Increased Immune Activation by Pathologic α-Synuclein in Parkinson’s Disease

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Objective: Excessive inflammation in the central nervous system (CNS) and the periphery can result in neurodegeneration and parkinsonism. Recent evidence suggests that immune responses in Parkinson disease patients are dysregulated, leading to an increased inflammatory reaction to unspecified triggers. Although α-synuclein pathology is the hallmark of Parkinson disease, it has not been investigated whether pathologic α-synuclein is a specific trigger for excessive inflammatory responses in Parkinson disease.

Methods: We investigated the immune response of primary human monocytes and a microglial cell line to pathologic forms of α-synuclein by assessing cytokine release upon exposure.

Results: We show that pathologic α-synuclein (mutations, aggregation) results in a robust inflammatory activation of human monocytes and microglial BV2 cells. The activation is conformation-dependent, with increasing fibrillation and early onset mutations having the strongest effect on immune activation. We also found that activation of immune cells by extracellular α-synuclein is potentiated by extracellular vesicles, possibly by facilitating the uptake of α-synuclein. Blood extracellular vesicles from Parkinson disease patients induce a stronger activation of monocytes than blood extracellular vesicles from healthy controls. Most importantly, monocytes from Parkinson disease patients are dysregulated and hyperactive in response to stimulation with pathologic α-synuclein. Furthermore, we demonstrate that α-synuclein pathology in the CNS is sufficient to induce the monocyte dysregulation in the periphery of a mouse model.

Interpretation: Taken together, our data suggest that α-synuclein pathology and dysregulation of monocytes in Parkinson disease can act together to induce excessive inflammatory responses to α-synuclein.

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important component of PD pathology, as microglial activation is observed already early in the disease course and observed in postmortem brains of PD patients even decades after onset of disease. Interestingly, peripheral α-synuclein (α-syn) in the substantia nigra is crucial for neuronal loss induced by peripheral inflammation in rodent models demonstrated that monocyte infiltration in the substantia nigra is crucial for neuronal loss induced by α-synuclein (α-syn), the key pathologic protein in PD. Moreover, recent reports from rodent models demonstrated that monocyte infiltration in the substantia nigra is crucial for neuronal loss induced by α-synuclein (α-syn), the key pathologic protein in PD. Moreover, recent reports from rodent models demonstrated that monocyte infiltration in the substantia nigra is crucial for neuronal loss induced by α-synuclein (α-syn), the key pathologic protein in PD.1,2}

However, much less is known about the role of α-syn-induced inflammation in human disease. In particular, 2 questions are indispensable: (1) How do human immune cells react to α-syn pathogenesis? (2) Is there a predisposition of PD immune cells to excessive inflammation? To address these questions, we thoroughly investigated the immune response of human monocytic cells and of a microglial cell line (BV2) to several pathologic α-syn triggers and compared the monocyte response induced by pathological α-syn in PD patients and healthy controls.

**Materials and Methods**

**Study Approval**

All experiments with human material were performed in accordance with the Declaration of Helsinki, and approved by the ethics committee of Ulm University. Animal experiments were performed in accordance with the Mayo Clinic Institutional Animal Care and Use Committee guidelines.

**Animal Experiments**

Thyl-αsyn C57BL/6-DBA/2 mice were originally provided by Eliezer Masliah and were maintained in the in-house breeding facility of the Mayo Clinic Jacksonville on the mixed C57BL/6-DBA/2 background, in a pathogen-free environment in open-top cages, with ad libitum access to food and water and a standardized 12 hour/12 hour light/dark cycle. Peripheral blood for phenotyping of blood monocytes was collected from the vena facialis by puncture with a 5mm lancet and in a tube rinsed with sodium heparin 25,000 (Ratiopharm, Ulm, Germany).

**Antibodies Used in the Study and Flow Cytometry**

Flow cytometric analysis of murine blood monocytes was performed as previously described with following antibodies: NK1.1-FTTC (PK136; BD Biosciences, Franklin Lakes, NJ), Ly6G-FTTC (1A8; BD Biosciences), CD43-PE (S7; BD Biosciences), Ly6C-PerCP-Cy5.5 (HK1.4; BioLegend, San Diego, CA), CD3e-FTTC (145-2C11; BD Biosciences), CD19-FTTC (6D5; BioLegend,), CD11b-APC (M1/70; BD Biosciences), CD45-APC-Cy7 (30-F11; BD Biosciences), CD115-Biotin (AFS98; eBioscience, San Diego, CA), and Streptavidin-PE-Cy7 (eBioscience).

**Cell Culture**

Human primary monocytes were isolated by positive selection with magnetic microbeads coupled to anti-CD14 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) as described previously from healthy volunteers with no known neurological, infectious, or chronic disease or from PD patients diagnosed according to standardized diagnostic criteria by a movement disorder specialist. Monocytes and BV2 cells (RRID: CVCL_0182, no entry in ICLAC) were maintained in Roswell Park Memorial Institute–1640 medium with L-glutamine (2.05mM) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; 20 mM; Sigma-Aldrich, St. Louis, MO), 10% fetal calf serum (Gibco, Life Technologies, Rockville, MD) and 1% penicillin/streptomycin (for monocytes; PAA Laboratories Inc) at a final concentration of 10^6 cells/ml. Monocytes and BV2 cells were rested for 24 to 72 hours in culture before stimulation, then treated for 24 hours before cell culture supernatant was collected for assaying cytokine release. For inhibition experiments, cells were pretreated for 2 hours with inhibitors or antibodies before treatment.

