

Lack of autophagy induces steroid-resistant airway inflammation

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Background: Neutrophilic corticosteroid-resistant asthma accounts for a significant proportion of asthma; however, little is known about the mechanisms that underlie the pathogenesis of the disease.

Objective: We sought to address the role of autophagy in lung inflammation and the pathogenesis of corticosteroid-resistant neutrophilic asthma.

Methods: We developed CD11c-specific autophagy-related gene 5 (*Atg5*)^{-/-} mice and used several murine models to investigate the role of autophagy in asthmatic patients.

Results: For the first time, we found that deletion of the *Atg5* gene specifically in CD11c⁺ cells, which leads to impairment of the autophagy pathway, causes unprovoked spontaneous airway hyperreactivity and severe neutrophilic lung inflammation in mice. We found that severe lung inflammation impairs the autophagy pathway, particularly in pulmonary CD11c⁺ cells in wild-type mice. We further found that adoptive transfer of *Atg5*^{-/-}, but not wild-type, bone marrow–derived dendritic cells augments lung inflammation with increased IL-17A levels in the lungs. Our data indicate that neutrophilic asthma in *Atg5*^{-/-} mice is glucocorticoid resistant and IL-17A dependent.

Conclusion: Our results suggest that lack of autophagy in pulmonary CD11c⁺ cells induces neutrophilic airway inflammation and hyperreactivity. (J Allergy Clin Immunol 2015;■■■■:■■■■-■■■■.)

Key words: Autophagy, asthma, lung inflammation, neutrophilic asthma, corticosteroid-resistance asthma

Asthma is a heterogeneous disease with different phenotypes of lung inflammation involving the large and small airways and alveoli, resulting in airway hyperreactivity (AHR), bronchoconstriction, and airway remodeling.¹⁻³ The prevalence

Abbreviations used

AEC:	Alveolar epithelial cell
AHR:	Airway hyperreactivity
BAL:	Bronchoalveolar lavage
BALF:	Bronchoalveolar lavage fluid
BM:	Bone marrow
BMDC:	Bone marrow–derived dendritic cell
COPD:	Chronic obstructive pulmonary disease
DC:	Dendritic cell
HDM:	House dust mite
PE:	Phycoerythrin
RT-PCR:	Real-time PCR
TCR:	T-cell receptor
WT:	Wild-type

of allergic disease and asthma has increased dramatically over the past 5 decades. Although treatment of mild-to-moderate asthma has dramatically improved inhaled corticosteroid and long-acting β_2 -agonist combination therapy, approximately 10% of asthmatic patients are unresponsive to conventional treatment and have severe refractory asthma.^{4,5} A recent cohort analysis has revealed that noneosinophilic inflammation is predominant in patients with mild-to-moderate asthma and that neutrophilic inflammation is dominant in patients with severe refractory asthma.⁶ In particular, patients with increased neutrophil counts, as well as eosinophil counts, in sputum have been found to have decreased lung function.⁷ Severe allergic asthma starts with a T_H2-mediated disease with secretion of IL-4, IL-5, and IL-13, and as the severity of the disease increases (through unknown mechanisms), other cytokines, such as IL-17A, mediate the recruitment of inflammatory cell types, such as neutrophils, which further contribute to the pathogenesis of the disease.⁸⁻¹⁰ Several reports suggest that IL-17 levels are increased in the lungs of patients with severe asthma and correlate with AHR severity.¹¹⁻¹³ Dendritic cells (DCs) might play an essential role in regulating IL-17A production and greatly contribute to the pathogenesis of asthma.^{14,15}

Autophagy is a critically important intracellular process through which damaged self-organelles are cleared and disassembled and their composing units are recycled.¹⁶⁻¹⁸ Interestingly, genetic polymorphisms in autophagy-related gene 5 (*Atg5*) have been associated with childhood asthma.^{19,20} However, the role of autophagy in the development of allergic asthma remains unknown. In the present study we demonstrate a critical role for autophagy in inducing neutrophilic lung inflammation in a murine model of allergic asthma by modulating CD11c⁺ cells through the axis of IL-23/IL-17A–producing T cells. This study will provide new evidence to understand the biological mechanisms of asthma pathogenesis

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that might be fundamental for the development of novel treatment options.

METHODS

Detailed methods are described in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Mice, sensitization, and measurement of airway hyperresponsiveness

Six- to 8-week-old female C57BL/6, BALB/c, CD45.1, OT-II, tamoxifen-induced *Atg5*^{-/-}, and CD11c-specific *Atg5*^{-/-} mice were purchased or generated, as described in the [Methods](#) section in this article's Online Repository. House dust mite (HDM) sensitization and measurement of airway hyperresponsiveness were done, as described previously and in the [Methods](#) section in this article's Online Repository. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Southern California.

Collection of bronchoalveolar lavage fluid, lung histology, and lung lysates

After measurements of AHR, bronchoalveolar lavage (BAL) cells were collected, and lung histologic sections were obtained, as described previously and in the [Methods](#) section in this article's Online Repository.^{21,22}

Flow cytometry

The composition of BAL cells was analyzed by using flow cytometry, as described previously.²³ The repertoire of pulmonary DCs was analyzed by using flow cytometry, as described elsewhere.²⁴ Intracellular cytokine production and T-cell subsets were identified, as described in the [Methods](#) section in this article's Online Repository.

Preparation of bone marrow–derived DCs, *in vitro* culture, and adoptive transfer

Bone marrow (BM) cells were harvested and cultured, as previously described.^{21,25} BMDCs were cocultured with naive CD4⁺ T cells or pulsed with HDM and adoptively transferred to naive C57BL/6J mice, as described in the [Methods](#) section in this article's Online Repository.

Real-time PCR assays

Relative gene expression levels were measured by using real-time PCR (RT-PCR), as described in the [Methods](#) section in this article's Online Repository.

Quantification of autophagy levels by using Western blotting and confocal microscopy

The autophagy pathway was analyzed in BALB/c, C57BL/6, and LC3-GFP mice by using Western blotting or confocal microscopy, as described in the [Methods](#) section in this article's Online Repository.

Statistical analysis

P values for lung function data were calculated by using repeated-measures ANOVA, and *P* values for other data were calculated by using the Student *t* test. *P* values of less than .05 were considered significant. All data are expressed as means ± SDs. Statistical analyses were performed with JMP Start Statistics (SAS Institute, Cary, NC).

RESULTS

Lack of autophagy augments neutrophilic airway inflammation

We used *Atg5*-deficient mice to study the role of the autophagy pathway in lung allergic inflammation because depletion of *Atg5* has been shown to efficiently disrupt the autophagy pathway.^{16-18,26-28} Constitutively, *Atg5*-deficient mice die soon after birth,²⁸ and therefore we used inducible conditional *Atg5* knockout mice in which injection of tamoxifen deletes approximately 80% to 90% of the *Atg5* gene, which we refer to as *Atg5*^{-/-} mice (see [Fig E1](#) in this article's Online Repository at www.jacionline.org). Wild-type (WT) and *Atg5*^{-/-} mice were sensitized and intranasally challenged with HDM extract according to the protocol shown in [Fig 1, A](#). One day after the last HDM challenge, lung function was evaluated by means of direct measurements of lung resistance and dynamic compliance, as described in the [Methods](#) section. The results showed that HDM-challenged *Atg5*^{-/-} mice had significantly higher lung resistance and dynamic compliance compared with HDM-challenged WT mice ([Fig 1, B](#)). HDM-challenged *Atg5*^{-/-} mice had significantly higher numbers of total cells, neutrophils, and macrophages in bronchoalveolar lavage fluid (BALF) compared with WT mice ([Fig 1, C](#)). Histologic examinations revealed marked peribronchial inflammatory responses in *Atg5*^{-/-} mice, with increased airway wall thickness and accumulation of inflammatory cells than seen in WT mice ([Fig 1, D](#)).

Levels of IL-17A, IL-1β, IL-4, and IL-13 were assessed in whole-lung lysates by means of ELISA and at the intracellular level by means of flow cytometry to explore the mechanism of autophagy-dependent neutrophilic airway inflammation. We found significantly higher levels of IL-17A and IL-1β, which can induce IL-17A production, in the lungs of HDM-sensitized *Atg5*^{-/-} mice than in WT mice ([Fig 1, E](#)). This finding is consistent with the role of IL-17A in recruitment of neutrophils in BALF ([Fig 1, C](#)). Interestingly, there was no difference in levels of the T_H2 cytokines IL-4 and IL-13 between *Atg5*^{-/-} and WT mice, which correlate with the comparable number of eosinophils in BALF found in WT and *Atg5*^{-/-} mice ([Fig 1, C and E](#)). The intracellular cytokine staining assay also revealed a significantly increased frequency of CD3⁺CD4⁺CD44⁺IL-17A⁺ pulmonary effector T cells in *Atg5*^{-/-} compared with WT mice, whereas the frequency of IL-4, IL-5, IL-13, and IFN-γ from the CD3⁺CD4⁺CD44⁺ population did not differ ([Fig 1, F](#), and see [Fig E2](#) in this article's Online Repository at www.jacionline.org). Because T-cell receptor (TCR) γδ T cells, TCRαβ cells, and innate lymphoid cells can produce IL-17A in the lungs, we further identified the source of IL-17A production in the lungs. We found that the TCRγδ T cells are the major source of IL-17A in the lungs of *Atg5*^{-/-} mice (see [Fig E3](#) in this article's Online Repository at www.jacionline.org). We further examined whether disruption of autophagy leads to enhanced viability of neutrophils in BALF and found no difference between *Atg5*^{-/-} and WT mice (see [Fig E4](#) in this article's Online Repository at www.jacionline.org).

