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Parkin Impairs Antiviral Immunity by Suppressing the Mitochondrial Reactive Oxygen Species-Nlrp3 Axis and Antiviral Inflammation

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HIGHLIGHTS
Loss of Parkin enhances viral clearance but does not affect type I IFN production
Parkin deletion promotes antiviral inflammation in vivo
Parkin deficiency enhances antiviral inflammation via the mtROS-NLRP3 axis
The expression of Parkin is downregulated following viral infection
Parkin Impairs Antiviral Immunity by Suppressing the Mitochondrial Reactive Oxygen Species-Nlrp3 Axis and Antiviral Inflammation

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SUMMARY

Although mitochondria are known to be involved in host defense against viral infection, the physiological role of mitophagy, a crucial mechanism for maintaining mitochondrial homeostasis, in antiviral immunity remains poorly defined. Here, we show that Parkin, a central player in mitophagy, has a vital function in regulating host antiviral responses. Parkin-deficiency knockouts mice exhibit improved viral clearance and survival after viral infection. However, Parkin deficiency does not affect antiviral signaling and interferon production. Instead, Parkin deficiency augments innate antiviral inflammation by enhancing mitochondrial ROS (mtROS)-mediated NLRP3 inflammasome activation and promoting viral clearance. Loss of NLRP3 can reverse the enhanced antiviral responses in Parkin knockout mice. Furthermore, we find that Parkin expression is downregulated in peripheral blood mononuclear cells of patients infected with virus. Collectively, our results suggest that Parkin plays an important role in antiviral immunity by controlling mtROS-NLRP3 axis-mediated inflammation. These findings provide physiological insight of the importance of mitophagy in regulating host antiviral response.

INTRODUCTION

The innate immune response plays an essential role in defense against viral infection by the induction of antiviral cytokines and inflammation (Takeuchi and Akira, 2010). Host recognition of viral infection is mediated by different pattern-recognition receptors (PRRs), including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and DNA sensors (Sun et al., 2010). Upon viral recognition, the PRRs trigger downstream signaling to activate transcription factors like nuclear factor (NF)-κB and IRFs and induce various inflammatory cytokines (Roers et al., 2016).

Mitochondria are the central hubs of cellular metabolism and have multifunctional roles in bioenergetic production, apoptosis, signal transduction, and the production of reactive oxygen species (ROS) (Chandel, 2014; Wallace and Fan, 2010). Mitochondria produce cellular ATP and metabolites, which are important in antiviral immune responses (Mills et al., 2017). Recent studies have shown that mitochondria participate in innate immune signaling to regulate antiviral responses (Weinberg et al., 2015; West et al., 2011). Mitochondria serve as a signaling platform in antiviral innate immunity. The mitochondrial membrane protein mitochondrial antiviral signaling protein (MAVS) is the central adaptor in RLR signaling and plays an important role in defense against RNA viruses (Kawai et al., 2005; Seth et al., 2005; Xu et al., 2005). NLRX1, another outer mitochondrial membrane (OMM) protein, functions as a negative regulator of inflammation by disrupting the MAVS and TRAF6-mediated pathways in response to viral infection (Allen et al., 2011; Moore et al., 2008). In addition, some published reports suggest that mitochondrial ROS (mtROS) can directly modulate antiviral signaling pathway, although its role remains controversial (Agod et al., 2017; Jin et al., 2010; Tal et al., 2009). Moreover, mtROS acts as an upstream signal to promote activation of the NLRP3 inflammasome (Zhong et al., 2018; Zhou et al., 2011). The NLRP3 inflammasome has been reported to drive antiviral inflammation and enhance the host immune response to viral infection (Allen et al., 2009; Ichinohe et al., 2009; Thomas et al., 2009; Wang et al., 2017).

Virus-induced mtROS is associated with mitochondrial dysfunction. Mitochondrial homeostasis is mediated by mitophagy, which decreases mtROS production by removing impaired mitochondria (Kubli and Gustafsson, 2012; Tal et al., 2009). Parkin, a cytosolic E3 ubiquitin ligase protein linked with Parkinson disease (PD), mediates mitophagy in conjunction with the ubiquitin kinase PINK1 that, like Parkin, is also a

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A

Survival %

WT
Park2^{−/−}

VSV 0 2 4 6 8 10 days

100%

50% **

B

Survival %

WT
Park2^{−/−}

HSV-1 2 4 6 8 10 days

100%

50% *

C

mtβ-mRNA expression fold

WT Park2^{−/−}

HSV-1 2 4 6 8 10 days

100%

50% NS

D

IFNβ-mRNA expression fold

WT Park2^{−/−}

HSV-1 2 4 6 8 10 days

100%

50% NS

E

Nrf2-mRNA expression fold

WT Park2^{−/−}

HSV-1 2 4 6 8 10 days

100%

50% NS

F

Ddx48-mRNA expression fold

WT Park2^{−/−}

HSV-1 2 4 6 8 10 days

100%

50% NS

G

Western Blot Analysis

VSV 0 3 6 9 h (KD)

p-TBK1
TBK1
p-IRF3
IRF3
cGAS
STING
Park2
β-actin

HSV-1 0 3 6 9 h (KD)

p-TBK1
TBK1
p-IRF3
IRF3
cGAS
STING
Park2
β-actin
Kin functions as a negative regulator of MAVS antiviral signaling (Khan et al., 2016). Next, we intravenously infected mice with herpes simplex virus 1 (HSV-1, a DNA virus) to investigate if Parkin has a role in the clearance of viral infection while also describing the regulatory role of Parkin-dependent mitophagy in antiviral immunity.

We now provide insight into the physiological function of Parkin in the host defense against viral infection while also describing the regulatory role of Parkin-dependent mitophagy in antiviral immunity.

RESULTS

Loss of Parkin Enhances Viral Clearance and Mice Survival but Does Not Affect Type I IFN Production

To study the physiological role of Parkin in viral infection, we utilized Parkin gene deletion mice (Park2−/−) for viral infection studies. First, we intranasally infected mice with vesicular stomatitis virus (VSV), a single-stranded RNA virus that activates RIG-I-MAVS antiviral signaling. We found that mice lacking Parkin exhibited significantly increased survival through 9 days post infection (dpi) and had lower viral loads in lung than wild-type (WT) mice after infection (Figure 1A), which is consistent with a previous report that Parkin functions as a negative regulator of MAVS antiviral signaling (Khan et al., 2016). Next, we intravenously infected mice with herpes simplex virus 1 (HSV-1, a DNA virus) to investigate if Parkin has a role in the response to DNA virus infection. We found that Parkin deficiency in mice also enhanced mice survival and reduced viral loads after viral infection compared with WT mice (Figure 1B). Given HSV-1 infection-induced innate antiviral response is independent of MAVS signaling (Paludan et al., 2011), these data suggest that Parkin-mediated effects on viral clearance and survival are not mediated via MAVS antiviral signaling.
suggest that Parkin can play a negative regulatory role in host defense against both RNA and DNA viruses, and we next addressed the mechanisms underlying these regulatory roles.

