

Cellular Interactions of Airway Smooth Muscle and Human Lung Mast Cells

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by

Lucy Woodman BSc (Hons)
Department of Infection, Immunity & Inflammation
University of Leicester

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Cellular Interactions of Airway Smooth Muscle and Human Lung Mast Cells

Lucy Woodman

Abstract

Asthma is characterized by variable airflow obstruction, airway hyperresponsiveness (AHR), inflammation and remodeling. Mast cells (MC) co-localise to airway structures such as epithelium and airway smooth muscle (ASM) - the latter is a key determinant of AHR. Our group has reported that the CXCL10/CXCR3 axis is important in MC migration towards ASM, MCs adhere to ASM predominately via CADM-1 and this interaction promotes MC survival and proliferation. Whether there are other important mechanisms driving MC localization, ASM migration, and how MCs affect ASM differentiation is uncertain.

We sought to examine i) chemokine concentrations in airway secretions in eosinophilic bronchitis (EB), and asthma, and their effects on MC migration ii) CCR3 mediated ASM migration, iii) effects of MC–ASM co-culture on ASM differentiation.

Bronchoalveolar lavage (BAL) CXCL10 and CXCL8 concentrations were increased in subjects with EB compared to asthmatics and controls; were chemotactic for MCs and was attenuated by CXCR1 or CXCR3 inhibition. CCL11 mediated ASM migration and wound healing, but had no effect on proliferation or survival. Co-culture with β -tryptase or MCs degraded CCL11, and inhibited CCL11-mediated ASM migration. *In vitro* co-culture of ASM cells with β -tryptase or MCs increased ASM-derived TGF- β 1 secretion, α -smooth muscle actin (α -SMA) expression and agonist-provoked contraction. Promotion to a more contractile phenotype was inhibited by leupeptin and TGF- β 1 neutralization, suggesting ASM differentiation was driven by the autocrine release of TGF- β 1 in response to β -tryptase. Importantly, *in vivo*, in asthmatic bronchial biopsies, intensity of α -SMA expression was strongly related to the number of MCs within or adjacent to ASM bundles.

In conclusion, CXCL8 and CXCL10 are important in MC migration to the epithelium, but CCL11 is unlikely to be important in ASM migration. MCs drive ASM differentiation to a more contractile phenotype via autocrine release of TGF- β 1, which may contribute to the disordered airway physiology in asthma.

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Saunders R, Sutcliffe A, Woodman L, Kaur D, Siddiqui S, Okayama Y, Wardlaw A, Bradding P, Brightling C. The airway smooth muscle CCR3/CCL11 axis is inhibited by mast cells. Allergy. 2008 Sep;63(9):1148-55

Woodman L, Sutcliffe A, Kaur D, Berry M, Bradding P, Pavord ID, Brightling C. Chemokine concentrations and mast cell chemotactic activity in BAL fluid in patients with eosinophilic bronchitis and asthma, and in normal control subjects. Chest. 2006 Aug;130(2):371-8

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Chapter One

Introduction

1.1 **Characterisation of Asthma**

1.1.1 **Historical Perspective**

From the time of the Greeks to the present day, the name asthma has changed significance several times. The classification of the disease has changed even more as physicians looked for different sets of symptoms that changed with their theoretical concepts of causality. One of the earliest recognized treatise was recorded in the 12th century, which was concerned with treating the disordered airway function according to the various supposed environmental and dietary causes (1). In the late 19th century the discussion evolved to further characterize asthma and connect the symptoms with a variety of pathological changes in the lung including bronchial mucosal oedema, and inflammation (2). In the early 20th century, proposals were made to classify asthma into 'extrinsic' and 'intrinsic' asthma based on etiology, and in the mid 20th century, subgroups of patients with asthma were identified according to the degree and severity of the disease (3).

Currently discussion about the characterization of asthma has moved onto cluster analysis in order to distinguish asthmatic phenotypes. Clinically important prognostic differences identified between the phenotypes within models may provide a reliable framework for exploratory molecular and genetic studies, presently undermined by population heterogeneity (4).

1.1.2 **Clinical Presentation and Definition**

Asthma has significant genetic and environmental components, but since its pathogenesis is not clear, much of its definition is descriptive. Based on the functional consequences of airway inflammation, an operational description of asthma could include that it is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyperresponsiveness (AHR) that leads to the clinical presentation of recurrent episodes of shortness of breath, wheezing, chest tightness and coughing - particularly at night or in the early morning. These episodes are usually associated with widespread, but variable airflow obstruction (VAO) that is often, but not always reversible either spontaneously or with treatment (5).

As there is no clear definition of asthma phenotype, clinical characteristics that can be measured objectively, such as atopy, airway inflammation and AHR can be used to classify the severity of asthma. (5). Various systems have used mild, moderate and severe to describe the severity of asthma exacerbations (6), or replaced the use of the term mild with intermittent in order to emphasize that even patients who have intermittent asthma can have severe exacerbations (7), and correspondingly used the terms mild, moderate and severe persistent (5).

Clinical classification of asthma symptoms is useful when decisions are being made about management at the initial assessment of the patient. It is important to recognize, however, that asthma severity involves both the severity of the underlying disease and its responsiveness to treatment. Therefore, subjects with asthma can present with severe symptoms and airflow obstruction and be classified

as severe persistent on presentation, but respond to treatment and then be classified as moderate persistent asthma (5).

As subjects with asthma can respond to treatment and the classification of asthma severity by clinical features can change, recent emphasis has been placed on the classification of asthma by level of control – defined as controlled, partly controlled or uncontrolled. Achieving and maintaining asthma control has been emphasized as the goal of asthma treatment (8).

Clinical control of asthma is defined as no (twice or less / week) daytime symptoms, no limitation of daily activities, including exercise, no nocturnal symptoms or awakening because of asthma, no (twice or less / week) need for reliever treatment, normal or near-normal lung function (in practical terms forced expiratory volume in 1 second (FEV₁) and / or peak expiratory flow (PEF) or more than 80% predicted or best), and no exacerbations (5).

There are other measures of control, including humanistic outcomes such as the quality of life score and satisfaction and the economic outcomes, including costs to patients, society, health care system, and the workplace. Because of the variability in these outcomes, it is likely that there is no single measurement that is the best indicator of asthma control, nor will one function as a universally applicable surrogate for all patients (9).

				STEP 5
			STEP 4	<p>Continuous or frequent use of oral steroids: Use daily dose steroid tablet in lowest dose providing adequate control. Maintain high dose inhaled steroid at 2000 mcg/day *. Consider other treatments to minimize the use of steroid tablets. Refer patient for specialist care.</p>
		STEP 3	<p><u>Persistent poor control:</u> Consider trials of • increasing inhaled steroid up to 2000 mcg/day * • addition of a fourth drug e.g. leukotriene receptor antagonist, SR theophylline, β_2 agonist tablet</p>	
	STEP 2	<p><u>Initial add-on therapy:</u> 1. Add inhaled long-acting β_2 agonist (LABA) 2. Assess control of asthma: • good response to LABA – continue LABA • benefit from LABA but control still inadequate – continue LABA and increase inhaled steroid dose to 800 mcg/day *. If control is still inadequate, institute trial of other therapies, leukotriene receptor antagonist or sustained release (SR) theophylline</p>		
STEP 1				
<p><u>Mild intermittent asthma:</u> Inhaled short-acting β_2 agonist as required</p>	<p><u>Regular preventer therapy:</u> Add inhaled steroid 200 – 800 mcg /day * 400 mcg is an appropriate starting dose for many patients</p>			

Table 1.1.2: Guidelines for the Management of Asthma in Adults (10)

mcg ; micrograms

* BDP (beclometasone given via a CFC-metered dose inhaler) or equivalent

A stepwise approach aims to abolish symptoms as soon as possible and to optimize peak flow by starting treatment at the level most likely to achieve this. Patients should start treatment at the step most appropriate to the initial severity of

asthma. This aim is to achieve early control and to maintain control by stepping up treatment as necessary and stepping down when control is good (10).

Evidence that non-pharmacological management is effective can be difficult to obtain and more well controlled intervention studies are required.

Interventions introduced before the onset of asthma and designed to reduce its incidence (primary prophylaxis) include the promotion of breastfeeding. There is conflicting evidence as to suggest whether aeroallergen and food allergen (particularly in pregnancy) avoidance, weaning, nutritional supplementation (fish oils) and microbial exposure in early life assist in reducing the incidence of asthma. Interventions introduced after the onset of disease and designed to reduce its impact include house dust mite avoidance (in sensitized individuals), animal and fungal allergen avoidance, but there is conflicting evidence to suggest that these interventions are effective. Other interventions include the cessation / avoidance of smoking, immunotherapy where a clinically significant allergen cannot be avoided and weight reduction in clinically obese asthmatics (10).

The diagnosis of asthma in early childhood is challenging and has to be based largely on clinical judgment and an assessment of symptoms and physical findings. Since the use of the label 'asthma' for wheezing children has important clinical consequences, it must be distinguished from other causes of persistent and recurrent wheeze (5). Quantitative parameters of respiratory function such as FEV₁ can be used effectively in most patients of 5 years of age or older (11). Diagnostic challenges include the assessment of cough-variant asthma, exercise-induced bronchoconstriction, and occupational asthma (6), and some symptoms of congestive heart failure, pulmonary embolism, and cystic fibrosis (12) may also be similar to those symptoms present in asthmatics.

1.1.3 Epidemiology

Asthma is a common disease and remains a significant cause of morbidity and mortality worldwide. Despite hundreds of reports on the prevalence of asthma, the lack of a precise and universally accepted definition of asthma makes a reliable comparison of reported prevalence problematic. Nonetheless, based on the application of standardized methods such as measuring FEV₁ or PEF to measure the prevalence of asthma and wheezing illness in children and adults, it appears that the global prevalence of asthma ranges from 1% to 18% of the population in different countries. Mortality does not appear to correlate well with prevalence, and there is insufficient data to determine the likely causes of the described variations in prevalence within and between populations (5). In the UK, it affects 9% of children and 5% of adults, and its prevalence continues to increase (13).

Direct costs of asthma includes costs to the NHS of £754 million a year (14), arising from GP consultations, prescriptions, and hospital admissions. There are also indirect costs which are more difficult to assess - such as resources lost, including a loss of productivity from lost work days, early retirement or premature death. Intangible costs include the impairment of the quality of a patient's life. (15).

Comparisons of the cost of asthma in different regions lead to a clear set of conclusions. The costs of asthma depend on the individual's patient's level of control and the extent to which exacerbations are avoided. Emergency treatment is more expensive than planned treatment. Non-medical economic costs of asthma are substantial. Guideline-determined asthma care can be cost effective. Families can suffer from the financial burden of treating asthma (5).

Although from the perspective of both the patient and society the costs to control asthma seem high, the cost of not treating asthma correctly is even higher. Proper treatment of the disease poses a challenge for individuals, health care professionals, health care organizations, and governments. There is every reason to believe that the substantial burden of asthma can be dramatically reduced through efforts by individuals, their health care providers, health care organizations, and local and national governments to improve asthma control (5).

1.1.4 Clinical Expression

Clinical features in adults that influence the probability that episodic respiratory symptoms are due to asthma include;

- More than one of the following symptoms:
 - wheeze, shortness of breath, chest tightness and cough,
 - particularly if symptoms are worse at night and in the early morning,
 - or in response to exercise, allergen exposure and cold air,
 - or after taking aspirin or β -blockers
- Widespread wheeze heard on auscultation of the chest
- Otherwise unexplained low ^(*) FEV₁ or PEF (historical or serial readings).
^(*) Predicted normal values for FEV₁ (measured in litres) and PEF are dependent on the subject's height, age, sex and weight.
- History of atopic disorder
- Family history of asthma and / or atopic disorder (16).

Initial diagnosis must be based on a careful assessment of symptoms and a measure of airflow obstruction:

- Patients with a high probability of asthma can be moved straight to a trial of treatment. Further testing can be reserved for those whose response to a trial of treatment is poor.
- The preferred approach in patients with an intermediate probability of having asthma is to carry out further investigations, including an explicit trial of treatments for a specified period, before confirming a diagnosis and establishing maintenance treatment.
- Patients with a low probability of asthma, whose symptoms are thought to be due to an alternative diagnosis must be investigated and managed accordingly. A diagnosis of asthma must be reconsidered in those patients who do not respond (10).

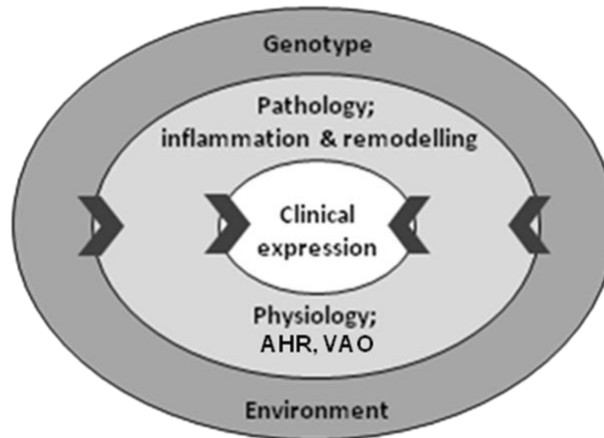


Figure 1.1.4: Factors Influencing the Clinical Expression of Asthma

Factors such as genotype and environment, physiology and pathology can influence the clinical expression of asthma.

1.1.5 Abnormal Physiology

The clinical presentation of recurrent episodes of wheezing, shortness of breath, chest tightness and coughing are associated with AHR and VAO. Investigations used to examine the abnormal physiology and which would be useful in determining the presence of airway narrowing and assist in the diagnosis of asthma include;

- The study of AHR through a methacholine PC_{20} challenge. PC_{20} is the provocative concentration of methacholine required to cause a 20% fall in FEV_1 . An application of more than 16mg/ml to induce 20% fall in FEV_1 could be interpreted as normal bronchial responsiveness (BR), 4 – 16mg/ml could be borderline BR, 1 – 4mg/ml could be mild BR, and less than 1mg/ml could be moderate to severe BR (17).

Investigations which would also be useful in examining the presence of inflammation and assist in the diagnosis of asthma include;

- The examination of FE_{NO} - exhaled nitric oxide concentration. An FE_{NO} of less than 25 parts per billion (ppb) is considered to be the normal range. More than 50ppb is indicative of eosinophilic airway inflammation (16).
- A sputum eosinophil count can also be examined. A normal range is considered to be less than 2% (of the total cell count) (16), but eosinophilia is not always present in asthmatics, and alternative classification methods include examining non-eosinophilic asthmatic phenotypes (18);

	Non-eosinophilic asthma	Eosinophilic asthma
	Normal eosinophil count	Raised eosinophil count (>2%)
Normal neutrophil count	Paucigranulocytic <ul style="list-style-type: none"> • Well controlled asthma • Intermittent asthma • Consider alternative diagnosis 	Eosinophilic <ul style="list-style-type: none"> • Typical asthma, frequently associated with atopic disease • May indicate inadequate corticosteroid therapy
Raised neutrophil count (>61%)	Neutrophilic <ul style="list-style-type: none"> • Associated with acute or chronic infection, smoking, pollutants, occupational antigens, endotoxin exposure, obesity. 	Mixed granulocytic <ul style="list-style-type: none"> • (Severe) asthma exacerbations • Refractory asthma

Table 1.1.5a: Classification Based on Induced Sputum Patterns of Cellular Inflammation in Asthma (18)

While many of the immunopathological features of asthma are observed in the airways of patients with eosinophilic bronchitis (EB) (19-21) there are crucial differences. EB is characterized by a corticosteroid responsive cough, eosinophilia detectable in sputum and unlike asthma, patients with EB present without VAO or AHR (22),(23). Critically, there are almost no mast cells present in the ASM in patients with EB (19). The number of mast cells per mm² of ASM is significantly higher in subjects with asthma than in EB (19;24). The infiltration of ASM by mast cells could be associated with the disordered airway function found in asthma, and indicates an important role for ASM and mast cells in this condition.

	Asthma	Eosinophilic bronchitis
Symptoms	Dyspnoea, cough, wheeze	Cough and associated upper airway symptoms.
Cough reflex hypersensitivity	Normal / increased	Increased
Atopy	Common	Same as general population
AHR	Present	Absent
VAO	Present	Absent
Mast cell – ASM colocalisation	Present	Absent
Sputum Eosinophilia	Usually	Always at diagnosis
Bronchial biopsy; eosinophilic infiltration	Yes	Unknown
Bronchoalveolar lavage; eosinophilia	Yes	Yes

Table 1.1.5b: Characteristics of Asthma & Eosinophilic Bronchitis (25)

1.1.6 Inflammation

AHR and VAO result in airway narrowing, and this is the final common pathway leading to symptoms and physiological changes in asthma. Abnormal physiology underlying the development of airway narrowing in asthma includes;

- Airway edema, which could be due to increased microvascular leakage, and mucus hypersecretion, which may lead to luminal occlusion. Both may be due to an increase in cytokine synthesis from inflammatory cells.
- Airway thickening due to structural changes such as increased smooth muscle mass, and is often termed “remodeling.”
- Airway smooth muscle (ASM) contraction in response to multiple bronchoconstrictor mediators, possibly from inflammatory cells. This is the predominant mechanism of airway narrowing (5).

There is a complex relationship between various triggers of airway inflammation, the pattern of inflammation generated, the diseases they are associated with, the outcomes in terms of disordered airway physiology and the associated pathological abnormality. Each phenotype is associated with a more or less specific pathological abnormality, which gives clues to pathogenesis and assists in treatment of the disorder (26).

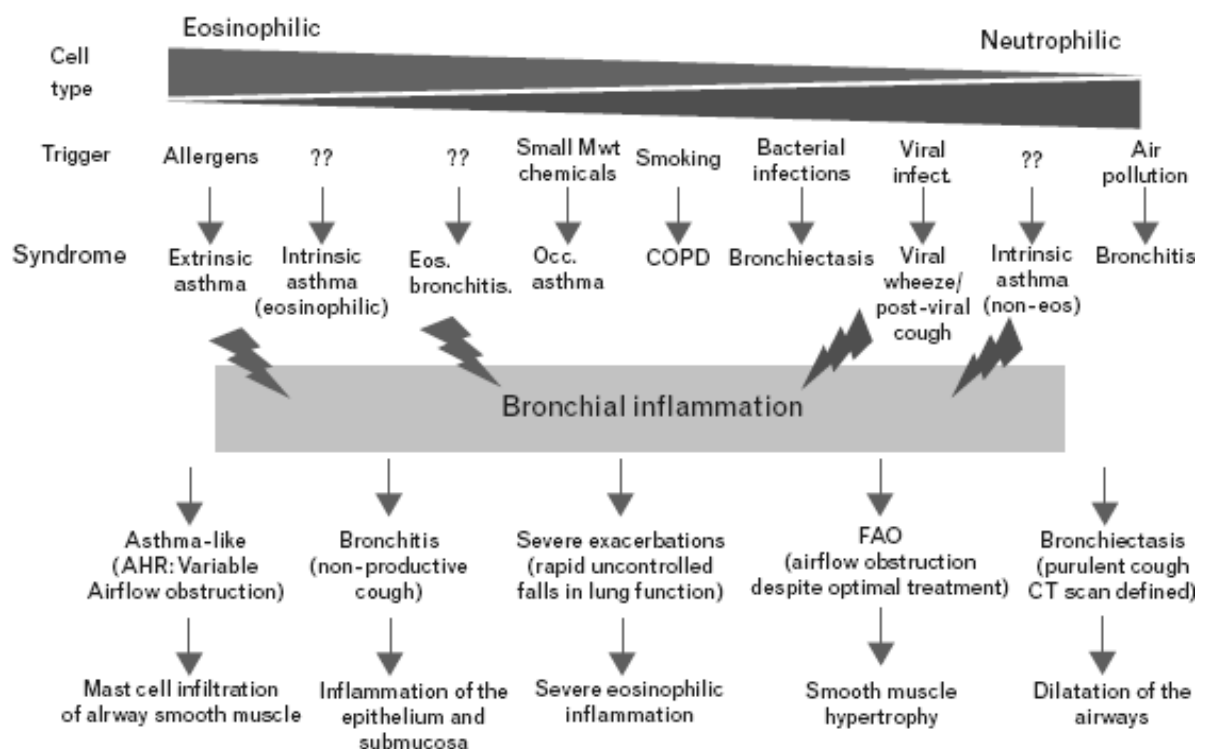


Figure 1.1.6: Airway Inflammation and Asthmatic Phenotypes (26)

In asthma, inflammation is a multicellular process involving mainly mast cells, T-cells, neutrophils, eosinophils and macrophages (27). The inflammatory process is largely restricted to the conducting airways but as the disease becomes more severe and chronic the inflammatory infiltrate spreads both proximally and distally to include the small airways and in some cases the adjacent alveoli. In the large airways, inflammation of the submucosa and mucous glands dominates, whereas in the small airways, inflammation appears to be predominantly outside the airway smooth muscle (28;29).

In relation to the epithelium, increased numbers of epithelial mast cells (30-32), T-cells, eosinophils and macrophages (30) have been observed in asthmatics compared to controls, along with increased numbers of the aforementioned cells with neutrophils after allergen challenge in asthmatics (33). Intraepithelial mast cells obtained from bronchial brushings of subjects with ICS-responsive chronic cough (a condition similar to EB) were found to be increased compared to asthmatics (23). Concentrations of mast cell mediators histamine and PGD₂ are reported to be significantly higher in sputum samples from subjects with EB and not asthma when compared to normal subjects (34), suggesting that mast cell infiltration into the superficial airway may be a particular feature of this condition.

In relation to the localization of inflammatory cells to mucosal glands in the proximal airways, increased numbers of mast cells and neutrophils were observed in asthmatics compared to controls, and the degree of mast cell degranulation increased with the severity of the disease (35).

In relation to the localization of inflammatory cells to ASM, increased numbers of mast cells (19;24) – particularly in the ASM of the small airways (36) and T-cells (24) were observed in asthmatics compared to subjects with eosinophilic bronchitis and normal controls.

Existing data suggests that there is (37;38) or isn't (39) a significant correlation between airway inflammation and AHR, but studies have been restricted to the examination of eosinophils or eosinophil products such as eosinophil cationic protein (ECP) as markers of inflammation, and have not examined the presence of cells within the ASM.

If patients use a short-acting β_2 -adrenoceptor agonist (Step 1 in the guidelines for asthma management) more than 1 – 2 times (puffs) a day, then the addition of inhaled corticosteroids (ICS) (Step 2 in the guidelines for asthma management) is recommended (10;40). While the efficacy of short acting β_2 -adrenoceptor agonists lie in bronchodilation (effective over 4 – 6 hours), the efficacy of ICSs is due primarily to the suppression of airways inflammation and associated AHR. Acting via the glucocorticoid receptor (GR), ICSs repress the expression of inflammatory cytokines, their receptors and inflammatory cell adhesion molecules. These effects of ICSs, and their ability to promote apoptosis of many cell types including the eosinophil, act to reduce the pulmonary leucocyte burden and attenuate inflammation (40).

1.1.7 Remodeling

Airway remodeling is negatively associated with prebronchodilator and postbronchodilator FEV₁ values in asthmatic subjects (41). Airway inflammation could cause the abnormal physiology and remodeling that is observed in asthma, therefore ultimately contributing towards AHR and VAO.

Features of remodeling in asthma include increased subepithelial basement membrane thickness in all severities of asthma (compared to controls). In mild to moderate and severe persistent asthmatics there is increased myofibroblast infiltration into the subepithelial basement membrane, mucous gland area and ASM area. In severe persistent asthmatics, there is increased collagen III deposition from the subepithelial fibroblasts and a reduced subepithelial basement membrane to ASM distance (41;42).

Epithelial thickness is greater in severe asthmatics compared to mild asthmatics or controls and is a feature of remodeling. There is no significant difference in epithelial desquamation between groups, but there are increased levels of epithelial proliferation and apoptosis in severe asthmatics (43). Mast cell tryptase could increase the proliferation of epithelial cells (44), while conversely T-cells (using IFN- γ and TNF- α) and eosinophils (using ECP) could also induce apoptosis (45) resulting in an increased turnover of the epithelial cells.

Along with T cells, mast cells are also capable of producing cytokines such as GM-CSF, IL-8, IL-13 as well as tryptase contributing to the airway inflammation and disorder that is observed in eosinophilic bronchitis and asthma. Tryptase from mast cells could activate the PAR-2 receptor on epithelial cells, and induce the release of GM-CSF. This could result in the promotion of survival of eosinophils (46). IL-8 has been reported to reduce cilia beat frequency in epithelial cells, reducing the potential for aeroallergen or particulate clearance (47), and prolonged IL-13 exposure contributes to subepithelial fibrosis (48).

Airway epithelium in asthma is fundamentally abnormal with goblet cell hyperplasia and increased mucin secretion resulting in an occluded airway, particularly in mild-moderate and severe persistent asthmatics (41;49;50), and EB (22;51).

The increased mucus secretion could be caused by mast cells after aggregation of Fc ϵ RI. This increases the production of amphiregulin, which in turn upregulates mucin gene expression in airway epithelial cells. Upregulation of amphiregulin expression has been observed in the mast cells of asthmatics compared to controls (52). Mast cell derived mediators such as IL-13 could also increase the expression of pendrin, which in turn also upregulates mucus production (53). Neutrophil elastase also increases mucus production (54).

ASM area has been found to be significantly increased in the airways of mild-to-moderate and severe asthmatic subjects (24;41), the severity of which has been found to increase with age (55).

1.2 The Role of Airway Smooth Muscle In Asthma

Airway smooth muscle plays a critical role in asthma through a variety of mechanisms;

- Contributing to the inflammatory process arising from the synthetic response of ASM.
- Contributing to airway remodeling through increases in ASM area.
- Contributing to AHR through mesenchymal cell differentiation and increased ASM contractility.

1.2.1 Inflammation and the Synthetic Response of ASM

The traditional view of ASM in asthma is that it is a passive partner in airway inflammation, contracting in response to proinflammatory mediators and neurotransmitters and relaxing in response to endogenous and exogenous bronchodilators. Therefore early studies of ASM function in asthma tended to concentrate on the contractile aspects to explain AHR. It has become apparent that this paradigm was a rather simplistic view and that ASM has several other important properties of relevance to obstructive lung diseases (56).

Transforming growth factor- β (TGF- β) (57), PDGF (58), connective tissue growth factor (CTGF) (59;60), basic fibroblast growth factor (bFGF), nerve growth factor (NGF) (61), vascular endothelial growth factor (VEGF) (60;62), prostaglandin E₂ (PGE₂) (63), CX₃CL1 (fractalkine) (64) CCL2 (MCP-1) (65), CCL5 (RANTES) (66-68), IL-6 (69), CXCL8 (65;70), CXCL10 (71;72), CCL11 (Eotaxin) (73) and CCL19 (MIP-3 β) (74) can all be produced by ASM cells.

A role for ASM cells as secretory cells involved in the recruitment of inflammatory cells is highlighted by studies showing intra-ASM inflammation with the localization of mast cells and T-cells to asthmatic ASM bundles (19;24;36). Mast cell migratory stimuli include CXCL8 (65;70) and CXCL10 (71;72), the latter of which is also a migratory stimuli for T-cells (75). Besides producing mast cell chemotaxins, asthmatic ASM may also be incapable of producing a mast cell-trafficking inhibitory factor that is produced in non-asthmatic ASM (73).

The production of cytokines by ASM can be influenced by mast cells and T-cells which co-localise with ASM (24). For example, histamine (66) and β -tryptase (76) from mast cells have both been reported to reduce RANTES released by ASM, while TNF- α alone and in combination with IFN- γ increases RANTES expression by ASM (67). β -tryptase has also been reported to increase TGF- β production (57;77), or alternatively increase the level of active TGF- β (78). IFN- γ and IL-1 β can increase NGF expression, while IL-4 can decrease IL-1 β -stimulated NGF expression (61), and IL-4 along with IL-5, IL-10, IL-13 and TGF- β can increase VEGF production. While IFN- γ can individually reduce and attenuate IL-4, IL-5, IL-13 and TGF- β mediated VEGF expression (62).

ASM derived cytokines can also act in an autocrine manner. For example, IL-6 can stimulate bFGF production (69), and TGF- β 1 can stimulate IL-8, PGE₂ (63), CTGF and VEGF production – the latter of which is augmented by PGE₂. (60).

In relation to the extracellular matrix, the deposition of collagen I, III, V, fibronectin, tenascin, hyaluronan, versican and laminin α 1 / β 2 is increased in asthmatics,

whereas the deposition of collagen IV and elastin is decreased (79). Interestingly, TGF- β can upregulate mRNA expression for collagen I and fibronectin along with collagen IV and elastin from ASM cells (80), but TGF- β can also upregulate CTGF from ASM cells, the production of which is increased in asthmatics (59). CTGF in turn upregulates the production of collagen I and fibronectin (81), and the increases in CTGF, collagen I and fibronectin can be attenuated by PGE₂ (60). While both TGF- β 1 and TGF- β 2 increase CTGF expression, only the latter isotype can induce elastin expression (82;83).

1.2.2 Airway Remodeling and Increases in ASM Area

ASM area has been found to be significantly increased in the airways of mild-to-moderate and severe asthmatic subjects (24;41), the severity of which has been found to increase with age (55).

Increases in ASM area are important in the pathogenesis of asthma as the effects of reducing the increased smooth muscle mass can be observed through examining the effects of bronchial thermoplasty. This involves the application of controlled radio frequency energy to selective areas of the airway wall in an attempt to reduce ASM mass. When this technique is applied to moderate or severe asthmatics, there have reported improvements in morning PEF, the percentage of symptom-free days and quality of life and symptom scores compared to the control group, but no difference between groups for airway responsiveness and FEV1. Adverse effects were also reported to be more common immediately after treatment, but similar during the period from 6 weeks - 12 months after treatment (84).

An increase in ASM area in asthma could arise from ASM hyperplasia, hypertrophy, increased cell survival or migration of ASM.

In relation to ASM hyperplasia, evidence indicates that in mild-to-moderate asthmatics, increased number of ASM cells may be responsible for the increase in ASM area (85), but there are conflicting reports as to whether there are (86) or aren't (24;41) increased levels of proliferation are present in asthmatic subjects. The presence of mast cells within the airway smooth muscle bundles could also contribute towards smooth muscle hyperplasia.

For example, histamine (87) and tryptase (88;89) have been reported to increase the proliferation of ASM. LTC₄ is produced by mast cells and is converted to extracellularly to Leukotriene D₄ (LTD₄) (90). LTD₄ has been reported increase the production of TGF- β (91) and to increase the proliferation of ASM cells, but as a result of the upregulation of the cysteinyl leukotriene type 1 receptor (CysLT₁R) by interleukin-4 (IL-4), interleukin-13 (IL-13) and TGF- β (92), and to also augment EGF mediated ASM proliferation (80). Platelet derived growth factor (PDGF) can also increase the proliferation of ASM cells, but its effect is inhibited by IL-4 (93;94). PDGF is also released by airway smooth muscle cells (58). TGF- β has been reported to promote proliferation in confluent ASM cells (95) or have no effect on proliferation in subconfluent cells (91;96;97), but other data suggests that TGF- β drives the proliferation of ASM regardless of confluence (98). TNF- α has been

reported to have no significant effect (99) on ASM cell numbers, but induces a proliferative response if IFN- β is neutralized (100). In contrast, heparin (101;102) has been reported to have anti-proliferative effects.

In relation to ASM hypertrophy, ASM cell size has been shown to be similar between mild-moderate asthmatics when compared to controls (41;85), but greater in subjects with severe asthma (24;41). Values of pre and postbronchodilator FEV₁ have been shown to negatively relate to ASM area and cell size (41). Significantly more mast cells are localized within the ASM bundles in asthmatic airways compared to non-asthmatics (19;24) – particularly in the ASM of the small airways (36). The presence of mast cells within the ASM bundles could contribute towards smooth muscle hypertrophy. Mediators from mast cells that can affect ASM cell size include histamine (87) and TGF- β (96). These mediators have been reported to induce hypertrophy, as measured by either an increase in cell size or in the protein content of the ASM cells.

Phenotyping of increased ASM in asthmatics has suggested that increases in ASM mass restricted to the central bronchi may be related to hyperplasia rather than hypertrophy, but if increases in ASM mass are observed throughout the airways then hyperplasia is mild and localized in the bronchi and hypertrophy is observed throughout the airway. This would suggest that hypertrophy may be a secondary feature of hyperplasia in mechanisms related to an increase in ASM mass (103).

In relation to the extracellular matrix (ECM), the deposition of collagen I, III, V, fibronectin, tenascin, hyaluronan, versican and laminin $\alpha 1 / \beta 2$ is increased in asthmatics, whereas the deposition of collagen IV and elastin is decreased (79). Research has shown that culture of airway smooth muscle on collagen I enhances the proliferative activity of the smooth muscle cells (104). Indeed, the state of proliferation of the smooth muscle cells can have a direct effect on the expression of contractile proteins as discussed further on in the introduction.

There are also suggestions that an increase in cell survival may be responsible for the increase in ASM mass as mediated by extra-cellular matrix factors such as fibronectin and collagen I as important anti-apoptotic elements (105).

Another possible contributing factor towards the increased smooth muscle mass observed in the airways of asthmatics is that smooth muscle progenitors may migrate from the blood to the smooth muscle bundles.

The presence of mast cells within the airway smooth muscle bundles could contribute towards smooth muscle migration. For example, airway smooth muscle migratory mast cell derived stimuli examined *ex vivo* include interleukin-1 β (IL-1 β), TGF- β (106), PDGF (106-109), basic fibroblast growth factor (bFGF) (108), chemokine ligand 11 (CCL11) (109), and CCL19 (74).

Evidence to support the idea of smooth muscle progenitor migration arises from a study in which allergen exposure induced the accumulation of fibrocyte like cells in the bronchial mucosa of patients with allergic asthma. These cells were CD34-positive; expressed collagen I, α -smooth muscle actin (α -SMA) and localized to areas of collagen deposition below the epithelium.

Labeled circulating fibrocytes were also tracked in a mouse model of allergic asthma, showing that fibrocytes are indeed recruited into the bronchial tissue following allergen exposure and differentiate into myofibroblasts. The researchers also illustrated how human circulating fibrocytes acquired the myofibroblast phenotype (reduced CD34 expression and increased α -SMA expression) under *in vitro* stimulation with fibrogenic cytokines (endothelin-1 and TGF- β) that are produced in exaggerated quantities in asthmatic airways. These results would suggest that circulating fibrocytes may function as myofibroblast precursors and may contribute to the genesis of subepithelial fibrosis in asthma (110).

1.2.3 AHR, Mesenchymal Cell Differentiation and Increased ASM Contractility

Smooth muscle is located around the walls of 'hollow' organs such as blood vessels, stomach, intestine, uterus, ureter and lungs. In the upper airway, smooth muscle is found in the posterior aspect of the trachea attached to the cartilage. In the lower airways, smooth muscle is orientated in a geodesic (111) / helix-antihelix pattern encasing the bronchi.

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. In the upper airways, smooth muscle accounts for approximately 25% of the cellular mass, while in the lower airways, smooth muscle constitutes 4 – 5% of the cellular mass (112).

ASM can be described as phenotypically plastic, differentiating from smooth muscle progenitors – labeled as fibrocytes. These can be identified as either CD34⁺ and collagen 1 double positive cells that stain positive for α -SMA after localization within the subepithelial layer of the airway wall (110).

Fibrocytes may then differentiate into fibroblasts to myofibrocytes, then into ASM and potentially de-differentiating depending on environmental influences. These phenotypically variable cells can be identified and characterized by the sequential appearance of cytoskeletal and contractile proteins to yield a phenotype that is characteristic of ASM cells.

	Fibrocyte	Fibroblast	Myofibroblast	ASM
CD34	++++	-	-	-
Collagen I	++++	+++	++	+
α -SMA	+	++	+++	++++
MHC (SM2)	?	++	+++	++++
MLCK	?	++	+++	++++
H1 calponin	?	++	+++	++++
H-caldesmon	?	++	+++	++++
L-caldesmon	?	++	++	++
α -Tropomyosin	?	++	++	++++
Desmin	?	++	++	++++


```

graph TD
    Fibrocyte --> Fibroblast
    Fibrocyte --> ASM
    Fibrocyte --> Myofibroblast
    Fibroblast --> ASM
    Fibroblast --> Myofibroblast
    ASM <--> Myofibroblast

```

+; low level of expression

++++; high level of expression

Table1.2.3: Differentiation of Fibrocytes

Two states of differentiation which are often described – synthetic and contractile – likely represent the ends of a spectrum of phenotypes rearranging from the undifferentiated state expressing few, if any, smooth muscle specific proteins, to the fully differentiated phenotype expressing a complete repertoire of contractile proteins (113;114).

1.2.4 Components of ASM

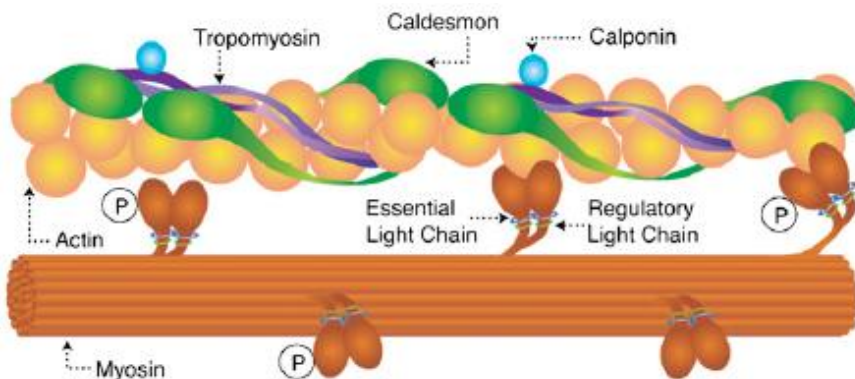


Figure 1.2.4: Components of ASM (115)

Actin

Among the proteins which can be used to identify ASM cells is the thin filament actin, which is contained in all eukaryotic cells. This is a globular protein (G-actin) which can polymerize to form thin myofilamentous structures (F-actin) which are important for cell motility and determination of cell structure as well as contraction

(116). In muscle cells, actin comprises of 10% by weight of the total cell protein (117). Six isoforms of actin have been identified, of which at least three isoforms; α , β and γ exist in tracheal smooth muscle. Northern blot analysis of acutely dissociated tracheal smooth muscle cells confirmed that transcripts encoding for smooth muscle α -actin and β / γ -actin were present and existed in a ratio of 75:25 (116). The 43kDa α -smooth muscle actin (α -SMA) has been reported to be of the first smooth muscle markers expressed, though its expression is not restricted to airway smooth muscle. It is also transiently expressed in the early stages of differentiation of both cardiac and skeletal myocytes, myofibroblasts in healing wounds and tumours, pericytes and juxtaglomerular cells (118;119), as well as hair follicle cells (120). α -SMA has been reported to be present in low quantities in rapidly proliferating *ex vivo* cultures, but increases as cultures reach confluence or are serum deprived (121;122).

Expression of α -SMA has been reported not to be increased in the airways of asthmatics (41;85), but the method of sampling cells must be taken into account. The intensity of α -SMA expression has been reported to vary in smooth muscle throughout the lung. For example, proximal airway fibroblasts have been reported to express less α -SMA than distal lung fibroblasts (114). Evidence suggests that lung fibroblasts which express higher levels of α -SMA possess an enhanced ability to wrinkle deformable silicone substrates and contract collagen gels than those which express lower levels of α -SMA. Also, only lung fibroblasts with higher levels of α -SMA were able to deform more rigid silicone substrates (123).

Myosin

Myosin is the primary protein of the thick filament in smooth muscle and is composed of two high molecular-weight subunits or heavy chains, and two each of two types of low molecular weight subunits, or light chains (124). The large asymmetric protein consists of the six polypeptide chains: two 205kDa heavy chains (MHC) that form a dimer and two pairs of light chains - the 20kDa regulatory chains and the 17kDa essential light chains. The myosin heavy chain dimer makes up the main body of the molecule, with each heavy chain containing a slightly elongated globular head at the amino terminus (or N-terminus). A single essential and regulatory chain is associated with each myosin head. There are two isoforms of the myosin heavy chain – SM1 (204 kDa) and SM2 (200kDa) specific to smooth muscle, the latter of which is considered to be a marker of mature airway smooth muscle (125). Expression of SM1 or SM2 has been reported not to be increased in the airway smooth muscle of asthmatics (41).

Myosin Light Chain Kinase

Myosin light chain kinase (MLCK) is a ubiquitous protein present in almost all eukaryotic cells and plays a role in a variety of actomyosin-related biological reactions (such as the abnormal contraction of arteries) (126). The smooth muscle specific isoform of MLCK is reported to be 130 kDa (127). The content of MLCK is

reported to be halved in proliferative airway smooth muscle in comparison to non-proliferating cells (128), and could therefore be considered to be a marker of mature smooth muscle cells. Expression of MLCK and phosphorylated myosin light chain (p-MLC) has been reported to be significantly increased in the airway smooth muscle of intermittent, mild-to-moderate and severe asthmatics (41;85), while other researchers report no difference in the expression of MLCK (85). Values of pre and postbronchodilator FEV₁ negatively related to MLCK expression (41).

Calponin

Basic calponin (H1) is a 34kDa protein expressed exclusively in smooth muscle cell rich tissues, and is considered to be a phenotypic marker of differentiated smooth muscle cells (129). It binds to actin and calmodulin and is therefore thin filament associated. It is able to inhibit the ATPase activity of myosin and is thought to play a role in smooth muscle contraction (130). Other varieties of calponin include the 34 kDa neutral H2 calponin which is expressed in low levels in smooth muscle and is also expressed in non-smooth muscle cells. The 29 kDa acidic calponin is expressed in both smooth muscle and non-smooth muscle cells such as the heart, placenta, lung, kidney, pancreas, spleen, testis and ovary (131;132).

Caldesmon

H-caldesmon is found on the actin thin filament beside tropomyosin, arranged continuously along the axis of the actin double helix (133). Interacting with calmodulin and in the presence of tropomyosin, caldesmon can inhibit actomyosin ATPase activity at the low ends of ratios of caldesmon to actin likely to occur *in vivo* (1:14) (134). The 150 kDa H-caldesmon is the primary isoform in smooth muscle (135), and is also located on the stress fibres of fibroblasts, while L-Caldesmon (70 – 80 kDa) is most abundant in non-muscle cells (136).

Tropomyosin

Tropomyosins (Tm) are rod-like helical proteins that dimerize and bind to actin. In smooth muscle cells, Tm's are likely to play a role in the stabilization of the smooth muscle α -actin contractile filaments. There are over 40 isoforms of Tm and most are found in non-muscle cells. Only two appear to be specific to smooth muscle – one from the β Tm gene (Tm1), and the other from the α Tm gene. α -smooth muscle Tm is a 40 kDa protein reported to be more specific to smooth muscle since it contains a unique exon 2a, not found in any other Tm, whereas β smooth muscle Tm uses the same 2b exon as other isoforms. α -smooth muscle Tm is also reported to be a late onset protein, appearing much later than actin and is a good marker of mature differentiated smooth muscle cells (137).

Desmin

In ASM the primary intermediate filament is desmin (138). The muscle specific 53 kDa protein (139) runs the length of the cell and exhibits ramifications to the cell membrane. They are less numerous than the myofilaments and are generally believed to play a structural role in maintaining the organization of the myofilament system (140). During development, levels of desmin expression vary, being present at low levels in the early stages of myofibroblast replication and increasing during terminal differentiation, therefore being expressed in both differentiating and mature muscle cells (141),(142). It is considered to be a marker of mature smooth muscle cells but is not specific for smooth muscle, as it is present in cardiac and skeletal smooth muscle as well (128).

1.2.5 Expression and Modulation of ASM Proteins

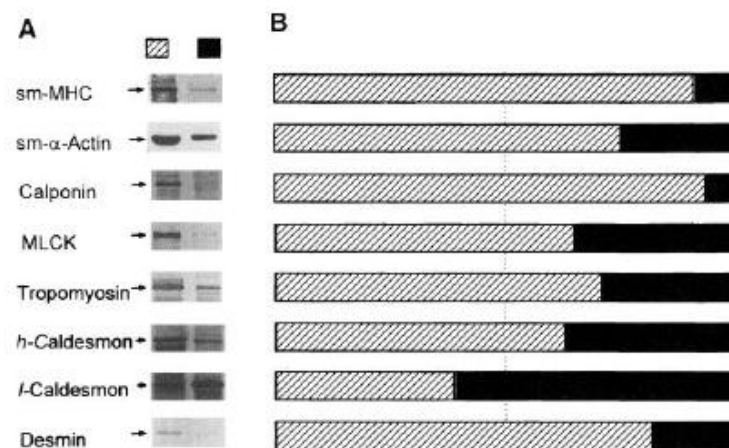


Figure 1.2.5: Expression of Markers of ASM Phenotype (128)

The content of these markers will change as cells are moved from *in vivo* to *ex vivo* conditions. As illustrated on the above figure, the hatched bars from the figure represent the abundance of specific smooth muscle proteins in freshly isolated tracheal smooth muscle cells in comparison with those in primary cultures just 7 days after seeding as represented by the solid bars (128). This analysis was achieved by using Western Blot analysis and scanning laser densitometry. Results show that there is a lower level of expression of almost all markers except in cultured cells, but the expression of α -SMA, tropomyosin and H-caldesmon can still be easily detected.

It has been reported that serum deprivation induces a distinct differentiation pathway in cultured myocytes, and allows the cells to reacquire the abundant contractile protein content, marked shortening capacity, and the elongated morphology characteristic of contractile cells within intact tissue (143). Serum deprivation would be useful when preparing cultured airway smooth muscle cells for experiments and investigations.

As described, ASM is a dynamic and complex cell type, whose normal contractile activity is controlled by internal components such as the contractile apparatus and

regulatory proteins. The inclusion of other resident airway cells such as mast cells could affect the expression of ASM contractile proteins.

HMC-1 cells induce α -SMA expression in dermal fibroblasts, and also stimulate the dermal fibroblast contraction of collagen cells, with the relative amount of contraction dependent upon the number of HMC-1 cells present (collagen gels containing HMC-1 cells alone did not contract). The same researchers also found that mast cell constituents such as histamine, tryptase and TGF- β all increased the α -SMA expression of the fibroblasts, but interestingly, only tryptase and TGF- β stimulated the dermal fibroblasts to contract the collagen gel (144).

In combination with the anti-proliferative effects, heparin increases α -SMA expression (102). IL-4 and TGF- β increase α -SMA expression in smooth muscle (145). PDGF-BB alone (122), and in combination with IL-1 β (146) has been reported to decrease α -SMA, sm-MHC and calponin expression. IFN- γ has been reported to decrease α -SMA expression (147). IFN- γ (148) and bFGF (149) both inhibit TGF- β mediated increases in α -SMA.

