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Microglia, inflammation and gut microbiota responses in a progressive monkey model of Parkinson’s disease: a case series

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Competing interests: Christopher J Barnum is the director of Neuroscience at the pharmaceutical company INmune Bio. Malú G Tansey is an ex-employee of and coinventor on the Xencor Inc. patents describing the dominant-negative TNFs and is a consultant to INmune Bio.
Abstract

Inflammation has been linked to the development of nonmotor symptoms in Parkinson’s disease (PD), which greatly impact patients’ quality of life and can often precede motor symptoms. Suitable animal models are critical for our understanding of the mechanisms underlying disease and the associated prodromal disturbances. The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated monkey model is commonly seen as a “gold standard” model that closely mimics the clinical motor symptoms and the nigrostriatal dopaminergic loss of PD, however MPTP toxicity extends to other nondopaminergic regions. Yet, there are limited reports monitoring the MPTP-induced progressive central and peripheral inflammation as well as other nonmotor symptoms such as gastrointestinal function and microbiota. We report 5 cases of progressive parkinsonism in non-human primates to gain a broader understanding of MPTP-induced central and peripheral inflammatory dysfunction to understand the potential role of inflammation in prodromal/pre-motor features of PD-like degeneration. We measured inflammatory proteins in plasma and CSF and performed $^{18}$F-FEPPA PET scans to evaluate translocator proteins (TSPO) or microglial activation. Monkeys were also evaluated for working memory and executive function using various behavior tasks and for gastrointestinal hyperpermeability and microbiota composition. Additionally, monkeys were treated with a novel TNF inhibitor XPro1595 (10mg/kg, n=3) or vehicle (n=2) every three days starting 11 weeks after the initiation of MPTP to determine whether XPro1595 would alter inflammation and microglial behavior in a progressive model of PD. The case studies revealed that earlier and robust $^{18}$F-FEPPA PET signals resulted in earlier and more severe parkinsonism, which was seen in male cases compared to female cases. Potential other sex differences were observed in circulating inflammation, microbiota diversity and their metabolites. Additional studies with larger group sizes of both sexes would enable confirmation and extension of these findings. If these findings reflect potential differences in humans, these sex differences have significant implications for therapeutic development of inflammatory targets in the clinic.

Keywords

Parkinson’s disease; MPTP; Tumor necrosis factor; rhesus monkey; inflammation; sex differences; microglia phenotypes; XPro1595; microbiota; nonmotor symptoms

Introduction

Numerous pre-clinical and epidemiological studies have demonstrated that peripheral and central inflammation accompany and may contribute to the progressive dopaminergic neuronal cell death in Parkinson’s disease (PD) (Gao et al., 2008; McGeer et al., 1988). The existence of ongoing and enhanced inflammatory processes in patients with idiopathic PD is supported by in vivo evidence of activated microglia imaged by positron emission tomography (PET) (Gerhard et al., 2006; Ouchi et al., 2009; Terada et al., 2016) and altered inflammatory markers measured in biofluids (Brodacki et al., 2008; Eidson et al., 2017). Moreover, genetic evidence from large genome-wide association studies (GWAS) has identified risk single nucleotide polymorphism (SNP) variants in genes that encode immune
function (Hamza et al., 2010; Pierce and Coetzee, 2017) and in genes enriched in distinct subsets of immune cells (Coetzee et al., 2016), further implicating the involvement of the immune system in the pathogenesis of PD. A range of nonmotor symptoms are well recognized to burden PD patients and have been detected years prior to a PD diagnosis (Abbott et al., 2005; Goldman and Postuma, 2014; Ross et al., 2006). It has been hypothesized that inflammation plays a role in the development of PD nonmotor symptoms (Barnum and Tansey, 2012), yet the pathophysiological mechanisms behind the emergence of nonmotor dysfunctions has not been determined. The purpose of this study is to gain a broader understanding of central and peripheral inflammatory dysfunction triggered by chronic exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).

The MPTP-treated rhesus macaque monkey model is commonly seen as a “gold standard” model that closely mimics the clinical motor symptoms and the nigrostriatal dopaminergic loss of PD, but the neuropathologic and behavioral effects of the drug extend beyond the ventral midbrain dopaminergic cell groups and parkinsonian motor signs when low doses of MPTP are administered chronically to monkeys and mice (Emborg, 2007; Masilamoni et al., 2011; Masilamoni et al., 2017; Masilamoni and Smith, 2018; Vivacqua et al., 2019). Chronic systemic MPTP administration over several weeks to months allows the slow development of parkinsonism that is more similar to PD, importantly creating a window of time to evaluate early premotor symptoms including inflammation and nonmotor manifestations that are alike impaired in PD. Non-human primates are valuable experimental models because of their genetic proximity to humans, the close resemblance behaviorally to humans, and microglia heterogeneity with gene expression patterns similar to humans (Geirsdottir et al., 2019). Dysregulated cytokines have been described in non-human primates treated with MPTP (Mondal et al., 2012; Roy et al., 2015) or systemically (Barcia et al., 2005; De Pablos et al., 2009), but the trajectory of the inflammatory environment has not been detailed throughout the gradual progression of MPTP-induced neuropathology and related behavioral changes. In addition to parkinsonian motor signs, chronically MPTP-treated monkeys display early cognitive dysfunction (Roeltgen and Schneider, 1991; Schneider and Kovelowski, 1990; Schneider et al., 2010; Slovin et al., 1999), alterations in sleep, social relationships, olfaction, and gastrointestinal pathologies (Almirall et al., 1999; Barraud et al., 2009; Chaumette et al., 2009; Durand et al., 2015; Phillips et al., 2017). In line with these complex neurobehavioral alterations, profound loss of central noradrenergic, serotonergic and thalamic neurons, and the development of synuclein aggregates have been described in chronically MPTP-treated monkeys and mice (Fornai et al., 2005; Masilamoni et al., 2011; Masilamoni et al., 2017; Masilamoni and Smith, 2018; Vivacqua et al., 2019). Another monkey study reported as much as 70% reduction in the number of catecholaminergic myenteric neurons (Chaumette et al., 2009); however, the effects of chronic systemic MPTP on in vivo gastrointestinal functions, inflammation, and bacterial composition have not been evaluated in non-human primates. With accumulating evidence highlighting the connection between the gut and the brain (van de Wouw et al., 2019), exploring the effects of nigrostriatal dopaminergic depletion on gastrointestinal symptoms will expand the relevance of these models in a multisystem disorder.

Increased levels of the pro-inflammatory cytokine tumor necrosis factor (TNF) have been described in postmortem brains and cerebrospinal fluid of PD patients (Eidson et al., 2017;
Mogi et al., 1994) and in neurotoxin animal models of nigrostriatal degeneration (Barcia et al., 2005; Mogi et al., 1999), implicating its role in PD pathophysiology. Soluble TNF (sTNF) mediates pro-inflammatory responses by preferentially binding to the TNF receptor 1 (TNFR1) demonstrated by the increased susceptibility of bacterial infections in TNFR1 knockout mice (Rothe et al., 1993). Mice deficient in TNFR1 or both soluble and transmembrane TNF ligands are protected from MPTP-induced nigrostriatal degeneration (Ferger et al., 2004; Sriram et al., 2002), suggesting that TNF has a role in mediating neurotoxin-induced dopaminergic degeneration. Moreover, our group has previously shown that selective blocking of sTNF with XPro1595 was sufficient to protect against acute 6-hydroxydopamine neurotoxicity of dopaminergic neurons and neuroinflammation in rats (Barnum et al., 2014). Yet it is not clear whether nonmotor symptoms are tied to sTNF/TNFR1 signaling and whether XPro1595 would alter inflammation and microglial behavior in a progressive model of PD.

Thus, the primary objective of this study was to perform a preliminary investigation into the evolution of central and peripheral inflammatory responses elicited in a small set of chronic low-dose MPTP monkeys. Secondly, given the presence of nonmotor symptoms in PD and the need for appropriate pre-clinical animal models that replicate these features, in this case series report we evaluated the gastrointestinal and cognitive behaviors in the slowly-progressing monkey model of PD. Finally, we evaluated the extent to which XPro1595 mitigated neuroinflammation and nonmotor symptoms elicited by MPTP.

Materials and Methods

Subjects and Ethics Statement

Five rhesus monkeys (Macaca mulatta, 5-7yrs of age, 3 females, 2 males) from the Yerkes National Primate Research Center (YNPRC) colony at Emory University were used in this study (Table 1). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Emory University and conducted in accordance with guidelines in the NIH Guide for Care and Use of Laboratory Animals (NIH Publications, 8th edition, revised 2011). The monkeys were housed in temperature-controlled rooms and exposed to a 12-hour light cycle. Animals were fed twice daily with Lab Diet monkey chow supplemented with fruits and vegetables and water ad libitum. To encourage eating at later stages of the study animals may have been fed ensure soaked chow or provided a Lab Diet high-protein chow.