To investigate whether disruption of autophagy influences the pulmonary DC repertoire, we evaluated numbers of different DC subsets in the lungs of *Atg5*^{-/-} and WT mice at steady state and after HDM stimulation using a previously described approach.²⁴ Numbers of alveolar macrophages, plasmacytoid DCs, and

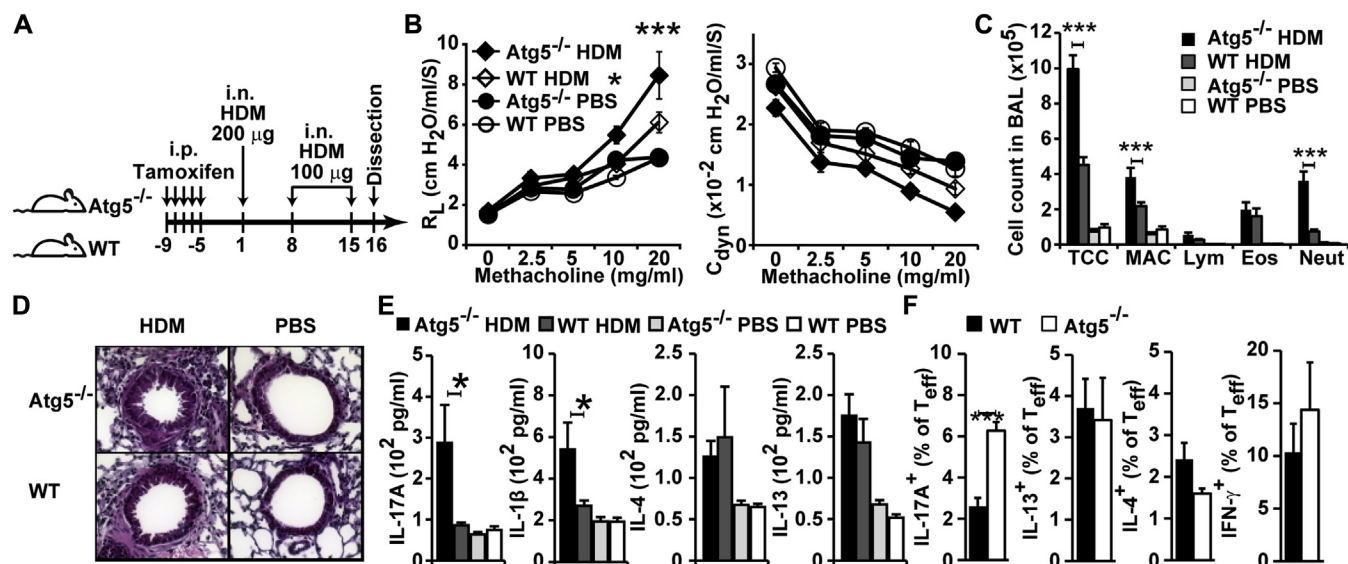


FIG 1. Lack of autophagy augments neutrophilic airway inflammation. **A**, Experimental timeline. *i.n.*, Intranasal; *i.p.*, intraperitoneal. **B**, Lung resistance (R_L ; left panel) and dynamic compliance (C_{dyn} ; right panel) measured on day 16. **C**, Differential cell counts in BALF. *Eos*, Eosinophils; *Lym*, lymphocytes; *MAC*, macrophages; *Neut*, neutrophils; *TCC*, total cell number. **D**, Hematoxylin and eosin-stained lung sections (magnification $\times 200$). **E**, Cytokine levels in whole-lung lysates. **F**, Frequency of IL-17A⁺, IL-13⁺, IL-4⁺, and IFN- γ -producing CD3⁺CD4⁺CD44⁺ effector T (T_{eff}) cells. Data are representative of 2 to 3 experiments and shown as means $\pm$ SEMs ($n = 5$ –10 per group). * $P < .05$ and *** $P < .001$.

CD103⁺ and CD11b⁺ DCs are comparable in *Atg5*^{-/-} and WT mice at steady state and after HDM stimulation (see Fig E5 in this article's Online Repository at www.jacionline.org). These results demonstrate that a lack of autophagy in *Atg5*^{-/-} mice causes severe lung inflammation and AHR, which is mediated by increased neutrophilic airway inflammation through secretion of IL-17A by T cells.

Lack of autophagy in immune cells, but not lung epithelial cells, contributes to induction of lung inflammation

Because a recent study revealed that epithelial autophagy is involved in the pathogenesis of cigarette smoke-induced chronic obstructive pulmonary disease (COPD),^{29,30} we investigated whether lack of autophagy in alveolar epithelial cells (AECs) might play a role in the development of severe AHR. We generated lung AECs specific for autophagy-deficient mice, as described previously and in the Methods section in this article's Online Repository.³¹ Our data showed that levels of lung inflammation and AHR, IL-1 β , or IL-17A in SPC-*Atg5*^{-/-} mice were similar to those of WT mice (see Fig E6 in this article's Online Repository at www.jacionline.org), suggesting a nonepithelial cell involvement for the development of autophagy-mediated severe lung inflammation.

Next, to examine the contribution of immune cells in autophagy-mediated severe lung inflammation, we injected BM of *Atg5*^{-/-} (CD45.2⁺) or WT (CD45.2⁺) mice into sublethally irradiated WT (CD45.1⁺) mice (Fig 2, A), which resulted in approximately 90% reconstitution of donor cells (Fig 2, B). Compared with WT BM-recipient mice, *Atg5*^{-/-} BM recipients displayed significantly higher AHR (Fig 2, C and D) and significantly higher neutrophil numbers in BALF (Fig 2, E). There was no significant induction of AHR in the recipients of WT BM.

Lung histology showed similar results to AHR and BAL findings (Fig 2, F). These results indicate that BM-derived immune cells rather than nonhematopoietic cells (including alveolar and bronchial epithelial cells) contribute to the severity of AHR induced by autophagy deficiency. To confirm our findings and exclude the role of autophagy in nonhematopoietic cells, we performed a new set of chimeric experiments in which irradiated *Atg5*^{-/-} hosts received either WT or *Atg5*^{-/-} BM, followed by intranasal sensitization (Fig 2, G). This was an inverse experiment to the aforementioned experiment and enabled us to address whether radioresistant *Atg5*^{-/-} host cells play an important role in induction of neutrophilic lung inflammation. Donor BM cells reconstituted more than 80% of irradiated host CD45⁺ hematopoietic cells (Fig 2, H). In agreement with our previous findings, our results show that intranasal HDM administration increased neutrophilic lung inflammation and AHR only in *Atg5*^{-/-} BM recipients but not in WT BM recipients (Fig 2, I–L). Taken together, these data show that lack of autophagy in hematopoietic cells underlies the augmented lung inflammation and AHR.

Induction of asthma impairs autophagy in lung CD11c⁺ cells

Because it has been shown that IL-4 and IL-13 can reduce autophagy in macrophages,³² we tested whether severe lung inflammation impairs autophagy in CD11c⁺ cells. To induce severe lung inflammation, we sensitized mice by means of intraperitoneal injection of HDM plus alum, followed by 4 intranasal administrations of HDM, as shown in Fig 3, A. Autophagy assessment was done according to recent guidelines for the use and interpretation of assays for monitoring autophagy.^{33,34} Interestingly, we observed that severe asthma impairs autophagy in pulmonary CD11c⁺ cells (Fig 3, B and C). We confirmed these findings by analyzing LC3 foci with