Type-1 IFNs play crucial roles in innate antiviral immunity by inducing the expression of hundreds of antiviral genes (Takeuchi and Akira, 2010). Because Park2−/− mice exhibit a significant increase in viral clearance in vivo, we next sought to assess the effects of Parkin deficiency on IFN-β production. Unexpectedly, no significant differences in IFN-β production from bronchoalveolar lavage fluid (BALF), sera, and lung homogenates were detected between WT and Park2−/− mice at early time points, such as 12, 24, and 72 h after VSV or HSV-1 infection (Figures S1A, 1B, and S1A), which is in contrast with the previously reported role of Parkin as a negative modulator of IFN production in cells (Xin et al., 2018). To further investigate any direct effect of Parkin on IFN-β production, we examined IFN expression in ex vivo Park2−/− mouse embryonic fibroblasts (MEFs) challenged with VSV and HSV-1. Parkin-deficient MEFs exhibited similar expression levels of Ifn transcripts and IFN-β protein as WT MEFs in response to infection with VSV or HSV-1 (Figures 1C and 1D). Previous study showed that Park2−/− mice following exhaustive exercise exhibit increased mitochondrial DNA (mtDNA) release from damaged mitochondria into cytosol, leading to activation of cGAS-STING-mediated IFN signaling (Sliter et al., 2018). We thus next investigate the abundance of mtDNA between WT and Park2−/− MEFs. The mtDNA copy number was enhanced after viral infection, whereas the amount of mtDNA and expression of IFN-stimulated genes was not affected by Parkin deficiency (Figures 1E and 1F). Moreover, we evaluated early antiviral innate signaling after viral stimulation and observed no obvious differences in virus-induced activation of TBK1 or IRF3 and the protein levels of cGAS and STING between WT and Park2−/− MEFs (Figure 1G). In addition, flow cytometry analysis showed that WT and Park2−/− cells had similar susceptibility to VSV-GFP and HSV-1-GFP viral infection (Figure S1B) at various time points, which further suggests that Parkin does not affect the susceptibility of cells to initial viral infection or their ability to respond to viral infection by production of type I IFN. To further investigate any effect of Parkin on IFN-β production in response to non-replicative IFN inducer such as RLR ligands, we examined it by using poly(I:C) transfection and found that Parkin deficiency does not affect IFN production in response to this MAVS-dependent PAMP (Figures S2C and SBD). To address potential cell type differences, we also assessed the effect of Parkin deficiency in bone marrow-derived dendritic cells (BMDCs) and bone marrow-derived macrophage cells (BMDMs). Like MEFs, there were no differences in virus-induced IFN production and IRF3-dependent signaling between WT and Park2−/− BMDCs or BMDMs (Figures S2A–S2E). Collectively, these results clearly indicate that Parkin has no direct influence on innate antiviral signaling that triggers type I IFN production.

**Parkin Deletion Promotes Antiviral Inflammation In Vivo**

During viral infection, the initial inflammatory response can contribute to viral clearance by recruiting and activating immune cells to combat viral invasion (Chen et al., 2011; Rouse and Sehrawat, 2010). Thus, we next assessed if Parkin can affect antiviral inflammation in an in vivo setting. Histological analysis revealed that lungs in Park2−/− mice contained more severe inflammation characterized by more infiltration of immune cells than lungs from WT mice after the early stage of VSV infection (at 3 dpi) (Figure 2A). This was further verified by immunohistochemical detection of increased infiltration of CD11b+ myeloid cells in lungs from Park2−/− mice (Figure 2B). Moreover, fluorescence-activated cell sorting (FACS) analysis showed that Park2−/− mice had markedly increased percentages and numbers of CD11b+ cells in lungs on day 3 after VSV infection compared with WT mice (Figure 2C). In relation to CD11b+ subsets of cells, immunofluorescence microscopy showed that CD11b+ cells were increased in Park2−/− mice. In addition, there were increases in the numbers of monocyte-macrophage DCs (dendritic cells) (CD11b+/CD11c+/MHC II+), NK cells (NK1.1+), T (Tcrβ+), and B (B220+) lymphatic cells in lungs of...
expression of IL-6 in cell infection studies, it is interesting to note from our earlier data that kines by Parkin applies to murine and human cells. Although Parkin deficiency does not affect viral induced antiviral inflammatory response, which contributes to enhanced viral clearance.

Thus, these results confirm that the selective targeting in the production of inflammasome-related cytokines, such as IL-6 and IL-12 between WT and Park2−/− mice show augmented lung levels of IL-6 in response to viral infection. Such differences in cell- and animal-based infection approaches are reconciled by previous studies that have shown inflammasome-related cytokines, such as IL-1β and IL-18, in response to VSV or HSV-1 infection, whereas there were no differences in the virus-induced production of other cytokines such as IL-6 and IL-12 during VSV infection (Varol et al., 2015), we next investigated the effect of Parkin on the production of virus-induced inflammatory cytokines in BMDCs. VSV and HSV-1 induced expression of mRNA transcripts of cytokines, such as Il1b, Il18, Il6, and Il12, were largely comparable between WT and Park2−/− cells after viral infection (Figure 4A). In addition, early signaling pathways, such as activation of NF-κB and p38 mitogen-associated protein kinase, that mediate virus-induced transcription of these genes, were unaffected in Parkin-deficient cells as evidenced by intact VSV and HSV-1 induced phosphorylation of IκBα and p38 in Park2−/− cells (Figure 4B). However, Park2−/− BMDCs produced higher amounts of inflammasome-related cytokines, including IL-1β and IL-18, in response to VSV or HSV-1 infection, whereas there were no differences in the virus-induced production of other cytokines such as IL-6 and IL-12 between WT and Park2−/− cells (Figure 4C). These findings suggest that Parkin in myeloid cells negatively regulates virus-induced production of inflammasome-related cytokines by targeting post-transcriptional regulation of their expression. To extrapolate these findings into a human setting, we further evaluated the effect of Parkin deficiency on the production of cytokines in the human monocyte cell line THP-1 after viral infection. THP-1 cells were transfected with small interfering RNA to knockdown Parkin expression (Figure S5A) and then infected with VSV or HSV-1. The production of IL-1β and IL-18 was significantly enhanced in Parkin knockdown cells as determined by ELISA assay, whereas virus-induced expression of other cytokines, such as IL-6 and IFN-β, was not affected (Figure S5B). Thus, these results confirm that the selective targeting in the production of inflammasome-related cytokines by Parkin applies to murine and human cells. Although Parkin deficiency does not affect viral induced expression of IL-6 in cell infection studies, it is interesting to note from our earlier data that Park2−/− mice show augmented lung levels of IL-6 in response to viral infection. Such differences in cell- and animal-based infection approaches are reconciled by previous studies that have shown inflammasome-related cytokines, such as IL-1β and IL-18, to induce the production of proinflammatory cytokines and chemokines (Brabcova et al., 2014; Cahill and Rogers, 2008; McGeough et al., 2012; Yoo et al., 2005). Therefore the enhanced
and caspase-1 in cell supernatants was significantly increased in virus-infected WT with this, the oxidized mtDNA (ox-mtDNA) significantly increased in the cytosol of (Figure 4D). This was further confirmed by western blot analysis showing that the release of mature IL-1

production of IL-6, CXCL1, and CCL2 in lungs of virus-infected Park2−/− mice is a likely secondary consequence of the higher amounts of IL-1β and IL-18.

