npj | parkinson's disease

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Published in partnership with the Parkinson's Foundation

https://doi.org/10.1038/s41531-025-01185-8

Interferon gamma stimulates coordinated changes in LRRK2, GCase, and cathepsin activities in idiopathic and genetic Parkinson's disease monocytes

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The immune system is increasingly recognized as playing an important role in the pathogenesis of the neurodegenerative movement disorder Parkinson's disease (PD). In particular, key PD risk genes *LRRK2* (which encodes leucine-rich repeat kinase 2) and *GBA1* (which encodes glucocerebrosidase) are highly expressed in monocytes and implicated in the regulation of immune inflammatory pathways and lysosomal function. While preclinical studies demonstrate that missense mutations in *LRRK2* and *GBA1* can modulate the response to inflammatory stimuli, studies in primary immune cells from PD patients harboring these mutations are few. Therefore, peripheral blood mononuclear cells were obtained from idiopathic PD patients, ($n = 42$), as well as PD patients with *GBA1* ($n = 15$) and *LRRK2* ($n = 13$) mutations, and neurologically normal controls ($n = 36$). Cells were stimulated with interferon gamma, which strongly induces expression of the LRRK2 protein, and treated with and without the LRRK2 kinase inhibitor MLi-2. Live and fixed cell flow cytometry panels were used to measure the activities of LRRK2 and glucocerebrosidase, as well as cathepsin activity and the expression of the human leukocyte antigen receptor (HLA-DR) in classical, intermediate, and non-classical monocytes. Interferon gamma stimulation had marked effects on LRRK2 levels and phosphorylation of the LRRK2 substrate Rab10, as well as effects on the expression of HLA-DR and cathepsin activity, with some mutation-specific and monocyte-type-specific outcomes. These results help to advance understanding of how risk genes may interact with immune stimuli in the context of PD.

Parkinson's disease (PD) is a common neurodegenerative movement disorder, characterized neuropathologically by the selective degeneration of dopamine-producing neurons in the midbrain. Exactly what causes PD remains unclear, and there are no current disease-modifying therapies. Increasing evidence indicates that the peripheral immune system may contribute to PD pathogenesis via interactions with the central nervous system (CNS)[1,2]. Altered populations of peripheral immune cells[3,4], altered cytokine profiles[5,6], and intestinal dysbiosis[7] have all been implicated in PD, and bacterial and viral pathogens can induce PD phenotypes in preclinical models[8]. Compelling evidence of a role for the immune system in PD also comes from genetic findings, in particular, missense mutations in the gene encoding leucine-rich repeat kinase 2 (LRRK2) can cause autosomal dominantly inherited PD[9,10], and variation in *LRRK2* is also a risk factor for Crohn's disease (CD), a form of inflammatory bowel disease (IBD)[11,12].

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Table 1 | Demographic table of study participants

	HC	iPD	GBA1-PD	LRRK2-PD	*p*-value
N	36	42	15	13	
Age (y) +/− SEM	66 ± 1.6[ab]	68 ± 1.3[a]	60 ± 2.7[b]	67 ± 2.3[ab]	0.0306
Male %	39	60	60	50	0.2885
MDS-UPDRSIII (mean +/− SEM)	1.9 ± 0.5[a]	18.0 ± 1.6[b]	16.7 ± 2.2[b]	13.0 ± 2.2[b]	<0.0001
MOCA (mean +/− SEM)	27.4 ± 0.4	27.0 ± 0.5	27.4 ± 0.5	25.7 ± 1.0	0.3273
Disease duration (y) +/− SEM		8 ± 1.1	8.1 ± 1.9	9.0 ± 1.8	0.9011
Post-thaw viability (mean %) +/− SEM	77.17 ± 2.2	77.48 ± 1.8	73.80 ± 2.2	84.25 ± 1.2	0.1341
Classical monocytes (mean %) +/− SEM	50 ± 2.5	52 ± 2.6	57 ± 3.3	50 ± 4.1	0.6154
Intermediate monocytes (mean %) +/− SEM	22 ± 2.2	19 ± 2.0	15 ± 2.5	24 ± 3.8	0.1735
Non-classical monocytes (mean %) +/− SEM	17 ± 1.3	14 ± 1.0	14 ± 2.8	18 ± 2.2	0.2794

Demographic and clinical data are shown for 106 study participants, categorised by PD and *LRRK2/GBA1* mutation status. MDS-UPDRSIII, Movement Disorder Society Unified Parkinson's Disease Rating Scale part 3; *MoCA* Montreal Cognitive Assessment. Values in each row which have different letters (a and b) are significantly different; one-way ANOVA with Tukeys post-hoc test.

Table 2 | Frequency of *GBA1* and *LRRK2* mutations present in study population

	GBA1-PD	*LRRK2*-PD
GBA1-N370S	9	
GBA1-L444P	2	
GBA1-E326K	2	
GBA1-T369M	1	
GBA1-84GG/E326K	1	
LRRK2-G2019S		13
Total	**15**	**13**

Table shows the frequency of known mutations in the GBA-PD and LRRK2-PD groups.

LRRK2 is a multidomain protein with both GTPase and protein kinase enzymatic activities[13]. LRRK2 is predominantly expressed in monocytes and neutrophils and has been linked to the regulation of innate immune pathways, including toll-like receptor[14], dectin[15], NFAT[16], inflammasome[17], and STING[18] signalling pathways. LRRK2 mutations have also been linked to impaired antigen presentation via major histocompatibility molecules, including HLA-DR[19,20], which is genetically implicated in PD[21]. Early research on the role of LRRK2 in immunity demonstrated that LRRK2 is an interferon gamma (IFN-γ) target gene, and LRRK2 protein levels are markedly upregulated in immune cells in response to IFN-γ stimulation[22]. In patients with idiopathic PD (iPD), IFN-γ stimulation of peripheral blood mononuclear cells (PBMCs) resulted in higher expression of LRRK2 compared to age- and sex-matched neurologically healthy controls, and this was associated with an inflammatory phenotype in PD patients[23]. As LRRK2 is advancing through clinical pipelines as a therapeutic target for PD[24,25], understanding the biological functions of LRRK2 and how mutations dysregulate this function is increasingly important.

The exact biological functions of LRRK2 are still being elucidated, but it is known to phosphorylate a subset of Rab GTPase proteins[26]. In particular, LRRK2 can be recruited to damaged lysosomes where it is activated and phosphorylates downstream substrates, including Rab10[27]. Lysosomal dysfunction is heavily implicated in PD, where aggregates of alpha-synuclein protein accumulate in neurons, at least in part due to failed lysosomal clearance mechanisms[28]. As well as LRRK2, missense mutations in *GBA1*, which encodes the lysosomal hydrolase glucocerebrosidase (GCase), are also a major genetic risk for PD[29]. GCase is also highly expressed in monocytes and is required for the hydrolysis of the lipid glucocerebroside to glucose and ceramide[30]. Genome-wide association studies have also identified that variation in *CTSB*, which encodes the alpha-synuclein-degrading lysosomal enzyme cathepsin B, is also a significant genetic risk factor for PD[31]. Importantly, recent studies employing induced pluripotent stem cell models clearly demonstrate that a functional relationship exists between LRRK2, GCase, and cathepsin enzymes, at least in neuronal cells[32–34]. However, the extent to which these enzymes interact in immune cells and how these interactions may be affected by specific immune stimuli, PD, or PD-associated mutations remains to be further explored.

