Charles University Faculty of Science

Study programme:
Biology

Branch of study:
Immunology

Bc. Mônica Jandová

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

Efekt Vliv bezlepkové diety na imunitní parametry u Parknsonovy nemoci

Diploma thesis

Supervisor:
MUDr. David Funda, Ph.D

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, 03. 01. 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 and giving me a chance to be part of his group. His guidance, invaluable insights, and continuous encouragement throughout the research process. His expertise and mentorship have significantly shaped the trajectory of this work.

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 colleaguesBc. 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 nuanced impact of a gluten-free diet (GFD) in comparison to a standard diet (STD) within the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of human Parkinson's disease, utilizing male C57Bl6 mice. The research involves the establishment of both acute and chronic MPTP mouse models, accompanied by a battery of flow cytometry assessments, including the proportions of regulatory T cells (Tregs), cytokines - interleukin-10 (IL-10), and interferon-gamma (IFNγ), gammadelta T cells and natural killer (NK) cells in mucosal and non-mucosal lymphoid organs.

To ascertain the behavioral impact, an open field test was conducted, providing valuable insights into the locomotor activity and exploratory behavior of the mice in response to the dietary interventions. Additionally, immunofluorescence was employed to validate the effects of MPTP, offering a visual confirmation of any neuroanatomical alterations induced by the neurotoxin.

Preliminary results suggest a subtle yet promising indication of the positive effects of the gluten-free diet. However, these findings warrant cautious interpretation, and further in-depth studies are imperative to establish a more comprehensive understanding of the observed effects. The integration of behavioral assessments, immunofluorescence histology, and immunological analyses underlines the multidimensional nature of this investigation, setting the stage for future research endeavors in the complex interplay among diet, gut, brain, immune responses, 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 v porovnání se standardní dietou na rozvoj Parkinsonovi nemoci 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 řadu navazujících flow cytometrických analýz zaměřených na regulační T buňky, cytokiny – interleukin 10 (IL-10) a interferon gama (IFN-γ), gamma/delta T buňky, a NK buňky ve slizničních a sytémových lyfatických orgánech.

Pro posouzení vlivu na chování jse použili test otevřeného pole, který nám umožnil posoudit lokomotorickou aktivitu a explorativní chování myší v závislosti na podávané dietě. Dále jsem použili také imunofluorescenční barveni pro validaci efektu MPTP, které nám poskytlo vizuální potvrzeni neuroanatomických změn indukovaných neurotoxinem.

První výsledky naznačují malé ale slibné známky pozitivních efektů bezlepkové diety. Nicméně tyto změny lze pouze opatrně interpretovat, protože je potřeba opakovaných a podrobnějších experimentů k posouzení zazanmenaných efektů. Spojení behaviorálních testů, imunohistologie a imunologických analýz představuje vícečetný metodicky přístup k této problematice, který nám může poodhalit komplexní interakce diety, střeva, mozku, a imunitnich reakcí u neurozánětlivých procesů.

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

Table of contents

List of abbreviations

GFD	Gluten free diet
GIT	Gastro Intestinal 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

1 Introduction

Parkinson's disease (PD) is a neurodegenerative disease typically causing impaired gait and muscle control and 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 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 instance, Type I Diabetes(Antvorskov et al., 2014), Psoriasis (Afifi et al., 2017). Several trials have shown that a gluten-free diet has 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 gluten could influence the permeability of the gut. Moreover, studies have not yet evidenced whether the accumulation of α-synuclein protein may start in the gut during gluten

intake and if this accumulation increase the activation of proinflammatory pathways. Thus, we hypothesize that PD's onset starts as a consequence 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 years of disability-adjusted life loss in 2019, an increase of 81% since 2000. PD was also responsible for 329,000 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 & 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 (Tolosa et al., 2021). 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) (Tolosa et al., 2021). This age gap may be observed because the illness was not properly diagnosed at its onset (Hirsch et al., 2016; 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 & Schwarzschild, 2016). For instance, in a Danish study of over 13,000 PD cases,

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 (Tolosa et al., 2021). 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). 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 (Tolosa et al., 2021).

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 & Westenberger, 2012).

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 (Jankovic & Tan, 2020; Klein & Westenberger, 2012).

Another type of rare PD may be caused by PINK1: A rare type of Parkinson's disease can also be brought on by mutations in this gene. The PINK1 protein 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 (Jankovic & Tan, 2020).

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

2.3 Mechanisms underlying PD symptoms

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 an important player in PD, according to several studies (Ho, 2019; Rocha et al., 2018; S. Wang et al., 2016). 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 (Grotemeyer et al., 2022).

It is known that PD is a degenerative condition that affects the brain's dopamine-producing cells, which are responsible for regulating movement. The deterioration of the neuronal cells occurs due to the accumulation of the misfolded α-synuclein protein. The α-synuclein is a small soluble protein, which 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 (Rocha et al., 2018).

However, it is also known that α-synuclein contributes to the onset of a number of neurodegenerative conditions. The protein clumps of α-synuclein can form Lewi Bodies (LB), which are responsible for neuronal death. Moreover, α-synuclein accumulation is a single mechanism that may cause neuronal death in PD as microglia and other immune cells may become activated in the brain, resulting in an immune reaction (Ho, 2019; Rocha et al., 2018).

PD is thought to progress because of a proinflammatory response. Thus, there are other mechanisms linked to the onset of PD, such as oxidative stress, inflammation, and mitochondrial dysfunction (Rocha et al., 2018).The α-synuclein aggregates trigger the brain's immune cells called microglia, which then release pro-inflammatory cytokines that can harm neurons and cause neuroinflammation.

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. Furthermore, it has been demonstrated that α-synuclein localizes 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-synuclein accumulation in neurons is thought to contribute to oxidative stress and ROS generation in PD (Borsche et al., 2021).

The brain's substantia nigra, 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 SNpc is the cause of many of the disease's motor symptoms, such as tremors, bradykinesia, gait disturbance, and rigidity (Ascherio & Schwarzschild, 2016; Borsche et al., 2021; Jankovic & Tan, 2020).

PD exhibits numerous types of non-motor symptoms. 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).

Recent research has shown that patients with PD exhibit greater intestinal permeability than healthy people. Furthermore, there is evidence that variations in the gut microbiota could be linked to the onset of PD. The capacity of the lining of the intestines to prevent harmful compounds from entering the bloodstream from the gut is referred to as gut permeability. It has been suggested that gut permeability may be increased in patients with PD, allowing chemicals from the gut to enter the bloodstream and potentially influencing the onset or progression of the condition (Romano et al., 2021; Scheperjans et al., 2015; Q. Wang et al., 2021).

Constipation may be a first-stage premorbid PD symptom. The evidence from laboratory experiments that suggested unusual a-synuclein 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).

A recent study has shown that the misfolded α-synuclein protein may begin to accumulate in the gut and then travel through the nervous system to the brain (Romano et al., 2021). Consequently, the misfolded protein might cause the gut to become inflamed, which would increase gut permeability and facilitate α-synuclein to enter the bloodstream.

2.4 Adaptive and Innate Immune Response in PD

2.4.1 Regulatory T cells (Tregs)

There is mounting data that suggests immune system dysregulation may play a 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 FOXP3, 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).

In a mouse model of PD, Tregs migrated into the brain and suppressed neuroinflammation (Badr et al., 2022). Moreover, the use of Treg therapy prevented the loss of dopamine-producing neurons and enhanced mouse motor performance. Also, Tregs may play a role in the pathogenesis of PD since diagnosed patients had lower blood levels of Tregs compared with healthy controls.

In summary, even though more investigation is required to completely understand the function of Tregs in PD, mounting evidence supports the possibility that these immune cells may act as a preventative measure by lowering inflammation and promoting neuronal survival.

2.4.2 Memory T cells

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 may also play a role in the onset and progression of PD, according to mounting data (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).

2.4.3 Natural Killer cells

Natural killer (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.

NK cells have the ability to homing to the CNS in neurological disorders such as PD, especially in conditions of heightened inflammation. Furthermore, their function 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.

2.4.4 Cytokines

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).

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 Dysbiosis

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). Thus, the gut dysbiosis may contribute to the mechanisms mentioned previously, 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 1), and the most common medications are as follows:

a. Levodopa is converted to dopamine, which can assist with the disease's motor symptoms.
b. Dopamine agonists imitate dopamine's effects in the brain and can support in the relief of motor symptoms.
c. MAO-B inhibitors are drugs that prevent the brain from breaking down dopamine and can help with improving movement symptoms (Lancet,2021).
d. COMT inhibitors are drugs that prevent the body from breaking down levodopa.

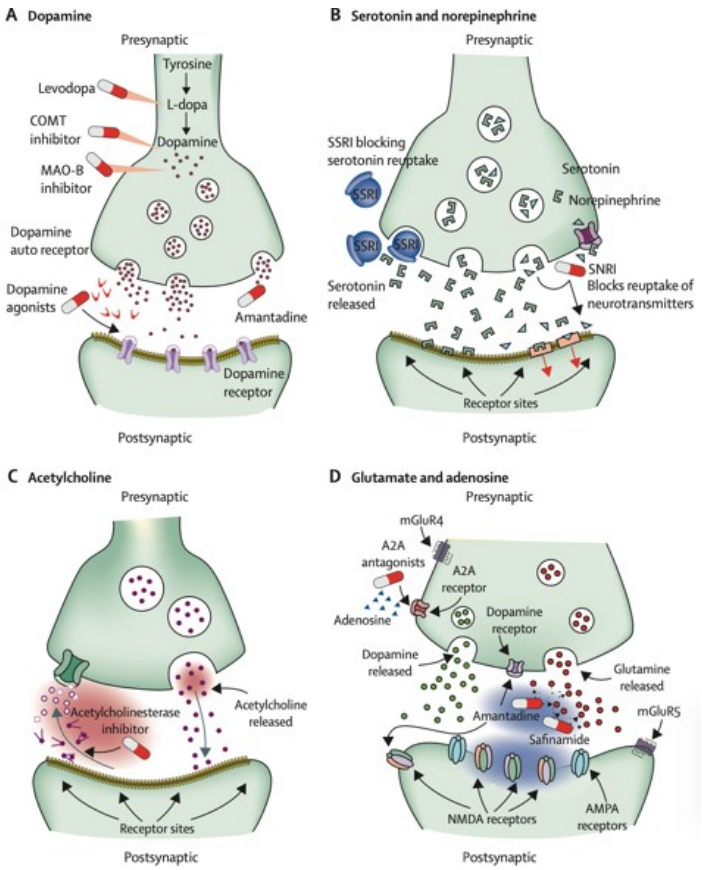

Figure 1 Sites of action for several antiparkinsonian medications (Lancet,2021)

2.6.1.1 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).

2.6.2 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. 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 health (Figure 2) (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).

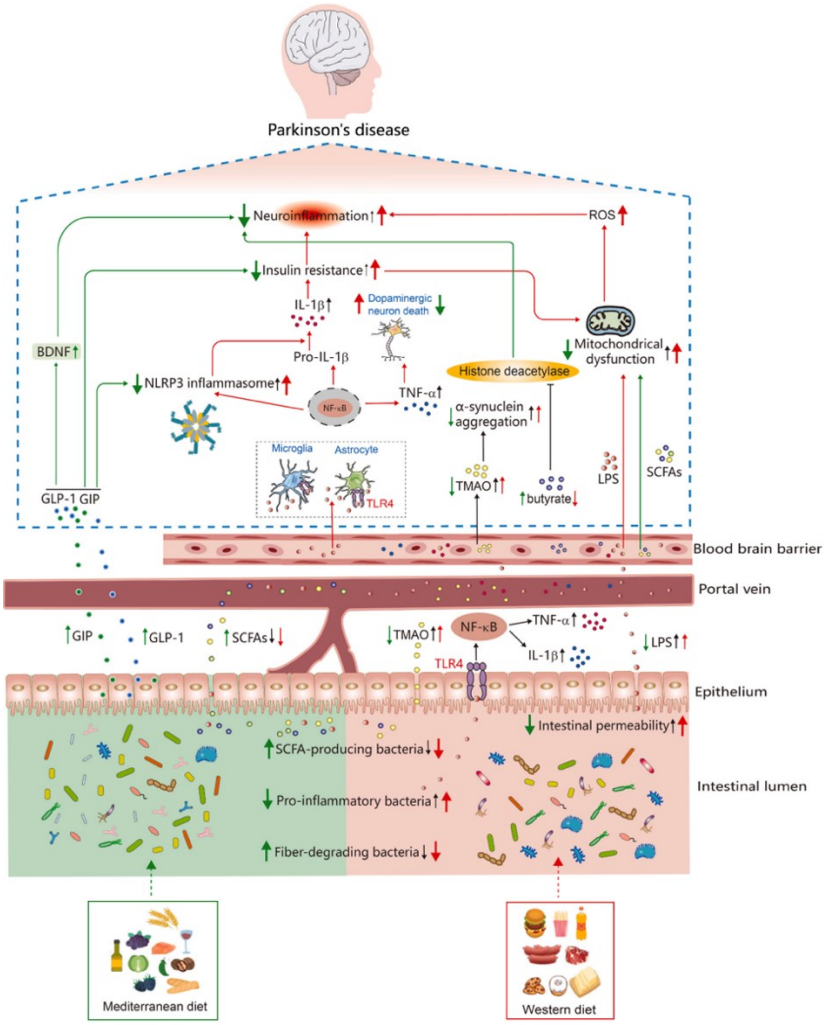

Figure 2 Demonstration of how possibly the western diets have been found to exacerbate neuroinflammation in PD through interactions involving the microbiota-gut-brain axis. Conversely, Mediterranean diets have demonstrated the ability to ameliorate neuroinflammation in this condition (Chu et al., 2021).

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 3).

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.

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).

