Autophagy functions in lung macrophages and dendritic cells to suppress T_H17 responses and neutrophil accumulation during allergic airway inflammation

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ABSTRACT

Asthma affects nearly 260 million people worldwide, where severe asthma cases represent the most difficult to treat due to corticosteroid insensitivity. Severe asthma is associated with higher levels of T_H17 and T_H1 responses, accompanied by neutrophil dominated inflammation. Better understanding of the immune responses to airway allergens that promote or protect against severe asthma is critical for identifying ways to treat these patients. Single nucleotide polymorphisms in the *ATG5* gene, which encodes for a protein required for the cellular recycling process of autophagy, has been associated with higher risk for developing severe asthma. However, the exact role for ATG5 during allergic inflammation and whether other autophagy proteins are involved remains elusive. Using genetic tools to dissect the roles for ATG5 in innate immune cells in house dust mite (HDM)-challenged mice, we have identified a specific role for ATG5 in $CD11c^+$ lung macrophages and dendritic cells for suppressing T_H17 responses and neutrophil accumulation. We found that this role for ATG5 in $CD11c^+$ cells to regulate neutrophil accumulation during allergic airway inflammation also required other autophagy proteins but did not involve regulation of

inflammasome, despite higher levels of IL-1β and Caspase 1 in the lungs of mice lacking autophagy in innate immune cells. Our data support a role for autophagy in $CD11c^+$ lung macrophages and dendritic cells to promote an immune response to airway allergens that is associated with less severe asthma by suppressing T_H17 responses and neutrophil accumulation in the lung.

INTRODUCTION

Asthma affects nearly 260 million people worldwide[1] and involves a deleterious inflammatory response that results in immune cell infiltration in the lungs, bronchoconstriction, airway remodeling, and airway hyperresponsiveness (AHR)[2]. Asthma and allergic airway inflammation occurs in response to a wide variety of substance called allergens that induce varying levels of immune hypersensitivity[3] The most prevalent form of asthma is associated with T_H2 responses signaled through airway epithelial cell-derived alarmins IL-33 and IL-25, which stimulate the production of IL-4, IL-5, IL-13, and TGF-β cytokines by airway cells and an influx of eosinophils and mast cells, along with other inflammatory cells, into the lungs[4–6]. Although this form of the disease is usually less severe and responsive to conventional corticosteroid therapy[4], asthma can also present with other distinct immunological and pathological features[7–9]. In particular, 5-15% of the total asthma cases involve a T_H17-dominated response, leading to neutrophil recruitment and accumulation in the lungs[10,11]. Increased accumulation of neutrophils is associated with severe asthma[10,11], which is clinically defined by resistance to corticosteroid therapy[8,12–14]. In addition, T_H1 responses mediated by interferon-γ (IFN-γ) production by $CD4^+$ T cells are also associated with severe asthma cases[15,16]. The association of heightened T_H17 responses, T_H1 responses, and neutrophil accumulation with AHR and corticosteroid resistance has been reported during severe asthma in both mice and humans[14–17].

A better understanding of the immunological processes that contribute to severe asthma is required to develop alternative therapies for when corticosteroids fail. The T_H17 polarization of T cells that is associated with severe asthma is stimulated by airway epithelial cell derived cytokines IL-6, IL-1β, TGF-β and IL33[12,18] that are produced upon activation of several pattern recognition receptors in response to the allergen[19–23]. Simultaneously, IL-23 produced by macrophages and dendritic cells (DCs) plays a critical role in differentiation of IL-17A (herein IL-17) producing T_H17 cells[24–26]. IL-17 produced by T cells further induces neighboring cells, including bronchial

epithelial cells and fibroblasts, to produce CXCL8 and IL-8 (KC), thereby leading to the recruitment of neutrophils to the lungs[27–29]. Studies in murine asthma models have shown that depletion of neutrophils or blocking IL-17 signaling can reduce neutrophil accumulation in the lungs, rescue the pathology associated with severe asthma, and restore the efficacy of corticosteroids[14,15,30–32]. In addition, adoptive transfer of antigen specific T_H17 cells resulted in excessive neutrophilic infiltration and higher AHR in ovalbumin (OVA) sensitized mice[13]. Together, these reports support a direct contribution of T_H17 responses and neutrophils to the development of severe asthma, highlighting the need for a better understanding of how neutrophil recruitment and accumulation during asthma is regulated.

Several studies attempting to map genetic associations with asthma have identified a link between the *ATG5* gene and susceptibility to severe asthma. A single nucleotide polymorphism (SNP) in the third intron of the *ATG5* gene (rs12212740, alleles G/A, minor variant: G) was associated with lower pre-bronchodilator FEV1 (forced expiratory volume) and a risk of moderate to severe asthma in non-Hispanic Caucasian children[33]. In a separate genome wide association study, a SNP in the 5' untranslated region (UTR)/annotated promoter region of the *ATG5* gene (rs510432, alleles G/A, minor variant: G) was associated with increased risk of asthma in children[34]. The G variant allele of SNP rs510432 results in increased promoter activity and higher expression of *ATG5* mRNA in nasal mucosal cells isolated from the children with acute asthma compared to non-acute and healthy controls. Another study reported the association of the G variant allele of SNP rs510432 with increased sputum neutrophil counts in adult asthma patients, establishing a correlation between dysregulated *ATG5* expression and neutrophil recruitment during asthma[35].

ATG5 is essential for the process of autophagy, which involves multiple protein complexes that target cytosolic components and organelles to the lysosome for degradation[36–38]. The ULK1 complex (ULK1/ULK2, ATG13, FIP200, ATG101) and PI3 kinase complex (ATG14L, BECN1, VPS15, and VPS34) initiate autophagy by generating a phagophore from the membranes of the endoplasmic reticulum[39,40]. Elongation of the autophagosomal double membrane depends on two ubiquitin-like conjugation systems. In the first system, ATG12 (ubiquitin-like protein) is activated by ATG7 (E1-like), transferred to ATG10 (E2-like), and covalently attached to ATG5. In the second system, LC3 (microtubule-associated protein 1 light chain 3) is conjugated to phosphatidylethanolamine to generate the membrane bound form LC3-II through the actions of

ATG7 (E1-like), ATG3 (E2-like), and the ATG5-ATG12/ATG16L complex[40,41]. The autophagosome membrane is then completed and targeted for fusion with the lysosome, where the autophagosome cargo are degraded. Several studies have reported that the expression of genes required for autophagy, including *ATG5*, are elevated in the lungs of asthma patients compared to non-asthmatic individuals[42–44]. Furthermore, LC3-II expression is increased in the sputum granulocytes from severe asthma patients compared to non-severe asthma patients[35,45]. Despite the genetic associations of ATG5 and autophagy with severe asthma, the precise roles for autophagy during asthma have remained elusive. Several experimental studies have implicated both deleterious and protective functions of autophagy during asthma[46], creating uncertainty in the field. There are likely multiple contributors to the discrepancies. For example, in most of the experimental studies so far, the interpretations were largely made based on pleiotropic chemical treatments that impact autophagy, among other pathways[47–53]. In addition, autophagy likely plays cell-type specific roles during exposure to allergens, which may complicate studies that that use chemical treatments or globally knockdown autophagy gene expression[14].