Other mechanisms that could account for the enhanced contractile ability of the ASM after co-culture with mast cells could include a role for cell to cell contact. The SCF / c-kit / CD117 receptor could be important after researchers discovered that smooth muscle – mast cell contact was necessary for the contraction of the collagen gels, and the contraction was inhibited by nearly 70% after antibodies against SCF and c-kit were added to the co-cultures (150). Gap junction intercellular communication (GJIC) could also be important after the addition of GJIC inhibitors were found to prevent the contraction of collagen gels seeded with dermal fibroblasts and HMC-1 cells (151)

1.2.6 The Effect of TGF- β in ASM Differentiation

TGF- β 1 is a chemoattractant and mitogen to fibroblasts (152;153), and induces fibroblasts to synthesize a variety of extracellular matrix proteins such as collagen (154). As previously mentioned, TGF- β 1 and TGF- β 2 are both known to increase α -SMA expression in smooth muscle (145;148;149;155).

Defects in TGF- β function are associated with a number of pathological states including over expression resulting fibrosis (156), and defects in receptors and signaling pathways resulting in aneurysm syndromes (157) and inflammation (158). The importance of TGF- β in mammalian biology is highlighted by the lack of viability in TGF- β ^{-/-} mice. TGF- β null mice die within 3 weeks due to widespread inflammatory disease (159).

TGF- β exists in three isoforms in mammals: TGF- β 1, TGF- β 2, and TGF- β 3 (160-162) with TGF- β 1 similar, albeit not identical, to TGF- β 3 and much more potent than TGF- β 2 (163). TGF- β 1 and TGF- β 2 appear to be the most common isoforms associated with the disorders characterized by inflammation and fibrosis (160), due to observations at the site of injury such as the levels in the BAL of asthmatics in comparison with normal controls (164)

TGF- β is synthesized as a 100-kDa pro-TGF- β (162), and before secretion, the pro-region - called the latency-associated peptide (LAP), is cleaved but remains non-covalently (162) but disulphide linked (165) to TGF- β . When TGF- β is secreted in association with LAP as latent TGF- β (L- TGF- β), it cannot be associated with its receptor (166) and is therefore inactive. As TGF- β and its receptors are so ubiquitously expressed, the most critical regulation of TGF- β action is the generation of a biologically active form of TGF- β by the removal of LAP (162).

Additionally, L- TGF- β is associated with the matrix high-molecular-weight latent TGF- β binding protein-1 (LTBP-1) (167;168), which binds to L-TGF- β 1 and targets TGF- β 1 to the extracellular matrix (ECM), a process that serves as a reservoir of TGF- β 1 (168).

The plasma serine protease - plasmin has been shown to release L- TGF- β 1 from its association with LTBP-1 (168) and has been demonstrated to activate L- TGF- β 1 by removal of LAP (169;169). Although there is data that suggests that knockout mice lacking the plasma serine protease plasmin proenzyme (plasminogen) display none of the pathological features of TGF- β knockout mice (170).

TGF- β is known to increase levels of TSP-1 production (171). There are also suggestions that TSP-1 production is increased after injury (172), and can activate TGF- β by inducing conformational changes in the latent complexes (173). However, the inflammatory changes seen in TSP-1 knockout mice are not as severe as those seen in TGF- β 1 knockout mice (173), which suggests that several overlapping mechanisms are responsible for TGF- β activation.

ASM has been reported to express α 5, β 1 and β 3 integrins (174), with L-TGF- β associating with α 5, β 1, β 3, β 5, β 6 (175) and β 8 integrins. The α 5 β 6 integrin has been suggested as another mechanism for the activation of latent TGF- β (176), and even though α 5 β 6 integrins recognize a RGD (Arginine-Glycine-Aspartic amino acid sequence) on LAP, a covalent interaction between LAP and LTBP-1, as well as LTBP-1 mediated fixation to the ECM is required for α 5 β 6-mediated latent TGF- β mediated activation (177).

In relation to TGF- β , signaling, the TGF-Beta type II and type I receptors are each made of one small cysteine-rich extracellular region plus an intracellular region consisting mainly of kinase domains. After ligand binding to the TGF-Beta type II receptor, recruitment and phosphorylation of the TGF-Beta type I receptor occurs (178). Type I receptors have a region rich in glycine and serine residues (GS domain) preceding the receptor kinase domain. In the absence of a ligand, type I and type II receptors exist as homodimers at the cell surface. Ligand binding allows the formation of a stable receptor complex, consisting of two receptors of each type and permitting phosphorylation of the type I receptor GS domain by the type II receptor kinases. This phosphorylation stimulates the type I receptor kinases, resulting in the phosphorylation of Smad proteins, and subsequent downstream signaling (178). Type I receptors specifically recognize and phosphorylate ligand-specific receptor-activated Smads (R-Smad). These R-Smads are recruited to interact with the type I receptors by a membrane bound cytoplasmic protein called SARA (Smad Anchor for Receptor Activation). R-Smads include Smad2 and Smad3

downstream of TGF-Beta. Phosphorylation of R-Smads by type I receptors occurs on two serine residues within a conserved –SS(M/V)S- motif at their C-terminus. Upon phosphorylation by type I receptors, R-Smads forms heteromeric complex with the Common-Smad (Smad4 in vertebrates) and translocates into the nucleus for further signaling (178;178).

In humans, the number of TGF-Beta ligands exceeds the number of type II and type I receptors. Thus combinatorial interactions of type I and type II receptors in functional receptor complexes allow for the diversity and selectivity in ligand binding as well as in intracellular signaling. Several accessory cell surface proteins further define the binding efficiency and specificity of the ligand to the receptor complex (178)

1.2.7 ASM Contraction

The early phase of asthma episodes are largely dependent on bronchoconstriction (179), occurring within minutes of allergen challenge and is initiated by ASM contraction (180).

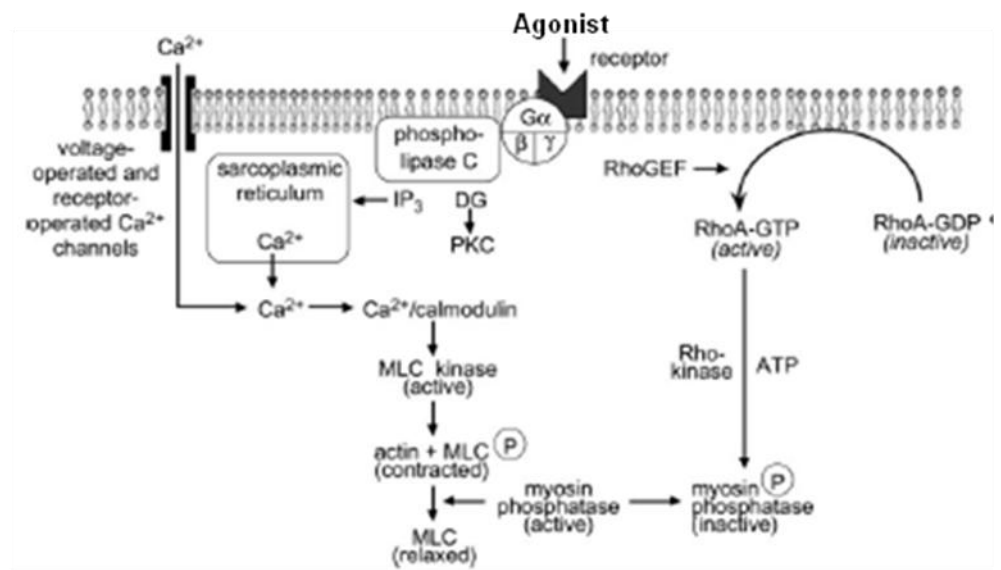


Figure 1.2.7: ASM Contraction (181)

- Various agonists such as acetylcholine bind to specific receptors such as M3 muscarinic receptors to activate contraction in ASM. Subsequent to this binding, phospholipase C activity is increased via binding through a G protein. Phospholipase C produces two potent second messengers from the membrane; lipid phosphatidylinositol 4,5-bisphosphate:diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP₃). IP₃ binds to specific receptors on the sarcoplasmic reticulum, causing the release of activator calcium (Ca²⁺). DG along with Ca²⁺ activates PKC, which phosphorylates specific target proteins. PKC has contraction-promoting effects such as the phosphorylation of Ca²⁺ channels or other proteins that regulate cross-bridge cycling (181).
- Ca²⁺ then binds to calmodulin, leading to activation of MLCK (181). This kinase phosphorylates the light chain of myosin on amino acid residue-serine 19 (182), and

in conjunction with actin, cross-bridge cycling occurs, initiating shortening of the smooth muscle cell (181).

- However, the elevation in Ca^{2+} concentration within the cell is transient, and the contractile response is maintained by a sensitizing mechanism brought about by the inhibition of myosin phosphatase activity by Rho kinase (181). This Ca^{2+} sensitizing mechanism is initiated at the same time that phospholipase C is activated, and it involves the activation of the small GTP-binding protein RhoA. The precise nature of the activation of RhoA by the G protein-coupled receptor is not entirely clear but involves a guanine nucleotide exchange factor (RhoGEF) and migration of RhoA to the plasma membrane. Upon activation, RhoA increases Rho Kinase activity, leading to inhibition of myosin phosphatase. This promotes the contractile state since the light chain of myosin cannot be dephosphorylated (181).

1.2.8 ASM Contractility, Compounding Factors and Airway Narrowing

If the environment present in asthmatics airways promotes the development of a contractile phenotype of ASM, then there are also other factors present which would work with the increased contractile phenotype of ASM to increase the effects of airway narrowing.

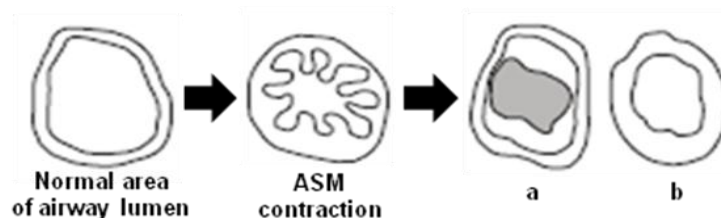


Figure 1.2.8: Schematic Cross-section of the Airway (183)

Airway narrowing due to contraction of ASM would be amplified by a) excessive mucus production, cells or other material within the lumen, and b) remodeling causing thickening of the airway wall that encroaches on the lumen.

The inflammation which contributes towards excessive mucus production and remodeling in the airway wall could cause a decoupling of the ASM from surrounding lung parenchyma and thus a decrease in the load against which ASM must constrict.

This is supported by the observation that in healthy subjects, deep inspirations reduce the level of pharmacologically induced airways obstruction (184), and prevention of deep inspirations in normal subjects can enhance the response to bronchoconstrictors (185). The bronchodilatory effect of deep inspirations (%) is reduced to 68 ± 5.4 % in intermittent asthmatics, 45 ± 7.2 % in mild persistent asthmatics, and 4 ± 15.6 % in moderate-severe asthmatics (186). In non-asthmatic or intermittent asthmatics, deep inspiration causes ASM to fully elongate and energy is dissipated in order to contract the ASM back to its original length – resulting in less ASM force and, by inference, less bronchoconstriction. This is

achieved through the stretch of the airways by the force of interdependence through which they are coupled to the lung parenchyma. In asthma, the airways may be less sensitive to the action of external forces like deep inspiration because the force of interdependence is reduced through peribronchial oedema attenuating the pulling effects of the parenchymal attachments on airway walls (187).

The lack of efficacy of deep inspiration has also been correlated with the increased presence of inflammatory cells such as T-cells in the lamina propria and mast cells in the ASM. The localization of mast cells and release of contractile mediators within the ASM could result in a physiologically altered intrinsic contractile function, leading to an increased formation of actin and myosin cross bridges which are more difficult to disrupt by deep inspiration-induced stretches of the airways (188)

To support this hypothesis, statistically significant increases in maximum shortening capacity and velocity have been observed in asthmatic ASM cells compared with normal cells. Increases in myosin light chain kinase were also observed in asthmatic cells – providing a mechanism for these increases (189). During the course of shortening, initial velocities are high and are functions of the activity of normally cycling (actin-myosin) crossbridges whose myosin light chain is phosphorylated by myosin light chain kinase.

1.3 The Role of Mast Cells in Asthma

1.3.1 Mast Cells

Mast cells may play important roles in adaptive immunity, for example in immune defences against certain parasites in sensitized hosts (190). Mast cells are also crucial in promoting certain autoimmune diseases such as arthritis (191), or play a role in the suppression of other autoimmune diseases such as lupus nephritis (192). Mast cells can also work with T_{REG} T-cells to suppress transplant rejection (193), and are reported to have both promoting and detrimental effects on tumours depending on the local stromal environment (194).

Mast cell numbers are significantly increased in the sputum of asthmatics, with mast cell counts being significantly higher from subjects developing an early and late phase response, compared to baseline. There was also a significant relationship between the maximum percent decrease in FEV₁ during the late response and the allergen induced increase in the number of sputum mast cells (195).

Mast cells arise from CD34⁺, CD117⁺ (c-kit⁺) and CD13 (aminopeptidase N / gp150⁺) progenitor cells from the bone marrow that circulate in the peripheral blood as undifferentiated mononuclear cells (196).

Lysophosphatidic (LPA) acid is important in the development but not survival of mast cells (197). IL-3, 4, 5 and 6 are important in the development and survival of mast cells (198;199). IL-6 can be produced by Interleukin-1 β or TNF- α activated ASM (99). SCF – the ligand for the c-kit receptor, is crucial in the growth and development of mast cells (199;200), as well as mast cell chemotaxis (201). Airway smooth muscle can produce SCF in a soluble and membrane bound form (202).

There are two main types of mast cells, the difference between which can be ascertained by examining their content and receptor expression. One type of mast cell contains tryptase and the chymotrypsin like proteinase chymase (MC_{TC}), and the other type of mast cell lacks chymase (MC_T) (203).

Originally, MC_{TC} cells were described as being found predominately in the skin and intestinal submucosa – corresponding more closely to a connective tissue location. MC_T cells were described as being found predominately in the intestinal mucosa and were reported to be more analogous to a mucosal tissue location (203). In relation to the lung, MC_{TC} cells have been reported to predominate in bronchial smooth muscle and glandular regions and markedly elevated levels of the MC_{TC} cell in the bronchial smooth muscle of patients with asthma have been found to correlate with levels of bronchial hyper reactivity (19). MC_T cells have been reported to predominate in the alveolar wall and epithelium of the lung and account for the majority of mast cells in the lung (204),(205). They are also the dominant phenotype recovered from bronchoalveolar lavage (BAL). MC_{TC} cells have also been reported to contain another chymotrypsin like proteinase – Cathepsin G (206), and also Carboxypeptidase A3 (207). These are not present in MC_T cells (206;207). The mature MC_{TC} cell has also been reported to express CD88, the receptor for the anaphylatoxin C5a, a protein fragment released from complement component C5 – whereas mature MC_T cells in the lung do not (205).

1.3.2 Mast Cell Chemotaxis and Localisation

CCL2 (MCP-1) (65;208), CCL5 (RANTES) (66-68), CXCL10 (71;72), nerve growth factor (61;209), fractalkine (64;210) along with TGF- β (77) all have the potential to act as mast cell chemoattractants. Mast cell mediators themselves can have an effect on mast cell chemoattractants. For example, histamine (66) and β -tryptase (76) have both been reported to reduce RANTES released by ASM, while the latter can increase TGF- β production (57;77).

CXCL8 (65;70;211) and CCL11 (212) are also potential mast cell chemoattractants. Mast cells have been shown to migrate towards IL-1 β , IL-4 and IL-13 (Th2) stimulated ASM from asthmatics but not non-asthmatics. Mast cell migration was mediated through the combined activation of CCR3 (a receptor for CCL11) and CXCR1 (a receptor for CXCL-8). CCL11 and CXCL8 expression by ASM increased markedly after stimulation, but was similar in those with and without asthma. ASM supernatants from non-asthmatics actually inhibited mast cell migration towards the asthmatic ASM supernatant (73). IL-1 β or TNF-activated ASM (213), as well as IL-4 and IL-13 (214) can activate CCL11 production. Conversely, chymase and β -tryptase have been shown to markedly reduce the immunoreactivity of CCL11 reducing its eosinophil chemotactic effects (76). What are the implications of this for mast cell chemotaxis to the ASM and epithelium?

The number of mast cells within the ASM bundle positively correlate with the degree of AHR (19), and have been shown to be degranulated (24) and in an activated state as evidenced by increased IL-4 and IL-13 production (215), which contribute towards increased ASM contraction and therefore AHR (216).

While many of the immunopathological features of asthma are observed in the airways of patients with EB (19-21) there are crucial differences. EB is characterized by a corticosteroid responsive cough, eosinophilia detectable in sputum and unlike asthma, patients with EB present without variable airflow obstruction or AHR (22),(23). Critically, there are almost no mast cells present in the ASM in patients with EB (19). The number of mast cells per mm² of ASM is significantly higher in subjects with asthma than in EB (19;24). The infiltration of ASM by mast cells could be associated with the disordered airway function found in asthma, and indicates an important role for ASM and mast cells in this condition.

Conversely, intraepithelial mast cells obtained from bronchial brushings of subjects with ICS-responsive chronic cough (a condition similar to EB) were found to be increased compared to asthmatics (23). Concentrations of mast cell mediators histamine and PGD₂ are reported to be significantly higher in sputum samples from subjects with EB and not asthma when compared to normal subjects (34), suggesting that mast cell infiltration into the superficial airway may be a particular feature of this condition.

1.3.3 Mast Cell Activation

Mast cell activation can occur when IgE is bound to the high-affinity IgE Fc receptor (FcεR1) on mast cells (217) and is cross-linked, triggering mast cell degranulation (218;219). Toll like receptors (TLR) 1, 2, 3, 4, 5, 6, 7, 8 and 9 have also been described on human cultured mast cells (220). Factors which activate these receptors include lipoproteins (for TLR1 / TLR2), peptidoglycans (TLR2 / TLR6) and lipoteichoic acid (TL2), viral double stranded RNA (TL3 / TL7) or a synthetic mimic such as polyinosine-polycytidylic acid (poly I:C), lipopolysaccharides (TL4), flagellin (TLR5) and CpG DNA (TLR9). The activation of these receptors can also cause the activation of the mast cell and subsequent release of mediators (221), or at least potentiate IgE-activated mediator (IL4, IL6 and IL8) release (222). Mast cell and ASM co-culture also resulted in mast cell activation (199).

Preformed mediators in granules include histamine, tryptase, chymase, heparin, carboxypeptidase A3 and MMP-3 (223), TNF and VEGF (224). There are also a variety of other cytokines and factors than can be released from mast cells (224);

Class of product	Products
Preformed	Histamine, serotonin (in rodents), heparin and/or chondroitin sulphates, tryptase, chymase, major basic protein, cathepsin, carboxypeptidase-A
Lipid-derived	PGD ₂ , PGE ₂ , LTB ₄ , LTC ₄ , PAF
Cytokines & growth factors	GM-CSF ^{a,b} , IFN-α ^{a,b} , IFN-β ^c , IFN-γ ^a , IL-1α ^{b,c} , IL-1β ^{a,b} , IL-1R antagonist ^b , IL-2 ^a , IL-3 ^{a,b} , IL-4 ^{a,b} , IL-5 ^{a,b} , IL-6 ^{a,b} , IL-8 (CXCL8) ^b , IL-9 ^{a,b} , IL-10 ^{a,b} , IL-11 ^b , IL-12 ^{a,d} , IL-13 ^{a,b} , IL-14 ^d , IL-15 ^d , IL-16 ^{b,c} , IL-17E (IL-25) ^a , IL-17F ^c , IL-18 ^d , IL-22 (IL-TIF) ^c , LIF ^d , LTβ ^d , M-CSF ^{c,d} , MIF ^d , SCF ^b , TGF-β1 ^{a,b} , TNF ^{a,b} , TSLP ^c , bFGF ^{a,b} , EGF ^b , IGF-1 ^a , NGF ^a , PDGF-AA ^d , PDGF-BB ^b , VEGF ^{a,b}
Chemokines	CCL1 (TCA3/1309) ^{b,c} , CCL2 (MCP-1) ^{a,b} , CCL3 (MIP-1α) ^{a,d} , CCL3L1 (LD78β) ^d , CCL4 (MIP-1β) ^{c,d} , CCL5 (RANTES) ^{a,b} , CCL7 (MCP-3) ^{c,d} , CCL8 (MCP-2) ^d , CCL11 (eotaxin) ^d , CCL13 (MCP-4) ^d , CCL16 (LEC/HCC-4) ^d , CCL17 (TARC) ^{a,d} , CCL20 (LARC) ^d , CCL22 (MDC) ^{a,d} , CXCL1 (Groα/KC) ^{a,d} , CXCL2 (Groβ/MIP-2) ^{a,d} , CXCL3 (Groγ) ^d , CXCL10 (IP-10) ^{b,c} , CXCL11 (I-TAC) ^d , XCL1 (lymphotactin) ^{b,c}
Free radicals	Nitric oxide ^{e,f} , superoxide ^{e,f}
Others	Corticotropin-releasing factor ^b , urocortin ^b , substance P ^a

Figure 1.3.3: Products Released by Activated Mast Cells (224)

Some of these cytokines, growth factors, and chemokines have only been detected at the mRNA level, only in studies of in vitro-derived mast cells and/or only from mast cells from a single species. For these products, the following apply: (a,b) Protein detected by ELISA or immunohistochemistry. (c,d) mRNA expression. (a,c,e) Rodent. (a,d,f) Human.

The early phase of asthma episodes are largely dependent on bronchoconstriction (179). This reaction occurs within minutes of the allergen challenge and is initiated by smooth muscle contraction as a consequence of the activation and mediator release from local mast cells (180).

Evidence that the mast cell undergoes degranulation and could participate in this response is underpinned by studies where concentrations of mast cell mediators such as TNF and IL-1 β , have been found to be increased in the bronchoalveolar lavage (BAL) of patients with symptomatic asthma in comparison to those with asymptomatic asthma (225). Histamine was found to be increased in atopic asthmatics after allergen challenge (226). LTD₄ is increased in the sputum of asthmatics (227), and PDGF was increased in the BAL of asthmatics (228). IL-4 and IL-13 are also increased in the BAL after allergen challenge (145).

TNF- α enhances smooth muscle Ca²⁺ responsiveness to bronchoconstrictor agents such as thrombin and bradykinin (229). Heparin increases smooth muscle contraction via a calcium independent mechanism (230).

Histamine has been reported to induce a contractile response in smooth muscle, which can be potentiated by LTD₄ (231). The latter of which can independently cause smooth muscle contraction (80;232). PDGF (233), IL-4, IL-13 (216) and tryptase (234;234) have also been reported to induce smooth muscle contraction.

Conversely in bronchial segments, IL-1 β inhibits acetylcholine, histamine, and potassium chloride (KCl) mediated contraction, but this inhibition is reversed if the epithelium is removed from the bronchial segments under investigation (235).

1.3.4 Tryptase

Application of tryptase to ASM cultures has been shown to increase ASM derived TGF- β 1 production (57), and activate TGF- β 1 (78), the latter of which has been observed to be a potent inducer of α -SMA expression in smooth muscle (145;148;149;155), therefore promoting a contractile phenotype of ASM (123).

There are two main types of tryptase; α and β tryptases, to which they have a 92% sequence homology. In relation to further subdivisions, there are α 1 and α 2 tryptases, along with β 1, β 2 and β 3 tryptases. β 2-tryptase is the isoform that is stored in the secretory granules of mast cells (236;237) in a catalytically active form (236;236).

Mast cell β -tryptase is a neutral serine protease with a tetrameric compound structure made up of 4 non-covalently bound subunits and it has a total molecular weight of 134-kDa (237). Each subunit has 1 enzymatically active site (238), but upon dissociation, these monomers lose their enzymatic activity (237). β -tryptase is concentrated within the secretory granules of mast cells where it is the most abundant mast cell protein, constituting up to 20 – 25% of the total mast cell protein in the granules (239;240). Upon degranulation, it is released along with chymase, histamine, heparin and other mast cell granule products (241). Tryptase requires

stabilization by heparin and other negatively charged proteoglycans (236). There is thought to be as much as $10.1 \pm 2.5 \mu\text{g}$ tryptase / 10^6 mast cells (242).

β -tryptase is able to cleave and degrade a number of proteins and extracellular substrates such as vaso-active intestinal protein (VIP), which is a bronchodilator (243), and it can activate fibrinogen, which belongs to a group of kinins, leading to broncho-constriction and airway hyper-responsiveness (243;244). β -tryptase is also a mitogenic (236) and contractile (234;236) agent for smooth muscle cells. Tryptase (20 nM) elicited 33 – 46% of the BrDU uptake occurring in the presence of 10% FCS. Exposure to tryptase (5 nM) for 4 days also produced significant increases in smooth muscle cell numbers, over that of cells maintained in serum free medium (245). Application of tryptase to rings of human bronchi in an organ bath did not produce any contractile responses at lower concentrations (1 nM), but did increase tone by an average 7%, 14% and 16% at higher concentrations (3, 10 and 30 nM), of the response to acetylcholine (1 mM) (234).

It is thought that most of the actions of tryptase are mediated by interactions with an extracellular amino terminus of a transmembrane-domain, G protein coupled receptor – protease activated receptor-2 (PAR-2). The protease cleaves a portion of the extracellular amino terminus to unmask a new N-terminal sequence, which then functions as a tethered ligand (246) binding to and interacting with the extracellular loop 2 on the receptor to initiate signal transduction (247). PAR-2 can also be activated by peptides corresponding to the tethered ligand domain such as by the synthetic PAR-2 ligand SLIGKV, without the need for the cleavage of the amino acid terminus (247), and also trypsin (234) and tissue factor and factor Xa (248).

The PAR-2 receptor is functionally expressed on airway smooth muscle cells, as well as the epithelium (234;249), bronchial glands, and the endothelium and smooth muscle of bronchial vessels (234).

Many extracellular proteins are glycosylated. Mono- or Oligo-saccharides can be attached to asparagine (N-linked) or to serine/threonine (O-linked) residues. The PAR2 receptor has 2 N-linked glycosylation sequons, one on the receptor N-terminus ($\text{Asn}^{30}\text{Arg}^{31}\text{Ser}^{32}$), and the other on the extracellular loop 2 (ECL2; $\text{Asn}^{222}\text{Ile}^{223}\text{Thr}^{224}$) (250). It has been hypothesized that N-linked glycosylation may be responsible for restricting tryptase access to the PAR-2 receptor, and varying states of glycosylation may be the reason for conflicting reports on whether tryptase is (57), or isn't (251) a good agonist for the PAR2 receptor.

Fibroblasts that have mutant N-terminal glycosylation-deficient receptors have displayed increased sensitivity towards tryptase (250). Treatment with agents that remove the sialic acid residues (such as with neuraminidase) from oligosaccharides also facilitate tryptase access to the cleavage / activation site of PAR2 (252). Sialic acid itself is not an inhibitor of tryptase. Removal of N-linked glycosylation has been reported to decrease the efficacy of either trypsin or the peptide agonist SLIGRL-NH₂ against PAR2 (250).

Cathepsin G, which is present in tryptase / chymase positive mast cells, has been reported to inhibit the response of the PAR2 receptor to trypsin, but not SLIGKV-NH₂, suggesting that proteolysis of the extracellular domain may occur downstream

of the trypsin cleavage / activation site, while leaving unmodified the SLIGKV-NH₂ binding site (253). Therefore *in vivo*, cathepsin G may promote tryptase access to PAR2 by means of removing the N-terminus glycosylation sequon (254).

Interestingly, although Cathepsin G may promote access to the PAR2 receptor, tryptase has been reported to increase TGF- β production from ASM cells (57). TGF- β is known to increase the production of Thrombospondin 1 (TSP-1) from airway smooth muscle (171), which is also a potent active site inhibitor of cathepsin G (255). This could form a feedback mechanism to prevent excessive signaling and resultant TGF- β production by tryptase.

Increases in cell confluence have been reported to down regulate cell-surface receptor expression of PAR2 by up to 71% (250).

The mitogenic effect of tryptase is still evident after the proteolytic actions of tryptase have been abolished (256), suggesting that there may also be nonproteolytic (non PAR2) mechanisms at play. This could arise from the several glycosylated residues on the outer surface of the crystal structure of tetrameric human β -tryptase - such residues would be accessible for binding to cell surface mannose receptors (257). To illustrate, in bovine airway smooth muscle cells, potent proliferative responses to β -hexosaminidase and other mannosyl-rich glycoproteins are mediated by specific mannose receptors (258).

1.4 Environmental and Genetic Influences in Asthma

On the basis of current understanding in asthma, it is clear that asthma has strong genetic components, but for those to manifest as chronic asthma, important environmental factors are also required, often beginning in pregnancy and early infancy, such as respiratory infections, environmental tobacco smoke exposure, dietary factors (259) and other environmental influences which include factors that can be incorporated into the non-pharmacological management of asthma, as illustrated in section 1.1.2.

The original hygiene hypothesis states that a lack of early childhood exposure to infectious agents, symbiotic microorganisms and parasites (underdeveloped Th1 immune response) increases susceptibility to allergic diseases by modulating immune system development and encouraging the overdevelopment of a Th2 immune response (260). Although it is now well established that being raised on a farm environment (and a consequent exposure to infectious agents) protects against hay fever and atopic sensitisation the evidence for an effect of living in a farm environment on asthma and wheeze remains conflicting. Differences in farming practices and hence in microbial exposures lead to discrepant results. It is therefore critical to assess whether distinct farm environment related exposures have distinct effects on asthma related phenotypes (261).

Genetic influences include the occurrence of polymorphisms within populations such as a genetic variation in the disintegrin and metalloprotease (ADAM) 33 gene which lead to its identification as a susceptibility gene for asthma, including an association with bronchial hyperresponsiveness. ADAM 33 is expressed in bronchial smooth muscle, and it is thought to alter the hypertrophic response of bronchial smooth muscle to inflammation (259). To date 55 single nucleotide polymorphisms (SNPs) for ADRB2 have been listed in public databases. A number of these SNPs are nonsynonymous resulting in amino acid changes and functional investigations have suggested that certain SNPs are of importance with regard to the cellular response of β agonists (262).

With the advances in genetics and genomics substantial steps have been undertaken in the last decade in understanding the genetic factors underlying asthma. With the application of genetic techniques the next decade will undoubtedly result in a further substantial increment in our understanding of the mechanisms underlying asthma (262).

1.5 Hypotheses and Aims

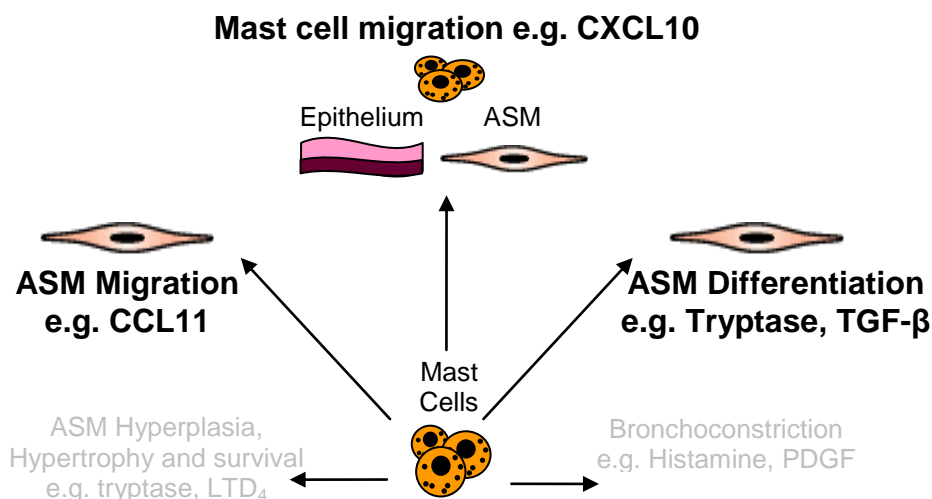


Figure 1.5.1: Cellular Interactions

Hypothesis One: The Concentration of Chemokines That Mediate Mast Cell Migration Are Increased in Airway Secretions in Asthma and EB.

To summarize, eosinophilic bronchitis (EB) is a condition which is similar to asthma. Both conditions can present with cough and increased cough reflex hypersensitivity, sputum and lavage eosinophilia, but AHR or VAO is absent in eosinophilic bronchitis. Airway epithelium in eosinophilic bronchitis and asthma is fundamentally abnormal with goblet cell hyperplasia and mucin secretion resulting in an occluded airway, particularly in mild-moderate and severe persistent asthmatics (41;49;50), and EB (22;51). Sputum mast cells are increased in both EB and asthma, but interestingly the number of mast cells in bronchial brushings and the sputum concentration of mast cell mediators were greater in EB than asthma (23;34), suggesting that mast cell infiltration into the superficial airway may be a particular feature of this condition.

The aims of this investigation involved the examination of mast cell mediator concentrations and mast cell chemotaxis activity in BAL in EB asthma and normal controls. In order to achieve this aim, the following criteria were examined;

- 1) BAL fluid concentration of (A) CXCL10 and (B) CXCL8 in subjects with EB and asthma, and in healthy control subjects.
- 2) CXCR1 and CXCR3 expression by HMC-1 cells .
- 3) Examination of whether the recombinant chemokines CXCL10, CXCL8, and CCL11 were chemotactic for HMC-1 cells.
- 4) HMC-1 chemotaxis to BAL fluid from subjects with EB, asthma and normal controls
- 5) Assessment of potential inhibition of HMC-1 chemotaxis to BAL from subjects with EB by neutralization Of CXCR1 & CXCR3.

Hypothesis Two: The CCR3 / CCL11 axis mediates ASM migration.

To summarize, Airway smooth muscle plays a critical role in asthma through a variety of mechanisms; • Contributing to the inflammatory process arising from the synthetic response of ASM. • Contributing to airway remodeling through increases in ASM area.

ASM area has been found to be significantly increased in the airways of mild-to-moderate and severe asthmatic subjects (24;41), the severity of which has been found to increase with age (55). Reductions in ASM area have resulted in improvements in PEF and quality of life scores (84). Increases in ASM mass could result from either increased ASM hypertrophy (24;41), hyperplasia (86), or an increase in ASM migration (263) under the influence of ASM derived CCL11 (109). The cause of ASM hyperplasia in asthma is unknown and is often attributed to increased proliferation, but several reports have been unable to demonstrate increased ASM proliferation *in vivo* (24;41;85). An alternative explanation is that ASM progenitors either located within the airway wall or derived from peripheral blood fibroblast progenitors (fibrocytes) (264), migrate to the ASM bundle and differentiate into ASM.

The aims of the investigation involved the examination of whether CCL11 mediated ASM migration. In order to achieve this aim, the following criteria were examined;

- 1) ASM expression of the CCL11 receptor - CCR3.
- 2) The effect of CCL11 on ASM proliferation and survival.
- 3) The effect of mast cells and β -tryptase on CCL11.

Hypothesis Three: Mast Cells Promote ASM to a More Contractile Phenotype.

To summarize, airway smooth muscle plays a critical role in asthma through a contributing to AHR through mesenchymal cell differentiation and increased ASM contractility. Fibrocytes may differentiate into fibroblasts to myofibrocytes, then into ASM and potentially de-differentiating depending on environmental influences. These phenotypically variable cells can be identified and characterized by the sequential appearance of cytoskeletal and contractile proteins to yield a phenotype that is characteristic of ASM cells. Evidence suggests that lung fibroblasts which express higher levels of α -SMA possess an enhanced ability to wrinkle deformable silicone substrates and contract collagen gels than those which express lower levels of α -SMA. Also, only lung fibroblasts with higher levels of α -SMA were able to deform more rigid silicone substrates (123).

The striking difference between the pathology of EB and asthma is that asthmatic ASM is infiltrated by mast cells, suggesting that this is a major determinant of the disordered airway physiology observed in asthma (19). The mechanisms that drive the development of disordered airway physiology in asthma as a consequence of mast cell-ASM interactions have not been fully elucidated. Indeed, to date whether mast cells promote ASM differentiation to a more contractile phenotype is unknown, and this remained the focus of this investigation.

Mediator release from mast cells could cause the ASM to contract (80;216;231-234), and along with the promotion of a more contractile phenotype of ASM, could contribute towards the disordered airway physiology observed in asthmatics.

The aims of the investigation involved the examination of the contractile phenotype of ASM. In order to achieve this aim, the following criteria were examined;

- 1) The expression of the contractile protein α -SMA in HASMCs after co-culture with HLMCs and mast cell products.
- 2) HASMC contractility after co-culture.

Chapter Two

Methods

2 **Methods**

2.1 **Clinical Characterisation**

Patients with eosinophilic bronchitis, asthma and non-asthmatic controls were recruited from respiratory outpatient clinics and from staff at the Glenfield Hospital, Leicester, UK.

Subjects with asthma had a consistent history and had objective evidence of asthma, as indicated by one or more of the following: (i) methacholine airway hyperresponsiveness (provocative concentration of methacholine causing a 20% fall in FEV₁ [PC₂₀], <8 mg/mL); (ii) greater than 15% improvement in FEV₁ 10 minutes after administration of 200µg of inhaled salbutamol; or (iii) greater than 20% of maximum within-day amplitude from twice daily peak expiratory flow measurements over 14 days.

The subjects with eosinophilic bronchitis had an isolated cough, no symptoms suggesting variable airflow obstruction, normal spirometric values, normal PEF variability, normal airway responsiveness (PC₂₀ > 16mg/ml), and sputum eosinophilia (> 3% nonsquamous cells). The healthy subjects gave no history of respiratory diseases, had negative allergen skin prick test results, normal spirometry findings, and normal airway responsiveness. None of the subjects had been treated with inhaled or oral corticosteroids, long-acting bronchodilators, or leukotriene antagonists for at least 1 month before entry into the study.

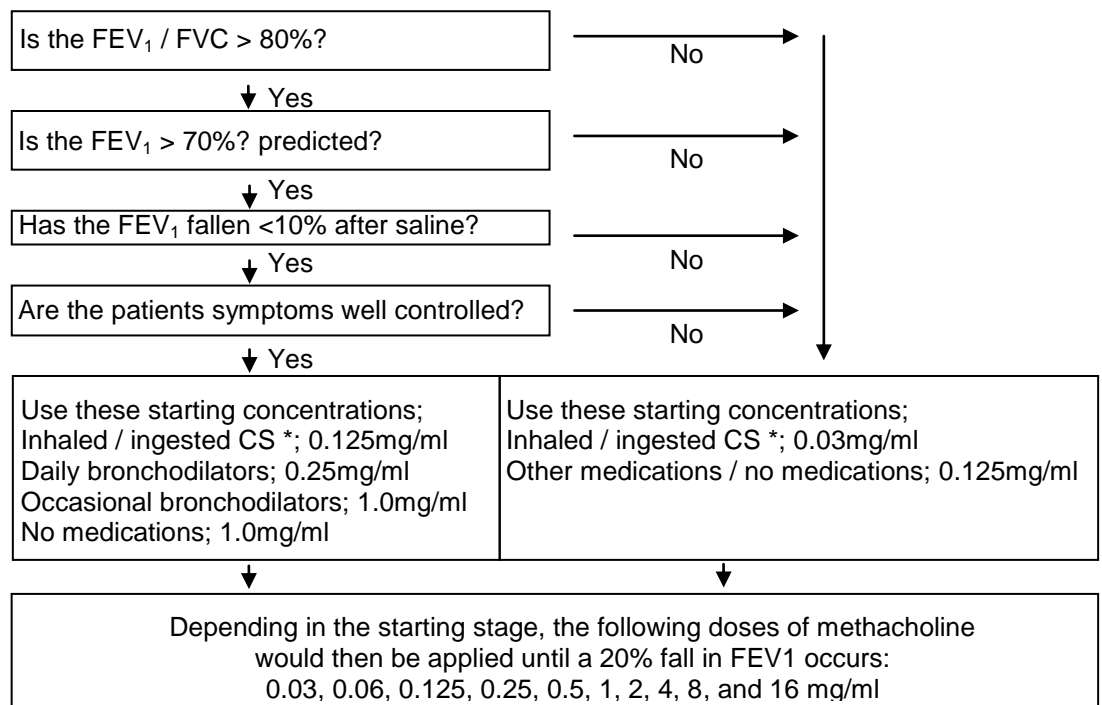
The healthy subjects gave no history of respiratory diseases, had negative skin prick test results, normal spirometry findings, and normal airway hyperresponsiveness.

Additional non-asthmatic subjects underwent lung resection.

The study was approved by the Leicestershire Ethics Committees and all patients gave their written informed consent.

2.2 **Clinical Measurements**

A selection of subjects underwent fiberoptic bronchoscopy and bronchial biopsy using current British Thoracic Society guidelines (265). Lignocaine was used as the local anesthetic, oxygen supplementation was used to achieve an oxygen saturation of at least 90% to reduce the risk of significant arrhythmias and asthmatic subjects were premedicated with a bronchodilator before bronchoscopy. A 20ml bronchial wash into the bronchial intermedius and a 180ml BAL into the right lower lobe was performed. Bronchial wash and BAL fluid samples were centrifuged at 790g for 10 minutes, and the supernatant was stored at -80°C for later analysis. Subjects also underwent spirometry, allergen skin prick tests for *Dermatophagoides pteronyssinus*, cat fur, grass pollen, and *Aspergillus fumigatus*, a methacholine inhalation test (Figure 2.2a) and sputum induction (Figure 2.2b). In relation to the methacholine inhalation test, after the inhalation of each dose of methacholine, FEV₁ measurements were made at 30 and 90 seconds after the last inhalation. The time between methacholine doses were standardized at 5 minutes to keep cumulative effect constant. After the first concentration, if the FEV₁ had not dropped by more than 5%, and there was no clinical evidence of bronchoconstriction, then the next dose was omitted. Concentrations were not omitted if a patient's FEV₁ fell by 5% or more after the diluent inhalation. If asthma symptoms did not appear to be well controlled, all patients were started at 0.03mg/ml. If FEV₁ had dropped > 16%, and the patient had asthmatic symptoms, then the test was stopped.



CS * ; Corticosteroids

Figure 2.2a: Methacholine Induction Test (266)

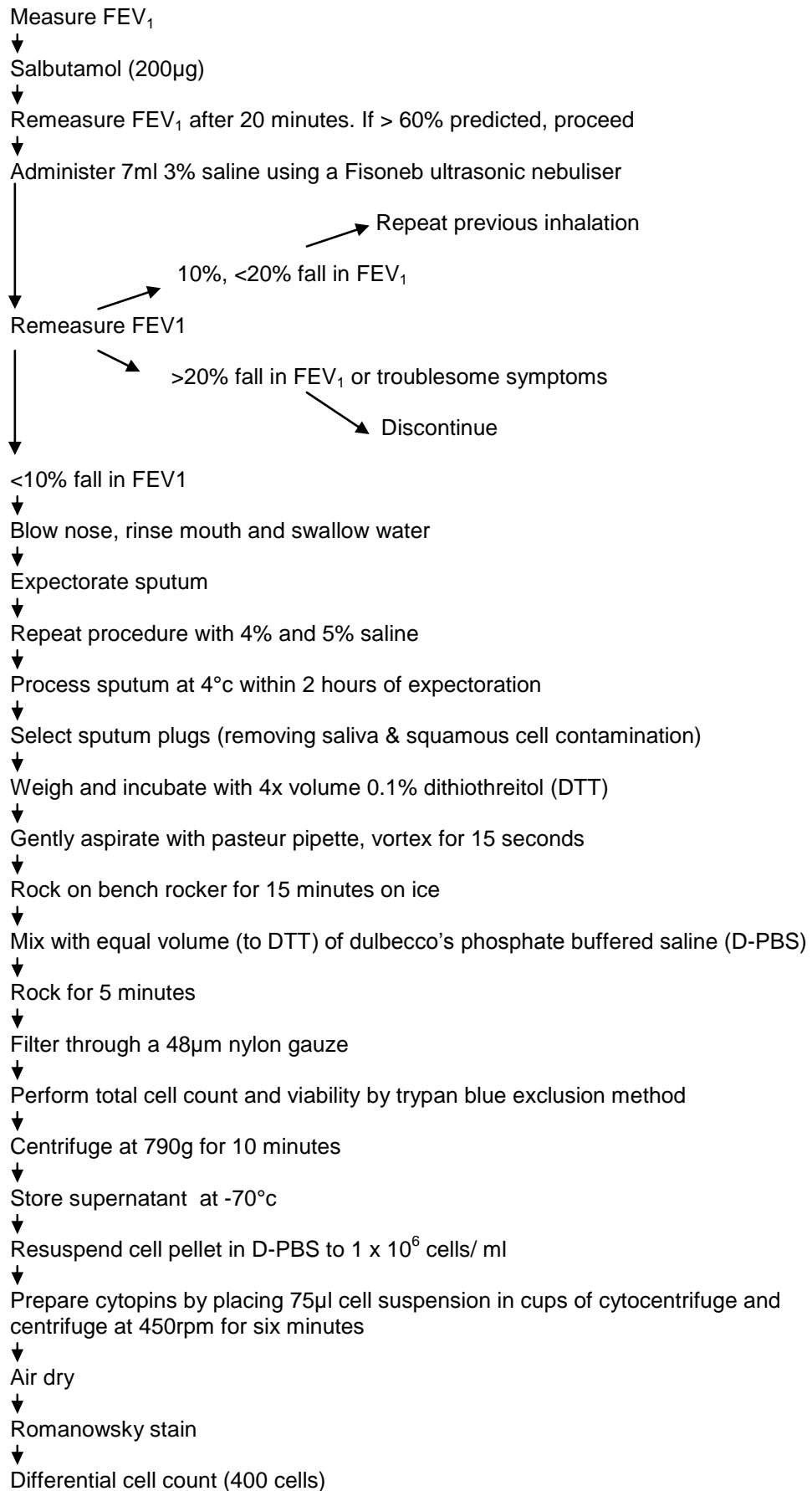


Figure 2.2b: Sputum Induction and Processing (267)

2.3 ASM Isolation and Culture

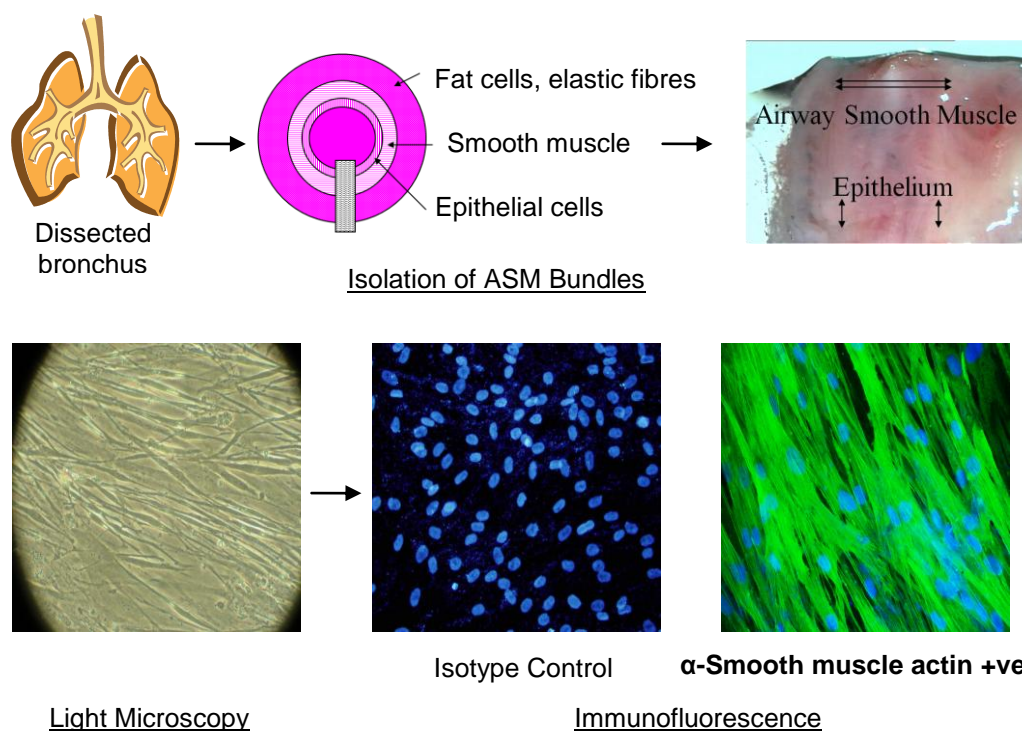


Figure 2.3: ASM Characterisation

Pure ASM bundles were isolated from bronchial biopsies obtained from fiberoptic bronchoscopy and additional airways isolated from lung resection were dissected free of surrounding tissue. Primary ASM was cultured in DMEM with Glutamax-1 supplemented with penicillin (100U/mL), streptomycin (100 μ g/mL), amphotericin (0.25 μ g/mL), non-essential amino-acids (100 μ M) (Invitrogen, Paisley, UK), sodium pyruvate (1mM) and fetal bovine serum (10%) (Sigma, Gillingham, UK). ASM cell characteristics were determined by immunofluorescence and light microscopy with α -smooth muscle actin-fluorescein isothiocyanate (FITC) direct conjugate, calponin and myosin indirectly conjugated with FITC (Sigma). ASM passage 2-6 was used for experiments.

