THE INFLUENCE OF INTERLEUKIN-13 ON FORCE GENERATION IN AIRWAY SMOOTH MUSCLE TISSUE

by

Nicholas Eric Swyngedouw

B.Sc., The University of Calgary, 2014

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Pathology and Laboratory Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

August 2016

Abstract

Airway smooth muscle (ASM) has been implicated in the pathophysiology of asthma by contributing to excessive airway narrowing and Airway Hyperresponsiveness (AHR). Furthermore, inflammation has also been suggested as a mechanism contributing to AHR in asthmatics. Levels of Interleukin-13 (IL-13), an inflammatory mediator, are increased in asthmatic sera and can alter the expression of specific contractile genes and proteins in cultured ASM cells. In cultured cells, IL-13 can cause increased ASM contractility and force generation in response to different contractile agonists such as acetylcholine (ACh), KCl, or histamine. However, there remains a lack of consensus regarding whether IL-13 can induce changes in mechanical properties of ASM tissue in response to all, or only some, contractile agonists. Our objective was to investigate the influence of IL-13 on the force generation of isolated ASM tissue in response to a variety of agonists. Ovine tracheal smooth muscle was isolated, bathed in Krebs saline solution, and then equilibrated using electrical field stimulation. In order to obtain baseline mechanical measurements, tissues were either contracted with a range of ACh concentrations, pre-stimulated with ACh then relaxed with progressively increasing doses of isoproterenol (ISO), or contracted with single a single concentration of KCl or histamine (n=5 per condition). Paired samples from each tissue were then pinned at constant (in situ) length and incubated for 24h or 72h with or without IL-13 (50 ng/mL) in serum-free DMEM. Tissue responses were compared to their baseline (t=0) measurements after incubation to determine the influence of IL-13. Compared to non-exposed tissues, IL-13 did not increase maximal force or sensitivity to a range of ACh concentrations after either 24 or 72h (n=5 each), nor did it impede the relaxation of ASM induced by ISO after 24h (n=5). Likewise, response to KCl was not changed by IL-13 after 72h (n=5). Response to histamine was ~120% higher compared to control

(t=72h) after treatment (% of baseline maximal force, n=5, p=0.03). These findings contrast with previous work done in ASM cell culture experiments. In tissue strips, IL-13 did not induce significant changes to ASM mechanics in response to ACh, ISO, or KCl treatment. However, IL-13 did influence histamine-induced contractile response suggesting a potential avenue by which airway inflammation influences ASM contraction.

Preface

This thesis is based on experiments performed by me in the laboratory of Dr. Chun Seow and Dr. Peter Paré at the Centre for Heart Lung Innovation, St. Paul's Hospital.

Chapter 1 is partially based on a published review by Chris D. Pascoe*, Nicholas E. Swyngedouw*, Chun Y. Seow, and Peter D. Paré. 2014. Gene expression in asthmatic airway smooth muscle: a mixed bag. *Canadian Journal of Physiology and Pharmacology* 93(2): 137-143. (* co-first author). This review was written in collaboration with lab members and certain sections were written by myself. Figure 1.1 has been reprinted with permission from NRC Research Press.

Experiments in this thesis were designed by Dr. Seow, Dr. Paré, Dr. Lu Wang, and myself. All experiments were performed by me. Data collected was analyzed by Dr. Seow, Dr. Paré, Dr. Wang, and myself. All figures were made by me.

The work in this thesis is covered under the Human Ethics Board of UBC certificate H12-02487, project title "Plasticity in airway smooth muscle".

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List of Abbreviations

AC	Alternating Current or Adenylyl Cyclase
ACh	Acetylcholine
AHR	Airway Hyperresponsiveness
AMP	Adenosine Monophosphate
ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
ASM	Airway Smooth Muscle
ATP	Adenosine Triphosphate
Ca^{2+}	Calcium ions
$CaCl_2$	Calcium Chloride
CaM	Calmodulin
cADPR	cyclic adenosine diphosphoribose
cAMP	cyclic adenosine monophosphate
CCh	Carbachol
CD38	cyclic ADP Ribose hydrolase
CO_2	Carbon dioxide
d	Day(s)
DG	Diacylglycerol
DI	Deep Inspiration
DMEM	Dulbecco's Modified Eagles Medium
DRC	Dose-Response Curve
EC_{50}	Effector concentration to elicit 50% of maximal response
EDTA	Ethylenediaminetetraacetic acid
EFS	Electrical Field Stimulation
EGTA	Triethylene glycol diamine tetraacetic acid

FEV_1	Forced expiratory volume in one second
F_{max}	Maximal isometric Force
FVC	Forced vital capacity
GPCR	G-protein coupled receptor
h	hour(s)
H_1	Histamine receptor 1
HASMC	Human Airway Smooth Muscle Cells
Hsp90	Heat-shock protein 90 kDa
Hz	Hertz
IC_{50}	Inhibitor concentration to elicit 50% of maximal response
IgE	Immunoglobulin E
IL	Interleukin
IL-13	Interleukin-13
IL-13R	Interleukin-13 receptor
IL-1β	Interleukin-1 β
IL-4Rα	Interleukin-4 receptor α subunit
INFγ	interferon γ
IP_3	Inositol 1,4,5-triphosphate
ISO	Isoproterenol
Jak1	Janus Kinase 1
K^+	Potassium ions
KCl	Potassium Chloride
kDa	kiloDalton
LABA	Long-acting β_2-agonists
LC_{20}	20 kiloDalton myosin light chain

L_{ref}	Reference length
M	Molar
M_3	Muscarinic receptor 3
MCh	Methacholine
min	minute
ml	millilitre
MLCK	Myosin Light Chain Kinase
MLCP	Myosin Light Chain Phosphatase
MMP	Matrix metalloproteases
mm	millimeter
mM	millimolar
mN	milliNewton
mRNA	messenger ribonucleic acid
MYH11	Myosin heavy Chain
NaCl	Sodium Choride
$NaHCO_3$	Sodium Bicarbonate
NaH_2PO_4	Sodium Phosphate monobasic
$NaVO_4$	Sodium orthovanadate
ng	Nanogram
NO	Nitrous oxide
O_2	Oxygen
OD	Optical Density
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein Kinase A
PLC	phospholipase C
pSTAT6	phosphorylated STAT6

SABA	Short-acting β_2-agonists
SEM	Standard Error of Means
siRNA	small interfering ribonucleic acid
smMLCK	smooth muscle Myosin Light Chain kinase
SR	Sarcoplasmic Reticulum
STAT6	Signal Transducer and Activator of Transcription 6
TGF-β	Transforming Growth Factor- β
T_H1	T helper cell type 1
T_H2	T helper cell type 2
TNF-α	tumor necrosis factor α
Tyk2	Tyrosine Kinase 2
V	Volts
α-SMA	α-smooth muscle actin
μM	micromolar

Acknowledgments

There are many influential people that I want to express gratitude and appreciation to that have helped me over the course of this degree.

First and foremost, I would like to thank my supervisor Chun Seow for giving me the opportunity to join his laboratory and pursue my graduate education. I admire your calm hard-working nature and strive to embody it myself.

Secondly, I would like to express my immense appreciation and gratitude to my co-supervisor Peter Paré for his guidance throughout my project. Your genuine curiosity and unique perspectives towards every aspect of research is truly inspiring.

Next, I would like to thank Lu Wang who was an invaluable resource for me in the lab and provided me with countless advice and tips to ensure my experiments ran smoothly. Thank you for sharing your expertise and being patient with me.

Additionally, I would like to thank my supervisory committee for their academic support and guidance.

I would also like to acknowledge my friends I have made while working in the lab. Chris Pascoe, without you, starting out in the lab would have been a lot more difficult. Your ideas and guidance for my project has helped me tremendously. Bo Lan, you are a genuine friend and I am continuously inspired by your work ethic and research productivity. Jeffrey Liu, your relaxed and humorous character have made my time here more enjoyable and fun. Finally, Brandon Norris, I am thankful for your well-timed advice during hard times and your generosity.

Dedication

I would like to primarily dedicate this work to my parents: Karen and Chris. Thank you for inspiring my interest in science and providing me with constant guidance and support.

Also, I dedicate this work to all the friends I have made since relocating to Vancouver for my masters degree. You have given me so many great memories during my time here and I will continue to cherish them.

Chapter 1: Introduction[1]

1.1 An overview of asthma and airway inflammation

The worldwide prevalence of asthma is steadily increasing with recent reports estimating 300 million asthmatics globally, or 4.3% of the entire population (Loftus & Wise, 2016). This rise has been documented in most areas of the world and is expected to continue into the foreseeable future, especially in urbanized countries (Lundbäck et al., 2016; Woodruff, Bhakta, & Fahy, 2016). In Canada however, the prevalence of asthma has held fairly steady from 2010-2014 at about 2.5 million diagnosed cases, yet this still represents 8.1% of the total population over 12 years of age (Statistics Canada, 2016). Furthermore, a recent systematic review examining the burden of asthma in Canada reported that the estimated direct costs of the disease ranged from $46 million for patients aged 5-55 years in British Columbia to $140 million for patients aged 0-9 years in Ontario over separate 4 year periods (adjusted to 2011 Canadian dollars)(Ismaila, Sayani, Marin, & Su, 2013). The indirect costs could be many times that of the direct costs.

Asthma is defined as a chronic inflammatory disorder of the airways that causes reversible airway obstruction (Mims, 2015). Although asthma is recognized as a heterogeneous disease, it can be generally characterized by four overarching hallmarks: bronchoconstriction, chronic inflammation of the airways, airway hyperresponsiveness (AHR), and airway remodeling (Jean Bousquet, Jeffery, Busse, Johnson, & Vignola, 2000). Common persistent

[1] Sections of this chapter are partially based on the published review by Chris D. Pascoe*, Nicholas E. Swyngedouw*, Chun Y. Seow, and Peter D. Paré. 2014. Gene expression in asthmatic airway smooth muscle: a mixed bag. *CJPP* 93(2): 137-143. (* co-first author).

symptoms leading to the clinical diagnosis of asthma are wheezing, cough, chest tightness, and/or shortness of breath together with reversible airway obstruction (Lougheed et al., 2012; Wenzel, 2006). In addition, pulmonary function tests are used to confirm this diagnosis, specifically spirometry, to demonstrate reversible airway obstruction, variability in peak expiratory flow, and/or a positive challenge test using methacholine (MCh) or exercise are useful diagnostic tools (Lougheed et al., 2012). According to the guidelines set by the Canadian Thoracic Society, spirometry results that are congruent with asthma diagnosis are reduced FEV_1/FVC ratio compared to normal (usually <0.75-0.8 in adults) and >12% increase in FEV_1 in response to a bronchodilator (FEV_1: forced expiratory volume in one second; FVC: forced vital capacity) (Lougheed et al., 2012).

Management of asthma utilizes treatments directed at either fast-acting relief of symptoms (relaxing of airway smooth muscle to open the airways) or the long-term control of symptoms (reducing airway inflammation) (Durrani & Busse, 2014). In the former category, bronchodilators, such as β_2-agonists, anticholinergic and anti-leukotriene drugs, are prescribed, whereas for the latter type of treatment, glucocorticoids are used to control inflammation (Barnes, 1995; Cazzola, Page, Calzetta, & Matera, 2012). While asthma is usually considered a manageable disease, with conventional therapies being generally effective treatment options, there remain about 5-10% of asthmatic patients who are relatively insensitive to glucocorticoid treatment (Durham, Adcock, & Tliba, 2011).

1.1.1 Asthma pathophysiology

In descriptions of the pathology of asthma, two cell types are particularly relevant: epithelial cells that predominantly contribute to initiating inflammation and producing mucus, and the airway smooth muscle (ASM) cells that are responsible for excessive airway narrowing (Erle & Sheppard, 2014). Of particular focus to this thesis is how ASM behaves in asthma, and as such, many of the following sections will concentrate on the ASM tissue and cells over other airway tissue types (see section 1.3). Besides bronchoconstriction and inflammation, which will be overviewed in Sections 1.2 and 1.1.2, respectively, asthma pathology imparts a hyperresponsiveness to the airways as well as structural changes (remodeling) (Woodruff et al., 2016).

Airway Hyperresponsiveness (AHR) is a phenomenon where relatively little provocation by a spasmogen, or contractile agonist, causes excessive contraction of ASM leading to bronchoconstriction and obstruction of the airways (Doeing & Solway, 2013). This increase in sensitivity is not specific for any types of spasmogen and can be visualized on a dose-response curve of a spasmogen inhalation challenge with a leftwards shift of the curve (more sensitive) accompanied by an increased slope and maximal response (reactivity) (West et al., 2013). While it is largely agreed that ASM mediates this hyperresponsive narrowing, it remains unclear if AHR is due to intrinsic differences in the ASM or because of the influence of the inflammatory milieu in which the muscle resides (Bossé, Chapman, Paré, King, & Salome, 2011; Lauzon & Martin, 2016; Pascoe, Swyngedouw, Seow, & Paré, 2014; West et al., 2013). The true cause of the exaggerated airway narrowing seen in AHR is unclear and is likely due to a complex mix of contributing factors such as increased ASM amount, increased ASM contraction, diminished

relaxation, and/or reduced response to deep inspiration (Lauzon & Martin, 2016; West et al., 2013). In addition, airway remodeling can amplify the airway narrowing produced by a given amount of airway smooth muscle shortening (Moreno, Hogg, & Paré, 1986). While ASM is likely a principal contributor to AHR, the airway inflammation present in asthma could further exacerbate AHR.

In healthy individuals, acute inflammation is a beneficial process for tissue repair and functional regeneration; however when this inflammation becomes chronic it can lead to altered structural repair (Jean Bousquet et al., 2000). Hence, the consequence of chronic inflammation in asthma is a remodeling of the airway tissues that further contributes to the worsening of symptoms (West et al., 2013). Remodeling in asthmatics is characterized as an overall increased thickness of the airway walls due to a prominent increase in ASM mass, goblet cell hyperplasia and subepithelial fibrosis (Jean Bousquet et al., 2000; Mims, 2015; Woodruff et al., 2016). In mild to moderate asthma, hyperplasia of the ASM can be observed while in severe cases of asthma hypertrophy of the ASM can be seen as well (West et al., 2013; Woodruff et al., 2016). Overall, the effect of airway remodeling on breathing is a composite of airflow resistance, airway blockage due to ASM contraction, mucous and inflammatory exudates plugging of the airways, and increasing surface tension favoring airway closure (Jean Bousquet et al., 2000).

1.1.2 Airway inflammation

Asthma is predominantly referred to as a chronic inflammatory disorder of the airways where many different cell types play a role in the pathology (Locksley, 2010; Mims, 2015).

Recently, more emphasis on the inflammatory aspect of asthma has been made such as designating particular phenotypes of asthma based on the types and populations of inflammatory cells found in asthmatic airways, or whether targeting specific inflammatory cells or cytokines are helpful treatment options (Doeing & Solway, 2013; Woodruff et al., 2009). Inflammation in asthma, can likely be attributed to a mixture of genetic predisposition, environmental factors and exposures, and perhaps even alterations in the microbiome of patients (Locksley, 2010; Mims, 2015).

Basic immune responses are usually distinguished as being either Type 1 or Type 2 and are regulated by $CD4^+$ T cells identified as T helper 1 (T_H1) or T helper 2 (T_H2) cells (Fahy, 2015). While the type 1 immune response is characterized by extensive phagocytic activity, type 2 immune responses are associated with increased IgE antibody production and eosinophilia stimulated by T_H2-produced cytokines (Fahy, 2015). In asthma, a major molecular mechanism for disease pathogenesis is type 2 inflammation and it is present in the majority of asthmatics (Fahy, 2015; Woodruff et al., 2009).

An airborne allergen is the usual culprit for inducing T_H2 inflammation. Many allergens possess protease activity or other properties that allow them to penetrate through mucus and epithelial barriers reaching myeloid cells (macrophages, dendritic cells, and/or mast cells) where an allergic reaction can occur (Fahy, 2015; von Bubnoff, Geiger, & Bieber, 2001). The first time the allergen is encountered, sensitization occurs whereby antigen presenting cells (APCs) take up the allergen and migrate to lymph nodes where they cause priming of naïve T cells to develop into T memory cells (von Bubnoff et al., 2001). When an allergen is encountered by an APC with the specific immunoglobulin E (IgE) it binds and initiates a reaction cascade ultimately

leading to chronic inflammation (von Bubnoff et al., 2001). These IgE-mediated responses, referred to as Type 1 hypersensitivity, are implicated as a cause for allergic asthma and provoke the release of stored histamine and other inflammatory mediators to surrounding tissues (Fuller et al., 1986).

1.1.2.1 Inflammatory mediators

Allergic inflammation involves the release of inflammatory mediators (e.g. cytokines, chemokines, growth factors, enzymes) that contribute to the development of asthma symptoms, nonspecific AHR, and remodeling (Fernandes et al., 2003; Pascoe et al., 2014). Some examples of asthmatic inflammatory mediators are cytokines [Interleukin(IL)-4, IL-13], chemokines, growth factors (transforming growth factor-β; TGF-β), histamine, leukotrienes, matrix metalloproteases (MMPs), and nitrous oxide (NO).

While not generally regarded as a cell involved in inflammation, ASM can participate in inflammatory mechanisms by interacting with T lymphocytes, eosinophils, and neutrophils. Additionally, ASM can respond to, and secrete, many inflammatory mediators to regulate immune responses (Xia et al., 2013; Zuyderduyn, Sukkar, Fust, Dhaliwal, & Burgess, 2008). In cultured cell experiments, inflammatory mediators, such as interleukin-13, have been reported to influence the expression of contractile genes and proteins in ASM (see section 1.3.1) (Pascoe et al., 2014).

