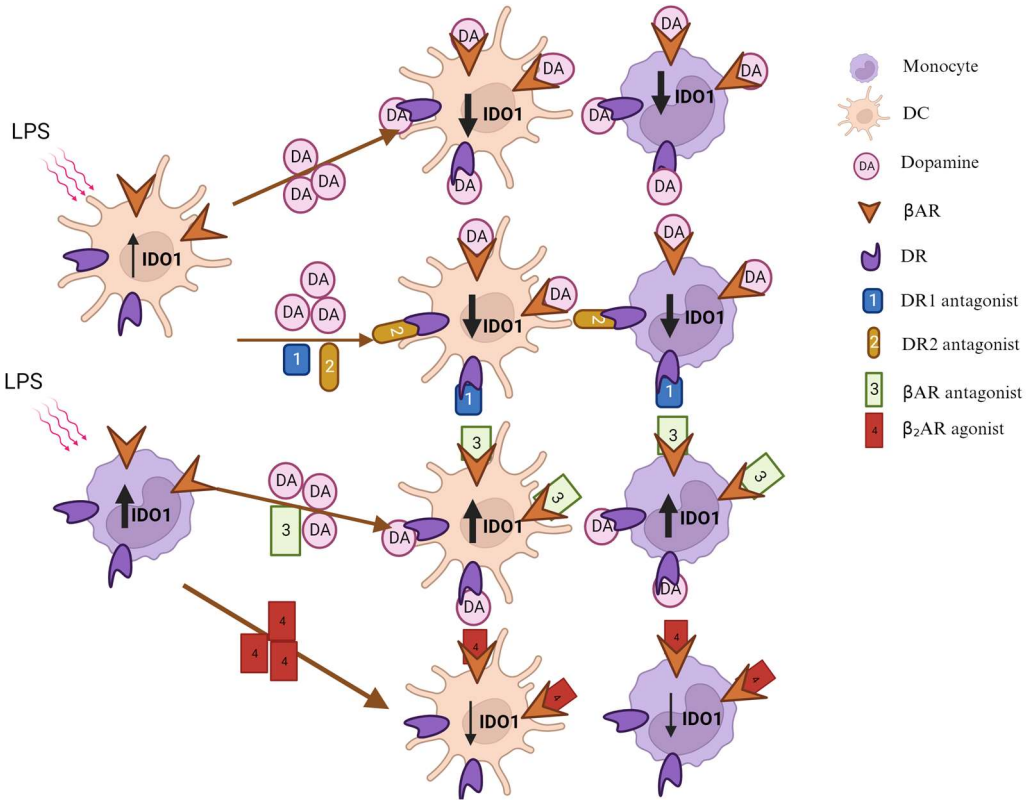

Abstract

Background: Levodopa (L-DOPA), a dopamine precursor, remains the gold standard of symptomatic treatment for Parkinson's Disease (PD). DA is a key transmitter that serves as an interface between the nervous system and the immune system. Moreover, this catecholamine is produced and released by dendritic cells (DCs) and monocytes. These cells are important immune response orchestrators that can express the enzyme indoleamine 2,3-dioxygenase 1 (IDO1), which is a key mechanism involved in immunosuppression. Our main goal is to understand the effect of DA on IDO1-expressing DCs and monocytes in PD.

Methods: Peripheral blood was stimulated with lipopolysaccharide (LPS, 1ng/mL) during 6h in the presence or absence of 100μM of DA. Pharmacological modulation of DCs and monocytes stimulated with LPS were made in the presence of (1) increasing concentrations of DA receptors (DRs) antagonists (SHC-223191 and domperidone, which are DR1-like and DR2-like antagonists, respectively); (2) the non-selective β-adrenoceptors (βAR) antagonist propranolol (20μM); (3) the β_2AR antagonist ICI118.551 hydrochloride (10μM) and (4) the β_2AR agonist salbutamol (1μM). Cells were acquired in FACS Canto II with DIVA software and supernatants were used to measure inflammatory cytokines levels.

Results: LPS was an important tool for characterizing circulating myeloid cells in PD patients, including their ability to induce IDO1. PD patients' monocytes under LPS stimulation showed a higher reduction of the relative frequency (RF) of non-classical monocytes (NC-Mo) and a stronger IDO1 induction in activated monocytes and their subpopulations in PD patients when compared with healthy subjects (HS). Importantly, DA seems to accentuate this NC-Mo reduction in PD patients. Moreover, DA also reduced IDO1 in DCs and monocytes through β_2AR. The effect of DA on cytokines suggests that it has an anti-inflammatory profile. Salbutamol seems to have a similar effect but is seemingly milder. Moreover, salbutamol showed a lower impact on PD patients' monocytes when compared with HS monocytes.

Conclusion: Our data suggest that monocytes show β_2AR signaling impairment.

Keywords: Parkinson's Disease; inflammation; indoleamine 2,3-dioxygenase 1, dendritic cells, monocytes, dopamine, β_2AR, salbutamol

1. Introduction

Increasing degeneration of the nigrostriatal dopaminergic pathway and consequent dopamine (DA) depletion in caudate putamen is a well-defined pathological characteristic of Parkinson's Disease (PD)[1]. The gold standard drug used to treat PD symptoms is levodopa (L-DOPA), a DA precursor. However, its continuous usage causes long-term complications, namely motor fluctuations, and dyskinesia[2]. DA imbalance seems to occur also at the periphery level, where the plasma DA levels are decreased in PD patients without dopaminergic substitution therapy when compared with HS[3]. DA is also a key transmitter between the nervous system and the immune system as well as a mediator produced and released by dendritic cells (DCs) and monocytes[3,4]. DA is a catecholamine that acts on DA receptors (DR) which can be divided into two families, as follows: *D1-like* receptors (D1 and D5) and *D2-like* receptors (D2, D3, and D4). The *D1-like* receptors are coupled to Gαs proteins and stimulate adenylyl cyclase (AC) with consequent increased production of cyclic AMP (cAMP) and elevated activity of protein kinase A (PKA). The *D2-like* receptors are coupled to Gi/o proteins and inhibit AC with consequent inhibition of the cAMP/PKA pathway[5]. However, in some situations and similarly to other catecholamines (norepinephrine (NE) and epinephrine (E)), DA can also activate α- and β-adrenergic receptors (αAR and βAR, respectively)[6] βAR, which are coupled to Gs proteins that stimulate the signaling pathway AC-cAMP-PKA, can be found on immune cells, including DCs and monocytes, and has an immunosuppressive action[6]. Moreover, β_2AR is the most highly and widely expressed receptor subtype in immune cells[6–9]. Importantly, catecholaminergic dysfunctions may also contribute to non-motor symptoms, including constipation, urinary dysfunction, depression, psychosis, apathy, and sleep disorders, in PD[10]. At the same time, DCs and monocytes also show the ability to induce indoleamine 2, 3 dioxygenase 1 (IDO1; this enzyme is induced by cytokines and lipopolysaccharide (LPS) and is the limiting enzyme of the kynurenine pathway (KP)[10,11] (please see Chapter 2 for further details). The expression of the IDO1 is a key mechanism involved in immunosuppression mediated by antigen-presenting cells (APCs)[12]. PD patients show both, KP and immune system (central and peripheral) dysfunctions, with the latter being considered a critical component of susceptibility and progression of PD[13–18]. We particularly showed that unstimulated DCs and monocytes are changed in PD (Chapter 4). Total DCs showed decreased RF, however with increased pro-inflammatory subpopulations, namely: plasmacytoid DCs and double negative (DN) classical DCs (cDCs) expressing CD16 are increased in PD patients when compared with healthy subjects. PD monocyte frequencies were not changed with statistical significance, however, non-classical monocytes (NC-Mo) and intermediate monocytes (I-Mo) of PD patients showed increased delta differences of *circa* 13 % and 25%, respectively, when compared with HS. Moreover, these

patients showed higher frequencies of monocytes (total, classical, and NC), expressing the adhesion molecule CD62L. Other pro-inflammatory cytokines are increased (IFNs, IL-6, IL-12p70, IL-17A, and IL-33), which may indicate immune dysfunction. However, IDO1^{+}DCs and IDO1^{+}monocytes were not significantly different between PD patients and HS. Furthermore, pharmacological manipulation of circulating myeloid cells may help to further disclose immune differences between PD patients and HS. IDO1 modulation has been pointed out as a target in autoimmune and neuroinflammatory diseases[19]. Altogether, this prompted us to first, characterize the impact of LPS on the following parameters: 1-DCs and monocytes subtypes including IDO1 induction and 2-released cytokines and second, to study the impact of catecholamine on IDO1-expressing DCs and monocytes in PD. To this end, we first set an optimal LPS stimulus (concentration and time) that would induce IDO1 on DCs and monocytes. Second, we analysed the impact of DA and salbutamol (β_2AR agonist) on LPS-induced IDO1 expression in these cells from PD patients.

2. Material and Methods

2.1. Subjects

Total blood samples were collected from PD patients and healthy age-matched individuals after signed informed consent to participate in the present study, which was approved by the Ethical Committee of the Coimbra Hospital and University Centre (Portugal; CHUC-131-19). PD patients were selected from the Movement Unit of Coimbra Hospital and University Centre and were characterized with a Unified Parkinson's Disease Rate Scale (UPDRS)III and a validated European Portuguese version of the 'non-motor symptoms questionnaire (NMSQ)[20] (appendix 1, Supplementary files, Chapter 4). The different assays employed herein used healthy subjects and PD patients with age mean, gender distribution, UPDRSIII score, and NMSQ score (if applicable) as described in Table 1. A schematic figure of the experimental design of the different experiences is represented in Supplementary Figure S1.

Table 1 – Sample characterization. The table includes the sample (number of individuals and percentage) characterization according to the experiment. Parkinson's Disease (PD) patient information is described as Unified Parkinson's Disease rating scale (UPDRS) III, Non-motor symptoms questionnaire (NMSQ) score (appendix 1, supplementary files), L-DOPA equivalent daily dose (LEDD), gender and risk factors. HS individuals' information includes gender, NMQS, and risk factors.

	Healthy subjects		**PD patients**	
	Number	%	Number	%
LPS time-course curve				

Subjects enrolled	5	100		
Age, years				
Mean	55			
Range	51-58			
Gender				
Female	3	60		
Male	2	40		
LPS concentration-response curve				
Subjects enrolled	5	100		
Age, years				
Mean	55			
Range	51-58			
Gender				
Female	3	60		
Male	2	40		
DA concentration-response curve				
Subjects enrolled	3	100		
Age, years				
Mean	58			
Range	57-58			
Gender				
Female	2	67		
Male	1	33		
The pharmacological effect of DA in PD				
Subjects enrolled	13	48	14	52
Age, years				
Mean	65		71	
Range	52-83		53-87	
Gender				
Female	5	39	8	57
Male	8	61	6	43
UPDRSIII score				
Mean			24	
SD			4	
NMSQ score				
Mean	4		14	
SD	4		4	
LEDD				
Mean	0		525	
SD			177	
Risk factors				
Diabetes	3	23	3	21
Low Literary abilities	10	77	12	86
Smoker	1	8	2	14
Urban Environment	1	8	4	29
Exposure risks (toxins and metals)	5	39	7	50
Brain injury	1	8	1	7
The pharmacological effect of DR antagonists (SHC-223191 and domperidone)				
Subjects enrolled	5	100		
Age, years				
Mean	43			

Range	32-58			
Gender				
Female	2	40		
Male	3	60		
The pharmacological effect of a non-selective βAR antagonist (propranolol)				
Subjects enrolled	5	100		
Age, years				
Mean	44			
Range	23-59			
Gender				
Female	1	20		
Male	4	80		
The pharmacological effect of a β_2AR agonist (salbutamol) and antagonist (ICI 118,551)				
Subjects enrolled	5	100		
Age, years				
Mean	44			
Range	23-59			
Gender				
Female	2	40		
Male	3	60		
The pharmacological effect of salbutamol on PD				
Subjects enrolled	5	100	6	100
Age, years				
Mean	57		67	
Range	52-63		48-87	
Gender				
Female	4	80	2	33
Male	1	20	4	67
UPDRSIII score				
Mean			11	
SD			5	
NMSQ score				
Mean	2		11	
SD	2		5	
LEDD				
Mean			569	
SD			212	
Risk factors				
Diabetes	0	0	1	17
Low Literary abilities	1	20	5	83
Smoker	1	20	0	0
Urban Environment	3	60	3	50
Exposure risks (toxins and metals)	4	80	3	50
Brain injury	0	0	1	17
Cytokine Assay				
Subjects enrolled	5	100	4	100
Age, years				
Mean	57		72	
Range	52-63		58-87	
Gender				

Female	4	80	1	25
Male	1	20	3	75
UPDRSIII score				
Mean			14	
SD			6	
NMSQ score				
Mean	2		10	
SD	2		5	
LEDD				
Mean			451	
SD			245	
Risk factors				
Diabetes	0	0	1	25
Low Literary abilities	1	20	3	75
Smoker	1	20	1	25
Urban Environment	3	60	1	25
Exposure risks (toxins and metals)	4	80	2	50
Brain injury	0	0	0	0

2.1. Reagents

RPMI 1640, heat-inactivated fetal bovine serum (FBS), glutamine, and penicillin/streptomycin were obtained from Gibco (Waltham, Massachusetts, USA). Lipopolysaccharide (LPS, Standard lipopolysaccharide from E. coli 0111:B4 strain; TLR4 ligand, tlrl-eblps) was obtained from Invivogen. DA, domperidone (D2R/D3R/D4R antagonist), propranolol hydrochloride (non-selective βAR antagonist), and Brefeldin A were purchased from Sigma-Aldrich® (St. Louis, Missouri, USA). SCH-23390 hydrochloride (D1R/D5R antagonist), ICI118.551 hydrochloride (β2AR antagonist), and salbutamol hemisulfate (β_2AR agonist) were acquired at Tocris Bioscience™ (Avonmouth, Bristol, UK). Phosphate buffer saline (PBS) was purchased from Invitrogen (Carlsbad, CA, USA). BD Cytofix/Cytoperm and 7-amino actinomycin D (7-AAD) were obtained from BD Biosciences. Human Inflammation Panel 1(13-plex) with V-bottom Plate, LEGENDplex™ Muli-Analyte Flow Assay Kit came from Biolegend (San Diego, CA, USA). Reagent C of NK test™ was obtained from Glycotope Biotechnology (Heidelberg, Germany). BD Lysing Solution was purchased from Becton Dickinson (San Jose, CA, USA).

2.2. Cell culture

Total blood was incubated with RPMI 1640 medium (1:1, 500µL of total blood + 500µL of RPMI) supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and 100 U/mL penicillin/streptomycin), and was stimulated with LPS (1 ng/ml) for 6h (at 37 °C in a moist atmosphere

of 5% CO_2. This LPS concentration and duration of incubation were set following performing an LPS time-course and an LPS concentration curve (Supplementary Figure 2S) brefeldin A at 1µg/mL was added 4h before the end of incubation aiming to measure the intracellular IDO1 expression by flow cytometry. Cytokine assay was performed without the addition of brefeldin A.

