Airway Epithelial Coordination of Inflammation and Smooth Muscle Phenotype

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Dedication

For Mom, Dad and Kristen, for your support as a family and encouragement over the years.

For Willa, for your friendship and love.

For my grandparents, for your admirable leadership and our countless memories together.

For Lucy and Guinness, for endless joy and entertainment.

Abstract

Background

Asthma is a chronic inflammatory disease of the airways for which there is no cure. The epithelium lines the lumen of airways creating a barrier to inhaled substances and protects the underlying tissue. However, it has been revealed over the past decades that the epithelium partakes in many features of asthma pathogenesis. Recruited inflammatory leukocytes contribute to airway responsiveness by increasing the mass of tissue underlying the epithelium that leads to narrowing of the airways, and also can drive airway structural changes over time. Release of pro-inflammatory cytokines by the epithelium is one way by which these cells are activated to translocate to the airways. Lipid mediators may contribute to the release of pro-inflammatory cytokines by epithelial cells. Furthermore, the epithelium may release many factors that can interact with the underlying smooth muscle; the functional tissue for regulating airway diameter. Increased smooth muscle mass is a hallmark feature of the disease and mediators of its mitogenesis may be released by epithelial cells. Finally, smooth muscle may be phenotypically regulated such that proliferative cells are not contractile. Epithelial derived factors may also modulate the contractility of this tissue.

Methods

Airway epithelial cells derived from primary donor patients as well as the cell line BEAS-2B were utilized. Primary smooth muscle cells were isolated from healthy control donors. BEAS-2B cells were stimulated to release the pro-inflammatory cytokine IL-8 with the lipid mediator sphingosine 1-phosphate (S1P). Pre-treatment of proteins associated with IL-8 release prior to stimulation with S1P were used to determine the cellular signalling events important in

mediating this process. Airway smooth muscle cells were co-cultured with or without epithelial cells prior to analysis of phenotype. The contractile phenotype was evaluated by examining traction force production in these cells, as well as intracellular calcium release to agonist stimulation. Pre-treatment with inhibitors of proteins known to mediate contraction were utilized to determine how co-culture modulates contractility. The proliferative phenotype was assessed by examining the incorporation of the thymidine analogue BrdU. Similarly, inhibitors of specific proteins were used to understand which pathways resulted in co-culture induced changes to the proliferative phenotype.

Results

S1P induced IL-8 release was mediated by S1P Receptor 2 signalling to NF-κB but not AP-1. The epidermal growth factor receptor (EGFR) transactivation by the generation of reactive oxygen species did not occur in this system. Co-culture with epithelial cells reduced the contractile phenotype, and the excitability of these cells was restored by inhibition of cyclooxygenase-1. Co-culture with epithelial cells increased the rate of proliferation that was also not sensitive to EGFR inhibition. Co-cultured cells expressed mRNA for proteins associated with pro-inflammatory cytokines and we observed increased expression of the pro-proliferative micro-RNA miR-210.

Conclusions

Airway epithelial cells have an increasingly important role in the pathogenesis of asthma. These cells release IL-8 after stimulation with S1P by virtue of S1PR2 signalling to Nf-κB. Furthermore, epithelial cells can phenotypically modulate airway smooth muscle cells towards a more proliferative and less contractile state.

Resume

Contexte

L'asthme est une maladie inflammatoire chronique des voies aériennes pour laquelle il n'y a pas de traitement curatif. L'épithélium du lumen des voies respiratoires créer une barrière aux substances inhalées et protège le tissu sous-jacent. Il a cependant été démontré au cours des dernières décennies que l'épithélium est impliqué dans l'apparition et le développement de l'asthme. Les leucocytes inflammatoires recrutés au niveau de l'épithélium contribuent à la réactivité des voies aériennes en augmentant la masse du tissu sous-jacent, conduisant ainsi au rétrécissement des voies respiratoires. De plus, leur recrutement peut entraîner des modifications structurelles des voies aériennes au cours du temps. La libération de cytokines pro-inflammatoires par l'épithélium est un moyen par lequel les leucocytes sont activés, pouvant ainsi se déplacer dans les voies respiratoires. Certains médiateurs lipidiques peuvent contribuer à la libération de cytokines pro-inflammatoires par les cellules épithéliales. En outre, l'épithélium peut libérer de nombreux facteurs qui peuvent interagir avec le muscle lisse sous-jacent, soit le tissu fonctionnel qui régule le diamètre des voies aériennes. L'augmentation de la masse musculaire lisse est une caractéristique importante de la maladie et des médiateurs de la mitogenèse des cellules de muscle lisse peuvent être libérés par les cellules épithéliales. En outre, le muscle lisse peut être régulé phénotypiquement de façon à ce que les nouvelles cellules qui prolifèrent ne soient pas contractiles. Finalement, des facteurs libérés par les cellules épithéliales peuvent également moduler la contractilité de ce tissu.

Méthodes

Des cellules épithéliales primaires de voie aérienne dérivées de patients donneurs ainsi que la lignée de cellules BEAS -2B ont été utilisées. Les cellules musculaires lisses primaires ont été isolées à partir de donneurs en bonne santé. Les cellules BEAS-2B ont été stimulées avec les médiateurs lipidiques de la sphingosine 1-phosphate (S1P) pour libérer la cytokine pro-inflammatoire IL-8. Des traitements influençant la relâche d'IL8 ont été effectués afin de découvrir les voies de signalisation impliqués dans les évènements médiés par la stimulation avec S1P. Les cellules musculaires lisses de voie aérienne ont été cultivées avec ou sans cellules épithéliales avant l'analyse du phénotype. Le phénotype contractile a été évalué en examinant la production d'une force de traction dans ces cellules, ainsi que la libération du calcium intracellulaire à l'agoniste de stimulation. Des inhibiteurs de protéines responsable de la contraction ont été utilisées afin de déterminer comment la co-culture module la contractilité. Le phénotype de prolifération a été évalué en examinant l'incorporation de BrdU, un analogue de la thymidine. En meme temps, des inhibiteurs ont été utilisés dans le modèle de co-culture pour comprendre quelles voies de signalisation ont entrainer les changements de phénotype prolifératif

Résultats

La sécrétion d'IL-8, suite au traitement par S1P, est médiée par le récepteur S1PR2 via l'activation de NF-kB, mais non par celle d'AP-1. La transactivation du récepteur du facteur de croissance épidermique (EGFR) par la génération d'espèces réactives de l'oxygène ne se produit pas dans ce système. La co-culture des cellules de muscle lisse avec des cellules épithéliales a réduit le phénotype contractile et l'excitabilité de ces cellules a été normalisée par l'inhibition de la cyclooxygénase-1. La co-culture avec des cellules épithéliales augmente le taux de prolifération et cette augmentation insensible à l'inhibition de l'EGFR. Les co-cultures cellulaires

ont exprimé l'ARNm pour les protéines associées à des cytokines inflammatoires et nous avons observé que l'expression du micro-ARN miR-210 pro-prolifératif est augmentée.

Conclusions

Les cellules épithéliales des voies aériennes jouent un rôle de plus en plus important dans la pathogenèse de l'asthme. Ces cellules libèrent de l'IL-8 après la stimulation du S1PR2 par S1P et via la voie de signalisation NF-kB. En outre, les cellules épithéliales peuvent moduler le phénotype des cellules musculaires lisses des voies aériennes en augmentant leur état prolifératif et en reduisant leur capacité de contraction.

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Table of Contents

Contribution of Authors

This manuscript-based thesis has been produced by manuscripts that have been published, submitted for publication or will be submitted for publication in peer reviewed journals.

Chapter 2. Sphingosine 1-Phosphate (S1P) Induced Interleukin-8 (IL- 8) Release Is Mediated by S1P Receptor 2 and Nuclear Factor kB in BEAS-2B Cells

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Michael O'Sullivan (PhD candidate) designed all experiments, conducted all experiments, interpreted the data and wrote the manuscript.

Dr. Nobuaki Hirota (Post-doctoral Fellow, PDF) contributed to the design of experiments, interpretation of the data and reviewed the manuscript.

Dr. James G. Martin (Principal Investigator, PI) contributed to the design of experiments, interpretation of the data and writing of the manuscript.

Chapter 3. Airway Epithelial Cells Reduce the Contractile Phenotype of Airway Smooth Muscle Cells through a COX-1 Dependent Mechanism

This article is in preparation for submission.

Michael O'Sullivan (PhD candidate) designed all experiments, conducted all experiments, interpreted the data and wrote the manuscript.

Elizabeth Gabriel (Research Technician, Harvard TH Chan School of Public Health) contributed to the conduct of traction force microscopy assays.

Dr. Chan Y. Park (Research Scientist, Harvard TH Chan School of Public Health) contributed to the design and conduct of traction force microscopy assays, interpretation of the data and revision of the manuscript.

Dr. Gijs Ijpma (Research Associate, McGill University) assisted with dissection of tissues and analysis of data.

Dr. Jeffrey Fredberg (Investigator, Harvard TH Chan School of Public Health) provided reagents, plates, equipment and technical support for the traction force microscope assays.

Dr. Anne-Marie Lauzon (Investigator, McGill University) provided human lung tissues for the primary culture of airway smooth muscle and epithelial cells.

Dr. James G. Martin (Principal Investigator, PI) contributed to the design of experiments, interpretation of the data and writing of the manuscript.

Chapter 4. Airway Epithelial Cells Increase Airway Smooth Muscle Cell Proliferation

This article is in preparation for submission.

Michael O'Sullivan (PhD candidate) designed all experiments, conducted all experiments, interpreted the data and wrote the manuscript.

Dr. Paul-Andre Risse (Post-doctoral Fellow, PDF) conducted ELISA for the measurement of heparin-binding epidermal growth factor.

Dr. Anne-Marie Lauzon (Investigator, McGill University) provided human lung tissues for the primary culture of airway smooth muscle and epithelial cells.

Dr. James G. Martin (Principal Investigator, PI) contributed to the design of experiments, interpretation of the data and writing of the manuscript.

Contribution to Original Knowledge

The work conducted in this thesis provides the scientific community with original findings regarding the pathogenesis of asthma. In chapter two, we explored the cellular signalling events leading to S1P induced IL-8 secretion. Specifically, we demonstrated that this occurs through the activation of S1PR2 and NF-κB, and not through the ROS induced EGFR transactivation. To our knowledge, we are the first to explore the receptor subtypes and transcription factors involved in this process. We provide molecular details surrounding a potentially key pathway involved in the recruitment of neutrophils to the asthmatic airway, drawing conclusions that S1PR2 antagonism may be beneficial for the treatment of certain subsets of asthma. We also ruled out a role of S1PR1 and S1PR3 in mediating this effect, turning the field away from future examination of these receptors as potential therapeutic targets for the treatment of S1P induced IL-8 release. Similarly, we disproved the role of the EGFR and the IL-8 transcription factor AP-1, again indicating that these molecules may not show promise for selective inhibition.

In Chapter three, we explored for the first time, the role that airway epithelial cells have in controlling airway smooth muscle cell excitability and contractility. Although previous work has been conducted in tissue preparations, we are the first to report that epithelial cell co-culture reduces the contractility of smooth muscle cells *in-vitro*. This system allowed us to probe molecular mechanisms by which this occurs. We discovered that the PGE_2 producing enzyme COX-1 mediates the reduction in excitability after co-culture with epithelial cells. We suggest that the phenotype modulation away from the contractile state may be mediated by COX-1 products and that activation of this enzyme may be of pharmacological interest for the reduction in ASM cell contractility in asthmatic patients.

In Chapter four we examined the mechanism by which epithelial cells increase ASM cell proliferation. We demonstrated that co-culture induces the pro-proliferative miRNA-210 and that this miR can augment the rate of DNA synthesis in ASM cells. We also demonstrated the novel finding that ASM cells express many pro-inflammatory cytokines after co-culture and suggest that these may act in an autocrine fashion to drive the proliferation of these cells. Furthermore, we observed that the EGFR was not involved in co-culture induced proliferation, although HB-EGF was induced in the muscle.

Abbreviations

5-LO: 5-lipoxygenase

AA: arachidonic acid

AHR: airway hyperresponsiveness

AP-1: activator protein-1

APC: antigen-presenting cell

ARE: anti-oxidant response element

ASM: airway smooth muscle

Bcl2: B cell lymphoma gene 2

cADPR: cyclic-ADP-ribose

cAMP: cyclic adenosine monophosphate

CC: (C-C motif) chemokine

CCL: chemokine ligand

CFTR: cystic fibrosis transmembrane conductance regulator

COX: cyclooxygenase

cPLA2: cytosolic phospholipase A2

CRAC: calcium release-activated channel

CSE: cigarette smoke extract

CXC: (C-X-C motif) chemokine

CXCL: chemokine ligand

CysLT: cysteinyl leukotriene

CysLTR: cysteinyl leukotriene receptor

DAMP: damage-associated molecular pattern

DC: dendritic cell

DMTU: dimethylthiourea

DUOX: dual oxidase

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

Elk1: E26-like protein 1

EMT: epithelial-mesenchymal transition

ERK: extracellular signal-regulated kinase

ET-1: endothelin-1

FEV_1: forced expiratory volume in one second

FGF-2: fibroblast growth factor 2

FGF: fibroblast growth factor

FLAP: 5-LO activating protein

FTY720: fingolimod

GM-CSF: granulocyte macrophage colony-stimulating factor receptor

GPCR: G-protein coupled receptor

GWAS: genome wide association study

HB-EGF: heparin binding EGF

HDM: house dust mite

HOCl: hypochlorous acid

HPETE: hydroperoxyeicosatetraenoic acid

IFN-γ: interferon-γ

Ig*X*: immunoglobulin *X*

IL: Interleukin

ILC: innate lymphoid cell

ILR: Interleukin receptor

iNOS: inducible nitric oxide synthase

IP3: inositol trisphospate

IP3R: IP3 receptor

Keap 1: Kelch-like ECH associating protein 1

KLF4: kruppel-like factor 4

LTC4: leukotriene C4

M1: classically activated macrophage

M2: alternatively activated macrophage

MAPK: mitogen-activated protein kinase

MAPK: mitogen-activated protein kinase

MCP-1: monocyte chemoattractant protein-1

MEJ: myoendothelial junctions

MIP: macrophage inflammatory protein

miRNA: micro-RNA

MLCK: myosin light chain kinase

MLCP: myosin light chain phosphatase

MMP: matrix metalloproteinase

MYD88: myeloid differentiation primary response gene 88

NES: nuclear export signal

NF-κB: nuclear factor-κB

NLS: nuclear localization signal

NO: nitric oxide

NOX: NADPH oxidase

Nrf-2: nuclear factor (erythroid-derived 2)-like 2

NSAID: non-steroidal anti-inflammatory drug

Orai: *olf186*-F protein

OVA: ovalbumin

PAMP: pathogen associated molecular pattern

PAR: protease activated receptor

PDGF: platelet derived growth factor

PDGF: platelet-derived growth factor

PGE_2: prostaglandin E_2

PI3K: phosphatidylinositol-4,5-bisphosphate 3-kinase

PKC: protein kinase C

PLC: phospholipase C

PRR: pattern recognition receptor

RANTES: regulated on activation, normal T cell expressed and secreted

ROS: reactive oxygen species

RTK: receptor tyrosine kinase

S1P: sphingosine 1-phosphate

S1PR: sphingosine 1-phosphate receptor

S1PR: sphingosine1-phosphate receptor

SERCA: Sarcolemmal-endoplasmic reticular ATPase

SNP: single nucleotide polymorphism

SOCE: store-operated calcium entry

SOD: superoxide dismutase

Sp1: simian virus 40 protein 1

SPHK: sphingosine kinase

Spl:sphingosine lyase

SRF: serum response factor

STIM: stromal interaction molecule

TGF: transforming growth factor

Th2: T-helper 2

TLR: Toll-like receptor

TNF: tumour-necrosis factor

TRAF: TNF receptor-associated factor

Treg: T regulatory cell

TRIF: TIR-domain-containing adapter-inducing interferon-β

TRP: transient receptor potential

TSLP: thymic stromal lymphopoietin

TXA_2: thromboxane A_2

VEGF: vascular endothelial growth factor

VSMC: vascular smooth muscle cell

CHAPTER 1:

Introduction & Review of the Literature

1.1 Introduction

Airway diseases are an increasing burden on healthcare systems. Asthma is one such disease for which the pathophysiology remains quite mysterious. Hallmark features of asthma include airway hyperresponsiveness to inhaled substances that cause narrowing of the airways as well as persistent inflammation of the airway wall. Both of these characteristics increase the work of breathing and the sensation of effort to breathe and they contribute to the symptoms experienced during an exacerbation. It is estimated that 300 million people worldwide are suffering from asthma [1]. Although research over the past several decades has contributed to novel therapies for patients suffering with asthma, there is an urgent need for better treatments to improve the quality of life of those suffering from the more severe forms of this disease. The work presented in this thesis aims to better understand the pathogenesis of asthma, and also to learn more about the interaction of structural cells within the airways. This disease is likely driven by the intricate communication between both inflammatory and airway structural cells. It is becoming more evident that airway wall changes that occur in asthmatic subjects may due to epithelial dysfunction [2] and so a better understanding of airway epithelial biology will contribute new knowledge on the pathogenesis of this disease.

1.2 Asthma

1.2.1 Overview

Asthma is a chronic inflammatory disease of the airways that affects millions of people worldwide. The prevalence of asthma has increased from 7.3% in 2001 to 8.4% in 2010 in the United States and thus costs healthcare systems more and more each year [3]. Asthma is

characterized by acute obstruction to airflow complemented by chronic inflammation of the bronchial tree [4]. In addition to airway inflammation, asthmatics possess a greater mass of smooth muscle within the airway walls [5], secrete more mucus into the airway lumen [6], deposit new matrix components and have increased airway vascularization [7]. It is apparent that diverse mechanisms drive the pathogenesis of this disease and it is becoming increasingly evident that patient-specific therapies are the future of clinical treatment. Cytokine production from airway structural cells, including epithelial cells, is responsible for the recruitment of leukocytes and the conditioning of cells at the interface between innate and adaptive immunity such as dendritic cells and innate lymphoid cells. Sustained inflammation likely drives airway remodeling [8]. The interaction of lung structural cells, their communication with one another, and the interface of immune and mesenchymal derived cells has created a fascinating environment to study. These cells and their interactions have driven research to pose intriguing questions and establish new concepts in this field.

1.2.2 Allergic Asthma

Asthma is a syndrome within which many subsets may be identified. This is evident through the descriptions of a variety of clinical phenotypes [9]. Allergic asthmatics are atopic individuals, indicating that they are predisposed to be hypersensitive to certain allergen(s) by mechanisms that are immunoglobulin E-related (IgE). Patients may present clinically with a wide variety of inflammatory markers. However it is quite common for allergic asthmatics to possess hallmark eosinophilia within the airway wall and in sputum. Severe asthmatics tend to possess increased bronchial infiltration by neutrophils in addition to eosinophilia [10]. The associated inflammation likely drives structural changes to the bronchial wall, which later contributes to the increased resistance observed in the airways of these patients. It has been evident for many years that there

is an association between susceptibility to allergy and the development of asthma with elevated serum IgE levels [11]. Furthermore, T-helper 2 (Th2) lymphocytes and their associated cytokines are frequently found within the asthmatic lung [12] and these are tightly linked with allergic responses. These and other arms of the immune system have been explored and mechanisms by which these immune effectors alter the structure of the airway and contribute to obstruction have become more evident.

Allergic asthma, the most prominent subset, involves the activation of Th2 cells, which secrete IL-4, IL-5 and IL-13 cytokines to coordinate a variety of events leading to airway hyperresponsiveness [13]. Allergic asthma is usually treated with corticosteroids. Corticosteroids bind to the glucocorticoid receptor which, when translocated to the nucleus, alter gene expression by controlling the rate of mRNA synthesis through effects mediated in the 5' promoter region of the glucocorticoid response element. Corticosteroids have been shown to prevent the binding of activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) to their respective promoter regions [14]. AP-1 and NF-κB are important transcription factors in mediating the production of pro-inflammatory cytokines [15]. Thus, treatment with corticosteroids has the ability to reduce infiltration of leukocytes to the airway wall and can lower the frequency of exacerbations. Innate lymphoid cells (ILCs) may also play a role in allergic asthma. ILCs do not recognize antigen, but are activated by innate cytokines, often epithelial-derived, to drive effector responses. There are currently three known members of the ILC family, named ILC1-3. It was shown that IL-13 producing ILC2s can infiltrate the airways and may drive "allergic" AHR [16]. Continued work in this new field should be conducted to better understand allergic asthma.

1.2.3 Non-Atopic Asthma

The immunopathology of non-allergic asthma, sometimes referred to as intrinsic asthma, shares many similarities to that of atopic asthma. Biopsies from intrinsic asthmatics demonstrated elevated bronchial infiltration by T-cells and macrophages, similar to atopic-asthmatic patients [17]. Furthermore, biopsies of non-atopic asthmatics showed increased mRNA and protein of the Th2 cytokines IL-4 and IL-5, suggesting the possibility that local IgE production could occur [18]. Intrinsic asthmatics do however possess increased numbers of cells positive for granulocyte macrophage colony-stimulating factor receptor-α (GM-CSFRα), a marker that is predominantly expressed on macrophages [19]. The presence of Th2 cytokines suggests that antigen presentation may occur, although the antigens that may be involved in driving Th2 type asthma in the absence of sensitization to usual aeroallergens are unknown. Potential candidates for such antigens may derive from viral infection or autoantibodies [18]. Alternatively, ILCs may be the source of these Th2 cytokines, as a non-atopic model of infection has demonstrated that ILC2s can generate IL-13 in a helminth parasite infection [20]. However the lack of robust models of non-atopic asthma has hindered the ability of scientists to understand this asthma subset. Recent work has given rise to the notion that ILCs can act as an effector cell within this subset of asthma. Most work exploring asthma pathogenesis in the context of ILCs has examined the role of ILC3s. It was demonstrated in a mouse model of steroid resistant asthma that IL-17 was necessary to drive the asthma phenotype [21]. Furthermore, ILC3s secrete IL-17 and have also been associated with obesity driven models of asthma through the induction of the inflammasome, a pro-inflammatory protein complex [22]. Although this is a poorly understood subset, there is new hope in expanding knowledge through continued exploration of ILCs and their role in non-allergic asthma.

1.3 Asthma Risk Factors

1.3.1 Hygiene Hypothesis

An interesting study described that the prevalence of asthma was increasing in developed Western nations [23]. Atopy is the predisposition to overreact to allergen exposures and the increase in atopy in western nations may be due to the combination of a variety of factors such as the fact that children are exposed to less antigenic diversity at a young age, changes in maternal diet, less infant infections and increased vaccination rates and antibiotic use [23]. Among the mechanisms for the increase in prevalence of asthma that have been postulated the one receiving the most attention in recent years is the "hygiene hypothesis". This hypothesis is based on the premise that children growing up in urban areas of developed nations are exposed to a reduced burden of potential allergens at a young age, or are exposed to less diverse allergens and are therefore more likely to develop allergic diseases such as asthma [24]. The first published observation supporting the hygiene hypothesis demonstrated that there was an increased rate of allergic rhinitis in children who grew up in homes with fewer siblings [25]. Children with older siblings are less likely to be atopic, which may reflect the increased exposure to pathogens due to more interaction with other young children [26]. Another study has shown that atopy is more prevalent in children that entered day care at an older age [27]. Furthermore, children who grew up on farms exhibited a downward trend in the susceptibility to develop asthma and were less likely to be atopic [28]. It was also shown that the quantities of endotoxin, a component of the bacterial cell wall, were increased in the farming environment [29]. Endotoxin sensing receptors, known as Toll-like receptors (TLRs) likely mediate the protective effect of endotoxin exposure throughout childhood against atopy [30]. It appears that air pollution such as diesel exhaust particles can act as an adjuvant and augment the host responses to allergen sensitization and may

further explain the increased rates of asthma [31]. The hygiene hypothesis requires more work to describe the physiological role that a lack of allergen exposure has on the development of asthma. Whatever the cause, researchers have uncovered a great deal regarding the immuno-pathology of this disease. It is well accepted that the immune system plays a critical role in regulating atopic asthma.

1.3.2 Genetic Component of Asthma

Examination of the risk of having asthma if one or both parents are affected by it suggest a significant heritable component to the disease [32]. Several genome wide association studies (GWAS) have revealed candidate genes that may be implicated in asthma predisposition. The fact that these studies have not produced a clear gene or group of genes that are definitively associated with asthma likely highlights the fact that there is an important environmental component of the disease. In one study exploring 2669 asthmatics and 4528 healthy control subjects revealed the interleukin-6 receptor (IL-6R) as an associated gene with atopic-asthma, adding interest to IL-6R antagonism as a treatment for allergic asthma [33]. Another GWAS identified a single nucleotide polymorphism (SNP) in *ORMDL3* linked to childhood asthma [34]. This gene was later discovered to be expressed by epithelial cells and to positively regulate metalloproteases and pro-inflammatory cytokines such as IL-8 [35]. Interleukin-33, an epithelial derived innate cytokine that drives Th2 responses, has been published as a gene with a SNP associated with asthma [36]. The receptor of IL-33, the interleukin 1 receptor-like 1 (IL1RL1), also contains a SNP that is linked to asthma in 10 different population studies [37]. Although these association studies have revealed some interesting genetic information, asthma remains an elusive disease with a need for new therapeutic targets. Future GWAS are required to consolidate

the data generated thus far in other populations, and some of these identified genes should be further explored as to their role in driving asthma pathology.

1.4 Innate Immunity of Asthma

1.4.1 Epithelium

Innate immunity plays an important role in asthma pathogenesis. A variety of asthma "triggers" include non-self peptides associated with viral, bacterial or aeroallergen components. The first line of defense against such insult is the airway epithelium. The epithelium consists of several cell types that collectively regulate airway surface liquid composition, clear mucus through coordinated cilia movement and maintain barrier integrity. These cells include the progenitor basal cells, ciliated cells, mucus secreting goblet cells, neuroendocrine cells and protective club cells. In the alveolar space, alveolar type I cells facilitate gas exchange and alveolar type II cells secrete surfactant into the luminal liquid to reduce surface tension.

Common aeroallergens include components of house dust mite, pollens, grasses, animal dander and moulds. When aeroallergens enter the lung, they interact with the airway epithelium. Airway epithelial cells express tight junctions that render the epithelium a tight barrier to inhaled molecules, preventing access to the underlying tissues. Tight junctions are formed by proteins such as occludin, claudin and junctional adhesion molecule between adjacent epithelial cells and the scaffold proteins zona-occludin proteins that connect the transmembrane proteins to the actin cytoskeleton; these structures define the apical/basolateral boundary [38]. Tight junctions are therefore important in the polarization of the epithelium, assisting in the coordination of membrane protein trafficking to either the apical or basolateral pole of the cell [39]. Below the

tight junction lies the adherens junction, which consists of E-cadherin and β-catenin [40]. The adherens junction tethers the two epithelial cells together and regulates the actin cytoskeleton [41]. It is appreciated that the epithelium has a myriad of mechanisms to contribute to innate and adaptive immune cascades. The epithelium will recognize pathogen associated molecular patterns (PAMPs) by virtue of expressing pattern recognition receptors (PRRs) such as TLRs. TLR activation by ligand activates two classical immune signalling pathways within the epithelial cells, being that of the TIR-domain-containing adapter-inducing interferon-β (TRIF) and myeloid differentiation primary response gene 88 (MYD88) pathways. MYD88 signalling leads to NF-κB activation which drives the transcription of many pro-inflammatory cytokines and chemokines [42]. Activation of PRRs on airway epithelial cells can lead to the release of chemokines, such as CXCL8 (IL-8) [43], a potent neutrophil chemoattractant [44]. SNPs of TLR2, a PRR expressed on airway epithelial cells, were found to be an important predictor of asthma in children growing up in rural areas [30]. TRIF effector signalling will activate Interferon Regulatory Factor 3 which drives type 1 interferon generation as well as NF-κB driven pro-inflammatory responses. Furthermore, the epithelium from asthmatic patients has been shown to be more permeable than that of controls [45], and this may be explained by the observation that epithelial cells derived from asthmatic patients possess less caveolin-1, a scaffold protein associated with epithelial barrier integrity [46]. The proteins responsible for creating tight junctions and maintaining barrier integrity are disturbed in asthmatic epithelial cells placed in culture [47], and this damaged asthmatic epithelium may allow allergens to more easily access the underlying antigen processing DCs. The epithelium from asthmatics also contains more basal cells, indicating a less-differentiated population that is associated with repair processes. These cells also secrete more pro-inflammatory cytokines than those derived from

healthy control subjects, upon exposure to both rhinovirus and particulate matter [48]. The epithelium can also release periostin in response to the Th2 cytokine IL-13, which stimulates fibroblasts to secrete collagen that may contribute to sub-epithelial fibrosis [49] as well as drive goblet cell metaplasia within the epithelium itself [50]. Asthmatic airways also possess more EGFR [51] and its ligand heparin binding EGF (HB-EGF) [52] likely to aid in the repair of barrier integrity. This EGFR ligand could drive proliferation of nearby airway smooth muscle (ASM), further contributing to airway remodeling. Activation of airway epithelial cells by S1P has been shown to lead to the release of IL-8 [53,54]. Epithelial cell activation by diesel exhaust particulate matter releases the dendritic cell maturation factor GM-CSF [55]. Each year, increasing evidence is pointing towards this tissue as an important culprit in mediating remodeling events within the airway, and for this reason, the majority of the work of this dissertation aimed to contribute knowledge in this area or research.

In chapters two to four of this dissertation, data from experiments examining the role of airway epithelial cells on release of pro-inflammatory chemokine and interaction with smooth muscle cells will be presented.

1.4.2 Dendritic Cells

Dendritic cells (DCs) are antigen-presenting cells (APCs) that survey the airway for the presence of potentially pathogenic molecules. DCs recognize antigens by expressing PRRs such as TLRs or C-type lectin receptors and upon activation of these receptors, the DC migrates to mediastinal lymph nodes where it encounters and activates naïve T-cells [56]. DCs may have the ability to extend protrusions through the epithelial barrier in order to sample the airway lumen [57]. Furthermore, the epithelial layer may allow small antigens to enter into the lymphatic vessels

where they could interact with resident lymphatic DCs such as plasmacytoid DCs [58]. This migration occurs through the expression of CCR2-like receptor (CCRL2) [59] and the sphingosine-1-phosphate receptor 1 (S1PR1) agonist SEW-2871 can down-regulate this receptor [60]. Epithelial derived signalling molecules will cause maturation of the underlying DCs through the production of cytokines such as thymic stromal lymphopoietin (TSLP) [61]. The release of TSLP can influence the DC to become a Th2 promoting APC via the up-regulation of OX40L, a co-stimulatory molecule in T cell activation [62]. As research moves forward, the importance of the DC in linking airway epithelial cell innate immune signalling and activation of the adaptive immune response in the asthmatic airway is becoming more evident.

