

Key words: Chronic rhinosinusitis, autophagy, eosinophil, macrophage, prostaglandin D₂

Chronic rhinosinusitis (CRS) is a common upper airway disease characterized by chronic inflammation of the sinonasal mucosa with significant health effects.¹⁻⁴ CRS is a multifactorial inflammatory disorder with heterogeneous clinical features, histopathology, and efficacy of medications.⁵⁻⁷ Such a heterogeneous nature of the disease confounds the identification of etiologic factors in patients with CRS. Defects in the innate immune system were recently proposed as important contributors to the initiation and amplification of inflammation in patients with CRS.²⁻⁵ Inflamed tissues from patients with CRS display a different spectrum of inflammatory mediators and infiltrated innate effector cells, including eosinophils, neutrophils, mast cells, and macrophages. Primary interest has centered on regulation of eosinophils in patients with CRS because eosinophilic inflammation is dominant in patients with severe refractory CRS.^{6,7} Thus there is growing attention to understanding the mechanisms that promote and perpetuate eosinophilic inflammation in patients with CRS.

Production of prostaglandin (PG), especially PGD₂, is dysregulated in patients with eosinophilic chronic rhinosinusitis (ECRS) and considered an important contributing factor to eosinophilic inflammation. Expression of prostaglandin D₂ synthase (PGDS) is increased in patients with chronic rhinosinusitis with nasal polyps (CRSwNP) and positively correlates with eosinophilic inflammation.⁸ Upregulation of PGD₂ in nasal polyps (NPs) strongly correlates with the number of mast cells that produce mainly PGD₂ and that play an important role in orchestrating eosinophil infiltration in patients with CRS.⁹⁻¹¹ Furthermore, PGDS overexpression that leads to overproduction of PGD₂ promotes pronounced lung infiltration of eosinophils, but not neutrophils, in a murine asthma model.¹² Despite its significance in ECRS pathogenesis, the mechanisms by which PGD₂ dysregulation occurs in patients with CRS remain unclear.

Autophagy is an evolutionarily conserved intracellular process through which compromised organelles and invading pathogens are sequestered and cleared.¹³ It also plays a pivotal role in shaping cellular immune responses and progression of diverse inflammatory diseases.¹⁴⁻¹⁶ Studies on genetic polymorphisms involving autophagy-related gene 5 (*Atg5*) and increased expression of *Atg5* in the nasal mucosa revealed a causal association of autophagy with asthma pathogenesis.^{17,18} Moreover, autophagy deficiency in CD11c⁺ cells promotes neutrophilic airway inflammation in a murine asthma model,¹⁹ supporting a direct contribution of autophagy to neutrophilic lung inflammation. Despite significant progress made regarding the role of autophagy as a pivotal contributor to inflammatory diseases, including asthma, few studies have been conducted on the association of autophagy with CRS pathogenesis. Recent reports provide some clues concerning the potential role of autophagy in patients with CRS by demonstrating autophagy deficiency in NPs and its inverse correlation with COX-2 expression.^{20,21} However, no study has sought to assess the direct role of autophagy in the development of CRS, especially ECRS. In the present study we obtained evidence indicating that autophagy deficiency in myeloid cells, particularly macrophages, is linked to PGD₂ dysregulation and eosinophilic inflammation in a murine model of ECRS.

Abbreviations used

Atg:	Autophagy-related gene
CRS:	Chronic rhinosinusitis
CRSwNP:	Chronic rhinosinusitis with nasal polyps
DAB:	Diaminobenzidine
DP2:	Chemoattractant receptor-like molecule on T _H 2 cells
ECRS:	Eosinophilic chronic rhinosinusitis
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
H-PGDS:	Hematopoietic prostaglandin D ₂ synthase
LC-MS/MS:	Liquid chromatography–tandem mass spectrometry
mPGES-1:	Microsomal prostaglandin E synthase 1
NP:	Nasal polyp
PG:	Prostaglandin
PGDS:	Prostaglandin D ₂ synthase
PGES:	Prostaglandin E ₂ synthase
PPARG:	Peroxisome proliferator-activated receptor γ
qRT-PCR:	Quantitative real-time RT-PCR
SPE:	Solid-phase extraction

METHODS

Animals

To generate *Atg7^{fl/fl};Lyz2-Cre* mice with myeloid cell–specific deletion of *Atg7*, we crossed *Lyz2-Cre* mice (stock number 4781; Jackson Laboratories, Bar Harbor, Me) with *Atg7^{fl/fl}* mice (kindly provided by Masaaki Komatsu of Niigata University, Niigata, Japan). Littermates were used in all experiments. The genotypes of the mice were analyzed with PCR by using established primers.²² All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Asan Institute for Life Science.

Murine CRS model

ECRS was induced by means of intranasal challenge with a mixture of *Aspergillus oryzae* protease (Sigma-Aldrich, St Louis, Mo) and ovalbumin (Worthington Biochemical, Lakewood, NJ), as described with minor modifications.²³ To deplete macrophages, mice were given an intranasal injection of clodronate liposome (ClodronateLiposomes.com, Amsterdam, the Netherlands). After CRS development, blood samples were collected, and nasal tissue sections were obtained and stained, as previously described.²³ Mast cell infiltration was detected by using acidic toluidine blue staining, as previously reported.²⁴

Quantitative real-time RT-PCR and RT² Profiler PCR Arrays

The mouse Allergy and Asthma RT² Profiler PCR Array (Qiagen/SA Biosciences, Frederick, Md) was used to study the effects of impaired autophagy on gene expression profiles related to eosinophilic inflammation in the setting of CRS, according to the manufacturer's recommendations. mRNA levels of hematopoietic prostaglandin D₂ synthase (H-PGDS), microsomal prostaglandin E synthase 1 (mPGES1), and COX-2 were determined by using quantitative real-time RT-PCR (qRT-PCR).

Immunofluorescence and immunohistochemistry

Tissue sections were immunostained for LC3B in combination with markers for macrophages (F4/80 and CD68), neutrophils (Ly6G), or eosinophils (Sirius red) to detect types of myeloid cells linked to autophagy deficiency. Association of H-PGDS levels with tissue eosinophilia was examined after staining of tissue sections with Sirius red, followed by anti-H-PGDS antibody.

PGD₂ measurement

PGD₂ was extracted from 1 mL of medium by using solid-phase extraction (SPE). A liquid chromatography–tandem mass spectrometry (LC-MS/MS)

system equipped with a 1290 HPLC (Agilent Technologies, Santa Clara, Calif), Qtrap 5500 (AB Sciex, Framingham, Mass), and a reverse-phase column (Pursuit 5, 200 × 2.0 mm) was used for PGD₂ analysis. PGD₂ was quantified with PGD₂-d₄ as the internal standard.

Statistical analysis

All data were analyzed with GraphPad Prism software (version 4.00; GraphPad Software, La Jolla, Calif). Groups were compared by using nonparametric Mann-Whitney *U* tests. Statistical significance was defined as a *P* value of less than .05.

RESULTS

Aggravation of ECRS by autophagy deficiency in myeloid cells

A previous study indicates an important contribution of autophagy in immune cells, specifically CD11c⁺ cells, to the induction of neutrophilic lung inflammation.¹⁹ However, its contribution to eosinophilic airway inflammation remains undefined. In this respect we studied the role of autophagy in the development of ECRS using mice with myeloid cell-specific deletion of *Atg7* (hereafter denoted as *Atg7*^{fl/fl}; *Lyz2*-Cre mice). In these mice the key autophagy gene *Atg7* is deleted in cells of myeloid origin, such as macrophages, neutrophils, and eosinophils, that infiltrate the sinonasal mucosa during CRS pathogenesis.²⁻⁵ *Atg7*^{fl/fl}; *Lyz2*-Cre mice exhibited no developmental abnormalities and had normal reproductive ability. Moreover, these mice displayed normal histology in the sinonasal tissues without noticeable infiltration of inflammatory cells (data not shown), even when evaluated in aged mice (10 months). Thus autophagy deficiency in myeloid cells appears not to disrupt the maintenance of normal sinonasal homeostasis.

Next, we investigated the effect of autophagy deficiency on eosinophilic inflammation by using a previously established murine model of ECRS. One day after the last challenge, the severity of blood and tissue eosinophilia was examined. Among leukocytes examined, we identified that the number of blood eosinophils was significantly increased and was greater in *Atg7*^{fl/fl}; *Lyz2*-Cre CRS mice than in *Atg7*^{fl/fl} CRS mice (*P* < .001; Fig 1, A). Histologic analyses revealed that epithelial hyperplasia and maximal mucosal thickness were more pronounced in *Atg7*^{fl/fl}; *Lyz2*-Cre mice with CRS than in *Atg7*^{fl/fl} mice with CRS (*P* < .05; Fig 1, B and C). In addition, the number of eosinophils infiltrating the sinonasal mucosa was significantly greater in *Atg7*^{fl/fl}; *Lyz2*-Cre mice with CRS than in *Atg7*^{fl/fl} mice with CRS (*P* < .05; Fig 1, B and C). Collectively, these results indicated a protective effect of myeloid cell autophagy on blood and tissue eosinophilia in a murine model of CRS.

Autophagy deficiency affects gene expression profiles related to eosinophilic inflammation and the PGD₂ pathway

To gain insight into underlying mechanisms, we next assessed gene expression in the sinonasal tissue of mice with CRS. To this end, we used a PCR array to monitor the expression of a selected set of 84 genes central to allergy and asthma. Differentially expressed genes were identified as those that were either upregulated or downregulated by more than 3-fold in *Atg7*^{fl/fl}; *Lyz2*-Cre mice with CRS compared with *Atg7*^{fl/fl} mice with CRS. Our analyses revealed that autophagy deficiency

caused substantial changes in gene expression profiles of sinonasal tissue from mice with CRS (Fig 2, A and B, and see Table E1 in this article's Online Repository at www.jacionline.org). Such an abundance of genes showing differential expression suggests a crucial role of autophagy in the regulation of allergic responses. Importantly, genes associated with eosinophils (eg, *Rnase2a*, *Rnase2b*, and *Itga4*) were upregulated markedly by autophagy deficiency, correlating with the increase in eosinophilic inflammation. A notable increase was also observed for genes related to eosinophil survival and activation (*Il3*, *Il5*, *Il13*, *Il3ra*, *Il5ra*, and *Il13ra2*), as well as recruitment (*Ccl5*, *Ccl11*, *Ccl24*, and *Ccl26*).

In support of this, substantial increases in mRNA expression were observed for genes encoding thymic stromal lymphopoietin (*TSLP*) and IL-1 receptor-like 1 (*IL1RL1*), which are linked to Th2 response and eosinophilia.²⁵⁻²⁷ Furthermore, there was an apparent increase in gene expression (*Arg1*, *Chil1*, and *Mrc1*) associated with alternatively activated (M2) macrophages that promote eosinophil recruitment.²⁸ Among others, genes (*Ptgsr2* and *Pparg*) that encode the receptor for PGD₂ and its metabolites were notably upregulated. These results raise the possibility that autophagy deficiency can provoke eosinophilic inflammation involving dysregulation of the PGD₂ pathway.

Autophagy deficiency is linked to PGD₂ dysregulation

Next, we evaluated whether the lack of autophagy could affect mRNA levels of PGDS, prostaglandin E₂ synthase (PGES), and COX-2, which are required for PG production and also implicated in CRS pathophysiology.⁸ Relative mRNA levels of H-PGDS, mPGES-1, and COX-2 in the sinonasal tissue of mice with CRS were determined by using qRT-PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA. We found a significant increase in mRNA levels of H-PGDS and COX-2 but significantly diminished mRNA levels of mPGES-1 by autophagy deficiency (*P* < .05; Fig 2, C). This result is consistent with a prior report showing an inverse correlation between PGDS and PGES expression and its correlation with tissue eosinophilia in the setting of CRS.^{8,29} The activation of peroxisome proliferator-activated receptor γ (*Pparg*), expression of which was notably increased by autophagy deficiency (Fig 2, A and B), promotes macrophage M2 polarization and mPGES-1 downregulation.³⁰⁻³² In support, we found a reciprocal regulation of H-PGDS and mPGES-1 expression in activated macrophages exposed to an agonist of *Pparg* (rosiglitazone, see Fig E1 in this article's Online Repository at www.jacionline.org). Moreover, immunohistochemical staining confirmed a significant increase in H-PGDS protein levels that was concomitant with tissue eosinophilia by autophagy deficiency (*P* < .001; Fig 2, D).

