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Role of autophagy and mitophagy of group 2 innate lymphoid cells in allergic and local allergic rhinitis

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ABSTRACT

Background: Roles of ILC2s in allergic rhinitis (AR) and local allergic rhinitis (LAR) are unclear. In this study, we are determined to find the levels of autophagy and mitophagy of ILC2s in allergic nasal inflammation.

Methods: ELISA was used to detect type 2 inflammatory cytokines. Hematoxylin and eosin (H&E) staining were used to compare the eosinophil (EOS) infiltration of nasal tissue specimens. Flow cytometry was used to detect the levels of ILC2s and Th2 cells. Immunohistochemistry (IHC) and Western blot (WB) were used to detect the levels of Beclin1, LC3, p62, PINK1, Parkin, FUNDC1, and BNIP3 in nasal mucosa. The levels of autophagy related proteins and mitophagy related proteins of the ILC2s were detected by WB. The number of autophagosomes of ILC2s was observed by transmission electron microscopy. The co-localization levels of GFP-LC3 and Mito tracker in ILC2s were observed by confocal microscopy using immunofluorescence.

Results: We found that the level of type 2 inflammation in AR and LAR mice was significantly increased. The levels of autophagy and mitophagy of AR and LAR mice in nasal mucosa and ILC2s were both increased.

Conclusions: ILC2s may be associated with the occurrence and development of nasal allergic inflammation. The abnormal increase of autophagy and mitophagy levels in the nose may be associated with the incidence of AR and LAR. Abnormal autophagy and mitophagy levels of ILC2s cells may be one of the causes of allergic nasal inflammation.

Keywords: Autophagy, Mitophagy, Allergic rhinitis, Local allergic rhinitis, Type 2 inflammation

INTRODUCTION

Allergic rhinitis (AR) is a mainly IgE-mediated allergic inflammation of the nasal mucosa, manifesting as nasal congestion, watery rhinorrhea, itching, and sneezing.[1] Its incidence worldwide is increasing, and it affects about 15% of the population.[2] Local allergic rhinitis (LAR) occurs in patients previously diagnosed with non-allergic rhinitis (NAR). LAR is characterized by a localized

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type 2 nasal mucosal allergic inflammation in which antigen-specific IgE (sIgE) is produced locally in the nasal mucosa and systemic atopy is absent.[3] LAR has similar clinical symptoms to AR but negative results on the skin prick test (SPT) and analyses of serum levels of sIgE and a positive result on the nasal allergen provocation test (NAPT).[4] However, the pathogeneses of AR and LAR are unclear. AR and LAR present as type 2 inflammation in the nasal mucosa, involving a variety of inflammatory cells, such as eosinophils, T cells, B cells, and group 2 innate lymphoid cells (ILC2s).[5]

ILCs are innate immune cells that do not express traditional antigen-specific receptors, instead expressing activated receptors for cytokines and other mediators.[6] ILCs are divided into ILC1s, ILC2s, and ILC3s, reflecting the cytokines released by Th1, Th2, and Th17 cells.[6] ILC2s play an important role in asthma and other type 2 inflammation[7] and promote airway remodeling.[8] They can be regulated by a variety of factors and are activated by the epithelial cytokines IL-25, IL-33, thymic stromal lymphopoietin (TSLP), and the leukotrienes (LT) C4 and D4, which are related to viral, bacterial, and fungal infections and allergen stimulation.[9] ILC2s in blood or nasal mucosa are associated with the severity of type 2 inflammation in AR.[10–12] ILC2s play a role in the initial stage of allergic inflammation and interact with T and B cells. Therefore, ILC2s might be involved in the pathogenesis of LAR.[5,13] However, little research has focused on the roles of ILC2s in LAR.

Autophagy is important for maintaining the survival and function of ILC2s.[14] Autophagy is a lysosomal-dependent degradation process in eukaryotic cells, which delivers cargo to the lysosome to facilitate the turnover of cellular components and is critical in organismal adaptation and homeostasis.[15] Three main types of autophagy have been characterized: chaperone-mediated autophagy, microautophagy, and macroautophagy.[16] The process is regulated by multiple genes and proteins, including autophagy-related genes (ATGs), microtubule-associated protein kinase light chain 3 (LC3), and Beclin1.[17] Mitophagy refers to the process by which damaged or dysfunctional mitochondria are selectively wrapped into the autophagosome, which combines with a lysosome in the event of hypoxia, mitochondrial depolarization, or bacterial or viral infection.[18] Mitophagy is regulated by ubiquitin-dependent and non-ubiquitin-dependent pathways.[19] The phosphatase and tensin homolog-induced putative kinase 1 (PINK1)/Parkin pathway is the major ubiquitin-dependent pathway. Non-ubiquitin-dependent pathways are mediated by mitophagy receptors (eg, FUN14 domain-containing protein 1 [FUNDC1] and BCL2-interacting protein 3 [BNIP3]).[19] Under physiological conditions, autophagy and mitophagy protect cells and tissues, and suppress inflammation, promoting clearance of cellular irritants and pathogens.[20] Abnormal autophagy and mitophagy are associated with diseases such as cancer, infections, inflammation, and neurodegenerative and autoimmune conditions.[21,22] Autophagy and mitophagy are implicated in the exacerbation of AR,[23–25] but this is controversial.[26] The roles of autophagy and mitophagy in LAR are unclear.

In this study, we assessed the role of autophagy and mitophagy in the pathogenesis of AR and LAR. Furthermore, we investigated the roles of ILC2s autophagy and mitophagy in local allergic inflammation.

MATERIALS AND METHODS

Ethics statement and animal model

This study was approved by the Research Ethics Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University (Zhejiang, China).

Allergic Rhinitis (AR) mouse model

Six-week-old female BALB/c mice, weight 18–20 g, were sensitized 0, 1, and 2 days after the start of the experiment by dropping 5% ovalbumin (OVA) (Sigma, St. Louis) solution into each side of the nasal cavity (5 μg/mouse/day). On day 22, 5% OVA solution (5 μg/mouse/day) was dropped into each side of the nasal cavity for provocation once daily for 1 week (Fig. 1A).

LAR mouse model 1

LAR mouse model 1 was established[13] by sensitizing 6-week-old female BALB/c mice by continuous nasal drops of l% OVA for 5 days,

