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Monocyte Activation by Serum Extracellular Vesicles from Parkinson's Disease Patients

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For my parents

Contents

Table of Contents

Contents ..... **I**

List of Abbreviations ..... **III**

1. Introduction **1**

1.1. Parkinson's Disease (PD) 1

1.1.1. Pathophysiology 1

1.1.2. Neuropathology 1

1.1.3. Diagnosis 2

1.1.4. Therapy 3

1.2. Inflammation in PD 4

1.2.1. Neuroinflammation 4

1.2.2. Peripheral Inflammation 6

1.3. Role of Monocytes in PD 7

1.3.1. Dysfunction of Monocytes 7

1.3.2. Monocytes and α-synuclein 9

1.4. Role of Extracellular Vesicles (EVs) in PD 10

1.4.1. EVs and α-synuclein 10

1.4.2. Activation of Immune Cells by EVs 12

1.5. Research Objectives 14

2. Materials and Methods **16**

2.1. Antibodies 16

2.1.1. Antibodies for Enzyme-Linked Immunosorbent Assay (ELISA) 16

2.1.2. Antibodies for Western Blot (WB) 16

2.1.3. Antibody for Monocyte Isolation 17

2.2. Chemicals and Reagents 17

2.3. Consumables 18

2.4. Technical Equipment 19

2.5. Buffers and Solutions 21

2.5.1. Solutions and Buffers for Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and WB 21

2.5.2. Buffer for Magnetic-Activated Cell Separation (MACS) 21

2.5.3. Solutions for ELISA 22

2.5.4. Enzymes and Proteins 22

2.6. Software 22

2.7. Ethical Approval and Study Cohort 23
2.8. Statistical Analysis 23
2.9. Monocyte Isolation and Culture 24
2.10. EV Isolation 25
2.11. Nanoparticle Tracking Analysis (NTA) 25
2.12. ELISA 25
2.13. SDS-PAGE 26
2.14. Transfer and Protein Detection by WB 27
2.15. Bicinchoninic Acid Assay (BCA) 28
2.16. Hemolysis Control of Serum 28
3. Results 29
3.1. Characterization of EVs Isolated from Human Serum 29
3.1.1. NTA Characterization of Serum EVs 29
3.1.2. WB Analysis of Serum EVs 30
3.2. Differential Monocytic Response to Healthy Control (HC) and PD EVs 31
3.2.1. Stimulation of *Aged* Healthy Donors' Monocytes with *Pooled* HC and PD EVs 32
3.2.2. Stimulation of *Young* Healthy Donors' Monocytes with *Pooled* HC and PD EVs 39
3.2.3. Stimulation of *Young* Healthy Donors' Monocytes with *Individual* HC and PD EVs 44
4. Discussion 47
4.1. Technical Limitations 47
4.1.1. Effectiveness and Quality of EV Isolation via Ultracentrifugation 47
4.1.2. Challenges of Monocyte Isolation 48
4.2. Weak Activation of Human Monocytes by Serum EVs 49
4.3. Identification of Responders to Pathologic PD Serum EVs 52
4.3.1. Age-dependency of Monocytic Response 52
4.3.2. Interindividual Differences in Monocytic Response to Stimulation 54
4.4. Two Reinforcing Factors: Pathologic PD EVs and Dysregulated PD Monocytes 56
4.5. Conclusions 58
5. Summary 59
6. References 61
Acknowledgments 78
Curriculum Vitae 79

List of Abbreviations

Abbreviation	Explanation
°C	Degree Celsius; unit of measurement of temperature
A	Ampere; unit of the electrical current
AACD	Aromatic amino acid decarboxylase, also known as DOPA decarboxylase
ALS	Amyotrophic lateral sclerosis
ANOVA	Analysis of variance
AP	Atypical parkinsonism
APS	Ammonium persulphate
Av-HRP	Avidin-horseradish peroxidase
BBB	Blood-brain barrier
BCA (assay)	Bicinchoninic acid (protein assay)
BSA	Bovine serum albumin (or “Fraction V”)
CD	Cluster of differentiation; cell surface molecules that act as targets for immunophenotyping of cells
CCL2	Chemokine C-C motif ligand 2, also called monocyte chemoattractant protein-1 (MCP-1)
CCR2	C-C Chemokine receptor type 2, also known as CD192
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
Con	Control (in experiments, only unconditioned media)
CSF	Cerebrospinal fluid
CX3CR1	C-C chemokine receptor type 3, also known as CD183
Da	Dalton; unified atomic mass unit; kDa = kilodalton
DAT	Dopamine (active) transporter, also known as SLC6A3
DBS	Deep brain stimulation
DLB	Dementia with Lewy bodies
DNA	Desoxyribonucleic acid
(D)PBS	(Dulbecco’s) Phosphate-Buffered Saline
DTT	Dithiothreitol

Abbreviation	Explanation
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
EV(s)	Extracellular vesicle(s)
F_C	Fragment crystallizable
FCS	Fetal calf serum
Fig.	Figure
g	Gravitational acceleration; standard gravity
GABAergic	Neurons that produce γ-aminobutyric acid as neurotransmitter
GC	Gastric cancer
GCase	Glucocerebrosidase
GFAP	Glial fibrillary acidic protein
GWAS	Genome-wide association studies
h	Hours
HT	Hashimoto's thyreoiditis
HC(s)	Healthy control(s)
HEK293	Human embryonic kidney 293 cells
HLA-DR	Human leukocyte antigen DR isotype
HSCs	Hematopoietic stem cells
HRP	Horseradish peroxidase
Hz	Hertz; unit of frequency, equal to 1 s^{-1}
IFN	Interferon; signaling molecules of different types
IgG	Immunoglobulin G; main type of antibody found in blood
IL	Interleukin; cytokines with different families
iPSCs	Induced pluripotent stem cells
ISEV	International Society for Extracellular Vesicles
L	Liter; unit of volume; mL = milliliter; µL = microliter
L-Dopa	L-3,4-dihydroxyphenylalanine, also known as Levodopa, also called L-DOPA
LCAM1	L1 cell adhesion molecule protein, a neural cell adhesion molecule, shortened L1 protein

Abbreviation	Explanation
LPS	Lipopolysaccharide
LRRK2	Leucin-rich repeat kinase 2
M	Molar concentration, also known as molarity; mM = millimolar
m	Meter; unit of length; µm = micrometer; nm = nanometer
MACS	Magnetic-activated cell sorting
MAO-B	Monoamine oxidase B
MCP-1	Monocyte chemoattractant protein-1, also called chemokine C-C motif ligand 2 (CCL2)
MHC(-II)	Major histocompatibility complex (class II)
min	Minutes
miRNA	Micro ribonucleic acid; small non-coding RNA molecules
MISEV2018	Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSA	Multiple system atrophy
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B cells; a family of transcription factors that control cell survival and cytokine production
NMDA	N-methyl-D-aspartate
NSAID	Non-steroidal anti-inflammatory drug
NTA	Nanoparticle Tracking Analysis
p.	page(s)
PBMCs	Peripheral blood mononuclear cells; encompass lymphocytes (T cells, B cells, NK cells) and monocytes
PBS-T	Phosphate-Buffered Saline with Tween
PD	Parkinson's Disease
PET	Positron emission tomography

Abbreviation	Explanation
pH	Power of hydrogen; scale of how basic or acidic a water-based solution is
REM	Rapid eye movement
RKU	University and Rehabilitation Clinics of Ulm
ROS	Reactive oxygen species
RT	Room temperature
SD	Standard deviation
SDS(-PAGE)	Sodium dodecyl sulfate (polyacrylamide gel electrophoresis)
SEM	Standard error of the mean
SN(Pc)	Substantia nigra (pars compacta)
TEMED	N, N, N', N' - Tetramethylethylenediamine
TBS(-T)	Tris-buffered saline (with tween)
TMB	3, 3', 5, 5' - Tetramethylbenzidine Substrate
TNFα	Tumor necrosis factor α
TLR	Toll-like receptor
UC	Ultracentrifugation
UPDRS	Unified Parkinson's Disease rating scale
V	Volt; unit of electric potential
WB	Western blot
x	Times; for example: four times = 4x, thousand-fold dilution = dilution (1000x)

1. Introduction

1.1. Parkinson's Disease (PD)

Parkinson's Disease is a common neurodegenerative disease with a chronic progressive course and a considerable socioeconomic burden. PD prevalence is increasing with age. PD affects up to 4% of the population above the age of 85 and is hence the second most common neurodegenerative disease after Alzheimer's disease [29]. Notably, PD, which affected approximately 6.1 million people worldwide in 2016 [45], is regarded as the fastest growing neurodegenerative disease [44].

PD was first described in James Parkinson's monograph "An essay on the shaking palsy" in 1817, where he wrote on a few patients presenting with the typical motor symptoms, namely tremor at rest, an absence of voluntary movements, stooped posture and festinating gait [82]. The cardinal symptoms are akinesia (slow or missing movements), rigor (muscle stiffness), tremor at rest with a frequency of about 5 Hertz (Hz) and postural instability. Beyond these motor symptoms, PD is characterized by non-motor symptoms such as vegetative and mental disorders, that often precede motor symptoms and contribute to severe disability, but also provide means to identify prodromal stages of PD [93].

1.1.1. Pathophysiology

The main pathologic feature of PD is the loss of dopaminergic neurons of the Substantia Nigra pars compacta (SNpc), which leads to a lack of dopamine at the striatal dopamine receptors causing an imbalance of the basal ganglia motor circuit. The degeneration of the SNpc decreases the activation of the direct pathway of movement and the inhibition of the indirect pathway. This results in a stronger inhibition of the thalamus due to subthalamic hyperactivity which consecutively causes a lower activation of the motor cortex [7].

At the time of disease onset, approximately 60% of the dopaminergic neurons of the SNpc are already lost and the striatal dopamine levels are reduced up to 80% [61].

1.1.2. Neuropathology

The histopathological hallmark of PD are Lewy bodies and Lewy neurites. These are eosinophilic homogenic inclusions mainly composed of α-synuclein within neuronal cells and cell processes [31]. PD-specific lesions and α-synuclein-immunopositive Lewy bodies

and Lewy neurites are not restricted only to the SNpc, but also affect other regions of the central nervous system (CNS), which may explain many of the non-motor symptoms. According to Braak et al., PD can be divided into six pathological stages [12]. At stage 1 Lewy-related pathology can be found in the dorsal motor nuclei of the glossopharyngeal and the vagal nerve. This can be regarded as putative underlying cause of the vegetative dysfunctions. At stage 2, there are additional lesions in caudal raphe nuclei, in gigantocellular nuclei of the reticular formation and at the coeruleus-subcoeruleus-complex. A typical feature of stage 3 is the involvement of the midbrain, particularly the SNpc. As the Lewy-related pathology propagates, the temporal mesocortex and the allocortex become affected at stage 4. Ultimately at stages 5 and 6, lesions occur in the neocortex, which is associated with cognitive disorders such as dementia. The Braak hypothesis suggests that Lewy-body pathology first arises at some sites at the brain stem or the olfactory bulb and then propagates to other brain regions such as the midbrain or even the neocortex [12].

As in other neurodegenerative diseases, the neuronal loss in PD comes along with reactive gliosis. Astrocytes become hypertrophic and microglial cells become activated, which causes them to express activation markers like human leukocyte antigen isotype DR (HLA-DR) – a class II major histocompatibility antigen (MHC-II) [72]. However, the microgliosis observed in PD patients may represent infiltration and activation of peripheral blood monocytes, which are indistinguishable from activated monocytes in this context. Indeed, Harms et al. demonstrated recently that infiltration of peripheral blood monocytes is not only taking place, but even required for an extensive neuronal loss in an α-synuclein-based animal model of PD [56].

1.1.3. Diagnosis

The diagnosis of PD primarily depends on the cardinal symptoms listed above. The “UK Parkinson’s Disease Society Brain Bank Clinical Diagnostic Criteria” [47] are the most widespread criteria in clinical studies. They use a three-step approach based on the cardinal motor symptoms. In addition to these motor symptoms, as step 2 there are some exclusion criteria such as a positive Babinski-sign, 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridin (MPTP) exposure or neuroleptic treatment at onset of disease. At step 3, the patient should be checked on prospective positive criteria for PD like an excellent response to Levodopa

or a unilateral onset. Definite histopathological confirmation of the diagnosis can only be obtained at autopsy [47, 49].

Although there can be warning symptoms such as hyposmia, insomnia, uncontrolled or sporadic movements while sleep, psychosis or constipation, an early diagnosis of PD is difficult and in the absence of motor symptoms often not possible.

Another possibility to diagnose PD is positron emission tomography (PET) imaging. PET neuroimaging enables to measure the dopamine transporter (DAT), aromatic amino acid decarboxylase (AADC) and vesicular monoamine transporter 2 (VMAT2) activity at the presynapse and to investigate the functionality of postsynaptic dopamine receptors. By using different radiotracers, early PD or even preclinical PD can be assessed. However, PET is very expensive and therefore often not available [70].

There are currently no biomarkers that are used routinely in daily clinical practice. Cerebrospinal fluid (CSF) levels of α-synuclein were shown to be capable of predicting cognitive decline but are not useful to estimate motor progression [103]. In addition, dermal phosphorylated α-synuclein was shown to be highly specific to PD but lacking high sensitivity [33]. Interestingly, phosphorylated α-synuclein in dermal nerve fibers can also be detected in some patients with rapid eye movement (REM) sleep behavior disorder, which probably represents prodromal PD [34]. Identifying people at risk of developing PD or PD patients in the prodromal stage of the disease is necessary for developing and testing disease-modifying therapies.

1.1.4. Therapy

Whereas there is no therapy available that can alter the course of the ongoing neurodegenerative process in PD, symptomatic therapies exist that improve patients' health-related quality of life.

The most widespread therapeutic strategy is to compensate dopaminergic deficiency by treatment with Levodopa (L-Dopa) or dopamine agonists like Pramipexol, Rotigotin and Ropinirol. Birkmayer and Hornykiewicz first published in 1961 that L-Dopa medication shows significant antiakinetic effects, which can even be augmented by additive treatment of Deprenyl, a specific monoamine oxidase B (MAO-B) inhibitor [5, 6]. Since then, the underlying principle of the therapeutic strategy did not change. There is still no neuroprotective drug. Although a potentially disease-modifying effect of Rasagiline, another MAO-B inhibitor, was shown [87], this was not definitely confirmed by further

clinical trials [79]. Other treatment options include catechol-O-methyltransferase (COMT) inhibitors and DOPA-Decarboxylase-inhibitors that reduce the degradation of L-Dopa mainly in the periphery. Thus, they increase concentration of L-Dopa and thereby of dopamine in the CNS. Anticholinergic drugs like Trihexyphenidyl and the N-methyl-D-aspartate (NMDA)-receptor-antagonist Memantine pose other treatment options, which also aim on correcting neurotransmitter imbalances. The long-term use of all these drugs comes along with distinct and severe side effects such as impulse control disorders under treatment with dopamine agonists [114], which need to be medically targeted on their own just as other adverse drug effects of current PD medication.

A non-pharmacological treatment option is deep brain stimulation (DBS) – preferably of the subthalamic nucleus. DBS is particularly useful in patients who do not respond to standard medical treatment any longer. DBS was shown to provide durable symptomatic relief but is not able to slow down disease progression [59].

Causal therapies, such as α-synuclein-based immunotherapies, are currently being investigated in clinical studies. Prasinezumab for example, a humanized monoclonal antibody against aggregated α-synuclein, is now entering phase 2 of clinical studies [62].

Another treatment option aiming at halting ongoing neurodegeneration is the transplantation of induced pluripotent stem cells (iPSCs). In a MPTP-based primate model, the transplantation of iPSCs into the midbrain could not only improve motor symptoms, but also did not cause any severe side effects like an overwhelming immune response or forming of tumors [64].

1.2. Inflammation in PD

1.2.1. Neuroinflammation

Neuroinflammation describes inflammatory responses that are centralized within the brain or spinal cord. Inflammation, which is part of the response of the immune system to injury or infection, is orchestrated by the production of mediators such as chemokines, cytokines, reactive oxygen species (ROS) or secondary messengers. In the CNS, these mediators are secreted by microglial cells, astrocytes, endothelial cells and peripherally derived immune cells. Whereas the connotation of the term neuroinflammation is in most of the cases negative, it is always context-dependent whether the inflammatory response is maladaptive or beneficial [32].

Understanding the underlying mechanisms of neuroinflammatory processes is crucial as neuronal damage often comes along with chronic activation of the innate immune response and neuroinflammation can be observed in many different neurodegenerative disorders such as Alzheimer's Disease (AD), Amyotrophic lateral sclerosis (ALS) or PD [102]. Georg Wilhelm Kreutzbergs' statement *"There is hardly any pathology in the brain without an involvement of glial cells"* on *page (p.) 312* in his publication "Microglia: a sensor for pathological events in the CNS" [68] underlines the major role that microglial and astroglial cells are playing in tissue homeostasis and integrity within the brain. Microglia are the primary innate immune cells and the resident macrophages of the CNS. They arise from mesodermal (myeloid) progenitors which originate from the yolk sac and which occur earlier than hematopoietic stem cells (HSCs) during brain development [50, 89]. This suggests a different origin of these cell types [86]. Although microglia can self-renew themselves in the healthy brain [1] and the microglia pool of the CNS is sustained via clonal expansion [110], depleted microglia can also be replenished by peripherally derived macrophages/monocytes. In a mouse model, these brain-engrafting macrophages maintain a distinct functional role and specific transcriptional identity [25].

Resting microglia show a ramified morphology with small somata and branched processes. They participate in immune surveillance by constantly monitoring their environment using their fine processes [76]. Different triggers can lead to a transformation of resting into activated microglial cells, which show an amoeboid morphology. Microglial responsiveness can for example be induced by the ligation of pattern recognition receptors such as Toll-like receptors (TLRs). The activation of microglia via TLRs causes an increased expression of MHC-II molecules, costimulatory molecules and cytokines [80]. Some of these factors have a neurotrophic function, while others such as nitrate oxides exaggerate oxidative stress and provoke apoptotic cascades.