HEK293 cells (RRID: CVCL_0045, no entry in ICLAC) were maintained in Dulbecco’s modified Eagle’s medium (Gibco, Life Technologies) supplemented with 10% fetal calf serum. For transient overexpression of α-syn and isolation of extracellular vesicles (EVs) from the cell culture supernatant, HEK293 cells were transfected with calcium phosphate as described previously. Overexpression of transgene was controlled by real-time quantitative polymerase chain reaction and by Western blot.

**Vectors**

Vectors for overexpression of transgenes were based on the mammalian expression vectors pcDNA3 (Invitrogen, Carlsbad, CA) and pSI (Promega, Madison, WI) and human α-syn Coding DNA sequence (CDS) (wild-type: NM_000345.3; A30P: NM_000345.3:c.88G>C, rs104893878; A53T: NM_000345.3:c.157G>A, rs104893877). Empty plasmid or plasmid encoding a myc-tag without a start codon was used for control transfections.

**EV Isolation, Characterization, and Treatment of Cells**

EVs were purified from the cell culture supernatant of HEK293 cells by sequential centrifugation and ultracentrifugation as described previously. For mass spectrometry–based analysis of EV proteome, EVs were washed 3 times with Dulbecco’s phosphate-buffered saline (DPBS). For isolation of EVs, serum was diluted 1:2 with sterile DPBS and plasma 1:10 with sterile DPBS. Nanoparticle tracking analysis (NTA) was performed with a NanoSight 2000 instrument (Malvern Instruments, Malvern, United Kingdom). EV protein was quantified by bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Waltham, MA). For imaging of EV uptake, EVs were labeled as described previously with 1μM DiI (Gibco, Life Technologies), and excess dye washed away by ultracentrifugation. EV-free DPBS was labeled
and used in parallel as a negative control to exclude bias by precipitated dye particles.

Overexpression of α-syn in HEK293 cells and EVs derived from cell culture supernatant was visualized by Western blot with the following primary antibodies: rabbit anti-β-actin (Cell Signaling Technology, Danvers, MA), mouse anti-β-tubulin-1 (BD Biosciences), mouse anti-α-syn clone 42 (BD Biosciences), rabbit polyclonal anti–α-syn (C-20)-R (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal anti–α-syn 211 (Santa Cruz Biotechnology).

For treatment of monocytes and BV2 cells, EVs were diluted in cell culture medium to a final concentration of 50μg/ml. EVs from cells overexpressing a transgene did not contain higher amounts of protein than control EVs. For treatment of cells with EVs isolated from human plasma, EVs isolated from 1ml of plasma were directly resuspended in 1ml of cell culture medium and applied to cells. For treatment of cells with plasma, cell culture medium was adjusted with 10% plasma.

Recombinant α-syn Species
Recombinant human α-syn species were prepared as previously described, where these species have been extensively characterized: oligomers DA and GA,10 oligomers type A and C,11 α-syn fibrils and ribbons,12–16 and α-syn fibrils-65 and fibrils-91.14 Endotoxin levels were lower than 0.02 endotoxin units per microgram as controlled using the Limulus Amebocyte Lysate Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific) following the manufacturer’s instructions. As an additional control, monomeric α-syn used for generating α-syn high-molecular-weight species was included for immune cell treatment. Recombinant monomeric human α-syn was purified as described previously.12 For the generation of α-syn oligomers type A and C, recombinant human wild-type α-syn was acquired commercially (rPeptide, Athens, GA). Protein concentration was quantified with a Pierce BCA assay (Thermo Fisher Scientific).

Analytical Ultracentrifugation
Sedimentation velocity measurements were carried using a Beckman Optima XL-A ultracentrifuge (Beckman-Coulter, Fullerton, CA) equipped with an ultraviolet visible detection system using an AN60-Ti 4-hole rotor and cells with 2-channel 12mm pathlength centerpieces. For higher molecular weight oligomers, the length heterogeneity was reduced by sonication for 20 minutes in 2ml Eppendorf tubes in a VialTweeter powered by a Beckman Coulter Genie II equipped with an ultraviolet visible detection system (National Institutes of Health, Bethesda, MD).17,18 The partial specific volume (0.7326ml/g for monomeric low- and high-molecular-weight oligomeric α-syn, 0.7326), the buffer viscosity (1.018cP for monomeric and oligomeric α-syn, 1.015cP for fibrillar α-syn, 1.029cP for α-syn oligomers A and C), and the buffer density (1.005g/ml for monomeric and oligomeric α-syn, 1.0067g/ml for fibrillar α-syn, and 1.0053g/ml for α-syn oligomers A and C) were calculated with the software Sednterp (http://bitc.sr.unh.edu/index.php/Main_Page). For all the measurements, the sedimentation coefficient values were corrected to s20,w (standard solvent conditions in water at 20°C).

Enzyme-Linked Immunosorbent Assay and Luminometry
Enzyme-linked immunosorbent assay was performed as described previously,9 according to the supplier’s instructions (BioLegend). All samples and standards were measured in duplicates or triplicates. Luciferase activity was measured as described previously.8,19

Mass Spectrometry
In-solution digestion, mass spectrometry, and data analysis were performed with the same equipment, tools, and settings as previously described,20 with following modification: mass spectra (MS) were acquired using 3E6 as an automatic gain control target, a maximal injection time of 20 milliseconds and a 60,000 resolution at 300m/z. Contaminants, reverse entries, and proteins that were only identified by site were filtered before further analysis.