2.3. Pharmacological modulation of IDO1$^+$cells

Total blood was stimulated with LPS (1 ng/ml) at 37 °C in a moist atmosphere of 5% CO_2 for 6h in the presence or absence of several drugs according to the different experiments outlined in the experimental timeline presented in the Supplementary Figure S1. DA was added 30 minutes before LPS stimulation of cultured blood at a concentration of 100µM (concentration-response curve, Supplementary Figure S4). The DR antagonists: SHC-223191 (DR1/DR5 antagonist) and domperidone (DR2/DR3/DR4 antagonist) were added to the cell cultures at the following concentrations: 1, 10, and 100µM. The non-selective βAR antagonist propranolol, the β_2AR antagonist ICI118.551 hydrochloride, and the β_2AR agonist salbutamol were added to the cell cultures at a concentration of 20µM, 10µM, and 1µM, respectively. All the antagonists were added 30 minutes before the DA addition. The β_2AR agonist salbutamol was added 30 minutes before the LPS stimulation in the absence of DA. Concentration-response curves of propranolol, ICI118.551 hydrochloride, and salbutamol are presented in the Supplementary Figure S6).

2.4. IDO1 analysis by flow cytometry

We collected 100 µL of each well content into 12 × 75 mm tubes and stained them with the extracellular antibodies (Table S1, Supplementary files). After 15 min of incubation in the dark at room temperature (RT), suspensions were treated with Fix and Perm A solution for 10 min, also in the dark and at RT. The suspension was then washed with 2 mL of 1 × PBS and centrifuged at 1500 rpm for 5 min. Next, cells were incubated with Fix and Perm B solution and the intracellular antibody IDO1 (Table S1, Supplementary files) for 20 min, in the dark at RT. Cell suspensions were washed twice with 2mL of 1 × PBS in the same conditions. In the end, cell suspensions were resuspended in 200µL of 1 × PBS and acquired in FACS Canto II with DIVA software (Becton Dickinson, San Jose, CA, USA).

2.5. Cytokine assay

Standard and reagent preparation was made according to the manufacturer's instructions (human Inflammation Panel 1(13-plex) with V-bottom Plate, LEGENDplex™ Muli-Analyte Flow Assay Kit). This kit allows the measurement of 13 cytokines simultaneously: IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL12p70, IL-17A, IL-18, IL-23, IL-33. Supernatants were obtained after centrifugation of the culture plate at 1500 rpm for 5 minutes and if necessary diluted with the assay buffer. Protocol was performed exactly as described in protocol instructions for the V-bottom plate. Briefly, 25µL of assay buffer and 25µL of previously diluted samples or prepared calibrator were placed in the respective wells simultaneously with mixed beads and incubated in a shaker for 2h, which was protected from light. The plate was then centrifuged at 1050 rpm for 5 minutes and the supernatant was discarded without disturbing the pellet. Wells were washed twice with a 1x wash buffer. 25µL of detection antibodies were added to each well followed by an incubation of 1 hour at room temperature protected from light. 25 µL of streptavidin-phycoerythrin (SA-PE) was added directly to each well without a previous wash and the plate was incubated in the same conditions for 30 minutes. A second wash was performed twice with a 1x wash buffer. Final pellets containing beads were resuspended in 150 µL of 1x wash buffer by pipetting and acquiring in FACS Canto II with DIVA software (Becton Dickinson, San Jose, CA, USA).

2.6. Data analysis and representation

The FCS files from the immunophenotyping assay were analysed with FlowJo™ v10.8.1 (BD, Becton Dickinson, San Jose, CA, USA). The identification of the DCs and monocyte populations as well as $IDO1^{+}$cells was made by gate strategy, Supplementary Figure S1, Chapter 4). Cells negative to the immunological markers CD3, CD19, CD20, CD14, and CD56, and positive to the HLA-DR were identified as total DCs. Inside total DCs the cells positive to CD11c were classified as myeloid DCs (mDCs) or classical DCs (cDCs) and cells positive to CD123 were considered plasmacytoid DCs (pDCs). cDCs were then divided into type 1 and type 2 cDCs (cDC1 and cDC2) according to the expression of CD141 (identify the cDC1 subpopulation) or CD1c (identify the cDC2 population). The subpopulation of cDCs negative to CD141 and CD1c was called double negative (DN). This DN subpopulation was also divided into $DNCD16^{+}$ and DNCD16-, according to the expression of CD16. IDO1 might be identified in all these DC subsets. On the other hand, monocytes were classified according to the expression of CD14 and CD16. We identified 3 types of monocytes: classical (C-Mo, $CD14^{++}CD16^{-}$), intermediate (I-Mo, $CD14^{+}CD16^{+}$), and non-classical (NC-Mo, $CD14^{-/+}CD16^{++}$). We also analysed the expression of adhesion molecules (CD43, CD62L, CD49) and 6-sulfo LacNAc

(SLAN) on total monocytes and their subpopulations. The flow cytometry standard (FCS) files acquired in cytokine assay were analysed using BioLegend's LEGENDplex™ data analysis software (biolegend.com/en-us/legendplex) online. Curve information about each cytokine is available in the Supplementary Table S2.

2.7. Statistical analysis

Data are reported as means ± standard deviation (SD) of the indicated number of experiments. The data acquired by flow cytometry is presented as RF (in percentage). When normalized, data was represented as follows: 1- the conditions without LPS stimulation (dashed line) were set as 100% and considered as control (Figures 1, 2, and 3); 2- conditions with LPS stimulation (dashed line) were set as 100% and were considered as control (Figures 4, 6 and 7). Data was represented as the percentage of control in both situations. Statistical analysis and graphic illustrations were performed using GraphPad Prism 8.0.1 software (San Diego, CA). Paired two-tailed Student's t-tests, ANOVA with posthoc analysis: Tukey test and Friedman test with post hoc analysis: Dunn test, one sample t-test, and Wilcoxon signed-rank test were used to calculate p values. A p-value <0.05 was considered significant. One sample t-test and Wilcoxon test were corrected by multiplication of the p values for the number of visualizations. $IDO1^+$DCs and $IDO1^+$monocytes represent the DCs or monocytes expressing IDO1 (represented as RF or percentage of HS). Images were created with Biorender.com.

3. Results

LPS changes DCs and monocytes' immune profile in PD patients and HS. Aiming to understand if there are differences in the immune profile between activated DCs and monocytes of PD patients and HS, we incubated their total blood with LPS (1ng/mL) for 6h at 37ºC, 5%CO_2. Cells were acquired by flow cytometry and identified using the gating strategy as described in section 2.7 (Supplementary Figure S1, Chapter 4). The condition without LPS stimulation was set as 100% and data are presented as means ± SD of the percentage of the HS.

LPS effect on DCs subpopulations

LPS decreased total DCs and cDCs in both groups (Figure 1, Table S3). Although there were no statistically significant differences between PD and HS regarding pDCs, there is a statistically significant increase of circa 20% in RF of pDCs in PD group (Figure 1, Supplementary Table S3). In turn, the analysis of subpopulations of cDCs by gating strategy (Supplementary Figure S1, Chapter 4)

showed increased numbers of cDCs type 2 and decreased numbers of DN and DNCD16^{+} subpopulations, in both PD patients and HS. cDC type 1 was found in very low numbers.

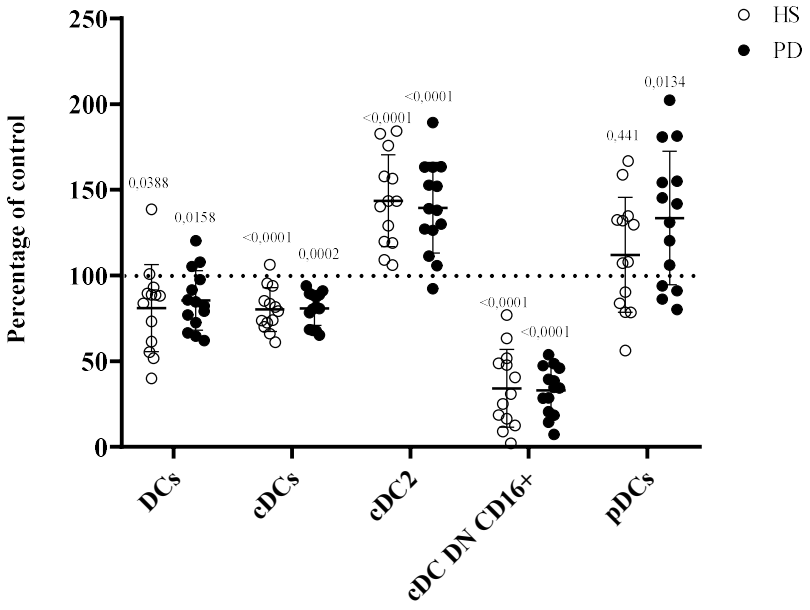

Figure 1. Lipopolysaccharide (LPS) effect on dendritic cells (DCs) subpopulations. Total blood of 13 healthy subjects (HS, with dots) and 14 PD patients (PD, black dots) were stimulated with LPS (1ng/mL) during 6h at 37ºC with 5% of CO_2. Total DCs and DCs subpopulations were identified by gate strategy on flow cytometry (as described in section 2.7). The conditions without LPS stimulation (dashed line) were set as 100% and data are presented as percentage of control. Data with a delta from a dashed line < 10% were not included in the graphs (cDC DN). Comparisons were done with 100% and the p-value was calculated using one sample t-test or one sample Wilcoxon test. p-values are indicated on the graphs and significant values were considered when $p<0.05$. Student T test and Mann-Whitney test were used to compare differences between control and PD groups. DCs – Dendritic cells, cDCs – classical DCs, pDCs – plasmacytoid DCs. DN – double negative.

LPS effect on monocyte subpopulations

The LPS induced a similar decrease in total monocytes in both groups. The impact of LPS on monocyte subpopulations in PD is the following: (1) decreased the NC-Mo subpopulation; (2) increased C-Mo subpopulation (Figure 2A) and (3) maintained I-Mo subpopulation (Table S3). There is no statistically significant difference in C-Mo and I-Mo between both groups. However, LPS triggered a delta increase of 15% in C-MO from PD patients when compared to HS. Additionally, LPS significantly reduced the RF of NC-Mo in PD patients when compared with the HS group. The expression of molecules related to monocyte adhesion or activation was also studied (CD62L, SLAN, CD43, CD49, and HLA-DR). LPS decreased, similarly, the number of total monocytes expressing CD62L in both groups (Figure 2B, Supplementary Table S3). Regarding the expression of CD62L in, LPS decreased C-Mo and I-Mo expressing CD62L and increased NC-Mo expressing CD62L, similarly, in both groups (Figure 2B,

Supplementary Table S3). About SLAN expression, LPS increased total monocytes and monocyte subpopulations expressing SLAN in both groups, in a similar fashion (Figure 2C, Supplementary Table S3). LPS also increased the cytokine levels in supernatants. Most cytokines from the panel were not detected in supernatants of resting condition (without LPS stimulation) and increased their levels in the presence of LPS (data not shown).

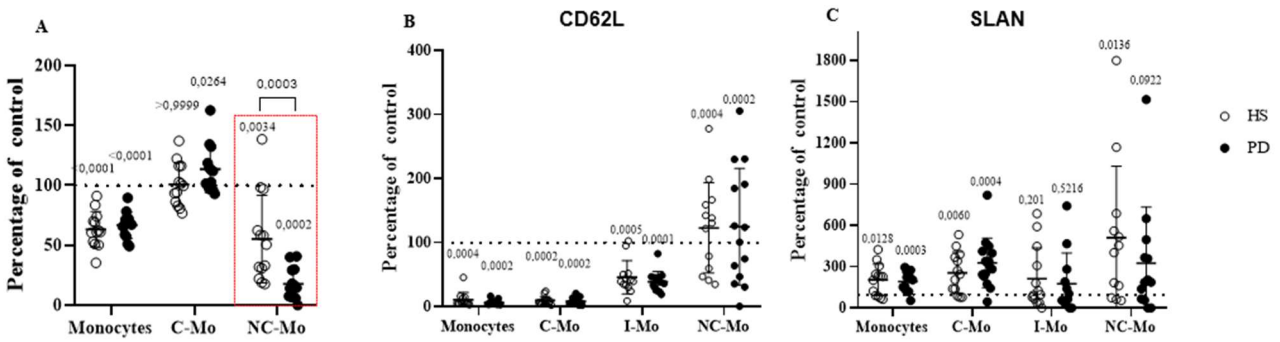

Figure 2. Lipopolysaccharide (LPS) effect on monocytes (Mo) subpopulations. Total blood of 13 healthy subjects (HS, withe dots) and 14 PD patients (PD, black dots) were stimulated with LPS (1ng/mL) during 6h at 37ºC with 5% of CO_2. The conditions without LPS stimulation (dashed line) were set as 100% and represent control. Data are presented as a percentage of control. Comparisons were done with 100% and the p-value was calculated using one sample t-test or one sample Wilcoxon test. p-values are indicated on the graphs and significant values are considered when $p<0.05$. Student T-test and Mann-Whitney test were used to compare differences between control and PD groups. C-Mo – Classical monocytes; I-Mo – Intermediate monocytes; NC-Mo – non-classical monocytes. (A) – Monocyte subpopulations; (B) expression of CD62L on monocytes subpopulations and (C) – expression of SLAN on monocytes subpopulations.

IDO1 induction in activated monocytes is higher in PD patients compared to HS. The IDO1 induced by LPS is increased in DCs, cDCs, and cDCs subpopulations without reaching statistical differences between experimental groups. However, LPS triggered a *circa* 30% increase in the frequencies of $IDO1^+$DCs, $IDO1^+$cDCs, and $IDO1^+DNCD16^+$ in the HS group compared to PD patients (Figure 3A, Table S3). Moreover, $IDO1^+$DN cells showed a higher increase in HS when compared to PD (61%). The numbers of cDC1 were very low and $IDO1^+$cDC1 were not analysed. pDCs do not induce IDO1 under our experimental conditions. On the other hand, the number of total monocytes expressing IDO1 in PD patients is higher than in the HS group under LPS stimulation (Figure 3B, Supplementary Table S3). Consistently, the number of $IDO1^+$C-Mo, $IDO1^+$I-Mo, and $IDO1^+$NC-Mo in PD patients is higher than in the HS group [all monocytes' subpopulations showed statistical significance ($p<0.05$), except C-Mo (67% increase, $p>0.05$)].