Mast cells mainly produce PGD₂ and play crucial pathogenic roles in the setting of ECRS. We counted the infiltration of mast cells into the sinonasal tissue of mice with CRS, which was visualized by using acidic toluidine blue staining. The number of infiltrated mast cells was increased in *Atg7*^{fl/fl}; *Lyz2*-Cre mice with CRS compared with that in *Atg7*^{fl/fl} mice with CRS (*P* < .01; Fig 2, E). Supporting this, genes associated with mast cells (*Cma1*, *Cpa3*, *Fcer1a*, and *Ms4a2*) were clearly upregulated by autophagy deficiency (Fig 2, A and B).

To probe the role of autophagy in the regulation of PGD₂ production, we used an established model of acute peritoneal

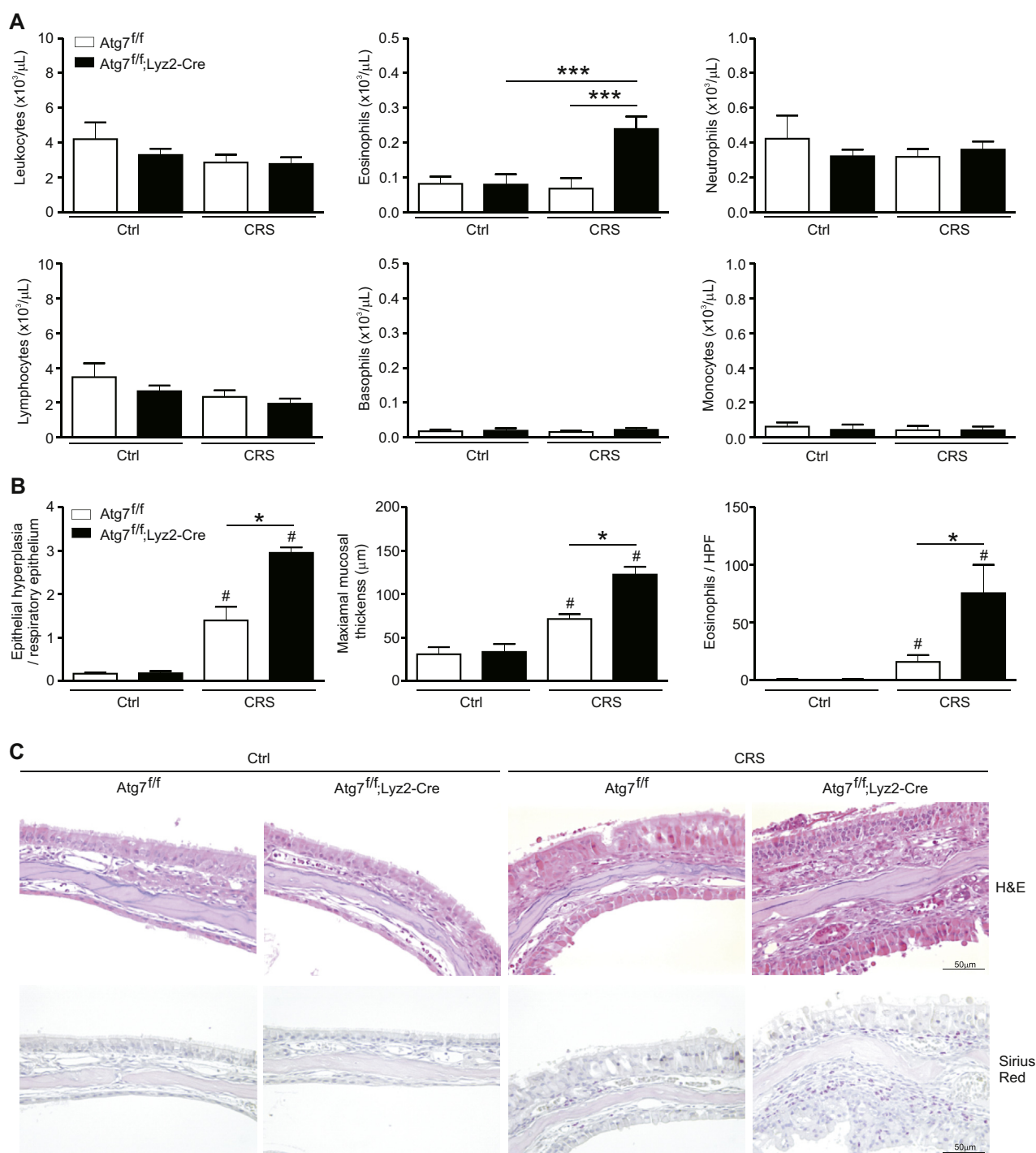
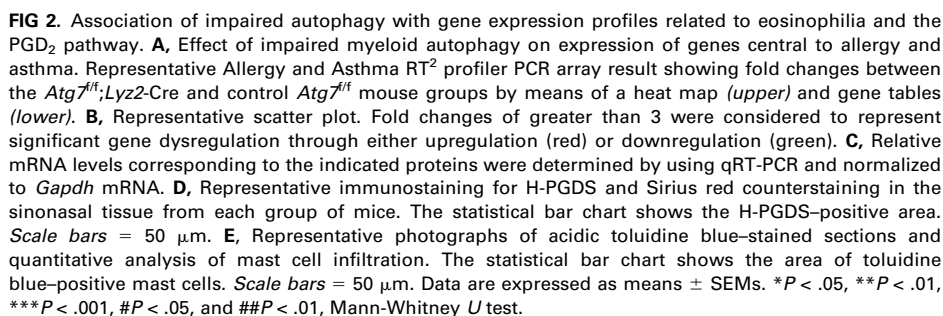


FIG 1. Impaired myeloid autophagy aggravates eosinophilic inflammation in mice with ECRS. **A**, Effect of impaired myeloid autophagy on leukocyte, eosinophil, neutrophil, lymphocyte, basophil, and monocyte counts in blood from each group of mice. **B**, Scores of epithelial hyperplasia (left), maximal mucosal thickness (middle) in hematoxylin and eosin–stained tissue sections, and eosinophil counts of the lamina propria (right) in Sirius red–stained tissue sections. **C**, Representative photographs of hematoxylin and eosin (H&E; upper)– and Sirius red (lower)–stained sections. Scale bars = 50 μm . Data are expressed as means $\pm$ SEMs ($n = 8$ –16 per group). * $P < .05$, *** $P < .001$, and # $P < .05$, Mann-Whitney U test.

inflammation induced by zymosan treatment.^{33,34} In this model PGD₂ derived from mast cells and macrophages contributes to the resolution of peritonitis, which enabled the assessment of PGD₂ production by autophagy deficiency despite the differences

in pathophysiology. Levels of PGD₂ in peritoneal exudates was determined by using LC-MS/MS. *Atg7^{fl/fl};Lyz2-Cre* mice had significantly higher levels of PGD₂ than *Atg7^{fl/fl}* mice ($P < .05$, see Fig E2 in this article's Online Repository at



www.jacionline.org). Collectively, our results suggest an association of autophagy deficiency with eosinophilic inflammation through an effect on PGD₂ dysregulation.

PGD₂ promotes eosinophil recruitment by acting through chemoattractant receptor-like molecule on T_H2 cells (DP2).³⁵ Eosinophilia in peripheral blood and sinonasal tissue caused by autophagy deficiency was significantly alleviated by an antagonist of DP2 (TM30089; see Fig E3, A and B, in this article's Online Repository at www.jacionline.org). Furthermore, epithelial hyperplasia and maximal mucosal thickness were significantly decreased in *Atg7^{fl/fl};Lyz2-Cre* mice with CRS (see Fig E3, B and C), an observation compatible with TM30089-mediated inhibition of peribronchial eosinophilia and mucus cell hyperplasia.³⁶ These results suggest that PGD₂ dysregulation associated with impaired autophagy might be an important contributing factor in aggravating eosinophilic inflammation in the setting of CRS.

Macrophages undergo autophagy in the sinonasal mucosa of mice with CRS

To investigate types of myeloid cells that undergo autophagy during CRS, we examined localization of LC3B, a widely used marker for autophagy, in association with markers for different types of myeloid cells. Immunohistochemical analysis revealed a marked increase in LC3B staining, including detectable LC3B puncta in the sinonasal tissue of mice with CRS compared with that of control mice. Of note, such LC3B staining was often colocalized with F4/80⁺ and CD68⁺ macrophages that infiltrated the sinonasal mucosa of *Atg7^{fl/fl}* CRS mice, which was hardly observed in *Atg7^{fl/fl};Lyz2-Cre* mice with CRS lacking autophagy in myeloid cells (Fig 3). These data reinforce the specificity of our immunohistochemical analysis and the role of macrophage autophagy in ECRS. In comparison, we observed less colocalization of LC3B staining with Ly6G⁺ neutrophils and Sirius red–positive eosinophils (Fig 3 and see Fig E4 in this article's Online Repository at www.jacionline.org).

Autophagy-deficient macrophages aggravate ECRS

To probe the pathogenic role of autophagy-deficient macrophages in the eosinophilic inflammation of CRS, we assessed the severity of blood and tissue eosinophilia by macrophage depletion with clodronate liposome treatment.³⁷ Efficacy of clodronate liposome–mediated depletion was confirmed in sinonasal mucosa stained for the presence of F4/80⁺ macrophages (see Fig E5 in this article's Online Repository at www.jacionline.org). We found that blood eosinophils in *Atg7^{fl/fl};Lyz2-Cre* mice with CRS were significantly decreased by macrophage depletion with clodronate liposome ($P < .05$; Fig 4, A), whereas blood eosinophils in *Atg7^{fl/fl}* mice with CRS remained unchanged by the same treatment. Histologic analyses revealed that clodronate liposome treatment also significantly decreased epithelial hyperplasia, maximal mucosal thickness, and numbers of eosinophils, but not Ly6G⁺ neutrophils, infiltrating the sinonasal mucosa of *Atg7^{fl/fl};Lyz2-Cre* CRS mice ($P < .01$; Fig 4, B and C, and see Fig E6 in this article's Online Repository at www.jacionline.org). These decreases were less significant in the sinonasal mucosa of *Atg7^{fl/fl}* mice with CRS after treatment with clodronate liposome (Fig 4, B

and C). Mice with CRS treated with control PBS-encapsulated liposomes had ECRS, similar to that noted in untreated CRS mice (Fig 4 and data not shown).

Having observed the contribution of autophagy deficiency to PGD₂ dysregulation, we next examined whether such depletion of autophagy-deficient macrophages could affect the expression of H-PGDS that is linked to eosinophilic inflammation of CRS.⁸ We found that clodronate liposome treatment significantly reduced H-PGDS levels in the sinonasal mucosa of *Atg7^{fl/fl};Lyz2-Cre* mice with CRS ($P < .05$; Fig 5, A). This decrease correlated with the reduced number of infiltrated mast cells in *Atg7^{fl/fl};Lyz2-Cre* mice with CRS ($P < .05$; Fig 5, B). Collectively, our results suggested a protective role of macrophage autophagy in the eosinophilic inflammation seen in patients with CRS through effects on PGD₂ regulation.

