Airway epithelial regeneration requires autophagy and glucose metabolism

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Abstract
Efficient repair of injured epithelium by airway progenitor cells could prevent acute inflammation from progressing into chronic phase in lung. Here, we used small molecules, genetic loss-of-function, organoid cultures, and in vivo lung-injury models to show that autophagy is essential for maintaining the pool of airway stem-like vClub cells by promoting their proliferation during ovalbumin-induced acute inflammation. Mechanistically, impaired autophagy disrupted glucose uptake in vClub progenitor cells, and either reduced accessibility to glucose or partial inhibition of glycolysis promoted the proliferative capacity of vClub progenitor cells and their daughter Club cells. However, glucose deprivation or glycolysis blockade abrogated the proliferative capacity of airway vClub cells and Club cells but promoted ciliated and goblet cell differentiation. Deficiency of glucose transporter-1 suppressed the proliferative capacity of airway progenitor cells after ovalbumin challenge. These findings suggested that autophagy and glucose metabolism are essential for the maintenance of airway epithelium at steady state and during allergic inflammation.

Introduction
In response to inhaled allergens, acute inflammation is initiated as a host defense; however, asthma develops when early acute inflammation progresses to the chronic and unremitting phase. Asthma is a hard-to-cure disease of the conducting airways that is characterized by airway epithelial damage and remodeling, goblet cell metaplasia, and an influx of eosinophils and neutrophils1,2. Tackling the disease during the early acute phase of inflammation represents a potentially promising strategy for curing patients by restoring lung homeostasis.

Airway epithelium plays a critical role in maintaining lung homeostasis3. In the previous two decades, much progress has been made on the characterization and functional identification of progenitor cells responsible for airway epithelial repair after injuries, with these findings attributed to the establishment of lineage-tracing transgenic mice and in vitro three-dimensional (3D)-organoid culture models4,5. Region-specific epithelial progenitor cells reside in the adult mouse airways4,6. As progenitor cells, Club cells (previously known as Clara cells) that express secretoglobin family member 1a (Scgb1a1) and cytochrome P450, family 2, subfamily f, polypeptide 2 (Cyp2f2) are capable of both proliferating and generating differentiated ciliated cells and goblet cells7,8. When loss of Club cells occurs (e.g., following naphthalene-induced lung injury), variant Club (vClub, previously called vClara) cells that also express Scgb1a1 in small airways are able to divide and replenish Club cells8,9. However, the mechanisms underlying the regulation of proliferation and differentiation of these two epithelial progenitor populations at steady state or during asthmatic inflammation are poorly understood.

Autophagy is a protective lysosome-dependent degradation process of cytoplasmic proteins and organelles by autophagosomes assembled by a series of autophagy-related (Atg) genes10. This process is activated in response...
to stress conditions, such as the deprivation of nutrients, including amino acids and glucose. AMP activated protein kinase (AMPK) has been shown to trigger autophagy in response to glucose starvation. Conversely, autophagy can be abrogated by the mammalian target of rapamycin (mTOR), a central regulator that integrates growth factors and nutrients. Reciprocally, autophagy promotes glucose uptake through glucose transporter-1 (Glut1). In adult stem/progenitor cells, autophagic activity changes during their differentiation. Loss of Atg7 by genetic targeting causes the senescence of satellite cells, which are considered as muscle stem cells, and inhibition of autophagy significantly blocks the proliferation and differentiation of epidermal stem cells, hematopoietic stem cells, and dermal stem cells. Therefore, autophagy appears to play an essential role in maintaining tissue homeostasis; however, it remains unexplored whether epithelial regeneration in conducting airways at steady state or during early acute allergic inflammation is governed by autophagy.

Here, we used immunofluorescence, flow cytometry, genetic loss-of-function experiments, and organoid cultures to investigate the regulation of airway epithelial progenitor cells by autophagy and glucose. We found that autophagy maintained the pool of vClub cells in mouse conducting airways and facilitated glucose uptake in vClub cells. In addition, we demonstrated that glucose withdrawal, glycolysis blockade, or Glut1 deletion significantly blocked the proliferation of both Club cells and vClub cells, and promoted the differentiation of Club cells into ciliated cells and goblet cells. Furthermore, the levels of autophagy were altered in Club cells and vClub cells during ovalbumin (OVA)-induced acute allergic conditions. These data suggested that autophagy maintains the airway progenitor cell pool by sustaining glucose at a moderate level. Alternation in the levels of autophagy and glucose in airway progenitor cells may not be favorable for the resolution of inflammation associated with airway epithelial damage in chronic phase of asthma.

