Human airway smooth muscle promotion of mast cell survival and proliferation, and altered state of activation in asthma

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Abstract

Asthma is a condition that is characterized by variable airflow obstruction, airway hyperresponsiveness (AHR), chronic airway inflammation and airway remodeling. There is microlocalisation of mast cells within the airway smooth muscle (ASM) bundle in asthma at a level significantly above health and other respiratory diseases. These cells are recruited and their survival promoted. However, their functional consequences have yet to be discovered. AHR in asthma is a result of increased responsiveness of the ASM to agonist and has found to increase with localized mast cell numbers. This phenomenon could be a result of an intrinsic abnormality or of local effects. So far, it has yet to be elucidated. We sort to examine this phenomenon of the presence of mast cells within the ASM and the increased responsiveness of the ASM; mechanisms involved in sustaining mast cell numbers, and the intrinsic differences of ASM between asthma and health.

ASM had the ability to maintain survival and promote proliferation of co-cultured mast cells, a mechanism supported by the actions of stem cell factor (SCF), interleukin (IL)-6 and cell adhesion molecule (CADM)-1. There was a physical interaction in mast cell adhesion to ASM between CADM1 and the SCF receptor. The co-culture also enhanced constitutive mast cell degranulation. Intracellular calcium levels of ASM from asthmatic patients at rest were found to oscillate to a great degree. Following stimulation with agonist, the ASM gave a reduced intracellular calcium response. However, their contractile ability measured still remained greater than ASM isolated from non-asthmatic subjects.

SCF, IL-6 and CADM1 supported the survival of mast cells co-cultured with ASM. Although ASM from asthmatic subjects produce a reduced intracellular calcium response to agonist, at baseline these cells are more activated and they still retain their increased contractile response. Mechanisms which may contribute to the altered airway physiology in asthma.

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List of Abbreviations

AHR	Airway hyperresponsiveness
ASM	Airway smooth muscle
Ca^{2+}	Calcium
CADM1	Cell adhesion molecule-1
cAMP	Cyclic adenosine monophosphate
COPD	Chronic obstructive pulmonary disease
CRAC	Calcium release-activated channels
DAG	Diacylglycerol
EB	Eosinophilic bronchitis
EGF	Epithelial growth factor
FCS	Foetal calf serum
FEV_1	Forced expiratory volume in the first second
FVC	Forced vital capacity
GTP	Guanosine triphosphate
HLMC	Human lung mast cells
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
IP_3	Inositol triphosphate
LTC_4	Leukotriene C_4
MC_T	Mast cell containing trypase
MC_{TC}	Mast cell containing both tryptase and chymase
MHC	Myosin heavy chain

MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
Na^+	Sodium
NGF	Nerve growth factor
PAF	Platelet activating factor
PAR	Protease-activated receptor
PDGF	Platelet-derived growth factor
PGD_2	Prostaglandin D_2
PIP_2	Phosphatidylinositol bisphosphate
PLC	Phospholipase C
pMLC	Phosphorylated myosin light chain
pMLCK	Phosphorylated myosin light chain kinase
RyR	Ryanodine receptor
SCF	Stem cell factor
SERCA	Sarco-endoplasmic reticulum calcium ATPase
SOCC	Store-operated calcium channels
SR	Sarcoplasmic reticulum
STIM1	Stromal interaction molecule-1
TGFβ	Transforming growth factor β
TLR	Toll-like receptors
TNF	Tumour necrosis factor
TRPC	Transient receptor potential calcium channel
VCAM-1	Vascular cell adhesion molecule-1

List of Abstracts, Publications and Prizes Arisen from this Thesis

Abstracts

- **F. Hollins**, D. Kaur, R. Saunders, L. Woodman, A. Sutcliffe, P. Bradding, C.E. Brightling. Airway smooth muscle supports mast cell survival. Presented at 5th International Young Investigators Symposium, "Smooth Muscle in Airway and Vascular Disease"; 9th September 2005, Malmö, Sweden.
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- D. Kaur, **F. Hollins**, L. Woodman, N. Neale, W. Yang, P. Monk, R. May, P. Bradding, C.E. Brightling. Mast cells express IL-13RαI and priming human lung mast cells with IL-13 increases FcεRI expression and proliferation. Thorax, 2005, 60:ii119.
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- D. Kaur, **F. Hollins**, R. Saunders, P. Bradding and C.E. Brightling. Human lung mast cells and human airway smooth muscle cell interactions facilitated by cell adhesion molecule and CD117 co-localization. Am J Respir Crit Care Med, 2008, 177:A66.

- D. Kaur, **F. Hollins**, R. Saunders, I. Akihiko, P. Bradding and C.E. Brightling. Human mast cell survival is promoted in co-culture with human airway smooth muscle cells. Am J Respir Crit Care Med, 2008, 177:A66.
- **F. Hollins**, G. Cruse, D. Kaur, C.E. Brightling and P. Bradding. Human lung mast cells in co-culture with airway smooth muscle demonstrate increased constitutive histamine release and decreased histamine content. Thorax, 2008, 63:A49-A50.
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Publications (Reviews)

- **F. Hollins,** P. Bradding, C.E. Brightling. "Leukocytes – Mast cells and basophils". Encyclopedia of Respiratory Medicine. G. J. Laurent & S. D. Spiro, eds., Oxford, UK: Elsevier 2006, Vol 2, 534-542.
- S. Siddiqui, **F. Hollins**, S. Saha, C.E. Brightling. Inflammatory cell microlocalisation and airway dysfunction: cause and effect? ERJ, 2007, Dec;30(6):1043-56
- S. Siddiqui, **F. Hollins**, C.E. Brightling. "Airway hyperresponsiveness: inflammatory mechanisms and clinical aspects". Allergy Frontiers: Epigenetics to Future Perspectives. R. Pawankar, S. Holgate, L. Rosenwaser, eds., Springer 2008, Vol 3:203-226.
- S. Siddiqui, **F. Hollins**, C.E. Brightling. What can we learn about airway smooth muscle from the company it keeps? ERJ 2008, July;32(1):9-11.

Publications (Original articles)

- D. Kaur, **F. Hollins**, L. Woodman, W. Yang, P. Monk, R. May, P. Bradding, C.E. Brightling. Mast cells express IL-13RαI and priming human lung mast cells with IL-13 increases FcεRI expression and proliferation. Allergy, 2006, Sep;61(9):1047-53.
- **F. Hollins**, D. Kaur, R. Saunders, G. Cruse, A. Sutclife A. Ito, P. Berger, C.E. Brightling, P. Bradding. Human airway smooth muscle supports the survival and proliferation of human lung mast cells through stem cell factor-, IL-6- and cell adhesion molecule-1-dependent mechanisms. J Immunol, 2008, 181(4):2772-80.

Prizes

- 1st runner up in British Thoracic Society Young Investigator of the Year Award, December 2005
- Winner of the Wendy Stannard Award at the Institute for Lung Health Research Day, May 2006
- Winner of the Barry Kay Award at the British Society for Allergy and Clinical Immunology, July 2007

Chapter 1:

Introduction

1.1 Airway disease

1.1.1 Definition of asthma and comparison to other chronic respiratory diseases

Diseases of the respiratory system are widespread in society today and are increasing in their prevalence globally, putting an increasing burden on health services. Asthma, a chronic respiratory disease affecting 10% of children and 5% of adults (1,2), is an inflammatory disorder of the conducting airways and is considered to be a heterogeneous disease with respect to immunopathology, clinical phenotypes and response to therapies.

Asthma is a condition that is characterized by variable and reversible airflow obstruction, airway hyperresponsiveness (AHR) and chronic airway inflammation that usually has an eosinophilic component with increased expression of Th2 cytokines (3). Airway remodelling in asthma encompasses several structural changes in the airway wall including reticular lamina and basement membrane thickening, an increased number of subepithelial myofibroblasts and increased airway smooth muscle (ASM) mass (4,5).

Other airway diseases include chronic obstructive pulmonary disease (COPD) and eosinophilic bronchitis (EB). The hallmark of COPD is progressive, irreversible airflow obstruction and chronic airway inflammation, which is predominately caused by smoking. COPD is the major cause of respiratory failure and is a common cause of chronic disability, and by 2020 it is predicted that COPD will be the third leading cause of mortality worldwide (6,7). This respiratory disease has generally been considered to be a neutrophilic disease, in contrast to asthma (8). However, there is increasing evidence that a significant subgroup exists consisting of patients with

stable COPD who have chronic airway eosinophilia with a more steroid-responsive disease (9-11).

EB is characterised by a corticosteroid responsive cough and the presence of sputum eosinophilia occurring in the absence of variable airflow obstruction and airway hyperresponsiveness, and accounts for 10-30% of cough cases referred to respiratory specialists (12-14). Asthma and EB share many immunopathological features including eosinophilic airway inflammation, Th2 cytokine expression and sub-basement membrane collagen deposition (3,12). However, EB does not present with the AHR and variable airflow obstruction (15), and (in conjunction with COPD), does not produce the increased concentrations of IL-13 that are seen in asthma (16,17).

1.1.2 Demographics

According to Asthma UK's report in 2004, there were 5.2 million people living within the United Kingdom with asthma and the prevalence is rising. One fifth of these asthmatic patients are children, meaning for every ten children living in the United Kingdom today, one is living with asthma. In 2002, there were over 1,400 deaths from asthma, averaging as one death every seven hours. Many of these deaths are thought to have been preventable with better routine and emergency care; avoiding delay in treatment during the final attack and use of prescribed medication. (18)

1.1.3 Health care burden

This same report also states asthma is costing the United Kingdom £2.3 billion every year, which is also thought to be rising. National Health Service comprises £889 million of this costing, which is consists of a combination of prescription, dispensing, general practitioner consultations and hospital admissions. Indirect costs comprise the remaining financial burden, including social security benefits and loss in work productivity due to absence. (18)

1.1.4 Immunopathology

Asthma is a complex chronic inflammatory disorder of the airways resulting in symptoms of coughing, wheezing, and shortness of breath, associated with evidence of bronchial reversibility and/or hyperreactivity. From a pathophysiological perspective, the two hallmarks of the disease are chronic airway inflammation and airway dysfunction, manifesting as airway hyperresponsiveness and variable airflow obstruction (19,20).

The asthmatic phenotype displays sub-basement membrane thickening due to collagen, fibronectin, tenascin and laminin deposition under the epithelium, thickening of the smooth muscle layer due to cell hyperplasia and hypertrophy, epithelial shedding, angiogenesis and mucus hypersecretion by goblet cells (20-22). There is also inflammatory cell infiltration by eosinophils, neutrophils, $CD4^+$ T lymphocytes and mast cells (19).

Asthma is becoming recognised as a heterogeneous disease with both allergic and non-allergic phenotypes (23). In allergic asthma, the airways predominantly develop eosinophilic inflammation with prominent oedema and mucus production in response to inhalation of antigen. In addition, smooth muscle cells of the airways become hyperreactive, leading to reversible bronchoconstriction in response to various stimuli. In non-allergic asthma, neutrophilic and a few immune forms have been described, but it is unclear if these represent distinct clinical phenotypes, as they often respond to conventional asthma therapy (23).

1.1.5 Airway hyperresponsiveness

Airway smooth muscle of the asthmatic phenotype is found to be at a more activated state than non-asthmatic airway smooth muscle (24,25), which contributes to the AHR, a consistent and defining feature of asthma. Clinically, AHR can be demonstrated by measuring FEV_1 or airway resistance after inhalation of incremental dose of non-specific airway smooth muscle direct agonist, such as histamine or methacholine, or indirect challenge to exercise or hyperventilation (26,27). There are two semi-independent components to AHR, variable or persistent (27). AHR can be transient or inducible, occurring after allergen exposure and improves following treatment with corticosteroids or environmental control. The transient AHR is more marked in response to indirect stimuli. Evidence has been collected linking AHR to airway inflammation (27); however the precise mechanisms involved have yet to be elucidated.

1.1.6 Treatments of Asthma

Asthma is characterized by airway narrowing secondary to chronic inflammation. Methods of treatment are targeted at controlling asthma symptoms, treatment of exacerbations and prevention of lung function decline.

Asthma symptoms are a cause of physical and psychological morbidity that impacts upon the patient's quality of life. Exacerbations are periods of poorly controlled asthma exhibiting an increase in symptoms and lung function deterioration. These exacerbations are usually precipitated by allergen exposure or viral infections (28). Severe exacerbations may lead to hospitalization and are the major cause of asthma mortality. A decline in lung function is usually the result of persistent airway inflammation exhibited in chronic asthma. However, the rate of decline in lung function is dependent on severity of disease, number of exacerbations and smoking history (29).

Inhaled pharmacological drugs used for treating asthmatic symptoms fall into two categories; bronchodilators and anti-inflammatories. Bronchodilators, (such as β_2-agonists, theophylline and anti-cholinergics), give immediate reversal of airway obstruction by relaxing the smooth muscle (30). These drugs act by a common pathway of increased intracellular cyclic AMP in smooth muscle cells that inhibits the contractility of the cells (31). The primary role of bronchodilators is short acting immediate relief of symptoms and the long acting prevention of further symptoms. Anti-inflammatory treatments, (including corticosteroids, anti-leukotrienes, anti-IgE, methotrexate and cyclosporine A), suppress the underlying disease process and provide long-term control of symptoms (30).

All these treatments are targeted at treating and suppressing the symptoms of asthma. They do not target the underlying disease, nor do they guarantee cessation of future exacerbations even if taken as prescribed. So despite their mechanisms of action; of relaxing smooth muscle and inhibiting the inflammatory response, AHR still remains, the underlying cause of which is still to be discovered. Asthmatic airway smooth muscle could be abnormal causing this unwanted and adverse contraction, and/or the inflammatory process could be faulty; in the recruitment and/or the mechanism of the cells and their subsequent respective interactions.

1.1.7 Lung mast cell and airway smooth muscle involvement

The defining feature of asthma is the presence of significantly increased numbers of mast cells located within the airway smooth muscle layer (15,32-35). These elevated numbers observed when compared to control have not been detected in patients with COPD (8,17,36) or in EB (15). These ASM-localised mast cells in asthma are also found in an activated secretary state (33), suggesting that their locality within the smooth muscle; through a process of recruitment with possible survival and proliferative effects, stimulates these cells to release their secretary granule products, which would then act locally on the airway smooth muscle.

1.2 Human lung mast cells

1.2.1 Introduction

In the late 1870s, Paul Ehrlich first identified the mast cell. These cells develop from pluripotential haematopoietic stem cells within the bone marrow. They enter the circulation as non-granular immature mast cell precursors, expressing $CD34^+$, $CD13^+$ and $CD117^+$ on their surface (37,38). They migrate to tissue and under the influence of local mediators they differentiate into mature tissue mast cells. Mature human mast cells are classified based on their serine protease content, into cells that contain both chymase and tryptase MC_{TC}, or cells that contain tryptase alone MC_T (38,39).

Mast cells have an important role in both the innate and the adaptive immune response to infections. These cells play a central role in the allergic inflammatory response and are implicated in the pathogenesis of a number of respiratory diseases (38).

1.2.2 Mast cell biology

1.2.2.1 Cell trafficking to the lung

Immature mast cell precursors are drawn into the circulation from the bone marrow by chemotactic factors and chemokines. Chemokines are a superfamily of low molecular weight heparin-binding molecules that serve as potent chemoattractants for cells of the immune system. The chemokine family is divided into 4 groups, designated CXC, CC, C, and CX3C; depending on the spacing of conserved cysteines, (where X represents an amino acid) (40). The CC and CXC chemokines are the major chemokine families consisting of more than 50 members. The C and

CX3C chemokines are much smaller families each consisting of only one or a few members (41).

Chemokines exert their effects through binding to heterotrimeric, seven-transmembrane spanning, G-protein-coupled receptors. Currently CCR1, 3, 4, 5, 7, CXCR1, 2, 3, 4 and 6 receptors have been identified on mast cells (42), which can bind a multitude of ligands. Some chemokines found to be important in the recruitment of mast cells include IL-8 (CXCL8 binding to CXCR1) (43), SDF-1α (CXCL12 binding to CXCR4) (44), IP-10 (CXCL10 binding to CXCR3) (45), RANTES (CCL5 binding to CCR1, 3 and 5) (46,47) and eotaxin (CCL11 binding to CCR3) (43). These chemokines, in conjunction with other chemotaxins such as stem cell factor (SCF), transforming growth factor (TGF)-β and nerve growth factor (NGF) (38), are involved in the migration of mast cell precursors into the circulation, and the movement of mast cells into tissue.

Mature mast cells localize to specific structures in the lung namely nerves, blood vessels, lymphatics, airway epithelium, glands and airway smooth muscle. This microlocalisation of mast cells is likely to be important in the pathogenesis of many lung diseases (38). The mechanisms involved in mast cell trafficking are summarized in Fig 1.1.

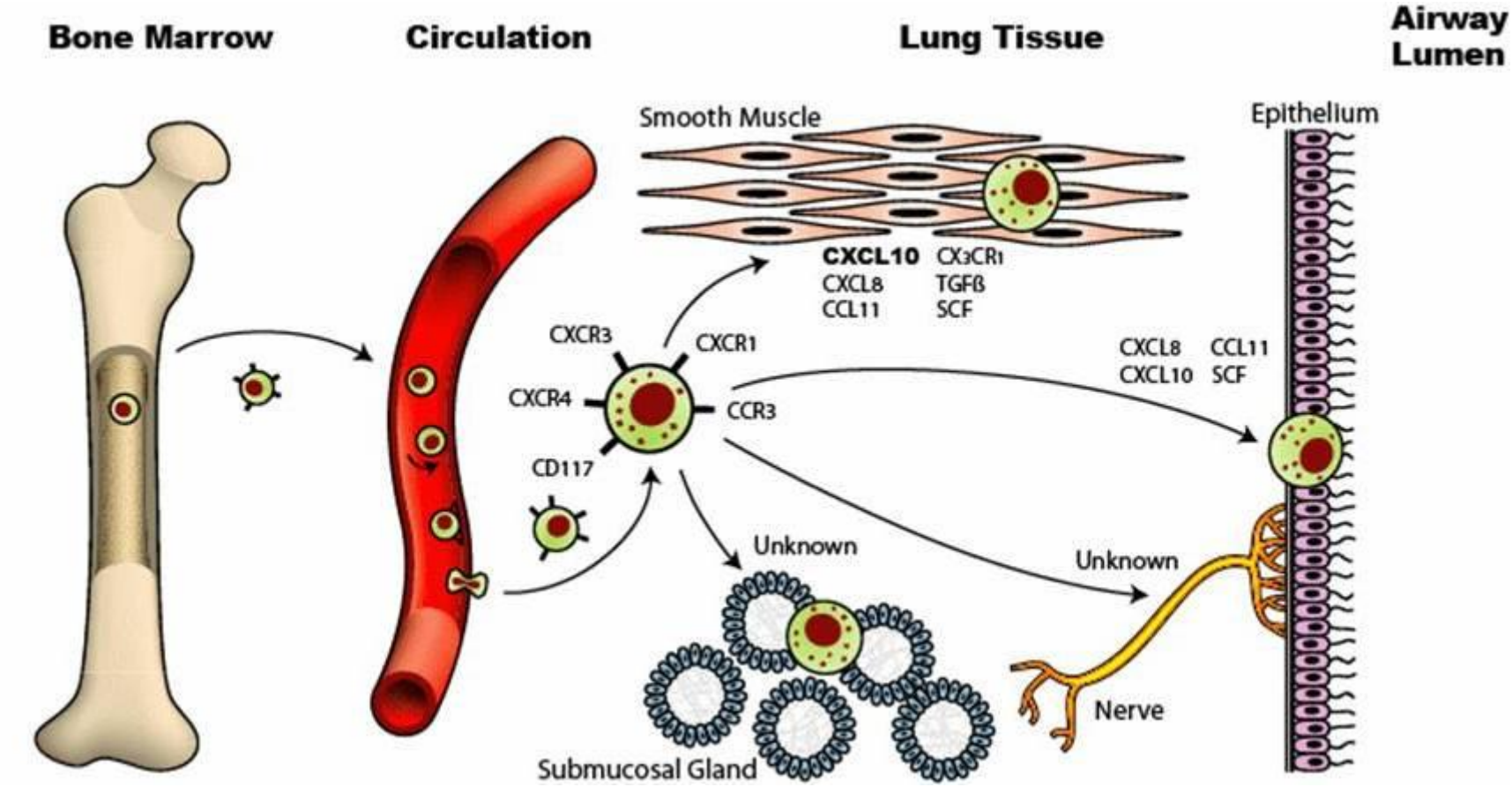


Figure 1.1 Mast cell trafficking to airway structures

In the airway mast cells traffic to airway structures namely the ASM-bundle, epithelium and mucus glands. The mechanisms known to be involved in this microlocalisation are as shown.

1.2.2.2 Mast cell differentiation and survival

Once within the tissues, mast cells can survive for a relatively long period of time, possibly days to weeks in comparison to neutrophils which survive only for hours. Mast cell survival is thought to occur through the action of cytokines, in particular SCF, which also plays an important role throughout the development and migration of mast cells (38,48). Several other growth factors and cytokines are also known to affect the growth and differentiation of mast cells, including interleukin (IL)-3, IL-4, IL-6, IL-9, IL-10 and NGF (48).

1.2.2.3 Mechanisms mediating degranulation

The extracellular release of both pre-formed and newly synthesized mediators by mast cells, a process termed degranulation, can be mediated by both IgE-dependent and IgE-independent mechanisms. IgE molecules produced by B-lymphocytes bind to high affinity IgE receptors ($Fc_{\varepsilon}RI$) on the surface of mast cells. IgE can also bind to $Fc_{\gamma}RII$, $Fc_{\gamma}RIII$ and to galectin-3, which are also found on the surface of these cells. However, IgE only elicits its functions through binding to $Fc_{\varepsilon}RI$ (49,50).

$Fc_{\varepsilon}RI$ is a heterotetrameric receptor composed of an IgE-binding α-subunit, a four-transmembrane-spanning β-subunit and two disulphide-bonded γ-subunits, ($\alpha\beta\gamma_2$) (49). The γ-subunits are signal-transducing molecules, producing a signal, which can then be amplified by the β-subunits. Crosslinking of the $Fc_{\varepsilon}RI$ with IgE and antigen induces activation of protein-tyrosine kinases, including those of the Src, Syk and Tec families (50-53). Pathways which involve the activation of these protein-tyrosine kinases, ultimately lead to degranulation.

In addition several IgE-independent mechanisms can cause degranulation: i) physical factors, i.e. high temperature, ionizing irradiation and mechanical trauma; ii) chemical substances, i.e. proteases, toxins and venoms; iii) endogenous mediators, i.e. tissue proteases and cationic proteins derived from eosinophils and neutrophils; iv) osmotic effects; v) adenosine, via activation of the adenosine A_{2B} receptor; and vi) anaphylatoxins C3a, C4a and C5a, formed during activation of complement can trigger degranulation through activation of the C5a-receptors on the surface of mast cells. However, it is important to note that human lung mast cells are devoid of the C5a receptor, hence it is unlikely that this mechanism of mast cell degranulation is important in the lung (54).

Following degranulation of mast cells there is an immediate release of granule products. Subsequently, there is synthesis and release of lipid mediators over several minutes, and the production and secretion of cytokines, chemokines and growth factors over several hours.

1.2.2.4 Pre-formed mediators

1.2.2.4.1 Histamine

A major source of histamine within the body is the mast cell, playing a pivotal role in allergic inflammation. Histamine, synthesized in the Gogli apparatus of the mast cell, is a vasodilator, a constrictor of smooth muscle, and a potent stimulant of vascular permeability, respiratory mucus, and gastric acid secretion. It exerts its effects on a variety of cell types including smooth muscle cells. Following mast cell degranulation, there is local and systemic increase in histamine concentration (55).

The histamine receptor family are a group of seven-transmembrane-spanning units that couple ligand binding with intracellular reactions through guanosine triphosphate (GTP)-binding heterotrimeric proteins. The histamine receptors are sub-classified into four groups: H_1, H_2, H_3 and H_4. The different receptor subtypes also have differing intracellular secondary messengers; H_1, H_3 and H_4-receptors mobilise intracellular calcium while H_2 and H_3-receptors utilize cyclic adenosine monophosphate (cAMP). Currently, there is evidence of H_1, H_2 and H_4 expression on human mast cells. These receptors act to induce or modulate cytokine synthesis in allergic inflammation, having varying effects on different cell types. (56,57)

1.2.2.4.2 Proteases

The mast cell granules contain neutral proteases. Four such proteases have been identified: tryptase, chymase, carboxypeptidase and cathepsin G.

Tryptase

Mast cell tryptase is a tetrameric neutral serine protease with a molecular weight of 134kDa and it composes 20% of the total cellular mast cell protein (58). Tryptase is found in negligible quantities in other cells making it a good mast cell marker both for identification of the cells and their causative properties (59). The enzyme is made up of four non-covalently bound subunits, each subunit having one enzymatically active site. There are two main types of mast cell tryptase, α-tryptase and β-tryptase, with approximately 90% sequence homology between these two types. α-tryptases are classified into αI- and αII-tryptases, and β-tryptases into βI-, βII- and βIII-tryptases. βII-tryptase is stored in the secretory granules of mast cells. In contrast, α-pro-tryptase is secreted constitutively from mast cells as an inactive proenzyme (60).

The activation of βII-pro-tryptase involves two proteolytic steps. The first is an autocatalytic intermolecular cleavage, which occurs optimally at an acidic pH and in the presence of heparin or dextran sulphate. The resulting product is a monomer, which is about 50 times less active than the final tetramer. The second step involves the removal of the remaining precursor dipeptide by dipeptidyl peptidase I, thus allowing the mature peptide to spontaneously form the active tetramer. This process also requires the presence of heparin or dextran sulphate (60).

Tryptase can activate a number of peptides by cleaving certain extracellular substrates such as vasoactive intestinal peptide, calcitonin gene-related peptide, fibronectin, kininogens and the protease activated receptor (PAR)-2 (61,62). Airway epithelial and smooth muscle cells, endothelial and vascular smooth muscle cells, terminal bronchial epithelium, type II pneumocytes and masts cells within the respiratory tract express PARs. Tryptase is a potent mitogen for epithelial cells, airway smooth muscle cells and fibroblasts (63). During inflammation, tryptase can stimulate the release of granulocyte chemoattractant IL-8, and up-regulate expression of intercellular adhesion molecule 1 (ICAM-1) on epithelial cells. It induces the expression of IL-1β mRNA, which could be important for the recruitment of inflammatory cells to the sites of mast cell activation. The release of tryptase also promotes the degranulation of neighbouring mast cells via the activation of PAR-2, thereby initiating a positive feedback mechanism (60).

Chymase

As outlined above there are two types of mast cells; MC_T and MC_{TC}. MC_T mast cells are the predominate mast cell subtype in the lung, comprising 90-95% of the lung

mast cell population. The MC_{TC} mast cells are found in increased number in the airway smooth muscle, epithelium and glands, and their number are increased in disease (64).

Mast cell chymase is a glycosylated chymotryptic serine protease with a molecular weight of 30kDa. It is stored in the same secretory granules as those that contain tryptase, but is released from mast cells in a macromolecular complex with carboxypeptidase and proteoglycans, which is distinct from tryptase. Like tryptase, chymase is present in the granules as a catalytically active form, but due to the acidic condition within the granules, this activity is suppressed.

Chymase has a net charge of +13 allowing is to bind strongly with negatively charged granule proteoglycans, to cells and to basement membranes. Angiotensin I is readily hydrolyzed by chymase to form angiotensin II, a reaction that is catalyzed more efficiently than with the angiotensin enzyme itself. Circulating α-antichymotrypsin and α1-antitrypsin, and the respiratory tract inhibitors bronchial secretion inhibitor and antileukoprotease inhibit chymase (65).

Chymase can modulate inflammatory processes. It can convert cytokines from inactive to their active form such as IL-1β and it can degrade others such as IL-4. In addition, chymase has a role in tissue destruction and matrix remodeling as chymase can activate stromelysin and interstitial collagenase, and can convert procollagen I to collagen-sized fragments and degrade type IV collagen. Interestingly, chymase like tryptase can activate cells via PARs. Chymase acts upon PAR-1, which is expressed

on several inflammatory and mesenchymal cells, and can lead to cell activation and proliferation (65,66).

Carboxypeptidase

Human mast cell carboxypeptidase is a zinc metallo-exopeptidase 34.5kDa monomer, only found in mast cells of the tryptase/chymase phenotype. It cleaves the carboxy terminal of the His-Leu bond of angiotensin I generating des-Leu_{10} angiotensin I, an inhibitor of angiotensin converting enzyme (67).

Cathepsin G

Various physiological effects are attributed to cathepsin G including antimicrobial activity, degradation of extracellular matrix, vasoregulation, activation of neutrophil elastase, processing of IL-8 and mast cell degranulation (65).

1.2.2.5 Synthesised mediators

1.2.2.5.1 Lipid-derived mediators

Arachidonic acid is released from the phospholipid membrane following activation; the metabolites of which are cyclooxygenase and lipooxygenase. The cyclooxygenase pathway results in the synthesis of prostaglandin D_2 (PGD_2), while the lipooxygenase pathway liberates leukotrienes and in particular leukotriene C_4, (LTC_4) (68). While initially recognized for their bronchoconstricting and vasoactive properties, these two eicosanoids are now known to serve diverse and pivotal functions in effector cell trafficking, antigen presentation, immune cell activation, matrix deposition, and fibrosis (68,69).

PGD_2, which is synthesized by mast cells, can bind to receptors on smooth muscle and can act as a vasodilator and a bronchoconstrictor. It can also cause chemotaxis and accumulation of inflammatory cells at the site of inflammation, which is aided by its effect on increasing the microvascular permeability. There are two types of receptor for PGD_2; DP_1 and DP_2 (CRTh2) (70). Both of these receptors are G-protein coupled rhodopsin-type receptors with seven transmembrane domains. DP_1 receptors are prostanoid receptors, found on various types of haematological and non-haematological cells, including platelets, neutrophils and smooth muscle cells. DP_2 or CRTh2 receptors are a member of the chemokine receptor family and are found exclusively on the haematological cells: basophils, eosinophils and Th2 cells (70,71).

Leukotrienes are produced upon the activation of mucosal mast cells. They bind to receptors on smooth muscle causing prolonged bronchoconstriction (10-1000 times more potent than histamine), induce prolonged wheal-and-flare response, enhance venular permeability, promote mucus secretion, and they act as chemotactic agents for neutrophils and eosinophils (72,73).

The other type of lipid mediator produced by mast cells is platelet-activating factor (PAF). PAF has a diverse range of physiological actions varying from aggregation and degranulation of platelets and neutrophils, to a variety of cellular effects including stimulation of chemotaxis, chemokinesis. It too can also result in bronchoconstriction and vasodilatation, and also may cause retraction of endothelial cells (73,74).

1.2.2.5.2 Cytokines and chemokines

Mast cells produce many different cytokines and chemokines that may contribute towards an inflammatory response. The cytokines and chemokines produced are illustrated in Table 1.1. Mast cell activation results in the *de novo* synthesis of these cytokines, however, tumour necrosis factor (TNF) and IL-4 may already be stored preformed in granules and rapidly released upon $Fc_{\varepsilon}RI$ cross-linking (75).

Table 1.1 Cytokines and chemokines produced by mast cells

Content of various proteases, lipid-derived mediators, cytokines and chemokines found within the resting and activated mast cell. (This is not an exhaustive list.)

+ Mediator present

- Mediator absent

MC_{TC} only; mediator only present in the tryptase/chymase mast cell subtype.

Table modified from: F. Hollins**,** P. Bradding, C.E. Brightling. "Leukocytes – Mast cells and basophils". Encyclopedia of Respiratory Medicine. G. J. Laurent & S. D. Spiro, eds., Oxford, UK: Elsevier 2006, Vol 2, 534-542.

	Mast Cell		Function
	Resting	**Activated**	
Histamine	+	+	Vascular permeability
Proteases			
Tryptase	+	+	Tissue remodelling
Chymase	MC_{TC} only	MC_{TC} only	
Carboxypeptidase	MC_{TC} only	MC_{TC} only	
Cathepsin G	MC_{TC} only	MC_{TC} only	
Lipid-derived mediators			
Prostaglandin D_2	-	+	Recruitment of effector cells, immune response regulation, and angiogenesis, bronchoconstriction and oedema promotion
Leukotrienes	-	+	
Platelet-activating factor	-	+	Effector cell activation and chemoattractant
Cytokines			
IL-1α and β	-	+	Inflammation induction
IL-6	-	+	
IFNα and β	-	+	
GM-CSF	-	+	
TNFα	+	+	
IL-3	-	+	Th2 cytokines
IL-4	+	+	
IL-5	-	+	
IL-13	-	+	
IL-12	-	+	Th1 cytokines
IFNγ	-	+	
IL-10	-	+	Inflammation and angiogenesis induction
TGFβ	-	+	
Chemokines			
CCL1 (TCA3/I309)	-	+	Recruitment of effector cells
CCL2 (MCP-1)	-	+	
CCL3 (MIP-1α)	-	+	
CCL4 (MIP-1β)	-	+	
CCL5 (RANTES)	-	+	
CCL20 (LARC)	-	+	
CXCL8 (IL-8)	-	+	Recruitment of effector cells and immune response regulation
CXCL10 (IP-10)	-	+	

1.2.3 Mast cell functions in the normal lung

Mast cells are found within tissues that interface with the local environment such as the skin, airways and intestine, and are therefore well placed to initiate and enhance early responses to a variety of challenges. In response to pathogens, mast cells produce cytokines, proteases and lipid-associated mediators (76). This leads to immune vascular permeability and recruitment of other inflammatory cells, such as neutrophils and eosinophils. The acquired immune response is enhanced through influencing the function and maturation of dendritic cells, and increasing recruitment of T lymphocytes in the lymph nodes through the actions of TNF-α, which optimizes the environment for effective antigen presentation (77).

Mast cells are able to detect and respond to pathogens through the use of direct or indirect receptors. Direct cell activation is essential for initiation of an early innate immune response as well as the generation of appropriate acquired immunity. For this direct interaction to occur, these cells have cell-surface receptors that can interact directly with pathogens. Toll-like receptors (TLRs) are an example of such a receptor type. TLRs recognize products from pathogens such as from Gram-positive and Gram-negative bacteria (78). Binding of these pathogen products to TLRs can result in their activation, which could ultimately lead to a pro-inflammatory response. In mammals, eleven TLRs have so far been identified, each having a distinct function with regard to its immune recognition properties. Mast cells have so far been found to express TLR1, 2, 3, 4, 6, and 9, each capable of activating mast cells upon binding of the appropriate ligand (79,80).

Indirect cell activation is important in the event of secondary or subsequent infections and can occur through several mechanisms including Fc-receptor-mediated activation (81).

1.2.3.1 Wound repair

Mast cells are resident in tissues, particularly in association with endothelial and epithelial cell basement membranes, and are increased at sites of inflammation, injury, and fibrosis (82). Although mast cells are known to both release and generate pro-inflammatory mediators in response to inflammatory stimuli, the cells express large amounts of mRNA for collagen IV, laminin, and heparin sulphate proteoglycan, suggesting that these cells may contribute to normal tissue repair (83).

1.3 Human airway smooth muscle

1.3.1 Introduction

Airway smooth muscle circumferentially surrounds the lumen of the bronchi and has a three-dimensional contractile ability. However, its precise function remains unclear. The orientation of smooth muscle in the lower airways suggests a highly efficient architectural structure that greatly enhances the ability of the muscle to sustain bronchoconstriction; a function that only appears to have a role in disease and not health (84,85).

ASM development occurs earlier than that of vascular smooth muscle during embryogenesis and originates from neural crest and mesenchymal cells. During development, ASM accumulates the cytosolic markers actin, myosin, myosin light chain (MLC), myosin light chain kinase (MLCK), calponin, caldesmon, tropomyosin and desmin (86).

Although the role of ASM in health remains unknown, evidence suggests that changes in ASM phenotype may play a fundamental role in the pathogenesis of lung diseases. The first detailed report of airway structural change in asthma was published over 80 years ago by Huber and Koessler (87), who demonstrated that patients with fatal asthma have substantial thickening of both the airway subepithelial and smooth muscle layers. Since then, focus has been on the airway structural changes accompanying asthma and the potential consequences of these changes for airway function and medical management. Nevertheless, the precise role of this airway remodelling in the pathogenesis of airflow obstruction remains unclear.

The increase in smooth muscle mass that was observed in fatal asthma has since been substantiated in further reports in non-fatal asthma (5,88,89). In addition, further differences have been identified including an increase in the proliferation rate (90,91) and an increase in the contractility (24,92) of the asthmatic smooth muscle cell type.

1.3.2 Human airway smooth muscle biology

1.3.2.1 Human airway smooth muscle trafficking to the lung

Cell migration could be in part responsible for the pathogenesis of airway smooth muscle hyperplasia and remodelling in asthma. ASM cells migrate in response to a number of physiological stimuli *in vitro*, including platelet-derived growth factor (PDGF), basic fibroblast growth factor, TGF-β and IL-1β (93,94). Recent studies suggest that chemokines, involved in the regulation of leukocyte trafficking, may also regulate ASM migration. For example, the CCR3 ligand eotaxin induces migration of airway smooth muscle cells *in vitro*, and airway smooth muscle CCR3 expression is increased in asthma (95). Airway smooth muscle cells also express CXCR1, CXCR2 and CCR7, and migrate in response to the CXCR1/2 ligand IL-8 and the CCR7 ligand CCL19 (96,97). While β-agonists and corticosteroids inhibit migration (93).

The signalling pathways regulating airway smooth muscle migration have been well studied. Activation of a p21-activating kinase/p38 mitogen-activated protein kinase heat shock protein 27 signalling pathway is required for the migration of canine tracheal smooth muscle cells (94,98), while activation of the tyrosine kinase Src is necessary and sufficient for the migration of cultured human ASM cells (99). In contrast, activation of protein kinase A has been shown to inhibit migration (93).

The source of the migrating ASM cells in vivo (i.e., the original airway smooth muscle layer, circulating fibrocytes, or bone marrow- or lung-derived mesenchymal stem cells) remains unknown. The presence of circulating fibrocytes and mesenchymal stem cells has been demonstrated that in the airways (100), suggesting that airway myofibroblasts may originate from distant sites. Allergen exposure induces the accumulation of CD34-, collagen I-, and α-smooth muscle actin-positive cells to areas of collagen deposition below the epithelium in patients with allergic asthma (100).

1.3.2.2 Human airway smooth muscle hyperplasia and hypertrophy

This increased proliferation rate of ASM from asthmatic patients has been well documented (90,91). Many inflammatory mediators are increased in the asthmatic lung, some of which are inducers of ASM proliferation; including growth factors, inflammatory mediators, contractile agonists, cytokines, and extra-cellular matrix proteins (101).

Cultured airway smooth muscle cells proliferate in response to a number of stimuli, including peptide growth factors ligating receptor tyrosine or serine/threonine kinases and bronchoconstrictor substances associated with G protein-coupled seven-transmembrane receptors. The former include PDGF, epidermal growth factor (EGF), and TGF-β (102-105), whereas the latter include histamine, thrombin, endothelin, elastase and tryptase (106-113).

In addition to smooth muscle proliferation, another component of the increase in smooth muscle mass is cell hypertrophy (5,90). Much less is known about this

increase in size of the airway smooth muscle cells, but increased levels of TGFβ (secreted by human lung mast cells [HLMC] and ASM) have been identified as potential hypertrophic influences (91,105,114).