2.4 HLMC Isolation and Culture

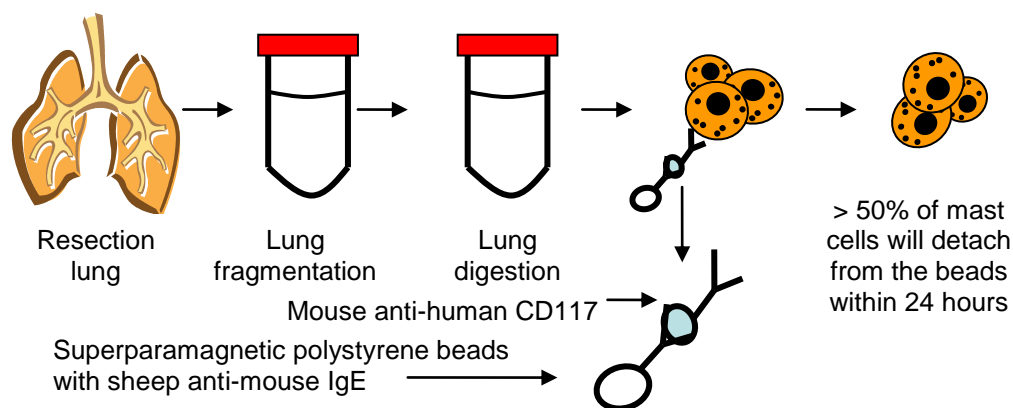


Figure 2.4: HLMC Isolation

HLMC were purified from macroscopically normal lung within 1 hour of resection for carcinoma using an enzymatic dispersal and immunomagnetic bead selection procedure. Lung tissue was finely diced and fragmented, then washed four times through two pieces of sterile 100µm nylon gauze (Fisher, Loughborough, UK) with Dulbecco's Modified Eagles Medium (DMEM) with GlutaMAXTM I, 4500 mg/L D-Glucose and 25mM HEPES (Invitrogen) supplemented with penicillin (100U/ml) streptomycin (100µg/mL), amphotericin (0.25µg/mL), non-essential amino-acids (100µM), (Invitrogen), sodium pyruvate (1mM) and 10% foetal calf serum (Sigma), herein referred to as HLMC media. The lung tissue was then cultured overnight in HLMC media (4ml/gram of tissue).

Enzymatic digestion of the tissue was performed through the addition of collagenase (75mg/10g tissue) and hyaluronidase (37.5mg/10g tissue) (both Sigma) followed by incubation at 37°C for 75 minutes on a magnetic stirrer. HLMC were then liberated by a two washes through a 100µm gauze (Fisher) and then a 50µm gauze (Precision Textiles, Bury, UK) after which the filtrate was centrifuged to pellet the cells. The cell pellet was then resuspended in 3ml HBSS/FCS protein (84.7% HBSS; 2% FCS; 10% horse serum – Invitrogen, 3.3% BSA – Sigma) and incubated for 30 minutes at 4°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 40µm cell strainer (BD Biosciences, Oxford, UK) and pelleting the cells.

The procedure of coating the immunomagnetic beads involved washing the sheep anti-mouse IgG Dynabeads (Invitrogen) with PBS / 0.1% BSA (Invitrogen and Sigma respectively) three times 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 PBS / 0.1% BSA (2ml) with 1% azide (Sigma). The suspension containing the mast cells were then resuspended with CD117-coated immunomagnetic beads at a 5:1 beads-cells ratio and incubated for 90 minutes at 4°C.

The beaded cells were then cultured in HLMC media supplemented with SCF (100ng/ml), IL-6 (50ng/ml) and IL-10 (10ng/ml) (R&D Systems, Abingdon, UK). Up to 50% of mast cells detached from the beads within 24 hours, and the two populations were separated with the use of a magnet.

2.5 **HMC-1 Culture**

The human mast cell line-1 (HMC-1) cell line was a generous gift from Dr J. Butterfield (Mayo Clinic, Rochester, Minn). HMC-1 cells were maintained in Iscove's modified DMEM; supplemented with penicillin (100U/mL), streptomycin (100µg/mL), amphotericin (0.25µg/mL), non-essential amino-acids (100µM) (Invitrogen) 10% iron-supplemented fetal calf serum and 1.2 mM α -thioglycerol (Sigma) (268).

2.6 **Concentration of Samples for ELISA**

As Vivaspin columns (Vivascience AG; Hanover, Germany) vary, the most appropriate membrane was selected for the concentration of the sample; for maximum recovery, a molecular weight cut off (MWCO) was selected that was at least 50% smaller than the molecular size of the species of interest. The concentrator was filled and the lid was sealed. The assembled concentrator was inserted into the centrifuge, and the centrifuge was operated at the appropriate speed for the recommended amount of time. Once the desired concentration was achieved, the assembly was taken apart by pinching the tube and twisting to remove the concentrator body. The sample was recovered from the bottom of the pocket with a pipette.

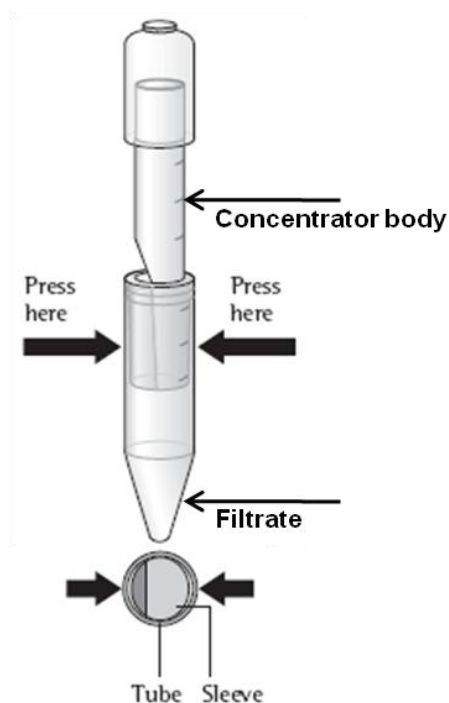


Figure 2.6: Vivaspin Column

2.7 Enzyme Linked Immunosorbent Assays

Enzyme linked immunosorbent assays (ELISAs) were conducted using the sandwich ELISA method. Briefly, a known quantity of capture antibody was bound to each well of a 96-well plate, then any non-specific binding sites on the well surface were blocked using a PBS; +/- BSA solution. The antigen containing samples, standards and control solutions were then added in duplicate to the plates, and following incubation the plates were washed to remove any unbound antigen. Detection antibodies; +/- Av-HRP conjugate were added, followed by washing to remove unbound detection antibody. Next 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (BD Biosciences) was added to each well, and following development, the reaction was stopped with the addition of H₂SO₄. Absorbance was read using a Wallac plate reader.

The mean absorbance for each set of duplicate standards and samples were calculated. The mean zero standard absorbance was then subtracted from each value. The standard curve was plotted on a log-log scale (figure 2.3), with the antigen concentration on the x-axis and absorbance on the y-axis to allow the construction of a trend line from which the concentrations of the unknowns were calculated (e.g. $\text{sum}((\text{absorbance of sample} - 0.1307) / 0.001)$). An example standard curve was as follows;

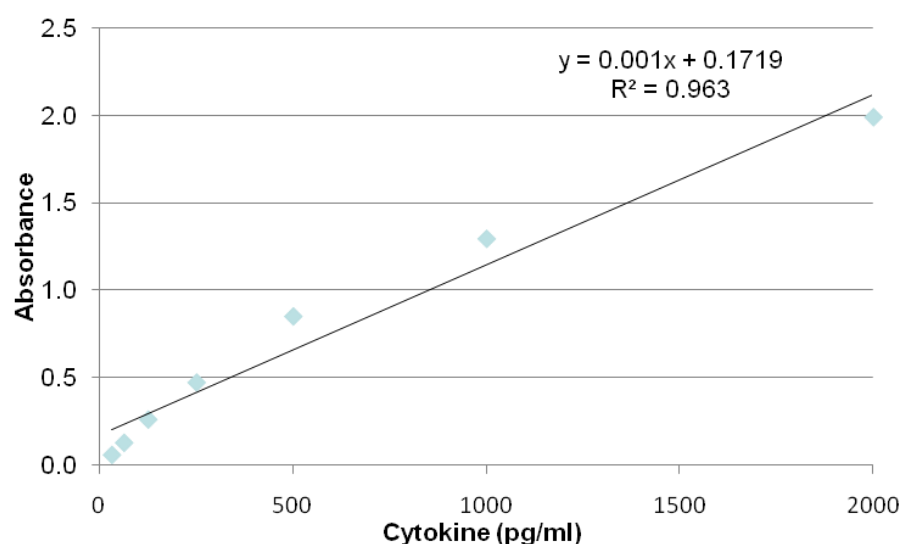


Figure 2.7: ELISA Standard Curve

2.8 Mast Cell Chemotaxis

Mast cell chemotaxis assays were performed using transwells with 8µm fibronectin-coated inserts in 24-well plates (BD Biosciences). 2×10^5 mast cells were placed in 100µl of culture medium into the top well, and 450µl of chemoattractant or a negative control into the bottom well. After incubating the cells for 4 hours at 37°C, the number of mast cells in the bottom well were counted using Kimura stain in a haemocytometer. Checkerboard analysis was used to distinguish chemotactic activity from chemokinetic activity. Dilutions of chemoattractant were added to the upper and lower wells, and mast cell migration increased as the concentration gradient between the upper and lower chambers increased. This indicated that the migratory activity was chemotactic rather than chemokinetic.

2.9 **Immunofluorescence**

HMC-1 (10^5 cells / well) were seeded onto fibronectin-coated chamber slides and cultured using Iscove's modified DMEM; with penicillin (100U/mL), streptomycin (100 μ g/mL), amphotericin (0.25 μ g/mL), non-essential amino-acids (100 μ M) (Invitrogen), 10% iron-supplemented fetal calf serum and 1.2 mM α -thioglycerol (Sigma) (268).

ASM cells were grown to confluence in chamberslides and growth arrested in DMEM with Glutamax-1 supplemented with penicillin (100U/mL), streptomycin (100 μ g/mL), amphotericin (0.25 μ g/mL), non-essential amino-acids (100 μ M), (Invitrogen) sodium pyruvate (1mM) and insulin-transferrin-selenium (1%) (Sigma) (ITS media) for 24 hours. Cells were fixed with methanol, labeled with appropriate antibodies, isotype controls and counterstained with DAPI (Sigma).

2.10 **Flow Cytometry**

HMC-1 (10^5 cells / well) were fixed with PFA (4%) in order to examine receptor expression.

ASM cells were grown to confluence and growth arrested in ITS media for 24 hours. Cells were either harvested with accutase (Insight biotechnology, London, UK) and fixed with PFA to examine receptor expression, or harvested with trypsin (0.25%) (Invitrogen) and fixed with PFA (4%) then re-suspended and permeabilised in PBS/0.5% BSA and 0.1% saponin (1×10^6 cells/ml) in order to examine intracellular protein expression using appropriate antibodies and isotype controls.

Antibody binding was detected using a FACScan flow cytometer with various colour detectors enabling the detection of different channels such as forward light scatter (cell size), side scatter (cell granularity), FL1; a 525nm band pass filter (green) for fluoro-chromes such as FITC, and FL2; a 575nm band pass filter (orange-red) for fluoro-chromes such as PE and PI. Data was analysed using CellQuest software (Becton-Dickinson). Cells were selected based on their side (SSC-H) and forward (FSC-H) scatter (Figure 2.6a) and in the case of single colour flow cytometry, antibody staining was quantified as the fluorescent intensity staining after subtraction of the isotype-matched control antibody fluorescence (Figure 2.6b).

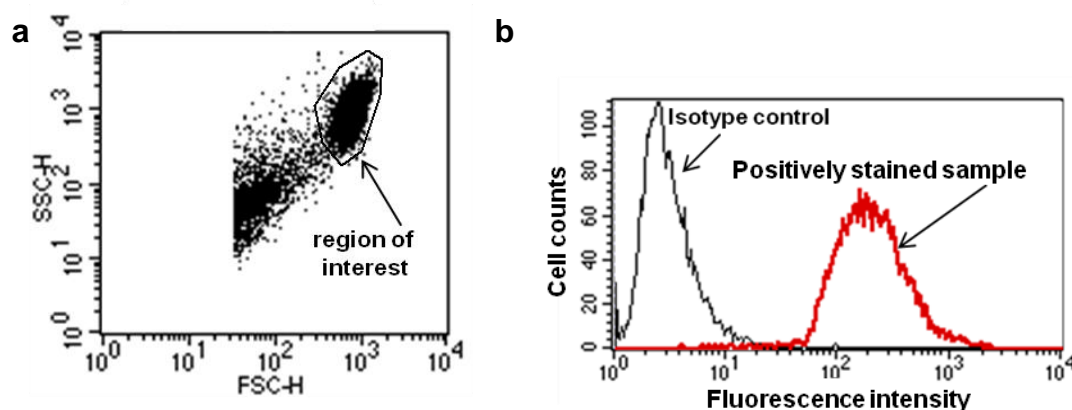


Figure 2.10: Flow Cytometry

Figures illustrating (a) the side (SSC-H) and forward (FSC-H) scatter of ASM cells, (b) the region of interest containing the ASM cells was then used to examine (b) the fluorescent intensity expression of the labeled cells.

2.11 Calcium Imaging

Freshly prepared phosphate saline solution (PSS 0 Ca^{2+} – containing H_2O , NaCl (118.4mM), KCl (4.7mM), D-glucose (11.1mM), Hepes (10mM), MgCl_2 (1.2mM) adjusted to pH 7.4 with NaOH) was used to prepare PSS 2mM Ca^{2+} . This was then used to construct the dye loading solution (PSS 2mM Ca^{2+} and FURA-2 (5 μM)).

The fluorescent dye, FURA-2 (Sigma), was then loaded into the cells. After stimulating the cells with an appropriate agent, cells were excited at altering wavelengths at 340nm (Ca^{2+} bound FURA-2 excitation wavelength) and 380nm (Ca^{2+} unbound FURA-2 excitation wavelength) (Figure 2.10a). Therefore, as signaling channels were activated and the Ca^{2+} concentration increased in the cytosol of each cell, the intensity of the dye at the 340nm wavelength increased and the intensity of the dye at 380nm decreased. The ratio of these two wavelengths allowed a fairly accurate measurement of the intracellular Ca^{2+} .

Fura-2 fluorescence emission was obtained at 510 nm by an intensified charge cooled device (CCD) (Hamamatsu, Japan) using a X40 fluor oil immersion lens (Nikon) and a 480-nm long-pass barrier filter using appropriate software (Openlab; Improvion; Coventry UK). This was converted to a $[\text{Ca}^{2+}]_i$ (cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), concentration using a commercially available calibration kit (Molecular Probes; Invitrogen; Paisley, UK) (Figure 2.10b).

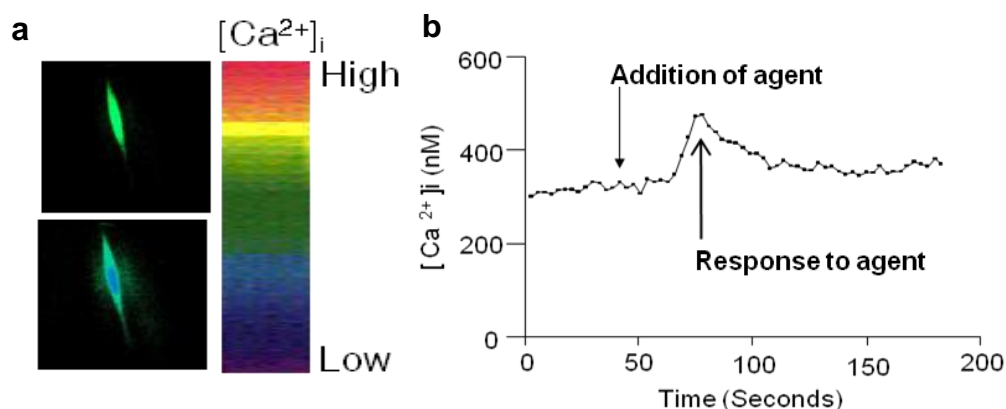


Figure 2.11: Calcium Imaging

Figures illustrating (a) increased cytosolic free Ca^{2+} ($[\text{Ca}^{2+}]_i$) after receptor activation, as illustrated by increased intracellular green fluorescence by FURA-2-loaded cells in the upper panel compared to the lower panel, and (b) a transient rise in $[\text{Ca}^{2+}]_i$ after receptor activation.

2.12 **ASM Chemotaxis**

ASM chemotaxis assays were performed using a validated chemotaxis assay (74). ASM cells were seeded onto 8-rectangular well plates coated with 10 μ g/ml fibronectin at a density of 0.25x10⁶ cells/well, allowed to adhere overnight, then serum deprived in ITS media for 24 hours prior to experimentation. Cells were removed by scraping between the top of the well and a line predrawn across the width of the well, on the underside of the plate, 22mm from the bottom of the well. Cell debris was removed by washing with ITS media. Blotting paper (25mm x 6mm; Sigma) was then placed along the upper edge of the well, secured in place using silicon grease. Migratory agent, where appropriate, was impregnated onto blotting paper from which it diffused into the media (Figure 2.11). The number of cells that moved towards the resultant concentration gradient of agent was enumerated after 6 hours by a blinded observer.

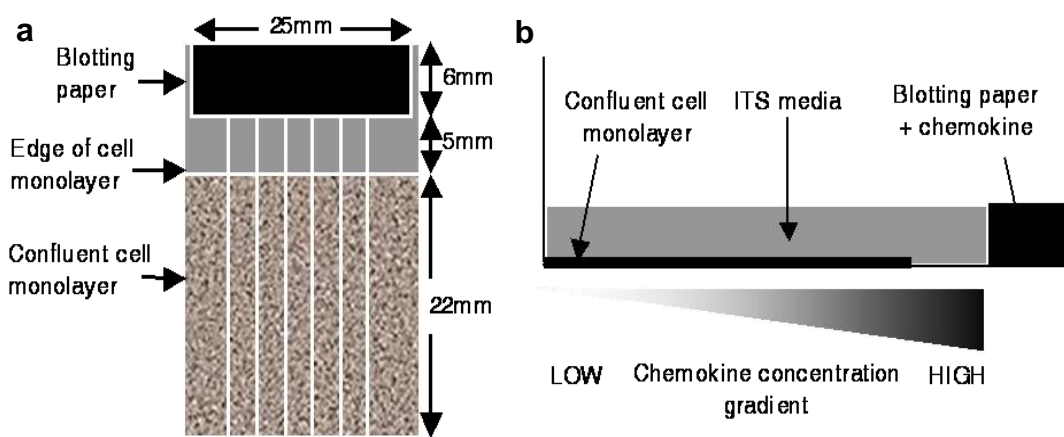


Figure 2.12: ASM Chemotaxis

Figures illustrating the (a) set-up of a chemotaxis well, and (b) the chemokine concentration gradient in the chemotaxis well.

2.13 **Wound Healing Assay**

ASM were seeded onto 8-rectangular well plates coated with 10 μ g/ml fibronectin at a density of 0.25x10⁶ cells/well, allowed to adhere overnight, then serum deprived in ITS media for 24 hours prior to experimentation. Wounds were introduced using a sterile 200 μ l pipette tip. The number of cells that moved into the wound in the presence of ITS control media and vehicle/agent were counted over 6 hours by a blinded observer.

2.14 **Cell Metabolic Activity**

1×10^4 ASM cells were seeded into each well of a 96-well plate, allowed to adhere overnight and stimulated with agents as appropriate for 24 hours. Cell metabolic activity was assessed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay; 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium inner salt (MTS) assay (Promega, Southampton, UK). The CellTiter 96® Aqueous One Solution reagent (20 μ l) was added to each well of a 96-well plate containing the samples in 100 μ l of culture medium. The plate was then incubated for 3 hours at 37°C in a humidified, 5% CO₂ atmosphere, and absorbance read at 490nm using a Wallac plate reader. The number of cells present in each well could be related to an increase in absorbance (Figure 2.13).

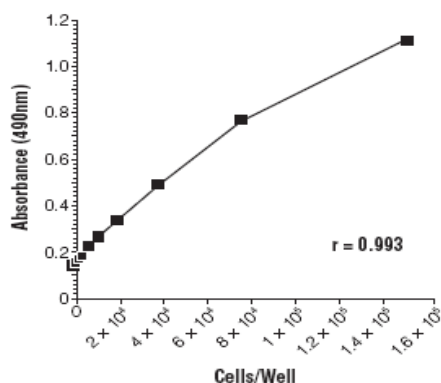


Figure 2.14: Cell Metabolic Activity Using an MTS Assay

2.15 **CFSE Cell Proliferation Assay**

ASM proliferation was assessed by using the CellTrace carboxyfluorescein diacetate, succinimidyl ester (CFSE) Cell Proliferation Kit (Invitrogen). ASM cells were seeded at a density of 5×10^4 cells / dish in 10% FBS media in 60mm tissue culture dishes and incubated at 37°C for 72 hours. Cells were then labeled with 2.5 μ M CFSE for 15 minutes in PBS, prior to incubation in 10% FBS media for a further 30 minutes to allow cleavage of the acetate groups to yield highly fluorescent carboxyfluorescein succinimidyl ester and incubated at 37°C for a further 24 hours. Cells were then exposed to agents as appropriate, prior to analysis using single colour flow cytometry on a FACScan (BD Biosciences). To illustrate (Figure 2.16), cells were harvested and stained with CFSE on day 0. A portion of the population was arrested at the parent generation using mitomycin C (open peak). The rest of the sample was stimulated with a proliferating agent and incubated for a further 5 days. The solid grey peak represents successive generations of proliferating cells.

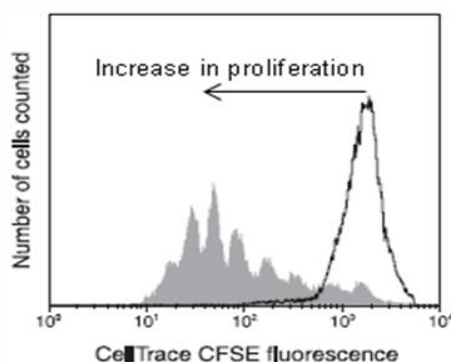


Figure 2.15: CFSE Cell Proliferation Assay

2.16 **Annexin V and Propidium Iodide Apoptosis Assay**

Apoptosis was detected using the annexin V-FITC apoptosis detection kit I (BD Biosciences). ASM cells were washed twice with cold PBS and then resuspended in 1X binding buffer at a concentration of 1×10^6 cells / ml. 100 μ l of the solution (1×10^5 cells) was then transferred to a 5ml culture tube along with the addition of annexin V-FITC (5 μ l) and propidium iodide (PI) (5 μ l). The cells were vortexed and incubated for 15 minutes at room temperature in the dark. 1X binding buffer (400 μ l) was added to each tube, and analysed by flow cytometry, as described in section 2.9, within one hour. Controls such as unstained cells, or cells stained with either annexin V-FITC alone (no PI), or PI alone (no annexin V-FITC) were used to set up compensation and quadrants.

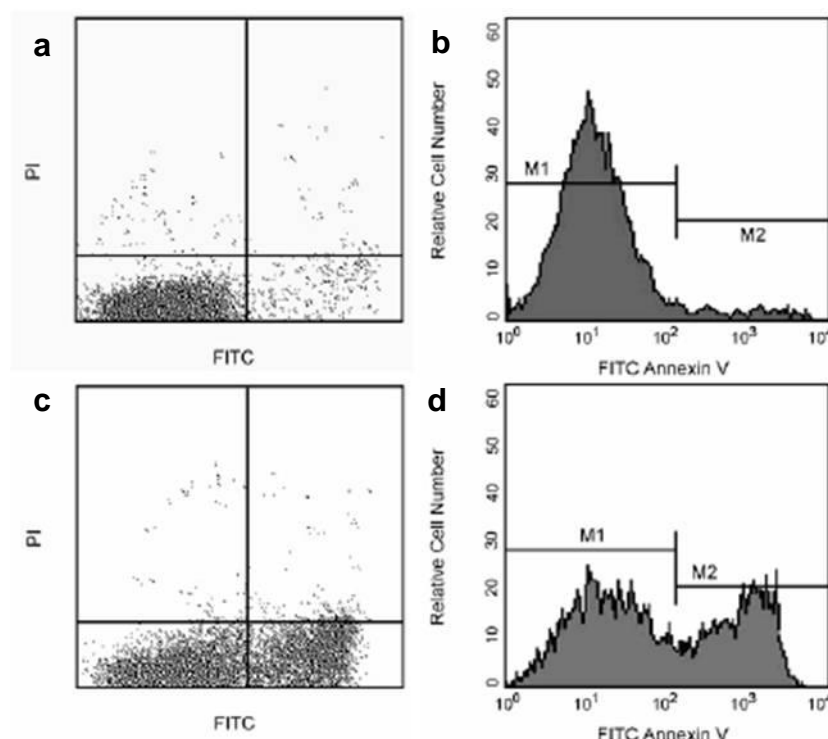


Figure 2.16: Annexin V and Propidium Iodide Apoptosis Assay

Cells were either left untreated (a, b) or treated for 4 hours with an agent (c, d). Cells were incubated with Annexin V-FITC in a buffer containing PI and analysed by flow cytometry. Untreated cells were primarily Annexin V-FITC and PI negative (a, b), indicating that they were viable and not undergoing apoptosis. After a 4 hour treatment (c, d), there were primarily two populations of cells; cells that were viable and not undergoing apoptosis (Annexin V-FITC and PI negative) (c; lower left quadrant and d; M1) and cells undergoing apoptosis (Annexin V-FITC positive and PI negative) (c; lower right quadrant and d; M2). A minor population of cells were observed to be Annexin V-FITC and PI positive (c; upper right quadrant), indicating that they were in end stage apoptosis or already dead.

2.17 Genechip Array Analysis

RNA expression levels were examined using the Human Genome U133A probe array (GeneChip, Affymetrix, Santa Clara, CA, USA).

RNA was prepared using TRIzol (1ml) (Invitrogen), which was added to adherent ASM cultures and incubated for 10 minutes at room temperature. Following TRIzol retrieval from the tissue culture vessels, chloroform (0.2ml) was added to the TRIzol, followed by agitation and centrifugation, retrieval of the colourless upper-aqueous phase, addition of isopropanol (600µl) and agitation and centrifugation. The resulting RNA pellet was then washed with 75% ethanol (1ml) followed by centrifugation and air-drying of the pellet. The concentration and purity of each sample were determined by analyzing spectrophotometric absorption at 260/280nm. Double stranded cDNA was synthesized using a Super-Script Choice system (Invitrogen Life Technologies) and a T7-(dT)24 primer (Amersham Pharmacia Biotech, Buckinghamshire, UK).

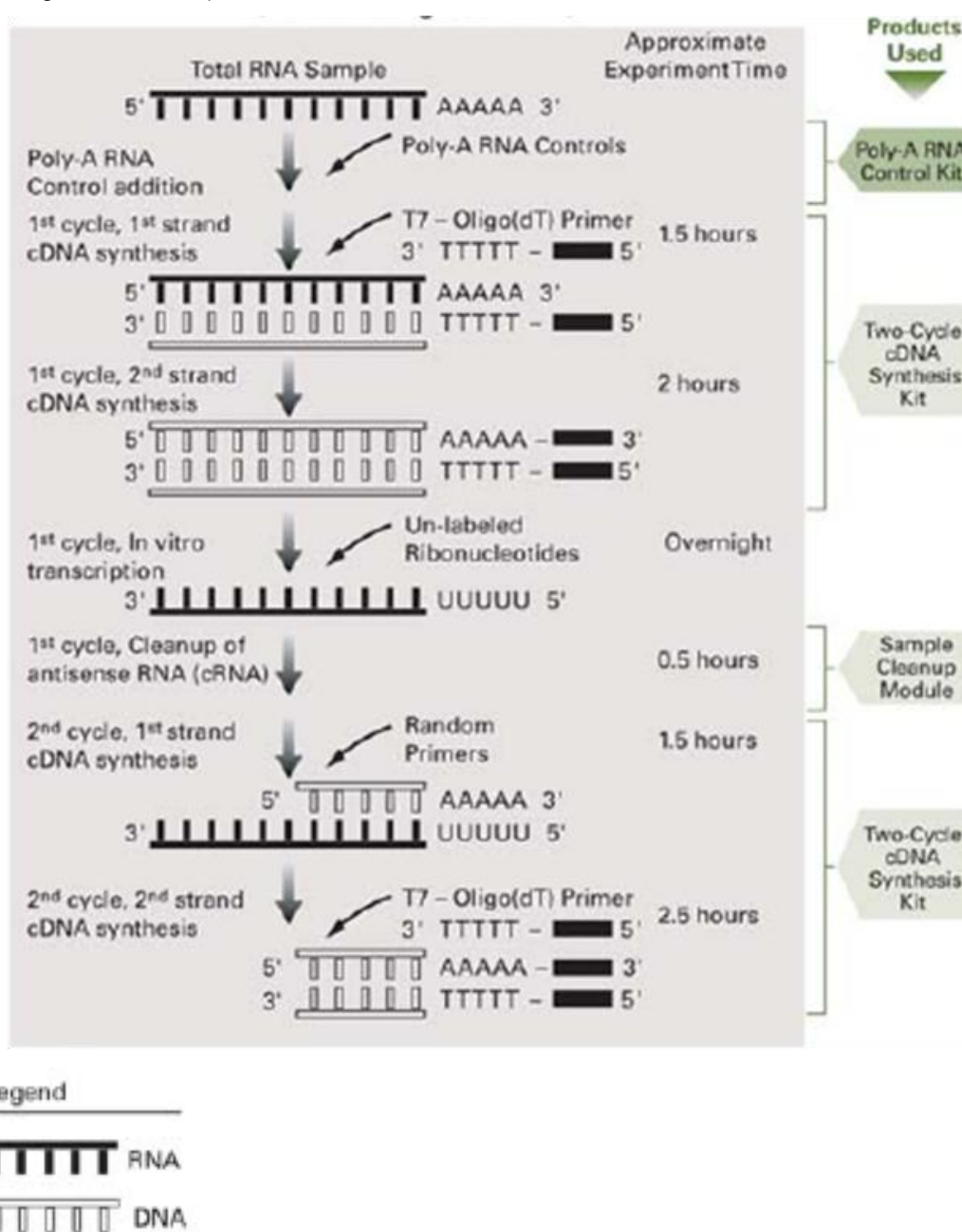


Figure 2.17a; cDNA Synthesis For Genechip Array Analysis

The cDNA was subjected to *in vitro* transcription in the presence of biotinylated nucleoside triphosphates using a BioArray high-yield RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY, USA). The biotinylated cRNA was hybridized with a probe array for 16 hours at 45°C. After washing, the hybridized, biotinylated cRNA was stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR, USA), and then scanned with a HP gene array scanner.

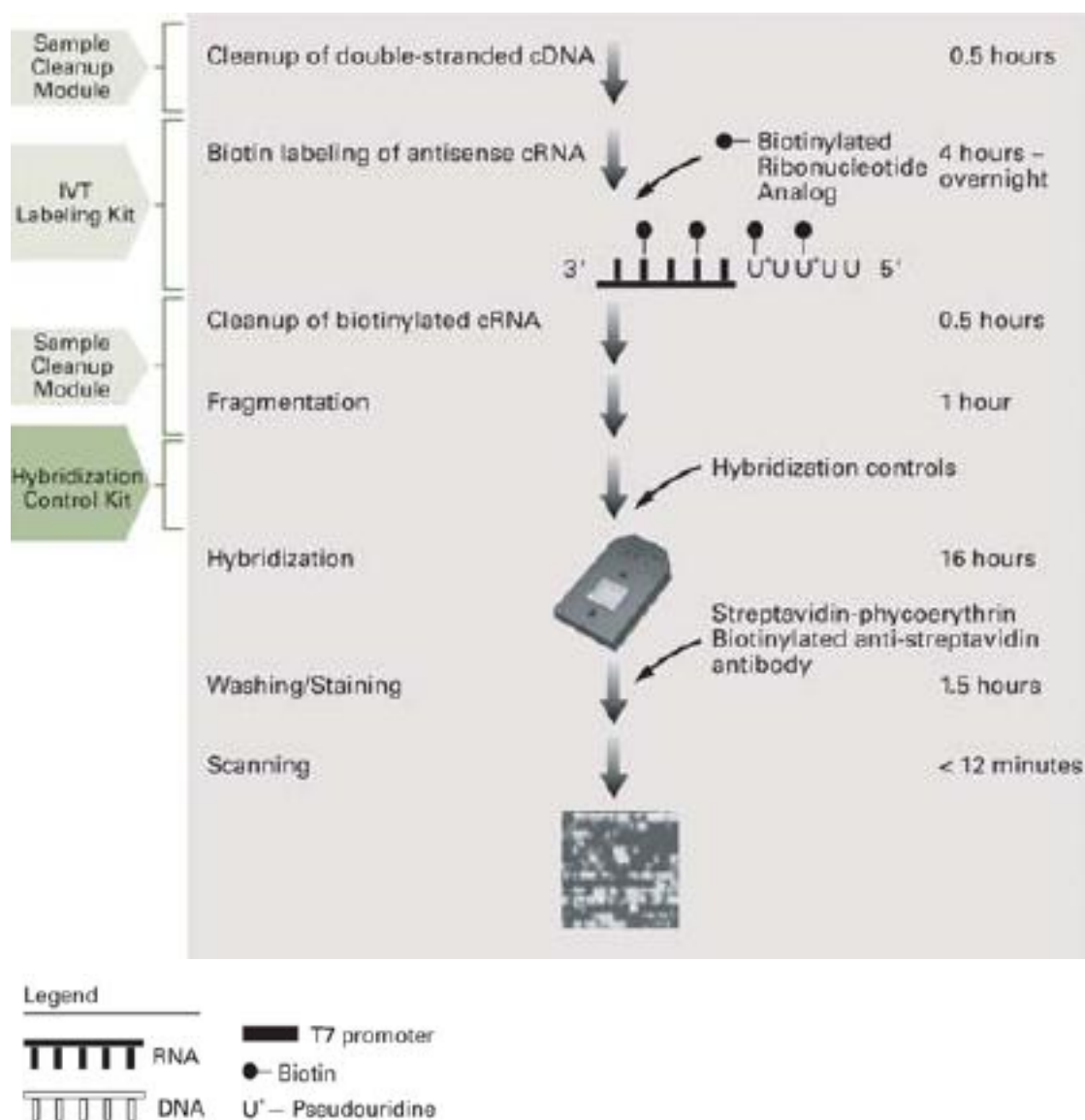


Figure 2.17b: Genechip Array Analysis

The fluorescence intensity of each probe was quantified using a computer program, GeneChip Analysis Suite 4.0 (Affymetrix). The expression level of a single mRNA was determined as the average fluorescence intensity among the intensities obtained by 11 paired (perfect-matched and single nucleotide –mismatched) probes consisting of 25-mer oligonucleotides. If the intensities of the mismatched probes were very high, gene expression was judged to be absent, even if a high average fluorescence was obtained with the GeneChip Analysis Suite 4.0 program. The level of gene expression was determined as the average difference (AD) using the GeneChip software. The percentages of the specific AD levels versus the mean AD level of six probe sets for housekeeping [β -actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] were then calculated (269).

2.18 GMA Embedding of Biopsy Specimens

Bronchial biopsies from subjects with asthma obtained through bronchoscopy and were fixed in acetone and embedded in glycomethacrylate (GMA). The biopsy was immediately placed into ice cold acetone containing phenyl methyl sulphonyl fluoride (2mM) and iodoacetamide (20mM) and fixed overnight at -20°C. After this, the fixative was replaced with acetone and incubated at room temperature for 15 minutes, and then the acetone was replaced with methyl benzoate and incubated at room temperature for 15 minutes. The biopsies were removed from the methyl benzoate and placed in the processing solution. This consisted of methyl benzoate (125µl) in glycol methacrylate (2370µl). Incubation followed at 4°C, and the biopsies were moved to fresh embedding solution every 2 hours for a total of 6 hours. A second embedding solution was then prepared which involved dissolving benzoyl peroxide (70mg) into GMA solution A, a polymer (10ml), then adding GMA solution B, an accelerator (250µl). Both GMA solutions A and B are glycomethacrylate based. The specimens were then embedded in the second embedding solution in Taab flat bottomed capsules. Polymerisation then occurred at 4°C for 48 hours. The specimens were stored in airtight boxes at -20°C with silica gel.

2.19 Immunohistochemistry

Sequential 2µm sections were cut from GMA embedded sections and floated on 0.2% ammonia water. Sections were collected onto saline coated slides and left to dry for 4 hours at room temperature. Slides were then incubated with sodium azide (0.1%) and hydrogen peroxide (0.3%) for 30 minutes and then washed with TBS. D-MEM with Glutamax 1 was then added to the slides for 30 minutes, followed by washing with TBS and labelling with appropriate antibodies and isotype controls.

2.20 Real-Time Reverse Transcription-Polymerase Chain Reaction

RNA was prepared using TRIzol (1ml) (Invitrogen), which was added to adherent ASM cultures and incubated for 10 minutes at room temperature. Following TRIzol retrieval from the tissue culture vessels, chloroform (0.2ml) was added to the TRIzol, followed by agitation and centrifugation, retrieval of the colourless upper-aqueous phase, addition of isopropanol (600µl) and agitation and centrifugation. The resulting RNA pellet was then washed with 75% ethanol (1ml) followed by centrifugation and air-drying of the pellet. The concentration and purity of each sample were determined by analyzing spectrophotometric absorption at 260/280nm.

Real-Time Reverse-Transcription Polymerase Chain Reaction (RT RT PCR) was performed using a single tube Full Velocity SYBR green kit (Stratagene, Stockport, UK). The reagent mixture consisted of experimental RNA (100ng), StrataScript RT/RNase block enzyme mixture, an upstream and downstream primer (250nM), FullVelocity SYBR Green QRT PCR master mix (reverse transcriptase, neutralizing hot start antibody to inactivate FullVelocity DNA polymerase during reaction set-up and cDNA synthesis, DNA polymerase, thermostable accessory protein, MgCl₂, nucleotides – GAUC, stabilizers, SYBR green I dye,) and the ROX passive reference dye (5-carboxy-X-rhodamine in 10mM Tris-HCl (pH 8.6), 0.1mM EDTA, and 0.01% Tween-20 at a final concentration of 30nM). The cycling program was as follows;

Segment		Cycles	Duration	Temperature
cDNA synthesis		1	30 min	50°C
Heat inactivation of reverse transcriptase		1	5 min	95°C
Amplification Step: Denaturation and annealing / extension step (fluorescence was recorded at the end of each cycle)		50	20 sec	95°C
			30 sec	60°C
Dissociation Curve	Melting of DNA	1	30 sec	95°C
	Annealing of DNA	1	30 sec	60°C
	Melting of DNA (fluorescence was recorded during the rise in temperature for this cycle)	1	30 sec	95°C
	Annealing of DNA	1	30 sec	60°C

Table 2.19: RT RT PCR Cycle Design

The fluorescence signal from the PCR reaction was monitored in real-time, and the results are displayed as an amplification plot (figure 2.17a), which reflects the change in fluorescence during cycling. This information was used to quantitate initial copy number based on threshold cycle (Ct), which is defined as the cycle at which fluorescence is statistically significant above background. The Ct value was then compared between various samples to compare the amount of template in each sample.

To determine if the fluorescence in the no template control reaction originated from true amplification of contaminating template in the reaction (primer stocks, master mix), or from the synthesis or primer-dimers or other non-specific products, a dissociation curve was

generated. The PCR samples were subjected to a stepwise increase in temperature from 55°C to 95°C and fluorescence measurements were taken at every temperature increment.

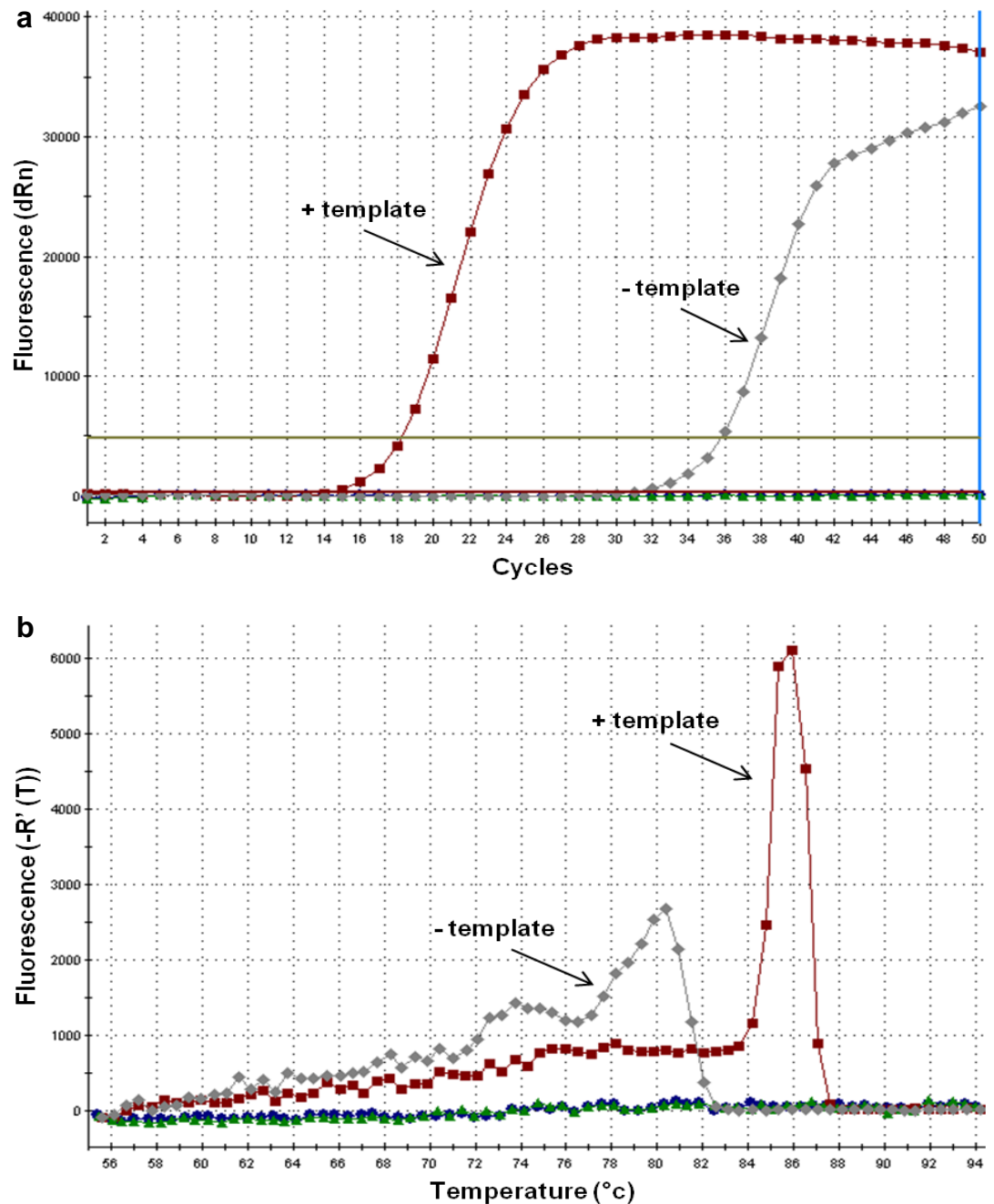


Figure 2.19: Amplification Plots and Dissociation Curves for RT RT PCR

Figures illustrating (a) amplification plots for β -actin with and without template RNA, showing an increase in fluorescence as the number of cycles increase, and (b) dissociation curve analysis, where the fluorescence peak corresponding to the amplicon (centered around 86°C) is distinguishable from the peak due to primer dimers (centered around 80°C).

2.21 Agarose Gels

Agarose (1.5g) was added to Tris-acetate-EDTA (TAE) buffer (50ml) and heated until the agarose had dissolved. The solution was cooled until it turned opaque, after which ethidium bromide (4 μ l) was then added to the solution. To construct the gel, the solution was poured into an appropriately sized tray to which a comb was added to leave space for the samples, and the gel was left to set. A 100bp DNA ladder (Promega), no template controls (NTC) and DNA samples and were individually combined with Blue/Orange Loading dye (Bio-Rad; Hemel Hempstead, UK). The gel was run at 80 volts and 55mA for approximately 45 minutes. The separated products in the gel were visualized with a UV transilluminator.

