

## LRRK2-Mediated Rab10 Phosphorylation in Immune Cells From Parkinson's Disease Patients

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**ABSTRACT:** **Background:** Leucine-rich repeat kinase 2 is a potential therapeutic target for the treatment of Parkinson's disease, and clinical trials of leucine-rich repeat kinase 2 inhibitors are in development. The objective of this study was to evaluate phosphorylation of a new leucine-rich repeat kinase 2 substrate, Rab10, for potential use as a target engagement biomarker and/or patient enrichment biomarker for leucine-rich repeat kinase 2 inhibitor clinical trials.

**Methods:** Peripheral blood mononuclear cells and neutrophils were isolated from Parkinson's disease patients and matched controls, and treated *ex vivo* with a leucine-rich repeat kinase 2 inhibitor. Immunoblotting was used to measure levels of leucine-rich repeat kinase 2 and Rab10 and their phosphorylation. Plasma inflammatory cytokines were measured by multiplex enzyme-linked immunosorbent assay.

**Results:** Mononuclear cells and neutrophils of both controls and Parkinson's disease patients responded the same to leucine-rich repeat kinase 2 inhibitor treatment. Leucine-rich repeat kinase 2 levels in mononuclear cells were the same in controls and Parkinson's disease patients, whereas leucine-rich repeat kinase 2 was signif-

icantly increased in Parkinson's disease neutrophils. Rab10 T73 phosphorylation levels were similar in controls and Parkinson's disease patients and did not correlate with leucine-rich repeat kinase 2 levels. Immune-cell levels of leucine-rich repeat kinase 2 and Rab10 T73 phosphorylation were associated with plasma inflammatory cytokine levels.

**Conclusions:** Rab10 T73 phosphorylation appears to be a valid target engagement biomarker for potential use in leucine-rich repeat kinase 2 inhibitor clinical trials. However, a lack of association between leucine-rich repeat kinase 2 and Rab10 phosphorylation complicates the potential use of Rab10 phosphorylation as a patient enrichment biomarker. Although replication is required, increased leucine-rich repeat kinase 2 levels in neutrophils from Parkinson's disease patients may have the potential for patient stratification. Leucine-rich repeat kinase 2 activity in peripheral immune cells may contribute to an inflammatory phenotype. © 2018 International Parkinson and Movement Disorder Society

**Key Words:** biomarker; blood; inflammation; Parkinson's disease; Rab GTPase

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Leucine-rich repeat kinase 2 (LRRK2) has emerged as a promising therapeutic target for the treatment of Parkinson's disease (PD). At least 6 missense mutations have been identified in LRRK2 that are pathogenic for autosomal-dominant familial PD, and collectively LRRK2 mutations contribute to ~1%-5% of all PD.<sup>1</sup> Importantly however, PD associated with LRRK2 mutations is largely clinically indistinguishable from common idiopathic PD.<sup>2</sup> Moreover, genetic variants in and around the LRRK2 locus are robustly associated with an increased risk of idiopathic PD.<sup>3,4</sup> These latter points are suggestive of a role for LRRK2 in the pathogenesis of both familial and idiopathic PD.

The first *in vitro* LRRK2 activity assays demonstrated that the most common pathogenic missense mutation, G2019S, increased the enzyme's catalytic kinase activity.<sup>5,6</sup> Substantial investment from both academia and industry has subsequently gone into the development of small-molecule inhibitors of LRRK2 (for recent reviews, see references 7-9). Indeed, LRRK2 inhibitor development has reached a point at which potent and selective compounds are available for clinical trials. *In vivo* studies have also now confirmed that all known pathogenic missense LRRK2 mutations increase the enzyme's kinase activity. Originally observed by incorporation of adenosine triphosphate into LRRK2 itself,<sup>10</sup> increased activity with pathogenic mutations was further quantified using an antibody to the LRRK2 serine 1292 autophosphorylation site.<sup>11</sup> Most recently, increased LRRK2 kinase activity with pathogenic mutations was again observed using newly identified Rab GTPase LRRK2 substrates.<sup>12</sup>

LRRK2 has been demonstrated both *in vitro* and *in vivo* to directly phosphorylate a subset of Rab GTPase family members.<sup>12,13</sup> The most robust LRRK2 substrate is Rab10, which is phosphorylated on threonine 73, a residue in the switch II region of Rab10 that appears important for regulating Rab10 protein interactions and subcellular localization.<sup>12</sup> Rab10 is expressed in peripheral blood cells including B-lymphocytes, monocytes, and neutrophils, and in healthy controls, Rab10 phosphorylation is decreased in these cell types by *ex vivo* LRRK2 inhibitor treatment.<sup>14,15</sup>

The observation that LRRK2 inhibitors reduce Rab10 phosphorylation is important. The current gold standard readout of LRRK2 inhibitor target engagement is to monitor reduced phosphorylation of serine 935, a residue on the LRRK2 protein that is required for interaction with 14-3-3 protein family members.<sup>16,17</sup> The use of LRRK2 serine 935 as a peripheral readout of LRRK2 inhibitor target engagement has been further validated in PD cohorts;<sup>18,19</sup> however, phosphorylation on this residue is complexly regulated, including phosphorylation by other kinases and counter-regulation by phosphatases.<sup>20-22</sup> For a clinical

trial of LRRK2 inhibitors, it would therefore be prudent to include a second readout of inhibitor target engagement.

In this study we have investigated Rab10 protein levels and Rab10 threonine 73 phosphorylation in both peripheral mononuclear cells and neutrophils from idiopathic PD patients and matched controls. We aimed to determine if Rab10 phosphorylation is increased in these cells in idiopathic PD patients. We also aimed to determine the extent to which Rab10 phosphorylation is reduced in idiopathic PD patients following *ex vivo* inhibitor treatment and the extent to which Rab10 phosphorylation associates with PD clinical variables.

## Materials and Methods

### Participants

Individuals with PD and age- and sex-matched healthy volunteers with no diagnosed neurological disease were recruited through 2 centers: the Brain and Mind Centre, University of Sydney, and the Parkinson's Institute and Clinical Centre, California. All participants gave informed consent, and the study was approved by the University of Sydney Human Research Ethics Committee (approval number 2016/657) and the El Camino Hospital Institutional Review Board (ECH-10-17) for sample collection at the Parkinson's Institute and Clinical Center. Participants with PD met the Movement Disorders Society criteria for clinically established disease and did not have an established family history of PD.<sup>23</sup> Motor symptom severity was measured using the Movement Disorders Society Unified Parkinson's Disease Rating Scale (MDS-UPDRS) part III.<sup>24</sup> The participant demographic data are shown in Table 1. Following clinical assessment, up to 40 mL of venous blood was collected from each participant by a trained phlebotomist and used for the isolation of neutrophils and peripheral blood mononuclear cells (PBMCs). A 4-mL blood sample was also collected into an ethylene-diaminetetraacetic acid (EDTA) tube for the collection of plasma, which was snap-frozen and stored at -80°C.