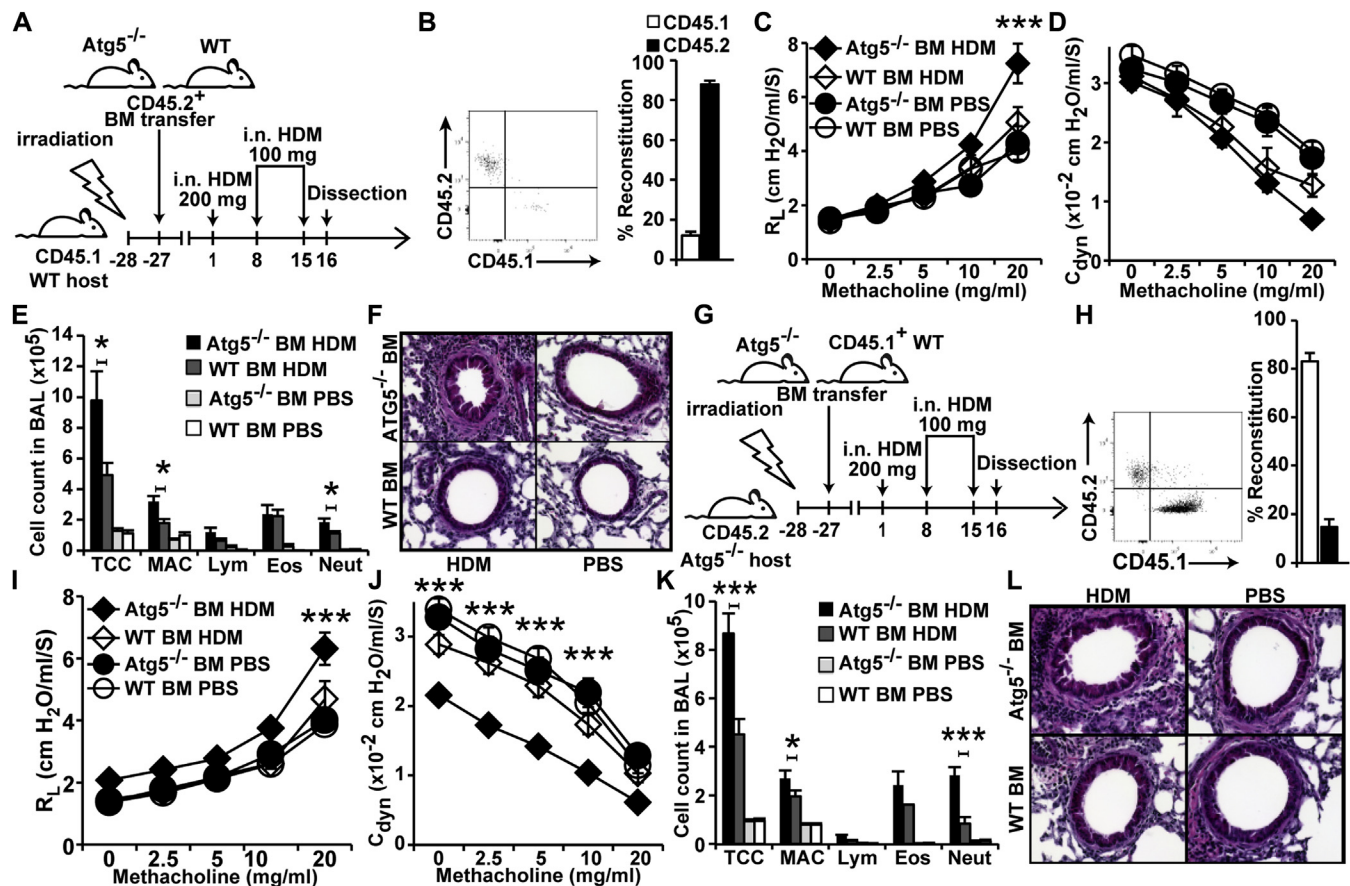


FIG 2. Lack of autophagy in immune cells contributes to induction of lung inflammation. **A**, WT or *Atg5*^{-/-} BM cells were adoptively transferred into irradiated CD45.1⁺ WT mice and then immunized and challenged with HDM, as indicated. **B**, Reconstitution rate of transferred cells, as assessed by using the frequency of CD45.1⁺ and CD45.2⁺ neutrophils in BALF. **C**, Lung resistance (R_L). **D**, Dynamic compliance (C_{dyn}). **E**, Differential cell counts in BALF. **F**, Hematoxylin and eosin-stained lung sections (magnification $\times 200$). **G**, *Atg5*^{-/-} mice were irradiated and received either WT or *Atg5*^{-/-} BM, followed by HDM sensitization, as indicated. **H**, Reconstitution rate of transferred cells. **I** and **J**, Lung resistance (R_L ; Fig 2, I) and dynamic compliance (C_{dyn} ; Fig 2, J) after sensitization, as shown in Fig 2, G. **K**, Differential cell counts in BALF. **L**, Hematoxylin and eosin-stained lung sections (magnification $\times 200$). *Eos*, Eosinophils; *i.n.*, intranasal; *Lym*, lymphocytes; *MAC*, macrophages; *Neut*, neutrophils; *TCC*, total cell number ($n = 5$ per group). Data are shown as means $\pm$ SEMs. * $P < .05$ and *** $P < .001$.

confocal microscopy and LC3-GFP mice. Severe asthma clearly decreased the numbers of LC3 foci in pulmonary CD11c⁺ cells (Fig 3, D and E).

Lack of autophagy in DCs induces IL-1 and IL-23 and T_H17 polarization

To investigate the mechanism by which impairment of autophagy in DCs contribute to T_H17 differentiation, we isolated and analyzed WT and *Atg5*^{-/-} BMDCs for production of IL-1 α , IL-1 β , IL-6, and IL-23.^{9,11-13,35,36} Interestingly, BMDCs from *Atg5*^{-/-} mice displayed higher amounts of IL-1 α , IL-1 β , and IL-23 production on LPS or HDM stimulation at both the protein and mRNA levels (Fig 4, A and B, and see Fig E7, A and B, in this article's Online Repository at www.jacionline.org). *Atg5*^{-/-} BMDCs significantly increased IL-17A levels in coculture with purified naive DO11.10 or HDM-sensitized CD4⁺ T cells compared with WT cells (Fig 4, C, and see Fig E7, C). To explore the mechanism underlying the observed phenotype in *Atg5*^{-/-} BMDCs, we evaluated the amount of caspase-1 in *Atg5*^{-/-} and

WT BMDCs cultured in the presence or absence of LPS for 24 hours and found that *Atg5*^{-/-} BMDCs express significantly more caspase-1 in the presence or absence of LPS (see Fig E7, D). Taken together, these results suggest that *Atg5*^{-/-} BMDCs enhance IL-17A production through increased IL-1 and IL-23 pathways.

CD11c⁺ *Atg5*^{-/-} cells induce severe neutrophilic lung inflammation

Because we found that impairment of autophagy increases IL-1 and IL-23 production by BMDCs and IL-17A production by T cells *in vitro*, we tested whether impairment of autophagy in DCs induces severe neutrophilic inflammation *in vivo*. Mice were immunized with HDM-loaded BMDCs, as shown in Fig 5, A. AHR was significantly higher in the mice immunized with HDM-loaded *Atg5*^{-/-} BMDCs compared with those immunized with WT BMDCs (Fig 5, B and C). Analysis of BALF contents showed a high number of neutrophils but not eosinophils in *Atg5*^{-/-} BMDC-immunized mice compared

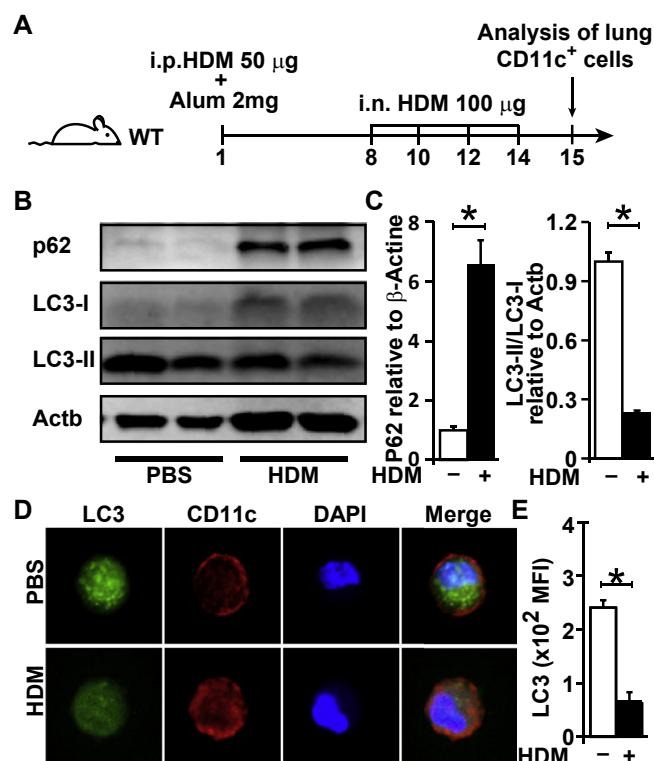


FIG 3. Induction of asthma impairs autophagy in lung CD11c⁺ cells. **A**, Timeline for sensitization and challenge. WT or LC3-GFP mice were immunized with HDM, as indicated. *i.n.*, Intranasal; *i.p.*, intraperitoneal. **B**, Western blot analysis of expression levels of p62, LC3-I, and LC3-II in purified pulmonary CD11c⁺ cells. **C**, Levels of the indicated proteins are expressed relative to the β-actin gene (*Actb*). **D**, Confocal microscopy of purified lung CD11c⁺ cells from LC3-GFP (magnification ×600). DAPI, 4',6-Diamidino-2-phenylindole dihydrochloride. **E**, Mean fluorescence intensity (MFI) of LC3. Data are expressed as means ± SEMs (n = 3-9 per group). *P < .05.

with WT BMDC-immunized mice (Fig 5, D). Analysis of cytokine levels in lung lysates showed higher IL-17A, but not IL-4, IL-1β, or IL-13, production in the lungs of *Atg5*^{-/-} BMDC-immunized mice compared with that in WT BMDC-immunized mice (Fig 5, E).

To confirm these findings, we generated CD11c-specific *Atg5*^{-/-} mice, as described in the Methods section in this article's Online Repository. Interestingly, we found that CD11c-specific *Atg5*^{-/-} mice had unprovoked asthma. Lung resistance is higher and dynamic compliance is lower in CD11c-specific *Atg5*^{-/-} than in WT mice at steady state (Fig 5, F and G). Furthermore, our results show that specific impairment of autophagy in CD11c⁺ cells leads to a substantially higher number of neutrophils and, to a lesser extent, eosinophils in BALF of mice at steady state (Fig 5, H).

Atg5^{-/-} mice have IL-17A-dependent steroid-resistant asthma

The presence of neutrophils in the lungs is predominant in patients with severe refractory asthma, and neutrophils do not respond to steroid treatment.^{7,8,10} We tested whether AHR in *Atg5*^{-/-} mice is IL-17A dependent and steroid resistant. Mice were immunized with HDM according to the protocol shown in

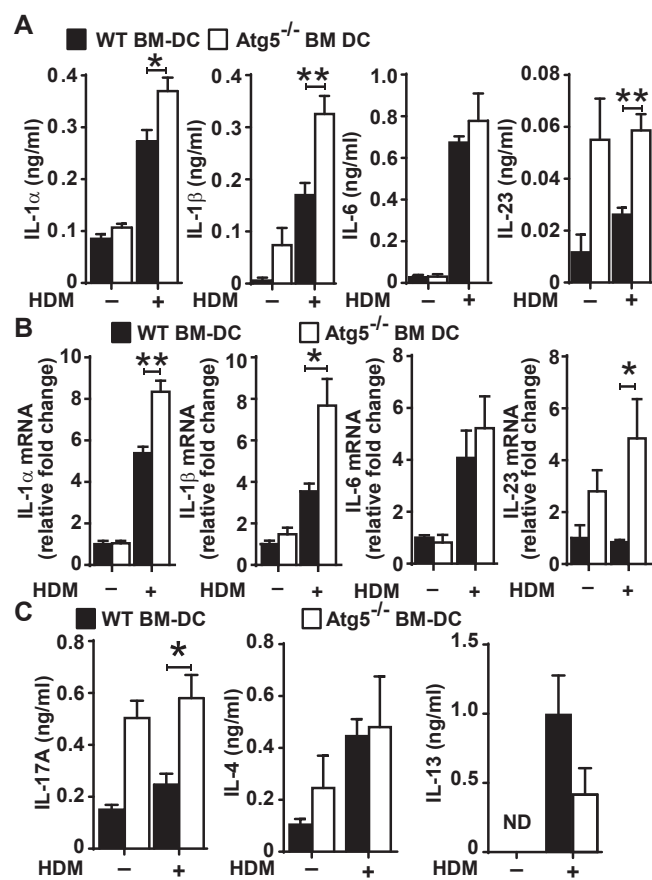


FIG 4. Lack of autophagy in DCs induces IL-1 and IL-23 and TH17 polarization. **A**, BMDCs from WT and *Atg5*^{-/-} mice were stimulated with or without HDM (50 µg/mL) for 36 hours. Cytokine levels in supernatants were measured by means of ELISA. **B**, Quantification of mRNA expression by means of RT-PCR. **C**, BMDCs from WT or *Atg5*^{-/-} mice were cocultured with CD4⁺ T cells isolated from lungs of HDM-sensitized mice in the presence of HDM (10 µg/mL) for 3 days. HDM sensitization was performed according to Fig 1, A. Cytokine levels were measured by means of ELISA. Data are representative of 2 experiments and expressed as means ± SEMs (n = 4-8 per group). *P < .05 and **P < .01.

