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## Involvement of autophagy in exacerbation of eosinophilic airway inflammation in a murine model of obese asthma

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### ABSTRACT

Obesity is a common comorbidity in patients with asthma, and obese asthma patients present the most refractory phenotype among patients with severe asthma. Similar to the observations in non-obese asthma patients, clinical studies have revealed heterogeneity in obese asthma patients, including the occurrences of T helper (Th)2-high and Th2-low phenotypes. However, the mechanisms underlying obesity-related asthma are not completely understood. Though macroautophagy/autophagy is involved in asthma and obesity, its role in obesity-associated asthma is unknown. We hypothesized that autophagy is involved in the pathogenesis of obese asthma. For our investigations, we used high-fat diet-induced *Atg5* (autophagy related 5)-deficient mice and epithelial cell-specific *atg5*<sup>-/-</sup> (*Scgb1a1/CCSP-atg5*<sup>-/-</sup>) obesity-induced mice. House dust mite (HDM)-sensitized *atg5*<sup>-/-</sup> obese mice exhibited marked eosinophilic inflammation and airway hyper-reactivity (AHR), compared to wild-type (WT) obese mice. Analyses of *atg5*<sup>-/-</sup> obese mice showed increased levels of Th2 cells but not ILC2s together with elevated expression of Th2 cytokines in the lung. In response to the HDM challenge, activated epithelial autophagy was observed in lean but not obese WT mice. Epithelium-specific deletion of *Atg5* induced eosinophilic inflammation in *Scgb1a1/CCSP-atg5*<sup>-/-</sup> obese mice, and genetic analyses of epithelial cells from HDM-immunized *atg5*<sup>-/-</sup> obesity-induced mice showed an elevated expression of thymic stromal lymphopoietin (TSLP) and IL33. Notably, HDM-sensitized *atg5*<sup>-/-</sup> mice developed TSLP- and IL33-dependent eosinophilic inflammation and AHR. Our results suggest that autophagy contributes to the exacerbation of eosinophilic inflammation in obese asthma. Modulations of autophagy may be a therapeutic target in obesity-associated asthma.

**Abbreviations:** AHR: airway hyper-reactivity; BAL: bronchoalveolar lavage; C<sub>dyn</sub>: dynamic compliance; BM: bone marrow; HDM: house dust mite; HFD: high-fat diet; ILC2s: type 2 innate lymphocyte cells; ROS: reactive oxygen species; R<sub>L</sub>: lung resistance; TSLP: thymic stromal lymphopoietin; TCC: total cell count; WT: wild type.

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### Introduction

Asthma and obesity are global health problems affecting adults, adolescents, and children [1–3]. Importantly, obesity is a common condition in asthma patients, and nearly 60% of patients with severe asthma are obese [4,5]. Obesity is intertwined with the pathogenesis of asthma via biological, physiological, and environmental factors. Epidemiological studies have revealed that obesity is a risk factor for the development of asthma [6] and vice versa [7]. Obese asthma patients exhibit the most severe phenotype with persistent symptoms, increased risk of hospitalization, and are refractory to standard medications [8–10]. Recently, obese patients with asthma have been shown to exhibit heterogeneous phenotypes similar to those of non-obese asthma patients [8]. In particular, obese asthma patients can be divided into two major phenotypes: late- and early-onset asthma [8]. Patients with late-onset asthma, who exhibit the classical features of obese asthma, are mostly females and have less allergic airway inflammation

(non-type 2 phenotype) involving neutrophilic inflammation [11] and oxidative stress [12]. Early-onset asthma, the most severe disease among obese asthma patients, is characterized by the type 2 phenotype, i.e., high expression of markers of allergic inflammation and airway hyper-reactivity (AHR), where eosinophils may play an important role [8]. However, the precise pathophysiology of obesity-associated asthma is not fully elucidated.

Autophagy is a fundamental cellular process that degrades and recycles damaged organelles, microorganisms, and denatured proteins [13–16]. This conserved process is essential for the maintenance of cellular homeostasis and organ function. Autophagy was originally identified as a cellular protective process in response to starvation [17]. Later, a growing body of evidence demonstrated that aberrant changes in autophagy contribute to various diseases, including aging, cancer, cardiovascular disease, diabetes mellitus, and obesity [13]. In addition, autophagy is also involved in asthma and allergic diseases via

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dendritic cell-, macrophage-, and type 2 innate lymphocyte cell (ILC2)-mediated adaptive and innate immune responses [18–21]. However, studies investigating the involvement of autophagy in obesity-associated asthma are lacking.

Here, we investigated the role of autophagy in the pathogenesis of obese asthma using a high-fat diet (HFD)-induced mouse model of obesity. We showed that aberrant autophagy contributed to deteriorating eosinophilic inflammation and AHR which was thymic stromal lymphopoietin (TSLP) - and IL33-dependent but did not respond to steroids. Defects in autophagy in epithelial cells are responsible for aggravated eosinophilic inflammation in mice with impaired autophagy. This study provides novel information for understanding the biological mechanisms of obese asthma and the importance of autophagy.

## Results

### Obese mice did not respond to house dust mite exposure

To investigate the role of autophagy in obese asthma, we generated HFD-induced obese mice. WT mice were fed HFD or normal chow for 16–18 weeks (Figure S1A–B), followed by sensitization and intranasal challenge with HDM extract according to the protocol shown in Figure S1C. Two days after the last HDM challenge, lung function was evaluated via direct measurements of lung resistance ( $R_L$ ) and dynamic compliance ( $C_{dyn}$ ) as described in the Methods section. Non-sensitized obese mice tended to have higher  $R_L$ , lower  $C_{dyn}$ , and higher total cell count (TCC) in bronchoalveolar lavage (BAL) than lean mice. Interestingly, HDM-sensitized obese mice exhibited less allergic inflammations, as was evident from the significantly lower  $R_L$  and higher  $C_{dyn}$ , together with lower eosinophils in BAL than in HDM-sensitized WT lean mice (Figure S1D–E). Interestingly, pharmacological inhibition of autophagy by 3-methyladenine (3-MA) reduced AHR and inflammation in HDM-sensitized WT obese mice (Figure S1F–G).

### Autophagy contributed to exacerbating airway inflammation in obese mice

The autophagy pathway consists of a cascade of essential genes, such as autophagy-related gene 5 (ATG5) [14–16,22]. Studies have shown that depletion of ATG5 efficiently disrupts the autophagy pathway; therefore, *Atg5*-deficient mice are important for studying the autophagy pathway [17,18,20,23]. As constitutively *Atg5*-deficient mice die soon after birth [17], we used inducible conditional *atg5* knockout mice in which tamoxifen injection deletes approximately 80–90% of *Atg5* expression (referred to as *atg5*<sup>-/-</sup> mice; Figure S2A). Alternatively, the impaired autophagy pathway in *atg5*<sup>-/-</sup> obese mice was further validated by assessing increased SQSTM1/p62 levels (Figure S2B).

First, we confirmed that *atg5*<sup>-/-</sup> lean mice exhibited higher AHR and eosinophil inflammation than WT lean mice (Figure S3). Then, WT and *atg5*<sup>-/-</sup> mice with HFD-induced obesity were sensitized and challenged with HDM (Figure 1A). Notably, HDM-sensitized *atg5*<sup>-/-</sup> obese mice

exhibited significantly higher AHR, representing higher  $R_L$  and lower  $C_{dyn}$  than the HDM-challenged WT obese mice (Figure 1B). Further, HDM-challenged *atg5*<sup>-/-</sup> obese mice showed significantly higher numbers of TCC, eosinophils, and neutrophils in BAL compared with the WT obese mice (Figure 1C). Histological analyses revealed significantly increased thickening of the bronchial wall and peribronchiolar cell infiltration, together with the presence of PAS<sup>+</sup> cells in HDM-challenged *atg5*<sup>-/-</sup> obese mice compared with the observations in WT obese mice (Figure 1D–F).

