Autophagy is selectively activated and correlates with airway remodeling in asthma

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Abstract

Current asthma therapies fail to target airway remodeling which correlates with asthma severity driving disease progression that ultimately leads to loss of lung function. Macroautophagy (here after autophagy) is a fundamental cell recycling mechanism in all eukaryotic cells; emerging evidence suggests that it is dysregulated in asthma. We investigated the interrelationship between autophagy and airway remodeling and assessed preclinical efficacy of a known autophagy inhibitor in murine models of asthma. Human asthmatic and non-asthmatic lung tissues were histologically evaluated and were immuno-stained for key autophagy markers. The percent area of positive staining was quantified in the epithelium and airway smooth muscle (ASM) bundles using ImageJ software. Furthermore, autophagy inhibitor chloroquine (CQ) was tested (*i.n.*) in prophylactic (3-weeks) and treatment (5-weeks) models of allergic asthma in mice. Human asthmatic tissues showed greater tissue inflammation and demonstrated hallmark features of airway remodeling displaying thickened epithelium ($p<0.001$) and reticular basement membrane ($p<0.0001$), greater lamina propria depth ($p<0.005$), and increase in ASM bundles ($p<0.001$) with higher expression of Beclin1 ($p<0.01$) and ATG5 ($p<0.05$) along with reduced p62 ($p<0.05$) compared to non-asthmatic controls. Beclin1 expression was significantly higher in asthmatic epithelium and ciliated cells ($p<0.05$) suggesting potential role of ciliophagy in asthma. Murine asthma models demonstrated effective preclinical efficacy (reduced key features of allergic asthma: airway inflammation, AHR and airway remodeling) of autophagy inhibitor CQ. Our data demonstrates cell-context dependent, and selective activation of autophagy in structural cells in asthma. Further, this pathway can be effectively targeted to ameliorate airway remodeling in asthma.

Key Words

Autophagy, asthma, remodeling, Beclin-1, immunohistochemistry

Introduction

Asthma is a complex and heterogeneous condition characterized by spontaneous bronchoconstriction accompanied by widespread but variable airflow obstruction. Various genetic and environmental factors interplay in an array of disorders amalgamating in the classical triadic asthma phenotype of airway inflammation, remodeling and airway hyperresponsiveness (AHR) (1). Therapeutically, only inflammation and airway hyperresponsiveness can be sufficiently attenuated. The third pathophysiological link in the triadic asthma phenotype is airway remodeling, which needs attention. Airway smooth muscle (ASM) mass, mucous gland hypertrophy, neo-angiogenesis in the submucosa, sub epithelial fibrosis, increased mucus secretion (by goblet cells), epithelial fragility and epithelial-mesenchymal transition (EMT) are defining features of airway remodeling in asthma (2). ASM hyperplasia and hypertrophy in the central airways are both indicators and traits of asthma severity (3). Current therapeutics do not effectively target progressive airway fibrosis and remodelling. Elevated basal concentrations of transforming growth factor β (TGFβ) in asthmatics has been associated with sustained characteristic airway remodeling (4, 5). Alongside having a multitude of effects in homeostasis and pathophysiology of disease, TGFβ is thought to drive airway remodeling through the activation of myofibroblasts and smooth muscle cells, subsequently inducing the release of fibrogenic extracellular matrix (ECM) proteins (6). Emerging data suggest that autophagy promotes and influences this classical TGFβ driven airway remodeling (7). Autophagy modulation may therefore be a novel effective therapy for unmanageable asthma.

Autophagy is a fundamental cellular and physiological process that occurs in all eukaryotic cells (8). The cellular process can be informally referred to as the inner recycling mechanism or a process of “self-eating”. Targeted components are sequestered in double-membrane autophagosomes and ultimately degraded upon fusion with a lysosome with the cytoplasmic components recycled (9). Autophagy is a crucial regulator in the pathogenesis of

human disease as it has the potential to influence innate immune responses and promote programmed cell death along with the ability to regularly remove aging proteins, large molecular complexes, and obsolete or damaged organelles (9). The Beclin-1/class III Phosphoinositide 3-kinase (PI3K) complex is integral in autophagosomal membrane nucleation (10). Autophagy-related gene 5 (ATG5) is covalently conjugated with ATG12 and interacts with ATG16 to form the ATG12-ATG5-ATG16 complex. This complex is associated with autophagosome elongation and is essential for autophagosome formation (9). During autophagy, a truncated cytosolic form of Microtubule-associated proteins 1A/1B light chain 3A (LC3), LC3-I is conjugated to Phosphatidylethanolamine (PE) to form the membrane bound LC3-II (9). Punctate LC3-II is visible with immunostaining and indicates complete formation of autophagosomes (11). Sequestosome-1 (SQSTM1/p62) is a ubiquitin-binding protein that targets and binds to other proteins for selective autophagy which are ultimately degraded in the lysosome (12). p62 accumulates when autophagy is inhibited and inversely, levels of p62 decrease when autophagy is induced (13, 14). Whilst autophagy routinely plays a protective role, its functions such as cell survival can be deleterious (15).