Fig 1, A. Dexamethasone or anti-IL-17A blocking antibody was administered 1 day before HDM challenge. In WT mice steroid treatment significantly reduced AHR and the presence of eosinophils. However, the levels of AHR and numbers of neutrophils in BALF of *Atg5*^{-/-} mice were resistant to steroid treatment, even though eosinophil counts were decreased (Fig 6, A and B). Blockade of IL-17A significantly reduced AHR and neutrophilic inflammation in *Atg5*^{-/-} mice but had no obvious effects on AHR and BAL analyses of WT mice (Fig 6, D and E). Histologic analyses also revealed that steroid treatment decreased airway thickness and infiltrated cell numbers in WT mice but only had a marginal effect on *Atg5*^{-/-} mice. In contrast, anti-IL-17A therapy reduced the inflammatory response with *Atg5*^{-/-} mice (Fig 6, C and F). These results indicate that *Atg5*^{-/-} mice had steroid-resistant IL-17A-dependent AHR with enhanced neutrophilic inflammation.

DISCUSSION

In the present study we examined the involvement of autophagy in the pathogenesis of neutrophilic lung inflammation. We

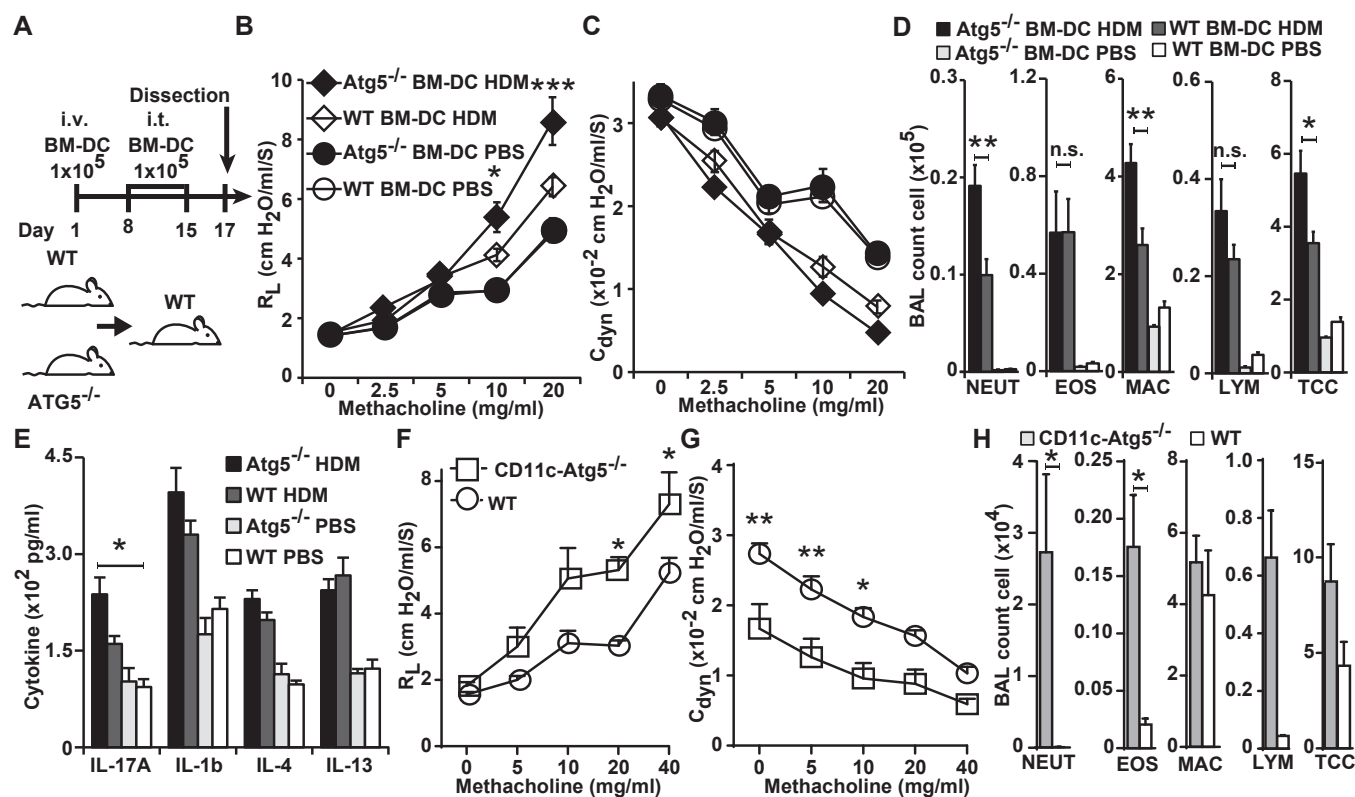


FIG 5. Lack of *Atg5* in CD11c⁺ cells causes unprovoked asthma and induces severe neutrophilic lung inflammation. **A**, Experimental timeline. Cultured BMDCs from WT or *Atg5*^{-/-} mice were stimulated with HDM for 6 hours and then intravenously injected to WT mice. Then mice were intratracheally challenged with HDM-stimulated BMDCs on days 8 and 15. *i.n.*, Intranasal; *i.t.*, intratracheal. **B** and **C**, Lung resistance (R_L ; Fig 5, **B**) and dynamic compliance (C_{dyn} ; Fig 5, **C**). **D**, Differential cell counts in BALF. **E**, Cytokine levels in whole-lung lysate. Pooled data from 2 experiments are shown ($n = 7-8$ per group). **F**, Lung resistance in CD11c-specific *Atg5*^{-/-} and WT mice was measured at steady state. **G**, Dynamic compliance. **H**, Differential cell counts in BALF. Values are expressed as means $\pm$ SEMs. * $P < .05$, ** $P < .01$, *** $P < .001$; n.s., Not significant. *Eos*, Eosinophils; *Lym*, lymphocytes; *MAC*, macrophages; *Neut*, neutrophils; *TCC*, total cell number.

found that a lack of autophagy causes severe IL-17A-mediated neutrophilic lung inflammation. We showed that impaired autophagy in BMDCs significantly increased levels of proinflammatory cytokines, in particular IL-1 and IL-23. Using BMDC immunization experiments and CD11c-specific *Atg5*^{-/-} mice, we showed that lack of autophagy in CD11c⁺ cells leads to severe neutrophilic airway inflammation and AHR. Importantly, we showed that lack of autophagy in CD11c⁺ cells leads to spontaneous asthma. Moreover, impairment of autophagy causes glucocorticoid resistance and IL-17A-dependent lung inflammation.

Our findings that impaired autophagy is involved in the pathogenesis of asthma are supported by several lines of research that showed association of *Atg5* polymorphisms with asthma in human studies^{19,20} and showed that T_H2 cytokines^{17,32} and viral infection inhibit autophagy,^{37,38} whereas T_H1 cytokines induce autophagy. Importantly, impaired autophagy itself can induce T_H17 polarization, resulting in refractory asthma. A randomized clinical trial showed that carbamazepine, an anticonvulsant drug and autophagy inducer, is significantly efficacious in patients with severe asthma,³⁹ underscoring the importance of the autophagy pathway in the pathogenesis of asthma. Taken together, autophagy plays a protective role in asthma, and

inducers of this pathway might represent novel therapeutic targets for treatment, in particular treatment of corticosteroid-resistant asthma.