In the current study, we aimed to determine how the activities of LRRK2, GCase, and Cathepsin are affected by stimulation of primary PD patient immune cells with the LRRK2-inducing cytokine IFN-γ. We employed our previously developed flow cytometry WHOPPA assay[35] to assess LRRK2, Rab10 phosphorylation, GCase activity, and cathepsin activity in monocyte subsets from PD patients with or without PD-associated missense mutations in *LRRK2* or *GBA1*, as well as matched controls. Experiments were also performed in the presence and absence of the LRRK2 protein kinase inhibitor, MLi-2. IFN-γ stimulation had the largest effect on LRRK2 levels, Rab10 phosphorylation, and cathepsin activity, with some mutation-specific and monocyte subset-specific outcomes. Collectively, these results advance our understanding of the functional significance of links between peripheral immune function and lysosome function in PD.

Results

Study cohort

The study cohort comprised iPD ($n = 42$), *GBA1*-PD ($n = 15$) *LRRK2*-PD ($n = 13$), and neurologically normal controls ($n = 36$) subjects (Table 1). Motor severity scores for participants with PD were significantly increased compared to controls (Table 1). The *LRRK2*-PD group contained only participants heterozygous for the *LRRK2 G2019S* mutation, whereas the *GBA1*-PD group was predominantly heterozygous carriers of the *GBA1 N370S* mutation, but also contained carriers with *L444P, E326K*, and *T369M* mutations (Table 2). The *GBA1*-PD group was, on average, younger than the control and iPD groups. The control group also contained more female participants, although not significantly different. For these reasons, age and sex were included as covariates in subsequent analyses. Average disease duration was similar across the PD groups, and MoCA scores were similar across all groups. The post-thaw viability of the cryopreserved PBMCs was not different across groups, and neither were the frequencies of the classical, intermediate, and non-classical monocyte subsets (Table 1).

Interferon gamma increases LRRK2 levels and phosphorylation of Rab10

Univariate analyses covarying for age and sex were used to identify significant effects of group and/or treatment in the different monocyte subsets. In classical monocytes the levels of LRRK2 did not significantly

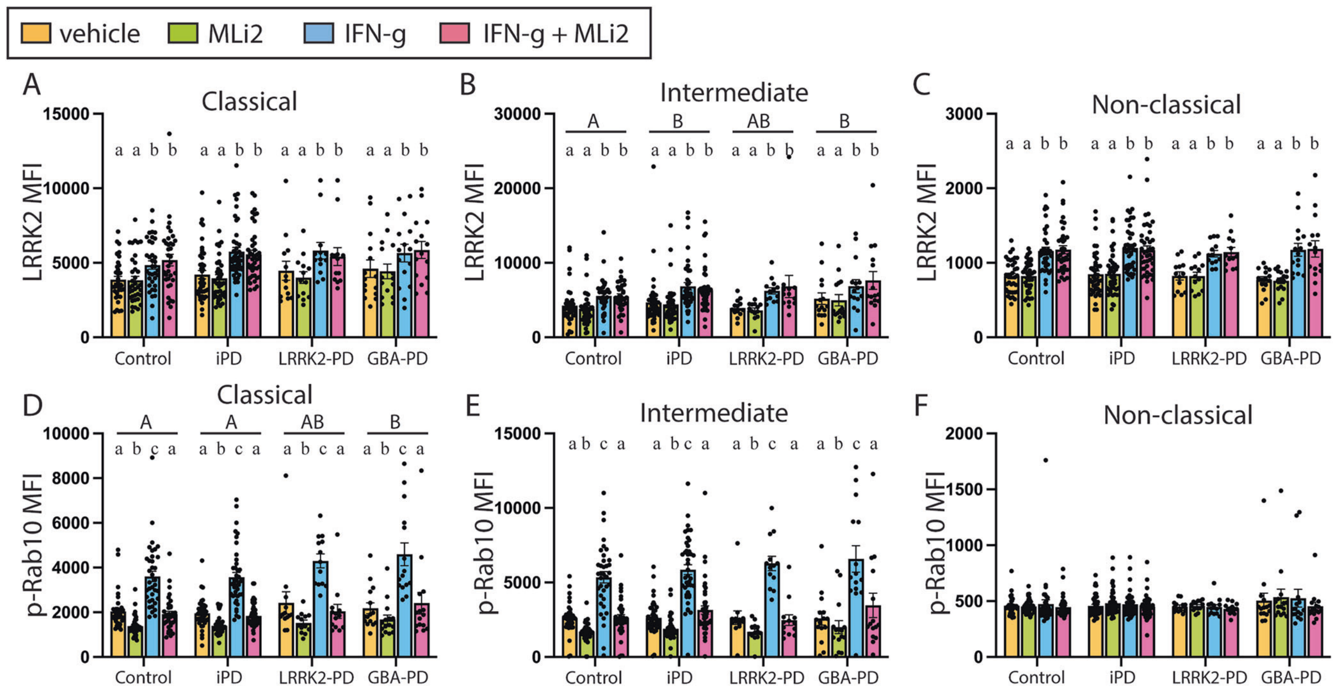


Fig. 1 | Interferon gamma increases LRRK2 levels and Rab10 phosphorylation. Cryopreserved PBMCs from iPD, *LRRK2*-PD, and *GBA1*-PD patients and matched control subjects were plated and treated for 18 h with 100 nM MLi-2 or vehicle control, both in the presence and absence of 100 U IFN-γ, and then stained with cell surface marker antibodies, viability stain, and antibodies directed to LRRK2 and p-Rab10. Data were acquired on a MACS Quant cytometer, and the median fluorescence intensity (MFI) of LRRK2 expression was calculated in (**A**) classical (**B**) intermediate, and (**C**) non-classical monocytes, and the MFI of p-Rab10 expression was calculated in (**D**) classical (**E**) intermediate, and (**F**) non-classical monocytes. Bars present the mean ± SEM with dots representing data from individual participants. Data were analyzed using a two-factor multivariate analysis covarying for age and sex. Post hoc pairwise comparisons were performed using Fisher's least significant difference. Capital letters denote group effect and lower-case letters denote treatment effect, with groups sharing the same letter not significantly different from each other ($p > 0.05$) whilst groups displaying different letters are significantly different from each other ($p < 0.05$).

differ between groups ($P = 0.06$, Fig. 1A). There was a significant effect of treatment ($P < 0.001$), with pairwise comparisons showing a significant effect of IFN-γ to increase LRRK2 ($P < 0.001$). In the intermediate monocytes there was a significant group effect ($P = 0.004$, Fig. 1B), with pairwise comparisons showing increased levels of LRRK2 in iPD ($P = 0.009$) and *GBA1*-PD ($P = 0.001$) groups compared to the controls. There was also a significant effect of treatment ($p < 0.001$), with IFN-γ again increasing the levels of LRRK2. In non-classical monocytes there was no effect of group ($P = 0.99$), but again a significant effect of treatment ($P < 0.001$), with IFN-γ significantly increasing LRRK2 levels (Fig. 1C). Neither age nor sex had a significant effect on LRRK2 levels in any of the monocyte populations. MLi-2 exposure did not significantly affect LRRK2 levels in any of the monocyte populations. The same analyses were also employed to look at levels of Thr73 Rab10 phosphorylation in the different monocyte subsets. In the classical monocytes there was a significant effect of both group ($P = 0.03$) and treatment ($P = < 0.001$) on the levels of phosphorylated Rab10, with pairwise comparisons showing that *GBA1*-PD group had higher Rab10 phosphorylation ($P = 0.02$) compared to the iPD and control groups (Fig. 1D). There was a significant increase in Rab10 phosphorylation with IFN-γ ($P < 0.001$), and a significant effect of MLi-2 to decrease Rab10 phosphorylation ($P < 0.001$). In the intermediate monocytes there was no effect of group ($P = 0.49$), but a significant effect of treatment ($P < 0.001$), with IFN-γ again increasing Rab10 phosphorylation ($P < 0.001$), and MLi-2 blocking Rab10 phosphorylation ($P = 0.006$, Fig. 1E). Interestingly, non-classical monocytes had a very low expression of P-Rab10 and there was not a significant effect of either group ($P = 0.51$) or treatment ($P = 0.31$, Fig. 1F). Sex was not a significant factor for Rab10 phosphorylation in any of the monocyte subsets. Age had a significant effect ($P = 0.02$) in the intermediate monocytes only.