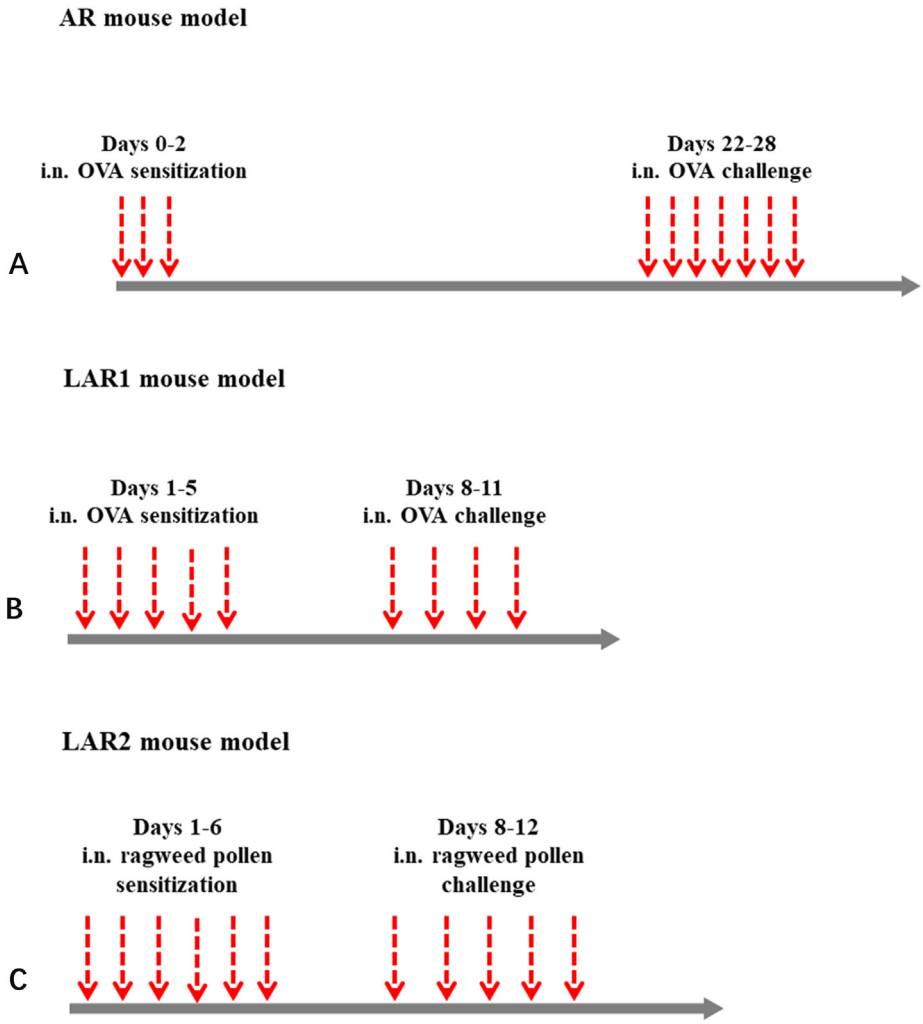

Fig. 1 AR and LAR mouse model. A: AR mouse model was built through nasal application OVA three times for sensitization and seven times for challenge. B: LAR1 mouse model was built through nasal application OVA five times for sensitization and four times for challenge. C: LAR2 mouse model was built through nasal application ragweed pollen six times for sensitization and five times for challenge.

followed by provocation by continuous nasal drops for 4 days in the second week after an interval of 2 days (5 µL per nostril) (Fig. 1B).

Local allergic rhinitis mouse model 2

Local allergic rhinitis (LAR) mouse model 2 was established[27] by sensitizing 6-week-old female BALB/c mice, by continuous nasal drops of ragweed pollen (20 µL phosphate buffer solution [PBS] containing 1 mg ragweed pollen) for 6 days. After a 1-day interval, ragweed pollen was nasally dropped at 20 µL per nostril for provocation for 5 days (Fig. 1C).

Control mice

The same volume of PBS was used for sensitization and stimulation in control mice. Twenty-four hours after the last provocation, mice were euthanized by intraperitoneal injection of pentobarbital. Blood samples were removed from the capillaries of the posterior ocular venous plexus, and turbinate mucosa tissues were obtained for paraffin-embedding, cell separation, and protein extraction.

Enzyme-linked immunosorbent assay

Serum levels of eosinophil cationic protein (ECP) (Cusabio, Wuhan, China), allergen-sIgE (Jianglaibio, Shanghai, China), nasal membrane IL-4, IL-5, IL-13 (NeoBioscience, Shenzhen, China), LTB4, LTC4, LTD4, and LTE4 (Jianglaibio) were measured via enzyme-linked immunosorbent assay (ELISA). Concentrations of the factors of interest were determined

by comparing sample optical densities (ODs) to standard curves.

Western blotting

The protein levels of Beclin-1, P62, and LC3II/LC3I (Abcam, Cambridge, UK) in nasal tissues were detected by Western blotting (WB). The levels of PINK-1, Parkin, FUNDC1, and BNIP3 (Abcam) (Proteintech, San Diego, CA) and mitochondrial proteins of nasal tissues and ILC2s were also determined by WB. Briefly, tissues or ILC2s were washed in PBS, added to phenylmethylsulfonyl fluoride (PMSF) containing cracking solution, and cracked on ice for 30 min. Samples were centrifuged at 12,000 rpm for 15 min at 4 °C, and the supernatant was retained. Next, 30 μg tissue lysate was mixed with 5 × sample buffer (15 g SDS, 15.6 mL 2 M Tris [pH 6.8], 57.5 g glycerol, and 16.6 mL β-mercaptoethanol), and samples were resolved by 10% SDS-PAGE and transferred to PVDF membranes. After sealing with 5% skim milk containing 0.1% Tween-20, the primary antibody was added, and the samples were incubated overnight at 4 °C. The samples were washed three times with PBS, and an HRP-labeled secondary antibody (Dianova, Hamburg, Germany) was added, followed by incubation for 2 h at room temperature and washing three times with PBS. ECL developer (Bio-Rad, CA) was added to the samples, which were imaged using the GelDoc Imaging System (Bio-Rad).

Flow cytometry and isolation of ILC2s

The ratio of ILC2s and Th2 cells in nasal membranes was analyzed by flow cytometry (Beckman Coulter, cytoFLEX, CA) according to the manufacturer's instructions. ILC2 markers were $Lin^{-}CD127^{+}CRTH2^{+}ckit^{\pm}CD56^{-}$ (Lin: CD3, CD4, CD8, CD14, CD15, CD16, CD19, CD20, CD33, CD34, CD203c, FcεR1), and Th2 cell markers were $CD4^{+}IL4^{+}$ (eBioscience, CA) (BD Pharmingen, Shanghai, China).

Hematoxylin and eosin staining

The number of eosinophils in nasal membranes per × 400 high-power field (HPF) was measured via hematoxylin and eosin (H&E) staining. After dewaxing, samples were stained with Mayer's hematoxylin solution for 5 min and washed with tap water. The sections were transferred to 1% hydrochloric acid-alcohol for 4 s and washed with tap water. The sections were then put in anhydrous ethanol I for 5min, anhydrous ethanol II for 5min, xylene I for 5min and xylene II for 5min. After air drying, the samples were sealed with neutral gum and visualized by microscopy.

Immunohistochemistry

Immunohistochemistry (IHC) was conducted to evaluate Beclin1, LC3II, p62, Parkin, PINK1, FUNDC1, and BNIP3 (Abcam, Cambridge, UK) protein levels in nasal mucosa as described previously.[28]

Transmission electron microscopy

Autophagosome formation in ILC2s was observed by transmission electron microscopy (TEM). ILC2s were fixed with 2.5% glutaraldehyde for 3 h and centrifuged at 1000 rpm. Fresh 2.5% glutaraldehyde was added, and the cells were fixed at 4C for 3 h followed by rinsing 3 times with 0.1 M phosphate buffer. Cells were fixed with 1% osmic acid 0.1 M phosphate buffer at room temperature for 2 h and rinsed 3 times (15 min each) with 0.1 M phosphate buffer. 50–70–80–90–95–100–100% ethanol -100–100% acetone dehydration, 15min each time. Acetone: 812 embedment agent = 1:1 penetration 3 h, acetone: 812 embedment agent = 2:1 overnight penetration, pure 812 embedment agent overnight penetration. Samples were polymerized at 60 °C for 48 h, sectioned to 80 nm thickness, double stained with uranium lead, dried overnight at room temperature, and observed under an electron microscope (HT7700-SS, Hitachi, Tokyo, Japan).

Immunofluorescence staining

For GFP-LC3 transfection, diluted Lipo6000™ reagent (Beyotime, Shanghai, China) and pCMV-GFP-LC3B plasmid (Beyotime) were placed at room temperature for 5 min, mixed, and allowed to stand at room temperature for 15 min. Cells were cultured for 4 h after transfection.

To label mitochondria in ILC2s, cells were cultured to a predetermined density and cell culture medium was removed. Mito-Tracker Red CMXRos working solution (Beyotime) was added, and the cells were incubated at 37°C for 30 min.

The co-localization of Mito-Tracker (red) and GFP-LC3 (green) was observed under a confocal laser microscope (Olympus FV3000, Takachiho, Japan).

Statistical analysis

GraphPad Prism 8.0 software was used for data analysis. One-way ANOVA was used for inter-group comparisons of data with a normal distribution, and nonparametric tests were used for non-normally distributed data. $P < 0.05$ was considered indicative of statistical significance.

RESULTS

AR and LAR mouse models

After the last provocation, the number of sneezes and grabbing around the nose of mice in 10 min were counted. Compared to the control group, mice in the AR and LAR groups showed significantly greater allergic symptoms, including more frequent sneezing and grabbing around the nose (Fig. 2A). Compared to the control group, AR mice, but not LAR mice, showed significantly upregulated serum levels of OVA-sIgE and ragweed pollen-sIgE (Fig. 2B). Furthermore, AR and LAR mice displayed markedly increased nasal tissue levels of allergen-sIgE (Fig. 2C). Notably, AR mice had higher nasal allergen-sIgE levels.

