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Blockade of CD16/32 phagocytic receptors in microglial-mediated dopaminergic elimination in MPTP experimental model: immunotherapeutic strategy for Parkinson's disease.

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Index

List of abbreviations

6OHDA	6-hydroxidopamine
AD	Alzheimer's disease
ANOVA	Analysis Of Variance
Ara-C	Cytosine Beta-D-arabinofuranoside
Aβ	Amyloid Beta
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
BSA	Bovine Serum Albumin
C3	Complement component 3
CA	California
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CNS	Central nervous system
COMT	catechol-o-methyl transferase
Cq1	Complement component q1
CR3	Complement receptor 3
CRT	Calreticulin
CSF	Cerebrospinal fluid
DA	Dopamine
DAB	Diaminobenzidine
DAG	diacylglycerol
DBS	Deep brain stimulation
DMEM	Dulbeccos Modified Eagle Medium

ER	Endoplasmic reticulum
FBS	Heat-inactivated fetal bovine serum
Fc	Fragment crystallizable
FcR	Fc-type immunoglobulin receptors
FcγR	FC-gamma immunoglobulin receptors
FUDR	5-Fluoro-2'-deoxyuridine
Gas-6	Growth-arrest specific 6
GDNF	Glial-derived neurotrophic factor
GEF	Guanine nucleotide exchange factor
GFAP	Glial fibrillary acidic protein
HLA-DR	Human Leukocyte Antigen D-related
HOCL	Hypochlorous acid
HS	Horse serum
IFN-γ	Interferon-gamma
IgG	Immunoglobulin G
IL	Interleukin
INc	Institut de Neurociències
iNOS	Inducible nitric oxide synthase
IP3	Inositol triphosphate
ITAM	Immune-tyrosine activation motifs
JNK	c-Jun N-terminal kinase
LAT	Linker for activation of T cells
LB	Lewy Bodies
LcK	Lymphocyte-specific protein tyrosine kinase

LN	Lewy Neurites
LPS	Lipopolysaccharide
LRP	Low density lipoprotein receptor related protein
LRRK2	Leucin-rich repeat kinase 2
LSD	Least significant difference
MA	Massachussetts
MAO-B	Monoamine oxidase B
MBP	Myelin Basic Protein
MerTK	MER receptor Tyrosine Kinase
MFG-E8	Milk fat globule EGF factor 8
MHC-II	Major Histocompatibility Complex II
MO	Missouri
MPTP	1-methyl-4-phenyl- 1,2,3,6-tetrahydropyridine
NB	Neuro Basal
NFκB	Nuclear Factor -Kappa B
NIH	National Institute Of Health
NK	Natural Killers
NM	Neuromelanin
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drugs
P/S	Penicillin-streptomycin
PBS	Phosphate-Buffered Saline
PD	Parkinson's disease
PET	positron emission tomography

PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-kinase
PIP3	Lipid phosphatidylinositol-3,4,5-triphosphate
PLCγ	Phospholipase C gamma
PS	Phosphatidylserine
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
rpm	Revolutions per minute
SNpc	Substantia Nigra *pars compacta*
Syk	Spleen tyrosine kinase
T lymphocytes	Thymus-derived lymphocytes
TGFβ1	Transforming growth factor beta-1
TNFα	Tumor necrosis factor alpha
TREM-2	Triggering Receptor Expressed on myeloid cells-2
UAB	Universitat Autònoma de Barcelona
UC	Ulcerative colitis
USA	United States of America
VNR	Vitronectin receptor
WASp	Wiskott-Aldrich Syndrome protein

List of Figures

List of tables

Summary

Parkinson's disease (PD) is a neurological syndrome characterized by the loss of mesencephalic dopaminergic neurons located in the Substantia nigra *pars compacta* (SNpc). The etiology of the disease is still unknown, although several evidences point to neuroinflammatory processes as important contributors of dopaminergic elimination. Microglial-mediated neuroinflammation contributes to the dopaminergic neuronal loss in PD, since activated microglia is present in the SNpc of post-mortem brains of PD patients and it is thought to be harmful for the surviving neurons. In addition, high circulating levels of IFN-γ in aging population may represent a risk for PD-related neuroinflammation, since it is considered that microglial cells are primed with IFN-γ, becoming susceptible to be fully activated and overreactive with a secondary stimulus like potential antigens, such as gut-derived lipopolysaccharide (LPS). Importantly, previous studies of our group demonstrated that IFN-γ is a critical cytokine particularly involved in microglial activation which leads to phagocytosis of stressed dopaminergic neurons in PD models in mice and monkeys. Because microglial activation and subsequent phagocytic capacity depends of Fc-gamma receptors (FcγR) and this receptor is overexpressed in pro-inflammatory conditions. In this thesis, we propose a new therapeutic strategy by using CD16/32 neutralizing antibody against FcγR to reduce microglial reactivity and phagocytic function *in vitro* and *in vivo* in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) experimental mouse model of PD. Our data, demonstrate that blockade of CD16/32 phagocytic receptor prevented the dopaminergic elimination *in vitro* and in the SNpc of the MPTP model of PD. Importantly, since CD16/32-mediated phagocytosis is tightly regulated by Cdc42, the inhibition of this important mechanistic factor downstream the FcγR, resulted in a similar outcome as blocking CD16/32, preventing the dopaminergic elimination *in vitro* in and in the MPTP model of PD.

These results suggest that, because microglial activation is an important factor in the inflammatory-mediated dopaminergic neurodegeneration, targeting their signaling with different approaches will help us to understand the role of the neuroinflammatory response and most importantly highlight the significance of the inflammatory component to design disease-modifying therapies for PD.

Introduction

Understanding Parkinson's disease

Parkinson's disease (PD) is a degenerative and progressive brain disorder manifested by specific movement disorders, such as akinesia/bradykinesia, gait alterations, tremor and postural overturn (Kalia & Lang, 2015; Kouli et al., 2018; Saavedra-López et al., 2016). However, a few non-motor symptoms are also related (olfactory disfunction, sleep disorders and gastrointestinal complications) some of them could even precede the motor symptoms (Kalia & Lang, 2015; Timeline et al., 2016). PD is estimated as the second most common neurodegenerative disorder, after Alzheimer's disease, of mid-to-late life in the western world, and it will become increasingly prevalent in developed countries in the present century (Kouli et al., 2018; Ray Dorsey et al., 2018). According to the PD Foundation and the European Parkinson's Disease Association (EPDA), an estimated to 10 million people worldwide are living with PD, being 1.2 million people just in Europe.

PD is a complex disease, with a combination of genetic and environmental factors playing a role in the cause.

PD affects people of all cultures, however, ethnicity could be a risk factor since in the USA, incidence is highest in people of Hispanic ethnic origins compared with non-Hispanic Whites, Asians and Blacks (Kalia & Lang, 2015). According to the Michael J Fox Foundation (MJFF), men are diagnosed with PD at a higher rate than women. However, age is the greater risk factor to PD, being 60 years the median age of diagnosis, although there are 10-20% of people with PD who experience symptoms before 50 years (Kouli et al., 2018).

Even though PD is an idiopathic disorder, 10-15% of the patients have familial forms, suggesting a genetic factor. Mutations in genes such as PARKIN, LRRK2, PINK1, SNCA, PARK 7 and also mutations in GBA and UHCL1 genes are linked to the familial form of PD (Belin & Ran, 2014; Schapira & Jenner, 2011; Schulte & Gasser, 2011).

Environmental factors also play a role in the risk of PD. Factors that increase the incidence include pesticide exposure, like rotenone or paraquat, prior head injury, beta-blockers use, among others. Factors associated with the decreased risk of PD are the intake of caffeine,

nicotine and the use of non-steroidal anti-inflammatory drugs (NSAID) like ibuprofen (Jiménez-Ferrer & Swanberg, 2018; Kalia & Lang, 2015).

Available therapies for PD only treat the symptoms without stopping or preventing the disease. Dopaminergic drugs designed to replace the striatal dopamine (DA) is the main treatment nowadays. The principal current treatment for PD is the DA-precursor L-DOPA, however prolonged use can lead to several side effects, like motor impairment (dyskinesias) and severe on-off motor fluctuations (Lees et al., 2009; Zahoor et al., 2018). Other therapies are used in order to replace the striatal DA such as DA agonists, monoamine oxidase B (MAO-B) inhibitors and catechol-o-methyl transferase (COMT) inhibitors (Poewe et al., 2017). Unfortunately, non-motor symptoms can fluctuate in response of dopaminergic therapies and these fluctuations can be even more disabling than the motor symptoms (Poewe et al., 2017). Therefore, a variety of non-dopaminergic treatments are available, such as benzodiazepine for sleep disorder, osmotic laxative for gastrointestinal problems, antidepressant, etc. (Kalia & Lang, 2015; Lees et al., 2009). Finally, deep brain stimulation (DBS), especially targeting the subthalamic nucleus, is a therapeutic option for patients with advanced PD with motor fluctuation and dyskinesias. However, patients with low response to L-DOPA treatment are not good candidates for this approach (Poewe et al., 2017) and PD advanced age may also hinder the adequacy.

The pathological hallmarks of PD are the loss of dopaminergic neurons, especially from the Substantia Nigra *pars compacta* (SNpc) and the presence of intraneural protein (α-synuclein) aggregates, located in the perikarya, termed as Lewy Bodies (LB) with cell processes termed Lewy Neurites (LN) (Dauer & Przedborski, 2003).

Dopaminergic neurons from the SNpc contain the pigment neuromelanin (NM) and their loss is reflected in the classical depigmentation of the SNpc (Dauer & Przedborski, 2003). These neurons project their axons to the striatum (composed by the caudate and putamen). Importantly, the loss of DA in this area the most probable cause of motor impairment in PD patients (Zinger et al., 2011). The great neuronal degeneration, even in early stages of the disease, suggest that neuronal depletion starts before the onset of motor symptoms (Poewe et al., 2017). Neuronal loss not only occurs in the SNpc. The locus coeruleus, nucleus basalis

of Meynert, raphe nucleus and dorsal motor nucleus of the vagus are also affected, as well as the olfactory bulbs and the autonomic nervous system (Kalia & Lang, 2015).

As the disease progresses, other non-dopaminergic neurotransmitters are also affected, such as, cholinergic, glutamatergic, GABAergic, noradrenergic, serotoninergic and histaminergic. Degeneration in those systems suggest the onset of some non-motor symptoms that do not respond well to dopamine therapies (Kalia et al., 2013; Kalia & Lang, 2015).

Another key component of PD pathogenesis is the intracellular accumulation of misfolded proteins, especially α-synuclein. These protein aggregates or LB are accompanied by neuronal processes or LN. Neurons containing LB go through neurodegenerative processes and finally die (Zinger et al., 2011). According to Braak and colleagues staging model, the Lewy pathology spreads rostrocaudally in the brain in a temporal sequence. At stage 1 and 2 enteric autonomic system is affected, as well as anterior olfactory nucleus and sleep-related dysfunctions. At stage 3 and 4 midbrain and basal prosencephalon is affected by the pathology, observed in the neuronal loss in the SN, finally reaching the neocortex at stage 5-6 (Braak et al., 2003).

After the staging of Braak's model, subsequent studies indicated that the distribution of LB does not follow a spreading pattern like prion pathologies. Withal, another critic is that the Braak system has not been focused on neuronal loss but only on LB distribution (Surmeier et al., 2017). Additionally, some PD patients have very few or no LB (Berg et al., 2014). Although Braak's assumptions are intriguing, it is clear that more studies should be done about the pathogenicity of LB and reevaluate the categorization of different subtypes that are affected in PD.

The cause of the dopaminergic degeneration is still unknown, however several mechanism have been implicated in PD pathogenesis: α-synuclein aggregation, mitochondrial dysfunction, oxidative stress, cytotoxicity, dysregulation of calcium homeostasis and, most importantly for the present thesis, neuroinflammation (Michel et al., 2016).

In the present work we are going to focus our attention to studying the neuroinflammatory features in PD, particularly microglial function in the parkinsonian-like-inflammatory scenario.

Neuroinflammation and PD

During the last decade, growing evidence suggests that neuroinflammatory processes are involved in the progression of PD. While initially was considered as a consequence of the degeneration, it has become clear that neuroinflammation seems to function as a synergic process, since neurodegeneration can trigger neuroinflammatory response and this reaction may provoke additional neurodegeneration (Hirsch et al., 2012; Saavedra-López et al., 2016).

In principal, neuroinflammation should be considered as a protective and beneficial response to initiate a healing process, promoting debris clearance, secreting neurotrophic factors and supporting tissue repair (Tansey & Goldberg, 2010). However, is now increasingly accepted as a double-edged sword, since exacerbated reaction can become detrimental, due to an excessive and prolonged reaction, which can lead to the increase of reactive oxygen species (ROS) and pro-inflammatory cytokines. In addition, the beneficial effects of neuroinflammation seems to be inadequate or insufficient in neurodegenerative diseases, since a reduction generating the favorable response may remove an important defense mechanism and consequently worsening the disease progression (Gao & Hong, 2008).

Brain inflammation occurs within the blood brain barrier (BBB) boundaries, where there is a relative absence of leukocytes and antibodies. However, local inflammation in the brain may trigger certain influx of peripheral blood cells into the brain parenchyma (Brown & Neher, 2010a). Therefore, neuroinflammation is characterized by three main phenomena: "1) the activation of local glial cells, 2) the release of specific cytokines and chemokines and 3) the recruitment and infiltration of peripheral blood cells into the brain parenchyma" (Saavedra-López et al., 2016) (Figure I). Generally, these three aspects are considered as elements of a protective response in order to initiate the healing process and restore homeostasis in most of brain injuries.
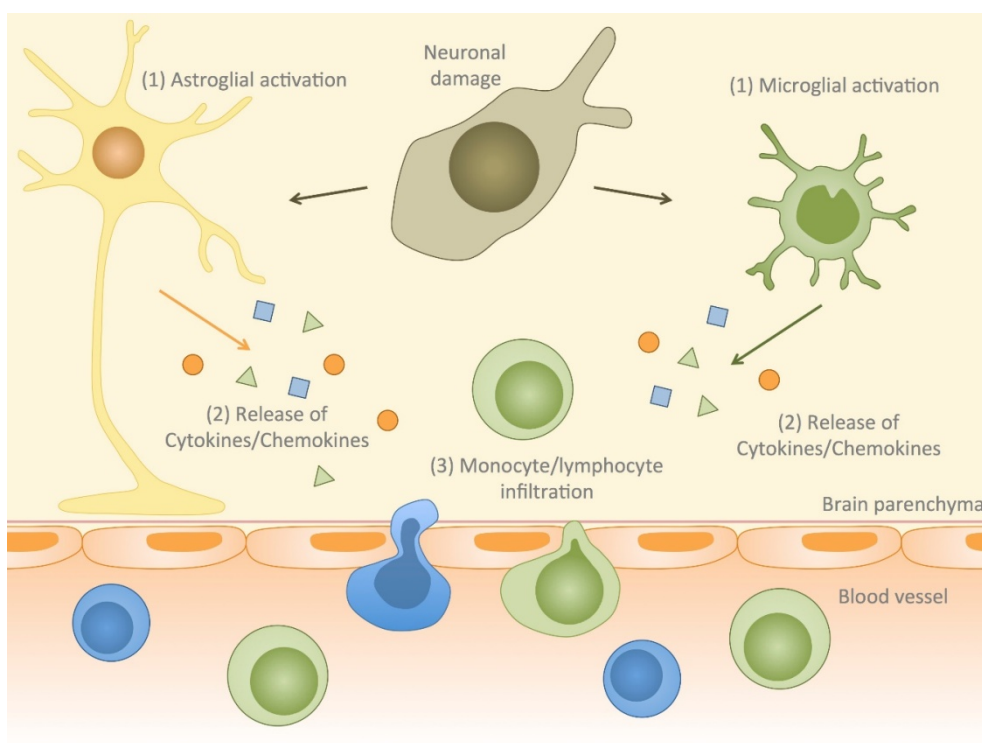
Figure I. **Cellular elements of neuro-inflammatory response in injured nerve tissue**. After neuronal damage (1) microglial and astroglial cells get activated. This activation causes (2) the release of pro-inflammatory cytokines and chemokines (3), which attract monocytes and lymphocytes to the brain parenchyma. The inflammatory environment seems to be damaging for remaining neurons. From: Saavedra et al., 2015.

However, the persistency of neuro-inflammatory response in *postmortem* studies of PD patients' brain suggests a negative contribution of neuroinflammation in neurodegeneration. In fact, decades ago, McGeer and colleagues found clear evidence of neuroinflammation in those brains, characterized by the activation of microglial cells expressing Human Leukocyte Antigen D-related (HLA-DR) in SNpc (McGeer et al., 1988).

This negative contribution of neuroinflammation was then supported by incidental findings in the conspicuous case of the frozen addicts (Langston & Palfreman, 1995) . In 1982 in Santa Clara, California several drug addicts were intoxicated with heroin contaminated with a neurotoxin called 1-methyl-4-phenyl- 1,2,3,6-tetrahydropyridine (MPTP). Interestingly these individuals exhibited parkinsonian symptoms, almost identical to idiopathic PD (Langston et al., 1999). In 1999 Langston and colleagues analyzed the brain of three of the deceased intoxicated persons, and the studies revealed that the neurons of the SNpc were depleted as PD patients. Moreover, the areas of dopaminergic degeneration presented active microglia expressing HLA-DR, identical to the studies performed by McGeer in PD brains in 1988 (Langston et al., 1999). This data demonstrated that active degeneration was ongoing many

years after the neurotoxic insult indicating that a single set of injections of MPTP, were able to initiate a persistent inflammatory response over the course of 15 years. Importantly, this phenomenon was simulated in experiments conducted with monkeys intoxicated with MPTP, revealing microglial activation in the SN years after the intoxication, confirming that neuroinflammatory response persists through the years (Barcia et al., 2004; Mcgeer et al., 2003) even without L-DOPA treatment. Also, studies indicated that injection of inflammatory agonists, such as lipopolysaccharide (LPS) to experimental animals, activates glial cells being deleterious to dopaminergic neurons (Herrera et al., 2000; W. G. Kim et al., 2000). Moreover, a combination of LPS with MPTP, is synergistic and exacerbate both glial activation and neurodegeneration indicating the role of microglia in degeneration of dopaminergic neurons (Gao et al., 2003).

Furthermore, *in vivo* analysis using the positron emission tomography (PET) with PK-11195 revealed elevated microglial activation in basal ganglia and mesencephalic areas of PD patients in early and late stages of the disease (Gerhard et al., 2006). Similar to these results, studies in monkeys with acute or chronic MPTP administration, showed similar inflammatory response, evidenced by HLA-DRalpha expression, mediated by microglial activation (Vázquez-Claverie et al., 2009).

Together with the histological evidence of *postmortem* studies, further biochemical analysis indicated a release of certain cytokines and chemokines probably mediated by glial cells (Barcia, 2013). Cytokines and chemokines are small proteins released in inflammatory process; they have several essential functions as part of the immune response. Both cytokines and chemokines are critical factors in the development of the central nervous system (CNS) inflammation since the local glial activation depends on cytokine levels and the recruitment of cells into the brain parenchyma is frequently driven by chemokines (Saavedra-López et al., 2016). Inflammatory cytokines such as interferon-gamma (IFN-γ), tumor necrosis factor (TNFα), interleukins (IL1β, IL6, IL2), CXCL12 and the anti-inflammatory transforming growth factor β1 (TGFβ1), are known to be increased in the striatum and SNpc of *postmortem* samples (Hunot et al., 1999; Mogi et al., 1994, 1996; Shimoji et al., 2009). In addition, studies in blood, plasma and cerebrospinal fluid (CSF) of PD patients showed upregulation of proinflammatory (IFN-γ, TNFα, IL1β, IL6, IL2) and anti-inflammatory (TGFβ1, IL4, IL10) cytokines (Brodacki et al.,

2008; Mogi et al., 1994, 1996; Nagatsu et al., 2000). These finding are important because revealed that inflammation in PD goes beyond the brain.

Overexpression of cytokines is also found in parkinsonian models. Some of the cytokines are elevated in mice after MPTP injection and seem to play a critical role in the inflammatory response (Hébert et al., 2003; Luchtman et al., 2009). Moreover proinflammatory cytokines such as TNF-α and IFN-γ are known to be elevated in humans and non-humans primates intoxicated with MPTP (Barcia et al., 2011).

The role of cytokines in PD is still poorly understood. Under precise regulation, inflammatory process mediated by release of cytokines, is normally self-limiting and essential for the CNS integrity (Gao & Hong, 2008). At first, the function of increased cytokines levels is actually beneficial, including restoration of damaged tissue, however the immune response can become exacerbated and destructive leading to a chronic persistent inflammatory state that becomes harmful for neurons (Barcia, 2013; Gao et al., 2016). Circulating cytokines induce glial activation, which leads to new cytokine release by neighboring glial cells. This response induces a vicious self-propagating cycle that may contribute to induce new neuronal degeneration, establishing a chronic process (Barcia, 2013). Evidence of detrimental effect of exacerbated cytokine release in parkinsonian macaques and mice proved that glial activation is mediated by IFN-γ signaling, aided by TNF-α and suggest that both cytokines are responsible for enhancing the activation of the surrounding glial cells contributing to neuronal degeneration (Barcia et al., 2011). Furthermore, genetic studies revealed the association of different loci with neuroinflammation in PD. For example, patients with sporadic PD show alterations in HLA-DR gene, which has been reported to significantly increase the risk of PD (Hamza et al., 2011; Jiménez-Ferrer & Swanberg, 2018). Likewise, Leucin-rich repeat kinase 2 (LRRK2) is one of the most associated genes with 13% of familial and 2% of sporadic PD cases (Jimenez-Ferrer & Swanberg, 2018; Russo et al., 2014) is involved in the regulation of IFN-γ immune response (Gardet et al., 2011). Moreover, LRRK2 gene also contributed to TNF-α production by microglia which is critical for glial activation (Nayak et al., 2014).

Although astrocytes and oligodendroglia are other important component of the neuroinflammatory response, we will review their contribution briefly, since the thesis is focused on microglial effect in the neuroinflammatory scenario of parkinsonian degeneration.

Evidence suggest that astrocytes are also activated in the *postmortem* tissues of PD patients (Forno et al., 1992). Similar to microglial cells, astrocytes are also observed activated in the areas of degeneration in parkinsonian models (Saavedra-López et al., 2016). Astrocytes are resident cells from the brain, their proper function is essential for normal development and plasticity of the CNS, including synapse formation and pruning through secreted factors and direct contact with the synapse (Gelders et al., 2018; Neal & Richardson, 2018). For instance, after acute trauma, inflammatory response mediated by astrocytes help to balance debris clearance with preservation of healthy tissue and restriction of the spread of additional cytotoxic inflammation (Liddelow & Barres, 2017). Likewise, astrocytes are able to release many factors such as Glial-derived neurotrophic factor (GDNF) and Brain-derived neurotrophic factor (BDNF) which may contribute to the restauration of the damaged tissue. Glial fibrillary acidic protein (GFAP) is a compound of the astrocytic cytoskeleton that indicates reactivity of astrocytes. This protein is found high expressed in the areas of degeneration in PD patients (Barcia, 2013; Forno et al., 1992). In addition, reactive astrocytes with high expression of GFAP are also present in the SNpc of chronic Parkinsonian macaques and in MPTP-treated mice (Barcia et al., 2011). However, like microglial cells, overreaction of astrocytes in neuroinflammation may become detrimental in pathological conditions such as PD (Gelders et al., 2018; Liddelow & Barres, 2017).

Astrocytes can release pro-inflammatory cytokines contributing to the inflammatory and neurodegeneration process. In ischemia it has been proposed that neuroinflammation can induce two types of reactive astrocytes, A1 and A2. Whereas A2 upregulate genes involved in synapse repair, survival and neuronal growth, suggesting a protective phenotype (Liddelow & Barres, 2017). In contrast, A1 are neurotoxic, they upregulate many classical cascade signals of the complement, previously shown to be destructive to synapse, loosing many of the normal astrocytic functions, like the ability to support synapse formation and tending to phagocytose neuronal elements. Moreover, there is evidence that microglia induce A2 to A1 by the release of IL-1α, TNF and Complement component 1q (C1q), indicating the importance of a microglia-astrocyte crosstalk considering that astrocytes activation relies on signals derived from the activation of other cells of the CNS, like microglia (Liddelow et al., 2017). Importantly, recent *in vivo* studies revealed that astrocytes and microglia acted in a highly coordinated fashion, "negotiating" the removal of apoptotic cells and debris, tightly regulated

by the receptor tyrosine kinase *Mertk* (Damisah et al., 2020). After reduction in microglial cell density, astrocytes compensated by engulfing cell bodies, but the overall process was less efficient than microglia (Damisah et al., 2020). These results evidenced the coordination between microglia and astrocytes and highlight the importance of the regulation of the microglial-astrocyte orchestration for recovery of homeostasis in age-related neurodegeneration.

Very few studies have been published regarding the contribution of oligodendroglia in the dopaminergic loss in PD. The presence of complement-activated oligodendroglia has been described in SN of postmortem PD tissue and the complement system opsonized damaged microglia for subsequent phagocytosis by microglia (Yamada et al., 1991). Moreover, inclusions of α-synuclein have been reported in oligodendroglia of PD patients (Wakabayashi et al., 2000). Experimental models of PD with MPTP have shown that mature oligodendrocytes, expressing Myelin Basic Protein (MBP) are overreactive, evidenced by the increase in the cell number and average size correlated with the reduction of dopaminergic fibers in the striatum (Annese et al., 2013). Importantly, these results are in accordance with microglial and astroglia reaction, suggesting an inflammatory-related phenomenon (Annese et al., 2013). Nevertheless, the mechanism driving the changes in oligodendrocytes after MPTP intoxication and its link with the proinflammatory environment are scarcely explored (Barcia, 2013).

Nowadays, some authors support the idea that neuroinflammation only occurs when the innate immune response is involved. However, the breakdown of the BBB and the presence of lymphocytes (such as T cells) in PD have been described (Brochard et al., 2009). Therefore, neuroinflammation should be considered as a process that includes all types of immune response in the CNS even though we are still too far to fully understand the implications of the adaptative immune response in PD (Saavedra-López et al., 2016).

As mentioned before, in *postmortem* tissue of PD patients, the presence of CD8+ and CD4+ T lymphocytes but not B cells or Natural Killers (NK), has been reported in the degenerating areas (Brochard et al., 2009). Importantly, these results were previously described in experimental models of PD in rat and mice, indicating an increase of CD4+ and CD8+ T cells after a neurotoxic insult (Kurkowska-Jastrzębska et al., 1999). Likewise, in Knock-out mice the

CD4+ T cells infiltration seems to contribute to the dopaminergic degeneration (Brochard et al., 2009). Some studies have suggested α-synuclein as a plausible antigen to be presented by the Major histocompatibility complexes II (MHC-II), which would support the hypothesis of an autoimmune disease, targeting the misfolded protein accumulated in dopaminergic neurons (Appel et al., 2010). This hypothesis would involve the phagocytosis of dopaminergic neurons by microglia/macrophages, the processing of the protein and the final presentation of the antigen in the microglial membrane, which remains to be elucidated (Barcia, 2013). The contribution and mechanism of adaptative immune response in PD, still remains unclear, therefore more detailed studies of the function of T lymphocytes represents a very promising field of research for the upcoming years (Barcia, 2013).

In summary, several rationales have been proposed about which mechanism are responsible for the induction of neuroinflammation and posterior degeneration in PD. On the one hand, α-synuclein has been postulated of one of the major triggers of neuroinflammation, this protein once in the extracellular space is phagocytosed by microglial cells, inducing microglial activation, increasing the expression of proinflammatory cytokines, NO and ROS contributing to the progression of dopaminergic degeneration (Hirsch & Hunot, 2009). On the other hand, the increase of pro-inflammatory cytokines released by the activated microglia have a direct effect on dopaminergic neurons by interacting with specific receptors, which can activate intracellular death pathways (Hirsch et al., 2012). Importantly, in pathological conditions such as PD there is evidence that overactivated microglia, as a consequence of inflammation, is able to phagocytose stressed and viable neurons, thus executing their death (Barcia et al., 2012; Neher et al., 2012; Vilalta & Brown, 2018). We will address in following sections, the features and mechanisms involved in microglial phagocytosis in neuroinflammation.

Microglia and PD

One of the most important cells participating in the neuroinflammatory process are microglia. Microglial cells are considered the macrophages of the brain since they are able, among other features, to phagocytose targets. However microglial and macrophages differ in their development, macrophages derived from monocytes originated in the bone marrow while microglial cells derived from the embryonic yolk sac (Bennett & Bennett, 2020; Ginhoux & Garel, 2018; Nayak et al., 2014). Microglial cells can be detected in the mice brain at embryonic days of 8 to 10 (E8 to E10). During the development, the microglial progenitor derived from the yolk sac does not display the typical ramified processes. However, trough developmental program they acquire a ramified phenotype commonly observed in the adult CNS. Once ramified, microglial cells are evenly spread across the parenchyma (Nayak et al., 2014).
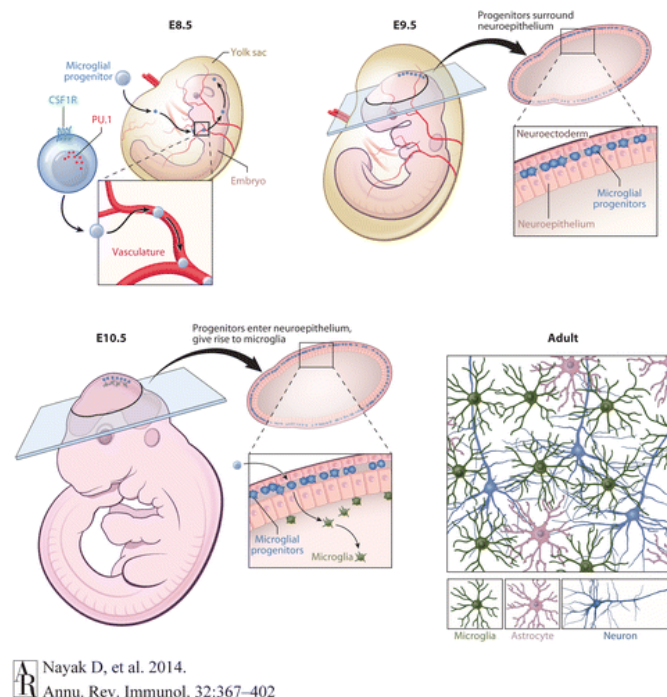
Figure II. Microglial origin and development. Recent studies have shown that microglia are ontologically distinct from bone marrow–derived monocytes/macrophages found in peripheral tissues. Microglia are instead derived from embryonic yolk sac during development and enter the brain rudiment via the circulatory system. Microglia at this stage of development have an amoeboid rather than a ramified morphology. Microglia become completely ramified throughout the brain by postnatal day 28. Development and survival of microglia are critically dependent on several factors, including the transcription factor PU.1 as well as CSF1R. From: Nayak et al., 2014.

