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Supplementary Information for

NK cells clear α-synuclein and the depletion of NK cells exacerbates synuclein pathology in a mouse model of α-synucleinopathy.

Rachael H. Earls[1*], Kelly B. Menees[1*], Jaegwon Chung[1*], Claire-Anne Gutekunst[2], Hyun Joon Lee[3], Manuel G. Hazim[2], Balázs Rada[4], Levi B. Wood[5], Jae-Kyung Lee[1†]

[†]Corresponding author: Jae-Kyung Lee, PhD
jamlee@uga.edu

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Supplementary Information Text

MATERIALS AND METHODS

Preparation of recombinant proteins and aggregates

Recombinant human α-syn (> 98 % purity) proteins were expressed in BL21(DE3)/RIL *E.coli* and purified by size exclusion chromatography, and Mono Q ion exchange chromatography as previously described (1). To further remove endotoxin contamination, it was purified by High S support cation exchange chromatography as described (2, 3) in the Bioexpression and Fermentation Facility (BFF) core at the University of Georgia. Final endotoxin tests resulted in less than 0.5 EU/mg. For α-syn fibril preparation, α-syn proteins were assembled into aggregates by incubating at 37°C at concentrations of 5 mg/mL with continuous shaking at 1100 rpm for 7 days. α-Syn fibrils were sonicated using a high intensity ultrasonic water bath (30 % power, 1 hr) at 4°C prior to surgical injections or addition in the primary hippocampal neuronal cultures. α-Syn uptake and degradation assays were performed using α-syn from rPeptide. α-Syn proteins (rPeptide, Bogart, GA, USA) were assembled into aggregates by incubating at 37°C at concentrations of 1 mg/mL with continuous shaking at 800 rpm for 7 days and aggregation was confirmed by a thioflavin T assay and TEM imaging.

Transmission Electron Microscope (TEM) imaging

A formvar, carbon-coated 400-mesh grid was floated onto a 40 µl drop of sample for 15 minutes. The grid was removed; excess sample is drained off with the edge of a filter paper and floated onto a drop of filtered 0.5 % aqueous uranyl acetate (UA) for 30 seconds. After draining excess UA from grid, the grid was allowed to dry on filter paper prior to viewing with the JEOL JEM-1011 Transmission Electron Microscope at an accelerating voltage of 80 kV. Representative digital images were taken with an XR80M Wide-Angle Multi-Discipline Mid-Mount CCD Camera from Advanced Microscopy Techniques (AMT) at varying magnifications. The Image-J software in the camera's system was used to measure the fibers.

Primary Hippocampal Culture

The hippocampus was dissected from mouse pups from postnatal day 0-2 (P0-2) and the meninges were removed. The tissue was enzymatically dissociated with a combination of dispase II (Roche), DNase I (Invitrogen), and papain (Sigma-Aldrich) for 15 min in a 37 °C, 5 % CO_2 incubator. The tissue was then mechanically dissociated by trituration with a fire polished glass pipet. The cell suspension was then filtered through a 40 µm cell strainer. Cells were counted and plated on poly-D-lysine coated coverslips in 24 well plates at 100,000 cells per well. On day 7 of culture, neurons were treated with 1 µg/mL of sonicated PFF α-syn, non-sonicated PFF α-syn, monomer α-syn, or the same volume of PBS. PFF α-syn was sonicated for 1 hr prior to addition to cell culture. Seven days following treatment cells were fixed with 4 % paraformaldehyde (PFA).

Cell lines

NK92 cells, a human NK cell line that was purchased from ATCC, were grown in RPMI 1640 culture medium (Corning Cellgro) supplemented with 10 % FBS (Gemini Bio-Product), 100 UI/mL of IL-2 (Peprotech), 2 mM L-glutamine (Gibco), penicillin (100 UI/mL) and streptomycin (0.1 µg/mL) (Gibco). K562 leukemia cells (human chronic myelogenous leukemia, CML), were kindly gifted by Dr. Mathew at University of North Texas Health Science Center at

Fort Worth, TX. Cells were cultured in complete medium consisting of RPMI 1640, 10 % FBS, 2 mM L-glutamine (Gibco), penicillin (100 UI/mL) and streptomycin (10 µg/mL) (Gibco).

Primary human NK cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of healthy donors using Ficoll-Paque PLUS density gradient medium (GE Healthcare) and centrifugation at 1500 rpm for 20 min with no brake. Following centrifugation, the PBMC layer was isolated. Primary human NK cells were isolated from PBMC suspension using the EasySep™ Human NK Cell Isolation Kit (StemCell Technologies). Briefly, PBMCs were suspended at 5×10^7 cells/mL and the Isolation Cocktail was added to the PBMC suspension and incubated at room temperature for 5 min. RapidSpheres™ were added to the PBMC mixture and the tube containing the cells was immediately placed into the magnet. Following 3 min of incubation at room temperature, the magnet was inverted, and the isolated NK cell suspension was poured off and ready to use.