Results

Autophagy maintains the pool of vClub progenitor cells during OVA-induced acute inflammation

We previously found that mouse airway vClub cells are characterized by epithelial cell-adhesion molecule (EpCAM) \(^ {+} \) CD31 \(^ {−} \) CD34 \(^ {−} \) CD45 \(^ {−} \) stem cell antigen (Sca)-1 \(^ {−} \) CD24low by fluorescence-associated cell sorting (FACS) \(^ {4} \) (Fig. 1a). Club cells express high levels of Sca-1 as compared

![Fig. 1 Autophagy maintains the pool of airway vClub progenitor cells during OVA-induced acute inflammation. a Sorting strategy of airway epithelial CD24lowSca-1− vClub cells (left) and CD24lowSca-1+ Club cells (right) from mouse lungs by FACS. b Quantification of vClub in total lung cells from PBS-treated or OVA-challenged wild type mice by flow cytometric analysis. c qPCR analysis of RNA expression of Atg5 (relative to β-actin) in vClub cells isolated from PBS-treated or OVA-challenged mice. d Autophagy induction in airway progenitor cells was analyzed using Cyto-ID labeling (fluorescent cationic amphiphilic tracer CAT) monitored with flow cytometry. e Quantification of vClub in total lung cells from tamoxifen-treated Atg5\(^ {−/−} \) or Scgb1a1-Atg5 mice during OVA-induced acute inflammation by flow cytometric analysis. Data represent the mean ± S.E.M. *p < 0.05, Student’s t test.](Image)
with vClub cells (Fig. 1a). Airway epithelial damage is usually observed in the chronic state of human asthma. We therefore determined whether epithelial reparative capacity is abrogated in the airways during the acute phase of inflammation. Following OVA-induced mouse acute allergic inflammation, we observed that the ratio of vClub cells to total lung cells decreased by 20% (Fig. 1b). Genetic variants in ATG5 have been associated with childhood asthma, and autophagosomes have been observed in bronchial epithelial cells and fibroblasts in asthmatic patients. Here, we observed that during OVA-induced acute inflammation, a significant decrease was observed in Atg5 expression in vClub cells (Fig. 1c). After OVA challenge, cationic amphiphilic tracer (CAT)-positive fraction was significantly reduced in vClub cells as shown in Cyto-ID Green labeling (Fig. 1d). Although loss of Atg5 in airway progenitor cells had little effect on the influx of eosinophils, neutrophils, and other inflammatory cells in bronchial epithelial cells in bronchoalveolar lavage fluid (BALF) or the expression of inflammatory cytokines associated with the recruitment of these inflammatory cells (Figs. S1 and S2), flow analysis indicated that the fraction of vClub cells was significantly decreased in Scgb1a1-CreER; Atg5fl/fl (Scgb1a1-Atg5) mice relative to that in Atg5fl/+ mice during OVA-induced acute inflammation (Fig. 1e). These data suggested that autophagy may be essential for maintaining the pool of mouse airway vClub progenitor cells, although it is not required for the development of OVA-induced acute inflammation.

**Autophagy promotes the colony-forming capacity of vClub progenitor cells**

Next, we investigated the possible role of autophagy in maintenance of the mouse airway epithelium. In vitro 3D organoid culture indicated that Atg5−/− vClub cells from tamoxifen-treated Scgb1a1-Atg5 mice displayed significantly lower the colony-forming efficiency (CFE) than vClub cells from Atg5fl/+ littermates (Fig. 2a, b). Consistent with this finding, vClub cells formed markedly smaller and

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**Fig. 2** Autophagy promotes the colony-forming ability of vClub progenitor cells. a Representative images of Matrigel cultures of vClub cells from tamoxifen-treated Atg5fl/+ or Scgb1a1-Atg5 mice at day 8 after plating. b CFEs of vClub cells isolated from tamoxifen-treated Atg5fl/+ or Scgb1a1-Atg5 mice. c CFE of vClub cells in the presence of spermidine (1 μM), 3-MA (3 mM) or bafilomycin (10 nM). d Diameter of colonies in vClub cell cultures under conditions described in (c). e Representative images of vClub cell-derived colonies stained with Ki67 (red), E-cadherin (green), and DAPI (blue). Scale bars, 50 μm. Arrowheads indicate Ki67+ epithelial cells. f Quantification of Ki67+E-cadherin+ cells in total E-cadherin+ cells from vClub cell cultures in the presence of spermidine. Data represent the mean ± S.E.M. *p < 0.05; **p < 0.01; ***p < 0.001, Student's t test.
fewer colonies in the presence of the autophagy inhibitors, either 3-MA or bafilomycin (Fig. 2c, d, Fig. S3). Treatment with the autophagy inducer spermidine increased the CFE of vClub cells, as well as the size of the colonies (Fig. 2c, d, Fig. S3). Immunofluorescence staining of epithelial colonies in organoid cultures indicated that the fraction of Ki67⁺ E-cadherin⁺ over total E-cadherin⁺ cells was higher in the presence of spermidine (Fig. 2e, f). Together, these data suggested that autophagy is essential for the proliferative capacity of mouse vClub progenitor cells.

