ORIGINAL RESEARCH

Parkin, an E3 ubiquitin ligase, enhances airway mitochondrial DNA release and inflammation

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ABSTRACT

Introduction Parkin (Park2), an E3 ubiquitin ligase, is critical to maintain mitochondrial function by regulating mitochondrial biogenesis and degradation (mitophagy), but recent evidence suggests the involvement of Parkin in promoting inflammation. In the present study, we determined if Parkin regulates airway mitochondrial DNA (mtDNA) release and inflammatory responses to type 2 cytokine interleukin (IL)-13 and allergens.

Methods We measured Parkin mRNA expression in brushed bronchial epithelial cells and mtDNA release in the paired bronchoalveolar lavage fluid (BALF) from normal subjects and asthmatics. Parkin-deficient primary human tracheobronchial epithelial (HTBE) cells generated using the CRISPR-Cas9 system were stimulated with IL-13. To determine the *in vivo* function of Parkin, Parkin knockout (PKO) and wild-type (WT) mice were treated with IL-13 or allergen (house dust mite, HDM) in the presence or absence of mtDNA isolated from normal mouse lungs.

Results Parkin mRNA expression in asthmatic airway epithelium was upregulated, which positively correlated with the levels of released mtDNA in BALF. IL-13-stimulated HTBE cells increased Parkin expression. Moreover, IL-13 induced mtDNA release in Parkin-sufficient, but not in Parkin-deficient HTBE cells. PKO (vs WT) mice attenuated airway mtDNA release and inflammation following IL-13 or HDM treatments. mtDNA amplified airway inflammation in mice treated with IL-13 or HDM. Notably, Parkin also mediated mtDNA-induced exacerbation of airway inflammation.

Conclusion Our research findings suggest that Parkin promotes mtDNA release and inflammation in airways, thus improving our understanding of the complex role of Parkin and mitochondrial dysfunction in asthma pathogenesis.

Key messages

What is the key question?

- Does mitochondrial dysfunction (eg, Parkin dysregulation) contribute to airway inflammation in asthma?

What is the bottom line?

- Excessive Parkin expression in type 2 inflammation high asthmatic airways increases the release of mtDNA and subsequently exacerbates inflammation.

Why read on?

- This is the first study to uncover a novel role of Parkin in promoting mtDNA release and inflammation in asthmatic airways.

INTRODUCTION

Mitochondrial dysfunction has been reported in various airway diseases including asthma. In asthmatic children, swollen mitochondria in bronchial epithelium was first reported by Konrádová and co-workers.[1] In mouse models of allergic asthma, several groups reported increased oxidative stress, decreased expression of proteins in the mitochondrial electron transport chain complex and reduction of cytochrome c oxidase activity.[2 3] These research findings highlight that mitochondrial dysfunction exists in asthma that may increase disease severity. Additionally, it has been suggested that production of reactive oxygen species (ROS) from inflammatory cells such as neutrophils and eosinophils[4] may contribute to mitochondrial damage and dysfunction. Neutrophils and/or eosinophils are increased in asthmatic airways that are characterised by type 2 inflammation (eg, interleukin (IL)-13) or non-type 2 inflammation (eg, IL-8). Although mitochondrial DNA (mtDNA) has been shown to induce proinflammatory responses,[5 6] it is still unclear about the levels of mtDNA release and its underlying mechanisms in asthmatic airways as well as the role of mtDNA in asthma pathogenesis.

Parkin (*Park2*) is expressed in various tissue cell types including airway epithelial cells.[7] It was initially identified as a cytosolic E3 ubiquitin ligase that ubiquitinates and delivers substrates for proteasomal degradation. Additionally, Parkin can maintain mitochondrial function by regulating mitochondrial biogenesis and degradation.[8] In response to oxidative stress, Parkin has been proposed to play a protective role by clearing damaged mitochondria via mitophagy,[9] in which it was reported that Parkin-mediated mitophagy prevented the release of circulating mtDNA.[10] However, Parkin overexpression also enhanced mtDNA replication and transcription,[11] which may facilitate mtDNA release in a mitophagy-independent manner. Therefore, the role Parkin in the release of mtDNA needs further investigation.

Moreover, recent publications suggest additional functions of Parkin that may not be directly related to its classical mitochondrial functions. For

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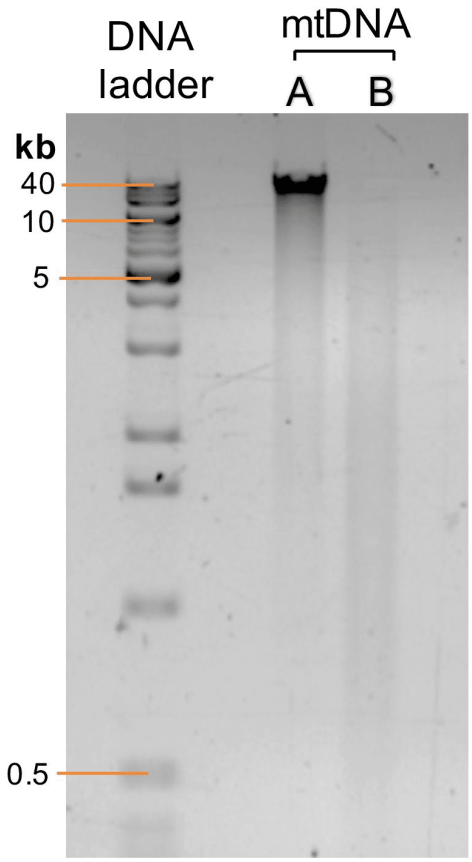

Figure 1 mtDNA isolated from normal C57BL/6 mouse lung tissues. (A) Unsonicated mtDNA; (B) sonicated mtDNA showing DNA fragments less than 5 kb. Both unsonicated and sonicated DNA was loaded at 250 ng/well and visualised on 0.7% agarose gel. mtDNA, mitochondrial DNA.

example, Parkin was found to promote apoptosis in response to valinomycin[12] and oxidative stress.[13] A study by Müller-Rischart *et al*[14] demonstrated that Parkin can activate the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling by ubiquitinating the NF-κB essential modulator. Interestingly, Parkin was shown to promote inflammation in lipopolysaccharides (LPS)-induced lung injury[15] as well as in rheumatoid arthritis via suppression of p53 and activation of NF-κB.[16] These studies suggest the potential function of Parkin as a proinflammatory modulator, but its expression and functions in asthmatic airways have not been explored.