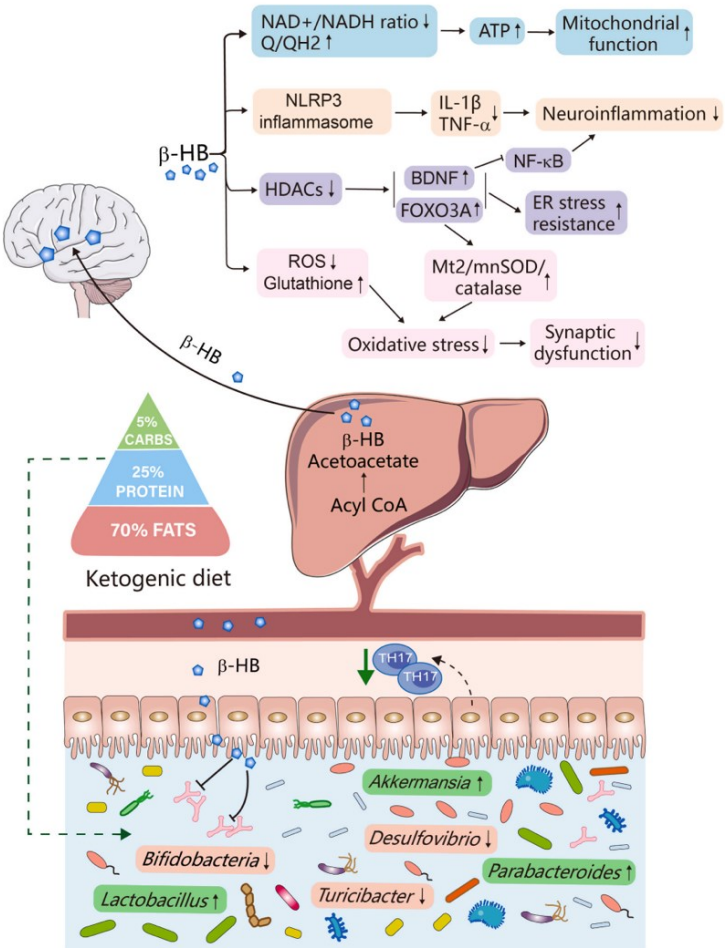

Figure 3 Ketogenic diet inducing a protective behavior in PD neurodegeneration (Goldberg et al., 2020)

2.6.2.1 Gluten, Autoimmunity and Parkinson

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 (Tan et al. 2020).

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. While a link between gluten and Type 1 Diabetes has previously been established (Julie C. 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.

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.

Studies have explored the potential connection between gluten, autoimmune responses, and diseases, leading to investigations into the impact of a gluten-free diet GFD on PD. Previously, a link was established between gluten and Type 1 Diabetes. However, conclusive evidence regarding the contribution of a GFD to PD onset and treatment is lacking, necessitating further research. The intricate interplay of genetics, the immune system, and diet calls for more investigation to clarify whether adopting a gluten-free diet could influence the development or management of PD.

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).

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 gastrointestinal tract (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).

2.7 Animal model for PD

Animal models enable researchers to study the early stages of Parkinson's disease 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 Parkinson's disease. Animal models can be used to investigate the role of microglial activation in neuroinflammation and its impact on disease progression. Furthermore, the impact of genetic factors on Parkinson's disease 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 Parkinson's disease patients. Rodents and non-human primates treated with MPTP show motor deficits and neuropathological changes similar to human Parkinson's disease, providing a valuable platform for studying the disease's progression.

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 Parkinson's disease. Similarly, the 6-OHDA model, which employs a neurotoxin to selectively destroy dopaminergic neurons, aids in understanding the neurochemical changes associated with Parkinson's disease.

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 Parkinson's disease pathology, allow researchers to study Lewy body formation, a hallmark of the disease. These models shed light on genetics' role in neurodegeneration.

Beyond chemical and genetic models, the role of inflammation in Parkinson's disease (PD) is investigated using the lipopolysaccharide (LPS) model, in which bacterial endotoxin induces neuroinflammation. This model aids researchers in their investigation of the relationship between inflammation and Parkinson's disease progression.

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

2.7.1 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) 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 4).

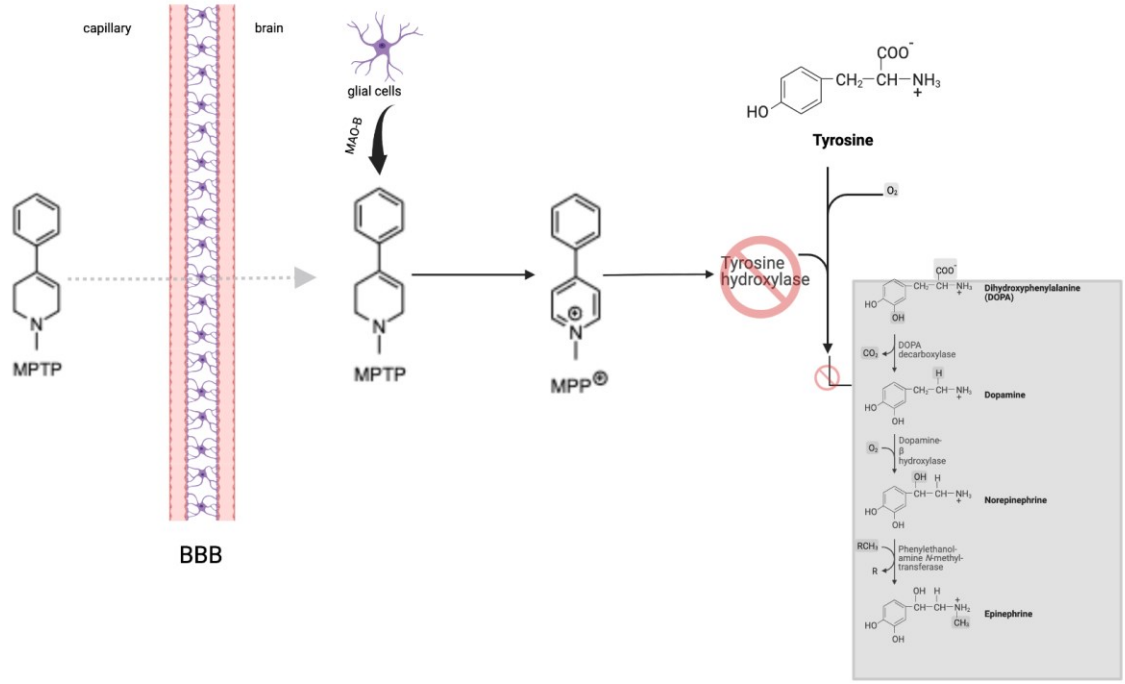

Figure 4 General MPTP pathway for PD animal model. (Created on BioRender)

2.7.1.1 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 used

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)

2.7.2 Other mouse models

Toxins like reserpine, haloperidol, and inflammogens namely lipopolysaccharide have been used, over the years, to simulate PD in animal models. The second most popular, after MPTP in mice and monkeys, is the application of the traditional 6-OHDA in rats (Blesa & Przedborski, 2014).

2.7.3 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).

The translational gap between animal findings and human applicability 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.

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 Parkinson's disease (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 **Error! Reference source not found.**.

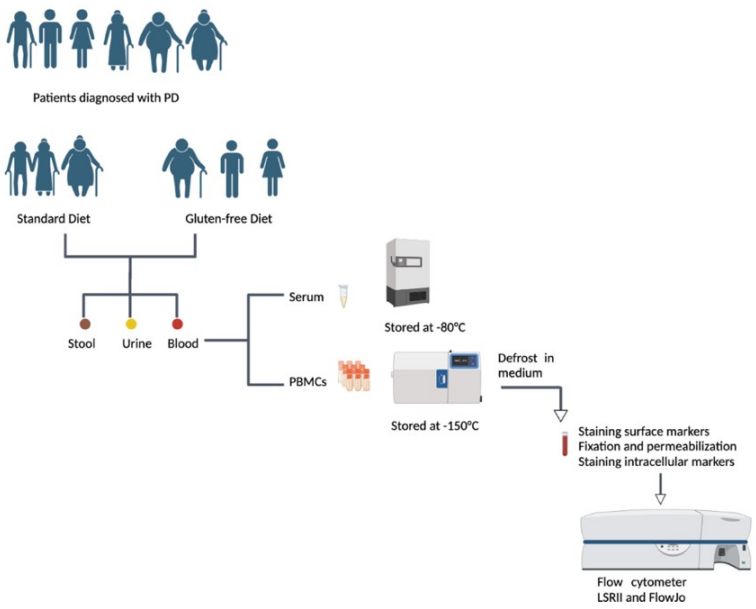

Figure 5 The experimental design of the human arm of this project (Created on BioRender)

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 preheated 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 was used to measure each sample, and the resulting data were examined using the FlowJo program.

4.2.2 Animal model samples

MPTP Acute model

Male C57BL/6 J mice (7–8 weeks old), obtained from the, were utilized for the MPTP intoxication study. The MPTP.HCl (20 mg MPTP/kg bodyweight of free base), dissolved in saline solution, was 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.

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 12 weeks for, completing the treatment within a single day. 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 6.

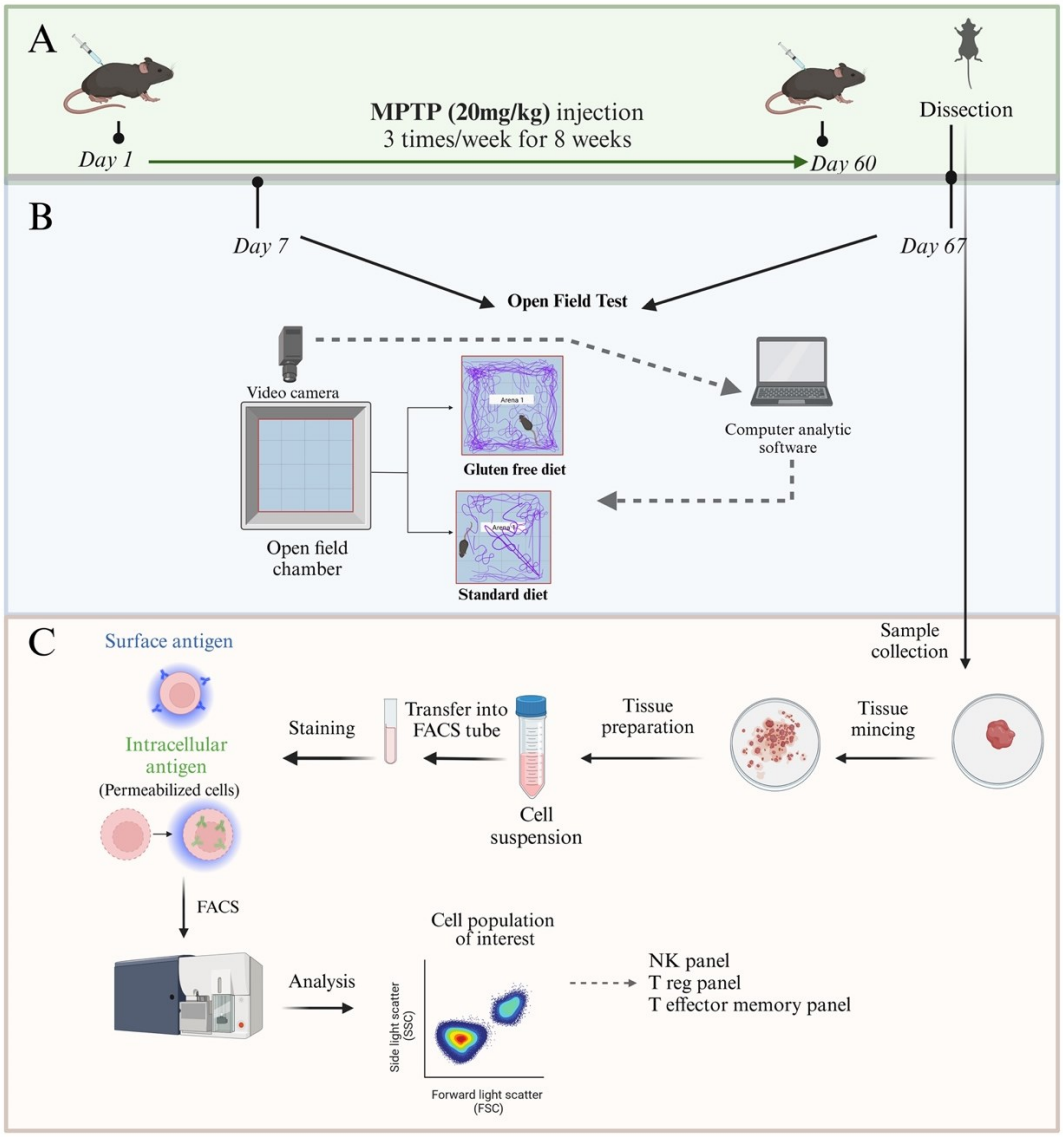

Figure 6 Experimental design of MPTP Mouse model in this project (Created on BioRinder)

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).

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 7)

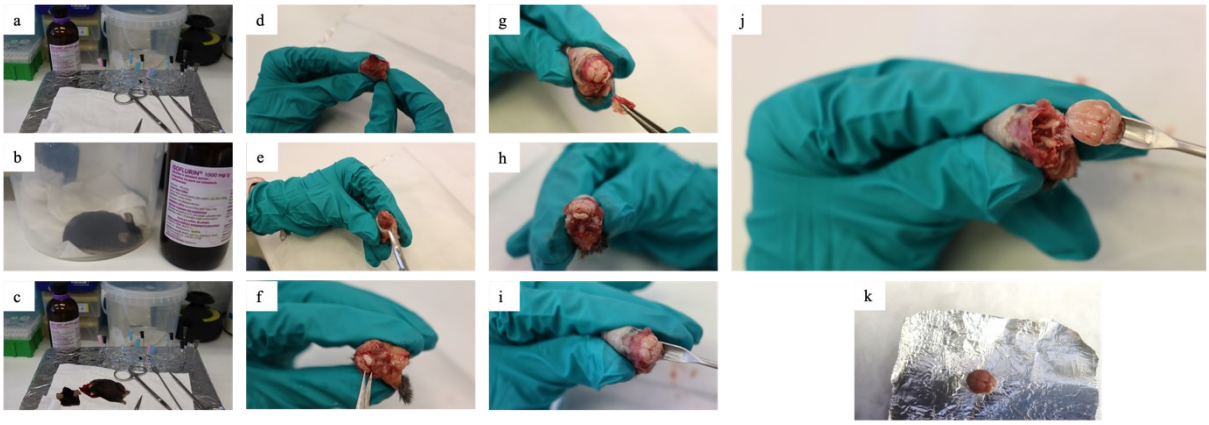

Figure 7 Brain extraction of C57Bl6 mouse for further analysis: a) Instruments and the anesthesia isoflurane; b) The mouse was placed in a closed recipient; c) Removal of the mouse's head; d) meticulous incisions are made, revealing the intricate structural nuances of the mouse brain; e-g) close-up perspective 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 60µm 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 are kept for later processing and analysis.