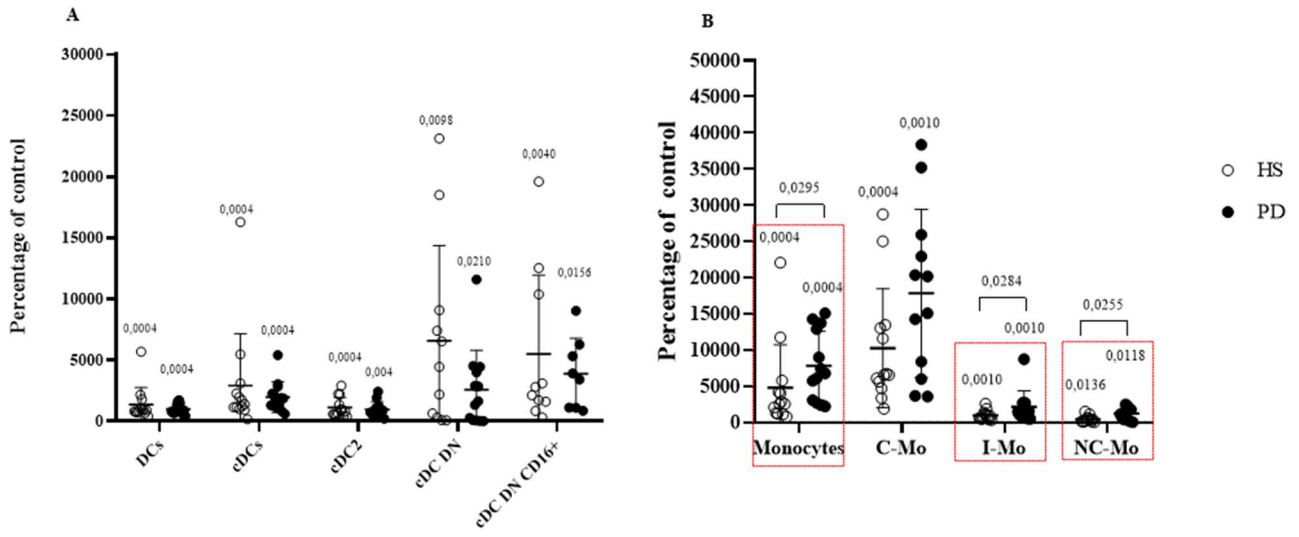

Figure 3. Lipopolysaccharide (LPS) effect on (A) Dendritic cells (DCs) and (B) monocytes (Mo) induced indoleamine 2,3-dioxygenase 1 (IDO1). The total blood of 13 healthy subjects (HS, with dots) and 14 PD patients (PD, black dots) were stimulated with LPS (1ng/mL) during 6h at 37ºC with 5% of CO_2. LPS can induce IDO1 on DCs and Mo. Conditions without LPS stimulation (not shown in the figure) were set as 100% and were considered as control. Data are presented as a percentage of control. Comparisons were done with 100% and the p-value was calculated using one sample t-test or one sample Wilcoxon test. p-values are indicated on the graphs and significant values are considered when $p<0.05$. Student T-test and Mann-Whitney test were used to compare differences between control and PD groups. cDCs – classical DCs; DN – double negative; C-Mo – classical monocytes; I-Mo – intermediate monocytes; NC-Mo – non-classical monocytes.

The impact of DA on activated IDO1$^+$DCs and IDO1$^+$monocytes is not altered in PD patients. To analyse the effect of DA in LPS-activated IDO1$^+$DCs and IDO1$^+$monocytes in PD patients and healthy subjects, we first performed a DA concentration-response curve in activated IDO1$^+$DCs and IDO1$^+$monocytes (Supplementary Figure S4). To this end, we added increasing concentrations of DA (0.01, 0.1, 1, 10, 100 µM) to the total blood cell culture media 30 minutes before LPS stimulation (1ng/mL, 6h). DA significantly decreased IDO1$^+$DCs and IDO1$^+$monocytes at a concentration of 10µM. At 100µM, both cell populations showed a reduction in IDO1 induction by more than half (Supplementary Figure S4). The RF of dead cells (Supplementary figure S5) incubated with higher concentrations of DA is not significantly different from the condition without DA. The DA concentration that was used thereafter was 100 µM. Importantly, this effect of DA on IDO1 was also verified in DCs (Figure 4A) and monocytes (Figure 4B) from PD patients. DA decreased total IDO1$^+$DCs and their subpopulations as well as IDO1$^+$monocytes and their subpopulations of IDO1$^+$C-Mo and IDO1$^+$I-Mo in both groups. On the contrary, DA showed a tendency to increase the RF of IDO1$^+$NC-Mo cells in both groups. Additionally, PD patients and HS were not significantly different ($p>0.05$).

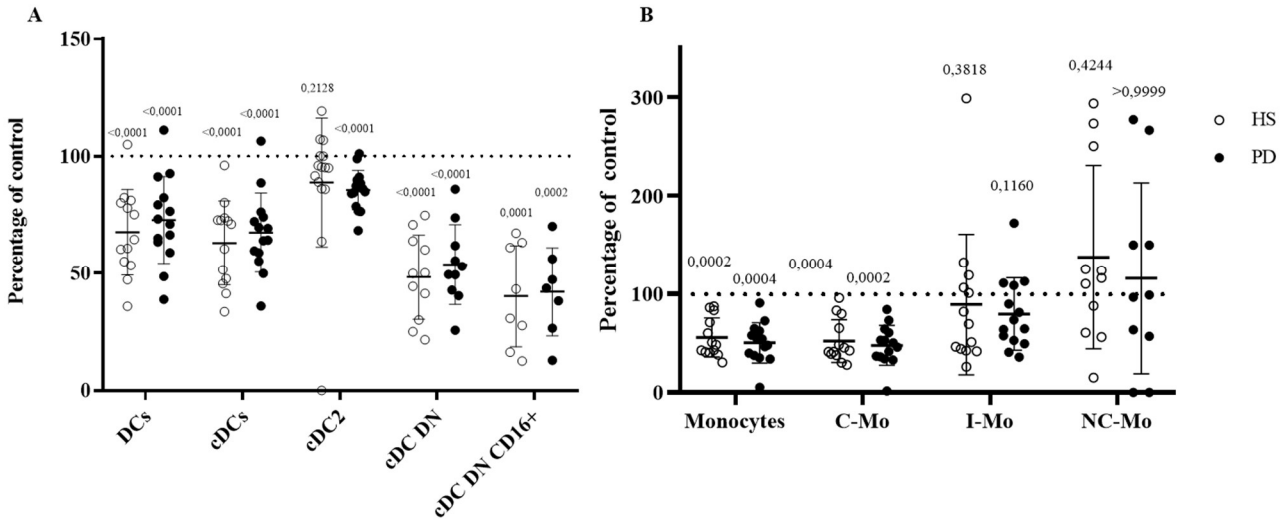

Figure 4. Dopamine (DA) effect on IDO^{+}1 DCs (A) and IDO1^{+}monocytes (B) stimulated with lipopolysaccharide (LPS). Total blood of 13 healthy subjects (HS, with dots) and 14 PD patients (PD, black dots) were stimulated with LPS (1ng/mL) in the presence or absence of DA (100µM) during 6h at 37ºC with 5% of CO_2. Conditions with LPS stimulation (dashed line) were set as 100% and were considered as control. Data are presented as a percentage of control. Comparisons were done with 100% and the p-value was calculated using one sample t-test or one sample Wilcoxon test. p-values are indicated on the graphs and significant values are considered when $p<0.05$. Student T-test and Mann-Whitney test were used to compare control and PD groups. DCs – dendritic cells; DN –double negative; C-Mo – classical monocytes; I-Mo - intermediate monocytes; NC-Mo – non-classical monocytes.

Furthermore, we analysed the impact of DA on the RF of DC cells and monocytes activated by LPS. Regarding DCs, DA further accentuated the effect of LPS by reducing even more the DCs in PD patients. However, no effect of DA was verified in total DCs of HS (PD vs HS with p=0.0291 with a delta difference of circa 14%, Supplementary Figure 7A). Oppositely, DA significantly increased the RF of cDC DN expressing CD16^{+} in circa 20% in both groups (Supplementary Figure 7A). It is also noteworthy to see that DA seems to have opposite effects in LPS-activated NC-Mo from patients and HS: it further decreases its frequency in PD patients but tends to increase it in HS (Supplementary Figure 7B). However, both groups are not significantly different. Considering that LPS induced changes in the expression of CD62L and SLAN, we analysed whether DA had any effect on these two molecules (Supplementary Figures 7C and 7D, respectively). DA has no impact on the effect of LPS on total monocytes expressing CD62L and a minor impact on the RF of C-Mo, NC-Mo expressing CD62L only in the HS group (*circa* 20% increase which did not reach statistical significance and I-Mo in both groups (*circa* 30% in HS and 37% in PD; $p>0.05$). Regarding SLAN, DA had a minor impact in total, and C-Mo expressed SLAN. However, DA increased SLAN expression on NC-Mo in both groups and I-Mo of the HS group. We should stress that although these differences were not

statistically significant, they reached a delta difference of *circa* 70% (Supplementary Figure 7D and Supplementary Table S3).

Disclosing the receptors that are responsible for the impact of DA on IDO1$^+$cells

DR antagonists are unable to revert the effect of DA on IDO1$^+$cells.

Due to the pronounced reduction of IDO1$^+$cells by DA, we further investigated which DA receptors were involved. To this end, we used different concentrations of SHC-2231191 (DR1/DR5 antagonist, 1, 10 and 100µM, Figure 5A) and domperidone (D2/D3/D4 antagonist, 1, 10 and 100µM, Figure 5B) in the presence of 100µM of DA. In this approach, we used total blood samples from healthy donors, which were stimulated with LPS (LPS, 1ng/mL; 6h). DA decreased the RF of IDO1$^+$DCs and IDO1$^+$monocytes. Increasing concentrations of SHC-223191 and domperidone were unable to prevent the effect of DA on both cells.

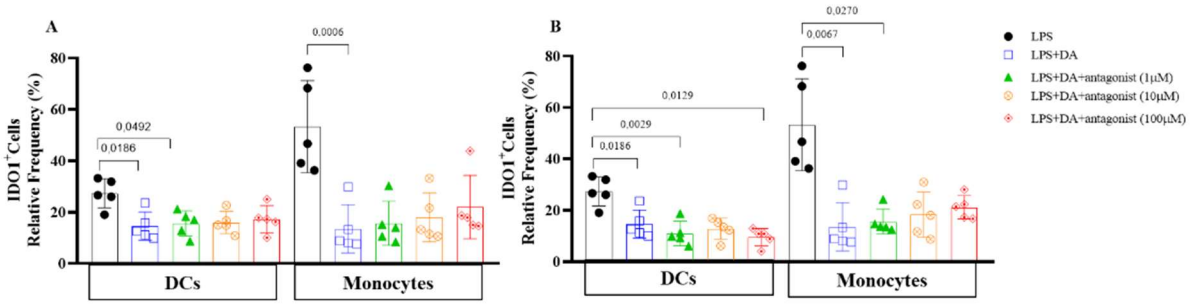

Figure 5. The effect of dopamine receptors type 1 (SHC-2231191) and type 2 (domperidone) blockade on IDO1$^+$dendritic cells (DCs) and IDO1$^+$monocytes. The total blood of 5 healthy donors was stimulated with lipopolysaccharide (LPS, 1ng/mL) or with LPS + dopamine (LPS+DA; DA, 100µM, bar with blue squares) during 6h at 37ºC with 5% of CO_2. The antagonists were added at different concentrations: 1µM (bar with green triangles), 10µM (bar with orange circles), and 100µM (bar with red hexagon) to the condition LPS+DA (LPS+DA+SHC-2231191; panel A and LPS+DA+domperidone; panel B). The relative frequency (RF) of IDO1$^+$DCs and IDO1$^+$monocytes are represented on the left and right of each panel. p-values are indicated on the graphs and significance data was considered when $p<0.05$. One-way ANOVA or Friedman test were used.

β_2AR is involved in the reduction of IDO1$^+$cells triggered by DA

To demonstrate that DA may be targeting βAR, we used propranolol which is a non-selective βAR antagonist at a concentration of 20µM. Propranolol was able to rescue the RF of IDO1$^+$DCs, and IDO1$^+$monocytes (Figure 6A) in HS.

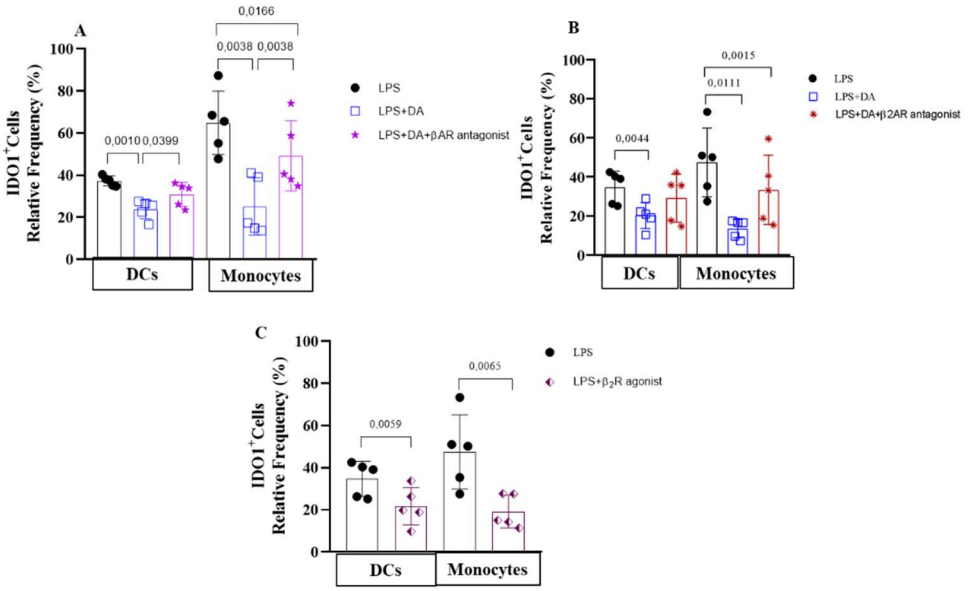

Figure 6. Dopamine (DA) decreased indoleamine 2, 3-dioxygenase 1 (IDO1) induced by LPS through β-adrenergic receptors (βAR) in dendritic cells (DCs) and monocytes. (A) The effect of the non-selective βAR antagonist propranolol (20µM, bar with violet stars) in the presence of LPS+DA (DA, 100µM, blue squares). (B) The Effect of β_2AR antagonist ICI118.551 hydrochloride (10µM, bar with red asterisk) in the presence of LPS+DA (DA, 100µM, blue squares). (C) The effect of β_2AR agonist salbutamol (1µM, bar with dark violet squares) on IDO1 induced by LPS. The total blood of 5 healthy donors was stimulated with lipopolysaccharide (LPS, 1ng/mL) during 6h at 37ºC with 5% CO_2 in the presence or absence of DA and adrenergic ligands. DA and the adrenergic ligands were added to the culture media for 30 min. before LPS. The relative frequency (RF) of IDO1$^+$DCs is represented on the left and the RF of IDO1$^+$monocytes is represented on the right of each panel. p-values are indicated on the graphs and data were considered significant when $p<0.05$. One-way ANOVA and paired t-tests were used to calculate significant differences.