1.1.2.1.1 Interleukin-13

Of all the inflammatory mediators involved in asthma, interleukin-13 (IL-13) is arguably the most well recognized cytokine with widespread affects in asthmatic airways and prominent involvement in disease pathology. IL-13 is produced by activated $CD4^+$ T_H2 lymphocytes and shares some functional similarities to IL-4 including a common receptor subunit (IL-4Rα) (Khurana Hershey, 2003; Zhu et al., 1999). Downstream of the polymeric IL-13 receptor (consisting of IL-4Rα and IL-13Rα1 subunits), IL-13 binding induces intracellular changes via activation of Janus kinases on the intracellular domain of its receptor which subsequently leads to the phosphorylation of Signal Transducer and Activator of Transcription 6 (STAT6) (See Figure 1.1) (Erle & Sheppard, 2014; Laporte et al., 2001). STAT6 then dimerizes and translocates to the nucleus to modulate transcription (Akiho, Blennerhassett, Deng, & Collins, 2002; Laporte et al., 2001). IL-13 has been shown to modulate the expression of eoxatin and RhoA in ASM (Chiba et al., 2009; Moore et al., 2002) plausibly via a STAT6-dependent mechanism similar to airway epithelial cells (Matsukura et al., 2001). In a transgenic mouse model, targeted pulmonary over-expression of IL-13 caused mononuclear and eosinophilic inflammation, mucus cell metaplasia, Charcot-Leyden-like crystal deposition, fibrosis, eotaxin production, airway obstruction and nonspecific AHR (Zhu et al., 1999). The mechanism by which IL-13 induces asthma pathology and inflammation, however, seems to be independent from IgE and eosinophils (Wills-Karp et al., 1998). It has become increasingly clear that IL-13 is a key mediator of allergen-induced asthma since it can cause many of the pathophysiological features of the disease. The effect of IL-13 on ASM gene expression and mechanics has been widely researched and will be further reviewed in Section 1.3.

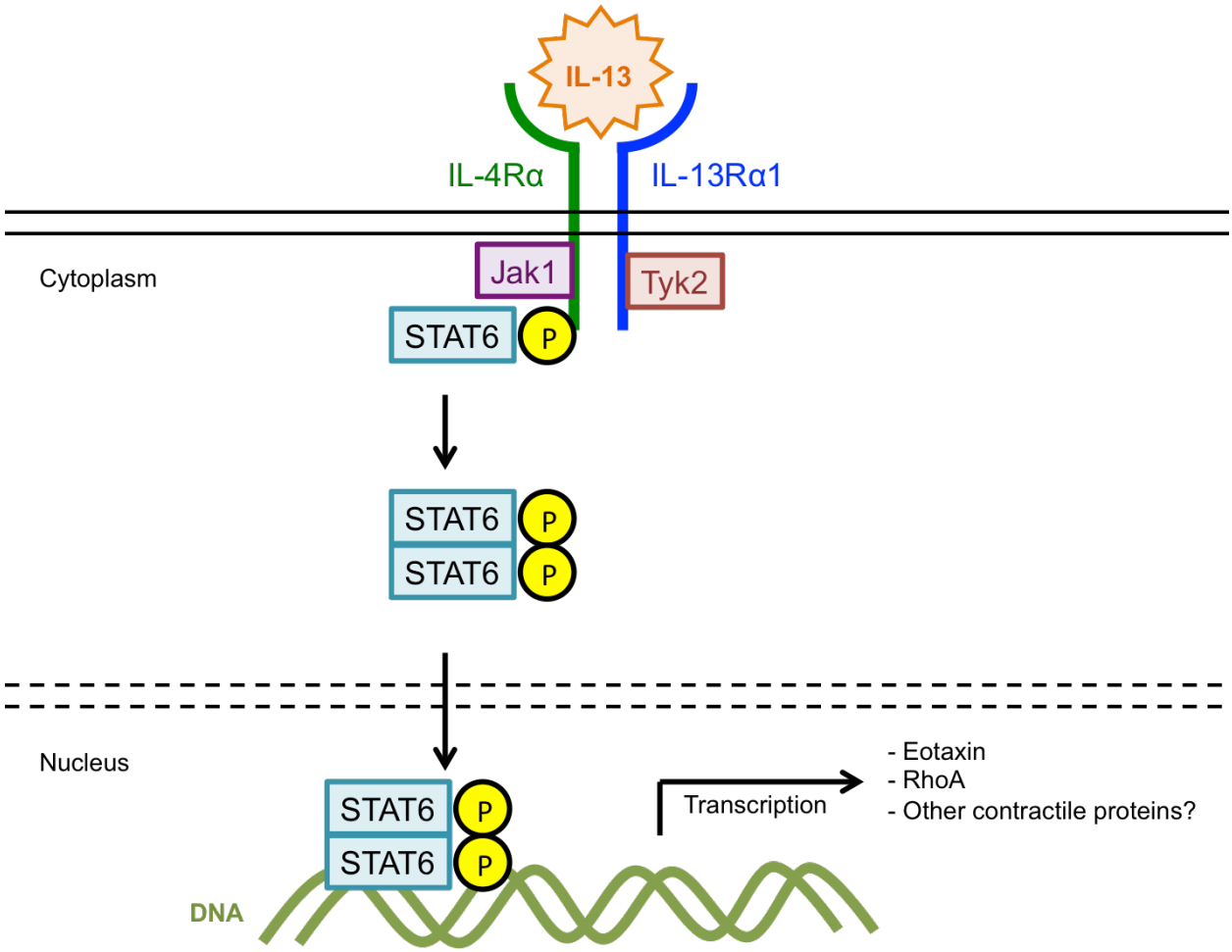

Figure 1.1 Signaling downstream of IL-13 leading to phosphorylation of STAT6. IL-13 binds to the cell membrane via its receptor that is composed of the IL-4Rα subunit and the IL-13Rα1 subunit. On the intracellular domain of the receptor, the Janus Kinases, Tyk2 and Jak1 mediate the signal transduction of IL-13 binding and lead to the phosphorylation of STAT6. Phosphorylated STAT6 (pSTAT6) then dimerizes in the cytoplasm and translocate to the nucleus where it induces transcription changes within the cell. Genes that have been reported to be induced by IL-13 in a STAT6-depended manner are Eotaxin and RhoA. Potentially STAT6 could induce other genes related to the contractile pathway in ASM.

1.2 Smooth muscle physiology

Smooth muscle cells line the walls of various organs in the body including the blood vessels, stomach, bladder, intestines, uterus, and airways (Webb, 2003). Like other forms of muscle, when activated the myocytes shorten in length to perform their primary physiological function: contraction. Smooth muscle contraction in hollow organs aids in regulating flow or expelling the contents of the lumen (Webb, 2003). Unlike skeletal or cardiac muscle, smooth muscle is not striated and receives autonomic neural innervation (Webb, 2003). A key unique feature of many types of smooth muscle, including ASM, is their ability to generate maximal force over a great range of lengths after experiencing an initial decrease in force due to a change in length in a phenomenon known as Length Adaptation (Bossé, Sobieszek, Paré, & Seow, 2012). As is pertinent to this thesis, the overview of smooth muscle physiology in section 1.2 will focus primarily upon ASM.

ASM does not fulfill a physiological function as obviously as its vascular or gastrointestinal counterparts. In adult lungs, the very existence of ASM seems paradoxical with an apparent role in asthma pathogenesis and no clear beneficial function (Mitzner, 2004; C. Y. Seow & Fredberg, 2001). While perhaps a vestigial organ akin to the appendix, there is however evidence to suggest a function for ASM in fetal development (Schittny, Miserocchi, & Sparrow, 2000). What is known about ASM is that it plays a critical role in the pathogenesis of asthma and an improved understanding and appreciation of its physiology is necessary for elucidating the relationship between airway inflammation and excessive contraction and how they contribute to AHR.

1.2.1 Smooth muscle contraction

In order to understand bronchoconstriction in asthma, we first require an overview of smooth muscle contraction and the signal transduction mechanisms therein. *In vivo*, the contractile process is regulated by agonist-receptor interaction and stretch activation (Webb, 2003). Similar to striated muscle, an increase in intracellular Ca^{2+} concentration causes contraction by initiating cyclic actin-myosin cross-bridge interactions (Hartshorne & Gorecka, 2011; Webb, 2003). This increase in intracellular (cytosolic) Ca^{2+} can be initiated in response to specific stimuli and is further amplified by release of Ca^{2+} stores from within the sarcoplasmic reticulum (SR) or from extracellular sources via sarcolemmal Ca^{2+} channels. Ca^{2+} then binds to the cytosolic protein calmodulin (CaM) forming a complex which goes on to activate myosin light chain kinase (MLCK) (Horowitz, Menice, Laporte, & Morgan, 1996; Ouedraogo & Roux, 2014). The initiation of the actomyosin cross-bridge interaction, which leads to muscle contraction, is directly regulated and enabled by the phosphorylation of the 20 kDa myosin light chain (LC_{20}) by MLCK (Horowitz et al., 1996). Thick (myosin) filament regulation in smooth muscle instead of thin (actin) filament regulation in striated muscle is an important feature distinguishing these two muscle types (Paul, 2009; Webb, 2003). The physical cycling of the cross-bridges is enabled by energy released from the myosin ATPase-mediated hydrolysis of ATP.

Extracellular agonists are the primary means of initiation for calcium-dependent contraction (see section 1.2.1.1) via specific G-protein coupled receptor (GPCR) mechanisms. Binding of agonists initiates a GPCR signaling cascade whereby a phospholipase C (PLC) cleaves the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate

diacylglyerol (DG) and inositol 1,4,5-triphosphate (IP_3) which both act as second messengers. IP_3 binds to its receptor on the SR and liberates calcium into the cytoplasm (Hakonarson & Grunstein, 1998; Webb, 2003). Selected contractile agonists relevant to asthma and this thesis will be reviewed in the following section.

1.2.1.1 Contractile agonists

Perhaps the two most well recognized contractile agonists involved in ASM-mediated bronchoconstriction are the physiological agonists acetylcholine (ACh) and histamine (Erle & Sheppard, 2014; Ouedraogo & Roux, 2014; Pelaia et al., 2008). Other large groups of contractile agonists are leukotrienes and prostaglandins (Krell et al., 1981; Madison, Jones, Sankary, & Brown, 1989). Bronchoconstriction is stimulated through efferent cholinergic parasympathetic innervation of ASM via the vagus nerve (Eglen, Hegde, & Watson, 1996). ACh is the cholinergic neurotransmitter released from the efferent nerve and binds to the M_3 muscarinic receptor on the ASM cell membrane initiating contraction via a GPCR mechanism (Erle & Sheppard, 2014; Pelaia et al., 2008). Histamine, which also plays the role of an inflammatory mediator, can cause contraction of ASM as well. In asthma, the primary source of histamine is storage granules within mast cells and basophils and it can be released leading to ASM contraction via the H_1 receptor (also a GPCR) (Erle & Sheppard, 2014). In asthmatics, increased sensitivity to these agonists is a characteristic of AHR. Additionally, ASM contraction can be initiated in vitro by increasing extracellular concentration of KCl in Krebs saline solution (also known as High K^+ solution) (Risse et al., 2011). This method of inducing ASM contraction

bypasses agonist-specific GPCR mechanisms and acts via voltage-operated Ca^{2+} channels which depolarizes the cellular membrane and increases intracellular [Ca^{2+}].

1.2.2 Smooth muscle relaxation

Like two sides of the same coin, what would muscle contraction be without relaxation? Muscle relaxation is especially relevant in asthma given that it is the desired outcome of a bronchodilator for a patient suffering from an asthma exacerbation – to relax the ASM and open the airways. Similar to contraction, relaxation of ASM centers around the regulation of the myosin light chain phosphorylation (Webb, 2003). Intuitively, the reverse of contractile mechanisms can generally cause relaxation: dephosphorylation of LC_{20} by myosin light chain phosphatase (MLCP) or a decrease in intracellular Ca^{2+} concentration. Intracellular Ca^{2+} can be lowered via the Ca,Mg-ATPase transporters either on the plasma membrane (removal of Ca^{2+} from the cell) or the SR membrane (sequestration of Ca^{2+} back into the SR lumen) (Webb, 2003).

Therapeutically-induced bronchodilation is generally achieved by inhaling agonists that stimulate the β_2-adrenergic receptors present on ASM (Kotlikoff & Kamm, 1996; Morgan et al., 2014). Stimulation of the β_2 receptor (a GPCR) causes the G_s protein to activate adenylyl cyclase (AC) which catalyzes the formation of cyclic AMP (cAMP) from ATP. cAMP then activates protein kinase A (PKA). PKA is thought to then promote relaxation via two mechanisms: 1) directly inhibiting MLCK thereby promoting dephosphorylation of LC_{20} by MLCP and 2) inhibiting PLC and therefore the IP_3-mediated Ca^{2+} release from the SR (Hakonarson & Grunstein, 1998; Ouedraogo & Roux, 2014). While the exact mechanism of how β_2-adrenergic

receptor agonists cause relaxation remains not entirely clear, PKA has been suggested as the key regulator by which the agonists exert their relaxant effects (Morgan et al., 2014). Additionally, antileukotrienes have been included in combination therapy for asthmatics along with corticosteroids due to their anti-inflammatory and bronchodilatory effects (Chun Y Seow & van Eeden, 2013).

1.2.2.1 Relaxant agonists

While their primary function remains essentially the same, β-agonists used to induce ASM relaxation for research purposes differ in structure and efficacy from those used as asthma medication. β_2-agonists used to treat asthma are grouped by their duration of action with short-acting β_2-agonists (SABAs) such as albuterol, fenoterol and terbutaline working for 4-6 hours and long-acting β_2-agonists (LABAs) such as salmeterol, and formoterol, having a duration up to 12h (Cazzola, Page, Rogliani, & Matera, 2013). For research purposes, the non-selective β-agonist isoproterenol (ISO) can be used to induce ASM relaxation however it is inappropriate for therapeutic use due to its cardiovascular effects and a short duration of action (Cazzola et al., 2012). When examining the effect of IL-13 on ASM relaxation, ISO has been used in many studies as a β-agonist (Grunstein et al., 2002; Laporte et al., 2001; Nino et al., 2012).

1.3 Airway smooth muscle in asthma

The recent notion that asthmatic ASM is "good muscle" in a "bad environment" has been suggested and as such would exonerate ASM of guilt and place the blame instead on inflammation-derived spasmogens or other factors related to the inflammatory environment (Bossé et al., 2011; Paré & Mitzner, 2012). Whether or not this is indeed true, it presents an interesting idea to set the stage for studies investigating the influence of inflammatory mediators found in asthmatic airways on ASM contraction. While it remains apparent that ASM contraction undoubtedly contributes to AHR, the muscle cell itself may not be inherently abnormal – just under a "bad" influence (Pascoe et al., 2014). To date there has been no clear consensus reached about whether or not AHR in asthma is due to phenotypic differences in ASM (Gunst & Panettieri, 2012; Paré & Mitzner, 2012). This section is meant to overview the cellular changes of ASM in asthma and to examine the effect an inflammatory environment has on the cell function with a particular emphasis on IL-13.

1.3.1 Mechanical changes in asthmatic airway smooth muscle

If excessive airway narrowing could be explained by a phenotypic change in ASM, increased force production would be the first parameter to investigate (Pascoe et al., 2014). However, a recent study demonstrated that if force is normalized to the amount of ASM present in the airways, asthmatic ASM does not produce higher force (per cross-sectional area of the muscle cells) than normal ASM (Chin et al., 2012). If differences in shortening capacity or velocity are analyzed the results have been more heterogeneous with no difference between

asthmatic and normal being reported in some studies (Bai, 1991; Chin et al., 2012; Ijpma et al., 2015) as well as an increase in shortening capacity/velocity in asthmatics in others (Ma et al., 2002; Matsumoto et al., 2007). An increase in shortening capacity could contribute to AHR by narrowing the airways more extensively while an increase in shortening velocity could possibly lead to faster shortening between breaths (Bullimore et al., 2011; Pascoe et al., 2014). Another mechanical difference between asthmatic and normal ASM is in the muscle's response to strain induced by deep inspirations (DIs) (Pascoe et al., 2014). In non-asthmatics, a DI can dilate airways after they have been narrowed via inhalation of a spasmogen (Pascoe et al., 2014). This bronchodilating effect of DI in non-asthmatics is absent or markedly lessened in asthmatic patients (Fish, Peterman, & Cugell, 1977; Lim, Ang, Rossing, Ingenito, & Ingram, 1989; Skloot & Togias, 2003). Further evidence for this was provided by Chin et al. who demonstrated that asthmatic ASM was less responsive to length oscillations that mimicked DIs than the muscle of non-asthmatics (Chin et al., 2012).

Several studies have examined the influence of IL-13 on the mechanical properties of ASM using various tissue sources, culture models, agonists, and measured variables. Tliba et al. demonstrated that in mouse tracheal rings, IL-13 (100 ng/mL; 24h) treatment significantly increased both the carbachol (CCh) and KCl-induced maximal force generation in ASM with no affect on sensitivity (Tliba et al., 2003). Farghaly et al. replicated this result in mouse tracheal rings finding that IL-13 treatment (10 or 100 ng/mL; 24h) increased contraction induced by CCh or KCl over control with no change in sensitivity (EC_{50}) (Farghaly, Blagbrough, Medina-Tato, & Watson, 2008). Also in mouse tracheal rings, Kudo et al. found that IL-13 treatment (100 ng/mL; 12h) significantly increased the force of contraction in response to methacholine (MCh) (Kudo et

al., 2013). Similarly, Sugimoto et al. found that IL-13 treatment (100 ng/mL;12h) increased MCh-induced contraction (force) in mouse tracheal rings (Sugimoto et al., 2012). In cultured murine bronchial tissue, Chiba et al. found that IL-13 (100 ng/mL; 12h) significantly increased ACh-induced contraction, however response to KCl-induced contraction was unchanged (Chiba et al., 2009). In mouse gastrointestinal smooth muscle, IL-13 (10ng/mL; 16h) has been found to increase contraction in response to CCh in cultured cells (Akiho et al., 2002). Using an in vivo model, Zhao et al. showed that injections of IL-13 (100 μg/mL per day for 7 days) into mouse jejunum resulted in increased contractility (tension) induced by ACh or electrical field stimulation (EFS) (Zhao et al., 2003).