1.3.2.3 Human airway smooth muscle differentiation

A broad heterogeneity of smooth muscle function and responsiveness exists between proximal and distal airways in receptor repertoire and in the pharmacological, electrophysiological and mechanical properties of smooth muscle. However, heterogeneity of myocytes at a single site may also be a feature in the airway wall identified by the expression of contractile proteins such as smooth muscle myosin heavy chains (MHCs) and smooth muscle α-actin. Two main phenotypes of smooth muscle exist, contractile and synthetic (86). Contractile cells are characterized by a high density of contractile proteins and few biosynthetic intracellular organelles; they are mitotically quiescent and retain their ability to contract in response to spasmogens. Synthetic smooth muscle cells have a low density of contractile proteins and high fraction of biosynthetic organelles, are mitotically active and may lose their ability to contract. However, these two cell phenotypes are not mutually exclusive and there may in fact be an intermediate phenotype whereby both contractile and synthetic functions are expressed. (86)

The synthetic function of ASM from an asthmatic patient differs to that of a non-asthmatic individual in many ways. Connective tissue growth factor expression is increased in ASM from an asthmatic patient (115), whereas culturing ASM from an asthmatic patient reduces prostaglandin E_2 production, while increasing the prostaglandin receptor and altering its extracellular matrix proteins (116-118).

Following stimulation with TNFα, expression of OX40L increases in the asthmatic phenotype (119), and following stimulation with Th1 and Th2 cytokines CXCL10 and eotaxin increases respectively (120,121). ASM from an asthmatic patient also displays a rhinovirus-specific increase in IL-6 (122).

1.3.2.4 Components of airway smooth muscle

The dynamics of the ASM contraction, rather than its force-generating capacity, have been postulated to be key features of AHR (123). The proteins of the contractile apparatus are responsible for the force and movement of muscle, and dictate the resulting mechanical properties of the ASM. The ASM contractile apparatus is composed of thick and thin filaments. The thick filaments are constituted of myosin molecules, while the thin filaments comprise α and γ actin; the regulatory proteins tropomyosin, caldesmon and calponin. (123).

1.3.2.4.1 Myosin

Myosin, the primary protein of the thick filament, is a hexameric protein made up of two heavy chains (approx 200 kD), each non-covalently associated with a pair of (17 kD) light chains and a pair of regulatory (20 kD) light chains (124). The myosin heavy chain dimers form globular amino terminal actin-binding heads and coiled-coil tails at their carboxyl terminus. ASM myosin heavy chain (MHC) comprises two isoforms, SM MHC-1 (SM1; 204kDa) and SM MHC-2 (SM2; 200kDa) (123,125), the latter of which is considered to be the marker of mature smooth muscle (126). However, expression of these two isoforms have not been found to differ between asthma and health (5).

1.3.2.4.2 Actin

The actin concentration in smooth muscle is approximately 0.9-1.6mM and accounts for 30-50% of the total non-collagenous proteins in smooth muscle actin, which is thought to exist primarily as filamentous F-actin in intact cells. Filamentous actin is formed of a double-helix of polymers of globular actin and comprises the thin filament. Contractile smooth muscle actin isoforms include the α and γ actin, also referred to as vascular and enteric, respectively (127).

1.3.2.4.3 Myosin light chain kinase

Phosphorylation and subsequent activation of myosin is accomplished by myosin light chain kinase (MLCK). Dephosphorylation is accomplished by myosin light chain phosphatase (MLCP). Smooth muscle MLCK consists of an actin-binding domain at the N-terminal, the catalytic domain in the central portion, and the myosin-binding domain at the C-terminal. The kinase activity is mediated by the catalytic domain that phosphorylates the myosin light-chain of 20kDa, activating smooth muscle myosin to interact with actin (128). The expression of MLCK has been reported to be increased in ASM from asthmatic subjects compared with healthy controls (5,89).

1.3.2.4.4 Tropomyosin

Tropomyosin is one of the major components of smooth muscle. It is a coiled-coil α-helix and has molar ratio of tropomyosin to actin monomers is ~1.2:7 in smooth muscle. Individual molecules interact head to tail, thereby creating a continuous strand along the actin filament (127). Tropomyosin is likely to play a role in the stabilization of the smooth muscle actin contractile filaments, similar to its role in

non-muscle cells. The four Tm genes produce many isoforms (>40) as a result of alternative exon usage. Most are found in non-muscle cells, but there are some specific to either striated or smooth muscle. Only two isoforms appear to be specific to smooth muscle; one from the β tropomyosin gene and the other from the α tropomyosin gene (129). Unfortunately, tropomyosin has not been widely studied in smooth muscle.

1.3.2.4.5 Caldesmon

Caldesmon is a myosin, actin, tropomyosin and calmodulin binding protein. This elongated protein consists of a C-terminal domain that is responsible for actin binding and inhibition of myosin ATPase activity *in vitro*. Binding of calmodulin or phosphorylation of sites between the two C-terminal actin binding domains can reverse some of the inhibitory actions of caldesmon *in vitro*. The N-terminal half of the molecule has been shown to bind myosin and, *in vitro*, tethers myosin to actin in conjunction with C-terminal actin binding domains of caldesmon (130).

1.3.2.4.6 Calponin

Calponin is a family of alleged actin regulatory proteins that includes three isoforms that are separate gene products. Basic calponin (h1CaP) is the predominant species in ASM cells, although it is also expressed in other cell types. In tissues and in cultured primary ASM, h1CaP associates with the central (potentially contractile) actin bundles, and is excluded from cell matrix adhesion sites. Neutral calponin (h2CaP) expression has been detected in both smooth muscle and non-muscle cells, and is involved in the regulation of cell proliferation and cytokinesis. Expression of acidic

calponin (h3CaP) appears mostly restricted to neuronal tissues, although it has also been detected in ASM and pancreatic cells (131).

1.3.2.5 Mechanisms mediating contraction

Spasmogens such as acetylcholine, bradykinin and histamine induce ASM contraction, and thereby regulate the tone of airways (Fig 1.2). This process requires elevation of intracellular calcium, which is brought about by the generation of a variety of second messengers inside the cell. The elevation of intracellular calcium concentration results from a multitude of mechanisms, including influx from the extracellular space and release from intracellular stores, principally the sarcoplasmic reticulum (SR). Calcium combines with calmodulin and this complex activates MLCK to phosphorylate myosin light chain (MLC) resulting in the actin cross-bridging causing the smooth muscle cell to shorten (132).

The agonist binds to a G-protein-coupled receptor stimulating phospholipase C (PLC), an enzyme specific for the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to catalyze the formation of two second messengers: inositol trisphosphate (IP_3) and diacylglycerol (DAG). The binding of IP_3 to its receptor on the SR results in the release of calcium into the cytosol. The resulting elevation of intracellular calcium activates ryanodine receptor (RyR) channels through a mechanism that is widely described as calcium-induced calcium release (132,133).

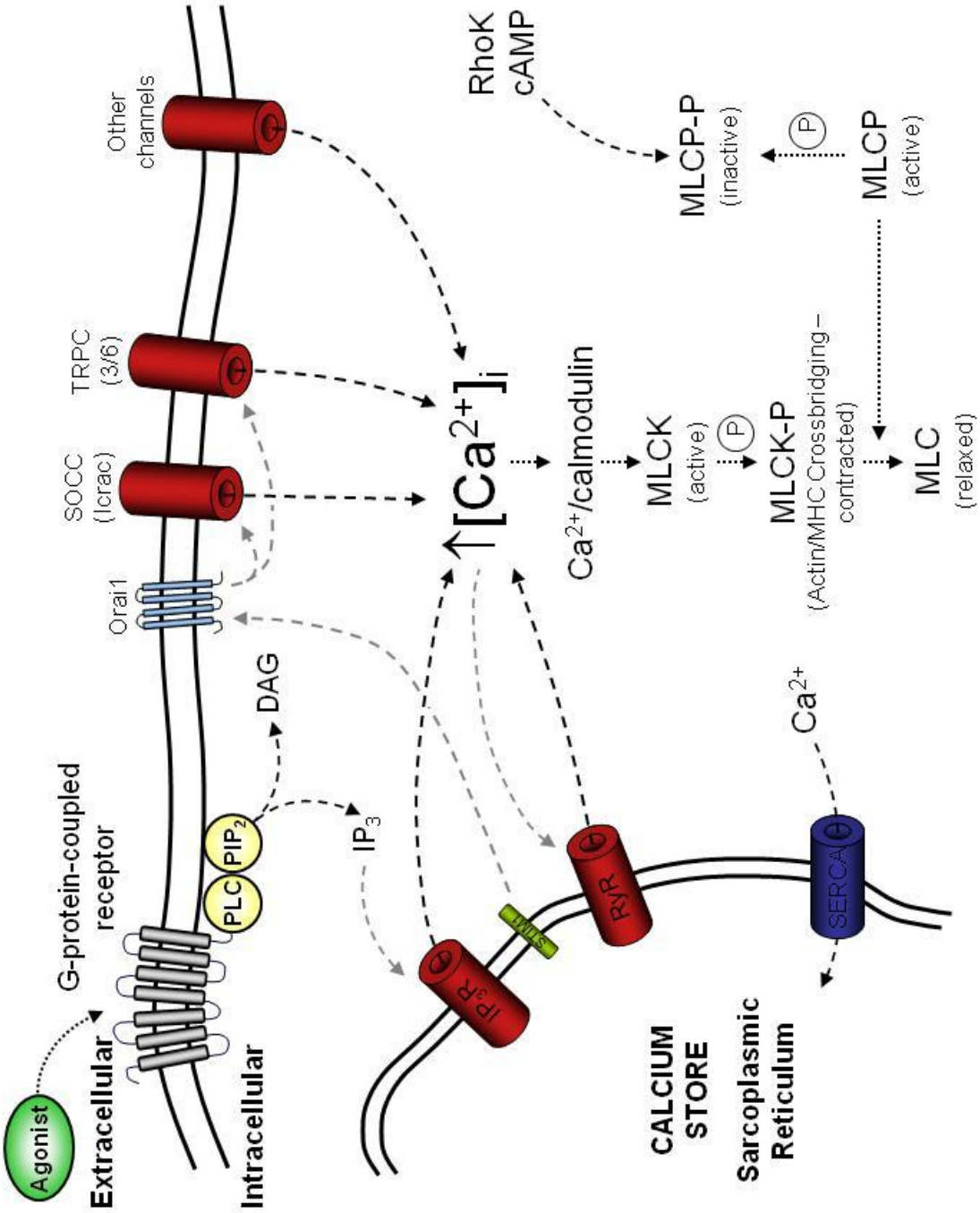
Fig 1.2 Airway smooth muscle contraction

Binding of an agonist (such as bradykinin or histamine) to the G-protein-coupled receptor in the cell membrane results in the activation of phospholipase C (PLC) and then phosphatidylinositol bisphosphate (PIP_2) to yield diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 then goes and activates its receptor in the sarcoplasmic reticulum (SR) allowing calcium release. There is also calcium-induced calcium release through ryanodine receptors (RyR).

The DAG combines with protein kinase C (PKC) to activate non-selective calcium channel allowing the entry of calcium from the extracellular environment. TRPC channels also become activated mainly by an unknown pathway, but DAG has been implicated in this. The depletion of calcium stores within the cell result in the activation of store-operated calcium channels possible via IP_3 allowing further influx of calcium.

The free calcium binds to calmodulin and the resulting calcium-calmodulin complex activates myosin light chain kinase (MLCK), an enzyme that phosphorylates myosin light chains (MLC) in the presence of ATP. MLC are 20-kD regulatory subunits found on the myosin heads. MLC phosphorylation leads to cross-bridge formation between the myosin heads and the actin filaments, and hence, smooth muscle contraction.

The calcium is then extracted out of the cell through a Na^+/Ca^{2+} exchanger and the calcium can be re-sequestered into the SR through a calcium-ATP pump in the membrane, SERCA.


Agonist
Extracellular
Intracellular
G-protein-coupled receptor
PLC
PIP2
DAG
IP3
Orai1
SOCC (Icrac)
TRPC (3/6)
Other channels
↑[Ca2+]i
Ca2+/calmodulin
RhoK
cAMP
MLCK (active)
MLCK-P (Actin/MHC Crossbridging – contracted)
MLC (relaxed)
MLCP-P (inactive)
MLCP (active)
P
IP3R
RyR
TMS
SERCA
Ca2+
CALCIUM STORE
Sarcoplasmic Reticulum

The depletion of calcium within the SR stimulates the translocation of stromal interaction molecule 1 (STIM1) to the plasma membrane where is combines with co-localized Orai1 proteins to activated store operated calcium channels (SOCC), including calcium release activated channels (CRAC), and transient receptor potential calcium (TRPC) channels to allow the influx of calcium from the extracellular environment (134,135). Other plasma membrane channels are also involved in the influx of extracellular calcium such as voltage-operated channels, which are activated in response to membrane depolarisation.

In addition to MLCK, the phosphorylation of MLC may also occur through MLCP. This is regulated by Rho kinase and cyclic adenosine monophosphate (cAMP). Rho kinase, a serine/threonine kinase, phosphorylates the myosin-binding subunit of MLCP, inhibiting its activity. (136)

1.3.3 Human airway smooth muscle functions in the normal lung

The role of airway smooth muscle in the normal non-diseased lung has long been speculated. It is thought that it has a no essential physiological function and instead can lead to serious medical problems. However, functional roles of airway smooth muscle have been hypothesised.

One such proposed role is a function in the peristaltic effect of the lung; this includes peristalsis to assist exhalation, peristalsis to assist mucus propulsion and peristaltic contraction in the foetal lung to generate fluid pressure. The smooth muscle has also been implicated in promoting lymphatic and venous flow, and ventilation/perfusion matching. Protection roles have been suggested such as protecting the peripheral

lung and the airway structure in addition to stabilizing the airways, enhancing the effectiveness of cough and optimizing anatomical dead space (84).

These proposed roles for airway smooth muscle in the non-diseased lung are speculative, not without contradictory reasoning and all without sufficient substantial evidence. It does seem that ASM is vestigial organ, but still having the capabilities to cause serious medical problems and will continue to be the target for many therapies in respiratory disease.

1.4 Mast cell and airway smooth muscle interactions in asthma

1.4.1 Inflammatory cell localisation to the ASM bundle

Although the features of the disordered airway physiology, inflammatory profile and structural changes in the airway in asthma are well described, the interactions between these components of airway disease are poorly understood. To date little attention has been paid to the localisation of inflammatory cells within structural compartments of the airway wall. Recently, enthusiasm for the view that microlocalisation is important in obstructive airway diseases has been fuelled by studies showing inflammatory cells within the ASM in asthma, but not in normal controls (15,32,34,35,137-139). Most notably, ASM from an asthmatic patient is infiltrated by mast cells and the number of mast cells within the smooth muscle bundle was associated with the degree of AHR (15,140), suggesting a role for the mast cells within the asthmatic lung.

Communication between cells within the airway predominately occurs across distances of only a few microns, as many inflammatory mediators are rapidly inactivated once they leave the cell and direct cell-cell is critical in modulating function. Therefore it is likely that microlocalisation between inflammatory and structural cells is a fundamental organising principle of airway inflammation and repair.

A key pathway in the recruitment of mast cells to the ASM bundles is through the CXCR3/CXCL10 axis. The chemokines receptor most abundantly expressed on ASM-localized mast cells is CXCR3, its ligand CXCL10 is preferentially expressed on ASM and furthermore, activation of CXCR3 induces mast cell migration (120).

The ASM bundle infiltrating mast cells in asthma are predominately of the chymase-positive phenotype (15,32,34,35,137-139). Mast cell-ASM interactions in asthma are summarised in Fig 1.3. Mast cells adhere avidly to ASM in-part via a cell adhesion molecule-1 (CADM1) (141), (previously known as tumour suppressor in lung cancer-1 [TSLC-1], immunoglobulin superfamily member 4 [IGSF4], spermatogenic immunoglobulin superfamily, synaptic cell adhesion molecule [SynCAM], nectin-like molecule 2 [Necl2], and RA175) (142). Adhesion to ASM is heterophilic and calcium independent (141). Mast cell numbers correlated inversely with AHR (15,140), suggesting that mast cell-ASM cell interactions are central in the development of the disordered physiology in asthma. The strength of this assertion was underpinned by the paucity of mast cells within the ASM bundle in eosinophilic bronchitis; a condition that presents with chronic cough and shares many of the immunopatholgical features of asthma but is not associated with airflow obstruction or AHR (12,14,15,143-147). Importantly, there is evidence that mast cells infiltrating the ASM bundle are activated with increased expression of Th2 cytokines IL-4 and IL-13 (148). One post mortem study of fatal and non-fatal asthma has shown that there was a marked increase in mast cell degranulation in the ASM bundle in both the large and small airways (34) and another demonstrated that increased numbers of mast cells (degranulated and intact) are associated with increased ASM shortening in fatal asthma (149). Interestingly, there was a lack of other inflammatory cells suggesting that mast cells are selectively recruited to the ASM bundle.

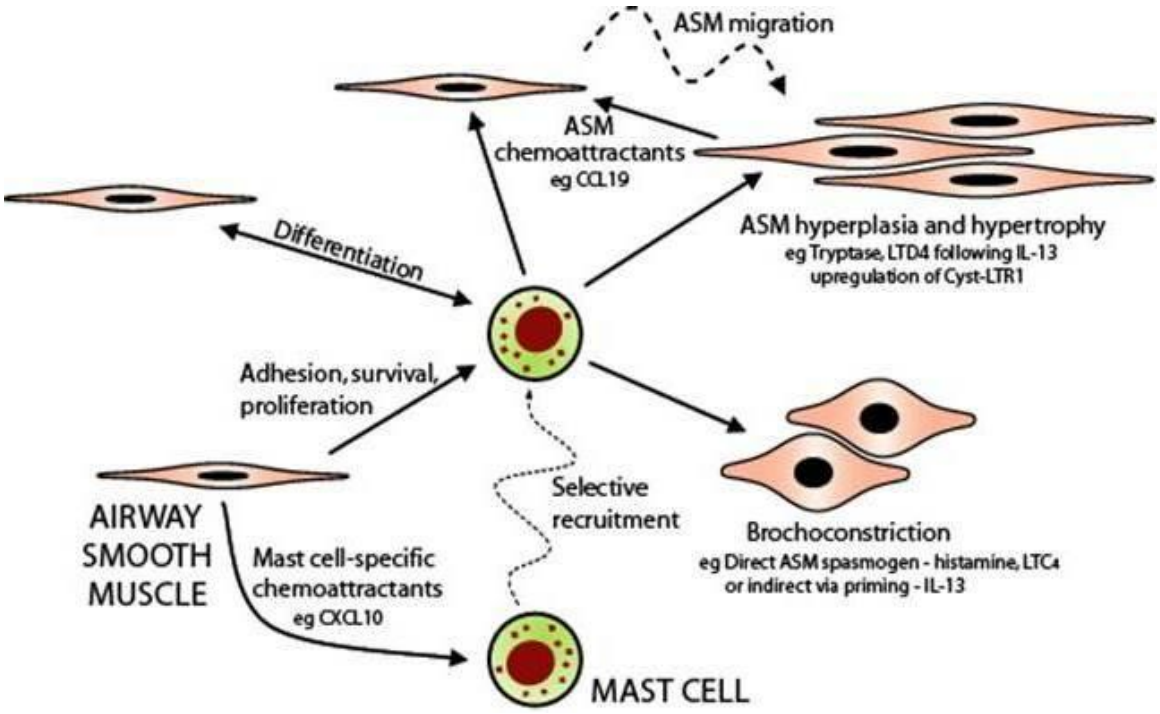


Figure 1.3 Mast cell-ASM interactions

Mast cells are recruited to the ASM under the influence of ASM-derived chemoattractants and avidly adhere to ASM. In the ASM-bundle there is an appropriate milieu to support mast cell survival and the cells interact resulting in cellular differentiation, ASM hyperplasia, recruitment of ASM progenitors and ASM contraction either directly or indirectly.

Activation of the inflammatory cells within the ASM bundle would be predicted to have important consequences on ASM function. Following mast cell degranulation the mediators histamine, PGD_2 and LTC_4 are released which are all potent agonists for ASM contraction (38). Mast cell cytokines may further contribute to airway hyperresponsiveness. The mast cells in the ASM bundles in asthma express IL-13 (150). IL-13 has been shown to attenuate relaxation to β-agonists and augment contractility to acetylcholine (150,151). The effect of neutrophil derived mediators on ASM function is less clear with conflicting reports from animal studies showing that elastase can increase and diminish smooth muscle responsiveness (152,153).

The interactions between inflammatory cells and ASM cells may have more long-term consequences. Mast cells co-cultured with ASM promote differentiation of the ASM with increased α-smooth muscle actin expression (154). Similarly, mast cell differentiation towards the chymase-positive phenotype observed within the ASM bundle may be mediated by mast cell-ASM interactions. Increased ASM mass is a well-established feature of asthma (88). A number of mast cell mediators including histamine (108), tryptase (155) and LTD_4 (156) as well as the neutrophil product elastase (107) promote ASM proliferation. Alternatively increased ASM mass may be a consequence of recruitment of ASM progenitors. This view is supported by the increased number of fibrocytes that migrate into the airway following allergen challenge (100). Recent evidence suggests that ASM migration towards the ASM bundle is mediated by activation of CCR7 by ASM- and mast cell-derived CCL19 (97). In asthma increased ASM mass occurs predominately in the large airway and in the small airways in COPD. Using a computational model, increased muscle mass has been shown to probably be the most important abnormality responsible for the

increased airflow resistance observed in response to bronchoconstricting stimuli (157). Consistent with this view, in a cross-sectional study of the immunopathology of asthma across severity using a multiple regression model, increased ASM mass including ASM hypertrophy were the features of remodelling that were associated most strongly with impairment in lung function (5).

Therefore mast cell interactions with ASM in asthma is critical in the development of AHR and may play a critical role in the development of increased ASM mass and the development of fixed airflow obstruction seen in severe disease.

1.5 Asthma and airway smooth muscle activity

Most immunopathological descriptions of asthma have focussed on the number of inflammatory cells that infiltrate the airway submuscosa. Often structural components of the airway are excluded. There is an increasing recognition that inflammatory cells selectively localise to different airway structures in disease and it is biologically plausible that this microlocalisation has important functional consequences. For example, bronchoconstriction is a consequence of ASM contraction. Thus the localisation of inflammatory cells within the ASM bundle is likely to modulate AHR and variable airflow obstruction.

The presence of lung mast cells within the airway smooth muscle was first identified in patients with fatal asthma (35). This observation has since been confirmed by several other reports, not only in fatal cases, but also in live patients and by a variety of methods (15,32,120,137-139,158). In addition, these smooth muscle-residing mast cells were also found to be activated in that degranulation of these cells had occurred (33).

Given the locality and the state of activation of mast cells within the smooth muscle, the released mediators may have a plethora of consequences on the adjacent smooth muscle (Fig 1.3). Such examples include inducing bronchoconstriction through the actions of histamine and LTC_4, or IL-13 attenuating β-agonist relaxation. Hyperplasia and hypertrophy may occur through the actions of tryptase and leukotriene D_4 release following IL-13 upregulation of Cyst-LTR1. Mast cells express CCL19 and following activation of its receptor CCR7 on ASM, induces smooth muscle migration into the local environment (97). Additionally, both cell

types may have a mutual effect on the cell differentiation whereby smooth muscle could effect the tryptase/chymase expression of mast cells and mast cells could effect the actin/myosin content of smooth muscle (154) (Fig 1.3).

Airway hyperresponsiveness, the excessive contraction of airway smooth muscle, is a hallmark of asthma and is poorly understood. Although it is due to a combination of inflammation, smooth muscle cell hypertrophy and hyperplasia, the primary event leading to AHR is the stimulation of smooth muscle contraction.

The location of the activated mast cells and their subsequent degranulation within the ASM results in the mediators histamine, PGD_2 and LTC_4 being released from the granules, which are potent agonists for airway smooth muscle contraction (38). Subsequent cytokine release from the mast cells may further contribute to this hyperresponsiveness of the airways. IL-13 is expressed by mast cells and found within the airway smooth muscle (148). Furthermore, IL-13 has been found to attenuate relaxation to β-agonists and to augment contractility to acetylcholine (150,151).

Benayoun, *et al.*, 2003, found that the airways of patients with severe asthma have greater α-smooth muscle actin and MLCK immunoreactivity than control subjects or patients with COPD (5). Woodruff, *et al.*, 2004 found a 50 to 83% increase in α-smooth muscle actin immunoreactivity in patients with mild to moderate asthma (89). Both these reports indicate a possible source for the increased contractility of asthmatic smooth muscle to stimulus.

1.6 Summary, hypotheses, and aims

Due to the presence of the mast cells within the airway smooth muscle bundles in the asthmatic lung and the knowledge of the adverse effects that the mast cell products may have on its local environment, it is logical to assume that they play a pivotal role in the pathogenesis of asthmatic symptomology. In turn, these cells must be recruited to the airway smooth muscle site and their survival then promoted; a mechanism that is not present in the non-asthmatic lung.

Therefore, the first hypothesis of this thesis is that asthmatic airway smooth muscle has the potential to promote survival of lung mast cells when cultured together without the addition of serum or exogenous cytokines that are normally used to sustain cultured mast cells.

Aims:

1. To co-culture human lung mast cells with airway smooth muscle from non-asthmatic subjects to investigate whether airway smooth muscle can promote survival of the mast cells to a greater degree than culturing them alone with serum and exogenous cytokines.
2. If smooth muscle from non-asthmatic subjects can support the survival of mast cells, to explore whether smooth muscle isolated from asthmatic patients can also support these cells and whether the survival signal is any different to that from non-asthmatic individuals.
3. If survival is observed in these mast cell dual cultures, to investigate the mechanisms mediating this survival.
4. To examine whether there is any difference in the activity of the mast cells following culture with airway smooth muscle.

Airway smooth muscle from asthmatic patients exhibits altered physiology compared with non-asthmatic smooth muscle in that it displays hypercontractility to stimuli and altered synthetic function.

Therefore, the second hypothesis of this thesis is that airway smooth muscle from an asthmatic patient has altered contractile response and intracellular calcium handling, which promotes its altered physiology.

Aims:

1. To investigate the intracellular calcium handling of airway smooth muscle and to determine whether there are any differences between asthmatic and non-asthmatic phenotypes.
2. To use collagen gel contraction assays to measure any differences in the contractile abilities between airway smooth muscle from asthmatic and non-asthmatic subjects.
3. Finally, to examine whether there is any differences in the levels of the intracellular proteins that are involved in the calcium-contraction coupling process.

Chapter 2:

Materials and Methods

2.1 Primary cell purification and culture

The study was approved by the Leicestershire Research Ethics Committee, and all tissue donors gave written informed consent. All experiments were performed using primary lung cells; human lung mast cells and airway smooth muscle cells.

2.1.1 Human lung mast cells (HLMC)

2.1.1.1 HLMC purification

HLMC were purified from macroscopically normal lung obtained within 24 hours of resection for lung cancer using an enzymatic dispersal procedure (Fig 2.1).

Lung tissue was finely diced then washed four times through two pieces of sterile 100μm nylon gauze (Fisher) with Dulbecco's Modified Eagles Medium (DMEM) with GlutaMAX™ I, 4500mg/L D-Glucose and 25mM HEPES (Invitrogen) supplemented with 10% foetal calf serum (FCS; Invitrogen), 1% antibiotic/antimycotic (Sigma; containing 100units/ml penicillin, 100mg/ml streptomycin sulphate, and 0.25μg/ml amphotericin B), 100μM non-essential amino acids (Invitrogen) and 1mM sodium pyruvate (Sigma), herein referred to as HLMC 10% media. The lung tissue was then cultured at 37^{o}C, 5% CO_2 overnight in HLMC 10% media (4ml/gram of tissue).

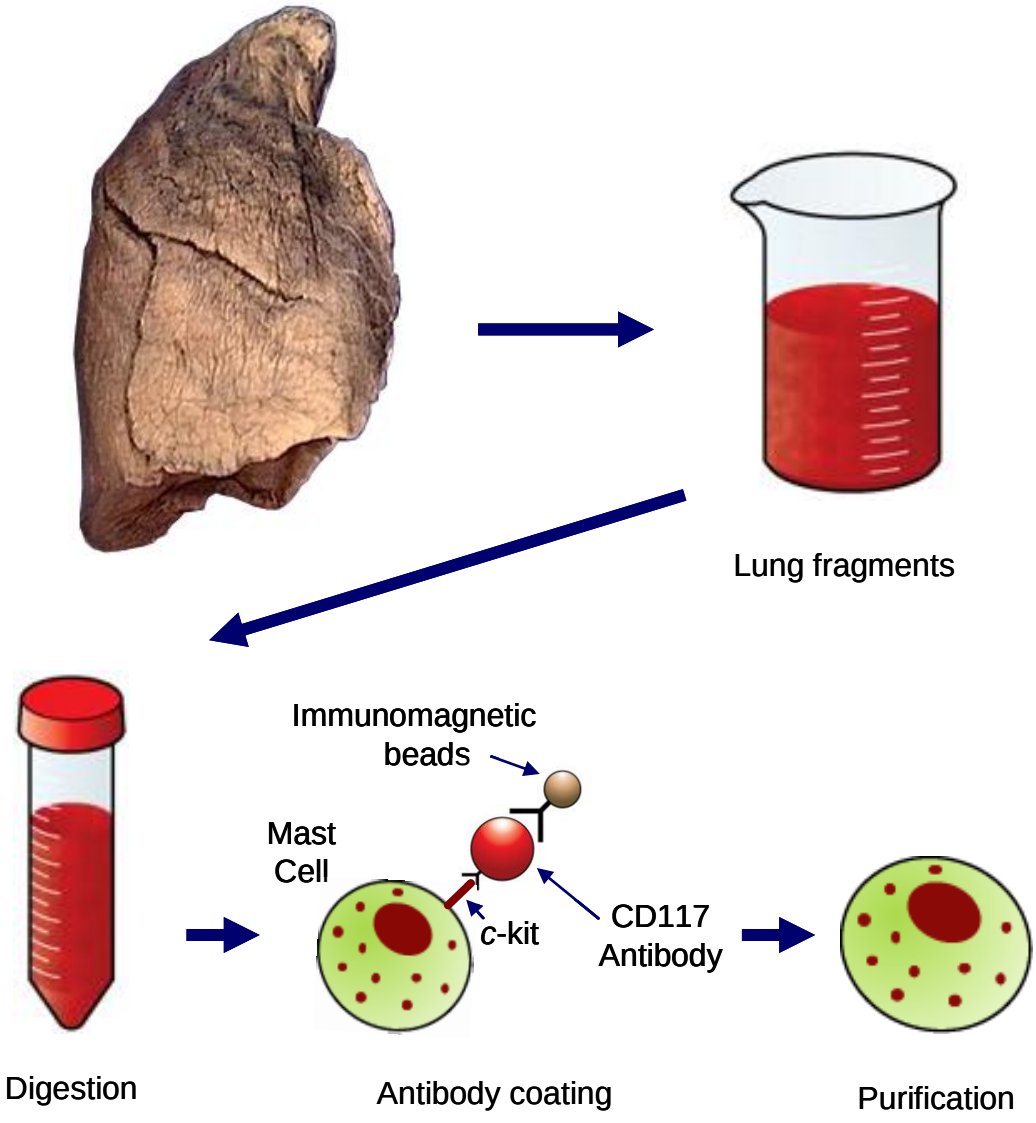


Figure 2.1 Human lung mast cell isolation

Lung tissue is chopped into fragments and digested using collagenase and hyaluronidase. Beads coated with a CD117 antibody are then used to magnetically remove the mast cells from the suspension.

Enzymatic digestion of the tissue was performed through addition of 75mg/10g tissue collagenase and 37.5mg/10g tissue hyaluronidase (both Sigma) and incubation at 37^{o}C, 5% CO_2 for 75 minutes on a magnetic stirrer. HLMC were then liberated by a series of washes with HLMC media containing 2% FCS, (herein referred to as HLMC 2% media), through 100μm gauze and then 50μm gauze (Precision Textiles), following which the filtrate was centrifuged to pellet the cells. The cell pellet was then resuspended in 2-4ml HBSS/FCS-protein (84.7% HBSS [Invitrogen]; 2% FCS; 10% horse serum [Invitrogen]; 3.3% BSA [Sigma]) and incubated for 30 minutes at 4^{o}C to prevent non-specific binding of the immunomagnetic beads in the next stage. The cell suspension was then diluted with HBSS + 2% FCS before passing through a 70μM cell strainer (Beckton Dickinson) and pelleting the cells. The cells were then resuspended with CD117-coated immunomagnetic beads (see below) at a 5:1 (beads:cells) ratio and incubated for 90 minutes at 4^{o}C.

The beaded cells were then removed from the suspension through positive magnetic selection using an MPC magnet (Dynal) and a series of washes with HLMC 2% media. Final mast cell purity was >99.4% and viability >98%.

HLMCs were cultured in 10% HLMC media supplemented with recombinant 100ng/ml stem cell factor (SCF), 50ng/ml interleukin(IL)-6 and 10ng/ml IL-10 (all R&D).

The freshly isolated HLMC was left in culture for 2-3 days to allow the cells to rest; following this the cells were used either with or without the accompanying beads. For experiments requiring bead-free cells, the HLMC were put on the magnet for two

successive periods of 10 minutes to obtain the cells where the beads have become detached.

2.1.1.2 Coating of anti-mouse IgG beads with CD117

The vial of sheep anti-mouse IgG Dynabeads (Dynal) was washed three times with PBS/0.1%BSA (Invitrogen and Sigma respectively) using a MPC magnet. The beads were then resuspended in PBS/0.1%BSA, to which 8μg CD117 (BD Biosciences) was added. This was incubated on a rotating mixer at room temperature for 30 minutes. The beads were washed a further three times before resuspending in 2ml PBS/0.1%BSA with 1% azide (Sigma).

2.1.1.3 HLMC culture

Purified HLMC were fed once weekly. Cells were resuspended in fresh 10% HLMC medium supplemented with recombinant 100ng/ml SCF, 50ng/ml IL-6, and 10ng/ml IL-10 (all R&D).

2.1.2 Human airway smooth muscle (ASM)

2.1.2.1 Subjects

Eleven subjects with asthma and fourteen non-asthmatic controls were recruited from Glenfield Hospital outpatients and staff and by local advertising. Asthma was defined by 1 or more of the following objective criteria: significant bronchodilator reversibility of FEV_1 >200ml, PC_{20} <8mg/ml, or a peak flow amplitude percentage mean over 2 weeks of more than 20%. Clinical characteristics of asthmatic patients and non-asthmatic controls subjects are shown in Table 2.1. The Leicestershire ethics committee approved the study and all patients gave their written informed consent.

	Non-asthmatic n=14	Asthmatic n=12	Combined n=26
Age (years)	57.6 (4.0)	52.9 (4.9)	55.7 (3.1)
Actin expression*	90.5 (83.0-95.5)	94.5 (88.0-95.0)	94.0 (86.0-95.0)
FEV_1 current (L)	2.1 (0.15)	2.4 (0.27)	2.2 (0.15)
FEV_1 predicted %	66.2 (4.6)	77.2 (7.8)	71.4 (4.5)
FEV_1/FVC %	74.2 (4.4)	68.9 (3.2)	71.7 (2.8)

Table 2.1 Patient clinical characteristics

Data expressed as means (SEM); *median (interquartile range).

F, female; *M*, male; *FEV_1*, forced expiratory volume in 1 second; *FVC*, forced vital capacity.

2.1.2.2 ASM isolation

Pure ASM bundles in airways isolated from lung resection and biopsy tissue were dissected free of surrounding tissue with the aid of a dissecting microscope from both asthmatic and non-asthmatic donors (Fig 2.2).

The small smooth muscle bundles that were intended for long term culture were individually seeded onto the wells of 6-well plates, allowed to adhere before the addition of DMEM with GlutaMAX™ I and 4500 mg/L D-Glucose (Invitrogen) supplemented with 10% FCS, 1% antibiotic-antimycotic (Sigma; containing 100units/ml penicillin, 100mg/ml streptomycin sulphate, and 0.25µg/ml amphotericin B), 100µM non-essential amino acids (Invitrogen) and 1mM sodium pyruvate (Sigma), herein referred to as ASM media.

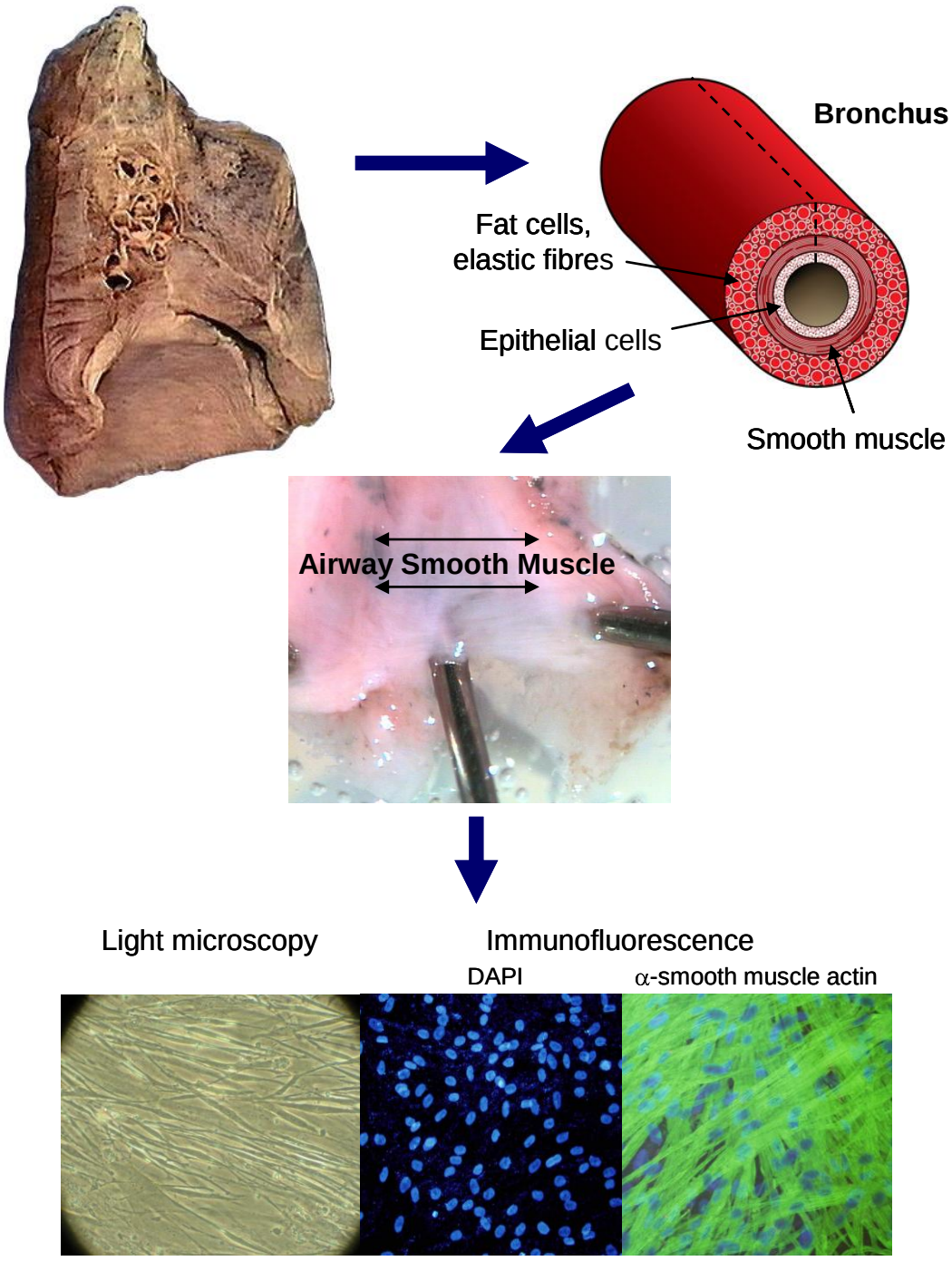


Figure 2.2 Airway smooth muscle isolation

Bronchus tissue is dissected from lung tissue. Surface epithelium is removed and small bundles of smooth muscle are removed and cultured before characterization.