To further understand the involvement of β_2AR on the DA effect on IDO1$^+$cells, we used the selective β_2AR antagonist (ICI 118.551 hydrochloride,10 µM, Figure 6B) and the β_2AR agonist (salbutamol, 1 µM, Figure 6C). Importantly, ICI 118.551 hydrochloride partially prevented the effect of DA on IDO1$^+$DCs and IDO1$^+$monocytes (Figure 6B). Salbutamol mimicked the effect of DA by reducing the RF of IDO1$^+$DCs and IDO1$^+$monocytes (Figure 6C).

The effect of β_2AR agonist salbutamol is less pronounced in IDO1$^+$monocytes from PD patients when compared to healthy subjects. To understand the role of β_2AR in the RF of IDO1$^+$DCs and IDO1$^+$monocytes in PD patients and HS, we incubated the total blood of 6 PD patients and 5 HS (Table 1) with LPS with or without salbutamol 1µM. The experimental conditions with LPS stimulation (black bar) were set as 100% and were considered as control. Data are presented as percentages of control (Figure 7). Salbutamol decreased IDO1$^+$DCs and IDO1$^+$monocytes in both groups.

Importantly, whereas there are no significant differences in IDO1^{+}DCs between groups (p=0.4855, PD vs HS), IDO1^{+}monocytes from PD patients were less responsive to salbutamol when compared to HS (p=0.0322, PD vs HS).

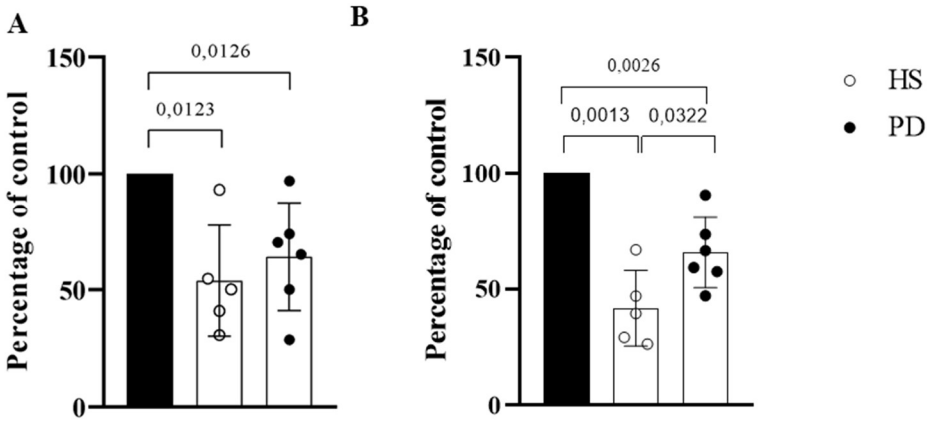

Figure 7. Salbutamol effect on IDO1^{+}DCs (A) and IDO1^{+}monocytes (B) stimulated with lipopolysaccharide (LPS) of Parkinson's Disease patients (PD) and healthy age-matched subjects (HS). Total blood of 5 HS (with dots) and 6 PD patients (PD, black dots) was stimulated with LPS (1ng/mL) or LPS with salbutamol (1µM) for 6h at 37ºC with 5% of CO2. Salbutamol was added to the culture media 30 min before LPS. The conditions with LPS stimulation (black bar) were set as 100% and were considered as control. Data are presented as a percentage of control. Comparations were done with 100% and the p-value was calculated using a one-sample t-test. p-values are indicated on the graphs and significance was set for $p<0.05$. Student t-test was used to compare control and PD groups.

The impact of DA and salbutamol on the cytokine profile

Cytokine levels were collected and analysed in culture supernatants of 4 PD patients and 5 healthy subjects, after 6h of LPS stimulation (1ng/mL) in the presence or absence of DA and salbutamol (Figure 8). With this experiment, we aimed to evaluate if these drugs can also change the peripheral immune profile of individuals and if those changes are different between HS and PD subjects. The cytokines from non-stimulated cells were below the detection limit and LPS increased the levels of all cytokines. Although without statistical significance (but with high delta differences), cytokine levels (IL-1β, TNF-α2, MCP-1, IL-6, IL-8, IL-10, IL18, IL-23) triggered by LPS are higher in PD patients when compared with HS, except for INF-α2 (Supplementary Table S4). The cytokines IFN-γ, IL-6, IL-8, IL12p70, IL17A, and IL-33 demonstrated values under the LOD in the presence of DA and salbutamol and are not included in this analysis. We found that DA, decreased IFN-α2, TNF-α, MCP-1, IL-10, IL-18, and IL-23 levels in HS. Although only TNF-α, MCP-1, and IL-23 showed statistically significance decreases, ranging from 20 to 73%. Interestingly, in PD patients only TNF-α, MCP-1 and IL-10 showed a decrease (22-27 %) which was lower when compared to HS and which did not reach statistical significance. Salbutamol triggered a statistically significant reduction (*circa* 60%) of TNF-

α and MCP levels which was similar to DA in HS. Also in HS, salbutamol decreased IL-23, however in a lower percentage when compared to DA (circa 44%) and without reaching statistical significance. In PD patients, salbutamol seemed to reduce TNF-α similarly to DA. Importantly and in contrast with HS, DA, and salbutamol seemed to increase the levels of IL-1β and INF-α2 in PD patients. When we compare cytokine levels from HS and PD patients, we found statistically significant differences between these two groups regarding TNF-α and MCP-1. DA (TNF-α, MCP−1) and salbutamol (MCP-1) had a greater impact on HS when compared to PD patients.

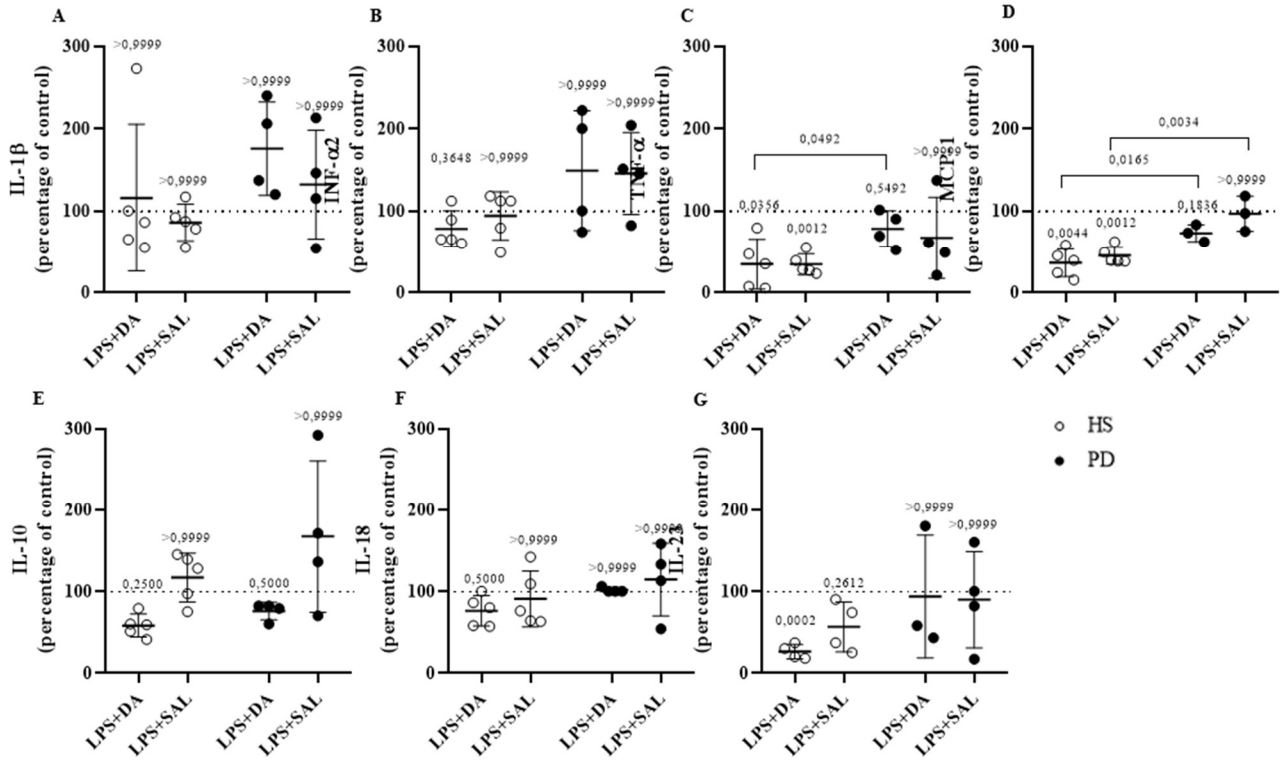

Figure 8. Dopamine (DA) and salbutamol (SAL) effect on cytokines measured in cell-cultured supernatants. The total blood of 5 healthy subjects (HS, with dots) and 4 PD patients (PD, black dots) was stimulated with LPS (1ng/mL) or with LPS+DA (100µM) or with LPS + salbutamol (SAL, 1µM) during 6h at 37ºC with 5% of CO2. The ligands were added to the culture media for 30 min. before LPS. Condition without LPS stimulation (resting) was below the limit of detection and is not included on graphs. Conditions with LPS stimulation (dashed line) were set as 100% and were considered as control. Cytokine concentrations are represented in pg/mL. p-value was calculated using a one-sample t-test. p-values are indicated on the graphs and significance was set for $p<0.05$. Student t-test was used to compare differences between control and PD groups.

4. Discussion

LPS: a pharmacological tool to characterize the immune response of DCs and monocytes in PD patients. LPS is an important stimulus in basic research to study acute and chronic inflammation[21].

LPS binds to the toll-like receptors (TLR) 4 and triggers NF-kB pathways in immune cells[22]. Considering that PD patients show central and peripheral immune dysfunctions[23] and that DCs and monocytes express TLR-4, LPS is a good tool to characterize the immune response mediated by these cells in PD patients[24]. Moreover, TLR4 activation by LPS culminates with a production of cytokines and chemokines, including IL-1β, IL-6, IL-8, TNF-α, PGE2, ROS, NO, and type I interferons[24]. Finally, and importantly, LPS also induces IDO1 in these circulating myeloid cells which allows us to study this immunoregulator mechanism on DCs and monocytes (Chapter 2). Moreover, we previously have shown that inflammatory stimuli such as LPS and/or anti-CD3/CD28 induced IDO1 on circulating myeloid cells (DCs and monocytes) (Chapter 3). We performed LPS time-course and concentration-response curves (Supplementary Figures S2 and S3) to optimize this stimulus aiming to obtain IDO1$^+$cells in *circa* 1/3 or 1/2 of total cells (around 33% of total DCs and 43% of total monocytes, under our experimental conditions). This allowed us to pharmacologically manipulate IDO1. Importantly, we show for the first time that stimulated monocytes, but not DCs displayed increased IDO1 induction in PD patients when compared to HS. This raises the following question: why DCs and monocytes behave differently regarding IDO1 induction in PD? Although unstimulated peripheral blood of PD patients did not show basal levels of IDO1$^+$cells significantly higher than in HS, monocytes but not DCs seemed to be primed by the inflammatory milieu in place. This may suggest that monocytes but not DCs have an upregulation of the TLR4 signaling pathways in PD. Additionally, RF of IDO1$^+$DC in PD patients seems to show a tendency to be lower than in HS. We and others have shown that DCs are reduced in the unstimulated blood of PD patients when compared with HS[25]. This may contribute to IDO1$^+$DCs RF under LPS stimulation in these patients. IDO1 has been associated with PD and rescuing IDO1 function on neurodegeneration has been pointed as a target in neurodegenerative diseases[19]. For example, it was suggested that rescuing IDO1 function in PBMCs of patients with multiple sclerosis (MS) with relapse might be useful as an anti-inflammatory strategy[19]. Considering monocytes and DCs as important immune response orchestrators, understanding the IDO1 regulation in these cells might constitute a strategy to control the inflammatory profile in PD, namely by increasing anti-inflammatory responses and controlling peripheral and central inflammation[26]. Moreover, LPS stimulation allowed us to find important delta differences between cells of both groups, which are not apparent in unstimulated cells. It is noteworthy to stress that not all differences are statistically significant, but they all are physiologically important. For example, we showed that a significantly higher reduction of RF of NC-Mo is in place when compared with HS and that LPS increased pDCs in PD patients (delta circa 20%) when compared with HS, without reaching significance. LPS might be used to create an inflammatory environment which

may recapitulate some of the inflammatory features seen in circulating myeloid cells of PD patients. Particularly, our *in vitro* data showed that LPS decreased total DCs and cDCs in both groups and significantly increased pDCs in PD patients. The reduction of total DCs was verified by us (Chapter 4) and others on unstimulated blood of PD patients. Moreover, we showed that pro-inflammatory cells such as pDCs and cDC-expressing CD16 were increased in PD patients. Similarly, our *in vitro* studies, showed that LPS increased pDCs in PD patients. This might indicate that pDCs of PD patients are more reactive to LPS than pDCs of HS. Furthermore, pDCs are multifaceted cells, which have the ability to produce high amounts of IFN-γ and present antigens to $TCD4^{+}$ [27]. Interestingly, pDCs and NK cells were associated with prion pathogenesis[28,29]. Our data supports higher pDCs RF, more reactive pDCs as well as higher levels of NK cells (data not shown) in PD patients. This may be aligned with the Braak's prion hypothesis , which considers that DCs play a role in spreading α-syn. However, more studies are warranted to support this hypothesis[30]. We also found opposite effects when comparing cells stimulated with LPS and the pro-inflammatory profile of PD patients. For example, we found higher expression of CD62L adhesion molecule in unstimulated PD monocytes when compared to HS. On the contrary, LPS triggered a reduction of this molecule in monocytes in both groups. Importantly, unstimulated blood data showed a tendency towards increased RF of NC-Mo in PD patients when compared to HS. However, LPS triggered a significantly decrease in these cells in PD when compared to HS. In any case, LPS highlights differences between HS and PD that were apparent in unstimulated blood. Functional studies are necessary to understand the relevance of this mild difference found on unstimulated cells. Monocytes-derived DC (Mo-DC), which are a distinct DC subset differentiated from monocytes, are an excellent example of immune cell plasticity[31]. This immune cell plasticity might explain *in vitro* decrease of DCs and may also contribute to its decrease *in vivo.* Migration of DCs to the inflamed brain is pointed also as a possible reason for DCs' decreased levels[25].