The pathological finding of microgliosis in PD patients' brains post-mortem [72] suggests an inflammatory component of PD, which is supported by the fact that activated microglial cells are mainly localized in the SN in close relationship to dying neurons [71]. Furthermore, an autopsy study that compared three PD patient's midbrains versus three controls' midbrains, who died without psychiatric or neurological disorders, pointed out that tumor necrosis factor α (TNFα) immunoreactivity occurred only in PD patients' microglial cells, whereas the controls did not show any TNFα immunoreactivity. TNFα immunoreactivity is

regarded as a marker of microglial activation. On top of that, cell bodies and processes of dopaminergic neurons of healthy controls (HCs) as well as PD patients stained positive for TNFα receptor suggesting a vulnerability of dopaminergic neurons to TNFα, which is able to provoke cell death [11]. Glial cells in PD patients are not only expressing higher levels of TNFα but also of interleukin-1β (IL1-β), interferon-γ (IFNγ) and cluster of differentiation 23 (CD23). As oxidative stress is thought to play an important role in the etiology of PD, a CD23/cytokine-dependent activation pathway of the inducible nitrate oxide synthase can be regarded as another evidence for the connection between microglial activation and dopaminergic cell death [11]. The role of microglial activation in PD could also be clearly confirmed by [11C](R)-PK11195 PET in living PD patients. [11C](R)-PK11195 is a PET marker of peripheral benzodiazepine sites, which are exclusively expressed by activated microglia. Gerhard et al. showed a widespread pathologic microglial activation in the pons, basal ganglia and temporal and frontal cortical regions in PD. This microglial activation seems to occur early in the disease and then remains stable during disease progression. Therefore, this could be a disease-driving mechanism [48]. The susceptibility of the nigrostriatal dopaminergic system to inflammatory processes is also shown by studies using an intranigral injection of lipopolysaccharide (LPS), an immunostimulant, to produce microgliosis and dopaminergic cell death. Interestingly, dopaminergic neurons seem to be particularly vulnerable to this kind of stress as serotonergic and GABAergic (γ-aminobutyric acid producing) neurons did not suffer lasting damage [18, 58].

1.2.2. Peripheral Inflammation

Whereas neuroinflammation is recognized as a hallmark of PD, the involvement of the peripheral immune system in pathogenesis and disease progression is only partially understood. However, data gained by genome-wide association studies (GWAS) link polymorphisms in the HLA genes to PD [116]. As the cell-surface proteins encoded by HLA genes regulate the immune system in humans, a widespread contribution of the immune system to PD seems likely. In addition, there is further epidemiological and experimental data which demonstrate a clear association of peripheral inflammation with PD. A meta-analysis by Gagne et. al suggests that there is a reduced incidence of PD among regular non-aspirin non-steroidal anti-inflammatory drug (NSAID) users [40]. Besides this epidemiological data, there are different PD mouse models and at least one rat model which show the infiltration of peripheral immune cells into the CNS and their contribution

to neurodegenerative processes [30, 42, 55, 56]. Furthermore, increased cytokine levels can not only be observed in the CSF of PD patients but also in the serum [10]. Elevated levels of serum cytokines such as IL-2, TNFα and IFNγ in PD patients hint at a general inflammatory process [14, 106]. There is evidence that a chronic inflammatory process contributes to PD because increased IL-6 serum levels can already be detected prior to disease onset [21]. The elevation of cytokines in the blood and in the CSF can be explained by an activation of immune cells in the blood and brain of PD patients. This can go hand in hand as there is a dysregulation of peripheral blood mononuclear cells (PBMCs) [52] and their precursors [38]. Although the blood brain barrier (BBB) is usually thought to be intact in PD, there is recent evidence for an altered BBB function in PD [4, 51]. This can consecutively lead to an increased infiltration of myeloid cells into the brain, which has been demonstrated in several PD animal models [30, 42, 55, 56]. Moreover, Couch et al. showed in a mouse model that the intranigral injection of extracellular α-synuclein is able to cause a severe inflammatory response if combined with a systemic LPS injection, which represents another hint that peripheral immune activation contributes the neuroinflammatory processes observed in PD [24].

1.3. Role of Monocytes in PD

1.3.1. Dysfunction of Monocytes

Next to macrophages and dendritic cells, monocytes are another type of mononuclear phagocytes that are responsible for maintaining tissue homeostasis. They originate from the bone marrow, where they share a common myeloid progenitor with neutrophils. They make up about 3-8% of all circulating leukocytes in humans and can migrate to different tissues, where they can differentiate in macrophages, dendritic cells or act as effector cells on their own.

In humans, at least three different subsets can be distinguished upon the expression of surface markers: classical $CD14^{++}$ $CD16^{-}$ monocytes, intermediate $CD14^{++}$ $CD16^{+}$ monocytes and non-classical $CD14^{+}$ $CD16^{++}$ monocytes [121]. In mice, equivalent subsets of monocytes exist: the so-called 'inflammatory' $Ly6C^{high}CCR2^{high}CX3CR1^{low}$ (CCR2 = C-C chemokine receptor type 2; CX3CR1 = C-X-C chemokine receptor type 3) expressing monocytes, that can quickly be recruited to inflamed tissue, can be seen as equivalent to the classical human monocytes, whereas the 'resident' $Ly6C^{low}CCR2^{low}CX3CR1^{high}$, that constitutively are

located in non-inflamed tissue and patrol along blood vessels, can rather be compared to the non-classical monocytes in humans [46].

As peripheral inflammation seems to be prominent in PD, monocytes may convey some of the inflammatory processes. Recently, there has been shown that an increased number of $CD14^{++}$ $CD16^{-}$ classical monocytes [52] as well as monocytic precursors [38] is present in the blood of PD patients. Chemokine C-C motif ligand 2 (CCL2) serum levels are elevated in PD [52] and classical monocytes, which are recruited to the site of inflammation in a CCR2-dependent manner, show an upregulation of CCR2 expression [38]. Furthermore, PD patients' monocytes show a proinflammatory gene expression pattern – even at an early stage of the disease [94]. This is also demonstrated by their hyperactivity in response to LPS stimulation, leading to an excessive IL-6 production. Strikingly, a correlation between PD disease severity and monocytic hyperactivity can be observed. PD patients' monocytes also showed a reduced phagocytic capability compared to those of HCs [43, 52]. PD patients' PBMCs, which are made up of monocytes and lymphocytes, do not only show an increase in IL-6 release upon LPS stimulation but also display increased basal and LPS-induced expression levels of chemokines such as monocyte chemoattractant protein-1 (MCP-1) or other cytokines such as TNFα or IFNγ. This comes along with a nuclear factor 'kappa-light-chain-enhancer' of activated B cells (NF-κB) activation in PD monocytes [88]. However, other studies with different designs suggest that PD patients' PBMCs less efficiently increase the release of IL-6 and other proinflammatory cytokines in response to LPS stimulation [57, 77]. These discrepancies might be traced back to differences in cell selection, duration and amount of LPS stimulation as well as cohort differences. It should be noted that the larger of these two studies indicating a decrease in monocytic IL-6 release in response to LPS was based on experiments with thawed PBMCs of study participants with unclear immune-related confounding factors (e.g. infectious diseases, autoimmune disorders and current intake of immunomodulatory medications), which might directly affect the immune system [77].

Whereas PD myeloid cells seem to produce higher levels of proinflammatory cytokines in response to stimuli, there is evidence that the production of anti-inflammatory cytokines such as IL-10 after LPS stimulation is reduced in PD patients' PBMCs. The study by Nissen et al. in 2019 points out that PD patients' PBMCS show a decreased viability and an abnormal CD163 expression and turnover *in vitro*, which in turn may affects other

components of the innate as well as adaptive immune system [77]. Moreover, PD patients' monocytes, but not lymphocytes, show a reduced glucocerebrosidase (GCase) activity, a lysosomal enzyme, even though these patients do not carry a GCase mutation [2]. As GCase mutations that lead to reduced GCase activity are strongly associated with PD [98], this is another hint that monocytes, that highly express GCase, are functionally altered and involved in PD pathogenesis. Another common cause of familial and sporadic PD is mutation in the leucin-rich repeat kinase 2 (LRRK2) [122], first described by Funayama in 2002 [37]. Interestingly, it has been shown that LRRK2 levels are increased in PD patients' monocytes but not in B cells [9]. While the exact pathomechanism of LRRK2 mutations and upregulation in PD remains unclear, triggering the dysfunction of immune cells seems to be a potential explanation.

1.3.2. Monocytes and α-synuclein

Considering that Lewy bodies and Lewy neurites are the pathological hallmark of PD, their major component – *α-synuclein* – is regarded as a decisive factor in PD etiology [101]. Whereas much is known about the interaction between α-synuclein and neuronal cell function, the exact impact of α-synuclein on immune cells remains to be unraveled.

Harms et al. showed recently in a mouse model that α-synuclein overexpression in the SNpc leads to an entry of proinflammatory monocytes from the periphery and to the activation of resident microglia. This activation of resident microglia seems to be largely dependent on monocytic infiltration as a CCR2 knockout, which was able to attenuate monocytic infiltration, has proven to be an effective strategy to prevent microglial activation. To go even one step further, CCR2 knockout and the consecutive absence of monocyte entry was successful in weakening α-synuclein-mediated neurodegeneration [56]. In another study, it was shown that especially α-synuclein fibrils in contrast to monomeric forms induce a strong microglial activation and an infiltration of peripheral immune cells. It is important to notice that the recruitment of peripheral immune cells to the SNpc occurred prior to neurodegeneration, showing that this event may be rather a cause than a consequence of the ongoing neurodegeneration in PD [55]. Asides from that, it is already known that α-synuclein and especially its pathologic strains induce a strong monocyte and microglial activation of primary cells as well as continuous cell lines (THP1 and BV2-cells) [53, 66].

To sum up, it can be stated that α-synuclein is a potent activator of human myeloid cells and this is especially true for fibrillar α-synuclein compared to α-synuclein monomers and oligomers [53, 55, 60, 77].

1.4. Role of Extracellular Vesicles (EVs) in PD

1.4.1. EVs and α-synuclein

"Extracellular vesicles" is a collective term covering different subtypes of membranous, cell-released structures: exosomes, microvesicles, microparticles and many others. Many publications use the terms "extracellular vesicles" and "exosomes" synonymously but do not conclusively distinguish exosomes as specific EV subtype. The International Society of Extracellular Vesicles (ISEV) recommended in their latest guidelines in 2018 to use "*extracellular vesicles as the generic term for particles naturally released from the cell that are delimited by a lipid bilayer and cannot replicate" (p. 8-9)* [111]. Moreover, the ISEV makes clear that it is in most cases not advisable to demonstrate that a function is specific to exosomes as EVs of endosomal origin as compared with other types of small EVs due to technical limitations, although this has been the practice for several years.
Therefore, the term "extracellular vesicles" is used according to the "Minimal information for studies of extracellular vesicles 2018 (MISEV2018) guidelines" in this thesis [111].

EV size ranges from under 100 nm to over 200 nm, where smaller EVs under 200 nm are likely of plasma membrane or endosomal origin, and they can contain a diverse cargo of desoxyribonucleic acid (DNA), messenger ribonucleic acid (mRNA), micro ribonucleic acid (miRNA), proteins and lipids. Although they can originate from nearly all kind of different cells, they share similar specific surface proteins. All EVs possess membrane-associated and/or endosome-derived proteins for example of the tetraspanin family (CD63, CD81, CD82). All EVs regardless of their tissue origin are characterized by specific cytosolic proteins such as Flotillin-1 and 2 [111]. EVs serve many different physiologic and pathologic functions, which are subject to ongoing research efforts, and range from delivering signals to target cells and mediating cell-to-cell communication to releasing toxic metabolites and thereby sustaining cell homeostasis.
Interestingly, EV-associated α-synuclein is considered as carrier for interneuronal transmission of pathology in PD. It has already been shown *in vitro* that EV-associated

α-synuclein oligomers are more likely to be internalized by adjacent cells and are more toxic than free α-synuclein oligomers [28]. In addition, EVs isolated from the CSF of PD patients were able to induce α-synuclein oligomerization in a reporter cell line [105]. The same study also found a positive correlation between EV-associated α-synuclein levels and their capability to cause α-synuclein oligomerization, using EVs from patients with PD and Dementia with Lewy bodies (DLB). Same correlation was not observed for control EVs [105]. By using the neural cell adhesion molecule 1 (L1CAM) antibody, which specifically identifies EVs from the CNS, Shi et al. showed that CNS-derived EVs can cross the BBB and be found in the blood [95]. Moreover, the α-synuclein level from CNS-derived EVs is higher in PD patients than in HCs. The α-synuclein content of CNS-derived plasma EVs also correlated with disease severity in PD and could therefore be exploited as biomarker [97]. In accordance with that, Cerri et al. have shown that EVs of PD patients carry greater levels of α-synuclein than those of healthy subjects [18]. EV-associated α-synuclein seems to be enriched in PD patients, as an increased ratio between EV-associated α-synuclein and total α-synuclein could be revealed in their plasma. Moreover, this ratio correlates negatively with GCase activity [19]. This could be due to the increased release of aggregated α-synuclein in the extracellular space because of GCase deficiency [3]. These results indicate that GCase activity plays a key role in the regulation of α-synuclein release through EVs. Regarding the strong association between GCase depletion and PD, EV-associated α-synuclein can be considered as potential mediator of this connection. Additionally, Picca et. al even demonstrated that PD patients show greater levels of circulating small EVs relative to HCs [85]. Although the amount of EVs was normalized for total serum protein concentration in this study, this data should be treated with caution as the absolute amount of EVs is difficult to determine due to technical limitations – especially because of the possible contamination with serum proteins. Furthermore, Han et al. recently showed that PD patients' EVs do not only carry increased levels of α-synuclein but also of the proinflammatory cytokines, IL-1β and TNFα [54], and of the phosphorylated synuclein form pS129, of which CSF levels positively correlate with "Unified Parkinson's Disease Rating Scale (UPDRS)" scores [104]. Interestingly, they could demonstrate that the isolated EVs of both HCs as well as PD patients exhibit NeuN, a neuronal marker, Iba-1, a microglial marker, and glial fibrillary acidic protein (GFAP), an astrocyte marker, indicating a possible origin of the CNS. The same study was able to show that intrastriatal injection of PD patients' EVs in

mice causes a Lewy body-like molecular phenotype and neurodegeneration in contrast to EVs from controls [54]. In 2020, Niu et al. published the first longitudinal study that links an increase in α-synuclein levels in plasma neuronal EVs to disease progression in PD, suggesting the pathophysiologic involvement of EV-associated α-synuclein in neurodegenerative processes. On top of that, this study demonstrated that α-synuclein in plasma neuronal EVs may serve as non-invasive biomarker to improve early diagnosis of PD [78]. Another study in 2020 carried out by Vacchi et al. showed by using flow cytometry of plasma-derived EVs combined with machine learning algorithms that distinct pools of cell surface markers of immune cells on EVs can be used as biomarkers for PD. Interestingly, the same study revealed that synucleinopathies like PD and multiple system atrophy (MSA) were characterized by a greater pool of overexpressed immune markers compared to atypical parkinsonism (AP) with tauopathy, indicating a major or at least different contribution of inflammatory pathways to PD and MSA in comparison to AP pathogenesis [112].

1.4.2. Activation of Immune Cells by EVs

As monocytes are dysregulated in PD [38, 52] and EVs, that are known to have an impact on monocytic activation, carry an altered cargo in PD patients [19, 97], modulation of monocytic function by serum EVs may contribute to the inflammatory phenotype of PD monocytes.

It has been shown that monocytes of patients suffering from ALS, a neurodegenerative disorder leading to the progressive loss of upper and lower motor neurons, exhibit an impaired cytokine secretion when stimulated with EVs as well of ALS patients as of HCs [123]. Interestingly, EVs secreted from red blood cells are not only able to bind to monocytes, but also induce the production of proinflammatory cytokines such as TNFα. This seems to be a specific interaction of smaller EVs such as exosomes as larger microvesicles (larger than 200 nm) were not able to induce such cytokine production [27]. Another example for the modulation of immune cells by EVs could be studied in Gastric Cancer (GC), where GC-derived EVs induced the differentiation of monocytes into programmed cell death protein 1 (PD1)-positive M2-like tumor-associated macrophages, which promote tumor progression [113]. A similar reaction of human monocytes could be observed upon stimulation with Glioblastoma stem cell-derived EVs [39].

Breast cancer-derived EVs were also able to activate macrophages, which share the same lineage with monocytes, resulting in an increased production of IL-6, CCL2 and TNFα. It has been shown that this activation is mainly conveyed by a TLR2-dependent activation of the NF-κB pathway [23]. Further evidence of the impact EVs can have on immune cells comes from a study in which EVs of patients suffering from Hashimoto Thyreoiditis (HT) were efficiently taken up by $CD14^+$ monocytes. After stimulation with HT-EVs, PBMCs showed an increased production of IFNγ und IL17A – both proinflammatory cytokines. This activation was dependent on TLR2 and TLR3 activation [26]. Furthermore, mesenteric lymph EVs isolated after trauma/hemorrhagic led to proinflammatory cytokine production in alveolar macrophages and acute lung injury in an animal model, which seemed to rely on a TLR4-dependent signaling pathway [67].

Recently, Grozdanov et al. showed that EVs derived from human embryonic kidney 293 cells (HEK293) overexpressing wild-type human α-synuclein lead to a higher activation of HC monocytes compared to EVs derived from control-transfected HEK293-cells indicating a decisive role of α-synuclein levels of EVs on monocytic activation. Interestingly, EV-associated α-synuclein induces a stronger activation of monocytes than free α-synuclein. On top of that, PD patients' monocytes responded with higher IL-6 release than HC monocytes upon stimulation with EVs from HEK293 which overexpressed, wild-type, luciferase-tagged α-synuclein. An increased activation of healthy monocytes by PD plasma EVs compared to activation by HC EVs was also found. The observed inflammatory response of HC monocytes to EVs isolated from PD patients' blood seems to show a strong interindividual variation. While monocytes from some healthy donors respond similarly to PD patients' and HCs' plasma EVs, monocytes from other healthy donors respond to PD patients' plasma EVs with elevated cytokine release [53].

Therefore, we hypothesize that there are distinct genetic and epigenetic polymorphisms that convey susceptibility to aggravated monocytic response to EV-associated α-synuclein. Recently, a similar phenomenon has been shown for T cells of PD patients which can recognize α-synuclein peptides bound to specific HLA-alleles of the MHC. These specific HLA-alleles are more likely to be found among PD patients and are thought to play a role in PD etiology [107].

1.5. Research Objectives

Given that monocytes seem to play an important role in PD, identification of pathways that lead to increased monocyte activation in PD is an important aspect with potential implications for diagnostics and therapy. The factors which trigger monocyte hyperactivity in PD are also relevant to the CNS as microglial cells and monocytes share many activation pathways and both seem to be equally involved in pathogenesis and progression of PD. EVs, which act directly in cell-cell communication and carry an altered cargo in PD, could be potential modulating factors of monocytic dysregulation in PD patients. We propose that two main factors act synergistically to result in the increased monocyte activation in PD: *increased activation by pathologic EV-associated α-synuclein and predisposition of PD monocytes* (illustrated in Figure [Fig.] 1).

