Charles UniversityFaculty of Science

Study programme:
Biology

Branch of study:
Immunology

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Effects of gluten-free diet on immune parameters in Parkinson's disease

Efekt vliv bezlepkové diety na imunitní parametry u Parknsonovy nemoci

Diploma thesis

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Prague, 2024

Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 29. 04. 2024

Mônica Jandová

Acknowledgment

I extend my heartfelt gratitude to my husband Martin, whose support and guidance have been essential in the completion of this thesis.

I am deeply thankful to my thesis advisor, MUDr. David Funda, for opening the door of his laboratory and giving me a chance to be part of his group. His guidance, invaluable insights, and continuous encouragement throughout the research process.

I am indebted to my parents, Jair and Iolanda, for their unwavering support, understanding, and encouragement throughout this academic journey. Their belief in my capabilities has been a constant and source of motivation.

I extend my gratitude to my dear friend Carolina Mattosinho, who is my person across the ocean.; as well to my colleagues Bc. Anna Zelenska, Dr. Peter Ergang, Dr. Karla Vagnerová, and others who provided a supportive academic and social environment, contributing to a fulfilling research experience.

Finally, I would like to extend heartfelt appreciation to my four-legged confidante and loyal friend, my dog, Pedro. Through countless late-night study sessions, thesis-induced stress meltdowns, and the occasional paw-on-the-laptop moments. Pedro has been a constant source of joy and comfort.

> Have I not commanded you? Be strong and courageous. Do not be afraid; do not be discouraged, for the Lord your God will be with you wherever you go.
> Joshua 1:9

Abstract

This study explores the possible, even subtle, impacts of a gluten-free diet (GFD) in comparison to a standard diet in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of human Parkinson's disease (PD), utilizing male C57Bl6 mice. The research involves the establishment of both acute and chronic MPTP mouse models, accompanied by a set of flow cytometry assessments, such as the proportions of regulatory T cells (CD4+Foxp3+ Tregs), cytokines - interleukin-10 (IL-10), and interferon-gamma (IFNγ) in CD4 and CD8 T cells, respectively, regulatory CD4+CD45RB-low T cells, γδ T cells as well as subsets of natural killer (NK) cells in mucosal and non-mucosal lymphoid organs in the chronic MPTP mouse model of PD.

Preliminary results indicate subtle positive effects of the gluten-free diet (GFD) in the chronic MPTP mouse model of PD. A tendency to increased proportion of CD4+Foxp3 Tregs in almost all lymphoid organs studied (spleen, mesenteric, inguinal lymph nodes, and Peyer's patches, but with exception of pancreatic lymph nodes), in animals on the GFD may indicate some subtle effect of the GFD on Tregs. In addition, similarly to the effect of GFD in some other immune-mediated diseases, this study also reveals generally increased proportion of γδ T cells (irrespective of CD8 expression) in the mice fed GFD, suggesting a potential role for immunomodulation through γδ T cells in PD. No changes were observed in proportions of CD4+CD45RB-low T cells, IL-10-positive CD4 and IFNγ-positive CD8 T cells as well as various subsets of NK cells.

Besides flow cytometry findings, this study also employed behavioral testing. Mice fed the GFD exhibited higher exploration values in the open field test, indicating increased willingness to engage with their environment. Further research is necessary to dissect the dietary effects on the neuroinflammatory conditions in PD, both in the clinical/neurological and immune parameters.

These first findings indeed warrant cautious interpretation and further in-depth studies are needed to establish a more comprehensive understanding of the observed dietary effects in PD. The integration of behavioral assessments, immunofluorescence histology, and immunological analyses underlines the multidimensional nature of this study that seems to be necessary for address the complex interplay among diet, gut, brain, and neuroinflammation.

Key words: Parkinson`s disease, gluten-free diet, MTPT mouse models, behavioral tests, immunohistochemistry, flow-cytometry, T cells, Tregs, NK cells

Abstrakt

Tato studie zkoumá možné, i malé, vlivy bezlepkové diety (GFD) v porovnání se standardní dietou na rozvoj Parkinsonovi nemoci (PN) s využitím 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridinem (MPTP) indukovaném myším modelu Parkinsonovi nemoci, a samců kmene C57Bl6. Tento výzkum zahrnuje zavedení jak akutního tak chronického myšího MPTP modelu, a navazující flow cytometrické analýzy zaměřené na detekci proporčních změn regulačních T buněk (CD4+Foxp3+ Tregs), cytokiny produkujících interleukin 10 (IL-10) CD4+ a interferon-gama (IFN-γ) CD8+ T buněk, γδ T buněk, a subpoulací NK buněk ve slizničních a systémových lymfatických orgánech na chronickém MPTP myším modelu PN.

První výsledky ukazují na možné malé, pozitivní efekty bezlepkové diety v chronickém MPTP myším modelu PN. Tendence ke zvýšenému procentu CD4+Foxp3+ Treg buněk ve skoro všech studovaných lymfatických orgánech (slezina, mezenterické uzliny, inguinální uzliny a Peyerovy pláty, - s výjimkou pankreatické uzliny), u myší na GFD může indikovat určitý malý efekt GFD na Treg buňky. Podobně jako byly popsány efekty GFD u jiných imunitně způsobených onemocnění, tato studie ukazuje celkově zvýšené zastoupení γδ T buněk (bez ohledu na jejich expresi CD8) u myší na GFD, naznačující též možný imunomudulační vliv γδ T buněk u PN. Nenalezli jsme žádné změny v proporčním zastoupení CD4+CD45RB-low T buněk, IL-10 produkujících CD4+ T buněk a IFN-γ produkujících CD8+ T buněk, ani v různých subpopulacích NK buněk.
Kromě flow cytometrické analýzy jsme v této studii použili také behaviorální testování. Myši krmené GFD vykázaly vyšší hodnoty explorativního chování v testu otevřeného pole, které indikují zvýšený zájem zvířat k průzkumu svého okolí. Další výzkum je potřebný pro rozlišení vlivu diety na neurozánětlivé procesy u PN, jak v klinicko/neurologických tak i imunitních parametrech.

Tyto první výsledky jistě vyžadují opatrnou interpretaci a další podrobnější výzkum pro komplexní porozumění vlivům diety u PN. Spojení behaviorálních testů, imunohistologie a imunologických analýz představuje vícečetný metodický přístup k této problematice, který nám může pomoci poodhalit komplexní interakce diety, střeva, mozku, a neurozánětlivých procesů.

Klíčová slova: Parkinsonova nemoc. Bezlepková dieta, MTPT myší modely, behaviorální testy, imunohistochemie, flow cytometrie, T buňky, T regulační buňky, NK buňky

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List of abbreviations

GFD	Gluten free diet
GIT	Gastrointestinal Tract
IL	Interleukin
ILN	Inguinal lymph node
MLN	Mesenteric lymph node
MPTP	1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine
NK cells	Natural Killer cells
PBMC	Peripheral Blood Mononuclear Cell
PD	Parkinson's Disease
PLN	Pancreatic lymph node
PP	Peyer's Patch
SPL	Spleen
STD	Standard diet
Tregs	T regulatory cells
TH	tyrosine hydroxylase
TCR	T cell receptor
OF	Open Field

1 Introduction

Parkinson's disease (PD) is a neurodegenerative disease typically causing impaired gait and muscle control also has cognitive and emotional features, such as depression, impaired memory, and cognition (Jankovic & Tan, 2020). The prevalence of PD doubled between the years 1990 and 2016(Hirsch et al., 2016). Due to financial and geographic barriers to healthcare, the underreporting of cases, incorrect diagnoses, lack of awareness of PD, and false perceptions related to PD, the data on incidence and prevalence are inconsistent, especially for low- and middle-income countries and for ethnic minorities in high-income countries (Pringsheim et al., 2014)(Brakedal et al. 2022). In the Czech Republic, the prevalence is estimated to be around 15,000 to 27,000 patients; however, more accurate statistics are not available.

A further PD hallmark is the loss of dopaminergic neurons, which leads to both motor and non-motor symptoms. This is known to be caused by the accumulation of misfolded α-synuclein in neuron cells, which destroys these cells and damages the substantia nigra pars compacta (SNpc). The protein α-synuclein is a crucial factor in the onset of PD and is present in the gastrointestinal tract (GIT) and the brain (Margaret S. Ho, 2019). The presence of α-synuclein in the GIT has raised the concern of whether increased intestinal permeability would contribute to the development or worsening of PD symptoms.

In PD, gastrointestinal dysfunctions are one of the common non-motor symptoms. These dysfunctions may be caused by a dysregulated microbiota in the gut-brain axis, which tends to manifest years before the diagnosis, supporting the idea that the disease process spreads from the gut to the brain (Lei et al., 2021). Environmental factors contribute to PD in a pathogenic manner as well, for instance, exposure to diets and factors that induce the activation of cytokine pathways may contribute to the development of chronic inflammation (Grover et al., 2019). In the case of diets, gluten plays an important role in the development of autoimmune diseases, for example, e.g. in type 1. diabetes (Antvorskov et al., 2014) and psoriasis (Afifi et al., 2017). This was demonstrated in several trials documenting that a gluten-free diet has probably influenced the course of autoimmune diseases by reducing the production of pro-inflammatory cytokines (Antvorskov et al., 2014).

The main impact of the gluten-free diet in autoimmune diseases occurs by reducing the inflammation in the gut. In PD, the augment of the intestinal permeability may increase the available concentration of α-synuclein protein in the gut-brain axis. However, it is unknown how mechanistically the gluten could influence the permeability of the gut and collaborate to

aggravating the neuroinflammation. Moreover, studies have not yet evidenced whether the accumulation of the pathological form of α-synuclein protein may start in the gut and spread to the brain travelling via vagus nerve.

Thus, we hypothesize that PD's onset starts because of an eventual damage in the intestine cell wall that would lead to a more proinflammatory cytokine profile and the release of α-synuclein, present in the gut, to the vagus nerve. Then, this release would lead to an accumulation of α-synuclein in the brain before the first motor symptoms.

This diploma thesis's primary aim is to fill a gap in the existing literature concerning the effects of a gluten-free diet on immunological parameters in patients with PD. In particular, our goal is to evaluate the alterations in populations of regulatory and potentially memory T cells and their cytokine profiles in peripheral blood mononuclear cells (PBMCs) of PD patients who underwent a 12-month prospective intervention study on a gluten-free diet together with a control group. This is a crossover study, meaning that after the 12 months period, the control group will exchange its diet to GFD and the GFD group would return to a regular diet containing gluten.

Furthermore, we employed a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) animal model to investigate the second and third goals of this thesis in both acute and chronic models of Parkinson's disease. Changes in immunological markers (Tregs and IL-10 cytokine) are also discussed. These aims were established to investigate how diet-related changes may affect the progression and severity of PD as well as immunological traits in the MPTP mice model of PD.

2 Literature Review

2.1 Prevalence and incidence of Parkinson's Disease

More people have become disabled and are dying across the globe because of PD than any other neurological condition (WHO 2022). For instance, in the previous 25 years, the prevalence of PD has doubled. Approximately 8.5 million people worldwide were estimated to have PD in 2019. According to current estimates, PD resulted in 5.8 million disability-adjusted life loss in 2019, an increase of 81% since 2000. PD was also responsible for 329000 deaths in 2019, an increase of over 100% since 2000 (WHO 2022). With a prevalence ratio of roughly 3:2, men are more prone to developing Parkinson's disease than women (Feigin et al. 2019).

The most recent data from the Czech Republic's Institute for Health Information and Statistics (IHIS CR) from 2012 showed that 26,680 patients were diagnosed with PD, and at that time, the prevalence was 2.53 per 1000 inhabitants. The onset of PD in young patients at diagnosis, established before age 40, comprised 15% of cases in the country (Búřil et al. 2021).

2.2 Disease characterization

The primary risk factor for PD's onset is thought to be age (Ascherio and Schwarzschild 2016). Prior to the age of 50, the prevalence of PD is relatively low, but it rapidly increases with aging and, according to the majority of studies, peaks around the age of 80, reaching a prevalence of 2.6% in people who are 85 to 89 years old (Grover et al. 2019).

A meta-analysis demonstrated that the prevalence of PD increased with age for both men and women. Incidence rates for females increased over time, steadily increasing from 3.26 per 100,000 person-years at ages 40 to 49 to 103.48 at ages 80 and beyond, peaking in the majority of studies between ages 70 and 79. From 3.57 per 100,000 person-years in the 40-49 age group to 258.47 in the 80+ age group, men's incidence rates rose. In about half of the studies, the incidence rose in men but not in women after age 80 (Hirsch et al. 2016). Women aged 40 and older had a total incidence rate of PD of 37.55 per 100,000 person-years (95% CI 26.20-53.83), whereas men aged 40 and older had an incidence rate of 61.21 (95% CI 43.57-85.99) (Hirsch et al. 2016). This age gap may be observed because the illness was not properly diagnosed at its onset (Pringsheim et al. 2014).

In addition to the age, exposure to certain environmental factors, such as pesticides, water pollutants, traumatic brain injury, and dairy intake may contribute to the disease onset (Ascherio and Schwarzschild 2016).

For instance, in a Danish study of over 13,000 PD cases, demonstrated the risk ratio of PD after a concussion was a) 1.9 (1.3 to 2.8) between 4 and 12 months, b) 1.8 (1.4 to 2.2) between 1 and 4 years, c) 1.4 (1.1 to 1.7) between 5 and 9 years, and d) 1.2 (0.98 to 1.5) between 10 and 14 years (Rugbjerg et al. 2009).

However, other habits, namely smoking, coffee, and physical exercise are linked to a reduced risk of PD development, providing protective mechanisms against the disease. Smoking appears to have a protective effect for PD's onset (odds ratio [OR]=0.71, 95% CI 0.56–0.89, p=0.0041)(Grover et al. 2019).

Regarding the age of onset, clinical manifestations, rate of progression, and treatment response, PD exhibits remarkable heterogeneity. There are numerous clinical subgroups that have been suggested. For instance, REM sleep behavior disorder subgroups, brain-first and body-first subtypes, genetic subtypes, and biological subtypes are all potential prodromal PD subtypes (Berg et al. 2021).

Misdiagnosis can also occur when a patient experiences PD-like symptoms that are consequence of another illness (Farotti et al. 2020; Tolosa et al. 2021; Hughes et al. 1992). For instance, others movement disorders can be confused with PD, such as essential tremor and spasm. In addition, some side effects of medicines can mimic signs of PD, which may lead to an incorrect diagnosis of the disease. Moreover, the accuracy of PD's diagnosis is strongly associated with expertise level (Rizzo et al. 2016).

The identification of genetically forms of PD, which differs from sporadic disease in a number of clinical characteristics, has also raised questions about the universalist perspective of PD and introduced a biological concept of sub entities within PD's spectrum (Nalls et al. 2019).

Several genes have been found to be linked to a higher risk of developing PD, despite the fact that no specific gene is known to cause the condition. It is possible to list a few genes that possibly contribute to the onset of PD, for instance we can start mentioning the production of alpha-synuclein, which is controlled by SNCA gene, the protein responsible for LB's formation in the brain of PD patients as it was mentioned before (Klein and Westenberger 2012; Nalls et al. 2019).

Mutations in the LRRK2 gene is the most frequently inherited through in PD, as the enzyme LRRK2 cellular is responsible for regulation of processes such as inflammation and

cell death. A rare early manifestation of PD might be related to mutation on PARK2 gene. The PARK2 protein aids in cell cleanup of injured mitochondria (Klein and Westenberger 2012; Nalls et al. 2019; Hernandez, Reed, and Singleton 2016).

A type of rare PD may be caused by PINK1 protein, which controls how well the mitochondria function. An uncommon type of early-onset Parkinson's disease can result from mutations in the DJ- gene. Cells are believed to be protected from oxidative stress by the DJ-1 protein (Ge, Dawson, and Dawson 2020).

It is critical to consider that while changes in these genes increase the chance of developing PD, they do not always result in the disease. The complex condition known as PD most likely results from a combination of genetic and environmental variables.

2.3 Mechanisms underlying PD symptoms

Neuroinflammation, comprehends both innate and adaptive immune systems in the Central nervous system (CNS), contributing together to usual brain development and neuropathological events. Despite a consistent link to neurodegenerative conditions like PD, the causal relationship with neuronal degeneration remains uncertain. Microglia activation increases NFKB and NLRP3, leading to upregulated NADPH-oxidase and cytokine release (Panicker et al. 2019).

Neuropsychiatric indications and non-motor symptoms are frequent throughout the course of PD patients. These symptoms may be as clinically significant and incapacitating as motor symptoms, and they can present in a manner that is comparable to or different from that of their counterparts in the overall population (Weintraub et al. 2022). Thus, neuroinflammation is, in fact, an important player in PD, according to several studies (Ho 2019; Rocha, Miranda, and Sanders 2018; J. Wang et al. 2020). For instance, a meta-analysis of over twenty-five clinical trials found a significant increase in inflammatory cytokines in the blood and cerebrospinal fluid (CSF) of PD patients (X.-Y. Qin et al. 2016).

The innumerous non-motor symptoms exhibited in PD comprehends impaired cognition, gastrointestinal problems, sleep disturbances, fatigue, memory loss, and mood changes that can accompany the well-known motor symptoms of PD are commonly observed. The impact of these non-motor symptoms on a person's quality of life can be equally severe as that of motor symptoms (Weintraub et al. 2022).

There is evidence suggesting that patients with PD may experience heightened gut permeability, which could allow chemicals from the gut to enter the bloodstream and

potentially impact the development or advancement of the condition (Romano et al. 2021; Scheperjans et al. 2015; Q. Wang et al. 2021).

Constipation may be a first stage of PD symptom. The evidence from laboratory experiments using human feces suggested unusual α-syn deposits resided within the submucosal and myenteric plexuses of the enteric nervous system supports the biological plausibility of this reverse cause (Scheperjans et al. 2015).

The misfolded α-syn protein may begin to accumulate and aggregate into pathological forms in the gut and then travel through the nervous system to the brain (Romano et al. 2021). As a result, heightened permeability in the gut could potentially facilitate the translocation of α-syn into the bloodstream, eventually allowing it to reach the brain.

Early stages of α-syn buildup typically affect neurons in the medulla oblongata particularly those in the dorsal motor nucleus of the vagus nerve and the reticular formation. The role of medulla oblongata neurons during the early stages of α-syn accumulation is not completely understood. Nonetheless, there is a hypothesis that suggests pathogenic variations of the protein may enter the medulla oblongata while being transported from peripheral areas (such as enteric plexi) to the CNS (Braak et al. 2003).

The α-syn, a small soluble protein, is predominantly located in the presynaptic terminals of neurons. This protein controls the release of neurotransmitters and is highly expressed in neurons. Its most significant role in maintaining the form and functionality of synaptic terminals is essential for neuronal communication. It is known that PD is a degenerative disorder that affects the brain's dopamine-producing cells, which are responsible for regulating movement. The deterioration of the neuronal cells occurs exactly due to the accumulation of the misfolded form α-syn protein (Rocha, Miranda, and Sanders 2018).

The brain's substantia nigra (SN), which is responsible for the process of producing dopamine, is the location where neuronal loss largely takes place in PD. Dopamine is an essential neurotransmitter for controlling movement, and the death of dopaminergic neurons in the substantia nigra pars compacta (SNpc) is the cause of many of the disease's motor symptoms, such as tremors, bradykinesia, gait disturbance, and rigidity (Ascherio and Schwarzschild 2016; Borsche et al. 2021; Jankovic and Tan 2020).

The α-syn has the ability to induce IL-1β production in LPS-primed microglia by triggering the NLRP3 inflammasome, as indicated by the Casp-1–, NLRP3-, and ASC-dependent generation of IL-1β. A key discovery presented in this study is that α-syn can act as both a priming and activating agent, facilitating the assembly of the NLRP3 inflammasome in microglia. Notably, in most prominent NLRP3 inflammasome investigations, achieving

these two steps typically involves distinct agents, with LPS commonly serving as the priming agent (Panicker et al. 2019)

The aggregation of misfolded α-synuclein not only represents a hallmark of PD, but also is implicated in the onset of various neurodegenerative conditions. The formation of protein clumps of α-syn, known as Lewy Bodies (LB), has been associated with neuronal death. Besides, the accumulation of α-syn represents just one mechanistic pathway that may lead to neuronal death in PD, as the activation of microglia and other immune cells in the brain can induce different immune responses (Ho 2019; Rocha, Miranda, and Sanders 2018).

PD is believed to advance due to a proinflammatory response, compounded by various contributing mechanisms including oxidative stress, inflammation, and mitochondrial dysfunction. As α-synuclein aggregates, it triggers microglia, the brain's resident immune cells, prompting the release of pro-inflammatory cytokines. These cytokines, in turn, can inflict harm upon neurons, contributing to neuroinflammation (Rocha, Miranda, and Sanders 2018).

Mitochondrial dysfunction is also a defining characteristic of PD. Cellular energy generation is carried out by mitochondria, whose dysfunction can result in reactive oxygen species (ROS) and oxidative stress. Additionally, it has been demonstrated that α-syn restricts to mitochondria and interferes with mitochondrial activity. ROS are produced in more quantities compared to the biological ability to eliminate them, which is referred to as oxidative stress. α-syn accumulation in neurons is thought to contribute to oxidative stress and ROS generation in PD (Borsche et al. 2021).

Breaking it down, when α-syn comes from struggling DA neurons, it sets astrocytes in motion, releasing complement C3. This sets off a domino effect, activating the C3a receptor in microglia and prompting them to release both complement C1q and inflammatory cytokines (W. Zhang et al. 2023).

This complex interplay between astrocytes and microglia not only accelerates the decline of DA neurons but also worsens the motor dysfunction seen in PD. In response to the stress, compromised DA neurons release α-synuclein, initiating a dialogue between astrocytes and microglia. This conversation leads to astrocytes releasing complement C3, and when microglia get going through the C3a receptor, they contribute even more to the breakdown of DA neurons, intensifying the motor challenges in PD (W. Zhang et al. 2023).

Partial deficiency of Cntnap4 expedites the progression of α-synuclein pathology, degeneration of nigrostriatal neurons, and the onset of motor disorders in mouse models of PD featuring α-synucleinopathy (Figure 1).