1.4.3 Neutrophils

Neutrophilia is a prominent feature of severe asthma [10]. Neutrophils are recruited to the airways through the local release of chemokines such as IL-8 [63] and the sputum levels of this chemokine have been shown to correlate with the severity of asthma [10]. In chapter two of this thesis, the release of IL-8 from airway epithelial cells was explored for its potential role in the recruitment of this cell type. Neutrophils reside predominantly in the bone marrow [64], spending only 6-8 hours as circulating peripheral cells [65]. Neutrophil chemotaxis from the blood to the airway occurs upon the release of a variety of chemokines including IL-8, chemokine (C-X-C motif) ligand 1(CXCL1/GRO-α), macrophage inflammatory protein 1α, chemokine (C-C motif) ligand 5 (CCL-5/RANTES) and chemokine (C-X-C motif) 5 (CXCL5/ENA-78) [66]. The release of these chemokines can occur when damage-associated molecular patterns (DAMPs), which are normally sequestered away from TLRs are released upon tissue damage, activating the TLR and inducing neutrophil chemokine production [67]. Uric acid is one such agonist that is released from dying cells [68]. In a study examining

competitive swimmers, it was demonstrated that chlorine by-products caused epithelial damage, increased DAMPs and augmented airway neutrophilia [69]. As discussed previously, TLR activation may also occur in the presence of PAMPs, recruiting neutrophils [43]. Neutrophils respond to IL-8 when the neutrophil CXCR1/2 receptor is activated at the leading edge of the cell, inducing G-protein coupled signalling and the release of intracellular calcium [70]. Neutrophil migration is driven by the activation of phosphatidylinositol (3,4,5)-trisphosphate kinase (PI3K) that in turn results in actin polymerization at the site of receptor activation [71]. In the early 1930's it was discovered that leukocytes consume more oxygen during phagocytosis [72]. For over three decades this "respiratory burst" remained a mystery. Eventually it was discovered that this increase in oxygen consumption was a key requirement for phagocytic leukocytes to destroy bacteria [73]. Patients with leukocytes lacking this oxidative burst capability are prone to infection and the term chronic granulomatous disease has been given to describe this pathology [74]. The NADPH oxidase (NOX) enzyme was discovered to be the protein complex responsible for the consumption of oxygen and producer of reactive oxygen species (ROS). The NOX complex consists of two membrane subunits, collectively termed cytochrome b558, and four cytosolic components, p47PHOX, p67PHOX, p40PHOX and Rac. The phosphorylation of p47PHOX is a crucial step in the recruitment of the four cytosolic subunits to cytochrome b558, and is regarded as a necessary event in the activation of the complex [75]. Protein kinase C (PKC) likely phosphorylates serine residues on the p47PHOX subunit [76]. Superoxide is generated by NADPH oxidase which can then be converted to hydrogen peroxide, another ROS, by superoxide dismutase (SOD). The leukocytes in asthmatics have been shown to produce more superoxide than those of healthy controls [77]. Neutrophils also secrete the enzyme myeloperoxidase (MPO), which converts hydrogen peroxide to

hypochlorous acid (HOCl), a strong oxidant. It is estimated that HOCl levels in the lung can reach 8mM [78]. MPO secretion from neutrophils isolated from asthmatic patients is increased compared to healthy controls [79]. HOCl can also be produced when chlorine gas is inhaled and combines with water in the airways [80]. Children who regularly attend chlorinated pools are more likely to develop asthma [81]. Although the role that oxidative stress plays in asthma pathogenesis is largely unclear, mechanisms are starting to be postulated.

Phagocytic leukocytes may produce ROS in the airways that can damage airway structural cells and may contribute to the pathophysiology of asthma. ROS may also play a role in activation of airway epithelial cells. Asthmatic airways have been shown to possess more ROS [82] but the source and significance is still unclear.

Oxidative stress is an emerging theme in asthma research. The oxidation state of a cell can play a critical role in signalling pathways, and asthmatics have been shown to have more 8-isoprostane, a marker of oxidative stress, in the breath condensate [83]. Asthmatics also have more hydrogen peroxide, a product of neutrophils, in their airways [84].

ROS and the Epithelium

When a cell is exposed to an oxidative environment, lipid peroxidation, DNA damage and protein oxidation can occur. Of course, airway epithelial cells express a variety of antioxidants to deal with such oxidative insults, including SOD, catalase, glutathione peroxidase and the non-enzymatic antioxidants such as glutathione, thioredoxin, ascorbic acid and tocopherol. SOD is expressed in a variety of isozymes with SOD1 being the most prevalent. SOD1 is expressed throughout the nucleus and cytoplasm [85], while SOD2 is expressed in the mitochondria [86], and SOD3 is a secreted, soluble form [87]. SOD converts superoxide to hydrogen peroxide,

which can then be converted to water by catalase. SOD activity in airway epithelial cells from asthmatics not on inhaled corticosteroids is decreased compared to controls or asthmatics receiving corticosteroids [88]. Another study has shown that asthmatic respiratory tract lining fluid contains less tocopherol and ascorbate, and more oxidized glutathione [89] , further evidence that the redox status of the asthmatic lung is unbalanced.

Airways are constantly exposed to ROS from air pollutants such as ozone and nitrogen oxides. During oxidative insult, cells can activate the master anti-oxidant transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf-2). Nrf-2 is a cap'n collar protein, retained in the cytoplasm by Kelch-like ECH associating protein 1 (Keap 1) [90]. Normally Nrf-2 is ubiquitinated and targeted for proteosomal degradation [91]. Keap-1 contains a nuclear export signal (NES), retaining Nrf-2 in the cytoplasm [92] and Nrf-2 contains a nuclear localization signal (NLS) [93]. Keap-1 contains cysteine residues that are oxidized during oxidative stress, a critical step in releasing Nrf-2 [94]. Nrf-2 can then translocate to the nucleus where it binds the anti-oxidant response element (ARE) [95]. Nrf-2 knockout mice have shown increased eosinophilia and IL-4 and IL-13 levels in the bronchoalveolar lavage after sensitization and challenge with allergen [96]. It is possible that Nrf-2 deficiencies, leading to anti-oxidant deficiency or increased oxidation status of the lung could contribute to the pathogenesis of asthma.

If the exposure to ROS overwhelms the cells' antioxidant capacity, the cells will either undergo apoptosis or necrosis. Apoptosis involves the activation of the caspase cascade, and ROS can induce caspase activity [97-99]. HOCl produced by MPO has been shown to cause endothelial and neuronal necrosis by activation of L-type and T-type calcium channels at the plasma membrane with downstream activation of calpain and loss of mitochondrial membrane potential

[100,101]. Cell viability after exposure to HOCl appears to be dependent on the cell type, with endothelial cells being more susceptible than cultured neurons [100,101]. During an oxidative insult, airway epithelial cells can be shed. Brown Norway rats pre-treated systemically with the anti-oxidant N-acetyl cysteine showed reduced airway dysfunction having been exposed to chlorine gas [102]. In another study, Balb/c mice were treated with the antioxidant dimethylthiourea (DMTU), which protected the lungs from lipid peroxidation and decreased airway hyperresponsiveness after chlorine gas exposure [103]. Airway epithelial cells have the ability to secrete a large amount of glutathione. Asthmatics secrete almost two-fold more glutathione into the epithelial lining fluid than healthy controls, which appears to prevent airway hyperreactivity to methacholine challenge [104]. Asthmatic airway redox status could play an important role in the development of disease.

ROS as Signalling Molecules

ROS have become of interest to medical research as their role as signalling molecules has been uncovered. ROS appear to mediate events downstream of growth factor receptor and cytokine receptor activation [105]. A seminal publication in the early 1990's showed that rabbit tracheal epithelial cells release hydrogen peroxide when activated with PMA [106]. Since, much work has been conducted studying ROS production in airway epithelial cells. Production of ROS by airway epithelial cells has been implicated in cell signalling, controlling apoptosis [107] and mucus secretion [108]. The amino acid cysteine is an oxidation target due to its sulfhydryl side chain. Proteins can undergo conformational changes upon cysteine oxidation and thus signalling pathways can be affected. Cysteine oxidation of tyrosine phosphatases can render them inactive causing proteins to remain phosphorylated. This is the case with the EGFR [109]. Matrix metalloproteinases (MMPs) can be activated when intracellular cysteine oxidation causes a

conformational change to the extracellular domain, exposing the catalytic site [110,111]. Since MMPs have the ability to cleave EGFR ligands from the cell membrane, oxidative activation of MMPs and the EGFR represent a potential mechanism by which airway epithelial cells become activated to produce pro-inflammatory chemokines such as IL-8, downstream of activation of the EGFR [112]. Hydrogen peroxide can cause activation of the transcription factors NF-κB and AP-1 [113]. Pre-treatment of airway epithelial cells with the anti-oxidant DMTU prevents tumour-necrosis factor-α (TNF-α) converting enzyme activation after stimulation with cigarette smoke extract (CSE) [114]. ROS has also been shown to mediate G-protein coupled receptor (GPCR) signalling, including transactivation of the EGFR [115] Endogenous ROS generation may play a crucial role in regulating airway epithelial repair after injury.

The main ROS producing enzyme in airway epithelial cells is the NOX family including NOX1-5 and the larger dual oxidases DUOX1-2, named for containing both NADPH oxidase and peroxidase homology domains [116]. NADPH oxidases produce superoxide by virtue of transferring one electron from cellular sources of NADPH across the lipid bilayer. Dual oxidases (DUOX) produce hydrogen peroxide. Increases in intracellular calcium have been shown to activate DUOX production of hydrogen peroxide by airway epithelial cells [106]. Calcium likely regulates DUOX function by binding to intracellular EF-hand domains [117]. EF-hand domains are sequences found in calcium binding proteins that possess two alpha helices, the “E” and “F” helices, separated by a calcium-binding loop. These domains are sensitive to millimolar calcium, and the sites are normally occupied by intracellular magnesium [118].

TRPA1, a ROS Sensitive Ion Channel

Another avenue by which ROS may play a role in asthma pathogenesis is through activation of the oxidant sensitive transient receptor potential (TRP) channel TRP ankyrin 1 (TRPA1), named for its 18 N-terminal ankyrin repeats. TRP channels are non-selective cation channels, permeable to calcium. Increases in intracellular calcium can initiate signalling cascades through proteins such as calmodulin [119]. C-fibers that innervate the airways appear to mediate oxidative stress induced lung dysfunction in the mouse [120]. Airway C-fiber activation can also induce neurogenic inflammation. Neuropeptide released from C-fibers, such as substance P and neurokinin A can bind to neurokinin receptors and cause a variety of pro-inflammatory events such as increasing lymphocyte proliferation [121] and increased vascular permeability in the airways [122]. Oxidative molecules have the ability to gate TRPA1. 4-hydroxynonenal, an oxidized lipid metabolite, has been shown to activate TRPA1 in airway sensory neurons, causing the release of substance P from these nerve endings and contributing to neurogenic inflammation [123]. Recently, TRPA1 was detected on small airway epithelial cells, and it appears to respond to oxidative ligands such as cinnamaldehyde, acrolein and CSE. The same study showed that acrolein and CSE can evoke the release of IL-8 from cultured human airway epithelial cells in a TRPA1-sensitive manner [124]. Since TRPA1 is activated by oxidative molecules such as acrolein, it is likely that this channel is gated more frequently in asthmatic lungs and causes calcium flux into airway structural cells.

1.4.4 Macrophages

The macrophage is a rather diverse cell, both driving inflammation as well as resolving it. Macrophages have the ability to engulf foreign pathogens and present antigen to adaptive

immune cells. It has long been known in the mouse that macrophages may exist in a classically activated (M1) or alternatively activated (M2) phenotype. Depending on the M1/M2 expression, macrophages influence adaptive immunity by altering T-cell phenotype [125]. M1 macrophages develop in the presence of interferon- γ (IFN- γ) and TNF and release a variety of pro-inflammatory cytokines [126]. The M2 variety mature in the presence of IL-4 [127] and secrete extracellular matrix proteins that may be utilized for tissue repair [128]. These macrophages are named for their differential processing of arginine through arginase rather than inducible nitric oxide (NO) synthase (iNOS) [129]. iNOS drives the synthesis of NO, a molecule associated with viral immune responses [130]. M1 macrophages have been associated with prevention of Th2 responses in a murine OVA model of asthma [131]. In a murine house dust mite model of allergic asthma, it was observed that M1 macrophages were increased in less severe forms of the disease whereas M2 was greater in more severe "asthma" [132]. The specific macrophage subsets that are expressed across the asthmatic disease severity spectrum are still unclear [133]. Due to the increased prevalence of Th2 cytokines IL-4 and IL-13, the induction of M2 cells may be expected in asthmatic airways. This may represent a homeostatic mechanism through which induced M2 cells by Th2 inflammation inhibit these responses.

1.5.3 Eosinophils

Eosinophilia is a common feature of asthma and the extent of eosinophilia correlates with the severity of the disease [134]. Th2 derived IL-5 and IL-9 greatly promote the presence of airway eosinophils through stimulating their recruitment and aiding in their proliferation [135,136]. IL-13 also generates eotaxin release from the epithelium that acts as a potent chemoattractant for eosinophils [137]. Eosinophil derived major basic protein is elevated in the sputum of asthmatics [138] and this molecule can also induce the constriction of airway smooth muscle cells [139].

Major basic protein causes augmented muscle reactivity through binding to inhibitory M2 muscarinic receptors, preventing the normal regulatory function of this receptor and causing augmented acetylcholine release [140]. Eosinophils secrete lipid mediators such as cysteinyl leukotrienes [141] which are potent bronchoconstrictive agonists [142]. Eosinophils also secrete a variety of cytokines that can sustain airway inflammation such as IL-4 [143], IL-13 [144] and eotaxin [145].

Eosinophil peroxidase-derived HOCl, eosinophil cationic protein and major basic protein can be damaging to the epithelium and could represent another deleterious effect of eosinophilia [146]. Furthermore, activated eosinophils can release the arachidonic acid metabolite leukotriene C4 (LTC_4) which can cause the constriction of airway smooth muscle cells and microvascular leak [147]. Preventing eosinophilia through IL-5 or IL-5 receptor specific antibodies is a strategy that has received a fair amount of attention recently. Severe asthmatics that possess a high number of airway eosinophils had fewer exacerbations when treated with mepolizumab, an antibody against IL-5 [148]..Future work exploring eosinophil-targeting therapies will likely lead to improved specific patient prognosis.

1.5 Adaptive Immunity in Asthma

1.5.1 T Cells

Adaptive immunity plays a key role in mediating allergic asthma. Inhalation of air-borne allergens, known as aeroallergens, causes sensitization of susceptible subjects and subsequent exposure initiates an inflammatory allergic reaction in the airways that contributes to airway

narrowing and remodeling. APCs such as dendritic cells present the peptide to T cells via the MHC class II molecule. Once presented with allergen, T cells with the appropriate antigen specific T cell receptor proliferate in local lymph nodes into the Th2 subset. The T cells will then recirculate, eventually homing towards the airway along a chemokine gradient of CCL17 and CCL22 which are secreted by epithelial cells [149]. An expanded population of allergen specific Th2 cells in the lung will secrete IL-4, IL-5 and IL-13. IL-4 also causes the induction of mucus secreting goblet cells in the airway epithelium [150]. IL-5 secretion from the Th2 cell causes activation [151] and recruitment of eosinophils [152].

Local T lymphocytes have been shown to be in intimate contact with airway smooth muscle cells and, in vitro, trigger proliferation [153]. This property may contribute to airway remodeling in asthma. Recruited T cells within the airway wall contribute to the "late" phase of the allergic asthmatic reaction and their cytokines orchestrate a variety of remodeling events. The Th2 cell will secrete four important asthma-related cytokines, namely IL-4, IL-5, IL-9 and IL-13. IL-4 and IL-13 can activate the airway epithelium to secrete the potent neutrophil chemoattractant IL-8 [154]. A recent clinical trial demonstrated that lebrikizumab, an antibody against IL-13, reduced the number of exacerbations in patients with moderate and severe asthma [155].

Asthmatic airways possess more mucus than those of healthy subjects and this secretion contributes to airway narrowing resulting in increased airway resistance [156]. Furthermore, mucus production by epithelial cells can be driven by the Th2 cytokine IL-13 in asthmatic airways [157].

Naive T cells may also develop into IL-17 secreting Th17 cells in the presence of naive T cell derived IL-23 [158]. This phenotype of T lymphocyte also matures in the presence of TGF-β and

IL-6 [159] and contributes to asthma pathology. IL-17A causes lung fibroblasts to secrete the pro-inflammatory cytokines IL-6, IL-8, IL-11 and Gro-α [160].

An emerging concept in the immuno-pathology of asthma is the role of resolution of inflammation. T regulatory cells (Tregs) are Foxp3 expressing cells that produce the immuno-suppressive cytokine IL-10. In an animal model of allergic asthma, it has been demonstrated that the adoptive transfer of Tregs was able to diminish airway hyperresponsiveness and that this reduction in the asthma phenotype depended on IL-10 [161]. Future work regarding these Foxp3 expressing cells may lead to novel mechanisms regarding asthma pathogenesis.

1.5.2 B Lymphocytes

B lymphocytes are key cells in humoral immunity as the antibody producing cells of the body. Th2 cytokines will influence B cells to class switch towards an IgE producing cell [162,163], which likely contributes to the "early" phase of the allergic responses in asthma. IgE binding to mast cell Fc receptor induces exocytosis of mediators of the allergic response [164]. Some mast cell derived mediators released include histamine, leukotrienes, proteases and cytokines, all of which contribute to asthma pathology [165]. Targeted blockade of IgE through the administration of an anti-IgE antibody is sometimes used clinically for the management of severe asthma [166]. B cells also have a role in antigen presentation to T lymphocytes through expression of the MHC class II molecule [167].

After the induction of class switching, B cells expressing specific IgE for the antigen may become memory B cells ready to respond to generate more robust responses to subsequent allergen exposures. Conflicting data exists as to the longevity of memory B cells. Data suggest that IgE specific B cells are not long-lived without the presence of continued antigen exposure in

the rat [168]. However in another publication, persistent memory cells were observed in the spleen and bone marrow 79 days after systemic sensitization of BALB/c mice [169]. There may be a discrepancy due to the species examined, although the former article did not explore the presence of memory B cells in the bone marrow or the spleen, rather it examined only the generation of serum IgE antibody production after allergen challenge.

B-regulatory cells, similarly to T-regulatory cells, inhibit rather than increase responses of the immune system. These cells were first described in the early 2000s as IL-10 expressing B-cells [170]. IL-10, as mentioned previously, is a cytokine that is able to suppress both Th1[171] and Th2 differentiation and is therefore it can act as a potent repressor of immunity. This cytokine can also suppress macrophage release of pro-inflammatory cytokines [172] and in an allergic mouse model of asthma, IL-10 was shown to inhibit airway inflammation but not AHR [173]. In a similar study, selective inhibition of this B cell subset acted to drive airway inflammation that was likely dependent on the loss of CD4+ cell inhibition [174]. Furthermore, regulatory B cells may increase the number of T regulatory cells and act together to suppress immunity [175]. Recently it has been suggested that B regulatory cells may be further differentiated into IL-10 secreting Br1 cells [176], transforming growth factor (TGF)-releasing Br3 cells [177], and Foxp3 expressing Bregs [178]. B lymphocytes have emerged as important cells in mediating the asthma pathogenesis, supported by the use of omalizumab to neutralize IgE in human asthma.

1.6 Lipid Mediators

1.6.1 Sphingosine-1-Phosphate

S1P derives from sphingosine, a prominent sphingolipid found in the plasma membrane of many cell types. Likely due to the "enigmatic structure" of these lipids, they are named after the Egyptian Sphinx [179]. Sphingolipids derive from ceramide, giving rise to a variety of sphingolipids [180]. Ceramide regulation is therefore critical to maintain sphingolipid homeostasis. When sphingosine is phosphorylated it becomes a biologically active lipid. Sphingosine phosphorylation occurs by one of two enzymes known as sphingosine kinase (SPHK) 1 and 2. SPHK1 is normally sequestered in the cytoplasm but will translocate to the inner leaflet of the plasma membrane upon binding to calmodulin [181]. Phosphorylation of serine 225 by ERK1/2 dependent signalling can induce a 14-fold increase in SPHK1 activity [182]. Ceramide and sphingosine signal apoptosis [183] in a variety of cell types, whereas the phosphorylated counterparts induce proliferation [184]. These opposing effects of ceramide and S1P, first discovered in the late 1990s [185], are now known as the sphingolipid rheostat. The phosphate moiety of S1P can be targeted for removal and conversion back into sphingosine by sphingosine phosphatase [186]. Furthermore, S1P can be irreversibly degraded by sphingosine lyase (Spl) [187]. S1P can pass into the cytoplasm through the cystic fibrosis transmembrane conductance regulator (CFTR) [188] and can be actively exported through the ATP-binding cassette transporter ABCC1 [189] and through the spinster homolog 2 [190]. Extracellular S1P binds to albumin and apolipoprotein M [191] in the high density lipoprotein fraction of the plasma [192].

S1P Immunology

S1P is considered to be bioactive due to five GPCRs that the sphingolipid can activate. S1P receptors$_{1\text{-}5}$ (S1PR$_{1\text{-}5}$) can mediate a wide variety of cellular responses and have implications in asthma immune-pathology. One such role is the control of the egress of T lymphocytes from the local lymph node or thymus that depends on a gradient of S1P between the lymphatic and the efferent vessel and the T cell movement from secondary lymphoid organs depends on the activation of S1PR$_1$ [193,194]. Indeed, S1P concentrations are higher in the plasma than in the lymphatic tissues [195]. Fingolimod (FTY720) is an analogue of sphingosine that is phosphorylated by SPHK2 [196]. Phosphorylated fingolimod potently binds to S1PR$_1$ causing internalization and down-regulation of this receptor [197]. FTY720 has been shown to reduce the emigration of lymphocytes from the lymphatic tissue [198]. B cell N,N-dimethyl sphingosine, is an inhibitor of SPHK and is able to reduce eosinophilia and airway hyperresponsiveness (AHR) to methacholine in a murine model of allergic asthma [199]. This inhibitory effect was further confirmed to be mediated by a reduction in Th2 cytokines [200]. S1P administered subcutaneously to mice also caused an increase in AHR upon acetylcholine challenge, and led to increased levels of IL-4 and IL-13 [201]. Fingolimod inhalation was able to prevent airway allergen challenge induced AHR and inflammation through the inhibition of dendritic cell induced T cell polarization [202]. Along these lines, T cells adoptively transferred from mice that had been previously treated with S1P had increased AHR to carbachol challenge [203].

In ASM cells, S1P has the ability to increase proliferation and cause calcium release through the activation of S1PR2/3 [204]. Besides driving remodeling, S1P may induce contraction and cause the assembly of stress fibers in these cells [205]. More studies regarding the regulation of ASM cell phenotype by S1P are necessary to understand the role of this sphingolipid in driving asthma

pathogenesis. Due to the role of S1P in promoting inflammatory processes, we examined how this lipid mediates the release of IL-8 from epithelial cells in chapter two.

Intracellular Targets of S1P

Recently, it has been demonstrated that S1P can act intracellularly as well. In the nucleus, S1P can bind to histone deacetylase 1 and 2, influencing the regulation of a variety of genes [206]. S1P may play a role in mitochondrial biogenesis, as it was discovered to bind to Prohibitin 2 [207]. The phospholipid also can act as a co-factor for the E3 ubiquitin ligase activity of TNF receptor-associated factor 2 and could activate NF-κB through this intracellular target [208]. Future work in this emerging field may define novel implications of S1P and its role in disease.

S1P and Other Diseases

Due to its role in regulating proliferation and survival, S1P has been associated with a variety of cancers. One study demonstrated that the use of neutralizing S1P antibodies is able to inhibit tumour growth progression in a variety of murine xenograft and allograft models [209]. Patients suffering from ovarian cancer have presented with greater S1P levels in the serum which were reduced upon removal of the tumours [210]. Furthermore, mutant SPHK1 lacking serine 225 fibroblasts showed less Ras-dependent transformation, indicating that membrane trafficking of SPHK1 is a requirement in driving tumour generation [211]. In gastric cancer cells, S1P was found to transactivate the EGFR through signalling of S1PR2 and metalloproteinase activation [212]. Endothelial cell survival was mediated by SPHK1 regulation of B cell lymphoma gene 2 (Bcl-2) [213], an oncogene commonly associated with a variety cancers [214]. S1P has also been shown to activate eicosanoid biosynthesis through the activation of cyclooxygenase 2 (COX2) [215], and PGE_2 has been associated with prostate cancer [216]. Besides asthma, atherosclerosis

is another inflammatory condition with which S1P is associated. S1PR3-/- mice present with less macrophage recruitment to the peritoneum [217], a prominent driver of this disease [218]. Similarly, another murine model of atherosclerosis showed that S1PR2-/- mice possess less recruited monocytes due to reduced NF-κB driven monocyte chemoattractant protein-1 (MCP-1) expression [219]. Diabetes has been linked to S1P. In a mouse model of this disease, plasma S1P levels were increased [220]. Furthermore, it appears that S1PR2 is involved in the apoptosis of pancreatic beta cells [221].

Clearly, S1P is an important lipid mediator associated with a variety of diseases. Future work exploring mechanisms by which this sphingolipid mediates its effects may prove useful in the identification of therapeutic targets.

1.6.2 Arachidonic Acid Metabolites

Arachidonic acid (AA) is a 20-carbon chain polyunsaturated fatty acid that is produced from phospholipid metabolism by phospholipase A2 in cellular membranes. Upon activation of cytosolic phospholipase A2 (cPLA2) by increased intracellular calcium levels, the enzyme migrates from the cytosol to the plasma membrane, producing AA [222]. Furthermore, ERK phosphorylation of cPLA2 mediates the enzyme's activation [223]. Metabolites of AA have been implicated in a variety of aspects of airway biology.

AA may be metabolized via a variety of bioactive products through one of two major pathways (**Figure 1**). These products include cyclooxygenase derived prostanoids or leukotrienes and lipoxins produced by 5-lipoxygenase (5-LO). 5-LO, together with an activating enzyme 5-LO activating protein (FLAP), 5-hydroperoxyeicosatetraenoic acid (5-HPETE) or 8-HPETE are synthesized. 12-lipoxygenase products include lipoxins, which can resolve inflammation in

airways [224]. Lipoxin A_4 has been shown to be reduced in severe asthmatics, which may represent another mechanism by which inflammation persists in the airways [225]. 5-HPETE can also be converted into LTA_4, which can then give rise to either the cysteinyl leukotrienes (LTC_4, LTD_4, LTE_4), or LTB_4. LTB_4 can act upon the BLT GPCR. BLT receptor expression was found to be required for the recruitment of CD8+ T cells in a mouse model of allergic asthma [226]. LTB_4 can also promote neutrophil adhesion to endothelial cells, increasing their ability to be recruited [227]. Several BLTR antagonists have been examined for their therapeutic potential to treat asthma [228]. The cysteinyl leukotrienes (CysLTs) also drive airway inflammation and exert their biological effects by binding to either the CysLT receptor 1 ($CysLTR_1$) or 2 ($CysLTR_2$). CysLTs induce both airway smooth muscle proliferation and contraction and thus these lipid mediators can induce both airway remodeling and exacerbation [142]. Leukotrienes can also promote increases in vascular permeability that likely contributes to increased inflammation [229]. Goblet cell hyperplasia is also driven by CysLTs, leading to increased mucus production [230]. Aspirin-intolerant asthmatic-derived sputum possesses more CysLTs than healthy control subjects [231] and there is increased expression of CysLTR1 in the bronchial mucosa of asthmatic subjects that will further augment the effects of this potent remodeling factor [232]. Three CysLTR1 antagonists are used clinically for the treatment of asthma.

The other arm of the AA metabolite pathway gives rise to the prostanoids. The constitutively expressed COX-1 and the inducible COX-2 (targets of selective non-steroidal anti-inflammatory drugs (NSAIDs)) convert AA to prostaglandin endoperoxide H_2 (PGH_2), which can then be further processed by a variety of enzymes to form prostaglandin D_2 (PGD_2), PGI_2, PGE_2, or thromboxane A_2 (TXA_2). In some asthmatic patients, the administration of NSAIDs to inhibit

cyclooxygenases shunts AA metabolism to the 5-LO pathway, increasing the levels of cysLTs and causing exacerbation [233,234]. These aspirin sensitive patients must turn to other anti-inflammatory drugs in order to prevent worsening symptoms. Prostanoids can elicit a myriad of signalling events in cells expressing GPCRs that recognize the ligands. In smooth muscle, PGE_2 can increase cyclic adenosine monophosphate (cAMP) via binding to PGE_2 receptors EP_2 or EP_4, where as EP_3 activation increases cytosolic calcium. PGI_2 can also increase cAMP, whereas PGD_2 and TXA_2 increase calcium levels. The respiratory epithelium can be a significant source of PGE_2 [235], as can smooth muscle cells [236]. PGE_2 can be both pro-inflammatory and anti-inflammatory, however it seems that it may protect against the asthmatic phenotype. Inhaled PGE_2 was shown to prevent the fall in forced expiratory volume in one second (FEV_1) in exercise-induced bronchoconstriction of asthmatic subjects [237], and this bronchodilation is mediated by EP2 receptors in the mouse [238]. Alternatively, TXA2 can induce airway constriction [239] as can $PGF_2\alpha$ that likely does so through the activation of TXA2 receptors [240]. In an allergic model of asthma, it was shown that PGI_2 reduces bronchial infiltration by leukocytes and Th2 cytokine production, pointing towards a protective role of this lipid [241]. Novel inhibitors of these pathways are beginning to emerge and may lead to new treatments for this disease. In chapter three of this dissertation, we explored the role of COX activity and its products on influencing epithelial induced changes to ASM cell phenotype.

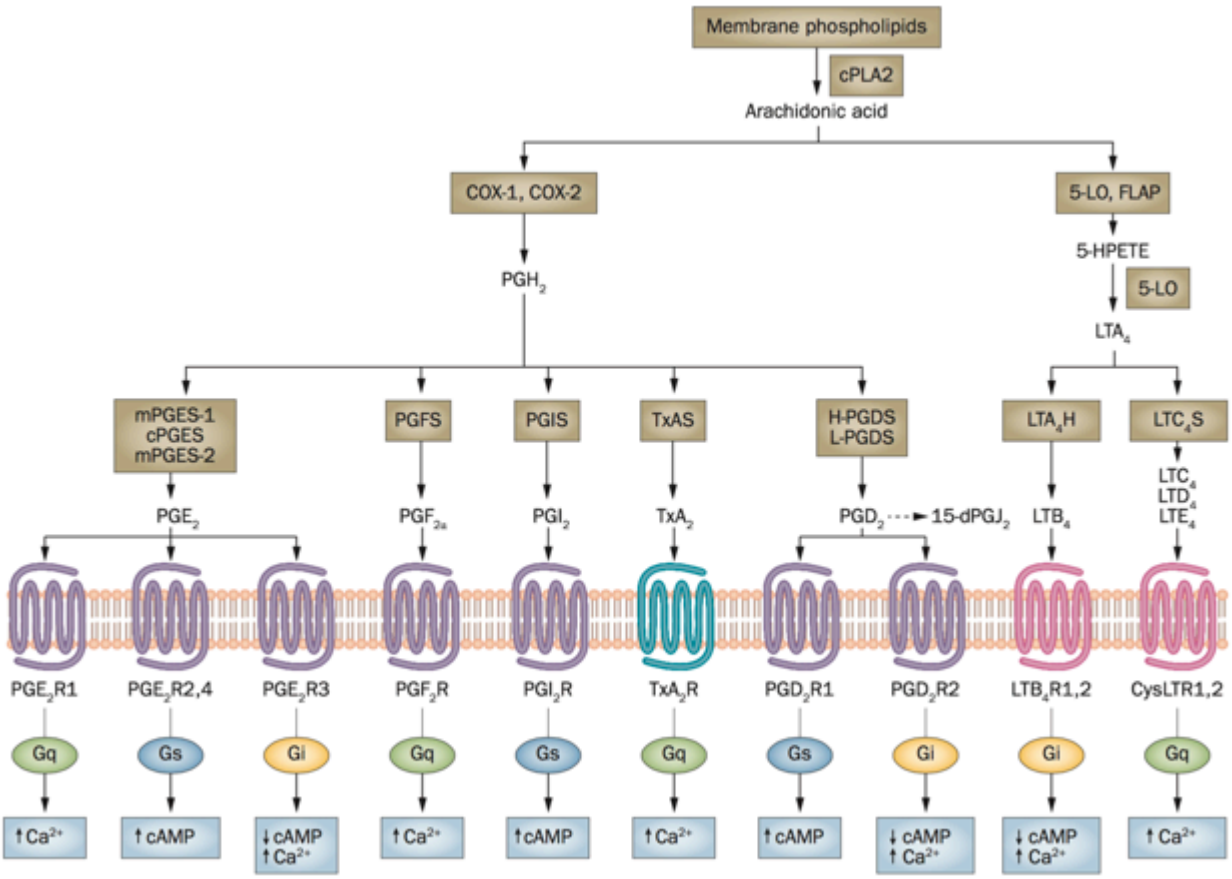


Figure 1. Overview of arachidonic acid metabolism. cPLA2 converts phospholipids into AA. AA is then processed into one of two pathways, the 5-LO/ FLAP to give rise to the leukotrienes (right), or through the COX-1/2 pathway synthesizing prostanoids (left) (figure from Korotkova, M et al. Nat Rev Rheumatol, 2014 (10)) [242].