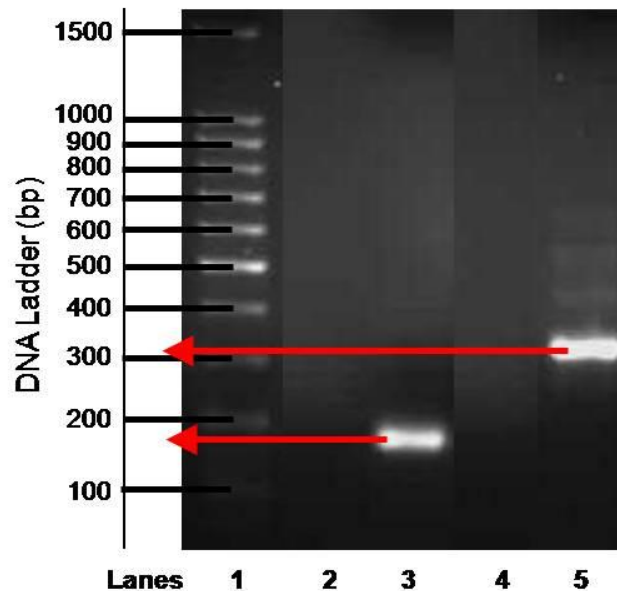


Figure 2.20: DNA Product Size Illustrated in an Agarose Gel

Lane 1; DNA ladder, lane 2; α -SMA NTC, lane 3; α -SMA amplification product (175bp), lane 4; β -actin NTC, lane 5; β -actin amplification product (300bp).

2.23 **Collagen Gels**

ASM cells were grown to confluence and growth arrested in DMEM with Glutamax-1 supplemented with penicillin (100U/mL), streptomycin (100µg/mL), amphotericin (0.25µg/mL), non-essential amino-acids (100µM), sodium pyruvate (1mM) and insulin-transferrin-selenium (1%) (Sigma) for 72 hours, and harvested with trypsin (0.25%).

A gel consisted of 0.125×10^6 ASM cells resuspended in DMEM with Glutamax-1 supplemented with penicillin (100U/mL), streptomycin (100µg/mL), amphotericin (0.25µg/mL), non-essential amino-acids (100µM) (Invitrogen), sodium pyruvate (1mM) and insulin-transferrin-selenium (1%) (Sigma) (144µl), with collagen (299ul resulting in 1.73mg/ml) (Inamed Biomaterials, San Diego, CA, USA), 10X D-MEM (37µl), sodium bicarbonate (20µl) (Invitrogen) including stimulus as required. The gel mixture was added to a precoated well (PBS + 0.5% BSA) on a 24-well plate and left to polymerize at 37°C for 90 minutes.

The gels were then detached and suspended in 500µl of DMEM with Glutamax-1 supplemented with penicillin (100U/mL), streptomycin (100µg/mL), amphotericin (0.25µg/mL), non-essential amino-acids (100µM) (Invitrogen), sodium pyruvate (1mM) and insulin-transferrin-selenium (1%) (Sigma) including stimulus as required and incubated for 48 hours. Histamine (Sigma) was then added to the appropriate wells to a final concentration of 100µM. Photographs were taken at appropriate time points and the surface area of each gel was measured using ImageJ (<http://rsb.info.nih.gov/ij/>) by a blinded observer. Preliminary investigations included the assessment of the inter-subject variability in the assessment of 5 different gels between 5 observers and the results demonstrated excellent agreement (intra-class correlation= 0.99).

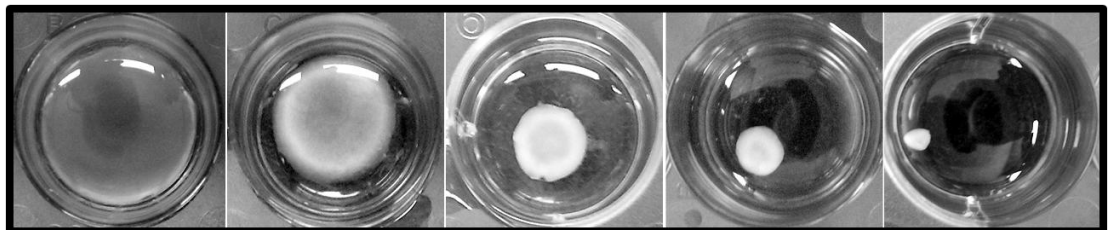


Figure 2.21: Collagen Gels

Representative pictures of a collagen gel embedded with ASM contracting over time.

Chapter Three

Studies

3.1 Chemokine Concentrations and Mast Cell Chemotactic Activity in Bronchoalveolar Lavage (BAL) in Eosinophilic Bronchitis (EB), Asthma and Normal Controls.

3.1.1 Introduction

Sputum mast cells are increased in both asthma and eosinophilic bronchitis, but interestingly the number of mast cells in bronchial brushings and the sputum concentration of mast cell mediators were greater in eosinophilic bronchitis than asthma (23;34), suggesting that mast cell infiltration into the superficial airway may be a particular feature of this condition.

The recruitment of mast cells into the bronchial epithelium in asthma and eosinophilic bronchitis is likely to be mediated by a chemotactic signal arising from the superficial airway. The C-C and C-X-C chemokines in particular are attractive candidates as inflammatory cell chemoattractants. Several chemokine receptors and their respective ligands have been implicated in mediating mast cell migration (223). The chemokine receptors most highly expressed by human lung mast cells are CCR3, CXCR1, 3 and 4 (212;270). The concentration of chemokines in airway secretions in eosinophilic bronchitis has not been reported and thus whether there are important differences between eosinophilic bronchitis and asthma is unknown.

Our hypothesis is that the concentration of chemokines that mediate mast cell migration is increased in airway secretions in asthma and eosinophilic bronchitis. To test our hypothesis we have measured the concentration of the chemokines CCL11 (eotaxin), CXCL8 (IL-8) and CXCL10 (IP-10) in bronchoalveolar lavage (BAL) from subjects with eosinophilic bronchitis, asthma and normal controls and investigated whether the BAL from these subjects is chemotactic for mast cells.

3.1.2 Methods

Subjects, Protocol and Clinical Measurements

Subjects with asthma (n=15), eosinophilic bronchitis (n=14) and healthy subjects (n=15) were selected and characterized as described in section 2.1 and 2.2.

Variables	EB Subjects (n=14)	Asthma Subjects (n = 14)	Healthy Control Subjects (n=15)
Age, † yr	51 (4)	53 (4)	42 (5)
Male	10	5	6
Atopy	6	9	2
PC ₂₀ , ‡ mg/ml	>16	1.1 (0.13)	>16
FEV ₁ , † % predicted	95 (4)	97 (3)	107 (4)
FEV ₁ / FVC ratio	82 (2)	76 (2)	80 (2)
Bronchodilator response †	0.7 (0.4)	5.4 (2.6)	0.9 (0.9)

Table 3.1.2: Clinical Characteristics

‡ Values are given as the geometric mean (log SEM).

† Values are given as the mean (SEM).

Mediator Measurements

The concentrations of CXCL8, CXCL10 and CCL11 in bronchoalveolar lavage (BAL) and bronchial wash samples were all measured by a commercial enzyme linked immunoassay (BD Biosciences) as described in section 2.7. The concentration of CCL11 in 59% of the BAL fluid samples was below the limit of detection, so these samples were concentrated x30 (Vivascience) as described in section 2.6, and the assay was repeated.

As CCL11 has a molecular weight of 8.4kDa, a membrane size of 3000 MWCO was selected. The columns were centrifuged at 12,000xg for 30 minutes.

An insufficient volume of bronchial wash was available to concentrate the samples. The interassay and intrassay variabilities were between 5% and 10%. The limit of detection was 3.1 pg/ml in BAL fluid or bronchial wash sample for CXCL8, 15.6pg/ml in BAL fluid or bronchial wash sample for CXCL10, and 0.21 pg/ml in BAL fluid or 6.25 pg/ml in bronchial wash sample for CCL11. There was an insufficient volume of the bronchial wash sample to measure any chemokines in one subject from each group. Supernatants with undetectable levels of cytokine were assigned a concentration of zero.

HMC-1 Chemotaxis

Chemotaxis assays were conducted as described in section 2.8. Mast cell migration toward recombinant CCL11, CXCL8, and CXCL10 (100ng/ml) (R&D Systems), and BAL fluid (culture medium with 2% fetal calf serum in the top well, and the BAL was diluted 1:2 with culture medium with 2% fetal calf serum in the bottom well) was investigated in 10 subjects with eosinophilic bronchitis, 10 subjects with asthma, and 8 healthy control subjects who had sufficient BAL fluid samples. Insufficient bronchial wash samples were available to assess HMC-1 migration toward these samples. The HMC-1 cells were maintained as described in section 2.5.

To assess the role of CXCR1 and CXCR3 in HMC-1 migration to BAL fluid, HMC-1 cells were preincubated with receptor blocking antibodies (R&D Systems) alone or in combination with the appropriate isotype controls (Dako; Ely, Cambridgeshire, UK) for 1 hour prior to the chemotaxis assay.

HMC-1 Expression of CXCR1 and CXCR3

HMC-1 cells were prepared for immunofluorescence as described in section 2.9. The HMC-1 cells were labeled with CXCR3 monoclonal antibody (MoAb), indirectly labeled with fluorescein isothiocyanate (FITC) and CXCR-1-PE MoAb (R&D Systems) or appropriate isotype controls. Cells were counterstained with 4',6'-diamidine-2-phenylindole (DAPI) (Sigma), and the proportion of positively stained cells was identified by immunofluorescence.

We had identified technical problems with the indirectly labeled CXCR1 MoAb that was used in an earlier report (270), leading us to believe that we had previously underestimated the CXCR1 expression by the HMC-1. Therefore we reanalyzed the expression of CXCR1 by HMC-1 using a conjugated CXCR1-PE MoAb (R&D Systems) with an appropriate isotype control by single-color flow cytometry as described in section 2.10. HMC-1 expression of the other chemokine receptors identified previously by flow cytometry was consistent and is not rereported here.

The HMC-1 functional response to CXCL8 and CXCL10 (100ng/ml) was assessed using calcium imaging as described in section 2.11.

Statistical Analysis

Bronchial wash sample and BAL fluid cell characteristics were described as the median and interquartile range (IQR). Bronchial wash and BAL fluid sample CXCL8 and CXCL10 concentrations were log-normally distributed and were described as geometric means (log SEM). Bronchial wash and BAL fluid sample CCL11 concentration was described as the median and IQR. Supernatants with undetectable levels of cytokine were assigned a concentration of zero. Mast cell migration was expressed as the mean (SEM) fold difference in migration compared with controls. HMC-1 cells were defined as responders to ligand activation if the increase in $[Ca^{2+}]_i$ of the responding cells was more than 2 SEMs above the baseline variation. The increase in $[Ca^{2+}]_i$ of the responding cells was described as the mean (SEM). Cytokine concentrations were compared across all groups by

analysis of variance, those between disease groups were compared by unpaired t test for parametric data, and those across groups were compared by the Kruskal – Wallis test and between groups by Mann-Whitney test for nonparametric data. CCL11 was measurable in about half of the samples, so the number of subjects who had measurable CCL11 concentrations was compared across groups by χ^2 test. Correlations between BAL fluid chemokine concentrations and HMC-1 migration toward BAL fluid were analyzed by the Spearman rank correlation coefficient. Significance was accepted at the level of 95%.

3.1.3 Bronchial Wash and BAL Characteristics

Variables	EB Subjects (n=14)	Asthma Subjects (n = 14)	Healthy Control Subjects (n=15)
BW cell counts			
Eosinophils, § %	3 (8)	4 (10)	0.5 (1)
Neutrophils, § %	62 (29)	42 (10)	40 (39)
Macrophages, § %	30 (20)	48 (26)	54 (24)
Lymphocytes, ‡ %	1 (2)	0 (1)	1 (2)
Total Cell Count, † x10 ⁶	0.5 (0.6)	0.56 (0.4)	0.6 (0.2)
BW chemokines			
CCL11, § pg/ml	3.9 (5)	0 (13)	0 (5)
CXCL8, ‡ pg/ml	689 (0.10)	307 (0.08)	370 (0.09)
CXCL10, ‡ pg/ml	839 (0.10)	208184 (0.18)	245 (0.18)
BAL cell counts			
Eosinophils, § %	2.2 (3)	1.2 (1.6)	0.6 (0.8)
Neutrophils, § %	6 (19)	10 (14)	3 (13)
Macrophages, § %	69 (28)	75 (32)	83 (32)
Lymphocytes, § %	8 (18)	7 (6)	4.9 (8.55)
Total cell count, † x10 ⁶	5.5 (0.6)	6 (1.1)	7.2 (1.1)
BAL Chemokines			
CCL11, § pg/ml	0 (7)	8 (38)	1 (17)
CXCL8, ‡ pg/ml	193 (0.11)	139 (0.08)	89 (0.08)
CXCL10, ‡ pg/ml	470 (0.09)	184 (0.08)	270 (0.07)

* BW = bronchial wash sample.

‡ Values are given as the geometric mean (log SEM).

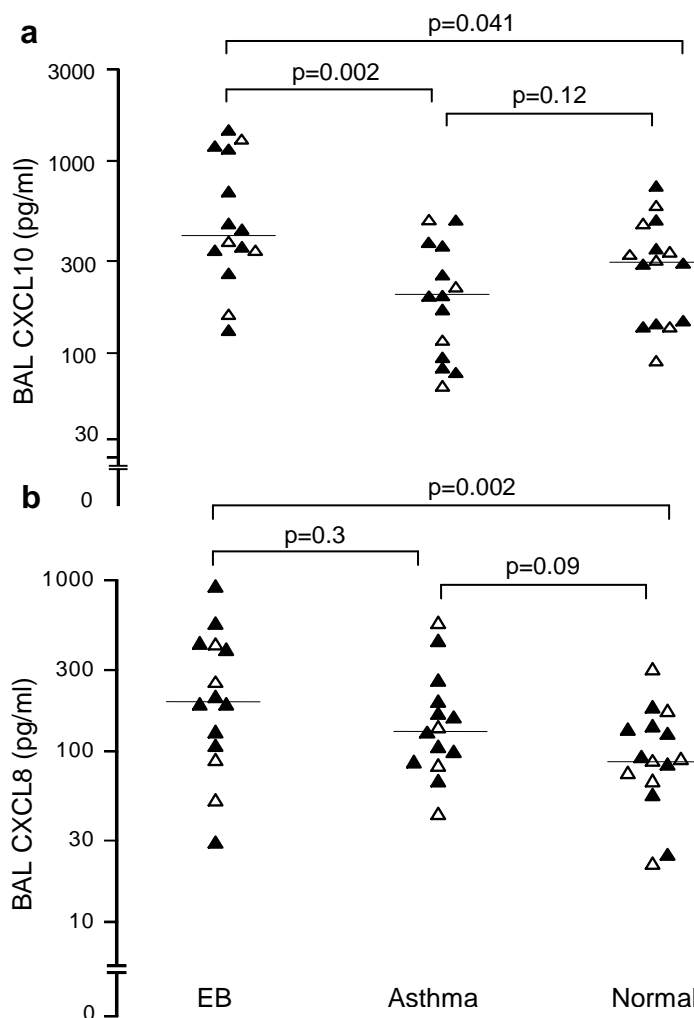
§ Values are given as the median (IQR).

† Values are given as the mean (SEM).

|| p < 0.05 (by analysis of variance).

The bronchial wash, BAL differential cell counts and chemokine concentrations were as shown (Figure 3.1.3). The bronchial wash samples CXCL8 and CXCL10 concentrations were increased in those subjects with eosinophilic bronchitis (p<0.05) (Figure 3.1.3). The individual BAL fluid concentrations of CXCL10 and CXCL8 for each subject were as shown (Figure 3.1.4).

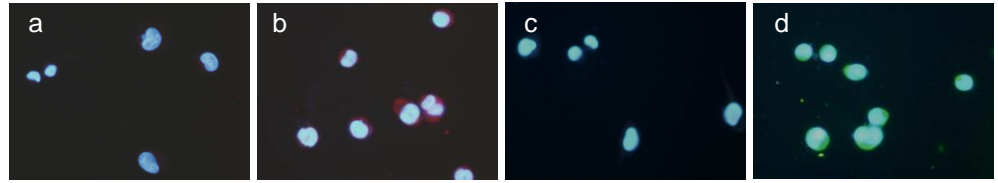
3.1.4 BAL Fluid Concentration of (A) CXCL10 and (B) CXCL8 in Each Subject With EB and Asthma, and in Healthy Control Subjects.



▲ = subjects who were included in the chemotaxis assays. Δ = subjects not included in the chemotaxis assays. Horizontal bars represent the geometric mean.

The BAL fluid CXCL10 concentration was significantly different between groups ($p=0.003$). The BAL fluid CXCL10 concentration was greater in those subjects with eosinophilic bronchitis than in those with mild asthma ($p=0.002$) and in healthy control subjects ($p=0.041$) (Figures 3.1.3 and 3.1.4a). There was no difference in the BAL fluid concentration between subjects with asthma and healthy control subjects. Similarly, the BAL fluid CXCL8 concentration was significantly different between groups ($p=0.038$) and was greater in those subjects with eosinophilic bronchitis than healthy control subjects ($p=0.02$) (Figures 3.1.3 and 3.1.4b). The BAL fluid CXCL8 concentration was not different in subjects with asthma compared to those with eosinophilic bronchitis or healthy control subjects. There were no between-group differences in the CCL11 concentrations in BAL fluid ($p=0.43$). The number of subjects with a measurable BAL fluid CCL11 concentration was not significantly greater in those with eosinophilic bronchitis (6 of 14 subjects), compared to those with asthma (7 of 14 subjects) and healthy control subjects (9 of 15 subjects; $p=0.65$).

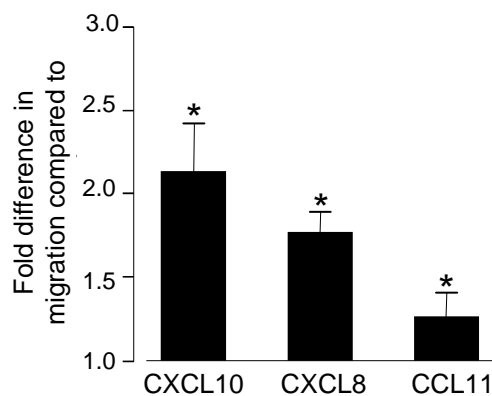
3.1.5 CXCR1 and CXCR3 Expression by HMC-1 Cells



(a) IgG2a isotype control, (b) red CXCR1+ cells, (c) IgG1 isotype control, (d) green CXCR3+ cells. Blue nuclear counterstain (original x 400).

HMC-1 cells highly expressed CXCR3 (mean 95%; SEM, 0.8%) by immunofluorescence and CXCR1 (by immunofluorescence: mean, 89%; SEM, 2.3%; by flow cytometry: mean, 84%; SEM, 5%; n=4) (Figure 3.3.4). HMC-1 $[Ca^{2+}]_i$ increased in response to activation by CXCL8 (geometric mean, 267 nmol/L; log SEM, 30 nmol/L; 81% of cells responded; n=27) and CXCL10 (geometric mean 80 nmol/L; log SEM, 11 nmol/L; 96% of cells responded; n=45).

3.1.6 The Recombinant Chemokines CXCL10, CXCL8, and CCL11 (100ng/ml) were Chemotactic for HMC-1 Cells

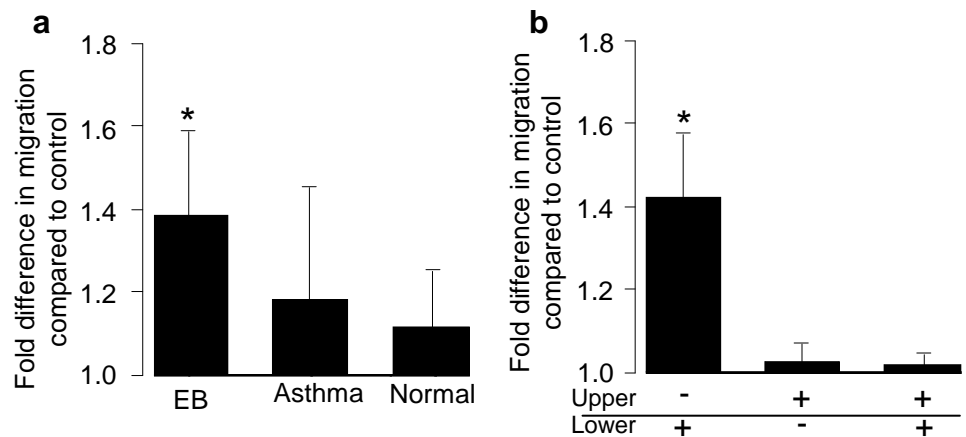


Error bars represent the mean \pm SEM of HMC-1 migration compared to controls.

* = $p < 0.05$ ($n = 6$).

HMC-1 cells migrated significantly towards recombinant CXCL8, CXCL10, and CCL11 ($n=6$) (Figure 3.1.6). This migration was inhibited by the chemokine receptor blocking antibodies CXCR1 (mean inhibition, 83%; SEM, 17%; $p=0.04$) CXCR3 (mean inhibition, 73%; SEM, 13%; $p<0.0001$), but not CCR3 (mean inhibition, 50%; SEM, 22%; $p=0.08$) compared to the appropriate isotype controls.

3.1.7 HMC-1 Chemotaxis to BAL Fluid from Subjects with EB, Asthma and Normal Controls

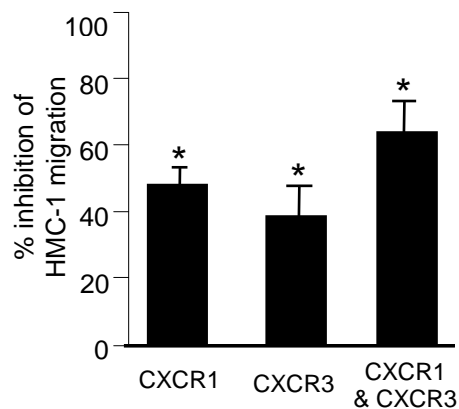


(a) HMC-1 chemotaxis to BAL fluid from subjects with eosinophilic bronchitis (EB) ($n = 10$), subjects with asthma ($n = 10$), and healthy control subjects ($n = 8$) (b) HMC-1 cells were analyzed for chemotactic and chemokinetic activity towards BAL fluid from subjects with EB ($n = 7$) by the addition of BAL fluid to the upper chamber or lower chamber, as indicated. Error bars represent the mean \pm SEM HMC-1 migration compared to controls.

The BAL fluid from subjects with eosinophilic bronchitis ($n=10$), exhibited chemotactic activity for HMC-1 (1.4 fold increase compared to the control; 95% confidence interval [CI], 1.1 to 1.9; $p=0.04$) (Figure 3.1.7a). However, there was no significant HMC-1 migration toward BAL fluid from subjects with asthma (1.1 fold increase; 95% CI, 0.67 to 1.98; $p=0.6$; $n=10$) or healthy controls (1.1 fold increase; 95% CI, 0.77 to 1.52; $p=0.6$, $n=8$).

Checkerboard analysis confirmed that HMC-1 migration toward BAL fluid from subjects with eosinophilic bronchitis was due to chemotaxis rather than chemokinesis ($n=7$) (Figure 3.1.7b).

3.1.8 Inhibition of HMC-1 Chemotaxis to BAL fluid from Subjects with EB by Neutralization of CXCR1 and CXCR3



The mean \pm SEM percentage inhibition of HMC-1 migration to BAL fluid from subjects with EB by blocking MoAbs to CXCR1 and CXCR3 alone or in combination (n=5) compared to appropriate isotype control. Error bars represent the mean \pm SEM HMC-1 migration compared to controls. * = $p < 0.05$.

The clinical characteristics and BAL fluid concentrations were not significantly different in the subgroup of patients whose BAL fluid was used for the chemotaxis assay compared to those for the whole group (Figure 3.1.3). In this subgroup, the BAL fluid CXCL10 concentration was increased in the subjects with eosinophilic bronchitis (geometric mean, 517 pg/ml; log SEM, 0.1 pg/ml) compared to those with asthma (geometric mean, 209 pg/ml; log SEM, 0.1 pg/ml) and healthy control subjects (geometric mean, 270 pg/ml; log SEM, 0.1 pg/ml; $p=0.02$). The CXCL8 BAL fluid concentration was increased in subjects with eosinophilic bronchitis (geometric mean, 224 pg/ml; log SEM, 0.1 pg/ml) compared to that in healthy control subjects (geometric mean, 109 pg/ml; log SEM, 0.06 pg/ml; $p=0.05$), but not compared to that in asthmatic subjects (geometric mean, 137 pg/ml; log SEM, 0.05 pg/ml; $p=0.17$).

As significant HMC-1 migration was only observed toward BAL fluid from subjects with eosinophilic bronchitis, BAL fluid from five of those subjects was used to assess the effect of chemokine receptor blockade on HMC-1 migration. The chemotactic effect of the BAL fluid was markedly reduced by CXCR1 antibodies (45% inhibition; 95% CI, 28 to 62% $p=0.002$) and CXCR3 blockade (38% inhibition; 95% CI, 5 to 72%; $p=0.034$) alone and in combination (65% inhibition; 95% CI, 46 to 85%; $p=0.01$) compared to the isotype control (Figure 3.1.8). There was a positive correlation between HMC-1 migration toward the BAL fluid and the BAL fluid concentration of CXCL8 ($r=0.42$; $p=0.031$) and CXCL10 ($r=0.52$; $p=0.007$).

3.1.12 Discussion

We describe for the first time that BAL fluid from subjects with eosinophilic bronchitis is chemotactic for mast cells. In contrast, BAL fluid from subjects with mild asthma and healthy control subjects did not demonstrate chemotactic activity for mast cells. Mast cell migration was significantly correlated with the BAL fluid concentration of CXCL8 and CXCL10. The BAL fluid concentrations of CXCL10 and CXCL8 were increased in subjects with eosinophilic bronchitis, and blocking CXCR1 and CXCR3, the receptors for these ligands, inhibited mast cell migration. The concentrations of CXCL8 and CXCL10 were similarly elevated in bronchial wash samples compared to those subjects with eosinophilic bronchitis. This suggests that CXCL8 and CXCL10 may promote mast cell migration into the airway lumen in subjects with eosinophilic bronchitis.

Mast cell localization to the superficial airway is a feature of asthma and eosinophilic bronchitis. In both conditions, there were increased numbers of mast cells in bronchial brushing and induced sputum samples (23;31;51). We found that BAL fluid from subjects with eosinophilic bronchitis was chemotactic for mast cells. However, we were unable to demonstrate mast cell chemotactic activity in the BAL fluid from asthmatic subjects. Similarly, Olsson and colleagues (271) found that BAL fluid from allergic asthmatic subjects outside of pollen season had chemotactic activity for mast cells in only 30% of case compared to 100% of cases from the same subjects during pollen season. This may provide a plausible explanation for our inability to show mast cell migration toward BAL fluid from asthmatic subjects as we recruited subjects with mild asthma who had minimal symptoms and perhaps BAL fluid from asthmatic subjects may be chemotactic for mast cells only when the subjects have more active disease. Of note, the mast cell chemotactic activity of the asthmatic BAL fluid obtained during the pollen season in this previous report (271) was increased 1.5 fold over that of control subjects, which was similar to that observed in our subjects with eosinophilic bronchitis, suggesting that this degree of chemotaxis is likely to be biologically relevant. An alternative explanation for the differential effect on mast cell chemotaxis toward BAL from subjects with asthma and eosinophilic bronchitis is that mast cell migration to the superficial airway may be a more prominent feature of eosinophilic bronchitis. In support of this view, the number of mast cells in bronchial brushing samples and the sputum concentration of mast cell mediators were greater in subjects with eosinophilic bronchitis than in those with asthma (23;34).

Several chemotaxins have been described for mast cells. Olsson et al (271) reported that mast cell migration to allergic asthmatic BAL fluid was mediated mainly by the activation of Gi-protein-coupled receptors, and to a lesser extent by stem cell factor and transforming growth factor- β , suggesting that chemokines contribute to the majority of mast cell chemotactic activity of the BAL fluid from these subjects. A number of chemokine receptors have been identified on human blood-derived mast cells and mast cells present in tissues, namely, CCR1, CCR2, CCR3, CCR4, CXCR1, CXCR2, CXCR3, CXCR4, and CX₃CR1 (reviewed in the study by Brightling et al (223)). We have previously described the fact that CXCR3

was the most highly expressed chemokine receptor by mast cells in bronchial biopsy specimens and by HMC-1 cells, and that CXCL10 was chemotactic for mast cells (71;270). In contrast to our earlier report (270), we found that CXCR1 was highly expressed by HMC-1 cells. This discrepancy in CXCR1 expression by HMC-1 was due to different receptor identification by the directly and indirectly labeled monoclonal antibodies used and the different techniques employed. The chemokine receptor profiles of HMC-1 and primary lung mast cells were similar, supporting the view that HMC-1 cells are a suitable model for lung mast cell migration. We are confident that HMC-1 CCR3, CXCR1 and CXCR3 are functional as the activation of HMC-1 cells by CXCL8 and CXCL10 resulted in a transient rise in $[Ca^{2+}]_i$ in a percentage of cells similar to the proportion that expressed CXCR1 and CXCR3, and that these ligands and CCL11 were chemotactic for HMC-1 cells.

All of these ligands are expressed by the airway epithelium (272-274). Therefore, all of the chemokines studied here have the potential to mediate mast cell migration to the superficial airway. We found that the BAL fluid concentrations of CXCL10 and CXCL8 were increased in subjects with eosinophilic bronchitis. BAL fluid from these subjects was chemotactic for mast cells, and blocking CXCR1, CXCR3, or both inhibited mast cell migration. The inhibition of migration was incomplete, and it is likely that other chemokines, such as CXCL12, the ligand for CXCR4, may also play a part in mast cell migration to the superficial airway. Mast cell migration toward the BAL fluid from subjects with eosinophilic bronchitis was reduced compared to migration toward recombinant CXCL8 and CXCL10. This probably reflects the lower concentrations of the chemokines in the BAL fluid. We did not find an increase in the BAL fluid chemokine concentrations in subjects with asthma, which may provide an explanation for the absence of mast cell chemotactic activity in these subjects. In addition, mast cell migration was significantly correlated with the BAL fluid concentration of CXCL8 and CXCL10.

Importantly, CXCL8 and CXCL10 concentrations were also elevated in the bronchial wash samples from those subjects with nonasthmatic eosinophilic bronchitis, suggesting that these chemokines may play a role in mast cell migration to the airway lumen in both large and small airways. In contrast to CXCL8 and CXCL10 concentrations, the BAL fluid and bronchial wash sample CCL11 concentration was detectable in less than half of the subjects, and no differences were observed between subjects with disease and healthy control subjects. This is in contrast with some previous reports that found that BAL fluid CCL11 concentration was increased in stable subjects with disease (275;276) and after allergen challenge (276). However, it is consistent with a report (277) that sputum CCL11 concentrations were not increased in subjects with mild asthma. The subjects with asthma in our study had mild disease, and it is likely that CCL11 concentrations increase with the severity of disease (277). Thus, our findings suggest that CXCL8 and CXCL10, but not CCL11, promote mast cell migration into the superficial airways in subjects with eosinophilic bronchitis.

The distribution of mast cells within particular airway structures is important in the development of specific features of airway diseases. In patients with eosinophilic

bronchitis and idiopathic cough, there was an increase sputum histamine level (278), supporting the view that superficial mast cells play a role in the genesis of chronic cough perhaps due to interactions with airway nerves (279). In mast cells from subjects with asthma, microlocalization within the airway smooth muscle (19) and mucosal glands (36) were associated with increased airway responsiveness and mucous plugging, respectively. We have recently found (71) that in subjects with asthma there was an upregulation of CXCL10 released by the airway smooth muscle, suggesting that the CXCR3/CXCL10 axis may be critical in the localization of mast cells to the airway smooth muscle in subjects with asthma. Therefore it is likely that there are common mechanisms that control the microlocalization of mast cells to different parts of the airway wall.

Eosinophilic bronchitis, like asthma, is generally regarded as a T helper (Th) type-2-driven disease (21), although there is sufficient evidence to suggest that the immunology in subjects with asthma is far more complex and that Th-1 cytokines play a role (280). Indeed, we report here that the BAL fluid concentration of the Th-1 chemokine CXCL10 was increased in subjects with eosinophilic bronchitis in contrast to very low levels of the Th-2 chemokine CCL11. Tumor necrosis factor- α and IFN- γ induce the expression of CXCL10 (72). Tumor necrosis factor- α expression is up-regulated in subjects with asthma (281) and IFN- γ is increased by several stimuli, including viral infection (282). Interestingly, in one report (283) CXCL10 expression by BAL fluid cells was increased in subjects with asthma, illustrating that this chemokine can be up-regulated in subjects with mild disease. In addition, CXCL10 production was increased markedly following segmental allergen challenge in subjects with asthma (284-286). Therefore CXCL10 may be an important chemokine in mast cell recruitment to the airway in response to a variety of stimuli.

3.1.10 Criticisms

One shortcoming of our study design was that the BAL fluid cytopins were not stained appropriately to be able to enumerate the number of mast cells in BAL fluid across the groups, and therefore the concentrations of these chemokines and BAL fluid mast cell numbers cannot be correlated.

3.1.11 Conclusion

In conclusion, we found that bronchial wash sample and BAL fluid concentrations of CXCL8 and CXCL10 were increased in subjects with eosinophilic bronchitis. BAL fluid from these subjects had chemotactic activity for mast cells that was inhibited by blocking CXCR1 and CXCR3. This suggests that the chemokines CXCL8 and CXCL10 may be important in mast cell localization to the superficial airway in subjects with eosinophilic bronchitis.

3.2 **The Airway Smooth Muscle CCR3/CCL11 Axis is Inhibited by Mast Cells**

3.2.1 Introduction

Asthma is characterised by typical symptoms, airway hyperresponsiveness (AHR) and variable airflow obstruction, which can become fixed in severe disease. In addition there is associated airway inflammation together with features of tissue repair known as remodeling (287). The airway inflammation in asthma is typically eosinophilic with increased expression of Th2 cytokines. Importantly the number of mast cells localised within the airway smooth muscle (ASM) bundle is increased in asthma and is related to the degree of AHR (19;57;71;288). Airway remodeling 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 ASM mass (42). This latter feature could be due to a combination of both ASM hyperplasia (85) and hypertrophy, which increases with disease severity and is associated with fixed airflow obstruction (41;42).

The cause of ASM hyperplasia in asthma is unknown and is often attributed to increased proliferation. Indeed ASM proliferation is increased in *ex vivo* asthmatic ASM (86;289), but several reports have been unable to demonstrate increased ASM proliferation *in vivo* (24;41;85). An alternative explanation is that ASM progenitors either located within the airway wall or derived from peripheral blood fibroblast progenitors (fibrocytes) (264), migrate to the ASM bundle and differentiate into ASM. In support of this view myofibroblasts expressing fibrocyte markers have been identified following OVA challenge in a mouse model of asthma and after allergen challenge in human disease (110). We have demonstrated that mast cell and ASM-derived CCL19, a CCR7 ligand, mediates ASM migration (74). The CCR3 ligand CCL11 (eotaxin) is released by ASM (73;290) and in bronchial biopsies the intensity of expression increases with disease severity (42) suggesting that CCR3-mediated ASM migration may be important in severe asthma. We hypothesised that the CCR3/CCL11 axis mediates ASM migration. To test our hypothesis we examined ASM CCR3 expression, function and its modulation by mast cells.

3.2.3 Methods

Subjects, Protocol and Clinical Measurements

Subjects with asthma (n=18) and non-asthmatic subjects (n=25) were selected and characterized as described in section 2.1 and 2.2.

	Non-asthmatic	Asthmatic
Number	25	18
Male (n)	18	11
Age (years)	62 (3)	53 (4)
FEV ₁	2.2 (0.1)	2.2 (0.2)
FEV ₁ % predicted	76 (5)	74 (6)
FEV ₁ /FVC	74 (4)	65 (3)

ASM and Mast Cell Isolation and Culture

Pure ASM bundles in bronchial biopsies obtained from fiberoptic bronchoscopy (n = 20, 18 asthmatic subjects, 2 nonasthmatic) and additional airways isolated from lung resection (n=23) were dissected and cultured as described in section 2.3. HLMCs were isolated and cultured from nonasthmatic lung (n=12) as described in section 2.4

CCR3 Expression

Immunofluorescence

ASM cells were prepared for immunofluorescence as described in section 2.9, labeled with CCR3 mAb or appropriate isotype control, and indirectly labeled with RPE.

Flow Cytometry

ASM were stained with CCR3 mAb or appropriate isotype control (R&D Systems), indirectly labelled with R-Phycoerythrin (RPE), then analysed using single colour flow cytometry on a FACScan (BD Biosciences) as described in section 2.10.

Functional Assessment of ASM CCR3

Calcium Imaging

Changes in cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in ASM in response to CCL11 (100ng/ml) were assessed as described in section 2.11.

Chemotaxis Assay

A chemotaxis assay was conducted as described in section 2.12 towards recombinant CCL11 (12.5-300ng; R&D Systems) or ITS control media, plus β -tryptase (0.5–1.25 μg , Europa Bioproducts). Where appropriate experiments were performed in the presence of CCR3 blocking antibody (8 $\mu\text{g}/\text{ml}$, Millennium, Cambridge, MA, USA) or isotype control (8 $\mu\text{g}/\text{ml}$, Dako).

Wound Healing Assay

ASM were subjected to a wound healing assay as described in section 2.13. The number of cells that moved into the wound in the presence of ITS control media and vehicle/CCL11 (25 – 200ng/ml) in the presence or absence of a CCR3 blocking antibody or isotype control (10 $\mu\text{g}/\text{ml}$ R&D) over 6 hours were counted by a blinded observer (74).

Cell Metabolic Activity

ASM cell metabolic activity was assessed using the MTS assay as described in section 2.14 following incubation with 12.5-100ng/ml CCL11 in 10% FBS media or ITS media for 24 hours.

Proliferation and Survival

ASM proliferation was assessed by cell counts following incubation with CCL11 (100ng/ml) in 10% FBS media or ITS media for 24 hours.

ASM proliferation was also assessed by preparing cells using the CellTrace CFSE Cell Proliferation Kit as described in section 2.15. Cells were then treated with 50 $\mu\text{g}/\text{ml}$ mitomycin C (Sigma) for 3 hours to mitotically arrest cells at the parent population, prior to readdition of 10% FBS media, were cultured in parallel to cells exposed to 10% FBS media \pm 100ng/ml CCL11 for 72 hours.

The percentage of apoptotic ASM cells exposed to 10% FBS media \pm CCL11 (100ng/ml) for 24 hours was identified by DAPI staining of cell nuclei to examine nuclear condensation and fragmentation characteristic of apoptosis. Staurosporine (STS) (1 μM) (Sigma) was incubated with the ASM for 20 hours as a positive control. The percentage of apoptotic ASM cells exposed to 10% FBS media \pm CCL11 (100ng/ml) for 24 hours was also identified by using the annexin V and propidium iodide apoptosis assay as described in section 2.16.

Effect Of β -Tryptase And HLMC On Recombinant And ASM-Derived CCL11

The concentration of CCL11 for in cell free experimental supernatants was measured using a commercial ELISA (BD Biosciences) as described in section 2.6, with a limit of detection of 6.25pg/ml and inter and intra-assay variation <5%.

β -tryptase (0.5 μ g/ml) (Europa Bioproducts) was incubated with recombinant CCL11 (6.25 – 200 pg/ml) (R&D) for 2 hours at 37°C. HLMC lysates and unstimulated whole cells (ratio of HLMC : ASM, 1:4) were also incubated with leupeptin (0-80nM/ml).

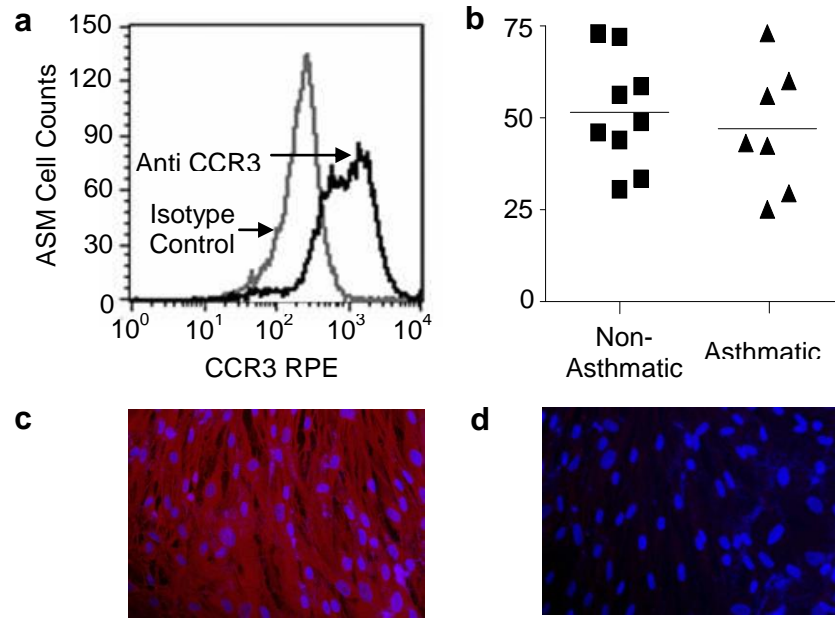
Serum deprived ASM cultures were co-cultured at 37°C (a) with or without β -tryptase (0.5 μ g/ml) and/or leupeptin (20nM/ml) (Sigma) for 3 days; or (b) for 1, 3 and 7 days with: i) HLMC lysates (at a ratio of HLMC : ASM, 1:2 - 1:16), ii) whole unstimulated HLMC, iii) HLMC sensitised with human myeloma IgE (2.5 μ g/ml) (Calbiochem-Novabiochem, Nottingham, UK) or iv) sensitised with IgE and activated with goat polyclonal anti-human IgE (1:1000 dilution) (Sigma). The ratio of HLMC:ASM for the whole cell co-cultures was 1:4.

To assess the effect of HLMC on CCL11 mRNA expression by ASM, supernatants from IgE sensitised /anti-IgE activated HLMC (10×10^6 cells pooled from 3 donors) were incubated with ASM from asthmatic (n=3) and non-asthmatic donors (n=3) for 6 and 24 hours. The proportion of HLMC:ASM cells was 1:4. RNA expression levels of CCL11 extracted from the ASM was examined using the Human Genome U133A probe array (GeneChip, Affymetrix) as described in section 2.17

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 4 (Graphpad Software, San Diego, CA, USA). Data is presented as mean + SEM. Data was analysed by ANOVA across groups and T-tests between groups with Tukey's Correction for multiple comparisons. Differences were considered significant when $p = < 0.05$.

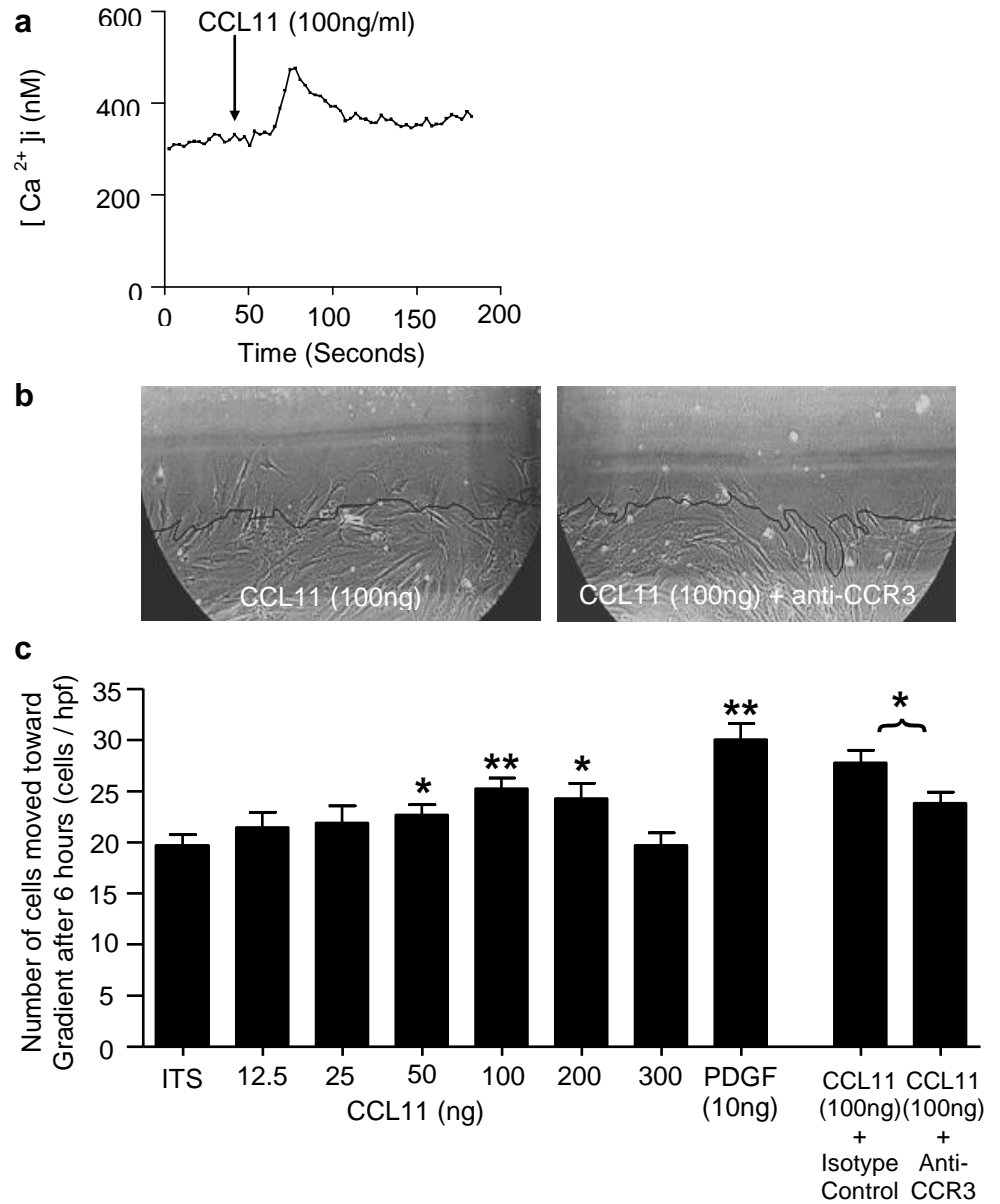
3.2.3 CCR3 is Expressed by ASM



As confirmed by flow cytometry, the example fluorescent histograms represent populations of (a) primary cultured ASM cells that were CCR3 positive, plotted with the corresponding isotype control, (b) percentage of CCR3 positive ASM cells from non-asthmatic control donors Vs. asthmatics. CCR3 expression by ASM was confirmed by immunofluorescence, (nuclei stained blue) with (c) red staining for CCR3 and (d) an isotype control.