In the present study, we sought to investigate the role of Parkin in airway inflammation. We hypothesised that Parkin expression is induced in asthma. We further hypothesised that Parkin in the context of type 2 inflammation increases the release of mtDNA and subsequently exacerbates airway inflammation. We tested our hypotheses by measuring Parkin expression and mtDNA release in asthmatic airway epithelial cells and bronchoalveolar lavage fluid, as well as examining Parkin expression, mtDNA release and pro-inflammatory responses in IL-13 and/or allergen-challenged human primary airway epithelial cells and mice.

MATERIALS AND METHODS

Human subjects

Brushed bronchial epithelial cells and bronchoalveolar lavage fluid (BALF) from normal healthy and asthma subjects were obtained through bronchoscopy as previously described.[17] Based on the American Thoracic Society criteria for asthma,[18] we have used FEV1% and medication (β2 agonists and corticosteroids) data to confirm asthma diagnosis and disease severity. Both normal and asthmatic subjects received methacholine test, spirometry test, fractional exhaled nitric oxide (FeNO) and blood tests for eosinophils.[17] Asthmatic patients with current or past smoking were excluded from the study.

De-identified donor lungs from fatal asthmatics and non-smokers without history of lung disease were obtained from the National Disease Research Interchange (Philadelphia, Pennsylvania, USA), the International Institute for the Advancement of Medicine (Edison, New Jersey, USA) or Donor Alliance of Colorado. The collected lung was donated for medical research and was approved by the Institutional Review Board at National Jewish Health. Human tracheobronchial epithelial (HTBE) cells were processed and isolated from the distal region of the trachea and proximal parts of the main bronchi as we previously described.[19] Freshly isolated uncultured HTBE cells were frozen at 5×10^6 cells/vial at −80°C until use for western blot.

HTBE cell culture

Freshly isolated HTBE cells were expanded on collagen-coated 60 mm dishes containing BronchiaLife with supplements (Lifeline Cell Technology, Frederick, MD, USA). Air–liquid interface (ALI) culture of HTBE cells was performed as we previously reported.[19 20] Briefly, cells were transferred onto collagen-coated 12-well transwell plates (Transwell 2460, Corning Incorporated, Corning, New York, USA) with PneumaCult-ALI medium (StemCell, Vancouver, British Columbia, Canada). After 7 days of submerged culture, cells were shifted to ALI for the next 14 days to induce mucociliary differentiation. Cells were stimulated with 10 ng/mL recombinant human IL-13 (R&D Systems, Minneapolis, Minnesota, USA) for three consecutive days starting on day 11 of ALI.[20] Basolateral supernatants and cells were harvested on day 14. Cells were lysed with RLT buffer for RNA extraction or with RIPA buffer for western blot analysis.

Generation of Parkin knockout (PKO) HTBE cells

PKO cells were generated using the CRISPR-Cas9 method as we previously described.[21] A single guide (sg) RNA (sgRNA sequence: 5′ AGTCTAAGCAAA-TCACGTGG 3′) was designed to target exon 7 of human Parkin in HTBE cells, while a scrambled sgRNA was used for the control CRISPR.[22]

Parkin or scrambled sgRNA was cloned into an 'all-in-one' pLenti-CRISPR vector coexpressing the scaffold RNA and Cas9 nuclease with a puromycin resistance gene. The construct was sequenced to confirm the presence of sgRNA sequence, and then packaged into lentivirus by cotransfection with VSV-G and psPAX2 plasmids in 293FT cells. The packaged lentivirus in the culture supernatant was transduced into 70% confluent HTBE cells. After 48 hours of recovery, the transduced cells were seeded onto irradiated, puromycin-resistant 3T3 fibroblasts for expansion and selection with puromycin (1 µg/mL) for 7 days. Cells were then transferred onto collagen-coated 12-well transwell plates (Transwell 2460) with PneumaCult-ALI medium (StemCell). After 7 days of submerged culture, cells were shifted to ALI for the next 14 days and stimulated with IL-13 on day 11 of ALI for three consecutive days.

Preparation of mtDNA from mouse lung tissue

mtDNA was isolated from naïve C57BL/6 mouse lungs using a Mitochondrial DNA Isolation Kit (Biovision, Milpitas, California, USA). The concentration of mtDNA was measured using

NanoDrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Sonication was done to generate mtDNA fragments for our *in vivo* mouse models. mtDNA was analysed on 0.7% agarose gel to confirm sonicated fragments (figure 1). The sonicated mtDNA from mouse lung tissue showed fragments less than 5 kb compared with the unsonicated mtDNA, which was consistent with the results reported by Patil *et al*.[23] We used the sonicated/fragmented mtDNA to mimic mtDNA from damaged and released mtDNA. Notably, a previous report demonstrated that intact mtDNA did not have any proinflammatory effect in mouse lungs.[24] Thus, the use of fragmented DNA is conceivably more physiologically relevant to test its potential proinflammatory effect.