1.8 Airway Smooth Muscle

Smooth muscle lines the walls of many tubular structures within the body. Smooth muscle differs from that of skeletal and cardiac muscle in two important ways. First, smooth muscle can undergo phenotypic de-differentiation from a well-differentiated contractile cell to a less

differentiated proliferative (synthetic) cell. Second, smooth muscle can maintain force for long periods of time with reduced consumption of ATP compared to non-sustained contraction, a phenomenon known as the "latch state" [243,244]. These cells are the target of one of the most common treatments of asthma. The administration of β-adrenergic agonists is a first-line therapeutic class that relaxes the airways to improve symptoms. ASM's main function is to control airway tone. Smooth muscle can maintain force for long periods of time by performing tonic contractions. It is also capable of performing faster contractions, termed phasic contractions. As the muscle contracts the airways narrow, causing increased airway resistance. Although there are other determinants of airway diameter, such as the elastic recoil of the lungs and the airway surface liquid, smooth muscle is by far the most important term in regards to asthma. As the role of ASM cells is to generate force, the molecular motor proteins actin and myosin are of crucial importance.

1.8.1 Contribution to sub-epithelial fibrosis

Thickening of the sub-epithelial layer through the deposition of extracellular matrix proteins is a hallmark feature of asthma and may occur by secretion of extracellular matrix proteins by myofibroblasts in the airways of asthmatic patients [245]. Stimulation of ASM cells from previously healthy subjects with asthmatic serum induced the secretion of a variety of extracellular matrix proteins, indicating a role for this cell type in driving sub-epithelial fibrosis [246]. Asthmatic ASM cells produced greater quantities of collagen I and perlecan than did those from healthy control subjects, and the extracellular matrix protein profile expressed by the asthmatic cells was able to augment the proliferation of both control and asthmatic ASM cells [247]. This indicates a potential role for these proteins in driving remodeling of the ASM layer. The story is not fully understood, as it has been shown that OVA induced matrix remodeling

actual prevented airway hyperresponsiveness in the rat when examining long exposures of the allergen, pointing towards a possible protective effect of these secreted proteins [248]. MMP modulation of extracellular matrix proteins may also play a role in asthma, and corticosteroid treatment was found to diminish collagen deposition in asthmatic airways via the up-regulation of MMP-9 and down-regulation of tissue inhibitors of metalloproteinase-1 expression [249]. Further work in this area is needed to truly understand the role of extracellular matrix proteins and their interaction with ASM cells in the asthmatic airway.

1.8.2 The Origins of New Airway Smooth Muscle

Airway smooth muscle is present underneath the basement membrane of the epithelial lining of the airways and is arranged in a helical fashion. (**Figure 2**)

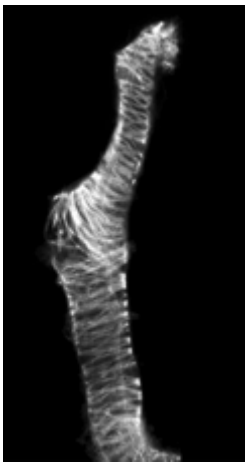


Figure 2. Airway stained with TRITC-phalloidin to image filamentous actin in airway smooth muscle bundles of a healthy control airway (figure from Ijpma, G et al. Shortness of Breath, 2013 (3)) [250].

The angle at which the muscle wraps around the airways is likely greater in the peripheral airways compared to that of the central ones [251]. It is possible that muscle that is oriented more in the longitudinal axis may have less impact, when contracted, on the airway diameter. Whether there is a difference in orientation of the muscle between asthmatic and control subjects needs to be evaluated. It is unclear where the excess smooth muscle mass originates from within the asthmatic airway, however several mechanisms have been postulated. New ASM cells could be generated from nearby precursors such as myofibroblasts that undergo differentiation in the presence of TGF-β [252]. It is also postulated that adjacent epithelial cells may undergo

epithelial-mesenchymal transition (EMT) to generate AMS cell progenitor cells [253]. In support of this idea, asthmatic cultured bronchial epithelial cells were shown to be more likely to undergo EMT when stimulated with TGF-β than those derived from control subjects [254]. Another plausible mechanism for the origin of increased ASM mass is through the proliferation of the ASM cell itself, representing a popular area of asthma research. Alternatively, less apoptosis of existing ASM cells may promote the increase mass found in asthmatics [255]. Mesenchymal stem cells may have the potential to generate ASM cells as the number of these cells increases in an OVA model of allergic asthma [256]. Fibrocytes derived from peripheral blood could also be a source of ASM cells as these cells localize to the airways of asthmatic patients [257], although their numerical contribution seems unlikely to be large.

1.8.3 Phenotype Regulation of ASM

Cellular phenotype refers to the plasticity of an individual cell, or a subpopulation of cells, that can differ from their neighbors with respect to their specific function. Smooth muscle is unique from other muscle types in its ability to change phenotype. ASM cells can exist as either a proliferative or a contractile phenotype. Phenotype switching provides an explanation for airway wall remodeling such that the muscle layer can proliferate to increase in mass before converting to the contractile phenotype and contributing to airway narrowing. The term “modulation” is used to describe ASM cell phenotype switching [258]. Myogenic phenotype variation was first discovered in vascular smooth muscle where subsets of cells with differing potassium channels were associated with resistive or conduit arteries [259].

Transcriptional Regulation

ASM cell phenotype is transcriptionally regulated by many proteins and small molecules. The transcription factor serum response factor (SRF) can drive the expression of both proliferative and contractile genes [260]. To achieve specificity, the cell utilizes co-transcription factors. E26-like protein 1 (Elk1) and kruppel-like factor 4 (KLF4) bind to SRF to drive the enzymes that contribute to cell cycle progression. Phosphorylated Elk1 induces the expression of c-fos [261] which is a well described oncogene [262]. JNK and p38 drive Elk1 phosphorylation to respond to cytokines and stress [263]. However, in an equine model of asthma, it was shown that ASM mass did not correlate with phosphorylation of Elk1[264]. It is possible Elk1 activity was indeed present prior to the examination of the equine airways in this study. Myocardin binds SRF to induce the expression of genes associated with the contractile apparatus. Furthermore, the contractile promoter contains two CArG boxes which respond to simian virus 40 protein 1 (Sp1), AP2 and TGF-β [265]. Elk1 over-expression has been shown to prevent the activation of smooth muscle specific genes driven by myocardin and is likely due to the competition for binding to SRF [266]. The activation of mitogen-activated protein kinase (MAPK) signalling by growth factor receptors can lead to the phosphorylation of ELK-1 and binding to SRF, which represses SRF:myocardin binding [267], highlighting the pleiotropic role of SRF. In chapters three and four of this work, we explored the expression of these co-transcription factors in mediating the phenotype switch that occurs in ASM cells after co-culture with the epithelium.

Micro-RNA

The regulation of co-transcription factors can occur through the generation of micro-RNA (miRNA). miRNAs are small non-coding RNA molecules, 18-22 nucleotides in length [268].

miRNA are generated in the nucleus as primary miRNA which is processed by RNase III Drosha forming precursor miRNA [269] and exported from the nucleus via the pore channel exportin-5 [270]. Once present in the cytoplasm, miRNA is processed further by Dicer, and will be bound by proteins such as Argonaute to form the RNA-induced silencing complex (RISC). RISC bound to miRNA will cause the reduction of proteins in three ways. miRNA/RISC can bind to the 5' untranslated region of genes and prevent the progression of the translational machinery from translating the gene. MiRNA binding also utilizes the RISC complex to enzymatically degrade mRNA transcripts, or they can become destabilized through 5' decapping [271]. miR-143 and miR-145 form a complex that has been shown to inhibit the expression of ELK1 and KLF4, thus reducing proliferation and allowing the cell to become more contractile (**Figure 3**) [272]. Furthermore, miR-145 was up-regulated in an OVA model of allergic asthma and its antagonism reduced airway hyperresponsiveness to methacholine [273]. miR-21, through the repression of PTEN [274], and miR-25, through inhibition of KLF-4 [275], allow for a more contractile cell. In fact, the most highly expressed miRNA in human ASM cells, miR-10a, regulates proliferation by targeting the PI3K pathway [276]. Recent work in asthmatic airway smooth muscle cells has shown that asthmatic cells possess more miR-155 than controls after stimulation with a mixture of cytokines and that this mi-RNA positively correlated with the amount of COX-2 expression [277]. Since the discovery of miRNA 20 years ago, they have been implicated in a great number of biological processes and diseases. Further research in the field of airway remodeling may uncover novel therapeutic targets. We examined the expression of miRNA constructs in ASM cells after co-culture with epithelial cells in chapter four of this thesis.

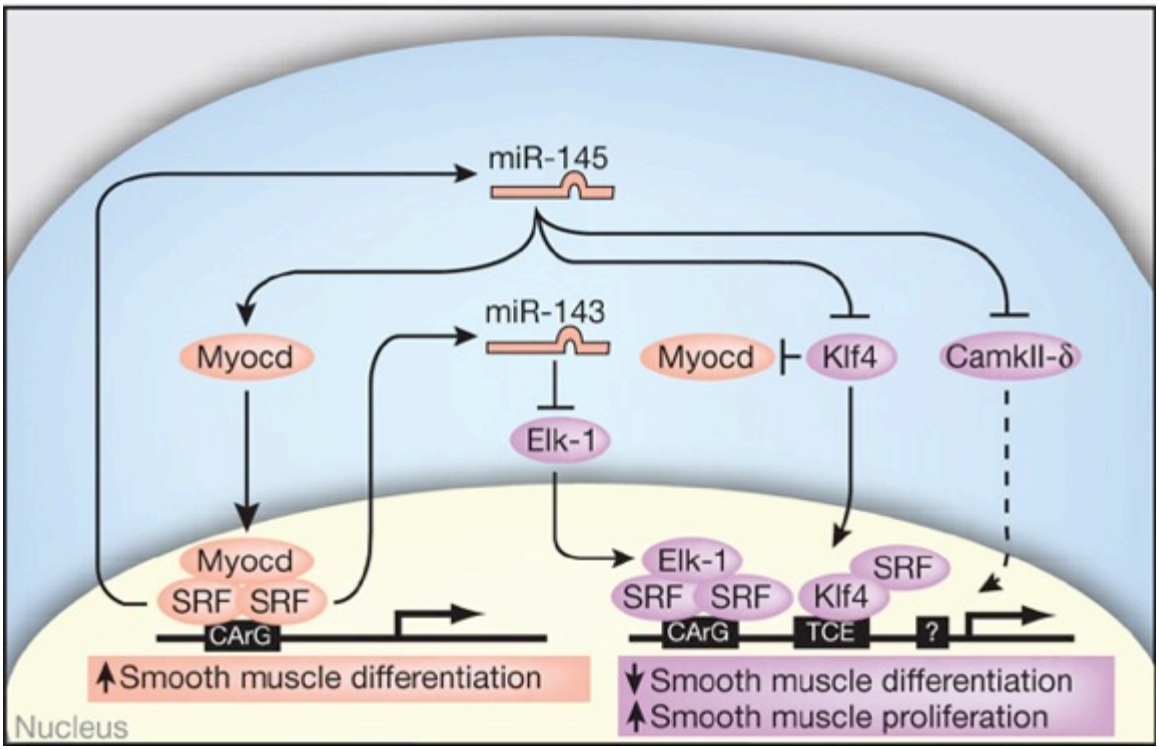


Figure 3. miR143/145 regulate smooth muscle phenotype. The expression of miR-143 suppresses Elk-1 while the expression of miR-145 reduces Klf4. The removal of these pro-proliferative co-transcription factors allows for Myocd to bind to SRF, driving the expression of smooth muscle specific genes (figure from Cordes, K et al. Nature, 2009 (460)) [272].

1.8.4 ASM Proliferation

Since airway remodeling features increased smooth muscle mass, mechanisms of ASM cell proliferation are of great interest. ASM cells, like many other cell types, respond to serum by actively proliferating. In asthma, the vascularity of the airways is increased [278]. Furthermore, the vascular permeability may be increased in asthmatic subjects, allowing for the possibility of greater quantities of serum to stimulate ASM cell growth [279]. ASM cells also respond to an inflammatory milieu. CD4+ T cells co-localize with actively proliferating ASM cells [280] and these lymphocytes potentially drive the proliferation [153]. Eosinophils have also been shown to

induce ASM cell proliferation through production of eosinophil derived cysteinyl leukotrienes [281].

The molecular mechanisms of ASM cell proliferation complement those of many other cell types. Of relevance to airway biology are the epidermal growth factor (EGF) family, insulin growth factor, fibroblast growth factor (FGF)-2 and platelet derived growth factor (PDGF). These ligands act upon tyrosine kinase receptors and GPCRs to elicit a variety of downstream signalling events. Tyrosine kinase receptor activation causes dimerization of the heterodimeric receptor, allowing autophosphorylation of specific tyrosine residues on the intracellular domains [282]. This phosphorylation event creates a site for effector proteins to dock. Protein tyrosine phosphatases such as SHP-1 can de-phosphorylate and negatively regulate EGFR signalling. Downstream signalling effectors are quite numerous, however some key pathways involved are the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK), and PKC/PI3K pathways [283-285]. GPCR signalling involves the activation of 7 transmembrane receptors. Upon ligand binding, the GPCR undergoes a conformational change between the third and sixth transmembrane domain [286]. Conversion of inactive GDP bound G protein, into active GTP bound G protein causes the α-subunit to dissociate from the β and γ subunits. Free Gα subunits are able to bind to other signalling proteins and allow for the activation of cascades [287]. Depending on the Gα subtype, different signalling cascades will be activated. Gαs stimulates the production of cAMP in the ASM cell via the activation of adenylyl cyclase, and this is the mechanism of action for β-agonist therapy in asthma. $G\alpha_i$ on the contrary, inhibits the generation of cAMP by inhibiting adenylyl cyclase. $G\alpha_q$ activates phospholipase C (PLC) and $G\alpha_{12/13}$ interacts with Rho guanine exchange factors to regulate cytoskeletal rearrangement and proliferation. After activation, the GPCR will be endocytosed to facilitate the removal of the

signalling complex, a phenomenon that depends on the binding of arrestin to the GPCR [288]. Tyrosine kinase and GPCR downstream effectors can cross-talk and regulate one another. EGFR signalling can activate PLC-γ that can stimulate calcium entry, whereas the GPCR can also cause calcium influx via a PLC-β mechanism [289]. Both tyrosine kinase and GPCR signalling activate p21ras, which, when bound to GTP, recruits Raf-1 [290]. Active Ras can also activate the PI3K pathway [291], which can drive the proliferation of ASM cells [292]. ERK signalling is known to induce cyclin D1 expression as this cyclin contains ERK dependent transcription factor regulatory sites such as Sp1 and AP-1 (**Figure 4**) [293,294]. Activation of the PI3K pathway can activate $p70^{S6}$ kinase, which drives DNA synthesis and induces ASM proliferation [295]. Furthermore, PI3K can induce Rac1 signalling [296], which has been shown to increase cyclin D1 expression in ASM cells [297]. The cyclin D1 promoter is also positively regulated by the Rho GTPase cdc42 in ASM cells [298]. This cyclin promoter contains binding sites for the transcription factors SP-1 and possesses a cAMP responsive element [299]. After cyclin-dependent kinase activation by cyclins such as cyclin D1, an important event in the proliferative cycle of ASM cells occurs. The phosphorylation of retinoblastoma protein occurs, causing the release of elongation E2F. The phosphorylation occurs by cyclin-dependent kinases and released E2F can go on to activate DNA polymerase [294]. Currently, there are no therapies targeting the prevention of smooth muscle proliferation in asthmatics. It has been shown that corticosteroid administration inhibits the growth of these cells *in vitro*, [300] however, in a follow-up study, it was shown that collagen prevents this beneficial effect and the extracellular matrix remodeling present in asthmatics may impair this therapeutic function [301]. Furthermore, some patients with severe asthma are candidates for a relatively new therapy known as bronchial thermoplasty, a treatment used to heat smooth muscle, inducing its apoptosis. It is likely however that the

prevention of muscle growth in patients with developing asthma would greatly improve future pathology and thus more knowledge regarding how these cells are stimulated to proliferate will be helpful in creating new therapies for the treatment of asthma.

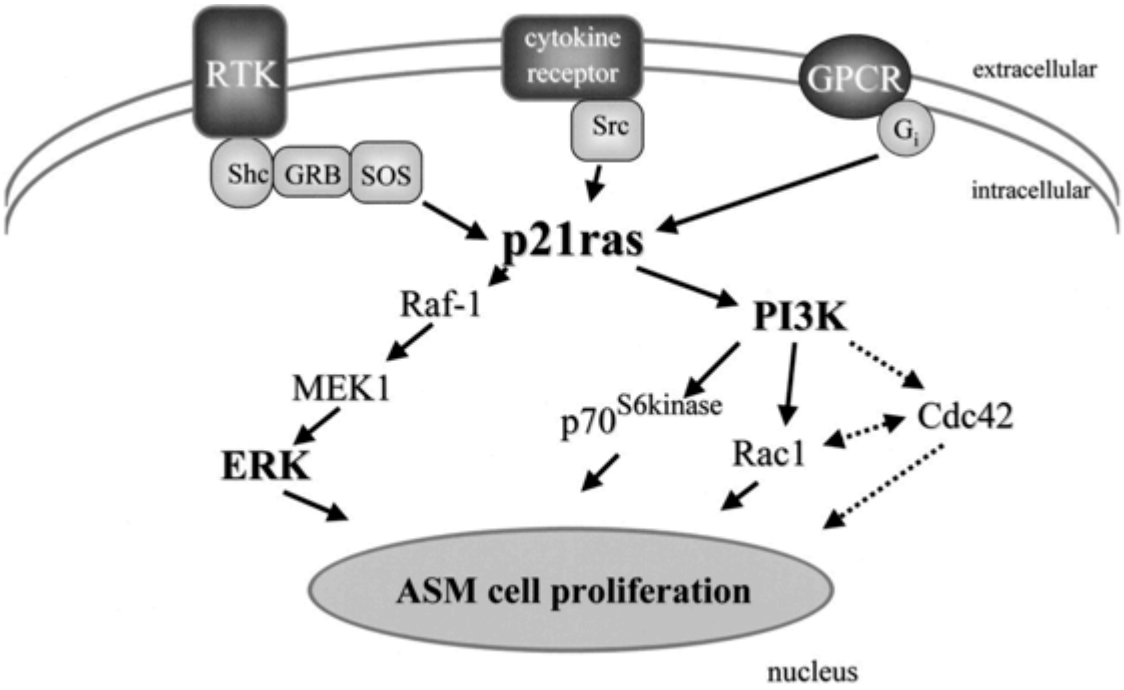


Figure 4. RTKs, cytokine receptors and GPCRs lead to the activation of ASM cell proliferation through the MAPK and PI3K pathways. p21ras plays a central upstream role in the initiation of these signalling events. (figure from Ammit, A et al. J Appl Physiol, 2001 (3)) [294].

Receptor mediated activation of airway smooth muscle proliferation involves receptor tyrosine kinase (RTK), cytokine receptor or GPCRs converging on p21ras. Downstream signalling through MAPK or the PI3K pathway signals nuclear events that drive cell cycle progression. RTK utilizes the effector proteins Shc, growth factor receptor-bound protein and Son of

Sevenless to activate downstream p21ras. In chapter four of this dissertation, we explored the role of the EGFR in mediating epithelial induced proliferation of ASM cells.

1.8.5 ASM Contractility

Halayko et al. revealed that freshly isolated canine trachealis smooth muscle was comprised of distinct populations of cells, being either high or low in content of contractile apparatus proteins [302]. The same authors first noted that phenotypic changes occur when ASM cells are placed in primary culture. Namely, smooth muscle myosin heavy chain, calponin, sm-α-actin, and desmin protein all decreased by more than 75% [303]. ASM cell cultures demonstrated increases in vimentin, PKC, CD44, caldesmon and non-muscle myosin heavy chain [303]. Another marker of contractile ASM cell maturation is the expression of dystrophin-glycoprotein complex, a transmembrane protein that anchors the actin cytoskeleton to extracellular laminin [304,305]. Recently, caveolin proteins have been demonstrated as markers of contractile ASM cells, as muscarinic receptors co-localize with caveolin-1 proteins to mediate calcium mobilization [306]. Changes in ASM cell length upon electrical field stimulation revealed that cells isolated from more proximal airways were more reactive than those isolated from lower airways [307]. Due to the importance of contractility in driving exacerbations in asthma, we were interested in exploring the effects of co-culture with epithelial cells on contractile properties and relevant signalling pathways.

Cross Bridge Cycling

To generate force, the ASM cells employ a variant of the sliding filament theory. Thin filaments of actin and thick filaments of myosin interact with each other to shorten the cytoskeleton. Two articles published in 1954 demonstrated that muscle bands can re-arrange upon changes in length

and that the contractile components actin and myosin generate the differences observed in light refraction [308,309]. In the resting state, myosin does not interact with actin. Myosin protein contains a "tail" domain that interacts with other myosin tail domains to form an α-helical, thick filament. There is also a "head" region for interaction with actin, and a regulatory "neck" region connecting the other two domains. It is in the regulatory region where phosphorylation of serine 19 [310] and threonine 18 [311] occurs, which is considered a key event in the cross-bridge cycling theory. Upon phosphorylation of myosin, it binds ATP, releasing the myosin head from the actin filament. After release, the ATP is hydrolyzed by the intrinsic ATPase activity of myosin causing the head to displace. ADP-bound myosin can again interact with the actin filament and move the filament approximately 6nm [312]. This returns the myosin to the beginning of the cross-bridge cycle.

This cycling mechanism relies on the presence of intracellular free calcium. Calcium release from the sarcoplasmic reticulum or influx from the extracellular space is considered to be a critical step in initiating cross-bridge cycling and ASM cell contraction. Calcium binds to four calcium-sensing domains of calmodulin that then activates myosin light chain kinase (MLCK). Calmodulin activity is a critical component for the initiation of cross bridge cycling [313]. Recent work points towards the importance of spontaneous calcium oscillations as the frequency of oscillations of cultured ASM cells correlated with asthma disease severity and therefore may play a role in AHR [314]. Calcium sensitization can occur such that the muscle will maintain contraction after the calcium levels are restored. This can be accomplished by the small GTPase protein Rho which phosphorylates CPI-17 and can inactivate myosin light chain phosphatase (MLCP), allowing the myosin to remain phosphorylated even with low levels (100nM) of calcium [315].

Calcium Release in Smooth Muscle Cells

An increase in intracellular free calcium is critical for cross-bridge cycling. Calcium can be toxic for cells and so its concentration is tightly regulated. At basal levels, the ASM cell maintains free calcium at approximately 80-200nM $[Ca^{2+}]_i$ [316] by virtue of the actions of several proteins. The sarcoplasmic reticular protein (Sarcolemmal-endoplasmic reticular ATPase (SERCA) pump) actively transports intracellular calcium across the sarcoplasmic reticulum membrane while consuming ATP. On the plasma membrane, calcium/sodium exchangers remove calcium by allowing sodium ions to enter. Upon activation, the cells may release calcium from intracellular stores or allow entry from the extracellular space and increase levels towards 1μM. The release of calcium in ASM cells is biphasic comprising an initial peak followed by a sustained elevation above the resting state [317]. The initial peak is likely mediated by release of calcium from SR stores[318], whereas the sustained phase appears to be dependent on extracellular calcium influx [317]. Release of calcium in ASM cells occurs when extracellular ligands activate intracellular PLC signalling. PLC cleaves membrane bound PIP2 to inositol trisphospate (IP3) and diacyl glycerol. IP3 then activates IP3 receptors (IP3Rs) expressed on the SR allowing calcium to flow down a concentration gradient into the cytosol. Furthermore, CD38 can generate cyclic-ADP-ribose (cADPR), which can activate the ryanodine receptor on the SR and can allow calcium release [319]. CD38 is also associated with store-operated calcium entry (SOCE)[320]. SOCE is a mechanism that exists to allow extracellular calcium to enter the cell upon release of calcium from intracellular stores [321]. SOCE involves activation of plasma-membrane calcium release-activated channels (CRAC) [322] by calcium sensing stromal interaction molecules (STIMs) [323] expressed on the SR. Upon depletion of SR calcium, STIM proteins oligomerize and re-locate within areas of proximity to the plasma membrane CRACs [324]. CRACs are made up of

olf186-F (Orai) proteins [325] and upon interaction with STIMs, the CRAC is activated, allowing calcium entry [326]. Recently it was shown that OVA challenged mice possess more ASM STIM1 and Orai1 and that these proteins mediate PDGF induced proliferation [327].

Calcium Sensitization

Due to the toxicity of calcium, after its release and activation of calmodulin, ASM cells actively pump the ion back into the SR. In order to maintain force, the smooth muscle actin-myosin interaction must continue to occur even without elevated calcium. In order to achieve this, the myosin must remain phosphorylated and in smooth muscle cells this sensitization occurs not by activation of myosin light-chain kinase but rather through the inhibition of myosin light-chain phosphatase [328]. Inhibition of the phosphatase occurs through the activation of the small GTPase Rho and Rho associated kinase, which phosphorylates the myosin binding subunit of MLCP rendering it unable to bind to and therefore de-phosphorylate myosin [329]. This occurs in intact smooth muscle and has been shown to be involved in driving hypertension [330] and in an allergic model of asthma [331]. This latter study demonstrates a role of the immune system and its ability to influence structural cell excitability and will be described below.

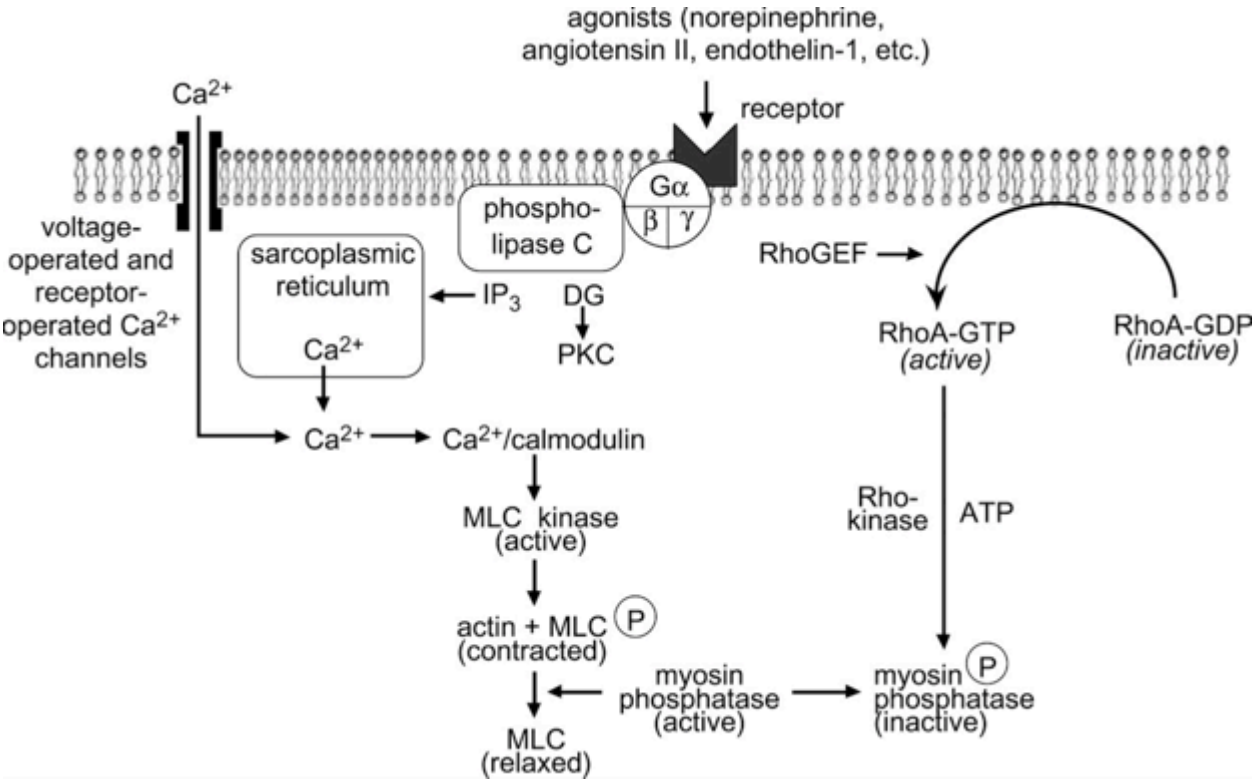


Figure 5. Calcium signalling in smooth muscle cells. Agonist stimulation of GPCRs induces the activity of PLC, cleaving PIP2 into IP3 and DAG. IP3 acts as a ligand on the SR IP3R releasing calcium into the intracellular compartment. This elevated calcium binds to calmodulin, activating MLCK, phosphorylating myosin light chain allowing cross bridge cycling to occur. Rho-kinase phosphorylation of MLCP inactivates the enzyme, allowing calcium sensitization to occur (figure from Webb, C. Adv Physiol Educ, 2003 (27)) [332].

Contractility and Asthma

The contractile status of ASM may play a key role in mediating the constriction of the airways of asthmatic patients. Many biochemical pathways have been described to alter ASM contractility. Several conflicting studies have demonstrated that ASM is either more contractile in asthmatic subjects, or it is the same as that of control subjects. In these studies, the methodologies differ

and these differences could explain the discrepancies. One key issue with comparing the studies performed is the fact that force generation per cross sectional area is often not considered and needs to be due to the expectation that a greater mass of muscle will produce more force [333]. One study exploring ASM cells in suspension showed that cells derived from asthmatic subjects had increased shortening velocity, however in this study, the cells contracted against no external load, and such a situation would not occur *in vivo.* The cells in this study were freshly isolated from human tissues and placed in a Kreb's solution perfused chamber and then stimulated to contract using electric field stimulation and length changes were monitored by microscopy. Furthermore, this study was performed at room temperature [334]. Furthermore, two other studies demonstrated that ASM cells embedded in collagen gels induced greater deformation of the gel upon agonist stimulation [335,336]. The later study also implicated reactive oxygen species through the increased expression of NOX4 to mediate this increase in asthmatic ASM cell contractility [336]. Another explanation for the increased contractility of asthmatic ASM cells may be increased MLCK [337-339]. Along this line of evidence, mast cell–ASM cell co-culture induced the up-regulation of smooth muscle α-actin in the ASM cell [340]. In a recent study examining eight asthmatics and 11 healthy control ASM tissue preparations, it was demonstrated that the tissues were not different in terms of contractility as measured through a variety of parameters with an array of stimuli [341]. This study was further confirmed in tracheal tissue of an equine model of asthma known as heaves, however in this study, the peripheral airway tissues of horses with heaves did demonstrate greater maximal shortening velocity [342]. This study adds evidence to the heterogeneity observed in airway diseases. More work should be conducted in this field to have a better understanding of whether or not asthmatic ASM is

different in terms of contractility. Several of these studies suffer from small numbers of subjects and the lack of uniform methodology.

Due to the importance of cytosolic calcium release in the initiation of cross-bridge cycling, researchers have explored calcium signalling in ASM cells derived from asthmatic subjects. It has been described that, in the presence of inflammatory cytokines, ASM cells up-regulate cADPR pathway of calcium release through the induction of the biphasic enzyme, CD38 [343,344]. It was also shown that there may be less mRNA and protein expression of SERCA, which could contribute to an accumulation of cytosolic calcium and contribute to cells being in a more excitable state [345]. Calcium sensitization may also be altered in asthmatic subjects' ASM. In hyperresponsive rats, increases in RhoA may contribute to a more contractile smooth muscle [331]. Finally, ORMDL3, a gene associated with asthma [34], was shown to inhibit SERCA and could lead to an increased cytosolic calcium concentration [346]. Although the verdict is not in, what is clear is that asthmatic patients do indeed experience exaggerated airway narrowing and that drugs such as β-agonists that relax the smooth muscle aid in reducing asthma symptoms.

1.8.6 ASM Immunologic Properties

The role of ASM cells is not limited to their structural and mechanical properties. These cells are known to synthesize and secrete pro-inflammatory cytokines. Cells synthesizing such cytokines do so when they are not in the contractile state, but rather can be secreted from cells that are actively proliferating [347]. ASM cells are able to release both Th1 and Th2 cytokines including IL-5, GM-CSF, IL-2, IL-12 and IFN-γ and the release of these cytokines increases after exposure of the ASM cells to human atopic asthma sensitized serum [348]. Upon stimulation of human

ASM cells with the pro-inflammatory cytokine TNF-α, NF-κB is activated and can drive the expression of IL-6 and CCL5 [349]. Furthermore, in the presence of the Th2 cytokines IL-4 and IL-13, ASM cells release the eosinophil chemoattractant eotaxin [350]. Exposure of ASM cells to IFN-γ causes the release of the neutrophil chemoattractant IL-8, which was inhibited in the presence of Th2 cytokines [351]. S1P stimulation of ASM cells can also induce the release of IL-6 from these cells [352]. In a similar fashion to the epithelium, the role of smooth muscle cells in promoting airway inflammation is becoming more evident.