Using rabbit tracheal rings, Grunstein et al. found that IL-13 treatment (20 ng/mL;24h) imparted enhanced ACh-induced contractility and diminished ISO-induced relaxation (Grunstein et al., 2002). Nino et al. used rabbit ASM tissue to show that IL-13 treatment (50 ng/mL; 24h) resulted in increased isometric force generation in response to ACh and impaired relaxation to ISO (Nino et al., 2012). In cultured human ASM cells (HASMC), Risse et al. found that IL-13 treatment (10 ng/mL; 24h) increased muscle stiffness induced by histamine over control, however KCl-induced stiffness was unchanged (Risse et al., 2011). Also in cultured HASMC, Laporte et al. found that IL-13 (50 ng/mL; 24h), but not IL-4, attenuated the ISO-induced decrease in stiffness over control (Laporte et al., 2001).

Taken as a whole, the above literature review on IL-13 treatment of ASM reveals two key themes: 1) no clear consensus exists on the effect of IL-13 treatment on ASM mechanics with inconsistency plaguing the literature ranging from a broad range of contractile agonists used to

induce contraction, to the types of output measured, and 2) the majority of studies generally use an IL-13 concentration in the range of 10-100 ng/mL and expose tissues to IL-13 for roughly 24h.

1.3.2 Gene and protein expression changes in asthmatic airway smooth muscle

If inflammation is able to influence the mechanical properties of ASM it would presumably do so by changing the expression of genes or proteins involved in its structure or in the contractile pathway. Genetically programmed or acquired expression changes for major contractile proteins such as MLCK, myosin heavy chain (MYH11), α-smooth muscle actin (α-SMA), or other proteins related to contraction such as CD38, RhoA, or the H_1 receptor could plausibly contribute to changes in ASM mechanical properties such as force generation, shortening capacity and velocity, and could present as interesting therapeutic targets for exaggerated airway narrowing in AHR. This section is meant to provide a brief overview of gene expression changes seen in ASM induced by inflammatory mediators with a particular focus on IL-13 and contractile genes. For an illustrative summary of literature review in this section, see Figure 1.2.

Recently, Balhara et al. found that stimulating cultured ASM cells with IgE leads to an upregulation of smooth muscle MLCK (smMLCK) mRNA and protein expression (Balhara, Redhu, Shan, & Gounni, 2014). IgE is consistently elevated in the sera of many asthmatics compared to non-asthmatics and plays a crucial role in allergic response (Borish et al., 2005). Balhara et al. showed that in cultured ASM, smMLCK mRNA levels were significantly

increased after 6h (1.5 fold), 24h (2.2 fold), and 48h (1.5 fold) of exposure to IgE. Similarly, smMLCK protein was significantly upregulated after 48h (2.5 fold) of culturing with IgE (Balhara et al., 2014). The authors further postulated that their results suggest a plausible role for IgE in regulating the contractile machinery of ASM through MLCK expression.

The myosin heavy chain (MYH11) is one of two major functional proteins responsible for contraction in ASM, the other being actin. The structure of MYH11 consists of a phosphorylatable neck region and a head region that binds to actin to shorten muscle (Sellers, Spudich, & Sheetz, 1985). Similarly to MLCK, the inflammatory environment in asthma could influence the expression of MYH11. Lee et al. conducted a microarray experiment to examine the expression of approximately 6500 genes in ASM and other airway cells in response to IL-13 treatment (Lee et al., 2001). In cultured HASMC, it was found that IL-13 treatment (100 ng/mL; 6h) caused a 3.61-fold increase in MYH11 gene expression (Lee et al., 2001). This study provides evidence to suggest that IL-13 from the inflammatory milieu could influence the contractile response of ASM and contribute to AHR.

As previously mentioned, in ASM, the other major contractile counterpart to myosin is actin (α-SMA) which together allow for contraction. Woodman et al. examined the expression of α-SMA in cultured HASMC either when co-cultured with mast cells or when mast-cell derived β-tryptase was added to the media (Woodman et al., 2008). In both instances, they found that treatment increased the HASMC expression of α-SMA. Furthermore, Woodman et al. observed that TGF-β secretion from ASM and agonist-induced contraction were increased in the presence

of β-tryptase (Woodman et al., 2008). The results of this study suggest that β-tryptase increases TGF-β secretion in ASM which leads to the upregulation of the α-SMA protein.

Cyclic ADP ribose (cADPR) hydrolase, otherwise known as CD38, is an enzyme present on the cytosolic cell surface that plays a role in intracellular Ca^{2+} regulation (Deshpande, Walseth, Panettieri, & Kannan, 2003; Malavasi et al., 2008). CD38 controls the degradation and synthesis of cADPR which is a regulator for Ca^{2+} release from the SR via the ryanodine receptor channel (Deshpande et al., 2003). Because of its control over Ca^{2+} release via cADPR, an increase in CD38 could feasibly lead to increased intracellular Ca^{2+} levels by modulating its release from the SR and altering contractile functions (Deshpande et al., 2003). Deshpande et al. exposed cultured HASMC to the inflammatory cytokines tumor necrosis factor α (TNF-α; 20 ng/mL), IL-1β (20ng/mL), or interferon γ (INFγ; 1 IU/mL) for 22h and found CD38 mRNA expression to be upregulated in all cases however only TNF-α was significantly higher than control (Deshpande et al., 2003). Similarly, CD38 protein was upregulated in all cases but only TNF-α caused a statistically significant increase. In their next follow-up study, Deshpande et al. investigated the effect of IL-13 (50 ng/mL; 22h) on CD38 expression in cultured HASMC and found that both mRNA and protein expression were increased 2.5 fold over control (Deshpande et al., 2004).

RhoA is a small G protein that targets the downstream Rho kinase pathway which directly regulates MLCP activity (Webb, 2003). Rho kinase phosphorylates MLCP inhibiting it from binding to MLC therefore promoting the phosphorylated (contractile) state of MLC (Webb, 2003). Goto et al. examined the effect of TNF-α (10 ng/mL; 12 and 24h) treatment on RhoA

mRNA and protein expression in cultured HASMC and found significant increases in the expression of both (Goto, Chiba, Sakai, & Misawa, 2009). The same group investigated the effect of IL-13 (100 ng/mL) treatment on cultured HASMC and found that after 12h there was ~3-fold increase in protein expression (Chiba et al., 2009). Mouse ASM tissues subject to identical IL-13 treatment displayed significantly increased contractility in response to ACh compared to controls (Chiba et al., 2009).

The histamine H_1 receptor is another protein involved in ASM contraction that could have its expression modulated by inflammatory mediators. Jarai et al. investigated the influence of IL-13 treatment (10 ng/mL; 4h and 24h) on the mRNA expression of the *H_1 receptor* and found that there was a 2.5-fold increase in expression over control (Jarai et al., 2004). Syed et al. found a similar result when they subjected cultured HASMC to IL-13 (50 ng/mL; 6 and 18h) although less pronounced with a 1.5 fold increase in *H_1 receptor* expression (Syed et al., 2005). An upregulation of the H_1 receptor expression could influence ASM contractility by two means: 1) more receptors present would lead to increased sensitivity to histamine-induced contractions and 2) ASM cells cultured with histamine were found to have a significant increase in intracellular Ca^{2+} stores (Kotlikoff, Murray, & Reynolds, 1987) and this could lead to augmented Ca^{2+} release in response to histamine and thus the extent of contraction as a result.

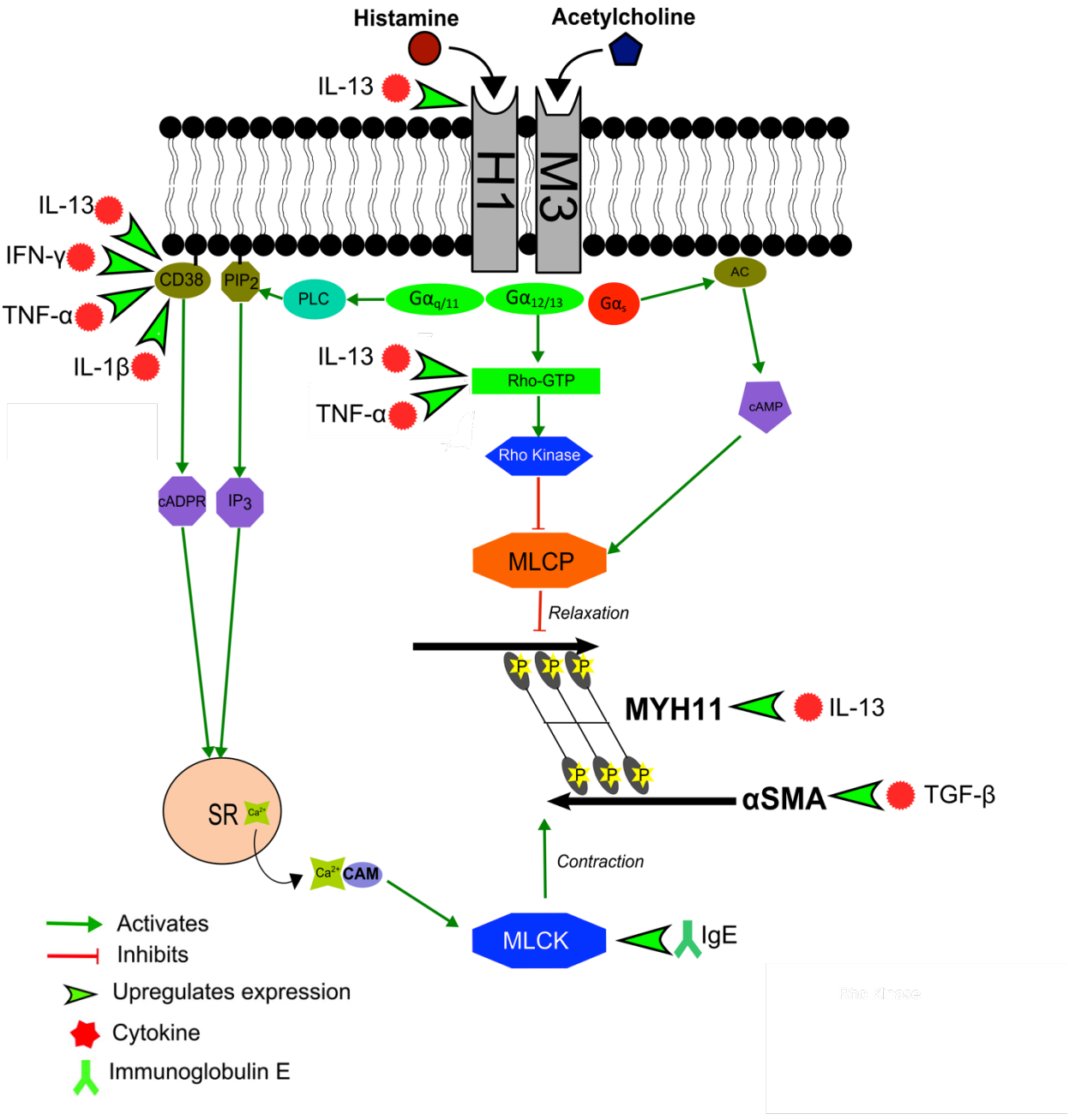

Figure 1.2 Canonical signal pathways in airway smooth muscle contraction. Green arrows indicate activation of the next step. Red stop arrows indicate inhibition of the next step. Green arrowheads indicate upregulation of gene or protein expression induced by the noted inflammatory mediator. In summary, airway smooth muscle (ASM) is stimulated by agonists binding to their respective G-protein coupled receptors on the cellular surface, histamine to the H_1 receptor, and acetylcholine to the M_3 receptor. Activation of the G-proteins leads to activation

of 3 pathways. The first, activated through the $G\alpha_{q/11}$ protein, leads to the generation of inositol trisphosphate (IP_3) from membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP_2) via phospholipase C (PLC). IP_3 then liberates calcium (Ca^{2+}) from the sarcoplasmic reticulum (SR) allowing Ca^{2+} to bind to calmodulin (CaM) to activate myosin light chain kinase (MLCK). MLCK phosphorylates myosin light chain 20 (LC_{20}), which is part of myosin, to activate cross-bridge cycling and subsequently muscle shortening or force development. In parallel, with this pathway, cyclic ADP ribose hydrolase (CD38) can produce the second messenger cyclic ADP ribose (cADPR) to liberate Ca^{2+} from the sarcoplasmic reticulum and eventually lead to muscle contraction. Activation of the $G\alpha_{12/13}$ protein activates Rho kinase to inhibit myosin light chain phosphatase (MLCP) and lead to what is known as calcium sensitization by preventing MLCP from dephosphorylating the LC_{20}. The G-protein $G\alpha_s$ is the protein that activates the relaxing pathway in airway smooth muscle. $G\alpha_s$ activation leads to cyclic AMP (cAMP) production from adenylyl cyclase (AC). cAMP activates MLCP to initiate dephosphorylation of MLC_{20} and cross-bridge detachment and eventual muscle relaxation. There are numerous inflammatory mediators or cytokines shown in the figure that can upregulate the expression of a number of these proteins potentially altering the signaling pathways they are a part of. Briefly, interleukin-13 (IL-13) has been shown to upregulate the expression of the H_1 receptor, myosin heavy chain (MYH11), RhoA (Rho-GTP), and CD38. CD38 expression has also been shown to be upregulated by interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukin 1-beta (IL-1β). There is also evidence that transforming growth factor beta (TGF-β) upregulates the expression of alpha smooth muscle actin (αSMA) and that immunoglobulin E (IgE) upregulates the expression of MLCK. (Figure reproduced with permission from Pascoe et al. 2014, NRC Research Press).

1.4 Cell and tissue cultures of airway smooth muscle

Admittedly, despite the many advantages of using freshly prepared ex vivo ASM strips, the limited viability of the tissue presents a problem for experiments lasting more than a few hours. Utilizing culturing techniques on ASM cells or tissue prolongs their viability and allows for the long-term exposure to agents like inflammatory mediators or siRNA. This technique has been particularly useful for allowing long-term studies of ASM that examine mitogenic effects of growth, division, and response to prolonged stimuli (Hall & Kotlikoff, 1995; Hirst, 1996). Nevertheless, there are obvious differences that should be kept in mind between the responses of freshly dissected muscle strips and cultured ASM cells, beyond cross-species variation (Hall & Kotlikoff, 1995). There has also been the notion that cultured ASM could be useful for developing new therapeutics by acting as a secondary screening system (Hall & Kotlikoff, 1995).

1.4.1 Phenotypes of cultured airway smooth muscle cells

Freshly isolated ASM retains its contractile phenotype however once cultured in serum-rich media, the cells rapidly switch to a non-contractile proliferative phenotype (sometimes called the synthetic phenotype) (Ma, Wang, & Stephens, 1998; Zuyderduyn et al., 2008). In 1996, Halayko et al. reported markers for specific ASM cell phenotypes in culture which were dependent on culturing length (of time) and cell density (Halayko, Salari, MA, & Stephens, 1996). In primary culture it was found that the cells with a contractile phenotype were rich in smMHC, α-SMA, calponin, desmin, MLCK, β-tropomyosin, and *h*-caldesmon, while the proliferative phenotype expressed relatively more non-muscle MHC, *l*-caldesmon, PKC and

CD44 (Halayko et al., 1996). The proliferative phenotype demonstrates not only a reduction in contractile proteins but also diminished responsiveness to contractile agonists, increased responsiveness to proliferative mitogens, and increased production of extracellular matrix proteins and inflammatory cytokines (Zuyderduyn et al., 2008). When investigating phenotypic changes in ASM cells, Gosens et al. found that culturing ASM in 10% fetal bovine serum caused a time-dependent decrease in MCh-induced contraction compared to serum-deprived cells (Gosens et al., 2002). Whether switching between these phenotypes occurs *in vivo* has not been definitively determined, yet the possibility of different ASM phenotypic populations in the lung contributing to AHR or remodeling in asthma remains an intriguing idea to be investigated in the future.

1.5 Introduction to thesis research

With the preceding literature review in mind, some research questions that set the scope for this project are: 1) Can contractile changes induced by IL13 seen in cultured ASM cells also occur in isolated tissue strips? 2) Can treatment with the inflammatory mediator IL-13 produce a measurable change in the force output of ASM tissue? 3) Does the effect of this inflammatory mediator depend on the agonist used to induce contraction or relaxation?

The research project that I pursued throughout my masters program was designed to improve our understanding of the role ASM plays within the complex relationship between the inflammatory and hyperresponsive components of asthma. Furthermore, this work sought to highlight the distinction between cultured cell experiments in the literature and fresh tissue. In

this project, the effect that IL-13 has on the force output of fresh ASM tissue strips was investigated using a variety of agonists.

The research hypothesis, specific aims, and rationale are outlined in detail in the following chapter. In Chapter 3, the methods used to culture ASM tissue and measure force output are provided with specific protocols for each experiment performed. In Chapter 4, the data from each experiment are presented along with statistical analyses. Finally, Chapters 5 and 6 provide discussion points and conclusions drawn from this set of experiments and summarize the contributions of this thesis to the field of ASM research.

Chapter 2: Hypothesis, specific aims, and rationale

2.1 Hypothesis

The hypothesis of this thesis is that incubation with IL-13 will induce "hypercontractile" properties in airway smooth muscle (ASM) tissue strips. Specifically, these properties will include increased maximal force generation and sensitivity to contractile agonists, and diminished relaxation and sensitivity to ASM relaxants.

2.2 Specific aims and rationale

1) Develop and optimize an experimental protocol/model that allows ASM tissue strips to be exposed to inflammatory mediators for extended time periods without compromising tissue viability.

   Rationale: Although cell and tissue culture models exist for ASM, an optimized protocol is still required for the tissue type (ovine) used and for the types of mechanical experiments proposed.

2) Investigate intracellular signalling changes induced by IL-13 (pSTAT6) using Western Blotting.

   Rationale: Using the protocol created, confirmation that IL-13 treatment induces intracellular changes via STAT6 phosphorylation is necessary. This experiment will also confirm the presence of the IL-13R in the ASM strips. Presumably, if IL-13 induces signaling via pSTAT6, downstream transcription could affect contractile genes resulting in measurable changes in the mechanical properties.

