LRRK2 protects immune cells against erastin-induced ferroptosis

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\begin{abstract}
Ferroptosis is an iron-dependent regulated cell death pathway characterized by excessive lipid peroxidation. It is implicated in many neurodegenerative diseases, including Parkinson's Disease (PD). Mutations and increased leucine-rich repeat kinase 2 (LRRK2) kinase activity are associated with both familial and idiopathic PD pathologies. Increased iron deposition was observed in the substantia nigra of LRRK2 mutation-carrying PD patients compared to healthy individuals, suggesting a potential link between LRRK2 and ferroptosis. However, the role of LRRK2 in the immune cells is still not well-understood. This study aims to investigate the effect of LRRK2 on ferroptosis-induced cell death in immune cells.

We used LRRK2 parental (WT) and LRRK2 KO (KO) RAW 264.7 murine macrophages. Cells were challenged directly with the ferroptosis inducer, erastin, and the kinase activity was investigated using the LRRK2 kinase inhibitor, MLi2. Cell metabolism and viability analysis showed that WT cells were more resistant to ferroptosis than the KO cells. Lipid peroxidation and cellular reactive oxygen species (ROS) generation were significantly elevated in the KO cells. Furthermore, mitochondrial membrane potential and mitochondrial respiration were decreased in the KO cells after erastin treatment compared to the WT cells. Inhibition of the LRRK2 kinase function resulted in increased cell sensitivity to erastin. Cell and mitochondrial substrates utilization were altered in the KO and kinase inhibited WT cells compared to WT cells. These results indicate a protective role of LRRK2 against erastin-induced ferroptosis in RAW macrophages and point towards the importance of LRRK2 kinase function in the protective mechanism.
\end{abstract}

1. Introduction

Ferroptosis is an iron-dependent programmed cell death characterized by excessive lipid peroxidation. Morphologically, ferroptotic cells are recognized by necrosis-like features, including loss of plasma membrane integrity along with small dysmorphic mitochondria (Vanden Berghe et al., 2014). In particular, mitochondrial morphology and structure change during ferroptosis, involving condensation, increased membrane density, reduced or absent cristae, as well as rupture of the outer membrane (Dixon et al., 2012; Friedmann Angeli et al., 2014; Yagoda et al., 2007). Besides mitochondrial morphological alterations, iron accumulation and lipid peroxidation are two important hallmarks of ferroptosis. Iron is an essential trace element in the cell and its homeostasis is precisely controlled. Disregulation of iron content or distribution disrupts the cell function. Through the Fenton process, it directly produces excessive reactive oxygen species (ROS) which

\textit{Abbreviations:} ALOX, arachidonic lipoxygenase; AUC, Area under curve; DAMP, Damage-associated molecular pattern; DPR1, Dynamin-related protein 1; Fer-1, Ferrostatin-1; GBA1, Glucocerebrosidase1; GPX4, Glutathione peroxidase 4; GSH, Glutathione; GSSG, Oxidized glutathione; LRRK2, Leucine-rich repeat kinase 2; MMP, Mitochondrial membrane potential; MTT, Thiazolyl Blue Tetrazolium Bromide; OCR, Oxygen consumption rate; PD, Parkinson's Disease; PGC-1\textalpha, Peroxisome proliferator-activated receptor-gamma coactivator-1\textalpha; PHD, Phenylalanine dehydrogenase; PI, Propidium iodide; PUFAs, Polyunsaturated fatty acids; ROS, Reactive oxygen species; SNpc, Substantia nigra pars compacta; System Xc\textsuperscript{−}, Glutamate-cystine antiporter; TCA, Tricarboxylic acid; TMRE, Tetramethylrhodamine, Ethyl Ester, Perchlorate.

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increases oxidative damage and can increase the activity of arachidonate lipoxygenase (ALOX) and phenylalanine dehydrogenase (PHD), two enzymes responsible for lipid peroxidation (Dixon et al., 2012). Lipid peroxidation is a free radical-driven chain reaction that oxidizes lipids, resulting in cell damage (Ayala et al., 2014). The most prominent and important type is the peroxidation of polyunsaturated fatty acids (PUFAs) by ALOXs (Kagan et al., 2017; Wenzel et al., 2017). Furthermore, the release of damage-associated molecular pattern (DAMP) signals and lipid oxidation products activates inflammatory reactions linking the immune function to the ferroptosis process (Yuan et al., 2016; Doll et al., 2017; Yang et al., 2014).

Chemically, ferroptosis can be induced by several stimuli, including erastin. Erastin, the classical inducer of ferroptosis, inhibits system Xc (glutamate-cystine antipporter) that in turn lowers glutathione (GSH) levels. Reduction in GSH, the main antioxidant of the cell, causes the accumulation of oxidized lipid. Erastin also causes the degradation of glutathione peroxidase 4 (GPX4). GPX4 converts GSH into oxidized glutathione (GSSG) and reduces the cytotoxic lipid peroxidases to the corresponding alcohols. Thus, degradation of GPX4 contributes to lipid peroxide accumulation (Li et al., 2020). Ferroptosis inhibitors such as ferrostatin-1 (Fer-1), liproxstatin-1 and vitamin E exert their protective effects against cell death by inhibiting lipid peroxide formation (Li et al., 2020).