Our findings also suggest that impaired autophagy induces IL-17A production by T-cell subsets, including TCR $\gamma\delta$ and TCR $\alpha\beta$ T cells, and IL-17A-mediated corticosteroid-resistant asthma. The importance of IL-17A is highlighted in certain patients with severe asthma who have increased IL-17A levels, which correlate with neutrophilic airway inflammation and AHR.^{11,13}

In addition to IL-6 and TGF- β , IL-1 α , IL-1 β , and IL-23 are secreted by BMDCs and play a crucial role in the differentiation of T_H17.^{9,36} Secretion of IL-1 β is controlled by caspase-1 through the inflammasome pathway and consequently promotes IL-17A production by T cells. This caspase-1-mediated IL-1 β production is negatively regulated by autophagy.⁴⁰ The observed neutrophilic airway inflammation seen in *Atg5*^{-/-} mice in our studies might be explained by the enhanced production of IL-1 α , IL-1 β , and IL-23 by DCs in *Atg5*^{-/-} mice. Autophagy also regulates IL-1 α secretion and is dependent on reactive oxygen species and the calpain pathway but independent of the inflammasome pathway.⁴¹

Airway epithelial cells act as an external barrier against foreign environmental antigens by secreting mucus and interacting with

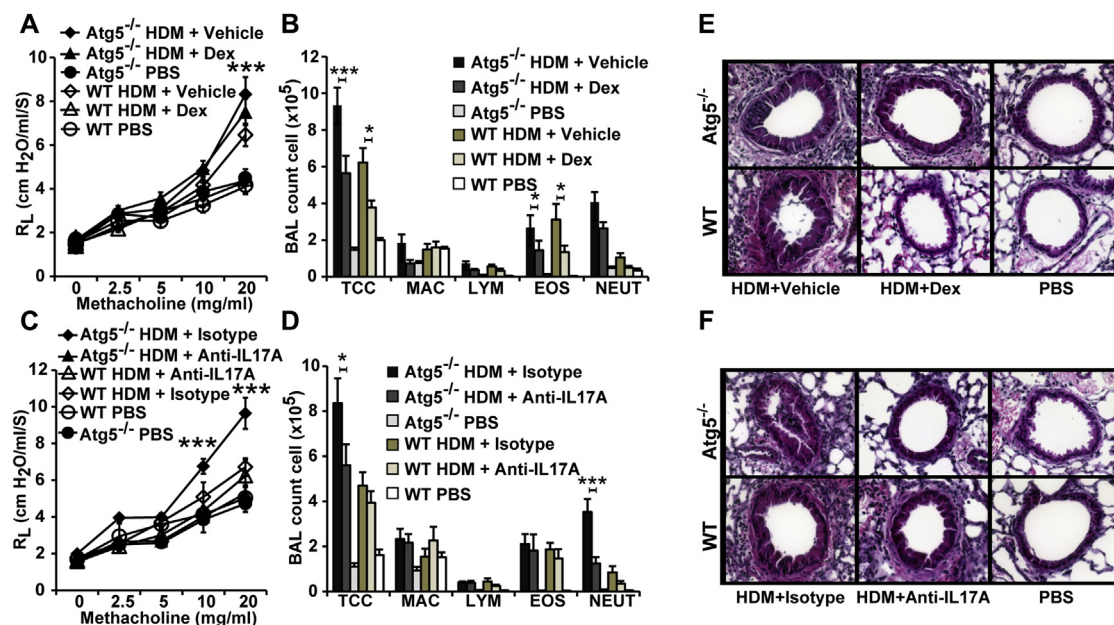


FIG 6. *Atg5*^{-/-} mice have IL-17A-dependent steroid-resistant airway inflammation. **A** and **D**, WT and *Atg5*^{-/-} mice were immunized and challenged with HDM, as described in Fig 1, A, and treated with dexamethasone (1 mg/kg) or anti-IL-17A antibody (300 μg) 1 day before HDM challenge. Mice were subsequently assessed for AHR by measuring lung resistance (*R_L*). **B** and **E**, BAL cells from the mice in Fig 6, A and D, were analyzed after AHR measurements. **C** and **F**, Lung tissue sections from WT and *Atg5*^{-/-} mice after dexamethasone or anti-IL-17A antibody treatment were stained with hematoxylin and eosin (magnification ×200). Data are expressed as means ± SEMs and representative of 2 independent experiments (n = 5-6 per group). **P* < .05 and ****P* < .001.

innate and adaptive immune cells. Interestingly, autophagy in epithelial cells plays a distinct role in the pathogenesis of COPD^{29,30} but is protective in patients with cystic fibrosis, lung fibrosis, and acute lung injury.^{16,42-46} Because pathogenesis is different in asthma and COPD, we found no evidence that nonhematopoietic cells, including AECs and lung bronchial epithelial cells, play a crucial role in induction of IL-17A-mediated corticosteroid-resistant asthma in *Atg5*^{-/-} mice.

We found that severe asthma impairs autophagy in pulmonary DCs and that autophagy-disrupted DCs more potently induced IL-17A secretion in T cells. Autophagy deficiency in DCs affects antigen presentation and induces hyperstable interactions and activation of T cells.⁴⁷⁻⁴⁹ Furthermore, blockade of autophagy inhibits degradation of the adapter protein B-cell lymphoma/leukemia 10, which plays a key role in transmitting signals from the TCR.⁵⁰ This might explain the increased number of effector T cells in *Atg5*^{-/-} mice with AHR. Furthermore, adoptive transfer of autophagy-deficient DCs enhanced AHR, and severe steroid-resistant AHR in *Atg5*^{-/-} mice was neutralized by anti-IL-17A antibody. The recipients of WT BM did not have increased AHR in our adoptive transferred experiments, suggesting that a more severe model of asthma is needed to induce AHR in these experiments.

In conclusion, the present study demonstrates that impaired autophagy causes severe neutrophilic inflammation and that this condition is mainly mediated by modulated DC function. We suggest a model in which initiation of asthma leads to impairment of autophagy in pulmonary CD11c⁺ cells through the T_H2 cytokines IL-4 and IL-13, and impairment of autophagy in turn causes neutrophilic lung inflammation and more severe asthma. Therefore these findings suggest a protective role for autophagy

in the pathogenesis of asthma, and improving the autophagy pathway in the lung might be an effective therapeutic target for patients with refractory severe asthma, especially those with IL-17A-mediated neutrophilic asthma.

Clinical implications: Our findings offer an explanation for pathogenesis of neutrophilic asthma. Severe asthma causes impairment of autophagy in pulmonary CD11c⁺ cells, which in turn causes neutrophilic asthma.

REFERENCES

- Wills-Karp M. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu Rev Immunol* 1999;17:255-81.
- Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. *Nature* 2008;454:445-54.
- Lambrecht BN, Hammad H. The airway epithelium in asthma. *Nat Med* 2012;18:684-92.
- Chung KF, Godard P, Adelroth E, Ayres J, Barnes N, Barnes P, et al. Difficult/therapy-resistant asthma: the need for an integrated approach to define clinical phenotypes, evaluate risk factors, understand pathophysiology and find novel therapies. ERS Task Force on Difficult/Therapy-Resistant Asthma. *European Respiratory Society. Eur Respir J* 1999;13:1198-208.
- American Thoracic Society. Proceedings of the ATS workshop on refractory asthma: current understanding, recommendations, and unanswered questions. American Thoracic Society. *Am J Respir Crit Care Med* 2000;162:2341-51.
- McGrath KW, Icitovic N, Boushey HA, Lazarus SC, Sutherland ER, Chinchilli VM, et al. A large subgroup of mild-to-moderate asthma is persistently noneosinophilic. *Am J Respir Crit Care Med* 2012;185:612-9.
- Hastie AT, Moore WC, Meyers DA, Vestal PL, Li H, Peters SP, et al. Analyses of asthma severity phenotypes and inflammatory proteins in subjects stratified by sputum granulocytes. *J Allergy Clin Immunol* 2010;125:1028-36.e13.
- Haldar P, Pavord ID. Noneosinophilic asthma: a distinct clinical and pathologic phenotype. *J Allergy Clin Immunol* 2007;119:1043-54.
- Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. *Annu Rev Immunol* 2009;27:485-517.