ECRS by autophagy deficiency is IL-1 dependent

Finally, we investigated the possible mechanism by which autophagy-deficient macrophages aggravate the eosinophilic inflammation seen in CRS. We focused on inflammatory mediators derived from macrophages and noticed a clear upregulation of *Il18* mRNA in sinonasal tissue of *Atg7^{fl/fl};Lyz2-Cre* mice with CRS (Fig 2, A and B). A significant increase in mRNA levels of IL-1 β and IL-6 but not IL-1 α and TNF- α , as determined by using qRT-PCR, were also observed (see Fig E7 in this article's Online Repository at www.jacionline.org), which is consistent with the enhanced production of IL-1 β and IL-18 by autophagy-deficient macrophages.^{22,38} Supporting this, IL-1 β staining colocalized with F4/80⁺ and CD68⁺ macrophages was significantly increased in *Atg7^{fl/fl};Lyz2-Cre* mice with CRS compared with that in *Atg7^{fl/fl}* mice with CRS, which was diminished by clodronate liposome treatment ($P < .001$, see Fig E8 in this article's Online Repository at www.jacionline.org).

IL-1 β is the cytokine produced primarily by macrophages and monocytes and significantly potentiates the production of PGD₂ and type 2 cytokines by mast cells under inflammatory conditions.^{39,40}

Thus we tested the involvement of IL-1 in the aggravation of eosinophilic inflammation and PGD₂ dysregulation by autophagy deficiency. Mice were administered IL-1 receptor blocking antibody during the development of ECRS. Blockade of IL-1 receptor significantly alleviated eosinophilia in blood and sinonasal mucosa of *Atg7^{fl/fl};Lyz2-Cre* mice with CRS compared with *Atg7^{fl/fl}* mice with CRS (Fig 6, A and B). Epithelial hyperplasia and maximal mucosal thickness were also significantly decreased by IL-1 blockade in *Atg7^{fl/fl};Lyz2-Cre* mice with CRS ($P < .01$; Fig 6, B and C). Furthermore, the same treatment significantly reduced levels of H-PGDS and numbers of mast cells in the sinonasal mucosa of *Atg7^{fl/fl};Lyz2-Cre* mice with CRS (Fig 6, D and E). Collectively, these results suggest IL-1, including IL-1 β , as a potential candidate mediator implicated in PGD₂ dysregulation and eosinophilic inflammation by autophagy deficiency, although we cannot exclude the possible contribution of other mediators.

DISCUSSION

Autophagy has been implicated in the regulation of diverse inflammatory diseases, including severe asthma, whereas its role in the setting of CRS, especially with respect to eosinophilic

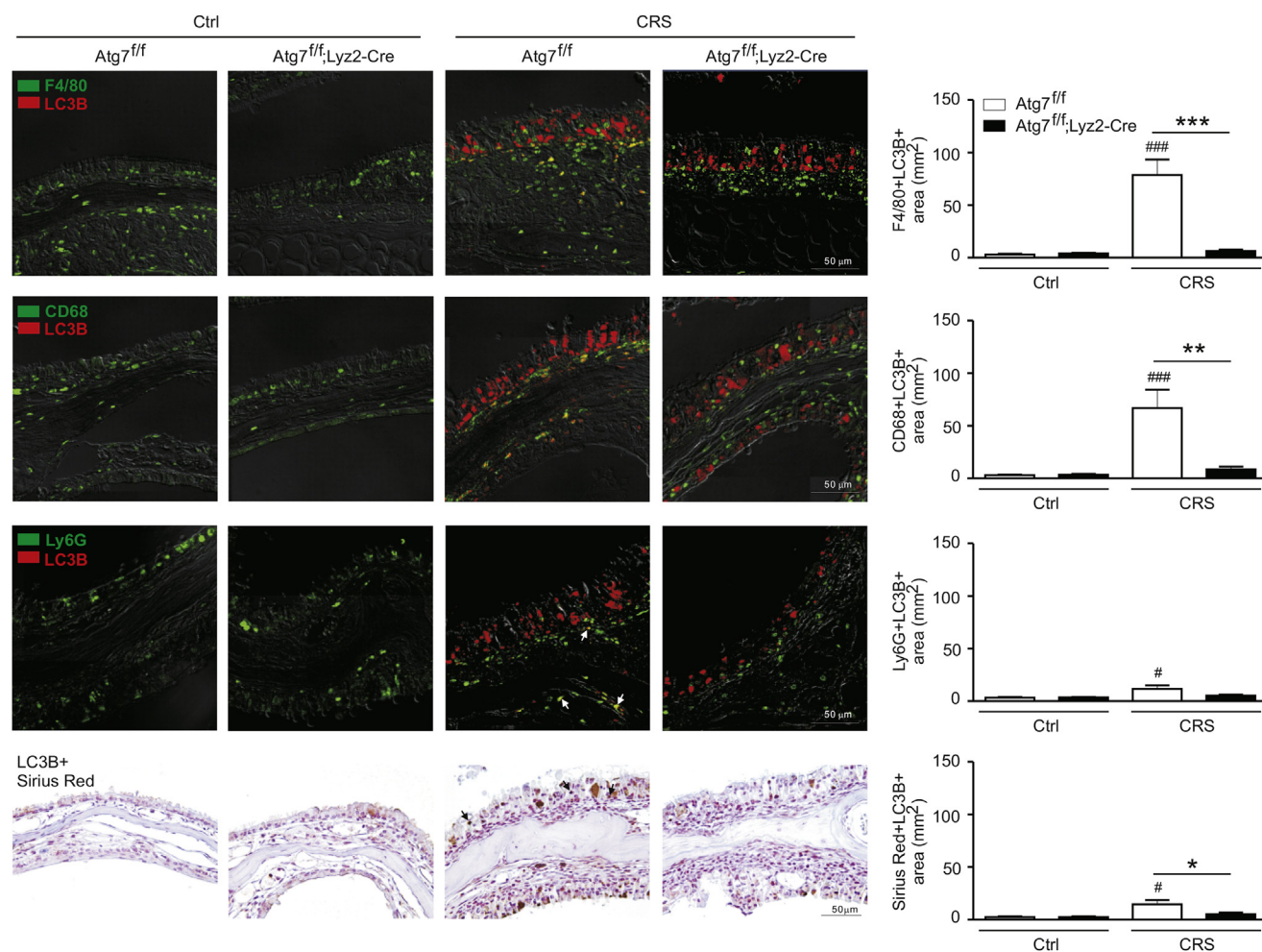


FIG 3. Localization of autophagy deficiency to macrophages among affected myeloid cells. Representative dual-immunofluorescence staining for LC3B and markers of different myeloid cells in the sinonasal tissue for each group of mice is shown. F4/80⁺ (upper) and CD68⁺ (middle) for macrophages and Ly6G⁺ (lower) for neutrophils are shown. Yellow signals indicate colocalization of 2 marker proteins. Representative immunostaining for LC3B and Sirius red counterstaining (bottom) is shown. The statistical bar chart shows the area of colocalization. Arrows indicate colocalization signals for neutrophils and eosinophils. Scale bars = 50 μ m. Data are expressed as means $\pm$ SEMs. * P < .05, ** P < .01, *** P < .001, # P < .05, and ### P < .001, Mann-Whitney U test.

inflammation, is yet to be explored. Recent studies revealed that levels of the autophagosome marker LC3 decreased in NPs of patients with CRS and that it correlates inversely with COX-2 expression,^{20,21} suggesting a potential role of autophagy in CRS pathogenesis. For the first time, our study findings demonstrate that autophagy deficiency in myeloid effector cells, particularly macrophages, aggravates eosinophilic sinonasal inflammation and blood eosinophilia in a murine model of CRS. Importantly, we showed that loss of autophagy is associated with augmented production of PGD₂, which is concomitant with the upregulation of multiple components in the PGD₂ pathway. This dysregulation of the PGD₂ pathway correlated with increased infiltration of mast cells and promoted aggravation of eosinophilic inflammation. Moreover, the findings of alleviation of eosinophilic inflammation and PGD₂ dysregulation through depletion of autophagy-deficient macrophages exhibiting high levels of IL-1 β suggests that macrophage autophagy has an important

role in regulating eosinophilic inflammation and ECRS development.

Eosinophilic inflammation is considered a major pathologic hallmark of CRS^{7,41} and is frequently associated with refractory CRS and recurrence of NPs after medical and surgical intervention.⁴² In patients with ECRS, peripheral blood eosinophilia correlates well with eosinophilic inflammation in the sinonasal mucosa.^{42,43} Considering its intractable features, eosinophilic inflammation is a major therapeutic target in patients with CRS,⁴⁴ as well as those with asthma.⁴⁵

To probe the role of autophagy in eosinophilic inflammation, we used a previously established ECRS mouse model induced by a combination of *Aspergillus* species protease and ovalbumin.^{23,46} This model exhibits blood eosinophilia and chronic eosinophilic sinonasal inflammation, including T_H2 polarization and epithelial hyperplasia. In our current study impaired autophagy in myeloid cells of mice with ECRS led to

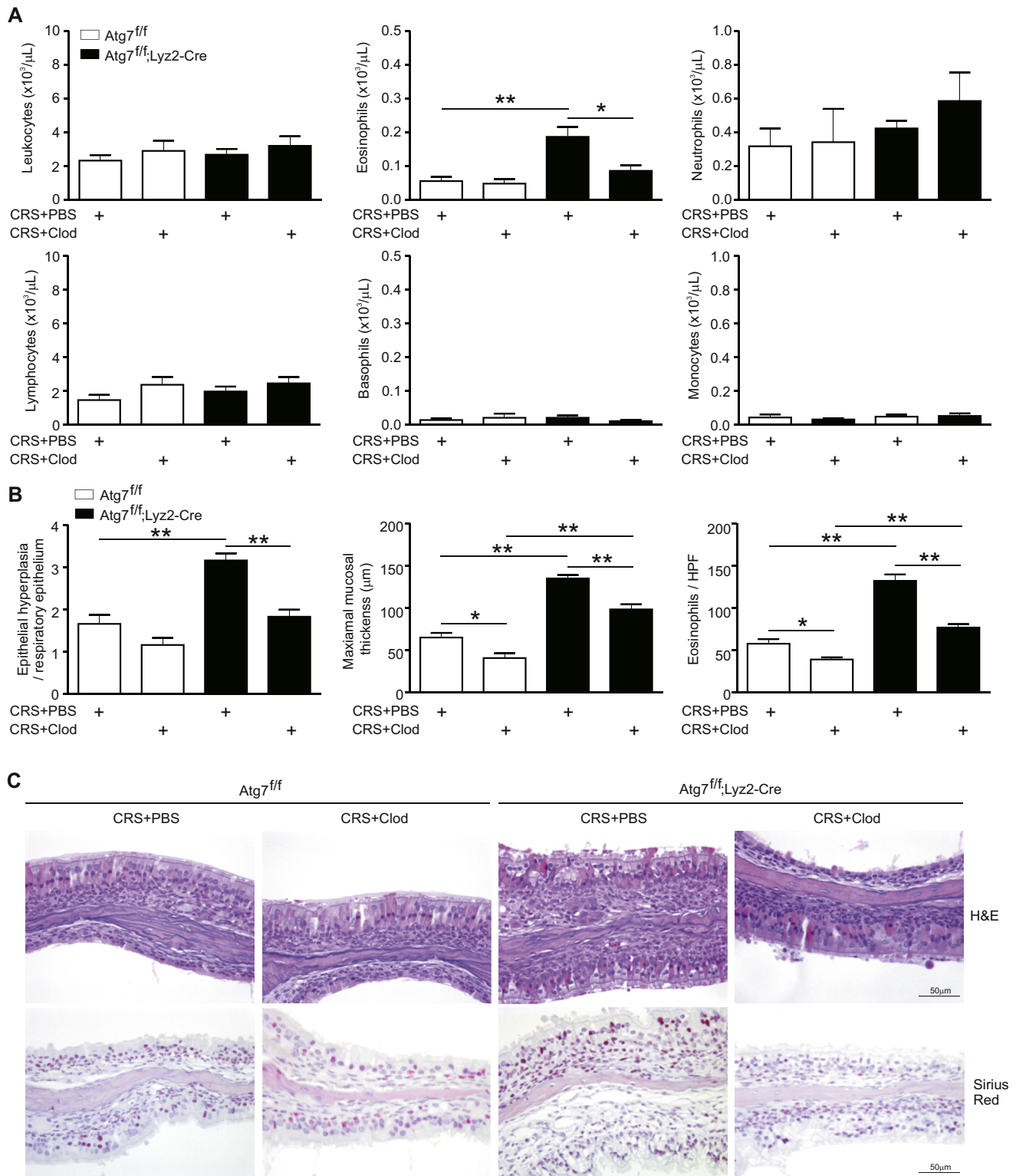


FIG 4. Macrophage autophagy regulates eosinophilic inflammation in mice with ECRS mice. **A**, Effect of macrophage depletion through clodronate liposome (*Clod*) treatment on leukocyte, eosinophil, neutrophil, lymphocyte, basophil, and monocyte counts in blood from each group of mice. **B**, Scores of epithelial hyperplasia (*left*), maximal mucosal thickness (*middle*) in hematoxylin and eosin-stained tissue sections, and eosinophil counts of the lamina propria (*right*) in Sirius red-stained tissue sections. **C**, Representative photographs of hematoxylin and eosin (*H&E*; *upper*)– and Sirius red (*lower*)–stained sections. Scale bars = 50 μm . Data are expressed as means $\pm$ SEMs ($n = 5$ –7 per group). * $P < .05$ and ** $P < .01$, Mann-Whitney U test.

