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*

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

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 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