2.1.2.3 ASM culture

ASM cultures were fed twice weekly with ASM media and washed as required. Upon reaching confluence, the cells were passaged. The ASM were harvested through trypsinisation; cells were washed twice with Hanks' balanced salt solution (HBSS; Invitrogen) before incubated for 4 minutes at 37^{o}C, 5% CO_2 with using 0.25% trypsin (Gibco). This was then neutralized with an equal volume of ASM media and the cells collected and washed, and the viable cells were counted on a haemocytometer using trypan blue and the cells were plated out into fresh T75 flasks at a minimum density of 1.7×10^5/10ml ASM media/T75 flask.

2.1.2.4 ASM characterization

ASM characteristics were determined by immunofluorescence and light microscopy with α-smooth muscle actin-FITC direct conjugate and myosin indirectly conjugated with FITC (Sigma-Aldrich).

The ASM cells were grown to confluence in Lab-Tek™ II CC2 8-well chamber slides (Nunc) and then serum deprived for 3 days using DMEM with GlutaMAX™ I and 4500 mg/L D-Glucose (Invitrogen) supplemented with 1% antibiotic-antimycotic (Sigma; containing 100units/ml penicillin, 100mg/ml streptomycin sulphate and 0.25μg/ml amphotericin B), 100μM non-essential amino acids (Invitrogen), 1mM sodium pyruvate (Sigma) and 1% ITS+3 liquid media supplement (Sigma; containing 1.0mg/ml insulin from bovine pancreas, 0.55mg/ml human transferrin [substantially iron-free], 0.5μg/ml sodium selenite, 470μg/ml linoleic acid, 470μg/ml oleic acid and 50mg/ml bovine serum albumin), herein referred to as ITS media.

The adhered cells were washed with PBS (Invitrogen) before fixing with ice cold methanol for 20 minutes. The wells were allowed to air dry for 10 minutes before blocking with PBS/3% BSA for 30 minutes. Appropriate primary antibodies were then added to each well, (see Table 2.2), and incubated on ice for 90 minutes.

Condition	Antibody	Company	Dilution
Actin negative control	Normal mouse IgG2a-FITC	DAKO	1:1000
Actin positive	Monoclonal anti-α-smooth muscle actin, IgG2a-FITC	Sigma	1:500
Myosin negative control	Normal mouse IgG1	DAKO	1:100
Myosin positive	Monoclonal anti-smooth muscle myosin IgG1	Sigma	1:500

Table 2.2 Primary antibodies for immunofluorescence

The wells were then washed three times with PBS/0.05% Tween 20 (Sigma-Aldrich) before the addition of secondary FITC antibody (1:10; DAKO) to the unconjugated wells and then incubation on ice for a further 90 minutes. The wells then underwent two sets of three washes with PBS/0.05% Tween 20 and PBS before addition of 0.1μg/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for 50 seconds. The cells were then washed six times with PBS. The chambers were removed from the slides and the slides were then mounted with photo bleach-retardant mounting medium (DakoCytomation Fluorescent mounting medium; DakoCytomation).

The cells were then assessed using a standard fluorescent microscope for their percentage actin and myosin expression. ASM donors used in experiments had actin expression >75%.

2.2 HLMC survival assays

2.2.1 ASM/HLMC co-culture assays

HLMCs taken from culture 3 days following isolation were washed with serum-free media and seeded onto confluent ASM that had been serum-deprived for 3 days in 6-well plates (Falcon) in ITS media, in the presence or absence of neutralizing antibodies (0.5μg/ml anti-human SCF/c-kit ligand and 0.6μg/ml anti-human IL-6; both R&D), individually and in combination, and/or the blocking antibody anti-CADM1 antibody (10 μg/ml; generated as described previously (159)).

ASM was also seeded onto 6-well plates suitable for cell culture inserts (BD Biosciences) and were grown to confluence. HLMC were added to the ASM cultures at 1:4 ratio in cell culture inserts with a high density translucent PET 0.4μm membrane (BD Biosciences), in the presence and absence of the isotype controls (0.5μg/ml anti-goat IgG1 and 0.6μg/ml anti-mouse IgG1; Dako), the neutralizing antibodies (0.5μg/ml anti-human SCF/c-kit ligand and 0.6μg/ml anti-human IL-6) individually and in combination. These experiments allowed for close co-culture of the cells while physically preventing their adhesion to one another.

The ability of soluble mediators released from ASM to maintain HLMC survival was also investigated through the culture of HLMC in ITS media taken from a three-day culture with ASM in the presence and absence of the isotype controls (0.5μg/ml anti-goat IgG1 and 1μg/ml anti-mouse IgG1), and the neutralizing antibodies (anti-human 0.5μg/ml SCF/c-kit ligand and 0.6μg/ml anti-human IL-6) individually and in combination.

HLMC control mono-culture controls were set up in parallel to all the co-culture experiments and included mast cells in serum- and cytokine-free media, mast cells with the exogenous growth cytokines SCF (100ng/ml) and IL-6 (50ng/ml), and mast cells with both the cytokines and 10% FCS.

All mono- and co-cultures were incubated at 37^{o}C, 5% CO_2 for 10 days and the HLMC within the cultures were evaluated on days 1, 3, 7 and 10. Cell number was assessed using mast cell-specific stain, Kimura (160) and enumerated using a haemocytometer.

2.2.2 Quantification of cell proliferation using CFSE staining

To quantify the proliferation of the HLMC with the mono- and dual-cultures, a cell tracing agent was used with which to stain the HLMC. Carboxyfluorescein diacetate succinimidyl ester (CFSE) passively diffuses into cells. It is colourless and non-fluorescent until the aceltate groups are cleaved by intracellular esterases to yield highly fluorescent carboxyfluorescein succinimidyl ester. The succinimidyl ester group reacts with intracellular amines, forming fluorescent conjugates that are well retained. The dye-protein adducts that form in labelled cells are retained by the cells throughout development and mitosis and can be used for *in vivo* tracing. The label is inherited by daughter cells after either cell division and is not transferred to adjacent cells in a population.

HLMC were stained with CFSE using a CellTrace CFSE cell proliferation kit (Molecular Probes). The 5mM CellTrace CFSE stock solution was prepared prior to use by dissolving the contents of one vial in 18μl of the supplied DMSO. The HLMC

were resuspended in warmed PBS/0.1% BSA (Gibco and Sigma respectively) at 1×10^6 HLMC/ml. 2μl of 5mM stock CFSE solution was added per millilitre of cell for a final working concentration of 10μM. This dye/cell suspension was incubated for 10 minutes at 37°C, 5% CO_2. The dye solution was then quenched through the addition of five-times the original volume of ice-cold HLMC media. This was then incubated on ice for 5 minutes before the cells were pelleted through centrifugation. The cells were washed a further 2 times in HLMC media before a final wash in serum-free media and counted. Cells were then used to either add to the ASM cultures or used in the control culture whereby the cells were incubated in HLMC media in the presence of SCF, IL-6 and IL-10 for 10 days after which these cells were analysed alongside the HLMC obtained following co-culture with ASM.

ASM cells were grown to confluence in T25 flasks and serum-deprived for 3 days before the addition of CFSE-stained HLMC at 1:4 ratio. These cells were incubated for 10 days at 37°C, 5% CO_2 before they were harvested through trypsinisation. These cells were dual stained with CD117-PE before analysis by two-colour flow cytometry with 488nm argon-ion laser.

2.2.3 Quantification of cell death

Propidium iodide (PI) binds to DNA by intercalating between the bases with little or no sequence preference and with a ratio of one dye per 4–5 base pairs of DNA. Once the dye is bound to nucleic acids, its fluorescence is enhanced 20-30-fold. PI is suitable for fluorescence microscopy, confocal laser scanning microscopy, flow cytometry, and fluorometry. PI is membrane impermanent and generally excluded

from viable cells, and is therefore commonly used for identifying dead, necrotic cells in a population and as a counterstain in multicolour fluorescent techniques.

A marker used for the identification of apoptotic cells is Annexin V. During the apoptosis pathway, the phospholipid phosphatidylserine, which is usually only found on the cytoplasmic surface of the outer membrane, is flipped onto the external side. Annexin V detects this external surface expression of phosphatidylserine. However, in HLMC, Annexin V may lead to false positives. During mast cell activation, intracellular vesicles containing preformed mediators fuse with the plasma membrane to release the vesicle contents into the extracellular environment. The fusion of the two membranes causes phosphatidylserine to become expressed on the cell's outer surface and is thus detected by Annexin V.

HLMC were cultured alone at a density of 0.5×10^6 cells per T75 flask in either ITS media, which is serum and cytokine free or ITS media supplemented with 10% FCS and exogenous cytokines (50ng/ml IL-6 and 100ng/ml SCF). HLMC were also co-cultured with ASM cells (at a ratio of 1:4) that had been grown to confluence in T75 flasks and serum deprived for 3 days. The mono and dual mast cell cultures were then incubated at 37ºC, 5% CO_2 over 10 days and their proportion of cell death quantified at days 1, 3, 7 and 10. At these time points, the cell supernatant from the cultures were removed and reserved while and the adhered cells were washed with HBSS, (which was also reserved) and harvested with Accutase. The detached cells along with their respective supernatants and washes (containing cells that may have detached the cells during the experiment) were pooled. The percentage of apoptotic or necrotic mast cells was assessed by three colour flow cytometry. The cultures

were stained with a monoclonal antibody against CD117 (clone:YB5.B8) followed with an APC secondary (to identify the HLMC). The cells were then stained with 1× Annexin binding buffer containing FITC-conjugated Annexin V (1µl/200µl). PI (0.5µg/ml) was added to the samples just prior to analysis on the BD FACSCanto flow cytometer. Appropriate isotype controls were used.

2.2.4 Morphological detection of HLMC apoptosis

One of the later stages of the apoptosis pathway is the fragmentation of the nucleus. Blue-fluorescent 4',6-diamidino-2-phenylindole, dilactate (DAPI) nucleic acid stain preferentially stains dsDNA; it appears to associate with AT clusters in the minor groove. This staining procedure allows visualisation of the nuclear morphology under fluorescent microscopy.

Nuclear morphology of HLMC was assessed by DAPI staining. For these experiments, HLMC were seeded at a density of 1×10^4 cells/well into 8 well chamber slides in either serum and cytokine free ITS media or in the presence of 10% FCS and exogenous cytokine IL-6 (50ng/ml), SCF (100ng/ml). ASM cells had been grown to confluence in 8 well chamber slides and serum deprived for 3 days in ITS media prior to co-culturing with HLMC (at a ratio of 1:4 HLMC:ASM). The mono or dual HLMC cultures were then incubated at 37ºC over 10 days and the HLMC nuclear morphology was assess at days 1, 3, 7 and 10. ASM cells co-cultured with HLMC were stained with a directly conjugated monoclonal antibody against RPE-CD117 (clone: 104D2) and indirectly labelled with secondary NorthenLights RPE secondary (to maintain CD117-RPE fluorescence). The cells were then counterstained with 0.1µg/ml DAPI and mounted with photo bleach retardant

mounting medium. CD117 was used to identify the HLMC in the presence of ASM cells. HLMC cultured alone were harvested and cytospun down onto slides and stained with DAPI alone and mounted. For each mono- or co-cultured HLMC, 6 random high powered fields (hpf) were examined for morphologic features of apoptosis such as nuclear condensation and fragmentation.

2.2.5 Immunofluorescence

Immunofluorescence is a technique allowing the visualization of a specific protein or antigen in cells or tissue sections by binding a specific antibody chemically conjugated with a fluorescent dye. There are two major types of immunofluorescence staining methods: 1) direct immunofluorescence staining in which the primary antibody is labeled with fluorescence dye, and 2) indirect immunofluorescence staining in which a secondary antibody labeled with fluorochrome is used to recognize a primary antibody.

ASM cells were grown to confluence in 8-well chamber slides and serum deprived for 3 days before the addition of 1×10^4 (1:4) HLMC in ITS media to the wells. These were then incubated for their appropriate length of time at 37°C, 5% CO_2. Following their incubation period, the cells were washed, fixed with pure methanol, blocked with PBS/3%BSA (see above) and labelled with mouse anti-human tryptase-biotin (Chemicon International) indirectly labelled with Texas Red streptavidin (Vector Laboratories) and either mouse anti-human α-smooth muscle actin (Sigma-Aldrich) or mouse anti-human mast cell chymase (Chemicon) indirectly labelled with polyclonal rabbit anti-mouse immunoglobulins/FITC rabbit $F(ab')_2$ (DakoCytomation). Cells were counterstained with 0.1μg/ml DAPI and mounted with

photo bleach-retardant mounting medium (DakoCytomation Fluorescent mounting medium; DakoCytomation). Appropriate isotype controls were performed.

The cells were then either studied by a standard fluorescent microscope or studied using an MRC 600 confocal laser scanning attachment (Bio-Rad Microsciences) linked to a Zeiss Axiovert epifluorescence microscope. Digital images and data files of immunofluorescence intensity transecting specimens in the x,y plane were recorded using COMOS software (Bio-Rad). Images and intensity scan graphs were rendered using Jasc Paint Shop Pro 8 and Adobe Photoshop CS.

2.2.6 Histamine analysis of co-culture supernatant and cell lysates

Histamine was measured by sensitive radioenzymatic assay based on the conversion of histamine to [^{3}H]methylhistamine in the presence of the enzyme histamine-*N*-methyl transferase. Radioenzymatic methods remain, arguably, the most sensitive for the detection of histamine.

HLMC were activated using IgE and anti-IgE stimulation. HLMC were harvested and resuspended in IgE (Chemicon; 24µg/ml) and incubated for 1 hour before resuspending in fresh medium containing anti-IgE (Sigma; 1/1000). After 1 hour incubation, supernatant was collected and the cell pellet was lysed in sterile de-ionized water both samples were analysed for histamine content (n=4).

Samples and control standards were incubated with rat kidney histamine methyl transferase (generous gift from Dr S Harper, AstraZeneca) and *S*-adenosyl-L-[*methyl*-^{3}H]methionine (Amersham Life Science). After organic extraction in a 4:1

mixture of toluene/isoamyl alcohol, the samples were read in a Tri-carb liquid scintillation analyzer (model 1500; Packard). Histamine concentrations were calculated from a standard curve that was run in triplicate in the range 500-7500pg. This assay has a lower detection limit of 50pg histamine, and is linear throughout the range 50-7500pg. Intra- and interassay coefficients of variation are 2-3%, respectively.

2.2.7 ELISA analysis of co-culture supernatant

Quantification of SCF and IL-6 within the single and co-culture conditions were measured using sandwich ELISA (enzyme-linked immunosorbent assay) analysis. Human SCF and IL-6 Quantikine sandwich ELISA kits (R&D Systems) were used and manufacturer's instructions followed. A monoclonal antibody specific for the target cytokine is pre-coated onto a microplate. Standards and samples are pipetted into the wells and any of the target cytokine is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for the target cytokine is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the amount of target cytokine bound in the initial step. The colour development is stopped and its intensity is measured at 450nm using a microplate reader.

Using the standard dilutions from 2000pg/ml to 31.25pg/ml, a standard curve was constructed by construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and drawing a best fit curve from which the concentrations of samples could be determined.

The ELISA analysis employed to quantify LTC_4 in the co-culture supernatant, was a competitive assay (Cayman Chemical Co.). The assay is based on the competition between cysteinyl leukotrienes (CysLT) and a CysLT-acetylcholinesterase (AChE) conjugate. A monoclonal antibody specific for the target cytokine is pre-coated onto a microplate. Standards and samples are pipetted into the wells and any of the target cytokine is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for the target cytokine is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the amount of target cytokine bound in the initial step. The colour development is stopped and its intensity is measured.

Using the standard dilutions from 1000pg/ml to 7.8pg/ml, a standard curve was constructed by construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and drawing a best fit curve from which the concentrations of samples could be determined.

2.3 ASM calcium/contraction assays

2.3.1 Single cell imaging

Changes in cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_i$) were monitored fluorometrically by use of the Ca^{2+}-sensitive probe Fura-2. Fura-2 is a highly fluorescent Ca^{2+} chelator, used to detect changes in intracellular Ca^{2+} concentration. Free Fura-2 emits maximally upon excitation at 380nm. Upon binding Ca^{2+} its excitation spectrum shifts, so that it emits at 340nm (161). The emission is measured at 509nm. By using the ratio of emission when the cells are excited at 340nm and 380nm, changes in intracellular Ca^{2+} concentration can be measured independent of the concentration of Fura-2 within the cell and the sensitivity of the instrument used to measure the emission. Free Fura-2 (the lipophobic pentacarboxylic form) is membrane impermeable, therefore cells are loaded using Fura-2 AM (the membrane permeable acetoxy-methyl form of the compound) allowing the fluorescent dye to enter the cells. The dye is prevented from leaking out of the cell by non-specific intracellular esterases which cleave off the acetoxy-methyl groups. Pluronic acid and probenecid also aid in the intracellular trapping of the dye by helping to disperse acetoxy-methyl (AM) esters of fluorescent ion indicators and by inhibiting organic-anion transporters in the plasma membrane, which extrude dyes respectively. This 340/380nm ratiometric technique allows accurate measurement of intracellular calcium to be performed, as it is independent from variations in dye concentration, illumination intensity, optical path length, uneven dye loading, dye leakage and photo bleaching.

2.3.1.1 Preparation of cells

25mm diameter glass coverslips (VWR) were sterilized through incubation for 20 minutes in pure ethanol. These were then allowed to dry before placing one coverslip/well in a 6-well plate. 2.5×10^4 HLMC/500µl/coverslip were added to the coverslips and incubated for 30 minutes at 37°C, 5% CO_2 to allow the cells to adhere to the glass. Following this, an additional 2.5ml/well was added to the plates and these were then cultured for up to a week to allow growth but not to reach confluency.

2.2.1.2 Fura-2 dye loading

Cells washed and then loaded with Fura-2 by incubation in normal physiological saline solution (PSS; 130mM NaCl, 5.6mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$, 11mM glucose, 10mM HEPES; pH 7.4) containing 2µM Fura-2-AM (Molecular Probes), 0.04% pluronic acid (Molecular Probes) and 50µM probenecid (Sigma) for 45 minutes at room temperature and then washed in PSS for 30 min. A cover slip with attached cells was mounted in a perfusion chamber and perfused with PSS at 1ml/min at 37 °C. Agonists were applied via the perfusion lines.

2.3.1.3 Imaging protocol

The recording system included a Nikon TE200 inverted microscope fitted with epifluorescence (Nikon) equipped with a ×40 Plan Fluor objective (Nikon). Light from a mercury arc lamp (Nikon) was passed alternately through excitation band-pass filters of 340 and 380 nm, which were exchanged by a computer-controlled filter wheel unit (Ludl Electronic Products). A 430 nm long-passdichroic mirror and 510 nm long-pass emission filter supplied light to the Hamamatsu CCD camera

(Hamamatsu Photonics). All optical filters were from Omega Optical (Brattleboro, VT, USA). Data were collected using Openlab version 3.1 software (Improvision). Data acquisition occurred at a rate of one dual-wavelength image every 6 seconds as the 340/380 nM ratio. This was converted to intracellular calcium concentration using the method of Grynkiewicz *et. al.*(161), and using Rmin and max values calculated using a calcium calibration kit (Molecular Probes).

2.3.2 Fluorometric imaging plate reader (FLIPR)

The Fluorometric Imaging Plate Reader (FLIPR) system (Molecular Devices) was developed to perform cell-based, high-throughput screening assays. The system provides functional data in real time and with such precision, replicate assays are often unnecessary.

Using a unique integration of optics, fluidics, and temperature control, the FLIPR system is ideal for homogeneous, kinetic, cell-based fluorometric assays, such as measuring intracellular calcium, membrane potential, and intracellular pH. The key advantage of the system is that it simultaneously delivers compounds to and images of all the 96 wells of a microplate within one second. The system can be used with both adherent and non-adherent cell lines.

The FLIPR system measures changes in cells that have been stimulated with compounds of interest. An argon-ion laser excites a fluorescent indicator dye suitable for the specific change being measured and the emitted light is detected using a proprietary optical system. A very sensitive, cooled CCD camera images the entire plate and integrates data signals over a time interval specified by the user.

Because the FLIPR system uses a combination of laser illumination and cooled CCD optical detection, the measurements are more sensitive, flexible, and faster than conventional plate readers. With the FLIPR system, data points can be taken from each of the 96 wells in one second. Sensitivity is further enhanced by proprietary cell-layer isolation optics that allow signal discrimination on a cell monolayer. This eliminates the undesirable extracellular background fluorescence found in most fluorescent assays.

The FLIPR system includes an integrated 96-well pipettor. The pipettor is used to rapidly aspirate, dispense, and mix precise volumes of fluid from microplates containing test compounds and the microplate containing cells, allowing simultaneous stimulation of all 96 wells. Up to two plates of test compounds can be used in each experiment. For example, one plate can hold antagonists, and the second plate can contain agonists. The pipettor uses disposable tips, so there is no risk of carryover between experiments.

To minimize experimental variability due to inconsistent environmental conditions, the FLIPR system's incubation chamber maintains the assay and reagent microplates at a constant temperature during experiments. In addition, to minimize evaporation at high temperature, the chamber is humidified. The temperature control and the highly accurate integrated pipettor provide the controlled experimental conditions necessary for performing reliable and reproducible cell-based assays.

2.3.2.1 Preparation of ASM

ASM cells from both an asthmatic and non-asthmatic donors were obtained from culture stocks through trypsinisation. ASM were plated onto collagen-treated, black-walled, 96-well plates (Becton Dickinson) at 1×10^4 cells/well/200µl of ASM media and incubated at 37°C, 5% CO_2 for one week. The media was removed and replaced with fresh media after 4 days.

2.3.2.2 Fluo-4 loading of ASM

ASM cells were washed twice with 100µl/well ASM media using a repeating multichannel pipette. Dye loading solution (ASM media, 20mM Hepes [Sigma], 25µM Probenecid [Sigma], 0.03% Pluronic acid [Molecular Probes], 0.1µg Fluo-4 (AM) [Tef Labs]; pH 7.4) was added to the cells at 70µl/well insuring no air bubbles before incubating at 37°C, 5% CO_2 for 50-55 minutes. Following this, the cells were washed a further three times with 100µl/well FLIPR buffer (Distilled water, 125mM NaCl, 25mM Hepes (pH 7.6), 5mM Glucose, 5mM KCl, 1.5mM $CaCl_2$, 1mM $MgCl_2$, 25µM Probenecid [all Sigma]; pH 7.4), before the addition of 70µl/well FLIPR buffer. The plates were wrapped in aluminium foil until they were needed to be run on the FLIPR.

2.3.2.3 FLIPR protocol

Agonist dilutions were prepared in FLIPR buffer at 3.3 times the final concentration required (as 30µl agonist added to 70µl FLIPR buffer).

Final optimised bradykinin concentrations used:

1nM, 200pM, 40pM, 8pM, 1.6pM, 0.32pM, 0.064pM.

Final optimised histamine concentrations used:

500μM, 100μM 20μM, 4μM, 0.8μM, 0.16μM, 0.032μM.

The agonist dilutions were added to a 96-well V-bottomed polypropylene plate (Greiner) in the same plate format in which the ASM cells have been prepared, which included a blank (buffer only) negative control.

2.3.3 Collagen gel contraction assay

The collagen gel contraction assay is an established physiological *in vitro* model that is used to examine the mechanism of cytoskeletal reorganisation or stress fibre formation in cells (162,163). Therefore, this assay was employed to examine the contractile capacity of ASM cells.

ASM was harvested by trypsinisation (see above) and resuspended in ITS media at 1.735×10^6/ml. Per individual collagen gel for one well of a 24-well plate, to 37μl 10× conc DMEM (Invitrogen) was added 299μl pure collagen (3mg/ml; INAMED Biomaterials) and 144μl cell suspension (2.5×10^6 cells/gel). 20μl sodium bicarbonate (7.5%; Gibco) was added just before plating out the gel suspension into the well. The collagen suspension was cast in one well of a 24-well plate, which had been pre-coated with PBS 2% BSA and the collagen was allowed to polymerize at 37^{o}C for 90 minutes. All gel conditions were performed in duplicate. After polymerization, 500μl ITS media was added to each well and the gel was detached from the plastic surface

to allow free contraction. A further 500µl media alone or 2× bradykinin (final concentration 1nM) was added as required to each gel. The percentage contraction was assessed by measuring the collagen gel area after incubation at specified time intervals throughout the length of the time course using photographs of the gel, then analysis using ImageJ software (National Institutes of Health).

2.3.4 Western blotting

Intracellular proteins involved in the calcium/cell contraction coupling process were measured for their levels of expression in ASM. Western blotting is a method of specific protein detection in a cell lysate sample. It using a combination of gel electrophoresis to separate proteins based on their size, transfer of these proteins onto a membrane to allow the antibody detection, which is then visualised using chemiluminescent detection.

2.3.4.1 Protein isolation

ASM was harvested through trypsinisation (described above) and washed. The ASM was lysed in 2× laemmli sample buffer (Sigma-Aldrich) diluted 1:1 with distilled water at a concentration of 7.5×10^5/100µl buffer (1.5×10^5/20µl loaded into wells). The cell suspension was then boiled at 90^oC for 5 minutes to denature the proteins.

2.3.4.2 Protein electrophoresis

Fresh resolving and stacking gels (see Table 2.3 and 2.4) were made. The resolving gel was loaded in the glass plates on top of which 500µl isobutanol was added to ensure the gel is level and this was allowed to set. The isobutanol was removed and washed with water before the addition of the stacking gel with the comb in place.

Once set, the comb was removed and the plates were set in the gel running unit so the smaller plate is on the inside, and the running buffer (see Table 2.5) was added. 10µl of SeeBlue Plus2 Pre-Stained marker (Invitrogen) was loaded along with 20µl of sample protein. The gel was run at 180V, 400mA for approximately 60-90 minutes (percentage density of gel dependent).

Resolving Gel	**8%**	**10%**	**12%**
Water	2.98ml	2.4ml	1.82ml
Tris, *pH 8.8*	3.36ml	3.36ml	3.36ml
10% SDS	90ul	90ul	90ul
10% APS	200ul	200ul	200ul
30% Acrylamide	2.33ml	2.92ml	3.5ml
TEMED	10ul	10ul	10ul

Table 2.3 Resolving gel

Stacking Gel	
Water	3.6ml
Tris, *pH 6.8*	625ul
10% SDS	50ul
10% APS	100ul
30% Acrylamide	665ul
TEMED	8ul

Table 2.4 Stacking gel

10× Running Buffer	Final Concentration	Add
Tris	25mM	30.3g
Glycine	192mM	144g
SDS	0.1%	10g
Water		1 litre
	pH 8.3, store at 4°C	

Table 2.5 Running buffer

2.3.4.3 Membrane blotting

Immobilion-P Transfer Membrane (Sigma-Aldrich) was cut to size and soaked in 100% methanol for 15 seconds, then Milli-Q grade water for 2 minutes, then in transfer buffer (see Table 2.6) for at least 5 minutes. The gel was removed from the glass plates and soaked along with filter paper and the scouring pads in the transfer buffer for at least 5 minutes. The transfer sandwich was constructed with the gel and transfer membrane in the middle and on either side the filter paper, scouring pads and plates, minimising air bubbles at each stage of construction. The sandwich was loaded into the transfer tank along with a magnetic flea and topped up with transfer buffer. The tank was put on a magnetic stirrer and run at 200mA, constant voltage for 2 hours.

Transfer Buffer	Final Concentration	Add
Tris	12.5mM	3.04g
Glycine	200mM	28.88g
Methanol		100ml
Water		900ml

Table 2.6 Transfer buffer

2.3.4.4 Detection of proteins

The sandwich was disassembled and the transfer membrane was placed in Ponceau S stain for 1-2 minutes to ensure transfer of proteins. The membrane was then washed with water then TBS + 0.1% Tween 20 (see Table 2.7) to remove staining. Using the SeeBlue Plus2 Pre-Stained marker as a guide, the membrane was divided at the appropriate molecular weights to enable probing with multiple antibodies detecting proteins at different molecular weights.

10× TBS Buffer	**Final Concentration**	**Add**
Tris	250mM	30.3g
NaCl	150mM	90.0g
Water		1 litre
	pH 7.5	

Table 2.7 Tris-buffered saline

The membranes were blocked in 10× TBS + 0.1% Tween 20 + 5% Marvel (for monoclonal antibodies) or 5% BSA (for polyclonal antibodies), for 1 hour (at room temp) or overnight (at 4^{o}C). The primary antibodies were then added in same solution as their blocking for 1 hour (at room temperature) or overnight (at 4^{o}C). The membranes were then washed three times in TBS + 0.1% Tween 20 before addition of the appropriate secondary HRP antibody in marvel/BSA solution as above at 1/1000. (For complete list of antibodies, see Table 2.8). The membranes were washed a further three times before developing using ECL detection reagents (Amersham Biosciences), a light emitting non-radioactive method for detection of immobilized specific antigens, directly or indirectly with horseradish peroxidase (HRP) labeled antibodies. Reagents 1 and 2 were added at a 1:1 ratio and added to

the membranes for 1 minute before being removed. Saranwrap is then used to enclose the membrane ready for detection. The maximum light emission at 428nm can then be detected by a short exposure to blue-light sensitive autoradiography film (Kodak).

Antibody	Molecular Weight (kDa)	Company	Dilution	Poly-/monoclonal	Secondary
MLCK	132/210	Santa Cruz	1:200	Polyclonal	Anti-Goat HRP
pMLCK	135/210	Santa Cruz	1:200	Polyclonal	Anti-Goat HRP
SERCA 2	110	Abcam	1:2500	Monoclonal	Anti-Mouse HRP
β-actin	43	Santa Cruz	1:20000	Monoclonal	HRP-Conjugated
CD38	45	Santa Cruz	1:200	Monoclonal	Anti-Mouse HRP
MLC	20	Abcam	1:5000	Monoclonal	Anti-Mouse HRP
pMLC	20	Abcam	1:5000	Polyclonal	Anti-Rabbit HRP
MLCP	19	Abcam	1:500	Polyclonal	Anti-Rabbit HRP

Table 2.8 Calcium/contraction protein antibodies

2.3.4.5 Secondary probing

Membranes to be stripped were left in blocking buffer at 4°C until required. The membranes were added to stripping buffer (see Table 2.9) and incubated at 50°C in a shaker bath for 30 minutes. They were then washed twice in TBS + 0.1% Tween 20 before blocking and incubating with antibodies as required.

Stripping Buffer	**Final Concentration**	**Add (50ml)**
2-β-mercaptoethanol	100mM	350µl
SDS	2%	10ml
Tris 1M pH 6.8	62.5mM	3.12ml
Water		36.53ml

Table 2.9 Stripping buffer

Chapter 3:

Airway smooth muscle supports the survival and proliferation of human lung mast cells

3.1 Introduction

In health, mast cells are recruited to sites of inflammation and their survival is maintained for a short period of time. Following resolution of the inflammation, the recruitment and survival signals are withdrawn, and signals to promote apoptosis are triggered. However, within the asthmatic lung, mast cell numbers within the airway smooth muscle bundles are maintained at a significantly higher degree (15). This sustained increase in cell number must be a result of a balance between promotion of recruitment to the site and subsequent survival and inhibition of apoptosis. To what degrees each of these pathways influences the numbers of mast cells found within the smooth muscle of the asthmatic lung has yet to be ascertained and will be addressed in this first chapter.

Lung mast cells have previously been maintained in an ex vivo culture setting (164). Their survival however is dependent on the addition of exogenous growth cytokines IL-16 and SCF, and the addition of serum to the culture. But using this method, mast cells do not necessarily have a short finite lifespan and may in fact survive for a period of months in this manner. Airway smooth muscle may also be maintained ex vivo in a similar manner with the addition of serum to the culture. But when exploring the survival of mast cells in an ex vivo co-culture model with ASM, the addition of growth cytokines and serum would negate any survival we did see. Therefore, a co-culture model without cytokines and without serum to artificially maintain the cells would be employed. Mast cells will be added to the cultured smooth muscle at a ratio of 1 mast cell to 4 smooth muscle cells. This concentration reflects the in vivo state where concentrations of mast cells to smooth muscle cells of 1:4 to 1:12 have been observed (15).

To address the first aim of the first hypothesis, we will co-culture human lung mast cells with airway smooth muscle isolated from non-asthmatic subjects without the addition of exogenous cytokines or serum, to observe whether smooth muscle may maintain the survival of the mast cells to a greater degree than culturing them alone with the required growth cytokines and serum.

Following this, we shall then investigate the second aim of the first hypothesis by culturing mast cells with airway smooth muscle isolated from asthmatic patients. We will explore whether asthmatically-sourced smooth muscle can promote the survival of mast cells to a greater degree than non-asthmatically sourced ASM.

The rates of proliferation and apoptosis of the mast cells will then be quantified. This will explore the balance between these two systems and examine any differences in mast cells cultured alone and those cultured within the co-culture system with airway smooth muscle.

3.2 Survival and proliferation of HLMC in co-culture with ASM

When human lung mast cells were cultured alone, without addition of serum or of exogenous growth cytokines (SCF and IL-6), their numbers diminished to zero by day 10 (Fig 3.1). In the presence of IL-6 and SCF, but in the absence of serum, $1.5\pm0.35\times10^4$ (38.1%) of cells survived by day 10 (Fig 3.1), while culturing them in 10% serum, SCF and IL-6 sustained survival with minor proliferation evident by day 10 ($5.2\pm0.67\times10^4$; 30.0% increase; P=0.05; Fig 3.1). Remarkably, when mast cells were co-cultured with airway smooth muscle in the absence of serum or exogenous cytokines, not only did they survive, but there was rapid proliferation evident from day 3, and at day 10 the number of mast cells had increased to $9.2\pm0.11\times10^4$ (130%; P=0.003; n=11; Fig 3.1). The increase in lung mast cells number at day 10 in ASM co-culture was significantly greater than the various mono-culture control conditions including the cytokine/serum-supplemented mono-culture (P=0.001). This survival and proliferative effect of the airway smooth muscle did not differ between asthmatic or non-asthmatic smooth muscle cells (P=0.34; Fig 3.2).

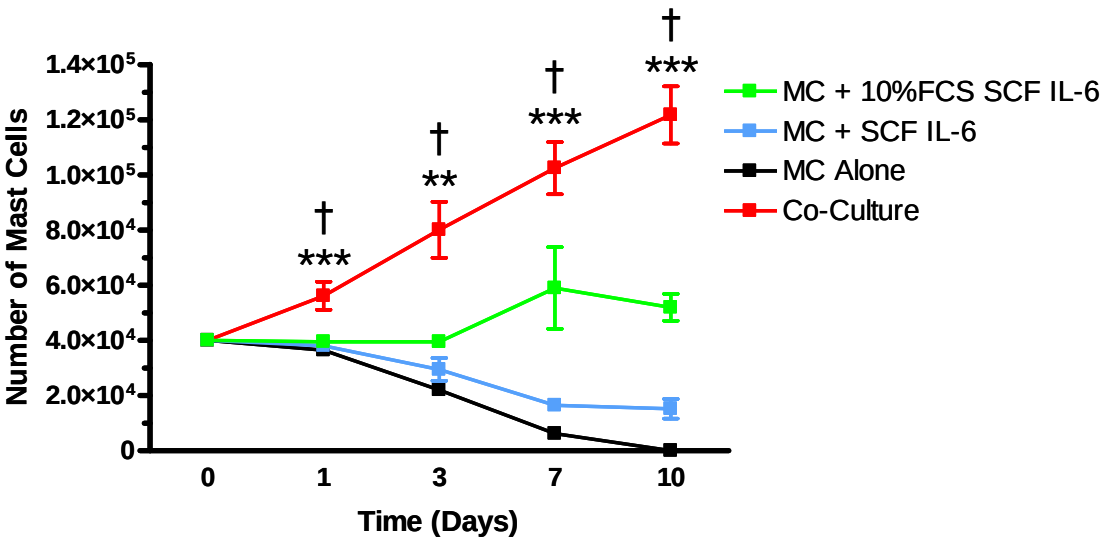


Figure 3.1 Human lung mast cell survival when co-cultured alone with airway smooth muscle

Numbers of metachromatic mast cells present over 10 days of culture in culture medium alone (black line), with SCF (100 ng/ml) and IL-6 (50 ng/ml) (blue line), medium plus SCF/IL-6/FCS (green line), and co-culture with ASM in the absence of SCF/IL-6/FCS (red line).

Data represented a mean±SEM. n=14 experiments using 8 HLMC donors and 14 ASM donors. ** $p<0.01$, *** $p<0.001$ by ANOVA. †$p<0.05$ by Tukey's multiple comparison test comparing HLMC-ASM co-culture to each of the other conditions.

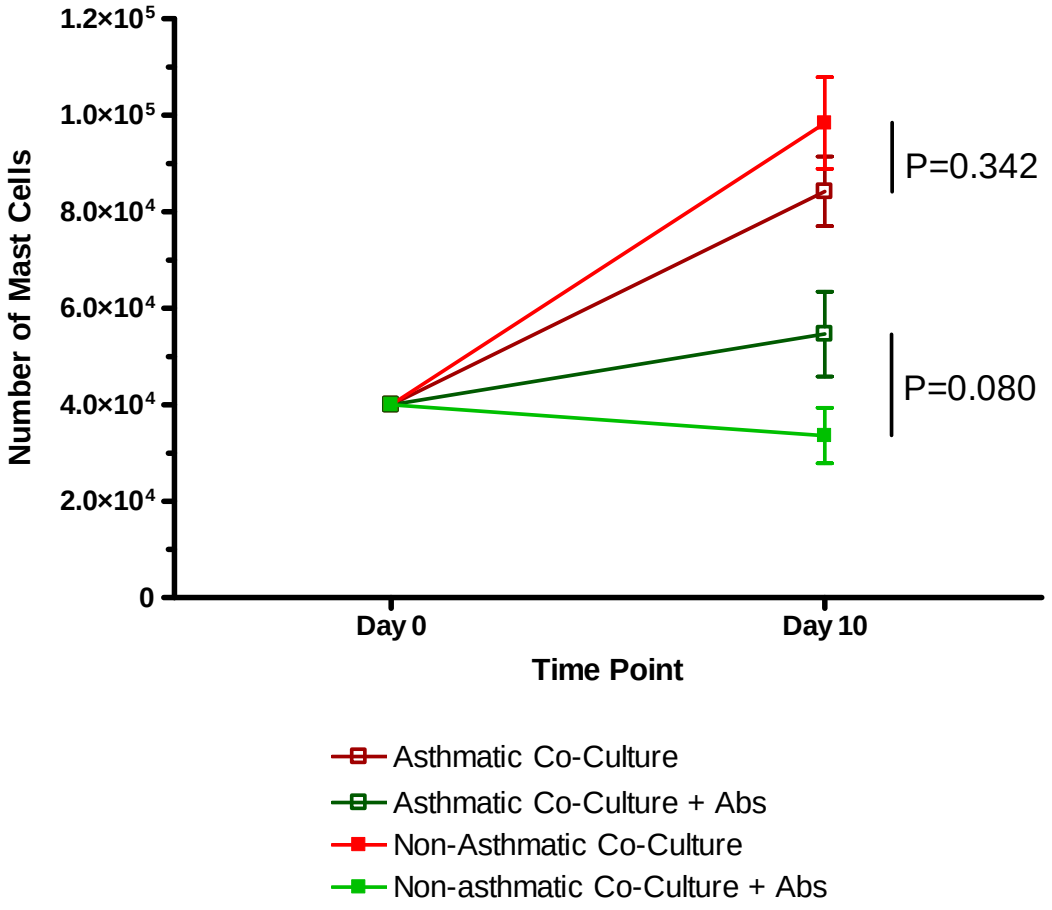


Figure 3.2 Proliferation comparison of human lung mast cells cultured with airway smooth muscle from asthmatic and non-asthmatic subjects

Numbers of metachromatic mast cells present after 10 days of culture in medium without the addition of SCF/IL-6/FCS with ASM from non-asthmatic subjects (red line, filled squares) and asthmatic subjects (dark red line, open squares), and in the presence of neutralising antibodies to SCF and IL-6 (green line, filled squares and dark green line, open squares respectively).