### Isolation of Neutrophils

Venous blood was collected into sodium citrate tubes (BD Bioscience, Franklin Lakes, NJ), and neutrophils were isolated by negative selection using EasySep Direct Human Neutrophil Isolation Kits (Stemcell Technologies, Vancouver, BC) as per the manufacturer's protocol. In brief, EDTA (in phosphate-buffered saline [PBS]) was added into 20 mL of blood for a final concentration of 1 mM. Isolation cocktail antibody and Rapid-Spheres magnetic beads (provided in kit) were added to the blood (both 50 µL/mL) and incubated for 5 minutes at room temperature. Samples were made up to 50 mL with 1 mM EDTA in PBS and placed into Easy 50 Easy-Sep magnets (Stemcell Technologies, Vancouver, BC)

**TABLE 1.** Demographic details for Parkinson's disease and control participants

	Recruited through PICC		Recruited through USYD		Combined demographics	
	Parkinson's disease	Control	Parkinson's disease	Control	Parkinson's disease	Control
Participant number	17	16	31	31	48	47
Age (y)	62 ± 3 (36-89)	62 ± 3 (35-80)	67 ± 1 (51-83)	66 ± 1 (48-80)	66 ± 1 (36-89)	66 ± 1 (35-80)
Sex (% male)	50%	31%	61%	61%	57%	51%
Disease duration (y)	7 ± 1 (0-19)		5 ± 1 (1-19)		6 ± 1 (0-19)	
Age at diagnosis (y)	55 ± 3 (36-82)		63 ± 2 (47-77)		60 ± 2 (36-82)	
Disease severity	17 ± 2 (5-37.5)		26 ± 2 (6-55)		23 ± 2 (5-55)	
LED	585 ± 81 (0-1855)		518 ± 78 (0 - 1297.5)		541 ± 61 (0-1855)	

Data are shown as mean ± SEM, with the range indicated underneath in parentheses. Disease severity was measured using the Movement Disorders Society Unified Parkinson's Disease Rating Scale (MDS-UPDRS) part III. LED is the calculated L-dopa-equivalent dose.

for 10 minutes at room temperature. The enriched cell suspension was transferred to a new tube, followed by the addition of 1 mL of RapidSpheres magnetic beads for 5 minutes. The tube was then placed in the magnet for 5 minutes. To ensure the removal of magnetic beads, enriched cells were again transferred into a new tube and placed in the magnet for 10 minutes for a third round of separation. Cells were then washed with PBS, centrifuged at 335 g for 5 minutes (both acceleration and deceleration at 5), and resuspended in Rosewell Park Memorial Institute (RPMI) medium supplemented with 10% heat-inactivated low-endotoxin fetal bovine serum (FBS) and 1× penicillin/streptomycin solution (all from Gibco, Thermo Fisher, Waltham, MA). Cell count and viability were determined using trypan blue and an automatic cell counter (Countess II-FL, Thermo Fisher, Waltham, MA). The average viability was 95% ± 0.4%. For neutrophil purity assessment, cells were resuspended in flow buffer (1× PBS, 1 mM EDTA, 25 mM HEPES, 1% heat-inactivated FBS, pH 7.0) and stained with anti-CD66b monoclonal antibody conjugated to fluorescein isothiocyanate (BD Biosciences, Franklin Lakes, NJ) for 30 minutes at 4°C. The appropriate isotype negative control antibody was included. Neutrophil purity was measured using a FACS Fortessa X-20 cytometer (BD Biosciences, Franklin Lakes, NJ) with neutrophils defined as the CD66b-positive population. Data were analyzed using FlowJo software version 10.2 (Tree Star Inc.) with neutrophil purity routinely >95% (Supplementary Fig. 1).

### Isolation of Peripheral Mononuclear Cells

Venous blood was collected into sodium heparin tubes (BD Bioscience), and PBMCs were isolated using Ficoll. In brief, 15 mL of blood was diluted with an equal volume of phosphate-buffered saline (PBS) containing 2% low-endotoxin FBS (Gibco, Thermo Fisher, Waltham, MA) and layered on top of Ficoll-Paque PREMIUM (GE Healthcare, Chicago, IL) in a SepMate-50 tube (Stem Cell Technologies, Vancouver, BC). The SepMate-50 tubes were processed according to the manufacturer's protocol by centrifugation at 1200g for 10 minutes at room temperature. PBMCs were transferred to a 15-mL

centrifuge tube and washed twice with PBS containing 2% FBS with centrifugation at 1000g for 2 minutes. The PBS was aspirated, and the PBMC pellet was resuspended in 10 mL of RPMI medium as described above for neutrophils. Cell count and viability were determined using trypan blue and an automatic cell counter (Countess II-FL, Thermo Fisher, Waltham, MA). The average viability was 80% ± 1.2%. Purity assessment was performed as described for neutrophils, with the CD66b-negative population considered PBMCs. PBMC purity was routinely >95% (Supplementary Fig. 2). The blood for neutrophil isolation was obtained first, and in 8 cases insufficient blood could subsequently be obtained for PBMC isolation.

### LRRK2 Inhibitor Treatment and Cell Lysis

PBMCs and neutrophils were both treated with 30 or 100 nM of the LRRK2 inhibitor MLi2<sup>25</sup> dissolved in dimethylsulfoxide (DMSO) or an equal volume of DMSO alone as control for 30 minutes at room temperature in the dark. MLi2 was kindly provided by Dario Alessi, University of Dundee. Cells were then spun down and pellets lysed in buffer containing 50 mM Tris-HCl (pH 7.5), 1% (v/v) Triton X-100, 1 mM ethylene glycol-bis(β-aminoethyl ether) N-N-N'-N'-tetraacetic acid, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 50 mM sodium fluoride, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 0.1 µg/mL mycroystin-LR, 0.27 M sucrose, 0.1% (v/v) β-mercaptoethanol, 1× complete EDTA-free protease inhibitor cocktail (Roche, Basal, Switzerland), and 1 mM di-isopropylfluorophosphate (Sigma, St Louis, MI). For every 10 million cells, 100 µL of lysis buffer was used. For robust lysis, cells were incubated on ice for 10 minutes and lysates then snap-frozen and stored at -80°C until analysis.