Experimental Design

Following the collection of all baseline measures, monkeys were given weekly doses of intramuscular MPTP (0.2–0.8mg/kg, Sigma-Aldrich) starting at 0.2 mg/kg for 18 weeks and increasing to 0.8 mg/kg to maintain a parkinsonian state. All animals, regardless of sex, received the same dose by weight until stable parkinsonian motor symptoms appeared and were maintained, at which time MPTP administration was halted (Masilamoni et al., 2011). At 11 weeks, animals were randomly assigned to two groups (Figure 1), with consideration of sex, and peripheral subcutaneous treatment of XPro1595 (10mg/kg, n=2 female and n=1 male) or vehicle (n=1 female and n=1 male) began and continued every 3 days. Based on the
clear differences in sensitivity to MPTP dosing between the 3 female and 2 male monkeys used in this study, once an animal in either sex group became mildly parkinsonian based on clinical rating, administration of MPTP was terminated to all animals of the same sex. These animals were followed for approximately 3-4 weeks to confirm the stability of the clinical status, and to conduct final endpoint procedures before euthanasia. Males received a total of 5.9 mg/kg of MPTP over 22 weeks (highest weekly dose was 0.5mg/kg) and were euthanized 26 weeks after the start of neurotoxin dosing, while females progressed more slowly than males and therefore received 15.2 mg/kg of MPTP over 37 weeks (highest weekly dose was 0.8mg/kg) and were euthanized at 40 weeks.

Clinical and Motor Evaluations

Clinical status was assessed using a validated 27-point scoring system modified from the Unified Parkinson’s Disease Rating Scale (Masilamoni et al., 2011) by two investigators blinded to the treatment history of the groups. Each category was scored between 0-3 including: gross motor activity, posture, balance, bradykinesia (arm and leg), hypokinesia (arm and leg) and tremor (arm and leg). Assessments were begun after monkeys habituated to the observation cage for 15 minutes. An intra-class variability analysis between the two-experimenter scores demonstrated significant rater agreement with ICC of 0.977 (95% CI: 0.969-0.983).

Cognitive behavior

For all cognitive behavior, animals were acclimated to a Wisconsin General Test Apparatus (WGTA) contained in a sound-shielded room with a white noise generator to mask external sound. Animals were tested with an intradimensional/extradimensional (ID/ED) cognitive behavior task which requires monkeys to update their responses based on changing reinforcement contingencies (Weiss et al., 2019). Prior to MPTP, animals were tested on a visual compound discrimination training with a positive-rewarded stimulus and a negative-unrewarded stimulus. Following compound discrimination tests, animals were tasked with an intradimensional shift (IDS1; attention shift within same dimension) to measure associative learning. Five weeks after the start of chronic MPTP dosing and prior to administration of XPro1595 (or vehicle), animals were retrained to perform the compound discrimination followed by an extradimensional shift task (EDS1; attention shift to new dimension) to measure cognitive flexibility and a series of extradimensional reversals to measure behavioral inhibition. Following 6 weeks of XPro1595 (or vehicle) treatment and 17 weeks of escalating doses of MPTP, animals were retrained on a compound discrimination test and tasked with a new extradimensional shift (EDS2) and reversal. Finally, starting 10 weeks after the beginning of the XPro1595 (or vehicle) treatment, animals were retrained with a compound discrimination task and retested on an intradimensional shift (IDS2). In order to complete a stage and move onto the next, 10 consecutive correct trials had to be performed with up to 60 trials in a day, but a stage was failed if the criterion was not met in 500 trials. The number of trials and errors to reach criterion in each stage were used for analysis.

Animals were also tested with an object self-ordered task (ObjSO) to assess working memory and measure the monkey’s ability to monitor their own responses over a series of
trials as a measure of working memory (Heuer and Bachevalier, 2011). Due to the shortened timeline to parkinsonism for the males, only female monkeys were evaluated for the ObjSO task at baseline and 30 weeks post-MPTP administration. A trio of unique objects were presented to the monkey in the WGTA. The animal displaced an object to retrieve a food reward. Next, the door was closed allowing the tester to rearrange the objects in a randomized fashion. The monkey was then given 3 trials with a 10-second inter-trial interval to displace an object with the goal of not repeating the placement of an object already selected. Primary errors were defined as the first incorrect choice on trial 2 or 3. Perseverative errors were defined as errors committed after a primary error on the same day. Criterion performance is 85% or better performance across 10 consecutive days of testing (i.e. 3 or less primary errors in 10 days), but task completion was deemed a failure if the criterion was not met in 50 sessions. The number of sessions to reach the criterion was recorded along with primary and perseverative errors during trials 2 and 3.

Gastrointestinal Assessments

A sugar permeability assay was conducted to determine intestinal barrier function; feces were collected for microbiome 16sRNA gene amplicon sequencing and obtained for inflammatory analysis at baseline, after 10 weeks of chronic MPTP administration and before drug treatment (MPTP) and prior to euthanasia after animals had been chronically challenged with MPTP and received either XPro1595 or vehicle treatment (MPTP + treatment). Additionally, lipopolysaccharide (LPS) and lipopolysaccharide binding protein (LBP) were evaluated from serum collected at baseline, 8 weeks after MPTP and prior to euthanasia as an indirect measure of circulating LPS due to gut leakiness.

Intestinal permeability assessment: Intestinal permeability was evaluated with the use of a cocktail that includes sugars of different sizes (sucrose, lactulose, mannitol, and sucralose) that are passively absorbed from the gut under either transcellular or paracellular mechanisms without considerable metabolism and then excreted into the urine (Forsyth et al., 2011). The quantitation of the uptake and excretion of these substrates in urine samples allow for a relatively precise determination of the integrity of intestinal epithelial cell barrier function or disruption of barrier integrity resulting in passage/leak of these compounds into the blood stream and urine (McOmber et al., 2010; Shaikh et al., 2015). Animals were anesthetized with either Ketamine (5-10 mg/kg IM) or Telazol (3-5 mg/kg IM), intubated and then maintained under isoflurane (1-3%) anesthesia. A bladder catheter was placed to collect a baseline urine sample and to expel the urine remaining after baseline was established. After a baseline sample was collected, the animal was orally gavaged through a gastric tube with a bolus of up to ~20 mL of a sugar solution (a combination of 40g sucrose, 7.5g lactulose, 2g manitol and 2g sucralose per 70kg weight all dissolved in water). The animals remained anesthetized for ~3 hours under the surveillance of the YNPRC veterinary staff. During this time, all urine was continuously collected via a bladder catheter. Three hours after administration of the sugar cocktail, the total urine volume from the bladder was measured and aliquots taken for gas chromatography analysis as previously described (Forsyth et al., 2011; Shaikh et al., 2015). Results are expressed as the amount of sugar excreted as a % of the oral dose.
**Feces collection:** Monkeys were anesthetized with either ketamine (5-10 mg/kg IM) or Telazol (3-5 mg/kg, IM) and placed in ventral or lateral recumbency. A lubricated anoscope was introduced in the rectum. Feces were removed and collected using sterile cotton-tipped applicators commonly used for stool collection for microbiome 16S rRNA gene sequencing and SCFA levels.

**Radiosynthesis of [18F]FEPPA and [18F]FECNT**

The preparation of [18F]FEPPA was similar to that previously described (Wilson et al., 2008). [18F]Fluoride was produced at YNPRC on a Siemens RDS 111 medical cyclotron, trapped and released, azeotropically dried, before undergoing a nucleophilic reaction with the tosylate precursor. The product was purified by reverse phase high-pressure liquid chromatography (HPLC) in buffered saline with 5-10% ethanol. The final product was passed through a 0.22-micron filter and formulated sterile, pyrogen free, to a pH of 5-8. Radiochemical purity was confirmed to be > 96% with specific activities greater than 1Ci/μmol.

The preparation of [18F]FECNT was conducted as previously described (Goodman et al., 2000; Masilamoni et al., 2010; Masilamoni et al., 2011). In the present study, we performed [18F]FECNT PET scans to determine the neuroprotective effects of XPro1595 on the nigrostriatal dopaminergic system during the course of escalating MPTP intoxication. In vivo PET imaging of dopamine markers is the most sensitive approach for longitudinal monitoring of the state of degeneration of the nigrostriatal dopaminergic system during the course of neuroprotective trials in PD (Pavese and Brooks, 2009). We have recently shown that the PET tracer [18F]FECNT, is a highly reliable ligand to estimate levels of striatal and nigral dopamine transporter in the MPTP-treated monkey model of PD (Masilamoni et al., 2010).

**MRI scans**

Animals were initially anesthetized with Telazol (3-5 mg/kg IM) and maintained using 12% isoflurane gas anesthesia for the duration of the imaging procedure. They were placed in a custom-made stereotaxic frame while in a supine position. A 3D T1-weighted MPRAGE (Mugler, 1999) of the brain was acquired with an Siemens Magnetom Trio 3-T (Siemens Medical Solutions USA, Malvern, PA) typically recommended for brain structure morphometric analyses (Lusebrink et al., 2013). The scan parameters were 0.5mm-thick images with a transverse plane isotropic pixel size of 0.5 mm (repetition time/echo time 2500/4.38 msec, inversion time of 900ms, flip angle of 10, acquisition field-of-view of 320 x 192 pixels, and a matrix of 320 x 320 pixels).