As assessed by flow cytometry and immunofluorescence, the proportion of primary cultured ASM cells that expressed CCR3 on their cell surface was $50 \pm 4\%$ (n=16) (Figure 3.2.3a). CCR3 expression by permeabilised ASM was $79 \pm 4\%$ (n=6). There was no difference in expression between ASM derived from asthmatics or non-asthmatic controls ($52 \pm 5\%$, n=9, versus $47 \pm 6\%$, n=7, respectively; p=0.59, Figure 3.2.3b) and CCR3 expression was not upregulated through activation by CCL11. CCR3 expression by ASM was also confirmed by immunofluorescence (Figure 3.2.3 c, d).

3.2.4 ASM CCR3 is Functional



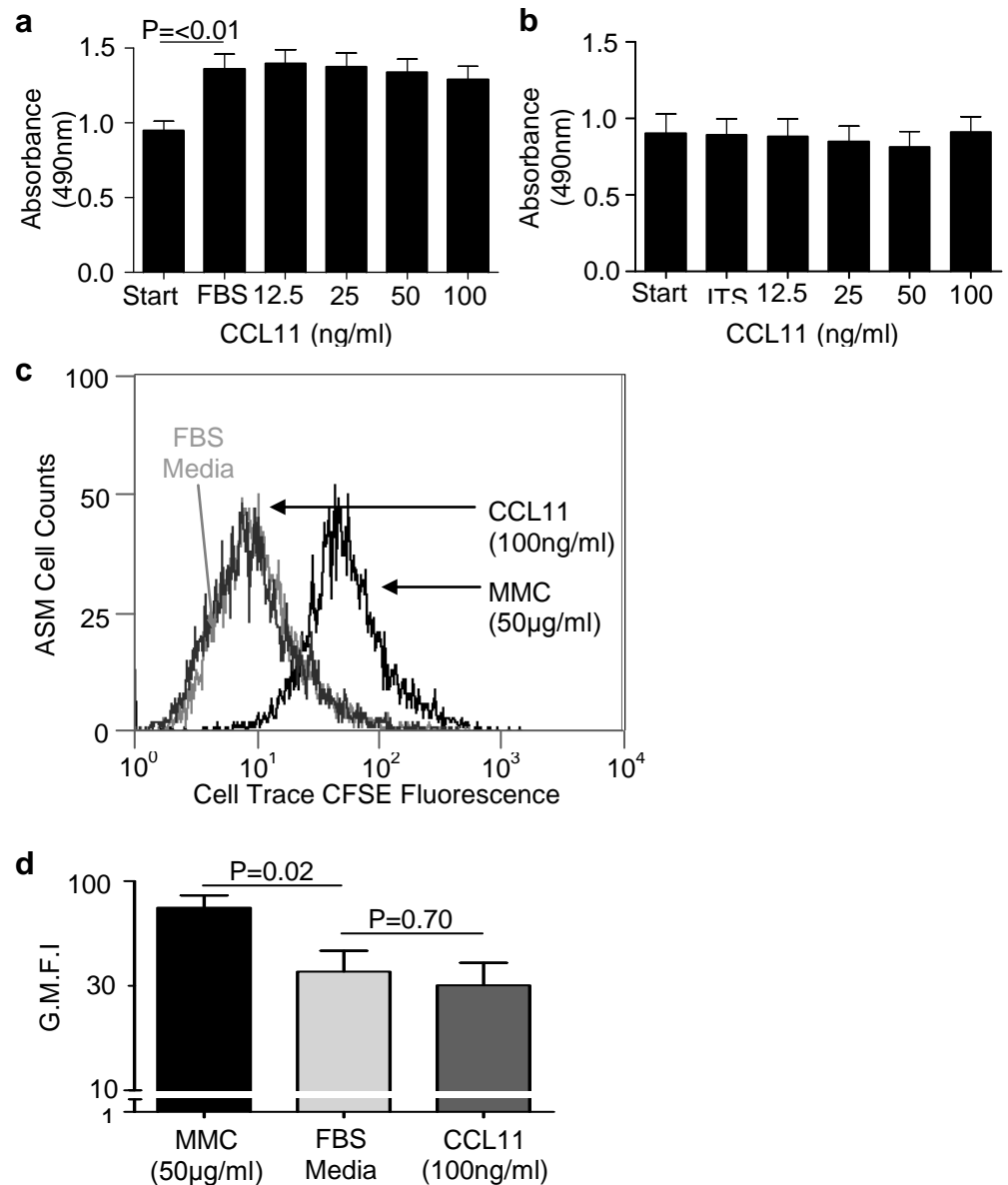
(a) Addition of 100ng/ml CCL11 (indicated by arrow) stimulated $[Ca^{2+}]_i$ elevation in ASM cells. (b) Representative pictures of ASM chemotaxis after 6 hours in one high powered field (hpf) towards CCL11 (100ng) or CCL11 (100ng) plus anti-CCR3. Black line represents position of cells at 0 hours. (c) Concentration-dependant chemotaxis of ASM towards CCL11 and PDGF (10ng) as a positive control. Data are presented as mean + SEM for 4 donors, comparisons made to ITS alone. * $p < 0.05$ ** $p < 0.01$.

As assessed by calcium imaging, following ASM activation by CCL11, 58% of cells responded with a transient mean increase in $[Ca^{2+}]_i$ of 244 ± 38 nM ($n=26$ individual cells, from 3 non-asthmatic donors, Figure 3.2.4a).

As assessed by chemotaxis, CCL11 mediated dose-dependent ASM ($n=4$, $p=0.01$) migration (figure 3.2.4b, c). The number of ASM cells per high-powered field that migrated to CCL11 (100ng on blot) was blocked using a CCR3 blocking antibody (23.8 ± 1.1 versus 27.8 ± 1.2 ; $p=0.02$; $n=4$). This directional movement was predominately due to chemotaxis rather than chemokinesis, as addition of CCL11 directly to the media did not result in significantly more ASM movement than ITS media alone ($n=5$). ASM from non-asthmatic donors was used for chemotaxis assays.

Wound healing was promoted by 10ng/ml PDGF (35.4 ± 2.0 cells/hpf versus 27.1 ± 1.1 in control; $p<0.001$; $n=4$). Recombinant CCL11 promoted wound healing in a concentration-dependent manner, but was only significant for CCL11 100ng/ml (32.6 ± 1.1 cells/hpf versus 27.1 ± 1.1 in control; $p=0.001$; $n=4$). Anti-CCR3 did not significantly inhibit wound healing by recombinant CCL11, but did significantly reduce the wound healing response (mean difference 6.0 ± 2.4 cells/hpf; $p=0.015$; $n=5$) in the presence of ITS media alone, suggesting that the endogenous release of CCL11 is important in mediating wound repair. ASM from non-asthmatic donors was used for wound healing assays.

3.2.5 CCL11 Has No Effect on ASM Cell Metabolic Activity and Proliferation

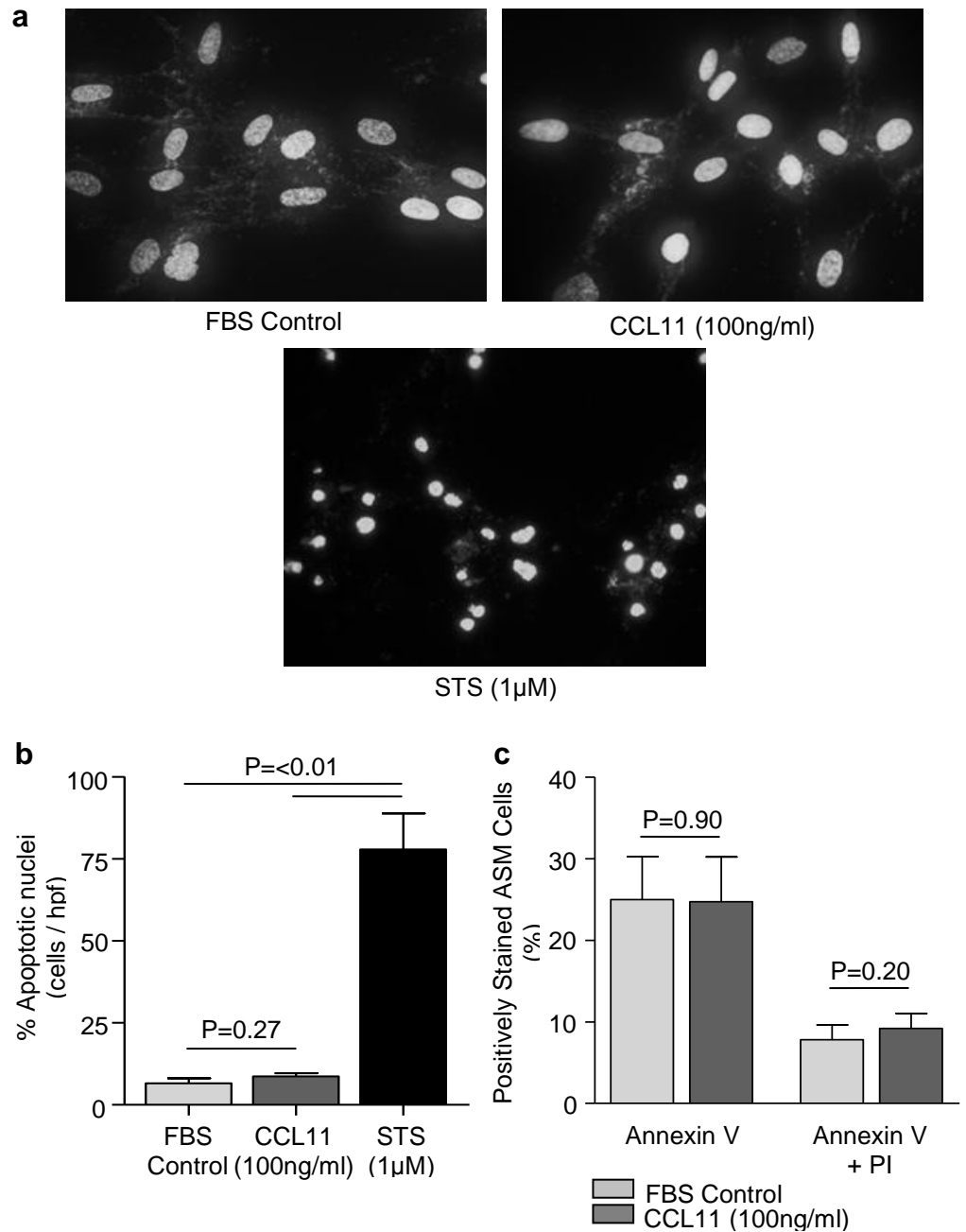


ASM cell metabolic activity in (a) serum free media vs FBS media±CCL11 (data are presented as mean±sem for 10 donors) and (b) serum free media vs ITS media±CCL11 (data are presented as mean±sem for 6 donors). (c) histogram illustrating CFSE fluorescence in ASM cells incubated with MMC (50µg/ml), black line, or FBS media ± CCL11 (100ng/ml) for 72 hours, light grey and dark grey lines respectively, d) ASM cell proliferation was observed after 72 hours (n=7), but was unaffected by incubation with CCL11 (n=6). Data are presented as geometric mean±sem.

In relation to the metabolic activity, the absorbance by formazan seen at 490 nm in the MTS assay was increased in ASM in media supplemented with FBS compared to ASM cells in serum free media (mean difference 0.39 ± 0.07 absorbance units, $p < 0.01$, $n=10$, figure 3.2.5a, b). Recombinant CCL11 had no effect on the MTS assay in the presence of FBS media ($n=10$, figure 3.2.5a) or ITS media ($n=6$, figure 3.2.5b). No difference in the effect of CCL11 was observed between ASM cells derived from non-asthmatic vs asthmatic subjects.

In relation to proliferation, by assessing cell numbers in the presence of FBS media ($n=8$) or ITS media ($n=6$) we saw no effect of 100ng/ml CCL11 after 24h, with no difference between non-asthmatic or asthmatic subjects. Using the Cell Trace CFSE Cell Proliferation assay cell proliferation was seen after 72h in the presence of 10% FBS media compared to MMC treated cells (mean decrease in fluorescence intensity 37.7 ± 4.0 , $p < 0.05$, $n=7$) however this was unaffected by incubation with 100ng/ml CCL11 ($n=6$) (figures 3.2.5c, d) with no difference observed between ASM cells derived from non-asthmatic vs asthmatic subjects.

3.2.6 CCL11 Has No Effect on ASM Survival



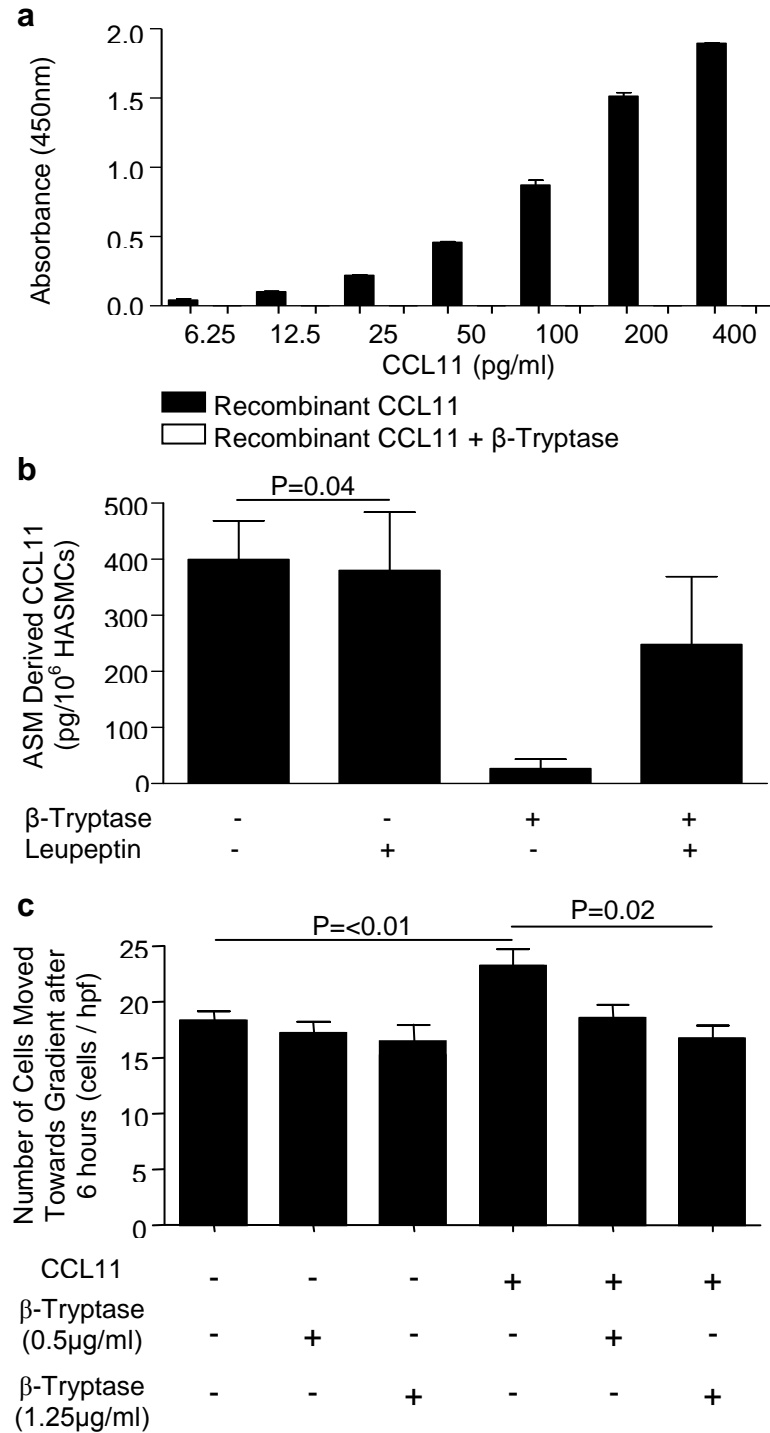
(a) representative photographs showing DAPI staining of nuclear morphology under control conditions, following incubation with: CCL11 (100ng/ml) for 24 hours, both showing normal nuclear morphology, or STS (1µM) as a positive control, (b) Percentage of apoptotic cells in each condition, n=8 donors, (c) Detection of annexin V⁺/PI⁻ and annexin V⁺/PI⁺ ASM cells using 2 colour flow cytometry, n=9 donors. Data are presented as mean ± sem. Statistical differences were assessed using unpaired t-tests.

In relation to survival, the percentage of ASM nuclei showing nuclear condensation and fragmentation characteristic of apoptosis, detected by DAPI staining, was unaffected by incubation with 100ng/ml CCL11 for 24h (control, $6.6 \pm 1.6\%$ vs 100ng/ml CCL11, 8.7 ± 1.0 , $n=8$). In marked contrast in the presence of staurosporine (STS, $1 \mu\text{M}$, 20h), a positive control, $77.9 \pm 10.9\%$ of ASM cells showed nuclear morphology characteristic of cells undergoing apoptosis ($n=8$) (figure 3.2.6a, b).

The above data was confirmed using annexin V/PI staining of ASM cells. The percentage of annexin V^+/PI^- (early apoptotic) ASM cells was unaffected following incubation with 100ng/ml CCL11 for 24h (control, $25.0 \pm 5.3\%$ vs 100ng/ml CCL11, $24.7 \pm 5.5\%$ cells, $n=9$), the same is seen for annexin V^+/PI^+ (late apoptotic/necrotic) ASM cells (control, $7.8 \pm 1.8\%$ vs 100ng/ml CCL11, $9.2 \pm 1.9\%$ cells, $n=9$) (figure 3.2.6c).

Using both DAPI and annexin V/PI staining no difference in the effect of CCL11 was observed between cells derived from asthmatic subjects compared to non-asthmatic controls.

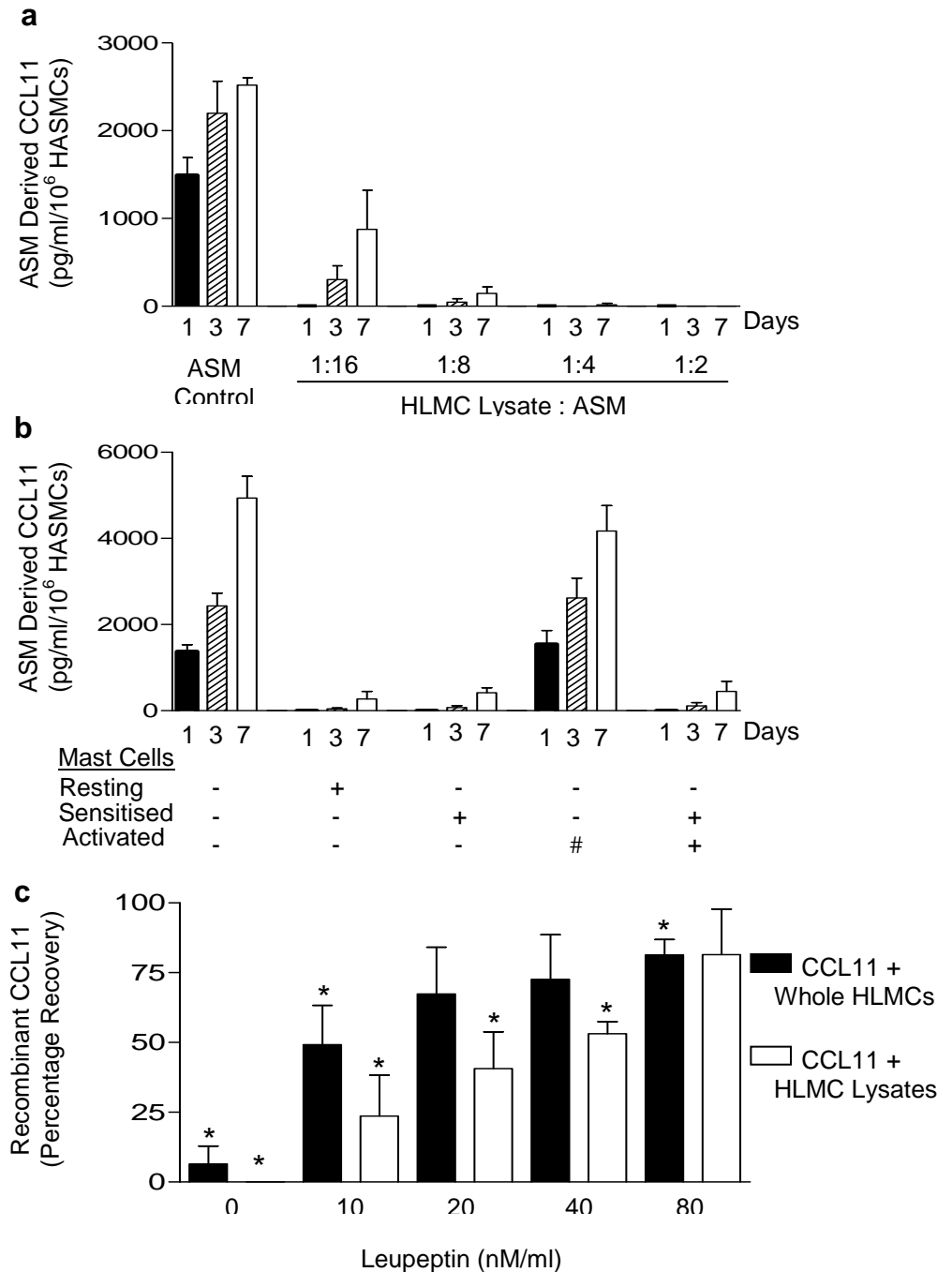
3.2.7 β -Tryptase Degrades CCL11 and Inhibits CCL11 Mediated ASM Migration



(a) The concentration of recombinant CCL11 \pm β -tryptase (0.5 μ g/ml) after 2 hours at 37 $^{\circ}$ c (n=3) (b) The concentration of ASM derived CCL11 (pg/10⁶cells) in supernatants from ASM cultures for 3 days alone, with β -tryptase (0.5 μ g/ml) and/or leupeptin (20nM) (n=3). (c) Chemotaxis of ASM towards recombinant CCL11 (100ng on blot) was inhibited by β -tryptase (n=4). Data are presented as mean \pm sem.

The concentration of CCL11 in ASM supernatant cultured for 3 days ($400 \pm 69 \text{ pg}/10^6 \text{ cells}$) was attenuated by the presence of β -tryptase ($27 \pm 17 \text{ pg}/10^6 \text{ cells}$; mean difference [95% CI] 373 [32-713]; $p=0.04$). This effect was attenuated by leupeptin (Figure 3.2.7a, b).

Chemotaxis assays confirmed that chemotaxis was promoted by 100ng CCL11 ($23.2 \pm 1.5 \text{ cells/hpf}$ versus 18.3 ± 0.8 in control; $p<0.01$) and β -tryptase reduced the migratory response significantly (mean difference $6.5 \pm 2.8 \text{ cells/hpf}$; $p=0.02$; $n=4$) (figure 3.2.7c). β -tryptase did not significantly inhibit chemotaxis in the absence of CCL11.



(a) The concentration of CCL11 in day 1, 3, and 7 ASM culture supernatants alone and co-cultured with HLMC lysates in the proportion of HLMC:ASM 1:2-1:16 ($n=3$; $p<0.001$), (b) co-cultured with whole HLMC (1:4) after sensitisation and/or activation ($n=6$, $p<0.001$) and (c) the percentage recovery of recombinant CCL11 (50pg/ml) after incubation with whole HLMC and lysates (HLMC:ASM 1:4 $\equiv 5 \times 10^4$ cells/ml) for 2hours \pm increasing concentrations of leupeptin. * $p<0.05$ paired t-test. Data are presented as mean \pm sem for 3 donors.

Co-culture of ASM with either HLMC lysates, unstimulated or stimulated whole HLMC reduced markedly the CCL11 concentration in the co-culture supernatant compared to ASM culture alone (figure 3.2.7a, b). The percentage of recombinant CCL11 recovery compared to control was also markedly reduced following incubation with HLMC lysates and whole HLMC. The addition of leupeptin inhibited this reduction in CCL11 concentration in a concentration-dependent manner (figure 3.2.7c).

Quantified by gene array analysis, CCL11 mRNA was present in all ASM donors ($75\pm 15\%$ of GAPDH mRNA, asthmatic, $n=3$ vs $24\pm 6\%$, non asthmatic, $n=3$, $p=0.04$), but was neither up nor down-regulated in co-culture with the HLMC supernatants.

3.2.9 Discussion

We found that ASM cells express CCR3 and that CCL11 mediates ASM migration. Importantly we found that β -tryptase, and stimulated or unstimulated HLMC degrade recombinant and ASM-derived CCL11 and that β -tryptase inhibited CCL11-mediated ASM migration. In addition HLMC supernatants did not up- or down-regulate CCL11 mRNA expression. We found that the CCR3/CCL11 axis has the potential to mediate ASM migration and repair in asthma, but mast cell localisation to the ASM-bundle is a feature of the disease and it is therefore likely that mast cells will have a profound effect on local CCL11 concentrations. Therefore, taken together our findings question the biological importance of ASM-derived CCL11 in ASM hyperplasia in asthma.

CCR3 is expressed preferentially, but not exclusively, by Th2-lymphocytes (291), basophils (292) and mast cells (212;270). The number of CCR3+ cells is increased in bronchial biopsies in asthma (293) and CCR3 is considered an important potential therapeutic target in asthma and other allergic diseases (294). There is increasing recognition that structural cells can express functional chemokine receptors. Indeed bronchial epithelial cells (295), vascular (296) and, in one report, ASM expresses CCR3 (109). Here we confirm that ASM from both asthmatics and non-asthmatics equally express functional CCR3.

However we found that CCL11 had no effect on ASM proliferation. There is a paucity of data on the effect of chemokine receptor activation on ASM proliferation with a single report that CCR7 activation did not affect proliferation (74). In contrast there are several studies of chemokine receptor activation modulating vascular smooth muscle proliferation with CCR2 (297), CXCR6 (298) and CX3CR1 (299) activation increasing proliferation and CCR3 having no effect (300). In addition we report for the first time that ASM metabolic activity and survival was not affected by CCR3 activation. In keeping with an earlier (109) report we found that CCL11 mediated increased $[Ca^{2+}]_i$ concentration-dependent migration and extended these findings to support a role in wound healing. The time course for the both assays was 6hours, which is not sufficient time to observe proliferation by cell counts. Therefore proliferation alone cannot explain our observations. Thus CCR3 has the potential to play a role in ASM repair and hyperplasia in asthma.

If CCR3 expression by ASM is important in asthma then the CCR3 ligands should be differentially expressed in health and disease. Primary ASM from asthmatics and non-asthmatics express CCL11, but it is contentious whether the expression is increased in disease with one report supporting (301) and two refuting that CCL11 release is increased in asthma (71;73) and here we found increased CCL11 mRNA expression in primary ASM from asthmatics compared to non-asthmatics. However perhaps more importantly, CCL11 expression in bronchial biopsies from asthmatics increases with worsening severity of disease (42). Even though CCL11 is expressed by ASM paradoxically there is a marked paucity of eosinophils in the ASM bundle. One possible explanation for this apparent anomaly is that mast cells are microlocalised within the ASM bundle and that mast cell products degrade

CCL11. This view is supported by one study suggesting that β -tryptase inactivates CCL11 (76). Here we have extended this earlier observation by examining the effect of mast cell-ASM co-culture upon CCL11 production and recovery. We confirm that β -tryptase, and for the first time show that mast cells degrade recombinant and ASM-derived CCL11. These effects were blocked by leupeptin. Importantly CCL11 mRNA expression was not affected by co-culture confirming that the reduction in CCL11 concentration in ASM supernatants is unlikely to be due to reduced synthesis. β -tryptase attenuated markedly CCL11-mediated ASM migration. These findings support the view that mast cells can modulate the functional consequences of CCL11 released by ASM.

Our observations question the role of the CCL11/CCR3 axis in the recruitment of ASM and its progenitors towards the ASM bundle in asthma. However, the control of the migration of mesenchymal cells within the airway is likely to be under the influence of a variety of mediators, which in turn are themselves controlled in terms of their synthesis, release and degradation. The concept that ASM hyperplasia may be a consequence of fibrocytes trafficking to the airway remains plausible and recent evidence supports a role for CCR7, in mediating ASM migration towards the ASM bundle (74). In contrast to CCL11, CCL19 a ligand for CCR7 is not degraded by mast cells but is expressed and released by ASM and mast cells. In addition recombinant and mast cell-derived CCL19 mediated ASM migration. Future studies that examine the role of other chemokine receptors involved in ASM migration need to consider the complexity of cellular interactions and the microenvironment relevant to the airway compartment to be studied.

3.2.10 Criticisms

One criticism of our study is that the HLMC were derived from lung resection tissue and not asthmatic subjects. Currently we are limited by our inability to isolate sufficient mast cells from the asthmatic airway. However, we feel that using HLMC that are sensitised and IgE/anti-IgE activated is likely to be reflective of asthmatic mast cells. The proportion of mast cells within the ASM-bundle is unknown, but we have estimated the ratio of mast cells : ASM cells based on our experience with the assessment of bronchial biopsies and we are confident that the range we have chosen captures the proportions of these cells in the asthmatic airway.

3.2.11 Conclusion

In conclusion, we have found that ASM express CCR3. The CCR3/CCL11 axis mediated ASM migration. Critically, recombinant and ASM-derived CCL11 was inactivated by β -tryptase and co-culture with mast cells. Mast cells are microlocalised to the ASM-bundle in asthma. Therefore our findings question the importance of ASM-expressed CCR3 in the development of ASM hyperplasia in asthma.

3.3 The Effect of HLMCs and Mast Cell Products on ASM Differentiation and Contractility

3.3.1 Introduction

Asthma is characterised by the presence of variable airflow obstruction, AHR, and an airway inflammatory response characterized by eosinophilic airway inflammation, Th2 cytokine expression and reticular basement membrane thickening, features which have been implicated in the development of the disordered airway physiology (302;303).

We have demonstrated that many of the immunopathological features of asthma are also observed in the airways of patients with eosinophilic bronchitis (19-21), a condition that presents with a corticosteroid responsive chronic cough, but which in contrast to asthma is not associated with airway hyperresponsiveness or airflow obstruction (22).

The striking difference we found in the pathology of these two diseases was the infiltration of asthmatic ASM by mast cells, suggesting that this is a major determinant of the disordered airway physiology observed in asthma (19).

Importantly the mast cells in the ASM-bundle are in an activated state as evidenced by increased IL-4 and IL-13 expression (215). Mast cells have the capacity to secrete many autacoids, cytokines and proteases, which have the ability to induce ASM proliferation and contraction (304). However, the mechanisms that drive the development of disordered airway physiology in asthma as a consequence of mast cell-ASM interactions have not been fully elucidated. Indeed, to date whether mast cells promote ASM differentiation to a more contractile phenotype is unknown.

The mast cell mediator tryptase has been shown to cause an upregulation in TGF- β production from ASM (57), and TGF- β is known to be increased in the asthmatic airways at a basal level as well as after allergen challenge (305). TGF- β has also been shown to increase expression of the contractile protein α -SMA (96), enhancing the ability of the airway smooth muscle to contract and contribute to variable airflow obstruction (306).

3.3.2 Methods

Subjects, Protocol and Clinical Measurements

Subjects with asthma (n=12) and non-asthmatic subjects (n=29) were selected and characterized as described in section 2.1 and 2.2.

	Asthma *	COPD	Controls
Number	12	11	18
Gender M/F	5/7	7/4	13/5
Age (years)	47 (6)	65 (12)	60 (13)
FEV ₁ (litres)	2.6 (0.3)	1.68 (0.12)	2.5 (0.11)
FEV ₁ % predicted	82.4 (9.8)%	61 (3)	85 (4)
FEV ₁ /FVC (%)	71.9 (5.3)	59 (3)	79 (2)

Mean (SEM) * = GINA treatment step; I=4, II-IV=5, V=3

Immunohistochemical Assessment of Proximal Airway

2µm sections were cut from embedded biopsies, processed as described in section 2.18, and stained sequentially using monoclonal antibodies against α-SMA, tryptase for mast cells and appropriate isotype controls (Dako).

Morphometry was assessed using a computerised image analysis system. In brief the ASM bundle was identified at x200 magnification and its area determined [mean (SEM) area 0.072 mm²]. The threshold (Hue, Saturation & Intensity) required to identify high intensity α-SMA staining within the bundle was then identified by visually selecting high intensity α-SMA stained pixels. The percentage of the ASM bundle area that was occupied by high intensity α-SMA staining was then determined. The corresponding sequential section stained for tryptase was then used to determine the number of mast cells within the bundle (x 400 magnification) and within a 30µm perimeter; determined by a line drawn perpendicular from the perimeter of the bundle to neighbouring mast cells. The total number of mast cells within the bundle and perimeter were corrected for bundle area and the area of the bundle plus the perimeter, respectively, and expressed as /mm².

ASM and Mast Cell Isolation and Culture

Pure ASM bundles in bronchial biopsies obtained from fiberoptic bronchoscopy (n = 14, 12 asthmatic subjects, 2 nonasthmatic) and additional airways isolated from lung resection (n=27) were dissected and cultured as described in section 2.3. HLMCs were isolated and cultured from nonasthmatic lung (n=18) as described in section 2.4

α -SMA Expression

Flow Cytometry. ASM were stained with α -SMA FITC direct conjugate or appropriate isotype control (Dako), and α -SMA expression was assessed by flow cytometry as described in section 2.10, and quantified as fold difference in geometric mean fluorescent intensity (GMFI) compared with isotype control or the proportion of positive cells.

Immunofluorescence. Cells were prepared for immunofluorescence as described in section 2.9. ASM were labeled with α -SMA mAb FITC direct conjugate (Sigma), and compared with an appropriate isotype control (Dako). ASM & HLMC co-cultures were counterstained with tryptase biotin (Chemicon, Watford, UK) indirectly conjugated with streptavidin texas red (Vector Labs, Peterborough, UK) and compared with an appropriate isotype control (Dako). Cells were counterstained with 4',6'-diamidino-2 phenylindole (DAPI, Sigma).

Real-time RT-PCR. Real-time PCR was performed using a SYBR green kit (Stragene) as described in section 2.19.

The internal normaliser gene used was β -actin, and was carried out with β -actin forward (TTCAACTCCATCATGAAGTGTGACGTG), and β -actin reverse (CTAAGTCATAGTCCGCCTAGAAGCATT) primers, while amplification of α -SMA was carried out with α -SMA forward (CTGTTCCAGCCATCCTTCAT), and α -SMA reverse (CCGTGATCTCCTTCTGCATT) primers (307). Amplification efficiency was evaluated using 10-fold serial dilutions of positive controls and calculated from the slopes of log input amounts (initial quantity; copies) plotted versus Ct values. The efficiency of the α -SMA and β -actin primer was confirmed to be high (97.88% and 98.12% respectively) and comparable (less than 2% difference between primers). Products were analysed on an agarose gel as described in section 2.20 in order to verify product size, reagent contamination and primer-dimer formation.

Assessment of ASM contraction by collagen gel analysis

Collagen gels were constructed as described in section 2.21. Following the addition of histamine, photographs were taken at 0.25, 0.5, 1, 2, 4 and 8 hours. The surface area of each gel was measured at each time point using ImageJ (<http://rsb.info.nih.gov/ij>).

Study Protocol

Modulation of α -SMA expression by mast cells and their products.

ASM was serum deprived for 72 hours in ITS medium (D-MEM with Glutamax-1 supplemented with 100U/mL penicillin, 100 μ g/mL streptomycin, 0.25 μ g/mL amphotericin, 100 μ M non-essential amino-acids and 1mM sodium pyruvate and 1% insulin-transferrin-selenium) and co-cultured for 1, 3, or 7 days with i) whole HLMC, ii) HLMC sensitised with 2.5 μ g/ml IgE (Calbiochem) for 1 hour, iii) sensitised HLMC activated with 1:1000 anti-IgE (Sigma), iv) HLMC or HMC-1 lysates and v) isolated 0.25, 0.5 or 1 μ g/ml human lung tryptase (Europa Bioproducts) a concentration equivalent to the tryptase concentration found in a 1:4 mast cell to ASM ratio (242). HLMC or lysates were co-cultured with ASM at a 1:4 mast cell:ASM ratio in order to reflect the ratio that is found *in vivo* (19). Experiments were performed in the presence and absence of the serine protease inhibitor leupeptin (Sigma) and lactoferrin (Sigma), a member of the transferrin class of proteins, at appropriate concentrations to inhibit the activity of the tryptase (308). After 1, 3 or 7 days, α -SMA protein and mRNA expression were examined by immunofluorescence, flow cytometry, and RT RT PCR.

ASM contractile activity was examined by collagen gel analysis (as described in section 2.19) after ASM and either HMC-1 lysate or tryptase \pm leupeptin co-cultures. Cell free supernatants from co-culture of ASM with HLMC, HLMC lysates or tryptase co-cultures were stored at -80°C for later analysis of TGF- β 1 and TGF- β 2 expression by ELISA (R&D Systems) with a limit of detection of 31.25pg/ml.

Modulation of α -SMA expression by TGF- β .

0.25ng/ml, 2ng/ml or 10ng/ml of recombinant TGF- β 1 or TGF- β 2 (R&D Systems) was added to ASM that had been serum deprived for 72 hours. After 1, 3 or 7 days, α -SMA protein expression and ASM contractile activity was examined by flow cytometry and collagen gel analysis.

TGF- β 1 was pre-incubated with or without the TGF- β 1 neutralizing antibody or isotype control (R&D Systems) for 30mins at 37°C before addition to the ASM that had been serum deprived for 72 hours. The concentration of TGF- β 1 neutralizing antibody used was based on the TGF- β 1 content measured in previous ASM co-culture supernatants. α -SMA protein expression and ASM contractile activity was examined by flow cytometry and collagen gel analysis.

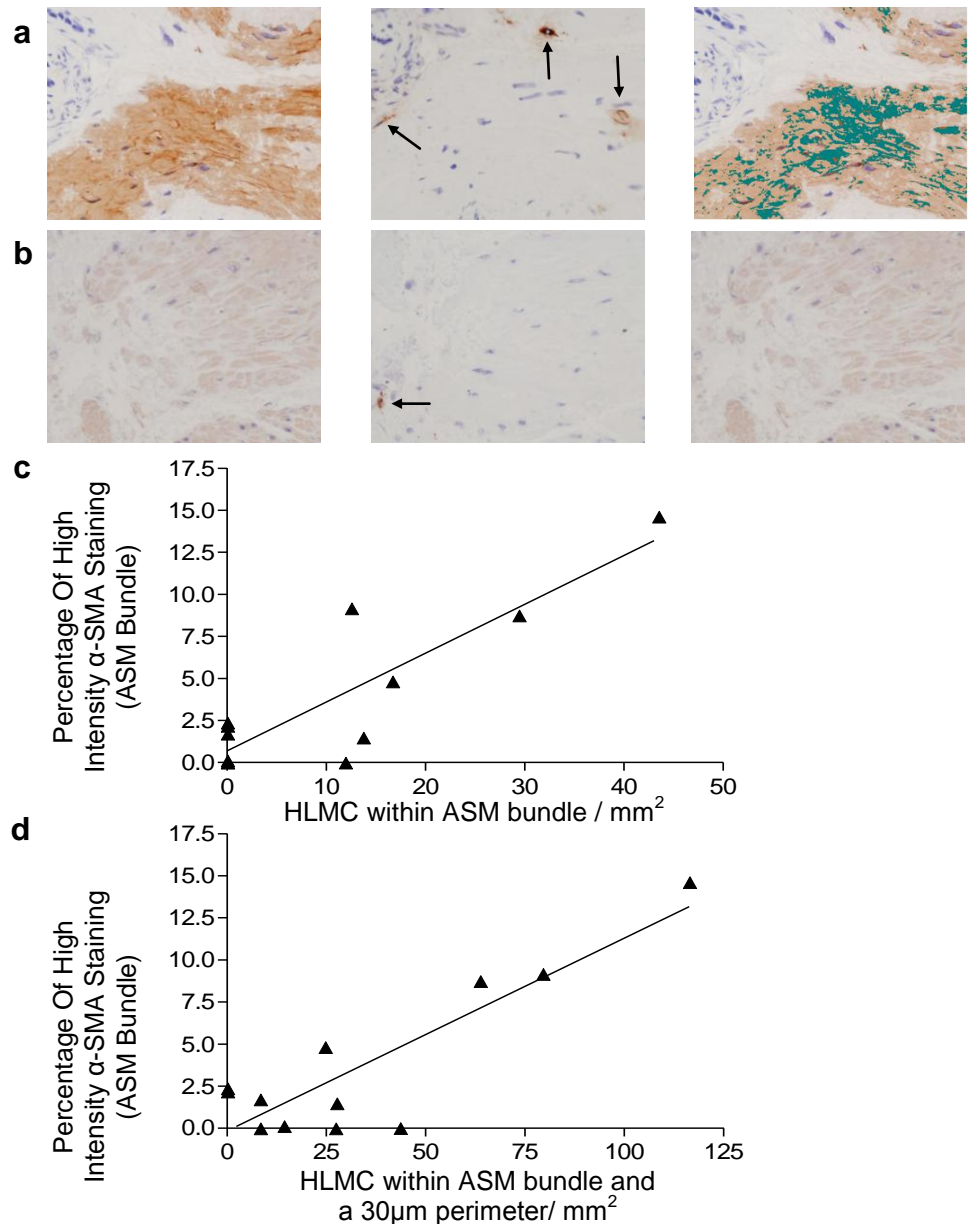
PAR 2 expression by ASM.

Surface and intracellular protein expression by ASM of PAR-2 was assessed by flow cytometry (as described in section 2.10) using an indirectly FITC labelled PAR-2 mouse mAb (R&D Systems) and appropriate isotype control (Dako).

Statistical Analysis.

Statistical analysis was performed using GrapdPad Prism 4. Data are presented as mean (+ SEM). Paired and unpaired data were analysed by using paired and unpaired t tests, respectively. Comparison across groups was assessed using ANOVA and Tukey's multiple comparison test was used for between group comparisons. Differences were considered significant when $p < 0.05$.

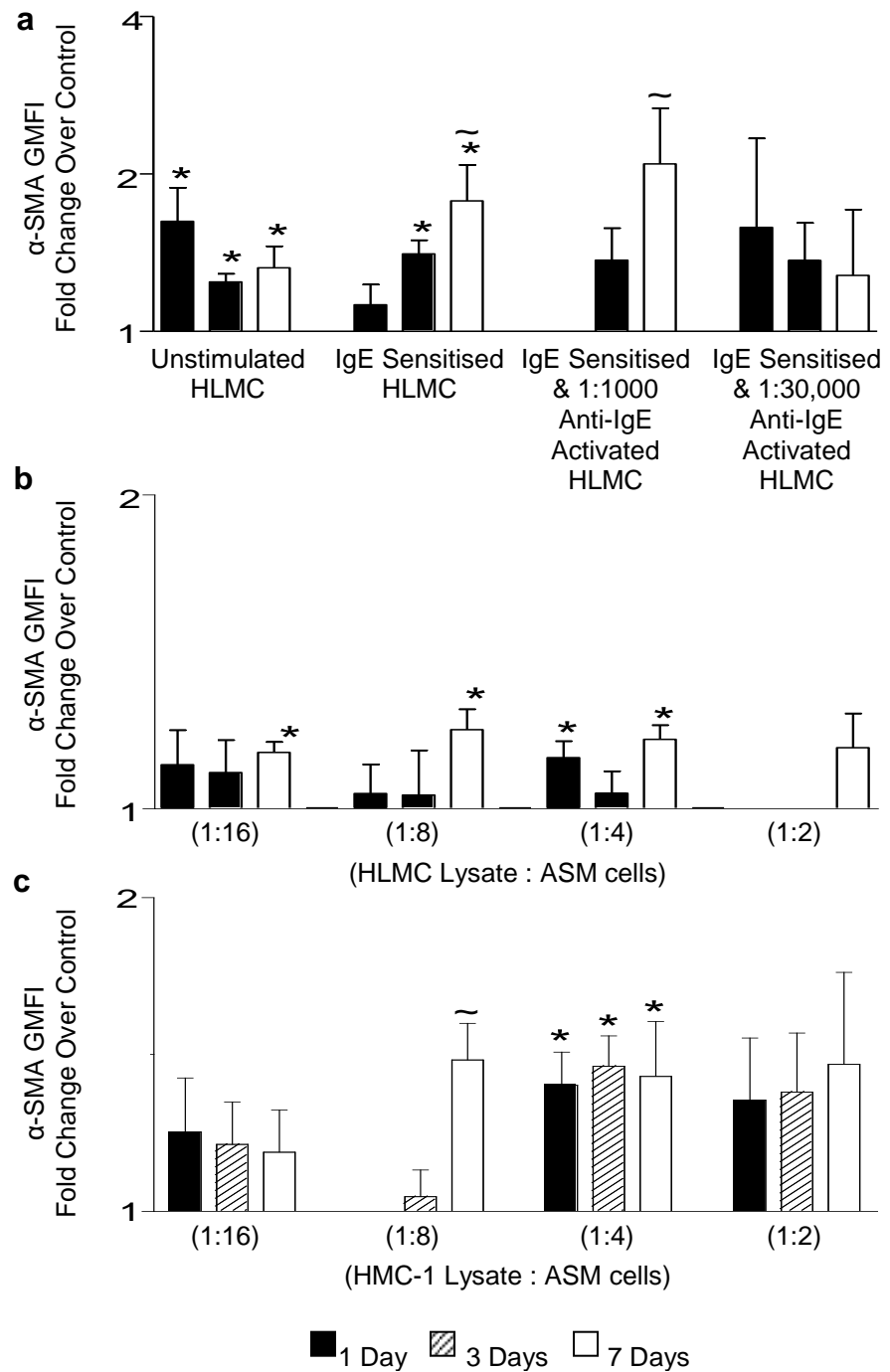
3.3.3 Increased ASM α -SMA Expression *in vivo* Localized with Mast Cells



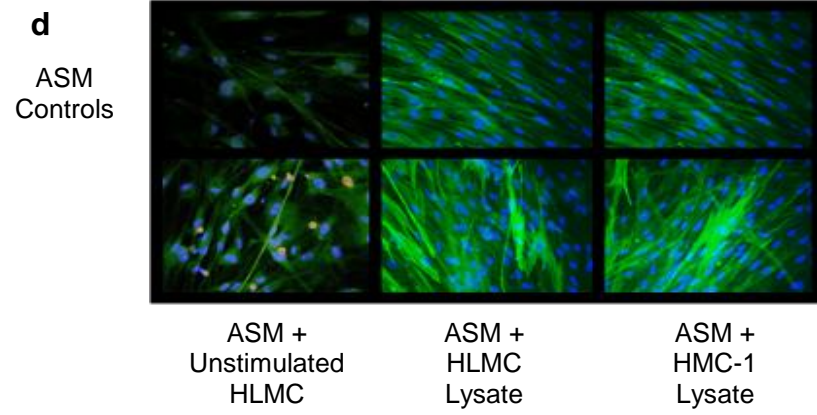
a) Photomicrographs of a bronchial biopsy from an asthmatic illustrating ASM-bundles stained for α -SMA (left), sequential tryptase stained section revealing 1 mast cell within the ASM-bundle and 2 in the 30 μ m perimeter of the bundle (middle), and high intensity thresholded α -SMA stained bundle covering 8.76% of the total ASM-bundle area (right) (x400). **b)** Photomicrograph of another asthmatic again illustrating α -SMA stained ASM-bundle (left), sequential tryptase stained section revealing 1 mast cell in the 30 μ m perimeter and none within the ASM-bundle (middle), and high intensity thresholded α -SMA stained ASM-bundle covering 0% of the total ASM-bundle area (right) (x400). **c)** Correlation between the percentage of high intensity α -SMA staining and the number of mast cells in the ASM-bundle alone ($r=0.87$; $p=0.0003$) and **d)** together with the number of mast cells in the 30 μ m perimeter of the ASM-bundle ($r=0.87$; $p=0.0002$).

