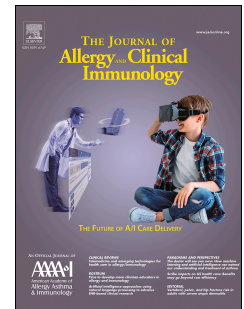


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CaMKII Oxidation Regulates Cockroach Allergen-Induced Mitophagy in Asthma

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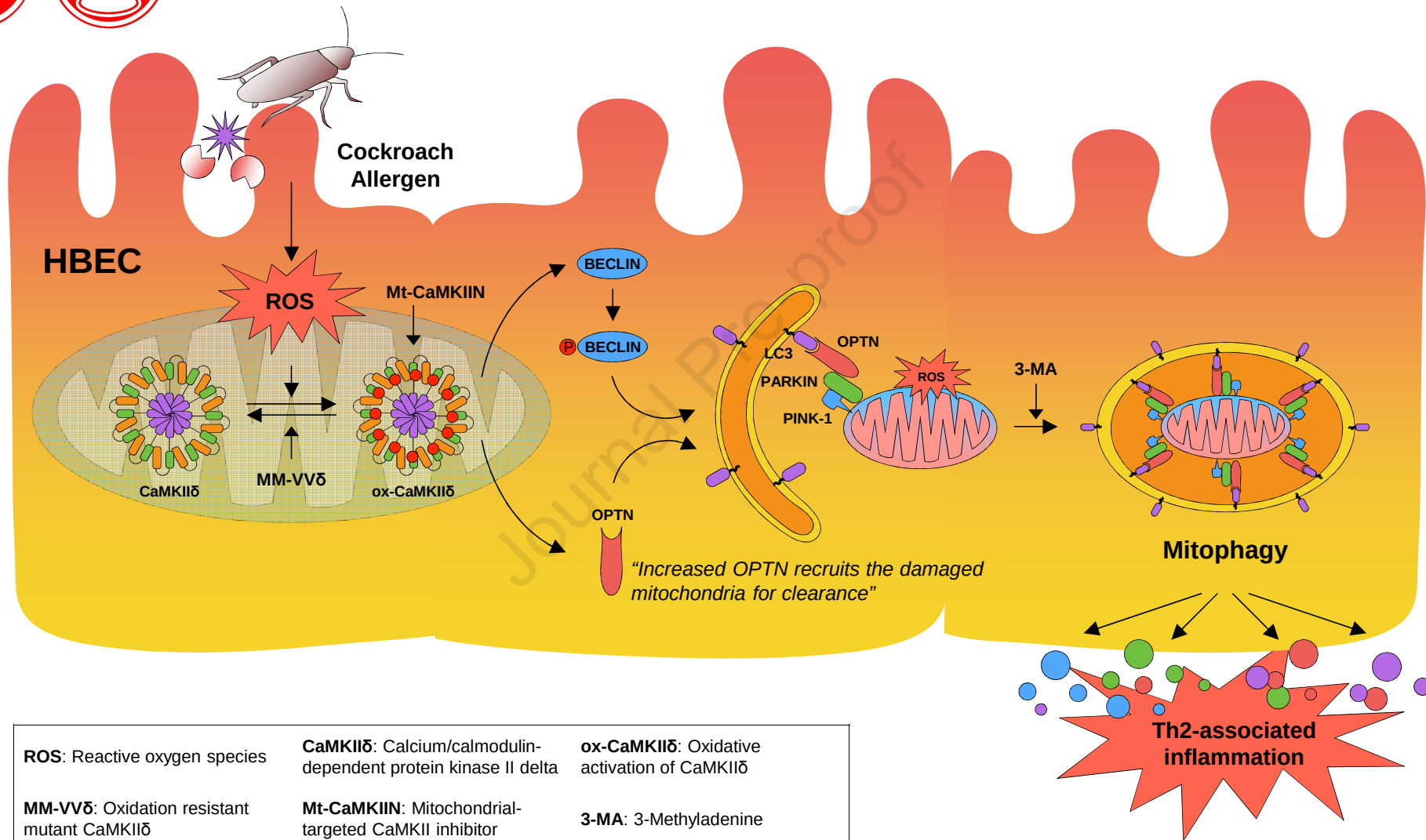
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ROS: Reactive oxygen species

MM-VVδ: Oxidation resistant mutant CaMKIIδ

LC3: Microtubule-associated protein 1A/1B-light chain 3

CaMKIIδ: Calcium/calmodulin-dependent protein kinase II delta

Mt-CaMKIIN: Mitochondrial-targeted CaMKII inhibitor

OPTN: Optineurin

ox-CaMKIIδ: Oxidative activation of CaMKIIδ

3-MA: 3-Methyladenine

HBEC: Primary human bronchial epithelial cells

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The authors declare that they have no relevant conflicts of interest.

25 ABSTRACT

26 **Background:** Autophagy plays an important role in causing inflammatory responses initiated by
27 environmental pollutants and respiratory tract infection.

28 **Objective:** We sought to investigate the role of cockroach allergen-induced excessive activation of
29 autophagy in allergic airway inflammation and its underlying molecular mechanisms.

30 **Methods:** Environmental allergen-induced autophagy was investigated in the primary human
31 bronchial epithelial cells (HBECs) and lung tissues of asthmatic mouse model and patients. The role of
32 autophagy in asthma development was examined by using autophagy inhibitor 3-Methyladenine
33 (3-MA) in an asthma mouse model. Further, the involvements of reactive oxygen species (ROS) and
34 oxidized Ca^{2+} /calmodulin-dependent protein kinase II (ox-CaMKII) signaling in regulating autophagy
35 during asthma were examined in allergen-treated HBECs and mouse model.

36 **Results:** Cockroach allergen activated autophagy in HBECs and in the lung tissues from asthmatic
37 patients and mice. Autophagy inhibitor 3-MA significantly attenuated airway hyper-responsiveness
38 (AHR), Th2-associated lung inflammation and ROS generation. Mechanistically, we demonstrated a
39 pathological feedforward circuit between cockroach allergen-induced ROS and autophagy that is
40 mediated through CaMKII oxidation. Furthermore, transgenic mice with ROS-resistant CaMKII
41 MM-VV δ showed attenuation of Th2-associated lung inflammation and autophagy. Mitochondrial
42 ox-CaMKII inhibition induced by adenovirus carrying mitochondrial-targeted inhibitor peptide
43 CaMKIIN suppresses cockroach allergen-induced autophagy, mitochondrial dysfunction, mitophagy,
44 and cytokine production in HBECs. Finally, mitochondrial CaMKII inhibition suppressed the
45 expression of one of the key ubiquitin binding autophagy receptors, optineurin (OPTN), and its
46 recruitment to fragmented mitochondria. OPTN knockdown inhibited cockroach allergy-induced
47 mitophagy.

48 **Conclusion:** Our data suggest a previously uncovered axis of allergen-ROS-ox-CaMKII-mitophagy in

the development of allergic airway inflammation and asthma.

Key words: Asthma, Cockroach allergen, ROS, CaMKII, Mitochondria, Autophagy, Mitophagy

Key Messages

- Cockroach allergen can induce autophagy and ROS in airway epithelial cells.
- There is a feedforward connection between cockroach allergen-induced ROS and autophagy.
- Excessive activation of autophagy contributes to airway hyper-responsiveness, ROS generation, and Th2-associated lung inflammation.
- ROS-activated CaMKII plays a role in downstream regulation of autophagy/mitophagy.

Capsule summary

Allergen-induced excessive activation of autophagy in airway epithelium leads to increased ROS generation and release of pathogenic cytokines through oxidized CaMKII that may contribute to the development of Th2-associated allergic airway inflammation and asthma.

73 **Abbreviations**

74	CRE	Cockroach extract
75	AHR	Airway hyper-responsiveness
76	ROS	Reactive oxygen species
77	mtROS	Mitochondrial ROS
78	NAC	N-Acetyl Cysteine
79	HBEC	Human bronchial epithelial cell
80	BALF	Bronchoalveolar lavage fluid
81	CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
82	ATG	Autophagy-related gene
83	3-MA	3- methyladenine
84	AMPK	AMP-activated protein kinase
85	PINK1	PTEN-induced putative protein kinase 1
86	CaMKIIN	Adenoviral transduction with the CaMKII peptide inhibitor
87	OPTN	Optineurin.
88		
89		

INTRODUCTION

Autophagy is an important homeostatic process by which damaged proteins and organelles are captured by autophagosomes and taken to lysosomes for degradation during cellular stress¹. Studies have demonstrated autophagy participation in inflammatory diseases including asthma^{2,3}. Increased autophagy has been observed in sputum granulocytes, peripheral blood cells, and peripheral eosinophils from patients with severe asthma⁴. Increased autophagy has also been associated with asthma immune mechanisms, extracellular matrix deposition, and airway remodeling^{5,6}. It has been reported that autophagy can be induced by reactive oxygen species (ROS), which facilitate cellular adaptation and diminish oxidative damage⁷. These findings raise the possibility that allergens may induce ROS as signaling molecules to initiate the process of the autophagic cycle. However, the molecular mechanisms underlying the interplay between ROS and autophagy in the development of asthma remain unclear.

The Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) is a serine/threonine-specific protein kinase that is initially activated by binding calcified calmodulin. Once activated by Ca²⁺/calmodulin, CaMKII activity can be sustained, even after Ca²⁺/calmodulin unbinding by oxidation of regulatory domain methionines 281/282 (ox-CaMKII)⁸. Increased expression of ox-CaMKII has been correlated with the severity of asthma⁹, and airway epithelial mitochondrial CaMKII plays an important role in mitochondrial ROS generation and allergic inflammation¹⁰. We have previously demonstrated that oxidant-resistant CaMKII, due to knock in replacement of methionine 281 and 282 with valines on the CaMKII δ isoform (MM-VV δ), protects against cockroach allergen-induced ROS generation and airway inflammation¹¹. Recent studies suggested that CaMKII can regulate autophagy by directly phosphorylating Beclin 1¹², a central player in the formation and maturation of autophagosome¹³. Beclin 1 also plays a critical role in the initiation of mitophagy by facilitating parkin translocation to mitochondria¹⁴. Mitophagy, also called mitochondrial autophagy, was first introduced by Lemasters *et*

114 *al* to describe the selective degradation of mitochondria by autophagy¹⁵. Mitophagy can specifically
115 target damaged mitochondria for degradation to maintain cellular homeostasis¹⁶. The PTEN-induced
116 putative kinase 1 (PINK1) and Parkin-mediated pathway has been recognized as a key regulatory
117 mechanism of mitophagy^{17, 18}.

118 In the current study, we provided new evidence that excessive autophagy plays a critical role in
119 cockroach allergen-induced airway epithelial ROS generation, cytokine release, and subsequent
120 allergic airway inflammation. By investigating the underlying mechanisms, we revealed a feedforward
121 connection between cockroach allergen-induced ROS and autophagy in airway epithelia cells. Our
122 studies suggest that CaMKII is one of the key signaling pathways downstream to ROS, and that
123 ox-CaMKII regulates allergic lung inflammation and autophagy/ mitophagy. We further identified
124 optineurin (OPTN) as a critical component in the signaling pathway regulating mitophagy that was
125 controlled by ox-CaMKII. Our data provide insights into the development of allergic airway
126 inflammation in asthma.

127

128 **METHODS**

129 **Animal**

130 All animal experimentations were carried out using 6-8-week-old mice. C57BL/6J (WT, stock: 000664)
131 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). ROS-resistant CaMKII-delta
132 (MM-VV δ) mutant mice with C57BL/6 background were provided by Professor Mark Anderson at
133 Johns Hopkins University¹¹. Animals were maintained under specific pathogen-free conditions at the
134 animal facility of the Johns Hopkins University School of Medicine. All experiments were carried out
135 with age- and gender-matched controls. The experimental protocol was reviewed and approved by the
136 Institutional Animal Care and Use Committee of the Johns Hopkins University, Baltimore, MD.

137 **Human lung tissues**

138 Dr. Kollarik at the Johns Hopkins Asthma Center Histology laboratory provided paraffin-embedded
139 human airway sections from asthmatic and healthy individuals. The Johns Hopkins Institutional
140 Review Board (IRB) has approved my study with an approval number NA_00046988.

141 **Cockroach Allergen-induced mouse model of asthma**

142 Cockroach Allergen-induced mouse model of asthma was generated as previously described¹⁹. In some
143 cases, mice were treated with 3-methyadenine (3-MA, 30 mg/kg, Sigma-Aldrich, St. Louis, MO) or
144 saline vehicle control by intraperitoneal administration 2 h prior to every single challenge.

145 **Airway hyper-responsiveness**

146 Airway responsiveness was measured as previously described¹⁹.

147 **Analysis of lung inflammation**

148 Cockroach allergen-induced lung inflammation was determined by the analyses of lung tissue
149 histology and inflammatory cells and cytokines in bronchoalveolar lavage (BAL) fluids as previously
150 described^{11, 19-21}.

Enzyme •linked immunosorbent assay (ELISA)

Levels of IL-4, IL-5, IL-13, IL-17, IL-10, TGF β 1, TSLP, IL-33, and IL-25 were measured by ELISA with Ready-Set-Go! ELISA Kits (ThermoFisher) according to the manufacturer's instructions. Serum levels of allergen specific IgE and IgG1 were also analyzed by ELISA as previously described¹⁹.

Immunofluorescence staining

Immunofluorescence staining was performed as previously reported¹⁹. Primary antibodies are provided in the Online Repository (Table E1). Fluorescent positive cells or positive staining of tissue sections were quantified in at least four different high-power fields (HPFs) per slide or lung section using Fiji ImageJ v2.00 (NIH) and presented as mean fluorescent intensity per square micrometer.