**PET imaging**

Neuroimaging was employed to monitor in vivo microglial activation and dopaminergic neuron health. TSPO expression was monitored as a proxy for inflammatory activity and related to DAT expression on PET scans with [18F]FEPPA and [18F]FECNT ligands respectively, at baseline and at approximately 8 weeks (PET I), 16 (PET II), 24 (PET III) and 38 (PET IV) after the initiation of MPTP administration (3 post-MPTP scans for males, and 4 for females). PET images were acquired in the YNPRC on a Siemens Focus 220.
microPET scanner (Siemens, Concorde Microsystems, Knoxville, TN), which has an 8/26 cm axial/transaxial FOV and reconstructed image resolution is 1.7 mm in all directions. Animals were initially anesthetized with ketamine and Telazol then intubated and maintained on 0.8-1.5% isoflurane anesthesia for the duration of the imaging procedure. An acute venous catheter was placed in the saphenous vein for intravenous administration of the radioligand. The catheter access was maintained with a sterile saline drip throughout the study. Animals were placed supine in the micro-PET scanner, fitted with pulse oximetry equipment and a rectal thermistor for physiological monitoring during the procedure. Prior to injection of the PET radiopharmaceutical, a transmission scan was obtained with a Co 57-point source to correct for attenuation in the image reconstruction. Approximately 5 mCi bolus of radiopharmaceutical was injected intravenously over 5 min with an infusion pump set at a rate of 1.0 mL/min. The initial acquisition was a 28-frame dynamic sequence, starting with 30-sec scans and ending with 20-min scans (total duration, 115 min). PET data were reconstructed with a 3D Ordered subset Maximum Expectation algorithm including corrections for random, scatter, attenuation and dead-time.

**PET data analysis**

All images were decay-corrected to the time of injection. $^{[18}F]FECNT$ time-activity curves were generated for each monkey. PET images were co-registered to their structural MRI using IDL software (ITT Visual Information Solutions) and averaged to draw regions of interest (ROI) so that the volumes would be equal and comparable over all treatment time points. ROIs were manually drawn as defined by the Paxinos atlas including the following striatal regions: putamen/associative (PA), putamen/motor (PM), putamen/limbic (PL), caudate nucleus/associative (CA), nucleus accumbens/limbic (AC) and the substantia nigra (SN) (Paxinos et al., 2000). The regions of interest were then superimposed onto the individual animal PET images to obtain time-activity curves. Because of the minimal expression of dopamine transporter in the cerebellum, we used it as the reference region, and expressed the FECNT uptake value as the non-displaceable binding potential, as described in our previous studies (Masilamoni et al., 2010; Votaw et al., 2002).

For quantification of $^{[18}F]FEPPA$, regions of interest were manually drawn and defined on the MRI within structural boundaries defined by the Paxinos atlas (Paxinos et al., 2000), including the cerebellum, frontal cortex, parietal cortex, occipital cortex, temporal cortex, putamen, substantia nigra, and lateral geniculate nucleus. The manually-drawn ROIs were superimposed onto the individual animal $^{[18}F]FEPPA$ PET images to obtain time-activity curves using IDL software (ITT Visual Information Solutions). $^{[18}F]FEPPA$ uptake was expressed as Standardized Uptake Values (SUV), accounting for body mass and injected activity of the tracer (Zhang et al., 2012). SUV allows precise assessment of activity changes in response to drug treatments (Masilamoni et al., 2016; Zhang et al., 2012). No reference brain region was used to assess background activity because systemic MPTP administration is likely to affect all regions of the brain, albeit differentially. $^{[18}F]FEPPA$ uptake was validated using a LPS-treated monkey model (Figure S1). The total binding was determined by calculating the mean SUV for $^{[18}F]FEPPA$ data from 85 – 115 minutes post-injection.
**Blood collection**

Animals were anesthetized with ketamine (10 mg/kg, IM) to collect blood and CSF monthly. Blood was collected in EDTA tubes from either femoral or saphenous veins. Plasma was extracted after a 15-minute centrifugation at 3000rpm at 4°C. CSF was obtained by lumbar puncture using sterile technique. All samples were stored at −80°C until assayed. Additional blood was collected at baseline, 8 weeks after MPTP and at endpoint to evaluate LPS and LPS binding protein (LBP) in a serum separator tube, allowed to remain at room temperature for at least 30 minutes to clot, and serum extracted after a 10-minute centrifugation at 1000g. Animals’ vital signs were monitored by YNPRC veterinary staff until animals fully recovered.

**Necropsy and Tissue Preparation**

Male and female animals were anesthetized with ketamine (10 mg/kg IM) and sodium pentobarbital (100 mg/kg IV) 26 or 40 weeks after the start of MPTP toxin dosing, respectively. The descending aorta was clamped and animals were trans-aortically perfused with cold oxygenated Ringer’s solution followed by 4% paraformaldehyde (PFA) and 0.1% glutaraldehyde in phosphate buffer (0.1M, pH 7.4) (Masilamoni et al., 2011). Prior to perfusion with PFA, a burr hole was drilled into the skull and a cortical biopsy taken using a skin biopsy tool for measurements of XPro1595 brain levels. The brain was removed and placed in 4% PFA for 48 hours and then cryoprotected in 30% sucrose. Sections of each segment of the small and large intestine were post-fixated in 4% PFA for 48 hours and then incubated in 70% isopropyl alcohol. Gut sections were processed and blocked in paraffin, cut on a rotary microtome at 5-micron thick sections and mounted on positive-charged glass microscope slides. The right hemisphere of the brain was cut in the coronal plane on a freezing microtome into 50-micron thick sections. All tissue was stored in cryoprotectant at −20°C before being processed for immunohistochemical or immunofluorescence analyses.

**Multiplexed immunoassays**

Plasma and CSF were analyzed for IFN-γ, IL-10, IL-1β, IL-2, IL-6, and IL-8 using a non-human primate V-PLEX pro-inflammatory panel (K15056D-1 MesoScaleDiscovery (MSD), Rockville, MD) multiplexed immunoassay with 1:2 dilutions for plasma. Biofluids were also measured for neutrophil gelatinase-associated lipocalin (NGAL), C-C motif chemokine ligand 2 (CCL2; also known as monocyte chemoattractant protein-1, MCP1) and TIMP-1 using a 3-PLEX cynomologous (catalog #K1519D-1, MSD) with 1:20 dilution for plasma, and for C-reactive protein (CRP) using a single analyte immunoassay (catalog #K151STD) with 1:1000 dilution for plasma. All CSF was analyzed using 1:1 dilution. All samples were measured in duplicate and immunoassays were performed as per the manufacturer’s instructions by the Emory Multiplexed Immunoassay Core.

**Immunohistochemistry and Immunofluorescence**

Brightfield immunological staining of coronal brain sections was performed as described previously (Masilamoni et al., 2011; Mathai et al., 2015) using antibodies against tyrosine hydroxylase (RRID:AB_572268; 1:10,000, Immunostar, Hudson WI), and macrophilin/CD68 clone KP1 (RRID:AB_10987212; 1:400, Thermofisher, Waltham, MA).
sections were further incubated in biotinylated secondary antibodies raised against the appropriate species, followed by avidin-biotin complex and developed in solution containing 0.025% 3,3’-diaminobenzidine tetrahydrochloride (Sigma-Aldrich), 10 mM imidazole, and 0.005% hydrogen peroxide in Tris buffer. Brain sections stained for CD68 were developed with nickel. Slides containing gut tissue were deparaffinized in xylene and rehydrated before performing antigen retrieval as previously published (Joers et al., 2014; Shultz et al., 2016). Gut tissue was similarly stained for CD68 without nickel enhancement. To assess microglial cell size, immunofluorescent staining was conducted using IBA1 (RRID:AB_839504; Wako, Richmond VA) and HLA-DR (B-lymphocyte LN-3; catalog 0893031, MP Biomedical, Santa Ana, CA) antibodies with a previously published protocol (Galvan et al., 2012).