Microglia are indispensable for normal brain development and maintenance. In physiological conditions, microglia retain a vivid capacity to survey the brain parenchyma, actively scanning the extracellular space, to detect different signals released from to the environment, in order to maintain brain homeostasis (Nimmerjahn et al., 2005). Environmental signals comprise invading pathogens, damaged or death cells, cellular debris, or the release of different compounds such as proinflammatory cytokines (Nimmerjahn et al., 2005).

During development, microglia contributes to synaptic pruning by engulfing axons and dendritic spines in order to eliminate excessive synapse and to promote the functional maturation of the appropriate ones (Kettenmann et al., 2013; Paolicelli et al., 2011). However, an exaggerated microglial pruning leads to abnormal synaptic development which may aggravate pathological diseases such as Alzheimer's disease (AD), schizophrenia, autism, or having detrimental effect in aging (Li & Barres, 2018; Vilalta & Brown, 2018).

About half of all immature neurons die during brain development primarily through programmed cell death. The reason for this programmed cell death is not fully understood, it has been proposed that the excess of defective neurons, resulting of a deficient differentiation and migration, requires elimination (Nayak et al., 2014). Microglial cells function as scavengers and phagocyte neuronal debris after programmed cell death, however microglial cells can induce cell death by the release of ROS, nerve growth factors and TNFα (Lull & Block, 2010; Marín-Teva et al., 2012). Thus, microglia are not simply accessory cells that respond to neuronal cell death during development, but rather are active promoters of the process (Marín-Teva et al., 2012; Nayak et al., 2014). Not only during development but also in the healthy adult brain, microglia develop their functions by removing synapses and cellular debris in the context of neuronal plasticity. Intriguingly, during adulthood, microglia are also able to remove apoptotic newborn neurons in the hippocampus (Sierra et al., 2010).

Morphologically, the ramified phenotype of microglial cells facilitates the constant scanning of the CNS parenchyma, using the filaments and projections to seek out alterations in the surroundings (Saavedra-López et al., 2016). Any disturbance in the CNS is sensed by microglial cells and induces their activation. Reaching this activated state microglia undergo dramatic biochemical and morphological changes, characterized by an augmentation of the entire cell body size and increased number of terminal tips retracting their filaments, consistent with the

classically named "ameboid" phenotype (Saavedra-López et al., 2016). Activated microglia rearrange surface molecules to induce cell motility, proliferate and move to the site of the lesion and release multiple factors with inflammatory effects, which activate neighboring glial cells (Barcia, 2013; Heneka et al., 2014). The term of microglial activation comprises a range of different "activated" stages, were the polarized cells were traditionally categorized as having either "toxic" (M1) or "protective" (M2) phenotype, depending on the expression of different markers (Heneka et al., 2014). However several evidence suggests that microglial polarization is multidimensional, with extensive overlap in gene expression, as opposed on a simplified linear expression (Dubbelaar et al., 2018; Li & Barres, 2018).

Consequent with the activation, as mentioned before, microglial cells release several inflammatory factors such as cytokines. Cytokines are categorized in proinflammatory and anti-inflammatory. Pro-inflammatory cytokines such as TNFα, IFN-γ, IL-1β and IL-6, facilitate the inflammatory response. However, as addressed above, their excessive and chronic release may result in detrimental consequences contributing to neurodegeneration (Barcia, 2013; Gao et al., 2016). These cytokines can have a direct effect on dopaminergic neurons, by interacting with their specific neuronal receptors, activating intracellular death pathways such Nuclear Factor -Kappa B (NFκB) (Hirsch & Hunot, 2009). In addition, these cytokines trigger the increase of inducible nitric oxide synthase (iNOS) expression, leading to the augmented production of ROS or NO, which can activate glial cells, and therefore a new increment of cytokine release, converting the process in a vicious cycle contributing to degeneration (Gao & Hong, 2008; Hirsch & Hunot, 2009).

In contrast, anti-inflammatory cytokines such as TGFβ1, IL4, IL10, control the proinflammatory response, promoting tissue repair and restraining the synthesis of pro-inflammatory cytokines (Hirsch & Hunot, 2009; Tansey & Goldberg, 2010).

Moreover, it has been suggested that activated glial cells can create a toxic oxidative environment that damage dopaminergic neurons (Hunot et al., 1996). Activated microglia can induce enzymatic complexes including nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) and iNOS. These enzymes form superoxide (O_2^-), NO and hypochlorous acid (HOCl) (Hunot et al., 1996; Wu et al., 2003). Each of these enzymes have been described in PD patients and models of PD and determined a role in toxicity to TH-positive cells of the SNpc. Postmortem tissue of idiopathic PD patients presented iNOS-immunoreactivity in the ameboid

microglia surrounding the fragmented neurons in the SNpc (Knott et al., 2000).Moreover, in MPTP rodents and postmortem tissue from sporadic PD patients, microglial activation in the SNpc is related with an induction of NADPH oxidase. In fact, the upregulation of the NADPH oxidase correlates topographically and temporarily with the dopaminergic degeneration seen in the MPTP and PD brains (Wu et al., 2003)

It is established that in the context of microglial activation, there is a process named "priming" in which microglial cells present a pre-activated state as a product of a primary inflammatory stimulus, susceptible to a secondary inflammatory stimulus in which become overreactive, triggering an exaggerated response (**Figure III**) (Perry & Holmes, 2014). The secondary stimulus can arise from the CNS (ie, protein aggregation, interaction with particular antigens genetic susceptibility, etc) but in aged individuals, the secondary stimulus most commonly appears from a systemic disease with inflammatory component (Perry & Holmes, 2014). Importantly studies have suggested that IFN-γ is increased in the aged brain (Maher et al., 2006) which may implicate IFN-γ as a candidate for microglial priming.

In the canonical proinflammatory stimulation, activation of macrophages, from which microglia share lineage, requires a "priming" stimulus such as IFN-γ and a "triggering" stimulus like LPS. The priming effect of IFN-γ is required to cause a phenotypic shift toward a more sensitized state characterized by increased expression of cell surface antigens, including MHC-II (Dilger & Johnson, 2008). This sensitized macrophage will then respond to the "triggering" stimulus more rapidly and in a greater degree than a non-primed macrophage (Dilger & Johnson, 2008; Schroder et al., 2006).

Several evidences suggest that systemic disease can affect the onset of degenerative diseases. In the healthy CNS, a transient peripheral infection, chronic exposure or low levels of infectious pathogens, can prime and also activate microglia (Cunningham, 2013; Perry, 2010). In the case of PD, lipopolysaccharide (LPS) derived from the bowel microbiome could play a role, especially in patients with constipation, a symptom very frequent in PD patients (Khalif et al., 2005). Then, increases of systemic exposure to LPS might further exacerbate neurodegenerative process through microglial priming (Perry & Holmes, 2014). Indeed, the progressive neuronal damage induce new microglia activation and priming by the increase of

cytokines or even misfolded proteins α-synuclein, establishing a vicious cycle, since primed microglia may be deleterious for the remaining neurons (Brown, 2019; Perry & Holmes, 2014).
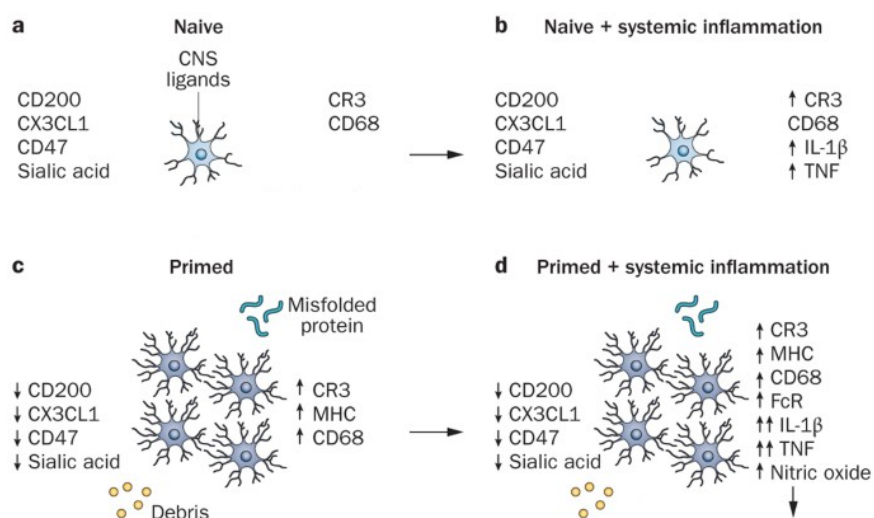
Figure III. Effect of systemic inflammation on microglia. **a**. In the absence of disease microglia kept in the quiescent state by regulatory factors in the brain parenchymal microenvironment and inhibitory ligands expressed on the surface of the neurons. **b.** Following an initial systemic inflammatory stimulus, cytokines and other inflammatory mediators in the blood signal to receptors on cerebral endothelial cells, which in turn signal to microglia. These stimuli transiently activate microglia, leading to release of cytokines and probably other molecules the brain that induce sickness behavior. **c.** During chronic neurodegeneration, neurotoxic misfolded proteins and debris from dying neurons accumulate. The resulting loss of inhibitory neuronal ligands and increase in neurodegenerative components lead to activation of microglia, indicated by altered morphology, increased proliferation and upregulation of cell surface molecules. **d.** In the setting of chronic neurodegeneration, the effect of systemic inflammatory stimulus on the CNS is amplified due to increased numbers of microglia, as well as the primed state of microglia. The resulting exaggerated release of cytokines and other inflammatory mediators lead to neurotoxicity. Modified from: (Perry & Holmes, 2014).

The phenomenon of microglia priming was first described within the brains of animals with prion disease (Combrinck et al., 2002; Cunningham et al., 2005). A systemic challenge with LPS to mimic aspects of a bacterial infection, led to an exacerbation of the symptoms of sickness behavior and later demonstrated that microglia was primed and responsible for the exaggerated response (Cunningham, 2013). Now, the phenomenon have been observed in in animal models of prion disease, AD, PD, stroke and multiple sclerosis (Cunningham, 2013; Perry & Holmes, 2014).

The pathways by which systemic inflammation leads to CNS inflammation have been mostly studied in rodent models, using either a peripheral challenge with LPS or with peripheral cytokines such as IFN-γ, either alone or in combination (Perry et al., 2010). However, the

sensitivity to LPS is much higher in humans than in rodents, meaning that the applicability of the results to humans should be cautiously considered (Fink, 2014). Besides, studies in humans and non-human primates challenged with lower doses of LPS were sufficient to induce microglial activation and sickness behavior (Brydon et al., 2008; Hannestad et al., 2012), therefore supporting the notion that systemic inflammation actually leads to microglial activation.

In the PD context, in animal models of 6-hydroxidopamine (6OHDA), injecting adenovirus expressing IL-1β systemically, provoked an increase of degeneration of dopaminergic cells in the SN accompanied by an increase of microglial activation (Pott Godoy et al., 2010).

Interestingly, those changes in systemic inflammation may come from gastrointestinal alterations as well as abnormal gut microbiota (Sampson et al., 2016). In fact, gastrointestinal disfunction is related to peripheral inflammation. For example, ulcerative colitis (UC) correlates with an increase of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6) in serum. And this peripheral inflammation, induced by UC, exacerbates the deleterious effects of LPS comprised by degeneration of dopaminergic neurons, microglial activation and alteration of the BBB permeability (Villarán et al., 2010). On the other hand, PD-derived gut microbiota promotes motor and gastrointestinal dysfunction triggering microglial activation (Sampson et al., 2016), which also illustrates that systemic changes may result in PD-related neuroinflammation.

Moreover, LPS-induced inflammation may also exacerbate the toxicity to pesticides, frequently related with PD prevalence. For example, it is known that toxicity with the herbicide paraquat causes oxidative stress and selective loss of dopaminergic neurons (Purisai et al., 2007). However, an initial paraquat exposure in naïve animals may not be sufficient to induce neurodegeneration, in contrast pre-treatment with LPS made a single exposure to paraquat capable of triggering loss of dopaminergic neurons accompanied by microglial activation (Purisai et al., 2007). In addition, the synergistic contribution of LPS and α-synuclein seems to be important in the exacerbation of microglial cells activation. Extracellular α-synuclein injected directly in the SNpc provoked a robust proinflammatory response, however when the animals were subsequently challenged with systemic LPS, levels of IL-1β in the SNpc became comparable to that induced by the direct injection of LPS into the brain (Couch et al., 2011).

In similar way, PD-microbiota exacerbates pathology in α-synuclein mice involving brain neuroinflammation (Sampson et al., 2016).

Similar to degenerative diseases, ageing has been proposed to converge in priming microglial cells (Dilger & Johnson, 2008; Perry, 2010). Activated microglia, evidenced by the expression of MHC-II, was found increased in the brain of healthy aged mice and systemic challenge of LPS resulted in exacerbated inflammatory cytokine response (Chen et al., 2008; Godbout et al., 2005). Hypothetically, this activated state of microglia during ageing may promote the susceptibility and vulnerability of some neuronal populations like DA neurons of the SNpc in PD.

The consequences of priming comprise an over-reactive inflammatory response which leads to neuronal loss mediated by apoptosis, as seen in the ME7 mice model of prion disease (Cunningham et al., 2005). These changes are associated with dramatic expression of cytokines such as IL-1, as well as NO in the brain (Cunningham et al., 2005). Another model which demonstrate the detrimental effect of priming indicated that multiple systemic challenge with poly I:C, in order to mimic viral infection in murine models of prion disease, lead to increased apoptotic cells in the hippocampus and thalamus and consequent sickness behavior characterized by an acute decline in motor performance of the animals, contributing to disease progression (Perry & Holmes, 2014). Whether these changes are translatable to PD is yet to be determined.

In addition to increased expression of cytokines that can damage neurons, primed microglia respond to further pro-inflammatory insults by altering their receptor repertoires. For example: In mice with chronic neurodegenerative disease mediated by prion disease, systemic inflammatory insult increases the expression of Fc-type immunoglobulin receptors (FcR) in microglia, with an expression many times greater than in the degenerative disease or systemic inflammation alone (Lunnon et al., 2011). Although immunoglobulin G (IgG) is not generally believed to enter the brain parenchyma, the BBB is apparently not entirely impenetrable to IgG (Perry & Holmes, 2014).

The overexpression of Fc-type receptor (FcR) becomes relevant to understand the contribution exacerbated microglia in neurodegeneration and for the current thesis. FcR is one of the multiple receptors expressed in a variety of cell types including CNS macrophages

and microglia (Fuller et al., 2014; Uribe-Querol & Rosales, 2020). Activation of these receptors initiate a range of biological response that includes triggering phagocytosis, degradation, and the release of cytokines and other mediators (Fuller et al., 2014; Okun et al., 2010)

Since these receptors are important for the onset of phagocytosis in microglial cells, and is a central topic for this thesis, in the next section of this work phagocytic process will be review in more detail.

Phagocytosis insight in PD

As mentioned above, there are increasing evidences that indicate the deleterious effect of exacerbated immune response in the CNS, particularly mediated by microglia and by the increased expression of cytokines that can directly damage neurons. However, besides cytotoxicity, another feature of microglial cells that entails the contact and physical interaction is phagocytosis. It was previously thought that neurons were phagocytosed only when dead or dying in order to maintain the CNS homeostasis. However, it is becoming clear that besides pathogens, viable synapses, dendrites, axons and whole neurons can also be phagocytosed contributing to neurodegeneration (Vilalta & Brown, 2018).

Phagocytosis is an essential process of the innate immune response, which functions relies in the defense against pathogens during infection and clearance of cellular debris produced during normal brain development and injuries (Brown & Neher, 2014; Fricker et al., 2012). Microglia, the resident brain macrophages, can become highly phagocytic when activated. Although other type of cells are able to accomplish phagocytosis, they are not as efficient as microglia, thus microglial cells are termed as professional phagocytes (Fricker et al., 2012; Uribe-Querol & Rosales, 2020). Phagocytosis is defined as the cellular process of sensing and engulfing particles larger than 0.5 µm, which also involves dramatic changes in the shape and movement of the cell (Barcia et al., 2012; Janda et al., 2018; Uribe-Querol & Rosales, 2020). The particle is internalized into a phagosome, this phagosome changes structure of the membrane and the composition of its contents in a process known as phagosome maturation. Next, the phagosome fuses with lysosomes to become a phagolysosome were containing enzymes can degrade the ingested particle. (Uribe-Querol & Rosales, 2020).

In basal conditions, microglial cells are constantly scanning the brain parenchyma and are susceptible to undergo morphological changes according to variations of the microenvironment. It is thought that microglia cells move to engulf particles as a key function of the immune response in the brain (Barcia, 2013; Nayak et al., 2014; Sierra et al., 2014). Previous *in vitro* studies reported that microglial cells are able to phagocytose fluorescent microspheres, opsonized beads or fluorescently labeled β-amyloid (Aβ), which addresses the phagocytic properties of microglia (Koenigsknecht-Talboo & Landreth, 2005; Majumdar et al., 2007; Ueyama et al., 2004). Moreover experiments performed in zebra fish using time-lapse

confocal microscopy, have demonstrated that microglia phagocytose portions of apoptotic neurons during embryo development *in vivo* and that the formation of phagosomes and phagolysosomes is mediated by de v0-ATPase a1 subunit (Peri & Nüsslein-Volhard, 2008). On the other hand, in adults, apoptotic newborn cells are phagocytosed by microglial filaments forming ball-and-chain structures which is a relevant process for the homeostasis of the brain parenchyma (Sierra et al., 2010). Apart from the stages of embryo development and the formation newborn cells, how the engulfing process takes place in neurodegeneration is still under investigation.

In the context of neurodegenerative diseases, in an *in vitro* model of AD, nanomolar concentrations of Aβ activate microglial cells leading to the phagocytosis of viable neurons and synapses, and the blocking of phagocytosis by blocking the exposure of phosphatidylserine (PS) on vitronectin receptor (VNR), the neuronal loss and death were prevented (Neher et al., 2011; Neniskyte et al., 2011). Similarly, *in vitro* experiments where neuroinflammation was induced by LPS, caused a slow progressive neuronal loss by phagocytosis. Blocking milk fat globule EGF factor 8 (MFG-E8), an opsonin which binds to exposed PS triggering the phagocytosis via VNRs, prevented the neuronal loss leaving viable neurons in the culture (Fricker et al., 2012). Analogously, LPS injection into the striatum of rats and mice caused a strong microglial inflammation and neuronal loss, this neuronal loss was reduced in *Mfge8* knockout mice and after co-injection with VNRs inhibitors (Fricker et al., 2012). In addition, focal brain ischemia produced delayed neuronal death, functional impairment and up-regulation of the expression of MFG-E8 in rats and mice. Interestingly, *Mfge8* knockout mice presented limited microglial phagocytosis therefore, prevented neuronal loss and brain atrophy, after focal brain ischemia (Neher et al., 2013).

Regarding PD, rotenone induced microglial phagocytosis of neurons *in vitro*, blocking phagocytosis prevented neuronal loss and death (Emmrich et al., 2013). Importantly, previous studies of our group, demonstrated that phagocytosis of neurons can be prevented by blocking RHO-associated protein kinase (ROCK) which is a protein involved in the microglial cell motility, in a PD model with MPTP-induced degeneration (Barcia et al., 2012). Moreover, the loss of dopaminergic neurons induced by repeated systemic LPS, was rescued in complement complement component 3 (C3)-deficient mice (Bodea et al., 2014).

The phagocytic process involves several phases, detection of the particle to be ingested, activation of the internalization process, formation of the phagosome and phagosome maturation and posterior degradation of the particle ingested (Uribe-Querol & Rosales, 2020). Within the detection of the particle, the expression and recognition of "find-me" and "eat-me" signals take place. Stressed and damaged neurons can release the soluble "find-me" signal ATP or fractalkine that attracts microglia by chemotaxis to such neurons via the P2Y12 receptor or CX3CR1 (fractalkine receptor) (Vilalta & Brown, 2018). Moreover, some reports demonstrate that damaged neurons may release calcium and induce calcium waves which attracts the local microglia into the area of damage (Sieger et al., 2012). Although different "eat-me" signals have been suggested, one of the most studied is the expression of PS, this "eat-me" signal is required for microglial phagocytosis of both dying and viable neurons (Brown & Neher, 2014; Fricker et al., 2012; Neher et al., 2011). PS is a phospholipid usually confined on the inner membrane leaflet of the plasma membrane and can be exposed by different stimuli such as the inhibition of the PS translocase due to oxidative stress, increase of calcium levels, etc. (Brown & Neher, 2014). Importantly, low levels of peroxynitrite, hydrogen peroxide or glutamate induces reversible PS exposure on viable neurons. In the absence of microglia, these neurons were able to recover and internalize PS, but in the presence of microglia, neurons with exposed PS were lost due to phagocytosis (Brown & Vilalta, 2015; Hornik et al., 2016). Another potential "eat-me" signal is calreticulin (CRT), is usually localized in the endoplasmic reticulum (ER), can be exposed in non-neuronal cells as a result of ER stress or apoptosis, and can act as a "eat-me" signal or opsonin (Gardai et al., 2005). On neurons, CRT binds to microglial LRP (low density lipoprotein receptor related protein) and triggers their phagocytosis *in vitro* (Fricker et al., 2012). In addition, phagocytosis of neurons with exposed PS can be mediated via several microglial receptors and opsonin, some of which are strongly upregulated by inflammation. Opsonin are soluble proteins that target cells in order to facilitate their phagocytosis. Classic opsonin include antibodies and complement components such as C1q and C3b, but also different proteins act as a bridge between PS and the phagocytic receptors (Vilalta & Brown, 2018). For example activated microglia after stimulation with LPS, release opsonin MFG-E8 (also known as lactadherin), which bound exposed PS and activate phagocytosis via VNR (Fricker et al., 2012; Neher et al., 2011; Neniskyte et al., 2011).

As mentioned above, the phagocytic process can result in the engulfment of dead or dying neurons as well as viable neurons. This process is defined as "phagoptosis" or primary phagocytosis (Brown & Neher, 2014). Phagoptosis is the process in which the cells die as a consequence of being phagocytosed while the secondary phagocytosis or "efferocytosis" the primary cause of death of target cell is apoptosis or necrosis and the dead of dying cells are phagocytosed. In the case of phagoptosis, the neurons or the target cell can express transiently "eat-me" signals such as PS, however when the phagocytosis is blocked this "eat.me" signals can be re-internalized. In the case of efferocytosis, the apoptotic neuron or cell express "eat-me" signals irreversibly which trigger phagocytosis of the dead cell. Experimentally, when inhibiting or blocking the phagocytosis, in the case of phagoptosis, will leave live neurons, whereas secondary phagocytosis will leave dead neurons (Brown & Neher, 2014; Brown & Vilalta, 2015).

The recognition and engulfment of particles are mediated by phagocytic receptors localized in the surface of the phagocytes, they are able to bind different "eat-me" proteins and opsonin in the target cell (Uribe-Querol & Rosales, 2020). Several phagocytic receptors have been described and characterized in microglia in the apoptotic cells context, such as VNR which binds to MFG-8, MER receptor Tyrosine Kinase (MerTK) which binds opsonin growth-arrest specific 6 (Gas-6) (Fricker et al., 2012; Neher et al., 2012; Vilalta & Brown, 2018), Triggering Receptor Expressed on myeloid cells-2 (TREM-2) which possible ligand may be apolipoprotein E (Atagi et al., 2015), complement receptor 3 (CR3) which binds to different molecules of the complement cascade such as Cq1 and C3b (Bodea et al., 2014; Hong et al., 2016). And finally FcγR which binds to IgG (Uribe-Querol & Rosales, 2020).

Since microglial cells are able to phagocytose viable neurons of the SNpc in an MPTP model of PD, contributing to the neurodegenerative process (Barcia et al., 2012), the role of FcγR seems to be important because neuroinflammation-mediated neurodegeneration, induced by the intranigral injection of IgG coming from patients with PD bonded to FcγR, resulted in microglial activation and dopaminergic degeneration (He et al., 2002). In addition, mice lacking FcγR did not present a significant loss of dopaminergic neurons in the SN after IgG injection, indicating the relevance of the FcγR in the potential elimination of opsonized dopaminergic neurons in this particular model (He et al., 2002). In the AD context, microglial cells fail to efficiently clear

deposits of Aβ in the brain, however this clearance is enhanced during Aβ immunotherapy which stimulate the FcγR-mediated phagocytic clearance of Aβ plaques (Koenigsknecht-Talboo & Landreth, 2005). The present thesis focused on blocking the phagocytosis of dopaminergic neurons by the administration of neutralizing antibodies against CD16/32 (murine subunits of FcγR), which are known to mediate phagocytosis and are overexpressed in primed microglia as a result of neuroinflammation, in an MPTP-induced degeneration model of PD. Therefore, the next section will address how phagocytosis mediated by FcγR takes place, specifically the signaling involved in the phagosome formation which leads to rearrangement of actin cytoskeleton.

When a particle of the target interacts with the phagocyte receptor FcγR , a series of signals are triggered to activate phagocytosis (Flannagan et al., 2012) Changes in the membrane and the actin cytoskeleton take place leading to the formation of the phagocytic cup and the pseudopods that cover the particle (Diakonova et al., 2002; Freeman & Grinstein, 2014). The first structure formed is a depression in the membrane named phagocytic cup, then the membrane surrounds the target particle and within few minutes it closes at the distal end (Diakonova et al., 2002; Flannagan et al., 2012; Freeman & Grinstein, 2014).

The signaling cascade FcγR-mediated phagocytosis is one of the most studied and described: FcγR (fragment-crystallizable γ receptors) are glycoproteins that specifically bind the Fc part of IgG molecules. In humans, three classes have been identified: FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16). In mice four classes: FcγRII, II (CD32), FcγRIII (CD16) and FcγRIV, all of them being opsonic receptors (Flannagan et al., 2012; F. Nimmerjahn & Ravetch, 2008; Uribe-Querol & Rosales, 2020). The phagocytic process starts when FcγR get activated when they bind to IgG of the opsonized target and get clustered in the phagocyte membrane. Receptor clustering triggers the signaling cascade which, among other processes, provokes the polymerization of actin, relevant for the formation of the phagosome (Freeman & Grinstein, 2014; Rosales & Uribe-Querol, 2017) (Figure IV). Moreover, the Fcγ chain contains immune-tyrosine activation motifs (ITAM). The activation of different FcγR (FcγRI, FcγRIIa, FcγRIII and FcγRIV) is very similar, after clustering the Src-kinase family (such as Lyn, LcK and HcK) phosphorylates the tyrosine of the ITAM. Then, Syk (spleen tyrosine kinase) binds to the phosphorylated ITAM and gets activated. Activation of Syk-family kinase, result in activation of various downstream

pathways including the LAT (linker for activation of T cells), PI3K (phosphatidylinositol 3-kinase) and PLCγ (phospholipase Cγ) (Okun et al., 2010). Activated PLCγ produces IP3 (inositol triphosphate) and DAG (diacylglycerol) (Figure 4). Phosphorylated PI3K generates the lipid phosphatidylinositol-3,4,5-triphosphate (PIP3) at the phagocytic cup. This lipid also regulates the activation of the GTPase Rac and contractile proteins such as Myosin. Active Rac is important in actin remodeling and activation of other signaling pathways such as JNK (c-Jun N-terminal kinase) and NF-κB (Flannagan et al., 2012; Uribe-Querol & Rosales, 2020). Moreover, the guanine nucleotide exchange factor (GEF) Vav activates GTPases of the Rho (Cdc42) and Rac family, which are involved in regulation of the actin nucleation complex Arp2/3, which induces the actin polymerization that drives pseudopod extension (Rosales & Uribe-Querol, 2013, 2017) (**Figure IV**).