Immunofluorescence

In the experimental procedure, the brain was meticulously extracted from the skull, as demonstrated in Figure 7 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 a square white box, in the center, and its behavior was recorded for 5 minutes. The AnimalTA software was used to process the recorded videos, which aided in the extraction of quantitative parameters such as speed and distance travelled. 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.

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 Dopamine Staining in acute model

A pilot experiment was conduct while stablishing the MPTP mouse model and the effect of the drug.

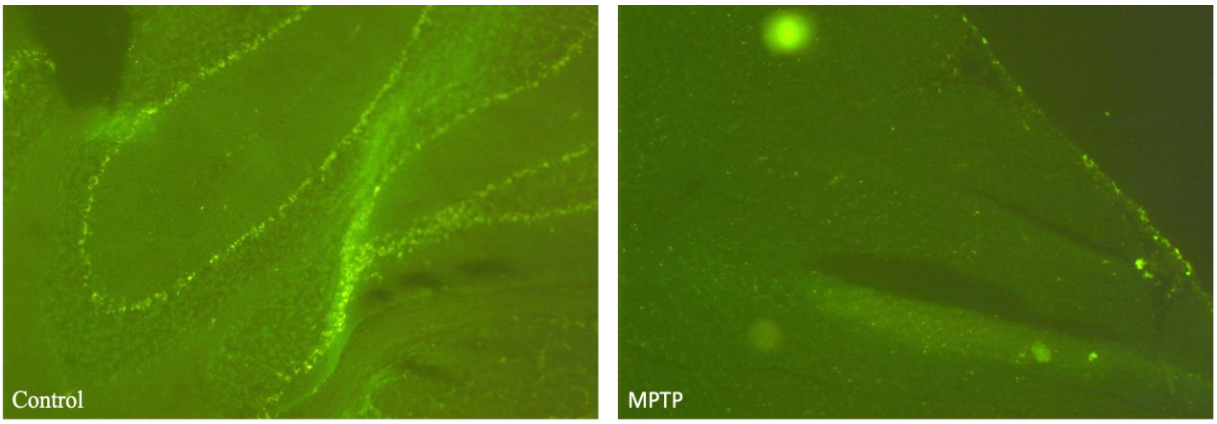

Figure 8 Control representing a brighter stronger signal of dopaminergic neurons and on the right can be observed the dopamine loss with lower signal after MPTP injection for the acute model.

5.1.2 Tyrosine Hydroxylase Staining with 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 9) revealed a decrease in tyrosine hydroxylase fluorescence in the MPTP model (Figure 9– 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.

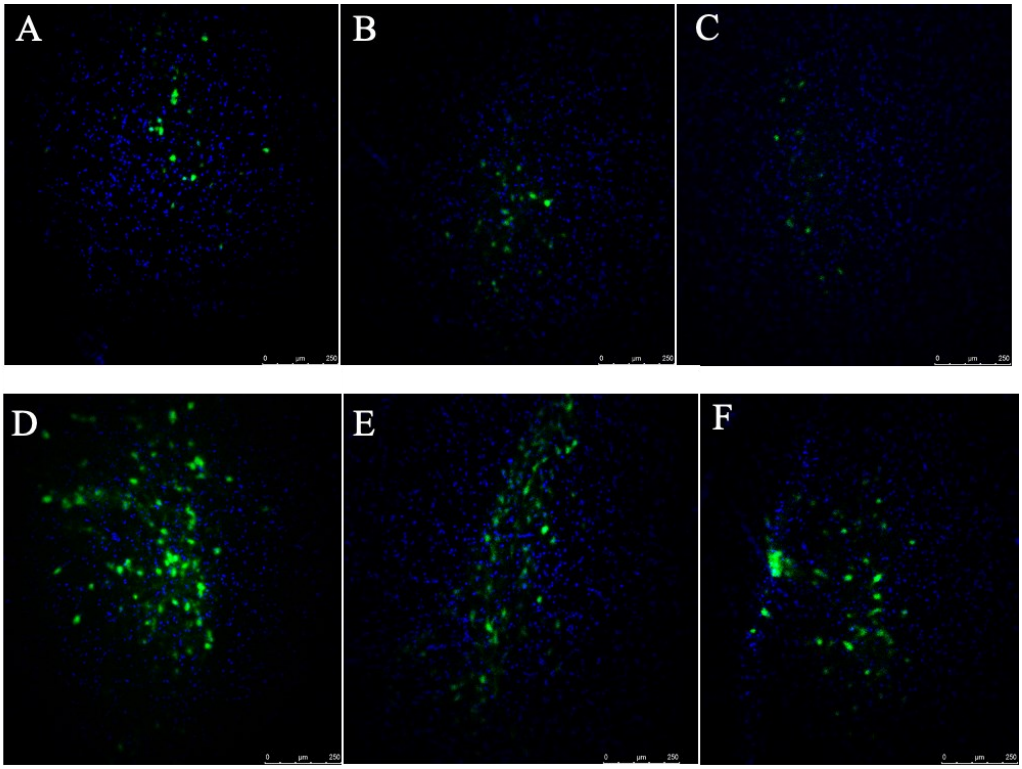

Figure 9 In panels A, B, and C, three mouse brains were subjected to the acute model of MPTP treatment, whereas in panels D, E, and F, another set of three brains underwent treatment with PBS, serving as the control group.

In contrast, the PBS-treated control group (Figure 9- Panels D, E and F) showed stronger tyrosine hydroxylase fluorescence and preserved nuclear architecture, 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 had decreased locomotor activity, based overall exploration within the open field arena at the end of the experiment (Figure 10).

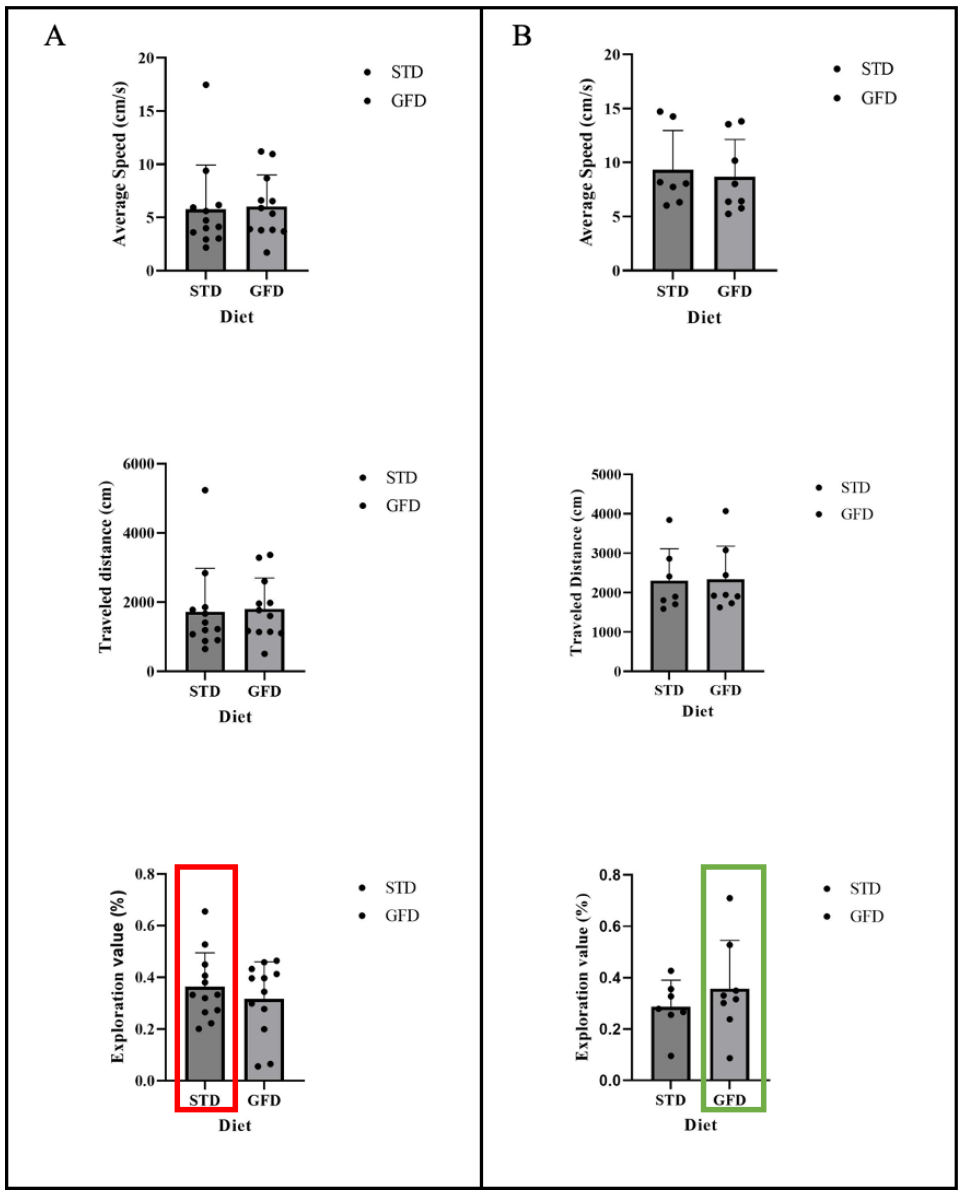

Figure 10 Plots depicting average speed, traveled distance, and exploration value of two groups treated with standard diet and gluten free diet. Panel A corresponds to the first Open Field test, performed 1 week after first MPTP injection; panel B corresponds to the second Open Field test performed one week after the last MPTP injection. The statistical analysis was done with Two-way ANOVA.

The open field test results show considerable difference in exploration behavior between the STD and the GFD. A visualization of the increased exploration value can be seen in the Figure 11 mice on the GFD explored more, covering a greater area within the open field arena than mice on the STD.

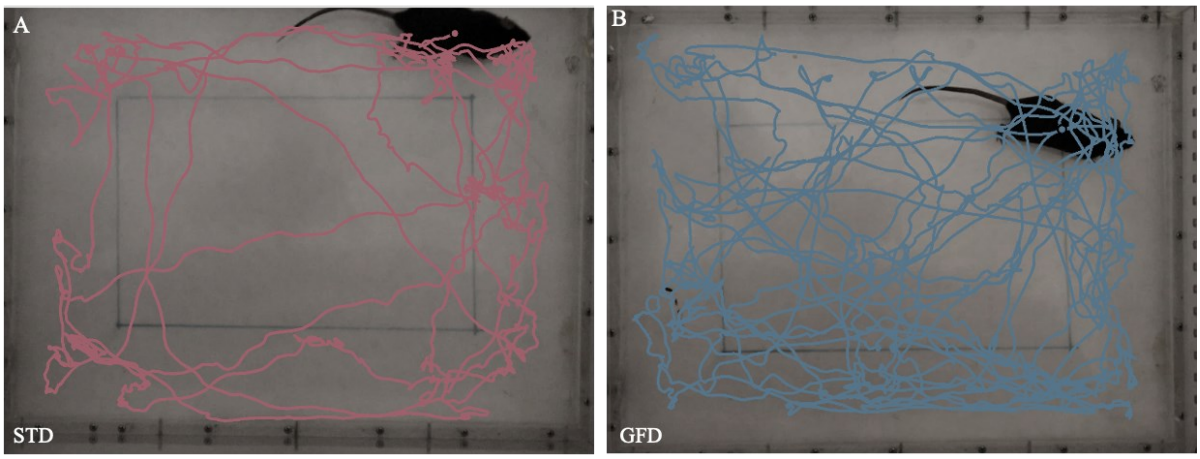

Figure 11 Illustration of the exploration exhibited through the trajectory of individual mice under the influence of two distinct diets at the end of the Chronic Model experiment; (A) Total trajectory of a mouse on STD; (B) Total trajectory of a mouse on GFD. (Image Generated during the analysis using the software AnimalTA)

5.3 FACS

To see if there was a 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 a thorough examination of γδ TCR and within this experimental setting.

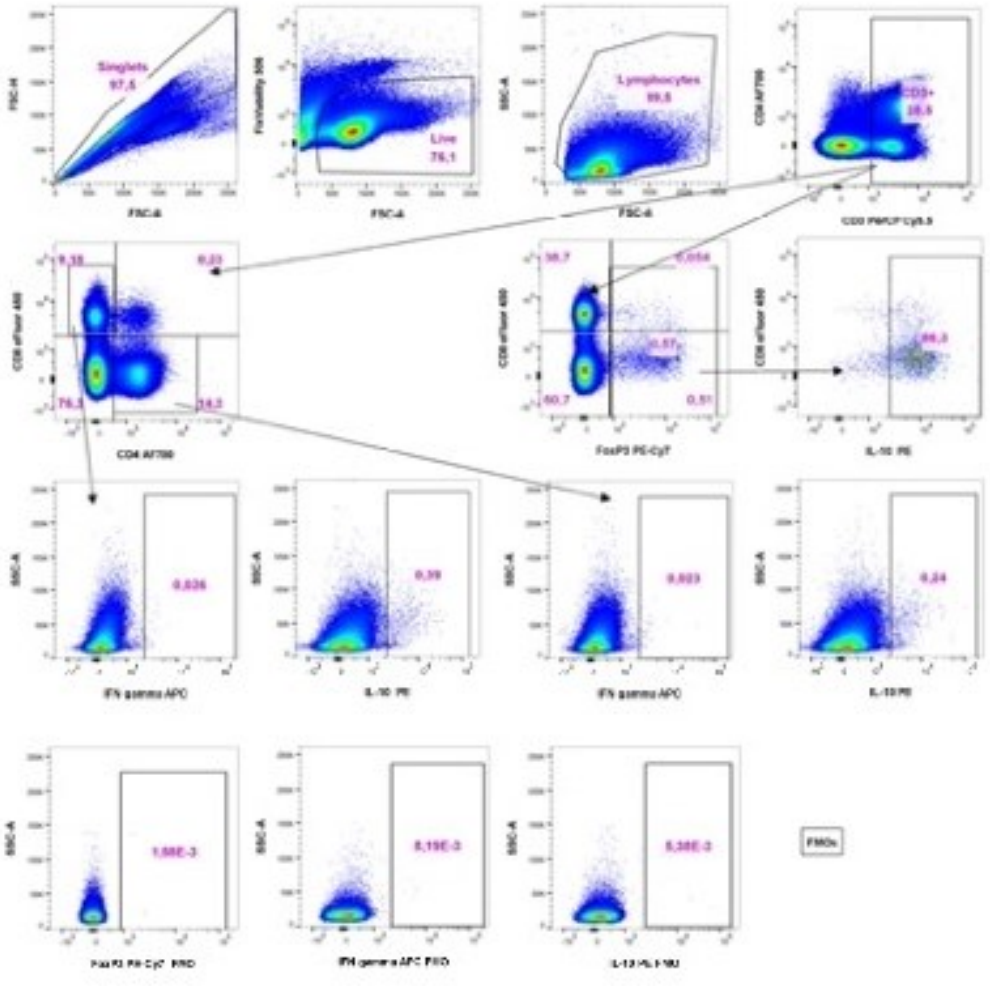

Figure 12 Gating strategies of IL-10, IFN-γ producing CD4+ T-cells. Gating strategies of the two cytokines were done similarly at the beginning as the first gate was set to exclude doublets, next only viable cells were included in the analysis, then lymphocytes and monocytes were gated, then the gate was set at CD3+CD4+ cells. As the last step, cells from the CD3+CD4+ gate were plotted according to the IL-10, IFN-γ staining. 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 CD3 FoxP3 in GFD mice compared to STD Figure 13, 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 expected death due to MPTP effect.