Mouse models of airway inflammation and mtDNA treatment

Wild-type (WT) BALB/c, WT C57BL/6 and Parkin knockout (PKO) mice on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). The mice were maintained and bred in the animal facility at the Biological Resources Center of National Jewish Health. All animals and procedures were approved by our Institutional Animal Care and Use Committee.

To determine if Parkin regulates IL-13-mediated airway inflammation, PKO and WT mice were administered intranasally with recombinant mouse IL-13 (Peprotech, Rocky Hill, New Jersey, USA) at 250 ng/mouse prepared in 0.1% bovine serum albumin (BSA) or 0.1% BSA (control) once daily for three consecutive days.[22] To test whether Parkin additionally contributes to mtDNA-induced inflammation in the context of IL-13 stimulation, sonicated mtDNA at 5 µg/mouse or 50 µL Tris-EDTA (TE) buffer was delivered to mice by oropharyngeal administration after 6 hours of the last IL-13 intranasal treatment.

To induce mouse allergic airway inflammation, mice were intranasally sensitised with house dust mite (HDM) extracts at 10 µg/mouse or 50 µL phosphate-buffered saline (PBS) (control) on days 0 and 7. Mice were then challenged once a day for three consecutive days (days 14–16) with 10 µg HDM or 50 µL PBS via intranasal inoculation. Moreover, to examine the role of mtDNA in modulating established allergic airway inflammation, mtDNA at 5 µg/mouse in 50 µL TE buffer or 50 µL TE buffer alone (control) was given via oropharyngeal administration after 6 hours of the last HDM intranasal challenge. We chose the mtDNA dose (5 µg/mouse) based on recent publications in several mouse models where mtDNA from 1 to 60 µg/mouse was delivered via intratracheal instillation.[5 25 26] In a pilot experiment in mice (n=4), we determined the mtDNA levels right after oropharyngeal administration of 5 µg mtDNA/mouse. We found that about 2 µg entered the lung of mice as measured by mtDNA levels in bronchoalveolar lavage fluid (data not shown). This dose is within the range of the three published mouse models as mentioned above.[5 25 26]

Mice were sacrificed after 24 hours of the mtDNA treatment with or without the last HDM or IL-13 administration. BALF was collected for leucocyte counts. Cell-free BALF was used to assess proinflammatory mediators using ELISA and release of mtDNA using real-time PCR.

Quantification of mtDNA in BALF and airway epithelial cell supernatants

mtDNA was extracted from mouse and human BALF using a DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. For the supernatant of airway epithelial cells in ALI culture, we isolated mitochondria first and then extracted mtDNA as described by Nerlich *et al*[27] since released mtDNA can be present in either intact free mitochondria and/or inside extracellular microparticles.[28] For initial primer selection of human mtDNA, we compared primers for the D-Loop (conserved, non-coding region) and ATPase6 (adenosine triphosphatase 6, coding region). Results using both D-Loop and ATPase6 primers were similar and significantly correlated (data not shown). Hence, mtDNA was quantified by real-time PCR for mitochondrial specific genes: human D-Loop (forward (FW) primer: TGCACGCGATAGCATTGC; reverse (RV) primer: AGGCAGGAATCAAAGACAGATACTG)[29] and mouse 16 s rRNA (FW primer: CCGCAAGGGAAAGATGAAAGAC; RV primer: TCGTTTGGTTTCGGGGTTTC)[30] using iQ SYBR Green Supermix (Bio-Rad). Real-time PCR standard curves were created to quantify mtDNA concentration using purified mtDNA isolated from human and mouse lungs, respectively.[29 31]

Quantitative real-time reverse transcription PCR (RT-PCR)

RNA extracted from uncultured and cultured human airway epithelial cells using the GenCatch Total RNA Extraction System (Epoch Life Sciences) was reversely transcribed to cDNA. Taqman qPCR assays of Parkin, eotaxin-3 and periostin were obtained from Applied Biosystems. To demonstrate relative mRNA expression levels, the comparative cycle of threshold (ΔΔCT) method was performed with the housekeeping gene GAPDH as an internal control.

Western blot

Cell lysates were prepared and analysed as previously described.[19] Blots were probed with antibody against Parkin and β-actin (1:500, Santa Cruz Biotechnology, Dallas, Texas, USA) following by horseradish peroxidase-conjugated secondary IgG (1:3000; EMD Millipore, Burlington, Massachusetts, USA). Densitometry was performed using the National Institutes of Health's ImageJ software.

ELISA

Mouse KC/CXCL1, mouse eotaxin-2, human IL-8, human IL-6 and human eotaxin-3 protein levels were measured using Duoset ELISA kits from R&D systems.

Statistical analyses

Parametric tests were used if data were normally distributed. For two-group comparisons, a paired Student's t-test was performed. Multiple comparisons were done using one-way analysis of variance with the Tukey's post hoc test. For non-parametric test wherein the data were not normally distributed, comparisons were done using the Mann-Whitney test. Correlation was assessed using the Spearman coefficient. A p value <0.05 was considered statistically significant.

RESULTS

Increased Parkin mRNA expression in uncultured asthmatic brushed airway epithelial cells and its association with mtDNA release in BALF

As expected, asthmatics had significantly lower FEV1 (% predicted) than healthy controls (table 1). Compared with healthy controls, patients with asthma demonstrated higher FeNO levels, suggesting the presence of type 2 inflammation, as FeNO is considered a type 2 inflammation biomarker in asthma.[32]

Importantly, brushed bronchial epithelial cells from asthmatics had significantly higher Parkin mRNA levels than healthy

Table 1 Characteristics of healthy and asthma subjects

Parameters	Healthy controls (n=9)	Patients with asthma (n=12)	P value
Age, year	37.0 (31.0–42.0)*	36.0 (24.5–56.8)	0.35
FEV1 (% predicted)	96.0 (92.5–102.0)	91.0 (75.5–93.8)	0.01
FeNO (ppb)	15.5 (9.7–28.7)	32.6 (17.1–43.8)	0.04
BMI	28.7 (25.1–32.5)	29.0 (23.6–36.8)	0.41
Gender	Male (n=3) Female (n=6)	Male (n=3) Female (n=9)	0.52

*Data expressed as median and IQR.
BMI, body mass index; FeNO, fractional exhaled nitric oxide; PPB, parts per billion.