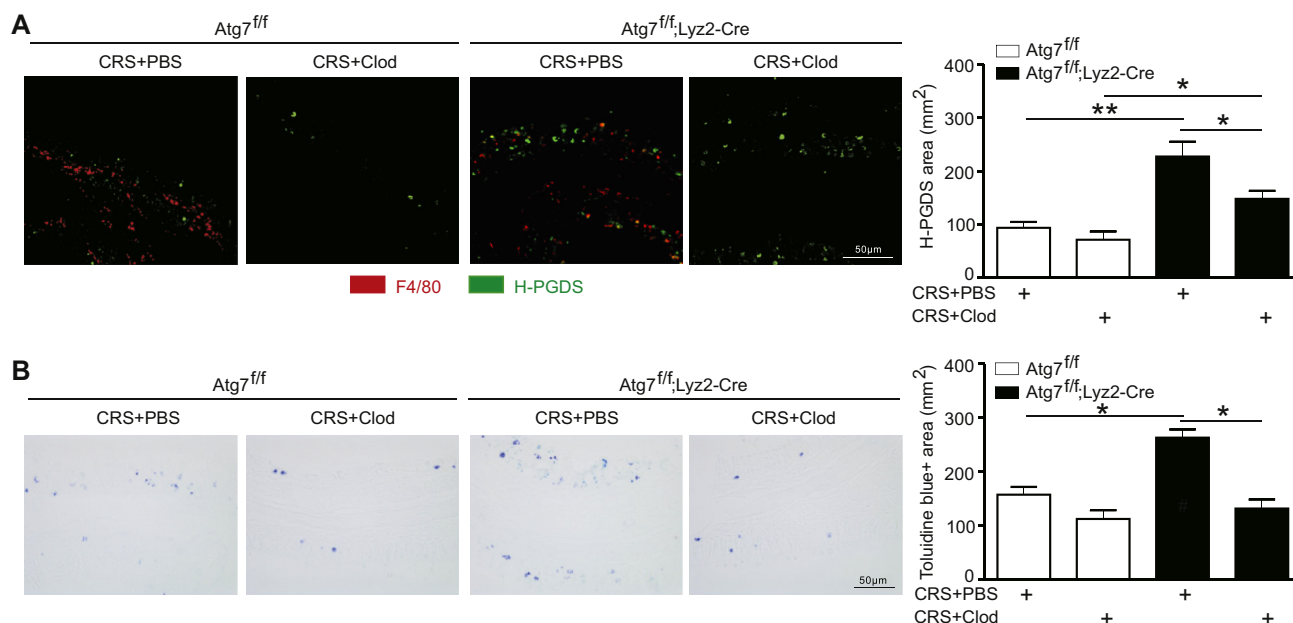


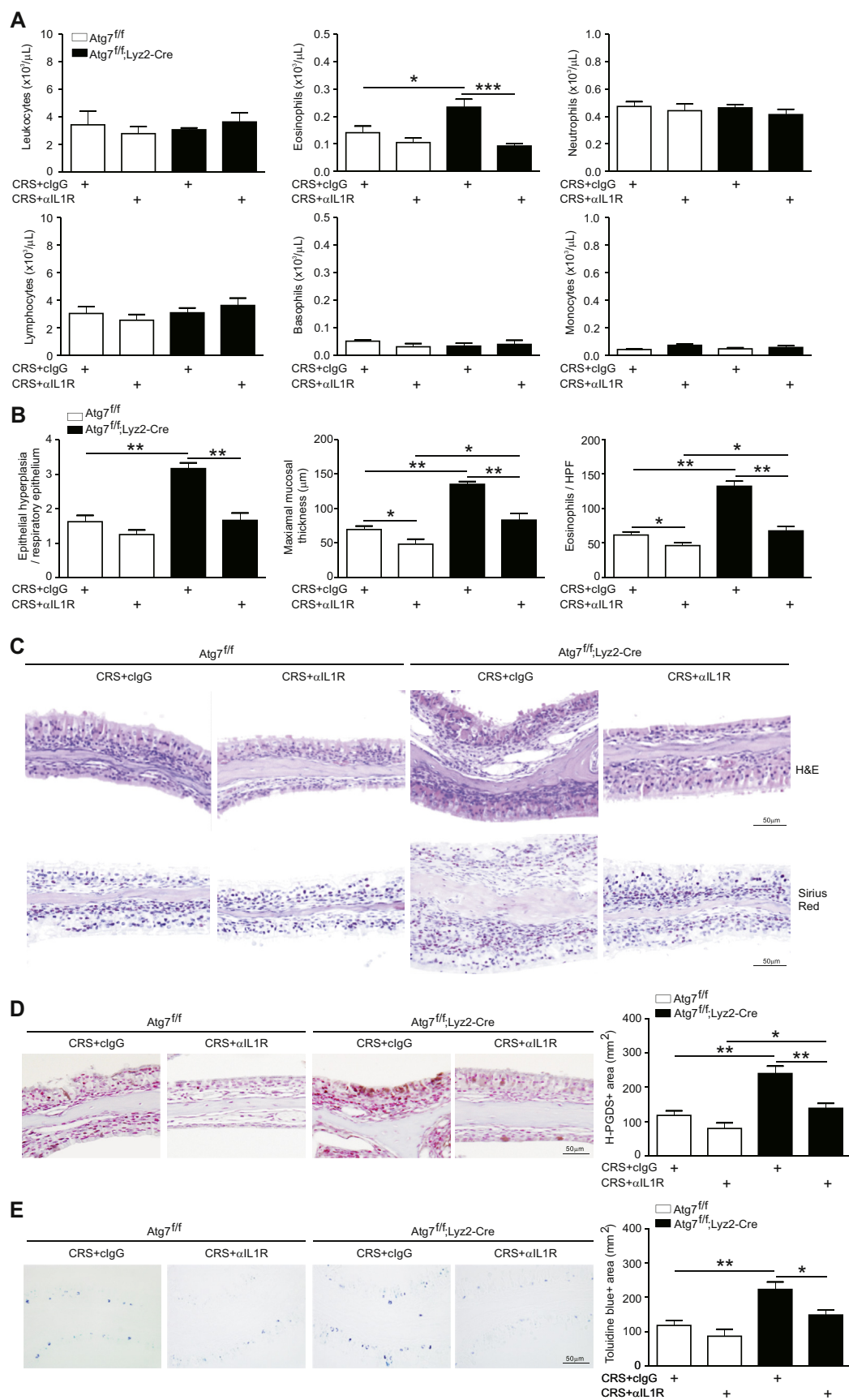
FIG 5. Macrophage autophagy regulates PGD₂ dysregulation in mice with ECRS. **A**, Effect of macrophage depletion by clodronate liposome (*Clod*) treatment on H-PGDS levels. Representative dual-immunofluorescence staining for H-PGDS and F4/80⁺ macrophages in sinonasal tissue for each group of mice. The statistical bar chart shows the H-PGDS-positive area. **B**, Representative photographs of acidic toluidine blue-stained sections and quantitative analysis of mast cell infiltration. The statistical bar chart shows the area of toluidine blue-positive mast cells. Scale bars = 50 μ m. Data are expressed as means $\pm$ SEMs. * P < .05 and ** P < .01, Mann-Whitney U test.

induction of blood eosinophilia and aggravation of eosinophilic sinonasal inflammation and tissue hyperplasia. In support, genes closely related to eosinophils (*Rnase2a*, *Rnase2b*, and *Itga4*) and T_H2 polarization (*Tslp* and *Il1rl1*) were markedly upregulated by the impaired autophagy. Moreover, a large increase was observed for genes encoding multiple cytokines (*Il3*, *Il5*, and *Il13*) and their receptors (*Il3ra*, *Il5ra*, and *Il13ra2*) that promote eosinophil survival and activation, as well as chemokines (*Ccl5*, *Ccl11*, *Ccl24*, and *Ccl26*) involved in the recruitment of CCR3-expressing eosinophils. Collectively, these results imply that impaired autophagy in myeloid cells can provoke pathologic changes in gene expression, which promote chronic eosinophilic inflammation in a murine model of ECRS.

Another important finding in our study is the role of autophagy in mediating effects on PGD₂ regulation. PGD₂, one of the major mast cell-produced PGs, is important in type 2 inflammation, primarily through recruitment of T_H2 cells, eosinophils, and basophils³⁵ and production of type 2 cytokines.⁴⁷ In addition, PGD₂ can promote eosinophilic inflammation through suppression of natural killer cell function and natural killer cell-mediated eosinophil apoptosis.²³ In a murine model of allergic asthma, PGD₂ and PGDS were responsible for triggering pronounced eosinophilic inflammation and T_H2 cytokine release.¹² Furthermore, direct application of PGD₂ induces eosinophilic airway inflammation.⁴⁸ In patients with CRSwNP, levels of mRNA encoding H-PGDS were significantly increased and correlated with the degree of eosinophil infiltration and disease severity.⁸ In

comparison, levels of mRNA encoding PGES and PGE₂ concentrations were downregulated.^{8,49} Of interest, impaired autophagy in myeloid cells of mice with ECRS could recapitulate the results of such an inverse correlation between H-PGDS and PGES levels and their correlation with the tissue eosinophilia seen in patients with CRSwNP. The association of autophagy with PGD₂ regulation is also supported by our findings that impaired autophagy provoked notable upregulation of genes (*Ptgdr2* and *Pparg*) encoding receptors for PGD₂ and its metabolite in addition to a significant increase in PGD₂ production. Moreover, autophagy deficiency was associated with an increased number of mast cells in the sinonasal mucosa of CRS mice. In this respect autophagy can coordinate levels of PGD₂ production and components related to the PGD₂ pathway, the underlying mechanism of which requires further study. Given a significant alleviation of eosinophilia by an antagonist of DP2, our results collectively suggest PGD₂ dysregulation by autophagy deficiency as a potential mechanism that drives eosinophilic inflammation in patients with CRS.

Our results also suggest that autophagy in macrophages among myeloid effector cells has a protective role against the development of ECRS. We identified that depletion of autophagy-deficient macrophages in mice with ECRS results in significant alleviation of blood and sinonasal eosinophilia, along with a reduction in H-PGDS levels and mast cell numbers in the inflamed sinonasal mucosa. Supporting this, blockade of IL-1, including IL-1 β , which was upregulated in autophagy-deficient macrophages, similarly reduced H-PGDS levels, mast cell



numbers, and eosinophilic inflammation associated with autophagy deficiency. Macrophages are major producers of inflammatory cytokines and chemokines and are known to be accumulated in significantly increased numbers in the nasal mucosa of patients with CRSwNP.^{50,51} Compared with eosinophils and mast cells, the role of macrophages in the pathogenesis of ECRS is still unclear. Alteration in macrophage phenotype can occur in the presence of the T_H2 cytokines IL-4 and IL-13, which are abundant in the sinonasal mucosa of patients with ECRS.⁷ These M2 macrophages were reported to be increased in NP tissue and positively correlated with IL-5 levels in the nasal mucosa,⁵¹ implicating their roles in ECRS pathogenesis. M2 macrophages can contribute to the recruitment of polarized T_H2 cells and eosinophils²⁸ and have thus been linked to the persistence of allergic disease and asthma.⁵² Recent studies suggest a potential link between M2 macrophages and eosinophilia in the setting of CRSwNP,^{4,53} in which eosinophil production of CCL23 recruiting macrophages and macrophage release of eotaxins (eg, CCL11 and CCL26) that recruit eosinophils form a positive feedback loop to amplify eosinophilia. Of interest, the loss of autophagy in myeloid cells led to an increase in the expression of genes encoding the T_H2 cytokines IL-4 and IL-13, which can suppress autophagy in macrophages.⁵⁴ Hence we speculate that impaired autophagy in macrophages, as demonstrated here, can occur during the course of ECRS, which in turn aggravates and perpetuates eosinophilic inflammation, possibly through an IL-1–dependent mechanism. In this respect further study will be required to address the exact underlying mechanisms involved.

