Title: The *R1441C-LRRK2* mutation induces myeloid immune cell exhaustion in an age- and sex-dependent manner

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Abstract: Considering age is the greatest risk factor for many neurodegenerative diseases, aging, in particular aging of the immune system, is the most underappreciated and understudied contributing factor in the neurodegeneration field. Genetic variation around the *LRRK2* gene affects risk of both familial and sporadic Parkinson's disease (PD). The leucine-rich repeat kinase 2 (LRRK2) protein has been implicated in peripheral immune signaling, however, the effects of an aging immune system on LRRK2 function have been neglected to be considered. We demonstrate here that the *R1441C* mutation induces a hyper-responsive phenotype in macrophages from young female mice, characterized by increased effector functions, including stimulation-dependent antigen presentation, cytokine release, phagocytosis, and lysosomal function. This is followed by age-acquired immune cell exhaustion in a Lrrk2-kinase-dependent manner. Immune-exhausted macrophages exhibit suppressed antigen presentation and hypophagocytosis, which is also demonstrated in myeloid cells from *R1441C* and *Y1699C*-PD patients. Our novel findings that *LRRK2* mutations confer immunological advantage at a young age but may predispose the carrier to age-acquired immune exhaustion have significant implications for LRRK2 biology and therapeutic development. Indeed, LRRK2 has become an appealing target in PD, but our findings suggest that more research is required to understand the cell-type specific consequences and optimal timing of LRRK2-targeting therapeutics.

One Sentence Summary: The *R1441C-LRRK2* mutation causes an age-acquired immune cell exhaustion in macrophages in sex-dependent manner

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INTRODUCTION

Parkinson's disease (PD) is a common, progressive neurodegenerative disease, affecting around 1-2% of the population over the age of 65 (*1*). PD prevalence will likely increase two-fold by the year 2030 (*2*). In addition, the projected total economic burden will surpass $79 billion by 2037 (*3*), underscoring the need to reduce PD incidence and delay disease progression. The fact that there are currently no disease-modifying drugs for people with PD indicates that knowledge gaps still need to be closed to identify ways to cure or prevent this disease. Although the etiology of PD is largely unknown, it is thought to involve a complex interaction between various genetic and environmental factors (*4*). Although typically thought of as a disease limited to the central nervous system (CNS), evidence suggests a crucial and fundamental role of inflammation in both the CNS and the periphery in PD pathogenesis (*5*).

Pathogenic variants in the gene encoding leucine-rich repeat kinase 2 (LRRK2) account for the majority of familial PD patients (*6*). LRRK2 is a large, complex protein containing six functional domains, two of which have known enzymatic activities; a ROC domain (GTPase activity) and a MAPKK domain (kinase activity). There are numerous single-nucleotide substitutions in each of these domains that have been shown to be pathogenic (*7-14*). The second most prevalent mutation, *R1441C*, located in the GTPase ROC domain, is present in about 46% of familial Parkinson's disease that has an origin in the Basque region in Spain (*15*) and leads to decreased LRRK2 GTPase hydrolysis and increased LRRK2 protein kinase activity (*16, 17*).

LRRK2 is expressed in innate and adaptive immune cells and its expression is tightly regulated by immune stimulation (*18-22*). Furthermore, LRRK2 is a member of the receptor interacting protein (RIP) kinase family, which is a group of proteins that detect and respond to cellular stress by regulating cell death and activation of the immune system (*23*). LRRK2 function has also been implicated in pathogen control based on observations that LRRK2 variants confer differential susceptibility to a number of bacterial infections, including *Mycobacterium tuberculosis* (*24, 25*), *Salmonella Typhimurium* (*26*), and *Listeria monocytogenes (27)*, as well as an increased risk of developing leprosy (*28*) and inflammatory bowel disease such as

Crohn's (*29*) and ulcerative colitis (*30*). Collectively such data strongly suggest a generalized role of LRRK2 in immune system regulation.

Although research has focused primarily on neuroinflammation in PD over the last two decades, it is becoming increasingly clear that peripheral inflammatory responses may play important roles in PD pathogenesis (*31, 32*). For example, immune signaling plays a role in the regulation of neurodegeneration in a *LRRK*2-PD pre-clinical model, with the *R1441C* mutation altering the type II interferon response in peripheral immune cells (*22*). Furthermore, neutralization of IL-6 over-production in the periphery of these mice prevents substantia nigra *pars compacta* (SNpc) dopaminergic (DA) neuron loss in the presence of peripheral lipopolysaccharide (LPS) exposure (*33*). Importantly, immune dysfunction in the periphery takes primacy over the CNS, as the presence of *Lrrk2* mutations in lymphocytes alone is sufficient to induce DA neuron loss in the midbrain of LPS-treated mice (*33*). Such data suggest a pertinent role of the peripheral immune system in *LRRK2*-PD, with immune dysfunction driving the development of the disease.

As well as a role in immune response, a vast amount of research has focused on the role of LRRK2 at the lysosome. In immune cells, specifically, LRRK2 has been shown to mediate a number of lysosome-dependent immune functions such as phagocytosis (*34*) and antigen presentation (*18, 21, 35*). Intriguingly, LRRK2 has been shown to alter lysosomal function and autophagy in kidneys in an age-dependent, biphasic manner, with enhanced autophagy levels in young *Lrrk2*-null mice, that are reduced and drop below control levels once the mice reach 20 months of age (*36*). Despite this intriguing observation, few studies have investigated the effects of *LRRK2* mutations on lysosomal processes in the context of aging. Considering age is the greatest risk factor for many neurodegenerative diseases, aging, in particular aging of the immune system (*5*), is the most underappreciated and understudied contributing factor to neurodegeneration in the field.

To close this important gap, we assessed the effects of *LRRK2* mutations on immune responses and lysosomal function in peripheral immune cells in young versus old mice. We used *R1441C-Lrrk2* mutant knock-in (KI) mice based on previous findings demonstrating an effect of the *R1441C/G* mutation on

peripheral immune responses (*22*, *33*) whilst evading limitations of an overexpression model and investigating the effects of the *R1441C* mutation in physiologically relevant levels. Peritoneal macrophages (pMacs) were isolated from these mice to examine the effects of the *R1441C* mutation on macrophage cell function at different ages. pMacs are not a homogenous population of cells, but rather a mix of small and large pMacs (SPMs and LPMs, respectively). LPMs are resident to the peritoneal cavity and are traditionally thought of as anti-inflammatory, phagocytic and responsible for the presentation of exogenous antigens (*37*). SPMs, on the other hand, are generated from bone-marrow-derived myeloid precursors which migrate to the peritoneal cavity in response to infection, inflammatory stimuli, or thioglycolate, and present a pro-inflammatory functional profile (*37*). We observed a bi-phasic, age-dependent effect of the *R1441C-Lrrk2* KI mice, with increased antigen presentation, production of anti-inflammatory cytokines, lysosomal activity, and pathogen uptake in peripheral macrophages from young (2-3 months) *R1441C-Lrrk2* KI mice examined *ex vivo*. Conversely, peripheral macrophages from aged (18-21 months) *R1441C-Lrrk2* KI mice exhibited a diminished capacity to present antigens in the presence of inflammatory insults, decreased lysosomal function and pathogen uptake, with concomitant increases in DNA fragmentation in the presence of pathogens. Importantly, we demonstrate that this is a sex-dependent phenotype, with male *R1441C-Lrrk2* KI failing to present this age-dependent phenotype. Furthermore, we demonstrate that the age-dependent hypophagocytosis and decreased antigen presentation in the *R1441C-LRRK2* KI females is due to an acquired immune cell exhaustion driven by increased Lrrk2 protein kinase activity and immune responses whilst young. These phenotypes were also observed in human peripheral myeloid cells, with monocyte-derived macrophages from *R1441C* and *Y1699C* patients exhibiting decreased pathogen uptake and increased programmed cell death protein 1 (PD1) expression, consistent with immune cell exhaustion.

RESULTS

LRRK2 R1441C mutant immune cells display age-dependent biphasic alteration in antigen presentation

LRRK2 protein expression has been shown to increase in immune cells from both murine pre-clinical models and patient cells in response to inflammatory stimuli (*18, 21, 22, 38-40*). To determine if whether this is age-dependent, Lrrk2 protein levels were assessed in pMacs from young and aged mice treated with 100U of IFNγ for 18h in the presence or absence of 100nM of the Lrrk2 kinase inhibitor, PF360 (PF-06685360). In pMacs from young females, IFNγ treatment significantly increased total Lrrk2 protein levels in both B6 and *R1441C* pMacs, with no effects of PF360 observed (Fig. 1A, B). Relative to pMacs from young female mice, pMacs from aged females expressed increased total Lrrk2 protein levels under vehicle stimulation, with aged *R1441C* pMacs expressing significantly less Lrrk2 protein levels relative to B6 aged pMacs. Furthermore, pMacs from aged females did not exhibit a significant increase in Lrrk2 protein expression with IFNγ treatment, with aged *R1441C* pMacs still expressing significantly less Lrrk2 protein levels relative to B6 aged pMacs in IFNγ-treatment. In pMacs from young male mice, the same observations were made as in the females (Fig. S1A, B). However, in aged male mice, although a lack of IFNγ-mediated Lrrk2 increase was observed as seen in females, the effect of the *R1441C* mutation was not observed. These observations indicate that Lrrk2 protein levels increase in macrophages as a function of age in both males and females, however, the *R1441C* mutation limits this increase in a sex-dependent manner.

As pMacs are not a homogenous population of cells, as previously discussed, we used multi-color flow cytometry-based methods to immunophenotype LPMs and SPMs, as the two populations can be distinguished based on CD11b expression (Fig. S1C) (*37*). No differences were seen in LPM count between genotypes in males or females at either young or aged (Fig. S1D-G). No significant differences in SPM count were seen in pMacs from young females or males (Fig. S1H, I), nor aged males (Fig. S1J). However, a significant increase in SPM count was observed in pMacs from *R1441C* aged females relative to B6 controls in IFNγ treatment conditions (Fig. S1K). As bone-marrow-derived myeloid precursors are known to differentiate into SPMs upon inflammation, it may be, therefore, *R1441C* bone-marrow-derived myeloid precursors present in these cultures have increased propensities to differentiate in such conditions.