Data represented a mean±SEM. n=3 experiments using 3 HLMC donors and 3 ASM donors.

3.3 Quantification of HLMC proliferation

To confirm the results obtained by cell counting, the proliferation of human lung mast cells in airway smooth muscle co-culture was quantified using mast cells that were preloaded with the fluorescent marker carboxyfluorescein diacetate, succinimidyl ester (CFSE). This stable dye is not passed between cells upon adhesion and is split equally upon cell mitosis, allowing detection using flow cytometry.

Loaded lung mast cells were co-cultured with airway smooth muscle, or in cytokine/serum-supplemented monoculture (positive control). Co-cultured mast cells demonstrated a population with lower fluorescence compared to the same fluorescence intensity of the positive control mast cells population (mean difference [95% CI] 16.4% [0.7-32.2%]; P=0.046; n=3; Fig 3.3). These fluorescence histograms were also analysed using Modfit LT software, which accurately approximates generation peaks within the histograms. Co-cultured lung mast cells were found to contain 6.00±0.58 generations following 10 days of culture, while the positive control lung mast cells contained 4.67±0.33 generations (P=0.057; n=3; Fig 3.4).

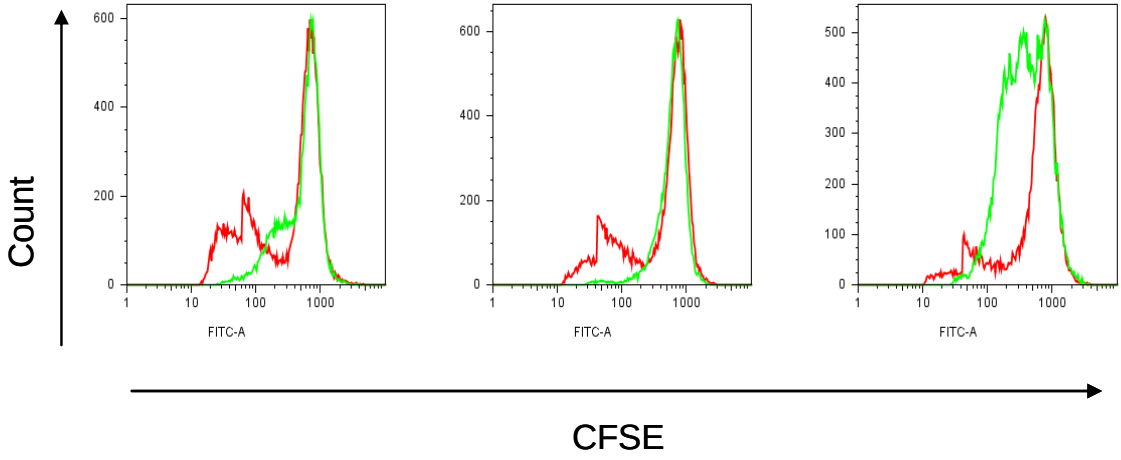
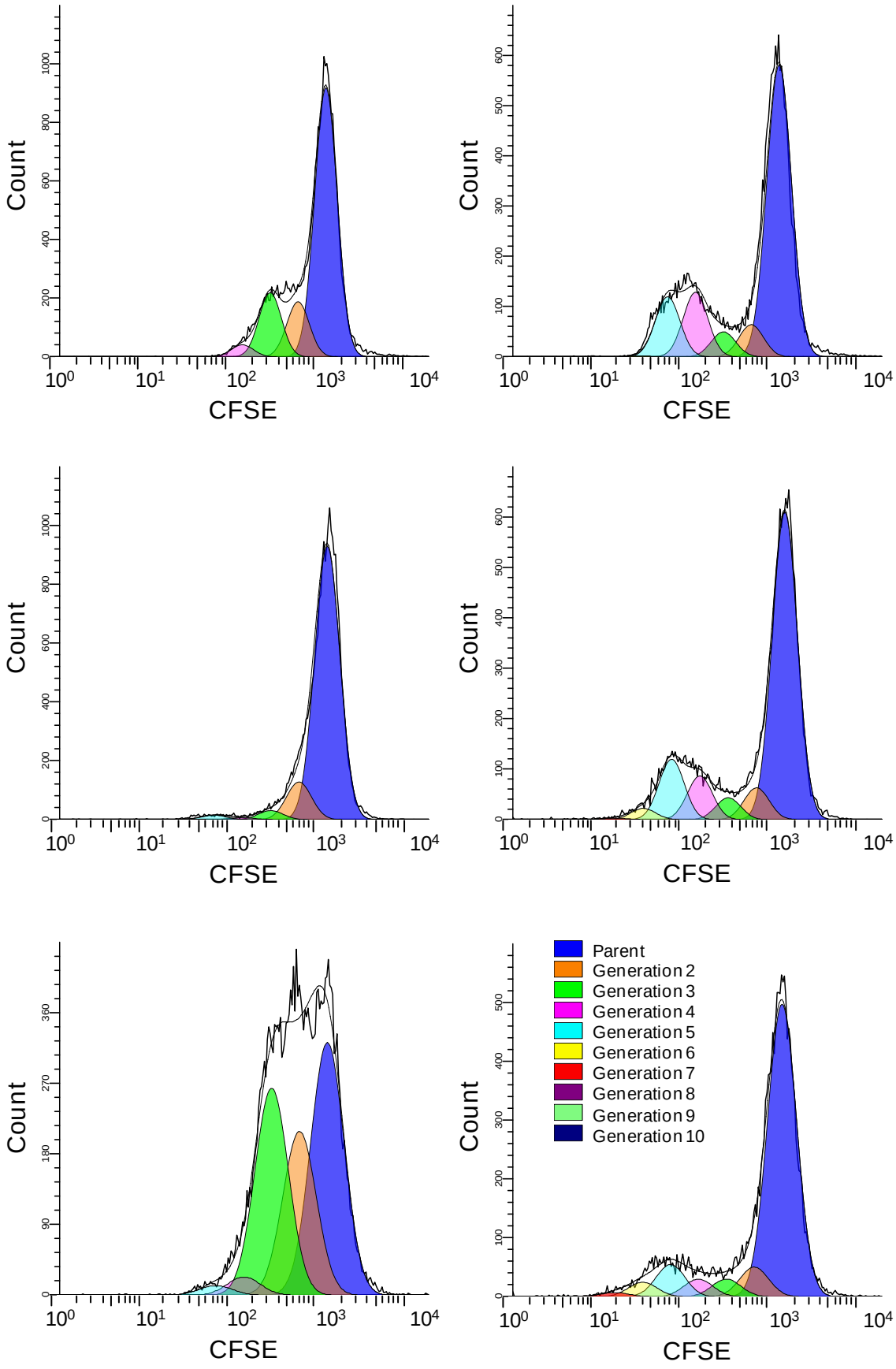


Figure 3.3 Proliferation of human lung mast cells cultured with airway smooth muscle as analysed by flow cytometry using intracellular CFSE staining

HLMC were loaded with CFSE before culturing in SCF/IL-6/FCS (green line) or co-culture with ASM in the absence of SCF/IL-6/FCS (red line) for 10 days, and then analysed using flow cytometry. Graphs shown of 3 separate experiments of 3 HLMC donors and 3 ASM donors. Co-cultured HLMC exhibit a sub-population with significantly reduced fluorescence beyond the control HLMC population, indicating the presence of a proliferating pool of cells ($p=0.046$ by paired t test; $n=3$).

Figure 3.4 Proliferation of human lung mast cells cultured with airway smooth muscle as analysed by flow cytometry using intracellular CFSE staining

HLMC were loaded with CFSE before culturing alone in SCF/IL-6/FCS (left-hand graphs) or co-culture with ASM in the absence of SCF/IL-6/FCS (right-hand graphs) for 10 days, and then analysed using Modfit LT software. Graph pairs shown are of 3 separate experiments of 3 HLMC donors and 3 ASM donors. Analysis of CSFE staining revealed the presence of a reduced number of generations following 10 days in monoculture compared to ASM co-culture ($P=0.057$ by paired t-test; $n=3$).


Count
CFSE
Parent
Generation 2
Generation 3
Generation 4
Generation 5
Generation 6
Generation 7
Generation 8
Generation 9
Generation 10

3.4 Analysis of HLMC death in co-culture

The extent of cell death in the various culture conditions was analysed using a combination of propidium iodide (PI) and Annexin V staining, with 4'-6-diamidino-2-phenylindole (DAPI) staining of the nuclei. PI and Annexin V analysis of cell death was assessed using flow cytometry in combination with CD117-APC to identify the human lung mast cell population (Fig 3.5). Following staining with PI, mast cell death in both the airway smooth muscle co-culture and the cytokine/serum-supplemented monoculture control was relatively low and stable (Fig 3.6). In contrast, lung mast cells in the cytokine/serum-free mono-culture demonstrated accelerating cell death which was significantly greater than the co-culture by day 7 ($P=0.002$; Fig 3.6).

Annexin V staining (Fig 3.7) demonstrated consistently increased expression in the cytokine/serum-supplemented control lung mast cell compared to the mast cells in airway smooth muscle co-culture suggesting a decreased rate of apoptosis in co-culture. There was a significant difference by day 7 ($P<0.001$). This was further supported by analysis of nuclear fragmentation using DAPI staining (Fig 3.8 and 3.9). Thus by day 3, there was a significant increase in the percentage of apoptotic nuclei in the cytokine/serum-supplemented control lung mast cells compared with the mast cells in airway smooth muscle co-culture ($P=0.011$). There was some discrepancy in the number of apoptotic cells analysed by Annexin V versus DAPI in the cytokine/serum-supplemented control mast cells. This is may be accounted for by the activation of lung mast cells following resuspension in 100ng/ml SCF which increases Annexin V staining (165). Nevertheless, the data demonstrate consistently greater cell death within the cytokine/serum-supplemented mast cells monoculture compared to the mast cells in airway smooth muscle co-culture

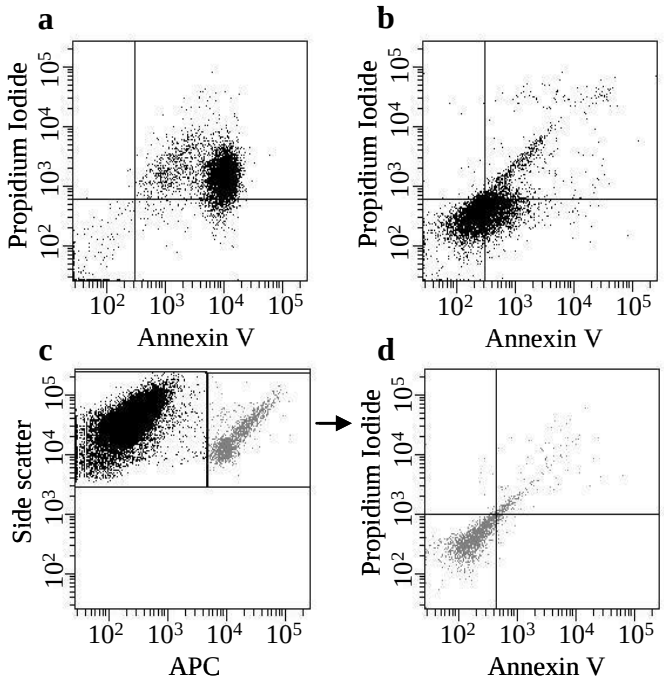


Figure 3.5 Percentage of necrotic and apoptotic cells within the mono- and duel-cultures as analysed by propidium iodide and Annexin V

Representative flow cytometric dot plots for 7 day HLMC cultures. FITC Annexin V/PI dual colour flow cytometry of HLMC in absence of exogenous cytokine and serum (a) and cultured with FCS and cytokines (b). Three colour flow cytometry of HLMC co-cultured with ASM in the absence of exogenous cytokines and serum labelled with CD117-APC (c) and gated CD117 cells then analysed for Annexin V and PI (d).

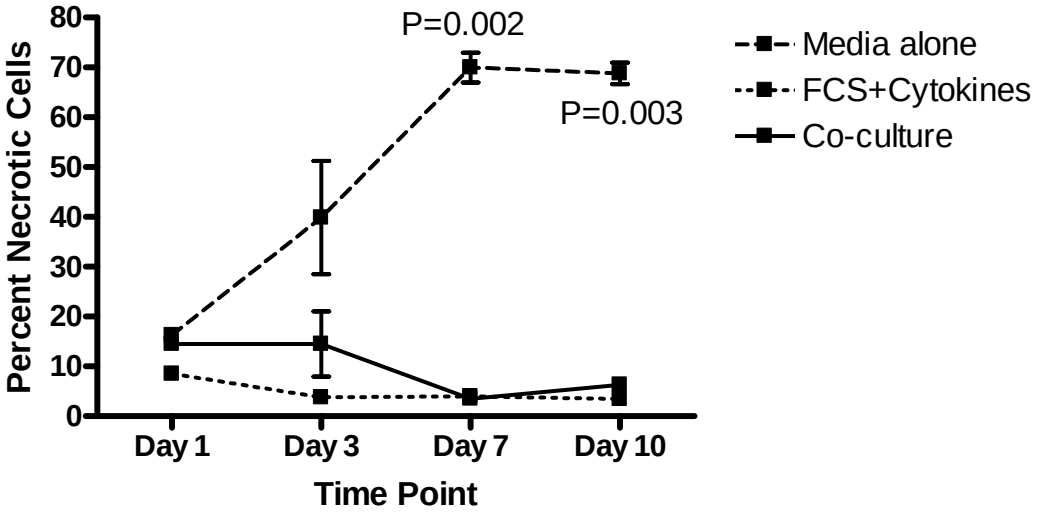


Figure 3.6 Percentage of necrotic cells within the mono- and duel-cultures as analysed by propidium iodide

Mast cells were either cultured alone in the presence or absence of serum, SCF and IL-6 (dotted and dashed lines respectively), or co-cultured with airway smooth muscle in the absence of exogenous cytokines (solid line). Necrosis measured in the mast cell mono-culture in the presence of growth cytokines and serum was similar to that seen in the co-culture. Necrosis measured in the mono-culture in the absence of the serum and growth cytokines was significantly increased compared with the co-cultured mast cells.

Data represented a mean±SEM. n=3 experiments using 3 HLMC donors and 3 ASM donors. Analysis by paired t test.

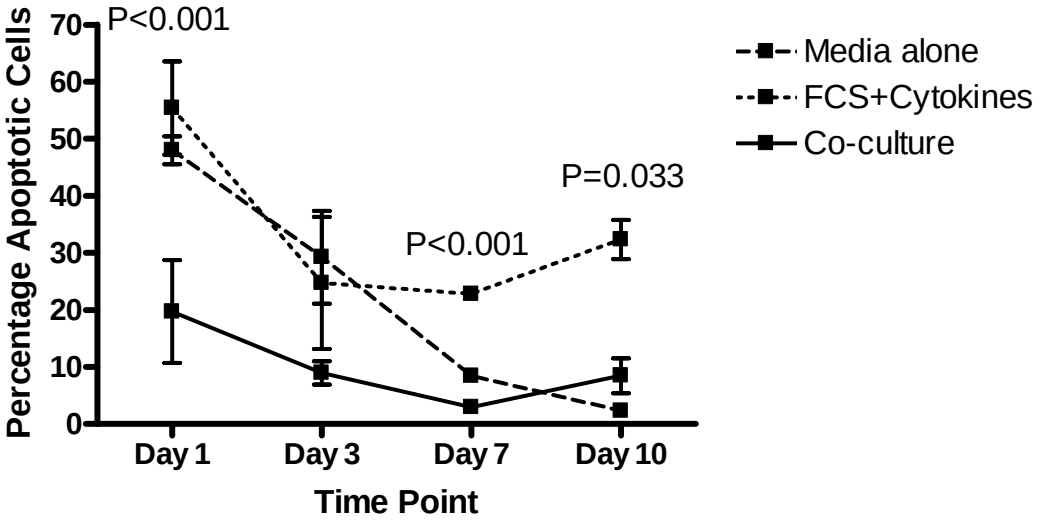


Figure 3.7 Percentage of apoptotic cells within the mono- and duel-cultures as analysed by Annexin V

Mast cells were either cultured alone in the presence or absence of serum, SCF and IL-6 (dotted and dashed lines respectively), or co-cultured with airway smooth muscle in the absence of exogenous cytokines (solid line). Apoptosis measured in the control mono-cultures was relatively high by day 1. During the culture, mono-cultured mast cells in the presence of exogenous cytokines and serum had consistently higher percentage apoptosis than mast cells cultured in the presence of airway smooth muscle.

Data represented a mean±SEM. n=3 experiments using 3 HLMC donors and 3 ASM donors. Analysis by paired t test.

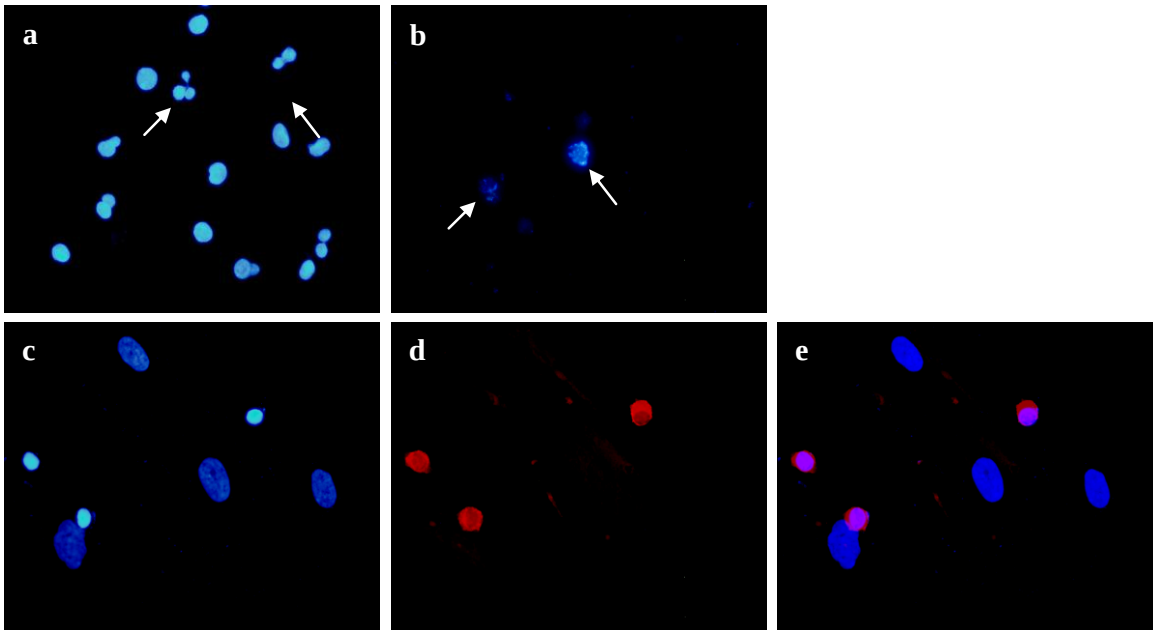


Figure 3.8 Immunofluorescence of apoptotic cells within the mono- and duel-cultures as analysed by nuclear morphology

DAPI staining of nuclear morphology was detected using fluorescent microscopy (magnification ×400). Representative micrographs of day 7 showing DAPI staining on mast cells (a) in the presence of serum and cytokines, (b) in absence of exogenous cytokine and serum. (c) HLMC and ASM co-culture stained with DAPI, (d) CD117 to identify the mast cells and then (e) overlaid to count HLMC DNA fragmentation and blebbing.

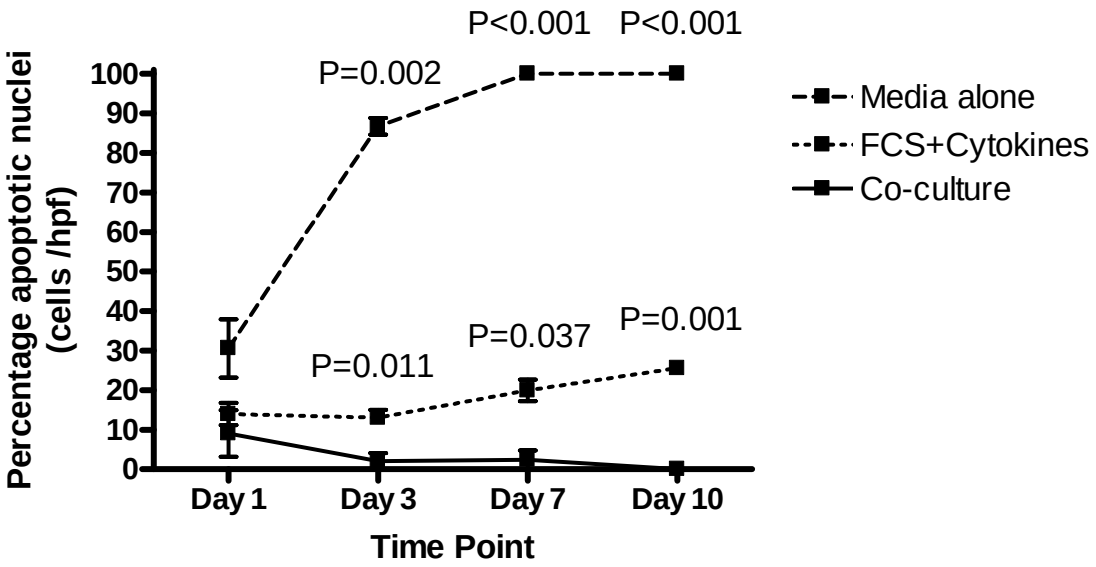


Figure 3.9 Percentage of apoptotic cells within the mono- and duel-cultures as analysed by nuclear DAPI staining

Mast cells were either cultured alone in the presence or absence of serum, SCF and IL-6 (dotted and dashed lines respectively), or co-cultured with airway smooth muscle in the absence of exogenous cytokines (solid line). Culture of mast cells alone; regardless of the presence or absence of exogenous cytokine and serum, exhibited significantly higher rates of apoptotic nuclei when compared with mast cells co-cultured with airway smooth muscle in media alone.

Data represented a mean±SEM. n=3 experiments using 3 HLMC donors and 3 ASM donors. Analysis by paired t test.

3.5 Summary

Airway smooth muscle, regardless of whether it has been sourced from a subject with or without asthma, has the ability to promote survival and even proliferation of human lung mast cell without the need for additional exogenous growth cytokines or serum. This increase in cell number over the 10 day culture period is a combination of promotion of cell proliferation and inhibition of cell apoptosis. However, maintaining and even promotion of cell number my not be the only mechanism of increased human lung mast cell numbers in airway smooth muscle in vivo, recruitment may also be an important player.

Human lung mast cells have widely been considered as terminally differentiated cells. Previous studies of culturing these cells ex vivo in cytokine and serum-supplemented media, has found their numbers to be maintained and even to increase slightly over time (166)(166)(166)(164,166). However, the quantity of proliferation observed within these studies did not reach the level we found following co-culture with airway smooth muscle.

Maintenance of mast cell survival by another cell type is not a phenomenon unique to airway smooth muscle. Previous reports have found mast cell numbers to be preserved when co-cultured with fibroblasts, human umbilical vein endothelial cells (HUVEC) and epithelial cells. Human gut fibroblasts maintained gut mast cell survival, but did not induce their proliferation (167). HUVEC were able to promote gut mast cell survival and over a long period of time induced a small amount of proliferation, much reduced than that seen in this co-culture model (168). Human bronchial epithelial cells maintained the survival of human cord blood-derived mast

cells but did not induce any proliferation (169). However, to our knowledge this is the first report of human airway smooth muscle maintaining human lung mast cell number and promoting proliferation to such a degree.

As lung mast cells are the only cell type to be found within the airway smooth muscle, little work has been done on the impact of airway smooth muscle survival effect on other cell types such as eosinophils. ASM does seem to be equipped for the promotion of mast cell survival due to the constitutive production of the essential growth cytokines SCF and IL-6 that we and others have found (48). As these cytokines are produced by airway smooth muscle, their involvement in the promotion of mast cell survival and proliferation seen in this model should be investigated. An addition, lung mast cells do adhere to airway smooth muscle (141), therefore the participation of this adhesion role should also be explored.

Chapter 4:

Airway smooth muscle-induced mast cell proliferation is dependent on SCF, IL-6 and CADM1-dependent adhesion

4.1 Introduction

SCF is an essential mast cell growth factor (170-172), and IL-6 promotes cord blood mast cell progenitor growth (173) and prevents human lung mast cell apoptosis (174). Both of these key mast cell cytokines have been found to be produced by airway smooth muscle (175,176). Indeed, ex vivo human lung mast cell numbers may be maintained in culture with the addition of these cytokines in combination with serum (164). Proliferation of these cultured lung mast cells was even evident in this supplemented mono-culture environment, but not to the extent that was observed in our ASM co-culture model, implying that these growth cytokines may not be the only pro-survival and proliferative signal occurring.

To investigate the third aim of the first hypothesis: examining the mechanisms underlying the ASM-induced mast cell survival and proliferation; in the first instance, the level of contribution that the cytokines SCF and IL-6 has in ASM survival and proliferative effect on the mast cells will be investigated through the use of neutralizing antibodies. In addition, as mast cells adhere to the smooth muscle to such a high degree and partly through the adhesion antibody CADM1 (141), these mechanisms will also be explored.

4.2 HLMC proliferation may be inhibited by blocking SCF and IL-6

IL-6 concentrations in the medium obtained from human lung mast cell and airway smooth muscle co-culture for 3 days were 12.2±0.67ng/ml, which is above the optimal 1ng/ml concentration of IL-6 required for the development of human mast cells from peripheral blood progenitors (172). SCF concentrations in this medium were only 0.29±0.06ng/ml, which exert only a weak survival capacity on cultured human mast cells (172). However, SCF also exists in a membrane-bound form generated by alternative mRNA splicing (177). Membrane-bound SCF was highly expressed as demonstrated by both immunofluorescent and flow cytometry staining in smooth muscle from both asthmatic and non-asthmatic patients (Fig 4.1).

Addition of neutralising antibodies targeting SCF or IL-6 inhibited HLMC proliferation in ASM co-culture to a similar degree, and employing both antibodies together had no significant additive effect (Fig 4.2). However, with cytokine neutralisation cell number at day 10 still remained similar to that present at day 0, suggesting that while both IL-6 and SCF play important roles in mediating HLMC proliferation, a further factor may also be supporting their survival. The effect of these antibodies in combination were also investigated in control cultures, HLMC cultured with the exogenous cytokines (Fig 4.3) and HLMC cultured with exogenous cytokine and serum (Fig 4.4). As expected, the addition of the neutralising antibodies significantly reduced the number of HLMC remaining in the culture by day 10 ($P=0.013$ and $P<0.001$ respectively).

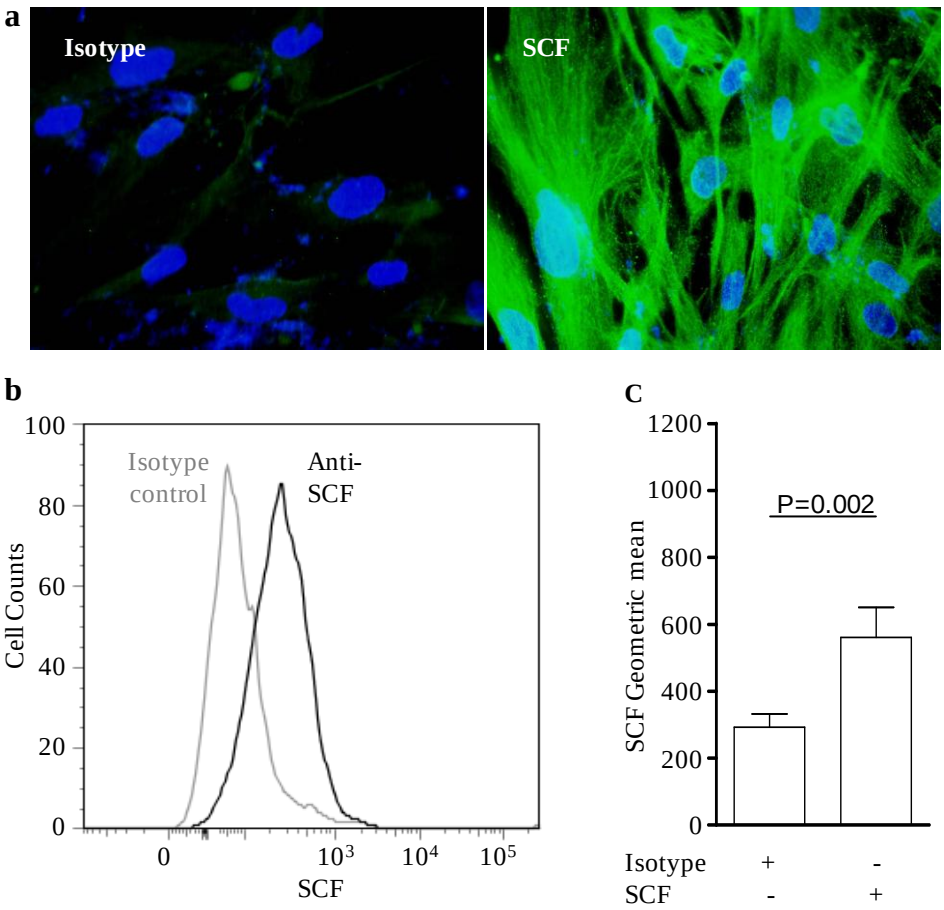


Figure 4.1 Human airway smooth muscle expresses membrane-bound SCF

This was measured by immunofluorescence, (a) showing positive staining for SCF (green) in smooth muscle monolayers; flow cytometry, (b) representative histogram of SCF expression on smooth muscle (black line) showing the shift in fluorescence compared to the isotype control (grey line); and (c) the geometric mean analysis of smooth muscle demonstrating surface SCF expression. (Data represented a mean±SEM). n=8 experiments.

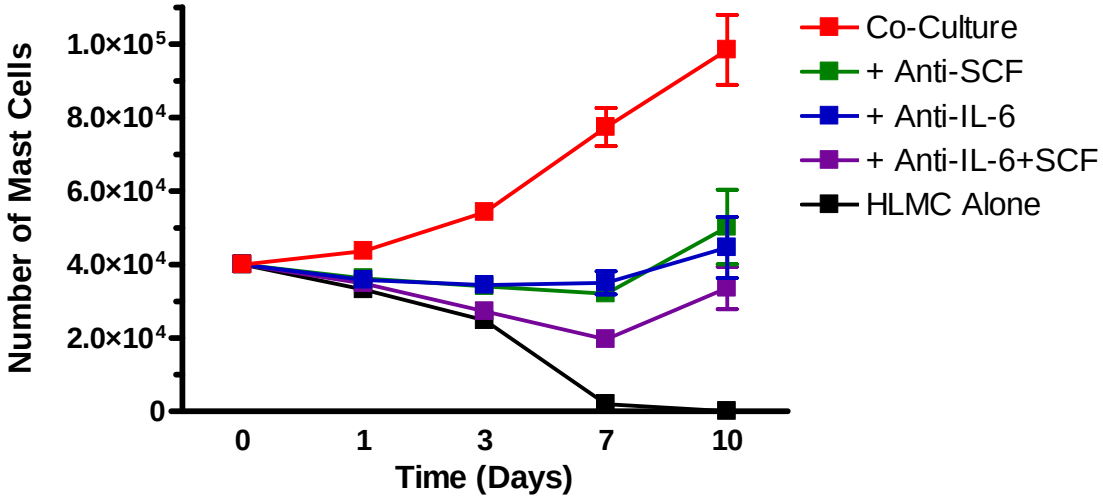


Figure 4.2 Co-cultured mast cell proliferation is dependent on SCF and IL-6 - effect of cytokine neutralisation

HLMC were cultured in media alone (black line) or co-cultured with ASM in the absence SCF/IL-6/FCS (red line), and in the presence of the neutralising antibodies to IL-6 (blue line), SCF (green line) or both (purple line). Addition of the neutralising antibodies significantly reduced the proliferation of the mast cells during the culture. n=5 experiments using 3 HLMC donors and 5 ASM donors. Cultures compared using one-way ANOVA. †$p<0.05$ by Tukey's multiple comparison test comparing co-cultured HLMC to addition of neutralising antibodies singularly and in combination. Data represented a mean±SEM.

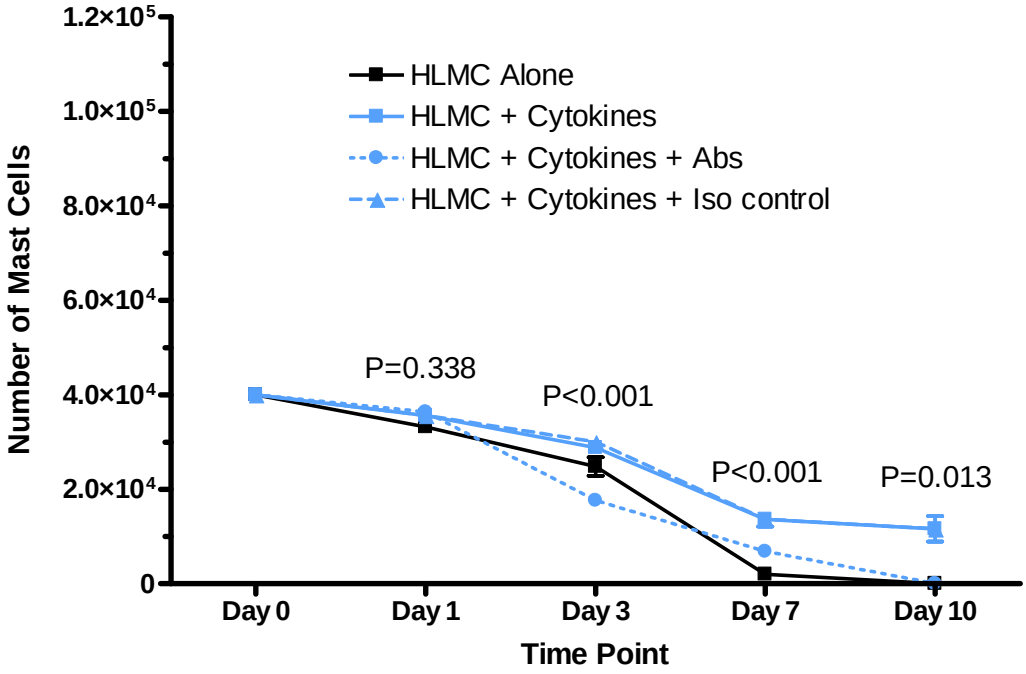


Figure 4.3 Control graph of effect of SCF and IL-6 antibodies on mast cells cultured with exogenous cytokines

Addition of the SCF and IL-6 neutralising antibodies to the control culture significantly reduced the number of mast cells during the 10 day experimental period. n=5 experiments (3 HLMC donors and 5 ASM donors) for control culture of HLMC incubated with exogenous cytokines (blue squares with solid line) and for control culture with the addition of the neutralising antibodies (blue circles with dashed line). Analysis by paired t test comparing the control culture with the respective culture with the addition of the antibodies. Data represented a mean±SEM.

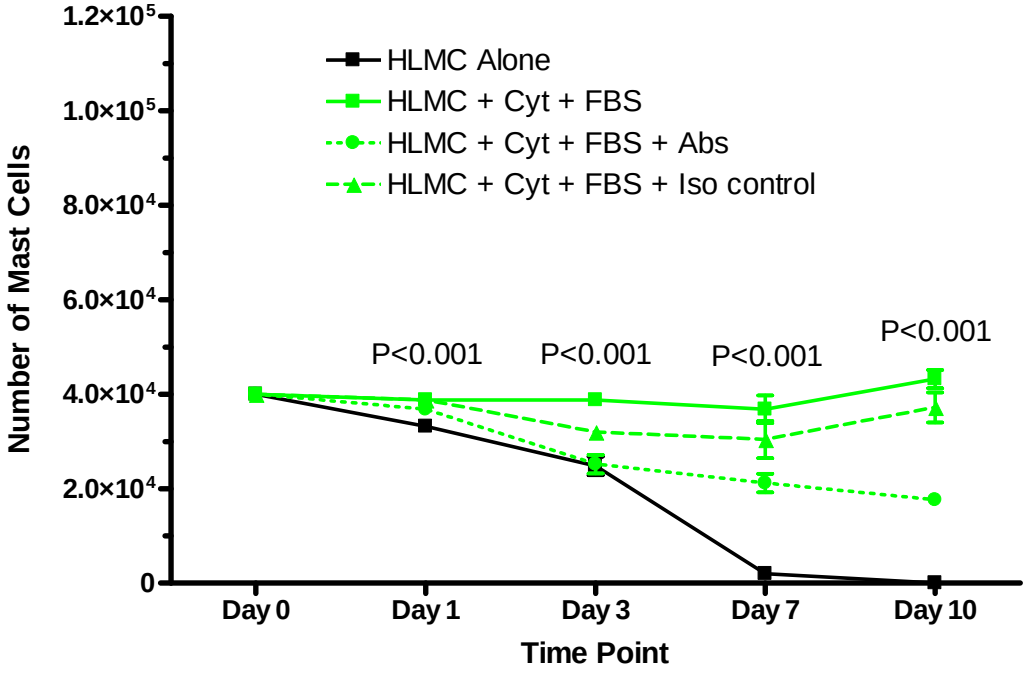


Figure 4.4 Control graph of effect of SCF and IL-6 antibodies on mast cells cultured with exogenous cytokines and serum

Addition of the SCF and IL-6 neutralising antibodies to the positive control culture significantly reduced the number of HLMC during the 10 day experimental period. n=5 experiments (3 HLMC donors and 5 ASM donors) for control culture of mast cells incubated with exogenous cytokines and serum (green squares with solid line) and for control culture with the addition of the neutralising antibodies (green circles with dashed line). Analysis by paired t test comparing the control culture with the respective culture with the addition of the antibodies. Data represented a mean±SEM.