Of interest is the finding that, in addition to macrophages, other types of myeloid cells, particularly eosinophils, also underwent autophagy in the sinonasal mucosa during ECRS, although not as clear as macrophages. Using cotreatment with LPS and rapamycin as a positive control (see Fig E9, A and B, in this article's Online Repository at www.jacionline.org), we found that a small fraction of eosinophils in the blood of *Atg7^{fl/fl}* CRS mice also showed increased LC3B expression, which was not apparent in autophagy-deficient *Atg7^{fl/fl};Lyz2-Cre* mice with CRS (see Fig E9, C and D). Autophagy was reported to be increased in sputum and blood eosinophils from patients with severe asthma.⁵⁵ Thus alteration in eosinophil autophagy might contribute to ECRS pathogenesis, given its role in counterregulation of eosinophil cytotoxicity,⁵⁶ which is linked to the severity of asthma exacerbation.⁵⁷ In support of this notion, there were still significant differences in eosinophil numbers and mucosal thickness between *Atg7^{fl/fl}* mice with CRS and *Atg7^{fl/fl};Lyz2-Cre* mice with CRS, even after macrophage depletion ($P < .01$; Fig 4, B), suggesting the possible contribution of autophagy deficiency in myeloid cells other than macrophages,

including eosinophils, to ECRS. In this respect further study will be required to dissect the role of autophagy in other types of myeloid cells in patients with ECRS.

Emerging evidence suggests that autophagy functions as an important regulator of immune responses and has multiple effects on inflammation, either proinflammatory or anti-inflammatory.^{14–16} Lack of autophagy in $CD11c^+$ cells, especially dendritic cells, results in severe IL-17–mediated neutrophilic lung inflammation, suggesting the protective role of autophagy in neutrophilic asthma.¹⁹ In our present study loss of autophagy in myeloid cells, particularly macrophages, exacerbated eosinophilic inflammation involving PGD_2 dysregulation, which suggests the protective role of autophagy in patients with ECRS. In contrast, autophagy deficiency in the same myeloid cells reduced neutrophil degranulation and the severity of neutrophil-mediated inflammatory and autoimmune diseases.⁵⁸ These studies indicate that inflammation affected by autophagy appears to be diverse and context dependent, according to the types of immune cells and disease models, thus supporting a diverse role of autophagy in immune cell functions. In addition to its role in inflammation, autophagy can affect T_H cell polarization, partly through control of innate immune cells.¹⁴ Autophagy-deficient myeloid cells, especially macrophages, significantly enhanced levels of the T_H1 cytokine IFN- γ during dextran sulfate sodium–induced colitis⁵⁹ and GalN/LPS-induced liver injury.⁶⁰ Impaired autophagy in myeloid cells also promoted T_H17 responses during *Mycobacterium tuberculosis* infection.⁶¹ In this respect our results of T_H2 cytokine regulation by myeloid autophagy in the context of ECRS suggest a broad effect of autophagy on diverse T_H responses according to the context and disease model involved.

In summary, we demonstrate here a previously unappreciated aspect of the protective effect of autophagy on the pathophysiology of CRS in the context of PGD_2 dysregulation and eosinophilic inflammation. Our results reveal that impaired autophagy provokes eosinophilic and type 2 inflammation in a murine model of ECRS and that this pathologic change largely relies on altered macrophage function. In support of autophagy as a versatile immune modulator, our results suggest further that autophagy plays a significant role in T_H2 -biased eosinophilic inflammation in CRS pathogenesis. Our findings also imply that enhancing the autophagy pathway might be an effective therapeutic strategy for the resolution of eosinophilic inflammation in patients with CRS.

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FIG 6. Macrophage autophagy regulates PGD_2 dysregulation and eosinophilia in an IL-1–dependent mechanism. **A**, Effect of IL-1 blockade through treatment with IL-1 receptor blocking antibody ($\alpha IL1R$) on leukocyte, eosinophil, neutrophil, lymphocyte, basophil, and monocyte counts in blood from each group of mice. **B**, Scores of epithelial hyperplasia (left), maximal mucosal thickness (middle) in hematoxylin and eosin–stained tissue sections, and eosinophil counts of the lamina propria (right) in Sirius red–stained tissue sections. **C**, Representative photographs of hematoxylin and eosin (H&E; upper)– and Sirius red (lower)–stained sections. **D**, Representative immunostaining for H-PGDS and Sirius red counterstaining in the sinonasal tissue from each group of mice. The statistical bar chart shows the H-PGDS–positive area. Scale bars = 50 μm . **E**, Representative photographs of acidic toluidine blue–stained sections and quantitative analysis of mast cell infiltration. The statistical bar chart shows the area of toluidine blue–positive mast cells. Scale bars = 50 μm . Data are expressed as means $\pm$ SEMs ($n = 6$ –8 per group). * $P < .05$ and ** $P < .01$, Mann-Whitney U test.

Key messages

- Autophagy deficiency in myeloid cells aggravates eosinophilic inflammation in a murine model of ECRS.
- Impaired autophagy was associated with dysregulation of IL-1 and the PGD₂ pathway and increased infiltration of mast cells.
- Depletion of autophagy-deficient macrophages led to alleviation of eosinophilic inflammation and PGD₂ dysregulation.

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METHODS

Animal

To generate *Atg7^{eff};Lyz2-Cre* mice with myeloid cell-specific deletion of *Atg7*, a key autophagy gene, we crossed *Lyz2-Cre* mice (stock no. 4781; Jackson Laboratories) with *Atg7^{eff}* mice (kindly provided by Masaaki Komatsu of Niigata University). Littermates were used in all experiments. PCR genotyping was conducted with the following primers: 5'-CCC AGA AAT GCC AGA TTA CG-3' for wild-type, 5'-CTT GGG CTG CCA GAA TTT CTC-3' for both genotypes, and 5'-TTA CAG TCG GCC AGG CTG AC-3' for Cre in *Lyz2-Cre* mice, and 5'-CCA CTG GCC CAT CAG TGA GCA TG-3' for wild-type, 5'-CAT CTT GTA GCA CCT GCT GAC CTG C-3' for common, and 5'-GCG GAT CCT CGT ATA ATG TAT GCT ATA CGA AGT TAT-3' for loxP in *Atg7^{eff}* mice.

Animals were bred at the Laboratory Animal Facility of the Asan Institute for Life Sciences under specific pathogen-free conditions and maintained at a constant ambient temperature ($22^{\circ}\text{C} \pm 1^{\circ}\text{C}$) with a 12-hour/12-hour light/dark cycle. Animals were housed 3 to 5 per cage with *ad libitum* access to food and water. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Asan Institute for Life Science.

Murine CRS model

ECRS was induced by means of intranasal challenge with a mixture of 1 U of protease from *A. oryzae* (Sigma-Aldrich) and OVA (Worthington Biochemicals) diluted in sterile PBS to a total volume of 20 μL 3 times a week for 5 weeks, as previously described, with slight modifications.^{E1} Control mice were intranasally challenged with PBS. To deplete macrophages, mice were administered an intranasal injection of 40 μL of clodronate liposomes (ClodronateLiposomes.com). Control mice were injected with control liposomes (ClodronateLiposomes.com). The first injections of clodronate or control liposomes were performed 1 day before intranasal challenge with protease combined with ovalbumin and then 2 times a week for 5 weeks until death. Mice were injected with anti-IL-1 receptor antibody administered intranasally (50 μg per mouse, JAMA-147; Bio X cell, West Lebanon, NH) and intraperitoneally (300 μg per mouse, JAMA-147) on alternate days in a total of 6 times a week for 5 weeks to block IL-1 receptor. Control mice were injected equally with Armenian hamster IgG (Bio X cell) on the same days. The first intranasal injections of anti-IL-1 receptor antibody or control IgG were performed 2 hours before intranasal challenge with protease combined with OVA. Mice were given an intranasal injection of 24 μL of TM30089 (1 mg/kg; Cayman Chemicals) to block the DP2 receptor. Control mice received vehicle (PBS containing 12% dimethyl sulfoxide). The first injections of TM30089 or vehicle were performed 1 hour before intranasal challenge with protease combined with OVA and then 6 times a week for 5 weeks until death.

Eosinophil cell counts in blood

Blood samples were taken by means of cardiac puncture, and differential cell counting was performed with the ADVIA 2120 Hematology System (Bayer HealthCare, Diagnostics Division, Tarrytown, NY).

Histologic analysis

Nasal tissue sections were stained with hematoxylin and eosin or Sirius red and analyzed by a pathologist from the Department of Pathology, Asan Medical Center, who was blind to the group assignments. Epithelial hyperplasia was scored on a scale of none (0), minimal (1), mild (2), moderate (3), and severe (4). Minimal was defined as barely detectable, mild as slightly detectable, moderate as easily detectable, and severe as very evident. The maximal mucosal thickness was measured at the transition zone of the olfactory and respiratory epithelia by using an image analysis system (CellSens Standard 1.7). Infiltration of eosinophils in the lamina propria was expressed as the number of cells per high-powered field. Slides of consecutive sections were dewaxed, rehydrated, and stained with acidified (0.02% in 0.25% glacial acetic acid) toluidine blue to detect mast cells in sinonasal tissues of mice with CRS.^{E2}

Detection of LC3B expression in eosinophils

Leukocytes were isolated from peripheral blood by means of centrifugation and lysis of red blood cells. Thereafter, cells (1×10^6 cells) were incubated with Fc γ receptor-binding inhibitor (eBioscience, San Diego, Calif) for 20 minutes at 4°C and stained for surface markers with anti-Siglec-F-phycoerythrin and anti-CCR3-fluorescein isothiocyanate antibodies for 1 hour in the dark at 4°C .^{E3} Cells were incubated with BD Cytotfix/Cytoperm solution (BD Biosciences, San Jose, Calif) for 20 minutes at 4°C to determine LC3B expression. Cells were incubated with anti-LC3B antibody (Novus Biologicals, Littleton, Colo) for 30 minutes at 4°C and then Alexa Fluor 647-conjugated goat anti-rabbit F(ab')₂ (Jackson ImmunoResearch, West Grove, Pa) for 30 minutes at 4°C . Before and after intracellular staining for LC3B, cells were washed twice with BD Perm/Wash buffer (BD Bioscience). LC3B expression was then analyzed by means of flow cytometry gated on Siglec-F⁺ side scatter-high or Siglec-F⁺CCR3⁺ cells. As a positive control, mice underwent intraperitoneal administration of a combination of LPS (5 mg/kg) and rapamycin (1.5 mg/kg) in a total volume of 400 μL for 4 hours.