ATG5 has recently been shown to function both dependent and independent of autophagy to regulate inflammatory responses in innate immune cells during infection[36,37,54–57]. Innate immune cells are also a primary contributor to lung inflammation during asthma. However, studies into the roles for autophagy specifically in innate immune cells during asthma and allergic airway inflammation have been limited. In the one study that focused on ATG5 in innate immune cells, loss of *Atg5* in hematopoietic cells was sufficient to lead to increased neutrophils and macrophages in the bronchoalveolar lavage (BAL), increased AHR, and higher levels of IL-17 and IL-1β in response to house dust mites (HDM) challenge in mice[14]. When *Atg5*-deficient bone marrow cells were cultured in the presence of GM-CSF to generate a mixed culture of DCs, macrophages, and granulocytes, and treated with HDM, the cells produced higher levels of IL-1α, IL-1β, IL-23, and caspase 1 as compared to wild type (WT) GM-CSF cultures. *Atg5*$^{-/-}$ GM-CSF cultures also caused a higher level of IL-17 production in a co-culture with CD4^{+} T cells as compared to WT GM-CSF cultures. Transfer of *Atg5*$^{-/-}$ GM-CSF cultured cells loaded with HDM into WT mice intratracheally resulted in increased neutrophilic inflammation and IL-17 production as compared to transfer of WT GM-CSF cultured cells, indicating that ATG5 is important to control neutrophil recruitment in response to HDM exposure. These studies support a role for ATG5 in innate immune cells to control neutrophilic inflammation during allergic challenge. However, it is still

unknown which innate immune cell types specifically require ATG5 and whether its role in controlling neutrophilic inflammation during allergic challenge is dependent on autophagy, since ATG5 and other autophagy proteins have been shown to function outside of autophagy to regulate inflammation[36,38,56,57]. To address these gaps in understanding, we used genetic tools to dissect the autophagy-dependent and independent roles for ATG5 in specific innate immune cells during HDM challenge *in vivo* in mice. We find that autophagy is specifically required in CD11c^{+} lung macrophages and dendritic cells to suppress T_H17 responses and control the accumulation of neutrophils during allergic airway inflammation. These findings highlight a protective role for autophagy in lung resident innate immune cells during allergic challenge.

RESULTS

Loss of *Atg5* expression in LysM^{+} cells results in increased neutrophil-dominated inflammation upon HDM challenge

The role of ATG5 and autophagy in specific innate immune cell types during asthma has yet to be tested since all prior work was done with general administration of pleiotropic and non-cell type specific treatments[43,47,51,52] or using cell cultures containing a mixed population of cell types[14]. To determine if ATG5 functions in innate immune cells during allergic airway inflammation, we used *Atg5*$^{fl/fl}$-LysM-Cre mice[36,54,58,59], which specifically delete *Atg5* in LysM^{+} monocytes, macrophages, neutrophils, and dendritic cells (DCs). Analysis of the immune cell populations in the lungs of naïve *Atg5*$^{fl/fl}$ control mice and *Atg5*$^{fl/fl}$*-LysM-Cre* mice by flow cytometry revealed no significant differences **(Figure 1, S1)**, similar to previous reports[36]. We then challenged the *Atg5*$^{fl/fl}$ and *Atg5*$^{fl/fl}$*-LysM-Cre* mice with HDM intranasally on day 1, day 8 and day 15 before analyzing lung inflammation on day 16 by flow cytometry **(Figure 1A)**, which was the same regimen and timeline used in the prior study that explored the effects of transferring Atg5$^{-/-}$ GM-CSF cultured cells into the lungs of mice[14]. HDM challenge resulted in an increase in the numbers of immune cells in the lungs of mice from both genotypes compared to naïve mice **(Figure 1, S1)**, providing evidence that we are achieving an allergic airway inflammatory response. The predominant innate immune cell type recruited to the lungs in both *Atg5*$^{fl/fl}$ and *Atg5*$^{fl/fl}$*-LysM-Cre* mice following HDM challenge was neutrophils **(Figure 1B)**. HDM-challenge of *Atg5*$^{fl/fl}$*-LysM-Cre* mice resulted in significantly increased recruitment and accumulation of neutrophils, non-alveolar macrophages, DCs, and inflammatory monocytes compared to *Atg5*$^{fl/fl}$ controls **(Figure 1B, C)**. There were no

differences in the numbers of eosinophils or alveolar macrophages in the lungs of HDM-challenged *Atg5*$^{fl/fl}$*-LysM-Cre* and *Atg5*$^{fl/fl}$ mice **(Figure 1C, S1).** Analysis of the lymphoid cell repertoire revealed higher total cell counts for CD4^{+} T cells, but no difference in the number of CD8^{+} T cells or B cells in the lungs of HDM-challenged *Atg5*$^{fl/fl}$*-LysM-Cre* mice compared to *Atg5*$^{fl/fl}$ controls **(Figure 1D, S1)**. Further analysis of the CD4^{+} T cell population revealed that the lungs of HDM-challenged *Atg5*$^{fl/fl}$*-LysM-Cre* mice had an increased number of activated CD4^{+} T cells (CD4^{+}CD44^{+}CD62L^{-}) and T regulatory (T_{reg}) cells (CD4^{+}CD25^{+}) compared to *Atg5*$^{fl/fl}$ mice **(Figure 1E, F)**. Together our results show that *Atg5* is required in LysM^{+} innate immune cells to control neutrophil, inflammatory monocyte, macrophage, and DC recruitment and accumulation in the lungs of mice challenged with HDM, which is associated with higher numbers of activated CD4^{+} T cells and T_{reg} cells in the lungs.

Loss of *Atg5* in innate immune cells results in increased production of IL-17 from CD4^{+} T cells

The increased accumulation of neutrophils, inflammatory monocytes, and recruited macrophages without a change in the numbers of eosinophils, along with the increase in activated CD4^{+} T cells, suggests that loss of ATG5 in innate immune cells results in increased T_H17 and/or T_H1 responses, rather than the T_H2 response traditionally associated with asthma. The increased inflammation in HDM-challenged *Atg5*$^{fl/fl}$*-LysM-Cre* mice was dominated by neutrophils **(Figure 1B)**, which is particularly suggestive of increased T_H17 responses[10,28]. To determine if the higher levels of neutrophil accumulation in *Atg5*$^{fl/fl}$*-LysM-Cre* mice is accompanied by elevated T_H17 responses, we crossed the *Atg5*$^{fl/fl}$*-LysM-Cre* and *Atg5*$^{fl/fl}$ mice with an IL-17 reporter mouse[60] (*Il17a*^{eGFP}, #018472, Jackson Laboratory) that expresses GFP as a proxy of IL-17, generating *Atg5*$^{fl/fl}$*-LysM-Cre/Il17a*^{eGFP} and *Atg5*$^{fl/fl}$/*Il17a*^{eGFP} mice. Following HDM challenge, we detected a similar increase in the abundance of neutrophils and activated CD4^{+} T cells in *Atg5*$^{fl/fl}$*-LysM-Cre/Il17a*^{eGFP} mice compared to *Atg5*$^{fl/fl}$/*Il17a*^{eGFP} controls **(Figure 2A, B**) as observed in the *Atg5*$^{fl/fl}$*-LysM-Cre* and *Atg5*$^{fl/fl}$ parent strains **(Figure 1B, E)**. We monitored GFP expression from immune cells following HDM challenge of *Atg5*$^{fl/fl}$*-LysM-Cre/Il17a*^{eGFP} and *Atg5*$^{fl/fl}$/*Il17a*^{eGFP} mice using flow cytometry and found that the only cell types positive for GFP were CD4^{+} T cells. A higher percentage of total CD4^{+} T cells, activated CD4^{+} T cells, and Treg cells expressed GFP in HDM-challenged *Atg5*$^{fl/fl}$*-LysM-Cre/Il17a*^{eGFP} mice compared to *Atg5*$^{fl/fl}$/*Il17a*^{eGFP} mice **(Figure 2C)**,

which reflected a higher number of GFP$^+$ activated CD4$^+$ T cells and T_{regs} in the lungs of HDM-challenged *Atg5$^{fl/fl}$-LysM-Cre/Il17a^{eGFP}* mice compared to controls **(Figure 2D)**. Based on mean fluorescence intensity, the CD4$^+$ T cells in *Atg5$^{fl/fl}$-LysM-Cre/Il17a^{eGFP}* mice also expressed more GFP per cell than CD4$^+$ T cells from *Atg5$^{fl/fl}$/Il17a^{eGFP}* mice following HDM challenge **(Figure 2E)**. These data indicate that loss of *Atg5* in LysM$^+$ cells results in increased IL-17 expression from CD4$^+$ T cells, which could represent increased T_H17 responses that would promote neutrophil recruitment during allergic airway inflammation. A higher T_H17/T_{reg} ratio is also associated with increased asthma severity, increased AHR, and refractory asthma[61–65]. Indeed, we observed an increase in the ratio of T_H17/T_{reg} cells in HDM-challenged *Atg5$^{fl/fl}$-LysM-Cre/Il17a^{eGFP}* mice compared to *Atg5$^{fl/fl}$/Il17a^{eGFP}* controls **(Figure 2F)**, supporting that loss of *Atg5* in myeloid cells results in a CD4$^+$ T cell imbalance that is associated with increased asthma severity[61,62,64].