To address whether ASM-induced HLMC survival and proliferation requires cell-cell contact or soluble factors only, HLMC were cultured in serum-free ASM-conditioned medium generated over 3 days. The soluble mediators within the media were sufficient to maintain the survival of the HLMC for 7 days but no proliferation was evident (Fig 4.5). By day 7, HLMC numbers began to decline, indicating a possible depletion of survival factors. Addition of the IL-6 and SCF neutralising antibodies independently to this culture reduced the number of surviving HLMC further, and addition of these antibodies in combination demonstrated a minor additive effect compared to the culture without the addition of the antibodies (P=0.036; Fig 4.5).

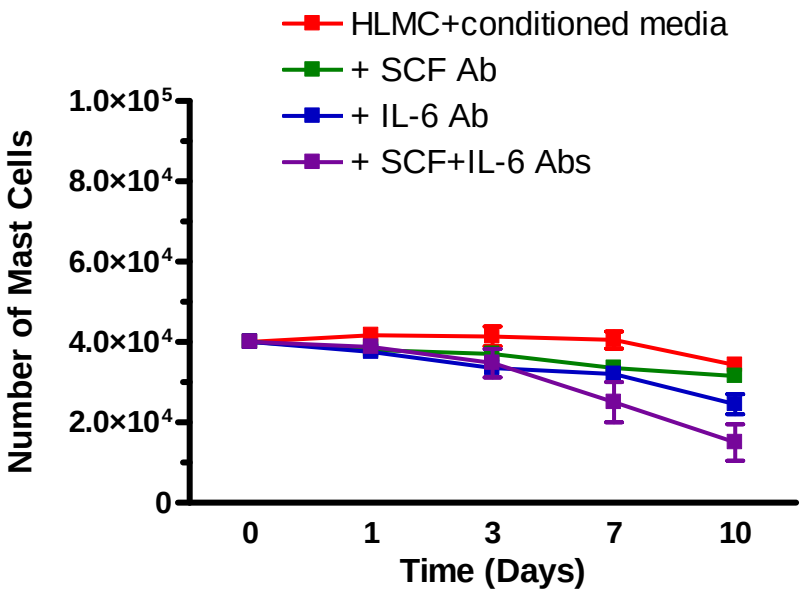


Figure 4.5 Mast cells cultured with ASM-conditioned media

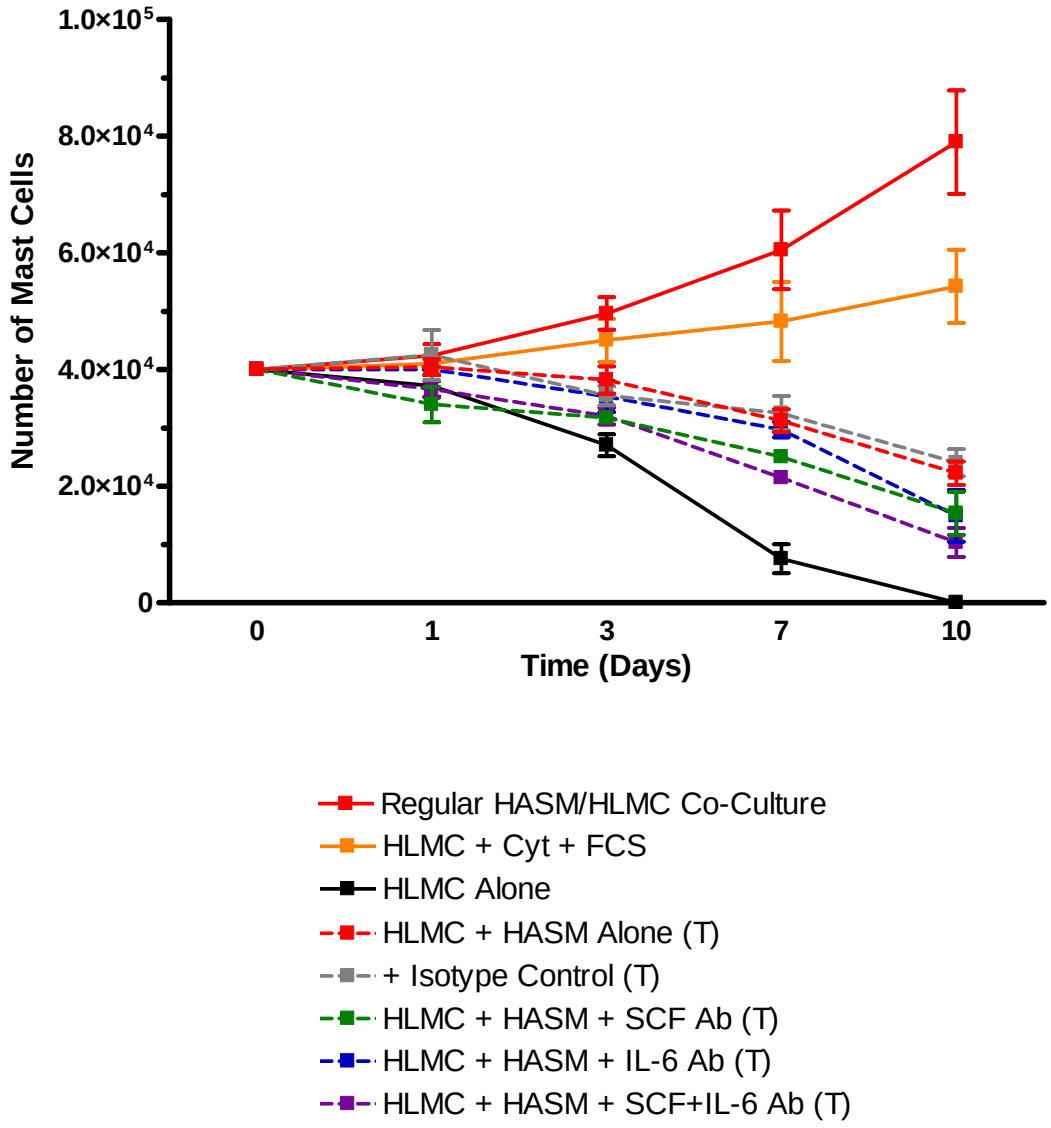
In the presence of ASM-conditioned media, HLMC survived for 7 days but did not proliferate (red line). This survival effect was attenuated equally by neutralising antibodies to IL-6 (blue line) and SCF (green line), with a non-significant additive effect evident with both SCF and IL-6 neutralisation (purple line). n=6 experiments using 3 HLMC donors and 6 ASM donors. Analysis by one-way ANOVA. † p=0.002 by Tukey's multiple comparison test comparing mast cells cultured in airway smooth muscle-conditioned medium to addition of combined SCF and IL-6 neutralising antibodies. Data represented a mean±SEM.

4.3 Complete prevention of cell adhesion arrests survival and proliferation of HLMC

We then examined ASM-dependent HLMC survival and proliferation using the Transwell system. ASM cells were seeded onto the plate surface and grown to confluence before a 0.4μM Transwell insert was placed inside the well in which the HLMC were added (4:1 ASM:HLMC ratio as previously). Use of the Transwell inserts significantly reduced survival and proliferation of the HLMC compared to the co-culture without inserts (Fig 4.6; P=0.021), comparable to the effects seen in ASM-conditioned media. Addition of IL-6 and SCF neutralising antibodies individually and in combination, further reduced this survival to $1.0\pm0.25\times10^4$ by 10 days, but this was not significant when allowing for multiple comparisons (Fig 4.6). These data confirm the importance of adhesion in combination with soluble IL-6 and SCF in airway smooth muscle-induced lung mast cell survival and proliferation.

Figure 4.6 Mast cell survival following incubation with ASM separated by Transwell inserts

Preventing contact of mast cells to smooth muscle using a 0.4mM Transwell insert inhibited their survival and proliferation. Mast cells were cultured in media alone (black solid line), with exogenous SCF/IL-6/FCS (light green solid line), or in the presence of smooth muscle without SCF/IL-6/FCS (red solid line). Mast cells were also co-cultured with smooth muscle separated by a 0.4μM Transwell insert (T) to block physical contact between the two cell types, in the presence and absence of isotype control antibody (red and grey dashed line respectively), neutralising anti-IL-6 antibody (blue dashed line), neutralising anti-SCF antibody (green dashed line) or both anti-IL-6 and -SCF (purple dashed line). n=5 experiments using 3 HLMC donors and 5 ASM donors. Analysis by one-way ANOVA. † $p<0.05$ by Tukey's multiple comparison test comparing co-cultured HLMC to the addition of Transwell inserts in the presence and absence of neutralising antibodies. Data represented a mean±SEM.


1.0×10⁵
8.0×10⁴
6.0×10⁴
4.0×10⁴
2.0×10⁴
0
Number of Mast Cells
0
1
3
7
10
Time (Days)
Regular HASM/HLMC Co-Culture
HLMC + Cyt + FCS
HLMC Alone
HLMC + HASM Alone (T)
+ Isotype Control (T)
HLMC + HASM + SCF Ab (T)
HLMC + HASM + IL-6 Ab (T)
HLMC + HASM + SCF+IL-6 Ab (T)

4.4 Inhibition of CADM-1 adhesion antibody attenuates HLMC proliferation

HLMC adhere to ASM in part through CADM1, an adhesion molecule highly expressed on HLMC but not airway smooth muscle (141). Interestingly, the adhesion-blocking anti-CADM1 monoclonal antibody 9D2 (141) significantly reduced the proliferation of HLMC in airway smooth muscle co-culture compared with the isotype control ($P<0.001$; Fig 4.7), indicating that adhesion via CADM1 is a critical factor for the induction of HLMC proliferation by airway smooth muscle. Any direct effect of anti-CADM1 on HLMC survival was negated by culturing the antibody with HLMC cultured with exogenous cytokines and serum (Fig 4.8).

Inhibiting CADM1, IL-6 and SCF together (Fig 4.9), reduced the survival and proliferation of HLMC further than that seen with the individual antibodies ($P=0.007$ at day 10 by one-way ANOVA). Nevertheless, this inhibition was not complete and 56.9%±25.5% of HLMC remained viable in the culture after 10 days, indicating that either blockade of these molecules was not complete or a further factor(s) may be important. There was no evidence of any physical or functional cross-reaction between the anti-SCF, anti-IL-6 or 9D2 anti-CADM1 antibodies. Taken together, the marked effects of inhibiting each of SCF, IL-6 and CADM1 in isolation, but relatively small additive effects seen when inhibiting them together, suggests the presence of a co-operative interaction between these three molecules.

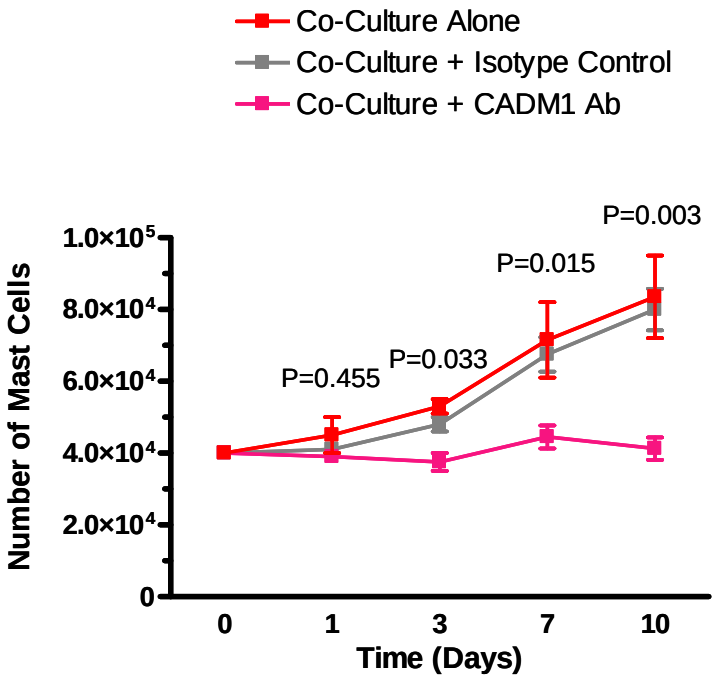


Figure 4.7 Co-culture of mast cells with ASM in the presence of the adhesion antibody to CADM1

In keeping with the Transwell experiments, addition of an adhesion-blocking antibody to CADM1 also prevented HLMC proliferation in co-culture with ASM, suggesting that CADM1 dependent adhesion is an important component of the ASM-induced HLMC proliferation pathway. n=5 experiments using 3 HLMC donors and 5 ASM donors. Statistical analysis by paired t test comparing isotype control co-cultured HLMC with the anti-CADM1 antibody. Data represented a mean±SEM.

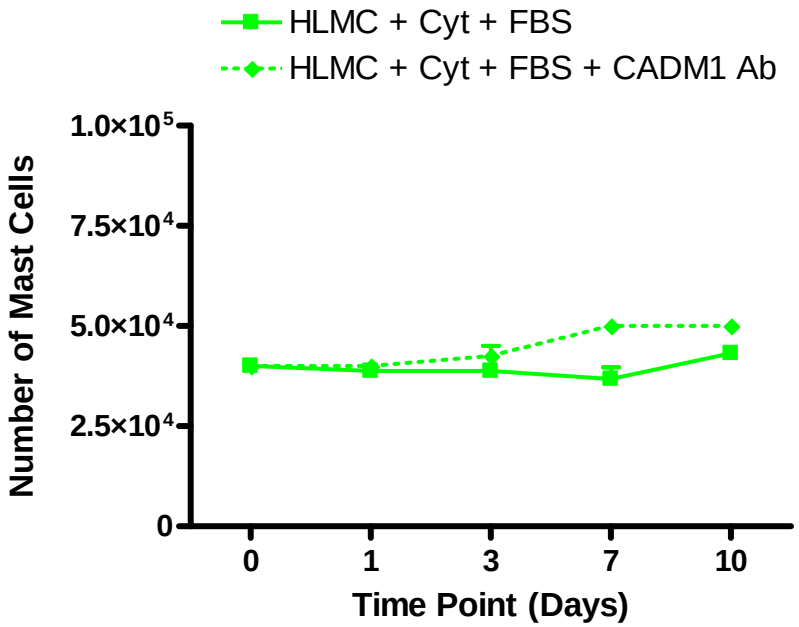


Figure 4.8 Control graph of effect of SCF and IL-6 antibodies on HLMC cultured with exogenous cytokines and serum

Addition of the CADM1 to the positive control culture did not effect the number of HLMC during the 10 day experimental period. n=5 experiments (3 HLMC donors and 5 ASM donors) for control culture of HLMC incubated with exogenous cytokines and serum (green squares with solid line). n=2 experiments (2 HLMC donors and 2 ASM donors) for control culture with the addition of the adhesion antibody CADM1 (green diamonds with dashed line). Data represented a mean±SEM.

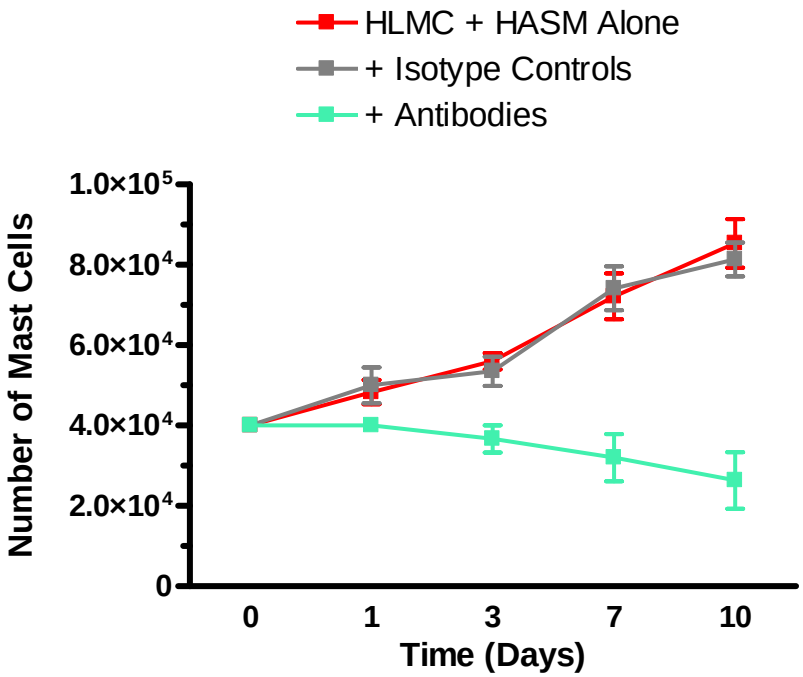


Figure 4.9 HLMC co-culture with ASM in the presence of antibodies to SCF, IL-6 and CADM1

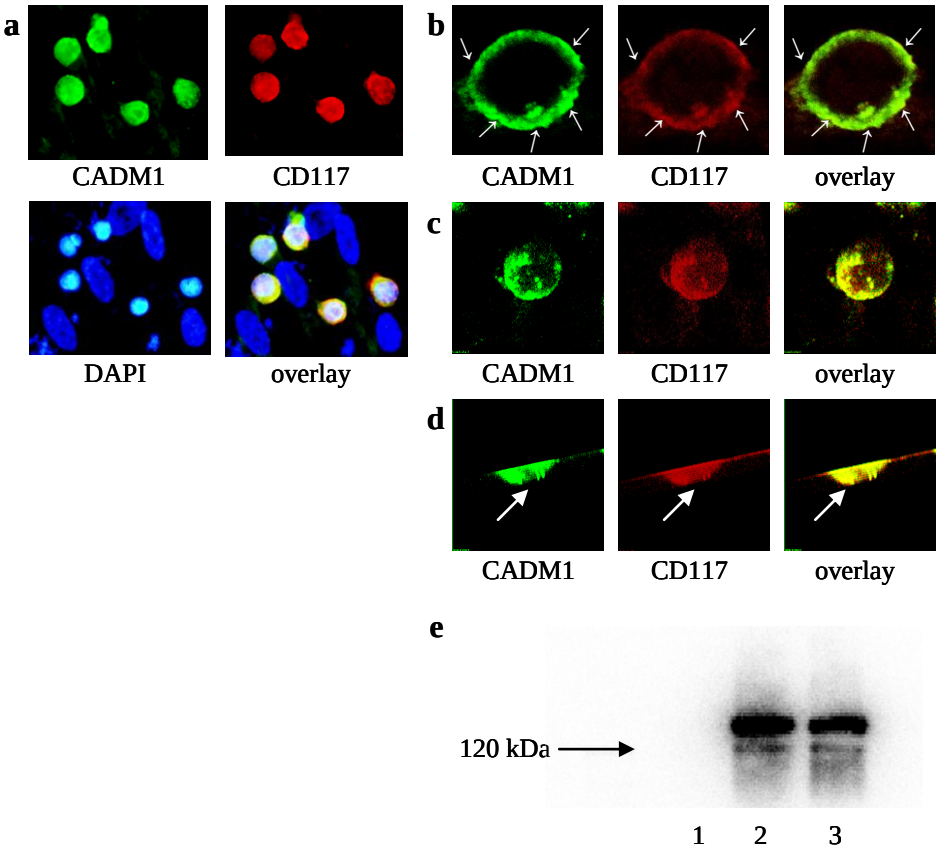
Lung mast cells co-cultured with airway smooth muscle in the presence of neutralising antibodies to SCF, IL-6 and CADM1 revealed a minor additional effect over neutralisation of each in isolation suggesting a co-operative effect between the 3 molecules. n=6 experiments using 3 HLMC donors and 6 ASM donors. Analysed by paired t test comparing isotype control co-cultured HLMC with anti-SCF/IL-6/CADM1-treated cells. Data represented a mean±SEM.

4.5 CD117 and CADM1 co-localise and physically interact in HLMC

Our data suggest that there is a co-operative interaction between SCF and CADM1. It is possible therefore that CADM1-dependent adhesion facilitates the interaction of membrane-bound SCF on human airway smooth muscle with its receptor CD117 (c-kit) on HLMC. Confocal immunofluorescence demonstrated strong co-localisation of CD117 and CADM1 in the HLMC plasma membrane (Fig 4.10). There was evidence for hotspots of both CADM1 and CD117 immunofluorescence which were 100% co-localised and present at points of adhesion with airway smooth muscle (Fig 4.10). Interestingly, immunoprecipitation of CADM1 resulted in the co-immunoprecipitation of CD117, indicating the presence of a direct physical interaction (Fig 4.10)

Figure 4.10 CD117 co-localises with CADM1 on co-cultured HLMC

HLMC were co-cultured with ASM cells over 3 days and stained with anti-human RPE-CD117 (a; red), anti-CADM1 (green) and DAPI (blue cell nuclei). The cells negative for CD117 and CADM1 are the airway smooth muscle cells (×400 magnification). Representative micrographs of confocal laser scanning microscopic sections of lung mast cells co-cultured on confluent airway smooth muscle for 3 days (b). Diffuse plasma membrane staining was punctuated by hotspots of staining (arrows). Notably the cell surface is the most intensely fluorescent and CD117 and CADM1 are co-localised in the mast cell plasma membrane. Mast cell XYZ plane images were captured and a 3D image cropping series animation was produced of CADM1 and CD117 staining (c). The displays of the measured 3D image stack are shown for CADM1, CD117 and the overlay, demonstrating strong co-localisation. The 3D image was then rotated and demonstrated that there were hot spots of co-localised staining present on the mast cells at the point of contact with the airway smooth muscle (d, arrow). Lane 2 - CADM1 was immunoprecipitated from the HMC-1 human mast cell line using the 3E1 anti-CADM1 antibody (e), run on an SDS gel, transferred to a PVDF membrane and then probed for CD117. CD117 was consistently present as a characteristic doublet of 145 and 122 kDa (representative of 3 experiments). Lane 1 – Negative control i.p. with anti-chicken IgY omitted. Lane 3 – Positive control for CD117 present in HMC-1 cell lysate.


a
CADM1
CD117
DAPI
overlay
b
CADM1
CD117
overlay
c
CADM1
CD117
overlay
d
CADM1
CD117
overlay
e
120 kDa
1
2
3

4.6 Summary

These data suggest that the survival and proliferative effect airway smooth muscle has upon the human lung mast cells is a combination of soluble and membrane-bound SCF, IL-6 and the adhesion molecule CADM1.

Mast cell contact with airway smooth muscle was an essential component mediating mast cell proliferation. This was demonstrated when contact between the two cell types was prevented through the use of Transwell inserts. The degree of impediment of mast cell proliferation observed using this method was similar to that seen when adhesion via CADM1 was blocked. This indicates that mast cell adhesion to ASM, and their subsequent survival and proliferation involves Ca^{2+}-independent adhesion through CADM1. This adhesion molecule, which is highly expressed on mast cells, also mediates adhesion of mast cells to fibroblasts (178) and nerves (159). Though the effect of CADM1 in mast cell proliferation at these sites is unknown, within ASM it does have a role in mediating mast cell proliferation; a mechanism that seems to involve a specific interaction with the SCF receptor CD117. This is supported by the observation that blocking both CD117 and CADM1 only had a marginal additive effect on the inhibition of mast cell proliferation. In addition, the SCF expressed by ASM was predominantly membrane-bound; there was strong co-localisation of CD117 and CADM1 at the plasma membrane, and CD117 co-immunoprecipitated with CADM1. All these data indicate that there is a physical interaction between these two molecules; an interaction that could be facilitating mast cell survival and proliferation in co-culture with airway smooth muscle.

IL-6 was also an important mediator of the mast cell proliferative response in the co-culture environment. The concentrations measured in the co-culture supernatant were above the optimal concentration required to promote cultured mast cell proliferation (172). But the source of the IL-6 may be either the smooth muscle or the mast cells ASM or indeed both (174,179). The IL-6 in this culture appeared to co-operate with SCF and CADM1, as its involvement in the ASM survival and proliferative effect was very similar to these molecules, and the effects of its blockade in conjunction were barely additive.

Mast cells have been shown previously to survival in the presence of other cell types including fibroblasts (167), HUVEC (168) and epithelial cells (169). Human gut fibroblasts maintain gut mast cell survival, but do not induce their proliferation. This did not require cell-cell contact and was not mediated via SCF. HUVEC promoted mast cell survival and also induced some proliferation. This however was found to be dependent on adhesion, which was through membrane-bound SCF on the mast cells and VCAM-1 expressed on HUVEC; which is not involved in the adhesion of mast cell to ASM. Human bronchial epithelial cells also maintained the survival of human cord blood-derived mast cells through a SCF-dependent mechanism, but no proliferation was observed in this model.

Mast cells not only proliferate in culture with ASM, but in previous studies mast cells found within the airway smooth muscle of asthmatic patients were degranulated and thus in an activated state (33). Therefore, the histamine content of the co-culture supernatants was investigated to further support or disprove this finding.

Chapter 5:

Human lung mast cells co-cultured with airway smooth muscle become activated and release histamine

5.1 Introduction

Human lung mast cell present within the airway smooth muscle bundles of patients with asthma demonstrate morphological features of activation with loss of granule contents (33); so-called piecemeal degranulation. Therefore, following migration of mast cells into the smooth muscle or proliferation of cells already resident, the mast cells become activated through either IgE-dependent or independent mechanisms causing subsequent loss of granule content. Release of mediators such as histamine and leukotrienes into the local environment could have detrimental effects and could even be contributing to the pathogenesis of the asthmatic phenotype. The mediators could be increasing migration, survival and proliferation of the mast cells, and hypertrophy, proliferation and contraction of the airway smooth muscle cells. So further understanding the cause of the mast cell activation could lead to uncovering a pathway to intercede, preventing granule loss.

Therefore, to address the fourth and last aim of the first hypothesis: examining the level of activation of co-cultured mast cells, collected co-culture supernatants and cells were analysed for their histamine and LTC_4 content. In addition, the co-cultured mast cells were assessed for their ability to become activated through IgE/anti-IgE mechanism and release further mediators. These were compared with fresh HLMC at day 0 and parallel HLMC cultured in SCF, IL-6 and serum.

5.2 HLMC constitutively release histamine when co-cultured with ASM

HLMC co-cultured with ASM released significantly more histamine into the culture supernatant over 10 days (1198±142ng/10^6 cells present at day 10) when compared to HLMC cultured alone with SCF/IL-6/FCS (88±67ng/10^6 cells present at day 10, P=0.0384; Fig 5.1). This suggests that HLMC in co-culture with airway smooth muscle are activated.

In keeping with the increased histamine content in the co-culture supernatant, the histamine content of HLMC in the co-culture was approximately half that of the control cells and the cells at baseline (1491±253 ng/10^6 cells in co-culture, 7101±1214 ng/10^6 cells at baseline, 4029±398 ng/10^6 cells in SCF/IL-6/FCS, both $p<0.001$; Fig 5.2). Although the 10-day co-cultured HLMC contained less histamine than the day 0 controls, when they were activated with IgE/anti-IgE for 30 min, the total amount of histamine released was similar to that at day 0 (Fig 5.3). Thus HLMC are constitutively activated whilst in co-culture with airway smooth muscle, and maintain their responsiveness to stimulation through the high affinity IgE receptor.

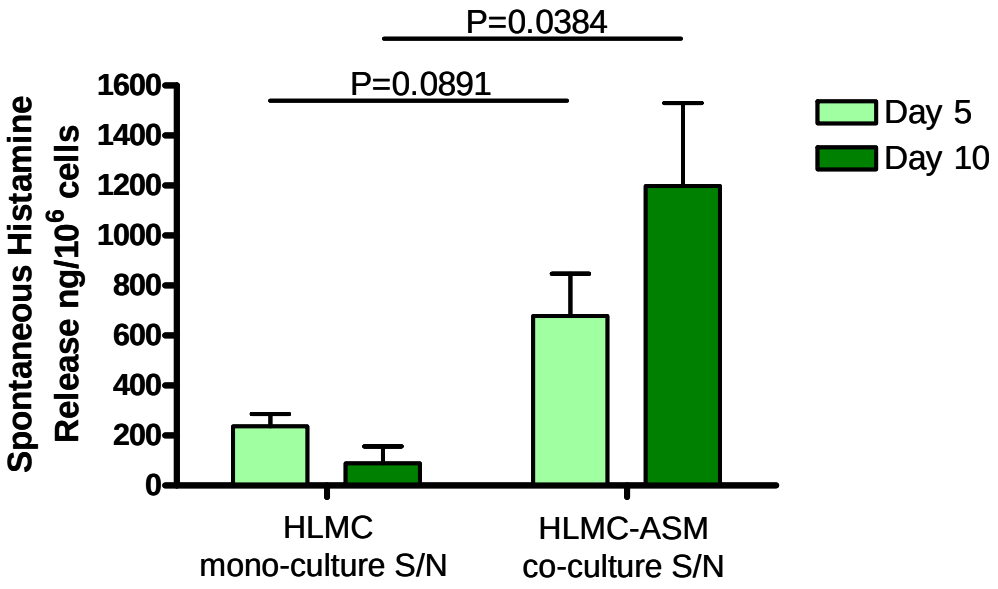


Figure 5.1 Constitutive histamine release from mono and duel mast cell cultures

HLMC cultured alone with SCF, IL-6 and serum or with ASM without the addition of supplements. Histamine concentrations were measured in the culture supernatant and corrected for the number of cells present at 5 and 10 days of culture. Analysed by paired t test. Data represented a mean±SEM.

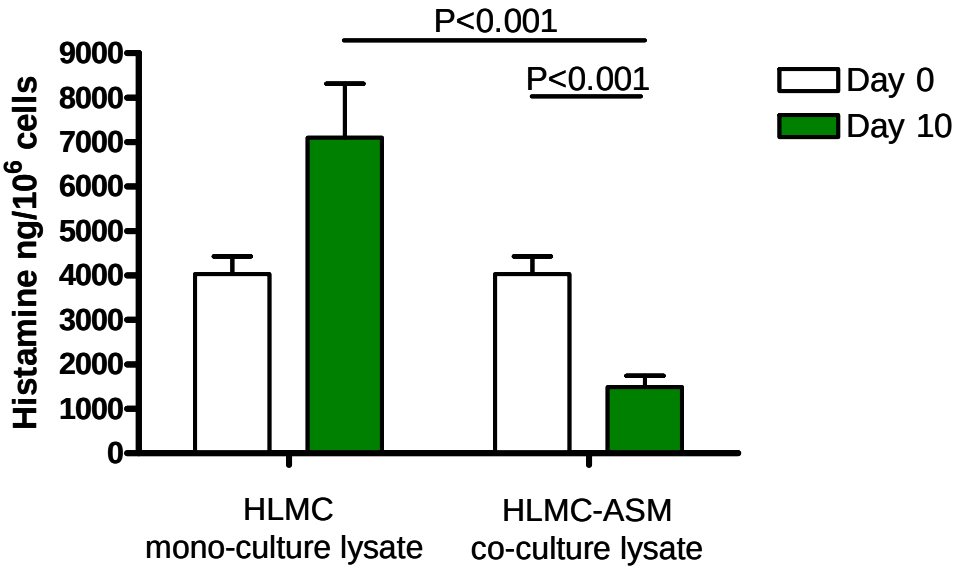


Figure 5.2 Histamine content of mono and duel cultured mast cells

Mast cells cultured alone with SCF, IL-6 and serum or with ASM without the addition of supplements were measured for their histamine content at 0 and 10 days of culture. Analysed by paired t test. Data represented a mean±SEM.

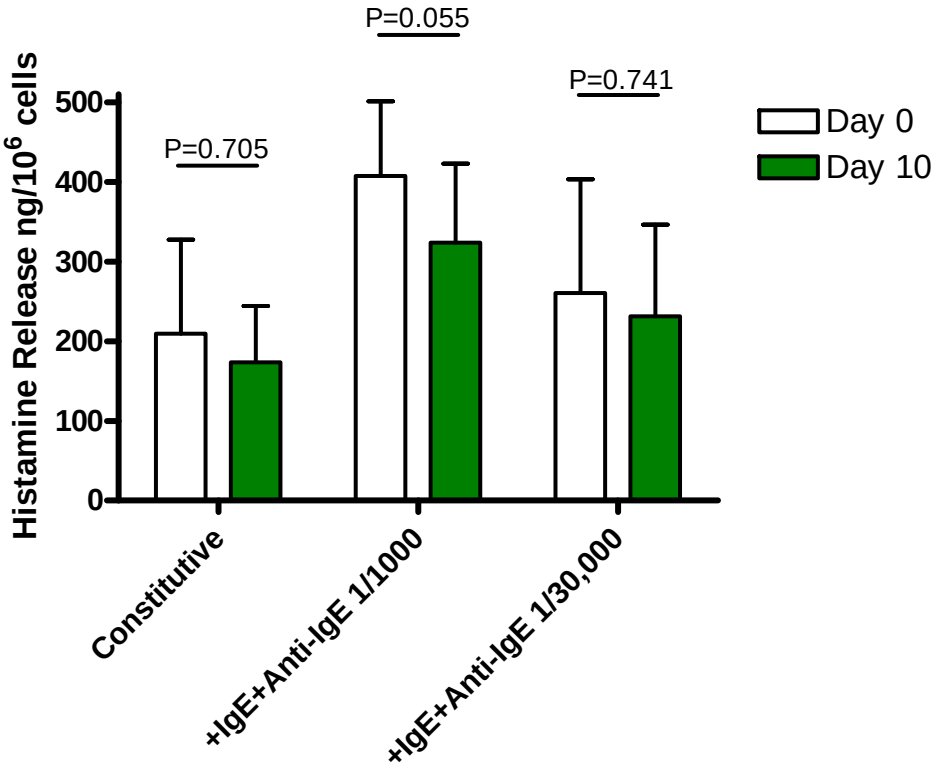


Figure 5.3 Histamine release of stimulated mast cells before or after culture with ASM

Total histamine release corrected for cell number was measured in mast cells at day 0 or after 10 days of culture with ASM in response to IgE-dependent activation for 30 min. Analysed by paired t test. Data represented a mean±SEM.

However, when the percentage release is calculated (Fig 5.4), whereby the amount available within the cell is taken into account, a significantly higher percentage of histamine is released following stimulation at day 10 compared with that at day 0 across all conditions (Fig 5.4; P=0.002). However, no significant difference was found in the histamine release between each stimulation condition.

The release of LTC_4 from the cultures was measured in difference culture and stimulation conditions, and also compared to the respective release of histamine (Fig 5.5). Although LTC_4 has a role in asthma and is involved in AHR (68), its relationship firstly with either spontaneous release in co-cultured mast cells compared with mono-cultured mast cells, and release following activation with IgE/anti-IgE did not have any significance. No significance relationship was found either when the release of LTC_4 was compared with the histamine release at the respective cultures and stimulation conditions.

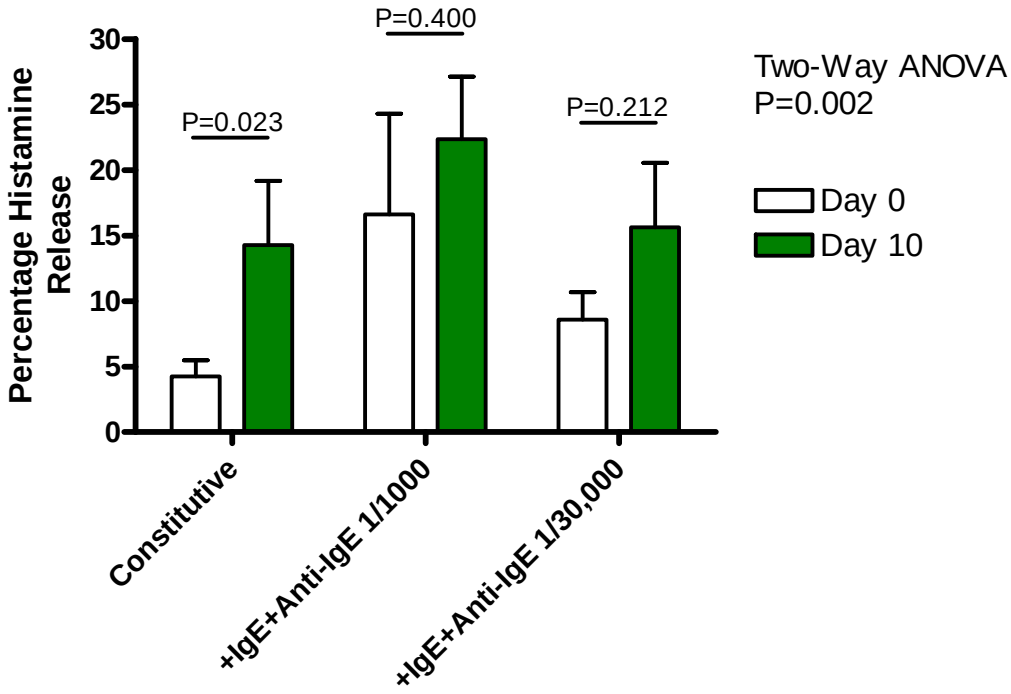


Figure 5.4 Percentage histamine release of mast cell before or after culture with ASM

Percentage histamine release corrected for cell number and histamine content was measured in mast cells at day 0 of after 10 days of culture with ASM in response to IgE-dependent activation for 30 min. Analysed by paired t test. Data represented a mean±SEM.

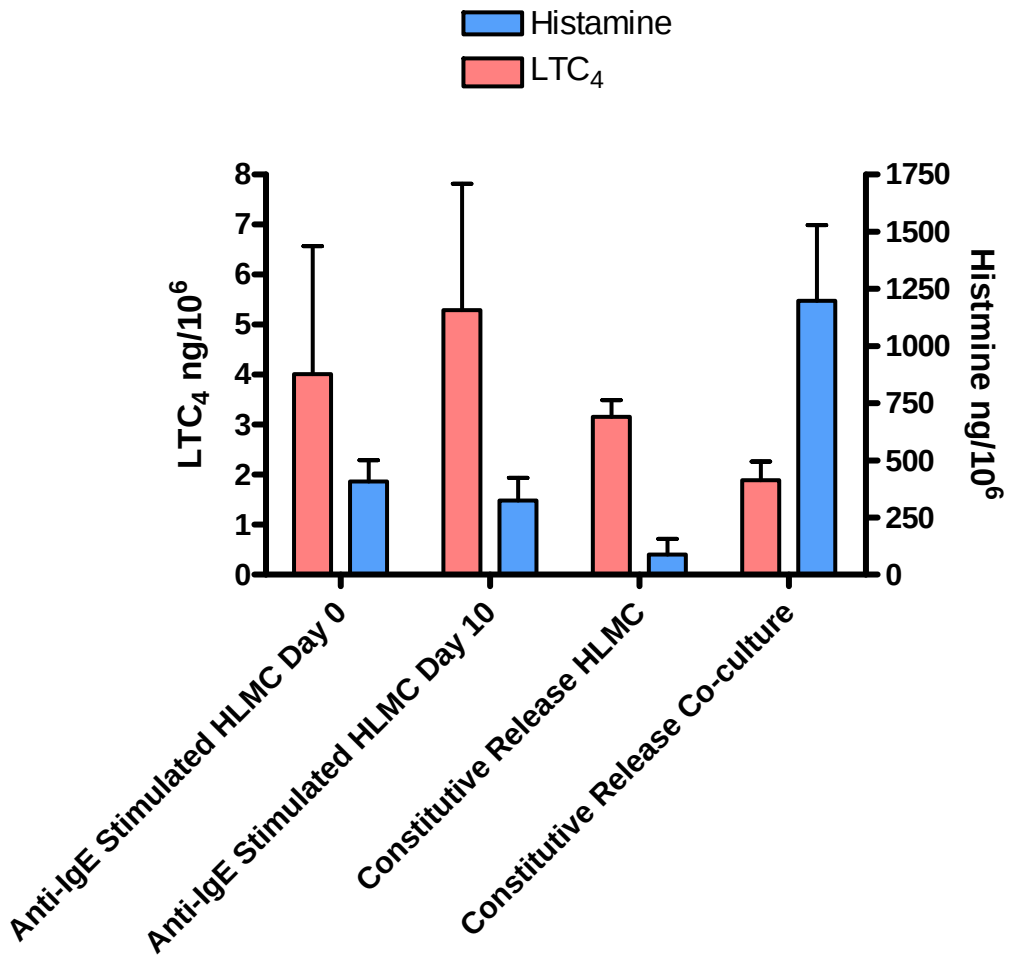


Figure 5.5 Comparison of the amounts of histamine and LTC_4 in cultures following stimulation or constitutive release cultures

Histamine and LTC_4 release corrected for cell number in response to IgE-dependent activation for 30 min and spontaneous release was compared in cells after 10 days in co- or mono-culture to observe any similarities. Data represented a mean±SEM.