To begin to investigate the effects of the *R1441C* mutation on pMac function at different ages, surface MHC-II expression was assessed on both LPMs and SPMs via multi-color flow cytometry as a measure of antigen presentation in both vehicle and IFNγ treated pMacs. In pMacs from young females, the *R1441C* mutation increases surface MHC-II expression on LPMs and percentage of MHC-II$^+$ LPMs in both vehicle and IFNγ treatments (Fig 1C, Fig. S1L). Interestingly, this effect of the *R1441C* mutation was not observed in LPMs from young male mice (Fig. 1D, Sup. Fig 1M). Regarding surface MHC-II expression on SPMs, a significant increase was seen in vehicle-stimulated SPMs from young *R1441C* females relative to B6 controls (Fig. S1N) which was not observed in SPMs from males (Fig. S1O). However, an increase in MHC-II$^+$ SPM percentage was observed in both male and female *R1441C* pMacs (Fig. S1P, Q).

When assessing surface MHC-II on vehicle and IFNγ treated pMacs from aged females it was observed that, although no effects of genotype were detectable in vehicle-treated LPMs, a significant reduction in surface MHC-II expression on LPMs from *R1441C* females relative to B6 controls was evident in IFNγ-treated conditions (Fig. 1E), indicative of decreased ability to engage in antigen presentation in these cells. As observed in young mice, no effects of genotype were observed in LPMs from male mice (Fig. 1F) suggesting a sex-dependent effect of the *R1441C* mutation on antigen presentation in macrophages. No significant genotype effects were observed regarding percentage of MHC-II+ LPMs or MHC-II+ SPMs (Fig. S1R-W).

With alterations in surface MHC-II expression, there is likely increased and decreased transport of MHC-II-complexes to the cell surface in pMacs from young and aged *R1441C* females, respectively. To test this, using pMacs treated with vehicle or IFNγ, we co-stained for extracellular vs intracellular MHC-II (ExMHCII vs IcMHCII) and monitored expression via fluorescence microscopy. pMacs were co-treated with 100nM PF360 to determine the effects of Lrrk2 kinase activity on surface MHC-II expression. PF360 treatment reduced levels of Lrrk2 phosphorylated at S935 (pS935 Lrrk2) in all groups (Fig. S2A, B). pMacs from young *R1441C* females exhibited increased Ex:IcMHCII ratio relative to B6 controls upon IFNγ treatment, which was ameliorated upon Lrrk2 kinase inhibition (Fig. S2C, D), with this treatment

decreasing Ex:IcMHCII ratio in both genotypes. Such data suggests that Lrrk2-kinase inhibition reduces the transport of IcMHCII to the cell surface to engage in antigen presentation. No effect of the *R1441C* mutation was observed in young males (Fig. S2E, F). Interestingly, in pMacs from aged *R1441C* females, a significant reduction in Ex:IcMHCII ratio was observed in IFNγ treatments relative to B6 controls, with no effect of the *R1441C* mutation observed in pMacs from aged males (Fig.1G-J). Lrrk2 kinase inhibition also had no significant effect on Ex:IcMHCII ratio in pMacs from *R1441C* mice, but significantly reduced Ex:IcMHCII ratio in pMacs from B6 controls. It seems therefore that the *R1441C* mutation exhibits a bi-phasic, age- and sex-dependent effect on antigen presentation in macrophages. The effect of Lrrk2 protein kinase inhibition on antigen presentation, however, does not appear to be bi-phasic nor age-dependent, with Lrrk2 kinase inhibition decreasing antigen presentation in pMacs from both young and aged mice.

LRRK2 R1441C **mutant immune cells display age-dependent biphasic alteration in cytokine release** ***ex vivo*** **and circulating cytokines in vivo**

To investigate the extent to which alterations in antigen presentation were accompanied by changes in cytokine release, media from vehicle- and IFNγ treated cells were collected and cytokine levels were quantified on a multiplexed immunoassay panel (Fig. 2A). In pMacs from young females, increases in IL6 and IL10 relative to B6 controls were observed, with no differences between genotypes in TNF release observed (Fig. 2B). IL6 and IL10 release were also increased in pMacs from young *R1441C* male mice relative to B6 controls with no differences between genotypes observed in TNF release (Fig. 2C). In pMacs from aged female mice, the genotype-dependent changes in IL6 seen in young mice were no longer observed (Fig. 2D) and a significant decrease in IL10 was observed, with a concomitant increase in TNF release, relative to B6 controls. With a decrease in the release of the anti-inflammatory cytokine, IL10, and an increase in the release of the pro-inflammatory cytokine, TNF, these findings indicate that pMacs from aged *R1441C* female mice promote a pro-inflammatory microenvironment. Interestingly, in pMacs from

aged male mice, the *R1441C*-associated increase in IL6 and IL10 seen in young mice was retained with age but *R1441C* macrophages secreted more TNF than B6 controls after stimulation (Fig. 2E).

To determine if these alterations in cytokine release associated with the *R1441C* mutation *ex vivo* was also detectable *in vivo*, plasma from whole blood was collected and cytokine levels were quantified (Fig. 2F). A significant increase in IL10, IL6, and TNF was observed in plasma from young *R1441C* female and male mice (Fig. 2G). Conversely, a decrease in circulating levels of IL10 was observed in the plasma of aged *R1441C* female and male mice relative to B6 controls (Fig. 2H). Similarly, IL6 was also increased in aged *R1441C* plasma relative to B6 controls, although this was only observed in females, and no significant differences in TNF levels were observed.

Regarding other cytokines measured in media collected from pMacs, although significant effects of genotype were observed, these differences were not as consistent between age and sex as was observed for IL10, IL6 and TNF. For example, an increase in keratinocyte chemoattractant (KC)/growth-regulated oncogene (GRO), a small cytokine that belongs to the CXC chemokine family, was observed in pMacs from young *R1441C* females, which was not observed in pMacs from males (Fig. S3A). However, no significant effects of genotype were observed in aged pMacs (Fig. S3B). Upon IFNγ treatment, pMacs from young *R1441C* females exhibited decreased levels of the pro-inflammatory cytokine, IL1β (Fig. S3C) which was not observed in pMacs from young male mice. Again, no significant differences between genotypes were observed in aged pMacs (Fig. S3D). Although no significant differences between genotypes were observed in the release of the anti-inflammatory cytokine, IL4, in young pMacs (Fig. S3E), a significant decrease was observed in pMacs from aged *R1441C* females relative to B6 controls, with no differences seen in aged males (Fig. S3F). Lastly, although no significant differences in IL5 were seen in pMacs from young mice (Fig. S3G), a decrease in IL5 release was observed in aged pMacs from *R1441C* females and males relative to B6 controls (Fig. S3H).

The R1441C Lrrk2 mutation is associated with alterations of monocyte populations and microglia activation in aged female mice

The observation of a biphasic immune response, with an initial hyperinflammation state proceeded by a subsequent downregulation of immune responses, prompted us to hypothesize the presence of the *R1441C* mutation elicited immune cell exhaustion. Immune exhaustion arises from chronic stimulation of immune cells, leading to a downregulation, or even absence, of an expected effector response (e.g. antigen presentation, cytokine release or phagocytosis) (*41*). It is known that monocytes pushed to exhaustion shift to express higher Ly6C levels and a reduction in Ly6C^{-} monocytes under resting conditions (*42*). We therefore assessed monocyte sub-populations in the blood of aged mice to determine if a shift to an exhausted state could be observed. Indeed, we observed a detectable increase in Ly6C^{+}:Ly6C^{-} in the peripheral blood mononuclear cells isolated from blood of aged *R1441C* female mice relative to B6 controls (Fig. S4A) with no significant change observed in aged male mice (Fig. S4B). Interestingly, no effects of genotype were observed in young mice (Fig. S4C, D), suggesting development of an age-acquired immune exhaustion phenotype in the *R1441C* females.

It has been suggested that circulating monocytes, disrupted in PD, may infiltrate the brain parenchyma and affect local glial and adaptive immune responses (*43*). To determine whether exhausted monocytes could be infiltrating the brain parenchyma of aged *R1441C* female mice, immune cells were isolated from whole brain and assessed via multi-color flow cytometry. Indeed, in aged *R1441C* females, an increase in overall Ly6C^{+} monocytes in the brain parenchyma was observed relative to B6 controls, whilst MHC-II/Ly6C^{+} were decreased (Fig. S4E, F), suggesting that whilst there is an increase in infiltrating Ly6C^{+} monocytes in the brains of aged *R1441C* females, they exhibit the same decrease in antigen presenting capabilities to those observed in pMacs *ex vivo*. Importantly, alongside this increase in infiltrating monocytes into the brain parenchyma, a concomitant increase in MHC-II expression on microglia was observed in aged *R1441C* females relative to B6 controls (Fig. S4G) with no changes in overall microglia number (Sup. Fig 4H). No changes were observed in the brains of male mice (Fig. S4I-L).

***LRRK2 R1441C* mutant immune cells display age-dependent biphasic alterations in lysosomal activity**

As previously discussed, loss of *Lrrk2* leads to biphasic alterations in lysosomal function in kidneys (*36*). Interestingly, lysosomal dysfunction has been implicated in immune cell exhaustion, with decreased autophagy levels and lysosomal function being associated with increased T cell exhaustion (*44*). We therefore hypothesized that the biphasic immune response observed in *R1441C* females would be associated with concomitant biphasic lysosomal function.