DA as an immunomodulator agent. The neurotransmitter DA has also been shown to have an immunomodulatory role[29]. Herein, DA showed mild effects on the modulation of the effect of LPS on DCs and monocyte populations and strong effects on $IDO1^{+}$DCs and $IDO1^{+}$monocytes. DA seems to further accentuate the effect of LPS on the reduction of DCs and NC-Mo frequency in PD patients with minimal effect in HS (Supplementary Figure 7B). This effect might suggest that DA signaling in both DCs, and monocytes is different between PD and HS. Interestingly, DA decreased LPS-induced cytokines more effectively in HS when compared to PD. The effect of DA on the frequency of immune cells seems to be the opposite regarding the impact of DA on cytokines and IDO1 in PD. Nonetheless, these data suggest alterations in DA signaling in peripheral immune cells in PD. Whether DA has a

pro or anti-inflammatory effect is debatable. However, the reduction of inflammatory cytokine levels supports an anti-inflammatory role[29]. Additionally, our work demonstrated that DA decreased IDO1 expression in a concentration-dependent way on both DCs and monocytes (Supplementary Figure 4). This further suggests an immunomodulatory role for this neurotransmitter. However, further studies are necessary to understand whether DA has a direct effect on IDO1 induction in these cells or if this IDO1 decrease reflects a reduction of the inflammatory environment, thus suggesting an anti-inflammatory role of DA. One cannot exclude that both hypotheses can co-exist. Our data indicates that β_2AR is implicated in DA reduction of IDO1 expression on DCs and monocytes. It was shown that these cells also express βAR, besides DR[32,33]. Also, since we are using very high DA concentrations (when compared to physiological circulating DA levels), it is not surprising that DA may be binding to β_2AR[32]. However, when we used the β_2AR agonist salbutamol at lower concentrations (100-fold) we saw a similar effect to the one showed by DA on IDO1. Nonetheless, one should have measured the DA concentration in our supernatants since DA is very labile in the culture medium. It would also be relevant to measure DA cell content (DCs and monocytes) to understand its contribution to the final effect seen in our experimental conditions. Interestingly, some studies have already addressed βAR modulation in PD as discussed by Hopfner and colleagues (2020). These authors considered that the epidemiological evidence for a causal relationship between the use of β_2AR antagonists (like propranolol) and the increased risk of developing PD is weak (observational studies)[32] Interestingly, Mittal *et al.,* 2017 discovered that salbutamol is a regulator of α-syn gene and was able to reduce this pathological hallmark in PD. Moreover, these authors suggested that the use of the non-selective βAR propranolol increased α-syn aggregates [34]. On the other hand, the impact of DA on activated IDO1$^+$DCs and IDO1$^+$monocytes are not altered in PD patients. However, we clearly show that the effect of salbutamol is less pronounced in IDO1$^+$monocytes from PD patients when compared to healthy subjects. On the other hand, DCs from both PD patients and HS responded similarly to salbutamol. This suggests that β_2AR signaling seems to be altered in monocytes in PD. The mechanism underlying the reduction of cytokines and IDO1 triggered by DA and by salbutamol may be shared. Overall, this suggests that DA has strong immunomodulatory effects that may have implications in inflammation. Since the blockade of β_2AR did not completely prevent the impact of DA on IDO1 induction, other receptors may contribute to the immunomodulatory effect of DA, including αAR, β_1AR, or β_3AR [7]. Moreover, the effect of salbutamol on cytokines seemed less robust than the one triggered by DA. This further indicates that β_2AR is not the only receptor-operated by DA in our experimental conditions.

Finally, it is important to notice that the patients enrolled in this study are taking L-DOPA daily. It was suggested that L-DOPA-induced dyskinesias (LIDs, long-term L-DOPA-induced complications) are underlined by the release of soluble proinflammatory mediators by reactive microglia[29]. Therefore, L-DOPA intake by patients may represent a confounding factor in our study. The inexistence of PD patients not taking L-DOPA is a limitation of this study.

5. Conclusion

Our findings showed that LPS might be an important tool: (1) to characterize changes in circulating myeloid cells from PD patients, and (2) to create an *in vitro* model that mimics a pro-inflammatory environment found in PD. We disclosed differences in monocyte RF and monocyte IDO1 induction in PD when compared to HS. Also, we showed that the effect of salbutamol in the modulation of IDO1 is impaired in monocytes of PD patients. This suggests that an altered adrenergic receptor signaling, or density is in place in monocytes in PD. Importantly, our data showed that salbutamol mimicked the anti-inflammatory role of DA, although salbutamol had shown a milder effect. The β_2AR antagonist did not completely revert the effect of DA, thus indicating that DA is exerting its effect by other receptors as well. This work underlines the pressing need to disclose the activity of IDO1 in circulating myeloid cells in PD patients and whether targeting IDO1 in circulating myeloid cells might be an anti-inflammatory strategy to be used in PD.

Data Availability Statement

All datasets generated for this study will be disclosed in response to a reasonable request.

Ethics Statement

Total blood samples were collected from healthy individuals after signed informed consent to participate in the present study approved by the Ethical Committee of the Coimbra Hospital and University Centre (Portugal; CHUC-131-19).

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Disclosers

No conflicts of interest, financial or otherwise, are declared by the authors.

Author Contributions

Milene Gonçalves: conceptualization, methodology, validation, investigation, formal analysis, writing the original draft. **Ana Morgadinho**: clinical selection of PD patients and manager of clinical information. **Paulo Rodrigues Santos**: methodology, resources, funding acquisition. **Jani Almeida**: methodology. **Vera Alves**: methodology. **Ivo Cruz:** methodology. **Isabel Costa**: research nurse, blood collection PD patients, and healthy subjects. **Cristina Januário**: clinical selection of PD patients, writing-review and editing. **Marco Cosentino**: writing-review and editing. **Frederico C. Pereira**: conceptualization, resources, writing-review and editing, supervision, funding acquisition.

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1. Supplementary Files

1.1. Supplementary Methods

1.1.1. LPS stimulation: time-course and concentration-response curve of LPS in IDO1 induction in DCS and monocytes

1.1.1.1. LPS time-course curve

Total blood from 5 healthy individuals was incubated as described in 2.3 (material and methods) and was stimulated with LPS (100 ng/ml) at 37 °C in a moist atmosphere of 5% CO_2 for 24h. Cells were collected and immunophenotyped by flow cytometry as described below at the following time points: 0, 1h, 3h, 6h, 12h, 18h, and 24h. This experiment allowed us to choose 6 as an optimal time point to measure IDO1 induction by LPS on DCs and monocytes. The following experiments will be performed with 6h LPS incubation.

1.1.1.2. LPS concentration-response curve

In this experiment, we used increasing concentrations of LPS (0, 0.1, 1, 10, 100, 1000 ng/ml) to stimulate the total blood of 5 healthy individuals for 6h at 37 °C in a moist atmosphere of 5% CO_2. This experiment allowed us to set 1ng/mL of LPS as the concentration to induce IDO1 on DCs and monocytes in the following experiments.

1.1.2. DA concentration-response curve

The total blood of healthy individuals (Table 1) was cultured as described above and stimulated with LPS (1 ng/ml) in the presence or absence of increasing DA concentrations (0, 1, 10, 100, 1000 µM) during 6h at 37 °C in a moist atmosphere of 5% CO_2. DA was added 30 minutes before the LPS stimulation. Cells were then collected and analysed by flow cytometry.

1.1.3. Cell viability assay

Cell viability was verified by using two different fluorescent reagents (Reagent C of NK test™ and 7-AAD) that intercalate the DNA and indicate the relative frequency (RF) of the dead cells. In the protocol using the reagent C of NK test, we resuspended cells in 100µL of PBS1x and we added 2 mL of RBC lysis buffer followed by a 20-minute incubation at room temperature. We then centrifuged the sample at 1500 rpm for 10 minutes and resuspended the pellet in 100 µL of PBS1x following the

addition of 10µL of reagent C of NK test with incubation of 5 minutes at an ice bath. The suspension was read within 30 minutes after the addition of reagent C to the NK test. In turn, when we used 7-AAD, we incubated 5µL of this reagent with 100µL of blood sample followed by a 30-minute incubation at 4ºC. We then washed cells with 2 mL of BD lysing solution and incubated them for 10 minutes followed by centrifugation at 1500 rpm for 5 minutes. After the incubation, we added 2mL of PBS1x followed by a centrifugation at 1500 rpm for 5 minutes. Finally, we resuspend the pellet in 200 µL of PBS and we read in FACS Canto II with DIVA software (Becton Dickinson, San Jose, CA, USA).

1.2. Supplementary Figures

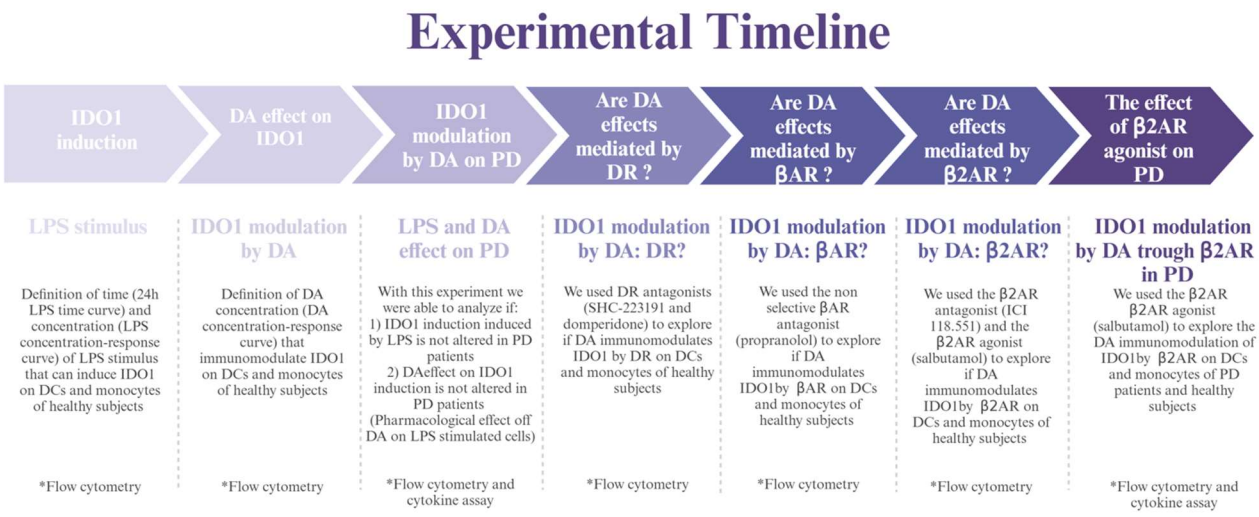

Figure S1. Experimental timeline. The figure shows the experiments done in chronological order as well as the questions answered by each experiment. The endpoints measured were IDO1 induction and cytokine release in supernatant by flow cytometry.

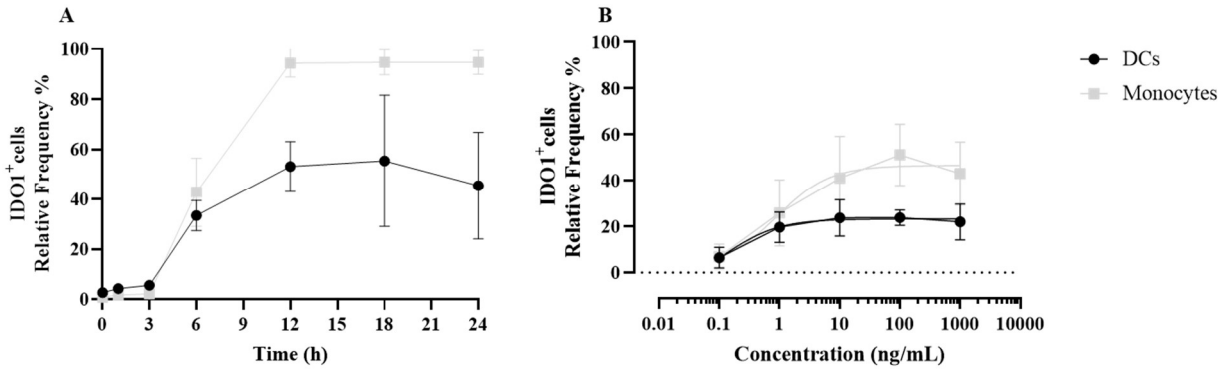

Figure S2. Time-course and concentration-response curves of the effect of LPS on IDO1 induction in DCc and monocytes. (A) IDO1^{+}DCs and IDO1^{+}monocytes under lipopolysaccharide (LPS, 100ng/mL) stimulation across 24h incubation. Data represented as mean±SD [DCs, 0h (2.70±0.41%), 1h (4.33±0.98 %) 3h (5.65±1.32%), 6h (33.48±6.13%), 12h (53.10±10.01%), 18h (55.40±26.30%) and 24h (45.42±21.40%). MO, 0h (0.49±0.19%), 1h (0.83±0.29%), 3h (0.73±0.33%), 6h (42.72±13.72%), 12h (96.94±2.21%), 18h (95.92±5.27%) and 24h (96.40±4.77%)] (B) Relative frequency of IDO1^{+}DCs and IDO1^{+}monocytes under increasing concentrations of LPS (0, 0.1, 1, 10, 100 and 1000 ng/mL) during 6h of incubation. DCs LPS EC50 = 0.14 ng/ml, R squared= 0.57, and monocytes LPS EC50 = 0.93 mg/ml, R squared = 0.93. 0 ng/mL (DCs=1.76±1.12% and monocytes= 0.46±0.51%); 0.1ng/mL (DCs=6.46±4.48%, monocytes= 6.93±5.53%); 1ng/mL (DCs=19.64±6.61%, monocytes=25.88±14.33%,); 10ng/mL(DCs=23.72±7.86%, monocytes= 41.04±18.12%,); 100ng/mL (DCs=23.82±3.40%, and monocytes= 51.12±13.41%,) and 1000ng/mL (DCs=21.96±7.80%, and monocytes= 43.02±13.75%). Cultured cells were collected at the following time points: 0, 1, 3, 6, 12, 18, and 24h (A) and 6h (B) and analysed by flow cytometry.