Western blotting

Western blotting was carried out as previously described¹¹. Primary antibodies are provided in the Online Repository (Table E1).

Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was performed as previously reported¹⁹. Primer sequences are provided in the Online Repository (Table E2).

Assessment of intracellular ROS production

Intracellular ROS were detected using the general and mitochondria-specific oxidative sensitive fluorescent dyes CM-H₂DCFDA (ThermoFisher) and MitoSOX Red (ThermoFisher) as previously reported²⁰.

Transfection of GFP-LC3 and CaMKIIN into airway epithelial cells

EGFP-LC3 was a gift from Karla Kirkegaard²². Approximately 3.0x10⁵ HBECs were seeded onto a 6-well tissue culture plate for 24 h prior to transfection with a plasmid expressing EGFP-LC3 or empty vector (EGFP-EV) control using FuGENE 6 Transfection Reagent (Promega) following the manufacturer's instructions. Similarly, Adenovirus containing the cDNA for mitochondrial-targeted

175 GFP (Mt-GFP, empty vector) or Mt-CaMKIIN (both at 100 MOI) were transfected for 24 h.

176 Transfection efficiency was assessed by GFP expression using flow cytometry.

177 **Mitochondrial respiration**

178 Mitochondrial respiration was determined by measuring oxygen consumption rates (OCRs) with

179 Seahorse extracellular flux assay kit (Agilent) on a seahorse XF96 Extracellular Flux Analyzer,

180 according to the manufacturer's protocol. Briefly, HBECs were plated onto wells of a 1% gelatin

181 coated XF96 plate for 24 h, and then treated with CRE or PBS control for 48 h. Oxygen consumption

182 rates (OCR) were measured using the mitochondrial stress test procedure in DMEM containing 10 mM

183 glucose, 4 mM L-glutamine, and 2 mM sodium pyruvate with pH 7.4. After calibration of the sensor

184 cartridge, the XF96 plate was placed into the seahorse instrument and OCR was measured as p mole

185 per minute per cell. Analysis of mitochondrial function was made using changes in OCR in the

186 presence of 1.0 μ M oligomycin (inhibits mitochondrial ATP synthase), 1.0 μ M FCCP (uncoupler), 0.5

187 μ M Rotenone and Antimycin A (inhibits both complex I and III).

188 **Mitophagy detection**

189 HBECs were transfected with 1 μ g of plasmid expressing the mitochondrially targeted Keima

190 (MT-mKeima-Red, addgene)²³ using FuGENE 6 Transfection Reagent (Promega) followed by

191 treatment with CaMKIIN or GFP control. The fluorescent protein Keima has an excitation spectrum

192 that changes according to pH. A short wavelength (440 nm) is predominant for excitation in a neutral

193 environment, whereas a long wavelength (586 nm) is predominant in an acidic environment that occurs

194 when mitochondria are fused with lysosomes. Mitophagy was quantified through the difference in

195 fluorescent wavelengths [586 nm (green) /440 nm (red)] from obtained images using Fiji ImageJ

196 v2.00-rc-69/1.52p (NIH). Nuclear staining was carried out using 0.5 μ g/ml of Hoechst 33258

197 (ThermoFisher).

198 **Statistical analysis**

199 All data were analyzed with Graph Pad Prism statistical software program (GraphPad Software, La
200 Jolla, CA) and are expressed as mean $\pm$ SEM. Statistical significance for normally distributed samples
201 was assessed by an independent two-tailed Student's *t* test or one-way analysis of variance (ANOVA)
202 followed by Turkey's post-hoc test. A $P < 0.05$ was considered statistical significance for all analyses.

203

RESULTS

Cockroach allergen induces autophagy in human airway epithelial cells and asthmatic lung tissues

To determine whether cockroach allergen (CRE) exposure can elicit autophagy, the expression of autophagy-associated genes was detected in CRE-treated human bronchial epithelial cells (HBECs). Western blot analysis illustrated a dose-dependent induction of autophagy by CE treatment in HBECs, as assessed by the ratio of microtubule-associated protein 1A/1B-light chain 3 (LC3) (Fig 1, A and B). Consistently, a dose-dependent increase in the expression of ATG5 and decrease in p62 were detected in the CRE-treated HBECs. The results were further confirmed by RT-PCR (Fig E1, A). To visualize the induction of autophagy, we transfected a GFP-LC3 construct in HBECs and imaged GFP-LC3 after CRE treatment (20 μ g/ml). As shown in Fig 1, C, the autophagy induction by CRE was increased as monitored by the number of LC3 puncta. In line with the finding, cockroach allergen-induced epithelial activation was observed as assessed by the increased levels of TGF- β 1, IL-25, and IL-33 in supernatants (Fig 1, D). Furthermore, to determine the role of autophagy in the pathogenesis of asthma, we detected the expression of autophagy markers in the lung tissues of patients with asthma and healthy controls by immunofluorescent staining. As expected, there was a significant increase in the expression of LC3B and ATG5 in the lung tissues of asthmatic patients compared with healthy controls (Fig 1, E and F). We also examined the expression of Lc3b and Atg5 in the lung tissues of mouse model of asthma^{20, 24}. Consistently, CRE-treated mice showed significantly increased expression of Lc3b in the lung tissues (Fig E1, B and C). The Lc3b was predominantly expressed in the airway epithelium as assessed by the co-localization of Lc3b and epithelial cell marker EpCAM. Similarly, increased expression of Atg5 was observed in the lung tissues of these mice (Fig E1, B and D). Together, these data indicate that cockroach allergen can induce autophagy in primary airway epithelial cells and lung tissues of asthmatic patients and mice.

Inhibition of autophagy suppresses allergen-induced airway hyper-responsiveness and lung inflammation

Next, we investigated the role of autophagy in cockroach allergen-induced airway hyper-responsiveness (AHR) and lung inflammation by using autophagy inhibitor 3-MA in our acute mouse model of asthma following the protocol illustrated in Fig 2, A. 3-MA is a class III group of phosphoinositide 3-kinase (PI3K) inhibitors that has been widely used as autophagy inhibitor²⁵. Compared to controls, CRE challenge induced an increased airway resistance, which was attenuated by 3-MA treatment (Fig 2, B). Consistent with our previous findings^{20, 21}, CRE challenge increased airway inflammation compared to PBS treatment. When these CRE-challenged mice were pre-treated with 3-MA, the increased lung inflammation was significantly abrogated. Specifically, 3-MA treatment suppressed cockroach allergen-induced peribronchial inflammation (H&E) and goblet cell hyperplasia (PAS) (Fig 2, C and D). Moreover, these 3-MA-treated mice showed a marked reduction in the number of total inflammatory cells, particularly eosinophils in the BAL fluids (Fig 2, E). Serum levels of cockroach allergen specific IgE and IgG1 (sIgE and sIgG1) were also reduced in 3-MA-treated mice (Fig 2, F). Furthermore, mice treated with 3-MA showed reduced IL-4, IL-5, IL13 and TGF- β 1, but unchanged IL-17 α and IL-10 levels in the BAL fluids (Fig 2, G). These results provide evidence that inhibition of autophagy can prevent cockroach allergen-induced Th2 inflammation. Additionally, 3-MA treated mice also showed reduced levels of epithelial-derived cytokines TGF β 1, IL-33, and TSLP in the BAL fluids (Fig E2, A), which were further supported by *in vitro* analyses of 3-MA treated CRE-challenged HBECs (Fig E2, B).

Feedforward connection between cockroach allergen-induced ROS and autophagy

Recent studies have suggested a cross talk between ROS and autophagy^{7, 26}. Thus, we asked whether inhibition of autophagy could suppress ROS generation in our mouse model of asthma. Indeed, we

found increased ROS expression in the lung tissues of CRE-treated mice (Fig 3, A and B), which was abolished when these mice were pre-treated with 3-MA. The results were further supported by *in vitro* analyses of intracellular and mitochondrial ROS production. Compared to untreated cells, CRE-treated HBECs displayed increased mitochondrial ROS as detected by immunostaining for the co-localization of MitoSOX, a fluorescent mitochondrial ROS reporter dye, with MitoTracker green (Fig 3, C and D). Of note, the increased mitochondrial ROS were significantly inhibited by 3-MA. Similar patterns were observed when intracellular ROS were detected by flow cytometry with CM-H₂DCFDA (Fig 3, E and F). These findings suggest that inhibition of autophagy can suppress cockroach allergen-induced ROS generation, particularly mitochondrial ROS.

In contrast, we investigated whether ROS can regulate the activation of autophagy by using antioxidant NAC or mitochondrial-targeted antioxidant MitoTEMPO. Compared to media control, CRE treatment led to an increase in the expression of LC3 and ATG5 as detected by Western blot (Fig 3, G and H), which were significantly inhibited by either NAC or MitoTEMPO. In contrast, the CRE-induced decrease in p62 was fully recovered by either NAC or MitoTEMPO treatment. Compared to CRE, no clear autophagy induction was observed for either NAC or MitoTEMPO (Fig E3, A and B). Collectively, these data suggest a feedforward connection between cockroach allergen-induced ROS and autophagy.

Cockroach allergen induces mitochondrial CaMKII oxidation in airway epithelial cells

Our previous studies suggested a critical role of CaMKII oxidation in mast cell activation and the development of asthma¹¹. Here, we examined whether cockroach allergen can induce CaMKII oxidation in airway epithelial cells. HBECs were treated with CRE at different concentrations for 24 h, and expression of ox-CaMKII was detected by immunostaining. We found a dose-dependent increase in the expression of ox-CaMKII in HBECs after CRE treatment (Fig E4, A and B). Furthermore, the

increased ox-CaMKII was predominantly localized in mitochondria as stained with TOM20, a specific marker for mitochondria. Our results indicate that cockroach allergen can induce CaMKII oxidation, specifically in mitochondria.

ROS-resistant CaMKII prevents allergen-induced autophagy

To better understand the role of ox-CaMKII in asthma, we used an oxidant-resistant CaMKII MM-VV δ (Fig 4, A) to generate a mouse model of asthma^{20, 24}. Histological analysis demonstrated an increased expression of ox-CaMKII in cockroach allergen-induced mouse model of asthma, particularly in the airway epithelial cells, by co-immunostaining of ox-CaMKII and EpCaM (Fig 4, B and C). As expected, ox-CaMKII expression was almost completely absent in the CaMKII MM-VV δ mice. Furthermore, these mice showed protection against CRE-induced peribronchial inflammation (H&E) and goblet cell hyperplasia (PAS) (Fig E5, A and B). These CaMKII MM-VV δ mice also showed less total inflammatory cells, particularly eosinophils, in the BAL fluids (Fig E5, C). We next investigated an un-explored link between CaMKII oxidation and autophagy in allergic asthma. Compared to PBS, CRE-treated WT mice showed increased expression of Lc3b in the airways (Fig 4, D and E), which was attenuated in CaMKII MM-VV δ mice. CRE-induced expression of Atg5 in the airways was similarly attenuated in the CaMKII MM-VV δ mice (Fig 4, D and F). In contrast, p62 expression was reduced in CRE-induced WT mice, but increased in CaMKII MM-VV δ mice (Fig 4, D and G). These findings were further supported by RT-PCR (Fig E5, D) and Western blot analyses (Fig 4, H and I). Collectively, these data suggest that CaMKII oxidation may regulate cockroach allergen-induced lung inflammation and autophagy.

Inhibition of mitochondrial CaMKII suppresses autophagy

To confirm the role of CaMKII in regulating autophagy, we investigated whether inhibiting

mitochondrial CaMKII can affect cockroach allergen-induced autophagy. HBECS were infected with adenovirus containing a mitochondrial-targeted inhibitor peptide CaMKIIN (Mt-CaMKIIN) or control adenovirus and confirmed by immunostaining (Fig E6, A and B). We found that CRE exposure increased both LC3 and ATG5, but decreased p62 in HBECS as detected by immunostaining (Fig E6, C and D). However, the increased expression of LC3 and ATG5 and decreased p62 were reversed in HBECS pre-transduced with Mt-CaMKIIN. Similar observations were also made by Western blot analysis (Fig 5, A and B). Thus, the data support a view that mitochondrial CaMKII regulates autophagy in bronchial epithelial cells.

We also explored the further downstream signal molecules that mediate CaMKII regulated autophagy. CaMKII has been shown to regulate autophagy by directly phosphorylating Beclin 1¹². Consistent with this finding, CRE induced an increase in the phosphorylation of Beclin 1, which was reversed by Mt-CaMKIIN treatment (Fig 5, C and D). Furthermore, we tested several significant signaling molecules [e.g., AMPK, mTOR] that are critical in the initiation of autophagosome²⁷. Similar to Beclin 1, CRE induced an increase in the phosphorylation of AMPK (p-AMPK) and a decrease in the phosphorylation of mTOR (p-mTOR). Of interest, the CRE-induced increase in p-AMPK and decrease in p-mTOR were reversed by Mt-CaMKIIN. Therefore, CaMKII may play a critical role in cockroach allergen-induced initiation of autophagosome via multiple signaling pathways.

