Julius-Maximilians-Universität Würzburg
Department of Neurology
University Hospital Würzburg

Targeting Regulatory T Cells by CD28 Superagonistic Antibodies Mitigates Neurodegeneration in the A53T-α-Synuclein Parkinson's Disease Mouse Model

Aktivierung Regulatorischer T-Zellen durch die Superagonistische CD28 Antikörper mindert die Neurodegeneration im A53t-α-Synuclein Parkinsonkrankheitsmausmodell

Doctoral thesis for a doctoral degree
at the Graduate School of Life Sciences,
Julius-Maximilians-Universität Würzburg,
Section Neuroscience

Submitted by

MOHAMMAD MAMDOUH ABDELWARETH MOHAMMAD BADR

From

Giza, Egypt

Würzburg, 2022

Members of Thesis Committee

Chairperson:	Prof. Dr. Carmen Villmann Institute of Clinical Neurobiology University Hospital Würzburg
Primary Supervisor:	Prof. Dr. med. Chi Wang Ip Department of Neurology University Hospital Würzburg
Second Supervisor:	Prof. Dr. Manfred Lutz Institute of Virology and Immunobiology University of Würzburg
Third Supervisor:	Prof. Dr. rer. nat. Rudolf Martini Department of Neurology University Hospital Würzburg

Affidavit

I hereby confirm that my thesis entitled: “Targeting Regulatory T Cells by CD28 Superagonistic Antibodies Mitigates Neurodegeneration in the A53T-α-Synuclein Parkinson’s Disease Mouse Model” is the result of my own work. I did not receive any help or support from commercial consultants. All source and/or materials are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Place, Date Signature

Eidesstaatliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation „ Aktivierung Regulatorischer T-Zellen durch die Superagonistische CD28 Antikörper mindert die Neurodegeneration im A53T-α-Synuclein Parkinsonkrankheitsmausmodell“ eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommeziellen Promotionsberater, angefertigt unde keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderem Prüfungsverfahren voregelegen hat.

Ort, Datum Unterschrift

Acknowledgements

I would like to express my sincere appreciation for my supervisor, Dr. Chi Wang Ip, who has the substance of a true leader. He stood by me during hard times, believed in my skills and ignited my motivation. He was patient in times of uncertainty and allowed me to grow through challenges and emerge as a better researcher. Without his persistent help, the goal of this project would not have been realized.

My gratitude goes equally to Dr. Manfred Lutz who was inspiring to me as I was still taking very first steps in the complex but beautiful world of immunology. He was keen to provide advice and recommendations to different stages of the project. Without his established knowledge, it would have been very difficult to navigate through this project.

I would like also to thank Dr. Rudolf Martini, who as my third supervisor, always provided interesting input during our annual discussion and had the knowledge and imagination to draw our attention to different angle while interpreting our data.

I am grateful for the support I received from my colleagues in the labs of Dr. Ip and Dr. Lutz. I would like to highlight here Louisa Frieß, Heike Menzel, Marion Heuer, Akua Karikari and Diyaaeldin Ashour who did not spare any effort to help me throughout the project with their knowledge and experience.

I wish also to acknowledge the members of the Graduate School of Life Sciences (GSLS) for their support in the course of my studies.

Finally, I would like to thank in this space my dear partner, my daughter and son, my parents, my sister, and my brother. Without their emotional support throughout this journey, I would have never surmounted the needed energy to wrap up this project. They provided me with meaning and aim and I am forever grateful for their presence in my life.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

Alzheimer's Disease:	AD
Amyotrophic Lateral Sclerosis:	ALS
3,3-Diaminobenzidine tetrahydrochloride dihydrate:	DAB
Empty AAV1/2 viral vector:	EV
AAV1/2 A53T-α-Synuclein viral vector:	haSyn
Interleukin:	IL
1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine:	MPTP
Multiple Sclerosis:	MS
6-hydroxydopamine:	6-OHDA
Parkinson's Disease:	PD
Rheumatoid Arthritis:	RA
Substantia Nigra	SN
Substantia nigra pars compacta	SNpc
Systemic Lupus Erythematosus:	SLE
Tyrosine Hydroxylase:	TH

Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disease with still no cure available. The prominent feature of PD is the loss of dopaminergic neurons at the Substantia nigra (SN). Genetic and environmental insults affecting the SNCA gene encoding the α-Synuclein (α-Syn) protein result into an aberrant form of the protein with higher propensity towards oligomerization becoming part of insoluble inclusions called Lewy Bodies (LB). LB impart cytotoxicity leading to neurodegeneration, activate resident microglia and escape to the periphery where they get captured by dendritic cells and presented to naïve T cells. Proliferating effector T lymphocytes invade the brain releasing proinflammatory cytokines and performing a cytotoxic effect on neurons.

In this study, we examine the hypothesis that the expansion of regulatory T cells (Treg) could exert an anti-inflammatory effect that averts neurodegeneration in the AAV1/2-A53T-α-Syn mouse model for PD.

Mice brains were transfected by a unilateral stereotaxic injection at the SN region with a chimeric Adeno-Associated Viral vector of serotypes 1 and 2 (AAV1/2) carrying the A53T-mutated human SNCA gene encoding the readily aggregating aberrant α-Syn (AAV1/2-A53T-α-Syn). One week after injection, mice were treated with the CD28 superagonistic antibody (CD28SA), known to significantly expand the Treg population. Mice were then analyzed by behavioral analysis using the Rotarod performance test and the Cylinder test. The impact of CD28SA on the immune system was examined by flow cytometry. The integrity of the nigrostriatal system was assessed by stereological quantification of Tyrosine hydroxylase (TH)-stained dopaminergic neurons in SN and optical density measurements of TH-stained striatum. The mechanism of action of CD28SA was analyzed by treating PD mice alternatively with a Treg adoptive transfer, while CD28SA effect on levels of neurotrophic factors was quantified by ELISA.

We observed an expansion of Treg by FACS analyses three days after CD28SA treatment, demonstrating target engagement. CD28SA treatment of AAV1/2-A53T-α-Syn mice provided neuroprotection evident through elevated numbers of dopaminergic neurons in the SN and higher optical density of TH-staining in the striatum, in CD28SA-treated mice compared to PBS-treated control mice, and that was reflected in an enhanced performance in behavioral studies. Additionally, brain infiltration of proinflammatory activated T lymphocytes ($CD4^+CD69^+$ and $CD8^+CD69^+$ cells), that were obvious in PBS-treated AAV1/2-A53T-α-Syn control mice, was augmented in PD mice receiving CD28SA. The alternative treatment with Treg adoptive transfer did replicate the beneficial effects of CD28SA indicating that Treg expansion is the main effector mechanism by which it exerts its neuroprotective effect. CD28SA treatment of PD mice led to an increase of GDNF and BDNF in some brain structures that was not observed in untreated mice.

We conclude that in the AAV1/2-A53T-α-Syn PD mouse model, CD28SA suppresses proinflammation, reverses behavioral deficits and is neuroprotective on SN dopaminergic cells.

Zusammenfassung

Die Parkinson-Krankheit (PD) ist die zweithäufigste neurodegenerative Erkrankung, für die es noch keine Heilung gibt. Das herausragende Merkmal von PD ist der Verlust von dopaminergen Neuronen an der Substantia nigra (SN). Genetische und umweltbedingte Schäden, die das SNCA-Gen betreffen, das für das α-Synuclein (α-Syn)-Protein kodiert, führen zu einer abweichenden Form des Proteins mit einer höheren Neigung zur Oligomerisierung, die Teil von unlöslichen Einschlüssen wird, die Lewy Bodies (LB) genannt werden. LB verleihen Zytotoxizität, die zu Neurodegeneration führt, aktivieren residente Mikroglia und entweichen in die Peripherie, wo sie von dendritischen Zellen eingefangen und naiven T-Zellen präsentiert werden. Proliferierende Effektor-T-Lymphozyten dringen in das Gehirn ein, setzen proinflammatorische Zytokine frei und üben eine zytotoxische Wirkung auf Neuronen aus.

In dieser Studie untersuchen wir die Hypothese, dass die Expansion regulatorischer T-Zellen (Treg) einen entzündungshemmenden Effekt ausüben könnte, der die Neurodegeneration im AAV1/2-A53T-α-Syn-Mausmodell für PD verhindert.

Mäusegehirne wurden durch eine unilaterale stereotaktische Injektion in die SN-Region mit einem chimären Adeno-Assoziierten viralen Vektor der Serotypen 1 und 2 (AAV1/2) transfiziert, der das A53T-mutierte humane SNCA-Gen trägt, das für das leicht aggregierende aberrante α-Syn (AAV1/ 2-A53T-α-Syn). Eine Woche nach der Injektion wurden die Mäuse mit dem superagonistischen CD28-Antikörper (CD28SA) behandelt, von dem bekannt ist, dass er die Treg-Population signifikant vergrößert. Mäuse wurden dann durch Verhaltensanalyse unter Verwendung des Rotarod-Leistungstests und des Zylindertests analysiert. Der Einfluss von CD28SA auf das Immunsystem wurde mittels Durchflusszytometrie untersucht. Die Integrität des nigrostriatalen Systems wurde durch stereologische Quantifizierung von Tyrosinhydroxylase (TH)-gefärbten dopaminergen Neuronen in SN und Messungen der optischen Dichte von TH-gefärbtem Striatum bewertet. Der Wirkungsmechanismus von

CD28SA wurde analysiert, indem PD-Mäuse alternativ mit einem adoptiven Treg-Transfer behandelt wurden, während die Wirkung von CD28SA auf die Spiegel neurotropher Faktoren durch ELISA quantifiziert wurde.

Wir beobachteten eine Expansion von Treg Zellen durch FACS-Analysen drei Tage nach der CD28SA-Behandlung, was ein biologisches Ansprechen der Treg auf CD28SA. Die CD28SA-Behandlung von AAV1/2-A53T-α-Syn-Mäusen lieferte bei CD28SA-behandelten Mäusen im Vergleich zu PBS-behandelten Kontrollmäusen eine Neuroprotektion, die durch eine erhöhte Anzahl von dopaminergen Neuronen im SN und eine höhere optische Dichte der TH-Färbung im Striatum ersichtlich ist. und das spiegelte sich in einer verbesserten Leistung in Verhaltensstudien wider. Darüber hinaus wurde die Hirninfiltration von proinflammatorisch aktivierten T-Lymphozyten ($CD4^+CD69^+$- und $CD8^+CD69^+$-Zellen), die bei PBS-behandelten AAV1/2-A53T-α-Syn-Kontrollmäusen offensichtlich war, bei PD-Mäusen, die CD28SA erhielten, verstärkt. Die alternative Behandlung mit dem adoptiven Treg-Transfer replizierte die vorteilhaften Wirkungen von CD28SA, was darauf hindeutet, dass die Treg-Expansion der Haupteffektormechanismus ist, durch den es seine neuroprotektive Wirkung ausübt. Die CD28SA-Behandlung von PD-Mäusen führte zu einem Anstieg von GDNF und BDNF in einigen Gehirnstrukturen, der bei unbehandelten Mäusen nicht beobachtet wurde.

Wir schlussfolgern, dass CD28SA im AAV1/2-A53T-α-Syn-PD-Mausmodell die Entzündungshemmung unterdrückt, Verhaltensdefizite umkehrt und auf SN-dopaminergen Zellen neuroprotektiv ist.

1.0 Introduction and Background

1.1.0 Overview of Parkinson's Disease

Parkinson's disease (PD) is a complex disease that is considered the most common neurodegenerative motor disorder worldwide (Kalia & Lang, 2015). First descriptions and characterizations of PD are credited to James Parkinson and Jean-martin Charcot. Ageing has an essential correlation with PD and that is why the fruit of modern medicine, namely less mortality, comes also with a cost since it endows a higher probability of developing PD and neurodegenerative diseases in general simply by the fact of living longer. The prevalence of PD in the age group of 65 – 80 years was established to fall between 0.5% to 3% (Nussbaum & Ellis, 2003; Tanner & Goldman, 1996) and is foreseen to increase to 30% by the year 2030 (R. C. Chen et al., 2001) presenting substantial social and economic costs.

1.2.0 Clinical Symptoms of Parkinson's Disease

Despite manifesting at early stages through some non-motor symptoms, clinically PD is classified by movement impairment in form of bradykinesia, gait dysfunction, rigidity, and resting tremor. Even though a single rooting cause for PD has not been elucidated, studies show on one hand that while genetic predisposition results in rare familial forms of PD, environmental factors like pesticide exposure and smoking may elevate the risks of developing sporadic PD. By the time, the patient is clinically diagnosed with PD, a lot of the dopamine depletion has already taken place irreversibly (Ma, Röyttä, Rinne, Collan, & Rinne, 1997). Therefore, clinical trials aiming at reversing the disease progress should ideally take place during the prodromal phase.

The clinical aspects of PD are a consequence of the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). The driving element for such loss is the aggregation of

alpha (α) synuclein proteins in pathological structures known as lewy bodies (LB). Several mechanisms have been proposed to explain neuronal death in PD, notably defective protein clearance, oxidative stress due to mitochondrial dysfunction and neuroinflammation.

Major PD treatments are palliative in nature focusing mainly on alleviating the symptoms by restoring the levels of dopamine in the striatum or engaging post synaptic dopamine receptors. Over time, motor symptoms become more pronounced, and complications of levodopa give rise to non-motor setbacks like psychosis, dementia, and hallucinations. At very advanced stages, symptoms grow resistant to therapy and postural instability develops leading to repeated falls and injuries (Martinez-Martin, Rodriguez-Blazquez, Kurtis, & Chaudhuri, 2011).

1.3.0 Etiology of Parkinson's Disease

The interplay of genetic and environmental influences renders PD as a classical example of a multifactorial condition, with age being the main risk factor (Lees, Hardy, & Revesz, 2009). There is also some ethnogeographical differences with PD more prevalent in Europe, North and South America, relative to African, Asia, and Arabic countries (Kalia & Lang, 2015).

Genetically, PD could be inherited both in autosomal dominant and recessive manner. About 10-15% of PD patients report a family history (Lees et al., 2009). The first identified gene to be involved in PD autosomal dominant inheritance was the SNCA gene encoding the α-synuclein protein. The first point mutation linked to SNCA was recorded in 1997 as an (A53T) point mutation (Polymeropoulos et al., 1997), after which four additional point mutations were described in addition to gene duplications and triplications (Appel-Cresswell et al., 2013; Chartier-Harlin et al., 2004; Krüger et al., 1998; Lesage et al., 2013; Singleton et al., 2003; Zarranz et al., 2004). However, the most common gene linked to familial PD is LRRK2 encoding Leucine rich repeat kinase 2. Six LRRK2 mutations have been identified, the most frequent of which is G2019S (Healy et al., 2008).

In 1983, some people injected themselves with a drug contaminated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and subsequently they developed PD symptoms, elevating the attention that PD could be caused by environmental toxins. MPTP is metabolized into MPP^+ which is a neurotoxin that inhibits mitochondrial complex I, interfering with cellular respiration and resulting in selective death of dopaminergic neurons in the SN (William Langston, Ballard, Tetrud, & Irwin, 1983; Xu et al., 2006). Moving forward, many studies showed a correlation between pesticides like paraquat and rotenone and PD, although mechanisms are not as well described as in the case of MPTP. The PD risk of different environmental agents has been quantified and it was shown that it increases with pesticides and head traumas and sinks with smoking and caffeine consumption (Bower et al., 2003; H. Chen et al., 2010; Hernán, Takkouche, Caamaño-Isorna, & Gestal-Otero, 2002). Equally important, genome-wide association studies confirmed a correlation between sporadic PD and numerous genetic polymorphisms affecting genes for proteins like α-synuclein and tau (Ross, 2013). This creates genetic vulnerability that when combined with specific environmental insults, precipitates the pathogenesis of PD.

1.4.0 Pathology of Parkinson's Disease

The most prominent morphological change in brains of PD patients is the loss of dark pigmentation in the SNpc which is a direct translation to the damage of dopaminergic neurons that contain neuromelanin (Dickson, 2012). By the onset of motor symptoms, it is estimated that 30% of DA neurons has already been lost, which deteriorates as the disease progress into more than 60% (Cheng, Ulane, & Burke, 2010; Fearnley & Lees, 1991; Giguère, Nanni, & Trudeau, 2018; Greffard et al., 2006; Kordower et al., 2013; Rudow et al., 2008). This neuronal death leads to diminishing dopamine levels in the striatum, which is believed to be the major contributing reason behind the PD motor symptoms. Some studies demonstrated that the loss of axon terminals projecting into the striatum may occur before the actual cell death (Kordower

et al., 2013). The main driving forces behind PD pathology are altered α-synuclein as well as neuroinflammation (Figure 1).

1.4.1 α-Synuclein

The defining feature of PD under the microscope is the irregular cytoplasmic disposition of protein aggregations composed mainly of α-synuclein protein which is widely expressed in the brain (Spillantini et al., 1997). Those pathological aggregations are known as lewy bodies. Proteinopathies are a class of disorders, where the misfolding of one or more proteins is the driving pathological mechanism. PD is considered accordingly α-synucleinopathy, with misfolded α-synuclein being the pathological hallmark. A common cause of proteinopathies is ageing, which even in isolation of any neurodegenerative disease, is considered a cause for accumulation of altered proteins (Elobeid, Libard, Leino, Popova, & Alafuzoff, 2016).

α-synuclein is present normally as an unfolded protein (Burré et al., 2013) which could interact with the phospholipids in the plasma membrane forming alpha helices (Desplats et al., 2011). In PD brains, it assumes different conformations rich in beta sheets and amyloid in nature. Subsequent modifications in form of ubiquitination, c-terminal truncation, or phosphorylation renders α-synuclein more liable to aggregation forming various species ranging from soluble oligomers to insoluble fibrils. Oligomeric forms are more toxic as they are capable of seeding acquiring in the process a characteristic ability to spread in a temporospatial fashion in the brain between adjacent cells through time (Barrett & Timothy Greenamyre, 2015; Fujiwara et al., 2002).

Toxicity of soluble misfolded α-synuclein could be attributed to mitochondrial dysfunction. Deficiencies in mitochondrial complex I are evident in postmortem studies of SNpc in PD brains (Schapira et al., 1990). This could be explained by the ability of α-synuclein to interact with the mitochondrial membrane and to reside within organelles, disrupting in the way

Complex-I activity and leading consequently to oxidative stress (Devi, Raghavendran, Prabhu, Avadhani, & Anandatheerthavarada, 2008; Luth, Stavrovskaya, Bartels, Kristal, & Selkoe, 2014). Moreover, parkinsonian symptoms arising following MPTP exposure were tracked to the fact that oxidized forms of MPTP inhibit Complex-I in DA neurons (Nicklas, Vyas, & Heikkila, 1985; William Langston et al., 1983). In both cases, this inhibition is significant because it unbalances energy metabolism and thus leads to cell death (Hattori & Mizuno, 2002). In normal conditions, soluble misfolded α-synuclein should be tagged and broken down by the ubiquitine-proteasome system or the autophagy lysosome pathway (Xilouri, Brekk, & Stefanis, 2013). In PD however, this process is interrupted where this machinery could be damaged contributing to the build-up of altered proteins and the resultant formation of toxic aggregates (Ebrahimi-Fakhari, Wahlster, & McLean, 2012; Lee, Khoshaghideh, Patel, & Lee, 2004).

1.4.2 Inflammation

Originally regarded as a secondary phenomenon, inflammation is recently gaining more traction as one driving mechanism rather than auxiliary consequence in PD. Typical immune proceedings, like microglial activation, lymphocyte infiltration and active involvement of proinflammatory cytokines have been demonstrated to take place in the striatum and SNpc of postmortem PD brains (E. C. Hirsch & Hunot, 2009; Hunot et al., 1999; Loeffler, Camp, & Conant, 2006; P. L. McGeer, Itagaki, Boyes, & McGeer, 1988). PD rodent models replicated the same conclusion and affirmed that inflammation could be induced through the engagement of microglia and α-synuclein (He, Appel, & Le, 2001; Su et al., 2008; Wu et al., 2002). Employing various models, it is now possible to construct a comprehensive view on the way lymphocytes and cytokine networks participate in neurodegeneration. A breakdown of those mechanisms follows in the next section.

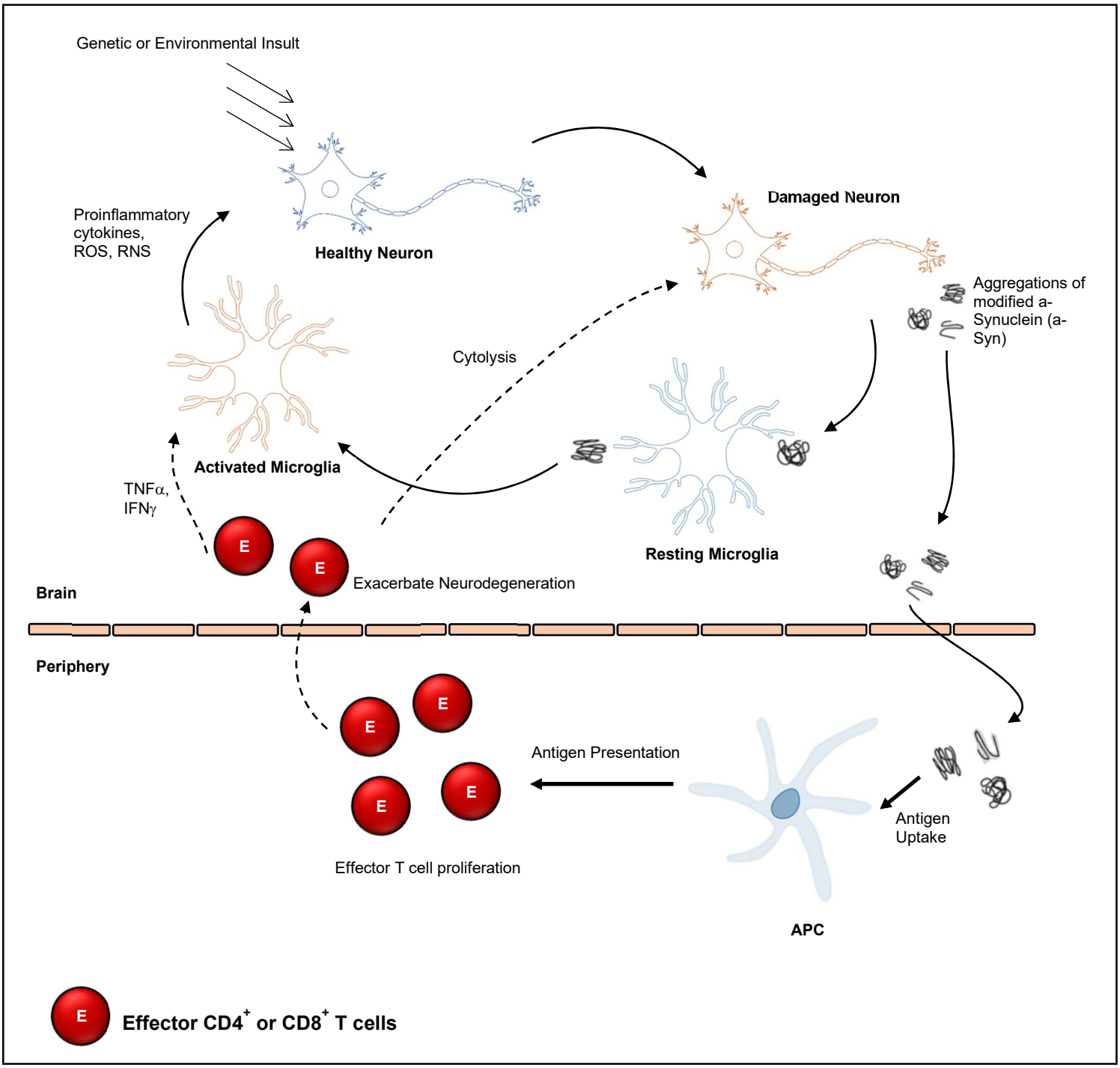

Figure 1: Mechanisms of neuroinflammation in PD pathology

1.5.0 Neuroinflammation in Parkinson's Disease

The central nervous system was believed for a long time to be an immune-privileged organ, meaning that it does not mount immune responses to antigens that reach the brain or spinal cord. That was backed then by longer survival of tissue grafts in the brain, that were otherwise quickly rejected by other organs (Mosley, Hutter-Saunders, Stone, & Gendelman, 2012). By now, this proved to be not exactly true since immune reactions do happen in the CNS and swift communication between the CNS and peripheral immune system is instrumental in brain development and infection control (Ferrari & Tarelli, 2011). Immune cells do not have, though, a free access to the brain since access is controlled by the blood brain barrier (BBB). BBB is viewed as a communication interface that coordinates two-way interaction between the immune system and CNS (Abbott, Patabendige, Dolman, Yusof, & Begley, 2010; Banks, 2015). During the last decade, a wealth of articles has explored the interplay of immune reactions and neurodegeneration with varying outcomes. Peripheral immunity was shown to exacerbate neurodegeneration in disorders like Alzheimer's disease (AD), Amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS) and PD (Arai, Furuya, Mizuno, & Mochizuki, 2006; Fiala & Veerhuis, 2010; Hasegawa, Inagaki, Sawada, & Suzumura, 2000; Heesen et al., 2010; Kamer et al., 2008; Perry, 2010; Reale et al., 2009; Teeling & Perry, 2009). Inflammation in those diseases disintegrates the BBB and allows leukocytes to patrol the brain parenchyma and provide neurodegenerative or neurotrophic influences. This knowledge is crucial if we are to consider neuroinflammation as a promising target for disease-modifying interventions of neurodegenerative disorders.

1.5.1 Access of Immune Cells to the Brain

While naïve T and B lymphocytes are normally excluded from entering the brain, activated lymphocytes monitor the CNS in few numbers. They are few because of the low expression of adhesion molecules on endothelial cells; they represent the effector mechanism that attracts

lymphocytes to the site of injury. When adhesion molecules become more surface-abundant on endothelial cells, they pave the way to brain access of T and B lymphocytes (Engelhardt & Ransohoff, 2005; Hickey, 2001; Togo et al., 2002). Microglia are the housekeeping agents of innate immune cells in the brain. If they are activated by CNS antigens, they release a host of proinflammatory mediators which raises the expression of adhesion molecules on endothelial cells and enhances the permeability of the BBB (Desai, Monahan, Carvey, & Hendey, 2007). Despite the absence of a clearly defined lymphatic system in the brain, CNS antigens can escape to the periphery through spinal nerve root ganglia, cranial nerves, and arachnoid villi (Cserr & Knopf, 1992). As soon as they travel in the lymph, they become on the way to interact with dendritic cells. Once there, antigens are internalized by dendritic cells, broken down and then presented at the surface whereby they engage and activate naïve T and B lymphocytes to mount an adaptive immune reaction. Activated lymphocytes can then move in a targeted way guided by endothelial cells through the leaky BBB to sites of CNS injury (E. G. McGeer & McGeer, 2003; Olson & Miller, 2004). While in the short term, this reaction could prove essential to restore homeostasis and recover normal brain activity following injury, on a longer term, it yields detrimental effects and predisposes neurodegeneration. Such chain of events, mobilizing immune effector cells to brain previously inaccessible sites, was described to take place in some neurological disorders like AD, PD and Neuro AIDS (Desai et al., 2007; Farkas et al., 2000; Petito & Cash, 1992).

1.5.2 Innate Immunity in Parkinson's Disease

At birth, innate immune scripts are written within germline cells and manifest in nonspecific and quick mechanisms that combat microbial infections. Physical hindrances like the skin are the first defensive ranks of a host and it regulates, on a cellular level, enzymes that eliminate pathogens and cell debris, phagocytose foreign agents, employ immune cells in sites of infection, trigger complement cascades and assist the adaptive immune system in recognizing

antigens as they process and present them to naïve lymphocytes. Such immune induction is carried out by common and nonspecific cell signaling pathways. Foreign material and cell debris interact with toll-like receptors that are part of the surface repertoire of microglia, astrocytes, and neurons. This interaction turns on signaling chains that culminate into the production of proinflammatory mediators and affect the brain tissue through glial cells and BBB leakage (Basith, Manavalan, Lee, Kim, & Choi, 2011; Filias et al., 2011; Goldmann et al., 2016; Greenwood et al., 2011; Kacimi, Giffard, & Yenari, 2011; Perry, 2012; Zurolo et al., 2011).

The innate immune system helps kick start the adaptive response by guiding the antigen presentation process to shape a specific response performed by activated T cells. Microglia are decisive in managing a harmonious network between innate and adaptive immune components that render the host capable of identifying various environmental signals and ultimately defending itself. Microglia secrete both anti- and- proinflammatory cytokines along with other factors that control the immune response, balance the neural environment, and maintains the neural activity. Following activation, microglia begin to release both neurotoxic mediators like IL-1α, IL-1β and TNFα, and neurotrophins like NT-3, NGF, TGFβ and IL-10. With continued activation, toxicity outweighs trophic factors and consequently aggravates the neurodegeneration. This inflammatory status and disrupted BBB open the doors for naïve T cells to infiltrate the brain and get exposed to activated microglia that acutely produce more proinflammatory mediators and chronically interact with activated T cells that has been primed and proliferated in the periphery and migrated to sites of neuronal injury (Desai et al., 2007; Mrdjen et al., 2018; Prinz & Priller, 2017; Qiu, Ye, Kholodenko, Seubert, & Selkoe, 1997; Wong, Prameya, & Dorovini-Zis, 1999).

Misfolded proteins are the main convict when it comes to the pathogenesis of PD as they are responsible for the involvement of microglia and antigen presenting cells (APC) and eventually the recruitment of the adaptive immune system. Some posttranslational modifications confer a certain liability for aggregation for α-synuclein protein. These aggregates form insoluble

inclusions known as LB that consist of α-synuclein, neurofilament and ubiquitin, and they are directly culpable for cytotoxicity in dopaminergic neurons. Within LB inclusions, α-synuclein is altered by phosphorylation, ubiquitination, nitration or oxidation (Cavallarin, Vicario, & Negro, 2012; Duffy et al., 2018; Fujiwara et al., 2002; Giasson et al., 2000; Jellinger, 2007; Shimura et al., 2001). Some autosomal dominant PD forms are initiated either by specific point mutations or gene duplications and triplications in the SNCA gene that increases its propensity to aggregate (Uversky, 2007). The previous remarks could be concisely narrowed into the "α-synuclein burden" hypothesis which postulates that the inability to clear those protein aggregates results into neuronal death (Patrick L. McGeer & McGeer, 2008). Considering PD from this angle, an ideal treatment strategy would be to reduce the α-synuclein production or preventing it from aggregation. Approaches like α-synuclein vaccination aims at enhancing the clearance of aggregated and misfolded proteins. Another angle to consider PD pathogenesis is a non-neuronal angle that is based on neuroinflammation as a driving mechanism that perpetuates neuronal death and whose modulation by immunotherapeutic means could eventually halt PD progression (Dawson, 2008).

Microglia, activated by aberrant α-synuclein, secretes reactive oxygen species (ROS) which elevates the nitration of α-synuclein further compounding the neuronal death. An MPTP PD model demonstrates that T cells patrolling the CNS are primed against aberrant α-synuclein antigens, and then they readily ingress to the sites of injury through a leaky BBB to heighten the neurotoxic activities of microglia and direct further a cytotoxic effect of their own towards the neurons expressing those antigens (Reynolds et al., 2008; Shavali, Combs, & Ebadi, 2006). In that sense, microglia, a component of innate immunity, can influence and employ the adaptive arm of immune system. Another possibility for this kind of crosstalk is the escape of CNS proteins to the periphery where they are processed and presented to T cells to create effector clones that migrate to the brain. Such possibility is supported by the fact that pathogenic

modified α-synuclein forms have been found in peripheral tissues like the gastrointestinal tract and cervical lymph nodes (Beach et al., 2010).

1.5.3 Adaptive Immunity in Parkinson's Disease

The adaptive immune system is tasked with providing targeted responses to foreign antigens by means of mobilizing specific subsets of T and B lymphocytes. The surveillance of CNS steady state is managed by naïve and memory T lymphocytes (Cose, Brammer, Khanna, Masopust, & Lefrançois, 2006; Krogsgaard & Davis, 2005; van der Most, Murali-Krishna, & Ahmed, 2003). PD patients show T cell infiltration of the brain, in addition to a PD-specific atypical immune response in the peripheral blood characterized by decreased total number, but not frequency, of lymphocytes (J Bas, 2001; Stevens et al., 2012). PD has a certain immune manifestation, uncommon with other neurological disorders, where the number of memory T cells is increased, while the number of naïve T cells shrinks. Moreover, the CD4:CD8 ratio becomes smaller and there is a shift to IFNγ$^-$:IL-4 producing T cells, which reflects a cytotoxic phenotype (Baba, Kuroiwa, Uitti, Wszolek, & Yamada, 2005).

α-synuclein, recombinantly expressed by AAV2, was used in some studies to overexpress α-synuclein in the MPTP PD mouse model. This overexpression results in extravasation of T and B cells into the brain as well as activation of microglia, proposing a central role for α-synuclein in the calling of effector immune cells to the CNS (Theodore, Cao, McLean, & Standaert, 2008). A further testament to this was a study that showed depletion of T and B lymphocytes in mice with α-synuclein knock-out, as compared to wild type mice (Shameli et al., 2016). FACS analysis of the CSF of PD patients uncovered a clear phenotypic change in intrathecal monocytes and elevation in activated T cells and proinflammatory mediators (Schröder et al., 2018). Sulzer et al., highlighted how particular T cells in PD patients are able to recognize and respond to peptides derived from specific regions within the α-synuclein protein (Sulzer et al., 2017). Contributing to those T cell subsets are IL-4-producing or- IFNγ-producing producing

CD4+ cells, and IFNγ-producing $CD8^+$ cells. Two PD-linked beta chain alleles of MHC class II are responsible for presenting the α-synuclein epitopes, although other non-PD alleles can also display those antigens.

1.5.4 T-Cell Mediated Effects in Parkinson's Disease

While the concept of CNS immune-privilege associated for a long time the mere presence of lymphocytes in the brain necessarily with a pathological manifestation, the latest decade of research challenged this hypothesis in many ways. Basic cognitive tasks like memory consolidation, hippocampal long-term potentiation and neurogenesis were shown to be controlled by T cells that access the meninges and are able to recognize brain-derived self-antigens (Baruch & Schwartz, 2013; Lewitus & Schwartz, 2009; Radjavi, Smirnov, & Kipnis, 2014; Yirmiya & Goshen, 2011; Ziv et al., 2006). Regardless of the presence or absence of inflammatory triggers, evidence suggest that this process is an active one. Naïve T cells are first exposed to CNS antigens in the draining cervical lymph nodes, then primed T cells migrate to the CSF and infiltrate the perivascular regions. In the normal state, the subarachnoid compartment contains numerous $CD4^+$ memory T cells and APC (Anandasabapathy et al., 2011; Baruch & Schwartz, 2013; Derecki et al., 2010; Hatterer, Touret, Belin, Honnorat, & Nataf, 2008; Laman & Weller, 2013). Upon some cognitive tasks, $CD4^+$ T cells are activated, and they activate glial cells by creating a specific cytokine environment and thus affect the CNS functionality. The conclusion is that the meninges are inhabited by adaptive components that support cognitive tasks in the CNS (Baruch & Schwartz, 2013; Derecki et al., 2010). Unlike such homeostatic tasks, inflammatory T cells could also infiltrate the brain and exert some neurotoxic effects creating neuroinflammation and contributing to neurodegeneration like what happens in PD and other diseases like AD, MS, and ALS (González & Pacheco, 2014). When oxidative stress manifests in dopaminergic neurons culminating in neuronal death, it creates an environment that modifies CNS antigens into neoantigens that engage toll-like receptors in

microglia; an example of which is nitrated alpha synuclein. Microglia activation by neo antigens contributes to an inflammatory milieu that phenotypically modifies APC in the CSF, prompting them to migrate to the cervical lymph nodes where they display those neoantigens to naïve T cells. Peripheral T cells, now neoantigen-specific, ingress to the brain fueling the microglial-driven inflammation and consequent dopaminergic neuronal depletion (Benner et al., 2008; González & Pacheco, 2014; Reynolds & Gendelman, 2010).

$CD4^+$ T cells have the unique feature of being activated by a specific antigen and thereby exert an effector anti- or- proinflammatory action. Damaging effects in PD are coordinated by proinflammatory $CD4^+$ T cell which manifest in two classes: T helper 1 (Th1) and T helper 17 (Th17) cells. While Th1 cells are mainly initiated by IL-2 and act through IFNγ and TNFα (Dardalhon, Korn, Kuchroo, & Anderson, 2008; Pacheco, Riquelme, & Kalergis, 2012), Th17 cells are promoted by IL-6 and TGFβ and release IL-12, IL-17 and IL-22 (Kebir et al., 2007). Employing those soluble effector mediators, both phenotypes induce the inflammatory M1 phenotype of microglia and peripheral macrophages in the CNS (Barcia et al., 2011; Codarri et al., 2011; Sica & Mantovani, 2012). As underlying effector arms for neuroinflammation, $CD4^+$ T cells can also assume anti-inflammatory phenotypes like T helper 2 (Th2) and regulatory T cells (Treg) (Appel, 2009). Development into Treg is potentiated by TGFβ (Burzyn, Benoist, & Mathis, 2013), and active Treg act through immunosuppressive mediators; soluble like IL-10 and TGFβ and membrane-bound like CTLA4. Employing their anti-inflammatory properties, Treg can reverse the activated M1 phenotype of microglia and macrophages and halt the induction of Th1 and Th17 cells (Badie, Bartley, & Schartner, 2002; Fontenot, Gavin, & Rudensky, 2017; Reynolds & Gendelman, 2010). Through a different palette of mediators, Th2 cells can mitigate inflammation by promoting the M2 phenotype in microglia which induces the release of neurotrophic factors, and they secrete IL-4, IL-5 and IL-13 that participate in the functional regulation of microglia and macrophages. The Th2 phenotype is turned on by

engagement with IL-4 (Appel, 2009; Derecki et al., 2010; Shechter et al., 2013). With that wide array of tools, $CD4^+$ T cells can mount a tailored response that could, depending on many factors, either advance neuroinflammation and perpetuate CNS injury, or advocate an immunosuppressive environment that decelerates neuroinflammation and aids in augmenting brain damage (Anderson et al., 2014). The latter effect could be harnessed for a therapeutic intervention to manage neurodegeneration in PD.