**Autophagy enhances vClub progenitor cell differentiation**

We then assessed the role of autophagy in the differentiation of vClub cells into Club cells. Expression of Club-cell markers, including Scgb1a1 and Cyp2f2, was promoted in the presence of spermidine, suggesting that autophagy favors the differentiation of vClub cells into Club cells (Fig. 3a, b). In accordance with this, autophagy inhibition by either 3-MA or bafilomycin significantly reduced Cyp2f2 expression (Fig. 3c). Naphthalene causes severe depletion of Club cells. As predicted, loss of body weight was observed within 2 days of naphthalene administration to Atg5⁺/⁻ mice, with a similar loss but insufficient recovery in Scgb1a1-Atg5 mice (Fig. 3d). qPCR analysis and immunofluorescence staining of Cyp2f2 expression in lung tissue confirmed the extensive loss of Club cells at 2 days after naphthalene administration in Atg5⁺/⁻ mice, with an even greater loss observed when autophagy was defective in Scgb1a1-Atg5 mice after tamoxifen treatment (Fig. 3e, f). In addition, recovery of Cyp2f2 expression was also inefficient in Scgb1a1-Atg5 mice than in Atg5⁺/⁻ mice (Fig. 3e, f). Collectively, these data suggested that autophagy is essential for the generation of Club cells by vClub progenitor cells.

**Distinct role of autophagy in the colony-forming ability and differentiation of Club cells**

Club cells are capable of proliferating and differentiating into ciliated cells and goblet cells [7,8]. Therefore, we determined whether the function of Club cells is also governed by autophagy. Club cells, characterized as EpCAM⁺ CD24low Sca-1⁺ (Fig. 1a) and grown in the presence of spermidine, generated larger colonies and exhibited increased CFEs (Fig. 4a, b). By contrast, those in the presence of 3-MA showed fewer CFEs and formed smaller colonies than those grown in control medium (Fig. 4c, d). Atg5⁻/⁻Club cells isolated from Scgb1a1-Atg5 mice after administration of tamoxifen showed markedly lower CFEs and smaller colony size than those from Atg5⁺/⁻ littermates (Fig. 4e, f). These data suggested that autophagy promoted the proliferation of Club cells. To evaluate the differentiation of Club cells into ciliated or goblet cells, we analyzed the gene expression of ciliated cell markers including α-tubulin, and forhead box J1 (Foxj1), and goblet cell markers including chloride channel, calcium activated, family member 3 (Clca3), SAM pointed domain-containing ETS transcription factor (Spdef), and forhead box A3 (Foxa3) in organoid cultures. α-tubulin expression was decreased in cultures supplemented with spermidine but increased in cultures with 3-MA or bafilomycin relative to control cultures (Fig. 4g, h). Foxj1 expression was increased in Club-cell cultures with bafilomycin treatment (Fig. 4i). Similarly, more α-tubulin mRNAs were detected in cultures of Club cells isolated from Scgb1a1-Atg5 mice after tamoxifen treatment than in cultures of those from littermate control mice (Fig. 4j), indicating that autophagy exhibited an inhibitory role in ciliated cell differentiation. Similar to α-tubulin, expression of Clca3, Spdef, or Foxa3 was reduced in the presence of spermidine, but was elevated in the presence of 3-MA, bafilomycin, or genetic loss of Atg5 (Fig. 4k–r). These data suggested that autophagy is essential for the proliferation differentiation of airway progenitor cells.

**Glut1 maintains the pool of airway progenitor cells**

Glucose deprivation was shown to induce autophagy, which, in turn, upregulates glucose uptake [17,23]. We determined whether autophagy promotes glucose uptake in vClub cells by 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG)-trafficking analysis. vClub cells assimilated glucose rapidly (Fig. S4a), and flow cytometric analysis indicated that 28.2% of vClub cells were positive for green fluorescent protein (GFP) at 1 h after incubation with 2-NBDG (Fig. S4b). Glucose uptake by Atg5⁻/⁻ vClub cells isolated from tamoxifen-treated Scgb1a1-Atg5 mice was reduced by 30% relative to that of Atg5⁺/⁺ vClub cells (Fig. 5a). Next, we sought to examine whether autophagy facilitates glucose uptake by impacting the expression of glucose transporters (GLUTs). Among 12 GLUT family members, we observed that Glut1 was the most abundant in both lung epithelial cells and purified airway progenitor cells (Fig. S5a). To evaluate the role of Glut1 in maintaining the function of airway progenitor cells, we established Scgb1a1-CreER:Glut1⁺/⁻ (Scgb1a1-Glut1) mice. As described, spermidine promoted the CFEs of vClub cells; however, this role was abolished in the absence of Glut1 (Fig. 5b). Quantitative PCR and flow cytometric analysis indicated that Glut1 expression was comparable between Atg5⁺/⁺ vClub and Atg5⁻/⁻ vClub cells isolated from tamoxifen-treated Atg5⁺/⁻ and Scgb1a1-Atg5 mice (Fig. S5b, c). These data suggested that autophagy facilitates glucose uptake in vClub cells by impacting endocytosis and/or recycling of GLUTs rather than increasing their surface expression.