Autophagy is involved in the pathogenesis of various diseases and links between autophagy and asthma are emerging (16-18). A positive correlation of ATG5 and collagen alpha-1 (V) gene expression in the airways of patients with refractory asthma supports this link between dysregulated autophagy and fibrosis in the airways (19). ECM-regulated autophagy is proposed to maintain tissue homeostasis and thus dysfunctional autophagy in the presence of increased TGFβ may propel the progression of airway remodeling (20). In this study we have found the evidence of activation of autophagy pathway in the small and large airways from asthmatic patients. The localization of autophagy proteins in the asthmatic airways is restricted to structural cells in the airway wall and is associated with features of airway remodeling in a TGFβ-dependent manner. We found that TGFβ concomitantly induced autophagy and

profibrotic signaling in ASM cells, this induction was prevented by CQ *in vitro*. Further, using mouse models of allergic asthma, we demonstrated that targeting autophagy pathway is an efficient way of providing therapeutic benefit in asthma.

Materials and methods

Acquisition of human lung tissue

Human lung tissue was obtained from surgical resection, explanted lungs and post mortem organ donors with ethical approval from Royal Prince Alfred Hospital, Concord Repatriation General Hospital and St Vincent's Hospital (# HREC14-0045, Sydney).

Human subject classification

See the data supplement for full subject classification.

Human Lung Tissue Processing and Section Preparation

Dissected lung tissues were fixed, processed and embedded in paraffin for analyses (21). Following microtome sectioning, Haematoxylin and Eosin (H&E) staining and masson's trichrome staining were used to assess structural integrity, inflammation and features of airway remodeling. See the data supplement for full methods.

Morphometric analysis of inflammation and airway remodeling features

Lamina propria depth was measured perpendicularly from multiple points at the base of the reticular basement membrane (RBM) to the outer edge of ASM bundles, and the proportion of ASM in the airway wall (ASM/LP, %) was calculated by measuring the total area of ASM mass per airway and dividing by the total area of lamina propria. Overall tissue inflammation in the lung was assessed and immune cells were counted manually in the lung tissue as described in data supplement.

Immunohistochemistry and immunofluorescent staining

Immunostaining for Beclin-1, ATG5, LC3B, p62 and sm-a-actin was performed as previously described (22-24). See the data supplement for full methods.

Image analysis

Computer-assisted image analysis was performed with a NanoZoomer-SQ Digital slide scanner (Hamamatsu, Hamamatsu City, Japan), Olympus BX51 upright epifluorescence microscope fitted with a DP70 CCD camera (Olympus, Shinjuku, Japan) and ImageJ software.

Cell culture

Human ASM cells were obtained from human lung by a method as described previously, see the data supplement for full methods.

Mouse models of allergic asthma

Experiments were conducted according to the institutional guidelines and the code for the care and use of animals. Animal Care Committee of Thomas Jefferson University and UTS approved the protocol. All surgeries were performed under tribromoethanol anesthesia, and all efforts were made to minimize suffering. BALB/c mice (female) were subjected to a sub-chronic (prophylactic) model of allergic asthma as described and shown in Fig 8A. 30 minutes prior to HDM challenges, selected mice were administered either chloroquine (CQ) intra nasally (50mg/kg) or saline as a vehicle. In a separate study BALB/c mice (female) were subjected to a treatment model (chronic allergic asthma model) of asthma as described and shown in Fig 9A. At week four and commencing for two weeks, 30 minutes prior to HDM challenges, selected mice were administered either chloroquine (CQ) intra nasally (50mg/kg) or vehicle (saline). In both studies, 24h after the last HDM challenge, lung function measurements were performed (flexiVent, Scireq, Montreal, Canada), bronchoalveolar lavage (BAL) fluid was collected, and lungs were formalin-fixed or flash frozen for histopathology and biochemical analysis. See the data supplement for full methods.

Mouse BAL immune cell staining, lung H&E, PAS and Masson's trichrome Staining

BAL sample cytospins were prepared and stained with Hema-3 staining kit (Fisher Scientific, Hampton, USA). The fixed lung tissues embedded in paraffin were cut and stained with H&E, PAS and Masson's trichrome staining using a protocol described previously (25-27). See the data supplement for full methods.

Measurement of TGFβ1

The content of TGFβ1 in BAL fluid was measured by Multiplexing LASER Bead Technology (Eve Technologies, Calgary, Canada) using a custom TGF-beta 3-Plex Cytokine Array.

Western blotting

Protein levels of Collagen 1A, pSMAD2/3, SMAD2/3, Beclin1 and LC3B in ASM cell lysates or murine lung tissues were measured by immunoblotting. All immunoblotting was carried out using protocols described previously (26, 28). See the data supplement for full methods.