Ferroptosis is implicated in neurodegenerative diseases, including Parkinson’s Disease (PD) (Do Van et al., 2016; Reichert et al., 2020). PD is the second most common neurodegenerative disease affecting elderly population and its pathology is associated with selective degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Lees et al., 2009). Recent findings showed that several characteristics of ferroptosis were reported in PD patients. For example, iron accumulation in the SN was significantly increased in PD patients compared to the controls (Dexter et al., 1992; Sofic et al., 1991). Increased lipid peroxidation was found not only in the SN (Dexter et al., 1989; Yoritaka et al., 1996) but also in the cerebrospinal fluid (Selley, 1998) and plasma (Selley, 1998; Nikam et al., 2009; de Farias et al., 2016) of PD patients. In addition, downregulation of system Xc- (Vallerga et al., 2020) and reduction of GSH (Pearce et al., 1997) were reported in PD brain.

Mutations and increased activity of Leucine-Rich Repeat Kinase 2 (LRRK2) have been shown to be involved in PD pathology, in idiopathic and autosomal dominant PD (Kachergus et al., 2005). LRRK2 is a multidomain protein with two enzymatic functions; a kinase and a GTPase (Mata et al., 2006). The increased kinase activity of LRRK2 is a shared feature in both familial and idiopathic PD (Shu et al., 2016; Lis et al., 2018; Wang et al., 2022). Importantly, idiopathic PD patients and LRRK2 mutation carriers have common pathophysiology; thus investigating the role of LRRK2 in PD is crucial (Kluss et al., 2019). LRRK2 is highly expressed in peripheral immune cells (Gardet et al., 2010; Hakimi et al., 2011), indicating a potential role of LRRK2 in immune cells that contribute to neuroinflammation and PD pathogenesis (Ahmadi Raste-gar and Dzamko, 2020; Cabezudo et al., 2020). Furthermore, LRRK2 is associated with several immune disorders such as inflammatory bowel disease (Franke et al., 2010) and leprosy (Fava et al., 2016), emphasizing the role of LRRK2 in peripheral immunity.

The link between LRRK2 and ferroptosis originates from a study that compared iron deposition in the SN of LRRK2 mutation-carrying PD patients (symptomatic and asymptomatic), idiopathic PD patients and healthy controls (Pyatigorskaya et al., 2015). In this study, iron deposition was significantly increased in the mutation-carrying and idiopathic PD patients compared to healthy individuals. Moreover, symptomatic mutation-carrying patients had higher iron deposition compared to idiopathic patients, suggesting that iron deposition is differentially regulated in PD, depending on the affected genes (Pyatigorskaya et al., 2015). Recently, the role of LRRK2 in iron uptake and storage was studied in microglia derived from Lrrk2 G2019S transgenic mice (Mamais et al., 2021). In patient-derived microglia, iron transport through transferrin was altered by sequestration of Rab8a and transferrin receptor to the lysosomes leading to disturbance in iron homeostasis following inflammatory stimuli. In addition, iron deposition was reported in microglia from Lrrk2 transgenic mice compared to controls upon lipopolysaccharides (LPS)-induced inflammation (Mamais et al., 2021). These data suggest a possible link between LRRK2 and ferroptosis in the CNS immune microglial cells, however, the role of LRRK2 in ferroptosis in the peripheral immune cells, in macrophages, has not been elucidated yet.

In this study, we investigated the role of LRRK2 in the ferroptosis process using murine macrophage cell lines; LRRK2 parental RAW 264.7 (WT) cells and LRRK2 KO RAW 264.7 (KO) cells. We showed that upon ferroptosis induction via erastin, the KO cells were significantly more vulnerable to ferroptotic stimuli compared to the WT cells. The mechanism underlying the reduced cell viability in the KO cells involves an increase in lipid peroxidation and cellular ROS levels. We further assessed the effects of ferroptosis on mitochondrial function and showed that the integrity of the mitochondrial membrane and mitochondrial respiration in LRRK2 deficient cells were highly compromised upon erastin treatment. LRRK2 and its kinase functions were shown to regulate metabolic pathways in the cells under unstimulated conditions. In addition, we proved that the protective effects of LRRK2 are kinase-dependent, indicating a beneficial role of LRRK2 under physiological conditions.

2. Results

2.1. LRRK2 and its kinase function increase the resilience against erastin-induced cell death

To investigate the role of LRRK2 in ferroptosis, LRRK2 parental (WT) and KO RAW 264.7 were treated with different concentrations of erastin, a ferroptosis inducer, ranging from 1 to 7.5 μM. Bright-field microscopy pictures showed cell death in the KO cells at concentrations that the WT cells seem unaffected (Fig. S1a). MTT assay was used to assess the metabolic function of the cells that reflects the cell viability. Following erastin treatment, the KO cells displayed a significant reduction in the metabolic activity in a dose-dependent manner compared to the WT cells (Fig. S1b). Based on the MTT measurements, we continued with 5 μM erastin for the rest of the measurements.