Interferon gamma increases cathepsin activity and has monocyte subset-dependent effects on lysosomal GCase activity

Flow cytometry using live cells was also employed to investigate the effects of IFN-γ stimulation on cathepsin and GCase activities. The reference control showed that the GCase activity index and cathepsin activity MFI were relatively stable between batches, with a coefficient of variation (CV) of 16.25% and 19.16%, respectively (Supplementary Fig. 1). In classical monocytes there was a significant effect of treatment on cathepsin activity ($P = 0.011$) but not a significant effect of group ($P = 0.19$, Fig. 2A). Pairwise comparisons show that the combination of IFN-γ and MLi-2 had a significant effect to increase cathepsin activity compared to the control ($P = 0.005$) and MLi-2 only treated ($P = 0.008$) groups. In the intermediate monocytes, there was a significant effect of both group ($P = 0.02$) and treatment ($P < 0.001$) on cathepsin activity (Fig. 2B). Pairwise comparisons show that cathepsin activity was elevated in *LRRK2*-PD compared to control ($P = 0.049$) and iPD ($P = 0.002$) groups. IFN-γ also significantly upregulated cathepsin activity ($P = 0.001$) and there was no additional effect of MLi-2. In non-classical monocytes there was again a significant effect of both group ($P = 0.002$) and treatment ($P < 0.001$) on cathepsin activity (Fig. 2C). Cathepsin activity was again elevated in the *LRRK2*-PD group compared to control ($P = 0.018$) and ($P = 0.008$) iPD groups, but this time also in *GBA1*-PD compared to control ($P = 0.005$) and iPD ($P = 0.002$) groups. Cathepsin activity in the non-classical monocytes was again significantly upregulated by IFN-γ ($P < 0.001$). Age did not have an effect on cathepsin activity, whilst sex did have a significant effect in both classical ($P = 0.003$) and intermediate ($P = 0.017$) monocytes. We next assessed GCase activity in the different monocyte subsets. In classical monocytes there was no significant effect of group ($P = 0.09$) but there was an effect of treatment ($P = 0.004$, Fig. 2D). Pairwise comparisons indicated that MLi-2 treatment reduced GCase activity compared to the control ($P = 0.004$) and IFN-γ treated ($P = 0.001$) groups. In contrast, in intermediate monocytes there was a significant effect

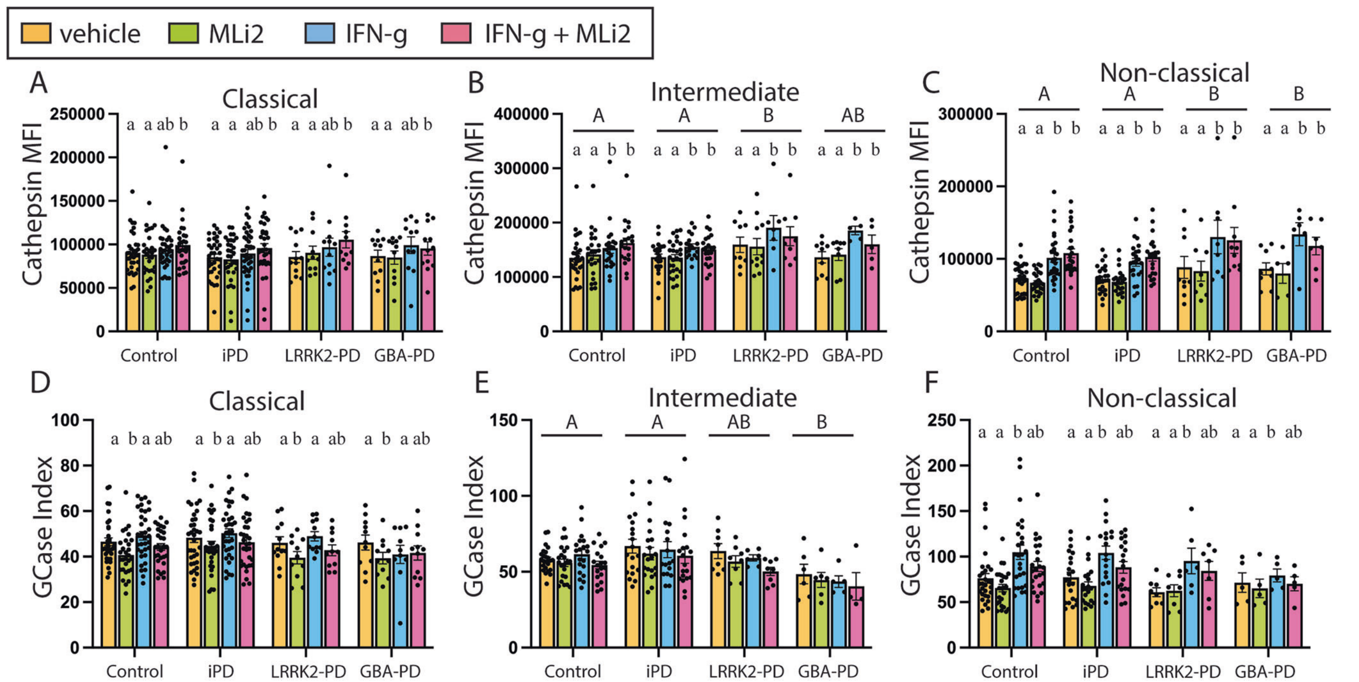


Fig. 2 | Interferon gamma increases cathepsin activity and has monocyte subset-dependent effects on lysosomal GCase activity. Cryopreserved PBMCs from iPD, *LRRK2*-PD, and *GBA1*-PD patients and matched control subjects were plated and treated for 18 h with 100 nM MLi-2, 100 nM CBE, or vehicle control, both in the presence and absence of 100 U IFN-γ. PBMCs were then incubated with the fluorogenic GCase substrate 5-(Pentafluorobenzoylamino) Fluorescein Di-β-D-Glucopyranoside (PFB-FDGlu) and fluorogenic cathepsin substrate BMV109 and stained with cell surface marker antibodies and viability stain. Data were acquired on a Cytek Aurora spectral cytometer, and the MFI of cathepsin activity was calculated in (**A**) classical (**B**) intermediate and (**C**) non-classical monocytes, and the GCase activity index was calculated in (**D**) classical (**E**) intermediate and (**F**) non-classical monocytes by dividing MFI of cells treated without CBE by the MFI of cells treated with CBE. Bars present the mean ± SEM with dots representing data from individual participants. Data were analyzed using a two-factor multivariate analysis covarying for age and sex. Post hoc pairwise comparisons were performed using Fisher's least significant difference. Capital letters denote group effect and lower-case letters denote treatment effect, with groups sharing the same letter not significantly different ($p > 0.05$) whilst groups displaying different letters are significantly different from each other ($p < 0.05$).

of group ($P < 0.001$), but not treatment ($P = 0.189$, Fig. 2E). Pairwise comparisons show that GCase activity was significantly decreased in the *GBA1*-PD group compared to all other groups ($P < 0.001$). The result from non-classical monocytes was different again, with a significant effect of treatment ($P < 0.001$) but not group ($P = 0.11$, Fig. 2F). This time, pairwise comparisons indicated a significant effect of IFN-γ to increase GCase activity ($P < 0.001$). Age did not influence GCase activity in any monocyte subsets, whilst sex had a significant effect ($P = 0.022$) on GCase activity in non-classical monocytes only. We also conducted a preliminary analysis in classical monocytes to determine the effect of GBA1 mutation type on the activity of GCase and cathepsin, as well as levels of LRRK2 and P-Rab10. Results indicated that different GBA1 mutation types did not impact any of the variables (Supplementary Fig. 2), however, since the sample sizes are very low, this should be interpreted with caution.