Imaging
For visualization of α-syn and EVs uptake, peripheral blood monocytes and BV2 cells were washed twice with DPBS, fixed with polyformaldehyde (4% in phosphate-buffered saline), permeabilized with methanol (100%) or saponin (0.1% in DPBS; Sigma-Aldrich) and blocked with 1% bovine serum albumin (Sigma-Aldrich). α-syn was visualized with anti-human α-syn antibody 15G7 (Enzo, rabbit polyclonal) and goat anti-rabbit-AlexaFluor-488 (Gibco, Life Technologies). Cell membranes were visualized with membrane stains DiI and DiO (Gibco, Life Technologies) per the supplier’s instructions. Cell nuclei were visualized with DAPI (Sigma-Aldrich) and Hoechst 33342 (Sigma-Aldrich). Images were acquired with a confocal fluorescence microscope (LSM 710; Zeiss, Gottingen, Germany), using AxioVision and Zen software (Zeiss). Collages were generated with ImageJ.21 For transmission electron microscopy (TEM), recombinant α-syn species were adsorbed on carbon-coated 200 mesh grids, negatively stained with 1% uranyl acetate and analyzed with a Jeol 1400 TEM, and images were acquired with a Gatan Orius CCD camera (Gatan, Pleasanton, CA).

The atomic force microscopy samples were characterized using tapping as well as Quantitative Imaging mode on JPK NanoWizard 3 Ultra with silicon nitride cantilevers (Olympus, Tokyo, Japan) with a spring constant of 2N/m.

Data Analysis
Statistical analysis was performed with Prism 7.03 (GraphPad Software, La Jolla, CA). Gaussian distribution was tested with D’Agostino and Pearson omnibus normality test, Shapiro–Wilk normality test, and Kolmogorov–Smirnov normality test. Unpaired t test (for Gaussian-distributed data) or Mann–Whitney test (for non–Gaussian distributed data) was used for single, unpaired comparisons. Paired t test (for Gaussian-distributed data) or Wilcoxon matched-pairs signed rank test (for non–gaussian distributed data) was used for single, paired comparisons. One-way analysis of variance (ANOVA) with Dunnett multiple
comparison correction was used for multiple comparisons and repeated-measures 1-way ANOVA for paired tests. All tests for significance were 2-tailed with α = 0.05. Normal data presented as mean average ± standard error of the mean, lognormal data as geometric mean with 95% confidence interval of the geometric mean, unless stated otherwise. Gene enrichment analysis was performed with the Gene Ontology database (2017-12-27, REF List [21042] GO biological process complete Homo sapiens) and the PANTHER tool (v11.0, released 20171205; http://www.pantherdb.org/tools/). Venn diagrams were generated with the Venn diagram drawing tool from University of Gent (http://bioinformatics.psb.ugent.be/webtools/Venn).

Results

α-synchon Pathology Enhances the Activation of Innate Immune Cells by α-syn

To investigate whether α-syn pathology has an effect on immune cell activation, we generated different forms of recombinant α-syn using well-established, previously published protocols (Fig 1) and used them for inflammatory activation of monocytes and BV2 cells. Increased expression of α-syn due to single-nucleotide polymorphisms/gene duplication/gene triplication, or expression of α-syn with different point mutations lead to familial-inherited PD. To test the effect of these pathologic changes in α-syn on immune cells, we stimulated peripheral blood monocytes and microglial BV2 cells with recombinant, monomeric wild-type and mutant human α-syn (Fig 2 A-C). As also reported by others, the cytokine response to monomeric, wild-type α-syn was dose dependent. To assess the effect of different inheritable PD point mutations, we chose a concentration of α-syn (10μg/ml = 690nM) at which monomeric wildtype α-syn does not elicit a strong immune activation. This concentration of α-syn does not strongly exceed CNS concentrations (up to 6μM for some areas of the brain) and is not enough for spontaneous aggregation in cell culture medium at 37°C. Remarkably, all but 1 familial mutation tested (A53T, A53E, E46K, H50Q, and G51D) resulted in increased activation of the immune cells. Interestingly, mutations associated with early onset PD (E46K, H50Q, G51D25)–28 induced the strongest immune responses by both monocytes and BV2 microglia.

Mutations and increased gene doses (gene duplications or triplications) are relatively rare causes of α-syn pathology.29 By contrast, all sporadic PD cases display aggregation of α-syn postmortem. Therefore, we next investigated if immune activation is also increased by α-syn aggregation. Aggregated, fibrillar α-syn is formed from

