Investigating autophagy as a target in asthma and understanding how autophagy can modulate allergen-induced airway remodelling

Thesis

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CERTIFICATE OF ORIGINAL AUTHORSHIP

I Kielan Darcy McAlinden declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the Graduate School of Health at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

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Abstract

Background:

Airway remodelling is an untreatable hallmark of asthma. Autophagy, the cellular homeostatic recycling mechanism has emerged as a factor playing a role in asthma and potentially airway remodelling.

Objectives:

To explore the involvement of autophagy in asthmatic airway remodelling and test autophagy inhibition as a novel therapeutic target in asthmatics.

Methodology:

Autophagy protein expression was measured in the airways of both human and mouse asthmatic tissue by immunohistochemistry. Autophagy inhibitors chloroquine (CQ) and bafilomycin A1 (BafA1) were tested in murine asthma models. Relevant lung function, cell counts, histological staining and protein expression were supported by *in vitro* experiments.

Results:

We have found increased autophagy protein expression involved in asthmatic airway remodelling in human and mice tissue. Transforming growth factor beta (TGF-β) concomitantly induces remodelling changes and the upregulation of autophagy. Autophagy inhibition reduced the pathophysiological symptoms of asthma.

Conclusion:

Autophagy contributes to airway remodelling in asthma and autophagy modulation is a promising approach in developing therapies that target remodelling.

Dissemination of Research

Peer reviewed publications

1. **KD McAlinden** et al. (2019). There can be smoke without fire. Warranted caution in promoting e-cigarettes and heat not burn devices as a safer alternative to cigarette smoking. European Respiratory Journal Open Research, Aug 12;5(3):00114

2. **KD McAlinden** et al. (2019). Pharmacologic Inhibition of Vacuolar H+ATPase Attenuates Features of Severe Asthma in Mice. American Journal of Respiratory Cell and Molecular Biology, Jan; 62(1):117-120.

3. **KD McAlinden** et al. (2019). Altered Calcium in Ciliary Dysfunction: Potential Role of ER stress and Ciliophagy. American Journal of Respiratory Cell and Molecular Biology, Dec; 61(6):794-795.

4. **KD McAlinden** et al. (2018). Autophagy Activation in Asthma Airways Remodelling. American Journal of Respiratory Cell and Molecular Biology, May; 60(5):541-553.

5. MS Eapen, A Kota, H Vindin, **KD McAlinden**, D Xenaki, BG Oliver, DA Deshpande, SS Sohal, P Sharma (2018). Apoptosis signal-regulating kinase 1 (ASK1) inhibition attenuates human airway smooth muscle growth and migration in chronic obstructive pulmonary disease (COPD). Clinical Science, July 13:1615-1627.

6. MS Eapen, **KD McAlinden**, PM Hansbro, RY Kim, C Ward, TL Hackett, EH Walters, SS Sohal (2017). Abnormal M1/M2 macrophage phenotype profiles in the small airway wall and lumen in smokers and chronic obstructive pulmonary disease (COPD). Scientific Reports Vol. 7 (1):13392.

7. MS Eapen, **KD McAlinden**, D Tan, S Weston, C Ward, Muller HK, EH Walters SS Sohal (2017). Profiling cellular and inflammatory changes in the airway wall of mild to moderate COPD. Respirology Vol. 22 (6):1125-1132.

Conference proceedings

1. **High fat diet in conjunction with electronic cigarette vaping worsens lung function and inflammation**

   American Thoracic Society Conference 2019 (Dallas, USA)

2. **Electronic-cigarette vaping in combination with a high fat diet augments lung function and inflammation**

   Thoracic Society of Australia and New Zealand Conference 2019 (Gold Coast, AUS)

3. **Autophagy is Selectively Activated and Correlated with Airway Remodelling in Asthma**

   American Thoracic Society Conference 2018 (San Diego, USA)

4. **Selective activation and targeting of autophagy in severe asthma**

   European Respiratory Society International Congress 2018 (Paris, FRA)

   (Unable to attend - work was presented by supervisor: Dr Pawan Sharma)

   * Awarded Best Abstract in Airway Diseases (supported by GlaxoSmithKline)

5. **Expression of Autophagy markers in the human asthmatic epithelium**

   Thoracic Society of Australia and New Zealand Conference 2018 (Adelaide, AUS)

6. **Maternal E-cigarette Vaping Enhances Development of Allergic Asthma in the Offspring**

   American Thoracic Society Conference 2017 (Washington, USA)

   (Unable to attend - work was presented by supervisor: Dr Pawan Sharma)

   * Awarded ATS Stuart J. Hirst Award (Top ranked scientific abstract in the RSF category submitted by a fellow or student that relates to airway biology and physiology).

7. **Maternal e-cigarette vaping enhances development of allergic asthma in the offspring**

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Abbreviations

3-MA 3-methyladenine

A Adenine

AAD allergic airways disease

AHR airway hyperresponsiveness

ALS Amyotrophic lateral sclerosis

AMPK 5' adenosine monophosphate- activated protein kinase

ASM airway smooth muscle

ASM/LP, % proportion of ASM in the airway wall

ATG Autophagy-related gene

ATG3 Autophagy-related gene – 3

ATG4 Autophagy-related gene – 4

ATG5 Autophagy-related gene – 5

ATG7 Autophagy-related gene – 7

ATG8 Autophagy-related gene – 8

ATG12 Autophagy-related gene – 12

ATG13 Autophagy-related gene – 13

ATG14 Autophagy-related gene – 14

ATG16 Autophagy-related gene – 16

ATG101 Autophagy-related gene – 101

ATP Adenosine triphosphate

BCG (Bacillus Calmette–Guérin) vaccine

BafA1 Bafilomycin A1

β2 agonists β2-adrenergic receptor agonists

BAL Bronchoalveolar Lavage

Ca^{2+} Calcium

cAMP Cyclic adenosine monophosphate

CD11c cluster of differentiation molecule 11c

CFA complete Freund's adjuvant

CO_2 Carbon dioxide

COPD chronic obstructive pulmonary disease

CQ Chloroquine

CS cigarette smoke

CSE cigarette smoke extract

CysLT Cysteinyl leukotrienes

DAB 3,3'-Diaminobenzidine

DALYs disability-adjusted life years

DMEM Dulbecco's modified Eagle's medium

ECM extracellular matrix

EMT epithelial-mesenchymal transition

ER Endoplasmic reticulum

ERK extracellular signal–regulated kinases

FDA The United States Food and Drug Administration

FEV1 Forced expiratory volume in one second

FP fluticasone propionate

$FSTL_1$ Follistatin-related protein 1

G Guanine

GFP green fluorescent protein

GRE glucocorticoid responsive elements

GWAS genome wide association studies

H&E Haematoxylin and Eosin

H_20_2 hydrogen peroxide

H2 relaxin human gene-2 relaxin

HCQ Hydroxychloroquine

HDAC histone deacetylases

HDAC-6 Histone Deacetylase 6

HDM House dust mite

HIF-1 hypoxia inducible factor

HRP horse-radish peroxidase

ICS inhaled corticosteroids

IFN-γ interferon-gamma

IgE Immunoglobulin E

IHC Immunohistochemistry

IMPase Inositol monophosphatase

IP_3 1,4,5-inositol trisphosphate

IL-1b Interleukin-1b

IL-4 Interleukin-4

IL-5 Interleukin-5

IL-8 Interleukin-8

IL-10 Interleukin-10

IL-13 Interleukin-13

IL-25 Interleukin-25

IL-33 Interleukin-33

IκBα nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

KC CXCL1

LABA long-acting β2 agonists

LC3 microtubule-associated protein light chain 3

LP Lamina propria

MAPK mitogen-activated protein kinase

MCC mucociliary clearance

MHC major histocompatibility complex

MIICs MHC class II-containing compartments

MYLK myosin light-chain kinase

MMP-2 matrix metallopeptidase-2

MMP-9 matrix metallopeptidase-9

mRNAs Messenger ribonucleic acids

MTECs mouse tracheal epithelial cells

mTOR mammalian (or mechanistic) target of rapamycin

mTORC1 mammalian target of rapamycin complex 1

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NIH National Institute of health

$NADP^+$ Nicotinamide adenine dinucleotide phosphate

NF-κB Nuclear factor-kappa B

NSCCa non-small cell carcinoma

NSCLC Non-small cell lung cancers

O_2 Oxygen

OCR oxygen consumption rate

ORAI3 ORAI calcium release-activated calcium modulator 3

OVA ovalbumin

PAS Periodic acid–Schiff

p62 Sequestosome-1

PB1 (Phox and Bem1) **domain**

PBCs peripheral blood cells

PDE phosphodiesterase

PE phosphatidylethanolamine

PI3K phosphatidylinositol 3-kinase

PI3P phosphatidylinositol-3-phosphate

PKA Protein kinase A

PKC Protein kinase C

PMN polymorphonuclear

RBM reticular basement membrane

ROS reactive oxygen species

SAECs small airway epithelial cells

SD Standard deviation

SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SABA short-acting β2 agonists

SQSTM1 sequestosome 1

SNP single-nucleotide polymorphism

TAS2R taste 2 receptor

TEM transmission electron microscopy

TGFβ Transforming growth factor-beta

T_H1 Type 1 Helper T cell

T_H2 Type 2 Helper T cell

TLSP thymic stromal lyphopoietin

TNF Tumour necrosis factor

ULK1 UNC-51-like kinase 1

UPR unfolded protein response

UPS ubiquitin-proteasome system

V-ATPase Vacuolar-type H+-ATPase

VEGF Vascular endothelial growth factor

WHO World Health Organisation

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Chapter 1

Introduction and background

1.1 Asthma

Asthma is a complex and heterogeneous condition characterized by inflammation, hyperresponsiveness and remodelling of the airways (Holgate 2008b). Asthma sufferers experience spontaneous bronchoconstriction accompanied by widespread but variable airflow obstruction. Ann Woolcock defined asthma as "an abnormal state of the airways which causes the airways to narrow too much and too quickly in response to a wide variety of provoking stimuli" (Woolcock 1996). Available treatments for asthma are most effective in alleviating symptoms in majority of patients but not completely comprehensive in treatment for some asthmatics. For some sufferers, current medications do not satisfactorily alleviate symptoms. Therapeutically, only airway hyperresponsiveness and inflammation can currently be sufficiently attenuated. Airway remodelling, however, needs greater attention. Airway obstruction, experienced by sufferers, is considered mostly reversible (Boulet 2009). The reversibility of airflow limitation however, is incomplete in some patients. The importance of treating remodelling changes in the asthmatic lung is therefore of great importance. Complexity and variability defines asthma, with differing inflammatory profiles accompanying a cause that is often unknown (Mims 2015).

Asthma affects 1 in 9 Australians and one in six Australian children under the age of 16 suffers from asthma. Between 300 and 350 million people worldwide (roughly 12 to 15 times the population of Australia) are estimated to experience some form of asthma. In Australia, asthma causes significant morbidity and mortality, with the death rates being among the highest in the world. The hidden annual health care costs of asthma in Australia were revealed to be $28 billion in 2016 (Deloitte 2015). Asthma was projected to cost the Australian government $4.0 billion over 2016-2019 in direct

costs (Deloitte 2015). The rate of mortality due to asthma in Australia is averaged to be around 1.5 deaths per 100,000 population. Indigenous Australians are faced with inexcusably higher mortality rates (roughly 2.3 times higher) of asthma than non-Indigenous Australians (Australian Institute of Health and Welfare (AIHW): Poulos LM 2014). The number of disability-adjusted life years (DALYs) lost due to asthma is globally significant with Australia joining the United Kingdom, Brazil and Thailand in having higher than average figures in this statistical area. The overall burden of asthma (measured by DALYs and premature death) is greatest in pre-adolescent children (10-14 years old) and the elderly (Chen et al. 2014).

Provocation of asthma occurs by a wide variety of environmental and endogenous stimuli (Holgate 2008a). Allergic stimuli such as house dust mite (HDM) (Peat et al. 1996), cat dander, aspirin (Samter & Beers 1968) and pollen can trigger an asthma attack (Passalacqua & Ciprandi 2008). Alternatively, asthma can be triggered by non-allergic stimuli such as exercise and cold temperatures (Strauss et al. 1977; Turcotte et al. 2003). In allergic asthma, immunoglobulin E (IgE) attached to mast cells trigger the activation of the mast cells upon contact with aeroallergens. Activated mast cells release their granule content initiating a complex inflammatory process (Passalacqua & Ciprandi 2008).

Exploring and expanding upon the definition of hypersensitivity, Arthur F. Coca and Robert A. Cooke were the first to introduce the term ‘Atopy’. This was initially defined as an abnormal form of hypersensitivity, further subdivided and expanded as an inherited form of hypersensitivity (Coca & Cooke 1923). Atopy refers to a genetic predisposition of hypersensitivity towards common environmental airborne antigens. Atopy can be clinically characterised as secreting elevated IgE levels in response to these aeroallergens.

Total serum IgE can be used as an estimate for the intensity of the allergic response in asthma (Burrows et al. 1989). Atopy has been considered the strongest identifiable risk factor for developing asthma. Interestingly though, a large population of the western world is atopic but only a small proportion of these people display asthmatic symptoms (Holgate 2008b). So arguably, a firm relationship between atopy and asthma does not exist and the importance of atopy as a cause of asthma is not as high as once thought (Pearce, Pekkanen & Beasley 1999).

Prevalence of asthma in developed countries has steadily and substantially increased over time (Ramsey & Celedon 2005). The hygiene hypothesis, as an explored explanation, centres on the proposed link between early exposures to microbial sources and the development of an allergic asthma response. Skewing of the T_H1/T_H2 balance in foetal and early postnatal immune responses is closely tied to the hygiene hypothesis and a delayed maturation of T_H1-cytokine responses is identified as a risk for children to become sensitised to allergens related to asthma (Martinez & Holt 1999). Environmental factors are core to the hygiene hypothesis, with children exposed to a diverse array of microbial material (such as a farming environment) identified as less likely to develop sensitisation towards potential allergens. Furthermore, maternal exposure to similar environments is shown to play a protective role in potential asthma development for the children (Riedler et al. 2001; Riedler et al. 2000). Asthma treatment during pregnancy often requires the use of inhaled beta 2 receptor agonists (β_2RA) and intravenous administration of these drugs may occasionally be used to suppress premature labour (Fox et al. 2008). There is an observed association with intrauterine exposure to β_2RA and increased risk of asthma in children at 5 years (Ogawa et al. 2017).

Ultimately, no gene or environmental factor solely accounts for the pathogenesis of asthma. Due to the vast heterogeneity of asthma, there is still a great degree of unknown in relation to why the development of asthma occurs in some, whilst others are unaffected. The clinical expression of asthma varies greatly. Numerous environmental factors interact with the airways, causing different degrees of inflammation, smooth muscle contraction, oedema, and remodelling. The heterogeneity of asthma is further accentuated in relation to differing responses to therapies. A ruthless mixture of genetic and environmental factors amalgamate in the classical triadic asthma phenotype of airway inflammation, airway remodelling and airway hyperresponsiveness (AHR) (Royce et al. 2012).

1.2 Inflammatory response

In allergic asthma, allergens are processed and presented via antigen-presenting cells to T lymphocytes, stimulating the classical T_H2 inflammatory response (Holgate 2008). This classic allergic asthma model revolves around mast cells and other mediators such as eosinophils, basophils, T helper 2 (T_H2) cells, and immunoglobulin E (IgE) producing B cells (Bradding 2003; Bradding et al. 2006; Locksley 2010; Fahy 2015). Activation of the T_H2 phenotype initiates the increased production of inflammatory cytokines (IL-4, IL-5 and IL-13). B-cells are stimulated by IL-4 and IL-13 to produce IgE, subsequently activating mast cells, with resultant amplification the inflammatory response (Bradding & Holgate 1999).

Mast cells have been observed to localise and infiltrate airway smooth muscle (ASM) bundles in asthma, promoting differentiation of the muscle into a more contractile phenotype (Woodman et al. 2008). Mast cell degranulation may determine the length of a fatal asthma attack, and the level of mast cell degranulation (particularly in the ASM

and the outer adventitia) found in these early phase deaths may ultimately be the culprit (Elliot et al. 2009).

Other master regulators such as thymic stromal lyphopoietin (TLSP), IL-25 and IL-33 are activated and produced in the epithelium. Expression and activation of these proteins increases the production of type 2 cytokines (including IL-4, IL-5 and IL-13) which activate an inflammatory cascade, leading to the activation of epithelial cells and chemoattraction of inflammatory mediators (Fahy 2015). Ultimately, dysregulation and chronic feedback of this cascade can result in airway remodelling.

Two major and distinct inflammatory profiles are mostly observed in asthma (particularly in severe asthma); the asthma subtypes of eosinophilic and neutrophilic asthma (Wenzel et al. 1999). These strict inflammatory subtypes were identified through the analysis of sputum samples from asthmatic patients: eosinophilic asthma (increased number of eosinophils in the sputum), neutrophilic asthma (increased neutrophils), mixed granulocytic asthma (both increased eosinophils and neutrophils), and paucigranulocytic (normal levels of both eosinophils and neutrophils) (Simpson et al. 2006). However, asthmatic airway inflammation may not simply be identified and diagnosed by only these two polymorphonuclear cell populations, but rather a combination of influences from macrophage and neutrophil systems accompanying eosinophil influence (Ward et al. 1990).

Within the last decade, another classification via molecular phenotype has emerged. Asthma can be defined as T_H2-High and T_H2-Low in accordance with the degree of type 2 inflammation. It was found that these subgroups differ significantly in the expression of IL-5 and IL-13 in bronchial biopsies as well as levels of subepithelial fibrosis, airway

hyperresponsiveness and mucin gene expression (Woodruff et al. 2009). T_H2-High Asthma can also be identified with eosinophilia and larger numbers of circulating airway mast cells (Dougherty et al. 2010). Asthma with a lack of eosinophilia can be used to predict a non-favourable response to glucocorticoid treatment (Berry et al. 2007). T_H2-Low asthma is characteristically unresponsive to inhaled corticosteroids (Samitas et al. 2017). However, the existence of a subgroup called "severe refractory asthma" or "refractory eosinophilic asthma" poses difficulty when treating patients that fall into this category. The subgroup is a characterisation that encompasses all the sub-phenotypes of asthma (including a proportion of T_H2-High Asthma patients) that do not respond to current therapies (Chung et al. 1999).

1.3 Airway Remodelling

The term "airway remodelling" envelops the adverse structural changes that occur in the progression of asthma. Fibroblasts, myofibroblasts and smooth muscle cells (mesenchymal cells) are the cells that predominantly contribute to remodelling of asthmatic airways. Increased and enlarged ASM mass, mucous gland hypertrophy, neoangiogenesis in the submucosa, subepithelial fibrosis, epithelial fragility, epithelial-mesenchymal transition (EMT), and goblet cell hyperplasia with increased mucus secretion are defining features of airway remodelling in asthma. These changes can each be indicators and traits of asthma severity (Benayoun et al. 2003). Current available therapies target inflammation and AHR, however in severe asthma, patients become non responsive and there is no treatment available to target remodelling.

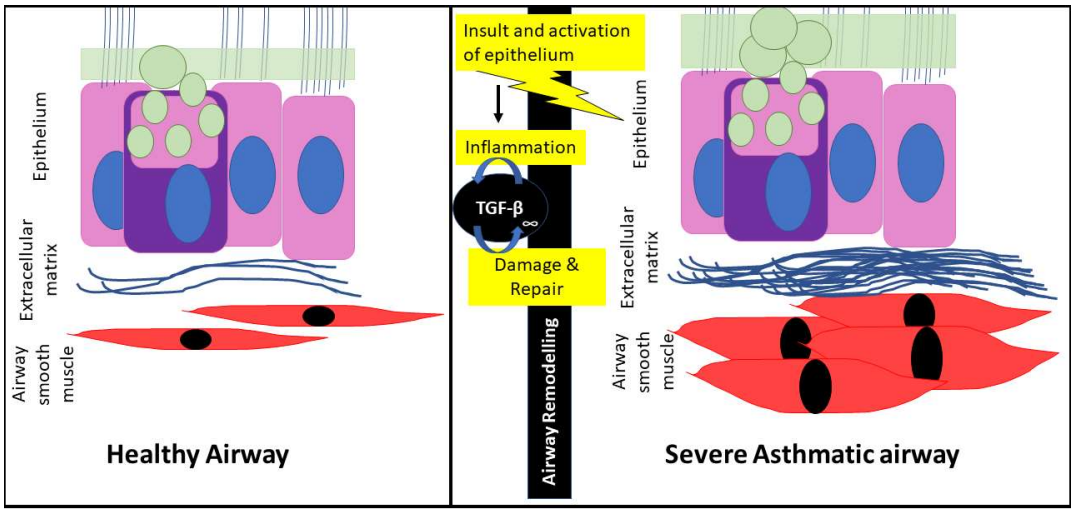


Figure 1. Airway remodelling in severe asthma

Insult and activation of the airway epithelium by various irritants leads to the cyclic state of inflammation and dysregulated tissue damage and repair. Strongly mediated by transforming growth factor-β (TGF-β), leading to the aggravated development of tissue remodelling changes. The severe asthmatic airway (right) in comparison to the healthy (left) airway displays greater amounts of enlarged ASM mass, increased mucus production and impaired mucociliary clearance, with increased accumulation within the extracellular matrix.

(Adapted from: Chronic inflammation and asthma, by J. R. Murdoch & C. M. Lloyd, 2010, Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. Copyright © 2009 Elsevier B.V. All rights reserved.)

1.3.1 Transforming growth factor beta - 1 (TGF-β1)

The activation of transforming growth factor-β-1 (TGF-β1) is fundamental to subsequent airway remodelling (Boxall, Holgate & Davies 2006; Howell & McAnulty 2006). TGF-β is a potent pro-fibrogenic cytokine in the airways. Basal concentrations of TGF-β in bronchoalveolar lavage (BAL) samples of atopic asthmatics are recognised as being elevated in comparison to non-asthmatic patient controls (Redington et al. 1997). A similar trend has also been reported in the asthmatic airways of bronchial biopsies through immunohistochemical methods (Chakir et al. 2003). Anti-IL-5 experiments and the

resulting depletion of eosinophils revealed that these eosinophils are a strong source of TGF-β (Flood-Page et al. 2003). Eosinophil-derived TGF-β increases as the result of eosinophilic airway inflammation. The activation and differentiation of fibroblasts is known to be stimulated by TGF-β which culminates into subepithelial fibrosis and results in airway wall stiffness. TGF-β activates sub-epithelial mesenchymal cells, which then proliferate and release matrix proteins. The number of fibroblasts in asthmatic bronchial biopsies correlates with the thickness of the basement membrane. Furthermore, the numbers of epithelial and submucosal cells expressing TGF-β was also found to correlate to basement membrane thickness (Vignola et al. 1997). Abnormal collagen deposition sequestering below the basement membrane in asthmatic bronchial biopsies supports the knowledge that the subepithelial fibrosis is driven by fibroblast activation (Roche et al. 1989). TGF-β mRNA expression has been found to correlate with the volume of subepithelial fibrosis and subsequently the severity of asthma (Minshall et al. 1997). Subepithelial fibrosis in asthma is linked to eosinophil-derived cytokines, most significantly IL-5 and TGF-β (Flood-Page et al. 2003; Humbles et al. 2004).

Methacholine, a cholinergic drug used to measure AHR has been shown to induce airway remodelling in asthmatics without any accompanying inflammation (Grainge et al. 2011). In mice, an overregulation of GATA-3 and an accompanied shift in the T_H1/T_H2 bias leads to an increase in ASM hyperplasia (Kiwamoto et al. 2006). Additionally, hypercontractility of asthmatic ASM cells has been observed in comparison with ASM cells from healthy donors (Matsumoto et al. 2007). A possible reason for this hypercontractility could be an increased oxidative stress burden in asthmatic ASM (Sutcliffe et al. 2012).

1.3.2 extracellular matrix (ECM)

Abnormal and excessive extracellular matrix (ECM) protein content is observed in asthmatics, with significant increases in laminin, fibronectin, tenascin, proteoglycans and collagens (I, III and V) and a reduction in elastic fibres and collagen IV observed in comparison with healthy lung specimens (Laitinen et al. 1997; Slats et al. 2008). Matrix metalloproteinase-2 (MMP-2) and Matrix metalloproteinase-9 (MMP-9) are gelatinase enzymes responsible for the degradation of many ECM components such as collagens, elastin, fibronectin and other proteins (Lagente et al. 2005). We see, in severe asthma that expression and regulation of both MMP-2 and MMP-9 is dysregulated and consequently the capacity for ECM degradation is reduced (Laliberte et al. 2001; Wenzel et al. 2003). The inability to clear ECM unbalances and shifts the asthmatic lung to a state in which the accumulation of ECM proteins is favoured and resultantly unfavourable.

1.3.3 Vasculature

A pro-angiogenic environment features in adult and childhood asthma which promotes submucosal neoangiogenesis. This bronchial vascular proliferation increases with disease severity (Salvato 2001). Vascular endothelial growth factor (VEGF) is a significant mediator of neoangiogenesis in asthma, promoting vascular remodelling. Secreted by eosinophils, macrophages and ASM cells; it is associated with airflow limitation and disease severity (Siddiqui et al. 2007). In samples from asthmatic patients, VEGF is upregulated and an increase in angiogenic “sprouts” is observed, which indicates an increase in angiogenic activity in asthmatics (Feltis et al. 2006).