In bronchial biopsies from asthmatics, mast cells were identified in and adjacent to ASM-bundles (n=12). Representative photomicrographs of sequential sections illustrating the association between the location of mast cells and the intensity of α -SMA are as shown in Figures 3.3.3a and b. The mean (SEM) number of mast cells within the ASM bundle was 10.6(4.05)/mm² and 34.4(10.3)/mm² in the bundle and perimeter. There was a strong correlation between the percentage high intensity α -SMA staining and the number of mast cells in the ASM bundle alone (r=0.87; p=0.0003; Figure 3.3.3c) and together with the number of mast cells adjacent to the bundle (r=0.87; p=0.0002; Figure 3.3.3d).

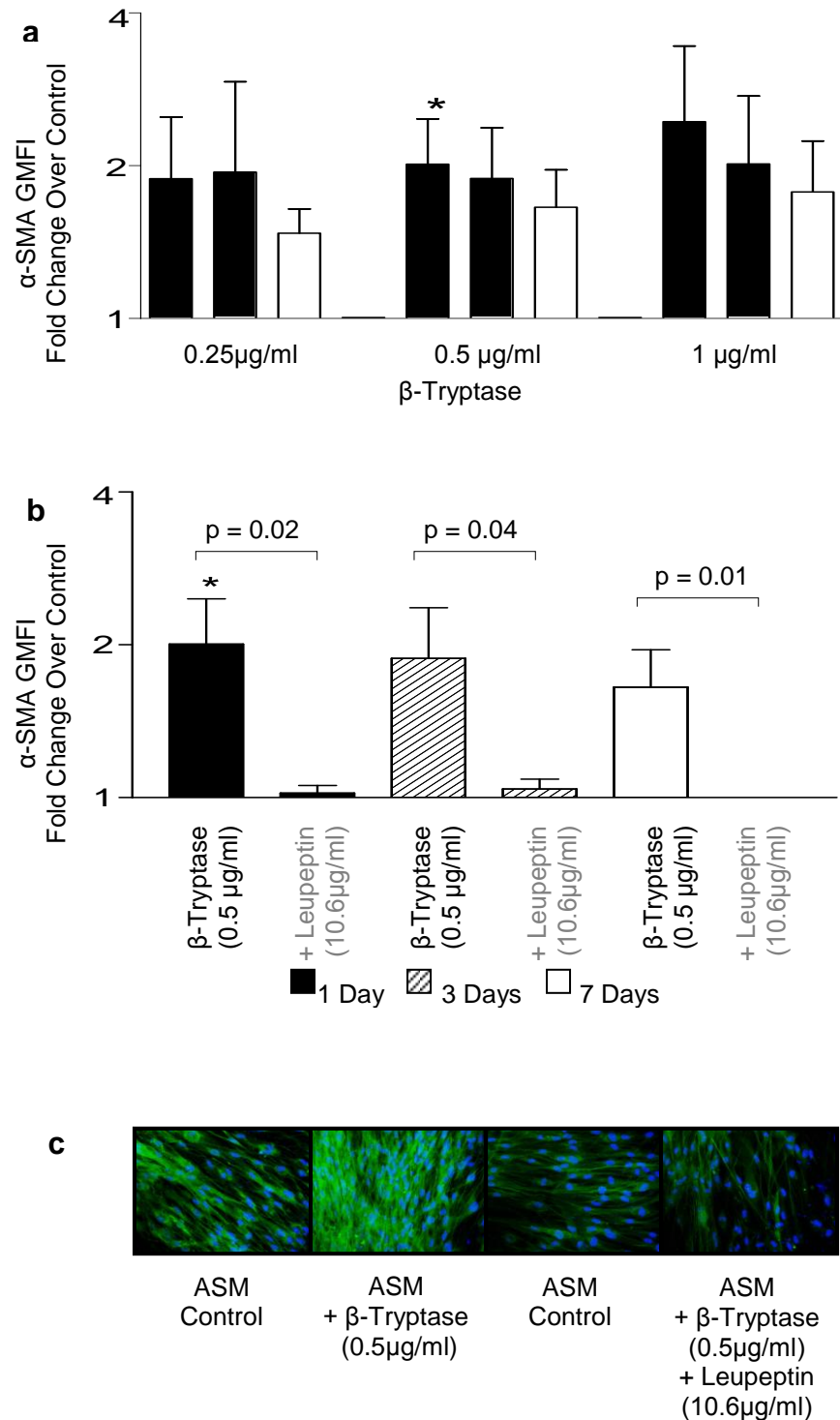
3.3.4 Mast Cells and Mast Cell Lysates Increase ASM α -SMA Expression



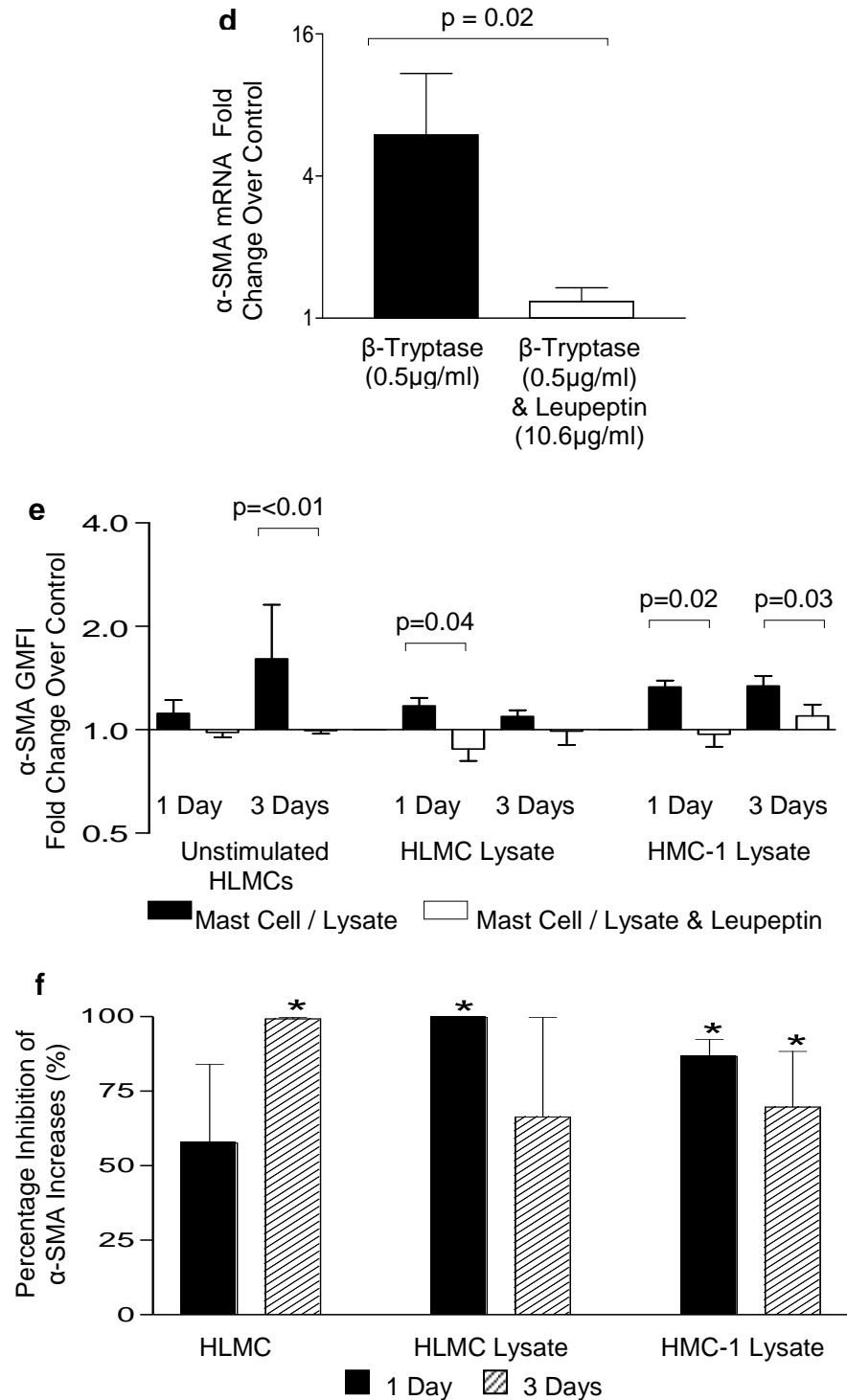
a) Bar charts illustrating the fold change over control of α -SMA GMFI expression by ASM as assessed by flow cytometry after co-culture with unstimulated, IgE sensitised (2.5 μ g/ml) (n = 9 ASM donors, n = 9 HLMC) or anti-IgE activated (1:1000 or 1:30,000) HLMCs (ratio 1 HLMC to 4 ASM) (n = 5 ASM donors, n = 7 HLMC) or b) HLMC (n = 8 ASM donors, n = 7 HLMC) or c) HMC-1 lysates (n = 16 ASM donors) at a ratio of 1:16, 1:8, 1:4 or 1:2 cell lysate to ASM for 1, 3 or 7 days. * p < 0.05 per time point ~ p < 0.05 across all time points.



d) Example immunofluorescence images of ASM co-cultured with unstimulated HLMC, HLMC or HMC-1 lysates for 7 days (1:4 cell / lysate to ASM) (n = 3 ASM donors) * p < 0.05 per time point. ~ p < 0.05 across all time points.



a) Bar chart illustrating the fold change over control of α -SMA GMFI expression by ASM as assessed by flow cytometry after stimulation with tryptase (0.25, 0.5 or 1 μ g/ml) for 1, 3 or 7 days. (n = 4 ASM donors) b) or stimulation with β -tryptase (0.5 μ g/ml) with or without leupeptin (10.6 μ g/ml) for 1, 3 or 7 days. (n = 4 ASM donors) c) Example immunofluorescence images of ASM co-cultured with β -tryptase (0.5 μ g/ml) with or without leupeptin (10.6 μ g/ml) for 7 days (n = 3 ASM donors) * p < 0.05 per time point .



d) Bar chart illustrating the fold change over control of α-SMA mRNA expression by ASM as assessed by RT RT-PCR after stimulation with β-tryptase (0.5 μg/ml) with or without leupeptin (10.6 μg/ml) for 7 days. (n = 3 ASM donors) e) Bar chart illustrating the fold change over control of α-SMA GMFI expression by ASM as assessed by flow cytometry after stimulation with mast cells or lysates (ratio 1 HLMC / lysate to 4 ASM) with or without leupeptin (10.6 μg/ml) for 1 or 3 days. (n = 4 ASM donors, n = HLMC) f) or as illustrated by the percentage inhibition of α-SMA increases observed in figure f. * p < 0.05 per time point .

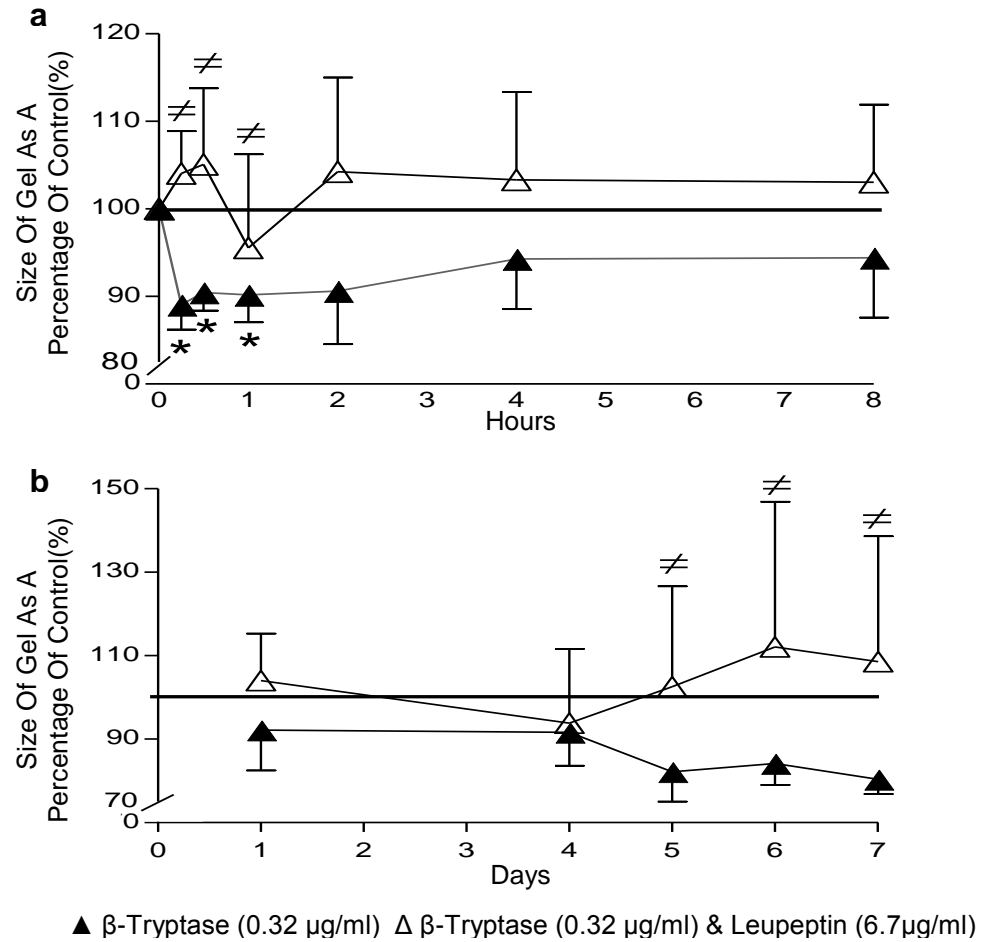
Measuring the GMFI of ASM α -SMA, mast cells or their lysates in co-culture with ASM increased ASM α -SMA expression markedly over 1, 3 and 7 days assessed by flow cytometry (Figures 3.3.4a, b, c). The increase in ASM α -SMA expression in response to co-culture with mast cells was not different between ASM co-cultured with unstimulated or IgE/anti-IgE stimulated HLMCs (Figure 3.1.5a; ANOVA $p > 0.05$ for all time points).

Flow cytometry findings were confirmed by immunofluorescence (Figures 3.3.4d, e). Similar effects on ASM differentiation were observed with the mast cell-derived serine protease β -tryptase. Human lung β -tryptase (0.25, 0.5 or 1 μ g/ml) increased α -SMA expression assessed by flow cytometry (Figure 3.3.5a, b), immunofluorescence (Figure 3.3.5c) and mRNA expression (Figure 3.3.5d).

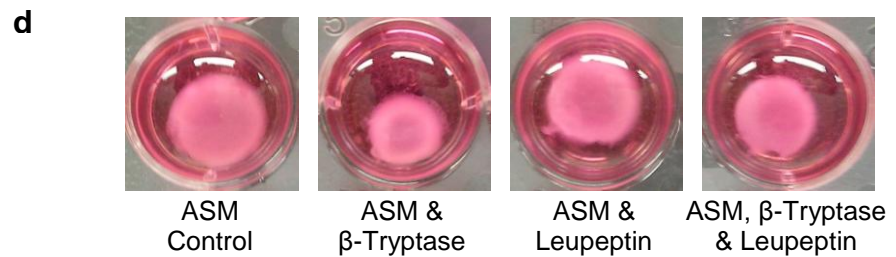
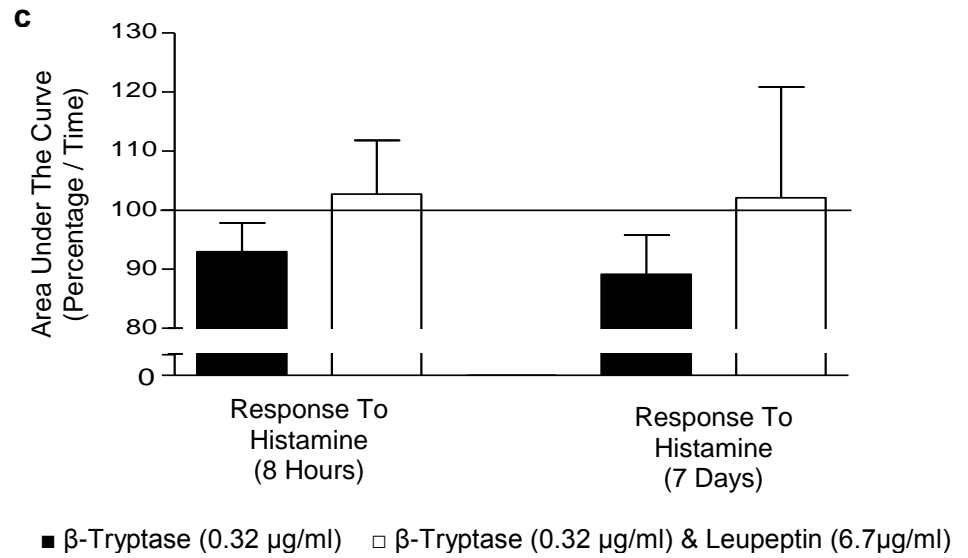
Both the β -tryptase-mediated increase in protein and mRNA α -SMA was significantly inhibited by the serine protease inhibitor leupeptin (10.6 μ g/ml) (Figures 3.3.5b and d).

Similarly, the increase in α -SMA protein following co-culture with whole unstimulated HLMC or HLMC or HMC-1 lysates was significantly inhibited by leupeptin (10.6 μ g/ml) as assessed by flow cytometry (Figures 3.3.5e and f). This suggests that the mast cell mediated up-regulation in ASM α -SMA expression was mediated by tryptase.

3.3.6 Enhanced ASM Contractility in Response to β -Tryptase



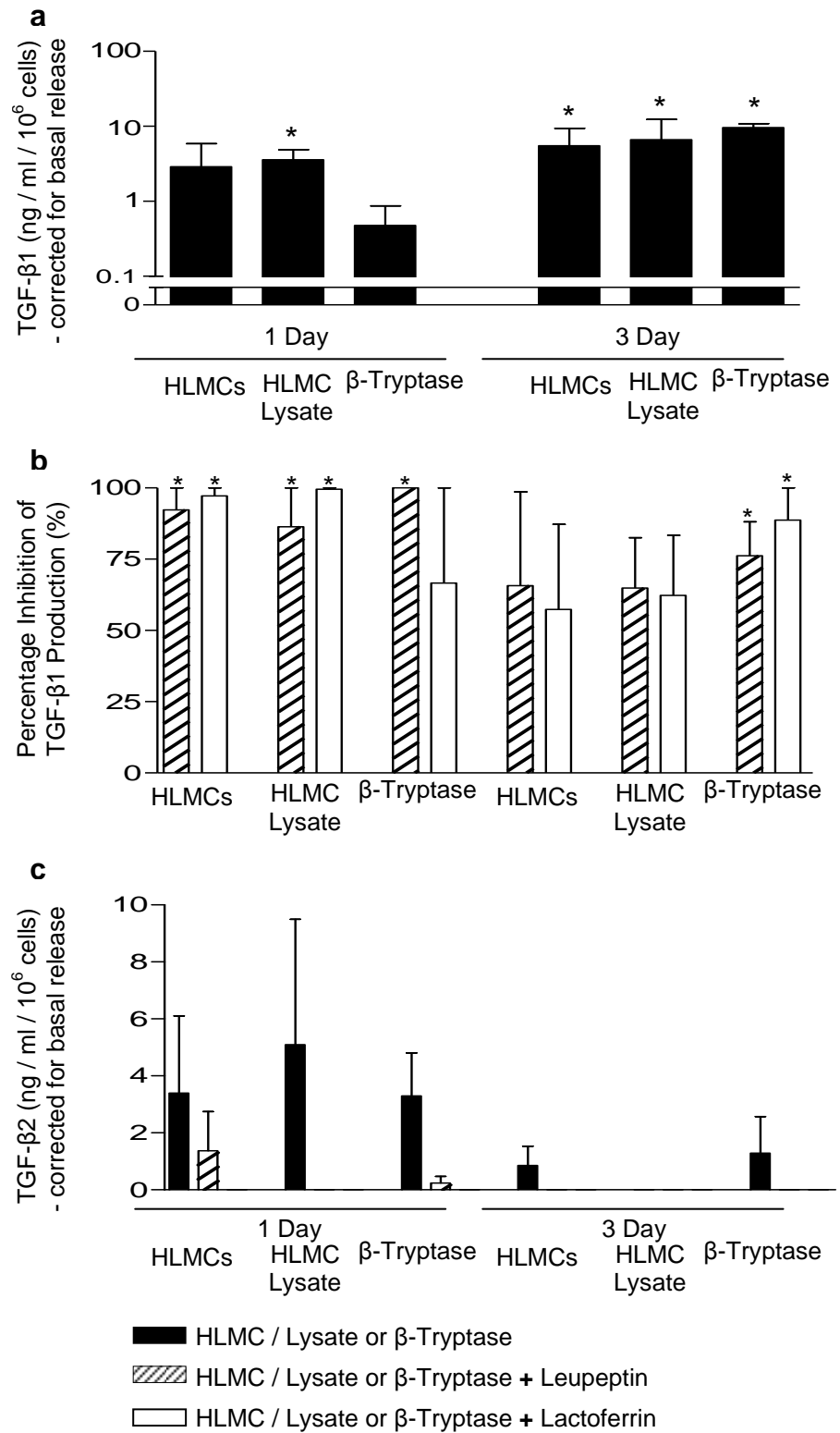
Time courses illustrating the size of collagen gels as a percentage of control after ASM primed with β -tryptase (0.32 μ g/ml) with or without leupeptin (6.7 μ g/ml) for 48 hours, and a subsequent application of histamine (100 μ M/ml) with observations of gel size (as a percentage of gel size after 48 hours of priming) over the following a) 8 hours or b) 7 days. (n = 5 ASM donors) * p < 0.05 compared to control; per time point. ≠ p < 0.05 β -tryptase Vs. β -tryptase & leupeptin.



d) Bar chart illustrating the area under the curve of collagen gel contraction assays after ASM priming with β -tryptase (0.32 μ g/ml) with or without leupeptin (6.7 μ g/ml) for 48 hours, and a subsequent application of histamine (100 μ M/ml) to the same gels with observations of gel size over the following 8 hours or 7 days. e) Images of example collagen gels 8 hours after the application of histamine (n = 5 ASM donors).

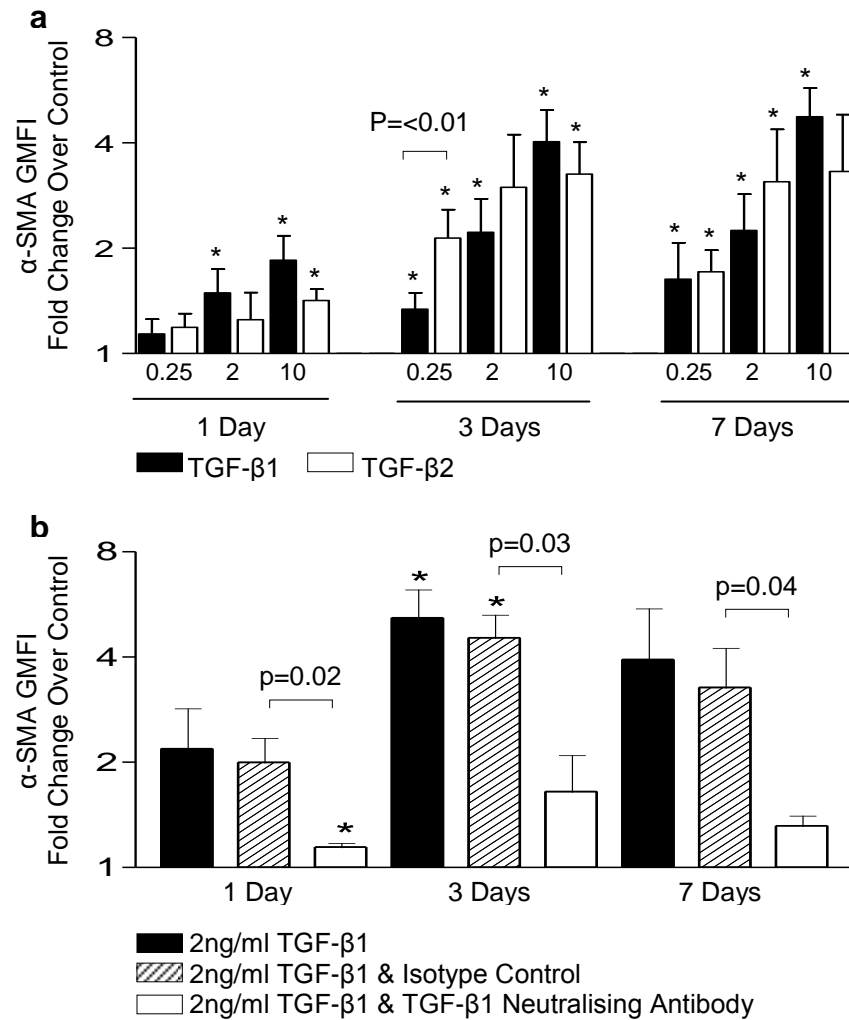
We assessed the functional consequence of the β -tryptase-mediated increased α -SMA expression by ASM using a gel contraction assay (Figure 3.3.6a, b). The contractile response of β -tryptase-primed ASM to histamine was increased compared to control (after 1 hour mean difference [95% CI] 90.2 [81.5 – 98.9]%; $p=0.03$) (Figure 3.3.6a). This increased contractility was also inhibited by leupeptin (Figures 3.3.6, a, b, c and d).

3.3.7 Mast Cells and Their Products Increase ASM TGF- β Production



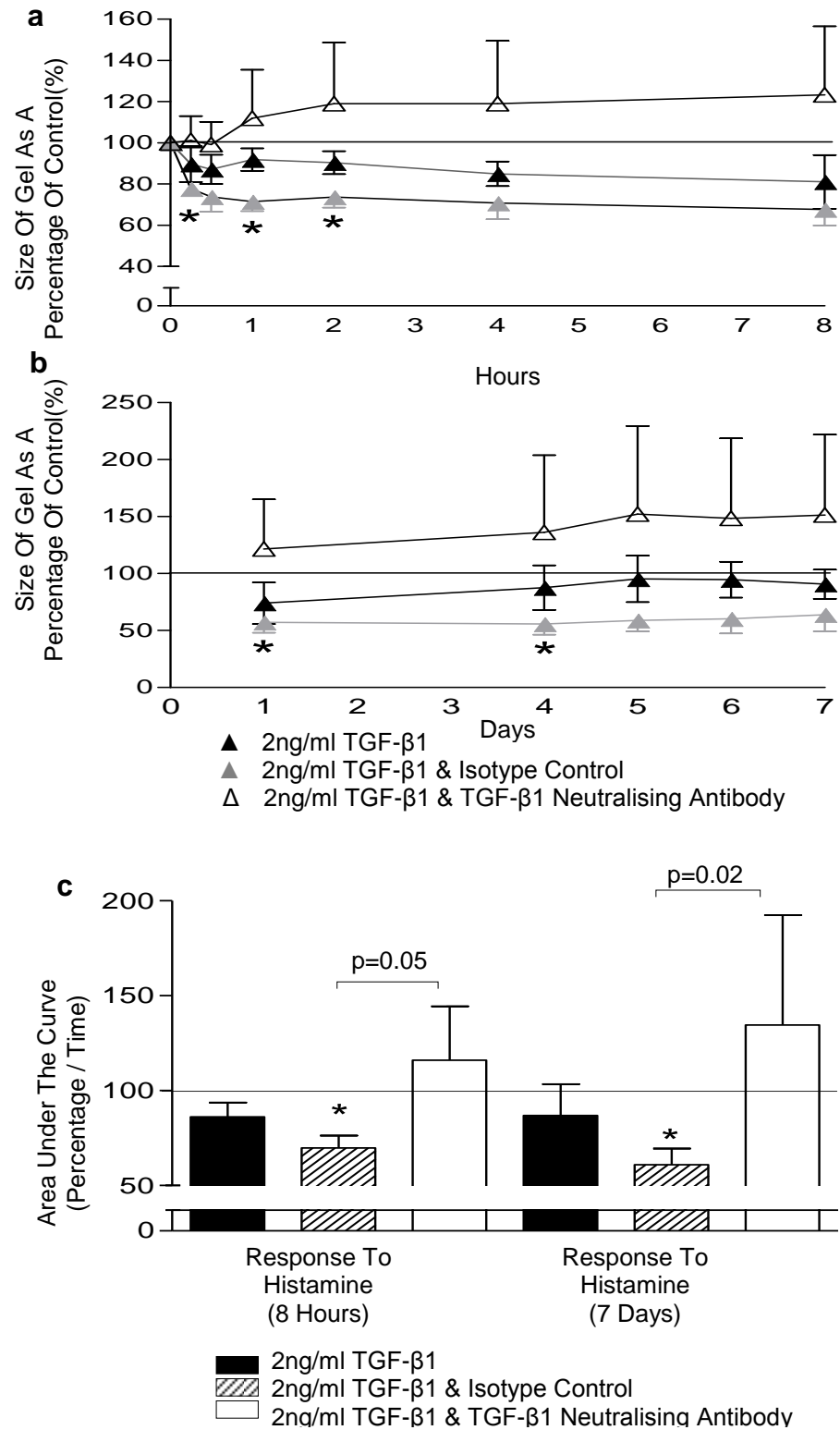
Bar charts illustrating the increase in a,b) TGF- β 1 or c) TGF- β 2 production by ASM above basal levels as assessed by ELISA after culture with HLMC, HLMC lysates or β -tryptase (ratio 1 mast cell to 4 ASM or β -tryptase (0.5 μ g/ml); n=3) for 1 or 3 days with or without leupeptin (10.6 μ g/ml) or lactoferrin (80.6ng/ml). * p < 0.05.

3.3.8 TGF- β 1 and TGF- β 2 Increase ASM α -SMA Expression



Bar charts illustrating the fold change over control of α -SMA GMFI expression by ASM as assessed by flow cytometry after co-culture with a) either TGF- β 1 or TGF- β 2 (0.25, 2 or 10ng/ml) or (n = 9 ASM donors) b) TGF- β 1 (2 ng/ml) with or without a TGF- β 1 neutralizing antibody or appropriate isotype control for 1, 3 or 7 days. (n = 3 ASM donors) * p < 0.05 per time point.

3.3.9 Enhanced ASM contractility in Response to TGF- β 1



Time courses illustrating the size of collagen gels as a percentage of control after ASM priming with TGF- β 1 (2 ng/ml) with or without a TGF- β 1 neutralizing antibody or isotype control for 48 hours, and a subsequent application of histamine (100 μ M/ml) with observations of gel size over the following a) 8 hours or b) 7 days. c) Also represented as area under the curve (n = 3 ASM donors). * p < 0.05 compared to control; p < 0.05 TGF- β 1 & Isotype Control Vs. TGF- β 1 & TGF- β 1 Neutralising Antibody.

The basal release of TGF- β 1 by ASM cells was geometric mean [95% CI] 0.54 [0.009 – 37.76] ng/ml/ 10^6 cells after 24 hours, and 3.57 [0.05 – 248.31] ng/ml/ 10^6 cells after 72 hours. The basal release of TGF- β 2 by ASM cells was 0.18 [0.004 – 7.87] ng/ml/ 10^6 cells after 24 hours, and 0.93 [5.81 – 1499.68] ng/ml/ 10^6 cells after 72 hours.

The TGF- β 1 concentration was increased in cell free supernatants as assessed by ELISA from ASM co-cultured for 1 and 3 days with HLMC (Day 3: 5.52 [0.55 – 55.59] ng/ml/ 10^6 cells, $p=0.03$) or HLMC lysates (Day 3: 6.65 [0.46 – 95.54] ng/ml/ 10^6 cells, $p=0.04$) or β -tryptase (Day 3: 9.62 [5.72 – 16.14] ng/ml/ 10^6 cells, $p<0.01$) corrected for basal release by ASM and mast cells (Figure 3.3.7a).

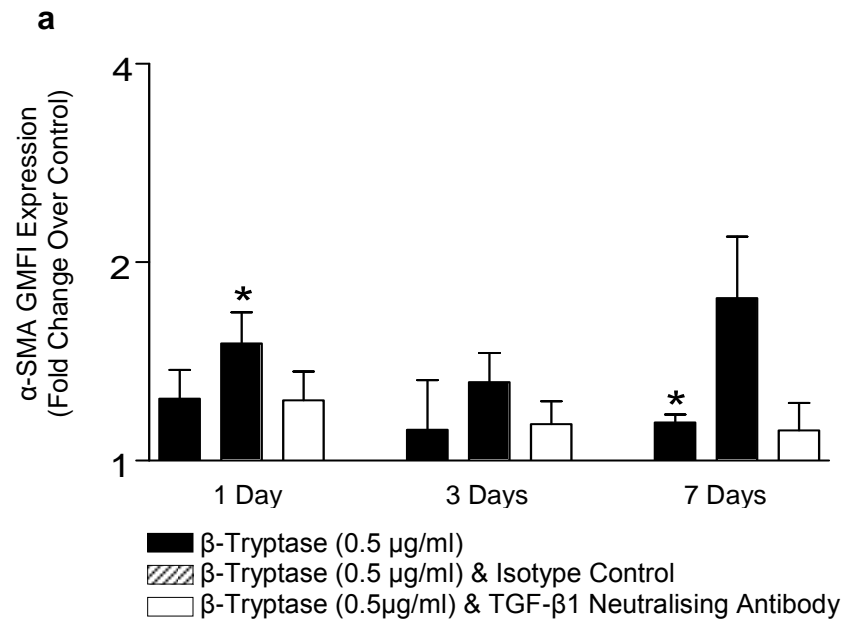
The increases in TGF- β 1 concentration in cell free supernatants from ASM & HLMC or β -tryptase co-cultures were significantly inhibited by the application of leupeptin or lactoferrin (Figure 3.3.7a, b).

There was a small non-significant increase in the TGF- β 2 concentration in cell free supernatants from ASM co-cultured for 1 and 3 days with HLMC (Day 3: 0.93 [0.1 – 8.58] / 10^6 cells) or HLMC lysates (Day 3: 0 [0 - 0] ml/ 10^6 cells) or β -tryptase (Day 3: 1.57 [0.23 – 10.86] ng/ml/ 10^6 cells) corrected for basal release by ASM and mast cells (Figure 3.3.7c).

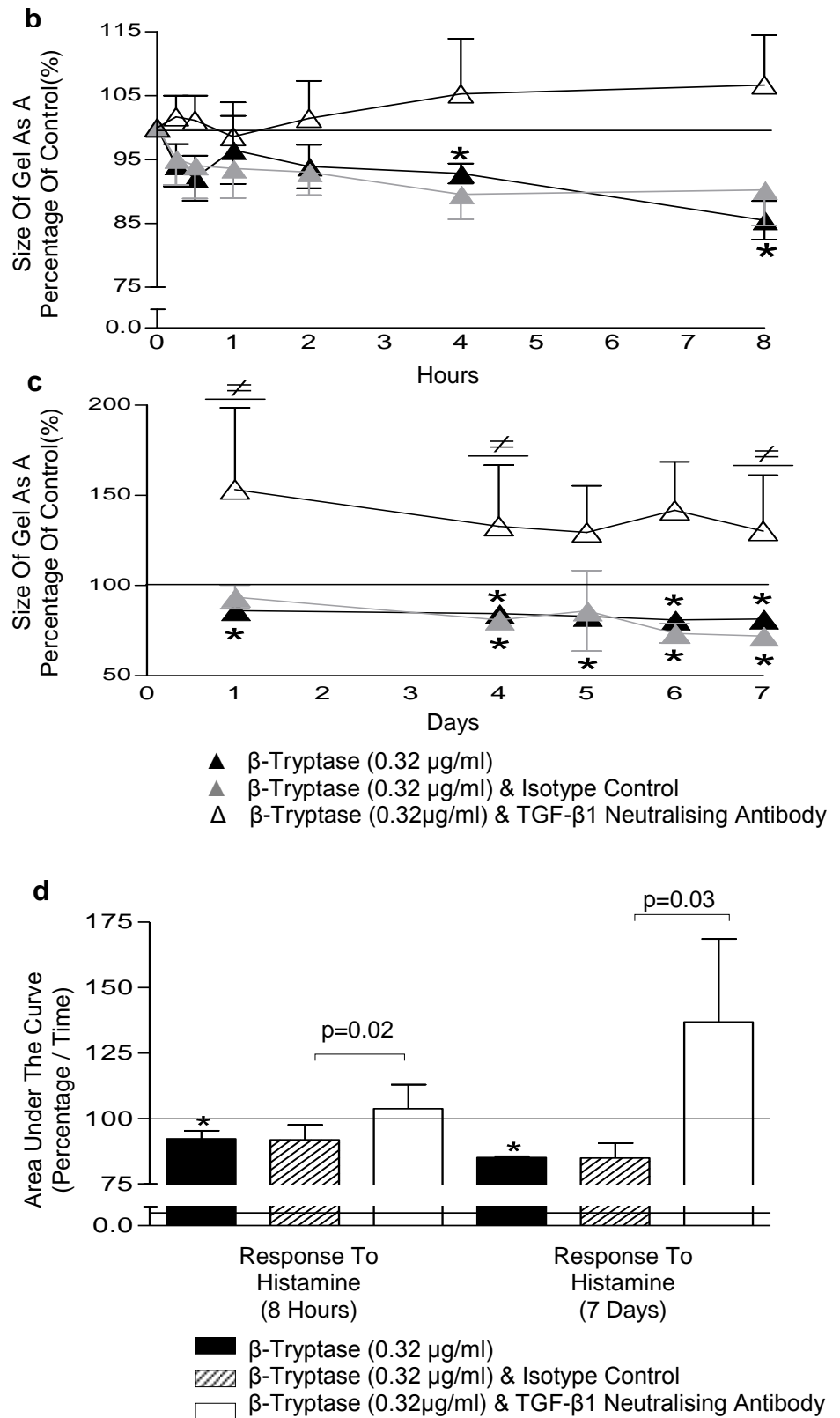
The expression of α -SMA in ASM significantly increased as a result of stimulation with both recombinant TGF- β 1 and TGF- β 2 (0.25-10ng/ml), after 1, 3 and 7 days ($p<0.05$) (Figure 3.3.8a), stimulation with TGF- β 2 was only significantly greater than TGF- β 1 at 3 days with 0.25ng/ml as assessed by flow cytometry. The increase in α -SMA expression mediated by TGF- β 1 (2ng/ml) was significantly inhibited by TGF- β 1 neutralizing antibody (Figure 3.3.8b) as assessed by flow cytometry.

TGF- β 1 also increased the contractile response of ASM to histamine (after 1h mean difference [95% CI] 74 [51.7-91.1]%; $p=0.02$) (Figure 3.3.9a), which was inhibited by the TGF- β 1 neutralizing antibody (Figures 3.3.9a, b, c) as assessed using a gel contraction assay.

3.3.10 The Neutralization of TGF- β 1 Inhibited β -Tryptase-Mediated Increased ASM α -SMA Expression and ASM Contraction

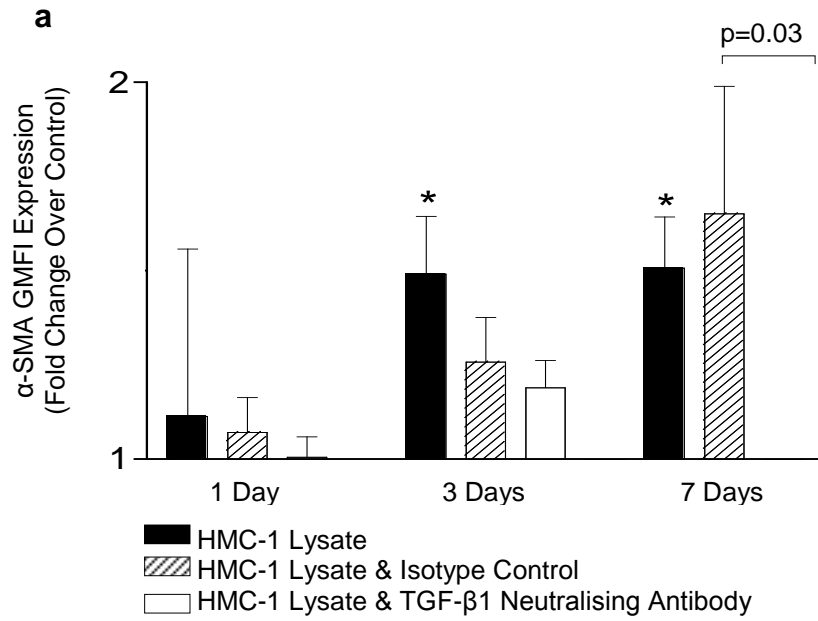


Bar chart illustrating the fold change over control of α -SMA GMFI expression by ASM as assessed by flow cytometry after co-culture with a) β -Tryptase (0.5 μ g/ml) with or without a TGF- β 1 neutralizing antibody or isotype control for 1, 3 or 7 days. (n = 4 ASM donors) * p < 0.05 per time point.



Time courses illustrating the size of collagen gels as a percentage of control after ASM priming with β -Tryptase (0.32 μ g/ml) with or without a TGF- β neutralizing antibody or isotype control for 48 hours, and a subsequent application of histamine (100 μ M/ml) with observations of gel size over the following b) 8 hours or c) 7 days. d) Also represented as area under the curve (n = 4 ASM donors). * p <0.05 compared to control; per time point. \neq p <0.05 β -tryptase & Isotype Control Vs. β -tryptase & TGF- β -1 Neutralising Antibody.

3.3.11 The Neutralization of TGF- β 1 Inhibited HMC-1 Lysate Mediated Increased ASM α -SMA Expression



Bar chart illustrating the fold change over control of α -SMA GMFI expression by ASM as assessed by flow cytometry after co-culture with a) HMC-1 Lysate (1:4 HMC-1 Lysate : ASM) with or without a TGF- β 1 neutralizing antibody (2ng/ml Antibody per 1pg/ml TGF- β) or isotype control for 1, 3 or 7 days. (n = 5 ASM donors) * p <0.05 per time point.

The neutralization of TGF- β 1 inhibited β -tryptase-mediated increased ASM contraction

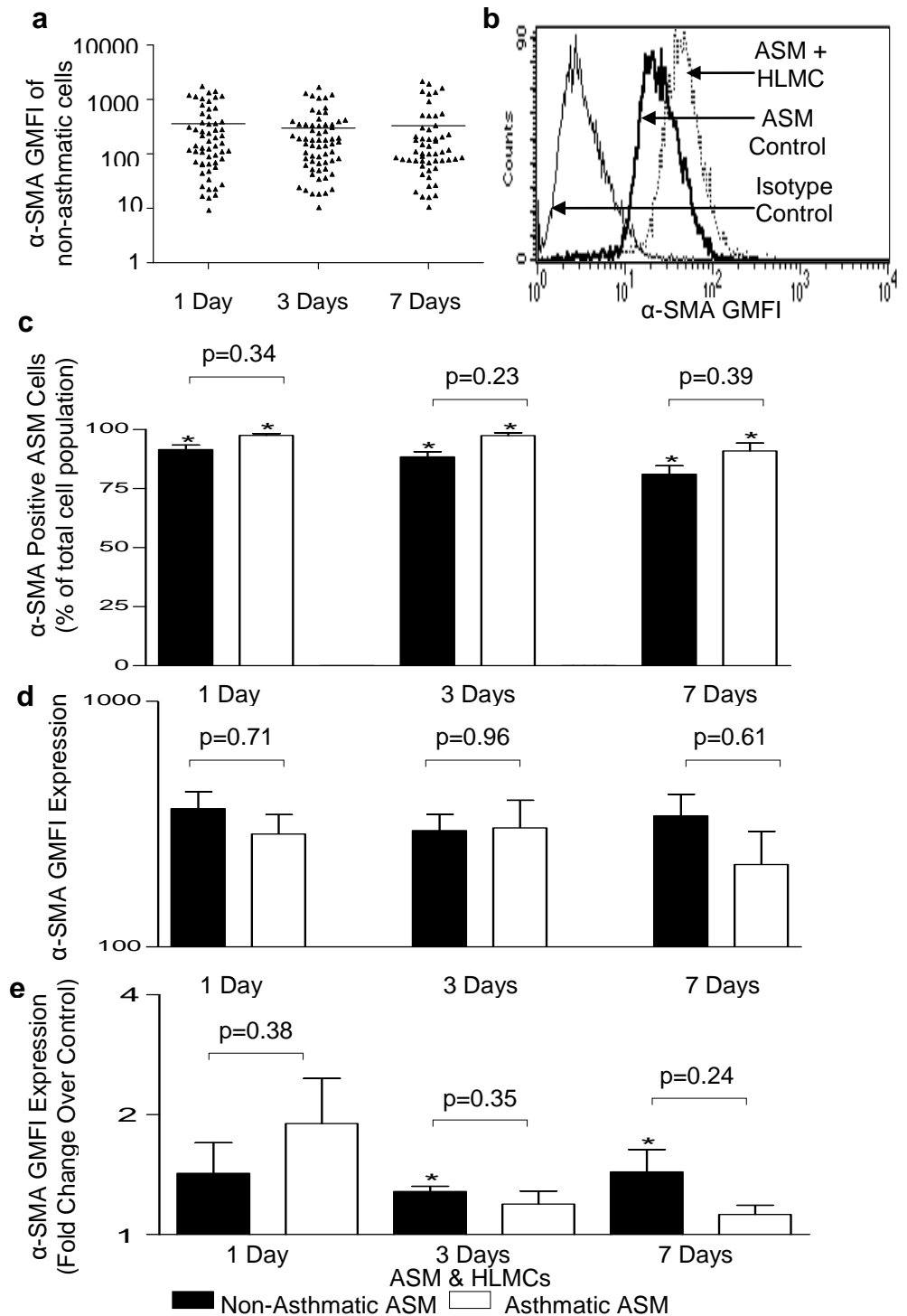
The increase in α -SMA expression mediated by β -Tryptase (0.5 μ g/ml) was inhibited by TGF- β 1 neutralizing antibody as assessed by flow cytometry (Figure 3.3.10a). The increase in ASM contraction in response to histamine mediated by β -tryptase priming was markedly inhibited by TGF- β 1 neutralization compared with isotype control as assessed using a collagen gel contraction assay (Figures 3.3.10b, c, d). This suggests that the β -tryptase induced up-regulation in ASM α -SMA may be attenuated by the inhibition of the TGF- β 1 released by the ASM in response to β -tryptase stimulation.