**Glut1⁻/⁻ vClub cells isolated from tamoxifen-treated Scgb1a1-Glut1 mice generated fewer and smaller colonies (Fig. 5c, d). Moreover, the expression of Club-cell markers**
Scgb1a1 and Cyp2f2 was significantly reduced in Glut1−/− vClub cell cultures relative to littermate controls (Fig. 5e, f), suggesting that the differentiation of vClub cells into Club cells was positively regulated by Glut1. Flow cytometric analysis of total disassociated lung cells indicated that the ratio of Club cells to vClub cells was lower in Scgb1a1-Glut1 mice than in Glut1ff mice at day 20 after naphthalene treatment (Fig. 5g). The CFEs of Glut1−/− Club cells were significantly lower than those of Club cells from littermate control mice (Fig. 5h). Furthermore, the expression of Foxj1 was induced in organoid cultures of Club cells isolated from Scgb1a1-Glut1 mice relative to

**Fig. 3** Autophagy enhances the differentiation of vClub cells. a, b qPCR analysis of Scgb1a1 and Cyp2f2 expression (relative to E-cadherin) in organoid cultures of vClub cells in the presence of spermidine at 10 days after seeding. c qPCR analysis of Cyp2f2 expression in organoid cultures of vClub cells in the presence of 3-MA or baflomycin. d, e Time course of relative body weight and Cyp2f2 expression were analyzed in the lungs of tamoxifen-treated Atg5ff or Scgb1a1-Atg5 mice after naphthalene administration. f Representative images showing the extent of Club-cell injury or repair in tamoxifen-treated Atg5ff or Scgb1a1-Atg5 mice after naphthalene administration according to immunofluorescence analysis: Cyp2F2 (green), DAPI (blue). Data represent the mean ± S.E.M. *p < 0.05; **p < 0.01; ***p < 0.001, Student’s t test.
Fig. 4 (See legend on next page.)
those from littermate controls (Fig. 5i). Expression of Foxa3 and Spdef was elevated in Club-cell cultures in the absence of Glut1 (Fig. 5, k). Leukocyte recruitment remained unchanged in the absence of Glut1 during OVA-induced acute inflammation (Fig. S6). These data suggested that Glut1 contributes to maintaining the pool of vClub and Club progenitor cells by promoting their proliferation and inhibiting ciliated and goblet cell differentiation.

Glucose level determines the function of airway progenitor cells

We next attempted to explore the regulatory role of glucose in the function of airway progenitor cells. When glucose supplementation decreased, an increase in CFEs and colony size of vClub cells was observed (Fig. 6a, b). However, both CFEs of vClub cells and the size of colonies were significantly reduced in cultures deprived of glucose (Fig. 6a, b). In a similar fashion, Sggb1a1 expression was promoted in vClub cultures with low amounts of glucose, followed by a significant reduction in cultures without glucose (Fig. 6c). Similarly, both CFEs and colony size were significantly promoted in Club-cell cultures with low amounts of glucose, but were reduced when glucose was deprived (Fig. 6d, e). Expression of Foxj1 was lower in Club organoid cultures with 0.5 g/L glucose than in the control group (Fig. 6f). Likewise, the expression of Clca3 and Foxa3 was also decreased in Club cultures with 0.5 g/L glucose as compared with the control group (4.5 g/L) (Fig. 6g, h). Further deprivation of glucose resulted in a rebound in the expression of Clca3 and Foxa3 (Fig. 6g, h). These data indicated that glucose is required for the colony-forming ability and differentiation of mouse airway progenitor cells including vClub cells and Club cells. A decrease in glucose accessibility promotes the colony-forming ability of vClub and Club cells, but inhibits ciliated cell and goblet cell differentiation. Deprivation of glucose may lead to arrested proliferation of airway progenitor cells or even cell death.

Glycolysis impacts the proliferation and differentiation of airway progenitor cells

Glycolysis is a series of reactions that extract energy from glucose. We adopted 2-DG to examine glucose metabolism in regulating the function of airway progenitor cells. Mouse vClub cells generated slightly larger colonies, while their CFEs remained unchanged at a lower dose of 2-DG (Fig. 7a, b). However, both CFEs and colony size of vClub cells decreased at a higher dose of 2-DG (Fig. 7a, b). Expression of Sggb1a1 remained unchanged in vClub cultures at a low dose of 2-DG, but was promoted at a high dose of 2-DG (Fig. 7c). Similar to vClub cells, the CFEs of Club cells were increased at a low dose of 2-DG, but were decreased at a higher dose (Fig. 7d). The size of Club colonies was smaller at a higher dose of 2-DG (Fig. 7e). Expression of a-tubulin, Clca3, Spdef, and Foxa3 remained unchanged at a low dose of 2-DG, but was significantly promoted at a higher dose (Fig. 7f–i). This was consistent with our previous findings that the proliferative potential of both vClub and Club cells was promoted at a lower glucose dose, but was inhibited when glucose was totally deprived (Fig. 6). These data suggested that the proliferation of vClub and Club cells are impacted by glycolysis in a two-way fashion.

Together, our data suggested that autophagy is essential for maintaining the pool of airway progenitor cells through sustaining glucose at a moderate level. Low level glucose or partial inhibition of glycolysis promotes the proliferation of both vClub and Club progenitor cells, and suppressing goblet cell differentiation. This may, in part, contribute to the resolving outcome during acute phase of asthmatic inflammation. Blockade of glucose uptake or complete inhibition of glycolysis may lead to a diminished pool of airway progenitor cells through inhibition and acceleration of their proliferation and differentiation, respectively. Eventually, failure to restore airway epithelia may not be favorable for the resolution of asthmatic inflammation in the chronic phase.