1.3.4 Epithelial cells, tight junctions, epithelial-mesenchymal transition (EMT)

The asthmatic airway epithelium is abnormal and fragile, with consequent defective cellular adherence and tight junctions. The resultant dysfunctional barrier enables dendritic cells to have better interactions with allergens in the lumen leading to greater levels inflammation of an undesirable manor (Jiang et al. 2007). Tight junction complexes have been shown to be compromised within the asthmatic epithelium (Xiao et al. 2011). This disruption of tight junctions contributes to the initiation of epithelial cells to undergo EMT in asthmatics (Hackett 2012). EMT is a process in which airway epithelial cells lose polarity, undergo cytoskeletal remodelling and differentiate to a mesenchymal phenotype. With the loss of their epithelial markers they subsequently migrate through the reticular basement membrane (RBM) to the subepithelial lamina propria. Excessive EMT contributes to fibrosis and the exceeding deposition of ECM proteins (Sohal et al. 2010; Willis, duBois & Borok 2006). Non-small cell lung cancers (NSCLC) enhance their invasion into surrounding connective tissue through EMT activity in cancer cells (Mahmood, Ward, et al. 2017). EMT activity is measured and analysed by the characteristic loss of E-cadherin coupled with an increase in mesenchymal markers such as vimentin and N-cadherin (Zeisberg & Neilson 2009). RBM fragmentation is caused by migrating epithelial cells digesting their way through the membrane. Distinguishing RBM fragmentation in conjunction with the expression of mesenchymal markers is considered the major criteria for identifying EMT (Zeisberg & Neilson 2009). Whilst the RBM found in chronic obstructive pulmonary disease (COPD) patients and smokers display classic fragmentation (Sohal et al. 2010), the asthmatic RBM is significantly thickened but fragmentation is not as visibly evident (Sohal, Ward & Walters 2014). TGF-β has been shown to induce EMT to a greater extent in asthmatic epithelial cells (Hackett et al. 2009;

Heijink et al. 2010). HDM extract and TGF-β also synergistically induce internalisation of E-cadherin and increase vimentin expression in human bronchial epithelial cells, which is a sign for EMT (Heijink et al. 2010). EMT intensifies subepithelial fibrosis, thus influencing the asthmatic remodelling process.

1.3.5 Mucus production

In the peripheral airways, where there is normally no presence of goblet cells, an increase in these mucus-producing cells is observed in asthmatics. The resulting increase in mucus production drives the progression and pathogenesis of chronic asthma (Jeffery 2001). Two mucins are predominately expressed in the airways; MUC5AC and MUC5B. Goblet cells produce MUC5AC, and the mucus glands produce MUC5B. Increases in mucus production and the gene expression of these mucins have been found to be associated with AHR, decline in lung function and an increase in airway resistance (Jinnai et al. 2010). Excessive asthmatic inflammation and goblet cell hyperplasia will further stimulate mucus hyper-production (Rogers 2003; Shim et al. 2001).

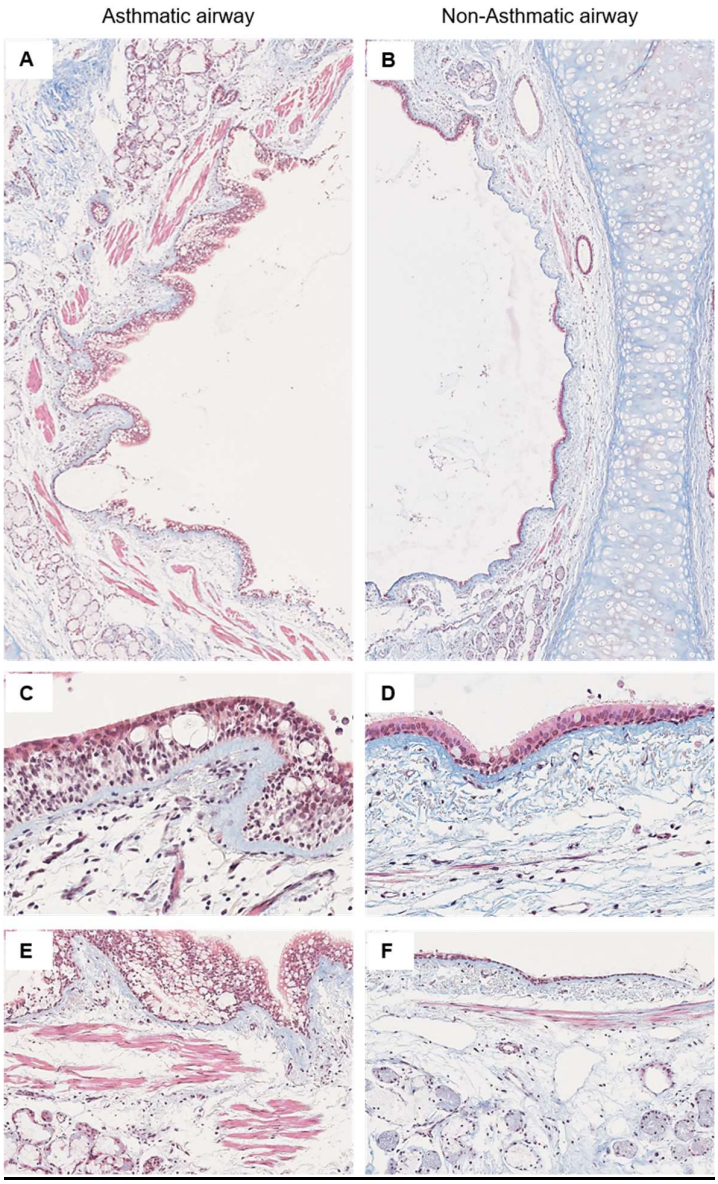


Figure 2. Asthmatic and Non-asthmatic airway structure and composition

Structural and compositional features of a human asthmatic airway compared with a human non-asthmatic airway. The asthmatic airway (A,C,E) in comparison to the non-asthmatic (B,D,F) airway displays greater amounts of enlarged ASM mass (E-F), increased mucus production, thickened pseudostratified epithelium and a thickened reticular basement membrane RBM (C-D) (McAlinden et al. 2018).

1.4 Current treatments for asthmatic patients

Current asthma therapies are a means of suppression, alleviating inflammation and mitigating AHR. The complexity of the asthma and the numerous presented phenotypes of asthma renders complete restoration from the condition a difficult objective. Inhaled β2-adrenergic receptor agonists were first approved for the treatment of asthma in 1969, followed by inhaled glucocorticoids in 1974.

1.4.1 β2-adrenergic receptor agonists or just β2 agonists

β2-adrenergic receptor agonists (β2 agonists) cause smooth muscle relaxation and ultimately achieve bronchodilation. Short-acting β2 agonists (SABA) include: fenoterol, isoproterenol, and albuterol. These are referred to as rescue medications in relation to asthma. β2-adrenergic receptors are coupled to adenylate cyclase through a trimeric G protein (Robison et al. 1967). Adenylate cyclase promotes the conversion of ATP to cyclic adenosine monophosphate (cAMP). Activation of these receptors mediates an increase in levels of cAMP. Increased intracellular cAMP levels catalyses the activation of protein kinase A (PKA), inhibits the release of calcium ions from intracellular stores and reduces calcium entry into cells. Depletion of intracellular calcium, phosphorylation of proteins such as myosin light-chain kinase (MYLK), and increased membrane potassium conductance contribute to the resultant relaxation of airway smooth muscle (Johnson 2001). β2 agonists have been engineered to improve the length of their activity, with the first long-acting β2 agonists (LABA) (Salmeterol) being introduced in the early 1990s. Due to their lipophilic nature, LABAs are able to sustain extended periods of bronchodilation.

For some patients, combination therapies are required to control their asthma as inhaled corticosteroids (ICS) alone may not provide clinical improvement or control. The addition

of a LABA alongside an existing low-dose ICS provides better clinical improvement than simply increasing the dose of ICS monotherapy (Tamm et al. 2012, Woolcock et al. 1996). However, it is advised not to use LABAs as a monotherapy, due to the association with exacerbations and increased risk of asthma death (Spitzer et al. 1992, Wijesinghe et al. 2008).

Greater engineering of longer acting or "ultra-long" beta agonists (indacaterol, oldaterol and vilanterol) began in the mid-2000s (Baur et al. 2010, Bouyssou et al. 2010, Procopiou et al. 2010). The formulation of these β2 agonists with much longer half-lives are being introduced with the goal of reaching greater practicality of once-daily dosing (Cazzola et al. 2011).

1.4.2 Glucocorticoids

In 1950, Philip Hench, Edward Kendall, and Tadeus Reichstein were awarded the Nobel Prize in the field of medicine for their early contributions to scientific research on glucocorticoids and their therapeutic applications (Eapen et al. 2015).

ICS are now the first utilised form of treatment for asthma and are likely to control the symptoms of an asthma attack. They display anti-inflammatory properties which are strongly useful in the treatment of asthma. The mechanism of action for glucocorticoids is through the modulation of several transcription factors, with a resultant inhibition of nuclear factor kappa B (NF-κB) proving to be broadly very effective in the reduction in inflammation (Kagoshima et al. 2001; Reichardt et al. 2001). Higher concentrations glucocorticoids lead to the activation of several anti-inflammatory genes such as Annexin-1, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) and IL-10. Lower concentrations of glucocorticoids can facilitate the

suppression of pro-inflammatory genes with the recruitment of histone deacetylases (HDAC) to the sites of inflammatory response (Barnes 2006).

Glucocorticoids have a positive effect on aspects of asthmatic vascular remodelling by acting on proangiogenic factors such as vascular endothelial growth factor (VEGF) and angiopoietin-1 (Chetta & Olivieri 2012; Kanazawa et al. 2007). The effect of glucocorticoids on VEGF expression leads to a reduction in vascularity and asthmatics treated with high doses of ICS have a reduced number of blood vessels in the airway wall (Orsida et al. 1999; Hoshino et al. 2001).

Prolonged treatment with ICS has contentiously been shown to lead to a reduction in RBM thickness but the reduction in RBM thickness was not observed after three months of treatment (Hoshino et al. 1998; Ward et al. 2002). Further studies have followed which investigate the potential for glucocorticoids to be used to reverse airway remodelling, with limited success (Vlahos et al. 2003; Lee et al. 2008; Chachi et al. 2015; Chetta et al. 2003). A major limitation is that targeting inflammation with ICS does not eliminate AHR (Lundgren et al. 1988).

1.4.3 Leukotriene receptor antagonists

Additional treatments can now be added in combination for asthma sufferers such as leukotriene modulators and IgE-targeting therapies. Leukotrienes are lipid eicosanoids produced in leukocytes and derived from arachidonic acid. They are key inflammatory mediators involved in the pathogenesis of asthma. Cysteinyl leukotrienes (CysLT) have an established role in maintaining adverse asthmatic airway inflammation and contributing to airway obstruction (Hallstrand & Henderson. 2010). Leukotrienes have also been shown to be powerful bronchoconstrictors, with great potency for ASM contraction (Barnes et al. 1984). CysLTs have also been implicated in the promotion of

ASM proliferation (Cohen et al. 1995; Panettieri et al. 1998). An increase in the receptor for CysLTs (CysLT1 receptor) is observed in mild asthma and proceeds to further increase with disease severity (Zhu et al. 2005). Leukotriene receptor antagonists (LTRA) include montelukast and zafirlukast; both of which are potent CysLT1 receptor antagonists. Their blockade of the CysLT1 receptor (demonstrated by montelukast) appears to improve lung function, AHR and control eosinophilic inflammation (Amlani et al. 2011); however, ICS are still considered to be a more efficacious monotherapy (Chauhan & Ducharme. 2012). Anti-leukotrienes are advised to be added in combination with ICS for adolescents and adults with persistent asthma. This combination is beneficial in further reducing exacerbations and improving lung function when compared with stand-alone ICS treatment (Chauhan et al. 2017).

1.4.4 Theophylline

Theophylline is a methylxanthine that has been used to treat airway disease for almost a century and was initially used as a bronchodilator (Nyhan et al. 1974; Tuft & Brodsky 1936). However, very high concentrations of theophylline are required for the desired mechanism of bronchodilation and these concentrations are commonly associated with frequent side-effects (Barnes 2013). Theophylline functions through the inhibition of phosphodiesterase (PDE) 3, increasing levels of cAMP, leading to airway smooth muscle relaxation (Rabe et al. 1995). Therapeutically, theophylline can be better utilised at low dose concentrations, at which there are known anti-inflammatory effects (Sullivan et al. 1994; Lim et al. 2001). This occurs via a stimulatory effect upon histone deacetylase-2 (HDAC-2) (Barnes 2009). Restored levels of HDAC-2 increase suppression of inflammatory gene expression which, in the treatment of asthma, can boost the responsiveness of ICS.

1.4.5 Omalizumab

Omalizumab is an FDA-approved (as of 2003) monoclonal antibody that directly depletes IgE, inactivating basophils and mast cells. Overall, through this mechanism it reduces the airway's response to inhaled allergens (Fahy et al. 1997; Boulet et al. 1997). It has been shown to improve the asthma control in patients with both mild and particularly severe conditions as well as improving asthma-related quality of life (Milgrom et al. 1999). Omalizumab as an add-on for the treatment of moderate to severe allergic asthma has been shown to be predominantly safe and highly tolerable (Corren et al. 2009).

1.4.6 Anti-IL5

Between 2015 and 2018, three other monoclonal antibody drugs have received FDA-approval as therapies for severe asthma (Ramonell & Iftikhar 2020). Mepolizumab, Benralizumab and reslizumab have been designed to target IL-5 (Ortega et al. 2014; Nair et al. 2017; Bjermer et al. 2016) which interferes with the maturation and release of eosinophils, making these therapies suitable in the treatment of patients with persistent eosinophilia that isn't suppressed by corticosteroids (Egan et al. 1999; Haldar et al. 2009; Ghazi et al. 2012; Shrimanker & Pavord 2017).

1.4.7 Experimental Therapies

Master regulators of asthma in the epithelium are thought to be TLSP, IL-25 and IL-33. They each play a role in the activation of immune cells and the subsequent secretion of IL-5 and IL-13 (Verstraete et al. 2017). Inhibitors of IL-25 and IL-33 are in the early stages of trials, whilst inhibitors of TSLP are being developed and tested. Tezepelumab is a human-specific monoclonal antibody that targets the interaction between TSLP and its receptor complex. In early experiments it was shown to reduce allergen-induced bronchoconstriction in early and late phase asthma as well as attenuating systemic and

airway inflammation, also in early and late phase asthma (Gauvreau et al. 2014). In a randomised control trial, the number of exacerbations was reduced in patients (with severe and uncontrollable asthma) who were given Tezepelumab, accompanied by improved lung function and asthma control compared with those who received a placebo (Menzies-Gow et al. 2021). AstraZeneca's Biologics License Application (BLA) for tezepelumab has since been accepted and granted Priority Review for the treatment of asthma from the FDA.

Relaxin is a hormone protein belonging in the insulin family and is thought to be naturally responsible for relaxing the pubic ligament and mediating several changes during pregnancy (Samuel et al. 1998). The recombinant form of relaxin is shown to have anti-fibrotic properties in a variety of tissues such as the heart, liver, kidneys and lung (Samuel et al. 2016). In a murine model of allergic airways disease (AAD), Royce et al. have shown that expression of relaxin is significantly lower than controls and treatment with human gene-2 relaxin (H2 relaxin) is able to reverse changes associated with airway remodelling such as collagen deposition back to baseline (Royce et al. 2009). Serelaxin (recombinant human relaxin) has been shown to consistently inhibit fibrosis from chronic inflammatory insult and dysregulated repair (Kanai et al. 2019). A peptidomimetic of recombinant H2 relaxin has recently been developed, advancing the possibility for a novel fibrosis and airway remodelling therapy in asthma (Hossain et al. 2016).

Bronchial thermoplasty is a controversial therapy currently approved to target airway remodelling, however there is no consensus on the scientific verification of the proposed clinical benefits (Berair & Brightling 2014). The mechanisms that underlie the proposed beneficial role of thermoplasty are still poorly understood. Bronchial thermoplasty involves applying radio frequency thermal energy directly to airways in a series of

bronchoscopies, targeting ASM for removal (Laxmanan & Hogarth 2015). It was initially trialled in dogs, returning positive results with significant improvement in AHR at temperatures of 65 and 75 degrees Celsius. The inverse correlation between ASM mass and AHR suggested that reducing ASM mass through thermoplasty could be an effective means of reducing AHR and airway obstruction experienced in severe asthmatics (Danek et al. 2004). The application of bronchial thermoplasty in humans was shown to be feasible and with a good degree of tolerance. Treatment of airways in areas scheduled for lung resection resulted in a reduction of ASM mass and no accompanying adverse clinical effects (Miller et al. 2005).

Despite advances in treating inflammation as an important hallmark feature of asthma, current therapeutics do not effectively target progressive airway fibrosis and remodelling.

1.5 Autophagy

Macroautophagy (autophagy) is a fundamental cellular and physiological process that occurs in all eukaryotic cells (Mizushima & Levine 2010). Informally it is referred to as the inner recycling mechanism of all cells. Autophagy maintains homeostasis, ensures cell survival and recycles cellular components. During infection, autophagy has the capability to clear invading microorganisms and toxic protein aggregates. More regularly autophagy removes aging proteins, large molecular complexes, and obsolete or damaged organelles. Autophagy also has the potential to influence inflammation, innate immune functions and promote programmed cell death. All of these influences on vital cellular processes establish autophagy as a crucial regulator in pathogenesis of human disease (Ryter et al. 2012). Autophagy involves the sequestration of targeted components in double-membraned autophagosomes (derived from the ER). The cargo-carrying autophagosomes then fuse with a lysosome, forming autophagolysosomes. This fusion is

integral for the resultant delivery, degradation and recycling of cytoplasmic components (Choi, Ryter & Levine 2013).

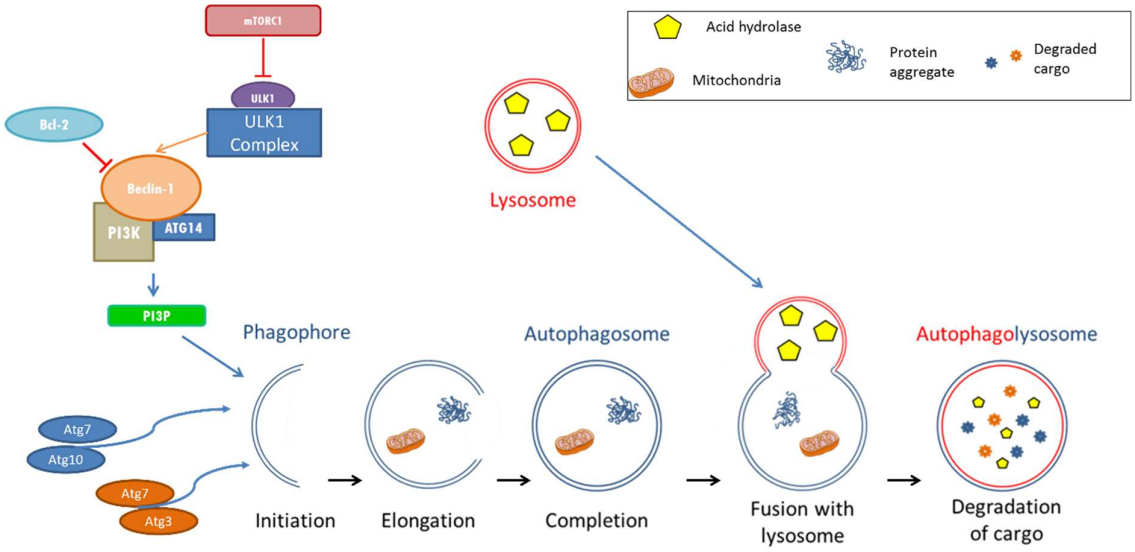


Figure 3. Autophagy process

Inhibition of mTORC1 due to starvation or energy exhaustion or stimulation of the class I phosphatidylinositol 3-kinase (PI3K)-AKT pathway contributes to the initiation of the autophagy process. Stimulation of the beclin-1-interacting complex is integral to autophagosomal membrane nucleation. Bcl-2 proteins interact with beclin-1 and inhibit autophagy, whilst the phosphorylation of Bcl-2 regulates the initiation or nucleation step of autophagy. Phagophore is formed (derived from the ER) and elongation requires the ATG5-ATG12 conjugation system and assembly of the microtubule-associated protein light chain 3 (LC3-ATG8) conjugation system. Cleavage of LC3 and subsequent conjugation to phosphatidylethanolamine (PE) is integral in the formation of the autophagosome. Unfolded/misfolded/aggregated protein and other targeted components are sequestered in double-membraned autophagosomes. The cargo-carrying autophagosomes then fuse with a lysosome, forming autophagolysosomes. This fusion is integral for the resultant delivery, degradation and recycling of cytoplasmic components.

The term “autophagy” was first coined in 1963 by Christian de Duve. Notably, de Duve also made the discovery of lysosomes (de Duve 1983; De Duve et al. 1955). Electron microscopy then became the key in unravelling the initial secrets of autophagy (De Duve & Wattiaux 1966). Evidence for the formation of a double-membraned phagophore and its closure into the autophagosome (Arstila & Trump 1968) provided great insight into the structures involved in the cellular process, but the true nature of autophagy function and mechanism would not be uncovered for another two decades. In the early 1990’s, future Nobel Prize recipient Yoshinori Ohsumi made the discovery of 15 genes of key importance for autophagy (Autophagy-related gene ATG) in yeast cells. His lab also described the mechanism of autophagic degradation of cytosolic components in the vacuoles of yeast cells (Baba et al. 1994; Takeshige et al. 1992; Tsukada & Ohsumi 1993). Several ATG genes were then cloned and the characteristics of their protein products were characterized (Funakoshi et al. 1997; Kametaka et al. 1996; Matsuura et al. 1997). The discovery of the important and essential autophagy related protein 12 (ATG12)-ATG5 conjugation system in autophagy followed by the conjugation of ATG8 to phosphatidylethanolamine (PE) and the understanding of their connection in the role of autophagosome formation truly propelled research in the greater understanding of autophagy mechanism (Ichimura et al. 2000; Mizushima et al. 1998; Xie, Nair & Klionsky 2008). In 2003, Levine B et al. showed that tumorigenesis was promoted in beclin-1 knockout mice, and in 2006, Mizushima N et al. showed neurodegeneration in brain-specific ATG5 knockout mice (Hara et al. 2006; Qu et al. 2003b). The role of autophagy in disease pathogenesis is emerging as a truly important path of research.

The process of autophagy consists of initiation, elongation, formation, maturation and ultimately, the degradation of contents.

1.5.1 Initiation

The mammalian target of rapamycin complex 1 (mTORC1) negatively regulates a complex consisting of UNC-51-like kinase 1 (ULK1), ATG13, ATG101 and FIP200. Inhibition of mTORC1 due to starvation or energy exhaustion allows for the activation of ULK1 and thus contributing to the initiation of the autophagy process (Choi, Ryter & Levine 2013). Stimulation of the class I phosphatidylinositol 3-kinase (PI3K)-AKT pathway is also linked to autophagy initiation. The other complex involved in the initiating steps of autophagy is the beclin-1-interacting complex. This complex consists of beclin-1, BCL-2 family proteins (which inhibit autophagy), the class III PI3K, and ATG-14. The beclin-1/class III phosphatidylinositide 3-kinase (PI3K) complex is integral in autophagosome nucleation (Kihara et al. 2001). Stimulation of this complex generates phosphatidylinositol-3-phosphate (PI3P), which promotes autophagosomal membrane nucleation (Choi, Ryter & Levine 2013). Bcl-2 proteins inhibit autophagy through the interaction with beclin-1 (Pattingre et al. 2005) and the phosphorylation of Bcl-2 regulates the initiation or nucleation step of autophagy (Wei et al. 2008).

1.5.1.1 Beclin-1

Stimulation of Beclin-1 is integral in the nucleation of the autophagosomal membrane and has a critical role in the regulation of autophagy. Through interactions with either Bcl-2 or PI3K it regulates the activation of autophagy. Beclin-1 is also important in the early stages of lymphocyte development (Arsov et al. 2011). It is also heavily involved in autophagic programmed cell death (Zhong et al. 2009). The beclin-1 protein is encoded by the BECN1 gene and an increased expression and activation of beclin-1 in certain complexes relates to an increase in autophagy (Kang et al. 2011).

1.5.2 Induction

Hypoxia is capable of rapidly inducing autophagy via the hypoxia inducible factor (HIF-1). In the most severe hypoxic conditions and states of anoxia, an autophagy response is induced independently of HIF-1. This occurs via the AMPK-mTOR and unfolded protein response (UPR) pathways. Starvation is a potent physiological regulator of autophagy. Through inhibition of the mammalian (or mechanistic) target of rapamycin (mTOR) autophagy is induced. mTOR is a serine/threonine protein kinase and suppresses autophagy in normal physiological conditions (Choi, Ryter & Levine 2013). Autophagy has also been shown to be induced by reactive oxygen species (ROS) (Chen et al. 2007; Jain et al. 2010). Upon activation by ROS, autophagy is employed to remove large quantities of oxidised proteins during conditions of elevated oxidative stress (Huang, Lam & Brumell 2011). Under extreme stress conditions autophagy may trigger cell death which is ultimately due to failed adaptation (Mazure & Pouyssegur 2010).

1.5.3 Elongation, formation & maturation and degradation

Autophagosomal elongation requires the ATG5-ATG12 conjugation system and the microtubule-associated protein light chain 3 (LC3-ATG8) conjugation system (Choi, Ryter & Levine 2013). ATG12 is first activated by via linkage by ATG7 followed by ATG10 before binding to ATG5. Introduction of ATG16 then forms the ATG5-ATG12-ATG16 complex which is present in the phagophore or autophagy isolation membrane (Farooq & Walsh 2016). The phagophore that this complex resides in is destined to form an autophagosome. ATG8 is also known as microtubule-associated protein light chain 3 (LC3). The terminal amino acid of LC3 is cleaved by ATG4. LC3 is then linked to ATG7, followed by ATG3, and finally conjugated to phosphatidylethanolamine (PE) (Choi,

Ryter & Levine 2013; Farooq & Walsh 2016). This cleaving step and ensuing conjugation to PE is integral in the formation of the autophagosome.

1.5.3.1 microtubule-associated protein light chain 3 (LC3)

The conversion of cytosolic LC3 (LC3-I) to the PE-conjugated form (LC3II), visible with immunofluorescence analysis as discrete puncta on the autophagosome membrane, indicates autophagosome formation (Choi, Ryter & Levine 2013). Upon fusion with the lysosome, LC3-II in the autophagolysosome is degraded along with the autophagosomal cargo. This lysosomal turnover of LC3-II is indicative of autophagy activity and can be utilised as a reliable method for monitoring autophagy flux (Klionsky et al. 2012). Another role for LC3B is the recruitment of Sequestosome-1 into the autophagosome (Shvets et al. 2008).