α-Synuclein Internalization Assays

NK92 and primary human NK cells were incubated with 0, 0.1, 1, or 5 µg/mL α-syn aggregates (rPeptide) as indicated for 1 hour in triplicates. Following the incubation period, cells were then washed 3 times in 1× PBS. Cells were then prepared for western blot analysis as described in *Protein sample preparation and Western blot analysis*. To investigate the mechanism through which NK cells internalize α-syn aggregates, NK92 cells were incubated with 0, 1, 5, or 10 µg/mL of TLR2-neutralizing (InvivoGen), 0, 1, 5, or 10 µg/mL of TLR4-neutralizing (InvivoGen) antibodies, or with heparan (100 ug/mL; Amsbio) for 1 hour. Cells were then incubated with 5 µg/mL α-syn aggregates for 1 hour and then prepared for western blot analysis as described in *Protein sample preparation and Western blot analysis.* To investigate the endocytosis mechanism of α-syn internalization by NK cells we utilized several inhibitors involved in endocytosis mechanisms. NK92 cells were incubated with Rottlerin (30µM; Calbiochem) cytochalasin D (1µM; Sigma), or dynasore (80µM; Sigma) for 1 hour and then incubated with 5 µg/mL α-syn aggregates for 1 hour. Cells were then prepared for western blot analysis as described in *Protein sample preparation and Western blot analysis*.

α-Synuclein Degradation Assays

NK92 and primary human NK cells were incubated with 5 µg/mL α-syn aggregates (rPeptide) for 1 hour. Cells were then washed 3 times in 1× PBS and incubated in fresh medium for 0, 1, 4, or 24 hours as indicated. Following the indicated incubation time cells were prepared for western blot analysis as described in *Protein sample preparation and Western blot analysis*.

NK cell cytotoxicity assay

Cytotoxicity activity was assessed using the calcein AM (Life Technologies) release assay. The K562 target cells were stained with 2 µg/mL calcein AM as described (4). Briefly, the K562 target cells were suspended at 1×10^6 cells/mL in calcein AM-containing media and incubated for 40 minutes at 37°C in a 5 % CO_2 incubator. The target cells were washed and resuspended at 1×10^6 cells/mL in RPMI complete media. Aliquots of 100 µl from each NK effector cell serial dilution were added per well in a 96 well round bottom plate in triplicates or quadruplicates. Aliquots of 100 µl of calcein AM loaded K562 cells were added (1×10^5 cells/well) to each of the wells to generate a different effector-to-target (E:T) ratio. Maximum and spontaneous release controls were set up in 4 replicates using 1 % Triton X-100 and media only, respectively. After a

4 hr incubation, the cells were gently mixed to evenly distribute the released calcein AM in the supernatant, and the plate was spun at 400 g for 2 min to pellet the cells and any debris. Then, 150 µl of the supernatant was recovered and transferred to a 96 well clear bottom black plate. The fluorescence was read using a BioTek Synergy 2 plate reader (Ex: 485 nm/Em: 530 nm). The percent specific lysis was calculated using the formula [(Test release−Spontaneous release)/(Maximum release−Spontaneous release)] × 100.

IFN-γ ELISA assay
NK92 cells were rested in complete medium without IL-2 for 24 hrs. NK92 cells were plated at 200,000 cells per well in a 96 well round bottom plate. α-Syn aggregates were treated in various concentrations for 24 hrs in triplicates. Cell supernatants were collected and analyzed by enzyme-linked immunosorbent assay (ELISA) using a human IFN-γ ELISA Ready-SET-Go!® kit from eBioscience (Affimatrix). Primary human NK cells were plated at 10,000 cells per well in a 96 well plate. α-Syn aggregates were treated in various concentrations for 24 hrs in triplicates. Cell supernatants were collected and analyzed by ELISA using a human IFN-γ ELISA Ready-SET-Go!® kit from eBioscience (Affimatrix).

Cell proliferation assay
Cell viability was measured by using the CellTiter 96 Aqueous Assay reagent (Promega). This reagent utilizes the tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt;MTS) and the electron coupling reagent, phenazine methosulfate (PMS). MTS is chemically reduced into formazan by cells, which is soluble in tissue culture medium. The measurement of the absorbance of formazan was performed in multi-titer 96-well plates at 492 nm during the last 4 hrs of a 72 hr culture. Each experimental condition was performed in quadruplicate, and three independent experiments were conducted to confirm the results.

Immunocytochemistry
Primary hippocampal cells were incubated with antibodies against MAP2 (Invitrogen) (1:1000) and p-α-syn (Abcam) (1:200) overnight followed by incubation with Alexa fluor 594- and Alexa fluor 488-conjugated secondary antibodies (Invitrogen) (1:1000) against primary antibodies for 2 hr at RT. Coverslips were then mounted on microscope slides with Fluoroshield Mounting Medium with DAPI (Abcam). NK92 cells were treated with 5 µg/mL of α-syn aggregates for 1 hour. Cells were washed 3 times with PBS and then fixed with 4 % PFA. NK92 cells were incubated with antibodies against α-syn (Cell Signaling Technology), LC3B (Novus Biologicals) or Rab7 (Cell Signaling) overnight followed by incubation with Alexa fluor 594- or 488-conjugated secondary antibodies (Invitrogen). For human postmortem brains, tissues were incubated in 0.2 M glycine for 1 hr to reduce autofluorescence. The samples were then permeabilized in TBS with 0.3 % Triton X-100 for 35 min followed by blocking in 1 % normal serum in TBS for 1 hr. Samples were incubated in primary antibodies for anti-Iba1 (Wako) and anti-human 2B4 (Biolegend) overnight followed by appropriate Alexa Fluor 488- and 594-conjugated secondary antibodies (Thermo Fisher). All digital images were acquired on a Nikon A1R confocal microscope (Nikon Eclips Ti-E inverted microscope).