LRRK2-PD classical monocytes displayed impaired upregulation of HLA-DR in response to interferon gamma

We also assessed the expression of the human leukocyte antigen receptor HLA-DR using the live cell imaging dataset. In classical monocytes, there was a significant upregulation of HLA-DR following treatment with IFN-γ ($P < 0.001$). There was also a significant overall group effect ($P = 0.013$), with pairwise comparisons showing an impaired upregulation of HLA-DR in the *LRRK2*-PD classical monocytes, compared to all other groups (Fig. 3A). A significant effect of IFN-γ to increase HLA-DR was also seen in intermediate monocytes ($P < 0.001$, Fig. 3B). There was also an overall group effect ($P = 0.02$) in this monocyte subset, with HLA-DR increased in the iPD group more than the control ($P < 0.013$) and *LRRK2*-PD groups ($P < 0.009$). A similar result was also seen for non-classical monocytes, with significant group ($P = 0.008$) and treatment ($P < 0.001$) showing an increase in HLA-DR with IFN-γ ($P < 0.001$, Fig. 3C), and a higher increase in iPD monocytes compared to controls ($P = 0.33$) and LRRK2 mutation groups ($P = 0.001$). Age and sex did not have a significant effect in any of the analyses. MLi-2 also had no effect on the expression of HLA-DR. An overall summary of the measured changes in monocytes subsets for the different groups compared to controls is shown in Fig. 4.

LRRK2 levels correlate to levels of phosphorylated Rab10 in classical and intermediate monocytes

Correlation analysis was performed using the unstimulated monocyte data to determine any associations of the measured variables with each other, or with the available clinical disease severity data. At baseline, there were no correlations of the measured variables to clinical data for any monocyte subsets. In classical monocytes, LRRK2 was significantly positively correlated with levels of phosphorylated Rab10 ($r = 0.541$; $P < 0.0001$, Fig. 5A). There was also a significant negative correlation between GCase and cathepsin activities ($r = -0.270$; $P = 0.0098$, Fig. 5B). In the intermediate monocytes the only significant correlation was between LRRK2 and Rab1o phosphorylation ($r = 0.408$; $P < 0.0001$, Fig. 5C), whilst in non-classical monocytes the only significant correlation was between cathepsin activity and HLA-DR ($r = 0.282$; $P = 0.015$, Fig. 5D).

Interferon gamma stimulation reveals associations between LRRK2 and GCase activities, and between cathepsin activity and HLA-DR expression

Correlation analysis was also performed to determine any associations of the measured variables with each other, or with the available clinical disease severity data following stimulation of the cells with IFN-γ. In the classical monocytes there was a significant positive correlation of LRRK2 levels to the

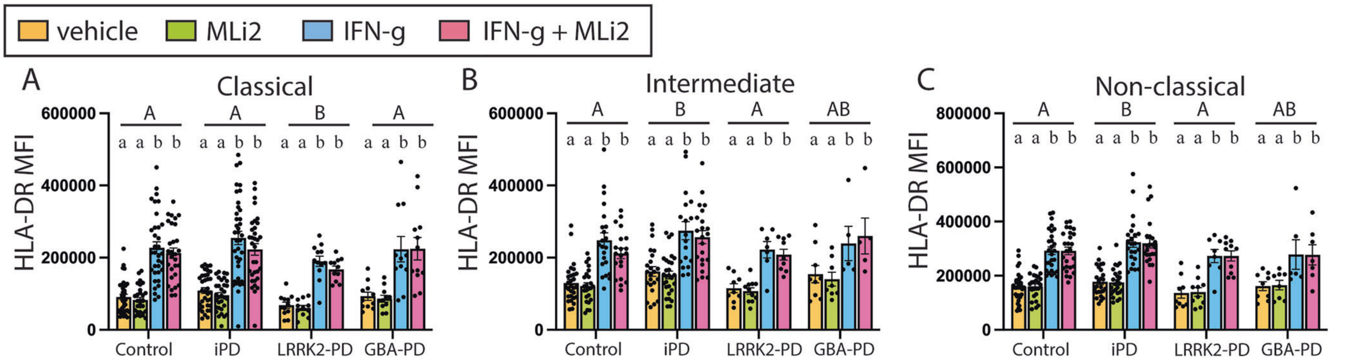
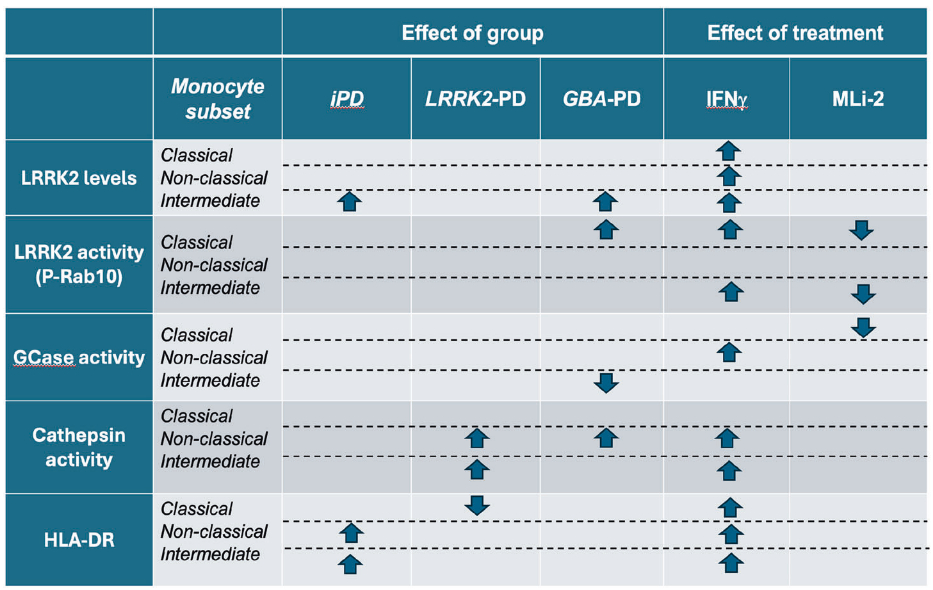


Fig. 3 | LRRK2 mutation cells have an impaired upregulation of HLA-DR in response to interferon gamma. Cryopreserved PBMCs from iPD, *LRRK2*-PD and *GBA1*-PD patients and matched control subjects were plated and treated for 18 h with 100 nM MLi-2 or vehicle control, both in the presence and absence of 100 U IFN-γ, and then stained with cell surface marker antibodies and viability stain. Data were acquired on a Cytek Aurora spectral cytometer and the MFI of HLA-DR expression was calculated in (**A**) classical (**B**) intermediate and (**C**) non-classical monocytes. Bars present the mean ± SEM with dots representing data from individual participants. Data were analyzed using a two-factor multivariate analysis covarying for age and sex. Post hoc pairwise comparisons were performed using Fisher's least significant difference. Capital letters denote group effect and lower-case letters denote treatment effect, with groups sharing the same letter not significantly different ($p > 0.05$) whilst groups displaying different letters are significantly different from each other ($p < 0.05$).

Fig. 4 | Summary of changes in different monocyte subsets. A summary of the group and treatment changes for the measured variables in different monocyte subsets. Arrows indicate the significantly different group and treatment effects and the direction of change for the previously measured variables.