Autophagy is required in innate immune cells to control neutrophil-dominated inflammation during HDM-challenge

ATG5 has been shown to control inflammatory responses via both autophagy-dependent and autophagy-independent pathways[36,56–58,66,67]. In particular, we previously reported that ATG5 can function independent of autophagy in innate immune cells to suppress neutrophil inflammation during pulmonary *Mycobacterium tuberculosis* infection in mice[36]. To determine whether the regulation of neutrophil recruitment by ATG5 in HDM-challenged mice was dependent on other autophagy proteins or represented an autophagy-independent role for ATG5, we monitored neutrophil abundance in the lungs of mice lacking expression of the essential autophagy genes *Atg16l1* or *Becn1* in LysM$^+$ cells following HDM challenge by flow cytometry. BECN1 operates during the nucleation step of the autophagosome membrane biogenesis, whereas ATG16L1 functions with ATG5 in the phagophore elongation complex[40]. In response to HDM, we observed higher numbers of neutrophils and activated CD4$^+$ T cells in the lungs of both *Atg16l1$^{fl/fl}$-LysM-Cre* and *Becn1$^{fl/fl}$-LysM-Cre* mice compared to the *Atg16l1$^{fl/fl}$* and *Becn1$^{fl/fl}$* controls, respectively **(Figure 3A, B)**. The lungs of HDM-challenged *Atg16l1$^{fl/fl}$-LysM-Cre* mice also accumulated significantly higher numbers of DCs and T_{reg} cells in comparison to *Atg16l1$^{fl/fl}$* mice **(Figure 3A, D)**, and the lungs of HDM-challenged *Becn1$^{fl/fl}$-LysM-Cre* mice accumulated a higher number of inflammatory monocytes compared to Becn1$^{fl/fl}$ mice **(Figure 3A)**. Similar to *Atg5$^{fl/fl}$-LysM-Cre* mice, we did not observe differences in eosinophils in the HDM-challenged *Atg16l1$^{fl/fl}$-LysM-Cre*

and *Becn1*$^{fl/fl}$*-LysM-Cre* mice compared to controls **(Figure 3B)**, indicating that the increased inflammation was representative of enhanced T_H17 or T_H1 responses and not T_H2 responses. Our results indicate that ATG5 is required in an autophagy-dependent manner to control neutrophil accumulation and activation of $CD4^+$ T cells in the lungs of mice during HDM challenge. Together, we show that autophagy has a protective role in innate immune cells during HDM-challenge.

Autophagy regulates neutrophil accumulation during HDM challenge independent of inflammasome activation

A key inflammatory process negatively regulated by autophagy is inflammasome activation[68–70]. Several reports have demonstrated a role for the NLRP3 (nucleotide-binding oligomerization domain-like Receptor Family Pyrin Domain Containing 3) inflammasome pathway, which catalyzes caspase-1 dependent release of pro-inflammatory cytokines IL-1β and IL-18, during severe allergic inflammation[71–74]. Elevated levels of caspase-1, IL-1β and IL-18 have been observed in patients and mice with severe asthma[68,72,73,75]. Genetic deletion of *Nlrp3* or treatment with NLRP3 inhibitor in mice alleviates the immune cell filtration and mucus secretion in the lungs of mice during asthma[68,71,72]. Considering that *Atg5*$^{-/-}$ bone marrow cells cultured in GM-CSF produced higher levels of IL-1β following exposure to HDM *in-vitro*[14], we sought to investigate if the higher neutrophil abundance and inflammation following HDM challenge is a consequence of inflammasome activation in the absence of autophagy in *Atg5*$^{fl/fl}$*-LysM-Cre* mice. Since caspase-1 is the key mediator of the inflammatory consequences of the NLRP3 inflammasome, we crossed the *Atg5*$^{fl/fl}$*-LysM-Cre* and *Atg5*$^{fl/fl}$ mice with *Casp1/11*$^{-/-}$ germline deletion mice, which are deleted for the genes encoding caspase-1 and caspase-11 (Jax #016621, Jackson Laboratory). We challenged *Atg5*$^{fl/fl}$/*Casp1/11*$^{-/-}$ and *Atg5*$^{fl/fl}$*-LysM-Cre/Casp1/11*$^{-/-}$ mice with HDM and analyzed the lung immune cell populations. Similar to *Atg5*$^{fl/fl}$*-LysM-Cre* mice, HDM-challenged *Atg5*$^{fl/fl}$*-LysM-Cre/Casp1/11*$^{-/-}$ mice accumulated more neutrophils, non-alveolar macrophages, DCs, activated $CD4^+$ T cells, and T_{reg} cells in their lungs than *Atg5*$^{fl/fl}$/Casp1/*11*$^{-/-}$ mice (**Figure 4A, B**), indicating that elevated caspase 1-dependent NLRP3 inflammasome activation in the absence of autophagy was not responsible for the increased neutrophil dominated inflammation during HDM challenge of *Atg5*$^{fl/fl}$*-LysM-Cre* mice. Therefore, the increased inflammasome activation reported previously in myeloid cells exposed to HDM[14] is not responsible for the neutrophilic inflammation in HDM-challenged *Atg5*$^{fl/fl}$*-LysM-Cre* mice.

Autophagy is required in CD11c$^+$ lung macrophages and dendritic cells to control neutrophil recruitment during exposure to HDM

Atg5$^{fl/fl}$-LysM-Cre mice delete *Atg5* in multiple different types of innate immune cells, including neutrophils, macrophages, inflammatory monocytes, and some DCs. Autophagy has been shown to function in many of these cell types, including neutrophils, macrophages, and DCs, to regulate inflammation[36,37,59,66,76]. Therefore, to determine specifically which LysM$^+$ cell type requires ATG5 to control neutrophil recruitment during allergic airway inflammation, we challenged *Atg5$^{fl/fl}$-CD11c-Cre* (delete *Atg5* in lung macrophages and DCs) and *Atg5$^{fl/fl}$-MRP8-Cre* (delete *Atg5* in neutrophils) mice[36] with HDM and monitored lung inflammatory responses. Flow cytometric analysis of the immune cell populations in the lung revealed a significantly higher abundance of neutrophils, non-alveolar macrophages, DCs, inflammatory monocytes, activated CD4$^+$ T cells, and T_{reg} cells in *Atg5$^{fl/fl}$-CD11c-Cre* mice (**Figures 5A-D**) but not in *Atg5$^{fl/fl}$-MRP8-Cre* mice (**Figures 5E-H**) compared to respective littermate *Atg5$^{fl/fl}$* controls. Therefore, loss of Atg5 in CD11c$^+$ cells was sufficient to phenocopy loss of Atg5 in LysM$^+$ cells, demonstrating that ATG5 functions in lung macrophages and DCs to control neutrophil recruitment during HDM challenge. We did not observe differences in any of the immune cell populations in the lungs of HDM-challenged *Atg5$^{fl/fl}$-MRP8-Cre* and *Atg5$^{fl/fl}$* littermate control mice, demonstrating that ATG5 is dispensable in neutrophils to control inflammation during HDM challenge.