Protein sample preparation and Western blot analysis
NK92 cells and human NK cells were separated into Triton X-100 soluble and insoluble fractions, then insoluble fractions were processed for western blot analysis. Cells were lysed in a

buffer containing 1 % Triton X-100 and 1 × protease inhibitor mix (Sigma) for 10 min on ice. Lysates were centrifuged at 16,000 g for 5 min at 4 °C. Triton X-100 supernatant was then transferred to a new tube and mixed with 4 × Laemmli sample buffer. The remaining pellet was then washed with ice cold PBS and centrifuged at 16,000 g for 5 min at 4 °C. The supernatant was then removed and the remaining Triton X-100 insoluble pellet was resuspended in 1 × Laemmli sample buffer. Triton X-100 insoluble samples were then sonicated using a high intensity ultrasonic water bath (50 % power, 5 sec pulses for 1 min) at 4 °C prior to being loaded on pre-cast 4-20 % SDS electrophoresis gels (Bio-Rad), transferred onto PDVF membranes (Millipore), and probed with anti-α-syn (MJFR1, Abcam), β-actin (Santa Cruz biotechnology) and the appropriate HRP-conjugated secondary antibody (1:2000; Jackson ImmunoResearch Lab).

For quantification of striatal TH levels, proteins were extracted from four coronal sections on the lesioned side of the striatum closely matching the following coordinates, relative to bregma: 1.26 mm, 0.98 mm, 0.70 mm, 0.42 mm, (n=6-7 mice/group). Protein extractions from fixed tissues were performed by a modified protocol described previously (5, 6). Briefly, striatum tissues were incubated in 150 µl of 300 mM Tris buffer (pH 8.0) containing 2 % SDS. Samples were incubated at 100 °C for 30 min and then 80 °C for 2 hrs. The extracts were centrifuged at 4 °C for 10 minutes at 14,000 g, and supernatants were transferred to fresh microcentrifuge tubes, and protein concentrations were determined using the BCA assay (Pierce, Thermo Scientific). Equal amounts of total protein (5 µg) were loaded on precast PAGE gels and transferred onto PVDF membranes (Millipore) and probed with anti-TH (Millipore), β-actin (Santa cruz biotechnology), and the appropriate HRP-conjugated secondary antibody (Jackson ImmunoResearch Lab). Immunoreactive bands were visualized with Super Signal West Femto horseradish peroxidase substrate (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer's instructions and imaged on a Syngene G:BoxChemi gel documentation station (Frederick, MD, USA).

Mononuclear cell isolation from the CNS

Mononuclear cells from the brain were isolated from the mouse as described previously (7). Briefly, the brains were removed and placed in 3 mL of ice-cold 1 × Hanks balanced salt solution (HBSS) and finely minced with a scalpel, then mixed with 3 mL of 2 × enzymatic dissociation medium containing sterile filtered DNase1 (1 µL/mL, Invitrogen), Dispase II (1.2 U/mL, Roche), and Papain (1 mg/mL, Sigma-Aldrich) dissolved in DMEM/F12 (Invitrogen). Cells from each brain were resuspended in 4 mL of 37 % isotonic percoll (Sigma), layered on a 30:37:70 (4 mL of each in 15-mL conical tube) percoll gradient, with 2 mL of HBSS was layered on the top of the gradient, and centrifuged for 30 minutes at 500 g. Immune cells were obtained from the 37:70 % interface, washed twice in 1x HBSS. Cells were counted and prepared for flow cytometry.

Flow Cytometry

For NK92 cell surface receptor staining, NK92 cells were washed with FACS buffer (1mM EDTA, 0.01 % sodium azide, 0.1 % BSA, 0.02 M phosphate, 0.15 M NaCl, pH 7.2) and then stained for 20 minutes with anti-FcR/anti-CD16+CD32/Fc Block (eBiosciences, Thermo Fisher Scientific) and fluorophore-conjugated antibodies for hCD107 (eBiosciences), hNKG2A (R&D System) and anti-CD314 (NKG2D) (eBiosciences). For cell surface receptor staining, mouse CNS mononuclear cells, splenoctyes, and inguinal lymph node cells were prepared. Cells were

suspended with FACS buffer and then stained for 20 minutes with anti-FcR/anti-CD16+CD32/Fc Block (ebiosciences) and fluorophore-conjugated antibodies for anti-NK1.1, anti-CD45, and anti-CD3 (Biolegend). After staining, cells were washed and then fixed with 1 % PFA for 30 minutes. Post fixation, cells were washed three times with 200 µl FACS buffer, and then 50 µl of 123count eBeads Counting beads (Thermo Fisher) were added to cells to allow for quantification of total number of NK cells following manufacturer's instructions. Data were acquired on a LSRII instrument (BD Biosciences). Analysis was performed using FlowJo software, version 10.0.8.

Stereotaxic Surgery
Mice were anesthetized with Ketamine/Xylazine (100 mg/10 mg/kg) and placed in a stereotaxic frame (KOPF) with ear bars. Animals received a unilateral injection of human PFF or monomer α-syn (5 µg in 1 µl) into the right striatum using stereotaxic coordinates relative to bregma and the dural surface at AP, +0.3 mm; ML, + 2.3 mm; DV, -3.5 mm bregma at the rate of 0.2 µl/min using a 29-gauge needle. The needle was left in place for five minutes before being retracted to allow for complete diffusion of α-syn, and then removed at a rate of one mm per minute to minimize tissue damage. Postoperatively and for the following 3 days, animals received subcutaneous injections of analgesics and were monitored meticulously for signs of pain and discomfort.

In vivo NK cell depletion
mAbs to mouse NK1.1 was produced from hybridomas (PK136) (American Type Culture Collection, Manassas, VA) at the BFF core at UGA. Mouse IgG2a (Sigma-Aldrich) was used as isotype control antibody. To deplete NK cells *in vivo*, 100 µg of anti-NK1.1 was administered through intraperitoneal (i.p.) injection 2 days prior to stereotaxic injection. Thereafter, 50 µg anti-NK1.1 mAb or IgG2a were injected every 5 days for the remainder of the experiment (8, 9). Selective depletion of NK cells was confirmed through flow cytometry analysis of spleen, lymph node, and brain and was consistently > 95 %.