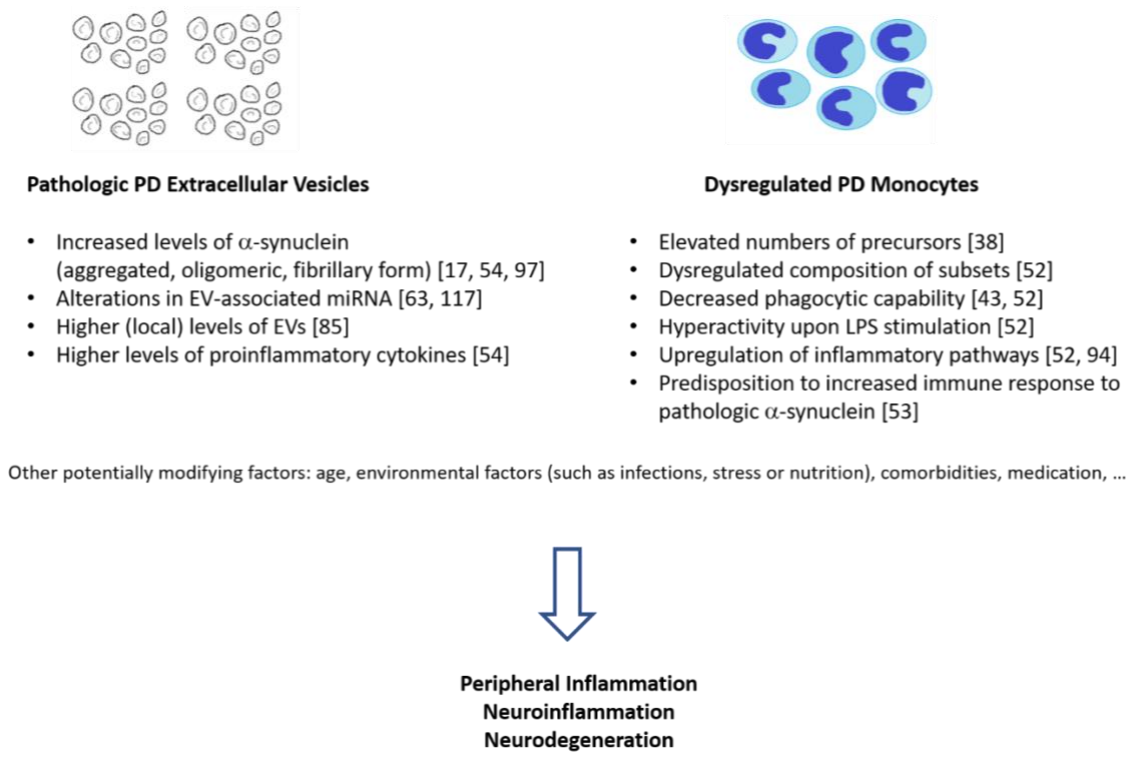

Figure 1: Proposed pathophysiologic mechanisms in PD. Pathologically altered EVs and dysregulation of PD monocytes both may act synergistically in inflammatory processes. PD patients' serum EVs display several differences compared to those of healthy controls. Interestingly, PD monocytes, which are overactive in response to PD EVs, show many abnormalities and contribute to neurodegeneration in PD. Moreover, EVs play an important role in monocyte dysregulation as there seem to be factors that make PD monocytes more vulnerable for a proinflammatory, detrimental response to PD EVs. EVs = extracellular vesicles, LPS = lipopolysaccharide, miRNA = micro ribonucleic acid, PD = Parkinson's Disease.

Consequently, this study is intended to investigate whether or not there are some healthy donors' monocytes that show increased IL-6 release upon stimulation with PD EVs similarly to PD patients' monocytes and therefore be classified as 'responders' in contrast to 'non-responders' whose monocytes react in the same way to EV stimulation regardless of the origin of EVs (PD or HC). In addition, young as well as older healthy monocyte donors should be compared to identify if there is any age-dependency of monocytic response to pathologic PD EVs. Furthermore, monocytes were stimulated with individual as well as pooled serum EVs of HCs and PD patients to examine interindividual differences in the capability of EVs to provoke a proinflammatory monocytic IL-6 release.

Simultaneously, interindividual differences in monocytic responsiveness to stimulation were assessed by using LPS as a positive control.

Identifying such 'responders' will allow further identification of the underlying genetic and epigenetic polymorphisms, which could then also be investigated in PD patients.

Studying EV-induced proinflammatory reactions of monocytes could eventually lead to a better understanding of the potential role of pathologic serum EVs in PD-associated immune dysregulation and could consequently serve as pharmacological target.

2. Materials and Methods

2.1. Antibodies

2.1.1. Antibodies for Enzyme-Linked Immunosorbent Assay (ELISA)

Antibodies used for ELISA

Antibodies	Clone	Application/dilution	Order number	Manufacturer
Biotin anti-human IL-6 Antibody	MQ2-39C3	ELISA/1:1000	#501101	BioLegend
Purified anti-human IL-6 antibody	MQ2-13A5	ELISA/1:1000	#501201	BioLegend

2.1.2. Antibodies for Western Blot (WB)

Antibodies used for WB

Antibodies	Application/dilution	Order number	Manufacturer
Anti-mouse IgG (H+L), HRP conjugate	WB/1:5000	W402B	Promega
Anti-rabbit IgG (H+L), HRP conjugate	WB/1:5000	W401B	Promega
Biotin anti-human IgG F_c Antibody	WB/1:5000	#409307	BioLegend
LEAF Purified anti-human CD63 Antibody	WB/1:5000	#353013	BioLegend
Purified Mouse Anti-Flotillin-1	WB/1:5000	#610820	BD Transduction Laboratories

2.1.3. Antibody for Monocyte Isolation

Antibody used for monocyte isolation

Antibody	Application/dilution	Order number	Manufacturer
CD14 Microbeads, human	Magnetic-activated cell sorting (MACS)-based positive selection of monocytes/1:10	130-050-201	Miltenyi Biotec

2.2. Chemicals and Reagents

Chemicals and reagents used during experiments

Substance	Order number	Manufacturer
Albumin Fraction V	8076.2	Carl Roth GmbH
Albumin Fraction V, very low endotoxin	CP77.2	Carl Roth GmbH
Albumin Standard 2 mg/mL	23209	Thermo Fisher Scientific
Ammonium persulphate (APS)	9592.3	Carl Roth GmbH
Ampuwa water	13LIP301	Fresenius
Cell Culture RPMI-1640-Medium	R7388	Sigma-Aldrich
Dithiothreitol (DTT)	A2948	AppliChem GmbH
Dulbecco's phosphate buffered saline (DPBS)	14190094 L 182-50	Gibco/Life Technologies (now: Thermo Fisher Scientific) Biochrom (now: Merck)
Ethanol	32205	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA)	ED-100G	Sigma-Aldrich
Fetal calf serum (FCS)	S0615	Merck Millipore
Glycine	LC-4522.2	NeoFroxx
Histopaque-1077	10771	Sigma-Aldrich
Isopropanol	2084.2.330	VWR International
LDS sample buffer (4x)	NP0007	Invitrogen
Lymphopure (density gradient medium)	426202	BioLegend
N, N, N', N' - Tetramethylethylenediamine (TEMED)	2367.3	Carl Roth GmbH

Ponceau S	5938.2	Carl Roth GmbH
Rotiphorese Gel 30	3029.2	Carl Roth GmbH
Roti-Block	A151.2	Carl Roth GmbH
Sodium dodecyl sulfate (SDS)	A0675.0500	AppliChem GmbH
Sulfuric Acid (H_2SO_4)	X.873.1	Carl Roth GmbH
3, 3', 5, 5' - Tetramethylbenzidine (TMB) Substrate	421101	BioLegend
Tris	T1503	Sigma-Aldrich
Trypan blue stain 0.4%	15250061	Gibco/Life Technologies (now: Thermo Fisher Scientific)
Tween 20	6F015840	AppliChem GmbH

2.3. Consumables

Used consumables

Product	**Order number**	**Manufacturer**
Blotting filter paper 2.5 mm	LC2008	Invitrogen
Cassettes 1.0 mm	NC2010	Novex
Filter tip 10 µL	07-612-8300	Nerbeplus
100 µL	07-642-8300	Nerbeplus
300 µL	07-672-8300	Nerbeplus
1250 µL	07-695-8300	Nerbeplus
Empty Gel Cassette Combs, 1.0 mm, 12 well	NC3012	Invitrogen
LS Columns 25	130-042-401	Miltenyi Biotec
Micro tube 1.5 mL SafeSeal	72.706.400	Sarstedt
Micro tube 2 mL SafeSeal	72.695.400	Sarstedt
Microtest plate 96 Well, round base	82.1582.001	Sarstedt
Nitrocellulose Western blotting membrane, 0.2 µm pore size	1060001	GE Healthcare

Pipette tips PP 20 µL	07-362-2015	Nerbeplus
1250 µL	07-369-2005	Nerbeplus
Pipette tips 200 µL	70.760.002	Sarstedt
Safety-Multifly Needle 23G 200 mm	85.1640.235	Sarstedt
Safety-Multifly Needle 21G 200 mm	85.1638.200	Sarstedt
Serological pipette 25 mL	86.1685.001	Sarstedt
Serological pipette 10 mL	86.1254.001	Sarstedt
Serological pipette 5 mL	86.1253.001	Sarstedt
S-Monovette 7.5 mL Z-Gel (Serum)	01.1602	Sarstedt
S-Monovette 9 mL Z-Gel (Serum)	02.1388.001	Sarstedt
S-Monovette 7.5 mL K3 EDTA	01.1605.100	Sarstedt
S-Monovette 9 mL K3 EDTA	02.1063.100	Sarstedt
TC plate 24 well	83.3922.005	Sarstedt
TC plate 48 well	83.3923.005	Sarstedt
Tubes 50 mL with conical base	62.547.004	Sarstedt
Tubes 50 mL with conical base with smear edge	62.559	Sarstedt

2.4. Technical Equipment

Used technical equipment

Technical equipment	**Product name**	**Manufacturer**
Autoclave	VX-95	Systec
Bacteria shaking incubator	GFL 3033	GFL – Gesellschaft für Labortechnik
Camera on Microscope	AxioCamMRm	Carl Zeiss Microscopy GmbH
Cell counting chamber	Neubauer-chamber	Marienfeld
Cell incubator	Hera cell	Heraeus
Centrifuge	Hereaus Biofuge stratos	Thermo Fisher Scientific
Centrifuge	Heraeus Fresco 21	Thermo Fisher Scientific
Centrifuge	MiniStar silverline	VWR

Centrifuge	Megafuge 1.0R	Heraeus Instruments
Clean bench	HERA Safe	Heraeus Instruments
Freezer -80°C	HERA Freeze HFUT Series	Thermo Fisher Scientific
Magnetic Separator	Quadro MACS Separator	Miltenyi Biotec
Magnetic Stirrer	RCT Classic	IKA
Microscope	Axio Observer A1	Carl Zeiss Microscopy GmbH
Microscope	DM IL LED	Leica
Pipetting	Pipetus	Hirschmann
Spectrophotometer	NanoDrop 2000	Thermo Fisher Scientific
Spectrophotometer	SPECTROstar Nano	BMG Labtech
Power supply	Peqpower 250V	Peqlab
Shaker	KS-15	Edmund Bühler GmbH
Thermomixer	Mixing Block MB-201	Bioer
Vortexer	RS-VA-10	Phoenix Instruments
Western Blot equipment	Image Quant LAS 4000	GE Healthcare
Western Blot equipment	Xcell II Blot module	Invitrogen
Western Blot equipment	Xcell Sure Lock Electrophoresis Cell	Novex
Western Blot equipment	Xcell Sure Lock Lid	Invitrogen

2.5. Buffers and Solutions

2.5.1. Solutions and Buffers for Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and WB

Solutions and Buffers used for SDS-PAGE and WB

Solution A	Rotiphorese 30
Solution B	1.5 M Tris, pH 8.8
Solution D	0.5 M Tris, pH 6.8
10% weights per volume APS	APS in Milli-Q water (H_20)
Running Buffer	- 25 mM Tris - 192 mM Glycine - 0.1% SDS
Blotting Buffer	- 25 mM Tris - 192 mM Glycine - 20% Ethanol
1x Tris-buffered saline with tween (TBS-T)	- 90% Milli-Q H_2O - 10% TBS - 0.05% Tween
Ponceau S	- 0.2% Ponceau S - 3% TCA

2.5.2. Buffer for Magnetic-Activated Cell Separation (MACS)

Buffer used for MACS

MACS-Buffer	- 1x PBS (Phosphate-buffered saline) 9.55 g/L, without Ca^{2+} and Mg^{2+} - 0.5% Bovine Serum Albumin (BSA; Albumin Fraction V, very low endotoxin) - 2 millimolar (mM) EDTA

2.5.3. Solutions for ELISA

Solutions used for ELISA

Blocking Solution	-	1% BSA (Albumin Fraction V)
	-	PBS without Ca^{2+} and Mg^{2+}
1x PBS-T	-	90% Milli-Q H_2O
	-	10% PBS
	-	0.05% Tween
TMB Substrate	-	50% TMB A
	-	50% TMB B

2.5.4. Enzymes and Proteins

Used enzymes and proteins

Enzyme/Protein	Application	Order number	Manufacturer
Avidin-Horseradish Peroxidase (Av-HRP)	ELISA	405103	BioLegend
PageRuler Pre-stained Protein Ladder	WB	26616	ThermoFisher Scientific

2.6. Software

Used software

Software	Manufacturer
GraphPad Prism 6	GraphPad Software
Microsoft Office, 2019 & MS Paint	Microsoft
Nanoparticle Tracking Analysis (NTA) Software	NanoSight Ltd.
RefWorks Citation Manager	Ex Libris, ProQuest
RStudio IDE	RStudio, PBC

2.7. Ethical Approval and Study Cohort

All human experiments were performed in accordance with the Declaration of Helsinki approved by the Ethics Committee of Ulm University *(Antrag Nr. 20/10 – Untersuchungen zur verbesserten Früh- und Differentialdiagnose entzündlicher und neurodegenerativer Erkrankungen des zentralen Nervensystems – einschließlich der Amyotrophen Lateralsklerose).* All study participants gave informed written consent to participate in the study. PD patients were recruited at the University and Rehabilitation Clinics of Ulm (RKU) and HCs were recruited at RKU as well as at Ulm University. A description of the patients' characteristics included in the experiments can be found next to the results of the corresponding experiments.

In general, HCs and PD patients with confounding factors with a direct influence on the immune system, e.g. infections or autoimmune diseases, were excluded from all experiments. Other comorbidities like arterial hypertension, hypothyroidism or depression were not excluded. PD patients were diagnosed using "UK PD Society Brain Bank Clinical Diagnostic Criteria" [47] at the RKU, which is a specialized center for PD.

Peripheral venous blood in non-fasting state was collected with Monovette™ blood drawing system using a standard procedure. The blood was processed within 3 hours (h) post collection. For isolation of PBMCs, EDTA was used as anticoagulant. For gaining serum for EV isolation, serum collection tubes were used.

2.8. Statistical Analysis

Statistical analyses were carried out using GraphPad Prism 6 for Windows, GraphPad Software, San Diego, California, USA, "www.graphpad.com" and RStudio IDE, RStudio, PBC. Gaussian distribution was tested with D'Agostino-Pearson omnibus normality test. Student's t-test with Welch's correction was used for unpaired data from Gaussian distribution (with unequal standard deviation [SD]). Mann-Whitney U-test (unpaired data) or Wilcoxon matched pairs signed-rank test (paired data) were used for data from non-Gaussian distribution. One-way analysis of variance (ANOVA) with Dunn's multiple comparison correction was used for multiple comparisons. All tests for significance were two tailed. All values in the figures are presented as mean ± standard error of the mean (SEM). Rank correlation was measured with Spearman's rank correlation coefficient.

2.9. Monocyte Isolation and Culture

PBMCs were isolated from blood drawn into EDTA-tubes. PBMCs were obtained by density centrifugation (500 g [standard gravity], 30 minutes [min], room temperature [RT], without brake) on Histopaque or on Lymphopure. Cells were taken from the interphase between Histopaque/Lymphopure and plasma layer. Consecutively, the PBMCs were resuspended in 50 mL DPBS at RT and centrifuged at 300 g for 10 min at RT. Afterwards, cells were centrifuged at 300 g for 10 min at 4°C and resuspended in 1 mL MACS-Buffer. After these two washes to get rid of contaminating platelets, magnetic beads coupled to monoclonal antibodies against CD14 (100 µL of CD14-antibodies per 1 mL cells resuspended in MACS-Buffer) were used to label $CD14^+$-cells. After 15 min of incubation, cells were centrifuged again at 300 g for 10 min at 4°C. Anti-CD14-beads-labeled cells were then separated in a column placed in a strong magnetic field. After removing the column, the cells that were held back were eluted with MACS-buffer. Cells were counted in a Neubauer cell counting chamber, while dead cells were stained with Trypan blue. Subsequently, cells were resuspended in cell culture medium (48-well plate: 200 µL per well, 2 x 10^5 cells per well, concentration of 1 x 10^6 cells/mL [Fig. 2]; RPMI1640, 10% FCS, 1% Penicillin/Streptomycin). Monocytes were allowed to rest for 24 h after isolation to avoid unspecific activation by the isolation process.

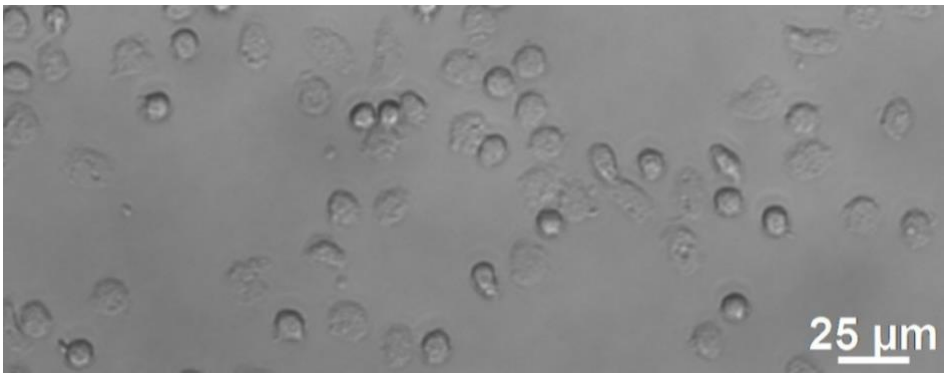

Figure 2: Primary human monocytes (400x magnification) isolated from peripheral blood. $CD14^+$ monocytes were isolated by MACS. Monocytes were seeded 10^6 cells/mL in a 48-well plate. MACS = Magnetic-activated cell sorting.

As preliminary studies performed by our group have already ruled out effects of DPBS or serum protein contamination of the EV fraction on monocytic behavior [53, 123], sterile

and unconditioned culture medium was used as negative control (Con). LPS stimulation served as positive control. Stimulations with EVs (resuspended in DPBS, diluted in cell culture medium) as well as controls were performed in triplicates. After 24 h of stimulation, cell culture supernatant was collected.