![Figure 1: Recombinant α-synuclein (α-syn) species for treatment of innate immune cells. (A) Full-length monomeric human α-syn (NP_000336.1), wild-type (wt), or familial Parkinson disease–associated mutants were used. (B) Full-length monomeric, wt α-syn was used to produce different types of small homogenous oligomers (DA, GA), larger heterogenous oligomers (type A, type C), or different strains of fibrillar α-syn: fibrils (physiological salt concentration), ribbons (low salt concentration), fibrils-65 (low pH), and fibrils-91 (high pH). (C) Fibrils-110 were assembled from C-terminally truncated α-syn (amino acids 1–110) under standard assembly conditions (physiological salt concentration). DA = dopamine; GA = glutaraldehyde; MES = 2-(N-morpholino)ethanesulfonic acid; PBS = phosphate-buffered saline; RT = room temperature. [Color figure can be viewed at www.annalsofneurology.org]]
monomeric α-syn in a process where species range from low- to large-molecular-weight oligomers, protofibrils, and fibrils. To investigate which molecular species of α-syn are capable of activating immune cells, we directly compared the immune response to monomeric recombinant α-syn, several types of low- and high-molecular-weight α-syn oligomeric species, as well as different α-syn fibrillar assemblies. We assessed oligomeric α-syn size and morphology by analytical ultracentrifugation and characterized DA and GA oligomers as relatively small and homogenous particles, whereas oligomers type A and C were more heterogeneous and contained very large oligomers resembling protofibrils of α-syn (see Fig 1D, Table). Stimulation of blood monocytes and BV2 microglial cells resulted in strong activation of the immune cells from all oligomeric α-syn species, but not from monomeric α-syn at the same concentration (see Fig 2E, F). Thus, monocyte activation is rising with increasing aggregation state or molecular weight of oligomeric α-syn, with mature α-syn fibrils inducing the strongest activation of monocytes.

Recent studies have demonstrated that α-syn assembles into distinct fibrillar α-syn polymorphs under different...
experimental conditions.\textsuperscript{12–14} These strains of \( \alpha \)-syn result in different features of pathology in animal models, and have different structures and physicochemical properties, resulting in the exposure of different epitopes to their surfaces.\textsuperscript{12,31} However, it is not known whether this affects the immunogenicity of \( \alpha \)-syn aggregates. To investigate whether conformation of \( \alpha \)-syn fibrils affects also the activation of immune cells, we stimulated blood monocytes and BV2 microglial cells with the same \( \alpha \)-syn concentration (10\( \mu \)g/ml) of \( \alpha \)-syn fibrils, ribbons, and 3 additional fibrillar \( \alpha \)-syn polymorphs (fibrils-65, fibrils-91, and C-terminally truncated \( \alpha \)-syn fibrils-110).\textsuperscript{12–15} We found that the strong activation of immune cells by \( \alpha \)-syn fibrils is not observed in response to \( \alpha \)-syn ribbons, but weaker activation is observed by 2 other strains of \( \alpha \)-syn (fibrils-91 and fibrils-110; see Fig 2H, I).

**Association of \( \alpha \)-syn to EVs Further Increases Activation of Immune Cells**

\( \alpha \)-syn is released into the extracellular space not only as free protein but also in association with EVs.\textsuperscript{32} EVs greatly increase the uptake of \( \alpha \)-syn by recipient cells and are believed to facilitate the cell-to-cell transmission of oligomeric \( \alpha \)-syn.\textsuperscript{19} Previously, we observed that peripheral blood monocytes can be activated by EVs depending on their protein cargo.\textsuperscript{33} To investigate in-depth the effect of \( \alpha \)-syn association to EVs on immune activation, we measured the immune response of healthy control blood monocytes to EVs secreted by HEK293 cells overexpressing human wild-type \( \alpha \)-syn and compared it to EVs from control-transfected HEK293 cells (vector backbone). We characterized the purified EVs by NTA, MS-based proteomic analysis, and Western blot (Fig 3A–D). We found that the proteome of all EV preparations corresponds to the proteomic signature of EVs (for a list of GO-terms and most abundant proteins, see Supplementary Table 2). Furthermore, EVs at the expected size (assessed by NTA) and contain \( \alpha \)-syn when isolated from the cell culture media of HEK293 cells overexpressing \( \alpha \)-syn. Interestingly, we found that EVs from cells overexpressing wild-type human \( \alpha \)-syn induce a significantly higher activation of healthy control monocytes (see Fig 3E) than EVs from cells transfected with control plasmid.

Next, we asked if there are differences in immune cell activation when \( \alpha \)-syn is associated with EVs or present in its free form. \( \alpha \)-syn–containing EVs induced much stronger activation of blood monocytes than the same protein amount from the EV-free fraction of the cell culture medium (see Fig 3F). To compare the amount of total \( \alpha \)-syn found in EVs and in the EV-free fraction, we utilized a very sensitive, previously described luciferase reporter system, in which \( \alpha \)-syn is fused to full-length luciferase.\textsuperscript{8,19} Using this approach, we observed a much stronger activation of monocytes upon exposure to \( \alpha \)-syn associated with EVs,}

### TABLE. Sedimentation Coefficients and Relative Abundance of the Different \( \alpha \)-syn Oligomeric Species Used Throughout this Study

| Species                  | Sedimentation Coefficient, \( s_{20,w} \) | Relative Abundance, % | Estimated MW, kDa | Estimated No. of \( \alpha \)-syn Monomers |
|--------------------------|------------------------------------------|-----------------------|-------------------|------------------------------------------|
| Monomer                  | 1.12                                     | 100                   | 14.5              | 1                                        |
| Oligomer (GA mediated)   | 4.82                                     | >95                   | 230               | 16                                       |
| Oligomer (DA mediated)   | 5.74                                     | >95                   | 310               | 20                                       |
| Oligomer (type A)        | 13,600–35,800                            | 50                    | 10,400,000–44,000,000 | 720,000–3,030,000               |
| Oligomer (type C)        | 5,075                                    | >90                   | 2,350,000         | 162,000                                  |
| Fragmented fibrils       | 110                                      | >95                   | 14,500            | 1,000                                    |
| Fragmented ribbons       | 84                                       | >95                   | 10,150            | 700                                      |
| Fragmented fibrils-65    | 110                                      | >95                   | 14,500            | 1,000                                    |
| Fragmented fibrils-91    | 110                                      | >95                   | 14,500            | 1,000                                    |
| Fragmented fibrils-110   | 120                                      | >95                   | 16,800            | 1,160                                    |