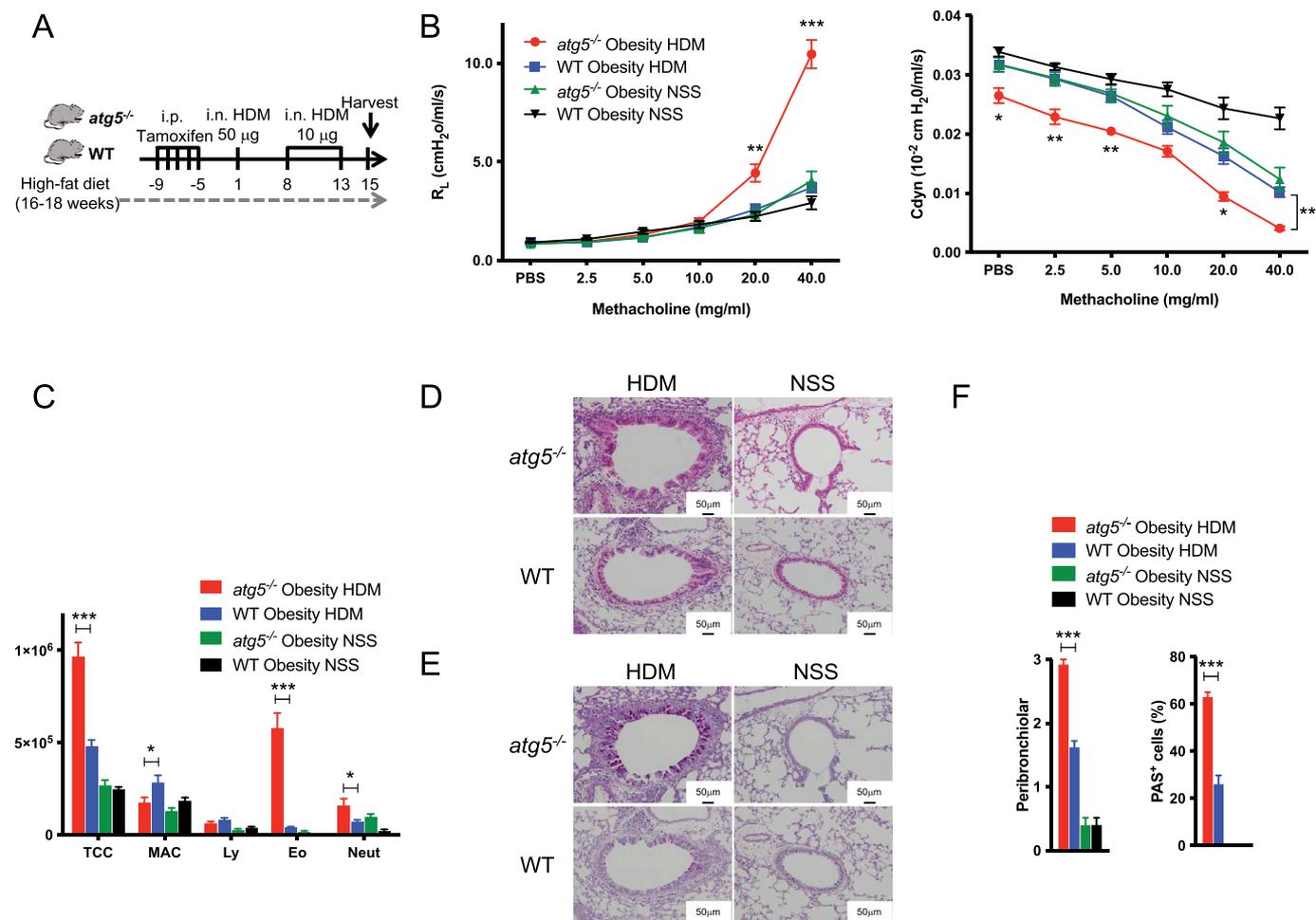
Next, cytokine levels in the lungs were assessed. Compared to WT obese mice, HDM-challenged *atg5*<sup>-/-</sup> obese mice exhibited significantly higher levels of IL5 (interleukin 5), IL13, IL33, and TSLP, along with ADIPOQ/adiponectin downregulation (Figure 2A–C). Meanwhile, the levels of IL17A, IL25, and LEP/leptin did not differ between *atg5*<sup>-/-</sup> obese mice and WT obese mice. The proportions of IL5- and IL13-producing CD4<sup>+</sup> T cells, but not IL17A-producing CD4<sup>+</sup> T cells, were significantly higher in HDM-challenged *atg5*<sup>-/-</sup> obese mice compared with those in WT obese mice (Figure 2D). In agreement with these results, GATA-3 and p-STAT6 expression was also elevated in HDM-challenged *atg5*<sup>-/-</sup> obese mice (Figure 2E). As both Th2 cells and ILC2s produce IL5 and IL13, we further analyzed ILC2s. As observed in a previous study [20], the number of ILC2s decreased in HDM-challenged *atg5*<sup>-/-</sup> obese mice (Figure S4). These results indicated the importance of Th2 cells in the development of severe allergic inflammation in *atg5*<sup>-/-</sup> obese mice.

### House dust mite-challenged *atg5*<sup>-/-</sup> obese mice were resistant to steroid treatment

Obese asthma is known to be resistant to steroid treatment [8–10]. In particular, early-onset obese asthma patients exhibited the most severe phenotype with high expression of markers of eosinophilic inflammation and AHR [8]. Further, increased infiltrations of eosinophils in the lungs of obese asthma have been reported [24,25]. Hence, we hypothesized that *atg5*<sup>-/-</sup> obese mice might represent obesity-associated asthma with a severe Th2 phenotype and investigated whether mice with HFD-induced obesity were resistant to steroid treatment. HDM-sensitized *atg5*<sup>-/-</sup> obese mice were treated with dexamethasone 1 day before and 1 day after the HDM challenge (Figure 3A). Notably, the results showed that dexamethasone did not ameliorate AHR and airway inflammation in HDM-sensitized *atg5*<sup>-/-</sup> obese mice (Figure 3B–C). Consistent with the AHR and BAL findings, lung histology showed that dexamethasone did not reduce bronchial wall thickening and the numbers of PAS<sup>+</sup> cells in *atg5*<sup>-/-</sup> obese mice (Figure S5D–E).

### Epithelial autophagy contributed to eosinophilic inflammation

As epithelial autophagy is considered to be involved in the pathogenesis of asthma [26,27], we investigated whether lack of autophagy in immune cells or epithelial cells might influence the development of severe asthma. Chimeric mice were generated to



**Figure 1.** Impaired autophagy exacerbates airway inflammation in house dust mite (HDM)-sensitized obese mice. (A) Experimental timeline. WT and *atg5*<sup>-/-</sup> mice were fed with high-fat diet, and then sensitized and challenged with house dust mite (HDM). (B) Lung resistance ( $R_L$ ) and dynamic compliance ( $C_{dyn}$ ), ( $n = 8-10$ /group) (C) Differential cell counts in BALF ( $n = 8-10$ /group). TCC, total cell count; MAC, macrophages; Ly, lymphocytes; Eo, eosinophils; Neut, neutrophils. (D) Hematoxylin and eosin staining. Original magnification:  $100\times$ . (E) PAS staining. Original magnification:  $100\times$ . (F) Quantifications of lung histopathology ( $n = 3-5$ /group). Data are representative of three independent experiments. Data are expressed as the mean  $\pm$  SEM. P-values were calculated using two-way ANOVA test post hoc test with the Tukey's multiple comparison test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