Next, to assess the potential effect of erastin on cell morphology and viability, real-time impedance measurements were performed on cells treated with 5 μM erastin. The cell morphology of WT and KO cells did not show differences for the first 24 h. However, KO cells showed a lower normalized cell index than the WT cells following erastin treatment (Fig. 1A). The reduction in the cell index reflects cell rounding and shrinking that occurs before ferroptotic cell death (Pedrera et al., 2021). A significant decrease in the area under curve (AUC) in the KO cells following erastin treatment (5 μM) was observed, while erastin treatment did not affect the WT cells (Fig. 1B). To further confirm that the alterations of cell morphology are leading to cell death, we assessed the incorporation of propidium iodide (PI) into damaged nuclei by flow cytometry measurements. Using annexin V-PI double staining kit, we assessed the erastin-induced cell death in both WT and KO LRRK2 macrophages. Analysis of the annexin V-PI double staining revealed that the percentage of living cells was not altered under control conditions. However, the percentage of living cells in the KO cells following erastin challenge was significantly reduced compared to WT cells (Fig. 1C + D).

To further elucidate whether the reduction in cell viability is caused by erastin-induced ferroptosis and not due to a different cell death mechanism, ferrostatin-1, a commonly used ferroptosis inhibitor, was applied to erastin-challenged cells. Ferrostatin-1 cotreatment was able to revert the decrease in the MTT values (Fig. 1E), the reduction in normalized cell index (Fig. S2a), and prevent cell death mediated by erastin in the KO cells (Fig. S2b + c). These findings indicate that the effects observed following erastin challenge in the RAW macrophages are ferroptosis dependent.
To exclude the possibility that apoptosis is playing a role in the observed erastin-induced cell death, the percentage of annexin V positive cells was determined by flow cytometry (Fig. 1C) and (Fig. S3a). Annexin V detects the exposure of phosphatidylserine at the cell surface which is a characteristic for apoptosis (Fadok et al., 1998; Koopman et al., 1994). Our results showed that erastin challenge did not affect apoptosis in the WT and KO cells (Fig. S3a). The sensitivity of the KO cells to ferroptosis was further confirmed by RSL3 challenge that causes ferroptosis through direct inhibition of GPX4 (Chen et al., 2021). Following 5 h of 0.25 μM RSL3 challenge, the KO cells showed significant reduction in the metabolic activity compared to the WT cells. The reduction in the metabolic activity was reverted by the cotreatment with ferrostatin-1 (Fer-1) (Fig. S3a). The sensitivity of the KO cells to ferroptosis was further confirmed by RSL3 challenge that causes ferroptosis through direct inhibition of GPX4 (Chen et al., 2021). Following 5 h of 0.25 μM RSL3 challenge, the KO cells showed significant reduction in the metabolic activity compared to the WT cells. The reduction in the metabolic activity was reverted by the cotreatment with ferrostatin-1 (Fer-1) (Fig. S3a).
ferrostatin-1 (Fig. S4). These results are similar to the erastin challenge and confirm the sensitivity of the KO cells to ferroptosis cell death in particular.

To examine the role of the LRRK2 kinase function in the ferroptosis cell death process, we studied whether ferroptotic pathways are affected in the WT cells pretreated with MLi2, a well-known kinase inhibitor of LRRK2 (Fell et al., 2015). WT cells pretreated with MLi2 did not alter the cell morphology compared to those without MLi2 pretreatment, while in presence of erastin, the cell morphology and size were reduced (data not shown). MTT measurements showed that MLi2 pretreatment alone did not affect the metabolic activity of the WT cells. However, MLi2 pretreatment followed by erastin significantly reduced the metabolic activity of the WT cells (Fig. 1F). In accordance with these data, flow cytometry measurement with annexin V-PI showed no effect of MLi2 alone on the percentage of viable cells. However, MLi2 in the presence of erastin challenge significantly reduced the cell viability when compared to the WT cells without MLi2 (Fig. S5 a + b). Analysis of apoptosis using Annexin V revealed no effect of MLi2 on apoptosis following erastin challenge (Fig. S3b). These results point towards the importance of the kinase function of LRRK2 in regulating the ferroptosis process and that an impaired kinase activity might increase the cell vulnerability towards ferroptotic cell death. Overall, our results suggest a protective role of LRRK2 kinase activity against erastin-induced cell death.