UPDRSIII motor severity scale (r = 0.229; $P = 0.047$, Fig. 6A), and there was also a significant negative correlation between cathepsin activity and the MoCA scale (r = 0.294; $P = 0.0094$, Fig. 6B). LRRK2 levels were also positively correlated to levels of phosphorylated Rab10 (r = 0.434; $P < 0.0001$, Fig. 6C), and phosphorylated Rab10 was negatively correlated to GCase activity (r = −0.2580; $P = 0.014$, Fig. 6D). There was also a significant negative correlation between cathepsin activity and HLA-DR expression (r = −0.213; $P = 0.038$, Fig. 6E). There were no significant correlations to clinical data in the intermediate monocytes, but again a significant positive correlation between levels of LRRK2 and phosphorylated Rab10 was observed (r = 0.721; $P < 0.0001$, Fig. 6F), as well as a significant negative correlation of both LRRK2 (r = −0.406; $P = 0.0042$, Fig. 6G) and phosphorylated Rab10 (r = −0.496; P = 0.0003, Fig. 6H) to GCase activity. In the intermediate monocytes, levels of LRRK2 were also positively correlated to cathepsin activity (r = 0.315; P = 0.023, Fig. 5I) and again a significant negative correlation between cathepsin activity and HLA-DR expression was observed (r = −0.336; $P = 0.015$, Fig. 6J). Interestingly, results in non-classical monocytes were somewhat different, with a significant negative correlation between levels of LRRK2 and phosphorylated Rab10 (r = −0.222; $P = 0.022$, Fig. 6K). There were no correlations to GCase activity or to clinical severity data in the non-classical monocytes. There was again a significant negative correlation between cathepsin activity and HLA-DR expression (r = −0.386; $P = 0.0015$, Fig. 6L), which was a result consistent across all three monocyte subsets.

Discussion

This study aimed to systematically compare LRRK2-PD and GBA1-PD at baseline across lysosomal and inflammatory parameters in different monocyte subsets, including a reference comparison with idiopathic PD. Following this baseline characterization, the effects of IFN-γ treatment and the combination of MLi-2 and IFN-γ on monocyte subsets was investigated. Our study revealed increased LRRK2 protein expression and kinase activity in monocytes following IFN-γ stimulation in alignment with previous reports showing induction of LRRK2 levels[22,23] and activity[36] in immune cells following stimulation. The IFN-γ-induced increase in LRRK2 kinase activity in our study was subset dependent, with robust increases observed in

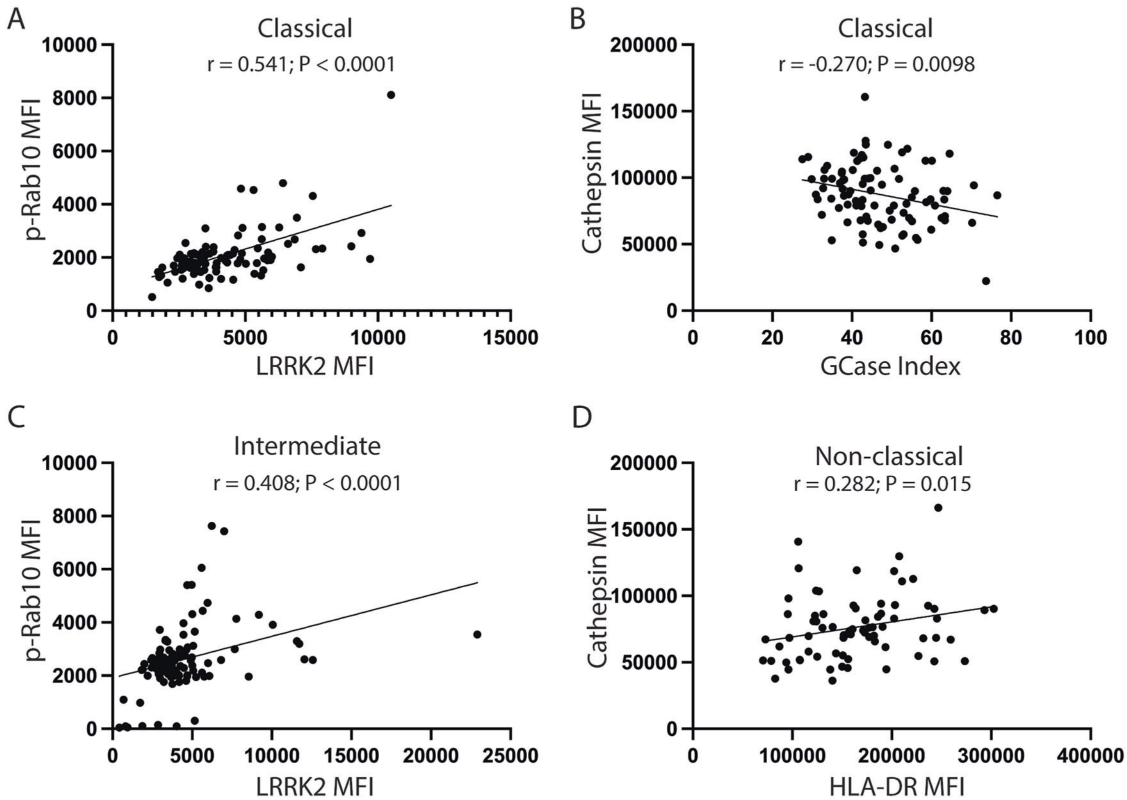


Fig. 5 | LRRK2 levels correlate to levels of phosphorylated Rab10 in classical and intermediate monocytes. The dataset was analyzed for correlations at baseline to determine any associations of the measured variables with each other, or with the available clinical disease severity data. **A** LRRK2 levels positively correlate with p-Rab10 levels in classical monocytes (r = 0.541, $p < 0.0001$); (**B**) GCase activity negatively correlates with cathepsin activity in classical monocytes (r = −0.270, $p = 0.0098$); (**C**) LRRK2 levels positively correlate with p-Rab10 levels in intermediate monocytes (r = 0.408, $p < 0.0001$); (**D**) cathepsin activity positively correlates with HLA-DR expression in non-classical monocytes (r = 0.282, $p = 0.015$). Correlations were assessed using Pearson's r, and statistical significance accepted at $P < 0.05$, $n = 106$.

classical and intermediate but not in non-classical monocytes. In addition to differential cell surface marker expression, classical, intermediate and non-classical monocyte subsets are functionally distinct. Classical monocytes comprise the majority of circulating monocytes, are highly phagocytic, and known to be important scavenger cells; intermediate monocytes are involved in the production of reactive oxygen species, antigen presentation, and the stimulation of T cells, and non-classical monocytes are important in maintaining vascular homeostasis and have both pro-and-anti-inflammatory roles[37,38]. These functional differences highlight the importance of assessing readouts in each immune cell subset separately and may have important implications in the design of therapeutics and target engagement assays. In the context of PD, classical monocytes can migrate to the brain and contribute to an inflammatory milieu and the phagocytosis of alpha-synuclein protein[39]. Intermediate monocytes also make a significant contribution to the inflammatory milieu of PD and may be important for the presentation of alpha-synuclein antigens to T-cells. Whereas non-classical monocytes may help to mitigate inflammation in PD and contribute to vascular health. Lysosomal biology is tightly linked to these cellular events and therefore, disruption of lysosomal signaling through changes in LRRK2, GCase, or cathepsins could have PD-relevant functional consequences.