Immunofluorescence and confocal analysis

To detect macrophages or neutrophils in the sinonasal tissues of mice with CRS, slides were incubated with primary antibodies against F4/80 (1:100, CI:A3-1; AbD Serotec, Oxford, United Kingdom), CD68 (1:100, FA-11; AbD Serotec), Ly6G (1:100, 1A8; BD Biosciences), IL-1 β (1:100, 3A6; Cell Signaling, Danvers, Mass), and against LC3B (1:250; Novus Biologicals) for 1 hour, followed by Alexa Fluor 488-conjugated goat anti-rat F(ab')₂ (1:250; Jackson ImmunoResearch) or Alexa Fluor 488-conjugated goat anti-mouse F(ab')₂ (1:250; Jackson ImmunoResearch) and Alexa Fluor 647-conjugated goat anti-rabbit F(ab')₂ (1:250; Jackson ImmunoResearch) for 30 minutes in PBS containing 1% BSA and 1% goat serum. All incubations were performed under coverslips at room temperature, followed by 3 washes with PBS. Coverslips were mounted with ProLong Gold anti-fade reagent (Molecular Probes, Eugene, Ore). Cells were imaged with an LSM 710 laser-scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). Colocalization of LC3B with eosinophils was also detected by using a fluorescent property of Sirius red. LC3B expression was localized by means of immunofluorescence staining with Alexa Fluor 647-conjugated goat anti-rabbit F(ab')₂ (1:250; Jackson ImmunoResearch). The excitation and emission wavelengths were 543 and 633 nm for Sirius red staining and 633 and 647 nm for LC3B staining, as previously described.^{E4}

Immunohistochemistry

To detect LC3B on eosinophil counts or H-PGDS levels in the sinonasal tissues of mice with CRS, slides were first stained with Sirius red to identify eosinophils and incubated with primary antibodies against LC3B (1:200; Novus Biologicals) or H-PGDS (1:100; Cayman Chemicals) for 1 hour, respectively, and stained with Vectastain ABC kits (peroxidase, rabbit IgG; Vector Laboratories, Burlingame, Calif) according to the manufacturer's instructions. The diaminobenzidine (DAB) signal was observed by using the DAB substrate kit (Roche Diagnostics, Mannheim, Germany).

Image quantification

Slides after immunohistochemical staining were scanned with the Vectra slide scanner (PerkinElmer, Waltham, Mass) to identify DAB staining, and the spectral library of hematoxylin or Sirius red was created. Then inForm image analysis software (PerkinElmer) was applied to quantify the spectra in the sinonasal tissues. Fluorescence was analyzed with ImageJ software (National Institutes of Health, Bethesda, Md) in digital fluorescence microscopic images in a total of 6 randomly chosen fields ($420 \times 420 \mu\text{m}$). Based on analysis of pixel fluorescence intensities, which ranged from 0 to 255, the signal for F4/80, CD68, Ly6G, IL-1 β , or LC3B was distinguished from background by empirically counting only those pixels above a threshold value, thus maximizing the inclusion of only those pixels with specific antigen staining. The area density was calculated as the percentage of total pixels with a fluorescence intensity value equal to or greater than the threshold.

qRT-PCR and RT² Profiler PCR Arrays

The skull was bisected from the snout and septal and turbinate mucosae were removed and placed immediately in RNAlater solution (Invitrogen) to harvest nasal tissue for RNA extraction. All harvested sinonasal tissues were included in RNA extraction. RNA was isolated from nasal tissue by using the RNeasy Microarray Tissue Mini Kit (Qiagen). qRT-PCR was used to quantify the amounts of mRNA in the nasal tissue by using mouse Allergy and Asthma RT² Profiler PCR Arrays (Qiagen/SA Biosciences). cDNA was synthesized from 2 µg of total RNA by using SuperScript III reverse transcriptase (Invitrogen). Thereafter, RT-PCR was performed with the SuperArray Master Mix (Qiagen) and a Roche LightCycler 480 instrument, according to the manufacturer's instructions. Experiments were performed on RNA pooled from 4 to 5 individual mice per group of mice. cDNA was synthesized from 1 µg of total RNA by using the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan), according to the manufacturer's instructions, to examine the mRNA levels of H-PGDS, mPGES-1, and COX-2. The following PCR primers were used: 5'-TGG GAA GAC AGC GTT GGA G-3' (forward) and 5'-AGG CGA GGT GCT TGA TGT G-3' (reverse) for mouse H-PGDS; 5'-CTG CTG GTC ATC AAG ATG TAC G-3' (forward) and 5'-CCC AGG TAG GCC ACG TGT GT-3' (reverse) for mouse mPGES-1; 5'-GCA TTC TTT GCC CAG CAC TT-3' (forward) and 5'-AGA CCA GGC ACC GAC CAA AGA-3' (reverse) for mouse COX-2; 5'-GCA ACG GGA AGA TTC TGA AG-3' (forward) and 5'-TGA CAA ACT TCT GCC TGA CG-3' (reverse) for mouse IL-1α; 5'-GAA TGA CCT GTT CTT TGA AGT-3' (forward) and 5'-TTT GTT CAT CTC GGA GCC-3' (reverse) for mouse IL-1β; 5'-TGG AGT CAC AGA AGG AGT GGC TAA G-3' (forward) and 5'-TCT GAC CAC AGT GAG GAA TGT CCA C-3' (reverse) for mouse IL-6; 5'-CCT GTA GCC CAC GTC GTA GC-3' (forward) and 5'-TTG ACC TCA GCG CTG AGT TG-3' (reverse) for mouse TNF-α; and 5'-AGT ATG ATG ACA TCA AGA AGG-3' (forward) and 5'-ATG GTA TTC AAG AGA GTA GGG-3' (reverse) for mouse GAPDH. To analyze the effect of *Pparg*, mouse peritoneal macrophages were stimulated with LPS (100 ng/mL; InvivoGen, San Diego, Calif) for 24 hours in the absence or presence of rosiglitazone (10 µmol/L; Sigma-Aldrich). The relative mRNA levels of H-PGDS and mPGES-1 were determined by using qRT-PCR and normalized to *Gapdh* mRNA.

Zymosan-induced peritonitis

Acute peritoneal inflammation was elicited in mice with an intraperitoneal injection of 0.5 mL of zymosan (2 mg/mL; Sigma-Aldrich). Six hours after injection, animals were killed, and the peritoneal cavity was washed with 2 mL of Dulbecco modified Eagle medium (Gibco, Carlsbad, Calif). Supernatants obtained from peritoneal lavage fluid was centrifuged at 300g for 5 minutes at 4°C and placed immediately in 10 µmol/L indomethacin (Cayman Chemicals).

Sample preparation for PGD₂

PGD₂ was extracted from 1 mL of medium by using SPE. A 60-mg Oasis HLB (Waters, Milford, Mass) SPE cartridge was washed and

preconditioned with ethyl acetate, methanol, and 0.1% acetic acid/5% methanol in H₂O, sequentially. Ten microliters of 0.2 mg/mL EDTA and butylated hydroxytoluene in methanol/H₂O (50:50) was added to the sorbent bed of SPE column. PGD₂-d4 was also added to samples as an internal standard. Sample solutions were added into the SPE column, washed with 2 column volumes of 0.1% acetic acid and 5% methanol in H₂O, and then dried under a vacuum. Finally, PGD₂ was eluted with 0.5 mL of methanol, followed by 1.5 mL of ethyl acetate. Sample solutions were dried with a vacuum centrifuge and then stored at -20°C until analysis. The desiccated material was reconstituted with 40 µL of 50% acetonitrile before LC-MS/MS analysis.

LC-MS/MS analysis for PGD₂

An LC-MS/MS system equipped with a 1290 HPLC (Agilent Technologies), Qtrap 5500 (ABSciex), and a reverse phase column (Pursuit 5, 200 × 2.0 mm) was used. The separation gradient for PGD₂ analysis used mobile phase A (0.1% acetic acid in H₂O) and mobile phase B (0.1% acetic acid in acetonitrile/methanol (84/16 vol/vol) and proceeded at 300 µL/min and 25°C. The separation gradient was as follows: 40% to 60% of B for 5 minutes, 60% to 100% of B for 0.1 minutes, hold at 100% of B for 4.9 minutes, 100% to 40% of B for 0.1 minutes, and then hold at 40% of A for 2.9 minutes. The multiple reaction monitoring mode was used in the negative ion mode, and the extracted ion chromatogram corresponding to the specific transition of each analyte was used for quantification (Q1/Q3 = 351/271 and 351/315, RT = 4.66 minutes for PGD₂; Q1/Q3 = 355/275 and 355/319, RT = 4.66 minutes for PGD₂-d4). The calibration range for each analyte was 0.1 to 1000 nmol/L ($r^2 \geq 0.99$).

Statistical analysis

All data were analyzed with GraphPad Prism software (version 4.00). Groups were compared by using nonparametric Mann-Whitney *U* tests. Statistical significance was defined as a *P* value of less than .05.

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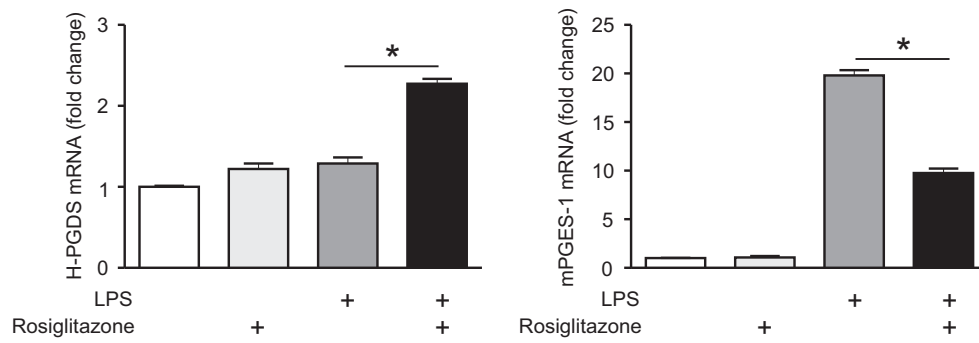


FIG E1. Effect of rosiglitazone on H-PGDS and mPGES-1 expression. Macrophages were incubated with LPS (100 ng/mL) for 24 hours in the absence or presence of rosiglitazone (10 μ mol/L). Relative mRNA levels corresponding to the indicated proteins were determined by using qRT-PCR and normalized to *GAPDH* mRNA. Data are expressed as means $\pm$ SEMs. * $P < .05$, Mann-Whitney U test.

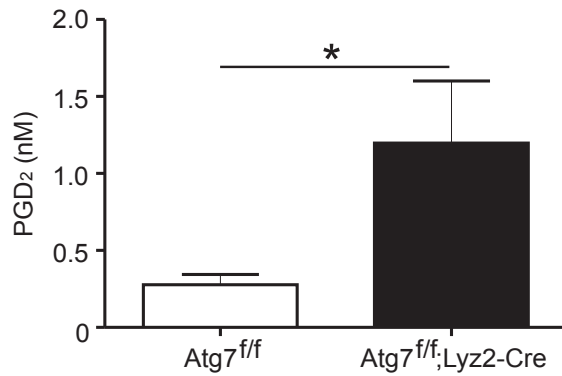


FIG E2. Myeloid autophagy regulates PGD₂ production. *Atg7^{fl/fl}* mice and *Atg7^{fl/fl};Lyz2-Cre* mice were challenged with zymosan to induce acute peritoneal inflammation. Levels of PGD₂ in peritoneal exudates from control *Atg7^{fl/fl}* mice and *Atg7^{fl/fl};Lyz2-Cre* mice were determined by using LC-MS/MS. Data are expressed as means ± SEMs (n = 4-5 per group). **P* < .05, Mann-Whitney *U* test.