1.5.3.2 Sequestosome-1 (SQSTM1/p62)

Sequestosome-1 (SQSTM1/p62) is a ubiquitin-binding protein that targets and binds to other proteins to undergo selective autophagy. p62 recognises and co-localises with polyubiquitinated protein aggregates (Kuusisto, Salminen & Alafuzoff 2001; Stumptner et al. 2007). p62 guides ubiquitinated protein to and through the autophagy process, where it is also ultimately degraded in the lysosome. Short-lived proteins are mostly degraded by the ubiquitin-proteasome system (UPS), where proteins conjugate to chains of ubiquitin and are directed to the 26S proteasome (Kleiger & Mayor 2014). Long-lived proteins and damaged organelles are more typically degraded by autophagy. Lysosomes are membrane-enclosed organelles that contain degradative enzymes that hydrolyse proteins, polysaccharides and lipids. Upon fusion with the autophagosome it is their role in autophagy to digest these damaged or obsolete intracellular components. Autophagy

contributes to the process of antigen processing and delivery to MHC class II-containing compartments (MIICs). It also facilitates extracellular antigen processing by lysosomal hydrolysis for MHC class II presentation (Jyothula & Eissa 2013). Augmentation of this mechanism has been experimentally exploited to improve efficacy of the Bacille Calmette-Guérin (BCG) vaccine (Jagannath et al. 2009).

p62 also has the ability to bind directly to LC3 (Bjorkoy, Lamark & Johansen 2006). Autophagy is upregulated via p62 blocking the interaction between Bcl-2 and beclin-1. Inhibiting or knocking down Bcl-2 leads to autophagy activation (Zhou et al. 2013). p62 is a key regulator of autophagy all throughout the process. Interactions with Bcl-2 and its influence on the beclin-1-interacting complex have key importance in the earliest stages of autophagy initiation. The ubiquitin-binding of p62 has brought focus to the protein with potential as an additional diagnostic marker for various diseases that feature inclusion bodies (Komatsu & Ichimura 2010). p62 accumulates when autophagy is inhibited and inversely, levels of p62 decrease when autophagy is induced. Studies of p62 in the autophagy process can be utilised effectively to monitor autophagy flux (Bjorkoy et al. 2009).

1.5.4 Measuring Autophagy

Transmission electron microscopy (TEM) was initially the accepted and appreciated way of measuring autophagy (Ashford & Porter 1962; Deter & De Duve 1967). While it continues to serve as an integral diagnostic tool in relation to autophagy measurement, it only allows for qualitative analysis and as such, further assays for monitoring autophagy flux have been developed (Klionsky et al. 2012).

ATG8/LC3 can be utilised as an autophagosome marker using the identification of its non-lipidated and lipidated forms (LC3-I and LC3-II). ATG8/LC3-I is approximately 18 kDa and ATG8–PE/LC3-II is approximately 16 kDa. LC3-II is localised on autophagosomes during their formation. The quantification of LC3 turnover to the PE conjugated form can help to identify the amount of complete autophagosomes (Klionsky et al. 2012; Mizushima & Yoshimori 2007). Caution must be taken when quantifying LC3 by western blot and using it for autophagy analysis. Ratios of the two forms of LC3 may not be accurate in determining the amount of autophagy flux, however the comparison of levels of LC3-II between samples may be a useful marker for autophagy (Klionsky et al. 2012). LC3-II is degraded by the lysosome after the formation of the autophagolysosome, therefore lysosomal turnover of LC3 may better reflect overall autophagy activity (Tanida et al. 2005). Combination of immunohistochemistry for autophagy markers LC3 and p62 (SQSTM1) are combined efficient methods for measuring autophagy (Daniels et al. 2013). The use of autophagy markers needs to be accompanied by complementing assays in order to estimate the overall autophagy flux (Klionsky et al. 2012). The green fluorescent protein (GFP)-LC3B assay has been used to measure flux. When the GFP-LC3B is degraded in the lysosome, GFP protein, which is relatively resistant to the lysosomal hydrolases, survives. Free GFP (in the lysosome) measured by western blot can indicate the lysis of the autophagosomal cargo (Shintani & Klionsky 2004). Fluorescence microscopy may also measure the delivery of GFP-LC3 to the lysosome (Kabeya et al. 2000), although both methods are difficult to quantify (Klionsky et al. 2012). Beclin-1 is inhibited and bound by Bcl-2 (Pattingre et al. 2005) and autophagy is induced upon the release of beclin-1. The detection of GFP-beclin-1 puncta via fluorescence microscopy or TEM can be utilised as a marker for autophagosomal nucleation and autophagy induction (Yue et al. 2002). Multispectral imaging flow

cytometry has been demonstrated as a fast and reliable method for counting punctate GFP-(LC3) in a large number of cells (Dolloff et al. 2011). Further methods of detection and quantification of autophagy flux have taken advantage of the fluorescence-activated cell sorter (FACS) with much success (Eng et al. 2010; Hundeshagen et al. 2011; Shvets, Fass & Elazar 2008). An alternative quantitative method using image-based cytometry has been developed, demonstrating similar detection of autophagy to flow cytometry methods in live cells (Chan et al. 2012). The assessment of steady-state levels of autophagosomes may not be sufficient. The monitoring of autophagy flux or the measurement of autophagosome cargo may be required. The key to studying autophagy is the use of multiple assays.

1.5.5 Role of autophagy in disease

Autophagy may prevent or fuel the progression of disease (Choi, Ryter & Levine 2013). Whilst autophagy routinely plays a protective role, its functions such as cell survival can be deleterious (Levine & Kroemer 2008). Lower levels of autophagy are said to promote the progression of cancer, and ovarian cancer with higher levels of autophagy is characteristically less aggressive and more responsive to chemotherapy (Valente et al. 2014). An increased number of autophagosomes has been observed in patients suffering from acute necrotising pancreatitis (Helin et al. 1980). This has been found to be due to impaired autophagosome-lysosome fusion (Fortunato et al. 2009). Aggrephagy, or the insufficient clearance of aggregated protein, has been implicated in cystic fibrosis (Luciani et al. 2010). The accumulation of polyubiquinated proteins and the insufficient clearance of aggresomes in airway epithelial cells of cystic fibrosis patients characteristically describe dysfunctional aggrephagy (Luciani et al. 2010). Autophagy deficiency has been shown *in vitro* to result in dysregulation of NF-κB signalling. The

accumulation of p62 is involved in this process, resulting in the promotion of tumorigenesis (Mathew et al. 2009). Mutations in p62 have been found to be associated with Amyotrophic lateral sclerosis (ALS) and Paget's disease of the bone (Cavey et al. 2006; Fecto et al. 2011; Hocking et al. 2002). Mallory bodies found in various chronic liver disorders can be analysed with immunohistochemical staining for p62 (Stumptner et al. 2002). Other inclusion bodies, positive for p62, have been identified in several neurodegenerative diseases (Kuusisto, Salminen & Alafuzoff 2001).

Increased autophagosome formation is observed via electron microscopy in lung tissue from COPD patients. The high levels of ROS in cigarette smoke exposure augments autophagy in epithelial cells (Poon, Eidelman, et al. 2012). Cigarette smoke extract (CSE) decreases histone deacetylase (HDAC) activity which creates the downstream effect of increased LC3B-II expression. Due to chronic exposure to cigarette smoke, autophagy is associated with a pro-pathogenic phenotype in COPD (Chen et al. 2008). Alveolar macrophages isolated from current smokers showed impaired autophagy (Monick et al. 2010). Autophagy is important in the host defence against intracellular pathogens. Pathogens may need to successfully antagonise host autophagy in order to achieve prime virulence. The autophagy pathway successfully eliminates invading extracellular pathogens such as group A streptococcus. In autophagy-depleted (Atg5-/-) cells, group A streptococcus survive, proliferate and are released from the cells (Nakagawa et al. 2004). Autophagy is linked to the pathogenesis of several pulmonary disorders such as COPD, cystic fibrosis, and tuberculosis (Haspel & Choi 2011).

1.5.5.1 Ciliophagy

Ciliated cells of bronchi and bronchioles have numerous motile cilia which clear the airways of excess mucus in a process called mucociliary clearance (MCC). Ciliophagy is the process in which ciliary proteins, following ubiquitination and forming ciliary protein aggregates, are degraded and recycled through the endoplasmic reticulum stress–dependent autophagy pathway. Dysregulation and an imbalance, with excessive ciliophagy can ultimately result in shortening of cilia and impaired MCC (Cloonan et al. 2014). Ciliophagy accompanying an increase in autophagy has been described in COPD lungs following cigarette smoke exposure and deletion of Beclin1 and LC3B was shown to mitigate these changes in mice (Cloonan et al. 2014). Regulators of autophagy can negatively impact the physical characteristics and function of cilia in the respiratory airways through increased levels of ciliophagy, and novel treatments targeting these pathways could provide a new approach in severe asthma treatment.

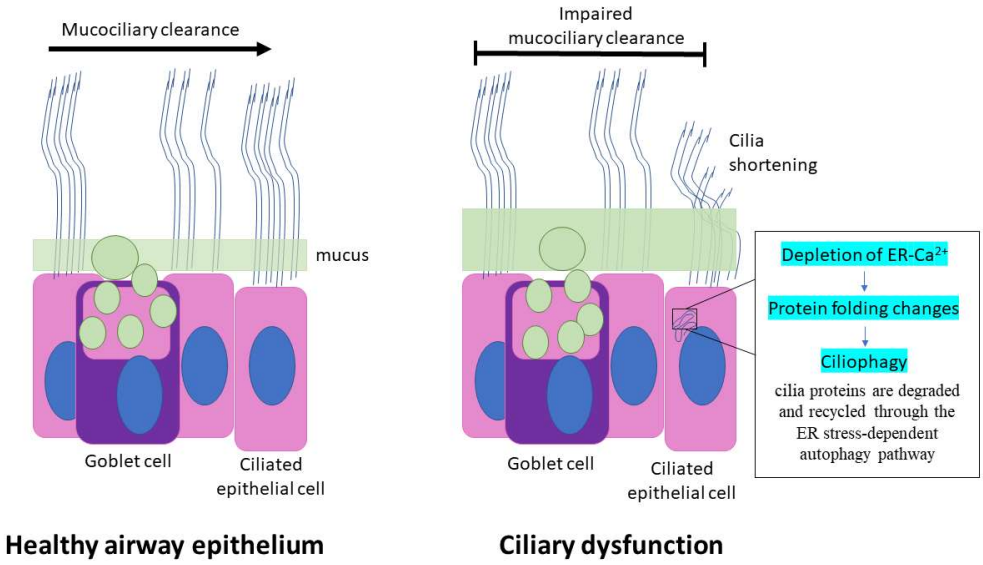


Figure 4. Ciliophagy in airway epithelium

Mucus-producing goblet cells and ciliated cells line the epithelium in the larger airways. In healthy individuals, cilia function to constantly beat and clear mucus up the respiratory tract and into the throat (mucociliary clearance (MCC)). Homeostatic disruption can lead to damage to epithelial cells and cilia. Depletion of endoplasmic reticulum (ER) – Ca^{2+} may result in protein misfolding and the formation of intracellular protein aggregates. Ubiquitinated Cilia protein aggregates accumulate in autophagosomes and delivered to the lysosome for degradation or recycling (ciliophagy). In the remodelled airways of sever asthmatics cases, chronic dysregulation can lead to degradation of cilia proteins, resulting in the shortening of cilia and impaired MCC.

(Adapted from: "Ciliophagy": The consumption of cilia components by autophagy, by S. M. Cloonan, H. C. Lam, S. W. Ryter, A. M. Choi , 2014, Autophagy. Copyright © 2021 Informa UK Limited.)

1.5.6 Role of autophagy in asthma

In a Canadian population, a Single-nucleotide polymorphism (SNP) of the ATG5 gene (rs12212740, G>A) was found to be associated with asthma and FEV1% predicted (Poon, Chouiali, et al. 2012). The same researchers, using electron microscopy, observed greater prevalence of autophagosomes in fibroblast and epithelial cells from the bronchial biopsy of an asthmatic patient, compared to cells from a healthy donor (Poon, Eidelman, et al. 2012). This raises questions about the state of autophagy flux in asthma. An increase in the number of autophagosomes could be due to an overall increase in autophagy flux or it could represent an impairment of a terminal step in the autophagy process (Jyothula & Eissa 2013). It has also been shown that ATG5 variants are associated with childhood asthma (Martin et al. 2012). Lack of autophagy in pulmonary CD11c+ cells has been shown to induce neutrophilic airway inflammation and hyperreactivity, driven by dendritic cells. This implies a protective role for autophagy in the pathogenesis of asthma (Suzuki et al. 2015). However, more autophagosomes were identified (by TEM) in asthmatic patients and ovalbumin (OVA)-challenged mice compared to healthy control (Liu et al. 2017). Autophagy may play opposing roles in asthmatic airway remodelling and inflammation in different cells of the lung. Exhaled levels of ROS correlate with the severity of asthma (Sanders et al. 1995). Production of ROS also correlates with airway hyperresponsiveness (de Boer et al. 2001). Autophagy is induced upon ROS exposure. Thus, in asthma with greater severity and elevated levels of ROS, the presence of autophagy may be amplified. Higher levels of autophagy were observed in sputum granulocytes and peripheral blood cells of severe asthmatics in comparison with mild

asthmatics and healthy donors (Ban et al. 2016). Dexamethasone treatment didn't affect these autophagy levels.

IL-13 has been shown to activate autophagy in epithelial cells. Prolonged exposure to IL-13 led to the increase of LC3-II expression. A mouse model of asthma showed that IL-33 stimulation resulted in IL-13-dependent formation of airway goblet cells. Mitigation of IL-13-mediated ROS generation was achieved through autophagy inhibition (Dickinson et al. 2016). IL-13 stimulation of epithelial cells isolated from asthmatic patients results in greater release of transforming growth factor-beta 1 (TGFβ1 (Wen et al. 2002) promoting TGF-β-dependent airway remodelling changes including IL-13-dependent goblet cell hyperplasia (Kondo et al. 2008). Autophagy is shown to be involved in IL-13-regulated increase in MUC5AC secretion (Dickinson et al. 2016). Therefore, upregulation of autophagy in the presence of increased TGF-β production may propel the progression of airway remodelling.

1.5.7 Role of autophagy in asthmatic airway remodelling

Fibrosis and remodelling in the heart, liver and kidneys has links to autophagic mechanisms (Ding & Choi 2014; Ghavami et al. 2015; Hernandez-Gea et al. 2012). TGF-β-induced autophagy has been shown to be required for a fibrotic response in ASM cells (Ghavami et al. 2011). Several ECM components can modulate autophagy signaling pathways and this ECM-regulated autophagy is proposed to maintain tissue homeostasis (Lock & Debnath 2008; Neill, Schaefer & Iozzo 2014). Dysfunctional autophagy in the presence of increased TGF-β may propel the progression of airway remodelling (Ghavami et al. 2015). A positive correlation of ATG5 and COL5A1 gene expression in the airways of patients with refractory asthma supports this link between dysregulated

autophagy and fibrosis in the airways (Poon et al. 2017). Creating a deeper understanding of how autophagy contributes to fibrosis and remodelling will unlock novel avenues for potential therapies (Kota et al. 2017).

In a murine model of asthma (OVA-challenged) the interaction between Follistatin-related protein 1 (FSTL1), autophagy, and EMT has been observed. Expression of FSTL1 and markers of autophagy were notably upregulated in the airways of OVA-challenged mice, which was further validated *in vitro*. Targeting FSTL1 pharmacologically may reduce EMT and airway remodelling through the inhibition of autophagy, or alternatively, inhibiting autophagy may reduce FSTL1 expression, FSTL1-induced EMT and airway remodelling (Liu et al. 2017).

1.5.8 Autophagy targeting mechanisms

Autophagy can be reprogrammed. Flux can be restored, or it can be completely inhibited (See Figures 2 and 3). Inflammation and airway remodelling display contradictory involvement and levels of autophagy, therefore autophagy may need to be compartmentally targeted separately.

1.5.8.1 3-methyladenine (3-MA)

3-methyladenine (3-MA) was first identified in 1982 as having the ability to suppress the formation of autophagosomes (Seglen & Gordon 1982). It specifically inhibits autophagy through the inhibition of class III PI3K. 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 (Wu et al. 2010). 3-MA has been shown to attenuate an increase in LC3-II expression in autophagy-induced epithelial cells (Ban et al. 2016).

1.5.8.2 Bafilomycins

Bafilomycins are macrolide antibiotics derived from the bacteria Streptomyces griseus. Bafilomycins inhibit Vacuolar-type H+-ATPase (V-ATPase) (Werner et al. 1984). V-ATPases are capable of forming a proton gradient and regulating intracellular pH. Inhibition of V-ATPase via bafilomycin A1 (BafA1) leads to significant cytosolic acidification. At high concentration, bafilomycin is capable of blocking late-phase autophagy. BafA1 has been shown to alter the effector functions of resident alveolar macrophages through this mechanism (Bidani & Heming 1995). Inhibiting chemotherapy-induced autophagy with bafilomycin is also a promising direction for future cancer intervention (Li et al. 2016). The inhibition of autophagy via bafilomycin led to the induced apoptosis of osteosarcoma cells through the upregulation of beclin-1 and p53 (an apoptosis-related protein) (Xie et al. 2014). At lower concentrations BafA1 has been shown to target both early and late phase autophagy as well as apoptotic pathways. It induced apoptosis by targeting mitochondria and the translocation of apoptosis-inducing factor as well as inhibiting the formation of autophagolysosomes. Autophagy was further inhibited through the induced binding of beclin-1 to Bcl-2, promoting cell death. These actions have highlighted BafA1 as a prime candidate for treating B-cell acute lymphoblastic leukaemia (Yuan et al. 2015). In experiments exploring the anti-mitogenic effect of bitter taste receptors, pre-treatment with BafA1, followed by a taste 2 receptor (TAS2R) agonist mitigated the TAS2R-induced cell death in human ASM cells (Pan et al. 2017). At low concentrations, BafA1 has been shown to have good antiviral properties with an absence of host cell cytotoxicity (Yeganeh et al.

2015). Furthermore, pre-treatment with BafA1 can reduce the amount of TGF-β-induced fibrogenesis in human atrial myofibroblasts (Ghavami et al. 2015) which indicates promising future potential in the treatment of severe asthma.

1.5.8.3 Vacuolar-type H+-ATPase (V-ATPase)

Vacuolar (V-)ATPases are large ATP-driven proton pumps which don't synthesize ATP as they located in organelles such as endosomes, lysosomes and the golgi apparatus where a sufficient electrochemical gradient cannot be generated. They operate through the hydrolysis of ATP, in a rotary mechanism, with resultant proton translocation and functioning to acidify intracellular compartments (such as the lysosome) (Futai 2007; Forgac 2007; Moriyama & Futai 1990). V-ATPases are involved in processes such as bone resorption, tumour metastasis, and autophagy (Futai 2007). Mutations in V-ATPases have been found and linked to osteoporosis (Sobacchi et al. 2001; Kornak et al. 2000), distal renal tubular acidosis (Stover et al. 2002; Karet et al. 1999), and a rare genetic disease called X-linked myopathy with excessive autophagy (Ramachandran et al. 2013). V-ATPases are sensitive to macrolide antibiotics such as Bafilomycin A1 (Bowman et al. 1988).

1.5.8.4 LY294002

LY294002, derived from the flavonoid quercetin (Vlahos et al. 1994), inhibits PI3K activity (Blommaart et al. 1997). It may however not be exclusively specific for PI3K (Gharbi et al. 2007). LY294002 has been shown to attenuate an increase in LC3-II expression in autophagy-induced epithelial cells (Ban et al. 2016). LPS-induced hepatic injury has been shown to be attenuated with LY294002 through immunoregulation and

the suppression of leukocyte infiltration (Chen et al. 2016). In mice, it has also been shown to protect against myocarditis (Liu et al. 2016).

1.5.8.5 Rapamycin

Rapamycin (also known as sirolimus) is a macrolide derived from streptomyces hygroscopicus (Sehgal, Baker & Vezina 1975). Rapamycin's mode of action is through the inhibition of the mTOR complex, promoting the induction of autophagy and an overall increase in autophagy flux. Rapamycin also acts as an immunosuppressant, blocking the proliferation and differentiation of T cells (Dumont & Su 1996). In neurodegenerative diseases, a dysfunctional autophagy process can result in deficient clearance of protein aggregates and therefore induction of autophagy in this scenario has been proposed as strategically relevant.

1.5.8.6 Genetic deletions

Beclin-1-/- knockout mice have been shown to die early in embryogenesis and beclin-1+/- mice have been shown to develop spontaneous tumours (Qu et al. 2003a). The apoptotic response of beclin-1-/- embryonic stem cells is unaltered, but autophagy flux is severely dysregulated (Yue et al. 2003b). This indicates a tumour suppressive role for autophagy. Mice deficient in beclin-1 also displayed pro-angiogenesis characteristics through the upregulation of HIF-2α (Lee et al. 2011). Therefore beclin-1 knockout mice may not be an efficient and side-effect free method for autophagy modulation.

Other deletions of autophagy-related genes have been shown to be embryonic lethal (*Atg9a*) (Yue et al. 2003a), neonatal lethal (*Atg3, Atg5, Atg7*) (Komatsu et al. 2005; Kuma

et al. 2004; Sou et al. 2008; Yoshii et al. 2016), and viable (*Map1lc3b*) (Cann et al. 2008). Mice deficient in ATGs that function upstream of ATG conjugation systems and autophagy initiation experience have the poorest outcomes, with early lethality (Kuma, Komatsu & Mizushima 2017).

1.5.8.5 inositol trisphosphate (IP3) receptor modulation

Independent of the mTOR pathway, autophagy is also regulated by the inositol trisphosphate (IP_3) receptor. Reduction of intracellular IP_3 levels promotes autophagy, and increased IP_3 levels can inhibit autophagy. Blocking and knocking down the IP_3 receptor has been shown to strongly induce autophagy (Criollo et al. 2007). Lithium has been used for many decades in the treatment of bipolar disorder. Lithium has the power to induce autophagy via the inhibition of inositol monophosphatase (IMPase) and resultant reduction in intracellular IP_3 (Sarkar & Rubinsztein 2006). Lithium has been shown to induce autophagosomes in cancer cells, and in combination with existing chemotherapy, lithium can improve efficacy and promote cell death in the apoptosis deficient cancer cells (O'Donovan et al. 2015). Lithium-induced autophagy has the ability to clear prion protein aggregates in neuronal and non-neuronal cells *in vitro* (Heiseke et al. 2009).

HDAC inhibitors also regulate the initiation and flux of autophagy (True & Matthias 2012). HDAC inhibitors may be a good consideration for addition to the repertoire of potential autophagy modulators (Choi, Ryter & Levine 2013).

1.6 Concluding and linking remarks

The studies contained within this thesis highlight the pathophysiological changes in the human severe asthmatic lung and in the mouse lung upon exposure to stimuli of allergic airways disease (*in vivo*). The studies belong to the emerging body of research identifying different aspects of airway remodelling. To reiterate, current therapeutics do not effectively target progressive airway fibrosis and remodelling. In this thesis, we also investigate the involvement of autophagy/mitophagy pathways in asthmatic airway remodelling. In addition, we investigate the possibility of autophagy as a novel therapeutic target in asthma. Changes in airway smooth muscle may be targeted through autophagy modulation as well as overcoming steroid insensitivity in the treatment of severe asthma. In this thesis, we show that autophagy promotes and influences the classical TGF-β driven airway remodelling. In severe asthma, autophagy also maintains oxidative stress and delays apoptosis. As autophagy is increasingly implicated in the pathogenesis of asthma, autophagy modulation may be a novel therapeutic target for difficult to treat asthma (Ban et al. 2016).