Inhibition of mitochondrial CaMKII protects against allergen-induced mitochondrial respiration

Elevated levels of mitochondrial ROS may lead to mitochondrial dysfunction. We investigated oxygen consumption rate (OCR) in CRE-treated HBECS, an indicator of mitochondrial respiration, as illustrated in Fig 6, A. Basal and maximal respiration, ATP production, and spare respiration capacity were measured in HBECS after 48 hours of CRE treatment (Fig E7, A). Compared to control cells, CRE-challenged cells showed substantially decreased basal respiration rate and ATP production, and

increased H^+ leak after blocking the proton channel subunit F₀ of the ATP synthase with oligomycin (FIG E7, B). Maximal respiration elicited by FCCP, which discharges the pH gradient across the inner mitochondrial membrane, was also substantially lower in CRE-treated HBECs. Similar patterns were observed for the spare respiration capacity. We next investigated the potential effects of ox-CaMKII on cockroach allergen-induced mitochondrial respiration by pre-treating HBECs with Mt-CaMKIIN as illustrated in Fig 6, B. Pre-treatment of HBECs with Mt-CaMKIIN reversed the CRE-induced reduction in basal and maximal respiration, ATP production, and spare respiration capacity in HBECs (Fig 6, C). Together, these data align with previous studies²⁸ indicate that inhibition of mitochondrial CaMKII protects against allergen-induced mitochondrial dysfunction in airway epithelial cells.

Inhibition of mitochondrial CaMKII suppresses cockroach allergen-induced mitophagy

Next, we examined the cockroach allergen-induced mitophagy using mitochondrially targeted Keima-Red (MT-mKeima-Red)²³, which can track the mitochondrial delivery to lysosomes (Fig 7, A). HBECs were transfected with plasmid MT-mKeima-Red and then treated with CRE or PBS. The mitochondria-localized MT-mKeima-Red was displayed in CRE, but not in PBS-treated HBECs (Fig 7, B), indicating an induction of mitophagy by cockroach allergen. We next investigated whether the inhibition of mitochondrial CaMKII can affect cockroach allergen-induced mitophagy. HBECs were transduced with the inhibitory peptide CaMKIIN, then transfected with plasmid MT-mKeima-Red. As expected, Mt-CaMKIIN significantly inhibited CRE-induced mitophagy (Fig 7, C). Furthermore, we examined whether inhibition of mitochondrial CaMKII can affect cockroach allergen-induced epithelial cytokine release. We found higher levels of TGF- β 1, IL-25, IL-33, and TSLP in supernatants of CRE-treated HBECs as compared to controls (Fig 7, D), whereas TGF β 1, IL-25 and TSLP, but not IL-33, were significantly reduced in Mt-CaMKIIN-transfected HBECs. Thus, these findings suggest that inhibition of mitochondrial CaMKII suppresses cockroach allergen-induced

mitophagy and cytokine release in the airway epithelial cells.

Inhibition of mitochondrial CaMKII suppressed mitophagy through controlling OPTN

Lastly, we explored the underlying mechanisms by which mitochondrial CaMKII regulates mitophagy. We found that CRE stimulated an increase in the expression of ULK1, PINK1 and PARKIN, the most studied pathways for autophagy. These increased expressions were suppressed in HBECs transfected with Mt-CaMKIIN (Fig 8, A). Furthermore, Western blot analyses showed a similar pattern for the expression of PINK1 and PARKIN (FIG E8, A and B). Optineurin (OPTN), one of the ubiquitin binding autophagy receptors, was enhanced by CRE treatment, whereas this enhancement was abolished by Mt-CaMKIIN (Fig 8, A). To confirm the involvement of OPTN in cockroach allergen-induced mitophagy, we transfected a GFP-labelled OPTN in HBECs. HBECs treated with CRE showed the recruitment of OPTN-GFP to fragmented mitochondria as assessed by the robust co-localization of OPTN-GFP with MitoTracker (Fig 8, B and C) and OPTN was stably enriched in over 50% of mitochondria (Fig 8, D). Of note, the recruitment of OPTN-GFP to the fragmented mitochondria was inhibited by Mt-CaMKIIN. Furthermore, we explored the role of OPTN in cockroach allergen-induced mitophagy by OPTN knockdown in HBECs. As expected, knockdown of OPTN attenuated the CRE-induced mitophagy in HBECs (Fig 8, E and F). These results suggest that OPTN is involved in the CaMKII-regulated cockroach allergen-induced mitophagy

DISCUSSION

In the present study, we examined whether cockroach allergen, one of the major triggers for developing asthma, can induce autophagy in the airway epithelial cells and explored the role of autophagy in cockroach allergen-induced airway inflammation and its underlying mechanisms.

Our *in vivo* results demonstrated that autophagy was increased in the lung tissues of cockroach allergen-treated mice, particularly in the airway epithelium of these mice. Further *in vitro* analysis in the human airway epithelial cells revealed that treatment with cockroach allergen led to increased autophagy as indicated by the increased LC3 and ATG5 and decreased p62 expression. Importantly, by visualizing autophagosome formation in real time in live cells using GFP-LC3, a mammalian expression vector containing the human LC3 gene fused at its 5' end to the GFP gene, we found a time-dependent increase in the number of autophagosomes in the airway epithelial cells exposed to cockroach allergen. These results demonstrated that cockroach allergen could directly stimulate autophagy in airway epithelial cells. Although we mainly focused on airway epithelial cells, which are the first line of defense against environmental pollutants and allergens, cockroach allergen could also induce autophagy in other structural and immune cells. Our results support previous findings on the association of autophagy with asthma and provide further evidence that environmental allergens can induce autophagy that may contribute to epithelial cell cytokine release and subsequent airway inflammation and asthma.

Despite of the increased autophagy in asthma, the exact role of autophagy in asthma remains still poorly understood. Our findings indicate that inhibition of autophagy using 3-MA, a widely used autophagy inhibitor that can block the formation of autophagosome, significantly attenuated AHR and lung eosinophilic inflammation and levels of Th2 cytokines. These results suggest that blockage of autophagy may prevent cockroach allergen-induced AHR and Th2-associated inflammation. Recent studies showed very similar results that treatment with 3-MA suppressed ovalbumin (OVA)-induced

eosinophilia, lung inflammation, oxidative stress, mitochondrial damage, and eosinophil extracellular traps formation in asthma²⁹. Furthermore, inhibition of autophagy attenuated the OVA-induced airway epithelial fibrosis³⁰ and HDM-induced airway inflammation, airway hyper-responsiveness, and features of airway fibrosis and remodeling⁵. These findings implicated that autophagy could exacerbate asthma. However, autophagy may also have a protective mechanism given that autophagy is a key homeostatic process in which cytosolic components are degraded and recycled through lysosomes. For example, autophagy deficient mice (*atg5*^{-/-} and *atg7*^{-/-}) develop spontaneous sterile lung inflammation³¹, and deficiency of cell specific autophagy (e.g., *CD11C-Cre:Atg5*^{fl/fl}, *ER-Cre:Atg7*^{fl/fl}) results in increased airway inflammation and airway hyper-reactivity³²⁻³⁴. The reasons for the dual roles of autophagy in asthma remain unclear. It is postulated that autophagy may have a protective role in maintaining homeostasis at baseline or excessive autophagy during acute infection may become detrimental in a prolonged exposure to environmental pollutants/allergens or inflammation³⁵. Furthermore, autophagy may play distinct roles in different cell types, thereby leading to different phenotypic changes.

Increased ROS have been shown to cause airway and lung damage and be associated with severity of asthma³⁶⁻³⁸. We have previously shown that environmental allergens can induce ROS, which are primary mediators to induce airway epithelial cytokine release (e.g., TSLP, IL-33)^{20, 39}. Similarly, ROS have been considered as a key checkpoint responsible for IL-33 release from damaged airway epithelium⁴⁰. Another important role of ROS is to activate autophagy⁷, and that persistent ROS over-production results in the dysregulation of autophagy, leading to airway epithelial cell damage, inflammatory cytokine release, and exacerbation of allergic inflammation in asthma. Thus, an attractive hypothesis would be that ROS, as signaling molecules, might contribute to epithelial barrier dysfunction, cytokine release, and allergic airway inflammation through activating autophagy. Indeed, we found that cockroach allergen-induced autophagy can be blocked by either antioxidant NAC or

mitochondrial-targeted antioxidant MitoTEMPO, suggesting that ROS, particularly mitochondrial ROS, may be critical in cockroach allergen induced autophagy. In contrast, autophagy can also regulate ROS generation. Autophagy is generally thought to eliminate intracellular sources of ROS; however, prolonged activation of autophagy or impaired autophagy under a disease state may promote ROS production⁴¹. Indeed, we found that inhibition of autophagy suppressed cockroach allergen-induced ROS generation in a mouse model of asthma and in the primary airway epithelial cells. Together, these studies suggest a pathological feedforward connection between ROS and autophagy in airway epithelium that may exacerbate cytokine released and allergic airway inflammation in asthma.

Our study also provides more detailed molecular mechanisms underlying ROS-autophagy interplay. It was suggested that ROS can induce autophagy by directly oxidizing autophagy-related proteins²⁶. CaMKII has been suggested to be one of the key signaling pathways downstream to ROS and can be activated to be oxidized CaMKII^{8, 42}. We have previously suggested a strong association for ox-CaMKII with asthma^{9, 11}. Our current studies demonstrated a novel role for ox-CaMKII in cockroach allergen-induced autophagy. First, cockroach allergen can induce the expression of ox-CaMKII in HBECs. Next, the increased ox-CaMKII was significantly abrogated by NAC or MitoTEMPO, indicating that cockroach allergen could induce ox-CaMKII through ROS generation and mitochondrial ROS. Furthermore, ox-CaMKII may serve as a key signaling molecule that contributes to cockroach allergen-induced lung inflammation and autophagy. Especially, CaMKII MM-VV δ mice showed reduced lung inflammation and autophagy in comparison to wild type controls. This raises the possibility that the reduced inflammation may be due to the reduced autophagy in CaMKII MM-VV δ mice and further suggests that ox-CaMKII may be critical in cockroach allergen-induced autophagy and subsequently airway inflammation and asthma.

To identify the underlying mechanisms by which CaMKII regulates autophagy, we tested several key signaling molecules [e.g., Beclin 1¹², AMPK⁴³⁻⁴⁵, mTOR^{43, 46}] for autophagy activation and

determined whether CaMKII is involved in controlling their expression. Of these signaling molecules, Beclin 1 was directly phosphorylated by CaMKII to promote K63-linked ubiquitination of Beclin 1 and activation of autophagy¹². We found that inhibition of CaMKII could attenuate cockroach allergen-induced phosphorylation of Beclin 1, supporting that CaMKII may play a role in cockroach allergen-induced autophagy through phosphorylation of Beclin 1. Similar effects were observed for the phosphorylation of AMPK and mTOR. Additionally, CaMKII may regulate autophagy through other signaling molecules. For example, several transcription factors (e.g., p53, Forkhead box O3 (FOXO3)⁴⁷, Transcription Factor EB (TFEB)⁴⁸] and histone modifications (histone H4K16 acetylation, H3K9 dimethylation, and H3K27 trimethylation)⁴⁹ have drawn attention in regulating autophagy.

Mitochondria are critical in maintaining cellular homeostasis, and the clearance of damaged or depolarized mitochondria are through mitophagy^{17, 50}. Mitophagy is a selective degradation of mitochondria by autophagy¹⁵. It often happens to the damaged mitochondria following the exposure to environmental pollutants/allergens or stress. Defective removal of damaged mitochondria can induce hyper-activation of inflammatory signaling pathways, leading to chronic inflammation. Both enhanced and impaired mitophagy have been associated with COPD and lung fibrosis^{51, 52}. Our current studies provide evidence that cockroach allergen can lead to mitochondrial dysfunction with decreased basal and maximal respiration and ATP turnover rate, and that inhibition of mitochondrial CaMKII can reverse this process. Furthermore, we demonstrated that cockroach allergen can induce mitophagy, and the epithelial mitochondria-targeted inhibition of CaMKII significantly suppressed cockroach allergen-induced mitophagy. Collectively, these data suggest that mitochondrial CaMKII may contribute to the cockroach allergen-mitochondrial dysfunction and mitophagy.

Pink1- and Parkin-mediated mitochondrial autophagy is currently the most well established molecular mechanism for the regulation of mitophagy in mammalian cells^{63, 64}. Active PINK1 accumulates on the outer mitochondrial membrane (OMM) to recruit Parkin to damaged mitochondria,

resulting in the phosphorylation and activation of Parkin. The activated Parkin triggers the poly-ubiquitination of several OMM proteins, which interact directly with LC3 to regulate mitochondrial elimination. Studies have also suggested other proteins that are involved in the molecular regulation of mitophagy, such as Nip3-like protein X (NIX), BCL2 interacting protein 3 (BNIP3), and Fun14 domain-containing protein 1 (FUNDC1)^{16, 53, 54}. In this study, we suggest one of the novel mechanisms that CaMKII modulates mitophagy through regulating OPTN, an ubiquitin binding autophagy receptor. OPTN can bind both ubiquitin and LC3 to target ubiquitinated substrates to newly forming autophagosomes for the autophagic clearance of damaged mitochondria¹. Our studies demonstrated that cockroach allergen can induce the recruitment of OPTN to fragmented mitochondria, and OPTN knockdown protects against cockroach allergy-induced mitophagy. Thus, one of the potential mechanisms could be that CaMKII regulates mitophagy through regulating OPTN. However, future studies are needed to explore the mechanisms for ox-CaMKII-regulated OPTN and mitophagy.