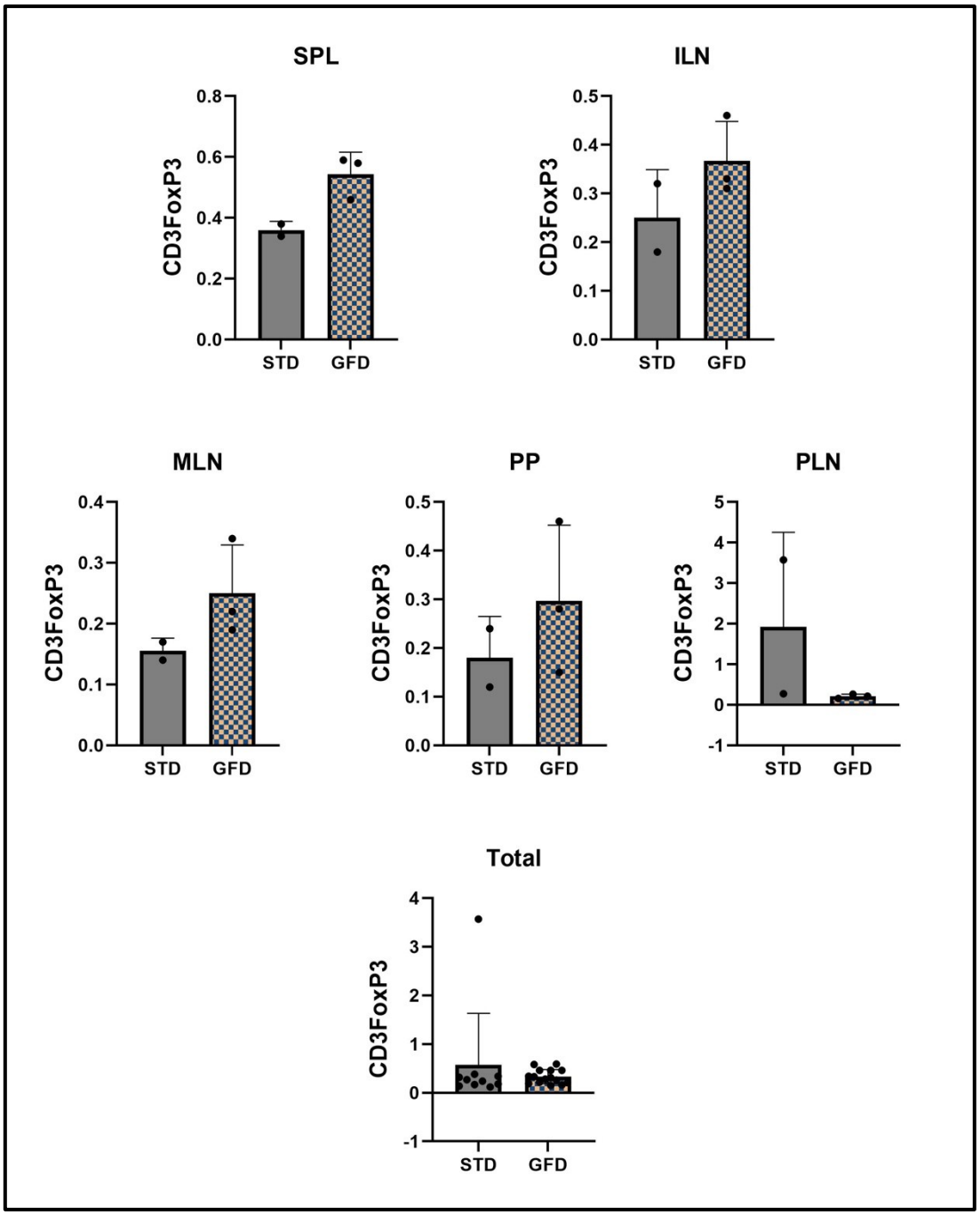

Figure 13 Percentage of CD3+FOXP3+ in STD and GFD groups in SPL, ILN, MLN, PLN and PP. As well the total percentage considering all organs. The statistical analysis was done with Two-way ANOVA and followed by Tukey's Multiple Comparisons Test.

In inguinal and pancreatic lymph nodes it was exhibited enhanced IL-10 production in GFD mice compared to STD Figure 14, 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 expected death due to MPTP effect.

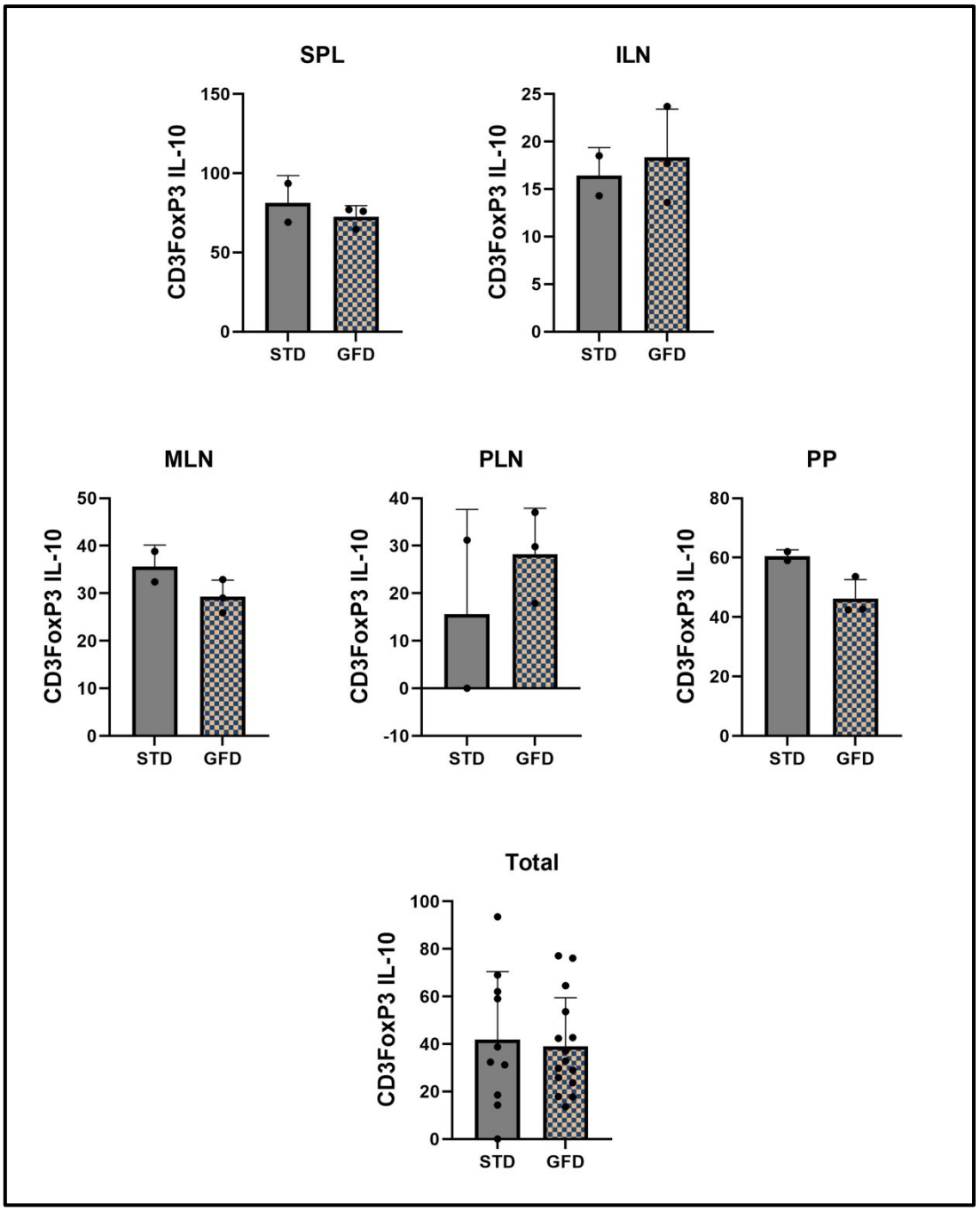

Figure 14 Percentage of CD3+FOXP3+ producing IL-10 in STD and GFD groups in SPL, ILN, MLN, PLN and PP. As well the total percentage considering all organs. The statistical analysis was done with Two-way ANOVA and followed by Tukey's Multiple Comparisons Test.

In spleen, inguinal lymph nodes and in mesenteric lymph nodes it was exhibited enhanced IL-10 production in CD8+ T cells (Figure 15-Panel A) in GFD mice compared to STD similar result was found in CD8- T cells (Figure 15- Panel B), 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 expected death due to MPTP effect.

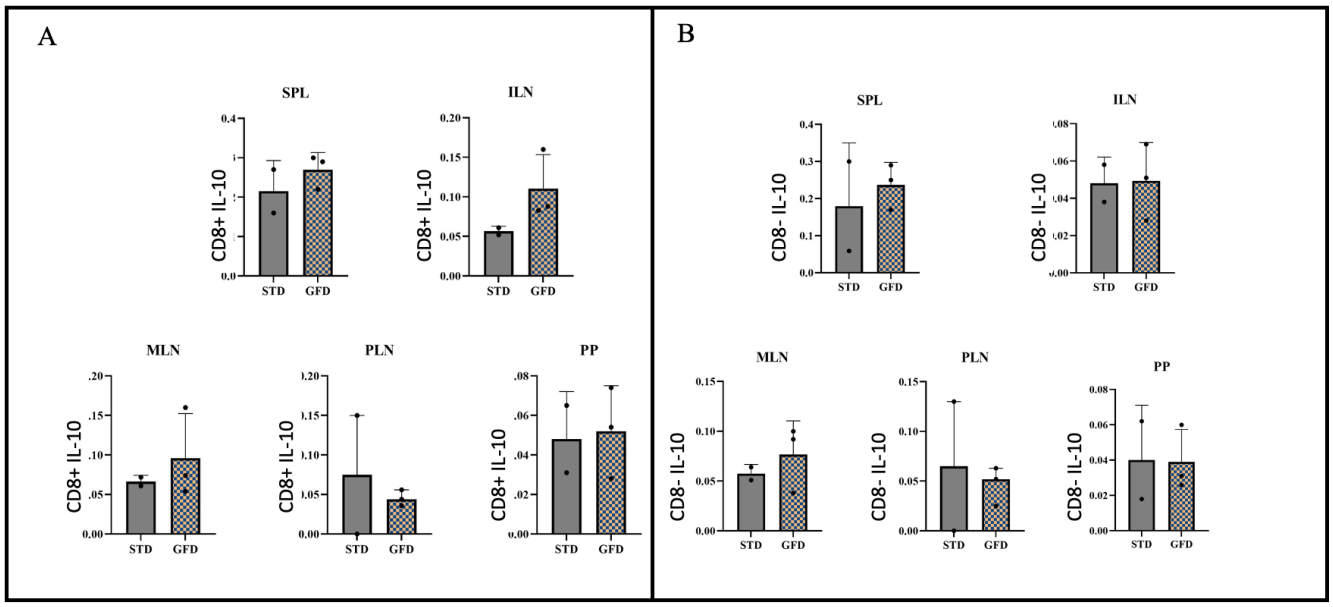

Figure 15 (A)Percentage IL-10 in CD8+ T cells STD and GFD groups in SPL, ILN, MLN, PLN and PP; (B) Percentage IL-10 in CD8- T cells STD and GFD groups in SPL, ILN, MLN, PLN and PP . The statistical analysis was done with Two-way ANOVA and followed by Tukey's Multiple Comparisons Test.

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.

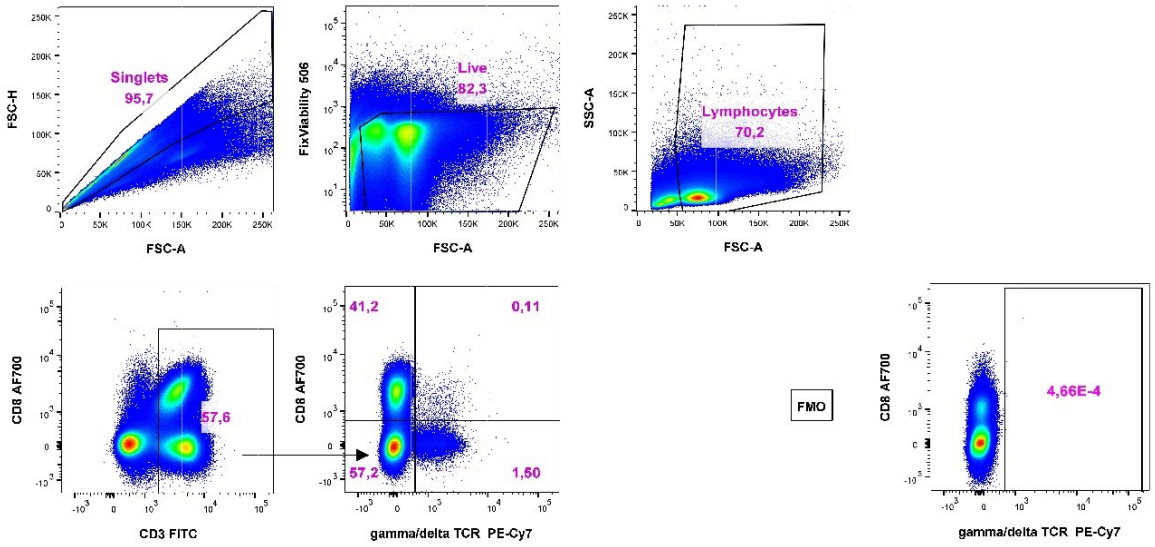

Figure 16 The gating strategy of γδ T-cells. Gates were set for single cells, lymphocytes and monocytes, CD3+ and at last for γδ T-cells. On the right side there is the FMO control. The gating was done in FlowJo software.