Soluble Collagen Assay

Total soluble collagen content in the lung lysates was assessed using Sircol collagen assay (Biocolor, Carrickfergus, UK) (29). See the data supplement for full methods.

Statistical analysis

Data was analyzed using unpaired t-tests or one-way or two-way ANOVA as appropriate and is presented as mean ± SD or ± SEM. All data was analyzed with PRISM V7.04 software (GraphPad, La Jolla, USA) and $p<0.05$ was considered statistically significant.

Results

Histological evidence of airway remodeling and inflammation

As the link between autophagy and airway remodeling has been established, we first chose to histologically measure remodeling features of our selected asthmatic and non-asthmatic patients. Asthmatic airways in comparison with non-asthmatic airways displayed greater features of airway remodeling. Gross remodeling changes between non-asthmatic and asthmatic large airways were observed utilizing trichrome staining (Fig. 1A-F). The average epithelium thickness was significantly greater in asthmatics than in non-asthmatics (A: 50.46μm ± 9.52 vs. NA: 27.46μm ± 9.16, $p<0.001$) and with aniline blue dye staining of the fibrous RBM we observed an overall increase in thickness of the RBM in asthmatics (A: 9.94μm ± 1.63 vs. NA: 5.32μm ± 0.93, $p<0.0001$) (Fig. 1G-H). The average lamina propria (LP) depth for asthmatics was significantly thicker than non-asthmatics (A: 235.71μm ± 81.97 vs. NA: 105.31μm ± 48.81, $p<0.05$) and staining of acidophilic tissue components (cytoplasm and muscle) with biebrich scarlet-acid fuchsin allowed us to measure and observe an increase in the percentage of ASM mass in asthmatic LP (A: 20.63% ± 7.01 vs. NA: 8.99% ± 3.16, $p<0.001$). (Fig. 1I-J). These measurements classify the selected asthmatic patients as having undergone associated airway remodeling and justify their inclusion into the immunohistological component of this study. We further assessed tissue inflammation in the lungs by scoring overall airway wall inflammation (Fig. 1K) which showed greater influx of immune cells in the asthmatic airways. Nature of immune cell influx was determined by counting eosinophils (Fig. 1L), neutrophils (Fig. 1M) and macrophages (Fig. 1N). As shown in Figure 1, asthmatic lungs demonstrated significantly higher influx of immune cells into the lungs.

Expression profile of autophagy marker proteins in the large airways of asthmatics

We have selected four key markers of autophagy to examine their expression in the epithelial and ASM components of small and large airway walls in conjunction with normal non-asthmatic histology and remodeled asthmatic histopathology. There were no significant differences in the large airway epithelium (Fig. 2); while in the large airway ASM bundles we observed a marked increase in the expression (% area ± SD) of Beclin1 (A: 16.78% ± 9.38 *vs*. NA: 5.16 % ± 3.07, $p<0.01$) and ATG5 (A: 4.23% ± 2.09 *vs*. NA: 1.94% ± 1.94, $p<0.05$), and a decrease in p62 expression (A: 1.65% ± 1.06 vs. NA: 3.41% ± 2.31, $p<0.05$) in asthmatics compared with non-asthmatics (Fig. 3).

Expression profile of autophagy markers in the small airways of asthmatics

In the small airway epithelium, we observed solely a marked increase in the expression (% area ± SD) of Beclin1 (A: 19.44% ± 7.31 vs. NA: 11.44% ± 4.63, $p<0.05$) (Fig. 4), with no significant differences in small airway ASM bundles (Fig. 5).

Expression of Beclin-1 in cilia lining the large airways of asthmatics

In accordance with our classification, five out of six asthmatic patients displayed strong expression of Beclin-1 in the cilia lining the large airway epithelium whilst only one out of ten non-asthmatic patients displayed any expression of Beclin-1 in the cilia of the large airway epithelium. Overall, we have demonstrated that strong expression of Beclin1 in cilia lining large airway epithelial cells occurs mostly in asthmatic patients with associated airway remodeling evident compared to non-asthmatics (Fig. 6 A-C). Other markers (ATG5, LC3B, p62) were not evident in the cilia lining of large airway epithelium from both asthmatic and non-asthmatics.

Concomitant induction of pro-fibrotic signaling and autophagy markers in human airway smooth muscle cells

Greater tissue inflammation in the asthmatic lungs is in congruence with the published literature and supports the tenet that inflammation can partly drive airway remodeling in asthma. Therefore, we tested this hypothesis whether TGFβ1(a pleiotropic cytokine, elevated in asthma) would concomitantly induce pro-fibrotic signaling and autophagy *in vitro.* As shown in Fig 7, we found that TGFβ1 in a time-dependent manner increased collagen-1 expression and SMAD2/3 phosphorylation (pro-fibrotic signaling) and induced autophagy in ASM cells as seen with increased expression of Beclin-1 and LC3BII (Fig. 7A). This demonstrates an association between the effects of TGFβ1, the accumulation of collagen and increased pro-fibrotic signaling in an autophagy-dependent manner.