In summary, we have provided evidence for the first time that cockroach allergen induces autophagy in airway epithelial cells. Inhibition of autophagy suppresses cockroach allergen-induced AHR, airway epithelial cytokine release, and Th2-associated lung inflammation. Most importantly, we found that there is a feedforward loop between cockroach allergen-induced ROS generation and autophagy, and identified CaMKII as a linkage molecule that is activated by ROS and lead to downstream regulation of autophagy/mitophagy. We further revealed one of the novel mechanisms that CaMKII modulates mitophagy through regulating OPTN. These results suggest an allergen-ROS-ox-CaMKII-mitophagy axis in the development of allergic airway inflammation, and CaMKII may be an attractive therapeutic target for asthma.

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Author contributions

Y.Z., D.D., J.W., Y.Z., S.M., X.Z., and X.H. performed experiments and analyzed data. Y. Z., D.D., S.M., and P.G. wrote the manuscript. M.W. and P.G. designed, supervised the study, and revised the manuscript. All authors read and approved the final manuscript.

REFERENCES

1. Sachdeva K, Do DC, Zhang Y, Hu X, Chen J, Gao P. Environmental Exposures and Asthma Development: Autophagy, Mitophagy, and Cellular Senescence. *Front Immunol* 2019; 10:2787.
2. Lee J, Kim HS. The Role of Autophagy in Eosinophilic Airway Inflammation. *Immune Netw* 2019; 19:e5.
3. Zeki AA, Yeganeh B, Kenyon NJ, Post M, Ghavami S. Autophagy in airway diseases: a new frontier in human asthma? *Allergy* 2016; 71:5-14.
4. Ban GY, Pham DL, Trinh TH, Lee SI, Suh DH, Yang EM, et al. Autophagy mechanisms in sputum and peripheral blood cells of patients with severe asthma: a new therapeutic target. *Clin Exp Allergy* 2016; 46:48-59.
5. McAlinden KD, Deshpande DA, Ghavami S, Xenaki D, Sohal SS, Oliver BG, et al. Autophagy Activation in Asthma Airways Remodeling. *Am J Respir Cell Mol Biol* 2019; 60:541-53.
6. Xia F, Deng C, Jiang Y, Qu Y, Deng J, Cai Z, et al. IL4 (interleukin 4) induces autophagy in B cells leading to exacerbated asthma. *Autophagy* 2018; 14:450-64.
7. Kaushal GP, Chandrashekar K, Juncos LA. Molecular Interactions Between Reactive Oxygen Species and Autophagy in Kidney Disease. *Int J Mol Sci* 2019; 20(150):3791.
8. Erickson JR, Joiner ML, Guan X, Kutschke W, Yang J, Oddis CV, et al. A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation. *Cell* 2008; 133:462-74.
9. Sanders PN, Koval OM, Jaffer OA, Prasad AM, Businga TR, Scott JA, et al. CaMKII is essential for the proasthmatic effects of oxidation. *Sci Transl Med* 2013; 5:195ra97.
10. Sebag SC, Koval OM, Paschke JD, Winters CJ, Jaffer OA, Dworski R, et al. Mitochondrial CaMKII inhibition in airway epithelium protects against allergic asthma. *JCI Insight* 2017; 2:e88297.
11. Qu J, Do DC, Zhou Y, Luczak E, Mitzner W, Anderson ME, et al. Oxidized CaMKII promotes asthma through the activation of mast cells. *JCI Insight* 2017; 2:e90139.
12. Li X, Wu XQ, Deng R, Li DD, Tang J, Chen WD, et al. CaMKII-mediated Beclin 1 phosphorylation regulates autophagy that promotes degradation of Id and neuroblastoma cell differentiation. *Nat Commun* 2017; 8:1159.
13. Kang R, Zeh HJ, Lotze MT, Tang D. The Beclin 1 network regulates autophagy and apoptosis. *Cell Death Differ* 2011; 18:571-80.
14. Choubey V, Cagalinec M, Liiv J, Safiulina D, Hickey MA, Kuum M, et al. BECN1 is involved in the initiation of mitophagy: it facilitates PARK2 translocation to mitochondria. *Autophagy* 2014; 10:1105-19.
15. Lemasters JJ. Selective mitochondrial autophagy, or mitophagy, as a targeted defense against oxidative stress, mitochondrial dysfunction, and aging. *Rejuvenation Res* 2005; 8:3-5.
16. Palikaras K, Lionaki E, Tavernarakis N. Mechanisms of mitophagy in cellular homeostasis, physiology and pathology. *Nat Cell Biol* 2018; 20:1013-22.
17. Matsuda N, Tanaka K. Uncovering the roles of PINK1 and parkin in mitophagy. *Autophagy* 2010; 6:952-4.
18. Koyano F, Okatsu K, Kosako H, Tamura Y, Go E, Kimura M, et al. Ubiquitin is phosphorylated by PINK1 to activate parkin. *Nature* 2014; 510:162-6.
19. Do DC, Mu J, Ke X, Sachdeva K, Qin Z, Wan M, et al. miR-511-3p protects against cockroach allergen-induced lung inflammation by antagonizing CCL2. *JCI Insight* 2019; 4.
20. Qiu L, Zhang Y, Do DC, Ke X, Zhang S, Lambert K, et al. miR-155 Modulates Cockroach Allergen- and Oxidative Stress-Induced Cyclooxygenase-2 in Asthma. *J Immunol* 2018; 201:916-29.
21. Ke X, Do DC, Li C, Zhao Y, Kollarik M, Fu Q, et al. Ras homolog family member

- 555 A/Rho-associated protein kinase 1 signaling modulates lineage commitment of mesenchymal
 556 stem cells in asthmatic patients through lymphoid enhancer-binding factor 1. *J Allergy Clin*
 557 *Immunol* 2019; 143:1560-74 e6.
- 558 22. Jackson WT, Giddings TH, Jr., Taylor MP, Mulinyawe S, Rabinovitch M, Kopito RR, et al.
 559 Subversion of cellular autophagosomal machinery by RNA viruses. *PLoS Biol* 2005; 3:e156.
- 560 23. Vargas JNS, Wang C, Bunker E, Hao L, Maric D, Schiavo G, et al. Spatiotemporal Control of
 561 ULK1 Activation by NDP52 and TBK1 during Selective Autophagy. *Mol Cell* 2019; 74:347-62
 562 e6.
- 563 24. Xu T, Zhou Y, Qiu L, Do DC, Zhao Y, Cui Z, et al. Aryl Hydrocarbon Receptor Protects Lungs
 564 from Cockroach Allergen-Induced Inflammation by Modulating Mesenchymal Stem Cells. *J*
 565 *Immunol* 2015; 195:5539-50.
- 566 25. Wu YT, Tan HL, Shui G, Bauvy C, Huang Q, Wenk MR, et al. Dual role of 3-methyladenine in
 567 modulation of autophagy via different temporal patterns of inhibition on class I and III
 568 phosphoinositide 3-kinase. *J Biol Chem* 2010; 285:10850-61.
- 569 26. Dickinson JD, Sweeter JM, Warren KJ, Ahmad IM, De Deken X, Zimmerman MC, et al.
 570 Autophagy regulates DUOX1 localization and superoxide production in airway epithelial cells
 571 during chronic IL-13 stimulation. *Redox Biol* 2018; 14:272-84.
- 572 27. Ghosh R, Pattison JS. Macroautophagy and Chaperone-Mediated Autophagy in Heart Failure:
 573 The Known and the Unknown. *Oxid Med Cell Longev* 2018; 2018:8602041.
- 574 28. Roy SJ, Koval OM, Sebag SC, Ait-Aissa K, Allen BG, Spitz DR, et al. Inhibition of CaMKII in
 575 mitochondria preserves endothelial barrier function after irradiation. *Free Radic Biol Med* 2020;
 576 146:287-98.
- 577 29. Silveira JS, Antunes GL, Kaiber DB, da Costa MS, Ferreira FS, Marques EP, et al. Autophagy
 578 induces eosinophil extracellular traps formation and allergic airway inflammation in a murine
 579 asthma model. *J Cell Physiol* 2019; 235:267-80.
- 580 30. Cho IH, Choi YJ, Gong JH, Shin D, Kang MK, Kang YH. Astragalin inhibits
 581 autophagy-associated airway epithelial fibrosis. *Respir Res* 2015; 16:51.
- 582 31. Abdel Fattah E, Bhattacharya A, Herron A, Safdar Z, Eissa NT. Critical role for IL-18 in
 583 spontaneous lung inflammation caused by autophagy deficiency. *J Immunol* 2015;
 584 194:5407-16.
- 585 32. Suzuki Y, Maazi H, Sankaranarayanan I, Lam J, Khoo B, Soroosh P, et al. Lack of autophagy
 586 induces steroid-resistant airway inflammation. *J Allergy Clin Immunol* 2016; 137:1382-9 e9.
- 587 33. Inoue D, Kubo H, Taguchi K, Suzuki T, Komatsu M, Motohashi H, et al. Inducible disruption
 588 of autophagy in the lung causes airway hyper-responsiveness. *Biochem Biophys Res Commun*
 589 2011; 405:13-8.
- 590 34. Pu Q, Gan C, Li R, Li Y, Tan S, Li X, et al. Atg7 Deficiency Intensifies Inflammasome
 591 Activation and Pyroptosis in Pseudomonas Sepsis. *J Immunol* 2017; 198:3205-13.
- 592 35. Racanelli AC, Kikkers SA, Choi AMK, Cloonan SM. Autophagy and inflammation in chronic
 593 respiratory disease. *Autophagy* 2018; 14:221-32.
- 594 36. Lee IT, Yang CM. Role of NADPH oxidase/ROS in pro-inflammatory mediators-induced
 595 airway and pulmonary diseases. *Biochem Pharmacol* 2012; 84:581-90.
- 596 37. Poon A, Eidelman D, Laprise C, Hamid Q. ATG5, autophagy and lung function in asthma.
 597 *Autophagy* 2012; 8:694-5.
- 598 38. Rogers LK, Cismowski MJ. Oxidative Stress in the Lung - The Essential Paradox. *Curr Opin*
 599 *Toxicol* 2018; 7:37-43.
- 600 39. Wang E, Liu X, Tu W, Do DC, Yu H, Yang L, et al. Benzo(a)pyrene facilitates
 601 dermatophagoides group 1 (Der f 1)-induced epithelial cytokine release through aryl
 602 hydrocarbon receptor in asthma. *Allergy* 2019; 74:1675-90.

- 603 40. Uchida M, Anderson EL, Squillace DL, Patil N, Maniak PJ, Iijima K, et al. Oxidative stress
604 serves as a key checkpoint for IL-33 release by airway epithelium. *Allergy* 2017; 72:1521-31.
- 605 41. Wible DJ, Bratton SB. Reciprocity in ROS and autophagic signaling. *Curr Opin Toxicol* 2018;
606 7:28-36.
- 607 42. Luczak ED, Anderson ME. CaMKII oxidative activation and the pathogenesis of cardiac
608 disease. *J Mol Cell Cardiol* 2014; 73:112-6.
- 609 43. Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct
610 phosphorylation of Ulk1. *Nat Cell Biol* 2011; 13:132-41.
- 611 44. Kim J, Kim YC, Fang C, Russell RC, Kim JH, Fan W, et al. Differential regulation of distinct
612 Vps34 complexes by AMPK in nutrient stress and autophagy. *Cell* 2013; 152:290-303.
- 613 45. Jang JE, Eom JI, Jeung HK, Cheong JW, Lee JY, Kim JS, et al. AMPK-ULK1-Mediated
614 Autophagy Confers Resistance to BET Inhibitor JQ1 in Acute Myeloid Leukemia Stem Cells.
615 *Clin Cancer Res* 2017; 23:2781-94.
- 616 46. Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. *J Pathol* 2010;
617 221:3-12.
- 618 47. Zhao J, Brault JJ, Schild A, Cao P, Sandri M, Schiaffino S, et al. FoxO3 coordinately activates
619 protein degradation by the autophagic/lysosomal and proteasomal pathways in atrophying
620 muscle cells. *Cell Metab* 2007; 6:472-83.
- 621 48. Napolitano G, Ballabio A. TFEB at a glance. *J Cell Sci* 2016; 129:2475-81.
- 622 49. Baek SH, Kim KI. Epigenetic Control of Autophagy: Nuclear Events Gain More Attention. *Mol*
623 *Cell* 2017; 65:781-5.
- 624 50. Gkikas I, Palikaras K, Tavernarakis N. The Role of Mitophagy in Innate Immunity. *Front*
625 *Immunol* 2018; 9:1283.
- 626 51. Bueno M, Lai YC, Romero Y, Brands J, St Croix CM, Kamga C, et al. PINK1 deficiency
627 impairs mitochondrial homeostasis and promotes lung fibrosis. *J Clin Invest* 2015; 125:521-38.
- 628 52. Tsubouchi K, Araya J, Kuwano K. PINK1-PARK2-mediated mitophagy in COPD and IPF
629 pathogeneses. *Inflamm Regen* 2018; 38:18.
- 630 53. Lazarou M, Sliter DA, Kane LA, Sarraf SA, Wang C, Burman JL, et al. The ubiquitin kinase
631 PINK1 recruits autophagy receptors to induce mitophagy. *Nature* 2015; 524:309-14.
- 632 54. Saito T, Sadoshima J. Molecular mechanisms of mitochondrial autophagy/mitophagy in the
633 heart. *Circ Res* 2015; 116:1477-90.
- 634

635 **FIGURE LEGENDS**

636 **FIG 1.** Cockroach allergen induces autophagy in human airway epithelial cells and asthmatic lung
 637 tissues. **A,** Western blot analysis of LC3-I, LC-3II, ATG5, and p62 in HBECs treated with cockroach
 638 extract (CRE) at different concentrations for 4 h. β -actin was measured as the loading control. **B,**
 639 Quantitative analysis of **(A)** (n=4). **C,** Representative immunofluorescence images of HBECs
 640 expressing GFP-LC3 after CRE treatment (20 μ g/ml), and the average number of puncta per cell was
 641 analysed in PBS and CRE-treated group (n=12, number of puncta per cell from four different
 642 high-power fields (HPFs) of three independent experiments). **D,** Quantitative analysis of IL-25, IL-33,
 643 and TGF β 1 levels in supernatants of PBS or CRE-treated HBECs for 4h (n=4-8). **E,** Representative
 644 immunofluorescence images of LC3B and ATG5 expression in lung tissues from asthmatics (n=5) and
 645 healthy controls (n=5). **F,** Quantitative analysis of **(E)**. Data represent mean $\pm$ SEM. * p <0.05, ** p
 646 <0.01, *** p <0.001.