The Eα: YAe model is used to monitor the antigen presentation capabilities of cells by incubating an endogenous peptide (Eα 52-68) which is subsequently phagocytosed, transported to the lysosome, loaded onto an MHC-II complex at the lysosome and transported back to the plasma membrane for antigen presentation (Fig. 3A). This Eα peptide-loaded MHC-II can subsequently be detected via flow cytometry using the YAe antibody (*45*). We observed that YAe median fluorescence intensity (MFI) was significantly increased in IFNγ-treated LPMs from young *R1441C* females relative to B6 controls, which was not observed in LPMs from young male mice (Fig. 3B, C). When co-treated with PF360, YAe MFI was significantly reduced in LPMs of both sexes, ameliorating the increased YAe MFI observed in LPMs from young *R1441C* females. Antigen presentation and pathogen sensing require protease action and sufficient lysosomal function in order to occur (*46*), which is why lysosomotropic agents have been shown to decrease peptide-loaded-MHC-II surface expression in antigen presenting cells (*45*). Indeed, when co-treated with the vacuolar H+ ATPase (V-ATPase) inhibitor, Bafilomycin A1, YAe MFI significantly decreased in both *R1441C* and B6 LPMs from both sexes (Fig. 3B, C). The same age-dependent biphasic phenotype in *R1441C* females was observed, with IFNγ-treated LPMs from aged females exhibiting decreased YAe MFI relative to B6 controls which was not observed in aged male mice (Fig. 3D, E). PF360 significantly reduced YAe in LPMs from aged B6 mice and aged *R1441C* males but had no significant effect on LPMs from aged *R1441C* females. Furthermore, the same effect of Bafilomycin A1 was observed in all genotypes. Due to

this observation and the crucial role of the lysosome in antigen presentation, we next sought to probe the effects of the *R1441C* mutation on lysosomal function in these pMacs.

pMacs were plated and incubated with the lysosomal degradation probe, DQ Red BSA, and the pan-cathepsin probe, BMV109, and collected for flow cytometry to quantify lysosomal activity. It was observed that LPMs from young *R1441C* females exhibited increased DQ-BSA and BMV109 MFI (Fig. 3F, G), indicative of increased lysosomal protein degradation and pan-cathepsin activity, respectively. Conversely, LPMs from aged *R1441C* females exhibited increased DQ-BSA and BMV109 MFI (Fig. 3H, I). No effects of genotype were observed in LPMs from male mice (Fig. S5A-D). These observations made via flow cytometry were confirmed with fluorescence microscopy (Fig. 3J-N; Fig. S5E-J). As was observed with YAe measurements, Lrrk2 kinase inhibition via treatment with PF360 significantly reduced both DQ-BSA and BMV109 MFI observed via microscopy in all groups except aged *R1441C* females, which exhibited decreased measurement of lysosomal activity which were unaffected by Lrrk2 kinase inhibition.

***R1441C* Lrrk2 mutation is association with age-acquired hypo-phagocytosis**

Lysosomal function in macrophages is crucial for a number of other immune-related functions in myeloid cells, including phagocytosis. Therefore, we posited that the observed biphasic effects of the *R1441C* mutation on lysosomal function would have downstream consequences on phagocytosis in pMacs. To explore this hypothesis, pMacs were plated and incubated with pHrodo™ Green *E. coli* BioParticles™, after which uptake and fluorescence intensity were monitored over 5 hours. An increase in both GFP^+ cells and GFP MFI was observed in pMac cultures from young *R1441C* females (Fig. 4A-C). In male mice, although the percent of GFP^+ cells was not significantly different between the two genotypes, pMacs from young *R1441C* males did exhibit increased GFP MFI relative to B6 controls (Fig. S6A-C). Lrrk2 kinase inhibition with PF360 significantly reduced GFP MFI in both *R1441C* males and female pMacs, however only pMacs from male *R1441C* mice exhibited significantly reduced percentage of GFP^+ cells in response to Lrrk2 kinase inhibition. It appears, therefore, that in young mice, the *R1441C* mutation induces an

upregulation in phagocytic uptake in pMacs from both males and females, as indicated by increased GFP MFI, which can be ameliorated upon Lrrk2 kinase inhibition.

In pMacs from aged females, a significant reduction in both percent GFP^+ cells and GFP MFI was observed in pMacs from *R1441C* females relative to B6 controls (Fig. 4D-F). Although Lrrk2 kinase inhibition with PF360 significantly increased percent GFP^+ cells in pMacs from *R1441C* females, no effect of Lrrk2 kinase inhibition was observed in GFP MFI. pMacs from aged male *R1441C* mice exhibited the same decrease in GFP^+ cells as females, relative to B6 controls, that was also partially ameliorated by Lrrk2 kinase inhibition (Fig. S6D). Interestingly, pMacs from aged *R1441C* male mice exhibit increased GFP MFI relative to B6 controls (Fig. S6E, F) which was unaffected by Lrrk2 kinase inhibition. Collectively, it seems that pMacs from *R1441C* females exhibit increased phagocytic uptake at a young age and develop an age-dependent hypophagocytic phenotype. pMacs from *R1441C* males, however, exhibit this same increased phagocytic uptake at a young age but retain this increase in old ages.

Hypophagocytic activity in *R1441C* macrophages is associated with aberrant pathogen-mediated DNA damage

Our data thus far suggests a potential age-acquired immune cell exhaustion in macrophages from *R1441C* female mice. In other disease states associated with cellular exhaustion, apoptotic depletion of immune cells is also observed (*47*). To investigate the level of pathogen-induced apoptosis in pMacs from *R1441C* mice, pMacs were plated in the presence of *E. coli* for 12 hours, harvested and stained using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), which detects DNA breaks formed during the final phase of apoptosis, and TUNEL fluorescence was assessed via flow cytometry. LPMs from young *R1441C* females exhibited decreased TUNEL MFI in response to *E. Coli* relative to B6 controls (Fig. 4G), whilst an increase was observed in LPMs from aged *R1441C* females (Fig. 4H). Reduced apoptosis could lead to prolonged presence of activated immune cells which should otherwise be eliminated, resulting in an increased and prolonged inflammatory response and, if sustained for prolonged times, could lead to immune

cell exhaustion. Indeed, LPMs from young *R1441C* males exhibit increased TUNEL MFI indicative of increased apoptosis in these cells (Fig. S6G), with no effect of genotype observed in aged males (Fig. S6H). These novel findings support the conclusion that *R1441C* males evade this female-specific, age-acquired immune exhaustion that is seen in females by increasing clearance of activated immune cells.

Immune exhaustion in *R1441C* Lrrk2 mutant immune cells is dependent on Lrrk2 kinase activity

Our data thus far suggest a potential age-acquired immune cell exhaustion in macrophages from *R1441C* female mice. We hypothesized that this age-acquired immune exhaustion was driven by the initial hyperinflammatory phase when these mice are young, characterized by increased cytokine release, increased antigen presentation, and reduced apoptosis of activated immune cells. We therefore hypothesized that pMacs from young *R1441C* female mice could be pushed to acquire this age-dependent immune exhaustion phenotype if subjected to chronic overstimulation *ex vivo.* To test this, pMacs were isolated and plated in the presence of a vehicle or 100ng/mL lipopolysaccharide (LPS) for a 5-day chronic treatment (Fig. 5A). We first sought to understand how Lrrk2 expression is altered by LPS-induced exhaustion and how age and the *R1441C* affects this. In pMacs from young females, an increase in Lrrk2 expression was observed in response to LPS-induced exhaustion (Fig. 5B, C). Importantly, there was no significant effect of treatment in pMacs from aged female mice, with aged *R1441C* pMacs expressing significantly less Lrrk2 relative to B6 controls. Interestingly, these same observations were made in pMacs from aged male mice (Fig. S7A, B) suggesting that, with an additional chronic inflammatory stress, aged *R1441C* males can exhibit some of the phenotypes observed in females.

A number of negative costimulatory markers that are associated with immune exhaustion have been identified, including the immune checkpoint molecule, programmed cell death-1 (PD1) (*48*). PD1 expression was quantified via multi-color flow cytometry in pMacs from aged mice to determine if pMacs from *R1441C* females were exhausted as hypothesized. Indeed, an increase in $PD1^{+}$ cells was observed in both vehicle and LPS-exhausted *R1441C* LPMs relative to B6 controls (Fig. 5D). As this was seen in both

vehicle and LPS conditions, these exciting findings suggest that pMacs from *R1441C* females are already exhibiting immune exhaustion markers, which is then exacerbated by chronic LPS-exhaustion. Interestingly, LPMs from aged *R1441C* male mice, although no significant effect of genotype was observed in vehicle conditions, when pushed to exhaustion with LPS, an increase in $PD1^+$ cells relative to B6 controls was observed (Fig. S7C). The lack of differences in $PD1^+$ cells between genotypes in aged male mice suggests there is no effect of the *R1441C* mutation on immune exhaustion in naïve animals. However, based on the increased $PD1^+$ cell counts observed in *R1441C* males when treated with LPS for 5 days, we conclude that a secondary, chronic inflammatory insult can push *R1441C* pMacs from male mice to acquire immune exhaustion like that seen in female mice.

When macrophages from young *R1441C* female mice were assessed for PD1 expression after a 5-day incubation with vehicle or LPS, a significant increase in $PD1^+$ cell count was observed in both treatment conditions relative to B6 controls, with a significant increase detectable in LPS-treated conditions relative to vehicle (Fig. 5E). Interestingly, when cells were pushed to exhaustion with Lrrk2 kinase inhibitor co-treatment, a significant reduction in $PD1^+$ cell count was observed in *R1441C* cells. These data suggest that *R1441C* macrophages from female mice may be susceptible to immune exhaustion in a Lrrk2-kinase dependent manner. No significant differences between genotypes were observed in LPMs from young male mice (Fig. S7D).

We next sought to determine if LPS-induced exhausted macrophages also exhibited the diminished antigen presentation capabilities that we observe in the aged *R1441C* macrophages. After a 5-day treatment with vehicle or LPS +/- PF30, macrophages were treated with IFNγ and MHC-II expression was quantified via multi-color flow cytometry. In LPMs subjected to a 5-day vehicle pre-treatment, the same increased MHC-II expression in *R1441C* macrophages relative to B6 controls was observed (Fig. 5F). As hypothesized, in LPMs pushed to exhaustion with a 5-day LPS pre-treatment, a significantly muted response to IFNγ was elicited in both genotypes; however, *R1441C* LPMs exhibited decreased MHC-II relative to B6 controls in response to IFNγ, phenocopying what was observed in macrophages with aged mice. Importantly, this could

be ameliorated by co-treatment with PF360 during the 5-day pre-treatment, suggesting that the push to immune exhaustion and vulnerability of *R1441C* macrophages is dependent on Lrrk2 protein kinase activity. Furthermore, we also observed that pMacs from young *R1441C* females pushed to exhaustion with a 5-day pre-treatment of LPS exhibited a significant reduction in phagocytosis as measured by uptake of pHrodo™ Green *E. coli* relative to B6 controls detected by GFP^+ cell percentage and GFP MFI which was ameliorated with PF360 co-treatment (Fig. 5G-I).

Based on the observation that the $PD1^+$ cell count was elevated in LPMs from aged *R1441C* males relative to B6 controls, we hypothesized that a 5-day LPS treatment would induce immune exhaustion in pMacs from aged *R1441C* males similar to that seen in female mice. After a 5-day treatment with vehicle or LPS +/- PF30, macrophages from both young and aged male mice were treated with IFNγ and MHC-II expression quantified via multi-color flow cytometry. No significant effects of genotype were observed in LPMs from young male mice (Fig. S7D-E). However, in LPMs from aged males pushed to exhaustion with 5-day LPS pre-treatment, *R1441C* LPMs exhibited decreased MHC-II relative to B6 controls in response to IFNγ, phenocopying what was observed in macrophages from aged female *R1441C* mice (Fig. S7F). Notably, this could be rescued with PF360 co-treatment during the 5-day LPS pre-treatment. Additionally, the same hypophagocytic phenotype observed in aged *R1441C* female pMacs was observed in males once pushed to exhaustion with a 5-day LPS treatment (Fig. S7G-I). Collectively such data support the hypothesis that, with a secondary hit in the form of a chronic, inflammatory stimulus, male *R1441C* mice exhibit increased immune cell exhaustion relative to B6 controls, similar to that seen in naïve *R1441C* female macrophages.