Effect of autophagy inhibitor on the concomitant induction of pro-fibrotic signaling and autophagy markers in human airway smooth muscle cells

Furthermore, we tested *in vitro* whether the treatment with autophagy inhibitor, CQ, would alleviate pro-fibrotic signaling and the induction of autophagy by TGFβ1 stimulation. As shown in Fig 7, we found that treatment with CQ (50μM) in conjunction with TGFβ1 stimulation reduced the expression of collagen 1A, Beclin-1 and LCB3-II, and the phosphorylation of SMAD2/3 in a time-dependent manner (Fig 7). This shows that CQ reduced TGFβ1 -induced airway remodeling markers in an autophagy dependent (inhibition) manner.

Effect of autophagy inhibitor in a prophylactic model of allergic asthma

To further investigate the interplay between autophagy and asthma we studied the effects of autophagy inhibition in an allergen (HDM)-induced asthma in mice (prophylactic model) as shown in Fig 8A. CQ is a known inhibitor of autophagy, increasing the pH of lysosomes and inhibiting fusion and formation of the autophagolysosome. Increased

inflammatory cell infiltration in both the airway wall and in the BAL, along with increased production of mucus are observed in the HDM-challenged mice in comparison with control mice (Fig. 8). Autophagy inhibitor effectively blocked influx of immune cells into the airways (Fig. 8 B, C, D, K) and significantly reduced eosinophils and neutrophils in the BAL fluid with no effect on macrophages (Fig. 8 L, M, N). Tissue inflammation was also reduced in the mouse lungs treated with CQ (Fig. 8 E, F, G: H&E staining) and there was a reduction in the mucus production in HDM-challenged mice treated with an autophagy inhibitor CQ (Fig. 8 H, I, J: PAS: airway mucus).

To examine the connection of autophagy with airway remodelling we performed assays to measure levels of TGFβ1 in the BAL, soluble lung collagen and immunoblot analysis of Collagen 1, Beclin-1 and ATG-5 expression in the lung lysates. The HDM-challenged group showed significant increase in the concentration of TGFβ1 in the BAL compared with controls ($p<0.001$), while treatment with CQ significantly reduced TGFβ1 levels when compared with the HDM group ($p<0.05$) (Fig. 8O). Further we found significantly greater amount of soluble lung collagen in tissue lysates of HDM-challenged group when compared to the controls ($p<0.001$), while lysates from the CQ treated group showed a significant reduction in the amount of soluble collagen in comparison with the HDM group ($p<0.05$) (Fig. 8P). Lung function measurements using flexivent showed increased AHR in HDM-challenged mice to methacholine (MCh) when compared with control animals (increase in airway resistance (Rn) at 25, 50 and 100 mg/ml) while CQ treatment prevented development of AHR in mice (significant reduction in Rn at 25, 50 and 100 mg/ml) when compared to HDM-challenged group (Fig. 8Q). Immunoblotting in lung lysates revealed higher and concomitant expression of ECM protein Collagen 1A and autophagy markers Beclin-1 and ATG-5 in the HDM-challenged mice when compared to controls, while CQ treatment prevented induction of

autophagy (as shown by the reduction in Beclin-1 and ATG-5) and accumulation of collagen in the lung (Fig. 8R).

Effect of autophagy inhibitor in a chronic model of allergic asthma

We further employed an allergen (HDM)-induced mouse model of chronic asthma (treatment model) to investigate the role of autophagy in airway remodeling in asthma (Fig. 9A). Mass inflammatory cell influx was observed in the airways of mice challenged with HDM and this was significantly attenuated in the CQ+HDM treated mice (Fig. 9B: H&E). Similar reduction was observed in the accumulation of fibrotic proteins in the CQ+HDM treated group when compared with HDM group (Fig. 9B: Mason trichrome). Increased production of mucus was observed in the HDM-challenged mice in comparison with saline-controls and was greatly reduced in CQ+HDM treated group (Fig. 9B: PAS). We further carried out immunofluorescent imaging for ASM marker: sm-**α**-actin; chronic allergen challenge increased ASM bundles which were significantly reduced by CQ treatment (Fig. 9B: sm-**α**-actin).