Given that Parkin does not appear to regulate the transcription of genes encoding IL-1β and IL-18, coupled to a recent report showing that Parkin inhibits ATP-induced NLRP3 inflammasome activation (Zhong et al., 2016), we next explored if Parkin could affect virus-induced inflammasome activation in BMDMs. To this end, Park2−/−MDDMs were challenged with VSV, HSV-1, and ATP as a second signal control for NLRP3 activation. ELISA analysis showed that IL-1β and IL-18 secretion was markedly augmented in Park2−/− cells relative to WT counterparts after viral infection, whereas the production of tumor necrosis factor (TNF)-α, an inflammasome-independent cytokine, was not affected by Park2 deletion (Figure 4D). This was further confirmed by western blot analysis showing that the release of mature IL-1β and caspase-1 in cell supernatants was significantly increased in virus-infected Park2−/− cells (Figure 4E).

Moreover, the deficiency of Parkin in BMDMs augmented virus-induced lactate dehydrogenase (LDH) release (Figure 4F), a measure of pyroptotic cell death that is also triggered by NLRP3 activation. Together, all these results indicate that Parkin in myeloid lineage cells has a unique role in inhibiting virus-induced inflammasome activation and subsequent production of IL-1β and IL-18 and pyroptosis.

**Parkin Restricts Mitochondrial Damage, mtROS Overproduction, and mtROS-Induced Inflammasome Activation During Viral Infection**

We next probed the mechanism by which Parkin targets activation of the NLRP3 inflammasome. Mitochondrial dysfunction induces the production of mtROS that can trigger NLRP3 inflammasome activation (Joule and Narendra, 2011). As a central mitophagy regulator, Parkin is linked to the removal of damaged mitochondria (Youle and Narendra, 2011). We therefore examined whether Parkin could control virus-induced mitochondrial dysfunction and mtROS production in BMDMs. To investigate this, we first performed cell fractionation to isolate mitochondrial and cytosolic fractions and used western blot analysis to monitor recruitment of autophagy proteins such as P62 and lipidated LC3 (LC3II) to damaged mitochondria that are destined for clearance by mitophagy. Infection of WT BMDMs with VSV or HSV-1 showed mitochondrial enrichment of the autophagy proteins P62, LC3II, and Parkin, whereas the mitochondrial recruitment of these proteins was abolished in Parkin-deficient cells (Figure 5A), thus confirming the central role of Parkin in virus-induced mitophagy. Moreover, we found that loss of Parkin led to more severe virus-induced loss of mitochondrial membrane potential as indicated by reduced mitochondrial accumulation of the fluorescence dye TMRM (Figure 5B), indicating the increased mitochondrial damage in Park2−/− cells. In addition, FACS analysis showed that viral infection induced more mtROS production in Park2−/− than WT cells (Figure 5C). Consistent with this, the oxidized mtDNA (ox-mtDNA) significantly increased in the cytosol of Park2−/− cells after viral infection (Figure 5D). These data strongly suggest that Parkin inhibits virus-induced mtROS production and ox-mtDNA release by restricting accumulation of damaged mitochondria.

ROS produced by damaged mitochondria is required for the production of ox-mtDNA, which is an important trigger of NLRP3 inflammasome activation (Yu et al., 2014; Zhong et al., 2018; Zhou et al., 2011). Thus, we reasoned that mtROS might be required for increased inflammasome activation in Parkin-deficient cells after viral infection. To test this hypothesis, we treated macrophages with the mitochondrial-specific antioxidant Mito-Q before viral challenge. Indeed, Mito-Q treatment blocked virus-induced mtROS production in WT and Park2−/− cells (Figure 5C). Interestingly, the virus-induced secretions of IL-1β and IL-18...
Figure 5. Parkin Restricts Mitochondrial Damage, mtROS Overproduction, and mtROS-Induced Inflammasome Activation during Viral Infection

(A) Immunoblot analysis of p62, LC3II, and Park2 in cytosolic and mitochondrial subcellular fractions from Pam3CSK4-primed WT and Park2+/− BMDMs infected with VSV at MOI 5 or HSV-1 at MOI 10 for 5 h.

(B) Microscopic analysis of mitochondrial membrane potential of cells stimulated with VSV or HSV-1 at MOI 1 by TMRM (tetramethylrhodamine, methyl ester) fluorescence. UI, uninfected. Scale bar, 100 μm.

(C) Flow cytometry analysis of the levels of mitochondria-associated ROS (mtROS) labeled by MitoSOX in Pam3CSK4-primed WT and Park2+/− BMDMs untreated or treated with 50 nM Mito-Q (mitochondrial-specific antioxidant) followed by VSV or HSV-1 infection at MOI 5 for 12 h.

(D) ELISA analysis of 8-OH-dG in the cytosol of Pam3CSK4-primed WT and Park2+/− BMDMs treated with VSV and HSV-1 at MOI 10 for 5 h.

(E) Immunoblot analysis of cleaved IL-1β, IL-18, and TNF-α in the supernatants of Park2+/− BMDMs untreated or treated with 50 nM Mito-Q and then infected with VSV at MOI 1 or HSV-1 at MOI 2 for 24 h (n = 3).

(F) Immunoblot analysis of cleaved IL-1β and caspase-1 in culture supernatants (SN) of Pam3CSK4-primed WT and Park2+/− BMDMs treated with 50 nM Mito-Q and then infected with VSV at MOI 5 or HSV-1 at MOI 10 for 24 h.

(G) LDH assay of Pam3CSK4-primed WT and Park2+/− BMDMs untreated or treated with 50 nM Mito-Q and then infected with VSV at MOI 1 or HSV-1 at MOI 2 for 24 h.

Enhanced Antiviral Inflammation and Viral Clearance in Parkin-Deficient Mice Is Dependent on NLRP3 Inflammasome

To further confirm if the effect of Parkin on virus-induced inflammasome activation is NLRP3 dependent, we crossed Park2+/− and Nlrp3−/− mice to make double knockout BMDMs and then characterized inflammasome activation in these cells. The absence of NLRP3 in Park2+/− BMDMs had no effect on virus-induced mtROS production (Figure 6A), and this is hardly surprising because mtROS production is upstream of NLRP3. However, VSV or HSV-1-induced release of IL-1β and IL-18 was blocked in Park2−/− Nlrp3−/− double knockout cells with this suppressive effect being selective for inflammasome-related cytokines because virus-induced production of TNF was fully intact in double knockout cells (Figure 6B). This was further confirmed by western blot analysis showing that NLRP3 deficiency blocked the secretion of mature IL-1β and caspase-1 in supernatant of Park2−/− cells after viral infection (Figure 6C). Furthermore, the absence of NLRP3 in double knockout cells also precluded the augmented virus-induced release of LDH that is normally observed in Park2−/− cells (Figure 6D). All these findings are fully consistent with Parkin deficiency promoting virus-induced inflammasome activation via the mtROS-NLRP3 axis.