Stereological estimation of TH+ and Nissl cells

The unbiased stereological estimation of the number of dopamine neurons in the ventral substantia nigra pars compacta (SNv), dorsal substantia nigra pars compacta (SNd), ventral tegmental area (VTA), locus coeruleus (LC) was achieved using the optical fractionator probe (Stereoinvestigator, MicroBrightField, Inc.), a stereological approach that combines the optical dissector with a fractionator sampling scheme. This sampling technique is not affected by tissue volume changes and does not require reference volume determinations. The random systematic sampling of counting areas was done using the Leica DMR microscope. TH-immunoreactivity (ir) and Nissl staining was used to sample cell number; these markers provide information as to whether reductions in the numbers of TH+ cell bodies are due to neuronal destruction or to a loss or downregulation of TH phenotype in the surviving neurons. To quantify midbrain TH+ and Nissl+ neuron number, slides were scanned at 20x using a ScanScope CS scanning system (Aperio Technologies, Vista, CA). Digital representations of the slides were saved and viewed using ImageScope software (Aperio Technologies). Low-power micrographs (1.8x) of TH, Nissl and calbindin-immunostained ventral midbrain sections were used to manually delineate the borders of the SNv, SNd, and ventral tegmental area, based on the presence or absence of calbindin-positive neurons (Masilamoni et al., 2010). Then, the borders of the different ventral midbrain regions were manually delineated on TH-immunolabelled and Nissl-stained slides adjacent to those immunostained for calbindin. Counts of TH+/Nissl+ cells were generated using a 100x oil-immersion objective. To perform unbiased stereology, counting frames (65 x 65 μm) were randomly placed by the stereology software within the chosen ROI. The software also controlled the position of the x–y stage of the microscope with a grid size of 300 x 300-μm using a dissector height of 27-μm and a 2-μm guard zone, so that the entire brain region could be scanned; 11 SN sections and 7 locus sections were used for stereological analyses. The software algorithm calculated the estimated total number of cells in each region of interest per hemisphere. The analysis was performed by one examiner blind with regard to the experimental condition.

Analysis of microglia size and phenotype

Imaris (version 7.2.2) reconstruction was used to quantify immunofluorescence staining of IBA1 and HLA-DR to assess microglial phenotype in the area of the SN. A ROI was placed around the DAT-expressing area and imaged using automatic tiling on an Olympus FV1000 confocal microscope at 20x (N.A 0.75, z-step: 1.00 μm). Within the SN ROI, 5 fluorescent
confocal stacks were collected at random for each piece of nigral tissue stained for DAT, IBA1 and LN3 (n=2 per animal). High-magnification image stacks were imported into Imaris and IBA1+ cells were identified using the “spot” function and assigned a designated number. Then, using a random number generator, a microglia cell was randomly selected from each image stack and a surface reconstruction performed of the IBA1 and HLA-DR based on fluorescence threshold. For quantification, the surface area and volumes of individual microglia were summed across all images and the percent coverage of HLA-DR per cell was calculated. CD68-positive particle counts were quantified using ImageJ software (version 1.52e).

Analysis of CD68-ir in gastrointestinal samples

Two sections from the Ileum and descending colon were evaluated for CD68-ir. Microphotographs were collected from two sites with intact villi avoiding those with large lacteal structures in the ileum and from colonic crypts in both the longitudinal and cross-section orientation. Two villi and longitudinal crypts per image were outlined, while a 100-mm² ROI was positioned over cross-section crypts for analysis. Images were analyzed using ImageJ software (version 1.52e) for optical density and area above threshold and averaged within animal.

Microbiota Profiling and Bioinformatics Analysis

Total DNA was extracted from the monkey fecal samples utilizing the FastDNA beadbeating Spin Kit for Soil (MP Biomedicals, Solon, OH, USA), and DNA concentrations verified with fluorometric quantitation (Qubit, Life Technologies, Grand Island, NY, USA). Sequencing libraries were generated using a two-stage “targeted amplicon sequencing (TAS)” protocol, as described previously (Naqib et al., 2018). Briefly, genomic DNA was PCR amplified with primers CS1_515F and CS2_806R (modified from the primer set employed by the Earth Microbiome Project (EMP; GTGYCAGCMGCCGCGGTAA and GGACTACHVGGGTWTCTAAT) targeting the V4 region of microbial small subunit ribosomal RNA genes. Subsequently PCR products were amplified using Access Array Barcodes for Illumina primers (Fluidigm, South San Francisco, CA) to incorporate Illumina sequencing adapters and sample-specific barcodes. Sequencing was performed using an Illumina MiSeq (Illumina, San Diego, CA, USA), with a V3 kit at the Sequencing Core at the University of Illinois at Chicago (UICSCQ). Raw sequence data (FASTQ files) were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA), under the BioProject identifier PRJNA597903.

Raw FASTQ files for each sample were merged using the software package PEAR (Paired-end-read merger) (v0.9.8)(Schmieder and Edwards, 2011; Zhang et al., 2014). Merged reads were quality trimmed, and primer sequences removed. Sequences shorter than 225 base pairs were discarded. Sequences were screened for chimeras (usearch8.1 algorithm), (Edgar, 2010) and putative chimeric sequences were removed from the dataset (QIIME v1.8) (Caporaso et al., 2010). Each sample was rarefied (34,000 sequences/sample) and data were pooled, renamed, and clustered into operational taxonomic units (OTU) at 97% similarity (usearch8.1 algorithm). Representative sequences from each OTU were extracted and classified using the uclust consensus taxonomy assigner (Greengenes 13_8 reference
A biological observation matrix (BIOM) (McDonald et al., 2012) was generated at each taxonomic level from phylum to species (“make OTU table” algorithm) and analyzed and visualized using the software packages Primer7 and the R programming environment (Clarke, 1993; R Development Core Team, 2013). Following sequencing, computational bioinformatics analysis was used to describe the bacterial diversity, community structure, and composition of the microbiota, as described previously (Bishehsari et al., 2018; Mahdavinia et al., 2018; Xiao et al., 2019). Dominant phylum, family, and genus bacteria were determined using a relative abundance threshold >2%, within the sex of monkeys. Both highly abundant (>2%) and low abundant (<2%) individual taxa were examined. Alpha diversity indices (i.e., Shannon, Simpson, richness, and evenness) were examined at different taxonomic levels (Phylum, class, order, family, genus, and species). To further compare microbial community structure, beta diversity (variance between animal samples) was conducted using Bray-Curtis pairwise dissimilarity analyses (0=similarity; 1=dissimilarity) to compare the overall microbial compositional differences.

**Short chain fatty acids levels: a marker of microbiota function**

SCFA are produced from gut bacteria fermentation of dietary carbohydrates and have been shown to inhibit inflammation (MacFabe, 2015). For SCFA measurements, stool was weighed, mixed with 3 parts carbonate-phosphate buffer and homogenized, and it was then centrifuged at 13000 rpm for 5 min. A mixture of 5% phosphoric acid containing 50mM 4-methylvaleric acid and 1.56 mg/mL copper sulfate in water was prepared as internal standard and was added to the supernatant.

Sample (4μl) was injected onto a capillary column in an Agilent 6890 GC with a flame ionization detector as described previously (Bishehsari et al., 2019; Y.E. et al., 2017). The GC column used was a Nukol (Supelco Bellefonte; PA) 30m length, inner diameter 0.25-mm ID, 0.25-μm bonded phase. The GC was run under the following conditions: injector temperature 240°C; detector temperature 230°C. The initial oven temperature 100°C was held for 10 min and then increased at 8°C to 192°C for total run time of 22 minutes. The carrier gas helium was maintained at 1mL/min flow rate.

**Statistical Analysis**

Due to the nature of the case series, minimal data was statistically analyzed and graphs were produced using Graphpad software (version 6f). A correlation between final [18F]FEPPA PET signal and microglia histology quantification was performed using a Pearson’s correlation coefficient. A Student’s t-test was used to analyze the difference between baseline and early MPTP time points of Bray Curtis beta diversity for female monkeys.

**Results**

**Rlk13 (male, vehicle drug treatment)**

Animal Rlk13 demonstrated a robust increase in [18F]FEPPA binding in the putamen motor (97% increase) and in the SN (32% increase) after 8 weeks of MPTP dosing prior to the start of vehicle treatment (PET I), corresponding to the peak of [18F]FEPPA binding for both regions throughout the study (Figure 2; Table 2). Central nhpTNF levels were evaluated at
baseline and 4 and 8 weeks after the initiation of MPTP and peaked at 4 weeks, the timepoint directly before the maximum $^{18}$FJEPPA uptake was identified at 8 weeks. Consistent with our previous reports (Masilamoni et al., 2010; Masilamoni et al., 2011), $^{18}$FECNT binding acutely increased after the start of MPTP and plummeted in the putamen motor (96% decrease) and SN (80% decrease) at endpoint scan (PET III; Table 2; SFigure 2). RIK13 presented with peak clinical impairment at 23 weeks, 3 weeks preceding the completion of their study. Immune mediators were measured every month throughout the study and followed a similar pattern as clinical status where CSF and plasma IL-6, NGAL and CRP were increased near endpoint (Figure 3). Chronically administered MPTP led to an expected severe reduction in TH+ nigral and locus coeruleus cell death as determined by stereological counting, and reduced putamen TH-ir as measured by optical density as compared to historical controls (SFigure 3). Postmortem quantification of dopaminergic TH-ir neurons and neuroinflammatory markers are presented within Table 2.