The next three aims (2-4) involve investigations of the effects of IL-13 on the mechanical properties of ASM in response to different agonists.

3) Investigate the influence of IL-13 on the contraction of ASM tissue strips induced by a range of ACh concentrations (the dose-response relationship). Specifically, maximal force produced at increasing concentrations of ACh will be compared before and after 24h and 72h of IL-13 treatment.

   Rationale: As reviewed in Chapter 1, previous studies have demonstrated that IL-13 can cause increased contractility in cultured ASM and/or tracheal rings relative to control (Chiba et al., 2009; Grunstein et al., 2002; Nino et al., 2012). The purpose of the experiments is to verify whether IL-13 has the same effect on bovine ASM tissue preparation cultured in serum-free media.

4) Investigate the influence of IL-13 on the relaxation of ASM tissue strips induced by increasing concentrations of isoproterenol (ISO) when pre-contracted with ACh. Specifically, the decrease in force produced at increasing concentrations of ISO will be compared before and after 24h of IL-13 treatment.

   Rationale: As reviewed in Chapter 1, previous studies have demonstrated that IL-13 can attenuate relaxation induced by ISO (isometric force or stiffness) in cultured ASM cells or tracheal rings relative to control (Grunstein et al., 2002; Laporte et al., 2001; Nino et al., 2012). The purpose of the experiments is to verify whether IL-13 has the same effect on bovine ASM tissue preparation cultured in serum-free media.

5) Investigate the influence of IL-13 on the contraction of ASM tissue strips induced by Histamine and KCl. Specifically, the maximal force produced at a single concentration of histamine or KCl will be compared before and after 72h of IL-13 treatment.

   Rationale: As reviewed in Chapter 1, previous studies have demonstrated that IL-13 can influence the contractility of ASM (force or stiffening), although there is no consensus on KCl-induced contraction (Chiba et al., 2009; Risse et al., 2011; Tliba et al., 2003). The purpose of the experiments is to determine whether any changes in the mechanical properties are receptor specific (histamine in this case) and whether there is a non-receptor-specific (KCl-induced) change in the mechanical properties of IL-13 treated bovine ASM tissue preparation. The results will help us understand how the signaling pathways governing ASM contraction could be altered by the presence of the inflammatory mediator IL-13 in the lung.

Chapter 3: Materials and Methods

3.1 Tissue preparation and equilibration

Sheep tracheas used for all experiments were obtained from a local abattoir. The use of these tissues was approved by the Committees of Animal Care and Biosafety of The University of British Columbia. Immediately following the sacrifice of the animals, tissues were placed into Krebs solution (pH 7.4; 118 mM NaCl, 4 mM KCl, 1.2 mM NaH_2PO_4, 22.5 mM $NaHCO_3$, 2mM $CaCl_2$ and 2 g/L Dextrose). Upon arrival to the laboratory, tracheas were cleaned of blood and gross external connective tissues then stored at 4°C in fresh Krebs solution until required. Before tissue processing, the *in situ* length of the trachealis smooth muscle band was recorded as the reference length (L_{ref}) and was maintained throughout the remainder of the processing and subsequent experiment. First, tracheal segments were cut open through the cartilage opposite the smooth muscle band, then pinned epithelial-side down into a wax-covered dissection tray filled with Krebs solution. In order to isolate a smooth muscle strip, both adventitial tissue and epithelium was carefully removed resulting in a smooth muscle strip approximately 7 mm in length, 1.5 mm wide, and 0.25 mm thick. Clips of aluminum foil were attached lengthwise to either end of the smooth muscle strip in order to maintain the *in situ* length. These clips allowed for the strip to be mounted vertically on a dual mode muscle lever system and myograph (Aurora Scientifics, Aurora, ON) that recorded force output and muscle length simultaneously. The bottom clip was attached to a stationary hook while the top clip was attached to leaver of the force-length transducer by surgical thread. The distance between the clips was then adjusted to the original L_{ref}. Next, tissue strips were submerged into an organ bath maintained at 37°C by an

exterior water jacket. The inner bath was filled with Krebs solution containing 50 μM Indomethacin (Enzo Life Sciences, Farmingdale, NY) and was aerated with a carbogen gas mixture of 95% oxygen and 5% carbon dioxide. Indomethacin is a nonselective cyclooxygenase inhibitor that is included to prevent or reduce the development of active muscle tone by blocking prostaglandin synthesis. Hereafter, as pertaining to these experiments, "Krebs" will denote Krebs solution at 37°C and supplemented with 50 μM Indomethacin.

ASM tissue strips were then equilibrated with the following protocol in order to allow them to recover from the trauma of 4°C storage and dissection. The tissue strip was subject to electrical field stimulation (EFS; 60 Hz AC, Voltage 15 V) for a period of 9 seconds and repeated every 5 minutes (one cycle). After every cycle, the Krebs solution in the bath was drained and replaced with fresh aerated Krebs. The tissue was deemed equilibrated once a stable maximal isometric force (F_{max}) was reached between at least 3 cycles. No strips with baseline resting tension higher than 2% of F_{max} or a baseline F_{max} less than 40 mN were used in any subsequent experiment.

3.2 Overview of experimental design

All experiments conducted in this study that subject ASM tissue to IL-13 follow a similar protocol (Figure 3.1). A high-level overview of the general sequence will be summarized below with more detailed descriptions found in the specific section for each protocol. The initial part of every experiment was the isolation and dissection of a viable ASM tissue strip and the following equilibration thereof (Section 3.1). Next, baseline (t=0) mechanical measurements of the ASM

strip were recorded using the agonist of choice (Section 3.5). Each strip was randomly designated a particular condition (paired strips from the same trachea were either control or IL-13 treated) then carefully removed from the organ bath and pinned on wax at L_{ref}. ASM strips were then separately cultured to subject some strips to IL-13 for a lengthy period of time without sacrificing tissue viability and contractility (Section 3.3). After the designated time had elapsed, ASM strips were removed from culture media and brought back to the organ bath in order to assess they mechanics. Mechanical measurements of ASM strips taken after culturing were then compared both to their initial baseline values and to their paired strip (taken from the same trachea; either control or treated) to assess the influence of IL-13.

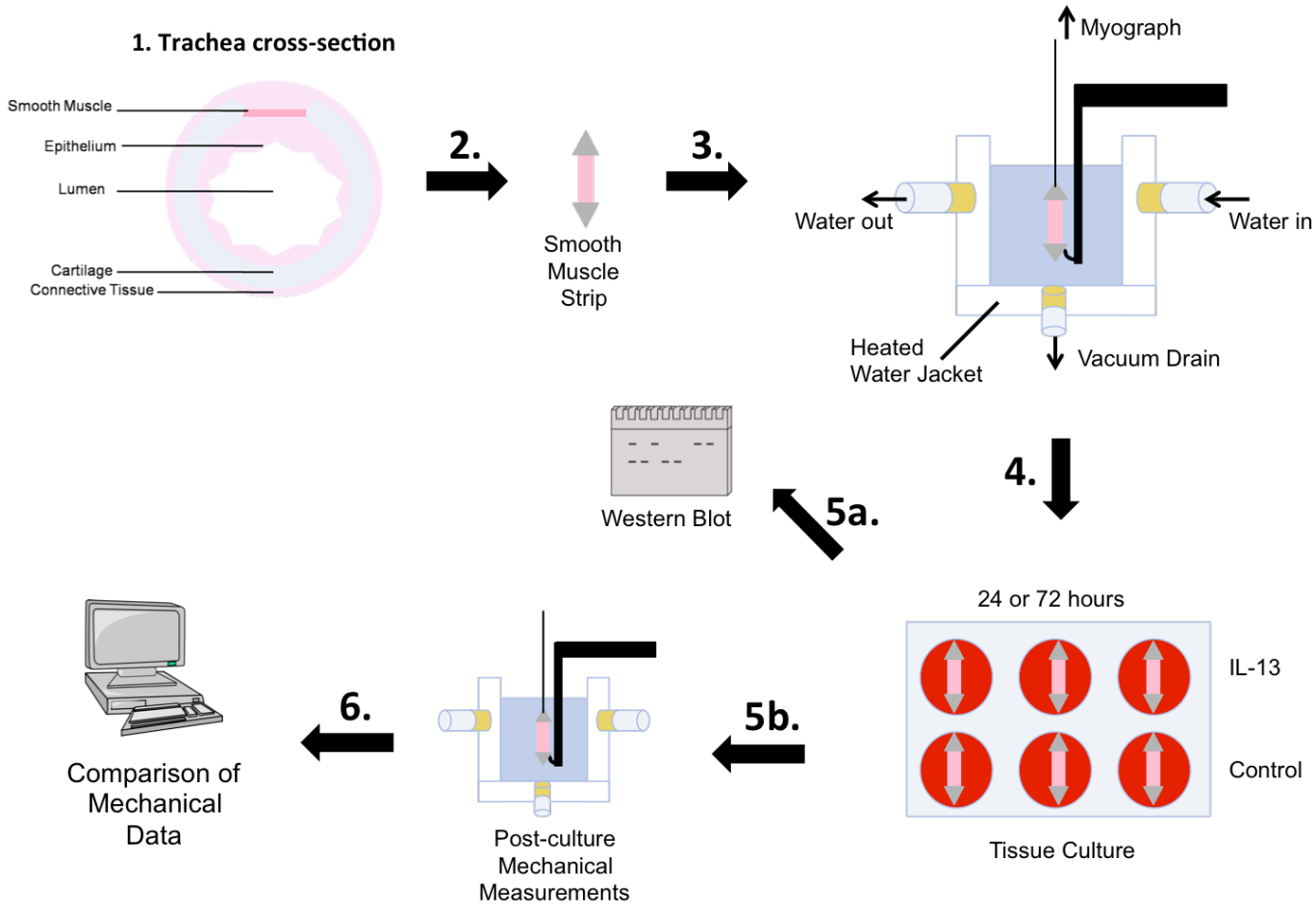

Figure 3.1 Schematic of methods used: overview of experimental design. 1) Dissection of ASM from fresh ovine trachea and removal of other tissues. 2) Preparation of smooth muscle strip. 3) Measurement of Force Generation at baseline (t=0) in response to agonist. 4) Tissue culturing with/without IL-13. 5a) Protein extraction for Western Blotting. 5b) Measurement of Force Generation after incubation 24/72h in response to agonist. 6) Comparison of mechanical data and statistical analysis of data.

3.3 Tissue culture

Once the baseline mechanical properties for each ASM strip had been measured, each strip was removed from its respective organ bath and pinned to wax in a small dish containing warmed Krebs. Using L-shaped metal pins, ASM strips were pinned at L_{ref} lengthwise through their clips onto wax pieces 10 mm by 20 mm in size. Once pinned, all samples were transported to the culture room in a test tube containing warmed Krebs. In a Type A/B3 Biological Safety Cabinet (Forma Scientific, Marietta, OH), each ASM strip pinned to wax was placed face-down into its own well of a 12-well culture plate filled with serum-free Dulbecco's Modified Eagles Medium (DMEM). DMEM was warmed to 37°C and supplemented with 10 mM HEPES buffer, and an antibiotic/antimycotic mixture (100 U/mL penicillin; 100 μg/mL streptomycin; 250 ng/mL amphotericin B). For IL-13-treated samples, 50 ng/mL recombinant human IL-13 (R&D Systems, Minneapolis, MN) was added into the culture media. For all experiments, samples were then placed into a Series II Water Jacketed CO_2 Incubator that maintained temperature at 37°C, humidity, and 5% CO_2 (ThermoForma, Marietta, OH). Samples remained in the incubator for the designated amount of incubation time, then removed, washed with Krebs, and either taken back the lab for mechanical measurements, or flash-frozen for protein extraction (see Section 3.4).

3.4 Measurement of phosphorylation of signal transducer and activator of transcription 6 (STAT6)

ASM strips post culturing (30 min) were removed from the incubator and snap frozen using pre-chilled acetone (-80°C). Tissue samples were stored in eppendorf tubes at -80°C until required for protein extraction.

To extract protein, tissue strips were first mechanically ground using mortar and pestle at -80°C then further homogenized in a STAT6 extraction buffer (1% v/v Triton X-100, 20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM $NaVO_4$) supplemented with cOmplete protease inhibitor (Sigma) and phospho-STOP (Sigma). Approximately 200 uL of extraction buffer was used per tissue strip. Next, samples were sonicated on ice using a Sonifier Cell Disruptor 350 (Branson Sonic Power Co., Danbury, Conn.). Samples were then rotated (7.5 rotations per minute) for 1 hour at 4°C then centrifuged at 13200 rpm for 30 min also at 4°C (Eppendorf, Germany). Supernatants were collected into new tubes and used for a protein assay to determine concentration (DC Protein Assay; BioRad Laboratories, Mississauga, Canada).

To determine the extent of STAT6 phosphorylation due to IL-13 treatment, SDS-PAGE and western blotting were performed. A 7.5 % polyacrylamide denaturing gel and a stacking gel were allowed to set then loaded with equal amounts and concentrations of protein samples. To separate the proteins, electrophoresis was run at 200V for 1 hour at room temperature. Protein was then transferred to a 0.2 μm nitrocellulose membrane at 100 V for 1 hour at 4°C. Membranes were then blocked separately overnight at 4°C using Odyssey Blocking Buffer PBS (LI-COR Biosciences, Lincoln, NE). For western blotting, monoclonal primary mouse antibodies specific for either phosphorylated STAT6 (pSTAT6; BD Biosciences, San Jose, CA; 1:1000, 1 hr) or Total STAT6 (BD Biosciences; 1:500, 2 hr) were allowed to incubate with their designated membrane at room temperature. For the hsp90 loading control, a specific primary antibody (BD Biosciences, 1:1000, 1 hr) was also added separately to each membrane at room temperature. A

goat anti-mouse IRDye 800 conjugated antibody (1:5000, 1 hr; Rockland, Gilbertsville, Penn.) was used as a secondary antibody for all blots. Between the addition of each antibody, membranes were washed with PBS-T four times for 5 minutes each, then once with PBS before imaging. In order to image blots, a LI-COR Odyssey 2.1 Infrared Imaging System (LI-COR Biosciences) was used to scan the membrane and quantify band optical density (OD). LI-COR associated software was used for obtaining OD measurements and subtracting background. All OD measurements were then normalized to their respective hsp90 loading control.

3.5 Mechanical measurements

Following a common tissue preparation and equilibration protocol (Section 3.1), tissues were subject to a specific protocol depending on the agonist-response under investigation. These protocols were used to collect the force-output of the ASM in response to a specific agonist and were carried out both before and after culturing (Section 3.3). Each protocol differed and will be distinguished below.

3.5.1 Acetylcholine protocol

After equilibration with EFS was complete, each strip was contracted with ACh (10^{-6} M) at least two times or until a plateau in maximal force was reached at this dose of ACh. After each stimulation, baths were washed with Krebs and allowed to rest for at least 15 minutes before the following stimulation. Next, a dose-response experiment was performed whereby progressively

higher doses of ACh were added and the force-output of the ASM was continuously measured. ACh from 10^{-8} to 10^{-3} M was added into the bath for each strip in progressive fashion with washing between doses. Strips were finally washed until force returned to baseline levels.

3.5.2 Isoproterenol protocol

After equilibration with EFS was complete, each strip was contracted with ACh (10^{-5} M) at least two times or until a plateau in maximal force was reached at this dose of ACh. After each stimulation, baths were washed with Krebs and allowed to rest for at least 15 minutes before the following stimulation. Then, all strips were contracted using 10^{-5} M ACh and once a plateau was reached, progressive doses of ISO (10^{-9} to 10^{-4} M) were added in a cumulative fashion in order to relax the muscle. Baths were not washed between additions of ISO. Strips were finally washed until force returned to baseline levels.

3.5.3 KCl (High K^+) protocol

After equilibration with EFS was complete, each strip was contracted with KCl (80 mM) at least two times or until a plateau in maximal force was reached at this dose of KCl. The maximal force reached for 80 mM of KCl was recorded at baseline and following culturing for comparison. Strips were finally washed until force returned to baseline levels.

3.5.4 Histamine protocol

After equilibration with EFS was complete, each strip was contracted with Histamine (10^{-4} M) two times in order to determine the maximal force response to this dose of Histamine. The maximal force reached for Histamine (10^{-4} M) was recorded at baseline and following culturing for comparison. Strips were finally washed until force returned to baseline levels.

3.6 Statistical analysis

All statistical tests were done using GraphPad Prism 5 or SigmaPlot 11. For direct comparisons between two means, a two-tailed paired t-test was performed. When comparing two curves (dose-responses), a repeated measures two-way analysis of variance (ANOVA) was used. Data is presented as a mean +/- Standard Error of Means (SEM) and n indicates the number of sheep (trachea) used per experiment. For statistical significance, a p value less than 0.05 was used to reject the null hypothesis.

Chapter 4 : Results

4.1 Phosphorylation of STAT6 in IL-13-treated smooth muscle preparations

The extent of STAT6 phosphorylation following 30 minutes of IL-13 treatment is visualized in Figure 4.1A (representative western blot; n=5). Optical Density (OD) for each band was measured for comparison and graphed below. Figures 4.1B and 4.1C show the mean OD for the phosphorylated, or total STAT6 bands, respectively (normalized to the OD for their respective hsp90 loading control). IL-13-treated ASM tissue demonstrated a significantly increased pSTAT6 OD compared with control (p=0.02). Total STAT6 OD was slightly increased over control although this was not a statistically significant difference (p=0.15).