Moreover, different PD mutations may also impact on monocyte subset biology. We observed increased LRRK2 protein expression in iPD and *GBA1*-PD intermediate monocytes and increased LRRK2 kinase activity in *GBA1*-PD classical monocytes. The increased LRRK2 levels in iPD and *GBA1*-PD intermediate monocytes along with the positive correlation between LRRK2 levels and the UPDRSIII motor severity score in stimulated classical monocytes suggest that the LRRK2 protein contributes to disease pathogenesis. Previous studies have also reported increased LRRK2 protein expression in peripheral immune cells from iPD patients including monocytes, T cells, B cells, and neutrophils[23,35,40]. The increased intermediate monocyte LRRK2 protein levels and classical monocyte kinase activity in *GBA1*-PD suggests an interplay between LRRK2 and GCase enzymatic activities in these monocyte subsets. In support of this concept is the IFN-γ-induced negative correlation between p-Rab10 levels and GCase activity in classical and intermediate monocytes and the negative correlation between LRRK2 levels and GCase activity in intermediate monocytes. Given that LRRK2 regulates a number of inflammatory pathways[22,41–43], these data may also indicate exacerbated peripheral inflammation in association with a *GBA1* mutation, and raise the possibility that LRRK2 kinase inhibitors may have clinical utility in the treatment of *GBA1*-PD. Interestingly, patients with a LRRK2 mutation did not significantly upregulate P-Rab10 compared to other groups in any of the monocyte subsets. The extent of LRRK2 activation may depend on the type of mutation present. For example, a study by Iannotta *et al* showed that mice carrying the *R1441C* mutation had increased Rab10 phosphorylation in the kidneys and lungs, while Rab10 phosphorylation levels in mice carrying the *G2019S* mutation were insignificantly different to those observed in wild-type mice[44]. Another study using human neutrophils found that the *LRRK2 R1441G* mutation in both PD patients and non-manifesting carriers significantly increased P-Rab10 levels four-fold compared with healthy control (HC), iPD, and *G2019S* mutation cases, while the *G2019S* mutation did not increase Rab10 phosphorylation compared with HC and iPD subjects[45]. As our study comprised only patients with the *G2019S* mutation, it would be of interest to repeat these experiments in the future with the inclusion of a subset of patients harbouring the *R1441G* mutation as a point of comparison.

Elevated cysteine cathepsin activity was detected in *LRRK2-PD* compared to control and iPD groups in intermediate and non-classical

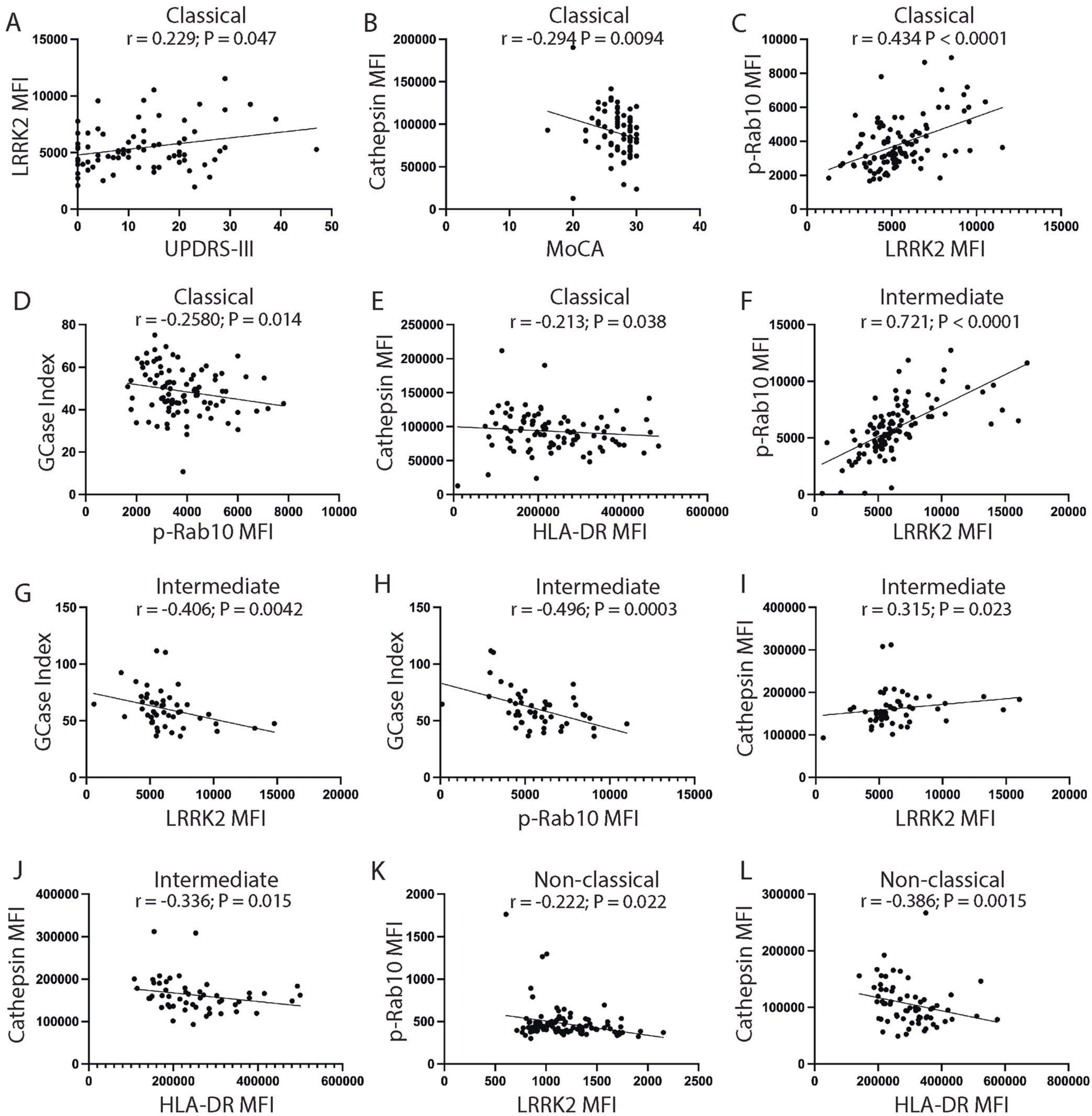


Fig. 6 | Interferon gamma stimulation reveals associations between clinical outcome measures and LRRK2 and GCase activities, and between cathepsin activity and HLA-DR expression. Correlation analysis was performed to determine any associations of the measured variables with each other, or with the available clinical disease severity data following stimulation of the cells with IFN-γ. **A** Classical monocyte LRRK2 levels positively correlate with UPDRSIII score (r = 0.229 $p = 0.047$); **B** classical monocyte cathepsin activity negatively correlates with MoCA score (r = 0.294 $p = 0.0094$); **C** p-Rab10 levels positively correlate with LRRK2 levels in classical monocytes (r = 0.434 $p < 0.0001$); (**D**) GCase activity negatively correlates with p-Rab10 levels in classical monocytes (r = −0.2580 $p = 0.014$); (**E**) cathepsin activity negatively correlates with HLA-DR levels in classical monocytes (r = −0.213 $p = 0.038$); (**F**) p-Rab10 levels positively correlate with LRRK2 levels in intermediate monocytes (r = 0.721 $p < 0.0001$); (**G**) GCase activity negatively correlates with LRRK2 levels in intermediate monocytes (r = −0.406 $p = 0.0042$); (**H**) GCase activity negatively correlates with p-Rab10 levels in intermediate monocytes (r = −0.496, $p = 0.0003$); (**I**) cathepsin activity positively correlates with LRRK2 levels in intermediate monocytes ($r = 0.315$, $p = 0.023$); (**J**) cathepsin activity negatively correlates with HLA-DR levels in intermediate monocytes (r = −0.336, $p = 0.015$); (**K**) p-Rab10 levels negatively correlate with LRRK2 levels in non-classical monocytes (r = −0.222, $p = 0.022$); (**L**) cathepsin activity negatively correlates with HLA-DR levels in non-classical monocytes (r = −0.386, $p = 0.0015$). Correlations were assessed using Pearson's r and statistical significance accepted at $P < 0.05$, $n = 106$.