Discussion

Alterations in airway epithelium are related to both the acute and chronic phases of asthma. Obstacles related to asthma treatment are attributable to insufficient understanding of the molecular mechanisms that regulate the pool of airway progenitor cells involved in repopulating the airway epithelium. A major goal of this study was to elucidate the asthma-related role of autophagy in the proliferation and differentiation of airway progenitor cells.
during tissue homeostasis and OVA-induced acute inflammation. Our main finding was that autophagy maintained the pool of airway Club cells and vClub cells by promoting their proliferation and inhibiting ciliated cell differentiation. Mechanistically, we observed that glucose uptake was facilitated by autophagy in airway vClub progenitor cells and that glucose deprivation, glycolysis inhibition, or Glut1 loss led to a diminished pool of airway progenitor cells by inhibiting and accelerating their proliferation and differentiation, respectively. A moderate level of glucose or glycolysis favors the maintenance of the pool of airway progenitor cells, which

Fig. 5 Atg5 loss inhibits glucose uptake by airway vClub progenitor cells. a The fraction of 2-NBDG-positive vClub cells (taking up glucose) in tamoxifen-treated Arg5f/f or Scgb1a1-Arg5 mice by flow cytometric analysis. b CFEs of vClub cells sorted from Glut1f/f mice in the absence or presence of spermidine and of vClub cells sorted from tamoxifen-treated Scgb1a1-Glut1 mice. c, d CFEs and mean diameters of epithelial organoids in Matrigel cultures of vClub cells isolated from tamoxifen-treated Glut1f/f or Scgb1a1-Glut1 mice. e, f Fold change of Scgb1a1 or Cyp2f2 mRNA expression in organoid cultures of vClub cells sorted from tamoxifen-treated Glut1f/f or Scgb1a1-Glut1 mice. g Flow cytometric analysis of total lung cells indicated that the ratio of Club cells to vClub cells was lower in tamoxifen-treated Scgb1a1-Glut1 mice than in Glut1f/f mice at day 20 after naphthalene administration. h CFEs of epithelial organoids in Matrigel cultures of Club cells isolated from tamoxifen-treated Glut1f/f or Scgb1a1-Glut1 mice. i–k qPCR analysis of fold change of expression of Foxj1, Foxa3, and Spdef in organoid cultures of Club cells isolated from tamoxifen-treated Glut1f/f or Scgb1a1-Glut1 mice. Data represent the mean ± S.E.M. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001, Student’s t test.
may be beneficial for resolving OVA-induced acute inflammation.

Our in vitro 3D organoid experiments demonstrated that induction of autophagy promoted the colony-forming ability of both vClub cells and Club cells in the airway epithelium. Inhibition of autophagy using selective inhibitors or Atg5 conditional-knockout mice favored the differentiation of Club cells into ciliated cells and goblet cells; this suggested that autophagy is active in maintaining the homeostasis of the slow-turnover epithelium, such as that found in the lung at steady state. Autophagy is usually considered a cellular process associated with stress conditions. Here, we observed that autophagy was protective during naphthalene-induced lung injury.

Autophagy appears to play differential roles in epithelial injury and repair in different organs. Loss of leucine-rich repeat-containing G-protein coupled receptor-5-expressing intestinal stem cells and impairment of epithelial recovery after irradiation were observed in mice lacking Atg5. In addition, Atg5 deletion in keratin-5-positive epithelial cells does not affect the morphology of the thymus, but disturbs the differentiation of the preputial gland in mice. Atg16L1 in the intestinal epithelium is critical for preventing the loss of Paneth cells and protecting against exacerbated intestinal injury after murine norovirus infection. Furthermore, autophagy in the proximal, but not distal, tubule epithelium protects against acute kidney injury. However, it remains unknown whether autophagy favors epithelial regeneration in the kidney at steady state and after injury, although some types of endogenous stem cells have been proposed.

Fig. 6 Regulation of airway progenitor cells by glucose. a, b CFEs and mean diameters of epithelial organoid cultures of vClub cells in the presence of glucose at day 10 after seeding. c qPCR analysis of fold change of Scgb1a1 expression in vClub organoid cultures in the presence of glucose. d, e CFEs and mean diameters of epithelial organoid cultures of Club cells in the presence of glucose. f-h qPCR analysis of fold change of Foxj1, Clca3, and Foxa3 expression in Club organoid cultures in the presence of glucose. Data represent the mean ± S.E.M. *p < 0.05; **p < 0.01; ***p < 0.001, Student’s t test.
Autophagy in inflammatory cells is also involved in OVA-induced acute inflammation. Mice with deletion of Atg5 in CD11c+ dendritic cells spontaneously develop severe neutrophilic lung inflammation and airway hyperreactivity, suggesting that promoting autophagy in dendritic cells is also critical for controlling asthmatic inflammation.