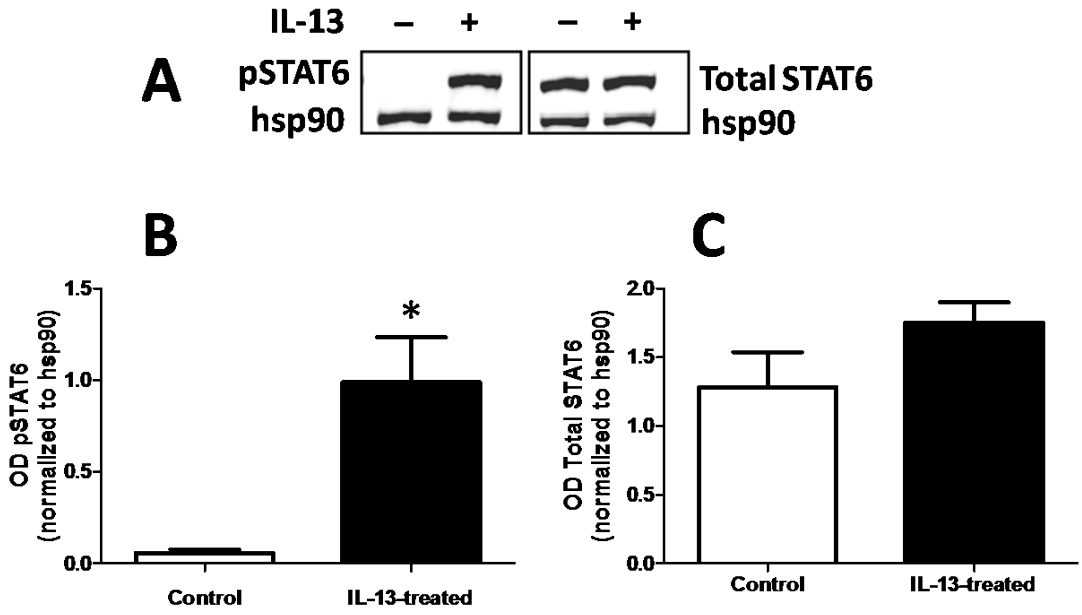

Figure 4.1 STAT6 phosphorylation following IL-13 treatment in airway smooth muscle tissue strips (A) Representative western blot for total and phospho-STAT6 expression in ASM after 30 minutes incubation with (+) or without (–) IL-13 (50 ng/mL). Mean optical density (OD) values for corresponding western blot bands for pSTAT6 (p=0.02; n=5) **(B)** and Total STAT6 **(C)** expression relative to hsp90 loading control (p=0.15; n=5). Data shown as mean +/- SEM.

4.2 Mechanics of control ASM samples for culturing alone

For control incubations (no IL-13), the mean dose-response curves to ACh, both before and after culturing, are shown in Figure 4.2. The difference between baseline vs. cultured for the whole curve was significant with a p=0.03 (n=8, two-way repeated measures ANOVA). Additionally, while curves diverge significantly at mid-concentrations of ACh (10^{-7} M, p=0.04; 10^{-6} M, p=0.02; 10^{-5} M, p=0.01), the curves do not differ in the lower and upper concentrations ranges (p>0.05). Using a Prism 5 built-in nonlinear fit equation of "log(agonist) vs. response – variable slope" for both ACh dose-response curves (t=0 and t=3d), the $logEC_{50}$ (the concentration of agonist that gives 50% maximal response), Hill slopes (describes the steepness of the curve) and R squared (goodness of fit) values were obtained. The $logEC_{50}$ values were -5.951 (t=0) and -5.706 (t=24h); p=0.054, while the respective Hill slopes were 0.529 (t=0) and 0.544 (t=3d); p=0.87. Furthermore, the R squared values demonstrated a good fit for each equation at 0.974 (t=0) and 0.938 (t=3d), respectively.

In Figure 4.3, the mean maximal force ([ACh] = 10^{-3} M) before and after culturing is compared for ASM tissue strips. The mean F_{max} after culturing was not significantly different than the initial baseline measurements (p=0.17; n=8). Taking into consideration both these results, the culturing process imparted diminished sensitivity to mid-range [ACh] in the ASM tissue strips (Figure 4.2), while not significantly affecting the maximal force that the tissue can produce (Figure 4.3).

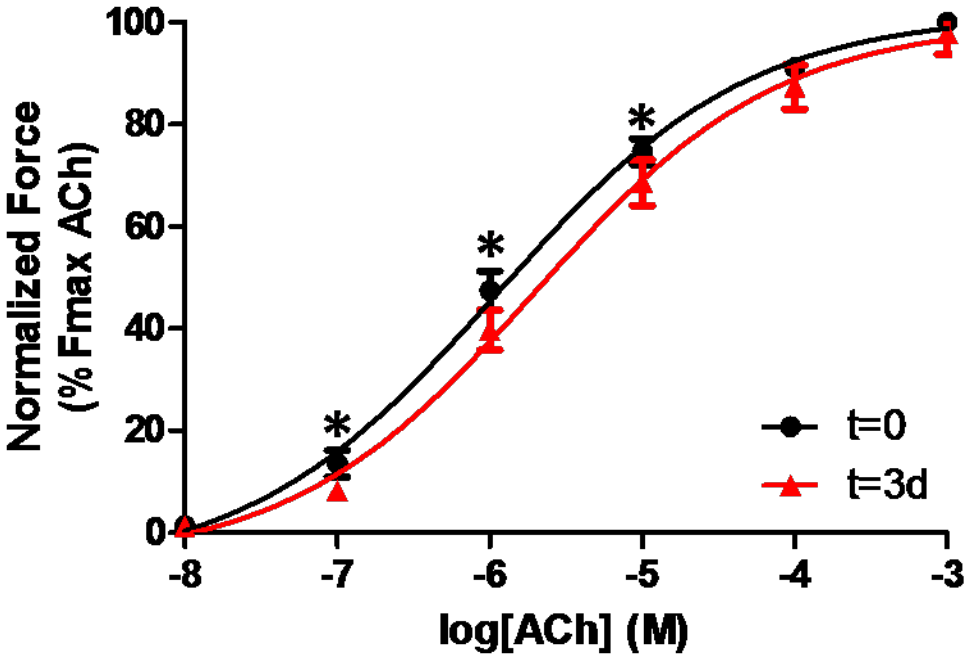

Figure 4.2 The influence of the culturing process (3 days;3d) on the dose-response of ACh-induced force in ASM tissue strips. Force-output is normalized to % of baseline F_{max} for each strip. n=8; p=0.03 whole curves; 2-way ANOVA. Individual points: 10^{-7} M, p=0.04; 10^{-6} M, p=0.02; 10^{-5} M, p=0.01. * p<0.05. Data shown as mean +/- SEM.

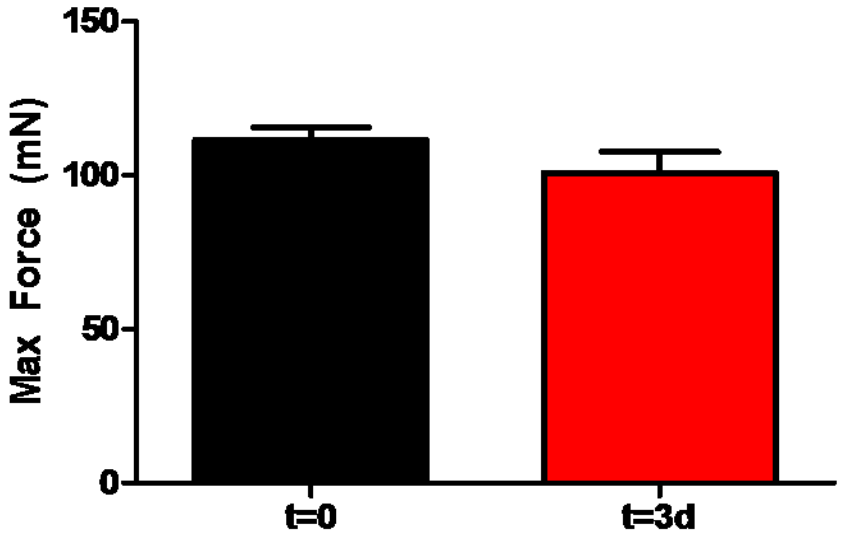

Figure 4.3 The influence of the culturing process (3 days;3d) on the mean max force (mN) produced by the ASM tissue strips in an ACh-induced contraction (10^{-3} M). Means were compared with a two-tailed paired t-test; $p>0.05$. n=8. Data shown as mean +/- SEM.

4.3 Influence of IL-13 on acetylcholine-induced contraction

To examine the influence of IL-13 (24h) on the ACh-induced ASM tissue force-output and sensitivity, dose-responses were plotted as a curve (DRC). In Figure 4.4, the ACh DRC for IL-13-treated tissues (24 hrs) is compared with the control untreated tissues and presented as means normalized as percentage of the initial baseline F_{max} for each dose. DRC curves of treated and untreated ASM were not significantly different (whole curve; p=0.30, n=5), nor was there any significant difference at any particular dose of ACh. Again using the Prism 5 built-in nonlinear fit equation of "log(agonist) vs. response – variable slope" for both ACh dose-response curves (control t=24h and IL-13-treated t=24h), the $logEC_{50}$, Hill slopes and R squared values were obtained. The $logEC_{50}$ values were -5.891 (Control) and -5.703 (IL-13); p=0.33, while the respective Hill slopes were 0.551 (Control) and 0.496 (IL-13); p=0.70. The R squared values demonstrated a good fit for each equation at 0.973 (Control) and 0.944 (IL-13), respectively.

In Figure 4.5, the mean F_{max} ([ACh] = 10^{-3} M) for control and treated tissues (IL-13) are compared as initial baseline values (t=0) and post-culture values (t=24h). After culturing, there was no significant difference between the mean F_{max} for treated vs. control tissues (t=24h; p=0.51, n=5) or between their respective baseline values (p=0.43).

When incubations were extended from 24h to 3 days (3d), a similar trend was observed. Figure 4.6 demonstrates mean DRC curves for treated and control ASM tissues (after 3 days of culturing) expressed as a percentage of baseline F_{max} values. Once again, DRC curves of treated and untreated ASM were not significantly different (p=0.09), and there was not any significant

difference at any individual dose of ACh. Treated samples tended to be more sensitive to ACh after 3d but this was not significant. For each DRC, The $\log EC_{50}$ values were -5.715 (Control) and -5.925 (IL-13); p=0.32, while the respective Hill slopes were 0.524 (Control) and 0.509 (IL-13); p=0.43. Additionally, the R squared values demonstrated a good fit for each equation at 0.926 (Control) and 0.955 (IL-13), respectively.

In Figure 4.7, the mean F_{max} ([ACh] = 10^{-3} M) for control and treated tissues (IL-13) are compared as initial baseline values (t=0) and post-culture values (t=3d). Again, after culturing, there was no significant difference between the mean F_{max} for treated vs. control tissues (t=3d; p=0.81, n=5) or between their respective baseline values (p=0.33, n=5).

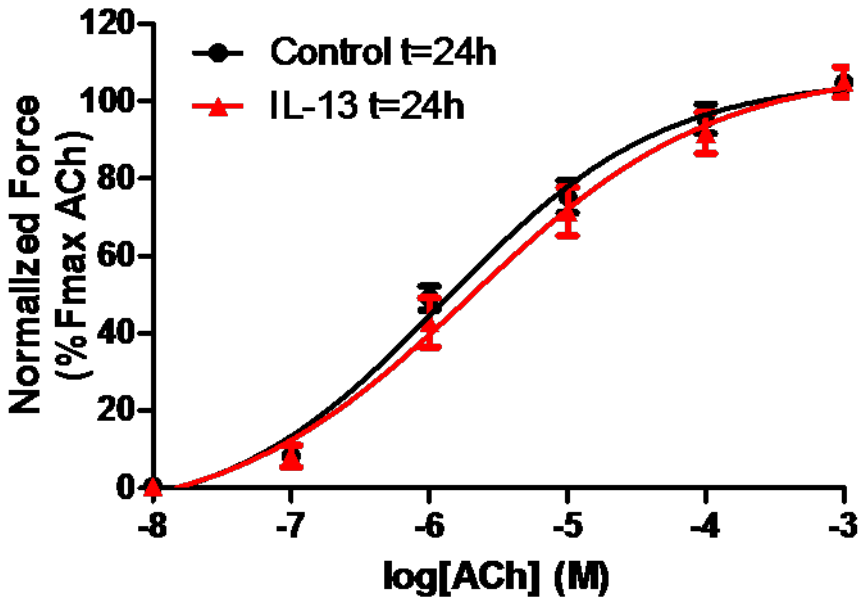

Figure 4.4 The influence of the IL-13 (24h) on the dose-response of ACh-induced force in ASM tissue strips. Force-output is normalized to % of baseline F_{max} for each strip. n=5; p=0.30 whole curves; 2-way ANOVA. All individual points have p>0.05. Data shown as mean +/- SEM.

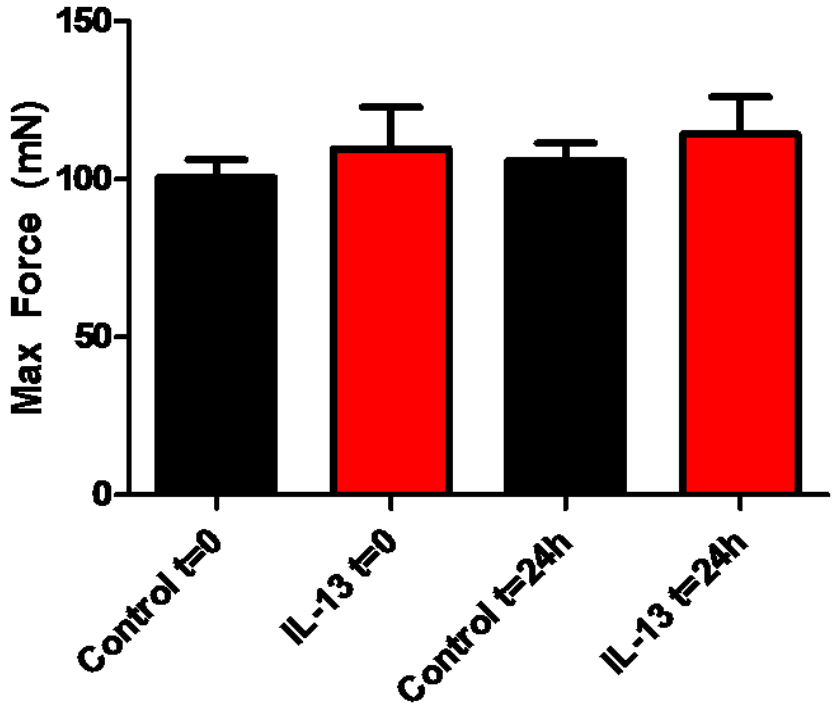

Figure 4.5 The influence of IL-13 (24h) on the mean max force (mN) produced by the ASM tissue strips in an ACh-induced contraction (10^{-3} M). Means were compared with a two-tailed paired t-test; $p>0.05$. n=5. Data shown as mean +/- SEM.

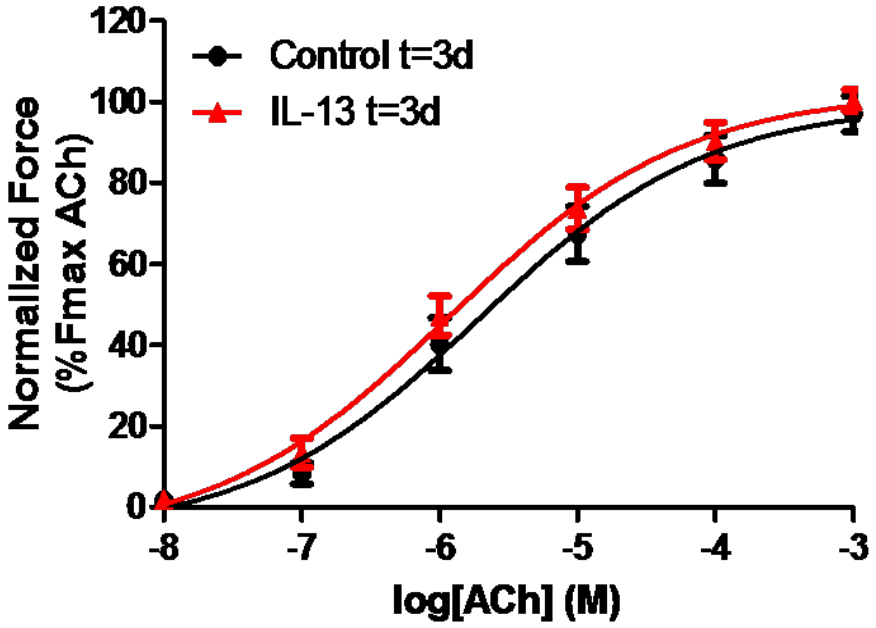

Figure 4.6 The influence of the IL-13 (3d) on the dose-response of ACh-induced force in ASM tissue strips. Force-output is normalized to % of baseline F_{max} for each strip. n=5; p=0.09 whole curves; 2-way ANOVA. All individual points have p>0.05. Data shown as mean +/- SEM.

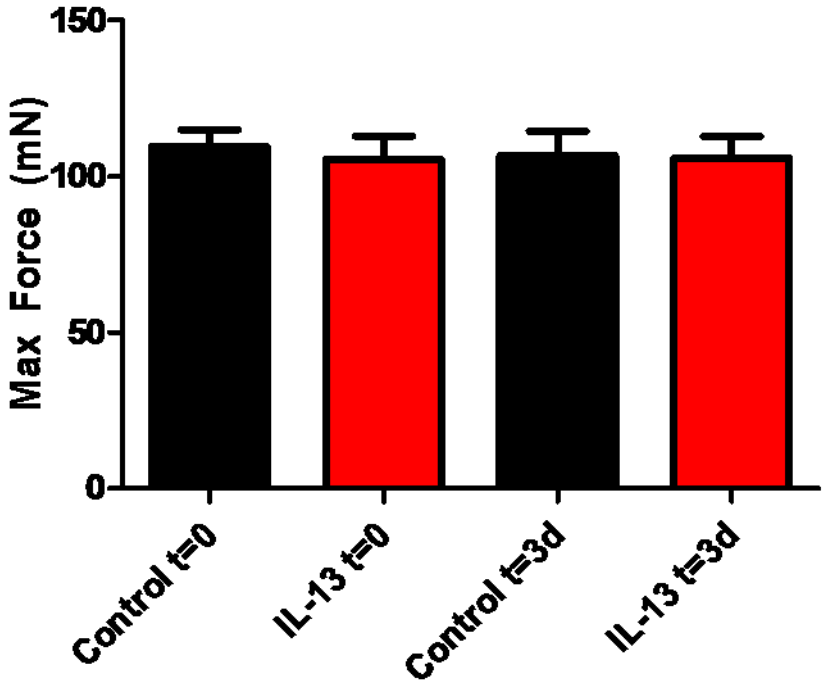

Figure 4.7 The influence of IL-13 (3d) on the mean max force (mN) produced by the ASM tissue strips in an ACh-induced contraction (10^{-3} M). Means were compared with a two-tailed paired t-test; $p>0.05$. n=5. Data shown as mean +/- SEM.