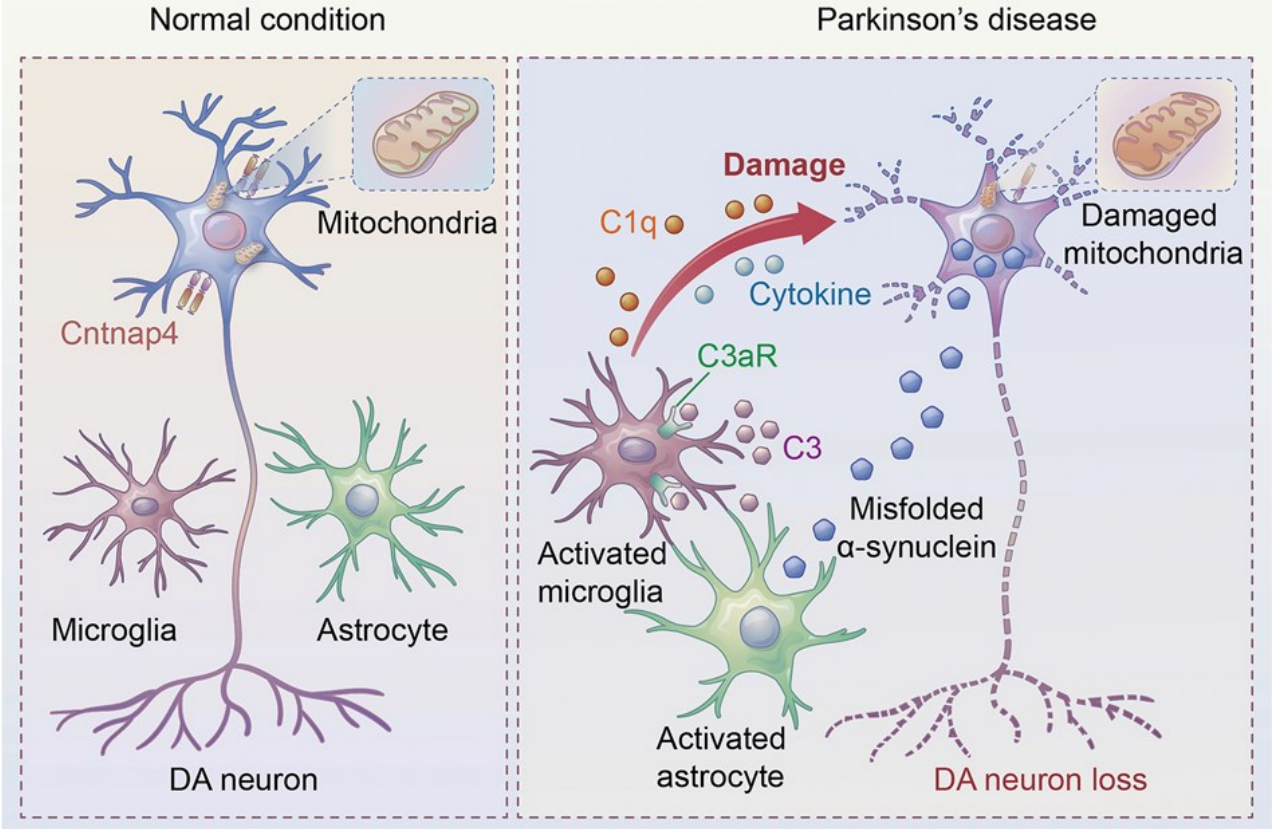

Figure 1 Cntnap4 deficiency intensifies α-synuclein-induced DA neuronal death, triggering astrocyte–microglia crosstalk. This cascade, involving astrocytic release of complement C3 and subsequent microglial activation, exacerbates motor dysfunction in PD. Adapted from W. Zhang et al. 2023

The exacerbation of α-syn pathology due to insufficient Cntnap4 is contingent on the astrocyte–microglia C3-C3aR signaling pathway, with microglia elimination and C3aR suppression mitigating these effects. Hence, the absence of Cntnap4 emerges as a crucial factor in PD pathogenesis, underscoring the potential of Cntnap4 as a promising target for further therapeutic exploration in PD (W. Zhang et al. 2023).

2.4 Adaptive and Innate Immune Response in PD

2.4.1 The involvement of immune cells in PD immune cells involvement

The impairment of immune system plays an important role in the onset and development of PD. For instance, Tregs are a subset of immune cells that are crucial for maintaining the homeostasis of the immune system and avoiding an autoimmune response. The presence of the transcription factor F3, which is essential for their growth and operation, distinguishes these cells from other T cells. By lowering inflammation and encouraging neuronal survival, Tregs are associated with protective effects in PD rodent models (Badr et al. 2022).

Under normal circumstances, T cells typically exhibit tolerance towards α-synuclein; however, in PD, this tolerance can be compromised, leading to an autoimmune response. Research has uncovered a correlation between α-synuclein epitopes found on specific MHC alleles and PD related to T-cell activity. Of note, the Y39 epitope of α-syn plays a critical role in provoking T-cell responses and cytokine secretion, implying that PD may be instigated by an autoimmune reaction involving α-syn protein. (Sulzer et al. 2017).

The Y39 antigenic region shows significant proximity to the α-syn mutations linked to PD (A30P, E46K, H50Q, G51D, A53T) (Hernandez, Reed, and Singleton 2016). The second antigenic region, which includes S129 and necessitates S129 phosphorylation—a form found in Lewy bodies (Fujiwara et al. 2002) exhibits less stringent restriction, allowing antigenic epitopes from this region to elicit immune responses even in patients lacking HLA alleles that recognize the Y39 region (Hernandez, Reed, and Singleton 2016; Klein and Westenberger 2012; Sulzer et al. 2017).

In the cohort studied, immune responses to α-syn epitopes were observed in approximately 40% of participants with PD. These responses may potentially indicate variations in disease progression or environmental influences (Hernandez, Reed, and Singleton 2016). Interestingly, similar observations of immune responses are often made in classic autoimmune disorders such as type-1 diabetes, rheumatoid arthritis, and multiple sclerosis, where the fraction of patients exhibiting such responses is typically in the range of 20% to 50% (Petrich De Marquesini et al. 2010; Arif et al. 2011).

Similar to type-1 diabetes, where epitopes are derived from both pre-proinsulin and additional proteins, it is possible that epitopes associated with PD are derived from α-syn and additional proteins. In conventional autoimmune disorders, the MHC class II response may be preceded by MHC class I, and it is observed that exposing microglia to α-syn results in

MHC class I expression being induced by dopamine neurons. The PD associated proteins parkin and PINK1 may be involved in regulating the antigenic presentation of mitochondrial peptides (Matheoud et al. 2016).

Within a mouse model of Parkinson's disease, Tregs were observed to migrate into the brain, effectively mitigating neuroinflammation. Notably, the application of Treg therapy not only averted the depletion of dopamine-producing neurons, but also improved motor performance in the mice (Badr et al. 2022).

PD patients had lower blood levels of Tregs compared with healthy controls. The observation that mice lacking CD4 exhibit partial protection against MPTP-induced injury implies the involvement of T-helper cell-mediated harmful mechanisms in the demise of DN. Cytokines like IFN-γ and membrane-bound ligands such as FasL are significant players in the effector functions of CD4 T-helper cells, particularly in the activation of macrophages. Intriguingly, prior studies indicate that IFN-γ plays a role in the death of DN by influencing microglial activity (Mount et al. 2007).

Using recombinant α-syn preformed fibrils (PFF) as a model for studying misfolded α-synuclein transmission between neurons, a screen of transmembrane proteins identified three candidates – lymphocyte-activation gene 3 (LAG3), neurexin 1β, and amyloid β precursor-like protein 1 (APLP1) – that bind α-synuclein PFF (Mao et al. 2016).

LAG3 exhibited the highest selectivity for α-synuclein PFF over the monomer (Figure 2). α-Syn PFF specifically bind to LAG3 while tau PFF, β-amyloid oligomer, and β-amyloid PFF do not. LAG3 is involved in the internalization of α-synuclein PFF, as its deletion reduces endocytosis. Neuron-to-neuron transmission of pathologic α-syn and associated neurotoxicity is significantly reduced by deleting LAG3 or using LAG3 antibodies. The absence of LAG3 also delays α-synuclein PFF-induced loss of dopamine neurons and associated deficits in vivo (Mao et al. 2016).

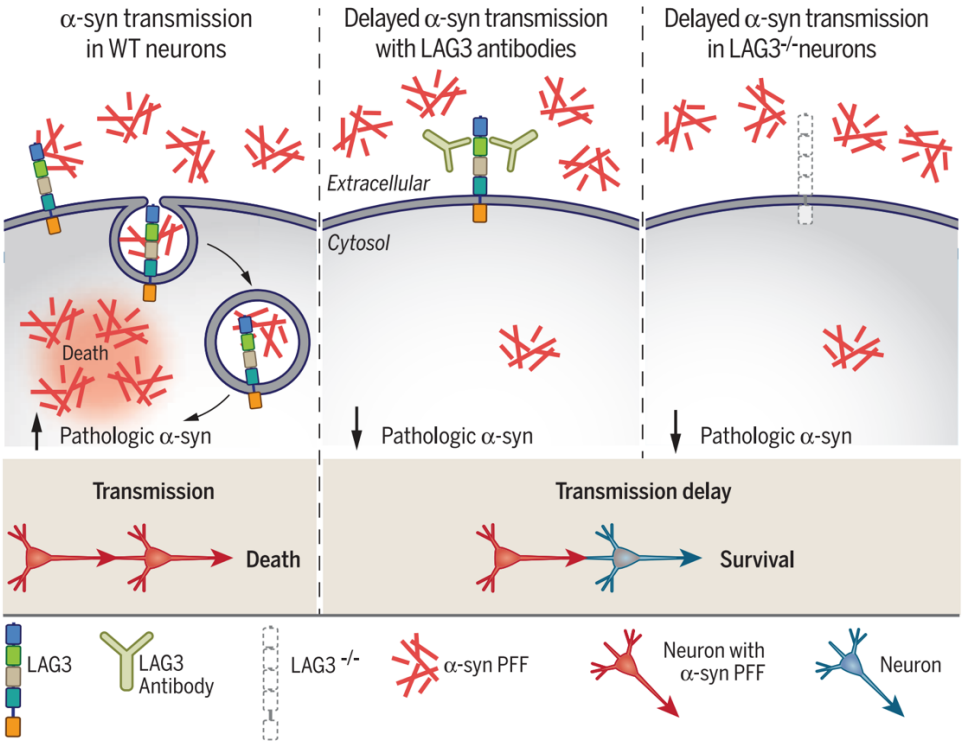

Figure 2 Binding and internalization of α-synuclein PFF is significantly diminished in neurons lacking LAG3 or treated with anti-LAG3 antibodies, leading to a delayed transmission and reduced toxicity compared to wildtype neurons (Mao et al. 2016)

The identification of LAG3 as a receptor which is capable of binding α-syn PFF provides an avenue for therapeutic development aimed at impeding the progression of PD and related α-synucleinopathies. This discovery suggests that targeted interventions could slow the progression of α-synuclein-related neurodegenerative disorders.

Translocator Protein (TSPO) is frequently associated with the activation of microglia, which are the immune cells native to the central nervous system. In a study examining immune cell types expressing TSPO in the context of PD, a comparison between the MPTP + vehicle group and the sham + vehicle group revealed heightened TSPO expression. Notably, the immune cell type with the highest TSPO levels was identified as CD45+CD11b+ microglia, surpassing the expression levels observed in CD45highCD11b+ and CD45highCD11b– immune cells (Girard et al. 2008).

A subset of T cells called memory T cells participate in the adaptive immune reaction to particular pathogens or antigens. They contribute significantly to long-term immunity and are distinguished by their capacity to quickly react to a disease upon re-exposure (Yan et al. 2021).

Memory T cells also has its part in the PD onset and progression (Dhanwani et al., 2022; Galiano-Landeira et al., 2020; Seledtsov & von Delwig, 2020). According to a North American study, patients with the disease had more memory T cells in their blood than healthy controls. The authors showed that memory T cells were enriched in PD patients' brains and were producing more pro-inflammatory cytokines. Memory T cells were also linked to an increased microglial activation and neuroinflammation (Yan et al. 2021).

The expression of neuronal MHC-I and the subsequent signaling to CD8 T-cells has also been associated with neurodegeneration resembling PD, particularly in the specific susceptibility of catecholaminergic neurons in rodents. Examination of peripheral blood lymphocytes in PD patients indicates dysfunction in CD4 T-helper (Th) cells and changes in the CD4:CD8 ratio (Hisanaga et al. 2001).

Regulatory T cells, identified by the markers CD4+CD25+ and expressing the transcription factor Forkhead box (FOXP3), serve to uphold self-tolerance and stave off autoimmune reactions (Sakaguchi et al. 2006). In PD, these Tregs are believed to exert a crucial role in restraining the activity of effector T cells, thus thwarting neuronal degeneration. An investigation explored the relationship between PD progression and the status of Tregs, revealing compromised immune suppression and neuroprotective capabilities of these cells in PD patients. This finding implies a potential neuroprotective role of Tregs in PD.

Employing mouse models, where Treg preparation was administered to ventral mesencephalic (VM) neurons prior to treatment with 1-methyl-4-phenylpyridinum (MPP+), allowed for an examination of the neuroprotective mechanisms of Treg. This investigation uncovered that dopaminergic neurons could be shielded from MPP+ toxicity via interactions involving CD47-signal regulatory protein alpha and the Ras-related C3 botulinum toxin substrate 1 (Rac1)/Akt pathway. (Yan Huang et al. 2017).

Th1 and Th17 cells utilize several pathways to induce neuronal death and advance PD progression. Conversely, augmenting the population of Tregs can counteract this neurotoxic effect. Therefore, there is a need for a comprehensive investigation into the regulatory mechanisms governing CD4+ T cells in PD (Reynolds et al. 2010).

The accumulated α -syn released from neurons binds to either Toll-like receptor 2 (TLR2) or Toll-like receptor 4 (TLR4), initiating a proinflammatory cascade. Concurrently, nuclear factor-kappa B (NF-κB) activation triggers the MyD88 pathway, leading to M1 microglia activation (L. Qin et al. 2005). The α -syn can also be transferred to microglia via exosomes. Activated M1 microglia release proinflammatory cytokines such as tumor necrosis

factor-alpha (TNF-α) and interleukin-1beta (IL-1β), contributing to neuronal death (L. Qin et al. 2005; George et al. 2019).

In PD, damage to the BBB allows infiltration of T cells, including CD4+ and CD8+ T cells, as well as regulatory T cells, from the peripheral blood into the CNS. Within the CNS, microglia and astrocytes serve as antigen-presenting cells (APCs), presenting alpha-synuclein to T cells. Activated CD4+ and CD8+ T cells release cytokines that promote neuronal death, whereas Tregs exert a protective effect on neurons (Baba et al. 2005; Galiano-Landeira et al. 2020). This is illustrated on Figure 3.

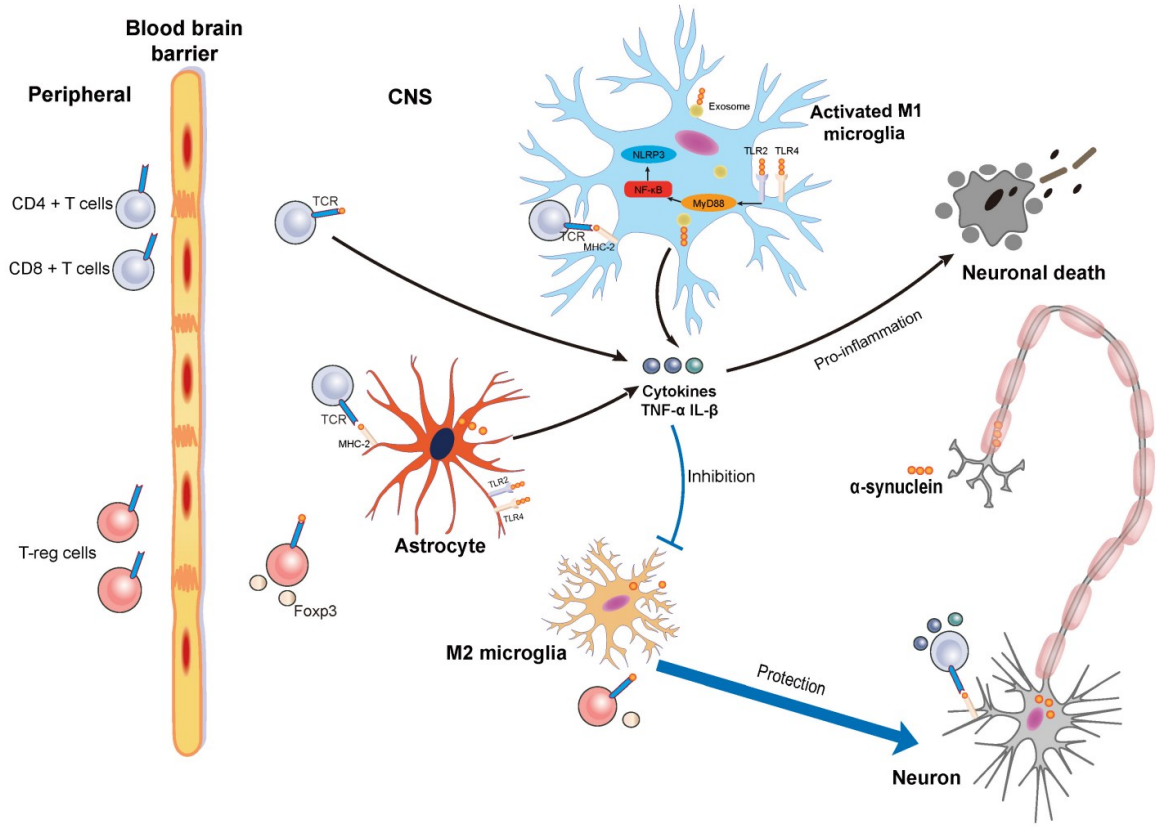

Figure 3 Illustration depicting the process of microglia-mediated neuronal death and protection via α-synuclein, and the correlation between α-synuclein and T cells (Su and Zhou 2021).

NK cells, known for their critical role in immune surveillance against cancer, they are as well important players in the complex landscape of PD (L. Zhang, Zhang, and Fan 2022). NK cells, which are classified as innate effector lymphocytes, are well known for their ability to target and eliminate cancerous cells, highlighting their critical role in immune defense. Beyond this primary function, NK cells have a wide range of abilities, including the ability to resolve inflammation and form immunological memory, as well as modulate antigen-presenting cell function (Lanier, Corliss, and Phillips 1997).

The BBB serves to prohibit the entry of peripheral immune cells into the CNS. However, in instances of infection or neurodegenerative conditions, the BBB may undergo disruption due to factors such as free radicals, pro-inflammatory cytokines and chemokines, proteolytic enzymes, and matrix metalloproteinases. This breakdown in the BBB's integrity can lead to the infiltration of immune cells, such as NK cells, into the CNS, particularly in neurological disorders like PD, especially when there is heightened inflammation present (Guo, Zeng, and Gao 2021; Earls and Lee 2020; L. Zhang, Zhang, and Fan 2022).

Furthermore, the function of NK cells includes the inhibition of hyperactivated microglia, which play an important role in neuroinflammatory responses. Recent advances in the field of neuroinflammation suggest that complex interactions between cells in the CNS and immune cells from the periphery contribute to PD pathogenesis. Nonetheless, the mechanism regulating peripheral cell recruitment into the CNS, as well as whether it involves passive migration or active participation, remains unknown (L. Zhang, Zhang, and Fan 2022; Earls and Lee 2020).

Pro-inflammatory cytokine concentrations have been found to be elevated in Parkinson's patients' blood and CSF, as well as in their brains. Interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-alpha), and Interleukin-6 (IL-6) are strongly associated with neuroinflammation and neuronal damage in PD. These are produced by activated microglia and astrocytes (Starhof et al., 2018).

It has been reported an increased proportion of Th1 cells in the peripheral blood of individuals with PD, with cytokines such as TNF-α, secreted by activated Th1 cells, potentially influencing neurons. Th1 cells are known to exacerbate inflammation by stimulating the secretion of cytokines, although their precise role in PD pathogenesis remains unclear, necessitating further investigation (Baba et al. 2005; Storelli et al. 2019; Kustrimovic et al. 2018) .

Similarly, elevated levels of circulating Th17 cells have been observed in the early stages of PD, with proinflammatory Th17 cells implicated in mediating PD pathology through the upregulation of cytokines such as IL-6, IL-23, and IL-1β (J. Fu et al. 2022; Storelli et al. 2019).

Furthermore, Th17 cells have been shown to directly induce the apoptosis of dopaminergic neurons by binding to membrane molecular proteins like leukocyte function-associated antigen (LFA)-1 and intercellular adhesion molecule (ICAM)-1, thereby promoting PD. Neurons derived from midbrain iPSCs of PD patients demonstrate increased

IL-17–IL-17R signaling and NFκB expression associated with Th17 activation, indicating the involvement of Th17-related pathways in PD pathogenesis (Storelli et al. 2019).

This evidence emphasizes the proinflammatory role of Th17 cells and their signaling pathways in promoting dopaminergic neuronal death, suggesting that inhibiting Th17 or its related functions may hold neuroprotective potential in PD.

Anti-inflammatory cytokines may also play a role in PD, in addition to pro-inflammatory cytokines. For instance, Interleukin-10 (IL-10) is an anti-inflammatory cytokine that has been demonstrated to have neuroprotective benefits in animal models (Starhof et al., 2018).

2.5 Gut insights into PD

The gut microbiota is composed by crucial bacteria that are responsible for maintaining the integrity of the gut barrier and immune system regulation.

An imbalance of these microbes, known as dysbiosis, has been linked to a number of illnesses, such as inflammatory bowel disease, metabolic problems, and neurodegenerative diseases, such as PD (Romano et al., 2021).