5.3 Summary

Human lung mast cells in co-culture with airway smooth muscle demonstrate increased constitutive histamine release and decreased histamine content. Therefore, not only do mast cells proliferate in co-culture with smooth muscle, but they are also activated. The basal histamine release during the co-culture period was increased, which was paralleled by a decrease in lung mast cell histamine content. This is particularly interesting because mast cells in the ASM bundle from an asthmatic patient are activated as shown by the presence of piecemeal degranulation. However, there was a lack a relationship of LTC_4 release from mast cells, indicating that where this cytokine is concerned, mast cells may not be the important source in asthma.

There is however a discrepancy in the amount of constitutive histamine released by the mast cells following 10 days of culture with ASM and the remaining content of histamine within these cells; being reduced compared with the amount of histamine that these mast cells contain at day 0. This could be explained by the histamine becoming metabolised. In vivo, histamine is readily metabolised either through oxidation by diamine oxidase or semicarbazide-sensitive amine oxidase, or methylated by histaminemethlytransferase (180,181). Whilst the production of these enzymes have not been thought to occur in the lung, they have not been measured and are thus have a possible role in explaining the loss of measurable histamine in the co-culture supernatant over time.

The LTC_4 assay is not specific, it also cross-reacts with leukotrienes D_4, E_4, D_5, C_5, E_5 and N-acetyl leukotriene E_4. Therefore, the results found with this assay should be classed as a general release of leukotrienes from the mast cells, rather than specific

release of LTC_4. In addition, in vivo following release of LTC_4 from activated mast cells is readily metabolised into LTD_4 and LTE_4 within minutes through the action of γ-glutamyltranspeptidase and dipeptidase (182). However, metabolism of LTC_4 within the *in vitro* setting along with expression, and release of enzymes from primary cultured ASM and mast cells has not been explored. Therefore, the lack of relationship in the LTC_4 release and culture time, condition and stimulation, may be a result of the lack of specificity of the assay and metabolism of LTC_4.

These results have shown that culturing mast cells with airway smooth muscle does result in their activation though a IgE-independent pathway as shown by their increased constitutive histamine release and decreased histamine content, which requires further investigation to fully elucidate the mechanisms. In addition, these activated cells may be further activated by IgE/anti-IgE pathway to release additional histamine at comparable levels to non-pre-activated cells.

Chapter 6:

Airway smooth muscle exhibits altered calcium handling

6.1 Introduction

Previous studies looking at the contractile ability of airway smooth muscle and the differences observed between asthmatic and non-asthmatic smooth muscle have been extensive. The main finding however was airway smooth muscle from an asthmatic subject was more contractile than that from a non-asthmatic subject. This result is in keeping to what is seen in vivo is response to agonists such as methacholine and indeed the response of the airways during an asthma attack. But in what way is the contractility of these two cell phenotypes different?

Cell contraction may be measured directly using real time videoing or collagen gel assays, or indirectly by measuring the intracellular calcium. The latter can be achieved on a single cell level using fluorescent microscopy or on a population of cells using a fluorescent imaging plate reader (FLIPR). Both these methods were employed to investigate the first aim of the second hypothesis: determining whether there are any differences in the intracellular calcium handling of airway smooth muscle from asthmatic and non-asthmatic subjects.

Firstly, intracellular calcium levels were monitored at baseline and during stimulation with agonist to determine whether the handling of intracellular calcium did differ between the cell phenotypes giving rise to the altered contractility of these cells reported previously. The smooth muscle cells were also exposed to ionomycin to completely deplete the intracellular calcium stores to quantify the calcium load within different cell phenotypes. These experiments were performed using Fura-2, which is a quantifiable fluorescent calcium indicator.

The intracellular calcium response in the airway smooth muscle cells was also assessed in a cell population rather than in individual cells. This was performed using Fluo-4, a non-quantifiable fluorescent calcium indicator using the FLIPR. This system was high-throughput, allowing large numbers of different conditions to be measured simultaneously. The ASM cells in this system were also pre-treated with IL-13. Previous work has found that IL-13 increases these responsiveness of airway smooth muscle cells to stimulus (151). Sputum analysis of asthmatic patients has found increased levels of IL-13 when compared with non-asthmatic subjects (16) and ASM-localised mast cells express IL-13 (148). In addition, anti-IL-13 therapy has improved asthma symptomology in studies with mouse models (183,184). Therefore, pre-treatment with IL-13 was added into this stimulation model to investigate whether it indeed had a role in increasing the responsiveness of ASM cells to stimulation through the measurement of the cells intracellular calcium.

6.2 Baseline calcium levels and baseline calcium oscillations

Intracellular calcium levels of airway smooth muscle were monitored to determine whether there were any differences in the handling of calcium homeostasis that could be indirectly related to the contractile ability of the cell. Cells were loaded with the ratiometric calcium dye Fura-2 and perfused with media (with or without the addition of 2mM calcium) at 37^{o}C to keep them as stable as possible. Cells were perfused for 5 minutes before recording began to allow the cells to stabilize and (in the case of calcium-free media) to allow the removal of free calcium.

The resting baseline calcium levels were measured in single ASM cells from both asthmatic and non-asthmatic donors in the presence and absence of extracellular calcium; firstly to observe any difference between the two cell phenotypes and secondly to see whether this was affected by the presence of extracellular calcium.

Cells were monitored at baseline and their intracellular calcium measured. The data was firstly exported as a ratio value, but to give the value a more physiological relevance, this ratio data was then converted to millimoles of calcium using the Grynkiewicz equation with the Rmin and Rmax calculated previously using a standard calcium calibration kit (Molecular Probes). Average baseline calcium values observed in asthmatic and non-asthmatic donors in the presence of extracellular calcium were similar and did not show any significant difference in the overall mean as analysed by an unpaired t test (Fig 6.1; P=0.098). In the absence of extracellular calcium, the baseline calcium levels in the asthmatic cells were significantly reduced compared with that of the non-asthmatic donor cells as analysed by an unpaired t test (Fig 6.1; P=0.013).

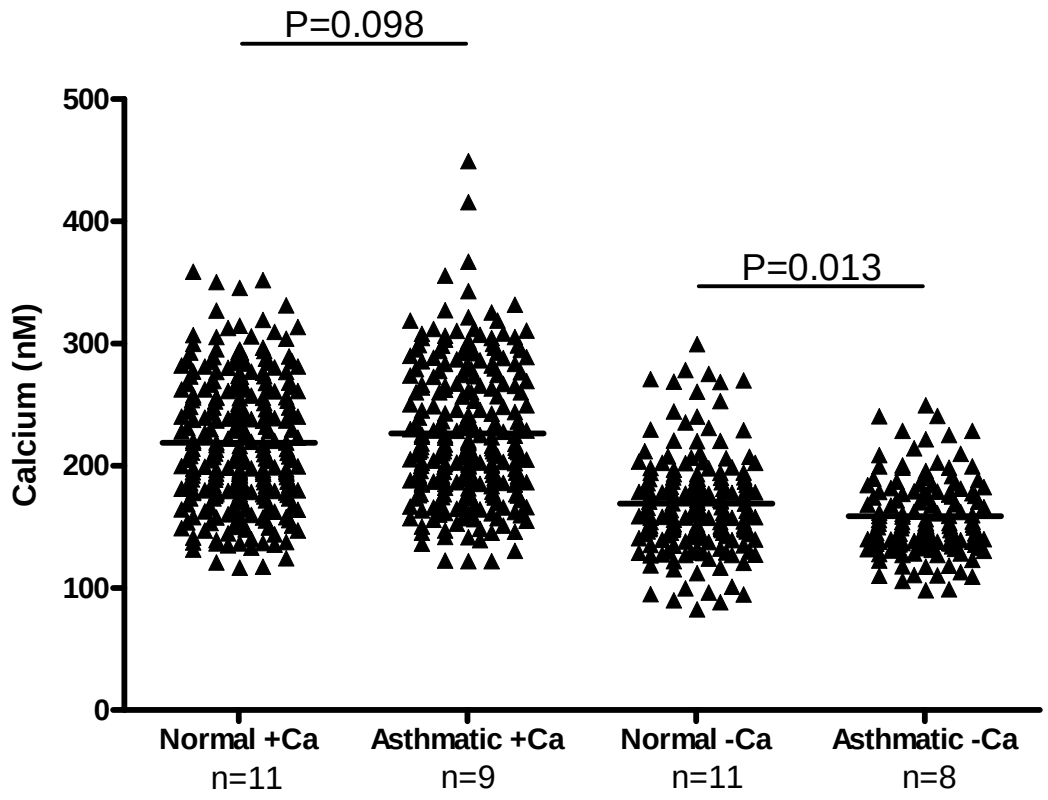


Figure 6.1 Average baseline concentration of ASM intracellular calcium per cell

The average baseline intracellular calcium concentration was measured for each airway smooth muscle cell from asthmatic and non-asthmatic patients in both the presence and absence of extracellular calcium. Analysis by unpaired t test. Bar represents mean of data points.

To observe whether the spread of data was in any way related to the individual airway smooth muscle donors, the data was then arranged into donor groups, whereby the average calcium value was calculated using all the individual cell calcium values for each donor. There was no significant difference in the baseline calcium levels of donors between the asthmatic and non-asthmatic phenotypes in the presence of extracellular calcium (Fig 6.2; P=0.182). However, in the absence of extracellular calcium, the significant difference that was observed previously when all data points were analysed, was lost upon segregating the data points into their respective donors as calculated using an unpaired t test (Fig 6.2; P=0.267). Therefore, these data indicates that at rest, there is no difference in the baseline calcium level between asthmatic and non-asthmatic cells regardless of the presence of extracellular calcium.

These latter statistical results, highlight that the significance previously observed when all calcium baseline data for all the donors was due to the shear number of data points rather than any specific difference between the two cell phenotypes. This is highlighted when taking into account the donor variability (Fig 6.3), whereby the spread of data for each donor in each condition can easily be observed to be extremely broad and overlaps considerably between donors.

When the average intracellular calcium baseline value was compared to its respective patient clinical characteristics (Table 6.1), no correlation was found between any of the groups. This indicates that the clinical presentation of the patients was not a result of the baseline intracellular calcium values, which as the baseline data were not significantly different between the phenotypes, was to be expected.

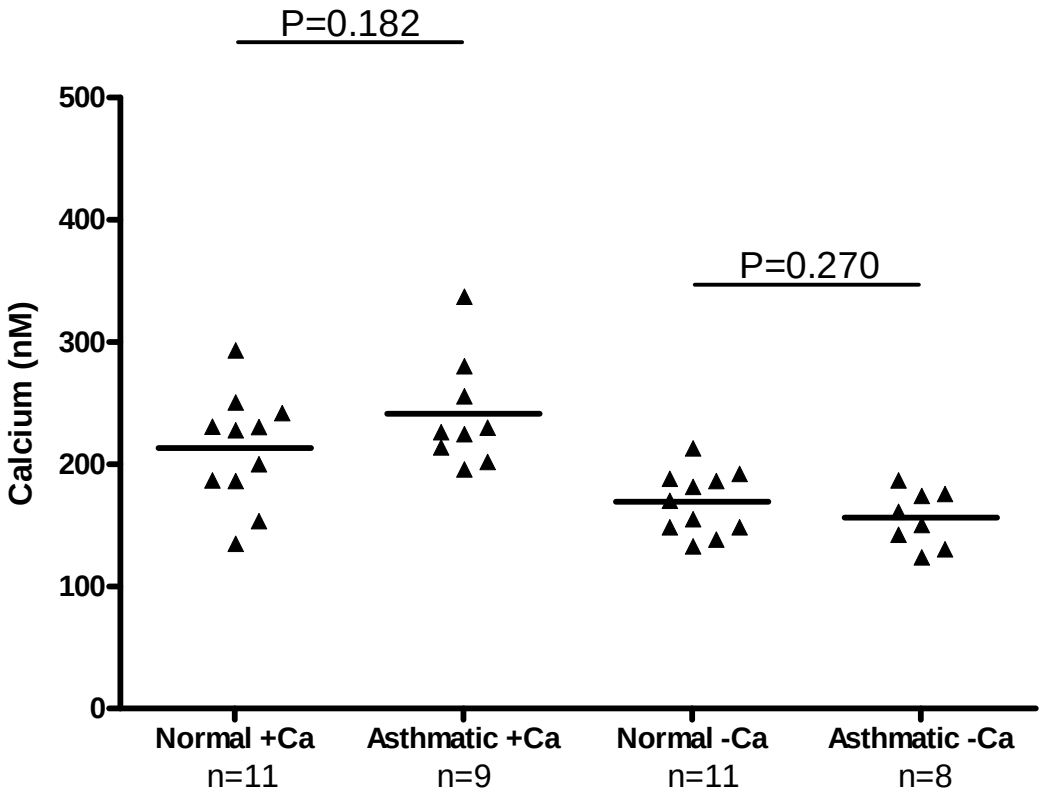


Figure 6.2 Average baseline concentration of ASM intracellular calcium per donor

The average baseline intracellular calcium concentration data for each airway smooth muscle donor, from asthmatic and non-asthmatic patients in both the presence and absence of extracellular calcium. Analysis by unpaired t test. Bar represents mean of data points.

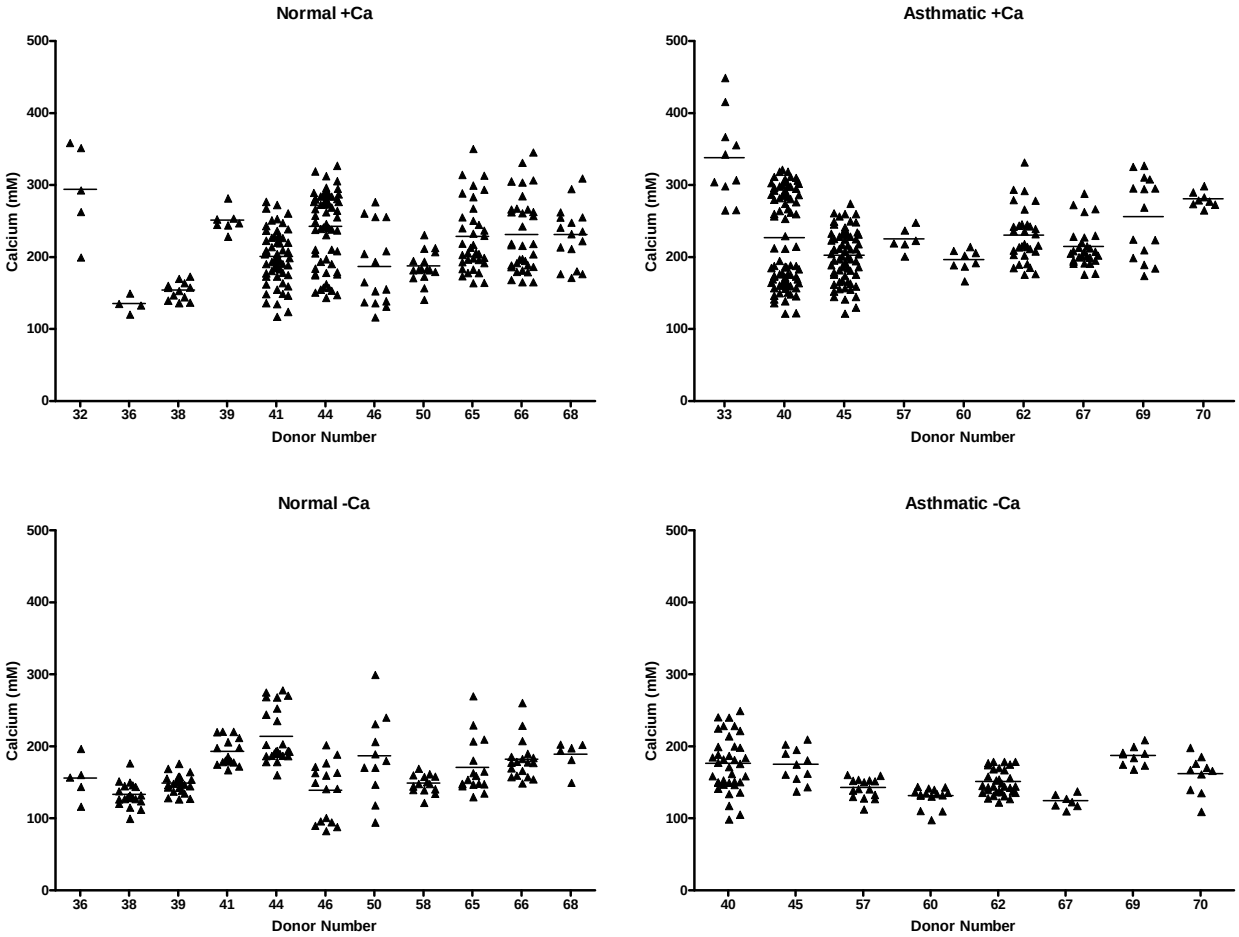


Figure 6.3 Average baseline concentration of ASM intracellular calcium per cell per donor

The average intracellular calcium concentration for each cell arranged into individual donors, to illustrate the broad variability of calcium measurements within each patient. Bar represents mean of data points.

However, a difference that was found to be dramatically different within the baseline was the presence of oscillations, (defined at a rise or fall in the baseline greater that 10% of the average calcium baseline value). An example of which is shown in Fig 6.4. Cell traces were run for a period of 10 minutes and the numbers of oscillations within that time were recorded for asthmatic and non-asthmatic cells in both the presence and absence of extracellular calcium (Fig 6.5). Asthmatic airway smooth muscle cells were found to be oscillating at a significantly greater degree than non-asthmatic cells only in the presence of extracellular calcium ($P<0.001$). Upon removal of extracellular calcium, these oscillations observed in both cell phenotypes were lost along with the significant increase of oscillations detected in the asthmatic smooth muscle ($P=0.118$). These data indicate firstly that the asthmatic smooth muscle cells are at a more activated state and secondly that extracellular calcium is pivotal to the incidence of these oscillations regardless of whether that are in asthmatic or non-asthmatic cells.

However, when the number of oscillations data was assigned to individual donors and the average was taken and plotted, the significant increase in oscillations between asthmatic and non-asthmatic smooth muscle measured from all data points was lost, (Fig 6.6, $P=0.129$). These data showed that out of the five asthmatic smooth muscle donors, only three were oscillating, an insufficient number to provide significance. These results do indicate however, that while non-asthmatic airway smooth muscle has a consistent low level of calcium oscillations, asthmatic smooth muscle either oscillates or not; a hypothesis that would require further investigation.

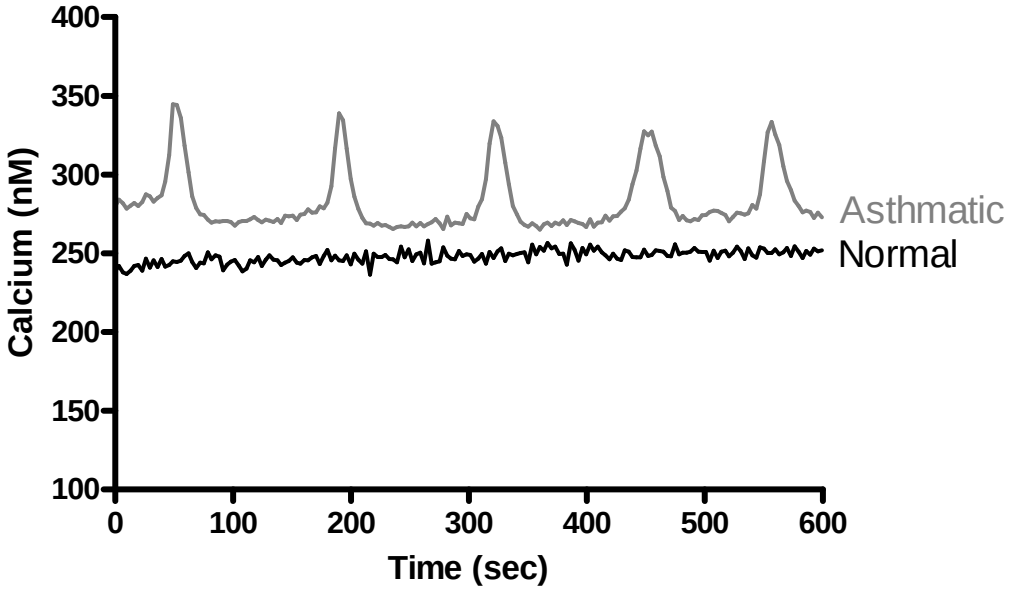


Figure 6.4 Example baseline calcium traces

Baseline calcium trace of an airway smooth muscle cell isolated from an asthmatic donor (grey line) and a non-asthmatic donor (black line) monitored over a 10 minute period perfused with calcium-containing media heated to 37^{o}C.

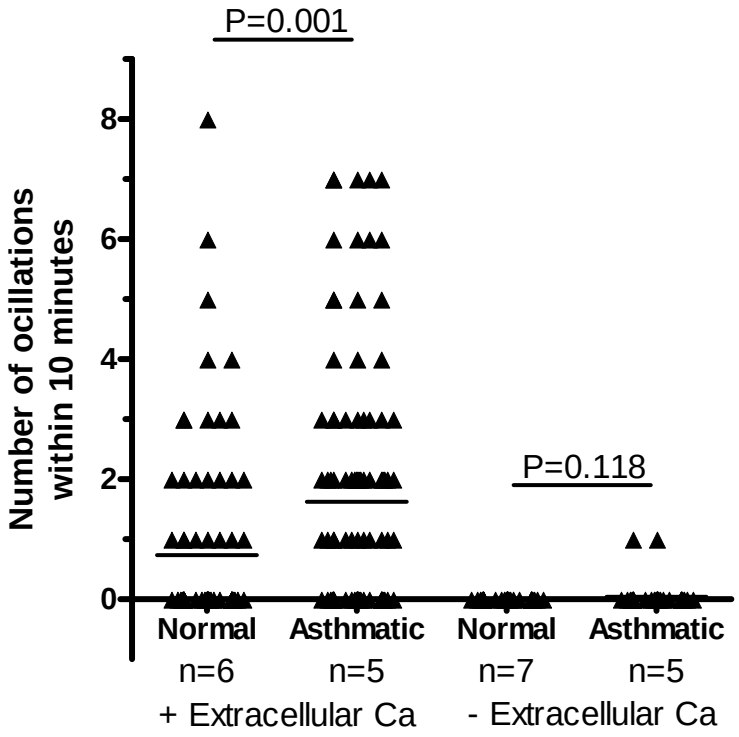


Figure 6.5 Number of oscillations within each ASM cell

Data points for each cell trace for the number of oscillations within the baseline calcium trace within a 10 minute period for asthmatic and non-asthmatic airways smooth cells in the presence and absence of extracellular calcium. Analysis by unpaired t test. Bar represents mean of data points.

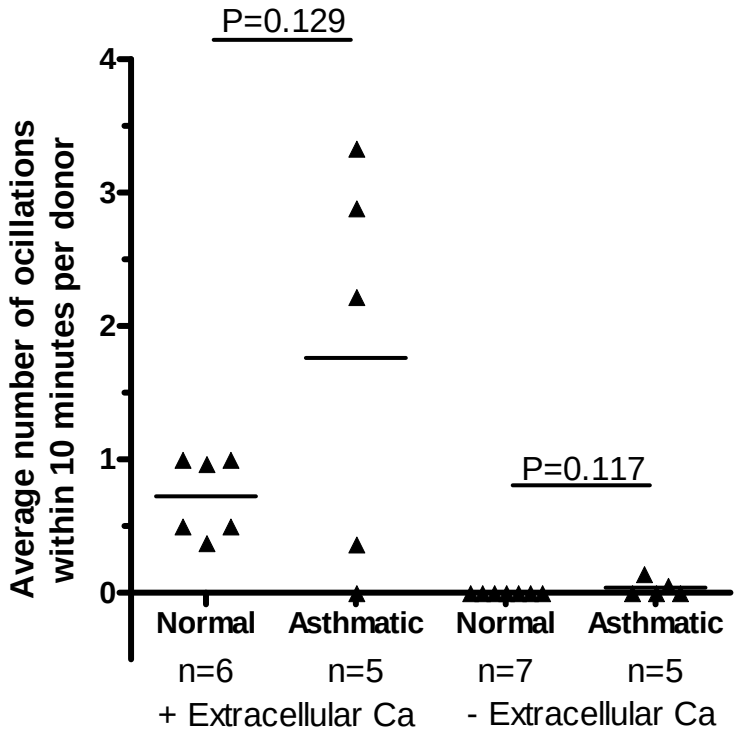


Figure 6.6 Number of oscillations within each ASM donor

Collated data points for the average number of oscillations within the baseline calcium trace during a 10 minute period per smooth muscle donor, for asthmatic and non-asthmatic airways smooth cells in the presence and absence of extracellular calcium. Analysis by unpaired t test. Bar represents mean of data points.

When the average number of oscillations per donor was compared to its patient clinical characteristics (Table 6.2), correlation was found when comparing the FEV_1/FVC% ratio only in the non-asthmatic cells (P=0.044). However, as there was a low level of oscillations in all these non-asthmatic donors that were all similar in value, a significant correlation when this is compared to FEV_1/FVC% is not an informative result; particularly when the asthmatic smooth muscle cells had no correlation in the comparison of these two data groups. A positive correlation was implied when the age of the patient was compared; indicating the increasing age increased the number of ASM oscillations. This is feasible as an increasing age correlates with reduced lung function and increased AHR (185). But due to donor number, this had not reached significance and therefore could not be confirmed.

6.3 Bradykinin and ionomycin stimulation

Responsiveness of airway smooth muscle cells to agonist was measured on an individual cellular basis by measuring the change in intracellular calcium levels as measured fluorometrically using the ratiometric dye Fura-2.

Firstly, response to a muscarinic agonist bradykinin was measured. Baseline calcium levels were monitored before perfusion of the cells with 1nM bradykinin, an example of the calcium trace obtained for both asthmatic and non-asthmatic airway smooth muscle is shown in Fig 6.7. To collate the data from individual cells of each donor, different methods were employed. The area under the curve value (Fig 6.8) was obtained from the start of the cell's response to the agonist, to it returning to baseline, with the respective average baseline calcium concentration removed. The peak response (Fig 6.9) was measured using the highest calcium concentration that the cells reached during their response. And the change in response (Fig 6.10) was calculated whereby the average baseline value was removed from the peak response value. These measurements were taken for each cell of every donor and collated in reference to their cell phenotype and presence or absence of extracellular calcium.

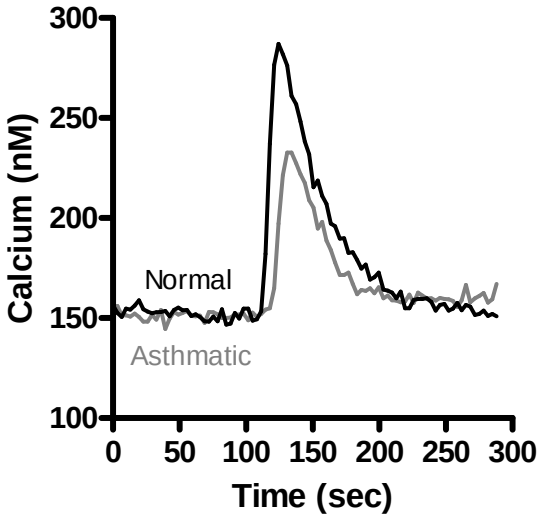


Figure 6.7 Example intracellular calcium trace following stimulation with bradykinin

Intracellular calcium trace for asthmatic (grey line) and non-asthmatic (black line) airway smooth muscle following stimulation with 1nM bradykinin in the presence of extracellular calcium and maintained at 37oC. Showing the initial activation of the cell followed by the recovery of the cell's intracellular calcium level.

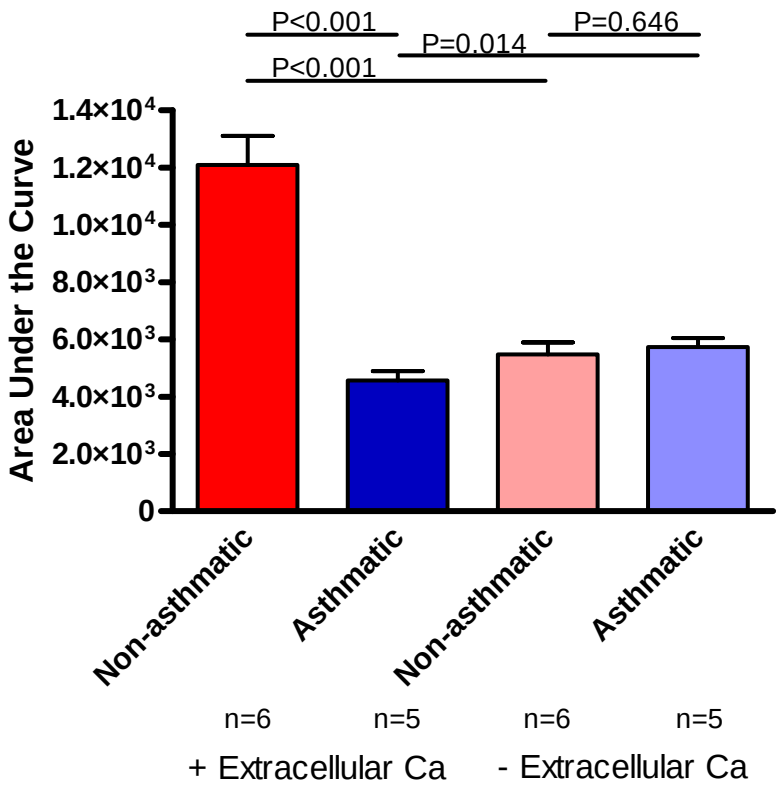


Figure 6.8 Area under the curve analysis following bradykinin stimulation

Collated area under the response curve values following stimulation with 1nM bradykinin of asthmatic (blue) and non-asthmatic (red) airway smooth muscle in the presence (deeper shades) and absence (lighter shades) of extracellular calcium. Analysis by unpaired t test. Data represented a mean±SEM.

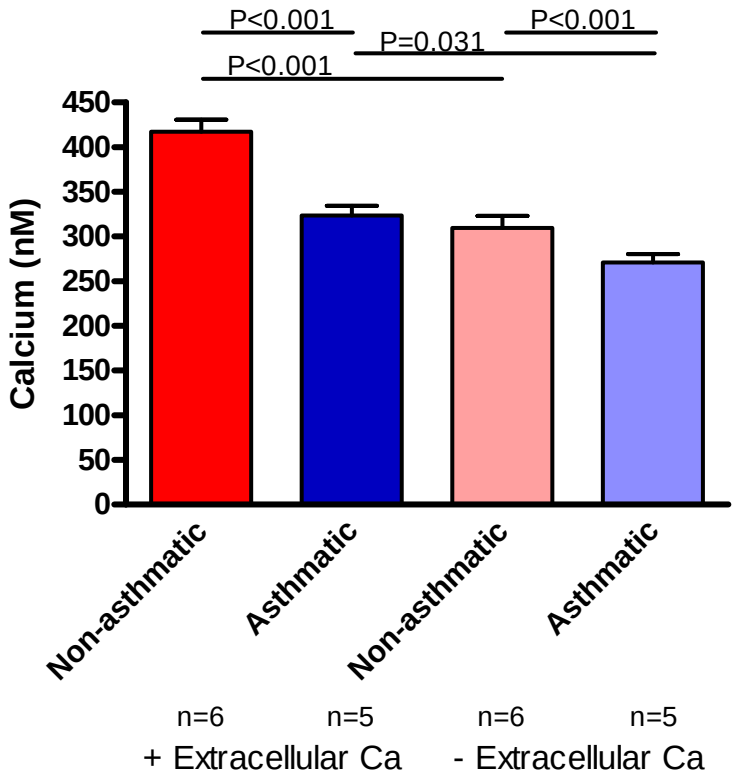


Figure 6.9 Peak calcium response analysis following bradykinin stimulation

Collated peak intracellular calcium response values following stimulation with 1nM bradykinin of asthmatic (blue) and non-asthmatic (red) airway smooth muscle in the presence (deeper shades) and absence (lighter shades) of extracellular calcium. Analysis by unpaired t test. Data represented a mean±SEM.

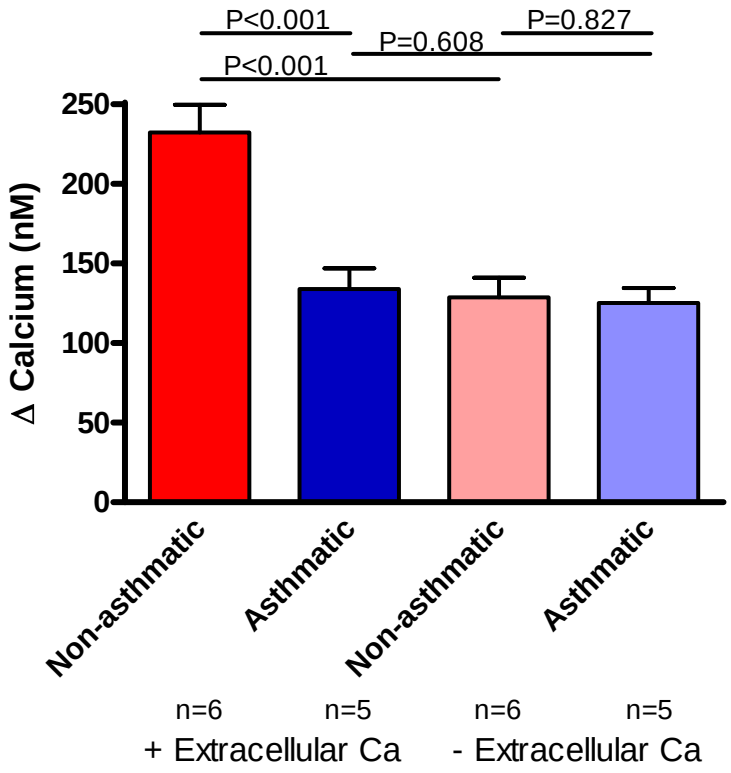


Figure 6.10 Change in intracellular calcium analysis following bradykinin stimulation

Collated change in intracellular calcium values following stimulation with 1nM bradykinin of asthmatic (blue) and non-asthmatic (red) airway smooth muscle in the presence (deeper shades) and absence (lighter shades) of extracellular calcium. Whereby the baseline calcium level is removed from the respective peak calcium response. Analysis by unpaired t test. Data represented a mean±SEM.

Asthmatic cells produced a significantly reduced response to stimulation with bradykinin compared with non-asthmatic cells when analysing their area under the curve, peak response and change in response (Figs 6.8, 6.9 and 6.10; all $P<0.001$). This significant difference was lost when the extracellular calcium was removed from the culture in all analysis methods apart from the peak response (Fig 6.9; $P<0.001$). The bradykinin response observed in non-asthmatic cells was reduced following removal of extracellular calcium, (all $P<0.001$). However, in asthmatic cells, removal of the extracellular calcium gave a variety of results. The absence of calcium resulted in an increase in the responsiveness of the cells when analysing area under the curve (Fig 6.8; $P=0.014$), a decrease when measuring the peak response (Fig 6.9; $P=0.030$) and no significant difference when the change in intracellular calcium was calculated (Fig 6.10; $P=0.608$). Therefore, it appears that in the case of asthmatic smooth muscle cells, the presence or absence of extracellular calcium has no effect of the intracellular responsiveness of the cells to agonist.

Individual data points were arranged into donor groups, giving an average value for area under the curve (Fig 6.11), peak response (Fig 6.12) and change in response (Fig 6.13) following stimulations with bradykinin per donor. When these conditions were analysed, the reduced significant response to agonist that was previously observed when cells were compared, was now lost. This again highlights the broad donor variability, but increasing donor numbers may restore this significant difference.

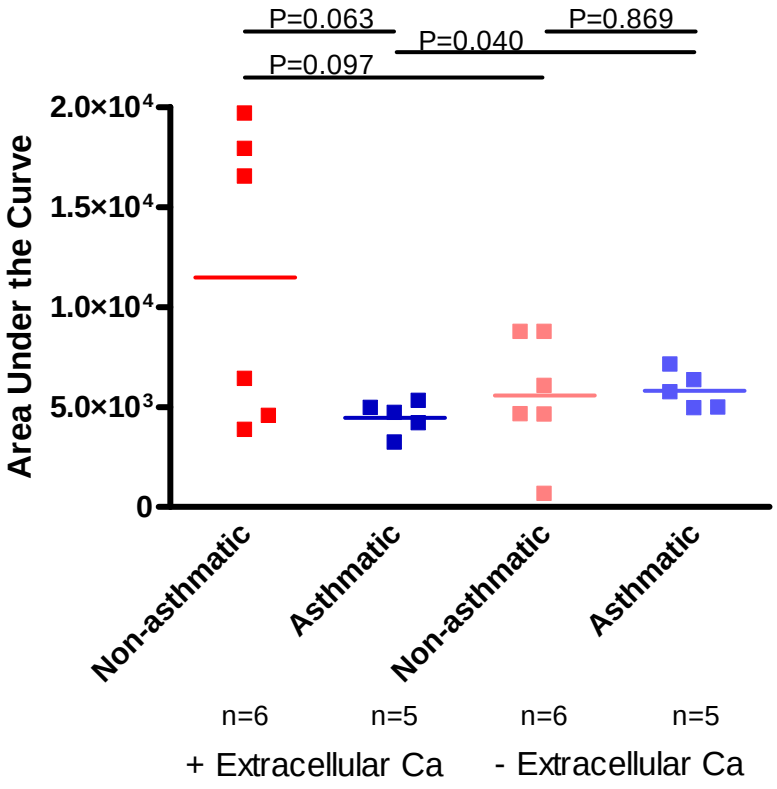


Figure 6.11 Area under the curve analysis following bradykinin stimulation per donor

Collated average area under the curve values per smooth muscle donor following stimulation with 1nM bradykinin of asthmatic (blue) and non-asthmatic (red) airway smooth muscle in the presence (deeper shades) and absence (lighter shades) of extracellular calcium. Analysis by unpaired t test. Bar represents mean of data points.