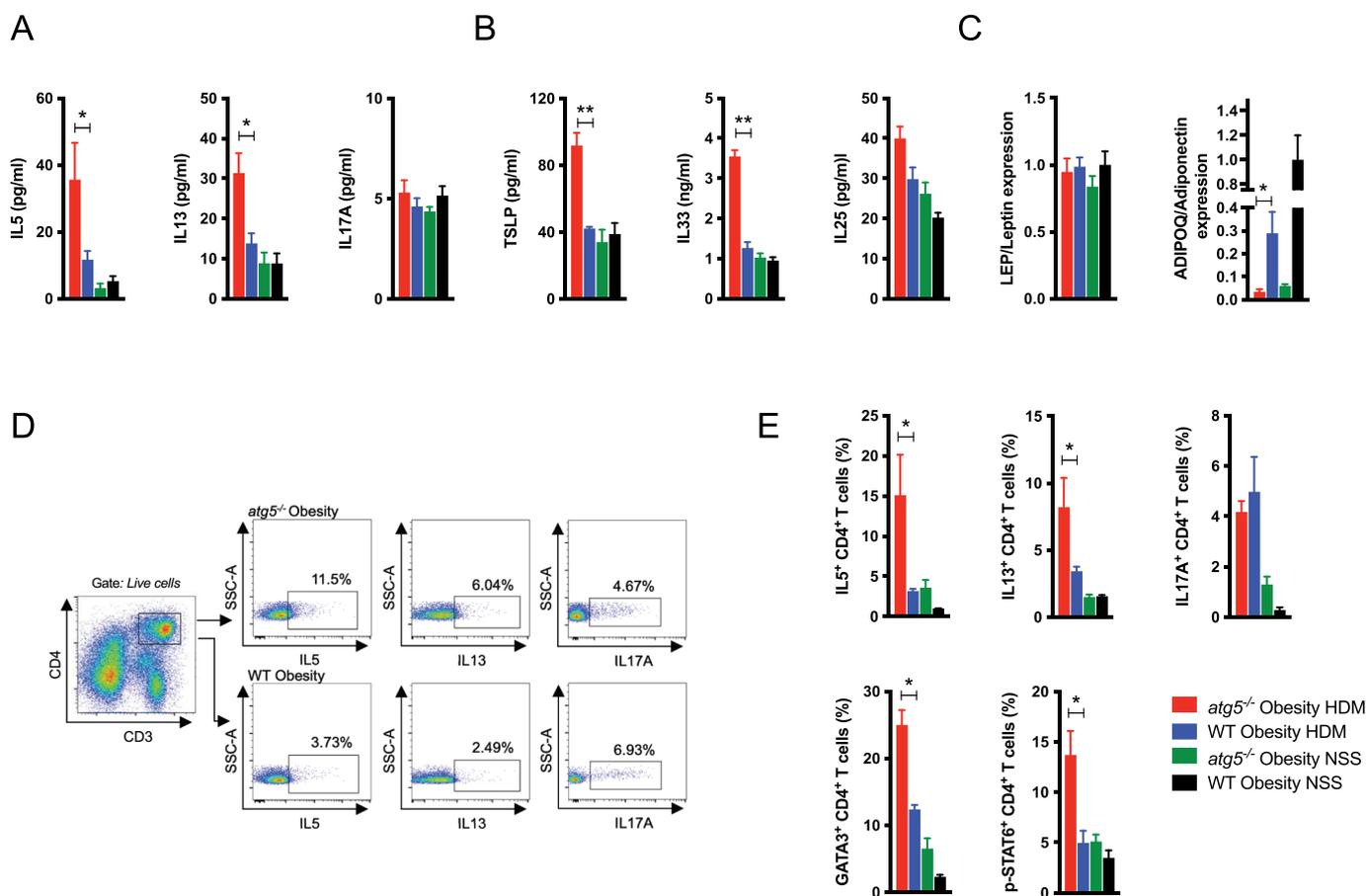
assess the role of immune cells and structural cells in autophagy-dependent eosinophilic airway inflammation. Bone marrow (BM) cells from WT or *atg5*<sup>-/-</sup> mice were injected into sublethally irradiated *atg5*<sup>-/-</sup> mice or WT mice. These mice were fed with HFD and then immunized with HDM (Figure 4A). A part of the BM cells was isolated from CAG-EGFP transgenic mice to determine the reconstitution rate. The results showed a reconstitution rate of approximately 80–90% by donor immune cells in the lungs (Figure 4B). Moreover, *atg5*<sup>-/-</sup> BM-injected *atg5*<sup>-/-</sup> obese mice (recipient, red) exhibited increased  $R_L$  and eosinophils in BAL compared to *atg5*<sup>-/-</sup> BM-injected WT obese mice (blue, Figure 4C–D). Similarly, another set of chimeric mice in which BM cells from WT mice were injected into irradiated *atg5*<sup>-/-</sup> mice (green) exhibited higher AHR and eosinophilic inflammation than WT obese mice generated by injecting WT BM cells (black, Figure 4C–D). HE and PAS staining are shown in Figure 4E–G. Collectively, these results suggested that *atg5*<sup>-/-</sup> structural cells are involved in eosinophilic inflammation in obese asthma rather than in immune cells.

Among the structural cells of the lung, epithelial cells act as external barriers against foreign antigens and can

mediate the innate and adaptive immune responses [28,29]. In this regard, the pivotal role of autophagy in the maintenance of epithelial homeostasis has been reported [30]. Hence, we investigated whether the lack of autophagy in epithelial cells may play a role in the development of persistent eosinophilic inflammation. We generated lung epithelial cell-specific autophagy-deficient mice by cross-matching *Atg5*<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup> mice with *Scgb1a1/CCSP-Cre* mice, in which *Atg5* is deleted in the lung epithelial cells under the control of *Scgb1a1* (secretoglobin, family 1A, member 1 (uteroglobin)). We observed that the eosinophils in BAL in HDM-sensitized *Scgb1a1/CCSP-atg5*<sup>-/-</sup> obese mice were significantly more abundant (Figure 5B), although this was not the case with AHR (Figure 5A).

#### Gene expression analyses in autophagy-deficient epithelial cells

Next, we investigated autophagy levels in lung epithelial cells using GFP-LC3 mice, which allow the analysis of LC3



**Figure 2.** Increase in Th2 cells in HDM-challenged *atg5*<sup>-/-</sup> obese mice. (A) Cytokine levels in BALF (n = 5/group). (B) Cytokine levels in lung homogenates (n = 5/group). (C) Relative lung cytokine mRNA expression in lung homogenates (n = 3–7/group). (D) Representative dot plot presentation of cytokine production by CD3<sup>+</sup> CD4<sup>+</sup> T cells in the lung. (E) Frequencies of IL-5-, IL13-, IL17A-producing CD3<sup>+</sup> CD4<sup>+</sup> T cells, and GATA3- and p-STAT6-expressing CD3<sup>+</sup> CD4<sup>+</sup> T cells in the lung (n = 3–5/group). Data are representative of two independent experiments. Data are expressed as the mean ± SEM. P-values were calculated using two-way ANOVA test post hoc test with the Tukey's multiple comparison test. \*, P < 0.05, \*\*, P < 0.01.

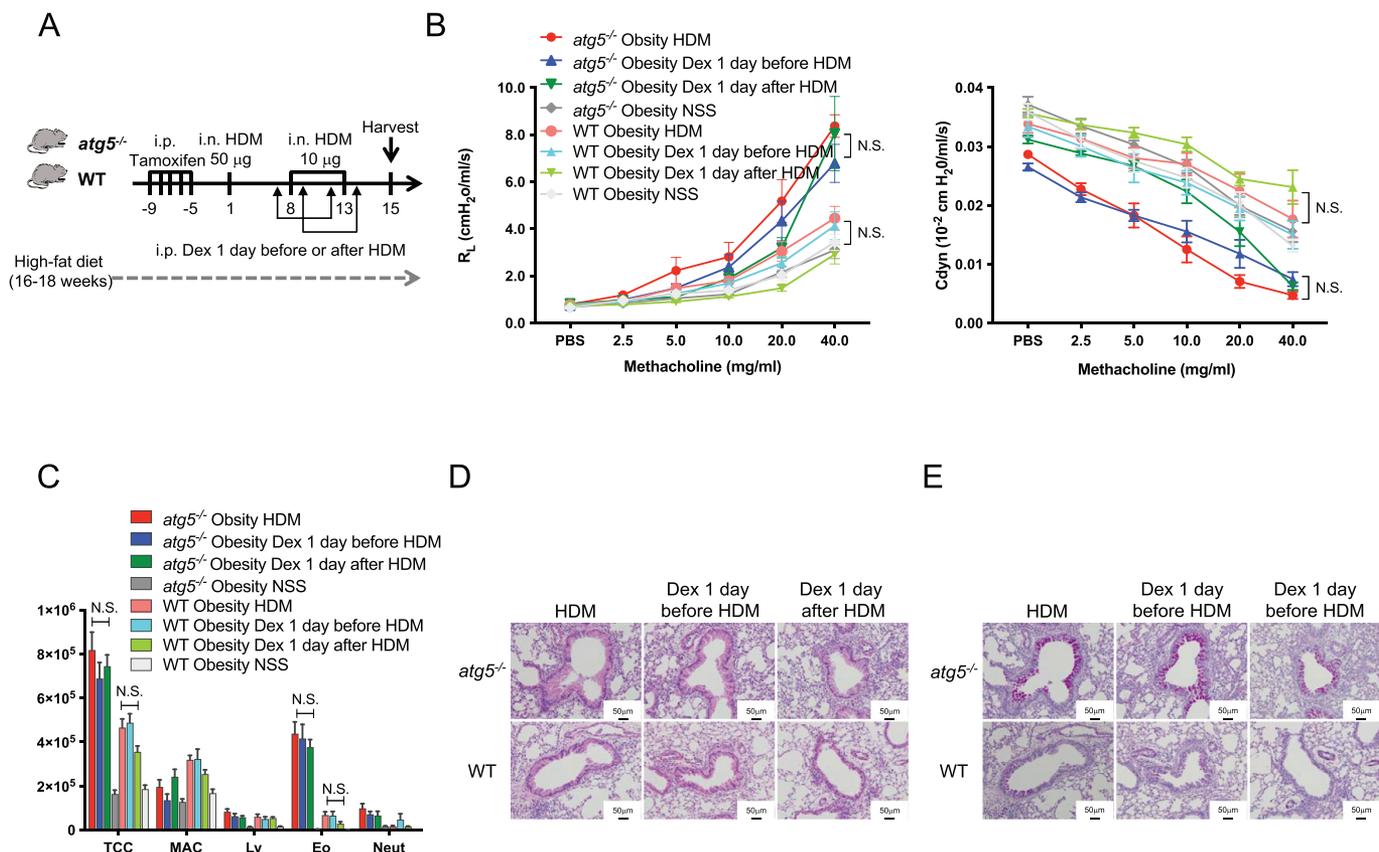
expression as a marker for autophagosomes [31,32]. Lung epithelial cells were identified as PTPRC/CD45<sup>-</sup> PECAM1/CD31<sup>-</sup> EPCAM<sup>+</sup> cells (Figure 6A), and LC3 expression in lean and obese mice was determined. In agreement with the induction of activated epithelial autophagy in response to IL13 *in vivo* [27], lung epithelial cells from HDM-sensitized lean WT mice exhibited increased LC3 expression compared with that in the lung epithelial cells of HDM-sensitized obese WT mice (Figure 6A). Confocal microscopic analyses of lung epithelial cells also showed a marked reduction in the GFP-LC3 puncta in HDM-sensitized obese mice compared with the observations in the HDM-sensitized lean mice (Figure 6B), suggesting that epithelial autophagy is induced to maintain epithelial homeostasis in response to allergic inflammation in HDM-sensitized lean mice.