Transcriptomic profiling reveals age- and sex-specific alterations in critical effector function pathways in *R1441C* macrophages

To explore the age and sexually dimorphic effects of the *R1441C* mutation on macrophage function, RNA was harvested from pMacs post-vehicle or IFNγ treatment and processed for RNA sequencing (RNAseq).

A principal component analysis (PCA) was conducted from the resulting dataset which revealed that groups in our dataset primarily cluster based on age and treatment with vehicle or IFNγ treatment (Fig. S8A, B).

We first sought to identify key cellular pathways altered under different treatment conditions in *R1441C* pMacs relative to B6 controls. In pMacs from young females treated with IFNγ, Gene Ontology (GO) enrichment analysis revealed that upregulated differentially expressed genes (DEGs) in *R1441C* macrophages were enriched in pathways such as regulation of phagocytosis, T cell differentiation, cytokine production and defense response to virus (Fig. S8C, D). In pMacs from aged females treated with IFNγ, GO enrichment analysis showed downregulated DEGs in *R1441C* macrophages were enriched in pathways such as detection of external biotic stimulus, acute inflammatory response, and regulation of MAP kinase activity (Fig. S8E, F). Collectively, such observations support our previous data that shows an effect of *R1441C* Lrrk2 on functional readouts such as increased phagocytosis and cytokine production in response to IFNγ in young females, whilst in aged mice, effector functions responsible for defense responses are significantly downregulated.

Overall, fewer genes were dysregulated in *R1441C* pMacs from young females treated with vehicle, relative to pMacs treated with IFNγ (Sup. Fig.9A), although these DEGs were enriched in similar pathways (Fig. S9B). Similar observations were made with pMacs treated with vehicle from aged females (Fig. S9C). As Lrrk2 protein levels increase in response to IFNγ, it follows that DEGs driven by the *R1441C* mutation would have a more significant biological impact in these conditions where Lrrk2 levels are increased. Furthermore, given that the majority of phenotypes we observe in pMacs from *R1441C* females are seen in response to an inflammatory insult or immunological challenge, such as IFNγ or *E. Coli,* it is logical that more *R1441C*:B6 DEGs would be observed in these conditions relative to vehicle treatments. Few *R1441C*:B6 DEGs were observed in pMacs from male mice at both ages and in both treatments (Fig. S9E-I). This is unsurprising, given that many phenotypes were not observed in *R1441C* male mice. pMacs from aged *R1441C* only begin to exhibit phenotypes similar to *R1441C* female mice when pushed to immune cell exhaustion and re-challenged with IFNγ. It is therefore likely that to identify key cellular pathways that

are altered by the *R1441C* mutation in male mice, a second, chronic immune insult is needed to elicit those changes.

***R1441C* Lrrk2 mutation alters transcriptional regulation of mitochondrial and oxidative stress genes in macrophages in a sex- and age-dependent manner**

Based on the observation that the *R1441C* mutation alters gene expression in pMacs from aged female mice, we next sought to determine the key cellular pathways that this mutation altered in the aging process. When comparing DEGs in pMacs from both B6 and *R1441C* aged mice treated with IFNγ relative to those from young pMacs, there was a significant degree of overlap between the DEGs identified in the two genotypes (Fig. 6A-C). However, 2012 genes were identified to be differentially expressed upon aging and novel to *R1441C* pMacs. KEGG pathway enrichment analysis revealed that these 2012 genes unique to aged R1441C pMacs were significantly enriched for pathways associated with neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease and Huntington's disease (Fig. 8D). Similarly, GO biological pathway enrichment analysis of these DEGs showed a significant enrichment in pathways related to mitochondria, oxidative stress and metabolism, including mitochondrial respiratory chain complex I assembly, glucose and pyruvate metabolic process and cellular respiration, which are all known key lysosomal and mitochondrial functions. Many DEGs observed in the Parkinson's Disease KEGG pathway were present in the GO biological pathways associated with mitochondrial function and oxidative stress, including many *Nduf* and *Cox* genes, *Park7*, *Pink1* and *Prkn.*

When comparing DEGs in aged pMacs from both B6 and *R1441C* female mice treated with vehicle from, unsurprisingly, different DEGs were identified relative to what was seen in IFNγ conditions (Fig. S10A-D). As opposed to pathways related to mitochondria and oxidative induced by IFNγ conditions, DEGs novel to *R1441C* in vehicle conditions were significantly enriched in pathways such as wound healing responses, and Wnt, ERK and PI3K signaling. It seems, therefore, that at rest, or under vehicle-treatment conditions, the *R1441C* mutation exerts its effects on aging through pathways crucial for metabolism, cell survival, and

cell growth. The Wnt signaling cascade is crucial in macrophages and serves as a regulator of macrophage inflammation-resolving state (*49*). However, during an inflammatory insult, the *R1441C* mutation exerts its effects on aging through pathways crucial for oxidative stress and cellular respiration.

Interestingly, when assessing DEGs in pMacs from aged male mice treated with IFNγ, 540 genes were identified as novel and significantly upregulated in *R1441C* macrophages that were not significantly affected by aging in B6 controls (Fig. S10E-G). Pathway enrichment analysis revealed that these 540 upregulated genes significantly enriched pathways related to type II interferon production, cytokine production, and NFkB and JNK signaling (Fig. S10H). We also observed that, with the 5-day LPS stimulation, pMacs from aged *R1441C* male mice could be pushed to exhibit phenotypes that paralleled those seen in aged female *R1441C* macrophages. It has previously been reported that sustained and chronic STAT1 signaling, known to be stimulated by cytokine signaling (*50*), is necessary to induce macrophage exhaustion (*42*). Collectively, we interpret our findings that the upregulated DEGs, including driving increased cytokine release in *R1441C* aged male macrophages, may predispose these macrophages to become exhausted upon LPS-stimulation.

***R1441C*- and *Y1699C* LRRK2 monocyte-derived patient macrophages exhibit hypophagocytosis and increased markers for exhaustion**

To assess the effects of PD-associated mutations on immune exhaustion in humans carrying *LRRK2* mutations, pathogen phagocytosis was quantified in monocyte-derived macrophages from 2 PD-*Y1699C*, 1 PD-*R1441C*, 7 healthy controls, and 3 non-manifesting carriers (NMC) of the *R1441C* pathogenic variant (Fig. 7A). Both *R1441C* and *Y1699C* variants are located in the GTPase domain of the LRRK2 protein, and both have been shown to perturb LRRK2's GTPase activity and have been reported to elevate its kinase activity (*8, 16, 17, 51, 52*). A hypophagocytic phenotype was observed in monocyte-derived macrophages relative to healthy controls and *R1441C* NMCs, as indicated by a reduced number of GFP^+ cells (Fig. 7B, C), with no differences in GFP MFI observed between groups (Fig. S11A). Next, increased markers of

immune exhaustion accompanied reduced rates of phagocytosis, PD1 expression was assessed in peripheral blood mononuclear cells (PBMCs). Consistent with our mouse data, we observed that PBMCs from both PD-*Y1699C* and PD-*R1441C* patients exhibited increased $PD1^+$ cell count and expression levels (Fig. 7D, E). When quantifying PD1 expression in specific immune-cell subsets (Fig. S11B), it was determined that this overall increase in PD1 expression was driven by expression in classical monocytes, with increased PD1 expression observed in PD-*Y1699C* and *R1441C* classical monocytes relative to healthy controls and *R1441C* NMCs (Fig. 7F), with no significant differences observed in other cell types (Fig. S11C-E). Similarly, increased PD1 expression was evident in monocyte-derived macrophages from PD-*Y1699C* and *R1441C* patients relative to healthy controls and *R1441C* NMCs (Fig. 7G). Collectively, the immune exhaustion phenotype observed in aged *R1441C* female mice is also associated with an exhaustion phenotype in cell from patients harboring the *LRRK2-Y1699C* or *R1441C* mutation.

DISCUSSION

Considering age is the greatest risk factor for many neurodegenerative diseases, aging, in particular aging of the immune system, is the most underappreciated and understudied contributing factor in the neurodegeneration field. Our novel findings reveal a bi-phasic age- and sex-dependent effect of the *R1441C* mutation in peripheral immune cells, with increased cytokine release, antigen presentation, phagocytosis and lysosomal activity in macrophages from young female *R1441C* mice, with many of these phenotypes ameliorated by Lrrk2 protein kinase inhibition. Our findings further reveal an age-acquired immune cell exhaustion in macrophages from *R1441C* females associated with increased Lrrk2 protein kinase activity, hypophagocytosis, decreased antigen presentation, and reduced lysosomal pan-cathepsin activation, protein degradation and increased pathogen-mediated DNA breakage, and increased PD1 expression indicative of immune cell exhaustion. Importantly, this immune cell exhaustion phenotype was also observed in monocytes and macrophages from human subjects with PD and harboring the *R1441C* and the *Y1699C* mutation. Furthermore, it was demonstrated that, although macrophages from male mice did not exhibit

this bi-phasic age-dependent phenotype, they could be pushed to exhaustion with chronic LPS stimulation, suggesting that, along with age, a secondary inflammatory insult is required to observe immune cell exhaustion phenotypes in macrophages from *R1441C* males. Finally, it was demonstrated that there is an age-acquired dysregulation in the expression of genes associated with PD risk, mitochondrial function, and oxidative stress in macrophages from female *R1441C* mice.