Compared to healthy individuals, people with PD have a more distinct gut microbiota. PD patients appear to have had a lower abundance of bacteria from the *Lachnospiraceae* family and a higher abundance of bacteria from the *Verrucomicrobiaceae* family compared to healthy controls (Scheperjans et al., 2015).

These alterations in the gut microbiota have been connected to the buildup of the alpha-synuclein protein in the gastrointestinal tract. Alpha-synuclein is thought to assemble in the gut as a result of gut dysbiosis, which may then spread to the brain via the vagus nerve and enhance the neurodegeneration in PD (Kim et al., 2019).

Gluten plays an important role in the gut microbiome since the incomplete digestion of this protein may lead to its accumulation in the small intestine (Zopf et al., 2018). Gliadin and glutenin are the two parts of the gluten protein. In contrast to glutenin, which is crucial for defining the elasticity of dough and bread quality, gliadin is a more significant source of the protein-digesting fragments (peptides) that can lead to unfavorable reactions in the intestines (Hausch et al., 2002).

The gliadin superstructures may disrupt the initial attachment and subsequent establishment of beneficial bacteria. Additionally, the structural resemblance to pathogenic bacteria might promote their adherence and colonization in the mucosal lining. Importantly,

both situations could result in dysbiosis, characterized by an imbalance in the gut's normal microbiota (Carding et al. 2015).

Several studies have highlighted alterations in the abundance of specific bacteria, such as *Prevotellaceae, Bifidobacterium, Akkermansia,* and *Lactobacillus*, among PD patients (Boertien et al. 2019). However, discrepancies in findings often arise due to variations in study methodologies, patient demographics, and control selection. While the precise mechanisms through which particular taxa of gut bacteria influence or initiate PD remain unclear, numerous investigations have revealed links between motor symptoms, disease progression, and early pre-motor stage conditions of PD with the relative abundance of specific bacterial families within fecal samples from PD patients (Boertien et al. 2019).

Additionally, shifts in gut bacterial composition have been associated with intestinal inflammation in PD. Elevated levels of various inflammatory markers, including IL-1α, IL-1β, CXCL8, CRP, and calprotectin, have been detected in the stool of PD patients compared to controls, with some of these markers inversely correlated with the age of PD symptom onset, suggesting potential involvement in disease development. Furthermore, correlations between levels of *Bacteroides* and *Verrucomicrobia* with plasma levels of TNF and IFNγ, respectively, support the notion that gut dysbiosis contributes to an inflammatory milieu, possibly initiating PD pathology. Nonetheless, strategies to effectively modulate the gut microbiome for delaying or attenuating PD development remain an active area of investigation (Schwiertz et al. 2018; Boertien et al. 2019; Lin et al. 2019).

Current literature suggests that the gut microbiota and the brain communicate via the GBA, when fecal samples from PD patients and matched controls were compared, it revealed differences in the gut microbiota between PD patients and their healthy counterparts. In addition, individuals with Parkinson's disease exhibit elevated intestinal permeability (Schwiertz et al. 2018; Unger et al. 2016; Hill-Burns et al. 2017).

Thus, the gut dysbiosis may contribute to the mechanisms mentioned earlier in the literature review, namely neuroinflammation and oxidative stress favoring the onset of the disease.

2.6 Interventions for PD

2.6.1 Medicaments available for the treatment

The first course of treatment for PD is commonly medication. The primary drug classes used to cope with PD are based on increasing the availability of dopamine (Figure 4), and the most common medications are:

Levodopa

A precursor to dopamine, undergoes conversion to dopamine within the brain, thereby playing a crucial role in mitigating the motor symptoms characteristic of Parkinson's disease (Isaacson et al. 2023).

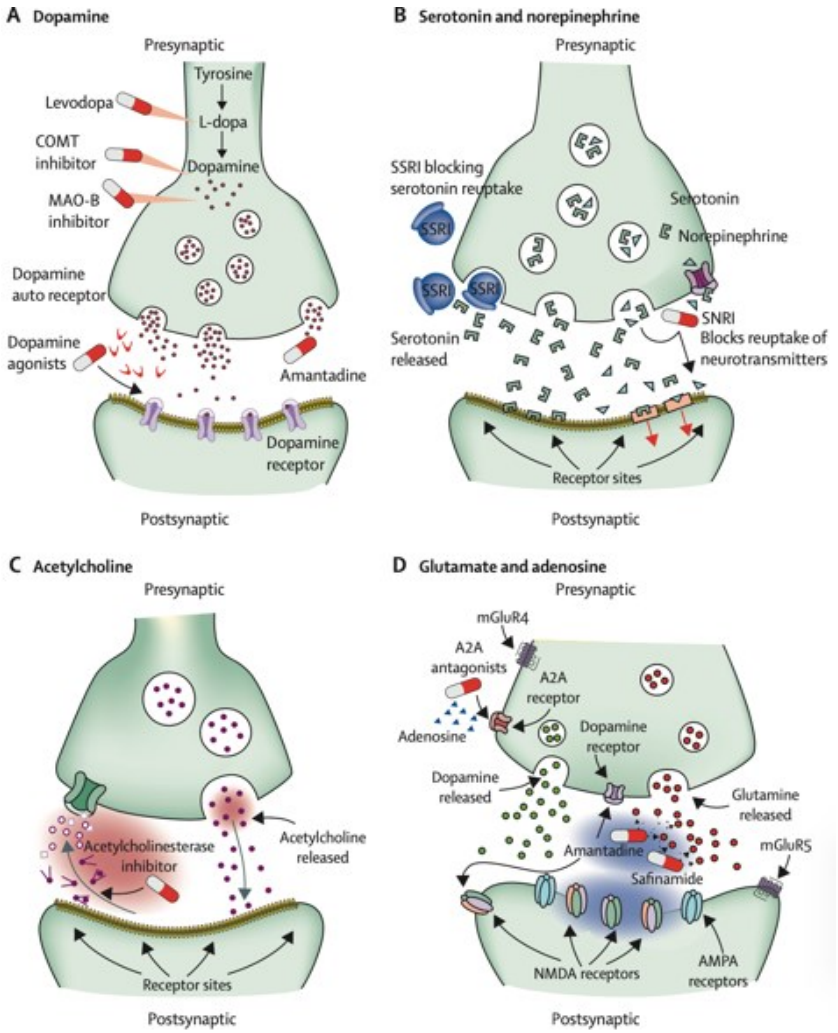

Figure 4 Sites of action for several antiparkinsonian medications

Dopamine agonists

Act by mimicking the effects of dopamine in the brain, thereby supplementing the deficient dopamine levels and contributing to the improvement of motor symptoms (Isaacson et al. 2023).

MAO-B inhibitors

A class of drugs, function by inhibiting the enzymatic breakdown of dopamine within the brain, thus helping to sustain adequate dopamine levels and improving movement symptoms (Regensburger et al. 2023).

Similarly, COMT inhibitors operate by impeding the peripheral breakdown of levodopa, thereby extending its availability and enhancing its therapeutic effects in managing Parkinson's disease symptoms. These pharmacological interventions collectively target different aspects of dopamine metabolism, providing a multifaceted approach to symptom management in PD (Regensburger et al. 2023).

2.6.2 Other molecules influencing the course of PD

Serotonin and norepinephrine

Although they may have some indirect effects on some aspects of Parkinson's disease (such as mood and emotional symptoms), serotonin and norepinephrine are not the main targets for treating the motor symptoms connected with Parkinson's disease. Instead, other neurotransmitters such as dopamine and glutamate are (Muñoz et al. 2020).

Acetylcholine

The correct functioning of the basal ganglia, a set of brain regions involved in movement regulation, depends on the balance between dopamine and acetylcholine. The imbalance between dopamine and acetylcholine caused by dopamine depletion in the basal ganglia of people with Parkinson's disease adds to the condition's motor symptoms.

Bringing dopamine and acetylcholine back into balance is one of the key therapeutic focuses for Parkinson's disease. This can be accomplished by taking drugs that either elevate dopamine levels or obstruct cholinergic receptors (Rizzi and Tan 2017).

Glutamine and adenosine

Glutamine is an amino acid that the body uses to construct several different types of proteins. Parkinson's disease is not immediately treated with it. However, some research has looked into the pathophysiology of Parkinson's disease in relation to the neurotransmitter glutamate, which is formed from glutamine. Cell damage and neurotoxicity can result from too much glutamate activation. Although glutamine-based treatments are being investigated as possible neuroprotective agents, glutamate-modulating drugs are not yet accepted as standard care for Parkinson's disease (Z. Zhang et al. 2019; J. Wang et al. 2020).

Adenosine

It is a chemical involved in a number of biological functions, including the transfer of energy within cells. It controls neuronal activity in the brain and functions as a

neurotransmitter. Adenosine receptors may be possible targets for the treatment of Parkinson's disease. In preclinical investigations, activation of specific adenosine receptors has demonstrated neuroprotective effects. Adenosine-based medicines, however, are still in the experimental stages and have not yet become accepted as routine Parkinson's disease therapy (Zhao, Liu, and Yang 2023).

Different approaches to treatment for PD patients are crucial because the medication does not always improve the patient's quality of life as expected. For instance, the levodopa response in PD patients is anticipated to decline after 5 years of therapy, and 40–50% of them will develop motor dyskinesias. For PD patients, the motor impairment is the main factor contributing to their disability and significantly lowering their quality of life (Zopf et al., 2018).

2.7 Influence of diets on PD

In general, a healthy diet is one that has been scientifically shown to improve health parameters, such as lowering mortality or the likelihood of diseases becoming worsened. In general, healthy diets place a high priority on enhancing a person's overall health.

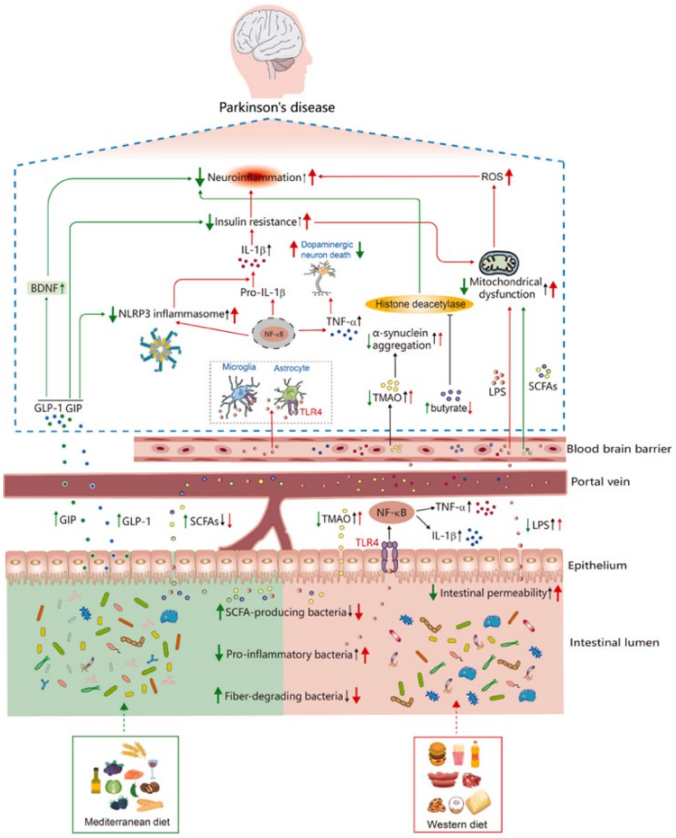

Figure 5 Demonstration of how possibly the western diets have been found to exacerbate neuroinflammation in PD through interactions involving the microbiota-gut-brain axis. (Chu et al., 2021).

Diets may be focused on one or more specific dietary groups, such as dairy, low-fat, low-carb, gluten-free, etc., or a combination of these and other groups not specifically stated (Bellou et al., 2016; Knight et al., 2022).In case of PD, it may be attributed to a specific eating pattern. Thus, several studies have investigated whether changes in diet could improve this disease's symptoms. For instance, Mediterranean diet has been shown to modulate the gut microbiota and to improve neuronal) (Solch et al., 2022).

The increased adherence to Mediterranean diet was linked to a lower chance of getting these diseases, according to a meta-analysis of a study examining the impact of this diet on Alzheimer's and PD. Yet, it was found in a pilot study including just women, the Mediterranean diet was associated with a lower risk for PD. Therefore, more research is required to determine whether this diet can prevent PD in people (Solch et al., 2022).

Several studies documented possible beneficial effects of dietary interventions in PD (Mischley, Lau, and Bennett 2017; Bisaglia 2022). As already mentioned chronic intestinal inflammation seems to be an important feature in PD, since the original but long time overlooked description of gastrointestinal sign of PD (Goetz 2011; Braak et al. 2006), the role of chronic intestinal inflammation has become recently appreciated in several immune mediated diseases (Barnabei et al. 2021) including PD (Mulak 2015) , also due to the existence of the gut-brain bidirectional axis (Bittinger, Barnert, and Wienbeck 1999).

Existing literature has established a correlation between exposure to gluten and/or gliadin and the manifestation of chronic pro-inflammatory effects reminiscent of lipopolysaccharide (LPS). Notably, gluten exhibits lectin-like attributes, as reported by Köttgen (Köttgen et al. 1983). Furthermore, gliadin, akin to LPS, directly triggers innate immune response (Jelínková et al. 2004; Palová-Jelínková et al. 2005). Although the precise signaling pathways are unknown, studies have shown that gliadin pepsin digestion induces inflammatory cytokines via the MyD88 signaling pathway (Thomas et al. 2006; Palová-Jelínková et al. 2013).

Our lab discovered, and others confirmed, that a gluten-free diet is a very effective environmental preventive factor in the NOD mouse model of T1D (D. P. Funda et al. 1999; Schmid et al. 2004). More recently, the lab took part in a clinical trial that found a beneficial effect of GFD in children who were put on it at the time of diagnosis for a year (Neuman et al. 2020). Several other groups also reported possible beneficial effect of GFD in human T1D (Pastore et al. 2003; Sildorf et al. 2012).

Ketogenic diet also provides a positive result in a short-term adherence. It is commonly referred as "keto diet", consisting in extremely high fat consumption and very low

carbohydrates intake, promoting the growth of tissue-protective γδ T lymphocytes in adipose tissue, while in PD patients, alterations were observed in the gut microbiota, inducing a potential mechanism of the ketogenic neuroprotective impact (Goldberg et al., 2020) (Figure 6).

In PD is known the dopaminergic neurodegeneration process from mitochondrial dysfunction, leading to reduced ATP synthesis. The ketogenic diet and β-HB contribute to heightened antioxidant levels through the elevation of glutathione, a decrease in ROS generation, and activation of KATP channels, which serve to safeguard synaptic function (Xu et al. 2022; Goldberg et al. 2020).

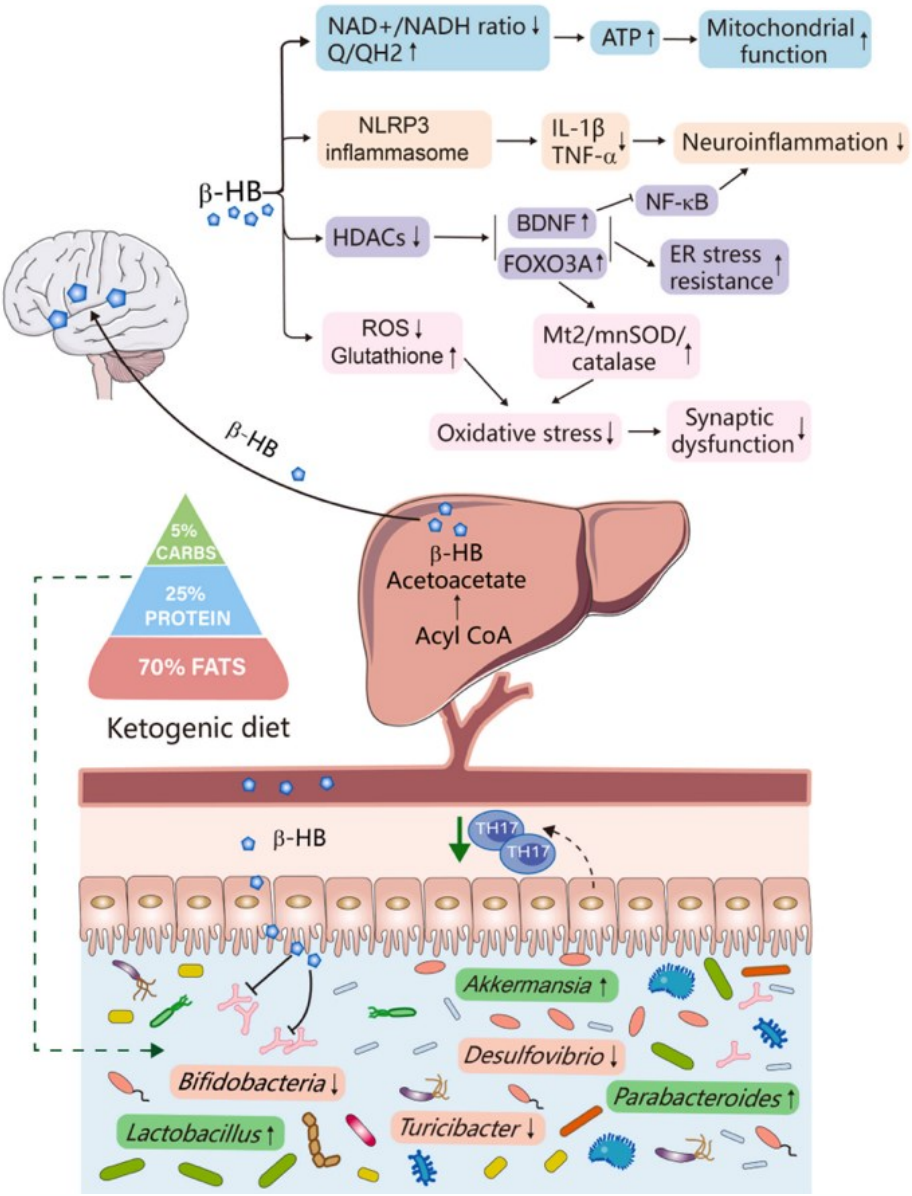

Figure 6 Ketogenic diet inducing a protective behavior in PD neurodegeneration (Goldberg et al., 2020)

Moreover, the inhibition of HDACs by β-HB promotes the production of brain-derived neurotrophic factor and Forkhead box O3 as well as enhances endoplasmic reticulum stress tolerance. BDNF further mitigates NFκB-mediated neuroinflammation and apoptosis, while simultaneously providing protection against oxidative damage (Bellou et al., 2016; Chu et al., 2021; Knight et al., 2022).

2.7.1 Gluten, Autoimmunity and Parkinson Disease

The link between autoimmune disorders and an increased risk of PD has prompted studies to investigate the potential role of autoimmunity in the disease's pathogenesis. A related line of research has looked into the possible link between gluten, autoimmune

responses, and various diseases, sparking interest in the effect of a GFD on PD. The complex interplay of genetics, the immune system, and dietary factors highlights the need for additional research to determine whether a gluten-free diet can affect the development or management of PD (Tan et al. 2020).

Growing evidence indicates that autoimmune mechanisms may be involved in the pathogenesis of Parkinson's disease. Autoimmune responses, which are triggered by a variety of environmental factors, may contribute to chronic inflammation in the brains of people with PD (Ascherio and Schwarzschild 2016).

The gut brain-axis (GBA), a bidirectional communication system that connects the gastrointestinal tract along with the central nervous system, is essential for maintaining homeostasis. Disruptions in this direction, including those seen in gluten-related disorders, might contribute to neuroinflammation and either the onset or progression of PD (Liu et al. 2017).

These fragments might contain core amino acid sequences that, in people with certain genetic predispositions, can cause immunological reactivity. For instance, gliadin is able to cross the intestinal barrier and reach the bloodstream. Moreover, other immunogenic peptides are generated as a product of gluten digestion, which contribute to the inflammation of the gut (Hausch et al., 2002). As a result, hazardous compounds may enter the bloodstream and cause an immunological reaction, possibly initiating autoimmune illnesses (Zopf et al., 2018).

Through the phenomenon of molecular mimicry, gluten peptides can resemble body proteins, especially those in the GIT, and the immune system may mistakenly attack those body proteins for gluten. It has been proposed that a process known as molecular mimicry may play a role in the emergence of autoimmune disorders (Neuman et al., 2020).

Thus, gluten is also associated with many autoimmune diseases. For instance, diabetes type I may be caused by the consumption of gluten, because this protein induces an immunological response in the pancreas by inhibiting insulin synthesis (Antvorskov et al., 2014; Neuman et al., 2020).

However conclusive evidence regarding the impact of a GFD on the onset and treatment of PD remains elusive, necessitating further investigation.

2.8 Animal model for PD

Animal models enable researchers to study the early stages of PD development, providing insights into the chain of events that leads to neuronal degeneration. These models assist in the identification of potential biomarkers, allowing for early disease detection and intervention. Furthermore, they allow for the evaluation of novel therapeutic strategies ranging from pharmacological interventions to gene therapies and deep brain stimulation.

The use of animal models also helps to understand the role of inflammation and the immune system in PD, being used to investigate the role of microglial activation in neuroinflammation and its impact on disease progression. Furthermore, the impact of genetic factors on PD can be investigated by using transgenic animals with specific genetic modifications associated with PD risk.

One widely used model involves the administration of MPTP, a neurotoxin that selectively damages dopamine-producing neurons, mimicking the neurodegeneration seen in PD patients. Rodents and non-human primates treated with MPTP show motor deficits and neuropathological changes similar to human PD, providing a valuable platform for studying the disease's progression (Przedborski and Vila 2003).

The rotenone model, which uses a pesticide that causes oxidative stress and neuronal damage, provides another avenue for researchers to investigate the role of environmental factors in PD. Similarly, the 6-OHDA model, which employs a neurotoxin to selectively destroy dopaminergic neurons, aids in understanding the neurochemical changes associated with PD (Kirik, Rosenblad, and Björklund 1998).

Genetic models involving the manipulation of specific genes associated with Parkinson's disease provide critical insights into the disease's genetic basis. Transgenic animals expressing human alpha-synuclein, a key protein in PD pathology, allow researchers to study Lewy body formation, a hallmark of the disease. These models shed light on genetics' role in neurodegeneration (Rockenstein et al. 2002).