Immunohistochemical staining was used to examine the expression of four key protein markers of autophagy in the airways of saline-control, HDM-challenged, and CQ+HDM treated mice. In the ASM bundles of the HDM-challenged mice we observed a marked increase in the expression (% area ± SD) of Beclin1 (HDM: 14.67% ± 3.02 *vs*. control: 6.98 % ± 3.08, $p<0.0001$) (Fig. 9D), ATG5 (HDM: 18.7% ± 3.99 *vs*. control: 11.27% ± 6.67, $p<0.05$) (Fig. 9E) and LC3B (HDM: 20.47% ± 14.67 *vs*. control: 2.97% ± 1.59, $p<0.05$)(Fig. 9F) in the HDM-challenged mice compared with control mice. Treatment with CQ significantly reduced the expression of Beclin1 (CQ+HDM: 7.5% ± 4.65 vs. HDM: 14.67% ± 3.02, $p<0.0001$)(Fig. 9D), ATG5 (CQ+HDM: 7.78% ± 7.18 *vs*. HDM: 18.7% ± 3.99, $p<0.001$)(Fig. 9E) and increased the expression of p62 (CQ+HDM: 7.27% ± 1.97 vs. HDM: 2.82% ± 2.09, $p<0.0005$) (Fig. 9G) in the CQ+HDM treated mice compared with HDM-challenged mice.

Discussion

The upregulation of markers of autophagy has been linked with fibrosis and remodeling in various organs (7, 30). However, there has been limited histopathological evidence to show similar trends in the lungs of asthmatic patients. Whilst there are early reports on increased gene expression of ATG5 with additional measurement of ATG5 protein expression in the airways of refractory asthmatics (16, 19), our study provides comprehensive analysis of multiple autophagy markers and its association with airway remodeling in asthma. More importantly our data on Beclin-1 expression in both large and small airways and in the ciliated cells provides novel insight by which autophagy pathway may regulate and control airway remodeling in asthma. Further, this is also replicated in the murine models where we found induction of autophagy in experimental asthma models with a concomitant expression of pro-fibrotic cytokines and collagen in the lung, which were attenuated in presence of an autophagy inhibitor.

Firstly, we confirmed that the tissues we used displayed classical features of airway remodeling (5). There are variety of factors that contribute to the pathogenesis of asthma and subsequent development of airway remodeling in asthma. It is suggested that persistent insult leading to chronic inflammation with time leads to the development of structural changes in the lung that tracks clearly with significant reduction in the lung function and increase in asthma severity (31). Expression of Beclin-1 and ATG5 were found to be increased in the large airway ASM of asthmatics compared with healthy non-asthmatics, along with reduced expression of p62 in the large airway ASM of asthmatics compared with healthy non-asthmatics. Therefore, in this cohort of patient population we found concomitant expression and association of autophagy with airway remodeling and inflammation. These findings are novel in asthma as there is no evidence to date suggesting expression of autophagy being closely associated with airway remodeling. This finding is reinforced by data from our chronic

HDM model of allergic asthma in mice in which we found increased expression of Beclin-1, ATG5 and LC3B in the ASM bundles of HDM-challenged mice when compared with the controls. This work clearly supports the published literature from kidney, liver and other organs where it has been shown that autophagy regulates tissue fibrosis (32, 33). On the other hand, it has been recently reported that autophagy is a necessary mechanism for changing the phenotype of lung epithelial cells to mesenchymal cells (34, 35) therefore current finding which shows incidence of autophagy in epithelium of asthmatic patient and HDM challenged mice might confirm the role of EMT in accumulation of mesenchymal cells in asthma pathogenesis.

Airway remodeling features such as thickening of basement membrane is a key indicator for the development of asthma later in life; basement membrane thickening has been seen at an early age in children at 3-4 years of age (36). This led us to think about various signaling pathways such as autophagy that can be aberrant and can contribute to disease development later in life. While this needs to be investigated in samples from children, our data from adult asthmatics uncover the novel fundamental mechanism (autophagy) that may act as a key determinant of airway remodeling in asthma. Similarly, airway smooth muscle mass is increased in asthma and correlates with poor lung function and increased airway responsiveness to variety of contractile agonists, allergens, pollens etc. Our data from human lungs is clearly demonstrating a link between increased accumulation of ASM mass and increased expression of autophagy markers in the asthmatic lung, a feature totally absent in the non-asthmatic human lung. If these results are replicated in a larger cohort of asthma patients then we can direct our research efforts to selectively target mesenchymal cells using novel formulation and chemistry approaches which will lead to reduced mesenchymal cell mass, reduction in release of ECM proteins, and ultimately reduction in the ability of the airways to contract to variety of triggers.

An increase in the protein expression of Beclin-1 was also observed in the small airway epithelium compared with healthy non-asthmatics. With access to tissue for a greater number of patients, then accompanying significant differences in protein expression of Beclin1 and ATG5 in the large airway epithelium may be observed. Interestingly asthmatic epithelium displayed strong expression of Beclin-1 in cilia, while non-asthmatic epithelium did not display these levels of expression. The comparative expression of Beclin-1 in cilia of asthmatic and non-asthmatic epithelium has a significant binary pattern. In chronic obstructive pulmonary disease (COPD), autophagy-dependent pathways regulate cilia length upon exposure to cigarette smoke (37). The identification of an over-expression of Beclin-1 in ciliated cells of asthmatics highlight a role for ciliophagy also in asthma and may have implications upon mucociliary clearance (MCC). A question that arises is whether airway wall changes in asthmatics are contributing to increased expression of Beclin1 in cilia, or whether the increased expression of Beclin1 in the cilia is influencing the cellular stimulation of airway remodeling. The effect that this upregulation of Beclin-1 in asthmatic ciliated cells has upon autophagy flux in other respiratory cells of the airway wall requires further investigation. One limitation of this study is the coincidence of all patients included being male, and this coincidence is unavoidable with the limited access to asthmatic and non-asthmatic tissue we have at this time. A further limitation is our lack of addressing the different phenotypes presented in asthma diagnosis such as eosinophilic, neutrophilic, or paucigranulocytic asthma. We believe the patient group in this study is reflective of mild-moderate asthma i.e. it represents a greater asthma population in the general public. In future we would like to investigate whether expression of autophagy is associated with asthma severity.