Consistent with findings in *ex vivo* studies in macrophages, we consistently observed elevated levels of both IL10 and IL6 in circulating plasma from young *R1441C* mice. It has recently been shown that RAW264.7 cells expressing *T1348N-LRRK2*, an artificial P-Loop null mutation that disrupts GTP binding (*53, 54*), produce significantly less IL10 relative to wild-type cells in response to LPS and Zymosan (*55*). IL10 is produced by macrophages and is critical in limiting immune-mediated pathology (*56*), and it was therefore suggested by Nazish *et al* that there may be a neuroprotective role of LRRK2 in immune signaling through regulation of IL10 secretion. In addition, we also observed elevations in IL6, which is known to be promptly and transiently produced in response to infections and tissue injuries during host defense through the stimulation of immune reactions (*57*). These observations implicate a protective role of Lrrk2 in young *R1441C* mice. The pro-inflammatory cytokine, TNF, was also increased in circulating plasma of young *R1441C* mice, a compensatory increase that may also contribute to organisms ability to fight infection given that mice lacking TNF exhibit increased susceptibility to infection with increased bacterial load (*58*). In support of the protective role of LRRK2 in immune signaling, LRRK2 has been demonstrated to provide increased pathogen control of *Salmonella typhimurium (26, 59)* and *Listeria monocytogenes* (*27*). Consistent with these reports, we observed increased *E. coli* uptake and decreased *E. coli*-mediated DNA damage in macrophages from young *R1441C* female mice, providing further support for a protective role of LRRK2 in immune signaling and defense against pathogens, at least in a young organism. Given that *LRRK2* mutations are associated with not only PD, but also Crohn's Disease (CD) (*2, 60, 61*) and leprosy (*62*), it is difficult to understand why these mutations would have persisted in the human population. Given the evidence discussed and the data presented here, it is plausible to speculate that these mutations may

have persisted due to the immunological advantage they confer to young organisms, including protection against certain infections.

For the first time, we have reported here an age-dependent, bi-phasic effect of LRRK2 on immune cell function, with the *R1441C* mutation inducing myeloid cell immune exhaustion in a sex-dependent manner. The concept of immune cell exhaustion was first described as the clonal deletion of virus-specific $CD8^+$ T-cells that occurs during high-grade chronic infections, and in T-cells is characterized by the stepwise and progressive loss of T-cell functions (*63*). Regarding exhausted myeloid cells, pathogenic inflammation and immunosuppression, characterized by decreased antigen presentation, hypophagocytosis and altered cytokine release, have been described more recently (*42*). The immune system is composed of numerous cell types with varying states of activation, and cell-to-cell communication during immune responses is crucial for host defense and resolution of inflammation. The observations made here in innate immune cells, therefore, likely have downstream effects on myeloid cell communication with adaptive immune cell types in the periphery. Indeed, alterations in the adaptive immune system have been reported in PD patients. For example, a meta-analysis of 943 cases of PD identified a reduction in circulating $CD4^+$ T cells in PD-patients (*64*, *65*). More specifically, HLA-DR^+ T cells and $CD45RO^+$ memory T cells have been shown to be increased in people with PD relative to healthy age- and sex-matched controls, while naive $CD4^+$ T cells are reduced (*65*-*67*). $CD4^+FOXP3^+$ Treg cells have increased suppressive activity in patients with PD (*68*). Interestingly, one characteristic of immune exhaustion is the dysregulated cross-talk between $CD4^+$ and $CD8^+$ T-cells due to the suppressive action of Tregs (*69*). Furthermore, Tregs directly inhibit the antigen-presenting function of monocytes and macrophages (*70*). Further research is required to understand the role of cell-to-cell communication in these myeloid cell phenotypes, and if immune cell exhaustion driven by LRRK2 is a contributing factor.

A sex-dependent effect of the *R1441C* mutation was reported here, with females exhibiting an age-acquired immune cell exhaustion phenotype which could only be elicited in macrophages from males upon a secondary, chronic inflammatory insult. Sex-dependent effects of *LRRK2* mutations have previously been

reported. A protective effect of the *G2019S* mutation against *S. typhimurium* has been observed in mice, with female mutant mice showing increased survival relative to B6 controls which was not observed in males (*59*). Such observations are also consistent with the sexual dimorphism reported in subjects with PD who also carry a heterozygous p.*G2019S* mutation in *LRRK2*, with female sex being more common amongst carriers than non-carriers (*71-73*). This sexual dimorphism has also been reported for the *R1441C/G/H* mutations (*72*). We observed an *R1441C*-dependent phenotype in macrophages from male mice only when a secondary, chronic inflammatory insult was applied. It is particularly interesting that genes that are significantly upregulated with age in *R1441C* macrophages from male mice are associated with pathways which are heavily implicated in the phenotypes we observed in macrophages from young *R1441C* females. An increase in gene expression in pathways associated with cytokine production is consistent with our previous observation of increased IL6, IL10 and TNF release in aged male pMacs relative to B6 controls. Further research is therefore required to determine the extent to which this sexual dimorphism is driven by altered immune system susceptibility to *LRRK2* mutations in males and females.

We observed alterations in genes enriched in the KEGG Parkinson's Disease pathway in *R1441C* female macrophages as a function of age, with these genes being mostly associated with mitochondrial respiration and oxidative stress. Not surprisingly, mitochondrial dysfunction has emerged as one of the key mechanisms underlying the pathogenesis of both sporadic PD and familial Parkinsonism (*74, 75*). LRRK2 has been heavily implicated in mitochondrial function and mitophagy, with *LRRK2* mutations causing reduced mitochondria potential and decreased ATP production, mitochondrial DNA damage, mitochondrial fragmentation and altered rates of mitophagy in various cell types and pre-clinical models (*76-79*). But importantly, our findings herein reveal that pathways once thought to be primarily important to neuronal demise (mitochondrial and oxidative stress) are also disrupted in peripheral immune cells by PD-associated mutations in a sex- and age-dependent manner and have important implications for monitoring risk, target engagement, and disease progression of PD and perhaps other NDs by assessing peripheral immune cell types *ex vivo*. It is known that, in T cells, mitochondrial stress induced by chronic antigen stimulation drives

immune exhaustion (*80*). Further research is therefore required to fully understand the role of mitochondria and mitophagy, and therefore lysosomal function, in the *R1441C*-mediated immune cell exhaustion phenotypes observed here.

It has previously been shown that LRRK2 exerts differential effects on inflammatory responses in a tissue-specific manner, and it has been suggested that there is a differential role of LRRK2 in the periphery versus the central nervous system (CNS) (*59, 81*). It therefore remains to be determined how the data reported here impacts our understanding of LRRK2 in myeloid cells in both the periphery and the CNS. However, alterations in type II interferon responses have been observed in both induced pluripotent stem cell (iPSC)-derived microglia and macrophages from patients harboring *G2019S* mutations (*82*), and similar effects of *LRRK2* mutations on lysosomal function have also been reported in patient iPSC-derived microglia and macrophages (*83*). However, it has been demonstrated that expression of *R1441C*-Lrrk2 in peripheral lymphocytes alone is sufficient to induce dopaminergic neuronal cell loss in the midbrains of LPS-treated mice (*33*), suggesting that the periphery plays a pertinent and perhaps primary role in *LRRK2*-PD, and future research into how immune exhaustion in the periphery impacts cross-talk with the CNS is needed to inform future immunomodulatory therapeutic interventions for PD.

We successfully demonstrated that, like macrophages from aged *R1441C* mice, myeloid cells from *R1441C* and *Y1699C* patients exhibited hypophagocytosis and increased expression of immune exhaustion markers relative to those from HCs and *R1441C*-NMCs. However, it is unclear if the same hyper-responsive phenotype that was observed in young *R1441C* female mice is exhibited in myeloid cells in *R1441C* carriers at a younger age or prior to disease onset. Indeed, we did show that *R1441C*-NMCs did not exhibit these immune exhaustion phenotypes, and it is noteworthy that the average age of the 3 *R1441C*-NMCs in this study was 30.5 years (see supplementary material), whilst the average age of the *R1441C* and *Y1699C*-PD subjects was 42 and 63.5, respectively. Further investigation is therefore required to determine if PBMCs from NMCs, upon follow-up after further aging or disease onset, exhibit immune exhaustion. Furthermore, further research is required to understand if these phenotypes are exclusive to *LRRK2*-pathogenic variants

located in the GTPase domain or if they are seen in other *LRRK2* mutant immune cells as well as in idiopathic PD or sporadic cases.

LRRK2-targeting therapies, including antisense oligonucleotides (ASOs) and kinase inhibitors, have been suggested as a potential therapeutic for *LRRK2*-PD, with many hypothesizing that targeting the increased LRRK2 levels and kinase activity will be beneficial to CNS-related pathology. Our findings suggest that more research is required to understand the location and optimal timing for the administration of these LRRK2-targeting therapeutics. Specifically, if LRRK2 plays a critical role in pathogen and infection control in a pathogen or tissue-specific manner, it may mean that LRRK2-targeting therapies may need to be limited to carriers of *LRRK2* pathogenic variants and/or may need to be delivered in a CNS compartment-targeted manner as opposed to systemically to avoid deleterious collateral damage to immune function. Indeed, the LRRK2 ASO currently being tested in the clinic is being delivered via intrathecal injection directly into the cisterna magna, thus limiting systemic exposure (NCT03976349). Lastly, perhaps somewhat surprisingly, we found that while LRRK2 kinase inhibition could rescue some of the effector functions of aged mutant-LRRK2 mice, it had no beneficial effects on the immune-cell exhaustion phenotypes observed here in macrophages from aged mice; however, it did prove to be protective in macrophages from young mice to prevent the development of an immune-cell exhaustive phenotype. Such observations suggest that LRRK2-targeting therapies would elicit the most beneficial effects during the prodromal phases of disease, and further research on PBMCs from prodromal patients with pre-motor features at risk for development of motor PD due to a family history will be required to investigate this possibility and inform future clinical trials.

MATERIALS AND METHODS

Study design

The primary objective of this research was to investigate the effects of the *R1441C LRRK2* pathogenic variant on myeloid cell effector function in a sex- and age-dependent manner. We hypothesized that a biphasic, age-dependent effect of the *R1441C* pathogenic variant would be observed, affecting antigen presentation, cytokine release, phagocytosis and lysosomal function in macrophages. To explore our hypothesis, we used *R1441C* knock-in mice and B6 controls and assessed myeloid cell effector function both *ex vivo* and *in vivo,* in males and females at 2-3 months and 18-21 months. All studies were performed by investigators blinded to genotype and treatment groups at the University of Florida, USA. Power analyses based on the labs pilot and published data indicated that n = 12 per group for flow-cytometry and cytokine release assays, and n = 6 for imaging based immunophenotyping and immunoblotting would be needed to yield 80% power with a α set to 0.05. The study used human PBMCs received from collaborators at University Hospital Schleswig-Holstein, University of Luebeck, Germany and the University Hospital Eppendorf, Hamburg, Germany. Samples were received at the University of Florida and investigators were unblinded after completion of analysis. Deidentified data provided included sex, age at collection, age of diagnosis, clinical diagnosis and genetic status.