Beyond chemical and genetic models, the role of inflammation in PD is investigated using the LPS model, in which bacterial endotoxin induces neuroinflammation. This model aids researchers in their investigation of the relationship between inflammation and PD progression (Castaño et al. 1998).

Specific models for PD can be created using virally induced models, which use viral vectors to deliver genes or toxins to specific brain regions for elucidating region-specific effects, paving the way for targeted therapeutic interventions.

2.8.1 MPTP (1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine) mouse model

The only known dopaminergic neurotoxin that can reproduce a clinical picture that is analogous to PD in both humans and monkeys is 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine (MPTP). Despite several safety precautions required for the use of MPTP, its use is not challenging. Because, unlike other known toxins, MPTP causes consistent and repeatable damage to the nigrostriatal dopaminergic pathway after systemic treatment in mice (Jackson-Lewis & Przedborski, 2007).

The MPTP neurotoxin damages dopaminergic neurons in the SNpc and striatum by triggering a series of mechanisms of cell damage, such as oxidative stress, mitochondrial apoptosis, inflammation, excitotoxicity, and the formation of inclusion bodies. These events are strongly associated with PD in humans, as described in sections above (Meredith & Rademacher, 2011).

The toxic MPTP metabolite (MPP+) continues to increase and aggregates in the synaptosome vesicles of dopaminergic neurons until reaching an excessive level in the cytoplasm. Then, MPP+ causes cell death in the striatum and SNpc (Meredith & Rademacher, 2011) (Figure 7).

This model offers a distinct approach to investigate non-cell autonomous pathomechanisms by replicating several key features of PD, such as the loss of dopaminergic neurons, decreased striatal dopamine levels, and inflammatory processes associated with glial cells.

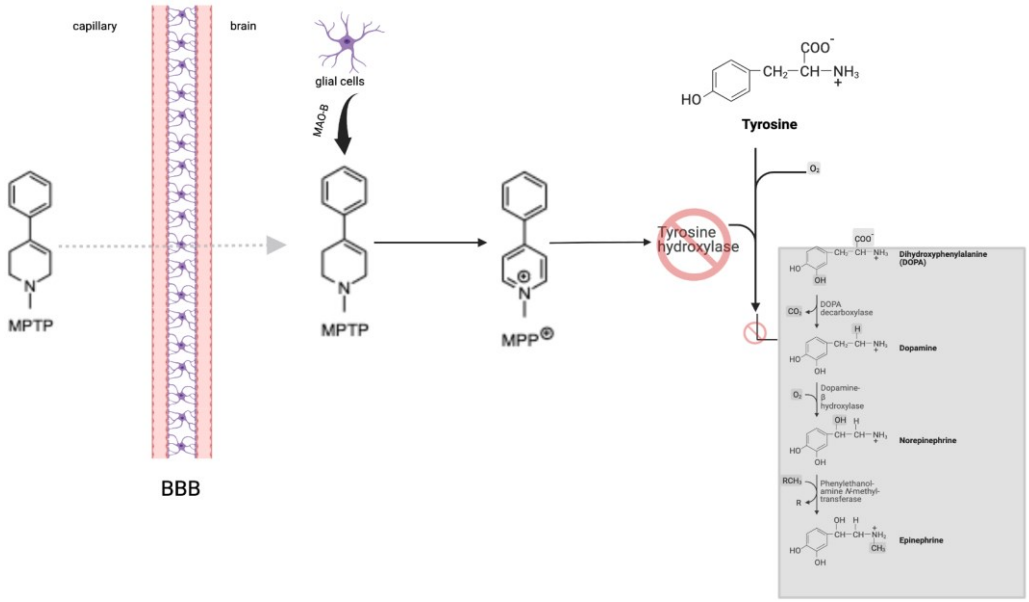

Figure 7 General MPTP pathway for PD animal model. (Figure created on BioRender)

Chronic and acute models of MPTP

The regime of the administration of MPTP can differ according to the desired effect of the drug. This variation can be not only in the frequency of the administration but in the dosage to be used, it can be seen in the table below Table 1.

Table 1 Different dosage of MPTP for each model

Model	**Dosage**	**Interval of Administration**	**Use**	**Reference**
Acute	10–20 mg/kg	Given four times at 1–2 h intervals	Commonly used in research for Parkinson's disease (PD)	(Jackson-Lewis and Przedborski 2007)
Subacute	30 mg/kg/ day	Given for 4–5 days	Attracts much attention for its short period and similarity to PD. However, it may not be a suitable model for studying parkinsonism	(Jackson-Lewis and Przedborski 2007)
Chronic	20-25 mg/kg	Given three times a week for 8-12 weeks	Results in a significant loss of dopaminergic neurons, which is accompanied by anxiety-like behaviors in addition to motor dysfunction	(Jackson-Lewis and Przedborski 2007)

Limitations of the MPTP mouse model

Although neurotoxic models appear to be the most appropriate for testing nigrostriatal pathway degeneration, some notable differences from PD must be mentioned: lesions are primarily or exclusively dopaminergic, and animals lack the typical PD proteinaceous inclusions known as Lewy bodies (Blesa & Przedborski, 2014).

There is a translational gap between animal findings and human applicability that poses challenges. PD is a disease that is unique to humans, and the extent to which animal models accurately mimic the complexity of human PD is still being studied.

The enrichment of categories associated with basal ganglia-related motor dysfunction and neurodegeneration in the SN of both PD patients and MPTP-treated mice, MPTP toxicity appears to induce similar phenotypic consequences as observed in human PD (Klemann et al. 2016).

However, disparities emerge in the specific dysregulated biological processes. PD patients exhibit enriched molecular and cellular functions related to neuronal and synaptic functions in the SN, while MPTP-treated mice predominantly show themes associated with cell growth and death (Blesa and Przedborski 2014).

This contrast may reflect the distinctions between the prolonged neurodegenerative processes and compensatory neuroplastic mechanisms in PD compared to the acute toxicity induced by MPTP in mice. Furthermore, common biological processes between the SN of PD patients and MPTP-treated mice primarily involve neuronal/synaptic function and (neuronal) cell death, with overlapping molecular signaling cascades regulating dopamine synthesis and recycling, endocytosis and exocytosis of (dopamine-containing) synaptic vesicles, and cytoskeleton-dependent synaptic remodeling (Klemann et al. 2016).

3 Aims

This diploma thesis is a part of a larger project, which was done in collaboration with Všeobecná fakultní Hospital in Prague, and Laboratory of Gnotobiology at the Institute of Microbiology in Nový Hrádek.

Specific aims related directly to this thesis were to study the influence of gluten-free diet on composition of immune system and thus to:

1. Establishment of a mouse model for Parkinson's disease (PD) aimed at discerning immune responses between mouse cohorts subjected to a gluten-free diet and those adhering to a standard diet.

a) Implementation of an acute model to evaluate neuroinflammation by quantifying dopamine reduction, ascertaining the efficacy of pharmaceutical intervention, and evaluating the animals' resilience.

b) Induction of a chronic model to instigate prolonged neuroinflammation for the comparative analysis of the impact of the two dietary regimens.

2. Evaluation of immunological characteristics within systemic lymphoid organs of C57Bl6 mice subjected to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration, comparing those on a gluten-free diet with their respective controls on a standard diet.

3. Extraction of peripheral blood mononuclear cells (PBMCs) from the circulatory system of individuals diagnosed with PD for subsequent analysis of immunological parameters.

4 Materials and Method

4.1 Materials

4.1.1 Disposable laboratory items

Test tubes 5 ml: Becton Dickinson, USA

Gloves: Nitrile nonsterile, 9018 – S, Vulkan Medical, a.s., Czech Republic

Petri dishes 60 mm: GAMA GROUP a.s, Czech Republic

Serological pipettes 10 ml: Jet Biofil, China

Pipette tips 1000 µl: Greiner bio-one, Austria

Pipette tips 200 µl: Greiner bio-one, Austria

Pipette tips 20 µl: Greiner bio-one, Austria

4.1.2 Solutions, buffers, antibodies

Viability Dye: Fixable Viability Dye, eFluor 780, eBiosciences, USA

Compensation beads: UltraComp eBeads™, Invitrogen, USA

Trypan Blue solution 0,4 %: Sigma-Aldrich, USA

Red Blood Cell Lysing Buffer Hybri-Max™: Sigma Life Science, USA

Fixation and Permeabilization Solution: BD Cytofix/Cytoperm™ Plus, BD Biosciences, USA

Complete medium: RPMI-1640 with L-glutamine (Lonza, USA), fetal bovine serum (FBS)(10%, Gibco-Life Technologies, USA)

FACS solution: PBS with added 0,1% sodium azide, 0,02% EDTA, 2% FBS FACS + monensin solution: FACS solution + protein transport inhibitor (0,66 µl/ml, BD GolgiStop, BD Biosciences, USA)

Perm/Wash solution: distilled water + BD Cytofix/Cytoperm™ Plus, Perm/Wash™ Buffer (10%, BD Biosciences, USA)

MPTP hydrochloride (Cat. No.: HY-15608Purity: 99.52%**)** sourced from MedChemExpress**,** Sweden

Antibodies: see

Table *2*

Table 2 Antibodies for mouse samples

	Surface staining markers							
Antigen	CD3	CD3	CD4	CD4	γδ	CD8	CD8	LAG3
Fluorochrome	FITC	FITC	PerCP Cy5.5	PE	PE-Cyanine 7	eFluor 450	Alexa Fluor 700	APC eFluor 780
Manufacturer	eBioscience	eBioscience	Invitrogen	Invitrogen	eBioscience	Invitrogen	Invitrogen	Invitrogen
Clone	0KT3	0KT3	RM4-5	RM4-5	eBioGL3	53-6-7	53-6-7	C9B7W
Antigen	CD44	CD45RB	CD62L	CD49b	NKG2D	CD27	CD11	NKp46
Fluorochrome	APC	PE	APC eFluor 780	FITC	PE	PerCP Cy5.5	eFluor 780	APC
Manufacturer	eBioscience	eBioscience	eBioscience	Invitrogen	Invitrogen	Invitrogen	Invitrogen	Invitrogen
Clone	IM7	C363.16A	MEL-14	DX5	CX5	LG.7F9	M1/70	29A1.4
			Intracelular staining markers					
			IL-17	IL-10	FoxP3	IFN-γ		
			PE	PE	PE-Cyanine 7	PE		
			eBioscience	eBioscience	eBioscience	Invitrogen		
			eBio17b7	JES3-9D7	FJK-16S	XMG1.2		

4.1.3 Laboratory equipment

Light microscope: MoticÒ BA310 Professional Light Microscope, Motic, USA

Bürker chamber: Assistent, Germany

Surgical instruments: scalpel, tweezers, scissors

Pipettes (0,5–10, 5-50, 20-200, 200-1000 µl): Finnpipette, Labsystems, Hungary

Manual repetitive pipette: HandyStep, BRAND, Germany

Combitip: Eppendorf, Germany

Pipette Controller: FastPette V-2, Labnet International, USA

Flowbox: Biocyt 150, Esi Flufrance, France

Centrifuge: Rotanta 460R, Hettich, Germany

Centrifuge: IEC CL31R Multispeed centrifuge, Thermo Electron, USA

Microcentrifuge: Micro-Centrifuge II, LabTech, Korea

Vortex: MS2 Minishaker, IKA Works, INC., USA

Flow cytometer: BD LSR II, BD Biosciences, USA

Open Field white box: 60cm x 60cm x 60cm

Camera: Sencor 3CAM 4K52WR

4.2 Methods

4.2.1 Human samples

This thesis was a part of a larger project, which was done in collaboration with the neurological clinic at Všeobecná fakultní Hospital in Prague, who recruited patients with diagnosis of PD into this study and divided them in a control group, which adhered to a standard diet and the intervention group, which was submitted to a gluten-free diet, the first part of the study lasts for 12 months.

After 12 months there was a crossover within the groups, meaning, intervention group became the control group having gluten back to their diet, and the previous control group was submitted to the gluten-free diet. The patients were monitored every 3 months, samples of blood, stool and urine were collected every visit, and cerebral spinal fluid (CSF) was collected at the first and last visit.

The blood samples were processed in our laboratory in order to obtain serum and isolate PBMCs. The serum and urine samples were frozen and stored at -80°C, while stool samples were kept frozen at -20°C and finally the PBMCs were stored at -150°C.

The experimental design of this part of the study is demonstrated in Figure 8.

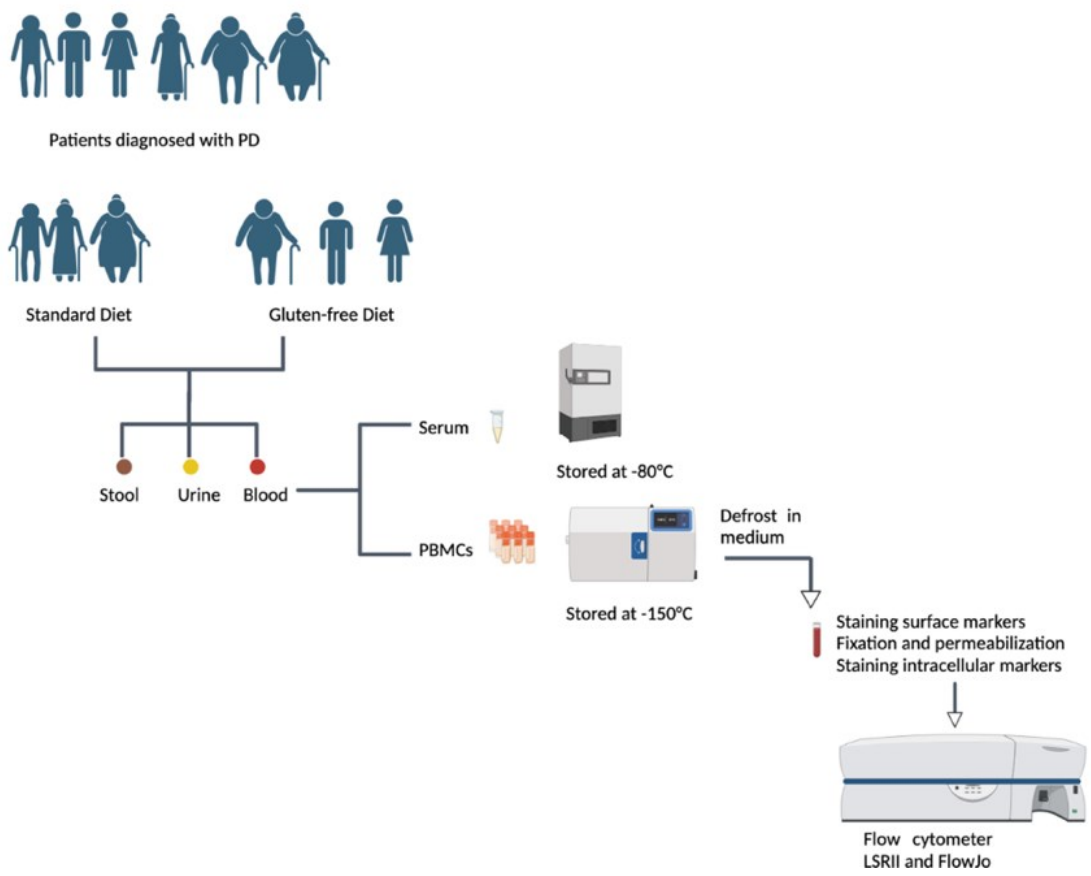

Figure 8 The experimental design of the human arm of this project (Created on BioRender)

4.2.1.1 Peripheral blood mononuclear cells isolation

Ficoll density gradient centrifugation was used to isolate peripheral blood mononuclear cells (PBMCs). In a sterile environment, whole blood samples were collected from human subjects in HEPA tubes and diluted with phosphate-buffered saline (PBS) to complete 35mL in a Falcon tube of 50 mL. Then proceeding with layering of diluted blood over Ficoll-Paque solution and being place in the centrifuge for the separation of blood components based on density via centrifugation at 1000g for 20 minutes, excluding the brake. The resulting whitish layer, known as PBMCs ring, was carefully harvested, and washed twice with PBS before centrifugation at 280g for 10 minutes. Trypan blue was used to assess viability and cell counts, respectively.

4.2.1.2 Defrosting of human PBMCs samples

PBMCs samples, which had been frozen at -150 C°, were defrosted in water bath at 37 °C. Each sample was then slowly, drop by drop, transferred by pipette to 10 ml of complete medium, which was also warmed to 37 °C. Samples were vortexed and centrifuged (1200 RPM, 20 °C, 10 minutes). Then the supernatant was poured off and the pellet resuspended in 2 ml of complete medium. Absolute number of viable cells per sample was counted by usage of 20 µl of the sample, trypan blue stain, Bürker chamber and light microscope. After that all samples were further processed for flow cytometry staining, which is described below.

4.2.1.3 Preparation of samples for flow cytometry

After resuspending the pellet and adding 1 ml of lysing solution, samples were incubated for 4 minutes to lyse erythrocytes. The lysis was halted by adding 30 ml of complete medium, and samples were once again centrifuged (1200 RPM, 20 °C, 5 minutes), with the supernatant being poured out. 4 ml of complete medium was added after the pellet was resuspended. The total number of live cells in each sample was determined using a light microscope, 20 µl of the sample, trypan blue dye, and Bürker chamber. All samples were then further prepared for flow cytometry staining, which is covered in more detail below.

According to the required number of cells per tube, the samples were divided within test tubes. Samples were centrifuged (1300 RPM, 4 °C, 4 minutes) with 0,5 ml of the FACS solution added, and the supernatant was emptied out. In accordance with the employed panels, the pellet was resuspended, samples were stained with Fixable Viability Dye, and fluorochrome conjugated antibodies were added to target surface markers. The samples were incubated for 25 minutes on ice in the dark with a 100µl final volume of FACS.

Fluorochromes were removed from samples after the incubation period by adding 1 ml of FACS, vortexing, centrifuging (1300 RPM, 4 °C, 4 minutes), and then draining the supernatant out.

The pellet was resuspended, fixed, and permeabilized with 250 µl of fixation and permeabilization solution (BD Cytofix/CytopermTM), vortexed for at least five seconds, and then incubated for 20 minutes on ice in the dark. By adding 1 ml of Perm/Wash solution (made from BD Perm/WashTM Buffer 10x solution), vortexing, and centrifuging (1300 RPM, 4 °C, 4 minutes), the solution was washed out.

The supernatant was then poured out. Following the addition of antibodies against intracellular markers to the pellet, samples were filled with 100 µl of Perm/Wash solution and incubated on ice in the dark for 25 minutes.

Following the incubation time, fluorochromes were lastly washed off by adding 1 ml of Perm/Wash solution, vortexing, centrifuging (1300 RPM, 4 °C, 4 minutes), and then draining out the supernatant. Finally, FACS solution was added to each sample until a final volume of 120 µl was reached, at which point the samples were prepared for measurement.

The BD LSR II flow cytometer will be used to measure each sample, and the resulting data will be examined using the FlowJo software.

4.2.2 Animal model samples

The mice were littermate and fed Gluten-free or Standard diet (Table 3) once they were separated from their mother, they were kept in 12h daylight/dark cycle. There were 4 to 5 animals placed per cage. If any signs of behavior aggressivity among the mice was observed, they were separated.

Table 3 Composition of Standard and Gluten free diet

Ingredients (g/100 g)	**Standard Altromin 1434**	**Gluten-free modified Altromin 1434**
Total protein	22.7	22.9
Meat protein	8.4	15.3
Cereal protein	3.5	–
Corn starch	3.2	–
Soybean protein	6.5	6.5
Milk protein	1.0	1.0
Crude Fat	8.2	8.2
Fibre	3.1	3.0
Minerals	5.5	5.4
Lysin	1.3	1.3
Methionin	0.6	0.6
Threonin	0.8	0.8
Tryptophan	0.2	0.2
Glutamic acid	2.8	1.5
Water	9.5	4.8
Energy (kcal/kg)	3308	3644

MPTP Acute model

Male C57BL/6 mice (7–8 weeks old) were utilized for the MPTP intoxication study using MPTP.HCl (20 mg MPTP/kg bodyweight of free base), dissolved in saline solution, administered via intraperitoneal (i.p.) injections.The mice received four injections, spaced two hours apart, completing the treatment within a single day.

On Day 7 following the MPTP treatment, the mice were subjected to further analyses. This model was applied mainly to establish the neuro-dopamine loss caused by the MPTP allowing to continue to use this drug for the long-term experiment for the MPTP Chronic Model which will be detailed next.

MPTP Chronic Model

Male C57BL/6 mice (7–8 weeks old) were utilized for the MPTP model for PD study. The MPTP.HCl (20 mg MPTP/kg body weight), dissolved in saline solution, was administered via intraperitoneal (i.p.) injections. The mice received three injections per week in a period of 8 weeks for in order to induce the chronic neuroinflammation.

On Day 7 following the MPTP treatment, the mice were subjected to further analyses. The timeline and overview of the MPTP Chronic Model can be observed in the Figure 9, which includes the behavior open field test at the beginning and the end of the MPTP injections.