To investigate the insufficient epithelial autophagy in obesity-associated asthma, we analyzed the gene expression profile of lung epithelial cells in *atg5*<sup>-/-</sup> and WT obese mice using Nanostring technology as described in the Methods section. Significant gene expression was depicted using a heat plot (Figure 6C). Eotaxin (CCL24 and CCL11) and inflammatory chemokines (CCL20, CXCL1, and CXCL3), as well as epithelial cell-derived cytokines (IL33 and TSLP), were upregulated

in *atg5*<sup>-/-</sup> obese mice compared to those in WT obese mice. Furthermore, IL18, IL1A/IL1 $\alpha$ , and TGFB/TGF- $\beta$  levels were reduced in *atg5*<sup>-/-</sup> obese mice. Among these, we validated the expression of epithelial cell-derived cytokines. Consistent with the array data, these observations showed increased expression of IL33 and TSLP, but not that of IL25, in epithelial cells from *atg5*<sup>-/-</sup> obese mice (Figure 6D).

### House dust mite-challenged *atg5*<sup>-/-</sup> obese mice developed TSLP- and IL33-dependent asthma

An increase in Th2 and decrease in ILC2s were observed in *atg5*<sup>-/-</sup> obese mice (Figure 2 and Figure S4), while impaired functions of autophagy-deficient ILC2s in response to IL33 have been reported [20]. Here, we investigated TSLP and IL33, as they can activate both type-2 skewed adaptive and innate immune systems. We determined whether AHR and eosinophilic inflammation in *atg5*<sup>-/-</sup> obese mice were TSLP- and IL33-dependent. Mice were immunized with HDM and treated with anti-TSLP blocking antibody or anti-IL33 neutralizing antibodies one day before the HDM challenge. As expected, both TSLP and IL33 blockade significantly



**Figure 3.** HDM-challenged *atg5*<sup>-/-</sup> obese mice were refractory to steroid treatment. (A) Experimental timeline. *atg5*<sup>-/-</sup> obese mice and WT obese mice were immunized as described in Figure 1A. The mice were intraperitoneally treated with dexamethasone (1 mg/kg) 1 day before or after HDM challenges. (B) Lung resistance ( $R_L$ ) and dynamic compliance ( $C_{dyn}$ ) (C) Differential cell counts in BALF. TCC, total cell count; MAC, macrophages; Ly, lymphocytes; Eo, eosinophils; Neut, neutrophils. (D) Hematoxylin and eosin staining. Original magnification: 100 ×. (E) PAS staining. Data are representative of two independent experiments ( $n = 5-7$ /group). Data are expressed as the mean ± SEM. P-values were calculated using two-way ANOVA test post hoc test with the Tukey's multiple comparison test.

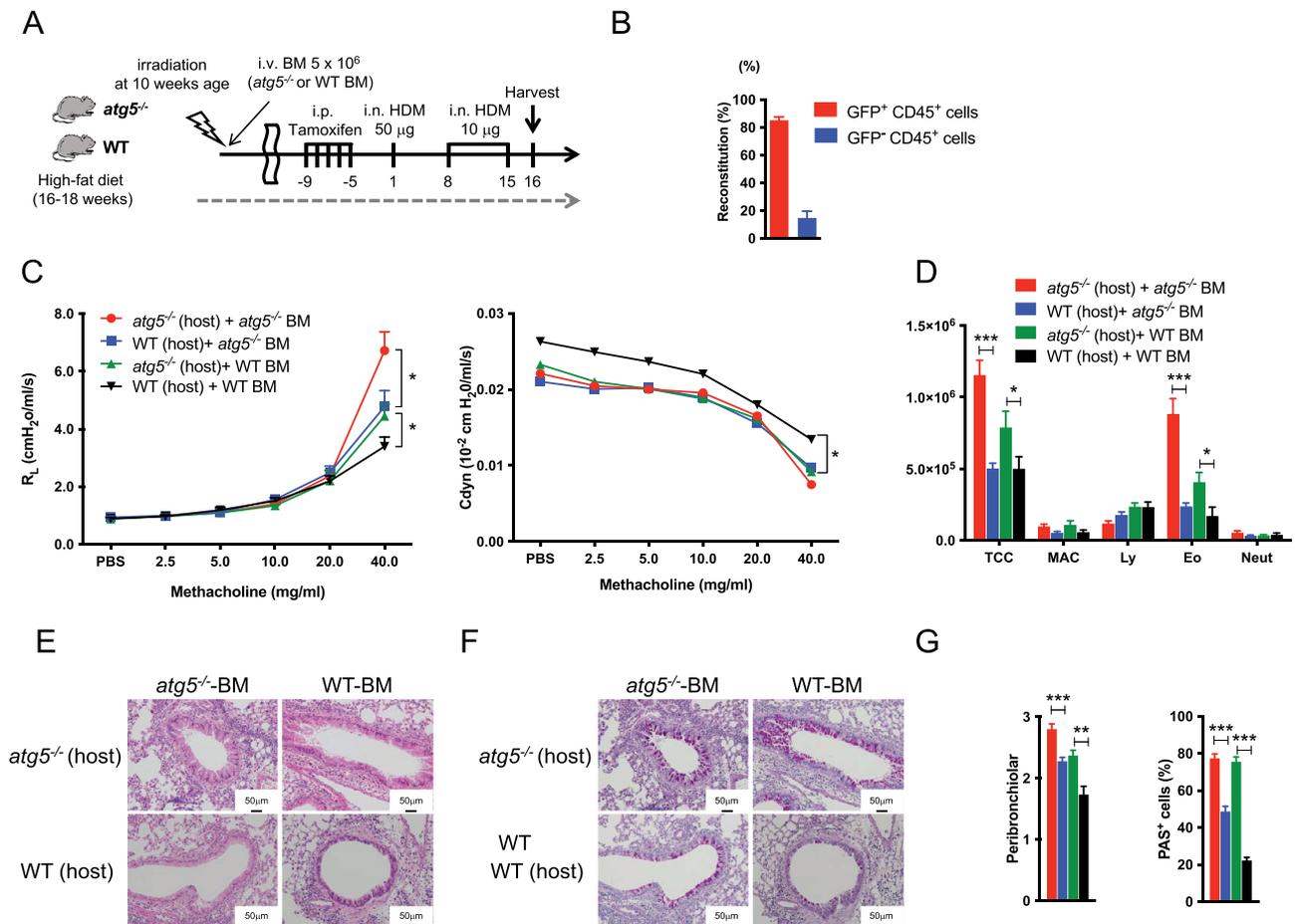
attenuated AHR and eosinophilic inflammation in *atg5*<sup>-/-</sup> obese mice, but not in WT obese mice (Figure 7). Histological analyses also showed that anti-TSLP and anti-IL33 treatment ameliorated airway thickness and reduced the number of infiltrated cells and PAS<sup>+</sup> cells in *atg5*<sup>-/-</sup> obese mice (Figure 7C, F). Collectively, these results indicated that *atg5*<sup>-/-</sup> obese mice developed TSLP- and IL33-dependent AHR with enhanced eosinophil inflammation. Our data suggested that autophagy may contribute significantly to the pathogenesis of severe obese asthma.