Animals

R1441C knock-in (Strain #009346; B6.Cg-Lrrk2tm1.1Shn/J) mice have previously been characterized (*84*) and were maintained in the McKnight Brain Institute vivarium (University of Florida) at 22°C at 60-70% humidity with a 12-hour light/dark cycle and *ad libitum* access to standard rodent chow and water. C57BL/6 (Strain #000664) controls were used for all studies. All animal procedures were approved by the University of Florida Institutional Animal Care and Use Committee and were in accordance with the National Institute of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publications No. 80-23) revised 1996. Female and male mice were aged to 2-3 or 18-21-months-old and sacrificed via cervical dislocation or rapid decapitation.

Human subjects

This study was reviewed and approved by the University of Lubeck Institutional Review Board (AZ16-039). Participants provided written informed consent to participate. During recruitment, a family history was used to assess history of disease and patients were screened for *LRRK2* variants. See Table S3 for patient demographics.

Statistics and data analysis

Data and statistical analyses were performed using IBM SPSS statistics 27 or GraphPad Prism 9. For assessing differences between groups, data were analyzed by either 1-way or 2-way analysis of variance (ANOVA), or by t-test. In instances when data did not fit parametric assumptions, Kruskal-Wallis non-parametric ANOVA was used. *Post hoc* tests following ANOVAs were conducted using Tukey HSD or Bonferroni correction. Two-tailed levels of significance were used and $p < 0.05$ was considered statistically significant. Graphs are depicted by means +/- standard error of the mean (SEM).

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Figures

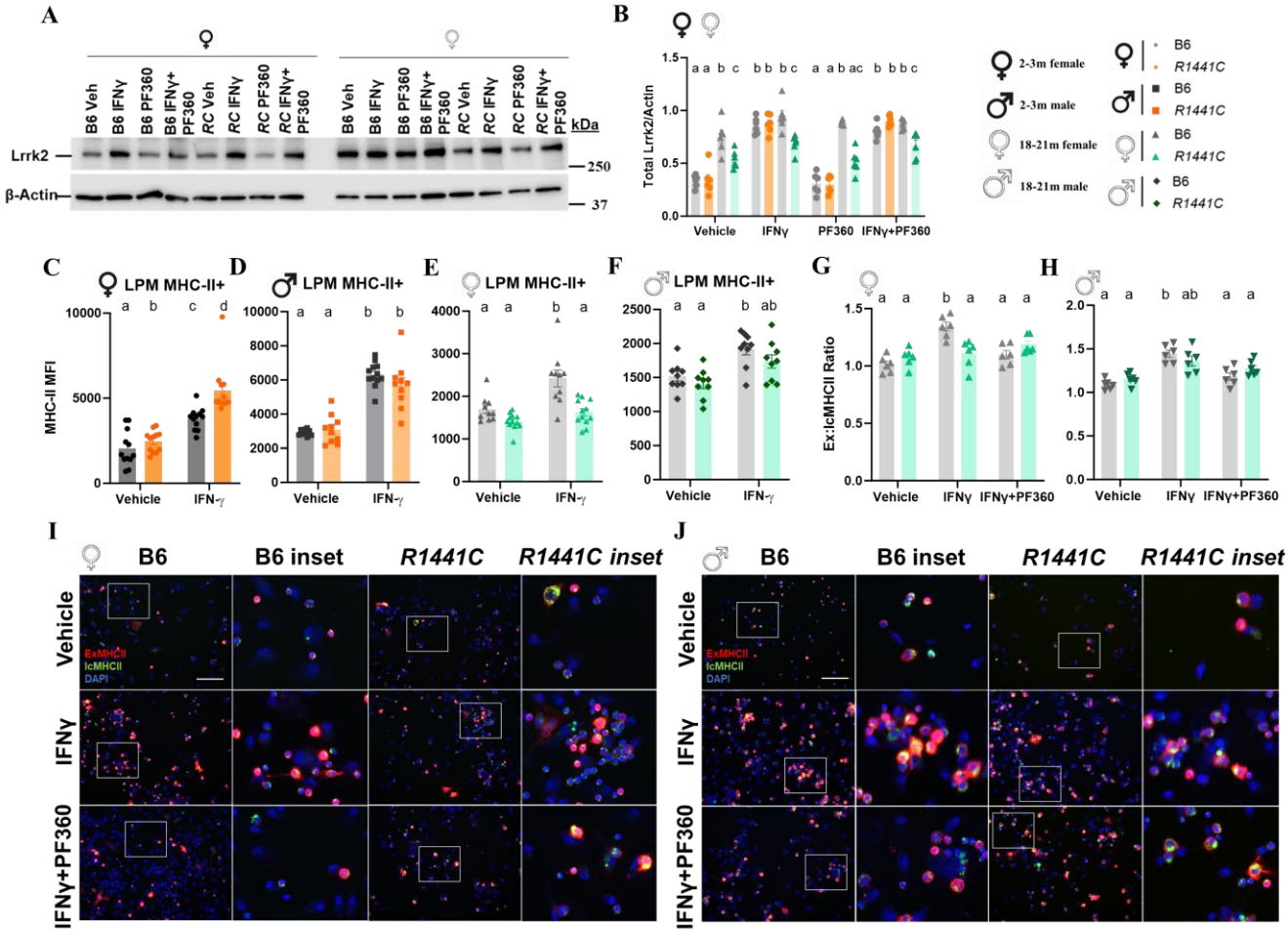

Figure 1. *R1441C* mutation leads to age dependent biphasic alteration in antigen presentation. pMacs from 2-3-month and 18-21-month-old female or male, B6 or *R1441C* mice were stimulated with 100U IFNγ for 18-hours +/- 100nM PF360. (A, B) Lrrk2 protein levels were assessed and normalized to β-Actin levels and quantified. Representative western blots shown. (C, D, E, F) Surface MHC-II median fluorescence intensity (MFI) was quantified on LPMs and via flow cytometry. (G-J) pMacs were stained for intracellular and extracellular MHC-II MFI and Ex:IcMHC-II ratio quantified. Scale bars, 100μM. Bars represent mean +/- SEM (N = 6-12). One/two-way ANOVA, Bonferroni *post hoc*, groups sharing the same letters are not significantly different ($p > 0.05$) whilst groups displaying different letters are significantly different ($p < 0.05$).

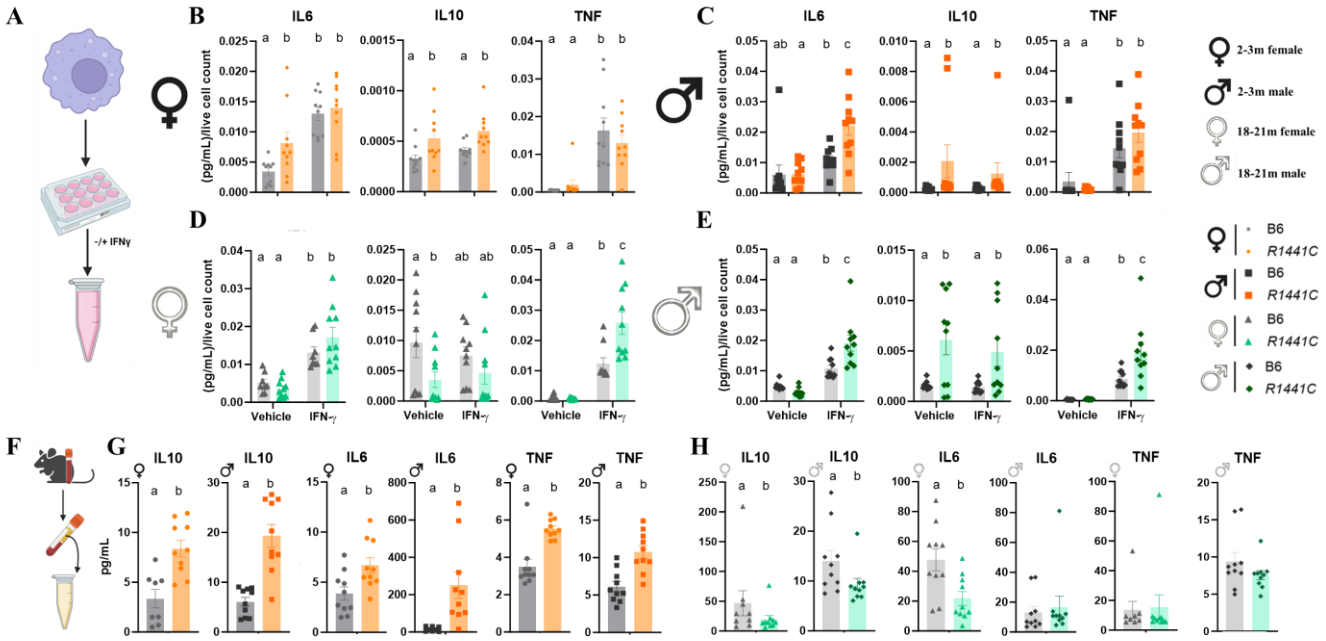

Figure 2. *R1441C* mutation leads to age dependent biphasic alteration in cytokine release ex vivo and circulating cytokines in vivo. (A) pMacs from 2-3-month and 18-21-month-old female or male, B6 or *R1441C* mice were stimulated with 100U IFNγ for 18-hours and media collected. (B, C, D, E) Cytokine levels of IL10, IL6 and TNF were quantified and normalized to live cell count. Bars represent mean +/- SEM (N = 8-10). Two-way ANOVA, Bonferroni *post hoc*. (F) Trunk blood was collected via trunk bleed and plasma was isolated. (G, H) Cytokine levels of IL10, IL6 and TNF were quantified. Bars represent mean +/- SEM (N = 8-10). Student's T-test. Groups sharing the same letters are not significantly different ($p > 0.05$) whilst groups displaying different letters are significantly different ($p < 0.05$).