Eosinophilic and type 2 inflammation are exacerbated in AR and LAR mice

Compared to the control group, AR mice showed a significantly increased serum level of ECP (Fig. 3A). AR and LAR mice had significantly upregulated levels of ECP, IL-4, IL-5, and IL-13 in nasal tissues (Fig. 3B–E). The nasal ECP and IL-13 levels were significantly decreased in LAR compared to AR mice. Therefore, systemic and local eosinophilic and type 2 inflammation were aggravated in AR mice, whereas only nasal eosinophilic and type 2 inflammation were exacerbated in LAR mice.

H&E staining showed that the eosinophil (EOS) level was higher in AR and LAR mice than in controls (Fig. 4A). Flow cytometry indicated that the numbers of ILC2s in AR and LAR mice were significantly and nonsignificantly increased compared to controls, respectively (Fig. 4B, D). The numbers of Th2 cells in AR and LAR mice were significantly greater (Fig. 4C, E). Compared to LAR mice, the numbers of ILC2s and Th2 cells were elevated in AR mice (Fig. 4D and E).

ELISA showed that AR and LAR mice had significantly increased levels of LTB4, LTC4, LTD4, and LTE4 compared to the controls (Fig. 5). The LTB4, LTC4, and LTD4 levels were elevated in AR mice compared to LAR mice.

These results suggest aggravated type 2 inflammation affecting EOS, ILC2s, and Th2 cells in AR mice and to a lesser degree, in LAR mice.

Autophagy and mitophagy in nasal tissues

IHC staining showed that Beclin1 and LC3II levels were significantly upregulated in AR and LAR mice (Fig. 6A–C), and the p62 level was significantly decreased in AR and LAR1 mice compared to the controls (Fig. 6D). WB also indicated higher levels of Beclin1 and LC3II/LC3I, and a lower level of p62 in AR and LAR mice compared to the controls (Fig. 6E–H). Notably, there were no differences in the levels of autophagy-related protein in nasal tissues between AR and LAR mice, except LC3II/LC3I (Fig. 6G).

IHC staining showed that AR and LAR mice had significantly increased PINK1, Parkin, FUNDC1, and BNIP3 levels in nasal tissues compared to controls (Fig. 7A–E). There were no significant differences in mitophagy-related protein levels in nasal tissues of AR and LAR mice.

WB showed that the levels of PINK1, Parkin, FUNDC1, and BNIP3 in nasal tissues were significantly increased in AR and LAR mice than in control mice, excluding the PINK1 and Parkin expressions in LAR2 mice (Fig. S1A-E). Compared to LAR mice, AR mice showed higher levels of PINK1, FUNDC1, and BNIP3 but not Parkin (Fig. S1A-E). Similarly, in mitochondrial proteins of the nasal tissue, the levels of PINK1, Parkin, FUNDC1, and BNIP3 were significantly elevated in AR and LAR mice compared to controls (Fig. S1F-J). AR mice also had higher levels of mitophagy-related proteins than LAR mice, except Parkin and FUNDC1 in LAR2 mice (Fig. S1H, I).

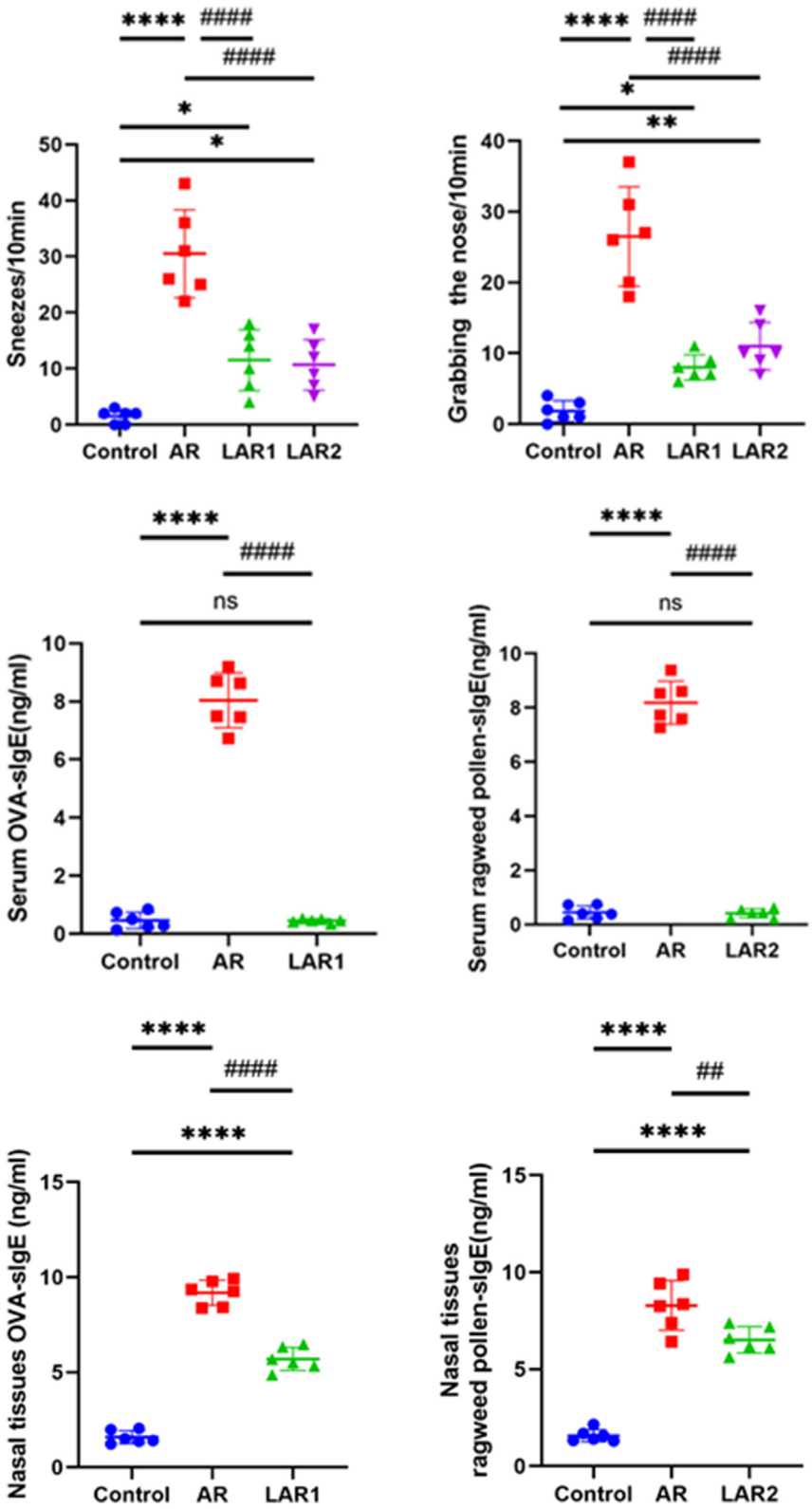

Fig. 2 The rhinitis symptoms and results of ELISA of allergen-sIgE. A: The rhinitis symptoms in different mice groups. B: Levels of serum allergen-sIgE in different mice groups. C: Levels of nasal allergen-sIgE in different mice groups. Compared with control, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Compared with AR, #p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001. ns : No significance.