10. Moore WC, Meyers DA, Wenzel SE, Teague WG, Li H, Li X, et al. Identification of asthma phenotypes using cluster analysis in the Severe Asthma Research Program. *Am J Respir Crit Care Med* 2010;181:315-23.
11. Chakir J, Shannon J, Molet S, Fukakusa M, Elias J, Laviolette M, et al. Airway remodeling-associated mediators in moderate to severe asthma: effect of steroids on TGF-beta, IL-11, IL-17, and type I and type III collagen expression. *J Allergy Clin Immunol* 2003;111:1293-8.
12. Molet S, Hamid Q, Davoine F, Nutku E, Taha R, Page N, et al. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J Allergy Clin Immunol* 2001;108:430-8.
13. Al-Ramli W, Prefontaine D, Chouiali F, Martin JG, Olivenstein R, Lemiere C, et al. T(H)17-associated cytokines (IL-17A and IL-17F) in severe asthma. *J Allergy Clin Immunol* 2009;123:1185-7.
14. Lewkowich IP, Lajoie S, Clark JR, Herman NS, Sproles AA, Wills-Karp M. Allergen uptake, activation, and IL-23 production by pulmonary myeloid DCs drives airway hyperresponsiveness in asthma-susceptible mice. *PLoS One* 2008;3:e3879.
15. Moreira AP, Cavassani KA, Ismailoglu UB, Hullinger R, Dunleavy MP, Knight DA, et al. The protective role of TLR6 in a mouse model of asthma is mediated by IL-23 and IL-17A. *J Clin Invest* 2011;121:4420-32.
16. Ryter SW, Nakahira K, Haspel JA, Choi AM. Autophagy in pulmonary diseases. *Annu Rev Physiol* 2012;74:377-401.
17. Deretic V, Saitoh T, Akira S. Autophagy in infection, inflammation and immunity. *Nat Rev Immunol* 2013;13:722-37.
18. Levine B, Mizushima N, Virgin HW. Autophagy in immunity and inflammation. *Nature* 2011;469:323-35.
19. Poon AH, Chouiali F, Tse SM, Litonjua AA, Hussain SN, Baglote CJ, et al. Genetic and histologic evidence for autophagy in asthma pathogenesis. *J Allergy Clin Immunol* 2012;129:569-71.
20. Martin LJ, Gupta J, Jyothula SS, Butsch Kovacic M, Biagini Myers JM, Patterson TL, et al. Functional variant in the autophagy-related 5 gene promoter is associated with childhood asthma. *PLoS One* 2012;7:e33454.
21. Akbari O, Stock P, Meyer E, Kronenberg M, Sidobre S, Nakayama T, et al. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. *Nat Med* 2003;9:582-8.
22. Lombardi V, Speak AO, Kerzerho J, Szely N, Akbari O. CD8alpha(+)beta(-) and CD8alpha(+)beta(+) plasmacytoid dendritic cells induce Foxp3(+) regulatory T cells and prevent the induction of airway hyper-reactivity. *Mucosal Immunol* 2012;5:432-43.
23. Maazi H, Patel N, Sankaranarayanan I, Suzuki Y, Rigas D, Soroosh P, et al. ICOS: ICOS-ligand interaction is required for type 2 innate lymphoid cell function, homeostasis, and induction of airway hyperreactivity. *Immunity* 2015;42:538-51.
24. Plantinga M, Williams M, Vanheerswynghe M, Deswarte K, Branco-Madeira F, Toussaint W, et al. Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. *Immunity* 2013;38:322-35.
25. Kerzerho J, Maazi H, Speak AO, Szely N, Lombardi V, Khoo B, et al. Programmed cell death ligand 2 regulates TH9 differentiation and induction of chronic airway hyperreactivity. *J Allergy Clin Immunol* 2013;131:1048-57, e1-2.
26. Haspel JA, Choi AM. Autophagy: a core cellular process with emerging links to pulmonary disease. *Am J Respir Crit Care Med* 2011;184:1237-46.
27. Castillo EF, Dekonenko A, Arko-Mensah J, Mandell MA, Dupont N, Jiang S, et al. Autophagy protects against active tuberculosis by suppressing bacterial burden and inflammation. *Proc Natl Acad Sci U S A* 2012;109:E3168-76.
28. Kuma A, Hatano M, Matsui M, Yamamoto A, Nakaya H, Yoshimori T, et al. The role of autophagy during the early neonatal starvation period. *Nature* 2004;432:1032-6.
29. Chen ZH, Lam HC, Jin Y, Kim HP, Cao J, Lee SJ, et al. Autophagy protein microtubule-associated protein 1 light chain-3B (LC3B) activates extrinsic apoptosis during cigarette smoke-induced emphysema. *Proc Natl Acad Sci U S A* 2010;107:18880-5.
30. Lam HC, Cloonan SM, Bhashyam AR, Haspel JA, Singh A, Sathirapongsasuti JF, et al. Histone deacetylase 6-mediated selective autophagy regulates COPD-associated cilia dysfunction. *J Clin Invest* 2013;123:5212-30.
31. Korfhagen TR, Glasser SW, Wert SE, Bruno MD, Daugherty CC, McNeish JD, et al. Cis-acting sequences from a human surfactant protein gene confer pulmonary-specific gene expression in transgenic mice. *Proc Natl Acad Sci U S A* 1990;87:6122-6.
32. Harris J, De Haro SA, Master SS, Keane J, Roberts EA, Delgado M, et al. T helper 2 cytokines inhibit autophagic control of intracellular *Mycobacterium tuberculosis*. *Immunity* 2007;27:505-17.
33. Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K, et al. Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy* 2012;8:445-544.
34. Mizushima N, Yoshimori T. How to interpret LC3 immunoblotting. *Autophagy* 2007;3:542-5.
35. Lajoie S, Lewkowich IP, Suzuki Y, Clark JR, Sproles AA, Dienger K, et al. Complement-mediated regulation of the IL-17A axis is a central genetic determinant of the severity of experimental allergic asthma. *Nat Immunol* 2010;11:928-35.
36. McGeachy MJ, Chen Y, Tato CM, Laurence A, Joyce-Shaikh B, Blumenschein WM, et al. The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells in vivo. *Nat Immunol* 2009;10:314-24.
37. Chen M, Hong MJ, Sun H, Wang L, Shi X, Gilbert BE, et al. Essential role for autophagy in the maintenance of immunological memory against influenza infection. *Nat Med* 2014;20:503-10.
38. Nakamoto M, Moy RH, Xu J, Bambina S, Yasunaga A, Shelly SS, et al. Virus recognition by Toll-7 activates antiviral autophagy in *Drosophila*. *Immunity* 2012;36:658-67.
39. Lomia M, Tchelidze T, Pruidze M. Bronchial asthma as neurogenic paroxysmal inflammatory disease: a randomized trial with carbamazepine. *Respir Med* 2006;100:1988-96.
40. Nakahira K, Haspel JA, Rathinam VA, Lee SJ, Dolinay T, Lam HC, et al. Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. *Nat Immunol* 2011;12:222-30.
41. Zhao Z, Fux B, Goodwin M, Dunay IR, Strong D, Miller BC, et al. Autophagosome-independent essential function for the autophagy protein Atg5 in cellular immunity to intracellular pathogens. *Cell Host Microbe* 2008;4:458-69.
42. Tanaka A, Jin Y, Lee SJ, Zhang M, Kim HP, Stolz DB, et al. Hyperoxia-induced LC3B interacts with the Fas apoptotic pathway in epithelial cell death. *Am J Respir Cell Mol Biol* 2012;46:507-14.
43. Mi S, Li Z, Yang HZ, Liu H, Wang JP, Ma YG, et al. Blocking IL-17A promotes the resolution of pulmonary inflammation and fibrosis via TGF-beta1-dependent and -independent mechanisms. *J Immunol* 2011;187:3003-14.
44. JIMA. Epidemiology of seasonal, avian and pandemic influenza. *J Indian Med Assoc* 2009;107:506-7.
45. Abdulrahman BA, Khweek AA, Akhter A, Caution K, Kotrange S, Abdelaziz DH, et al. Autophagy stimulation by rapamycin suppresses lung inflammation and infection by *Burkholderia cenocepacia* in a model of cystic fibrosis. *Autophagy* 2011;7:1359-70.
46. Luciani A, Vilella VR, Esposito S, Brunetti-Pierri N, Medina D, Settembre C, et al. Defective CFTR induces aggresome formation and lung inflammation in cystic fibrosis through ROS-mediated autophagy inhibition. *Nat Cell Biol* 2010;12:863-75.
47. Lee HK, Mattei LM, Steinberg BE, Alberts P, Lee YH, Chervonsky A, et al. In vivo requirement for Atg5 in antigen presentation by dendritic cells. *Immunity* 2010;32:227-39.
48. Cooney R, Baker J, Brain O, Danis B, Pichulik T, Allan P, et al. NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. *Nat Med* 2010;16:90-7.
49. Wildenberg ME, Vos AC, Wolfkamp SC, Duijvestein M, Verhaar AP, Te Velde AA, et al. Autophagy attenuates the adaptive immune response by destabilizing the immunologic synapse. *Gastroenterology* 2012;142:1493-503.e6.
50. Paul S, Kashyap AK, Jia W, He YW, Schaefer BC. Selective autophagy of the adaptor protein Bcl10 modulates T cell receptor activation of NF-kappaB. *Immunity* 2012;36:947-58.

METHODS

Mice

Female C57BL/6, BALB/c, CD45.1, and OT-II mice (6–8 weeks old) were purchased from Jackson Laboratory (Bar Harbor, Me). *Atg5^{fllox/flox}* and *LC3-GFP* mice were a gift from Dr Noboru Mizushima (Tokyo Medical and Dental University, Tokyo, Japan). CD11c-specific *Atg5^{-/-}* mice were generated by crossing *Atg5^{fllox/flox}* mice to *CD11c-Cre* mice. Mice were screened by means of PCR, and *Atg5^{fllox/flox}* homozygote *CD11c-Cre* hemizygote mice were selected for the experiments. *Atg5^{fllox/flox}* mice were backcrossed to *rosa26Cre ER^T* mice, and *sftpc-cre* mice (Jackson Laboratory) were bred in our facility at the Keck School of Medicine, University of Southern California, under protocols approved by the Institutional Animal Care and Use Committee. Mice received tamoxifen (800 μ g per mouse per day) for 5 consecutive days to induce deletion of *Atg5* (Fig 1, A).

HDM-induced AHR and measurement of airway hyperresponsiveness

Mice were sensitized intranasally with 200 μ g of HDM (Stallergenes, Norwell, Mass) on day 1, followed by 100 μ g of HDM administered intranasally on days 8 and 15. In some experiments mice were treated intraperitoneally with dexamethasone (Sigma-Aldrich, St Louis, Mo) or PBS and anti-mouse IL-17A antibody (clone 17F3; BioXcell, West Lebanon, NH) or IgG₁ isotype control antibody before each HDM challenge. One day after the last HDM challenge, lung resistance and dynamic compliance were measured with the Fine Pointe RC System (Buxco Research Systems, Wilmington, NC), as previously described.

Collection of BALF, lung histology, and lung lysates

After measurement of AHR, the trachea was cannulated, and lungs were lavaged 3 times with 1 mL of ice-cold PBS to collect BAL cells, as previously described. Transcardial perfusion of the lungs with cold PBS was then performed to remove red blood cells, and the lungs were fixed and harvested for histology with 4% paraformaldehyde in PBS, as described previously. In some experiments the lungs were collected and homogenized in 3 mL of RIPA Buffer (Millipore, Temecula, Calif). The homogenates were analyzed for cytokines, as described elsewhere.

Flow cytometry

The composition of BAL cells was analyzed by using flow cytometry, as described previously. The repertoire of pulmonary DCs was analyzed by using flow cytometry, as described elsewhere.

The following antibodies and reagents were used to identify T-cell subsets and innate lymphoid cells: fluorescein isothiocyanate anti-CD45 (clone 30F11; BioLegend, San Diego, Calif), phycoerythrin (PE) anti-TCR $\gamma\delta$ (clone GL3, BioLegend), peridinin-chlorophyll-protein complex/Cy5.5 anti-TCR β (clone H57-597; eBioscience, San Diego, Calif), biotin anti-Gr-1 (clone RB6-8C5, BioLegend), anti-B220 (clone RA3-6B2, BioLegend), anti-CD11c (clone N418, BioLegend), anti-TER119 (clone TER-119, BioLegend), anti-CD11b (clone M1/70, BioLegend), anti-FCeRI (clone MAR-1, BioLegend), Brilliant Violet 510 Streptavidin (BioLegend), and eFluor 780 dead cell discrimination dye (eBioscience).