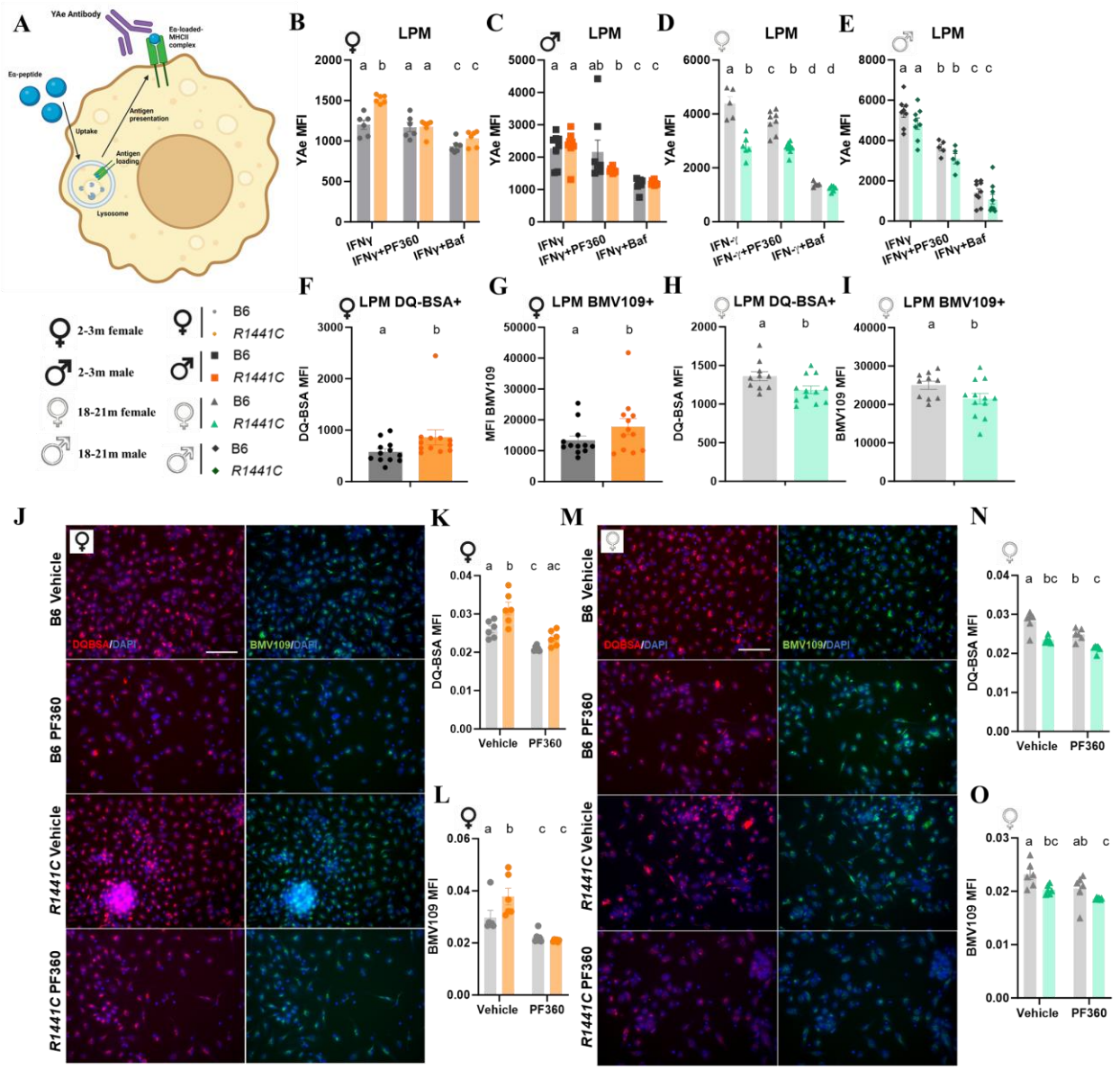

Figure 3. *R1441C* mutation leads to age dependent biphasic alteration in lysosomal activity. pMacs from 2-3-month and 18-21-month-old female or male, B6 or *R1441C* mice were stimulated with 100U IFNγ for 18-hours. (A) Schematic of YAe assay. (B-E) Cells were co-treated with 100nM PF360 or 50nM Bafilomycin A1 (baf) and YAe MFI was quantified on LPMs via flow-cytometry. (F-H) DQ-BSA and BMV109 MFI was quantified in LPMs via flow cytometry. (J-O) DQ-BSA and BMV109 MFI was quantified in pMacs via microscopy. Scale bars, 100µM. Bars represent mean +/- SEM (N = 6-12). Two -way ANOVA, Bonferroni *post hoc*, or Student's T-test. Groups sharing the same letters are not significantly different ($p > 0.05$) whilst groups displaying different letters are significantly different ($p < 0.05$).

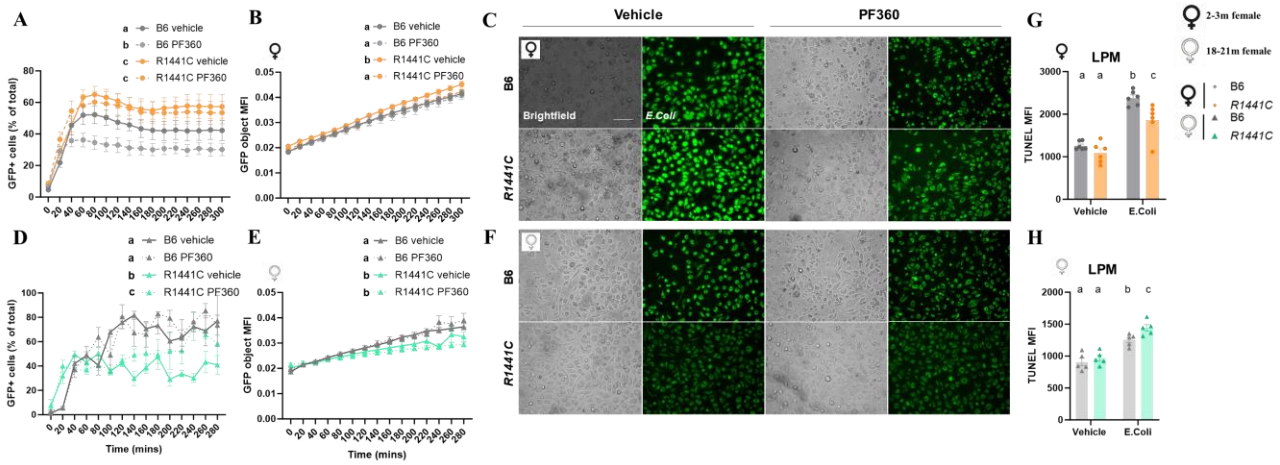

Figure 4. *R1441C* Lrrk2 mutation alters pathogen uptake and pathogen-mediated DNA damage in age- and sex-dependent manner. pMacs from 2-3-month and 18-21-month-old female, B6 or *R1441C* mice were incubated with 100µg of pHrodo *E. coli*. +/- 100nM PF360 (A-F) pMacs were imaged every 20 minutes for 5 hours, and GFP+ cells (% of total) and GFP MFI was measured. Scale bars, 100µM. Three-way ANOVA, Bonferroni *post hoc*. Main effects of groups are reported in each graph key. Groups sharing the same letters are not significantly different ($p > 0.05$) whilst groups displaying different letters are significantly different ($p < 0.05$). (G, H) TUNEL MFI was quantified in LPMs via flow cytometry. Bars represent mean +/- SEM (N = 6-12). One-way ANOVA, Bonferroni *post hoc*. Groups sharing the same letters are not significantly different ($p > 0.05$) whilst groups displaying different letters are significantly different ($p < 0.05$).