## Discussion

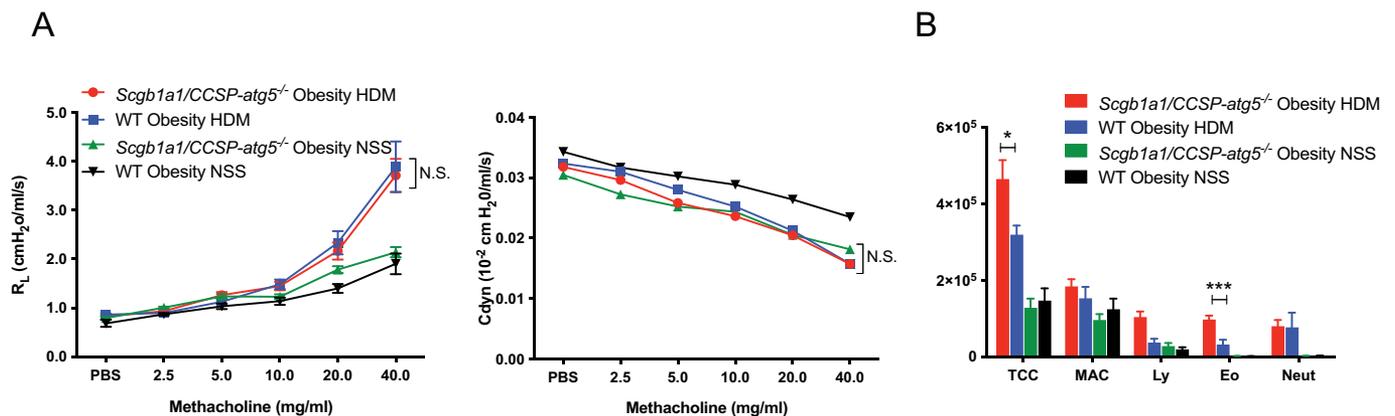
In this study, we investigated the role of autophagy in obese asthma using HFD-induced murine models of obesity. We observed that autophagy disruption in obese mice markedly increased eosinophil-mediated inflammation and elevated AHR in response to HDM sensitization. This autophagy-mediated type 2 inflammation in obese mice was augmented by Th2 cells but not by ILC2s. Furthermore, HDM-sensitized WT obese mice showed lower allergic inflammation. Importantly, epithelial autophagy was activated upon allergic inflammation in HDM-sensitized lean WT mice but not in HDM-sensitized obese WT mice. Conversely, obese mice with specific deletion of *Atg5* in lung epithelial cells

exhibited enhanced eosinophilic inflammation, and epithelial cells in HDM-sensitized *atg5*<sup>-/-</sup> obese mice exhibited an increased expression of TSLP and IL33. Finally, we showed that HDM-sensitized *atg5*<sup>-/-</sup> obese mice were refractory to steroid treatment and developed TSLP- and IL33-dependent asthma. Collectively, our findings suggest that the dysregulation of autophagy exacerbates obesity-associated asthma, particularly that with the Th2-high phenotype.

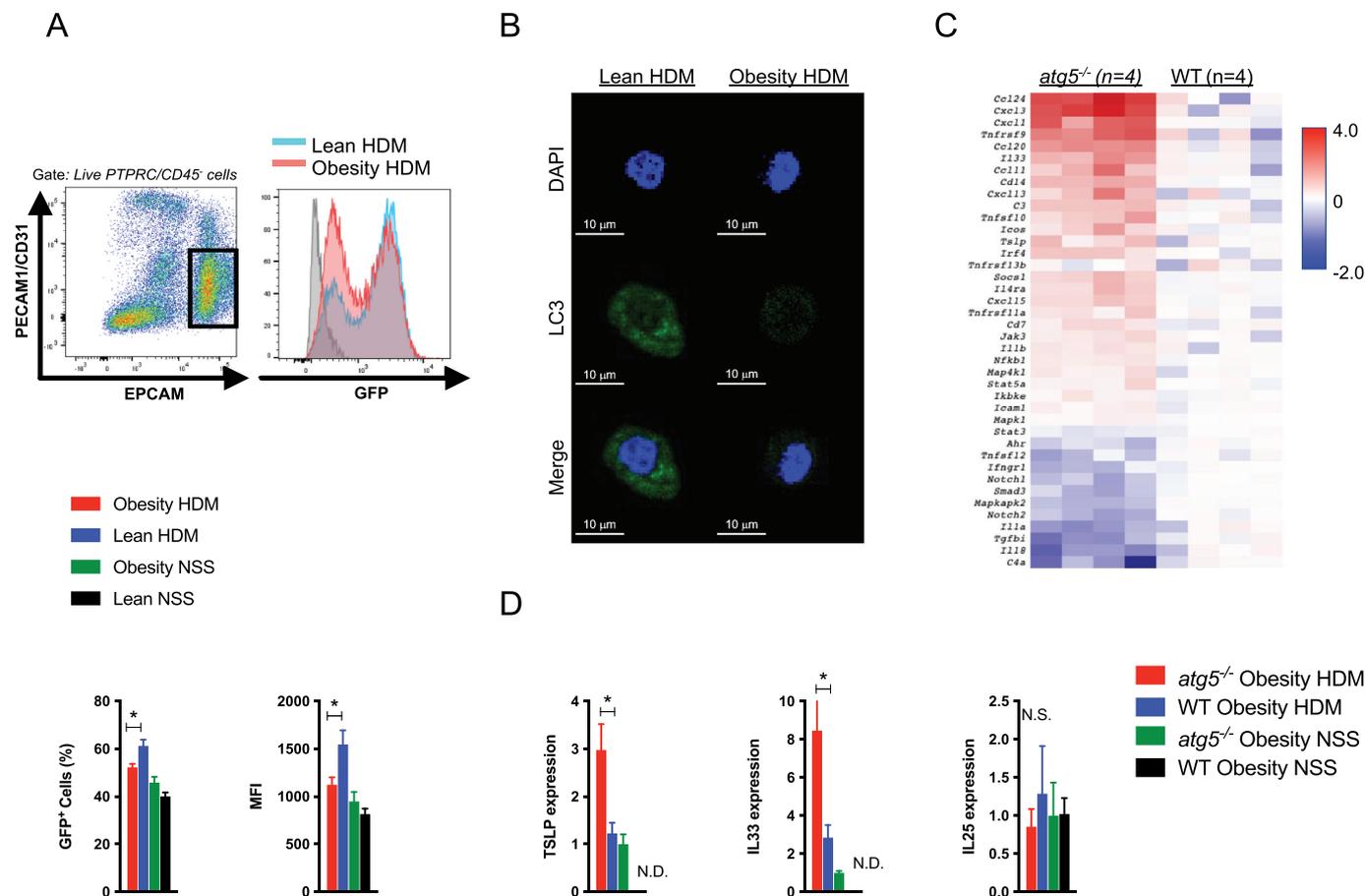
Obesity causes a chronic low-grade inflammatory state that autophagy-associated pathways can potentially modulate [33,34]. Lipid overload can affect autophagy, which can decrease lipophagy and mitochondrial turnover and increase ER stress, low-grade inflammation, and finally lead to insulin resistance, although differences in autophagy regulation can be cell- and organ-specific; autophagy is upregulated in adipose tissue, whereas it is mostly down-regulated in the liver, heart and pancreas [34]. However, previous studies have not investigated the effects of HFD on autophagy in the lungs. As described previously, increased autophagosome formation in the epithelium and sputum was observed in patients with asthma [26,35,36]. In agreement with this, we showed that HDM-sensitized lean mice expressed significantly higher levels of LC3 compared with non-sensitized lean mice. Meanwhile, LC3 expressions in HFD treated obese mice (both HDM-sensitized and non-



**Figure 4.** Impaired autophagy in structured cells contribute to autophagy-mediated airway inflammation in obesity asthma. (A) Experimental timeline. WT or *atg5*<sup>-/-</sup> bone-marrow (BM) cells were adaptively transferred into irradiated WT or *atg5*<sup>-/-</sup> mice. The mice were fed with HFD, and then immunized with HDM. A part of the BM cells was isolated from CAG-EGFP transgenic mice to assess the reconstitution rate. (B) Reconstitution rate. (C) Lung resistance (R<sub>L</sub>) and dynamic compliance (C<sub>dyn</sub>) (D) Differential cell counts in BALF (n = 8-9/group). TCC, total cell count; MAC, macrophages; Ly, lymphocytes; Eo, eosinophils; Neut, neutrophils. (E) Hematoxylin and eosin-staining. Original magnification: 100 × . (F) PAS staining. (G) Quantifications of lung histopathology (n = 5/group). Data are representative of two independent experiments. Data are expressed as the mean ± SEM. P-values were calculated using the Student's *t* test (*atg5*<sup>-/-</sup> (host) + *atg5*<sup>-/-</sup> BM vs. WT (host) + *atg5*<sup>-/-</sup> BM, and *atg5*<sup>-/-</sup> (host) + WT BM vs. WT (host) + WT BM). \*, P < 0.05, \*\*, P < 0.01, \*\*\*, P < 0.001.



**Figure 5.** Impaired autophagy in epithelial cells induce eosinophilic inflammation in obese asthma. *Scgb1a1/CCSP-atg5*<sup>-/-</sup> obese mice and WT obese mice were immunized as described in Figure 1A. (A) Lung resistance (R<sub>L</sub>) and dynamic compliance (C<sub>dyn</sub>) (B) Differential cell counts in BALF (n = 5-6/group). TCC, total cell count; MAC, macrophages; Ly, lymphocytes; Eo, eosinophils; Neut, neutrophils. Data are representative of two independent experiments. Data are expressed as mean ± SEM. P-values were calculated using two-way ANOVA test post hoc test with the Tukey's multiple comparison test. \*, P < 0.05, \*\*, P < 0.01.