Multiplex Cytokine Analysis
Serum content was analyzed for chemokines and cytokines (IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, KC (CXCL1), IL-10, IL-12p70, and TNF-α) using a multiplexed immunoassay per the manufacturer's instructions (Meso-Scale Discovery, Rockville, MD).

Clinical Scoring
To quantify overall motor deficits at the conclusion of our study, we assigned a score to each mouse at 10 weeks after stereotaxic surgeries, immediately preceding euthanasia. The clinical scoring system was as follows: 0 = normal or healthy; 1 = unsteady gait; 2 = limb weakness/occasional tripping; 3 = severe limb weakness, still able to move and reach food; 4 = severe limb weakness/paralysis, unable to move; 5 = moribund or dead. Incidence was determined by quantifying the number of animals per group with clinical scores of one or greater at the time of euthanasia.

Clasping Task
Behavioral assessment of motor and postural abnormalities was assessed through the clasping task, a marker of disease progression in a number of mouse models of neurodegeneration (10, 11). Briefly, mice were suspended by the base of their tails and the extent of hind limb retraction

in 30 seconds was quantified. Three trials were performed one hour apart every other week beginning 3 weeks after surgery to allow ample recovery time. The average score of three trials was recorded. Clasping scores range from 0 to 3 with 0 representing the absence of clasping, 1 representing any hind limb retracted at least 50 % of the observation period, 2 representing both hind limbs partially retracted at least 50 % of the observation period, 3 representing both hind limbs withdrawn for at least 50 % of the observation period.

Immunohistochemistry

Mice were euthanized and perfused with glucose-containing buffered saline followed by 4 % PFA for fixation. The brains were removed and fixed in 4 % PFA for 24 hrs then cryopreserved in 30 % sucrose solution. Brains were sectioned in a coronal plane in a cryostat at 40 µm thickness and collected to generate eight series of each brain region, i.e. striatum, midbrain and brainstem/cerebellum. For immunofluorescence experiments, brain sections were fixed for 15 min in 4 % PFA. Sections were incubated in 0.2 M glycine for 30 min to reduce tissue autofluorescence. Then brain sections were permeabilized for 30 min in TBS containing 0.3 % Triton X-100, followed by blocking for 1 hr in 1 % normal serum in TBS. Sections were incubated in primary antibodies for anti-Iba1 (Wako), anti-NK1.1, anti-GFAP (Thermo Fisher Scientific), and anti-tyrosine hydroxylase (TH) (Chemcon/Millipore) followed by Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies (Thermo Fisher). For bright field IHC, brain sections were quenched in 3 % hydrogen peroxide (H_2O_2), then blocked in 10 % normal serum for 1 hr each. Following blocking, sections were immunostained with antibodies for TH (Chemicon/Millipore) or phospho-serine129 α-syn (p-α-syn) (Abcam) and incubated overnight in 4°C. Following washes, sections were incubated with biotinylated secondary antibody and then with Vector ABC standard detection kit (Vector Laboratories). TH and p-α-syn staining was visualized by development in 3, 3′ diaminobenzidine (DAB).

Proteinase-K Digestion

One set from an eight series of striatum, SNpc, or brainstem/cerebellum tissue was treated with 10 µg/ml proteinase-K for 15 min at RT, followed by three washes in TBS containing 0.2 % Triton X-100. Then, sections were processed for bright field IHC with an antibody for p-α-syn (Abcam, Ab51253).

Confocal Microscopy

Fluorescence images for p-α-syn, NK1.1, TH, GFAP, and Iba-1 were acquired using a Nikon A1R Confocal microscope (12 bits/channel). Optical sectioning for a Z-series was obtained using a CFI Plan APO VC 60X Oil NA 1.4 WD 0.13mm objective lens with 8 slices at 1.804 µm intervals at a sample speed of 21.6 µs/pixel and processed for analysis using NIS-Elements AR analysis 4.00.12 64-bit software.

Image Analysis

The person who performed the quantification analysis was blinded to all experimental conditions. For quantification analysis of α-syn pathology, we analyzed sections (one set from an eight series) closely matching the following coordinates, relative to bregma: (1) for striatum: 1.30 mm, 1.02 mm, 0.74 mm, 0.46 mm; (2) for SNpc: -2.70 mm, -2.98 mm, -3.26 mm, -3.54 mm; (3) for brainstem/cerebellum: -5.80 mm, -6.08 mm, -6.36 mm. 4-6 images at 40× from each section were randomly captured using a Zeiss Axio Scope A1 microscope. Quantificational analysis was performed using the freeware NIHImageJ1.43 software (National Institute of

Health). For quantification of total α-syn pathology, the average optical densities (ODs) on the lesioned side were calculated. Data were plotted as the average OD value normalized to untreated M83 Tg brain ± SEM for each animal (n=6-7 mice/group). For quantification of PK-resistant α-syn inclusions, the average numbers of α-syn inclusions with size ≥ 7μm per region of interests (ROIs) on the lesioned side were counted. Data were plotted as the average number of p-α-syn inclusions per ROIs in the each brain region normalized to untreated M83 Tg brain ± SEM (n=4 mice/group). For quantification of TH in the striatum, four sections from one set from an eight series of striatum closely matching the coordinates (see coordinates above) were stained with TH. Four images at 10 × for each striatal section were captured. The average ODs for TH positive staining on the lesioned side of striatum were analyzed. The data were plotted as the average OD value for each animal (n=6-7 mice/group). For quantification of astrocytes and microglia, images of GFAP+ and Iba-1+ staining in striatum, SNpc, brainstem, and cerebellum were acquired from using a Nikon A1R Confocal microscope (12 bits/channel). 4-6 images at 40 × from each section were captured within each ROI. The average ODs for GFAP+ or Iba-1+ immunoreactivities (i.r.) were calculated. The data are presented as average OD per brain region normalized to untreated M83 Tg brain ± SEM (n=4 mice/group). Untreated M83 Tg mice brain (3 month-old) was used as the baseline control.