DISCUSSION

The association of genetic polymorphisms in the *ATG5* gene with susceptibility to asthma in humans has drawn attention to the roles for autophagy in regulating inflammatory responses during allergen challenge and asthma. In the present study, we directly investigate the roles for ATG5 and autophagy in innate immune cells during asthma using a series of conditional knockout mouse lines and an HDM challenge model[14]. Our data support a model where ATG5 functions in an autophagy-dependent process in CD11c$^+$ lung macrophages and/or dendritic cells to suppress T_H17 responses and neutrophil recruitment to the lungs during HDM challenge. These studies establish a protective role of autophagy in CD11c$^+$ cells in a murine model of allergic airway inflammation.

Our findings build off of the observations reported previously by Suzuki *et al*., where tamoxifen-inducible deletion of *Atg5* in HDM-challenged mice or intratracheal instillation of HDM-primed *Atg5*$^{-/-}$ GM-CSF myeloid cell cultures led to neutrophil recruitment to the lungs, increased AHR, and increased production of IL-1β and IL-17[14]. In our study, we determine that ATG5 and other autophagy proteins are specifically required in lung macrophages and DCs to suppress the activation of T_H17 cells and the accumulation of T_{reg} cells. In addition, despite the higher levels of IL-1β and caspase-1 previously reported, we find that inflammasome activation is not responsible for the increased accumulation of neutrophils in HDM-challenged *Atg5*$^{fl/fl}$*-LysM-Cre* mice.

There are multiple possible mechanisms by which autophagy could be functioning in CD11c$^+$ DCs and lung macrophages to impact T cell polarization during HDM challenge. Both DCs and lung macrophages are antigen-presenting cells (APCs), and allergen antigen presentation by APCs is associated with CD4$^+$ T cell activation during asthma[77–79]. Autophagy plays a central role in transferring antigen to vesicles that contain MHC class II molecules for antigen presentation to CD4$^+$ T cells[80,81]. In addition to direct roles in MHC class II antigen presentation, autophagy also regulates the production of cytokines that can impact the polarization of T cells during activation. Loss of autophagy can result in increased production of IL-23 by macrophages and DCs[82], and IL-23 can induce the differentiation of T cells into T_H17 cells[24,83]. Macrophage polarization also impacts T cell responses, where M1-like macrophages produce T_H1 associated cytokines (TNF-α, IL-6, IL-1β, and IL-23) and induce a T_H1/T_H17 type inflammatory response, while M2-like macrophages produce type 2 cytokines (IL-5 and IL-13), activating a T_H2 type asthmatic response[84]. M1 polarized macrophages also express higher levels of the transcription factor IRF5, which was demonstrated to drive T_H1/T_H17 responses in culture[85]. In addition, deletion of *Irf5* in mice causes a reduction in IFN-γ, IL-17, and neutrophil recruitment in HDM-challenged mice[86]. Loss of autophagy in macrophages promotes pro-inflammatory M1-like macrophage polarization in inflammatory conditions such as aging-associated hepatic injury and obesity[66,87]. Therefore, autophagy in macrophages and DCs can drive differential T cell responses via antigen presentation and the production of inflammatory signals. In addition, autophagy in macrophages and DCs suppresses pro-inflammatory responses independent of T_H17 and inflammasome activation, including through mitophagy[88–91], clearance of damage-associated molecular patterns (DAMPs) and PAMPs[92–94], ER-phagy[95,96], as well as through direct interaction

with MyD88[97,98]. These roles for autophagy could also impact neutrophil recruitment and accumulation in the lungs during HDM challenge. While we ruled out a neutrophil intrinsic role for ATG5 in our study using the *Atg5*$^{fl/fl}$*-MRP8-Cre* mice, it is likely that the neutrophils that accumulate in the lungs also exacerbate the T_H17 response and inflammation. For example, neutrophil-derived antimicrobial peptide cathelicidin can induce T_H17 differentiation in a TGF-β dependent manner in purified cells and induce IL-17 production in mice[99].

Loss of autophagy in lung macrophages and DCs also resulted in the accumulation of T_{regs} in the lungs following HDM challenge. Although T_{regs} are well-known to suppress the inflammatory responses during asthma[100–103], T_{reg} accumulation is also reported to be increased during severe asthma[104,105]. Higher T_{reg} accumulation during severe asthma is associated with higher T_H17 responses, where the transfer of purified T_H17 cells promoted the expansion of T_{regs} in mice[106]. Therefore, it is possible that the increased T_H17 responses directly contribute to the increase in T_{regs} during HDM challenge of *Atg5*$^{fl/fl}$*-LysM-Cre* mice. Loss of autophagy in DCs is associated with dysregulated T_{reg} function in mice due to an accumulation of ADAM10 metalloprotease on the surface of DCs in the absence of autophagy[107]. Accumulation of ADAM10 leads to cleavage of ICOS-L, which is required for the efficient maturation of T_{regs} in steady state. Moreover, in the absence of proper maturation, FOXP3$^+$ CD25$^+$ T_{regs} transition to IL-17$^+$ CD4$^+$ T cells[107], which may explain the increased IL-17 expression in T_{regs} that we observed in HDM-challenged *Atg5*$^{fl/fl}$*-LysM-Cre* mice.

Together our studies highlight a protective role for autophagy in controlling neutrophil accumulation during asthma, suggesting that inducing autophagy may hold promise as a therapeutic for treating severe asthma cases. Given that autophagy can perform different functions in different cell types, this will need to be tested directly, which is currently limited by the absence of autophagy-specific inducers. Nonetheless, these studies establish the role for autophagy in lung macrophages and DCs during exposure to an allergen, which may also be applicable to other lung inflammatory diseases.

METHODS

<u>Mouse strains</u>

All flox mice (*Atg5*$^{fl/fl}$, *Becn1*$^{fl/fl}$, *Atg16l1*$^{fl/fl}$) used in this study have been described previously[54,59,108,109] and are maintained in an enhanced barrier facility. LysM-Cre (Jax #004781),

CD11c-Cre (Jax #007567), and MRP8-Cre (Jax #021614) mice from the Jackson Laboratory were crossed to specific flox mice, respectively. *Atg5* $^{fl/fl}$ and *Atg5* $^{fl/fl}$*-LysM-Cre* mice were crossed with *Casp1/11*$^{-/-}$ (Jax #016621) germline deletion mice to generate *Atg5*$^{fl/fl}$*/Casp1/11*$^{-/-}$ and *Atg5*$^{fl/fl}$*-LysM-Cre/Casp1/11*$^{-/-}$ mice. *Atg5*$^{fl/fl}$ and *Atg5*$^{fl/fl}$*-LysM-Cre* mice were crossed with *Il17a*^{eGFP} (Jax #018472) mice to generate *Atg5*$^{fl/fl}$*/Il17a*^{eGFP} and *Atg5*$^{fl/fl}$*-LysM-Cre/Il17a*^{eGFP} mice. Adult male and female mice (aged 8–12 weeks) were used for the experiments. All the experiments were performed with respective littermate flox controls. All the mice have been backcrossed to C57BL/6J background. The mice were housed and bred at Washington University in St. Louis in specific pathogen-free conditions in accordance with federal and university guidelines. All procedures involving animals were conducted following National Institute of Health guidelines and protocols were approved by the Institutional Animal Care and Use Committee of Washington University in St. Louis. Animals were euthanized by CO_2 asphyxiation, approved by Euthanasia of the American Veterinary Association.

Asthma model

Mice were challenged with HDM as described previously[14]. Briefly, mice were challenged intranasally with HDM in PBS (Greer Laboratories). The first primary dose of HDM (200 µg) was administered on Day 1 and two secondary (100 µg) doses were administered on day 8 and day 15 respectively. Mice were sacrificed on day 16 and the whole lung except the post caudal lobe was used in flow cytometry experiments.