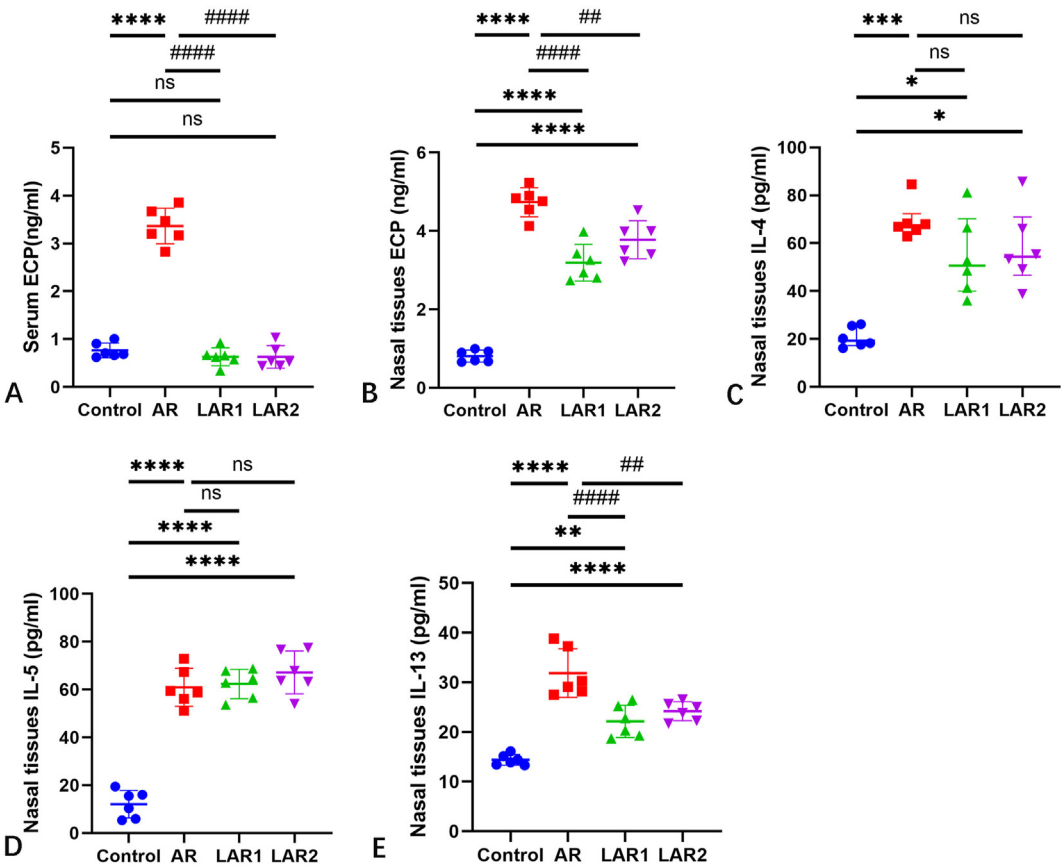

Fig. 3 The results of ELISA of type 2 cytokines. A: Levels of serum ECP in different mice groups. B: Levels of nasal ECP in different mice groups. C: Levels of nasal IL-4 in different mice groups. D: Levels of nasal IL-5 in different mice groups. E: Levels of nasal IL-13 in different mice groups. Compared with control, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Compared with AR, #p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001. ns : No significance.

Taken together, these results suggest that autophagy and mitophagy have an important role in the pathogenesis of AR and LAR. The mitophagy pathways including PINK1/Parkin, FUNDC1, and BNIP3 are involved in the onset of AR and LAR. Meanwhile, AR mice had a higher level of mitophagy.

Autophagy and mitophagy of nasal ILC2s

First, we isolated ILC2s from nasal tissues by flow cytometry. Then, WB assays indicated that AR and LAR mice had significantly increased Beclin1 and LC3II/LC3I levels and a decreased p62 level in ILC2s compared to controls (Fig. S2A-D). In addition, there were significant differences in autophagy-related proteins levels between AR and LAR mice (Fig. S2B-D).

In addition, AR and LAR mice had significantly higher overall levels of PINK-1, Parkin, FUNDC1, and BNIP3 in total protein (Fig. S3A-E). AR mice also had elevated levels of mitophagy-related proteins compared with LAR mice in total protein, except PINK-1 and BNIP3 (Fig. S3B-E). In mitochondrial proteins, the levels of PINK1, Parkin, FUNDC1, and BNIP3 were significantly increased in AR and LAR mice compared to controls (Fig. S3F-J). The mitophagy-related protein levels in mitochondrial protein differed significantly between AR and LAR mice (Fig. S3G-J).

TEM showed that AR and LAR mice had more autophagosomes than control mice (Fig. 8). Immunofluorescence staining and laser confocal microscopy indicated that AR and LAR mice had elevated colocalization of GFP-LC3 and Mito-Tracker than control mice (Fig. S4).

These results implicate ILC2 autophagy and mitophagy in the pathogenesis of AR and LAR. The PINK1/Parkin, FUNDC1, and BNIP3 pathways in ILC2s participate in the pathogenesis of AR and

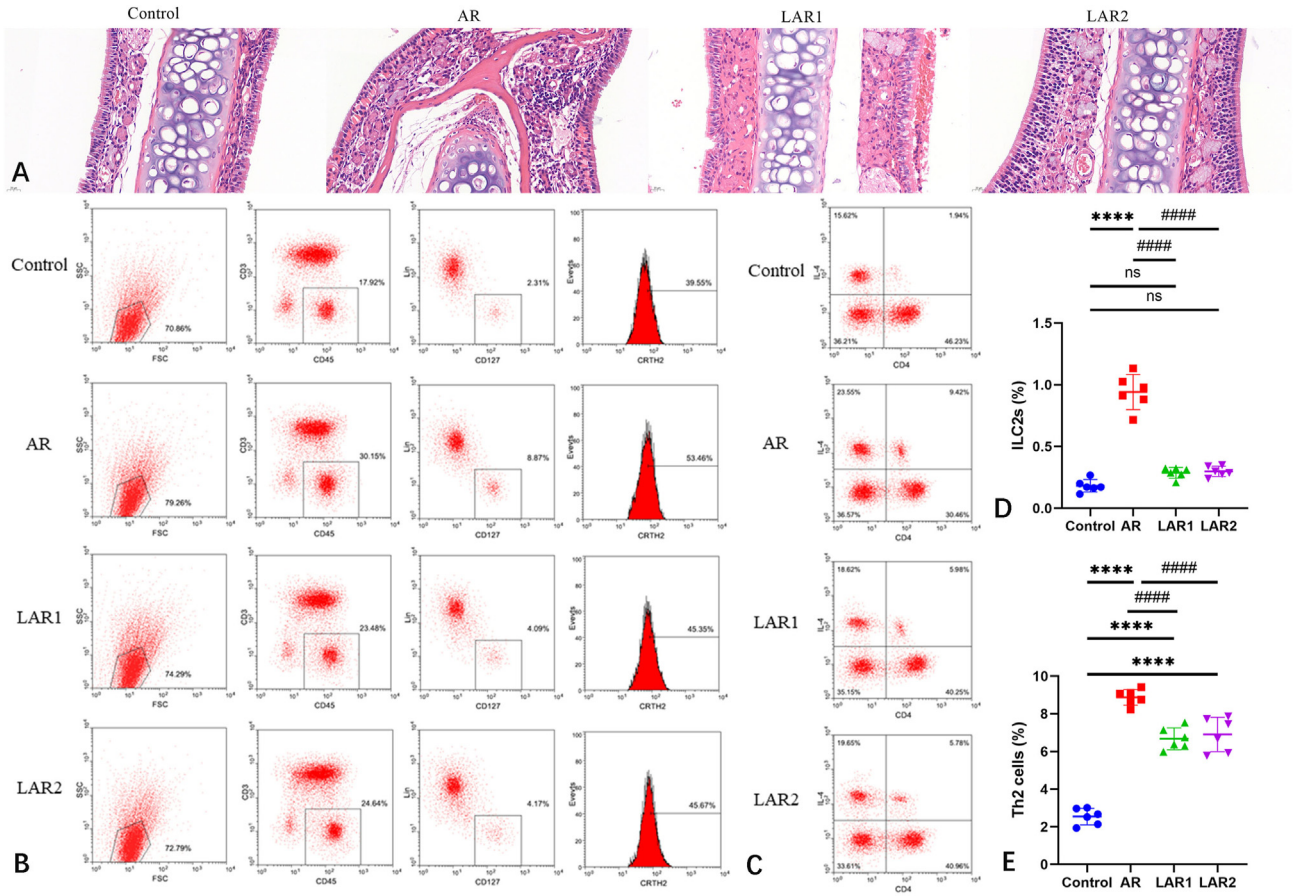

Fig. 4 The count of infiltrating inflammatory cells. A: The infiltration of EOS in nasal tissues detected by H&E staining in different mice groups. B, D: The count of ILC2s in nasal tissues detected by flow cytometry in different mice groups. C, E: The count of Th2 cells in nasal tissues detected by flow cytometry in different mice groups. Compared with control, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Compared with AR, #p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001. ns : No significance.

LAR. We speculate that the upregulation of autophagy and mitophagy levels in ILC2s promoted ILC2s to produce more type 2 inflammatory cytokines and exacerbate local type 2 inflammation of the nasal cavity. The differences in the autophagy and mitophagy levels of ILC2s might be part of the reason that AR and LAR are unique diseases.