Although T cell involvement in neuroinflammation have been mostly studied in animal models of MS and EAE (Ben-Nun et al., 2014), T cell roles in PD have received increased focus through the latest two decades. In PD patients, peripheral blood $CD4^+$ T cell subsets show altered frequencies, in addition to phenotypic shifts in different $CD4^+$ T cell subsets (Jordi Bas et al., 2001; Fiszer et al., 1994; Romero-Ramos, von Euler Cheplin, & Sanchez-Guajardo, 2014). Analysis of postmortem brain sections showed extravasation of $CD4^+$ and $CD8^+$ T lymphocytes. The same remark has been replicated in PD models employing mice and rats(Brochard & Hunot, 2009; Sanchez-Guajardo, Febbraro, Kirik, & Romero-Ramos, 2010). PD models utilizing mouse strains that lack T cells, like TCR b chain-deficient, SCID, or RAG1KO mice, exhibited a considerable rescue of dopaminergic neurons in MPTP-based PD models, what further stresses the crucial contribution of T cells in PD pathogenesis (Benner et al., 2008; Brochard & Hunot, 2009). When mice that selectively lack either the $CD4^+$ or $CD8^+$ T cell populations are examined, it appears that the neuroprotective outcome manifests only with $CD4^+$-deficient but not with $CD8^+$ deficient mice, which points to the assumption that in PD mice, $CD4^+$ T cells orchestrate the neuroinflammation and loss of dopaminergic neurons in SN (Brochard & Hunot, 2009).

Activation of $CD4^+$ T cells exposes their effector helper functions. One helper function of $CD4^+$ T cells is the induction of CD8-controlled reactions, whereby they interact with APC prompting antigen presentation to $CD8^+$ T cells, while also releasing appropriate cytokines to aid the priming of naïve $CD8^+$ T cells (Bennett et al., 1998; Ridge, Di Rosa, & Matzinger, 1998).

Another helper function of $CD4^+$ T cells is to support the differentiation of B cells into antibody-producing plasma cells. Such helper functions explain the underlying hypothesis that $CD4^+$ T cells are the main factor when it comes to neurodegeneration even when final effector acts are also carried out by $CD8^+$ T cells and B cells (Parker, 1993).

As well as coordinating the adaptive immune response, $CD4^+$ T cells also potentiate microglial transformation into the M1-like proinflammatory phenotype that create an inflammatory milieu by secretion of TNFα, IL-1β and superoxide, further worsening the neurodegeneration (Appel, 2009).

The functions of $CD4^+$ T cells are proposed to take place only when naïve $CD4^+$ T cells are primed through antigen presentation by APC in an MHC class II-dependent interaction. This claim was examined by employing PD models using MHCII-deficient mice, and the results confirmed the proposed reduced microglial activation and increased neuronal rescue. At the human level, MHCI- and- II polymorphisms have been linked with elevated potential of PD development (Harm et al., 2013).

Due to the unavailability of live PD brains and the multifactorial nature of PD, the study of the exact mechanisms of T cell-mounted response in PD is always complicated in patients and is only doable in PD animal models. Data from the MPTP mouse model shows that Th1 and Th17 responses are mostly involved with neurodegeneration in this model (González et al., 2013; Reynolds et al., 2010). Th1 cells implement their effect on the molecular level by secreting proinflammatory cytokines like TNFα and IFNγ. TNFα can directly induce cell death by overstimulation of TNFR receptors expressed by neurons. Moreover, TNFα can also promote the inflammatory condition of microglia and astrocytes which perpetuates further neurodegeneration (Barcia et al., 2011; McCoy et al., 2006; Sriram et al., 2002). Contrary to the defined role of TNFα, the involvement of IFNγ in neurodegeneration in PD is still disputable. On one hand, T cells that infiltrate the SN in the MPTP-PD-model produce high

amounts of IFNγ and mice in this model that are deficient in D3 dopamine receptor, which promotes $CD4^+$ T cells to release IFNγ, exhibited protection against neurodegeneration (González et al., 2013). On the other hand, the transfer of splenocytes from IFNγ-deficient mice to RAG1KO mice was unsuccessful in attenuating the MPTP-induced neurodegeneration (Brochard & Hunot, 2009). Th17 cells, the second arm of $CD4^+$ T helper effects, have been also implicated in the MPTP-PD mouse model. When different T cells subsets are adoptively transferred to wild type mice that receive afterwards MPTP injections, both Th1 and Th17 were indicated to be the most associated with lethal outcome in SN neurons. Yet, Th17 adoptive-cell-transfer appeared to be the most potent in this regard (Reynolds et al., 2010). The exact underlying mechanisms for this action are undefined but work on TH17 cells in EAE shows that their effector function could be performed by secretion of mediators like TNFα and GM-CSF (Codarri et al., 2011). However, in PD-context, GM-CSF has been shown to have rather neuroprotective properties following MPTP insults in mice (Kosloski & Gendelman, 2013). To sum it up, evidence favors an active participation of Th1 and Th17 cells in neuroinflammation in PD, with neurodegeneration precipitated on a molecular level mainly by TNFα, in addition to possible, yet still unconfirmed, roles of IFNγ and GM-CSF.

1.6.0 Immunomodulatory Approaches in Parkinson's Disease

Despite the extensive research to elucidate the etiology of PD, a well-rounded explanation for the loss of dopaminergic neurons in SNpc, that manifests in deteriorating motor and non-motor functions, continues to be lacking. The outcome of this research suggests interactions between genetics, environment, immune system, and age play decisive roles in PD emergence and development. No treatments have been successfully able to halt or decelerate the development of PD. All current clinical treatments aim merely at alleviating the symptoms and enhance the patients' quality of life (Olanow, Bartus, Volpicelli-Daley, & Kordower, 2015). Present medications like dopamine or dopamine precursors, like L-DOPA, are totally ineffective

against the neurodegenerative process. That is why, it is imperative that new PD therapies be developed, that not only relieve the symptoms, but also can confront disease progression. Yet, the chronic and ongoing deterioration of PD and the fact that extensive neuronal loss is already irreversible by the time of clinical manifestation renders any disease-modifying treatments ineffective as to modify the disease prognosis. Intervention at early preclinical time points is, thus, indispensable, if we are to provide any efficacious therapies. Though such early diagnosis is not yet available, findings on early PD biomarkers are gradually shaping a possible future where PD pathology can be spotted early enough to be stopped and reversed. Whenever early detection is feasible, safe and targeted treatments could be quickly channeled to clinical trials. Neuroinflammation has been established to have pivotal roles in exasperating the neurodegeneration in PD and therefore it represents a target for such disease-modifying interventions (Anderson et al., 2014; Mosley et al., 2012). Immunomodulation is the collective term describing any therapies that stimulate, improve, or suppress the immune system in a host-beneficial manner to combat a specific disease. Neurodegenerative disease is one category where immunomodulatory treatments have been employed. As for PD, some immunomodulatory approaches have been utilized with some success in animals and they are briefly reviewed in the following paragraphs.

1.6.1 Non-Steroidal Anti-Inflammatory Drugs

One of the early candidates for immunomodulation in PD was the application of non-steroidal anti-inflammatory drugs (NSAIDS). Analysis of large groups of individuals showed less potential for PD development among ibuprofen regular users. Ibuprofen also displayed neuroprotective properties in rodent models employing MPTP and 6-hydroxydopamine (6-OHDA), suggesting a link between PD risk and anti-inflammatory treatments (Rees et al., 2011). Translation of such effect to the clinic was, however, elusive, as the case of Minocyclin proved to be. Minocyclin was utilized in PD rodent MPTP and 6-OHDA models showing

effectiveness in modulating the inflammation and reducing the neurodegeneration. When administered to patients however, it failed to exhibit any significant motor benefits, although it did lessen the microglial activation (Du et al., 2001; H. S. Kim & Suh, 2009). Consequently, anti-inflammatory agents do not seem like the go-to strategy for a clinical improvement in PD.

1.6.2 Natural Compounds

Some natural compounds have inherent anti-inflammatory and antioxidant features. Such agents could perturb the M1 microglial phenotype, reduce the secretion of proinflammatory cytokines and suppress NF-kB activation. Examples for such compounds include resveratrol and tanshinone who both can reduce IL-1β, IL-6 and TNFα levels. Resveratol can raise the expression of the suppressor of cytokine signaling (SOCS-1), while tanshinone can impede nitric oxide (NO) secretion and downregulate iNOS and NADP expression (Lofrumento et al., 2014; Ren et al., 2015). The consensus is that natural anti-inflammatory products show some promise in modulating inflammation in PD, although this is still restricted to animal models and is yet to be employed in clinical settings.

1.6.3 α-Synuclein Vaccination Strategies

Another way to halt PD progression by immunomodulation is to target the aberrant α-synuclein proteins. Misfolded and neurotoxic α-synuclein aggregates are secreted from dying neurons where they induce a detrimental inflammatory reaction. The aim of immunomodulatory therapies in this realm is to increase the clearance efficiency of the modified α-synuclein proteins. Both the N- and- C- terminals of α-synuclein could be targeted by those therapies. In a rodent model that overexpresses a syn through an AAV-α-syn, a vaccination strategy was to deliver anti human α-synuclein N terminal peptide antibodies that proved to be effective in sparing nigrostriatal neurons and lowering the microglial activation. Such vaccinations can also modify the IgG secretion, improve MHC-II expression, and promote $CD4^+$ T cells to migrate

to the CNS (Shahaduzzaman et al., 2015). α-synuclein C terminal can also be targeted, and this could suspend protein spread, rescue dopaminergic neurons, and reverse motor deficits in PD rodent models (Games et al., 2014; Tran et al., 2014; Vaikath et al., 2015). Several clinical trials have been running through the last decade to investigate the potential of α-synuclein-based immunotherapies (Mandler et al., 2014; Sanchez-Guajardo, Annibali, Jensen, & Romero-Ramos, 2013; Schneeberger, Mandler, Mattner, & Schmidt, 2012). To conclude, these vaccinations show a real prospective in modulating neuroinflammation and leading consequently to management of nigrostriatal neuronal loss. Meanwhile, some setbacks may arise while targeting α-synuclein, because antibody-based therapies mediate their effects through antigen-specific T cell reactions that could elevate, rather than halt, the inflammatory status in PD. This is probable to happen, based on AD studies showing that stimulating the immune system to better clear amyloid beta aggregations brought about sustained T cell activation and autoimmune meningeal encephalitis (Orgogozo et al., 2003). Therefore, an eventual successful vaccination strategy consists in mounting an α-synuclein-targeted immune reaction that excludes any cross-reactivity to other non-pathogenic α-synuclein species or proteins from related families.

1.6.4 Regulatory T Cells for Immunomodulation

Treg represent a subset of T cells that display unique and powerful immunosuppressive properties. Treg cells undertake the key task of endowing self-tolerance and combating autoimmunity, ensuring that the immune system always mounts weighted beneficial responses according to the available stimuli. This key role is a feat of Treg's ability to inhibit the activation and effector functions of T cells, natural killer cells, APC and B cells (Onishi, Fehervari, Yamaguchi, & Sakaguchi, 2008). Phenotypically, Treg are identified by their expression of IL2R alpha chain (CD25) and the fork head box protein 3 (FoxP3) transcription factor. Although the latter could be also expressed on macrophages and B cells, FoxP3 is universally

regarded the specific marker of Treg which also greatly influences Treg development and functions (Fontenot et al., 2017; Gavin et al., 2007; Hori, Nomura, & Sakaguchi, 2017). In terms of origin, Treg are either thymus-derived and labelled as natural Treg (nTreg) or peripherally derived and labelled as inducible Treg (iTreg) (Mills, 2004).

Treg utilize various arms to manifest their suppressive functions. Treg can display direct cytotoxic functions and kill effector T cells (Teff) by secretion of perforin and granzyme (Cao et al., 2007; Gondek, Lu, Quezada, Sakaguchi, & Noelle, 2005). They can also inhibit APC whereby they inhibit their maturation and leave them unable to activate T cell (Tadokoro et al., 2006). Additionally, Treg secrete anti-inflammatory cytokines like IL-10 and TGFβ, that engage receptors on activated T cells and lower signaling activity (Vignali, Collison, & Workman, 2008). Metabolically, Treg can deprive activated T cells from IL-2 thanks to their expression of high-affinity IL-2 receptors that compete for the cytokines needed by T cells for survival and proliferation (Pandiyan, Zheng, Ishihara, Reed, & Lenardo, 2007). Furthermore, Treg express the cytotoxic T lymphocyte antigen 4 (CTLA4) that interacts competitively with CD80 or CD86, expressed on professional APC, leading to negative regulation of the immune response (Onishi et al., 2008; Wing et al., 2008). These Treg mechanisms make them an attractive approach for immunomodulation in inflammatory or disorders with autoimmune components like PD (Figure 2).

Awareness of the immune surveillance in the CNS presented opportunities for employing the immune system to achieve better outcomes for autoimmunity and CNS pathologies. Treg are candidate immune tools that could implement such strategy, though not without inevitable complexity. Whereas Treg provide self-tolerance and augment harmful immune reactions (Kronenberg & Rudensky, 2005), they could also suppress the activity of effector T cells that slow neurodegeneration (Moalem et al., 1999; Schwartz & Baruch, 2014; Walsh & Kipnis, 2011). It has been generally established that Treg dysregulation or depletion is evident in certain CNS pathologies like MS, ALS, ischemic stroke, and PD (Saunders et al., 2012; Schuhmann et

al., 2015). We learnt from EAE models of MS that Treg depletion or adoptive transfer could respectively progress or improve EAE. Treg have been found to be reduced in ALS patients, and Treg intravenous injection in SOD1 transgenic mouse models have halted disease exacerbation. In stroke models, increased expression of Treg controlled lesion volume and enhanced locomotor deficits, while their depletion expanded infarct volume and worsened sensorimotor dysfunction. Reports on Treg roles in Alzheimer's disease show both beneficial and detrimental effects. While Treg expansion with IL-2 therapy supported better cognitive function and Treg depletion resulted in higher cognitive deficits, early transient Treg depletion supported plaque clearance and cognitive improvement. In PD mouse models (MPTP), higher numbers of Treg protected dopaminergic neurons in SN. In PD patients, there are conflicting accounts of better and worse immunosuppressive functions of Treg cells (Rosenkranz et al., 2007; Saunders et al., 2012).

In 2007, Reynolds et al., postulated that activated Treg could infiltrate the CNS in MPTP-models interacting with reactive microglia and shifting them to a neuroprotective phenotype promoting astrocyte expression of neural growth factors like BDNF and GDNF that support neuron survival (Reynolds, Banerjee, Liu, Gendelman, & Mosley, 2007). Treg treated mice showed higher levels of IL-10 and TGF-β that halt neuroinflammation by inhibiting microglial activation and synthesis of proinflammatory cytokines and provide another mechanism for Treg-imparted neuroprotection (Arimoto et al., 2007; W.-K. Kim et al., 2004).

In 2012, Chung et al., showed that the anti-inflammatory effects of bee venom result from heightened peripheral Treg response that modulates adaptive immunity reducing the secretion of Th1/Th17 cytokines from the TCR-stimulated CD4 T cells and limiting the infiltration of CD4 T cells into the SN. Bee venom showed no neuroprotective affects in Treg-depleted mice (E. S. Chung et al., 2012).

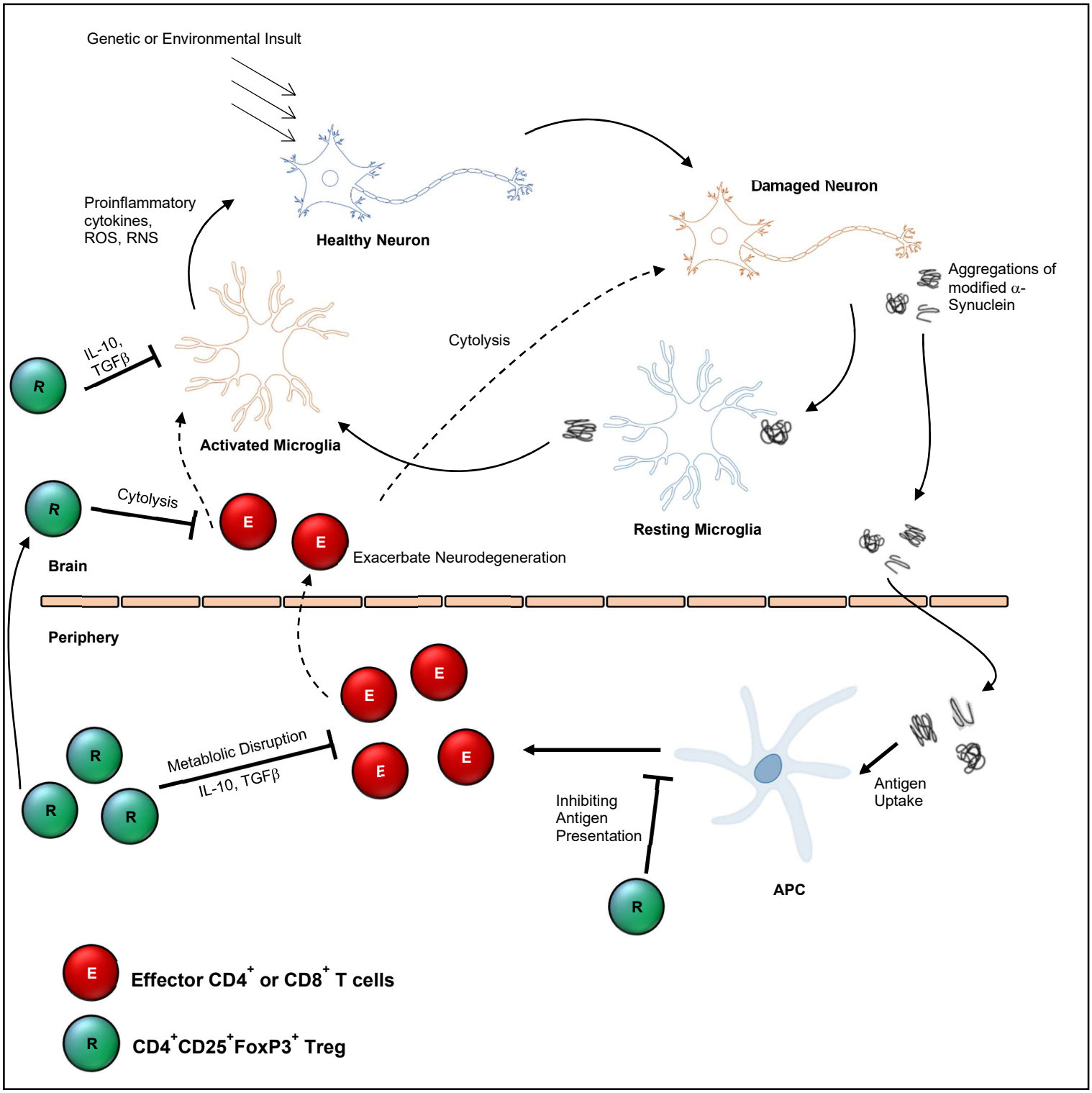

Figure 2: Treg mechanisms to mitigate neuroinflammation in PD

1.7.0 Expansion of Treg by CD28 Superagonistic Antibody

T cells can perform its important roles in adaptive immunity through the expression of the T cell receptor (TCR), which enables them to recognize specific antigens. T cells can only recognize their antigens when they are loaded on an MHC (major histocompatibility complex) molecule. MHC molecules are expressed primarily in APC like dendritic cells or B cells. These cells process self and non-self proteins into peptides, that are loaded on MHC molecules at the cell surface which eventually present the peptides to T cells (Blum, Wearsch, & Cresswell, 2013). The binding between MHC molecules and TCR is also affected by coreceptors like CD4 and CD8. Mature T cells express either CD4 or CD8 and according to their binding with MHC-II or MHC-I, they are classified as either helper or killer T cells, respectively (Smith-Garvin, Koretzky, & Jordan, 2009).

In addition to the interaction between a TCR-complex and a peptide-loaded MHC-molecule (1st signal), T cell activation requires a 2nd costimulatory signal, without which T cell activation does not take place. The absence of costimulation turns T cells either into an anergic or apoptic state (Sharpe & Freeman, 2002). The most significant and well-characterized costimulatory receptor is the homodimeric glycoprotein CD28 (Hara, Fu, & Hansen, 1985). CD28 is a member of the Immunoglobin (IG) superfamily and is expressed on all T cells. Its ligands are the homodimeric CD80 (B7-1) and the monomeric CD86 (B7-2) that also belong to the IG superfamily and are expressed by activated APC (Sharpe & Freeman, 2002).

TCR and CD28 could also be stimulated by monoclonal antibodies. In 1985 (Hara et al., 1985), the first human monoclonal CD28 antibody (mAB) was isolated. This antibody exhibited costimulatory properties in the presence of polyclonal T cell activator PMA (phorbol 12-myristate 13-acetate). Five years later, naïve T cells were activated in vitro with a combination of murine CD28 mAB and TCR-specific mAB (Gross, St John, & Allison, 1990). In isolation, these conventional CD28 mABs could not activate naïve T cells in vitro. In 1997, a new class of CD28 mABs was observed along with conventional CD28 mAB. The new class was named

"superagonistic (SA) mAB" due to their unique ability to activate naïve T cells without TCR stimulation (Tacke, Hanke, Hanke, & Hünig, 1997).

Luhder et al., showed that while the conventional CD28 mAB binds the CD28 receptor at a position near the binding site of CD80/CD86 ligands, CD28SA mAB binds the lateral IG-like domain. This difference was later confirmed in studies employing murine and human CD28 mABs (K. M. Dennehy et al., 2006; Evans et al., 2005; Lühder et al., 2003). CD28SA mABs were found to bind laterally to CD28 receptors in a bivalent manner.

Although CD28SA activates T cells independent from TCR engagement, the resulting stimulatory outcome is not independent of the TCR signaling pathway (Kevin M. Dennehy et al., 2007, 2003; Lühder et al., 2003). Contact between T cells and self-antigen-loaded MHC molecules provides a sort of weak or tonic TCR signal that becomes amplified when CD28SA binds CD28 (Kevin M. Dennehy et al., 2007; Levin, Zhang, Kadlecek, Shokat, & Weiss, 2008; Štefanoví, Dorfman, & Germain, 2002).

The injection of CD28SA in rats showed a dose-dependent transient increase in lymphocyte count and proliferation of $CD4^+$ T cells, that was not observed using CD28 mAB (Tacke et al., 1997). No pathogenic symptoms were noticed in the treated rats, while a cytokine analysis showed an elevation of anti-inflammatory IL-10 that was mainly secreted by $CD4^+CD25^+Foxp3^+$ Treg (Rodríguez-Palmero, Hara, Thumbs, & Hünig, 1999). Treg frequency within the $CD4^+$ population increased from 5% to 20%. A mouse-specific CD28SA, D665, has been show to induce similar outcome (N. Beyersdorf, Hanke, Kerkau, & Hünig, 2005; Niklas Beyersdorf et al., 2005; Lin & Hünig, 2003).

Building on this knowledge, CD28SA has been used to expand Treg and harness the immunoregulatory mechanisms, like the production of anti-inflammatory IL-10 and TGF-β, that suppress conventional T cell activation (Tang & Bluestone, 2008). The expanded Treg produced immunosuppressive effects that are stronger than unstimulated Treg (N. Beyersdorf et al., 2005; Niklas Beyersdorf et al., 2005; Lin & Hünig, 2003). Preclinical data in animal

models of autoimmune disorders have exhibited the positive effect of CD28SA-induced Treg expansion on the mitigation and prevention of autoimmunity.

Positive immunomodulatory effects of CD28SA in rodent models created a consensus that their therapeutic application in human inflammatory diseases would be successful. Indeed in 2003, the first mouse anti-human CD28 mABs were generated and labelled as TGN1412 (Lühder et al., 2003). Preclinical studies into TGN1412 employed the long-tailed macaque as a primate in vivo model as well as human peripheral blood mononuclear cells (PBMCs) as an in vitro model. These models were selected due to the high conservation of Fc receptors in both species and because TGN1412 exhibited an affinity to its CD28 receptor that is similar to its affinity to human CD28 (Hanke, 2006). Despite high dosage, no signs of toxicity or side effects were observed in the primate model. Similar results were observed after stimulating human PBMCs with TGN1412 (Duff, 2006).

Following these promising data, a first-in-human clinical trial for TGN1412 took place in 2006 by a collaboration of the University of Würzburg and the company TeGenero. As a shocking surprise, the six volunteers who conducted the study developed a life-threatening cytokine release syndrome (CRS) with multiorgan failure. Unlike in macaques, the human volunteers developed in few hours high levels of proinflammatory cytokines like TNFα and IFNγ. It was proposed that CD28SA has activated effector T cells that were responsible for the immediate release of proinflammatory cytokines that were observed in the blood samples of volunteers in few hours after drug administration (Duff, 2006; Suntharalingam et al., 2006).

In the following years, TGN1412 has been subjected to extensive studies to understand why the reported toxicity in humans was not observed nither in in vitro studies nor in cynomolgus macaques. As for the PBMCs, the explanation was that the effect of CD28SA on CD28 signal transduction requires the presence of weak tonic activation of TCR, that is then amplified by CD28 signals (Kevin M. Dennehy et al., 2007). Such tonic TCR activation is normally detected when T cells reside in tissues as they crawl against the surface of other cells looking for antigen

presentation by MHC molecules. In the peripheral blood, such tonic inhibition of T cells is shut down as it is not any more needed due to the absence of interactions. Standard PBMC culture mimic the conditions in peripheral blood and therefore T cells show no tonic activation of TCR and when they engage with CD28SA, T cells are much less activated as they would have been if the PBMC culture better imitated the condition in tissues. That is why higher doses of CD28SA were added to the PBMCs ex vivo without inducing a CRS. In order to simulate the tissue environment, PBMCs were cultured for 2 days at high density and, with that, were able to maintain a weak TCR signal and thereby responded to the TGN1412 dose, similar to the 2006 trial, with CRS (Römer et al., 2011). As for the absence of CRS in the primate model in response to TGN1412, it was demonstrated that CD28 is downregulated in CD4 effector memory cells in cynomolgus macaques, unlike humans, and therefore it cannot be targeted by CD28SA (Eastwood et al., 2010) and that explains the failure of cynomolgus macaques to respond to high doses of TGN1412.

Equipped with better understanding for the reasons of failure of TGN1412, CD28SA made a come-back through the Russian company TheraMAB and it was renamed to TAB08. Preclinical work on in vitro PBMCs has been resumed and TAB08 showed a dose-dependent activation of different T cell populations. At lower doses, TAB08 showed Treg activation without triggering the release of proinflammatory cytokines (Tabares et al., 2014). A new clinical trial employing TAB08 was permitted, and it used doses that were 1000-fold less than the ones applied in the 2006 study. The results showed no CRS and no elevation of any cytokines but the ant-inflammatory IL-10 which is the key cytokine produced by Treg. More clinical trials are performed to establish the efficacy in treating autoimmune disorders like rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and psoriasis.

1.8.0 Animal Models of Parkinson's Disease

Despite the availability of various PD medications aimed at enhancing the quality of life for patients, the absence of therapies that can slow or prevent the advancement of neuronal loss remains a challenge for PD management. This challenge is predicated on the lack of an understanding of the exact mechanisms regulating the onset and progression of neurodegeneration. While a lot of light has been shed since the 1990s on some underlying pathways, many gaps still await to be filled. There is a pressing need to establish and refine in vivo models that captures the clinical presentation of PD while also representing the probable pathogenetic mechanisms (Bezard, Yue, Kirik, & Spillantini, 2013).

1.8.1 Neurotoxin Models

Throughout the last 40 years, experimental animal models employing neurotoxins have been a standard tool in PD research. Neurotoxins are selected based on their ability to induce selective neuronal death, thereby reproducing some PD hallmarks without necessarily reflecting the actual mechanistic features recognized clinically.

The most remarkable PD neurotoxin models are based on either 6-ODHP usually used in rats, but also in mice, or MPTP that is employed with mice and some non-human primates. Not only does MPTP models show loss of dopaminergic neurons that mirrors progressive stages of PD, but they also exhibit PD-resembling motor dysfunction (M. Gerlach, Desser, Youdim, & Riederer, 1996; Manfred Gerlach, Riederer, Przuntek, & Youdim, 1991; Heikkila & Sonsalla, 1992; Zigmond & Stricker, 1989). However, the absence of the characteristic intraneuronal LB in most of MPTP-induced models harms the validity of these models.

LB are proteinaceous cytoplasmic inclusions that represent a unique pathological feature of PD. These LB are formed by intracellular aggregation of proteins, most notably α-synuclein. The mechanisms of formation of these inclusions remain to be fully elucidated (Crowther, Daniel, & Goedert, 2000). In some studies, α-synuclein pathology has been correlated with death of

dopaminergic neurons in MPTP monkey models (McCormack et al., 2010, 2008; Purisai, McCormack, Langston, Johnston, & Di Monte, 2005). It has been suggested that primate dopaminergic neurons are more prone to degenerate upon toxic onslaughts, as they possibly lack rodent counteracting mechanisms (Herrero et al., 1993; E. Hirsch, Graybiel, & Agid, 1988; Paris, Lozano, Perez-Pastene, Muñoz, & Segura-Aguilar, 2009).

1.8.2 Etiologic Models

A newer decade-long trend in modeling PD emerged based upon a gene-based approach. These so-called etiologic models aim at reproducing the neuronal lesions of PD not through a direct insult to the cells, through oxidative stress for example, but rather by triggering the death cascade with downregulation or overexpression of disease-related genes. The most targeted of these genes is the α-synuclein gene (SNCA).

In familial PD, duplications, and triplications of SNCA as well as missense mutations have been documented as a causing factor (Corti, Lesage, & Brice, 2011; Polymeropoulos et al., 1997). α-synuclein is also the principal protein in the LB inclusions that characterize PD (Spillantini, Crowther, Jakes, Hasegawa, & Goedert, 1998; Spillantini et al., 1997). The previous facts encouraged the development of α-synuclein-based transgenic animal models that induce PD through the overexpression of wild-type or mutant α-synuclein to produce insoluble aggregates of α-synuclein which is key to replicate PD in animal models.

Generally, mouse models are preferred in mimicking human genetic disorders as they feature analogous neuronal networks and gene homologs (Waterston et al., 2002). Different genetic mouse models have been generated and enhanced throughout the years. Transgenic models indicate promoter-controlled overexpression of a gene in the brain that could be tissue-or-cell-type-specific or could be temporally induced by specific drugs (Landel, Chen, & Evans, 1990). To simulate PD, dominant α-synuclein mutants like A53T, A30P are employed. Mice, that overexpress α-synuclein, lose neurons in the SNpc and the nigrostriatal pathway, develop

synucleinopathies and experience motor dysfunctions (M. Decressac, Mattsson, Lundblad, Weikop, & Björklund, 2012; Kirik et al., 2003, 2002; Lo Bianco, Ridet, Schneider, Déglon, & Aebischer, 2002). These outcomes stemming from α-synuclein overexpression could be traced back to mitochondrial impairment, oxidative stress and induction of cell death cascades (Farrer et al., 2004; Ibáñez et al., 2004; Miller et al., 2004; Singleton et al., 2003). Despite the overall utility of transgenic mouse models in advancing our understanding of α-synuclein pathology, they are limited by the fact they require long time to develop relevant clinical symptoms and that they do not express a marked death of dopaminergic neurons in the SN.

Alternatively, transgenes could also be consistently induced by the stereotaxic viral injection into particular regions in the brain. The choice of a viral vector to model PD is based on the aim of the experiment and the size and structure of the gene. While early studies employing the recombinant adeno-associated virus (rAAV) were able deliver wild-type and mutant alpha synuclein by direct injection into the SNpc and led to α-synuclein aggregation in the SN, dopamine depletion in the striatum and loss of DA (Kirik et al., 2003; Lo Bianco et al., 2002), only low expression was observed without consistent dopaminergic degeneration which normally required longer period (8-10 weeks) (C. Y. Chung, Koprich, Siddiqi, & Isacson, 2009; Maingay, Romero-Ramos, Carta, & Kirik, 2006). Different rAAV serotypes confer different advantages; while AAV2 is more specific for neurons, AAV1 and AAV5 are highly diffusible but not specific. To harvest the benefits of different serotypes, new hybrid viral vectors were developed that combine different serotypes, notably the AAV1/2 viral vector. AAV1/2 maintains high expression of α-synuclein in dopaminergic nerurons, lewy-like pathology with intraneuronal inclusions of α-synuclein in the SN and dystrophic neurites in the striatum, loss of dopaminergic neurons in SN and behavioral motor deficits (Ip et al., 2017; Akua A. Karikari, Koprich, & Ip, 2019; Akua Afriyie Karikari et al., 2019; Koprich, Johnston, Reyes, Sun, & Brotchie, 2010).

1.9.0 Hypothesis and Aim

The aim of the work presented in this study is to test a specific immunomodulatory therapeutic intervention in a clinically relevant preclinical PD mouse model. We established our mouse model through the unilateral stereotaxic injection in the SNpc of C57B/L6 mice delivering the AAV1/2 viral vectors which harbors the human mutant α-synuclein-A53T (haSyn).

Our therapeutic agent of choice was CD28SA that has been shown to induce the expansion on Treg in mice. Our hypothesis is that the elevation of Treg levels after one week of the viral vector delivery would mitigate the inflammatory processes that accompany the onset of synucleinopathy and neurodegeneration in the AAV1/2 PD mouse model.

Through their myriad mechanisms, we are postulating that Treg would be able to inhibit the antigen presentation of pathogenic forms of α-synuclein to $CD4^+$ T cells, deprive peripheral effector T cells of IL-2, halting their proliferation, secrete ani-inflammatory cytokines like IL-10 and extravasate through the BBB into the brain to suppress immune activation as well as and engage with cytotoxic $CD8^+$ T cells.

As we discussed earlier, since neuroinflammation is well established as key player in the progression of PD as evidenced by the presence of activated microglia, CD4 and CD8 T cells in SNpc of PD patients as well as different animal models, modulation by Treg could be a viable strategy to halt or slow the progression of dopaminergic neurodegeneration and locomotor dysfunction in the AAV1/2 mouse model.

To that end, our treatment consisted of the administration of a single i.p. dose of CD28SA (D665), one week after the unilateral delivery of mutant asyn-A53T into SNpc. Mice are monitored throughout 12 weeks, with behavioral tests performed at 3 distinct time points. After 12 weeks, mice are sacrificed and immunohistochemical studies are performed to assess neurodegeneration in the nigrostriatal pathway as well as the infiltration of T cells into SN and striatum (Figure 3). In addition, flow cytometric analyses are carried out to evaluate the changes

in main T cell subsets in the cervical lymph nodes, spleen, and brain in response to the CD28SA treatment.

To explore the mechanism of CD28SA effects, we performed another study whereby we adoptively transferred Treg to haSyn PD mice to check whether we could replicate any effects we might observe with CD28SA treatment (Figure 4).

Our findings indicate a beneficial outcome by immunomodulation through Treg expansion by CD28SA in the AAV1/2-α-Syn-A53T mouse model. CD28SA treatment resulted in less activation of T cells and less infiltration into brain. We observed protection of SNpc dopaminergic neurons as well as sparing of the dopaminergic striatal terminals. Behaviorally, mice had higher latency to fall off the rotating rod, and they showed improved usage of paws corresponding to the side of viral vector injection. Similar outcomes were also observed when adoptive transfer of Treg was employed instead of CD28SA to provide neuroprotective and anti-inflammatory actions in haSyn PD mice.

We are looking forward to replicating these findings in different animal models, especially nonhuman primate models, to gather data from various angles that could eventually support the application of CD28SA in PD to enhance the disease prognosis.

1.10.0 Declaration on Participation

This study was conducted in the working group of PD. Dr. Chi Wang Ip of the Department of Neurology of the University of Würzburg. The experiments were carried out by me unless otherwise stated. DAT binding assay and dopamine neurochemical analysis with HPLC were conducted by the Dr. James B. Koprich lab of the Krembil Research Institute, Toronto Western Hospital, Canada.

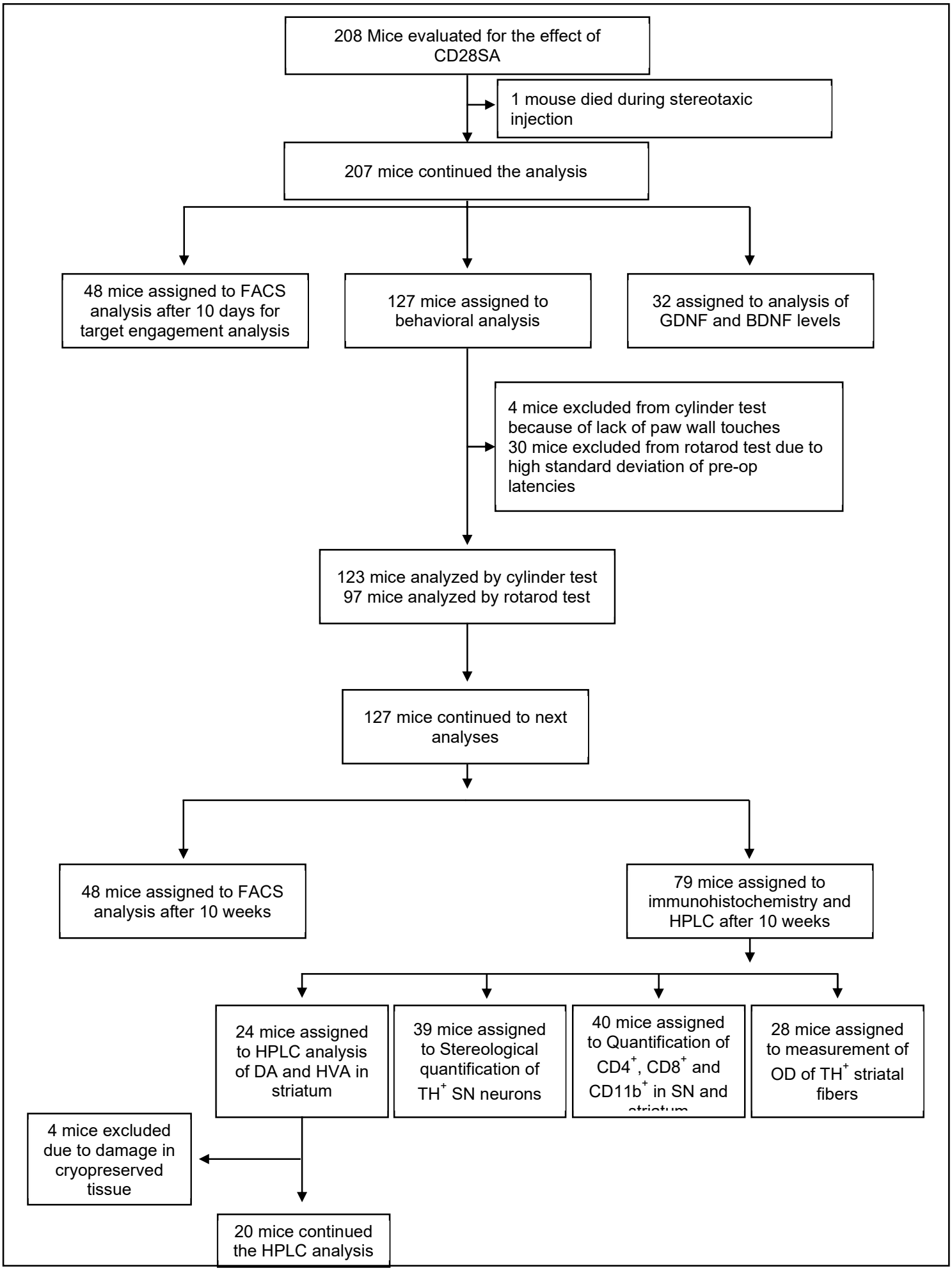

Figure 3: Experimental design of CD28SA treatment of haSyn PD mice.