Small GTPase of the Rho family are important regulators of the actin cytoskeleton. The GTPase Rac and Cdc42 are activated and recruited to the forming phagosome during FcγR-mediated phagocytosis (Park & Cox, 2009). The relevance of Cdc42 during FcγR-mediated phagocytosis was evidenced in experiments in leukocytes where FcγR-mediated phagocytosis was impaired, trhough the deficient formation of rich actin "phagocytic cup" when RAC1 and Cdc42 were inhibited (Cox et al., 1997). Furthermore, experiments in macrophages also demonstrated the importance of Cdc42, since reducing the expression of Cdc42 by using RNA-mediated interference, resulted in a dramatic impaired phagocytic capacity, due to defects in phagocytic cup formation, actin assembly and pseudopod extension (Park & Cox, 2009). However the activation of Cdc42 is done by a mechanism that still remains unknown (Rosales & Uribe-Querol, 2017). Cdc42 and Rac participate in regulating the localized formation of actin fibers, necessary for pseudopod extension, by activating the nucleation-promoting factors WASp (Wiskott-Aldrich Syndrome protein). WASp in turn activate the Arp2/3 complex for actin polymerization (Flannagan et al., 2012; Park & Cox, 2009; Rosales & Uribe-Querol, 2017).
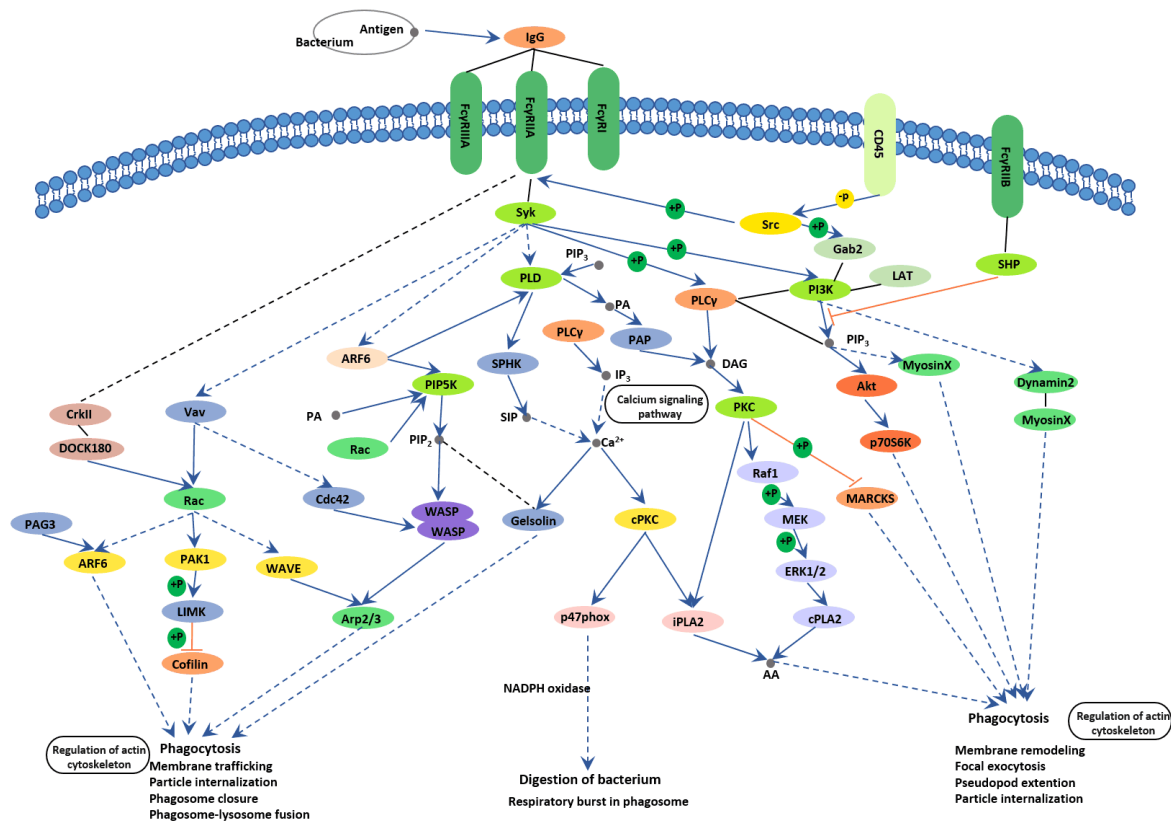
Figure IV: **FcγR receptor signal transduction**. FcγRIIa crosslinking by immunoglobulin (IgG) bound to a particle induces activation of Src family kinases (SFK), which phosphorylate tyrosine residues in the ITAMs of the cytoplasmic tail of the receptor. Then, Syk associates with phosphorylated ITAMs and leads to phosphorylation and activation of protein LAT (linker for activation of T cells) interacting with various proteins. Some of these proteins are phospholipase C gamma (PLCγ), which produces inositol-trisphosphate (IP3) and diacylglycerol (DAG). These second messengers cause calcium release and activation of protein kinase C (PKC), respectively. PKC leads to activation of ERK and p38. The guanine nucleotide exchange factor Vav activates the GTPase Rac and **Cdc42** by an unknown mechanism and which induces actin polymerization by activating the nucleation-promoting factor WASp (Wiskott-Aldrich Syndrome protein). Illustration by Cusabio Technology LLC

Besides the formation of the phagocytic cup, small GTPases also play an important role in cell migration and motility, features that in microglia are essential for an efficient neuroinflammatory response. Cell migration has largely been studied in vitro. During motility, cells undergo dramatic changes in the membrane and initiate a dynamic organization of the actin cytoskeleton where the small GTPase Rho family (including Rac1, Cdc42 and Rho) critically regulates this process (Barcia et al., 2012). Rac1 organizes the formation of lamellipodia while Cdc42 promotes the formation of pseudopods (Cox et al., 1997; Park & Cox, 2009). For the case of microglia, previous studies of our group demonstrated that inhibiting Rho-GTPase prevents the neurodegeneration in a model of PD. Therefore, since the small GTPase Rho family is so important for cell migration, chemotaxis, and phagocytosis, it seems logical to target these signaling pathways to tarnish the neuroinflammatory response in

parkinsonism. Most importantly for this thesis, because FcγR-mediated phagocytosis is regulated by Cdc42 (Park & Cox, 2009), we also explored the effects of targeting Cdc42 as well. Since Cdc42 signaling is supposedly downstream of FcγR activation, we blocked this pathway by the administration of a Cdc42 inhibitor, named ML141, which also resulted in neuroprotection of dopaminergic neurons in an MPTP-induced degeneration PD model. Having similar outcomes, the results of both strategies suggest that the specific targeting of FcγR and its Cdc42-mediated downstream signaling, involved in cell motility and phagocytic cup formation, could be a promising therapeutic approach for PD.

With the present work we aimed to highlight the relevance of neuroinflammation and phagocytosis in parkinsonian neurodegeneration. Most importantly, we propose the use of neutralizing antibodies as a potential immunotherapy tool to halt dopaminergic degeneration PD.

Objectives

The main objective of the present thesis is to determine whether microglia when fully activated are able to increase their phagocytic domain on dopaminergic neurons under characteristic neuroinflammatory conditions of a parkinsonian scenario and propose potential therapeutic use of neutralizing antibodies to prevent the dopaminergic elimination *in vivo* and *in vitro.* Thus, to achieve this aim, the specific objectives were:

- To analyze the microglial activation in a parkinsonian-like neuroinflammatory scenario *in vitro*.

- To standardize the maximal microglial activation with pro-inflammatory stimulus such as IFN-γ and LPS *in vitro* mimicking a neuroinflammatory environment.

- To determine the mechanism underlying the IFN-γ-mediated priming of microglia in parkinsonian-like neuroinflammation *in vitro.*

- To determine the consequences of full microglial activation after priming for dopaminergic neuron-like cells *in vitro*.

- To clarify the outcome of the interactions between dopaminergic neuron-like cells and microglial cells within their phagocytic activity after activation and priming.

- To manipulate microglial phagocytic capacity to avoid engulfing events and reduce dopaminergic elimination *in vitro*.

- To study the effects of a pure neuroinflammatory model induced by LPS on dopaminergic neurons of the SNpc *in vivo*.

- To evaluate the contribution of neuroinflammation and microglial activation in an MPTP-induced degeneration model of PD.

- To demonstrate the phagocytic activity of microglia in the SNpc of animals in an MPTP model of PD.

- To propose a new therapeutic approach by using neutralizing antibodies against CD16/32 to reduce microglial reactivity and their phagocytic function to protect dopaminergic neurons in an MPTP-induced degeneration model of PD.

- To determine the effect of inhibiting Cdc42, mechanistic factor of phagocytosis signaling, on dopaminergic neurons of the SNpc in an MPTP model of PD.

Materials and Methods

In vitro studies

Cell cultures

In order to study the behavior of microglial and dopaminergic cells in a PD-like neuroinflammatory scenario, we used the well-known cell lines BV-2 and PC12.

BV-2 cell line, from the Cell Culture unit of the Institut de Neurociències of the Universitat Autònoma de Barcelona (INc-UAB), is an immortalized mouse microglial cell line that exhibits the morphological and functional characteristics of microglia (He et al., 2002). PC-12 cell line, also provided by the INc-UAB Cell Culture unit, derived from a transplantable rat pheochromocytoma and closely resembles dopaminergic neurons, as they synthesize and store dopamine (DA), and sometimes noradrenaline as well as they express dopamine transporter (DAT) in their membranes (Westerink & Ewing, 2008). BV-2 cells were maintained at 37°C and 5% CO_2 in Roswell Park Memorial Institute (RPMI) medium (Sigma Aldrich; Saint Louis, MO, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 0.1% penicillin-streptomycin (P/S) while PC12 cells were maintained at 37°C and 5% CO_2 in Dulbeccos Modified Eagle Medium (DMEM) (Sigma Aldrich; Saint Louis, MO, USA), supplemented with 7% heat-inactivated fetal bovine serum (FBS), 7% horse serum (HS), 1.14% HEPES 1M pH=6.8 and 0.2% P/S.

The maintenance of the cell culture was done every 2 or 3 days. When the cells were confluent, 3 mL of trypsin (Sigma Aldrich; Saint Louis, MO, USA) was added to the flasks, previously washed with PBS. The flask was placed for 3 minutes at 37°C in order to activate the trypsinization and break the bonding between cells. Subsequently, centrifugation at 1000 rpm (revolutions per minute) during 5 minutes was done and then the cells were resuspended in fresh RPMI or DMEM and seeded in new flask until their use.

Treatment with interferon - gamma and lipopolysaccharide

In order to see the effect of proinflammatory inductors, microglial cells were seeded on coverslips in 24-well culture plates at a density of 20,000 cells per milliliter (cells/mL). The cells were then exposed to increasing concentrations of interferon-gamma (IFN-γ) (0.05, 0.5, 5, 25, 50, 75 and 100 ng/mL) or combined with proinflammatory stimulus lipopolysaccharide (LPS)

(Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 100 ng/mL. After 24 hours, day *in vitro* 1, supernatant was collected for further studies. New fresh medium was added and 24 hours later (day *in vitro* 2) supernatant was collected, then the cells were fixed with 4 % paraformaldehyde (PFA) for immunostaining.

Priming

Concerning the understanding of microglial priming, the order of the stimulus was alternated to see if there were variations in the level of activation of the BV-2 cells. In this case, microglial cells were seeded on coverslips in 24-well culture plates at a density of 20,000 cells cells/mL, 24 hours later IFN-γ was added to the medium at a final concentration of 0.5 ng/mL. After 24 hours, the supernatant with IFN-γ was collected for further studies and new fresh medium with LPS, at a final concentration of 100 ng/mL, was administered to each well. After 24 hours, supernatant was collected and the cells were fixed with 4% PFA for immunostaining.

Detection of microglial activation by Griess assay

The Griess assay (Sigma-Aldrich, St. Louis, MO, USA), a colorimetric analysis, was used to detect the presence of nitrite compounds in the supernatant, in this case, product of the activation of BV-2 cells. The Griess reactive consists of sulfanilic acid and N- (1-naftil) ethildiamide (NED). Sulfanilic acid reacts with the nitrites in an acidic solution and then becomes a diazonized salt that binds to NED, resulting in a colored compound which can be spectrophotometrically quantified at 540 nm. A calibration curve was determined using $NaNO_2$ (sodium nitrite) in a range between 0.78 µM and 100 µM. Then in a 96-microwell plate Griess reactive (100 µL/well) with 100 µL of each of the samples was added. After 15 minutes of incubation at room temperature, absorbance was read at 540 nm and the values were interpolated with the calibration curve to obtain the concentration of nitrites in the supernatants.

BV-2 and PC12 interaction

In order to see the interaction and the effect of activation between the microglial and dopaminergic cells, a co-culture with BV-2 and PC12 cells was set up. Both cell lines were incubated at 37 °C in culture flasks. BV-2 cells were seeded in a 24-well plate at a concentration of 300,000 cells/mL. Afterwards, the cells were stimulated with LPS 100 ng/mL plus IFN-γ 0.5 ng/mL, 24 hours later the medium with the treatment was removed from the wells. Previously

trypsinized PC-12 cells were seeded on top of the BV-2 cells with fresh DMEM medium at a density of 1,200,000 cells/mL. The co-culture was then fixed with 4% PFA after 10, 20 and 30 minutes of interaction and after 1, 4 and 10 hours of interaction. Fixed cells were preserved in PBS to later perform multicolor immunocytofluorescence.

BV-2 and PC12 interaction after priming

In order to see the effect of the highest activation of microglial cells on the interaction with dopaminergic cells, both cell lines were incubated at 37° C in culture flasks. BV-2 cells were seeded in a 24-well plate at a concentration of 300,000 cells/mL. After 24 hours of incubation BV-2 cells were stimulated with IFN-γ at a concentration of 0.5 ng/mL. 24 hours later the supernatant was retired and then BV-2 cells were challenged with LPS at 100 ng/mL. After 24 hours of incubation the supernatant was removed and previously trypsinized PC-12 cells were seeded on top of BV-2 cells with fresh DMEM medium at a density of 1,200,00 cells/mL. The co-culture was fixed with 4% PFA at 10, 20 and 30 minutes of interaction and at 1, 4 and 10 hours. Fixed cells were preserved in PBS until performing immunocytofluorescence with multiple markers.

Cdc42 inhibition in BV-2 and PC12 cell co-cultures

To analyze how the inhibition of the protein CdC42, affects the preservation of dopaminergic cells, the inhibitor ML141 (Cdc42/Rac1 GTPase Inhibitor, Calbiochem, San Diego, CA, USA) was used in BV-2 PC12 co-cultures. This experiment was divided in two scenarios. In the first one, the inhibition with the ML141 was done before the activation with IFN-γ and LPS and in the second one; the inhibition was done after the activation. According to the aforementioned protocol of culture, BV-2 cells were seeded at a concentration of 50,00 cells/mL and incubated at 37ºC and 5% CO_2.

Concerning the inhibition before the activation, ML141 was added at a concentration of 10 μM with new fresh medium to the BV-2 cells after 24 hours of being seeded. 1 hour later, the medium was removed and new RMPI medium was added with IFN-γ at 0.5 ng/mL. After 24 hours, the medium was removed and LPS 100 ng/mL was added, according to the protocol of priming described above. PC12 cells were seeded on top of the BV-2 cells at a concentration

of 200,000 cells/mL, after 1 hour of incubation, cells were fixed with 4% PFA and preserved in PBS to conduct immunocytofluorescence.

Regarding the second scenario, the Cdc42 inhibition was done after the activation following the protocol of priming explained above. After the activation of BV-2 cells with IFN-γ and LPS, ML141 was added at 10 µM concentration and 1 hour afterwards the medium was removed and PC12 cells were added at a concentration of 200,000 cells/mL. 1 hour later the cells were fixed with 4 % PFA and preserved in PBS.

Inhibition of CD16/32 in BV-2 and PC12 cells interactions

As described for Cdc42, we followed the same procedure of inhibition in a pre-activation and post-activation scenario. Cells were treated with CD16/CD32 blocking antibody (Ultra-LEAF Purified anti-mouse CD16/32 Antibody, Biolegend, San Diego, CA, USA) to avoid phagocytosis or its Isotype (Ultra-LEAF Purified Rat IgG2a, κ Isotype Ctrl Antibody). BV-2 and PC12 cells were seeded proceeding as described above and the cells were activated with IFN-γ and LPS before and after being treated with either CD16/32 neutralizing antibody or the isotype at a concentration of 10 µg per 10^6 cells. After 1 hour of interaction, cells were fixed and preserved in PBS.

Immunocytofluorescence

In the case of the IFN-γ/LPS treatment, microglial cells were stained to visualize their morphology variations. After fixation, cells were washed with PBS to eliminate PFA residues. Cells were then treated for permeabilization and antigen retrieval with PBS with saponine 0.02% during 7 minutes. After washing, a blocking solution was added (PBS with 0.01% saponine, 10mM Glycine) for 15 minutes incubation at room temperature. Then, a more concentrated solution of blocking was added (PBS with 0.01% saponine, 10 mM Glycine, 5% BSA) for 1 hour at room temperature. The primary antibody anti-Iba1 (1:500, rabbit polyclonal, WAKO Cytomation, Glostrup, Denmark) was diluted in PBS with 0.01% saponine, 1% BSA (Sigma-Aldrich, St. Louis, MO, USA), and placed in a humidity chamber overnight at room temperature. After washing with PBS, a secondary antibody Alexa Fluor 488 (1:1000, Goat anti-rabbit, Life technologies; Carlsbad, CA, USA) was added for 45 minutes. Nuclei were counterstained with DAPI (1:1000) for 5 minutes before washing with PBS. Finally, antifading

reagent was used to mount the coverslips on glass slides (Prolong Gold, Invitrogen; Carlsbad, CA, USA).

To analyze the microglia-dopaminergic cells interactions, BV-2 and PC12 co-cultures were immunostained with anti-Iba-1 or anti-CD11b (1:100, rat monoclonal IgG2b; AdB Serotec, Bio-Rad laboratories, Hercules, CA, USA) and anti-TH (1:500, sheep polyclonal IgG1, SIGMA, Saint Louis, MO, USA) respectively.

In addition, the presence of CdC42 was characterized by immunostaining with anti-CdC42 antibody (1:500 mouse monoclonal MAb, Cytoskeleton, Inc. Denver, CO, USA). Regarding the experiment of blocking CdC42 using ML141, the microglia-DA cells interaction was identified as described above, using CD11b and TH antibodies. Furthermore, the same antibodies were used to characterize the intercellular interactions in CD16/32 blocking experiment. Besides, the presence of CD16/32 receptor in BV2 cells was detected using anti-CD16/32 antibody (Rat Anti-mouse Fc Block, BD Pharmigen, San Jose, CA, USA). After washing following the incubation with primary antibodies, secondary antibodies Alexa Fluor 555 (1:1000, Goat anti-rabbit, Life technologies; Carlsbad, CA, USA) was used to visualize Iba-1, Alexa Fluor 488 (1:1000, Donkey anti-sheep, Life technologies; Carlsbad, CA, USA) for TH, Alexa Fluor 555 (1:1000, Goat anti-rat, Life technologies; Carlsbad, CA, USA) for CD11b, Alexa Fluor 488 (1:1000, Goat anti-mouse, Life technologies; Carlsbad, CA, USA) for CdC42, and Alexa Fluor 488 (1:1000, Goat anti-rat, Life technologies; Carlsbad, CA, USA) for CD16/32. After washing the secondary antibodies, nuclei were counterstained with DAPI for 5 minutes before mounting on microscope slides.

Note: Table.1 for primary antibodies.

Table 1. List of primary antibodies used in immunofluorescence and immunohistochemistry

Primary antibodies

Antibody	Source	Dilution	Company
anti-TH	sheep polyclonal	1:500	Sigma
anti-Iba1	rabbit polyclonal	1:500	Wako
anti-Cd11b	rat monoclonal	1:100	Bio-rad
anti-CD16/32	rat	1:250/200	BD Pharmigen
Anti-Cdc42	mouse monoclonal	1:500	Cytoskeleton
anti-MAP-2	chicken polyclonal	1:500	Abcam
Anti-NeuN	mousse monoclonal	1:500	Merck

Microscopy, stereology and quantification

The number of cells expressing Iba-1 or TH in the experiments of increasing concentration of IFN-γ and co-cultures was quantified by stereology. Each coverslip was systematically sampled using a fluorescence microscope (Nikon Eclipse 90i, Nikon, Tokyo, Japan) attached to a digital camera (DXM 1200F) and software (ACT-1 version 2.70, Nikon corporation) with a 20X objective, taking 10 sample pictures per coverslip, per condition. Once the pictures were taken, Iba-1 and TH positive cells were quantified with image-analysis software (Image J version 1.47, NIH, USA), as well as their interactions and phagocytic events, always following stereological criteria: each picture was considered as a physical dissector and only particles in focus in one Z optical plane were quantified. The area occupied by Iba-1 and DAPI was also measured with the afore-mentioned Image J software establishing a threshold for every fluorophore. Threshold was critical to include all the positive material and exclude the background.

In addition, representative images were taken with a confocal laser-scanning microscope (LSM 700; Carl Zeiss, Oberkochen, Germany) and a Plan-Apochromat 40x/1,3 Oil DIC M27 objective lens (Carl Zeiss; Oberkochen, Germany), processed with the ZEN 2010 software (Carl Zeiss;

Oberkochen, Germany). A series range for each section was determined by setting an upper and lower threshold using the Z/Y Position for Spatial Image Series setting. Then, the microscope captured pictures at a fixed distance all over the volume of the cells. Stacks of the images were taken with an optical section interval, from 0.5μm. Pictures were analyzed with appropriate image analysis software (Image J version 1.47, NIH, USA).

Primary cultures

Extraction of primary microglia from the mesencephalon

According to the protocol approved by the Ethics Committee on Animal and Human Research of the UAB, newborn Sprague Dawley rats from the animal house at the UAB, were sacrificed at days 0-2 post-natal in order to obtain the glia from their mesencephalon.

After carefully removing the scalp, brain extraction was performed following the isolation of the mesencephalon after separating cortex and cerebellum. Meninges at this level were carefully removed and subsequently the mesencephalon was chopped into small pieces.

Five solutions were done previously to perform the culture and all of them were filtered with a 0.2 μm filter; Solution 1, containing 50 mL of Krebs-Ringer Buffer 1X (120 mM NaCl, 4.8 mM KCl, 1.2 mM KH2PO4, 25 mM NaHCO3, 14.3 mM Glucose) with 0.15 g BSA and 0.4 mL $MgSO_4$ 3.8%, Solution 2, containing 10 mL of solution 1 with 2.5 mg of trypsin, Solution 3, containing 10 mL of solution 1 with 0.8 mg DNase (Sigma-Aldrich, St. Louis, MO, USA), 5.2 mg of trypsin inhibitor (Gibco; Thermo Fisher Scientific; Waltham, MA, USA) and 0.1 mL $MgSO_4$ at 3.8%, Solution 4 containing 8.4 mL of solution 1 and 1.6 mL of solution 3, and Solution 5, containing 5 mL solution 1, 40 μL $MgSO_4$ 3.8% and 6 μl $CaCl_2$ 1.2%.

Once all the solutions were filtered, the chopped mesencephalon were resuspended in 15 mL of Solution 1. After centrifugation of 1500 rpm for 30 seconds, the pellet was resuspended in Solution 2 and incubated at 37 °C for 10 minutes. The enzymatic digestion was stopped adding the Solution 4, followed by 1500-rpm centrifugation. The pellet was resuspended in 3 mL of Solution 3 and disaggregation was performed by gently pipetting up and down 10 times with a Pasteur pipette through a cell net. The cell suspension was added to Solution 5 and centrifuged for 5 minutes at 100 rpm, the supernatant was discarded and cells were

resuspended in 10 mL of DMEM with 1% P/S and 10% FBS. After resuspension, the cells were counted and seeded at 300,000 cells/mL in a culture flask.

Isolation of microglia was done when the primary culture was already confluent, normally 2 or 3 weeks after the dissection, with regular changes of medium. In order to extract the microglia, the flask containing the cells was agitated during 3 hours at 300 rpm. The supernatant was collected and centrifuged for 5 minutes at 1000 rpm and the pellet was resuspended in DMEM with 50% conditioned medium (medium collected from the first centrifugation after the desegregation of the tissue). Cells were counted and seeded in a 24-well plate at a cell density of 100,000 cells/mL and kept at 37 °C and 5% CO_2.

Cells remaining in the flask were maintained adding Neuro Basal medium (NB) (GIBCO from Thermo Fisher Scientific; Waltham, MA, USA) supplemented with 2% of B27 (GIBCO from Thermo Fisher Scientific; Waltham, MA, USA) , 1% of Glutamax (Thermo Fisher Scientific; Waltham, MA, USA) and 0.5 % of P/S. After one week, the supernatant was collected and centrifuged, the pellet discarded and the supernatant of the NB medium was stored and identified as NB conditioned for the culture of primary neurons.

Primary microglia were stimulated with pro-inflammatory inductors as described above for cell lines cultures. After the maintenance of microglia in the 24-well plate, cells were treated with either increasing concentrations of IFN-γ (10 ng/mL, 25 ng/mL and 50 ng/mL), LPS at a concentration of 10 ng/mL or the combination of LPS and IFN-γ (LPS 10 ng/mL plus IFN-γ 10 ng/mL; LPS 10 ng/mL plus IFN-γ 25 ng/mL and LPS 10 ng/mL plus IFN-γ 50 ng/mL). After 24 hours, the supernatant was collected for Griess assay and the cells were fixed with 4% PFA.

Once the optimal concentration of IFN-γ to obtain microglial activation was known, primary microglial cells were treated either with IFN-γ (25 ng/mL) or LPS (10 ng/mL). 24 hours later, after removing the medium, IFN-γ or LPS was added as in the previous protocol described above as priming. Appropriated controls consisted in cells with no treatment, treated with only LPS or with only IFN-γ. As opposed to the cell lines, in primary cultures the neuroinflammatory inductors were added in medium containing half fresh and half conditioned media. With every treatment, the supernatant was removed and collected for Griess assay. After 48 hours of experiment, cells were fixed with 4% PFA.

Detection of microglial activation by Griess assay

In order to detect the released nitrites by primary microglial cells, therefore the indication of their activation, a calibration curve was determined using $NaNO_2$ (sodium nitrite) in a range between 0.78 µM and 100 µM. Then in a 96-microwell plate we added Griess reactive (100 µL/well) with 100 µL of each of the samples. After 15 minutes of incubation at room temperature, absorbance was read at 540 nm and the values were interpolated with the calibration curve to obtain the concentration of nitrites in the supernatants

Extraction of primary neurons from the mesencephalon

For the culture of primary dopaminergic neurons, a pregnant E17 Sprague Dawley rat from the animal house of the UAB was used. The rat was sacrificed according to the protocol approved by the Ethics Committee on Animal and Human Research of the UAB. The abdomen was cleaned with 70 % ethanol and the embryos were removed and placed in a petri dish with PBS. The rostral and the distal part of the brain were removed, isolating the midbrain. The meninges were carefully removed and the ventral part of the midbrain was taken and collected in PBS. All the five solutions used in the culture of primary microglia, were previously prepared and filtered; Solution 1, containing 50 mL of Krebs-Ringer Buffer 1X (120 mM NaCl, 4.8 mM KCl, 1.2 mM KH2PO4, 25 mM NaHCO3, 14.3 mM Glucose) with 0.15 g BSA and 0.4 mL MgSO$_4$ 3.8%, Solution 2, containing 10 mL of solution 1 with 2.5 mg of trypsin, Solution 3, containing 10 mL of solution 1 with 0.8 mg DNase, 5.2 mg of trypsin inhibitor and 0.1 mL MgSO$_4$ at 3.8%, Solution 4, containing 8.4 mL of solution 1 and 1.6 mL of solution 3, and Solution 5, containing 5 mL solution 1, 40 µL MgSO$_4$ 3.8% and 6 µl CaCl$_2$ 1.2%. Once all the mesencephala were mixed together, were chopped in small pieces. The same protocol to extract primary microglia was followed but with two small differences, as the centrifugations were only of 2 minutes instead of 5, and the cell net was not used to desegregate the cells. Finally, the cells were suspended in DMEM medium, and seeded in 24-well plate at 300,000 cells/mL density. After 3 hours of being seeded, the cells were cultured into three conditions to evaluate the optimal setting for dopaminergic neurons. The first condition, as the control, contained only NB fresh medium. The second condition contained NB fresh medium with GDNF (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 10 ng/mL and the third condition contained a mixture of NB fresh medium and NB conditioned (obtained from the

culture of primary microglia). Every 3 or 4 days, half medium was changed including the antimitotic Ara-C at a concentration of 2µM (Cytosine β-D-arabinofuranoside) (Sigma-Aldrich, St. Louis, MO, USA) or FUDR (5-Fluoro-2'-deoxyuridine) (Sigma-Aldrich, St. Louis, MO, USA). The seventh day in vitro, the cells were fixed with 4% PFA for further staining.

Immunocytofluorescence of primary culture

The protocol described above for immunocytofluoresece of cell lines was used. Primary microglia and/or primary neurons were washed with PBS to eliminate de PFA residues. Cells were then treated for permeabilization and antigen retrieval with PBS with 0.02% saponine during 7 minutes. After washing, blocking of nonspecific antibodies was done by adding PBS with 0.01% saponine and 10mM Glycine for 15 minutes at room temperature. Then, a more concentrated solution of blocking solution was then added (PBS with 0.01% saponine, 10 mM Glycine, 5% BSA) for 1 hour at room temperature. Primary antibodies used were anti-Iba1 (1:500) for primary microglia, TH (1:500), anti-MAP-2 (1:500, chicken polyclonal, Abcam, Cambridge, CB2 0AX, UK) and anti-NeuN (1:500, mouse IgG1, Merck Millipore, Darmstadt, Germany) for primary neurons. Primary antibodies were diluted in PBS with 0.01% saponine, 1% BSA, and cells were incubated with this solution overnight at room temperature. After washing with PBS, secondary antibodies were added for 45 minutes. Alexa Fluor 488 (1:1000, Goat anti-rabbit) for Iba-1, Alexa Fluor 488 (1:1000, Donkey anti-sheep) for TH, Alexa Fluor 555 (1:1000, Goat anti-chicken, Life technologies; Carlsbad, CA, USA) for MAP-2 and Alexa Fluor 555 (1:1000, Goat anti-mouse) for NeuN. Nuclei were counterstained with DAPI (1:1000) for 5 minutes before washing with PBS and mounting the coverslips on glass slides using antifade reagent (Prolong).
Note: Table.1 for primary antibodies.

Microscopy, stereology and quantification

Primary microglial cells expressing Iba-1 were visualized using a fluorescence microscope (Nikon Eclipse 90i, Nikon, Tokyo, Japan) attached to a digital camera and software (DXM 1200F, ACT-1 version 2.70, Nikon corporation) using a 20X objective. In this case, the presence of Iba-1 expression was considered as an indicator of the effectiveness of the extraction of microglial cells, therefore, no quantification by stereology was done for this characterization.

In the case of the extraction of primary neurons, cells were stained with TH and MAP-2. Quantification was done using a fluorescence microscope (Nikon Eclipse 90i, Nikon, Tokyo, Japan) attached to a digital camera and specialized software (DXM 1200F ACT-1 version 2.70, Nikon corporation) using a 20X objective, taking 10 sample pictures per coverslip and condition. Once the pictures were taken, MAP-2 and TH positive cells were quantified with image-analysis software (Image J version 1.47, NIH, USA) always following stereological criteria: each picture was considered as a physical dissector and only particles in focus in one Z optical plane were quantified. In this case, the percentage of MAP-2 and TH was used to indicate which condition was optimal for further cultures of primary mesencephalic neurons.

In vivo studies

Intraperitoneal administration of LPS

For this experiment 25 male mice from the strain C57BL/6 were purchased from Janvier Labs at an age of 3 weeks. The animals were kept and maintained at the UAB animal house facilities for two more weeks, distributed in five groups of five mice per cage, and every cage was labelled with an experimental condition being, Saline, Corn oil (Sigma Aldrich; Saint Louis, MO, USA), LPS, ML141, and ML141/LPS.

First, the solutions were prepared in advance to follow the administration the same day. ML141 was diluted in corn oil up to a concentration for a 10-mg/Kg dose, and LPS was diluted in saline solution to a concentration for a 500-µg/Kg dose. The same volumetric amount of corn oil, in which was diluted the ML141 (104 µL), or the same volumetric amount of saline, in which LPS was diluted (100 µL), were injected per animal in control groups Corn oil and Saline respectively.