DISCUSSION

Traditionally, chronic rhinitis is classified as AR or LAR based on allergy. However, a special form of chronic rhinitis, LAR, manifests as allergic and type 2 inflammation localized to the nasal mucosa.[29] The term LAR was first suggested by Rondón et al 15 years ago[30] but its pathogenesis is unclear. Several studies have focused on LAR patients[31-33] but few have focused on LAR animal models. Kato et al established an LAR mouse model using nasal ragweed pollen sensitization and challenge.[27] Chen et al developed an LAR mouse model by nasal OVA sensitization and challenge.[13] In this study, we created two LAR mouse models based on these previous works. Our LAR mice showed aggravated rhinitis-like symptoms and elevated nasal allergen-sIgE levels but normal serum levels of allergen-sIgE. These results indicate the successful establishment of an LAR mouse model, in agreement with a prior report.[34]

AR is a primarily IgE-mediated type 2 inflammatory disease of the nasal mucosa and activated Th2 cells produce type 2 inflammatory cytokines.[35] In this study, the levels of ECP, IL-4, IL-5, and IL-13 were all elevated in both serum and nasal tissues of AR mice. The numbers of nasal Th2 cells and EOSs were also increased. These results confirm that systemic and local type 2 inflammation were

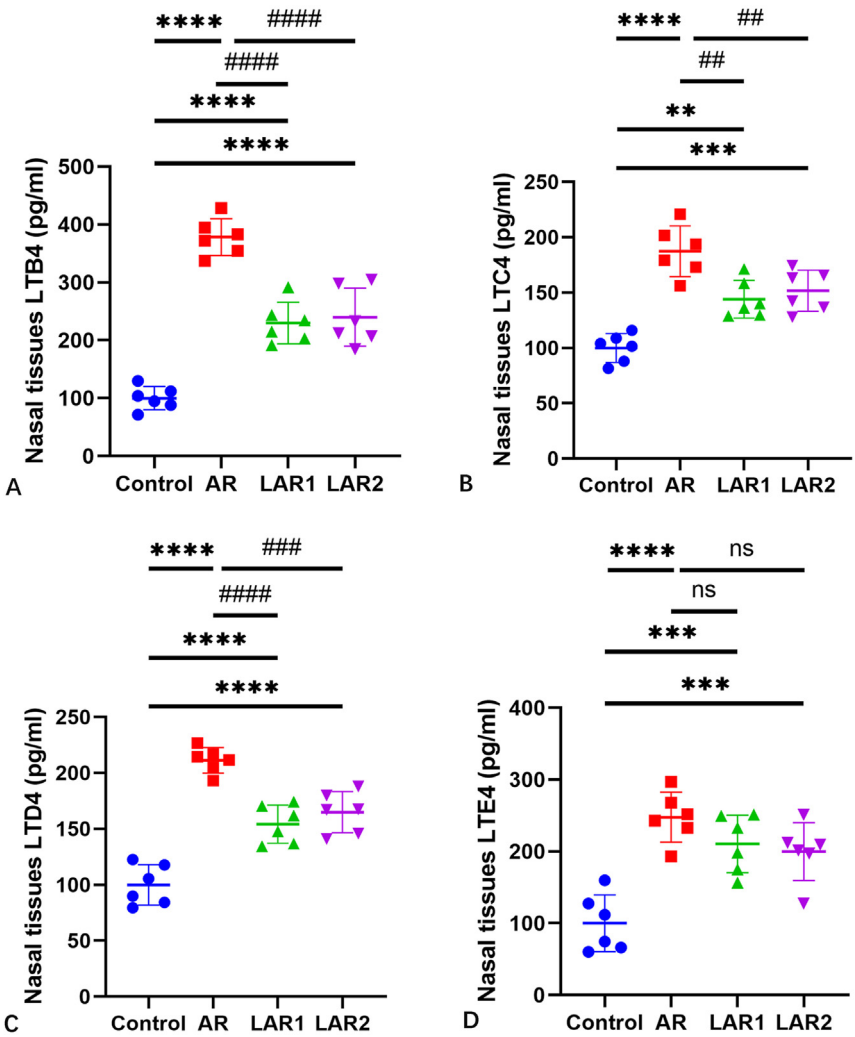

Fig. 5 The results of ELISA of LT. A: Levels of nasal LTB4 in different mice groups. B: Levels of nasal LTC4 in different mice groups. C: Levels of nasal LTD4 in different mice groups. D: Levels of nasal LTE4 in different mice groups. Compared with control, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Compared with AR, #p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001. ns : No significance.

aggravated in AR. Meanwhile, in the present study, LAR mice had higher ECP, IL-4, IL-5, and IL-13 levels, and Th2 cell and EOS counts, in nasal tissues. These findings suggest nasal type 2 inflammation in LAR, in line with a previous study.[36] Because our AR mice showed upregulation of nasal ECP, IL-13 levels, and Th2 cell counts, compared to LAR mice, we can assume that type 2 inflammation be more severe in AR than in LAR.

ILC2s play an important role in nasal type 2 inflammation.[37] They produce type 2 inflammatory cytokines, such as IL-4, IL-5, and IL-13, to promote eosinophilic inflammation in nasal polyps.[38] In addition, they regulate the differentiation, proliferation, and function of Th2 cells and promote type 2 inflammation in nasal polyps.[39] Furthermore, they may participate in tissue remodeling of nasal polyps by producing amphiregulin and promoting fibroblast activation.[40] Lin et al reported that ILC2s have a proinflammatory effect in AR mice.[41] Tojima et al reported that AR patients have elevated levels of ILC2s in the nasal mucosa, and that prostaglandin D2 and cysteinyl LT promote the secretion of type 2 inflammatory cytokines by ILC2s.[42] Chen et al suggested the involvement of ILC2s in the initial or acute inflammatory response to allergen stimulation in AR and LAR patients.[13] ILC2s have been detected in the nasal mucosa of LAR mice and may produce IL-5 and IL-13,[27] but this is controversial.[43] In our study, AR mice had more ILC2s in the nasal mucosa than control and LAR mice. The levels in LAR mice