It is clear from the above ex vivo-based results that Parkin deletion augments virus-induced inflammation through the activation of mtROS-NLRP3 axis. To further explore whether the enhanced survival and viral clearance in Parkin-deficient mice are dependent on NLRP3-activated antiviral inflammation, we next performed infection studies in Parkin and NLRP3 double knockout mice. Notably, we observed that like NLRP3+/− mice, Park2−/− Nlrp3−/− mice exhibited substantial reduction in survival and higher viral loads in lung after VSV (Figure 7A) and HSV-1 (Figure 7B) challenge compared with WTs, and this is markedly in contrast to the protected phenotype of Park2−/− mice. Histological analysis revealed that there was much less infiltration of immune cells in lungs of Park2−/− Nlrp3−/− mice compared with WT and Park2+/− mice on day 3 after viral infection (Figure 7C), indicating the impairment of antiviral inflammation in Park2−/−NLRP3+/− mice. In accordance with these results, VSV- (Figure 7D) and HSV-1- (Figure 7E) induced production of cytokines and chemokines, including IL-1β, IL-18, IL-6, CXC1, and CCL2, were significantly inhibited in BALF or sera from Park2−/− Nlrp3−/− mice, further suggesting an absolute NLRP3 dependence for the enhanced antiviral inflammation in Park2−/− mice. Altogether, these results clearly demonstrate that loss of Parkin in mice promotes viral clearance through NLRP3-mediated antiviral inflammation.

Parkin Gene Expression Is Downregulated in PBMCs from Virus-Infected Patients

To further determine the relationship of Parkin with virus infection, we analyzed by qPCR transcriptional changes in the gene expression of Parkin in BMDMs after virus infection. Unlike upregulated expression...
Figure 6. NLRP3 Deficiency Inhibits the Overproduction of Inflammasome-Related Cytokines in Parkin-Deficient Cells during Viral Infection

(A) Flow cytometry analysis of mtROS in Pam3CSK4-primed WT, Park2<sup>−/−</sup>, Nlrp3<sup>−/−</sup>, and Park2<sup>−/−</sup>Nlrp3<sup>−/−</sup> BMDMs infected with VSV or HSV-1 at MOI 5 for 12 h.

(B) ELISA analysis of IL-1β, IL-18, and TNF-α in the supernatants of Pam3CSK4-primed WT, Park2<sup>−/−</sup>, Nlrp3<sup>−/−</sup>, and Park2<sup>−/−</sup>Nlrp3<sup>−/−</sup> BMDMs infected with VSV at MOI 1 or HSV-1 at MOI 2 for 24 h.

(C) Immunoblot analysis of cleaved IL-1β and caspase-1 in culture supernatants (SN) of Pam3CSK4-primed WT, Park2<sup>−/−</sup>, Nlrp3<sup>−/−</sup>, and Park2<sup>−/−</sup>Nlrp3<sup>−/−</sup> BMDMs infected with VSV at MOI 5 or HSV-1 at MOI 10 for 24 h.

(D) LDH assay of Pam3CSK4-primed WT, Park2<sup>−/−</sup>, Nlrp3<sup>−/−</sup>, and Park2<sup>−/−</sup>Nlrp3<sup>−/−</sup> BMDMs infected as in (B). Data are pooled from three (B and D) independent experiments or are representative of two independent experiments (A and C). Error bars show means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, NS, not significant. Two-way ANOVA with Tukey’s multiple comparisons test. Related to Figure S7.
Figure 7. NLRP3 Deficiency Reverses the Enhanced Antiviral Inflammation and Viral Clearance in Parkin-Deficient Mice

(A) WT, Park2−/−, Nlrp3−/−, and Park2−/− Nlrp3−/− mice (n = 8 mice per group) were intranasally infected with VSV at 2 × 10^6 plaque-forming unit (PFU) per mouse or intravenously infected with HSV-1 at 6 × 10^7 PFU per mouse, and then the survival rates of mice are observed and recorded on the indicated days.
of antiviral cytokines, the expression of Parkin and another mitophagy regulator Pink1 was markedly reduced in response to VSV or HSV infection (Figure S6A), and this may serve to remove a negative regulator of antiviral inflammation and facilitate an enhanced antiviral response. This is also fully consistent with our earlier data showing that Parkin expression was downregulated at protein level after viral infection (Figure 1G). To further explore the relationship of decreased Parkin expression following viral infection with IFN-β, we treated WT MEFs and BMDMs with IFN-β. However, the Parkin expression did not decrease, suggesting the independent role of type I IFNs in regulating Parkin expression after viral infection (Figures S8A and S8B). Thus, how Parkin expression is regulated by host cells following viral infection remains to be investigated in future. To further explore the relevance of Parkin in the context of clinical infections, we next sought to assess Parkin expression in white blood cells of humans with virus infection. We isolated PBMCs from uninfected healthy donors and patients infected with hepatitis C virus (HCV, an RNA virus). Although our studies question the role, if any, of Parkin in regulating the type I IFN pathway, we clearly highlight its function in controlling inflammasome-mediated antiviral immunity. Several recent studies have

**DISCUSSION**

Increasing evidence has demonstrated that mitochondria play broad roles in innate immune response, including their functioning as signal transduction platforms, providing energy and metabolites for inflammation, and generating ROS as modulators of immune response (Weinberg et al., 2015; West et al., 2011). Mitophagy is a specific form of autophagy that can remove damaged mitochondria and maintain mitochondrial homeostasis (Kubli and Gustafsson, 2012). Some studies have reported that mitophagy can control inflammasome-related inflammation by mediating the clearance of damaged, mtROS-generating mitochondria (Nakahira et al., 2011; Zhong et al., 2016). We now provide a major insight into the physiological role of mitophagy and its regulation of inflammasome-mediated inflammation in the context of antiviral immunity.

Parkin, a central player in mitophagy, can be recruited to damaged mitochondria and ubiquitylate OMM proteins to initiate mitophagy (Narendra et al., 2008). A recent study showed that Parkin recruits LUBAC to mitochondria via interaction with MAVS and inhibits RLR-MAVS signaling (Khan et al., 2016), whereas another recent study reported the converse result that Parkin fails to interact with MAVS but regulates antiviral signaling by controlling TRAF3 stability (Xin et al., 2018). However, these studies were restricted to cell-based infection models and lacked data to evaluate the physiological or pathophysiological relevance of Parkin in viral infections. We now provide insight into the role of Parkin in the in vivo response to viral infections. We discover a critical role for Parkin in regulating viral clearance in vivo. Parkin deficiency enhances mouse survival in cases of infection with RNA and DNA viruses, and this is associated with improved viral clearance. Intriguingly, Parkin deficiency did not affect early virus-induced expression of type I IFN production either in vivo or in primary cells and the early signaling pathways, like IRFs, that act as upstream drivers of IFN induction, were also intact in Parkin knockout cells. This contrasts with previous reports using cell models, which proposed Parkin to be a negative modulator of virus-induced type I IFN production. It is possible that this difference might be caused by using carcinoma cell lines in previous studies. A recent study demonstrated that Parkin controlled mtDNA release into cytosol and cGAS-STING-dependent activation of IFN following exhaustive exercise (Sliter et al., 2018). Although during viral infection Park2−/− cells exhibit the increased release of ox-mtDNA (Figure 5D), much more nuclear acids from virus can mask the effect of more mtDNA release by Parkin deficiency on cGAS-sting activation, which might explain why Parkin does not regulate mtDNA-cGAS-STING axis-mediated IFN production after viral infection.