### Immunoblotting

Samples were thawed on ice and clarified by centrifuging at 10,000g for 20 minutes at 4°C. Protein concentration was determined by bicinchoninic protein assay (Thermo Fisher, Waltham, MA) and samples made up

in 1× LDS sample buffer (Thermo Fisher, Waltham, MA). Up to 20 µg of lysate was separated using 4%-12% Novex Tris-glycine gels (Thermo Fisher, Waltham, MA) and transferred to a nitrocellulose membrane (Biorad, Hercules, CA). Membranes were blocked with 5% skim milk powder in Tris-buffered saline with 0.1% (v/v) Tween 20 (TBST). Membranes were cut into strips based on molecular weight markers and probed overnight at 4°C for phosphorylated Rab10 P-T73 (Abcam, Cat ab230261 [MJF-R21], diluted 1:1000), Rab10 (Cell Signaling Technologies, diluted 1:1000), LRRK2 (N241A/34, Neuromab, diluted 1:1000), phosphorylated LRRK2 P-S935 (UDD2, Abcam, diluted 1:1000), and β-actin (Abcam, diluted 1:50,000) as loading control. All membrane strips were processed identically and imaged simultaneously. The LRRK2 and Rab antibodies have been validated using relevant knockouts.<sup>13,20,26</sup> After overnight incubation, membranes were washed in TBST. Antirabbit and antimouse horseradish peroxidase secondary antibodies (Abcam, Cambridge, UK) at 1:5000 dilution in 2.5% skim milk in TBST or antimouse fluorescent secondary antibodies (Abcam, Cambridge, UK) in TBST were then used. Enhanced chemiluminescence reagent (GE Healthcare, Chicago, IL) was used for detection using a Chemidoc MP digital imaging system (Biorad). Immunoblot quantification was performed using ImageJ software 5.2.1 (Biorad, Hercules, CA). Representative cropped immunoblots are shown in the figures. Representative uncropped immunoblots are available as Supplementary Figures 3-6.

### Inflammatory Cytokine Enzyme-Linked Immunosorbent Assay

A panel of 27 inflammatory cytokines was measured in plasma using a Bioplex human cytokine multiplex enzyme-linked immunosorbent assay, exactly as we have performed previously.<sup>27</sup>

### Statistical Analysis

All statistical analyses were performed using either SPSS Statistics software (IBM, Armonk, NY) or R, with significance set at  $P < 0.05$ . Analysis of variance, with age and sex included as covariates, was used to compare levels and phosphorylation of LRRK2 and Rab10 in control and PD patient cells at baseline. Data were checked for normal distribution using the Shapiro-Wilk test. Nonnormally distributed data were transformed using either log<sub>10</sub> or square root functions. To analyze the effect of LRRK2 inhibition on LRRK2 and Rab10 phosphorylation in the control and PD patient cells, 2-way repeated-measures analysis of variance (ANOVA) with Tukey's post hoc test was used. An independent-samples *t* test was used to compare inflammatory cytokine levels between control and PD. Pearson and/or Spearman correlations were used to determine any associations between levels

and phosphorylation of LRRK2 and Rab10 and participant demographic variables or inflammatory cytokines. Bonferroni-adjusted *P* values were employed for multiple comparisons. Graphs were generated using Prism software version 7.0 (GraphPad, San Diego, CA) or R.

## Results

### LRRK2 Inhibitor Treatment Reduces Rab10 Phosphorylation in PD Patient Neutrophils

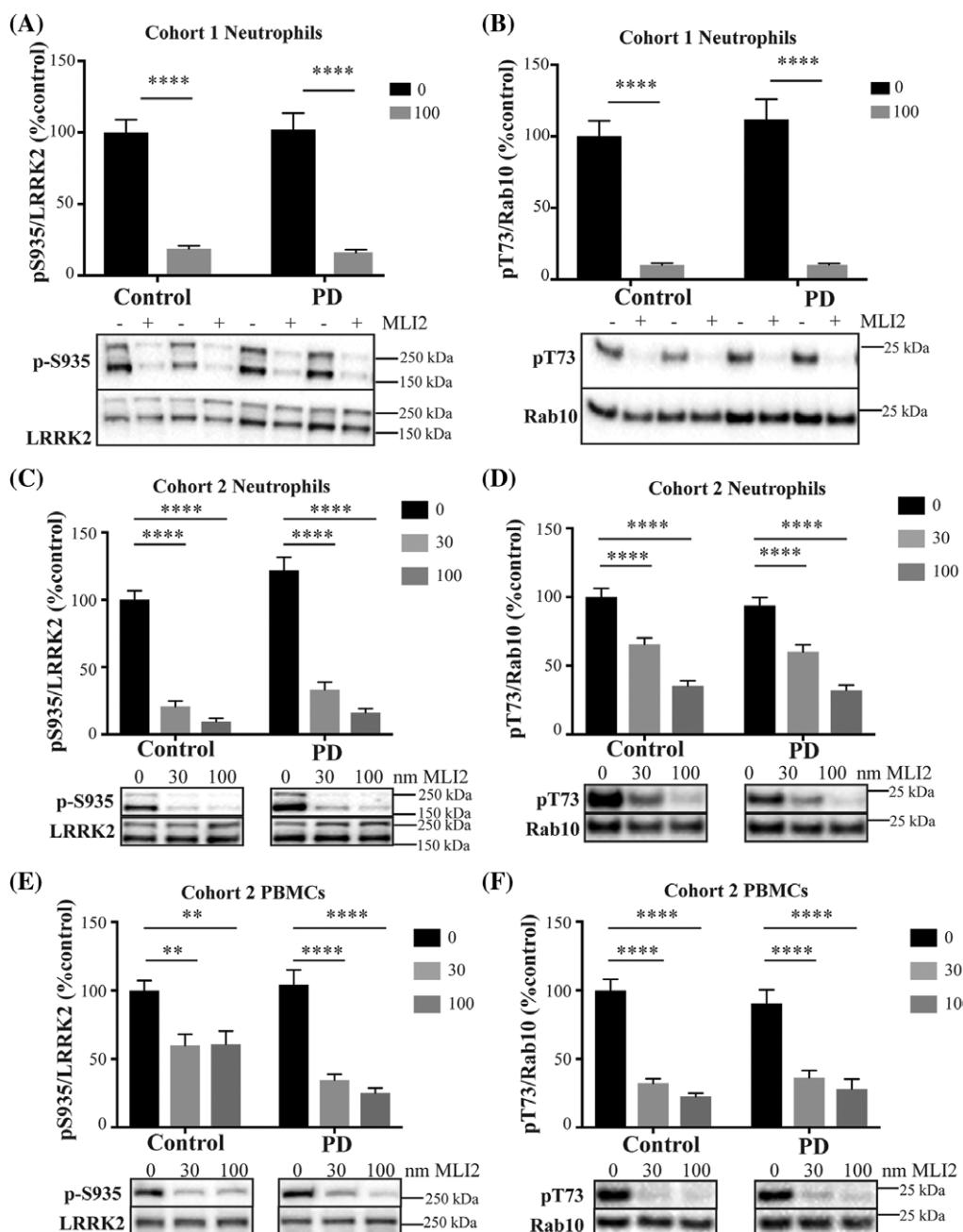
LRRK2 has recently been reported as highly expressed in neutrophils; thus, we conducted an initial study to assess the effect of *ex vivo* LRRK2 inhibitor treatment on Rab10 T73 phosphorylation in control ( $n = 16$ ) and PD patient ( $n = 17$ ) neutrophils. Consistent with the aforementioned report,<sup>14</sup> LRRK2 in neutrophil immunoblots was present as 2 bands, the expected full-length band at ~280 kDa and a more prominent band at ~170 kDa (Fig. 1A). Both these bands were also detected with the LRRK2 S935 phosphorylation antibody, with signal significantly reduced following LRRK2 inhibitor treatment (Fig. 1A). As both bands were sensitive to LRRK2 inhibitor treatment, we quantified both bands together for each participant. Consistent with the loss of LRRK2 S935 phosphorylation, Rab10 T73 phosphorylation was also significantly reduced to a similar extent in both control and PD patient neutrophils following LRRK2 inhibitor treatment (Fig. 1B).