Monomeric and oligomeric (native, DA, and GA) \( \alpha \)-syn samples (400\( \mu \)l in phosphate-buffered saline, pH 7.4) were spun at 55,000\( \times \)g (220,000 \( \times \)g) and 25,000\( \times \)g (45,500 \( \times \)g), respectively, at 20°C. The sonication-fragmented fibrillar assemblies were spun at 3,000\( \times \)g (655 \( \times \)g) at 20°C. Oligomers A and C were spun at 3,000 \( \times \)g (655 \( \times \)g) at 20°C. Sample displacement profiles were obtained by recording the absorbance at 280nm every 5 minutes. DA = dopamine; GA = glutaraldehyde; MW = molecular weight; \( \alpha \)-syn = \( \alpha \)-synuclein.
as compared to identical amounts of free α-syn. Furthermore, we measured the release of 10 different cytokines after stimulation with free, recombinant α-syn or α-syn EVs, and found a similar cytokine signature, including release of proinflammatory cytokines (interleukin [IL]-1β, IL-6, IL-8, IL-12p70, and tumor necrosis factor α [TNF]-α), but not anti-inflammatory cytokines (IL-4, IL-13).

To further investigate if immune activation by EVs depends on α-syn conformation, we tested the effect of 2 PD familial mutations: A30P and A53T.34,35 We compared the immune responses to EVs of cells overexpressing wild-type or mutant α-syn, and found that mutations significantly increased the activation of monocytes and BV2 cells (see Fig 3H–I) by α-syn EVs.

**Cellular Uptake Is Important for Immune Cell Activation by EV-Associated α-syn**

We have previously shown that EVs markedly enhance the uptake of α-syn oligomers.19 Therefore, we next investigated whether the increased immune activation by EV-associated α-syn is linked to its uptake. By labeling EVs with a fluorescent membrane probe, we found that EVs are efficiently internalized both by primary human monocytes and by BV2 microglia (Fig 4), but not by non-phagocytic cells (primary human lymphocytes, data not shown). Previously, we found that phagocytosis inhibitor cytochalasin D efficiently blocks EVs uptake by phagocytic cells.8 Interestingly, blocking uptake with cytochalasin D significantly reduced the activation of BV2 cells by EV-associated α-syn, but not by lipopolysaccharide (LPS), suggesting that activation of immune cells by EV-associated α-syn is at least in part due to its uptake. Also, with human monocytes stimulated with EV-associated α-syn, a dose-dependent decrease of activation was observed with both cytochalasin D or an inhibitor of endocytosis (dynasore). Furthermore, we observed a trend for reduced immune activation of monocytes by EVs by blocking toll-like receptor 2 and toll-like receptor 4 with antibodies. These data further strengthen the idea that uptake and toll-like receptor signaling are important for the activation of innate immune cells by EV-associated α-syn.

**Increased Activation of Healthy Blood Monocytes by EVs from PD Blood**

To address the question of whether the sensitivity of human monocytes to α-syn pathology is relevant for the disease in humans, we next turned to patient-derived material. While there is still no consensus on if levels of free α-syn are increased in PD plasma,36 3 independent recent studies demonstrated increased α-syn in EVs from PD blood.37–39 We isolated EVs from the blood of PD patients or healthy controls as recently described33 (the characteristics of the study cohorts are summarized in Supplementary Table 1), and assessed monocyte activation by the EVs (Fig 5). Interestingly, EVs from PD plasma induced a 2.5-fold increase in IL-6 release when compared to EVs from healthy controls. To rule out effects of contamination with plasma proteins, we treated monocytes with full plasma from PD patients and healthy controls and found no difference in monocyte activation. To verify these results, we also stimulated monocytes with extensively washed EVs from serum and found a similar trend for increased activation by PD EVs. Furthermore, we found a trend for increased activation of PD monocytes both by EVs from healthy control blood and from PD blood.

**Monocytes from PD Patients Are Predisposed to Increased Immune Response to Pathologic α-syn**

Several lines of genetic, epidemiologic and experimental evidence suggest that inflammatory predisposition is associated with higher risk for PD.40–42 We and others have previously demonstrated that monocytes from PD patients are dysregulated and present with a characteristic subset composition and increased cytokine response to LPS.9,43 Thus, we hypothesized that α-syn pathology and inflammatory predisposition act synergistically to result in excess inflammatory activation in PD. To investigate this hypothesis, we compared the immune response of healthy control monocytes and PD patient monocytes to α-syn. PD monocytes secreted significantly higher levels of IL-6 than healthy control monocytes in response to wild-type, luciferase-tagged α-syn-EVs and to untagged wild-type and untagged mutant α-syn-EVs (Fig 6A, B). To assess whether PD monocytes are also hyperactive in response to free α-syn, and whether hyperactivity is specific to distinct species of aggregated α-syn, we stimulated monocytes from healthy controls and PD patients with α-syn oligomers type A and C and with the different fibrillar strains (see Fig 6C, D). We found that monocytes from PD patients are hyperactive in response to all α-syn assemblies that induced significant monocyte activation.