In spleen, inguinal lymph nodes and pancreatic lymph nodes it was exhibited enhanced FoxP3 CD8+ T cells in GFD mice compared to STD (Figure 17- Panel A); FoxP3 CD8- T cells was enhanced in all organs of GFD group (Figure 17- Panel B). It was not statistically significant as the total number of mice in the experiment was limited by expected death due to MPTP effect.

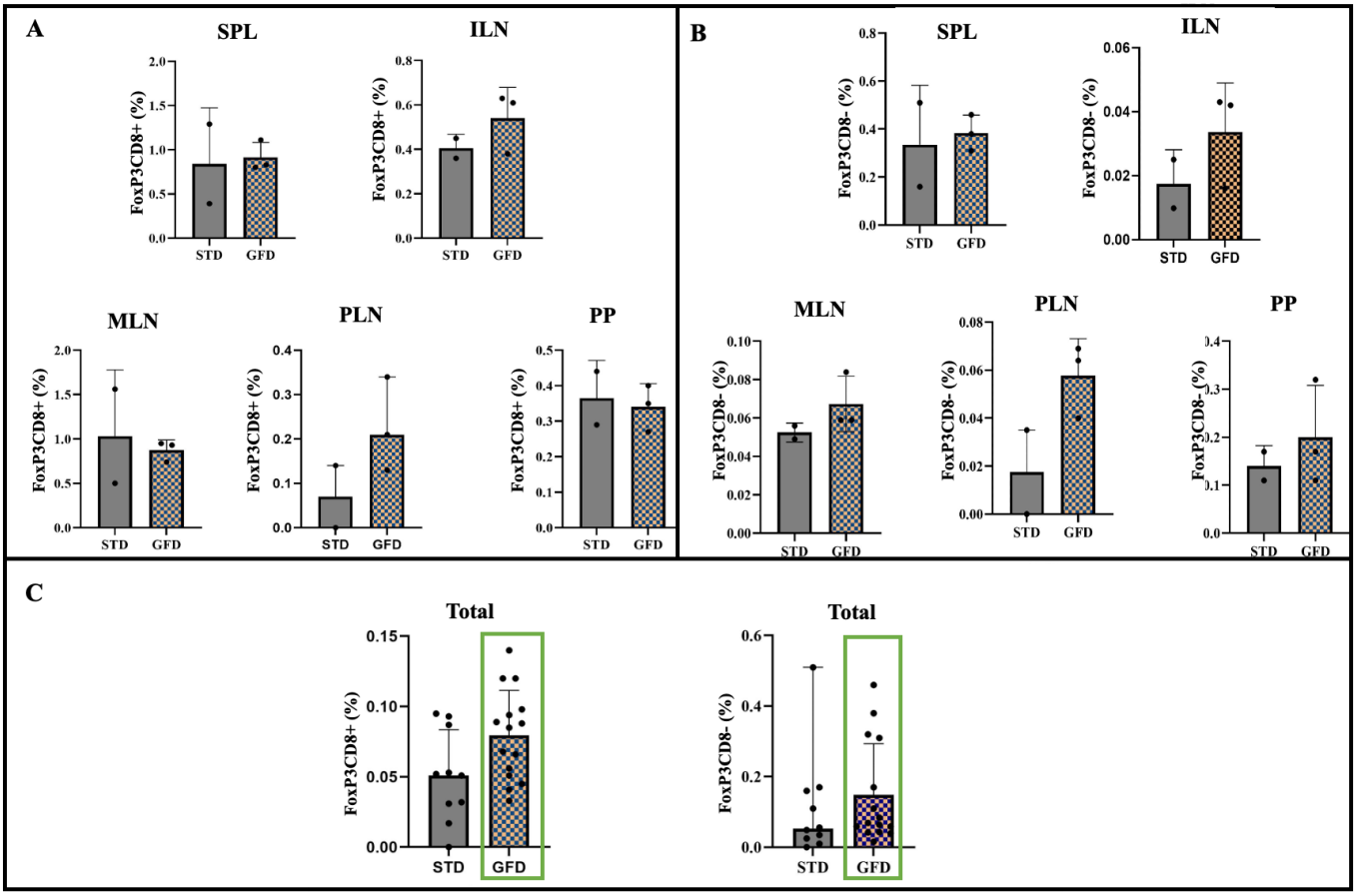

Figure 17 The percentages of γδ T-cells. (A) Percentage of FoxP3CD8+ γδ T-cells in SPL, ILN, MLN, PLN, and PP; (B) Percentage of FoxP3CD8- γδ T-cells in SPL, ILN, MLN, PLN, and PP. (C) Total percentage of γδ T-cells in all organs, on the left it was FoxP3CD8+ and on the right FoxP3CD8-. The statistical 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.

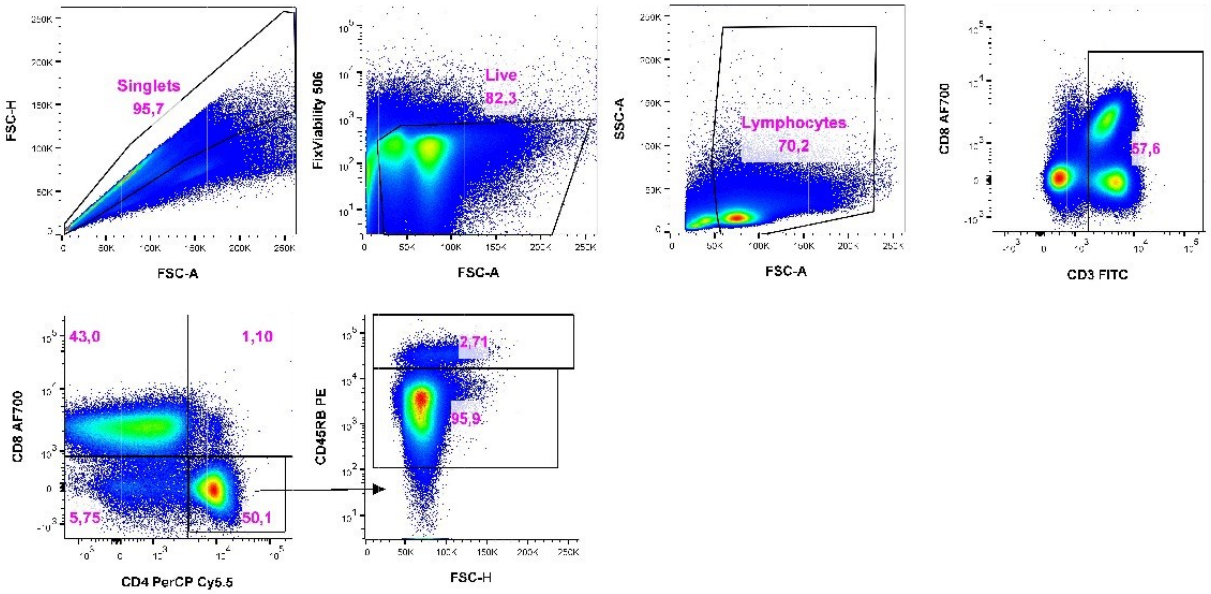

Figure 18 The gating strategy of CD45RB. Gates were set for single cells, next only viable cells were included in the analysis, then lymphocytes and monocytes were gated, then the gate was set at CD3+, then setting the gate for CD4+CD8- to finally gating the CD45RB subset population. The gating was done in FlowJo software.

In spleen, inguinal lymph nodes and Peyer's patches it was exhibited similar presence of CD3+CD4+CD45RB$_{low}$ T cells in GFD mice compared to STD (

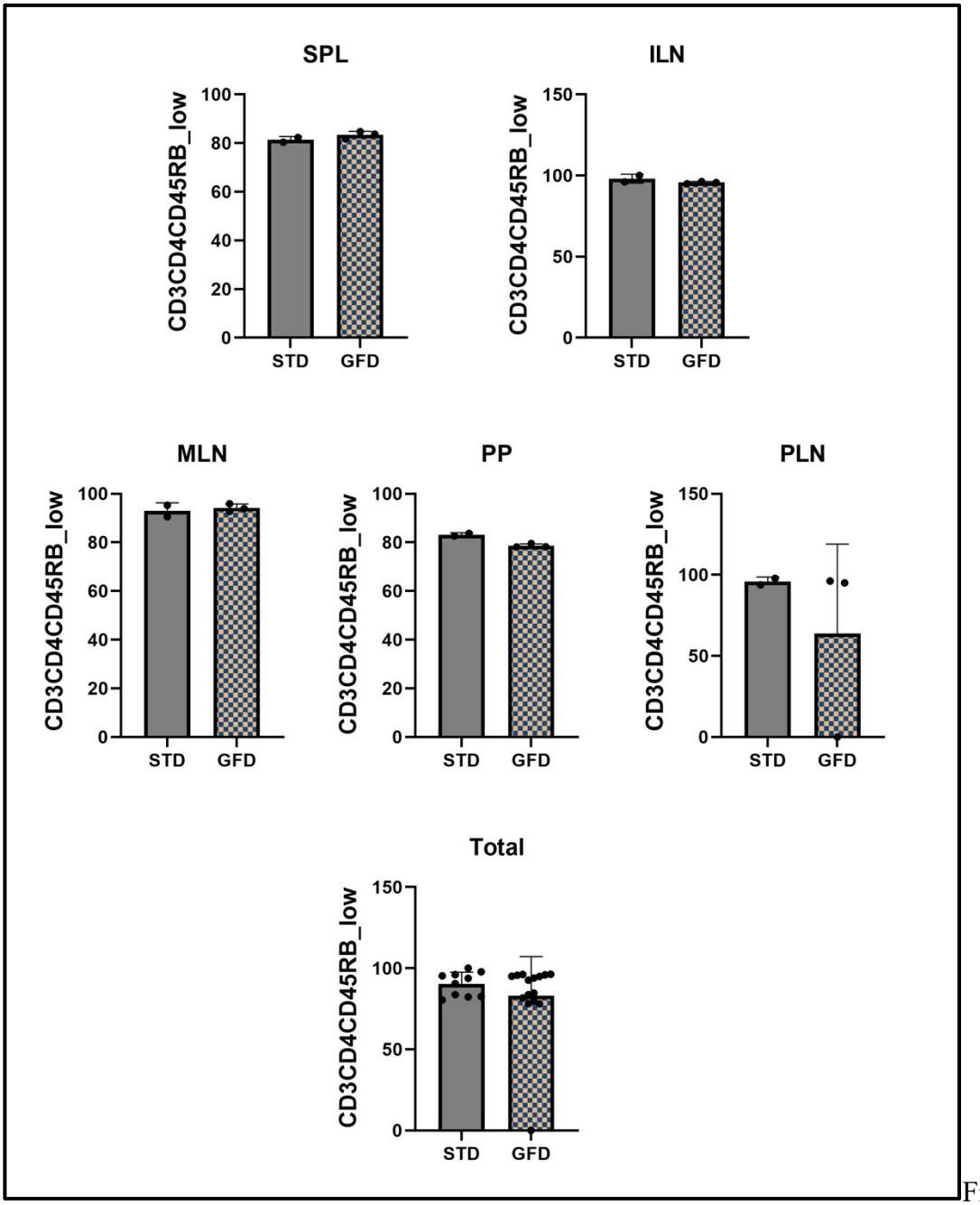

Figure *19*). It was not statistically significant as the total number of mice in the experiment was limited by expected death due to MPTP effect.

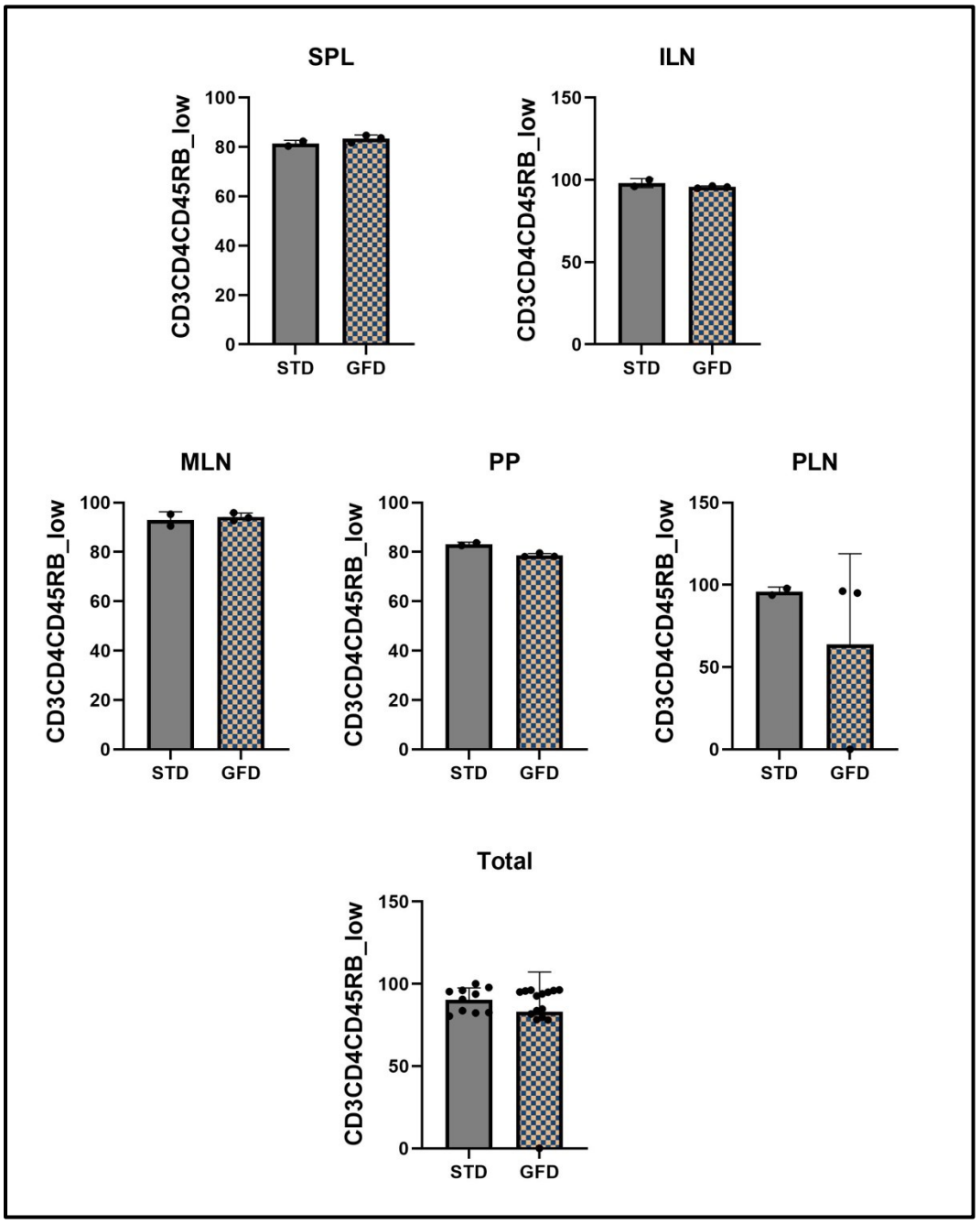

Figure 19 The percentage of CD3+CD4CD45RB$_{low}$ in STD and GFD groups in SPL, ILN, MLN, PLN and PP. As well the total percentage considering all organs. The statistical analysis was done with Two-way ANOVA and followed by Tukey's Multiple Comparisons Test.