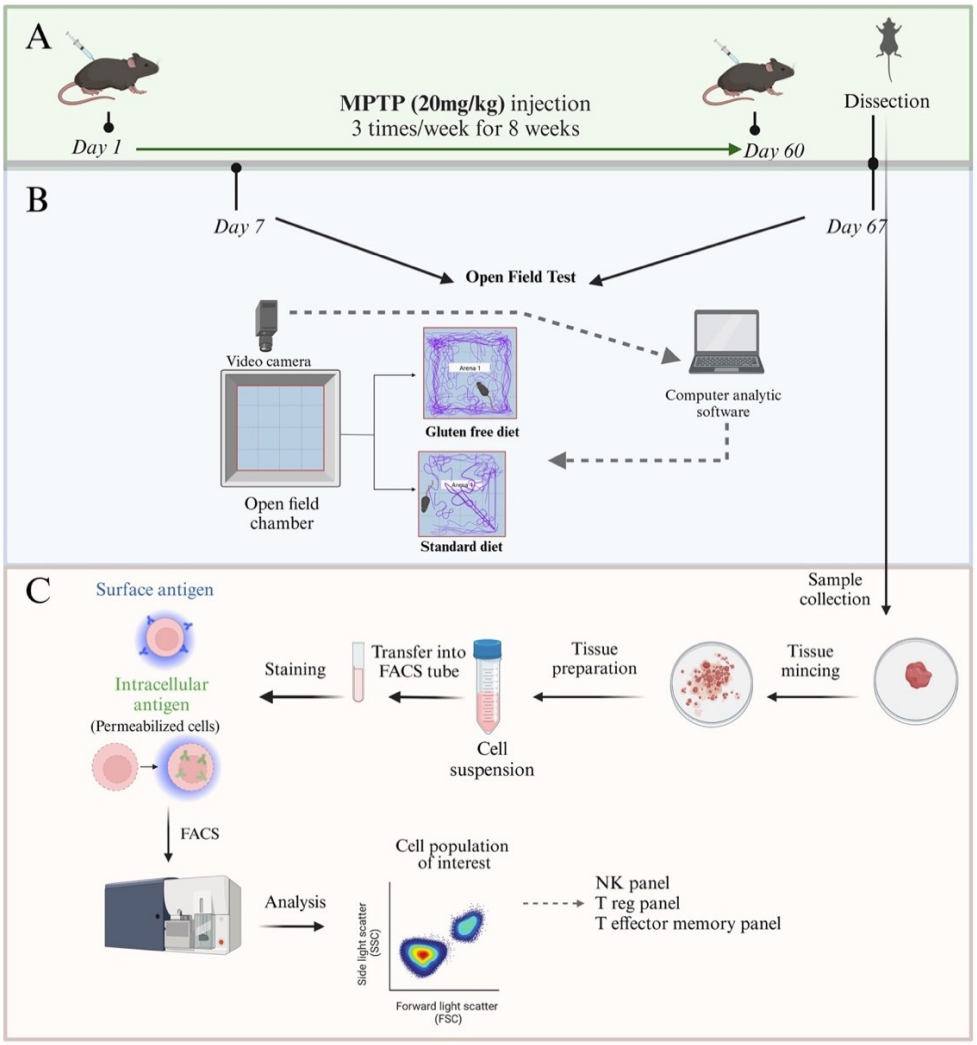

Figure 9 Experimental design of MPTP Mouse model in this project (Created on BioRinder)

Dissection of MPTP mice

At day 7th after the last injection mice were put down and dissected for the necessary organs. The spleen, as well as the inguinal, mesentery, and pancreatic lymph nodes, were placed into the petri dish which contained 3 mL of complete medium (500mL RPMI + 50 mL FBS).

The organs were next mechanically but delicately triturated by rubbing them on a rough area of a microscope slide with a tweezer. A pipette was used to wash the microscope slide with 400 µl of FACS medium, and a filter was used to filter the material into a test tube. Erythrocytes were eliminated after additional processing of spleen sample. Prior to pouring off the supernatant, the tubes were centrifuged (1200 RPM, 20 °C, 5 minutes). The procedure performed following the same protocol for both models.

Collection mouse's brain

C57Bl6 mice were anesthetized using an isoflurane in order to ensure they were unconscious and insensitive to pain and the head is cut. Small scissors or a scalpel were used to make a midline incision on the scalp, revealing the skull. Using small forceps and bone-cutting scissors, the skull is carefully opened to reveal the brain inside the cranial cavity. During this process, care was taken to avoid harming the brain. Using fine spatula, the brain was gently removed from the skull cavity, taking care to save any nearby tissues, it was placed in an aluminum foil and stored in dry ice (Figure 10). The procedure performed following the same protocol for both models.

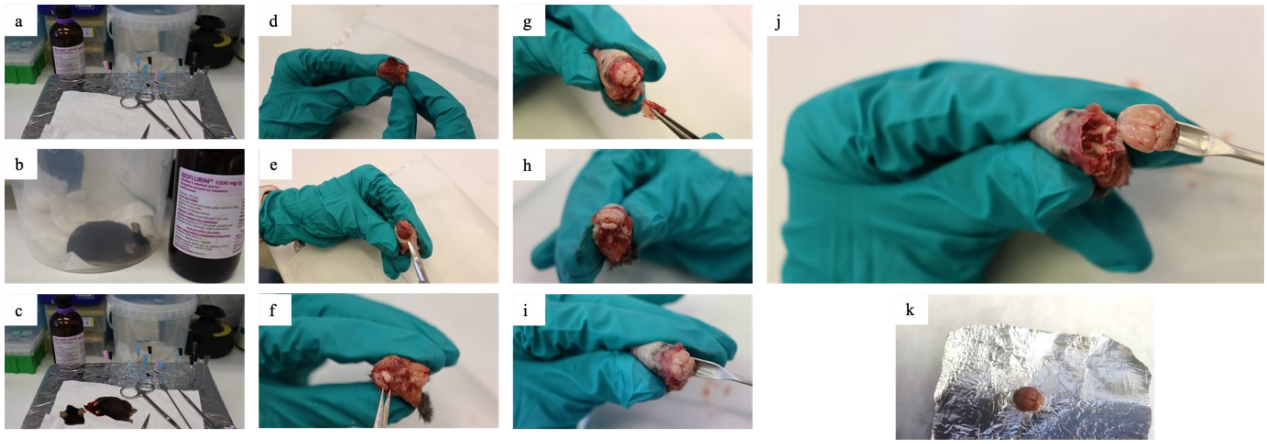

Figure 10 Brain extraction of C57Bl6 mouse for further analysis: a) Instruments and the anesthesia isoflurane; b) The mouse was placed in a closed recipient; c) Removal of the mouse's head; d) meticulous incisions are made, revealing the intricate structural nuances of the mouse brain; e-g) close-up perspective captures the delicate extraction process while removing ensuring minimal disturbance to surrounding tissues; h-j) Using a spatula gentle extraction the mouse brain from its cranial cavity; k) Carefully, the brain is placed in aluminum foil to be rapidly frozen.

Transferring the frozen brain block to a cryostat, a specialized tool for slicing frozen tissues, follows. The brain is kept at a very low temperature using the cryostat, allowing for precise and thin sectioning of 60 μm thick. Serial brain sections are cut using the cryostat's microtome, and adjustments are made to get the desired region of interest, in this case the substantia nigra. On glass slides, the substantia nigra-containing brain tissue is meticulously assembled. Then, these segments were kept for later processing and analysis.

Immunofluorescence

In the experimental procedure, the brain was meticulously extracted from the skull, as demonstrated in Figure 10 and promptly placed on dry ice, followed by storage at -80°C. Subsequently, 12-µm thick slices were precision-cut using a Leica 1800 cryocut at -17°C, and these slices were affixed to super frost glass, allowing for a one-hour drying period. The glass slides were then fixed in freshly prepared 4% paraformaldehyde (PFA) for 30 minutes at room temperature. Afterward, a sequence of steps ensued: washing the slides three times for 5 minutes each in PBS with a pH of 7.2, permeabilizing the slides in 0.2% PBS-Triton 100 without agitation, followed by four washes of 5 minutes each in PBS with agitation.

Subsequently, tissue pieces were demarcated using a Barrier pen, and the slides underwent blocking with 5% normal rabbit serum in 0.1% PBS-Tween 20 within a humidified chamber for one hour at room temperature. The blocking solution was then removed, and the slides were subjected to an overnight staining procedure using Anti-Tyrosine Hydroxylase Antibody, clone LNC1, Alexa Fluor™ 488 Conjugate (MAB318-AF488 Sigma Aldrich) in 0.1% PBS Tween 20 with 5% normal rabbit serum, at a dilution of 1:50.

Following staining, the slides were washed five times for 5 minutes each in 0.1% PBS-Tween 20 with agitation. The slides were subsequently placed in a humidified chamber, and the tissue pieces were exposed to a 200 µl solution of DAPI in PBS (concentration: 10 µg/ml) for 15 minutes. Afterward, the glass slides underwent a 2x5 minute wash in 0.1% PBS-Tween 20 with agitation.

Finally, cover slips were mounted on the glass slides using Vectashield antifade mounting medium H-1000, and the edges of the glass slides were sealed with colorless nail polish, allowing for drying.

Open Field Test

The open field test was used to evaluate MPTP-treated C57Bl/6 mice's movements and exploratory behavior. Each mouse was placed individually in the center of a square white box, and its behavior was recorded for 5 minutes, then the animal was placed back to the cage.

Prior the start of the experiment the animals were climatized in the environment for several minutes to avoid more stress and interreference in the result

The *AnimalTA* software was used to process the recorded videos, which aided in the extraction of quantitative parameters such as speed, distance travelled and exploration value. This methodology allowed for a detailed analysis of motor function and exploratory behavior in a controlled experimental setting, as well as a comprehensive evaluation of the impact of the GFD on the mice's spontaneous locomotor activity.

The use of the *AnimalTA* software ensured the precision and reliability of the obtained behavioral metrics, which aided in the rigorous characterization of the experimental results.

The parameters setup for analysis were adjusted as follows:

Correct brightness: NO

Correct flickering: NO

Threshold: 50

Erosion: 9

Dilation: 2

Area: 3.75- 396.45 mm^2

Distance threshold: 42 mm

Individuals per arena: 1

5 Results

5.1 Assessment of the effect of MPTP during acute model

The use of an acute model of MPTP is necessary for verifying MPTP's efficacy and is crucial in understanding its neurotoxic effects, because it enables the rapid induction of neurotoxic effects, allowing to observe and analyze the immediate effects of MPTP exposure on the nervous system.

5.1.1 Tyrosine hydroxylase staining

A pilot experiment was conduct while stablishing the MPTP mouse model and the effect of the drug, it was observed a less bright and less intense signal on the MPTP treated brain when compared with the control, which was treated solely with PBS (Figure 11).

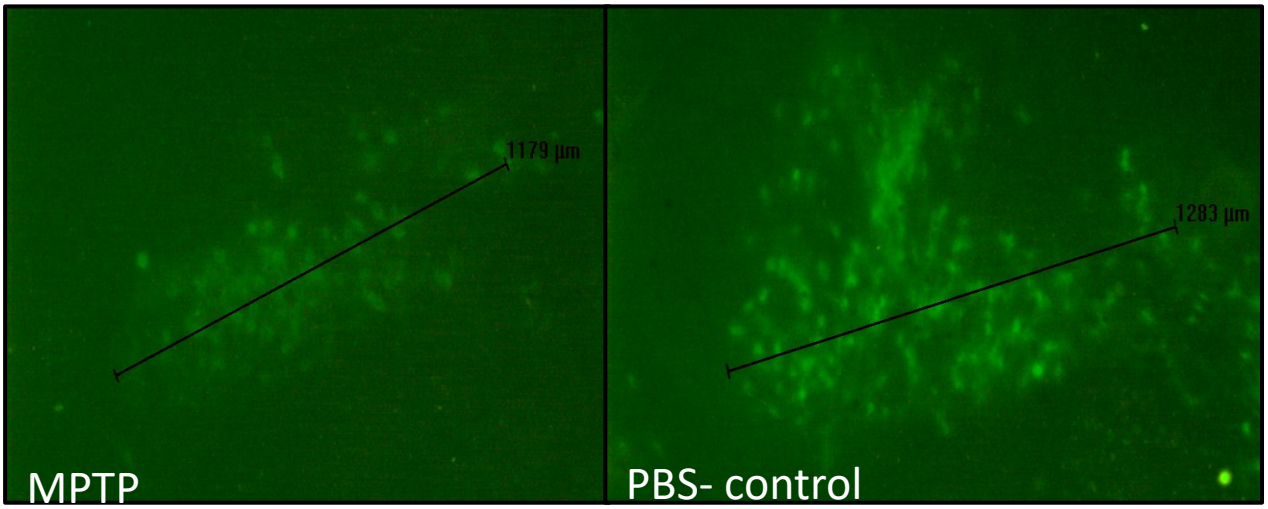

Figure 11 On the left, it is observed the dopamine loss with lower signal after MPTP injection for the acute model. Control (PBS) representing a brighter stronger signal of dopaminergic neurons

5.1.2 Staining with Tyrosine Hydroxylase and DAPI

Tyrosine is converted to dopamine as a precursor of catecholamines by L-Dopa and the enzymes tyrosine hydroxylase (TH) and aromatic l-amino acid decarboxylase. The immunofluorescence results showed that tyrosine increases dopamine availability.

DAPI staining revealed changes in nuclear morphology, which could indicate changes in cellular integrity or density. The immunofluorescence results (Figure 12) revealed a decrease in tyrosine hydroxylase fluorescence in the MPTP model (Figure 12– Panels A, B and C) indicating a decrease in the expression of this key enzyme involved in dopamine synthesis. Changes in DAPI staining also suggested potential changes in cellular structure.

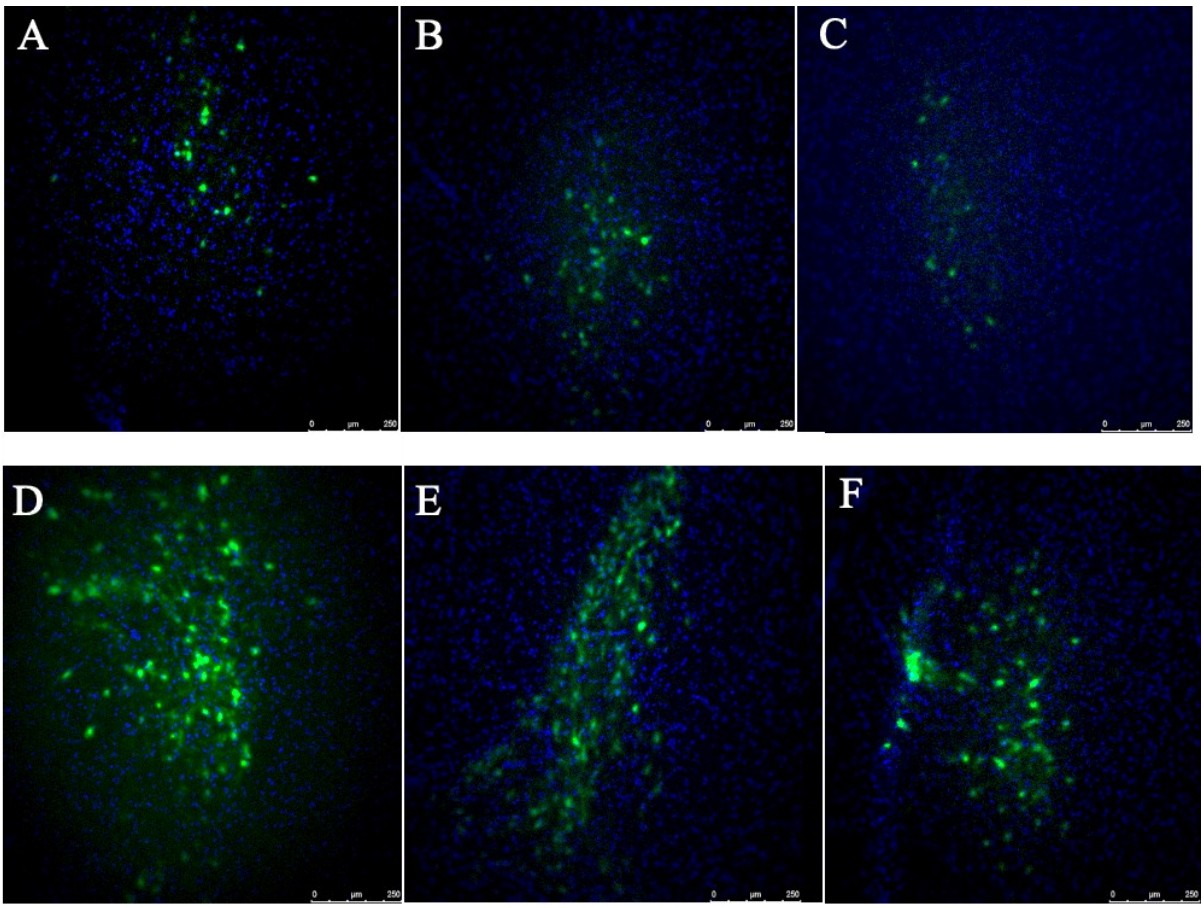

Figure 12 In the panels A, B, and C, three mice's brains were subjected to the acute model of MPTP treatment, whereas in panels D, E, and F, another set of three brains underwent treatment with PBS, serving as the control group.

In contrast, the PBS-treated control group (Figure 12- Panels D, E and F) showed stronger tyrosine hydroxylase fluorescence (green dots) and preserved nuclear architecture (blue dots), indicating that dopaminergic neurons and overall cellular homeostasis were maintained.

5.2 Open Field Test

A comparison of those on a standard diet (STD) versus those on a gluten-free diet (GFD) revealed slightly distinct behavioral outcomes. Mice fed the standard diet demonstrated slightly decreased locomotor activity, based in the overall exploration within the open field arena at the end of the experiment (Figure 13).

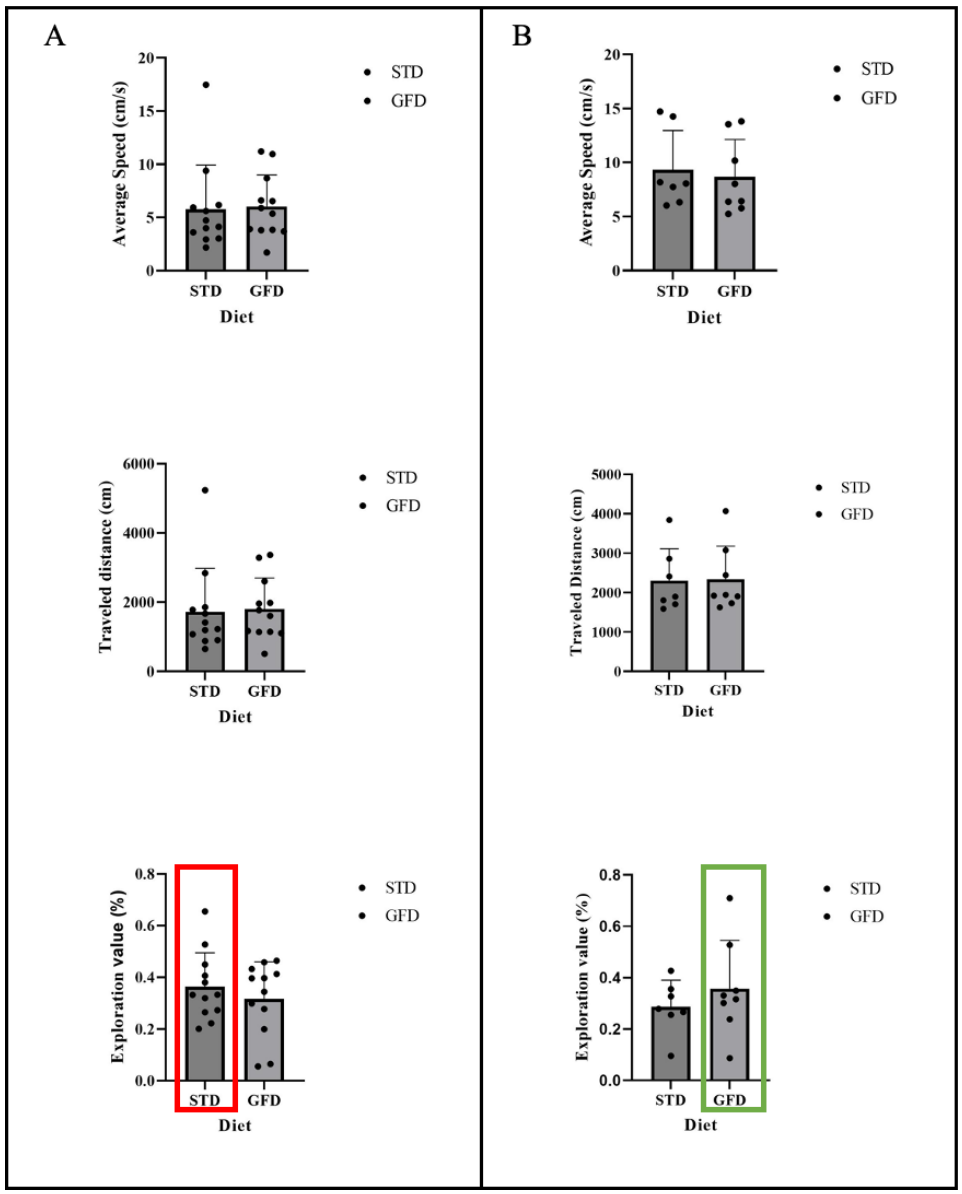

Figure 13 Plots depicting average speed, traveled distance, and exploration value of two groups treated with standard diet and gluten free diet. Panel A corresponds to the first Open Field test, performed 1 week after first MPTP injection; panel B corresponds to the second Open Field test performed one week after the last MPTP injection. The statistical analysis was done with Two-way ANOVA.

The open field test results show a small difference in exploration behavior between the STD and the GFD. A visualization of the increased exploration value can be seen in the Figure 14 mice on the GFD explored more, covering a greater area within the open field arena than mice on the STD.