Stereological Analysis

To estimate the total dopaminergic neuron number within the SNpc, we performed unbiased stereology using the Cavalieri method. Stereology was performed using a Leica DM2500 light microscope, in conjunction with Stereologer Version 3.0 CP – Version 2. Injected and non-injected sides were analyzed separately, as identified using a visible punch made prior to the cutting of the sections. The person who performed the counts was blinded to all experimental conditions. For each SNpc, 40 μm coronal sections spanning the entire SNpc were used for the count for a total of eight sections per SNpc separated by 120 μm (every fourth section). The area of SNpc was outlined at 5 × according to the Paxinos and Franklin atlas (12), and the TH-positive neurons were counted at 40 ×. The parameters used for optical dissection are as follows: frame size (25 % of screen height^2 or 2425 μm^2), frame area (25 % of screen height^2 or 2425 μm^2), frame height (20 μm), guard height (2 μm), frame spacing (150 μm). The results displayed an average Coefficient of Error (CE) of 0.1109 and an average slice thickness of 21.1142 μm.

Statistical Analysis

Statistical analysis and graphs were performed and created with Graphpad Prism 6.0 software. Differences in treatments between the different groups were analyzed by two-way ANOVA followed by the Bonferroni *post-hoc* test for p values significance. Differences in treatments within the group were analyzed by one-way ANOVA followed by the Tukey *post-hoc* test for p values significance. Values expressed are the group mean ± SEM. Mean of clinical scores compared PFF α-syn + NK1.1 group to PFF α-syn + IgG, ***, $p<0.001$, determined by Mann-Whitney *U*-test.

Figure S1.