2.10. EV Isolation

EVs were isolated from serum by sequential centrifugation steps. Whereas isolation of EVs by ultracentrifugation (UC) is time-consuming and not suitable for small volumes, it is recommended for EV-based treatment studies [83]. Serum was drawn into serum-tubes and after drawing centrifuged at 2000 g for 10 min at 4°C and then stored at -80°C to prevent degradation.

To increase EV yield, serum viscosity was reduced by diluting serum 1:5 with DPBS. Serum was centrifuged at 2000 g for 30 min at 4°C. Hereafter, the supernatant was centrifuged at 12 000 g for 45 min at 4°C. The first two steps were performed to get rid of cells and cell debris. In the next step, the supernatant was passed through a 0.45 µm and then through a 0.22 µm filter. This was followed by UC at 186 000 g for 90 min at 4°C. Finally, EV pellets were resuspended in DPBS and stored at -80°C for further applications.

2.11. Nanoparticle Tracking Analysis (NTA)

The NTA technique (NanoSight, part of Malvern Instruments Ltd, Malvern, UK) enables its user to measure the size distribution and concentration of particles in solution. To this end, vesicles are visualized by their light scattering using a camera. The NTA software tracks down the individual movement of each particle and calculates the size of each vesicle based on Brownian motion [35].

Serum, EVs and supernatant of EV isolation were diluted 1:10 in DPBS and 300 µL of the samples were then applied to the laser chamber. Samples were recorded in triplicates and 60 second videos were taken under automated settings. The concentration and size distribution of the particles were automatically analyzed by the NTA software.

2.12. ELISA

Levels of IL-6 in cell culture supernatant were measured by ELISA. A purified IL-6 antibody (Clone MQ2-13A5, 0.5 µg/mL in coating buffer) was used to coat the ELISA plates overnight.

After washing with PBS-T four times (4x), unspecific binding sites were blocked by 1% BSA in DPBS for 1 h at RT. Consecutively, the plate was washed again 4x with PBS-T. Then IL-6 standard and samples were added – diluted in blocking solution – and could bind to the immobilized capture antibody during an incubation time of 2-4 h at RT or overnight at 4°C. After washing with PBS-T 4x, the biotinylated IL-6 antibody (Clone MQ2-39C3) diluted in blocking solution (0.5 µg/mL) was added for 1 h at RT as detection antibody. After the next washing step, Av-HRP diluted (1000x) in blocking solution was added. Avidin binds with very high affinity to the biotin-labeled IL-6 antibodies. After 30 min of incubation time, the plate was finally washed 5x with PBS-T and then substrate solution (TMB substrate set) was added. Oxidation of TMB by HRP results in a blue water-soluble complex of TMB-diimine, which turns yellow upon acidification with sulfuric acid. The signal that can now be measured spectroscopically is directly proportional to the concentration of IL-6 in cell culture supernatant.

2.13. SDS-PAGE

To separate proteins depending on their polypeptide chain length, SDS-PAGE was performed before WB analysis. Starting with the resolving gel, gels were cast subsequently. After applying the mixture to a gel cast system, isopropanol was added to prevent formation of bubbles. Consecutively, the stacking gel was prepared and added after discarding the isopropanol. A comb was inserted to get gel pockets for sample application. The mixture of the gels can be seen below:

Composition of the gels used for WB

	Stacking Gel (5%)	**Separating Gel (12%)**
H_2O	2.925 mL	1.05 mL
Rotiphorese 30 = solution A	750 µL	3.75 mL
0.5 M Tris pH 6,8 = solution D	1.25 mL	/
1.5 M Tris pH 8,8 = solution B	/	5 mL
20% SDS	25 µL	50 µL
10% APS	50 µL	100 µL
TEMED	5 µL	10 µL

100 µL of each sample was prepared as follows:

- 25 µL 4x LDS sample buffer (25%)
- 10 µL DTT (10%)
- 65 µL sample with lowest protein concentration

All other samples were diluted to the same protein concentration and volume was adjusted with Milli-Q H_20. After vortexing, samples were incubated at 96°C for 5 min and either stored at -20°C or immediately used. 5 µL of the protein ladder and 15 µL of the samples were loaded in the gel pockets, the gel was run for 2 h at 125 Volt (V) and 0.02 – 0.04 Ampere (A).

2.14. Transfer and Protein Detection by WB

The separated proteins were transferred on a nitrocellulose membrane by electro transfer. The blot sandwich consisting out of two sponges, Whatman blotting paper, gel membrane and again Whatman blotting paper and two sponges was implemented into the Xcell II™ Blot module. The blot module was inserted into the Xcell Sure Lock™ Electrophoresis-Cell and filled up with blotting buffer. To transfer the proteins from the gel to the membrane, an electric field was applied and the blot was run for 2-3 h at 25 V and 0.23 – 0.25 A. After the transfer, the membrane was washed 3x with TBS-T to remove rests of the buffer. To check if protein transfer was successful, membranes were stained with Ponceau S for 2 min, which binds reversibly to positively charged amino acids. The membrane was washed afterwards several times with Milli-Q water. The membrane was consecutively blocked with 1x Roti-Bloc for 1 h at RT. Primary antibodies were diluted 1:5000 in Roti-Bloc and the membrane was then incubated with 1.5 mL of diluted primary antibody at 4°C overnight using a hydrophobic Teflon plate. Before incubating with the secondary antibody, the membrane was washed 3x for 15 min at RT with TBS-T. The membrane was then incubated for 1-2 h at RT with 1.5 mL diluted secondary antibody – the dilution ranged from 1:1000 to 1:5000 depending on the antibody used. Thereafter, the membrane was washed 4-5x with TBS-T for at least 30 min to remove unbound antibody. As the secondary antibody is coupled with HRP, the chemiluminescence signal arising after adding an appropriate substrate (Luminata™ Forte Western HRP Substrate) can be detected.

2.15. Bicinchoninic Acid Assay (BCA)

The BCA assay was used for determining the total protein concentration in a sample by using the Pierce™ BCA Protein Assay Kit. Firstly, peptide bonds, especially the amino acid residues cysteine, tyrosine and tryptophan, reduce Cu^{2+} to Cu^{+}. Secondly, Cu^{+} ions couple to the colorimetrically detectable reagent bicinchoninic acid. As the reduction of Cu^{2+} to Cu^{+} is proportional to the amount of protein in the solution, the amount of the purple-colored complex which forms after the reaction of bicinchoninic acid with Cu^{+} ions is also proportional to the protein concentration of the sample. This purple reaction product strongly absorbs light at a wavelength of 562 nm [100]. A BSA protein standard was prepared in a range from either 7.8125 µg/mL or 125 µg/mL to 2000 µg/mL. For the BCA working solution, BCA reagents A and B were mixed at the ratio of 50:1. Each sample was measured in duplicates in a 96-well-plate. Either 10 µL or 20 µL of standard or sample were mixed with 100 µL or 200 µL (ratio 1:10) of working solution, respectively. After 20 min of incubation at 37°C, the absorbance at a wavelength of 562 nm was detected at a BMG SPECTROstar Nano spectrometer. The protein concentration was then calculated based on a standard curve.

2.16. Hemolysis Control of Serum

As hemolysis can affect the composition of a serum sample by releasing low- and high-molecular cell contents, the degree of hemolysis of the serum samples was controlled. This was especially important because it is known that erythrocytes contain particularly high levels of α-synuclein. For this purpose, hemolysis was measured by using the ratio between the absorbance peaks at 414 nm and 375 nm to exclude any effects of hemolysis on EV purity. An elevated absorbance at 414 nm is regarded as sign of increased free oxyhemoglobin. All samples with a hemolysis ratio below 2 can be regarded as hemolysis-free [65, 118]. Absorbance of 100 µL serum at these wavelengths was determined by using BMG SPECTROstar Nano and measurements were performed in duplicates.

3. Results

3.1. Characterization of EVs Isolated from Human Serum

To investigate human monocyte response to EVs from human serum, EVs were isolated by ultrafiltration and ultracentrifugation. To check the quality and purity of the EV yield, NTA and WB for EV markers were performed.

3.1.1. NTA Characterization of Serum EVs

Serum EVs of a HC as well as a PD patient were prepared and the content of the EV fractions as well as HCs' supernatant gained after UC and the pure HC serum were analyzed using NTA (Table 1, Fig. 3). Analysis of serum showed the highest concentration of particles (Table 1) and a high polydispersity (presence of many particle of different sizes), as expected (Fig. 3 A). The size of particles with highest concentration in whole human serum was 34 nm (Fig. 3 A). By contrast, purified EV samples from a HC and a PD patient contained particles of uniform size (single peak) around 116 nm and 87 nm (HC EVs respectively PD EVs; Fig. 3 C, D). The supernatant from UC also showed several peaks and a highest peak around 26 nm (Fig. 3 B).

Table 1: Results of NTA characterization of isolated EVs, serum and supernatant

(EVs = extracellular vesicles, HC = healthy control, NTA = nanoparticle tracking analysis, PD = Parkinson's Disease)

	Mode	Total concentration
HC EVs	116 ±6.1 nm	82.23 ±5.90 particles/frame 13.84 ±1.15 x 10^8 particles/mL
PD EVs	87 ±4.7 nm	136.91 ±2.20 particles/frame 23.91 ±0.45 x 10^8 particles/mL
HC Serum	34 ±4.6 nm	148.74 ±3.12 particles/frame 24.38 ±0.51 x 10^8 particles/mL
HC Supernatant	26 ±5.9 nm	87.14 ±1.24 particles/frame 14.22 ±0.31 x10^8 particles/mL

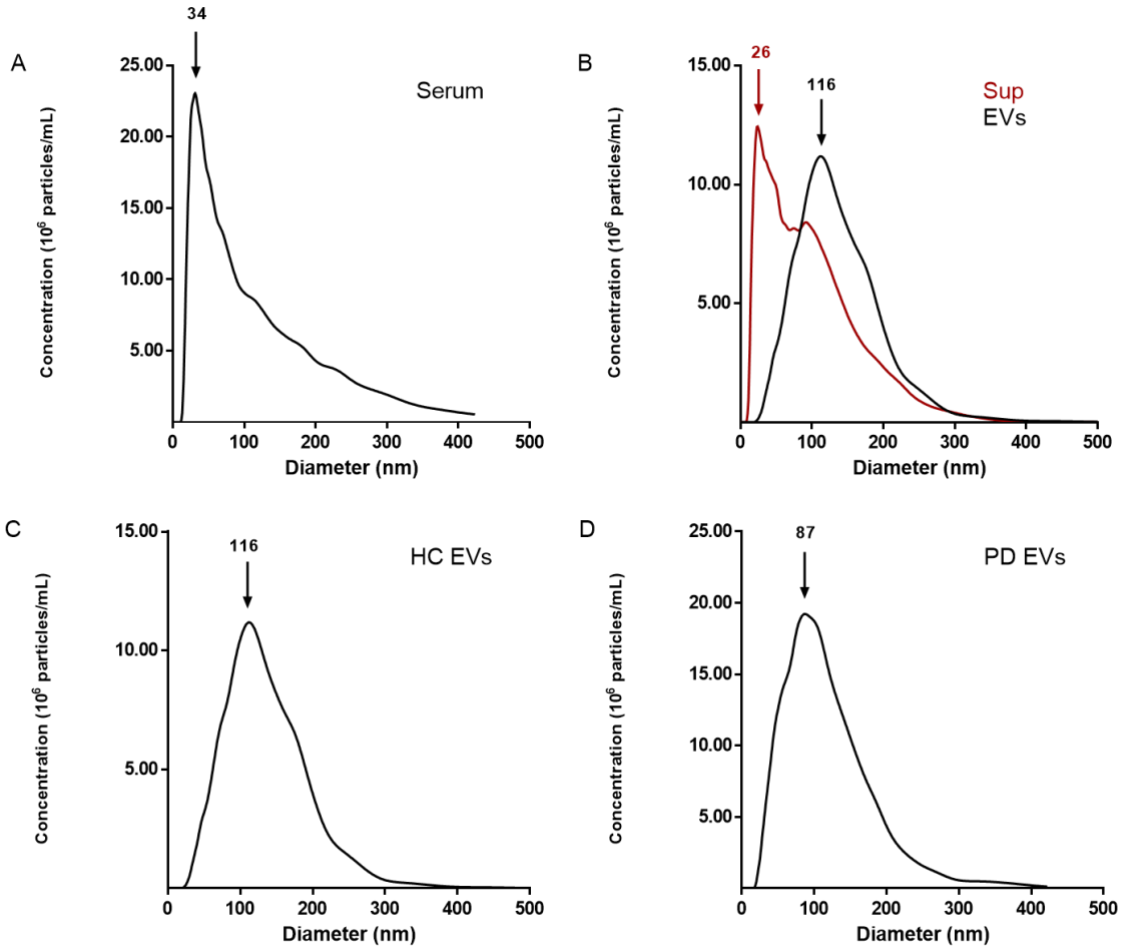

Figure 3: NTA analysis of EV purification from human serum. Representative graphs from NTA analysis. ***A, B:*** A single particle type at the expected size is enriched in the EV fraction, whereas multiple particle types of mostly lower size are present in serum and supernatant of EV isolation. ***C, D:*** EV purification yielded similar results with HC and PD serum. EV(s) = extracellular vesicle(s), HC = healthy control, NTA = nanoparticle tracking analysis, PD = Parkinson's Disease, Sup = supernatant.

3.1.2. WB Analysis of Serum EVs

To verify the purity of the isolated EVs, equal amounts of protein of purified EVs, supernatant from the EV isolation and human serum were used for WB analysis of EV markers. Using an antibody against Flotillin-1, which is an EV marker with an estimated molecular weight of 49 kilodaltons (kDa), a band was observed at around 50 kDa in the EV preparation (Fig. 4 A). On the contrary, no bands could be observed neither in the supernatant nor in the serum sample. CD63, a member of the tetraspanin family and another EV marker with a molecular weight of approximately 25 kDa, could be detected in the EV fraction as well as in serum and to a lesser extent in supernatant (Fig. 4 B).

A WB using an antibody against the fragment crystallizable (F_C) region of Immunoglobulin G (IgG) was used to assess contamination of EVs with serum proteins, as IgG is abundant in serum and accounts for approximately 15% of all serum proteins. IgG levels were drastically reduced in the EV fraction compared to whole serum and the supernatant of the EV isolation (Fig. 4 C).

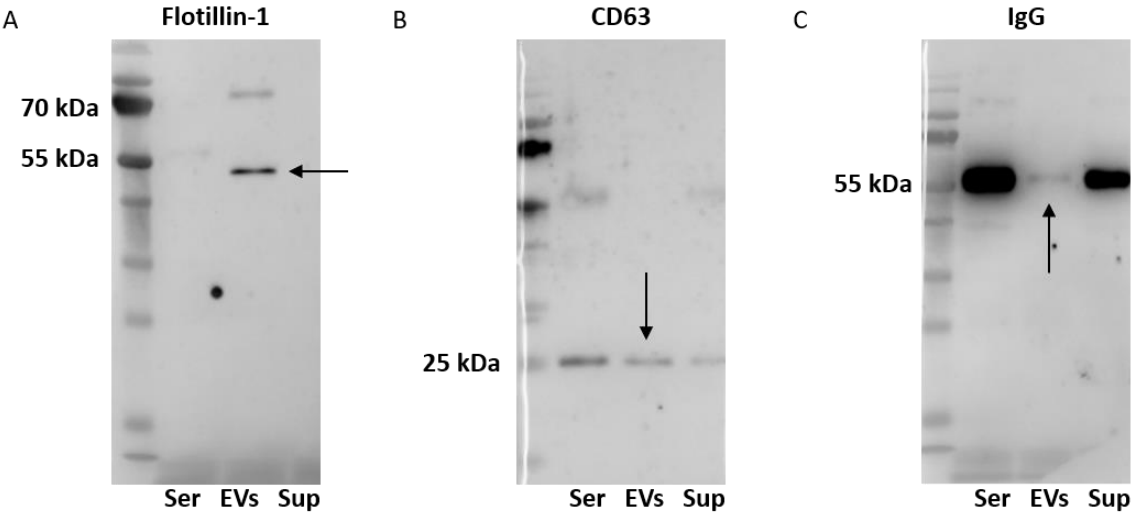

Figure 4: Characterization of EVs by WB. Representative images from WB analysis of EVs. ***A,*** WB using a purified mouse anti-Flotillin-1 antibody. Flotillin-1, an EV marker, is clearly enriched in the EV fraction (marked by an arrow). ***B,*** CD63, another EV marker, is enriched in the EV fraction (marked by an arrow) in comparison to the supernatant collected after UC. ***C,*** IgG, which is abundant in human serum, is strongly reduced in the EV fraction (marked by an arrow). CD63 = cluster of differentiation 63, EV(s) = extracellular vesicle(s), IgG = Immunoglobulin G, kDa = kilodaltons, Ser = serum, Sup = supernatant, UC = ultracentrifugation, WB = Western Blot.

Taken together, NTA and WB results indicate that isolating EVs by sequential centrifugation and UC according to our laboratory protocol is an effective way to gain purified human serum EVs with a low amount of contamination with serum proteins.

3.2. Differential Monocytic Response to Healthy Control (HC) and PD EVs

To investigate the hypothesis that monocytes from some healthy donors ('responders') respond to PD serum EVs with increased cytokine (*IL-6*) release, we used several experimental paradigms. The main challenge was posed by the limited availability of serum from each HC or PD patient. To overcome this obstacle, we pooled the serum of all HCs or all PD patients and isolated EVs. The amount of EVs isolated this way was enough to

compare the monocyte response by twelve aged donors to the *same* HC and PD EVs (*see 3.2.1*). Moreover, we again isolated HC and PD EVs and assessed the relative contribution of each serum donors' EVs to the EV pool. After that, we stimulated six young healthy donors' monocytes with these HC and PD EVs (*see 3.2.2.*). To evaluate the impact of individual EVs, we analyzed the response of two young healthy donors' monocytes to EVs from individual HCs and PD patients (see *3.2.3.*).

3.2.1. Stimulation of *Aged* Healthy Donors' Monocytes with *Pooled* HC and PD EVs

Monocytes from different donors respond to inflammatory stimulation with different levels of cytokine release. To be able to distinguish the specific monocyte response to serum EVs from a general high 'reactivity' of some donors' monocytes, we employed LPS as a positive control. To this end, we first assessed the dose-dependent response to LPS with monocytes from two healthy controls (gender/age in years: male/22, male/25). All LPS concentrations (1, 10 & 100 ng/mL) induced a strong, statistically significant increase in IL-6 release (Fig. 5). We selected the 1 ng/mL LPS concentration for the further experiments, as it was in the linear range of the dose response.