The neutralization of TGF- β 1 inhibited HMC-1 Lysate-mediated increased ASM α -SMA Expression

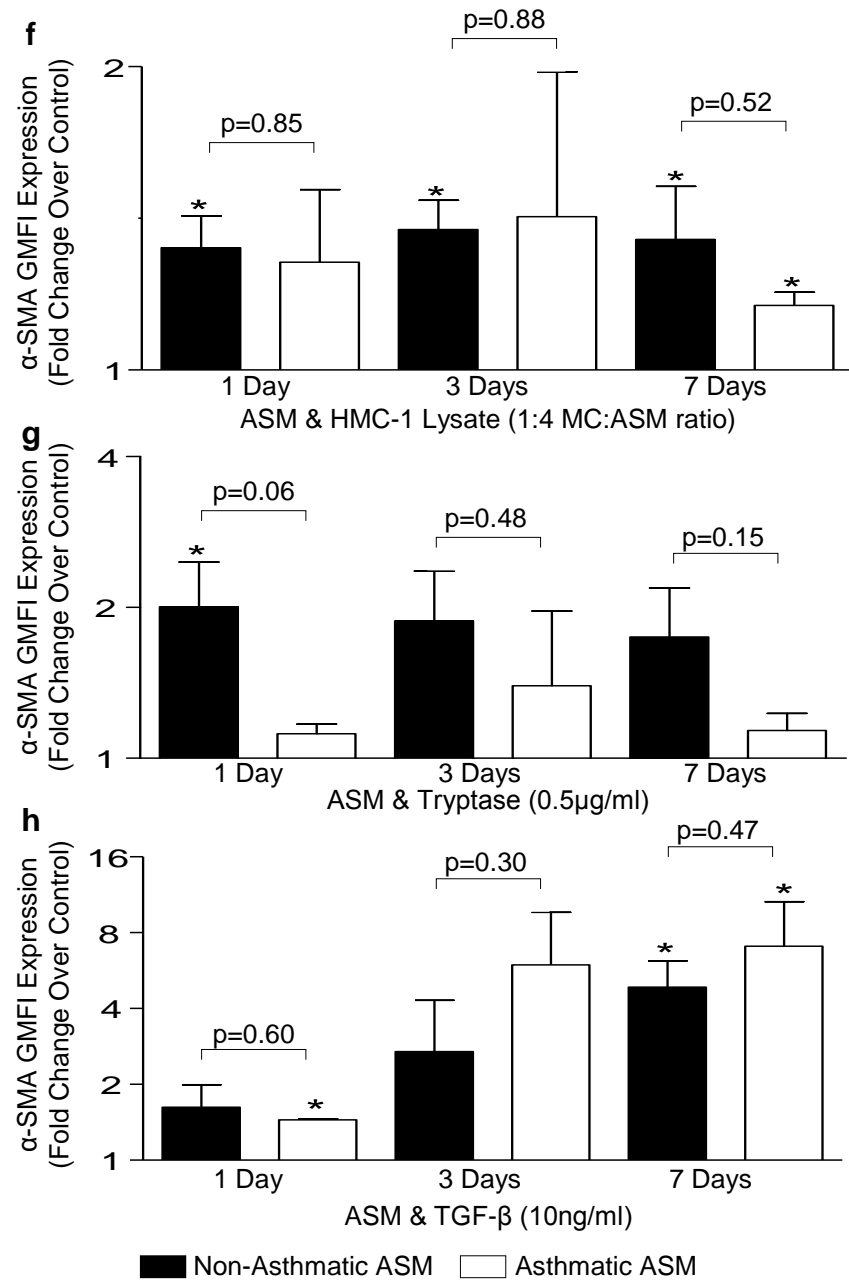
The increase in α -SMA expression mediated by HMC-1 lysate (1:4 cell lysate : ASM) was significantly inhibited by TGF- β 1 neutralizing antibody after 7 days as assessed by flow cytometry (Figure 3.3.11a).

PAR-2 expression by ASM

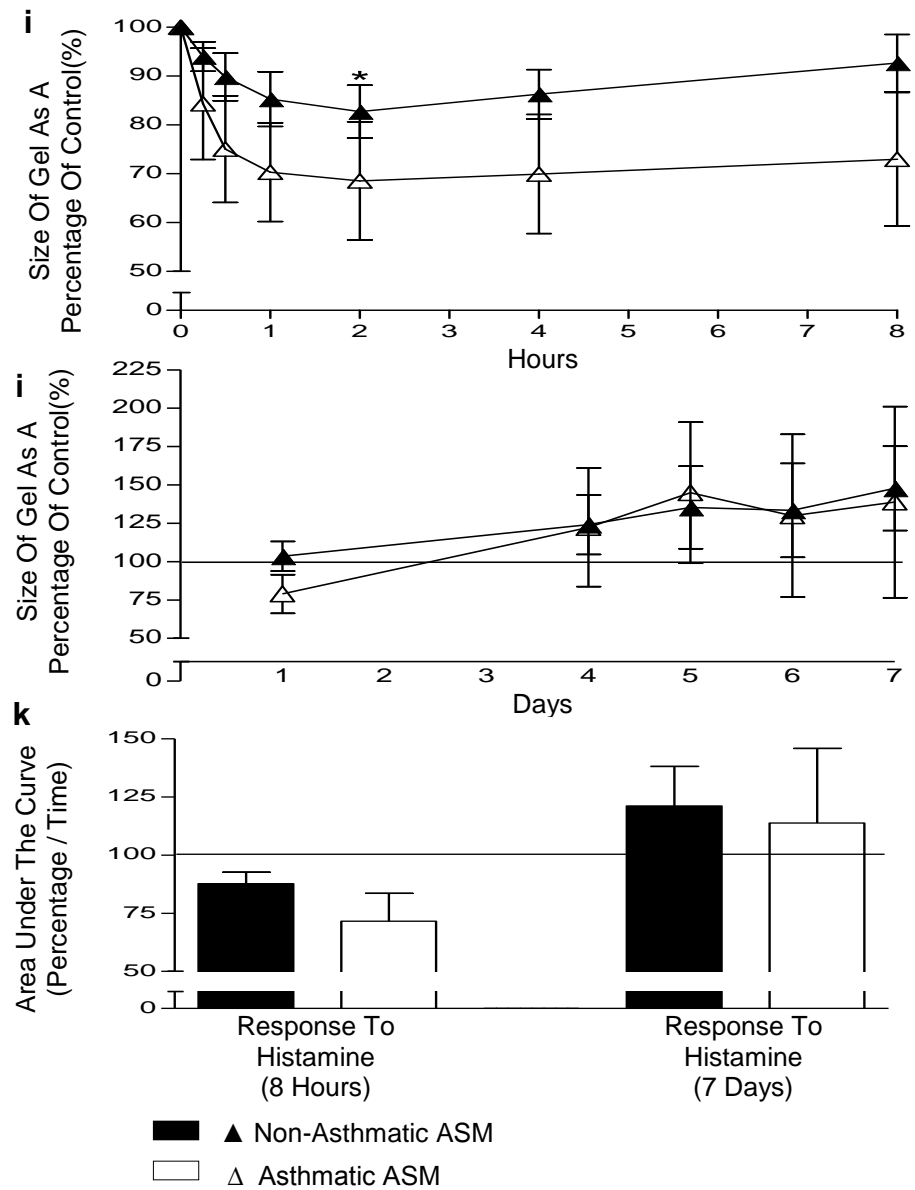
The proportion of primary cultured ASM that expressed PAR-2 on their cell surface was (mean \pm SEM) 7.6 \pm 0.99%; p=0.02 (n=3) and the PAR-2 expression by permeabilized ASM was 24.53 \pm 4.7%; p=0.03 (n=3).



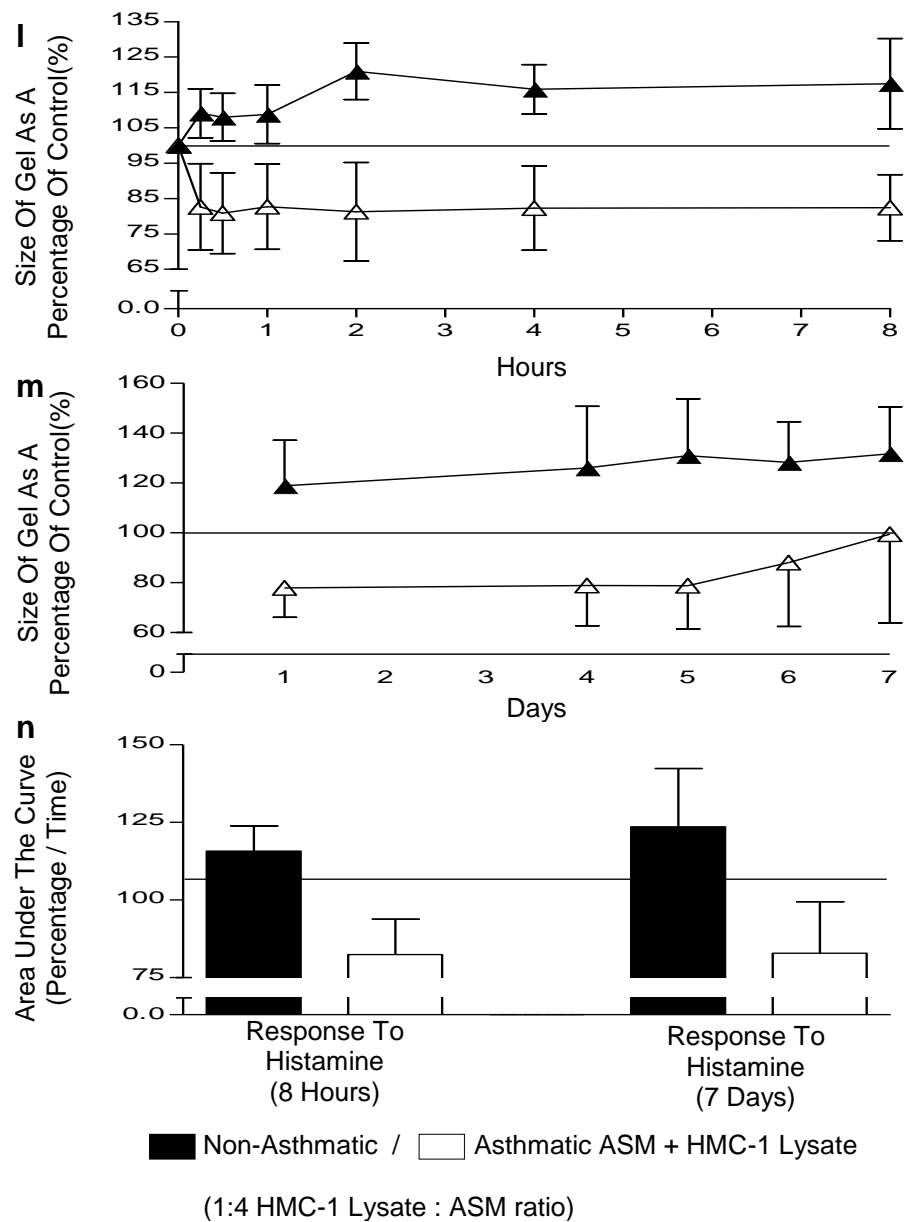
Graphs illustrating (a) the α -SMA GMFI of serum deprived non-asthmatic ASM cells over 1, 3 and 7 days. (b) A histogram illustrating the change in the α -SMA GMFI of non-asthmatic ASM cells after a 7 day co-culture with HLMCs (1:4 HLMC : ASM ratio). Bar charts illustrating (c) α -SMA positive ASM cells as the percentage of the total cell population or (d) the α -SMA GMFI expression of unstimulated non-asthmatic and asthmatic serum deprived ASM cells after 72 hours and (e) the fold change over control of α -SMA GMFI expression of non-asthmatic and asthmatic serum deprived ASM cells stimulated with unstimulated HLMCs (1:4 MC:ASM ratio) over 1, 3 and 7 days. (n = 6 ASM donors, n = 6 HLMC) *p<0.05 t-test.



Graphs illustrating the fold change over control of α -SMA GMFI expression of serum deprived ASM cells stimulated with (f) HMC-1 lysate (1:4 mast cell : ASM ratio) (n = 16 ASM donors), (g) tryptase (0.5μg/ml) (n = 4 ASM donors) or (h) TGF-β1 (10ng/ml) over 1, 3 and 7 days. (n = 9 ASM donors) *p<0.05 t-test.



Time courses illustrating the size of collagen gels as a percentage of control after serum deprived ASM for 48 hours, and a subsequent application of bradykinin (1 ng/ml) with observations of gel size over the following (i) 8 hours or (j) 7 days. (k) Bar chart illustrating the area under the curve of collagen gel contraction assays as illustrated in i and j. (n = 3 ASM donors) *p<0.05 t-test.



Time courses illustrating the size of collagen gels as a percentage of control after ASM primed with HMC-1 Lysate (1:4 HMC-1 lysate : ASM ratio) for 48 hours, and a subsequent application of bradykinin (1 ng/ml) with observations of gel over the following (l) 8 hours or (m) 7 days. (n) Bar chart illustrating the area under the curve of collagen gel contraction assays as illustrated in j and k. (n = 3 ASM donors) * p < 0.05 per time point.

In relation to stimulated ASM, there was no significant difference in the expression of α -SMA positive ASM cells or α -SMA GMFI between non-asthmatic and asthmatic donors after serum deprivation for 72 hours (Figure 3.3.12c, d). Stimulation of ASM with tryptase (0.5 μ g/ml) resulted in a significant difference in the expression of α -SMA GMFI between non-asthmatic and asthmatic donors after 1 day, but no other significant differences were noted after stimulation of ASM with either HLMCs, HMC-1 lysates, Tryptase (at 3 and 7 days) and TGF- β 1 (Figures 3.3.12e, f, g, h). Specifically, TGF- β 1 (10ng/ml) mediated up-regulation of ASM α -SMA after 7 days in those with (geometric mean [95% CI] 7.0 [1.2-41.3] fold increase; $p=0.04$) or without asthma (4.9 [1.7-13.8] fold increase; $p=0.04$), but there was no difference between groups (mean difference [95% CI] asthma versus non-asthma 1.44 [0.4-5.4] fold; $p=0.49$) (Figure 3.3.12h). Investigations were conducted to examine the contractility of non-asthmatic and asthmatic ASM. There was a trend towards increased contractility in unstimulated asthmatic ASM, compared to non-asthmatic ASM following the subsequent application of bradykinin (Figures 3.3.12 i, j, k), along with a similar trend observed in the contractility between unstimulated non-asthmatic and asthmatic ASM primed with HMC-1 lysates following the subsequent application of bradykinin (Figures 3.3.12 l, m, n).

3.3.13 Discussion

This is the first report to demonstrate that mast cells promote ASM differentiation to a more contractile phenotype. Since mast cell localization to the ASM-bundle is a key feature of the immunopathology of asthma, we have identified a novel mechanism whereby mast cell-ASM interactions may aggravate the disordered airway physiology that characterizes asthma.

Mast cells increased ASM α -SMA mRNA and protein expression. This was mediated by β -tryptase and was attenuated by the serine protease inhibitor leupeptin. We confirmed that mast cell derived β -tryptase increased ASM TGF- β secretion (57), which itself increased α -SMA expression (96). Importantly the increased ASM α -SMA expression by β -tryptase was inhibited by TGF- β neutralisation suggesting that its effects were predominately exerted via the autocrine activation of ASM by TGF- β . However, we cannot exclude the possibility that some of the β -tryptase mediated effects on ASM may have been direct and TGF- β independent. We are confident that our *in vitro* findings are relevant in asthma as *in vivo* we found that in bronchial mucosal biopsies from asthmatics, the intensity of α -SMA expression was strongly related to the number of mast cells within or adjacent to an ASM-bundle. Although we present here the first evidence of human lung mast cells driving ASM differentiation, the importance of mast cell-mesenchymal interactions is not restricted to the airway. Current evidence suggests that colocalization of mast cells with pericryptal fibroblasts in the lamina propria of the duodenum is important in maintaining normal villous morphology (309) and co-culture of dermal fibroblasts and HMC-1 cells result in fibroblast differentiation (310).

Mast cell microlocalisation to the ASM-bundle is a feature of asthma across severities. Mast cell number correlates positively with the degree of AHR (19) and with the bronchoconstrictor response to a deep inspiration (188) supporting the view that mast cell-ASM cell interactions are central in the development of the disordered physiology in asthma. Importantly, there is evidence that mast cells infiltrating the ASM-bundle are activated with increased expression of Th2 cytokines IL-4 and IL-13 (215). Post mortem studies of fatal and non-fatal asthma have demonstrated that there was a marked increase in mast cell degranulation in the ASM-bundle in both the large and small airways (36) and that increased numbers of mast cells are associated with increased ASM shortening in fatal asthma (311). Our data suggest that mast cell β -tryptase is central in the development of ASM dysfunction, which is supported by early clinical trials of tryptase inhibitors in sheep models of asthma (312) and in human disease (247;313). However, the link between mast cell-ASM interactions and disordered airway physiology has been questioned by the inconsistent effect of anti-IgE therapy on AHR (314-317). Importantly, our findings indicate that mast cell derived β -tryptase from human lung mast cells can promote ASM differentiation independently of IgE. We have therefore identified a novel IgE-independent mast cell mediated mechanism that may be an important driver in the development of AHR in asthma.

Smooth muscle contraction is under the control of a well-described multi-step process (140). Specifically, ASM contraction occurs due to myosin-actin cross-bridging following phosphorylation of smooth muscle myosin light chain by myosin light chain kinase, which in turn is activated by cytoplasmic Ca^{2+} bound to calmodulin. Although α -SMA is a key contractile protein involved in ASM contraction this process can be modulated by the content and activation of several proteins and kinases. We restricted our investigations to α -SMA expression as there is a considerable body of evidence that there is close relationship between the α -SMA content and mesenchymal cell contraction (318). Fibroblasts from an ovalbumin-challenged mouse model of asthma demonstrated augmented gel contraction and expressed more α -SMA than fibroblasts from control animals (319). In α -SMA null mice there is diminished vascular tone (320) and reduced contractile force by bladder smooth muscle (321). In humans there was a very strong correlation between α -SMA expression and contraction of bone marrow stroma-derived mesenchymal stem cells (322). We are therefore confident that the changes we observed in the upregulation of α -SMA expression by ASM in response to co-culture with mast cells is critical in the transition of the ASM to a more contractile phenotype.

3.3.14 Criticisms

One possible criticism of our study is that our *in vitro* findings are limited to primary cells rather than *ex vivo* bronchial rings or *in vivo* whole animal studies. To date there is not a good animal model of asthma to study mast cell-ASM interactions (27). Contraction studies using bronchial rings have the advantage over gel contraction assays in that the ASM-bundle and the microenvironment are intact, but are limited in their ability to examine interactions between cell types. However, in support of our findings sensitized bronchial rings primed with β -tryptase demonstrate increased contractility to histamine (323). Critically the mechanisms proposed by our ASM-mast cell co-cultures are strengthened by two lines of evidence. Firstly, we observed a remarkably strong correlation between the intensity of α -SMA expression and the number of mast cells within an ASM-bundle, and secondly improvements in lung function in asthmatics following treatment with tryptase inhibitors are encouraging (247;324).

3.3.15 Conclusion

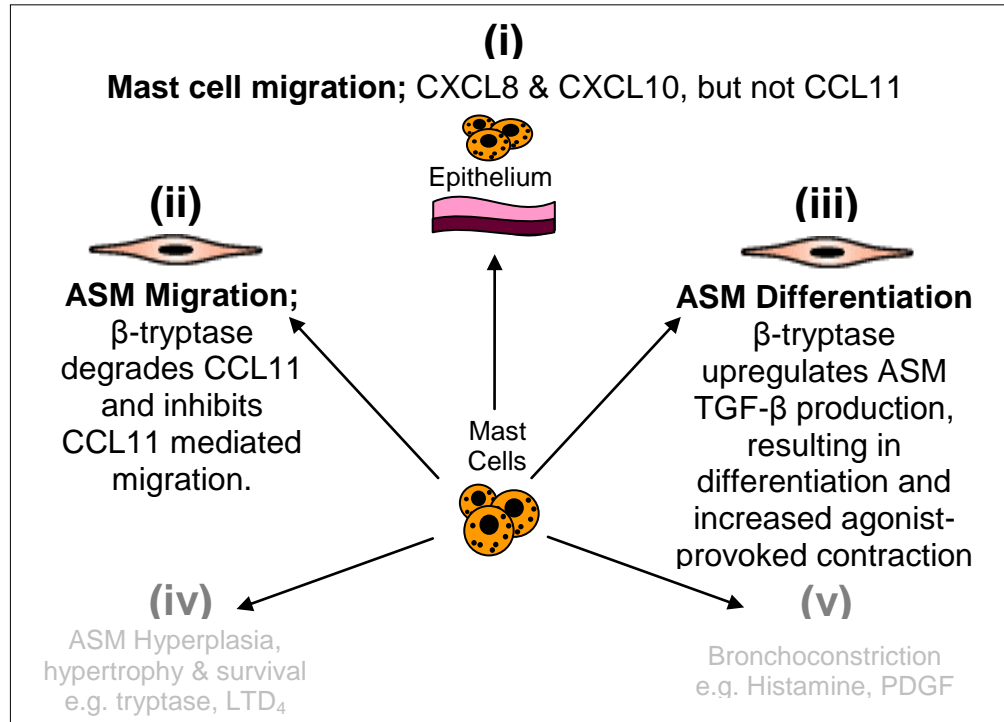
Asthma is a major cause of morbidity and mortality worldwide. It is characterized by airway dysfunction and inflammation. A key determinant of the asthma phenotype is infiltration of airway smooth muscle bundles by activated mast cells. We hypothesised that interactions between these cells promotes airway smooth muscle differentiation into a more contractile phenotype.

In vitro co-culture of human airway smooth muscle cells with β -tryptase, or mast cells with or without IgE/anti-IgE activation, increased airway smooth muscle-derived TGF- β secretion, α -smooth muscle actin expression and agonist-provoked

contraction. This promotion to a more contractile phenotype was inhibited by both the serine protease inhibitor leupeptin and TGF- β neutralization, suggesting that the observed airway smooth muscle differentiation was driven by the autocrine release of TGF- β in response to activation by mast cell β -tryptase.

Importantly, *in vivo* we found that in bronchial mucosal biopsies from asthmatics the intensity of α -smooth muscle actin expression was strongly related to the number of mast cells within or adjacent to an airway smooth muscle bundle. These findings suggest that mast cell localization in the airway smooth muscle bundle promotes airway smooth muscle cell differentiation into a more contractile phenotype, thus contributing to the disordered airway physiology that characterizes asthma.

In conclusion, mast cell localization to the ASM-bundle facilitates interactions between these cell types and leads to an altered ASM phenotype with increased α -SMA expression and contraction. This ASM differentiation to a more contractile phenotype mediated by mast cell-derived β -tryptase presents a novel target to improve the disordered airway physiology that is the hallmark of asthma.

4.1 **Summary**

Asthma is a major cause of morbidity and mortality worldwide. It is characterized by variable airflow obstruction and airway hyperresponsiveness (AHR), inflammation and remodeling. Mast cell co-localisation to airway smooth muscle (ASM) is a key determinant of airway hyperresponsiveness and mast cell localization to other structures such as the epithelium has been demonstrated.

To date our group has elucidated that the CXCL10 / CCR3 axis is important in mediating mast cell migration towards the ASM; mast cells adhere to ASM via CADM-1 and interactions between mast cells and ASM promotes mast cell survival and proliferation. Whether there are other important mechanisms driving mast cell localization to airway structures, ASM migration and repair, and how mast cells affect ASM differentiation is uncertain.

We therefore sought to examine;

- i) the concentration of chemokines in bronchial wash samples and bronchoalveolar lavage fluid (BAL), and the mast cell chemotactic activity in BAL fluid from subjects with asthma and eosinophilic bronchitis, and from healthy control subjects.
- ii) CCR3 mediated ASM migration. CCR3-mediated recruitment of ASM progenitors towards the ASM bundle has been proposed as one possible mechanism involved in ASM hyperplasia. Mast cells are microlocalized to the ASM bundle and whether mast cells influence CCR3-mediated migration is uncertain.
- iii) The investigation of whether interactions between ASM and mast cells promote ASM differentiation into a more contractile phenotype.

The bronchial wash sample and BAL fluid concentrations of CXCL10 and CXCL8 was increased in subjects with eosinophilic bronchitis compared to those in subjects with asthma and healthy control subjects ($p < 0.05$). The CCL11 concentration was below the limit of detection in most subjects. BAL fluid from subjects with eosinophilic bronchitis was chemotactic for mast cells (1.4-fold migration compared to a control, 95% confidence interval, 1.1 to 1.9; $p = 0.04$) and was inhibited by blocking CXCR1 (45% inhibition; $p = 0.002$), CXCR3 (38% inhibition; $p = 0.034$), or both (65% inhibition; $p = 0.01$). BAL fluid from the subjects with asthma and healthy control subjects was not chemotactic for mast cells. Mast cell migration to BAL fluid was correlated with the concentration of CXCL8 ($r = 0.42$; $p = 0.031$) and CXCL10 ($r = 0.52$; $p = 0.007$).

ASM expressed CCR3. ASM CCR3 activation by CCL11 mediated intracellular calcium elevation, concentration-dependent migration and wound healing, but had no effect on proliferation or survival. Co-culture with β -tryptase or mast cells degraded recombinant and ASM-derived CCL11, and β -tryptase inhibited CCL11-mediated ASM migration.

In vitro coculture of human ASM cells with β -tryptase, or mast cells with or without IgE/anti-IgE activation, increased ASM-derived TGF- β 1 secretion, α -smooth muscle actin (α -SMA) expression and agonist-provoked contraction. This promotion to a more contractile phenotype was inhibited by both the serine protease inhibitor leupeptin and TGF- β 1 neutralization, suggesting that the observed ASM differentiation was driven by the autocrine release of TGF- β 1 in response to activation by mast cell β -tryptase. Importantly, *in vivo* we found that in bronchial mucosal biopsies from asthmatics the intensity of α -SMA expression was strongly related to the number of mast cells within or adjacent to an ASM bundle.

In conclusion, CXCL8 and CXCL10 play an important role in mast cell migration to the epithelium. CCL11 is not important for airway smooth muscle migration. Mast cells drive ASM differentiation through the autocrine release of TGF- β 1 in response to activation by β -tryptase and may drive AHR.

Targeting mast cell migration towards the epithelium and smooth muscle progenitor migration, differentiation and co-localisation of mast cells with ASM could provide an effective treatment in the control of asthma.

4.2 Critique

In relation to the first study (i), subjects with mild asthma who had minimal symptoms were included and this may have been a limiting factor to the chemotactic potential of the asthmatic airway secretions studied. BAL fluid cytopins were also not stained appropriately to be able to enumerate the number of mast cells in BAL fluid across the groups, and therefore the concentrations of CXCL10, CXCL8 and BAL fluid mast cell numbers could not be correlated. Chemokines other than CXCL10 and CXCL8 could also have been examined, but the examination of the number of chemokines was limited by the volume of bronchial wash and BAL available. Mast cell migration towards BAL was also

conducted using an immortalized cell line rather than primary cells, although the chemokine receptor profiles of HMC-1 and primary lung mast cells are similar, supporting the view that HMC-1 cells are a suitable model for lung mast cell migration.

A limitation of the second and third studies (ii, iii) include the application of a mast cell : ASM cell ratio which reflects that found *in vivo*. The ratio used is based on our experience with the assessment of bronchial biopsies and we are confident that the range we have chosen captures the proportions of these cells in the asthmatic airway. Primary mast cells were also derived from lung resection tissue and not asthmatic subjects. Currently we are limited by our inability to isolate sufficient mast cells from the asthmatic airway. However, we feel that using human lung mast cells that are sensitised and IgE/anti-IgE activated is likely to be reflective of asthmatic mast cells.

In relation to the third study (iii), although α -SMA is a key contractile protein involved in ASM contraction, this process can be modulated by the content and activation of several protein kinases. We restricted our investigations to α -SMA expression as there is a considerable body of evidence that there is close relationship between the α -SMA content and mesenchymal cell contraction (318). Fibroblasts from an ovalbumin-challenged mouse model of asthma demonstrated augmented gel contraction and expressed more α -SMA than fibroblasts from control animals (319). In α -SMA null mice there is diminished vascular tone (320) and reduced contractile force by bladder smooth muscle (321). In humans there was a very strong correlation between α -SMA expression and contraction of bone marrow stroma-derived mesenchymal stem cells (322). We are therefore confident that the changes we observed in the upregulation of α -SMA expression by ASM in response to co-culture with mast cells is critical in the transition of the ASM to a more contractile phenotype. Other limitations include that our *in vitro* findings are limited to primary cells rather than *ex vivo* bronchial rings or *in vivo* whole animal studies. To date there is not a good animal model of asthma to study mast cell-ASM interactions. Contraction studies using bronchial rings have the advantage over gel contraction assays in that the ASM-bundle and the microenvironment are intact, but are limited in their ability to examine interactions between cell types. However, in support of our findings sensitized bronchial rings primed with β -tryptase demonstrate increased contractility to histamine (323). Critically the mechanisms proposed by our ASM-mast cell co-cultures are strengthened by two lines of evidence. Firstly, we observed a remarkably strong correlation between the intensity of α -SMA expression and the number of mast cells within an ASM-bundle, and secondly improvements in lung function in asthmatics following treatment with tryptase inhibitors are encouraging (247; 324).

4.3 Future Directions

In relation to the first study (i), chemokine concentrations and mast cell migration towards airway secretions from severe asthmatics could be studied to examine whether there is an altered potential in comparison with mild asthmatics.

BAL fluid cytospins could also be stained to identify mast cells enabling the correlation of BAL fluid mast cell numbers and chemokine concentrations.

Taking into account the limitation of obtaining suitable tissue, further studies could include the examination of the migration of HLMCs rather than HMC-1 cells towards airway secretions from subjects with EB, asthma and normal controls. It would be particularly interesting to use asthmatic mast cells for all the studies described here.

Using the same volume of experimental samples, a wider variety of chemokines could be examined through utilizing techniques such as mesoscale discovery for all the studies described here. Further investigations could also be conducted to see if there is an inhibitory factor present in non-asthmatic airway secretions or cell culture supernatants that could affect mast cell, ASM migration or ASM differentiation. Different contractile markers such as myosin light chain kinase could also be used to examine the ASM contractile phenotype.

Co-culture assays using transwells and collagen gels could also be used to examine the interaction of smooth muscle cells and mast cells – and possibly other cells present in EB and asthma such as T-cells, neutrophils, eosinophils and macrophages enabling the development of a complete co-culture system which would more accurately reflect conditions found *in vivo*.

Results from these studies have also indicated that along with the influence of increased ASM mass and co-localised mast cells, asthmatic ASM may be more intrinsically contractile than non-asthmatic ASM – but this would require further investigation to confirm this observation.

Existing literature also suggests that the development and characterisation of asthmatic phenotypes may result in a more tailored and effective treatment of the disease. This practice could also be used when selecting the subjects that are used in future investigations.

Additional areas of investigation which were not covered in these studies, but which could be examined in future include the examination of ASM hyperplasia, hypertrophy and survival in response to the interaction between ASM and mast cells. In relation to bronchoconstriction, would the co-localisation of ASM and mast cells affect the contractile response of ASM to β -agonists? The examination of the synthetic response of ASM and mast cells upon these factors could also be investigated. The effects of current therapies such as corticosteroids and beta-agonists on our findings in this thesis need to be examined and then extended to include the effects of novel emerging therapies. This will inform the translation of our laboratory findings into the clinic.

5 **References**

- (1) Rosner F. Moses Maimonides' treatise on asthma. *Thorax* 1981; 36(4):245-251.
- (2) Osler W. *Bronchial Asthma. Principles and Practice of Medicine*. New York, D. Appleton & Co., 1892: 497-501.
- (3) Green RH, Brightling CE, Bradding P. The reclassification of asthma based on subphenotypes. *Curr Opin Allergy Clin Immunol* 2007; 7(1):43-50.
- (4) Haldar P, Pavord ID, Shaw DE, Berry MA, Thomas M, Brightling CE et al. Cluster analysis and clinical asthma phenotypes. *Am J Respir Crit Care Med* 2008; 178(3):218-224.
- (5) Global Initiative For Asthma (GINA). *Global Strategy For Asthma Management And Prevention*. <http://www.ginasthma.org>. 12-10-2007.
- (6) Global Initiative For Asthma (GINA). *Pocket Guide For Asthma Management And Prevention*. www.ginaasthma.com . 12-12-2006.
- (7) National Heart LaBl. Expert Panel Report 3 (EPR 3): Guidelines for the Diagnosis and Management of Asthma. NIH Publication no. 08-4051, 2007. 2007.
- (8) Bateman ED, Hurd SS, Barnes PJ, Bousquet J, Drazen JM, FitzGerald M et al. Global strategy for asthma management and prevention: GINA executive summary. *Eur Respir J* 2008; 31(1):143-178.
- (9) Luskin AT. What the asthma end points we know and love do and do not tell us. *J Allergy Clin Immunol* 2005; 115(4 Suppl):S539-S545.
- (10) Scottish Intercollegiate Guidelines Network, British Thoracic Society. *British Guideline on the Management of Asthma - A National Clinical Guideline*. Scottish Intercollegiate Guidelines Network and the British Thoracic Society . 2008.
- (11) Makino S, Adachi M, Ago Y, Akiyama K, Baba M, Egashira Y et al. Definition, diagnosis, disease types, and classification of asthma. *Int Arch Allergy Immunol* 2005; 136 Suppl 1:3-4.
- (12) King CS, Moores LK. Clinical asthma syndromes and important asthma mimics. *Respir Care* 2008; 53(5):568-580.
- (13) Soriano JB, Kiri VA, Maier WC, Strachan D. Increasing prevalence of asthma in UK primary care during the 1990s. *Int J Tuberc Lung Dis* 2003; 7(5):415-421.
- (14) Gupta R, Sheikh A, Strachan DP, Anderson HR. Burden of allergic disease in the UK: secondary analyses of national databases. *Clin Exp Allergy* 2004; 34(4):520-526.
- (15) Barnes PJ, Jonsson B, Klim JB. The costs of asthma. *Eur Respir J* 1996; 9(4):636-642.
- (16) *British Guideline on the Management of Asthma*. *Thorax* 2008; 63 Suppl 4:iv1-121.
- (17) McCormack MC, Enright PL. Making the diagnosis of asthma. *Respir Care* 2008; 53(5):583-590.
- (18) Haldar P, Pavord ID. Noneosinophilic asthma: a distinct clinical and pathologic phenotype. *J Allergy Clin Immunol* 2007; 119(5):1043-1052.
- (19) Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-cell infiltration of airway smooth muscle in asthma. *N Engl J Med* 2002; 346(22):1699-1705.
- (20) Brightling CE, Symon FA, Birring SS, Bradding P, Wardlaw AJ, Pavord ID. Comparison of airway immunopathology of eosinophilic bronchitis and asthma. *Thorax* 2003; 58(6):528-532.

- (21) Brightling CE, Symon FA, Birring SS, Bradding P, Pavord ID, Wardlaw AJ. TH2 cytokine expression in bronchoalveolar lavage fluid T lymphocytes and bronchial submucosa is a feature of asthma and eosinophilic bronchitis. *J Allergy Clin Immunol* 2002; 110(6):899-905.
- (22) Brightling CE, Ward R, Goh KL, Wardlaw AJ, Pavord ID. Eosinophilic bronchitis is an important cause of chronic cough. *Am J Respir Crit Care Med* 1999; 160(2):406-410.
- (23) Gibson PG, Zlatich K, Scott J, Sewell W, Woolley K, Saltos N. Chronic cough resembles asthma with IL-5 and granulocyte-macrophage colony-stimulating factor gene expression in bronchoalveolar cells. *J Allergy Clin Immunol* 1998; 101(3):320-326.
- (24) Begueret H, Berger P, Vernejoux JM, Dubuisson L, Marthan R, Tunon-de-Lara JM. Inflammation of bronchial smooth muscle in allergic asthma. *Thorax* 2007; 62(1):8-15.
- (25) Brightling CE, Pavord ID. Eosinophilic bronchitis - what is it and why is it important? *Clin Exp Allergy* 2000; 30(1):4-6.
- (26) Wardlaw AJ, Silverman M, Siva R, Pavord ID, Green R. Multi-dimensional phenotyping: towards a new taxonomy for airway disease. *Clin Exp Allergy* 2005; 35(10):1254-1262.
- (27) Siddiqui S, Hollins F, Saha S, Brightling CE. Inflammatory cell microlocalisation and airway dysfunction: cause and effect? *Eur Respir J* 2007; 30(6):1043-1056.
- (28) Holgate ST. Pathogenesis of asthma. *Clin Exp Allergy* 2008; 38(6):872-897.
- (29) Kraft M, Djukanovic R, Wilson S, Holgate ST, Martin RJ. Alveolar tissue inflammation in asthma. *Am J Respir Crit Care Med* 1996; 154(5):1505-1510.
- (30) Laitinen LA, Laitinen A, Haahtela T. Airway mucosal inflammation even in patients with newly diagnosed asthma. *Am Rev Respir Dis* 1993; 147(3):697-704.
- (31) Gibson PG, Allen CJ, Yang JP, Wong BJ, Dolovich J, Denburg J et al. Intraepithelial mast cells in allergic and nonallergic asthma. Assessment using bronchial brushings. *Am Rev Respir Dis* 1993; 148(1):80-86.
- (32) Foresi A, Bertorelli G, Pesci A, Chetta A, Olivieri D. Inflammatory markers in bronchoalveolar lavage and in bronchial biopsy in asthma during remission. *Chest* 1990; 98(3):528-535.
- (33) Montefort S, Gratziau C, Goulding D, Polosa R, Haskard DO, Howarth PH et al. Bronchial biopsy evidence for leukocyte infiltration and upregulation of leukocyte-endothelial cell adhesion molecules 6 hours after local allergen challenge of sensitized asthmatic airways. *J Clin Invest* 1994; 93(4):1411-1421.
- (34) Brightling CE, Ward R, Woltmann G, Bradding P, Sheller JR, Dworski R et al. Induced sputum inflammatory mediator concentrations in eosinophilic bronchitis and asthma. *Am J Respir Crit Care Med* 2000; 162(3 Pt 1):878-882.
- (35) Carroll NG, Mutavdzic S, James AL. Increased mast cells and neutrophils in submucosal mucous glands and mucus plugging in patients with asthma. *Thorax* 2002; 57(8):677-682.
- (36) Carroll NG, Mutavdzic S, James AL. Distribution and degranulation of airway mast cells in normal and asthmatic subjects. *Eur Respir J* 2002; 19(5):879-885.
- (37) Bradley BL, Azzawi M, Jacobson M, Assoufi B, Collins JV, Irani AM et al. Eosinophils, T-lymphocytes, mast cells, neutrophils, and macrophages in bronchial biopsy specimens from atopic subjects with asthma: comparison with biopsy specimens from atopic subjects without asthma and normal control subjects and relationship to bronchial hyperresponsiveness. *J Allergy Clin Immunol* 1991; 88(4):661-674.
- (38) Pizzichini E, Pizzichini MM, Efthimiadis A, Evans S, Morris MM, Squillace D et al. Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid-phase measurements. *Am J Respir Crit Care Med* 1996; 154(2 Pt 1):308-317.

- (39) Crimi E, Spanevello A, Neri M, Ind PW, Rossi GA, Brusasco V. Dissociation between airway inflammation and airway hyperresponsiveness in allergic asthma. *Am J Respir Crit Care Med* 1998; 157(1):4-9.
- (40) Giembycz MA, Kaur M, Leigh R, Newton R. A Holy Grail of asthma management: toward understanding how long-acting beta(2)-adrenoceptor agonists enhance the clinical efficacy of inhaled corticosteroids. *Br J Pharmacol* 2008; 153(6):1090-1104.
- (41) Benayoun L, Druilhe A, Dombret MC, Aubier M, Pretolani M. Airway structural alterations selectively associated with severe asthma. *Am J Respir Crit Care Med* 2003; 167(10):1360-1368.
- (42) Pepe C, Foley S, Shannon J, Lemiere C, Olivenstein R, Ernst P et al. Differences in airway remodeling between subjects with severe and moderate asthma. *J Allergy Clin Immunol* 2005; 116(3):544-549.
- (43) Cohen L, E X, Tarsi J, Ramkumar T, Horiuchi TK, Cochran R et al. Epithelial cell proliferation contributes to airway remodeling in severe asthma. *Am J Respir Crit Care Med* 2007; 176(2):138-145.
- (44) Cairns JA, Walls AF. Mast cell tryptase is a mitogen for epithelial cells. Stimulation of IL-8 production and intercellular adhesion molecule-1 expression. *J Immunol* 1996; 156(1):275-283.
- (45) Trautmann A, Schmid-Grendelmeier P, Kruger K, Cramer R, Akdis M, Akkaya A et al. T cells and eosinophils cooperate in the induction of bronchial epithelial cell apoptosis in asthma. *J Allergy Clin Immunol* 2002; 109(2):329-337.
- (46) Vliagoftis H, Befus AD, Hollenberg MD, Moqbel R. Airway epithelial cells release eosinophil survival-promoting factors (GM-CSF) after stimulation of proteinase-activated receptor 2. *J Allergy Clin Immunol* 2001; 107(4):679-685.
- (47) Allen-Gipson DS, Romberger DJ, Forget MA, May KL, Sisson JH, Wyatt TA. IL-8 inhibits isoproterenol-stimulated ciliary beat frequency in bovine bronchial epithelial cells. *J Aerosol Med* 2004; 17(2):107-115.
- (48) Malavia NK, Mih JD, Raub CB, Dinh BT, George SC. IL-13 induces a bronchial epithelial phenotype that is profibrotic. *Respir Res* 2008; 9:27.
- (49) Aikawa T, Shimura S, Sasaki H, Ebina M, Takishima T. Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack. *Chest* 1992; 101(4):916-921.
- (50) Ordonez CL, Khashayar R, Wong HH, Ferrando R, Wu R, Hyde DM et al. Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression. *Am J Respir Crit Care Med* 2001; 163(2):517-523.
- (51) Gibson PG, Dolovich J, Denburg J, Ramsdale EH, Hargreave FE. Chronic cough: eosinophilic bronchitis without asthma. *Lancet* 1989; 1(8651):1346-1348.
- (52) Okumura S, Sagara H, Fukuda T, Saito H, Okayama Y. FcepsilonRI-mediated amphiregulin production by human mast cells increases mucin gene expression in epithelial cells. *J Allergy Clin Immunol* 2005; 115(2):272-279.
- (53) Nakao I, Kanaji S, Ohta S, Matsushita H, Arima K, Yuyama N et al. Identification of pendrin as a common mediator for mucus production in bronchial asthma and chronic obstructive pulmonary disease. *J Immunol* 2008; 180(9):6262-6269.
- (54) Park JA, He F, Martin LD, Li Y, Chorley BN, Adler KB. Human neutrophil elastase induces hypersecretion of mucin from well-differentiated human bronchial epithelial cells in vitro via a protein kinase C{delta}-mediated mechanism. *Am J Pathol* 2005; 167(3):651-661.
- (55) Bai TR, Cooper J, Koelmeyer T, Pare PD, Weir TD. The effect of age and duration of disease on airway structure in fatal asthma. *Am J Respir Crit Care Med* 2000; 162(2 Pt 1):663-669.

- (56) Howarth PH, Knox AJ, Amrani Y, Tliba O, Panettieri RA, Jr., Johnson M. Synthetic responses in airway smooth muscle. *J Allergy Clin Immunol* 2004; 114(2 Suppl):S32-S50.
- (57) Berger P, Girodet PO, Begueret H, Ousova O, Perng DW, Marthan R et al. Tryptase-stimulated human airway smooth muscle cells induce cytokine synthesis and mast cell chemotaxis. *FASEB J* 2003; 17(14):2139-2141.
- (58) Raines EW, Dower SK, Ross R. Interleukin-1 mitogenic activity for fibroblasts and smooth muscle cells is due to PDGF-AA. *Science* 1989; 243(4889):393-396.
- (59) Burgess JK, Johnson PR, Ge Q, Au WW, Poniris MH, McParland BE et al. Expression of connective tissue growth factor in asthmatic airway smooth muscle cells. *Am J Respir Crit Care Med* 2003; 167(1):71-77.
- (60) Burgess JK, Ge Q, Poniris MH, Boustany S, Twigg SM, Black JL et al. Connective tissue growth factor and vascular endothelial growth factor from airway smooth muscle interact with the extracellular matrix. *Am J Physiol Lung Cell Mol Physiol* 2006; 290(1):L153-L161.
- (61) Kemi C, Grunewald J, Eklund A, Hoglund CO. Differential regulation of neurotrophin expression in human bronchial smooth muscle cells. *Respir Res* 2006; 7:18.
- (62) Wen FQ, Liu X, Manda W, Terasaki Y, Kobayashi T, Abe S et al. TH2 Cytokine-enhanced and TGF-beta-enhanced vascular endothelial growth factor production by cultured human airway smooth muscle cells is attenuated by IFN-gamma and corticosteroids. *J Allergy Clin Immunol* 2003; 111(6):1307-1318.
- (63) Fong CY, Pang L, Holland E, Knox AJ. TGF-beta1 stimulates IL-8 release, COX-2 expression, and PGE(2) release in human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2000; 279(1):L201-L207.
- (64) Sukkar MB, Issa R, Xie S, Oltmanns U, Newton R, Chung KF. Fractalkine/CX3CL1 production by human airway smooth muscle cells: induction by IFN-gamma and TNF-alpha and regulation by TGF-beta and corticosteroids. *Am J Physiol Lung Cell Mol Physiol* 2004; 287(6):L1230-L1240.
- (65) Watson ML, Grix SP, Jordan NJ, Place GA, Dodd S, Leithead J et al. Interleukin 8 and monocyte chemoattractant protein 1 production by cultured human airway smooth muscle cells. *Cytokine* 1998; 10(5):346-352.
- (66) Chhabra J, Li YZ, Alkhouri H, Blake AE, Ge Q, Armour CL et al. Histamine and tryptase modulate asthmatic airway smooth muscle GM-CSF and RANTES release. *Eur Respir J* 2007; 29(5):861-870.
- (67) John M, Hirst SJ, Jose PJ, Robichaud A, Berkman N, Witt C et al. Human airway smooth muscle cells express and release RANTES in response to T helper 1 cytokines: regulation by T helper 2 cytokines and corticosteroids. *J Immunol* 1997; 158(4):1841-1847.
- (68) Juremalm M, Olsson N, Nilsson G. Selective CCL5/RANTES-induced mast cell migration through interactions with chemokine receptors CCR1 and CCR4. *Biochemical and Biophysical Research Communications* 2002; 297(3):480-485.
- (69) Rodel J, Woytas M, Groh A, Schmidt KH, Hartmann M, Lehmann M et al. Production of basic fibroblast growth factor and interleukin 6 by human smooth muscle cells following infection with *Chlamydia pneumoniae*. *Infect Immun* 2000; 68(6):3635-3641.
- (70) John M, Au BT, Jose PJ, Lim S, Saunders M, Barnes PJ et al. Expression and release of interleukin-8 by human airway smooth muscle cells: inhibition by Th-2 cytokines and corticosteroids. *Am J Respir Cell Mol Biol* 1998; 18(1):84-90.
- (71) Brightling CE, Ammit AJ, Kaur D, Black JL, Wardlaw AJ, Hughes JM et al. The CXCL10/CXCR3 axis mediates human lung mast cell migration to asthmatic airway smooth muscle. *American Journal of Respiratory and Critical Care Medicine* 2005; 171(10):1103-1108.