2.2. LRRK2 protects against erastin-induced lipid peroxidation and ROS levels

Lipid peroxidation and ROS production are hallmarks of ferroptosis. Accumulation of lipid hydroperoxides due to glutathione depletion and inactivation of glutathione peroxidase increases cellular oxidative stress leading to cell death (Stockwell et al., 2017). We next tested whether the increased vulnerability of KO cells to ferroptotic stimuli is also reflected in the production of lipid peroxidation. To evaluate lipid peroxidation in the WT and KO cells after erastin application, measurements of the C11-Bodipy sensor by flow cytometry were performed. Since lipid peroxidation occurs prior to ferroptotic cell death (Pedrera et al., 2021), we evaluated lipid peroxidation after 5 h of erastin treatment. The analysis of the FACS measurements showed that erastin treatment in the KO cells induced a significant increase in lipid peroxidation compared to the WT cells (Fig. 2 A + B). The increased lipid peroxidation in the KO cells was completely inhibited by ferrostatin-1 cotreatment (Fig. S2d + e), confirming that ferroptosis is the source of increased lipid peroxidation. MLi2 in itself increased the amount of lipid peroxidation under basal conditions and following erastin treatment the levels of lipid

Fig. 2. LRRK2 protects against erastin-induced lipid peroxidation and cellular ROS levels. A. & B. Flow cytometry measurement of lipid peroxidation using C11-bodipy sensor after 5 h of 5 μM erastin treatment. A. Representative FACS sample analysis of the WT and KO cells with and without erastin treatment. The y-axis represents the red channel while the x-axis represents the green channel. B. Bar graph showing a significant increase in the lipid peroxidation in the KO cells compared to the WT cells after erastin treatment. C&D. Cellular ROS measurement by flow cytometry using DCFDA dye in the WT and KO cells after 18 h of erastin treatment. C. Representative FACS sample analysis of the WT (blue) and KO (red) cells after erastin treatment. The graph shows the shift in the DCF fluorescence in the KO cells to the right indicating higher ROS levels. D. Statistical analysis shows significantly higher mean DCF fluorescence in the KO cells compared to the WT cells after erastin treatment. Data are presented as mean ± SD, statistical significance was determined using one-way ANOVA test, each experiment was performed at least 3 times. p-values indicating statistically significant differences between the mean values are defined as follows: *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
peroxidation were higher in the WT cells pretreated with MLi2 compared to the non-treated WT cells (Fig. 3A+B). Together, these data indicate that LRRK2 could provide protection against erastin-induced lipid peroxidation.

Lipid peroxidation is highly associated with cellular ROS production. Measurement of ROS production following erastin challenge was performed using flow cytometry measurement of DCFDA. H$_2$DCFDA is a non-fluorescent dye, which upon cleavage of the acetate group by intracellular esterases, an oxidation-sensitive form of the dye is formed and retained inside the cells. Upon oxidation by cellular ROS, fluorescent product is formed which can be measured by flow cytometry (Jakubowski and Bartosz, 2000). The higher the fluorescence detected, the more ROS is produced. Similar to lipid peroxidation, ROS levels under control conditions were not altered in both WT and KO cells. Conversely, KO cells produced significantly higher ROS levels than the WT following erastin treatment (Fig. 2C-D). Pretreatment of the WT cells with MLi2 did not change the total ROS level, while after erastin application the ROS levels were significantly increased in MLi2 pretreated WT cells (Fig. 3C-D). In contrast, pretreatment of the KO cells with MLi2 does not change metabolic activity, cell viability and lipid peroxidation levels (Fig. S6), indicating that the MLi2 effects are due to the inhibition of LRRK2. MLi2 pretreatment in WT cells showed a small, yet significant increase in lipid peroxidation after 5 h. Meanwhile cellular ROS levels after 18 h were unaffected by MLi2. However due to the different duration of MLi-2 treatment in the two experiments we cannot exclude that MLi2 might cause an initial increase in ROS that is not detected after 18 h.

These data indicate increased sensitivity of the KO cells to erastin challenge and point towards the mechanism of LRRK2 protection against erastin-induced ferroptosis.

2.3. LRRK2 preserves mitochondrial integrity and respiration from ferroptotic damage

Changes in mitochondrial morphology and function have been linked to ferroptosis cell death (Gao et al., 2019; Wu et al., 2021; Krabbendam et al., 2020; Maher et al., 2018) and several studies showed that ferroptosis induction affects mitochondrial membrane potential (MMP) and mitochondrial respiration (Neitemeier et al., 2017). To address the effect of LRRK2 on mitochondrial membrane following erastin treatment, we measured MMP using TMRE staining. The MMP of the WT cells was not changed following erastin treatment at 5 μM, while the KO cells displayed a significant reduction in the MMP (Fig. 4A+B), indicating higher sensitivity of the KO cells to ferroptosis. Likewise, MLi2 in itself did not affect the MMP of the WT cells. However, following erastin challenge, a significant reduction in the MMP was observed in the MLi2-pretreated WT cells (Fig. 4C&D). Previous work from our group reported changes in mitochondrial respiration between WT and KO macrophages under basal conditions (Rosenbusch et al., 2021). To further investigate the effect of LRRK2 on mitochondrial bioenergetics following erastin challenge, measurements of the extracellular fluxes using Seahorse XF mitochondrial stress assay were performed. Oxygen consumption rate (OCR) were analyzed in both WT and KO macrophages following 18 h erastin challenge. The analysis of several mitochondrial parameters linked to mitochondrial respiration revealed that the KO cells displayed a significant reduction in the maximum...
uncoupled respiration, which was measured after uncoupling the respiratory chain with protonophore FCCP. The reduction was more pronounced when compared to WT cells following erastin treatment (Fig. 5A+B). In addition, the spare respiratory capacity was lower in erastin-treated KO cells compared to erastin-treated WT cells (Fig. 5B). Consistently, OCR measurement in the WT cells pretreated with MLi2 followed by erastin showed a reduction in maximal respiration and spare respiratory capacity (Fig. 5C+D). This reduction in both maximum uncoupled respiration and spare respiratory capacity in KO cells suggests the inability of the cells to cope with increased energy demand and reduced cell fitness. These LRRK2 effects on mitochondrial respiration further indicate the potential role of LRRK2 in preserving mitochondrial functions.