**Fig. 7** Glycolysis is essential for the function of airway progenitor cells. a, b CFEs and mean diameters of epithelial organoids in vClub cell cultures with or without 2-DG at day 10 after seeding. c qPCR analysis of fold change of Scgb1a1 expression in organoid cultures of vClub cells in the presence of 2-DG. d, e CFEs and mean diameters of epithelial organoids in Club-cell cultures with or without 2-DG. f-i qPCR analysis of fold change of expression of α-tubulin, Clca3, Spdef, and Foxa3 in organoid cultures of Club cells in the presence of 2-DG. Data represent the mean ± S.E.M. *p < 0.05, **p < 0.01, ***p < 0.001, Student’s t test. j Schematic model describing a possible contribution of autophagy and glucose metabolism to the proliferation and differentiation of airway progenitor cells. Autophagy maintains glucose at low level to sustain the pool of airway progenitor cells. Reduced autophagy in airway progenitor cells may result in insufficient repair of airway epithelia, which is not favorable for the resolution of asthmatic inflammation.
inflammation. However, a recent study suggested that autophagy is enhanced in B cells by IL4 in the lungs of asthma-prone mice, and that autophagy deficiency in B cells attenuates asthmatic symptoms. We observed that autophagy in airway epithelial progenitor cells maintains the pool of these cells. Insufficient repair of airway epithelium is not favorable for the resolving of inflammation associated with airway epithelial damage in asthma. But this effect may not be observable until the chronic phase of inflammation as loss of Atg5 or Glut1 has little role in the recruitment of inflammatory cells in acute phase. The findings indicate that the contributions of autophagy to asthmatic inflammation are cell-specific; therefore, selective induction of autophagy in epithelial and dendritic cells might be a potential solution.

Genetic variations in ATG5 are associated with childhood asthma, and genetic polymorphisms in ATG5 and ATG7 contribute to neutrophilic airway inflammation in adult asthma. Double-membrane autophagosomes were detected in fibroblasts and epithelial cells in bronchial biopsy tissue from asthma subjects; however, additional information is needed to adequately evaluate the contribution of autophagy in fibroblasts to asthma pathogenesis. Here, we observed that basal level of autophagy in vClub cells was higher than that in Club cells. Although increased autophagy levels were observed in total lung tissue during OVA-induced acute inflammation, autophagy was reduced in vClub cells to a level that was similar to that in Club cells. Possible induction of autophagy in Club cells during OVA-induced acute inflammation sustained the pool of airway progenitor cells by inhibiting the differentiation of ciliated cells and goblet cells. Loss of Atg5 resulted in reduced uptake of glucose in airway progenitor cells; while low accessibility to glucose favored the proliferation of airway progenitor cells. Our data suggested that autophagy is induced when glucose is exhausted; whether proliferative potential of airway progenitor cells is eventually promoted may also be determined by the function of supportive fibroblast cells.

Autophagy is capable of reprogramming cellular metabolism in multiple tissues. A previous study showed that either pharmacological inhibition of autophagy with 3-MA or Atg5 knockdown significantly increased triglyceride content in hepatocytes. In addition, autophagy maintains the stemness and regenerative potential of hematopoietic stem cells by removing activated mitochondria and controlling oxidative metabolism. Moreover, autophagy facilitates glucose uptake through two pathways in mouse embryonic fibroblasts (i.e., promoting cell-surface expression of Glut1 and enabling the endocytosis and exocytosis of Glut1 trafficking). In the present study, we discovered that glucose uptake by airway epithelial progenitor cells was promoted by autophagy, possibly through regulating the endocytosis and/or exocytosis of Glut1 trafficking, given that Glut1 expression was not altered in the absence of autophagy. At steady state, deprivation of glucose, inhibition of glycolysis, or knockdown of Glut1 expression showed that a moderate level of glucose metabolism was critical for the proliferation and differentiation of airway epithelial progenitor cells, which enter a differentiated state when glucose metabolism is blocked. During OVA-induced acute inflammation, Glut1 expression in airway progenitor cells remained unchanged, although autophagy was altered. However, we cannot rule out the possibility that glucose uptake by airway progenitor cells might be adjusted through other GLUTs during OVA-induced acute inflammation.

In summary, these data suggested autophagy is essential for regenerative capacity of airway progenitor cells. A moderate level of glucose or partial inhibition of glycolysis is beneficial for maintaining airway progenitor cells. However, complete blockade of glucose uptake or glycolysis largely abrogated proliferation but promoted goblet cell differentiation. Failure to restore airway epithelia may accelerate the progression into the chronic state during asthma. Therefore, future investigations are needed to determine whether such progression can be prevented by sustaining glucose at a moderate level in airway progenitor cells, and whether the ability of glucose uptake is abrogated in chronic phase of asthma given that high glucose levels are usually observed in BALF from asthmatic patients.

Materials and methods
Reagents
Reagents and their sources are available in the online version of the paper.