Although our studies question the role, if any, of Parkin in regulating the type I IFN pathway, we clearly highlight its function in controlling inflammasome-mediated antiviral immunity. Several recent studies have
described a role for the NLRP3 inflammasome in mediating innate immunity to virus. In all of these cases, NLRP3 promotes antiviral inflammation and viral clearance (Allen et al., 2009; Ichinohe et al., 2009; Thomas et al., 2009). Moreover, a study in IL-1R-deficient mice has shown that the animals have reduced acute airway inflammation associated with influenza virus infection and have significantly decreased survival (Schmitz et al., 2005). A recent study suggested that inflammasome activation can negatively regulate IFN-β response to DNA viruses through caspase-1-mediated cleavage of cGAS (Wang et al., 2017), whereas our studies did not find that virus-induced IFN-β was affected in Nlrp3-deficient BMDM (Figures S7A and S7B). We reasoned that the other inflammasome complex, for example AIM2, but not NLRP3, might be involved in this regulatory process. Our present studies indicate that Parkin deficiency increases virus-induced NLRP3 inflammasome activation and related inflammation through defective mitophagy. Impaired mitophagy results in overproduction of mtROS, which is correlated with increased antiviral inflammation in vivo, better viral clearance, and decreased mortality in mice. Notably, recent studies clearly demonstrate that in macrophages, the Parkin-p62-mitophagy pathway can dampen NLRP3 inflammasome activation by NLRP3 agonists by the removal of damaged mitochondria to control mtROS overproduction (Zhong et al., 2016). We now discover an important role for the mitophagy-inflammasome signaling axis in the context of antiviral immunity. We propose that in response to viral infection, mtROS is produced by damaged and depolarized mitochondria leading to NLRP3 activation and antiviral inflammation that is driven by IL-1β and IL-18. However, Parkin suppresses this antiviral response by promoting the clearance of damaged mitochondria by the process of mitophagy. Notably we show that under conditions of viral infection in both experimental and clinical cases the expression of Parkin is suppressed by the virus, thus relieving a braking system that will lead to accumulation of damaged mitochondria, high levels of mtROS and NLRP3 activation, and the triggering of NLRP3-driven antiviral inflammation. It is interesting to speculate that the negative role of Parkin in antiviral inflammation may fill a physiological function ensuring that persistent infection does not trigger a dysregulated inflammatory response and pathological tissue injury.

PD is associated with accumulation of damaged and defective mitochondria, which can be the major cause of neuroinflammation (Winklhofer and Haass, 2010). Indeed, although parkinsonism exhibits age-related degeneration of dopaminergic neurons, increasing evidence show that excessive inflammation may exacerbate neuronal cell death and disease development (Wang et al., 2015). In addition, it has been suggested that viruses, and in particular influenza virus, can be a precipitating factor in the development of PD (Jang et al., 2009). We found that viral infections in humans were associated with significant decreases in the expression levels of Parkin. Given that some genetic forms of PD are associated with mutations in the Parkin gene, virus-induced suppression of Parkin may phenocopy the pathology associated with Parkin-related PD. Indeed, impaired mitophagy resulting from Parkin deficiency or virus-induced suppression may link excessive NLRP3 inflammasome activation in microglia to chronic neuroinflammation that exacerbates neuronal cell death. Thus the present studies strongly prompt future studies to explore the potential role of Parkin in virus-related PD.

Taken together, our findings demonstrate a vital role for Parkin and mitophagy in regulating antiviral inflammation. These pathways act as a brake on virus-induced NLRP3 inflammasome activation by controlling mtROS. Our studies thus promote Parkin as a potential therapeutic target in the promotion of antiviral immunity.

**Limitations of the Study**

Our study clearly demonstrates that Parkin plays an important role in antiviral immunity by controlling mtROS-NLRP3 axis-mediated inflammation. The decreased expression of Parkin following viral infection is observed in mice and humans, but how Parkin expression is regulated by host cells following viral infection remains unknown. The known PD-related Parkin mutants majorly include splice site mutations and missense mutations. Our study in Parkin-deficient mice can well reflect the effect of splice site mutants on antiviral response. However, we do not know the relationship of antiviral response with missense mutations, which will lead to a loss of Parkin function in E3-ligase catalytic activity. Thus, these issues need to be investigated in future studies.

**METHODS**

All methods can be found in the accompanying Transparent Methods supplemental file.
SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.06.008.

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AUTHOR CONTRIBUTIONS

J.L., C.M., D.Y., X.L., Y.H., and C.W. designed and performed the experiments, analyzed the data, and prepared the figures; F.L. and T.P. provided the key technical mentoring and resources; M.W. and G.L. collected blood samples from humans and did clinical phenotyping of patients; B.W. and P.N.M. contributed to the experimental design and edited the manuscript; Y.C. and J.Z. provided the key research reagents and mice; T.P. and S.Y. supervised the project. S.Y. conceived the study and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

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Supplemental Information

Parkin Impairs Antiviral Immunity by Suppressing
the Mitochondrial Reactive Oxygen Species-Nlrp3
Axis and Antiviral Inflammation

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Supplementary information

Transparent methods

Mice

*Park2*<sup>−/−</sup> mice (Strain ID: 006582, C57BL/6 background) were provided by Dr. Jiawei Zhou from the Jackson Laboratory. *Nlrp3*<sup>−/−</sup> mice on C57BL/6 background were a gift from Dr. V. Dixit (Genentech). *Park2*<sup>−/−</sup> *Nlrp3*<sup>−/−</sup> mice were established by crossing *Nlrp3*<sup>−/−</sup> with *Park2*<sup>−/−</sup> mice. Mice used in this study (8-10-week-old male mice) were kept and bred under specific-pathogen-free conditions. All experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of Nanjing Medical University, Guangzhou Medical University and Shanghai Institute of Neuroscience, Chinese Academy of Sciences.