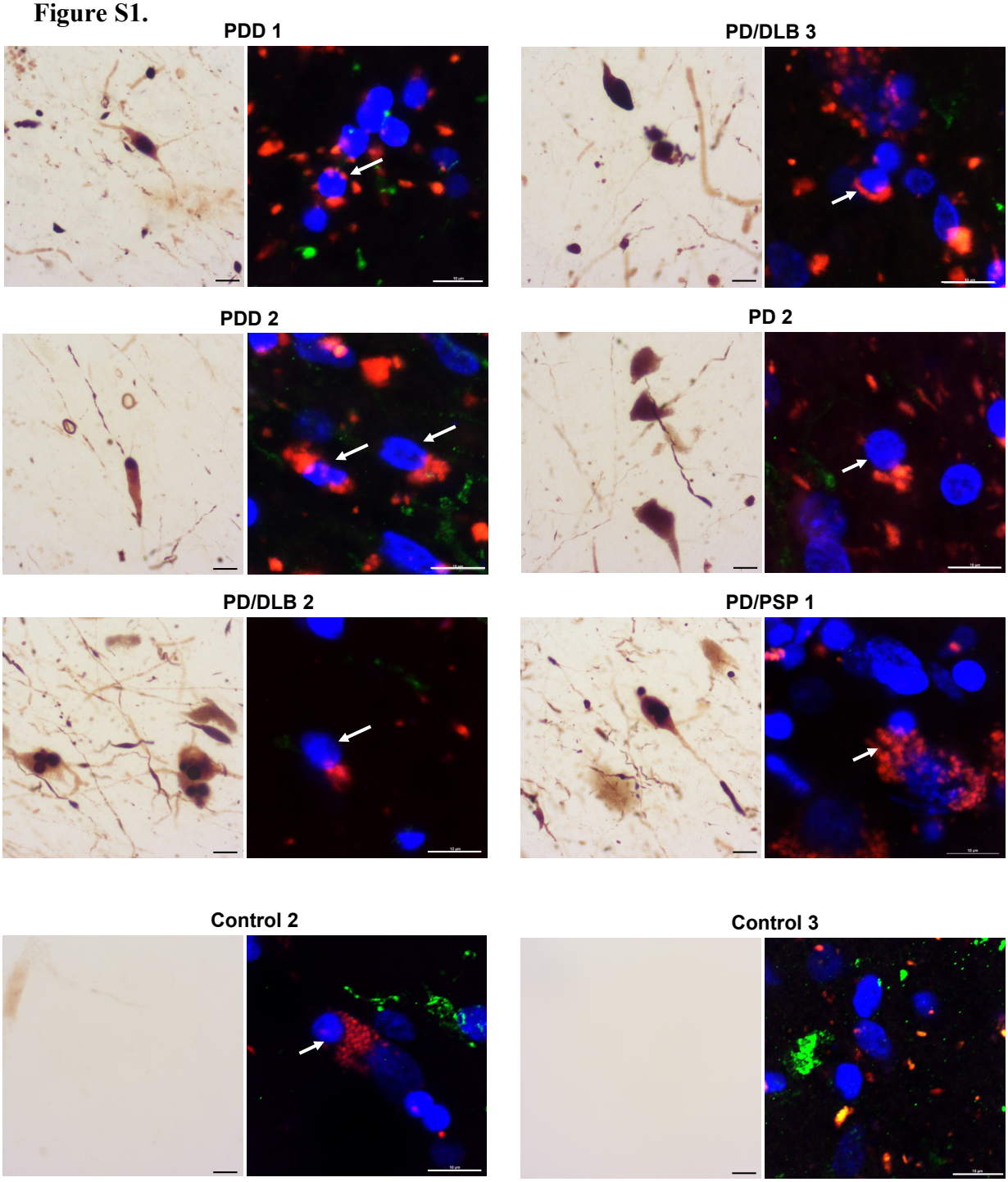

Figure S1. NK cells are present in the brains of synucleinopathy patients. The substantia nigra from synucleinopathy patients and age-matched controls were stained with antibodies against p-a-syn (bright field images; left panels) and the human NK cell marker 2B4 (red)/Iba1 (green)/DAPI (blue) (immunofluorescent images; right panels). Scale bars represent 10μm. Parkinson's disease dementia (PDD); Dementia with Lewy bodies (DLB); Parkinson's disease (PD); Progressive supranuclear palsy (PSP)

Figure S2.

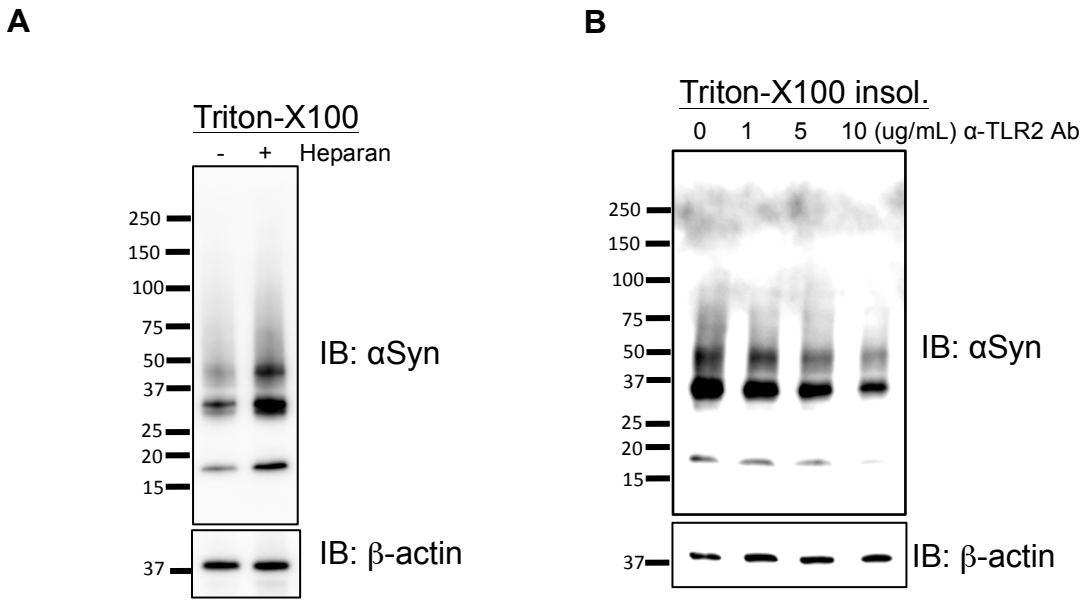

Figure S2. α-Syn uptake in NK92 cells is partially inhibited by blocking TLR2 but not by heparan. (A) NK92 cells were incubated with or without heparan (100 ug/mL) for 1 hr. Cells were then incubated with 5 μg/mL of α-syn aggregates for 1 hr. Triton X-100 insoluble fractions were analyzed by western blot analysis for α-syn and β-actin. **(B)** NK92 cells were pre-treated with varying concentrations of TLR2 neutralizing antibody for 1 hr. Cells were then incubated with α-syn aggregates (5 μg/mL) for 1 hr. Triton X-100 insoluble fractions were analyzed by western blot analysis for α-syn and β-actin.

Figure S3.