647 **FIG 2.** Inhibition of autophagy suppresses allergen-induced lung inflammation. **A,** Protocol for
 648 cockroach allergen-induced mouse model of asthma in the presence or absence of 3-MA during the
 649 challenge phase. **B,** Lung resistance in response to increasing concentrations of methacholine using the
 650 forced oscillation technique (FlexiVent, SCIREQ) (n=6). **C,** Histological examination of mouse
 651 paraffin lung sections stained with hematoxylin and eosin (H&E, upper panel) and Periodic acid–Schiff
 652 (PAS, lower panel) staining. **D,** Percentage of PAS⁺ area among total lung area. **E** Bronchoalveolar
 653 lavage (BAL) fluid total and differential (eosinophil, macrophage, neutrophil, and lymphocyte) cell
 654 counts as assessed by flow cytometry. **F,** Serum levels of cockroach allergen-specific IgE and IgG1. **G,**
 655 BAL fluid levels of cytokines. E-G, n=6-9. Data represent mean $\pm$ SEM from two-independent
 656 experiments. * p <0.05, ** p <0.01, *** p <0.001.

657 **FIG 3.** Feedforward connection between cockroach allergen-induced ROS and autophagy. **A,**
 658 Representative immunofluorescence images of ROS expression with dihydroethidium (DHE) in lung

659 tissues from cockroach allergen-induced mouse model of asthma treated with or without 3-MA (30
 660 mg/kg, *i.p.*). **B**, Quantitative analysis of florescent signal in lung tissue sections (n=8). **C**,
 661 Representative immunofluorescence images of mitochondrial ROS expression with MitoSOX in
 662 cockroach allergen-treated HBECs with or without 3-MA. **D**, Quantitative analysis of florescent signal
 663 in HBECs (n=8). **E**, Flow cytometry analysis of intracellular ROS production with CM-H2DCFDA. **F**,
 664 Quantitative analysis of flow cytometry data (n=5). **G**, Western blot analysis of LC3-I, LC-3II, ATG5,
 665 and p62 expression in CRE-treated HBECs in the presence or absence of either NAC or MitoTEMPO.
 666 β -actin was measured as the loading control. **H**, Quantitative analysis of western blots. Data represent
 667 mean $\pm$ SEM from two independent experiments. * p <0.05, ** p <0.01, *** p <0.001.

668 **FIG 4.** ROS-resistant CaMKII prevents allergen-induced autophagy in asthma mouse model. **A**,
 669 Scheme of ROS-resistant CaMKII MM-VV δ (281/282) mice generation. **B**, Representative
 670 immunofluorescence images of ox-CaMKII expression (green) in the airway epithelial cells (EpCAM,
 671 red) of CRE-induced mouse model of asthma with wild-type (WT) or CaMKII MM-VV δ (MM-VV δ)
 672 mice. **C**, Quantitative analysis of florescent signal (n=5-6). **D**, Representative immunofluorescence
 673 images of LC3B, Atg5, and p62 expression (red) in the airway epithelial cells (EpCAM, red) of
 674 CRE-induced mouse model of asthma with wild-type (WT) or CaMKII MM-VV δ (MM-VV δ) mice.
 675 **E-G**, Quantitative analysis of florescent in **(D)** for LC3 **(E)**, ATG5 **(F)**, and p62 **(G)** in lung tissues of
 676 CRE-induced mouse model of asthma with WT or MM-VV δ mice. n=6-8 from two independent
 677 experiments. **H**, Western blot analysis of LC3-I, LC-3II, ATG5, and p62 in lung tissues of
 678 CRE-induced mouse model of asthma with WT or MM-VV δ mice. β -actin was measured as the
 679 loading control. **I**, Quantitative analysis of western blots. Data represent mean $\pm$ SEM of two
 680 independent experiments. * p <0.05, ** p <0.01, *** p <0.001

681 **FIG 5.** Inhibition of mitochondrial CaMKII suppressed autophagy. **A**, Western blot analysis of LC3-I,
 682 LC-3II, ATG5, and p62 in Empty-GFP or Mt-CaMKIIN transfected HBECs with PBS or CRE

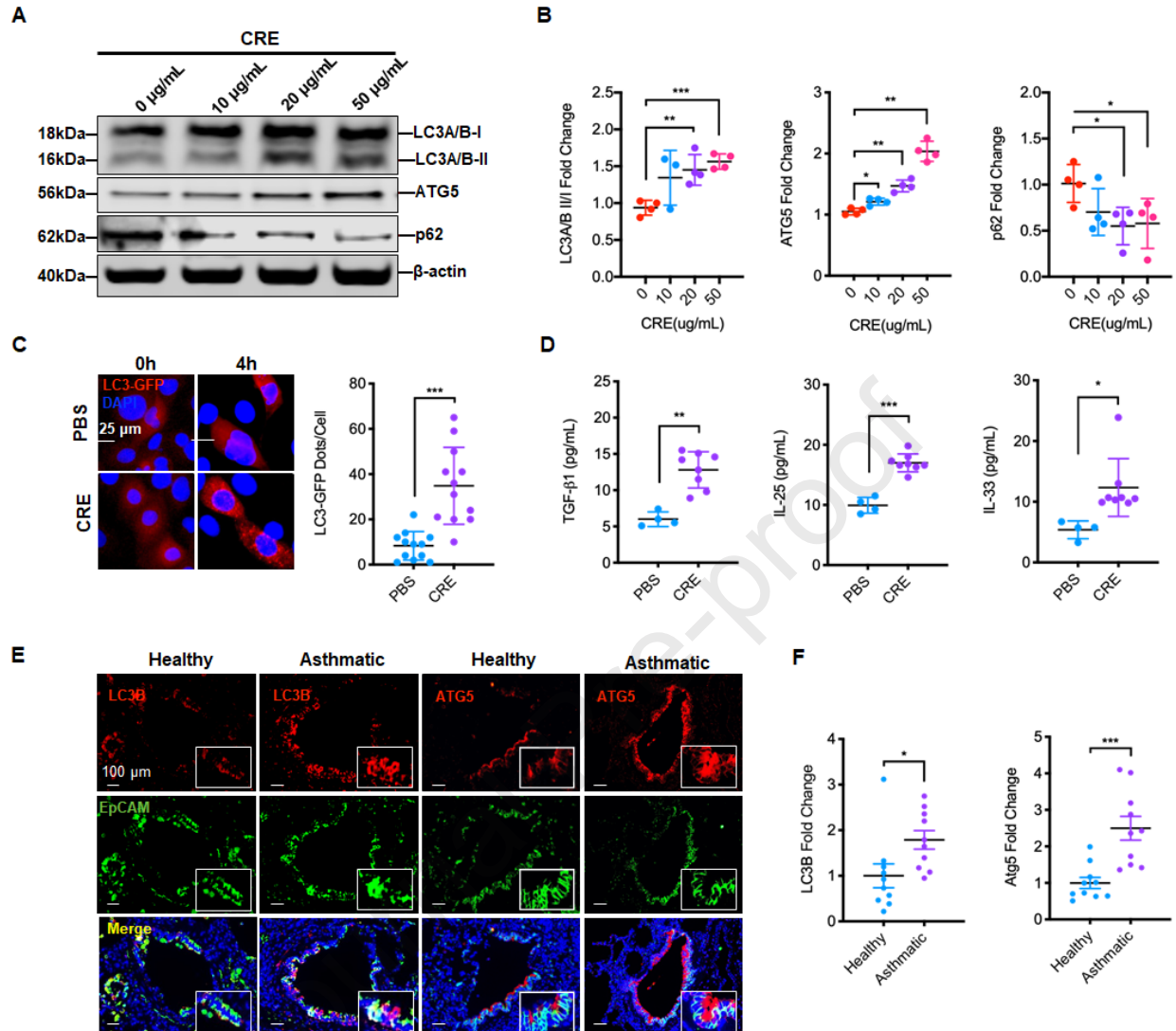
683 treatment. β -actin was measured as the loading control. **(B)** Quantitative analysis of western blots
684 (n=3). **C**, Western blot analysis of p-AMPK, AMPK, p-BECLIN 1, BECLIN 1, p-mTOR, and mTOR
685 expression in Empty-GFP or Mt-CaMKIIN transfected HBECs with PBS or CRE treatment. β -actin
686 was measured as the loading control. **D**, Quantitative analysis of western blots. Data represent mean $\pm$
687 SEM of two independent experiments. * p <0.05, ** p <0.01, *** p <0.001.

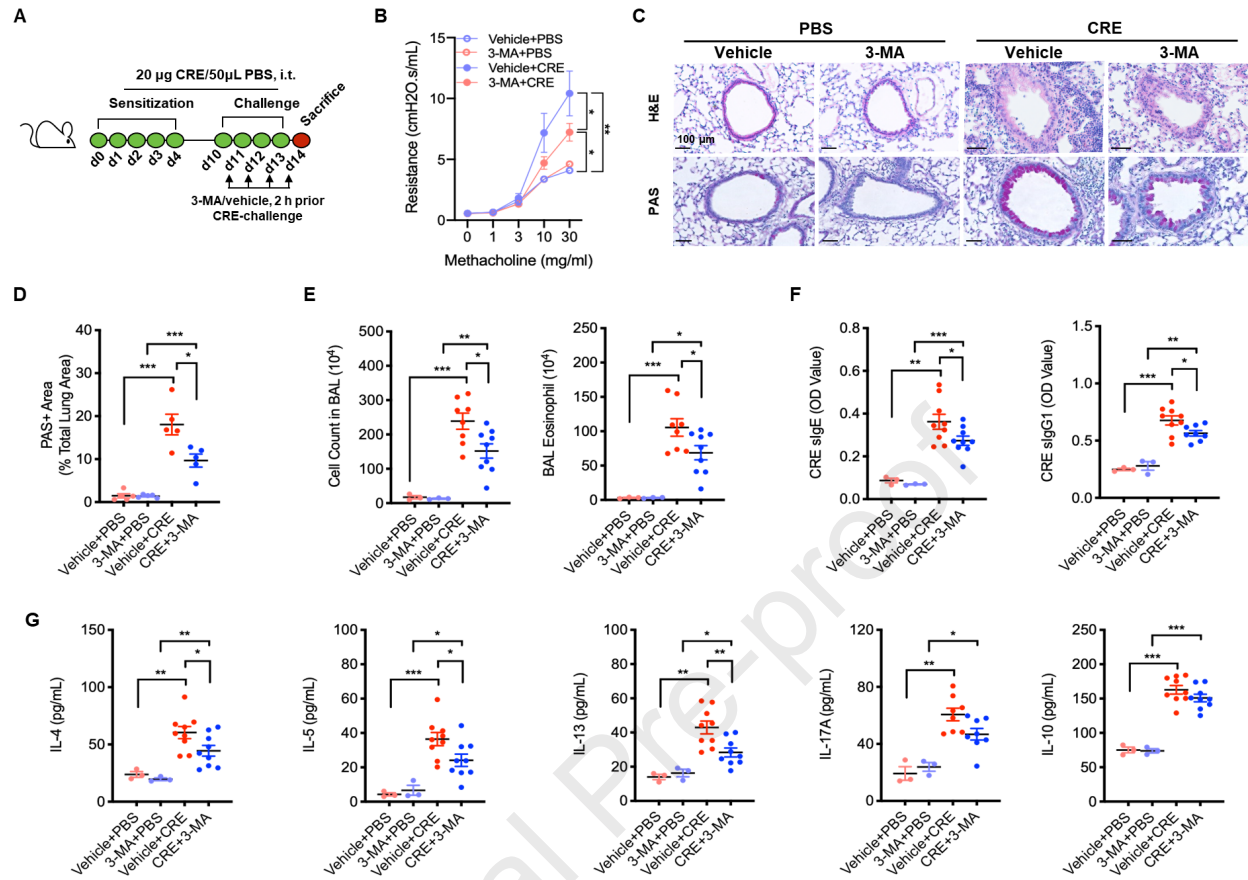
688 **FIG 6.** Inhibition of mitochondrial CaMKII protects against allergen-induced mitochondrial
689 respiration. **A**, Oxygen consumption rate (OCR) under basal conditions followed by the sequential
690 measurements after addition of oligomycin, FCCP, rotenone and antimycin A were determined by Mito
691 Stress test. **B**, HBECs were transfected with Mt-CaMKIIN and then treated with CRE for OCR
692 measurement. **C**, Measurement of basal respiration, ATP production, H leak, maximal respiration, and
693 spare respiration capacity in CRE-treated HBECs. n=7-8. Data represent mean $\pm$ SEM of two
694 independent experiments. p <0.05, ** p <0.01, *** p <0.001.