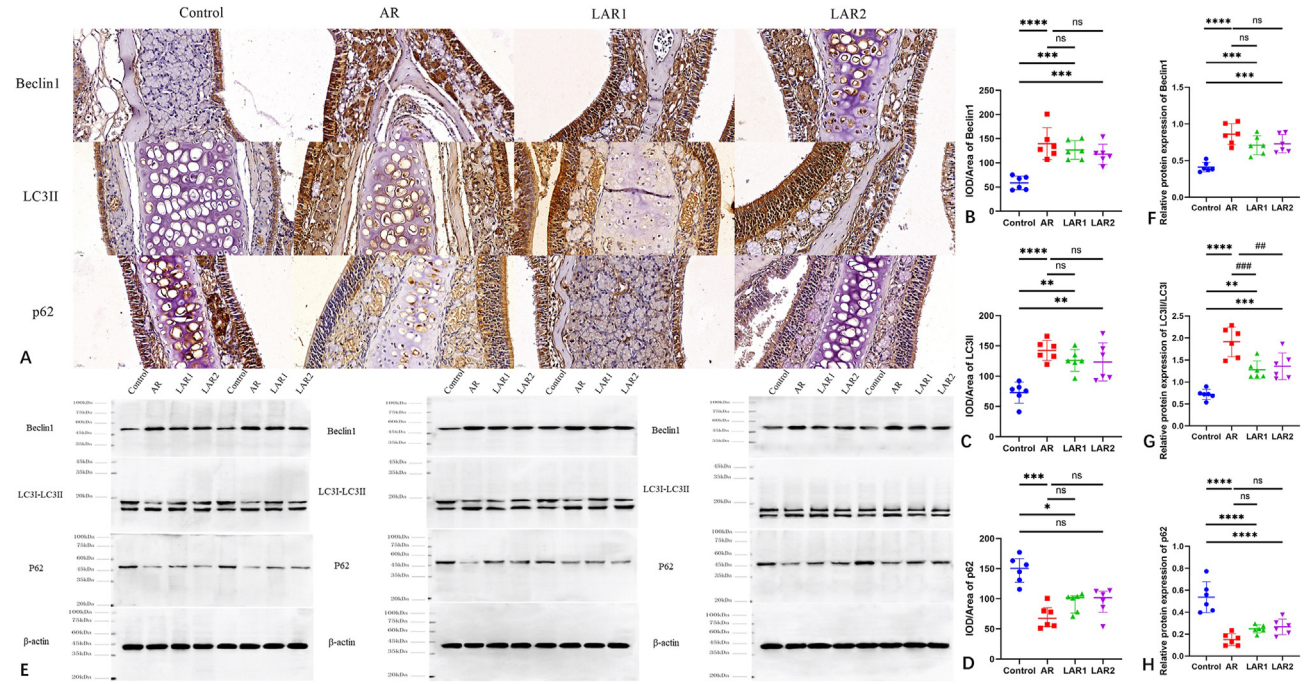

Fig. 6 The expression of autophagy related protein in nasal tissues. A: Levels and distribution of autophagy related protein in nasal tissues detected by IHC in different mice groups. B: The results of IHC of nasal Beclin1 in different mice groups. C: The results of IHC of nasal LC3II in different mice groups. D: The results of IHC of nasal p62 in different mice groups. E: The expression of autophagy related protein in nasal tissues detected by WB assay. F: The results of WB of nasal Beclin1 in different mice groups. G: The results of WB of nasal LC3II/LC3I in different mice groups. H: The results of WB of nasal p62 in different mice groups. Compared with control, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Compared with AR, #p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001. ns : No significance.

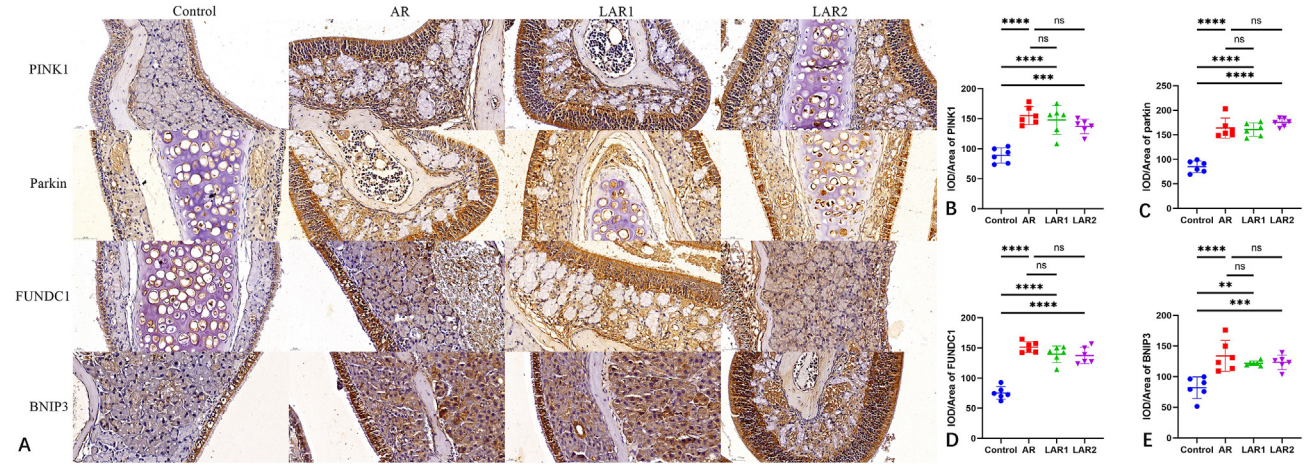

Fig. 7 The IHC results of mitophagy related protein in nasal tissues. A: The expression and distribution of nasal mitophagy related protein detected by IHC. B: Levels of IHC of PINK1 in different mice groups. C: Levels of IHC of Parkin in different mice groups. D: Levels of IHC of FUNDC1 in different mice groups. E: Levels of IHC of BNIP3 in different mice groups. Compared with control, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Compared with AR, #p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001. ns : No significance.

increased, albeit nonsignificantly. These results implicate ILC2s in AR and LAR. In addition, LTB4, LTC4, LTD4, and LTE4 levels were elevated in AR and LAR mice. Therefore, LTs might activate Th2 cells and ILC2s to promote type 2 inflammation in AR and LAR. Further studies are needed to confirm the effects of LT and ILC2s in LAR.

Autophagy plays an important role in nasal inflammation.[44–47] Simsek et al found that LC3 expression was decreased in nasal polyps.[44] Qi et al showed that Beclin1, Beclin1 mRNA, and LC3II mRNA levels were decreased and p62 and p62 mRNA levels were increased in nasal polyps.[45] By contrast, LC3II and Beclin1 are

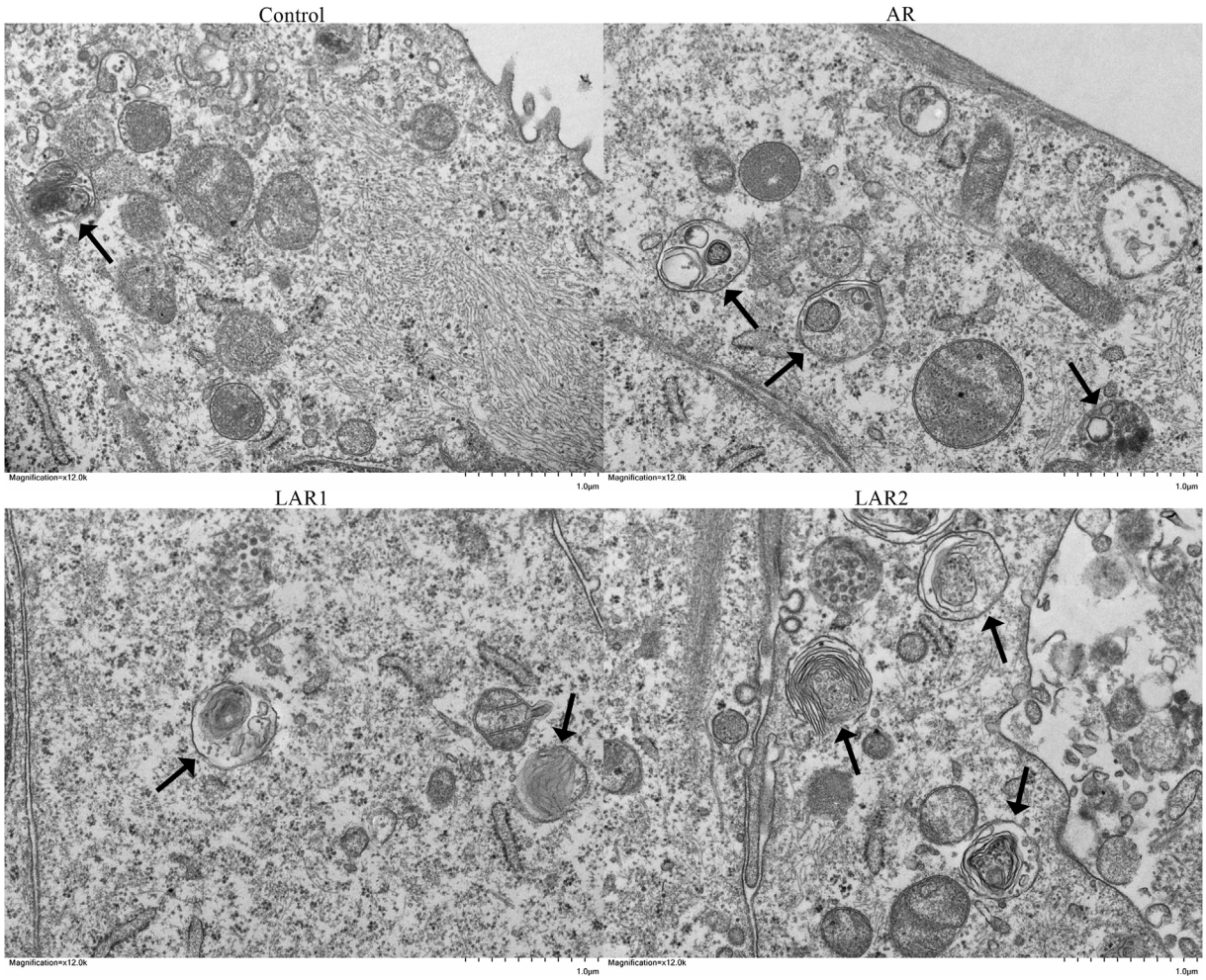

Fig. 8 The formation of autophagosome in ILC2s of different mice groups under transmission electron microscopy. Black arrow: autophagosome

reportedly upregulated in nasal polyps and epithelial cells.[46,47] Li et al reported that autophagy is enhanced in AR patients and is related to tissue remodeling.[23] Zhang et al found elevated autophagy in AR mice.[48] In our study, Beclin1 and LC3II/LC3I protein levels and Beclin1 and LC3II distributions were increased, and the level and distribution of p62 were decreased, in AR and LAR mice. These results implicate an elevated autophagy level in AR and LAR. Our study first investigated the role of autophagy in LAR.