Figure 5. Pathways of Autophagy Inhibition

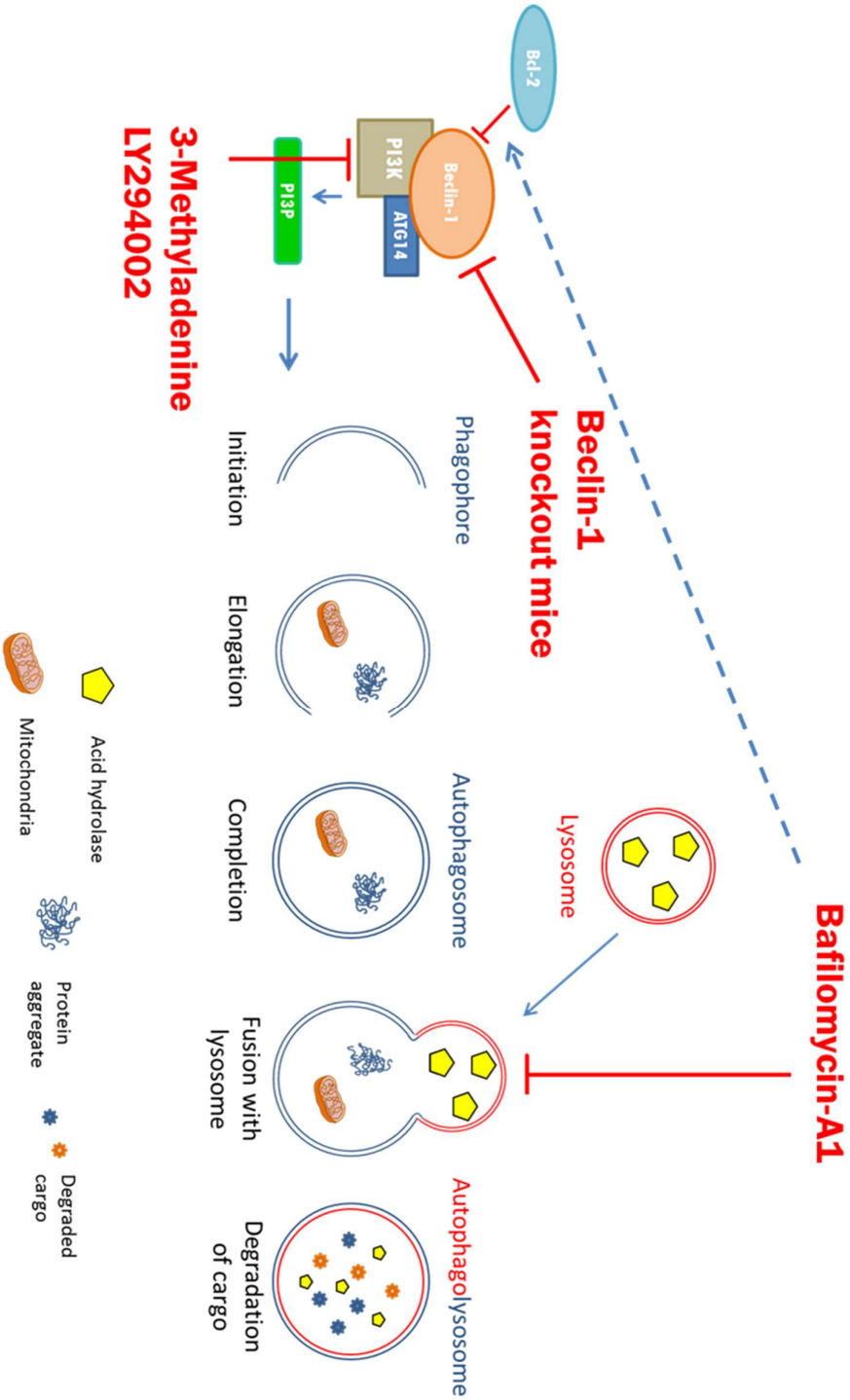
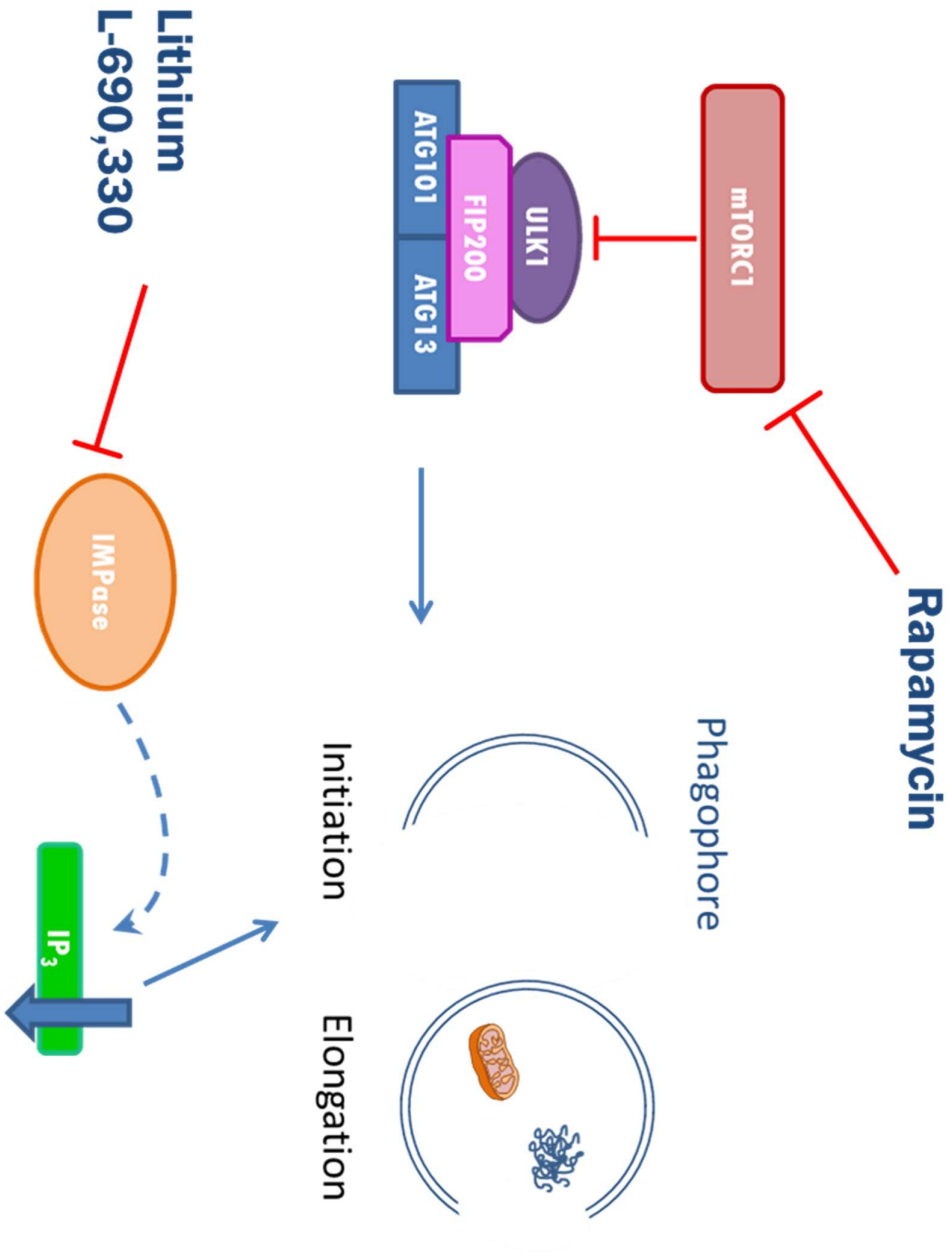


Figure 6. Pathways of Autophagy Induction



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Hypotheses and Aims

We hypothesize that autophagy induction is a central mechanism in driving airway remodelling in severe asthma.

The aims of this thesis are

1. To comprehensively characterize autophagy markers with respect to airway remodelling in human asthma.
2. To explore the role of ciliophagy in asthmatic airways.
3. To test the preclinical efficacy of autophagy inhibition in murine models of asthma (prophylactic and a treatment model).
4. To explore the link between autophagy and airway remodelling in vitro and to test autophagy inhibition in a murine model of severe asthma.

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Chapter 2

Autophagy Activation in Asthma Airways Remodelling

Autophagy Activation in Asthma Airways Remodeling

McAlinden K.D, Deshpande D.A, Ghavami S, et al. Autophagy Activation in Asthma Airways Remodeling. Am J Respir Cell Mol Biol. 2019;60(5):541-553.

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Abstract

Current asthma therapies fail to target airway remodelling 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 remodelling and assessed preclinical efficacy of a known autophagy inhibitor in a murine model of asthma. Human asthmatic and non-asthmatic lung tissues were histologically evaluated and were immuno-stained for 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 intranasally in prophylactic (3 wk) and treatment (5 wk) models of allergic asthma in mice. Human asthmatic tissues showed greater tissue inflammation and demonstrated hallmark features of airway remodelling displaying thickened epithelium (p<0.001) and reticular basement membrane (p<0.0001), greater lamina propria depth (p<0.005), and increased airway smooth muscle bundles (p<0.001) with higher expression of beclin-1 (p<0.01) and ATG5 (autophagy-related gene 5) (p<0.05) together with reduced p62 (p<0.05) compared to non-asthmatic controls. Beclin-1 expression was significantly higher in asthmatic epithelium and ciliated cells (p<0.05), suggesting a potential role of ciliophagy in asthma. Murine asthma model demonstrated effective preclinical efficacy (reduced both airway inflammation and airway remodelling) of the autophagy inhibitor chloroquine. Our data demonstrate cell context-dependent, and selective activation of autophagy in structural cells in asthma. Furthermore, this pathway can be effectively targeted to ameliorate airway remodelling in asthma.

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, remodelling and airway hyperresponsiveness (AHR) (Royce et al. 2012). Therapeutically, only inflammation and airway hyperresponsiveness can be sufficiently attenuated. The third pathophysiological link in the triadic asthma phenotype is airway remodelling, 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 remodelling in asthma (James et al. 2012). ASM hyperplasia and hypertrophy in the central airways are both indicators and traits of asthma severity (Benayoun et al. 2003). 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 remodelling (Redington et al. 1997; Chakir et al. 2003). Alongside having a multitude of effects in homeostasis and pathophysiology of disease, TGFβ is thought to drive airway remodelling through the activation of myofibroblasts and smooth muscle cells, subsequently inducing the release of fibrogenic extracellular matrix (ECM) proteins (Boxall et al. 2006). Emerging data suggest that autophagy promotes and influences this classical TGFβ driven airway remodelling (Ghavami et al. 2015). 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 (Mizushima et al. 2010). Autophagy is evolutionarily conserved and is involved in an immense variety of processes which have different effects on pathophysiology. 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-membraned autophagosomes and ultimately degraded upon the fusion with a lysosome with the cytoplasmic components recycled (Choi et al. 2013). 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 (Choi et al. 2013). The beclin-1/class III PI3K complex is integral in autophagosome nucleation (Kihara et al. 2001). Generation of PI3P occurs upon stimulation of beclin-1, and autophagosomal membrane nucleation is developed. 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 (Choi et al. 2013). During autophagy, a truncated cytosolic form of LC3 (LC3-I) is conjugated to PE to form LC3-II (Choi et al. 2013). This PE-conjugated form of LC3 is recruited to and associated with the autophagosomal membrane. Punctate LC3-II is visible with immunostaining and indicates complete formation of autophagosomes (Klionsky et al. 2012). Upon fusion with the lysosome, LC3-II in the autophagolysosome is degraded along with the autophagosomal cargo. Sequestosome-1 (SQSTM1/p62) is a ubiquitin-binding protein that targets and binds to other proteins for selective autophagy. p62 may guide ubiquitinated protein to and through the autophagy process, where it is also ultimately degraded in the lysosome (Stumptner et al. 2007). p62 accumulates when autophagy is

inhibited and inversely, levels of p62 decrease when autophagy is induced (Bjorkoy et al. 2009). LC3 recruits Sequestosome-1 into the autophagosome (Shvets et al. 2008). Whilst autophagy routinely plays a protective role, its functions such as cell survival can be deleterious (Levine & Kroemer 2008).

Autophagy is involved in the pathogenesis of various diseases and links between autophagy and asthma are emerging (Poon et al., Martin et al., Poon et al., 2012). 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 (Poon et al. 2017). ECM-regulated autophagy is proposed to maintain tissue homeostasis and thus dysfunctional autophagy in the presence of increased TGF-β may propel the progression of airway remodelling (Neil, Schaefer & Iozzo 2014). 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 associated with features of airway remodelling in a TGF-β-dependent manner. We found that TGF-β concomitantly induced autophagy and profibrotic signalling in ASM cells. This induction was prevented by chloroquine (CQ) *in vitro*. Furthermore, using mouse models of allergic asthma, we demonstrated that targeting the 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.

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	6/0

Histology

Human Lung Tissue Processing and Section Preparation

Dissected lung tissues were fixed, processed and embedded in paraffin for analyses (Eapen et al. 2017). After microtome sectioning, haematoxylin and eosin (H&E) staining and masson's trichrome staining were used to assess structural integrity, inflammation and features of airway remodelling. See the data supplement for full methods.

Morphometric analysis of inflammation and airway remodelling features

Lamina propria depth was measured perpendicularly from multiple points at the base of the RBM to the outer edge of ASM bundles, and the proportion of ASM in the airway wall (ASM/LP as a percentage) 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 the data supplement.

Immunohistochemistry and immunofluorescence staining

Immunostaining for beclin-1, ATG5, LC3B, p62 and ACTA2 was performed as previously described (Eapen et al. 2017; Sohal et al. 2010; Sharma et al. 2015). 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 using a method described previously. See the data supplement for full methods.

Mouse models of allergic asthma

Experiments were conducted according to the guidelines of the Australian code for the care and use of animals. The animal Care Committees of Thomas Jefferson University and University of Technology Sydney approved the protocol. All surgeries were performed under tribromoethanol anaesthesia, and all efforts were made to minimize suffering.

BALB/c mice (female) were subjected to a subchronic (prophylactic) model of allergic asthma as described. Thirty minutes before HDM challenges, selected mice were administered either chloroquine (CQ) intranasally (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. At Week 4 and commencing for 2 weeks, 30 minutes before HDM challenges, selected mice were administered either CQ intranasally (50 mg/kg) or vehicle (saline). In both studies, 24 hours 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 histopathological and biochemical analysis. See the data supplement for full methods.

Mouse BAL Immune Cell Staining, Lung H&E and PAS 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 stains using a protocol described previously (Sharma et al. 2012; Sharma et al. 2010; Sharma et al. 2014). 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, beclin-1, and LC3B in ASM cell lysates or murine lung tissues were measured by immunoblotting. All immunoblotting was performed using protocols described previously (Sharma et al., Deshpande et al., 2010). 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) (Schaafsma et al. 2011). See the data supplement for full methods.

Statistical analysis

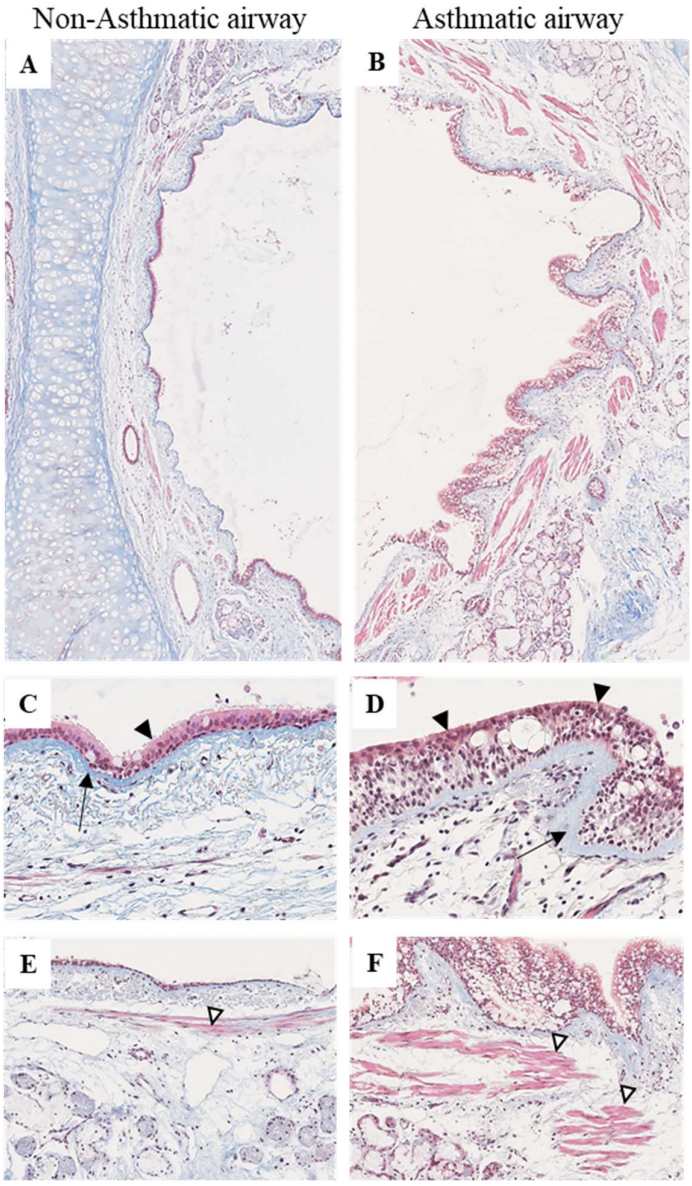
Data were analyzed using unpaired t-tests or one-way or two-way ANOVA as appropriate and are presented as mean ± SD or mean ± SEM. All data were analyzed with

PRISM V7.04 software (GraphPad, La Jolla, USA) and $p<0.05$ was considered statistically significant.

Results

Histological evidence of airway remodelling and inflammation

As the link between autophagy and airway remodelling has been established, we first chose to histologically measure remodelling features of our selected asthmatic and non-asthmatic patients. Asthmatic airways in comparison with non-asthmatic airways displayed greater features of airway remodelling. Gross remodelling changes between non-asthmatic and asthmatic large airways were observed utilizing trichrome staining (Fig. 4A-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. 4G-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. 4I-J). These measurements classify the selected asthmatic patients as having undergone associated airway remodelling and justify their inclusion into the immunohistological component of this study. As is also evident in Figure 4, asthmatic lungs also demonstrated greater influx of immune cells into the lungs.


Non-Asthmatic airway
Asthmatic airway
A
B
C
D
E
F

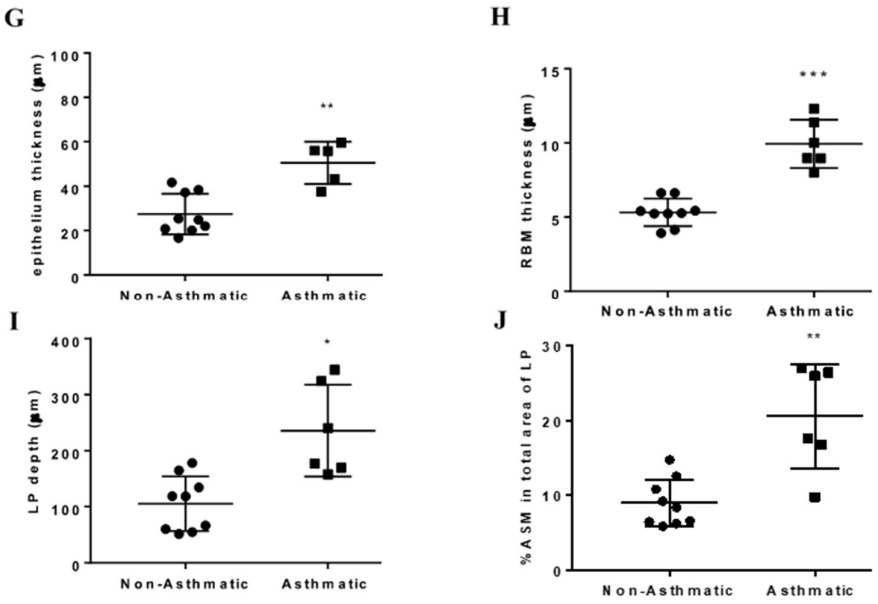


Figure 7. Histological evidence of airway remodelling in the large airways of the selected asthmatic patients. Non-asthmatic (A) and asthmatic (B) airways were stained with masson's trichrome staining as described in the methods. Original magnification, ×50 (A, B). Arrows indicate the RBM and arrowheads 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) and the proportion of ASM in the asthmatic airway wall (J) were quantified. (Data is expressed as mean ± SD; *p<0.005, ***p*<0.001, ****p*<0.0001).

Expression profile of autophagy marker proteins in the large airways of patients with asthma

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 remodelled asthmatic histopathology. There were no significant differences in the large airway epithelium (Figure 5), whereas in the large airway ASM bundles we observed a marked increase in the expression of beclin-1 (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 patients with asthma compared with those without asthma (Fig. 6).

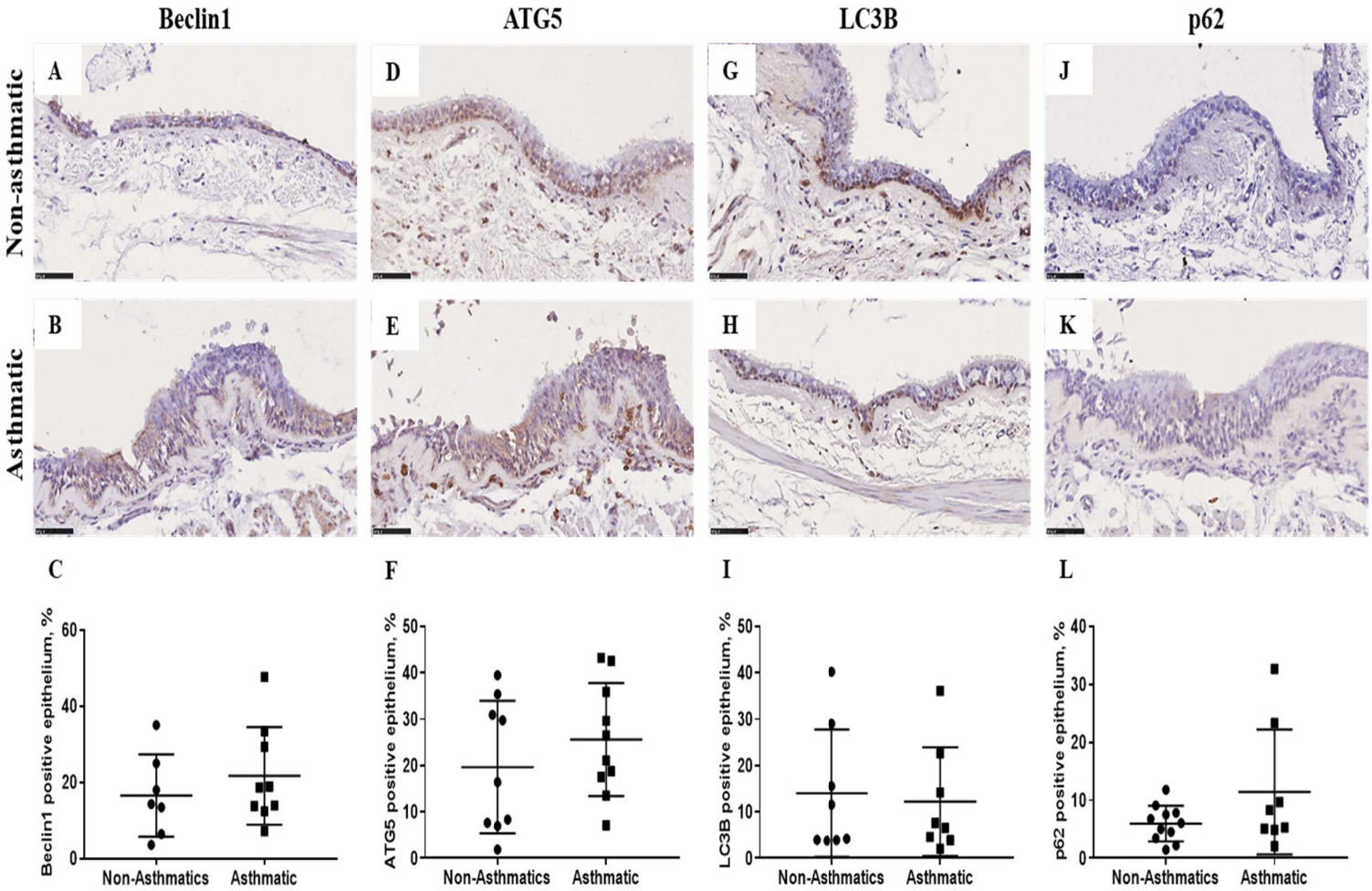

Beclin1
ATG5
LC3B
p62
Non-asthmatic
Asthmatic
A
B
C
D
E
F
G
H
I
J
K
L
Beclin1 positive epithelium, %
ATG5 positive epithelium, %
LC3B positive epithelium, %
p62 positive epithelium, %
Non-Asthmatics
Asthmatic

Figure 8. Expression of autophagy markers in the large airway epithelium of asthmatic and non-asthmatic tissue. Asthmatic and non-asthmatic tissue were immunohistochemically stained for beclin-1 (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).

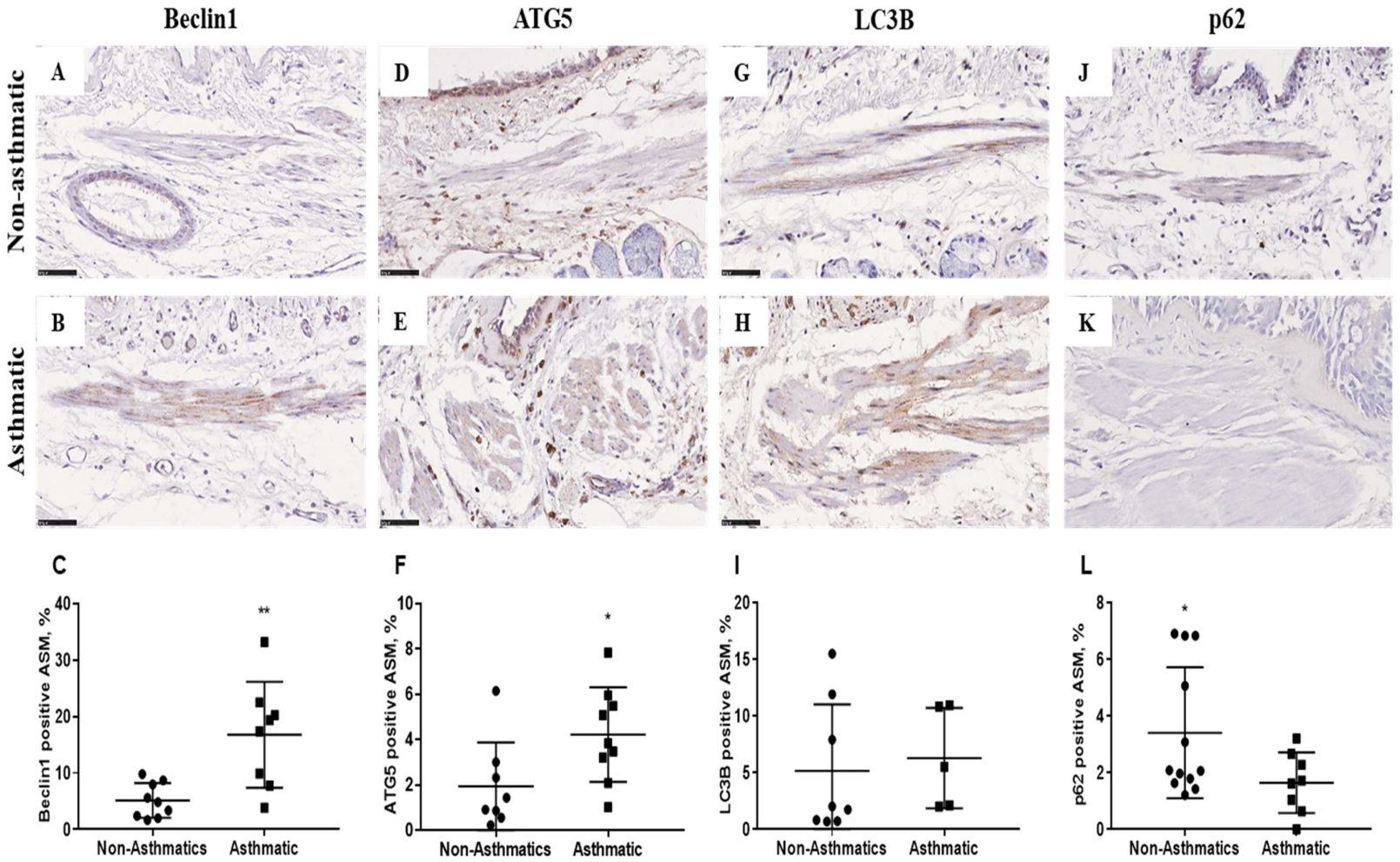

Beclin1
ATG5
LC3B
p62
Non-asthmatic
Asthmatic
A
B
D
E
G
H
J
K
C
F
I
L
Beclin1 positive ASM, %
ATG5 positive ASM, %
LC3B positive ASM, %
p62 positive ASM, %
Non-Asthmatics
Asthmatic
**
*
*
*

Figure 9. 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 beclin-1 (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).