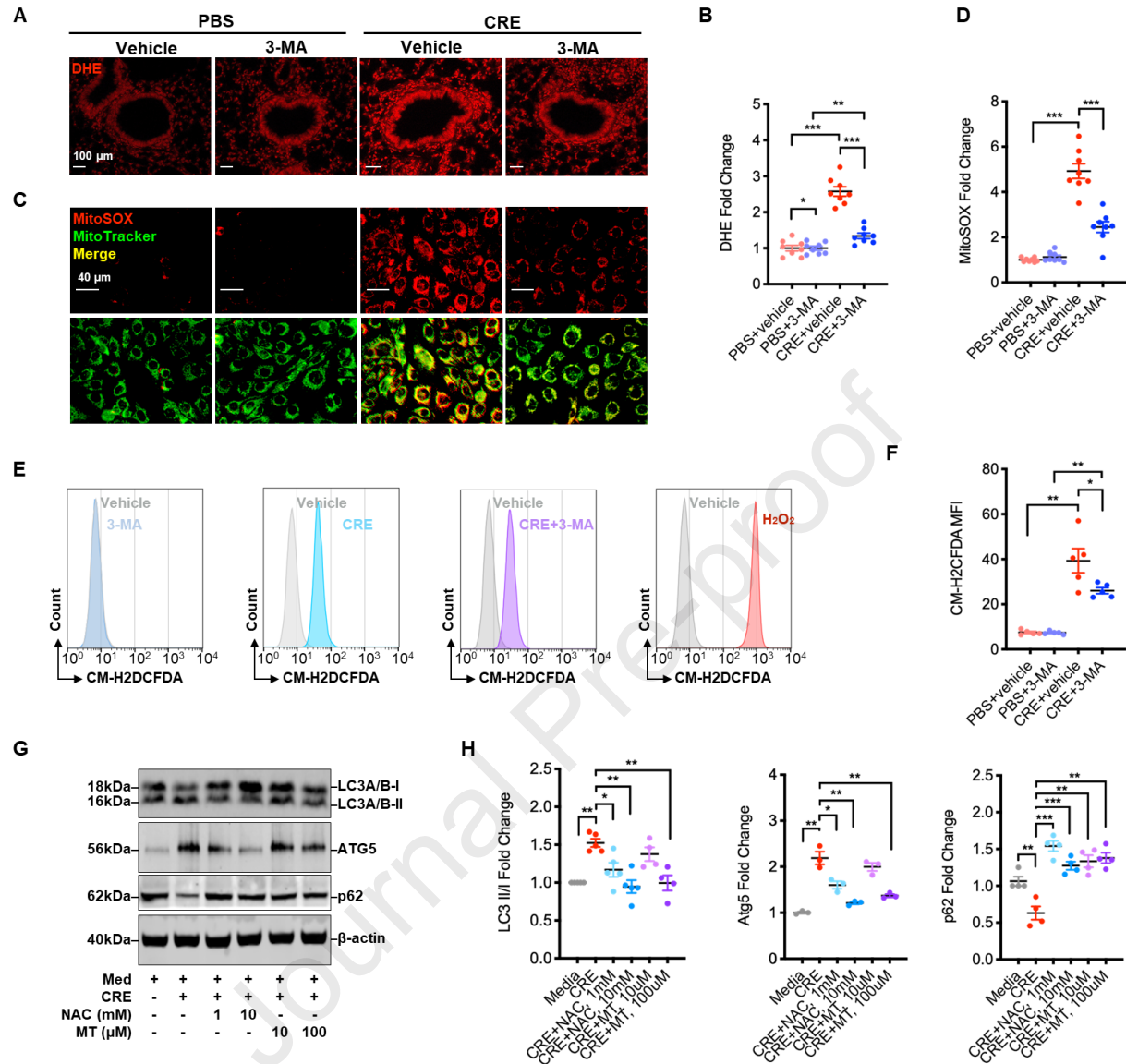
695 **FIG 7.** Inhibition of mitochondrial CaMKII suppressed cockroach allergen-induced mitophagy in
696 airway epithelial cells. **A**, Scheme of mitophagy detection with mitochondrially targeted Keima-Red
697 (MT-mKeima-Red). **B**, Representative immunofluorescence images of mitophagy with
698 MT-mKeima-Red (red) and mitochondria (Mitotracker, green) in Empty-GFP or Mt-CaMKIIN
699 transfected HBECs with PBS or CRE treatment. **C**, Quantitative analysis of florescent signals in
700 HBECs (n=10-18). **D**, Quantitative analysis of IL-25, IL-33, and TGF β 1 levels in supernatants of in
701 Mock or CaMKIIN transfected HBECs with PBS or CRE treatment for 4h (n=4). Data represent mean
702 $\pm$ SEM. * p <0.05, ** p <0.01, *** p <0.001).

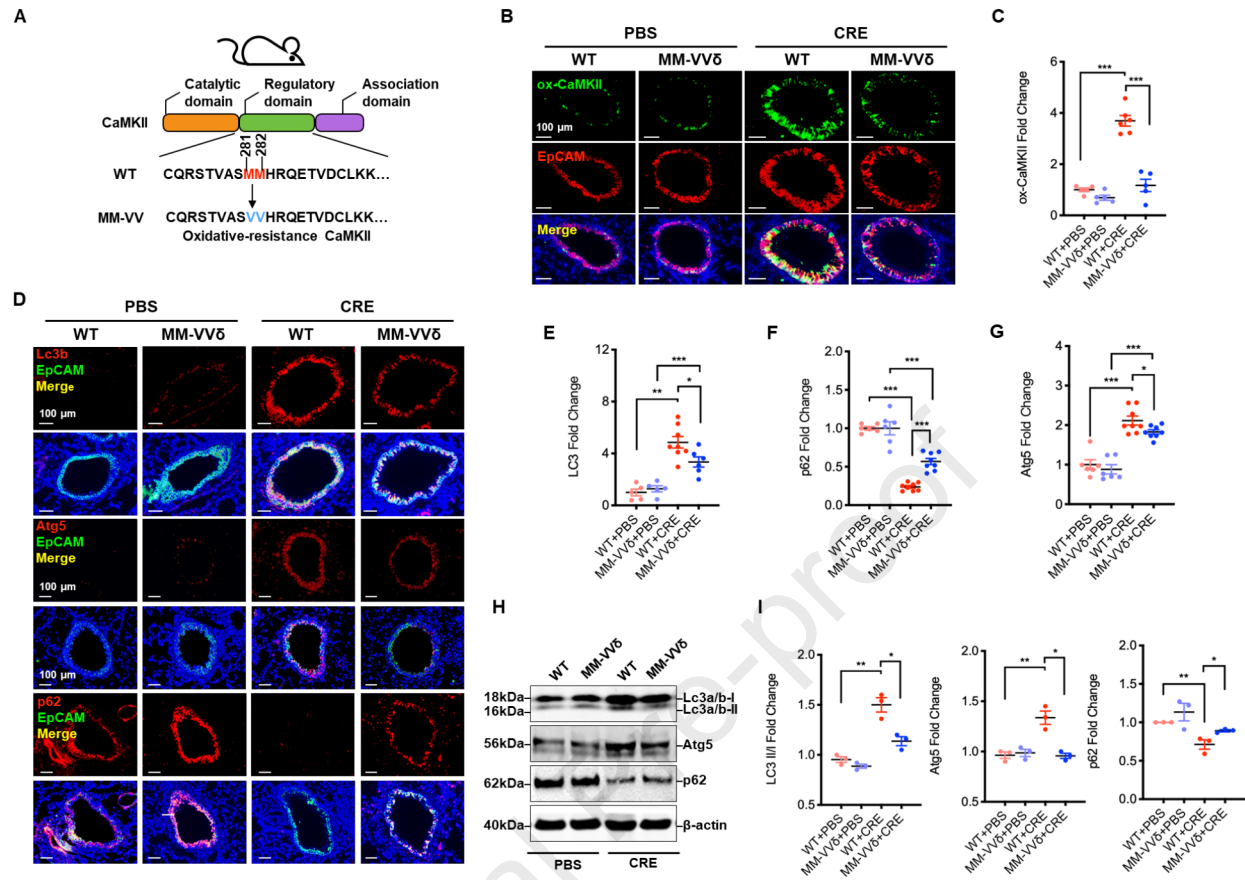
703 **FIG 8.** Inhibition of mitochondrial CaMKII suppressed mitophagy through controlling OPTN. **A**,
704 qPCR analysis of ULK1, PINK1, PARKIN, and OPTN in Empty-GFP or Mt-CaMKIIN transfected
705 HBECs with PBS or CRE treatment (n=6). **B**, Representative immunofluorescence images of HBECs
706 expressing OPTN-GFP after CRE treatment in the presence or absence of Mt-CaMKIIN transfection.

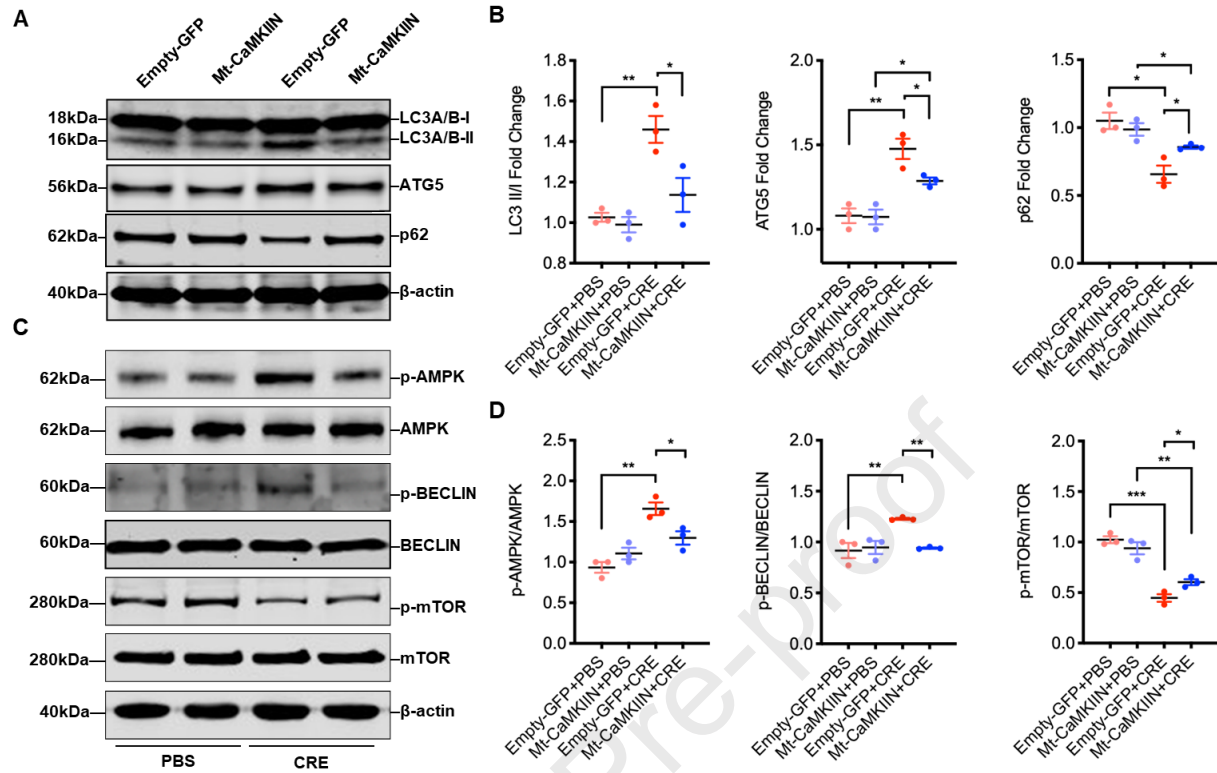
707 **C-D**, Number of OPTN foci (**C**) and percentage (**D**) of OPTN-MitoTracker were quantified in PBS
708 and CRE-treated group. n=10-18. **E**, Representative images of mitophagy with MT-mKeima-Red (red)
709 and mitochondria (Mitotracker, green) in LV-shRNA-Mock or LV-shRNA-OPTN transfected HBECs
710 with PBS or CRE treatment. **F**, Quantitative analysis of florescent signals in HBECs (n=16). Data
711 represent mean $\pm$ SEM from two independent experiments. * p <0.05, ** p <0.01, *** p <0.001.