Since the administration was intraperitoneal and safe, there was no need to anesthetize the animals. Observe **Figure V**: In day 1, (A) Corn oil group animals were administered (with a syringe with hypodermic needle) with 104 l of corn oil, (B) Saline group animals received 100 l of saline solution, animals of the (D) ML141 group received a 10 mg/Kg dose of ML141 diluted in corn oil, and the (C) ML141/LPS animals were only administered 10 mg/Kg dose of ML141. In day 2, animals of the (E) LPS group were injected with saline and 30 minutes later with 500-µg/Kg LPS dose. (A) The animals of the Corn oil group were injected with a dose of

corn oil and 30 minutes later another dose of the same corn oil. Animals of (D) ML141 group received the second dose of ML141 10 mg/Kg, the (B) Saline group received a first dose of saline and a second one 30 minutes later. In the case of (C) ML141/LPS, animals received a dose of 10 mg/kg of ML141 and 30 minutes later 500-µg/Kg of LPS.
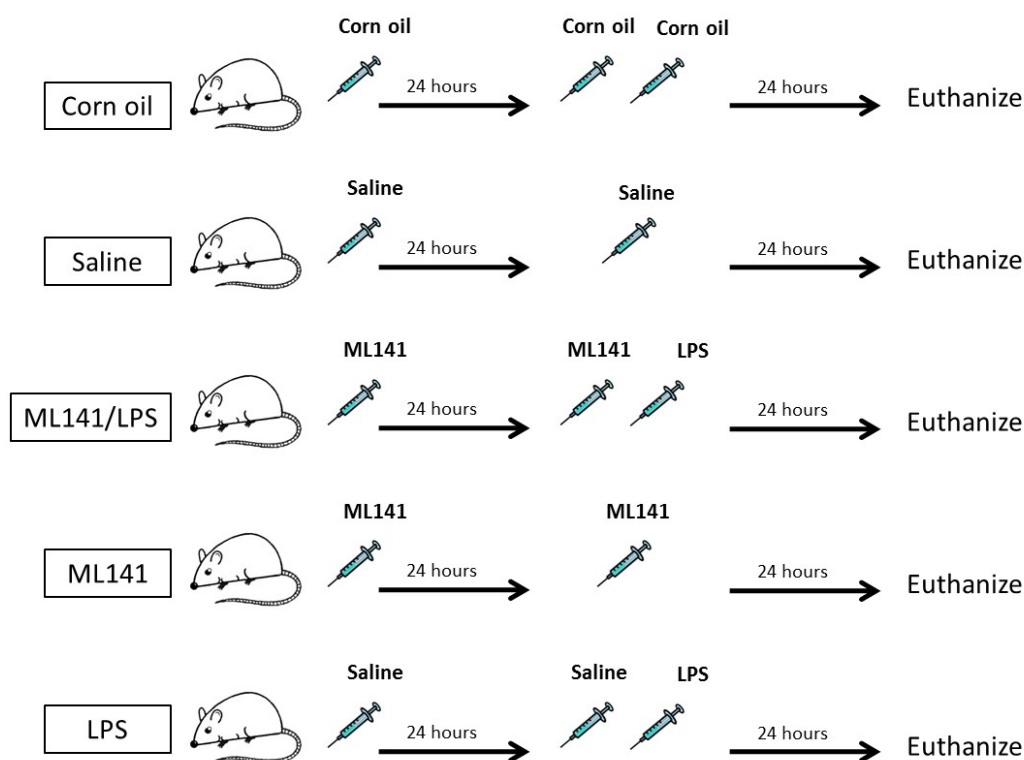
Figure V. Illustration of the protocol of intraperitoneal administration of LPS. Schematic draw to illustrate days of the experiment and the different treatments and respective controls: In the **ML141** group first dose ML141 was injected on day 1 and second dose was injected on day 2. In the **ML141/LPS** group first dose of ML141 was injected on day 1, second dose was injected on day 2 and 30 minutes later the LPS. **Saline** group, first dose was administered on day one and second dose on day 2. In the **LPS** group, the day 1 only saline was injected and day 2 saline was injected and 30 minutes later the LPS. In the Vehicle of ML141 (**corn oil**) was administered the day 1, then the day 2 a double dose of corn oil was injected with 30 minutes between doses.

After 24 hours animals were euthanized according to the guidelines of the Ethics Committee of the UAB. First, mice were anesthetized with a mixture of ketamine (Imalgene, Merial Laboratorios, Barcelona, Spain), at a dose of 100 mg/kg; and Medetomidine (Medetor, Ecuphar, Barcelona, Spain), at a dose of 0.5 mg/kg, then were placed on a shallow tray. Dissection was done, cutting the abdominal cavity and separating the rib cage until exposing the heart. A small incision of the left ventricle of the heart was done and a perfusion needle was inserted in the heart of the animal. First, a syringe of 50 mL was filled with Tyrode solution to achieve a complete blood perfusion and clearance. Then, a second syringe of 50 mL of

4% PFA was plugged to the end of the perfusion needle, the plunger was pushed at a constant speed in order to obtain an uniform flux through the animal vascular system until fixation of tissues was achieved. The brain was then extracted from the perfused mice and post-fixed in tubes with 4% PFA during 48 h. After post fixation, brains were stored in PBS and NaN$_3$, until sectioning.

Brain sectioning

Stored brains were prepared for sectioning as follows; 0.5 cm of the rostral part of each brain was sectioned by vibratome (Leica Microsystems, Germany) after removing the olfactory bulbs. Six series of sections of 40 μm were obtained, setting the vibratome at a speed of 0.225 mm/s and a vibrating frequency of 60 Hz. Once sectioned, the brain tissue was stored in PBS with NaN$_3$ until staining.

Immunohistochemistry

Immunohistochemistry by diaminobenzidine (DAB) detection was performed on brain sections to visualize microglial cells and dopaminergic neurons using anti-Iba-1 and anti-TH antibodies respectively. Antigen retrieval was conducted with a pre-treatment with citrate (10mM, pH 6, 60ºC) for 20 minutes. After PBS washing, a solution of H$_2$O$_2$ 0.3% was added to the tissue sections during 15 minutes to inactivate the endogenous peroxidase. Then, a blocking of nonspecific binding sites was done by incubating the sections with 0.5% Triton X-100 with 10% horse serum (HS) (Sigma-Aldrich; St. Louis, MO, USA) during 1 hour. After blocking, the incubation with primary antibodies was done during 48 hours, diluting the antibodies in Trizma Base Saline (TBS)-0.5% Triton X-100, 1% HS and 0.1% NaN$_3$. (Trizma Base Sigma-Aldrich; St. Louis, MO, USA). Then secondary detection was carried out with specific biotinylated antibodies. Secondary antibodies were; biotinylated goat anti-rabbit, for Iba-1 or biotinylated donkey anti-sheep, for TH (1:1000; Dako; Glostrup, Denmark), diluted in 0.5% Triton X-100 with 1% HS without NaN$_3$. Biotin of secondary antibodies was detected by using the Vectastain Elite ABC horseradish peroxidase method (Vector Laboratories; CA, USA) following the manufacturer instructions. After DAB detection, sections were placed on gelatinized glass slides and were dehydrated through graded ethanol solutions (70%, 80%, 90% and 100%) and submerged in Xylene before putting the coverslips with appropriated

mounting media (Xylene substitute mounting medium. Thermo Fisher Scientific; Waltham, MA, USA). Note: Table.1 for primary antibodies.

Microscopy, stereology and quantification

To evaluate the morphology variation of microglial cells, expressing Iba-1 cells were analyzed following a similar strategy for cell cultures. In this case, using a bright field microscope (Eclipse 80i microscope, Nikon; Tokyo, Japan) and taking the required sampling with a digital camera connected to the microscope (DXM 1200F Digital Camera, Nikon, Tokyo, Japan). According to the anatomy of the mouse brain defined by the Mouse Brain Atlas (Paxinos & Frankin, 2001)motor cortex and striatum were analyzed. Therefore, six randomized dissector photo-samples of the motor cortex and six of the striatum were taken with 20X objective. Once the pictures were taken, the area occupied by Iba-1 was measured with the afore-mentioned software tools (Image J version 1.47, NIH, USA) establishing a threshold for every stained cell of the field taken covering approximately XX cells per animal (??).

Intracranial injection of LPS

For this experiment, 3-week old C57BL/6 male mice (n = 10) were purchased (Janvier Labs). Animals were kept and maintained in the UAB housing facilities for two more weeks. Every mouse was labelled in the tail for each experimental condition: 8 animals for LPS and 2 animals for saline.

The procedure was carried out in the appropriate surgical room at the UAB Animal House. Animals were first anesthetized with a mixture of anesthesia and analgesia with ketamine at a dose of 100 mg/kg; and Medetomidine, at a dose of 0.5 mg/kg. The animals were placed on a heated mat in order to keep an appropriate and stable temperature of each mouse during the procedure. After the standard absence of reaction when pinching the paw of the mouse, LPS at a concentration of 1 mg/mL was inoculated stereotaxically in the striatum at the specific coordinates; Anteroposterior (AP): + 0.5 mm; Mediolateral (ML): + 2.1 mm; Dorsoventral (DV): - 3 mm, according to Bregma. The injection was carried out slowly in a total time of 2 minutes, to avoid high pressure or any disruption of the brain tissue. After the inoculation and careful needle removal, the incision was stitched back and once the mice were recovered, were returned to the cage in normal conditions with normal feeding and maintained until the

euthanasia. The same procedure was done with a control group, injecting intra-cranially 0.5 µL of saline solution.

The animals were euthanized 3 or 7 days after the intracranial injection (n = 5 and 3 respectively) by perfusion following the protocol described for the intraperitoneal administration of LPS in the previous sections. The brain was extracted from the perfused mice and post-fixed in tubes with 4% PFA during 48 h. After post fixation, brains were stored in PBS and NaN_3, until further sectioning.

Sectioning

Sectioning was performed as described above in the protocol for the intraperitoneal injection of LPS. Six series of sections of 40 µM were obtained with vibratome, setting the vibratome speed at 0.225 mm/s and 60 Hz of vibration frequency. Once sectioned, the brain tissue was stored in PBS with NaN_3 until DAB staining or multicolor immunofluorescence.

Immunohistochemistry

In order to understand how LPS administration affects neuroinflammation and degeneration of dopaminergic neurons, immunohistochemistry by diaminobenzidine (DAB) detection was performed on brain sections to visualize microglial cells and dopaminergic neurons with Iba-1 and TH detecting antibodies respectively. As described above for the protocol of intraperitoneal administration of LPS, 20 minutes pre-treatment with citrate (10mM, pH 6, 60ºC) was done to facilitate antigen retrieval. After this pre-treatment and PBS washing, a solution of 0.3% H_2O_2 was used to incubate the sections for 15 minutes to inactivate the endogenous peroxidase. After proper washing, blocking of nonspecific binding sites was carried out by incubating the tissue with 0.5% Triton X-100 with 10% HS, during 1 hour. After blocking, 48 hours incubation with primary antibodies was done using a solution of TBS-0.5% Triton X-100, 1% HS and 0.1% NaN_3. Secondary biotinylated antibodies goat anti-rabbit, for Iba-1 or donkey anti-sheep, for TH, diluted in 0.5% Triton X-100 with 1% HS without NaN_3 were used accordingly. Secondary detection and amplification was done by using the Vectastain Elite ABC horseradish peroxidase method, incubating with AB (avidin-biotin) solution for 3 hours and obtaining DAB precipitation. After DAB detection, the sections were placed on gelatinized glass slides and were dehydrated through graded ethanol solutions (70%, 80%,

90% and 100%) and submerged in Xylene before putting the coverslips with appropriate mounting media (Xylene substitute mounting medium. Thermo Fisher Scientific; Waltham, MA, USA) for histological examination.

Variations to this procedure were done for the protocol to detect CD16/32 positive cells in the tissue, including that primary anti-mouse CD16/32 antibody was used at 1:250 dilution instead of 1:500, and the citrate was added at a temperature of 80 ºC.
Note: Table.1 for primary antibodies.

Immunohistofluorescence

To detect changes in microglia due to neuroinflammation and its effect on dopaminergic neurons, anti-Iba-1 antibody was used to detect and visualize microglial cells in combination with anti-TH antibodies to detect dopaminergic neurons. Routinely, antigen retrieval was done as described before with citrate at 60 °C, and blocking of nonspecific antibody binding sites during 1 hour with 10% HS. Primary antibodies were diluted in TBS-0.5% Triton X-100, 1% HS and 0.1% NaN_3 and incubated for 48 hours. Suitable secondary antibodies, diluted in TBS-0.5% Triton X-100, 1% HS and 0.1% NaN_3, were used accordingly (AlexaFluor 555 Goat-Anti rabbit 1:1000 to recognize Iba-1 and AlexaFluor 488 Donkey-Anti sheep 1:1000 to recognize TH) and incubated overnight. Finally, DAPI (1:1000; Life technologies; Carlsbad, CA, USA) was used to stain the nuclei of the cells. Sections were mounted on glass slides and coverslipped using appropriate antifading reagent (Prolong Gold, Invitrogen; Carlsbad, CA, USA). Note: Table.1 for primary antibodies.

Confocal microscopy, stereology and quantification

In order to visualize and analyze changes in the amount of dopaminergic neurons, the substantia nigra *pars compacta* (SNpc) was identified with the Paxino's Mouse Brain Atlas (Paxinos & Frankin, 2001) and positively stained brain tissue was imaged with a confocal laser-scanning microscope (LSM 700; Carl Zeiss, Oberkochen, Germany) and a Plan-Apochromat 20x/0.8 Ph2 M27 objective lens (Carl Zeiss; Oberkochen, Germany), processed with specialized software (ZEN 2010, Carl Zeiss; Oberkochen, Germany). Using the 20X objective, the optical field was able to cover the SNpc visualized in each section, therefore, two to four serial sections were imaged in each hemisphere and the number of dopaminergic neurons was

quantified (with Image J software), obtaining the average number of cells per SNpc section, per animal.

MPTP intoxication and Cdc42 inhibition

For the induction of the experimental model of Parkinson's disease, male mice (n = 25) with C57BL/6 MPTP susceptible background were acquired at 3 weeks of age (Janvier Labs). The animals were maintained in special plastic disposable cages/boxes in an isolated room from the UAB Animal House, which conditions were adequate for MPTP administration. The animals were separated into five boxes with five mice per box (n=5 mice). First group of animals were injected with a single dose of 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) dissolved in saline solution at a concentration of 20 mg/Kg, second group with the same amount of saline solution, third group with the CdC42 inhibitor (ML141) dissolved in corn oil at a concentration of 10 mg/Kg, fourth group with the same amount of corn oil and the final group was injected with ML141 30 minutes prior MPTP injection. No method of anesthesia and analgesia was indicated or recommended.

After 72 hours, animals were euthanized, perfused with Tyrode solution and fixed with 4% PFA. Brains were extracted and post-fixed in 4% PFA during 48h. After post fixation, brains were stored in PBS and NaN$_3$, until further sectioning.

MPTP intoxication and CD16/32 blocking

In order to see the effect of blocking CD16/32 Fcγ receptors in a parkinsonian model, 3-week-old C57BL/6 male mice (n = 25) were obtained commercially (Janvier Labs). As described in the previous section, in experiments using MPTP, the animals were kept and maintained in special and isolated conditions due to the toxicity of MPTP. Six cages of five animals each were used (n=5 mice). First group was injected with MPTP dissolved in saline solution, the second group with the same volumetric amount of saline solution. Third group was injected with blocking CD16/32 antibody (Ultra-LEAF Purified anti-mouse CD16/32 Antibody, Biolegend, San Diego, CA, USA) 200 µL/mouse (10 mL/Kg) 24 hours prior to MPTP administration (20 mg/Kg). Fourth group was injected with the control Isotype rat antibody (Ultra-LEAF Purified Rat IgG2a, κ Isotype Ctrl Antibody) 200 µL/mouse (10 mL/Kg) also 24 hours prior to the MPTP injection. The fifth group, as control of the antibody, was injected with a single dose of CD16/32 blocking antibody 24 hours before the injection of saline solution (n= 3 mice), and the final group,

labeled as control Isotype, an injection of the isotype rat antibody was injected 24 hours before an injection of saline solution (n= 2 mice).

After 72 hours, animals were euthanized by intracardiac perfusion of Tyrode solution and fixed with 4% PFA. Then, brains were extracted and post-fixed during 48h in 4% PFA. After post fixation, brains were stored until further sectioning in tubes PBS and NaN$_3$.

Sectioning

Brain sectioning was performed as described above for the intraperitoneal and intracranial injection of LPS experiments. Six series of 40-µm thick sections were obtained with a vibratome (Leica Microsystems, Germany) after removing the olfactory bulbs and setting the vibratome at a speed of 0.225 mm/s and 60 Hz vibrating frequency. Once sectioned, brain tissue was stored in PBS with NaN$_3$ until staining.

Immunohistofluorescence

In order to evaluate changes in degeneration of dopaminergic neurons and neuroinflammation due to administration of Cdc42 inhibitor ML141, and anti-CD16/32 neutralizing antibodies, floating sections were treated following the protocols of immunohistochemistry described in previous sections. Antigen retrieval pretreatment with citrate was done at 80 °C during 20 minutes. After this, blocking of unspecific binding sites was carried out with 0.5% Triton X-100 with 10% horse serum during 1 hour. Primary antibodies were also diluted in TBS-0.5% Triton X-100, 1% HS and 0.1% NaN$_3$. The primary antibodies used for this set of experiments were; anti-Iba 1 (1:500) to label microglial cells, anti-TH (1:500) to identify dopaminergic neurons, and anti-CD16/32 (1:250) in order to mark phagocytic microglia/macrophages. After 48 hours of incubation with the primary antibodies and proper subsequent washes, secondary antibodies diluted in TBS-0.5% Triton X-100, 1% HS and 0.1% NaN$_3$ (AlexaFluor 555 GoatAnti-rabbit 1:1000 to recognize Iba-1, AlexaFluor 488 Donkey Anti-sheep 1:1000 to recognize TH and AlexaFluor Goat Anti-Rat to recognize CD16/32) were used accordingly for an overnight incubation of the sections. Finally, DAPI (1:1000) was used to stain the nuclei of the cells. Sections were mounted on glass slides and coverslipped using antifading reagent (Prolong Gold, Invitrogen; Carlsbad, CA, USA).

Note: Table.1 for primary antibodies.

Confocal microscopy, stereology and quantification

To properly visualize detailed changes in the amount of dopaminergic neurons, sections of the SNpc, identified by the Mouse Brain Atlas criterion (Paxinos & Frankin, 2001)were imaged with high resolution in a confocal laser-scanning microscope (LSM 700; Carl Zeiss, Oberkochen, Germany), using Plan-Apochromat 20x/0.8 Ph2 M27 objective lens (Carl Zeiss; Oberkochen, Germany), and processed with the proper software of capture (ZEN 2010 Carl Zeiss; Oberkochen, Germany). At 20X objective level, the optical dissector was able to cover the extent of the SNpc, therefore, two to four serial sections were imaged for both hemispheres and the number of dopaminergic neurons was quantified (with Image J software), obtaining the average number of cells per SNpc, per animal.

A magnification of 1X was applied to the scanning field using the 20X objective in order to achieve higher definition to image Iba-1 and CD16/32 positive cells. Image analysis software and plugin (Image J software) allowed the transformation of images to black and white, and with the adequate threshold the area of every cell was measured and calculated.

Statistics

All the data were expressed as the mean with the SEM (standard error of the mean). Regarding the cell cultures and the Griess Assay, every experiment was performed at least 5 times. Normal distribution of the data was tested with the Shapiro-Wilk and Pearson normality test, being the P value bigger than the α value (α=0.05) to avoid discarding the gaussian distribution of the data. Once the normality was tested, a one-way ANOVA and multiple comparisons were conducted to compare the mean of every treatment, both between them and, especially, against the control. Tukey posthoc was also performed, being the α=0.05 and P value smaller than the α to accept the significant difference.

Regarding the in vivo experiments, for the intraperitoneal administration of ML141, every group counted with n=5. One of the groups did not follow a normal distribution; therefore, logarithmic modification was applied to every data set to analyze the data with one-way ANOVA with Tukey's multiple comparison posthoc.

Concerning the MPTP experiments, the data from the quantification, both the number of neurons and the area of Iba-1 were tested with Pearson's, Shapiro-Wilk and Kolmogorov-

Smirnov, indicating that normality should not be dismissed. Therefore, assuming variances were equal, comparison was done by one-way ANOVA and the LSD (least significant difference) post-hoc. In all cases, $P \leq 0.05$ was considered significant. All statistical analyses were performed using Prism 6 software (GraphPad Software; LaJolla, CA, USA) and SPSS software (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp).

Results

Microglial cells get activated and show morphological changes in a neuroinflammatory-like environment.

First off, since proinflammatory cytokines such as IFN-γ is known to be elevated in Parkinson's disease (PD) patients (Mount et al., 2007) and being crucial for microglial activation in PD animal models (Barcia et al., 2011), we set up a series of experiments in vitro to further understand the proinflammatory activation in parkinsonism. Then, BV-2 cells were exposed to increasing concentrations of IFN-γ alone, or combined with LPS, as a canonical proinflammatory inductor, added to the fact that it is a common bacterial antigen that people are exposed to, in order to mimic the neuroinflammatory-like environment in a parkinsonian scenario.

In the first day *in vitro*, as an evaluation of activation, the treatment with IFN-γ alone resulted in a release of nitrites in low concentrations (**Figure 1 A**). However, the release of nitrites was elevated when combining IFN-γ with LPS, obtaining the highest activation of the BV-2 microglial cells (**Figure 1 B**). Second day *in vitro*, BV-2 cells followed the same behavior, increasing concentrations of IFN-γ resulted in a significant release of nitrites but in low concentrations (**Figure 1 C**), whereas the combination with LPS, evidenced a higher release of nitrites indicating the highest activation of BV-2 cells (**Figure 1 D**).
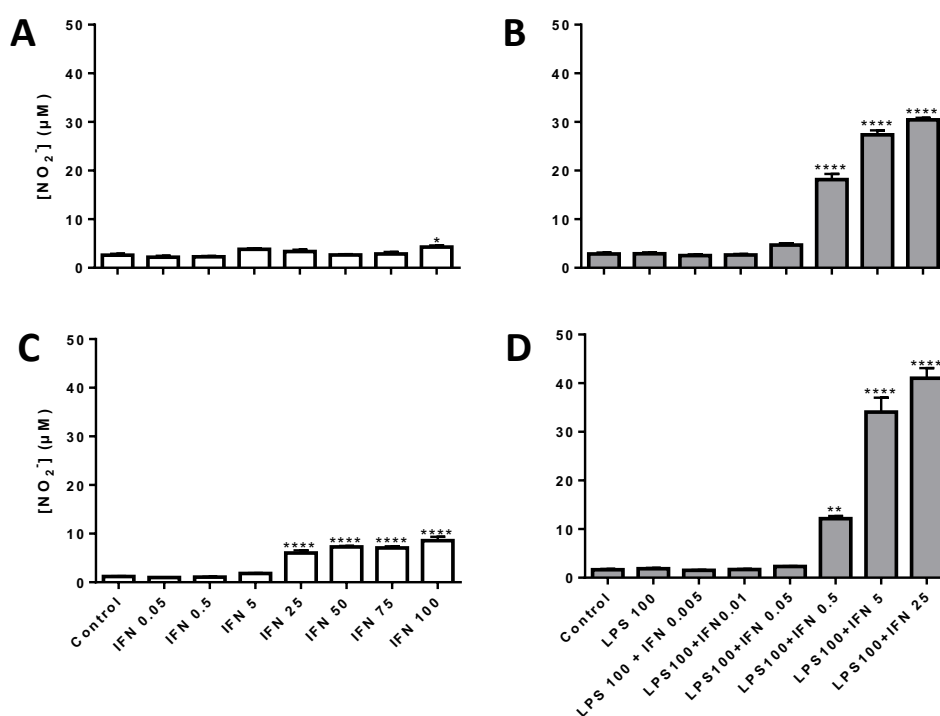
Figure 1. Microglial activation is reached when combining LPS and IFN-γ. Griess assay was used to measure the release of nitrites which indicates the level of activation of BV-2 microglial cells. (A) First day *in vitro*, the activation of microglial cells is significantly elevated (compared with the control) when stimulating with the highest concentration of IFN-γ. (B) Combination with LPS, resulted in even higher release of nitrites than the stimuli alone. Second day *in vitro,* the release of nitrites continued to be superior when challenging the cells with IFN-γ and LPS than with the stimuli alone. Comparisons were made against the control of every treatment (** $p < 0.05$, *** $p <0.001$, **** $p < 0.0001$).

To better understand the effect of proinflammatory inductors, the number of Iba1^{+} cells was evaluated in the different conditions (**Figure 2 A**), the BV-2 cell density increased as the concentration of IFN-γ augmented (**Figure 2 B**). Since the IFN-γ seems to be increasing the microglial cell density, in order to explore the possible proliferative effect of IFN-γ, a preliminary estimation was done by stereologically quantifying the number mitoses per concentration of IFN-γ. Interestingly, the number of mitoses per field increased, as the concentration of IFN-γ increased, concomitant to the microglial cell density (**Figure2 C and D**), suggesting that this cytokine might play a potential role in microglial cell proliferation. Increased cell density was not appreciable when combining LPS and IFN-γ, instead, the cell density significantly decreased (**Figure 2 E**), indicating, that the highest concentrations of IFN-γ combined with LPS may be deleterious for the BV-2 cells.

A

B

C

D
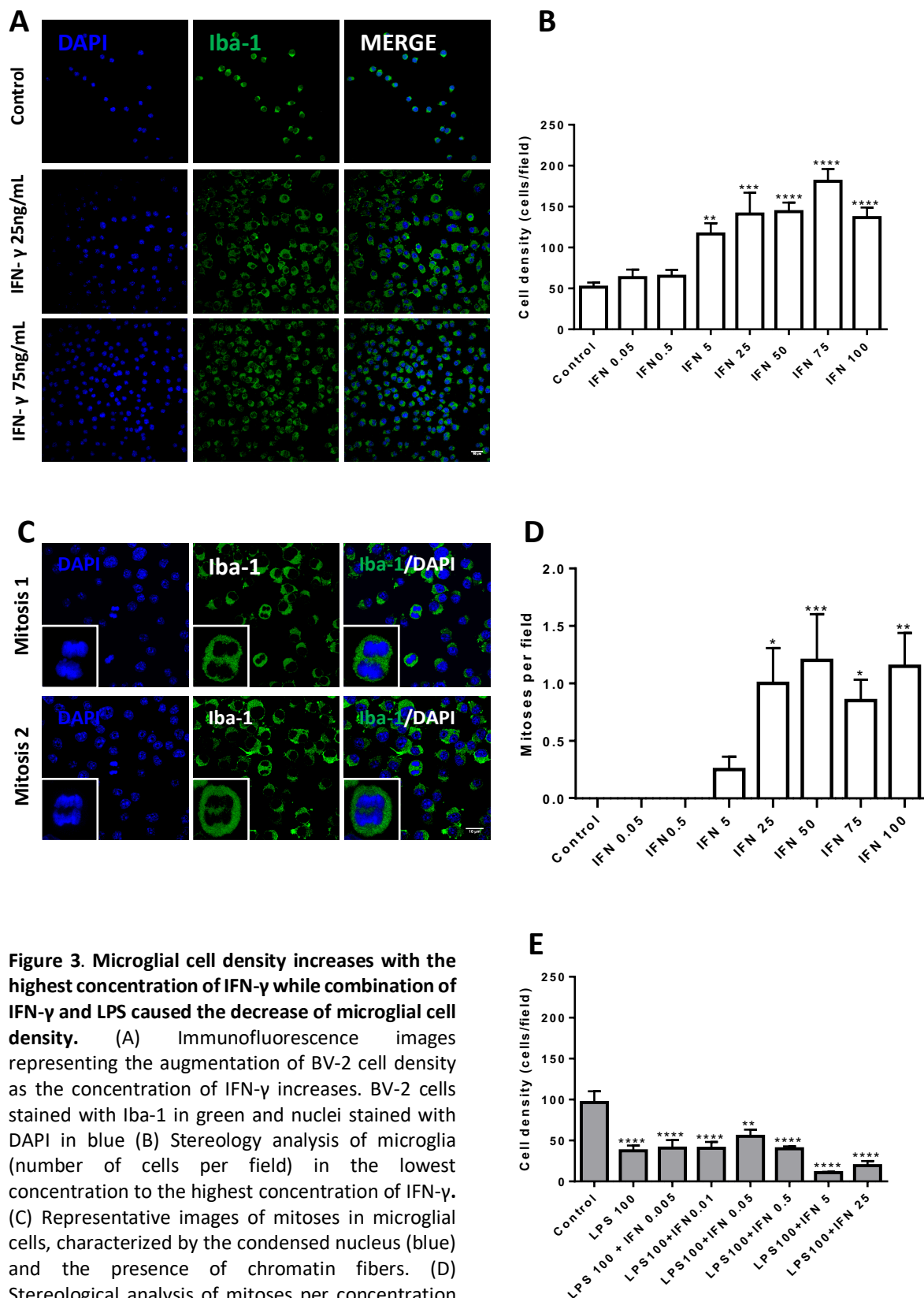
Figure 3. **Microglial cell density increases with the highest concentration of IFN-γ while combination of IFN-γ and LPS caused the decrease of microglial cell density.** (A) Immunofluorescence images representing the augmentation of BV-2 cell density as the concentration of IFN-γ increases. BV-2 cells stained with Iba-1 in green and nuclei stained with DAPI in blue (B) Stereology analysis of microglia (number of cells per field) in the lowest concentration to the highest concentration of IFN-γ. (C) Representative images of mitoses in microglial cells, characterized by the condensed nucleus (blue) and the presence of chromatin fibers. (D) Stereological analysis of mitoses per concentration of IFN-γ. (E) Stereological analysis of BV-2 microglia (number of cells per field) with fixed concentration of LPS and increasing concentrations of IFN-γ (*** $p < 0.001$ vs Control and IFN-γ 0.05) Scale bar: 10 μm

E

In addition, to assess changes in the morphology of the BV-2 microglial cells due to activation, the area of Iba-1 per cell was measured (**Figure 3**). On the one hand, results indicated a significant augmentation in the area of Iba-1 at a minimum concentration of IFN-γ 5 ng/mL, represented by the increase of cell bodies and thicker processes, consistent with an activated phenotype (**Figure 3 A**). On the other hand, BV-2 cells exposed to IFN-γ and LPS also exhibited a significant increase of the area of Iba-1. However, is important to remark the different morphology presented by microglial cells at the highest concentrations of IFN-γ plus LPS, characterized by a fusiform-like shape cell expressing Iba-1 (**Figure 3 A, last panel**). For that reason, the two maximum concentrations of IFN-γ plus LPS were statistically analyzed separately from the rest due to the potential high toxicity at these conditions.