1.9 Airway Smooth Muscle – Epithelial Interaction

1.9.1 Epithelial Derived Relaxing Factor

In the early 1980s, it was determined that the vascular endothelium released a relaxing factor that reduced tension of the vascular smooth muscle after muscarinic agonist stimulation [353]. Many laboratories focused their attention on this field, which eventually led to a 1998 Nobel Prize awarded to three investigators for the discovery of the soluble gas, NO, as a critical regulator of vascular tone [354,355]. In the mid 1980s, researchers began exploring whether such an interaction existed in the airway. It was demonstrated that, indeed, removal of the airway epithelium augmented the contractile response to several agonists in the canine airway [356]. Another study explored this phenomenon in bovine trachealis preparations, but observed no change in the contractility of ASM tissue preparations that were lacking the epithelial layer [357]. However, these experiments revealed that the epithelium released measurable quantities of both NO and PGE_2 upon stimulation with histamine [357]. In rat detrusor smooth muscle, the urothelium also has the ability to prevent contractions to carbachol through an NO and COX dependent mechanism [358]. Furthermore, in guinea-pig airway preparations, arachidonic acid

induced contraction of the smooth muscle in epithelial denuded samples, but caused relaxation in those with the epithelium present [359]. Similarly, in guinea-pig airway preparations, histamine induced the release of PGE_2 in epithelial intact samples and caused a greater contraction in the denuded group [360]. Besides agonist-induced release of COX metabolites, mechanical irritation of the epithelium may also cause the secretion of PGE_2 [361]. Some researchers have postulated that the absence of the epithelium may allow for a greater quantity of contractile agonists to reach the smooth muscle and this could explain another mechanism by which the epithelium causes a less contractile smooth muscle [362].

However, this line of research has not produced clear results. One study demonstrated that epithelial cells can induce increases on contraction of tracheal rings in the presence of eosinophilic derived major basic protein [363]. Another group showed that cultured guinea-pig respiratory epithelial cells may cause the constriction of tracheal preparations *in vitro* [364]. Furthermore, tracheal contractions to capsaicin were augmented in guinea-pig preparations that contained intact epithelium [365].

Although the majority of publications point towards consensus that a relaxing factor is indeed secreted by the epithelium, the jury is still out with regards to what that factor(s) may be. Vanhoutte has extensively reviewed the possibilities [366], and will be briefly described here. Besides the role of arachidonic acid metabolites such as PGE_2, NO was the first candidate of an epithelial derived relaxing factor due to its importance as a relaxant of the vasculature [354]. NO can indeed cause relaxation of the airway [367]. Through the use of inhibitors of the NO producing enzyme NO synthase, Munakata et al demonstrated that NO was not responsible for the relaxing effect of the epithelium [368]. Another study showed that NO was responsible for

the ASM cell relaxing properties of brain natriuretic peptide, but that it was not epithelial derived, postulating the involvement of an intermediate cell [369].

Cytokines may play an important role in modulating the ASM cell tone and many are associated with asthma. Pro-inflammatory cytokines may activate COX-2 [370,371] or NOS [372] to modulate the smooth muscle contraction.

Finally, neurotransmitters have been postulated to modulate ASM cell contractility. Acetylcholine can be released by airway epithelial cells [373] and can cause bronchial relaxation at low doses on isolated bronchi that have been pre-contracted with histamine that is likely due to the release of NO from the epithelium [369].

1.9.2 Epithelial Induced Proliferation

The epithelium has the ability to produce a variety of mitogens including EGFR ligands amphiregulin, HB-EGF, EGF, transforming growth factor-α (TGF- α), epiregulin, β-cellulin and heregulin [374-377]. Furthermore, the airway epithelium has been shown to secrete vascular endothelial growth factor (VEGF) [378], PDGF [379], FGF [380] and insulin-like growth factor [381]. All of these ligands have the ability to induce proliferative responses of ASM cells and may be important drivers of asthma pathogenesis. VEGF expression is augmented in epithelial cells derived from asthmatic subjects [382]. In 2009 Malavia et al described the proliferative effect of epithelial cells on airway smooth muscle cells *in vitro*, demonstrating that injured bronchial epithelial cells further augment the rate of proliferation of ASM cells. The effect of injured epithelial induced ASM cell proliferation was dependent on IL-6, IL-8, MCP-1 and MMP-9 whereas uninjured epithelial cell induced proliferation was only sensitive to 10μM GM6001 (MMP inhibitor) [383]. However it is important to note that the inhibition constant for

GM6001 against MMPs is in the low nanomolar range and that the dose utilized in this study is rather high [384]. Furthermore, the authors did not describe how the metalloproteinase caused an up-regulation in ASM cell proliferation [383]. Potential mechanisms by which this could occur are through the cleavage of growth factors from the plasma membrane by the MMP [385], or by direct activation of the protease activated receptor (PAR) [383,386]. It has been shown that chitinase-3-like protein 1 can be expressed by epithelial cells, it is increased in a model of airway exacerbation [387] and it can induce proliferation of ASM cells that is greater in myocytes derived from asthmatic subjects [388]. Another study that explored epithelial and ASM cell co-cultures showed that if the epithelial cells received house dust mite (HDM) extract induced greater induction in ASM cell proliferation after co-culture if they were derived from severe asthmatic donors. This study also demonstrated that the evoked response to HDM stimulation was mediated by increased activation of the $CysLTR_1$ and the authors reported augmented CysLT producing enzyme 5-LO and FLAP in epithelial cells subjected to HDM stimulation [389]. To date, the mechanism by which epithelial cells induce ASM cell proliferation is largely unknown. Besides the secretion of soluble growth factors by the epithelium, it is possible that cargo within microvesicles and exosomes could induce smooth muscle proliferation. For example, tissue factor can induce PAR-2 activation and is secreted in exosomes derived from epithelial cells [390]. The role of miRNAs in epithelial induced proliferation of ASM cells has yet to be explored and represents a line of investigation that motivated experiments discussed later in this dissertation.

1.9.3 Endothelial : Smooth Muscle Interactions

Parallels can be drawn when comparing the vasculature and the airways. As discussed previously, the endothelium regulates vascular smooth muscle cell (VSMC) tone through the

release of NO [354]. Upon reacting with VSMCs, NO induced guanylate cyclase generates cGMP which is the effector molecule mediating relaxation [355]. cGMP activates protein kinase G which phosphorylates Ca^{2+}-activated K^+ channels, causing membrane hyperpolarization and inactivation of voltage gated Ca^{2+} channels [391]. Interestingly, this relaxation step involved the release of sarcoplasmic reticulum intracellular calcium by ryanodine receptor activation, an event thought to trigger contraction in muscle cells. Besides inducing relaxation in VSMCs, NO can also reduce proliferation through decreased cyclin A and increased nuclear translocation of the cyclin-dependent kinase inhibitors p21 and p27 [392]. PKG can also attenuate proliferation through the induction of myocardin expression which may modulate the phenotype of the VSMC to be more contractile [393]. Besides NO, endothelin-1 (ET-1) is an important peptide that regulates VSMC contractility. VSMCs express both ET_A and ET_B receptors which are G-protein coupled and respond to the agonist by inducing contractions within the cell [394]. After release of ET-1, the protein can bind to ET_B receptors expressed on the endothelium itself, allowing internalization of the receptor representing a mechanism by which homeostasis is maintained after a release of ET-1 [395]. Work has been conducted exploring the interaction of NO and ET-1. In porcine blood vessels, NO induced cGMP reduced the secretion of ET-1 [396] and this likely occurs through a reduction in ET-1 mRNA production [397].

One important difference between the epithelial:ASM cell and the endothelial:VSMC is the anatomical relationship of the cell types. Endothelial cells have the ability to communicate with neighbouring VSMCs through myoendothelial junctions (MEJs). MEJs allow for small molecules of less than 1kDa to be transmitted from one cell to the other without the need for the molecule to be secreted [398]. Interestingly, MEJs have been examined in the context of endothelial regulation of VSMC phenotype. One group showed that the co-culture of endothelial

cells with pulmonary vascular smooth muscle cells on opposing sides of a Transwell® permeable support allowed for the formation of direct contact between the two cell types. Furthermore, the cell contacts contained MEJs that were responsible for mediating TGF-β transfer from the endothelial cell, which skewed the myocytes towards a more contractile, differentiated cell [399]. In a similar study, serotonin was shown to travel through the MEJ, inducing the expression of contractile gene expression through TGF-β activity within the myocyte [400]. These studies provide insights regarding the importance of direct contact co-culture of the two cell types and may represent an important difference when considering culture models of tubular structures. In the airway, there is probably no direct contact between ASM cells and epithelial cells and so the importance of cellular junctions may not be of importance in this system.

Finally, endothelial cells have been shown to communicate with VSMCs through the release of microvesicular bodies. Exosomes are endocyte-derived vesicles that can carry cargo including protein, mRNA and miRNA. VSMCs have been shown to secrete exosomes containing miR-143 that may drive angiogenesis in a model of pulmonary hypertension [401]. Due to the lack of direct contact of airway epithelial cells and ASM cells, the relative importance of exosomal communication is possibly greater in the airway structural cell communication.

1.10 Summary

Clearly, asthma is a diverse disease with a wide variety of cells and signalling molecules that have been implicated. Better understanding of this syndrome will lead to more personalized clinical approaches to improve symptoms and prevent exacerbations. Due to the limitations of experimentation on human subjects, researchers often turn to cell culture models to uncover insights into asthma pathogenesis.

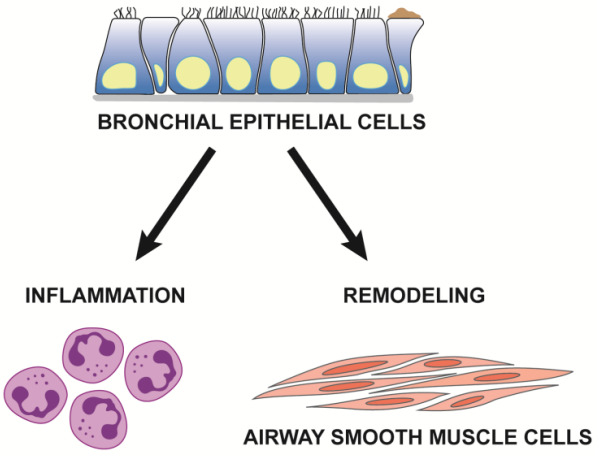
In the work of this dissertation, we aimed to better understand airway epithelial cell cultures derived from human subjects in regards to how they signal and interact with other cell types. More specifically, the first part of the experimental work explores the bioactive signalling lipid S1P and how this molecule instructs the epithelium to release the pro-inflammatory cytokine, IL-8. This was of interest due to the fact that persistent neutrophilia in the airways of severe asthmatics likely plays a significant role in driving airway remodeling. This sub-set of asthma is poorly treated and can lead to mortality and thus new therapeutic targets are necessary to treat such patients. The experiments conducted were to examine the intracellular signalling events that occur within epithelial cells upon introduction of S1P, and to understand which receptors and downstream signalling targets are involved in releasing the neutrophil chemoattractant, IL-8.

The second part of this dissertation has a two-fold purpose. We have continued to examine airway epithelial cells, but focused on their ability to alter smooth muscle properties. Of interest to asthma pathology, ASM is increased in mass and this may be due to proliferation of pre-existing smooth muscle cells. All mitogens of ASM cells are therefore of great interest to the field of asthma research and we have explored how epithelial cells stimulate proliferation within these cells. The second aim of this work was to understand how the epithelium modulates smooth contractility. Regulating airway tone is the functional property of smooth muscle and in asthma it is possible that the contractility of this tissue is modified. We were therefore interested in understanding how epithelial cells modulate the force generating ability of these cells and we explored the phenotypic regulation after co-culture with epithelial cells.

1.11 Hypothesis

Airway epithelial cells contribute to the release of IL-8 through S1P activation of specific S1PRs and downstream signalling. Furthermore, epithelial cells modulate the phenotype of ASM cells away from the contractile, and towards the proliferative phenotype.

Figure 6. Epithelial cells contribute to inflammation and ASM cell structural and molecular remodeling.



CHAPTER 2:

Sphingosine 1-Phosphate (S1P) Induced Interleukin-8 (IL- 8) Release Is Mediated by S1P Receptor 2 and Nuclear Factor kB in BEAS-2B Cells

2.1 Prologue

The purpose of this study was to examine the molecular mechanisms by which S1P induces IL-8 release from airway epithelial cells. Specifically, we were interested in identifying S1PRs that mediate this process to explore the potential for novel antagonists as therapeutic agents for the treatment of asthma. We also wished to elucidate the transcription factors involved in this process as well as to understand the role of the ROS/MMP/EGFR transactivation signalling pathway in mediating this event. To do this, we utilized the bronchial epithelial cell line, BEAS-2B, and measured protein secretion into the cell culture supernatant as a model of epithelial derived cytokine release.

Hypothesis: S1P-induced IL-8 release involves the activation of specific S1PRs, transactivation of the EGFR and is mediated through transcription factor activity in BEAS-2B cells.

2.2 Abstract

The airway epithelium may release pro-inflammatory cytokines and chemokines in the asthmatic airway. Sphingosine 1-phosphate (S1P) is a bioactive lipid, increased in the airways of asthmatics that may trigger the release of the potent neutrophil chemoattractant Interleukin-8 (IL-8) by epithelial cells. S1P is a ligand for 5 G protein-coupled receptors, S1PR1-5. We wished to explore the mechanisms of S1P induced IL-8 secretion with regard to the receptor(s) and downstream signalling events involved. Our results indicate that S1P induced IL-8 release is mediated by S1PR2 and the transcription factor NF-κB. Since the Epidermal Growth Factor Receptor (EGFR) and reactive oxygen species (ROS) have been implicated in IL-8 release in response to activation of other G protein-coupled receptors, we examined their importance in S1P induced IL-8 release and established that they are not involved. This study reveals S1PR2 and NF-κB as potential therapeutic targets in neutrophilic airway diseases such as severe asthma.

2.3 Introduction

S1P is a bioactive lipid important in immune system regulation, angiogenesis, migration and proliferation [402-405]. S1P is produced when sphingosine, derived from ceramide, is phosphorylated by sphingosine kinase I or II. There are currently five known S1P receptors (S1PR1-5) and these are G protein-coupled. S1P binding to these receptors can elicit diverse signalling mechanisms owing to the heterogeneity of these receptors and their coupling to different G proteins. Synthesis of S1P occurs in many cell types including platelets and mast cells [406,407]. S1P has been shown to be increased in the bronchoalveolar lavage of asthmatics upon segmental allergen challenge when compared to healthy control subjects [352]. S1P has also been shown to induce contraction of airway smooth muscle cells, strengthening its potential role as an important lipid mediator in the asthmatic airway [205].

Neutrophils, among other leukocytes, play an important role in asthma pathogenesis. When compared to healthy control subjects, severe asthmatics possess more neutrophils in the induced sputum [10]. It is well established that neutrophils undergo chemotaxis towards an increasing gradient of the chemokine interleukin 8 (IL-8), as reviewed by Baggiolini et al. [63]. IL-8 release from structural cells in the lung is therefore a possible avenue by which neutrophil recruitment occurs in the asthmatic airway.

S1P has previously been shown to induce IL-8 release from airway epithelial cells in a phospholipase D dependent manner [53,54]. We wished to explore which S1P receptor(s) are involved in S1P induced IL-8 release from airway epithelial cells, as receptor inhibition could reveal novel therapeutic targets for the treatment of severe asthma. Transactivation of the EGFR

is a requirement for leukotriene D4 (LTD_4)-induced release of IL-8 from airway epithelial cells [408]. LTD_4 also induces transactivation of the EGFR in airway smooth muscle cells and this phenomenon is dependent on the generation of ROS [115]. Because LTD_4 is an agonist of the cysteinyl leukotriene Receptors 1 and 2 (CysLTR1/2) and these receptors are G protein-coupled, we hypothesized that S1P may mediate IL-8 release via ROS dependent transactivation of the EGFR and explored this hypothesis in the context of the airway epithelium. Finally we examined the role of IL-8 transcription factors in the process of S1P induced IL-8 release.

2.4 Materials and Methods

Reagents

W 123 (10μM), JTE 013 (1-10μM), CAY 10444 (10μM), specific inhibitors of S1PR1, S1PR2, S1PR3 respectively, S1P (0.1-10μM), and the EGFR inhibitor tryphostin AG-1478 (0.3-3μM) were all obtained from Cayman Chemical (Ann Arbor, MI, USA). Helenalin (1μM), inhibitor of NF-κB, pEGFR antibody (p-Tyr-845 SC-23420-R) and total EGFR antibody (SC-03) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). SR 11302 (1μM), inhibitor of activator protein-1 (AP-1) and SEW 2871 (10μm), agonist of S1PR1 were obtained from Tocris Bioscience (Bristol, UK). Dichlorodihydrofluorescein diacetate (DCFH-DA) (10μM) and N-acetyl cysteine (NAC) (1mM) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Luciferase reporter lysis buffer was obtained from Promega (Madison, WI, USA). GM6001 (25μM), the broad-spectrum hydroxamic acid inhibitor of matrix metalloproteinases (MMPs) and TAPI-1 (10μM), inhibitor or MMPs and tumor necrosis factor-α converting enzyme (TACE) were obtained from Calbiochem (La Jolla, CA). Fura-2 AM (10μM), and pluronic F127 (0.02%) were obtained from Life Technologies (Carlsbad, Ca).

Cell Culture

Human BEAS-2B cells (ATCC, Manassas, VA) were grown in DMEM:F12 10%FBS 100 U/ml penicillin, 100μg/ml streptomycin and 2500ng/ml amphotericin B (PSA) (Invitrogen, Carlsbad, CA, USA) in 75cm^2 tissue culture flasks at 37° C and 5% CO_2. Culture medium was changed every 2 days and cells were seeded into new flasks when approximately 80% confluent. Cells were detached by incubation with 0.25% trypsin (Sigma-Aldrich). For experimentation, cells were seeded in 6 well plates at a density of 50 000 cells per well and grown for 3 days in culture medium. Cells were serum-starved for 24 hours in DMEM:F12 0.1%BSA (Sigma-Aldrich) with

PSA. Starvation medium was changed prior to all experiments and culture supernatant was collected at the end of the incubation period.

For NF-κB luciferase reporter assays, cells were grown in DMEM 10%FBS 100 U/ml penicillin, 100μg/ml streptomycin and hygromycin B. Starvation medium (CnT-17 basal medium) (CellNTec, Bern, Switzerland) was not changed prior to S1P stimulation. For intracellular calcium measurements, primary human airway smooth muscle cells obtained from lung transplant donors were cultured in DMEM 10%FBS with PSA and used between passages 3 and 5.

Measurement of IL-8

IL-8 concentration in the culture supernatant was measured after a 4 hour incubation, with or without S1P, and respective inhibitors by ELISA using the CXCL8 Duoset (R&D Systems, Minneapolis, MN, USA).

Measurement of ROS

Cells were seeded in dark-walled 96 well plates at a density of 10 000 cells per well in starvation medium for 24 hours. The cells were washed with Hanks balanced salt solution (HBSS) (137mM NaCl, 4.2 mM $NaHCO_3$, 10mM glucose, 3 mM Na_2HPO_4, 5.4 mM KCl, 0.4mM KH_2PO_4, 1.3mM $CaCl_2$, 0.5mM $MgCl_2$, 0.8 mM $MgSO_4$, 5mM HEPES) and incubated with fresh HBSS containing 10μM 2',7'-Dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich) for 30 minutes. Cells were washed with fresh HBSS and the baseline fluorescence intensity was read using a fluorescent plate reader (Tecan iControl, Männedorf, Schweiz, Switzerland) (Excitation = 485nm, Emission = 530nm). The cells were stimulated with S1P 1μM or vehicle and fluorescence intensity was read every 5 minutes for 1 hour.

EGFR Knockdown by siRNA Transfection

BEAS-2B cells were seeded in 6 well plates at a density of 25 000 cells per well in DMEM:F12 10%FBS (Invitrogen) without antibiotics. 12 hours later the medium was aspirated and 9pmols of scrambled (SC-37007) or EGFR specific (SC-29301) siRNA with 2µl of siRNA transfection reagent (SC-29528) in 1ml transfection medium (SC-36868) was added to the cells for 5 hours. (Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1ml of DMEM:F12 20%FBS with PSA (Invitrogen) was added and the cells were incubated for another 18 hours. Medium was changed into DMEM:F12 10%FBS with PSA (Invitrogen) for 24 hours and cells were serum starved in DMEM:F12 0.1%BSA with PSA (Invitrogen) for 24 hours. Starvation medium was replaced and cells were stimulated with 1µM S1P or vehicle for 4 hours.

Western Blot

Cells were washed with ice-cold PBS (Invitrogen) following experimentation and lysed with ice-cold protein extraction buffer containing 50mM TrisHCl (pH 8), 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS. Cell lysates were centrifuged at 13 000 RPM for 3 minutes and total protein supernatant was collected for SDS-PAGE (8%). 20 to 30µg of protein diluted in distilled water and loading dye were boiled together for 6 minutes and equal volumes of sample were loaded onto the separating gel. After separation, proteins were transferred to a PVDF membrane (BioRad, Hercules, CA, USA) for immunoblotting. The membrane was blocked for 1 hour at room temperature in a 2% BSA in Tris-buffered saline (TBS). Membranes were incubated with primary antibody (total-EGFR) diluted 1:2000 in TBS containing Tween-20 (TBST) overnight at 4ºC. Membranes were incubated with secondary antibody (goat, anti-rabbit-HRP) diluted 1:5000 in TBST for 1 hour at room temperature. Western blots were quantified using the ECL Plus Western blotting detection system (GE Healthcare, Little Chalfont, UK).

NF-kB Luciferase reporter assay

BEAS-2B cells were stably transfected with a plasmid containing a κB (GGGGACTTTCC) response element upstream of a hygromycin B resistance gene and a firefly luciferase construct. These cells were seeded into 24 well plates at a density of 50 000 cells per well in DMEM 10% FBS hygromycin B for 24 hours. Cells were serum starved overnight in CNT-17 basal medium (CellNTec) and stimulated with 1μM S1P for 4 hours. Other cells were pretreated for 30 minutes with 1μM JTE 013 and then stimulated with 1μM S1P for 4 hours. Reporter lysis buffer (Promega, Madison, WI, USA) was used to lyse the cells. Whole cell lysates were collected and spun at 13 000 RPM for 3 minutes. 10μl of supernatant was transferred to a 96 well plate for reading in the Tecan iControl luciferase system.

Measurement of Intracellular Calcium

Human airway smooth muscle cells were seeded onto sterilized glass cover slips at a density of 25 000 cells per well in 6 well tissue culture plates in DMEM 10% FBS with PSA (Invitrogen). After one day, cells were serum deprived in DMEM 0.5% FBS 1%PSA medium for 3 days before analysis of intracellular calcium responses to 1μM S1P using 10μM Fura2-AM. To mediate loading of the Fura-2 AM, pluronic F127 (0.02%), along with the 10μM Fura-2 AM was dissolved in HBSS for 30 minutes at 37^{o}C. Any unloaded Fura-2 AM was washed out with HBSS. Cover slips were loaded into a Leiden chamber (Medical Systems, Greenville, NY) and an inverted fluorescent microscope with a 40X oil-immersion objective (Olympus, Tokyo, Japan) was used to measure signals emitted at 510nm using a CCD camera (CoolSnapPro; Media Cybernetics, Bethesda, MD) controlled by Image Master software (Photon Technology International, Birmingham, NJ). Data was acquired as previously described [409].

Statistical Analysis

Statistical analysis was carried out in the GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA). All data are expressed as means + 1SE, with ≥ 3 independent observations per experiment. To test for statistical differences, one-way ANOVA with Tukey's post hoc test was applied to experiments with ≥2 groups. For experiments with only 2 groups, we applied Student's unpaired T-test. For comparison of fluorescence intensity curves, baseline values were normalized prior to S1P stimulation and a repeated measures ANOVA was applied. P values < 0.05 were considered to be significant.

2.5 Results

S1P induces IL-8 release

To confirm that S1P induces IL-8 release from BEAS-2B cells, the cells were incubated with various concentrations of S1P for 4 hours. ELISA analysis of the culture supernatant showed a dose-dependent increase in IL-8 release with S1P stimulation that was significant at a concentration of 1μM (Fig. 1). For subsequent experiments, 1μM S1P was used to stimulate IL-8 release.

S1P induced IL-8 release is mediated by S1PR2

To determine if S1P induced IL-8 release from airway epithelial cells is mediated by a specific S1P receptor, or group of receptors, BEAS-2B cells were pre-treated with specific S1P receptor inhibitors for 30 minutes prior to stimulation with S1P. W123, a competitive antagonist of S1PR1 (K_i=0.69μM), JTE 013, a selective antagonist of S1PR2 (IC_{50}=17nM), and CAY10444, a selective antagonist of S1PR3 (IC_{50}=4.6μM) were used as inhibitors of their respective receptors. Pre-treatment with JTE 013, but not with W123 or CAY10444, significantly inhibited S1P induced IL-8 release (Fig. 2 A). To ensure that S1PR1 and S1PR3 were not involved in this pathway, we confirmed these results by further experimentation. SEW 2871, a selective agonist of S1PR1 (EC_{50}=13nM) failed to elicit an increase in IL-8 release above vehicle treated cells (Fig 2 A). To ensure that CAY 10444 was active as a S1PR3 inhibitor at 10μM, we cultured human airway smooth muscle cells on glass coverslips and measured their intracellular calcium responses to S1P (1μM) stimulation. Pre-treating the cells with CAY 10444 abolished the S1P induced calcium responses in these cells, suggesting that the inhibitor was indeed active at these concentrations (Fig. 2 E). CAY 10444 treatment appeared to cause an increase in resting

intracellular calcium levels in human airway smooth muscle cells, again indicating the biological effect of CAY 10444 at a concentration of 10μM (Fig 2 D).

NF-κB Mediates S1P Induced IL-8 Release

IL-8 synthesis is driven primarily by the transcription factors NF-κB and AP-1. We wished to assess if pharmacological inhibition of these transcription factors affected IL-8 release. BEAS-2B cells were pre-treated for 30 minutes with Helenalin or SR11302, specific inhibitors of NF-κB [410] and AP-1 [411] respectively, prior to S1P stimulation for 4 hours. Pre-treatment with Helenalin but not with SR11302 inhibited IL-8 release (Fig. 3 A-B). To further confirm that AP-1 is not involved in S1P induced IL-8 release, we pre-treated cells with a cell permeant peptide fragment of the AP-1 monomer c-JUN. The peptide fragment contained amino acids 33-57 of the JNK binding domain and disrupts the interaction of JNK and c-JUN, preventing c-JUN phosphorylation. The peptide inhibitor did not significantly reduce S1P induced IL-8 release (Fig. 3 C). However there was a trend towards a decrease in IL-8 secretion, raising the possibility that AP-1 may play a minor role in S1P induced IL-8 release. We conclude from these experiments that NF-κB is the dominant transcription factor determining the magnitude of S1P induced IL-8 release.

S1P induced NF-κB activity is dependent on S1PR2

To further confirm that NF-κB drives S1P induced IL-8 release, we used a BEAS-2B NF-κB luciferase reporter cell line. Stimulation of the reporter cells with S1P for 4 hours induced a significant increase in luciferase activity (Fig 4). Since S1P induced IL-8 release was dependent on S1PR2, we evaluated whether S1P induced NF-κB activation was also dependent on S1PR2. Pre-treatment of the BEAS-2B NF-κB luciferase reporter cells with JTE 013 significantly

reduced the S1P induced luciferase activity (Fig 4). These results indicate that S1P mediates its activation of NF-κB via S1PR2.

S1P induced IL-8 release is not dependent on the epidermal growth factor receptor

Since activation of the EGFR can be upstream of IL-8 release [408] we wished to explore if EGFR transactivation is induced by S1P stimulation. Pretreatment with EGFR tyrosine kinase inhibitor AG1478 for 30 minutes prior to S1P stimulation failed to reduce S1P induced IL-8 release at reasonable concentrations for EGFR inhibition (Fig 5 A). To confirm these results we used siRNA against the EGFR and knocked down 54% of the constitutively expressed protein (Fig 5 B). Upon S1P stimulation, there was no difference in IL-8 release between siEGFR and scrambled siRNA groups (Fig 5 B). We conclude that the transactivation of the EGFR is not involved in mediating S1P induced IL-8 secretion from airway epithelial cells. In support of this conclusion, we failed to measure an increase in phosphorylation of tyrosine-845 by western blot after stimulation with S1P (data not shown).

We sought further supportive evidence that EGFR transactivation does not occur in S1P induced IL-8 release exists. Matrix metalloproteinases (MMPs) often play a role in transactivating the EGFR by causing the release of pro-forms of EGFR ligands [408,412,413]. GM6001 and TAPI-1, inhibitors of MMPs did not inhibit S1P induced IL-8 release (Fig 5 D) pointing towards an EGFR-independent mechanism.

S1P induced IL-8 release is not mediated by reactive oxygen species

To determine if the generation of ROS mediates S1P induced IL-8 release, we measured the production of ROS in BEAS-2B cells using the fluorescent ROS probe DCFH, a non-specific probe for a variety of oxidants. S1P did not induce significant increases in fluorescence (Fig 6

A). To further confirm that S1P does not induce IL-8 release through the production of ROS, we incubated BEAS-2B cells with the ROS scavenger N-acetyl cysteine for 30 minutes and then stimulated with S1P for 4 hours. N-acetyl cysteine can increase the intracellular levels of glutathione and thus act as an anti-oxidant [414]. ELISA analysis for IL-8 revealed that pretreatment with the antioxidant NAC is unable to decrease S1P induced IL-8 release (Fig 6 B). Pretreatment for 30 minutes with the general NADPH oxidase inhibitor DPI was also unable to inhibit S1P induced IL-8 release (Fig 6 C). Taken together, these results indicate that S1P induced IL-8 release is not dependent on the generation of ROS.