4.4 Influence of IL-13 on isoproterenol-induced relaxation

To examine the influence of IL-13 culturing (24h) on the Isoproterenol (ISO)-induced ASM relaxation, the maximal force reduction and sensitivity were plotted as dose-response curves (DRC). In Figure 4.8, the ISO DRC for IL-13-treated tissues is compared with the control untreated tissues after 24h and presented as means normalized as a percentage of the initial baseline F_{max} reached (initial contraction with ACh = 10^{-5} M) for each dose. Similar to contraction, relaxation DRC curves of treated and untreated ASM were not significantly different (p=0.77), nor was there any significant difference at any particular dose of ISO. Using the Prism 5 built-in nonlinear fit equation of "log(inhibitor) vs. response – variable slope" for both ISO dose-response curves (control t=24h and IL-13-treated t=24h), the $logIC_{50}$ (the concentration of inhibitor that gives 50% maximal response), Hill slopes and R squared values were obtained. The $logIC_{50}$ values were -7.314 (Control) and -7.267 (IL-13); p=0.58, while the respective Hill slopes were -1.189 (Control) and -1.192 (IL-13); p=0.27. The R squared values demonstrated a good fit for each equation at 0.941 (Control) and 0.916 (IL-13), respectively.

In Figure 4.9, the mean minimal force (F_{min}; [ISO] = 10^{-5} to 10^{-4} M) for control and treated tissues (IL-13) are compared as initial baseline values (t=0) and post-culture values (t=24h). After culturing, there was no significant difference between the mean F_{min} for treated vs. control tissues (t=24h; p=0.79, n=5) or between their respective baseline values (p=0.50, n=5).

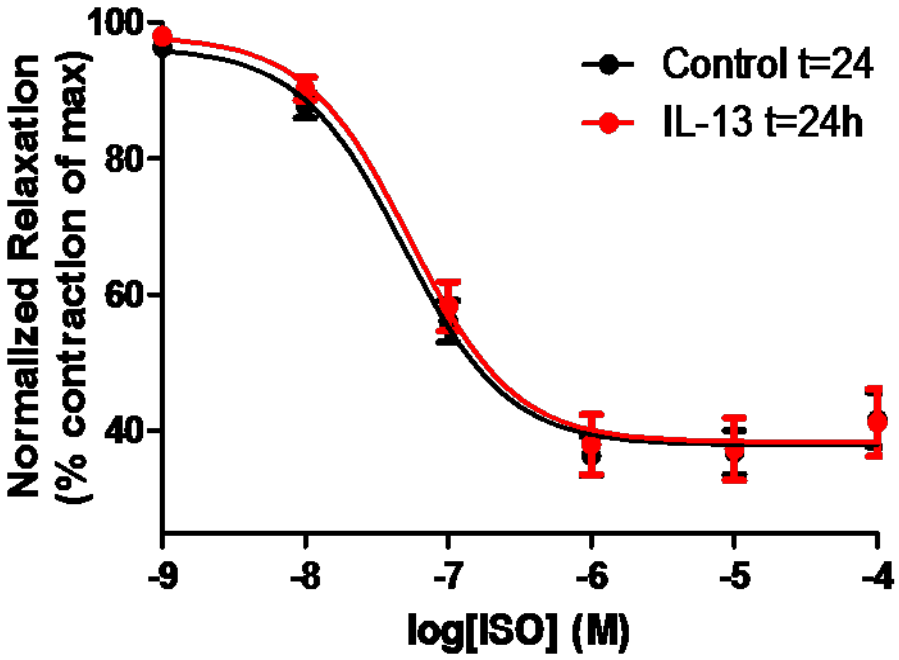

Figure 4.8 The influence of the IL-13 (24h) on the dose-response of ISO-induced relaxation in ASM tissue strips (10^{-9} – 10^{-4} M). Force-output is normalized to % of baseline F_{max} reached for each strip during an initial contraction with ACh 10^{-5} M. n=5; p=0.77 whole curves; 2-way ANOVA. All individual points have p>0.05. Data shown as mean +/- SEM.

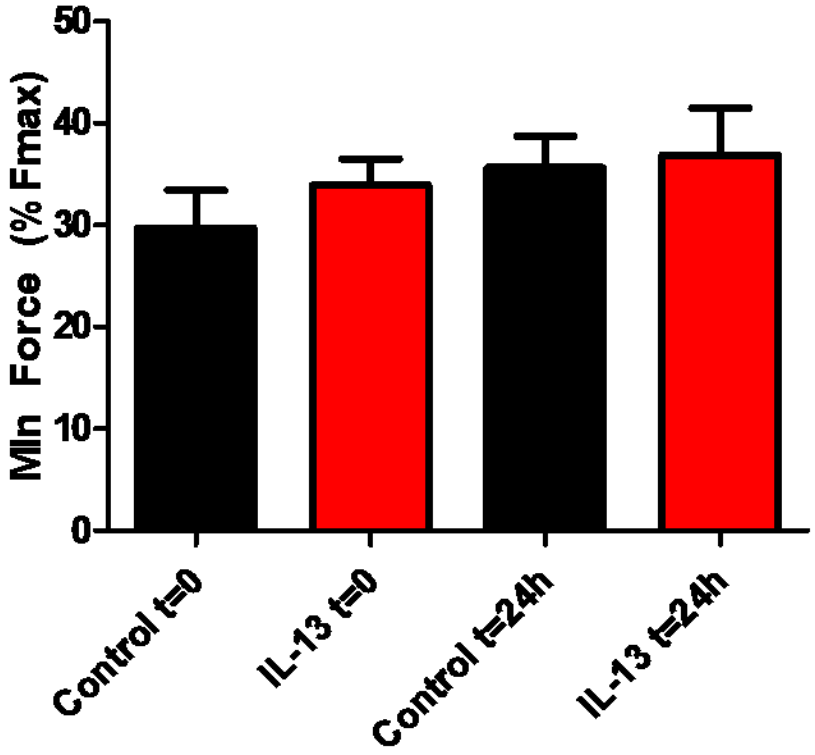

Figure 4.9 The influence of IL-13 (24h) on the mean minimum force (Min Force; % of F_{max} reached) produced by the ASM tissue strips in ISO-induced relaxation (10^{-9} – $^{-4}$ M). Means were compared with a two-tailed paired t-test; $p>0.05$. n=5. Data shown as mean +/- SEM.

4.5 Influence of IL-13 on KCl-induced contraction

To examine the influence of IL-13 on KCl-induced contraction, ASM strips were contracted with 80 mM High K^+ Krebs before and after 3 days of exposure to IL-13. In Figure 4.10 the maximal force post-culture (3 days) are shown as a percentage of their baseline values for control and treated samples. Treatment with IL-13 caused no significant change to the maximal force produced by ASM tissue strips after 3 days for KCl-induced contraction (p=0.85; n=5).

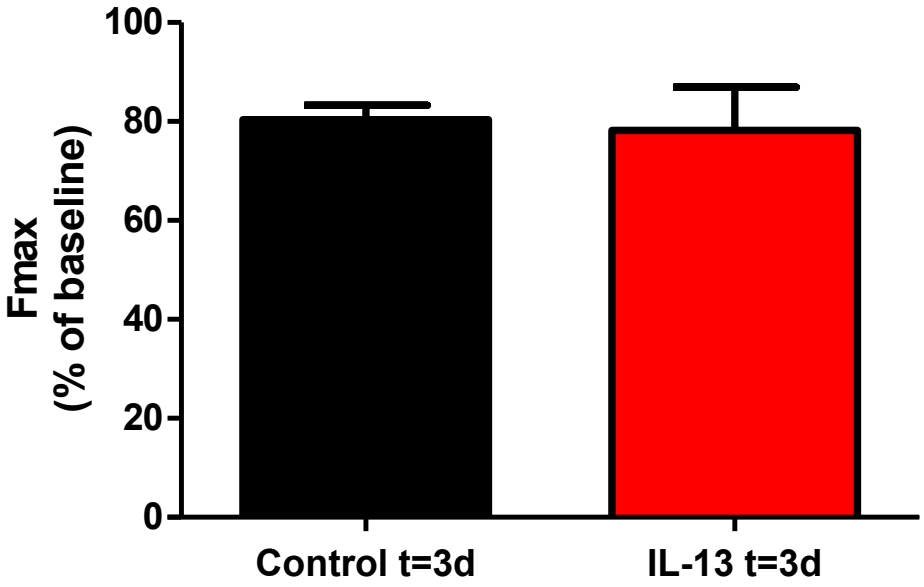

Figure 4.10 The influence of IL-13 (3d) on the force-output (F_{max}; % of F_{max} reached at baseline) produced by the ASM tissue strips in KCl-induced contraction (80 mM). Means were compared with a two-tailed paired t-test; p=0.85. n=5. Data shown as mean +/- SEM.

4.6 Influence of IL-13 on histamine-induced contraction

To examine the influence of IL-13 on Histamine-induced contraction, ASM strips were contracted with 10^{-4} M Histamine before and after 3 days of exposure to IL-13. In Figure 4.11 the maximal force post-culture (3 days) are shown as a log percentage of their baseline values for control and treated samples. Data was log transformed to normally distribute the data. Treatment with IL-13 caused a maintenance of force compared to control tissues in which maximal force decreased markedly after 3 days. After 3 days, compared to their respective baseline measurements, IL-13-treated ASM tissue exhibited significantly higher force than control tissues ($p=0.03$; $n=5$). The mean histamine-induced force produced by IL-13 treated tissue was roughly 120% higher than control after 3 days (Figure 4.11).

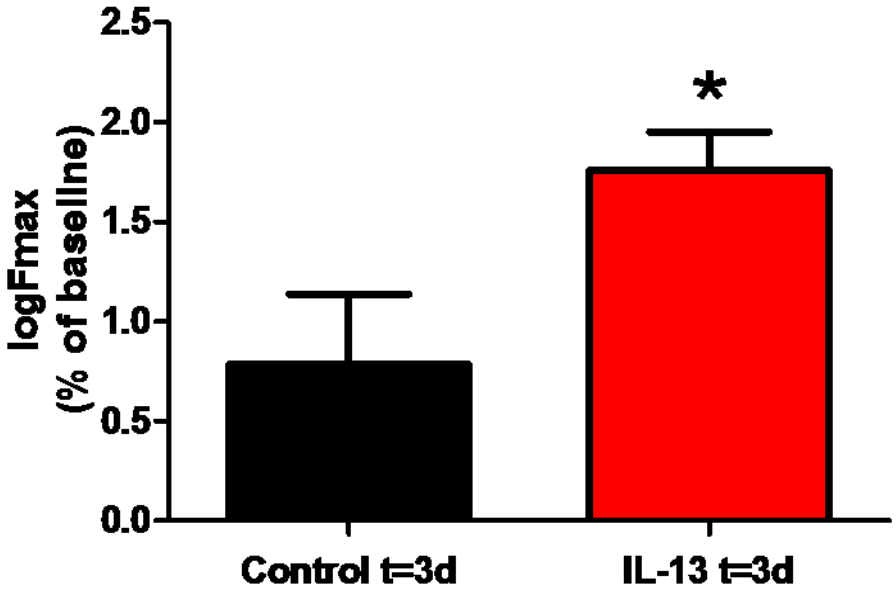

Figure 4.11 The influence of IL-13 (3d) on the force-output ($logF_{max}$; % of F_{max} reached at baseline) produced by the ASM tissue strips in histamine-induced contraction (10^{-4} M). Means were compared with a two-tailed paired t-test; p=0.03; n=5. Data shown as mean +/- SEM.

Chapter 5 : Discussion

Using cell culture methods has provided scientists with a means to examine the effect of long-term exposure of ASM to inflammatory mediators. However, the phenotype change known to be associated with cell culturing (Halayko et al., 1996) casts doubts on the relevance of the results from cell-culture experiments with respect to the real behavior of ASM in situ. Culturing freshly dissected ASM tissue instead of cells in a serum-free media preserves the contractile phenotype, thus avoiding the shortcomings of cell culture and allows for direct measurement of mechanical properties. The experiments described in this thesis offer a unique insight into the direct effects of IL-13 on force output in ASM tissue strips. The most important findings from these experiments are that IL-13 treatment does not influence the force output of ASM in response to ACh- or KCl, nor does it affect ISO-induced relaxation. On the other hand, IL-13 increased the force output in response to histamine-induced contractions after tissue culturing. The results stand in contrast to many cell culture studies and are discussed in the following sections.

One contribution of this study is the development of a methodological model that maintains tissue contractility and viability for extended periods of time, permitting studies that examine the treatment of ASM tissue with inflammatory mediators, or other biological or chemical agonists and inhibitors. While minor differences in sensitivity were found at mid-range concentrations of ACh after culturing (Figure 4.2), the maximal force the ASM strips produced remained unchanged (Figure 4.3). Western Blot analysis (Figure 4.1) confirmed that IL-13 treatment in this model induced intracellular signaling changes via STAT6 phosphorylation.

From this result it can be inferred that 1) the IL-13R exists on the ASM cell membrane and 2) that IL-13 can modulate transcription via pSTAT6. This transcriptional regulation could potentially lead to changes in contractile gene expressions that could manifest themselves as physiological changes in the contractile behavior of the ASM.

5.1 Discrepancies with previous studies on cultured ASM cells and IL-13

Whether asthmatic ASM is phenotypically different from that of non-asthmatic individuals remains a hotly debated topic (Gunst & Panettieri, 2012; Paré & Mitzner, 2012). So far, there has been no clear consensus on the effect that IL-13, a key inflammatory mediator associated with asthma, has on ASM contractility. Several cell culture studies suggest that IL-13 treatment enhances the contractility of ASM, yet there is no consistency with respect to the mechanical parameter measured (force output, tension, stress, etc.) or contractile agonist used (MCh, CCh, ACh, KCl, histamine, etc.).

In the present study, we hypothesized that in ASM tissue strips, IL-13 would increase force output and sensitivity to contractile agonists and attenuate relaxation in response to the relaxant agonist ISO. This prediction was based on several previous studies that demonstrated increased contractility of cultured ASM cells, or other ASM tissue preparations, when treated with IL-13 (see section 1.3.1) (Chiba et al., 2009; Farghaly et al., 2008; Grunstein et al., 2002; Kudo et al., 2013; Laporte et al., 2001; Nino et al., 2012; Risse et al., 2011; Sugimoto et al., 2012; Tliba et al., 2003). We expected tissue to behave similarly and exhibit “hypercontractile” properties after treatment with IL-13. However, we found that after 24h or 72h of IL-13

treatment, the force output from ACh-induced contractions was not significantly different than control over a range of concentrations (Figures 4.4-4.7). These findings differ from two previous studies examining the effect of IL-13 treatment on ACh-induced contraction (isometric force) using rabbit tracheal rings; they found increased contractility over untreated controls (Grunstein et al., 2002; Nino et al., 2012). It is possible that the discrepancy between the results of previous studies and those of this thesis can be attributed to the tissue type and source; both Grunstein et al. and Nino et al. used rabbit tracheal rings while this thesis used ovine ASM strips, a pure preparation of smooth muscle bundle. Using tracheal rings, or other tissue preparations that contain more than just smooth muscle (ex. epithelium) could introduce a confounding variable into the research and compromise the ability to investigate the direct effects of IL-13 on ASM alone. It is possible that in a scenario where ASM and epithelium are cultured together (such as a whole tracheal ring) that IL-13 could act on the epithelium which then could act on the smooth muscle by releasing bronchoactive substances and impart an indirect influence to contractiliy (Bossé, Paré, & Bossé, 2012). Overall, the results examining ACh-induced contraction contradicted our original hypothesis and suggest that IL-13 does not influence ACh-induced contraction in ASM strips (at least within this model system).

Next we examined ASM relaxation. In tissue strips, IL-13 treatment did not reduce the muscle's capacity to relax in response to ISO (Figures 4.8, 4.9). This finding is another discrepancy between the results of this research thesis and previous studies where three groups found that IL-13 treatment of ASM attenuated ISO-induced relaxation (Grunstein et al., 2002; Laporte et al., 2001; Nino et al., 2012). These results again contradicted our original hypothesis.

Using a pure muscle preparation, we have shown that IL-13 does not attenuate relaxation of ovine ASM induced by the non-specific β-agonist ISO.

KCl-induced contraction also remained unchanged after IL-13 treatment for 3 days (Figure 4.10). The findings from this thesis research are in contrast with three previous studies that found IL-13 increased the KCl-induced force of contraction (Farghaly et al., 2008; Kudo et al., 2013; Tliba et al., 2003). On the other hand, our results were similar to the findings of two studies, both found that IL-13 did not increase contractility, or stiffness, in KCl-induced contractions (Chiba et al., 2009; Risse et al., 2011).

The reason for the aforementioned discrepancies is not clear. As previously alluded to, a tracheal ring likely contains cells other than ASM cells, such as epithelial and immune cells. Co-culturing of ASM cells with other cells may lead to changes in ASM properties that are not present in pure ASM bundles cultured alone. Species difference could also be part of the explanation.