B

C
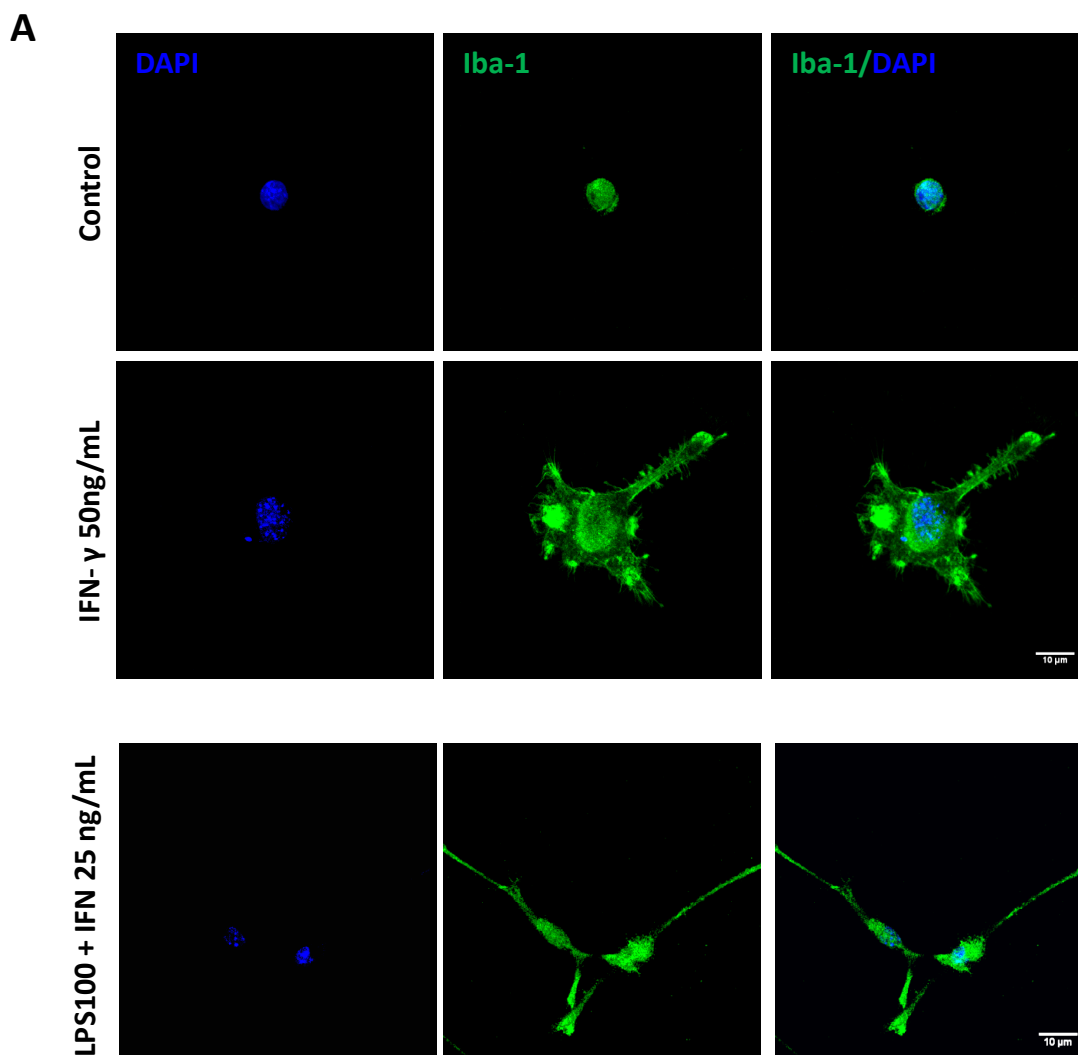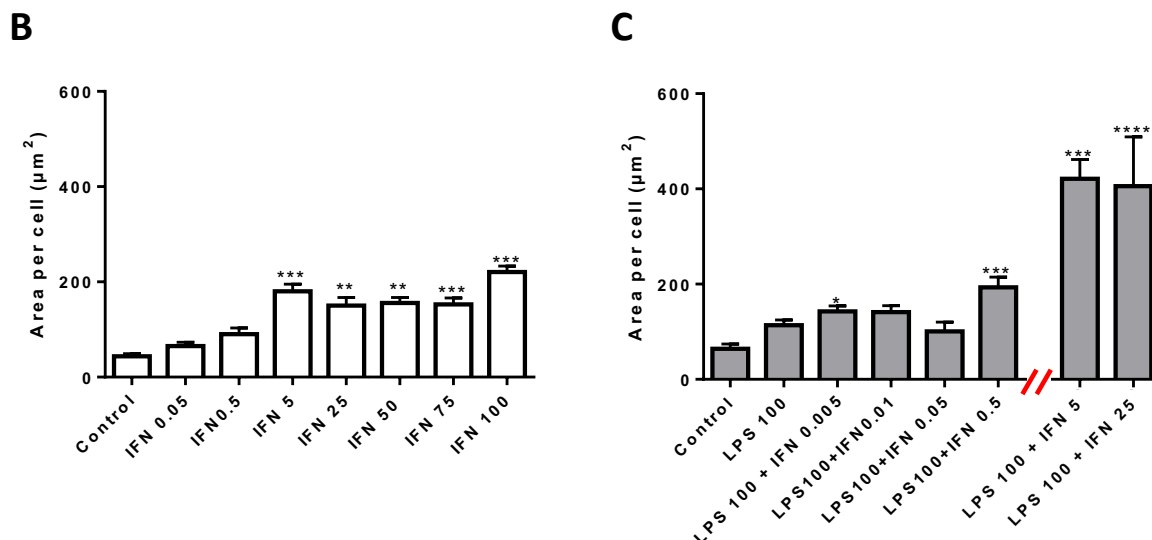
Figure 3. BV-2 cells increase the area of Iba-1 due to microglial activation. (A) Representative images of a resting and an activated BV-2 microglial cell stained with Iba-1 (green). Third panel represents a fusiform-like shape microglial cell, when combining LPS and the maximum concentration of IFN-γ. (B) Stereological analysis of BV-2 cells. Area of the expression of Iba-1 was measured per cell, indicating a significant augmentation while the concentration of IFN-γ increases. (C) Similar trend is observed when treating the cells with IFN-γ and LPS, with the particularity of the presence of significant bigger elongated microglial cells. Comparisons were made against the control (** $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$).

Results described above evidenced that BV-2 microglial cells achieved a higher activation when treated with the proinflammatory cytokine IFN-γ and the antigenic stimulus LPS with lower toxicity. Therefore, a concentration of LPS 100 ng/mL combined with 0.5 ng/mL IFN-γ was selected, simulating an inflammatory environment, since the concentration can activate the cells without being deleterious.

Microglial activation promotes dopaminergic degeneration in neuroinflammatory conditions

Because IFN-γ and LPS induces a solid microglial activation, we wanted to study how this inflammatory-mediated process affects dopaminergic degeneration as it is one of the major neuropathological features of PD. Therefore, we hypothesized that the inflammatory environment characterized by the stimuli of IFN-γ and LPS might promote dopaminergic elimination. Thus, an *in vitro* experiment was set up with BV-2 microglial and PC12 dopaminergic cells to simulate a parkinsonian-like proinflammatory scenario.

Microglial cells previously treated with LPS 100 ng/mL and INF 0.5 ng/mL were co-cultured at different timepoints (4, 10 and 24 hours) with PC12 dopaminergic cells. The analysis revealed that the classical inflammatory stimulation, by the combination of IFN-γ and LPS, activated microglial cells without changing significantly their cell density through time (**Figure 4 B**). Importantly de number of PC12 cells decreased significantly when the microglial cells were activated with LPS/IFN-γ (**Figure 4 C**). Moreover, to study whether the cell-cell interactions and potential phagocytosis may intervene in this process of elimination, we analyzed the close appositions between cell types. Our analyses revealed that BV-2 cells interacted with PC12 cells, establishing cell-to-cell contacts through time regardless of the treatment (**Figure 5 A and B**). However, although is not significant, this interaction seems to be decreasing over time, probably due to the actual decrease of dopaminergic cells (**Figure 4 B**). To better understand if these interactions may result in phagocytosis, we analyzed the phagocytic events in our *in vitro* set up. The phagocytic events were characterized as a either pyknotic nucleus (**Figure 5 C**) or PC12 cell TH-expressing debris, inside a microglial cell (**Figure 5 E**). Interestingly the number of phagocytic events increased as the PC12 cell density decreased, only occurring when the BV-2 cells were previously activated with LPS and IFN-γ (**Figure 5 D**) suggesting that phagocytosis of dopaminergic cells may be an important cause of the evident PC12 elimination.

A
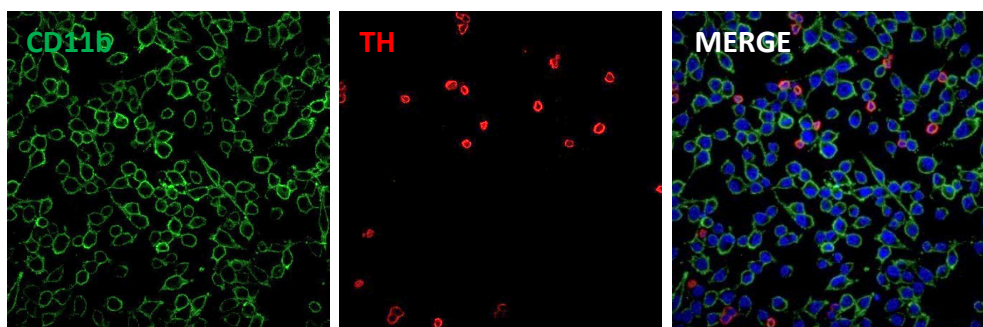
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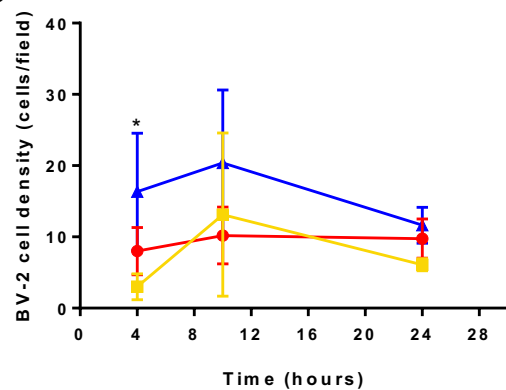
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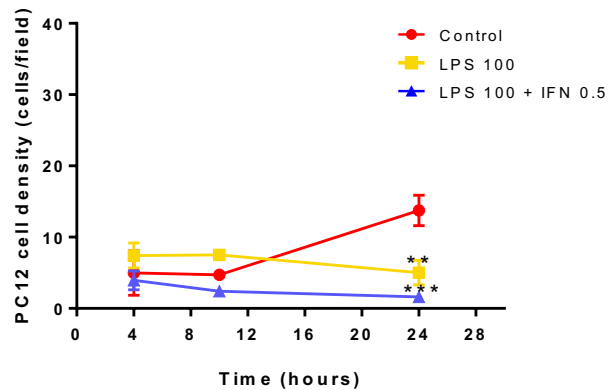
Figure 4. Dopaminergic cell density decreases when microglial cells are activated, while BV-2 cell density remains constant through time. (A) Representative image of a co-culture with BV-2 microglial cells immunostained for Cd11b (green) and PC12 dopaminergic cells immunostained for TH (red). (B) BV-2 cell density remains without significant changes over time, despite the activation. (C) PC12 cell density diminishes when BV-2 cells are activated with LPS and IFN-γ.

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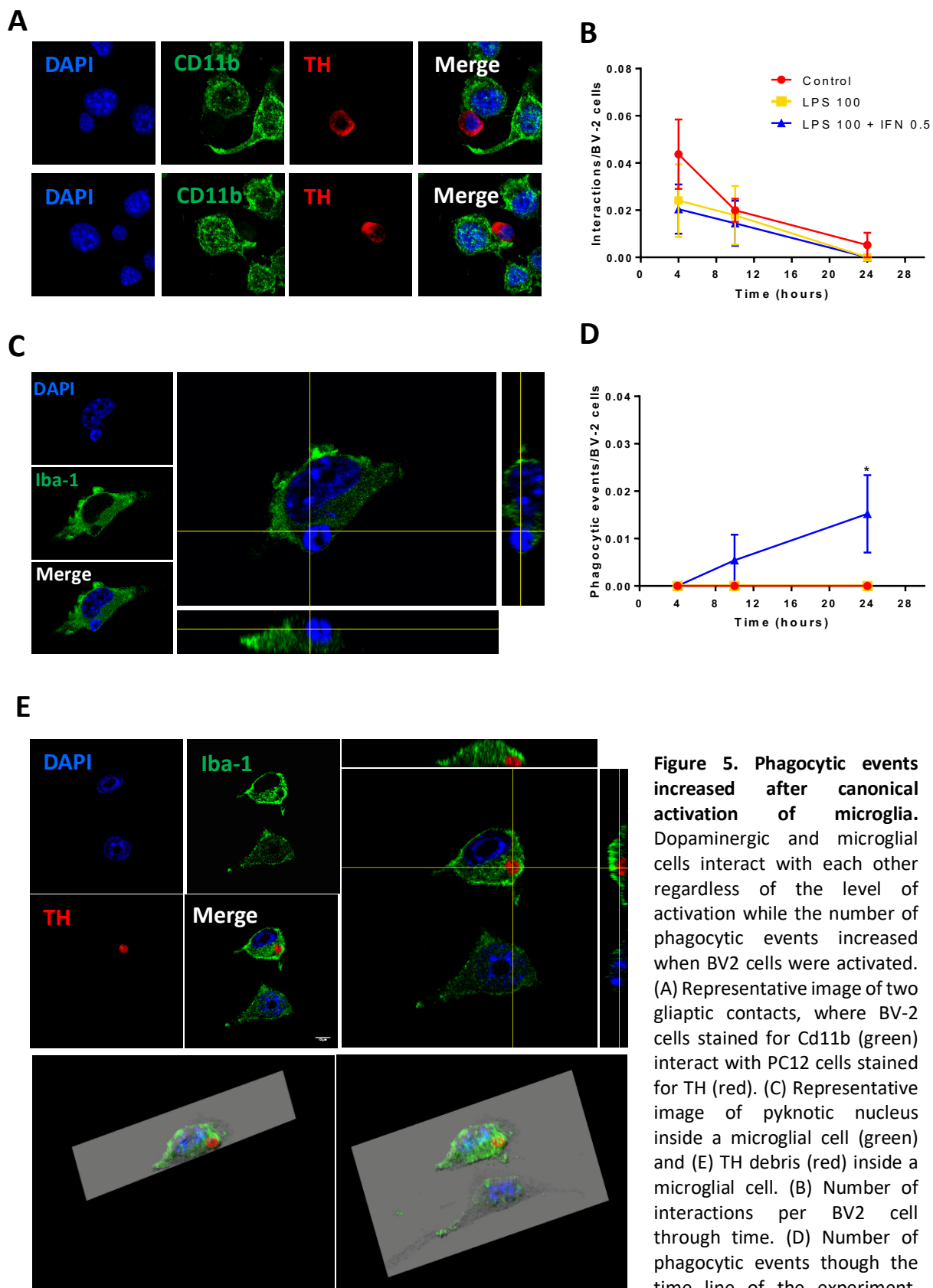
Figure 5. Phagocytic events increased after canonical activation of microglia. Dopaminergic and microglial cells interact with each other regardless of the level of activation while the number of phagocytic events increased when BV2 cells were activated. (A) Representative image of two gliaptic contacts, where BV-2 cells stained for Cd11b (green) interact with PC12 cells stained for TH (red). (C) Representative image of pyknotic nucleus inside a microglial cell (green) and (E) TH debris (red) inside a microglial cell. (B) Number of interactions per BV2 cell through time. (D) Number of phagocytic events though the time line of the experiment. (*p< 0.05 LPS/IFN-γ vs other conditions)

Primary exposure to IFN-γ and secondary challenge with LPS induces full microglial activation

Microglial priming has been defined as a pre-activated state, as a result of a primary inflammatory stimulus, susceptible to receive a secondary inflammatory stimulus leading to an exacerbated microglial response which can become deleterious for the bystander cells, including neurons (Perry & Holmes, 2014).

Knowing that the higher activation of microglial cells is achieved when stimulating with IFN-γ and LPS compared with IFN-γ or LPS alone, we analyzed the effect of the sequential stimulation in different orders. As aforementioned, activation was measured with the expression of nitric oxide, therefore the release of nitrites by microglial cells. In this case, stimuli were separated in primary or secondary, changing the order of administration in every condition, and waiting 24 hours between one and the other. Finally, after 24 hours of the last stimulus, activation was measured by the release of nitrites. In order to study which one of the stimuli generates the maximum priming, and the highest activation as a consequence, we used both BV-2 microglia and also primary mesencephalic rat microglia to closely mimic the anatomical conditions of parkinsonism. Our results indicated that priming of microglial cells takes place with both options. But most importantly, the maximal activation was reached when the microglial cells were first stimulated with IFN-γ and later challenged with LPS (**Figure 6 A**). Interestingly this result was replicated both in BV-2 cell line and in primary cultures of mesencephalic microglia (**Figure 6 B**). Primary microglia was successfully extracted and seeded in plates, from the rat mesencephalon (**Figure 7 A**). Once seeded, primary microglia were submitted to priming, stimulating first with LPS or IFN-γ and 24 hours later again with LPS or IFN-γ. Moreover, dopaminergic neurons from rat mesencephalon were also extracted and seeded in plates, normally this process has low efficiency, but our goal was to standardize the procedure to obtain a proper number of dopaminergic neurons to control the anatomical location of our cultures (**Figure 7 B and C**).

Thus, these results are the confirmation that the phenomenon of priming takes place in both BV-2 and primary microglia, being IFN-γ the most effective primary stimulus to induce an overreactive response and potentially having detrimental effects in a neuroinflammatory environment.

Note: Primary cultures were extracted from rat since primary cultures from mice mesencephalon have poor efficiency.
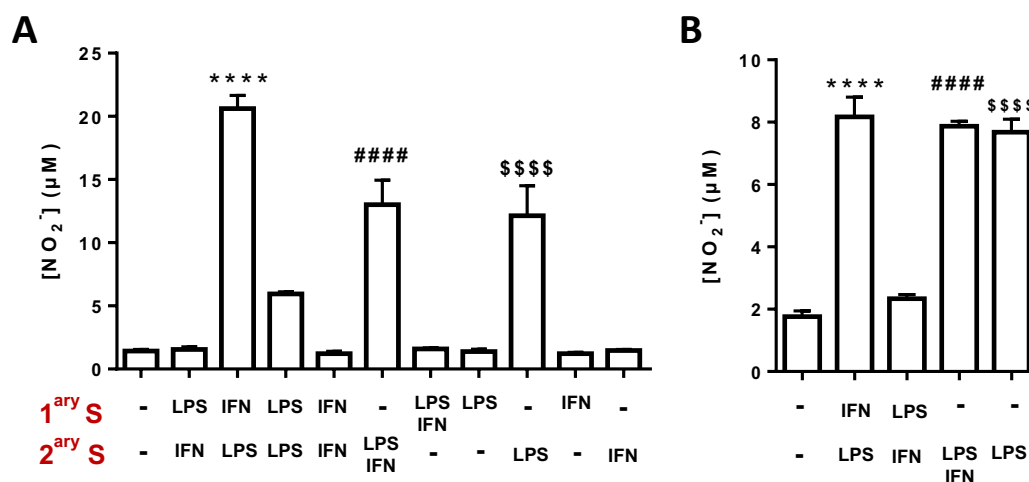
Figure 6. IFN-γ priming causes the highest activation of microglial cells. Nitrites released by microglial cells are measured by Griess assay, indicating that sequential stimulation with IFN-γ and subsequent LPS challenge is necessary to achieve the highest microglial activation in (A) BV-2 cell cultures and in (B) primary cultures of mesencephalic microglia. (A) ****p<0.0001 vs every condition; #### p<0.0001 vs every condition except - + LPS; $$$ p<0.0001 vs every condition except - + (LPS/IFN). (B) **** p<0.0001 vs -/- and LPS/IFN; #### p<0.0001 vs -/- and LPS/IFN-γ; $$$$ p<0.0001 vs -/- and LPS/IFN.
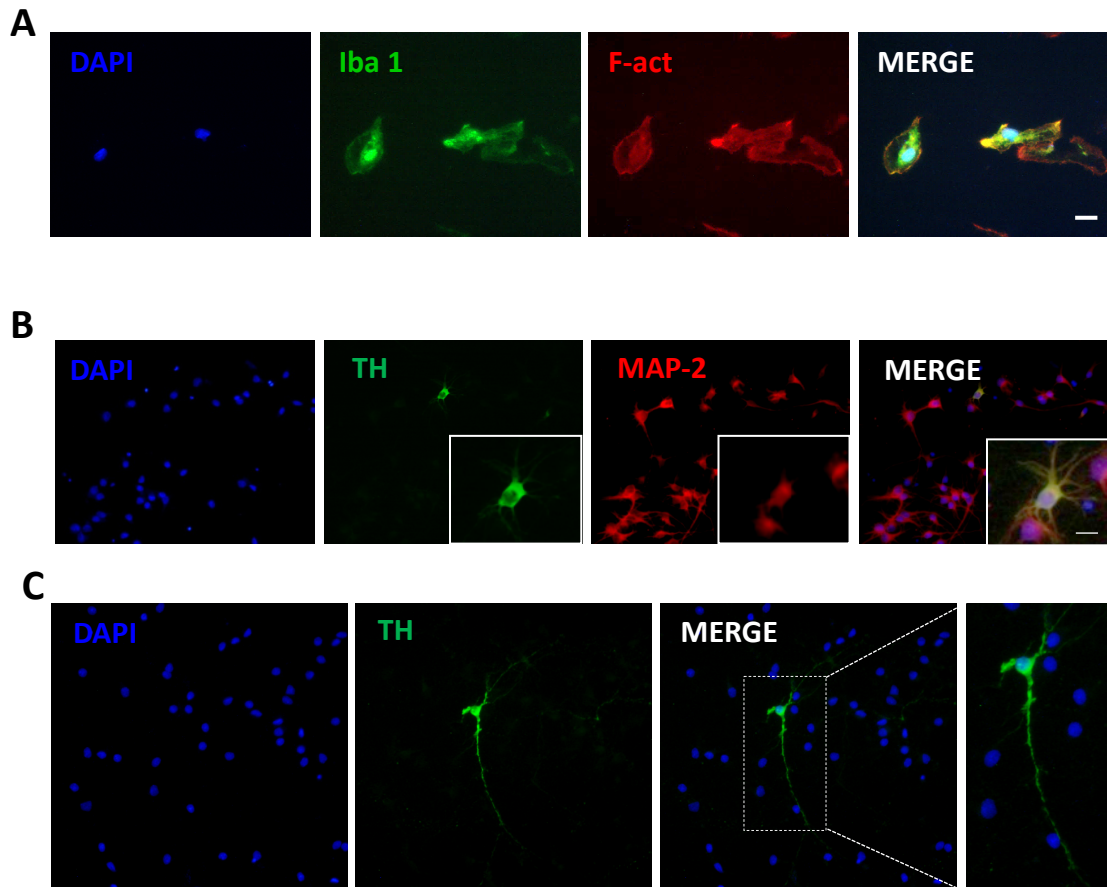
Figure 7. Mesencephalic cultures generate microglia and dopaminergic neurons. Immunofluorescence of primary culture expressing Iba1 (green), F-actin (red) and nucleus counterstained with DAPI (blue) (scale bar: 15 μm). (B) Microscope image from neurons immunostained for MAP-2 (red) and dopaminergic neurons immunostained for TH (green) (scale bar: 30 μm). (C) Zoom image of a dopaminergic neuron expressing TH (green) (scale bar: 30 μm).

Microglial priming causes rapid dopaminergic elimination after full activation

Since the sequential stimulation of IFN-γ and LPS induces the maximum microglial activation, we set up a new co-culture experiments with BV-2 and PC12 cells in order to mimic the neuroinflammatory environment in PD where microglia and dopaminergic neurons interact. BV-2 were first stimulated with IFN-γ and 24 hours later challenged with LPS, 24 hours later PC12 cells were added to the culture. Immunofluorescence technics allowed us to quantify cell-to-cell contacts and visualize phagocytic events.

Several evidences support that phagocytic process is very fast. In fact, experiments with opsonized beads indicate that total engulfing of the target takes no longer than 10 minutes (Schlam et al., 2015; Uribe-Querol & Rosales, 2020).Hence, in this experiment the timepoints of interaction were shorter than the previous experiment (10, 20, 30 until 60 minutes) since the cells may establish the first contacts at least at 10 minutes.

First off, being a cell line culture, the quantification allowed us to observe that cell density of PC12 was highly dynamic, increasing and decreasing through time, due to the proliferative nature of the cells (**Figure 8 A**). For that reason, the number of interactions was also variable through time regardless of the treatment (**Figure 8 C**). Importantly, the analysis revealed that full microglial activation resulted in a rapid loss of dopaminergic-like cells at 60 minutes, evidenced by the decrease in PC12 cell density (**Figure 8 B**) when BV-2 microglial cells were primed by IFN-γ, followed by LPS. Interestingly, we also observed evidences of phagocytic events (**Figure 8 CIII**). Moreover, cell-cell interactions where categorized in 1-1 (one BV-2 contacting one PC12), 1-2 (one BV-2 contacting two PC12), 1-3 (one BV2 contacting three PC12) and 2-1 (two BV-2 contacting one PC12). Importantly, we found that the predominant types of contact were the 1-1 in contrast with other types in every timepoint (**Figure 8 D and E**). With these results we hypothesized that the elimination of dopaminergic neurons may be by primary phagocytosis (or phagoptosis), starting with a contact (**Figure 8 CI**) followed by the formation of a phagocytic cup (**Figure 8 CII**) and the total engulfment where a pyknotic nucleus is visualized inside microglial cell labeled membrane (**Figure 8 CIII**).
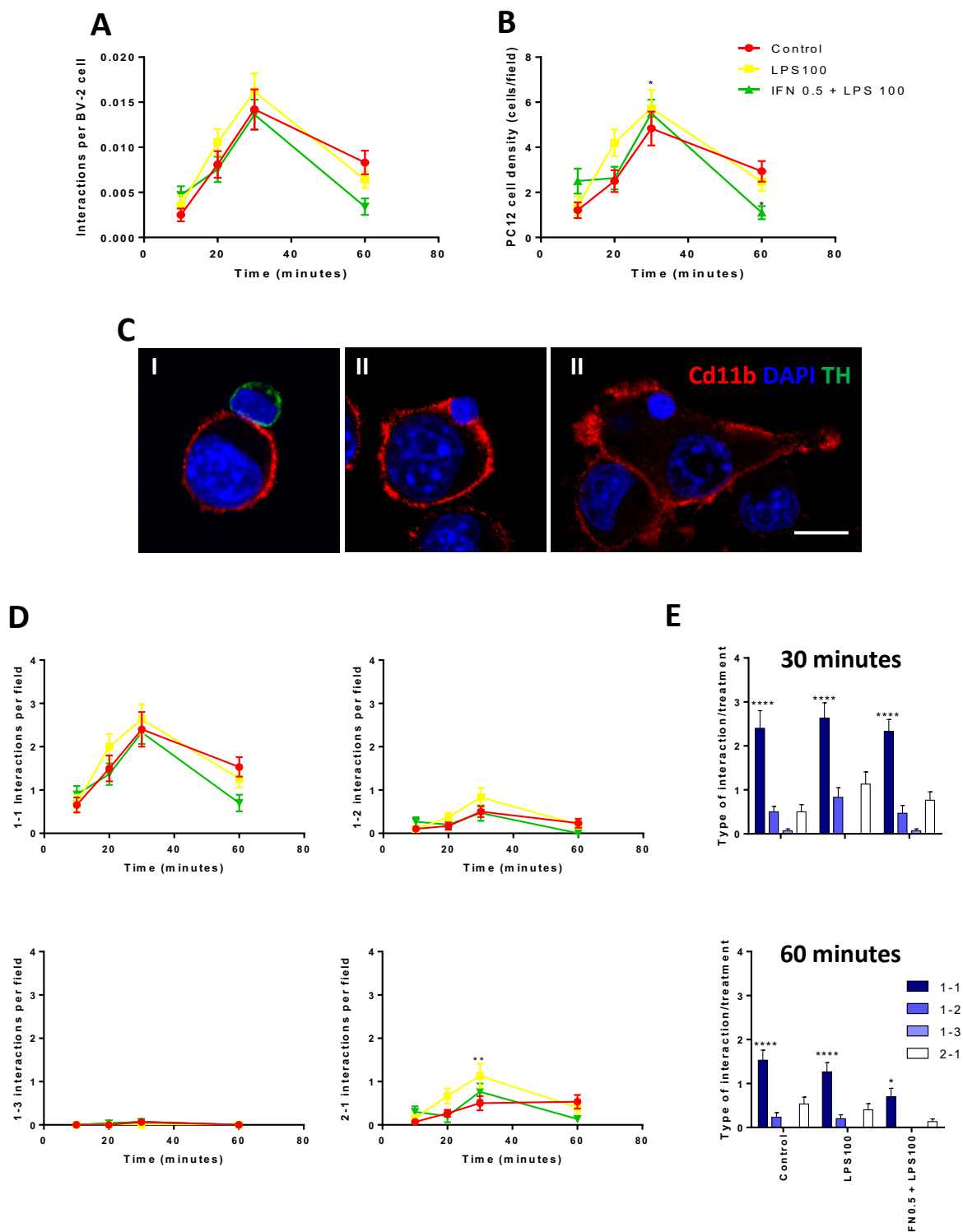
Figure 8. Microglial full activation after IFN-g priming results in one-to-one dopaminergic elimination. Rapid dopaminergic degeneration takes place when microglial cells are fully activated and it is predominantly correlated with 1-1 cell-to-cell contacts. (A) The number of interactions between PC12 and BV-2 cells varied with the same pattern through time in all the conditions. (B) PC12 cell density decreased significantly at 60 minutes when microglial cells were primed (* p< 0.05 IFN-γ 0.5 + LPS vs LPS100 and Control). (C) Confocal images illustrating a characteristic contact (I) between BV-2 microglial cell stained for Cd11b (red) and PC12 cell stained for TH (green) (Scale bar: 10 μm). (II) BV-2 (red) forming a phagocytic cup containing a pyknotic nucleus stained with DAPI (blue). (III) BV-2 cell (red) with pyknotic nuclei (blue) inside the cell. (D) Types of interactions (1-1, 1-2, 1-3 and 2-1) and their amount through the timepoints of the culture. (E) Quantification of type of contacts at 30 and 60 minutes. (**** p<0.0001 vs every other treatment).

Neutralizing antibody CD16/32 prevents dopaminergic degeneration

Because full microglial activation after IFN-γ priming induced elimination of dopaminergic cells, and being exacerbated phagocytosis a critical contributor of dopaminergic degeneration in a pro-inflammatory environment, we aim to target a central phagocytic receptor in microglia to block phagocytosis. Hence, we proposed the use of neutralizing antibodies against CD16/32 FcγR subunits, first to assess the contribution of phagocytosis in this scenario and then as a potential therapy to avoid the exacerbated microglial-mediated phagocytosis of dopaminergic neurons. First of all, we tested the presence of the receptor by means of immunofluorescence techniques using a CD16/32 staining antibody and demonstrated the expression of CD16/32 in the membrane and protruding filaments of BV-2 microglial cells (**Figure 9 A**).