monocytes, and in *GBA1-PD* compared to control and iPD groups in non-classical monocytes. An IFN-γ-induced increase was observed across all groups in intermediate and non-classical monocytes. The induction of cathepsin activity with IFN-γ and the IFN-γ-dependent correlations of cathepsin activity with LRRK2 levels in intermediate monocytes and with antigen presentation in all monocyte subsets indicates a role for cathepsins in inflammatory responses. Further, the elevated cathepsin activity in LRRK2-PD intermediate and non-classical monocytes and *GBA1-PD* intermediate monocytes may reflect an underlying state of inflammation in these groups, and the increased cathepsin activity could potentially contribute to disease development or progression. The cathepsin probe utilised in our study is cleaved by cathepsins B, L, S, and X, (CTSB, CTSL, CTSS and CTSX, respectively), and each of these cathepsins has been linked to PD pathogenesis or other neurodegenerative disease. CTSB has been shown to promote intracellular alpha-synuclein aggregate formation in cells[46], while CTSL contributed to dopaminergic neuron cell death in a rodent model of PD[47]. Additionally, levels of CTSL were elevated in a 6-hydroxydopamine (6-OHDA)-induced model of PD in SH-SY5Y cells in association with NF-κB activation and cytotoxicity[48]. In PD patients, CTSL expression in the substantial nigra was increased compared to controls[49], and increased *CTSL* mRNA has been detected in PD patient PBMCs relative to controls, and levels positively correlated with inflammatory markers[50]. CTSX levels and activity were increased in pathologically-affected brain tissue in a 6-OHDA-induced rodent model of PD[51], and increased CTSX expression and activity in microglia resulted in microglial activation-induced neurodegeneration[52]. However, neuroprotective effects of cysteine cathepsins have also been reported[53,54], thereby making it difficult to conclude the role of these proteins in PD. Cysteine cathepsins may constitute a potential therapeutic target, however, further work is required to resolve discrepancies in the literature and to elucidate precise mechanisms by which cathepsins may promote PD pathogenesis.

Interestingly, the effect of IFN-γ stimulation on monocyte GCase activity was also subset dependent, with increases observed in non-classical monocytes only, indicating a potential association between GCase enzyme activity and functional responses in this cell type. Reduced GCase activity was observed in intermediate monocytes from *GBA1*-PD patients but not from iPD patients. Decreased GCase activity has previously been identified in *GBA1*-PD patient blood spots relative to iPD and *LRRK2*-PD[55], and in PBMCs from *GBA1*-PD patients compared to iPD and control subjects, albeit with a considerable degree of overlap between groups[56,57]. Compared to controls, reduced GCase activity has also been observed in brain tissue from *GBA1*-PD patients[58,59]. Comparatively, alterations to GCase activity in PD patients without *GBA1* mutations are less clear. Lower GCase activity in the caudate and substantia nigra of iPD patients relative to controls has been identified[60], and reduced GCase activity in CSF has been observed in PD patients independent of *GBA1* mutation carrier status compared with controls[61,62]. In addition to the CNS, reduced GCase activity has also been observed in peripheral blood monocytes[63] and dried blood spots[64] from iPD patients compared to matched controls. However, other studies report no difference between iPD and control GCase activity in brain tissue[58], PBMCs[57], and plasma[56]. Interestingly, increased GCase activity in PBMCs from *LRRK2*-PD patients compared to control, iPD and *GBA1*-PD subjects has also been reported[65]. Given the high variability of peripheral GCase activity, observing significant differences between groups requires a large sample size. The sample size of the cohort presented in this chapter was relatively small in comparison to previous published studies by our group[63,66] which detected decreased monocyte GCase activity in iPD patients compared to matched controls. Moreover, the decreases in GCase activity in *GBA1*-PD patient monocytes in this study were relatively modest compared with previous reports. It has been shown that different mutations impact GCase activity to different extents, with *N370S* typically having a mild effect[67]. As the *GBA1*-PD cohort studied in this chapter primarily harboured the *N370S* mutation, it would be interesting to repeat the experiments detailed in this chapter in a larger number of patients harbouring a more severe mutation in the *GBA1* gene, such as *L444P* as a point of comparison.

As expected, we observed a robust increase in antigen presentation following stimulation with IFN-γ in all monocyte subsets. It is well established in the literature that IFN-γ induces antigen presentation to facilitate subsequent T cell activation and initiate adaptive immunity[68–70]. Interestingly, reduced expression of HLA-DR was observed in *LRRK2*-PD classical monocytes relative to control, iPD, and *GBA1*-PD groups. In patients with multiple sclerosis, reduced expression of HLA-DR on circulating monocytes is associated with active disease[71], and isolated rapid-eye-movement (REM) sleep behaviour disorder (iRBD) monocytes exhibit reduced HLA-DR expression compared to controls[72]. These findings may indicate an association between altered HLA-DR expression, which may potentiate monocyte dysregulation in the periphery, and neurodegenerative disease. Studies investigating HLA-DR in the context of PD have revealed no significant difference in expression between iPD and control subjects[73], while another study found IFN-γ-induced *HLA-DRA1* (the alpha subunit of HLA-DR) gene expression in monocytes was reduced in PD-linked *LRRK2* mutation cases compared to sporadic PD[19]. The reduction in HLA-DR exhibited by *LRRK2*-PD patients in our study may reflect an immune exhaustion phenotype with decreased antigen presentation capacity and a loss of immune competency consistent with previous studies from our group[74], or may be associated with the enhanced proliferative capacity and enrichment of monocyte precursors observed in PD patients, yielding a population of newly generated HLA-DR-negative monocytes[73,75]. The immune exhaustion concept is supported by findings that decreased monocyte HLA-DR is associated with systemic inflammatory response syndrome[76,77] and severe sepsis due to infection, which was associated with higher pro-inflammatory cytokine levels. Importantly, a reduction in monocyte HLA-DR expression has been observed in patients with CD and ulcerative colitis (UC)[78], diseases which LRRK2 has been pathologically linked to, and reduced monocyte HLA-DR levels were found to be associated with disease severity and predictive of outcome in IBD patients[79]. These data may present a link between mutant LRRK2 and HLA-DR alterations in both PD and IBD. Further studies investigating the relationship between HLA-DR and LRRK2 in peripheral immune cells may provide mechanistic insight into how PD-associated LRRK2 mutations may alter antigen presentation and immune responses in monocytes and the extent to which this may promote PD pathogenesis.

This study has a number of limitations and potential next steps to acknowledge. First, further work is still required to understand how changes in LRRK2, GCase, and cathepsin relate to functional changes in lysosomal function and inflammatory phenotypes. This could include applying additional unbiased approaches such as single-cell RNA sequencing to more broadly understand pathway dysfunction. The study could also be expanded to different imaging paradigms that can provide information on subcellular localisation, as this is important for the function of the enzymes like LRRK2 and GCase. Further work to elucidate specifically which cathepsins are altered in this study is also warranted. Treatments in this study were acute, and longer-term effects of chronic IFN-γ or MLi-2 exposure were not investigated, moreover, different concentrations of IFN-γ and/or analysis at different time points may give different outcomes. Importantly, this study was only powered to detect large effect sizes, and the inclusion of larger sample sizes would allow for replication and potentially uncover additional phenotypes. A larger cohort would also allow for investigation of demographic-specific effects such as ethnic background. A relatively small sample size may also limit the strength of the conclusions of the correlation analysis. It is also evident that mutations in LRRK2 and GCase affect these enzymes in different ways, and the inclusion of additional genetic variants would clarify the generalisability of results. Finally, IFN-γ itself is also likely to be a very simplistic tool in modelling the complex inflammatory milieu of PD. Nonetheless these findings contribute to a growing body of evidence integrating lysosomal function and immunity to the pathobiology of PD.

In summary, our findings suggest an interplay between enzymes associated with PD that can be monocyte subset dependent. IFN-γ increased levels of LRRK2 and HLA-DR in all monocyte subsets, but increased the phosphorylation of Rab10 only in the more inflammatory classical and intermediate monocytes, in line with known effects of LRRK2 to modulate inflammatory signalling. Elevated levels of LRRK2 and Rab10 phosphorylation were also found in intermediate and classical monocytes respectively in PD patients with a GBA1 mutation. In contrast, IFN-γ increased GCase and cathepsin activities in non-classical monocytes, indicating a distinct role for lysosomal function in these homeostatic monocytes. In particular, cathepsin activity was increased in non-classical monocytes from PD patients with a LRRK2 or GBA1 mutation. LRRK2 mutation was also associated with reduced expression of HLA-DR in classical monocytes. These data suggest that LRRK2, GCase, and cathepsin proteins may contribute to PD pathogenesis by modulating lysosomal/inflammatory conditions in different monocyte subsets.