Reagents

IL-18 ELISA capture antibody (D047-3), detection antibody (D048-6) and standard protein (12405-1) were from R&D; IFN-β ELISA capture antibody (sc-57201) was from Santa Cruz; IFN-β detection antibody (32400-1) and standard protein (12400-1) were from R&D. Anti-P62(5114), anti-LC3B(2775), anti-myco(2276), anti-phosphorylated IRF3(4947s), anti-IRF3(4302s), anti-TBK1(3013s), anti-phosphorylated TBK1(5483s), anti-phosphorylated IκBα(9246s) and anti-phosphorylated P38(9212s) were from Cell Signaling Technology; Anti-flag (F3165), anti-β-actin(AC-15; A 1978) and anti-α-tubulin(T9026) were from Sigma; Anti-Parkin (sc-32282) and anti-Traf3(sc-949) were from Santa Cruz; Anti-IL-1 β (AB-401-NA) was from R&D; Anti-caspase1 (AG-20B-0042-C100) was from Adipogen. Anti-mouse-HRP and anti-rabbit-HRP were from Jackson ImmunoResearch. Anti-CD45-AF700(30-F11,85-11-0112-81), anti-CD11b-FITC(M1/70,85-12-0114-81), anti-CD11c-PE (N418, 48-9688-82), anti-Ly6C-PE-Cy7(HK1.4,25-5932-82), anti-Ly6G- eFlour450 (1A8-LY6G, 48-9668-82), anti-TCRβ–eFlour450(H57-597, 48-5961-80), anti-B220-Percp-Cy5.5 (Ra3-6B2,45-0452-82) and anti-NK1.1-PE-Cy7(PK136, 25-5941-81) antibodies were from eBioscience; MHC II (M5/114.15.2, 107627.0) was from Biolegend. Pam3CSK4 (tlrl-pms) and Poly(I:C) (tlrl-picw) were from Invivogen. Mito-Q was from MCE(HY-100116). Ficoll was from GE Health (45-001-749).

Viruses

VSV (Indiana strain) and HSV-1 (KOS strain) were obtained from Dr. Yichuan Xiao (Shanghai Institutes for Biological Sciences, China). VSV-GFP and HSV-1-GFP were from Dr. Tao Peng. All viruses were amplified by infection of a monolayer of African green monkey kidney cells (Vero). Briefly, 24-48 h after infection, the infected cells were frozen and thawed for three times to release virus. Then the cell lysate and culture supernatant were harvested and clarified by centrifugation. Viral titers were determined by standard plaque assay on confluent monolayers of Vero cells.

Viral infection in vivo

Age matched male mice (8-10-week old) were intranasally infected with VSV at 2×10<sup>8</sup> pfu per mouse or intravenously infected with HSV-1 at 6×10<sup>7</sup> pfu per mouse. Lungs from inoculated animals were homogenized in PBS and assayed for viral loads by plaque assay on Vero cells. Serum, BALF and lungs were also collected for Elisa, histology and FACS analysis.
Plaque Assay for Virus Titer
Viral infectivity was quantified using Vero cell monolayer. In brief, several 10-fold serial dilutions of the samples were made, added to Vero cell monolayer and incubated for 1–2 days. Then the cells were fixed and stained with 0.1% crystal violet solution for 10 min. Plaques were counted and viral titers were determined as plaque-forming units per ml by multiplying the dilution factor. For assay of viral load in mouse organs, after mice were sacrificed organs were collected individually, disrupted with a tissue homogenizer, frozen, and thawed three times to release the virus. Serial dilutions were made of the organ homogenates and then subjected to plaque assay.

Cell preparation and stimulation
MEFs were generated from E12-14 embryos, maintained in DMEM supplemented with 10% FBS, and sub-cultured no more than five passages before experiments. For isolation of BMDMs and BMDCs, tibias and femurs were removed from mice by sterile techniques and bone marrow was flushed with fresh medium. BMDMs were cultured in DMEM supplemented with 10% FBS in the presence of 10% L929 conditioned medium. BMDCs were cultured in RPMI-1640 complemented with 10% FBS in the presence of 10% J558 culture medium. After 5 days culture, 1.5×10^6 BMDMs and BMDCs were plated overnight in 12-well plates and were infected with VSV and HSV-1 as indicated in legends. For inducing inflammasome activation, BMDMs were primed with 1μg/mL Pam3CSK4 for 3 h and then stimulated with ATP (2.5 mM) for 1 h or with VSV and HSV-1 as indicated MOI for 24 h. THP-1 cells were cultured in RPMI-1640 medium plus GlutaMAX-I medium (Gibco) and transfected with control or Parkin-specific siRNA for 48hr. The following siRNA sequences were used: Con-siRNAs UUCUCCGAACGUGUCACGUTT, Con-siRNAas ACGUGACACGUUCGGAGAATT; Park2-siRNAs CGACCCUCAACUUGCUACTT, Park2-siRNAas GUAGCCAAGUUGAGGGUCGTT. The knockdown cells were then infected with VSV and HSV-1 for indicated times.

Immunoblotting
Cells were grown in 12-well plates and after treatment were then collected in 120μl NP-40 lysis buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1% (vol/vol) Igepal, 10% (wt/vol) glycerol, 50 mM NaF, 1 mM Na3VO4, 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride and complete protease-inhibitor 'cocktail' (Sigma)), followed by incubation for 30 min at 4 °C. Cell lysates were initially precleared by centrifugation at 14000 rpm. An aliquot of 6X SDS-PAGE sample buffer (1M Tris-HCl, pH 6.8, 20% (w/v) glycerol, 4% (w/v) SDS, 3% (w/v) DTT and 0.02% (w/v) bromophenol blue) was added to cell lysates. Samples were resolved by SDS-PAGE, transferred to nitrocellulose membranes and analyzed by immunoblot with the appropriate antibodies. Immunoreactivity was visualized by the Odyssey Imaging System (LI-COR Biosciences) or enhanced chemiluminescence.

Measurement of cytosolic mtDNA
WT and Park2^-/- MEFS were infected with VSV and HSV-1 (MOI=10) for 4h. Total DNA was isolated from the indicated cells using E.Z.N.A.™ MicroElute Genomic DNA kit (OMEGA) according to manufacturer's instructions. mtDNA was quantified by qPCR using primers specific for the mitochondrial D-loop region or a specific region of mtDNA that is not inserted into nuclear DNA (non-NUMT). Nuclear DNA encoding Tert and B2m was used for normalization.
D-loop F: 5’AATCTACCATCCTCCGTGAAACC3’
D-loop R: 5’TCAGTTTAGCTACCCTCCAAGTTTAA3’
Tert F: 5’CTAGCTCATGTGTCGAGACCCCTC3’
Tert R: 5’GCCAGCAGTTTCTCTTGG3’
B2m F: 5’ATGGGAAGCAGCGAATCTG3’
B2m R: 5’CAGTCTCAGTGGGGAAT3’
non-NUMT F: 5’CTAGAAACCCCGAAACCAAA3’
non-NUMT R: 5’CCAGCTATCACCAAGCTCGT3’

Measurement of cytosolic 8OH-dG
WT and Park2-/- BMDMs were primed with 1µg/mL Pam3CSK4 for 3 hr and then stimulated with VSV and HSV-1(MOI=10) for 5h. Cellular fractionation was then performed using a mitochondrial isolation kit for cultured cells according to manufacturer’s instructions. Cytosolic protein concentration and volume of the supernatant were normalized, DNA was isolated from 300ul cytosolic fractions using E.Z.N.A. MicroElute Genomic DNA kit according to manufacturer's instructions. The 8OH-dG was quantified using 8OH-dG ELISA kit (Elabscience), as the manufacturer's instruction.