### Replication of Inhibitor Reduced Rab10 Phosphorylation in a Larger PD Cohort

We next sought to replicate these findings in a second, larger independent cohort. For this study we also added a second, lower dose of the LRRK2 inhibitor and simultaneously collected and treated both neutrophils ( $n = 31$  per group) and PBMCs ( $n = 27$  per group) from control and PD patients. In the neutrophils, LRRK2 inhibition again significantly reduced phosphorylation of LRRK2 S935 (Fig. 1C) and Rab10 T73 (Fig. 1D), and, again, there was no significant difference in how control or PD patients responded to inhibitor treatment with either of the doses used. The same result for LRRK2 (Fig. 1E) and Rab10 T73 (Fig. 1F) phosphorylation was also observed for PBMCs, indicating that either sample type would be appropriate for monitoring target engagement in a clinical trial. Of note for PBMCs was that LRRK2 predominantly migrated at the expected 280-kDa size on immunoblot.

### Total LRRK2 Is Increased in PD Patient Neutrophils

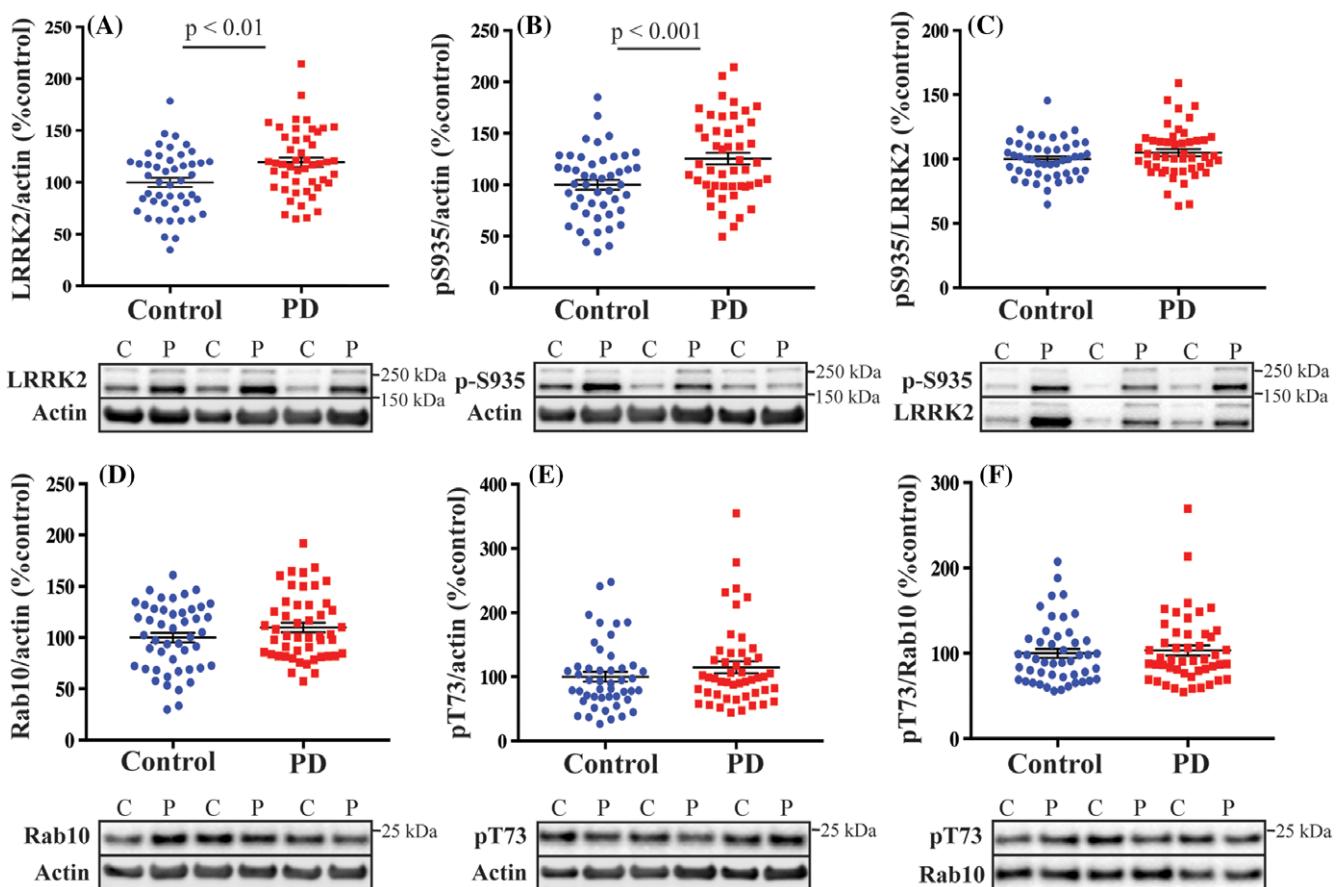
We were also interested in the extent to which increased Rab10 T73 phosphorylation could be detected