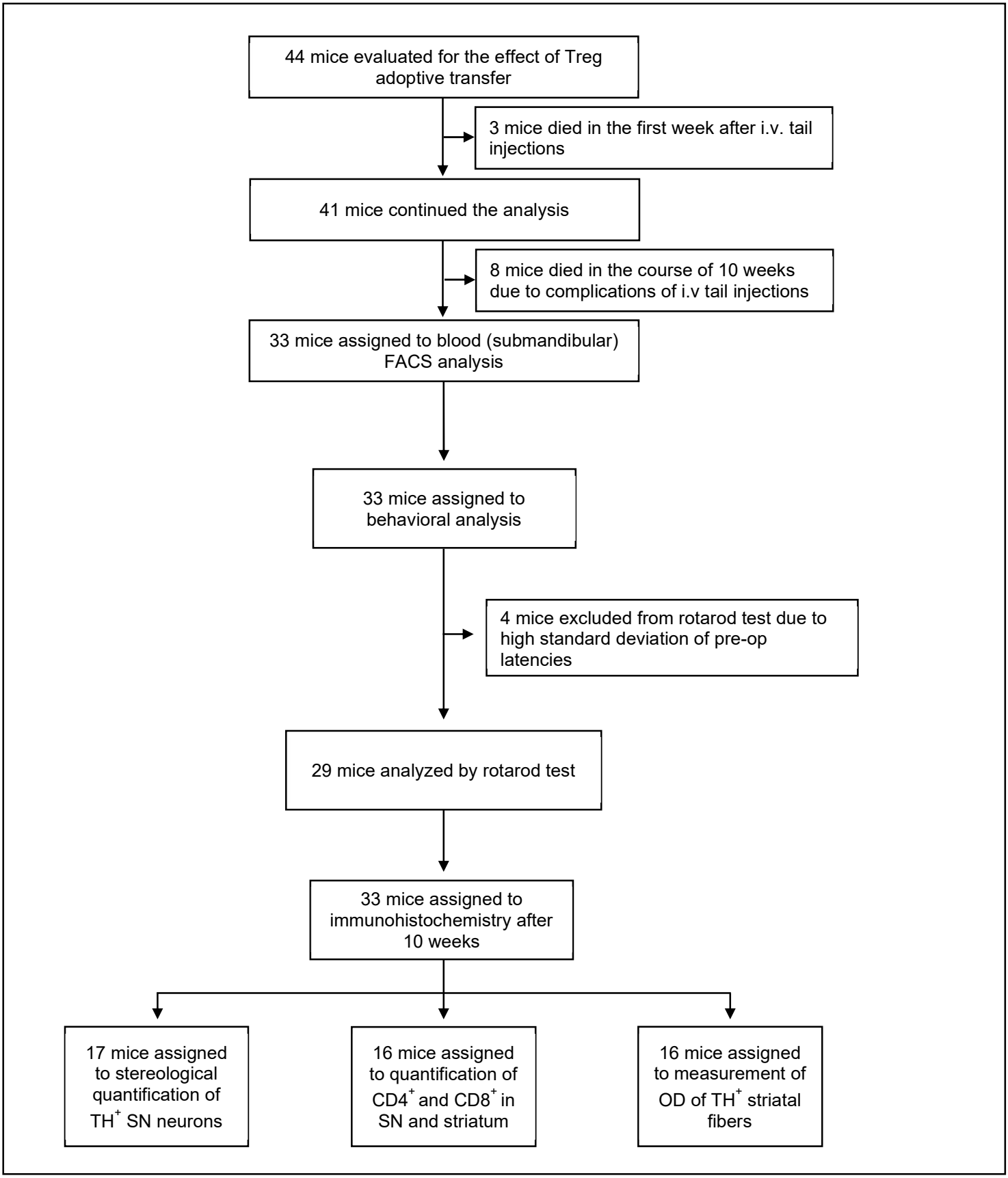

Figure 4: Experimental design of Treg adoptive transfer to haSyn PD mice.

2.0 Materials

2.1.0 Chemical and Reagent List

Table 1: Chemical and reagent list

Chemical	Company
2-Methylbutane	Roth
Bovine serum Albumin (BSA)	Sigma
Collagenase NB_4 Standard Grade	Serva
D-Sucrose (>99.5%, p.a.)	Roth
Ethanol (EtOH) (70%)	Fisher
Glycerol	Merck
Heparin	Rathiopharm
Hydrogen Chloride (HCl, 1N)	Fluka
Hydrogen peroxide 30% (H_2O_2)	Merck
Isoflurane	CP Pharma
Liquid blocker (Pap pen)	Science Services
Normal goat serum (NGS, 100%)	Dako
Ocular lubricant (Bepanthen)	Bayer
Optimal cutting temperature (OCT) Compund (Tissue Tek)	Sakura
Paraformaldehyde (PFA)	Merck
Percoll	GE Healthcare
Phosphate buffered saline (Dulbecco's PBS - low endotoxin)	Biochrom
Potassium Chloride (KCl)	Merck
Rimadyl	Zoetis

Sodium azide (NaN_3)	Sigma
Sodium Chloride (NaCl)	Sigma-Aldrich
Sodium hydroxide NaOH	Merck
TAB08m (CD28SA)	InVivo BioTech Services, Hennigsdorf, Germany
Triton X	Sigma

2.2.0 Solution Composition and Preparation

- 4% Paraformaldehyde (PFA): 10 g PFA was dissolved in 250 mL 0.01 M PBS. While continuously stirring at 60°C, 5 M NaOH is dropped twice into the solution. After the PFA solution becomes clear, it was cooled on ice and pH was adjusted to 7.4 utilizing HCL. The PFA solution is filtered afterwards to obtain the final working solution.
- 10% BSA solution: 100g of BSA dissolved in 100 ml 1x PBS and filtered.
- 30% sucrose solution: 30 g of sucrose was dissolved in 100 ml 1x PBS.
- 10x (0.1 M) phosphate buffered saline (PBS): 80 g NaCl, 14.2 g $NaHPO_4$ x $2H_2O$ and 2 g KCl dissolved in distilled water. Bring pH to 6.8 with HCl. The 10x PBS was diluted to 1x M) PBS with distilled water and pH adjusted to 7.4.
- Antibody diluent for immunohistochemistry: 2% NGS, 2% BSA, 0.5% Tx in 0.01 M PBS.
- Antifreeze cryoprotectant: consisted of 30% glycerol, 30% EtOH, 40% 0.01 M PBS.
- Blocking solution: 10% normal goat serum (NGS), 2% BSA 0.5% Triton X (Tx) in 0.01 M PBS.
- BSS (Balanced Salt Solution I, II)
    - BSS I: 50 g of glucose, 3 g KH_2PO_4, 11.9 g Na_2HPO_4, 0.5 g phenol red all dissolved in 5 L distilled H2O.

- BSS II: 9.25 g $CaCl_2$, 20 g KCl, 320 g NaCl, 10 g $MgCl_2$, 10 g $MgSO_4$ were dissolved in 5 L distilled H_2O. Then 125 ml of BSS I was mixed with 125 ml of BSS II in 1 l of distilled water H_2O.

- BSS/BSA: 0.2% BSA was added to BSS solution prepared above.
- FACS-buffer: 1000 ml PBS with 0.1% BSA, 250 mg NaN_3
- PBS with heparin solution: to 150 ml of 1x PBS, 0.5 ml of heparin was added for perfusion of mice.

2.3.0 Reagent Kits

- 3,3-Diaminobenzidine tetrahydrochloride dihydrate (DAB) peroxidase substrate kit (Vector Labs, #SK4100, Burlingame, CA, USA)
- BDNF ELISA kit (MyBioSource, #MBS355435, San Diego, USA)
- $CD4^+CD25^+$ selection kit and magnetic separation columns (Miltenyi Biotech, Bergisch Gladbach, Germany)
- FoxP3 /Anti-Mouse/Rat staining set APC (eBioscienc-Thermo Fisher Scientific, # 77-5775-40 Waltham, MA, USA).
- GDNF ELISA kit (MyBioSource, #MBS2020326, San Diego, USA)
- LEGENDplex™ Mouse Inflammation Panel (13-plex) with Filter Plate (BioLegend, San Diego, CA, USA).
- Ultra-sensitive avidin/biotin complex (ABC) peroxidase standard staining kit (1:56, Thermo Fisher Scientific cat # 32050, Waltham, MA, USA).

2.4.0 Materials for Stereotaxic Injection

- 75N 5-µl microinjection syringe (Hamilton Co., Reno, NV, USA)
- C57BL/6N wild type mice (Charles River Laboratories, Sulzfeld, Germany)
- Cotton swaps

- Drill with handpiece (Foredom Co., Bethel, CT, USA)
- Electric razor
- Gauze
- Human AAV1/2-A53T- α-synuclein (Genedetect, Sarasota, FL, USA)
- Human empty AAV1/2- viral vector (Genedetect, Sarasota, FL, USA)
- Induction box (Custom made)
- Isoflurane (CP Pharma)
- Isoflurane vaporizer (Dräger AG, Lübeck, Germany)
- Mouse stereotaxic frame (Stoelting, Wood Dale, IL, USA)
- Mouse anesthesia stereotaxic mask apparatus (Stoelting, Wood Dale, IL, USA)
- Motorized microinjection pump (Stoelting, Wood Dale, IL, USA)
- Oxygen tank
- Rimadyl as pain medication (Pfizer)
- Scalpel Nr. 10 (Feather Co., Osaka, Japan)
- Suture materials, V97D9, (Ethicon Inc., Somerville, NJ, USA)
- Tissue forceps

2.5.0 **Materials for Tissue Processing**

- 25 G needle
- 2-Methylbutane
- Aluminum foil
- Aquatex (aqueous mounting media)
- Bone scissors
- Brain matrix (mouse)
- Cover slips

- Cryomold
- Cryostat
- Distilled water
- Dry ice
- Heparin
- Object slides
- OCT compound (Tissue Tek)
- Tissue pincers

2.6.0 Antibodies for Immunohistological Staining

- Biotinylated rabbit anti-rat (1:100, Vector Laboratories cat# BA-4001, Burlingame, CA, USA)
- Biotinylated goat anti-rabbit (1:100, Vector Laboratories cat# BA-1000, Burlingame, CA, USA)
- Rat anti-mouse CD4 (1:1000, Serotec, cat # MCA1767, Oxford UK)
- Rat anti-mouse CD8 (1:500, Serotec, cat # MCA609G Oxford UK)
- Rat anti-mouse CD11b (1:100, Serotec, cat #MCA74G, Oxford UK)
- Rabbit anti-mouse TH (1:1000, Abcam cat # 112, Cambridge, UK)

2.7.0 FACS Antibodies

- CD4 Alexa Fluor 647, CD8 PerCP/Cy5.5, CD25 Alexa Fluor 488, CD69 Alexa Fluor 488 and FoxP3-PE antibody (Biolegend, San Diego, CA, USA)
- Fixation/Permeabilization buffer (eBioscience, San Diego, CA, USA)

2.8.0 Software and Equipment

- Rotating rod (RotaRod Advanced, TSE systems, Bad Homburg, Germany)

- Stereo Investigator software package (version 11.07; MicroBrightField Biosciences, Williston, VT, USA)
- BX53 microscope (Olympus Corporation, Tokyo, Japan)
- National Institutes of Health (NIH) ImageJ software (NIH, Bethesda, MD, USA)
- FlowJo software (FLOWJO LLC, Ashland, OR, USA)
- BDTM LSR II (Beckton Dickinson, Franklin Lakes, NJ, USA),
- GraphPad Prism (version 6.0; GraphPad Software, Inc., San Diego, CA, USA).

3.0 Methods

3.1.0 Animals and Stereotaxic Surgery

Male wildtype C57BL/6 mice are obtained from Charles River Laboratories, Sulzfeld, Germany, and kept at near pathogen-free environment under standard conditions (21°C, 12 h/12h light/dark cycle). At the age of thirteen weeks, a total of 242 mice are unilaterally injected in the right SN (Bregma: AP -3.1 mm; ML -1.4 mm; DV -4.2 mm) with 2 µl of human mutant A53T-α-Synuclein AAV1/2 (haSyn) or Empty Vector AAV1/2 (EV) as described by Ip et al. (Ip et al., 2017). AAV1/2 solutions were infused by a microinjector (Stoelting Co., Wisconsin, USA) at a rate of 0.2 µl/min using a 75N 5µl Hamilton Syringe at a concentration of 5.16 x 10^{12} genomic particles (gp)/ml. Genedetect® provided AAV1/2 and controlled an equal number of genomic particles in EV and haSyn AAV1/2 (Figure 5). General anesthesia was applied in all surgical procedures using isoflurane. All mice experiments were performed with respect to the German animal protection law and approved by the government of Unterfranken, Würzburg.

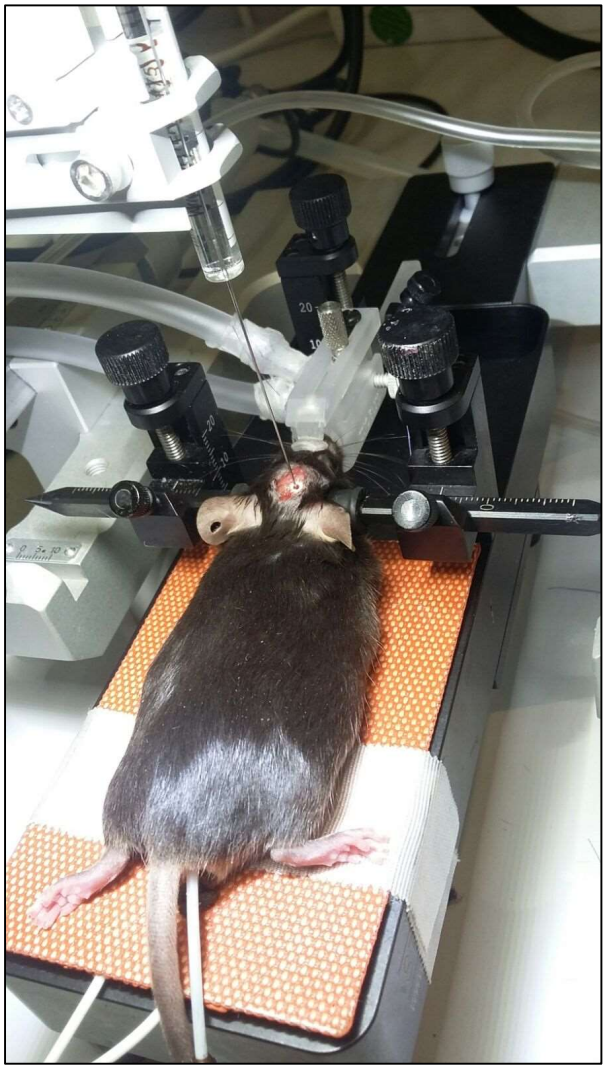

Figure 5: Set-Up of stereotaxic injection of AAV1/2 viral vectors, haSyn and EV.

3.2.0 In Vivo Delivery of CD28SA

One week after stereotaxic surgeries, all mice received intraperitoneal injections of either 200 µl of D665, which is a mouse anti-mouse CD28SA (commercially known as TAB08m like in Figure 6), in PBS solution at a concentration of 1 mg/ml (InVivo BioTech Services, Hennigsdorf, Germany) or 200 µl of 0.1M sterile PBS (Biochrome, Berlin, Germany).

Figure 6: TAB08m from InVivo Biotech Services GmbH

3.3.0 Adoptive Transfer of Treg

Treg were isolated from 10 donor mice whose Treg population was previously expanded by i.p. injections of CD28SA. Isolated Treg were injected intravenously into mice that received haSyn stereotaxic injection one week earlier.

3.3.1 Treg Isolation and Sorting

Spleen and lymph nodes of Treg-enriched donor mice were processed by forcing cells through 70 µm cell strainers. Red blood cells in spleens were lysed by 2-min incubation in an NH^4Cl-based RBS lysis buffer. Harvested cells were subject to a $CD4^+$ negative selection, that was followed by a $CD4^+CD25^+$ positive selection. Treg isolation was done using $CD4^+CD25^+$ selection kit from Miltneyi Biotech and magnetic separation was done using MS columns. A sample of Treg was analyzed by FACS to check purity.

3.3.2 Treg Adoptive Transfer

After isolation, Treg were intravenously (i.v.) injected into recipient mice through the tail vein. One week later, blood samples of recipient mice were drawn from facial vein to check the percentage of $CD4^+$ $CD25^+$ Treg by FACS analysis, compared to mice that received only a PBS

i.v. injection. I performed all i.v. injections with close supervision of Dr. Ip, who intervened when needed to ensure right delivery.

3.4.0 Behavioral Tests

Behavioral performance is assessed serially using the cylinder test and the rotarod test with a pre-operative baseline testing and follow-up testing until week 9 after surgery.

3.4.1 Accelerating Rotarod Performance Test

Motor coordination of mice is analyzed by the rotarod test. Mice were trained for two days before they were subjected to pre-operative testing. At the time of testing, mice are placed on a rotating rod (RotaRod Advanced, TSE systems, Bad Homburg, Germany) with an accelerating speed from 5 rpm to 50 rpm for a duration of 300 s. As animals fall, latency to fall off the rod is recorded automatically, and they are allowed afterwards to rest for 180 s. Pre-operative measurements were performed on the 3rd day after the two-day training, while post-operative measurements were captured in the 5th and 9th weeks. For each mouse, a total of 5 runs are carried out per measurement and latency time is calculated as the average excluding the highest and lowest values. Data are shown as the percentage of latency time at the 5th week and 9th week relative to pre-operative values.

3.4.2 Cylinder Test

The extent of asymmetry in mice spontaneous use of forepaws was analyzed by the cylinder test pre-operatively and post-operatively at the 5th and 9th week. Each mouse was placed in a transparent plexiglass cylinder (12 cm diameter, 30 cm height) in front of two vertical mirrors and video recorded for 12 minutes. During each mouse rearing, the number of touches of the ipsilateral, the contralateral and both forepaws concurrently on the inner surface of the cylinder and while landing is scored by a blinded observer as described by Schallert et al. (Schallert, Fleming, Leasure, Tillerson, & Bland, 2000). Forepaw use is calculated as the percentage of

ipsilateral forepaw use employing this equation: ((ipsilateral paw + 0.5 both paws) / (ipsilateral paw + contralateral paw + both paws)) x 100. Eventual data represent the bias of forepaw use as 100% = symmetric use of both forepaws; <100% = preference of the left forepaw; >100% = preference of the right paw.

3.5.0 Immunohistochemical Staining and Cell Quantification in Mouse Brain

Immunohistochemical staining of mouse tissue for $CD4^+$, $CD8^+$ and $CD11b^+$ profiles were performed using 10 µm fresh coronal cryo-sections of the SN and striatum. 40 µm PFA-fixed cryosections were processed for unbiased stereology (TH) covering the whole SN.

After acetone (for lymphocytes) or 4% PFA fixation (for $CD11b^+$), sections were incubated with either rat anti-mouse $CD4^+$ (1:1000, Serotec, cat # MCA1767), rat anti-mouse $CD8^+$ (1:500, Serotec, cat # MCA609G) or rat anti-mouse CD11b (1:100, Serotec, cat # MCA74G) antibodies followed by biotinylated rabbit anti-rat and goat anti-rabbit secondary antibodies (Vector Labs, cat # BA-4001 and BA-1000). Development was done using (DAB)-HCl-peroxidase (Vector Labs).

For unbiased stereology, PFA-fixed brains are coronally and serially cut in 40 µm cryo-sections. Free floating sections were pre-incubated for 1 hour on a shaker in a solution of 10% Normal goat serum (NGS), 2% BSA, 0.5% Triton X-100 in 0.1 M PBS and subsequently incubated with rabbit anti-mouse TH antibody (1:1000; abcam, Cambridge, UK) overnight at 4°C. This was followed by incubation, first with biotinylated goat anti-rabbit antibodies at 1:90 dilution, then with Avidin-Biotin-Peroxidase reagent and finally with DAB and H_2O_2 (Vector Labs). All diluent solutions consisted of 2% Normal goat serum (NGS), 2% BSA, 0.5% Triton X-100 in 0.1 M PBS. TH-stained sections are mounted on object slides, allowed to dry and incubated in Cresyl Violet for 30 min. Afterwards, sections are washed in distilled water, subjected to multiple one-minute incubations in ethyl alcohol in the following sequence: three times in 70%,

2 times in 95% and 2 times in 100% ethyl alcohol. Later, sections are placed in Xylol for two 5-min incubations and are cover-slipped using Vitrocloud.

T cells ($CD4^+$ or $CD8^+$ cells) and microglia ($CD11b^+$ cells) were quantified at a magnification of 200x in the region of the SN and striatum as depicted by consecutive sections stained for TH on a BH2 light microscope (Olympus). Nigral neurons were quantified by unbiased stereology according to the optical fractionator method using a 100x/1.25 numerical aperture objective on a BX53 microscope (Olympus), and a Stereo Investigator software package (version 11.07; MicroBrightField Biosciences, Williston, VT). 40 µm sections covering the extent of the SN and separated by 200 µm (1/5 series) were used for counting dopaminergic (TH^+) neurons and total ($Nissl^+$) neurons in both pars compacta and pars reticulate regions of the SN. Parameters of quantification were grid size 110 x 110 µm, counting frame 50 x 50 µm and 2-µm guard zone. Actual mounted thickness was calculated as an average of the measured thickness of each counting site. A Gundersen coefficient of error (for m=1) of <0.1 was accepted.

3.6.0 Catecholamine Quantification by High-Performance Liquid Chromatography (HPLC)

Brain sections were homogenized in 200–750 µl of 0.1 M TCA (10−2 M sodium acetate, 10−4 M EDTA, 10.5% methanol). Samples were centrifuged at 10,000 g for 20 min, supernatants were collected, and the pellets were stored for protein analysis. Catecholamines were evaluated using a specific HPLC assay with an Antec Decade II (oxidation: 0.5) electrochemical detector operated at 33 °C. Supernatant samples were injected by a Water 717+ autosampler onto a Phenomenex Nucleosil (5u, 100A) C18 HPLC column (150x4.60 mm). Analytes were eluted with a mobile phase of 89.5% 0.1 M TCA, 10−2 M sodium acetate, 10−4 M EDTA, 10.5% methanol, followed by delivery of the solvent at 0.8 ml/min with a Waters 515 HPLC pump. Analytes were examined in the following order: 3,4-dihydroxyphenylacetic acid (DOPAC), dopamine (DA), homovanillic acid (HVA). Waters Empower software was used for HPLC

control and data acquisition. Total protein for each sample was determined with Pierce BCA protein assay, and the amount of catecholamines was expressed as ng analyte/mg total protein.

3.7.0 Fluorescence-Activated Cell Sorting (FACS) and Cytokine Assay

3.7.1 Perfusion of Mice and Organ Harvest

Ten weeks post- AAV1/2 injection, mice are perfused with 0.1 M phosphate buffered saline (PBS). Cervical lymph nodes (CLN), spleens and brains were harvested. Single cell suspensions of CLN and spleens are prepared by forcing cells through 70 µm cell strainers. Red blood cells in spleens were lysed by 2-min incubation in an NH^4Cl-based RBS lysis buffer. Brains were homogenized and incubated with Collagenase (Serva Electrophoresis) and DNase I (AppliChem) for 30 minutes at 37°C. Later, brain homogenates were forced through 70 µm cell strainers and washed with BBS/BSA medium. Afterwards, cell suspensions are subjected to density gradient centrifugation for 30 min at RT at 2000 rpm in a medium of the silica colloid Percoll (GE Healthcare Life Sciences) comprising layers of 70% and 30% Percoll. Eventually, cellular layers are aspirated and suspended in BSS/BSA medium.

3.7.2 Flow Cytometry

Cell suspensions from CLN, spleen and brain are incubated with CD4 Alexa Fluor 647, CD8 PerCP/Cy5.5, CD25 Alexa Fluor 488 and CD69 Alexa Fluor 488 (Biolegend) for 30 minutes, subsequently fixed and permeabilized by eBioscience Fix/Perm buffer for 30 mins and then incubated with FoxP3-PE antibody (Biolegend) for 1 hour. Acquisition was performed on an LSRII flow cytometer (BD) and data analysis was done using FlowJo software (FLOWJO, LLC).

3.8.0 Estimation of BDNF and GDNF

Neurotrophic factors BDNF and GDNF were estimated in the motor cortex (M1), striatum and SN. fresh mouse brains were dissected in coronal plane with a brain matrix slicer at the region of +0.14 mm, +2.14 and -1.86 mm from Bregma (figure 30 Paxinos and Franklin mouse brain atlas). M1 cortex, striatum and SN are dissected from both hemispheres on ice-cold glass dishes and transferred immediately in cryo-tubes into liquid nitrogen and are subsequently stored at -80°C. Tissues were thawed in ice, sonicated in 0.1M PBS at a ratio of 1:10 (w/v). Total protein concentrations were determined using Lowry protein assay. An ELISA kit (MyBioSource, San Diego, USA, Cat. MBS355435) was used to estimate BDNF concentrations with a detection range of 31.2-2000 pg/ml, while GDNF concentrations were measured by an ELISA kit (MyBioSource, San Diego, USA, Cat. MBS2020326) with a detection range of 78–5000 pg/ml. Manufacturer's instructions were followed, and protein concentrations are showed as pg/mg of total protein.

3.9.0 Statistical Analysis

Normality of each data set was investigated by Q-Q-Plots. Parametric methods were employed for normally distributed data, and non-parametric tests were used in the instances of non-normal distributed data or unequal variances. For analysis of more than two groups in striatal TH^+ optical density, stereological estimation of SN cell numbers, cylinder and rotarod tests, HPLC analyses, estimation of lymphocyte numbers, FACS analyses, cytokine analyses, the parametric one-way ANOVA was used, followed by Tukey's multiple comparison test. Unpaired, two-tailed Student's t test was used for comparing BDNF and GDNF concentrations, striatal TH^+ optical density, stereological estimation of SN cell numbers, rotarod tests and estimation of lymphocyte numbers, between two groups. *P<0.05, **P<0.01, ***P<0.001 were considered as significant P-values.

4.0 Results

4.1.0 CD28SA Target Engagement: Expansion of Treg Three Days after CD28SA Injection

To evaluate the engagement of CD28SA with its target, CD28, on T cells, we studied the Treg numbers as well as IL-10 levels after the administration of CD28SA. The CD28SA dosage was i.p. injected into mice one week after the stereotaxic injection of AAV1/2 viral vector. The lag time of one week was selected to ensure a restoration of BBB integrity following the AAV1/2 delivery and exclude an early BBB leakage for T cell infiltration into the brain. The CD28SA dose per mouse was 200 μg, based on previous studies employing a similar dose for Treg expansion (Liang et al., 2017). Three days after the CD28SA administration, mice were sacrificed, and FACS analysis was performed on cells extracted from tissue homogenates of cervical lymph nodes (Figure 7A), spleen (Figure 8A), and brain (Figure 9A). The cell population in question in this FACS analysis was $CD4^+CD25^+FoxP3^+$ Treg.

Results displayed an increase of Treg percentage in control PBS-injected haSyn PD mice compared with control PBS-injected EV mice. That indicates a dysregulation of Treg by haSyn that is independent of CD28 treatment (Figure 7B and 8B). Experimental groups that received CD28SA whether haSyn or EV mice showed in both cases marked increase of Treg as indicated by significant increase in the percentage of $CD4^+CD25^+FoxP3^+$ T cells in cervical lymph nodes and spleen as compared to control groups that received PBS instead (Figure 7B and 8B). This indicates a Treg expansion in peripheral secondary lymphoid organs. Centrally, Treg were only observed in brains of haSyn PD mice, but not in EV mice, which further implicates a specific mechanism involving Treg activation and infiltration into the brain in response to haSyn only (Figure 9B).

We also measured IL-10 levels employing a cytokine multiplex assay as an additional Treg marker since IL-10 is one of the signature cytokines of Treg. Our data demonstrate a clear elevation of IL-10 in in the cervical lymph nodes and spleen of EV and haSyn PD mice that

received CD28SA. IL-10 was not observed to increase in the brain in any of the groups whether EV or haSyn. Taken together, these data indicate successful target engagement and Treg expansion (Figure 7C, 8C, 9C).

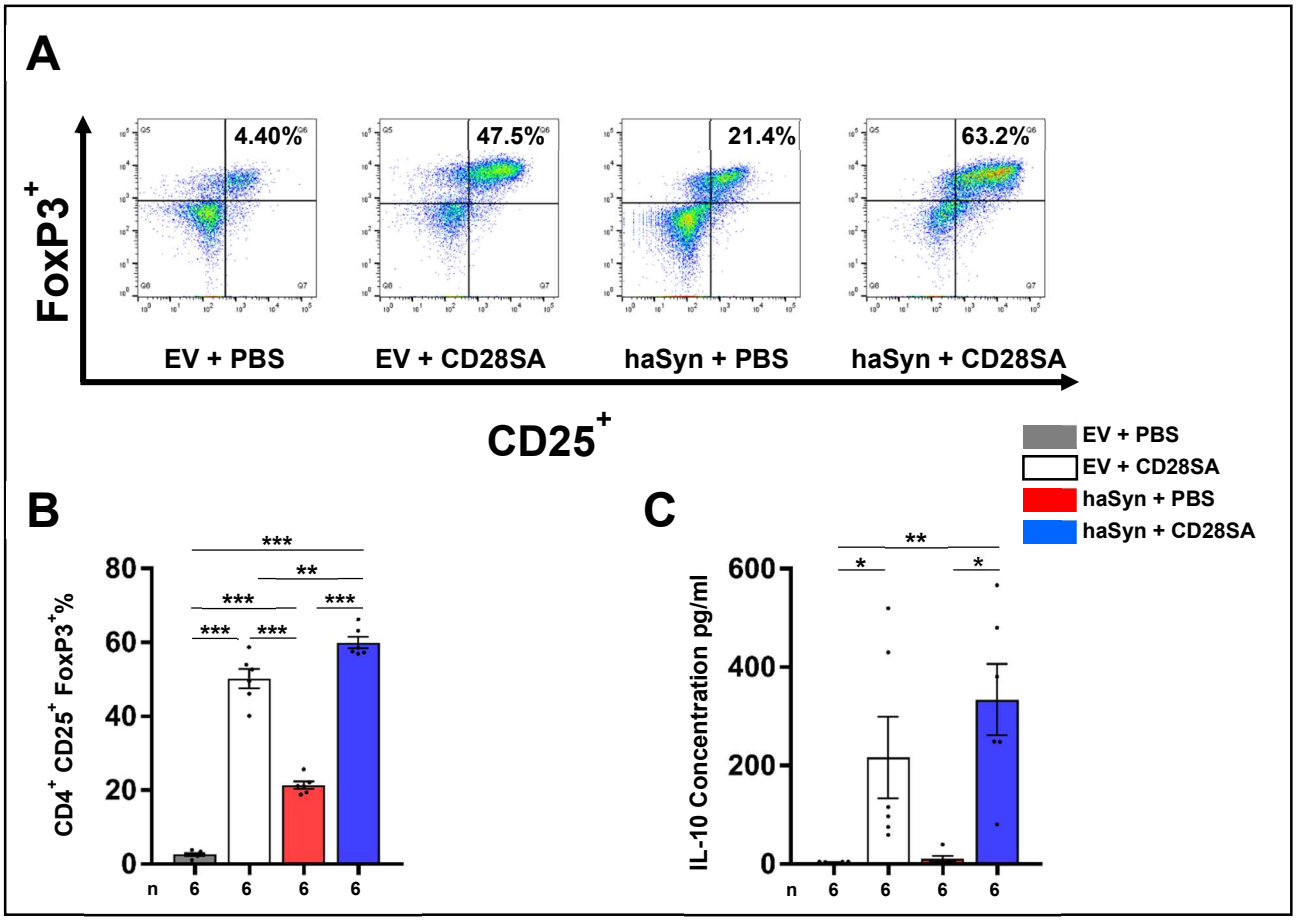

Figure 7: Treg Expansion in Cervical Lymph Nodes (CLN) after CD28SA injection

*A, FACS analysis of Treg in CLN three days following an i.p. injection of CD28SA. B, Treg percentages in CLN. Statistical analysis by one-way ANOVA followed by Tukey's multiple comparisons test: F(3, 20)=255.7, P<0.0001. C, IL-10 concentrations in CLN. Statistical by Kruskal-Wallis test followed by Dunn's multiple comparisons: H=17.93, P=0.0005; *P<0.05, **P<0.01, ***P<0.001. All data are shown as mean ± SEM. n = number of biologically independent animals.*

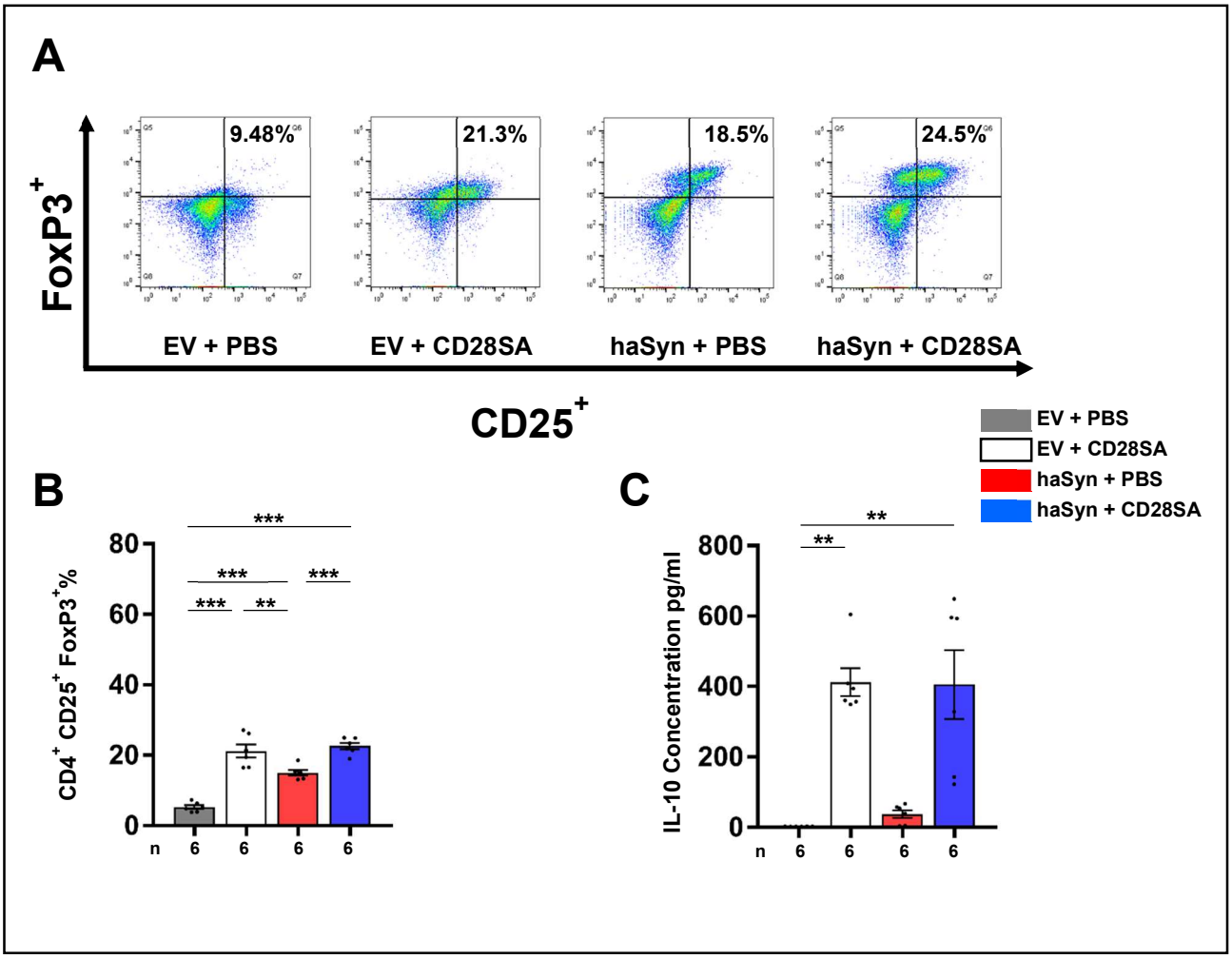

Figure 8: Treg Expansion in Spleen after CD28SA injection

*A, FACS analysis of Treg in spleen three days following an i.p. injection of CD28SA. B, Treg percentages in spleen. Statistical analysis by one-way ANOVA followed by Tukey's multiple comparisons test: $F(3, 20)=46.26$, $P<0.0001$. C, IL-10 concentrations in spleen. Statistical by Kruskal-Wallis test followed by Dunn's multiple comparisons: $H=19.47$, $P=0.0002$; *$P<0.05$, **$P<0.01$, ***$P<0.001$. All data are shown as mean ± SEM. n = number of biologically independent animals.*

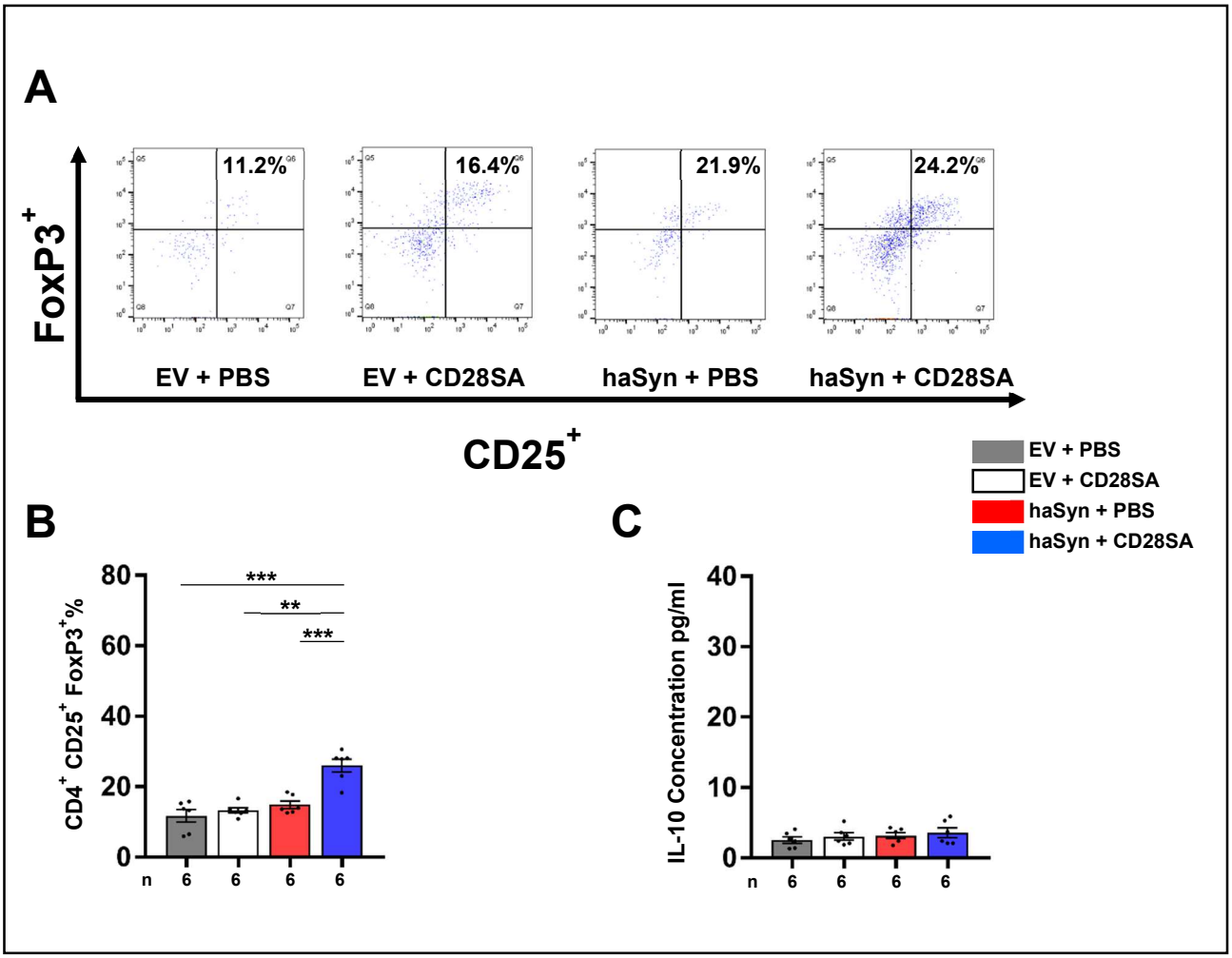

Figure 9: Treg Expansion in Brain after CD28SA injection

*A, FACS analysis of Treg in brain three days following an i.p. injection of CD28SA. B, Treg percentages in brain. Statistical analysis by one-way ANOVA followed by Tukey's multiple comparisons test: $F(3, 20)=20.22$, $P<0.0001$. C, IL-10 concentrations in spleen. Statistical analysis by one-way ANOVA followed by Tukey's multiple comparisons test: $F(3, 20)=0.643$, $P=0.5963$; *$P<0.05$, **$P<0.01$, ***$P<0.001$. All data are shown as mean ± SEM. n = number of biologically independent animals.*

4.2.0 CD28SA Rescues Nigrostriatal Dopaminergic Degeneration in hasyn PD Mice

After demonstration of successful CD28SA engagement, we analyzed the effect of one dosage of CD28SA on the behavioral deficits as well as the neurodegeneration in the nigrostriatal pathway of treated mice ten weeks after the stereotaxic injection of haSyn.