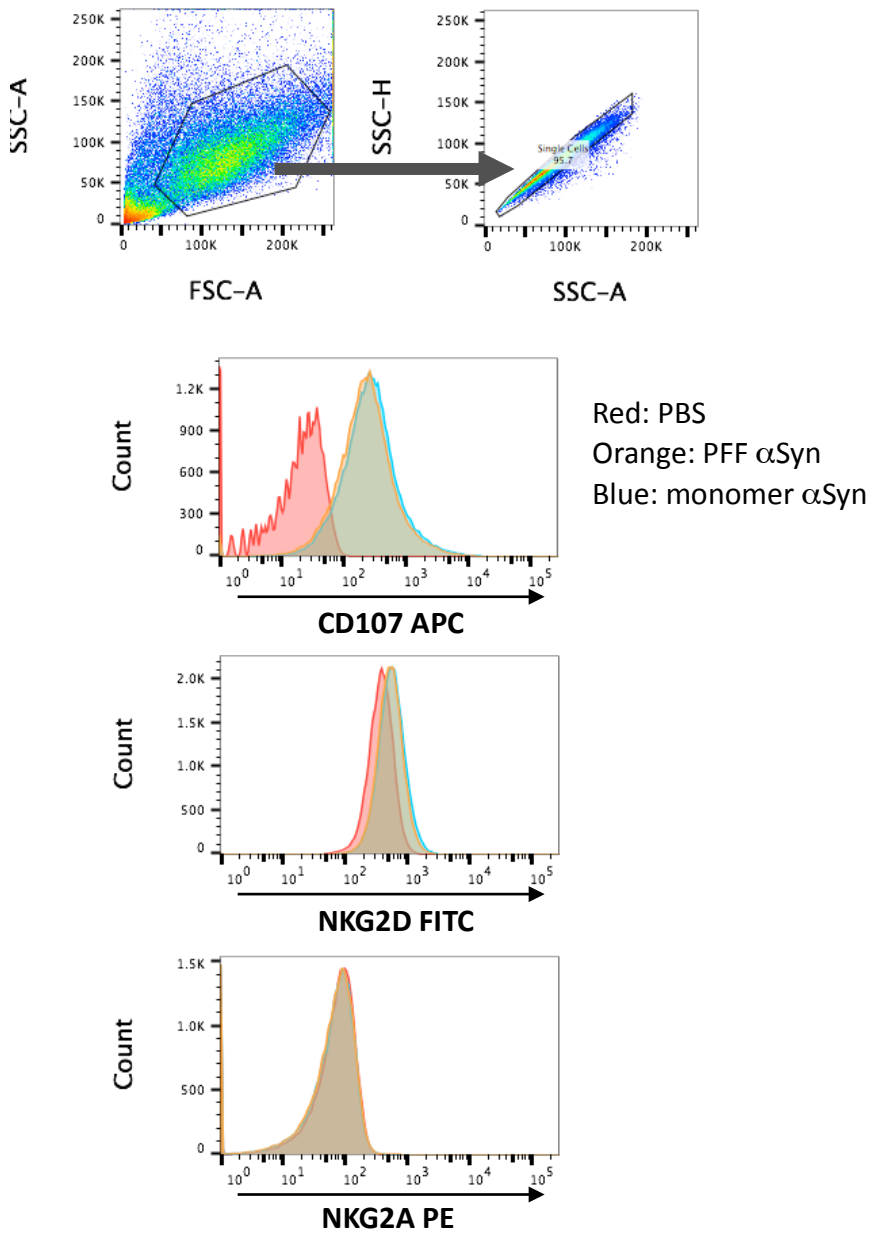

Figure S3. Extracellular α-syn aggregates do not alter the levels of surface expression of NKG2D (activating) or NKG2A (inhibitory) receptors on NK92 cells. NK92 cells were treated with PBS, monomer α-syn (5 μg/mL) or PFF α-syn (5 μg/mL) for 4 hr. Expression of NKG2D and NKG2A were evaluated by FACS analysis. (blue: NK92 cell with no Abs, orange: NK92 cells + vehicle, red: NK92 cells + α-syn aggregates 4 hrs). The data shown are representative of two independent experiments.

Figure S4.

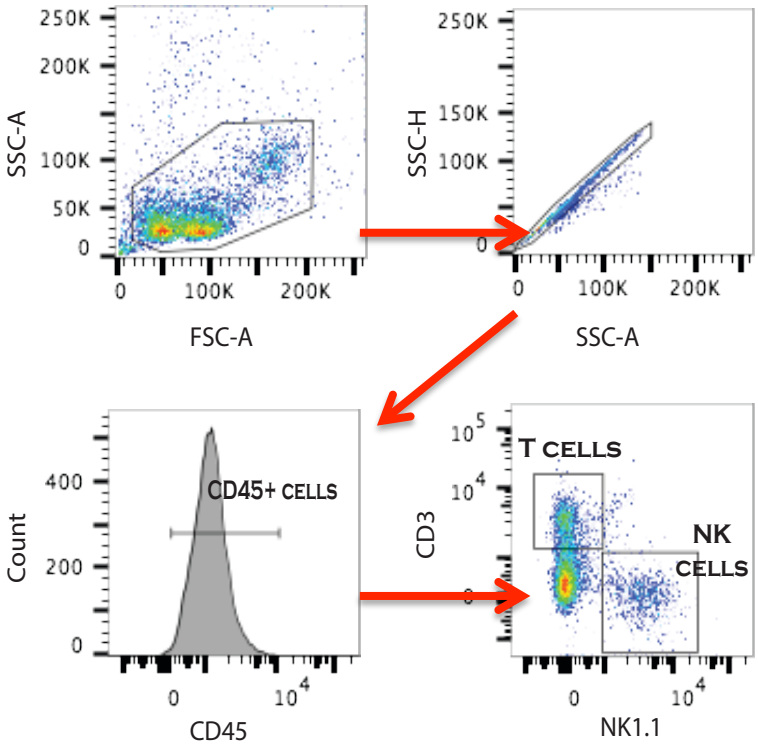

Figure S4. Gating strategy of CD45+CD3-NK1.1+ NK cells for flow cytometer analysis. Cells were gated first on a forward (FSC) and side scatter (SSC), then total CD45 positive leukocytes were gated. CD3 positive and NK 1.1 negative T cells or CD3 negative and NK 1.1 positive NK cells were gated from CD45+ parental population.

Figure S5.