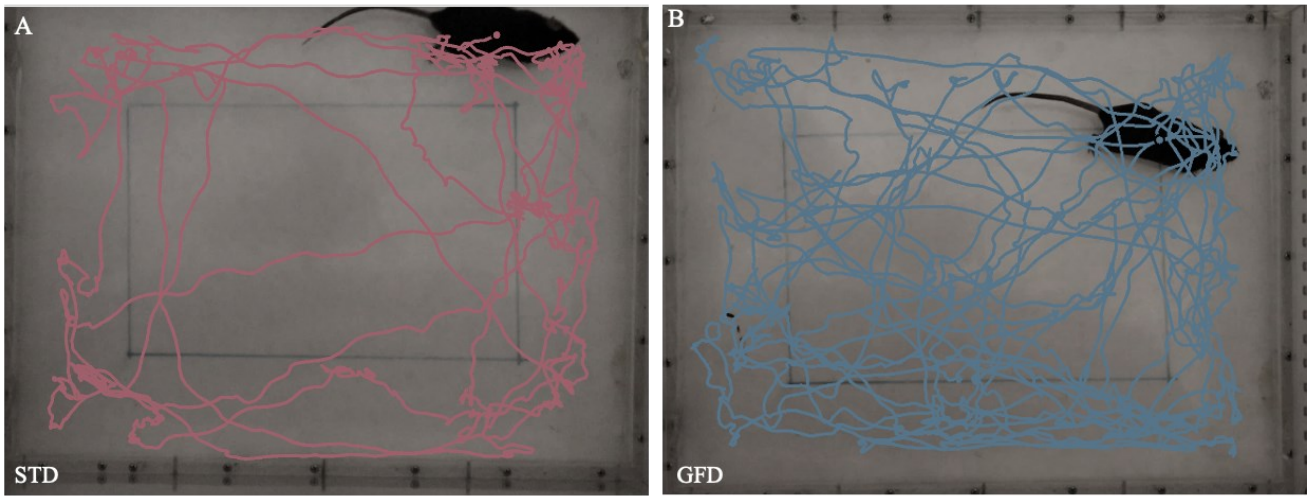

Figure 14 Illustration of the exploration exhibited through the trajectory of individual mice under the influence of two distinct diets at the end of the Chronic Model experiment; (A) Total trajectory of a mouse on STD; (B) Total trajectory of a mouse on GFD. (Image Generated during the analysis using the software AnimalTA)

5.3 Assessment of immunological parameters on MPTP Chronic model mice through FACS

To verify the possible beneficial effect of GFD in the immunological parameters among the MPTP-mice, mice were sacrificed one week after the last injection of MPTP dissected for SPL, ILN, MLN and PLN. Organs were then processed and stained for flow cytometry and measured for differences in T-cells and cytokine production.

5.3.1 Assessing the percentage of T cells and cytokines in induced chronic MPTP mice

The comprehensive assessment of immune parameters in the context of mice subjected to chronic MPTP induction. This investigation focuses on an examination of CD4+IL-10 and CD8+IFNγ+ T cells.

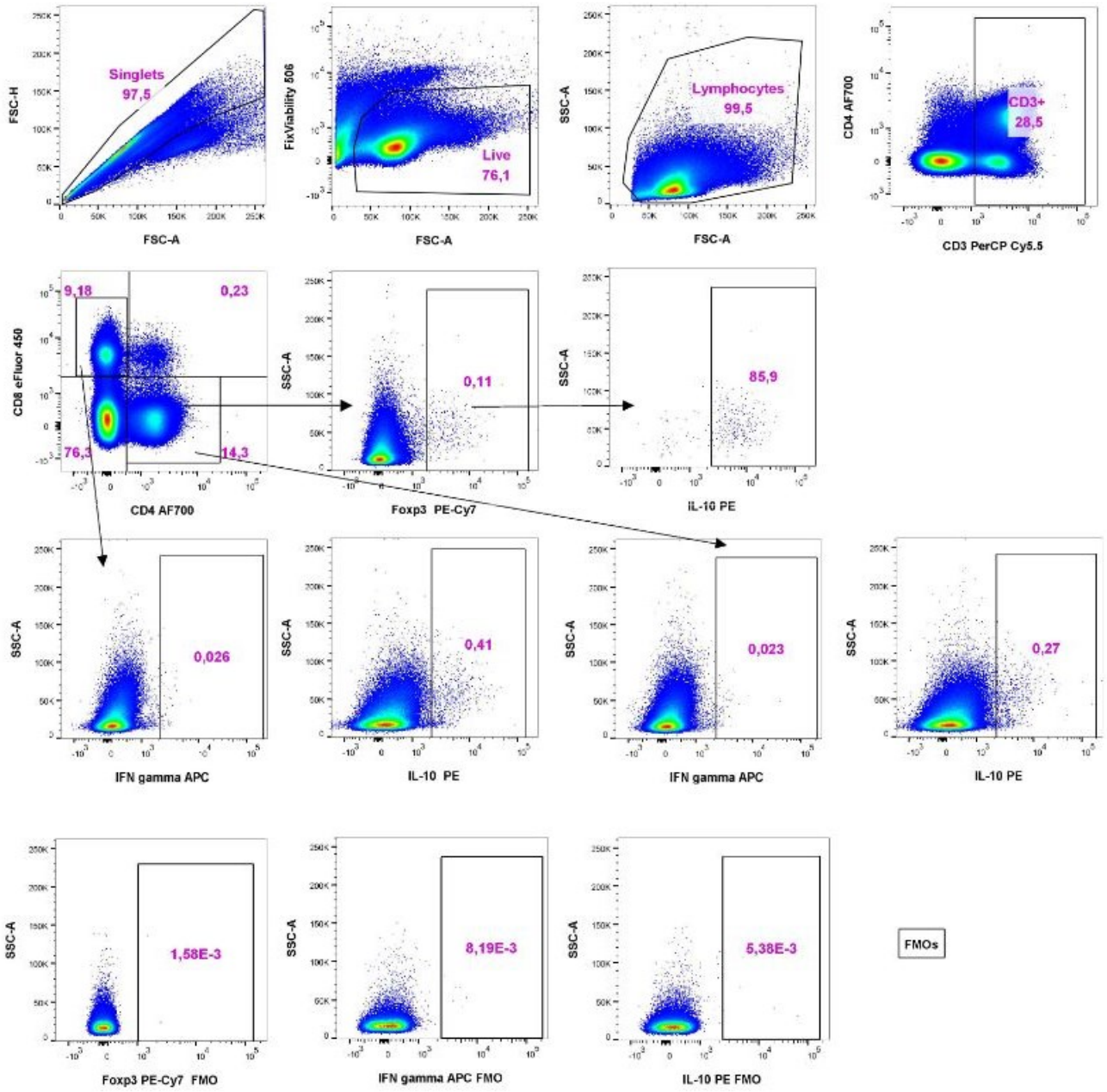

Figure 15 Gating strategies of IL-10+CD4+ and IFN-γ+CD8+ T cells. Gating strategies of the two cytokines were done similarly at the beginning as the first gate was set to exclude doublets, next only viable cells were included in the analysis, then lymphocytes and monocytes were gated, then the gate was set at CD3+ cells. As the last step, cells from the CD4+ and CD8+ gate were plotted according to the IL-10 and IFN-γ staining, respectively. The gating was done in FlowJo software.

In spleen, inguinal lymph nodes, mesenteric lymph nodes and Peyer's Patches it was observed enhanced presence of CD4+FoxP3+ T cells in GFD mice compared to STD Figure 16, however this trend was not confirmed by the. It was not statistically significant as the total number of mice in the experiment was limited by unexpected death due to the MPTP effect.

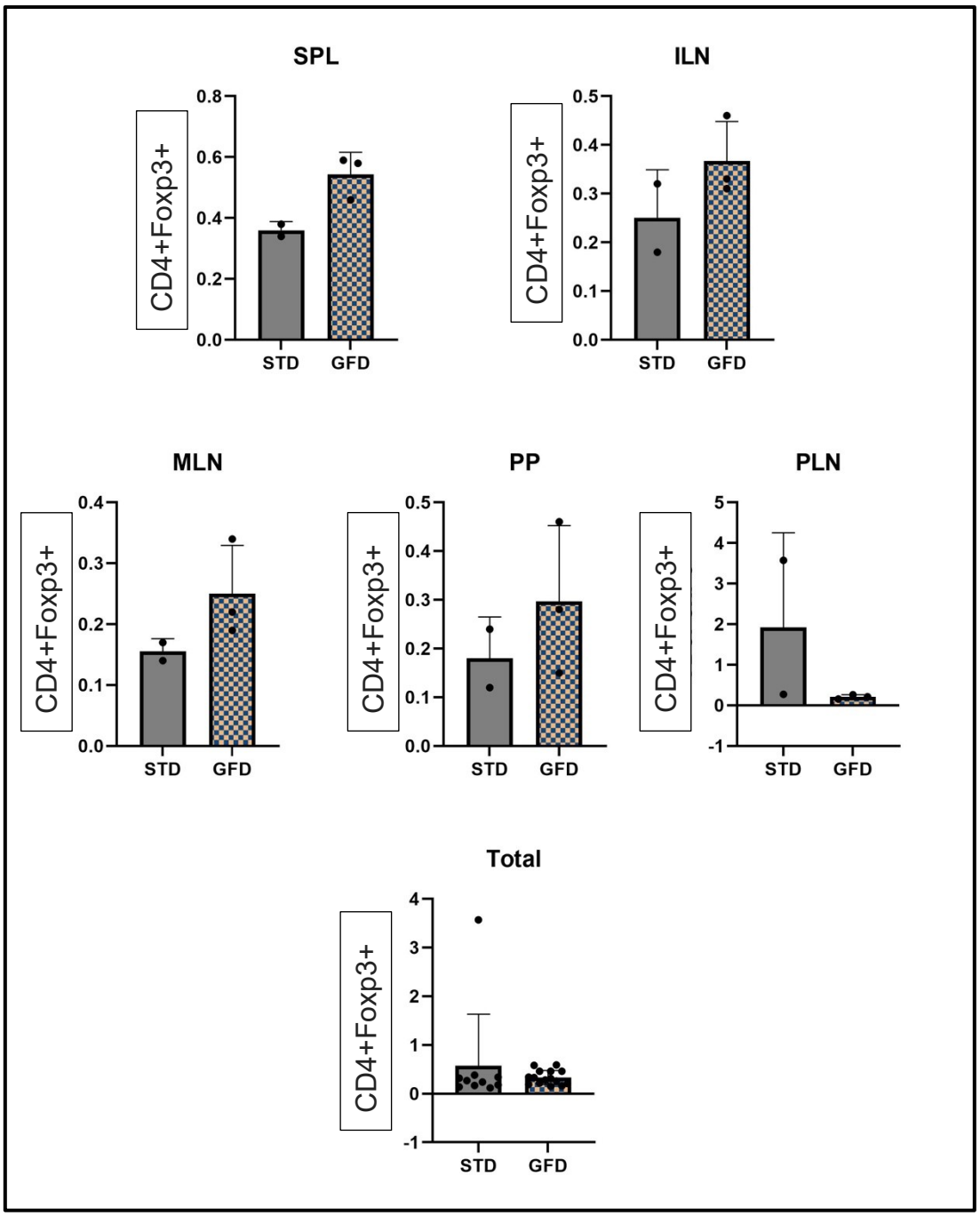

Figure 16 Percentage of CD4+FoxP3+ T cells in STD and GFD groups in SPL, ILN, MLN, PLN and PP, as well the total percentage considering all organs. The statistical analysis was done with Two-way ANOVA and followed by Tukey's Multiple Comparisons Test.

In inguinal and pancreatic lymph nodes there was a tendency to an enhanced IL-10 expression in GFD mice compared to STD, Figure 17, however this trend was not confirmed in the other organs. It was not statistically significant as the total number of mice in the experiment was limited by unexpected death due to the MPTP effect.

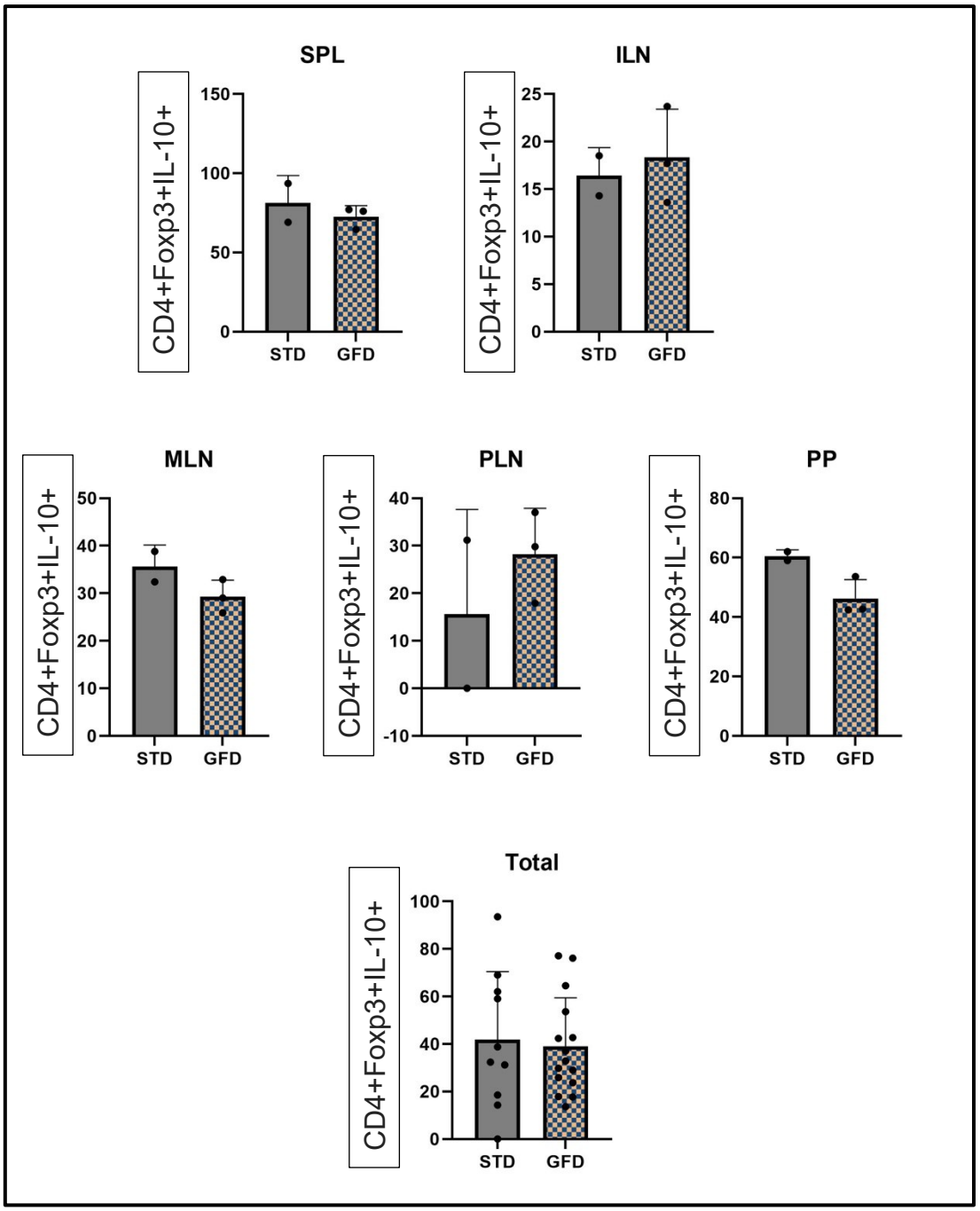

Figure 17 Percentage of CD4+FoxP3+ T cells producing IL-10 in STD and GFD groups in SPL, ILN, MLN, PLN and PP, as well the total percentage considering all organs. The statistical analysis was done with Two-way ANOVA and followed by Tukey's Multiple Comparisons Test.

In the spleen and in mesenteric lymph nodes there was a tendency towards enhanced proportion of IL-10-positive CD4+ T cells (Figure 18) in GFD mice compared to STD, however this trend was not confirmed on Peyer's patches. It was not statistically significant as the total number of mice in the experiment was limited by their death due to MPTP effect.

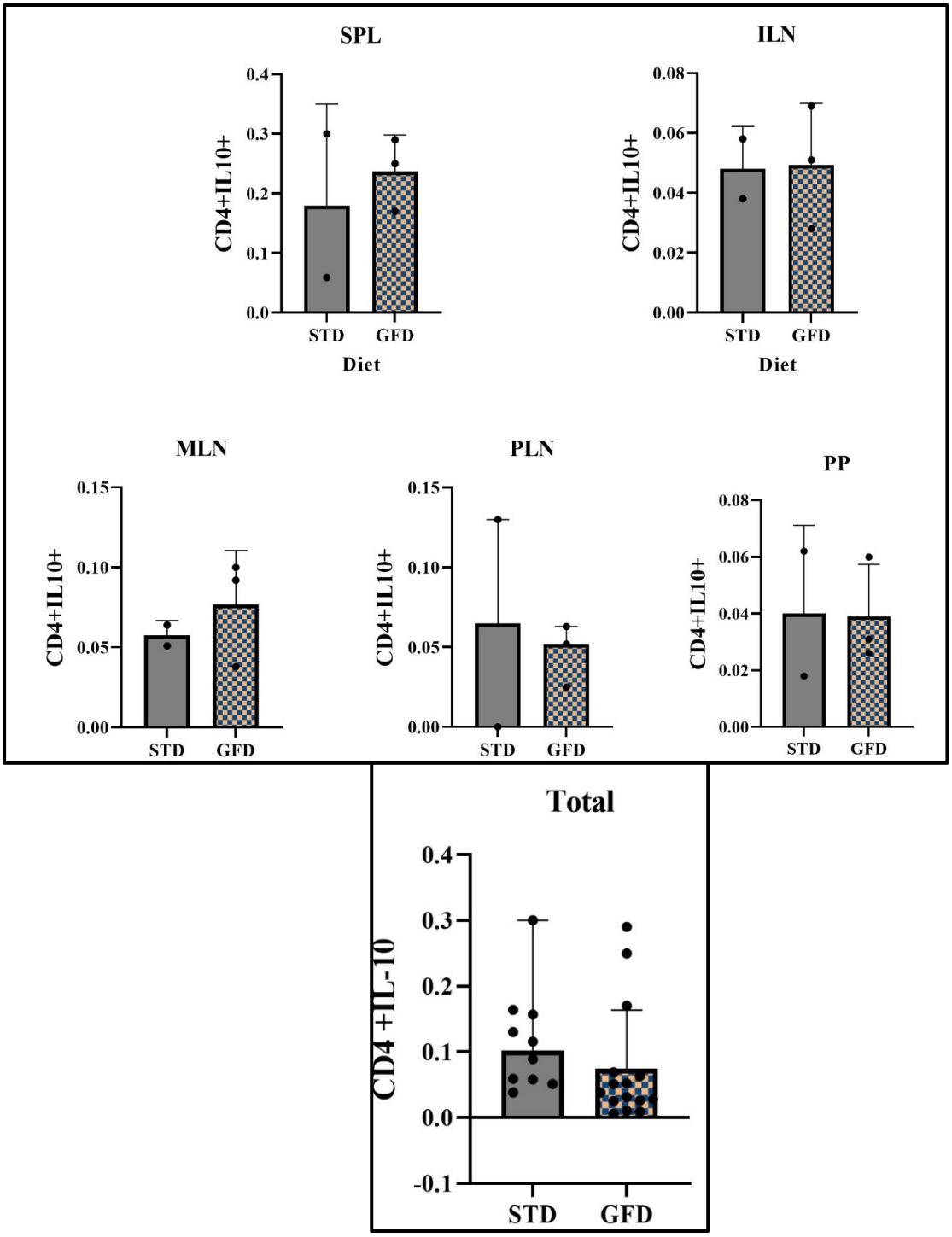

Figure 18 Percentage IL-10+CD4+ T cells in STD and GFD groups in SPL, ILN, MLN, PLN and PP, as well the total percentage considering all organs. The statistical analysis was done with Two-way ANOVA and followed by Tukey's Multiple Comparisons Test.

In inguinal lymph nodes and in mesenteric lymph nodes there was an increased proportion of IFN-γ-positive CD8+ T cells (Figure 19) in GFD compared to STD mice, however this trend was not confirmed in other organs. It was not statistically significant as the total number of mice in the experiment was limited by their death due to the MPTP effect.

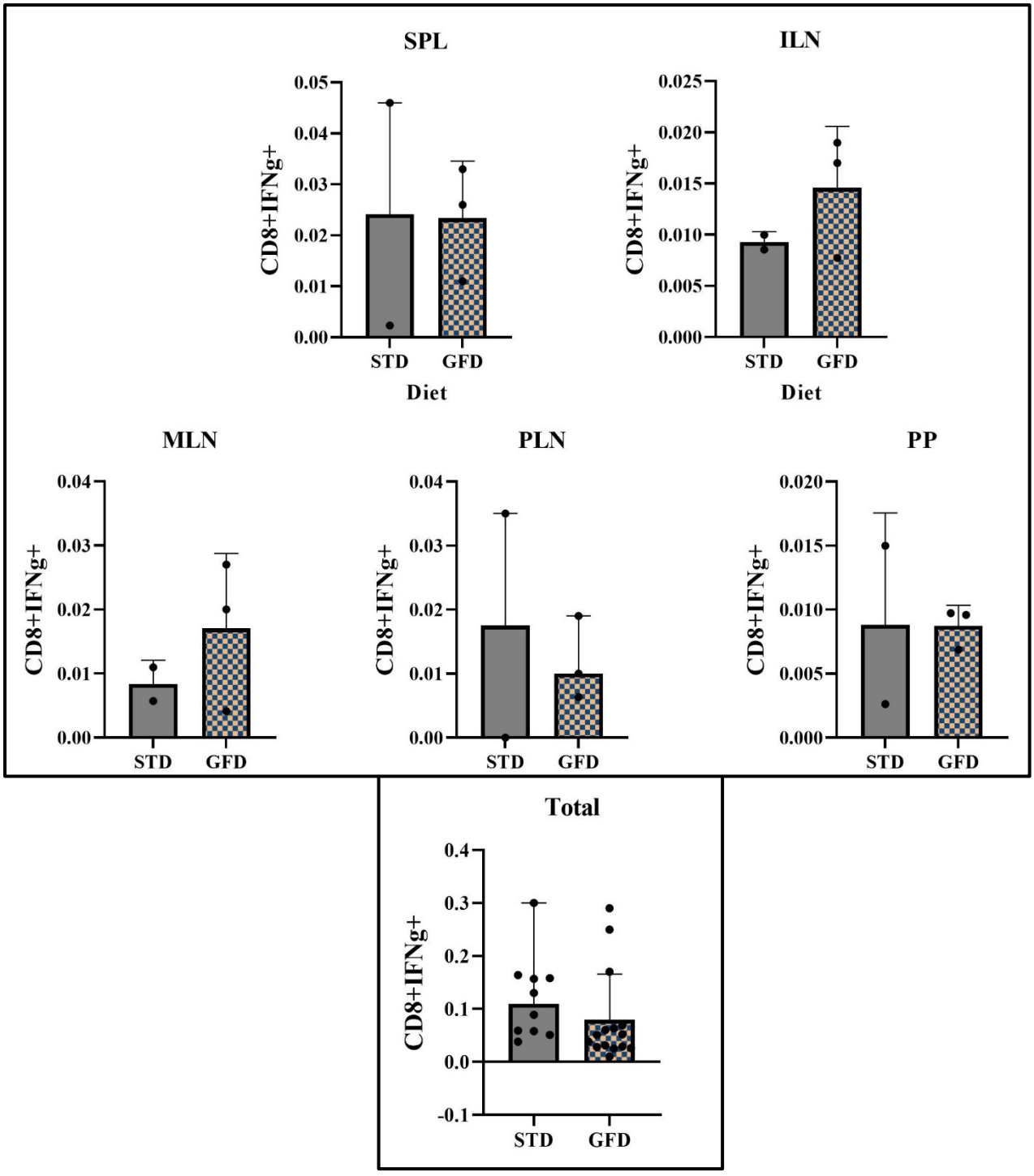

Figure 19 Percentage of IFN-γ+CD8+ T cells in STD and GFD groups in SPL, ILN, MLN, PLN and PP, as well the total percentage considering all organs The statistical analysis was done with Two-way ANOVA and followed by Tukey's Multiple Comparisons Test.