Finally, to investigate whether α-syn pathology itself can contribute to the inflammatory predisposition of monocytes, we used a well-described mouse model of PD, in which α-syn is overexpressed under the control of the Thy1 promoter. We applied 6-color flow cytometry to characterize subpopulation composition of blood monocytes, which is a reliable marker in several neurodegenerative models34,45 and parallels the dysregulation of monocyte subsets in human PD.43,45 Whereas the total number of monocytes did not differ between wild-type and α-syn transgenic mice (see Fig 6E), we found an enrichment of proinflammatory Ly6C-high monocytes and...
concurrently a reduction in less-aggressive Ly6low monocytes in the peripheral blood of α-syn transgenic mice compared to wild-type mice. This switch in the phenotype of monocytes was observed around the onset of motoric symptoms, but not in younger α-syn transgenic mice (see Fig 6F). These results suggest that the overexpression of
α-syn and concomitant pathology in the mouse CNS is sufficient for changes in monocytes’ phenotype in the periphery.

**Discussion**

In the present study, we show that innate immune cells are sensitive to pathologic changes in α-syn and its association to EVs. Moreover, we demonstrate that the hyperactivity of PD monocytes to inflammatory stimuli is found also in response to pathologic α-syn.

The α-syn polymorphs that were investigated in this study induce cell toxicity and seeded assembly of α-syn to different extents. The specific spatial orientation of α-syn monomers in these polymorphs, and possibly distinct α-syn folds, lead to the exposure of strain-specific surface epitopes, which likely result in the different extent of immune activation reported in our study. Whereas the activation of innate immune cells by high-molecular-weight, fibrillar α-syn is widely recognized, the role of lower-molecular-weight α-syn assemblies (eg, oligomers)
FIGURE 5: Extracellular vesicle (EVs) from Parkinson disease (PD) patient plasma activate peripheral blood monocytes. (A) EVs from healthy control (HC) plasma induce a slight activation in HC monocytes (control [Con] vs HC Plasma EVs, 0.03 vs 0.13, \( p = 0.33 \); Con vs HC Plasma, 0.03 vs 0.07, \( p = 0.58 \), 1-way analysis of variance [ANOVA]). (B) EVs from HC serum induce a moderate activation in HC monocytes (Con vs HC Serum EVs, 0.01 vs 1.34, \( p < 0.001 \); Con vs HC Serum sup, 0.01 vs 0.19, \( p = 0.88 \), 1-way ANOVA). (C) Stimulation of peripheral blood monocytes with EVs derived from the blood of different donors (allogenic, same species but different individuals) does not significantly influence the monocyte response when compared to EVs of the same donor (autologous, same individual; autologous vs allogenic, 1.29-fold, \( n = 7/6 \), \( p = 0.57 \), t test). (D, E) Stimulation of HC monocytes with EVs (HC/PD, \( n = 11/12 \); Mann–Whitney test; D) or unfractionated plasma (10%; HC/PD, \( n = 23/24 \); Mann-Whitney test; E) from HCs and PD patients. Line indicates geometric mean ± 95% confidence interval of geometric mean. (F) Washed serum EVs from PD patients show a trend for increased activation of HC monocytes (HC/PD, \( n = 6/6 \), \( p = 0.19 \), t test). (G, H) PD monocytes show a trend for increased immune response to EVs from both HC serum (\( p = 0.08 \), t test) and PD serum (\( p = 0.72 \); \( n = 3/3 \) for monocyte donors and \( n = 6/6 \) for serum donors). (I) Characterization of EVs with atomic force microscopy (scale bar = 100nm). (J) Serum EVs from PD patients do not significantly differ in size from serum EVs from HCs, as assessed by nanoparticle tracking analysis (HC/PD = 12/12). *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \), ****\( p < 0.0001 \). IL = interleukin; LPS = lipopolysaccharide, sup = supernatant. [Color figure can be viewed at www.annalsofneurology.org]
remains controversial.\(^{46,47}\) We investigated the immune response to 4 different types of prebrillar oligomers, and found that all types of oligomers potently activate monocytes and microglia. We carefully controlled the activation of immune cells by oligomers, by testing the immune response to the monomer used for the preparation of oligomers and to the buffers specific to 2 of the protocols. Furthermore, we avoided the use of 4-hydroxynonenal, a compound commonly used to produce \(\alpha\)-syn oligomers, as this compound inhibits TNF-\(\alpha\) release even at very low concentrations.

The different \(\alpha\)-syn assemblies and polymorphs we used have distinct molecular weights and are thus constituted of different numbers of \(\alpha\)-syn monomers per particle. In the present study, we exposed immune cells to the same total amounts of \(\alpha\)-syn in different assemblies and polymorphs. Furthermore, high-molecular-weight polymorphs were fragmented by sonication prior to use to ensure that particle concentrations are comparable. Still, we observed that high-molecular-weight assemblies and polymorphs induced a stronger immune response than monomers and low-molecular-weight assemblies. Furthermore, the immune response depended on \(\alpha\)-syn high-molecular-weight assemblies’ conformation. This strongly suggests that the immune response depends on the surfaces of \(\alpha\)-syn assemblies rather than only on the number of monomers in the protein particle. Interestingly, our findings are in agreement with a recent study where a set of peptides originating from \(\alpha\)-syn were shown to act as epitopes for the activation of cytotoxic and helper T cells.\(^{40}\) This and other studies suggest that the adaptive immunity is capable of a specific immune response to \(\alpha\)-syn pathology; however, little is known about the specificity of the innate immune response, which is the first line of defense and not only