Evaluation of microbiome suggests very little phylum alpha diversity (variance within-sample) changes after 10 weeks of MPTP and an observable shift to decreased diversity measures by endpoint (following MPTP and vehicle drug treatment; Figure 4). Given the impact of PD on colonic bacterial composition reported by multiple groups (Keshavarzian et al., 2015; Scheperjans et al., 2015; Unger et al., 2016), we further evaluated the relative abundance of individual taxa at different taxonomic levels. Review of the phylum relative abundance suggests overall reduction of phylum diversity was largely attributed to an appreciable increase in *Firmicutes* phylum (Figure 5). Similar alpha diversity patterns were seen in class, order, and family taxa, while genus Shannon index decreased after 10 weeks of MPTP and remained at those levels at endpoint. Genus relative abundance identified a large reduction in *Prevotella* at MPTP that rebounds with excess at endpoint (Figure 6). Interesting, SCFA concentrations (acetate, propionate and butyrate) started at low levels and further dropped after 10 weeks of MPTP. Yet acetate, propionate and butyrate increased above baseline levels by 129%, 139%, and 257%, respectively after MPTP and vehicle drug treatment (Figure 7). Despite the post-MPTP reduced excreted sugar in the urine compared to baseline suggesting no disruption of barrier permeability, serum LPS and LBP levels markedly increased after continued MPTP and vehicle drug treatment (Figure 8).

Postmortem assessment of CD68-ir (Figure 9) in the lower colon crypts averaged a percent coverage of 7.12% (196% of control) and optical density of 0.19 (122% of control).

**RHg13 (male, XPro1595 drug treatment)**

Animal RHg13 presented peak $^{18}$FJEPPA binding in the putamen motor (84% increase) and SN (7.6% increase) at 8 weeks, equivalent to the time as the peak seen in RIK13 and 3 weeks prior to the start of XPro1595 drug administration (Figure 2). Levels of nhpTNF were greatest 4 weeks before the height of TSPO binding (Table 2). Despite XPro1595 drug treatment, a marked decline in $^{18}$FECNT binding was found in the putamen motor (99% decrease) and SN (91% decrease) 25 weeks after MPTP initiation (Table 2; SFigure 2). The high degree of DAT loss as measured by PET signal is paralleled by the highest clinical impairment at 24 weeks of MPTP dosing. Cytokine and immune mediators often peaked near endpoint (following MPTP and XPro1595 drug treatment), yet with the exception of
NGAL levels in CSF, the peak concentration did not reach the level seen from RIk13 (Figure 3).

Alpha diversity indices indicated that stool samples obtained after 10 weeks of low-dose MPTP displayed elevated measurements when compared to baseline and largely remained at those levels after MPTP combined with XPro1595 drug treatment (Figure 4). Gut microbiota relative abundance of multiple phyla were increased including Verrucomicrobia, WPS-2, and Tenericutes resulting in a considerable decrease in Firmicutes at both MPTP timepoints (Figure 5). Relative abundance of genus taxa demonstrated reduced levels of Prevotella (Prevotellaceae family) after MPTP that appreciably increased after XPro1595 drug treatment (Figure 6). SCFA acetate and propionate levels remained relatively stable throughout study, and butyrate stool concentrations dropped 61% and 56% from baseline following MPTP alone or with MPTP and XPro1595 treatment, respectively (Figure 7). RHg13 SCFA concentrations were often the lowest of all animals involved in the study. In vivo intestinal permeability at the final timepoint demonstrated increased excretion of lactulose and the lactulose:mannitol ratio compared to baseline, suggestive of small intestine leakiness (Figure 8). In line with permeability results and elevated circulating cytokines, robust increases in circulating LPS and LBP were found after MPTP and XPro1595. Lower colon crypt area coverage (110% of control) and optical density (99% of control) of CD68-ir were approaching levels of control animals (100%, Figure 9).

**RPd13 (female, vehicle drug treatment)**

The pattern of $^{18}$F]FEPPA binding was markedly different from that seen in the two male monkeys. Instead, animal RPd13 $^{18}$F]FEPPA binding did not increase above baseline levels in the putamen motor, however the same level of binding at baseline was matched at 38 weeks (Figure 2; Table 2). Whereas, the SN $^{18}$F]FEPPA binding elevated above baseline levels and peaked at 38 weeks (19% increase). Central nhpTNF concentration peaked at 8 weeks, but was the lowest “peak” concentration seen across all animals in the study. Similarly to the male animals, the $^{18}$F]FECNT standard uptake values increased shortly after the initiation of MPTP and were lowest at the final timepoint of 38 weeks in the putamen motor (83% decrease) and SN (59% decrease; Table 2; Figure 2). RPd13 presented with peak motor deficits as measured with the CRS at 31 weeks. Interestingly, inflammatory levels of NGAL and CRP in the plasma and CSF and levels of MCP1 plasma remained stable throughout the study (Figure 3). IL-6 in the plasma and CSF and MCP1 CSF were most responsive to MPTP with concentrations that crest in the final weeks of the study corresponding to the loss of $^{18}$F]FECNT binding. Shannon alpha diversity remained relatively steady at different taxonomic levels (Phylum, class, order, and species), yet demonstrated slight increases in family and genus taxa after MPTP (Figure 4). Evaluation of fecal microbial genus community highlighted a uniform decrease in a known beneficial SCFA-producing bacteria Blautia after MPTP. And after vehicle drug treatment there was an observable decrease in Prevotella (both Prevotellaceae and Paraprevotellaceae family) and Lactobacillus and increased unclassified Ruminococcaceae (Figure 5). After 10 weeks of MPTP administration, an observable drop (76 – 78%) in all SCFA levels was seen in stool and rebounded back toward baseline levels after continued MPTP and vehicle drug treatment (Figure 7). The intestinal permeability assay identified increased mannitol excretion at the
final evaluation point, yet LPS and LBP serum levels did not change throughout the study (Figure 8). By endpoint, the lower colon crypts expressed heightened CD68-ir area (203% of control) and optical density (120% of control) as the male treated with vehicle (Figure 9).

**RWo13 (female, XPro1595 drug treatment)**

$[^{18}F]$FEPPA binding dropped globally below baseline levels after MPTP administration began and gradually increased to maximum levels at 38 weeks in the putamen motor (3.2% increase) and SN (11% increase; Figure 2). The nhpTNF central concentration was greatest at baseline, suggesting RWo13 did not experience elevated levels of TNF in the early stages of chronic MPTP administration (Table 2; Figure 3). Similar to all other animals in the study, despite XPro1595 treatment, serious loss of $[^{18}F]$FECNT binding was identified at 38 weeks in the putamen motor (89% decrease) and SN (63% decrease; Table 2; SFigure2). Clinical rating scores were highest at 30 weeks and persisted near this level of impairment until the end of the study (Figure 3). Cytokine levels were consistent overtime for NGAL, CRP, and MCP1 in CSF and plasma, yet pro-inflammatory IL-6 and anti-inflammatory IL-10 responded most to MPTP/XPro1595 treatment (Figure 3).

Shannon diversity index demonstrated small fluctuations across taxonomic levels, such that phylum and family diversity altered only after MPTP and XPro1595 drug treatment, while class and order diversity decreased with MPTP alone and returns to baseline levels after drug treatment (Figure 4). Increased relative abundance of the phylum *Tenericutes* and *Verrucomicrobia* after XPro1595 complemented the overall increase in alpha diversity at that time point (Figure 5). Although genus Shannon diversity did not generally shift across the study, there was a detectable decrease in *Prevotella (Paraprevotellacase)* and *Blautia*, and increased *Lactobacillus* with XPro1595 drug treatment (Figure 6). Acetate and propionate SCFA decreased in all timepoints from baseline, while an observable drop in butyrate (37%) was only seen following drug treatment (Figure 7). There were no notable increases compared to baseline found in excreted sugar levels at either MPTP measurements, parallelising the unaltered circulating LPS and LBP concentrations (Figure 8). CD68 immunostaining of the lower colon crypts demonstrated area (155% of control) and optical density (113% of control) levels lower than vehicle-treated animals (Figure 9).

**RKo13 (female, XPro1595 treatment)**

Animal RKo13 presented with highest $[^{18}F]$FEPPA radioligand binding at 8 weeks in the putamen motor (37% increase) and SN (25% increase), at the same time as peak levels were seen in the male subjects but to a lesser intensity (Figure 2). Corresponding to the height of $[^{18}F]$FEPPA binding, RKo13 exhibited a peak concentration of nhpTNF in the CSF; a concentration that was highest amongst all monkeys (Table 2). As with all other case subjects and despite drug treatment, $[^{18}F]$FECNT binding in the putamen motor (98% decrease) and SN (86% decrease) substantially plummeted at the final timepoint or 38 weeks for RKo13 (Table 2; SFigure2). The largest deficit in clinical motor presentation was noted at 31 weeks, and was remarkably the highest among all female subjects (Figure 3). Of the evaluated cytokines and immune mediators, IL-6 was the most sensitive to clinical impairment, and IL-10 was considerably increased during the period of XPro1595 drug treatment, similar to other XPro1595-treated monkeys. Alpha diversity measured by
Shannon index slightly increased after 10 weeks of MPTP in all taxonomic levels with the exception of family taxa, and only returned back to baseline levels in genus and species taxa following MPTP and drug treatment (Figure 4). *Proteobacteria* and *Actinobacteria* were the most observable increased phylum at 10 weeks of MPTP, and the decreased abundance of *Firmicutes* compensated for those increases (Figure 5). Following XPro1595 treatment, *Prevotella* (*Paraprevotellacase)* dramatically decreased (Figure 6). All SCFA levels demonstrated a step increase throughout the study with butyrate levels increasing the most (95% after MPTP and 183% increase after MPTP and XPro1595 drug treatment), reaching the highest levels across all animals in the study (Figure 7). RKo13 demonstrated the most dramatic increases in excreted sugars (lactulose, mannitol and sucrose) after MPTP and XPro1595 treatment compared to baseline and to the other monkeys (Figure 8). Nonetheless, serum levels of LBP and LPS largely were not increased throughout the study. Lower colon CD68-ir area (134% of control) and optical density (110% of control) were elevated compared to controls, but remained prominently lower than the vehicle-treated animals (Figure 9).