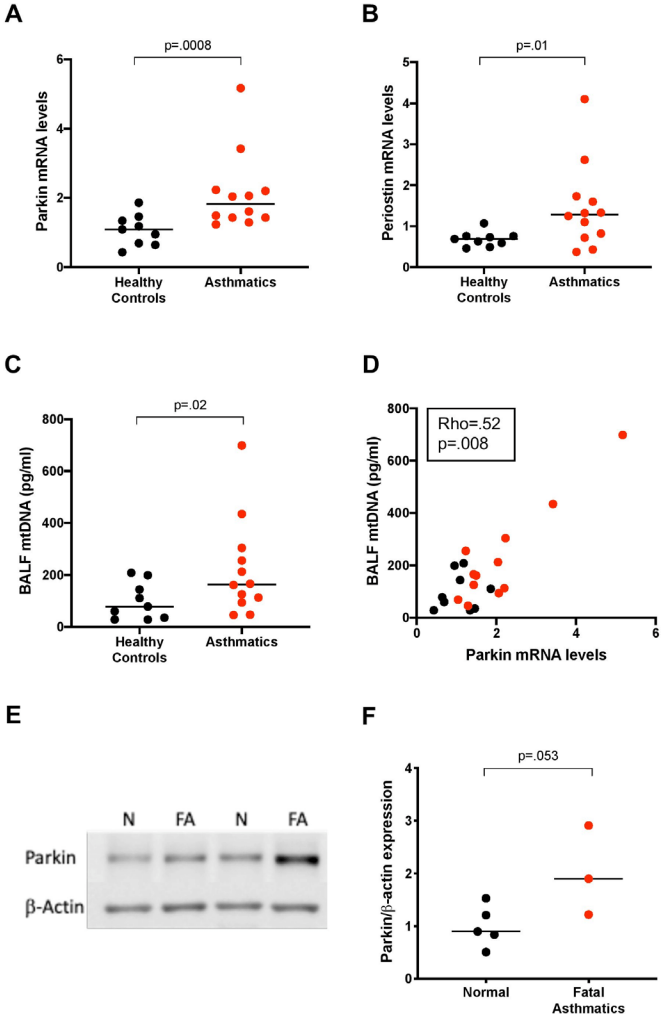

Figure 2 Upregulation of Parkin in brushed uncultured human bronchial epithelial cells was associated with mtDNA release in bronchoalveolar lavage fluid (BALF). (A) Parkin and (B) periostin mRNA levels in brushed bronchial epithelial cells were quantified by RT-PCR and normalised to housekeeping gene GAPDH. (C) mtDNA in the paired BALF was measured by real-time PCR for human mitochondrial D-Loop and normalised to a standard curve of purified human lung tissue-derived mtDNA. (D) Correlation between epithelial Parkin mRNA and mtDNA release from the BALF. (E) Representative Western blot images and (F) densitometry of Parkin protein from uncultured HTBE cells. Data were analysed using the Mann-Whitney test. The horizontal bars represent medians. Correlation was analysed using Spearman test. mtDNA, mitochondrial DNA; RT-PCR, reverse transcription PCR.

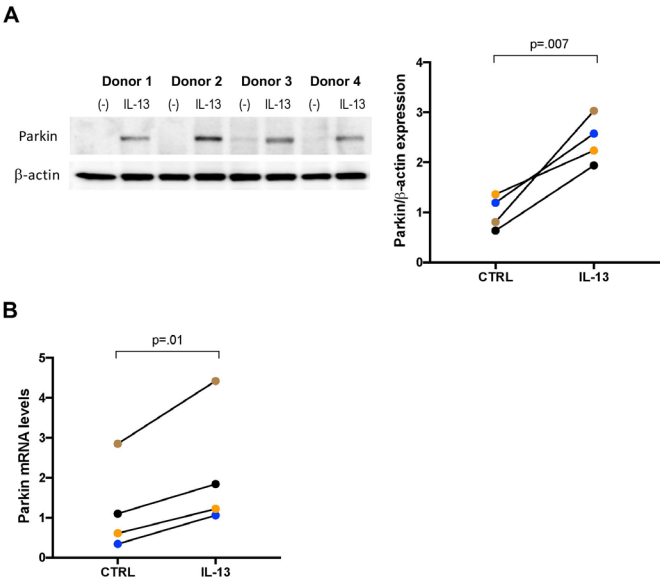

Figure 3 IL-13 increased parkin expression in human tracheobronchial epithelial (HTBE) cells. HTBE cells (n=4 subjects, as represented by different colour symbols) grown at air–liquid interface were stimulated with 10 ng/mL IL-13 or left untreated (medium control, CTRL). (A) Representative western blot images and densitometry of Parkin protein. (B) Parkin mRNA levels were quantified by RT-PCR and normalised to housekeeping gene GAPDH. Data were analysed using the paired t-test. IL, interleukin.

controls (figure 2A). Patients with asthma showed higher periostin mRNA, an epithelial type 2 inflammation marker, further supporting a type 2 inflammation endotype (figure 2B).

Whether Parkin is associated with the release of mtDNA into the airways has not been previously investigated. Using the paired BALF from the same subjects, we measured extracellular mtDNA in BALF and correlated the levels of BALF mtDNA with Parkin mRNA in airway epithelial cells. Patients with asthma had significantly higher mtDNA levels in BALF than healthy controls (figure 2C), which positively correlated with epithelial Parkin mRNA expression levels (figure 2D).