RT-qPCR assays
Total RNA was extracted with Trizol reagent (Invitrogen) and reversed-transcribed with Reverse Transcription System (Vazyme). Reverse transcription products of different samples were amplified by Steponeplus (Applied Biosystems) using Brilliant SYBR Green QPCR Master mix (Vazyme) according to the manufacturer's instructions and data were normalized by the level of HPRT expression in each individual sample. \(2^{-\Delta \Delta Ct}\) method was used to calculate relative expression changes. Heatmap analysis was carried out using MeV Experiment View software. The following primers were used:

| Mouse        | S    | As               |
|--------------|------|------------------|
| Mouse Il1b   | 5 ATTGCACTTTTGACAGTGATG 3  | 5 GTTGATGTGCTGCTG  |
| Mouse Il6    | 5 CTTGGGACTGATGCTGGTGA 3  | 5 GCCATTGCACAACCTT |
| Mouse Il8    | 5 TCAAATGCGCCAGTGAAACC 3  | 5 AGGGTCACAGCGACT |
| Mouse Il12   | 5 TGGTCCATCGTTTGTGC 3     | 5 GGGAGTCAGCCACCTT |
| Mouse Irf7   | 5 CAGCACAGGCCGTTTTTCTCT 3 | 5 TCTCCCTATTTTCG |
| Mouse Isg15  | 5 AGCAGATTGGCGAGAGATTG 3  | 5 CCCCTTTGTTTCACCA |
| Mouse Ifna   | 5 CTTCTCATGTCCCTGTCAATG 3 | 5 AATCCAAAATCCCTG |
| Mouse Ifnb   | 5 CCGTTCGGAGAGACTGAGGAGA 3 | 5 CCCAGTGCGGAAATTG |

Primary mouse cells were stimulated as indicated. Conditioned media were collected and measured for levels of IL-1β (DY401), TNF-α (DY410) and IL-12 (DY2398) according to manufacturer’s instructions (R&D Systems). The level of IL-18 and IFN-β was assayed by an in-house sandwich ELISA system. The serum, BALF and lung from wild-type and Park2−/− mice were collected after infection as indicated and the levels of IL-1β, IL-18, IL-6 (DY406), CXCL1 (DY453) and CCL2 (DY479) in these samples were measured by sandwich ELISA (R&D Systems). Conditioned supernatants from human THP-1 siRNA-transfected cells were assayed for IL-1β (DY201, R&D), IL-6 (DY206, R&D), IL-18(70-EK1181, Multi Sciences) and IFN-β (70-EK1236-48, Multi Sciences).

### ELISA

Primary mouse cells were stimulated as indicated. Conditioned media were collected and measured for levels of IL-1β (DY401), TNF-α (DY410) and IL-12 (DY2398) according to manufacturer’s instructions (R&D Systems). The level of IL-18 and IFN-β was assayed by an in-house sandwich ELISA system. The serum, BALF and lung from wild-type and Park2−/− mice were collected after infection as indicated and the levels of IL-1β, IL-18, IL-6 (DY406), CXCL1 (DY453) and CCL2 (DY479) in these samples were measured by sandwich ELISA (R&D Systems). Conditioned supernatants from human THP-1 siRNA-transfected cells were assayed for IL-1β (DY201, R&D), IL-6 (DY206, R&D), IL-18(70-EK1181, Multi Sciences) and IFN-β (70-EK1236-48, Multi Sciences).

### Cytotoxicity Assay (LDH)

Conditioned medium from treated BMDMs was assessed for LDH release using Pierce LDH Cytotoxicity Assay Kit (Promega) according to the manufacturer’s instructions. Relative LDH release was calculated as LDH release [%] = 100 * (measurement – unstimulated control)/ (lysis control – unstimulated control).

### Fluorescence and Confocal microscopy

BMDMs were seeded on slides overnight and then were stimulated as indicated. After being stained with TMRM (20 nM) in the dark for 30 minutes at 37 °C, the cells were fixed, washed three times with PBS and then followed by Hoechst for 5 min. Images were captured using Nikon50i fluorescent microscope.
Flow cytometry
For isolation of lung infiltrating immune cells, mice on day 3 after viral infection were
anesthetized and perfused with 10-15 mL of PBS (ice cold) through the right ventricle until lungs
cleared of blood. Lungs were harvested, cut into small pieces and incubated in collagenase type IV
(0.5mg/ml) and DNase I (10U/ml) at 37°C under agitation (200 rpm) conditions for 90 min. The
digested tissues were filtered through a 100 μm filter and the plunger end of syringe was used to
push the cells over the filter. Homogeneous cell suspensions were centrifuged over a 40%/80%
discontinuous Percoll gradient (GE Healthcare), mononuclear cells were isolated from the
interface. The cells were suspended in PBS containing 2% (w/v) FBS. Once lysed in RBC lysis
buffer, cells were washed with the suspension buffer and stained with fluorochrome-conjugated
surface marker antibodies for FACS analysis. The following antibodies were used: CD45, CD11b,
ly6C, ly6G, MHC II+, CD11c, TCRβ, B220 and NK1.1. Mitochondria-associated ROS levels were
measured by staining cells with MitoSOX (Invitrogen) at 2.5 μM for 30 min at 37 °C. Cells were
then washed with PBS solution and re-suspended in cold PBS solution containing 1% FBS for
FACS analysis. All flow cytometry was performed on an Attune NxT flow cytometer
(Thermofisher) and data were analyzed by FlowJo 7.6.1 software.

Histology and Immunohistochemistry
Lungs from control or virus-infected mice were fixed in 10% phosphate-buffered formalin,
embedded into paraffin and sectioned. Sections of lung 5 μm in thickness were collected on
superfrost slides, deparaffinized in xylol and rehydrated, and stained with hematoxylin-eosin
(H&E) solution y. For immunohistochemical staining, sections were blocked and incubated with
primary antibodies and horseradish peroxidase-conjugated secondary antibodies after heat induced
antigen retrieval. Diaminobenzidine was used for detection. Images were captured with Nikon 50i
microscope.

Analysis of PBMC samples from patients with viral infection
Individuals diagnosed with HCV infection and healthy volunteers were enrolled in this study.
Clinical parameters and characterization of these patients were determined by a physician (GY.
Liu). Blood samples were collected during severe illness. PBMCs were isolated by Ficoll-
density gradient centrifugation (GE Healthcare). Total RNA was extracted with Trizol reagent
(Invitrogen) and reversed-transcribed with Reverse Transcription System (Vazyme). This study
was approved by the institution review board at the First Affiliated Hospital of Nanjing Medical
University. All patient managements and blood sample collection were carried out in
accordance with the relevant guidelines. The following table was information of human subjects.

| Name | State | The sex | Age |
|------|-------|---------|-----|
| Hu   | HCV   | female  | 32  |
| Jiang| HCV   | female  | 41  |
| Lu   | HCV   | female  | 27  |
| Han  | HCV   | female  | 28  |
| Su   | HCV   | female  | 37  |
| Sun  | HCV   | female  | 26  |
| Name   | Status | Gender | Age |
|--------|--------|--------|-----|
| Wang   | HCV    | female | 49  |
| Lv     | HCV    | female | 33  |
| Liu    | Healthy| female | 24  |
| Wu     | Healthy| female | 26  |
| Hu     | Healthy| male   | 25  |
| Zhang  | Healthy| male   | 26  |
| Rao    | Healthy| male   | 27  |
| Long   | Healthy| male   | 26  |
| Yang   | Healthy| male   | 23  |
| Wang   | Healthy| male   | 26  |

**Statistical Analysis**

Data are presented as the mean ± standard error of the mean (SEM). Samples were analyzed using unpaired t-test for two groups and ANOVA for multiple groups. Survival curves were generated via the product-limit method of GraphPad. In all cases, a P value of less than 0.05 was considered statistically significant.
Figure S1. Loss of Parkin doesn’t affect IFN production in viral-infected mice, susceptibility of MEFs to VSV or HSV-1 infection, Related to Figure 1.