We further investigated whether an inhibitor of autophagy: CQ has any therapeutic value in both acute (sub-chronic) and chronic (treatment) model of allergen (HDM)-induced asthma in mice. A few clinical studies in the early 80’s and 90’s have demonstrated that

hydroxychloroquine can be used as an effective treatment for severe asthma as it has a steroid-sparing effect (38). Since then there has been a lack of big prospective clinical trials to further validate these original observations. Our preclinical data clearly demonstrates that CQ is effective in blocking the influx of immune cells both in BAL and lung tissue. Further our data uncover novel therapeutic effects of CQ in asthma as it reduces features of airway remodeling by preventing accumulation of mucus and ECM matrix protein collagen-1 in the lung. The inhibition of autophagy by CQ was supported with our immunohistochemical analysis in the chronic mouse model showing decreased expression in of Beclin-1 and ATG5, and increased expression of p62 in the ASM bundles of CQ+HDM-treated mice in comparison with HDM-challenged mice. We also found reduction in ASM mass as shown by reduced staining for sm-a-actin in the airways of mice treated with CQ. The mechanisms of the beneficial effect of CQ relies on blocking the release of pro-fibrotic cytokine TGFβ1 in the BAL of HDM-challenged mice after treating with CQ. It is well known that TGFβ1 drives fibrotic changes in the lung and its levels are elevated in asthma (39) and it is emerging that the autophagy pathway may interact with the ECM and affect cytokine secretion (7, 40). We have shown *in vitro* that upon stimulation with TGFβ1, human ASM cells produced greater amounts of collagen 1A, have increased expression of Beclin-1 and LC3B-II, and greater phosphorylation of SMAD2/3 in a time-dependent manner, which supports the growing notion that TGFβ1 concomitantly induces autophagy and drives pro-fibrotic signaling. CQ acts by inhibiting autophagy and reducing TGFβ1-dependent pro-fibrotic signaling in asthma. This was confirmed with the co-treatment of CQ and TGFβ1 resulting in a time-dependent reduction in collagen A1, diminished expression of Beclin-1 and LC3B-II, and reduced phosphorylation of SMAD2/3. These beneficial effects of modulating autophagy using CQ in disease setting (mouse models) also translated in reducing development of AHR (data not shown for chronic model) to MCh which is another hallmark feature of allergic asthma.

Further our mouse models demonstrated clear initiation (in the 3-week model) and establishment (in 5-week model) of airway remodeling as seen by increase in mucus release, accumulation of collagen-1 in the lung and activation of autophagy pathway, reflected by an increase in autophagy markers in the lung. CQ treatment in HDM-challenged mice clearly blocked features of airway remodeling in the lung with a concomitant reduction in autophagy as seen with reduction in Beclin-1 and ATG-5 proteins. This clearly states that CQ works at multiple levels to provide therapeutic benefit in asthma: it blocked inflammation, reduced mucus secretion, inhibited TGFβ1 release in BAL, and reduced release and accumulation of ECM proteins in the lung, leading to reduction in AHR. This is achieved by modulation of autophagy pathway as CQ inhibits overall autophagy flux by increasing the pH in the lysosome, preventing the fusion of the autophagolysosome and the subsequent degradation of lysosomal components (41). Though autophagy modulation with CQ however is not highly selective as it has a number of other pharmacological effects, at the concentrations used in this study, Beclin-1 and ATG-5 were reduced by CQ. Therefore, we are highly confident of these findings that CQ works in an experimental asthma model by modulation of autophagy pathway. Originally an anti-malarial and anti-arthritis drug (42), CQ has been shown to also target inwardly rectifying potassium (K_{ir}) channels (43), and by disrupting the blood-retinal barrier it has the potential to cause retinopathy through the binding to melanin and toxic sequestration in the eye (44). Compounds with greater specificity are now being considered in the target of the autophagy pathway. Bafilomycins are macrolide antibiotics derived from Streptomyces griseus bacteria that inhibit Vacuolar-type H+-ATPase (V-ATPase). At high concentration, bafilomycin is capable of blocking late-phase autophagy through significant cytosolic acidification (45). 3-methyladenine (3-MA) also has the ability to suppress the formation of autophagosomes specifically targeting autophagy through the inhibition of class III PI3K (46). However, 3-MA is found to have a dual role in autophagy modulation. It surprisingly promotes

autophagy flux with prolonged treatment under nutrient-rich conditions as well as having the capability of suppressing starvation-induced autophagy (47). LY294002, derived from the flavonoid Quercetin (48), inhibits PI3K activity (49) and belongs with the emerging safe therapeutics with the potential to treat airway remodeling. LY294002 treatment in mice challenged with Follistatin-related protein 1 (FSTL1) (linked with the promotion of both EMT and autophagy) has shown early signs of implication in the attenuation of airway remodeling (50).