**Discussion**

The overwhelming amount of evidence implicating a role for neuroinflammation in PD pathophysiology now compels our field to enrich for and enroll PD subjects for immunomodulatory clinical trials to better identify prognostic biomarkers, robust outcome measures, and therapeutic interventions. This is especially critical as various inflammatory targets become validated and/or drugs shown to be beneficial in autoimmune diseases are repurposed for use in the PD clinic. While a multitude of studies in rodents including several from our group suggest neuroinflammation starts early, precedes degeneration of the nigrostriatal pathway and, if blocked early, could mitigate degeneration of vulnerable populations, there is valid concern that results from those studies may have limited translational value to humans due to immunological differences between species; therefore, studies in non-human primates are merited to improve the probability that findings will translate from the lab to the clinic. Here we report the first attempt to evaluate the impact of progressive parkinsonism (modeled by chronic low doses of MPTP) on central and peripheral inflammatory responses in a case series report in rhesus monkeys. Additionally, we investigated nonmotor symptoms that arise in these animals including the first assessment of microbiome in MPTP monkeys, and the extent to which a sTNF-specific biologic XPro1595 (presently in clinical trials in early Alzheimer’s disease) could mitigate inflammatory responses and ameliorate nonmotor symptoms.

Our preliminary results highlight that monkeys RRI13, RRIg13, and RKo13 exhibited earlier peak TSPO upregulation as measured by \([^{18}F]FEPPA\) PET, which was associated with a poorer clinical phenotype and a greater loss of nigrostriatal \([^{18}F]FECNT\) binding at endpoint, regardless of accumulative MPTP. CRS impairment was echoed in the fine motor assessments using the FMS task, such that animals with the highest clinical score exhibited slower times to retrieve a treat (Figure S4). Furthermore, consistent with an earlier and more robust microglia PET response, these animals also exhibited higher levels of endogenous TNF in the CSF compared to the remaining two cases, RPd13 and RWo13. Enhanced neuroinflammation is critical in promoting neuronal damage. Mice with pharmacological
depletion of microglia using PLX3397 dramatically reduced MPTP neurotoxicity (Yang et al., 2018), and similarly, genetic deletion of TNF showed partial protection of nigral neurons following MPTP (Ferger et al., 2004). Therefore, a robust and early inflammatory response to MPTP that does not resolve would be expected to contribute to a faster rate of disease progression.

The earlier nigral [18F]FEPPA signal (8 weeks) seen in monkeys Rlk13, RHg13, and RKo13 was correlated with lower microglia IBA1+ cell size at endpoint compared to peak [18F]FEPPA signal seen in RPd13 and RWo13 at endpoint which was associated to larger IBA1 cell size (R²=0.837, p=0.0295). Our results indicate that IBA1 surface area is a good surrogate measurement for in vivo [18F]FEPPA binding. Previous studies have found good correlations between TSPO PET or autoradiography binding and histological immunoreactivity of innate immune cells positive for CD68 (Venneti et al., 2007; Vowinckel et al., 1997) and CD11b (Converse et al., 2011; Dickens et al., 2014). However, when evaluating nigral levels of antigen presentation proteins (HLA-DR) within the IBA1+ microglia or CD68+ particles (Table 2), we found no relationship with [18F]FEPPA binding; but that is not surprising as not all microglia effector functions are likely to be reflected in the activation status measured by upregulation of TPSO protein detected by [18F]FEPPA. Interestingly, the animals within this study expressing highest levels of HLA-DR tended to be associated with lower levels of CD68-ir particles (R²=0.7, p=0.079), potentially suggesting that MPTP may uncouple antigen presentation and lysosomal function. Future analyses to evaluate lysosomal function using BMV109 or cathepsin D may provide further incite on the lysosomal effects of chronic MPTP toxicity. While there are significant limitations to the use of TSPO ligands as specific indices of microglia activation, obtaining consistent outcomes from independent neuroimaging and neurohistological approaches give us additional confidence in the findings.

Notably, the most robust increase in [18F]FEPPA was identified in the putamen of the male monkeys (Rlk13 and RHg13) compared to any of the 3 female case studies. In another study, a MPTP-induced increase in TSPO was detected in the brains of male baboons (females not evaluated) using [11C]PK11195 PET (Chen et al., 2008). Interestingly, the pattern of [11C]PK11195 binding paralleled that found in the males in this study with an early pronounced increase and gradual decline to near baseline levels. In this study, male monkeys developed earlier and acute parkinsonism as measured by motor deficits and were sacrificed at 26 weeks, while females required additional weeks of MPTP to develop observable parkinsonian symptoms and were sacrificed at 40 weeks (Figure 3). There is a sexual dimorphic bias in the incidence of PD, such that it presents more in men than women with an age of onset 2 years earlier in men (Twelves et al., 2003). This feature has been recognized in preclinal models showing that male mice develop more severe MPTP-induced neurotoxicity than females (Antzoulatos et al., 2010; Przedborski et al., 2001).

Interestingly, women often have less severe motor deficits than men in advanced PD stages and respond better to levodopa therapy (Horstink et al., 2007; Zappia et al., 2005). However, the mechanisms underlying the development of PD regulated by sex remain unclear and merit further investigation.
Results from this case series may suggest that non-human primates display sex-specific differences in neuroinflammatory responses to MPTP as measured by circulating levels of inflammatory markers (Figure 3). In female monkeys, NGAL (or lipocalin-2) and CRP levels measured in biofluids did not increase during the course of the chronic MPTP delivery, while in males, these immune mediators demonstrated marked increases near the time of peak clinical status. Interestingly, male monkeys have intrinsically higher levels of NGAL and central MCP1 at baseline compared to female monkeys. NGAL has shown to heighten the sensitivity of neurons to amyloid beta toxicity in murine primary cortical cultures (Naude et al., 2012). While, MCP1 signaling recruits peripheral immune cells into the brain, and the crossing of peripheral monocytes has shown to be detrimental as demonstrated in animals models of multiple sclerosis and PD (Ajami et al., 2011; Harms et al., 2018); however, their exact role in the CNS is not clear. Taken together, our results may suggest that increased basal levels of NGAL and CCL2 influenced the male vulnerability to MPTP toxicity. There have been previous reports of heightened circulating cytokine levels in MPTP-treated monkeys. Specifically, levels of circulating TNF and IFNγ were still increased in male macaques when measured at least one year after MPTP administration, with highest levels in severely parkinsonian animals (Barcia et al., 2005; Barcia et al., 2011). Very few female monkeys were represented in these studies and cytokine analysis was not evaluated for sex effects. Although these observations must be confirmed in larger cohorts of animals, we speculate that perhaps females are able to regulate the inflammatory response that contributed to neurodegeneration more effectively than males, consistent with a slower progression of DA loss and motor symptoms. Yet the impact of sex on immune system responses and how the latter affect development of PD is not known.

Several studies have identified an influence of sex on the inflammatory state of PD patients (Houser et al., 2018; Manocha et al., 2017). Additionally, sex-biased expression of genes related to neuron and immune function have been identified in nigral tissue of PD patients (Mariani et al., 2018), highlighting the complex effects of sex and immunity on disease pathogenesis. In animal models, striatal dopamine depletion induced by MPTP was mitigated following estradiol hormone replacement in ovariectomized mice and monkeys (Liu and Dluzen, 2007; Miller et al., 1998; Morissette and Di Paolo, 2009), suggesting that estrogen may exert a neuroprotective role. Because estrogen influences multiple systems, the mechanism of its neuroprotective properties is not well understood. The monkeys used in this study are considered adults and although we did not measure circulating hormones, we expect the females to have had normal estrous cycles and this may have contributed to the sex-specific differences at baseline and as a response to chronic administration of MPTP.