When examining the influence of IL-13 on histamine-induced contractions, it was found that IL-13 treated tissues produced significantly more force than control after 3 days ($p=0.03$; Figure 4.11), however force was not increased over its own baseline measurements, the difference was related to a decline in force over time in the controls. The results indicate that there is a decrease in sensitivity or responsiveness to histamine during the culturing process under control conditions, and that IL-13 prevented this from happening and thus preserved the force. Another explanation is that the decrease in sensitivity and/or responsiveness to histamine

occurs under both the control and test conditions, perhaps due to a declining number of histamine receptors, but in the test condition IL-13 induces synthesis of histamine receptors in the ASM cells. This explanation can be supported by two studies that show an upregulation of the histamine H1 receptor mRNA after treatment of IL-13 (Jarai et al., 2004; Syed et al., 2005). Yet another explanation is that IL-13 treatment increases calcium release due to histamine stimulation. This explanation is supported by the study of Tliba et al who found that IL-13 treatment (24h) of cultured HASMC caused a 35% increase in calcium response to histamine (Tliba et al., 2003). Another study found that IL-13 treatment of cultured HASMC increased the histamine-induced stiffness compared to control (Risse et al., 2011), however, whether the stiffness increase is due to an increased calcium release is not known. Our study produced similar results, but since we did not measure calcium response in our ASM tissue preparations, we cannot exclude all the possible mechanisms discussed above.

In the unique case of histamine-induced contractions, it seems that cultured ASM cells and tissue can be similarly influenced by IL-13. With respect to our original hypothesis, this scenario was the only one in which IL-13 imparted “hypercontractile” (i.e. increased maximal force) properties to ASM tissue strips when stimulated with histamine. The proposed mechanism by which IL-13 treatment results in increased maximal force is discussed further in relation to some relevant literature in the following section.

5.2 Proposed mechanism of IL-13 influence on histamine-induced contraction

In order for IL-13 to influence histamine-induced contraction in ASM, and ultimately contribute to AHR, it could do so by regulating the expression of a histamine-specific gene related to contraction. The obvious candidate in this case is the H_1 receptor for histamine. Using both a gene microarray approach and a RT-PCR analysis, Syed et al. found that IL-13 treatment (50 ng/mL) increased the H_1 receptor gene expression after 6 hours in cultured HASMC (Syed et al., 2005). Similarly, Jarai et al. found that in cultured HASMC, IL-13 treatment increased H_1 receptor expression by 2.6-fold after only 4 hours (Jarai et al., 2004). In light of these cultured cell studies, we propose that IL-13 treatment induces the increased expression of the H_1 receptor gene early in the culturing processes and leads to increased expression of the receptor protein by 3 days. This increase in receptor expression would result in increased or preserved sensitivity to histamine and contractile response likely through increased calcium signaling in response to histamine. Another scenario is that the number of H_1 receptors is not altered, but the subsequent signaling pathway leading to calcium release is somehow enhanced by IL-13. There is no data in support of this alternative explanation.

During the culturing process in this thesis study, there was likely a down-regulation of the receptor number perhaps due to cellular autophagy in the serum-free environment and this could account for the decreased response to histamine in control tissues after 3 days. The mechanism by which IL-13 increases H_1 receptor expression would partially counter this de-sensitization from the culturing process and could result in a histamine response that is greater than control tissues after 3 days but lower than baseline measurements when the tissue was fresh.

Ultimately, the effect that IL-13 has on histamine-induced contraction suggests that the original hypothesis can still be correct under certain circumstances and depends on the agonist used. Additionally, this proposed mechanism presents a plausible avenue by which IL-13 can influence the contractile response of ASM and perhaps contribute to AHR in asthmatics.

5.3 Implications to asthma pathophysiology and the role of ASM

Taking into consideration the main results from this thesis, it seems clear that IL-13 can influence ASM tissue contraction; however it depends on the agonist used. This finding highlights the importance of distinguishing between factors like tissue type, model system, presence of serum, contractile agonist used, length of culturing/treatment, and mechanical parameter measured when comparing studies reported in the literature regarding the influence of inflammatory cytokines on ASM contractility.

Using freshly prepared ASM tissue strips in this study allowed us to examine the influence of IL-13 on ASM tissue and not cultured cells, thus moving one step closer to its physiological state. Evidence collected in this thesis suggests that ASM under the influence of the asthma-associated inflammatory mediator IL-13 is, for the most part, not different than untreated tissues. These findings differ from those reported by previous studies that used cultured cells or whole tracheal rings (with other tissue types together with ASM) that draw conclusions about ASM contractility in an inflammatory milieu. Perhaps the serum-rich media in cultured cells, or the contribution of other tissue types (epithelial, connective), may have influenced the effect of IL-13 on the ASM contraction in these studies.

With respect to asthma pathophysiology, it seems that ASM force generation is predominantly unchanged under the influence of IL-13, with the notable exception of histamine-induced contractions. It appears that IL-13 alone does not induce changes in the contractile apparatus of ASM in our experiments; the changes are likely related to the histamine receptors and the associated signaling pathways. The exact mechanisms of AHR pathogenesis remain complex and multifactorial. In the case of chronic inflammation seen in asthma, histamine, which is itself an inflammatory mediator and product of inflammation, could have its effect on ASM amplified by the presence of IL-13 in asthmatic airways.

5.4 Limitations of ASM culturing model

No model is perfect and each one has its own set of limitations; the model used in this thesis is no exception. The culturing model optimized and presented as part of this thesis was created to allow the long-term treatment of ASM tissue strips without compromising tissue viability. In order to accurately measure mechanical properties the tissue must be sufficiently contractile and ideally this contractility should not diminish during the treatment or culturing process. Additionally, in order to preserve the contractile phenotype for ASM cells within the tissue strips, a serum-free media was required. While the DMEM culture media did contain a basal amount of nutrients for the tissue, it is possible (even likely) that some autophagy occurred with "unnecessary" proteins degraded to support the cells. It is plausible that the difference measured between the force output of strips post-culture compared to baseline (Figure 4.2) can be attributed to some loss of contractile protein or M_3 receptor manifesting itself in a slight loss of sensitivity.

Another limitation of this model, or perhaps using strips of ASM tissue in general, is that there was no guarantee that IL-13 treatment was able to influence all ASM cells equally within the strip. Cells on the surface of the strip could have received more exposure to IL-13 than cells within the core of the strip not exposed to media resulting in an uneven exposure, or treatment, of the ASM. Using histological methods it could be possible to stain for IL-13 itself, or even pSTAT6, within a section of tissue to visualize the extent that IL-13 penetrates the tissue strips in culture. This type of confirmation experiment could be performed in the future should this model be used for further investigation.

5.5 Relevance to asthma therapeutics and research

Given the apparent influence of IL-13 on histamine-induced contraction it seems pertinent that future asthma therapeutics and research should focus more on the role of histamine in AHR. First generation antihistamines were used as H_1 blockers but showed limited efficacy in the treatment of asthma with high concentrations necessary and potentially adverse side-reactions (J Bousquet, Godard, & Michel, 1992). Second generation antihistamines have produced mixed results in clinical trials showing both beneficial and adverse effects of certain treatments (Wilson, 2006). With further drug development and clinical trials, antihistamines could show an improved benefit in asthma management for patients. In terms of future asthma research, specifically on smooth muscle contraction, more emphasis should be made on examining histamine-induced contractions. These studies could be conducted using normal ASM tissue in a model system for asthma (i.e. in the presence of inflammatory mediators), or by comparing behaviors observed in normal and asthmatic tissues.

Chapter 6: Conclusions

The pathogenesis of asthma remains complex, and so too does the interrelationship between airway inflammation and AHR. This study sought to examine the influence of IL-13 on the force generation of ASM using a variety of agonists. In the context of the experiments performed, IL-13 treatment did not influence the force generation for ACh- or KCl-induced contractions, nor did it impede relaxation induced by ISO in pre-contracted tissue. Force generation was significantly higher in IL-13 treated tissues when contracted with histamine after 72 h, highlighting the importance of histamine, its receptors, and the associated signaling pathways in the pathogenesis of asthma. This work also presents an experimental model for the long-term exposure of ASM tissue strips to agents, such as inflammatory mediators, that allow the tissues to retain their contractility. This model can have potential applications for any study examining the effect of a chemical treatment on the mechanical properties of ASM tissue strips over an extended period of time. Further elucidation of the complex relationship between airway inflammation and ASM function will be necessary to identify novel targets for therapeutic intervention to alleviate exacerbations for patients suffering from asthma.

Bibliography

Akiho, H., Blennerhassett, P., Deng, Y., & Collins, S. M. (2002). Role of IL-4, IL-13, and STAT6 in inflammation-induced hypercontractility of murine smooth muscle cells. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *282*(2), G226–G232.

Bai, T. R. (1991). Abnormalities in airway smooth muscle in fatal asthma. A comparison between trachea and bronchus. *The American Review of Respiratory Disease*, *143*(2), 441–3. http://doi.org/10.1164/ajrccm/143.2.441

Balhara, J., Redhu, N. S., Shan, L., & Gounni, A. S. (2014). IgE regulates the expression of smMLCK in human airway smooth muscle cells. *PLoS ONE*, *9*(4). http://doi.org/10.1371/journal.pone.0093946

Barnes, P. J. (1995). Inhaled Glucocorticoids of Asthma. *New England Journal of Medicine*, *332*(13), 868–875. http://doi.org/10.1056/NEJM199503303321307

Borish, L., Chipps, B., Deniz, Y., Gujrathi, S., Zheng, B., & Dolan, C. M. (2005). Total serum IgE levels in a large cohort of patients with severe or difficult-to-treat asthma. *Annals of Allergy, Asthma & Immunology : Official Publication of the American College of Allergy, Asthma, & Immunology*, *95*(3), 247–53. http://doi.org/10.1016/S1081-1206(10)61221-5

Bossé, Y., Chapman, D. G., Paré, P. D., King, G. G., & Salome, C. M. (2011). A "Good" muscle in a "Bad" environment: The importance of airway smooth muscle force adaptation to airway hyperresponsiveness. *Respiratory Physiology and Neurobiology*, *179*(2-3), 269–27. http://doi.org/10.1016/j.resp.2011.09.003

Bossé, Y., Paré, P. D., & Bossé, Y. (2012). Airway smooth muscle in asthma symptoms: culprit

but maybe innocent. *Lung Diseases - Selected State of the Art Reviews*, 3–54. Retrieved from http://cdn.intechopen.com/pdfs/30242/InTech-Airway_smooth_muscle_in_asthma_symptoms_culprit_but_maybe_innocent.pdf

Bossé, Y., Sobieszek, A., Paré, P. D., & Seow, C. Y. (2012, December 20). Length Adaptation of Airway Smooth Muscle. *Proceedings of the American Thoracic Society*. American Thoracic Society. Retrieved from http://www.atsjournals.org/doi/abs/10.1513/pats.200705-056VS#.Vw0tTvnNrgU

Bousquet, J., Godard, P., & Michel, F. B. (1992). Antihistamines in the treatment of asthma. *The European Respiratory Journal*, *5*(9), 1137–42. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1358673

Bousquet, J., Jeffery, P. K., Busse, W. W., Johnson, M., & Vignola, A. M. (2000). Asthma - From Bronchoconstriction to Airways Inflammation and Remodeling. *Am J Respir Crit Care Med*, *161*, 1720–1745.

Bullimore, S. R., Siddiqui, S., Donovan, G. M., Martin, J. G., Sneyd, J., Bates, J. H. T., & Lauzon, A.-M. (2011). Could an increase in airway smooth muscle shortening velocity cause airway hyperresponsiveness? *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *300*(1), L121–31. http://doi.org/10.1152/ajplung.00228.2010

Cazzola, M., Page, C. P., Calzetta, L., & Matera, M. G. (2012). Pharmacology and Therapeutics of Bronchodilators. *Pharmacological Reviews*, *64*(3), 450–504. http://doi.org/10.1124/pr.111.004580

Cazzola, M., Page, C. P., Rogliani, P., & Matera, M. G. (2013). β2-agonist therapy in lung disease. *American Journal of Respiratory and Critical Care Medicine*, *187*(7), 690–6. http://doi.org/10.1164/rccm.201209-1739PP

Chiba, Y., Nakazawa, S., Todoroki, M., Shinozaki, K., Sakai, H., & Misawa, M. (2009). Interleukin-13 augments bronchial smooth muscle contractility with an Up-regulation of RhoA protein. *American Journal of Respiratory Cell and Molecular Biology*, *40*(2), 159–167. http://doi.org/10.1165/rcmb.2008-0162OC

Chin, L. Y. M., Bossé, Y., Pascoe, C., Hackett, T. L., Seow, C. Y., & Paré, P. D. (2012). Mechanical properties of asthmatic airway smooth muscle. *The European Respiratory Journal*, *40*(1), 45–54. http://doi.org/10.1183/09031936.00065411

Deshpande, D. A., Dogan, S., Walseth, T. F., Miller, S. M., Amrani, Y., Panettieri, R. A., & Kannan, M. S. (2004). Modulation of calcium signaling by interleukin-13 in human airway smooth muscle: role of CD38/cyclic adenosine diphosphate ribose pathway. *American Journal of Respiratory Cell and Molecular Biology*, *31*(1), 36–42. http://doi.org/10.1165/rcmb.2003-0313OC

Deshpande, D. A., Walseth, T. F., Panettieri, R. A., & Kannan, M. S. (2003). CD38/cyclic ADP-ribose-mediated Ca2+ signaling contributes to airway smooth muscle hyper-responsiveness. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *17*(3), 452–4. http://doi.org/10.1096/fj.02-0450fje

Doeing, D. C., & Solway, J. (2013). Airway smooth muscle in the pathophysiology and treatment of asthma. *Journal of Applied Physiology*, *114*(7), 834–43. http://doi.org/10.1152/japplphysiol.00950.2012

Durham, A., Adcock, I., & Tliba, O. (2011). Steroid Resistance in Severe Asthma: Current Mechanisms and Future Treatment. *Current Pharmaceutical Design*, *17*(7), 674–684. http://doi.org/10.2174/138161211795428984

Durrani, S. R., & Busse, W. W. (2014). Management of Asthma in Adolescents and Adults. In

N. F. Adkinson (Ed.), *Middleton's Allergy: Principles and Practice* (8th ed., pp. 902–922). Philadelphia, PA: Elsevier Inc. Retrieved from https://www.clinicalkey.com/#!/content/book/3-s2.0-B9780323085939000565

Eglen, R. M., Hegde, S. S., & Watson, N. (1996). Muscarinic receptor subtypes and smooth muscle function. *Pharmacological Reviews*, *48*(4), 531–565. Retrieved from http://pharmrev.aspetjournals.org/content/48/4/531.full.pdf

Erle, D. J., & Sheppard, D. (2014). The cell biology of asthma. *Journal of Cell Biology*, *205*(5), 621–631. http://doi.org/10.1083/jcb.201401050

Fahy, J. V. (2015). Type 2 inflammation in asthma--present in most, absent in many. *Nature Reviews. Immunology*, *15*(1), 57–65. http://doi.org/10.1038/nri3786

Farghaly, H. S. M., Blagbrough, I. S., Medina-Tato, D. A., & Watson, M. L. (2008). Interleukin 13 increases contractility of murine tracheal smooth muscle by a phosphoinositide 3-kinase p110delta-dependent mechanism. *Molecular Pharmacology*, *73*(5), 1530–7. http://doi.org/10.1124/mol.108.045419

Fernandes, D. J., Mitchell, R. W., Lakser, O., Dowell, M., Stewart, A. G., & Solway, J. (2003). Do inflammatory mediators influence the contribution of airway smooth muscle contraction to airway hyperresponsiveness in asthma? *Journal of Applied Physiology (Bethesda, Md. : 1985)*, *95*, 844–853. http://doi.org/10.1152/japplphysiol.00192.2003

Fish, J. E., Peterman, V. I., & Cugell, D. W. (1977). Effect of deep inspiration on airway conductance in subjects with allergic rhinitis and allergic asthma. *The Journal of Allergy and Clinical Immunology*, *60*(1), 41–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/874208

Fuller, R. W., Morris, P. K., Richmond, R., Sykes, D., Varndell, I. M., Kemeny, D. M., …

Dollery, C. T. (1986). Immunoglobulin E-dependent stimulation of human alveolar macrophages: significance in type 1 hypersensitivity. *Clin. Exp. Immunol*, *65*, 416–426.

Gosens, R., Meurs, H., Bromhaar, M. M., McKay, S., Nelemans, S. A., & Zaagsma, J. (2002). Functional characterization of serum- and growth factor-induced phenotypic changes in intact bovine tracheal smooth muscle. *British Journal of Pharmacology*, *137*(0007-1188; 4), 459–466. http://doi.org/10.1038/sj.bjp.0704889

Goto, K., Chiba, Y., Sakai, H., & Misawa, M. (2009). Tumor Necrosis Factor–α (TNF-α) Induces Upregulation of RhoA via NF-κB Activation in Cultured Human Bronchial Smooth Muscle Cells. *Journal of Pharmacological Sciences*, *444*(110), 437–444.

Grunstein, M. M., Hakonarson, H., Leiter, J., Chen, M., Whelan, R., Grunstein, J. S., & Chuang, S. (2002). IL-13-dependent autocrine signaling mediates altered responsiveness of IgE-sensitized airway smooth muscle. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *282*(3), L520–8. http://doi.org/10.1152/ajplung.00343.2001

Gunst, S. J., & Panettieri, R. A. (2012). Point: alterations in airway smooth muscle phenotype do/do not cause airway hyperresponsiveness in asthma. *Journal of Applied Physiology (Bethesda, Md. : 1985)*, *113*(5), 837–9. http://doi.org/10.1152/japplphysiol.00483.2012

Hakonarson, H., & Grunstein, M. M. (1998). Regulation of second messengers associated with airway smooth muscle contraction and relaxation. *American Journal of Respiratory and Critical Care Medicine*, *158*(5 Pt 3), S115–22. http://doi.org/10.1164/ajrccm.158.supplement_2.13tac700

Halayko, A. J., Salari, H., MA, X., & Stephens, N. L. (1996). Markers of airway smooth muscle cell phenotype. *The American Journal of Physiology*, *270*(6 Pt 1), L1040–51. Retrieved from http://ajplung.physiology.org/content/270/6/L1040.abstract

Hall, I. P., & Kotlikoff, M. (1995). Use of cultured airway myocytes for study of airway smooth muscle. *The American Journal of Physiology*, *268*(1 Pt 1), L1–11. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7840213

Hartshorne, D. J., & Gorecka, A. (2011). Biochemistry of the contractile proteins of smooth muscle. In *Handbook of Physiology - the cardiovascular system II* (pp. 93–120).