Figure 1

S1P induces IL-8 release in BEAS-2B cells. BEAS-2B cells were stimulated with various concentrations of S1P for 4 hours. Culture supernatant was analyzed for concentration of IL-8 by ELISA (n=5). Data are representative of means + SE. ANOVA with Tukey post hoc pairwise comparisons. *P<0.05

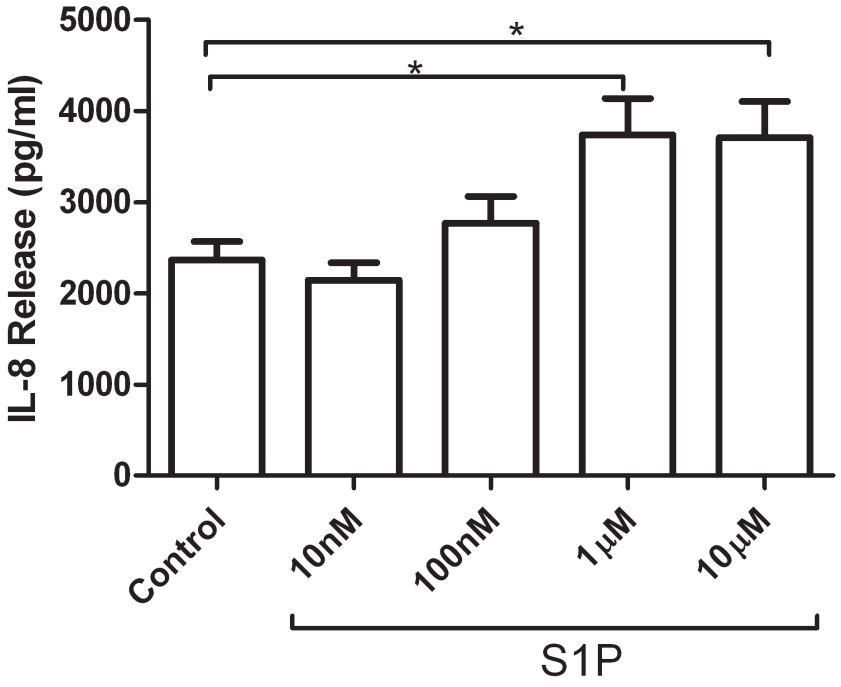


Figure 2

S1P induced IL-8 release is mediated by S1PR2 in BEAS-2B cells. BEAS-2B cells were pretreated for 30 minutes with (A) W 123 (n=5), (B) JTE 013 (n=5) or (C) CAY 10444 (n=8), specific inhibitors of S1PR1, S1PR2 and S1PR3 respectively and then stimulated with 1μM S1P or vehicle for 4 hours. Culture supernatant was analyzed for IL-8 concentration by ELISA. Human airway smooth muscle cells were loaded with 10μM Fura 2-AM, then treated with CAY10444 or vehicle (n=5) for 30 minutes and intracellular calcium was measured by ratiometric fluorescence microscopy (E). Cells were stimulated with S1P (n=5) and increases in resting intracellular calcium were recorded (F). Data are representative of means + SE. ANOVA with Tukey post hoc pairwise comparisons. **P<0.01, ***P<0.001

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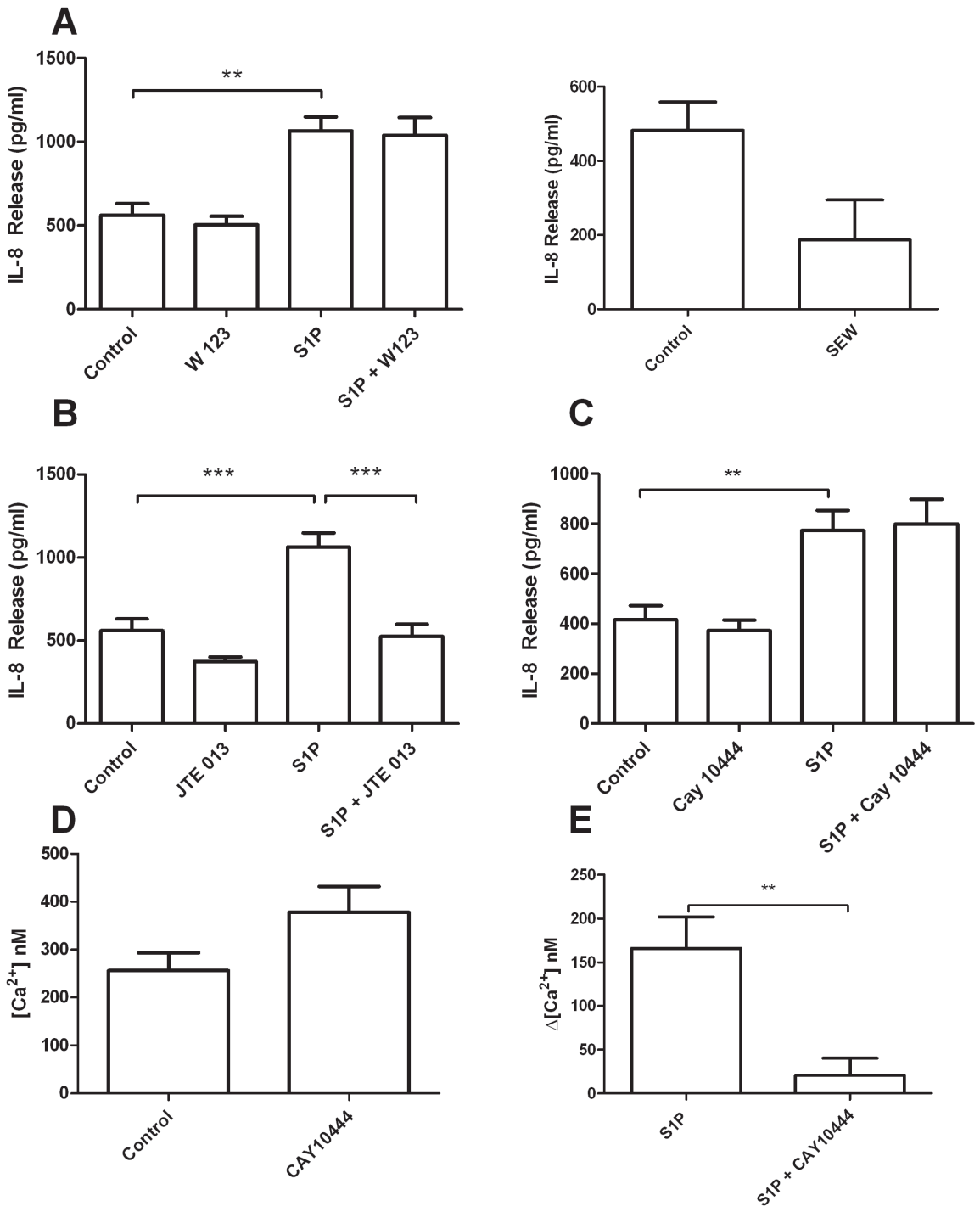
A
IL-8 Release (pg/ml)
1500
1000
500
0
**
Control
W 123
S1P
S1P + W123
600
400
200
0
Control
SEW
B
***
***
JTE 013
S1P + JTE 013
C
**
Cay 10444
S1P + Cay 10444
D
[Ca2+] nM
500
400
300
200
100
0
Control
CAY10444
E
Δ[Ca2+] nM
250
200
150
100
50
S1P
S1P + CAY10444

Figure 3

S1P induced IL-8 release is mediated by NF-κB in BEAS-2B cells. BEAS-2B cells were pretreated with (A) Helenalin (n=3), (B) SR 11302 (n=3), or c-JUN peptide (n=3) inhibitors of NF-κB, AP-1 and AP-1 respectively, for 30 minutes and then stimulated with S1P for 4 hours. Culture supernatants were analyzed for IL-8 concentration by ELISA. Data are representative of means + SE. ANOVA with Tukey post hoc pairwise comparisons. *P<0.05, ***P<0.001

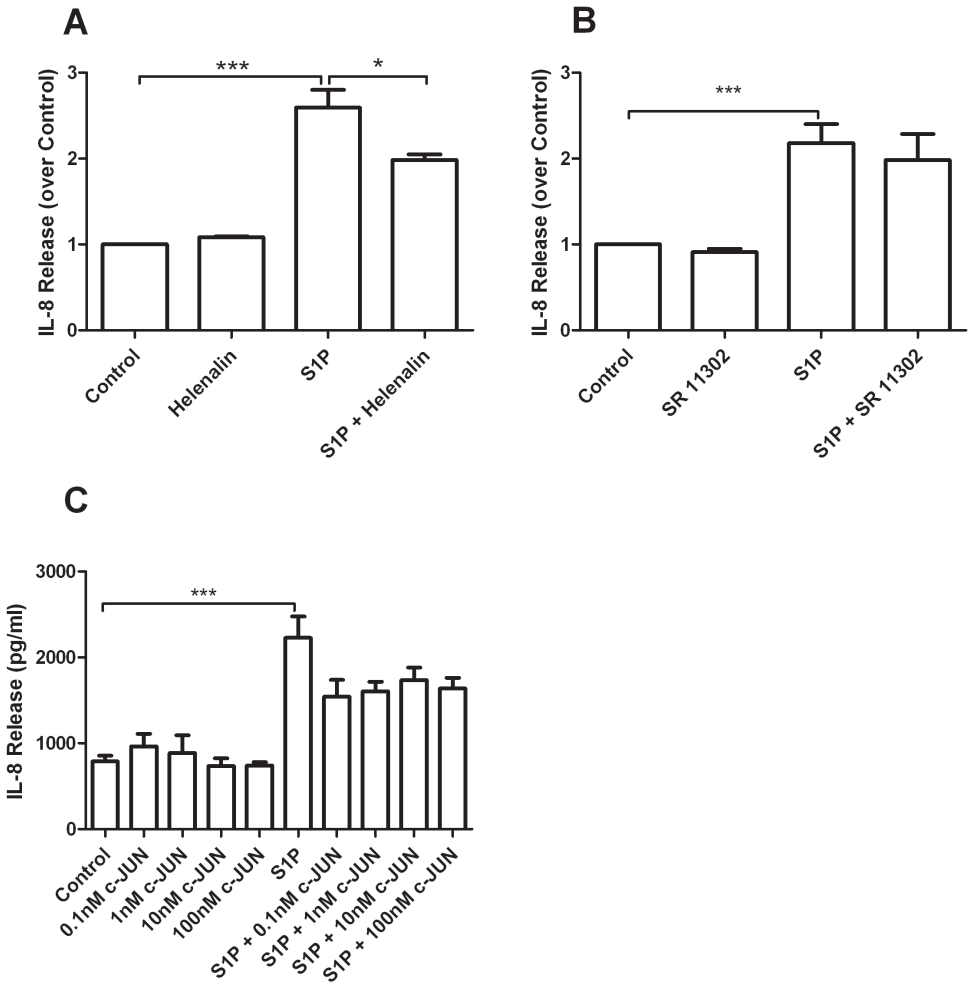

Figure 4

S1P induced Nf-κB activity is mediated by S1PR2 in BEAS-2B cells. Nf-κB luciferase reporter BEAS-2B cells were pretreated with S1PR2 inhibitor JTE 013 for 30 minutes before stimulation with S1P for 4 hours (n=3). Cell lysates were analyzed for luciferase activity by Tecan iControl plate reader. Data are representative of means + SE. ANOVA with Tukey post hoc pairwise comparisons. ***P<0.001

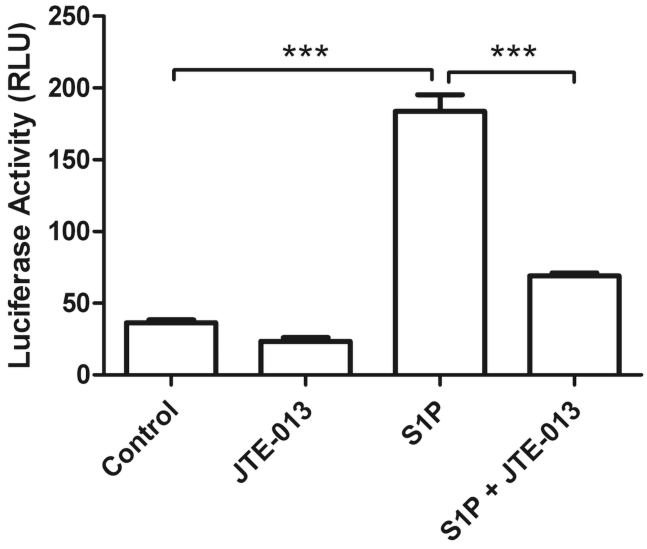

Figure 5

S1P induced IL-8 release is not dependent on the EGFR in BEAS-2B cells. (A) BEAS-2B cells were pretreated for 30 minutes with the specific EGFR inhibitor AG 1478 and then stimulated with S1P for 4 hours (n=7). Culture medium was assessed for IL-8 concentration by ELISA. (B) BEAS-2B cells transfected with control or EGFR specific siRNA were stimulated with S1P for 4 hours (n=3). Culture medium was assessed for concentrations of IL-8 by ELISA (left). Knock-down efficacy is shown by western blot for total EGFR (170kDa) with GAPDH loading control (37kDa) (right). Quantification of total EGFR bands is shown (n=4) (bottom left). (C) BEAS-2B cells were pretreated for 30 minutes with MMP inhibitors GM6001 or TAPI-1 and then stimulated with S1P for 4 hours (n=3). Culture medium was assessed for IL-8 concentration by ELISA. Data are representative of means + SE. ANOVA with Tukey post hoc pairwise comparisons. *P<0.05, **P<0.01, ***P<0.001.

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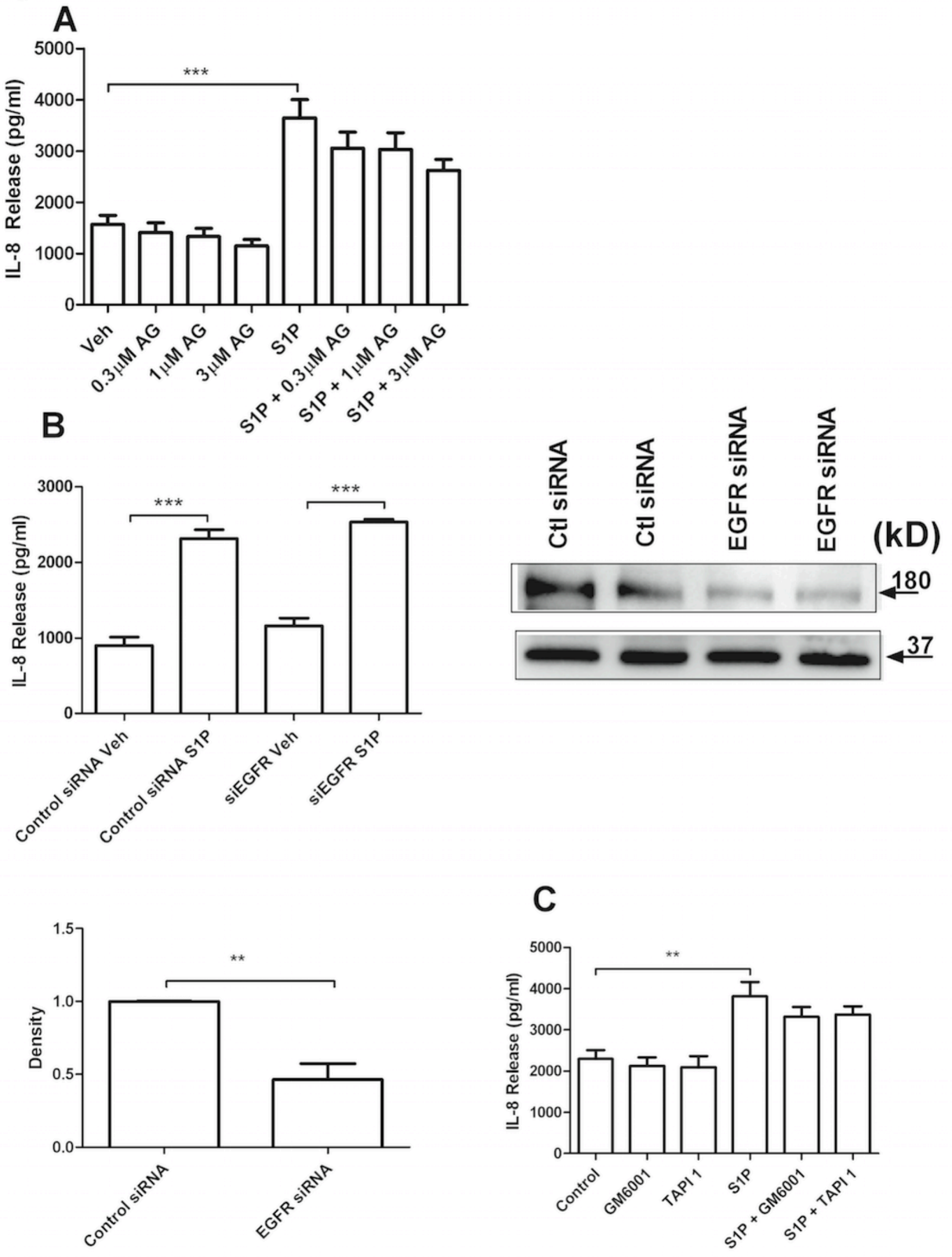
A
IL-8 Release (pg/ml)
5000
4000
3000
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0
***
Veh
0.3μM AG
1μM AG
3μM AG
S1P
S1P + 0.3μM AG
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S1P + 3μM AG
B
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Control siRNA Veh
Control siRNA S1P
siEGFR Veh
siEGFR S1P
Ctl siRNA
Ctl siRNA
EGFR siRNA
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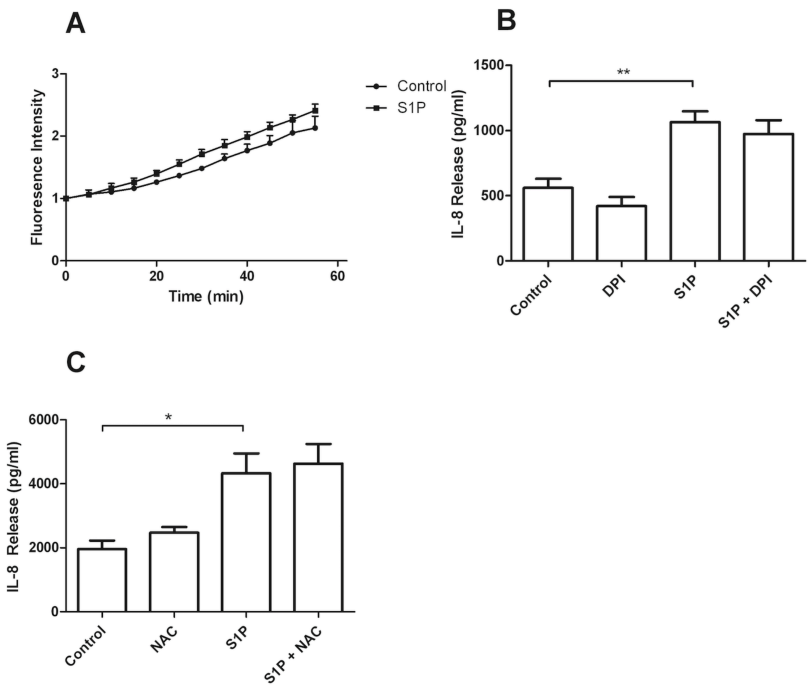
(kD)
180
37
Density
1.5
1.0
0.5
0.0
**
Control siRNA
EGFR siRNA
C
IL-8 Release (pg/ml)
5000
4000
3000
2000
1000
0
**
Control
GM6001
TAPI 1
S1P
S1P + GM6001
S1P + TAPI 1

Figure 6

S1P induced IL-8 release is not dependent on the production of reactive oxygen species in BEAS-2B cells. (A) BEAS-2B cells were incubated for 30 minutes with 10μM DCFH-DA. Free probe was washed with Hank's buffer and baseline fluorescence was measured at 530nm. Cells were then stimulated with S1P or vehicle and fluorescence intensity was measured every 5 minutes for one hour (n=3). Analysis using repeated measures ANOVA revealed no difference between the two curves. (B) BEAS-2B cells were pretreated for 30 minutes with the general antioxidant N-acetyl cysteine (NAC) and then stimulated with S1P for 4 hours (n=5). Culture medium was assessed for IL-8 concentration by ELISA. (C) BEAS-2B cells were pretreated for 30 minutes with the NADPH oxidase inhibitor DPI and then stimulated with S1P for 4 hours (n=5). Culture medium was assessed for IL-8 concentration by ELISA. (n=5). Data are representative of means + SE. ANOVA with Tukey post hoc pairwise comparisons *$P<0.05$, **$P<0.01$

(Figure on subsequent page):


A
Fluoresence Intensity
Time (min)
Control
S1P
B
IL-8 Release (pg/ml)
**
Control
DPI
S1P
S1P + DPI
C
IL-8 Release (pg/ml)
*
Control
NAC
S1P
S1P + NAC

2.6 Discussion

The purpose of this study was to evaluate the potential role of various S1P receptors and the mechanisms of transduction of the pro-inflammatory response of the airway epithelium to S1P. S1P induced IL-8 secretion represents an important avenue for the recruitment of neutrophils to the airways, particularly in asthma. Inhibition of this phenomenon could lead to improvement of lung function in severe asthmatic patients, and improve their symptoms. We have identified S1PR2 as a receptor responsible for transducing the S1P signal from the cell exterior, causing IL-8 secretion from BEAS-2B cells.

S1P is an important lipid mediator that has been implicated in a number of biological processes. It has been shown to be present in increased concentrations in the airways of asthmatic subjects, where it may act on the lining epithelial cells. S1P has been shown previously to trigger IL-8 secretion by cultured epithelial cells [53,54]. To confirm these previous findings, we stimulated BEAS-2B cells with various concentrations of S1P to construct a concentration-response relationship. Our results demonstrate that 1μM S1P is sufficient to cause a significant increase in IL-8 secretion, consistent with previously published data [53].

A study by Milara et al. examined the effect of S1P on IL-8 secretion from another airway epithelial cell line, A549. Milara et al. demonstrated that S1P induced the release of IL-8 from A549 cells at later time points than we show here in BEAS-2B cells. Also, A549 cells secreted much more IL-8 at basal levels than did BEAS-2B cells in this study. The conditioned medium from A549 cells recruited neutrophils in a Boyden chamber assay [415]. Milara et al. also implicated phospholipase D signalling in S1P induced IL-8 release in A549 cells, which has been described in BEAS2-B cells by Wang et al [54].

Following identification of S1PR2 and NF-κB as important mediators of S1P induced IL-8 release, the question of how this receptor signals to the transcription factor arises. Others have described the effect of S1PR2 blockade on various physiological processes; for example antagonizing S1PR2 in human bronchial epithelial cells inhibits the extrusion of apoptotic cells [416]. S1P receptor G protein coupling is complex, with S1PR2 coupling to a variety of Gα subunits including $G\alpha_s$, $G\alpha_i$, $G\alpha_{12/13}$, $G\alpha_q$ and G_0 [417]. Increased cell survival mediated by NF-κB stimulation by S1P in HeLa cells is transduced through S1PR2 coupling with $G\alpha_i$ [418]. Furthermore, evidence exists that S1P induced IL-8 secretion is $G\alpha_i$ and Rho dependent which in turn drives phospholipase D activation [53]. Lipopolysaccharide (LPS), a ligand for toll-like receptor 4 (TLR4), stimulates RhoA, which then activates NF-κB to release IL-8 in cervical stromal cells [419]. In the endothelium, activation of S1PR2 leads to activation of the small GTPase Rho [420]. Recently it was shown that LPS and tumor necrosis factor-alpha (TNF-a)-induced endothelial inflammation is mediated by S1PR2, coupling to the transcription factor NF-κB [421]. This literature supports the model that S1PR2 activation can drive NF-κB to up-regulate IL-8 synthesis and secretion in airway epithelial cells.

S1P administration in vivo to mice has been shown to increase airway reactivity to methacholine challenge, increase airway eosinophil recruitment, and increase interleukin (IL)-4, IL-13 and IL-17 in the BAL. In this study, mice were treated with S1P subcutaneously before analysis of airway function [201]. This study did not examine the production of chemokines associated with airway neutrophil recruitment, but adds strength to the rationale for studying this molecule in the context of lung disease by demonstrating that S1P itself can induce airway hyperresponsiveness. Inhibition of mouse lung S1PR2 with JTE 013 inhibited S1P induced pulmonary vasoconstriction, another S1PR2/Rho kinase dependent phenomenon [422].

S1P can induce bronchial smooth muscle contraction which has been shown to be dependent on S1PR2 and rho kinase [423]. Rho kinase has also been implicated in airway smooth muscle contraction from ovalbumin challenged mice, linking rho kinase activity to an allergic model of asthma [424]. This literature provides evidence that S1PR2/Rho kinase signalling could drive asthma pathogenesis not only by augmenting neutrophilic inflammation, but also by increasing airway smooth muscle contractility. Inhibition of S1PR2 in the lung of severe asthmatics could therefore cause relaxation of the bronchial smooth muscle and resolve inflammation strengthening the rationale for the use of JTE 013 as a pharmacological tool for the treatment of non-eosinophilic asthma.

Oxidative stress is an important mediator of other GPCR pathways [425,426] and is involved in asthma pathogenesis [427,428]. The NADPH oxidase enzyme is a large producer of oxidative stress in airway epithelial cells. A functional NADPH oxidase has been demonstrated to be essential in NF-κB activation in *Pseudomonas aeruginosa* infected mouse phagocytic leukocytes [429]. It has been previously shown that oxidative stress from NADPH oxidase activation can transactivate the EGFR [115]. ROS generation within the cell is able to inactivate protein tyrosine phosphatases by oxidation of cysteine residues, shifting the EGFR to a more activated state [430]. Specific inhibition of the NADPH oxidase, or use of a general anti-oxidant failed to inhibit S1P induced IL-8 secretion, nor did we measure any significant increase in cellular oxidative stress after S1P administration. In contrast hematopoietic progenitor cells egress the bone marrow under the influence of S1P in a ROS dependent manner via signalling through S1PR1 [431]. Similarly cardiac fibrosis is mediated by S1PR3 and oxidative stress in sphingosine kinase 1 transgenic mice [432]. Since the signal for IL-8 release in our experiments

was S1PR2-dependent, it suggests that the role of ROS in S1P receptor signalling may be receptor specific.

We have tested and rejected the hypothesis that EGFR transactivation mediates S1P induced IL-8 release in BEAS-B cells. Pharmacological inhibition of the EGFR with the tyrosine kinase inhibitor AG 1478 failed to inhibit S1P induced IL-8 secretion at concentrations appropriate for selective EGFR inhibition. The IC_{50} of AG1478 for inhibition of the EGFR is 3nM [433] Knockdown of the EGFR using small interfering RNA failed inhibit IL-8 release despite a 54% reduction in EGFR protein. Other studies have noted that AG 1478 may have non-specific effects [434,435]. We also failed to detect any increases in phosphorylation of tyrosine 845 of the EGFR after stimulation with S1P, a further indication that transactivation of the EGFR by S1P does not occur. This demonstrates that there is not a common mechanism of IL-8 secretion induced by either S1P or LTD_4 in BEAS-2B cells.

This study has shown that, *in vitro*, pharmacological inhibition of S1PR2 decreases S1P induced IL-8 release from BEAS-2B cells, and that S1PR2 is upstream of NF-κB in this phenomenon. Future translational work *in vivo* using S1PR2 antagonists in animal models of asthma will be of great interest.

CHAPTER 3:

Airway Epithelial Cells Reduce the Contractile Phenotype of Airway Smooth Muscle Cells

3.1 Prologue

Upon examining processes by which epithelial cells are stimulated to release pro-inflammatory cytokines, we wished to explore the effects that mediators derived from these cells may have on airway smooth muscle cell properties relevant to asthma. Muscle cells are important regulators of airway diameter and as such their contraction is linked to excessive airway narrowing in asthma and for the phenomenon of airway hyperresponsiveness to inhaled methacholine. Since the epithelium is the first tissue to experience the effects of inhaled airborne triggers of asthma, it is logical to address the interactions that this tissue has with ASM. To further this area of research, we turned to co-culture models in which ASM cells were cultured together with both the epithelial cell line BEAS-2B as well as with normal human bronchial epithelial cells. Since ASM cell force generation is a critical component of airway constriction, we were interested in examining the effect of epithelial co-culture on ASM cell contractile properties.

Hypothesis: ASM cells lose the contractile phenotype when co-cultured with airway epithelial cells.

3.2 Abstract

The airway epithelium is thought to participate in airway wall remodelling. Since asthma is associated with airway narrowing, the contractile status of airway smooth muscle (ASM) may be an important determinant in the susceptibility of exacerbation. ASM is likely phenotypically regulated, existing in either a proliferative or a contractile state. Epithelial cells are able to drive the proliferation of ASM cells and thus we hypothesized that the epithelium may reduce the contractile phenotype of these cells. We utilized *in-vitro* co-cultures of primary human ASM cells with epithelial cells deriving from primary bronchial epithelium or the cell line BEAS-2B. After co-culture or incubation with conditioned medium from epithelial cultures, we examined three markers of the contractile phenotype. We measured force production by traction microscopy, gene and protein expression by qPCR and western blot and calcium release by Fura-2 ratiometric imaging. After incubation with epithelial derived medium, we observed less force production after histamine stimulation than ASM cells that received control medium. There was a reduced expression of mRNA of myocardin, the master regulator of contractile apparatus proteins. Furthermore, we observed less α-smooth muscle actin mRNA and protein as well as reduced calponin mRNA. Finally, there was diminished peak calcium release upon histamine regulation that depended on cyclooxygenase-1 (COX-1) activity in ASM cells. Here we demonstrate the epithelial cells reduce the contractile phenotype of ASM cells *in-vitro*. The reduced peak calcium release is not transcriptionally regulated by altered calcium handling constructs, but rather depends on ASM cell COX-1 products. This demonstrates that epithelial derived relaxing factor may, in part, come from autocrine production of AMS cell products.

3.3 Introduction

Asthma is a chronic disease of which airway wall of which airway wall remodeling is a cardinal feature [436,437]. Excessive airway narrowing is mediated by airway smooth muscle (ASM) and motivates the treatment of asthma with β-adrenergic receptor agonists to relieve the tone generated by ASM contraction. A prominent element of the histopathology of asthma is increased mass of ASM [438]. How ASM mediates excessive airway narrowing remains controversial and consequently, mechanisms regulating ASM cell proliferative and contractile status are of great interest. One such mechanism involves the stimulation of proliferation by mediators derived from the nearby epithelial tissue [383]. The airway epithelium has been implicated as an important driving force of asthma pathogenesis [439] through the secretion of many pro-remodelling factors such as chemokines [440] and growth factors [374]. Maturation of smooth muscle generates cells expressing proteins of the contractile apparatus [441]. This differentiation is the result of nuclear translocation of the co-transcription factor myocardin, which acts by binding to the transcription factor serum response factor (SRF) [442,443]. Myocardin competes for SRF binding with the pro-proliferative co-transcription factors Elk-1 and KLF4[267,444]. Smooth muscle cells that are actively proliferating down-regulate proteins of the contractile apparatus, such as α-smooth muscle actin (αSMA) and calponin [445]. ASM cells that are co-cultured with airway epithelial cells were previously shown to increase the rate of proliferation of the muscle [383]. Furthermore, in *ex-vivo* tissue assays, it has been demonstrated that the removal of the epithelium increases the responsiveness of isolated bronchi [356,446]. We therefore hypothesized that ASM cells lose the contractile phenotype when cultured with epithelial cells.

Due to the importance of ASM cell force generation in mediating airway narrowing, we set out to explore phenotypic regulation in the context of its ability to create tension *in-vitro*. It is well established that calcium ions play a critical role in regulating the initiation of cross bridge cycling. Therefore, we explored the role of epithelial co-culture on calcium release within ASM cells. To induce calcium transients, the agonist histamine was utilized due to its relevance in driving asthma exacerbations [447,448]. Intracellular calcium concentration is tightly regulated and the release of this ion upon histamine stimulation can depend on proteins regulating histamine receptor signal transduction [318], calcium handling proteins, or other mechanisms such as cAMP generation within the cell [449]. PGE_2 is known to induce cAMP in ASM cells [450]. Here, we examined the effect of epithelial-derived mediators on histamine induced calcium release and contraction of ASM cells. We questioned whether or not ASM cells release factors associated with relaxation in an autocrine manner after co-culture with epithelial cells.

3.4 Materials and Methods

Reagents

Histamine dihydrochloride (1μM), Collagenase type IV from *Clostridium histolyticum*, general COX inhibitor indomethacin (3μM) and the COX-2 specific inhibitor celecoxib (100nM) were obtained from Sigma-Aldrich (St. Louis, MI, USA). The specific COX-1 inhibitor SC560 (100nM) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Fura 2-AM (10μM), Pluronic F-127 (0.02%) and qPCR primers were obtained from Thermo Fisher Scientific (Waltham, MA, USA).

Cell Culture

Primary human ASM cells were cultured from transplant grade lungs procured by the International Institute for the Advancement of Medicine. Protocols were approved by an Institutional Review Board. Dissected ASM tissue was digested overnight in collagenase (0.4mg/ml) dissolved in Dulbecco's Modified Eagle's Medium (DMEM) containing streptomycin, penicillin and amphotericin B (Anti-Anti) (Thermo Fisher Scientific). The next day, the tissue was suspended in the digestion medium by gentle agitation with a serological pipette. Cells were maintained in DMEM supplemented with Anti-Anti and 10% fetal bovine serum (FBS)(growth medium)(Thermo Fisher Scientific). Medium was replaced every second day. For long term storage, cells were cryo-stored in 90% FBS:10% dimethyl sulfoxide (DMSO) in liquid nitrogen. Prior to all experiments, medium was changed to DMEM supplemented with 0.5% FBS containing Anti-Anti overnight prior to epithelial co-culture or stimulation with conditioned medium. Cells were studied between passages 2 to 6.

Normal human bronchial epithelial (NHBE) cells were also obtained from lungs of donors. Micro-dissected tissue was digested overnight in Pronase (1.5mg/ml) (Sigma Aldrich) dissolved

in DMEM containing Anti-Anti. The following day, tissues were rinsed with Ham's F12 medium (Thermo Fisher) supplemented with 20% FBS and Anti-Anti. After 3 washes, undigested tissue was discarded. Cell suspensions were pooled, and centrifuged. Resulting cells were cultured in Bronchial Epithelial Growth Medium (BEGM) (Lonza, Basel, Switzerland) containing Anti-Anti and medium was changed every other day. Cells were cryo-stored in freezing medium and studied until passage 5. Prior to co-culture or medium conditioning, cells were deprived of growth factors in 50:50 medium containing DMEM 0.5%FBS Anti-Anti with Bronchial Epithelial Basal Medium (BEBM) (Lonza) containing Anti-Anti. In other experiments, the bronchial epithelial cell line BEAS-2B was utilized as a representative model of primary cells. For these experiments, BEAS-2B cells were maintained similarly to ASM cells, and serum deprived in DMEM containing 0.5% FBS with Anti-Anti.