Expression profile of autophagy markers in the small airways of patients with asthma

In the small airway epithelium, we observed solely a marked increase in the expression (percentage area ± SD) of beclin-1 (A: 19.44 ± 7.31% vs. NA: 11.44 ± 4.63%, $p<0.05$) (Fig. 7), with no significant differences in small airway ASM bundles (Fig. 8).

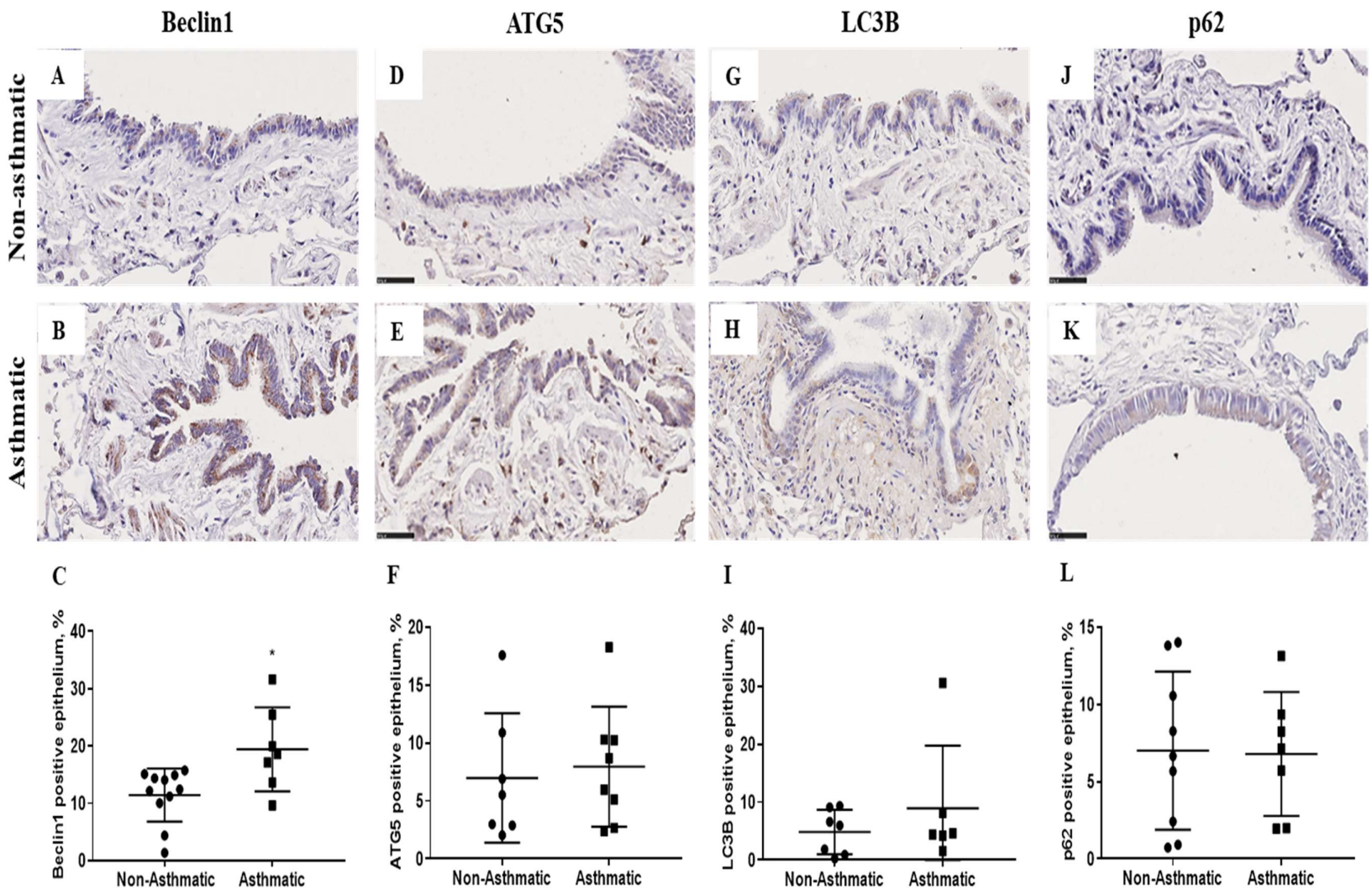

Beclin1
ATG5
LC3B
p62
Non-asthmatic
Asthmatic
A
B
D
E
G
H
J
K
C
F
I
L
Beclin1 positive epithelium, %
ATG5 positive epithelium, %
LC3B positive epithelium, %
p62 positive epithelium, %
Non-Asthmatic
Asthmatic
*

Figure 10. Expression of autophagy markers in the small airway epithelium of asthmatic and non-asthmatic tissue. Asthmatic and non-asthmatic tissue were immunohistochemically stained for beclin-1 (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; *$p<0.05$).

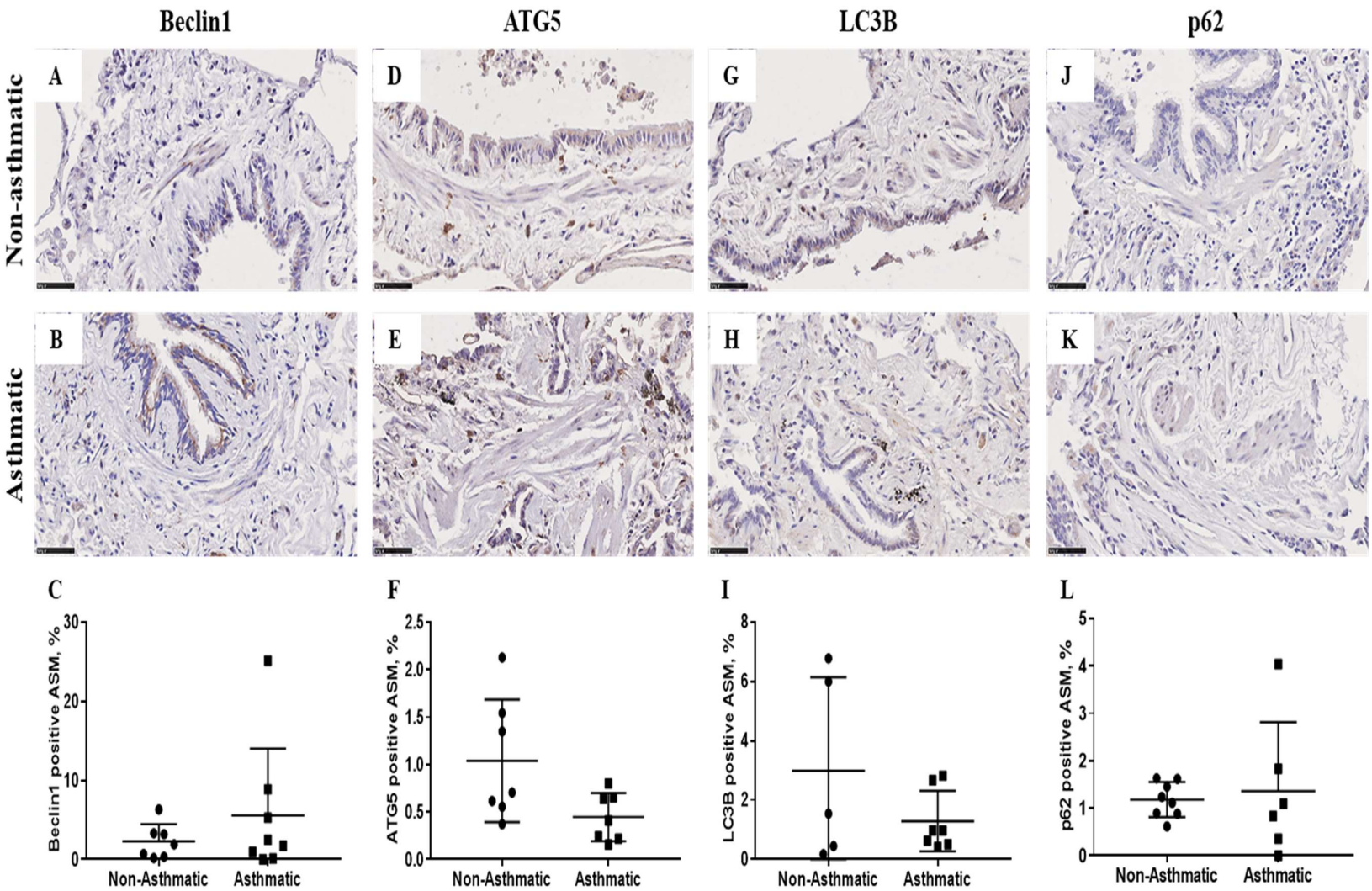

Beclin1
ATG5
LC3B
p62
Non-asthmatic
Asthmatic
A
B
D
E
G
H
J
K
C
F
I
L
Beclin1 positive ASM, %
ATG5 positive ASM, %
LC3B positive ASM, %
p62 positive ASM, %
Non-Asthmatic
Asthmatic

Figure 11. 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 beclin-1 (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).

Expression of beclin-1 in cilia lining the large airways of patients with asthma

In accordance with our classification, 5 of 6 patients with asthma displayed strong expression of beclin-1 in the cilia lining the large airway epithelium, whereas only 1 of 10 patients without asthma displayed any expression of beclin-1 in the cilia of the large airway epithelium. Overall, we have demonstrated that strong expression of beclin-1 in cilia lining large airway epithelial cells occurs mostly in patients with asthma with associated airway remodelling evident compared with patients without asthma (Fig. 9A-C). Other markers (ATG5, LC3B, p62) were not evident in the cilia lining of large airway epithelium from both patients with and without asthma.

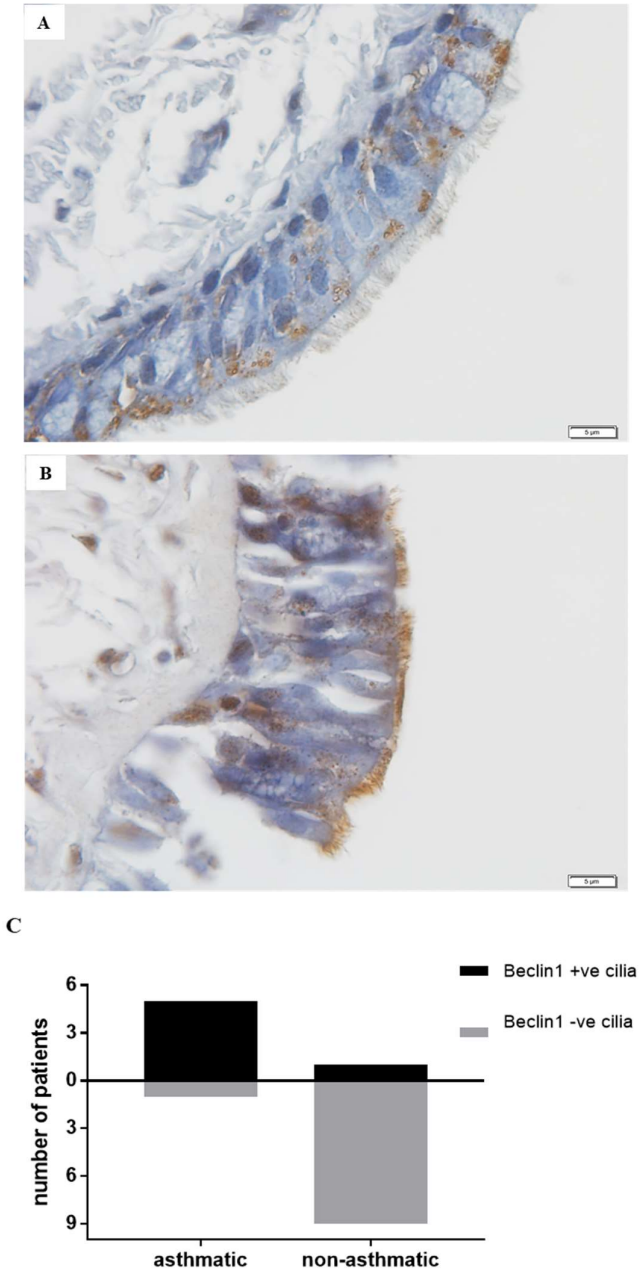


Figure 12. Expression of beclin-1 in cilia lining large airway epithelium of asthmatics. Non-asthmatic large airway epithelium (A) and asthmatic large airway epithelium (B) immunohistochemically stained for beclin-1. Arrowheads indicate cilia. Original magnification, ×1000. Representation of the number of patients (A v NA) with beclin-1 positivity in cilia (C).

Concomitant induction of profibrotic signalling and autophagy markers in human ASM 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 remodelling in asthma. Therefore, we tested this hypothesis that whether TGF- β1 (a pleiotropic cytokine, elevated in asthma) would concomitantly induce profibrotic signalling and autophagy *in vitro*. As shown in Figure 10, we found that TGF- β1, in a time-dependent manner, increased collagen I expression and SMAD2/3 phosphorylation (profibrotic signalling) and induced autophagy in ASM cells as seen with increased expression of beclin-1 and LC3B-II (Figure 10A). This demonstrates an association between the effects of TGF- β1, the accumulation of collagen, and increased profibrotic signalling in an autophagy-dependent manner.

Effect of autophagy inhibitor on the concomitant induction of profibrotic signalling and autophagy markers in human ASM cells

Furthermore, we tested *in vitro* whether the treatment with autophagy inhibitor CQ would alleviate profibrotic signalling and the induction of autophagy by TGF-β1 stimulation. As shown in Figure 10, we found that treatment with CQ (50 mM) in conjunction with TGF-β1 stimulation reduced the expression of collagen IA, beclin-1, and LCB3-II, as well as the phosphorylation of SMAD2/3, in a time-dependent manner (Figure 10). This shows that CQ reduced TGF- β1–induced airway remodelling markers in an autophagy-dependent (inhibition) manner.

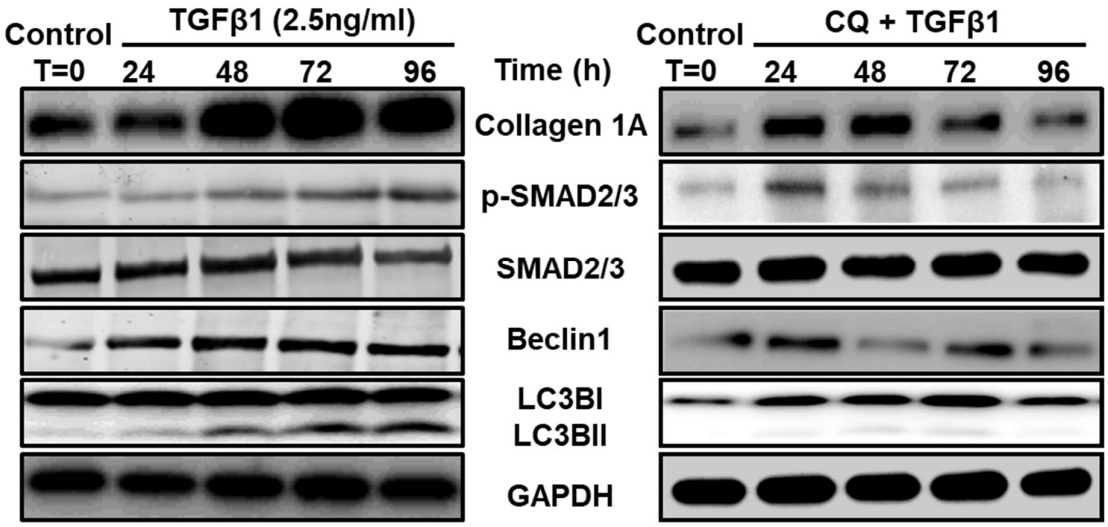


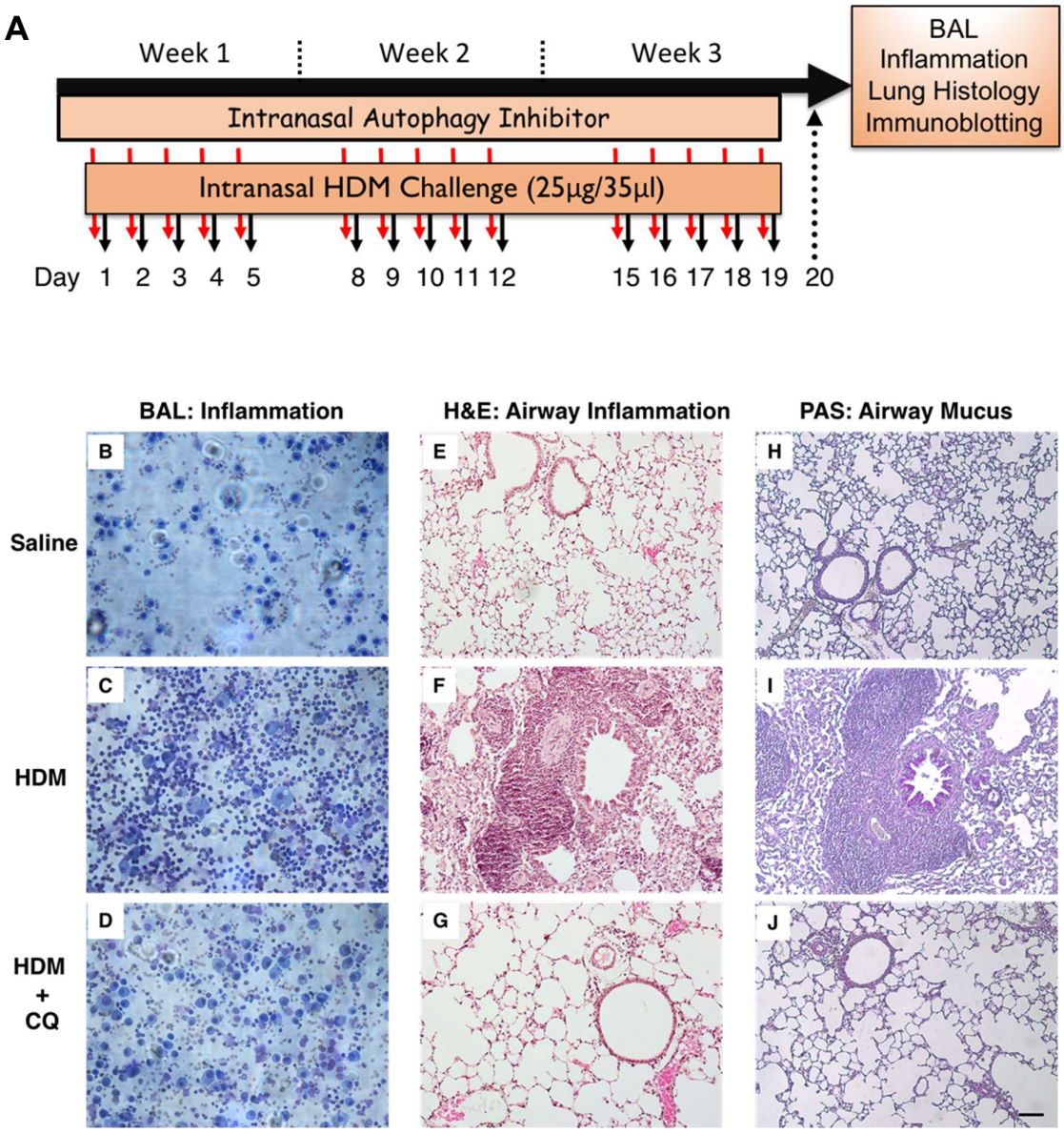
Figure 13. Concomitant induction of profibrotic signalling and autophagy by transforming growth factor (TGF)-β1*in vitro*. Human airway smooth muscle cells were treated with either TGF-β1 (2.5ng/ml) or TGF-β1 (2.5 ng/ml) + chloroquine (CQ) (50 mM) for 0, 24, 48, 72, and 96 hours. Cell lysates were prepared, and immunoblotting was performed for collagen IA, phospho-SMAD2/3, totalSMAD2/3, beclin-1, and LC3B-I/II. GAPDH was used as a loading control. Data shown are representative of four independent primary human airway smooth muscle cells. Western blot performed by co-authors and I am not in possession of the raw data relating to these figures.

Effect of autophagy inhibitor in a prophylactic model of allergic asthma

To further investigate the interplay between autophagy and airway remodelling we studied the effects of autophagy inhibition in allergen (HDM)-induced asthma in mice (prophylactic model) as shown in Figure 11A. 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, together with increased production of mucus, was observed in the HDM-challenged mice in comparison with control mice (Fig. 11). Autophagy inhibitor effectively blocked influx of immune cells into the airways (Fig. 11B-11D and 11K) and significantly reduced eosinophils and neutrophils in the BAL fluid with no effect on macrophages (Figures 11L–11N). Tissue inflammation was also reduced in the mouse lungs treated with CQ (Figures 11E–11G; H&E staining), and there was a reduction in mucus production in HDM-challenged mice treated with autophagy inhibitor CQ (Figures 11H–11J; PAS staining of airway mucus).

To examine the connection of autophagy with airway remodelling we performed assays to measure levels of TGFβ1 in the BAL and soluble lung collagen, and we performed immunoblot analysis of COL1A1, beclin-1 and ATG5 expression in the lung lysates. The HDM-challenged group showed significant increase in the concentration of TGFβ1 in the BAL compared with controls ($p<0.001$), whereas treatment with CQ significantly reduced TGFβ1 levels when compared with the HDM group ($p<0.05$) (Fig. 11O). Furthermore, we found a significantly greater amount of soluble lung collagen in tissue lysates of the HDM-challenged group than in the control group ($p<0.001$), whereas 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. 11P). Lung function measurements using flexiVent showed increased AHR in HDM-challenged mice in response to methacholine (MCh) when compared with control animals (increase in airway resistance at 25, 50, and 100 mg/ml), whereas CQ treatment prevented development of AHR in mice (significant reduction in airway resistance at 25, 50, and 100 mg/ml) when compared with the HDM-challenged group (Figure 11Q). Immunoblotting in lung lysates revealed higher and concomitant expression of ECM protein Collagen 1A and autophagy markers beclin-1 and ATG5 in the HDM-challenged mice when compared with control animals, whereas CQ treatment prevented induction of autophagy (as shown by the reduction in beclin-1 and ATG5) and accumulation of collagen in the lung (Fig. 11R).


A
Week 1
Week 2
Week 3
BAL
Inflammation
Lung Histology
Immunoblotting
Intranasal Autophagy Inhibitor
Intranasal HDM Challenge (25µg/35µl)
Day 1 2 3 4 5 8 9 10 11 12 15 16 17 18 19 20
BAL: Inflammation
H&E: Airway Inflammation
PAS: Airway Mucus
Saline
HDM
HDM + CQ
B
C
D
E
F
G
H
I
J

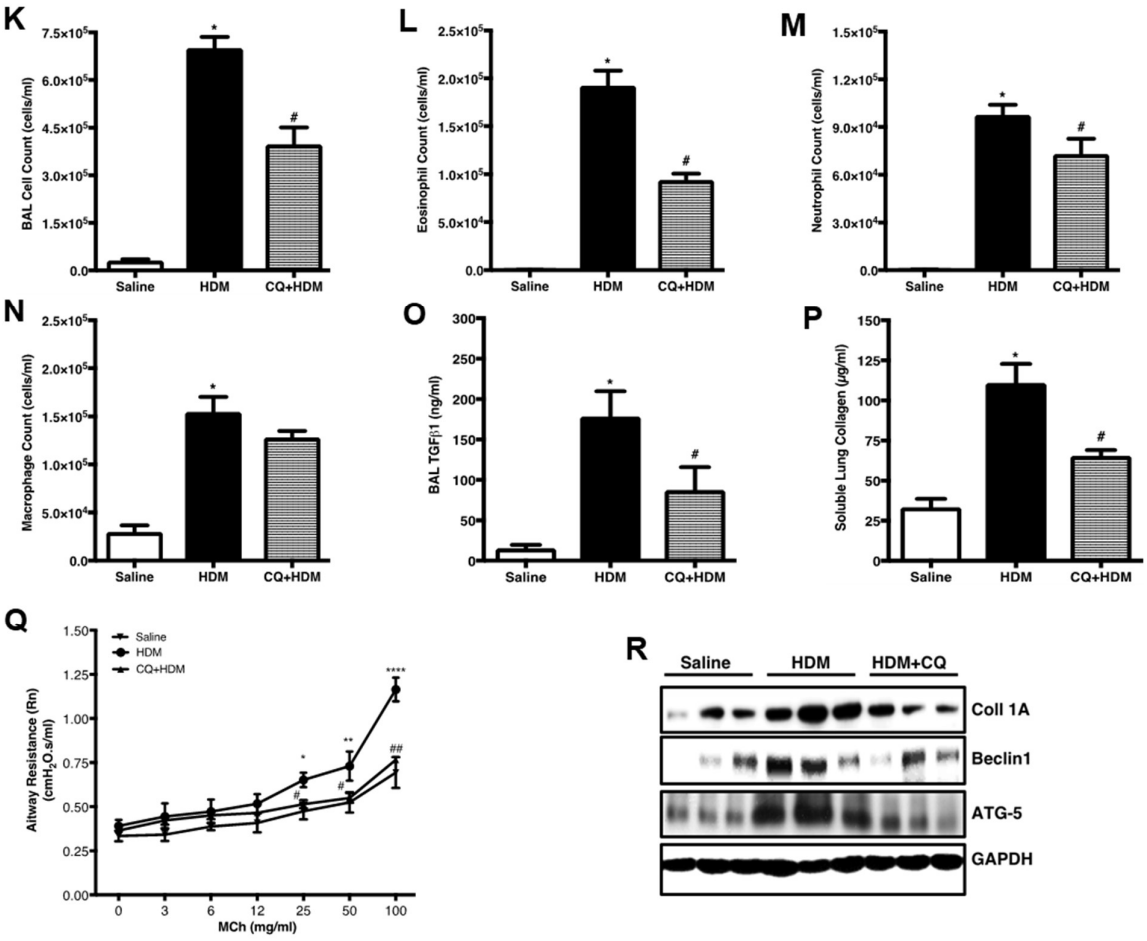
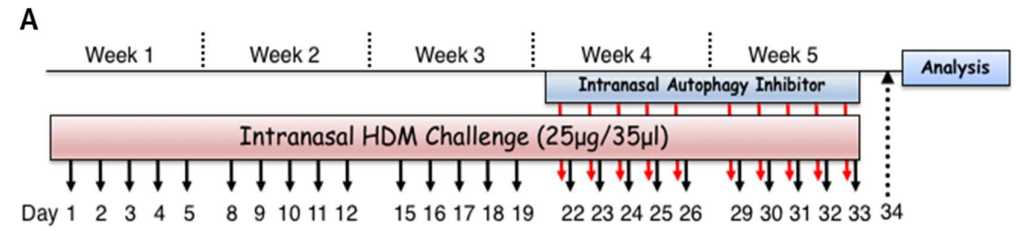