Animal models
C57BL/6 mice were purchased from Huafukang Bioscience Co. Inc. (Beijing, China). Scgb1a1-CreER mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Atg5f/f mice were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT (Tokyo, Japan). Scgb1a1-CreER;Atg5f/f (Scgb1a1-Atg5) mice were created by crossing Scgb1a1-CreER mice with Atg5f/f. Glut1f/f mice, generated as previously described, were crossed with Scgb1a1-CreER to generate Scgb1a1-CreER;Glut1f/f (Scgb1a1-Glut1) mice. Mice were randomly grouped and age- and sex-matched. Eight- to twelve-week-old mice of both sexes were used in experiments involving OVA-induced acute lung inflammation. All mice were housed in a specific pathogen-free facility at Haihe Clinic College of Tianjin Medical University. All mice were used in
accordance with the guidelines approved by the Institutional Animal Care and Use Committee of the Haihe Clinic College of Tianjin Medical University.

**Cell lines**

The mouse lung fibroblast cell line MLg was purchased from ATCC (Manassas, VA, USA). MLg cells were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37 °C and 5% CO2.

**Tamoxifen and naphthalene administration**

Tamoxifen (Sigma-Aldrich, St. Louis, MO, USA) was prepared in corn oil (Sigma-Aldrich) at 20 mg/mL as a stock solution. Atg5 deletion in Scgb1a1-Atg5 mice or Glut1 deletion in Scgb1a1-Glut1 mice was induced by intraperitoneal injection of 200 mg/kg tamoxifen (Sigma-Aldrich) every other day up to three times. Atg5/− littermates or Glut1/− littermates received tamoxifen in the same way. Scgb1a1-Atg5 mice were administered naphthalene (Sigma-Aldrich) at a dose of 250 mg/kg dissolved in corn oil via intraperitoneal injection. At the indicated time points, lung tissues were collected for histological analysis or for isolating lung cells.

**OVA-induced acute lung inflammation**

Mice were sensitized by intraperitoneal injection on days 0 and 7 of 100 μL of 10 mg/mL chicken OVA (Sigma-Aldrich) in phosphate-buffered saline (PBS) emulsified with alum. On days 14, 15, and 16, the mice inhaled aerosolized 1% OVA diluted in PBS for 1 h daily. Matched littermate mice used as controls underwent the same procedures, but with OVA replaced by PBS. The mice were sacrificed 2 days after the final challenge, after which BALF was collected and centrifuged. Cells were spread on slides for Diff-Quick staining (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer protocol in order to analyze the abundance of infiltrated leukocytes, including eosinophils, neutrophils, and macrophages. Lung tissue was fixed for further immunostaining or was lysed in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for subsequent gene-expression or western blot analysis. In some experiments, lung tissue was digested with elastase (Worthington Biochemical Corporation, Lakewood, NJ, USA) to allow the sorting of airway epithelial cells.

**Lung dissociation and flow cytometry**

Lung tissue was dissociated as described previously. Briefly, lungs were perfused with 5 mL PBS and digested with 4 U/mL elastase and Dnase I (Sigma-Aldrich) to yield single-cell suspensions for flow cytometry. Lung cells were then resuspended in Hank’s balanced saline solution (HBSS) supplemented with 2% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10 mM HEPES. Cells were then incubated with primary antibodies, including anti-CD31-biotin (1:40), anti-CD34-biotin (1:20), anti-CD45-biotin (1:67), anti-CD24-phycocerythrin (PE, 1:25), anti-EpCAM-PE-Cyanine7 (PE-Cy7, 1:100), and Sca-1-allophycocyanin (APC, 1:100), followed by incubation with the secondary antibody streptavidin APC-eFluor 780 (1:100). 7-Aminoactinomycin D was added to discriminate dead cells (1:20). Autophagosome formation was analyzed using a CYTO-ID autophagy detection kit with a cationic amphiphilic tracer (CAT) as a fluorescence reagent (Enzo Life Sciences). Flow cytometric analysis or FACS was performed using a FACS Aria III sorter (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software (v10.0.7r2; FlowJo, LLC, Ashland, OR, USA).

**Glucose-uptake assays**

For flow cytometric glucose-uptake assays, 1 × 10^4 cells were resuspended in 100 μL glucose-free medium and incubated for 20 min at 37 °C in a humidified atmosphere with 5% CO2. 2-NBDG (100 μM) dissolved in glucose-free medium (Life Technologies; Thermo Fisher Scientific) was then added to a final concentration of 50 μM, and cells were incubated for an additional 1 h. Cells were washed three times with HBSS-plus (supplemented with 2% FBS, 10 mM HEPES, 100 IU/mL penicillin, and 100 μg/mL streptomycin) and resuspended in 100 μL of HBSS-plus solution before measuring fluorescence by flow cytometry. For fluorescence-microscope-based glucose-uptake assays, cells were washed three times with HBSS-plus, transferred to 96-well plates (Costar; Corning, Corning, NY, USA), and photographed using an Olympus DP80 fluorescence microscope (Olympus, Tokyo, Japan).