As the number of brushed/uncultured bronchial epithelial cells was limited to assess Parkin expression by western blot, we instead used uncultured HTBE cells from normal controls and patients with fatal asthma to demonstrate Parkin protein expression. Similar to the mRNA level, we showed that patients with fatal asthma had higher Parkin expression compared with normal controls (figure 2E,F). However, this increase (p=0.053) did not research statistical significance due to the small sample size from the fatal asthmatic group.

Parkin was upregulated by IL-13 in cultured primary human airway epithelial cells and promoted mtDNA release

To determine if type 2 inflammation induces Parkin expression, primary HTBE cells grown at ALI were stimulated with IL-13 to mimic type 2 inflammation in asthma. Parkin expression at the baseline was very low. However, IL-13 significantly upregulated Parkin protein expression (figure 3A). We also observed increased Parkin mRNA expression by IL-13 (figure 3B). Together, our cell culture data were similar to what we found in the uncultured asthmatic airway epithelial cells, further suggesting a role of a type 2 cytokine setting in Parkin induction.

To test whether Parkin promotes mtDNA release and subsequent proinflammatory responses to IL-13, PKO was established

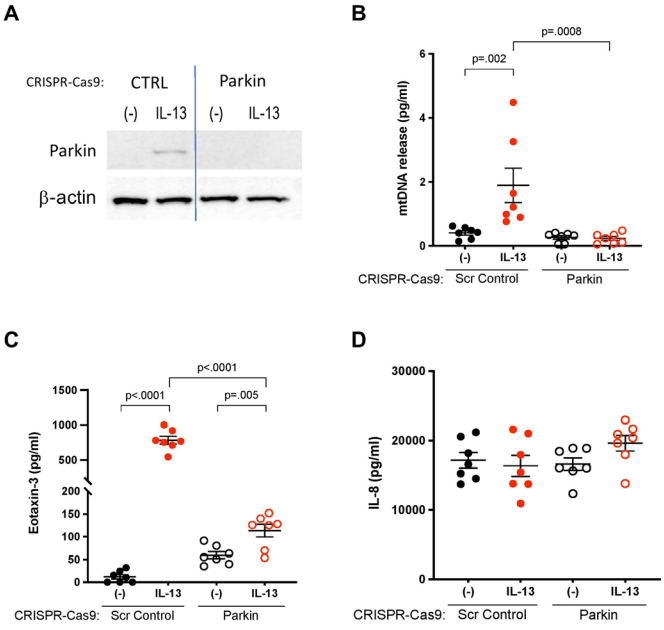

Figure 4 Parkin knockout in IL-13-stimulated human tracheobronchial epithelial (HTBE) cells inhibited mtDNA release. HTBE cells under the submerged condition were transduced with lentivirus containing the Parkin sgRNA or scramble (SCR) control sgRNA plasmid construct, and then moved to air–liquid interface (ALI) culture. Cells at day 11 of ALI culture were stimulated with 10 ng/mL IL-13 for 3 days or left untreated (–). (A) Representative western blot images of parkin protein. (B) mtDNA was measured in the basal supernatant using real-time PCR for human mitochondrial D-Loop, followed by normalisation to a standard curve of purified human lung mtDNA. (C) Proinflammatory cytokines eotaxin-3 and (D) IL-8 from the basal supernatants were quantified using ELISA. Data were presented as means with SEM and analysed using analysis of variance. IL, interleukin; mtDNA, mitochondrial DNA.

in HTBE cells using the CRISPR-Cas9 system. In line with the above data, IL-13 induced Parkin protein expression in scramble control CRISPR cells. Parkin protein was not detectable in IL-13-stimulated Parkin CRISPR cells, confirming PKO (figure 4A). Importantly, mtDNA release was increased in the basolateral supernatant of the scramble control CRISPR cells but not in that of Parkin CRISPR cells stimulated with IL-13 (figure 4B). Consistent with the mtDNA data, IL-13-mediated eotaxin-3 production was significantly higher in scramble control CRISPR cells than in Parkin CRISPR cells (figure 4C). IL-8 levels were similar in both CRISPR cells with or without IL-13 treatment (figure 4D).

Lack of Parkin-attenuated airway inflammation and mtDNA release in mice exposed to IL-13 or HDM

To investigate the *in vivo* function of Parkin in IL-13-mediated airway inflammation and in an allergic setting, WT and PKO C57BL6/J mice were intranasally inoculated with IL-13 or HDM. Parkin deficiency was confirmed in the lungs of PKO mice (figure 5A).

As expected, WT mice treated with IL-13 significantly increased the numbers of both neutrophils and eosinophils in BALF (figure 5B,C). Interestingly, PKO mice showed significantly lower numbers of neutrophils following IL-13 treatment than WT mice. Treatment with IL-13 also increased mtDNA release and KC/CXCL1 (neutrophil chemokine) levels in BALF of WT mice compared with PKO mice (figure 5D,E). However,