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**FIGURE 6:** Monocytes from Parkinson disease (PD) patients are hyperactive in response to stimulation with pathologic \(\alpha\)-synuclein (\(\alpha\)-syn). (A, B) PD monocytes are hyperactive in response to stimulation with extracellular vesicles (EVs) from cells overexpressing wild-type (wt), luciferase (luc)-tagged \(\alpha\)-syn (A; \(n = 9/9\), Mann–Whitney test), or wt and mutant, untagged \(\alpha\)-syn (B; \(n = 3/3\), 1-way analysis of variance [ANOVA]; bars = mean ± standard error of the mean [SEM]). (C, D) PD patient monocytes are hyperactive in response to stimulation with oligomeric (C) and fibrillar (D) \(\alpha\)-syn (PD/healthy controls [HCs] \(n = 3/3\), 1-way ANOVA, same internal control group [lipopolysaccharide (LPS)], bars = mean ± SEM. Unconditioned RPMI-1640 medium was used as control (10% fetal calf serum); LPS, 1ng/ml; bovine serum albumin, 1mg/ml; \(\alpha\)-syn, 10\(\mu\)g/ml. (E, F) Dysregulation of monocyte subsets in Thy-1 mouse model of PD. Expression of \(\alpha\)-syn in the central nervous system results in dysregulation of the ratio of Ly6Chigh to Ly6C\(^{\text{low}}\) monocytes (F) in the peripheral blood of Thy-1 mice around onset of symptoms (14 months), without a concomitant net increase in total monocyte numbers (E; bars = mean ± SEM, \(n = 10/20\) [8 months], \(n = 10/22\) [14 months]). *\(p < 0.05\), **\(p < 0.01\), ****\(p < 0.0001\). IL = interleukin; mon. = monomeric. [Color figure can be viewed at www.annalsofneurology.org]
It is now widely recognized that monomeric α-syn associates to cellular membranes and microvesicles and can be released by cells through EVs. In the present study, we demonstrate that association of α-syn to EVs dramatically increases its potency to activate monocytes and microglia. Interestingly, α-syn concentration in EVs is very low in both cell culture- and blood-derived EVs (unpublished observations), but is still sufficient to induce a robust inflammatory response. The concentration is approximately 1,000 times lower than the concentration of recombinant α-syn oligomers and fibrils necessary for a similar response. An important question, therefore, is whether α-syn itself, or some other component of EVs, results in the immune activation by α-syn-EVs. Several lines of evidence strongly suggest that α-syn is responsible for the immune activation: First, EVs from naïve or mock-transfected cells failed to induce the same immune response. Second, our proteomic analysis identified the same protein composition independent of whether EV’s came from α-syn-overexpressing cells or not. Third, by employing 3 different overexpression strategies, we excluded that the luciferase tag or the coexpressed regulatory sequences mediate the effects we observed. Fourth, the same mutation (A53T) that increases the strongest immune response by purified, recombinant α-syn also increases the strongest immune response to EVs from cells overexpressing α-syn. Taken together, this evidence strongly suggests that EV-associated α-syn is the trigger for the activation of innate immune cells.

We have previously demonstrated that microglia take up EV-associated α-syn much more efficiently than free α-syn, whereas in the present study we found that EVs dramatically increase immune activation by α-syn. The activation by EVs was significantly reduced after blocking cellular uptake. Thus, we hypothesize that EVs could serve as a vector for shuttling α-syn that triggers inflammation into immune cells. In line with this, Matsumoto and colleagues recently observed that application of EVs in vivo results in upregulation of iNOS only in microglia that take-up the EVs. Furthermore, the increased activation of monocytes by PD patient-derived blood EVs confirms that EVs may also play a role in vivo.

In summary, we show that monocytes from PD patients are hyperactive in response to free- and EV-associated α-syn, thus providing a link between immune dysregulation and α-syn aggregation. We demonstrate that monocytes and microglia are sensitive to pathologic α-syn, and are possibly an important determinant of early pathology in PD and other synucleinopathies. As recently demonstrated, monocytes are not just passive bystanders in model synucleinopathies, but have an active role in the events that lead to loss of dopaminergic neurons. Thus, if monocytes prove a similar role in human disease, the inflammatory predisposition could be an important, therapeutically relevant factor for PD etiology.

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Author Contributions

V.G., P.J.M., J.K., C.Be., A.C.L., J.H.W., R.M., and KD contributed to the conception and design of the study. V.G., L.B., M.H., C.Bl., C.M., K.M., L.P., and M.K. contributed to the acquisition and analysis of the data. V.G., L.B., M.H., J.H.W., R.M., and K.M.D. contributed to drafting the text and preparing the figures.
Nothing to report.

References

1. Harms AS, Delic V, Thome AD, et al. alpha-Synuclein fibrils recruit peripheral immune cells in the rat brain prior to neurodegeneration. Acta Neuropathol Commun 2017;5:85.

2. Harms AS, Thome AD, Yan Z, et al. Peripheral monocyte entry is required for alpha-synuclein induced inflammation and neurodegeneration in a model of Parkinson disease. Exp Neurol 2018;300:179–187.