For co-culture experiments, Transwell® permeable supports (Corning, Corning, NY, USA) were utilized. Confluent epithelial cultures were serum deprived for 24 hours prior to co-culture with ASM cells. For conditioned medium experiments, supernatant from serum deprived confluent cultures was collected after 24 hours of conditioning with fresh starvation medium (0.5% FBS, Anti-Anti, DMEM), centrifuged at 1500 RPM for 5 mins and stored at -80ºC.

RT-qPCR

ASM cells were seeded at a density of 100 000 cells per well in 6 well plates in growth medium. The next day, ASM cells were serum deprived for 24 hours in starvation medium after which they were placed in co-culture with BEAS-2B cells. After 24 hours of co-culture, ASM cells were washed once with PBS (Thermo Fisher Scientific) and mRNA was extracted using an RNeasy mini-kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed on 100ng of total RNA with AffinityScript qPCR cDNA synthesis kit (Agilent Technologies, Santa Clara,

CA, USA). qPCR was performed using iTaq SYBR green supermix (Bio-Rad Laboratories, Hercules, CA, USA). Primer sequences are reported in Table 1. Amplification of cDNA was performed using a StepOnePlus realtime PCR system (Applied Biosystems, Foster City, CA, USA). Relative mRNA expression was calculated using the ΔΔCt method and all gene expression was normalized to S9.

Gene Array

mRNA was submitted to Genome Quebec for analysis by Illumina HT-12 Expression BeadChip Kit (Illumina, San Diego, CA, USA). Data was analyzed by CyberT followed by Benjamani-Hochberg false discovery rate P value correction for multiple comparisons.

PGE_2 ELISA

After co-culture, supernatant was collected and centrifuged at 1500 RPM for 5 minutes at 4^{o}C to remove any cell debris. Samples were analyzed using DetectX Prostaglandin E_2 Enzyme Immunoassay Kit (Arbor Assays, Ann-Arbor, MI, USA).

cAMP Assay

After stimulation of ASM cells for 24 hours with BEAS-2B conditioned medium, ASM cells were washed with ice-cold PBS, lysed and assayed for intracellular cAMP concentration as per manufacturer's protocol (Cyclic AMP XP Assay Kit, Cell Signaling Technology, Danvers, MA, USA). To prevent the degradation of cAMP, cells were incubated with 0.5mM 3-isobutyl-1-methylxanthine (IBMX) during the 24 hours of conditioned medium stimulation. Lysis buffer was also supplemented with 0.5mM IBMX.

Western Blot

After co-culture, AMSCs were washed with ice cold PBS, and protein extraction was performed using protein lysis buffer containing 50mM TrisHCl (pH 8), 150mM NaCl, 1% NP-40, 0.5%

sodium deoxycholate and 0.1% SDS. The lysis buffer was supplemented with protease inhibitor cocktail (Sigma-Aldrich). Upon mechanical disruption, cell lysates were centrifuged at 13 000 RPM for 3 minutes and total protein within the supernatant was measured by Quick Start Bradford Protein Assay (Bio-Rad). 20µg of protein diluted in double distilled water was loaded per lane into a separating gel. After separation, protein was transferred to a PVDF membrane (Bio-Rad). Membranes were blocked with 5% bovine serum albumin (Sigma-Aldrich) for one hour at room temperature prior to antibody incubation overnight at 4ºC. Primary antibodies included anti αSMA (1A4, Sigma-Aldrich, 1:1000) and anti GAPDH (6C5, EMD Millipore, Billerica, MA, USA, 1:3000). Membranes were washed three times in Tris-buffered saline solution containing 0.1% TWEEN 20 (TBS-T). Membranes were incubated with secondary antibodies for one hour and room temperature. Membranes were washed 3 more times in TBS-T and then once with TBS prior to development with chemiluminescent techniques (ECL, Bio-Rad, Hercules, CA, USA) and imaging.

Measurement of Intracellular Calcium

Human ASM cells were seeded on #2 glass cover slips and cultured in growth medium for 24 hours. Medium was changed for starvation medium, after which the cells were co-cultured or stimulated with epithelial cells. For COX inhibitor experiments, ASM cells were treated with inhibitor during the starvation protocol and for the duration of conditioned medium stimulation. ASM cells were washed with Hanks Balanced Salt Solution (HBSS) (Thermo Fisher Scientific), and loaded with 10µM Fura 2-AM containing 0.1% Pluronic F-127 (Thermo Fisher Scientific) for 30 minutes. Cells were washed twice with fresh HBSS and after 15 minutes, coverslips were transferred to a Leiden chamber (Medical Systems, Greenville, NY, USA) and imaged using an Olympus IX71 inverted fluorescent microscope equipped with a 40X oil immersion objective

(Olympus, Tokyo, Japan). In some experiments, PGE_2 or its vehicle (0.1%DMSO) was added to the ASM cells during the 15 minute Fura 2-AM washout and to the Leiden chamber during the recording. Intracellular calcium concentration was measured as previously described [409]. Briefly, cells were illuminated using a DeltaRAM (Horiba Scientific, Kyoto, Japan) stimulating with 340nm and 380 nm alternatively. Emission at 510nm was measured through a CCD camera (CoolSnapPro, Media Cybernetics, Bethesda, MD) controlled with Image Master software (Photon Technology International, Birmingham, NJ, USA). 340/380 fluorescence intensity ratios were converted to calcium concentrations as previously describe using Grynkiewicz' equation [451].

Traction Microscopy

Cells were seeded at a density of 8,000 cells per well on collagen coated acrylamide (Young's modulus: 8kPa) gels in 96 well plates as previously described [452]. After 24 hours, ASM cells were serum deprived in 50:50 medium for 24 hours. Fresh starvation medium or starvation medium that had been incubated with HBE cells was added to the ASM cells for 24 hours after which baseline traction was recorded. Medium was replaced with HBSS for 1 hour and then cells were stimulated with 1μM histamine for 1 hour prior to measuring traction. Data are presented as a fold change in root mean square traction between baseline and post-histamine treatment..

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). Data are presented as mean +1SE with ≥4 independent experiments. Independent experiments were considered ASM cells deriving from different patients, or from different passage numbers measured on a different day. In each experiment, a minimum of three independent patient's ASM cells were studied. In experiments where >2 groups were compared,

one-way ANOVA with Tukey's post-hoc test was utilized. For experiments where only two groups were compared, a paired Student's T-test was employed. P values <0.05 were considered to be significant. Calcium data were analyzed for normality by Smirnov-Kolmogorov test and 16 of 18 groups were non-normally distributed. Calcium data were therefore compared with non-parametric Mann-Whitney or Kruskal Wallis with Dunn's post-hoc test as appropriate. Data are presented as box plots showing medians and with whiskers showing group maxima and minima.

Table 1

Primer sequences for qPCR ractions:

Gene	Forward Primer	Reverse Primer
S9	CTGCTGACGCTTGATGAGAA	CAGCTTCATCTTGCCCTCA
CNN1	AGCAGGAGCTGAGAGAGTGG	AAAGCCAGGAGGGTGGACTG
ACTA2	TCATGATGCTGTTGTAGGTGGT	CTCTTCCAGCCATCCTTCAT
MYOCD	TCAGCAATTTCAGAGGTAACACA	TGACTCCGGGTCATTTGC
MYLK	TGGGGCTCTTATGACCTACAGT	CCTGAAGTTGCTCTGAACTGC
ITPR1	CCTTTTCCGTTTCAAGCATC	AGGCATTCTTCCTCAAAGTCAG
H1R	AAGTCACCATCCCAAACCCCCAAG	TCAGGCCCTGCTCATCTGTCTTGA
SERCA	TCGAACCCTTGCCAGTAAGT	CACACAGGGAAGACGTCTCA
CD38	CAGCAACAACCCTGTTTCAGT	CCATTGAGCATCACATGGAC
PLC-β	TCCAAGAAGAAGTGGCCAAG	ATGCATCCCTGGACATGTTT
PTGS2	CTTCACGCATCAGTTTTTC	TCACCGTAAATATGATTTAAGTCCAC
PTGES	CGCTGCTGGTCATCAAGA	TCCGTGTCTCAGGGATC

3.5 Results

Airway epithelial cells reduce histamine-induced contraction

To examine the force producing ability of ASM cells *in vitro,* we stimulated the cells with 1μM histamine. AMSCs that had been treated with conditioned medium derived from NHBE cells for 24 hours demonstrated less force-generation than those that had been incubated with control starvation medium (Fig 1).

Gene expression of contractile apparatus proteins is altered in epithelial co-cultured ASM cells

To further investigate this force reduction after co-culture with epithelial cells, we explored a variety of proteins associated with the contractile phenotype. Myocardin, the master regulator of contractile apparatus proteins [453], had reduced transcript expression after co-culture with BEAS-2B cells (Fig 2A). We examined downstream targets of myocardin, observing a reduction in mRNA of αSMA (Fig 2B), calponin (Fig 2C) and a downward trend of myosin light chain kinase (Fig 2D). Primer sequences used to probe gene expression are presented in Table 1. Furthermore, αSMA protein was reduced after 96 hours of co-culture with BEAS-2B cells (Fig 2E). These results further confirm a phenotypic change of ASM cells after epithelial co-culture.

Co-culture reduces agonist induced calcium release

Since intracellular calcium release is an important feature of the initiation of cross-bridge cycling in ASM cells, we examined the effect of epithelial co-culture on histamine induced calcium release. After 24 hours of co-culture with either BEAS-2B (Fig 3A) or NHBE cells (Fig 3B), peak calcium responses to stimulation with 1μM histamine were diminished.

Calcium release is not transcriptionally regulated

Calcium release may be regulated transcriptionally via calcium handling proteins. We hypothesized that ASM cells co-cultured with epithelial cells would have altered transcriptional

regulation of the mRNA of these enzymes. However, we observed no reduction in the expression of mRNA of the histamine receptor (H_1R) (Fig 4A), in the calcium handling proteins phospholipase C-β (*PLCβ*)(Fig 4B), inositol trisphophate receptor (*IP_3R*)(Fig 4C), or an increase in the expression of the calcium reducing sarcoplasmic reticulum Ca^{2+}-ATPase pump (*SERCA*)(Fig 4D). There was an increase in cyclic ADP ribose hydrolase (*CD38*)(Fig 4E) mRNA after co-culture, a finding which cannot explain the reduction in intracellular calcium release as CD38 is associated with increased activation of the calcium releasing ryanodine receptor [319]. These data indicate that the reduction in histamine-stimulated calcium release is not transcriptionally regulated by calcium handling proteins.

Prostaglandin E_2 diminishes agonist-induced calcium release in ASM cells

Since the calcium reduction by co-culture with epithelial cells was not dependent on calcium handling proteins, we explored the role of arachidonic acid metabolites. Prostanoids are capable of generating cAMP within ASM cells that can be associated with both reduced calcium and diminished tone. Gene array data indicated an increase in the PGE_2 producing enzymes cyclooxygenase-2 (COX-2) (Fig 5A) and membrane-associate prostaglandin E synthase-1(mPGES-1)(Fig 5B) after co-culture with BEAS-2B cells. To confirm these gene array findings, PCR was conducted on ASM cells co-cultured with BEAS-2B cells, which also demonstrated increased expression of these arachidonic acid metabolizing enzymes (Fig 5C-D). Analysis of secreted PGE_2 into the cell culture medium demonstrated that co-cultured ASM cells generate more PGE_2 than those that were not co-cultured (Fig 5E). The cell culture supernatant from BEAS-2B cells alone did not produce detectable quantities of PGE_2. Furthermore, incubation of ASM cells for 24 hours with BEAS-2B conditioned medium increased the concentration of intracellular cAMP within the ASM cells (Fig 5F). To examine the role of PGE_2 in regulating

ASM cell calcium responses to histamine stimulation, we pre-treated the cells with 10μM PGE_2 prior to stimulation with histamine. Preatment of ASM cells with PGE_2 diminished the agonist induced peak calcium concentration (Fig 5 G). These data indicate that epithelial cells cause ASM cells to up-regulate enzymes associated with PGE_2 synthesis as well as release this lipid mediator and its downstream effector molecule, cAMP.

Reduction in calcium release by epithelial cells is dependent on cyclooxygenase-1

ASM cells were pre-treated for 24 hours with the non-selective COX inhibitor indomethacin. COX inhibition restored the reduction in agonist-induced peak calcium release caused by BEAS-2B cell conditioned medium, indicating the role of a COX metabolite such as PGE_2 in reducing ASM cell excitability (Fig 6 A). To prevent the inhibition of COX within the epithelium, we utilized conditioned medium from epithelial cells rather than co-culture. This allowed the drug treatment to inhibit its target within the ASM cell alone. Next, we examined the inducible COX isoform, COX-2, by inhibition with the selective COX-2 inhibitor celecoxib. Pre-treatment of ASM cells with celecoxib did not restore the excitability after incubation with BEAS-2B conditioned medium (Fig 6 B). Since indomethacin restored excitability after incubation with conditioned medium, we hypothesized that this effect must be mediated by the non-inducible COX isoform, COX-1. Treatment with the COX-1 specific inhibitor SC560 restored ASM cell excitability after treatment with conditioned medium of BEAS-2B cells (Fig 6 C). These data indicate that airway epithelial cell-dependent reductions in ASM cell calcium release after agonist stimulation are mediated by ASM cell specific COX-1 products.

Figure 1

Airway epithelial cells reduce histamine-induced contraction. ASM cells were stimulated to ASM cells were stimulated to contract with 1μM histamine or vehicle (HBSS). Control cells were incubated for 24 hours with 50:50 medium that had not been conditioned with epithelial cells. Conditioned medium (C.M.) of NHBE cells was utilized to deliver epithelial derived mediators to the ASM cells. Average contractile forces of confluent ASM cells were measured using Traction Microscopy. Relative forces were defined as the fold change between baseline and post-histamine treatment. Data are presented as means + SE. ANOVA with Tukey post hoc pairwise comparisons were conducted and p values are reported.

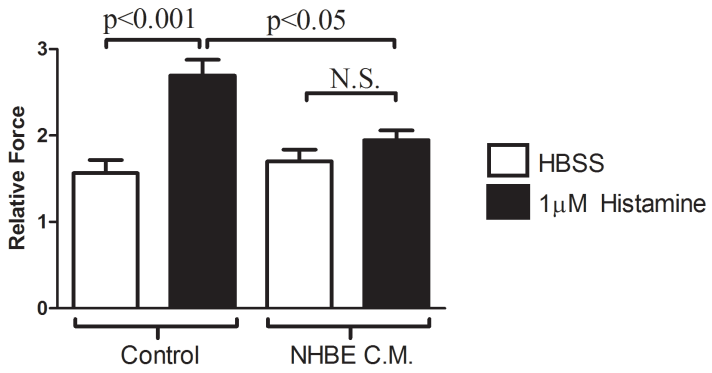
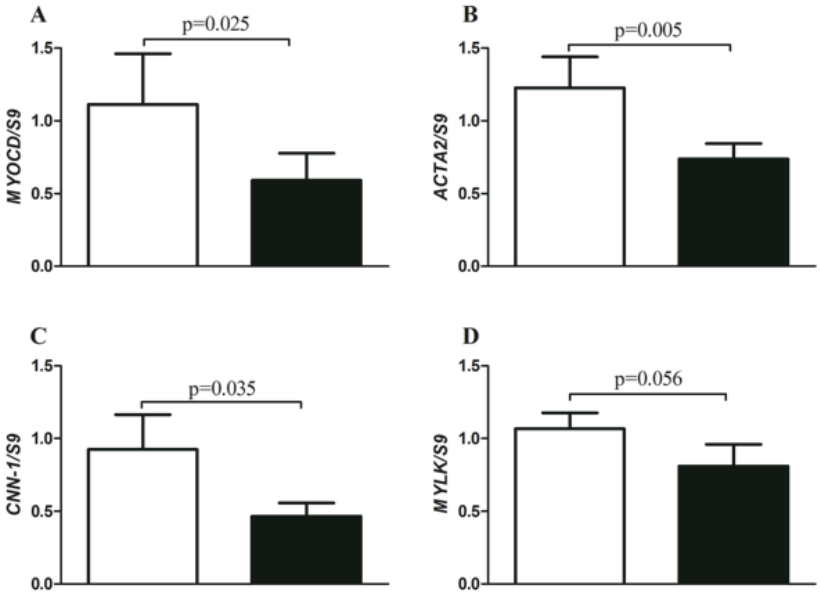


Figure 2

Gene expression of contractile apparatus proteins is altered in epithelial co-cultured ASM cells. ASM cells were co-cultured with BEAS-2B cells for 24 hours. mRNA was extracted to perform RT-qPCR examining myocardin (*MYOCD*)(A), α-smooth muscle actin (*ACTA2*)(B), calponin (*CNN-1*)(C) and myosin light chain kinase (*MYLK*). White bars = control ASM cells, black bars = co-cultured ASM cells. Protein lysate was separated in a poly-acrylamide gel before transfer to PVDF membrane and blotted for αSMA protein (E). Pixel densitometry (F) of αSMA blotting. Data are presented as means + SE. Student's paried t-test was used to compare samples with p values reported above the bars.



E

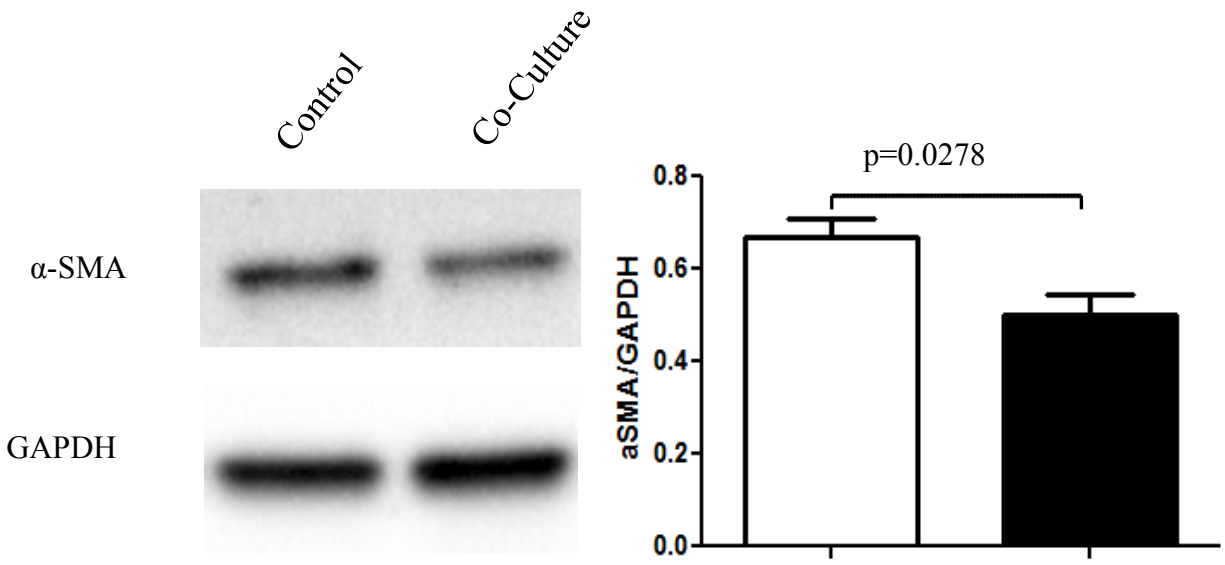

Control
Co-Culture
α-SMA
GAPDH
p=0.0278
aSMA/GAPDH
0.8
0.6
0.4
0.2
0.0

Figure 3

Co-culture reduces agonist induced calcium release. ASM cells were cultured with BEAS-2B (A) or NHBE (B) cells for 24 hours prior to Fura-2 AM calcium imaging. Peak calcium release after 1μM histamine stimulation is reported. Data are presented as medians +/- maxima and minima. (A) Control=72 cells across 5 donors Co-Culture=128 cells across 5 donors, (B) Control=34 cells across 4 donors, Co-Culture=36 cells across 4 donors. Student's paried t-test was used to compare samples with p values reported above the bars.

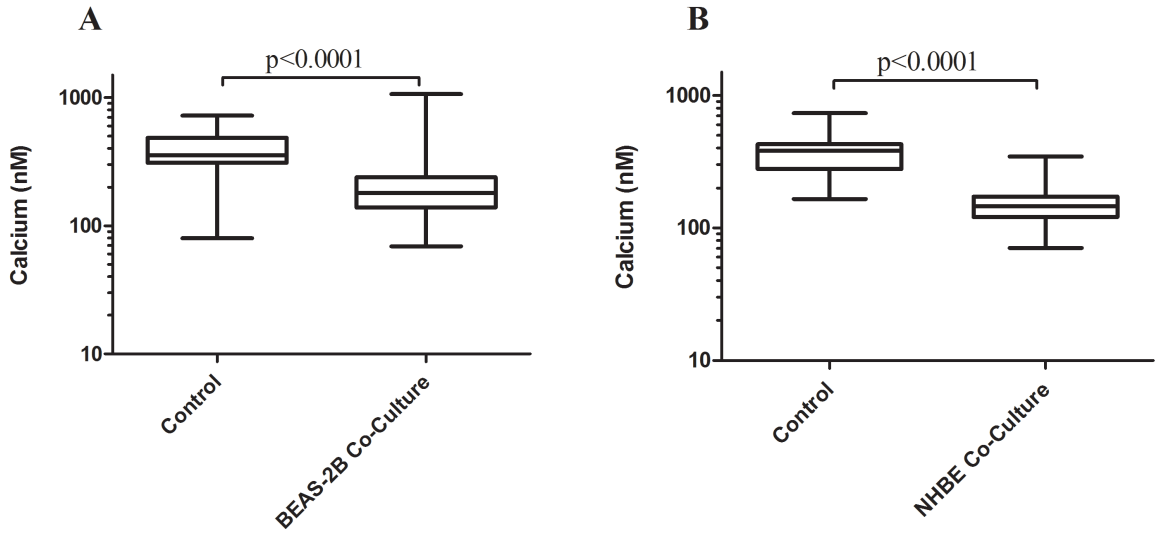


Figure 4

Calcium release is not transcriptionally regulated. ASM cells were co-cultured with BEAS-2B cells for 24 hours. mRNA was extracted to perform RT-qPCR examining histamine receptor (*H1R*)(A), phospholipase C-β (*PLCβ*)(B), inositol trisphosphate receptor 1 (*ITPR1*)(C), sarco/endoplasmic reticulum Ca^{2+}-ATPase (*SERCA*)(D) and cyclic ADP ribose hydrolase (*CD38*)(E). White bars = control ASM cells, black bars = co-cultured ASM cells. Data are presented as means +SE. Student's paired t-test was used to compare samples with p values reported above the bars.

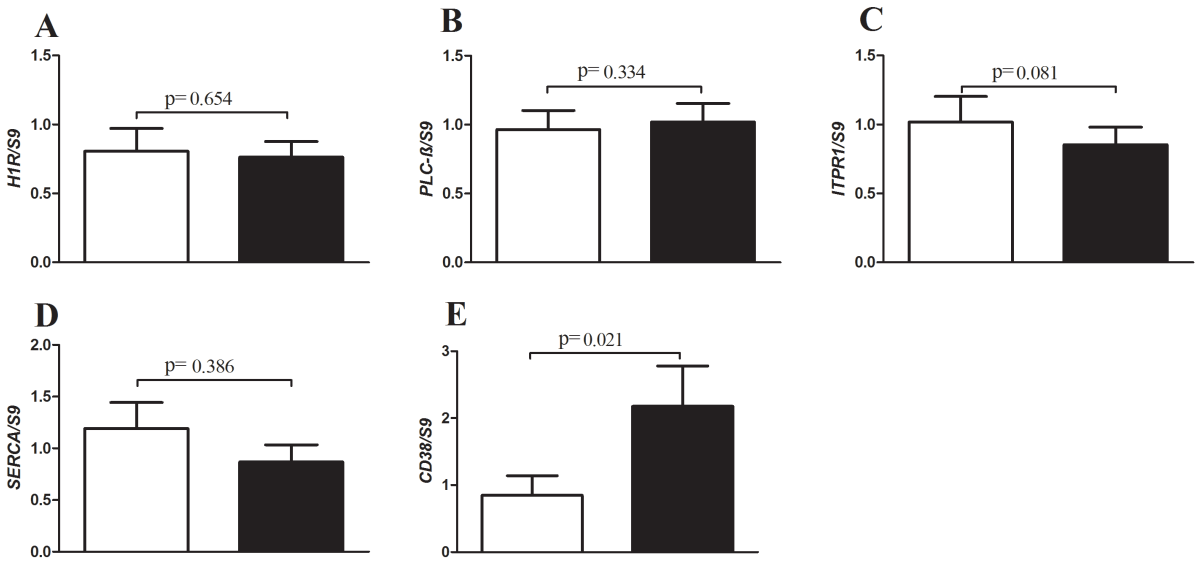


Figure 5

Prostaglandin E_2 diminishes agonist-induced calcium release in ASM cells.

ASM cells were co-cultured with BEAS-2B cells for 24 hours. Illumina HT-12 gene array was performed and raw signal intensity is plotted for COX-2 (*PTGS-2*) (A) and mPGES-1 (*PTGES*)(B). P values are adjusted for multiple comparisons (number of trancripts = 34 695). RT-qPCR was performed on the samples to confirm the increase (C-D). Cell culture supernatant was analyzed by enzyme immunoassay for PGE_2 concentration from ASM cells alone or those that had been co-cultured with BEAS-2B cells for 24 hours (E). ASM cells were treated with BEAS-2B conditioned medium for 24 hours prior to measurement of intracellular cAMP (F). ASM cells were incubated with 10 μM PGE_2 or vehicle (0.1% DMSO) for 15 minutes prior to stimulation with 1μM histamine to induce intracellular calcium release (G). Data are presented as means +SE. Student's paired t-test was used to compare samples and p values are reported above the bars. Calcium data are presented as medians with whiskers showing group maxima and minima and the Mann-Whitney test was utilized.

(Figure on subsequent page):

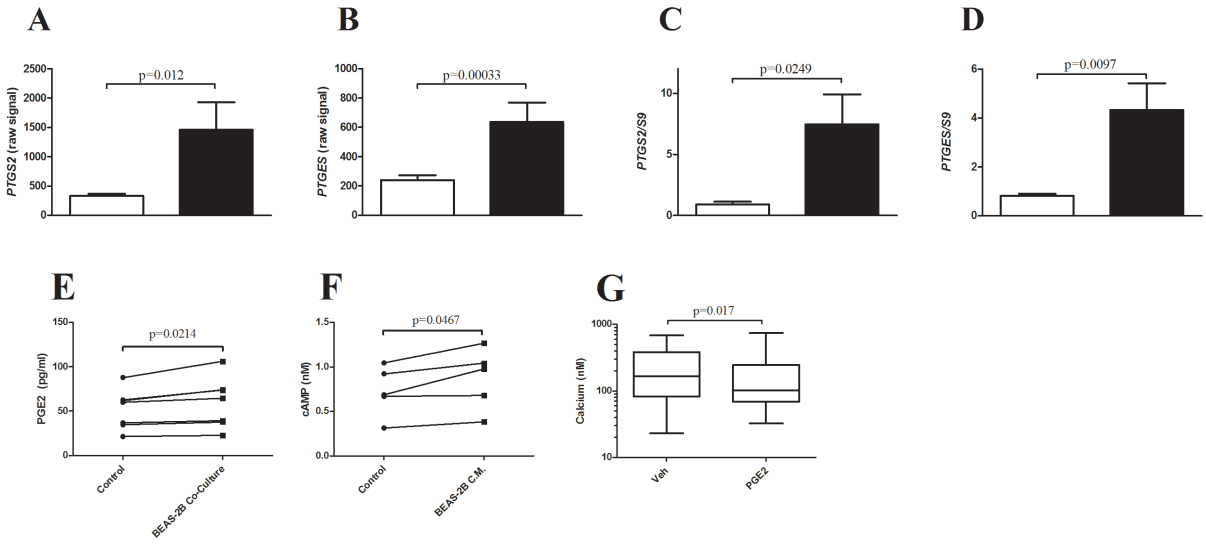
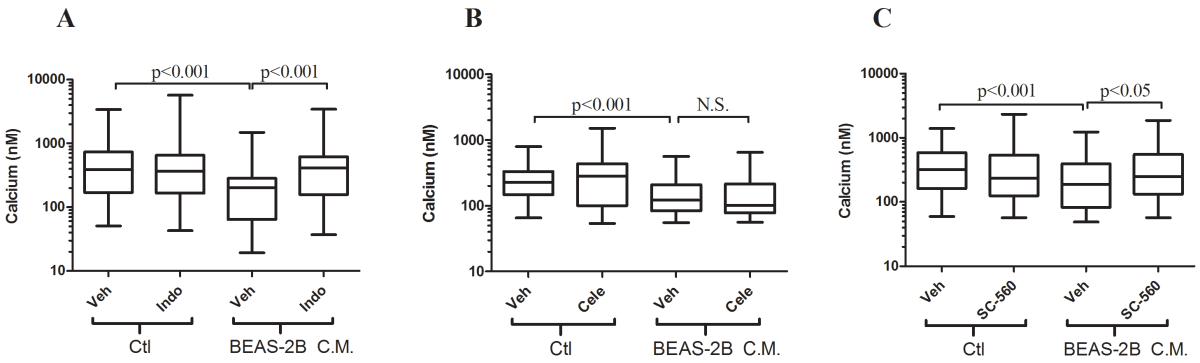

A
B
C
D
p=0.012
p=0.00033
p=0.0249
p=0.0097
PTGS2 (raw signal)
PTGES (raw signal)
PTGS2/S9
PTGES/S9
E
F
G
p=0.0214
p=0.0467
p=0.017
PGE2 (pg/ml)
cAMP (nM)
Calcium (nM)
Control
BEAS-2B Co-Culture
Control
BEAS-2B C.M.
Veh
PGE2

Figure 6

Reduction in calcium release by epithelial cells is dependent on ASM cell cyclooxygenase-1. ASM cells were pre-treated for 24 hours with vehicle (0.1%DMSO) or inhibitors indomethacin (A), Celecoxib (B) or SC560 (C). Cells were then treated with conditioned medium from BEAS-2B cells with respective drug or vehicle present for 24 hours. Cells were loaded with 10 μM Fura 2-AM and peak intracellular calcium release in response to 1μM histamine was measured. Data are presented as medians with whiskers showing group maxima and minima and comparisons were made with the Kruskall Wallis and Dunn's post-hoc test with p values reported. Calcium was performed on 70-157 cells per group across 4-10 donors.



3.6 Discussion

ASM contraction contributes directly to airway narrowing and therefore mediates exacerbations of asthma. The purpose of this study was to examine the role of airway epithelial cells in modulating ASM cell phenotype. Due to the importance of ASM in mediating asthmatic exacerbations and the putative role of the epithelium in asthma [454], it is important to understand mechanisms by which the properties of ASM cells are altered by epithelial mediators. Our results demonstrate a potent down-regulation of calcium signals in ASM cells stimulated with histamine when co-cultured with epithelium. Furthermore, we identified COX-1 as a regulator of the loss of ASM cell excitability after co-culture with epithelial cells. PGE_2 increased in the medium of co-cultured cells and exogenous PGE_2 reduced histamine-induced calcium transients, suggesting that it may mediate the reduction in calcium signals attributable to co-culture of AMSC with epithelium.

Although asthma is often considered an epithelial disease, rather than augmenting the excitability of ASM cells, the epithelium reduced its responses to stimulation. The idea of an epithelial derived relaxing factor is not a novel concept. Others have shown that tissue preparations contract more when the airway is denuded of its epithelial layers [356]. Much of this work followed the discovery of endothelial derived nitric oxide and its role in vascular tone [455]. However, in the airway epithelial studies, it is difficult to separate the mechanical resistance to airway narrowing that the epithelium creates for smooth muscle force generation. The present study confirms that epithelial cells have the ability to reduce the contractile phenotype *in vitro*, and we present data suggesting that smooth muscle is the source of the products that exercise this relaxing function. While others have explored the concept of a direct

effect of an epithelial-derived relaxing factor on ASM, our data suggest that the epithelium secretes factors influencing the contractile phenotype of ASM and that the relaxing factor could be ASM-derived and acting in an autocrine fashion.

We explored the effect of epithelial mediators on ASM cell contractions using a traction microscopy as previously described [452]. The assay, which is based on measuring the displacement of elastic substrates by cellular contractile force, demonstrated a reduction in histamine-induced force generation by the ASM cells when treated with medium derived from primary NHBE cells. The effect of NHBE conditioned medium on baseline traction was negligible in our studies, however it may be of interest to examine the effect that medium derived from diseased NHBE cells has on both baseline and stimulated ASM cells.