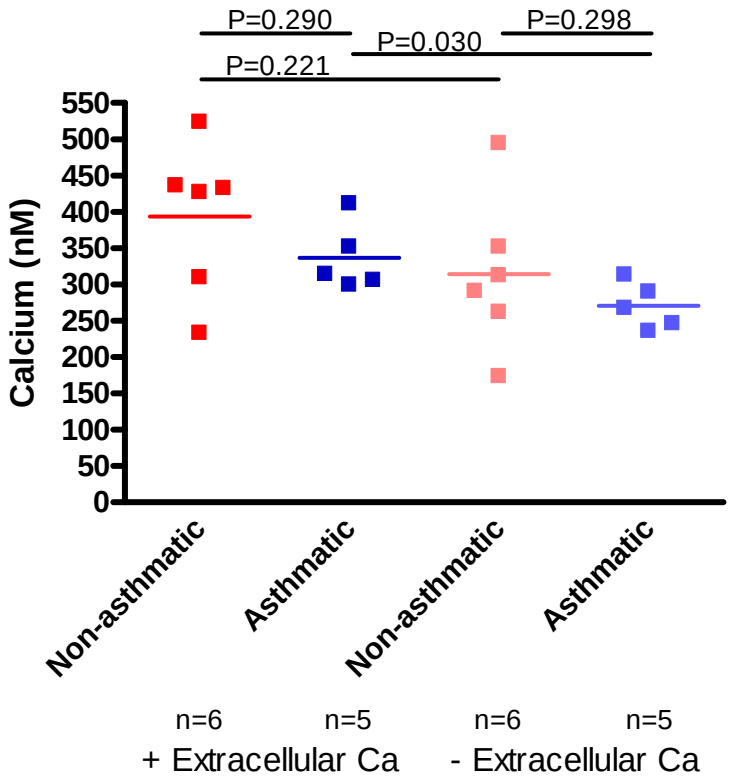


Figure 6.12 Peak calcium response analysis following bradykinin stimulation per donor

Collated average peak intracellular calcium response values per smooth muscle donor following stimulation with 1nM bradykinin of asthmatic (blue) and non-asthmatic (red) airway smooth muscle in the presence (deeper shades) and absence (lighter shades) of extracellular calcium. Analysis by unpaired t test. Bar represents mean of data points.

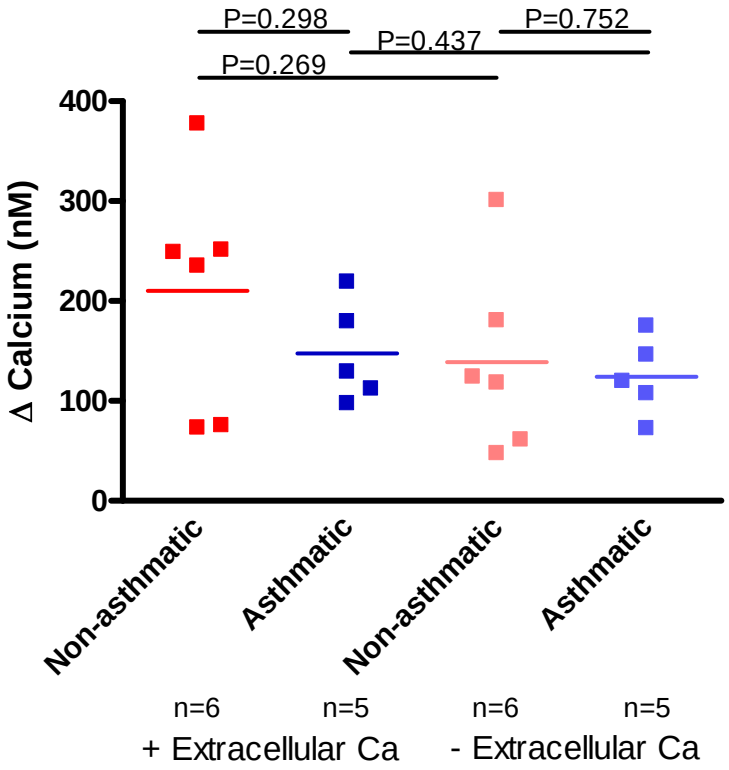


Figure 6.13 Change in intracellular calcium analysis following bradykinin stimulation per donor

Collated average change in intracellular calcium values per smooth muscle donor following stimulation with 1nM bradykinin of asthmatic (blue) and non-asthmatic (red) airway smooth muscle in the presence (deeper shades) and absence (lighter shades) of extracellular calcium. Whereby the baseline calcium level is removed from the respective peak calcium response. Analysis by unpaired t test. Bar represents mean of data points.

When the average area under the curve per donor was compared to its respective patient clinical characteristics (Table 6.3), minor correlations were found when comparing the FEV_1% and age in the asthmatic cells, and comparing FEV_1% in the non-asthmatic cells. However, this was not significant and requires additional donors to allow any definitive confirmations.

To assess the total calcium content of the cells, ionomycin was used to deplete all intracellular stores. Baseline calcium levels were monitored before addition of 1μM ionomycin to the cells, an example of the calcium trace obtained for both asthmatic and non-asthmatic airway smooth muscle is shown in Fig 6.14. The data was collated from individual cells donors from asthmatic and non-asthmatic subjects, in both the presence and absence of extracellular calcium. The area under the curve value (Fig 6.15) was obtained from the start of the cell's response to ionomycin, to it returning to baseline, with the respective average baseline calcium concentration removed. The peak response (Fig 6.16) was measured using the highest calcium concentration that the cells reached during their response. The change in response (Fig 6.17) was calculated whereby the average baseline value was removed from the peak response value. These measurements were taken for each cell of every donor and collated in reference to their cell phenotype and presence or absence of extracellular calcium.

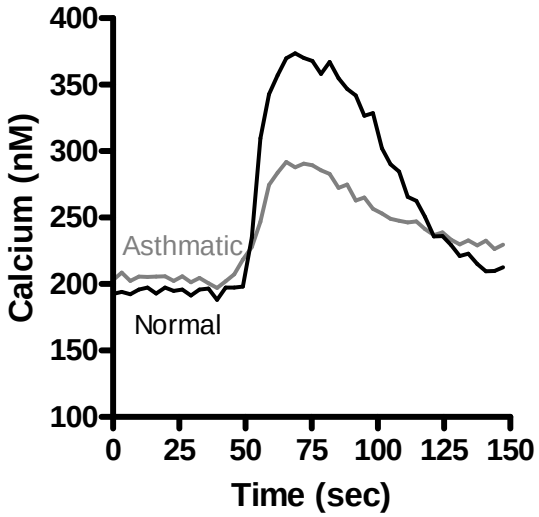


Figure 6.14 Example intracellular calcium trace following stimulation with ionomycin

Intracellular calcium trace for asthmatic (grey line) and non-asthmatic (black line) airway smooth muscle following stimulation with 1mM ionomycin in the presence of extracellular calcium. Shows the initial activation of the cell followed by the recovery of the cell's intracellular calcium level.

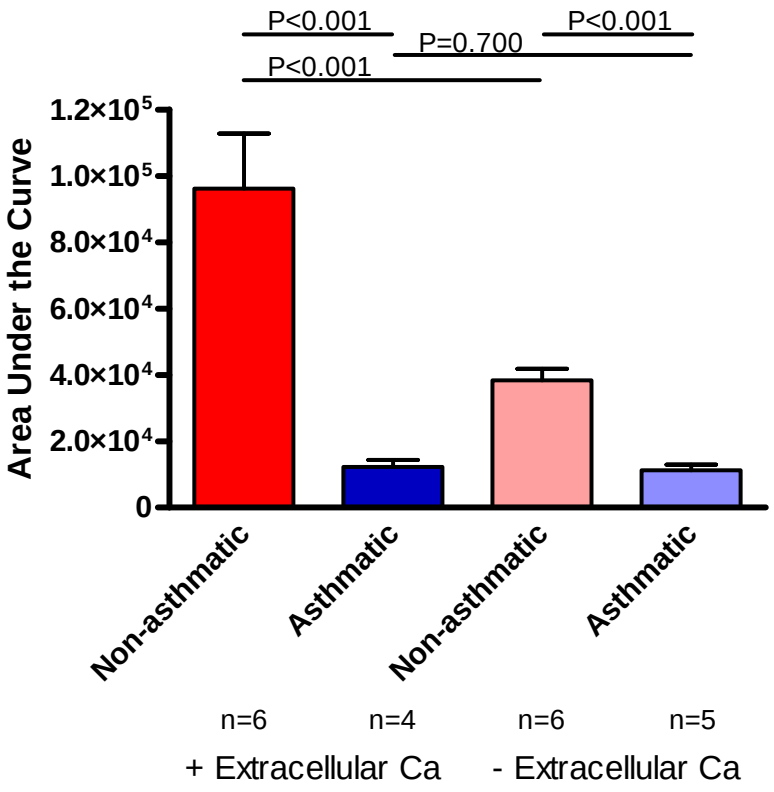


Figure 6.15 Area under the curve analysis following ionomycin stimulation

Collated area under the curve values following stimulation with 1mM ionomycin of asthmatic (blue) and non-asthmatic (red) airway smooth muscle in the presence (deeper shades) and absence (lighter shades) of extracellular calcium. Analysis by unpaired t test. Data represented a mean±SEM.

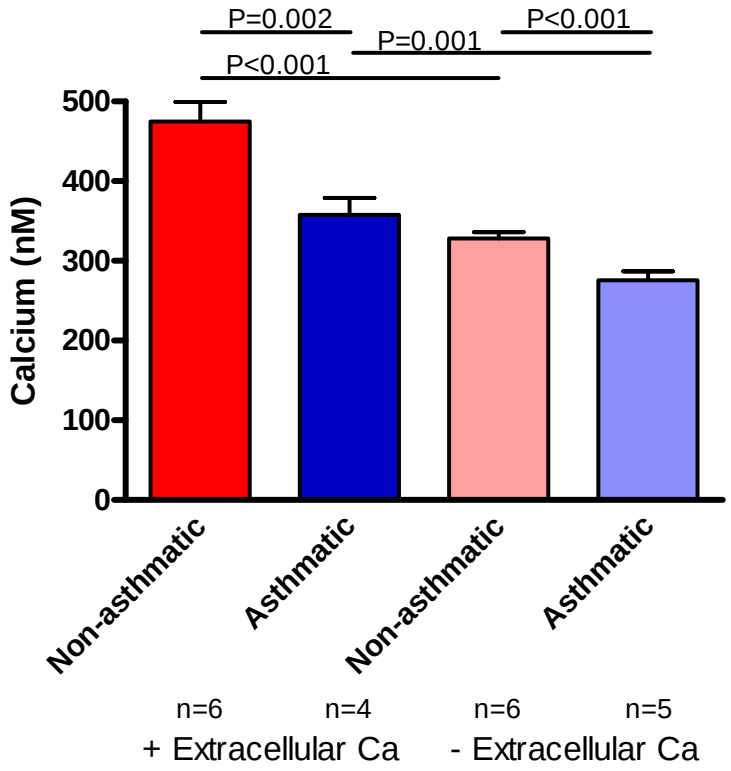


Figure 6.16 Peak calcium response analysis following ionomycin stimulation

Collated peak intracellular calcium response values following stimulation with 1mM ionomycin of asthmatic (blue) and non-asthmatic (red) airway smooth muscle in the presence (deeper shades) and absence (lighter shades) of extracellular calcium. Analysis by unpaired t test. Data represented a mean±SEM.

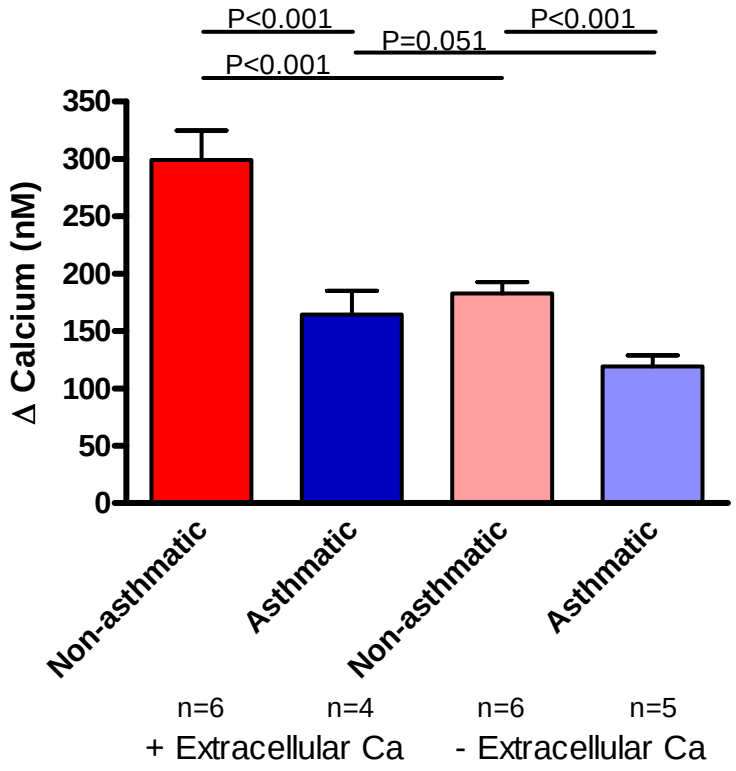


Figure 6.17 Change in intracellular calcium analysis following ionomycin stimulation

Collated change in intracellular calcium values following stimulation with 1mM ionomycin of asthmatic (blue) and non-asthmatic (red) airway smooth muscle in the presence (deeper shades) and absence (lighter shades) of extracellular calcium. Whereby the baseline calcium level is removed from the respective peak calcium response. Analysis by unpaired t test. Data represented a mean±SEM.

Asthmatic cells produced a significantly reduced response to stimulation with bradykinin compared with non-asthmatic cells when analysing their area under the curve, peak response and change in response (Figs 6.15, $P<0.001$; Fig 6.16, $P=0.002$ and Fig 6.17, $P<0.001$). This significant difference remained when the extracellular calcium was removed from the culture in all analysis methods (Fig 6.15, Fig 6.16 and Fig 6.17; all $P<0.001$). The bradykinin response observed in non-asthmatic cells was reduced following removal of extracellular calcium, (all $P<0.001$). However, in asthmatic cells, removal of the extracellular calcium was only significant when the peak response was assessed (Fig 6.16; $P=0.001$), questioning the importance of the presence of extracellular calcium for the responsiveness of the cells to store depletion to ionomycin.

Individual data points were arranged into donor groups, giving an average value for area under the curve (Fig 6.18), peak response (Fig 6.19) and change in response (Fig 6.20) following store depletion with ionomycin per donor. When these conditions were analysed, the reduced significant response to ionomycin that was previously observed when cells were compared, was now lost. This once again highlights the broad donor variability.

When the average area under the curve per donor was compared to its respective patient clinical characteristics (Table 6.4), minor correlations were found when comparing the FEV_1/FVC% ratio, FEV_1% and age in the asthmatic cells. However, this was not significant and requires additional donors to allow any convincing verification.

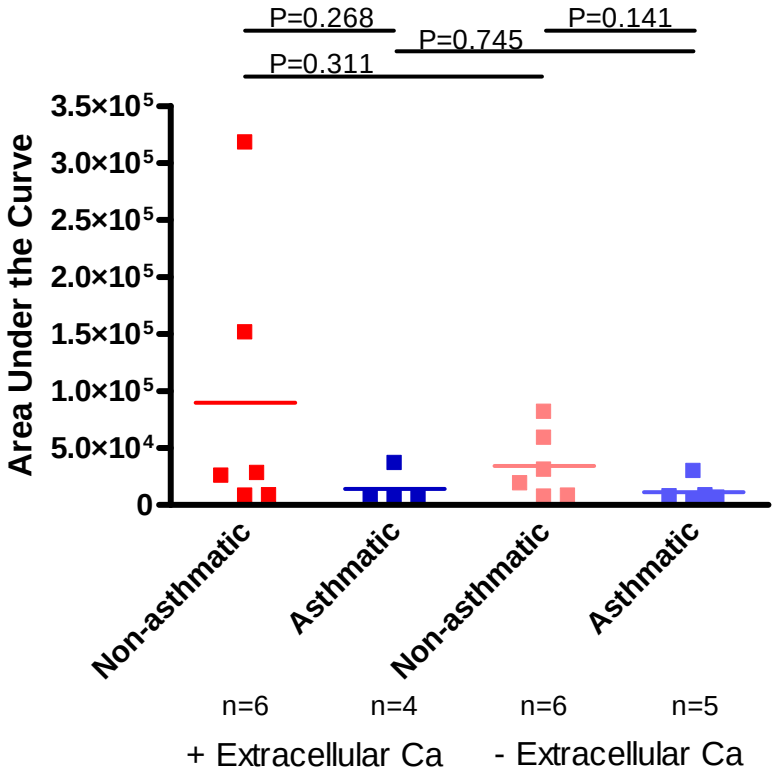


Figure 6.18 Area under the curve analysis following ionomycin stimulation per donor

Collated average area under the curve values per smooth muscle donor following stimulation with 1mM ionomycin of asthmatic (blue) and non-asthmatic (red) airway smooth muscle in the presence (deeper shades) and absence (lighter shades) of extracellular calcium. Analysis by unpaired t test. Bar represents mean of data points.

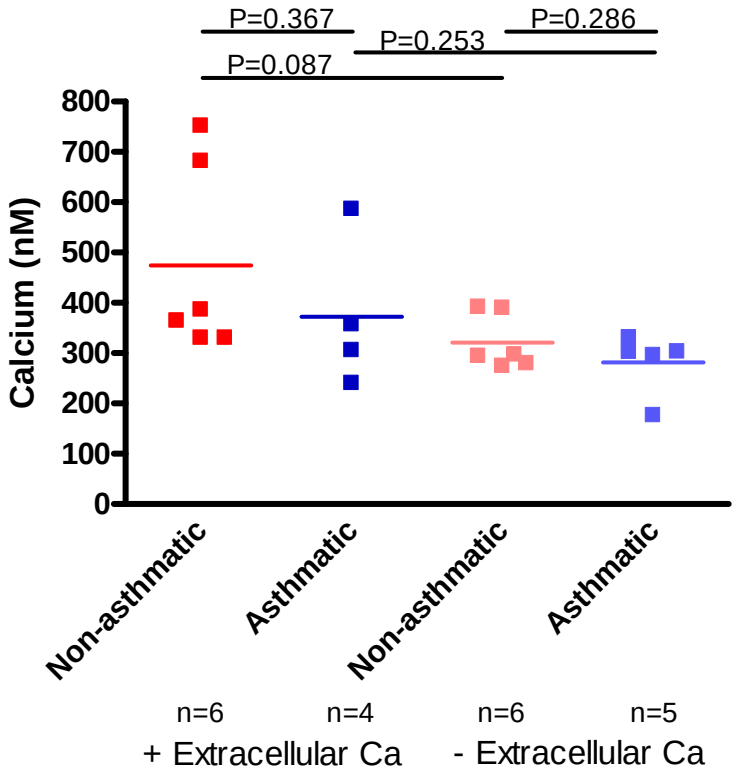


Figure 6.19 Peak calcium response analysis following ionomycin stimulation per donor

Collated average peak intracellular calcium response values per smooth muscle donor following stimulation with 1mM ionomycin of asthmatic (blue) and non-asthmatic (red) airway smooth muscle in the presence (deeper shades) and absence (lighter shades) of extracellular calcium. Analysis by unpaired t test. Bar represents mean of data points.

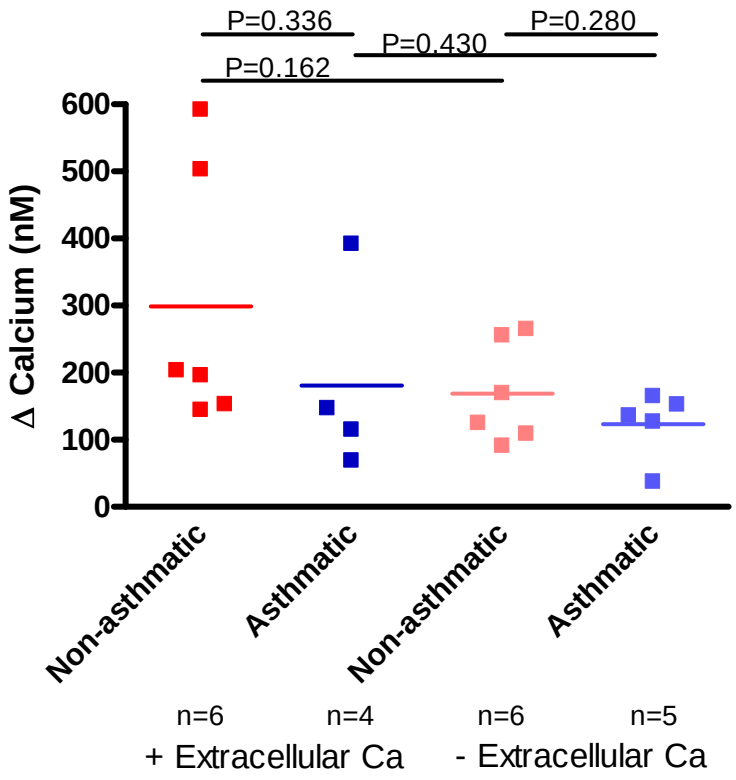


Figure 6.20 Change in intracellular calcium analysis following ionomycin stimulation per donor

Collated average change in intracellular calcium values per smooth muscle donor following stimulation with 1mM ionomycin of asthmatic (blue) and non-asthmatic (red) airway smooth muscle in the presence (deeper shades) and absence (lighter shades) of extracellular calcium. Whereby the baseline calcium level is removed from the respective peak calcium response. Analysis by unpaired t test. Bar represents mean of data points.

6.4 FLIPR stimulation with bradykinin and histamine

The fluorometric imaging plate reader (FLIPR) detects changes in intracellular calcium of cells loaded with a non-ratiometric dye (such as Fluo-4) following stimulation with agonist. The advantage of this system is that is monitors the calcium flux of a whole population of cells and many different culture conditions can be ran simultaneously. The disadvantage of this system is only changes in calcium levels are detected, not the baseline level and due to the use of a non-ratiometric dye, the calcium concentration can not be calculated, it is instead a measure of fluorescence.

Confluent airway smooth muscle from both asthmatic and non-asthmatic sources were pre-incubated with IL-13 (0, 5, and 10ng/ml) for 18 hours. Cells were loaded with Fluo-4 and dose responses to bradykinin (0.064pM – 1nM) and histamine (0.032µM – 500µM) were performed using the automated machine.

Pre-incubation with IL-13 did increase the responsiveness of the cells to agonist in a dose-dependent manner (as analysed using linear regression) in non-asthmatic cells but not asthmatic cells following stimulation with bradykinin (Fig 6.4.1; P=0.013 and P=0.915 respectively; Fig 6.25), and in both non-asthmatic and asthmatic cells following stimulation with histamine (Fig 6.4.2; P=0.016 and P=0.045 respectively; Fig 6.26). In keeping with the results from the single cell data, calcium response to both bradykinin and histamine was significantly reduced in asthmatic cells compared with non-asthmatic (Fig 6.25; P<0.001 and Fig 6.26; P<0.001 respectively using two-way ANOVA).

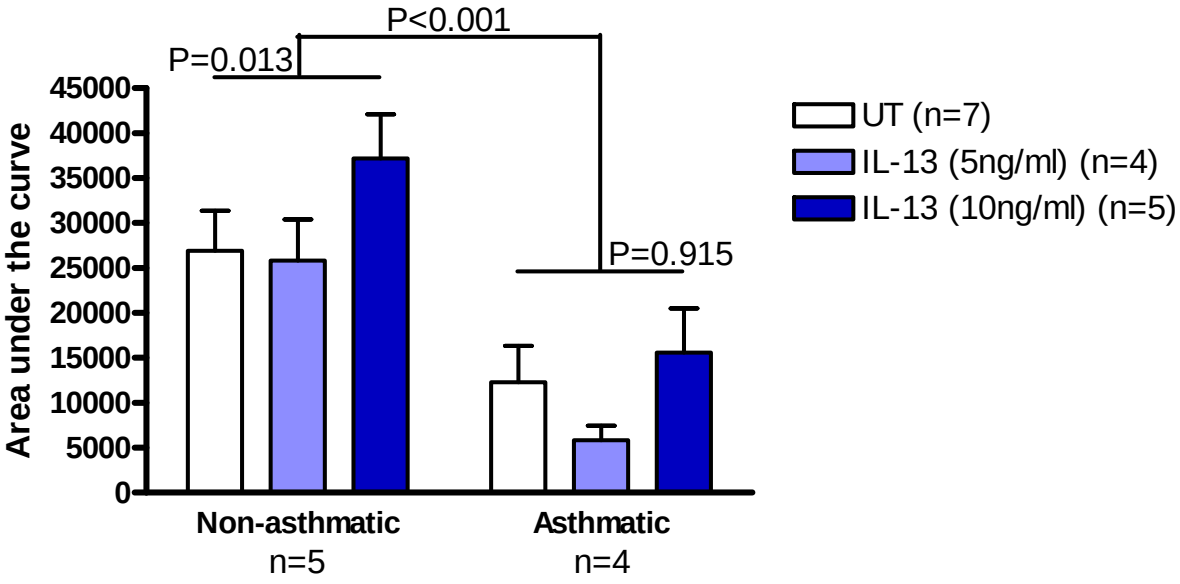


Figure 6.21 Bradykinin stimulation of cell populations pre-incubated with IL-13

Airway smooth muscle cells from non-asthmatic (left-hand side) and asthmatic (right-hand side) patients were pre-incubated with IL-13 at 0ng/ml (white bars), 5ng/ml (light blue bars) or 10ng/ml (dark blue bars) for 18 hours before a dose response to bradykinin (0.064pM – 1nM) was performed and the change in fluorescence of the loaded Fluo-4 intensity measured using a fluorometric imaging plate reader (FLIPR). The area under the curve values from the resulting dose response curves were collated. Analysis by unpaired t test and linear regression. Data represented a mean±SEM. UT – untreated.

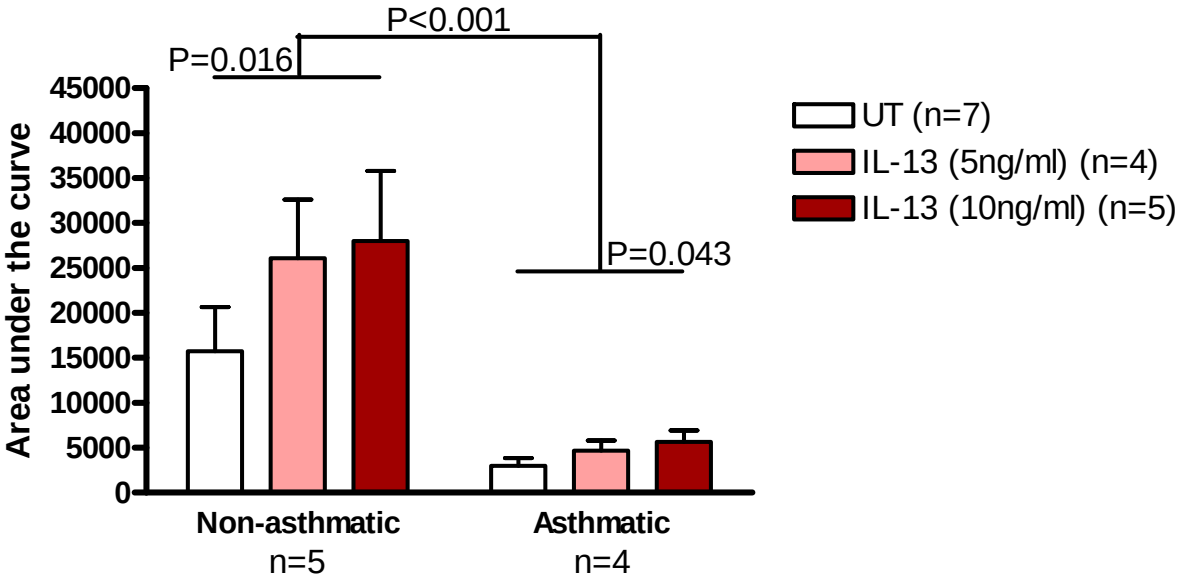


Figure 6.22 Histamine stimulation of cell populations pre-incubated with IL-13

Airway smooth muscle cells from non-asthmatic (left-hand side) and asthmatic (right-hand side) patients were pre-incubated with IL-13 at 0ng/ml (white bars), 5ng/ml (light blue bars) or 10ng/ml (dark blue bars) for 18 hours before a dose response to histamine (0.032µM – 500µM) was performed and the change in fluorescence of the loaded Fluo-4 intensity measured using a fluorometric imaging plate reader (FLIPR). The area under the curve values from the resulting dose response curve were collated. Analysis by unpaired t test and linear regression. Data represented a mean±SEM. UT – untreated.

6.5 Summary

Regardless from whether the ASM was isolated from a subject with or without asthma, the average resting baseline intracellular calcium did not differ. These measurements were found to vary enormously between donors and even between cells of the same donor, so any difference in the baseline intracellular calcium level that would lead to a difference in the normal functioning of the cell would be expected to be large. However, a significant difference that was observed between the two ASM phenotypes at rest was the presence of calcium oscillations. These were present to a significantly higher degree in ASM from an asthmatic patient than that from a non-asthmatic subject indicating that the "asthmatic" cells were at a constant level of activation. These spontaneous oscillations ceased upon removal of extracellular calcium indicated the importance of the presence of calcium and a possible abnormality in the extracellular membranous calcium channels that could lead to these oscillations. Previous work has been published on calcium oscillations in airway smooth muscle cells, but this has been following stimulation such as with acetylcholine on porcine ASM (186). But in our model, no initial stimulation took place, all oscillation observed were spontaneous. Intracellular calcium oscillations are thought to be a crucial control mechanism in many cell types. The temporal and spatial information encoded by these oscillations controls many processes, including secretion, gene expression, differentiation, muscular contraction, cell movement, and apoptosis (187). The exact mechanism for which these oscillation code for is yet to be elucidated and required further work.

In response to agonist, bradykinin or histamine, intracellular calcium flux in ASM from an asthmatic patient is significantly reduced regardless of whether the peak

response, change in response or the area under the curve (which takes duration and peak into account) is measured. In all circumstances, ASM from asthmatic patients produced significantly reduced calcium fluxes. This observation is reduced when extracellular calcium is removed from the culture and in the case of the area under the curve measurement; the significant difference is lost completely, indicating again the importance of extracellular calcium to this phenomenon. These results conflict with previous work where following stimulation with acetylcholine or histamine, the intracellular calcium response was considerably larger in ASM isolated from an asthmatic subject compared with that from a non-asthmatic subject (188).

Pre-treatment of airway smooth muscle cells with IL-13 did enhance the responsiveness of these cells to stimulation to bradykinin and histamine in a dose-dependent manner, which complements previous findings (151). This ability to enhance responsiveness was not limited to ASM from non-asthmatic individuals; responsiveness of smooth muscle from asthmatic patients was also increased following pre-treatment with IL-13, though this increase was less prominent. However, these data do indicate that targeting IL-13 for therapeutic targets in asthma could have beneficial effects. But the overall intracellular calcium response to agonist in this population setting; following stimulation with bradykinin or histamine, resulted in a reduced calcium response in ASM from asthmatic patients compared with that from non-asthmatic individuals, a result which confirms the calcium responses measured in the single cell setting.

However, previous reports of the contractile ability of smooth muscle cells from an asthmatic patient indicate that they are significantly more contractile in response to

agonist when compared to that from a non-asthmatic subject (189). In addition, previous studies have used intracellular calcium as a measure of cell contraction. But if this is to be believed, then it follows that the reduced intracellular calcium response seen following stimulation in ASM from an asthmatic patient results in a reduced contractile response of the cell, which does not concur with already published data. There is also published data to support that there is no difference in the contractile ability of smooth muscle cells from asthmatic and non-asthmatic individuals (163). But this also does not support the implied intracellular calcium data that this investigation has yielded. Therefore, direct measurement of cellular contraction and measurement of the intracellular calcium/contraction coupled proteins needs to be performed to address this connection.

Chapter 7:

Relationship between intracellular calcium and airway smooth muscle contraction

7.1 Introduction

Previous reports have suggested that ASM from an asthmatic patient is more contractile than that from a non-asthmatic subject (189). Intracellular calcium can be used as an indirect measure of cell contraction, but the data collected seems to indicate that the ASM from an asthmatic patient is less contractile than that from a non-asthmatic individual due to the significantly reduced intracellular calcium response measured following stimulation of the cell with agonist. Therefore, to directly measure the contractile ability of the cell, to address the second aim of the second hypothesis and to determine whether the intracellular calcium findings are a correct measure of the ability of the cell to contract, a collagen gel contraction assay was employed.

The collagen gel assay is an established physiological *in vitro* model used to assess the contraction of ASM in response to a stimulus; a system that has been used previously to assess the contractile response of ASM (162,163). Using this model, ASM from asthmatic patients have previously been found to be more contractile in response to stimulus compared with that from non-asthmatic subjects (163).

To address the third and final aim of the second hypothesis: to examine any differences in the proteins involved in the coupling of the calcium and contraction processes, Western blotting of lysed cell samples were performed. Previous studies have indicated that MLCK is increased in ASM isolated from asthmatic subjects (190), but data on these intracellular proteins as a whole is sparse and further investigation is required to understand the link between intracellular calcium response and cell contraction.

7.2 Contractile ability of asthmatic cells in response to bradykinin

Asthmatic (n=5) and non-asthmatic (n=4) ASM was loaded into collagen gel mixture at a concentration of 2.5×10^6/ml/well of a 24-well plate, and allowed to set. The gel was carefully detached from the well and allowed to float in 1ml media. A further 1ml media (+/- 2× agonist) was added to the gels and photos were taken at time points during the 24 hour culture. An example of which may be found in Fig 7.1.

The percentage of original gel size was significantly reduced in the gel containing ASM from an asthmatic patient when compared with that containing ASM from a non-asthmatic subject at 15 minutes following stimulation with 1nM bradykinin (P=0.035; Fig 7.2a). The remaining time points where the gel size was measured saw no further significant difference between the two ASM gel phenotypes. The overall difference in ASM contraction over the 5 hours was not found to be significantly different between ASM isolated from asthmatic and non-asthmatic subjects when the area under the curve was analysed (P=0.476; Fig 7.2b and c).

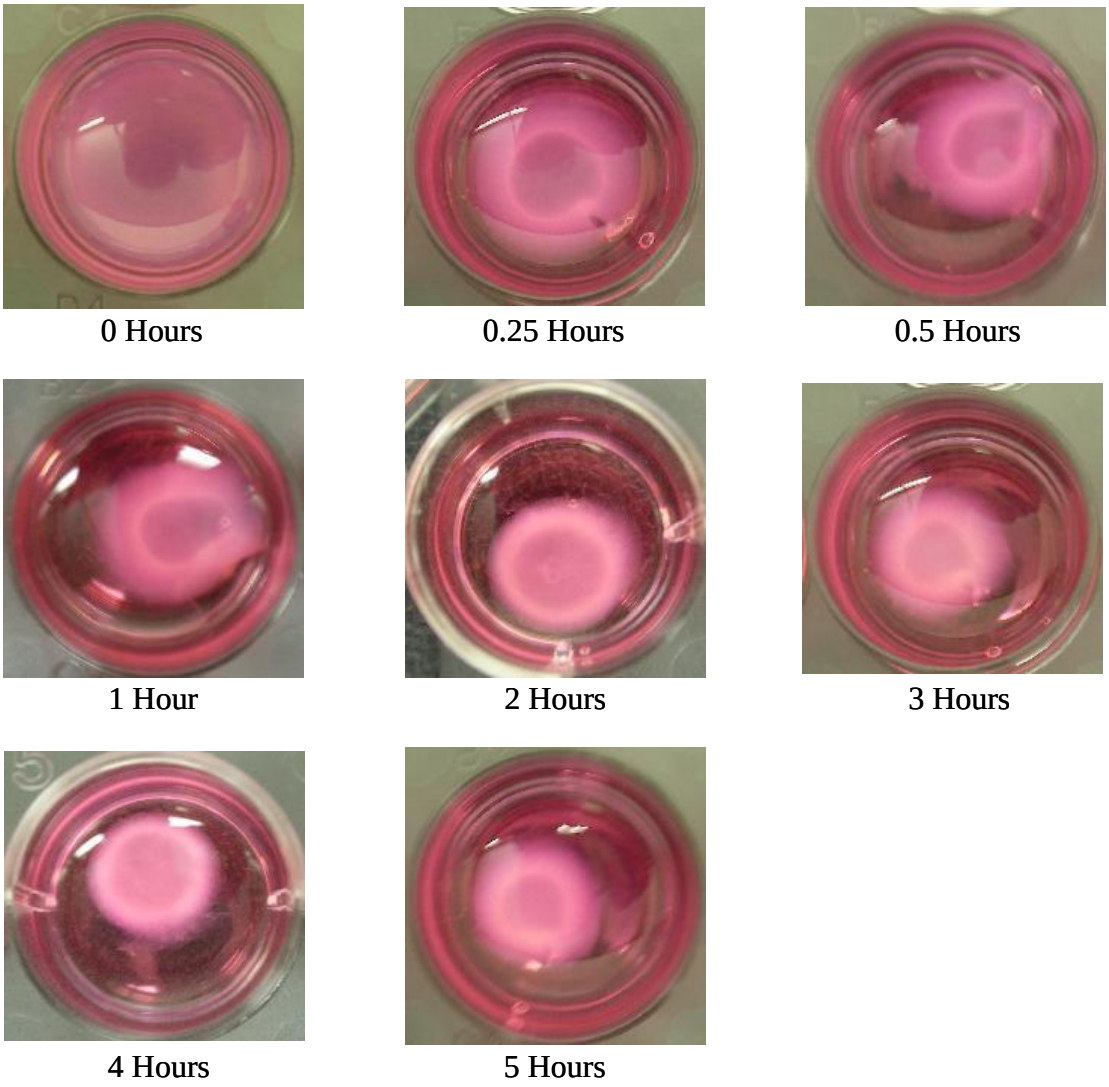


Figure 7.1 Example collagen gel photographs

Gel Photographs taken at specified time point demonstrating contraction of the gel over time following stimulation with 1nM bradykinin. The original gel size covered the entire base of the well. Therefore, the area of the base of the well is used to which the contracting gel area is compared against and a percentage contraction may be calculated.