While PBS-treated haSyn PD mice showed motor impairment as assessed by reduced latency to fall off the rotating rod and preference to use the right paw in the cylinder test, (ipsilateral to the side of the stereotaxic injection in the SN), CD28SA-treated mice and all EV mice displayed stable motor function as assessed by higher latency on the rotating rod and a balance paw usage in the cylinder test (Figure 10).

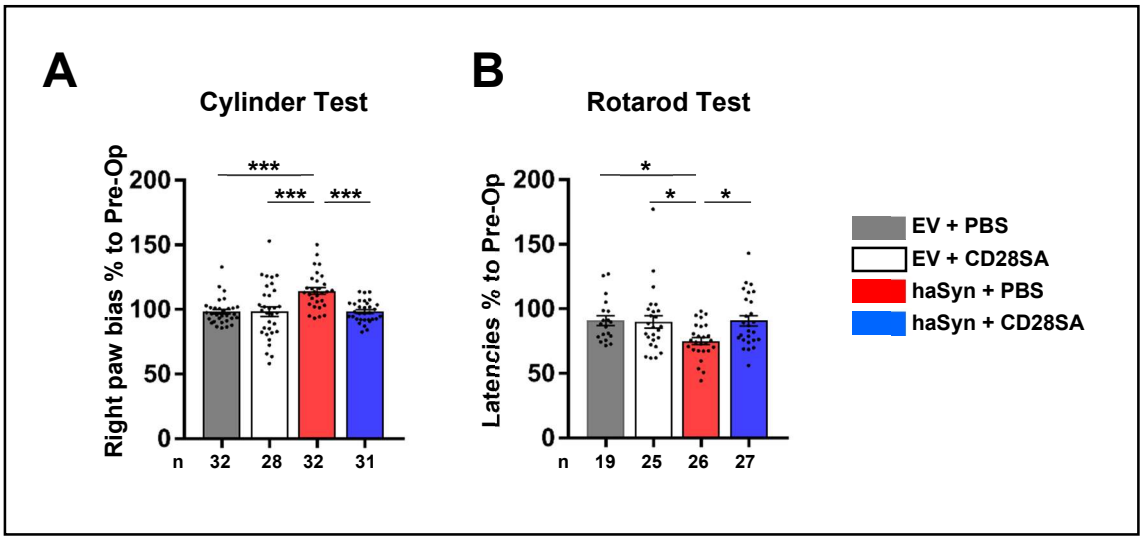

Figure 10: CD28SA augments behavioral deficits in haSyn PD mice.

*Evaluation of behavioral analyses using cylinder test (A) and rotarod performance (B) of EV-injected wildtype mice with PBS or with CD28SA treatment (EV+PBS and EV+CD28SA, respectively), as well as haSyn-injected wildtype mice with PBS or with CD28SA (haSyn+PBS and haSyn+CD28SA, respectively). Statistical analysis for Cylinder test by Kruskal-Wallis test followed by Dunn's multiple comparisons test: H=26.36, P<0.0001; Statistical analysis for Rotarod test by one-way ANOVA followed by Tukey's multiple comparisons test: F(3, 92)=3.921, P=0.0110; *P<0.05, **P<0.01, ***P<0.001. All data are shown as mean ± SEM. n = number of biologically independent animals.*

At the mentioned time point, we did a quantification of dopaminergic TH^+ neurons in the SN (Figure 11) as well as an analysis of the optical density of TH^+ fibers in the striatum (Figure 12). In PBS-treated haSyn PD mice, we observed a reduced number of dopaminergic neurons in the SN and diminished density of dopaminergic fibers in the striatum, while CD28SA-treated haSyn PD mice were consistent with control EV groups in showing higher numbers of

dopaminergic neurons in SN and higher optical density of TH^+ fibers in the striatum (Figure 11B and 12B). A parallel Nissl staining of SN neurons was performed to always ensure that any changes in neuron count are results of total neuron count differences ($Nissl^+$ neurons) and exclude that the increase or decrease is based only on the enzymatic activity of TH (Figure 11C).

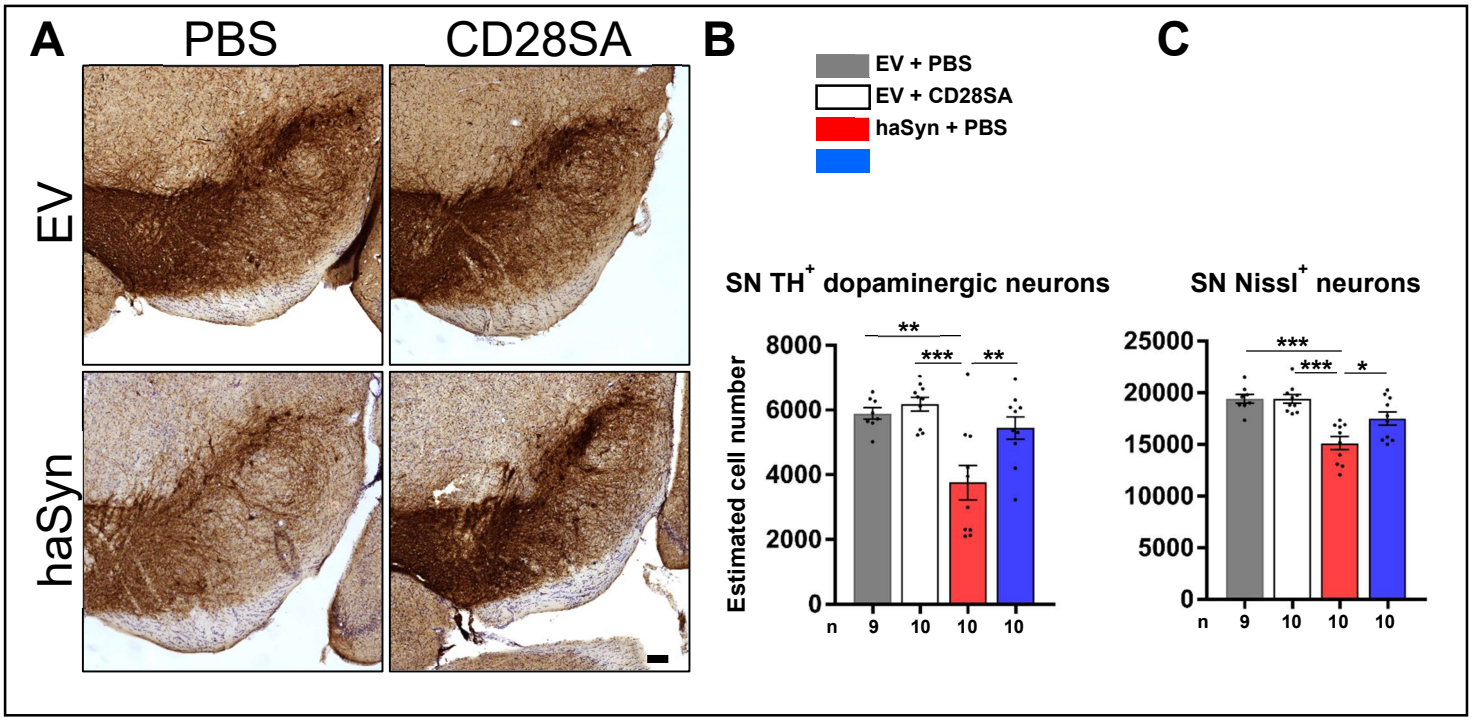

Figure 11: CD28SA rescues SN dopaminergic degeneration in haSyn PD mice.

*A, Representative images of TH^+/$Nissl^+$ dopaminergic neurons in the SN of EV-injected wildtype mice with PBS or with CD28SA treatment (EV+PBS and EV+CD28SA, respectively), as well as haSyn-injected wildtype mice with PBS or with CD28SA (haSyn+PBS and haSyn+CD28SA, respectively). B, Estimated cell number by unbiased stereology for TH^+ SN neurons. Statistical analysis by one-way ANOVA followed by Tukey's multiple comparisons test: TH^+, $F(3, 34)=9.209$, $P=0.0001$; $Nissl^+$, $F(3, 34)=13.77$, $P<0.0001$. *P<0.05, **P<0.01, ***P<0.001. All data are shown as mean ± SEM. n = number of biologically independent animals. Scale bars: 100 µm each.*

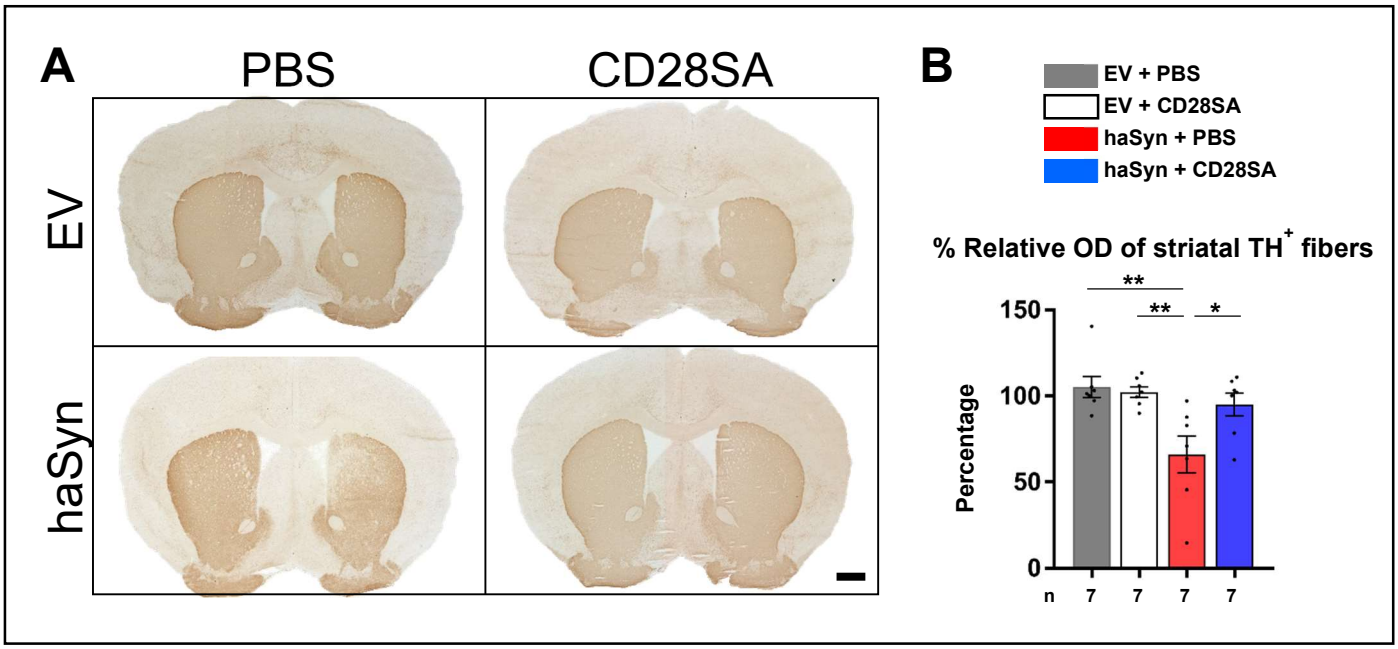

Figure 12: CD28SA rescues striatal dopaminergic degeneration in haSyn PD mice.

*A, Representative images from EV-injected wildtype mice with PBS or with CD28SA treatment (EV+PBS and EV+CD28SA, respectively), as well as haSyn-injected wildtype mice with PBS or with CD28SA (haSyn+PBS and haSyn+CD28SA, respectively) showing the dorsolateral striatum after TH⁺ immunostaining. B, Results from optical density measurements. Statistical analysis by one-way ANOVA followed by Tukey's multiple comparisons test: F(3, 24)=6.195, P=0.0029. *P<0.05, **P<0.01, ***P<0.001. All data are shown as mean ± SEM. n = number of biologically independent animals. Scale bars: 500 µm each.*

To demonstrate further the effects on the dopaminergic terminals in the striatum, we measured striatal dopamine (DA) and homovanillic acid (HVA) levels. Where PBS-treated mice haSyn PD mice exhibit reduced striatal dopamine and pathological DA/HVA ration, CD28SA treatment was able to restore normal dopamine levels in the striatum and show a healthier DA/HVA ratio (Figure 13). These data indicate that CD28SA treatment in this haSyn PD mouse model was successful in providing neuroprotection, fixing behavioral impairments, and maintaining normal dopamine levels.

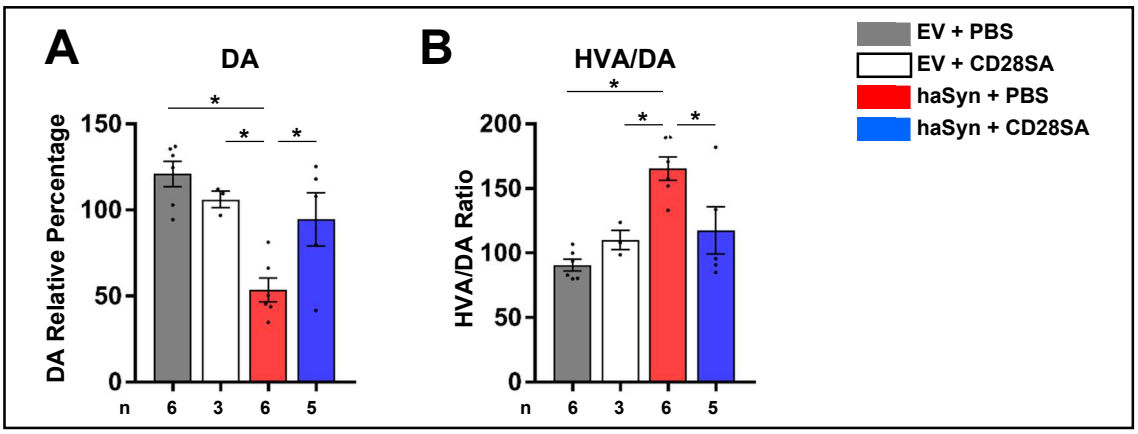

Figure 13: CD28SA effects on striatal dopamine levels in haSyn PD mice.

*Analyses of striatal dopamine levels (A) and HVA/DA ratio (B). Statistical analysis by one-way ANOVA followed by Tukey's multiple comparisons test: DA, F(3, 16)=9.805, P=0.0007; HVA/DA, F(3, 16)=9.361, P=0.0008. *P<0.05, **P<0.01, ***P<0.001. All data are shown as mean ± SEM. n = number of biologically independent animals.*

4.3.0 CD28SA Effect on Neuroinflammation in the Nigrostriatal Tract of haSyn PD Mice

To explain the mechanisms by which CD28SA drove the positive outcomes regarding neurodegeneration in haSyn PD mouse model, we sought to examine the status of neuroinflammation in our model in response to the CD28SA treatment after 10 weeks of haSyn stereotaxic delivery.

Our data indicate persistent neuroinflammation in haSyn PD mice that is absent in EV mice. That is demonstrated by higher counts of $CD4^+$ T cells (Figure 14), $CD8^+$ T cells (Figure 15), as well as $CD11b^+$ myeloid cells (Figure 16) in the SN and striatum of PBS-treated haSyn PD mice.

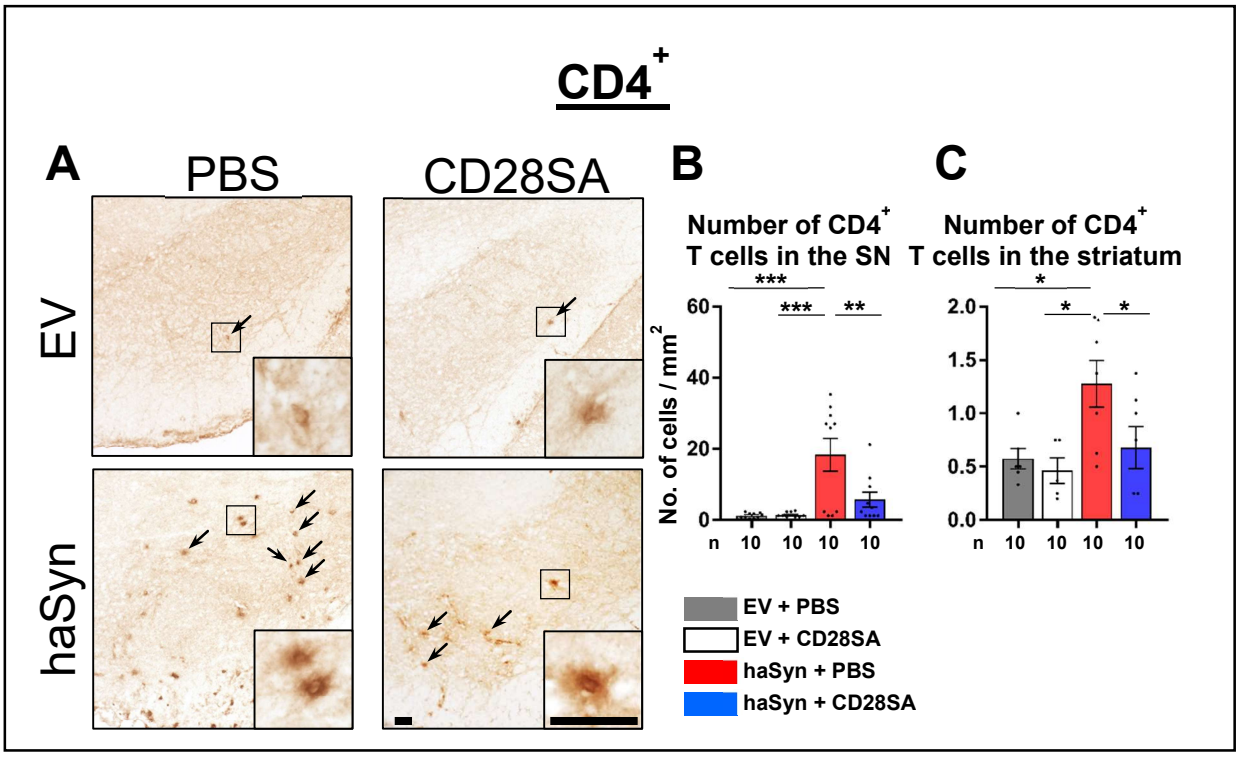

Figure 14: Effect of CD28SA on $CD4^+$ T cells in the nigrostriatal tract of haSyn PD mice

*A, Immunohistochemical staining for $CD4^+$ T cells in the SN of EV-injected wildtype mice with PBS or with CD28SA treatment (EV+PBS and EV+CD28SA, respectively), as well as haSyn-injected wildtype mice with PBS or with CD28SA (haSyn+PBS and haSyn+CD28SA, respectively). B-C, Quantification of $CD4^+$ T cells in SN (B) and striatum (C) of indicated groups of mice. Statistical analysis by one-way ANOVA followed by Tukey's multiple comparisons test: SN, $F(3, 36)=10.16$, $P<0.0001$; Striatum, $F(3, 21)=4.263$, $P=0.0168$; *$P<0.05$, **$P<0.01$, ***$P<0.001$. All data are shown as mean ± SEM. n = number of biologically independent animals. Scale bars: 20 µm each.*

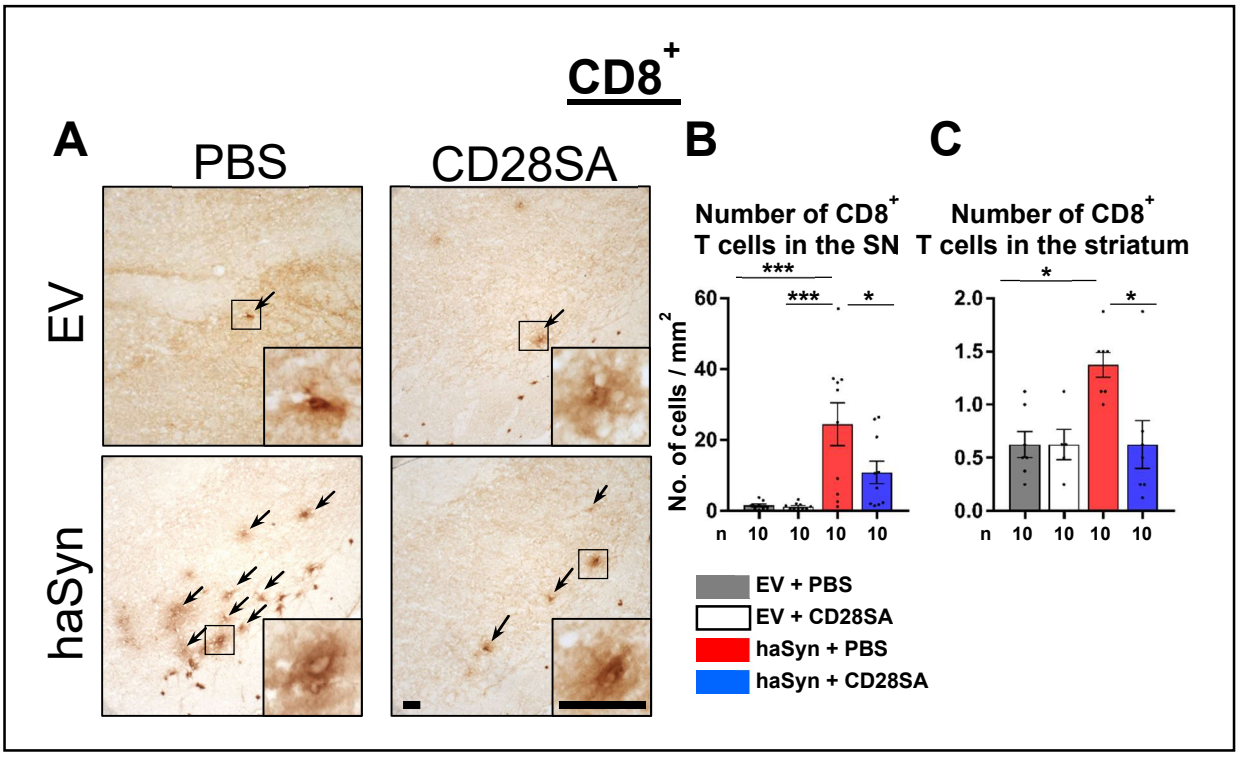

Figure 15: Effect of CD28SA on CD8⁺ T cells in the nigrostriatal tract of haSyn PD mice

*A, Immunohistochemical staining for CD8⁺ T cells in the SN of EV-injected wildtype mice with PBS or with CD28SA treatment (EV+PBS and EV+CD28SA, respectively), as well as haSyn-injected wildtype mice with PBS or with CD28SA (haSyn+PBS and haSyn+CD28SA, respectively). B-C, Quantification of CD8⁺ T cells in SN (B) and striatum (C) of indicated groups of mice. Statistical analysis of CD8⁺ count in SN by one-way ANOVA followed by Tukey's multiple comparisons test F(3, 35)=9.74, P<0.0001; *P<0.05, **P<0.01, ***P<0.001. Statistical analysis of CD8+ count in striatum by Kruskal-Wallis test followed by Dunn's multiple comparisons test, H=10.56, P=0.0144; *P<0.05, **P<0.01, ***P<0.001. All data are shown as mean ± SEM. n = number of biologically independent animals. Scale bars: 20 µm each..*

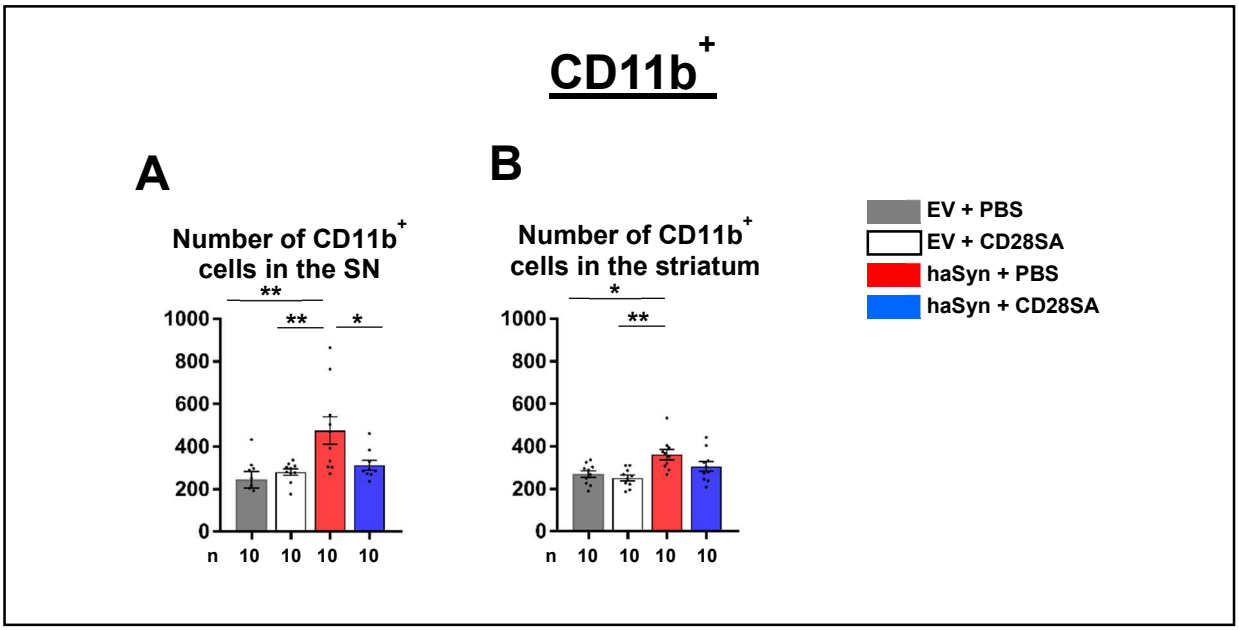

Figure 16: Effect of CD28SA on CD11b⁺ cells in the nigrostriatal tract of haSyn PD mice

*Quantification of CD11b⁺ cells in SN (B) and striatum (C) of EV-injected wildtype mice with PBS or with CD28SA treatment (EV+PBS and EV+CD28SA, respectively), as well as haSyn-injected wildtype mice with PBS or with CD28SA (haSyn+PBS and haSyn+CD28SA, respectively). Statistical analysis by one-way ANOVA followed by Tukey's multiple comparisons test: SN CD11b+, F(3, 34)=6.461, P=0.0014; Striatum CD11b⁺,F (3, 36), P=.0.0019. *P<0.05, **P<0.01, ***P<0.001. All data are shown as mean ± SEM. n = number of biologically independent animals.*

That is also shown by higher activation of T cells in the brain of haSyn PD mice as evidenced by the higher percentages of $CD4^+CD69^+$ T cells (Figure 17) and $CD8^+CD69^+$ T cells (Figure 18) infiltrating the brain as analyzed by FACS. CD28SA treatment of haSyn mice succeeds in reducing the counts of $CD4^+$ and $CD8^+$ T cells, and $CD11b^+$ myeloid cells and it reduces the percentages of $CD4^+CD69^+$ and $CD8^+CD69^+$ T cells (Figures 14-18).

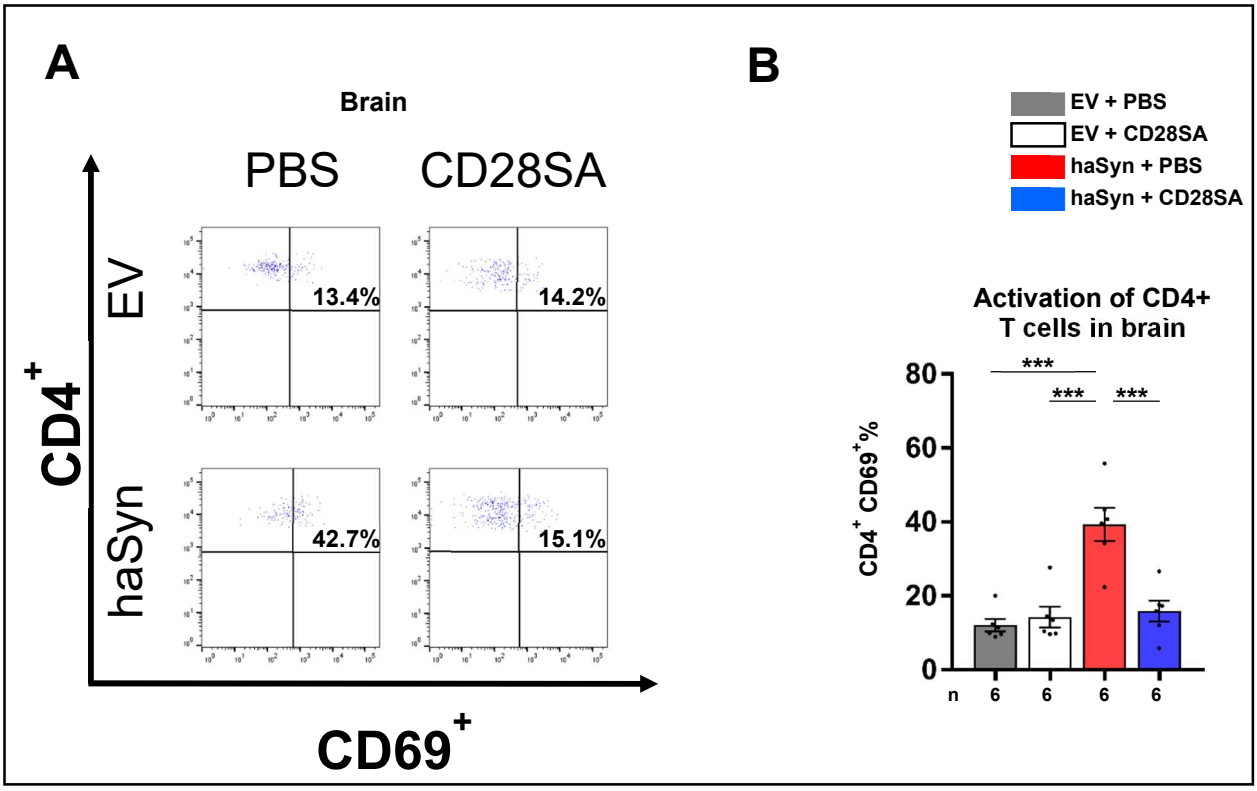

Figure 17: CD28SA effect on $CD4^+$ T-cell Activation in the Brain, 10 weeks after injection of viral vector.

*A, Representative FACS analysis images showing the infiltration of $CD4^+CD69^+$ T cells into the brain of EV-injected wildtype mice with PBS or with CD28SA treatment (EV+PBS and EV+CD28SA, respectively), as well as haSyn-injected wildtype mice with PBS or with CD28SA (haSyn+PBS and haSyn+CD28SA, respectively). B, percentages of $CD4^+CD69^+$ in the brain as analyzed by FACS. Statistical analysis by one-way ANOVA followed by Tukey's multiple comparisons test: $F(3, 20)=16.82$, $P<0.0001$; *$P<0.05$, **$P<0.01$, ***$P<0.001$. All data are shown as mean ± SEM. n = number of biologically independent animals.*

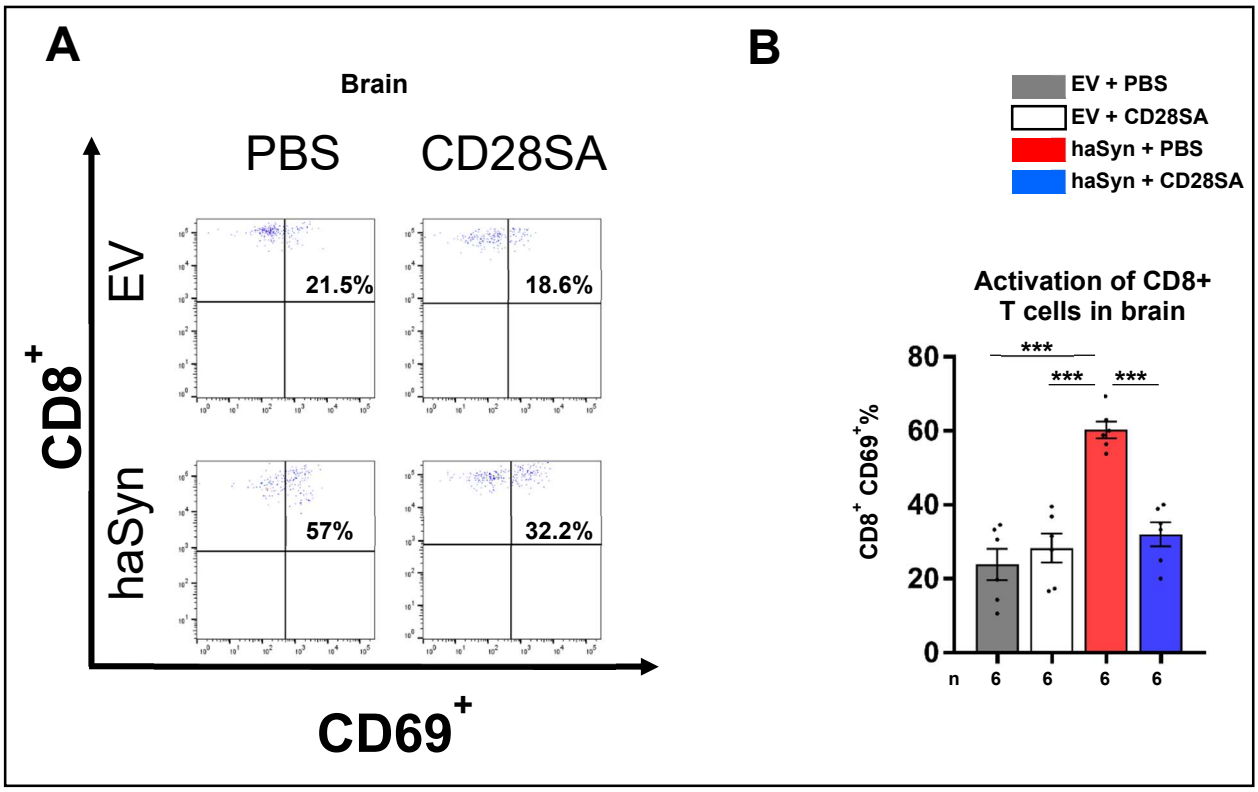

Figure 18: CD28SA effect on $CD8^+$ T-cell Activation in the Brain, 10 weeks after injection of viral vector.

*A, Representative FACS analysis images showing the infiltration of $CD8^+CD69^+$ T cells into the brain of EV-injected wildtype mice with PBS or with CD28SA treatment (EV+PBS and EV+CD28SA, respectively), as well as haSyn-injected wildtype mice with PBS or with CD28SA (haSyn+PBS and haSyn+CD28SA, respectively). B, percentages of $CD8^+CD69^+$ in the brain as analyzed by FACS. Statistical analysis by one-way ANOVA followed by Tukey's multiple comparisons test: $F(3, 20)=22.16$, $P<0.0001$; *$P<0.05$, **$P<0.01$, ***$P<0.001$. All data are shown as mean ± SEM. n = number of biologically independent animals.*

As for cytokine levels, while PBS-treated haSyn PD mice showed elevation in IL-2, IL-4, IL-5, IL-13, IL-17, and IFN-γ, CD28SA treatment was effective in preventing this elevation (Figure 19). Nevertheless, it is to be noted that only IL-2 reduction by CD28SA treatment showed up with statistical significance (Figure 19A).