Flow cytometry

Mouse lungs were excised after PBS perfusion, placed in DMEM containing 10% FBS, minced finely, and digested at 37ºC for an hour with mechanical disruption with a stir bar and enzymatic digestion with collagenase D (Roche 11088875103) and DNase I (Sigma D4527). Digested tissue was treated with ACK buffer to remove red blood cells and were passed through a 70 µm cell strainer to generate single cell suspension in 1 ml of semi-facs buffer (1X PBS pH 7.4 supplemented with 2% FBS and 2 mM EDTA). 100 µl of cells were stained with Zombie-NIR before staining with the antibody cocktail mix. Cells were resuspended in semi-facs buffer in the presence of Fc receptor blocking antibody (BioLegend, 101302) and stained with antibodies at a 1:200 dilution with the myeloid or lymphoid antibody cocktails. The innate immune cell staining

antibody cocktail included the following antibodies: Ly6G-BV421 (Biolegend, 1A8), SiglecF-BV480 (Biolegend, E50-2440(RUO), CD45-BV570 (Biolegend, 30-F11), CD11b-BV650 (Biolegend, M1/70), CD103-BV750 (Biolegend, M290), Ly6C-BV785 (Biolegend, HK1.4), MHCII-SparkBlue550 (Biolegend, M5/114.15.2), CD64-PerCP-eFluor710 (Biolegend, X54-5/7.1), MerTK-PE-Cy7 (Biolegend, DS5MMER), CD206-eF660 (AF647) (eBiosciences, C068C2), CD11c-APC/Fire750 (Biolegend, N418). The lymphoid cell staining antibody cocktail included the following antibodies: CD8α-Pacific blue (Biolegend, 53-6.7), CD44-BV510 (Biolegend, IM7), CD45-BV570 (Biolegend, 30-F11), CD19-BV605 (Biolegend, 6D5), CD11b-BV650 (Biolegend, M1/70), NK1.1-BV785 (Biolegend, PK136), TCRβ-FITC (Biolegend, H57-597), NKp46-PerCP-eFluor710 (eBiosciences, 29A1.4), CD62L-PE/Cy5 (Biolegend, MEL-14), CD25-PE-Cy7 (Biolegend, PC61), and CD4-APC (Biolegend, 100516). Flow cytometric analysis was performed on an Cytek Aurora and data was analyzed with FlowJo software (Tree Star). The gating strategies are shown in **Figure S2**. Cell populations are represented as counts per total lung suspension (1 ml) excluding the post-caudal lobe.

<u>Statistical analysis for biological experiments</u>

All data from HDM-challenged mice are from at least two independent experiments. Samples represent biological (not technical) replicates of mice randomly sorted into each experimental group. No blinding was performed during animal experiments. If presented on a log scale, the data was log transformed before statistical analysis. Statistical differences were calculated using Prism 9.0 (GraphPad Software) using unpaired Welch's t-tests or one-way ANOVA as denoted in the figure legends. Sample sizes were sufficient to detect differences as small as 10% using the statistical methods described. When used, center values and error bars represent means ± S.E.M. $P < 0.05$ was considered significant. $P < 0.05$ was denoted *, ** for $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. Comparisons that were not significantly different are designated as ns.

AUTHOR CONTRIBUTIONS

N.D. and C.L.S. designed the project, analyzed the data, and wrote the manuscript. N.D., S.R.M., N.R.E., and R.W. performed the experiments. N.D., A.R., A.L.K, and C.L.S. designed the asthma protocols. D.K. performed mouse crossing, breeding, and housing. A.L.K. and C.L.S. advised project design. All authors read and edited the manuscript.

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DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

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FIGURE LEGENDS

Figure 1. Loss of *Atg5* expression in LysM$^+$ cells results in increased neutrophil-dominated inflammation upon HDM challenge

(A) Schematic of asthma model used in study, made in part with BioRender. Mice were challenged with house dust mites intranasally. **(B)** Representative flow gating of neutrophils (left) and graph of neutrophil numbers per lung (right) from *Atg5$^{fl/fl}$* and *Atg5$^{fl/fl}$-LysM-Cre* mice either before HDM challenge (unchallenged) or post-HDM challenge. Number of **(C)** non-alveolar macrophages, eosinophils, DCs, inflammatory monocytes, **(D)** CD4$^+$ T cells, **(E)** activated CD4$^+$ T cells, naïve CD4$^+$ T cells, and **(F)** T_{reg} cells per lung. The legend for **(B-F)** is shown in **(B)**. **(B-F)** Each datapoint is from an individual mouse and the HDM samples are compiled from 2 independent experiments. The bar graphs represent the mean +/- S.E.M. One-way ANOVA was used to determine the statistical significance of differences between the groups, where only the differences within a single condition are shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Comparisons that were not significantly different are designated as ns.

Figure 2. Loss of *Atg5* in innate immune cells results in increased production of IL-17 from CD4$^+$ T cells

(A-B) Number of **(A)** neutrophils and **(B)** activated CD4$^+$ T cells in *Atg5$^{fl/fl}$/Il17a^{eGFP}* (n=5) and *Atg5$^{fl/fl}$-LysM-Cre/Il17a^{eGFP}* (n=10) mice following HDM challenge. **(C)** Percent or **(D)** number of CD4$^+$ T cells, activated CD4$^+$ T cells, and T_{regs} expressing IL-17 based on GFP expression in HDM-challenged *Atg5$^{fl/fl}$/Il17a^{eGFP}* and *Atg5$^{fl/fl}$-LysM-Cre/Il17a^{eGFP}* mice. **(E)** Mean fluorescence intensity for GFP (IL-17) in CD4$^+$ T cells, activated CD4$^+$ T cells, and T_{regs}. **(F)** Ratio of IL-17$^+$ CD4$^+$ T cell numbers to total T_{reg} cell numbers. **(A-F)** The legend for all panels is shown at the top of the figure. Each datapoint is from an individual mouse from at least 2 independent experiments. The bar graphs represent the mean +/- S.E.M. Unpaired Welch's t-test was used to determine the

statistical significance of differences between the groups. * P < 0.05 and ** P < 0.01. Comparisons that were not significantly different are designated as ns.

Figure 3. Autophagy is required in innate immune cells to control neutrophil-dominated inflammation during HDM-challenge

Number of **(A)** neutrophils, non-alveolar macrophages, eosinophils, DCs, inflammatory monocytes, **(B)** $CD4^+$ T cells, **(C)** activated $CD4^+$ T cells, naïve $CD4^+$ T cells, and **(D)** T_{reg} cells per lung in HDM-challenged *Atg16*$^{fl/fl}$ (n=4), *Atg16l1*$^{fl/fl}$*-LysM-Cre (*n=5), *Becn1*$^{fl/fl}$ (n=7), and *Becn1*$^{fl/fl}$*-LysM-Cre* (n=10) mice. The legend for **(A-D)** is shown in **(A)**. Each datapoint is from an individual mouse from at least 2 independent experiments. The bar graphs represent the mean +/- S.E.M. Unpaired Welch's t-test was used to determine the statistical significance of differences between the groups. * P < 0.05. Comparisons that were not significantly different are designated as ns.

Figure 4. Autophagy regulates neutrophil accumulation during HDM challenge independent of inflammasome activation

Number of **(A)** neutrophils, non-alveolar macrophages, eosinophils, DCs, inflammatory monocytes, **(B)** $CD4^+$ T cells, **(C)** activated $CD4^+$ T cells, naïve $CD4^+$ T cells, and **(D)** T_{reg} cells per lung in HDM-challenged *Atg5*$^{fl/fl}$*/Casp1/11*$^{-/-}$ (n=13) and *Atg5*$^{fl/fl}$*-LysM-Cre/Casp1/11*$^{-/-}$ (n=17) mice. The legend for **(B-D)** is shown above **(A)**. Each datapoint is from an individual mouse from at least 2 independent experiments. The bar graphs represent the mean +/- S.E.M. Unpaired Welch's t-test was used to determine the statistical significance of differences between the groups. * P < 0.05, ** P < 0.01, *** P < 0.001. Comparisons that were not significantly different are designated as ns.