To demonstrate the effect of the immunotherapy *in vitro*, we set up a new co-culture of BV-2 microglial cells and PC12 dopaminergic cells. We tested the blocking antibody against CD16/32 before and after classical priming of microglial cells, and then we let them interact with the dopaminergic cells during 60 minutes. The results indicated that in both conditions, blocking CD16/32 receptor before and after the activation was successful (**Figure 9 B**). PC12 cell density significantly decreased when BV-2 were previously activated with IFN-γ + LPS. However, this decrease was prevented when the cells were treated with CD16/32 blocking antibody. Interestingly, the effect of neutralizing antibody CD16/32 in PC12 cell density was very similar in both conditions, protecting the dopaminergic cells from phagocytic elimination (**Figure 9 B**). This result suggests that the contribution of phagocytosis in dopaminergic degeneration may be more prominent than previously thought and targeting phagocytic receptors may protect dopaminergic cells.

A

B
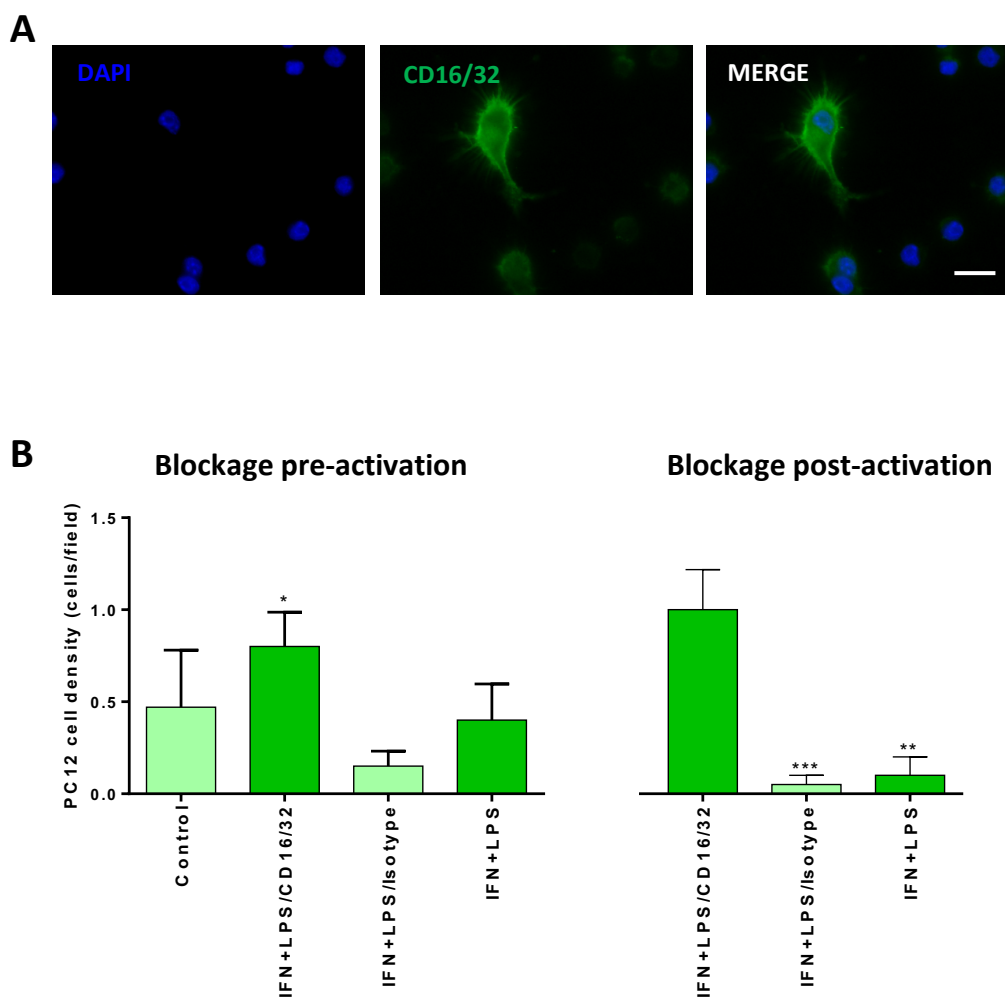
Figure 9. CD16/32 neutralizing antibody prevents dopaminergic cells elimination. (A) Representative image of BV-2 expressing CD16/32 (green) in the membrane and filopodia. (B) Quantification of the number of PC12 cells after 60 min of interaction with BV-2, previously primed and 24 hours later treated with the neutralizing antibody CD16/32 (**Blockage pre-activation**). Blockage post-activation indicates the number of PC12 cells after 60 min of interaction with BV2, previously treated with the neutralizing antibody and 24 later primed and activated. In both cases, when BV-2 microglia were blocked with CD16/32 neutralizing antibodies, PC12 dopaminergic cells were protected from phagocytic elimination. *** $p < 0.001$ (IFN-γ+LPS-CD16/32 significant against every other treatment). Scale bar: 12 μm.

Inhibiting Cdc42 protects dopaminergic cells from microglial-mediated phagocytosis

Since it has been described that CD16/32-mediated phagocytosis is regulated by Cdc42 signaling, we proposed to explore whether modifications in the Cdc42 signaling pathway resulted in similar consequence for the dopaminergic cells than blocking CD16/32. Cdc42 is a small GTPase of the Rho family which tightly regulates the organization of the actin cytoskeleton in the process of cell motility and is specially involved in the in the formation of the phagocytic cup (Cox et al., 1997; May & Machesky, 2001). Thus, in order to achieve this aim, we used ML141 a non-competitive inhibitor of Cdc42 GTPases. Firstly, we tested whether ML141 was innocuous for BV-2 microglia by measuring the cell viability with a colorimetric assay MTT (4 Succinate dehydrogenase activity) using H_2O_2 as a control. The MTT indicated the percentage of viability of BV-2 cell with different concentrations of ML141, indicating that up to a concentration of 20 µM of ML141, the cell viability remains unaltered compared with vehicle and H_2O_2 toxicity, indicating that administration of ML141 result harmless for BV-2 microglial cells (**Figure 10**).
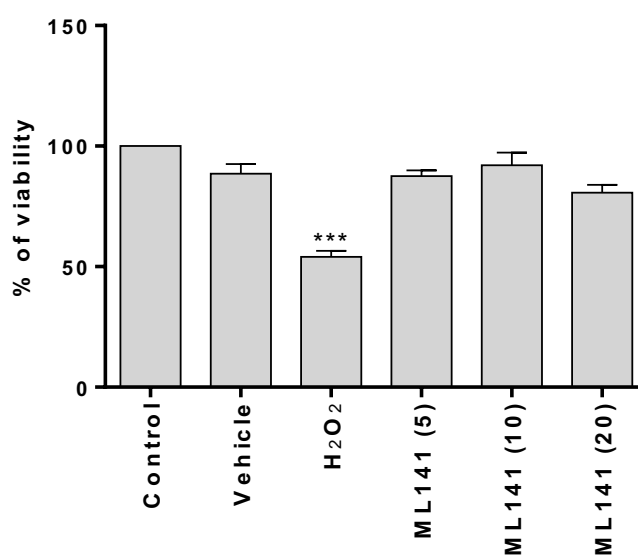
Figure 10. Increasing concentrations of ML141 resulted safe for BV-2 cell viability. MTT cell viability assay for BV-2 microglial cells submitted to increasing concentrations of ML141 in order to test their safety. 500 µg of H_2O_2 resulted in a decrease of approximately 50 % of the cell viability, however, no significant decrease was appreciated after the administration of ML141 (*** $p<0.001$ H_2O_2 significant against every other treatment).

Since our aim is to inhibit the motility-mediated phagocytic process of microglial cells by Cdc42, we also tested the expression of this small GTPase, using an antibody against Cdc42 to detect their microanatomical location. The fluorescent images showed that Cdc42 accompanied F-actin in the formation of protrusion of BV-2 microglia (**Figure 11 A**). Once we knew that the administration of ML141 was safe for microglial cells, we set up a co-culture with BV-2 and PC12 to evaluate the phagocytic capacity. We tested the effect of inhibiting Cdc42, by using ML141, before and after the microglial activation by IFN-γ priming, then we added the PC12 dopaminergic cells and let them interact for 60 minutes (a timepoint stablished in the previous experiments). After the time of interaction, the co-culture was fixed followed by immunocytofluorescence. BV-2 cells were stained for CD11b, a classical marker for microglial cells, and PC12 were stained for TH, the common marker for dopaminergic cells. The number of PC12 were quantified in every condition to evaluate the effect of the Cdc42 inhibitor in the survival of PC12 cells. Quantification resulted in diminished numbers of PC12 dopaminergic cells when microglial cells were activated with IFN-γ and LPS. Importantly, when microglia were treated with ML141, the decrease of PC12 was significantly halted. This protection happened in both conditions, when inhibition of Cdc42 was done either before or after microglial activation. The effect was evident, comparing with the activated microglia when the inhibition was done before the activation (Pre-activation inhibition) (**Figure 11 B**). On the other hand, when inhibiting Cdc42 was done after the activation, although also resulted in a protection of PC12 cells, the effect was not as efficient as inhibiting before the activation (Post-activation inhibition) (**Figure 11 B**). Even though there were slight differences regarding the order of the inhibition, these results show a similar outcome when blocking CD16/32 on BV-2 cells suggesting that inhibition of microglial cell motility also prevent microglial-mediated phagocytosis of PC12 dopaminergic cells.
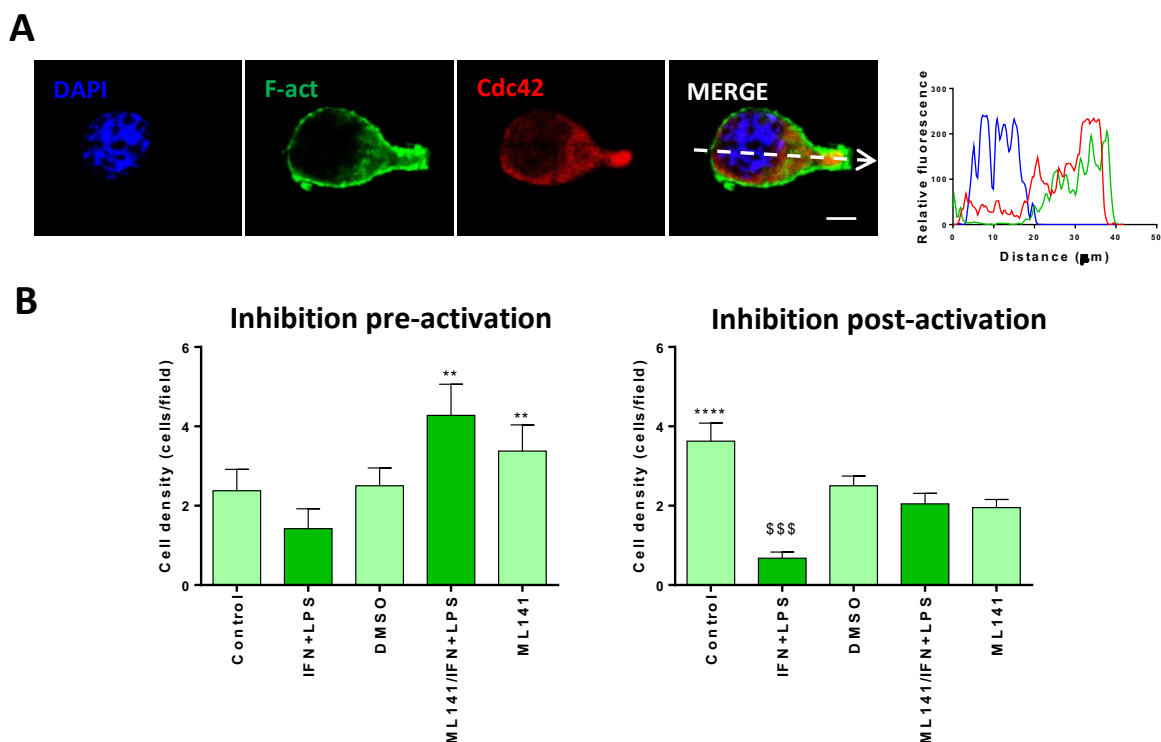
Figure 11. Cdc42 inhibition protects dopaminergic cells from elimination. (A) Representative image of a BV-2 microglial cell expressing high density of Cdc42 (red) in a F-actin-rich protrusion. The nucleus was counterstained with DAPI (blue). Plot profile of relative fluorescence displays high fluorescence of Cdc42 corresponding with high fluorescence of F-act. (B) Quantification of the number of PC12 cells at 60 min of interaction with BV-2. BV-2 were treated before and after activation with Cdc42 inhibitor ML141. Decrease of PC12 cells was prevented when BV-2 cells were incubated with ML141. (**Pre-activation inhibition** ** $p<0.01$ IFN-γ + LPS vs ML141/IFN-γ +LPS and ML141. **Post-activation inhibition** \$\$\$ $p<0.001$ IFN-γ + LPS vs every condition and **** $p<0.0001$ control vs IFN-γ + LPS). Scale bar: 3 μm

Intrastriatal LPS injection induces ipsilateral neuroinflammation characterized by CD16/32 expression and induces dopaminergic degeneration in the SNpc

Since *in vitro* results, indicated that the inflammatory environment can induce dopaminergic cell elimination, we first proposed to test the effect *in vivo* in a pure neuroinflammatory model by using LPS. Previous studies of our group demonstrated that intranigral administration of LPS resulted in a massive inflammatory response in the SN causing a parenchymal damage not equivalent to the parkinsonian scenario (Heman-Bozadas et al, in preparation). For that reason, we used a different approach by injecting LPS in the striatum. Since dopaminergic neurons of the SNpc project to the striatum, we obtained a contained neuroinflammation which can induce dopaminergic neuronal elimination. To achieve this aim, we injected LPS in the striatum of mice, and after 3- and 7-days brains were extracted after perfusion/fixation and sectioned. Immunofluorescence of the SN sections was done, staining microglial cells for Iba-1, dopaminergic neurons and fibers for TH (**Figure 12 B**) and nuclei counterstained with DAPI. Coherently, immunohistochemistry of the striatum and SN showed a significant neuroinflammation evidenced by the increased area of Iba-1. In fact, the difference between both hemispheres can be appreciated with the naked eye (**Figure 12 C**). Interestingly, at 3 days post-injection, in the ipsilateral hemisphere, the neuroinflammation was the highest compared with 7 days and the contralateral hemisphere (**Figure 12 D**). This phenomenon correlated with the neuronal elimination already visible at 3 days in the ipsilateral hemisphere. The number of TH positive neurons in the SN were quantified normalizing with the number of nucleus expressing DAPI. This result showed, a significant decrease in the number of TH neurons at 3- and 7-days post injection in the ipsilateral hemisphere, suggesting this model as suitable to analyze dopaminergic degeneration in the SN (**Figure 12 D**).

Importantly, staining antibody anti-CD16/32 was also tested in the striatum which resulted in a high expression of CD16/32 positive microglia (**Figure 12 A**), indicating that neuroinflammation induced by LPS administration may produce the externalization of FcγR such as CD16/32. In addition, high-resolution images obtained with z-scan confocal microscope, showed phagocytic structures of the ball-and-chain type (**Figure 13**), where microglial branches contain pyknotic nucleus, evidencing phagocytic events in the SN of animals treated with LPS. Overall, these experiments suggest that neuroinflammation by itself can induce neuronal death, probably by the contribution of CD16/32-mediated phagocytosis.

Therefore, these results paved the way to test the immunotherapeutic approach of using neutralizing antibody against CD16/32 in a established PD model such as MPTP intoxication.
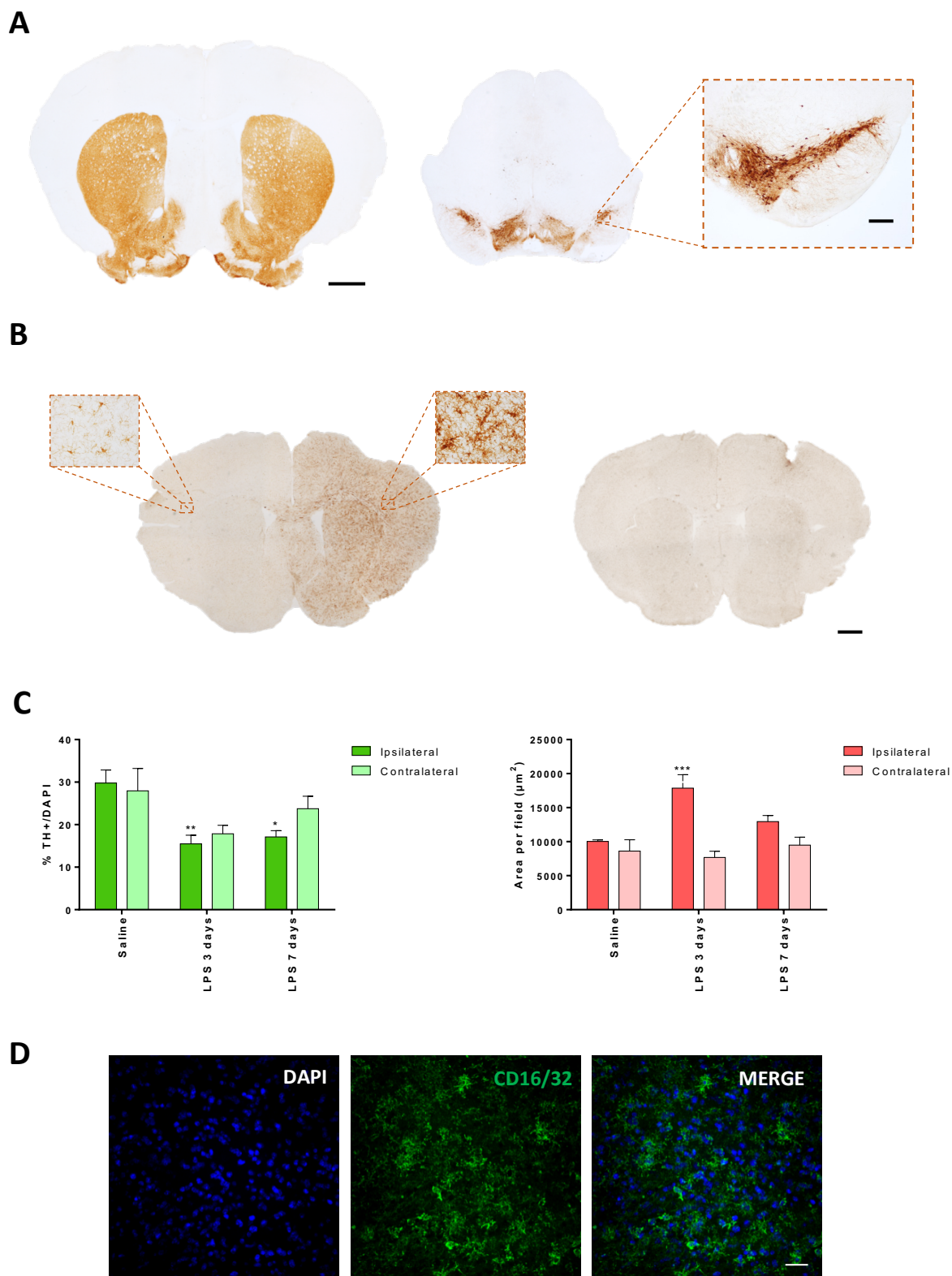
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Figure 12. Striatal administration of LPS induces dopaminergic degeneration in the SNpc. (**A**) Representative image of the striatum with dopaminergic fibers stained for TH (Scale bar: 500 µm). Mesencephalon with dopaminergic neurons of the SN stained for TH (scale bar: 200 µm). (**B**) Representative image of mice striatum with high expression of Iba-1 in microglia (and potentially macrophages at the site of injection) in the ipsilateral hemisphere where LPS was injected (right hemisphere). Right panel displays a control striatum injected with saline showing low expression of Iba-1 (scale bar: 500 µm). (**C**) Left panel: quantification of the number of dopaminergic neurons of the SN expressing TH. Significant decrease of neurons is appreciated at 3 days and 7 days in the ipsilateral hemisphere (** $p<0.01$ and * $p<0.05$ vs saline). Right panel: Quantification of the area of Iba-1 expressed in microglia in the SN indicated significant increase at 3 days post-injection in the ipsilateral hemisphere. (*** $p<0.001$ vs contra and ipsilateral saline, vs LPS 3 days contralateral and LPS 7 days contralateral). (**D**) Confocal image representing expression of CD16/32 in the striatum after intrastriatal injection of LPS (scale bar:20 µm).
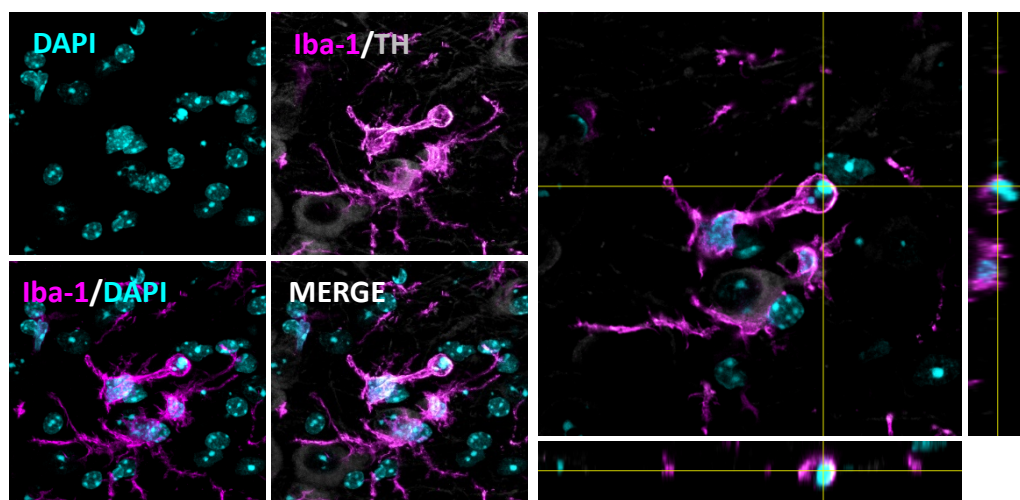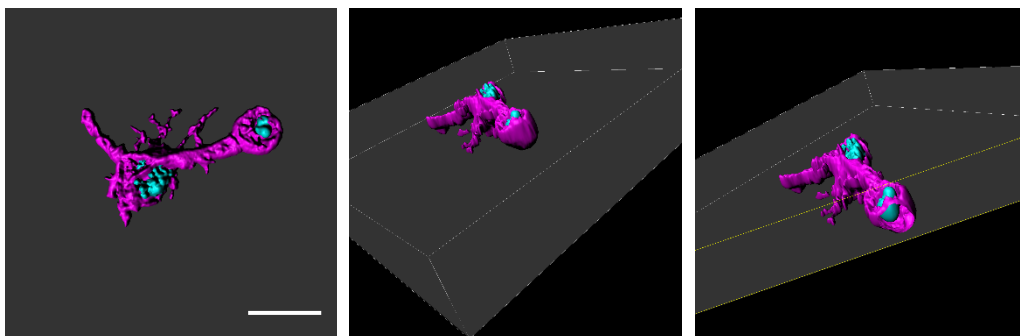
Figure 13: Phagocytic event with "ball and chain" structure in the SNpc: (A) High resolution stack image of microglial branch containing a pyknotic nucleus. Microglia immunostained with Iba-1, dopaminergic neuron with TH and nuclei counterstained with DAPI. Orthogonal views evidenced the engulfing along the z stack showing the pyknotic nuclei inside an Iba-1 ⁻ space, consistent with the phagosome structure. (B) 3D-reconstruction at central clipping plane show the phagosome containing the pyknotic nucleus (Scale bar: 10 µm).

Neutralizing antibody against CD16/32 protects dopaminergic neurons of the SNpc in an MPTP-induced degeneration model of PD

After testing the effectiveness of CD16/32 blocking microglial phagocytosis *in vitro*, we evaluated the effect of this immunotherapy in the MPTP model of PD. Following our research line, it has been described that MPTP induces the specific degeneration of dopaminergic neurons of the SNpc. Moreover, the administration of MPTP also induces an early microglial-mediated neuroinflammation with the consequent phagocytosis of dopaminergic neurons (Barcia et al., 2012). Coherently, in the above described experiment, we demonstrated that a pure neuroinflammatory model, as LPS, induced the expression of phagocytic receptor FcγR CD16/32 concomitant to dopaminergic elimination. Therefore, we also tested whether MPTP was also able to increase CD16/32 expression after MPTP.

Serial histological sections of the SNpc were obtained in the vibratome and subsequent immunofluorescence was done. We then tested the expression of CD16/32 in the SNpc with a specific antibody against CD6/32 (**Figure 14**). The stereological analysis of the sections indicated that MPTP intoxication resulted in an increase of microglial phagocytic receptor CD16/32, compared with the saline, indicating that a neurotoxic insult with MPTP produced neuroinflammation, with the increase in the expression of FcγR such as CD16/32 (**Figure 14**).

Figure 14: MPTP-induced degeneration induces expression of CD16/32. (A) Representative image of immunohistochemistry demonstrating the expression of CD16/32 in the MPTP-treated mice. (B) Stereological quantification of the number of CD16/32 positive cells indicating a higher number of CD16/32 labelled microglia in the MPTP group compared with the control (* $p < 0.05$).
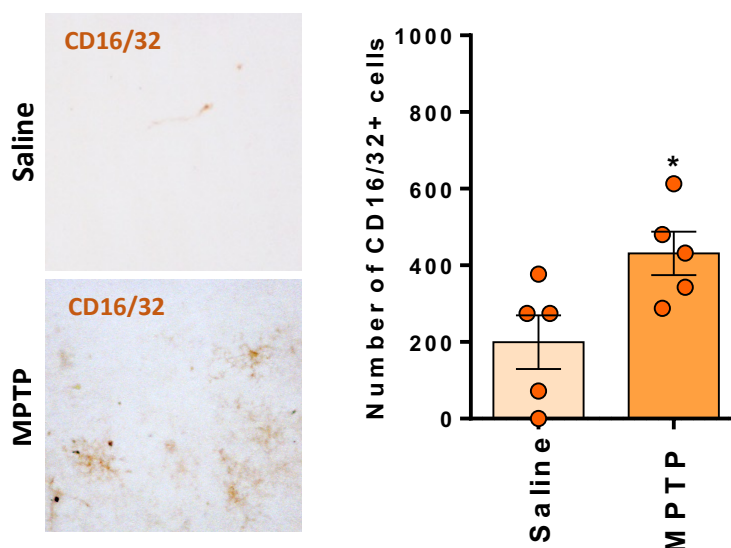
Then to test the effectiveness of the treatment of CD16/32 neutralizing antibodies, the immunotherapeutic dose was administered by an intraperitoneal injection in mice 24 hours before the intoxication with MPTP and animals were sacrificed 72 h after the neurotoxic insult. Immunofluorescence techniques allow us to stain the histological sections of the SNpc. Dopaminergic neurons were visualized by TH immunostaining in order to assess the dopaminergic degeneration, while Iba-1, a classical microglial marker allowed us to analyze the outcome of neuroinflammation in the SNpc (**Figure 15 A**). Knowing that activated microglial phagocytose entire neurons in a neuroinflammatory environment induced by MPTP intoxication (Barcia et al., 2012). The analysis of microglial activation in this scenario is relevant to understand their contribution in the process of neurodegeneration. For that reason, Iba-1 was used to stain microglial cells in the SNpc of the sections. Through image analysis software, the area of the expression of Iba-1 was measured, as a classical method to assess microglial activation in tissue. However, the evaluation of the Iba-1, although showed a slight increase, did not revealed significant changes when the animals were intoxicated with MPTP. In contrast, Iba-1 area slightly decreased when the animals were treated with the CD16/32 neutralizing antibody (**Figure 15 B and C**)