For intracellular cytokine evaluation, lung single-cell suspension was made by using collagenase D (Worthington Biochemical, Lakewood, NJ). Lung cells were then cultured in RPMI with 10% FCS in the presence of phorbol 12-myristate 13-acetate/ionomycin (50/500 ng/mL) for 5 hours. Cells were then fixed with BD Cytofix/Cytoperm kit (BD Biosciences, San Jose, Calif), according to the manufacturer's instructions. IL-17A intracellular staining was performed, and live CD45⁺IL-17⁺ single cells were further analyzed for the expression of TCR β and TCR $\gamma\delta$ and the lack of expression of CD11b, CD11c, Gr-1, Ter-119, B220, and FCeRI for innate lymphoid cells. In some experiments the cell surface was first stained with APC-Cy7-labeled anti-CD3 (clone 145-2-C11, BioLegend), eFluor 450-labeled anti-CD44 (clone IM7, eBioscience), and Brilliant Violet-510 (BV510)-labeled

anti-CD4 (clone RM4-5, BioLegend). After fixation and permeabilization, cells were then stained with fluorescein isothiocyanate-labeled anti-IFN- γ (clone XMG1.2, eBioscience), PE-labeled anti-IL-5 (clone TRFK5, eBioscience), PE-labeled anti-IL-13 (clone eBio13A, eBioscience), Alexa Fluor 647-labeled anti-IL-4 (clone 11B11, eBioscience), and PE-Cy7-labeled anti-IL-17A (clone TC11-18H10.1, BioLegend). Flow cytometry was carried out on the FACSCanto II (BD Biosciences), and data were analyzed with FlowJo version 8.6 software (TreeStar, Ashland, Ore).

Preparation of BM chimeric mice

Mice were irradiated with 600 rad and injected the following day with 5×10^6 BM cells. Reconstitution of lung leukocytes was approximately 90% by using flow cytometry with PE-Cy7-conjugated anti-CD45.1 (clone A20, BioLegend).

Preparation of BMDCs and adoptive transfers

BM cells were harvested and cultured, as previously described. After 8 days in culture with GM-CSF, BMDCs were harvested and incubated with HDM (80 μ g/mL) for 6 hours. BMDCs (1×10^5) were then adoptively intravenously transferred into naive C57BL/6 mice (day 1) and challenged intratracheally with 1×10^5 BMDCs on days 8 and 15 and killed on day 17.

In vitro culture of BMDCs

Naive CD4⁺ T cells were obtained from OT-II murine spleens by using CD4-conjugated magnetic beads (Miltenyi Biotec, Auburn, Calif) and positively sorted by means of magnetic cell sorting (Miltenyi Biotec). Purified CD4⁺ T cells (5×10^5 /mL) were cocultured with BMDCs (1×10^5 /mL) for 3 days in the presence of the ovalbumin peptide OVA₃₂₃₋₃₃₉ (10 μ g/mL; InvivoGen, San Diego, Calif). In some experiments BMDCs (2.5×10^6 /mL) were incubated with HDM (50 μ g/mL) and LPS (1 μ g/mL; Sigma-Aldrich, St Louis, Mo) for 1 or 2 days.

For generating HDM-specific T cells, WT mice were sensitized with HDM, according to the protocol described in Fig 1, A, and 1 day after sensitization, total CD4⁺ T cells were isolated from the lung by using magnetic isolation (Miltenyi Biotec). Purified CD4⁺ cells (1×10^1 cells/mL) were cocultured with BMDCs from CD11c-specific *Atg5^{-/-}* or WT mice (2×10^5 cells/mL) in the presence or absence of HDM (10 μ g/mL) for 3 days.

Measurement of cytokines

Cytokine levels were measured by means of ELISA (eBioscience), according to the manufacturer's instructions.

RT-PCR assays

For measuring *Atg5* or SPC-Cre expression, mRNA was extracted and converted to cDNA for each mouse and quantified by using RT-PCR. Total RNA was extracted from BMDCs incubated for 1 day with LPS (1 μ g/mL) by using the RNeasy Mini Kit (Qiagen, Valencia, Calif) and cDNA generated with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, Calif). RT-PCR was performed by using the CFX96 thermal cycler (Bio-Rad Laboratories, Hercules, Calif), and the $\Delta\Delta C_t$ method was used for data analysis.

Quantification of autophagy levels by using Western blotting and confocal microscopy

BALB/c, C57BL/6, and LC3-GFP knock-in transgenic mice were immunized with HDM with alum (Al[OH]₃; Thermo, Hanover Park, Ill) on day 1 and then challenged with HDM or PBS intranasally on days 8, 10, 12, and 14. On day 15, the lungs were either isolated for lung lysates or for lung CD11c⁺ cells by using magnetic beads and sorting with autoMACS (Miltenyi Biotec). Autophagy levels were quantified by means of Western

blotting and confocal microscopic analysis. For Western blot analysis, expression of p62 (MBL, Nagoya, Japan), LC3 (Cosmo, Tokyo, Japan), and the β -actin gene (*Actb*; Santa Cruz Biotechnology, Santa Cruz, Calif) was assessed. Lung CD11c⁺ cells isolated from LC3-GFP mice and LC3 expression were analyzed by using confocal microscopy (Nikon Instruments, Melville, NY).

Statistical analysis

P values for lung function data were calculated by means of repeated-measures ANOVA, and *P* values for other data were calculated by using the Student *t* test. *P* values of less than .05 were considered significant. All data are expressed as means $\pm$ SDs. Statistical analyses were performed with JMP Start Statistics (SAS Institute).

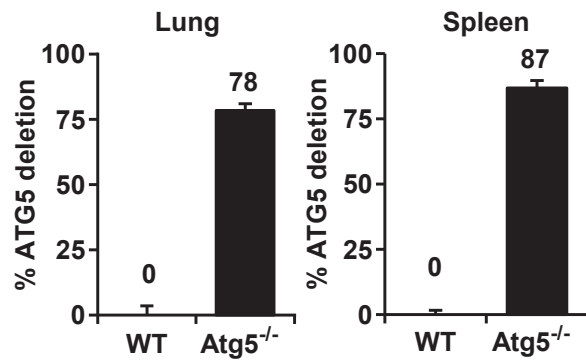


FIG E1. Confirmation of depletion of *Atg5* in the lungs and spleens of *Atg5*^{-/-} mice. ROSA CRE ERT-*Atg5* and WT mice received tamoxifen (800 μ g per mouse per day) for 5 consecutive days. Six days after the last tamoxifen injection, mice were killed, and relative gene expression of *Atg5* in lungs and spleens of ROSA CRE ERT-*Atg5*^{-/-} and WT mice was assessed by using RT-PCR. Values are expressed as means $\pm$ SEMs.

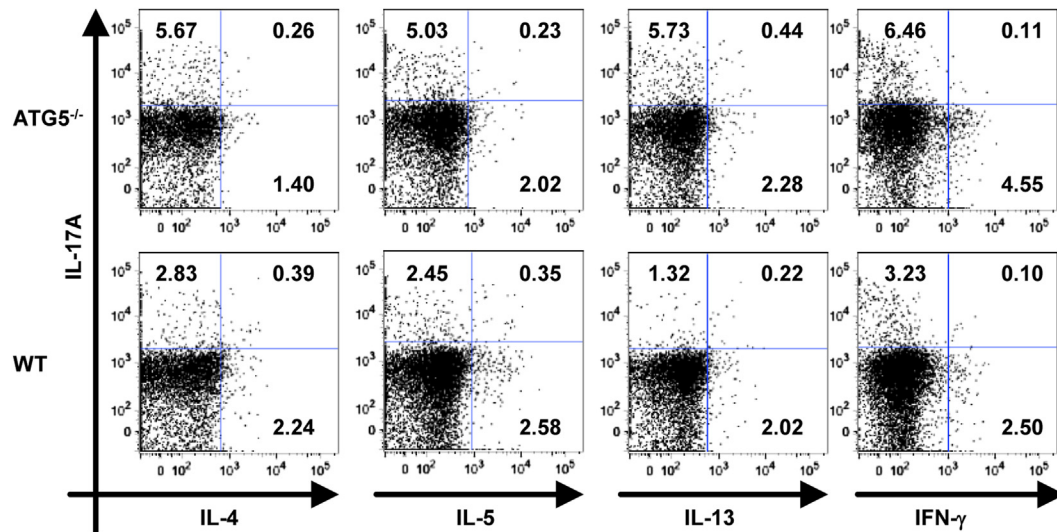


FIG E2. Representative dot plot presentation of cytokine production by T cells in the lung. *Atg5*^{-/-} and WT mice were sensitized to HDM, as described in Fig 1, A. One day after the last sensitization, lung single cells were cultured in the presence of phorbol 12-myristate 13-acetate/ionomycin, as described in the Methods section, followed by analysis of cytokine production by means of flow cytometry.

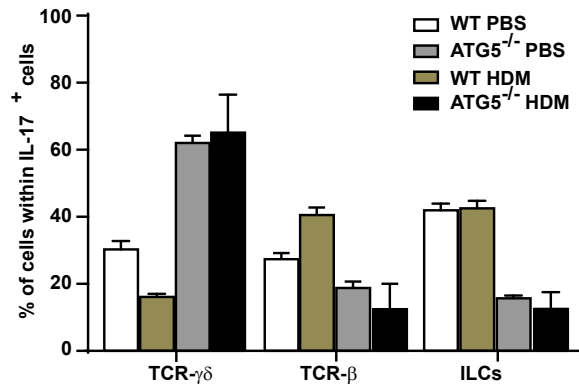


FIG E3. T cells are the major source of IL-17 production in lungs of *Atg5*^{-/-} mice. *Atg5*^{-/-} and WT mice were sensitized, as mentioned in Fig 1, A. One day after the last sensitization, lungs were harvested and cultured in the presence of phorbol 12-myristate 13-acetate/ionomycin for 5 hours, followed by evaluation of IL-17 production with flow cytometry. Live IL-17⁺CD45⁺ single cells were further gated to identify the source of IL-17A production. Bar graphs show the percentage of each subset within IL-17⁺ cells, as indicated. ILCs, Innate lymphoid cells.