5.3.2 Assessing the percentage of γδ TCR and cytokines in induced chronic MPTP mice

The comprehensive assessment of immune parameters in the context of mice subjected to chronic MPTP induction. This investigation focuses on a thorough examination of γδ TCR and within this experimental setting.

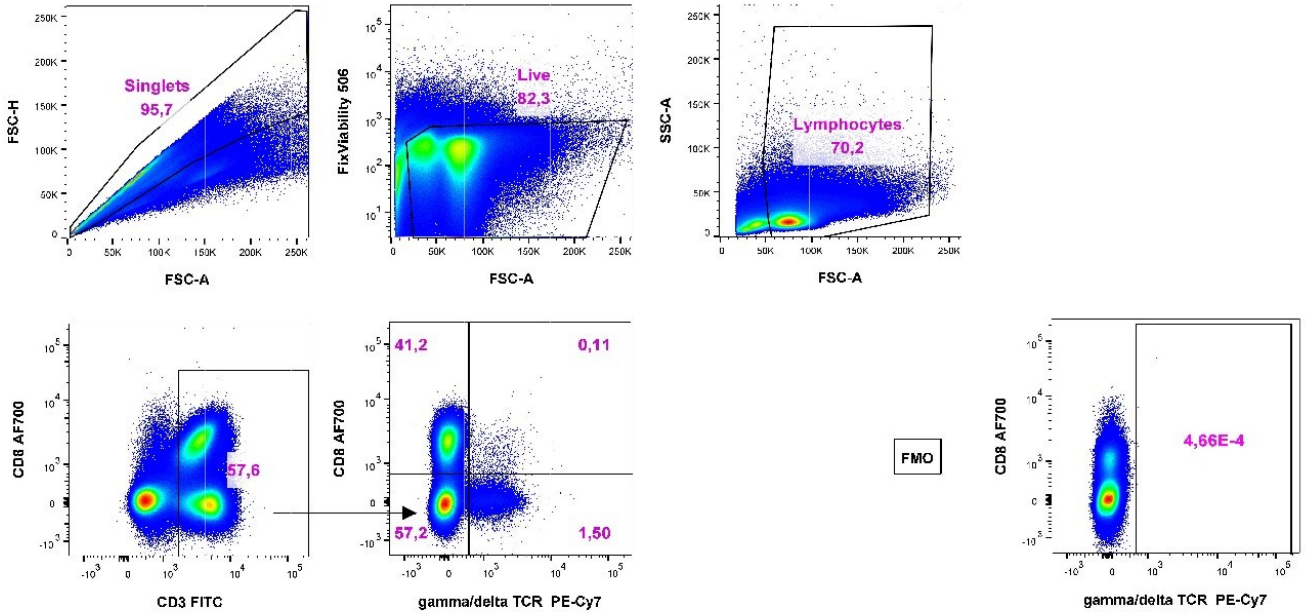

Figure 20 The gating strategy of γδ T cells. Gates were set for single cells, lymphocytes and monocytes, CD3+ and at last gate for the γδ T cells. On the right side there is the FMO control. The gating was done in FlowJo software.

In inguinal lymph nodes and pancreatic lymph nodes there was a tendency to increased proportion of CD8+ γδ T cells in GFD mice compared to STD (Figure 21- Panel A); on the other hand proportion of CD8- γδ T cells was increased in all organs of GFD group (Figure 21- Panel B). It was not statistically significant as the total number of mice in the experiment was limited by unexpected death of mice due to the MPTP effect.

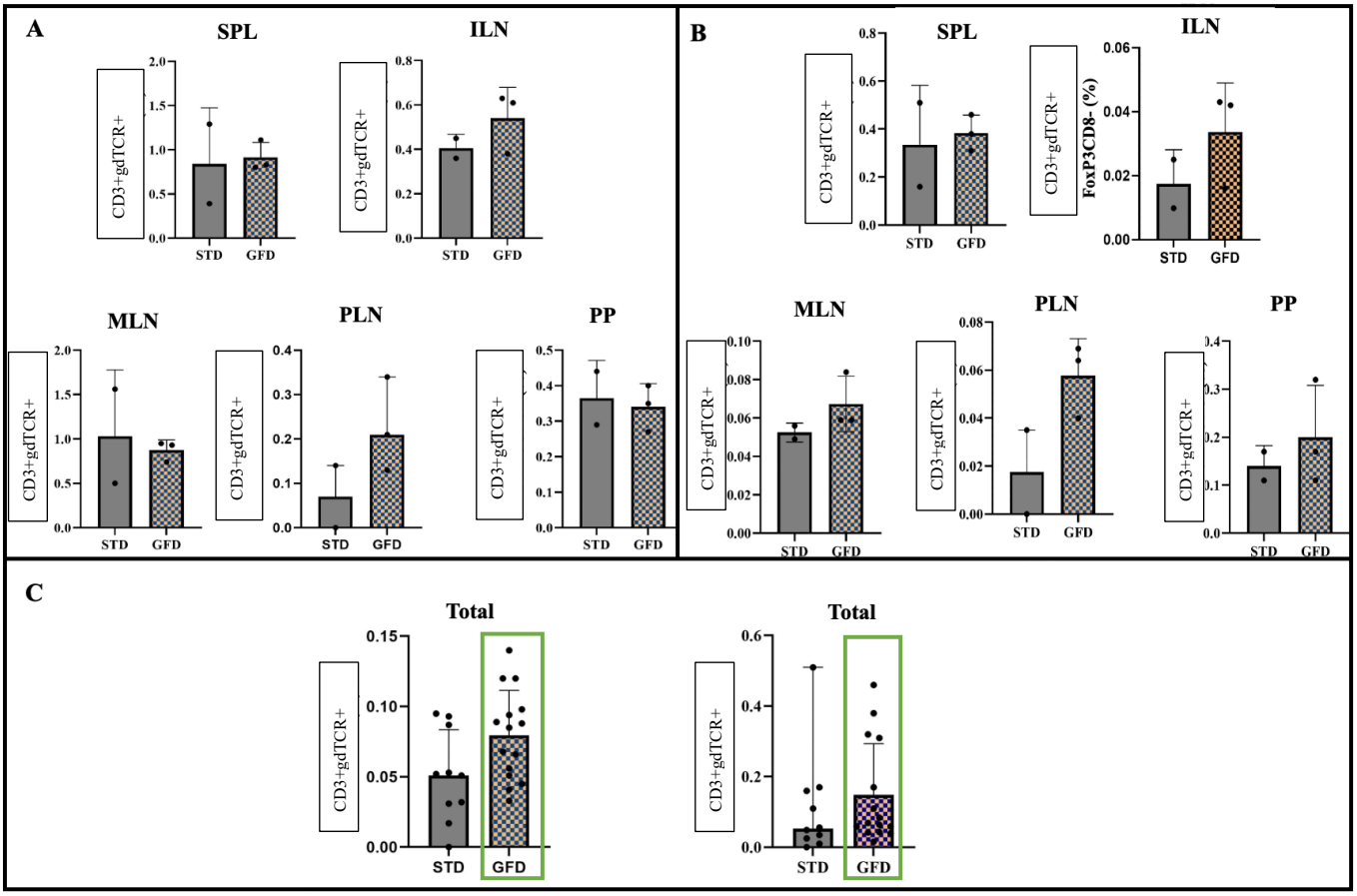

Figure 21 The percentages of γδ T cells. (A) Percentage of CD8+ γδ T-cells in SPL, ILN, MLN, PLN, and PP; (B) Percentage of CD8- γδ T-cells in SPL, ILN, MLN, PLN, and PP. (C) Total percentage of γδ T-cells in all organs, on the left it was are CD8+ and on the right CD8- γδ T cells. The statistical analysis was done with Two-way ANOVA and followed by Tukey's Multiple Comparisons Test.

5.3.3 Gating strategy for assessment of CD45RB

The comprehensive assessment of immune parameters in the context of mice subjected to chronic MPTP induction. This investigation focuses on a thorough examination of CD45RB and within this experimental setting.

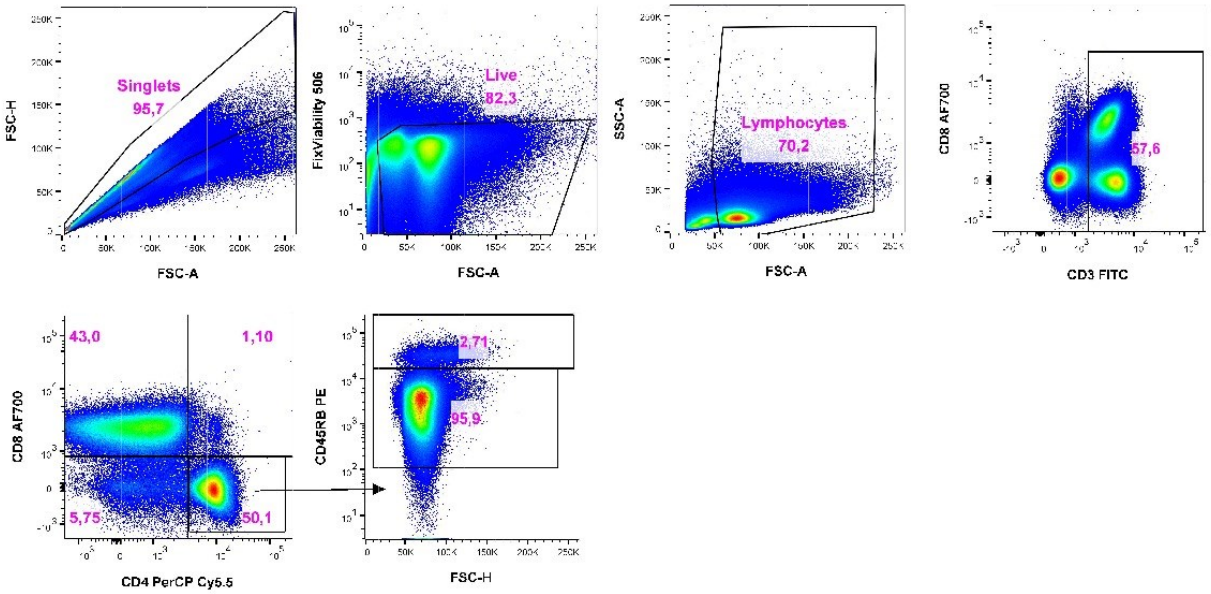

Figure 22 The gating strategy for CD4+CD45RB-low T cells. Gates were set for single cells, next only viable cells were included in the analysis, then lymphocytes and monocytes were gated, then the gate was set at CD3+, then CD3+CD4+CD8- cells were analyzed for their CD45RB expression (CD45RB-high, CD45RB-low). The gating was done in FlowJo software.

In the spleen, inguinal lymph nodes, mesenteric lymph nodes, pancreatic lymph nodes and Peyer's patches there was a similar proportion of CD3+CD4+CD45RB$_{low}$ T cells in GFD compared to STD fed mice (Figure 23). No statistically significant differences were found, the total number of mice in the experiment was limited by unexpected death due to the MPTP effect.

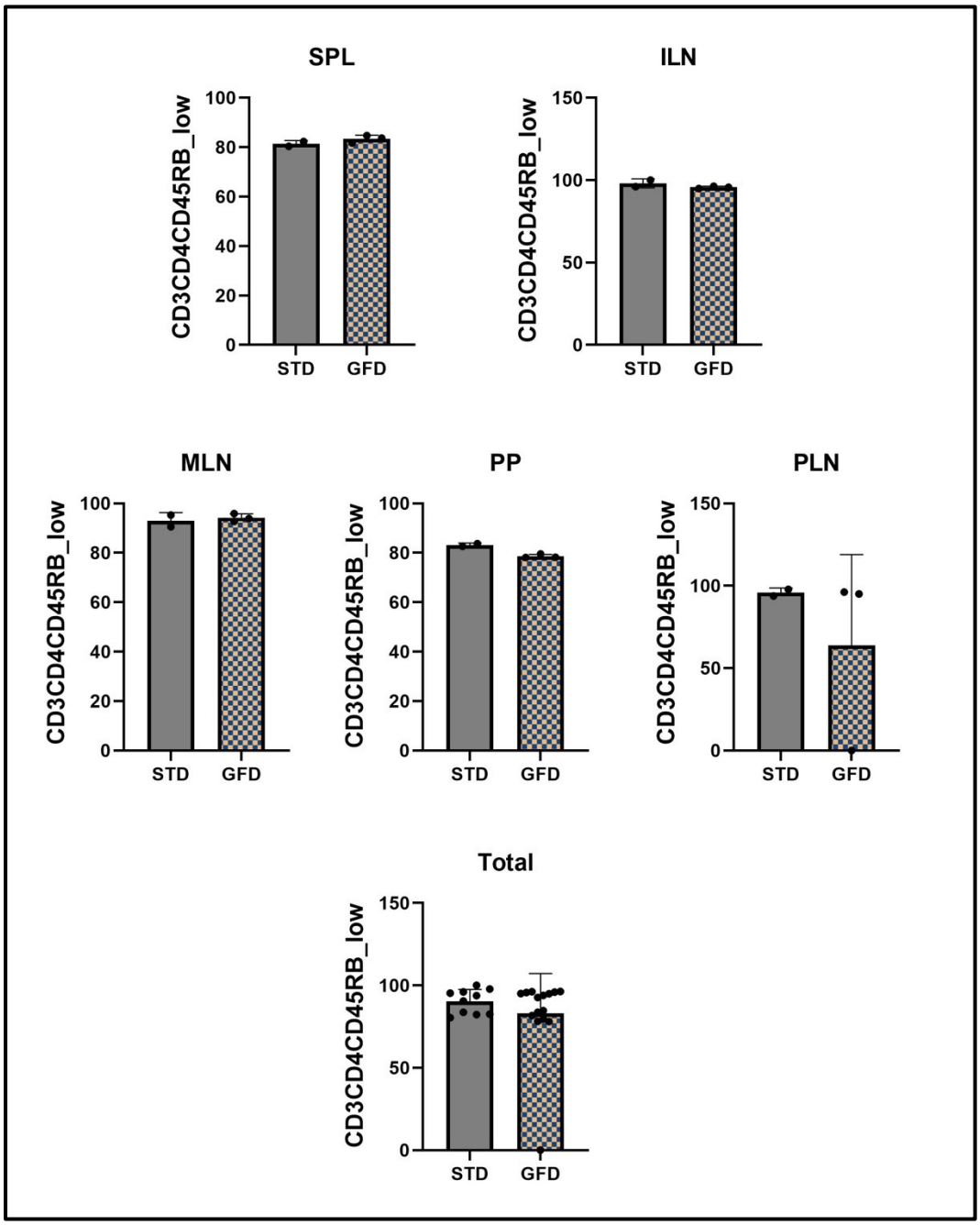

Figure 23 The percentage of CD3+CD4CD45RB$_{low}$ in STD and GFD groups in SPL, ILN, MLN, PLN and PP. As well the total percentage considering all organs. The statistical analysis was done with Two-way ANOVA and followed by Tukey's Multiple Comparisons Test.

5.3.4 Gating strategy for assessment of NK-cell subsets

The comprehensive assessment of immune parameters in the context of mice subjected to chronic MPTP induction. This experiment focused on a thorough examination of levels of NKG2D expression in NK cells, based on their maturation (CD27) and within this experimental setting – GFD vs. STD diets (Figure 24).

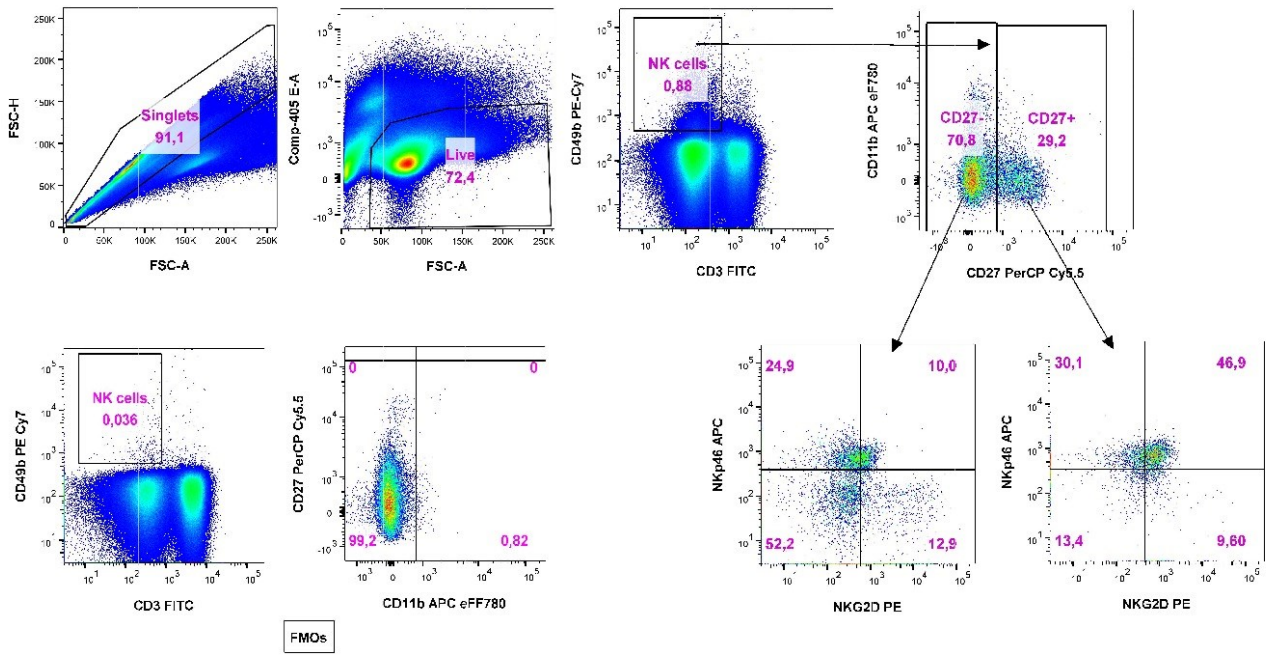

Figure 24 The gating strategy for NKG2D low and high expression on subsets of immature and mature NK cells. Gates were set for single cells, next only viable cells were included in the analysis, then lymphocytes and monocytes were gated, then the gate was set for CD3-CD49b+ NK cells, that were further divided to CD27- (immature) and CD27+ (mature) mouse NK cells, that were further assessed for their levels of NKG2D expression. The gating was done in FlowJo software.

In the spleen and inguinal lymph nodes a tendency to slightly increased proportion of matured NK CD27+ cells was noted in GFD compared to STD mice (Figure 25), however this trend was not confirmed in the other organs. The changes were not statistically significant. The total number of mice in the experiment was limited by unexpected death due to the MPTP effect.