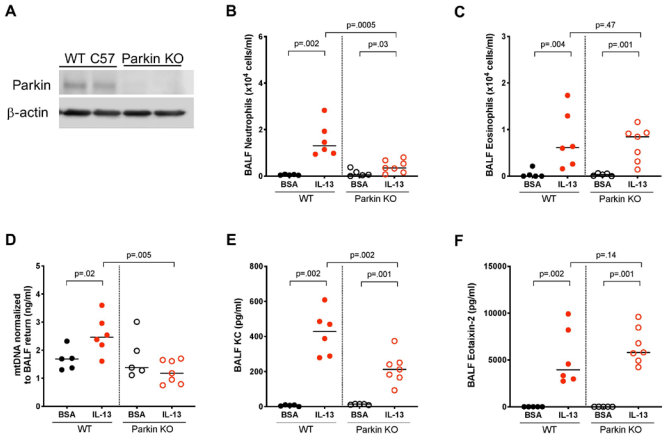

Figure 5 Reduced airway inflammation in IL-13-exposed Parkin knockout (PKO) mice. Wild-type (WT) and PKO C57BL/6 mice were intranasally inoculated with three consecutive doses of IL-13 (250 ng/mouse) or bovine serum albumin (BSA). After 24 hours, bronchoalveolar lavage fluid (BALF) was collected and analysed. (A) Representative western blot images of Parkin protein. (B) Neutrophil and (C) eosinophil counts in BALF. (D) mtDNA in cell-free BALF was measured using the mouse mitochondrial gene 16S by real-time PCR and then normalised to a standard curve of purified mouse lung tissue derived mtDNA. (E) Proinflammatory markers KC/CXCL1 and (F) eotaxin-2 were measured in the cell-free BALF using ELISA. Data were analysed using the Mann-Whitney test. The horizonal bars represent medians. IL, interleukin; mtDNA, mitochondrial DNA.

IL-13-induced eotaxin-2 (eosinophil chemokine) levels were similar between the two groups (figure 5F). Similar to what we found in the IL-13-treated mice, HDM-challenged PKO mice had decreased numbers of neutrophils and eosinophils in

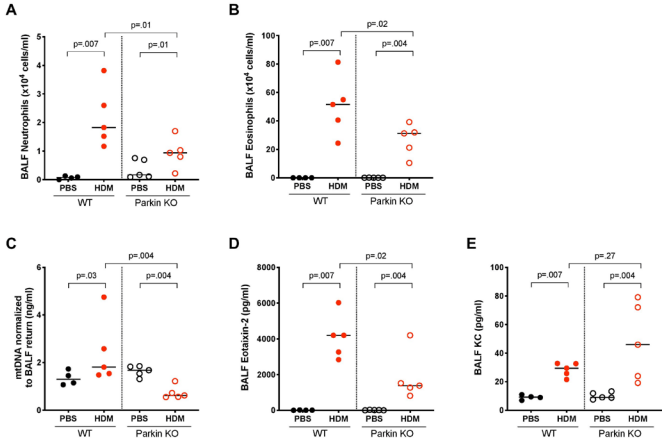

Figure 6 Airway inflammation was reduced in HDM-challenged Parkin knockout (PKO) mice. Wild-type (WT) and PKO C57BL/6 mice were sensitised with two doses of house dust mite (HDM) (10 µg/mouse) or PBS, followed by three daily challenges of HDM (10 µg/mouse) or PBS via intranasal inoculation. After 24 hours, bronchoalveolar lavage fluid (BALF) was collected and analysed. (A) Total neutrophil and (B) eosinophil counts in BALF. (C) mtDNA in cell-free BALF was measured using the mouse mitochondrial gene 16S by real-time PCR and then normalised to a standard curve of purified mouse lung tissue-derived mtDNA. (D) Proinflammatory markers eotaxin-2 and (E) KC/CXCL1 were measured in the cell-free BALF using ELISA. Data were analysed using the Mann-Whitney test. The horizonal bars represent medians. mtDNA, mitochondrial DNA.

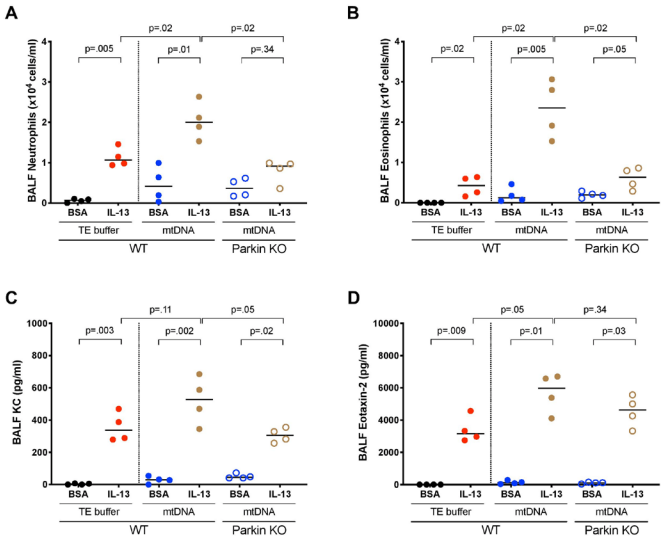

Figure 7 Parkin was necessary to mtDNA-induced lung inflammation in IL-13-treated mice. Wild-type (WT) C57) and Parkin knockout (PKO) mice were intranasally inoculated with three consecutive doses of IL-13 (250 ng/mouse) or BSA. On the final IL-13 treatment, sonicated mtDNA (5 µg/mouse) or TE buffer was administered via the oropharyngeal route. After 24 hours, bronchoalveolar lavage fluid (BALF) was collected and analysed. (A) Total neutrophil and. (B) eosinophil counts in BALF. (C) Proinflammatory markers KC/CXCL1 and (D) eotaxin-2 were measured in the cell-free BALF using ELISA. Data were analysed using the Mann-Whitney test. The horizonal bars represent medians. BSA, bovine serum albumin; mtDNA, mitochondrial DNA.