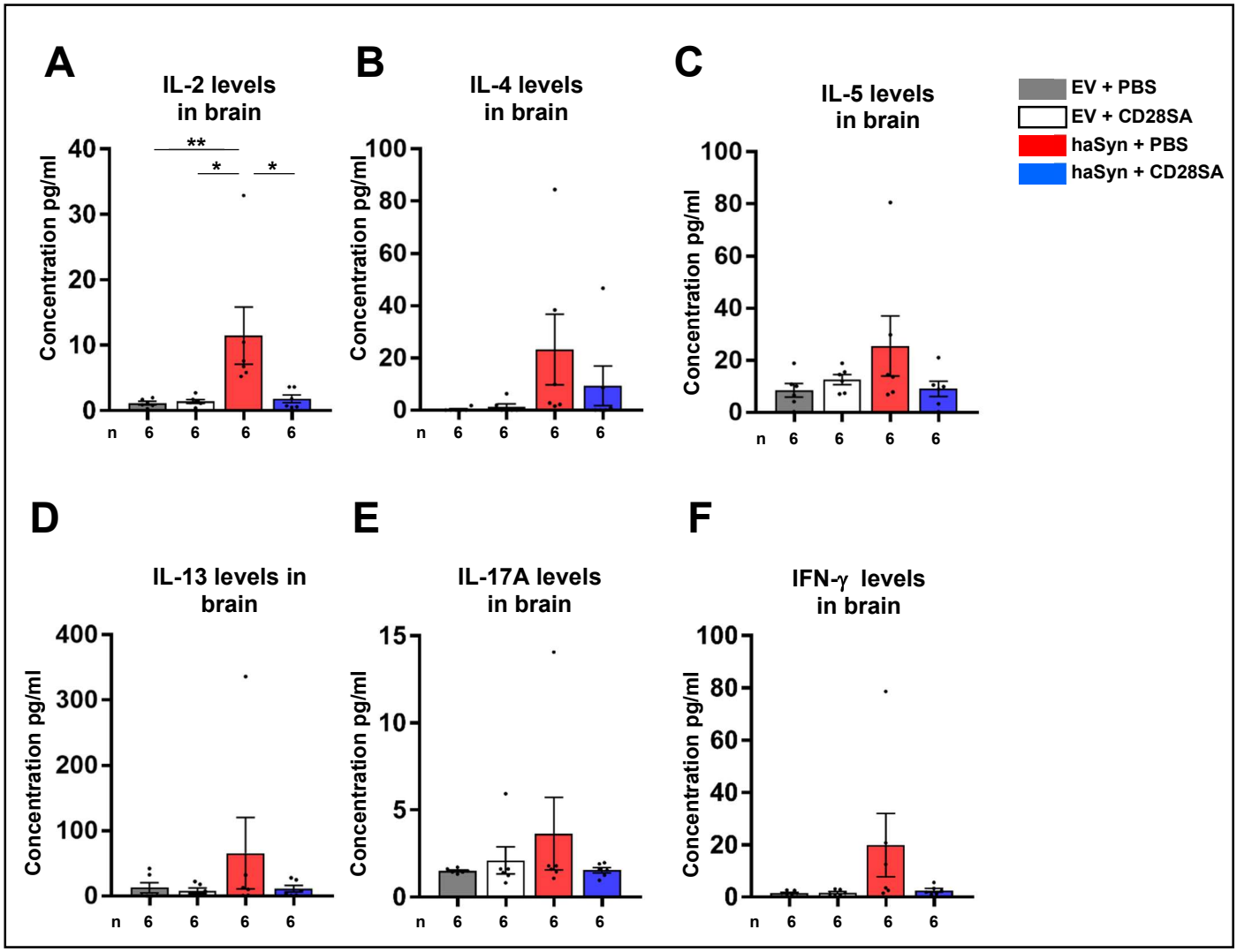

Figure 19: CD28SA effect on cytokine levels in the Brain, 10 weeks after injection of viral vector.

*A-F, Cytokine concentrations in the brain: IL-2 (A), IL-4 (B), IL-5 (C), IL-13 (D), IL-17A (E) and IFN-g (F). Statistical analysis by Kruskal-Wallis Test followed by Dunn's multiple comparisons test: IL-2, H=13.16, P=0.0043. *P<0.05, **P<0.01, ***P<0.001. All data are shown as mean ± SEM. n = number of biologically independent animals.*

These results indicate, the potential of CD28SA in controlling and neutralizing long-term neuroinflammation by one single dosage at an early stage.

4.4.0 Effect of Treg Adoptive Transfer on Nigrostriatal Dopaminergic Degeneration in haSyn PD Mice

We wanted to explore whether the neuroprotective effects of CD28SA are mediated only through Treg expansion or if there might be some special direct effect of CD28SA that is not based on Treg. To answer this question, we decided to run a parallel trial in which we adoptively transferred Treg to the haSyn PD mice instead of treating them with CD28SA. The adoptive transfer took place through a tail vein injection into haSyn PD mice one week after the delivery of the haSyn viral vector by stereotaxic injection.

Donor mice from which Treg were isolated were treated with CD28SA to expand their Treg population before isolating lymphocytes from their lymph nodes and spleen. Treg were then sorted and isolated using a $CD4^+$ negative selection followed by $CD4^+CD25^+$ positive selection using magnetic resonance columns. Isolated Treg were tested for purity before the adoptive transfer, and it stood at around 93% (Figure 20).

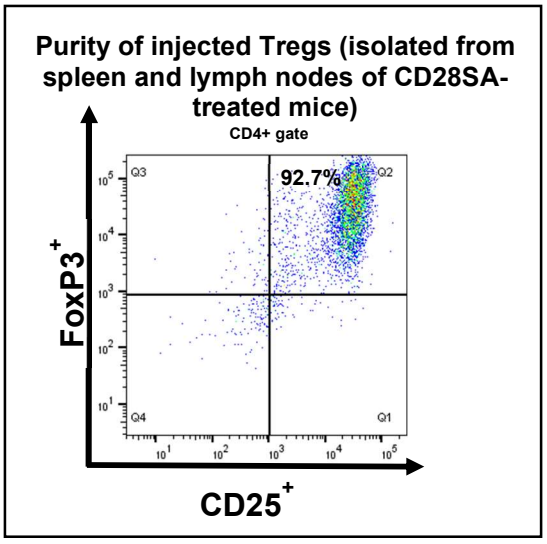

Figure 20: Representative FACS images of the Treg population in the adoptive cells.

In this focused study we ran two experimental groups of mice haSyn PD mice with Treg adoptive transfer or control PBS injection. To test the reconstitution of Treg after adoptive transfer, we drew blood from facial vein of mice and analyzed by FACS the percentage of $CD4^+CD25^+FoxP3^+$ Treg in the $CD4^+$ population and established accordingly higher Treg percentage in mice that received the Treg injection (around 10.4%) vs mice that received PBS (around 5.1%) (Figure 21).

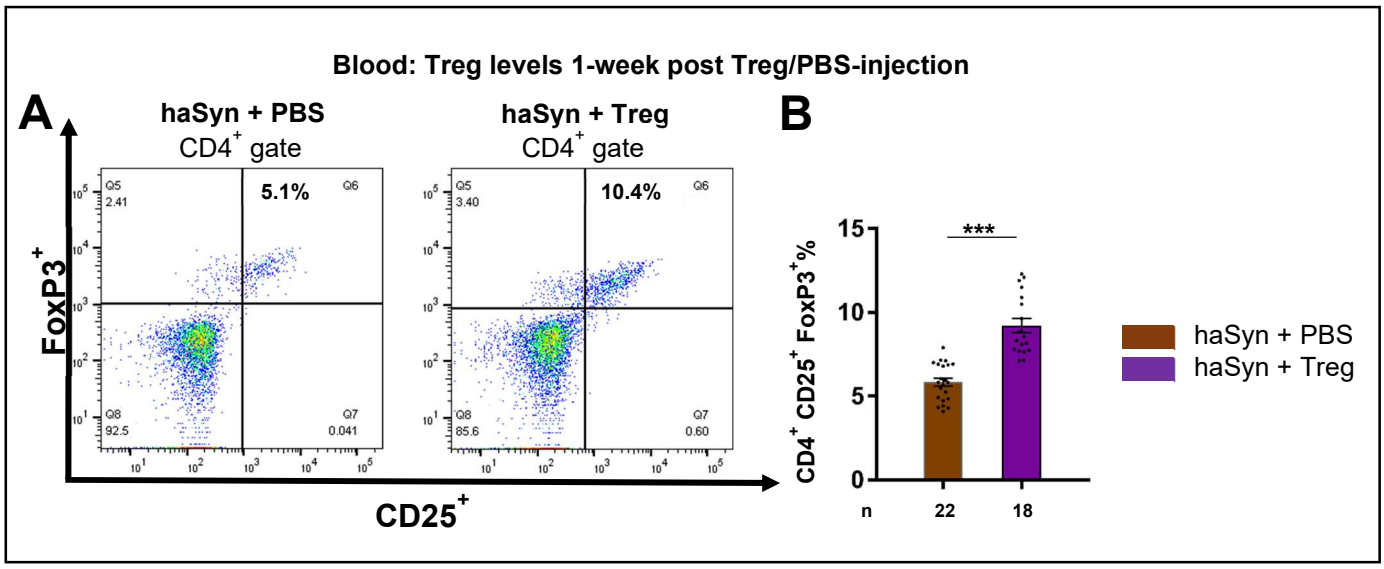

Figure 21: Treg levels 1-week post Treg/PBS-injection

*Comparison of Treg percentages in mice blood, one week after PBS or Treg injection of haSyn-injected wildtype mice (haSyn+PBS and haSyn+Treg, respectively), showing representative images of the FACS analysis (A) as well as statistical comparison (B). Statistical analysis by unpaired two-tailed t-test: t(38)=7.201 and P<0.0001. *P<0.05, **P<0.01, ***P<0.001. All data are shown as mean ± SEM. n = number of biologically independent animals.*

As an additional control, a different group of mice received adoptive transfer of $CD4^+CD25^-$ (Treg-free) cells that were isolated during the positive selection of $CD4^+CD25^+$ Treg, to exclude any the presence of any residual CD28SA that might contribute to cell expansion after the adoptive transfer. Analysis of Treg levels in the spleen in this control group showed low percentage of Treg (Figure 22), which comes contrary to the extensive Treg expansion after CD28SA treatment of haSyn PD mice and EV mice (Figure 8A).

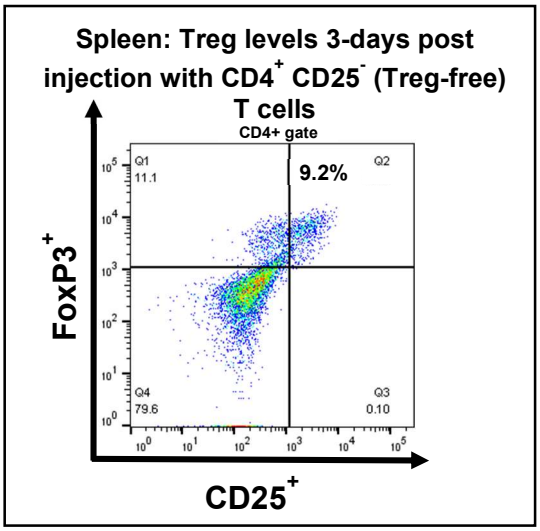

Figure 22: Representative FACS images of the Treg levels 3-days post injection with $CD4^+$ $CD25^-$ (Treg-free)

Performing our analysis at the ten-week time point, results of this experiment confirm that the Treg adoptive transfer was able to replicate the benefits that we established using the CD28SA in the main study. Treg adoptive transfer in haSyn PD mice enhanced the motor deficits in the rotarod performance test (Figure 23), preserved higher numbers of dopaminergic neurons in the SN and rescued the striatal dopaminergic terminals and as compared to control PBS treatment (Figure 24).

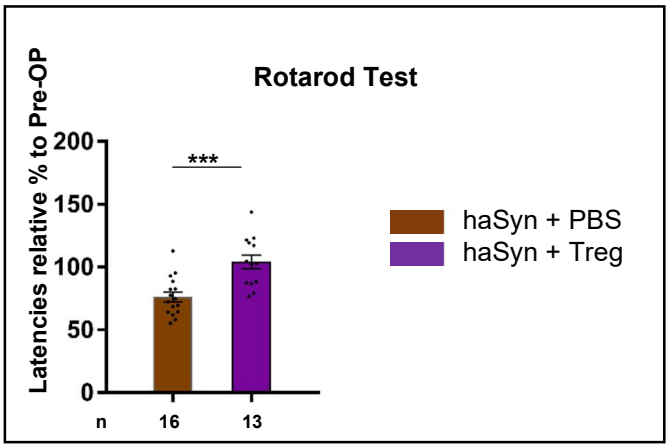

Figure 23: Treg adoptive transfer augments behavioral deficits in haSyn PD mice.

*Evaluation of behavioral analysis using rotarod performance in haSyn-injected wildtype mice (haSyn+PBS and haSyn+Treg, respectively). Statistical analysis by unpaired two-tailed t-test: t(27)=4.204 and P=0.0003. *P<0.05, **P<0.01, ***P<0.001. All data are shown as mean ± SEM. n = number of biologically independent animals.*

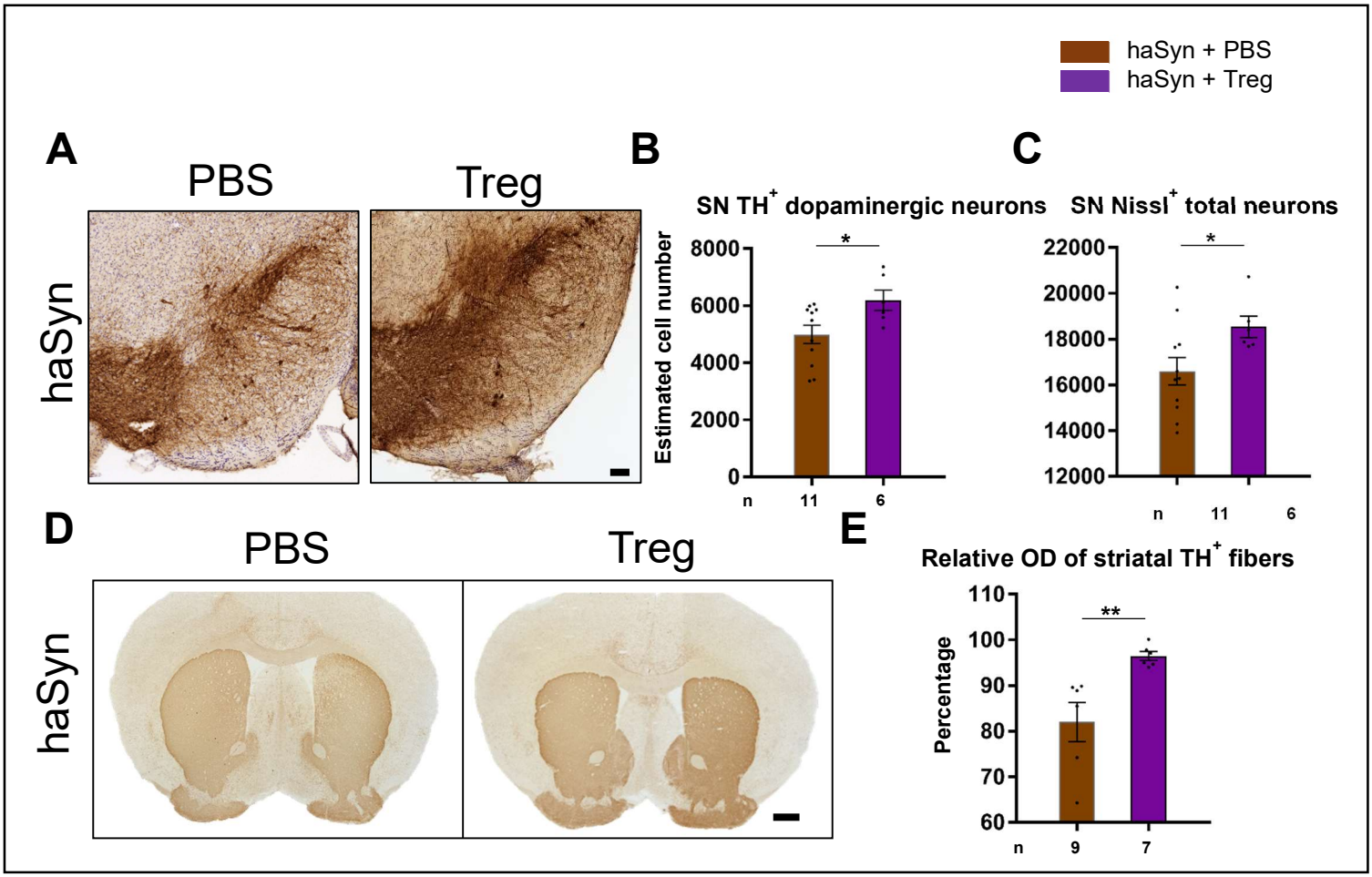

Figure 24: Effect of Treg adoptive transfer on nigrostriatal dopaminergic degeneration in haSyn PD mice.

*A-C, Representative images of TH⁺/Nissl⁺ dopaminergic neurons in the SN of haSyn-injected wildtype mice (haSyn+PBS and haSyn+Treg, respectively) (A) and estimated cell number by unbiased stereology for TH⁺ SN neurons (B) and Nissl⁺ SN neurons (C). Statistical analysis by unpaired two-tailed t-test: TH⁺, t(15)=2.378 and P=0.0311; Nissl+, t(15)=2.171 and P=0.0464. *P<0.05, **P<0.01, ***P<0.001. D-E, Representative images from the indicated groups of mice showing the dorsolateral striatum after TH⁺ immunostaining (D). Results from optical density measurements are shown (E). Statistical analysis by unpaired two-tailed t-test: t(10)=3.279 and P=0.0083. *P<0.05, **P<0.01, ***P<0.001. All data are shown as mean ± SEM. n = number of biologically independent animals. Scale bars: A 100 µm each, D 500 µm each.*

Treg enriched haSyn PD mice also showed amelioration of neuroinflammation as evidenced by reduced number of $CD4^+$ T cells (Figure 25) and $CD8^+$ T cells (Figure 26) in the SN and striatum.

These results show that Treg adoptive transfer replicated the neuroprotective, behavioral, and anti-inflammatory effects that was observed using CD28SA.

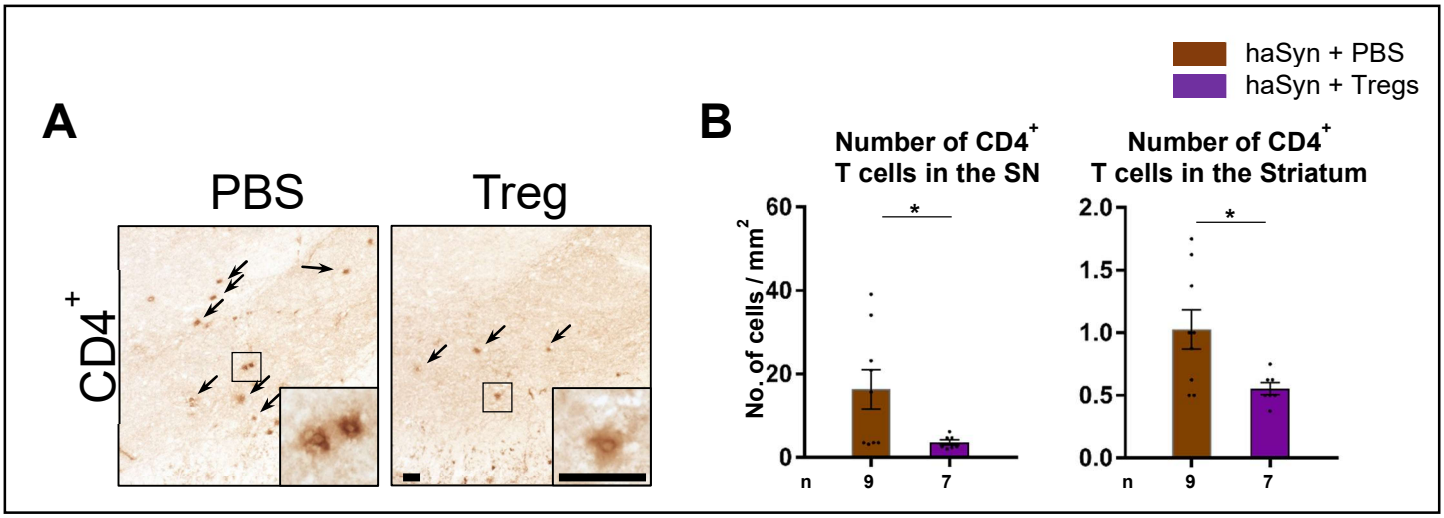

Figure 25: Effect of Treg adoptive transfer on $CD4^+$ T cells in the nigrostriatal tract of haSyn PD mice

*A, Immunohistochemical staining and quantification for $CD4^+$ T cells in the SN of haSyn-injected wildtype mice with PBS or Treg treatment (haSyn+PBS and haSyn+Treg, respectively). Statistical analysis by unpaired two-tailed t-test: SN, t(14)=2.367 and P=0.0329; striatum, t(14)=2.601 and P=0.0209. *P<0.05, **P<0.01, ***P<0.001. All data are shown as mean ± SEM. n = number of biologically independent animals. Scale bars: 20 μm each.*

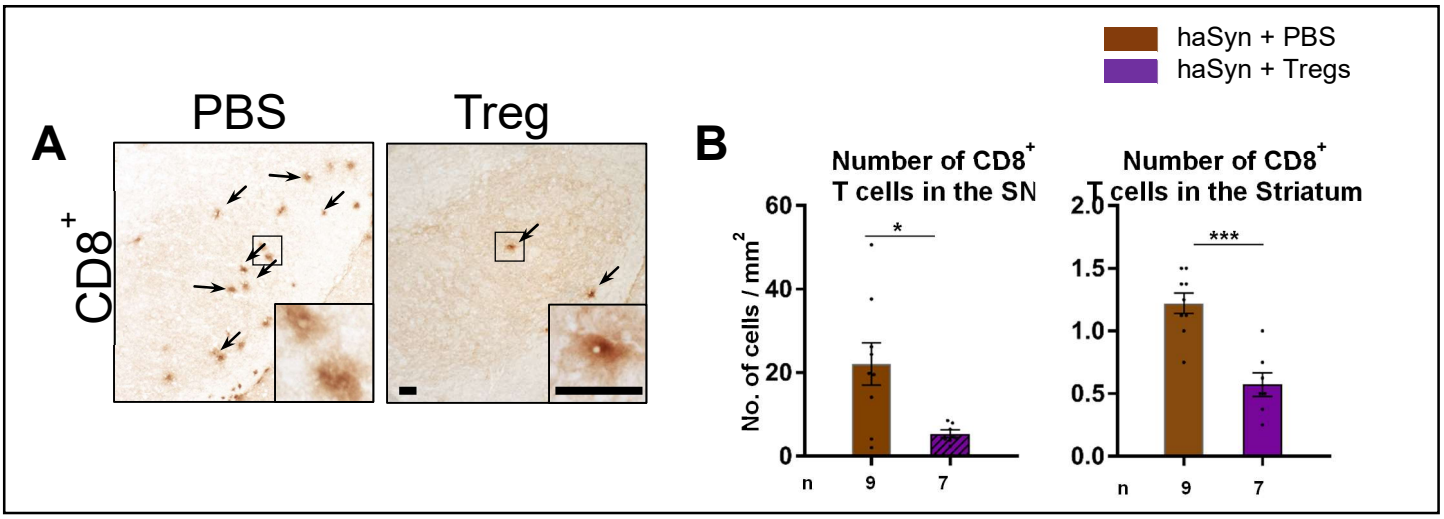

Figure 26: Effect of Treg adoptive transfer on $CD8^+$ T cells in the nigrostriatal tract of haSyn PD mice

*A, Immunohistochemical staining and quantification for $CD8^+$ T cells in the SN of haSyn-injected wildtype mice with PBS or Treg treatment (haSyn+PBS and haSyn+Treg, respectively). Statistical analysis by unpaired two-tailed t-test: SN, t(14)=2.835 and P=0.0132, striatum, t(14)=5.2 and P=0.0001. *P<0.05, **P<0.01, ***P<0.001. All data are shown as mean ± SEM. n = number of biologically independent animals. Scale bars: 20 μm each.*

4.5.0 CD28SA Effect on Early Inflammation in haSyn PD Mice

We wanted to analyze the effect of CD28SA treatment on the early inflammation associated with haSyn injection and PD induction. For that purpose, we used $CD69^+$ as a marker for T cell activation and we looked at its percentages within the $CD4^+$ and $CD8^+$ T cell populations ten days after the haSyn viral vector delivery, which corresponds to three days after CD28SA treatment (Figures 27-29).

In haSyn PD mice, T cell activation is observed, as shown by higher percentages of $CD4^+CD69^+$ cells and $CD8^+CD69^+$ T cells respectively within $CD4^+$ and $CD8^+$ T cell population in cervical lymph nodes (Figure 28) and spleen (Figure 29), and while in the brain, $CD4^+CD69^+$ T cells significantly increased, the $CD8^+CD69^+$ T cells did not (Figure 27). CD28SA treatment of haSyn PD mice can mitigate this T cell activation to a great extent, reducing $CD4^+CD69^+$ cell percentages in the cervical lymph nodes, spleen, and brain, and reducing $CD8^+CD69^+$ cell percentages in the cervical lymph nodes and the spleen but not in the brain (Figures 27-29).

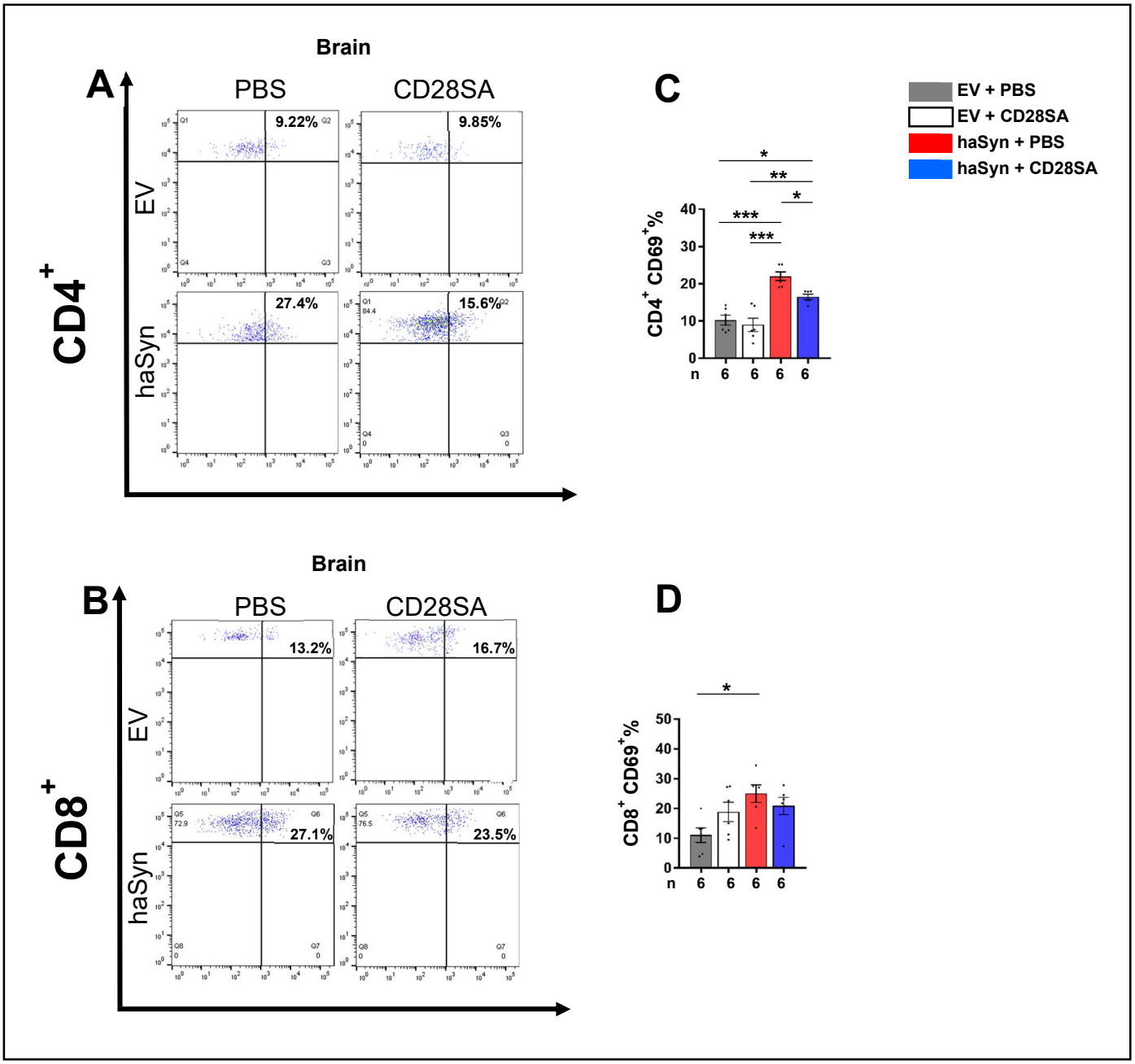

Figure 27: CD28SA effect on T-cell Activation in brain, 10 days after injection of viral vector.

*A-B, Representative FACS analysis images showing the percentages of $CD4^+CD69^+$ (A) and $CD8^+CD69^+$ (B) T cells in brain of EV-injected wildtype mice with PBS or with CD28SA treatment (EV+PBS and EV+CD28SA, respectively), as well as haSyn-injected wildtype mice with PBS or with CD28SA (haSyn+PBS and haSyn+CD28SA, respectively). C, percentages of $CD4^+CD69^+$ cells in brain as analyzed by FACS. Statistical analysis by one-way ANOVA followed by Tukey's multiple comparisons test: $F(3, 20)=21.46$, $P<0.0001$; *$P<0.05$, **$P<0.01$, ***$P<0.001$. D, percentages of $CD8^+CD69^+$ in brain as analyzed by FACS. Statistical analysis by one-way ANOVA followed by Tukey's multiple comparisons test: $F(3, 20)=4.073$, $P=0.0207$; *$P<0.05$, **$P<0.01$, ***$P<0.001$. All data are shown as mean ± SEM. n = number of biologically independent animals.*

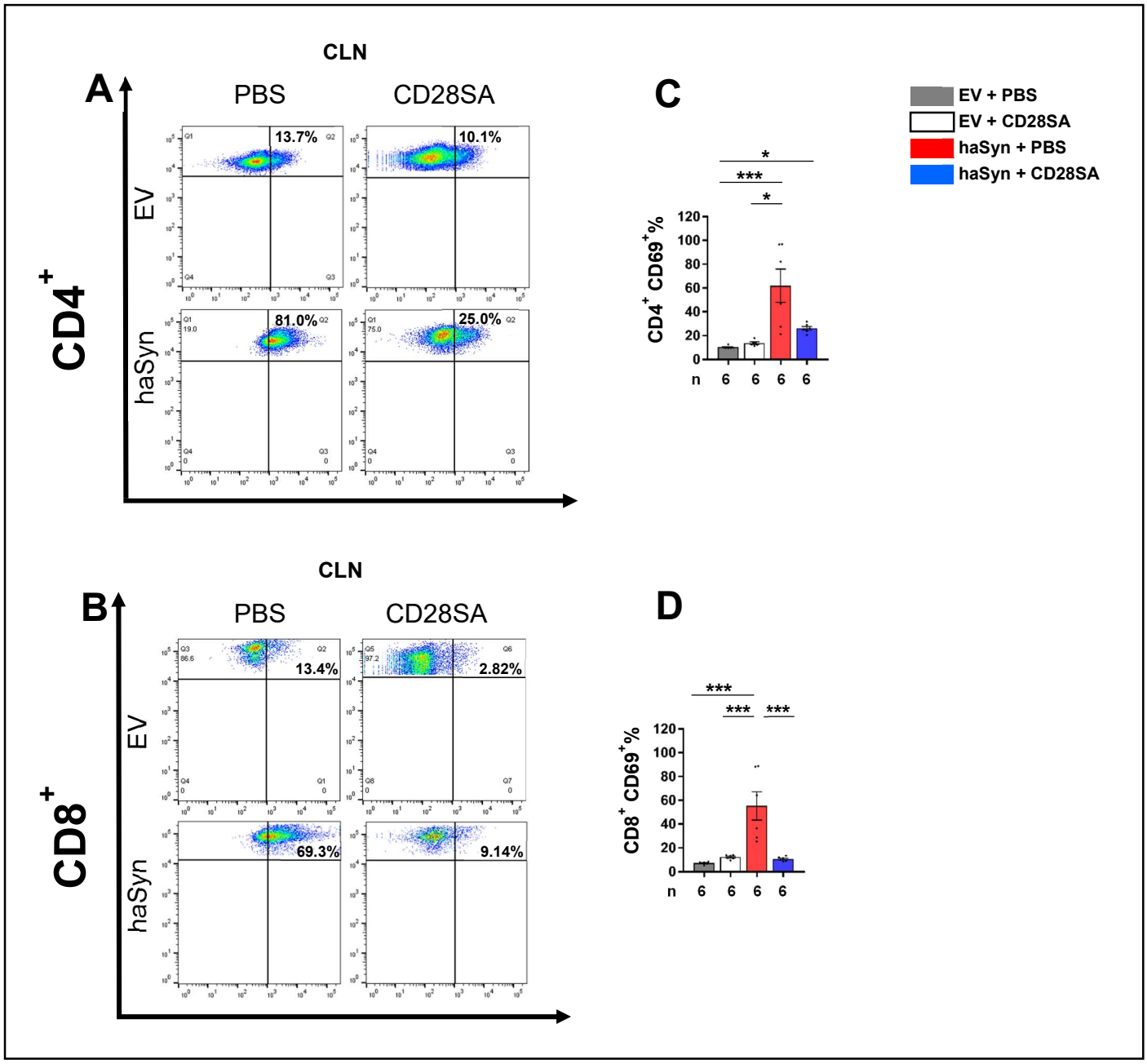

Figure 28: CD28SA effect on T-cell Activation in CLN, 10 days after injection of viral vector.

*A-B, Representative FACS analysis images showing the percentages of $CD4^+CD69^+$ (A) and $CD8^+CD69^+$ (B) T cells in CLN of EV-injected wildtype mice with PBS or with CD28SA treatment (EV+PBS and EV+CD28SA, respectively), as well as haSyn-injected wildtype mice with PBS or with CD28SA (haSyn+PBS and haSyn+CD28SA, respectively). C, percentages of $CD4^+CD69^+$ cells in CLN as analyzed by FACS. Statistical analysis by Kruskal-Wallis test followed by Dunn's multiple comparisons test: H=19.59, P=0.0002; P<0.0001; *P<0.05, **P<0.01, ***P<0.001. D, percentages of $CD8^+CD69^+$ in CLN as analyzed by FACS. Statistical analysis by one-way ANOVA followed by Tukey's multiple comparisons test: F(3, 20)=14.43, P<0.0001; *P<0.05, **P<0.01, ***P<0.001. All data are shown as mean ± SEM. n = number of biologically independent animals.*

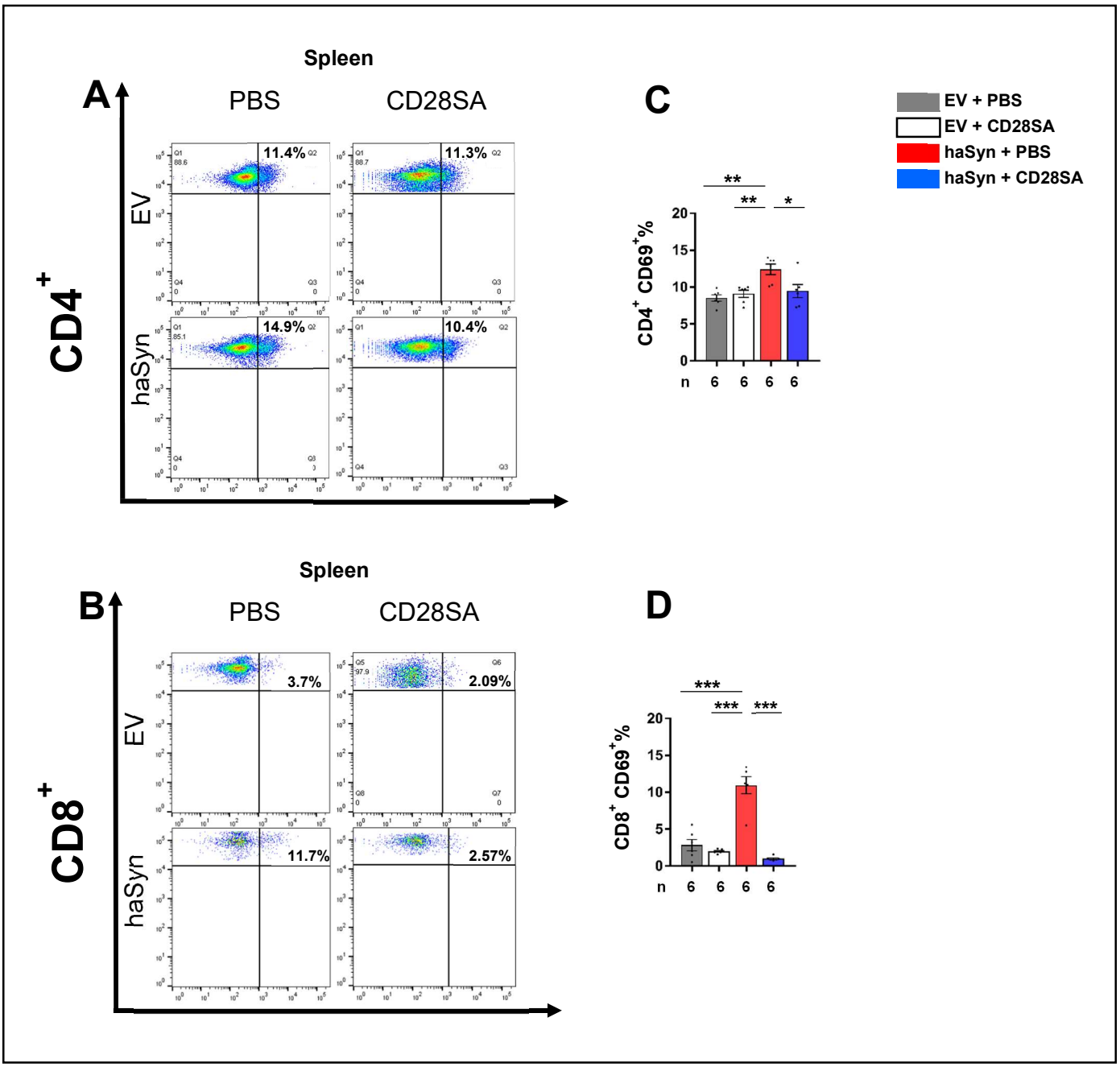

Figure 29: CD28SA effect on T-cell Activation in spleen, 10 days after injection of viral vector.