5.3.4 Gating strategy for assessment of NKG2D subset

The comprehensive assessment of immune parameters in the context of mice subjected to chronic MPTP induction. This investigation focuses on a thorough examination of NKG2D and within this experimental setting.

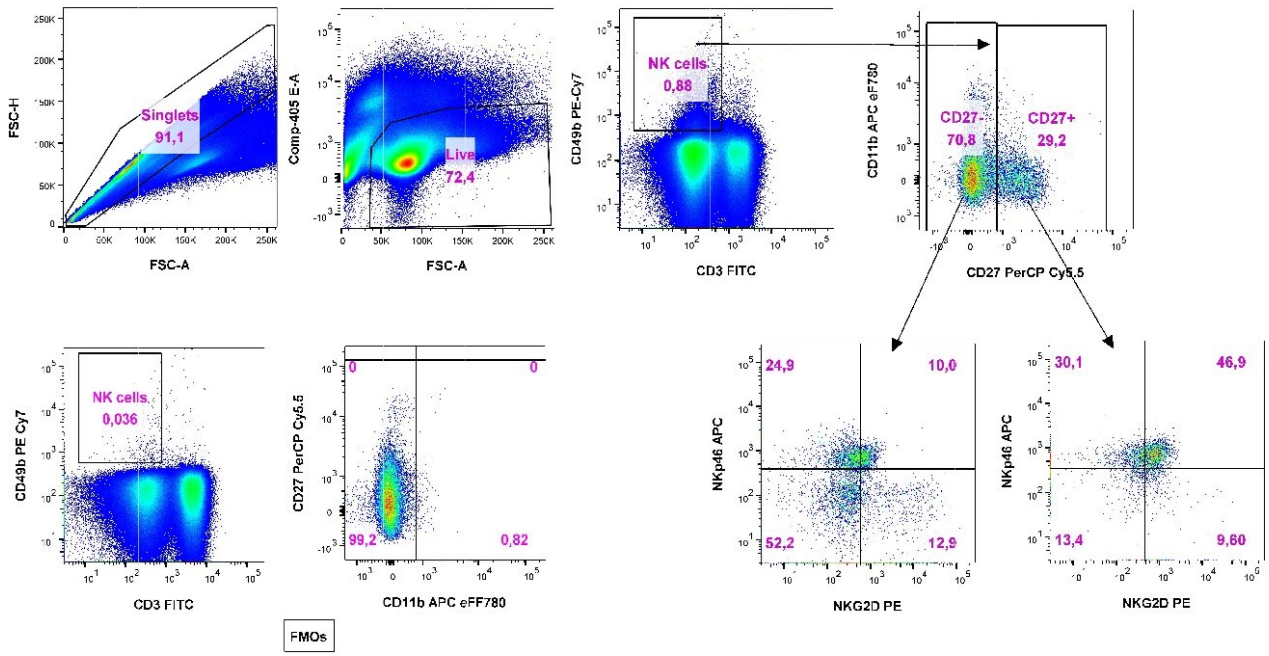

Figure 20 The gating strategy of NKG2D. Gates were set for single cells, next only viable cells were included in the analysis, then lymphocytes and monocytes were gated, then the gate was set at CD27- and CD27+ and then gating for NKG2D. The gating was done in FlowJo software.

In spleen and mesenteric lymph nodes slightly increased presence of NK CD27+ cells in GFD mice compared to STD (Figure 21), 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 expected death due to MPTP effect.

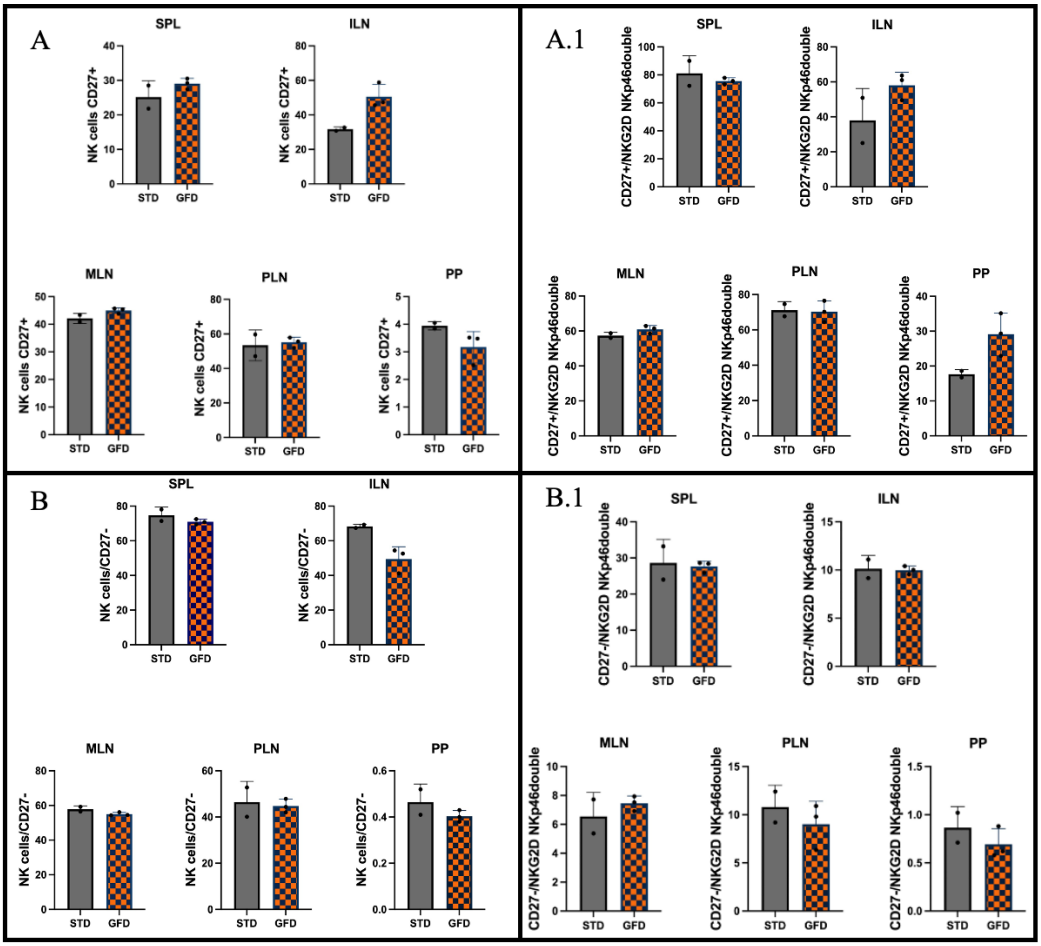

Figure 21 The percentages for every organ respectively: (A)The percentages of NKCD27+ in SPL, ILN, MLN, PLN and PP; (A.1) The percentages CD27+NKG2D NKp46 in SPL, ILN, MLN, PLN and PP; (B)The percentages of NKCD27- in SPL, ILN, MLN, PLN and PP; (B.1) The percentages CD27-NKG2D NKp46 in SPL, ILN, MLN, PLN and PP. The statistical analysis was done with Two-way ANOVA and followed by Tukey's Multiple Comparisons Test.

In all organs, according the Figure 22, it was exhibited slightly increased populations of NKCD27+ and CD27+NKG2DNKp46 in GFD mice compared to STD, however this trend was not confirmed by NKCD27-, CD27-NKG2DNKp46 subsets. It was not statistically significant as the total number of mice in the experiment was limited by expected death due to MPTP effect.

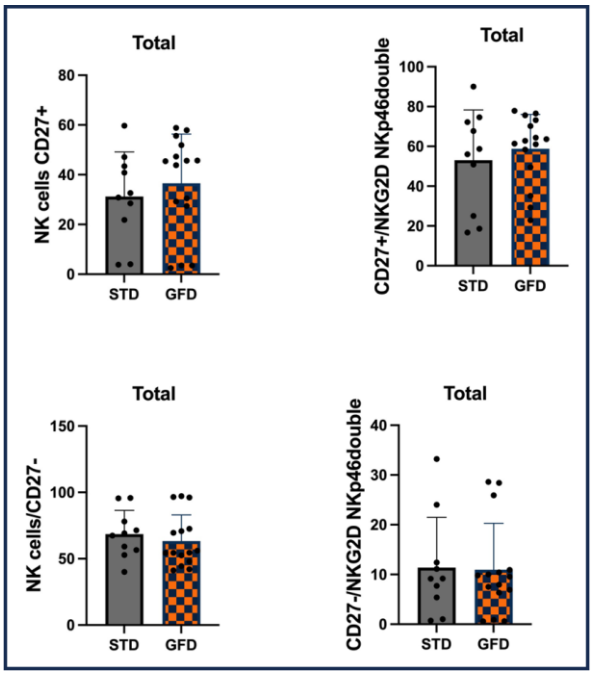

Figure 22 The percentages of NKCD27+, CD27+NKG2DNKp46, NKCD27-, CD27-NKG2D NKp46 for all organs jointly. The statistical analysis was done with Two-way ANOVA and followed by Tukey's Multiple Comparisons Test.

6 Discussion

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

The comparison of the effects of the STD and GFD on dopamine levels and dopaminergic neurons is critical in understanding the dietary influence on the dopaminergic system in the context of MPTP-induced neurodegeneration. Immunohistochemistry showed dopamine and tyrosine hydroxylase (TH) in relevant brain regions. To systematically assess dopaminergic neurons loss is necessary, for instance high-performance liquid chromatography (HPLC) for dopamine measurements, cell counting, and stereology, which will be a complementary analysis to be performed in the future.

The results of immunofluorescence staining for MPTP-treated specimens and control samples with PBS demonstrated effective staining procedures for both TH and DAPI. In both the MPTP and control groups, the IF staining protocol for tyrosine hydroxylase produced distinct signals indicating TH-positive cells Figure 9 identified by the green dots.

DAPI staining successfully marked cell nuclei in the examined tissues at the same time. The combined staining method enabled the visualization of tyrosine hydroxylase expression as well as nuclear morphology in relevant brain regions, visible in the Figure 9 as blue dots.

These findings validate the IF staining procedures, providing a solid foundation for future research into dopaminergic neuronal populations and their potential alterations in the MPTP-induced model compared to the PBS-treated control group.

When the MPTP-injected brains were compared to the control brains Figure 8, 9 the staining intensity and overall density of dopaminergic neurons were significantly reduced. This evidence highlights the successful induction of dopaminergic loss following MPTP injection, substantiating the experimental model's efficacy in replicating key neurochemical alterations associated with Parkinson's disease. The observed changes in dopaminergic populations strengthen the model's reliability and provide critical insights into MPTP's neurodegenerative effects, setting an adequate basis for subsequent analyses and interpretations in the context of Parkinsonian pathophysiology.

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 10. However, noteworthy trends emerged upon further examination (Figure 10-Panel B).

Nevertheless, over an extended 12-week duration under the chronic MPTP model, a significant 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 11.

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) what was observed

In 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 extended trajectory and exploration inferred from the results may imply that the Gluten-Free Diet can contribute reducing motor symptoms on 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 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 production of T cells

We have assessed the possible changes in proportions of Foxp3 Tregs and their Il-10 production in in mucosal and non-mucosal lymphoid organs from our first experiment using the chronic MPTP mouse model of PD Figure 14.

In addition, changes in proportions of CD4 and CD8 T cells and their cytokine profiles (Il-10, IFN g) were examined.

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 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 an immune response's effector and regulatory arms, which may contribute to the development of various immune-mediated diseases in 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 CD3 FoxP3 in GFD mice in spleen, inguinal lymph nodes, mesenteric lymph nodes and Peyer's Patches; in inguinal and pancreatic lymph nodes it was exhibited enhanced IL-10 production in GFD mice compared to STD (see Figure 13; Figure 14).

These changes hold particular significance when observed in situ, specifically within the mucosal immune system as represented by mesenteric lymph nodes (MLNs) and peripheral 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

Initially identified as regulatory T cells in inflammatory bowel disease (IBD), CD45RB-low CD4 T cells have been characterized as such (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 Parkinson`s disease (Saunders et al. 2012).

In the spleen, inguinal lymph nodes, and Peyer's patches, mice following a GFD displayed a similar presence of CD3+CD4+CD45RB-low T cells when contrasted with those on a STD (Figure 18). This observation underscores a noteworthy consistency in the distribution of this specific T cell subset within these immune niches.

While the exact implications of this uniformity merit further exploration, this consistent distribution across vital immune organs raises questions about the role of GFD in influencing the regulatory behavior of these T cells within these specific tissues. Further investigations are warranted to unravel the precise implications of this uniformity.

γδ T-cells

Effector immune responses of γδ T cells and also their possible pathogenic roles prevail in the literature, although in the field of T1D, from which we obtained the rational for testing the use of GFD in PD , mucosal gamma delta T cells were several times shown to display regulatory properties and to prevent type 1 diabetes.

Diabetic NOD mice display a lower proportion of γδ T-cells T cells (D. Funda, Peter Stenvang, and Buschard 1995) and protect NOD mice from development of diabetes via TGF-beta mechanisms (Han et al. 2010).