K
BAL Cell Count (cells/ml)
7.5x10^5
6.0x10^5
4.5x10^5
3.0x10^5
1.5x10^5
0.0
Saline
HDM
CQ+HDM
L
Eosinophil Count (cells/ml)
2.5x10^5
2.0x10^5
1.5x10^5
1.0x10^5
5.0x10^4
0.0
M
Neutrophil Count (cells/ml)
1.5x10^5
1.2x10^5
9.0x10^4
6.0x10^4
3.0x10^4
N
Macrophage Count (cells/ml)
O
BAL TGFβ1 (ng/ml)
300
250
200
150
100
50
P
Soluble Lung Collagen (μg/ml)
150
125
100
75
50
25
Q
Airway Resistance (Rn) (cmH2O.s/ml)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Saline
HDM
CQ+HDM
0
3
6
12
25
50
100
MCh (mg/ml)
R
Saline
HDM
HDM+CQ
Coll 1A
Beclin1
ATG-5
GAPDH

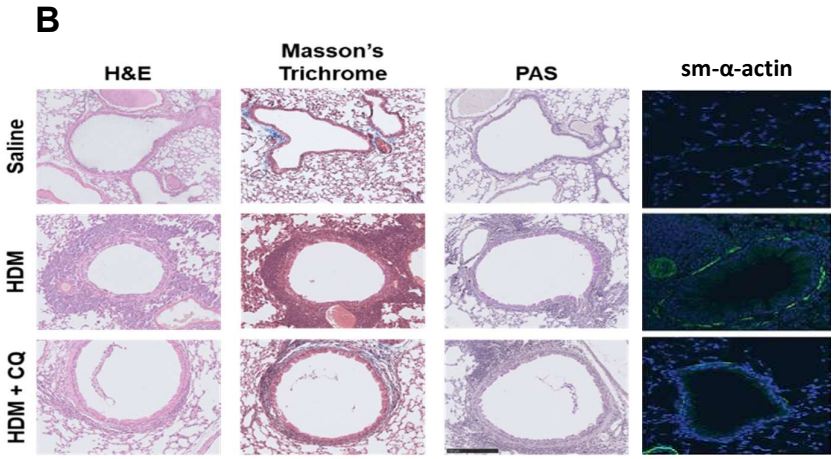
Figure 14. (A) Mouse model of allergic asthma. Female mice at 8 weeks old were challenged intranasally with house dust mite (HDM) allergen 5 d/wk for 3 weeks. Autophagy inhibitor CQ (50 mg/kg) was administered 30 minutes before each HDM challenge. Twenty-four hours after the last challenge, BAL and lung tissue were collected for further analysis. Inflammation in the BAL fluid was measured in (B) saline control mice, (C) HDM-challenged mice, and (D) the HDM+CQ-treated group. H&E staining was used to measure tissue inflammation in (E) saline control mice, (F) HDM-challenged mice, and (G) the HDM+CQ-treated group. Periodic acid–Schiff (PAS) staining was performed to measure mucus production in (H) saline control mice, (I) HDM-challenged mice, and (J) the HDM+CQ-treated group. Scale bar: 100 mM. CQ treatment in the HDM-challenged group reduced influx of (K) total immune cells, (L) eosinophils, (M) and neutrophils. (N) Macrophages remained unchanged. Furthermore, inhibition of autophagy reduced (O) profibrotic cytokine TGF-β1 concentrations in BAL and also (P) the amount of soluble lung collagen in tissue lysates. One-way ANOVA: $^{*}P<0.001$ for saline versus HDM; $^{\#}P<0.05$ for HDM versus HDM+CQ with Bonferroni multiple comparisons test. (Q) FlexiVent analysis revealed reduction in methacholine (MCh)-induced airway hyperresponsiveness in the CQ-treated group compared with the HDM-challenged group alone. Two-way ANOVA: $^{*}P<0.05$, $^{**}P<0.01$, and $^{****}P<0.0001$ for saline versus HDM (at 25, 50, and 100 mg/ml); $^{\#}P<0.05$ and $^{\#\#}P<0.01$ for HDM versus HDM+CQ (at 25, 50, and 100 mg/ml). (R) Representative protein immunoblots for collagen IA, beclin-1, and ATG5 in lung tissue lysates from saline-, HDM-, and HDM+CQ-treated mice. Mouse data shown represent mean ± SEM from n = 6 or 7 per group. Experiments performed by co-authors and I am not in possession of the raw data relating to these figures.

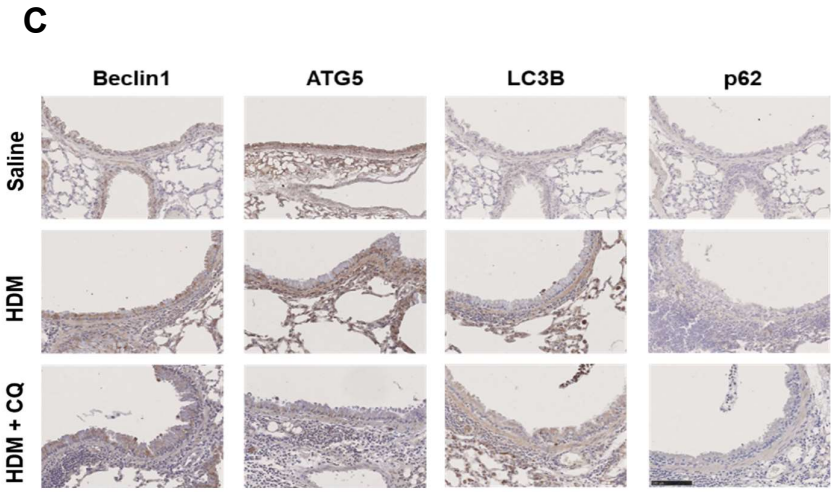
Effect of autophagy inhibitor in a chronic model of allergic asthma

We further employed an allergen (HDM)-induced mouse model of chronic asthma (treatment model) to investigate the role of autophagy in airway remodelling in asthma (Figure 12A). 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 (Figure 12B; H&E stain). Similar reduction was observed in the accumulation of fibrotic proteins in the CQ+HDM-treated group when compared with the HDM group (Figure 12B; Masson's trichrome stain). Increased production of mucus was observed in the HDM-challenged mice in comparison with saline-treated control animals and was greatly reduced in the CQ1HDM-treated group (Figure 12B; PAS stain). We further performed immunofluorescence imaging for ASM marker ACTA2; chronic allergen challenge increased ASM bundles, which were significantly reduced by CQ treatment (Figure 12B; ACTA2 stain). Immunohistochemical staining was used to examine the expression of four key protein markers of autophagy in the airways of saline-treated 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 (percentage area ±SD) of beclin-1 (HDM, 14.67±3.02%; control, 6.98±3.08%; $P<0.0001$) (Figure 12D), ATG5 (HDM, 18.7±3.99%; control, 11.27±6.67%; $P<0.05$) (Figure 12E), and LC3B (HDM, 20.47±14.67%; control, 2.97±1.59%; $P<0.05$) (Figure 12F) in the HDM-challenged mice compared with control mice. Treatment with CQ significantly reduced the expression of beclin-1 (CQ+HDM, 7.5±4.65%; HDM, 14.67±3.02%; $P<0.0001$) (Figure 12D) and ATG5 (CQ+HDM, 7.78±7.18%; HDM, 18.7±3.99%; $P<0.001$)

(Figure 12E) and increased the expression of p62 (CQ+HDM, 7.27±1.97%; HDM, 2.82±2.09%; $P<0.0005$) (Figure 12G) in the CQ+HDM-treated mice compared with HDM-challenged mice.


A
Week 1
Week 2
Week 3
Week 4
Week 5
Analysis
Intranasal Autophagy Inhibitor
Intranasal HDM Challenge (25µg/35µl)
Day 1 2 3 4 5 8 9 10 11 12 15 16 17 18 19 22 23 24 25 26 29 30 31 32 33 34


B
H&E
Masson's Trichrome
PAS
sm-α-actin
Saline
HDM
HDM + CQ


C
Beclin1
ATG5
LC3B
p62
Saline
HDM
HDM + CQ

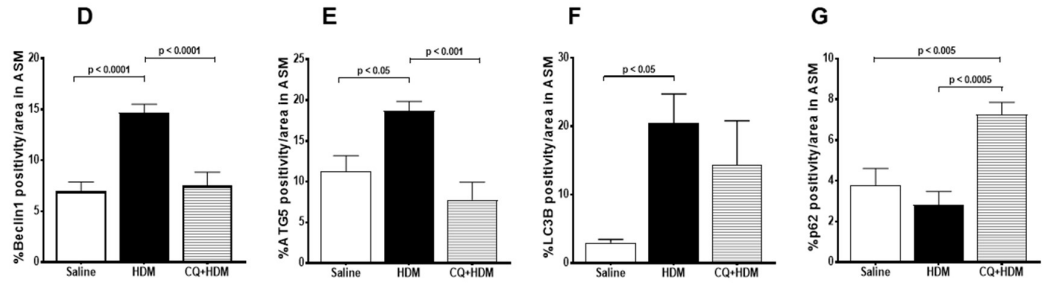

D
%Beclin1 positivity/area in ASM
p < 0.0001
p < 0.0001
Saline
HDM
CQ+HDM
E
%ATG5 positivity/area in ASM
p < 0.05
p < 0.001
F
%LC3B positivity/area in ASM
p < 0.05
G
%p62 positivity/area in ASM
p < 0.005
p < 0.0005

Figure 15. (A) Mouse model of chronic allergic asthma. Female mice at 8 weeks old were challenged intranasally with HDM allergen 5d/wk for 5 weeks. Starting at week 4 and for the remaining 2 weeks, autophagy inhibitor CQ (50 mg/kg) was administered 30 minutes before each HDM challenge. Twenty-four hours after the last challenge, BAL and lung tissue were collected for further analysis. (B) H&E staining was used to measure tissue inflammation in the saline control, HDM-challenged, and HDM+CQ-treated mice. Masson's trichrome staining was used to measure connective tissue deposition and smooth muscle mass in the saline control, HDM-challenged mice, and HDM+CQ-treated mice. PAS stain was used to measure mucus production in the saline control, HDM-challenged, and HDM+CQ-treated mice. Smooth muscle a-actin (sm-a-actin) was used to stain smooth muscle cells in the saline control, HDM-challenged, and the HDM+CQ-treated mice. Scale bar: 250 mm. (C) Immunohistochemical staining was used to measure the expression of autophagy proteins in the airway smooth muscle bundles of the mice. Beclin-1, ATG5, LC3B, and p62 expression were measured in the saline control, HDM-challenged, and HDM+CQ-treated mice. Scale bar: 150 mm. (D–G) Positive area for each of these markers in ASM was quantified. Data are expressed as mean ± SD or mean ± SEM. n = 4–6 mice. Mouse model carried out and tissues supplied by co-authors.

Discussion

The upregulation of markers of autophagy has been linked with fibrosis and remodelling in various organs (Ghavami et al. 2015; Hernandez-Gea et al. 2012). 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 (Poon et al. 2012; Poon et al. 2017), our study provides comprehensive analysis of multiple autophagy markers and its association with airway remodelling 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 remodelling in asthma. Furthermore, this is also replicated in the murine model where we found increased beclin-1 in experimental asthma model with a concomitant expression of pro-fibrotic cytokines and collagen in the lung, which was attenuated in presence of an autophagy inhibitor.

First, we confirmed that the tissues we used displayed classical features of airway remodelling (Chakir et al. 2003). There are variety of factors that contribute to the pathogenesis of asthma and subsequent development of airway remodelling 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 (Busse et al. 1999). 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 remodelling. These findings are novel in asthma as there is no evidence to date suggesting expression of autophagy being closely associated with airway remodelling. This finding is reinforced by data derived from studying 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 control animals. This work clearly supports the published literature on the kidney, liver, and other organs, where it has been shown that autophagy regulates tissue fibrosis (Aranguiz-Urroz et al. 2011; Koesters et al. 2010). However, it was recently reported that autophagy is a necessary mechanism for changing the phenotype of lung epithelial cells to mesenchymal cells (Alizadeh et al. 2018; Alizadeh et al. 2017); therefore, the present finding that shows incidence of autophagy in epithelium of patients with asthma and HDM-challenged mice might confirm the role of EMT in accumulation of mesenchymal cells in asthma pathogenesis.

Airway remodelling 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 (Payne et al. 2003). This led us to think about various signalling pathways such as autophagy that can be aberrant and can contribute to disease development later in life. Although 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 remodelling 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 studying 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, release of ECM proteins, and ultimately reduction in the ability of the airways to contract in response to a variety of triggers.

An increase in the protein expression of beclin-1 was also observed in the small airway epithelium compared with that in healthy individuals without asthma. With access to tissue for a greater number of patients, accompanying significant differences in protein expression of beclin-1 and ATG5 in the large airway epithelium may be observed. Interestingly, asthmatic epithelium displayed strong expression of beclin-1 in cilia, whereas nonasthmatic epithelium did not display these levels of expression. The comparative expression of beclin-1 in cilia of asthmatic and nonasthmatic epithelium has a significantly binary pattern. In chronic obstructive pulmonary disease (COPD), autophagy-dependent pathways regulate cilia length upon exposure to cigarette smoke (Cloonan et al. 2014). The identification of an over-expression of beclin-1 in ciliated cells of asthmatics highlights a role for ciliophagy 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 beclin-1 in cilia, or whether the increased expression of beclin-1 in the cilia is influencing the cellular stimulation of airway remodelling. 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 nonasthmatic 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; that is, it represents a greater asthma population in the general public. In the 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 (subchronic) and chronic (treatment) models 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 (Charous et al. 1990). 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 in lung tissue. Furthermore, our data uncover novel therapeutic effects of CQ in asthma because it reduces features of airway remodelling by preventing accumulation of mucus and ECM matrix protein collagen 1 in the lung. The inhibition of autophagy by CQ was supported by our immunohistochemical analysis in the chronic mouse model showing decreased expression 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 ACTA2 in the airways of mice treated with CQ. The mechanisms of the beneficial effect of CQ rely on blocking the release of profibrotic cytokine TGFβ1 in the

BAL of HDM-challenged mice after treatment with CQ. It is well known that TGFβ1 drives fibrotic changes in the lung and that its concentrations are elevated in asthma (Halwani et al. 2011), and it is emerging that the autophagy pathway may interact with the ECM and affect cytokine secretion (Ghavami et al. 2015; Lock & Debnath 2008). We have shown *in vitro* that upon stimulation with TGF-β1, human ASM cells produced greater amounts of collagen IA and had 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 TGF-β1 concomitantly induces autophagy and drives profibrotic signalling. CQ acts by inhibiting autophagy and reducing TGFβ1-dependent pro-fibrotic signalling in asthma. This was confirmed by the cotreatment of CQ and TGF-β1 resulting in a time-dependent reduction in alpha-1 type I collagen, diminished expression of beclin-1 and LC3B-II, and reduced phosphorylation of SMAD2/3. These beneficial effects of modulating autophagy using CQ in a disease setting (mouse models) also translated to reducing development of AHR (data not shown for chronic model) in response to MCh, which is another hallmark of allergic asthma.

Furthermore, our mouse models demonstrated clear initiation (in the 3-wk model) and establishment (in the 5-wk model) of airway remodelling, 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 remodelling in the lung with a concomitant reduction in autophagy, as seen with reduction in beclin-1 and ATG5 proteins. This clearly demonstrates 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 because 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 (Suzuki et al. 2002). Though autophagy modulation with CQ is not highly selective, owing to the fact that is has a number of other pharmacological effects, at the concentrations used in this study, beclin-1 and ATG5 were reduced by CQ. Therefore, we are highly confident of these findings that CQ works in an experimental asthma model by modulation of the autophagy pathway. Originally an antimalarial and antiarthritis drug (Homewood et al. 1997), CQ has been shown also to target inwardly rectifying potassium channels (Marmolejo-Murillo et al. 2017), and by disrupting the blood-retina barrier, it has the potential to cause retinopathy through binding to melanin and toxic sequestration in the eye (Bernstein & Ginsberg 1964). 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 (Werner et al. 1984). 3-Methyladenine also has the ability to suppress the formation of autophagosomes specifically targeting autophagy through the inhibition of class III PI3K (Seglen & Gordon 1982). However, 3-Methyladenine is found to have a dual role in autophagy modulation. It surprisingly promotes autophagy flux with prolonged treatment under nutrient-rich conditions and also has the capability of suppressing starvation-induced autophagy (Wu et al. 2010). LY294002, derived from the flavonoid Quercetin (Vlahos et al. 1994), inhibits PI3K activity (Blommaart et al. 1997) and belongs among the emerging safe therapeutics with the potential to treat airway remodelling. 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 remodelling (Liu et al. 2017).

Inhibition of autophagy, as shown here with CQ treatment, is entwined with an upstream TGFβ response and the amount of collagen production. As previously shown in hepatic cells and cardiomyocytes, TGFβ concomitantly influences features of remodelling and regulates degrees of autophagy (Ghavami et al. 2015; Hernandez-Gea et al. 2012), and we introduce and provide evidence that a similar concomitant pathway occurs both *in vivo* and *in vitro*. This pathway, we believe, has the greatest influence in the ASM of the large airways, as seen in asthmatic lungs, and this was also replicated *in vitro* using primary human ASM cells, where we found TGF-β1 concomitantly induced both profibrotic signalling 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 on autophagy in murine models can attenuate inflammation, clear mucus production, reduce concentrations of TGF-β1 in the BAL, and ultimately reduce airway remodelling and prevent bronchoconstriction. Thus, our study indicates that in asthmatic airways, autophagy is enhanced, which we believe contributes to remodelling in a TGFβ-dependent manner, and that inhibition of autophagy is an attractive target for alleviation of airway remodelling in asthma.

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Materials and Methods Online Supplement

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 (RPAH), Concord Repatriation General Hospital and St Vincent's Hospital (# HREC14-0045, Sydney) (Faiz et al. 2013). All patients provided consent for their lung tissue to be used for scientific research and in the case of post mortem samples, consent was obtained from the next of kin. Tissue used as non-asthmatic controls were from non-smoking donors with healthy lungs or from macroscopically normal and isolated regions of lungs from patients with non-small cell carcinoma (NSCCa) and free of respiratory or systemic diseases. Non-asthmatic tissue that were deemed to be in close vicinity to cancerous regions were excluded from the study. Patients with a known smoking history were excluded from the non-asthmatic group.

Human subject classification

The mean age of the non-asthmatic subjects was 48 years (SD = 16.41) and in the asthmatic subjects the mean age was 50 years (SD = 21.37) with 100% of the subjects being male. Airways with a diameter of greater than 2mm and identifiable pseudostratified epithelium were deemed to be large airways, whilst airways with columnar and cuboidal epithelium plus a diameter less than 2mm were classified as small airways. Asthmatic subjects were selected on the basis of confirmed clinical diagnosis and associated airway remodelling features determined histologically.

Histology

Human Lung Tissue Processing and Section Preparation

Dissected lung tissues were fixed in 10% neutral buffered formalin (Sigma-Aldrich, St. Louis, USA). With the use of an automated tissue processor (Excelsior AS Tissue Processor, Thermo Scientific, Waltham, USA), both small and large airway samples underwent dehydration process in ascending grades of ethanol and two periods of xylene. Tissue samples were embedded in paraffin for analyses (HistoStar Embedding Workstation, Thermo Scientific, Waltham, USA) (Eapen et al. 2017). Tissue sections cut at 4-micron thickness were prepared using a microtome sectioner (Microm HM325 Rotary Microtome, Thermo Scientific, Waltham, USA) and heated water bath. Following mounting on coated slides (PRO-03; Matsunami, Osaka, Japan), sections were deparaffinised in xylene and rehydrated in graded ethanol prior to staining. H&E staining was used to assess the structural integrity, inflammation and the absence or presence of additional pathologies.

Trichrome staining

Sections were incubated in Bouin's Solution (Sigma-Aldrich, St. Louis, USA) for 24hours at room temperature and Masson's trichrome staining with Weigert's haematoxylin counterstain (Sigma-Aldrich, St. Louis, USA) was performed to assess features of airway remodelling (HT15-1KT; Sigma-Aldrich, St. Louis, USA).

Morphometric analysis of inflammation and airway remodelling features

Epithelium and reticular basement membrane (RBM) thickness was measured at multiple points of each airway which are averaged and represented as means. Lamina propria depth was measured perpendicularly from the base of the RBM to the outer edge of ASM bundles at multiple points of each airway. These values are averaged and represented as means. The proportion of ASM in the airway wall of each individual

airway (ASM/LP, %) was calculated by measuring the total area of ASM mass per airway and dividing by the total area of the lamina propria. Non-asthmatic airways were excluded if they displayed inflammatory infiltrates, if the epithelium was damaged, the RBM was thickened or fragmented, and ASM mass was disproportionate.

Immunohistochemistry

Heated antigen epitope retrieval was performed by placing slides in pre-heated (60-90°C) 0.01M citrate buffer, pH 6.0 for 10 minutes and cooling for 30 minutes. After rinsing in water, sections were identified with a hydrophobic Dako Pen (Agilent, Santa Clara, USA) and endogenous peroxidase activity was quenched with incubation in 3% H_20_2 for 10 minutes (Eapen et al. 2017). Following rinses with water and TRIS buffer (1X tris-buffered saline (TBS)/0.1% tween-20), sections were incubated in DAKO serum-free Protein Block (Agilent, Santa Clara, USA) for 10 minutes. Sections were then washed in tris-buffered saline with tween-20 (TBST) and incubated for 40 minutes with the following diluted primary antibodies: rabbit polyclonal anti-beclin-1 (1:250, ab62557; Abcam, Cambridge, UK), rabbit monoclonal anti-APGL/ATG5 (1:200, ab109490; Abcam, Cambridge, UK), rabbit polyclonal anti-sequestesome-1/p62 (1:25, ab155686; Abcam, Cambridge, UK), and rabbit polyclonal anti-MAP1LC3B (1:125, L7543; Sigma-Aldrich, St. Louis, USA). Bound antibodies were elaborated with horse-radish peroxidase (HRP)-labelled EnVision+Rabbit secondary antibody (K4011, Agilent, Santa Clara, USA) incubation for 30 minutes (Sohal et al. 2010). For each case, the primary antibody was replaced by a species-appropriate isotype-matched immunoglobulin (Rabbit Immunoglobulin Fraction, X0936; Agilent, Santa Clara, USA) for a negative control (6). The sections were washed firstly in TBST, distilled water, then the antibodies were visualized with the addition of liquid 3,3'-Diaminobenzidine (DAB, Agilent, Santa Clara,

USA) and incubated for 10 minutes (Mahmood et al. 2017). The sections were then washed sufficiently in distilled water before nuclei were counter-stained with Mayer's haematoxylin (Fronine, Riverstone, Australia), blued with ammoniated water (Chem-Supply, Gillman, Australia), and dehydrated and cleared through graded ethanol solutions and xylene. Sections were mounted with Permount (Fisher Scientific, Hampton, USA).

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. Prior to image analysis observer was blinded to subject and diagnosis. After a minimum of 6-7 images per tissue were taken, out of which four images were randomized per patient to be quantified with the assistance of a random number generator. The percentage staining was quantified in the epithelium and ASM using ImageJ software. Cell staining in the epithelium and airway smooth muscle bundles was separately quantified and presented as per mm^2 in both small and large airways. Data from four random images is presented as means for each corresponding patient.

Human airway smooth muscle cell culture

Human ASM cells were obtained from human lung by a method as described previously (Chen et al. 2013; Penettieri et al. 1989; Sharma et al. 2008). Human ASM bundles were microdissected from approximately 4th-6th- order bronchii and were initially cultured in growth medium comprised of Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 1% antibiotics (Invitrogen, Carlsbad, CA, USA). All the cells tested negative for the

presence of mycoplasma before they were set up for experiments and were used between passages 2 and 5. ASM cells were seeded in six-well plates (BD Biosciences, New Jersey, USA) in growth medium and incubated at 37°C/5% CO2. Cells were starved in quiescing medium consisting of DMEM:F12 media supplemented with 1% ITS (R&D Systems, Minneapolis, MN, USA) for 48h before any treatment.

Mouse models of allergic asthma

All animal procedures were approved by the Institutional Animal Care Committee of Thomas Jefferson University, Philadelphia, USA and University of Technology Sydney. All methods were performed in accordance with the relevant guidelines and regulations of the institutions. All surgeries were performed under tribromoethanol (Avertin, 250 mg/kg) anesthesia, and all efforts were made to minimize suffering. All animals were given standard chow and maintained on a 12 h dark and light cycle within the animal facility.

Prophylactic model

BALB/c mice (female) at 8 weeks were intranasally challenged 5-days/week for three consecutive weeks with house dust mite (HDM) extract (Dermatophagoides pteronyssinus, Greer Labs, USA) (25µg/35µL) to develop a sub-chronic model of allergic asthma as shown in Fig 11A (Sharma, Yi & Deshpande 2015). Thirty min prior the HDM challenges, a select set of mice were administered either chloroquine (50mg/kg) or saline vehicle. Twenty-four hours after the last HDM challenge, lung function measurements were performed using a small animal ventilator (flexiVent, Scireq, Montreal, Canada). Bronchoalveolar lavage (BAL (2x0.5 ml)) fluid was collected to assess cellular influx into the airways and BAL supernatants were frozen for cytokines and chemokine, and

lungs were formalin-fixed or flash frozen and stored at -80°C for histopathology and biochemical analysis.