*A-B, Representative FACS analysis images showing the percentages of $CD4^+CD69^+$ (A) and $CD8^+CD69^+$ (B) T cells in spleen of EV-injected wildtype mice with PBS or with CD28SA treatment (EV+PBS and EV+CD28SA, respectively), as well as haSyn-injected wildtype mice with PBS or with CD28SA (haSyn+PBS and haSyn+CD28SA, respectively). C, percentages of $CD4^+CD69^+$ cells in spleen as analyzed by FACS. Statistical analysis by one-way ANOVA followed by Tukey's multiple comparisons test $F(3, 20)=46.26$, $P<0.0001$; *$P<0.05$, **$P<0.01$, ***$P<0.001$. D, percentages of CD8+CD69+ in spleen as analyzed by FACS. Statistical analysis by one-way ANOVA followed by Tukey's multiple comparisons test: $F(3, 20)=42.57$, $P<0.0001$; *$P<0.05$, **$P<0.01$, ***$P<0.001$. All data are shown as mean ± SEM. n = number of biologically independent animals.*

We also analyzed the cytokine profile in each case at the same early timepoint. The delivery of haSyn into PBS- treated PD mice led to sharp increase in IL-2 levels in the cervical lymph nodes and spleen, which was neutralized and reduced in PD mice that received CD28SA treatment. In the brain, there was no significant difference at that early stage between all mice groups (Figure 30A). We also measured levels of IL-4, IL-5, IL-13 and IFNγ but we reported no difference in cytokine levels between all mice groups (Figure 30B-F).

These data demonstrate that CD28SA was efficient in reducing the peripheral T cell activation at this early timepoint.

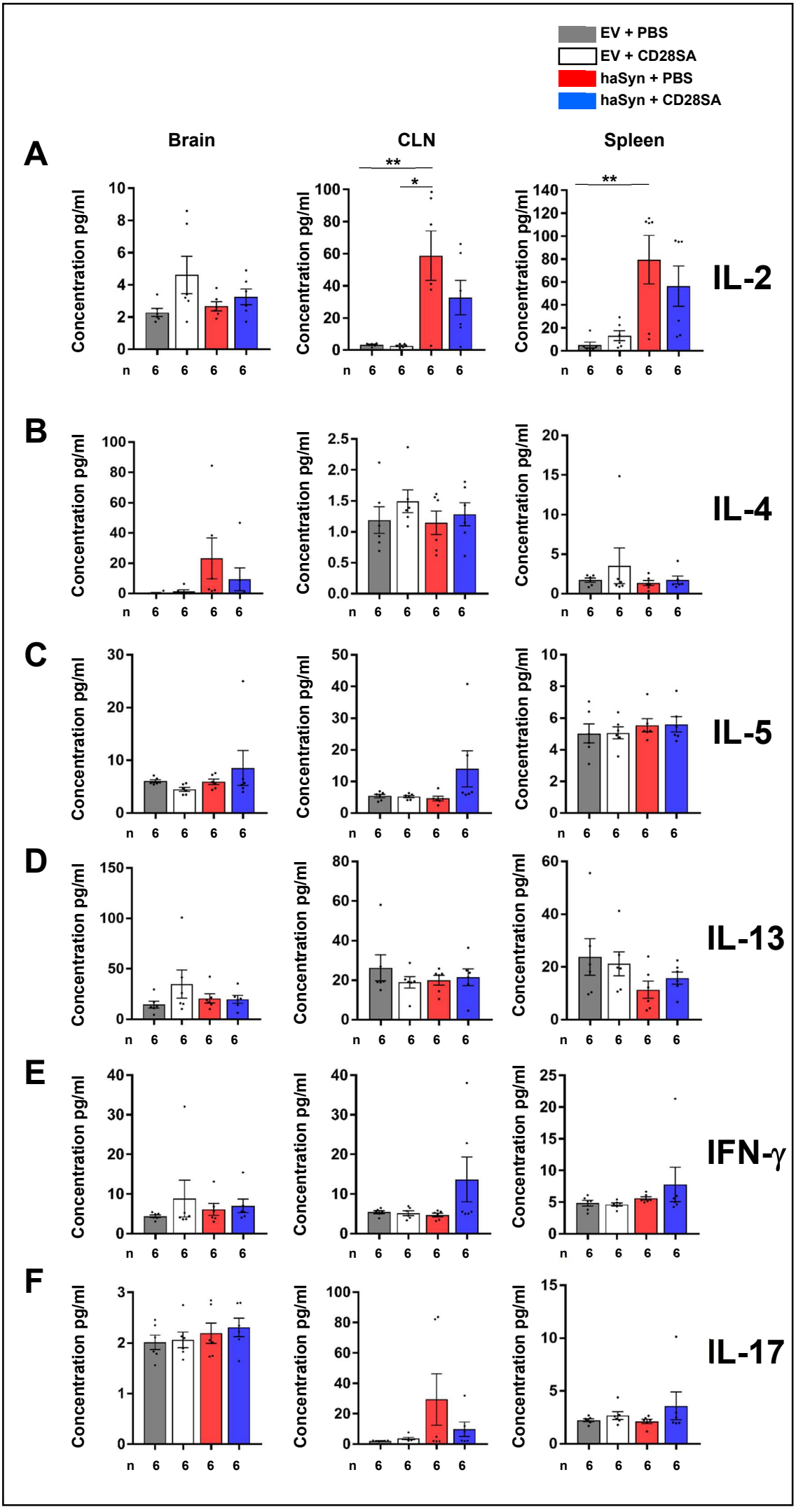

Figure 30: CD28SA Effect on cytokine levels in peripheral lymphatic organs and brain, 10 days following the viral vector injection.

*A-F, Concentrations of key cytokines in brain, CLN and spleen of EV-injected wildtype mice with PBS or with CD28SA treatment (EV+PBS and EV+CD28SA, respectively), as well as haSyn-injected wildtype mice with PBS and with CD28SA (haSyn+PBS and haSyn+CD28SA, respectively): IL-2 (A), IL-4 (B), IL-5 (C), IL-13 (D), IL-17A (E) and IFN□ (F). Statistical analysis for CLN by one-way ANOVA followed by Tukey's multiple comparisons test: IL-2 CLN, $F(3, 20)=8.186$, $P=0.0009$. Statistical analysis for spleen by Kruskal-Wallis test followed by Dunn's multiple comparisons test: IL-2 Spleen, $H=12.7$, $P=0.0053$; *$P<0.05$, **$P<0.01$, ***$P<0.001$. Concentrations are shown as mean ± SEM. n = number of biologically independent animals.*

4.6.0 CD28SA Effect on Neurotrophic Factor Levels in Different Brain Structures in haSyn PD Mice

After establishing that Treg are the main arm by which CD28SA treatment resulted in neuroprotection, motor benefits and ani-inflammatory effects in haSyn PD mice, we showed that the major mechanism for these effects was the suppression of activation of effector T cells. In addition, we did an experiment to further probe if Treg could also be supporting the survival of neurons in the brains of haSyn PD mice, through promoting the expression of neurotrophic factors in the brain, namely brain-derived neurotrophic factor (BDNF) and glial cell-line-derived neurotrophic factor (GDNF).

In this experiment, we used two groups of haSyn PD mice treated with either CD28SA or PBS, similar to what was previously described. Ten weeks after the delivery of haSyn viral vector, we sacrificed the mice and isolated the following brain structures: SN, striatum and M1 cortex. Afterwards, we analyzed the levels of BDNF and GDNF in these structures by ELISA. CD28SA treatment in haSyn PD mice showed significant increase of BDNF and GDNF in both the striatum and M1 cortex as compared to PBS treatment. In the SN, GDNF still showed significant increase in CD28SA haSyn PD mice, but BDNF showed no difference in its levels in both groups (Figure 31).

These data point toward a possible correlation between Treg increase and increased levels of BDNF and GDNF which have important roles in the support of dopaminergic neurons. That could be another mechanism by which CD28SA-induced Treg expansion delivers neuroprotection.

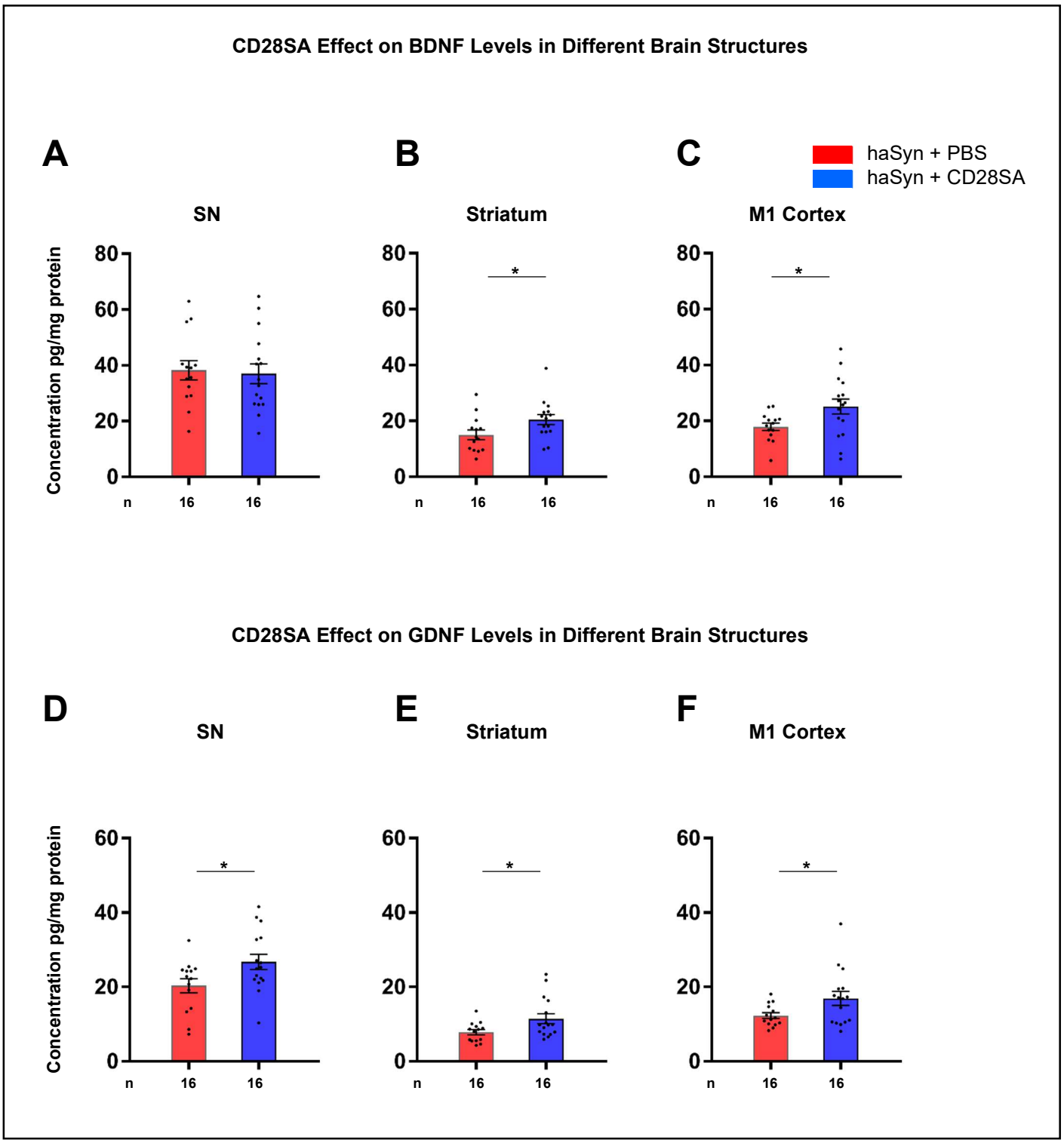

Figure 31: CD28SA effect of BDNF and GDNF levels in different brain structures in haSyn PD mice.

*A-C, Concentrations of BDNF in SN (A), striatum (B) and M1 cortex (C) of haSyn-injected wildtype mice with PBS or CD28SA treatment (haSyn+PBS and haSyn+CD28SA, respectively). Statistical analysis by unpaired two-tailed t-test: SN, t(28)=0.2352 and P=0.8158; striatum, t(27)=2.152 and P=0.0405; M1, t(28) and P=0.0301. *P<0.05, **P<0.01, ***P<0.001. D-F, Concentrations of GDNF in SN (D), striatum (E) and M1 cortex (F). Statistical analysis for SN and M1 Cortex by unpaired two-tailed t-test: SN, t(28)=2.278 and P=0.0306; M1, t(28)=2.154 and P=0.04. Statistical analysis for striatum by Mann-Whitney test, U=64, P=0.0472; *P<0.05, **P<0.01, ***P<0.001. All data are shown as mean ± SEM. n = number of biologically independent animals.*

5.0 Discussion

Symptomatic treatment of PD provides patients especially in early phases of the disease with relatively acceptable relief of symptoms and good quality of life. Since these treatments do not influence the causative factors of PD, as the years progress, the response to such treatments starts to be less remarkable and the side effects begin to annoy the patients and the question benefits versus drawbacks makes them ultimately unsatisfying solutions to relieve the suffering of PD patients.

Currently there is no therapies capable of ameliorating neurodegeneration or slowing is progression in PD. There is still a gap to be filled when it comes to treatments that can modify the course of the disease, rather than just addressing the symptoms. In the past, we were hampered by scarcity of data regarding the mechanism of PD onset and dynamics of its progression. However, in the latest decade, outstanding steps have been taken to deepen our understanding of PD pathology. Postmortem studies and advancement of animal models of PD elucidated a lot about PD etiology, the α-synuclein pathogenesis, as well as the underlying neuroinflammation and how it contributes to worsening of neurodegeneration. Accumulated knowledge paved the way for treatment approaches that target the actual disease mechanisms and thereby hold promise of enhancing disease prognosis in PD.

While neuroinflammation may not be the main culprit in the onset of PD, evidence supports a role of immune response in PD exacerbation. In addition to increased PD risk has been genetically linked to the MHC-II locus (Nalls et al., 2011; Saiki et al., 2010), proinflammatory cytokines in patients' blood predict worse course of PD progression (Williams-Gray et al., 2016). PD patients exhibit Th1-biased $CD4^+$ T cell profile as well as disrupted $CD8^+$ cell profile (Kustrimovic et al., 2018, 2016). α-synuclein promotes T cells to extravasate into the brain, aggravating the existing α-synuclein pathology and increasing the toxic load on dopaminergic neurons leading to further neurodegeneration (Ferreira & Romero-Ramos, 2018; Akua Afriyie

Karikari et al., 2019; Sommer et al., 2016, 2018). Autoimmunity in PD is now a principle that is gaining a lot of traction, especially with reports within the last few years showing that T cells isolated from PD patients could mount an immune response against α-synuclein (Garretti, Agalliu, Arlehamn, Sette, & Sulzer, 2019; Sulzer et al., 2017). Targeting neuroinflammation could provide then possible novel therapeutic strategies (Hutter-Saunders, Mosley, & Gendelman, 2011; Sommer, Winner, & Prots, 2017).

One of the major recorded disruptions of the immune system in PD is dysregulation of Treg leading to the onset of a proinflammatory environment in PD (Jordi Bas et al., 2001; Rosenkranz et al., 2007; Saunders et al., 2012). In this study, we employed a haSyn PD mouse model (Akua A. Karikari et al., 2019), and we show that inducing Treg expansion by a CD28SA at an early stage of the PD model could modulate the immune milieu so that inflammation is averted dopaminergic neurons in the nigrostriatal tract of haSyn PD mice is spared from degeneration.

As reviewed earlier, Treg display strong immunosuppressive qualities. This allows them to coordinate immune reactions to ensure they are proper and beneficial relative to the corresponding stimulus. There is evidence of Treg dysfunction or depletion in various autoimmune diseases like RA and SLE (Miyara et al., 2011). Improvement in chronic experimental autoimmune encephalomyelitis (EAE), as model of MS, has been linked with increased number of Treg in the CNS (Korn et al., 2007; Matsushita, Horikawa, Iwata, & Tedder, 2010), while Treg depletion increased disease severity in experimental autoimmune neuritis (EAN), a model of Acute Inflammatory Demyelinating Polyradiculoneuropathy (ADIP) (Meyer Zu Hörste et al., 2014). Furthermore, Treg were found to suppress neuropathic pain in several animal models (Austin, Kim, Perera, & Moalem-Taylor, 2012; Lahl & Sparwasser, 2011; Liu et al., 2014). In ischemic CNS injuries, circulating Treg were found to be diminished in patients with acute ischemic stroke, with increase in TH17 T cells (Hu, Zheng, Wu, Ni, & Shi, 2014).

In the realm of PD, previous studies aimed at harnessing the suppressive capacities of Treg. In the MPTP PD mouse model, Treg were demonstrated to show a neuroprotective and anti-inflammatory effect. Employing Treg was done by adoptive transfer (Reynolds et al., 2007, 2010) or by expansion of Treg by agents like bee venom phospholipase A2 (E. S. Chung et al., 2015) or BCG (Laćan et al., 2013; Yong et al., 2011). The translational value of these treatments in clinical trials remain rather inconclusive. As for adoptive transfer, despite the repeated trials for Treg therapy for some conditions like graft-vs-host disease as well as other autoimmune disease, the production and administration of clinical grade Treg is still a difficult domain (Esensten, Muller, Bluestone, & Tang, 2018). Regarding bee venom, and in spite of promising results in preclinical mice, it showed neither symptomatic nor disease-modifying effects when employed in a randomized controlled clinical trial (Hartmann et al., 2016). When it comes to BCG administration, it is also rather complicated since the BCG vaccine contains live-attenuated bacteria and could have serious consequences when delivered to patients with impaired immune responses. BCG could induce Treg responses, but could equally initiate proinflammatory reactions, and this ambiguity renders its clinical application uncertain (Boer et al., 2015).

In the present work, we tested the hypothesis that inducing Treg expansion at an early stage by one dosage of CD28SA could protect against or slow the loss of dopaminergic neurons in the haSyn PD mouse model. Karikari et al. has well described this model and showed that it reflected pathological α-synuclein features, neuroinflammation and motor impairment that is characteristic for human PD (Akua Afriyie Karikari et al., 2019). The potential of Treg immunomodulation in this model has not been hitherto studied. We showed that there is an increase in $CD4^+CD25^+FoxP3^+$ Treg within the $CD4^+$ population the secondary lymphoid organs in the periphery of haSyn PD mice ten days after the stereotaxic injection of haSyn viral vector to induce the PD model. This increase could be a sign of early Treg dysregulation that is consistent with what has been reported in PD patients regarding changes in Treg numbers (Jordi

Bas et al., 2001; Rosenkranz et al., 2007; Saunders et al., 2012). There is a possibility that the delivered pathological human A53T α-Syn could be acting as a self-antigen, since it differs from the physiological mouse α-Syn only in 6 positions (Figure 32). The elevation of Treg could then be understood in the frame of compensatory mechanisms aiming at neutralizing the proinflammatory immune reaction to the haSyn PD vector.

We showed that IL-10 increases in cervical lymph nodes and spleen of haSyn PD and EV mice three days after the CD28SA injection and this aligns with previous studies that demonstrated the utility of CD28SA in the treatment of EAE and G6PI-induced arthritis (Niklas Beyersdorf et al., 2005; Langenhorst et al., 2012; Win, Kühl, Sparwasser, Hünig, & Kamradt, 2016). Our data establish successful target engagement and shows that Treg in PD haSyn mouse model could be a target for treatment using CD28SA, despite the observed Treg dysregulation.

Unlike in the cervical lymph nodes and spleen, where CD28SA induced an expansion of Treg, centrally in the brain this increase in number was recorded only on haSyn PD mice. Neither in the CD28SA-treated mice nor in the control mice that received PBS injections did we witness any elevation of Treg. We suggest that T cells in haSyn PD mice are primed in the periphery against the haSyn proteins that possibly leaked from the brain through lymphatic vessels to the cervical lymph nodes as has been previously demonstrated (Akua Afriyie Karikari et al., 2019). We would suppose that activated microglia in the brain could secrete proinflammatory cytokines that could then upregulate the expression of adhesion molecules on microvascular endothelial cells (Wong et al., 1999), and thereby primed T cells could then home towards the CNS site of injury and extravasate into the brain. The possibility of a BBB leakage as the driving reason behind this early elevation of Treg number in haSyn PD mouse brain could be excluded based on a previous study (NH et al., 2013) that demonstrated restoration of BBB integrity one week following intracerebral surgery in a tumor implantation model.

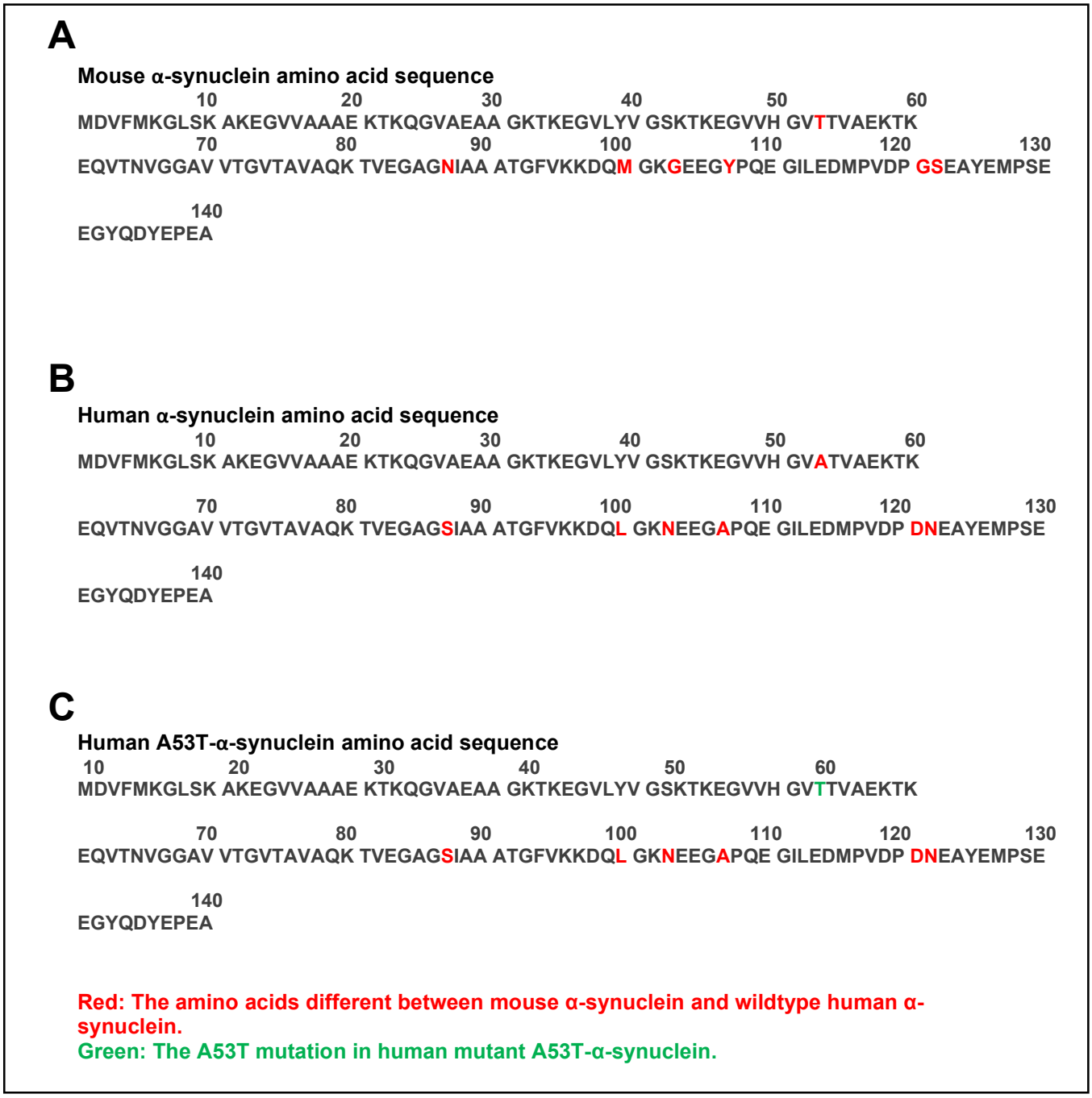

Figure 32: Comparison between amino acid sequences of human and mouse α-synuclein.

Treatment with one dosage of CD28SA protected haSyn PD mice from loss of dopaminergic neurons in the SN, while sparing dopaminergic terminals from neurodegeneration. PBS-treated haSyn PD mice showed neurodegeneration both in the SN and striatum. On a behavioral level, CD28SA-treated haSyn exhibited higher performance on the rotarod and had better scores for the cylinder test as compared with control PBS-injected haSyn PD mice. As for neuroinflammation, CD28SA administration reduced the number of $CD4^+$ and $CD8^+$ T cells in the SN and striatum, while the percentage of $CD69^+$ activated T cells within the $CD4^+$ and $CD8^+$ populations was markedly less than with PBS controls. IL-2 levels that are elevated in PBS-injected haSyn PD mice were brought down in CD28SA-treated haSyn PD mice.

The ability of CD28SA to augment the inflammatory response and reverse the behavioral deficits have been shown also in other rodent models of RA, MS and stroke (Niklas Beyersdorf et al., 2005; SY, E, A, T, & R, 2015; Win et al., 2016). In models of ischemic stroke, CD28SA treatment conferred neuroprotection in form of smaller infarct size (SY et al., 2015).

In our lab, Karikari et al., showed that mice that genetically lack functional lymphocytes are protected against induction of neurodegeneration in the SN by haSyn viral vector (Akua Afriyie Karikari et al., 2019). Also, in our lab, Musacchio et al, demonstrated that deep brain stimulation of subthalamic nucleus in haSyn PD rat model was successful in providing neuroprotection of dopaminergic neurons in the SN. Nevertheless, in the previously mentioned two studies, the imparted neuroprotection was limited to the SN and did not extend to the rest of the nigrostriatal pathway, as evidenced by the degeneration of the dopaminergic axon terminals in the striatum. We could deduce from these observations, that CD28SA provides an added advantage that was absent in the other models; namely that it protects the integrity of the nigrostriatal tract both in the SN and the striatum.

One question that we proposed was whether the observed neuroprotective effects of CD28SA are due to induce Treg expansion or whether there was some other direct action of CD28SA. To explore this proposition, we performed an adoptive transfer of Treg, previously expanded

by CD28SA, into haSyn PD mice. A replication of our results would confirm the Treg-based action of CD28SA, while failing to do so would have implicated another Treg-independent effect of CD28SA. According to our data, we have indeed found out that the single-time adoptive transfer of Treg rescued dopaminergic perikarya in SN and axonal terminals in striatum when observed after 10 weeks, while it also augmented motor deficits motor deficits observed in PBS-injected haSyn PD mice. Neuroinflammation was also ameliorated by Treg administration to haSyn PD mice, as evidenced with fewer numbers of $CD4^+$ and $CD8^+$ T cells both in the SN and striatum. These results lead to believe that Treg expansion is the main outcome of CD28SA treatment that is responsible for neuroprotection.

To better elucidate the initial mechanism by which the CD28SA-expanded Treg mitigate neuroinflammation, we looked at the percentages of activated $CD69^+$ T cells within the $CD4^+$ and $CD8^+$ populations at the timepoint of three days after CD28SA treatment of haSyn PD mice. At that early stage, we demonstrated that T cell activation is markedly prevented in haSyn PD mice by CD28SA, with low percentages of $CD4^+CD69^+$ and $CD8^+CD69^+$ T cells withing the $CD4^+$ and $CD8^+$ populations, respectively, in the cervical lymph nodes as compared with PBS-treated haSyn PD mice. The reduction amounted to around 40% in both populations. In the brain of haSyn PD mice, CD28SA treatment suppressed $CD4^+CD69^+$ cells by around 5%, while it did not affect the percentage of $CD8^+CD69^+$ cells. The previous outcome was reflected also in the cytokine profile, where CD28SA-treated haSyn PD mice show reduced levels of IL-2 in the cervical lymph nodes and spleen, but not the brain, when compared to PBS-treated haSyn PD mice. Taken together, these results confirm an early peripheral anti-inflammatory effect of CD28SA treatment in haSyn PD mice.

The translation of our results in humans looks like a promising aspect. If we can envision a future in which PD biomarkers are well developed that they facilitate an early diagnosis of the disease, we could well be optimistic of the potential of immunomodulation using CD28SA, when applied at early disease stages. For example, it was demonstrated that in some PD patients,

immune markers, like α-syn-specific T cell responses, could be measured already before the onset of motor symptoms (Lindestam Arlehamn et al., 2020). Such early-stage proinflammatory manifestation of PD makes it conceivable, that early anti-inflammatory interventions would yield disease modify outcomes, as compared with late interventions when neurodegeneration has already taken the upper hand. Tackling neurological diseases that have an inflammatory component with early immunotherapeutic treatments demonstrated clinical benefits in the long run in several clinical trials, especially for MS (Ontaneda, Tallantyre, Kalincik, Planchon, & Evangelou, 2019). Furthermore, in a study applied on a large population that employed corticosteroids and inosine monophosphate dehydrogenase inhibitors, it was observed that immune suppression correlates with a lower risk of PD (Racette et al., 2018). The findings support the premise of the results of our present study, that treatment approaches aimed at halting the proinflammatory immune reactions in PD could be a successful clinical strategy to protect against worse long-term outcomes. CD28SA appears as a good candidate to provide such immune suppression through the induction of Treg expansion.

In an additional set of experiments, we decided to delve further into the possible mechanisms governing the observed neuroprotective effects of CD28SA-induced Treg expansion in haSyn PD mice. We wanted to test if there is a correlation between the Treg expansion in our model and increase in neurotrophic factors which are known to support the survival and growth of neurons (Huang & Reichardt, 2001). We focused mainly on BDNF and GDNF.

Using ELISA, we measured the levels of BDNF and GDNF in SN, striatum and M1 cortex ten weeks after the delivery of the haSyn viral vector to mice that were treated afterwards with either CD28SA or PBS. Our data demonstrate a correlation between CD28SA treatment and an increase of GDNF levels in SN, striatum and M1 cortex. There was also a correlation between CD28SA treatment and elevated BDNF levels in striatum and M1 cortex, but not in the SN.

It was shown previously that there is a possible connection between Treg and increased expression of neurotrophic factors in the brain. Treg could bring neurotrophic factors into the locale of injury and thereby support survival (Greenberg, Xu, Lu, & Hempstead, 2009). In a study on acute ischemic stroke, it was shown that patients who had higher percentages of $CD4^{+}BDNF^{+}$ Treg had better clinical presentation after 6 months compared with patients who lacked this increase (Chan, Yan, Csurhes, Greer, & McCombe, 2015). GDNF and BDFN have been extensively reviewed in their potential as therapeutic agents in PD. Using a 6-OHDA rat model, Quintino et al. showed that GDNF, delivered by lentiviral vectors, was able to preserve the integrity of the nigrostriatal pathway, but only in early stages of the disease when there is still an adequate number of neurons that can engage with the treatment (Quintino et al., 2019). Zhao et al., demonstrated that the in vivo administration of GDNF-overexpressing macrophages to 6-OHDA mice has the potential to prevent the loss of dopaminergic neurons as compared with the administration of control macrophages (Zhao et al., 2014). Sun et al. employed GDNF and BDNF therapy in 6-OHDA rats using Herpes Simplex Virus-1 as a delivering viral vector and showed that both were able rescue dopaminergic neurons as well as reverse behavioral deficits, although GDNF was more effective in that regard (Sun et al., 2005).

Despite the seemingly successful utilization of GDNF in neurotoxin mouse models, the one study that tried to replicate that success in an α-synuclein model declared that their data could not show any special neuroprotective effects for GDNF in their model, which was based on overexpression of wildtype human α-synuclein using an AAV2 viral vector (Mickael Decressac et al., 2011). A similar outcome was observed in an early clinical trial where no benefits was observed when PD patients received GDNF injections at the lateral ventricle compared with placebo injections (Nutt et al., 2003).

Taken together, despite the correlation that our data show between GDNF and BDNF with CD28SA treatment, the utility of these neurotrophic factors as therapeutic agents in PD is still

debated and the exact mechanisms by which Treg deliver neurotrophic factors is not fully elucidated.

6.0 Conclusion

PD is a long-term disorder that affects millions of people and is characterized by the loss of dopaminergic neurons in the SN and their axon terminals in the striatum. A report in 2016 estimated that between 1990 and 2015, PD affected more than 6.2 million people worldwide (Vos et al., 2016). PD comes with motor deficits and a myriad of non-motor symptoms that adversely affect the quality of life of patients. Despite the availability of treatments that aim at enhancing the resting tremor, bradykinesia, and gait dysfunction, they fail to address the actual causes of PD. There is an unmet need for novel therapies that are based on a sound understanding of the PD pathogenesis mechanisms and aim at reversing or augmenting those mechanisms.

In the present study we aimed at delivering a preclinical model of a disease-modifying therapy for PD. While α-synuclein has been the center of many reports as a target in PD therapy, we focused on neuroinflammation as a driving force that aggravates neurodegeneration following α-synuclein pathology. Many reports recorded the infiltration of T cells into the brain in various animal models, altered cytokine and immune cell profiles in PD patients and presence of $CD4^+$ and $CD8^+$ T cells and activated microglia in postmortem SN of PD patients. It is now well established that inflammation plays a crucial rule in PD progression.

Equipped with that knowledge, we aimed at applying an immunomodulatory approach for the treatment of PD. Our agent of choice was CD28SA that is capable of inducing Treg expansion. Our spearhead is Treg which have been shown in numerous accounts to have neuroprotective effects in various neurological conditions, among which PD. Our model of choice was the AAV1/2 A53T-α-Synuclein mouse model, which is a genetic etiologic model that was shown to capture the loss of dopaminergic neurons in SN and the ablation of dopaminergic fibers in

striatum as well as the development of characteristic α-synuclein pathology with lewy-like protein aggregations and showing behavioral deficits consistent with PD.

Our data showed that CD28SA successfully engaged with T cells in haSyn PD mice leading to expansion of Treg that are able afterwards to counteract neuroinflammation by reducing the T cell activation and the levels of proinflammatory cytokines as well as increasing the levels of the anti-inflammatory IL-10 cytokine. Our results show that this amelioration of neuroinflammation paves the path towards neuroprotection as evidenced by the preserved numbers of dopaminergic neurons in the SN along with their terminals in striatum as well as the reversal of behavioral deficits of mice in the rotarod performance test and the cylinder test. Since CD28SA was already employed safely in clinical trials to treat other inflammatory disorders like RA, the data of our results represent a very attractive treatment approach that could be translated into the clinic with PD patients. That would still be dependent on our ability to intervene in early disease stages to be able to halt or slow the progression of PD. With continuous development of early PD biomarkers, there is reasonable hope that CD28SA could be employed at clinical stages where it still could enhance the outcome with respect to neurodegeneration and motor impairment.

6.1.0 Future Perspectives

The next steps that would bring CD28SA therapy in PD closer to the clinic would be replicating the results of this study in different models. Employing transgenic mice overexpressing known human PD mutations like A53T or A30P could be an option. Employing a nonhuman primate model could be a second option with the prerequisite of accurate comparison of species differences when it comes to CD28 expression.

Another avenue worth exploring would be to establish better understanding of Treg mechanisms in providing neuroprotection and elucidate the possible pathways of Treg delivery of neurotrophic factors.