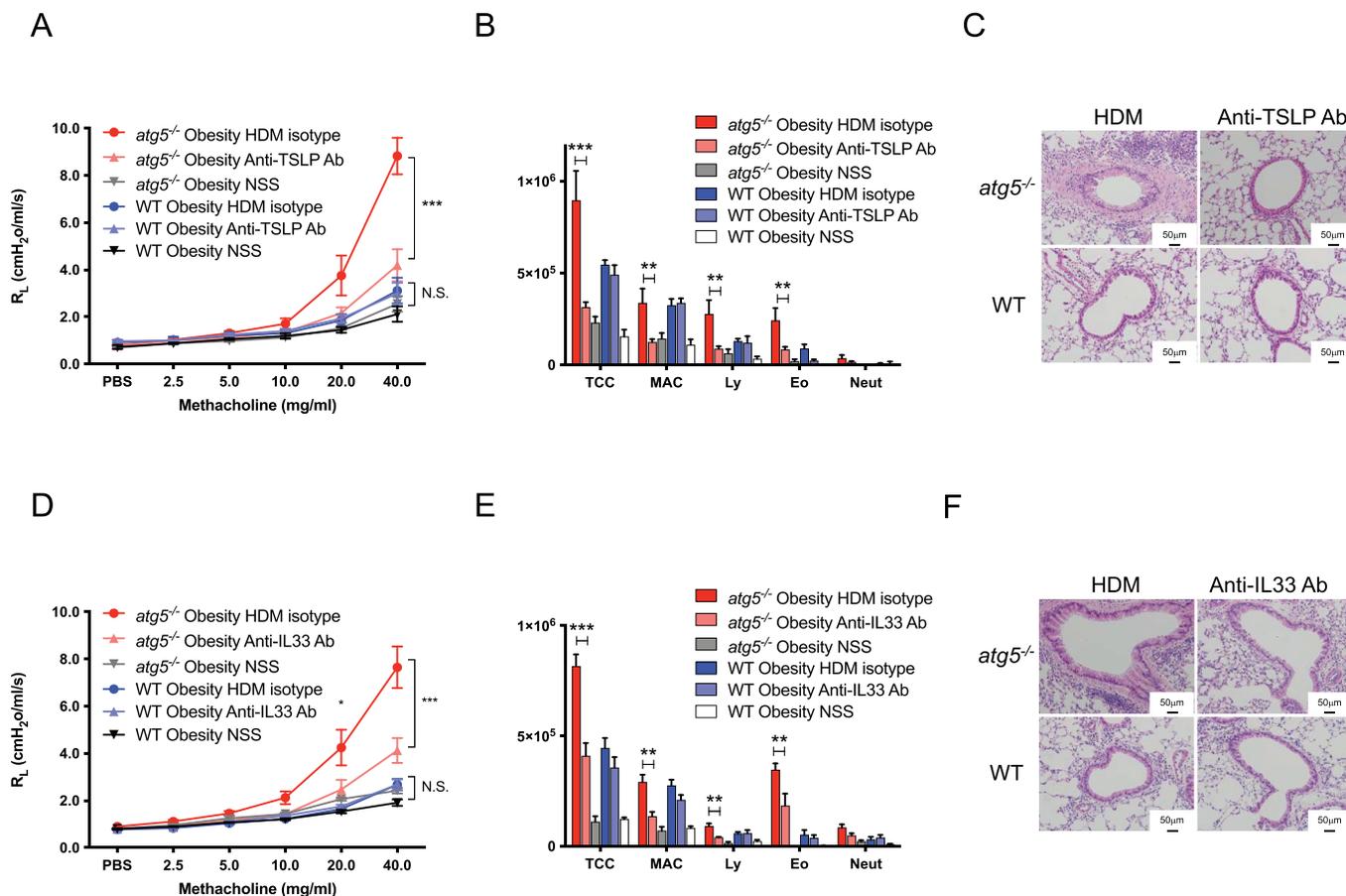
**Figure 6.** Gene expression analyses in autophagy-deficient epithelial cells. (A) GFP-LC3 mice were immunized as described in Figure E1C. Identification of lung epithelial cells (PTPRC/CD45<sup>-</sup> PECA1/CD31<sup>-</sup> EPCAM<sup>+</sup> cells). Representative FACS histogram plots of GFP-LC3 expression in lung epithelial cells. Frequencies of GFP-LC3-positive cells and mean fluorescence intensity (MFI) of GFP-LC3 in lung epithelial cells (n = 4–5/group). (B) Confocal microscopy images of isolated lung epithelial cells from GFP-LC3 mice. (C) Heat plot showing alteration of depicted genes in lung epithelial cells in *atg5*<sup>-/-</sup> obese mice and WT obese mice (P < 0.05, n = 4). FACS-purified lung epithelial cells from *atg5*<sup>-/-</sup> and WT obese mice immunized with HDM were quantified using the Nanostring nCounter technology. (D) Relative mRNA expression of lung epithelial cells (n = 3–5/group). Data are expressed as the mean ± SEM. P-values were calculated using two-way ANOVA test post hoc test with the Tukey's multiple comparison test. \*, P < 0.05.

sensitized) were similar to those in non-sensitized lean mice. Collectively, these results suggested that autophagy was activated in response to allergic inflammation, but overnutrition induced by HFD alone did not significantly affect epithelial autophagy levels.

Autophagy is a homeostatic cellular process, perturbations of which lead to various human diseases [13–16]. Recent evidence has suggested that autophagy is involved in asthma and allergy; in addition to activated autophagy in the lungs of asthma patients, autophagy was also involved in the pathogenesis of both childhood and adult asthma with respect to genetic polymorphism of *ATG5* [26,37], reduced forced expiratory volume in 1.0 s (FEV<sub>1.0</sub>) [26], and airway remodeling [38,39]. Importantly, obesity is common in patients with asthma and exacerbates the symptoms of asthma [4,8,9]. Furthermore, although autophagy mediates the shared underlying pathogenesis of obesity and asthma, studies analyzing its role in obese asthma are lacking. Here, we showed for the first time that autophagy exacerbates eosinophilic inflammation using a murine model. Obesity-associated asthma is

heterogeneous and includes Th2-high (eosinophilic) and Th2-low asthma (neutrophilic) phenotypes [4,8]. Particularly, Th2-high obese asthma is characterized by early-onset of symptoms and is the most refractory phenotype in patients with severe asthma. Additionally, increased eosinophil accumulation in the lung [40] and elevated submucosal eosinophilia in obese asthma were reported [24,25], suggesting the importance of eosinophilic inflammation in obesity-associated asthma. Collectively, though our murine model did not mimic early-onset asthma, our results suggest the involvement of autophagy in eosinophilic inflammation in obese asthma.

Autophagy plays cell-specific roles in asthma and allergic diseases. Indeed, previously distinct and often opposing effects of autophagy have been reported elsewhere [41–43]. Cell-specific *Atg5* deletion in ITGAX/CD11c<sup>+</sup> cells augmented lung inflammation with an increase in IL17A, leading to severe neutrophilic asthma in HDM-sensitized mice [18]. *atg7*<sup>-/-</sup> myeloid cell lineages exhibited increased eosinophil inflammation in a murine model of chronic rhinosinusitis, possibly mediated by aggravated IL1B and prostaglandin D2



**Figure 7.** HDM-challenged *atg5*<sup>-/-</sup> obese mice develop TSLP-dependent and IL33-dependent asthma. (A) Experimental timeline. *atg5*<sup>-/-</sup> obese mice and WT obese mice were immunized as described in Figure 1A. Anti-thymic stromal lymphoprotein (TSLP) antibody (20  $\mu$ g), anti-IL33 antibody (6  $\mu$ g), or isotype controls were intranasally administered before HDM challenges. (A and D) Lung resistance ( $R_L$ ), (B and E) Differential cell counts in BALF. TCC, total cell count; MAC, macrophages; Ly, lymphocytes; Eo, eosinophils; Neut, neutrophils. (C and F) Hematoxylin and eosin-staining. Original magnification: 100 $\times$ . (E) PAS staining. Data are representative of two independent experiments ( $n = 5-7$ /group). Data are expressed as the mean  $\pm$  SEM. P-values were calculated using two-way ANOVA test post hoc test with the Tukey's multiple comparison test. \*,  $P < 0.05$ , \*\*,  $P < 0.01$ , \*\*\*,  $P < 0.001$ .

production from macrophages and mast cells, respectively [21]. Conversely, autophagy is essential for the homeostasis and survival of ILC2s [19,20], and *Atg5*-deficient ILC2s showcase impaired ability to secrete IL5 and IL13, resulting in attenuation of AHR and eosinophil inflammation in IL33-treated mice [20]. Thus, these distinct roles of autophagy point to its ability to modulate different cellular processes, and not only direct control of the turnover of specific proteins [13,14,41]. In this study, we observed that deletion of *Atg5* in epithelial cells exacerbated eosinophil inflammation in obese asthma but was not sufficient to induce AHR. Epithelial cells act as the first line of mucosal defense against pathogens and innocuous antigens. Autophagy is induced in epithelial cells together with mucus secretion in response to various stimuli such as IL13 [27,44], IFNG/IFN- $\gamma$  [45-47], and particulate matter [48]. Recently, autophagy has been determined to be essential for the maintenance of homeostasis of the airway epithelium during steady-state and allergic inflammation [30,41]. Impaired autophagy also leads to epithelial cell dysfunction and lung fibrosis [49]. These pieces of evidence indicate that autophagy plays pivotal roles in maintaining epithelial homeostasis and recovery from epithelial injuries, suggesting that the dysregulation of

autophagy in epithelial cells exacerbates allergic airway inflammation.