Treatment model

BALB/c mice (female) at 8 weeks were intranasally challenged 5-days/week for five consecutive weeks with HDM extract (Dermatophagoides pteronyssinus, Greer Labs, USA) (25µg/35µL) to develop a chronic model of allergic asthma as shown in Fig 12A. From week four onwards, thirty min prior to the HDM challenges, a select set of mice were administered either chloroquine (50mg/kg) or saline vehicle for the remaining two weeks of the model. Twenty-four hours after the last HDM challenge, lung function measurements were performed using a small animal ventilator (flexiVent, Scireq, Montreal, Canada). BAL (2x0.5 ml) fluid was collected to assess cellular influx into the airways and BAL supernatants were frozen for cytokines and chemokine, and lungs were formalin-fixed or flash frozen and stored at -80°C for histopathology and biochemical analysis.

Mouse BAL Immune Cell Staining, Lung H&E, PAS and Masson's trichrome Staining

BAL samples were subjected to centrifugation and cell pellet resuspended in 1 ml PBS. The cells were stained with Hema-3 staining kit (Fisher Scientific, Hampton, USA). The lung tissues fixed in 10% formalin, embedded in paraffin were cut and stained with H&E, PAS and masson's trichrome staining using a standard histological protocol as described previously (Sharma, Yi & Deshpande 2015; Sharma et al. 2012; Sharma et al. 2010; Sharma et al. 2014), using 5-µm sections mounted on Superfrost Plus slides. Image acquisition and analysis was performed using a NanoZoomer-SQ Digital slide scanner (Hamamatsu, Hamamatsu City, Japan), a brightfield microscope and ImageJ software.

Mouse immunohistochemistry staining

Embedded lung tissues from the chronic 5-week HDM model were also cut and stained immunohistochemically for markers of autophagy (beclin-1, ATG5, LC3B, and p62, sm-a-actin) as described previously (Sohal et al. 2010; Sharma et al. 2015; Mahmood et al. 2017), using 5-µm sections mounted on coated slides (PRO-03; Matsunami, Osaka, Japan). Image acquisition and analysis was performed using a NanoZoomer-SQ Digital slide scanner (Hamamatsu, Hamamatsu City, Japan) and ImageJ software. Images were analysed and quantified using the same method as employed with the human tissue samples analysed within this paper.

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.

Preparation of Lung Lysates

Mouse lungs were cut into small pieces and approximately half of the lungs were preserved in 250 µl of lysis buffer (composition: 40 mM Tris, 150 mM NaCl, 1% IgepalCA-630, 1% deoxycholic acid, 1 mM NaF, 5mM β-glycerophosphate, 1 mM Na_3VO_4, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 7 µg/ml pepstatin A, 1 mM PMSF, pH 8.0) and stored at – 80˚C for protein analysis. Frozen lung tissues in the lysis buffer were slowly thawed in ice and were transferred into 5 ml tubes for homogenization using a polytron. The lysate was transferred to 1.5 ml plastic tube, centrifuged (760 × g, 5 min) and the supernatant stored at -80°C for subsequent protein assay and immunoblot analyses.

Immunoblotting

Protein concentration was determined by Pierce BCA assay kit (Thermo Scientific, Rockford, USA) and subjected to immunoblot analysis using protocols described previously (Sharma, Yi & Deshpande 2015; Sharma et al. 2010; Deshpande et al. 2010) with primary antibodies noted above (1:1000), followed by incubation with respective secondary antibodies (LI-COR Biosciences, Lincoln, USA) (1:5000). Immunoblots were visualized and bands quantified using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, USA).

Soluble Collagen Assay

Total soluble collagen content in the lung lysates was assessed using Sircol collagen assay (Biocolor, Carrickfergus, UK) according to the manufacturer's protocol as per (Sharma, Yi & Deshpande 2015; Schaafsma et al. 2011). Collagen assay was performed by mixing lung homogenates with Sircol Dye reagent and measuring absorbance using a plate reader. Collagen content was quantified using a standard curve generated by reference standards and was normalized to the total lung protein content in each sample.

Statistical analysis

Morphometric data was analyzed using unpaired t-tests assuming Gaussian distribution and is presented as mean ± SD. Immunohistochemistry data was analyzed using unpaired t-tests with Welch's correction assuming Gaussian distribution and is presented as mean ± SD with normal distribution. For the parametric statistical analyses carried out for immunohistochemistry data, normal (gaussian) distribution must be assumed. Normality of these data has been tested and the data does not differ significantly from that which is normally distributed. One-way or two-way ANOVA was used appropriately with Bonferroni's multiple comparisons test and data expressed as ±SEM.

All data was analyzed with PRISM V7.04 software (GraphPad, La Jolla, USA) and $p<0.05$ was considered statistically significant.

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Chapter 3

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Altered Calcium in Ciliary Dysfunction: Potential Role of ER stress and Ciliophagy

Altered Calcium in Ciliary Dysfunction: Potential Role of ER stress and Ciliophagy

McAlinden KD, Eapen MS, Ghavami S, Sohal SS, Sharma P. Altered Calcium in Ciliary Dysfunction: Potential Role of Endoplasmic Reticulum Stress and Ciliophagy. Am J Respir Cell Mol Biol. 2019;61(6):794-795

We read with great interest the recent original research article by Petit et al. (Petit et al. 2019) and the accompanied editorial (Wittekindt 2019) published in this journal (American Journal of Respiratory Cell and Molecular Biology) showing that epithelial cell calcium influx is decreased in smokers with impaired Ca^{2+} signalling and highlighting the importance of calcium in regulating ciliary beat frequency. The study showed fewer cells with beating cilia from smokers than controls. They point out that previously Ca^{2+} signalling was not sufficiently studied in primary bronchial epithelial cells and demonstrate that Ca^{2+} influx via store-operated Ca^{2+} is impaired in airway epithelial cells of smokers with and without COPD. *ORAI3*, a gene involved in calcium signalling and a Ca^{2+} pore-forming subunit was identified as being less expressed in the airway epithelial cells of smokers and COPD patients. Authors attributed the decrease in Ca2+-influx to the decreased levels of ORAI3 in smokers of which also has a role in cilia beat frequency. Pre-incubation of ALI cultures with an ORAI antagonist decreased the percentage of epithelium covered by functional beating cilia. Accompanied editorial (Wittekindt 2019) delves into discussion of the activation of store-operated calcium (SOC) entry being triggered by inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release and depletion of Ca^{2+}-stores in the endoplasmic reticulum (ER). Dr Wittekindt's editorial highlights that the paper from Petit et al. links SOC entry with ciliary beat of normal epithelial cells whilst also proposing additional experiments which would aid in the gain of insight into the signalling pathways and changes to cilia beat frequency observed in smokers with and without COPD. In addition to the mechanisms proposed by Petit et al. and the accompanying editorial, we believe that protein folding changes driven by depletion of ER-Ca^{2+} and resultant ciliophagy is also of great interest. Further understanding of these pathways which mediate cilia dysfunction could be greatly beneficial in the development of new therapies targeting muco-ciliary clearance (MCC).

Several recent investigations have shown that Ca^{2+}-depletion in the ER damages the protein folding pathway because it changes the functional activities of chaperones which depend on cellular Ca^{2+} (Gorlach, Klappa & Kietzmann 2006). After Ca^{2+} depletion, the unfolded protein response (UPR) pathway is initiated in cells as a result of accumulation of misfolded protein in the ER (Gorlach, Klappa & Kietzmann 2006). This calcium depletion and supported UPR pathway is integral in the initiation of cell death (Sehgal et al. 2017). UPR also regulates autophagy in a number of ways (Rashid et al. 2015), and it is the involvement of autophagy that we bring to attention in understanding the dysfunctional cilia observed in COPD.

Ciliophagy is the process in which cilia proteins, upon exposure to cigarette smoke, are degraded and recycled through the ER stress-dependent autophagy pathway, ultimately resulting in shortening of cilia and impaired MCC (Cloonan et al. 2014). Choi and colleagues first described the role of ciliophagy, accompanying an increase in autophagy in COPD lungs (Cloonan et al. 2014). Cigarette smoke exposure both *in vivo* and *in vitro*, via protein ubiquitination and cilia protein aggregation, induces autophagy and ultimately reduces cilia length. Deletion of crucial autophagy genes (beclin-1 and LC3B) were shown to protect mice exposed to cigarette smoke from impaired MCC, and MCC-impairment due to enhanced ciliophagy was shown to be mediated by Histone Deacetylase 6 (HDAC6). Autophagy-impaired mice and differentiated mouse tracheal epithelial cells (MTECs) showed resistance to cigarette smoke (CS)-induced cilia shortening. Lam et al. also showed that CS boosted the mechanism of autophagic recycling of ciliary proteins, highlighting the potentially crucial role of autophagy in the regulation of cilia homeostasis (Lam et al. 2013). Therefore, we need to additionally discuss the role of mechanisms by which cilia are shortened and lost, including whether

dysregulated autophagy can also affect ciliary beat function. We believe that excessive autophagy flux induced by stimulus such as cigarette smoke can contribute to respiratory pathogenesis through cilia shortening. We recently found the increased expression of beclin-1 in the cilia of severe asthmatics (McAlinden et al. 2018). Beclin-1 proteins are integral in autophagosome nucleation and autophagy initiation. The importance of such a binary expression of beclin-1 in ciliated cells may be of functional importance in regulating both ciliary length and beating which upon deeper investigation may have greater Ca^{2+} homeostasis is known to regulate the UPR and subsequently autophagy flux. Changes in calcium metabolism in bronchial epithelial cells of COPD patients could also change the cytokine processing and secretion in these cells and certainly affect immunomodulation of the COPD lung. A question we ponder is whether the altered expression of autophagy markers in the ciliated epithelium is influencing the airway wall and smooth muscle environment, or inversely are the structural changes influencing ciliophagy and cilia shortening. Perhaps changes in ciliary beat frequency and cilia length in COPD and asthma have downstream effects in epithelial and mesenchymal cells and altering autophagy flux in these different cellular environments is an important novel avenue to be explored. The potential interplay between calcium homeostasis and autophagy flux in ciliated epithelial cells and their role in pathogenesis is yet to be thoroughly explored.

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Chapter 4

Pharmacologic Inhibition of Vacuolar H^+-ATPase Attenuates Features of Severe Asthma in Mice

Pharmacologic Inhibition of Vacuolar H^+ATPase Attenuates Features of Severe Asthma in Mice

McAlinden, K.D[*], Kota A[*], Haghi H, Ghavami S, Sharma P. 2019. *Am J Respir Cell Mol Biol*.

Author Contributions: Anudeep Kota and I (Kielan McAlinden) contributed equally to this work. Dr. Pawan Sharma.: conceived of the idea for the study; Myself (Kielan McAlinden), Anudeep Kota and Dr. Pawan Sharma.: performed the experiments and data analysis; I (Kielan McAlinden) wrote the manuscript; Prof. Saeid Ghavami, Dr. Mehra Haghi and Dr Pawan Sharma.: reviewed, edited, and revised the manuscript and agreed to the final content.

To the editor,

Asthma can be defined as "an abnormal state of the airways which causes the airways to narrow too much and too quickly in response to a wide variety of provoking stimuli" (Woolcock 1996) and is associated with numerous genetic and environmental interactions. The complexity and heterogeneity of asthma is such that some patients experiencing spontaneous bronchoconstriction may not achieve complete reversibility of airflow obstruction, of which is experienced in severe asthmatics (Boulet 2009). Many asthmatic patients also have comorbidities such as obesity and metabolic dysfunction, further complicating the disease phenotype and making treatment more difficult (Chapman et al. 2014). The factors that determine these associations are not well understood. Current therapies targeting airway narrowing and inflammation are not entirely comprehensive or universally effective, with many patients remaining symptomatic. This has led to studies phenotyping asthma and development of biologic therapies to aid in precise and personalized management of the disease (Bousquet et al. 2017). Despite the advances in biological therapies, the decision criteria for clinicians when selecting a biological for each patient remains uncertain and thus, alternative novel therapies need to be developed to provide new avenues in the pursuit of severe asthma control. In this study, we tested the therapeutic efficacy of bafilomycin A1 (BafA1) compared to fluticasone propionate (FP) in a steroid insensitive mouse model of severe asthma. BafA1, a macrolide produced by *Streptomyces griseus Sulphurus* (*ssp*), is a highly selective inhibitor of vacuolar type H^+-ATPase (V-ATPase) (Klionsky et al. 2016; Yeganeh et al. 2015). We further verified our findings in primary human smooth muscle cells *in vitro,* where we looked at overcoming steroid insensitivity by BafA1 treatment.

Methods

Female BALB/c mice were subjected to a standard model of complete Freund's adjuvant and house dust mite (CFA+HDM) sensitization and challenge in order to induce severe asthma in presence and absence of intra nasal BafA1 or FP (Figure13A). Lung function measurements were performed using flexiVent (Scireq, Canada) to increasing concentration of methacholine (MCh). Airway inflammation was assessed by counting total immune cell influx in bronchoalveolar lavage (BAL) fluid, Multiplexing LASER Bead Technology was used to measure BAL cytokine expression, qPCR was used to measure key cytokines mRNA, and haematoxylin and eosin (H&E) staining was used to evaluate inflammation in the lung tissue, as previously described (McAlinden et al. 2018). Primary human airway smooth muscle (ASM) cells were transfected with GRE reporter constructs as described (Moodley et al. 2013). Steroid insensitivity was induced by a combined treatment of TNF-α (10 ng/ml) and IFN-γ (500 IU/ml) for 6 hours (Tilba et al. 2008) in presence or absence of FP (100 nM) ± BafA1 (20 nM) (2h prior TNF-α +IFN-γ treatment). Cells were then lysed, and the luciferase activity was measured. One-way or two-way ANOVA was used appropriately with Bonferroni's multiple comparisons test and data expressed as ±SEM. PRISM V8.0.1 software was used for analysis (GraphPad, La Jolla, USA) and $p<0.05$ was considered statistically significant.

Results

Using the model of severe asthma as shown in Figure 13A we measured the effects of BafA1 or FP on airway inflammation and hyperresponsiveness. Total immune cells were increased in the BAL of CFA+HDM mice (****$p<0.0001$) compared with saline, which failed to be attenuated with FP treatment but was significantly reduced in CFA+HDM+BafA1 mice (**$p=0.0065$) (Figure 13B). Differential cell counting revealed that BafA1 was able to reduce the number of eosinophils (*$p=0.0106$) (Figure 13C) and neutrophils (*$p=0.0233$) (Figure 13D) when compared with CFA+HDM, whilst FP did not reduce these numbers. We also found that CFA+HDM+BafA1 decreased (****$p<0.0001$) lung inflammation while FP was (*$p=0.0178$) less effective in reducing tissue inflammation in this model (Figure 13E-F). BAL cytokine analysis revealed increases in protein concentrations of IL-4, IL-5, IL-13, and CXCL1 (KC) (****$p<0.0001$) in CFA+HDM mice compared with the saline mice (Table 2). BafA1-treatment significantly reduced the expression of IL-4 (**$p=0.0022$), IL-5 (****$p<0.0001$) and KC (**$p=0.0038$) when compared with CFA+HDM mice, whilst FP only reduced the level of IL-5 (**$p=0.0087$) but did not change the concentration of IL-4, IL-13 and KC (Table 2). The fold change of IL-4, IL-5 and IL-13 lung tissue mRNA was increased in HDM+CFA mice in comparison with saline. IL-13 was greatly reduced with BafA1-treatment, whereas FP-treatment resulted in greater reduction in IL-4 and IL-5 mRNA (Table 2). Repeated HDM-exposure induced airway hyperresponsiveness (AHR) to inhaled methacholine (MCh) in mice when compared with saline and CFA+HDM+BafA1 animals (****p<0.0001 at 6, 12, 25 and 50 mg/ml MCh). BafA1 treatment prevented AHR, whilst FP treated mice still experienced significant AHR (***p=0.0001 at 6 mg/ml MCh and $^{\$}p$<0.0001 at 12, 25 and 50 mg/ml) (Figure 13G). In

ASM cells GRE-reporter activity was enhanced in the presence of FP alone (****$p< 0.0001$), while TNF-α +IFN-γ treatment rendered cells steroid-insensitive (***$p=0.0004$). BafA1 treatment was able to overcome steroid insensitivity in ASM cells (**p=0.0068) (Figure 13H).

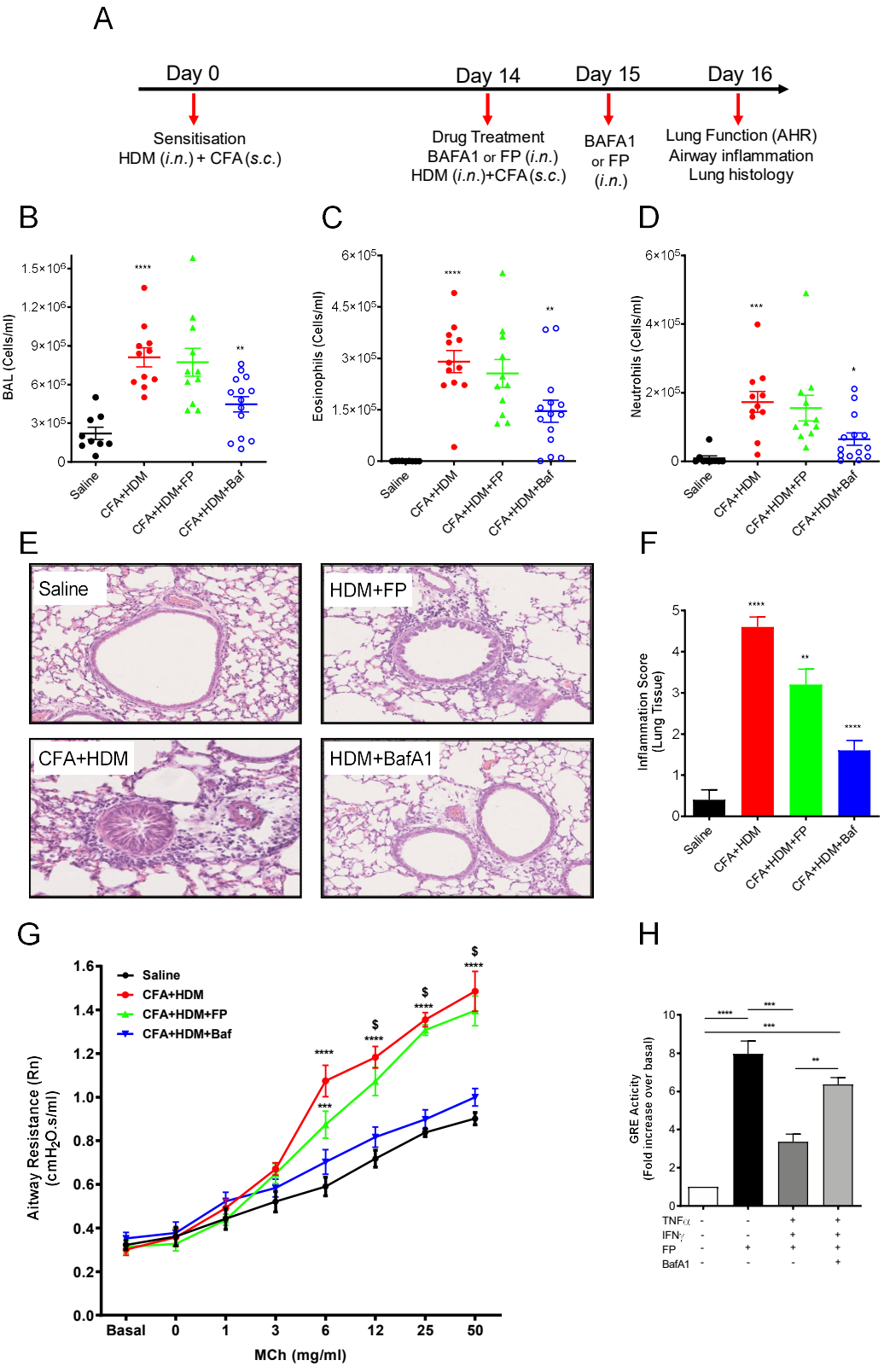
A
Day 0
Day 14
Day 15
Day 16
Sensitisation
HDM (i.n.) + CFA (s.c.)
Drug Treatment
BAFA1 or FP (i.n.)
HDM (i.n.)+CFA (s.c.)
BAFA1
or FP
(i.n.)
Lung Function (AHR)
Airway inflammation
Lung histology
B
BAL (Cells/ml)
C
Eosinophils (Cells/ml)
D
Neutrophils (Cells/ml)
Saline
CFA+HDM
CFA+HDM+FP
CFA+HDM+Baf
E
Saline
HDM+FP
CFA+HDM
HDM+BafA1
F
Inflammation Score
(Lung Tissue)
G
Saline
CFA+HDM
CFA+HDM+FP
CFA+HDM+Baf
Aitway Resistance (Rn)
(cmH2O.s/ml)
Basal
0
1
3
6
12
25
50
MCh (mg/ml)
H
GRE Acticity
(Fold increase over basal)
TNFα
IFNγ
FP
BafA1

Figure 16. Symptoms of severe allergic asthma model in mice are alleviated by Bafilomycin (BafA1). (A) Mouse model of house dust mite (HDM) and complete Freund's adjuvant (CFA) (100μg/animal)-induced severe asthma with fluticasone propionate (FP, 10μg) or BafA1 (3mg/kg) treatment. (B) Airway inflammation was assessed in the bronchoalveolar lavage (BAL) fluid by counting total cell influx. Total immune cells in the BAL fluid were significantly increased in the CFA+HDM-treated animals (****$p<0.0001$) when compared with saline where the immune cells are significantly reduced with BafA1 treatment (**p=0.0065) but not FP. Cell differentials revealed greater eosinophil (C) and neutrophil (D) influx with HDM challenge (****$p<0.0001$; ***$p=0.0009$), with reductions observed with BafA1 treatment (*$p=0.0106$; *$p=0.0233$) but not PF. (E) Hematoxylin and eosin (H&E) staining was carried out to highlight increased tissue inflammatory cell infiltration in the HDM-challenged mice (****$p<0.0001$) when compared to saline mice. Inflammatory cell infiltration was substantially reduced with BafA1-treatment (****$p<0.0001$) but not completely cleared in FP-treated mice (*$p=0.0178$) (F). (G) Lung function data shows differences in airway resistance (Rn) derived using constant phase model in response to increasing concentrations of contractile agonist methacholine (MCh). HDM-exposed mice were found to be hyperresponsive when compared to saline and BafA1-treated mice at 6, 12, 25 and 50 mg/ml MCh (****p<0.0001). BafA1-treatment presented lower airway resistance than HDM-challenged mice, whilst FP-treated mice were still subject to significant airway hyperresponsiveness in comparison with saline (***p=0.0001 at 6 mg/ml MCh and $p<0.0001 at 12, 25 and 50 mg/ml). (H) (GRE) activity was reduced in the presence of FP and TNF-α +IFN-γ treatment in comparison with FP alone (***$p=0.0004$) and FP+BafA1+ TNF-α +IFN-γ (**$p=0.0068$). Combined treatment with BafA1, FP and TNF-α +IFN-γ resulted in no significant changes in GRE activity in comparison with FP alone. Data represent n=8 mice and one or two-way ANOVA was used with Bonferroni's multiple comparison test. Mouse model and measurements in (figures B-D&G) performed by co-author and joint first author (Anudeep Kota) and I am not in possession of the raw data relating to these figures.

BAL cytokine protein expression				
Cytokine/ Chemokine	**Saline** pg/mL (SEM ±)	**CFA+HDM** pg/mL (SEM ±)	**CFA+HDM+FP** pg/mL (SEM ±)	**CFA+HDM+Baf** pg/mL (SEM ±)
IL-4	5.2 (2.86)	269 (27.86)	194.2 (15.97)	139.8 (24.89)
IL-5	6.6 (2.23)	187.4 (12.87)	121 (18.2)	72.8 (9.81)
IL-13	1 (0.55)	124.8 (24.62)	73.8 (8.42)	78.6 (9.4)
KC	8.6 (4.5)	375.4 (35.52)	302.2 (49.41)	142.2 (48.56)
Lung tissue cytokine mRNA levels				
Cytokine	**Saline** fold change (SEM ±)	**CFA+HDM** fold change (SEM ±)	**CFA+HDM+FP** fold change (SEM ±)	**CFA+HDM+Baf** fold change (SEM ±)
IL-4	1.0 (0.16)	20.7 (4.18)	10.3 (2.68)	17.8 (1.88)
IL-5	1.0 (0.15)	8.7 (1.77)	3.3 (2.22)	7.7 (1.18)
IL-13	1.5 (0.63)	316 (53.56)*	284.3 (61.32)	215 (29.08)

Table 2. Cytokine/chemokine protein and mRNA expression in BAL and lung tissue

Discussion

Severe asthma remains difficult to treat with current therapies therefore, potential targets for effective treatment of severe asthma are being investigated (Poon et al., Martin et al., 2012). We have recently demonstrated that autophagy can be targeted in chronic asthma (McAlinden et al. 2018). In this study, we show that treatment with BafA1 alleviates airway inflammation and prevents development of AHR associated with HDM-challenge and steroid-resistant severe asthma phenotype *in vitro* and *in vivo*. Bafilomycins inhibit Vacuolar-type H+-ATPase (V-ATPase), which at high concentrations are capable of blocking late-phase autophagy and inhibiting the formation of autophagolysosomes (Yamamoto et al. 1998). At lower concentrations, bafilomycins have been shown to target both early and late phase of autophagy and demonstrate antiviral properties with the absence of host cytotoxicity (Yeganeh et al., Yuan et al., 2015). Pre-treatment with BafA1, followed by a taste 2 receptor (TAS2R) agonist mitigated the TAS2R-induced cell death in human airway smooth muscle cells (Pan et al. 2017). Transforming growth factor beta (TGF-β) simultaneously induces autophagy and fibrosis, as shown in human atrial myofibroblasts and airway smooth muscle (McAlinden et al. 2018; Ghavami et al. 2015). It has been shown that BafA1 treatment can reduce TGF-β induced fibrogenic response in atrial fibroblasts (Ghavami et al. 2015). The findings of this study highlight the therapeutic potential of BafA1 in severe asthma. Our results indicate that exploring the role of autophagy modulation in severe asthma therapy could potentially lead to novel treatment opportunities for severe asthma.

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Chapter 5

Discussion

The prevalence and incidence of asthma has increased in recent history, attributed to a combination of genetic, lifestyle, and environmental factors (Nunes et al. 2017). In 2017-2018 there were 38,792 hospitalisations resulting from asthma (AIHW 2019). Globally, the direct and indirect cost of asthma vary significantly, but in some countries the mean cost per patient is rather costly (Nunes et al. 2017). In Australia the mean cost per patient was estimated at $11,740 per person with asthma and ultimately 436 Australians died from asthma in 2019 (Deloitte Access Economics 2015; AIHW 2019). In Australia the gap between indigenous and non-indigenous health sadly remains, between the years of 2010-2014, the mortality rate for asthma among Indigenous Australians was twice that of non-Indigenous Australians (AIHW 2018).