3. Byler SL, Boehm GW, Karp JD, et al. Systemic lipopolysaccharide plus MPTP as a model of dopamine loss and gait instability in C57Bl/6J mice. Behav Brain Res 2009;198:434–439.

4. Couch Y, Alvarez-Erviti L, Sibson NR, et al. The acute in

5. McGeer PL, Itagaki S, Boyes BE, McGeer EG. Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson’s and Alzheimer’s disease brains. Neurology 1988;38:1285–1291.

6. Cunningham C, Wilcockson DC, Campion S, et al. Central and systemic endotoxin challenges exacerbate the local inflammatory response and increase neuronal death during chronic neurodegeneration. J Neurosci 2005;25:9275–9284.

7. Gath J, Bousset L, Habenstein B, et al. The precursor protein of non-A beta component of Alzheimer’s disease amyloid is a presynaptic protein of the central nervous system. Neuron 1995;14:467–475.

8. Grozdanov V, Bliederhaeuser C, Grozdanov V, Speidel A, et al. Age-dependent defects of alpha-synuclein oligomer uptake in microglia and monocytes. Acta Neuropathol 2016;131:379–391.

9. Grozdanov V, Bliederhaeuser C, Grozdanov V, Rup WF, et al. Inflammatory dysregulation of blood monocytes in Parkinson’s disease patients. Acta Neuropathol 2014;128:651–663.

10. Melki R. How the shapes of seeds can influence pathology. Neurobiol Dis 2018;109(pt B):201–208.

11. Lee HJ, Patel S, Lee SJ. Intravesicular localization and exocytosis of alpha-synuclein and its aggregates. J Neurosci 2005;25:6016–6024.

12. Zarranz JJ, Alegre J, Gomez-Esteban JC, et al. The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. Ann Neurol 2004;55:164–173.

13. Zarranz JJ, Alegre J, Gomez-Esteban JC, et al. The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. Ann Neurol 2004;55:164–173.

14. Potroka C, Dudzik CG, Brier T, et al. A novel alpha-synuclein missense mutation in Parkinson disease. Neurology 2013:80:1062–1064.

15. Singleton AB, Farrer MJ, Bonifati V. The genetics of Parkinson’s disease: progress and therapeutic implications. Mov Disord 2013;28:14–23.

16. Kamyoshihara T, Kojima M, Ueda K, et al. Observation of multiple intermediates in alpha-synuclein fibril formation by singular value decomposition analysis. Biochim Biophys Res Commun 2007;355:398–403.

17. Delic V, Thome AD, Yan Z, et al. Peripheral monocyte entry is required for alpha-synuclein induced inflammation and neurodegeneration in a model of Parkinson disease. Exp Neurol 2018;300:179–187.

18. Schuck P. Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and lamrn equation modeling. Biophys J 2000;78:1606–1619.

19. Schuck P. Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and lamrn equation modeling. Biophys J 2000;78:1606–1619.

20. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 2012;9:671–675.

21. Shih M, Liu C, Cook TJ, et al. Plasma exosomal alpha-synuclein is associated with glucocerebrosidase activity and correlates with measures of disease severity in PD patients. Front Cell Neurosci 2018;12:125.

22. Zhao ZH, Chen ZT, Zhou RL, et al. Increased DJ-1 and alpha-synuclein in plasma neural-derived exosomes as potential markers for Parkinson’s disease. Front Aging Neurosci 2018;10:438.

23. Sulzer D, Alcalay RN, Garretti F, et al. T cells from patients with Parkinson’s disease recognize alpha-synuclein peptides. Nature 2017;546:656–661.
41. Hamza TH, Zabetian CP, Tenesa A, et al. Common genetic variation in the HLA region is associated with late-onset sporadic Parkinson’s disease. Nat Genet 2010;42:781–785.

42. Chen H, Jacobs E, Schwarzschild MA, et al. Nonsteroidal antiinflammatory drug use and the risk for Parkinson’s disease. Ann Neurol 2005;58:963–967.

43. Cook DA, Kannarkat GT, Cintron AF, et al. LRRK2 levels in immune cells are increased in Parkinson’s disease. NPJ Parkinsons Dis 2017;3:11.

44. Zondler L, Muller K, Khalaji S, et al. Peripheral monocytes are functionally altered and invade the CNS in ALS patients. Acta Neuropathol 2016;132:391–411.

45. Bliederhaeuser C, Zondler L, Grozdanov V, et al. LRRK2 contributes to monocyte dysregulation in Parkinson’s disease. Acta Neuropathol Commun 2016;4:123.

46. Hoffmann A, Ettle B, Bruno A, et al. Alpha-synuclein activates BV2 microglia dependent on its aggregation state. Biochem Biophys Res Commun 2016;479:881–886.

47. Kim C, Ho DH, Suk JE, et al. Neuron-released oligomeric alpha-synuclein is an endogenous agonist of TLR2 for paracrine activation of microglia. Nat Commun 2013;4:1562.

48. Alvarez-Erviti L, Seow Y, Schapira AH, et al. Lysosomal dysfunction increases exosome-mediated alpha-synuclein release and transmission. Neurobiol Dis 2011;42:360–367.

49. Matsumoto J, Stewart T, Sheng L, et al. Transmission of alpha-synuclein-containing erythrocyte-derived extracellular vesicles across the blood-brain barrier via adsorptive mediated transcytosis: another mechanism for initiation and progression of Parkinson’s disease? Acta Neuropathol Commun 2017;5:71.