Supplemental material and methods:

Part I Human study

Collection of colon biopsy, fecal, and blood samples

Out of eight mucosal biopsy samples, four samples were placed in either 10% formalin or in optimal cutting temperature compound (OCT) to perform histology studies. The remaining sigmoid biopsies were snapped frozen in liquid nitrogen for mucosal microbiota and gene array (RNAlater biopsies) analysis. Fecal samples were collected by subjects using a home collection kit (BD Gaspak, Becton Dickinson and Company, Sparks, MD). Fecal samples were collected within four weeks after the sigmoidoscopy. Biopsy and fecal samples were stored at -80°C until use.

Immunocytochemistry and Microscopic analyses

Primary antibody incubation was performed using the following antibodies: (TLR4: 1:500, ab22048, Abcam; ZO-1: 1:200, 617300, Abcam and CD3: 1:200, ab5690, Abcam). Immunofluorescence staining was performed for ZO-1 and TLR4 markers using fluorophore tagged secondary antibody (donkey anti-rabbit 488: 1:400, A21206, Thermofisher Scientific or donkey anti-mouse 488, 1:400, A21202, Thermofisher Scientific). Immunoperoxidase staining was performed for TLR4 and CD3 markers using biotinylated secondary antibodies (horse anti-mouse: 1:200, BA2000, Vector lab or goat anti-rabbit: 1:200, BA1000, Vector lab). Immunofluorescence sections were counter stained using DAPI and immunoperoxidase sections with hematoxylin (SH26-500D, Fisher Scientific).

Fluorescence images were taken using confocal microscope (LSM700, Zeiss). Levels of laser intensity, confocal aperture, photomultiplier voltage, offset, scan speed, image size, filter and magnification were kept at constant levels to keep consistencies among samples. These settings were maintained for analysis of each marker. Using a 488nm excitation source, in combination with 405nm (DAPI) excitation source, images were acquired at each sampling site from lamina propria of all subjects. Confocal microscopic analyses were performed to evaluate ZO-1 tight junction barrier integrity. Data for tight junction barrier ZO-1 integrity analyses was performed, as described before.[1] Sections were assigned a code and analysed in a blind manner. Arbitrary scoring scale 0-3 was used to evaluate tight junction integrity as: 0 = no ZO-1 immunofluorescence; 1 = very light and discontinuous ZO-1 immunofluorescence; 2 = intense and discontinuous ZO-1 immunofluorescence; 3 = smooth continuous and well-organized ZO-1 immunofluorescence. Mean values were collected for comparisons between groups and represented at Mean±SEM. Unbiased stereology was performed for TLR4 and CD3+ cells[1] using light microscope attached to stereoinvestigator cell count probe (S.I.-V.1.5, MBF Bioscience).

mRNA expression analyses

Genes specific for microbiota-induced intestinal inflammation and gut dysfunction were evaluated according to a published protocol.[2] The 55 genes measured are listed in Fig 3. Tissue homogenates were prepared from N=6 PD and N=4 HC using Affymetrix lysis buffer and processed according to the manufacturer's instructions. Tissue homogenates were not available form N=2 HC subjects. Levels of mRNA were determined using a Luminex-based custom multiplex bead array. Values that were less than or equal to 0 were

set to 1 (floor effect), and all values were log 2 transformed. These values were then normalized to the housekeeping. Selection of housekeeping gene was based on a screen plot of eigenvalues with two variables being consistent with an inflection break in the scree plot; thus, *HGPRT* was selected. Genes that did not have more than 50% of the samples in the detectable range (above background) were excluded based on unreliable gene expression data. Samples were analyzed via Significance Analysis of Microarrays (SAM) software and $P<0.05$ was selected for significant differences.

Part II Mice study

Motor function assessment

Mice were placed on an accelerating rod with speeds starting with 2 rpm and gradually increasing to 20 rpm. Time to fall was recorded for a maximum of 300s and reported as latency to fall. Mice were tested at baseline and every 7 days until sacrifice.