(A) ELISA analysis of IFN-β of lung homogenates from WT and Park2−/− mice infected with the indicated viruses for 3 days (n=8 for viral infection, n=3 for mock infection).

(B) Flow cytometry analysis of GFP expression in WT and Park2−/− MEFs infected with VSV-GFP and HSV-1-GFP at MOI 1 for 6,12 and 24 h. NT, No treatment
Figure S2. Parkin deficiency doesn’t affect antiviral signaling and IFN production in BMDCs and BMDMs, Related to Figure 1.
(A) RT-qPCR analysis of Ifnb mRNA of WT and Park2^-/- BMDCs infected with VSV or HSV-1 for 4, 8 and 12h. NT, No treatment
(B) ELISA of IFN-β in the supernatants of WT and Park2^-/- BMDCs infected with VSV or HSV-1 for 12 and 24h. NT, No treatment
(C) Immunoblot analysis of phosphorylated (p-) and total IRF3, STING and cGAS in WT and Park2^-/- BMDCs infected for 0, 3 or 6h with VSV or HSV-1.
(D) RT-qPCR analysis of Ifnb and Il6 mRNA of WT and Park2^-/- BMDMs infected with VSV or HSV-1 for 4, 8 and 12h. NT, No treatment
(E) Immunoblot analysis of phosphorylated (p-) and total IRF3, STING, cGAS and β-actin (loading control) in WT and Park2^-/-BMDMs infected for 0, 3 or 6h with VSV or HSV-1.
Data are pooled from three independent experiments (A, B and D) or are representative of two independent experiments (C and E). Error bars show means ± SEM. NS, not significant. two-way ANOVA with Sidak’s multiple comparisons test.
Figure S3

A

VSV

B

HSV-1
Figure S3. Parkin deficiency enhances viral-induced expression of pro-inflammatory cytokines in lung, Related to Figure 2 and 3.
(A) ELISA analysis of IL-1β, IL-18, IL-6, CXCL1 and CCL2 of lungs from WT and Park2−/− mice infected with VSV for 3 days (n=6 for viral infection, n=3 for mock infection).
(B) ELISA analysis of IL-1β, IL-18, IL-6, CXCL1, CCL2 of lungs from WT and Park2−/− mice infected with HSV-1 for 3 days (n=6 for viral infection, n=3 for mock infection).
Data are pooled from three independent experiments. Error bars show means ± SEM. * * P < 0.01, * * * P < 0.001. Two-way ANOVA with Sidak’s multiple comparisons test.
Figure S4. Parkin deficiency doesn’t affect lymphoid cells, Related to Figure 2 and 3.
Flow cytometry analysis of percentages and numbers of lymphoid cells in thymus and spleen of WT or Park2−/− mice at 10 weeks of age.
(A) CD4⁺, CD8⁺ and CD4⁺CD8⁺ T cells in thymus.
(B) CD19⁺ B cells in spleen.
(C) CD4⁺ CD44⁺ and CD4⁺ CD62l⁺ T cells in spleen.
(D) CD8⁺ CD44⁺ and CD8⁺ CD62l⁺ T in spleen.
N=3. Error bars show means ± SEM. NS, not significant. Unpaired t test.
Figure S5

(A) RT-qPCR analysis of Park2 mRNA of THP1 cells transfected with control scramble or Park2-specific siRNA for 48h.

(B) ELISA analysis of IL-1β, IL-18, IL-6 and IFN-β in the supernatants of THP1 cells transfected with control scramble or Parkin-specific siRNA for 48h and then infected with VSV at MOI 1 or HSV-1 at MOI 2 for 24h. Data are pooled from three independent experiments. Error bars show means ± SEM. * P < 0.05, ** P < 0.01, NS, not significant. Two-way ANOVA with Sidak’s multiple comparisons test.

Figure S5. Parkin knockdown in THP1 cells doesn’t affect IFN production but increases the production of inflammasome-related cytokines in response to viral infection, Related to Figure 4.
Figure S6

A

Log2 expression ratio value

B

Park2

Pink1

Log2 expression ratio value

Healthy HCV

Healthy HCV
Figure S6. Parkin gene decrease in response to virus infection in BMDMs and PBMCs of patients, Related to Figure 1.

(A) Heatmap visualization of gene expression for Park2, Pink1, P62, Irf7, Isg15, Ifnb, Ifna, Il1b, Il6, Cxcl1, Tnfa, Ndufab1 and Ndufa6 in BMDMs infected with VSV or HSV-1 for 6h determined by qPCR (log2 fold values). The abundance of each mRNA was normalized relative to PCR of the housekeeping gene hprt.

(B) RT-qPCR analysis of Park2 and Pink1 mRNA expression of PBMCs from healthy and HCV patients (log2 fold values).
Figure S7. NLRP3 deficiency in BMDMs doesn’t affect IFN production in response to viral infection, Related to Figure 6.

(A) RT-qPCR analysis of *Ifnb* mRNA of WT and *Nlrp3*-/- BMDCs infected with VSV or HSV-1 at MOI 1 for 12h.

(B) ELISA of IFN-β in the supernatants of WT and *Nlrp3*-/- BMDCs infected with VSV or HSV-1 at MOI 1 for 24h.

Data are pooled from three independent experiments. Error bars show means ± SEM. NS, not significant. Two-way ANOVA with Sidak’s multiple comparisons test.
Figure S8

Figure S8. The independent role of type I IFNs in regulating Parkin expression in response to virus infection, and IFN production in response to RLR non replicative IFN inducer, Related to Figure 1.

(A) Immunoblot analysis of phosphorylated (p-) STAT1, Park2 and β-actin (loading control) in WT BMDMs treated with IFNβ (500U/ml) for 0, 0.5, 1, 3 and 6h.

(B) Immunoblot analysis of phosphorylated (p-) STAT1, Park2 and β-actin (loading control) in WT MEFs treated with IFNβ (500U/ml) for 0, 0.5, 1, 3 and 6h.

(C) RT-qPCR analysis of Ifnb mRNA expression of WT and Park2−/− MEFs transfected with Poly(I:C) (10ug/ml) for 6h. NT, No treatment. TF: Transfect.

(D) ELISA analysis of IFN-β protein expression of WT and Park2−/− MEFs transfected with Poly(I:C) (10ug/ml) for 18h. NT, No treatment. TF: Transfect.

Data are pooled from three independent experiments. Error bars show means ± SEM. NS, not significant. Two-way ANOVA with Sidak’s multiple comparisons test.