The role of obesity in development of eosinophilic inflammation in murine models were different by the reports even using same antigens. We found less eosinophilic inflammation in HDM-sensitized WT obese mice, which was similar to previous study [50]. Meanwhile, we detected elevated levels of TSLP and IL33, epithelial cell-derived cytokines, in *atg5*<sup>-/-</sup> obese mice, indicative of TSLP and IL33-dependent AHR and eosinophil inflammation. Proteolytic and mechanistic epithelial damage induced by various allergens and pathogens is a key trigger of allergic inflammations [51-53]. Importantly, viral infections, the most common causes of asthma exacerbation, can also modulate the autophagic flux and occasionally hijack the autophagy pathway for their survival [41,54]. Recently, Liu *et al.* demonstrated that co-exposure of benzo-pyrene and dermatophagoides group 1 allergen (Der f 1), but not benzo-pyrene or Der f 1 alone, facilitated the generation of epithelial reactive oxygen species (ROS), a regulator of autophagy, which in turn promoted TSLP and IL33 production, resulting in eosinophil inflammation and AHR [55].

The present study demonstrated alteration of autophagy by genetic deletion and HDM-sensitization. However, obesity

could also affect autophagy flux. Consequently, increased SQSTM1/p62 levels, an autophagy substrate, were found in lungs of HDM-sensitized *atg5*<sup>-/-</sup> obese mice indicating impaired autophagy pathway. A number of stress signaling pathways, including ER stress, were activated during *Atg5* gene deletion, HDM-sensitization and obesity-associated low grade inflammation. Cellular stress induced by external or internal cues activate well-orchestrated processes to mitigate damage caused by such circumstances and maintain cellular homeostasis. The close and complex link between autophagy unfolded protein response (UPR) and mitochondrial functions were noted; both can either be stimulated or inhibited by feedback mechanisms [34,56,57]. Therefore, we speculated that aberrant autophagy in HDM-sensitized *atg5*<sup>-/-</sup> obese mice triggered changes in UPR or mitochondrial function, which could modulate autophagy flux, initiating crosstalk between the two processes. Defects in autophagy result in the inability to recycle damaged organelles, potentially causing the release of ROS and other cellular components [58]. Dysregulation of autophagy leads to impaired apoptosis and clearance of dying cells [58]. Considering these essential roles of autophagy in maintaining cellular homeostasis, genetic deletion of *Atg5* gene, overnutrition, and allergen exposure synergistically induce massive ROS generation and exacerbate eosinophilic airway inflammation in obese asthma.

The modulation of autophagy appears to be a novel therapeutic strategy for translating our results into a clinical setting. Indeed, viral infection is the most common cause of asthma exacerbation and can disrupt the autophagic flux [41,54]. Metformin, an autophagy inducer, reportedly curtails asthma exacerbation in patients with asthma also having diabetes mellitus; as such patients might be obese [59]. In addition, several autophagy inducers, including rapamycin and simvastatin, also reduced AHR and eosinophilic inflammation in a murine model [60–63]. Conversely, autophagy inhibitors can inhibit allergic inflammation [64], which was further confirmed with the observations in our HDM-sensitized WT obese mice. These bidirectional effects of autophagy on asthma can be explained by their multiple roles. For instance, autophagy is essential for antigen presentation; hence, autophagy inhibitors attenuate allergic inflammation. Meanwhile, rapamycin and metformin preserve energy metabolism by regulating mitophagy and mitochondrial biogenesis [42]. Furthermore, as described above, distinct cell-specific organ-specific roles of autophagy have been reported. Therefore, cell-specific and airway-targeted modulation of autophagy, which ultimately avoids detrimental systemic adverse effects, is required to apply our results in a clinical setting. However, in addition to the known mechanisms of obese asthma [65–67], the present study advances current understanding regarding obese asthma and proposes autophagy and epithelial cell-derived cytokines as therapeutic targets for Th2-high obese asthma.

In conclusion, this study showed that autophagy is involved in exacerbation of obese asthma using HDM-sensitized *atg5*<sup>-/-</sup> obese mice. Aberrant autophagy triggered eosinophil inflammation and AHR in a murine model of obese asthma. Importantly, this study identified that epithelial cell-derived cytokines, including TSLP and IL33, are involved

in the pathogenesis of this autophagy-mediated disease and may act as therapeutic targets for obesity-associated asthma.

## Materials and methods

### Mice

Female C57BL/6 mice (4 weeks old) were purchased from Nippon SLC. *Atg5*<sup>fllox/fllox</sup> and GFP-LC3 mice were gifts from Dr. Noboru Mizushima (Tokyo University). The CAG-EGFP transgenic mouse was a gift from Tetsumei Urano (Hamamatsu University School of Medicine). *atg5*<sup>-/-</sup> mice and *Scgb1a1/CCSP*-specific *atg5*<sup>-/-</sup> mice were generated by crossing *Atg5*<sup>fllox/fllox</sup> mice with *rosa26 Cre ER<sup>T</sup>* mice and *Scgb1a1/CCSP-Cre* mice, respectively. Mice were screened using polymerase chain reaction (PCR) and *atg5*<sup>fllox/fllox</sup> homozygote *Rosa-Cre* hemizygote mice and *atg5*<sup>fllox/fllox</sup> homozygote *Scgb1a1/CCSP-Cre* hemizygote mice were selected for experiments. *atg5*<sup>fllox/fllox</sup> mice were backcrossed with *rosa26 Cre ER<sup>T</sup>* mice, and *Scgb1a1/CCSP-Cre* mice were bred in our facility at Hamamatsu University School of Medicine using protocols approved by the Institutional Animal Care and Use Committee.

Mice (4 weeks old) were fed either a 60% high-fat diet (HFD; D12492) from Research Diets or normal chow (10% fat; PMI Nutrition International, 5L37) for 16 to 18 weeks before the experiments. Mice received tamoxifen (800 µg per mouse per day; Bayer, 341,110,680) intraperitoneally (*i.p.*) for five consecutive days to induce the deletion of the *Atg5* gene. The cellular analyses of BAL and AHR in WT obese mice treated with tamoxifen were similar to those of WT obese mice without tamoxifen (data not shown).

### House dust mite-induced AHR and measurement of airway hyper-responsiveness

Mice were sensitized intranasally (*i.n.*) with 50 µg of HDM (*Dermatophagoides pteronyssinus*; Greer Laboratories, XPB82D3A2.5) on day 1, followed by 10 µg of HDM (*i.n.*) on days 8 and 13. In some experiments, mice were treated with 3-MA (*i.p.*, 300 mg; Sigma-Aldrich, M9281), dexamethasone (*i.p.*, 1 mg/kg; Sigma-Aldrich, D4902) or phosphate-buffered saline (PBS; Gibco, 14,190), anti-mouse IL33 antibody (*i.n.*, 6 µg/mice, clone 396,118; R&D, AF3626) or Goat IgG isotype controls (R&D, AB-108-C), and anti-mouse TSLP antibody (*i.n.*, 20 µg/mice, clone 152,614; R&D, MAB555) or Rat IgG2A isotype control antibody (R&D, MAB006), one day before each HDM challenge. Two days after the last HDM challenge, mice were anesthetized using 300 µl *i.p.* injection of ketamine (10 mg/ml) and xylazine (1 mg/ml). Measurements of airway resistance and dynamic compliance were conducted using the Fine Pointe RC system (Buxco Research Systems), in which mice were mechanically ventilated using a modified version as described previously [18,68]. Mice were sequentially challenged with aerosolized PBS (baseline), followed by increasing doses of methacholine (2.5 mg/ml, 5.0 mg/ml, 10 mg/ml, 20 mg/ml and 40 mg/ml); Sigma-Aldrich, A2251.  $R_L$  and  $C_{dyn}$  values were recorded during a 3-min period after each methacholine challenge.