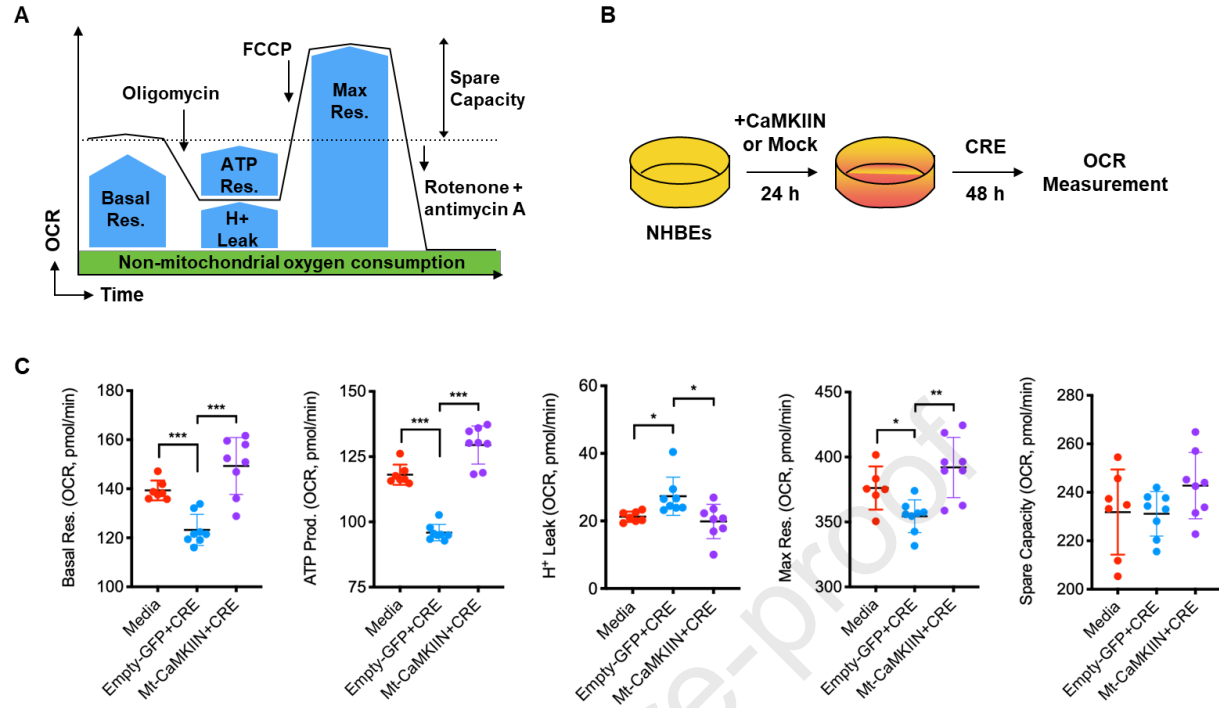




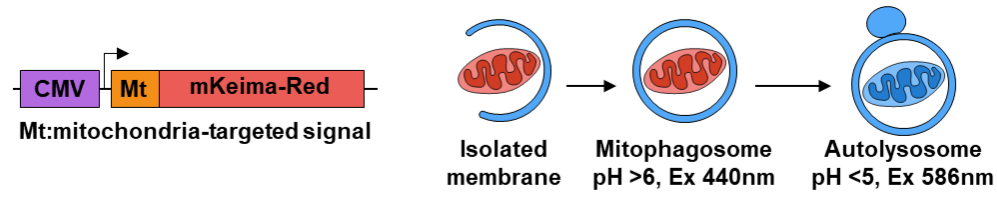




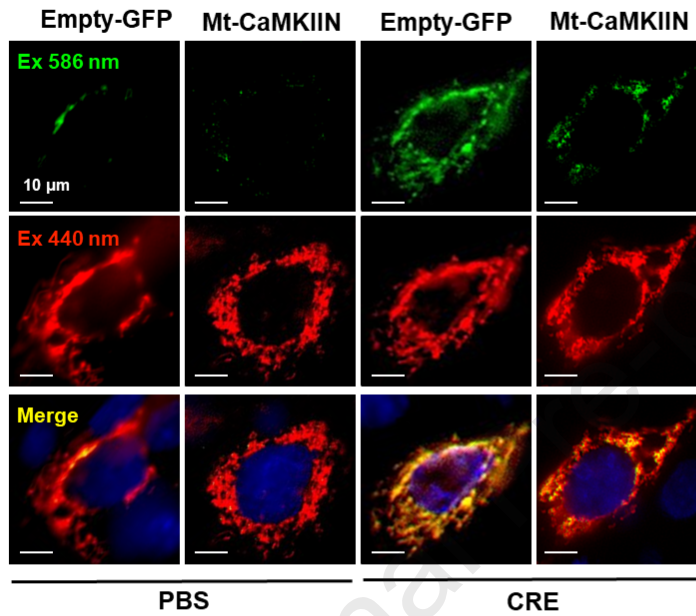




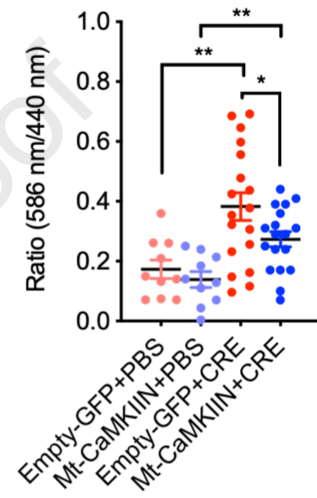
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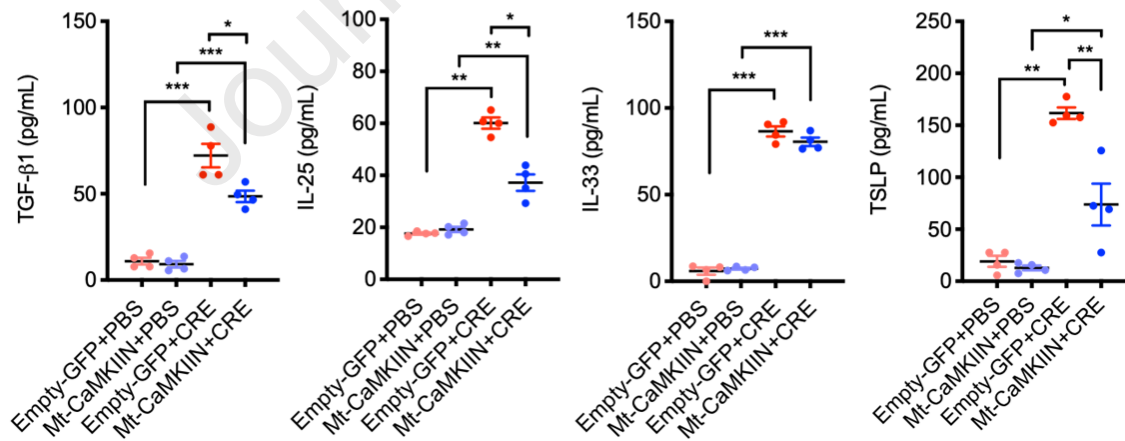
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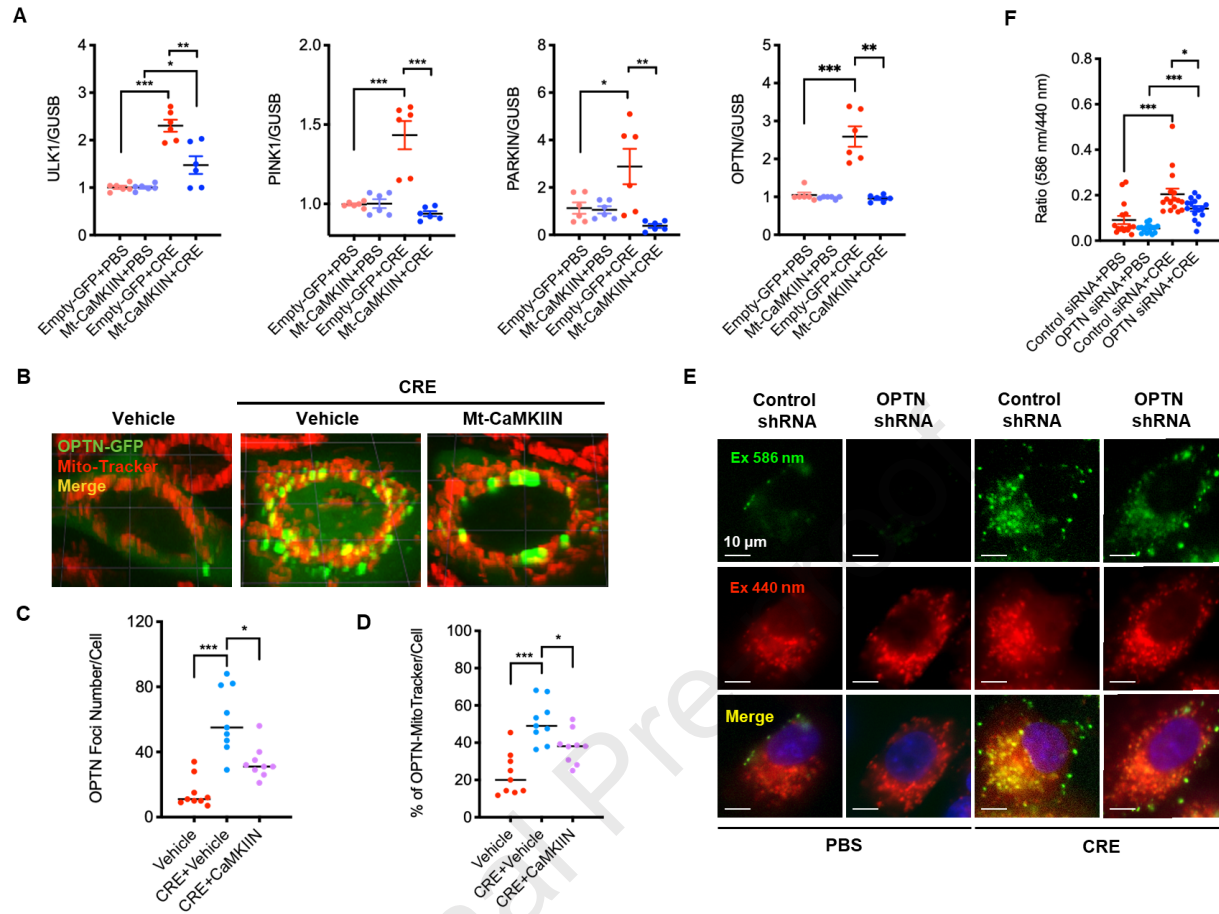


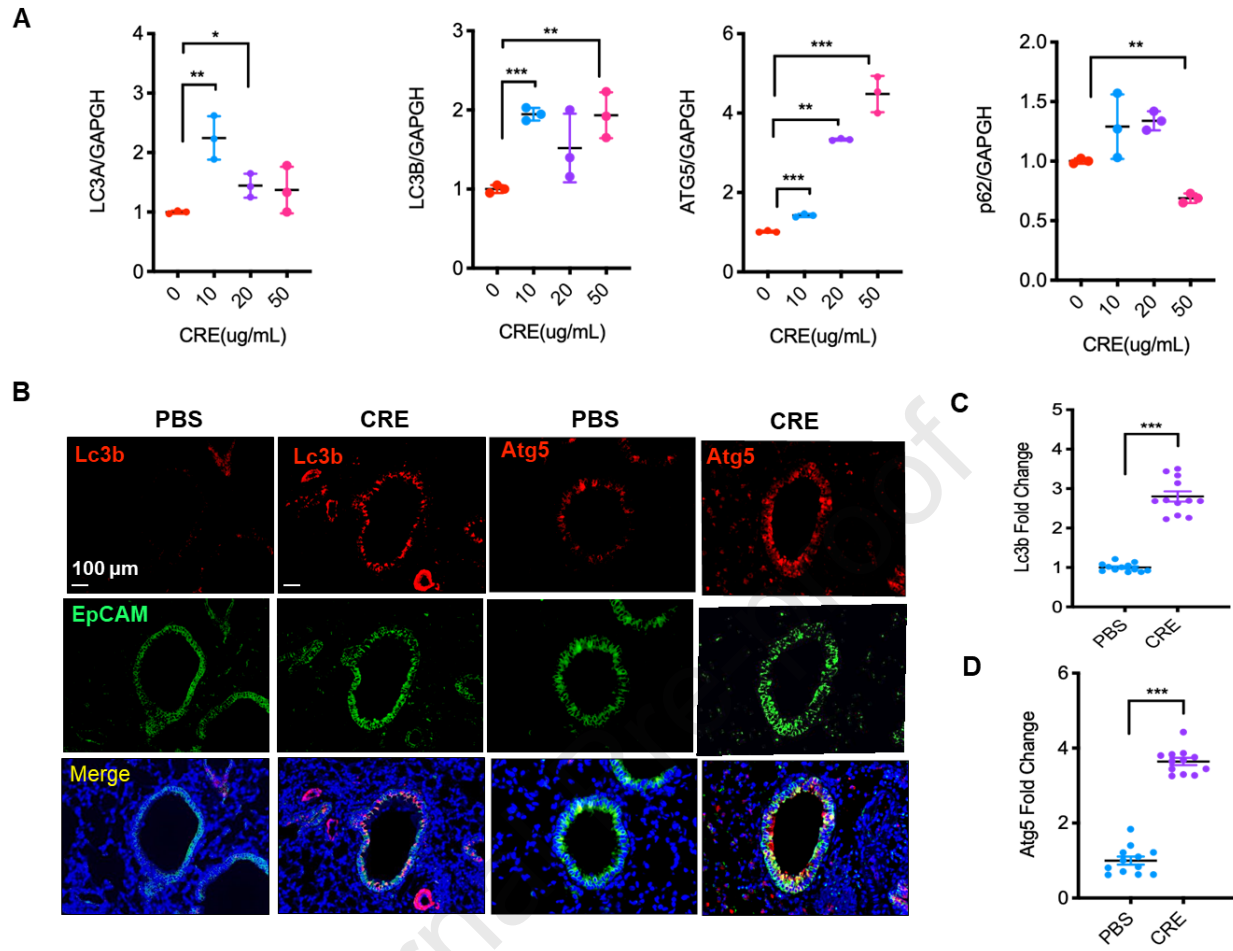
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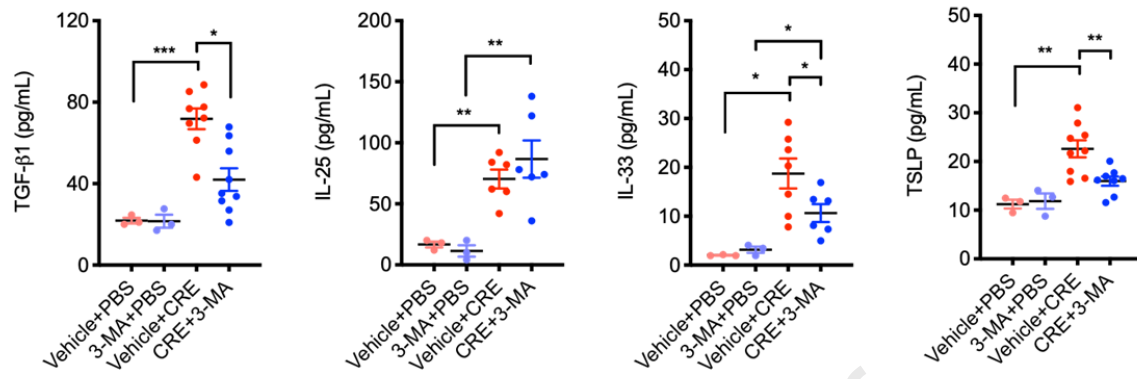
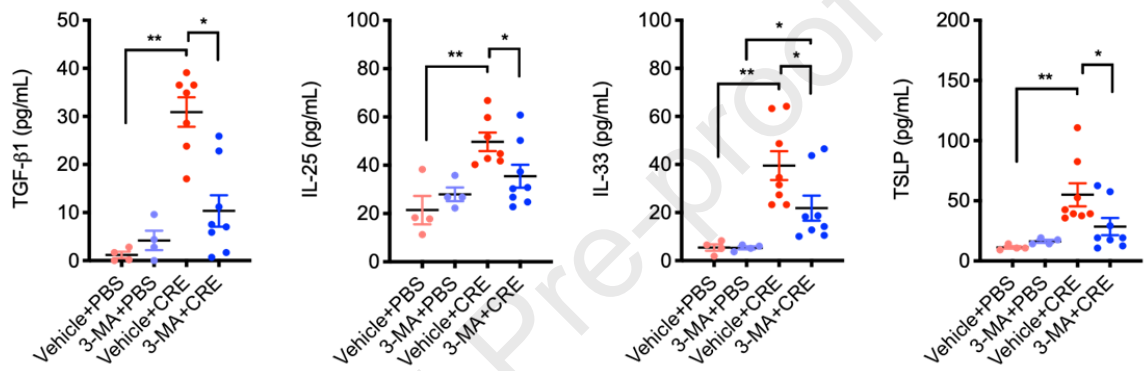