Figure 5. Autophagy is required in $CD11c^+$ lung macrophages and DCs to control neutrophil recruitment during exposure to HDM

(A-D) Number of **(A)** neutrophils, non-alveolar macrophages, eosinophils, DCs, inflammatory monocytes, **(B)** $CD4^+$ T cells, **(C)** activated $CD4^+$ T cells, naïve $CD4^+$ T cells, and **(D)** T_{reg} cells per lung from HDM-challenged *Atg5*$^{fl/fl}$ (n=10) and *Atg5*$^{fl/fl}$*-CD11c-Cre* (n=12) mice. The legend above **(A)** is shared for **(A-D)**. **(E-H)** Number of **(E)** neutrophils, non-alveolar macrophages,

eosinophils, DCs, inflammatory monocytes, **(F)** $CD4^+$ T cells, **(G)** activated $CD4^+$ T cells, naïve $CD4^+$ T cells, and **(H)** T_{reg} cells per lung in HDM-challenged *Atg5*$^{fl/fl}$ (n=6) and *Atg5*$^{fl/fl}$*-MRP8-Cre* (n=7) mice. The legend above **(E)** is shared for **(E-H)**. Each datapoint is from an individual mouse from at least 2 independent experiments. The bar graphs represent the mean +/- S.E.M. Unpaired Welch's t-test was used to determine the statistical significance of differences between the groups. * $P < 0.05$ and ** $P < 0.01$. Comparisons that were not significantly different are designated as ns.

Figure 1

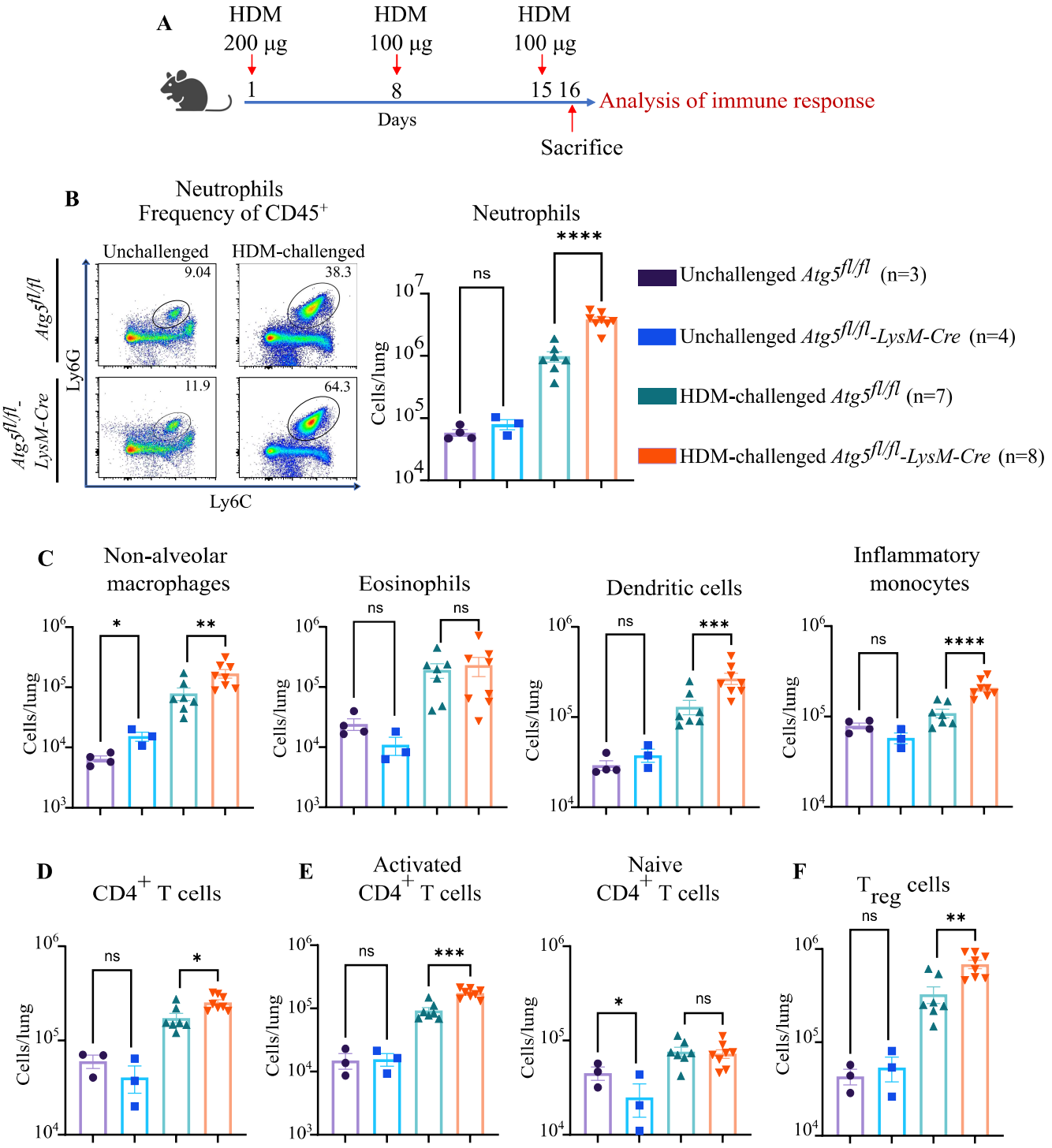

A
HDM
200 µg
HDM
100 µg
HDM
100 µg
1
8
15 16
Days
Analysis of immune response
Sacrifice
B
Neutrophils
Frequency of CD45+
Unchallenged
HDM-challenged
Atg5fl/fl
Atg5fl/fl-LysM-Cre
Ly6G
Ly6C
9.04
38.3
11.9
64.3
Neutrophils
Cells/lung
ns
****
Unchallenged Atg5fl/fl (n=3)
Unchallenged Atg5fl/fl-LysM-Cre (n=4)
HDM-challenged Atg5fl/fl (n=7)
HDM-challenged Atg5fl/fl-LysM-Cre (n=8)
C
Non-alveolar macrophages
Eosinophils
Dendritic cells
Inflammatory monocytes
D
CD4+ T cells
E
Activated CD4+ T cells
Naive CD4+ T cells
F
Treg cells