Inhibition of autophagy, as shown here with CQ treatment, is entwined with an upstream TGFβ1 response and the level of collagen production. As it has previously been shown in hepatic cells and cardiomyocytes, TGFβ concomitantly influences features of remodeling as well as regulating levels of autophagy (7, 30), we introduce and provide evidence that a similarly concomitant pathway is occurring both *in vitro* and *in vivo*. This pathway, we believe, has the greatest influence in the ASM of the large airways as seen in asthmatic lungs which is also replicated *in vitro* using primary human ASM cells where we found TGFβ1 concomitantly induced both pro-fibrotic signaling and autophagy. We have shown an increase (and supporting decrease for p62) in several vital autophagy-linked proteins in the large airway ASM bundles. We have shown that inhibition of autophagy in murine models can; attenuate inflammation, clear mucus production, reduce levels of TGFβ1 in the BAL, and ultimately reduce airway remodeling and prevent bronchoconstriction. Thus, our study indicates that in asthmatic airways autophagy is enhanced, which we believe contributes to remodeling in a TGFβ1-dependent manner, and inhibition of autophagy is an attractive target to alleviate airway remodeling in asthma.

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Table 1. Demographic data for the asthmatic patients and non-asthmatic control subjects (Data expressed as numbers of subjects or medians (range))

Large airway Demographics		
Groups (numbers)	Non-Asthmatic (10)	Asthmatic (6)
Age, median (range)	55 (19-67)	48 (15-80)
Male/Female	10/0	6/0
Small airway Demographics		
Groups (numbers)	Non-Asthmatic (10)	Asthmatic (7)
Age, median (range)	52 (25-69)	53.5 (15-80)
Male/Female	10/0	7/0

Figure Legends

Figure 1. Histological evidence of airway remodeling in the large airways of the selected asthmatic patients. Non-asthmatic (**A**) and asthmatic (**B**) airways were stained with masson's trichrome staining as described in the methods. Original magnification, ×50 (**A**, **B**). Arrows indicate the RBM and arrowheads indicate the epithelium (**C**, **D**). ASM bundles are indicated by white arrowheads (**E**, **F**). Smooth muscle bundles are stained (red) and connective tissue (blue). Original magnification, ×400 (**C**, **D**, **E**, **F**). The epithelium (**G**), RBM thickness (**H**), Lamina propria depth (**I**), the proportion of ASM in the asthmatic airway wall (**J**), inflammation score in sub-mucosa (**K**); eosinophil (**L**), neutrophil (**M**) and macrophage (**N**) counts per mm^2 were quantified. (Data is expressed as mean ± SD or SEM; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Figure 2. Expression of autophagy markers in the large airway epithelium of asthmatic and non-asthmatic tissue. Asthmatic and non-asthmatic tissue were immunohistochemically stained for Beclin1 (**A**, **B**), ATG5 (**D**, **E**), LC3B (**G**, **H**), and p62 (**J**, **K**). Original magnification, ×400. Positive area for each of these markers in the epithelium was quantified (**C**, **F**, **I**, **L**).

Figure 3. Expression of autophagy markers in the large airway ASM bundles of asthmatic and non-asthmatic tissue. Asthmatic and non-asthmatic tissue were immunohistochemically stained for Beclin1 (**A, B**), ATG5 (**D**, **E**), LC3B (**G**, **H**), and p62 (**J**, **K**). Original magnification, ×400. Arrowheads indicate ASM bundles. Positive area for each of these markers in the ASM was quantified (**C**, **F**, **I**, **L**). Data is expressed as mean ± SD; *p<0.05, **p<0.01.

Figure 4. Expression of autophagy markers in the small airway epithelium of asthmatic and non-asthmatic tissue. Asthmatic and non-asthmatic tissue were immunohistochemically stained for Beclin1 (**A**, **B**), ATG5 (**D**, **E**), LC3B (**G**, **H**), and p62 (**J**, **K**). Original magnification, ×400. Arrowheads indicate epithelium. Positive area for each of these markers in the epithelium was quantified (**C**, **F**, **I**, **L**). Data is expressed as mean ± SD; **p*<0.05.