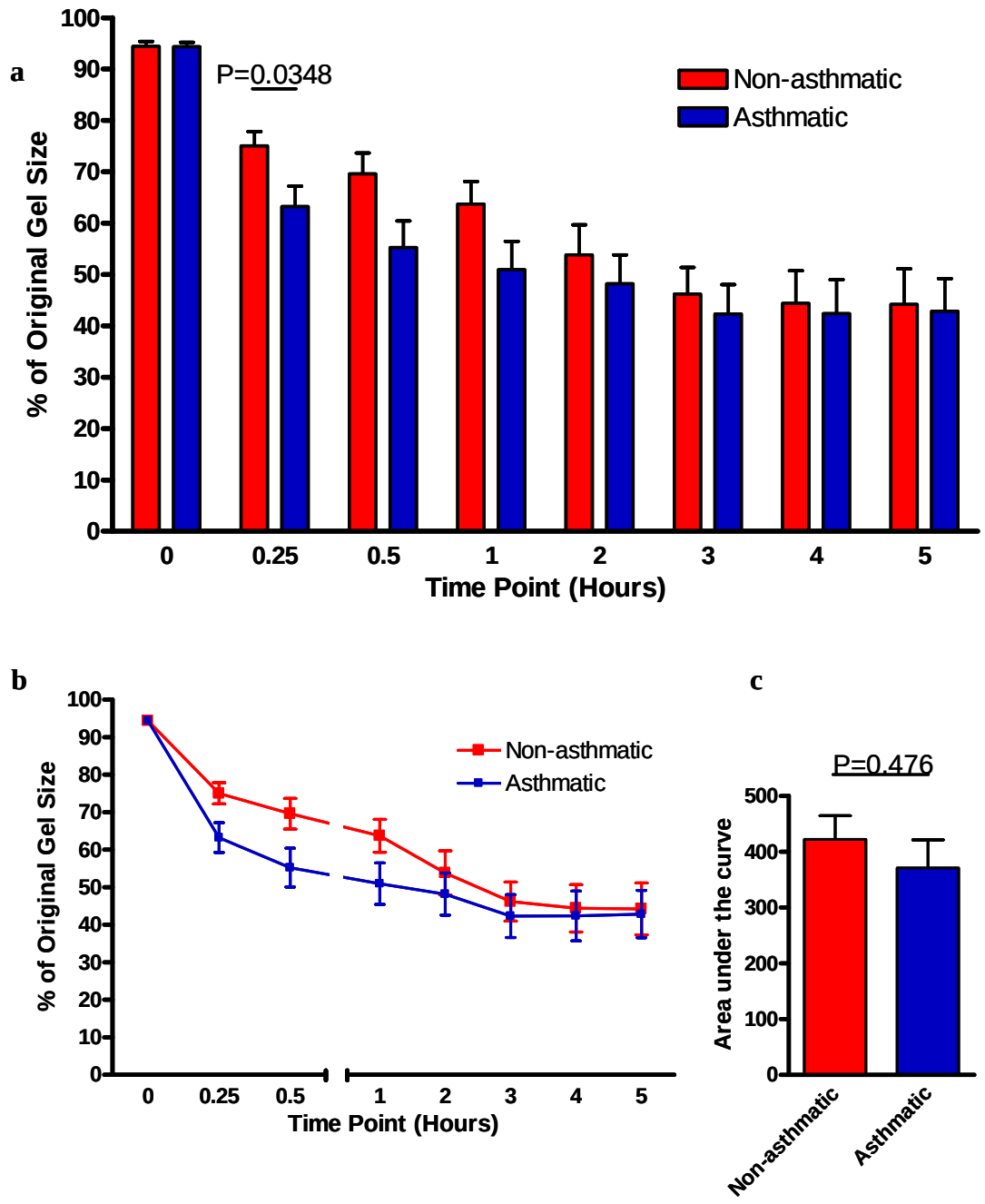


Figure 7.2 Percentage contraction of collagen gels following stimulation

Collated data for the cumulative percentage of contraction of the collagen gels observed when compared to the original gel size following stimulation with 1nM bradykinin, in asthmatic and non-asthmatic smooth muscle (a). This data expressed as a graph (b), from which area under the curve values were taken (c). Analysis by paired t test. Data represented a mean±SEM.

When the control, non-stimulated cell measurements were removed from the respective stimulated cell measurements (Fig 7.3), the significant increase in contraction observed at 15 minutes following contraction was more pronounced (P=0.013; Fig 7.3a). Significant difference in the contraction was also observed 1 hour following stimulation with bradykinin (P=0.002). The remaining time points where the gel size was analysed saw no difference in contraction between asthmatic and non-asthmatic ASM, and the overall gel size 5 hours after stimulation saw no significant difference either indicating that the important period showing the increased contraction of the ASM isolated from asthmatic patients was in the first hour following stimulation. When the amount of contraction above control was compared over the entire 5 hour culture (Fig 7.3b and c), no significant difference was measured (P=0.391).

The amount of contraction above control observed was split into time intervals, where by the previous amount of contraction measured was removed from the current time point so just the amount of contraction occurring within two adjacent time points was measured. During the first 15 minutes following addition of the agonist 1nM bradykinin, ASM isolated from asthmatic patients contracted to a significantly greater degree than ASM from a non-asthmatic individual (P=0.004; Fig 7.4a). After the first 15 minutes however, no significant difference in the amount of contraction between the two cell phenotypes was observed. Comparing the whole 5 hour collagen gel contraction between the ASM isolated from asthmatic and non-asthmatic individuals saw now difference in the area under the curve (P=0.564; Fig 7.4b and c).

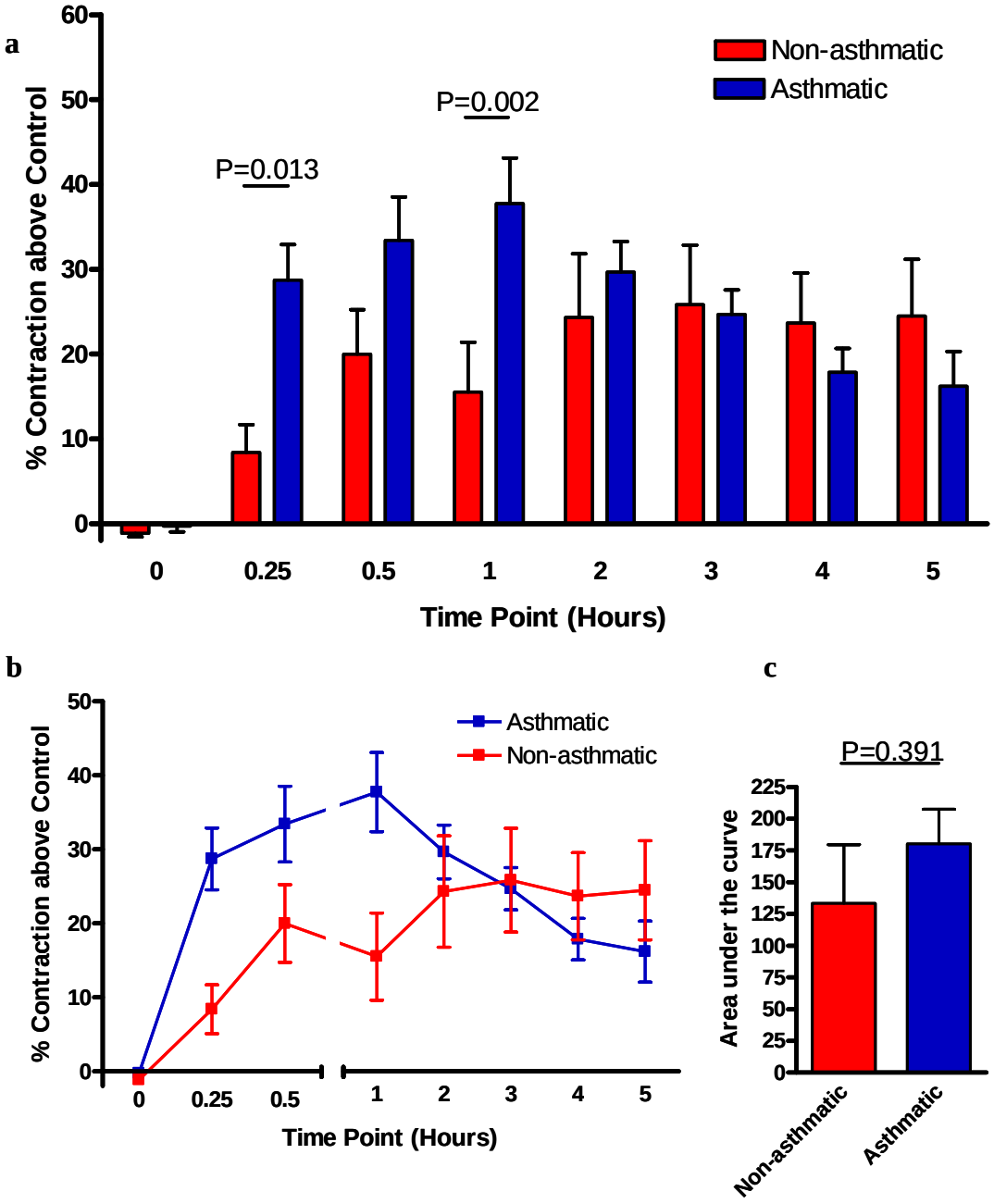


Figure 7.3 Percentage contraction of collagen gels above control

Collated data for the percentage of contraction of the collagen gels following stimulation with 1nM bradykinin measured above the respective control, in both asthmatic and non-asthmatic smooth muscle (a). This data expressed as a graph (b), from which area under the curve values were taken (c). Analysis by paired t test. Data represented a mean±SEM.

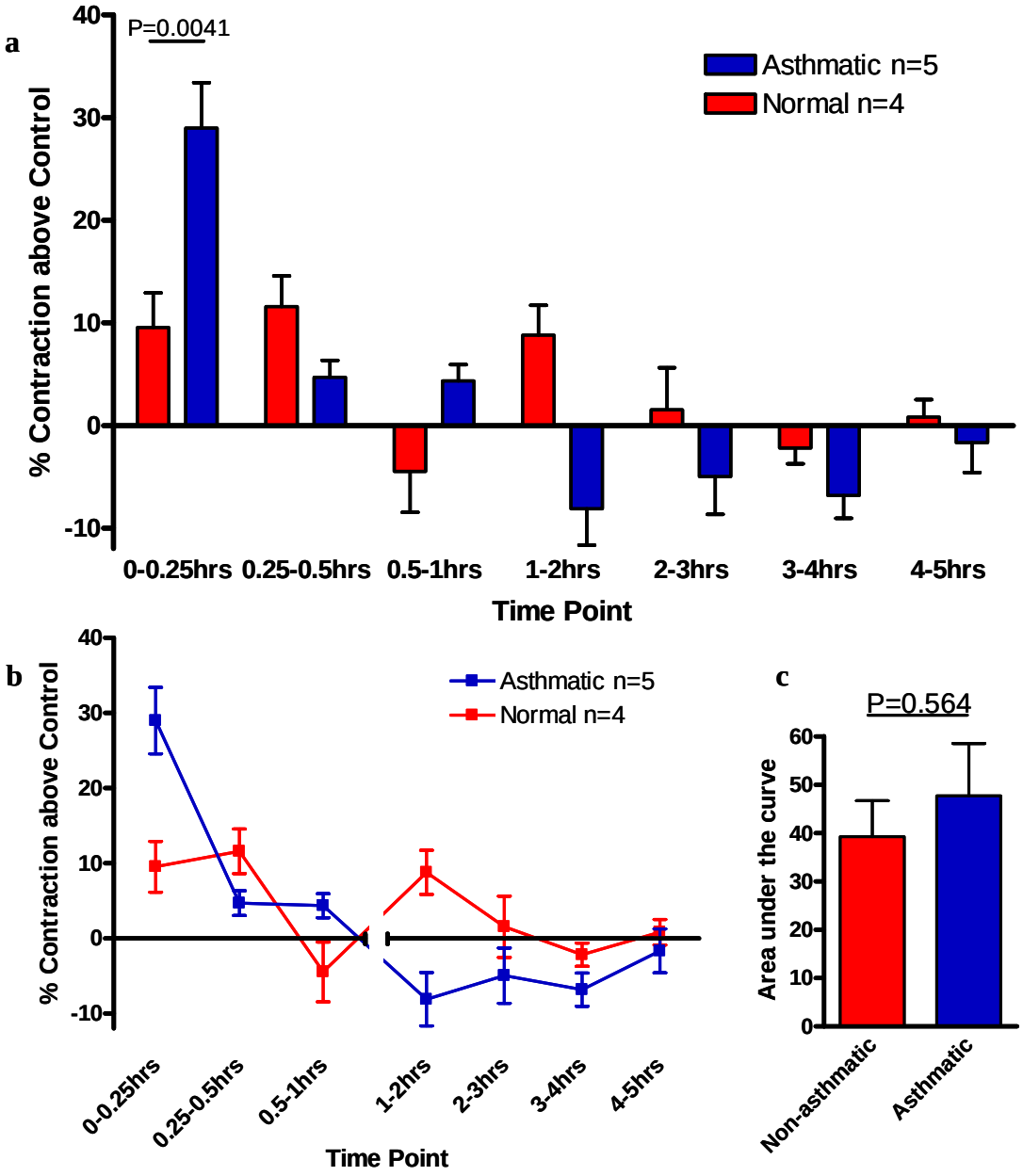


Figure 7.4 Percentage contraction of gels above control and previous time point

Collated data for percentage contraction of collagen gels following stimulation with 1nM bradykinin above control contraction and between specified time points, in both asthmatic and non-asthmatic smooth muscle (a). This data expressed as a graph (b), from which area under the curve values were taken (c). Analysis by paired t test. Data represented a mean±SEM.

When the control gel was analysed to assess whether there was any difference in the amount of spontaneous contraction observed over the 24 hour time course which were run in parallel with the respective stimulated ASM gels, ASM from non-asthmatic subjects was found to spontaneously contract to a significantly higher degree at 15 minutes and 1 hour into the incubation, ($P=0.018$ and $P<0.001$ respectively; Fig 7.5a). The remaining observed time points did not see any significant difference in this spontaneous contraction and there was no overall difference ($P=0.581$; Fig 7.5b).

These data indicate that ASM isolated from an asthmatic patient initially contracts to a significantly higher degree than that from a non-asthmatic subject following stimulation with agonist. But this does not correlate with measuring intracellular calcium flux following stimulation with 1nM bradykinin, the indirect method of measuring cell contraction. So what is the relation between intracellular calcium and cell contraction in ASM? The next step was to measure the cell's calcium/contraction coupling proteins.

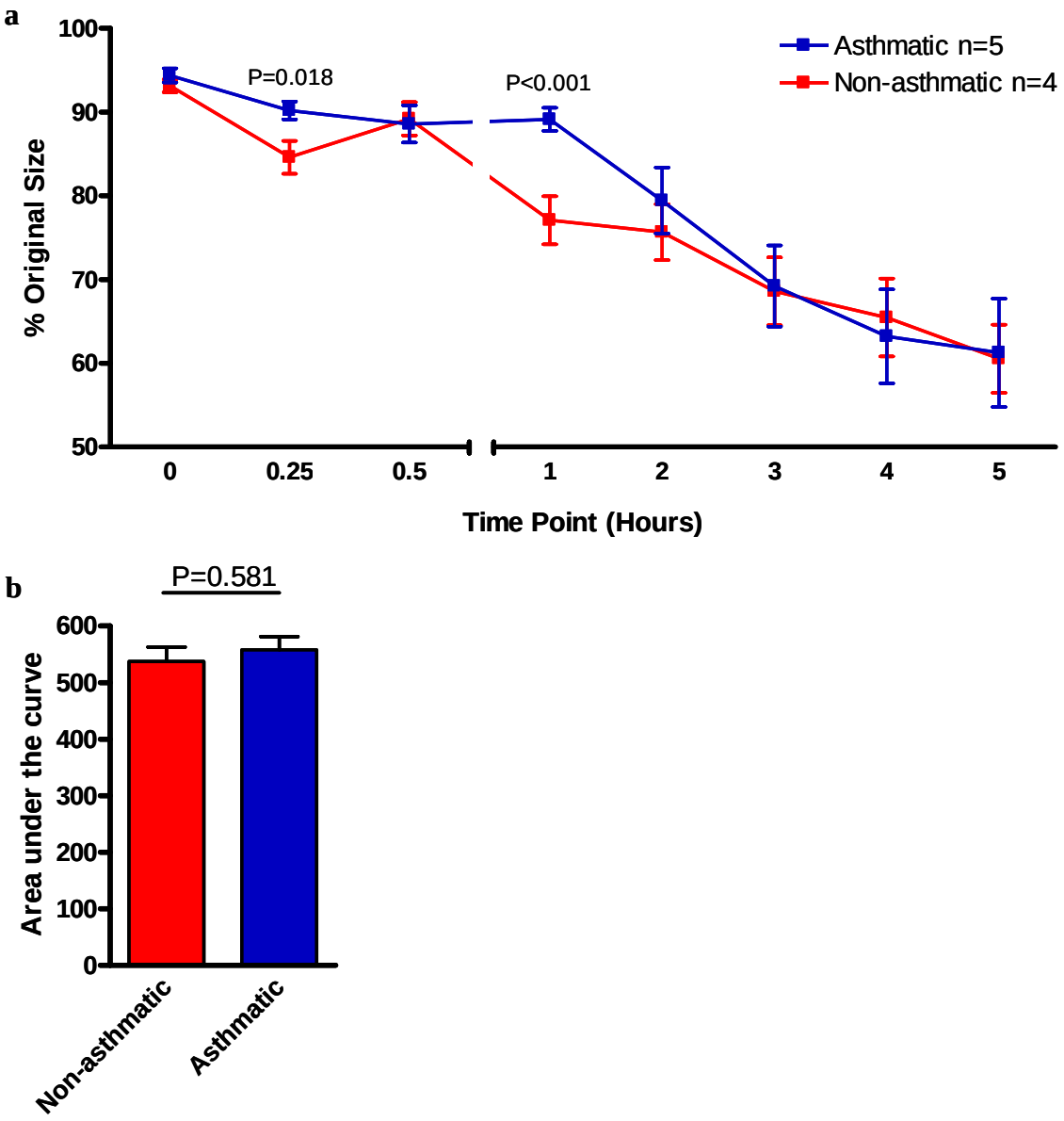


Figure 7.5 Spontaneous contraction of airway smooth muscle collagen gels

Collated data of spontaneous contraction of control gels that have not been stimulated with bradykinin cultured in parallel to their stimulated counterparts over the time course, displayed as a percentage of the original gel size in both asthmatic and non-asthmatic smooth muscle (a). Area under the curve values measured from these graphs (b). Analysis by paired t test. Data represented a mean±SEM.

7.3 Comparison of calcium/contraction proteins

A selection of intracellular proteins that are involved in coupling intracellular calcium to cell contraction were measured by Western Blotting in airway smooth muscle from both asthmatic and non-asthmatic airway smooth muscle (n=3 for each; Fig 7.6), 1.5×10^5 cells were loaded per well.

The following intracellular proteins were measured for their content in asthmatic and non-asthmatic airway smooth muscle (Fig 7.6): myosin light chain (MLC), phosphorylated myosin light chain (pMLC), myosin light chain kinase (MLCK), phosphorylated myosin light chain kinase (pMLCK), myosin light chain phosphatase (MLCP), smooth muscle sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2) and ADP-ribosyl cyclase (CD38).

Upon initial inspection, there appears to be a slightly lower expression of pMLCK in ASM isolated from asthmatic patients compared with non-asthmatic ASM, but this requires further investigation. It is believed that there is an increase in expression of MLCK in ASM from asthmatic patients (5,163), however, after preliminary inspection; we did not find any difference in expression with MLCK or any of the other intracellular calcium/contraction proteins measured between the asthmatic and non-asthmatic cellular phenotypes. However, further investigation needs to be carried out in order to fully determine any differential protein expression.

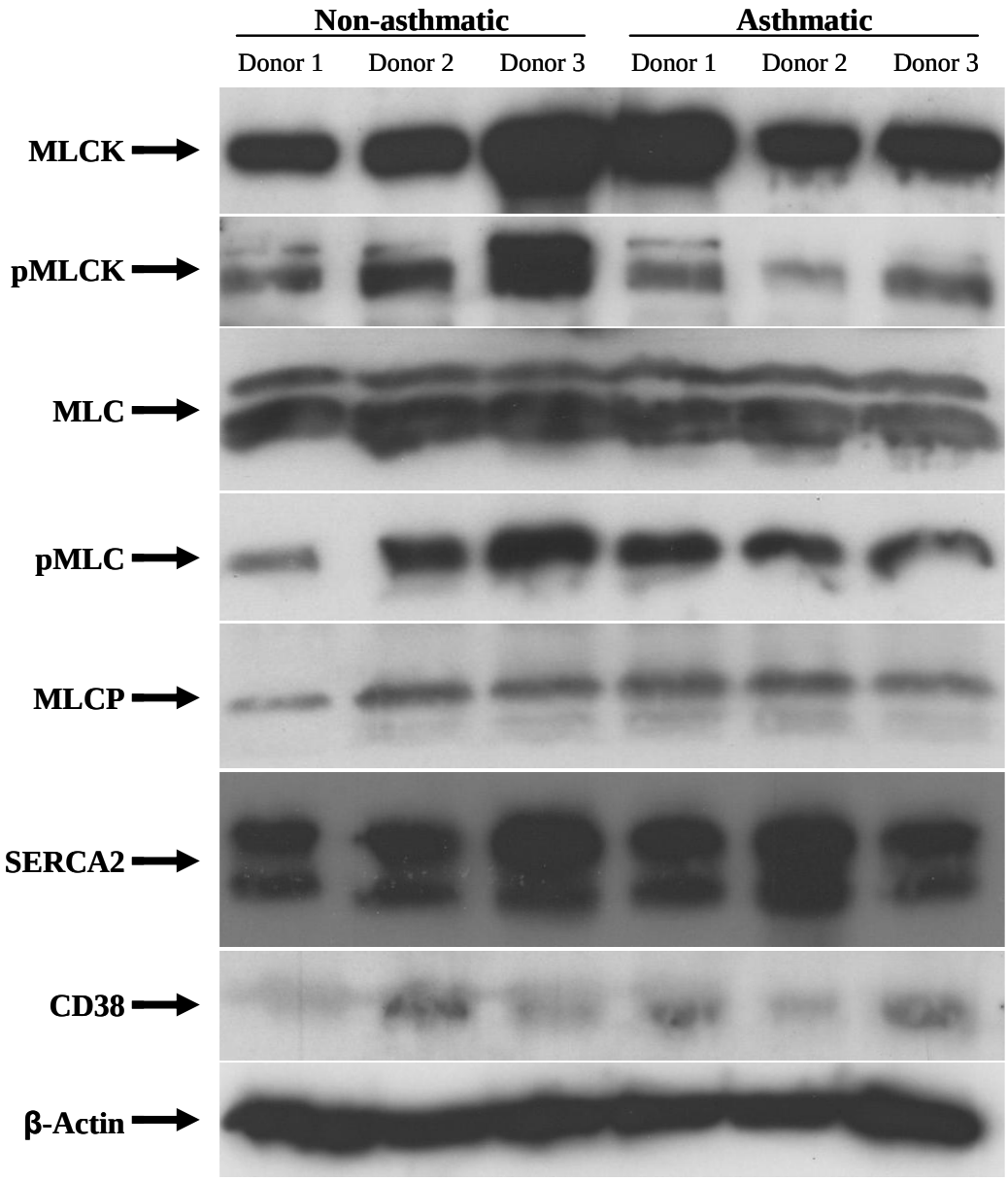


Figure 7.6 Examples of Western blots of calcium/contraction proteins

Western blot analysis of a selection of proteins involved in the calcium/contraction process. Each blot was performed on three asthmatic and non-asthmatic smooth muscle donors. An example of the β-actin blot (performed for each) is shown at the bottom.

7.4 Summary

Asthmatic airway smooth muscle contracts to a significantly greater degree than non-asthmatic airway smooth muscle within the first 15 minutes following stimulation with the agonist bradykinin, supporting previous findings (163). However, these data do not correlate with the intracellular calcium data collected during this thesis. One method of addressing this was to quantify the intracellular protein content of some of the main proteins involved in the calcium contraction process by Western Blotting.

Although the Western Blotting experiments have not yet been fully investigated and in most circumstances only performed once, it is hard to draw any conclusive differences, though initial inspection does imply that there is no difference in those intracellular proteins that have so far been explored. Therefore, what is the relationship between intracellular calcium and cell contraction, as intracellular calcium has been used to be an indirect measure of cell contraction. The significantly reduced intracellular calcium flux measured in response to agonist does not seem to correlate with the significantly increased initial cell contraction in asthmatic ASM. The expected link with the intracellular calcium/contraction proteins, an increased expression compared with non-asthmatic ASM, was not found, indicating that the link between calcium and cell contraction lies elsewhere. However, as these protein expression data are in its initial stages, it would be prudent to further investigate these intracellular proteins as well as other proteins involved in the calcium/contraction process and extracellular membrane protein channels.

Chapter 8:

Discussion

8.1 General discussion

We have shown for the first time that human airway smooth muscle supports the survival and proliferation of human lung mast cells. The effects of which are mediated through a co-operative interaction between ASM membrane-bound SCF, HLMC membrane-expressed CADM1, and soluble IL-6. Furthermore, mast cells co-cultured with airway smooth muscle demonstrate enhanced constitutive activation as measured by the release of histamine.

Human ASM was predicted to maintain HLMC survival because it produces SCF, an essential mast cell growth factor (175). However, the rapid proliferation of the lung mast cells was remarkable, particularly as they have long been considered to represent terminally differentiated cells. Nevertheless, HLMC will survive and proliferate slowly over many weeks in culture with SCF, IL-6 and IL-10 (191,192), but this is in marked contrast to the rapid rate of HLMC proliferation in the ASM co-culture. High resolution dye tracking confirmed the results observed by metachromatic counting, while DAPI and Annexin V staining indicated a decreased rate of HLMC apoptosis in the ASM co-culture compared to the HLMC SCF/IL-6/FCS-supplemented mono-culture. The increased mast cell numbers evident in airway smooth muscle co-culture compared to the cytokine mono-culture is therefore due to a combination of both proliferation and protection from apoptosis.

Cell-cell contact was an essential requirement for HLMC proliferation in co-culture with ASM. The effect of cell separation by a Transwell membrane was largely mimicked by an adhesion-blocking antibody to CADM1, a Ca^{2+}-independent adhesion molecule known to mediate, in part, the adhesion of HLMC to ASM (141),

and the adhesion of mouse mast cells to fibroblasts (178) and nerves (159). Homophilic CADM1 interactions in epithelial cells promote its tumour suppressor activity, but its heterophilic ligation in HLMC clearly promotes cell proliferation; a potential mechanism that would explain this specific interaction with the SCF receptor CD117 (c-kit). Several lines of evidence support this hypothesis. Firstly, the effects of blocking CD117 and CADM1 were only marginally additive suggesting a co-operative interaction. The SCF expressed by ASM was predominantly membrane-bound and we also observed strong co-localisation of CD117 and CADM1 in the HLMC plasma membrane. Furthermore, and perhaps most importantly, CD117 co-immunoprecipitated with CADM1 indicating a physical interaction. Interestingly in mouse mast cells, CD117-dependent signalling through PI-3 kinase has been shown to be important for CADM1-dependent adhesion (193) although this does not appear to apply to humans (141). Finally, there is a precedent for the interaction of CADM1-like molecules with other tyrosine kinase receptors. For example, CADM1 binds a membrane guanylate kinase family member, Pals2 which binds to Lin-7. Lin-7 is required for the correct membrane localisation of an epidermal growth factor receptor homologue in C. elegans (142). Thus the ability of CADM1 to mediate HLMC adhesion to ASM, its direct physical interaction with CD117, and its apparent co-operative role in CD117-dependent signalling suggests that CADM1 plays a fundamental role in many CD117-dependent mast cell processes. We therefore envisage a model whereby CADM1-dependent adhesion and CADM1-dependent CD117 membrane localisation facilitates the interaction of CD117 with membrane-bound SCF on ASM and other stromal cells.

IL-6 was also an important mediator of the lung mast cell proliferative response in the co-culture environment. The concentrations present in the co-culture supernatant were above the optimal concentration required to promote cultured mast cell proliferation (172). The source of this IL-6 might be ASM, but could also arise from HLMC which release this cytokine in large quantities (174,179). IL-6 appeared to co-operate with SCF and CADM1, as its contribution was similar to these molecules and the effects of its blockade were barely additive. Determining how IL-6 interacts with SCF and CADM1 requires further investigation.

Human gut fibroblasts maintain gut mast cell survival, but do not induce their proliferation. This did not require cell-cell contact and was not mediated via SCF (194), which is surprising as SCF- and CADM1-dependent adhesion mediates mouse mast cell adhesion to fibroblasts and promotes their survival (178,193). The gut fibroblast survival activity had a mass between 10 and 100kDA, suggesting that IL-6, which was not measured may have been partially responsible. In contrast, co-culture of human gut mast cells with HUVECs not only promoted mast cell survival but also induced their proliferation (168). However the proliferative effect was much slower than that seen in our experiments with ASM and the mechanism was distinct. Although gut mast cell-HUVEC co-culture required adhesion for proliferation, the adhesive process was mediated via membrane-bound SCF and the immunoglobulin superfamily molecule VCAM-1 expressed on HUVECs (168). Neither of these are involved in the adhesion of HLMC to ASM (141). Furthermore, blocking SCF in co-culture with HUVECs killed all mast cells, whereas in our experiments proliferation was attenuated but the cells remained viable. Human bronchial epithelial cells also maintain the survival of human cord blood-derived mast cells through a SCF-

dependent mechanism, but over 4 days did not induce proliferation (169). Determining whether HLMC interact with airway epithelial cells, endothelial cells and fibroblasts through via CADM1 will be of great interest.

Not only do HLMC proliferate in co-culture with ASM, but they are also activated. There was increased basal histamine release during the co-culture period, which was mirrored by a decrease in HLMC histamine content. This is particularly interesting because mast cells in the ASM bundle from asthmatic patients are activated as shown by the presence of piecemeal degranulation (33). Our data therefore suggests that this can occur as a result of the interaction of mast cells with ASM independently of allergen and/or monomeric IgE (195). This might explain why anti-IgE therapy is able to inhibit airway inflammation but have no effect on AHR (196). The mechanisms behind this require investigation, but could be mediated in part through the interaction of HLMC CD117 with membrane-bound SCF expressed by ASM. It will also be important to investigate the role of CADM1 in this activation process. Of further importance, these activated, histamine-depleted HLMC are still able to release equivalent amounts of histamine following IgE-dependent activation, indicating that they can still respond to allergen inhalation. These findings are in contrast to those within human airway epithelium, which actually inhibits constitutive and IgE-dependent mast cell activation (141,169), further highlighting the potential importance of the ASM-HLMC interaction.

We have shown that airway smooth muscle isolated from asthmatic individuals display altered intracellular calcium homeostasis and reduced calcium flux in response to agonist when compared to non-asthmatic smooth muscle. Intracellular

calcium homeostasis is coupled to contractile ability of the cell, but our results are inconsistent with this dogma. The ASM from asthmatic patients produce a reduced intracellular calcium response to stimulus, but an increase in initial contraction as shown by gel contraction assays. And preliminary experiments quantifying the linking calcium/contraction proteins shown no difference between the to cell phenotypes to link this phenomenon.

Increased contractility of asthmatic smooth muscle has long been implicated in airway hyperresponsiveness (24,25). Indeed, there have been previous studies reporting that smooth muscle isolated from asthmatic patients is hypercontractile compared with that from non-asthmatic subjects (163). Intracellular calcium has long been established as an indirect measure of a cell's contractile ability (133,197). But if this is the case, our reduced intracellular calcium results of ASM from asthmatic patients were contradictory of previous findings.

Baseline intracellular calcium handling of ASM cells were monitored for any differences compared with non-asthmatic cells. The average baseline did vary considerably between cells of the same donor and between donors, but when collated, no overall significance was identified. Although the average baseline intracellular calcium value did not significantly differ between the two cell phenotypes, the presence of significantly increased numbers of oscillations within the baseline in the ASM from asthmatic patients was a phenomenon isolated to the asthmatic phenotype. These intracellular calcium oscillations were lost when the extracellular calcium was removed from the culture, indicating that the abnormality may lie within the plasma membrane of the asthmatic ASM.

When the response to stimulus was measured, ASM from asthmatic patients gave a consistently reduced intracellular calcium response regardless of the method used for analysis. Indicating the ability of these cell to either release calcium from intracellular stores or to obtain it from the extracellular environment is somewhat hindered. When the extracellular calcium was removed from these cultures, the reduced calcium flux measured in the asthmatic cells was either reduced or completely lost its significance in comparison to control, indicating that the abnormality lies in both the ability of the SR to release calcium and the plasma membrane to allow calcium entry form the extracellular environment. Due to the donor variability, collating individual donors resulted in the loss of significance, although the difference was still evident. However, these data conflict with previous observations where following stimulation with acetylcholine and histamine when the area under the curve was measured, a significantly greater response was measured in the asthmatic smooth muscle cells compared with control (188).

Orai1 and STIM1 roles within calcium influx through SOC channels are well recognized (134,135). Previously, siRNA knockdown of these proteins have found reduced intracellular calcium flux in response to thapsigargin and cyclopiazonic acid (198), indicating a possible source and target for future investigations.

Gel contraction assay is a method that has previously been employed to measure the contraction rate and maximal contraction of many cell types including smooth muscle cells (162,163) and fibroblasts (199,200). One previous study has already shown the increased contractile ability of ASM from asthmatic patients (163), which compliments studies performed on ex vivo smooth muscle tissue (24). In support of

these findings, we found that the ASM isolated from asthmatic patients contracted to a significantly greater degree immediately after administration of the stimulus, but overall there was no difference in the maximal contraction. But it is the initial force exuded and the sensitivity of the cells to stimulus that is the key components of AHR rather than the maximal force generation. In cells that have a reduced intracellular calcium response but are still able to contract significantly greater than their control counterparts, the possibilities of altered calcium-independent mechanisms involved are vast.

One such explanation is a difference in the expression of the calcium-contraction proteins within the asthmatic and non-asthmatic phenotype. Previous studies have resulted in varying answers. MLCK protein expression has been found in ASM from asthmatic individuals to be increased (190) and no different (163) when compared with that from non-asthmatic subjects, and in rat ASM after repeated allergen challenge over a 35 day period showed even decreased expression (201). Our results showed no difference in the protein expression of MLCK between ASM isolated from asthmatic and non-asthmatic individuals. A slightly lower expression of pMLCK in ASM isolated from asthmatic individuals compared with non-asthmatic was observed in the initial investigation but requires further examination. The remaining calcium-contraction proteins MLC, pMLC, MLCP, SERCA2 and CD38 did not show any difference in their expression. However, these data are only preliminary and further work needs to be carried out on the calcium-contraction proteins to fully establish any differences and to decipher their precise role in calcium-linked cell contraction.

In summary, we have demonstrated that human ASM supports HLMC survival and proliferation, which may contribute to the mast cell myositis evident in asthma. Furthermore this interaction activates mast cells, which supports the view that they aggravate smooth muscle contractile dysfunction in asthma. Disturbing the SCF-CADM1-IL-6-dependent pathway through which these effects are achieved may offer a new approach to asthma therapy.

We have also demonstrated that human airway smooth muscle from asthmatic subjects is altered from non-asthmatic in its ability in handling intracellular calcium at baseline and in response to stimuli. In addition, the calcium-independent contractile response of these cells is raised and following preliminary investigations, this is not related to the calcium-contraction proteins. However, finding the source of the altered calcium handling and/or cell contraction, may offer new approaches to future treatment of AHR in asthma.

8.2 Critique

The experiments performed in this thesis have used primary cell lines of smooth muscle cell cultures from passages 3 to 6. This cell population is a small, highly proliferative subset of the original biopsy, rather than a true representation of the in vivo state. The human lung mast cells are not from asthmatic nor healthy subjects. They are obtained from patients undergoing lung resection, usually for lung cancer, so cannot be classed as from a healthy source, so any differences in their response to the local environment in which they are cultured, compared with mast cells from health or a disease state, is not known.

Co-culture of mast cells with airway smooth muscle isolated from asthmatic patients was only completed with three donors, compared to culture with airway smooth muscle from non-asthmatic subjects, which had fourteen donors. Although comparison was made between the ability of these two ASM phenotypes to maintain survival and promote proliferation, to adequately evaluate asthmatic and non-asthmatic ASM, the experiments must have comparable donor numbers.

Comparisons were made of the clinical characteristics to the ASM results (data not shown), with respect to intracellular calcium and cell contraction. However, some of the clinical data was incomplete resulting in some statistics unable to be performed. In addition, to obtain reliable statistics, adequate donor numbers are required. Therefore, as comparing the results obtained to the clinical characteristics would give an insight into the clinical-biological relationship, at this point any correlations that might be present are currently overlooked as significance is not reached due to the data being underpowered.

The protein expression analysis work completed was only in its initial stages at the time of completion of the thesis project. The experiments performed were limited in number and only performed using the Western Blot technique. Although this is a good method when looking at presence or absence protein expression, or even large differences in expression, it is usually only used as an indication of variation in the amount of expression between cells and in most cases, particularly when small differences in the concentration of protein is being investigated, a quantitative protein assay is required.

8.3 Future studies

We have shown that the presence of mast cells within the airway smooth muscle of asthmatic patients is in-part due to a process of survival and proliferation. But how do these cells come to be localised to the smooth muscle to begin with? These cells must be recruited; for example through the CXCR3/CXCL10 axis (120), but it is an area which requires further exploration to deduce the exact processes involved. Mast cells are still present in health and other disease states, just at reduced amounts compared with asthma, so the processes in place in these other conditions such as inhibition of proliferation and survival, and promotion of apoptosis must be in place to prevent the mast cells proliferating to the extent observed in asthma. Therefore, similar experiments could be carried out in other disease states, such as eosinophilic bronchitis and chronic obstructive pulmonary disease to observe the comparison of such cellular processes.

Due to experiments being conducted with ASM primary cell lines, experiments need to be repeated on freshly isolated cells. The source of HLMC is not from health or disease, however the availability of tissue from a certain state of disease or health is limited, but post-mortem acquirement of lung from asthmatic or non-asthmatic subjects while is possible, would be a highly infrequent and would therefore be a long process to complete.

This mast cell-smooth muscle model is only two-dimensional and does not include the same matrix composition found in the airways. Therefore including the correct matrix composition could better evaluate particularly whether there is a difference in

the survival and proliferative effect on the mast cells between ASM sourced from asthmatic and non-asthmatic subjects.

As asthmatic subjects undergo pharmacological treatments, the mast cell survival and proliferation model, ASM calcium handling model or intracellular protein expression measurement could be further expanded with either direct response to agents such as corticosteroids and β_2-agonists on the cultures, or obtaining primary cells from patients before and after treatment to assess the effect of these drugs on the pro-proliferative and anti-apoptotic effect the ASM has on the mast cells, which may lead to new therapies.

Indeed, what is the effect of these drugs on the cells? Are they able to reduce the mast cell number in airway smooth muscle of asthmatic subjects? Can they reduce oscillations within the intracellular calcium baseline of ASM or amend the calcium response to agonist to return it to that seen in the non-asthmatic ASM? If these effects do occur following treatment, then this mast cell-ASM model is redundant. If this is the case, other potential causes of asthma need to be identified as disease still persists in patients following pharmacological treatment.

Although siRNA would be a valuable approach in investigating the actions of certain cytokines and chemokines, previous experimentation into this area was not successful. Different methods of siRNA knockdown were investigated resulting in transfection and cell survival being negatively correlated. However, upon discovery of a method that is more successful with regard to both transfection and cell survival,

siRNA knockdown would be an excellent next step in identifying the exact contribution of different agents.

Quantitative protein assays are required to fully determine any differing expression of intracellular proteins that couple the calcium-contraction pathways. In addition to the proteins already analysed, quantification of Orai1, STIM1 and 2, TRPC channel and plasma membrane ion channel protein expression could be investigated in the asthmatic and non-asthmatic smooth muscle phenotypes. As well as measuring the relative quantity of these proteins, their sequence and confirmation could also be investigated to see whether there is any abnormality that could thus be hindering their function.

In addition to the possibility that any of the calcium-contraction proteins could vary in their expression between these two cell phenotypes, another option is rate at which these proteins are phosphorylated. Phosphorylation assays produce a precise quantification of phosphorylated and non-phosphorylated cell signaling proteins involved in phosphorylation cascades and could answer whether it is the rate at which the proteins are phosphorylated that result in the increased initial contraction of the cell.

The data presented in this thesis extends the view that ASM in asthma is intrinsically different and underlies the importance of mast cell-airway smooth muscle interactions in the development of disturbed airway physiology. This opens avenues of future work to further our understanding of the pathophysiology of asthma and the development of new therapies.

Chapter 9:

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