The expression of contractile apparatus genes was also diminished after co-culture with epithelial cells. Myocardin is a previously described co-transcription factor and driver of the contractile phenotype in ASM cells [453]. In equine asthma, myocardin nuclear staining in ASM cells was associated with the disease [264]. Here we observe decreased expression of this gene, as well as myocardin dependent genes αSMA and calponin. Furthermore, αSMA protein content was diminished after co-culture with epithelial cells and the reduced expression of these genes may play a role in the lack of force production upon histamine stimulation.

We observed also that both BEAS-2B and NHBE cells reduced the excitability of ASM cells, as reflected in peak calcium release, when stimulated by histamine. Although the modulation of force can be mediated through a variety of pathways [330,456], the concentration of intracellular

calcium is considered to be coupled to the magnitude of force production in smooth muscle cells [457]. Due to reduced intracellular calcium release by agonist stimulation after co-culture, we anticipated a possible reduction in calcium handling proteins, which we hypothesized would have mediated this effect. However, we found no transcriptional change in several key enzymes, leading us to explore alternative mechanisms of smooth muscle cell relaxation. A transcriptomic analysis demonstrated an increase in expression of the eicosanoid producing enzymes COX-2 and mPGES-1. We therefore focussed on the potential role of eicosanoids as causes of functional antagonism of calcium release of ASM cells.

Human ASM cells have been shown to express $EP_{2\text{-}4}$ [458] and it is established that PGE_2 can induce relaxation of this tissue [459,460]. Furthermore, EP_2 has been demonstrated to mediate PGE_2 induced relaxation of ASM [461,462]. PGE_2 was recently shown to inhibit the transcription and protein expression of αSMA during myofibroblast differentiation through a reduction in SRF expression [463], which adds further evidence supporting the relaxing effect of this prostanoid. PGE_2 also increases cAMP production within these cells [464,465], a molecule that has been described to target the IP3R in ASM cells thereby causing a reduction in calcium mobilization [466]. β-agonist treatment for asthma exacerbation relies on GPCR generation of cAMP induced calcium inhibition. cAMP can also activate PKA in these cells, and may further reduce the contractile phenotype through calcium sensitization of the contractile apparatus [467]. Roscioni and colleagues recently reported that PKA regulates phenotype switching in ASM cells and it may play a role in modulating proliferation [468].

Besides exploring the molecular physiology of PGE_2 mediated effects on ASM cells, there have been clinical investigations examining the efficacy of this prostanoid as a therapeutic agent. The fall in FEV1 by allergen challenge in asthmatic patients was prevented by inhalation of PGE_2 prior to challenge [469]. Another study demonstrated that inhalation of this prostanoid can cause an initial bronchoconstriction, followed by potent dilation in healthy control subjects and the authors describe that this effect depends on the initial tone of the tissue, where previously dilated airways were less responsive the relaxation phase induced by the prostanoid [470]. In our present study, the ASM cells were not previously relaxed and thus the cells should be expected to respond to the relaxing effect of PGE_2. In the rat, PGE_2 has been shown to reduce cysteinyl-leukotriene production and Th2 activation after allergen challenge, demonstrating a role of this molecule as an immunomodulatory agent for the treatment of allergic asthma [471]. It was proposed by Delamere et al. that PGE_2 produced by bovine ASM cells could act as a regulatory mechanism to control airway inflammation [472]. Although the observed increase in PGE_2 release was modest, it is known that the half-life of this prostanoid is on the order of seconds [473]. 15-PGDH degrades PGE_2 in the airway [474] and we may observe larger increases in PGE_2 release in cells lacking this enzyme.

Here, we demonstrate that reduced excitability of ASM cells due to treatment with epithelial conditioned medium can be restored by inhibition of ASM cell specific COX-1, an enzyme known to produce PGE_2. More specifically, this constitutively expressed protein has been implicated in generating PGE_2 in human ASM cells [475]. Recently, it was demonstrated that COX-1 products mediate the relaxing effect of glucagon in tracheal tissue preparations [476]. Similarly, selective COX-1 inhibition prevented tracheal ring relaxation of murine airways by

proteinase-activated receptor-2 agonism [477]. Furthermore, The selective inhibitor of COX-1, SC-560 augmented histamine triggered tracheal ring contractions [478]. However, inhibition of COX-1 has also been shown to reduce the stretch-induced contraction of ASM [479] and therefore the outcome of blockade of this molecule may depend on the stimulus studied. Our data provide further evidence that SC-560 increases the responsiveness of ASM to a contractile agonist. Although the inducible isoform of COX-2 is often described to modulate inflammatory processes, others have provided evidence supporting a role of COX-1 in driving asthmatic responses [480]. Future exploration of this enzyme as a therapeutic target will be of great interest.

This study shows that the epithelium modulates ASM cells away from the contractile phenotype, a phenomena that likely accompanies the previously described increase in the proliferative phenotype [383]. The reduced excitability of these cells is not transcriptionally regulated, but rather depends on COX-1 products. We observed a transcriptional diminution in myocardin, the master regulator of transcriptional apparatus proteins that further represents a down-regulation of the contractile phenotype. We also observed that epithelial cells reduce transcripts of contractile apparatus genes as well as reduce α-SMA protein upon four days of co-culture. There are thus broad effects of epithelial-derived mediators on ASM phenotypic regulation. The exploration of this interaction will be important to extend to the examination of cells derived from asthmatic patients as these cells retain significant differences in properties ex vivo. ASM cells are therefore capable of producing PGE_2, a prostanoid capable of reducing peak calcium responses to histamine after co-culture with epithelial cells. This reduced excitability is mirrored by a

reduction in gene expression of contractile apparatus transcripts as well as diminished force production after agonist stimulation.

CHAPTER 4:

Airway Epithelial Cells Increase Airway Smooth Muscle Cell Proliferation

4.1 Prologue

Due to the reduced contractility associated with co-culture of ASM cells with epithelial cells, we wished to explore the proliferative phenotype, hypothesizing an increase rate of proliferation in co-cultured ASM cells. We continued to utilize the BEAS-2B:ASM cell co-culture model, and examined the molecular mechanisms by which ASM cells are stimulated to proliferate by co-culture. We again probed the importance of the EGFR in mediating this effect and we explored the role of miRNA in this process. We examined phenotypic changes to the ASM cells after co-culture through gene and miRNA arrays and uncovered novel therapeutic targets for the inhibition of ASM cell proliferation.

Hypothesis: Epithelial co-culture induces the proliferative phenotype through EGFR signalling and miRNA modulation.

4.2 Abstract

Increased airway smooth muscle (ASM) mass in the airways of asthmatic patients may contribute to the pathobiology of this disease through its role in modulating airway caliber. The airway epithelium has a potential role in ASM remodeling. To investigate mechanisms by which airway epithelial cells induce ASM cell proliferation, we have employed a co-culture model, exploring markers of ASM proliferative phenotype. ASM incorporated increased quantities of the thymidine analogue bromodeoxyuridine (BrdU), indicating augmented proliferation and these cells expressed increased mRNA of the pro-proliferative co-transcription factor Elk1. Although the mitogen heparin-binding epidermal growth factor (HB-EGF) was augmented in ASM cells that had been co-cultured with BEAS-2B cells, the epidermal growth factor receptor (EGFR) did not mediate epithelial-induced proliferation. Within this ASM cells, co-culture increased the expression of mRNA for the pro-inflammatory cytokines IL-6 and IL-8 as well as the pro-proliferative micro-RNA miR-210. The transcriptional repressor Max-binding protein (Mnt), a putative target of miR-210, was transcriptionally repressed in co-cultured ASM cells. Together, these data indicate that the airway epithelium induced the proliferative phenotype within ASM cells is not driven by EGFR signalling, but rather may be dependent on miR210 targeting of tumor suppressor Mnt.

4.3 Introduction

Asthma is a chronic disease of the airways that is estimated to affect an estimated 300 million individuals globally [1]. The pathophysiology of this disease involves airway wall remodeling including increased mass of smooth muscle [5,481]. Airway smooth muscle (ASM) is the predominant mediator of airway constriction and is, therefore, a key tissue in driving exacerbation. Increased ASM mass may be the most important factor contributing to increased airway resistance in asthmatic airways [482]. One plausible source of increased ASM in the asthmatic airway is through increased proliferation of pre-existing ASM cells [438,481,483].

ASM cells are phenotypically regulated such that they may either exist in a proliferative state, or a contractile state, as serum deprivation induces the contractile phenotype [484,485]. The proliferative phenotype of ASM cells can be induced by the transcription factor serum response factor (SRF) binding to Elk1 to transcribe *c-fos* [267]. Furthermore, the interaction of SRF with Elk1 displaces SRF binding to myocardin, a smooth muscle specific co-transcription factor [267]. Induction of the pro-proliferative phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) or mitogen activated protein kinase (MAPK) pathways both drive proliferation in ASM cells and their induction represses smooth muscle tissue tension generation by methacholine stimulation [486]. Akt signalling has additional effects such as the induction of the expression of the anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) [487].

Micro-RNA (miRNA) are small (18-22 nucleotide), non-coding RNA that negatively regulate gene expression through binding to mRNA constructs preventing translation, or targeting the mRNA for degradation. Previously it has been shown that miRNA controls smooth muscle phenotype; miR-143 and miR-145 repress the expression of both Elk1 and Kruppel-like factor 4 (KLF4), another pro-proliferative co-transcription factor [272]. Recently, miR-10a was

demonstrated to inhibit ASM cell proliferation through targeting the PI3K pathway, and this miRNA was shown to be the most abundantly expressed miR in ASM cells [276]. Furthermore, miR-138 has been shown to regulate ASM cell proliferation via inhibition of 3'-phophoinositide dependent kinase-1 (PDK1), a protein in the of PI3K/Akt signalling cascade [488]. MiR-25 was repressed in ASM by inflammatory cytokine stimulation, a miR that normally prevents KLF4 expression [275].

Myc is a proto-oncogene transcription factor that, along with its binding partner Max [489], drives the expression of genes associated with cell cycle progression. Myc induces proliferation in ASM cells [490] and may represent a therapeutic target in asthmatic myocytes. Max binding protein (Mnt) also binds to Max and antagonizes the activity of Myc:Max [491], thereby inhibiting proliferation. Finally, in airway derived fibroblast cultures, miR-210 has been shown to negatively regulate Mnt, leading to increased rates of proliferation [492].

Another category of stimuli for proliferation of ASM that is of relevance to asthma pathology is the pro-inflammatory cytokines. Tumour necrosis factor (TNF)-α was shown to induce ASM cell methyl-[^{3}H]thymidine incorporation in a PI3K/Akt dependent manner [493]. Another study demonstrated that ASM cell stimulation with the pro-inflammatory cytokines eotaxin, regulated on activation, normal T cell expressed and secreted (RANTES), interleukin (IL)-8, and macrophage inflammatory protein (MIP)-1α all increased DNA synthesis [494]. The dual role of pro-inflammatory cytokines as effector molecules for recruiting leukocytes and directly driving airway remodeling is beginning to emerge.

It is also becoming increasingly evident that the airway epithelium plays an important role in driving airway remodeling in asthma and has been extensively reviewed by Lambrecht et al [454]. Airway epithelial cells can release ligands of the epidermal growth factor receptor (EGFR)

as well as other mitogens, including heparin-binding epidermal growth factor (HB-EGF) [374,495] and amphiregulin [496]. EGFR signalling also plays a role in driving the proliferative response of ASM in a rodent model of allergic asthma [497].

Recently it has been demonstrated that airway epithelial cells in culture can induce proliferation of ASM cells [383]. However the mechanism by which this occurs is largely unknown. Increased rate of proliferation may be due to secreted growth factors from the epithelium, however the possibility that ASM cells are phenotypically modulated and secrete mitogens that act in an autocrine manner is possible and un-explored. We wished to elucidate the molecular basis by which airway epithelial cells induce ASM cell proliferation *in vitro* by exploring their interaction in a co-culture model. Due to the involvement of growth factor receptor signalling, and the novel role of miRNA in governing ASM cell proliferation, we sought to examine the potential role of these molecules in epithelial-induced ASM cell growth.

4.4 Materials and Methods

Reagents

Collagenase type IV from *Clostridium histolyticum* was obtained from Sigma-Aldrich (St. Louis, MI, USA). EGFR inhibitor tryphostin AG1478 (0.3μM) was obtained from Cayman Chemical (Ann-Arbor, MI, USA). BrdU flow kit was obtained from BD Biosciences (Franklin Lakes, NJ, USA). The EGFR inhibitor afatinib (0.5μM) was obtained from Santa Cruz (Santa Cruz, CA, USA). qPCR primers for mRNA targets and lipofectamine 2000 were obtained from Thermo Fisher Scientific (Waltham, MA, USA). qPCR primers for miRNA targets and miR-210 mimic and inhibitors were obtained from Exiqon (Vedbaek, Denmark).

Cell Culture

Primary human airway smooth muscle cells (ASM cells) were obtained from lung transplant donors or bronchial biopsies. Protocols were approved by an Institutional Review Board. Micro-dissected tissue was digested overnight in collagenase (0.4mg/ml) dissolved in Dulbecco's Modified Eagle's Medium (DMEM) containing streptomycin, penicillin and amphotericin B (Anti-Anti,Thermo Fisher Scientific). The next day, the tissue was suspended in the digestion medium by gentle passing through a serological pipette several times. Cells were maintained in DMEM supplemented with Anti-Anti and 10% fetal bovine serum (FBS)(Thermo Fisher Scientific). Medium was replaced every second day. For long-term storage, cells were cryo-stored in 90% FBS:10% dimethyl sulfoxide (DMSO) in liquid nitrogen. Prior to all experiments, medium was changed to DMEM supplemented with 0.5% FBS containing Anti-Anti overnight prior to epithelial co-culture or stimulation with conditioned medium. Cells were studied between passage 2 and 6.

Bronchial epithelial cell line BEAS-2B cells were maintained similarly to ASM cells, and serum deprived in DMEM containing 0.5% FBS with Anti-Anti.

For co-culture experiments, Transwell ® permeable supports obtained from Corning (Corning, NY, USA) were utilized. Confluent epithelial cultures were serum deprived for 24 hours prior to co-culture with ASM cells. For conditioned medium experiments, supernatant from serum deprived confluent cultures was collected after 24 hours of conditioning with fresh starvation medium, centrifuged at 1500 RPM for 5 mins and stored at -80ºC.

RT-qPCR

ASM cells were seeded at a density of 100 000 cells per well in 6 well plates in growth medium. The next day, ASM cells were serum deprived for 24 hours in starvation medium after which they were placed in co-culture with BEAS-2B cells. After 24 hours of co-culture, ASM cells were washed once with PBS (Thermo Fisher Scientific) and mRNA was extracted using an RNeasy mini-kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed on 100ng of total RNA with AffinityScript qPCR cDNA synthesis kit (Agilent Technologies, Santa Clara, CA, USA). qPCR was performed using iTaq SYBR green supermix (Bio-Rad Laboratories, Hercules, CA, USA). Primer sequences are reported in Table 1. Amplification of cDNA was performed using a StepOnePlus realtime PCR system (Applied Biosystems, Foster City, CA, USA). Relative mRNA expression was calculated using the ΔΔCt method and all gene expression was normalized to S9.

For experiments examining miRNA expression, total RNA was extracted using Exiqon's miRCURY RNA Isolation kit – Cell and Plant according to manufacturer's protocol (Exiqon). miRNA cDNA libraries were generated with 20ng RNA using Exiqon's Universal cDNA synthesis kit II and RT-qPCR was performed with miRCURY LNA^{TM} Universal RT microRNA

PCR kit according to manufacturer's protocol (Exiqon). Amplification of cDNA was performed using a StepOnePlus realtime PCR system (Applied Biosystems, Foster City, CA, USA). Relative mRNA expression was calculated using the ΔΔCt method and all miRNA expression was normalized to miR-103a-3p.

mRNA Gene Array

Co-cultured or control ASM cell derived mRNA was analyzed for gene expression by HumanHT-12 Expression BeadChip Kit (Illumina, San Diego, CA, USA). Gene array was performed by Genome Quebec (Montreal, QC, Canada) and results were analyzed using FlexArray and cyber-T followed by Benjamini-Hochberg false discovery rate p-value correction for multiple comparisons.

miRNA Gene Array

Co-cultured or control ASM cell derived total RNA was analyzed for miRNA expression by miRCURY LNA^{TM} Array microRNA 7th generation profiling services (Exiqon). P value correction utilizing Benjamini-Hochberg false discovery rate was applied and data analysis was conducted by Exiqon.

Proliferation Assay

ASM cells were seeded in 6-well plates at a density of 25 000 cells per well in growth medium. The following day, medium was changed for starvation medium. 24 hours later, ASM cells were either co-cultured with confluent BEAS-2B cultures that had been serum deprived for 24 hours or not co-cultured. Six hours after the initiation of co-culture, BrdU was added to the culture medium according to manufacturers protocol (BD Biosciences, Franklin Lakes, NJ, USA). 24 hours after the initiation of co-culture, ASM cells were rinsed with PBS and collected using

trypsin to fix and permeabilize for analysis of BrdU incorporation by anti-FITC-BrdU staining and flow cytometry according to manufacturer's protocol (BD Biosciences). As negative controls, ASM cells received growth medium to induce proliferation but did not receive BrdU. As positive controls, ASM cells received growth medium along with BrdU. Viable smooth muscle populations were selected for and gates were established based on negative and positive controls.

MiR-210-3p Mimic

ASM cells were seeded in 6-well plates at a density of 25 000 cells per well in growth medium. The following day, cells were transfected with 50 nM of miR-210-3p mimic (Exiqon), along with 2µl of Lipofectamine® 2000 in 1ml Opti-MEM (Thermo Fisher Scientific). As a control transfection for miR-210-3p mimic, cel-miR-39-3p (Exiqon) was utilized. After one hour, one ml of starvation medium without antibiotics was added to all wells. Six hours later, medium was changed for fresh starvation medium containing antibiotics. BrdU assays were performed from 72 hours post-transfection.

HB-EGF Enzyme Linked Immunosorbant Assay

Cell culture supernatant was collected from ASM cells that had either been co-cultured with BEAS-2B cells or had not been co-cultured (control). Supernatant was centrifuged at 1500 RPM for 5 minutes to remove any potential cells and the culture medium was assayed for the presence of HB-EGF with the Human HB-EGF DuoSet ELISA kit (R&D Systems, Minneapolis, MN, USA).

Western Blot

After stimulation with epithelial derived conditioned medium, AMS cells were washed with ice cold PBS and protein extraction was performed using protein lysis buffer containing 50mM TrisHCl (pH 8), 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS. The lysis buffer was supplemented with protease inhibitor cocktail (Sigma-Aldrich). Upon mechanical disruption, cell lysates were centrifued at 13 000 RPM for 3 minutes and total protein within the supernatant was measured by Quick Start Bradford Protein Assay (Bio-Rad, Hercules, CA, USA). 5µg of protein diluted in double distilled water was loaded per lane into a separating gel. After separation, protein was transferred to a PVDF membrane (Bio-Rad). Membranes were blocked with 5% bovine serum albumin (Sigma-Aldrich) in TBS-T for one hour at room temperature prior to antibody incubation overnight at 4ºC. Primary antibody dilutions included: pEGFR (12A3, Santa Cruz Biotechnology, 1:3000) and pAKT, (D9E, Cell Signaling Technology, 1:2000) in 5%BSA TBS-T overnight at 4^{o}C. Membranes were washed three times with TBS-T prior to one-hour incubation with HRP -linked secondary antibody (7074, Cell Signaling Technology, 1:1000). Membranes were washed three more times in TBS-T and then once with TBS prior to development with chemiluminescent techniqes (ECL, Bio-Rad) and imaging.

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). All data are presented as mean +1SE with ≥4 independent experiments. Independent experiments were considered as ASM cells derived from different patients, or from different passage numbers measured on a different day. Each experiment included data deriving from at least three independent patients' ASM cells. In experiments where >2 groups were compared, one way ANOVA with Tukey's post-hoc test was utilized. For experiments where

only two groups are compared, a paired Student's T-test was employed. P values <0.05 were considered to be significant.

4.5 Results

Co-Culture with epithelial cells induces the proliferative phenotype in airway smooth muscle cells

To determine if BEAS-2B cells increased the proliferation of ASM cells, the two cell types were co-cultured for 24 hours prior to assessment of incorporation of the thymidine analogue BrdU. After treatment with BEAS-2B cells, ASM cells demonstrated increased rates of proliferation (Fig 1A). Due to the role of the pleiotropic transcription factor SRF in regulating ASM phenotype, we examined its expression and observed no change after co-culture (Fig 1B). However, the pro-proliferative co-transcription factor and binding partner of SRF, Elk1 was increased after co-culture (Fig 1C). KLF4 was unchanged (Fig 1D). These results indicate that ASM cells are not only stimulated to proliferate by the epithelial co-culture, but also differentially express a transcription factor associated with driving this phenotype.

Co-culture induced proliferation is not mediated by an EGFR ligand

Due to the importance of mitogen stimulation in ASM cell proliferation, we explored the EGFR as a potential target mediating co-culture induced proliferation. Within the ASM cells after co-culture, we examined the expression of the EGFR ligand HB-EGF, observing increases in this construct (Fig 2A). Furthermore, we observed a trend towards an increase in the concentration of this EGFR ligand in the supernatant of co-cultured ASM cells (Fig 2B). However, pre-treatment with the tyrosine kinase inhibitors tryphostin AG1478 (3μM) (Fig 2C) or afatinib (0.5μM) (Fig 2D) did not prevent the induction in proliferation by co-culture as assessed by BrdU incorporation. Finally, stimulation of ASM cells with conditioned medium derived from BEAS-2B cells for 15 minutes did not appear to activate phosphorylation of the EGFR at tyrosine 1068 although this medium did activate AKT (Fig 2E). These data indicate that although the ASM

cells express HB-EGF, it does not appear to activate the receptor to drive the increased proliferation rate.

Co-culture induces the expression of inflammatory cytokines

To have a more complete understanding of how airway epithelial cells may augment ASM cell proliferation, we ran a gene array (Illumina HT-12 version 4) exploring the differential expression of genes by ASM cells that had either been co-cultured with BEAS-2B cells or had not (control). We observed significant increases in the expression of CXCL1, IL-6 and IL-8 (Fig 3). These results indicate that the phenotypic skewing towards a more proliferative state is also accompanied by the expression of pro-inflammatory molecules after co-culture. For a complete list of genes that are two-fold differentially expressed after co-culture see supplement S1.

Co-culture with epithelial cells increases the expression of miR-210-3p

To assess the expression of miRNAs previously described to regulate ASM cell phenotype, we co-cultured ASM cells with BEAS-2B cells and performed RT-qPCR. We observed no change in miR-143-3p/145-5p (Fig 4A,B). To further study differential miR expression in co-cultured ASM cells, we performed a miRNA microarray (Exiqon). After Benjamini-Hochberg false discovery rate p-value correction for multiple comparisons, three candidate miRNAs were close to significant up-regulation (Fig 4C). To further examine these miRNAs, we performed RT-qPCR on co-cultured ASM cells, and observed a significant increases in miR-210-3p (Fig 4D), but not in miR-1246 (Fig 4E). MiR-4732-5p primers did not amplify a product (data not shown).

MiR-210 regulates tumour suppressive Mnt and can drive proliferation in vitro

Because of miR-210's role in driving fibroblast proliferation [492], we explored the functional role of this miRNA in ASM. In co-cultured ASM cells, expression of miR-210's putative target

Max-binding protein (Mnt), a Myc inhibitor, was reduced (Fig 5A). Furthermore, transfection of ASM cells with miR-210 mimic increased the rate of proliferation of ASM cells, indicating a potential role for this miR in driving airway remodeling (Fig 5B). These results imply, for the first time, that miRNA-210 expression can modulate ASM cell proliferation.

Figure 1

Co-culture with epithelial cells induces a proliferative phenotype in airway smooth muscle cells.

(A) ASM cells were cultured with (co-culture) or without (control) BEAS-2B cells for 24 hours. 6 hours after the initiation of co-culture, cells were pulsed with BrdU. BrdU incorporation by flow cytometry was then performed to mark ASM cells that had entered into S-phase (n=19). (B,C,D) RT-qPCR was performed on ASM cells to assess the expression of Elk1 (n=7), KLF4 (n=6) and SRF (n=4) after 24 hours of co-culture with BEAS-2B cells, or without co-culture (control). Data are presented as means +SE. Student's paired T-test was utilized to compare groups. P values are reported, N.D.=not different

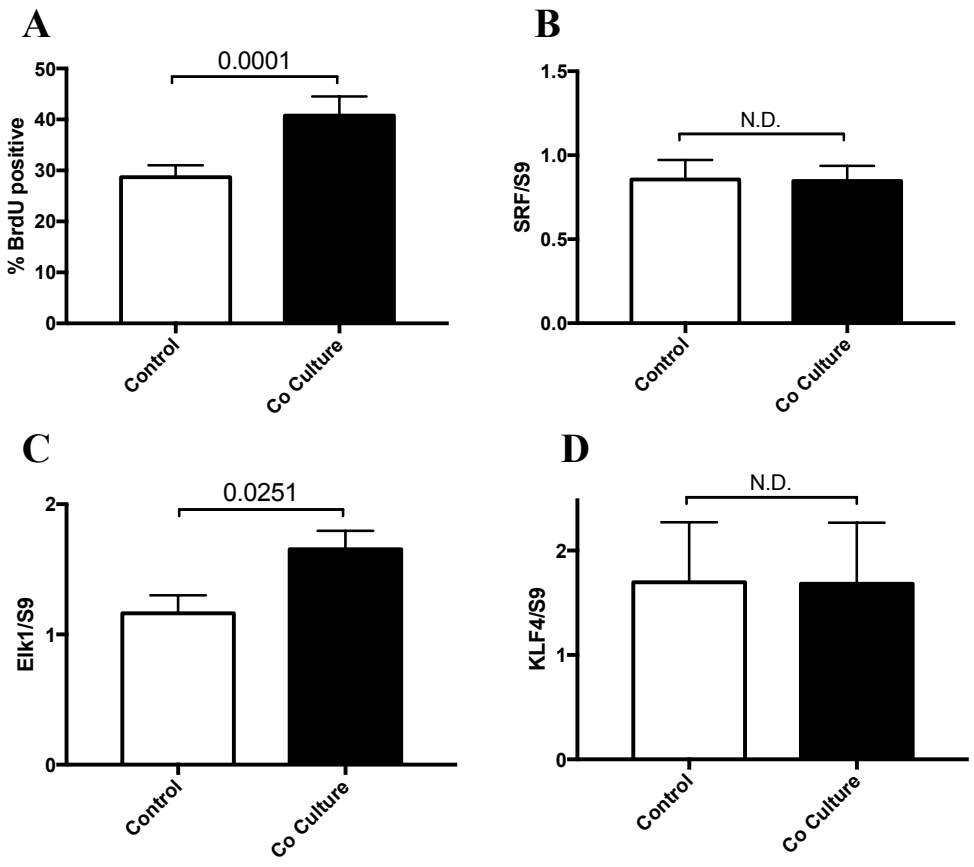
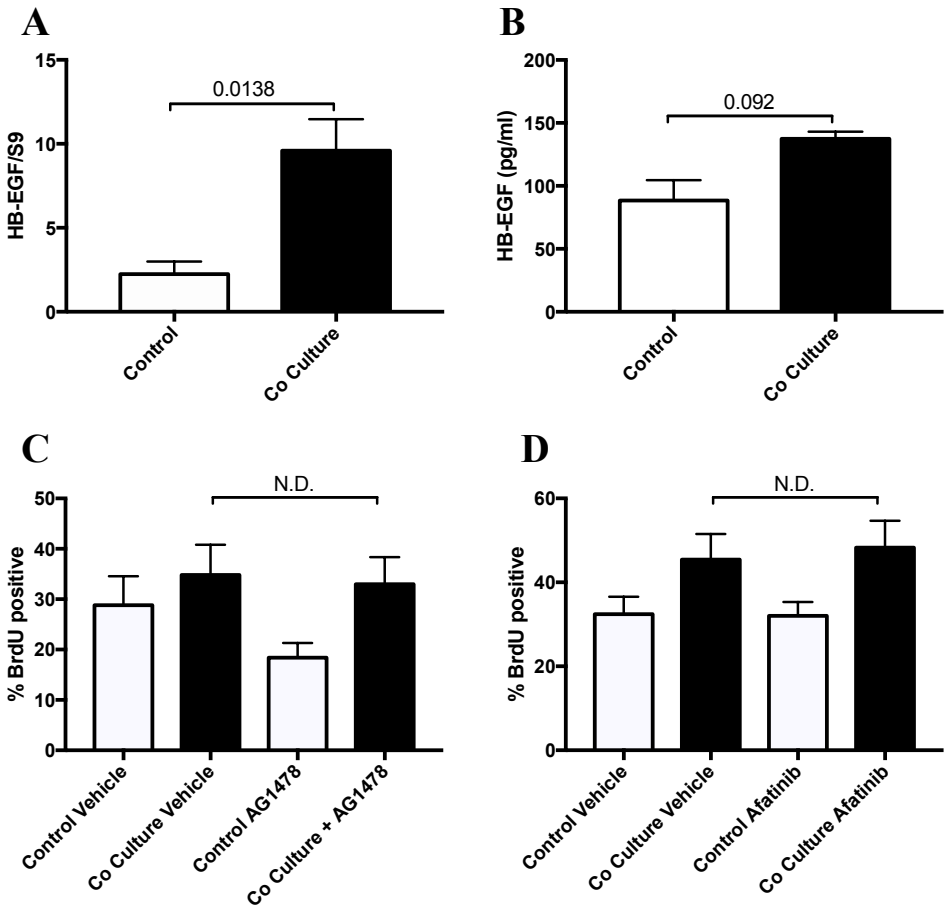


Figure 2

Co-culture induced proliferation is not mediated by an EGFR ligand. (A) ASM cell expression of HB-EGF was examined by RT-qPCR 24 hours after co-culture with or without (control) BEAS-2B cells (n=4). (B) HB-EGF protein concentration was assayed for in the cell culture supernatant of control or co-cultured ASM cells by ELISA after 24 hours (n=4). (C, D) Pre-treatment of ASM cells with 0.3μM tryphostin AG1478 (C, n=4) or 0.5μM afatinib (D, n=5) did not prevent BEAS-2B induced proliferation of ASM cells. Vehicle utilized was 0.1% DMSO (E) Treatment for 15 minutes with conditioned medium (C.M.) of BEAS-2B cells did not induce phosphorylation of EGFR tyrosine 1068 however Akt was phosphorylated by C.M. treatment. Cells were pre-treated with afatinib or vehicle (DMSO) for 1 hour prior to C.M. stimulation. Data are presented as means +SE. ANOVA with Tukey post hoc pairwise comparisons was employed for comparisons with more than two groups, otherwise Student's paired T-test was utilized. P values are reported.

(Figure on subsequent page):


A
HB-EGF/S9
0.0138
Control
Co Culture
B
HB-EGF (pg/ml)
0.092
Control
Co Culture
C
% BrdU positive
N.D.
Control Vehicle
Co Culture Vehicle
Control AG1478
Co Culture + AG1478
D
% BrdU positive
N.D.
Control Vehicle
Co Culture Vehicle
Control Afatinib
Co Culture Afatinib

E

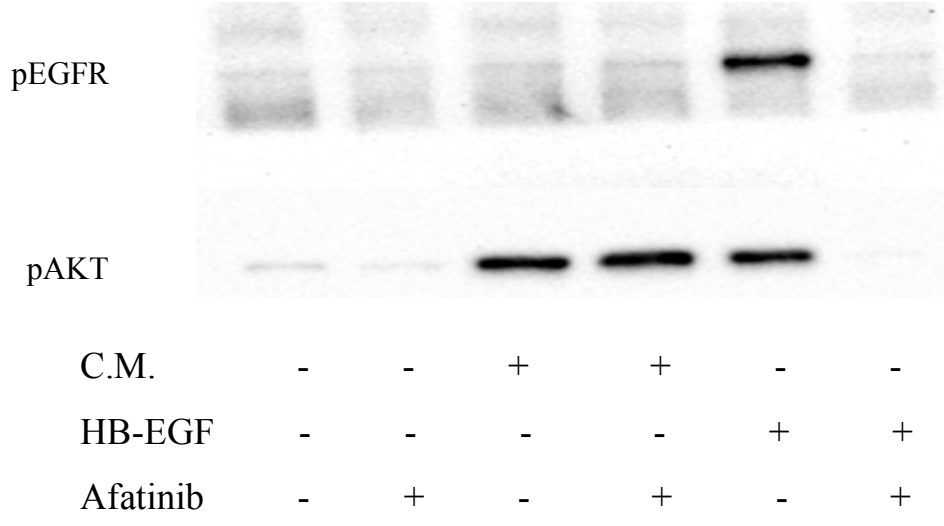

pEGFR
pAKT
C.M. - - + + - -
HB-EGF - - - - + +
Afatinib - + - + - +

Figure 3

Co-culture induces the expression of inflammatory cytokines. Messenger RNA was extracted from co-cultured or not co-cultured (control) ASM cells and gene array analysis was performed by Illumina HT-12 version 4 Expression BeadChip (Illumina) (n=6). Gene array analysis was performed using FlexArray software and a Cyber-T test followed by a Benjamini-Hochberg p-value correction for multiple comparisons. Genes that were up or down-regulated two-fold or more and possessed corrected P values<0.05 were labelled on the volcano plot. Data are presented as mean log fold change.