By means of stereology quantification, we determined the number of TH positive cells in every section of SN, obtaining an estimation of dopaminergic neuron density per SNpc, per treatment. As expected, quantification resulted in a significant decrease of the number of dopaminergic neurons when animals were intoxicated with MPTP. Importantly, this decrease was avoided when the animals were treated with the neutralizing antibody CD16/32 (Fc subunits) (**Figure 15 D and E**). For that reason, these results indicate that the elimination of neurons, in this context of experimental parkinsonism, may take place by phagocytosis and can be prevented by the administration of specific CD16/32 immunotherapy. Moreover, to show evidence of phagocytosis, thanks to high-resolution analyses performed with confocal microscopy, we were able to find specific phagocytic events in the SNpc of MPTP-treated animals (**Figure 16**). Importantly this phagocytic event presented a "ball-and-chain" structure, characterized by microglia filaments containing a pyknotic nucleus (**Figure 16 A and B**), similarly to the event found in the intrastriatal injection of LPS experiment, which contribute to demonstrate the phagocytic process in the SNpc of MPTP-induced degeneration model of PD.
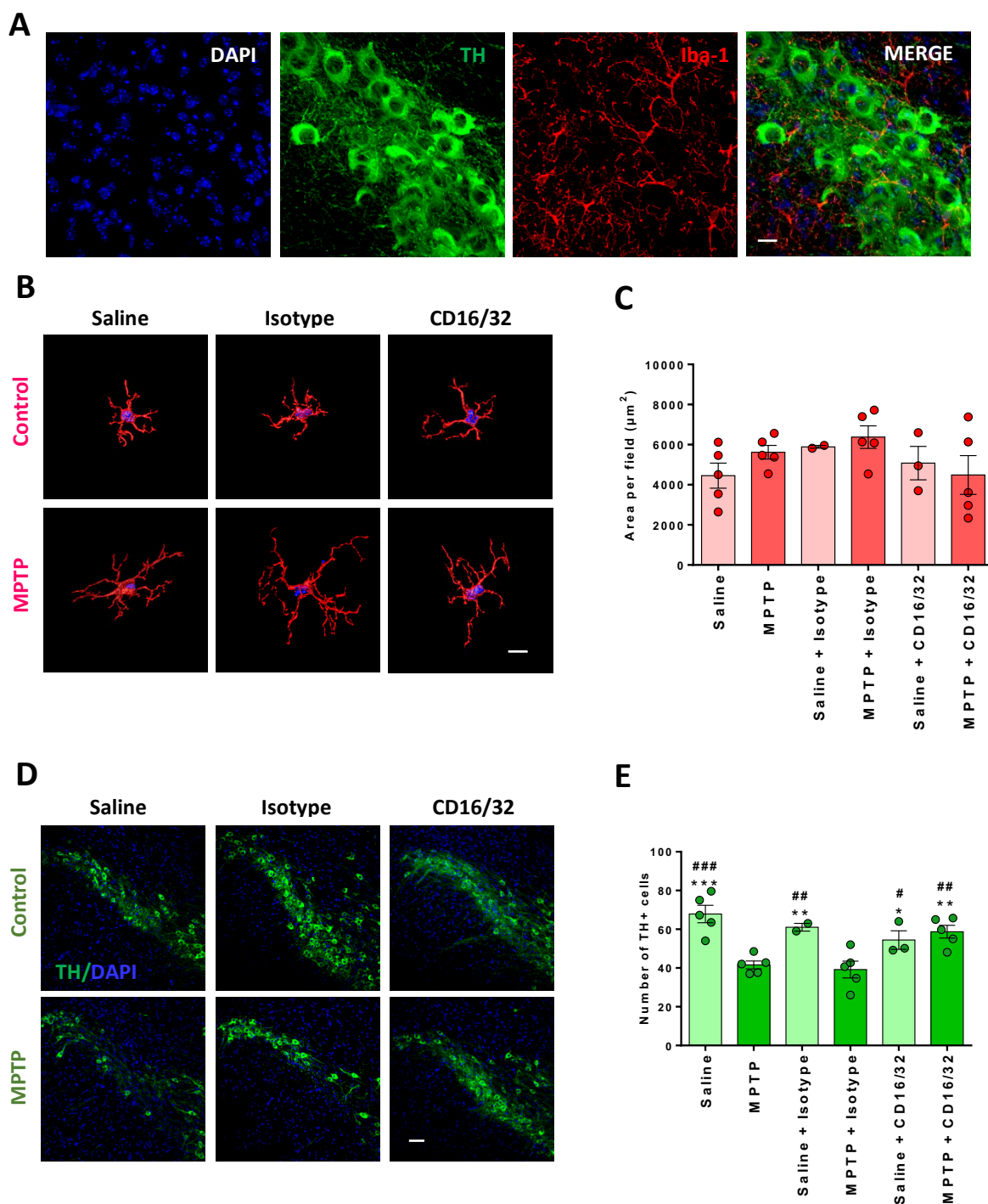
Figure 15. CD16/32 blockade protects dopaminergic neurons of the SNpc in MPTP parkinsonism. (A) Representative image of SNpc with microglia expressing Iba-1 (red), dopaminergic neurons expressing TH (green) and nuclei DAPI (blue) (Scale bar: 10 μm). (D) Representative confocal images illustrating dopaminergic neurons in the SNpc (scale bar: 30 μm). (E) Quantification of the number of TH positive cells in the SNpc demonstrating a significant decrease in the MPTP group which is prevented by the administration of CD16/32. From high resolution images microglial cells were extracted in order to create a 3D reconstruction. (B) 3D reconstruction of representative microglial cells expressing Iba-1 in different treatments of the experiment (Scale bar: 10 μm). (C) Quantification of the area of Iba-1 in the SNpc, indicating variation of microglial size when the animals were intoxicated with MPTP. (*** $p<0.001$ MPTP vs every treatment except MPTP+Isotype, ### $p<0.001$ MPTP + Isotype vs every treatment except MPTP).
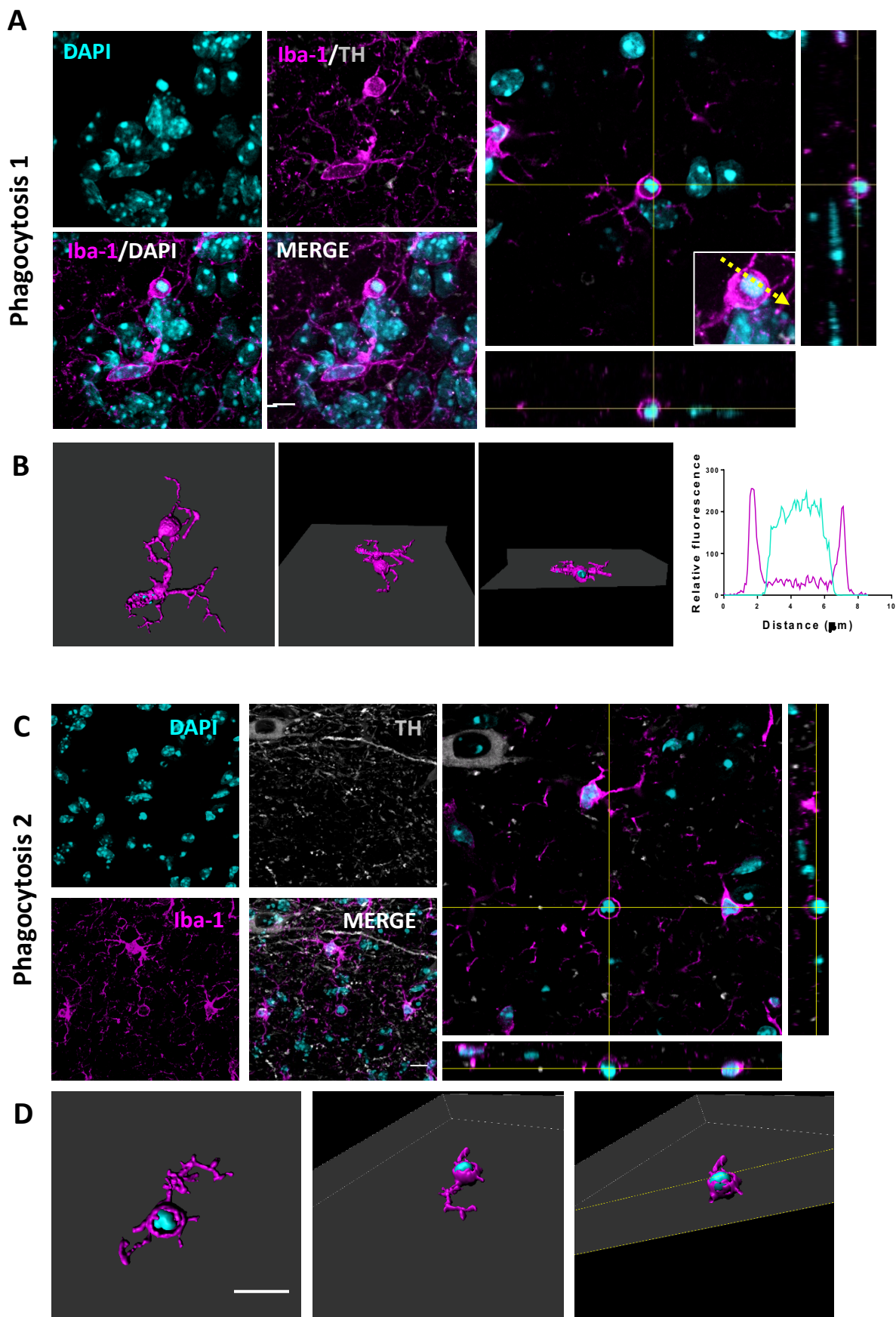
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Figure 16: Evidence of phagocytosis in the SNpc of MPTP-treated animals: Two different phagocytic events showing a potential microglial phagosome containing a pyknotic nucleus. (A) High resolution stack image of microglia branch containing pyknotic nucleus. Microglia immunostained with Iba-1, dopaminergic neurons immnuostained with TH and nuclei counterstained with DAPI. Orthogonal view evidencing the engulfing along the z-stack showing the pyknotic nucleus inside a an Iba-1⁻ space, consistent with the phagosome structure. Scale bar: 8 µm (B) 3-D reconstruction and clipping plane showing the phagosome containing the pyknotic nucleus Plot profile of relative fluorescence displays high fluorescence of DAPI consistent with the condensed pyknotic nucleus. High fluorescence of Iba-1 surrounds the pyknotic nucleus showing the phagosome. (C) Maximum intensity projection of the z-stack with microglia immunostained with Iba-1, dopaminergic neurons with TH and nuclei counterstained with DAPI. Orthogonal view evidencing a phagosome structure with pyknotic nucleus inside an Iba-1⁻space in microglia. 3D reconstruction and clipping plane demonstrating pyknotic nucleus inside the phagosome. Scale bar: 10 µm

Cdc42 inhibitor reduced neuroinflammation in the brain of animals challenged with systemic LPS injection

The analysis of the neuroinflammatory scenario becomes relevant to understand dopaminergic degeneration since they are tightly related. In this context, knowing that Cdc42 inhibitor, ML141, is able to protect dopaminergic-like cells *in vitro*, we used a well-known inflammatory model of systemic LPS to examine if after the challenge, peripheral administration of Cdc42 inhibitor is able to reduce the neuroinflammation. For these experiments, intraperitoneal administration of LPS was done after the intraperitoneal injection of ML141. After 24 hours, brains were extracted and sectioned. Immunohistochemistry was done in the histological sections to visualize microglial cells using anti-Iba-1. The morphology of microglial cells was analyzed by measuring the area of Iba-1, which indicated the activation of microglial cells. Results showed that the group of mice treated with LPS presented a higher inflammation evidence by the increased area of Iba-1 in microglia, both in the cortex (**Figure 17 A and B**) and in the striatum (**Figure 17 A and C**). Importantly, the area of Iba-1 decreased significantly when the animals were pre-treated with ML141, in the cortex (**Figure 17 A and B**) as well as in the striatum (**Figure 17 A and C**). These results suggest that Cdc42 inhibition reduces the neuroinflammation produced by a systemic insult of LPS. Importantly, since ML141 is able to reach the brain and reduce neuroinflammation, we proposed to test this inhibitor in a classical MPTP-induced degeneration model of PD to examine if neuroinflammation can be reduced in the SNpc.
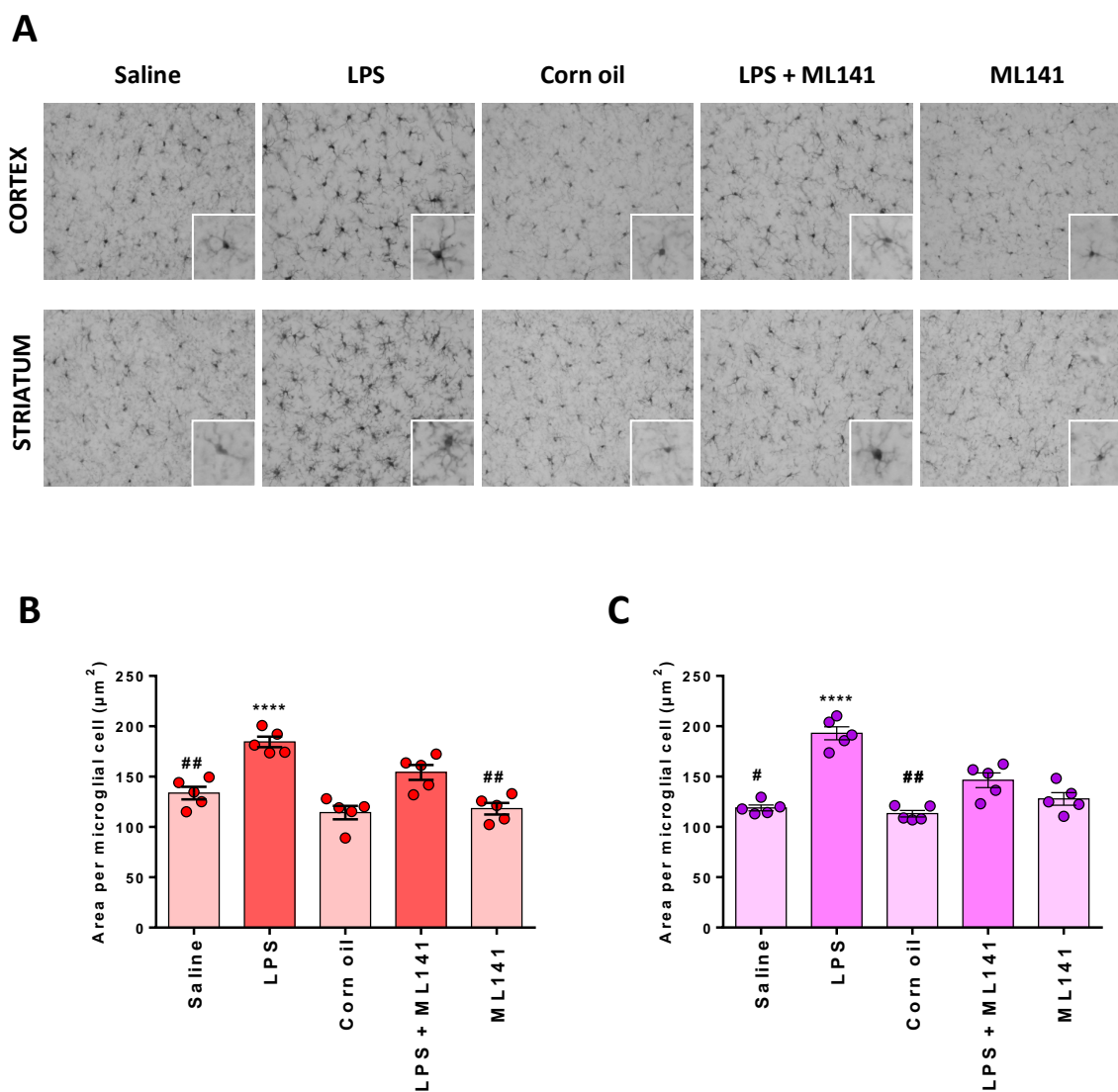
Figure 17. Cdc42 inhibition reduces neuroinflammation in the cortex and striatum. Representative images of immuno-staining for Iba-1 positive microglia indicate changes in size and morphology in every treatment, both in the cortex and in the striatum. The area of Iba-1 microglial cells in the cortex revealed a significant increase in size in the group of animals treated with LPS, however this increase was halted with the Cdc42 inhibitor ML141, reducing the activation of microglia. (C) The same phenomenon happened in the striatum, microglial activation was significantly increased in the animals challenged with systemic LPS, and activation was decreased when the mice were pre-treated with Cdc42 inhibitor (*** $p<0.001$ LPS against every other group)

Dopaminergic elimination is halted in an MPTP-induced model of PD after Cdc42 inhibition

Knowing that ML141 is able to prevent neuronal elimination *in vitro* and that is able to reach the brain after intraperitoneal administration reducing neuroinflammation *in vivo*, we tested ML141 in a well-known PD model to analyze if blocking Cdc42, a mechanistic factor downstream the CD16/32 signaling pathway, results in protection of dopaminergic neurons. For that reason, using an MPTP-induced degeneration model, we injected ML141 before the intoxication with MPTP in mice. After 72 hours of neurotoxin administration the brains were extracted. Serial histological sections were done from the mesencephalon in order to analyze the SNpc. By means of multifluorescence techniques we stained microglial cells with Iba-1 antibodies, dopaminergic neurons for TH, and nuclei with DAPI (**Figure 18 A**).

 By image analysis software, we measured the area of Iba-1 as an indicator of microglial activation due to neuroinflammation. Measurements resulted in increased area of Iba-1 in the group of animals intoxicated with MPTP, indicating that intoxication induced inflammation in the SNpc (**Figure 18 B**). Interestingly, the area of Iba-1 decreased when the animals were pre-treated with Cdc42 inhibitor, indicating that ML141 is able to reduce the neuroinflammation produced by a neurotoxic insult (**Figure 18 B**). In fact, animals treated with just ML141 also presented a decrease in the area of Iba-1 since inhibiting Cdc42 results in impaired motility of microglial cells. In order to assess the dopaminergic degeneration, the number of TH positive neurons were quantified in every condition. Our results demonstrated that there was a significant reduction of dopaminergic neurons in the MPTP group (**Figure 18 C**). Importantly, this reduction was prevented when the animals were previously treated with the Cdc42 inhibitor (**Figure 18 C**), meaning that avoiding microglial cell motility, phagocytosis of dopaminergic neurons becomes defective with a similar effect as obtained blocking FcγR subunits CD16/32. Importantly, high resolution images demonstrated the presence of phagocytic events in the SNpc demonstrating the phenomenon of phagocytosis in the SNpc after MPTP intoxication (**Figure 19**).

These results suggest that, because microglial activation is an important factor in the inflammatory-mediated dopaminergic neurodegeneration, targeting their signaling with different approaches will help us to understand the role of the local immune response and

most importantly highlight the significance of the inflammatory component to design new therapeutic strategies PD
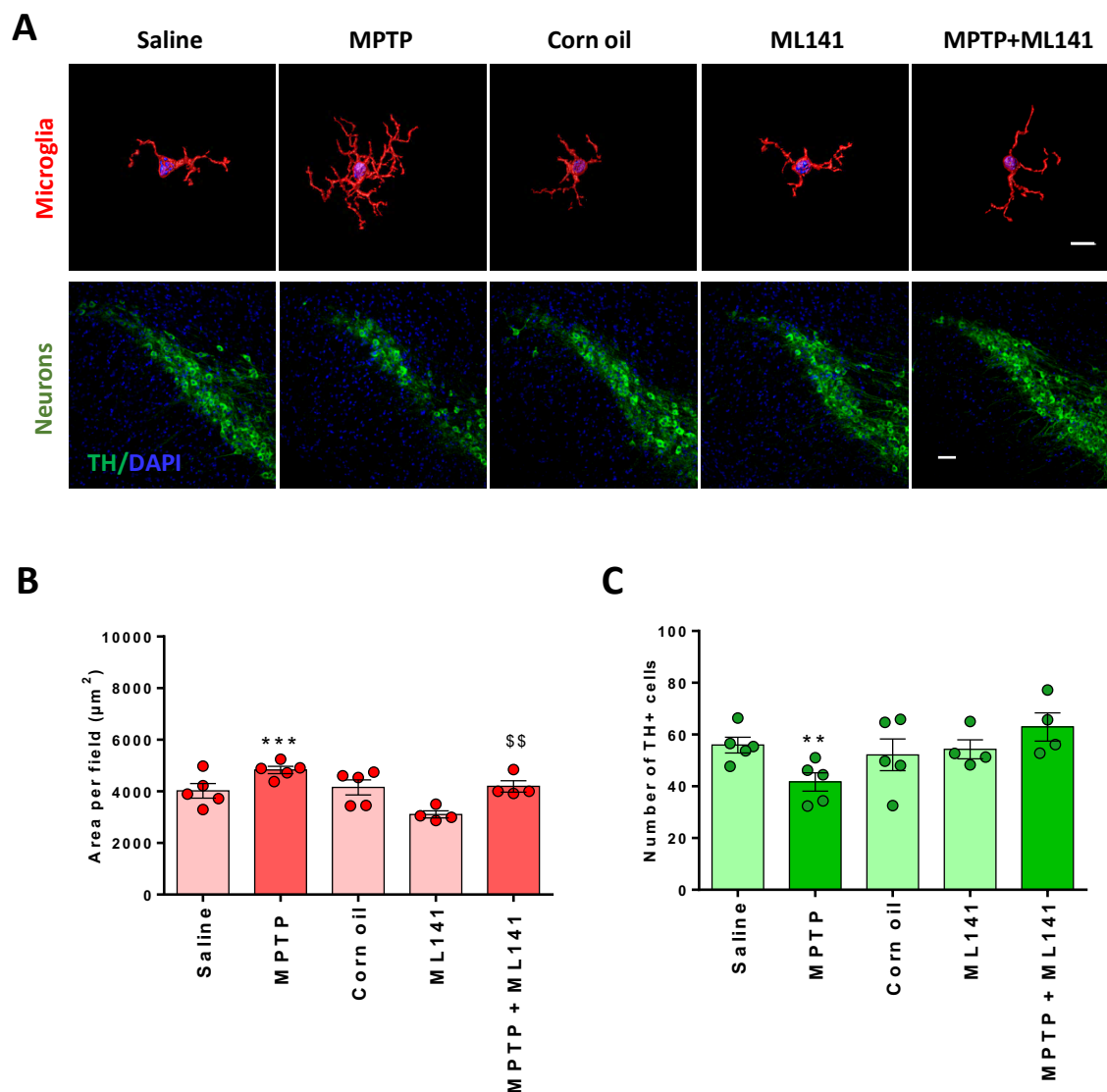
Figure 18. Blocking Cdc42-mediated microglial cell motility prevents dopaminergic degeneration. (A) Representative confocal images. Upper panel: 3D reconstructions illustrating morphology and changes of size in microglial cells expressing Iba-1 (scale bar: 10 μm). Lower Panel: Representative images of TH positive neurons of the SNpc in every treatment (scale bar: 30 μm). (B) Quantification of the area of Iba-1, which represents changes in microglia size. Microglial activation is present in the MPTP group evidenced by the increased area of Iba-1, which is decreased in the animals intoxicated with MPTP but previously treated with ML141. (C) Quantification of TH positive neurons indicating a significant decrease in the MPTP group and prevented by the previous administration of Cdc42 inhibitor ML141.
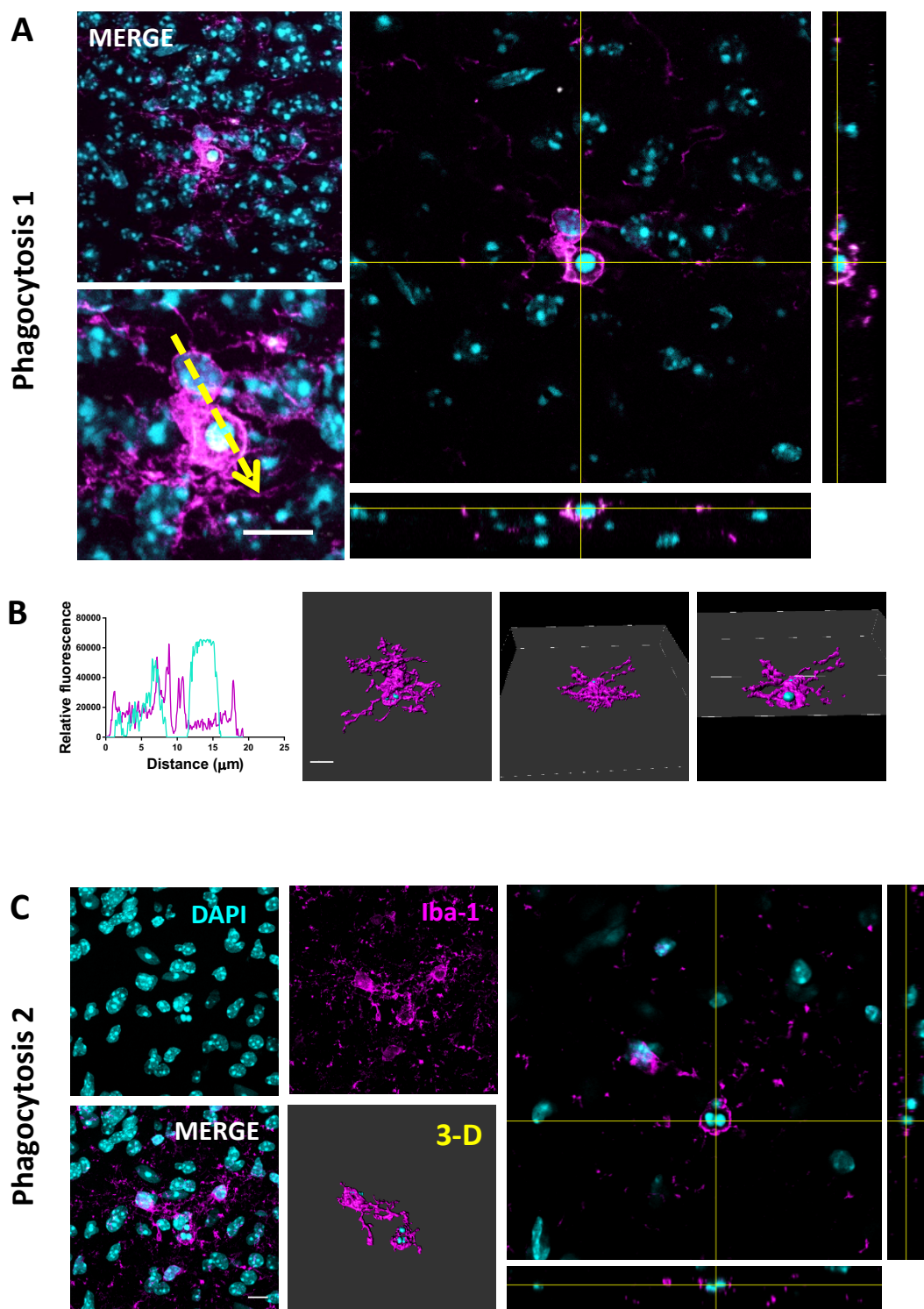
Figure 19: Phagocytic events in the SNpc of animals intoxicated with MPTP. Two different phagocytic events in the SNpc. Both, characterized by the presence of pyknotic nucleus, stained with DAPI inside microglia stained with Iba-1. (A) Maximum projection intensity of the Z-stack and orthogonal view evidencing the phagosome structure. (B) Plot profile of relative fluorescence along the yellow arrow, showing higher fluorescence in the pyknotic nucleus than the normal nucleus. 3-D reconstruction showing the phagosome containing pyknotic nucleus. (C) Maximum projection of microglia branch (Iba-1) containing pyknotic nucleus (DAPI). Orthogonal view showing the phagosome structure and 3-D reconstruction indicating the pyknotic nucleus inside Iba-1⁻ space.

General discussion

In normal conditions, neuroinflammation should be a protective process in response to injurious stimuli. Well regulated, neuroinflammation promotes debris clearance, tissue repair and restores homeostasis of affected tissues (H. Gao & Hong, 2008; Hirsch et al., 2012). However, sustained and exacerbated inflammatory response may become detrimental for the surrounding parenchyma, contributing to the harmful stimuli (H. Gao & Hong, 2008).

The first part of the present work was entailed to analyze the activation of microglial cells since they are the main mediators of the neuroinflammatory process. By using a well-known cell line named BV-2, we assessed microglial activation after IFN-γ and LPS proinflammatory stimuli. Selection of IFN-γ is supported by previous reports, since it is an important cytokine known to be elevated post-mortem tissue of PD patients (Hunot et al., 1999; Mogi et al., 1994, 1996), in blood plasma and CSF of PD patients (Brodacki et al., 2008; Mogi et al., 1994, 1996; Nagatsu et al., 2000) and chronic parkinsonian non-humans primates (Barcia et al., 2011). In the case of LPS, first, it is a common bacterial antigen that people are exposed to. Moreover, it has been recently described that alterations in the gut microbiota, rich in LPS, may affect the progression of PD (Sampson et al., 2016). Besides, these proinflammatory inductors are established as the canonical stimulation to switch microglia or macrophages to a proinflammatory phenotype *in vitro* (Dubbelaar et al., 2018; Ransohoff, 2016).

Microglial cells undergoing activation release pro-inflammatory cytokines, nitric oxide (NO), and reactive oxygen species (ROS) in response to inflammatory stimulus (Liu et al., 2002; Moss & Bates, 2001). Thus, we measured the release of nitrites as an indicator of NO production, therefore as the activation of microglial cells. Moreover, because microglia suffer dramatic phenotypic changes according to the stimuli within the environment, displaying an activated phenotype (Barcia et al., 2012; Nimmerjahn et al., 2005), we used Iba-1, a calcium binding protein specific to microglia/microphages (Hirsch & Hunot, 2009), to visualize morphological changes in the BV-2 microglial cells.

First of all, our results indicated that microglial cells achieve the highest activation after being treated with LPS/IFN-γ combination, instead of one or the other stimulus alone. This activation was also, confirmed with changes in the morphology of microglial cells, presenting an increased size, evidenced by the area of Iba-1. These results suggest that a combination of a

circulating cytokine combined with an antigenic stimulus could be a potential trigger for microglial activation.

Additionally, BV-2 cell density significantly augments with increasing concentrations of IFN-γ, which correlate with the increased number of mitoses as the concentration of IFN-γ augments, indicating that IFN-γ may induce proliferation of BV-2 cells, as happens with the expansion of other immune cells, however further experiments are required to further understand it. Even though proliferation of microglia and astroglial cells seem to play a role in the inflammatory process, enhancing the local activity (Brown & Neher, 2010b; Gómez-Nicola et al., 2013), there is no previous evidence suggesting that IFN-γ may be responsible for the proliferation of microglial cells in vivo. Nevertheless, there is evidence of astroglial proliferation in animal models and primary cultures (Yong et al., 1991).

In normal conditions, microglial have a vivid capacity to survey the brain parenchyma, actively scanning the surroundings in order to maintain brain homeostasis (Nimmerjahn et al., 2005; Saavedra-López et al., 2016). However, overactivated microglia can become dysfunctional and, in fact, harmful for neurons by producing an excess of cytotoxic factors, ROS and pro-inflammatory cytokines (Hirsch et al., 2012; S. Hunot et al., 1996; Saavedra-López et al., 2016). In this context, we believe that exacerbated microglia, in addition to releasing cytokines and cytotoxic factors that can be deleterious for the neurons, may contribute to degeneration by phagocytosis. Hence, to explore this assumption we performed a co-culture with activated microglia and dopaminergic neuron-like cells, such as PC12.

Our results indicated that PC12 cell density decreased as the number of phagocytic events increased, particularly, when the microglia achieved the highest activation with the combination of LPS/IFN-γ. These results suggest that microglial cells, activated with pro-inflammatory factors, are able to eliminate dopaminergic cells by increasing their phagocytic capacity.

Emphasizing the presence of "phagocytic events", defined as pyknotic nucleus with condensed DAPI expression, or TH debris inside a BV-2 microglial cell in an "Iba-1 negative space" suggesting the formation of a phagosome engulfing a PC12 dopaminergic neuron-like cell, as previously described for animal models of PD (Barcia et, al 2013).

Regarding the process of activation of microglia, we rely on the "priming" hypothesis. Perry et, al 2014, defined microglial priming as a pre-activated state, as a result of a primary

87

inflammatory stimulus, susceptible to receive a secondary inflammatory stimulus in which microglia becomes fully activated and overreactive in pathological conditions such as PD.

For that reason, in the following experiments we intend to answer the question of whether the order of the stimuli becomes relevant for the outcome of microglial activation and subsequent phagocytosis.

To solve this question, knowing that LPS/IFN-γ induce a higher activation of microglial cells than one stimulus alone, we analyzed the activation of BV-2 cells undergoing a sequential stimulation, changing the order of the stimuli.

The results indicated that the full activation of microglial cells was achieved when stimulating first with IFN-γ and later challenging with LPS, consistent with the canonical pro-inflammatory stimulation of macrophages *in vitro* (Perry & Holmes, 2014; Schroder et al., 2006). Importantly, primary mesencephalic microglia exposed to sequential stimulation, also achieved the full activation when exposed to IFN-γ and later challenged with LPS, indicating that the phenomenon of priming occurs in microglia in cell lines such as BV2 and in primary cultures. The priming effect of IFN-γ is required to cause a phenotypic shift towards a sensitized state, that will respond to a "triggering" stimulus, such as LPS, more rapidly and in a greater degree that a non-primed myeloid cell (Dilger & Johnson, 2008; Schroder et al., 2006). Interestingly, this suggest that previous elevated circulating levels of IFN-γ in elderly people, accompanied by a secondary antigenic stimulus, being LPS or other agents, may be behind the activation of microglia in potentially parkinsonian individuals.

The molecular pathways by which priming prepares for full activation have previously been described in macrophages *in vitro*, however, the information may be helpful to understand the heightened response of microglia *in vitro*. Since microglia and macrophages share lineage (Ginhoux & Garel, 2018; Nayak et al., 2014), it makes sense that the process of priming may be similar.