Immunocytochemistry and image analysis in mice

Antibodies for TLR4 (ab13867, abcam), ZO-1 (ab59720, abcam), GFAP (Z0334, Dako), CD3 (ab49943, abcam) and α-syn (04-1053, Millipore) were used to evaluate the gut pathology. Data collection for tight junction barrier integrity analyses (ZO-1 integrity) and stereology were performed, as previously described.[1] The brains were stained with tyrosine hydroxylase (TH) (sc-14007, Santa Cruz Biotechnology) and iba-1 (019-197410, Wako) antibodies to assess the amount of dopaminergic neurons and microglia morphology in the

SN. TH-immunopositive neurons were assessed using stereology counting. For microglia analysis z-stacks were imaged at 1µm step and analysed with Image-J software.

Microbiota analysis

Total DNA was extracted from both mice cecum mucosa and cecum luminal content samples, as well as human sigmoid colonic mucosa and fecal samples, utilizing the FastDNA bead-beating Spin Kit for Soil (MP Biomedicals, Solon, OH, USA). DNA concentrations were measured via fluorometric quantitation (Qubit, Life Technologies, Grand Island, NY). Primers 515F/806R targeting the V4 variable region of microbial small subunit (SSU or 16S) ribosomal RNA (rRNA) genes were used for PCR, and prepared for Illumina MiSeq high-throughput amplicon sequencing (2x151bp reads) at Argonne National Laboratory. The same sequence processing, quality assessment, and taxonomic assignment methodologies were implemented for both mice and human samples, as previously described.[3, 4] Mouse sample were rarefied to 25,000 sequences and human samples were rarefied to 8,700 sequences, as previously reported.[3, 4] Raw sequence data (FASTQ files) were deposited in the NCBI Sequence Read Archive under projects PRJNA268515 (human samples) and PRJNA387564 (mice samples).

Similar statistical analysis was performed on mice microbiome's alpha and beta diversity data for both mice cecum mucosa and luminal content samples (n=9-10 per group), as previously described.[4] Analysis of similarity (ANOSIM) and non-metric multidimensional scaling (nMDS) plots were performed at the genus taxonomic level, using square-root transformed data. The differences in the relative abundance of individual genus taxa

between defined mice groups were assessed and accepted for significance using Kruskal-Wallis test controlling for false-discovery rate (FDR) corrected p-value (FDR-P<0.05).

Volcano plots were generated using the R programming language (v.3.3.2) implementing the packages ggplot2 and ggrepel. Within each volcano plot, the log2 fold change (Log2FC) between vehicle (WT or TLR4-KO) and rotenone, as well as between TLR4-KO and WT (vehicle or rotenone) groups were examined using the mice cecum mucosa and content samples. The Log2FC between the respective intervention and corresponding control was calculated for each mouse group comparison, using the general formula Log2FC=Log2(mean of intervention/mean of control). An FDR-P<0.05 (q.value) was used to determine significant individual taxa differences (above red dotted line), at the taxonomic level of genus.

Additionally, the Log2FC was also implemented between human PD and HC genera, where Log2FC=Log2(mean of PD/mean of HC). We specifically highlighted decreased Log2FC trending changes in short-chain-fatty-acid (SCFA)-producing bacteria changes in PD compared to HC subject, as previously reported.[3]

References:

1. Engen PA, Dodiya HB, Naqib A, et al. The Potential Role of Gut-Derived Inflammation in Multiple System Atrophy. J Parkinsons Dis 2017.
2. Voigt RM, Keshavarzian A, Losurdo J, et al. HIV-associated mucosal gene expression: region-specific alterations. AIDS 2015;29:537-46.
3. Keshavarzian A, Green SJ, Engen PA, et al. Colonic bacterial composition in Parkinson's disease. Mov Disord 2015.
4. Perez-Pardo P, Dodiya HB, Engen PA, et al. Gut bacterial composition in a mouse model of Parkinson's disease. Benef Microbes 2018;9:799-814.