Mitophagy is the selective degradation of mitochondria by autophagy, which may play a critical role in environmental pollutant/allergen-induced mitochondrial dysfunction in allergic airway inflammation.[49] However, few studies have focused on the role of mitophagy in nasal inflammation. Li et al. showed that excessive mitophagy leads to adenosine triphosphate insufficiency, mitochondrial dysfunction, and nasal epithelial cell (NEC) apoptosis.[50] Dong et al found that the number of mitochondria in NECs increased, the body crest disappeared, and there was swelling, vacuolation, and autophagosomes in an AR rat model.[25] Allergens could aggravate these changes. In this study, we found elevated mitophagy-related protein levels in AR and LAR mice, suggesting that the mitophagy pathways involving PINK1/Parkin, FUNDC1, and BNIP3 are involved in the pathogenesis of AR and LAR. In addition, AR mice also showed higher mitophagy related protein levels than LAR mice in WB detection. These results suggest that different levels of mitophagy might in part explain the differences between AR and LAR.

Autophagy and mitophagy are essential for the functioning and survival of innate immune cells.[51] O'Sullivan et al reported that ATG5 knockout leads to mitochondrial damage in natural killer cells and other ILCs, the accumulation of reactive oxygen species (ROS), cell death, and disturbance of ILC maturation.[52] Galle-Treger et al reported that ATG5 knockout in allergic asthma mice suppressed autophagy and mitophagy, leading to metabolic disorders of ILC2s and accumulation of dysfunctional mitochondria and ROS, thus reducing the survival of ILC2s and the production of type 2 inflammatory cytokines.[14] In our study, the autophagy and mitophagy of ILC2s were increased in AR and LAR mice. We first investigated the role of autophagy and mitophagy of ILC2s in the pathogenesis of AR and LAR. Meanwhile, we speculated that the differences in the autophagy and mitophagy levels of ILC2s might be part of the reason that AR and LAR are unique diseases. In addition, we found that the levels of Beclin1, LC3II/LC3I, PINK1, Parkin, FUNDC1, and BNIP3 had positive correlations with type 2 cytokines. Whereas, the level of p62 had negative correlations with type 2 cytokines. (Data were not shown). Those implied that the autophagy and mitophagy levels of ILC2s were important for their functions.

This study had several limitations. First, the sample size was small (6 mice per group). Second, we did not evaluate autophagic flux using autophagy inhibitors or agonists; therefore, further research is needed.

CONCLUSIONS

Autophagy and mitophagy levels in nasal tissues were increased in AR and LAR mouse models. Furthermore, autophagy and mitophagy of nasal ILC2s were elevated in AR and LAR mice. Therapeutic targeting of autophagy has been applied in many fields, such as cancer,[53] asthma,[54] and neurodegenerative disorders.[55] Our findings suggest that autophagy and mitophagy have potential as therapeutic targets in AR and LAR.

Abbreviations

AR, allergic rhinitis; LAR, local allergic rhinitis; NAR, non-allergic rhinitis; sIgE, specific IgE; SPT, skin prick test; NAPT, nasal allergen provocation test; ILC2s, group 2 innate lymphoid cells; TSLP, thymic stromal lymphopoietin; LT, leukotrienes; ATGs, autophagy-related genes; LC3, light chain 3; PINK1, phosphatase and tensin homolog-induced putative kinase 1; FUNDC1, FUN14 domain-containing protein 1; BNIP3, BCL2-interacting protein 3; OVA, ovalbumin; ECP, eosinophil cationic protein; PBS, phosphate buffer solution; LT, leukotriene; IL, interleukin; ELISA, enzyme-linked immunosorbent assay; WB, Western blotting; HPF, high-power field; H&E, hematoxylin and eosin; IHC, immunohistochemistry; NEC, nasal epithelial cell; ROS, reactive oxygen species

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Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions

Conceptualization, C.W. and K.C.; Methodology, C.W.; Validation, J.Z. and W.L.; Formal Analysis, M.Z.; Data Curation, C.W.; Writing – Original Draft Preparation, C.W.; Writing – Review & Editing, K.C.; Project Administration, J.Z.; Funding Acquisition, K.C.

Ethics approval

This study was approved by the Research Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University (Zhejiang, China).

Authors' consent for publication

All authors approved the publication of this article.

Declaration of competing interest

None to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.waojou.2023.100852.