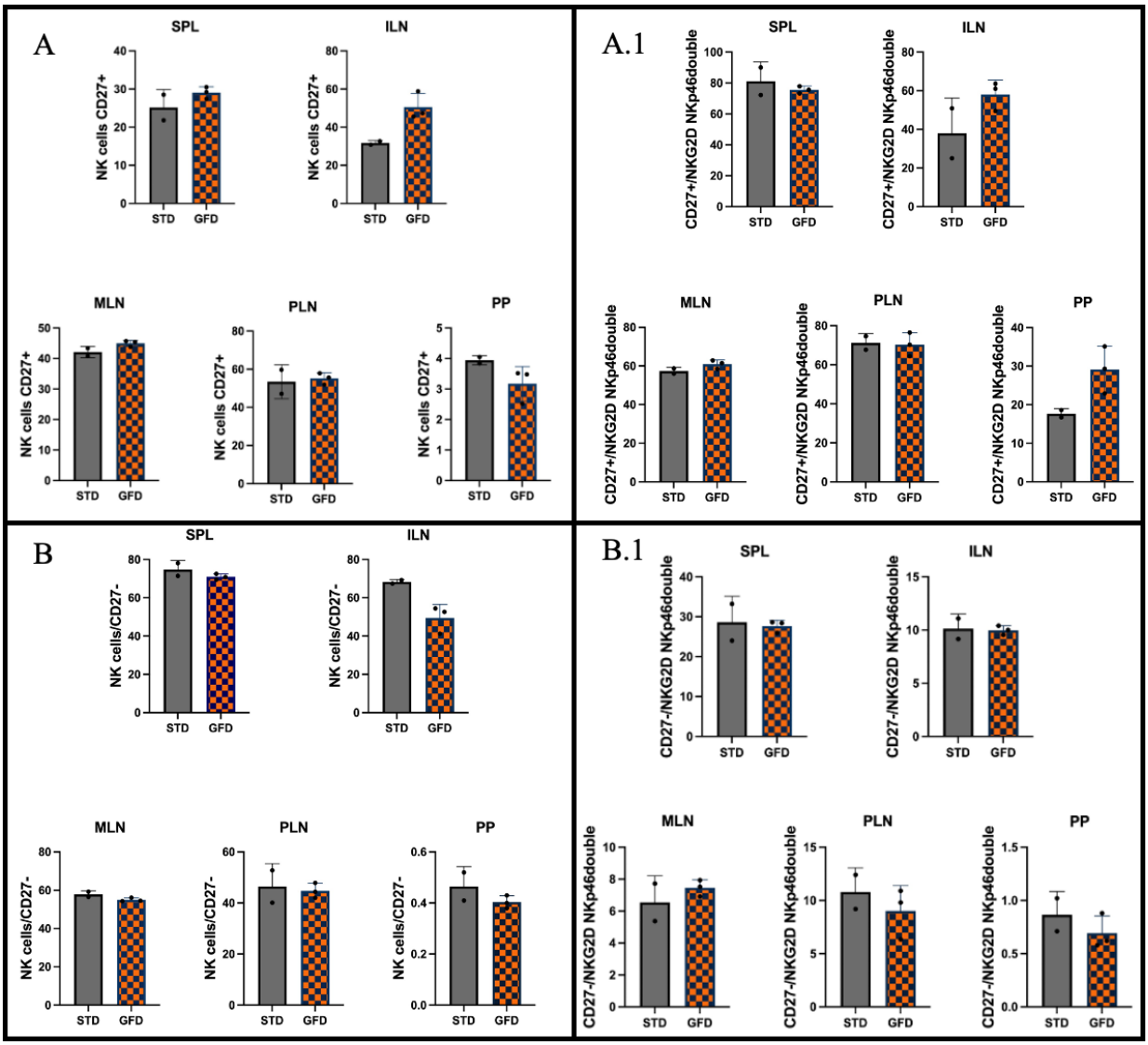

Figure 25 The percentages for every organ respectively: (A)The percentages of CD27+ NK cells in SPL, ILN, MLN, PLN and PP; (A.1) The percentages of NKG2D+NKp46+ double positive, CD27+ NK cells in SPL, ILN, MLN, PLN and PP; (B)The percentages of CD27- NK cells in SPL, ILN, MLN, PLN and PP; (B.1) The percentages of NKG2D+NKp46+ double positive, CD27- NK cells in SPL, ILN, MLN, PLN and PP. The statistical analysis was done with Two-way ANOVA and followed by Tukey's Multiple Comparisons Test.

The summary data from all organs, according to the Figure 26, showed slightly increased proportions of CD27+ and CD27+NKG2D+NKp46+ subsets of NK cells in GFD compared to STD fed mice, however this trend was not mirrored in CD27- and CD27-NKG2D+NKp46+ subsets. There were no statistically significant differences.

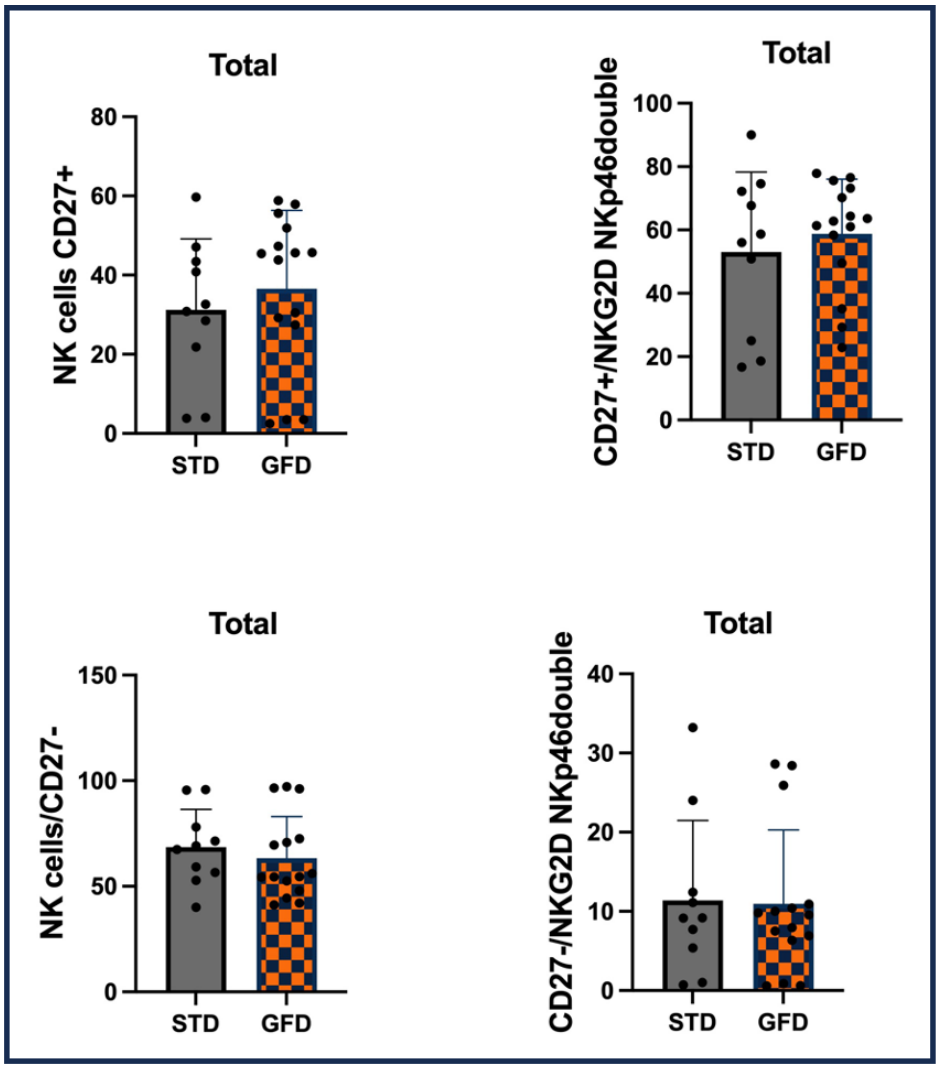

Figure 26 The percentage of CD27+ NK cells, NKG2D+NKp46 double positive CD27+ NK cells, CD27- NK cells, and NKG2D+NKp46 double positive CD27- NK cells for all organs jointly. The statistical analysis was done with Two-way ANOVA and followed by Tukey's Multiple Comparisons Test.

5.4 Assessment of immunological parameters on PD patients through FACS

Since this thesis constitutes a segment of a broader project, the study concerning the human arm is presently in progress due to its long-term nature. The analysis will commence upon reaching a sufficient number of patients in each group and upon completion of the diet crossover phase. PBMCs have been extracted from 25 patients, with 8 nearing the conclusion of the crossover period.

6 Discussion

6.1 Efficacy of the MPTP drug by Immunofluorescence (IF) staining

Comparing the effects of STD and GFD on dopamine levels and dopaminergic neurons is critical in the MPTP mouse model. It allows to assess directly, the dietary impact on the dopaminergic system during MPTP-induced neurodegeneration.

Immunohistochemistry showed dopamine and tyrosine hydroxylase (TH) in relevant brain regions. In order to systematically and semi-quantitatively assess the loss of dopaminergic neurons it is necessary, for instance to use high-performance liquid chromatography (HPLC) quantification of dopamine or its metabolites from standardized brain regions and amount of tissue, that are in progress in a collaborative laboratory.

Immunofluorescence staining of MPTP-treated specimens and control samples with PBS showed expected TH and DAPI staining results.

In both the MPTP and control groups, the IF staining protocol for tyrosine hydroxylase produced distinct signals indicating TH-positive cells in Figure 12 identified by the green dots. DAPI staining successfully marked cell nuclei in the examined tissues. The combined staining method enabled the visualization of tyrosine hydroxylase expression as well as nuclear morphology in relevant brain regions, visible in the Figure 12 as blue dots.

Brains from MPTP-injected mice displayed lower staining intensity and dopaminergic neuron density than control brains in Figure 12.

The findings confirm the IF staining results expected from the literature. They show a reduction in dopaminergic neurons in the MPTP-induced model compared to the PBS-treated control group. This confirmed the effect of the MPTP treatment and with this result we could proceed to the chronic model, using the same batch of MPTP.HCl.

In summary, this evidence shows the induction of dopaminergic loss following MPTP injection(s), substantiating the experimental model's efficacy in replicating neuroinflammation associated with PD. Next step for the establishment of a sensitive MPTP model in our lab is the confirmation of the dopamine quantification between MPTP-treated and PBS-control groups.

6.2 Open Field Test (OFT)

In the initial analysis of the OFT, the outcomes of the open field test administered to mice subjected to the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model indicated inconclusive findings, as the disparity in average speed (cm/s) and traveled distance (cm) between the Standard Diet (STD) and Gluten-Free Diet (GFD) groups did not attain statistical significance as it can be seen in the Panel A of Figure 13. However, trends emerged visually upon further examination (Figure 13-Panel B).

Nevertheless, over an extended 12-week duration under the chronic MPTP model, a reversal in this trend was observed. The GFD group exhibited superior exploration values, evident in increased total area covered in the arena demonstrated on Figure 14.

According to the literature, a mouse with a higher exploration value in the OFT indicates increased locomotor activity. This indicates that the mouse is moving around more, which could indicate that the mouse is more explorative and active (Gould, Dao, and Kovacsics 2009).

The STD group manifested impaired rearing behavior and heightened immobility, indicative of compromised motor coordination and diminished exploratory behavior. Conversely, mice fed a GFD displayed relatively preserved locomotor activity, characterized by slightly increased total distance traveled and heightened exploratory behavior.

The slightly higher exploration inferred from the results may imply that the Gluten-Free Diet can contribute reducing motor symptoms in the MPTP mouse model for Parkinson’s disease.

For instance, a four-month ketogenic diet had a positive effect on spatial learning, spatial memory, and working memory in 5XFAD mice. The observed cognitive improvement was linked to the regeneration of neurons and synapses in both the hippocampus and cortex, resulting in neuroinflammation reduction. Importantly, the ketogenic diet's efficacy in improving cognitive functions was discovered to be dependent on both the timing of initiation and the duration of the dietary intervention (Xu et al. 2022).

However, in the Open Field test it is critical to consider additional factors that may influence observed behaviors in the nuanced analysis of open field test results, thereby increasing the method's reliability. Individual exploratory tendencies can be influenced by social dynamics among mice within the testing environment, as well as their hierarchical structures.

6.3 Immunological parameters assessed by FACS.

Regulatory cells - Foxp3 Tregs and cytokine producing CD4 and CD8 T cells

We have assessed the possible changes in proportions of Foxp3 Tregs and their IL-10 positivity in mucosal and non-mucosal lymphoid organs from our first experiment using the chronic MPTP mouse model of PD, Figure 17.

In addition, changes in proportions of CD4 and CD8 T cells and their cytokine profiles (IL-10 and IFN-γ, respectively) were examined (Figure 18 and 19).

An inclination towards a higher ratio of CD4+FOXP3 Tregs was observed in nearly all examined lymphoid organs (spleen, mesenteric, inguinal lymph nodes, and Peyer's patches) in animals fed the GFD, except in the pancreatic lymph nodes. This finding may indicate a potential subtle influence of the GFD on Tregs, emphasizing the need for a thorough examination of regulatory T cells, including the LAG3-positive peripheral Tr1 regulatory cells in forthcoming studies.

FoxP3 Tregs have been studied in a wide range of autoimmune or immune-mediated disease (Sakaguchi et al. 2020), they are also therapeutic targets and one of the most commonly used immune biomarkers in clinical trials (Mallone and Roep 2013; Sharma and Rudra 2019).

Shifts in FoxP3 Tregs have been documented in other autoimmune diseases such as type 1 diabetes (T1D), multiple sclerosis (MS), and rheumatoid arthritis (RA), where experimental depletion led to disease acceleration and improved outcomes in animal models (Danikowski, Jayaraman, and Prabhakar 2017; Liston, Dooley, and Yshii 2022; Jiang et al. 2021; Petzold et al. 2013; Yang et al. 2022).

In the context of various models of immune-mediated diseases, the importance of these cells in preserving immune homeostasis is evident. It is suggested that, within this framework, the emphasis on their functionality may take precedence over the significance attributed to their relative numerical abundance (Sakaguchi et al. 2020; Y. Zhang, Bandala-Sanchez, and Harrison 2012).

The effect of a GFD on Foxp3 Tregs has been studied more thoroughly in the context of autoimmune diseases, specifically type 1 diabetes (T1D). Notably, no significant changes in Foxp3 Tregs were observed after GFD exposure in immunocompetent Blab/c (Julie Christine Antvorskov et al. 2012) and NOD mice (Haupt-Jorgensen et al. 2018). While, other groups reported increased proportions of FoxpP3 Tregs (Hansen et al. 2014; Marietta et al. 2013).

Consuming dietary gluten has been linked to an immune response that promotes inflammation. In rats fed a gluten-free diet based on casein, there was a noticeable shift from a Th1 to Th2 cytokine pattern, indicating a change in the immune system's inflammatory profile (G. S. Wang et al. 2000). Furthermore, the inclusion of dietary gluten resulted in a heightened pro-inflammatory cytokine pattern within T cells, correlating with an elevated proportion of Th17 cells (Julie Christine Antvorskov et al. 2012; Julie C. Antvorskov et al. 2013).

In Parkinson disease expression of pro-inflammatory cytokines is increased in colonic biopsies (Devos et al. 2013) and a functional defect of regulatory T cells was reported in PD patients (Saunders et al. 2012).

Several other studies reported dysfunction of T regs in early phase of PD (reviewed in (He and Balling 2013)) and their role in the brain and gut – along the gut-brain axis (Choi et al. 2022)

A very recent paper by Li J et al. (Li et al. 2023) indicates that elevated alpha-synuclein levels contribute to enhanced RORC transcription, resulting in heightened Th17 differentiation and a concurrent reduction in Tregs in PD.

Even though mucosal FoxP3 Tregs are not direct markers for decreased chronic inflammation in the gastrointestinal tract, they are one of the most extensively studied cell subsets. They effectively document shifts in the balance of the effector and regulatory arms of the immune response, which may contribute to the development of various immune-mediated diseases in genetically predisposed individuals, including those with PD.

Consistent with previously documented instances of increased or beneficial effects of Tregs following preventive interventions in various immune-mediated diseases, alterations noted in the chronic MPTP mouse model for PD suggest a slightly elevated presence of CD4+FoxP3+ Tregs in the spleen, inguinal lymph nodes, mesenteric lymph nodes and Peyer's Patches of mice fed GFD. Inguinal and pancreatic lymph nodes exhibited enhanced IL-10 production in GFD compared to STD fed mice (see Figure 16; Figure 17).

These changes may hold particular significance when observed in situ, specifically within the mucosal immune system as represented by mesenteric lymph nodes (MLNs) and pancreatic lymph nodes (PLNs). Peyer's patches (PP), along with gut-associated lymphoid tissue (GALT) and nasopharynx-associated lymphoid tissue (NALT), serve as mucosal inductive sites for immune responses.

CD4+CD45RB-low regulatory T cells

CD45RB-low CD4 T cells have been initially identified as regulatory T cells in inflammatory bowel disease (IBD), (Powrie et al. 1993). Subsequently, their characterization was refined based on the expression of CD38 (Read et al. 1998) and their function documented e.g. by their depletion, that led to the disease progression in the SCID transfer model of IBD (Read and Powrie 2001).

Nevertheless, the presence of CD4+CD45RB-low T cells is not extensively validated in other immune-mediated or autoimmune conditions. These cells exhibited a slight reduction across all organs in BALB/c mice on a GFD (Julie Christine Antvorskov et al. 2012) and they were also shown to prevent transfer of type 1 diabetes in the adoptive co-transfer NOD-SCID model (Shimada et al. 1996).

To the best of my knowledge, the mouse models of PD have not been investigated with respect to CD4+CD45RB-low T cells. In humans a lower proportion of naive CD45RA T cells was reported in PD (Saunders et al. 2012).

In the spleen, inguinal lymph nodes, and Peyer's patches, a similar proportion of CD4+CD45RB-low T cells was observed between the mice fed GFD and STD diet (Figure 22). While the exact implications of this uniformity merit further exploration, this consistent distribution across immune organs raises questions about their role as Tregs in relation to the GFD. Further investigations are warranted to unravel the precise implications of this uniformity.

γδ T-cells

Effector immune responses of γδ T cells also their possible pathogenic roles prevail in the literature, although in the field of T1D, from which we obtained the rationale for testing the GFD in PD, mucosal γδ T cells were several times clearly shown as important regulatory cells required e.g. for induction of oral tolerance (Locke NR et al. 2006) and also preventing development of type 1 diabetes after mucosal (i.n.) administration of an autoantigen in NOD mice (Harrison LC et al. 1996). With respect to administration of GFD, members of our lab found substantially increased proportions of γδ T-cells in both mucosal and non-mucosal lymphoid organs of mice, who were fed GFD since from uterus (Julie Christine Antvorskov et al. 2012). Similarly, ketogenic diet promoted mucosal, gamma/delta T cells in the lungs that protected mice from lethal influenza A infection (Goldberg et al. 2019).

Only limited number of studies addressed the γδ T-cells in PD. The first study from 1994, reported an increased proportion of γδ T-cells in patients with Parkinson disease

(Fiszer et al. 1994). More recently, a study by Zhou C et al. (Zhou et al. 2020) referred to reduction of both γδ T-cells and iNKT cells in PD patients.

In addition, Huang et al. 2021 also reported statistically significant decrease of γδ T cells in patients with PD (Yilin Huang et al. 2021). These recent observations may support protective or regulatory roles of γδ T cells in PD.

Our data, upon examining the outcomes for individual lymphoid organs, showed distinctly higher proportions of γδ T cells from animals fed GFD vs STD. This trend becomes more apparent when data from all lymphoid organs are plotted together (Figure 21 -Panel C).

This effect is consistent with previous reports indicating that dietary patterns influence the composition and functionality of T cell populations in various tissues.

NK cells

Mouse CD27+ (mature) and CD27- (immature) NK cells subsets were analyzed in lymphoid organs of MPTP treated mice fed the GFD and STD diets. In addition, we assessed the level of expression of activation markers NKG2D and NKp46. The CD27+ NK cells are sometime referred as regulatory (B. Fu, Tian, and Wei 2014) whereas CD27- NK cells are considered as highly cytotoxic (Vossen et al. 2008).

Low energy diets were reported to lower proportions of circulating NK cells (Kelley et al. 1994). With respect to the effect of GFD on NK cells the original paper studying the effect of GFD in BALB/c mice reported no substantial changes in NK cells (Julie Christine Antvorskov et al. 2012). However, GFD was reported to decrease expression of NKG2D activation marker and its ligands (Adlercreutz et al. 2014).

NK cells were reported elevated in PD and the increase is more pronounced with the disease progression (Cen et al. 2017). Recently, an increase in blood NK cells was reported and confirmed by Huan Y et al. (Yilin Huang et al. 2021)

NK cells have been identified in the brains of patients with alpha-synucleopathies, including PD. Even more intriguing is the fact, that the NK cells were found close to the alpha-synuclein aggregates (Earls and Lee 2020). In a mouse model of alpha-synucleinopathy, NK cells clear alpha-synuclein, and NK cell depletion exacerbates synuclein pathology (Earls et al. 2020).

Nigrostriatal injection of preformed alpha-synuclein fibrils alters central and peripheral immune cell profiles in non-transgenic mice. These data may indicate important, protective role of NK cells in PD such as in scavenging and degrading alpha-synuclein (Earls and Lee 2020).

Observing our dataset, it becomes evident that the analysis of each organ does not reveal a distinct trend in NK cells. Notably, in the spleen and mesenteric lymph nodes, there is a marginal increase in the presence of CD27+ NK cells in mice on a GFD compared to those on a standard diet (STD) (Figure 25). However, this observed trend is not consistently corroborated in other organs.

Examining a more detailed analysis in Figure 26, a subtle elevation in the populations of CD27+ and CD27+NKG2D+NKp46+ is discernible across all organs in GFD compared to SDT fed mice.

Interestingly, this trend is not mirrored in the CD27- NK cells, andCD27-NKG2D+NKp46+ subsets. It is essential to note that these observations did not attain statistical significance, primarily due to the limited number of mice in the experiment, a constraint imposed by mortality associated with the chronic MPTP effect.

7 Conclusion

In conclusion, our investigation into the immunomodulatory effects of a gluten-free diet in Parkinson's disease MPTP mouse models has yielded interesting insights although a low number of mice has been studied in the long-term chronic model so far.

We have established a chronic and acute MPTP mouse model of PD and verified it by immunofluorescence visualization of the decreased dopamine content due to the MPTP's induction of neuroinflammation.

Immune phenotyping by flow cytometry has so far documented only a few differences. Our study demonstrates that in the chronic MPTP mouse model, mice fed GFD show signs of slightly increased proportions of CD4+FoxP3+ Tregs across various lymphoid organs. While mucosal FoxP3 Tregs may not directly indicate reduced chronic inflammation, they are frequently used as a biomarker in clinical trials to monitor shifts in a balance of immune responses and are essential for maintaining immunological homeostasis and development and maintenance of immune tolerance. Interestingly, increased proportion of γδ T cells was documented in various lymphoid organs from the MPTP-treated mice on the GFD diet compared to mice fed the standard diet. This finding is in line with previous reports on the regulatory role of l γδ T cells originating above all from the field of type 1 diabetes research. On the other hand no substantial changes were found in proportion of CD4+CD45RB-low T cells, IL-10+ CD4 T cells, IFNγ+ CD8 T cells as well as in various developmental (CD27+ or CD27-) and activated (e.g. NKG2D-high) subsets of NK cells.

In addition to the results obtained by flow cytometry, this study includes also a behavioral approach that may be more sensitive than changes in immune parameters. In the open field test, mice fed the GFD showed higher exploration ratings, indicating a greater eagerness to interact with their surroundings.

To completely understand these effects, especially in neuroinflammatory conditions, more research is indeed required. Our results highlight the significance of behavioral evaluations in addition to immunological analyses and provide some novel and first data suggesting subtle beneficial effects of the gluten-free diet in the chronic MPTP mouse model of PD.

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