**FIG. 1.** Effect of LRRK2 inhibitors on LRRK2 and Rab10 phosphorylation in peripheral immune cells. Neutrophils were isolated from controls ( $n = 16$ ) and Parkinson's disease patients ( $n = 17$ ) and treated *ex vivo* with 100 nM of the LRRK2 inhibitor MLI2. Immunoblotting was performed to determine the effect of LRRK2 inhibition on the phosphorylation of LRRK2 S935 (A) and Rab10 T73 (B). In a second study, neutrophils and PBMCs were isolated from controls and Parkinson's disease patients and treated with either 30 or 100 nm of the LRRK2 inhibitor MLI2 or an equivalent amount of DMSO alone as a control.  $n = 27$  per group per treatment for PBMCs and  $n = 31$  per group per treatment for neutrophils. Immunoblotting was performed to determine the effect of LRRK2 inhibition on LRRK2 S935 (C) and Rab10 T73 (D) phosphorylation in neutrophils and on LRRK2 S935 (E) and Rab10 T73 phosphorylation in PBMCs (F). The percent inhibition relative to the DMSO-alone-treated cells was calculated. Graphs show mean  $\pm$  SEM. A 2-way ANOVA was used to compare the effect of LRRK2 inhibition on control and Parkinson's disease immune cells. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  compared with controls, which was set at 100%. Representative immunoblots are shown with larger examples available in the supplementary material.

in PD patient immune cells. To facilitate this analysis, immunoblots were repeated using only the non-inhibitor-treated samples. For the neutrophils, samples from both cohorts were combined to give a sample size of 47 controls and 48 PD patients. For each participant, we again quantified both LRRK2 bands together and

included age, sex, and collection site as covariates in the analysis. We found a significant increase in total LRRK2 in the PD neutrophils compared with controls (20% increase,  $P < 0.01$  Fig. 2A). The same results of increased LRRK2 in PD neutrophils were still obtained when both the 280- and 170-kDa LRRK2 bands were



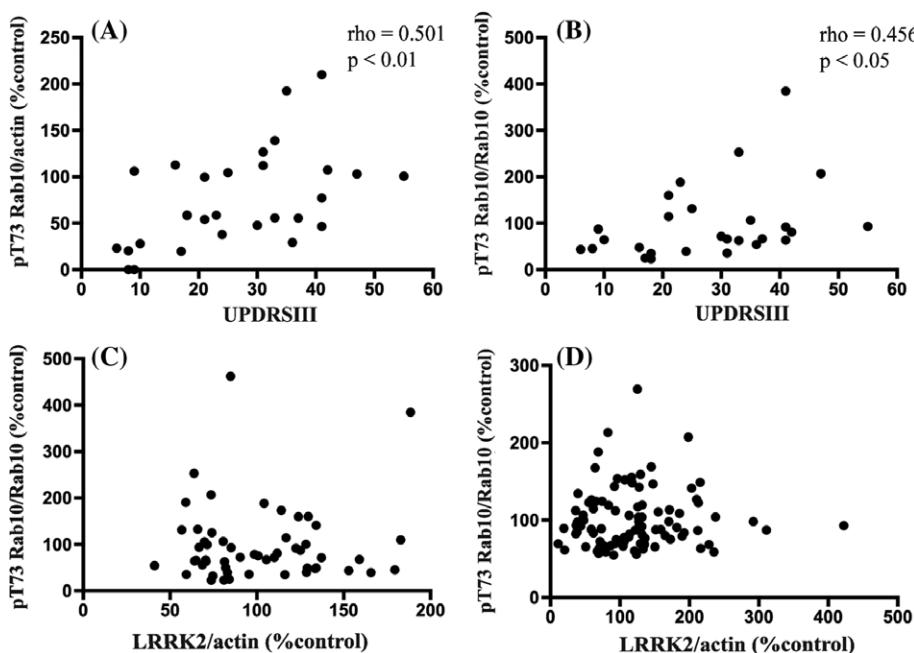
**FIG. 2.** LRRK2 and Rab10 phosphorylation in neutrophils. Non-inhibitor-treated neutrophil lysates from controls ( $n = 47$ ) and Parkinson's disease patients ( $n = 47$ ) were immunoblotted for total levels of LRRK2 (A) and S935 phosphorylated LRRK2, which was normalized to either  $\beta$ -actin (B) or total LRRK2 (C). Non-inhibitor-treated samples were immunoblotted for total levels of Rab10 (D) and T73 phosphorylated Rab10, which was normalized to either  $\beta$ -actin (E) or total Rab10 (F). Individual data points from immunoblot quantification are shown on the graphs, as well as the mean  $\pm$  SEM. Univariate analyses with age and sex as covariates were used to compare the 2 groups. Representative cropped immunoblots are shown with larger examples available in the supplementary material.

quantified separately (Supplementary Fig. 7). When normalized to  $\beta$ -actin, levels of Ser935 phosphorylated LRRK2 were also significantly increased in PD neutrophils (25% increase,  $P < 0.001$ , Fig. 2B), demonstrating that increased LRRK2 is measurable with 2 distinct antibodies. However, when normalized to levels of total LRRK2, Ser935 phosphorylation was no longer significantly increased in PD neutrophils (Fig. 2C), indicating that increased Ser935 phosphorylation, shown in Figure 2B, can be explained by the increased total LRRK2. In contrast to LRRK2, total Rab10 did not differ between PD patients and controls (Fig. 2D). In addition, despite the increased LRRK2, the phosphorylation of Rab10 at T73 also did not differ between PD and control neutrophils following normalization to either  $\beta$ -actin (Fig. 2E) or total Rab10 (Fig. 2F). In contrast to neutrophils, the same analysis in PBMCs revealed no significant difference in LRRK2 level between the 2 groups (Supplementary Fig. 8A). The phosphorylation of LRRK2 at S935 also did not differ between control and PD participants when normalized to either  $\beta$ -actin (Supplementary Fig. 8B) or total LRRK2 (Supplementary Fig. 8C). Similar

to neutrophils, protein levels of Rab10 in PBMCs did not differ between control and PD participants (Supplementary Fig. 8D). Neither did the phosphorylation of Rab10 at T73 following normalization to either  $\beta$ -actin (Supplementary Fig. 8E) or total Rab10 (Supplementary Fig. 8F).