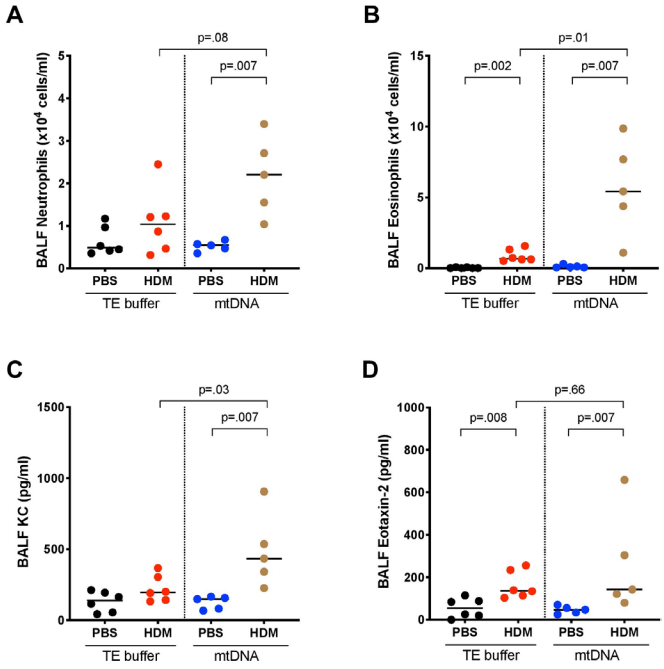

Figure 8 Exogenous fragmented mtDNA amplified airway inflammation in allergic mice. BALB/c mice were sensitised with two doses of house dust mite (HDM) (10 µg/mouse) or PBS, followed by three daily challenges of HDM (10 µg/mouse) or PBS via intranasal inoculation as well as three daily challenges of sonicated mtDNA (5 µg/mouse) extracted from a normal mouse lung or TE buffer via oropharyngeal administration. After 24 hours, bronchoalveolar lavage fluid (BALF) was collected and analysed. (A) Neutrophil and (B) eosinophil counts in BALF. (C) Proinflammatory markers KC/CXCL1 and (D) eotaxin-2 were measured in the cell-free BALF using ELISA. Data were analysed using the Mann-Whitney test. The horizonal bars represent medians. mtDNA, mitochondrial DNA.

the BALF (figure 6A,B). Importantly, mtDNA release as well as eotaxin-2 levels in the BALF of the allergic WT mice were higher than the allergic PKO mice (figure 6C,D). However, KC/CXCL1 levels showed no significant difference between the two groups (figure 6E). Overall, these results suggest that Parkin promotes mtDNA release and airway inflammation following IL-13 or allergen exposures.

Parkin-enhanced mtDNA-mediated inflammation in a mouse model of IL-13 challenge

We have shown that Parkin is critical to mtDNA release in the airways. To determine whether Parkin also regulates mtDNA-mediated inflammation in IL-13 exposed airways, mtDNA was instilled into airways of IL-13-exposed WT and PKO mice. mtDNA treatment alone in both WT C57 and PKO mice did not exert any significant proinflammatory effect. However, mtDNA in IL-13-treated WT mice significantly increased neutrophils and eosinophils (figure 7A,B). Notably, the loss of Parkin significantly reduced the recruitment of both neutrophils and eosinophils triggered by exogenous mtDNA. Interestingly, PKO mice (vs WT mice) treated with both IL-13 and mtDNA significantly reduced the levels of neutrophil chemokine KC/CXCL1, but not eotaxin-2, in BALF (figure 7C,D).

mtDNA amplified neutrophilia and eosinophilia in a mouse model of airway allergic inflammation

We used the HDM challenge model in WT BALB/c mice to test if mtDNA exacerbates airway inflammation in an allergic/type 2 inflammation setting. As expected, HDM challenges significantly increased the number of eosinophils (figure 8B) as well as the levels of eotaxin-2 (figure 8D). Although mtDNA treatment alone (vs compared with the TE buffer control) did not significantly change airway inflammation, mtDNA instillation in conjunction with HDM resulted in a significant increase of neutrophils and eosinophils (figure 8A,B), which was paralleled by the enhanced levels of KC/CXCL1 and eotaxin-2 (figure 8C,D). Taken together, these data indicate that mtDNA in an allergic setting exacerbate both neutrophilic and eosinophilic inflammation.

DISCUSSION

The present study shows the first evidence of Parkin dysregulation and function in allergic airway inflammation. We have demonstrated that in a type 2 cytokine/allergic setting, Parkin is upregulated and promotes the release of mtDNA. Notably, we have found that Parkin contributes to mtDNA release and in turn mediates mtDNA-induced exacerbation of airway inflammation.

The two most common endotypes of asthma are the type 2 high inflammation and the type 2 low inflammation. The type 2 high is characterised by airway and blood eosinophilia and the presence of type 2 cytokines IL-4, IL-5 and IL-13,[33] while the type 2 low exhibits a neutrophil-dominant disease process.[34] Mitochondrial dysfunction has been reported in both endotypes of asthma,[3 4] which was attributed to oxidative stress, including increased ROS formation,[35] inactivation of the electron transport chain[3] and reduced production of antioxidants.[36] The increased oxidative stress can trigger mitophagy (mitochondrial autophagy),

a process of removing excessive or damaged mitochondria. Our present study focused on the regulation and function of Parkin, a key protein involved in mitophagy, in airway inflammation. We demonstrated that Parkin upregulation in asthmatic airway epithelium is coupled with increased FeNO and periostin expression, markers of type 2 inflammation, and the release of airway mtDNA. We also discovered that type 2 cytokine IL-13 increased Parkin expression in airway epithelium, suggesting the contribution of the type 2 cytokine environment to Parkin upregulation in asthma. Future studies are warranted to determine how IL-13 regulates Parkin as well as whether oxidative stress modulates Parkin expression in airway epithelial cells.