Figure 2

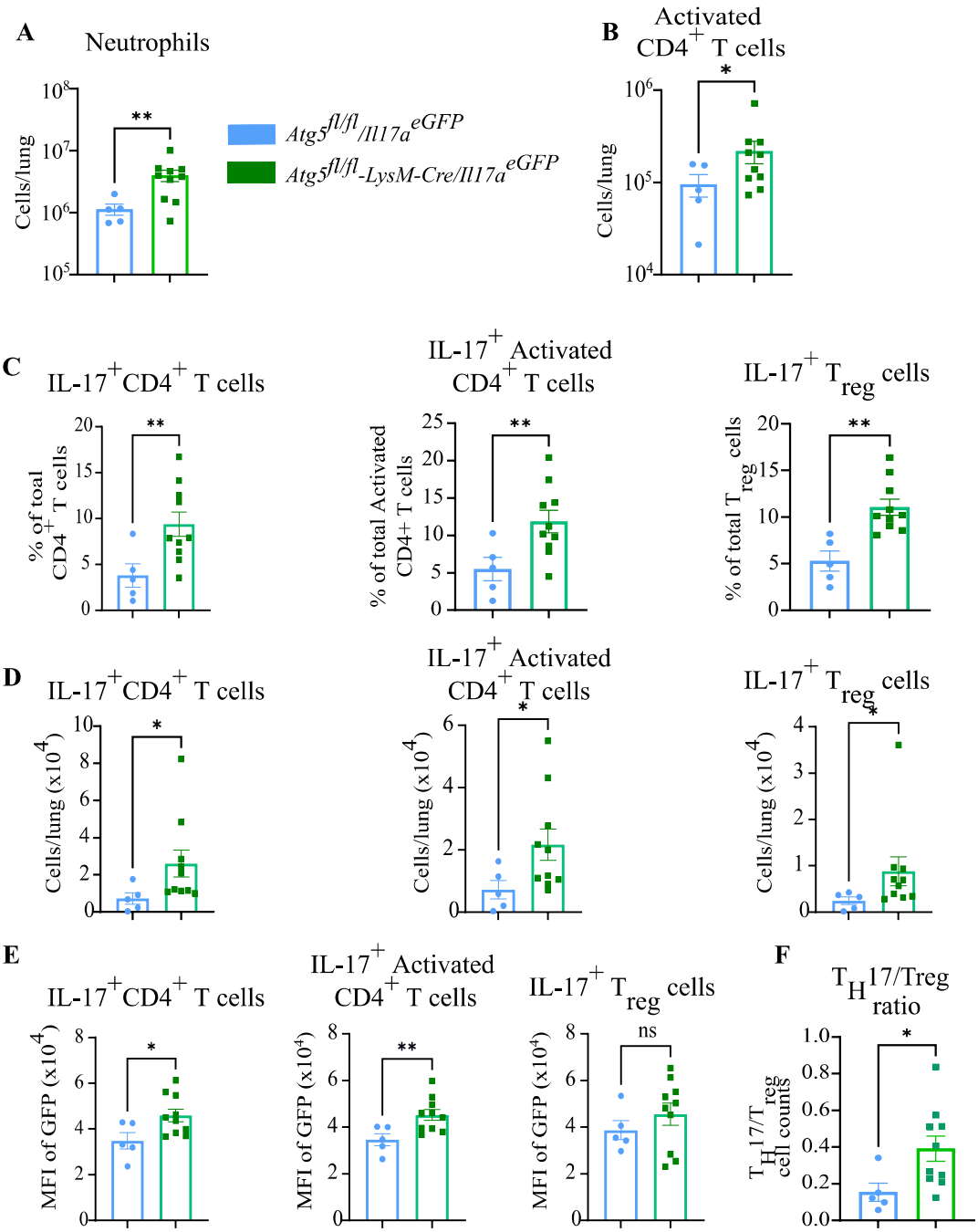

A
Neutrophils
Cells/lung
**
Atg5fl/fl/Il17aeGFP
Atg5fl/fl-LysM-Cre/Il17aeGFP
B
Activated CD4+ T cells
Cells/lung
*
C
IL-17+CD4+ T cells
% of toal CD4+ T cells
**
IL-17+ Activated CD4+ T cells
% of total Activated CD4+ T cells
**
IL-17+ Treg cells
% of total Treg cells
**
D
IL-17+CD4+ T cells
Cells/lung (x10^4)
*
IL-17+ Activated CD4+ T cells
Cells/lung (x10^4)
*
IL-17+ Treg cells
Cells/lung (x10^4)
*
E
IL-17+CD4+ T cells
MFI of GFP (x10^4)
*
IL-17+ Activated CD4+ T cells
MFI of GFP (x10^4)
**
IL-17+ Treg cells
MFI of GFP (x10^4)
ns
F
TH17/Treg ratio
TH17/Treg cell counts
*

Figure 3

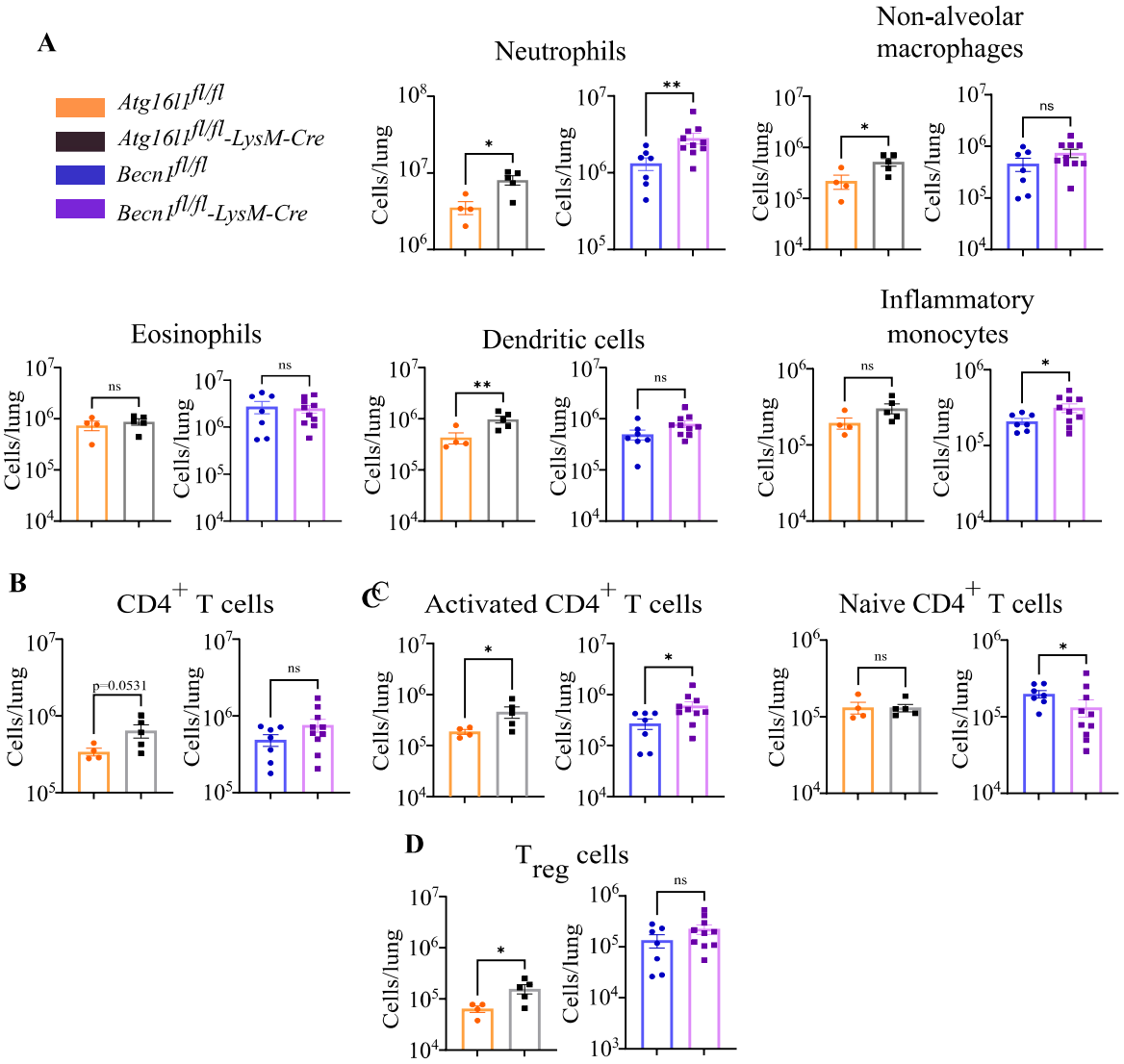

A
Atg16l1fl/fl
Atg16l1fl/fl-LysM-Cre
Becn1fl/fl
Becn1fl/fl-LysM-Cre
Neutrophils
Non-alveolar macrophages
Eosinophils
Dendritic cells
Inflammatory monocytes
Cells/lung
ns
*
**
B
CD4+ T cells
p=0.0531
C
Activated CD4+ T cells
Naive CD4+ T cells
D
Treg cells