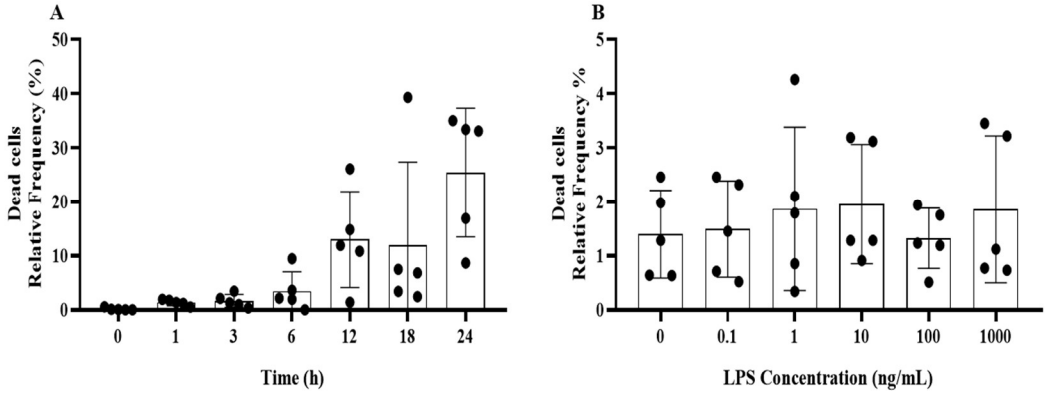

Figure S3. Relative frequency in percentage (%) of dead cells in blood cultures. A - Total blood was stimulated with lipopolysaccharide (LPS, 100ng/mL) across 24h incubation. Culture cell content was collected at the following time points: 0, 1, 3, 6, 12, 18 and 24h. B - Total blood was stimulated with growing concentrations of LPS (0, 0.1, 1, 10, 100, 1000 ng/mL during 6h incubation at 37ºC with 5% CO_2. Culture cell content was collected on the following time points: 0, 1, 3, 6, 12, 18, and 24h, labeled with NK t-test reagent C and acquired by flow cytometry to calculate the relative frequency of dead cells by flow cytometry.

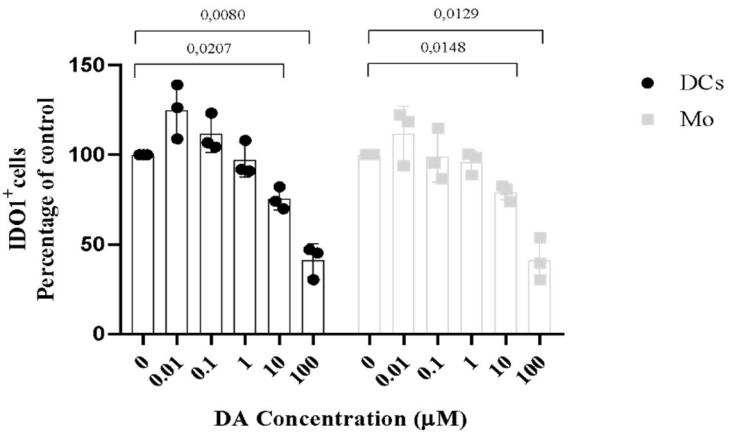

Figure S4. Dopamine (DA) decreased indoleamine 2,3-dioxygenase (IDO1)$^+$cells. Percentage of control of IDO1$^+$dendritic cells (black dots) and IDO1$^+$monocytes (grey squares) under lipopolysaccharide (LPS, 1ng/mL) stimulation and in the presence of increasing DA concentrations (0, 0.01, 0.1, 1, 10, 100µM). The total blood of 3 healthy donors was incubated for 6h at 37ºC with 5%CO_2 with LPS+DA. LPS condition (stimulated with LPS) was set as 100% and data are given as means ± SD of the percentage of the control. One sample t-test or Wilcoxon test was applied.

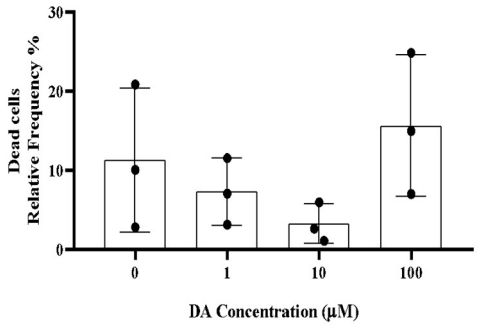

Figure S5. Relative frequency in percentage (%) of dead cells in blood cultures. Total blood was stimulated with lipopolysaccharide (LPS, 1ng/mL) during 6h at 37ºC with 5% CO_2. Growing concentrations of DA (0, 0.01, 0.1, 1, 10, 100µM) were added to the cell culture 30 minutes before LPS stimulation. Culture cell content was labeled with NK t-test reagent C and acquired by flow cytometry to calculate the relative frequency of the dead cells by flow cytometry.

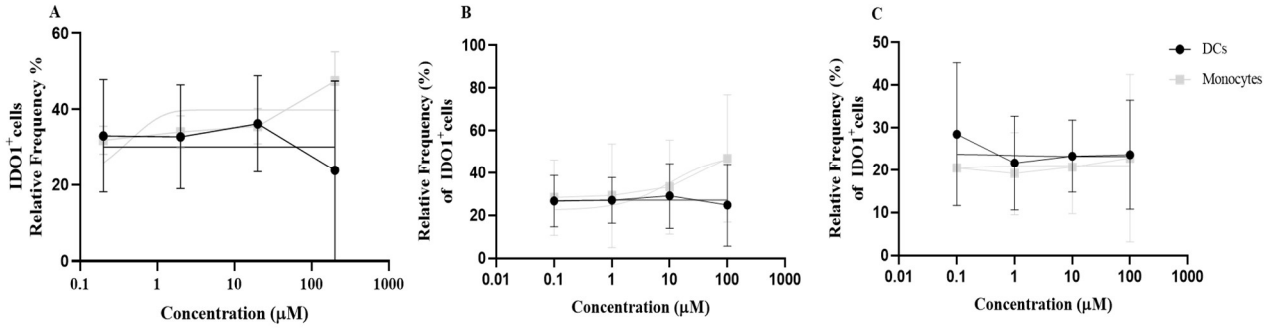

Figure S6. Concentration-response curves for adrenergic ligands. A- the non-selective beta-adrenergic receptor (βAR) antagonist propranolol, n=3, concentrations - 0.2, 2, 20, 200µM; B- the β2AR antagonist ICI118.551 hydrochloride, n=5, concentrations: 0.1, 1, 10, 100µM; C - the β2AR agonist salbutamol, n=5, concentrations: 0.1, 1, 10, 100µM. Total blood of healthy subjects was cultured under lipopolysaccharide (LPS, 1ng/mL) stimulation during 6h at 37ºC with 5% of CO_2

in the absence (LPS) or presence of dopamine (LPS+DA; DA, 100µM). The antagonists were added 30 minutes before DA. The RF of IDO^{+}DCs is represented by black dots and the RF of IDO1^{+}Monocytes is represented by grey squares.

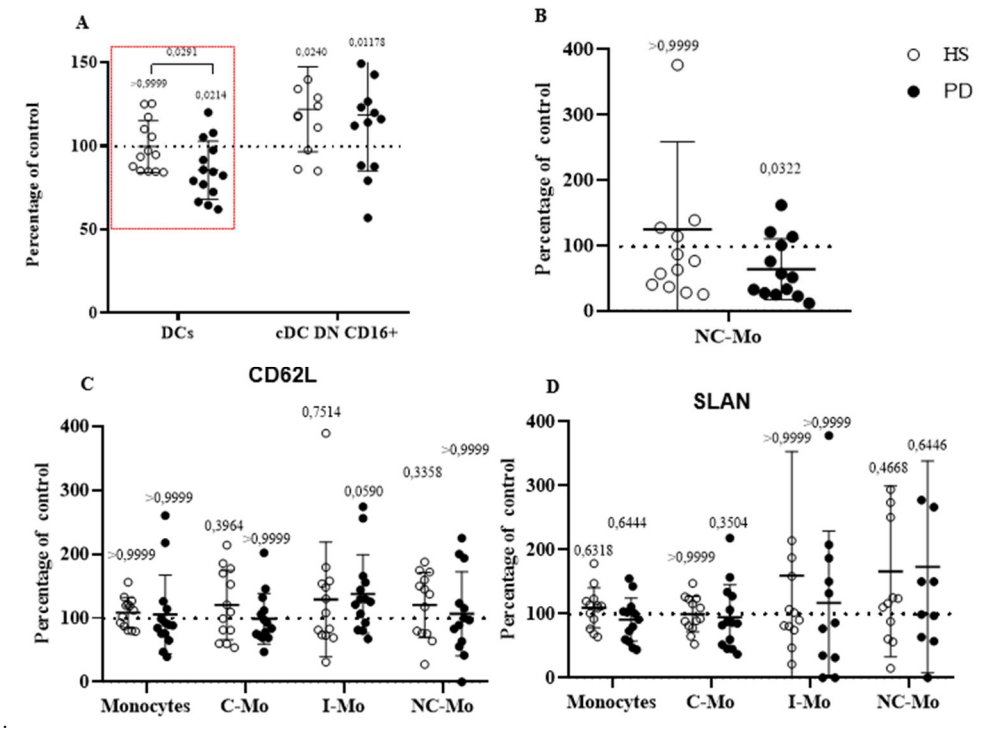

Figure S7. Dopamine (DA) effect on DCs (A), monocytes (B), monocyte populations expressing CD62L (C), and monocyte populations expressing SLAN (D) stimulated with lipopolysaccharide (LPS). The total blood of 13 healthy subjects (HS, with dots) and 14 PD patients (PD, black dots) was stimulated with LPS (1ng/mL) in the presence or absence of DA (100µM) during 6h at 37ºC with 5% of CO_2. Conditions with LPS stimulation (dashed line) were set as 100% and were considered as control. Data are presented as a percentage of control. Comparations were done with 100% and the p-value was calculated using one sample t-test or one sample Wilcoxon test. p-values are indicated on the graphs and significant values are considered when $p<0.05$. Student t-test and Mann-Whitney test were used to compare control and PD groups. DCs – dendritic cells; DN –double negative; C-Mo – Classical monocytes; NC-Mo – non-classical monocytes.

1.3. Supplementary Tables

Table S1. Monoclonal antibodies are used for flow cytometry studies.

Specificity	Fluorochrome	Clone	Isotype	Brand
CD1c	BV421	F10721A3	IgG_1	BD
CD3	APC-H7	SK7	IgG_1	BD
CD11c	PE-Cy7	BLY6	IgG_1	BD
CD14	V450	MOP9	IgG_2	BD
CD16	FITC	B73.1	IgG_1	BD
CD19	FITC	HIB19	IgG_1	BD
CD19	APC-H7	SJ2501	IgG_1	BD
CD20	APC-H7	2H7	IgG_1	BD
CD43	PE	EBlo84-3C1	IgG_1	eBioscience
CD49d	PerCp-Cy5.5	9F10	IgG_1	BD
CD56	PE	MY31	IgG_1	BD
CD62L	PE-Cy7	DREG-56	IgG_1	Biolegend
CD123	PerCp-Cy5.5	7G3	IgG_1	BD
CD141	PE	M80	IgG_1	BD
IDO1	AF647	V50-1886	IgG_1	BD
HLA-DR	V500	G46-6	IgG_2	BD
SLAN	FITC	DD-1	IgG_1	Milteryl
LIN2	FITC	CD3 – SK7, CD19 SJ25C1, CD20 – L27, CD14 MoP9, CD56 NCAM 16.2	IgG_1	BD

Table S2. Curve details of a cytokine assay. LOD – Limit of detection (pg/mL); LOQ – limit of quantification (pg/mL); 5PL- five parameter logistic; IFN - interferon

Target	Fit	IC50	R^2	LOD	LOQ
IL-1β	5PL	11.57	0.999	3.595	14.67
IFN-α2	5PL	227.6	0.999	0.452	0.951
IFN-g	5PL	3226	0.999	0.403	1.286
TNF-α	5PL	3.015	0.994	58.57	408.8
MCP-1	5PL	5.353	0.997	33.61	115.1

IL-6	5PL	52.94	0.998	1.28	9.32
IL-8	5PL	8.067	0.994	64.54	672.7
IL-10	5PL	13.55	0.998	3.867	24.9
IL-12p70	5PL	7.682	0.998	3.178	17.19
IL-17A	5PL	1.885	0.999	0.413	1.531
IL-18	5PL	27.65	0.998	8.24	60.24
IL-23	5PL	60.83	0.998	12.6	99.83
IL-33	5PL	159.1	0.998	77.69	368.5

Table S3. Statistics of circulating myeloid cells and cytokines (normalized data)

	HS		**PD**		**PD vs HS**	
	Mean%	**SD**	**Mean %**	**SD**	**p-value**	**Delta (%)**
DCs	80.98	**25.43**	**87.27**	**17.35**	0.5947	**7.77**
cDCs	80.15	12.76	80.67	9.848	0.9989	0.65
cDC2	143.6	26.78	139.5	26.42	0.6920	-2.86
DN	90.99	4.461	91.07	6.029	0.7251	0.09
DNCD16^{+}	34.12	22.75	32.88	13.98	0.8644	-3.63
pDCs	112	33.54	133.5	38.96	0.1388	19.2
IDO1^{+}DCs	1355	1383	1013	411.7	0.7203	-25.24
IDO1^{+}cDCs	2903	4230	1988	1218	0.7930	-31.52
IDO1^{+}cDC2	1111	818.6	941.1	593.2	0.9051	-15.29
IDO1^{+}DN	6574	7784	2590	3217	0.1674	-60.6
IDO1^{+}DNCD16^{+}	5496	6451	3870	2906	0.9654	-29.59
Monocytes	63.29	14.75	67.46	10.89	0.4086	6.59
C-Mo	100.8	17.93	116.1	21.04	0.0526	15.18
NC-Mo	55.21	36.56	17.81	12.82	0.0003	-67.74
I-Mo	95.1	55.46	92.03	51.63	>0.9999	-3.23
CD43^{+}Mo	99.85	0.257	99.96	0.1639	0.1978	0.11
CD49^{+}Mo	103.1	4.724	107.2	6.954	0.2869	3.98
CD62L^{+}Mo	10.27	11.36	5.896	3.91	0.1279	-42.59
HLA-DR^{+}Mo	101	3.81	102.2	2.456	0.3266	1.19
IDO1^{+}Mo	4776	5951	7571	4661	0.0255	58.52
SLAN^{+}Mo	204.2	113.9	191.7	69.78	0.7316	-6.12
CD62L-C-Mo	9.251	6.341	7.632	5.532	0.4020	-17.5
IDO1-C-Mo	10247	8200	17067	11437	0.1014	66.56
SLAN-C-Mo	255.6	151.3	327.8	180	0.2654	28.25
CD62L-NC-Mo	122.5	70.6	124.3	91.23	0.9561	1.47
IDO1-NC-Mo	428.4	450.5	1200	864.1	0.0192	180.11
SLAN-NC-Mo	510.6	519.5	325.1	407.5	0.3396	-36.33
CD62L-I-Mo	45.42	26.1	38.31	15.96	0.5186	-15.65
IDO1-I-Mo	949.2	667.8	2040	2127	0.0284	114.92
SLAN-I-Mo	211.6	226.1	176.5	223.7	0.6111	-16.59
Under LPS stimulation on the presence of DA						
DCs	99.63	15.45	85.46	17.35	0.0291	-14.22