Severe asthma can be devastating for those whose airway remodelling changes are too great. Whilst airway obstruction can be reversible for most asthma sufferers, the reversibility of airflow limitation is incomplete in some severe asthmatics. Therefore, this thesis focuses on the investigation of a novel approach of reversing or limiting airway remodelling in severe asthma. Therapeutically, only inflammation and airway hyperresponsiveness can be sufficiently attenuated in relation to asthma. Airway remodelling, the third pathophysiological link in the asthma phenotype needs attention. The therapeutic targeting of progressive airway fibrosis and remodelling has yet to be effectively established. As a result, severe asthma remains difficult to treat and novel treatments for severe asthma are being investigated (Martin et al. 2012; Poon, Chouiali, et al. 2012). Our studies (chapter 2) made the ground-breaking findings of increased expression of autophagy proteins in the human asthmatic airway wall along with two murine models of asthma showing the reduction in airway remodelling and fibrosis upon

treatment with autophagy inhibitors (CQ and BafA1). In chapter 4 of this thesis, we show that novel treatment with BafA1, an autophagy inhibitor, results in promising outcomes in the treatment of steroid-resistant severe asthma. We show that BafA1 alleviates airway inflammation and prevents development of AHR both *in vitro* and *in vivo*. We have shown here that the novel use of an autophagy inhibitor can be efficacious in the treatment of severe asthma in a multifaceted fashion and believe it to be one of the first murine models published showing these results. Acute (subchronic) and chronic (treatment) models of HDM-induced asthma in mice in chapter 2 showed more significant reduction in eosinophilic inflammation compared with neutrophilic inflammation in the BAL with chloroquine treatment. Airway remodelling changes were clearly initiated in the acute model and strongly established in the chronic model of HDM-induced asthma. In chapter 4 the efficacy of bafilomycin A1 (BafA1) was tested in a steroid insensitive mouse model of severe asthma and compared with fluticasone propionate (FP). BafA1 was able to reduce both the number of eosinophils and neutrophils significantly and was more effective than FP alone. Treatment with BafA1 also reduced the expression of IL-4, IL-5, and KC cytokines in the BAL. In vitro experiments also revealed that BafA1 can overcome steroid insensitivity in airway smooth muscle cells. BafA1 may therefore be a great candidate for treatment of severe asthma of various inflammatory phenotypes including those who suffer from steroid insensitivity. Insufficient inflammation data for the small human tissue cohort contained in chapter 2 make it difficult for any remarks about the results in relation to asthma phenotype. Greater cohorts of human tissue and further separation of phenotypic groups for in vivo studies can further elucidate effectiveness of treatments in future studies. Combined findings of my published work suggest autophagy modulation to be an exciting avenue for research into severe asthma.

Autophagy's role in pathogenesis is widely known as being a double-edged sword (Choi, Ryter & Levine 2013), now even more specifically in respiratory disease (Li, Wu, et al. 2017). The varying and complex involvement of autophagy in many different diseases is expanded in the introduction of this thesis. Cancer cells may be less aggressive and more responsive to chemotherapy if the levels of autophagy flux are higher (Valente et al. 2014). Alternatively, therapeutic selective inhibition of autophagy is being intensively explored in overcoming chemoresistance and tumorigenesis in glioblastomas (Raymond et al. 2018). Reductions in autophagy has also been implicated in impaired NF-κB signalling of which can result in tumorigenesis (Mathew et al. 2009). Diminished aggrephagy and the incomplete clearance of accumulated polyubiquitylated proteins has been documented in the airway epithelial cells of cystic fibrosis patients (Luciani et al. 2010). Autophagy depletion in cells also proves rather detrimental in effective host defence against intracellular pathogens (Nakagawa et al. 2004). An increased number of autophagosomes is observed in lung tissue from COPD patients and this increase in autophagy is associated with a pro-pathogenic phenotype (Chen et al. 2008). The high levels of ROS triggered by prolonged cigarette smoke exposure augments the levels of autophagy in COPD epithelial cells *via* a reduction in HDAC activity and resultant increase in LC3B-II expression (Poon, Eidelman, et al. 2012). Autophagy expression in a myriad of diseases, cell types and models has now been measured and continues to be measured, resulting in the acceleration of our understanding of a vital cellular process and the role it has in our longevity. However, the simple way of looking at this cellular process in disease as "up" or "down" may simply not be sufficient. We need to make multiple measurements within the autophagy cycle in order to measure possible dysfunction or dysregulation of autophagy flux. For example, an increased number of autophagosomes has been observed in patients suffering from acute necrotising pancreatitis (Helin et al.

1980) which has been found to be due to impaired autophagosome-lysosome fusion (Fortunato et al. 2009) or dysfunctional autophagy flux. The differing autophagy expression profiles in various diseases and disorders means that separate deep investigation of the process must be undertaken for lung pathogenesis and highlights the importance for targeted and selective modulation of autophagy.

Greater understanding of the autophagy mechanism and autophagosome structure wasn't reached until the studies in yeast by Nobel Prize recipient Yoshinori Ohsumi in which the molecular machinery of autophagy was extensively characterized for the first time (Ohsumi 1999). Ohsumi made the key discovery of 15 Autophagy-related genes, along with vital involvement in the description of the mechanism for autophagic degradation of cytosolic components (Baba et al. 1994; Takeshige et al. 1992; Tsukada & Ohsumi 1993). Fast-forward to modern scientific investigation and researchers such as Daniel Klionsky, Beth Levine and Augustine Choi lead the way in unravelling the disruption and dysregulation of integral autophagy proteins and stages of autophagy flux (Klionsky et al. 2012) and interplay with the development and progression of disease. Choi et al. found increased expression of autophagy proteins in COPD specimens and uncovered the interaction of autophagy and apoptosis in the lungs upon prolonged exposure and stimulus with cigarette smoke (Chen et al. 2010; Kim et al. 2008; Ryter, Lee & Choi 2010).

The research contained within this thesis is rather novel, as the exploration of autophagy expression and modulation in human asthma tissue and murine asthma models has yet to substantiate and we are joining the growing community investigating this pathway in the development of potential therapeutics in respiratory disease. Since autophagy is a homeostatic process, complete inhibition may not be the suitable approach for modulation, also autophagy modulation may not be appropriate for all patients perhaps

given their comorbidities or genetic predisposition to various other diseases. Our exciting contribution to the field is a step in the development of greater understanding of how autophagy is entwined with airway remodelling, igniting further research (Kota et al. 2017).

Current literature suggests autophagy to be a promoter of classically TGFβ driven airway remodelling (Ding & Choi 2014; Ghavami et al. 2015; Hernandez-Gea et al. 2012). Several proteins and markers of autophagy have been found to be upregulated in variety of organs other than the lungs, of which has been linked to fibrosis and remodelling in the heart and kidney (Aranguiz-Urroz et al. 2011; Boyle et al. 2011; Koesters et al. 2010). Measurement of human serum and BAL reveals that in asthmatics the concentration of TGFβ is elevated and has been associated with sustained airway remodelling which is characteristic of severe asthma (Chakir et al. 2003; Redington et al. 1997). TGFβ drives asthmatic airway remodelling *via* the activation of myofibroblasts and ASM cells, ultimately inducing the cellular release of ECM proteins (Boxall, Holgate & Davies 2006). A key feature of airway remodelling is the thickening of the basement membrane that is strongly linked to the susceptibility to asthma later in life, and these changes to the RBM can be seen as early as in children of the age of 3-4 (Cutz, Levison & Cooper 2002; Payne et al. 2003; Tsartsali et al. 2011). Another feature of asthmatic airway remodelling is the increased and thickened airway smooth muscle mass which correlates with poorer lung function. Our histopathological correlation of the increased accumulation of ASM mass and increased autophagy protein expression is a novel demonstration of the link between autophagy and remodelling in the human asthmatic lung which does not feature in the non-asthmatic human lung. In our published work entitled "Autophagy Activation in Asthma Airways Remodelling" we firstly confirmed that our selected human asthmatic

lung tissues (of both large and small airways) displayed classical features of airway remodelling, of mostly a severe degree (McAlinden et al. 2019). We measured RBM thickening, thickening of the epithelium, lamina propria depth, area of muscle mass, and the percentage of muscle per lamina propria; all of which were significantly greater than non-asthmatic controls in our selected tissue (McAlinden et al. 2019). The development of airway remodelling in asthma is due to a variety of factors and one suggestion is that persistent insult leading to chronic inflammation will lead to the development of these structural changes that correlate with decline in lung function and increase in asthma severity (Busse et al. 1999; Page et al. 2001; Pascual & Peters 2005). Autophagy could also emerge as being one of these factors that interplays with other factors to drive the structural changes that occur in severe asthmatic airway remodelling. A positive correlation of ATG5 and collagen alpha-1(V) gene expression in the airways of asthmatic patients was the first evidence to support the link between autophagy and fibrosis in asthma (Poon et al. 2017). ECM-regulated autophagy has been proposed to maintain tissue homeostasis and thus fuelling our hypothesis that dysfunctional autophagy in the presence of increased TGF-β may propel the progression of airway remodelling (Neill, Schaefer & Iozzo 2014). Our study delivers data and analysis of autophagy protein expression profiles in human and mouse tissue and provides comprehensive association with asthmatic airway remodelling. Furthermore, our human data showing increased beclin-1 protein expression in large and small airways is novel in understanding how the autophagy pathway regulates and initiates airway remodelling in asthma, with particular focus on the expression of beclin-1 localised in cilia and apical region of ciliated epithelial cells (McAlinden et al. 2019). We have shown that the increased expression of autophagy proteins, particularly beclin-1, along with the concomitant expression of collagen and pro-fibrotic cytokines can be attenuated with the administration of an autophagy inhibitor

(McAlinden et al. 2019). One of the greatest limitations of this study is that we only had access to a small amount of fixed adult remodelled asthmatic airway tissue and the n-number of patient tissue for large and small airways is quite low for the human component of this study. If these results were to be replicated with a larger number of large and small tissue from both asthmatic and non-asthmatic patients, then the true impact and drive of these results would become evident.

To support and further the findings in human asthmatic lungs we investigated whether CQ, an autophagy inhibitor, has any effect in reducing the features and symptoms of asthma in a murine model. CQ has long previously been shown to have potential as replacement treatment of asthma for its anti-inflammatory properties (Charous 1990, 1991; Charous, Halpern & Steven 1998; Goldstein 1983). The supporting mouse data in our manuscript shows promising results for the inhibition of autophagy through intranasal treatment with CQ. We show that CQ is effective in significantly reducing inflammatory cell influx, preventing mucus accumulation, reducing TGFβ1 production in the BAL, and preventing the production and deposition of ECM matrix proteins (McAlinden et al. 2019). This opens an avenue to investigate this autophagy-inhibiting drug (CQ) as a novel approach in targeting airway remodelling along with inflammation that proves to be rather elusive in the treatment of severe asthma. We believe that this therapeutic effect of CQ relies on the inhibition of TGFβ1 and reducing TGFβ1-dependent asthmatic pro-fibrotic signalling. The levels of TGFβ1 are elevated in asthmatics and key in driving remodelling changes in the lung (Halwani et al. 2011). It has previously been shown that TGFβ concomitantly influences features of remodelling along with regulating levels of autophagy in hepatic cells and cardiomyocytes (Ghavami et al. 2015; Hernandez-Gea et al. 2012).

Our murine model of asthma also demonstrates the activation of the autophagy pathway, as shown by an increase in beclin-1 protein expression. CQ treatment in this model reduces the protein expression of beclin-1 in the HDM-challenged mice with concomitant alleviation of features of airway remodelling (McAlinden et al. 2019). The multifaceted nature of CQ may be the commencement of exploration into autophagy modulation for severe asthma treatment. CQ modulates the autophagy pathway as it prevents the fusion of the autophagolysosome by increasing the lysosomal pH, ultimately stopping the completion of autophagy flux and the degradation of cargo destined for recycling in the lysosome (Ahlberg et al. 1985; Suzuki et al. 2002). The concentrations of CQ used in our study effectively inhibited the expression of beclin-1, however CQ has a number of other pharmacological effects, CQ was originally an anti-malarial and anti-arthritis drug (Homewood et al. 1972; Thome et al. 2013). CQ can also target rectifying potassium (Kir) channels (Marmolejo-Murillo et al. 2017), by disrupting the blood-retinal barrier CQ can bind to melanin resulting in toxic sequestration and retinopathy (Bernstein & Ginsberg 1964; Raines, Bhargava & Rosen 1989). These concentrations however are much higher than the concentrations we have used in our study, and with appropriate localisation of delivery CQ effectively modulates autophagy in asthma resulting in the reduction in airway remodelling and inflammation.

Our investigations into the involvement of the autophagy pathway in the asthmatic lung steers towards ASM bundles of the large airways, which we can observe the greatest impact on airway remodelling and fibrotic responses. In the human large airway ASM bundles of asthmatics, we have shown the increased expression of several integral autophagy proteins, accompanied by the supporting decrease in SQSTM1. In order to complement the human histopathological results we have shown that inhibition of

autophagy in a HDM-model of murine asthma attenuated inflammation, cleared mucus production, reduced TGFβ in the BAL, and ultimately reduced airway remodelling.

We then explored the use of other compounds with greater specificity for autophagy modulation such as BafA1. Bafilomycins are macrolides that inhibit Vacuolar-type H+-ATPase (V-ATPase) (Klionsky et al. 2016; Yeganeh et al. 2015). Higher concentrations of BafA1 are capable of inhibiting the fusion and formation of the autophagolysosome via cytosolic acidification, effectively preventing the completion of late-phase autophagy (Werner et al. 1984). Interestingly, at lower concentrations BafA1 has the ability to target both late and early phases of autophagy with no cytotoxicity, and at these lower concentrations it has shown various antiviral properties (Yeganeh et al. 2015; Yuan et al. 2015). Previous fibrosis and remodelling studies have shown that BafA1 treatment can reduce TGF-β triggered fibrogenesis in atrial fibroblasts, highlighting the therapeutic potential perhaps for severe asthmatic airway remodelling (Ghavami et al. 2015). Our results demonstrated that treatment with BafA1 alleviates airway inflammation and prevents development of AHR associated with the HDM-induced severe asthma phenotype *in vivo*. Our *in vitro* results from this study showed that treatment with BafA1 overcame steroid-insensitivity in TNF-α +IFN-γ treated ASM cells, providing more promising results for the development of future autophagy modulating therapeutics.

Our initial findings in fixed human tissue reveal the most binary expression protein of heavily expressed beclin-1 in the cilia of heavily remodelled asthmatic large airway epithelium in comparison with the absence of expression in non-asthmatic airways (McAlinden et al. 2018). The term ciliophagy was coined by Choi and colleagues following the identification of autophagy-dependent regulation of the shortening of cilia in the airways of COPD patients which is induced via the exposure to cigarette smoke

(Cloonan et al. 2014). In light of the establishment of the role for ciliophagy in COPD with the identification of beclin-1 expression profile in asthmatic cilia, we highlight the novel role and emerging research topic of the role of ciliophagy in severe asthmatic airway remodelling. Over expression of autophagy proteins in ciliated asthmatic epithelial cells may drive autophagy-dependent pathways involved in regulating cilia length and function with implications upon mucociliary clearance (MCC). One important question has arisen from these findings: Do airway remodelling changes in the airway wall and the increased autophagy protein expression profile contribute to the increased expression of beclin-1 in cilia, or does the increased cilia expression of beclin-1 in asthmatics influence the initiation and progression of airway remodelling? Another thought is that ciliophagy and dysfunctional MCC through various cellular stresses may alter the downstream levels of autophagy flux in various cell types and cellular environments in the airway wall. One recent study has also shown that smokers have fewer cells with beating cilia than non-smoking controls (Petit et al. 2019). They demonstrate that epithelial cell calcium influx and signalling is impaired in smokers with and without COPD whilst stating that Ca2+ signalling has not been sufficiently studied previously in primary bronchial epithelial cells. Reduced Ca^{2+} influx and depletion of Ca^{2+} in the ER can be attributed to the reduced expression of *ORAI3* which regulates cilia function and results in dysfunctional cilia beat frequency in COPD patients. The changes in Ca^{2+} metabolism observed in these cells may also alter the cytokine processing and secretory ability, affecting immunomodulation in the lung.

Ca^{2+} signalling and homeostasis is known to regulate the UPR and subsequently autophagy flux (Gorlach, Klappa & Kietzmann 2006; Rashid et al. 2015; Sehgal et al. 2017). UPR is an adaptive mechanism evoked by internal and external cellular stressors

that aims to maintain ER homeostasis, helping the cell adapt to ER stress (Walter & Ron 2011). When dysregulation of UPR occurs, the development of disease may ensue. The way in which autophagy flux in cilia, along with changes in the ASM and airway wall drives pathogenesis is yet to be completely understood and further understanding of these pathways and their involvement in ciliophagy and cilia dysfunction could greatly benefit novel therapeutic MCC targets.

Our studies into the modulation of autophagy demonstrate this pathway to be a promising avenue for treatment of severe asthmatic remodelling. It is also important to acknowledge several other airway remodelling pathways and therapies that are currently being investigated *in vitro* and *in vivo*. One of the promising studies has examined the role of the hormone protein relaxin in fibrosis and airway remodelling. Relaxin belongs in the insulin family and is naturally responsible for relaxing the pubic ligament and mediating several changes during pregnancy. The recombinant form of relaxin is shown to have anti-fibrotic properties in a variety of tissues such as the heart, liver, kidneys and lung (Samuel, Summers & Hewitson 2016). Royce *et al.* have shown that expression of relaxin is significantly lower in an allergic asthma murine model than in controls and treatment with human gene-2 relaxin (H2 relaxin) is able to reverse changes associated with airway remodelling such as collagen deposition (Royce et al. 2009). Development of a peptidomimetic of recombinant H2 relaxin has since pursued, advancing the possibility for another novel therapy for fibrosis in asthma (Hossain et al. 2016).

The process of EMT, where airway epithelial cells lose polarity and transform into cells with a mesenchymal phenotype, has been identified in both asthmatics and COPD patients (Hackett 2012; Sohal et al. 2010) with excessive EMT promoting fibrosis and the increased deposition of ECM proteins (Sohal et al. 2010; Willis, duBois & Borok 2006;

Willis et al. 2005). Markers of EMT have been shown to be reduced upon inhibition (with Bafilomycin A1) of autophagy in non-small cell lung cancer (NSCLC) cells (Alizadeh J et al. 2018) and autophagy-induced EMT has been shown to promote the invasion of carcinoma cells through activated TGF-β/Smad3-dependent signalling (Li et al. 2013). Grassi et al. have shown that a deficiency of autophagy reduces the expression of epithelial markers and increases the extent of mesenchymal biomarkers; whilst inducing EMT with TGFβ has a significant impact of autophagy flux (Grassi et al. 2015). The processes of autophagy and EMT are increasingly indicated as regulating each other, with crosstalk of great complexity (Colella et al. 2019).

We have shown increased expression of autophagy proteins in the epithelium of patients with severe asthma and in HDM-challenged mice, but further research is needed to confirm the interrelated roles of autophagy and EMT in the pathogenesis of severe asthma.

Sohal *et al.* continue to uncover the role of EMT in respiratory disease and the expansion in understanding of this process may grow into the development of successful airway remodelling therapeutics (Mahmood et al. 2017; Sohal, Ward & Walters 2014).

The only therapy currently approved to target airway remodelling is bronchial thermoplasty, however there is no consensus on the scientific verification of the clinical benefits as the proposed beneficial mechanisms of thermoplasty are poorly understood (Berair & Brightling 2014). Bronchial thermoplasty involves applying radio frequency thermal energy directly to airways in a series of bronchoscopies, targeting ASM for removal (Laxmanan & Hogarth 2015). It was initially trialed in dogs, returning positive results with significant improvement in AHR at temperatures of 65 and 75 °Celsius. The inverse correlation between ASM mass and AHR suggested that reducing ASM mass

through thermoplasty could be an effective means of reducing AHR and airway obstruction experienced in severe asthmatics (Danek et al. 2004). The application of bronchial thermoplasty in humans was shown to be feasible and with a good degree of tolerance as treatment of airways in areas scheduled for lung resection resulted in a reduction of ASM mass and no accompanying adverse clinical effects (Miller et al. 2005). However, the procedure and nature of bronchial thermoplasty is accompanied by strongly rooted contention and controversy with treatment through thermoplasty remaining an unfavorable procedure for many.

Severe asthmatic airways in children can be found to be remodelled at an age as early as 3 to 4 years old (Cutz, Levison & Cooper 2002; Payne et al. 2003; Tsartsali et al. 2011). Intervention therefore must occur at the earliest possible stage in order to prevent the future frequency of exacerbations and even limit the number of asthmatic deaths. Each year in Australia there are approximately 400 deaths due to asthma, one of the highest rates in the world, and this mortality rate doesn't seem to be declining (Australian Institute of Health and Welfare (AIHW): Poulos LM 2014). The work contained in this thesis proudly contributes to the effort in reducing asthma mortality and morbidity by assisting in identification of associated and supporting genes involved in airway remodelling which can be utilised in the development of targeted biologic therapies. The combination of this novel area of autophagy modulation, particularly autophagy inhibition, with other emerging procedures and biological therapies will boost our knowledge and repertoire in tackling airway remodelling.

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Chapter 6

Summary and Future Directions

In our studies we comprehensively characterised the expression of autophagy proteins with respect to airway remodelling in human tissue. We sufficiently tested the preclinical efficacy of autophagy inhibition in three murine models of asthma. With the use of both epithelial (Beas-2B) and ASM cells in vitro, we measured the interplay and mechanisms of autophagy and TGF-β-driven remodelling.

Signalling pathways such as autophagy can be aberrant and contribute to disease development later in life. Our data from adult asthmatics uncovers the novel mechanistic finding that autophagy may act as a key determinant of airway remodelling in asthma. As airway remodelling may occur at a very young age, this also needs to be investigated in samples from children. Studies of patients with mild to moderate severity of asthma may also be examined. Further investigation is also required into how the upregulation of beclin-1 expression in asthmatic ciliated cells affects autophagy flux regulation in other cells of the airway wall. Is increased expression of beclin-1 in the cilia influencing the cellular stimulation of airway remodelling, or are airway wall changes and airway remodelling in asthmatics contributing to increased expression of beclin-1 in cilia? The potential interplay between calcium homeostasis and autophagy flux in ciliated epithelial cells and their role in pathogenesis should also be thoroughly explored in the future. We would also wish to delve deeper into modulating the autophagy pathway by selectively targeting ASM cells in a novel attempt to limit airway remodelling and resultant airway constriction through the careful inhibition of selected autophagy proteins. Another subject for future investigation would be an endeavour to further understand the exact underpinnings of mitochondrial-based mechanisms and mediation by autophagy that determines asthma development and progression. In future studies we can include protein expression profiles of mitofusins (MFN1 & MFN2) and mitochondrial fission 1 protein

(Fis1) along with the utilisation of transmission electron microscopy (TEM) for a more robust measurement of mitochondria morphology, organisation, and function. TEM will also prove useful in the measurement of autophagosome formation in both human and mouse tissue in an expansion of the measurement of autophagy in severe asthma and the connection to airway remodelling and disease progression.

Autophagy is enhanced in asthmatic airways, which we believe contributes to remodelling in a TGFβ-dependent manner.

In future studies I wish to explore the intracellular changes which may drive ASM proliferation, result in airway wall remodelling and loss of lung function. I wish to broaden the focus to the impact of mitochondrial dysfunction and autophagy-mediated mitophagy.

Mitochondrial dysfunction has been identified as driving pathogenesis in various diseases and has now emerged as playing a role in airway disease pathophysiology (Prakash, Pabelick & Sieck 2017). In asthma, the increased number of mitochondria along with increased mitochondrial biogenesis in asthmatic ASM have been positively correlated with the contribution to AHR, ASM hyperplasia and ultimately poorer asthma control (Girodet et al. 2016; Trian et al. 2007). Alterations in mitochondrial homeostasis, can enhance airway smooth muscle contractility via increases in Ca^{2+} and lead to the development of AHR. I would therefore like to incorporate calcium and mitochondrial assays into future studies.

Finally, this thesis is not absent of limitations. To complement our studies, we require to further assess autophagy flux and protein expression. We have not sufficiently measured autophagy flux in these papers, yet we have measured the integral autophagy protein expression profiles in tissue known to have undergone airway remodelling and in comparison with non-asthmatic airways we have found autophagy expression to be increased in these tissues. Alongside TEM, to measure autophagy flux appropriately we would require to complement our existing autophagy protein measurements (particularly LCB-I conversion to the lipid-conjugated LC3B-II) with assays such as tandem mRFP/mCherry-GFP fluorescence microscopy or GFP-tracking lysosomal delivery and proteolysis (Klionsky et al. 2016). In order to further enhance the scientific might of these papers the n-number of human tissues in the second chapter (particularly asthmatic samples) requires to be increased and accompanied by correlative lung function data which could be acquired with assistance from the donating hospitals. A limitation of the study outlined in Chapter 2 is the fact that the numbers are small; non-asthmatic (10) and asthmatic (6) and further validation studies are warranted in a larger dataset.

During this time, we have contributed to the exploration of the autophagy pathway in regards to severe asthma and we hope that along with collaborators we will continue to delve deeper into uncovering disease stimulation from dysregulated autophagy. The summation of this thesis directs that inhibition of autophagy in the airways is of great interest in alleviating airway remodelling. In the future we wish to delve further into uncovering the mechanisms of autophagy in asthma and through collaboration map the intricacies of the autophagy pathway, crosstalk with other signalling pathways, and TGF-β in resultant asthma airway remodelling and disease progression.

The use of gene silencing techniques both *in vitro* and *in vivo* will be advantageous in developing targeted autophagy modulation therapies and novel respiratory formulations. Drugs with select antigen specificity could be developed to deliver these therapies and specifically target desired cell types within the airway wall in an attempt to reduce airway remodelling. Future studies and novel treatments derived from these ideas and data could potentiate in relieving remodelling changes in the airways of severe asthma sufferers.