References

Abbott, N. J., Patabendige, A. A. K., Dolman, D. E. M., Yusof, S. R., & Begley, D. J. (2010, January). Structure and function of the blood-brain barrier. *Neurobiology of Disease*. Neurobiol Dis. https://doi.org/10.1016/j.nbd.2009.07.030

Anandasabapathy, N., Victora, G. D., Meredith, M., Feder, R., Dong, B., Kluger, C., … Liu, K. (2011). Flt3L controls the development of radiosensitive dendritic cells in the meninges and choroid plexus of the steady-state mouse brain. *Journal of Experimental Medicine*, *208*(18), 1695–1705. https://doi.org/10.1084/jem.20102657

Anderson, K. M., Olson, K. E., Estes, K. A., Flanagan, K., Gendelman, H. E., & Lee Mosley, R. (2014). Dual destructive and protective roles of adaptive immunity in neurodegenerative disorders Translational Neurodegeneration Dual destructive and protective roles of adaptive immunity in neurodegenerative disorders. https://doi.org/10.1186/2047-9158-3-25

Appel-Cresswell, S., Vilarino-Guell, C., Encarnacion, M., Sherman, H., Yu, I., Shah, B., … Farrer, M. J. (2013). Alpha-synuclein p.H50Q, a novel pathogenic mutation for Parkinson's disease. *Movement Disorders*, *28*(6), 811–813. https://doi.org/10.1002/mds.25421

Appel, S. H. (2009, January 5). CD4+ T cells mediate cytotoxicity in neurodegenerative diseases. *Journal of Clinical Investigation*. J Clin Invest. https://doi.org/10.1172/JCI38096

Arai, H., Furuya, T., Mizuno, Y., & Mochizuki, H. (2006, April). Inflammation and infection in Parkinson's disease. *Histology and Histopathology*. Histol Histopathol. https://doi.org/10.14670/HH-21.673

Arimoto, T., Choi, D. Y., Lu, X., Liu, M., Nguyen, X. V., Zheng, N., … Bing, G. (2007). Interleukin-10 protects against inflammation-mediated degeneration of dopaminergic neurons in substantia nigra. *Neurobiology of Aging*, *28*(6), 894–906.

https://doi.org/10.1016/j.neurobiolaging.2006.04.011

Austin, P. J., Kim, C. F., Perera, C. J., & Moalem-Taylor, G. (2012). Regulatory T cells attenuate neuropathic pain following peripheral nerve injury and experimental autoimmune neuritis. *Pain*, *153*(9), 1916–1931. https://doi.org/10.1016/j.pain.2012.06.005

Baba, Y., Kuroiwa, A., Uitti, R. J., Wszolek, Z. K., & Yamada, T. (2005). Alterations of T-lymphocyte populations in Parkinson disease. *Parkinsonism and Related Disorders*, *11*(8), 493–498. https://doi.org/10.1016/j.parkreldis.2005.07.005

Badie, B., Bartley, B., & Schartner, J. (2002). Differential expression of MHC class II and B7 costimulatory molecules by microglia in rodent gliomas. *Journal of Neuroimmunology*, *133*(1–2), 39–45. https://doi.org/10.1016/S0165-5728(02)00350-8

Banks, W. A. (2015, February 1). The blood-brain barrier in neuroimmunology: Tales of separation and assimilation. *Brain, Behavior, and Immunity*. Academic Press Inc. https://doi.org/10.1016/j.bbi.2014.08.007

Barcia, C., Ros, C. M., Annese, V., Gómez, A., Ros-Bernal, F., Aguado-Year, C., … Herrero, M. T. (2011). IFN-γ signaling, with the synergistic contribution of TNF-α, mediates cell specific microglial and astroglial activation in experimental models of Parkinson's disease. *Cell Death and Disease*, *2*(4). https://doi.org/10.1038/cddis.2011.17

Barrett, P. J., & Timothy Greenamyre, J. (2015). Post-translational modification of α-synuclein in Parkinson's disease. *Brain Research*, *1628*(Pt B), 247–253. https://doi.org/10.1016/j.brainres.2015.06.002

Baruch, K., & Schwartz, M. (2013, November). CNS-specific T cells shape brain function via the choroid plexus. *Brain, Behavior, and Immunity*. Brain Behav Immun. https://doi.org/10.1016/j.bbi.2013.04.002

Bas, J. (2001). Lymphocyte populations in Parkinson's disease and in rat models of parkinsonism. *Journal of Neuroimmunology*, *113*(1), 146–152.

https://doi.org/10.1016/S0165-5728(00)00422-7

Bas, Jordi, Calopa, M., Mestre, M., Molleví, D. G., Cutillas, B., Ambrosio, S., & Buendia, E. (2001). Lymphocyte populations in Parkinson's disease and in rat models of parkinsonism. *Journal of Neuroimmunology*, *113*(1), 146–152. https://doi.org/10.1016/S0165-5728(00)00422-7

Basith, S., Manavalan, B., Lee, G., Kim, S. G., & Choi, S. (2011, June). Toll-like receptor modulators: A patent review (2006 - 2010). *Expert Opinion on Therapeutic Patents*. Expert Opin Ther Pat. https://doi.org/10.1517/13543776.2011.569494

Beach, T. G., Adler, C. H., Sue, L. I., Vedders, L., Lue, L. F., White, C. L., … Walker, D. G. (2010). Multi-organ distribution of phosphorylated α-synuclein histopathology in subjects with Lewy body disorders. *Acta Neuropathologica*, *119*(6), 689–702. https://doi.org/10.1007/s00401-010-0664-3

Ben-Nun, A., Kaushansky, N., Kawakami, N., Krishnamoorthy, G., Berer, K., Liblau, R., … Wekerle, H. (2014, November 1). From classic to spontaneous and humanized models of multiple sclerosis: Impact on understanding pathogenesis and drug development. *Journal of Autoimmunity*. Academic Press. https://doi.org/10.1016/j.jaut.2014.06.004

Benner, E. J., Banerjee, R., Reynolds, A. D., Sherman, S., Pisarev, V. M., Tsiperson, V., … Gendelman, H. E. (2008). Nitrated α-synuclein immunity accelerates degeneration of nigral dopaminergic neurons. *PLoS ONE*, *3*(1). https://doi.org/10.1371/journal.pone.0001376

Bennett, S. R. M., Carbone, F. R., Karamalis, F., Flavell, R. A., Miller, J. F. A. P., & Heath, W. R. (1998). Help for cytotoxic-T-cell responses is mediated by CD4O signalling. *Nature*, *393*(6684), 478–480. https://doi.org/10.1038/30996

Beyersdorf, N., Hanke, T., Kerkau, T., & Hünig, T. (2005). Superagonistic anti-CD28 antibodies: Potent activators of regulatory T cells for the therapy of autoimmune diseases. In *Annals of the Rheumatic Diseases* (Vol. 64). Ann Rheum Dis.

https://doi.org/10.1136/ard.2005.042564

Beyersdorf, Niklas, Gaupp, S., Balbach, K., Schmidt, J., Toyka, K. V., Lin, C.-H., … Gold, R. (2005). Selective targeting of regulatory T cells with CD28 superagonists allows effective therapy of experimental autoimmune encephalomyelitis. *The Journal of Experimental Medicine*. https://doi.org/10.1084/jem.20051060

Bezard, E., Yue, Z., Kirik, D., & Spillantini, M. G. (2013). Animal models of Parkinson's disease: Limits and relevance to neuroprotection studies. *Movement Disorders*. https://doi.org/10.1002/mds.25108

Blum, J. S., Wearsch, P. A., & Cresswell, P. (2013, March). Pathways of antigen processing. *Annual Review of Immunology*. Annu Rev Immunol. https://doi.org/10.1146/annurev-immunol-032712-095910

Boer, M. C., Prins, C., Van Meijgaarden, K. E., Van Dissel, J. T., Ottenhoff, T. H. M., & Joosten, S. A. (2015). Mycobacterium bovis BCG vaccination induces divergent proinflammatory or regulatory T cell responses in adults. *Clinical and Vaccine Immunology*, *22*(7), 778–788. https://doi.org/10.1128/CVI.00162-15

Bower, J. H., Maraganore, D. M., Peterson, B. J., McDonnell, S. K., Ahlskog, J. E., & Rocca, W. A. (2003). Head trauma preceding PD: A case-control study. *Neurology*, *60*(10), 1610–1615. https://doi.org/10.1212/01.WNL.0000068008.78394.2C

Brochard, V., & Hunot, S. (2009). Infiltration of CD4+ lymphocytes into the brain contributes to neurodegeneration in a mouse model of Parkinson disease.

Burré, J., Vivona, S., Diao, J., Sharma, M., Brunger, A. T., & Südhof, T. C. (2013). Properties of native brain α-synuclein. *Nature*, *498*(7453). https://doi.org/10.1038/nature12125

Burzyn, D., Benoist, C., & Mathis, D. (2013). Regulatory T cells in nonlymphoid tissues. *Nature Immunology*, *14*(10), 1007–1013. https://doi.org/10.1038/ni.2683

Cao, X., Cai, S. F., Fehniger, T. A., Song, J., Collins, L. I., Piwnica-Worms, D. R., & Ley, T. J. (2007). Granzyme B and Perforin Are Important for Regulatory T Cell-Mediated

Suppression of Tumor Clearance. *Immunity*, *27*(4), 635–646. https://doi.org/10.1016/j.immuni.2007.08.014

Cavallarin, N., Vicario, M., & Negro, A. (2012). The Role of Phosphorylation in Synucleinopathies: Focus on Parkinsons Disease. *CNS & Neurological Disorders - Drug Targets*, *9*(4), 471–481. https://doi.org/10.2174/187152710791556140

Chan, A., Yan, J., Csurhes, P., Greer, J., & McCombe, P. (2015). Circulating brain derived neurotrophic factor (BDNF) and frequency of BDNF positive T cells in peripheral blood in human ischemic stroke: Effect on outcome. *Journal of Neuroimmunology*. https://doi.org/10.1016/j.jneuroim.2015.06.013

Chartier-Harlin, M. C., Kachergus, J., Roumier, C., Mouroux, V., Douay, X., Lincoln, S., … Destée, A. (2004). α-synuclein locus duplication as a cause of familial Parkinson's disease. *Lancet*, *364*(9440), 1167–1169. https://doi.org/10.1016/S0140-6736(04)17103-1

Chen, H., Huang, X., Guo, X., Mailman, R. B., Park, Y., Kamel, F., … Blair, A. (2010). Smoking duration, intensity, and risk of Parkinson disease. *Neurology*, *74*(11), 878–884. https://doi.org/10.1212/WNL.0b013e3181d55f38

Chen, R. C., Chang, S. F., Su, C. L., H. Chen, T. H., Yen, M. F., Wu, H. M., … Liou, H. H. (2001). Prevalence, incidence, and mortality of PD. *Neurology*, *57*(9), 1679. https://doi.org/10.1212/WNL.57.9.1679

Cheng, H. C., Ulane, C. M., & Burke, R. E. (2010, June). Clinical progression in Parkinson disease and the neurobiology of axons. *Annals of Neurology*. https://doi.org/10.1002/ana.21995

Chung, C. Y., Koprich, J. B., Siddiqi, H., & Isacson, O. (2009). Dynamic changes in presynaptic and axonal transport proteins combined with striatal neuroinflammation precede dopaminergic neuronal loss in a rat model of AAV α-synucleinopathy. *Journal of Neuroscience*, *29*(11), 3365–3373. https://doi.org/10.1523/JNEUROSCI.5427-08.2009

Chung, E. S., Kim, H., Lee, G., Park, S., Kim, H., & Bae, H. (2012). Neuro-protective effects of bee venom by suppression of neuroinflammatory responses in a mouse model of Parkinson's disease: Role of regulatory T cells. *Brain, Behavior, and Immunity*, *26*(8), 1322–1330. https://doi.org/10.1016/j.bbi.2012.08.013

Chung, E. S., Lee, G., Lee, C., Ye, M., Chung, H., Kim, H., … Bae, H. (2015). Bee Venom Phospholipase A 2 , a Novel Foxp3 + Regulatory T Cell Inducer, Protects Dopaminergic Neurons by Modulating Neuroinflammatory Responses in a Mouse Model of Parkinson's Disease . *The Journal of Immunology*, *195*(10), 4853–4860. https://doi.org/10.4049/jimmunol.1500386

Codarri, L., Gyülvészii, G., Tosevski, V., Hesske, L., Fontana, A., Magnenat, L., … Becher, B. (2011). RORγ3t drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. *Nature Immunology*, *12*(6), 560–567. https://doi.org/10.1038/ni.2027

Corti, O., Lesage, S., & Brice, A. (2011). What genetics tells us about the causes and mechanisms of Parkinson's disease. *Physiological Reviews*, *91*(4), 1161–1218. https://doi.org/10.1152/physrev.00022.2010

Cose, S., Brammer, C., Khanna, K. M., Masopust, D., & Lefrançois, L. (2006). Evidence that a significant number of naive T cells enter non-lymphoid organs as part of a normal migratory pathway. *European Journal of Immunology*, *36*(6), 1423–1433. https://doi.org/10.1002/eji.200535539

Crowther, R. A., Daniel, S. E., & Goedert, M. (2000). Characterisation of isolated α-synuclein filaments from substantia nigra of Parkinson's disease brain. *Neuroscience Letters*, *292*(2), 128–130. https://doi.org/10.1016/S0304-3940(00)01440-3

Cserr, H. F., & Knopf, P. M. (1992). Cervical lymphatics, the blood-brain barrier and the immunoreactivity of the brain: a new view. *Immunology Today*. Immunol Today. https://doi.org/10.1016/0167-5699(92)90027-5

Dardalhon, V., Korn, T., Kuchroo, V. K., & Anderson, A. C. (2008). Role of Th1 and Th17 cells in organ-specific autoimmunity. *Journal of Autoimmunity*, *31*(3), 252–256. https://doi.org/10.1016/j.jaut.2008.04.017

Dawson, T. M. (2008, June). Non-autonomous cell death in Parkinson's disease. *The Lancet Neurology*. Lancet Neurol. https://doi.org/10.1016/S1474-4422(08)70099-1

Decressac, M., Mattsson, B., Lundblad, M., Weikop, P., & Björklund, A. (2012). Progressive neurodegenerative and behavioural changes induced by AAV-mediated overexpression of α-synuclein in midbrain dopamine neurons. *Neurobiology of Disease*. https://doi.org/10.1016/j.nbd.2011.12.013

Decressac, Mickael, Ulusoy, A., Mattsson, B., Georgievska, B., Romero-Ramos, M., Kirik, D., & Björklund, A. (2011). GDNF fails to exert neuroprotection in a rat α-synuclein model of Parkinson's disease. *Brain*. https://doi.org/10.1093/brain/awr149

Dennehy, K. M., Elias, F., Zeder-Lutz, G., Ding, X., Altschuh, D., Luhder, F., & Hunig, T. (2006). Cutting Edge: Monovalency of CD28 Maintains the Antigen Dependence of T Cell Costimulatory Responses. *The Journal of Immunology*. https://doi.org/10.4049/jimmunol.176.10.5725

Dennehy, Kevin M., Elias, F., Na, S.-Y., Fischer, K.-D., Hünig, T., & Lühder, F. (2007). Mitogenic CD28 Signals Require the Exchange Factor Vav1 to Enhance TCR Signaling at the SLP-76-Vav-Itk Signalosome. *The Journal of Immunology*, *178*(3), 1363–1371. https://doi.org/10.4049/jimmunol.178.3.1363

Dennehy, Kevin M., Kerstan, A., Bischof, A., Park, J. H., Na, S. Y., & Hünig, T. (2003). Mitogenic signals through CD28 activate the protein kinase Cθ-NF-κB pathway in primary peripheral T cells. *International Immunology*, *15*(5), 655–663. https://doi.org/10.1093/intimm/dxg063

Derecki, N. C., Cardani, A. N., Yang, C. H., Quinnies, K. M., Crihfield, A., Lynch, K. R., & Kipnis, J. (2010). Regulation of learning and memory by meningeal immunity: A key

role for IL-4. *Journal of Experimental Medicine*, *207*(5), 1067–1080. https://doi.org/10.1084/jem.20091419

Desai, B. S., Monahan, A. J., Carvey, P. M., & Hendey, B. (2007). Blood-brain barrier pathology in Alzheimer's and Parkinson's disease: Implications for drug therapy. In *Cell Transplantation* (Vol. 16, pp. 285–299). Cognizant Communication Corporation. https://doi.org/10.3727/000000007783464731

Desplats, P., Spencer, B., Coffee, E., Patel, P., Michael, S., Patrick, C., … Masliah, E. (2011). α-synuclein sequesters Dnmt1 from the nucleus: A novel mechanism for epigenetic alterations in Lewy body diseases. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.C110.212589

Devi, L., Raghavendran, V., Prabhu, B. M., Avadhani, N. G., & Anandatheerthavarada, H. K. (2008). Mitochondrial import and accumulation of α-synuclein impair complex I in human dopaminergic neuronal cultures and Parkinson disease brain. *Journal of Biological Chemistry*, *283*(14), 9089–9100. https://doi.org/10.1074/jbc.M710012200

Dickson, D. W. (2012). Parkinson's disease and parkinsonism: Neuropathology. *Cold Spring Harbor Perspectives in Medicine*, *2*(8). https://doi.org/10.1101/cshperspect.a009258

Du, Y., Ma, Z., Lin, S., Dodel, R. C., Gao, F., Bales, K. R., … Paul, S. M. (2001). Minocycline prevents nigrostriatal dopaminergic neurodegeneration in the MPTP model of Parkinson's disease. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(25), 14669–14674. https://doi.org/10.1073/pnas.251341998

Duff, G. (2006). *Expert Scientific Group on phase one clinical trials : final report.* TSO.

Duffy, M. F., Collier, T. J., Patterson, J. R., Kemp, C. J., Luk, K. C., Tansey, M. G., … Sortwell, C. E. (2018). Lewy body-like alpha-synuclein inclusions trigger reactive microgliosis prior to nigral degeneration. *Journal of Neuroinflammation*, *15*(1). https://doi.org/10.1186/s12974-018-1171-z

Eastwood, D., Findlay, L., Poole, S., Bird, C., Wadhwa, M., Moore, M., … Stebbings, R.

(2010). Monoclonal antibody TGN1412 trial failure explained by species differences in CD28 expression on CD4 + effector memory T-cells. *British Journal of Pharmacology*, *161*(3), 512–526. https://doi.org/10.1111/j.1476-5381.2010.00922.x

Ebrahimi-Fakhari, D., Wahlster, L., & McLean, P. J. (2012, August). Protein degradation pathways in Parkinson's disease: Curse or blessing. *Acta Neuropathologica*. Acta Neuropathol. https://doi.org/10.1007/s00401-012-1004-6

Elobeid, A., Libard, S., Leino, M., Popova, S. N., & Alafuzoff, I. (2016). Altered proteins in the aging brain. *Journal of Neuropathology and Experimental Neurology*, *75*(4), 316–325. https://doi.org/10.1093/jnen/nlw002

Engelhardt, B., & Ransohoff, R. M. (2005, September). The ins and outs of T-lymphocyte trafficking to the CNS: Anatomical sites and molecular mechanisms. *Trends in Immunology*. Trends Immunol. https://doi.org/10.1016/j.it.2005.07.004

Esensten, J. H., Muller, Y. D., Bluestone, J. A., & Tang, Q. (2018). Regulatory T-cell therapy for autoimmune and autoinflammatory diseases: The next frontier. *Journal of Allergy and Clinical Immunology*, *142*(6), 1710–1718. https://doi.org/10.1016/j.jaci.2018.10.015

Evans, E. J., Esnouf, R. M., Manso-Sancho, R., Gilbert, R. J. C., James, J. R., Yu, C., … Davis, S. J. (2005). Crystal structure of a soluble CD28-Fab complex. *Nature Immunology*, *6*(3), 271–279. https://doi.org/10.1038/ni1170

Farkas, E., De Jong, G. I., Apró, E., De Vos, R. A. I., Jansen Steur, E. N. H., & Luiten, P. G. M. (2000). Similar ultrastructural breakdown of cerebrocortical capillaries in Alzheimer's disease, Parkinson's disease, and experimental hypertension: What is the functional link? In *Annals of the New York Academy of Sciences* (Vol. 903, pp. 72–82). New York Academy of Sciences. https://doi.org/10.1111/j.1749-6632.2000.tb06352.x

Farrer, M., Kachergus, J., Forno, L., Lincoln, S., Wang, D. S., Hulihan, M., … Langston, J. W. (2004). Comparison of Kindreds with Parkinsonism and α-Synuclein Genomic Multiplications. *Annals of Neurology*, *55*(2), 174–179. https://doi.org/10.1002/ana.10846

Fearnley, J. M., & Lees, A. J. (1991). Ageing and parkinson's disease: Substantia nigra regional selectivity. *Brain*, *114*(5), 2283–2301. https://doi.org/10.1093/brain/114.5.2283

Ferrari, C. C., & Tarelli, R. (2011). Parkinson's disease and systemic inflammation. *Parkinson's Disease*. Parkinsons Dis. https://doi.org/10.4061/2011/436813

Ferreira, S. A., & Romero-Ramos, M. (2018, August 6). Microglia response during Parkinson's disease: Alpha-synuclein intervention. *Frontiers in Cellular Neuroscience*. Frontiers Media S.A. https://doi.org/10.3389/fncel.2018.00247

Fiala, M., & Veerhuis, R. (2010, January). Biomarkers of inflammation and amyloid-β phagocytosis in patients at risk of Alzheimer disease. *Experimental Gerontology*. Exp Gerontol. https://doi.org/10.1016/j.exger.2009.08.003

Filias, A., Theodorou, G. L., Mouzopoulou, S., Varvarigou, A. A., Mantagos, S., & Karakantza, M. (2011). Phagocytic ability of neutrophils and monocytes in neonates. *BMC Pediatrics*, *11*. https://doi.org/10.1186/1471-2431-11-29

Fiszer, U., Mix, E., Fredrikson, S., Kostulas, V., Olsson, T., & Link, H. (1994). γδ+ 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

Fontenot, J. D., Gavin, M. A., & Rudensky, A. Y. (2017). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Journal of Immunology*, *198*(3), 986–992. https://doi.org/10.1038/ni904

Fujiwara, H., Hasegawa, M., Dohmae, N., Kawashima, A., Masliah, E., Goldberg, M. S., … Iwatsubo, T. (2002). α-synuclein is phosphorylated in synucleinopathy lesions. *Nature Cell Biology*, *4*(2), 160–164. https://doi.org/10.1038/ncb748

Games, D., Valera, E., Spencer, B., Rockenstein, E., Mante, M., Adame, A., … Masliah, E. (2014). Reducing C-terminal-truncated alpha-synuclein by immunotherapy attenuates neurodegeneration and propagation in Parkinson's disease-like models. *Journal of Neuroscience*, *34*(28), 9441–9454. https://doi.org/10.1523/JNEUROSCI.5314-13.2014

Garretti, F., Agalliu, D., Arlehamn, C. S. L., Sette, A., & Sulzer, D. (2019). Autoimmmunity in parkinson's disease: The role of α:-synuclein-specific T cells. *Frontiers in Immunology*. Frontiers Media S.A. https://doi.org/10.3389/fimmu.2019.00303

Gavin, M. A., Rasmussen, J. P., Fontenot, J. D., Vasta, V., Manganiello, V. C., Beavo, J. A., & Rudensky, A. Y. (2007). Foxp3-dependent programme of regulatory T-cell differentiation. *Nature*, *445*(7129), 771–775. https://doi.org/10.1038/nature05543

Gerlach, M., Desser, H., Youdim, M. B. H., & Riederer, P. (1996). New horizons in molecular mechanisms underlying Parkinson's disease and in our understanding of the neuroprotective effects of selegiline. *Journal of Neural Transmission, Supplement*. Springer-Verlag Wien. https://doi.org/10.1007/978-3-7091-7494-4_2

Gerlach, Manfred, Riederer, P., Przuntek, H., & Youdim, M. B. H. (1991, December 12). MPTP mechanisms of neurotoxicity and their implications for Parkinson's disease. *European Journal of Pharmacology: Molecular Pharmacology*. Eur J Pharmacol. https://doi.org/10.1016/0922-4106(91)90073-Q

Giasson, B. I., Duda, J. E., Murray, I. V. J., Chen, Q., Souza, J. M., Hurtig, H. I., … Lee, V. M. Y. (2000). Oxidative damage linked to neurodegeneration by selective α-synuclein nitration in synucleinopathy lesions. *Science*, *290*(5493), 985–989. https://doi.org/10.1126/science.290.5493.985

Giguère, N., Nanni, S. B., & Trudeau, L. E. (2018, June 19). On cell loss and selective vulnerability of neuronal populations in Parkinson's disease. *Frontiers in Neurology*. Frontiers Media S.A. https://doi.org/10.3389/fneur.2018.00455

Goldmann, T., Wieghofer, P., Jordão, M. J. C., Prutek, F., Hagemeyer, N., Frenzel, K., … Prinz, M. (2016). Origin, fate and dynamics of macrophages at central nervous system interfaces. *Nature Immunology*, *17*(7), 797–805. https://doi.org/10.1038/ni.3423

Gondek, D. C., Lu, L.-F., Quezada, S. A., Sakaguchi, S., & Noelle, R. J. (2005). Cutting Edge: Contact-Mediated Suppression by CD4 + CD25 + Regulatory Cells Involves a

Granzyme B-Dependent, Perforin-Independent Mechanism . *The Journal of Immunology*, *174*(4), 1783–1786. https://doi.org/10.4049/jimmunol.174.4.1783

González, H., Contreras, F., Prado, C., Elgueta, D., Franz, D., Bernales, S., & Pacheco, R. (2013). Dopamine Receptor D3 Expressed on CD4 + T Cells Favors Neurodegeneration of Dopaminergic Neurons during Parkinson's Disease . *The Journal of Immunology*, *190*(10), 5048–5056. https://doi.org/10.4049/jimmunol.1203121

González, H., & Pacheco, R. (2014). T-cell-mediated regulation of neuroinflammation involved in neurodegenerative diseases. *Journal of Neuroinflammation*. https://doi.org/10.1186/s12974-014-0201-8

Greenberg, M. E., Xu, B., Lu, B., & Hempstead, B. L. (2009). New insights in the biology of BDNF synthesis and release: implications in CNS function. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *29*(41), 12764–12767. https://doi.org/10.1523/JNEUROSCI.3566-09.2009

Greenwood, J., Heasman, S. J., Alvarez, J. I., Prat, A., Lyck, R., & Engelhardt, B. (2011). Review: Leucocyte-endothelial cell crosstalk at the blood-brain barrier: A prerequisite for successful immune cell entry to the brain. *Neuropathology and Applied Neurobiology*, *37*(1), 24–39. https://doi.org/10.1111/j.1365-2990.2010.01140.x

Greffard, S., Verny, M., Bonnet, A. M., Beinis, J. Y., Gallinari, C., Meaume, S., … Duyckaerts, C. (2006). Motor score of the unified Parkinson disease rating scale as a good predictor of lewy body-associated neuronal loss in the substantia nigra. *Archives of Neurology*, *63*(4), 584–588. https://doi.org/10.1001/archneur.63.4.584

Gross, J. A., St John, T., & Allison, J. P. (1990). The murine homologue of the T lymphocyte antigen CD28. Molecular cloning and cell surface expression. *The Journal of Immunology*, *144*(8), 3201. Retrieved from http://www.jimmunol.org/content/144/8/3201.abstract

Hanke, T. (2006, November 4). Lessons from TGN1412. *Lancet*. Elsevier B.V.

https://doi.org/10.1016/S0140-6736(06)69651-7

Hara, T., Fu, S. M., & Hansen, J. A. (1985). Human T cell activation: II. A new activation pathway used by a major t cell population via a disulfide-bonded dimer of a 44 kilodalton polypeptide (9.3 antigen). *Journal of Experimental Medicine*, *161*(6), 1513–1524. https://doi.org/10.1084/jem.161.6.1513

Harm, A. S., Cao, S., Rowse, A. L., Thome, A. D., Li, X., Mangieri, L. R., … Standaert, D. G. (2013). MHCII is required for α-Synuclein-induced activation of microglia, CD4 T cell proliferation, and dopaminergic neurodegeneration. *Journal of Neuroscience*, *33*(23), 9592–9600. https://doi.org/10.1523/JNEUROSCI.5610-12.2013

Hartmann, A., Müllner, J., Meier, N., Hesekamp, H., Van Meerbeeck, P., Habert, M. O., … Schüpbach, M. (2016). Bee venom for the treatment of Parkinson disease - A randomized controlled clinical trial. *PLoS ONE*, *11*(7). https://doi.org/10.1371/journal.pone.0158235

Hasegawa, Y., Inagaki, T., Sawada, M., & Suzumura, A. (2000). Impaired cytokine production by peripheral blood mononuclear cells and monocytes/macrophages in Parkinson's disease. *Acta Neurologica Scandinavica*, *101*(3), 159–164. https://doi.org/10.1034/j.1600-0404.2000.101003159.x

Hatterer, E., Touret, M., Belin, M. F., Honnorat, J., & Nataf, S. (2008). Cerebrospinal fluid dendritic cells infiltrate the brain parenchyma and target the cervical lymph nodes under neuroinflammatory conditions. *PLoS ONE*, *3*(10). https://doi.org/10.1371/journal.pone.0003321

Hattori, N., & Mizuno, Y. (2002). Mitochondrial dysfunction in Parkinson's disease. *Nippon Rinsho. Japanese Journal of Clinical Medicine*. Exp Neurobiol. https://doi.org/10.5607/en.2015.24.2.103

He, Y., Appel, S., & Le, W. (2001). Minocycline inhibits microglial activation and protects nigral cells after 6-hydroxydopamine injection into mouse striatum. *Brain Research*,

909(1–2), 187–193. https://doi.org/10.1016/S0006-8993(01)02681-6

Healy, D. G., Falchi, M., O'Sullivan, S. S., Bonifati, V., Durr, A., Bressman, S., … Wood, N. W. (2008). Phenotype, genotype, and worldwide genetic penetrance of LRRK2-associated Parkinson's disease: a case-control study. *The Lancet Neurology*, *7*(7), 583–590. https://doi.org/10.1016/S1474-4422(08)70117-0

Heesen, C., Schulz, K. H., Fiehler, J., Von der Mark, U., Otte, C., Jung, R., … Gold, S. M. (2010). Correlates of cognitive dysfunction in multiple sclerosis. *Brain, Behavior, and Immunity*, *24*(7), 1148–1155. https://doi.org/10.1016/j.bbi.2010.05.006

Heikkila, R. E., & Sonsalla, P. K. (1992). The MPTP-treated mouse as a model of Parkinsonism: How good is it? *Neurochemistry International*, *20*(SUPPL.), 299–303. https://doi.org/10.1016/0197-0186(92)90256-Q

Hernán, M. A., Takkouche, B., Caamaño-Isorna, F., & Gestal-Otero, J. J. (2002). A meta-analysis of coffee drinking, cigarette smoking, and the risk of Parkinson's disease. *Annals of Neurology*, *52*(3), 276–284. https://doi.org/10.1002/ana.10277

Herrero, M. T., Hirsch, E. C., Kastner, A., Ruberg, M., Luquin, M. R., Laguna, J., … Agid, Y. (1993). Does neuromelanin contribute to the vulnerability of catecholaminergic neurons in monkeys intoxicated with MPTP? *Neuroscience*, *56*(2), 499–511. https://doi.org/10.1016/0306-4522(93)90349-K

Hickey, W. F. (2001). Basic principles of immunological surveillance of the normal central nervous system. *GLIA*, *36*(2), 118–124. https://doi.org/10.1002/glia.1101

Hirsch, E. C., & Hunot, S. (2009). Neuroinflammation in Parkinson's disease: a target for neuroprotection? *The Lancet Neurology*. https://doi.org/10.1016/S1474-4422(09)70062-6

Hirsch, E., Graybiel, A. M., & Agid, Y. A. (1988). Melanized dopaminergic neurons are differentially susceptible to degeneration in Parkinson's disease. *Nature*, *334*(6180), 345–348. https://doi.org/10.1038/334345a0

Hori, S., Nomura, T., & Sakaguchi, S. (2017). Control of regulatory T cell development by the transcription factor Foxp3. *Journal of Immunology*, *198*(3), 981–985. https://doi.org/10.1126/science.1079490

Hu, Y., Zheng, Y., Wu, Y., Ni, B., & Shi, S. (2014). Imbalance between IL-17A-producing cells and regulatory T cells during ischemic stroke. *Mediators of Inflammation*, *2014*. https://doi.org/10.1155/2014/813045

Huang, E. J., & Reichardt, L. F. (2001). Neurotrophins: roles in neuronal development and function. *Annual Review of Neuroscience*, *24*, 677–736. https://doi.org/10.1146/annurev.neuro.24.1.677

Hunot, S., Dugas, N., Faucheux, B., Hartmann, A., Tardieu, M., Debré, P., … Hirsch, E. C. (1999). FcεRII/CD23 is expressed in Parkinson's disease and induces, in vitro, production of nitric oxide and tumor necrosis factor-α in glial cells. *Journal of Neuroscience*, *19*(9), 3440–3447. https://doi.org/10.1523/jneurosci.19-09-03440.1999

Hutter-Saunders, J. A. L., Mosley, R. L., & Gendelman, H. E. (2011). Pathways towards an effective immunotherapy for Parkinson's disease. *Expert Review of Neurotherapeutics*, *11*(12), 1703–1715. https://doi.org/10.1586/ern.11.163

Ibáñez, P., Bonnet, A. M., Débarges, B., Lohmann, E., Tison, F., Pollak, P., … Brice, P. A. (2004). Causal relation between α-synuclein gene duplication and familial Parkinson's disease. *Lancet*, *364*(9440), 1169–1171. https://doi.org/10.1016/S0140-6736(04)17104-3

Ip, C. W., Klaus, L., Karikari, A. A., Visanji, N. P., Brotchie, J. M., Lang, A. E., … Koprich, J. B. (2017). AAV1 / 2-induced overexpression of A53T- α - synuclein in the substantia nigra results in degeneration of the nigrostriatal system with Lewy-like pathology and motor impairment : a new mouse model for Parkinson ' s disease, 1–12. https://doi.org/10.1186/s40478-017-0416-x

Jellinger, K. A. (2007, September). More frequent Lewy bodies but less frequent Alzheimer-type lesions in multiple system atrophy as compared to age-matched control brains. *Acta*

Neuropathologica. Acta Neuropathol. https://doi.org/10.1007/s00401-007-0227-4

Kacimi, R., Giffard, R. G., & Yenari, M. A. (2011). Endotoxin-activated microglia injure brain derived endothelial cells via NF-κB, JAK-STAT and JNK stress kinase pathways. *Journal of Inflammation*, *8*. https://doi.org/10.1186/1476-9255-8-7

Kalia, L. V., & Lang, A. E. (2015, August 29). Parkinson's disease. *The Lancet*. Lancet Publishing Group. https://doi.org/10.1016/S0140-6736(14)61393-3

Kamer, A. R., Dasanayake, A. P., Craig, R. G., Glodzik-Sobanska, L., Bry, M., & De Leon, M. J. (2008). Alzheimer's disease and peripheral infections: The possible contribution from periodontal infections, model and hypothesis. *Journal of Alzheimer's Disease*. IOS Press. https://doi.org/10.3233/JAD-2008-13408

Karikari, Akua A., Koprich, J. B., & Ip, C. W. (2019). Generation and analysis of viral vector-mediated rodent models for parkinson's disease. In *Methods in Molecular Biology* (Vol. 1948, pp. 271–286). Humana Press Inc. https://doi.org/10.1007/978-1-4939-9124-2_21

Karikari, Akua Afriyie, Gehmeyr, M., Ribechini, E., Volkmann, J., Brotchie, J. M., Koprich, J. B., … Ip, C. W. (2019). CD4+ T-lymphocytes exacerbate neurodegeneration in a new A53T-alpha synuclein expressing mouse model for Parkinson's disease. *JOURNAL OF NEURAL TRANSMISSION*, *126*(5), 668–669.

Kebir, H., Kreymborg, K., Ifergan, I., Dodelet-Devillers, A., Cayrol, R., Bernard, M., … Prat, A. (2007). Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. *Nature Medicine*, *13*(10), 1173–1175. https://doi.org/10.1038/nm1651

Kim, H. S., & Suh, Y. H. (2009, January 23). Minocycline and neurodegenerative diseases. *Behavioural Brain Research*. Behav Brain Res. https://doi.org/10.1016/j.bbr.2008.09.040

Kim, W.-K., Hwang, S.-Y., Oh, E.-S., Piao, H. Z., Kim, K.-W., & Han, I.-O. (2004). TGF-β1 Represses Activation and Resultant Death of Microglia via Inhibition of Phosphatidylinositol 3-Kinase Activity. *The Journal of Immunology*, *172*(11), 7015–

7023. https://doi.org/10.4049/jimmunol.172.11.7015

Kirik, D., Annett, L. E., Burger, C., Muzyczka, N., Mandel, R. J., & Björklund, A. (2003). Nigrostriatal α-synucleinopathy induced by viral vector-mediated overexpression of human α-synuclein: A new primate model of Parkinson's disease. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(5), 2884–2889. https://doi.org/10.1073/pnas.0536383100

Kirik, D., Rosenblad, C., Burger, C., Lundberg, C., Johansen, T. E., Muzyczka, N., … Björklund, A. (2002). Parkinson-like neurodegeneration induced by targeted overexpression of α-synuclein in the nigrostriatal system. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *22*(7), 2780–2791. https://doi.org/20026246

Koprich, J. B., Johnston, T. H., Reyes, M. G., Sun, X., & Brotchie, J. M. (2010). Expression of human A53T alpha-synuclein in the rat substantia nigra using a novel AAV1/2 vector produces a rapidly evolving pathology with protein aggregation, dystrophic neurite architecture and nigrostriatal degeneration with potential to model the pat. *Molecular Neurodegeneration*, *5*, 43. https://doi.org/10.1186/1750-1326-5-43

Kordower, J. H., Olanow, C. W., Dodiya, H. B., Chu, Y., Beach, T. G., Adler, C. H., … Bartus, R. T. (2013). Disease duration and the integrity of the nigrostriatal system in Parkinson's disease. *Brain*. https://doi.org/10.1093/brain/awt192

Korn, T., Reddy, J., Gao, W., Bettelli, E., Awasthi, A., Petersen, T. R., … Kuchroo, V. K. (2007). Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. *Nature Medicine*, *13*(4), 423–431. https://doi.org/10.1038/nm1564

Kosloski, L. M., & Gendelman, H. E. (2013). GM-CSF induces neuroprotective and anti-inflammatory responses in 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine intoxicated mice.