**Organoid culture of airway epithelial progenitor cells**

As described previously, mouse EpCAM+CD24low Sca-1− airway vClub cells (5 × 10^3 cells/well) and EpCAM+CD24low Sca-1+ Club cells (5 × 10^3 cells/well) were resuspended in 100 μL of Matrigel (BD Pharmingen, San Diego, CA, USA) and basic medium (1:1) in the presence of MLg lung fibroblasts (2 × 10^5 cells/well). The mixture was then added to 24-well Transwell filter inserts (Greiner Bio-One, Kremsmünster, Austria), and the lower chambers were filled with 410 μL. DMEM/F12 (Gibco; Thermo Fisher Scientific) supplemented with 10% FBS, penicillin/streptomycin, insulin/transferrin/selenium (Sigma-Aldrich), and 10 μM SB431542 (Sigma-Aldrich). Cultures were maintained in a humidified 37 °C incubator with 5% CO2, and fresh culture medium was replaced every other day. Colonies were visualized with an Olympus DP80 inverted fluorescence microscope (Olympus). Colonies with a diameter ≥ 50 μm were counted, and CFE was determined by the number of colonies in each insert.
as a percentage of the number of input epithelial cells at day 10 after seeding. For gene-expression analysis, TRIzol (Invitrogen) was added to the Matrigel discs, and RNA was extracted from cultures according to the standard protocol. For paraffin embedding, the Matrigel discs were fixed with formalin at 4 °C overnight and then removed and embedded into paraffin according to the standard protocol. Paraffin-embedded colony discs were then sliced at 5 μm for staining.

**Immunofluorescence staining**

Lung tissues were routinely perfused, inflated, and fixed in formalin for 2 h at room temperature. Culture colonies were fixed with 4% paraformaldehyde for 1 h at 4 °C, immobilized in O.C.T. compound (Sakura, Torrance, CA, USA), and frozen with liquid nitrogen. Paraffin sections (5 μm) and cyrosections (6 μm) were used for staining. Antigen retrieval was performed in citric acid (10 mM; pH 6), followed by blocking with 5% BSA in 0.2% Triton-X/PBS for 30 min at room temperature. The number of E-cadherin+ cells was counted in three or more random views of each colony section, and the percentage of E-cadherin+ cells in the total E-Cadherin population of each colony section was calculated and Ki67+ and E-cadherin+ cells was counted in three or more random 20 × views of each colony section, and the percentage of E-cadherin+ cell population of each colony section was calculated and averaged.

**RNA extraction and qPCR**

Total RNA was extracted from lung tissues, airway epithelial cells, or MLg cells using TRIzol reagent (Invitrogen). For qPCR analysis, 0.2 μg total RNA was used for reverse transcription using M-MLV reverse transcriptase (Promega, Madison, WI, USA). cDNA (2.5 μL) was subjected to real-time PCR using SYBR Select master mix (Applied Biosystems, Foster City, CA, USA) and the Roche LightCycler 96 real-time PCR system (Roche, Basel, Switzerland). The amplifying conditions were as follows: 95 °C for 2 min and 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s. The relative expression level of each gene was determined against β-actin levels in the same sample, and fold change in target-gene expression was calculated using the 2ΔΔCT method, with β-actin used for normalization. Primer sequences were as follows: β-actin-F: 5′-GGCCAACCGTGAAGATGTA-3′; β-actin-R: 5′-CA GCCTGGATGCTACAGTA-3′; E-cadherin-F: 5′-TGCT GCTCTCCTA6TTC-3′; E-cadherin-R: 5′-TCT TCTTCCACCTCTCTCT-3′; Sgb1a1-F: 5′-ATCA GAGTCTGTTATG-3′; Sgb1a1-R: 5′-ATCCACAG TCTCTCCAG-3′; Cyp2f2-F: 5′-CGACTGCTTCCAAAAGA-3′; Cyp2f2-R: 5′-GTCATAGAGGGATCATCATATT-3′; Clea3-F: 5′-GGCATGTCATCCGCATAG-3′; Clea3-R: 5′-CACCATGTCCTTATGTTGAGT-3′; Spdef-F: 5′-GACTGTGATTTCTGCGGGG-3′; Spdef R: 5′-ATGCTGGCAGAGCGAGAC-3′; Foxa3-F: 5′-CT TGGTGAGGTTGGTGAG-3′; Foxa3-R: 5′-ACAGGC AGATTCGCCAGCC-3′; a-tubulin-F: 5′-GGTGATG TGCTTCCAAAAGA-3′; a-tubulin-R: 5′-GTGGAGG CGTGGTGTATAC-3′; Foxj1-F: 5′-AGAGAGTGAGGG CAAGAGAC-3′; Foxj1-R: 5′-GGCGGCTTACAGGCA TTC-3′.

**Statistical analysis**

Data are representative of at least three independent experimental replicates and expressed as the mean ± S.E. M. P-values between the experimental and control groups were calculated using two-tailed unpaired Student’s t test. *P*-values (*p < 0.05; **p < 0.01; ***p < 0.001). Age-matched mice were assigned to control or treatment groups without a method of randomization. Sample size for animal experiments was determined based on pilot experiment. Outside of technical errors, no animals were excluded from the statistical analyses. Data were normally distributed, and no significant variance between groups was observed. Variance was similar between the groups that were being statistically compared.

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**Conflict of interest**

The authors declare that they have no conflict of interest.