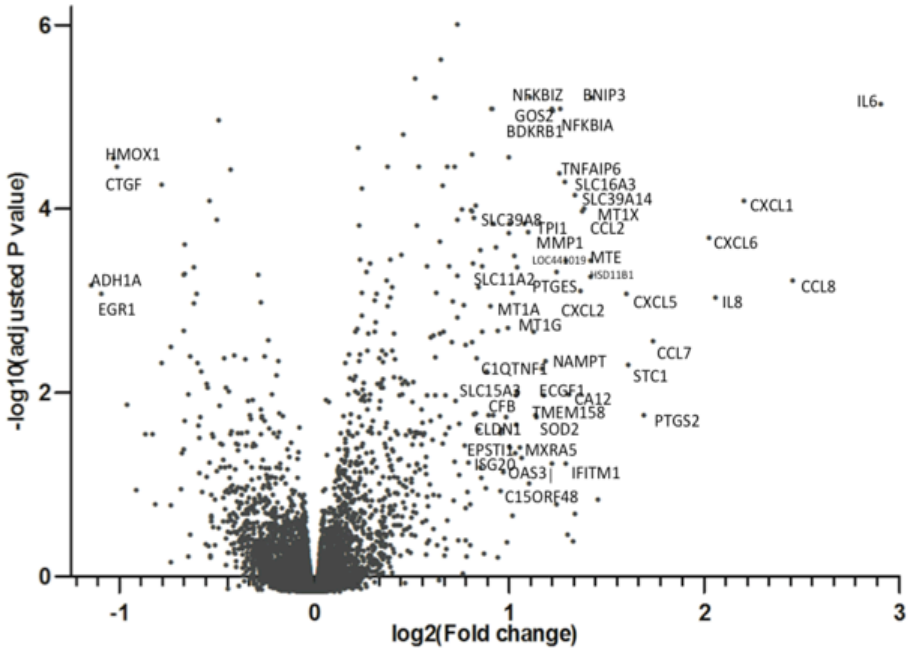


Figure 4

Co-culture of epithelial cells increases the expression of miR-210-3p without affecting miR-143/145. Total RNA was extracted from co-cultured or not co-cultured (control) ASM cells and RT-qPCR was performed to examine the expression of (A) miR-143 (n=5) (B) miR-145 (n=4). MiR-103a-3p was used to normalize the data. MiRNA microarray was performed by Exiqon miRCURY LNA^{TM} Array microRNA 7th generation profiling services. (C) Volcano plot analysis demonstrates 5 miRNAs that may be differentially expressed (n=6). RT-qPCR was performed and expression of (D) miR-210-3p (n=9), (E) miR-1246 (n=9) is presented with P values reported. Data are presented as means +SE. For RT-qPCR, Student's paired T-test was utilized. P values are reported.

(Figure on subsequent page):

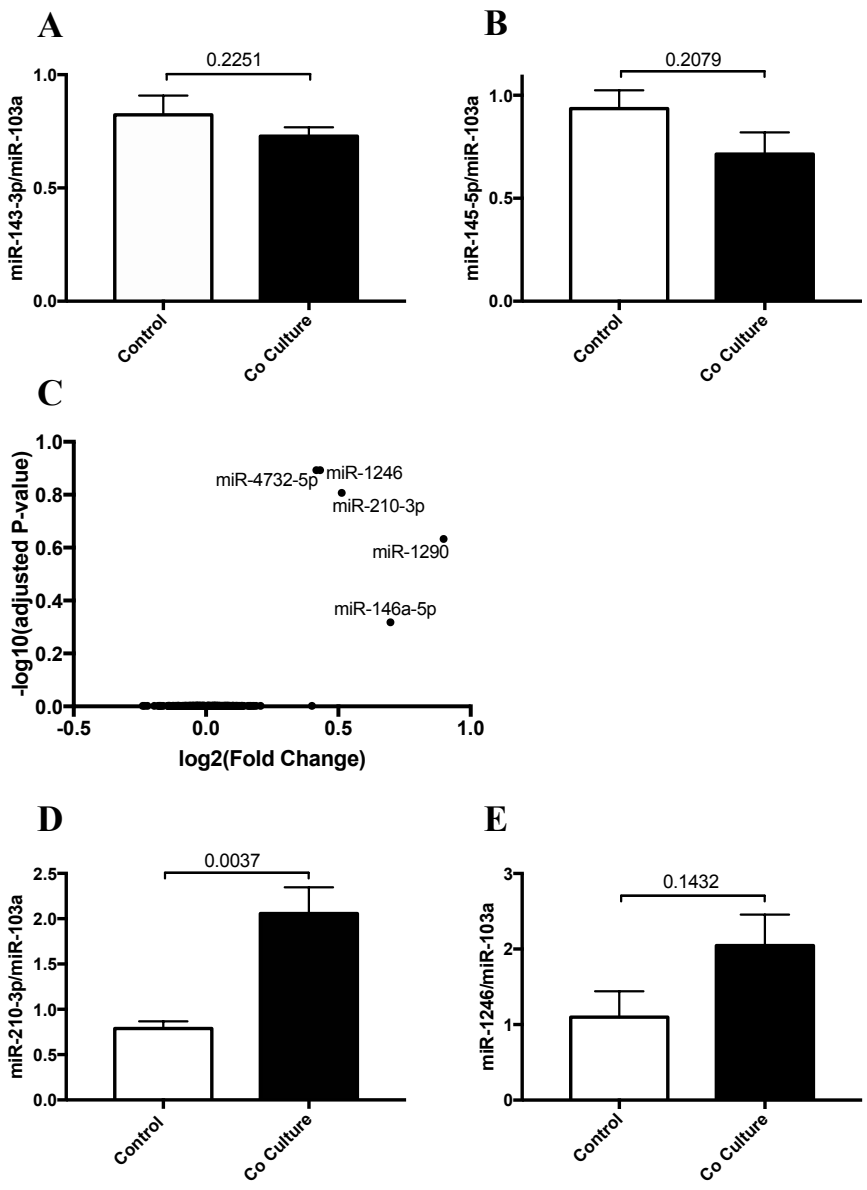
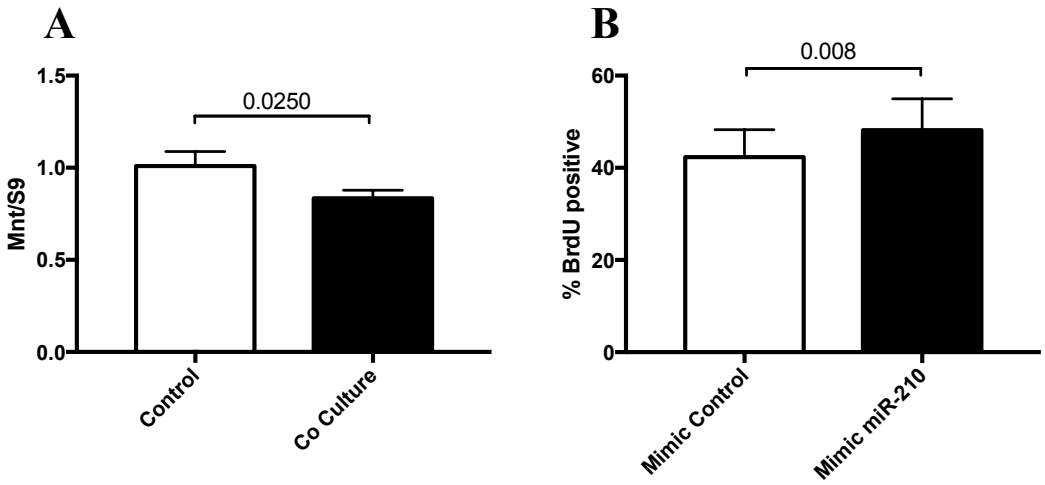

A
0.2251
miR-143-3p/miR-103a
1.0
0.5
0.0
Control
Co Culture
B
0.2079
miR-145-5p/miR-103a
1.0
0.5
0.0
Control
Co Culture
C
-log10(adjusted P-value)
1.0
0.8
0.6
0.4
0.2
0.0
miR-4732-5p
miR-1246
miR-210-3p
miR-1290
miR-146a-5p
-0.5
0.0
0.5
1.0
log2(Fold Change)
D
0.0037
miR-210-3p/miR-103a
2.5
2.0
1.5
1.0
0.5
0.0
Control
Co Culture
E
0.1432
miR-1246/miR-103a
3
2
1
0
Control
Co Culture

Figure 5

MiR-210s role in co-culture induced proliferation. (A) RT-qPCR was performed on mRNA of ASM cells that had been co-cultured or not (control) with BEAS-2B cells for 24 hours to assess the expression of Max-binding protein (Mnt) (n=9). (B) ASM cells were transfected with a mimic of miR-210-3p or cel-miR-39-3p (control) prior to BrdU assay to assess the role of miR-210-3p in proliferation (n=5). Data are presented as means +SE. ANOVA with Tukey post hoc pairwise comparisons was employed for comparisons with more than two groups, otherwise Student's paired T-test was utilized. P values are reported.



4.6 Discussion

The purpose of this study was to examine mechanisms by which airway epithelial cells induce the proliferation of ASM. ASM growth may represent an important mechanism by which the airway is remodelled in asthma and understanding the mitogenesis of this tissue is an important area of research. We have identified that ASM cells undergo increased rates of proliferation after culture with epithelial cells. The proliferation was accompanied by the appearance of a pro-inflammatory phenotype with expression of several chemokines and cytokines. The proliferation was associated with expression of miR-210, not previously associated with ASM proliferation and providing a potential mechanism for this observation.

Smooth muscle cells differ from other muscle in their ability to de-differentiate towards a less contractile, more proliferative state [498]. This ability to revert back into a proliferative cell provides a potential explanation for the source of increased mass of muscle surrounding the asthmatic airway. The proliferation of ASM cells derived from asthmatic patients is indeed increased [438] and the mass of this tissue surrounding the airways is augmented [339,499]. Although the presumed functional consequence of asthmatic ASM is increased airway reactivity, it has been observed that ASM cells may exist in discrete populations, where there are cells actively proliferating at the same time as others that are expressing proteins of the contractile apparatus [302]. If the smooth muscle remodeling that occurs in the asthmatic airway does so inwards towards the epithelium, the proximity of pro-proliferative factors released by epithelial cells may have an impact on driving further remodeling. Janssen proposed that inwardly growing muscle would not only contribute to the increased mass observed in asthma, but may also contribute to decreased luminal area [500]. However, it is unclear at this time as to which direction the muscle grows, or whether there is directionality at all.

Due to the potential of airway epithelial cells to secrete EGFR ligands [374,496], we explored the possibility that this tyrosine kinase receptor is responsible for mediating co-culture induced proliferation. Although there was increased expression of mRNA for HB-EGF in ASM cells after co-culture as well as augmented protein in the supernatant, inhibition of the receptor with appropriate concentrations of AG1478 and afatinib did not limit the induced proliferation. The increase in expression of this EGFR ligand without an obvious functional role may imply that HB-EGF could act as a marker of the proliferative phenotype, but does not necessarily drive proliferation itself. HB-EGF has been previously proposed as a biomarker of proliferating ASM, and the expression of this ligand in the muscle was associated with asthma severity [501].

It has been previously demonstrated that co-culture with epithelial cells drives the proliferation of ASM [383]. Malavia et al explored the hypothesis that injury to epithelial cells may drive the proliferation of ASM cells. Asthmatic airway epithelial cells are likely more fragile than those cells derived from healthy controls [502]. Injured epithelial cells induced a further increase in ASM cell proliferation than epithelial cells alone, and may depend on matrix metalloproteinases [383]. The mechanism by which un-injured epithelial cells drive increased proliferation has remained elusive. Although treatment of the co-cultures with the anti-inflammatory steroid dexamethasone prevented co-culture induced proliferation of ASM cells, it also significantly reduced the rate of proliferation in non co-cultured ASM cells [383]. Furthermore, the possibility of ASM cells participating in driving this proliferation in an autocrine manner has not been explored and it has been demonstrated that this phenomenon is an important driver of proliferation [247,503].

Other recent work has indicated a role for miRNA in regulating ASM cell proliferation [276]. We observed increased miR-210-3p within ASM cells after co-culture with BEAS-2B cells.

Previous work demonstrating a role of miR-210 in driving proliferation of fibroblasts in a Mnt specific manner [492] led us to explore this mechanism. Indeed, we observed reduced expression of Mnt in co-cultured ASM cells. Furthermore, other miRNAs have been associated with ASM cell phenotype regulation. MiR143 and miR145 target the pro-proliferative co-transcription factor Elk1 [272] and thus we tested the hypothesis that these miRs were reduced after co-culture. However we did not observe diminished expression of these two miRs. MiR-25 targets KLF4 in ASM cells therefore regulates the proliferative phenotype [275] but was not differentially expressed by co-culture, which was consistent with the lack of change in KLF4 mRNA in co-cultured cells.

Motivated by previous literature that miR-210 can negatively regulate the tumour suppressive Mnt [492], we explored its expression after co-culture of ASM cells with epithelial cells. The reduction we observed in ASM cell Mnt mRNA construct is consistent with the notion that this tumour-suppressor may regulate proliferation of this cell type.

Examination of models that drive the growth of ASM may help to uncover therapeutic targets for the treatment of this disease. This study has demonstrated that, *in-vitro*, co-culture with epithelial cells increases the rate of proliferation and expression of the pro-proliferative co-transcription factor Elk1 in ASM cells. Although this was accompanied with augmented expression of HB-EGF within the ASM cells, the EGFR did not appear to mediate the induced proliferative response. We observed increased expression of the pro-proliferative miRNA-210, and reduction of its target tumour suppressive protein Mnt. Furthermore, overexpression of miR-210 in ASM cells increased the rate of proliferation, implicating this miRNA as a candidate mediator of airway wall remodeling. Others have successfully administered miRNA antagonists to *in vivo* models of allergic asthma, indicating promise for the use of miRNA antagonists for the treatment

of asthma [273,504]. Future *in-vivo* work exploring this miRNA in models of asthma will be necessary to further understand the importance of this miR in driving pathogenesis of this disease.

Supplemental S1

Upregulated				Downregulated	
Gene	**Fold Change**	**Gene**	**Fold Change**	**Gene**	**Fold Change**
IL6	7.49	PTGES	2.37	ADH1A	0.45
CCL8	5.49	BDKRB1	2.34	EGR1	0.47
CXCL1	4.61	G0S2	2.33	HMOX1	0.49
IL8	4.16	C15ORF48	2.33	CTGF	0.49
CXCL6	4.07	NAMPT	2.27		
CCL7	3.34	ECGF1	2.26		
PTGS2	3.23	C1QTNF1	2.25		
STC1	3.06	SOD2	2.2		
CXCL5	3.04	TMEM158	2.19		
ISG15	2.75	MT1G	2.18		
BNIP3	2.68	NFKBIZ	2.15		
MTE	2.67	MX2	2.15		
HSD11B1	2.67	LOC644774	2.14		
MT1X	2.61	TPI1	2.11		
CCL2	2.6	OAS3	2.09		
CXCL2	2.58	MXRA5	2.08		
SLC39A14	2.53	SLC11A2	2.06		
IFI44L	2.53	SLC15A3	2.06		
MX1	2.51	CLDN1	2.05		
CA12	2.47	CFB	2.05		
CXCL10	2.46	ISG20	2.04		
IFITM1	2.45	MMP1	2.04		
LOC441019	2.45	MT1E	2.02		
SLC16A3	2.44	MT1A	2.02		
NFKBIA	2.4	SLC39A8	2		
TNFAIP6	2.39	EPSTI1	2		
IFI27	2.37				

CHAPTER 5:

General Discussion and Conclusions

5.1 Discussion and Conclusions

The purpose of this dissertation was to examine the role of the airway epithelial cells in coordinating specific aspects of airway remodeling. It has become increasingly evident that airway epithelial cells play a critical role in modulating airway inflammation and structural cell remodeling. More specifically, the work conducted in this dissertation aimed to understand how epithelial cells respond to the lipid mediator S1P, as well as to increase knowledge regarding the role of epithelial cells in modulating airway smooth muscle cell phenotype.

In the first chapter of experimental work presented here, the airway epithelial cell line, BEAS-2B, was stimulated with the asthma-associated lipid mediator S1P. S1P was of interest due to its potent properties as a ligand of GPCRs and its role in innate immunity. We confirmed that stimulation of epithelial cells with S1P generates the release of the pro-inflammatory chemokine IL-8 in a dose-dependent manner. IL-8 and neutrophils have been extensively associated with certain asthma subsets and therefore, this finding represents a potential mechanism in the airway for which neutrophils are recruited to the airway. Corticosteroids are used clinically for the treatment of asthma, however in some patients, the disease is poorly controlled, and thus novel mechanisms to inhibit inflammation may be of great interest in understanding how to treat asthma. S1P is bioactive signalling lipid produced by platelets and mast cells [406,407]. S1P has a prominent role in regulating vascular permeability [505]. Since we demonstrate a role for S1P in driving a pro-inflammatory response from the epithelium, we were interested in potential targets of this molecule, and to better understand the molecular pathways by which S1P induces IL-8 release. S1Ps previously described role as a regulator of airway inflammation [53,54] and its presence in the asthmatic sputum [352] makes it an attractive mediator to study. Since S1P is a GPCR ligand, and GPCR inhibition is an avenue that has generated great promise in the

treatment of many diseases, we were interested in better understanding which S1P receptor(s) mediated this phenomenon. We observed that specific inhibition of S1PR2, but not S1PR1 or S1PR3, inhibited IL-8 release by BEAS-2B cells. Furthermore, we confirmed that S1PR3 antagonist was indeed functionally active by examining the inhibition of calcium responses in airway smooth muscle cells after stimulating these cells with S1P. Cells that were pre-treated with the same concentration of the inhibitor CAY10444 that was used on the epithelial cells released significantly less calcium, indicating that the drug was active. To be sure that the S1PR1 antagonist W123 was active, we utilized an agonist of this receptor, stimulating the BEAS-2B cells with SEW2871. Stimulation with this specific agonist did not induce the release of IL-8, indicating no role of this receptor in mediating this process.

To better understand the transcriptional activation of IL-8 after S1P stimulation, we explored the sensitivity of this pathway to pre-treatment with the NF-κB inhibitor helenalin or the AP-1 inhibitor SR 11302. Inhibition of NF-κB but not AP-1 prevented the release of S1P induced IL-8. We therefore examined the translocation of NF-κB to the nucleus through the use of luciferase reporter BEAS-2B cells and we observed increased luciferase activity in cells that were stimulated with S1P. Furthermore, inhibition of S1PR2 prior to stimulation with S1P prevented the translocation of NF-κB, adding more evidence that this receptor does indeed initiate this signalling process.

Examination of transactivation of the EGFR revealed that this receptor was not important in mediating S1P induced IL-8 release. Using siRNA against this tyrosine kinase receptor, or inhibitors at reasonable concentrations did not prevent IL-8 release. Furthermore, inhibition of the metalloproteinases with either GM6001 or TAP-1 also did not prevent IL-8 secretion. Since MMPs cleave EGFR ligands, this further indicates no role of the EGFR in mediating this event.

Finally, due to the potential of ROS to mediate GPCR signalling, we explored the generation of ROS in BEAS-2B cells with the oxidative sensitive dye DCFH-DA and we observed no significant increase in the production of such species. We also did not observe the inhibition of IL-8 release with pre-treatment of the BEAS-2B cells with the antioxidants N-acetylcysteine or DPI.

With this knowledge, the use of the S1PR2 inhibitor JTE-013 may now be applied to understand if there may be a functional role of this receptor in driving neutrophilia in animal models of asthma. This will help to elucidate the clinical potential of JTE-013.

In the next chapter of experimental work, the potential role of the airway epithelium in modulating airway smooth muscle phenotype was examined. ASM cells are critical cells in regulating the diameter of the airways and are important contributors to airway narrowing in asthmatic patients. It is well established that this tissue is remodelled in asthmatic airways and thus understanding mitogenic stimuli of these cells is an important task for respiratory researchers. Furthermore, it is known that ASM cells are phenotypically regulated to be either contractile or proliferative and so we explored both of these phenotypes.

On the contractile side, we showed for the first time, that ASM cells placed in co-culture with epithelial cells demonstrate a reduced contractile phenotype. We observed less force generation of these cells by histamine stimulation after incubation with medium conditioned by primary human bronchial epithelial cells as well as less excitability as demonstrated through calcium responses to histamine. It is possible that asthmatic epithelial cells reduce the contractile phenotype of ASM cells less than that of epithelial cells deriving from healthy control subjects. Perhaps the property of the epithelium as a mitogenic source to drive the increase in mass of this tissue is the major mechanism by which it induces disease. Alternatively asthmatic epithelium

may have different properties and we have explored only epithelium derived from the lungs of previously healthy subjects.

Due to the importance of intracellular calcium release in mediating the initiation of cross bridge cycling of the actinomysosin machinery, we hypothesized that proteins related to the handling of calcium may be transcriptionally modified after co-culture with epithelial cells. After examination of several of these proteins, we concluded that co-culture does not transcriptionally regulate calcium handling.

Prostanoids may also modulate calcium signalling in ASM cells and so we sought to determine whether or not these lipid mediators had a role in co-cultured reduced excitability and force generating ability after stimulation with histamine. Indeed, we observed augmented PGE_2 producing enzymes COX-2 and mPGES-1. We also observed augmented production of PGE_2 in the co-cultured supernatant compared to either BEAS-2B cells or ASM cells alone. Pre-treatment of the ASM cells with the general COX inhibitor indomethacin prior to incubation with medium conditioned by BEAS-2B cells restored the calcium release back to levels similar to cells that received no treatment with conditioned medium. COX-1 inhibition with SC-560, but no COX-2 inhibition with Celecoxib, also restored the release of calcium after stimulation with histamine in cells that had been incubated with conditioned medium. This indicates a functional role of COX-1 in mediating epithelial modulation of ASM cell excitability and points towards ASM cell derived PGE_2 as the mediator of this phenomenon. This is of interest due to the lack of literature examining the constitutively active COX-1 enzyme in mediating biological processes. It is much more common that the inducible isoform COX-2 is utilized by cell signalling systems and this data builds on a somewhat small body of literature indicating that COX-1 may be of greater relevance in such systems than was previously thought.

The co-culture system explored in these assays was of great interest to understand airway biology. However, it is true that *in vivo* the smooth muscle tissue always resides nearby the epithelium. It is possible in asthma and other lung diseases where the ASM layer is remodelled that this remodeling occurs in the direction away from the airway lumen. This could imply that any epithelial derived mediators that modulate the phenotype of ASM cells are less active due to the greater distance required to diffuse to the added ASM. This is one explanation as to the clinical significance of these findings. Another interesting discovery was that COX-1 plays a role in ASM cell excitability. Inhibition of COX-1 led to a more contractile cell, and so it may be possible that selective activation of this enzyme could lead to a more relaxed tissue and represents a possible area for follow up studies to demonstrate clinical potential. This could have implications for aspirin-sensitive asthmatics who are intolerant to NSAIDs as the inhibition of COX-1 may further augment the excitability of the ASM cells. Indeed, aerosol administration of PGE_2 to aspirin-sensitive asthmatic patients prevented the reduction in FEV_1 after aspirin challenge [506].

On the proliferative side of the second part of this dissertation, we observed augmented mitogenesis of ASM cells after co-culture with BEAS-2B cells. This was determined by examining DNA synthesis after co-culture and demonstrating that co-cultured ASM cells incorporate more BrdU than do those that were not co-cultured. Furthermore, co-cultured ASM cells possessed more mRNA for the pro-proliferative co-transcription factor Elk1. We observed no change in the expression of the pleiotropic transcription factor SRF, nor KLF4.

Due to the previously established importance of the EGFR in mediating the mitogenesis of ASM cells, we determined the role that this tyrosine kinase receptor had in mediating co-culture induced proliferation. Although we observed increased mRNA for HB-EGF within the ASM

cells, as well as increased protein in the cell culture supernatant of the co-cultured cells, the amount of BrdU incorporation after co-culture was not reduced after pre-treatment with two EGFR inhibitors. Furthermore, stimulation of the ASM cells with BEAS-2B conditioned medium did not activate phosphorylation of the EGFR, however it did induce AKT phosphorylation. AKT phosphorylation was not sensitive to pre-treatment of the ASM cells with the EGFR tyrosine kinase inhibitor Afatinib.

We did observe augmented quantities of mRNA for several pro-inflammatory proteins including IL-6 and IL-8 within the ASM cells after co-culture ASM cells have been well described to synthesize pro-inflammatory chemokines [507]. Gene array data indicated a pro-inflammatory phenotype implying regulation of genes associated with the NF-κB system and other cytokines such as CXCL-1, CXCL5, CCL2, and COX-2. Cytokines have the ability to induce proliferation of ASM cells and this therefore represents an avenue by which the tissue is remodelled. Furthermore, IL-8 secretion from asthmatic ASM cells is increased due to augmented NF-κB [508]. The effect of co-culture induced pro-inflammatory chemokine release by asthmatic ASM cells remains to be determined.

The regulation of miRNA in ASM cells has emerged as a novel mechanism by which ASM cell phenotype can be modulated. We examined the expression of previously established miRNAs that regulate ASM cell phenotype, observing no changes in miR-143/145. We next explored the expression of global miRNAs after co-culture with BEAS-2B cells through miRNA array analysis and observed near-significant increases in miR-210-3p, miR-1246 and miR-4732-5p. However, by qPCR analysis, only miR-210-3p was significantly increased by co-culture.

One of miR-210's putative target proteins, Mnt, was reduced after co-culture with BEAS-2B cells. miR-210's inhibition of this protein has been previously described to augment the

proliferation of airway fibroblasts. We determined the ability of miR-210 to increase ASM cell proliferation by transfection with a mimic of miR-210-3p. Cells given exogenous miR-210 mimic were indeed more proliferative, demonstrating for the first time that this miRNA may play a role in ASM cell remodeling. Future work exploring miR-210 as a target for therapeutic efficacy in the treatment of asthma disease will be of great interest. It will also be of interest to examine the expression of miR-210 in asthmatic ASM cells as well as in epithelial tissue. It may be possible that the miRNA is delivered to ASM cells by the epithelium. This trans-cellular delivery, if true, would represent a novel mechanism by which these two cells communicate and should be explored in future work.

Again, the airway epithelium is always present *in vivo* and so the relative importance of epithelial derived proliferative signals may depend on the direction in which airway smooth muscle remodeling occurs. For example, if the ASM growth occurs towards the lumen of the airway in asthmatic patients, it may be expected that these cells receive greater concentrations of epithelial derived mitogenic factors. The directionality of the growth in asthmatic patients has not been well characterized but is of great interest.

In experiments throughout this thesis, BEAS-2B cells were utilized as a model of the airway epithelium. BEAS-2B cells are widely used as a representative culture of the airway epithelium. These cells possess anti-oxidant capacity similar to that of primary cells [509], and possess many of the same surface molecules that are also expressed on primary bronchial epithelial cells [510]. In comparison to our quantities of IL-8 released from BEAS-2B cells, it was shown that NHBE cells cultured at an air-liquid interface produced between 700-1500 pg/ml IL-8 [511], which is similar to what was observed in chapter two. Furthermore, in chapter three, we observed a larger reduction in calcium release after histamine stimulation in ASM cells that were co-cultured with

NHBE cells compared to BEAS-2B cells. However, the BEAS-2B model has its limitations. BEAS-2B cells do not form the same increase in trans-epithelial electrical resistance when compared to primary epithelial cells [512]. As research moves forward, the use of primary epithelial cells cultured at an air –liquid interface is becoming more widely utilized. Further work examining primary cells cultured at the air-liquid interface and the relationships between S1P-induced IL-8 release and epithelial modulation of ASM cell phenotype will be of interest.

In summary, data presented in this thesis provides support for the growing awareness of the role of the airway epithelium in governing airway processes related to the asthmatic phenotype. We have observed a molecular target in S1PR2 as a receptor that mediates S1P induced IL-8 release, a process that likely occurs in the asthmatic airway. We have also explored the effect of epithelial cell co-culture with ASM cells, and observed decreased contractility and increased proliferation.

5.2 Future Directions

Given the efficacy of S1PR2 antagonism for the prevention of IL-8 release by the epithelium after stimulation with S1P, it is of great interest to test the JTE 013 compound in animal models of asthma with the aim of preventing neutrophilia. The prevention of the release of this pro-inflammatory chemokine could have therapeutic benefit given the impact of airway inflammation of driving exacerbation. In fact, a recent study has demonstrated that systemic pre-treatment with JTE 013 in an IgE induced model of allergic lung inflammation reversed serum CCL5 concentration and prevented T cell migration [513]. Furthermore, a monoclonal antibody diminished mast cell derived IL-6 release after allergen challenge [513]. Alternatively, the administration of aerosolized helanalin as an anti-inflammatory agent may prove useful as a drug

treatment for these asthmatic patients. However NF-κB, the target of helenalin, is involved in a myriad of signalling pathways and it would likely be safer to target more upstream proteins such as the S1PR2. To bridge the gap between the findings reported here and the use of JTE 013 in the clinic, it would be useful to examine primary airway epithelial cells cultured at an air-liquid interface and the role that this drug plays in the prevention of cytokine release. Additionally, other cytokines such as IL-6 should be explored to further understand the capabilities of this treatment. Neutrophil migration measurements towards the conditioned medium of S1P stimulated air-liquid interface primary epithelial cells will uncover more information regarding the biological significance of the reduced quantities of IL-8 secretion. Finally, a vehicle to specifically deliver JTE 013 to the airway epithelium would be useful due to the variety of cell types that express the S1PR2 receptors. Aerosolization may be the most appropriate mechanism for such delivery.

Future work examining the reduction of the contractile phenotype in ASM cells that derive from asthmatic donors will be of great interest. It is possible that the relaxing effects of the epithelium may be reduced in cells deriving from diseased subjects however this remains to be explored. It is also possible that asthmatic derived epithelial cells may be less effective at reducing the contractility of the ASM. Further examination of the role of COX-1 in mediating this phenotype should be explored *in vivo*, potentially through the use of an inducible knock-out mouse lacking the COX-1 gene specifically in ASM. These animals may be expected to have greater airway hyperresponsiveness to challenge with contractile agonists. If this were true, it would be anticipated that overexpression of COX-1 may further augment the ability of the epithelium to induce a relaxed phenotype of the muscle. Delivery of recombinant COX-1 could also

recapitulate these results and may become an attractive area to explore in using next-generation gene editing techniques.

Due to the results indicating that epithelial co-culture stimulates ASM proliferation, future work should be conducted examining the quantity of miR-210 as a pro-remodelling molecule. It would be interesting to evaluate the ability to suppress this miR through the use of complementary RNA *in vivo* and also to explore the effect of this treatment on the expression of Mnt and rate of proliferation. Given the ability to use miRNAs as biomarkers, it may be interesting to examine miR-210 expression in a variety of asthmatic endotypes to examine the potential of this small non-coding RNA as such an indicator of disease. It would also be useful to explore the role of miR-210 in other tissues such as the vasculature or other diseases such as cystic fibrosis to better understand the role of this miRNA. Furthermore, if this miRNA is indeed epithelial derived as discussed above, it would be of great interest to examine the effect of epithelial injury and repair on the release of miR-210, given that the asthmatic epithelium appears to be more fragile.

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