cDCs	108.2	108.2	104.4	104.4	0.1879	-3.51
cDC2	91.78	26.03	95.69	11.28	0.6132	4.26
DN	102.6	5.583	102	4.719	0.7687	-0.58
DNCD16$^+$	122	25.41	118.5	33.48	0.7698	-2.87
pDCs	101.2	16.47	96.78	19.33	0.4020	-4.37
IDO1$^+$DCs	67.62	18.31	72.78	18.58	0.4749	7.63
IDO1$^+$cDCs	63.02	17.96	67.46	16.96	0.5150	7.05
IDO1$^+$cDC2	95.16	13.27	86.82	7.209	0.0511	-8.76
IDO1$^+$DN	48.36	18.01	53.75	17.12	0.4919	11.15
IDO1$^+$DNCD16$^+$	40.23	21.65	42.09	18.83	0.8622	4.62
Monocytes	112.6	19.87	110.5	20.94	0.9598	-1.87
C-Mo	103.9	9.55	106.3	5.759	0.4335	2.31
NC-Mo	124.1	133.9	63.58	46.62	0.1389	-48.77
I-Mo	111.6	47.86	105.1	41.97	0.7134	-5.82
CD43$^+$Mo	99.95	0.3256	100	0.2326	0.4991	0.05
CD49$^+$Mo	100.8	2.243	98.04	4.66	0.0764	-2.74
CD62L$^+$Mo	108.5	23.76	105.5	61.71	0.1852	-2.76
HLA-DR$^+$Mo	100	1.678	99.73	2.064	0.8697	-0.27
IDO1$^+$Mo	55.65	19.87	50.24	20.4	0.6848	-9.72
SLAN$^+$Mo	109.1	31.33	90.81	33.43	0.1557	-16.76
CD62L-C-Mo	120.5	54.16	98.84	39.77	0.3499	-17.98
IDO1-C-Mo	52.09	21.7	47.68	20.18	0.9430	-8.47
SLAN-C-Mo	99.35	27.47	94.47	50.76	0.7612	-4.91
CD62L-NC-Mo	120.7	50.74	106.7	65.68	0.5788	-11.6
IDO1-NC-Mo	137.5	93.36	116	97.09	0.6115	-15.64
SLAN-NC-Mo	166.2	133.4	173.2	165.4	>0.9999	4.21
CD62L-I-Mo	129.2	89.55	137.9	61.26	0.6160	6.73
IDO1-I-Mo	89.33	71.5	79.58	36.9	0.8674	-10.91
SLAN-I-Mo	159.4	193.8	116.7	112.6	0.5541	-26.79
Cytokines under LPS stimulation on the presence of DA						
Il-1β	115.8	89.62	175.8	56.72	0.1905	51.81
IFN-α2	78.2	22.02	149	72.93	0.0753	90.54
TNF-α	34.6	30.67	78	21.83	0.0492	125.43
MCP-1	36.2	17.02	72.67	10.5	0.0165	100.75
IL-10	58	14	75.75	10.59	0.0635	30.6
IL-18	76.2	18.55	101.5	3	0.0397	33.2
IL-23	26.25	8.884	93.67	75.14	0.1254	256.84
Cytokines under LPS stimulation on the presence of Salbutamol						
Il-1β	85,8	22,49	132	66,16	0,1823	35,00
IFN-α2	94	29,47	145,5	49,95	0,0935	35,40
TNF-α	34,4	12,95	67	49,58	0,1949	48,66
MCP-1	45,2	10,47	96,67	21,5	0,0034	53,24
IL-10	116,8	29,8	167,3	93,11	0,2851	30,19
IL-18	90,8	34,14	114,5	44,34	0,3933	20,70
IL-23	56,5	30,56	89,75	58,86	0,3547	37,05

Table S4. Cytokine levels in supernatants from blood cells stimulated with LPS (without normalization).

	HS		PD		PD vs HS	
	Mean (pg/mL)	**SD**	**Mean (pg/mL)**	**SD**	**p-value**	**Delta (%)**
IL-1β	823.3	96.1	1128	1406	0.875	27.01
INF-α2	9.32	1.391	6.758	3.05	0.375	-37.91
TNF-α2	2495	454.2	18462	14397	0.125	86.49
MCP-1	823.3	96.1	1128	1406	0.875	27.01
IL-6	823.3	96.1	1128	1406	0.875	27.01
IL-8	3798	930.8	18641	14527	0.226	79.63
IL-10	121.7	24.57	234.4	206.4	0.625	48.08
IL-18	190.9	17.71	291.5	131.2	0.25	34.51
IL-23	231.5	16.72	294	344.5	>0.9999	21.26

CHAPTER 6

General Discussion

Limitations of the study

Future perspectives

General Discussion

The main purpose of this thesis is to outline the importance of exploring PD beyond the brain. There is no doubt that PD is a neurodegenerative disease with an inflamed brain which contributes to perpetuating the neurodegenerative process[1]. However, understanding the multifactorial components of this disease, including peripheral immunity, is instrumental to fully characterize PD. Moreover, this complex disease demands multifactorial approaches to improve disease motor and non-motor symptoms and PD patients' quality of life[2]. The focus on IDO1 aimed first: to explore if the immune system is compromised in PD and second: to modulate this immune regulatory mechanism in the context of inflammation and PD. There is previous evidence of the following: 1) altered peripheral immune cells in PD, including DCs and monocytes and 2) changes in KP metabolites in PD[3–5]. However, there is a lack of information about the induction of IDO1 by DCs and monocytes in PD. We used different inflammatory stimuli addressing specific immune cell subsets (LPS: myeloid cells stimulation and anti-CD3/CD28: T lymphocytes stimulation) aiming to induce *in vitro* IDO1 in Chapter 3. The measurement of PBMCs proliferation showed us that we were able to replicate an inflammatory ecosystem. Simultaneously, the measurement of KP metabolites (Trp and KYN) by HPLC-EC showed us that different inflammatory conditions increased differently KP metabolites. Moreover, IDO1 induction was confirmed by RT-PCR. These experiments importantly showed the following: 1) circulating myeloid cells like DCs and monocytes but not T and B lymphocytes and NK cells can induce IDO1; 2) there is an important cell-to-cell interaction between T cells and myeloid cells; the use of PBMCs instead of isolated cell subsets (DCs: T cell) disclosed this; 3) strong pro-inflammatory stimuli may push the immune system towards its maximum response; thus hampering its pharmacological manipulation. For example, when we abolished IDO1 induction in our experimental model (by using the inhibitor epacadostat), we failed to have an impact on proliferation. Therefore, this work prompted us to optimize an *in vitro* model that allowed us to pharmacologically manipulate IDO1 in DCs and monocytes (Chapter 5). We should stress that this protocol optimization led us to use 1ng/mL of LPS instead of 100 ng/mL of LPS and 6h of LPS stimulation instead of 24 or 120h of LPS or LPS+anti-CD3/CD28 stimulation. This means a 100-fold difference in concentration and a much shorter time point. Importantly, with a LPS stimulation of 1ng/mL during 6h we were able to induce IDO1 in DCs (circa 33% of total DCs) and monocytes (circa 43% of total monocytes). This means that we have a very high percentage of cells producing IDO1 *in vitro* which was not seen in circulating myeloid cells (Chapter 4). We showed reduced percentages of $IDO1^{+}$DCs (1,77%) and $IDO1^{+}$monocytes (1,05%) in unstimulated blood samples from PD patients (Chapter 4). This suggests that the altered immune phenotype and RF of PD patients compared to HS were not sufficient to

significantly induce IDO1 in PD patients at the mild stage of the disease. The data obtained in Chapter 4 further fueled our interest in studying IDO1 in PD under stimulatory conditions. Therefore, we performed *in vitro* studies by using the pro-inflammatory stimuli LPS to characterize IDO1 induction in PD patients. Importantly, PD patients showed higher RF of $IDO1^{+}$monocytes (total and subpopulations) than HS when stimulated with LPS. Simultaneously, our data suggested that that DA and salbutamol have anti-inflammatory properties. Importantly, the effect of β_2AR agonist salbutamol is less pronounced in $IDO1^{+}$monocytes from PD patients when compared to HS. This means that under an inflammatory stimulus like LPS, PD patients showed higher percentages of $IDO1^{+}$monocytes, which are less responsive to the activation of β_2AR. These suggest that monocytes from PD patients have an upregulation of TLR4 signaling and a compromised β_2AR signaling. The TLR4 signaling alterations may be relevant regarding the sensing of circulating synuclein aggregates that have also been identified in blood from PD patients[6]. The β_2AR signaling deregulation may be aligned with autonomic dysfunction seen in PD patients[7]. However, these alterations are not evident in DCs. Therefore, monocytes maybe important mediators of immune dysfunction in PD. In the end, this highlights the importance of exploring the role of monocytes on PD. Particularly, IDO1+monocytes can be the foundation of diagnostics and therapeutic avenues in PD. Non-classical monocytes should also be further studied in PD. It is noteworthy to acknowledge that it was suggested that non-classical subtypes display 'inflammatory' characteristics on activation and display properties for antigen presentation[8]. Microglia display some similar features with monocytes including the ability to express some KP enzymes. Therefore, it is mandatory to study $IDO1^{+}$microglia in the brain. Understanding the effect of DA on KP enzymes on microglia might also be useful to prevent complications such as LIDs that have been associated with microglia pro-inflammatory phenotypes[9,10]. Finally, this thesis also highlights the importance of understanding the role of catecholamines as a putative disease-modifying strategy in PD. This will be important to manage PD medication long-term complications.

Limitations of the study

The lack of treatment-naïve patients (meaning patients without dopaminergic replacement) constitutes an important limitation of this study. These patients are very hard to enroll because the patients arriving at our Movement Unit are already taking dopaminergic medication. Moreover, most of our patients were in a mild stage, which could increase the difficulties in drawing correlations with clinical information. Therefore, studying PD patients in different disease stages, including the prodromal stage, is also fundamental to understanding immune changes across disease progression. Functional studies that included IDO1 activity and DA concentration are also lacking.

Future perspectives

Neurodegenerative diseases are no longer seen as brain diseases alone. The immune system, which is in constant communication with CNS, represents an open window to study these neurodegenerative diseases. Moreover, studying the immune system changes that may be present in the prodromal stages of the disease also seems to be instrumental in establishing disease-modifying strategies.

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Appendix

Abstract in English

Parkinson's Disease (PD) is a neurodegenerative disease where central and peripheral immune dysfunctions have been pointed out as a critical component of susceptibility and progression of this disease. Dendritic cells (DCs) and monocytes are key players in promoting immune response regulation and can induce the immunomodulatory enzyme indoleamine 2, 3-dioxygenase 1 (IDO1) under pro-inflammatory environments. IDO1 is a rate-limiting enzyme of the kynurenine pathway (KP) that converts tryptophan (Trp) into kynurenine (KYN) as the first step of the pathway. The immune functions of the pathway, which are extensively described in cancer, are unexplored in neurodegeneration. The dysfunctions of KP that have been described in PD are associated with neurotoxic functions. This thesis focuses on IDO1^{+}DCs and IDO1^{+}monocytes in PD aiming to explore the immune role of IDO1 in PD. We also highlight the importance of exploring the role of dopaminergic therapeutics in the immune system including IDO1 modulation. We first induced IDO1 in myeloid cells with different pro-inflammatory stimuli. The myeloid stimulus lipopolysaccharide (LPS), as well as the lymphoid stimulus anti-CD3/CD28, were able to induce IDO1 in DCs and monocytes (and not in B, T, and NK cells) supporting an important immune cell-to-cell interaction. This knowledge allowed us to optimize an in vitro model that enabled us to induce and manipulate pharmacologically IDO1^{+}cells. Before characterizing *in vitro*, the circulating myeloid cells of PD patients, including their ability to induce IDO1, we immunophenotyped these unstimulated cells of patients. DCs and monocytes of PD patients displayed inflammatory features including increased levels of pro-inflammatory subpopulations [plasmacytoid DCs and double negative (DN) classical DCs (cDCs) expressing CD16)] and higher frequencies of monocytes (total, classical, and NC), expressing the adhesion molecule CD62L. Importantly, these patients also showed increased expression of IL-10, which is known to induce T regulatory (Tregs) cells. This is another immunoregulatory mechanism. Other pro-inflammatory cytokines were increased (IFNs, IL-6, IL-12p70, IL-17A, and IL-33) indicating immune dysfunction. However, IDO1^{+}DCs and IDO1^{+}monocytes from PD patients remained at low levels. This is suggestive that the inflammatory profile of our mild-stage PD patients was not sufficient to significantly induce this enzyme. *In vitro* characterization of monocytes and DCs showed that LPS-stimulated monocytes from PD patients had a higher reduction of the relative frequency (RF) of non-classical monocytes (NC-Mo) and a higher IDO1-induction when compared with healthy subjects (HS). Importantly, DA seems to accentuate this NC-Mo reduction in PD patients. Moreover, DA decreased IDO1-induction in DCs and monocytes through βeta 2 adrenergic receptors (β_2AR). The effect of DA on cytokines and IDO1 suggests an anti-inflammatory profile of DA by attenuating the effect of LPS stimulation. Salbutamol, which is a β_2AR

agonist, seems to have a similar effect to DA, but milder. Salbutamol showed a significantly lower impact on PD patients' monocytes when compared with HS. Overall, our data suggest that β2AR signaling is impaired in IDO1^{+}monocytes from PD patients. The immunomodulatory effects of DA may also be mediated by other adrenoceptors including αAR, β_1AR, or β_3AR. This thesis highlighted IDO1^{+}monocytes as diagnostics and therapeutic avenues in PD. We also argue that further understanding the role of catecholamines as immunomodulators is of paramount importance to optimize current therapeutic strategies in PD.