### Associations Between LRRK2, Rab10, and PD Demographic Variables

Correlation analyses were then performed to determine any associations between LRRK2, Rab10, and their phosphorylation and available PD clinical variables. In the PBMCs, Rab10 T73 phosphorylation was significantly correlated with UPDRS motor score, when corrected to either  $\beta$ -actin ( $\rho = 0.501$ ;  $P < 0.01$ ; Fig. 3A) or total Rab10 ( $\rho = 0.456$ ;  $P < 0.05$ ; Fig. 3B). There were no correlations in PBMCs between Rab10 or Rab10 T73 phosphorylation and disease duration, age at diagnosis, or dopamine medication dose (Supplementary Table 1). In the PBMCs, LRRK2 level did not correlate with any available PD clinical



**FIG. 3.** Correlations with PD clinical variables. Spearman correlation analysis revealed a significant association between PBMC Rab10 phosphorylation and disease severity in the Parkinson's disease participants ( $n = 27$ ) when normalized to either (A)  $\beta$ -actin or (B) total Rab10. (C) In PBMCs, there was no correlation between LRRK2 level and phosphorylation of Rab10 in the whole cohort ( $n = 54$ ). (D) In neutrophils, there was also no correlation between levels of LRRK2 and the phosphorylation of Rab10 in the whole cohort ( $n = 62$ ).

variables, and S935 phosphorylated LRRK2 only correlated with dopamine medication dose (Supplementary Table 1). Interestingly, there was no correlation between LRRK2 level and Rab10 T73 phosphorylation in the participant PBMCs (Fig. 3C). In neutrophils, there were no correlations between LRRK2 or S935 phosphorylated LRRK2 and disease duration, disease severity, age at diagnosis, or dopamine medication (Supplementary Table 2). Phosphorylation of T73 Rab10 also did not correlate with any available PD clinical variables, whereas total Rab10 correlated with disease duration ( $r = 0.374$ ,  $P < 0.05$ ; Supplementary Table 2). In the neutrophils, there was also no correlation between LRRK2 level and Rab10 T73 phosphorylation (Fig. 3D).

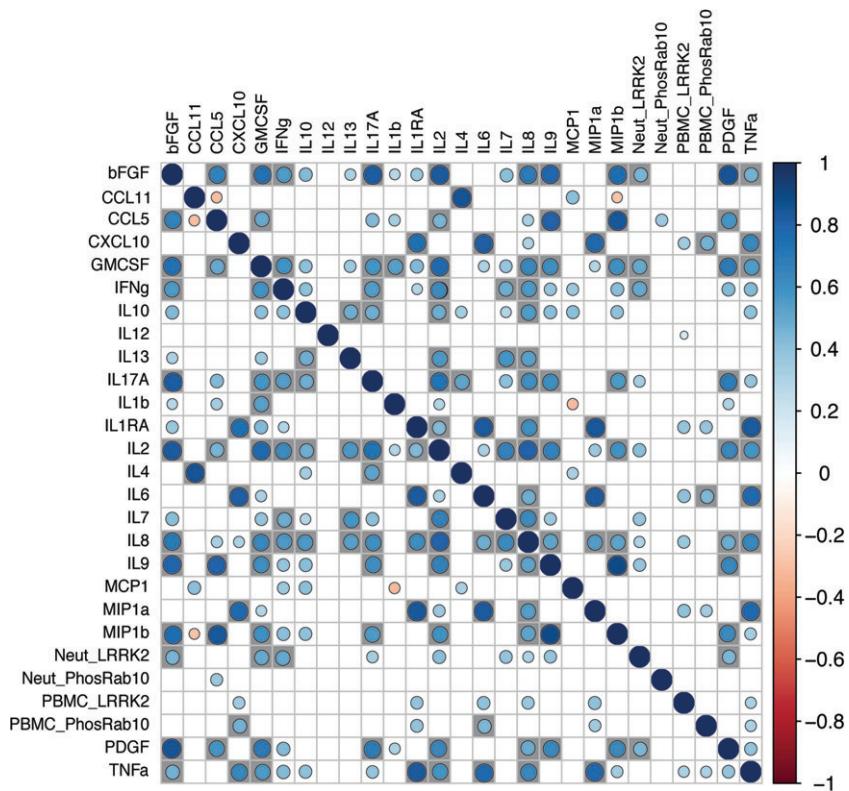
### Associations Between Immune Cell LRRK2, T73 Rab10, and Plasma Inflammatory Cytokines

LRRK2 has been linked to immune system function and inflammation; thus, we also aimed to determine if LRRK2 level or its activity based on Rab10 T73 phosphorylation in PBMCs or neutrophils was associated with plasma inflammatory cytokines. Of the 27 cytokines measured, 23 were detectable in plasma (Supplementary Table 3). Consistent with a mild inflammatory phenotype, a number of cytokines trended higher in PD patients, with 3 cytokines: tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), chemokine c-c like motif 5 (CCL5), and granulocyte-macrophage colony stimulating factor (GMCSF) significantly increased

with PD (Supplementary Table 3). TNF- $\alpha$  was significantly correlated with both LRRK2 level and Rab10 T73 phosphorylation in PBMCs, along with interleukin 6 (IL-6), interleukin 1 receptor antagonist, c-x-c motif chemokine 10, and macrophage inflammatory protein 1  $\alpha$  (Fig. 4). In contrast, CCL5 was significantly correlated with neutrophil Rab10 T73 phosphorylation (Fig. 4). Neutrophil levels of LRRK2 further correlated with basic fibroblast growth factor (bFGF), GMCSF, interferon gamma (IFN- $\gamma$ ), platelet-derived growth factor (PDGF), and interleukins 17A (IL-17A), 2 (IL-2), 7 (IL-7), 8 (IL-8), and 9 (IL-9); see Figure 4. The association between neutrophil LRRK2 level and bFGF, GMCSF, IFN- $\gamma$ , and PDGF was particularly robust, remaining significant following  $P$ -value adjustment for multiple comparisons (Fig. 4).

### Discussion

LRRK2 is considered a compelling target for the treatment of PD, to the point that therapeutic LRRK2 inhibitors are in advanced development and have even entered into phase 1 clinical trials.<sup>28</sup> An important aspect of clinical trials is to demonstrate target engagement. For LRRK2 preclinical studies, this has relied on measuring the S935 phosphorylation site on LRRK2 itself. However, S935 is not an LRRK2 autophosphorylation site, with S935 phosphorylation level indirectly regulated by LRRK2, and also by kinases other than LRRK2.<sup>17,20,22,29</sup> Thus, a complementary LRRK2



**FIG. 4.** Correlations with plasma inflammatory cytokines. A Pearson correlation matrix with a significance level of  $P < 0.05$  was plotted to identify associations between different plasma cytokines and the levels and activity (Rab10 T73 phosphorylation) of LRRK2 in neutrophils and PBMCs. Positive correlations are displayed in blue, and negative correlations are in red. Color intensity and the size of the circle are proportional to the correlation coefficients. Blank cells indicate no significant correlation. Gray-shaded cells indicate a still significant correlation following Bonferroni adjustment for multiple comparisons of the significance level to  $P < 0.002$  ( $n = 51$ ).

target engagement assay would be prudent for high-stakes clinical trials. The phosphorylation of Rab10 at T73 has recently been demonstrated to be directly mediated by LRRK2,<sup>12</sup> and T73 phosphorylation is reduced following LRRK2 inhibitor treatment of both PBMCs<sup>15</sup> and neutrophils<sup>14</sup> from healthy control volunteers. We now extend these studies and demonstrate that LRRK2 inhibitor treatment of both PBMCs and neutrophils from PD patients results in a loss of Rab10 T73 phosphorylation. Moreover, the response of PD and control immune cells to LRRK2 inhibition was the same, suggesting that Rab10 T73 phosphorylation in immune cells is indeed a valid peripheral target engagement biomarker.