2.4. LRRK2 influences the energy production in RAW macrophages

Mitochondria are the main source of energy production in the cell via ATP generation through the tricarboxylic acid (TCA) cycle. Besides their crucial role in energy production, mitochondria regulate gene expression under both physiological and pathological conditions. Mitochondria adjust cellular processes in different ways, including the release of cytochrome C, ROS production, the release of mitochondrial DNA, activation of AMPK, and release of TCA cycle metabolites (Martínez-Reyes and Chandel, 2020). The substrates for the TCA cycle are provided through different metabolic pathways including glycolysis, amino acid metabolism, and fatty acid oxidation. Since we showed that LRRK2 preserves mitochondrial respiration following ferroptosis induction, we hypothesized that changes in main metabolic pathways are present between the WT, KO and LRRK2 kinase inhibited WT cells. In order to investigate the role of different mitochondrial substrates/intermediates in the metabolism of RAW macrophages, biolog mitoplate S1 was used to analyze 31 mitochondrial substrates/intermediates that are precoated into the plate wells. The assay quantifies the utilization of different substrates/intermediates by measuring the rates of electron flow through the electron transport chain from NADH and FADH$_2$-producing substrates. The electrons pass through complex I and II to the tetrazolium redox dye (MC) that acts as a final electron acceptor and turns purple upon reduction. Since each substrate has different transporters to enter the mitochondria and different hydrogenases to produce NADH and FADH$_2$, analysis of different metabolic pathways can be performed.

Our results showed that under basal conditions, TCA cycle substrates are the most utilized substrates in WT, and KO cells (Fig. S7). Comparing the fold regulation of different substrates in the WT and KO cells, the KO cells showed a generalized reduction in substrate utilization except for pyruvate (Fig. 6A). Whereas, kinase inhibited WT cells increased utilization of some substrates including cis-aconitic acid and pyruvic acid compared to WT cells. The utilization of the rest of the substrates in different pathways was reduced in cells where LRRK2 kinase activity was inhibited (Fig. 7A).

We focused our analysis on four main metabolic pathways, namely TCA cycle, glycolysis, amino acid metabolism and fatty acid oxidation (Table S1). Out of these four pathways, the KO cells showed reduced utilization of the glycolysis substrates (α-Glucose-6-phosphate, D,L-α-glycerol phosphate and lactic acid) and amino acid metabolism (L-
leucine) (Fig. 6B+C). Although utilization of other substrates was also impaired, the change was not significant (Figure S8). Recent studies revealed that higher glycolysis increased ferroptosis resistance (Krab-bendam et al., 2020; Ma et al., 2022; Zhang et al., 2022) which could explain increased ferroptosis resistance in the WT showing higher utilization of glycolytic substrates/intermediates. Reduced glycolysis and non-glycolytic acidification in LRRK2 KO macrophages under basal conditions was previously reported using Seahorse Extracellular Acidification Rate measurements (Weindel et al., 2020). In addition, human microglia of LRRK2 KO cells showed reduced glycolysis compared to WT after LPS and interferon-gamma stimulation (Panagiotakopoulou et al., 2020).

Reduction in L-leucine (amino acid metabolism) and palmitoyl-D,L-carnitine (fatty acid oxidation) utilization was also observed in cells with kinase activity inhibition (Fig. 7B+C). However, other investigated metabolic pathways were not altered (Fig. S9). L-leucine is a member of branched-chain amino acids and is converted into acetyl-CoA by transamination and oxidative decarboxylation. Acetyl-CoA then participates in energy production via the TCA cycle (Ye et al., 2020). Leucine treatment has been shown to increase the basal OCR (Rivera et al., 2020) and mitochondrial biogenesis via upregulating the gene expression of the master regulator of mitochondrial biogenesis, peroxisome
proliferator-activated receptor-gamma coactivator-1alpha (PGC-1α) (Ye et al., 2020).

The reduction in palmitoyl D,L carnitine utilization in cells with kinase activity inhibition supports the role of LRRK2 kinase activity in energy metabolism through fatty acid oxidation. Differences in mitochondrial substrates/intermediates utilization between different cells indicate an influence of LRRK2 and its kinase function on the regulation of different metabolic pathways in macrophages.