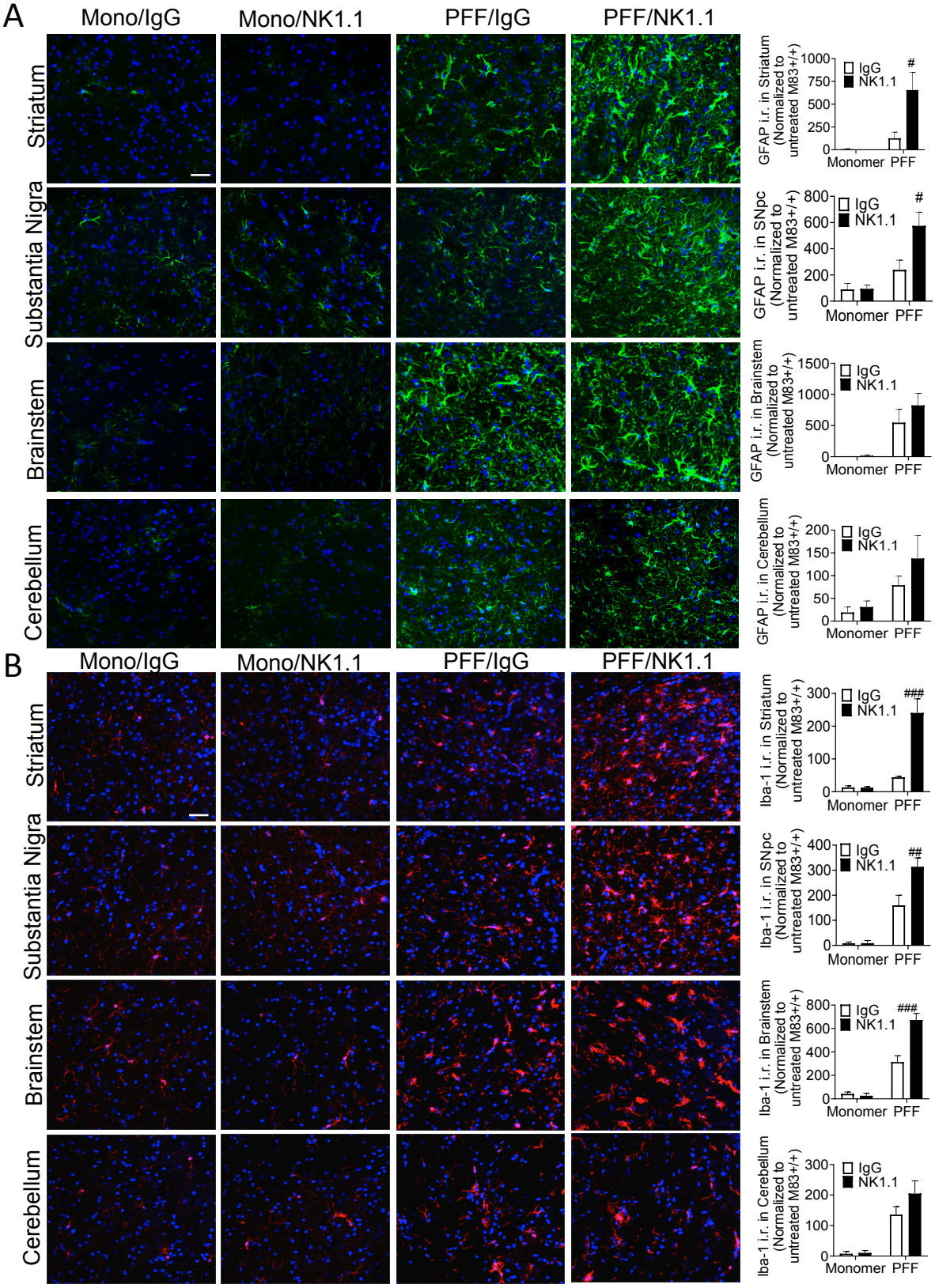
A
Mono/IgG
Mono/NK1.1
PFF/IgG
PFF/NK1.1
Striatum
Substantia Nigra
Brainstem
Cerebellum
GFAP i.r. in Striatum (Normalized to untreated M83+/+)
GFAP i.r. in SNpc (Normalized to untreated M83+/+)
GFAP i.r. in Brainstem (Normalized to untreated M83+/+)
GFAP i.r. in Cerebellum (Normalized to untreated M83+/+)
IgG
NK1.1
Monomer
PFF
#
B
Iba-1 i.r. in Striatum (Normalized to untreated M83+/+)
Iba-1 i.r. in SNpc (Normalized to untreated M83+/+)
Iba-1 i.r. in Brainstem (Normalized to untreated M83+/+)
Iba-1 i.r. in Cerebellum (Normalized to untreated M83+/+)
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Figure S5. NK cell depletion exacerbates astrogliosis and induction of microgliosis of PFF α-syn M83 Tg mice. **(A)** Representative immunohistological images and quantitation data of GFAP immunoreactivity (green) in the striatum, SNpc, brainstem, and cerebellum of M83 Tg mice injected with PFF α-syn (5 μg) or α-syn monomers (5 μg) with NK1.1 or IgG2a mAb injections (n = 4 mice/group) at 10 weeks p.i.. DAPI for nuclei staining shown in blue. Scale bars represent 10 μm. Graphs represent the average ODs of GFAP positive staining i.r per ROIs within the striatum, SNpc, brainstem, or cerebellum. **(B)** Representative immunohistological images and quantitation data of Iba-1 (red) immunoreactivity in the striatum, SNpc, brainstem, and cerebellum of M83 Tg mice injected with PFF α-syn (5 μg) or α-syn monomers (5 μg) with NK1.1 or IgG2a mAb injections (n = 4 mice/group) at 10 weeks p.i.. DAPI for nuclei staining shown in blue Graphs represent the average ODs of Iba-1 positive i.r. per ROIs within the striatum, SNpc, brainstem, or cerebellum. All data was analyzed with a two-way ANOVA. # $p < 0.05$, ## $p < 0.01$, ### $p<0.001$ comparing IgG vs. NK1.1 groups. Error bars represent ± SEM.

Movie S1. Representative animal with clinical motor score 2 of PFF α-syn injected M83 Tg mouse.

Movie S2. Representative animal with clinical motor score 4 of PFF α-syn injected M83 Tg mouse.

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