In regard to LRRK2 inhibitor clinical trials, there is also substantial interest in determining whether idiopathic PD patients with high LRRK2 activity can be identified and potentially stratified into inhibitor trials. Therefore, we also assessed whether LRRK2-mediated Rab10 T73 phosphorylation could potentially serve as a patient enrichment biomarker for such a purpose. However, Rab10 phosphorylation level did not differ between control and PD in either PBMCs or neutrophils. Importantly for interpretation, we did not observe a correlation between LRRK2 level and Rab10

phosphorylation, potentially suggesting a more complex relationship. Phos-tag studies of Rab10 show that only a very small proportion of Rab10 is phosphorylated in immune cells by LRRK2 at baseline.<sup>13</sup> Moreover, a limited analysis of neutrophils from LRRK2 G2019S mutation carriers suggests that Rab10 phosphorylation is not significantly increased in these patients.<sup>14</sup> This suggests a potential dissociation between LRRK2 level/activity and Rab10 phosphorylation. However, within the idiopathic PD cohort there were indeed individuals with high Rab10 phosphorylation, and a correlation was found between increased PBMC Rab10 phosphorylation and disease severity as measured by UPDRS motor score. In regard to PBMCs, it is noteworthy that LRRK2 has higher expression in monocytes compared with lymphocytes,<sup>14,30</sup> and it would be of interest to determine if correlations show population-specific effects in the different types of immune cells. Thus, although Rab10 phosphorylation appears a valid biomarker of reduced LRRK2 activity via kinase inhibition, the extent to which Rab10 phosphorylation is applicable as a biomarker of increased LRRK2 activity is not yet clear, at least in peripheral immune cells. The physiological conditions by which LRRK2 mediates Rab10 phosphorylation are currently

unknown, but their elucidation will be important for helping to clarify this issue. Indeed, a recent study has suggested that LRRK2-mediated Rab phosphorylation has complex regulation requiring membrane localization and guanine nucleotide binding,<sup>31</sup> highlighting the potential for additional rate-limiting steps that could contribute to the lack of correlation we observed.

A final aspect of our current study was that LRRK2 was significantly increased in idiopathic PD patient neutrophils, and both LRRK2 and Rab10 phosphorylation levels in neutrophils and PBMCs were associated with peripheral inflammatory cytokines. LRRK2 has been robustly linked to innate immunity,<sup>32,33</sup> with the immune system and inflammation potentially important for PD progression.<sup>34,35</sup> In PBMCs, LRRK2 and Rab10 phosphorylation both correlated with PD-implicated cytokines including TNF- $\alpha$  and IL-6 (for review, see reference 34), with TNF- $\alpha$  significantly increased in the PD patients. In the neutrophils, Rab10 phosphorylation associated with levels of CCL5, another PD-implicated cytokine<sup>36</sup> that was also increased in the PD patients in our study. Finally, neutrophil levels of LRRK2 were associated with a different subset of cytokines including interleukins and growth factors such as GM-CSF, which was significantly increased in the PD patients, and PDGF, which we have previously shown is increased in the serum of PD patients with the activating LRRK2 G2019S mutation.<sup>27</sup> It is important to note however, that these are associations, and the extent to which LRRK2 level and activity in different immune populations directly contribute to inflammatory profiles remains to be determined. Moreover, at least 1 study has also suggested an increase in neutrophils in PD patients,<sup>37</sup> and  $\alpha$ -synuclein has been implicated in neutrophil recruitment<sup>38</sup> and function,<sup>39</sup> but the extent to which neutrophils contribute to PD is largely unexplored. Therefore, it would be of interest to replicate our findings of increased LRRK2 in PD patient neutrophils in additional cohorts and extend the studies to include immunophenotyping of granulocyte populations and measures of neutrophil numbers and function. It also remains to be determined why 2 distinctly sized LRRK2 bands can be detected in neutrophil immunoblots. It has been shown with epitope-defined antibodies that the 170-kDa LRRK2 band is lacking the N-terminus<sup>14</sup>; however, whether this represents a proteolytic degradation product or a differential splice variant is still unknown. If neutrophil LRRK2 can be more functionally linked to idiopathic PD, then this may be a means to enrich suitable patients for LRRK2 inhibitor trials or to provide a potential tissue source for efficacy biomarkers during clinical trials. ■