Krogsgaard, M., & Davis, M. M. (2005). How T cells “see” antigen. *Nature Immunology*. Nat Immunol. https://doi.org/10.1038/ni1173

Kronenberg, M., & Rudensky, A. (2005, June 2). Regulation of immunity by self-reactive T cells. *Nature*. Nature. https://doi.org/10.1038/nature03725

Krüger, R., Kuhn, W., Müller, T., Woitalla, D., Graeber, M., Kösel, S., … Riess, O. (1998). Ala30Pro mutation in the gene encoding α-synuclein in Parkinson’s disease. *Nature Genetics*, *18*(2), 106–108. https://doi.org/10.1038/ng0298-106

Kustrimovic, N., Comi, C., Magistrelli, L., Rasini, E., Legnaro, M., Bombelli, R., … Cosentino, M. (2018). Parkinson’s disease patients have a complex phenotypic and functional Th1 bias: Cross-sectional studies of CD4+ Th1/Th2/T17 and Treg in drug-naïve and drug-treated patients. *Journal of Neuroinflammation*, *15*(1). https://doi.org/10.1186/s12974-018-1248-8

Kustrimovic, N., Rasini, E., Legnaro, M., Bombelli, R., Aleksic, I., Blandini, F., … Cosentino, M. (2016). Dopaminergic Receptors on CD4+ T Naive and Memory Lymphocytes Correlate with Motor Impairment in Patients with Parkinson’s Disease. *Scientific Reports*, *6*. https://doi.org/10.1038/srep33738

Laćan, G., Dang, H., Middleton, B., Horwitz, M. A., Tian, J., Melega, W. P., & Kaufman, D. L. (2013). Bacillus Calmette-Guerin vaccine-mediated neuroprotection is associated with regulatory T-cell induction in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson’s disease. *Journal of Neuroscience Research*, *91*(10), 1292–1302. https://doi.org/10.1002/jnr.23253

Lahl, K., & Sparwasser, T. (2011). In vivo depletion of FoxP3+ Treg using the DEREG mouse model. *Methods in Molecular Biology (Clifton, N.J.)*, *707*, 157–172. https://doi.org/10.1007/978-1-61737-979-6_10

Laman, J. D., & Weller, R. O. (2013, September). Drainage of cells and soluble antigen from the CNS to regional lymph nodes. *Journal of Neuroimmune Pharmacology*. J

Neuroimmune Pharmacol. https://doi.org/10.1007/s11481-013-9470-8

Landel, C. P., Chen, S., & Evans, G. A. (1990). Reverse genetics using transgenic mice. *Annual Review of Physiology*. Annu Rev Physiol. https://doi.org/10.1146/annurev.ph.52.030190.004205

Langenhorst, D., Gogishvili, T., Ribechini, E., Kneitz, S., McPherson, K., Lutz, M. B., & Hünig, T. (2012). Sequential Induction of Effector Function, Tissue Migration and Cell Death during Polyclonal Activation of Mouse Regulatory T-Cells. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0050080

Lee, H. J., Khoshaghideh, F., Patel, S., & Lee, S. J. (2004). Clearance of α-Synuclein Oligomeric Intermediates via the Lysosomal Degradation Pathway. *Journal of Neuroscience*, *24*(8), 1888–1896. https://doi.org/10.1523/JNEUROSCI.3809-03.2004

Lees, A. J., Hardy, J., & Revesz, T. (2009). Parkinson's disease. *The Lancet*. Elsevier B.V. https://doi.org/10.1016/S0140-6736(09)60492-X

Lesage, S., Anheim, M., Letournel, F., Bousset, L., Honoré, A., Rozas, N., … Brice, A. (2013). G51D α-synuclein mutation causes a novel Parkinsonian-pyramidal syndrome. *Annals of Neurology*, *73*(4), 459–471. https://doi.org/10.1002/ana.23894

Levin, S. E., Zhang, C., Kadlecek, T. A., Shokat, K. M., & Weiss, A. (2008). Inhibition of ZAP-70 kinase activity via an analog-sensitive allele blocks T cell receptor and CD28 superagonist signaling. *Journal of Biological Chemistry*, *283*(22), 15419–15430. https://doi.org/10.1074/jbc.M709000200

Lewitus, G. M., & Schwartz, M. (2009). Behavioral immunization: Immunity to self-antigens contributes to psychological stress resilience. *Molecular Psychiatry*, *14*(5), 532–536. https://doi.org/10.1038/mp.2008.103

Liang, Y., Li, Y., Kuang, Q., Ding, X., Wei, Z., & Fang, Y. (2017). Superagonistic CD28 Protects against Renal Ischemic Injury by Expansion of Regulatory T-Cell. *American Journal of Nephrology*, *45*(5), 389–399. https://doi.org/10.1159/000470918

Lin, C. H., & Hünig, T. (2003, March 1). Efficient expansion of regulatory T cells in vitro and in vivo with a CD28 superagonist. *European Journal of Immunology*. Eur J Immunol. https://doi.org/10.1002/eji.200323570

Lindestam Arlehamn, C. S., Dhanwani, R., Pham, J., Kuan, R., Frazier, A., Rezende Dutra, J., … Sette, A. (2020). α-Synuclein-specific T cell reactivity is associated with preclinical and early Parkinson's disease. *Nature Communications*, *11*(1). https://doi.org/10.1038/s41467-020-15626-w

Liu, X. J., Zhang, Y., Liu, T., Xu, Z. Z., Park, C. K., Berta, T., … Ji, R. R. (2014, November 5). Nociceptive neurons regulate innate and adaptive immunity and neuropathic pain through MyD88 adapter. *Cell Research*. Nature Publishing Group. https://doi.org/10.1038/cr.2014.106

Lo Bianco, C., Ridet, J. L., Schneider, B. L., Déglon, N., & Aebischer, P. (2002). α-synucleinopathy and selective dopaminergic neuron loss in a rat lentiviral-based model of Parkinson's disease. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(16), 10813–10818. https://doi.org/10.1073/pnas.152339799

Loeffler, D. A., Camp, D. M., & Conant, S. B. (2006). Complement activation in the Parkinson's disease substantia nigra: An immunocytochemical study. *Journal of Neuroinflammation*, *3*. https://doi.org/10.1186/1742-2094-3-29

Lofrumento, D. D., Nicolardi, G., Cianciulli, A., Nuccio, F. De, Pesa, V. La, Carofiglio, V., … Panaro, M. A. (2014). Neuroprotective effects of resveratrol in an MPTP mouse model of Parkinson's-like disease: Possible role of SOCS-1 in reducing pro-inflammatory responses. *Innate Immunity*, *20*(3), 249–260. https://doi.org/10.1177/1753425913488429

Lühder, F., Huang, Y., Dennehy, K. M., Guntermann, C., Müller, I., Winkler, E., … Hünig, T. (2003). Topological requirements and signaling properties of T cell-activating, anti-CD28 antibody superagonists. *Journal of Experimental Medicine*, *197*(8), 955–966.

https://doi.org/10.1084/jem.20021024

Luth, E. S., Stavrovskaya, I. G., Bartels, T., Kristal, B. S., & Selkoe, D. J. (2014). Soluble, prefibrillar α-synuclein oligomers promote complex I-dependent, Ca2+-induced mitochondrial dysfunction. *Journal of Biological Chemistry*, *289*(31), 21490–21507. https://doi.org/10.1074/jbc.M113.545749

Ma, S. Y., Röyttä, M., Rinne, J. O., Collan, Y., & Rinne, U. K. (1997). Correlation between neuromorphometry in the substantia nigra and clinical features in Parkinson's disease using disector counts. *Journal of the Neurological Sciences*, *151*(1), 83–87. https://doi.org/10.1016/S0022-510X(97)00100-7

Maingay, M., Romero-Ramos, M., Carta, M., & Kirik, D. (2006). Ventral tegmental area dopamine neurons are resistant to human mutant alpha-synuclein overexpression. *Neurobiology of Disease*, *23*(3), 522–532. https://doi.org/10.1016/j.nbd.2006.04.007

Mandler, M., Valera, E., Rockenstein, E., Weninger, H., Patrick, C., Adame, A., … Masliah, E. (2014). Next-generation active immunization approach for synucleinopathies: Implications for Parkinson's disease clinical trials. *Acta Neuropathologica*, *127*(6), 861–879. https://doi.org/10.1007/s00401-014-1256-4

Martinez-Martin, P., Rodriguez-Blazquez, C., Kurtis, M. M., & Chaudhuri, K. R. (2011). The impact of non-motor symptoms on health-related quality of life of patients with Parkinson's disease. *Movement Disorders*, *26*(3), 399–406. https://doi.org/10.1002/mds.23462

Matsushita, T., Horikawa, M., Iwata, Y., & Tedder, T. F. (2010). Regulatory B Cells (B10 Cells) and Regulatory T Cells Have Independent Roles in Controlling Experimental Autoimmune Encephalomyelitis Initiation and Late-Phase Immunopathogenesis. *The Journal of Immunology*, *185*(4), 2240–2252. https://doi.org/10.4049/jimmunol.1001307

McCormack, A. L., Mak, S. K., Henderson, J. M., Bumcrot, D., Farrer, M. J., & Di Monte, D. A. (2010). α-synuclein suppression by targeted small interfering rna in the primate

substantia nigra. *PLoS ONE*, *5*(8). https://doi.org/10.1371/journal.pone.0012122

McCormack, A. L., Mak, S. K., Shenasa, M., Langston, W. J., Forno, L. S., & Di Monte, D. A. (2008). Pathologic modifications of α-synuclein in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated squirrel monkeys. *Journal of Neuropathology and Experimental Neurology*, *67*(8), 793–802. https://doi.org/10.1097/NEN.0b013e318180f0bd

McCoy, M. K., Martinez, T. N., Ruhn, K. A., Szymkowski, D. E., Smith, C. G., Botterman, B. R., … Tansey, M. G. (2006). Blocking soluble tumor necrosis factor signaling with dominant-negative tumor necrosis factor inhibitor attenuates loss of dopaminergic neurons in models of Parkinson's disease. *Journal of Neuroscience*, *26*(37), 9365–9375. https://doi.org/10.1523/JNEUROSCI.1504-06.2006

McGeer, E. G., & McGeer, P. L. (2003). Inflammatory processes in Alzheimer's disease. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*. Elsevier Inc. https://doi.org/10.1016/S0278-5846(03)00124-6

McGeer, P. L., Itagaki, S., Boyes, B. E., & McGeer, E. G. (1988). Reactive microglia are positive for HLA-DR in the: Substantia nigra of Parkinson's and Alzheimer's disease brains. *Neurology*, *38*(8), 1285–1291. https://doi.org/10.1212/wnl.38.8.1285

McGeer, Patrick L., & McGeer, E. G. (2008, March 15). Glial reactions in Parkinson's disease. *Movement Disorders*. Mov Disord. https://doi.org/10.1002/mds.21751

Meyer Zu Hörste, G., Cordes, S., Mausberg, A. K., Zozulya, A. L., Wessig, C., Sparwasser, T., … Kieseier, B. C. (2014). FoxP3+ regulatory T cells determine disease severity in rodent models of inflammatory neuropathies. *PLoS ONE*, *9*(10). https://doi.org/10.1371/journal.pone.0108756

Miller, D. W., Hague, S. M., Clarimon, J., Baptista, M., Gwinn-Hardy, K., Cookson, M. R., & Singleton, A. B. (2004). α-synuclein in blood and brain from familial Parkinson disease with SNCA locus triplication. *Neurology*, *62*(10), 1835–1838.

https://doi.org/10.1212/01.WNL.0000127517.33208.F4

Mills, K. H. G. (2004, November). Regulatory T cells: Friend or foe in immunity to infection? *Nature Reviews Immunology*. Nat Rev Immunol. https://doi.org/10.1038/nri1485

Miyara, M., Gorochov, G., Ehrenstein, M., Musset, L., Sakaguchi, S., & Amoura, Z. (2011, October). Human FoxP3 + regulatory T cells in systemic autoimmune diseases. *Autoimmunity Reviews*. Autoimmun Rev. https://doi.org/10.1016/j.autrev.2011.05.004

Moalem, G., Leibowitz-Amit, R., Yoles, E., Mor, F., Cohen, I. R., & Schwartz, M. (1999). Autoimmune T cells protect neurons from secondary degeneration after central nervous system axotomy. *Nature Medicine*, *5*(1), 49–55. https://doi.org/10.1038/4734

Mosley, R. L., Hutter-Saunders, J. A., Stone, D. K., & Gendelman, H. E. (2012). Inflammation and adaptive immunity in Parkinson's disease. *Cold Spring Harbor Perspectives in Medicine*. https://doi.org/10.1101/cshperspect.a009381

Mrdjen, D., Pavlovic, A., Hartmann, F. J., Schreiner, B., Utz, S. G., Leung, B. P., … Becher, B. (2018). High-Dimensional Single-Cell Mapping of Central Nervous System Immune Cells Reveals Distinct Myeloid Subsets in Health, Aging, and Disease. *Immunity*, *48*(2), 380-395.e6. https://doi.org/10.1016/j.immuni.2018.01.011

Nalls, M. A., Plagnol, V., Hernandez, D. G., Sharma, M., Sheerin, U. M., Saad, M., … Wood, N. W. (2011). Imputation of sequence variants for identification of genetic risks for Parkinson's disease: A meta-analysis of genome-wide association studies. *The Lancet*, *377*(9766), 641–649. https://doi.org/10.1016/S0140-6736(10)62345-8

NH, O., R, M., SD, S., CJ, B., GM, H., & DW, M. (2013). Examination of blood-brain barrier (BBB) integrity in a mouse brain tumor model. *Journal of Neuro-Oncology*, *111*(2), 133–143. https://doi.org/10.1007/S11060-012-1006-1

Nicklas, W. m. do. J., Vyas, I., & Heikkila, R. E. (1985). Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenyl-pyridine, a metabolite of the neurotoxin, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. *Life Sciences*, *36*(26), 2503–

2508. https://doi.org/10.1016/0024-3205(85)90146-8

Nussbaum, R. L., & Ellis, C. E. (2003). Alzheimer's Disease and Parkinson's Disease. *N Engl J Med*, *348*(14), 1356–1364. https://doi.org/10.1056/NEJM2003ra020003

Nutt, J. G., Burchiel, K. J., Comella, C. L., Jankovic, J., Lang, A. E., Laws, E. R. J., … Wooten, G. F. (2003). Randomized, double-blind trial of glial cell line-derived neurotrophic factor (GDNF) in PD. *Neurology*, *60*(1), 69–73. https://doi.org/10.1212/wnl.60.1.69

Olanow, C. W., Bartus, R. T., Volpicelli-Daley, L. A., & Kordower, J. H. (2015). Trophic factors for Parkinson's disease: To live or let die. *Movement Disorders*. https://doi.org/10.1002/mds.26426

Olson, J. K., & Miller, S. D. (2004). Microglia Initiate Central Nervous System Innate and Adaptive Immune Responses through Multiple TLRs. *The Journal of Immunology*, *173*(6), 3916–3924. https://doi.org/10.4049/jimmunol.173.6.3916

Onishi, Y., Fehervari, Z., Yamaguchi, T., & Sakaguchi, S. (2008). Foxp3+ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(29), 10113–10118. https://doi.org/10.1073/pnas.0711106105

Ontaneda, D., Tallantyre, E., Kalincik, T., Planchon, S. M., & Evangelou, N. (2019). Early highly effective versus escalation treatment approaches in relapsing multiple sclerosis. *The Lancet. Neurology*, *18*(10), 973–980. https://doi.org/10.1016/S1474-4422(19)30151-6

Orgogozo, J. M., Gilman, S., Dartigues, J. F., Laurent, B., Puel, M., Kirby, L. C., … Hock, C. (2003). Subacute meningoencephalitis in a subset of patients with AD after Aβ42 immunization. *Neurology*, *61*(1), 46–54. https://doi.org/10.1212/01.WNL.0000073623.84147.A8

Pacheco, R., Riquelme, E., & Kalergis, A. (2012). Emerging Evidence for the Role of

Neurotransmitters in the Modulation of T Cell Responses to Cognate Ligands. *Central Nervous System Agents in Medicinal Chemistry*, *10*(1), 65–83. https://doi.org/10.2174/187152410790780154

Pandiyan, P., Zheng, L., Ishihara, S., Reed, J., & Lenardo, M. J. (2007). CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nature Immunology*, *8*(12), 1353–1362. https://doi.org/10.1038/ni1536

Paris, I., Lozano, J., Perez-Pastene, C., Muñoz, P., & Segura-Aguilar, J. (2009). Molecular and neurochemical mechanisms in PD pathogenesis. *Neurotoxicity Research*, *16*(3), 271–279. https://doi.org/10.1007/s12640-009-9059-4

Parker, D. C. (1993). T cell-dependent B cell activation. *Annual Review of Immunology*. Annu Rev Immunol. https://doi.org/10.1146/annurev.iy.11.040193.001555

Perry, V. H. (2010, September). Contribution of systemic inflammation to chronic neurodegeneration. *Acta Neuropathologica*. Acta Neuropathol. https://doi.org/10.1007/s00401-010-0722-x

Perry, V. H. (2012). Innate inflammation in Parkinson's disease. *Cold Spring Harbor Perspectives in Medicine*, *2*(9). https://doi.org/10.1101/cshperspect.a009373

Petito, C. K., & Cash, K. S. (1992). Blood-brain barrier abnormalities in acquired immunodeficiency syndrome: Immunohistochemical localization of serum proteins in postmortem brain. *Annals of Neurology*, *32*(5), 658–666. https://doi.org/10.1002/ana.410320509

Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., … Nussbaum, R. L. (1997). Mutation in the α-synuclein gene identified in families with Parkinson's disease. *Science*, *276*(5321), 2045–2047. https://doi.org/10.1126/science.276.5321.2045

Prinz, M., & Priller, J. (2017). The role of peripheral immune cells in the CNS in steady state and disease, *20*(2). https://doi.org/10.1038/nn.4418

Purisai, M. G., McCormack, A. L., Langston, W. J., Johnston, L. C., & Di Monte, D. A. (2005). α-Synuclein expression in the substantia nigra of MPTP-lesioned non-human primates. *Neurobiology of Disease*, *20*(3), 898–906. https://doi.org/10.1016/j.nbd.2005.05.028

Qiu, W. Q., Ye, Z., Kholodenko, D., Seubert, P., & Selkoe, D. J. (1997). Degradation of amyloid β-protein by a metalloprotease secreted by microglia and other neural and non-neural cells. *Journal of Biological Chemistry*, *272*(10), 6641–6646. https://doi.org/10.1074/jbc.272.10.6641

Quintino, L., Avallone, M., Bränstrom, E., Kavanagh, P., Lockowandt, M., Garcia Jareño, P., … Lundberg, C. (2019). GDNF-mediated rescue of the nigrostriatal system depends on the degree of degeneration. *Gene Therapy*, *26*(1–2), 57–64. https://doi.org/10.1038/s41434-018-0049-0

Racette, B. A., Gross, A., Vouri, S. M., Camacho-Soto, A., Willis, A. W., & Searles Nielsen, S. (2018). Immunosuppressants and risk of Parkinson disease. *Annals of Clinical and Translational Neurology*, *5*(7), 870–875. https://doi.org/10.1002/acn3.580

Radjavi, A., Smirnov, I., & Kipnis, J. (2014). Brain antigen-reactive CD4+ T cells are sufficient to support learning behavior in mice with limited T cell repertoire. *Brain, Behavior, and Immunity*, *35*, 58–63. https://doi.org/10.1016/j.bbi.2013.08.013

Reale, M., Iarlori, C., Thomas, A., Gambi, D., Perfetti, B., Di Nicola, M., & Onofrj, M. (2009). Peripheral cytokines profile in Parkinson's disease. *Brain, Behavior, and Immunity*, *23*(1), 55–63. https://doi.org/10.1016/j.bbi.2008.07.003

Rees, K., Stowe, R., Patel, S., Ives, N., Breen, K., Clarke, C. E., & Ben-Shlomo, Y. (2011). Non-steroidal anti-inflammatory drugs as disease-modifying agents for Parkinson's disease: evidence from observational studies. *Cochrane Database of Systematic Reviews*, (11). https://doi.org/10.1002/14651858.cd008454.pub2

Ren, B., Zhang, Y. X., Zhou, H. X., Sun, F. W., Zhang, Z. F., Wei, Z. F., … Si, D. W. (2015).

Tanshinone IIA prevents the loss of nigrostriatal dopaminergic neurons by inhibiting NADPH oxidase and iNOS in the MPTP model of Parkinson's disease. *Journal of the Neurological Sciences*, *348*(1–2), 142–152. https://doi.org/10.1016/j.jns.2014.11.026

Reynolds, A. D., Banerjee, R., Liu, J., Gendelman, H. E., & Mosley, R. L. (2007). Neuroprotective activities of CD4ƨCD25ƨ regulatory T cells in an animal model of Parkinson's disease. https://doi.org/10.1189/jlb.0507296

Reynolds, A. D., & Gendelman, H. E. (2010). Regulatory T cells attenuate Th17 cell mediated nigrostriatal dopaminergic neurodegeneration in a model of Parkinson's disease.

Reynolds, A. D., Glanzer, J. G., Kadiu, I., Ricardo-Dukelow, M., Chaudhuri, A., Ciborowski, P., … Gendelman, H. E. (2008). Nitrated alpha-synuclein-activated microglial profiling for Parkinson's disease. *Journal of Neurochemistry*. https://doi.org/10.1111/j.1471-4159.2007.05087.x

Reynolds, A. D., Stone, D. K., Hutter, J. A. L., Benner, E. J., Mosley, R. L., & Gendelman, H. E. (2010). Regulatory T Cells Attenuate Th17 Cell-Mediated Nigrostriatal Dopaminergic Neurodegeneration in a Model of Parkinson's Disease. *The Journal of Immunology*. https://doi.org/10.4049/jimmunol.0901852

Ridge, J. P., Di Rosa, F., & Matzinger, P. (1998). A conditioned dendritic cell can be a temporal bridge between a CD4 + T-helper and a T-killer cell. *Nature*, *393*(6684), 474–478. https://doi.org/10.1038/30989

Rodríguez-Palmero, M., Hara, T., Thumbs, A., & Hünig, T. (1999). *Triggering of T cell proliferation through CD28 induces GATA-3 and promotes T helper type 2 differentiation in vitro and in vivo*.

Römer, P. S., Berr, S., Avota, E., Na, S. Y., Battaglia, M., Ten Berge, I., … Hünig, T. (2011). Preculture of PBMCs at high cell density increases sensitivity of T-cell responses, revealing cytokine release by CD28 superagonist TGN1412. *Blood*, *118*(26), 6772–6782.

https://doi.org/10.1182/blood-2010-12-319780

Romero-Ramos, M., von Euler Cheplin, M., & Sanchez-Guajardo, V. (2014). Vaccination strategies for Parkinson disease.

Rosenkranz, D., Weyer, S., Tolosa, E., Gaenslen, A., Berg, D., Leyhe, T., … Stoltze, L. (2007). Higher frequency of regulatory T cells in the elderly and increased suppressive activity in neurodegeneration. *Journal of Neuroimmunology*. https://doi.org/10.1016/j.jneuroim.2007.05.011

Ross, O. A. (2013). A Prognostic View on the Application of Individualized Genomics in Parkinson's Disease. *Current Genetic Medicine Reports*, *1*(1), 52–57. https://doi.org/10.1007/s40142-012-0003-1

Rudow, G., O'Brien, R., Savonenko, A. V., Resnick, S. M., Zonderman, A. B., Pletnikova, O., … Troncoso, J. C. (2008). Morphometry of the human substantia nigra in ageing and Parkinson's disease. *Acta Neuropathologica*, *115*(4), 461–470. https://doi.org/10.1007/s00401-008-0352-8

Saiki, M., Baker, A., Williams-Gray, C. H., Foltynie, T., Goodman, R. S., Taylor, C. J., … Goris, A. (2010). Association of the human leucocyte antigen region with susceptibility to Parkinson's disease. *Journal of Neurology, Neurosurgery and Psychiatry*, *81*(8), 890–891. https://doi.org/10.1136/jnnp.2008.162883

Sanchez-Guajardo, V., Annibali, A., Jensen, P. H., & Romero-Ramos, M. (2013). α-synuclein vaccination prevents the accumulation of parkinson disease-like pathologic inclusions in striatum in association with regulatory T cell recruitment in a rat model. *Journal of Neuropathology and Experimental Neurology*, *72*(7), 624–645. https://doi.org/10.1097/NEN.0b013e31829768d2

Sanchez-Guajardo, V., Febbraro, F., Kirik, D., & Romero-Ramos, M. (2010). Microglia acquire distinct activation profiles depending on the degree of α-synuclein neuropathology in a rAAV based model of Parkinson's disease. *PLoS ONE*, *5*(1).

https://doi.org/10.1371/journal.pone.0008784

Saunders, J. A. H., Estes, K. A., Kosloski, L. M., Allen, H. E., Dempsey, K. M., Torres-Russotto, D. R., … Gendelman, H. E. (2012). CD4+ regulatory and effector/memory T cell subsets profile motor dysfunction in Parkinson's disease. *Journal of Neuroimmune Pharmacology*. https://doi.org/10.1007/s11481-012-9402-z

Schallert, T., Fleming, S. M., Leasure, J. L., Tillerson, J. L., & Bland, S. T. (2000). CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. *Neuropharmacology*. https://doi.org/10.1016/S0028-3908(00)00005-8

Schapira, A. H. V., Cooper, J. M., Dexter, D., Clark, J. B., Jenner, P., & Marsden, C. D. (1990). Mitochondrial Complex I Deficiency in Parkinson's Disease. *Journal of Neurochemistry*, *54*(3), 823–827. https://doi.org/10.1111/j.1471-4159.1990.tb02325.x

Schneeberger, A., Mandler, M., Mattner, F., & Schmidt, W. (2012). Vaccination for Parkinson's disease. *Parkinsonism and Related Disorders*, *18*(SUPPL. 1). https://doi.org/10.1016/s1353-8020(11)70006-2

Schröder, J. B., Pawlowski, M., Meyer zu Hörste, G., Gross, C. C., Wiendl, H., Meuth, S. G., … Warnecke, T. (2018). Immune Cell Activation in the Cerebrospinal Fluid of Patients With Parkinson's Disease. *Frontiers in Neurology*, *9*. https://doi.org/10.3389/fneur.2018.01081

Schuhmann, M. K., Kraft, P., Stoll, G., Lorenz, K., Meuth, S. G., Wiendl, H., … Kleinschnitz, C. (2015). CD28 superagonist-mediated boost of regulatory T cells increases thrombo-inflammation and ischemic neurodegeneration during the acute phase of experimental stroke. *Journal of Cerebral Blood Flow and Metabolism*, *35*(1), 6–10. https://doi.org/10.1038/jcbfm.2014.175

Schwartz, M., & Baruch, K. (2014, November 1). Breaking peripheral immune tolerance to CNS antigens in neurodegenerative diseases: Boosting autoimmunity to fight-off chronic

neuroinflammation. *Journal of Autoimmunity*. Academic Press. https://doi.org/10.1016/j.jaut.2014.08.002

Shahaduzzaman, M., Nash, K., Hudson, C., Sharif, M., Grimmig, B., Lin, X., … Bickford, P. C. (2015). Anti-human α-synuclein N-terminal peptide antibody protects against dopaminergic cell death and ameliorates behavioral deficits in an AAV-α-synuclein rat model of Parkinson's disease. *PLoS ONE*, *10*(2). https://doi.org/10.1371/journal.pone.0116841

Shameli, A., Xiao, W., Zheng, Y., Shyu, S., Sumodi, J., Meyerson, H. J., … Maitta, R. W. (2016). A critical role for alpha-synuclein in development and function of T lymphocytes. *Immunobiology*, *221*(2), 333–340. https://doi.org/10.1016/j.imbio.2015.10.002

Sharpe, A. H., & Freeman, G. J. (2002). The B7-CD28 superfamily. *Nature Reviews Immunology*. European Association for Cardio-Thoracic Surgery. https://doi.org/10.1038/nri727

Shavali, S., Combs, C. K., & Ebadi, M. (2006). Reactive macrophages increase oxidative stress and alpha-synuclein nitration during death of dopaminergic neuronal cells in co-culture: Relevance to parkinson's disease. *Neurochemical Research*, *31*(1), 85–94. https://doi.org/10.1007/s11064-005-9233-x

Shechter, R., Miller, O., Yovel, G., Rosenzweig, N., London, A., Ruckh, J., … Schwartz, M. (2013). Recruitment of Beneficial M2 Macrophages to Injured Spinal Cord Is Orchestrated by Remote Brain Choroid Plexus. *Immunity*, *38*(3), 555–569. https://doi.org/10.1016/j.immuni.2013.02.012

Shimura, H., Schlossmacher, M. G., Hattori, N., Frosch, M. P., Trockenbacher, A., Schneider, R., … Selkoe, D. J. (2001). Ubiquitination of a new form of α-synuclein by parkin from human brain: Implications for Parkinson's disease. *Science*, *293*(5528), 263–269. https://doi.org/10.1126/science.1060627

Sica, A., & Mantovani, A. (2012, March 1). Macrophage plasticity and polarization: In vivo veritas. *Journal of Clinical Investigation*. J Clin Invest. https://doi.org/10.1172/JCI59643

Singleton, A. B., Farrer, M., Johnson, J., Singleton, A., Hague, S., Kachergus, J., … Gwinn-Hardy, K. (2003). α-Synuclein Locus Triplication Causes Parkinson's Disease. *Science*, *302*(5646), 841. https://doi.org/10.1126/science.1090278

Smith-Garvin, J. E., Koretzky, G. A., & Jordan, M. S. (2009). T cell activation. *Annual Review of Immunology*. Annu Rev Immunol. https://doi.org/10.1146/annurev.immunol.021908.132706

Sommer, A., Fadler, T., Dorfmeister, E., Hoffmann, A. C., Xiang, W., Winner, B., & Prots, I. (2016). Infiltrating T lymphocytes reduce myeloid phagocytosis activity in synucleinopathy model. *Journal of Neuroinflammation*, *13*(1). https://doi.org/10.1186/s12974-016-0632-5

Sommer, A., Maxreiter, F., Krach, F., Fadler, T., Grosch, J., Maroni, M., … Winner, B. (2018). Th17 Lymphocytes Induce Neuronal Cell Death in a Human iPSC-Based Model of Parkinson's Disease. *Cell Stem Cell*, *23*(1), 123-131.e6. https://doi.org/10.1016/j.stem.2018.06.015

Sommer, A., Winner, B., & Prots, I. (2017, October 27). The Trojan horse - Neuroinflammatory impact of T cells in neurodegenerative diseases. *Molecular Neurodegeneration*. BioMed Central Ltd. https://doi.org/10.1186/s13024-017-0222-8

Spillantini, M. G., Crowther, R. A., Jakes, R., Hasegawa, M., & Goedert, M. (1998). α-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with Lewy bodies. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(11), 6469–6473. https://doi.org/10.1073/pnas.95.11.6469

Spillantini, M. G., Schmidt, M. L., Lee, V. M. Y., Trojanowski, J. Q., Jakes, R., & Goedert, M. (1997). α-synuclein in Lewy bodies [8]. *Nature*. Nature. https://doi.org/10.1038/42166

Sriram, K., Matheson, J. M., Benkovic, S. A., Miller, D. B., Luster, M. I., & O'Callaghan, J. P. (2002). Mice deficient in TNF receptors are protected against dopaminergic neurotoxicity: implications for Parkinson's disease. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *16*(11), 1474–1476. https://doi.org/10.1096/fj.02-0216fje

Štefanoví, I., Dorfman, J. R., & Germain, R. N. (2002). Self-recognition promotes the foreign antigen sensitivity of naive T lymphocytes. *Nature*, *420*(6914), 429–434. https://doi.org/10.1038/nature01146

Stevens, C. H., Rowe, D., Morel-Kopp, M. C., Orr, C., Russell, T., Ranola, M., … Halliday, G. M. (2012). Reduced T helper and B lymphocytes in Parkinson's disease. *Journal of Neuroimmunology*, *252*(1–2), 95–99. https://doi.org/10.1016/j.jneuroim.2012.07.015

Su, X., Maguire-Zeiss, K. A., Giuliano, R., Prifti, L., Venkatesh, K., & Federoff, H. J. (2008). Synuclein activates microglia in a model of Parkinson's disease. *Neurobiology of Aging*, *29*(11), 1690–1701. https://doi.org/10.1016/j.neurobiolaging.2007.04.006

Sulzer, D., Alcalay, R. N., Garretti, F., Cote, L., Kanter, E., Agin-Liebes, J., … Sette, A. (2017). T cells from patients with Parkinson's disease recognize α-synuclein peptides. *Nature*. https://doi.org/10.1038/nature22815

Sun, M., Kong, L., Wang, X., Lu, X. G., Gao, Q., & Geller, A. I. (2005). Comparison of the capability of GDNF, BDNF, or both, to protect nigrostriatal neurons in a rat model of Parkinson's disease. *Brain Research*. https://doi.org/10.1016/j.brainres.2005.05.072

Suntharalingam, G., Perry, M. R., Ward, S., Brett, S. J., Castello-Cortes, A., Brunner, M. D., & Panoskaltsis, N. (2006). Cytokine Storm in a Phase 1 Trial of the Anti-CD28 Monoclonal Antibody TGN1412. *New England Journal of Medicine*, *355*(10), 1018–1028. https://doi.org/10.1056/nejmoa063842

SY, N., E, M., A, L., T, H., & R, V. (2015). Amplification of regulatory T cells using a CD28 superagonist reduces brain damage after ischemic stroke in mice. *Stroke*, *46*(1), 212–220.

https://doi.org/10.1161/STROKEAHA.114.007756

Tabares, P., Berr, S., Römer, P. S., Chuvpilo, S., Matskevich, A. A., Tyrsin, D., … Hünig, T. (2014). Human regulatory T cells are selectively activated by low-dose application of the CD28 superagonist TGN1412/TAB08. *European Journal of Immunology*, *44*(4), 1225–1236. https://doi.org/10.1002/eji.201343967

Tacke, M., Hanke, G., Hanke, T., & Hünig, T. (1997). CD28-mediated induction of proliferation in resting T cells in vitro and in vivo without engagement of the T cell receptor: Evidence for functionally distinct forms of CD28. *European Journal of Immunology*, *27*(1), 239–247. https://doi.org/10.1002/eji.1830270136

Tadokoro, C. E., Shakhar, G., Shen, S., Ding, Y., Lino, A. C., Maraver, A., … Dustin, M. L. (2006). Regulatory T cells inhibit stable contacts between CD4+ T cells and dendritic cells in vivo. *Journal of Experimental Medicine*, *203*(3), 505–511. https://doi.org/10.1084/jem.20050783

Tang, Q., & Bluestone, J. A. (2008, March). The Foxp3+ regulatory T cell: A jack of all trades, master of regulation. *Nature Immunology*. Nat Immunol. https://doi.org/10.1038/ni1572

Tanner, C. M., & Goldman, S. M. (1996). EPIDEMIOLOGY OF PARKINSON'S DISEASE. *Neurol Clin.*, *14*(2), 317–335. https://doi.org/10.1016/S0733-8619(05)70259-0

Teeling, J. L., & Perry, V. H. (2009, February 6). Systemic infection and inflammation in acute CNS injury and chronic neurodegeneration: Underlying mechanisms. *Neuroscience*. Neuroscience. https://doi.org/10.1016/j.neuroscience.2008.07.031

Theodore, S., Cao, S., McLean, P. J., & Standaert, D. G. (2008). Targeted overexpression of human alpha-synuclein triggers microglial activation and an adaptive immune response in a mouse model of Parkinson disease. *Journal of Neuropathology and Experimental Neurology*, *67*(12), 1149–1158. https://doi.org/10.1097/NEN.0b013e31818e5e99

Togo, T., Akiyama, H., Iseki, E., Kondo, H., Ikeda, K., Kato, M., … Kosaka, K. (2002).

Occurrence of T cells in the brain of Alzheimer's disease and other neurological diseases. *Journal of Neuroimmunology*, *124*(1–2), 83–92. https://doi.org/10.1016/S0165-5728(01)00496-9

Tran, H. T., Chung, C. H. Y., Iba, M., Zhang, B., Trojanowski, J. Q., Luk, K. C., & Lee, V. M. Y. (2014). α-Synuclein Immunotherapy Blocks Uptake and Templated Propagation of Misfolded α-Synuclein and Neurodegeneration. *Cell Reports*, *7*(6), 2054–2065. https://doi.org/10.1016/j.celrep.2014.05.033

Uversky, V. N. (2007, October). Neuropathology, biochemistry, and biophysics of α-synuclein aggregation. *Journal of Neurochemistry*. J Neurochem. https://doi.org/10.1111/j.1471-4159.2007.04764.x

Vaikath, N. N., Majbour, N. K., Paleologou, K. E., Ardah, M. T., van Dam, E., van de Berg, W. D. J., … El-Agnaf, O. M. A. (2015). Generation and characterization of novel conformation-specific monoclonal antibodies for α-synuclein pathology. *Neurobiology of Disease*, *79*, 81–99. https://doi.org/10.1016/j.nbd.2015.04.009

van der Most, R. G., Murali-Krishna, K., & Ahmed, R. (2003, January 1). Prolonged presence of effector-memory CD8 T cell in the central nervous system after dengue virus encephalitis. *International Immunology*. Int Immunol. https://doi.org/10.1093/intimm/dxg009

Vignali, D. A. A., Collison, L. W., & Workman, C. J. (2008, July). How regulatory T cells work. *Nature Reviews Immunology*. Nat Rev Immunol. https://doi.org/10.1038/nri2343

Vos, T., Allen, C., Arora, M., Barber, R. M., Bhutta, Z. A., Brown, A., … Murray, C. J. L. (2016). Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015. *The Lancet*, *388*(10053), 1545–1602. https://doi.org/10.1016/S0140-6736(16)31678-6

Walsh, J. T., & Kipnis, J. (2011, October). Regulatory T cells in CNS injury: The simple, the

complex and the confused. *Trends in Molecular Medicine*. Trends Mol Med. https://doi.org/10.1016/j.molmed.2011.05.012

Waterston, R. H., Lindblad-Toh, K., Birney, E., Rogers, J., Abril, J. F., Agarwal, P., … Lander, E. S. (2002). Initial sequencing and comparative analysis of the mouse genome. *Nature*, *420*(6915), 520–562. https://doi.org/10.1038/nature01262

William Langston, J., Ballard, P., Tetrud, J. W., & Irwin, I. (1983). Chronic parkinsonism in humans due to a product of meperidine-analog synthesis. *Science*, *219*(4587), 979–980. https://doi.org/10.1126/science.6823561

Williams-Gray, C. H., Wijeyekoon, R., Yarnall, A. J., Lawson, R. A., Breen, D. P., Evans, J. R., … Barker, R. A. (2016). Serum immune markers and disease progression in an incident Parkinson's disease cohort (ICICLE-PD). *Movement Disorders*, *31*(7), 995–1003. https://doi.org/10.1002/mds.26563

Win, S. J., Kühl, A. A., Sparwasser, T., Hünig, T., & Kamradt, T. (2016). In vivo activation of Treg cells with a CD28 superagonist prevents and ameliorates chronic destructive arthritis in mice. *European Journal of Immunology*, *46*(5), 1193–1202. https://doi.org/10.1002/eji.201546104

Wing, K., Onishi, Y., Prieto-Martin, P., Yamaguchi, T., Miyara, M., Fehervari, Z., … Sakaguchi, S. (2008). CTLA-4 control over Foxp3+ regulatory T cell function. *Science*, *322*(5899), 271–275. https://doi.org/10.1126/science.1160062

Wong, D., Prameya, R., & Dorovini-Zis, K. (1999). In vitro adhesion and migration of T lymphocytes across monolayers of human brain microvessel endothelial cells: Regulation by ICAM-1, VCAM-1, E- selectin and PECAM-1. *Journal of Neuropathology and Experimental Neurology*, *58*(2), 138–152. https://doi.org/10.1097/00005072-199902000-00004

Wu, D. C., Jackson-Lewis, V., Vila, M., Tieu, K., Teismann, P., Vadseth, C., … Przedborski, S. (2002). Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-

1,2,3,6-tetrahydropyridine mouse model of Parkinson disease. *Journal of Neuroscience*, *22*(5), 1763–1771. https://doi.org/10.1523/jneurosci.22-05-01763.2002

Xilouri, M., Brekk, O. R., & Stefanis, L. (2013). α-Synuclein and protein degradation systems: a reciprocal relationship. *Molecular Neurobiology*. Mol Neurobiol. https://doi.org/10.1007/s12035-012-8341-2

Xu, K., Xu, Y., Brown-Jermyn, D., Chen, J. F., Ascherio, A., Dluzen, D. E., & Schwarzschild, M. A. (2006). Estrogen prevents neuroprotection by caffeine in the mouse 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. *Journal of Neuroscience*, *26*(2), 535–541. https://doi.org/10.1523/JNEUROSCI.3008-05.2006

Yirmiya, R., & Goshen, I. (2011). Immune modulation of learning, memory, neural plasticity and neurogenesis. *Brain, Behavior, and Immunity*, *25*(2), 181–213. https://doi.org/10.1016/j.bbi.2010.10.015

Yong, J., Lacan, G., Dang, H., Hsieh, T., Middleton, B., Wasserfall, C., … Kaufman, D. L. (2011). BCG vaccine-induced neuroprotection in a mouse model of Parkinson's Disease. *PLoS ONE*, *6*(1). https://doi.org/10.1371/journal.pone.0016610

Zarranz, J. J., Alegre, J., Gómez-Esteban, J. C., Lezcano, E., Ros, R., Ampuero, I., … De Yebenes, J. G. (2004). The New Mutation, E46K, of α-Synuclein Causes Parkinson and Lewy Body Dementia. *Annals of Neurology*, *55*(2), 164–173. https://doi.org/10.1002/ana.10795

Zhao, Y., Haney, M. J., Gupta, R., Bohnsack, J. P., He, Z., Kabanov, A. V., & Batrakova, E. V. (2014). GDNF-transfected macrophages produce potent neuroprotective effects in parkinson's disease mouse model. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0106867

Zigmond, M. J., & Stricker, E. M. (1989). Animal Models of Parkinsonism Using Selective Neurotoxins: Clinical and Basic Implications. *International Review of Neurobiology*,

31(C), 1–79. https://doi.org/10.1016/S0074-7742(08)60277-9

Ziv, Y., Ron, N., Butovsky, O., Landa, G., Sudai, E., Greenberg, N., … Schwartz, M. (2006). Immune cells contribute to the maintenance of neurogenesis and spatial learning abilities in adulthood. *Nature Neuroscience*, *9*(2), 268–275. https://doi.org/10.1038/nn1629

Zurolo, E., Iyer, A., Maroso, M., Carbonell, C., Anink, J. J., Ravizza, T., … Aronica, E. (2011). Activation of toll-like receptor, RAGE and HMGB1 signalling in malformations of cortical development. *Brain*, *134*(4), 1015–1032. https://doi.org/10.1093/brain/awr032