Interestingly, mucosal intranasal administration of insulin was shown to induce regulatory CD8+ γδ T cells and their adoptive transfer prevented development of T1D (Phillips, Trucco, and Giannoukakis 2011). Furthermore, in mouse, intraepithelial γδ T-cells are essential for establishing mucosal, oral tolerance (Locke et al. 2006).

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 Parkinson`s disease. The first study from 1994, reported an increased proportion of γδ T-cells in patients with Parkinson disease (Fiszer et al. 1994). More recently, 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 (Huang et al. 2021). These recent observations may support protective or regulatory roles of γδ T cells in PD.

Our data, aligns with the reported information, as a higher percentage of FoxP3-expressing CD8+ T cells were found in mice fed a GFD compared to those fed a STD (Figure 17- Panel A). Furthermore, in the GFD group, there was an increase in FoxP3-expressing CD8- T cells across all organs (Figure 17- Panel B). Upon examining the outcomes for individual organs, distinctly categorized by the dietary regimens, a discernible influence of the gluten-free diet (GFD) on the expression of γδ T cells becomes apparent when considering the graph plotted with all organs together.(Figure 17 -Panel C).

This effect is consistent with previous research indicating that dietary patterns influence the composition and functionality of T cell populations in various tissues. Additional study is needed to understand the underlying mechanisms and potential implications of this observed effect in the context of immunomodulation.

NK cells

CD27+ and CD27- NK cells subset was analyzed in lymphoid organs of MPTP treated mice on the GFD and STD diets. In addition, we assessed the expression of activation markers NKG2D and NKp46. The CD27+ sometime referred as regulatory (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. (Huang et al. 2021)

NK cells have been identified in the brains of patients with alpha-synucleopathies, including Parkinson's disease. Even more intriguing, 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).

Intrastriatal 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 natural killer (NK) cells. Notably, in the spleen and mesenteric lymph nodes, there is a marginal increase in the presence of NK CD27+ cells in mice on a gluten-free diet (GFD) compared to those on a standard diet (STD) (Figure 21). However, this observed trend is not consistently corroborated in other organs.

Examining the detailed breakdown in Figure 22, a subtle elevation in the populations of NKCD27+ and CD27+NKG2DNKp46 is discernible in GFD mice across all organs when juxtaposed with the STD group.

Interestingly, this trend is not mirrored in the NKCD27-, CD27-NKG2DNKp46 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 anticipated mortality associated with the MPTP effect.

7 Conclusion

In conclusion, our exploration into the immunomodulatory effects of a gluten-free diet (GFD) in the context of Parkinson's disease (PD) mouse models has yielded noteworthy findings. Mucosal FoxP3 Tregs, though not direct markers for decreased chronic inflammation, play a pivotal role in documenting shifts within the immune response. The chronic MPTP mouse model exhibits alterations indicative of a slightly elevated presence of CD3 FoxP3 and enhanced IL-10 production in GFD mice across various lymphoid organs. This is further supported by successful immunofluorescence, affirming the efficacy of MPTP in inducing neuroinflammation.

The uniform distribution of CD3+CD4+CD45RB-low T cells in the spleen, inguinal lymph nodes, and Peyer's patches under GFD conditions raises intriguing questions about the regulatory influence of GFD on these T cell subsets. Moreover, the study introduces novel insights into the unexplored territory of CD4+CD45RB-low T cells in PD mouse models, establishing a foundation for future investigations.

The data aligns with existing knowledge, showcasing increased FoxP3-expressing CD8+ and CD8- T cells in GFD mice. Additionally, the discernible influence of GFD on γδ T cells suggests a potential avenue for immunomodulation. Concurrently, the open field test reveals a higher exploration value in GFD mice, adding a behavioral dimension to our observations.

Despite the observed trends in natural killer (NK) cells lacking consistent replication across all organs, the successful immunofluorescence and enhanced exploration values in GFD mice underscore the complexity of the immune response in the context of dietary interventions. Further research is imperative to unravel the intricacies of these observed effects and ascertain their significance in the broader landscape of neuroinflammatory conditions and dietary modulation of the immune system.

Notably, the results revealed a significantly higher exploration value among mice on the GFD compared to those on the STD. This higher exploration value indicates an increased willingness of GFD mice to explore and engage with their environment. Such behavioral observations hold potential implications for the neurobehavioral effects associated with the gluten-free diet, suggesting a positive influence on exploratory behavior. The Open Field Test was an interesting approach to assess beyond molecular results, with a careful examination, it proved to be worthy for this project.

8 References

Adlercreutz, E. H., C. Weile, J. Larsen, K. Engkilde, D. Agardh, K. Buschard, and J. C. Antvorskov. 2014. "A Gluten-Free Diet Lowers NKG2D and Ligand Expression in BALB/c and Non-Obese Diabetic (NOD) Mice." *Clinical and Experimental Immunology* 177 (2): 391–403. https://doi.org/10.1111/cei.12340.

Antvorskov, Julie C., Petra Fundova, Karsten Buschard, and David P. Funda. 2013. "Dietary Gluten Alters the Balance of Pro-Inflammatory and Anti-Inflammatory Cytokines in T Cells of BALB/c Mice." *Immunology* 138 (1): 23–33. https://doi.org/10.1111/imm.12007.

Antvorskov, Julie C., Knud Josefsen, Kåre Engkilde, David P. Funda, and Karsten Buschard. 2014. "Dietary Gluten and the Development of Type 1 Diabetes." *Diabetologia*. Springer Verlag. https://doi.org/10.1007/s00125-014-3265-1.

Antvorskov, Julie Christine, Petra Fundova, Karsten Buschard, and David P. Funda. 2012. "Impact of Dietary Gluten on Regulatory T Cells and Th17 Cells in BALB/c Mice." *PloS One* 7 (3): e33315. https://doi.org/10.1371/journal.pone.0033315.

Barnabei, Laura, Emmanuel Laplantine, William Mbongo, Frédéric Rieux-Laucat, and Robert Weil. 2021. "NF-κB: At the Borders of Autoimmunity and Inflammation." *Frontiers in Immunology* 12: 716469. https://doi.org/10.3389/fimmu.2021.716469.

Bisaglia, Marco. 2022. "Mediterranean Diet and Parkinson's Disease." *International Journal of Molecular Sciences* 24 (1): 42. https://doi.org/10.3390/ijms24010042.

Bittinger, Maximilian, Jürgen Barnert, and Martin Wienbeck. 1999. "Autonomic Dysfunction and the Gastrointestinal Tract." *Clinical Autonomic Research* 9 (2): 75–81. https://doi.org/10.1007/BF02311763.

Braak, Heiko, Rob A.I. De Vos, Jürgen Bohl, and Kelly Del Tredici. 2006. "Gastric α-Synuclein Immunoreactive Inclusions in Meissner's and Auerbach's Plexuses in Cases Staged for Parkinson's Disease-Related Brain Pathology." *Neuroscience Letters* 396 (1): 67–72. https://doi.org/10.1016/j.neulet.2005.11.012.

Brakedal, Brage, Lilah Toker, Kristoffer Haugarvoll, and Charalampos Tzoulis. 2022. "A Nationwide Study of the Incidence, Prevalence and Mortality of Parkinson's Disease in the Norwegian Population." *Npj Parkinson's Disease* 8 (1): 19. https://doi.org/10.1038/s41531-022-00280-4.

Cen, Luan, Chaohao Yang, Shuxuan Huang, Miaomiao Zhou, Xiaolu Tang, Kaiping Li, Wenyuan Guo, et al. 2017. "Peripheral Lymphocyte Subsets as a Marker of Parkinson's Disease in a Chinese Population." *Neuroscience Bulletin* 33 (5): 493–500. https://doi.org/10.1007/s12264-017-0163-9.

Choi, Juli, Bo-Ram Kim, Begum Akuzum, Leechung Chang, June-Yong Lee, and Ho-Keun Kwon. 2022. "TREGking From Gut to Brain: The Control of Regulatory T Cells Along the Gut-Brain Axis." *Frontiers in Immunology* 13 (June): 916066. https://doi.org/10.3389/fimmu.2022.916066.

Danikowski, K. M., S. Jayaraman, and B. S. Prabhakar. 2017. "Regulatory T Cells in Multiple Sclerosis and Myasthenia Gravis." *Journal of Neuroinflammation* 14 (1): 117. https://doi.org/10.1186/s12974-017-0892-8.

Devos, David, Thibaud Lebouvier, Bernard Lardeux, Mandy Biraud, Tiphaine Rouaud, Hélène Pouclet, Emmanuel Coron, et al. 2013. "Colonic Inflammation in Parkinson's Disease." *Neurobiology of Disease* 50 (February): 42–48. https://doi.org/10.1016/j.nbd.2012.09.007.

Earls, Rachael H., and Jae-Kyung Lee. 2020. "The Role of Natural Killer Cells in Parkinson's Disease." *Experimental & Molecular Medicine* 52 (9): 1517–25. https://doi.org/10.1038/s12276-020-00505-7.

Earls, Rachael H., Kelly B. Menees, Jaegwon Chung, Claire-Anne Gutekunst, Hyun Joon Lee, Manuel G. Hazim, Balázs Rada, Levi B. Wood, and Jae-Kyung Lee. 2020. "NK Cells Clear α-Synuclein and the Depletion of NK Cells Exacerbates Synuclein Pathology in a Mouse Model of α-Synucleinopathy." *Proceedings of the National Academy of Sciences* 117 (3): 1762–71. https://doi.org/10.1073/pnas.1909110117.

Fiszer, U., E. Mix, S. Fredrikson, V. Kostulas, T. Olsson, and H. Link. 1994. "Gamma Delta+ T Cells Are Increased in Patients with Parkinson's Disease." *Journal of the Neurological Sciences* 121 (1): 39–45. https://doi.org/10.1016/0022-510x(94)90154-6.

Fu, Binqing, Zhigang Tian, and Haiming Wei. 2014. "Subsets of Human Natural Killer Cells and Their Regulatory Effects." *Immunology* 141 (4): 483–89. https://doi.org/10.1111/imm.12224.

Funda, D. P., A. Kaas, T. Bock, H. Tlaskalová-Hogenová, and K. Buschard. 1999. "Gluten-Free Diet Prevents Diabetes in NOD Mice." *Diabetes/Metabolism Research and Reviews* 15 (5): 323–27. https://doi.org/10.1002/(sici)1520-7560(199909/10)15:5<323::aid-dmrr53>3.0.co;2-p.

Funda, David, Jens Peter Stenvang, and Karsten Buschard. 1995. "Age-Related Changes in Tγδ Cells of NOD Mice." *Immunology Letters* 45 (3): 179–84. https://doi.org/10.1016/0165-2478(95)00003-N.

Goetz, Christopher G. 2011. "The History of Parkinson's Disease: Early Clinical Descriptions and Neurological Therapies." *Cold Spring Harbor Perspectives in Medicine* 1 (1): a008862. https://doi.org/10.1101/cshperspect.a008862.

Goldberg, Emily L., Ryan D. Molony, Eriko Kudo, Sviatoslav Sidorov, Yong Kong, Vishwa Deep Dixit, and Akiko Iwasaki. 2019. "Ketogenic Diet Activates Protective Γδ T Cell Responses against Influenza Virus Infection." *Science Immunology* 4 (41): eaav2026. https://doi.org/10.1126/sciimmunol.aav2026.

Gould, Todd D., David T. Dao, and Colleen E. Kovacsics. 2009. "The Open Field Test." In *Mood and Anxiety Related Phenotypes in Mice*, edited by Todd D. Gould, 42:1–20. Neuromethods. Totowa, NJ: Humana Press. https://doi.org/10.1007/978-1-60761-303-9_1.

Han, Gencheng, Renxi Wang, Guojiang Chen, Jianan Wang, Ruonan Xu, Liyan Wang, Jiannan Feng, et al. 2010. "Interleukin-17-producing $\Gamma\delta^+$ T Cells Protect NOD Mice from Type 1 Diabetes through a Mechanism Involving Transforming Growth Factor-β." *Immunology* 129 (2): 197–206. https://doi.org/10.1111/j.1365-2567.2009.03166.x.

Hansen, Camilla Hartmann Friis, Lukasz Krych, Karsten Buschard, Stine B. Metzdorff, Christine Nellemann, Lars H. Hansen, Dennis S. Nielsen, Hanne Frøkiær, Søren Skov, and Axel K. Hansen. 2014. "A Maternal Gluten-Free Diet Reduces Inflammation and Diabetes Incidence in the Offspring of NOD Mice." *Diabetes* 63 (8): 2821–32. https://doi.org/10.2337/db13-1612.

Haupt-Jorgensen, Martin, Jesper Larsen, Knud Josefsen, Tina Z. Jørgensen, Julie Christine Antvorskov, Axel K. Hansen, and Karsten Buschard. 2018. "Gluten-Free Diet during Pregnancy Alleviates Signs of Diabetes and Celiac Disease in NOD Mouse Offspring." *Diabetes/Metabolism Research and Reviews* 34 (4): e2987. https://doi.org/10.1002/dmrr.2987.

He, Feng, and Rudi Balling. 2013. "The Role of Regulatory T Cells in Neurodegenerative Diseases." *Wiley Interdisciplinary Reviews. Systems Biology and Medicine* 5 (2): 153–80. https://doi.org/10.1002/wsbm.1187.

Huang, Yilin, Huisheng Liu, Jiaqi Hu, Chongyin Han, Zhenggang Zhong, Wei Luo, Yuhu Zhang, and Fei Ling. 2021. "Significant Difference of Immune Cell Fractions and Their Correlations With Differential Expression Genes in Parkinson's Disease." *Frontiers in Aging Neuroscience* 13: 686066. https://doi.org/10.3389/fnagi.2021.686066.

Jackson-Lewis, Vernice, and Serge Przedborski. 2007. “Protocol for the MPTP Mouse Model of Parkinson’s Disease.” *Nature Protocols* 2 (1): 141–51. https://doi.org/10.1038/nprot.2006.342.

Jelínková, Lenka, Ludmila Tučková, Jana Cinová, Zuzana Flegelová, and Helena Tlaskalová-Hogenová. 2004. “Gliadin Stimulates Human Monocytes to Production of IL-8 and TNF-α through a Mechanism Involving NF-κB.” *FEBS Letters* 571 (1–3): 81–85. https://doi.org/10.1016/j.febslet.2004.06.057.

Jiang, Qi, Guocan Yang, Qi Liu, Shengjun Wang, and Dawei Cui. 2021. “Function and Role of Regulatory T Cells in Rheumatoid Arthritis.” *Frontiers in Immunology* 12: 626193. https://doi.org/10.3389/fimmu.2021.626193.