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## References

- Paisan-Ruiz C. LRRK2 gene variation and its contribution to Parkinson disease. *Hum Mutat* 2009;30(8):1153-1160.
- Healy DG, Falchi M, O'Sullivan SS, et al. Phenotype, genotype, and worldwide genetic penetrance of LRRK2-associated Parkinson's disease: a case-control study. *Lancet Neurol* 2008;7(7):583-590.
- Nalls MA, Pankratz N, Lill CM, et al. Large-scale meta-analysis of genome-wide association data identifies six new risk loci for Parkinson's disease. *Nat Genet* 2014;46(9):989-993.
- Sharma M, Ioannidis JP, Aasly JO, et al. Large-scale replication and heterogeneity in Parkinson disease genetic loci. *Neurology* 2012;79(7):659-667.
- Jaleel M, Nichols RJ, Deak M, et al. LRRK2 phosphorylates moesin at threonine-558: characterization of how Parkinson's disease mutants affect kinase activity. *Biochem J* 2007;405(2):307-317.
- Nichols RJ, Dzamko N, Hutt JE, et al. Substrate specificity and inhibitors of LRRK2, a protein kinase mutated in Parkinson's disease. *Biochem J* 2009;424(1):47-60.
- Atashrazm F, Dzamko N. LRRK2 inhibitors and their potential in the treatment of Parkinson's disease: current perspectives. *Clin Pharmacol* 2016;8:177-189.
- West AB. Ten years and counting: moving leucine-rich repeat kinase 2 inhibitors to the clinic. *Mov Disord* 2015;30(2):180-189.
- Christensen KV, Smith GP, Williamson DS. Development of LRRK2 Inhibitors for the Treatment of Parkinson's Disease. *Prog Med Chem* 2017;56:37-80.
- West AB, Moore DJ, Biskup S, et al. Parkinson's disease-associated mutations in leucine-rich repeat kinase 2 augment kinase activity. *Proc Natl Acad Sci U S A* 2005;102(46):16842-16847.
- Sheng Z, Zhang S, Bustos D, et al. Ser1292 autophosphorylation is an indicator of LRRK2 kinase activity and contributes to the cellular effects of PD mutations. *Sci Transl Med* 2012;4(164):164ra161.
- Steger M, Tonelli F, Ito G, et al. Phosphoproteomics reveals that Parkinson's disease kinase LRRK2 regulates a subset of Rab GTPases. *Elife* 2016;5.
- Ito G, Katsemonova K, Tonelli F, et al. Phos-tag analysis of Rab10 phosphorylation by LRRK2: a powerful assay for assessing kinase function and inhibitors. *Biochem J* 2016;473(17):2671-2685.
- Fan Y, Howden AJM, Sarhan AR, et al. Interrogating Parkinson's disease LRRK2 kinase pathway activity by assessing Rab10 phosphorylation in human neutrophils. *Biochem J* 2018;475(1):23-44.
- Thirstrup K, Dachsel JC, Oppermann FS, et al. Selective LRRK2 kinase inhibition reduces phosphorylation of endogenous Rab10 and Rab12 in human peripheral mononuclear blood cells. *Sci Rep* 2017;7(1):10300.
- Nichols RJ, Dzamko N, Morrice NA, et al. 14-3-3 Binding to LRRK2 is disrupted by multiple Parkinson's disease-associated mutations and regulates cytoplasmic localization. *Biochem J* 2010;430(3):393-404.
- Dzamko N, Deak M, Hentati F, et al. Inhibition of LRRK2 kinase activity leads to dephosphorylation of Ser(910)/Ser(935), disruption of 14-3-3 binding and altered cytoplasmic localization. *Biochem J* 2010;430(3):405-413.
- Perera G, Ranola M, Rowe DB, Halliday GM, Dzamko N. Inhibitor treatment of peripheral mononuclear cells from Parkinson's disease patients further validates LRRK2 dephosphorylation as a pharmacodynamic biomarker. *Sci Rep* 2016;6:31391.
- Dzamko N, Chua G, Ranola M, Rowe DB, Halliday GM. Measurement of LRRK2 and Ser910/935 phosphorylated LRRK2 in peripheral blood mononuclear cells from idiopathic Parkinson's disease patients. *J Parkinson's Dis* 2013;3(2):145-152.
- Dzamko N, Inesta-Vaquera F, Zhang J, et al. The IkappaB kinase family phosphorylates the Parkinson's disease kinase LRRK2 at Ser935 and Ser910 during Toll-like receptor signaling. *PLoS One* 2012;7(6):e39132.
- Lobbestael E, Zhao J, Rudenko IN, et al. Identification of protein phosphatase 1 as a regulator of the LRRK2 phosphorylation cycle. *Biochem J* 2013;456(1):119-128.

22. Chia R, Haddock S, Beilina A, et al. Phosphorylation of LRRK2 by casein kinase 1alpha regulates trans-Golgi clustering via differential interaction with ARHGEF7. *Nat Commun* 2014;5:5827.
23. Postuma RB, Berg D, Stern M, et al. MDS clinical diagnostic criteria for Parkinson's disease. *Mov Disord* 2015;30(12):1591-1601.
24. Goetz CG, Tilley BC, Shaftman SR, et al. Movement Disorder Society-sponsored revision of the Unified Parkinson's Disease Rating Scale (MDS-UPDRS): scale presentation and clinimetric testing results. *Mov Disord* 2008;23(15):2129-2170.
25. Fell MJ, Mirescu C, Basu K, et al. MLI-2, a potent, selective, and centrally active compound for exploring the therapeutic potential and safety of LRRK2 kinase inhibition. *J Pharmacol Exp Ther* 2015; 355(3):397-409.
26. Lis P, Burel S, Steger M, et al. Development of phospho-specific Rab protein antibodies to monitor in vivo activity of the LRRK2 Parkinson's disease kinase. *Biochem J* 2018;475(1):1-22.
27. Dzamko N, Rowe DB, Halliday GM. Increased peripheral inflammation in asymptomatic leucine-rich repeat kinase 2 mutation carriers. *Mov Disord* 2016;31(6):889-897.
28. Alessi DR, Sammler E. LRRK2 kinase in Parkinson's disease. *Science* 2018;360(6384):36-37.
29. Deng X, Dzamko N, Prescott A, et al. Characterization of a selective inhibitor of the Parkinson's disease kinase LRRK2. *Nat Chem Biol* 2011;7(4):203-205.
30. Cook DA, Kannarkat GT, Cintron AF, et al. LRRK2 levels in immune cells are increased in Parkinson's disease. *NPJ Parkinsons Dis* 2017;3:11.
31. Liu Z, Bryant N, Kumaran R, et al. LRRK2 phosphorylates membrane-bound Rabs and is activated by GTP-bound Rab7L1 to promote recruitment to the trans-Golgi network. *Hum Mol Genet* 2018;27(2):385-395.
32. Dzamko NL. LRRK2 and the immune system. *Adv Neurobiol* 2017; 14:123-143.
33. Dzamko N, Halliday GM. An emerging role for LRRK2 in the immune system. *Biochem Soc Trans* 2012;40(5):1134-1139.
34. Dzamko N, Geczy CL, Halliday GM. Inflammation is genetically implicated in Parkinson's disease. *Neuroscience* 2015;302:89-102.
35. Deleidi M, Gasser T. The role of inflammation in sporadic and familial Parkinson's disease. *Cell Mol Life Sci* 2013;70(22):4259-4273.
36. Rentzos M, Nikolaou C, Andreadou E, et al. Circulating interleukin-15 and RANTES chemokine in Parkinson's disease. *Acta Neurol Scand* 2007;116(6):374-379.
37. Horvath S, Ritz BR. Increased epigenetic age and granulocyte counts in the blood of Parkinson's disease patients. *Aging* 2015;7(12):1130-1142.
38. Stolzenberg E, Berry D, Yang, et al. A role for neuronal alpha-synuclein in gastrointestinal immunity. *J Innate Immun* 2017;9(5):456-463.
39. Azevedo EP, Guimaraes-Costa AB, Torezani GS, et al. Amyloid fibrils trigger the release of neutrophil extracellular traps (NETs), causing fibril fragmentation by NET-associated elastase. *J Biol Chem* 2012;287(44):37206-37218.

## Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.