3. Discussion

In the current study, we revealed a critical protective role of LRRK2 against erastin-induced ferroptosis in RAW macrophages. The WT cells showed higher metabolic activity and cell viability after erastin challenge compared to the KO cells. In addition, the lipid peroxidation and ROS levels were elevated in the KO cells following erastin application. Besides, LRRK2 protected the mitochondrial function against erastin-induced ferroptosis illustrated by the significant increase in the MMP and OCR levels, namely maximal respiration and mitochondrial spare capacity, in the WT cells following erastin treatment. Our results showed that the kinase function of LRRK2 is crucial for the protective effect of LRRK2 against ferroptosis since upon the specific inhibition of the kinase function of LRRK2 using MLi2, the WT cells showed similar behavior as in the KO cells.

Our data showed that the presence and absence of LRRK2 did not change cell viability and ROS levels in RAW cells under non-stimulating conditions, while following erastin treatment both the KO and WT cells pretreated with MLi2 kinase inhibitor displayed lowered viability and increased levels of ROS in comparison to the WT cells. In agreement with our data, LRRK2 rescued cell viability against oxidative stress induction (Toyofuku et al., 2020; Liou et al., 2008; Pereira et al., 2014) in different cell models. A possible explanation for the protective effect of LRRK2 could be represented by ERK1/2 pathway activation that was confirmed in HEK293 and SH-SY5Y cells (Liou et al., 2008). The activation in ERK1/2 was found to be kinase-dependent since kinase domain deletion in WT LRRK2 increased the cell sensitivity to oxidative stress (Liou et al., 2008), supporting the importance of the kinase function of LRRK2 in the protection against stress. Another study supporting LRRK2 role in oxidative stress reported increased induction of the antioxidant enzyme SOD2 in LRRK2 WT microglia upon treatment with α-synuclein fibrils, while the opposite was found in LRRK2 KO microglia (Russo et al., 2019). Conversely, studies in neuronal cells demonstrated that LRRK2 KO cells had lower ROS compared to the WT cells (Bahashaw et al., 2013; Heo et al., 2010). These data point to the regulatory function of LRRK2 in cell survival and oxidative stress and indicate distinctive LRRK2 effects in neuronal versus non-neuronal cells.
An attractive explanation for the protective effect of LRRK2 in macrophages is through the regulation of endosomal sorting complex required for transport-III (ESCRT-III)-mediated membrane repair. In macrophages, lysosomal damage induction activates LRRK2. Activated LRRK2, in turn, phosphorylates and recruits Rab8A and CHMP4B (a component of ESCRT-III machinery) to the damaged membrane, favoring ESCRT-III repair. On the other hand, LRRK2 deficient macrophages could not recruit ESCRT-III components and damaged membranes are targeted for lysophagy (Herbst et al., 2020). The role of ESCRT-III in ferroptosis has been recently described (Pedrera et al., 2021; Marmolejo-Garza and Dolga, 2021). Ferroptosis induction caused a sustained increase in cytosolic calcium, which is important for ESCRT-III activation and recruitment of CHMP4B. Since LRRK2 deficiency reduces intracellular calcium and inhibits mitochondrial calcium efflux (Bedford et al., 2016; Nabar et al., 2022; Ludtmann et al., 2019), LRRK2 may activate ESCRT-III repair through modulation of calcium homeostasis.

Our data showed that LRRK2 selectively protects macrophages against ferroptosis cell death induced by erastin or RSL3 (Fig. 1 and S4) and that apoptosis does not contribute to ferroptosis death (Fig. S3). However, when cell death was induced using manganese, macrophages carrying LRRK2 showed enhanced apoptosis and higher ROS production compared to the KO cells and kinase-inhibited cells (Kim et al., 2019). These data point to a general regulatory function of LRRK2 in cell survival and oxidative stress.

The significant increase in lipid peroxidation in the erastin-challenged KO and kinase-inhibited WT cells implies that the protective effects of LRRK2 could be related to lipid metabolism. Previous reports have linked LRRK2 function to lipid metabolism/signaling pathways. Recent research using an untargeted lipidomic approach reported significant alteration in different lipid species in PD patients and LRRK2 mutation carriers compared to matched controls. Besides, alteration in glycerophospholipid and sphingolipid metabolism and mitochondrial function were the most affected pathways in PD patients (Galper et al., 2022). In a recent study on HepG2 cells, it was found that overexpression of LRRK2 promotes β-oxidation through carnitine palmitoyl transferase 1A. However, in LRRK2 KO cells, β-oxidation was suppressed (Lin et al., 2020). Elevation of lipid metabolism in the LRRK2 expressing cells through β-oxidation lessened the inflammation generated by palmitic acid, whereas knocking out LRRK2 inhibited β-oxidation leading to increased inflammation (Lin et al., 2020).