Hirst, S. (1996). Airway smooth muscle cell culture: application to studies of airway wall remodelling and phenotype plasticity in asthma. *Eur. Respir. J.*, *9*(4), 808–820. Retrieved from http://erj.ersjournals.com/content/9/4/808.short

Horowitz, A., Menice, C. B., Laporte, R., & Morgan, K. G. (1996). Mechanisms of smooth muscle contraction. *Physiol Rev*, *76*(4), 967–1003. Retrieved from http://physrev.physiology.org/content/76/4/967.full-text.pdf+html

Ijpma, G., Kachmar, L., Matusovsky, O. S., Bates, J. H. T., Benedetti, A., Martin, J. G., & Lauzon, A.-M. (2015). Human trachealis and main bronchi smooth muscle are normoresponsive in asthma. *American Journal of Respiratory and Critical Care Medicine*, *191*(8), 884–93. http://doi.org/10.1164/rccm.201407-1296OC

Ismaila, A. S., Sayani, A. P., Marin, M., & Su, Z. (2013). Clinical, economic, and humanistic burden of asthma in Canada: a systematic review. *BMC Pulmonary Medicine*, *13*(1), 1–23. http://doi.org/10.1186/1471-2466-13-70

Jarai, G., Sukkar, M., Garrett, S., Duroudier, N., Westwick, J., Adcock, I., & Chung, K. F. (2004). Effects of interleukin-1beta, interleukin-13 and transforming growth factor-beta on gene expression in human airway smooth muscle using gene microarrays. *European Journal of Pharmacology*, *497*(3), 255–65. http://doi.org/10.1016/j.ejphar.2004.06.055

Khurana Hershey, G. K. (2003). IL-13 receptors and signaling pathways: An evolving web.

Journal of Allergy and Clinical Immunology, *111*(4), 677–690. http://doi.org/10.1067/mai.2003.1333

Kotlikoff, M. I., & Kamm, K. E. (1996). Molecular mechanisms of beta-adrenergic relaxation of airway smooth muscle. *Annual Review of Physiology*, *58*, 115–41. http://doi.org/10.1146/annurev.ph.58.030196.000555

Kotlikoff, M. I., Murray, R. K., & Reynolds, E. E. (1987). Histamine-induced calcium release and phorbol antagonism in cultured airway smooth muscle cells. *The American Journal of Physiology*, *253*(4 Pt 1), C561–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3661694

Krell, R. D., Osborn, R., Vickery, L., Falcone, K., O'Donnell, M., Gleason, J., … Bryan, D. (1981). Contraction of isolated airway smooth muscle by synthetic leukotrienes C4 and D4. *Prostaglandins*, *22*(3), 387–409. http://doi.org/10.1016/0090-6980(81)90101-5

Kudo, M., Khalifeh Soltani, S. M. A., Sakuma, S. A., McKleroy, W., Lee, T.-H., Woodruff, P. G., … Atabai, K. (2013). Mfge8 suppresses airway hyperresponsiveness in asthma by regulating smooth muscle contraction. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(2), 660–5. http://doi.org/10.1073/pnas.1216673110

Laporte, J. C., Moore, P. E., Baraldo, S., Jouvin, M. H., Church, T. L., Schwartzman, I. N., … Shore, S. a. (2001). Direct effects of interleukin-13 on signaling pathways for physiological responses in cultured human airway smooth muscle cells. *Am J Respir Crit Care Med*, *164*, 141–148. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11435252

Lauzon, A.-M., & Martin, J. G. (2016). Airway hyperresponsiveness; smooth muscle as the

principal actor. *F1000Research*, *5*. http://doi.org/10.12688/f1000research.7422.1

Lee, J. H., Kaminski, N., Dolganov, G., Grunig, G., Koth, L., Solomon, C., … Sheppard, D. (2001). Interleukin-13 induces dramatically different transcriptional programs in three human airway cell types. *American Journal of Respiratory Cell and Molecular Biology*, *25*(4), 474–85. http://doi.org/10.1165/ajrcmb.25.4.4522

Lim, T. K., Ang, S. M., Rossing, T. H., Ingenito, E. P., & Ingram, R. H. (1989). The effects of deep inhalation on maximal expiratory flow during intensive treatment of spontaneous asthmatic episodes. *The American Review of Respiratory Disease*, *140*(2), 340–3. http://doi.org/10.1164/ajrccm/140.2.340

Locksley, R. M. (2010). Asthma and Allergic Inflammation. *Cell*, *140*(6), 777–783. http://doi.org/10.1016/j.cell.2010.03.004

Loftus, P. A., & Wise, S. K. (2016). Epidemiology of asthma, 1–5. http://doi.org/10.1097/MOO.0000000000000262

Lougheed, M. D., Lemiere, C., Ducharme, F. M., Licskai, C., Dell, S. D., Rowe, B. H., … Boulet, L.-P. (2012). Canadian Thoracic Society 2012 Guideline Update: Diagnosis and management of asthma in preschoolers, children and adults: executive summary. *Can Respir J*, *19*(6).

Lundbäck, B., Backman, H., Lötvall, J., Rönmark, E., Lundbäck, B., Backman, H., … Lötvall, J. (2016). Is asthma prevalence still increasing?, *10*(March), 39–51. http://doi.org/10.1586/17476348.2016.1114417

Ma, X., Cheng, Z., Kong, H., Wang, Y., Unruh, H., Stephens, N. L., & Laviolette, M. (2002). Changes in biophysical and biochemical properties of single bronchial smooth muscle cells from asthmatic subjects. *American Journal of Physiology. Lung Cellular and Molecular*

Physiology, *283*(6), L1181–9. http://doi.org/10.1152/ajplung.00389.2001

Ma, X., Wang, Y., & Stephens, N. L. (1998). Serum deprivation induces a unique hypercontractile phenotype of cultured smooth muscle cells. *Am J Physiol Cell Physiol*, *274*(5), C1206–1214. Retrieved from http://ajpcell.physiology.org/content/274/5/C1206.short

Madison, J. M., Jones, C. A., Sankary, R. M., & Brown, J. K. (1989). Differential effects of prostaglandin E2 on contractions of airway smooth muscle. *Journal of Applied Physiology (Bethesda, Md. : 1985)*, *66*(3), 1397–407. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2468642

Malavasi, F., Deaglio, S., Funaro, A., Ferrero, E., Horenstein, A. L., Ortolan, E., … Aydin, S. (2008). Evolution and function of the ADP ribosyl cyclase/CD38 gene family in physiology and pathology. *Physiological Reviews*, *88*(3), 841–86. http://doi.org/10.1152/physrev.00035.2007

Matsukura, S., Stellato, C., Georas, S. N., Casolaro, V., Plitt, J. R., Miura, K., … Schleimer, R. P. (2001). Interleukin-13 Upregulates Eotaxin Expression in Airway Epithelial Cells by a STAT6-Dependent Mechanism. *American Journal of Respiratory Cell and Molecular Biology*, *24*(6), 755–761. http://doi.org/10.1165/ajrcmb.24.6.4351

Matsumoto, H., Moir, L. M., Oliver, B. G. G., Burgess, J. K., Roth, M., Black, J. L., & McParland, B. E. (2007). Comparison of gel contraction mediated by airway smooth muscle cells from patients with and without asthma. *Thorax*, *62*(10), 848–54. http://doi.org/10.1136/thx.2006.070474

Mims, J. W. (2015). Asthma: Definitions and pathophysiology. *International Forum of Allergy and Rhinology*, *5*(April), S2–S6. http://doi.org/10.1002/alr.21609

Mitzner, W. (2004). Airway smooth muscle: the appendix of the lung. *American Journal of Respiratory and Critical Care Medicine*, *169*(7), 787–90. http://doi.org/10.1164/rccm.200312-1636PP

Moore, P. E., Church, T. L., Chism, D. D., Panettieri Jr., R. A., Shore, S. A., Panettieri Jr., R. A., & Shore, S. A. (2002). IL-13 and IL-4 cause eotaxin release in human airway smooth muscle cells: a role for ERK. *Am J Physiol Lung Cell Mol Physiol*, *282*(4), L847–53. http://doi.org/10.1152/ajplung.00245.2001

Moreno, R. H., Hogg, J. C., & Paré, P. D. (1986). Mechanics of Airway Narrowing. *American Review of Respiratory Disease*, *133*(6), 1171–1180. Retrieved from http://www.atsjournals.org/doi/abs/10.1164/arrd.1986.133.6.1171#.Vyo9p_nNrgU

Morgan, S. J., Deshpande, D. A., Tiegs, B. C., Misior, A. M., Yan, H., Hershfeld, A. V, … Penn, R. B. (2014). β-Agonist-mediated relaxation of airway smooth muscle is protein kinase A-dependent. *The Journal of Biological Chemistry*, *289*(33), 23065–74. http://doi.org/10.1074/jbc.M114.557652

Nino, G., Hu, A., Grunstein, J. S., McDonough, J., Kreiger, P. A., Josephson, M. B., … Grunstein, M. M. (2012). G Protein βγ-subunit signaling mediates airway hyperresponsiveness and inflammation in allergic asthma. *PloS One*, *7*(2), e32078. http://doi.org/10.1371/journal.pone.0032078

Ouedraogo, N., & Roux, E. (2014). Physiology of Airway Smooth Muscle Contraction: An Overview. *Pulmonary & Respiratory Medicine*, *4*(6), 221. http://doi.org/10.4172/2161-105X.1000221

Paré, P. D., & Mitzner, W. (2012). Counterpoint: alterations in airway smooth muscle phenotype do not cause airway hyperresponsiveness in asthma. *Journal of Applied Physiology*

(Bethesda, Md. : 1985), *113*(5), 839–42. http://doi.org/10.1152/japplphysiol.00483.2012a

Pascoe, C. D., Swyngedouw, N. E., Seow, C. Y., & Paré, P. D. (2014). Gene expression in asthmatic airway smooth muscle: a mixed bag. *Canadian Journal of Physiology and Pharmacology*, *93*(2), 137–143. http://doi.org/10.1139/cjpp-2014-0390

Paul, R. J. (2009). Regulation of smooth muscle contraction/relaxation: paradigm shifts and quantifying arrows. *The Journal of Physiology*, *587*(Pt 14), 3413–4. http://doi.org/10.1113/jphysiol.2009.176107

Pelaia, G., Renda, T., Gallelli, L., Vatrella, A., Busceti, M. T., Agati, S., … Marsico, S. A. (2008). Molecular mechanisms underlying airway smooth muscle contraction and proliferation: implications for asthma. *Respiratory Medicine*, *102*(8), 1173–81. http://doi.org/10.1016/j.rmed.2008.02.020

Risse, P. A., Jo, T., Suarez, F., Hirota, N., Tolloczko, B., Ferraro, P., … Martin, J. G. (2011). Interleukin-13 inhibits proliferation and enhances contractility of human airway smooth muscle cells without change in contractile phenotype. *Am.J.Physiol Lung Cell Mol.Physiol*, *300*(6), L958–L966. http://doi.org/10.1152/ajplung.00247.2010

Schittny, J. C., Miserocchi, G., & Sparrow, M. P. (2000). Spontaneous peristaltic airway contractions propel lung liquid through the bronchial tree of intact and fetal lung explants. *American Journal of Respiratory Cell and Molecular Biology*, *23*(1), 11–8. http://doi.org/10.1165/ajrcmb.23.1.3926

Sellers, J. R., Spudich, J. A., & Sheetz, M. P. (1985). Light chain phosphorylation regulates the movement of smooth muscle myosin on actin filaments. *The Journal of Cell Biology*, *101*(5 Pt 1), 1897–902. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2113978&tool=pmcentrez&ren

dertype=abstract

Seow, C. Y., & Fredberg, J. J. (2001). Signal Transduction in Smooth Muscle: Historical perspective on airway smooth muscle: the saga of a frustrated cell. *J Appl Physiol*, *91*(2), 938–952. Retrieved from http://jap.physiology.org/content/91/2/938.short

Seow, C. Y., & van Eeden, S. (2013). Airway remodeling in asthma: tumor of the airway? *Respiration; International Review of Thoracic Diseases*, *86*(5), 364–6. http://doi.org/10.1159/000353914

Skloot, G., & Togias, A. (2003). Bronchodilation and bronchoprotection by deep inspiration and their relationship to bronchial hyperresponsiveness. *Clinical Reviews in Allergy & Immunology*, *24*(1), 55–72. http://doi.org/10.1385/CRIAI:24:1:55

Statistics Canada. (2016, March 7). Asthma, by sex, provinces and territories (Number of persons). Retrieved March 29, 2016, from http://www.statcan.gc.ca/tables-tableaux/sum-som/l01/cst01/health50a-eng.htm

Sugimoto, K., Kudo, M., Sundaram, A., Ren, X., Huang, K., Bernstein, X., … Sheppard, D. (2012). The αvβ6 integrin modulates airway hyperresponsiveness in mice by regulating intraepithelial mast cells. *The Journal of Clinical Investigation*, *122*(2), 748–58. http://doi.org/10.1172/JCI58815

Syed, F., Panettieri, R. A., Tliba, O., Huang, C., Li, K., Bracht, M., … Amrani, Y. (2005). The effect of IL-13 and IL-13R130Q, a naturally occurring IL-13 polymorphism, on the gene expression of human airway smooth muscle cells. *Respiratory Research*, *6*, 9. http://doi.org/10.1186/1465-9921-6-9

Tliba, O., Deshpande, D., Chen, H., Van Besien, C., Kannan, M., Panettieri Jr., R. A., & Amrani, Y. (2003). IL-13 enhances agonist-evoked calcium signals and contractile responses in

airway smooth muscle. *British Journal of Pharmacology*, *140*(7), 1159–62. http://doi.org/10.1038/sj.bjp.0705558

von Bubnoff, D., Geiger, E., & Bieber, T. (2001). Antigen-presenting cells in allergy. *J Allergy Clin Immunol*, *108*, 329–339. http://doi.org/10.1067/mai.2001.117457

Webb, R. C. (2003). Smooth muscle contraction and relaxation. *Advances in Physiology Education*, *27*(1-4), 201–6. Retrieved from http://advan.physiology.org/content/27/4/201.abstract

Wenzel, S. E. (2006). Asthma: defining of the persistent adult phenotypes. *Lancet (London, England)*, *368*(9537), 804–13. http://doi.org/10.1016/S0140-6736(06)69290-8

West, A. R., Syyong, H. T., Siddiqui, S., Pascoe, C. D., Murphy, T. M., Maarsingh, H., … Bossé, Y. (2013). Airway contractility and remodeling: Links to asthma symptoms. *Pulmonary Pharmacology and Therapeutics*, *26*(1), 3–12. http://doi.org/10.1016/j.pupt.2012.08.009

Wills-Karp, M., Luyimbazi, J., Xu, X., Schofield, B., Y, N. T., Karp, C. L., & Donaldson, D. D. (1998). Interleukin-13 : Central Mediator of Allergic Asthma. *Science*, *282*(December), 2258–2261. http://doi.org/10.1126/science.282.5397.2258

Wilson, A. M. (2006). The Role of Antihistamines in Asthma Management. *Treatments in Respiratory Medicine*, *5*(3), 149–158. http://doi.org/10.2165/00151829-200605030-00001

Woodman, L., Siddiqui, S., Cruse, G., Sutcliffe, A., Saunders, R., Kaur, D., … Brightling, C. (2008). Mast cells promote airway smooth muscle cell differentiation via autocrine up-regulation of TGF-beta 1. *Journal of Immunology (Baltimore, Md. : 1950)*, *181*(7), 5001–7. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3992381&tool=pmcentrez&ren

dertype=abstract

Woodruff, P. G., Bhakta, N. R., & Fahy, J. V. (2016). Asthma: Pathogenesis and Phenotypes. In *Murray and Nadel's Textbook of Respiratory Medicine* (7th ed., pp. 713–730). Philadelphia, PA: Elsevier Inc. Retrieved from https://www.clinicalkey.com/#!/content/book/3-s2.0-B9781455733835000415?scrollTo=%23hl0000196

Woodruff, P. G., Modrek, B., Choy, D. F., Jia, G., Abbas, A. R., Ellwanger, A., … Fahy, J. V. (2009). T-helper Type 2–driven Inflammation Defines Major Subphenotypes of Asthma. *American Journal of Respiratory and Critical Care Medicine*, *180*(5), 388–395. http://doi.org/10.1164/rccm.200903-0392OC

Xia, Y. C., Redhu, N. S., Moir, L. M., Koziol-White, C., Ammit, A. J., Al-Alwan, L., … Clifford, R. L. (2013). Pro-inflammatory and immunomodulatory functions of airway smooth muscle: Emerging concepts. *Pulmonary Pharmacology and Therapeutics*, *26*(1), 64–74. http://doi.org/10.1016/j.pupt.2012.05.006

Zhao, A., McDermott, J., Urban, J. F., Gause, W., Madden, K. B., Yeung, K. A., … Shea-Donohue, T. (2003). Dependence of IL-4, IL-13, and nematode-induced alterations in murine small intestinal smooth muscle contractility on Stat6 and enteric nerves. *Journal of Immunology (Baltimore, Md. : 1950)*, *171*(2), 948–54. http://doi.org/10.4049/jimmunol.171.2.948

Zhu, Z., Homer, R. J., Wang, Z., Chen, Q., Geba, G. P., Wang, J., … Elias, J. A. (1999). Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *Journal of Clinical Investigation*, *103*(6), 779–788. http://doi.org/10.1172/JCI5909

Zuyderduyn, S., Sukkar, M. B., Fust, A., Dhaliwal, S., & Burgess, J. K. (2008). Treating asthma

means treating airway smooth muscle cells. *The European Respiratory Journal*, *32*(2), 265–74. http://doi.org/10.1183/09031936.00051407