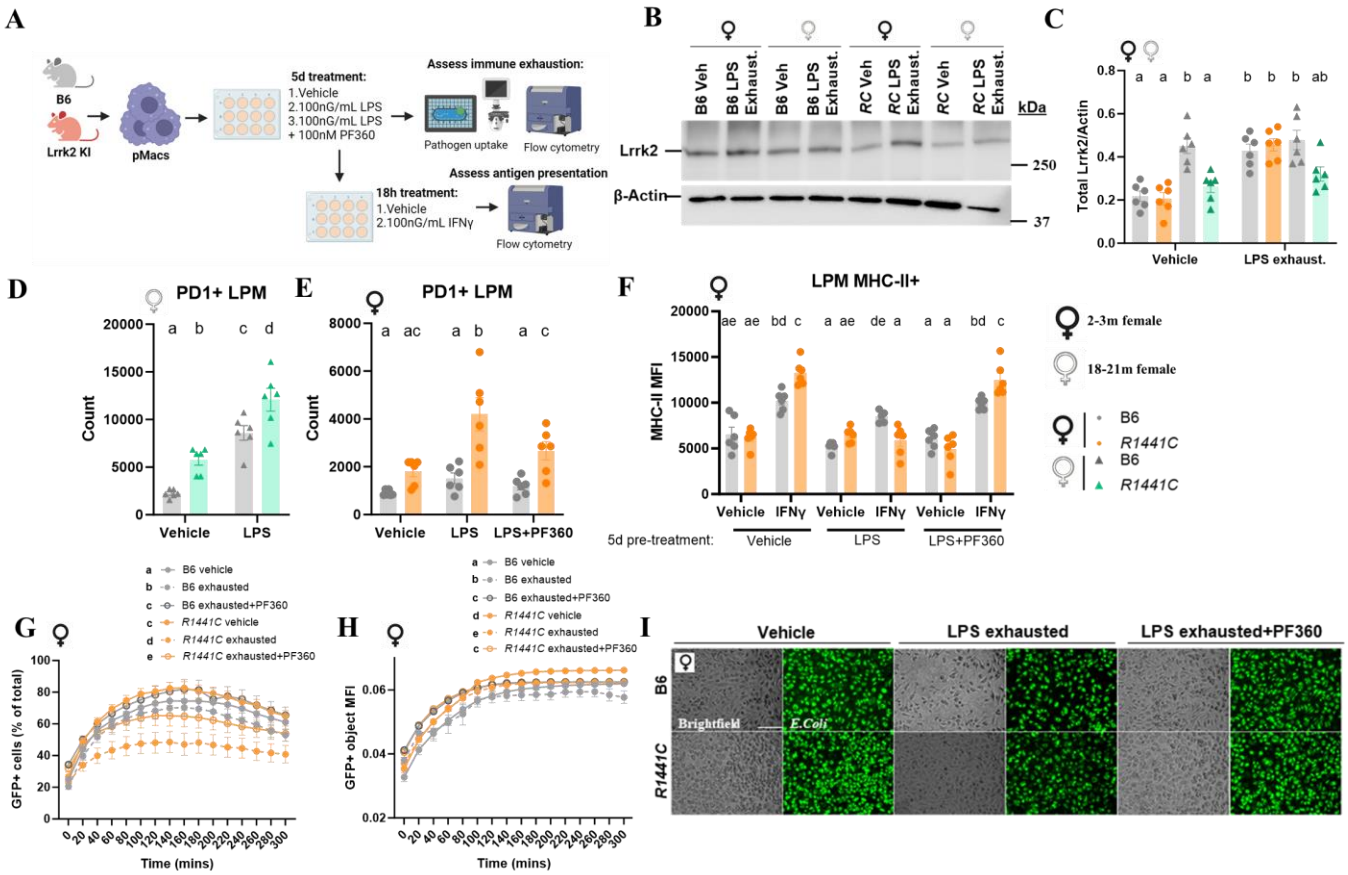

Figure 5. *R1441C* Lrrk2 mutation induces immune exhaustion in a kinase-dependent manner. pMacs from 2-3-month and 18-21-month-old female, B6 or *R1441C* mice were incubated with 100ng/mL of LPS for 5 days +/- 100nM PF360. (A) Schematic of study design. (B, C) Lrrk2 protein levels were assessed and normalized to β-Actin levels and quantified. Representative western blots shown. (D, E) PD1+ LPM count was quantified via flow cytometry. (F) After 5-day LPS or vehicle treatment, pMacs were stimulated with 100nG/mL of IFNγ for 18-hours and MHC-II MFI expression was quantified via flow cytometry. Bars represent mean +/- SEM (N = 6-12). Two -way ANOVA, Bonferroni *post hoc*. (G, H, I) After 5-day LPS or vehicle treatment, pMacs were incubated with 100µg of pHrodo *E. coli*. +/- 100nM PF360. pMacs were imaged every 20 minutes for 5 hours, and GFP+ cells (% of total) and GFP MFI was measured. Scale bars, 100µM. Three-way ANOVA, Bonferroni *post hoc*. Main effects of groups are reported in each graph key. Groups sharing the same letters are not significantly different ($p > 0.05$) whilst groups displaying different letters are significantly different ($p < 0.05$).

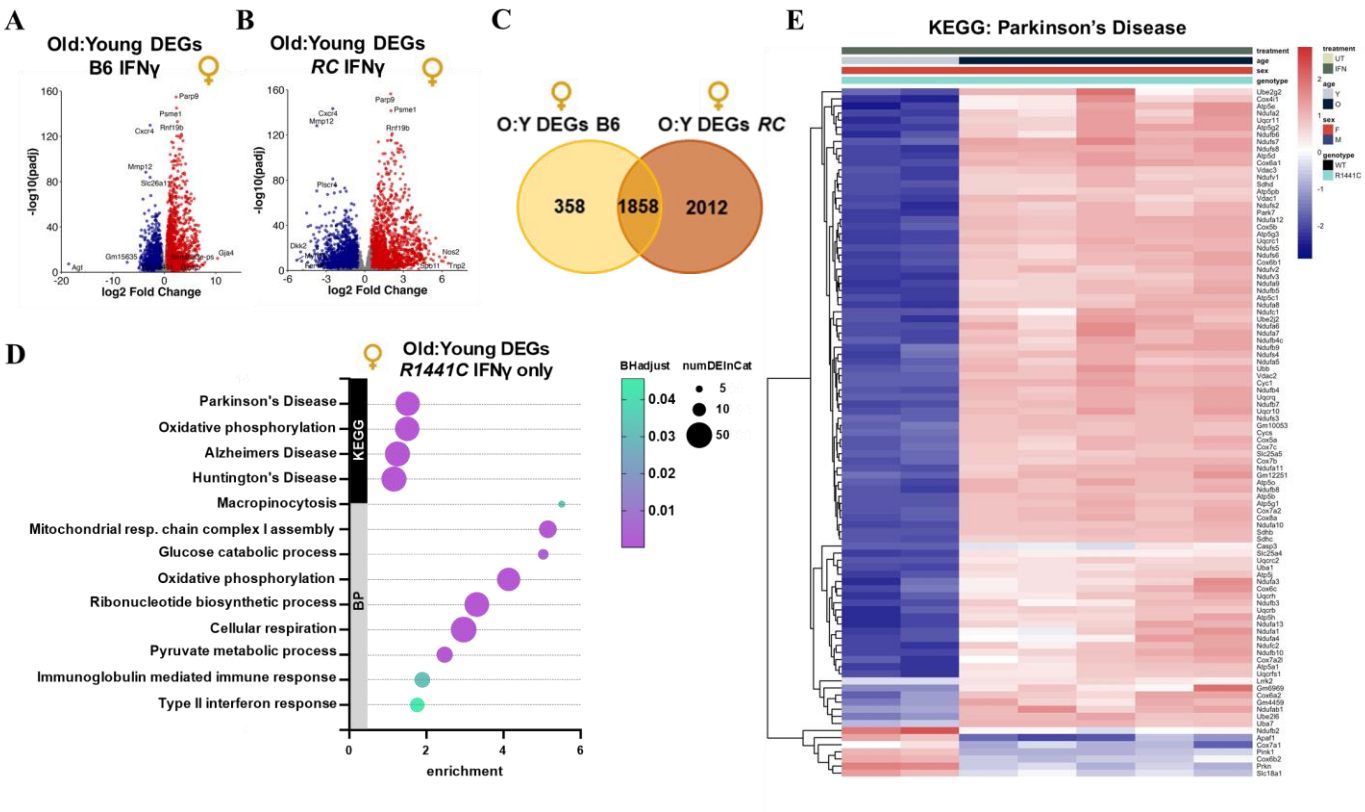

Figure 6. *R1441C* Lrrk2 mutation alters transcriptional regulation of mitochondrial and oxidative stress genes as a function of age in female macrophages. Transcriptomic analysis from B6 or *R1441C* vehicle and IFNγ-treated pMacs, harvested from 2-3-month or18-21-month-old. (A, B, C). Significant Old:Young DEGs in IFNγ-treated pMacs were counted and compared across B6 and *R1441C.* Volcano plots show genes with $\log_2$-transformed fold change > 0.5 and an adjusted p-value ≤ 0.05. (D) KEGG pathway and GO BP enrichment analysis identifies pathways in Old:Young DEGs seen only in *R1441C* samples. (F). Heat maps show KEGG: Parkinson's Disease pathway with significant Old:Young DEGs seen only in *R1441C* samples.

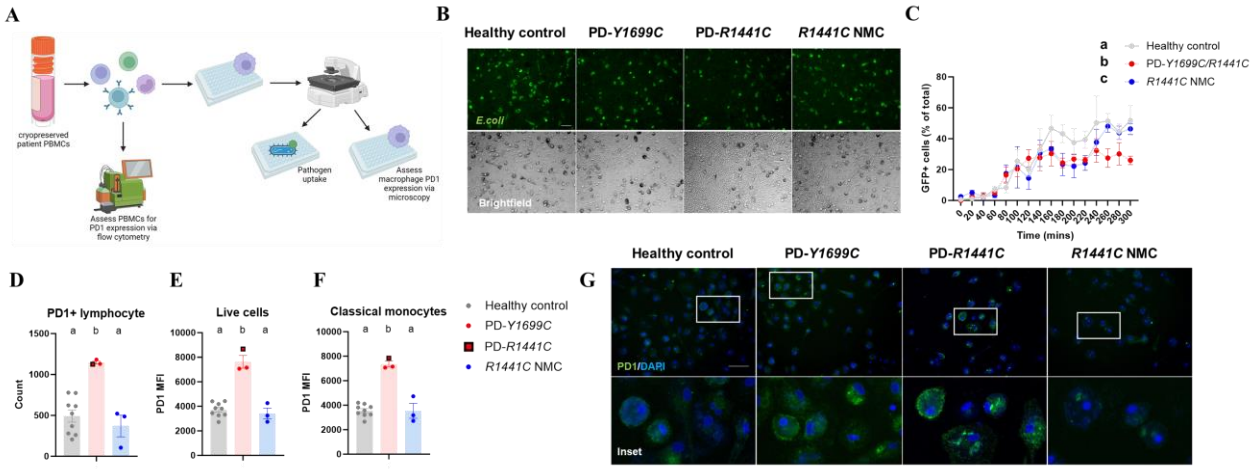

Figure 7. *R1441C* and *Y1699C-LRRK2* monocyte-derived patient macrophages exhibit hypophagocytosis and increased markers for exhaustion. (A) Schematic of study design. (B, C) Cryopreserved PBMCs were cryorecovered and plated in the presence of hM-CSF to differentiate myeloid cells to macrophages. Macrophages were incubated with 100µg of pHrodo *E. coli* and imaged every 20 minutes for 5 hours, and GFP+ cells (% of total) quantified. . Scale bars, 100µM. Three-way ANOVA, Bonferroni *post hoc*. Main effects of groups are reported in each graph key. Groups sharing the same letters are not significantly different ($p > 0.05$) whilst groups displaying different letters are significantly different ($p < 0.05$). (D, E, F) PD1+ cell count and PD1 expression was quantified in total PBMCs via flow cytometry. Bars represent mean +/- SEM (N = 6-12). One-way ANOVA, Bonferroni *post hoc*. Groups sharing the same letters are not significantly different ($p > 0.05$) whilst groups displaying different letters are significantly different ($p < 0.05$). (G) Monocyte-derived macrophages were stained for PD1 and microscopy images captured. Scale bars, 100µM.

List of Supplementary Materials

Materials and Methods

Supplementary figures:

Figure S1. Characterization of ex vivo pMac cultures.

Figure S2. *R1441C* mutation leads to age dependent biphasic alteration in antigen presentation.

Figure S3. *R1441C* mutation alters cytokine release from pMacs.

Figure S4. The *R1441C* Lrrk2 mutation causes alterations of monocyte population and microglia activation in aged female mice.

Figure S5. *R1441C* mutation leads to age dependent biphasic alteration in lysosomal activity.

Figure S6. *R1441C* Lrrk2 mutation alters pathogen uptake and pathogen-mediated DNA damage in age- and sex-dependent manner.

Figure S7. *R1441C* Lrrk2 mutation induces immune exhaustion in a kinase-dependent manner.

Figure S8. Transcriptomic profiling reveals alterations in critical effector function pathways in *R1441C* macrophages in age- and sex- dependent manner.

Figure S9. R1441C Lrrk2 mutation alters biological processes in macrophages in a sex- and age-dependent manner.

Figure S10. R1441C Lrrk2 mutation alters transcriptional changes associated with age in sex dependent manner.

Figure S11. *R1441C*- and *Y1699C*-LRRK2 monocyte derived macrophages exhibit hypophagocytosis, suppressed cytokine release and increased markers for exhaustion.

Supplementary tables:

Table S1. Flow cytometry marker antibody panel (mouse)

Table S2. Antibodies for immunoblotting

Table S3. Patient demographics

Table S4. Flow cytometry marker antibody panel (human)

References (*85-88*)