Effects of pathogenic mutations of LRRK2 on mitochondrial function were widely studied (Rosenbusch et al., 2021; Singh et al., 2019), while the influence of LRRK2 on mitochondrial function under ferroptotic conditions has not been studied before. Our study demonstrates that LRRK2 and its kinase function protect the mitochondrial membrane and mitochondrial respiration against ferroptotic damage. These results are
in line with another study showing that LRRK2 KO macrophages had reduced MMP compared to the WT cells in the presence of depolarizing agents as well as under baseline conditions. The reduced MMP in LRRK2 KO cells was due to hyperphosphorylation of dynamin-related protein 1 (DPR1), leading to increased mitochondrial fission and oxidative stress (Weindel et al., 2020). In the same study, maximal OCR and mitochondrial spare capacity were reduced in LRRK2 KO cells compared to WT. The reduction in maximal respiration and ATP production was also detected in LRRK2 \textit{KO} and LRRK2 G2019S MEFs compared to WT and kinase-dead MEF cells (Toyofuku et al., 2020). This finding illustrates that mitochondrial respiration is regulated by LRRK2 in a kinase-dependent manner.

In conclusion, our results suggest a protective role of LRRK2 against erastin-induced ferroptotic cell death in RAW macrophages. In addition, these findings emphasize the regulatory function of LRRK2 in mitochondrial pathways and cellular energy metabolism. Although increased kinase function of LRRK2 contributed to PD pathogenesis (Oun et al., 2022), our study highlighted the importance of the kinase function of LRRK2 in the protection against ferroptotic damage in peripheral immune cells. Our results provide more insights into the complex functions of LRRK2 and help define possible molecular pathways by which LRRK2 exerts beneficial effects.

4. Materials and methods

4.1. Cell culture

LRRK2 parental RAW 264.7 (ATCC® SC-6003™) and LRRK2 KO RAW 264.7 (ATCC® SC-6004™) were obtained from ATCC. LRRK2 KO RAW 264.7 have been modified by zinc finger nuclease gene editing to knock out LRRK2 expression. The cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (ATCC® 30–2002™), supplemented with 10% Fetal Bovine Serum (ATCC® 30–2020™), 1% penicillin-streptomycin (5,000 U/mL) (Gibco™, 15070063). The cells were regularly checked for mycoplasma using MycoAlertTM PLUS Mycoplasma Detection Kit (Lonza, LT07–710). Cells used in this study were between passage 3–15.

4.2. Cell treatment

LRRK2 parental and KO RAW 264.7 cells were treated with erastin 5 μM (Tocris, 5449) for 16–18 h (unless otherwise stated). Cotreatment with ferrostatin-1 (2 μM) (Sigma, SML0583) was used to inhibit erastin-induced ferroptosis. To check the effect of the kinase function of LRRK2, ML2 (Tocris, 5756) 1 μM was used by pretreating the cells for 90 min before erastin treatment. Mli2 was maintained in the medium during the treatment time.

4.3. Metabolic activity measurement

The metabolic activity of LRRK2 parental and KO RAW 264.7 cells was examined using Thiazolyl Blue Tetrazolium Bromide (MTT) (Sigma, M-2128). 20,000 cells/well were seeded for 24 h in a 96-well plate before the treatment with erastin. After treatment, the cells were incubated with 0.5 mg/mL MTT for 1 h at 37 °C. Then the medium containing MTT was removed and the plate was stored at −80 °C for at least 1 h. The insoluble formazan product was solubilized with dimethyl sulfoxide and measured using Synergy™H1 (Bad Friedrichshall, Germany) Hybrid Multi-Mode Reader at 570 nm and 630 nm as a reference filter. The metabolic activity of the cells treated with vehicle (control condition) was considered as 100% metabolic activity and the rest of the treatments were normalized to control solvent-treated cells.

4.4. Real-time cell impedance measurements

Morphological changes in LRRK2 parental and KO RAW 264.7 cells were measured using a label-free, real-time cell impedance-based system; xCELLigence® RTCA MP system (ACEA BIO) (Diemert et al., 2012). 20,000 cells/well were seeded in a 96-well E-plate (Agilent, S232368001) containing gold microelectrodes fused to the bottom surface of the well plate. The impedance of the electron flow caused by cell attachment to the well (cell index) was measured every 30 min. The cells were seeded one day before treatment and the measurement of the cell index was performed 20–24 h following erastin treatment. The cell index was normalized to 1, before the addition of various compounds.

4.5. Flow cytometry measurement

Parameters related to ferroptosis cell death were measured using flow cytometry via the CytoFLEXS benchtop flow cytometer (Beckman Coulter Life Sciences). LRRK2 parental and KO RAW 264.7 cells were seeded in a 24-well plate at a density of 80,000 cells/well one day before erastin challenge. After 16–18 h of erastin incubation, the cells were collected by trypsinization and incubated with various dyes, as described in the following sections and measured by flow cytometry. We used flow cytometry to analyze how LRRK2 affects ferroptotic pathways related to cell death, lipid peroxidation, ROS levels and mitochondrial membrane potential. At least three independent experiments were performed with three technical replicates per condition. 30,000 events were counted per each technical replicate and data were analyzed with flowJo-V10 software.