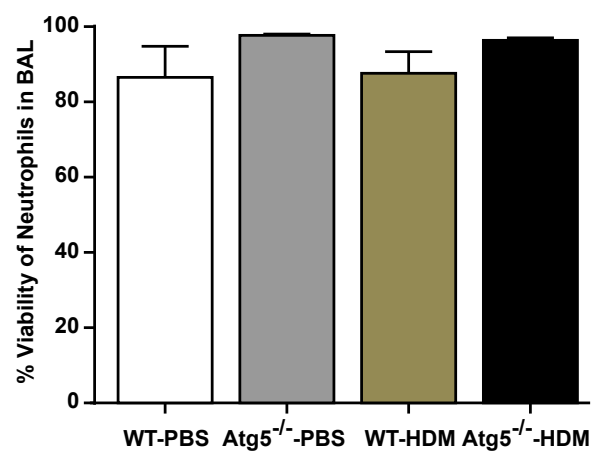


FIG E4. BAL neutrophils show similar viability in *Atg5*^{-/-} and WT mice. BALF of HDM-sensitized or control *Atg5*^{-/-} and WT mice were analyzed for neutrophil viability by using flow cytometry.

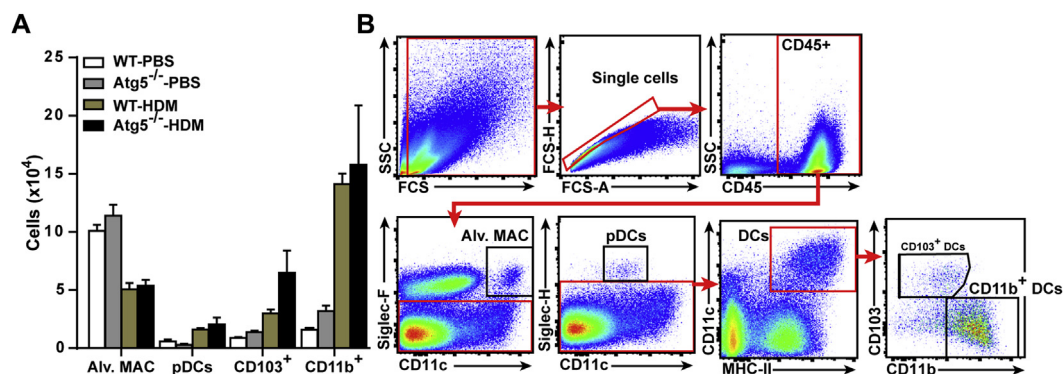


FIG E5. Proportions of different subsets of antigen-presenting cells in lungs of *Atg5*^{-/-} and WT mice are similar. Deletion of the *Atg5* gene was induced in ROSA-*Atg5*^{-/-} mice by using tamoxifen. Subsequently, *Atg5*^{-/-} and WT mice were sensitized, as mentioned in Fig 1, A. One day after the last HDM sensitization, lungs of HDM-sensitized or control mice were analyzed for different subsets of antigen-presenting cells. **A**, Bar graph showing numbers of alveolar macrophages, plasmacytoid DCs, and CD103⁺ and CD11b⁺ DCs in the lungs. **B**, Dot plots show the gating strategy. Red rectangles and arrows show the gating hierarchy. Data are expressed as means ± SEMs. Alv. MAC, Alveolar macrophages; pDCs, plasmacytoid DCs.

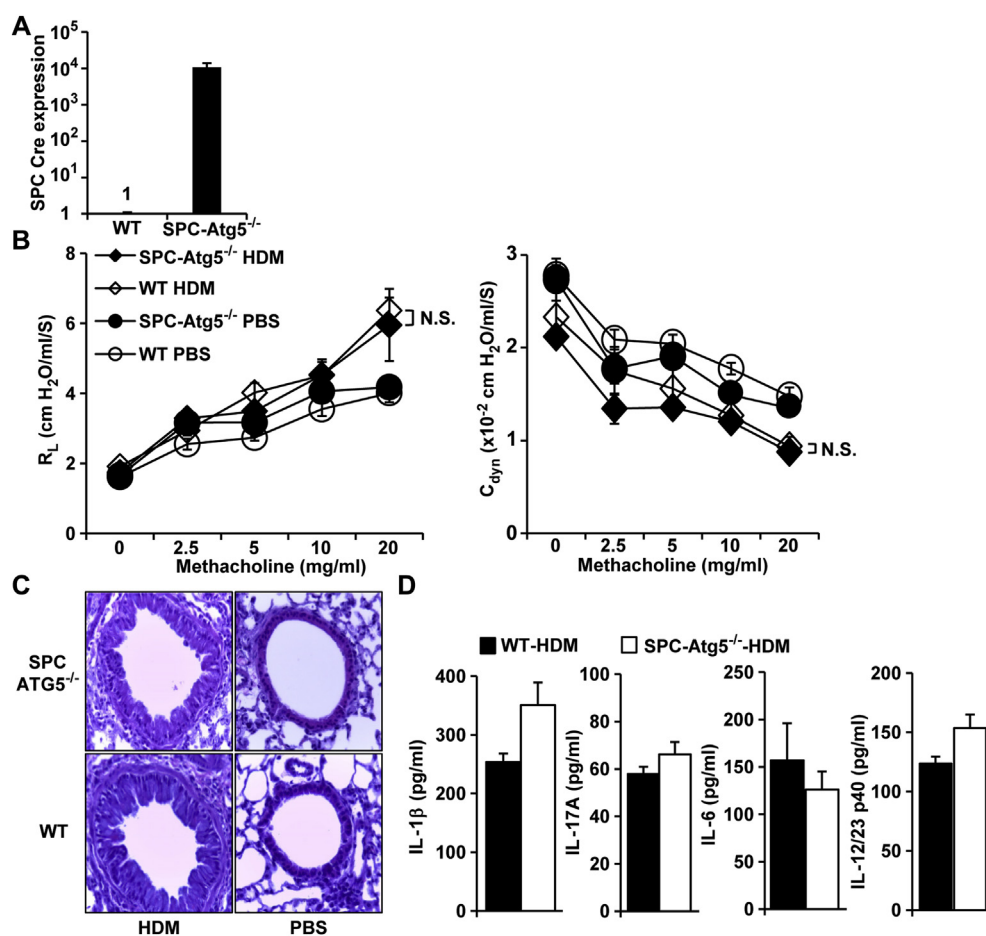


FIG E6. Lack of autophagy in lung epithelial cells does not contribute to AHR induction. **A**, Relative expression levels of the SPC-Cre gene were assessed by using RT-PCR. **B**, WT and SPC-Atg5^{-/-} mice were immunized intranasally with HDM, as described in Fig 1, A. The mice were subsequently assessed for AHR. Pooled data from 2 experiments are shown (n = 8 mice per group). **C**, Lung tissues from WT and Atg5^{-/-} mice were stained with hematoxylin and eosin (original magnification ×200). **D**, Cytokine levels in whole-lung lysates of asthmatic WT and SPC-Atg5^{-/-} mice immunized as in Fig 1, A (n = 5 per group). Values are expressed as means ± SEMs. C_{dyn}, Dynamic compliance; N.S., not significant; R_L, lung resistance.

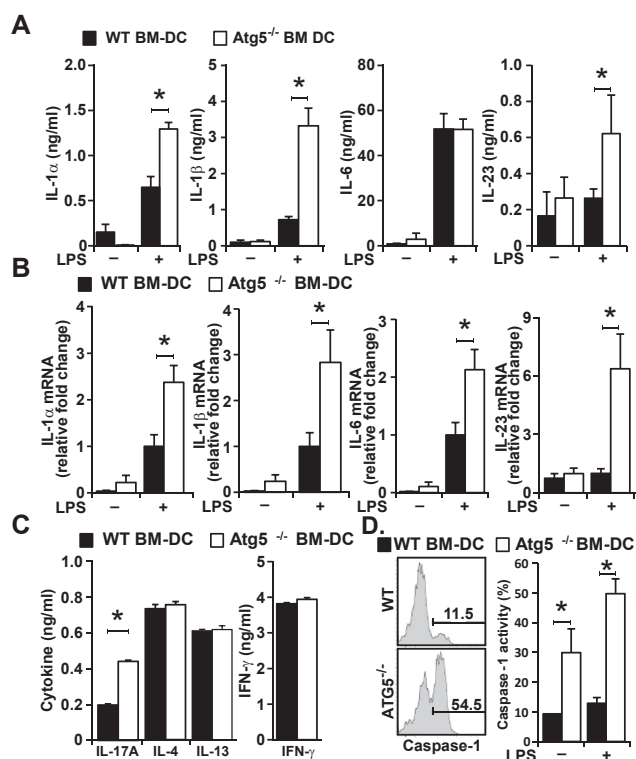


FIG E7. Lack of autophagy in DCs induces IL-1 and IL-23 and TH17 polarization. **A**, BMDCs from WT and *Atg5*^{-/-} mice were stimulated with or without LPS (1 μg/mL) for 1 or 2 days. Cytokine levels in supernatants were measured by using ELISA (n = 6-8 per group). **B**, Quantification of mRNA expression by using RT-PCR (n = 6 per group). **C**, BMDCs from WT or *Atg5*^{-/-} mice were cocultured with DO11.10 CD4⁺ T cells in the presence of the ovalbumin peptide OVA₃₂₃₋₃₃₉ for 3 days. Cytokine levels in supernatants were measured by using ELISA. **D**, Caspase-1 level, as measured by using flow cytometry in *Atg5*^{-/-} and WT BMDCs, in the presence or absence of LPS (1 μg/mL) for 24 hours. One of 2 representative experiments is shown (n = 4 per group). Data are expressed as means ± SEMs. P values were calculated with the Student t test. *P < .05.