The function of Parkin in airway inflammation of asthma has not been investigated. In the present study, we found that Parkin promotes airway eosinophilic and/or neutrophilic inflammation by using the airway epithelial ALI culture system and mouse models of IL-13 treatment and allergen challenge. Mechanistically, we have shown that Parkin promoted IL-13-mediated release of mtDNA in human airway epithelial cells and in BALF of IL-13-challenged and allergic mice. Furthermore, we demonstrated that administration of exogenous lung tissue-derived mtDNA to IL-13-treated or HDM-challenged WT mice significantly amplified both neutrophilic and eosinophilic inflammation. Our findings have explicitly revealed the ability of mtDNA in amplifying airway inflammation and extended previous studies suggesting the association of excessive airway mtDNA with disease severity of asthma,[37–39] idiopathic pulmonary fibrosis[40] and COPD.[5 41]

The classical function of Parkin is an E3 ubiquitin ligase that ubiquitinates and delivers substrates for proteasomal degradation. At the same time, Parkin maintains mitochondrial homeostasis by removing damaged mitochondria via mitophagy, which likely can reduce mtDNA release. Therefore, our finding that Parkin promoted mtDNA release in asthma seems to be contradictory to previous belief. IL-13 has been shown to induce mitochondrial damage such as decreased membrane potential, increased oxidative stress and cytochrome c release in human airway epithelial cells.[42] As the mixed airway neutrophilic and eosinophilic inflammation represents a more severe endotype of asthma,[43] our data support that Parkin upregulation may serve as one of the mechanisms underlying persistent airway inflammation in severe asthma where oxidative stress is one of the common features.[44] Although we propose that increased Parkin expression may lead to mitochondrial dysfunction and subsequent release of mtDNA, the present study did not focus on measuring mitophagy (eg, PINK1), mitochondrial dysfunction (eg, ROS production) and structural changes under IL-13 treatment in cultured cells and mouse models. These mechanisms will be pursued in our future experiments.

How mtDNA is increasingly released in asthmatic airways or in IL-13-stimulated airway epithelial cells remains unclear. mtDNA can be released from either passive (ie, cell death due to mechanical and biological injury) or active (ie, microparticles/vesicles and exocytosis) mechanisms.[45] Based on our lactate dehydrogenase assay (data not shown), IL-13 stimulation *in vitro* and *in vivo* did not induce cell death. Thus, cell death unlikely serves as the major mechanism of passive mtDNA release under IL-13 stimulation. In addition, calcium overloading, a secondary active mechanism of mtDNA release, facilitates the opening of the mitochondrial permeability transition pore[46] that can lead to cell death as well as release of mtDNA.[45] Whether active mtDNA release is involved in increased mtDNA release in IL-13-stimulated cells with Parkin upregulation deserves further investigation.

An exciting and 'unexpected' finding in our study is on the role of Parkin in enhancing mtDNA-mediated airway inflammation *in vivo*. We found that Parkin is necessary to promote mtDNA release and is critical to maintain IL-13-mediated airway neutrophilic and eosinophilic inflammation. We still do not know how Parkin-mediated mtDNA release induces the inflammatory response. It has been proposed that mtDNA activates proinflammatory responses through Toll-like receptor 9, NLRP3 inflammasome and STING signalling.[6 47] It will be interesting to examine which of these signalling pathways may be responsible for mtDNA-induced proinflammatory responses in the context of type 2 cytokines or allergic inflammation.

Our study has several limitations. First, we observed no difference of IL-8 levels in PKO (vs control) human airway epithelial cells stimulated with IL-13, which is not supportive of decreased KC/CXCL1 in BALF of IL-13 exposed PKO mice. We speculate that this might be due to the difference of cell population/composition *in vitro* versus *in vivo* where additional types of lung cells including macrophages may also produce KC/CXCL1. Thus, it will be interesting to determine whether Parkin may have different regulatory roles in neutrophil chemoattractant production among various types of cells. Second, we examined Parkin protein expression but not Parkin activity (eg, ubiquitin-protein ligase activity) in our samples. Oxidative and nitrosative stress observed in asthma[2 48] may alter Parkin activity. Our future work will investigate if Parkin upregulation correlates with Parkin activity in asthma. Third, we still observed the inflammatory response in HDM-challenged PKO mice in which mtDNA was not increased. We speculate that Parkin/mtDNA-mediated inflammation is not fully responsible for HDM-induced airway inflammation. In the absence of Parkin, the inflammatory responses may be mediated by other pathways such as NF-κB[14 49] or NLRP3 inflammasome[50] that could be activated during allergen challenges. It will be interesting to determine if and how mtDNA signalling interact other inflammatory mechanisms in allergic airways. Fourth, we understand the relatively small sample size in some experiments. Nonetheless, given the nature of consistent and robust data shown in figures 3 and 7, our findings support a role of excessive Parkin expression in promoting airway inflammation. Fifth, our mouse models of IL-13 treatment and allergen challenges are of acute nature, which were aimed to examine the effects of Parkin and mtDNA in acute exacerbations of asthma. How Parkin plays a role in chronic or stable asthma deserves future studies using chronic mouse models of asthma and repeated treatments of IL-13 in human airway epithelial cell cultures. Additionally, we will investigate whether Parkin-mediated inflammation leads to airway hyper-responsiveness and airway remodelling, two key features in asthma pathogenesis. Finally, although we demonstrated that mtDNA amplifies airway inflammation in IL-13-treated or allergen-treated mice, the role of mtDNA in human asthma pathogenesis needs to be further established in a longitudinal cohort.

In conclusion, we demonstrated for the first time that Parkin promotes mtDNA release and subsequently induces airway inflammation. Our findings may inspire scientists to further study the role of Parkin in the pathogenesis of asthma. Targeted therapy towards Parkin and associated mtDNA release and function may be effective in preventing or reversing asthma features.

Contributors KGD and HWC drafted the manuscript and designed the experiments. KGD performed the experiments and data analysis. NS helped with the mouse models. RJM, CK and NP provided the human samples. RAG and FH reviewed the manuscript. All authors have read, reviewed and approved the final manuscript.

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