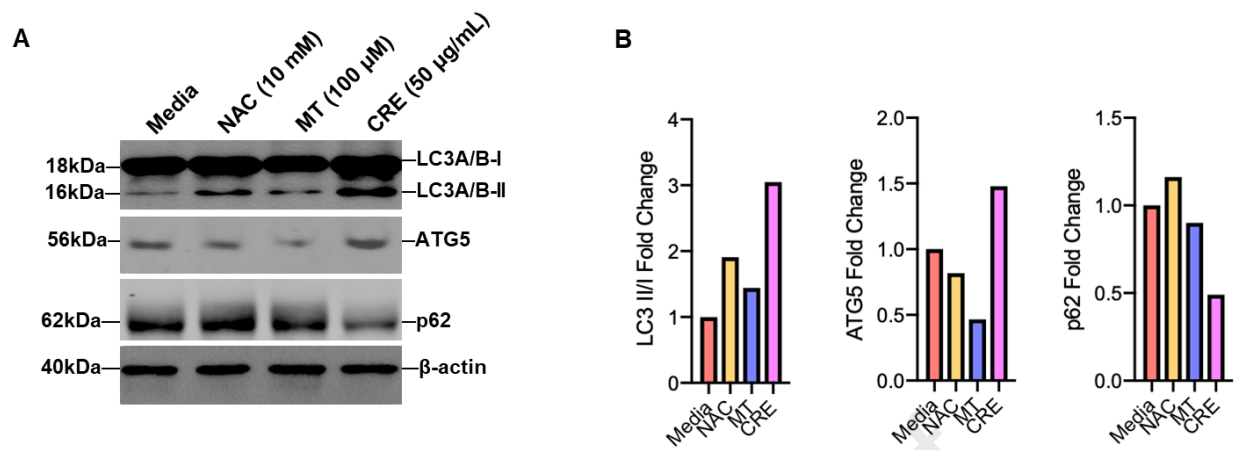
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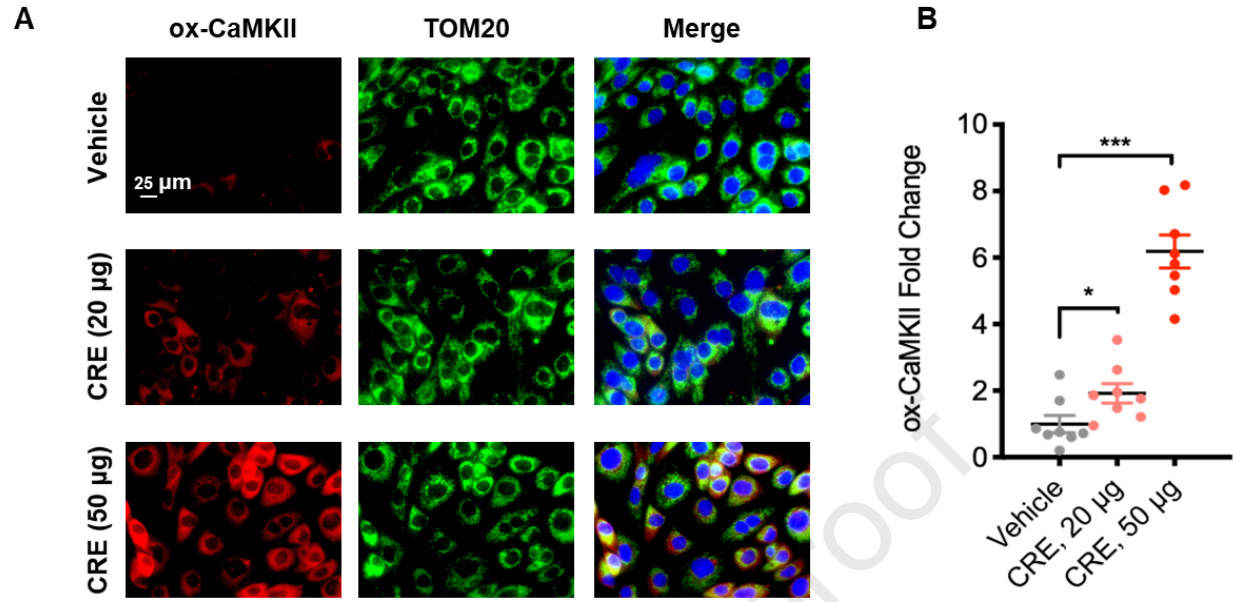


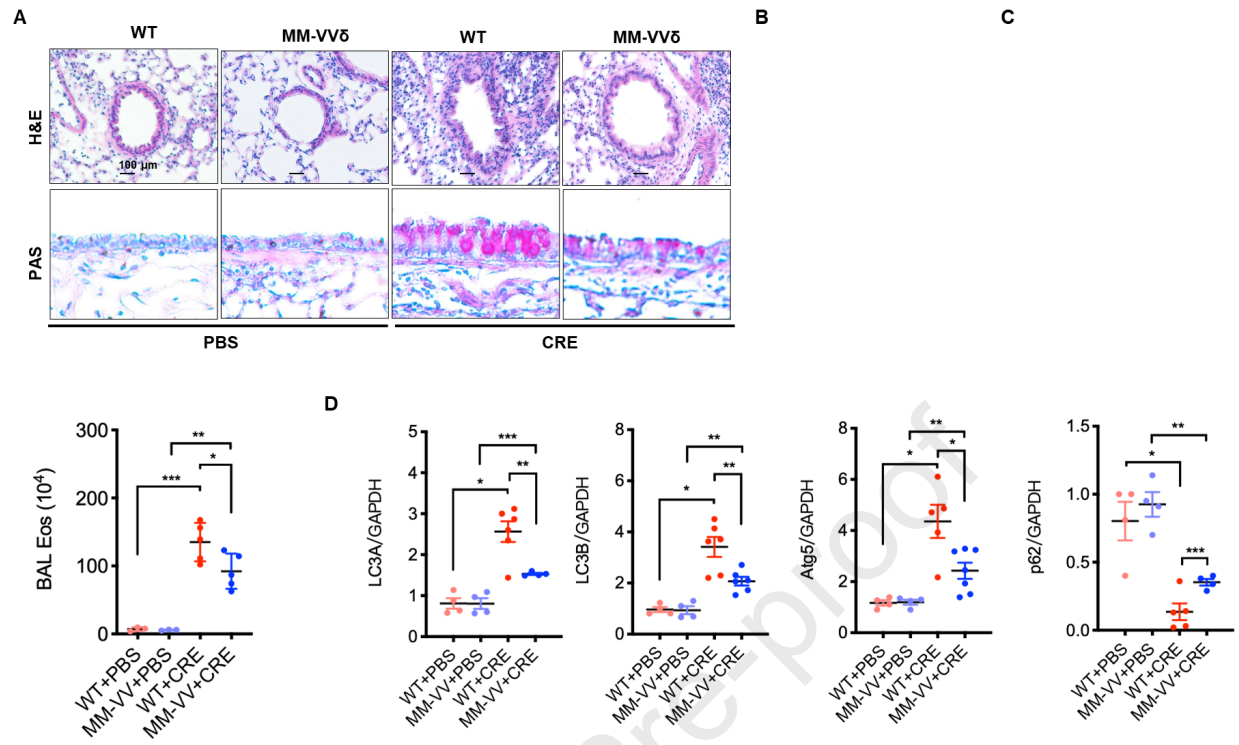


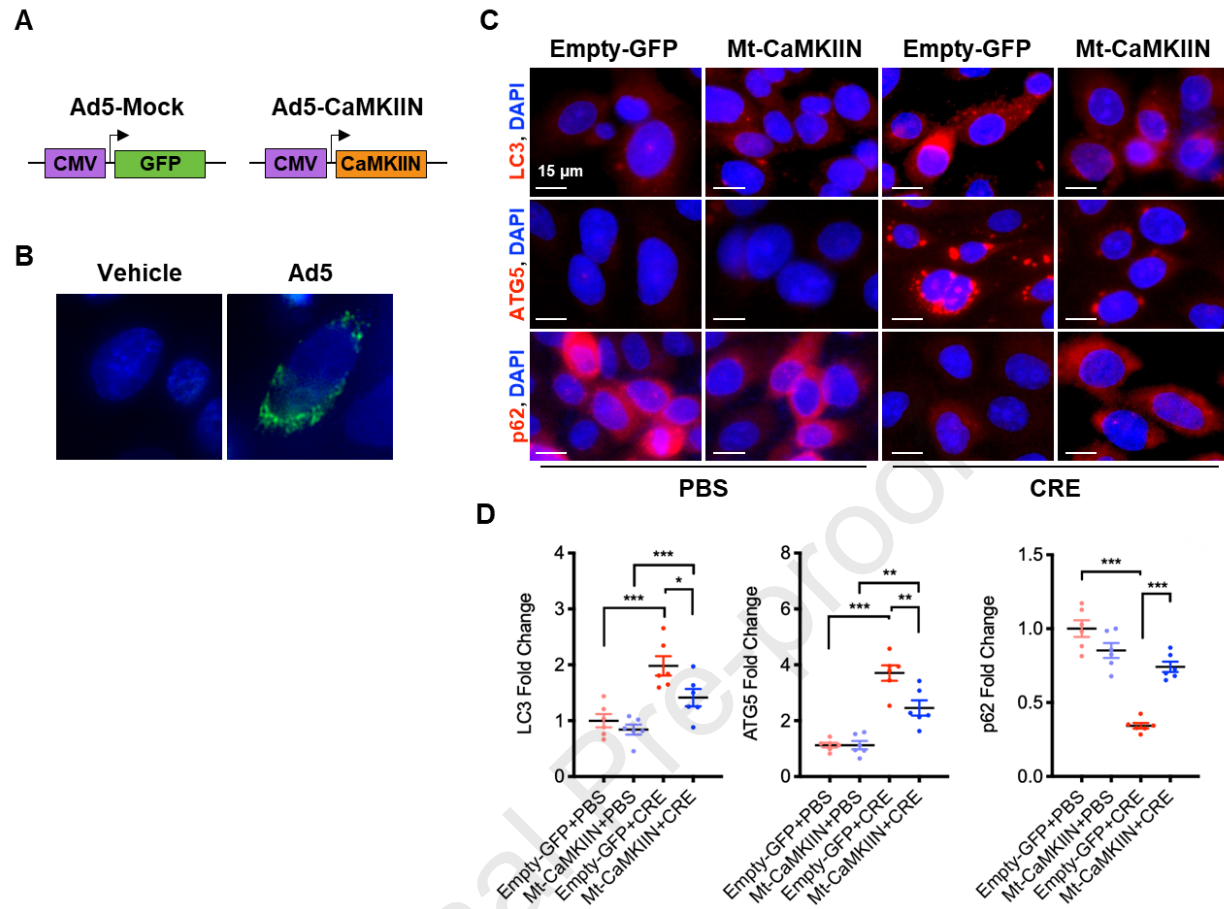


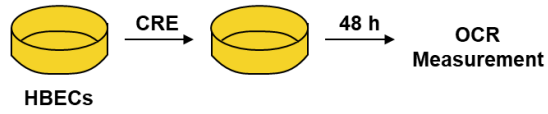
A**B**









A**B**