First off, in the case of LPS, efficient ligand recognition requires the signaling receptor TLR4 (Toll-like Receptor 4) as well as the CD14 (Cluster of Differentiation Antigen 14) co-receptor and MD2 (Myeloid Differentiation Factor 2) accessory molecule (Brown, 2019; Bryant et al., 2010; S. J. Kim & Kim, 2017). In addition, the LPS signal transduction requires a number of signaling adaptors (eg. MyD88) (Schroder et al., 2006) which leads to NF-KB transcriptional activation of several inflammatory genes including iNOS, as well as the production of nitric

oxide, typical of macrophage activation (Held et al., 1999). Pre-exposure of macrophages to IFN-γ upregulates expression of TLR2 and TLR4 in the cell surface and increase the LPS-binding ability of macrophages (Schroder et al., 2006). Moreover, IFN-γ also promotes TLR signaling by upregulating the expression of CD14 and signaling adaptor molecules such as MyD88 which potentiate LPS-signaling capabilities (Bosisio et al., 2002).Besides upregulation of receptors and accessory molecules of LPS-signaling, IFN-γ and LPS signaling also converge to the signal-transducing activator of transcription 1 (STAT1) signaling pathway, which enhances the macrophage response (Held et al., 1999). IFN-γ signaling is largely mediated by cytosolic factor STAT1 that is activated during the JAK-STAT signaling (Schroder et al., 2006). STAT1 activity is modulated by two phosphorylation signals at Y701 (tyrosine 701) and S727 (serine 727) (Schroder et al., 2006). Interestingly, TLR4 signaling potentiate the transcriptional effects of STAT1 via the phosphorylation of S727 and Y701 promoting the IFN-γ signaling capabilities (Lee & Sullivan, 2001). Furthermore, TLR signaling is mediated by adapter proteins, which lead to the activation of IRF1 (IFN-γ regulatory factor 1), which constitute one of the main components of IFN-γ signaling and essential for iNOS induction in macrophages (Kamijo et al., 1994).

In summary, these evidences suggest that priming is a complex process where pre-exposure of IFN-γ results in an enhanced response of TLR4. In addition, signaling pathways of both TLR4 and IFN-γ converge to STAT1, which also potentiates the activation of macrophages. Coherently, our experiments in microglial cells, seem to correspond with this notion, since IFN-γ was the most effective primary stimulus to induce an overreactive response in microglia. This is also coherent with the implication of IFN-γ and the subsequent STAT1 signaling seen in experimental models of PD (Barcia et al., 2011), and the elevated levels of IFN-γ in PD patients (Mount et al., 2007), pointing to IFN-γ as an essential cytokine in microglial-mediated parkinsonian neuroinflammation.

Since priming of microglia and further challenge with LPS produces microglial full activation by sequential stimulation, we intended to study if this exacerbated microglial response due to IFN-γ priming, have consequences in the dopaminergic elimination. For that reason, we set up co-cultures with fully activated BV-2 microglia after priming combined with PC12 dopaminergic neuron-like cells. In this scenario, we expected to visualize rapid phagocytic phenomena in fully activated microglia. In a parallel set, by combining macrophages with

opsonized beads or neutrophils with bacteria, it has been described that the phagocytic process is very fast, starting with the recognition of the target, and the subsequent engulfing, taking approximately 10 minutes (Hampton et al., 1994; Rosales & Uribe-Querol, 2017; Schlam et al., 2015). Thus, we selected shorter timepoints of interactions between BV-2 and PC12 cells.

Since microglial cells are highly dynamic, constantly surveying the environment, it is reasonable that the number of interactions per cell may increase through time in our culture set ups. Most interestingly, the most predominant type of contacting interactions where 1-1, meaning each microglia interacting with only one dopaminergic cell. This finding indicates that microglia focus their phagocytic machinery exclusively to one target polarizing to one dopaminergic cell. Moreover, we observed that overactivation of microglia, due to IFN-γ/LPS sequential stimulation, resulted in rapid elimination of dopaminergic cells compared with the activation produced by LPS alone. Importantly, we were able to visualize phagocytic events, which seems to support the notion that an exaggerated microglial response may be detrimental for the dopaminergic cells by phagocytosis. Whether BV-2 execute PC12 cell before the engulfing is yet to be determined, however, we suggest that dopaminergic elimination may be by primary phagocytosis (phagoptosis) starting with a 1-1 contact. After the contact, microglial cell starts embracing the dopaminergic cell, promoting the activation of actin cytoskeleton mediated by Rho GTPase such as Cdc42 to form the "phagocytic cup" and finish with the total engulfing of the target. Our images show evidences of the several steps of phagocytosis with the final steps showing single pyknotic nuclei present inside the microglial cell's phagosomes.

However, further experiments with innovative techniques, such as time-lapse confocal microscopy, are needed to investigate if phagocytosis is actually the cause of cell death. As well as the analysis of pro-inflammatory cytokines and potential toxic factors harmful for the dopaminergic cells.

Because full activation of microglial cells results in accelerated dopaminergic elimination, most probably by phagocytosis and knowing that exacerbated phagocytosis is critical contributor of dopaminergic degeneration in a pro-inflammatory environment. We proposed to target a phagocytic receptor in microglial membrane in order to assess the contribution of phagocytosis in this pro-inflammatory environment, and as a potential therapy to block

phagocytosis to protect dopaminergic neurons. Actually, several reports in different scenarios, such as ischemia, indicate that neuronal death caused by phagoptosis can be prevented by inhibiting phagocytosis, leaving live but stressed neurons in the environment (Brown & Neher, 2010b; Fricker et al., 2012; Neher et al., 2011, 2013).

Besides the advantage of FcγR being one of the most known phagocytic receptors and which pathways are described in detail, data report that primary microglia display enhanced expression of FcγR upon proinflammatory treatment with IFN-γ or LPS (Loughlin et al., 1992). In addition, in the same context of priming, systemic insult with LPS resulted in an overexpression of FcγR in microglia in the brain (Lunnon et al., 2011). Thus, we confirmed the presence of these receptor's subunits by CD16/32 staining antibodies and similarly, to block FcγR activity we used anti-CD16/32 (CD16 FcγRIII/CD32FcγRII) neutralizing antibody, to decrease microglial reactivity and phagocytic function in a co-culture setting of fully activated BV-2 microglia in combination with PC12 cells.

In this experiment, we demonstrated that dopaminergic elimination induced by microglial full activation was prevented when CD16/32 neutralizing antibodies were administered indicating that FcγR-mediated phagocytosis is crucial for dopaminergic elimination. Furthermore, the effectiveness of the antibody was tested before and after microglial activation and in both scenarios, dopaminergic elimination was prevented. In general, our data contributed to confirm that in a neuroinflammatory-like scenario, the cause of dopaminergic neuronal death is phagocytosis that can be prevented blocking the phagocytic process, consistent with the results found *in vitro* and in animal models of AD (Fuhrmann et al., 2010; Neniskyte et al., 2011), ischemia (Neher et al., 2013) and previous studies of our group in PD (Barcia et al., 2012).

The particular mechanisms by which CD16/32 may intervene in dopaminergic elimination in a parkinsonian scenario are yet to be elucidated. In the present work, we picture a clear link between microglial motility and the subsequent phagocytic interaction. It has been described that FcγR-mediated phagocytosis is regulated by Cdc42 signaling which is specially involved in the formation of the phagocytic cup (May & Machesky, 2001; Park & Cox, 2009). As we described above, Cdc42 is a small GTPase of the Rho family that plays an important role in cell migration and motility. Particularly in microglia, these functions are essential for efficient neuroinflammatory response. During motility and activation, cells undergo dramatic changes

in the membrane and initiate a dynamic organization of the actin cytoskeleton where Cdc42 tightly regulate this process (Barcia et al., 2012; Roser et al., 2017). Thus, our results suggest that modifications in the Cdc42 signaling pathway may affect the CD16/32-mediated phagocytosis and also the other way around. To shed light to this aspect, the use of non-competitive inhibitor of Cdc42 GTPases ML141 in our co-cultures, combining fully activated BV-2 and PC12 cells, demonstrated to exert a reduced phagocytic activity of microglia. ML141, besides being safe for the cells, appears to be interfering in the formation of protruding lamellas and filaments. This was confirmed by the presence of Cdc42 protein in BV-2 microglial cells. Importantly, Cdc42 seems to accompany F-actin in the formation of the motility leading protrusion, determined by the high fluorescence cluster surrounded by F-actin in the protrusion's edge, coherent with the established concept that Cdc42 mediates the polymerization of the actin cytoskeleton in cell motility.

Most importantly, Cdc42 inhibition by ML141 administration significantly halted dopaminergic elimination. However, in our cultures, when the inhibition was done after the activation (post-activation inhibition) the protecting effect of ML141 seem to be less efficient than the pre-activation inhibition. Thus, we propose the following mechanistic steps to potentially explain these events (**Figure VI**).
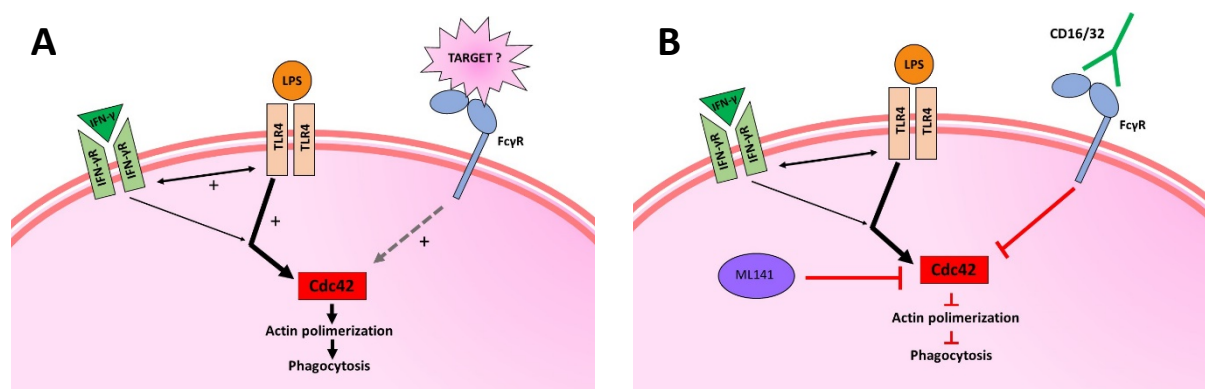
Figure VI. Schematic representation of canonical microglial activation and phagocytosis signaling in our *in vitro* model. IFN-γ receptors (IFN-γR) represented in green, Toll-like receptor 4 (TLR4) represented in orange. Each one of the receptors were illustrated with their specific ligand in the respective colors. FcγR (CD16/32) represented in blue, bonded to specific cell target. Signal integration of TLR4 and IFN-γ is represented with thick black arrow, converging in Cdc42. In the case of FcγR discontinuous arrow towards Cdc42 indicate that signaling cascade is not fully described. Signaling of all the receptors in the images, converge in the activation of Cdc42 which leads to actin polymerization and subsequent phagocytosis. Thick red lines represent the inhibition of the specific signaling.

Signal of IFN-γR and TLR4 converge in Cdc42 (**Figure VI**), that represents morphological changes in microglia due to full activation and following the most efficient sequential

stimulation. As mentioned above, these morphological changes are regulated by the activation of Cdc42 which mediate actin polymerization, and subsequent phagocytosis. Moreover, FcγR signal also converge in Cdc42, which represents that FcγR-mediated phagocytosis is tightly regulated by the activation of Cdc42.

In the case of post-activation inhibition, BV-2 cells were stimulated with IFN-γ and 24 hours later challenged with LPS [This is represented in the schematic illustration (**Figure VI A**) by a thin arrow starting in IFN-γR and converging with a thicker arrow from TLR4 and activating Cdc42]. After 24 hours of the sequential proinflammatory stimuli, ML141 was administered. [Panel B of the diagram (**Figure VI B**) represents the administration of ML141]. ML141 inhibits Cdc42 (represented in the diagram by the red lines) which subsequently inhibits the actin polymerization avoiding the cell motility and consequent phagocytosis. We suggest that the time between activation and posterior inhibition of Cdc42 with ML141 (24 hours), was enough to induce an initial dopaminergic elimination, that later was ceased, however some of the PC12 dopaminergic cells may have been eliminated in that time window. In contrast, in the pre-activation inhibition, ML141 inhibits the Cdc42 before IFN-γ and TLR4 stimulation, therefore initially impeding the morphological changes and motility of BV-2 cells. Then, static microglial cells, were not able to properly be activated by IFN-γ and LPS, therefore actin polymerization and the subsequent phagocytosis becomes impaired. As a conclusion, this sequence of mechanistic events provides an explanation why if the inhibition of Cdc42 is done before the process of activation of microglia, the dopaminergic elimination is prevented more successfully. In addition, since FcγR signaling converge in Cdc42, blocking CD16/32 results in a similar outcome than the inhibition of Cdc42, indicating that targeting a mechanistic factor of the FcγR-mediated phagocytosis results in a similar effect as blocking the phagocytic receptor but probably in a less efficient fashion regarding some of the activation features of microglia.

Altogether our *in vitro* results, indicate that a neuroinflammatory environment by itself can induce the elimination of dopaminergic neuron-like cells by phagocytosis due to an overreactive microglia. Additionally, the dopaminergic elimination can be prevented by the use of neutralizing antibodies against CD16/32 receptors. Furthermore, the inhibition of specific GTPase Cdc42 in the signaling pathway of CD16/32 FcγR-mediated phagocytosis, results in a defective phagocytic process, avoiding dopaminergic elimination.

Having a clear picture of our aims *in vitro*, this mechanistic exploration helped us to understand the biology behind our studies *in vivo*. Since neuroinflammation can contribute to degeneration *in vitro*, we used a pure neuroinflammatory model, where a single dose of LPS was administered by stereotaxic injection in mice brain. As recent results of our group show, the direct stereotaxic injection of LPS in the SNpc generates a massive inflammatory response producing parenchymal damage, which does not correlate with the parkinsonian scenario (Heman-Bozadas et al., in preparation). Then, we proposed to use a different approach by injecting LPS in the striatum, since dopaminergic neurons of the SNpc project their fibers to the striatum. With this approach, the indirect neuroinflammatory damage can be seen but with the preservation of the parenchymal integrity.

This strategy is fairly unexplored since stereotaxic injections of LPS to model PD in mice are not as common as in rat, and more so, as indirect analysis of the damaged produced through the nigrostriatal pathway (Deng et al., 2020). In a previous report in mouse model, a single intrastriatal injection of LPS can induce degeneration of dopaminergic neurons in the SNpc, visible at 1, 4 and 12 weeks after injection, being the concentration of LPS in the order of 10 mg/mL (Hunter et al., 2009). In our model, to avoid an extensive damage, we injected a lower dose of LPS (1mg/mL) and assessed the effects at 3- and 7-days post-injection to visualize the neuroinflammatory responses in tissue.

One of the most interesting findings in this experiment was the reliable and significant microglia-mediated neuroinflammation contained in the ipsilateral hemisphere. Importantly, 3 days after the injection, the neuroinflammation in the ipsilateral hemisphere was highly visible compared with the contralateral hemisphere and also when compared with 7 days. Further studies should be performed at longer time points (more days, or even weeks), to assess if the decreasing tendency of neuroinflammation through time, reaches basal levels beyond 7 days. This phenomenon of neuroinflammation correlated with the neuronal elimination evident at 3 days post injection in the ipsilateral hemisphere. Notably at 3 days post injection, dopaminergic neurons in the contralateral hemisphere also show a tendency to decrease, which may suggest that neuroinflammation-mediated degeneration may not remain confined to the ipsilateral hemisphere. However, and most important, at 7 days post injection, neurons of the contralateral hemisphere seem to recover, showed by the evident increase in the TH positive cells. Therefore, this phenomenon can be potentially explained as

a downregulation of TH in the contralateral hemisphere. In contrast, this effect of recovery was not visualized in the ipsilateral hemisphere which may indicate a real dopaminergic depletion in the SNpc.

Particularly, we tested the expression of CD16/32 in the striatum, resulting in a high expression of CD16/32 positive cells in the ipsilateral hemisphere. This result indicated that neuroinflammatory process induced by LPS injection produce the externalization of FcγR like CD16/32. In coherence with the CD16/32 expression, high-resolution images obtained with z-scan confocal microscope, showed phagocytic structures of the "ball-and-chain" type, evidencing phagocytic events in the SNpc of animals treated with LPS. Thus, these results suggest that neuroinflammation by itself (produced by lower doses of LPS) induces neuronal death, most probably by the contribution of CD16/32-mediated phagocytosis. Therefore, these results open new avenues to target the neuroinflammatory arm of neurodegeneration and particularly with an immunotherapeutic approach by using neutralizing antibody to mitigate the effects of the CD16/32-mediated phagocytosis of dopaminergic neurons.

As we determined the effectiveness of CD16/32 blocking microglial phagocytosis *in vitro,* we evaluated the effect of this immunotherapy in a well-known MPTP model of PD.

MPTP models have been extensively used to study mechanisms of dopaminergic degeneration since MPTP administration produces damage in the nigrostriatal dopamine similar to the one seen in PD, with the exception of protein aggregates such as LW (Vila & Przedborski, 2003). We particularly used an acute-short term MPTP model (Meredith & Rademacher, 2011), importantly because neuroinflammation can be reliably assessed without causing an excessive influence of neurotoxicity (Barcia et al., 2011,2012). Interestingly, we first demonstrated that a pure neuroinflammatory model, such as LPS, induced the expression of FcγR CD16/32 and consequent dopaminergic elimination. Secondly, knowing that the administration of MPTP also induces a microglial-mediated neuroinflammation and subsequent phagocytosis of dopaminergic neurons (Barcia et al., 2012), we also demonstrated by means of immunohistological techniques, that MPTP intoxication produced a significant increase of microglial FcγR CD16/32. Overall, both models suggest that neuroinflammation caused either by a neurotoxic insult or the administration of proinflammatory stimulus, induce the overexpression of FcγR such as CD16/32.

Importantly, in this context, our results revealed that blocking CD16/32-mediated phagocytosis, prevented MPTP-induced dopaminergic degeneration by inhibiting the neuroinflammatory arm of the process. Moreover, phagocytic events of the "ball-and-chain" type were visualized in the SNpc, similarly to the type found in the intrastriatal injection of LPS experiment, which contribute to demonstrate the phagocytic process may be crucial for the elimination of dopaminergic neurons in the SNpc of this PD models. In contrast, the evaluation of neuroinflammation due to microglial activation changed slightly, probably due to experimental variations quantifying microglia.

Since it has been described that TLR4 pathway is involved in the outcome of MPTP-degeneration in experimental PD (Noelker et al., 2013) and knowing that after MPTP intoxication, activated microglia increases the expression of pro-inflammatory cytokines such as IFN-γ (Barcia et al., 2011) we can also follow the mechanistic steps described above for the in vivo experiments (**Figure VI).** As previously reported MPTP-induced neurodegeneration is, in part, dependent on TLR4 (Noelker et al., 2013) since animals with defective TLR4 expression are less vulnerable to MPTP toxicity. We do not actually know what the intrinsic ligand of TLR4 in the MPTP scenario may be, but it is known that IFN-γ is a critical cytokine for the activation of microglia in MPTP mouse model (Barcia et al., 2011). Thus, the activation of microglia, by the binding to the IFN-γR may exacerbate the TLR4-dependent response in the MPTP model as we pictured *in vitro* (**Figure VI**). Thus, full microglial activation is achieved with the consequent downstream activation of Cdc42. Potentially, the phagocytic process may start when the target neuron tethered the Cd16/32 FcγR, which triggers signaling pathway activating Cdc42 and promoting actin polymerization and further the phagocytic cup formation. Theoretically, the therapeutic neutralizing antibody binds to CD16/32 FcγR, and thus the recognition and attachment to the target dopaminergic neuron, may be prevented. Therefore, Cdc42 may not be activated and subsequently the phagocytic process impaired (**Figure VI**). However, Cdc42 can also be independently activated by the TLR4 and IFN-γR pathway which could maintain some level of microglial activation regardless of CD16/32 blockage. In this context, the concomitant activated morphology of microglia may be visible even though the CD16/32 sites may be blocked by CD16/32 neutralizing antibodies and consequently the phagocytosis of dopaminergic neurons may be avoided. These mechanistic alternatives may explain the probable cause of seeing slight changes in microglial activation

after administration of CD16/32 neutralizing antibody in the MPTP experimental model. Therefore, as concluding remark, we suggest that the use of neutralizing antibody against CD16/32 results in an efficient protection of the dopaminergic neurons of the SNpc after MPTP intoxication, however, the effect may not be totally efficient in reducing some extent of the neuroinflammatory process.

Overall, the results of this thesis evidenced the contribution of phagocytosis in MPTP-induced degeneration model of PD and suggests the immunotherapeutic use of neutralizing antibody against FcγR, to prevent phagocytosis and protect dopaminergic neurons. Nevertheless, many questions are still to be answered and further studies should be conducted to analyze the long-term effects of these therapies and the consequences of remaining microglial activation.

Nowadays, although the contribution of neuroinflammation in dopaminergic degenerations is considered relevant, yet further analyses are needed to fully understand the tight relation between both phenomena. In this context, our above reported experiments of inhibiting Cdc42, by intraperitoneal administration of ML141, in the inflammatory model of systemic LPS may shed light to the interplay occurring between the systemic and central inflammation.

One of the first questions that comes to mind in this inflammatory model, is whether inflammation can reach the brain after systemic challenge with LPS. The passage of LPS into the brain is limited by the BBB, and it is likely that LPS causes neuroinflammation indirectly (Qin et al., 2007). Brown and colleagues in 2019, summarized the mechanism by which doses of systemic LPS can affect the brain: First, LPS activation of peripheral nerves acting centrally. For example, systemic administration of LPS may lead to activation of TLR4 in peritoneal macrophages/dendritic cells resulting in the release pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1 (Deng et al., 2020). Particularly IL-1, produced by macrophages/dendritic cells can activate the IL-1 receptor of the vagal nerve, which stimulates the CNS (Block et al., 2007). Second, inflammatory cytokines such as TNF-α, IL-6 and IL-1 in the bloodstream signal the CNS via the activation of circumventricular organs (Perry, 2010). Lastly, high doses of systemic challenge can induce an increase in BBB permeability, potentially resulting in neuroinflammation and potentially allowing LPS entering into the brain (Brown, 2019).

Logically, this information allows us to state that systemic insults with LPS may cause neuroinflammation in mouse brain, directly or even indirectly. Hence, according to our results

we can suggest that inhibition of Cdc42 with ML141 is able to reduce the neuroinflammation induced by LPS administration, both in the striatum and in the cortex.

Importantly, since our results suggest that ML141 is able to reach the brain, reducing the neuroinflammation caused by LPS challenge, it is plausible that this Cdc42 inhibitor may also arrive to the brain in the MPTP-induced degeneration model of PD. Moreover, we determined that Cdc42 inhibition by ML141 is able to prevent neuronal elimination *in vitro* by blocking motility-dependent activation of microglia. Following the same line of experiments a similar outcome was seen in the MPTP model of PD, which may imply the affectation of the same cellular target.

We reviewed above that Cdc42 plays a crucial role in the CD16/32-mediated phagocytosis (Park & Cox, 2009) and in the process of migration and activation (Barcia, 2013;Nimmerjahn et al., 2005). Therefore, we demonstrated that the inhibition of Cdc42 resulted in reduction of most of the microglial activation properties, such as cell body size, measured by the area of Iba-1 and also reflected in the increase of apparent ramifications. These results are coherent with previous data described by Barcia and colleagues in 2012 in MPTP-induced degeneration model of PD, where MPTP was seen to induce microglial polarization toward intoxicated neurons. This polarization was indeed prevented through the inhibition of Rho- associated kinase (ROCK) activity (Barcia et al., 2012). Interestingly, the major finding of this section of the thesis is that the inhibition of Cdc42 also resulted in protection of dopaminergic neurons pointing to the impairment of phagocytosis as the major cause. Notably, the presence of specific phagocytic events in the SNpc of animals treated with MPTP at the endpoint contributed to demonstrate the phagocytic process in the SNpc in this parkinsonian model.

In order to deliver a possible mechanistic explanation of the effect of Cdc42 inhibitor in the SNpc we will follow again the diagram indicated above (**Figure VI**). Besides cell motility and migration, Cdc42 is essential in the reorganization of actin cytoskeleton in the process of phagocytosis. In fact, the formation of the phagocytic cup around the target is specially mediated by the activation of Cdc42 (Park & Cox, 2009). In contrast, the inactivation of Cdc42 is crucial for the closure of the phagosome around the target particle (Uribe-Querol & Rosales, 2020). Thus, following our scheme, microglia is activated by the MPTP administration probably through IFN-γ (Barcia et al., 2011) in synergistic combination with TLR4 (Noelker et al., 2013) to reach full activation. In this context, the treatment with ML141 directly inhibits the

activation of Cdc42 (**Figure VI**). Then, as a consequence, the actin polymerization would become defective as well as the subsequent phagocytosis. Therefore, having a defective phagocytic process, the dopaminergic elimination may be prevented.

Regarding the involvement of the phagocytic process, both of our MPTP-induced degeneration experiments presented evidence of phagocytosis in the SNpc (**Figure 17 and 20**). In addition, dopaminergic elimination can be prevented by the immunotherapeutic use of CD16/32 neutralizing antibodies blocking FcγR-mediated phagocytosis or by targeting mechanistic factors in the signaling pathway of phagocytic process, such as Cdc42, demonstrating the relevance of this biological process. Although MPTP, metabolized into MPP$^+$, which is toxic to dopaminergic neurons in the nigrostriatal pathway (Deng et al., 2020; Y. He et al., 2002), is the primary cause of dopaminergic neurons in our PD models, we believe that the phagocytic process might also directly influence the neuronal degeneration. In this context, the contribution of microglia executing neurons has been described as "phagoptosis", in which microglia phagocytose stressed but viable neurons in a neuroinflammatory environment, being the phagocytic process itself, the cause of neuronal death (Emmrich et al., 2013; Fricker et al., 2012; Neher et al., 2011). In consequence, in our experiments, both neurotoxicity and active phagocytosis may play a part in the dopaminergic neuronal death.

As stated above, neuroinflammation is important and necessary to maintain a proper homeostasis in the brain and it represents the normal response to harmful stimuli. Microglial phagocytosis is one of the features of the neuroinflammatory process, which in normal conditions should exert a beneficial effect by engulfing pathogens, debris, protein aggregates, dead and dying cells (Vilalta & Brown, 2018). Therefore, inhibiting phagocytosis, either by blocking directly phagocytic receptors such as FcγR or by inhibiting the factors in the signaling pathway such as Cdc42 may, on the one hand, be beneficial to prevent the elimination of dopaminergic neurons by phagocytosis. On the other hand, it may result problematic in the long term, since a deficient clearance of apoptotic cells due to MPTP administration, may result in an overactive toxicity for the remaining cells in the brain parenchyma. However, increasing evidences point that microglia might be overreactive in PD contributing to the pathology and accelerated neurodegeneration (Hirsch & Hunot, 2009; Perry & Holmes, 2014). Therefore, reducing microglial activity and phagocytosis might be beneficial in the progress of the disease. Indeed, in the PD scenario immunotherapeutic use of neutralizing antibodies

targeting CD16/32 to block microglial phagocytosis, may represent a novel and safe strategy to reduce the exacerbated response mediated by microglia, particularly protecting neurons from phagocytosis.

Moreover, we must emphasize that neurotoxic model such as MPTP, although consider gold standard to model PD, cannot replicate all features of PD, such as the progressive degeneration of dopaminergic neurons and the α-synuclein aggregations, among other characteristics (Dauer & Przedborski, 2003; Meredith & Rademacher, 2011; Vila & Przedborski, 2003). Therefore, as near future experiments we proposed our immunotherapeutic approach to be tested in different context, to evaluate the effect, in experimental models with protein aggregations, such as α-synuclein.

In conclusion, in the context of neuroinflammation and neurodegeneration, microglial activation might be exacerbated by circulating IFN-γ and TLR4 stimulation in a synergistic way, phagocytosing neurons unnecessarily and contributing to the pathology. In this thesis we then propose that blocking or reducing the exaggerated phagocytosis by neutralizing antibodies against CD16/32 could be beneficial. Therefore, we suggest CD16/32 as a potential target to develop immunotherapeutic tools to avoid neuronal elimination in PD. Alternatively, the inhibition of CD16/32 downstream signaling, Cdc42 as possible anti-inflammatory therapy, that could as well prevent neuronal elimination. Finally, as neuroinflammation gets increasing attention and it is noted as a crucial contributor of dopaminergic degeneration, we firmly believe that targeting microglia may become a crucial disease-modifying approach to treat PD.

Conclusions

The main conclusions of this thesis are:

- Classical pro-inflammatory stimulus such as IFN-γ and LPS induce microglial activation *in vitro*.

- Classical pro-inflammatory stimulation of BV-2 microglia induces phagocytosis of PC12 dopaminergic neuron-like cells.

- Priming with IFN-γ and subsequent challenge with LPS induces the full activation of microglial cells in cell lines and primary cultures.

- *In vitro*, BV-2 microglial cells are able to increase the phagocytic potential over PC12 dopaminergic cells when they are fully activated after priming.

- Blockade of phagocytosis with CD16/32 neutralizing antibodies against the Fc portion of FcγR of microglial cells, protects dopaminergic cells, indicating that dopaminergic elimination might be mediated by phagoptosis *in vitro.*

- Inhibition of CD16/32 downstream signaling, with a Cdc42 inhibitor, results in the preservation of PC12 dopaminergic cells from elimination.

- Intrastriatal injection of LPS can be a potential model of neuroinflammation-induced degeneration, since low doses of LPS causes neuroinflammation confined to the ipsilateral hemisphere and subsequent dopaminergic elimination in the SNpc.

- Neuroinflammation caused by intrastriatal injection of LPS, produces the externalization of CD16/32 phagocytic receptors.

- Evidence of phagocytosis is visualized in the SNpc of animals challenged with intrastriatal injection of LPS by the presence of phagocytic events.

- Specific phagocytic events with "ball-and-chain" structure are present in the SNpc of MPTP-treated animals, evidencing the contribution of phagocytosis in dopaminergic elimination in a parkinsonian scenario.

- CD16/32 neutralizing antibodies against the Fcγ portion of FcγR phagocytic receptor protects dopaminergic neurons of the SNpc in an MPTP-induced degeneration model of PD.

- Inhibition of downstream signaling pathway of CD16/32-mediated phagocytosis with Cdc42 inhibitor ML141 prevents dopaminergic elimination in a MPTP model of PD, with a similar outcome as CD16/32 blockade.

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