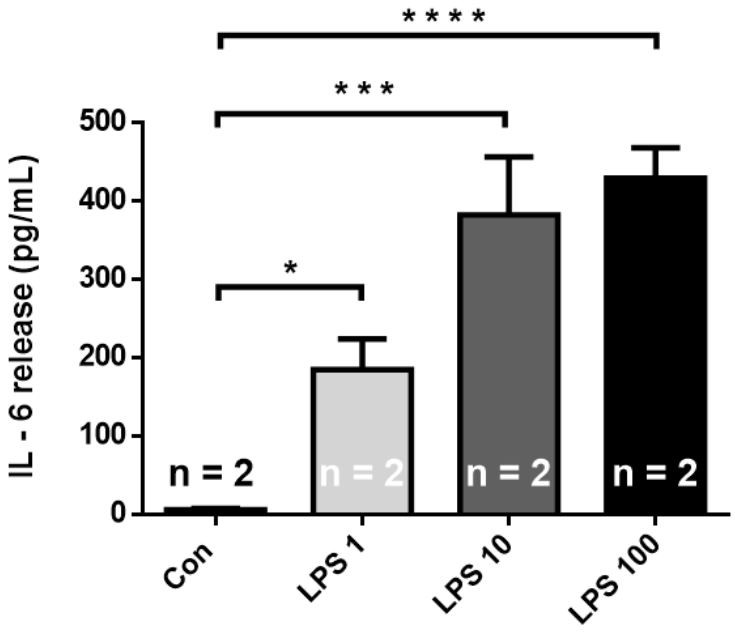

Figure 5: LPS-induced IL-6 release of healthy monocytes. Healthy donors' monocytes react to LPS stimulation with a dose-dependent increase of IL-6 secretion compared to basal IL-6 release. *p < 0.05, ***p < 0.001, ****p < 0.0001, ANOVA with Dunn's multiple comparisons correction, same internal control (Con). ANOVA = analysis of variance, Bars: mean ± standard error of the mean. Con = control (unconditioned media) IL-6 = interleukin-6, LPS 1, 10, 100 = lipopolysaccharide 1, 10, 100 ng/mL.

Next, we isolated monocytes from twelve aged healthy donors. Healthy controls were defined as not suffering from any acute or chronic neurological disease or any infectious disease. Participants > 60 years were defined as 'aged'. Monocyte donors (n = 12) had a mean age of 71.5 (±5.8 SD) years with a male/female-ratio of 10/2 (83.3%/16.7%). A detailed description of the study cohort can be found in Table 2.

Table 2: **Characteristics of blood donors for monocyte isolation – stimulation of aged donors' monocytes**

HC = healthy control, M/F-ratio = Male-to-Female-ratio. Mean average ± standard deviation; "Currently under medication": the participant received at least one drug only available on prescription at the time the blood was drawn.

Characteristics	**HC** **n = 12**
M/F-ratio	10/2 (83.3%/16.7%)
Average age in years	71.5 (±5.8)
Infectious disease	none at all
Included comorbidities	• Ankylosing spondylitis (Morbus Bechterew) • Arterial hypertension • Atrial fibrillation • Chronic obstructive pulmonary disease • Diabetes mellitus type 2 • Hypothyreosis • Hyperthyreosis • Status post prostate cancer
Currently under medication	10 of of 12 (83.3%)

On average, basal IL-6 secretion was low (mean: 50.50 ±6.98 pg/mL, ±SEM), while the mean monocyte response to LPS (1 ng/mL) was strong, statistically significant (mean: 234.60 ±17.77 pg/mL, ±SEM, Fig. 6 A). However, the individual responses varied significantly (Fig. 6 B). In detail, basal rate of IL-6 secretion in unconditioned media ranged from 18.96 pg/mL (±2.22 SEM, *HC8*) to 177 pg/mL (±65.47 SEM, *HC10*), whereas IL-6 release upon LPS stimulation ranged from 56.50 (±3.44 SEM, *HC8*) to 615 pg/mL (±26.86 SEM, *HC11*).

Monocytes which showed high basal IL-6 release without stimulation (*HC10*) did not respond with further IL-6 release upon stimulation, a phenomenon termed 'refractory state'.

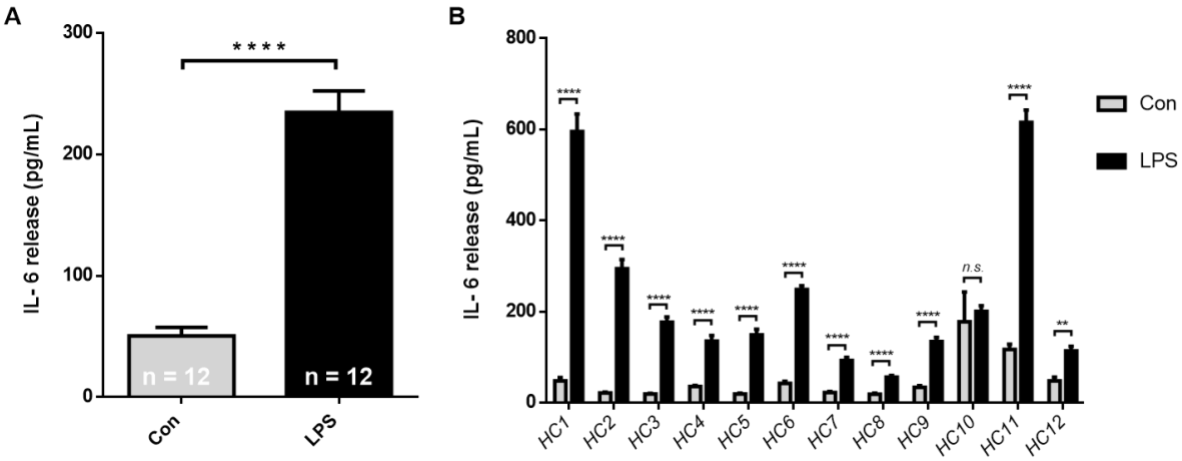

Figure 6: IL-6 response to LPS by monocytes from aged healthy donors. ***A, B:*** Aged healthy donors' monocytes react to LPS stimulation with a significant release of proinflammatory IL-6. However, the response to LPS strongly differs interindividually. **p < 0.01, ****p < 0.0001, n.s. = not significant, Wilcoxon matched-pairs signed rank test (A), Mann-Whitney U-test (B). Bars: mean ± standard error of the mean. Con = control (unconditioned media), *HC1-HC12* = healthy controls 1 to 12, IL-6 = interleukin-6, LPS = lipopolysaccharide 1 ng/mL.

Spontaneous activity and responsiveness of monocytes to LPS were not biased by donor gender (Fig. 7) and did not correlate significantly with age (Con ~ Age: ρ = 0.251, p = 0.431; LPS ~ Age: ρ = 0.0318, p = 0.922; Spearman correlation).

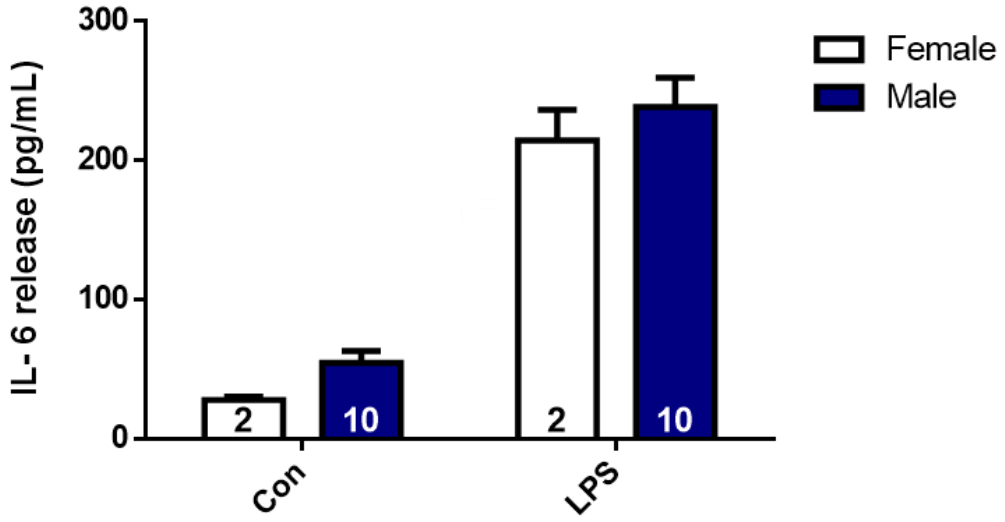

Figure 7: Basal and LPS-stimulated IL-6 release is not biased by gender of monocyte donors. Comparing the average basal and LPS-triggered IL-6 secretion of monocytes of aged female (n = 2) and male (n = 10) donors, no significant difference could be observed. Con: p = 0.22, LPS: p = 0.38, Mann-Whitney U-test. Bars: mean ± standard error of the mean. Con = control (unconditioned media), IL-6 = interleukin-6, LPS = lipopolysaccharide 1 ng/mL.

To analyze the IL-6 response of monocytes to EV stimulation, EVs were isolated from pooled HC and pooled PD serum (n = 12 per group; 5 mL from each proband, 60 mL total pooled serum for each group). The experimental design is shown in Fig. 8 and a detailed description of the cohort can be found in Table 3. Mean age of HC and PD EV donors (n = 12/12) was comparable (HC 62.6 ±14.1 years; PD 68.6 ±11.4 years, ±SD). Male/female-ratio was 3/9 (25%/75%) among HC and 8/4 (66.7%/33.3%) among PD patients. Monocyte donors (n = 12) had a mean age of 71.5 (±5.8 SD) with a male/female-ratio of 10/2 (83.3%/16.7%). HC serum donors were not the same as HC monocyte donors. Serum EVs were purified with two consecutive washing steps to get rid of serum protein contamination and resuspended with sterile PBS. Protein concentration was measured with a BCA assay after the second UC step to quantify EV yield. Protein concentration of HC EVs was 236.2 µg/mL and protein concentration of PD EVs was under the limit of detection (73.6 µg/mL). EVs were then diluted 1:5 in cell culture media and used for stimulation of the aged healthy donors' monocytes.

Monocytes rested for 24 h before stimulation and cell culture supernatant was collected 24 h post stimulation. IL-6 ELISA was performed with the collected cell culture supernatant to quantify monocytic IL-6 release.

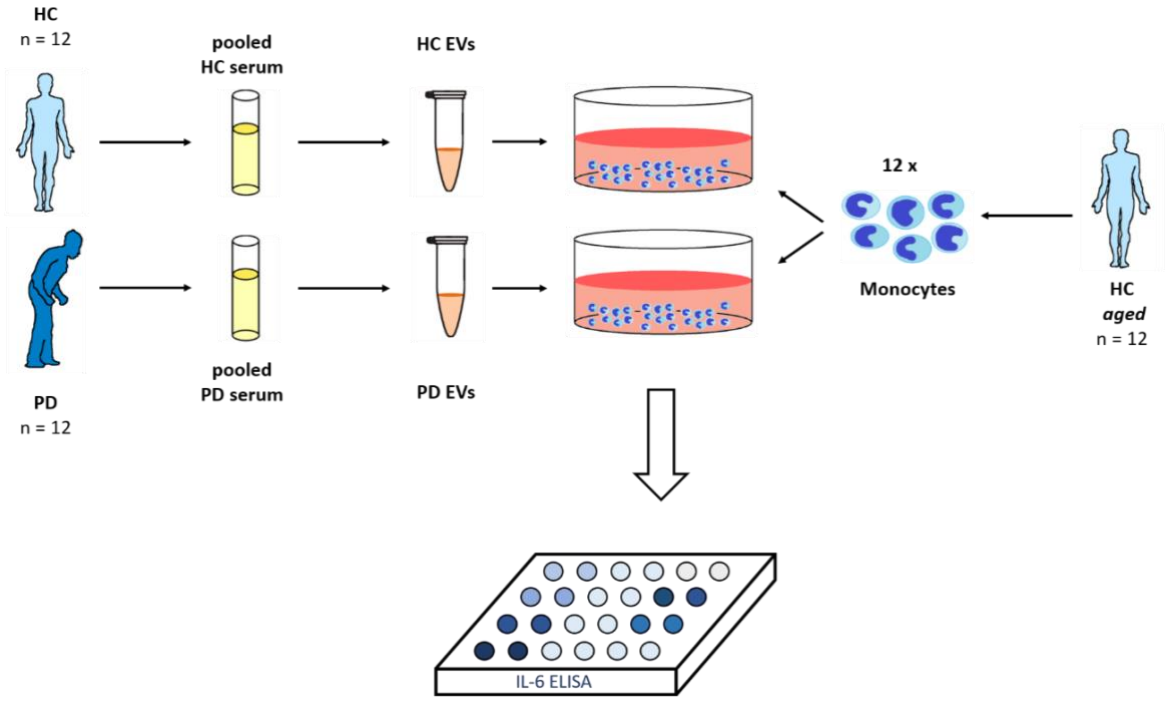

Figure 8: Experimental design – stimulation of aged healthy donors' monocytes with pooled HC and PD EVs. For EV isolation, 5 mL serum from each of twelve HCs and twelve PD patients were pooled together and EVs were isolated according to protocol. Following this, monocytes were isolated from EDTA-blood of another group of twelve HCs. After resting for 24 h, these monocytes were stimulated with pooled HC EVs and pooled PD EVs. 24 h post-stimulation cell culture supernatants were collected, and IL-6 levels were quantified via ELISA. EDTA = ethylenediaminetetraacetic acid, ELISA = Enzyme-linked immunosorbent assay, EV(s) = extracellular vesicle(s), h = hours, HC(s) = healthy control(s), IL-6 = interleukin-6, PD = Parkinson's Disease (patients).

Table 3: Characteristics of serum donors for EV isolation – stimulation of aged healthy donors' monocytes

EV = extracellular vesicle, HC = healthy control, M/F-ratio = Male-to-Female-ratio, PD = Parkinson's Disease (patients). Mean average ± standard deviation.

Characteristics	**HC n = 12**	**PD n = 12**
M/F-ratio	3/9 (25%/75%)	8/4 (66.7%/33.3%)
Average age in years	62.6 (±14.1)	68.6 (±11.4)
Infectious disease	none at all	none at all
Included comorbidities	• Arterial hypertension • Atrial fibrillation • Coagulopathy • Hyperparathyreoidism • Athyreosis, Hypothyreosis	• Aortic valvular stenosis • Arterial hypertension • Asthma bronchiale • Atrial fibrillation • Depression • Hyperuricemia • Impulsive control disorder • Migraine • Morbus Meniére • Peripheral neuropathy • Restless legs syndrom • Ulcerative colitis

On average, stimulation with serum EVs induced a statistically significant activation of aged healthy donors' monocytes (Fig. 9 A). However, on an individual level, the response to stimulation with serum EVs varied significantly (Fig. 9 B), similarly to the response to LPS. The response by monocytes from three donors (*HC9*, *HC10* and *HC12*) was below the limit of detection of the IL-6 ELISA. Both HC and PD EVs significantly activated human monocytes (Con vs PD EVs: * $p < 0.05$, Con vs HC EVs: ** $p < 0.01$, ANOVA with Dunn's multiple comparisons correction). The average response to PD EVs did not differ from the response to HC EVs (Fig. 10 A). Monocytes from most donors released similar or even lower levels of IL-6 in response to PD EVs (Fig. 10 B). Monocytes from one healthy donor, *HC2*, showed an increased response to PD EVs (potential 'responder'), but the difference was not statistically significant.

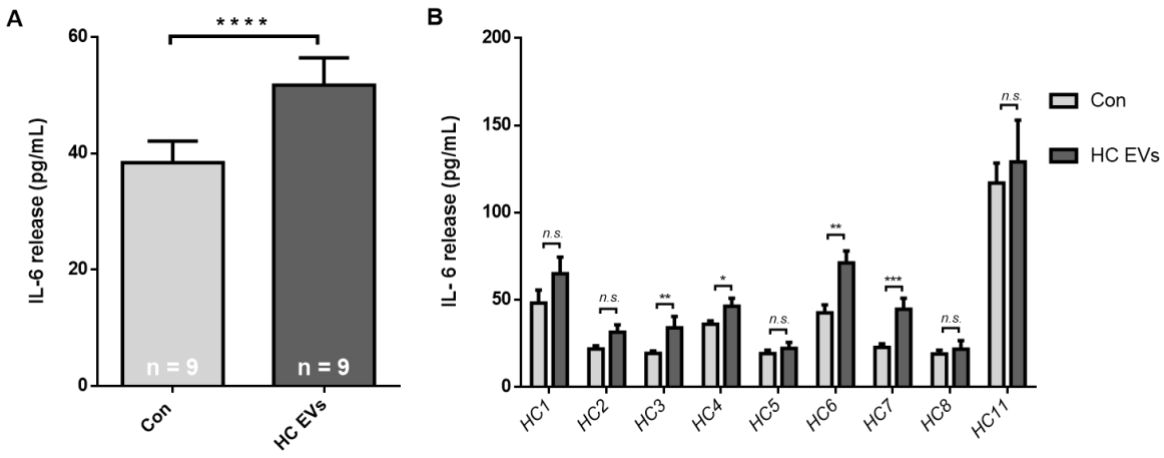

Figure 9: IL-6 response to HCs' serum EVs by monocytes from aged healthy donors. *A,* Average IL-6 response to EVs from HCs' serum. ***B,*** Individual IL-6 response by all nine aged healthy control monocyte donors. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, n.s. = not significant, Wilcoxon matched-pairs signed rank test (A), Mann-Whitney U-test (B). Bars: mean ± standard error of the mean. Con = control (unconditioned media), EVs = extracellular vesicles, *HC1-8, 11* = healthy controls 1 to 8 and 11, HC(s) = healthy control(s), IL-6 = interleukin-6.

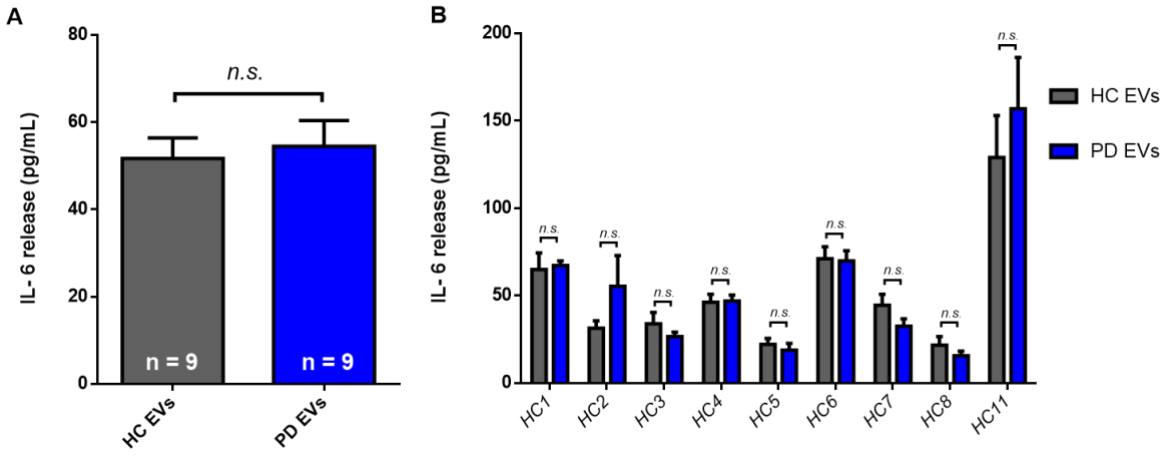

Figure 10: Comparison of IL-6 response to EVs from HCs and from PD patients. *A,* Average IL-6 response by monocytes from aged healthy controls to HC and PD serum EVs. ***B,*** Individual IL-6 response by all nine aged healthy control monocyte donors. n.s. = not significant, Wilcoxon matched-pairs signed rank test (A), Mann-Whitney U-test (B). Bars: mean ± standard error of the mean. EVs = extracellular vesicles, *HC1-8, 11* = healthy controls 1 to 8 and 11, HC(s) = healthy control(s), IL-6 = interleukin-6, PD = Parkinson's Disease.