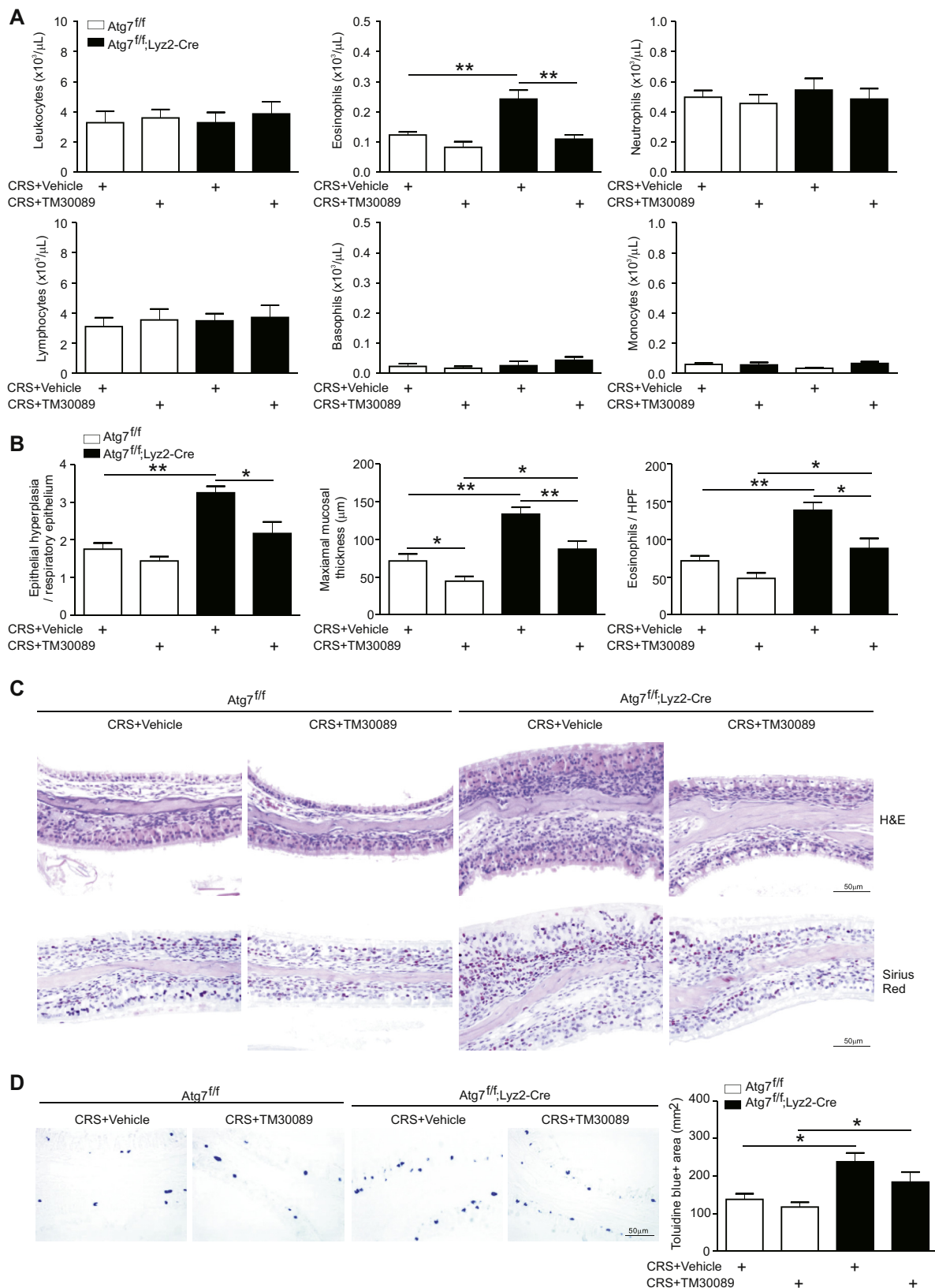


FIG E3. DP2 mediates eosinophilic inflammation by autophagy deficiency. **A**, Effect of DP2 blockade through treatment with an antagonist of DP2 (TM30089) on leukocyte, eosinophil, neutrophil, lymphocyte, basophil, and monocyte counts in blood from each group of mice. **B**, Scores of epithelial hyperplasia (left), maximal mucosal thickness (middle) in hematoxylin and eosin–stained tissue sections, and eosinophil counts of the lamina propria (right) in Sirius red–stained tissue sections. **C**, Representative photographs of hematoxylin and eosin (H&E; upper)– and Sirius red (lower)–stained sections. **D**, Representative photographs of acidic toluidine blue–stained sections and quantitative analysis of mast cell infiltration. The statistical bar chart shows the area of toluidine blue–positive mast cells. Scale bars = 50 μ m. Data are expressed as means $\pm$ SEMs ($n = 5$ –7 per group). * $P < .05$ and ** $P < .01$, Mann-Whitney U test.

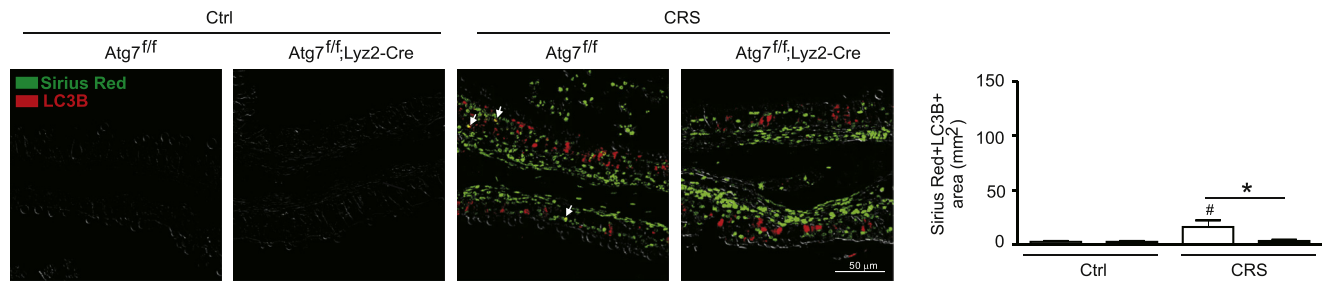


FIG E4. Colocalization of LC3B with Sirius red–positive eosinophils. Representative immunofluorescence staining for LC3B and Sirius red counterstaining in sinonasal tissue for each group of mice. The statistical bar chart shows the area of colocalization. *Scale bars* = 50 μ m. Data are expressed as means $\pm$ SEMs. * P < .05 and # P < .05, Mann-Whitney U test.

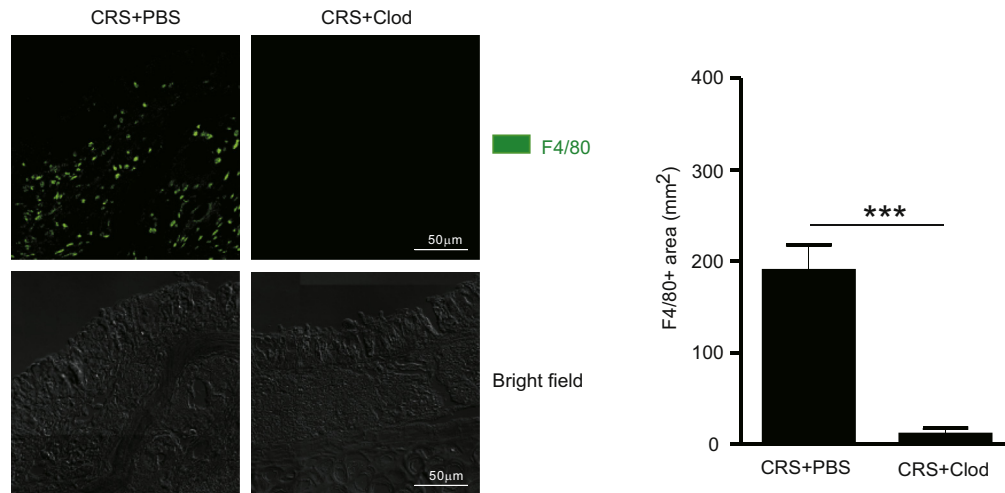


FIG E5. Macrophage depletion with clodronate liposome (*Clod*) treatment. Representative immunofluorescence staining for F4/80⁺ macrophages in the sinonasal tissue from mice with CRS treated with control PBS-encapsulated liposomes (PBS) or clodronate liposome is shown. The statistical bar chart shows the area of F4/80⁺ macrophages. Scale bars = 50 μm. Data are expressed as means ± SEMs. *** $P < .001$, Mann-Whitney U test.

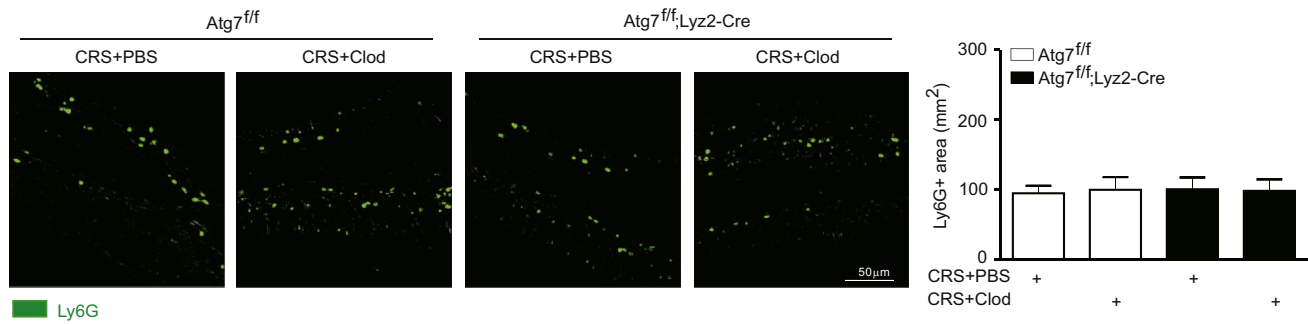


FIG E6. Effect of clodronate liposome (*Clod*) treatment on neutrophils. Representative immunofluorescence staining for Ly6G⁺ neutrophils in sinonasal tissue from mice with CRS treated with control PBS-encapsulated liposomes (PBS) or clodronate liposome is shown. The statistical bar chart shows the area of Ly6G⁺ neutrophils. *Scale bars* = 50 μm. Data are expressed as means ± SEMs.

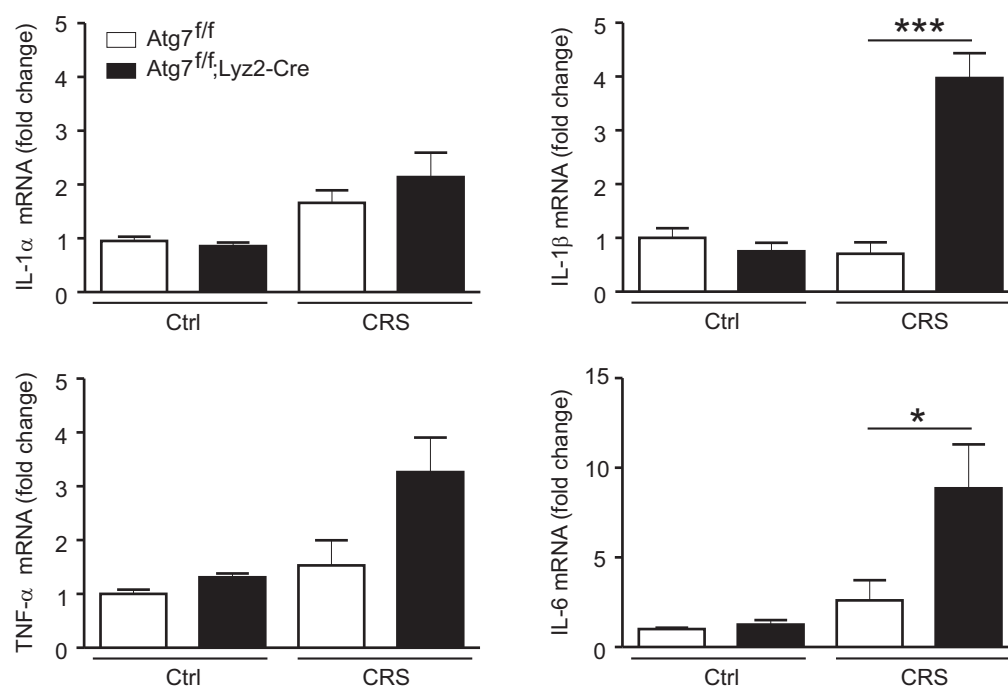


FIG E7. Cytokine expression in sinonasal tissue of mice with CRS. Relative mRNA levels corresponding to the indicated proteins were determined by using qRT-PCR and normalized to *GAPDH* mRNA. Data are expressed as means $\pm$ SEMs. * $P < .05$ and *** $P < .001$, Mann-Whitney U test.