4.5.1. Cell death

Cell death was measured using the dead cell apoptosis kit with Annexin V FITC and propidium iodide (PI) (Invitrogen™, V13242) according to the manufacturer’s protocol. After 18 h of erastin treatment and cell collection, the cells were incubated in 100μL of the staining solution containing 1 μL of Annexin V FITC and 1 μL of 100 μg/mL PI in the binding buffer for 30 min at room temperature. The fluorescence emission was measured at 530 nm and 575 nm using 488 nm excitation.

4.5.2. Lipid peroxidation

Lipid peroxidation was detected using BODIPY™ 581/591 C11 (Invitrogen™, D3861). The dye (2 μM) was added to the cell for 30 min following erastin treatment. We measured lipid peroxidation at various time points following erastin application and we choose 5 h as optimal for this type of measurement. Lipid peroxidation results in a shift of the fluorescence emission peak from 590 nm to 510 nm, detected using FITC and PE filters.

4.5.3. Reactive oxygen species (ROS) levels

ROS levels were determined via the cell-permeant 2’,7’-dichlorodihydrofluorescein diacetate H2DCFDA (Invitrogen™, D399). Following 18 h of erastin treatment, the cells were incubated with 2.5 μM of the dye in serum-free DMEM for 30 min. Fluorescence was detected using FITC filter.

4.5.4. Mitochondrial membrane potential

Changes in the mitochondrial membrane potential were detected using Tetravethylrhodamine, Ethyl Ester, Perchlorate (TMRM) (Invitrogen™, T669). 200 nM of the dye in PBS was added to the collected cells for 20 min. PE filter was used to detect the fluorescence signal.

4.6. Mitochondrial respiration measurement

Measurement of oxygen consumption rate (OCR) was performed using XFe extracellular flux analyzer (Seahorse Bioscience, Billerica, MA) and mitochondrial stress test kit (Richter et al., 2015). 20,000 cells/well of LRRK2 parental and KO RAW 264.7 cells were seeded in XFe96 cell culture microplate one day before cell treatment. 18 h following addition of various compounds, the medium was replaced by Seahorse XF base medium (Agilent, 102353–100) supplemented with 10 mM oxydase.
glucose, 1 mM Sodium pyruvate and 2 mM glutamine and incubated for 1 h in CO₂-free incubator at 37 °C. The following substances were injected in order; port A: Oligomycin (2.5 μM), port B: FCCP (0.5 μM) and port C: rotenone (0.5 μM) and antimycin A (0.5 μM). Three baseline OCR measurements (3 min mix, 0 min delay, 3 min measure = 3/0/3) were recorded, followed by assessment of mitochondrial metabolism by injection of oligomycin (3/0/3), FCCP (3/0/3), and a combination of rotenone and antimycin A (3/0/3). The following parameters were deduced: basal respiration (OCR values, used to provide ATP under baseline conditions), ATP-linked respiration (following oligomycin injection, a reduction in OCR values represents the part of basal respiration used to produce ATP), maximal respiration (the maximal OCR values following FCCP injection, representing the physiological energy demand), and spare respiratory capacity (the difference between the maximal and basal respiration, representing the cell fitness to high energy demand). The OCR values were normalized to the protein content/ well using Pierce™ BCA Protein Assay Kit (Thermo Scientific™, 23225). At least 3 independent experiments were performed. Two-way ANOVA test was used to determine statistical significance for different mitochondrial parameters.

4.7. Mitochondrial substrate utilization measurement

Biolog mitoplate-S1 (Biolog Inc., BLG.14105) was used to measure the rates of consumption of various mitochondrial and cytoplasmic substrates in LRRK2 parental and KO RAW 264.7 cells and LRRK2 parental cells pretreated with 1 μM ML2 for 90 min according to the manufacturer’s protocol. Briefly, the mitoplate-S1 was hydrated with 30 μL/ well of permeabilizing buffer (Biolog MAS (BLG.72303), supplemented with 75 μg/mL Saponin (Sigma-Aldrich, SAE0073) and Biolog Redox Dye Mix MC (BLG.74353)) for 1 h at 37 °C. Afterward, the cells were seeded to the plate (60,000 cells in 30 μL Biolog MAS/ well) and the absorbance was measured at 590 nm every 5 min for a period of 4 h at 37 °C using BioTek Synergy H1 hybrid plate reader. Mitochondrial substrate utilization in different metabolic pathways was analyzed by comparing with the LRRK2 parental RAW cells, indicated as control.

4.8. Statistics

Statistical analysis was performed using GraphPad prism. One-way ANOVA with Tukey’s post-test was used to determine significance between different groups using a single parameter, while two-way ANOVA with Bonferroni’s post-test was performed to determine the significance between two groups with multiple parameters/substrates. t-test was used to compare between 2 groups. p-values indicating statistically significant differences between the mean values are defined as follows: ns for not significant, *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2022.105917.

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