3.2.2. Stimulation of *Young* Healthy Donors' Monocytes with *Pooled* HC and PD EVs

We hypothesized that two factors may hinder the discovery of 'responders' to PD EVs among *aged* healthy donors: First, aged HCs and PD patients may reflect the 'non-responders' and 'responders' (respectively) that we observed in younger monocyte donors (unaffected by PD) in previous works. Thus, selecting the aged HCs and excluding the PD patients as monocyte donors will effectively exclude 'responders'. Second, the monocyte response to stimulation from aged donors might be generally lower than that from younger donors, hindering the sensitive detection of differences between HC and PD EVs. Indeed, we found that young donors' (age: 23 ±1 years, mean ±SD) monocytes reacted more heavily in response to LPS than aged donors' (age: 71.5 ±5.8 years, mean ±SD) monocytes (Fig. 11: LPS, young monocytes: 636 ±234 pg/mL; aged monocytes: 234 ±53 pg/mL, mean ±SEM; p = 0.047, Mann-Whitney U-test). In contrast to that, aged monocytes displayed a higher basal rate of IL-6 secretion (Fig. 11: Con, young monocytes: 11.00 ±2.42 pg/mL; aged monocytes: 50.50 ±13.95, mean ±SEM; p = 0.0003, Mann-Whitney U-test).

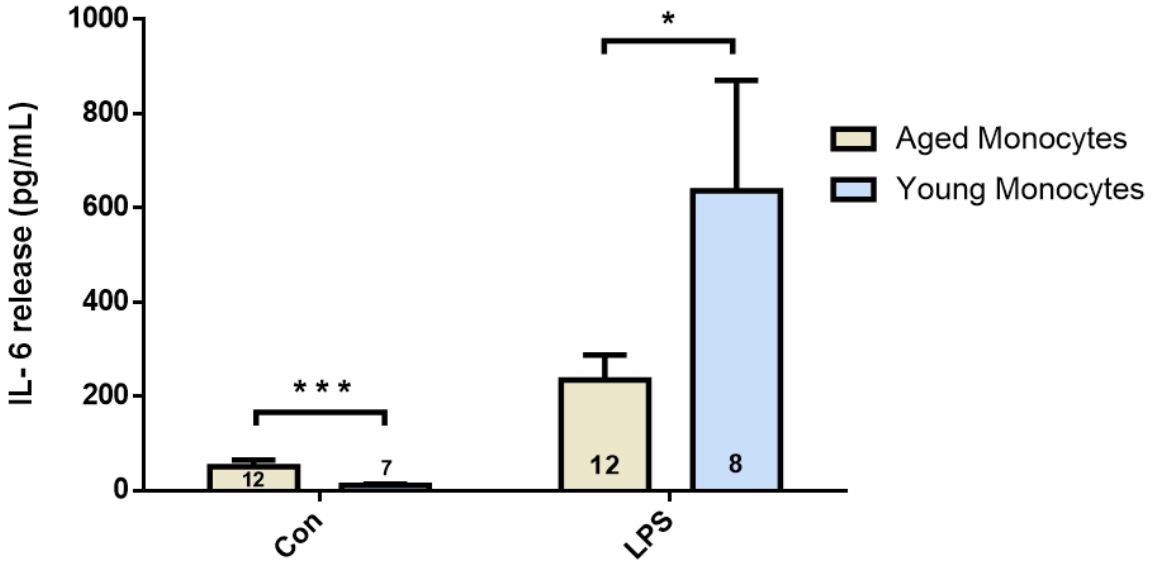

Figure 11: Monocyte donors' age influences basal and LPS-triggered IL-6 release. Comparing the average basal and LPS-triggered IL-6 secretion of aged (n = 12) and young (Con: n = 7, LPS: n = 8) donors' monocytes, different response modes could be observed. Con: p = 0.0003, LPS: p = 0.0473, *p < 0.05, ***p < 0.001, Mann-Whitney U-test. Bars: mean ± standard error of the mean. Con = control (unconditioned media), IL-6 = interleukin-6, LPS = lipopolysaccharide 1 ng/mL.

Thus, we next investigated the response to serum EVs by monocytes from *young* monocyte donors. To this end, we isolated peripheral blood monocytes from six young healthy donors with no infectious or chronic disease (male/female: 4/2; age: 23 ±0.8 years, mean ±SD). Similarly to monocytes from aged healthy donors, LPS (1 ng/mL) induced a strong, statistically significant IL-6 release by monocytes from young healthy donors (Fig. 12 A) with strong interindividual variation (Fig. 12 B, [minimum: 323 ±65 pg/mL *HC6*; maximum: 2221 ±426 pg/mL *HC3*]).

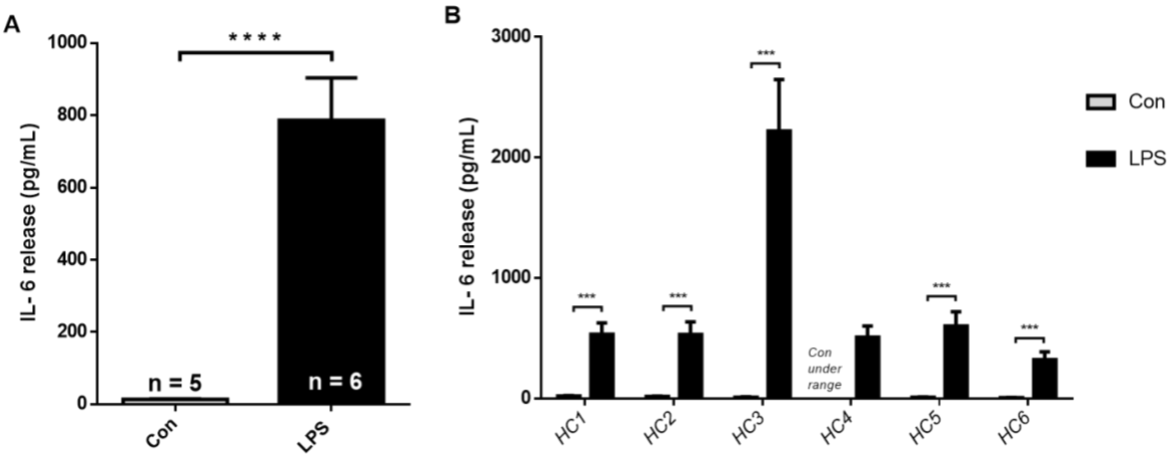

Figure 12: IL-6 response to LPS by monocytes from young healthy donors. Average ***(A)*** and individual ***(B)*** IL-6 response to LPS stimulation by monocytes from six young healthy donors. ***p < 0.001, ****p < 0.0001, Wilcoxon matched-pairs signed rank test (A), Student's t-test with Welch's correction. Bars: mean ± standard error of the mean. Con = control (unconditioned media), *HC1-6* = healthy controls 1 to 6, IL-6 = interleukin-6, LPS = lipopolysaccharide 1 ng/mL.

For purification of serum EVs, serum from each five HCs and five PD patients was collected. Mean age of HC and PD EV donors (n = 5/5) was comparable (HC 72.0 ±3.2 years; PD 71.2 ±7.4 years, mean ±SD). Male/female-ratio was 1/4 (20%/80%) among HCs and 5/0 (100%/0%) among PD patients. Further cohort details can be found in Tables 4 and 5.

Table 4: Characteristics of HC EV donors for stimulation of young monocytes

HC1-5 = healthy control EV donors 1 to 5, aHT = Arterial hypertension, EV = extracellular vesicle, f = female, HC = healthy control, m = male; Hemolysis ratio: ratio of absorbance of serum at 414 and 375 nm wavelength; under 2 means no hemolysis.

Characteristics	Gender	Age	Chronic disease	Protein concentration (µg/mL)	Hemolysis ratio
HC1	m	73	aHT	1567.93	0.93
HC2	f	77	no	1432.82	0.98
HC3	f	69	no	1313.08	1.49
HC4	f	68	aHT	1423.28	1.37
HC5	f	73	no	1463.11	1.06

Table 5: Characteristics of PD EV donors for stimulation of young monocytes

PD1-5 = Parkinson's Disease EV donors 1 to 5, m = male, EV = extracellular vesicle, PD = Parkinson's Disease, PCa = Prostate cancer, RBD = Rapid eye movement sleep behavior disorder, s.a. CABG = status after coronary artery bypass graft; Hemolysis ratio: ratio of absorbance of serum at 414 and 375 nm wavelength; under 2 means no hemolysis.

Characteristics	Gender	Age	Chronic disease	Protein concentration (µg/mL)	Hemolysis ratio
PD1	m	63	PD, PCa	3434.88	1.17
PD2	m	66	PD	2214.32	1.38
PD3	m	67	PD, RBD	1086.98	1.64
PD4	m	78	PD, BPH	1292.47	1.68
PD5	m	82	PD, s.a. CABG	1155.38	1.03

For this experiment, the EVs were isolated with a modified protocol (Fig. 13), pooling the EVs from individual serum samples after the first washing step, and not the serum samples at the beginning. This was possible due to the higher availability of serum for this experiment (10 mL) and allowed us to assess the relative contribution of each serum sample to the pool. To this end, 10 mL of each individual serum sample were diluted with 40 mL PBS and EV isolation was performed according to protocol. After the first washing step, EV yield was assessed by measuring protein amount of the EV pellet by BCA assay. The protein concentration of the EV samples revealed a high contamination with serum proteins (confirming the need for a second washing step) but a relatively comparable contribution of each HC and PD serum sample.

To exclude any contamination of EVs with erythrocyte-derived proteins due to hemolysis, hemolysis was measured before further processing the serum samples by using the ratio between absorbance peaks at 414 nm and 375 nm. Samples with hemolysis ratio under 2 were considered as hemolysis-free. All used serum samples were hemolysis-free. After pooling 5 x 2 mL of each HC and PD EVs, these EV fractions were each diluted in 15 mL PBS and EVs were again isolated by UC (second washing step). After this, pellets were resuspended with 1 mL PBS and the EV suspension as well as supernatant were collected.

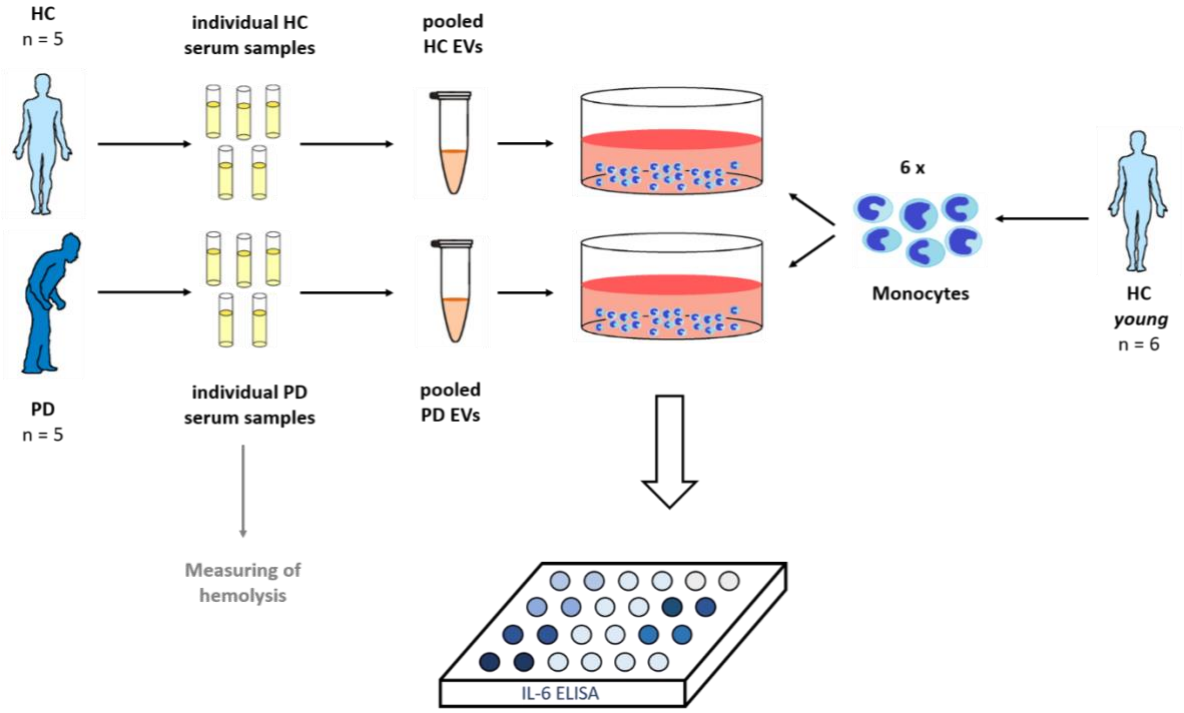

Figure 13: **Experimental design – stimulation of young healthy donors' monocytes with pooled HC and PD EVs.** For EV isolation, 10 mL serum from each of five HCs and five PD patients were used and EVs were isolated with a modified protocol, pooling the EVs from individual serum samples after the first washing step to assess the relative contribution of each serum sample to the pool. Additionally, hemolysis of serum samples was measured to exclude any contamination of EVs with erythrocyte-derived proteins. Following EV isolation, monocytes were isolated from EDTA-blood of six young healthy donors. After resting for 24 h, these monocytes were stimulated with pooled HC and pooled PD EVs. 24 h post-stimulation cell culture supernatants were collected, and IL-6 levels were quantified via ELISA. EDTA = ethylenediaminetetraacetic acid, ELISA = Enzyme-linked immunosorbent assay, EV(s) = extracellular vesicle(s), h = hours, HC(s) = healthy control(s), IL-6 = interleukin-6, PD = Parkinson's Disease (patients).

A BCA assay was performed to assess EV yield. As the results of the BCA assay indicated that there were still some serum proteins left, it was not possible to determine the exact, pure EV concentrations reliably. EVs were then diluted 1:5 (as *in 3.2.1.*) in cell culture media.

On average, HC EVs induced a statistically significant increase in IL-6 secretion of young healthy donors' monocytes (Fig. 14 A), with strong interindividual differences (Fig. 14 B). Overall, no difference between HC and PD EV-induced IL-6 response of young monocytes could be observed (Fig. 15 A). Interestingly, one healthy donors' monocytes (*HC6*) showed a statistically significant increase in IL-6 release in response to PD EVs compared to HC EVs (Fig. 15 B). Moreover, another donor's monocytes (*HC1*) displayed a trend for increased activation caused by PD EVs (p = 0.16, Mann-Whitney U-test).

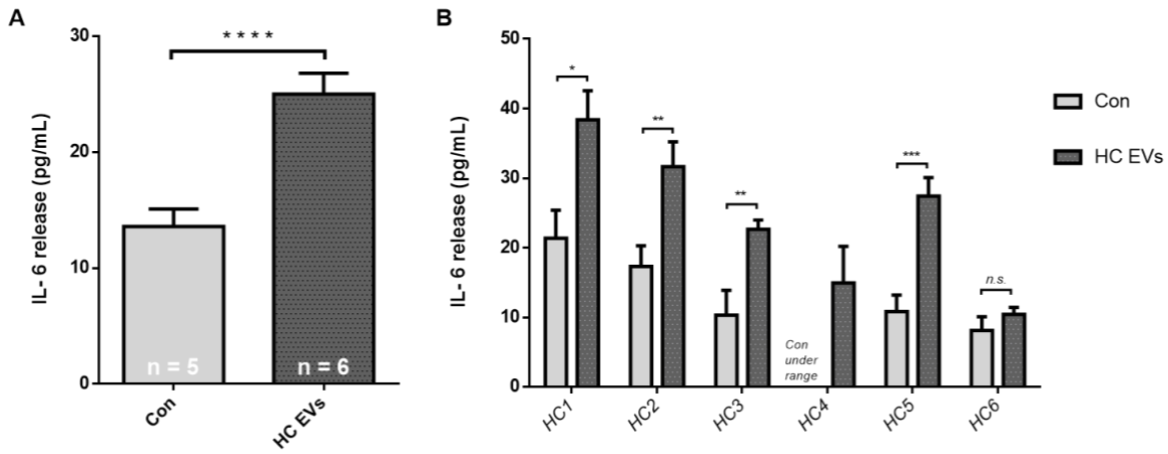

Figure 14: IL-6 response to HCs' serum EVs by monocytes from young healthy donors. ***A,*** Average IL-6 response to EVs from HCs' serum. ***B,*** Individual IL-6 responses by all six young healthy monocyte donors to HC EVs. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, n.s. = not significant, Wilcoxon matched-pairs signed rank test (A), Mann-Whitney U-Test (B). Bars: mean ± standard error of the mean. Con = control (unconditioned media), EVs = extracellular vesicles, *HC1-6* = healthy controls 1 to 6, HC(s) = healthy control(s), IL-6 = interleukin-6.

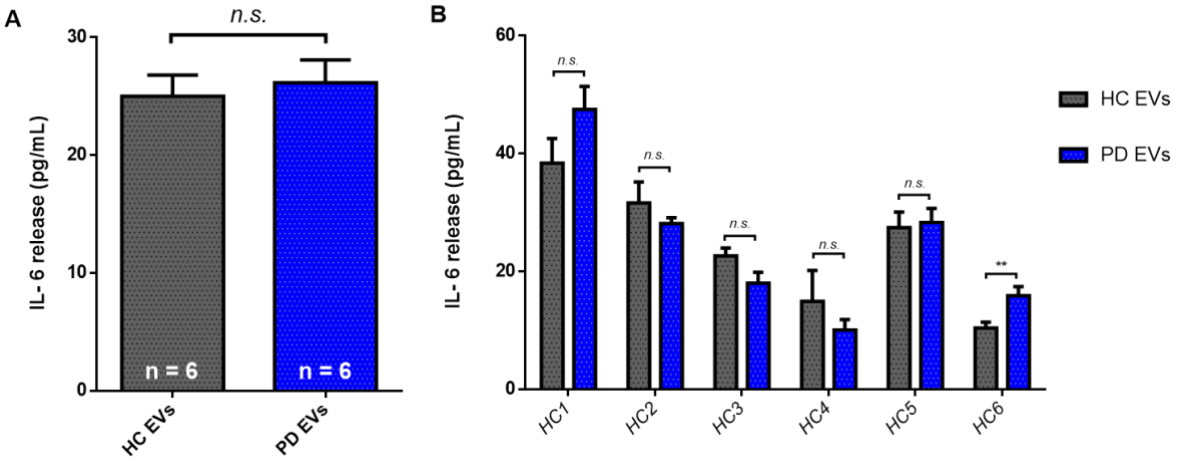

Figure 15: IL-6 response to HCs' and PD patients' serum EVs by monocytes from young healthy donors. ***A,*** Average IL-6 response to EVs from HCs' and PD patients' serum. ***B,*** Individual IL-6 responses by all six young healthy monocyte donors to HC and PD EVs. **p < 0.01, n.s. = not significant, Wilcoxon matched-pairs signed rank test (A), Mann-Whitney U-Test (B). Bars: mean ± standard error of the mean. EVs = extracellular vesicles, *HC1-6* = healthy controls 1 to 6, HC(s) = healthy control(s), IL-6 = interleukin-6, PD = Parkinson's Disease.