Figure 5. Expression of autophagy markers in the small airway ASM bundles of asthmatic and non-asthmatic tissue. Asthmatic and non-asthmatic tissue were immunohistochemically stained for Beclin1 (**A**, **B**), ATG5 (**D**, **E**), LC3B (**G**, **H**), and p62 (**J**, **K**). Original magnification, ×400. Positive area for each of these markers in the epithelium was quantified (**C**, **F**, **I**, **L**).

Figure 6. Expression of Beclin1 in cilia lining large airway epithelium of asthmatics. Non-asthmatic large airway epithelium (**A**) and asthmatic large airway epithelium (**B**) immunohistochemically stained for Beclin-1. Arrowheads indicate cilia. Original magnification, ×1000. Representation of the number of patients (Asthma v Non-asthma) with Beclin-1 positivity in cilia (**C**).

Figure 7. Concomitant induction of pro-fibrotic signaling and autophagy by TGFβ1 *in vitro*. Human airway smooth muscle cells were treated with either TGFβ1 (2.5ng/ml) or TGFβ1 (2.5ng/ml) + CQ (50μM) for 0, 24, 48, 72 and 96 hours. Cell lysates were prepared, and immunoblotting was performed for collagen-1A, phospho-SMAD2/3, total SMAD2/3, Beclin-1, LC3BI/II while GAPDH was used as a loading control. Data shown is representative of 4-independent primary human ASM cells.

Figure 8. Mouse model of allergic asthma (**A**). Female mice at 8-weeks were challenged intra nasally (*i.n.*) with HDM 5 days/week for 3 weeks. Autophagy inhibitor chloroquine (CQ, 50mg/kg) was administered 30 mins prior to each HDM challenge, 24h after the last challenge, BAL and lung tissue was collected for further analysis. Inflammation in the BAL fluid was measured in saline controls (**B**), HDM-challenged mice (**C**), and the HDM+CQ group (**D**). H&E staining was used to measure tissue inflammation in the saline controls (**E**), HDM-challenged mice (**F**), and the HDM+CQ group (**G**). Periodic Acid-Schiff (PAS) measured mucus production in the saline controls (**H**), HDM-challenged mice (**I**), and the HDM+CQ group (**J**). CQ treatment in the HDM-challenged group reduced influx of total immune cells (**K**), eosinophils (**L**), neutrophils (**M**), macrophages remain unchanged (**N**). Further, inhibition of autophagy reduced profibrotic cytokine TGFβ1 (**O**) levels in BAL and also reduced the amount of soluble lung collagen in tissue lysates (**P**). One-way ANOVA: saline *vs* HDM **p*<0.001; HDM *vs* HDM+CQ $^{\#}p$<0.05 with Bonferroni multiple comparisons test. Flexivent analysis revealed reduction in MCh-induced AHR in CQ-treated group when compared to HDM-challenged group alone (**Q**). Two-way ANOVA: saline vs HDM **p*<0.05; ***p*<0.01; *****p*<0.0001 (at 25, 50 and 100 mg/ml); HDM *vs* HDM+CQ #*p*<0.05; ##*p*<0.01 (at 25, 50 and 100 mg/ml). Representative protein immunoblots for Collagen1A, Beclin1 and ATG-5 in lung tissue lysates from Saline, HDM- and HDM+CQ-treated mice (**R**). Mouse data shown represent Mean ± SEM from n=6-7 per group.

Figure 9. Mouse model of chronic allergic asthma (A). Female mice at 8-weeks were challenged intra nasally (i.n.) with HDM 5 days/week for 5 weeks. At week 4 for the remaining 2 weeks, autophagy inhibitor chloroquine (CQ, 50mg/kg) was administered 30 mins prior to each HDM challenge, 24h after the last challenge, BAL and lung tissue was collected for further analysis. (**B**) H&E staining was used to measure tissue inflammation in the saline

controls, HDM-challenged mice, and the HDM+CQ group. Masson's trichrome staining was used to measure connective tissue deposition and smooth muscle mass in the saline controls, HDM-challenged mice, and the HDM+CQ group. Periodic Acid-Schiff (PAS) measured mucus production in the saline controls, HDM-challenged mice, and the HDM+CQ group. α-Smooth muscle actin staining was used to stain smooth muscle cells in the saline controls, HDM-challenged mice, and the HDM+CQ group. (**C**) Imunohistochemical staining was used to measure the expression of autophagy proteins in the ASM bundles of the mice. Beclin-1 expression was measured in the saline controls, HDM-challenged mice, and the HDM+CQ group. ATG5 expression was measured in the saline controls, HDM-challenged mice, and the HDM+CQ group. LC3B expression was measured in the saline controls, HDM-challenged mice, and the HDM+CQ group. p62 expression was measured in the saline controls, HDM-challenged mice, and the HDM+CQ group. Positive area for each of these markers in the ASM was quantified (**D**, **E**, **F** & **G**). (Data is expressed as mean ± SD or SEM, n=4-6).