### Collection of BAL, lung histology, and lung lysates

After measurements of AHR, BAL cells were obtained as described previously [18,68]. The lung histological section and lung lysate were obtained and quantified. After measurements of AHR, the trachea was cannulated and the lungs were lavaged 3 times with 1 ml ice-cold PBS to collect BAL cells. Transcardial perfusion of the lungs with PBS was performed to remove red blood cells, and the lungs were fixed and harvested for histology using 4% paraformaldehyde in PBS. After fixation, the lungs were embedded in paraffin, cut into 4- $\mu$ m sections, and stained with hematoxylin-eosin (HE) and periodic acid-Schiff (PAS). In some experiments, the lungs were collected and homogenized in RIPA buffer (Santa Cruz Biotechnology, sc-24,948) or RNA stabilization reagent (Qiagen, 76,104). The homogenates were analyzed for cytokines, as described elsewhere.

An inflammation score was assigned in a blinded fashion according to a previous study [69]. The score of peribronchiolar inflammation from HE staining was determined as follows: 0, normal; 1, few cells; 2, a ring of inflammatory cells one cell layer deep; 3, a ring of inflammatory cells 2–4 cells deep; and 4, a ring of inflammatory cells more than four cells deep. PAS staining was performed by examining at least 20 consecutive fields. Numerical scores for the abundance of PAS-positive goblet cells in each airway were determined as follows: 0, < 5% goblet cells; 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4, > 75%, with 0 being negative and 1–4 being positive for PAS staining.

### Identification of lung epithelial cells and ILC2s

Lung epithelial cells were identified as live, PTPRC/CD45<sup>-</sup> PECAM1/CD31<sup>-</sup> EPCAM<sup>+</sup> cells, while lung ILC2 cells were live cells lacking the classical lineage markers (CD3E, PTPRC/CD45R, GSR/Gr-1, ITGAX/CD11c, ITGAM/CD11b, LY76/Ter119, KLRB1/NK1.1, TRB/TCR- $\beta$ , TRD-TRG/TCR- $\gamma\delta$ , and FCER1A/FC $\epsilon$ RI $\alpha$ ), PTPRC/CD45<sup>+</sup>, IL7R/CD127<sup>+</sup>, and IL1RL1/IL33Ra<sup>+</sup> populations.

### Flow cytometry and intracellular analyses

BAL cells were stained with phycoerythrin (PE)-labeled anti-Siglec-F (clone E50-2440; BD Pharmingen, 562,068), allophycocyanin (APC)-labeled anti-Ly-6 G/Ly-6C (clone RB6-8C5; BioLegend, 108,412), PE-Cy (PE-Cy7)-labeled anti-PTPRC/CD45 (clone 30-F11; BioLegend, 103,114), APC-Cy7-labeled anti-ITAGX/CD11c (clone N418; BioLegend, 117,324), peridinin-chlorophyll-protein complex-Cy5.5 (PerCP-Cy5.5)-labeled anti-CD3E (clone17A2; BioLegend, 100,218), Alexa Fluor-labeled anti-CD19 (clone 6D5; BioLegend, 101,505), and Pacific Blue-labeled anti-ITGAM/CD11b (clone M1/70; BioLegend, 101,224).

The lung epithelial cells were isolated as follows. The lungs were digested with collagenase (Worthington, LS004196) and DNase (Worthington, LS002139) using a GentleMACS<sup>TM</sup> dissociator (Miltenyi Biotec), and a single cell suspension was prepared. The cells were incubated with anti-mouse PTPRC/CD45-coated magnetic beads (clone 13/2.3; BioLegend,

76,746) and negatively sorted via magnetic cell sorting (BioLegend, 480,019). The cells were stained using the live/dead fixable near-IR dead cell stain kit (Invitrogen, L10119), PE-Cy7-labeled anti-PTPRC/CD45 (clone 30-F11; BioLegend, 103,114), APC-labeled anti-PECAM1/CD31 (clone 390; BioLegend, 102,410), and PE-labeled anti-EPCAM (clone G8.8; BioLegend, 118,205). The lung epithelial cells were sorted as live, PTPRC/CD45<sup>-</sup>, PECAM1/CD31<sup>-</sup>, and EPCAM<sup>+</sup> cells using FACSARIA SORP (BD Bioscience). For analyses of ILC2 cells, the following antibodies were used: biotinylated anti-mouse lineage (CD3E (145–2C11; BioLegend, 79,751), PTPRC/CD45R (RA3-6B2; BioLegend, 79,752), GSR/Gr-1 (RB6-8C5; BioLegend, 79,750), ITGAX/CD11c (N418; BioLegend, 117,304), ITGAM/CD11b (M1/70; BioLegend, 79,749), LY76/Ter119 (TER-119; BioLegend, 79,748), KLRB1/NK1.1 (PK136; BioLegend, 108,704), TRB/TCR- $\beta$  (H57-597; BioLegend, 109,204), TRD-TRG/TCR- $\gamma\delta$  (BioLegend; 118,103), and FCER1A/FC $\epsilon$ RI $\alpha$  (MAR-1; BioLegend, 134,304), Brilliant Violet 510-labeled anti-PTPRC/CD45 (clone30-F11; BioLegend, 103,127), APC-labeled anti-IL7R/CD127 (clone A7R34; BioLegend, 135,011), PE-Cy7-labeled anti-IL7R/CD127 (clone A7R34; BioLegend, 135,014), PerCP-Cy5.5-labeled anti-IL1RL1/IL33Ra (clone DIH9; BioLegend, 145,312), and streptavidin-FITC (BioLegend, 405,201).

The following antibodies were used for intracellular staining; live/dead fixable near-IR dead cell stain kit (Invitrogen, L34965), PerCP-Cy5.5-labeled anti-CD3E (clone17A2; BioLegend, 100,218), PE-Cy7-labeled anti-CD3E (clone 17A2; BioLegend, 100,219), Pacific Blue-labeled anti-CD4 (clone GK1.5; BioLegend, 100,428), PerCP CD4 (clone RM4-5; BioLegend, 100,537), Brilliant Violet-510 (BV510)-labeled anti-CD44 (clone IM7; BioLegend, 103,043), eFluor 450-labeled anti-PTPRC/CD45 (clone 30 F-11; ebioscience, 40–0451-82), PE-labeled anti-IL5 (clone TRFK5; BoLegend, 504,304), PE-Cy7-labeled anti-IL13 (clone eBio13A; ebioscience, 25–7133-82), APC-labeled anti-IL17A (clone TC11-18H10.1; BioLegend), PE-labeled anti-GATA3 (clone 16E10A23; BioLegend, 653,804), and APC-labeled anti-p-STAT6 (clone CH12S4N; ebioscience, 17–9013-41). Flow cytometry was performed using Gallios (Beckman Coulter) and the data were analyzed using the FlowJo version 8.6 software (TreeStar).

Intracellular staining was performed using a BD Cytofix/Cytoperm kit (BD Bioscience, 554,714) and fixation/permeabilization buffer kit (eBioscience, 88–8824-00) according to the manufacturer's instructions.

### Gene expression analysis using Nanostring nCounter technology

The difference in the abundance of transcripts between lung epithelial cells purified from HDM-sensitized *atg5*<sup>-/-</sup> obese mice or WT obese mice were analyzed using Nanostring nCounter technology (Immunology Panel). Nanostring nCounter technology employs unique fluorescent barcode and enables direct and digital detection of the abundance of transcripts with no amplification. Heat plots were generated using the nSolver software.

### Preparation of bone marrow chimeric mice

Mice were irradiated with 600 rad and injected the following day with  $5 \times 10^6$  BM cells. In some experiments, BM cells were collected from CAG-EGFP mice, and the reconstitution rate was confirmed. Reconstitution of lung leukocytes was approximately 90% in flow cytometry quantification of GFP-labeled CD45<sup>+</sup> cells.

### Elisa

The levels of cytokines and SQSTM1/p62 were measured by ELISA (Thermo Fisher Scientific, 88-7054-22, 88-7137-22, 77-7371-22, 88-7002-22, 88-7333-22, 88-7490-22 and Enzo Life Science, ADI-900-212-0001), according to the manufacturer's instructions.

### Reverse transcription-PCR

Total RNA was extracted using the RNeasy mini kit (Qiagen, 74,104) and cDNA was synthesized using the HighCapacity cDNA reverse transcription kit (Applied Biosystems, 4,368,814). RT-PCR was performed using StepOnePlus (Applied Biosystems) and the  $\Delta\Delta C_t$  method [70] was used for data analysis.

### Quantification of autophagy levels using flow cytometry and confocal microscopy

GFP-LC3 knock-in transgenic mice were fed HFD or normal diet and then immunized with HDMs as described above (Figure S1). Lung epithelial cells were sorted as described above. MAP1LC3/LC3 (microtubule-associated protein 1 light chain 3) expression was quantified using FACS and confocal microscopy (Leica, TCS SP8).

### Statistical analysis

The Student's *t*-test and two-way ANOVA test post hoc test with the Tukey's multiple comparison test were used.  $P < 0.05$  was considered statistically significant. All data are expressed as mean  $\pm$  SD. Statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA).

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### Disclosure statement

No potential conflict of interest was reported by the author(s).

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