3.2.3. Stimulation of *Young* Healthy Donors' Monocytes with *Individual* HC and PD EVs

As HCs as well as PD patients are an inhomogeneous group, it is likely that the biological constitution of EVs and consequently the capability of EVs to provoke a modulation of immune cells shows a wide range within these two groups. To address the question whether the sensitivity of human monocytes to EVs is not only dependent on a specific predisposition of monocytes, but also modulated by the constitution of EVs, monocytes were treated with individual HC and PD EVs (Fig. 16).

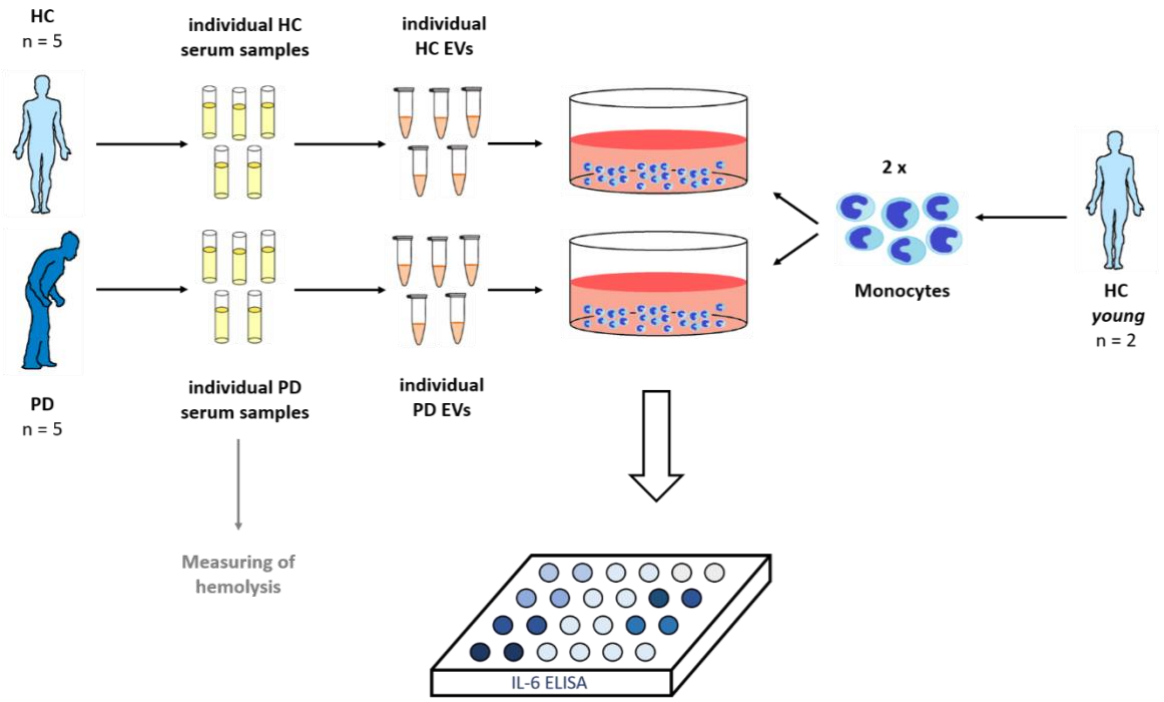

Figure 16: **Experimental design – stimulation of young healthy donors' monocytes with individual HC and PD EVs.** For EV isolation, 10 mL of hemolysis-free serum from each of five HCs and five PD patients were used. EVs were not pooled. Monocytes were isolated from EDTA-blood of two young healthy donors. After resting for 24 h, these monocytes were stimulated with single HC and PD EVs, respectively. 24 h post-stimulation cell culture supernatants were collected, and IL-6-levels were quantified via ELISA. EDTA = ethylene-diaminetetraacetic acid, ELISA = Enzyme-linked immunosorbent assay, EVs = extracellular vesicles, h = hours, HC(s) = healthy control(s), IL-6 = interleukin-6, PD = Parkinson's Disease (patients).

The characteristics of the serum donors for EV isolation are listed in Tables 4 and 5. Monocytes were isolated according to the established protocol from two young healthy donors: a 24-year-old male and a 23-year-old male. Both did not suffer from any infectious or chronic disease and did not receive chronic medication.

On average, first healthy donors' monocytes showed an increased activation by PD EVs relative to HC EVs without reaching statistical significance (Fig. 17 A, p = 0.087, Mann-Whitney U-test). Especially, *PD2* and *PD4* EVs triggered monocytic activation (Fig. 17 B).

Second donors' monocytes displayed no increased immune activation following EV stimulation regardless of the origin of EVs (Fig. 17 C, p = 0.65, Mann-Whitney U-Test).

LPS served as a positive control and induced a significant monocytic activation (first donor: 536.20 pg/mL ±39.17 SEM; second donor: 507.93 pg/mL ±20.89 SEM).

Overall, monocytes showed a differential reaction to individual HC and PD EVs indicating that EVs of some donors increase IL-6 release, whereas EVs of other donors do not have an impact on immune activation or may even inhibit IL-6 secretion.

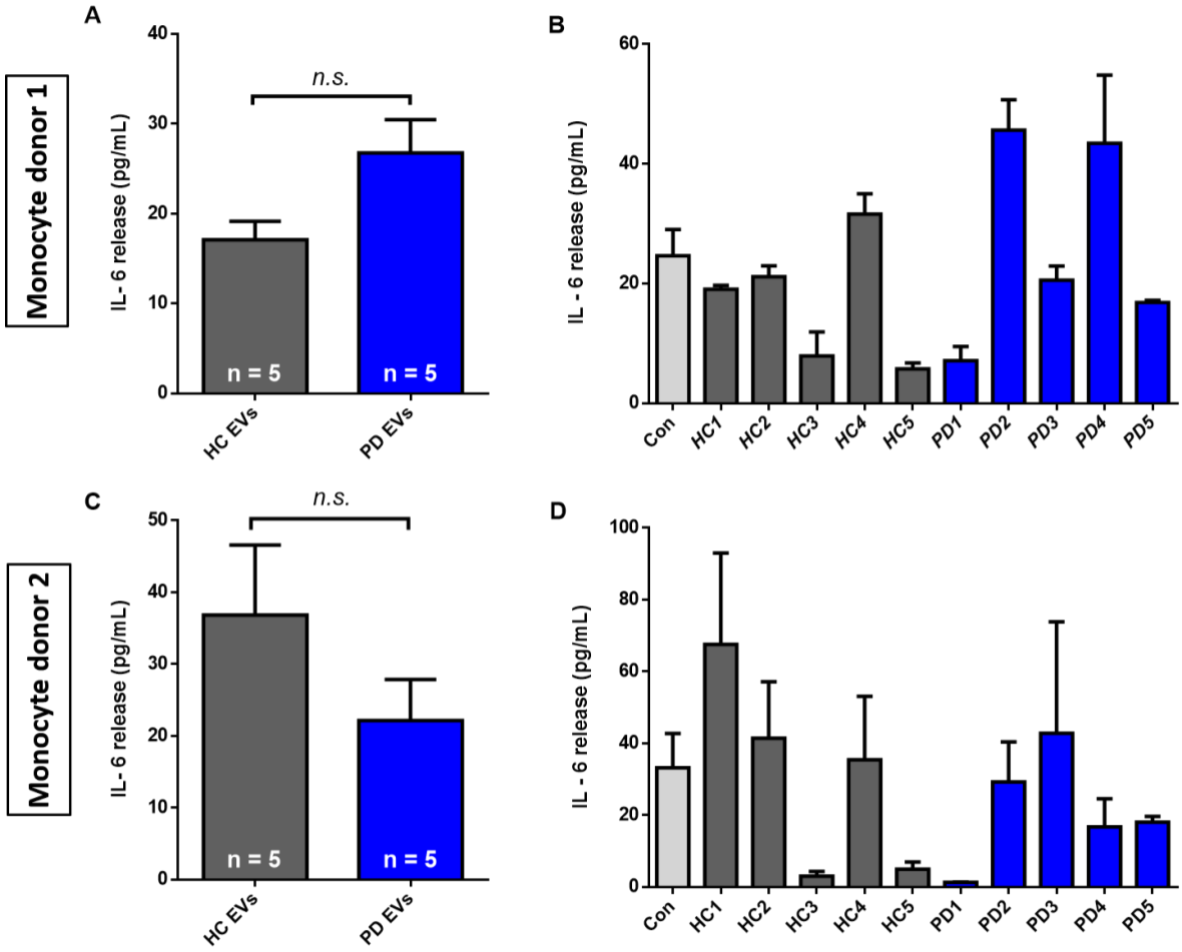

Figure 17: Comparison of the IL-6 response to EVs from individual serum donors. The response of monocytes (two young healthy donors) to EVs from different serum donors was compared. ***A, C:*** Average response to HC and PD EVs**.** ***B, D:*** IL-6 response to EVs from different individuals (five HCs, five PD patients). n.s. = not significant, Mann-Whitney U-test (A, C). Bars: mean ± standard error of the mean. Con = control (unconditioned media), *HC1-5* = healthy control EV donors 1 to 5, *PD1-5* = Parkinson's Disease EV donors 1 to 5, EVs = extracellular vesicles, HC(s) = healthy control(s), IL-6 = interleukin-6, PD = Parkinson's Disease.

4. Discussion

In the present work, we show that HCs' as well as PD patients' serum EVs can activate healthy donors' peripheral blood monocytes. The release of the proinflammatory cytokine IL-6 by monocytes shows a large interindividual difference and is influenced by monocyte donors' age as well as by the origin of the EVs (HC or PD serum). PD EVs potentially induce an increased IL-6 secretion in some healthy donors. As monocytes play an important role in neuroinflammatory and neurodegenerative processes, increased monocytic IL-6 response to pathologic PD EVs could be a relevant pathomechanism in PD.

4.1. Technical Limitations

4.1.1. Effectiveness and Quality of EV Isolation via Ultracentrifugation

Before stimulating monocytes, EVs were isolated from HCs' and PD patients' serum by differential UC. Whereas there are many different kits for EV isolation available, which are based on size exclusion chromatography, on precipitation, or on using immunomagnetic beads, conventional UC is recommended for EV-based treatment studies [83]. The main drawback of UC is that it is time-consuming and not effective for small volumes as it does not provide the highest yield of all EV isolation techniques. Limited availability of serum samples with limited amount of serum (approximately 12 mL of whole blood for 5 mL serum) and therefore limited numbers of EV preparations were the main obstacles this study had to deal with. To control the quality of our EV isolation protocol and to characterize EV size distribution and dispersity, EVs were analyzed by NTA, which is a method for particle quantification and size determination relying on single particle analysis. Importantly, particle size is solely determined by its Brownian motion and not influenced by particle density or refractive index [35]. HC as well as PD EVs comprised a homogenous population with low dispersity and similar peaks in particle size at the expected size (around 100 nm). Whereas a single particle type at the expected size was enriched in our EV preparation, multiple particle types were present in the analyzed serum samples. This is because serum contains far more lipoproteins with a lower size than EVs, whereas in the purified EV sample EVs have been enriched. With reference to the publication of Chernyshev et al. in 2015, size distribution and dispersity of the particles in the EV preparation indicate that our isolation protocol is an effective way to gain human serum EVs [22]. To provide evidence that our isolation method is specific for EVs and is not

accompanied by a high amount of contamination with other serum components, the content of the samples was examined by WB for EV markers and IgG as serum protein. Both EV markers – Flotillin-1 and CD63 – were highly enriched in the EV fraction. This EV characterization fulfills the requirements of the MISEV2018 guidelines to demonstrate EV nature and the degree of purity of an EV preparation, namely the analysis of at least one protein of three different categories [111]:

1. Transmembrane or GPI-anchored proteins associated to plasma membrane and/or endosomes: non-tissue specific – tetraspanin **CD63** (*via WB*)
2. Cytosolic proteins recovered in EVs: **Flotillin-1** (*via WB*)
3. Major components of non-EV co-isolated structures as purity control: **lipoproteins** (*indirectly via NTA*)

To claim specific analysis of a distinct EV subtype or to document functional activities of specific EV-associated proteins or functional components of EVs, even more extensive characterization of EVs would be required.

On the contrary, IgG, which is abundant in serum, was reduced in the EV samples. The MISEV2018 guidelines do not explicitly mention IgG as a purity control but it does mention albumin [111]. As albumin and IgG are the two most abundant human serum proteins, IgG seems (with some restrictions) a reliable purity control as well. However, why is there still some IgG left in the EV preparation? There are two reasons for this: Firstly, contamination of EV samples with serum proteins is a common problem. Secondly, B-cell-derived EVs contain and express immunoglobulins – predominantly IgM and IgD, but also detectable levels of IgG [90]. Consequently, the IgG content of the EV preparation can only be to some extent considered as serum protein contamination because IgG can physiologically be expressed on EVs. Taking this into account, our EV isolation protocol, which has already been used in several studies published by our group [52, 53, 123] is not only effective but also provides high purity.

4.1.2. Challenges of Monocyte Isolation

As monocytes only account for 3-8% of all human leukocytes, at least 30 mL of EDTA-blood of each donor are required to isolate enough monocytes for all treatments and controls (Con, LPS, HC EVs, PD EVs), including replicates. As we are interested in the underlying mechanisms of monocytic responsiveness to pathologic PD serum EVs, it would be highly interesting to additionally isolate RNA/chromatin for sequencing from monocytes from the

same sample. However, the sole amount of EDTA-blood needed for this from *one* voluntary participant is difficult to obtain in daily practice. Furthermore, another major challenge regarding monocyte isolation is the isolation process itself. It is important to avoid any unspecific activation of the monocytes during the isolation as this could lead to a decrease in IL-6 response due to a phenomenon called 'refractory state', which makes it even more difficult to measure small differences in IL-6 secretion. In Fig. 6 an example for this mechanism can be observed: *HC10* showed a high basal rate of IL-6 secretion that could not be boosted by LPS stimulation. Basically, this phenomenon also translates into clinical practice and is referred to as 'endotoxin tolerance'. During the initial phase of a sepsis, monocytes contribute to the excessive inflammatory response, whereas during the later phase of the disease downregulation of monocytic cytokine production, which is believed to be an important reason of sepsis-induced immunosuppression, can be observed [75, 115]. We used basal IL-6 levels as a quality control for a proper isolation process.

Even though CD14 acts as a coreceptor for bacterial LPS [120], monocytes were isolated by CD14-beads and did not stay 'untouched' during the isolation process. However, monocytic responsiveness to LPS stimulation was not impaired, as we have previously shown [52]. This is because CD14 shows a high turnover and monocytes had 24 h to rest post isolation and prior to stimulation.

4.2. Weak Activation of Human Monocytes by Serum EVs

We aimed to investigate the immune response of human monocytes towards pathologic PD serum EVs by measuring IL-6 levels in cell culture supernatant via ELISA. IL-6 was chosen as it is one of the main proinflammatory cytokines in humans and increased levels of IL-6 have been repeatedly linked to PD [10, 21, 88, 92]. Moreover, quantification of IL-6 levels by ELISA is an easy and very robust method for the assessment of monocyte activation [52, 53].

To rule out vehicle effects (PBS) and effects of EV contamination with proteins, our group has already performed preliminary experiments, in which monocytes were exposed solely to PBS, supernatant of the EV isolation and full plasma of PD patients and HCs (all diluted in cell culture media). No significant activation or difference between HC and PD plasma could be observed [53, 123].

Modulation of immune cells by EVs has been described for several disease entities [23, 26, 39, 67, 113] – as well as PD [53]. Whereas it is unclear if levels of free α-synuclein are increased in PD plasma [20], increased α-synuclein levels in EVs from PD patients' blood could be repeatedly detected [19, 97, 119]. As it is known that monocytes are pathologically overactive in PD [52] and are sensitive to the α-synuclein pathology [53], modulation of monocytic function by EVs could play an important role in peripheral inflammation in PD. This is supported by the fact that EVs facilitate the cell-to-cell transmission of the more immunogenic oligomeric α-synuclein [28]. Furthermore, inflammatory predisposition and α-synuclein pathology have been shown to act synergistically [53].

But what was our rationale to specifically look at *monocytes*? Monocytes are part of the innate immune system, which is inevitable for the initiation and proper functioning of the adaptive immune response. The crosstalk between innate and adaptive immune system is modulated by EVs. In 2013, Danesh et al. described in detail how EVs can act via antigen-presenting cells like monocytes to potentiate T-cell survival and boosting T-cell response [27]. It has been already demonstrated that $CD4^+$ T cells as part of the adaptive immune system invade the human brain in PD and contribute to neuronal degeneration [13]. Recent evidence shows that monocytes are not only passive bystanders, that may play a subsidiary role in the inflammatory processes observed in PD by supporting T-cell response, but also play an active role in neurodegeneration and are required for neuronal loss in model synucleinopathies [55, 56]. Another reason for specifically investigating monocytic response to PD EVs is the fact that monocytes share many features and display similarities with microglial cells. It is shown in humans that microglial activation is an early event in PD [81], that also can be detected after decades of disease onset in postmortem PD patients' brains [72]. As human microglia are hardly accessible, studying human peripheral monocytes is a valuable tool to gain insight in microglia functions in PD. Therefore, blood samples can be regarded as possible window to global functions of the immune system, allowing even distal processes in the brain to be reflected in a readily accessible peripheral blood sample.