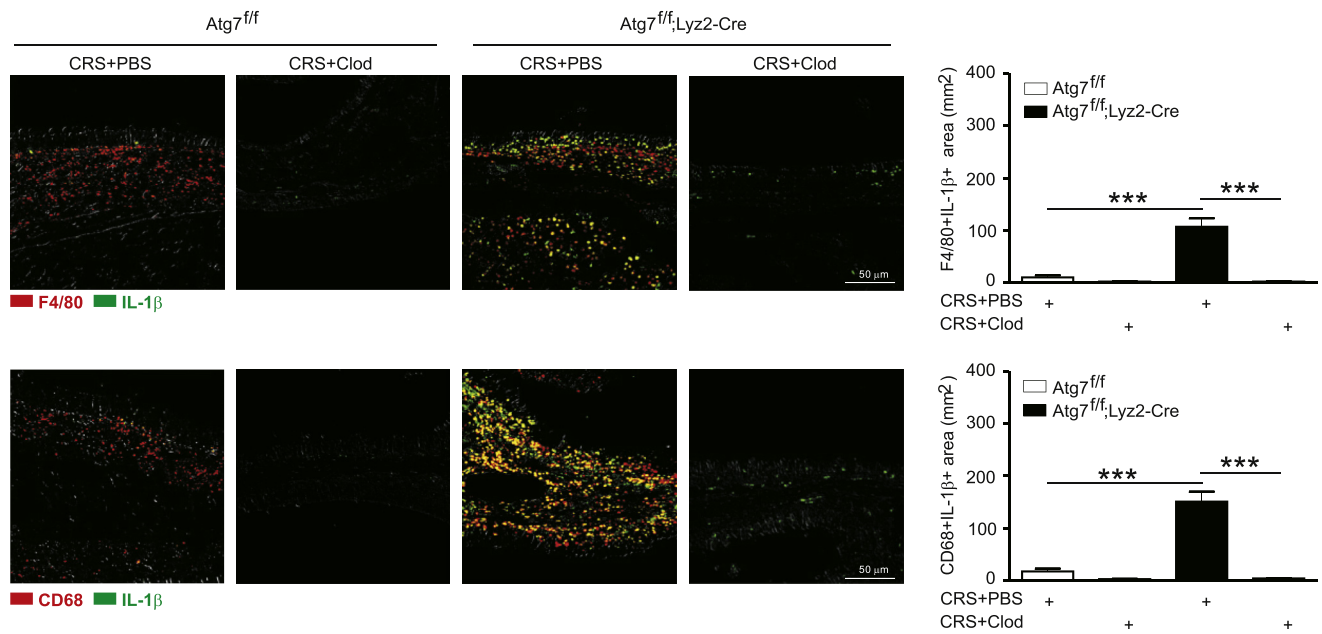


FIG E8. Colocalization of IL-1 β with autophagy-deficient macrophages. Representative dual-immunofluorescence staining for IL-1 β and markers of macrophages in sinonasal tissue from each group of mice is shown. F4/80⁺ (*upper*) and CD68⁺ (*lower*) are shown for macrophages. Yellow signals indicate colocalization of 2 marker proteins. The statistical bar chart shows the area of colocalization. *** $P < .001$, Mann-Whitney U test.

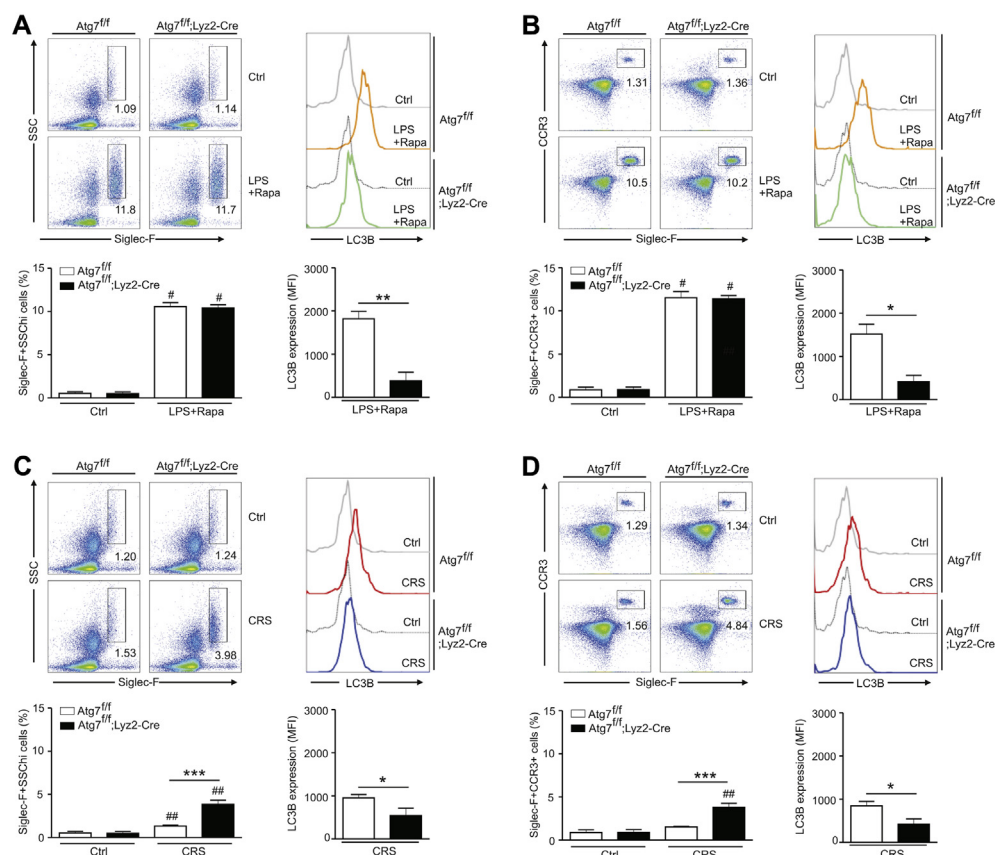


FIG E9. Increased expression of LC3B in eosinophils of mice with CRS. The presence of eosinophils was determined by analyzing Siglec-F expression on side scatter (SSC)^{hi} (A and C) and on CCR3⁺ (B and D) cells in peripheral blood from mice cotreated with LPS and rapamycin (Fig E9, A and B) and mice with CRS (Fig E9, C and D). Representative fluorescence-activated cell sorting profiles (upper left) and statistical bar charts (lower left) showing the percentages of Siglec-F⁺SSC^{hi} or Siglec-F⁺CCR3⁺ eosinophils. Representative fluorescence-activated cell sorting profiles (upper right) and statistical bar charts (lower right) show intracellular expression of LC3B in Siglec-F⁺SSC^{hi} or Siglec-F⁺CCR3⁺ eosinophils. Data are expressed as means $\pm$ SEMs. * $P < .05$, ** $P < .01$, *** $P < .001$, # $P < .05$, and ## $P < .01$, Mann-Whitney U test.

TABLE E1. Genes changed more than 2-fold in sinonasal tissue of *Atg7^{fl/fl};Lyz2-Cre* mice with CRS versus *Atg7^{fl/fl}* mice with CRS

Position	Gene name	Encoded protein	Fold change	RT ² catalog
A04	<i>Areg</i>	Amphiregulin	3.2134	PPM02976E
A05	<i>Arg1</i>	Arginase 1	3.3978	PPM31770C
A07	<i>Ccl11</i>	C-C motif chemokine 11 (eotaxin-1)	2.8697	PPM02967G
A08	<i>Ccl12</i>	C-C motif chemokine 12 (MCP-5)	3.5170	PPM02977E
A11	<i>Ccl24</i>	C-C motif chemokine 24 (eotaxin-2)	2.3229	PPM03159F
A12	<i>Ccl26</i>	C-C motif chemokine 26 (eotaxin-3)	4.1159	PPM59110E
B01	<i>Ccl4</i>	C-C motif chemokine 4 (MIP-1 β)	4.7147	PPM02948F
B02	<i>Ccl5</i>	C-C motif chemokine 5 (RANTES)	2.6723	PPM02960F
B04	<i>Ccr4</i>	C-C motif chemokine receptor 4	4.3991	PPM03147A
B05	<i>Ccr8</i>	C-C motif chemokine receptor 8	7.7021	PPM02975A
B06	<i>Cd40lg</i>	CD40 ligand	2.1563	PPM03226C
B07	<i>Chil1</i>	Chitinase 3-like 1	4.9533	PPM28528C
B10	<i>Cma1</i>	Chymase	5.6502	PPM24635B
B11	<i>Cpa3</i>	Mast cell carboxypeptidase A	2.7524	PPM24623A
C02	<i>Csf3r</i>	Colony-stimulating factor 3 receptor	2.1016	PPM03734A
C04	<i>Rnase2a</i>	Ribonuclease, Rnase A family, 2A	13.1152	PPM31749A
C05	<i>Rnase2b</i>	Ribonuclease, Rnase A family, 2B	17.9054	PPM60751A
C08	<i>Fcer1a</i>	Fc fragment of IgE receptor 1a	3.6024	PPM04613F
C09	<i>Foxp3</i>	Forkhead box P3	3.6353	PPM05497F
C11	<i>Ptgsdr2</i>	PGD ₂ receptor 2	4.1159	PPM04864A
C12	<i>Icos</i>	Inducible T-cell costimulator	10.3656	PPM03618B
D01	<i>Ifng</i>	IFN- γ	3.1356	PPM03121A
D03	<i>Il10</i>	IL-10	3.2930	PPM03017C
D05	<i>Il12b</i>	IL-12 subunit β	4.1159	PPM03020E
D06	<i>Il13</i>	IL-13	5.0613	PPM03021B
D08	<i>Il13ra2</i>	IL-13 receptor subunit α -2	6.4466	PPM03556C
D09	<i>Il17a</i>	IL-17A	4.1159	PPM03023A
D11	<i>Il18</i>	IL-18	3.2240	PPM03112B
D12	<i>Il1rl1</i>	IL-1 receptor–like 1 (ST2)	120.1666	PPM03546A
E01	<i>Il21</i>	IL-21	4.1159	PPM03761F
E03	<i>Il2ra</i>	IL-2 receptor subunit α	2.3353	PPM03125C
E04	<i>Il3</i>	IL-3	4.1159	PPM03012F
E06	<i>Il3ra</i>	IL-3 receptor subunit α	4.1159	PPM03128E
E07	<i>Il4</i>	IL-4	2.5161	PPM03013F
E09	<i>Il5</i>	IL-5	2.3541	PPM03014F
E10	<i>Il5ra</i>	IL-5 receptor subunit α	3.6980	PPM03026F
E12	<i>Itga4</i>	Integrin subunit α 4 (CD49d)	176.1503	PPM03242F
F03	<i>Ltb4r1</i>	Leukotriene B ₄ receptor 1	2.6334	PPM04902A
F06	<i>Mrc1</i>	Macrophage mannose receptor 1 (CD206)	2.3394	PPM24735A
F07	<i>Ms4a2</i>	Membrane spanning 4-domains A2	2.0952	PPM60291B
F10	<i>Pmch</i>	Promelanin concentrating hormone	2.4734	PPM63316F
F12	<i>Pparg</i>	Peroxisome proliferator–activated receptor γ	320.5331	PPM05108C
G07	<i>Tbx21</i>	T-box 21	4.1159	PPM03727A
G10	<i>Tnfsf4</i>	OX40 ligand	3.1539	PPM03240A
G12	<i>Tslp</i>	Thymic stromal lymphopoietin	215.4976	PPM31540A
A10	<i>Ccl22</i>	C-C motif chemokine 22	–2.1772	PPM02950B
B09	<i>Clca1</i>	Chloride channel accessory 1	–9.2097	PPM04053A
B12	<i>Crlf2</i>	Cytokine receptor–like factor 2	–2.3691	PPM28360E
D10	<i>Il17rb</i>	IL-17 receptor B	–2.4196	PPM03757A
E11	<i>Il9</i>	IL-9	–4.7851	PPM03110A