Methods

Clinical samples

PBMCs were obtained from clinical sites at the University of Barcelona and the Columbia University (CUIMC IRB number AAA9604). Participant demographic and clinical data which included age, sex, PD status, *GBA1/LRRK2* mutation status, time since diagnosis, Movement Disorder Society Unified Parkinson's Disease Rating Scale part 3 (MDS-UPDRSIII) score, and Montreal Cognitive Assessment (MoCA) score were collected and are summarised in Table 1. Blood samples were collected and PBMCs isolated and cryopreserved following our previously optimized WHOPPA protocol[35]. Aliquots of the same participant PBMCs were shipped on dry ice to the University of Sydney for measurement of GCase and cathepsin activities, and to the University of Florida for the measurement of LRRK2 and Rab10 phosphorylation. The project was approved by the University of Sydney Human Research Ethics Committee (2018/1017) and the University of Florida Institutional Review Board (IRB201500437).

Recovery and treatment of PBMCs

PBMC cryovials were rapidly thawed in a 37 °C water bath until a small piece of ice remained, added to warmed (37 °C) culture media (RPMI 1640 (Thermo Fisher) supplemented with 10% v/v fetal bovine serum (FBS, Thermo Fisher), 50 U/mL penicillin/streptomycin (Thermo Fisher) and 2 mM L-glutamine (Thermo Fisher) and centrifuged at 300 x *g* for 10 min at room temperature (RT). Supernatant was removed and PBMCs resuspended in culture media for cell counting and viability assessment using Trypan blue (Thermo Fisher) and an automated cell counter (Countess II-FL, Life Technologies). PBMCs were diluted to 1×10^6 per mL in culture media and 5×10^5 cells plated per well in 24-well non tissue culture treated plates (Sigma-Aldrich), rested for 2 h in a 37 °C 5% CO_2 incubator and then treated for flow cytometry assays. Treatment conditions were 18 h with or without 100 U IFN-γ (Peprotech), 100 nM MLi-2 (Tocris), 100 μM conduritol B epoxide (CBE, Sigma-Aldrich), 10 μM NCGC00188758 (NCGC758, Merck), or an equivalent volume of dimethyl sulfoxide (DMSO, Sigma-Aldrich) as a vehicle control.

Fixed cell flow cytometry of LRRK2 and P-Rab10

PBMCs were suspended in 50 μL per sample of master mix comprising phosphate-buffered saline (PBS, Thermo Fisher) with Fc receptor (FcR) blocking solution, viability stain, and antibodies directed to cell surface markers at dilutions specified in Supplementary Table 1, and incubated at 4 °C for 30 min, shielded from light. Samples were washed in fluorescence-activated cell sorting (FACS) buffer, PBS with 1% FBS, 1 mM ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA, Sigma-Aldrich) and 25 mM HEPES (Sigma-Aldrich) and resuspended in 100 μL BD Phosflow™ Fix Buffer I (BD Biosciences) and incubated for 20 min at RT. Samples were washed in FACS buffer and resuspended in 100 μL of 1 x Perm/Wash buffer (BD Biosciences) containing phosphorylated Rab10 at Thr73 (P-Rab10) antibody and incubated at 4 °C for 45 min. Cells were then washed twice with Perm/Wash buffer, resuspend in 100 μL of 1 x Perm/Wash buffer containing donkey anti-rabbit secondary antibody and incubated at 4 °C for 30 min. After washing twice with Perm/Wash buffer, cells were resuspended in 100 μL Perm/Wash buffer containing LRRK2 antibody and incubated at 4 °C for 30 min. Cells were washed twice in Perm/Wash buffer, resuspended in 200 μL FACS buffer, and passed through a cell strainer cap into a 5 mL polypropylene tube and kept on ice, shielded from light until data acquisition.

Live cell flow cytometry of GCase and Cathepsin activities

PBMCs were resuspended in a master mix of culture media containing 0.375 mM of the fluorogenic GCase substrate 5-(Pentafluorobenzoylamino) Fluorescein Di-β-D-Glucopyranoside (PFB-FDGlu, Thermo Fisher) and 1 μM of the fluorogenic cathepsin probe BMV109 (Vergent Bioscience) and incubated for 1 h at 37 °C, 5% CO_2. A sample pre-treated with 100 μM of the lysosomal GCase inhibitor CBE was included for each subject to enable calculation of GCase activity as an index. FACS buffer was added to terminate the reaction, and cells were pelleted via centrifugation, followed by a second wash with FACS buffer. Cells were resuspended in 50 μL per sample of master mix containing FcR blocking reagent, viability stain and surface marker antibodies, as described above. Cells were washed, resuspended in 200 μL FACS buffer, passed through a cell strainer cap into a 5 mL polypropylene tube and kept on ice, shielded from light until data acquisition. GCase activity was quantified and expressed as an index, obtained by dividing the median fluorescence intensity (MFI) of cells treated without CBE by the MFI of cells treated with CBE. Cryopreserved PBMCs from one healthy donor were used as a reference control, with one vial from this same person thawed and included in each flow cytometry batch to assess the stability of the GCase and cathepsin activity readouts across multiple batches.

Flow cytometry data acquisition

Data were acquired on an Aurora Spectral Analyser (Cytek Biosciences) at the University of Sydney, or a MACS Quant Analyser (Miltenyi Biotec) at the University of Florida. In most instances a minimum of 100,000 events were collected per sample. Results were excluded if the number of events was less than 50, which occurred in some instances for the intermediate and non-classical monocyte subsets. Flow Cytometry Standard (FCS) files were then imported into FlowJo software (BD Biosciences) for analysis. Example gating strategy for the different monocyte subsets is shown in Supplementary Fig. 3.

Data analysis

Clinical and demographic data were analyzed by one-way analysis of variance (ANOVA) and Tukey's posthoc testing. Group and treatment effects across monocyte subsets were determined using 2-factor univariate analyses, covarying for age and sex. Normal distribution of data was assessed using the Kolmogorov-Smirnov test. LRRK2 and P-Rab10 MFI data was log transformed to improve normality. Spearman correlations were performed to look for associations between the different variables. Data analysis was performed using SPSS (IBM) with significance accepted at $P < 0.05$. Graphs were generated using Prism Software (GraphPad).

Data availability

The datasets generated and/or analysed during the current study are available from the corresponding author(s) on reasonable request.

Received: 7 August 2025; Accepted: 21 October 2025;
Published online: 26 November 2025

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Acknowledgements

This study was funded by the Michael J Fox Foundation and the Shake it Up Australia Foundation (MJFF#16778 and MJFF#16778.01). We thank the Sydney Cytometry core research facility for access to flow cytometers and technical support. We thank the Dzamko and Tansey labs for useful discussions.

Author contributions

Conceived the idea and secured funding – N.D., M.G.T.; Planned the experiments – L.H., R.L.W., N.D., M.G.T.; Patient recruitment, cell collection and clinical data – A.G., R.N.A., J.A.L.; Performed the experimental work – L.H., R.L.W.; Data analysis and figures – L.H., R.L.W., N.D.; Drafted the manuscript – N.D., L.H., R.L.W., M.G.T.; Edited and approved the final version – all authors.

Competing interests

Nicolas Dzamko is an Associate Editor for npj Parkinson's Disease, and Malu Tansey is Editor in Chief for npj Parkinson's Disease, but they both had no involvement in the editorial review or publication decision of this article. All authors declare no other financial or non-financial competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41531-025-01185-8.

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