To our knowledge, this study is the first to evaluate intestinal permeability, microbiota and targeted SCFA metabolomics from MPTP-treated monkeys. Previous studies in patients with PD have reported hyperpermeable intestinal epithelium as evinced by increased excreted sucralose 24 hours following ingestion of an oral sugar solution (Forsyth et al., 2011). Forsyth et al also reported that 5-hour urinary lactulose and mannitol were normal in these PD patients, suggesting that disruption of intestinal brier integrity (“leaky gut”) in early PD is primarily in the colon. In this study, it was not possible to collect urine for 24 hours; and therefore, we could not assess colonic permeability. Regardless, we did not find any consistent leakiness of small intestine across the case subjects. Robust increased levels of...
serum LBP and LPS were also detected in RIk13 and RHg13 (male monkeys) at the final sample collection (post-MPTP and post-drug treatment), suggesting bacterial translocation into the circulation of male but not female monkeys following MPTP-induced parkinsonism.

Throughout the study, both pre- and post-MPTP, we observed a distinct difference in the diversity of microbiome not only across subjects but also between sexes. This was seen across multiple taxonomic levels for both alpha diversity (Figure 4) and for relative abundances of specific intestinal bacterial RNA (Figure 5 and 6). For example, the relative abundance of genus Blautia and Prevotella were both distinctly higher in females compared to males at baseline and after 10 weeks of MPTP. Yet, Blautia, an anti-inflammatory SCFA-butyrate producing bacteria, appeared less abundant during prodromal stages in female monkeys compared to their baseline levels, which has also been reported in PD patients (Keshavarzian et al., 2015). Indeed, multiple clinical studies have identified gut microbial dysbiosis in PD (Hasegawa et al., 2015; Keshavarzian et al., 2015; Scheperjans et al., 2015), accompanied by alterations in bacterial metabolites or SCFA profiles (Unger et al., 2016), which are known to have anti-inflammatory properties (Li et al., 2018). Although fecal samples from these monkeys treated with MPTP did not reveal homogenous changes in the SCFA metabolite concentrations, there was a notable difference in the basal concentrations of all SCFA metabolites between sexes (Figure 7), where the three female subjects (RPd13, RWo13, RKo13) presented remarkably higher levels of SCFAs. Yet, the small number of cases precludes conclusions on the causal relationships between gastrointestinal measurements and MPTP-induced toxicity. Results from this study warrant further investigation into the evaluation of sex-specific effects on gut dysbiosis in PD, specifically during early prodromal stages of the disease.

We previously reported the neuroprotective effects of XPro1595 in a rat model of PD when drug dosing began 3 days after an intrastriatal injection of 6-OHDA. However, delaying treatment to 14 days after the dopaminergic insult also mitigated inflammation but did not result in neuroprotection, suggesting that early blockade of neuroinflammation is required for neuroprotection of nigral dopaminergic neurons in this acute rodent model of parkinsonism (Barnum et al., 2014). In this study, XPro1595 was not able to protect against MPTP-induced neurodegeneration. The escalating and weekly administration of MPTP in this monkey model may not be ideal when assessing neuroprotective properties of immunomodulatory therapies because of the compounded oxidative toxic effect overtime (in particular increased ROS and decreased mitochondrial function). Additionally, the early and robust increase in TSPO measured by PET scans prior to the beginning of XPro1595 treatment to inhibit sTNF indicates that the window for therapeutic brain effects derived from sTNF neutralization may have been missed in these animals. On the other hand, the lack of robust [18F]FEPPA PET signal in female monkeys throughout the study prevented us from assessing the effects of sTNF neutralization on global brain inflammation using PET, despite detectable levels of peripheral and central levels of XPro1595 (Figure S5). Because female monkeys largely displayed less reactive circulating cytokines compared to males, it is difficult to interpret the immunomodulatory effects of XPro1595. Evaluation of RIk13 and RHg13 (males) do show that despite animal RHg13 displaying a greater clinical impairment following MPTP and XPro1595 drug treatment, the peripheral and central cytokine levels remained lower than the vehicle-treated male counterpart RIk13. Additionally, the highest
levels of the anti-inflammatory cytokine, IL-10, were found in the plasma of monkeys treated with XPro1595 compared to vehicle drug treatment, independent of sex (Figure 3). And finally, animals treated with XPro1595 demonstrated lowest levels of CD68-ir in the lower colon by histology, despite colorectal biopsy cytokine levels (Figure S6). We interpret these findings as evidence that neutralization of sTNF with XPro1595 can dampen numerous ongoing inflammatory processes, regardless of the stage of motor symptoms.

We were not able to determine whether blockade of sTNF had an impact on nonmotor symptoms including gastrointestinal function and cognitive symptoms, due to the low number of animals and the variable responses to MPTP administration. In this study, both male and female monkeys presented with MPTP-induced deficits in attention set-shifting (extra-dimensional shift task), but not affect associative learning (intra-dimensional shift task), while female monkeys also demonstrated MPTP-induced deficits in working memory (Object retrieval task, Table 2). Our data extend results of previous studies reporting deficits in attention and executive function in rhesus monkeys given low doses of intravenous MPTP (Decamp and Schneider, 2004).

**Conclusion**

This novel and detailed case series report revealed animal-specific immune sensitivity to MPTP with earlier microglial activation by PET and CSF TNF that resulted in earlier parkinsonism as measured by motor deficits, which was more severely found in males compared to female monkeys. Sex differences were also identified in microbiota diversity and their targeted SCFA metabolites at basal levels and often in response to chronic MPTP. Therefore, this study underscores the importance of considering sex as a biological variable in preclinical animal models of PD. The effects of XPro1595 were difficult to interpret because of the sexually dimorphic responses and the small animal numbers, but the preliminary findings suggest soluble TNF neutralization may have been associated with attenuated inflammation in biofluids and reduced CD68 expression in the colon. Yet, it was clear that XPro1595 was not able to counteract the escalating dose delivery of MPTP and failed to protect against dopaminergic nigrostriatal loss. This conclusion is based on a low number of animals that displayed significantly different experimental timelines due to the sex differences in MPTP sensitivity. Additional studies with larger group sizes of both sexes would enable confirmation and extension of these findings; alternatively, a different study design where XPro1595 administration is begun earlier and the MPTP dose is not escalated may afford more neuroprotection. Nevertheless, our results lay the foundation for the exciting possibility that inflammatory responses to neurotoxic stimuli may be sexually dimorphic and should be taken into consideration when initiating or assessing therapeutic immunomodulatory interventions in the clinic.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.
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Animals performed a weekly fine motor skill (FMS) task and were assessed weekly for clinical parkinsonism status using a clinical rating scale (CRS). Weekly systemic peripheral MPTP injections (0.2 – 0.8 mg/kg, im) were administered to achieve and maintain mildly parkinsonian features by CRS. Drug treatment with either XPro1595 (10 mg/kg sq) or vehicle began 11 weeks after the start of MPTP injections and continued every 3 days until the end of the study. PET scans for $^{18}$F-FECNT and $^{18}$F-FEPPA were performed 1 week apart at the times denoted by “PET”. Bi-monthly blood and CSF samples were collected to analyze circulating markers of inflammation and levels of XPro1595. Cognitive behavior was tested throughout the study using an intradimensional (IDS) and extradimensional (EDS) shift task and an object self-order (ObjSO) task. Gastrointestinal measurements (GI) including functional analyses for permeability and specimen collection of colorectal biopsies and feces for microbiome analyses were conducted at baseline, ~10 weeks after the start of MPTP treatment (prior to drug/vehicle treatment) and again at endpoint following either XPro1595 or vehicle treatment. Male and female monkeys were euthanized 26 and 40 weeks after the start of MPTP injections, respectively. Unless otherwise noted, procedures were done on both male and female monkeys.
Figure 2.