Kelley, D. S., P. A. Daudu, L. B. Branch, H. L. Johnson, P. C. Taylor, and B. Mackey. 1994. “Energy Restriction Decreases Number of Circulating Natural Killer Cells and Serum Levels of Immunoglobulins in Overweight Women.” *European Journal of Clinical Nutrition* 48 (1): 9–18.

K�ttgen, E., F. Kluge, B. Volk, and W. Gerok. 1983. “The Lectin Properties of Gluten as the Basis of the Pathomechanism of Gluten-Sensitive Enteropathy.” *Klinische Wochenschrift* 61 (2): 111–12. https://doi.org/10.1007/BF01496664.

Li, Jingyi, Jingwei Zhao, Longmin Chen, Hongling Gao, Jing Zhang, Danlei Wang, Yuan Zou, et al. 2023. “α-Synuclein Induces Th17 Differentiation and Impairs the Function and Stability of Tregs by Promoting RORC Transcription in Parkinson’s Disease.” *Brain, Behavior, and Immunity* 108 (February): 32–44. https://doi.org/10.1016/j.bbi.2022.10.023.

Liston, Adrian, James Dooley, and Lidia Yshii. 2022. “Brain-Resident Regulatory T Cells and Their Role in Health and Disease.” *Immunology Letters* 248 (August): 26–30. https://doi.org/10.1016/j.imlet.2022.06.005.

Locke, Natasha R., Sanda Stankovic, David P. Funda, and Leonard C. Harrison. 2006. “TCRγδ Intraepithelial Lymphocytes Are Required for Self-Tolerance.” *The Journal of Immunology* 176 (11): 6553–59. https://doi.org/10.4049/jimmunol.176.11.6553.

Mallone, Roberto, and Bart O. Roep. 2013. “Biomarkers for Immune Intervention Trials in Type 1 Diabetes.” *Clinical Immunology (Orlando, Fla.)* 149 (3): 286–96. https://doi.org/10.1016/j.clim.2013.02.009.

Marietta, Eric V., Andres M. Gomez, Carl Yeoman, Ashenafi Y. Tilahun, Chad R. Clark, David H. Luckey, Joseph A. Murray, Bryan A. White, Yogish C. Kudva, and Govindarajan Rajagopalan. 2013. “Low Incidence of Spontaneous Type 1 Diabetes in Non-Obese Diabetic Mice Raised on Gluten-Free Diets Is Associated with Changes in the Intestinal Microbiome.” *PloS One* 8 (11): e78687. https://doi.org/10.1371/journal.pone.0078687.

Mischley, Laurie K., Richard C. Lau, and Rachel D. Bennett. 2017. “Role of Diet and Nutritional Supplements in Parkinson’s Disease Progression.” *Oxidative Medicine and Cellular Longevity* 2017: 6405278. https://doi.org/10.1155/2017/6405278.

Mulak, Agata. 2015. “Brain-Gut-Microbiota Axis in Parkinson’s Disease.” *World Journal of Gastroenterology* 21 (37): 10609. https://doi.org/10.3748/wjg.v21.i37.10609.

Muñoz, Ana, Andrea Lopez-Lopez, Carmen M. Labandeira, and Jose L. Labandeira-Garcia. 2020. “Interactions Between the Serotonergic and Other Neurotransmitter Systems in the Basal Ganglia: Role in Parkinson’s Disease and Adverse Effects of L-DOPA.” *Frontiers in Neuroanatomy* 14 (June): 26. https://doi.org/10.3389/fnana.2020.00026.

Neuman, Vit, Stepanka Pruhova, Michal Kulich, Stanislava Kolouskova, Jan Vosahlo, Martina Romanova, Lenka Petruzelkova, et al. 2020. “Gluten-free Diet in Children with Recent-onset Type 1 Diabetes: A 12-month Intervention Trial.” *Diabetes, Obesity and Metabolism* 22 (5): 866–72. https://doi.org/10.1111/dom.13974.

Palová-Jelínková, Lenka, Klára Dáňová, Hana Drašarová, Miloš Dvořák, David P. Funda, Petra Fundová, Anna Kotrbová-Kozak, et al. 2013. "Pepsin Digest of Wheat Gliadin Fraction Increases Production of IL-1β via TLR4/MyD88/TRIF/MAPK/NF-κB Signaling Pathway and an NLRP3 Inflammasome Activation." Edited by Yolanda Sanz. *PLoS ONE* 8 (4): e62426. https://doi.org/10.1371/journal.pone.0062426.

Palová-Jelínková, Lenka, Daniela Rožková, Barbara Pecharová, Jiřina Bártová, Anna Šedivá, Helena Tlaskalová-Hogenová, Radek Spíšek, and Ludmila Tučková. 2005. "Gliadin Fragments Induce Phenotypic and Functional Maturation of Human Dendritic Cells." *The Journal of Immunology* 175 (10): 7038–45. https://doi.org/10.4049/jimmunol.175.10.7038.

Pastore, Matteo-Rocco, Elena Bazzigaluppi, Cristina Belloni, Claudia Arcovio, Ezio Bonifacio, and Emanuele Bosi. 2003. "Six Months of Gluten-Free Diet Do Not Influence Autoantibody Titers, but Improve Insulin Secretion in Subjects at High Risk for Type 1 Diabetes." *The Journal of Clinical Endocrinology & Metabolism* 88 (1): 162–65. https://doi.org/10.1210/jc.2002-021177.

Petzold, Cathleen, Julia Riewaldt, Deepika Watts, Tim Sparwasser, Sonja Schallenberg, and Karsten Kretschmer. 2013. "Foxp3(+) Regulatory T Cells in Mouse Models of Type 1 Diabetes." *Journal of Diabetes Research* 2013: 940710. https://doi.org/10.1155/2013/940710.

Phillips, Brett, Massimo Trucco, and Nick Giannoukakis. 2011. "Current State of Type 1 Diabetes Immunotherapy: Incremental Advances, Huge Leaps, or More of the Same?" Edited by Aziz Alami Chentoufi. *Clinical and Developmental Immunology* 2011 (July): 432016. https://doi.org/10.1155/2011/432016.

Powrie, F., M. W. Leach, S. Mauze, L. B. Caddle, and R. L. Coffman. 1993. "Phenotypically Distinct Subsets of CD4+ T Cells Induce or Protect from Chronic Intestinal Inflammation in C. B-17 Scid Mice." *International Immunology* 5 (11): 1461–71. https://doi.org/10.1093/intimm/5.11.1461.

Read, S., S. Mauze, C. Asseman, A. Bean, R. Coffman, and F. Powrie. 1998. "CD38+ CD45RB(Low) CD4+ T Cells: A Population of T Cells with Immune Regulatory Activities in Vitro." *European Journal of Immunology* 28 (11): 3435–47. https://doi.org/10.1002/(SICI)1521-4141(199811)28:11<3435::AID-IMMU3435>3.0.CO;2-P.

Read, S., and F. Powrie. 2001. "Induction of Inflammatory Bowel Disease in Immunodeficient Mice by Depletion of Regulatory T Cells." *Current Protocols in Immunology* Chapter 15 (May): Unit 15.13. https://doi.org/10.1002/0471142735.im1513s30.

Rizzi, Giorgio, and Kelly R. Tan. 2017. "Dopamine and Acetylcholine, a Circuit Point of View in Parkinson's Disease." *Frontiers in Neural Circuits* 11 (December): 110. https://doi.org/10.3389/fncir.2017.00110.

Sakaguchi, Shimon, Norihisa Mikami, James B. Wing, Atsushi Tanaka, Kenji Ichiyama, and Naganari Ohkura. 2020. "Regulatory T Cells and Human Disease." *Annual Review of Immunology* 38 (April): 541–66. https://doi.org/10.1146/annurev-immunol-042718-041717.

Saunders, Jessica A. Hutter, Katherine A. Estes, Lisa M. Kosloski, Heather E. Allen, Kathryn M. Dempsey, Diego R. Torres-Russotto, Jane L. Meza, et al. 2012. "CD4+ Regulatory and Effector/Memory T Cell Subsets Profile Motor Dysfunction in Parkinson's Disease." *Journal of Neuroimmune Pharmacology: The Official Journal of the Society on NeuroImmune Pharmacology* 7 (4): 927–38. https://doi.org/10.1007/s11481-012-9402-z.

Scheperjans, Filip, Velma Aho, Pedro A. B. Pereira, Kaisa Koskinen, Lars Paulin, Eero Pekkonen, Elena Haapaniemi, et al. 2015. "Gut Microbiota Are Related to Parkinson's

Disease and Clinical Phenotype." *Movement Disorders* 30 (3): 350–58. https://doi.org/10.1002/mds.26069.

Schmid, Sandra, Kerstin Koczwara, Susanne Schwinghammer, Vito Lampasona, Anette-G. Ziegler, and Ezio Bonifacio. 2004. "Delayed Exposure to Wheat and Barley Proteins Reduces Diabetes Incidence in Non-Obese Diabetic Mice." *Clinical Immunology (Orlando, Fla.)* 111 (1): 108–18. https://doi.org/10.1016/j.clim.2003.09.012.

Sharma, Amit, and Dipayan Rudra. 2019. "Regulatory T Cells as Therapeutic Targets and Mediators." *International Reviews of Immunology* 38 (5): 183–203. https://doi.org/10.1080/08830185.2019.1621310.

Shimada, Akira, Patricia Rohane, C. Garrison Fathman, and Brett Charlton. 1996. "Pathogenic and Protective Roles of CD45RBlow CD4+ Cells Correlate with Cytokine Profiles in the Spontaneously Autoimmune Diabetic Mouse." *Diabetes*, January 1996. Gale Academic OneFile.

Sildorf, S. M., S. Fredheim, J. Svensson, and K. Buschard. 2012. "Remission without Insulin Therapy on Gluten-Free Diet in a 6-Year Old Boy with Type 1 Diabetes Mellitus." *Case Reports* 2012 (jun21 1): bcr0220125878–bcr0220125878. https://doi.org/10.1136/bcr.02.2012.5878.

Tan, Eng-King, Yin-Xia Chao, Andrew West, Ling-Ling Chan, Werner Poewe, and Joseph Jankovic. 2020. "Parkinson Disease and the Immune System — Associations, Mechanisms and Therapeutics." *Nature Reviews Neurology* 16 (6): 303–18. https://doi.org/10.1038/s41582-020-0344-4.

Thomas, Karen E., Anna Sapone, Alessio Fasano, and Stefanie N. Vogel. 2006. "Gliadin Stimulation of Murine Macrophage Inflammatory Gene Expression and Intestinal Permeability Are MyD88-Dependent: Role of the Innate Immune Response in Celiac Disease." *The Journal of Immunology* 176 (4): 2512–21. https://doi.org/10.4049/jimmunol.176.4.2512.

Vossen, Mireille T. M., Mourad Matmati, Kirsten M. L. Hertoghs, Paul A. Baars, Mi-Ran Gent, Georges Leclercq, Jörg Hamann, Taco W. Kuijpers, and René A. W. van Lier. 2008. "CD27 Defines Phenotypically and Functionally Different Human NK Cell Subsets." *Journal of Immunology (Baltimore, Md.: 1950)* 180 (6): 3739–45. https://doi.org/10.4049/jimmunol.180.6.3739.

Wang, G. S., H. Gruber, P. Smyth, O. Pulido, L. Rosenberg, W. Duguid, and F. W. Scott. 2000. "Hydrolysed Casein Diet Protects BB Rats from Developing Diabetes by Promoting Islet Neogenesis." *Journal of Autoimmunity* 15 (4): 407–16. https://doi.org/10.1006/jaut.2000.0453.

Wang, Ji, Fushun Wang, Dongmei Mai, and Shaogang Qu. 2020. "Molecular Mechanisms of Glutamate Toxicity in Parkinson's Disease." *Frontiers in Neuroscience* 14 (November): 585584. https://doi.org/10.3389/fnins.2020.585584.

Xu, Yunlong, Chenyu Jiang, Junyan Wu, Peidong Liu, Xiaofei Deng, Yadong Zhang, Bo Peng, and Yingjie Zhu. 2022. "Ketogenic Diet Ameliorates Cognitive Impairment and Neuroinflammation in a Mouse Model of Alzheimer's Disease." *CNS Neuroscience & Therapeutics* 28 (4): 580–92. https://doi.org/10.1111/cns.13779.

Yang, Soo Jung, Akhilesh K. Singh, Travis Drow, Tori Tappen, Yuchi Honaker, Fariba Barahmand-Pour-Whitman, Peter S. Linsley, et al. 2022. "Pancreatic Islet-Specific Engineered Tregs Exhibit Robust Antigen-Specific and Bystander Immune Suppression in Type 1 Diabetes Models." *Science Translational Medicine* 14 (665): eabn1716. https://doi.org/10.1126/scitranslmed.abn1716.

Zhang, Le, Yingshuang Zhang, and Dongsheng Fan. 2022. "Pathological Role of Natural Killer Cells in Parkinson's Disease: A Systematic Review." *Frontiers in Aging Neuroscience* 14 (May): 890816. https://doi.org/10.3389/fnagi.2022.890816.

Zhang, Yuxia, Esther Bandala-Sanchez, and Leonard C. Harrison. 2012. “Revisiting Regulatory T Cells in Type 1 Diabetes.” *Current Opinion in Endocrinology, Diabetes, and Obesity* 19 (4): 271–78. https://doi.org/10.1097/MED.0b013e328355a2d5.

Zhang, Zhu, Shiqing Zhang, Pengfei Fu, Zhang Zhang, Kaili Lin, Joshua Ka-Shun Ko, and Ken Kin-Lam Yung. 2019. “Roles of Glutamate Receptors in Parkinson’s Disease.” *International Journal of Molecular Sciences* 20 (18): 4391. https://doi.org/10.3390/ijms20184391.

Zhao, Yuan, Xin Liu, and Guofeng Yang. 2023. “Adenosinergic Pathway in Parkinson’s Disease: Recent Advances and Therapeutic Perspective.” *Molecular Neurobiology* 60 (6): 3054–70. https://doi.org/10.1007/s12035-023-03257-3.

Zhou, Chao, Xinhua Zhou, Dan He, Zhen Li, Xufang Xie, and Yue Ren. 2020. “Reduction of Peripheral Blood iNKT and γδT Cells in Patients With Parkinson’s Disease: An Observational Study.” *Frontiers in Immunology* 11: 1329. https://doi.org/10.3389/fimmu.2020.01329.