Why did we study *healthy donors'* monocytes? PD patients' monocytes show an abnormal gene expression pattern [52, 94], are pathologically hyperactive to unspecific stimuli [52] and display a predisposition to increased activation in response to pathologic α-synuclein

species [53]. As we aimed to investigate monocytic behavior to pathologic PD serum EVs, we needed to study monocytes that are not *intrinsically/a priori* dysregulated. Analyzing healthy donors' monocyte response to PD EVs could provide insights on monocytic vulnerabilities to PD EVs, that may be a predisposing factor for PD if we believe monocytic activation by α-synuclein-rich EVs to be an early event in PD pathogenesis. We hypothesized that genetic and epigenetic polymorphisms confer susceptibility to aggravated monocyte response to α-synuclein-rich EVs. If so, we should be able to identify 'responders' among healthy donors. A recent study has shown an analogous phenomenon in T cells, which can recognize α-synuclein peptides bound to specific HLA-alleles of the MHC in an autoimmune manner [107]. These HLA-alleles are enriched in PD patients and believed to confer increased risk of PD. Such phenomenon is not known for monocytes or microglia so far.

However, it was difficult to prove our paradigm. On average, pooled HC as well as PD serum EVs induced an increase in IL-6 release of young as well as aged healthy donors' monocytes. Unfortunately, monocytic response to EVs was 5-10 times lower compared to LPS-stimulated IL-6 secretion. This weak activation of human monocytes by serum EVs made it difficult to investigate significant differences in IL-6 response to PD compared to HC EVs. One probable solution would be the use of more EVs. However, serum for EV isolation was a limiting factor.

Why do we believe that these small changes in IL-6 secretion induced by serum EV are still *relevant*? As plasma EV-associated α-synuclein, which is a potent activator of monocytes [53], is likely to be CNS-derived [97], local concentrations of EV-associated α-synuclein in the CNS, where the neurodegeneration in PD takes place, are likely to be much higher than in peripheral blood. Release of EVs is an important mechanism of damaged or stressed cells to maintain cellular homeostasis [109]. Therefore, it seems logical that the micromilieu of the vulnerable brain regions in PD favors high levels of EV-associated α-synuclein. This hypothesis is supported by the fact that early PD-affected regions in human brains express high levels of α-synuclein [108], which is known to be released into the extracellular space not only as free form but also in association with EVs [69]. Beyond that, PD monocytes only become functionally overactive if triggered by a so-called 'second hit', as two independent studies indicate [41, 52]. Hence, higher local levels of α-synuclein-rich EVs combined with other trigger factors such as infections or environmental cues could contribute to the

peripheral inflammation and especially to the neuroinflammation seen in PD. Interestingly, GCase activity negatively correlates with EV-associated α-synuclein levels [19] and PD patients' monocytes show a reduced GCase activity [2]. This could also lead to a higher concentration of EV-associated α-synuclein in the proximity of peripheral and/or brain-invading monocytes. Furthermore, even a weak activation of monocytes could result in a substantial inflammatory response as monocytes are not only effectors cells but are also able to attract and activate other immune cells such as T cells, thereby provoking an amplifying cascade of inflammatory processes.

4.3. Identification of Responders to Pathologic PD Serum EVs

Although serum EVs induced only a slight increase in monocytic IL-6 release, we could identify one putative 'responder' to pooled PD EVs among young healthy monocyte donors (Fig. 15, *HC6*). In two more cases a trend for increased monocytic activation upon stimulation with PD compared to HC EVs could be observed (Fig. 10, *HC2*; Fig. 15, *HC1*). While studying monocytic response to HC and PD EVs, it became clear that there are two main factors that play a role in monocyte activation: *age-dependency* and *interindividual differences*.

4.3.1. Age-dependency of Monocytic Response

Whereas gender did presumably not influence monocytic IL-6 response, monocyte donors' age influenced basal and LPS-triggered IL-6 release. Lower cytokine secretion rate of unstimulated cells and hyperactivation upon LPS challenge both indicate a more specific and distinct IL-6 secretion pattern among monocytes from younger donors. As the stimulated monocyte response from aged donors might be generally lower than that from young donors, it seems to be even more difficult to detect differences between HC and PD EVs among aged donors, which is in line with our results. Even though we could identify one possible 'responder' among aged healthy donors, it seems unlikely that factors that may lead up to an increased monocytic activation in response to PD EVs and that may be causally relevant in PD are highly enriched in the participating aged healthy monocyte donors. This is because these aged healthy donors did not suffer from PD or any early symptom of PD and therefore may reflect to a large extent the 'non-responders' (*see 3.2.2.*). However, one possible 'responder' out of twelve could be reasonable, as PD pathogenesis and disease manifestation are influenced by many more factors other than

monocytic susceptibility to pathologic EVs. On top of that, our possible 'responder' *HC2* was only 68 years old and still could develop PD, as onset of idiopathic PD is uncommon before an age of 50 years and a sharp increase in PD incidence is observed after age 60 years [29].

Our hypothesis that 'responders' to pooled PD EVs should be more easily and more frequently detectable among young monocyte donors was supported by our findings. We could identify two putative 'responders' out of six young monocyte donors. Showing that monocytes of donors, whose age is far from the average age of onset of idiopathic PD, already display a predisposition to abnormal activity upon stimulation with α-synuclein-rich EVs is in line with the findings that monocytic invasion and/or microglial activation play an active and early role in PD pathogenesis [56, 81]. Once EVs carry an altered cargo – especially higher levels of α-synuclein – monocytes in the periphery and/or monocytes/microglia of the brain could become activated and initiate the proinflammatory response.

Investigating these age-dependent differences in monocytic behavior patterns seems to be extremely valuable in the context of PD as aging is undoubtedly the most significant risk factor for idiopathic PD, which is unambiguously demonstrated by epidemiological data [29] as well as animal models [84, 91]. Chronic, low-grade, sterile inflammation is widely accepted as part of the aging process and sometimes referred to as 'inflammaging' as aging comes along with an increase in plasma concentrations of proinflammatory cytokines [16]. Coexisting neuroinflammation and neurodegeneration could potentiate each other in PD. Beyond the general changes of the innate as well as adaptive immune system associated with age, which are often summarized under the term 'immunosenescence', there exist specific age-dependent defects of monocytes and microglia in PD. It was demonstrated that human monocytes from elderly individuals display phagocytic defects regarding free α-synuclein as well es EV-associated α-synuclein uptake. This could lead to a local increase in EV-associated α-synuclein levels (*see 4.2.*). It is known that EV-associated α-synuclein can be taken up more efficiently [8]. Considering the phagocytic defects of aged donors' monocytes, the more efficient uptake of EV-associated α-synuclein could be even more important in the setting of PD. As it was already shown that monocytic activation is dependent on TLR-signaling as well as uptake of EVs [8], PD EV-mediated increase in monocytic IL-6 release is likely to be a relevant mechanism, especially in aged individuals

with a certain predisposition. Moreover, the same study illustrated an increase of non-classical monocytes in aged healthy donors, whereas numbers of classical monocytes did not change [8]. While only classical monocytes were considered as proinflammatory for a long time, recent evidence indicates a possible involvement of non-classical monocytes, that display many inflammatory features, in inflammatory autoimmune diseases such as systemic lupus erythematosus [74]. Even though PD predominantly affects the elderly, distribution of monocytic subpopulations during PD pathogenesis and course of disease seems to be more complicated as it is known that classical monocytes are enriched in PD while non-classical monocytes are reduced [52]. To elucidate the role of monocyte subpopulations in EV-mediated inflammation in PD, it would be necessary to separate/sort classical from non-classical and possibly intermediate monocytes prior to EV stimulation.
Unfortunately, it is difficult to assess the effects of age on monocytic response to EV stimulation based on our data as young and aged donors' monocytes were stimulated with different EVs, that did not have the same concentrations and aroused from different serum donors, and consequently are not comparable.

4.3.2. Interindividual Differences in Monocytic Response to Stimulation

Independently of monocyte donors' age, we could observe considerable interindividual differences in monocytic response regarding basal, LPS-stimulated and EV-mediated IL-6 release. Interestingly, those aged donors' monocytes that seem to react to LPS stimulation with a higher IL-6 release also secrete higher levels of IL-6 upon treatment with HC and PD EVs. As HCs as well as PD patients are no homogenous group, we assumed that their EVs show differences regarding their cargo and consecutively regarding their capability of activating human monocytes. To further investigate this hypothesis, we stimulated monocytes from two young healthy donors with individual serum EVs. Strikingly, not all EV samples could induce an increase in monocytic IL-6 release compared to basal IL-6 release. Some donors' EVs even inhibited IL-6 release, suggesting that EVs cannot only functions as proinflammatory but also as anti-inflammatory immunomodulators. It has been shown for diseases other than PD that EVs can suppress inflammation [95, 99]. Moreover, EVs which induce an increase in IL-6 release in one donors' monocytes do not influence or even reduce activation of another donors' monocytes. This further supports our hypothesis that a so-far unknown variation in immune receptors, downstream effectors or transcription

factors results in the increased response of some healthy individuals to pathologic PD EVs. As already mentioned, an analogous phenomenon was described in T cells [107].

We identified two putative 'responders' among young monocyte donors. Now one could ask if monocytic responses remain stable over a long period of time, which would be necessary for our findings in young healthy blood donors to be a relevant factor in PD onset many years later. There is some data that shows that monocytic cytokine response remains relatively stable over the course of three years [96]. This indicates that the human immune system remains to some part stable in its response patterns over time within a given person, although the immune system is highly variable between individuals. However, there is no data that has longitudinally investigated monocytic cytokine response over a longer period. Considering that genetic and/or epigenetic factors such as polymorphisms in immune receptors could confer the increased responsiveness of our 'responders', it seems likely that these factors could have a life-lasting influence on the reaction of our immune system to α-synuclein-rich EVs.

As the human immune system shows a wide variation from individual to individual and even some variation within one individual over time (age-dependent, seasonal and circadian immune system variation), it is not surprising that we could observe a differential behavior of our human monocytes upon stimulation. Population immunology aims at understanding diversity of immune states and responses across a large population. There is recent evidence from this relatively new scientific field that variation in the human immune system is mainly shaped by non-heritable influences such as the microbiota, viruses and other environmental factors. Interestingly, it was also demonstrated in a twin-study that several cytokine-induced signaling responses across multiple immune cell populations including monocytes are highly variable between individuals. Responses induced by cytokines like IL-6, IL-10, TNFα and IFNγ showed limited heritability [15].

4.4. Two Reinforcing Factors: Pathologic PD EVs and Dysregulated PD Monocytes

Besides *age-dependency* and *interindividual differences* as modifying variables, we believe that our data suggest that the two main factors underlying increased responsiveness of monocytes to PD EVs are*: increased activation by pathologic* α*-synuclein* and *predisposition of PD monocytes* (illustrated in Fig. 1). We could show that even some healthy donors' monocytes are hyperactive in response to PD EVs. This inflammatory predisposition could play a causal and therapeutically relevant role in PD pathogenesis and could become even more relevant during disease progression as PD comes along with several changes in the composition and function of monocytes. To what extent these changes are primary or secondary, and in what way they are related to PD EVs, still needs to be scrutinized more closely. Stimulation of young healthy donors' monocytes with individual HC and PD EVs suggests that both composition of EVs and functioning of monocytes contribute equally to the inflammatory response. We believe that our 'responders' to PD EVs possess the same predisposition to pathologic α-synuclein as PD patients do. We assume that investigating the role of EV-associated α-synuclein seems to be highly relevant as EV-associated α-synuclein oligomers are more easily taken up by cells, more toxic and increase activation of immune cells stronger than its free form [28, 53]. In PD, EVs show several abnormalities:

1. CNS-derived and serum EVs carry higher levels of α-synuclein [54, 97].
2. Salivary EVs show higher levels of α-synuclein oligomers [17].
3. Serum EVs contain more inflammatory cytokines such as TNFα [54].
4. Higher EVs concentrations are expected locally [85].
5. EVs carry a different miRNA-cargo [63, 117].

These pathologically altered PD EVs could contribute to and maintain the widespread dysregulation of PD monocytes [9, 38, 52, 53, 94], which could *per se* be hyperactive upon stimulation with pathologic EVs. Interestingly, we could find 'responders' even among young healthy donors' suggesting that monocytic predisposition could play a lasting role in the inflammatory events and thereby having an impact on PD pathogenesis. As we could find one possible 'responder' among aged healthy donors, we could conclude that monocyte predisposition does not necessarily lead up to PD, which we also had not assumed. The pathogenesis of idiopathic PD is thought to be multifactorial after all.

Our immune system is influenced by many different factors that are difficult to control in studies with humans. No participant at all suffered from any infectious disease while participating in our study. However, many infectious – especially viral – diseases run a subclinical course with effects on our immunity. Such subclinical infections could mislead us as they may attenuate or trigger monocytic response to some or even all our treatments. Furthermore, we defined intake of immune-modulating drugs as another exclusion criterion. Many of our PD serum donors were under L-Dopa treatment. One could now argue that L-Dopa could influence monocytic behavior. However, it was already shown that L-Dopa had no effect on IL-6 release of healthy donors' monocytes [52]. Additionally, chronic stress is known to interfere with monocytic function, which could also lead to a different cytokine secretion pattern [73]. Even though there are many factors affecting the immune system we could not control, we believe that this is rather an advantage than a disadvantage, as 'real life patients' are exposed to different environmental factors all the time. That is why a potential therapeutic target should not be influenced excessively by the constantly changing parameters of the immune system. One potential therapeutic target could be TLR2 as it is known that immunomodulation by EVs is dependent of TLR2 in breast cancer [23], inhibition of TLR2 decreases monocytic activation upon stimulation with α-synuclein-rich EVs [53] and TLR2 is known to be involved in PD α-synuclein pathology [36]. Another therapeutic target, that is already used in clinical trials, is to disrupt the spread of α-synuclein-associated neurodegeneration by directly targeting α-synuclein pathology with monoclonal antibodies such as Prasinezumab (PRX002/RG7935) [62]. To further investigate the role of pathologic EV-associated α-synuclein species in immune activation, it would be highly interesting to study the behavior of monocytes of patients on Prasinezumab therapy. Questions to be answered in this context are:

- Does anti-α-synuclein-antibody-therapy reverse dysregulation of monocytes regarding their subsets, functioning and gene expression pattern?
- Does anti-α-synuclein-antibody-therapy change the levels and the composition of EV-associated α-synuclein?

More insights on the potential inhibition of immune activation by EV-associated α-synuclein by α-synuclein-antibodies could be gained through treatment (for example via co-treatment of cells with EV-associated α-synuclein and specific antibodies) studies in cell culture or animal models.

4.5. Conclusions

In summary, this study shows evidence that pathologic PD serum EVs can induce a proinflammatory IL-6 release of some healthy donors' monocytes. Monocytic response to PD as well as HC EVs seems to be simultaneously dependent on monocytic vulnerability *and* composition of EVs. Moreover, monocyte donors' age and interindividual differences modulate the degree of proinflammatory IL-6 secretion upon stimulation. As it is known that dysregulated monocytes contribute to neurodegeneration and EVs show several abnormalities in PD, our data suggest that EV-mediated activation of PD monocytes could be a disease-driving mechanism, that could be present already before disease onset in some young healthy individuals. Monocytes play a central role in neuroinflammatory processes in PD and have widespread effects on other cells. Therefore, targeting EV-mediated monocyte activation pharmacologically by reducing EV-associated α-synuclein levels or inhibiting potentially involved receptors such as TLR2 could provide means to interrupt the vicious circle of neuroinflammation and neurodegeneration in PD. Whereas this study could shed some light on the interaction of monocytes and PD EVs, further questions that need to be investigated in more detail remain:

1. What are the exact underlying molecular mechanisms that determine responsiveness of monocytes to PD EVs?
2. How does monocytic response to EVs changes during PD pathogenesis and disease progression?
3. How do different strains and levels of EV-associated α-synuclein exactly contribute to monocyte activation by EVs?
4. What role do other abnormalities in PD EVs cargo play, such as increased levels of proinflammatory cytokines or altered miRNA?
5. To what extent and in what manner is EV-mediated activation of monocytes involved in neurodegeneration?
6. What factors – besides EV stimulation – lead up to the dysregulation of PD monocytes?

On a long term, the main goal is to establish immunomodulatory drugs that can delay, stop or even reverse peripheral and inflammatory processes that lead up to, maintain or aggravate PD.

5. Summary

Peripheral inflammation and neuroinflammation can result in neurodegeneration and parkinsonism. There is recent evidence that immune responses – especially monocytic cytokine secretion patterns – in Parkinson's Disease (PD) patients are dysregulated. It is known that serum extracellular vesicles (EVs) can induce proinflammatory reactions of immune cells. Moreover, PD EVs seem to carry an altered cargo – specifically containing higher levels of α-synuclein. Interestingly, α-synuclein pathology seems to act synergistically with monocytic dysregulation in PD to trigger excessive inflammatory responses.

As it is known that monocytes can contribute to PD neurodegeneration and immune dysregulation plays an early event in PD, we investigated the predisposition of primary human monocytes of healthy donors to pathologic PD serum EVs by assessing release of proinflammatory interleukin-6 (IL-6) upon EV exposure.

We isolated serum EVs of healthy controls (HCs) and PD patients by differential ultracentrifugation (UC) and checked the quality and purity of our EVs by Nanoparticle Tracking Analysis (NTA) and Western Blot (WB). NTA as well as WB proved our EV isolation protocol to be not only effective but also providing high purity. Prior to treatment, we isolated human monocytes of young as well as aged healthy donors by positive, CD14-based selection of monocytes from peripheral blood mononuclear cells. Monocytes were then stimulated with lipopolysaccharide (LPS) as a positive control and with HC EVs as well as with PD EVs. To rule out any unspecific activation of monocytes during isolation or cultivation and to quantify basal IL-6 release, negative controls with only unconditioned media were performed. We could show that treatment with pooled HC and PD EVs results in an inflammatory activation of young as well as aged human monocytes. The activation shows widespread interindividual differences. We also found that young monocytes seem to be more suitable for detecting slight changes in cytokine secretion as they show a more distinct cytokine secretion pattern. Young monocytes react to triggers such as LPS with a higher increase in IL-6 secretion but show a lower basal cytokine secretion rate than aged monocytes. Most importantly, we could demonstrate that there is a predisposition of some healthy donors' monocytes to react excessively to PD EVs. Our findings indicate that this

predisposition is more likely to be enriched in young healthy donors' monocytes than in those of aged healthy individuals. On average, no differences in monocytic activation upon stimulation with HC and PD EVs could be observed – neither in young nor in aged monocytes. Furthermore, we could show that monocytic activation by HC as well as PD EVs is not only influenced by monocytic vulnerability but also by the composition of EVs. Stimulating young healthy donors' monocytes with individual EVs showed that EVs that can increase cytokine secretion in one donor do not necessarily boost another donors' immune response. The opposite may be even the case as we could show that some donors' EVs do not influence or even inhibit monocytic IL-6 release.

Taken together, our data suggest that there are indeed 'responders' among healthy individuals to pathologic PD EVs as some healthy donors' monocytes seemingly reacted to PD EVs with an increased IL-6 release. Additionally, our data prompt that pathologic PD EVs and dysregulation of PD monocytes could potentiate each other and could play a detrimental role in initiating and maintaining neuroinflammatory processes in PD.

6. References

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Curriculum Vitae

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