Estratto in italiano

La malattia di Parkinson (MP) è una malattia neurodegenerativa in cui le disfunzioni immunitarie centrali e periferiche sono state indicate come una componente critica della suscettibilità e della progressione di questa malattia. Le cellule dendritiche (CD) i monociti sono attori chiave nel promuovere la regolazione della risposta immunitaria e possono indurre l'enzima immunomodulatore indolammina 2,3-diossigenasi 1 (IDO1) in ambienti proinfiammatori. IDO1 è un enzima limitante la velocità della cascata della chinurenina che converte il triptofano in chinurenina. Le funzioni immunitarie della cascata, ampiamente descritte nel cancro, sono inesplorate nella neurodegenerazione. Tuttavia, disfunzioni della cascata del segnale della chinurenina sono state descritte nel MP e associate a funzioni neurotossiche. Questa tesi si concentra sulle CD e sui monociti esprimenti l'enzima IDO1. Inoltre, viene esplorato il ruolo delle terapie dopaminergiche nel sistema immunitario, inclusa la modulazione di IDO1. Per prima cosa abbiamo indotto IDO1 nelle cellule mieloidi con diversi stimoli proinfiammatori. Il lipopolisaccaride (LPS) per lo stimolo della serie mieloide, così come lo stimolo linfoide anti-CD3/CD28, sono stati in grado di indurre IDO1 nelle DC e nei monociti (e non nelle cellule B, T e NK) supportando l'ipotesi di um ruolo importante sistema d'interazione immunitaria cellula-cellula. Questa conoscenza ci ha permesso di ottimizzare un modello *in vitro* che ci ha permesso di indurre e manipolare farmacologicamente le cellule IDO1^{+}. Prima di caratterizzare *in vitro* le cellule mieloidi circolanti dei pazienti con malattia di Parkinson, inclusa la loro capacità di indurre IDO1, abbiamo immunofenotipizzato queste cellule non stimolate dei pazienti. Le DC e i monociti dei pazienti con PD mostravano caratteristiche infiammatorie, tra cui livelli aumentati di sottopopolazioni pro-infiammatorie [DC plasmacitoidi e DC classiche doppio negative (DN) (cCD) che esprimono CD16)] e frequenze più elevate di monociti (totali, classici e non classici), che esprimono la molecola di adesione CD62L. È importante sottolineare che questi pazienti hanno anche mostrato una maggiore espressione di IL-10, che è nota per indurre le cellule T regolatorie (Tregs). Questo è un altro meccanismo immunoregolatorio. Altre citochine proinfiammatorie sono risultate aumentate (IFN, IL-6, IL-12p70, IL-17A e IL-33) indicando una disfunzione immunitaria. Tuttavia, le IDO1^{+}DC e i monociti IDO1^{+} dei pazienti con MP sono rimasti a livelli bassi. Ciò suggerisce che il profilo infiammatorio dei nostri pazienti con MP in stadio lieve non era sufficiente per indurre in modo significativo questo enzima. La caratterizzazione in vitro di monociti e DC ha mostrato che i monociti stimolati con LPS di pazienti con MP avevano una maggiore riduzione della frequenza relativa dei monociti non classici e una maggiore induzione di IDO1 rispetto ai soggetti sani. È importante sottolineare che la dopamina sembra accentuare questa riduzione dei monociti non classici nei pazienti con MP. Inoltre, la dopamina è risultata associata all'induzione di IDO1 nelle DC

e nei monociti attraverso i recettori adrenergici βeta 2 (RAβ_2). L'effetto del dopamina sulle citochine e sull'IDO1 suggerisce un profilo antinfiammatorio del dopamina attenuando l'effetto della stimolazione dell'LPS. Il salbutamolo, che è un agonista RAβ2, sembra avere un effetto simile alla dopamina, ma più lieve. Inoltre, il salbutamolo ha mostrato un impatto significativamente inferiore sui monociti dei pazienti con MP rispetto ai pazienti sani. Nel complesso, i nostri dati suggeriscono che la segnalazione RAβ_2 è compromessa nei monociti IDO1$^+$ di pazienti con MP. Inoltre, gli effetti immunomodulatori della dopamina possono anche essere mediati da altri adrenorecettori tra cui RAα, RAβ_1 o RAβ_3. In definitiva, questa tesi ha evidenziato il potenziale ruolo del monociti IDO1$^+$ nel *setting* diagnostico e terapeutico nella MP. Sosteniamo inoltre che comprendere ulteriormente il ruolo delle catecolamine come immunomodulatori è di fondamentale importanza per ottimizzare le attuali strategie terapeutiche nella MP.

Sumário em português

A doença de Parkinson (DP) é uma doença neurodegenerativa onde as disfunções do sistema imune, a nível central e periférico, têm sido sugeridas como uma componente importante na suscetibilidade e progressão da doença. As células dendríticas (CDs) e os monócitos são componentes imunes responsáveis pela promoção e regulação das respostas imunes. Em ambientes pró-inflamatórios, estas células têm a capacidade de induzir a enzima imunomoduladora indoleamina 2, 3-dioxigenase 1 (IDO1). Esta enzima é a enzima limitante da via da quinurenina e converte o aminoácido triptofano (Trp) em quinurenina, o que constitui o primeiro passo desta via. As funções imunes desta via, as quais são extensivamente descritas no cancro, são pouco exploradas nas doenças neurodegenerativas. No contexto destas doenças, as disfunções na via têm sido essencialmente associadas à neurotoxicidade produzida pelos seus metabolitos. Esta tese foca-se na função imune da IDO1 na DP através do estudo das CDs e dos monócitos produtores de IDO1. Para além disso, este trabalho explora o papel da dopamina na modulação do sistema imune, incluindo a imunomodulação da IDO1. Para tal, primeiro induzimos a IDO1 nas células mieloides circulantes usando diferentes estímulos pró-inflamatórios. O lipopolissacarídeo (LPS) foi usado como estímulo mieloide e o anti-CD3/CD28 como estímulo linfoide e ambos tiveram a capacidade de induzir a IDO1 nas CDs e nos monócitos (mas não nas células B, T e NK), demonstrando a importância da interação entre as células imunes. Esta informação, permitiu-nos otimizar um modelo *in vitro* no qual conseguimos induzir e manipular farmacologicamente as células IDO1^{+}. As células mieloides circulantes não estimuladas dos doentes de Parkinson foram fenotipadas e a sua capacidade de induzir IDO1 foi determinada, antes da caracterização destas células *in vitro*. As CDs e os monócitos dos doentes de Parkinson apresentam características inflamatórias, incluindo níveis elevados de subpopulações pró-inflamatórias [(CDs plasmacitóides e CDs clássicas duplas negativas que expressam CD16) e elevadas frequências de monócitos (totais, clássicos e não clássicos) que expressam a molécula de adesão CD62L]. Estes doentes também expressam níveis elevados de IL-10, a qual tem a capacidade de induzir células T reguladoras (Tregs), o que constitui outro mecanismo imunorregulador. A disfunção imune também é suportada por níveis elevados de citocinas pró-inflamatórias (IFNs, IL-6, IL-12p70, IL-17A e IL-33). No entanto, as CDs-IDO1^{+} e os monócitos-IDO1^{+} dos doentes de Parkinson mantêm-se em níveis baixos, o que sugere que o perfil infamatório dos nossos doentes não é suficiente para induzir a IDO1 de forma significativa. A caracterização *in vitro* das CDs e dos monócitos mostrou que os monócitos dos doentes de Parkinson estimulados com LPS têm uma maior redução da frequência relativa dos monócitos não clássicos e uma maior indução da IDO1 quando comparados com os indivíduos saudáveis. A exposição destas células à dopamina parece acentuar esta redução dos monócitos não

clássicos nos doentes. Para além disso, a dopamina diminuiu a indução de IDO1 nas CDs e nos monócitos por ação nos recetores adrenérgicos βeta$_2$ (RAβ_2). O efeito da dopamina nas citocinas e na IDO1 sugere um perfil anti-inflamatório desta, com capacidade de atenuar o efeito do LPS. O Salbutamol, um agonista dos RAβ_2, parece ter um efeito semelhante ao da dopamina, embora menos marcado. O impacto do salbutamol nos monócitos dos doentes de Parkinson é significativamente menor quando comparado com o efeito nos indivíduos saudáveis. Os nossos resultados sugerem que a sinalização pelos RAβ_2 está comprometida nos monócitos IDO1$^+$ dos doentes de Parkinson. Além disso, os efeitos imunomoduladores da dopamina podem também ser mediados por outros recetores adrenérgicos tais como RAα, RAβ_1 ou RAβ_3. Em suma, esta tese, enaltece a putativa importância terapêutica e de diagnóstico dos monócitos-IDO1$^+$ na DP. Além disso, a compreensão do papel das catecolaminas como agentes imunomoduladores é um parâmetro de extrema importância para a otimização das estratégias terapêuticas atualmente em uso na DP.

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O doutoramento é realmente um processo individual, mas impossível de realizar sem vós. Obrigada a todos.

Biography

Milene Vieira Gonçalves was born on 12th January 1988 in Leiria, Portugal. When she was 14 years old, she lost her older brother in a car accident and that was the moment when she decided to follow a profession related to health. She received her degree in biomedical science in October 2010, with a merit scholarship for the academic year 2008/2009. From February to May 2009, she was an ERASMUS student at Turku University of Applied Sciences, Finland, where she performed a microbiology internship at the National Institute for Health and Welfare, Turku, Finland. During this internship, she got her first contact with research being amazed for it. Arriving in Portugal, she looked for research laboratories and integrated the Immunology and oncology laboratory from the Center of Neuroscience and Cell Biology in Coimbra. Simultaneously, she performed a Biochemistry master's at the Faculty of Sciences and Technology of the University of Coimbra, Portugal. She finished her master's in 2012 presenting her thesis with the title 'Characterization of Peripheral Immune Response in Alzheimer's Disease'. From January to December 2012, she was also a research fellow in the project 'LRRK2 role on auto-antibody production by human B cells, funded by the Michael J Fox Foundation. From May 2013 to July 2018, she integrated the routine laboratory 'Laboratório de São José, Lda' as a laboratory technician and quality manager director where she consolidated her laboratory skills. From October 2018 to the present moment, she is a PhD student awarded with an individual scholarship from the Portuguese Foundation for Science and Technology (FCT). She is enrolled in a double PhD agreement (since 2019) between the University of Coimbra, Portugal (PhD Programme in Experimental Biology and Biomedicine) and the University of Insubria, Italy (PhD Programme in Clinical and Experimental Medicine and Medical Humanities). She is performing her thesis in Neuroscience and Disease: 'IMMU-KYN-PARK: exploring indoleamine 2, 3-dioxygenase 1 (IDO1) in peripheral immune system in Parkinson's Disease' under the supervision of Professor Frederico Guilherme da Costa Pereira, Professor Marco Cosentino, and Professor Maria Cristina Januário dos Santos. From January to November 2020, she was an ERASMUS student at the University of Insubria in the Center of Research in Medical Pharmacology under Professor Marco Cosentino's co-supervision. During the PhD Milene had the opportunity to participate in the GreenLabsCIBB initiative (Coimbra, Portugal), which is committed to foster ecological sustainability, as a greenkeepersTeam member. This team contain members from each research group that divided into small working groups dedicated to sustainable tasks. The aim of this initiative is reducing the ecological footprint of the laboratories at the national level. PhD also allowed Milene to get in touch with patients during their appointments, which increased the curiosity and passion about clinical research. Milene 'felt' the patients' symptoms

and discovered that she needed more field knowledge to make research that suppress patients' needs. She is currently a second-year medical student at the Faculty of Medicine from the University of Coimbra. Simultaneously, Milene is a daughter of a father with PD and a PD caregiver mother, which become this cause more personal. Since May2023, Milene is a laboratory technician at the blood service and medical transfusion in Coimbra Hospital and University Center. She is also married to André Rafael Agudo Cardoso (since May 2015) and mother of two amazing children: Laura Gonçalves Cardoso (7 years old) and Noah Gonçalves Cardoso (2 years old). She believes her family is the secret pillar that allows her to keep being passionate about science and the study of the human body.

Scientific Dissemination

- Gonçalves M, IDO1 activation decreased T cell proliferation. Oral presentation at the Settima Giornata Scientifica 2020, Corso di Dottorato di ricerca in Medicina Clinica e Sperimentale e Medical Humanities, Università degli Studi dell'Insubria.

- Gonçalves M, Exploring IDO1 in PD: immune cellular distribution and dopamine interaction. Oral presentation at the Ottava Giornata Scientifica 2021, Corso di Dottorato di ricerca in Medicina Clinica e Sperimentale e Medical Humanities, Università degli Studi dell'Insubria.

- Gonçalves M, Morgadinho A, Rodrigues-Santos P, Almeida J, Alves V, Santos Rosa M, Carlos Fontes ribeiro A, Cosentino M, Januário C, Pereira FC. IDO1 - a druggable target in immune dysregulation in Parkinson's Disease. Best poster presentation at CIBB – Center for Innovative Biomedicine and Biotechnology meeting 2022.

- Gonçalves M, Morgadinho A, Rodrigues-Santos P, Almeida J, Alves V, Santos Rosa M, Carlos Fontes ribeiro A, Cosentino M, Januário C, Pereira FC. Are $IDO1^+$ myeloid cells a druggable target in Parkinson's Disease. Guest speaker at the brain corner session of moving on series – what contributes to the well-being in PD? Event promoted by Portuguese society of Neurology, Portuguese society of Movement Diseases and BIAL.

- Gonçalves M, Morgadinho A, Rodrigues-Santos P, Almeida J, Alves V, Santos Rosa M, Carlos Fontes ribeiro A, Cosentino M, Januário C, Pereira FC. Are $IDO1^+$Dendritic cells a druggable target in Parkinson's Disease? Virtual poster presentation 284.11 at the Neuroscience meeting from Society for neuroscience 2022.

- Gonçalves M, Morgadinho A, Rodrigues-Santos P, Almeida J, Alves V, Santos Rosa M, Carlos Fontes ribeiro A, Cosentino M, Januário C, Pereira FC. Dopamine reduces indoleamine 2,3-dyoxigenase-1 inducibility in circulating myeloid cells through b-adrenergic receptors: is this relevant in Parkinson's Disease? Oral presentation at the Portuguese Society of Pharmacology, 2023.

- Gonçalves M, Morgadinho A, Rodrigues-Santos P, Almeida J, Alves V, Santos Rosa M, Carlos Fontes ribeiro A, Cosentino M, Januário C, Pereira FC. SOS Salbutamol: are β2 Adrenergic receptors druggable

targets to modulate inflammation in Parkinson's Disease? Oral presentation at the 1st Immune Parknet Annual Conference, Türkiye 2023.

- Gonçalves M, Morgadinho A, Rodrigues-Santos P, Almeida J, Alves V, Santos Rosa M, Carlos Fontes ribeiro A, Cosentino M, Januário C, Pereira FC. Dopamine as an immunomodulator agent in Parkinson's disease. Oral presentation at the Annual congress of Portuguese Society of movement disorders 2023.

- Gonçalves M, Furgiuele A, Rasini E, Legnaro M, Ferrari M, Rodrigues-Santos P, Caramelo F, Pereira FC, Cosentino. Evaluating the expression and activity of IDO1 in human circulating cells under a specific proinflammatory milieu: is this a possible in vitro Parkinson´s Disease model? Oral presentation at the 2nd Immune Parknet Annual Conference, Sevilha.

- Gonçalves M, Morgadinho A, Rodrigues-Santos P, Almeida J, Alves V, Santos Rosa M, Carlos Fontes ribeiro A, Cosentino M, Januário C, Pereira FC. SOS Salbutamol: are β_2 receptors druggable targets to modulate inflammation in Parkinson's Disease? Poster presentation at the 19th World Congress of Basic and Clinical Pharmacology, Glasgow.