[$^{18}$F]FEPPA PET reveal sex differences in response to chronic systemic MPTP injections. Representative coronal images of [$^{18}$F]-FEPPA uptake in a post-commissural striatal slice of chronically MPTP-treated male and female rhesus monkeys treated systemically with vehicle (A) or XPro1595 (10mg/kg) at baseline, 8 (PET I), 16 (PET II), 24/27 (PET III) and 39 weeks (PET IV) after the start of MPTP administration. Coronal MRI image of the post-commissural striatum that matches the plane of PET images. Individual [$^{18}$F]FEPPA quantification as it relates to [$^{18}$F]FECNT binding in the putamen and substantia nigra shows distinct patterns of [$^{18}$F]FEPPA binding between sexes with no discernable effect of XPro1595 treatment.
Figure 3.
Chronic MPTP induces central and peripheral immune responses in monkeys. Individual weekly assessment of clinical rating score (CRS) demonstrates increased impairment and a greater sensitivity to MPTP in males leading to a shorter project timeline (A). Plasma IL-6 levels show a similar pattern as CRS, revealing increased levels were associated with greater clinical impairment, suggesting their potential use as a biomarker of MPTP-induced parkinsonism. (B). Cytokine levels across time for IL-6 (C), NGAL (D,E) and CRP (F,G) MCP1 (H,I), and IL-10 (J). Solid lines represent vehicle-treated animals and dashed lines represent XPro1595-treated animals. Male animals represented by blue lines.
Figure 4. Alpha diversity increased in males compared to females following low doses of MPTP. Alpha diversity measures of Shannon, Simpson, Evenness and Richness indices for phylum taxa (A-D), are increased in males compared to females after low doses of systemic MPTP when animals did not present with motor phenotype. Similar patterns were found in identical alpha diversity indices at lower taxonomic levels including class, order and family (not genus nor species, data not shown). The baseline genus microbial composition in female display trends towards dissimilarity (p=0.0676), when compared to their corresponding MPTP measures (E). Only trend lines can be compared between male timepoints (n=1; F). Values closest to 1 represent dissimilarity in species populations while values closest to 0 are
most similar. MPTP = 10 weeks of MPTP dosing and pre-drug treatment; MPTP + treatment = endpoint measurement post-MPTP and post-drug treatment after 26 weeks of MPTP in males and 40 weeks of MPTP in females. Solid lines represent monkeys treated with vehicle and dashed lines represent monkeys treated with XPro1595. Male animals represented by blue lines.
Gut microbiota relative abundance of phylum individual taxa differs between sexes. Stacked column bar charts display the relative abundance (%) of bacterial phylum individual taxa in each subject for baseline, MPTP (after 10 weeks of MPTP) and MPTP + treatment timepoints and reveal greater phylum diversity in males than females. MPTP administration has a greater effect in males than females showing an observable reduction in the abundances of Firmicutes and Firmicutes to Bacteroidetes ratio and increased Verrucomicrobia. Solid lines represent monkeys treated with vehicle and dashed lines represent monkeys treated with XPro1595 at MPTP + treatment. Male animals represented by blue lines.

Figure 5.
Figure 6. Gut microbiota relative abundance of genus individual taxa differs between sexes.
Stacked column bar charts display the relative abundance (%) of bacterial genus individual
taxa in each subject for baseline, MPTP (after 10 weeks of MPTP) and MPTP + treatment
timepoints. Collectively, following 10 weeks of MPTP dosing (MPTP) males display a
decreased relative abundance of Prevotella (Prevotellaceae), while females display a
decreased relative abundance of the bacterial genus Blautia. Following treatment, males
increased Prevotella in both family taxa and blautia, while females noticeably decreased
abundance of Prevotella (Paraprevotellaceae). No pattern emerged for vehicle-treated (solid

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lines) compared to XPro1595-treated (dashed lines) monkeys. Male animals represented by blue lines.
Figure 7. Individual short chain fatty acids in stool.
SCFA acetate, butyrate, propionate and total SCFA levels in RIk13 and RHg13 (males) and RPd13, RWo13 and RKo13 (females) at baseline, MPTP (10 weeks of MPTP) and MPTP + treatment (at endpoint).
Figure 8. Gastrointestinal permeability.
Serum levels of lipopolysaccharide (A) and lipopolysaccharide binding protein (B) reveal male monkeys (Rlk13, RHg13) have markedly higher levels at baseline and at endpoint (MPTP + treatment) compared to female monkeys (RPd13, RWo13, RKO13). Gut permeability was analyzed by the sugar excreted as a % of the oral dose is graphed for lactulose (C), mannitol (D), lactulose:mannitol (E), and sucrose (F) for each animal at baseline, MPTP and post-MPTP/post-drug treatment timepoints.
Figure 9.
Treatment with XPro1595 reduced CD68-ir in the lower colon. Representative microphotographs of CD68 staining in the crypts of the lower colon either in longitudinal (A) or cross-sectional orientation (B). Quantification of CD68-ir optical density (C) and % area coverage (D) demonstrate reductions in the colon of XPro1595-treated monkeys (RHg13, RWo13, RKo13) compared to vehicle-treated (RIk13, RPd13). Solid colored bars represent animals treated with vehicle; Patterned bars represent animals treated with XPro1595. Male animals represented by blue bars.
Table 1.

Case study characteristics

<table>
<thead>
<tr>
<th>Case ID</th>
<th>Sex</th>
<th>Drug treatment started at 11 weeks</th>
<th>Length of study (weeks)</th>
<th>Accumulative MPTP (mg/kg)</th>
<th>Total weeks of MPTP</th>
<th>Total weeks of drug treatment</th>
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</thead>
<tbody>
<tr>
<td>Rlk13</td>
<td>Male</td>
<td>Vehicle</td>
<td>26</td>
<td>5.9</td>
<td>23</td>
<td>15</td>
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<tr>
<td>RHg13</td>
<td>Male</td>
<td>XPro1595</td>
<td>26</td>
<td>5.9</td>
<td>23</td>
<td>15</td>
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<tr>
<td>RPd13</td>
<td>Female</td>
<td>Vehicle</td>
<td>40</td>
<td>15.2</td>
<td>38</td>
<td>29</td>
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<tr>
<td>RWo13</td>
<td>Female</td>
<td>XPro1595</td>
<td>40</td>
<td>15.2</td>
<td>38</td>
<td>29</td>
</tr>
<tr>
<td>RKo13</td>
<td>Female</td>
<td>XPro1595</td>
<td>40</td>
<td>15.2</td>
<td>38</td>
<td>29</td>
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</table>
Table 2.

<table>
<thead>
<tr>
<th>Study outcome measures</th>
<th>Rlk13</th>
<th>RHg13</th>
<th>RPd13</th>
<th>RWo13</th>
<th>RKo13</th>
</tr>
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<tbody>
<tr>
<td>Peak PUT FEPPA SUV (wk)</td>
<td>2.41 (8)</td>
<td>2.30 (8)</td>
<td>1.10 (0)</td>
<td>1.23 (38)</td>
<td>1.21 (8)</td>
</tr>
<tr>
<td>Peak SN FEPPA SUV (wk)</td>
<td>0.79 (8)</td>
<td>0.72 (8)</td>
<td>0.88 (38)</td>
<td>0.92 (38)</td>
<td>0.75 (8)</td>
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<tr>
<td>Peak CRS (wk)</td>
<td>10 (23)</td>
<td>18.75 (24)</td>
<td>8.5 (31)</td>
<td>9.75 (30)</td>
<td>14.75 (31)</td>
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<tr>
<td>Peak nhpTNF pg/mL (wk)</td>
<td>3.43 (4)</td>
<td>2.83 (4)</td>
<td>1.94 (8)</td>
<td>2.51 (0)</td>
<td>3.86 (8)</td>
</tr>
<tr>
<td>Lowest PUTm FECNT SUV (wk)</td>
<td>0.5 (25)</td>
<td>0.009 (25)</td>
<td>1.41 (38)</td>
<td>0.85 (38)</td>
<td>0.15 (38)</td>
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<tr>
<td>Lowest SN FECNT SUV (wk)</td>
<td>0.64 (25)</td>
<td>0.32 (25)</td>
<td>1.00 (38)</td>
<td>0.78 (38)</td>
<td>0.30 (38)</td>
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<td>dSNpc cell # (% Ctrl)</td>
<td>28,686 (73.7%)</td>
<td>21,869 (56.2%)</td>
<td>24,923 (64.1%)</td>
<td>20,449 (52.6%)</td>
<td>14,201 (36.5%)</td>
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<td>vSNpc cell # (% Ctrl)</td>
<td>51,124 (35.7%)</td>
<td>30,390 (21.2%)</td>
<td>46,153 (32.2%)</td>
<td>31,526 (22.0%)</td>
<td>15,053 (10.5%)</td>
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<td>LC cell # (% Ctrl)</td>
<td>15,384 (68.4%)</td>
<td>13,412 (59.6%)</td>
<td>10,650 (47.4%)</td>
<td>16,370 (72.8%)</td>
<td>9,861 (43.9%)</td>
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<td>EDS2 errors to criterion (% of EDS1)</td>
<td>243 (155%)</td>
<td>202 (136%)</td>
<td>113 (314%)</td>
<td>285 (328%)</td>
<td>267 (988%)</td>
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<tr>
<td>ObjSO-2 Trial3 perseverative errors (% of ObjSO-1)</td>
<td>n/a</td>
<td>n/a</td>
<td>55 (220%)</td>
<td>54 (540%)</td>
<td>62 (248%)</td>
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<tr>
<td>IBA1 cell size um² (% Ctrl)</td>
<td>780.9 (105.5%)</td>
<td>474.3 (64.1%)</td>
<td>1033.6 (139.7%)</td>
<td>1233.4 (166.7%)</td>
<td>716.2 (96.8%)</td>
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<td>HLA-DR cell size um² (% Ctrl)</td>
<td>43.1 (137%)</td>
<td>95.7 (305%)</td>
<td>72.1 (230%)</td>
<td>26.7 (85%)</td>
<td>40.7 (130%)</td>
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<tr>
<td>CD68-ir particle count (% Ctrl)</td>
<td>1487 (385%)</td>
<td>1284 (332%)</td>
<td>1243 (322%)</td>
<td>2178 (564%)</td>
<td>1703 (441%)</td>
</tr>
</tbody>
</table>