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REFERENCES

1. Okano M, Fujieda S, Gotoh M, et al. Executive summary: Japanese guidelines for allergic rhinitis 2020. *Allergol Int*. 2023;72:41–53.
2. Avdeeva KS, Fokkens WJ, Reitsma S. Towards a new epidemiological definition of chronic rhinitis: prevalence of nasal complaints in the general population. *Rhinology*. 2021;59:258–266.
3. Vardouniotis A, Doulaptsi M, Aoi N, Karatzanis A, Kawauchi H, Prokopakis E. Local allergic rhinitis revisited. *Curr Allergy Asthma Rep*. 2020;20:22.
4. Melone G, Giorgis V, Di Pino M, et al. Local allergic rhinitis: lights and shadows of a mysterious entity. *Int Arch Allergy Immunol*. 2023;184:12–20.
5. Eguiluz-Gracia I, Pérez-Sánchez N, Bogas G, Campo P, Rondón C. How to diagnose and treat local allergic rhinitis: a challenge for clinicians. *J Clin Med*. 2019;8:1062.
6. Kortekaas Krohn I, Shikhagaie MM, Golebski K, et al. Emerging roles of innate lymphoid cells in inflammatory diseases: clinical implications. *Allergy*. 2018;73:837–850.
7. Verma M, Verma D, Alam R. Role of type-2 innate lymphoid cells (ILC2s) in type-2 asthma. *Curr Opin Allergy Clin Immunol*. 2022;22:29–35.
8. Liu Y, Liu Z. [Function and modulation of type II innate lymphoid cells and their role in chronic upper airway inflammatory diseases]. *Zhonghua er bi yan hou tou jing wai ke za zhi*. 2017;52:130–135 (Chinese).
9. Olguín-Martínez E, Ruiz-Medina BE, Licona-Limón P. Tissue-specific molecular markers and heterogeneity in type 2 innate lymphoid cells. *Front Immunol*. 2021;12, 757967.
10. Wang X, Shen Y, Ke X, Kang H, Hong S. Role of leptin/ILC2 axis in allergic rhinitis in obese children. *Int J Pediatr Otorhinolaryngol*. 2022;157, 111127.
11. Wang X, Shen Y, Hong S, Kang H, Ke X. Changes in type 2 innate lymphoid cells and serum cytokines in sublingual immunotherapy in pediatric patients with allergic rhinitis. *BMC Pediatr*. 2023;23:13.
12. Xie Y, Zhang Y, Zhu T, et al. CD226 deficiency alleviates murine allergic rhinitis by suppressing group 2 innate lymphoid cell responses. *Mediat Inflamm*. 2022;2022, 1756395.
13. Chen BW, Qu SH, Li M, et al. [A murine model of local allergic rhinitis]. *Zhonghua er bi yan hou tou jing wai ke za zhi*. 2016;51:533–537 (Chinese).
14. Galle-Treger L, Hurrell BP, Lewis G, et al. Autophagy is critical for group 2 innate lymphoid cell metabolic homeostasis and effector function. *J Allergy Clin Immunol*. 2020;145:502, 17.e5.
15. Allen EA, Baehrecke EH. Autophagy in animal development. *Cell Death Differ*. 2020;27:903–918.
16. Perrotta C, Cattaneo MG, Molteni R, De Palma C. Autophagy in the regulation of tissue differentiation and homeostasis. *Front Cell Dev Biol*. 2020;8, 602901.
17. Zhou J, Li XY, Liu YJ, et al. Full-coverage regulations of autophagy by ROS: from induction to maturation. *Autophagy*. 2022;18:1240–1255.
18. Wang H, Zheng Y, Huang J, Li J. Mitophagy in antiviral immunity. *Front Cell Dev Biol*. 2021;9, 723108.
19. Zhang T, Liu Q, Gao W, Sehgal SA, Wu H. The multifaceted regulation of mitophagy by endogenous metabolites. *Autophagy*. 2022;18:1216–1239.
20. Deretic V, Levine B. Autophagy balances inflammation in innate immunity. *Autophagy*. 2018;14:243–251.
21. Nelson MP, Shacka JJ. Autophagy modulation in disease therapy: where do we stand? *Curr Pathobiol Rep*. 2013;1:239–245.
22. Panigrahi DP, Praharaj PP, Bhol CS, et al. The emerging, multifaceted role of mitophagy in cancer and cancer therapeutics. *Semin Cancer Biol*. 2020;66:45–58.
23. Li J, Li Y. Autophagy is involved in allergic rhinitis by inducing airway remodeling. *Int Forum Allergy Rhinol*. 2019;9:1346–1351.
24. He YQ, Qiao YL, Xu S, et al. Allergen induces $CD11c^{+}$ dendritic cell autophagy to aggravate allergic rhinitis through promoting immune imbalance. *Int Immunopharm*. 2022;106, 108611.
25. Dong M, Chen XW, Xi KH, et al. Influence of 18-β-glycyrrhetinic acid on mitochondria of nasal mucosa epithelial cells in rats with allergic rhinitis. *Journal of Otolaryngology Shandong University*. 2014;3:1–6 (Article in Chinese).
26. Kim HY, Nam SY, Kim HM, Jeong HJ. Exposure of ovalbumin during pregnancy prevents the development of allergic rhinitis in offspring through the induction of mast cell autophagy. *Cell Immunol*. 2021;365, 104362.
27. Kato Y, Akasaki S, Muto-Haenuki Y, Fujieda S, Matsushita K, Yoshimoto T. Nasal sensitization with ragweed pollen induces local-allergic-rhinitis-like symptoms in mice. *PLoS One*. 2014;9, e103540.
28. Wang C, Zhou ML, Liu YC, Cheng KJ. The roles of autophagy, mitophagy, and the akt/mTOR pathway in the pathogenesis of chronic rhinosinusitis with nasal polyps. *J Immunol Res*. 2022;2022, 2273121.
29. Terada T, Kawata R. Diagnosis and treatment of local allergic rhinitis. *Pathogens*. 2022;11:80.
30. Rondón C, Fernández J, López S, et al. Nasal inflammatory mediators and specific IgE production after nasal challenge with grass pollen in local allergic rhinitis. *J Allergy Clin Immunol*. 2009;124:1005–10011.e1.
31. Testera-Montes A, Palomares F, Jurado-Escobar R, et al. Sequential class switch recombination to IgE and allergen-induced accumulation of IgE+ plasmablasts occur in the nasal mucosa of local allergic rhinitis patients. *Allergy*. 2022;77:2712–2724.
32. Bozek A, Zalejska Fiolka J, Krajewska Wojtys A, Galuszka B, Cudak A. Potential differences between local and systemic allergic rhinitis induced by birch pollen. *Int Arch Allergy Immunol*. 2020;181:831–838.
33. Bozek A, Scierski W, Ignasiak B, Jarzab J, Misiolek M. The prevalence and characteristics of local allergic rhinitis in Poland. *Rhinology*. 2019;57:213–218.
34. Jung AY, Kim YH. Reversal of olfactory disturbance in allergic rhinitis related to OMP suppression by intranasal budesonide treatment. *Allergy Asthma Immunol Res*. 2020;12:110–124.
35. Liu W, Deng L, Chen Y, et al. Anti-inflammatory effect of IL-37b in children with allergic rhinitis. *Mediat Inflamm*. 2014;2014, 746846.

36. Campo P, Rondón C, Gould HJ, Barrionuevo E, Gevaert P, Blanca M. Local IgE in non-allergic rhinitis. *Clin Exp Allergy*. 2015;45:872–881.

37. Cheng KJ, Liu YC, Zhou ML, Zhou SH. The role of innate lymphoid cells in nasal inflammation and cancer. *Eur Rev Med Pharmacol Sci*. 2019;23:10588–10599.

38. Stevens WW, Kato A. Group 2 innate lymphoid cells in nasal polyposis. *Ann Allergy Asthma Immunol*. 2021;126:110–117.

39. Li Y, Wang W, Ying S, Lan F, Zhang L. A potential role of group 2 innate lymphoid cells in eosinophilic chronic rhinosinusitis with nasal polyps. *Allergy Asthma Immunol Res*. 2021;13:363–374.

40. Ealey KN, Moro K, Koyasu S. Are ILC2s jekyll and hyde in airway inflammation? *Immunol Rev*. 2017;278:207–218.

41. Lin L, Dai F, Wei JJ, Tang XY, Chen Z, Sun GB. Allergic inflammation is exacerbated by allergen-induced type 2 innate lymphoid cells in a murine model of allergic rhinitis. *Rhinology*. 2017;55:339–347.

42. Tojima I, Matsumoto K, Kikuoka H, et al. Evidence for the induction of Th2 inflammation by group 2 innate lymphoid cells in response to prostaglandin D2 and cysteinyl leukotrienes in allergic rhinitis. *Allergy*. 2019;74:2417–2426.

43. Tenn MW, Thiele J, Steacy LM, Adams DE, Ellis AK. Peripheral group 2 innate lymphoid cells are decreased following nasal allergen challenge in allergic rhinitis. *Allergy*. 2019;74:388–391.

44. Simsek F, Eren E, Bahceci S, Aladag I. High PI3K/mTOR and low MAPK/JNK activity results in decreased apoptosis and autophagy in nasal polyposis. *Braz J Otorhinolaryngol*. 2021;87:572–577.

45. Qi JJ, Han XF, Cai XL, et al. [Expression and clinical significance of autophagy-related gene Beclin1 and P62 in nasal polyps]. *Zhonghua er bi yan hou tou jing wai ke za zhi*. 2016;51:428–432 (Chinese).

46. Ye Y, Zhao J, Ye J, et al. The role of autophagy in the overexpression of MUC5AC in patients with chronic rhinosinusitis. *Int Immunopharm*. 2019;71:169–180.

47. Wang BF, Cao PP, Wang ZC, et al. Interferon-γ-induced insufficient autophagy contributes to p62-dependent apoptosis of epithelial cells in chronic rhinosinusitis with nasal polyps. *Allergy*. 2017;72:1384–1397.

48. Zhang S, Yan ZY, Wang D, Li SN, Xu Z, Tang QF. [Experimental study on the effects of tumor necrosis factor-α monoclonal antibody on autophagy level in allergic rhinitis mice]. *Zhonghua er bi yan hou tou jing wai ke za zhi*. 2019;54:517–523 (Chinese).

49. 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.

50. Li M, Yang X, Wang S. PTEN enhances nasal epithelial cell resistance to TNFα-induced inflammatory injury by limiting mitophagy via repression of the TLR4-JNK-Bnip3 pathway. *Mol Med Rep*. 2018;18:2973–2986.

51. Germic N, Frangez Z, Yousefi S, Simon HU. Regulation of the innate immune system by autophagy: neutrophils, eosinophils, mast cells, NK cells. *Cell Death Differ*. 2019;26:703–714.

52. O'Sullivan TE, Geary CD, Weizman OE, et al. Atg5 is essential for the development and survival of innate lymphocytes. *Cell Rep*. 2016;15:1910–1919.

53. Elshazly AM, Gewirtz DA. Making the case for autophagy inhibition as a therapeutic strategy in combination with androgen-targeted therapies in prostate cancer. *Cancers*. 2023;15:5029.

54. Wang L, Yuan X, Li Z, Zhi F. The role of macrophage autophagy in asthma: a novel therapeutic strategy. *Mediat Inflamm*. 2023;2023, 7529685.

55. Mishra J, Bhatti GK, Sehrawat A, et al. Modulating autophagy and mitophagy as a promising therapeutic approach in neurodegenerative disorders. *Life Sci*. 2022;311, 121153.