Figure 4

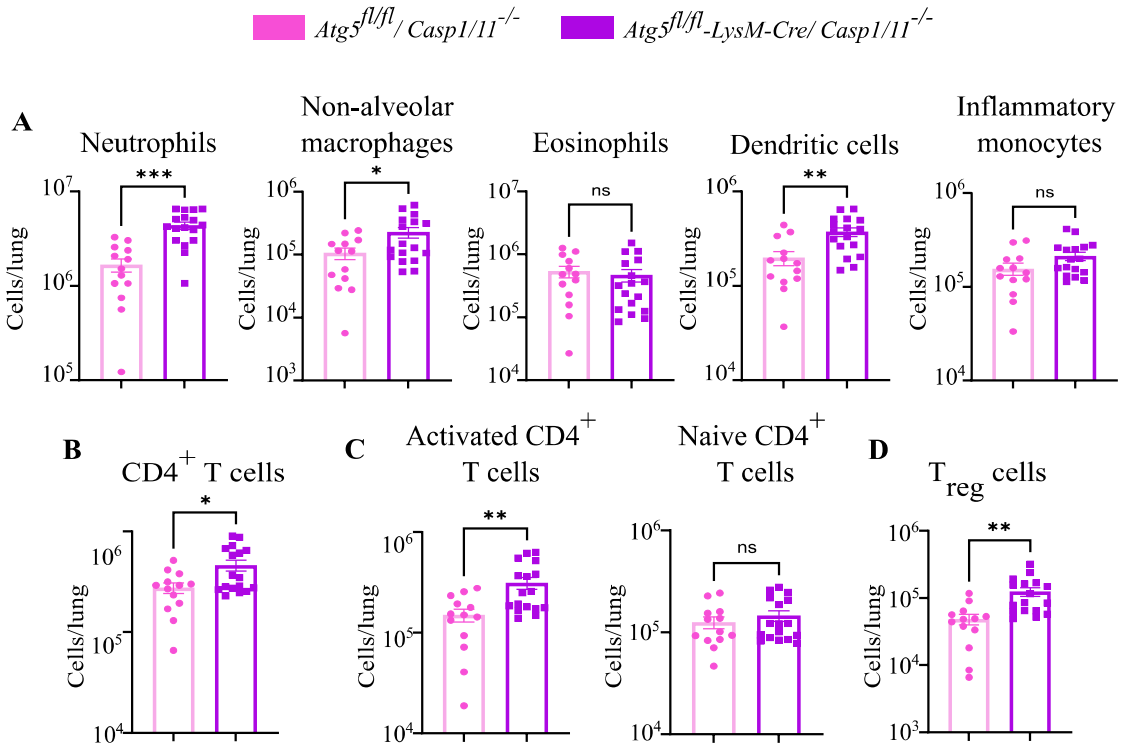
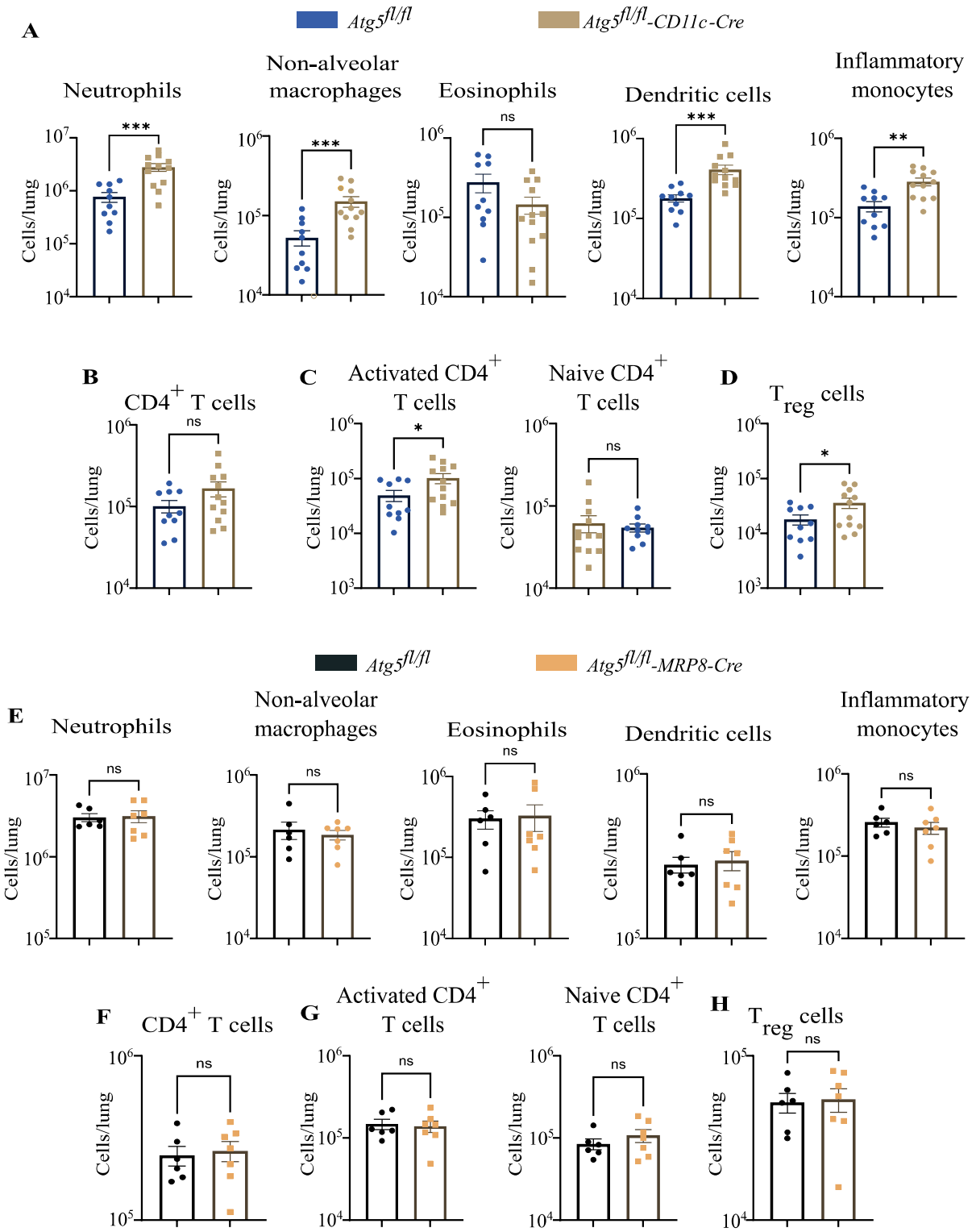

Atg5fl/fl/ Casp1/11-/-
Atg5fl/fl-LysM-Cre/ Casp1/11-/-
A
Neutrophils
Non-alveolar macrophages
Eosinophils
Dendritic cells
Inflammatory monocytes
Cells/lung
***
*
ns
**
ns
B
CD4+ T cells
C
Activated CD4+ T cells
Naive CD4+ T cells
D
Treg cells
*
**
ns
**

Figure 5


A
Atg5fl/fl
Atg5fl/fl-CD11c-Cre
Neutrophils
Non-alveolar macrophages
Eosinophils
Dendritic cells
Inflammatory monocytes
Cells/lung
***
***
ns
***
**
B
CD4+ T cells
C
Activated CD4+ T cells
Naive CD4+ T cells
D
Treg cells
ns
*
ns
*
Atg5fl/fl
Atg5fl/fl-MRP8-Cre
E
Neutrophils
Non-alveolar macrophages
Eosinophils
Dendritic cells
Inflammatory monocytes
ns
F
CD4+ T cells
G
Activated CD4+ T cells
Naive CD4+ T cells
H
Treg cells