- (72) Hardaker EL, Bacon AM, Carlson K, Roshak AK, Foley JJ, Schmidt DB et al. Regulation of TNF-alpha- and IFN-gamma-induced CXCL10 expression: participation of the airway smooth muscle in the pulmonary inflammatory response in chronic obstructive pulmonary disease. *FASEB J* 2004; 18(1):191-193.
- (73) Sutcliffe A, Kaur D, Page S, Woodman L, Armour CL, Baraket M et al. Mast cell migration to Th2 stimulated airway smooth muscle from asthmatics. *Thorax* 2006; 61(8):657-662.
- (74) Kaur D, Saunders R, Berger P, Siddiqui S, Woodman L, Wardlaw A et al. Airway smooth muscle and mast cell-derived CC chemokine ligand 19 mediate airway smooth muscle migration in asthma. *Am J Respir Crit Care Med* 2006; 174(11):1179-1188.
- (75) Campanella GS, Medoff BD, Manice LA, Colvin RA, Luster AD. Development of a novel chemokine-mediated in vivo T cell recruitment assay. *J Immunol Methods* 2008; 331(1-2):127-139.
- (76) Pang L, Nie M, Corbett L, Sutcliffe A, Knox AJ. Mast cell beta-tryptase selectively cleaves eotaxin and RANTES and abrogates their eosinophil chemotactic activities. *J Immunol* 2006; 176(6):3788-3795.
- (77) Olsson N, Piek E, Sundstrom M, ten Dijke P, Nilsson G. Transforming growth factor-beta-mediated mast cell migration depends on mitogen-activated protein kinase activity. *Cellular Signalling* 2001; 13(7):483-490.
- (78) Tatler AL, Porte J, Knox A, Jenkins G, Pang L. Tryptase activates TGFbeta in human airway smooth muscle cells via direct proteolysis. *Biochem Biophys Res Commun* 2008; 370(2):239-242.
- (79) Johnson PR. Role of human airway smooth muscle in altered extracellular matrix production in asthma. *Clin Exp Pharmacol Physiol* 2001; 28(3):233-236.
- (80) Panettieri RA, Tan EM, Ciocca V, Luttmann MA, Leonard TB, Hay DW. Effects of LTD4 on human airway smooth muscle cell proliferation, matrix expression, and contraction In vitro: differential sensitivity to cysteinyl leukotriene receptor antagonists. *Am J Respir Cell Mol Biol* 1998; 19(3):453-461.
- (81) Johnson PR, Burgess JK, Ge Q, Poniris M, Boustany S, Twigg SM et al. Connective tissue growth factor induces extracellular matrix in asthmatic airway smooth muscle. *Am J Respir Crit Care Med* 2006; 173(1):32-41.
- (82) Hong HH, Uzel MI, Duan C, Sheff MC, Trackman PC. Regulation of lysyl oxidase, collagen, and connective tissue growth factor by TGF-beta1 and detection in human gingiva. *Lab Invest* 1999; 79(12):1655-1667.
- (83) Neumann C, Yu A, Welge-Lussen U, Lutjen-Drecoll E, Birke M. The effect of TGF-beta2 on elastin, type VI collagen, and components of the proteolytic degradation system in human optic nerve astrocytes. *Invest Ophthalmol Vis Sci* 2008; 49(4):1464-1472.
- (84) Cox G, Thomson NC, Rubin AS, Niven RM, Corris PA, Siersted HC et al. Asthma control during the year after bronchial thermoplasty. *N Engl J Med* 2007; 356(13):1327-1337.
- (85) Woodruff PG, Dolganov GM, Ferrando RE, Donnelly S, Hays SR, Solberg OD et al. Hyperplasia of smooth muscle in mild to moderate asthma without changes in cell size or gene expression. *Am J Respir Crit Care Med* 2004; 169(9):1001-1006.
- (86) Johnson PR, Roth M, Tamm M, Hughes M, Ge Q, King G et al. Airway smooth muscle cell proliferation is increased in asthma. *Am J Respir Crit Care Med* 2001; 164(3):474-477.
- (87) Panettieri RA, Yadvish PA, Kelly AM, Rubinstein NA, Kotlikoff MI. Histamine stimulates proliferation of airway smooth muscle and induces c-fos expression. *Am J Physiol* 1990; 259(6 Pt 1):L365-L371.

- (88) Berger P, Perng DW, Thabrew H, Compton SJ, Cairns JA, McEuen AR et al. Tryptase and agonists of PAR-2 induce the proliferation of human airway smooth muscle cells. *J Appl Physiol* 2001; 91(3):1372-1379.
- (89) Chambers LS, Black JL, Poronnik P, Johnson PR. Functional effects of protease-activated receptor-2 stimulation on human airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2001; 281(6):L1369-L1378.
- (90) Kanaoka Y, Boyce JA. Cysteinyl leukotrienes and their receptors: cellular distribution and function in immune and inflammatory responses. *J Immunol* 2004; 173(3):1503-1510.
- (91) Bosse Y, Thompson C, McMahon S, Dubois CM, Stankova J, Rola-Pleszczynski M. Leukotriene D4-induced, epithelial cell-derived transforming growth factor beta1 in human bronchial smooth muscle cell proliferation. *Clin Exp Allergy* 2008; 38(1):113-121.
- (92) Espinosa K, Bosse Y, Stankova J, Rola-Pleszczynski M. CysLT1 receptor upregulation by TGF-beta and IL-13 is associated with bronchial smooth muscle cell proliferation in response to LTD4. *J Allergy Clin Immunol* 2003; 111(5):1032-1040.
- (93) Hawker KM, Johnson PR, Hughes JM, Black JL. Interleukin-4 inhibits mitogen-induced proliferation of human airway smooth muscle cells in culture. *Am J Physiol* 1998; 275(3 Pt 1):L469-L477.
- (94) Hirst SJ, Barnes PJ, Twort CH. PDGF isoform-induced proliferation and receptor expression in human cultured airway smooth muscle cells. *Am J Physiol* 1996; 270(3 Pt 1):L415-L428.
- (95) Chen G, Khalil N. TGF-beta1 increases proliferation of airway smooth muscle cells by phosphorylation of map kinases. *Respir Res* 2006; 7:2.
- (96) Goldsmith AM, Bentley JK, Zhou L, Jia Y, Bitar KN, Fingar DC et al. Transforming growth factor-beta induces airway smooth muscle hypertrophy. *Am J Respir Cell Mol Biol* 2006; 34(2):247-254.
- (97) Bosse Y, Thompson C, Stankova J, Rola-Pleszczynski M. Fibroblast growth factor 2 and transforming growth factor beta1 synergism in human bronchial smooth muscle cell proliferation. *Am J Respir Cell Mol Biol* 2006; 34(6):746-753.
- (98) Xie S, Sukkar MB, Issa R, Khorasani NM, Chung KF. Mechanisms of induction of airway smooth muscle hyperplasia by transforming growth factor-beta. *Am J Physiol Lung Cell Mol Physiol* 2007; 293(1):L245-L253.
- (99) McKay S, Hirst SJ, Haas MB, de Jongste JC, Hoogsteden HC, Saxena PR et al. Tumor necrosis factor-alpha enhances mRNA expression and secretion of interleukin-6 in cultured human airway smooth muscle cells. *Am J Respir Cell Mol Biol* 2000; 23(1):103-111.
- (100) Tliba O, Tliba S, Da Huang C, Hoffman RK, DeLong P, Panettieri RA, Jr. et al. Tumor necrosis factor alpha modulates airway smooth muscle function via the autocrine action of interferon beta. *J Biol Chem* 2003; 278(50):50615-50623.
- (101) Kanabar V, Hirst SJ, O'Connor BJ, Page CP. Some structural determinants of the antiproliferative effect of heparin-like molecules on human airway smooth muscle. *Br J Pharmacol* 2005; 146(3):370-377.
- (102) Desmouliere A, Rubbia-Brandt L, Gabbiani G. Modulation of actin isoform expression in cultured arterial smooth muscle cells by heparin and culture conditions. *Arterioscler Thromb* 1991; 11(2):244-253.
- (103) Ebina M, Takahashi T, Chiba T, Motomiya M. Cellular hypertrophy and hyperplasia of airway smooth muscles underlying bronchial asthma. A 3-D morphometric study. *Am Rev Respir Dis* 1993; 148(3):720-726.
- (104) Bonacci JV, Harris T, Wilson JW, Stewart AG. Collagen-induced resistance to glucocorticoid anti-mitogenic actions: a potential explanation of smooth muscle hyperplasia in the asthmatic remodelled airway. *Br J Pharmacol* 2003; 138(7):1203-1206.

- (105) Freyer AM, Johnson SR, Hall IP. Effects of growth factors and extracellular matrix on survival of human airway smooth muscle cells. *Am J Respir Cell Mol Biol* 2001; 25(5):569-576.
- (106) Hedges JC, Dechert MA, Yamboliev IA, Martin JL, Hickey E, Weber LA et al. A role for p38(MAPK)/HSP27 pathway in smooth muscle cell migration. *J Biol Chem* 1999; 274(34):24211-24219.
- (107) Carlin SM, Roth M, Black JL. Urokinase potentiates PDGF-induced chemotaxis of human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2003; 284(6):L1020-L1026.
- (108) Goncharova EA, Billington CK, Irani C, Vorotnikov AV, Tkachuk VA, Penn RB et al. Cyclic AMP-mobilizing agents and glucocorticoids modulate human smooth muscle cell migration. *Am J Respir Cell Mol Biol* 2003; 29(1):19-27.
- (109) Joubert P, Lajoie-Kadoch S, Labonte I, Gounni AS, Maghni K, Wellemans V et al. CCR3 expression and function in asthmatic airway smooth muscle cells. *J Immunol* 2005; 175(4):2702-2708.
- (110) Schmidt M, Sun G, Stacey MA, Mori L, Mattoli S. Identification of circulating fibrocytes as precursors of bronchial myofibroblasts in asthma. *J Immunol* 2003; 171(1):380-389.
- (111) Jeffery PK. Remodeling in asthma and chronic obstructive lung disease. *Am J Respir Crit Care Med* 2001; 164(10 Pt 2):S28-S38.
- (112) Amrani Y, Panettieri RA. Airway smooth muscle: contraction and beyond. *Int J Biochem Cell Biol* 2003; 35(3):272-276.
- (113) Low RB, White SL. Lung smooth muscle differentiation. *Int J Biochem Cell Biol* 1998; 30(8):869-883.
- (114) Kotaru C, Schoonover KJ, Trudeau JB, Huynh ML, Zhou X, Hu H et al. Regional fibroblast heterogeneity in the lung: implications for remodeling. *Am J Respir Crit Care Med* 2006; 173(11):1208-1215.
- (115) Leguillet R, Lauzon AM. Molecular mechanics of smooth muscle contractile proteins in airway hyperresponsiveness and asthma. *Proc Am Thorac Soc* 2008; 5(1):40-46.
- (116) Fatigati V, Murphy RA. Actin and tropomyosin variants in smooth muscles. Dependence on tissue type. *J Biol Chem* 1984; 259(23):14383-14388.
- (117) Lodish BZMBD. *Molecular Cell Biology*. W.H. Freeman & Company, 2000.
- (118) Owens GK. Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev* 1995; 75(3):487-517.
- (119) Raeburn D, Giembycz M. *Airway Smooth Muscle : Development and Regulation of Contractility*. Basel, Switzerland.: Birkhausen Verlag, 1994.
- (120) Jahoda CA, Reynolds AJ, Chaponnier C, Forester JC, Gabbiani G. Smooth muscle alpha-actin is a marker for hair follicle dermis in vivo and in vitro. *J Cell Sci* 1991; 99 (Pt 3):627-636.
- (121) Wong JZ, Woodcock-Mitchell J, Mitchell J, Rippetoe P, White S, Absher M et al. Smooth muscle actin and myosin expression in cultured airway smooth muscle cells. *Am J Physiol* 1998; 274(5 Pt 1):L786-L792.
- (122) Blank RS, Thompson MM, Owens GK. Cell cycle versus density dependence of smooth muscle alpha actin expression in cultured rat aortic smooth muscle cells. *J Cell Biol* 1988; 107(1):299-306.
- (123) Hinz B, Celetta G, Tomasek JJ, Gabbiani G, Chaponnier C. Alpha-smooth muscle actin expression upregulates fibroblast contractile activity. *Mol Biol Cell* 2001; 12(9):2730-2741.
- (124) Kamm KE, Stull JT. The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. *Annu Rev Pharmacol Toxicol* 1985; 25:593-620.

- (125) Woodcock-Mitchell J, White S, Stirewalt W, Periasamy M, Mitchell J, Low RB. Myosin isoform expression in developing and remodeling rat lung. *Am J Respir Cell Mol Biol* 1993; 8(6):617-625.
- (126) Kohama K, Ye LH, Hayakawa K, Okagaki T. Myosin light chain kinase: an actin-binding protein that regulates an ATP-dependent interaction with myosin. *Trends Pharmacol Sci* 1996; 17(8):284-287.
- (127) Adelstein RS, Klee CB. Purification and characterization of smooth muscle myosin light chain kinase. *J Biol Chem* 1981; 256(14):7501-7509.
- (128) Halayko AJ, Salari H, MA X, Stephens NL. Markers of airway smooth muscle cell phenotype. *Am J Physiol* 1996; 270(6 Pt 1):L1040-L1051.
- (129) Kitami Y, Maguchi M, Nishida W, Okura T, Kohara K, Hiwada K. The unique 5-flanking region of the human basic calponin gene. *Hypertens Res* 1999; 22(3):187-193.
- (130) Strasser P, Gimona M, Moessler H, Herzog M, Small JV. Mammalian calponin. Identification and expression of genetic variants. *FEBS Lett* 1993; 330(1):13-18.
- (131) Hossain MM, Hwang DY, Huang QQ, Sasaki Y, Jin JP. Developmentally regulated expression of calponin isoforms and the effect of h2-calponin on cell proliferation. *Am J Physiol Cell Physiol* 2003; 284(1):C156-C167.
- (132) Hossain MM, Crish JF, Eckert RL, Lin JJ, Jin JP. h2-Calponin is regulated by mechanical tension and modifies the function of actin cytoskeleton. *J Biol Chem* 2005; 280(51):42442-42453.
- (133) Lehman W, Sheldon A, Madonia W. Diversity in smooth muscle thin filament composition. *Biochim Biophys Acta* 1987; 914(1):35-39.
- (134) Gerthoffer WT, Pohl J. Caldesmon and calponin phosphorylation in regulation of smooth muscle contraction. *Can J Physiol Pharmacol* 1994; 72(11):1410-1414.
- (135) Nucci MR, O'Connell JT, Huettner PC, Cviko A, Sun D, Quade BJ. h-Caldesmon expression effectively distinguishes endometrial stromal tumors from uterine smooth muscle tumors. *Am J Surg Pathol* 2001; 25(4):455-463.
- (136) Burkhard FC, Lemack GE, Zimmern PE, Lin VK, McConnell JD. Contractile protein expression in bladder smooth muscle is a marker of phenotypic modulation after outlet obstruction in the rabbit model. *J Urol* 2001; 165(3):963-967.
- (137) Vrhovski B, McKay K, Schevzov G, Gunning PW, Weinberger RP. Smooth muscle-specific alpha tropomyosin is a marker of fully differentiated smooth muscle in lung. *J Histochem Cytochem* 2005; 53(7):875-883.
- (138) Park S, Rasmussen H. Carbachol-induced protein phosphorylation changes in bovine tracheal smooth muscle. *J Biol Chem* 1986; 261(33):15734-15739.
- (139) Paulin D, Li Z. Desmin: a major intermediate filament protein essential for the structural integrity and function of muscle. *Exp Cell Res* 2004; 301(1):1-7.
- (140) Gunst SJ, Tang DD. The contractile apparatus and mechanical properties of airway smooth muscle. *Eur Respir J* 2000; 15(3):600-616.
- (141) Pieper FR, Schaart G, Krimpenfort PJ, Henderik JB, Moshage HJ, van de KA et al. Transgenic expression of the muscle-specific intermediate filament protein desmin in nonmuscle cells. *J Cell Biol* 1989; 108(3):1009-1024.
- (142) Raats JM, Pieper FR, Vree Egberts WT, Verrijp KN, Ramaekers FC, Bloemendal H. Assembly of amino-terminally deleted desmin in vimentin-free cells. *J Cell Biol* 1990; 111(5 Pt 1):1971-1985.

- (143) Halayko AJ, Camoretti-Mercado B, Forsythe SM, Vieira JE, Mitchell RW, Wylam ME et al. Divergent differentiation paths in airway smooth muscle culture: induction of functionally contractile myocytes. *Am J Physiol* 1999; 276(1 Pt 1):L197-L206.
- (144) Gailit J, Marchese MJ, Kew RR, Gruber BL. The differentiation and function of myofibroblasts is regulated by mast cell mediators. *J Invest Dermatol* 2001; 117(5):1113-1119.
- (145) Batra V, Musani AI, Hastie AT, Khurana S, Carpenter KA, Zangrilli JG et al. Bronchoalveolar lavage fluid concentrations of transforming growth factor (TGF)-beta1, TGF-beta2, interleukin (IL)-4 and IL-13 after segmental allergen challenge and their effects on alpha-smooth muscle actin and collagen III synthesis by primary human lung fibroblasts. *Clin Exp Allergy* 2004; 34(3):437-444.
- (146) Chen CN, Li YS, Yeh YT, Lee PL, Usami S, Chien S et al. Synergistic roles of platelet-derived growth factor-BB and interleukin-1beta in phenotypic modulation of human aortic smooth muscle cells. *Proc Natl Acad Sci U S A* 2006; 103(8):2665-2670.
- (147) Tanaka K, Sano K, Nakano T, Yuba K, Kinoshita M. Suppression of alpha smooth muscle actin expression by IFN-gamma in established myofibroblast cell lines. *J Interferon Cytokine Res* 2007; 27(10):835-839.
- (148) Tanaka K, Sano K, Yuba K, Katsumura K, Nakano T, Tanaka K et al. Inhibition of induction of myofibroblasts by interferon gamma in a human fibroblast cell line. *Int Immunopharmacol* 2003; 3(9):1273-1280.
- (149) Kawai-Kowase K, Sato H, Oyama Y, Kanai H, Sato M, Doi H et al. Basic fibroblast growth factor antagonizes transforming growth factor-beta1-induced smooth muscle gene expression through extracellular signal-regulated kinase 1/2 signaling pathway activation. *Arterioscler Thromb Vasc Biol* 2004; 24(8):1384-1390.
- (150) Yamamoto T, Hartmann K, Eckes B, Krieg T. Mast cells enhance contraction of three-dimensional collagen lattices by fibroblasts by cell-cell interaction: role of stem cell factor/c-kit. *Immunology* 2000; 99(3):435-439.
- (151) Moyer KE, Saggars GC, Ehrlich HP. Mast cells promote fibroblast populated collagen lattice contraction through gap junction intercellular communication. *Wound Repair Regen* 2004; 12(3):269-275.
- (152) Garrett B, Garrett SB. Cellular communication and the action of growth factors during wound healing. *J Wound Care* 1997; 6(6):277-280.
- (153) Khalil N, Bereznay O, Sporn M, Greenberg AH. Macrophage production of transforming growth factor beta and fibroblast collagen synthesis in chronic pulmonary inflammation. *J Exp Med* 1989; 170(3):727-737.
- (154) McCartney-Francis NL, Wahl SM. Transforming growth factor beta: a matter of life and death. *J Leukoc Biol* 1994; 55(3):401-409.
- (155) Gailit J, Marchese MJ, Kew RR, Gruber BL. The differentiation and function of myofibroblasts is regulated by mast cell mediators. *J Invest Dermatol* 2001; 117(5):1113-1119.
- (156) Bonniaud P, Kolb M, Galt T, Robertson J, Robbins C, Stampfli M et al. Smad3 null mice develop airspace enlargement and are resistant to TGF-beta-mediated pulmonary fibrosis. *J Immunol* 2004; 173(3):2099-2108.
- (157) Loeys BL, Schwarze U, Holm T, Callewaert BL, Thomas GH, Pannu H et al. Aneurysm syndromes caused by mutations in the TGF-beta receptor. *N Engl J Med* 2006; 355(8):788-798.
- (158) Kelley TJ, Elmer HL, Corey DA. Reduced Smad3 protein expression and altered transforming growth factor-beta1-mediated signaling in cystic fibrosis epithelial cells. *Am J Respir Cell Mol Biol* 2001; 25(6):732-738.

- (159) Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M et al. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* 1992; 359(6397):693-699.
- (160) Border WA, Noble NA. Targeting TGF-beta for treatment of disease. *Nat Med* 1995; 1(10):1000-1001.
- (161) Khalil N, O'Connor RN, Flanders KC, Shing W, Whitman CI. Regulation of type II alveolar epithelial cell proliferation by TGF-beta during bleomycin-induced lung injury in rats. *Am J Physiol* 1994; 267(5 Pt 1):L498-L507.
- (162) Munger JS, Harpel JG, Gleizes PE, Mazzei R, Nunes I, Rifkin DB. Latent transforming growth factor-beta: structural features and mechanisms of activation. *Kidney Int* 1997; 51(5):1376-1382.
- (163) Cheifetz S, Hernandez H, Laiho M, ten Dijke P, Iwata KK, Massague J. Distinct transforming growth factor-beta (TGF-beta) receptor subsets as determinants of cellular responsiveness to three TGF-beta isoforms. *J Biol Chem* 1990; 265(33):20533-20538.
- (164) Redington AE, Madden J, Frew AJ, Djukanovic R, Roche WR, Holgate ST et al. Transforming growth factor-beta 1 in asthma. Measurement in bronchoalveolar lavage fluid. *Am J Respir Crit Care Med* 1997; 156(2 Pt 1):642-647.
- (165) Taipale J, Miyazono K, Heldin CH, Keski-Oja J. Latent transforming growth factor-beta 1 associates to fibroblast extracellular matrix via latent TGF-beta binding protein. *J Cell Biol* 1994; 124(1-2):171-181.
- (166) Wakefield LM, Smith DM, Masui T, Harris CC, Sporn MB. Distribution and modulation of the cellular receptor for transforming growth factor-beta. *J Cell Biol* 1987; 105(2):965-975.
- (167) Sinha S, Nevett C, Shuttleworth CA, Kielty CM. Cellular and extracellular biology of the latent transforming growth factor-beta binding proteins. *Matrix Biol* 1998; 17(8-9):529-545.
- (168) Taipale J, Lohi J, Saarinen J, Kovanen PT, Keski-Oja J. Human mast cell chymase and leukocyte elastase release latent transforming growth factor-beta 1 from the extracellular matrix of cultured human epithelial and endothelial cells. *J Biol Chem* 1995; 270(9):4689-4696.
- (169) Coutts A, Chen G, Stephens N, Hirst S, Douglas D, Eichholtz T et al. Release of biologically active TGF-beta from airway smooth muscle cells induces autocrine synthesis of collagen. *Am J Physiol Lung Cell Mol Physiol* 2001; 280(5):L999-1008.
- (170) Bugge TH, Flick MJ, Daugherty CC, Degen JL. Plasminogen deficiency causes severe thrombosis but is compatible with development and reproduction. *Genes Dev* 1995; 9(7):794-807.
- (171) Sajid M, Lele M, Stouffer GA. Autocrine thrombospondin partially mediates TGF-beta1-induced proliferation of vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol* 2000; 279(5):H2159-H2165.
- (172) Morishima Y, Nomura A, Uchida Y, Noguchi Y, Sakamoto T, Ishii Y et al. Triggering the induction of myofibroblast and fibrogenesis by airway epithelial shedding. *Am J Respir Cell Mol Biol* 2001; 24(1):1-11.
- (173) Crawford SE, Stellmach V, Murphy-Ullrich JE, Ribeiro SM, Lawler J, Hynes RO et al. Thrombospondin-1 is a major activator of TGF-beta1 in vivo. *Cell* 1998; 93(7):1159-1170.
- (174) Nguyen TT, Ward JP, Hirst SJ. beta1-Integrins mediate enhancement of airway smooth muscle proliferation by collagen and fibronectin. *Am J Respir Crit Care Med* 2005; 171(3):217-223.
- (175) Ludbrook SB, Barry ST, Delves CJ, Horgan CM. The integrin alpha v beta 3 is a receptor for the latency-associated peptides of transforming growth factors beta 1 and beta 3. *Biochem J* 2003; 369(Pt 2):311-318.

- (176) Munger JS, Huang X, Kawakatsu H, Griffiths MJ, Dalton SL, Wu J et al. The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell* 1999; 96(3):319-328.
- (177) Annes JP, Chen Y, Munger JS, Rifkin DB. Integrin alphaVbeta6-mediated activation of latent TGF-beta requires the latent TGF-beta binding protein-1. *J Cell Biol* 2004; 165(5):723-734.
- (178) Verrecchia F, Mauviel A, Farge D. Transforming growth factor-beta signaling through the Smad proteins: role in systemic sclerosis. *Autoimmun Rev* 2006; 5(8):563-569.
- (179) Kay AB. Mediators of hypersensitivity and inflammatory cells in the pathogenesis of bronchial asthma. *Eur J Respir Dis Suppl* 1983; 129:1-44.
- (180) Wu J, Kobayashi M, Sousa EA, Liu W, Cai J, Goldman SJ et al. Differential proteomic analysis of bronchoalveolar lavage fluid in asthmatics following segmental antigen challenge. *Mol Cell Proteomics* 2005; 4(9):1251-1264.
- (181) Webb RC. Smooth muscle contraction and relaxation. *Adv Physiol Educ* 2003; 27(1-4):201-206.
- (182) Macklem PT. A theoretical analysis of the effect of airway smooth muscle load on airway narrowing. *Am J Respir Crit Care Med* 1996; 153(1):83-89.
- (183) James AL, Wenzel S. Clinical relevance of airway remodelling in airway diseases. *Eur Respir J* 2007; 30(1):134-155.
- (184) Scichilone N, Permutt S, Togias A. The lack of the bronchoprotective and not the bronchodilatory ability of deep inspiration is associated with airway hyperresponsiveness. *Am J Respir Crit Care Med* 2001; 163(2):413-419.
- (185) Skloot G, Permutt S, Togias A. Airway hyperresponsiveness in asthma: a problem of limited smooth muscle relaxation with inspiration. *J Clin Invest* 1995; 96(5):2393-2403.
- (186) Scichilone N, Marchese R, Soresi S, Interrante A, Togias A, Bellia V. Deep inspiration-induced changes in lung volume decrease with severity of asthma. *Respir Med* 2007; 101(5):951-956.
- (187) Brusasco V, Crimi E, Pellegrino R. Airway hyperresponsiveness in asthma: not just a matter of airway inflammation. *Thorax* 1998; 53(11):992-998.
- (188) Slat AM, Janssen K, van Schadewijk A, van der Plas DT, Schot R, van den Aardweg JG et al. Bronchial inflammation and airway responses to deep inspiration in asthma and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2007; 176(2):121-128.
- (189) MA X, Cheng Z, Kong H, Wang Y, Unruh H, Stephens NL et al. Changes in biophysical and biochemical properties of single bronchial smooth muscle cells from asthmatic subjects. *Am J Physiol Lung Cell Mol Physiol* 2002; 283(6):L1181-L1189.
- (190) Villasenor-Cardoso MI, Salaiza N, Delgado J, Gutierrez-Kobeh L, Perez-Torres A, Becker I. Mast cells are activated by *Leishmania mexicana* LPG and regulate the disease outcome depending on the genetic background of the host. *Parasite Immunol* 2008.
- (191) Kneilling M, Hultner L, Pichler BJ, Mailhammer R, Morawietz L, Solomon S et al. Targeted mast cell silencing protects against joint destruction and angiogenesis in experimental arthritis in mice. *Arthritis Rheum* 2007; 56(6):1806-1816.
- (192) Lin L, Gerth AJ, Peng SL. Susceptibility of mast cell-deficient W/W^v mice to pristane-induced experimental lupus nephritis. *Immunol Lett* 2004; 91(2-3):93-97.
- (193) Lu LF, Lind EF, Gondek DC, Bennett KA, Gleeson MW, Pino-Lagos K et al. Mast cells are essential intermediaries in regulatory T-cell tolerance. *Nature* 2006; 442(7106):997-1002.
- (194) Theoharides TC, Conti P. Mast cells: the Jekyll and Hyde of tumor growth. *Trends Immunol* 2004; 25(5):235-241.

- (195) Gauvreau GM, Lee JM, Watson RM, Irani AM, Schwartz LB, O'Byrne PM. Increased numbers of both airway basophils and mast cells in sputum after allergen inhalation challenge of atopic asthmatics. *Am J Respir Crit Care Med* 2000; 161(5):1473-1478.
- (196) Kirshenbaum AS, Goff JP, Semere T, Scott LM, Metcalfe DD. Demonstration that human mast cells arise from a bipotential progenitor cell that is CD34+, c-kit+, and expresses aminopeptidase N (CD13). *Journal of Allergy and Clinical Immunology* 1999; 103(1):S170.
- (197) Bagga S, Price KS, Lin DA, Friend DS, Austen KF, Boyce JA. Lysophosphatidic acid accelerates the development of human mast cells. *Blood* 2004; 104(13):4080-4087.
- (198) Yanagida M, Fukamachi H, Ohgami K, Kuwaki T, Ishii H, Uzunaki H et al. Effects of T-helper 2-type cytokines, interleukin-3 (IL-3), IL-4, IL-5, and IL-6 on the survival of cultured human mast cells. *Blood* 1995; 86(10):3705-3714.
- (199) Hollins F, Kaur D, Yang W, Cruse G, Saunders R, Sutcliffe A et al. Human airway smooth muscle promotes human lung mast cell survival, proliferation, and constitutive activation: cooperative roles for CADM1, stem cell factor, and IL-6. *J Immunol* 2008; 181(4):2772-2780.
- (200) Bischoff SC, Sellge G, Schwengberg S, Lorentz A, Manns MP. Stem cell factor-dependent survival, proliferation and enhanced releasability of purified mature mast cells isolated from human intestinal tissue. *Int Arch Allergy Immunol* 1999; 118(2-4):104-107.
- (201) Sundstrom M, Alfredsson J, Olsson N, Nilsson G. Stem cell factor-induced migration of mast cells requires p38 mitogen-activated protein kinase activity. *Experimental Cell Research* 2001; 267(1):144-151.
- (202) Kassel O, Schmidlin F, Duvernelle C, Gasser B, Massard G, Frossard N. Human bronchial smooth muscle cells in culture produce stem cell factor. *Eur Respir J* 1999; 13(5):951-954.
- (203) Irani AA, Schechter NM, Craig SS, DeBlois G, Schwartz LB. Two types of human mast cells that have distinct neutral protease compositions. *Proc Natl Acad Sci U S A* 1986; 83(12):4464-4468.
- (204) Schwartz LB, Irani AM, Roller K, Castells MC, Schechter NM. Quantitation of histamine, tryptase, and chymase in dispersed human T and TC mast cells. *J Immunol* 1987; 138(8):2611-2615.
- (205) Oskeritzian CA, Zhao W, Min HK, Xia HZ, Pozez A, Kiev J et al. Surface CD88 functionally distinguishes the MCTC from the MCT type of human lung mast cell. *J Allergy Clin Immunol* 2005; 115(6):1162-1168.
- (206) Schechter NM, Irani AM, Sprows JL, Abernethy J, Wintroub B, Schwartz LB. Identification of a cathepsin G-like proteinase in the MCTC type of human mast cell. *J Immunol* 1990; 145(8):2652-2661.
- (207) Irani AM, Goldstein SM, Wintroub BU, Bradford T, Schwartz LB. Human mast cell carboxypeptidase. Selective localization to MCTC cells. *J Immunol* 1991; 147(1):247-253.
- (208) Conti P, Pang X, Boucher W, Letourneau R, Reale M, Barbacane RC et al. Monocyte chemotactic protein-1 is a proinflammatory chemokine in rat skin injection sites and chemoattracts basophilic granular cells. *Int Immunol* 1997; 9(10):1563-1570.
- (209) Sawada J, Itakura A, Tanaka A, Furusaka T, Matsuda H. Nerve growth factor functions as a chemoattractant for mast cells through both mitogen-activated protein kinase and phosphatidylinositol 3-kinase signaling pathways. *Blood* 2000; 95(6):2052-2058.
- (210) Papadopoulos EJ, Fitzhugh DJ, Tkaczyk C, Gilfillan AM, Sasseti C, Metcalfe DD et al. Mast cells migrate, but do not degranulate, in response to fractalkine, a membrane-bound chemokine expressed constitutively in diverse cells of the skin. *Eur J Immunol* 2000; 30(8):2355-2361.

- (211) Inamura H, Kurosawa M, Okano A, Kayaba H, Majima M. Expression of the interleukin-8 receptors CXCR1 and CXCR2 on cord-blood-derived cultured human mast cells. *International Archives of Allergy and Immunology* 2002; 128(2):142-150.
- (212) Romagnani P, De Paulis A, Beltrame C, Annunziato F, Dente V, Maggi E et al. Tryptase-chymase double-positive human mast cells express the eotaxin receptor CCR3 and are attracted by CCR3-binding chemokines. *Am J Pathol* 1999; 155(4):1195-1204.
- (213) Ghaffar O, Hamid Q, Renzi PM, Allakhverdi Z, Molet S, Hogg JC et al. Constitutive and cytokine-stimulated expression of eotaxin by human airway smooth muscle cells. *Am J Respir Crit Care Med* 1999; 159(6):1933-1942.
- (214) Moore PE, Church TL, Chism DD, Panettieri RA, Jr., Shore SA. IL-13 and IL-4 cause eotaxin release in human airway smooth muscle cells: a role for ERK. *Am J Physiol Lung Cell Mol Physiol* 2002; 282(4):L847-L853.
- (215) Brightling CE, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID, Bradding P. Interleukin-4 and -13 expression is co-localized to mast cells within the airway smooth muscle in asthma. *Clin Exp Allergy* 2003; 33(12):1711-1716.
- (216) Ohta Y, Hayashi M, Kanemaru T, Abe K, Ito Y, Oike M. Dual Modulation of Airway Smooth Muscle Contraction by Th2 Cytokines via Matrix Metalloproteinase-1 Production. *J Immunol* 2008; 180(6):4191-4199.
- (217) Cruse G, Kaur D, Yang W, Duffy SM, Brightling CE, Bradding P. Activation of human lung mast cells by monomeric immunoglobulin E. *Eur Respir J* 2005; 25(5):858-863.
- (218) Dvorak AM, Hammel I, Schulman ES, Peters SP, MacGlashan DW, Jr., Schleimer RP et al. Differences in the behavior of cytoplasmic granules and lipid bodies during human lung mast cell degranulation. *J Cell Biol* 1984; 99(5):1678-1687.
- (219) Reischl IG, Coward WR, Church MK. Molecular consequences of human mast cell activation following immunoglobulin E-high-affinity immunoglobulin E receptor (IgE-FcεRI) interaction. *Biochem Pharmacol* 1999; 58(12):1841-1850.
- (220) Kulka M, Alexopoulou L, Flavell RA, Metcalfe DD. Activation of mast cells by double-stranded RNA: evidence for activation through Toll-like receptor 3. *J Allergy Clin Immunol* 2004; 114(1):174-182.
- (221) Fukuishi N, Yoshioka M, Akagi M. [The role of mast cells in allergic inflammation--from the view point of innate immune system involvement]. *Nippon Yakurigaku Zasshi* 2005; 125(5):259-264.
- (222) Hosoda M, Yamaya M, Suzuki T, Yamada N, Kamanaka M, Sekizawa K et al. Effects of rhinovirus infection on histamine and cytokine production by cell lines from human mast cells and basophils. *J Immunol* 2002; 169(3):1482-1491.
- (223) Brightling CE, Bradding P, Pavord ID, Wardlaw AJ. New insights into the role of the mast cell in asthma. *Clin Exp Allergy* 2003; 33(5):550-556.
- (224) Galli SJ, Nakae S, Tsai M. Mast cells in the development of adaptive immune responses. *Nat Immunol* 2005; 6(2):135-142.
- (225) Broide DH, Lotz M, Cuomo AJ, Coburn DA, Federman EC, Wasserman SI. Cytokines in symptomatic asthma airways. *J Allergy Clin Immunol* 1992; 89(5):958-967.
- (226) Casale TB, Wood D, Richerson HB, Zehr B, Zavala D, Hunninghake GW. Direct evidence of a role for mast cells in the pathogenesis of antigen-induced bronchoconstriction. *J Clin Invest* 1987; 80(5):1507-1511.
- (227) Pavord ID, Ward R, Woltmann G, Wardlaw AJ, Sheller JR, Dworski R. Induced sputum eicosanoid concentrations in asthma. *Am J Respir Crit Care Med* 1999; 160(6):1905-1909.

- (228) Chanez P, Vignola M, Stenger R, Vic P, Michel FB, Bousquet J. Platelet-derived growth factor in asthma. *Allergy* 1995; 50(11):878-883.
- (229) Amrani Y, Krymskaya V, Maki C, Panettieri RA, Jr. Mechanisms underlying TNF-alpha effects on agonist-mediated calcium homeostasis in human airway smooth muscle cells. *Am J Physiol* 1997; 273(5 Pt 1):L1020-L1028.
- (230) Yamakage M, Matsuzaki T, Tsujiguchi N, Mori T, Namiki A. The direct effects of heparin and protamine on canine tracheal smooth muscle tone. *Anesth Analg* 1999; 88(6):1232-1238.
- (231) Carbajal V, Vargas MH, Flores-Soto E, Martinez-Cordero E, Bazan-Perkins B, Montano LM. LTD4 induces hyperresponsiveness to histamine in bovine airway smooth muscle: role of SR-ATPase Ca²⁺ pump and tyrosine kinase. *Am J Physiol Lung Cell Mol Physiol* 2005; 288(1):L84-L92.
- (232) Saegusa S, Tsubone H, Kuwahara M. Leukotriene D(4)-induced Rho-mediated actin reorganization in human bronchial smooth muscle cells. *Eur J Pharmacol* 2001; 413(2-3):163-171.
- (233) Schaafsma D, Gosens R, Bos IS, Meurs H, Zaagsma J, Nelemans SA. Role of contractile prostaglandins and Rho-kinase in growth factor-induced airway smooth muscle contraction. *Respir Res* 2005; 6:85.
- (234) Schmidlin F, Amadesi S, Vidil R, Trevisani M, Martinet N, Caughey G et al. Expression and function of proteinase-activated receptor 2 in human bronchial smooth muscle. *Am J Respir Crit Care Med* 2001; 164(7):1276-1281.
- (235) Tamaoki J, Yamawaki I, Takeyama K, Chiyotani A, Yamauchi F, Konno K. Interleukin-1 beta inhibits airway smooth muscle contraction via epithelium-dependent mechanism. *Am J Respir Crit Care Med* 1994; 149(1):134-137.
- (236) Abraham WM. Tryptase: potential role in airway inflammation and remodeling. *Am J Physiol Lung Cell Mol Physiol* 2002; 282(2):L193-L196.
- (237) Payne V, Kam PC. Mast cell tryptase: a review of its physiology and clinical significance. *Anaesthesia* 2004; 59(7):695-703.
- (238) Fukuoka Y, Schwartz LB. Human beta-tryptase: detection and characterization of the active monomer and prevention of tetramer reconstitution by protease inhibitors. *Biochemistry* 2004; 43(33):10757-10764.
- (239) Caughey GH. Of mites and men: trypsin-like proteases in the lungs. *Am J Respir Cell Mol Biol* 1997; 16(6):621-628.
- (240) Compton SJ, Cairns JA, Holgate ST, Walls AF. Human mast cell tryptase stimulates the release of an IL-8-dependent neutrophil chemotactic activity from human umbilical vein endothelial cells (HUVEC). *Clin Exp Immunol* 2000; 121(1):31-36.
- (241) Schwartz LB, Lewis RA, Seldin D, Austen KF. Acid hydrolases and tryptase from secretory granules of dispersed human lung mast cells. *J Immunol* 1981; 126(4):1290-1294.
- (242) Patella V, Marino I, Lamparter B, Arbustini E, Adt M, Marone G. Human heart mast cells. Isolation, purification, ultrastructure, and immunologic characterization. *J Immunol* 1995; 154(6):2855-2865.
- (243) Temkin V, Kantor B, Weg V, Hartman ML, Levi-Schaffer F. Tryptase activates the mitogen-activated protein kinase/activator protein-1 pathway in human peripheral blood eosinophils, causing cytokine production and release. *J Immunol* 2002; 169(5):2662-2669.
- (244) Compton SJ, Cairns JA, Holgate ST, Walls AF. The role of mast cell tryptase in regulating endothelial cell proliferation, cytokine release, and adhesion molecule expression: tryptase induces expression of mRNA for IL-1 beta and IL-8 and stimulates the selective release of IL-8 from human umbilical vein endothelial cells. *J Immunol* 1998; 161(4):1939-1946.

- (245) Laura R, Robison DJ, Bing DH. (p-Amidinophenyl)methanesulfonyl fluoride, an irreversible inhibitor of serine proteases. *Biochemistry* 1980; 19(21):4859-4864.
- (246) Dery O, Corvera CU, Steinhoff M, Bunnett NW. Proteinase-activated receptors: novel mechanisms of signaling by serine proteases. *Am J Physiol* 1998; 274(6 Pt 1):C1429-C1452.
- (247) Cairns JA. Inhibitors of mast cell tryptase beta as therapeutics for the treatment of asthma and inflammatory disorders. *Pulm Pharmacol Ther* 2005; 18(1):55-66.
- (248) Camerer E, Huang W, Coughlin SR. Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. *Proc Natl Acad Sci U S A* 2000; 97(10):5255-5260.
- (249) Knight DA, Lim S, Scaffidi AK, Roche N, Chung KF, Stewart GA et al. Protease-activated receptors in human airways: upregulation of PAR-2 in respiratory epithelium from patients with asthma. *J Allergy Clin Immunol* 2001; 108(5):797-803.
- (250) Compton SJ, Sandhu S, Wijesuriya SJ, Hollenberg MD. Glycosylation of human proteinase-activated receptor-2 (hPAR2): role in cell surface expression and signalling. *Biochem J* 2002; 368(Pt 2):495-505.
- (251) Alm AK, Gagnemo-Persson R, Sorsa T, Sundelin J. Extraprostatic trypsin-2 cleaves proteinase-activated receptor-2. *Biochem Biophys Res Commun* 2000; 275(1):77-83.
- (252) Compton SJ, Renaux B, Wijesuriya SJ, Hollenberg MD. Glycosylation and the activation of proteinase-activated receptor 2 (PAR(2)) by human mast cell tryptase. *Br J Pharmacol* 2001; 134(4):705-718.
- (253) Dulon S, Cande C, Bunnett NW, Hollenberg MD, Chignard M, Pidard D. Proteinase-activated receptor-2 and human lung epithelial cells: disarming by neutrophil serine proteinases. *Am J Respir Cell Mol Biol* 2003; 28(3):339-346.
- (254) Uehara A, Muramoto K, Takada H, Sugawara S. Neutrophil serine proteinases activate human nonepithelial cells to produce inflammatory cytokines through protease-activated receptor 2. *J Immunol* 2003; 170(11):5690-5696.
- (255) Hogg PJ, Owensby DA, Chesterman CN. Thrombospondin 1 is a tight-binding competitive inhibitor of neutrophil cathepsin G. Determination of the kinetic mechanism of inhibition and localization of cathepsin G binding to the thrombospondin 1 type 3 repeats. *J Biol Chem* 1993; 268(29):21811-21818.
- (256) Brown JK, Jones CA, Rooney LA, Coughley GH, Hall IP. Tryptase's potent mitogenic effects in human airway smooth muscle cells are via nonproteolytic actions. *Am J Physiol Lung Cell Mol Physiol* 2002; 282(2):L197-L206.
- (257) Pereira PJ, Bergner A, Macedo-Ribeiro S, Huber R, Matschiner G, Fritz H et al. Human beta-tryptase is a ring-like tetramer with active sites facing a central pore. *Nature* 1998; 392(6673):306-311.
- (258) Lew DB, Songu-Mize E, Pontow SE, Stahl PD, Rattazzi MC. A mannose receptor mediates mannosyl-rich glycoprotein-induced mitogenesis in bovine airway smooth muscle cells. *J Clin Invest* 1994; 94(5):1855-1863.
- (259) Holgate ST, Yang Y, Haitchi HM, Powell RM, Holloway JW, Yoshisue H et al. The genetics of asthma: ADAM33 as an example of a susceptibility gene. *Proc Am Thorac Soc* 2006; 3(5):440-443.
- (260) Strachan DP. Family size, infection and atopy: the first decade of the "hygiene hypothesis". *Thorax* 2000; 55 Suppl 1:S2-10.
- (261) Vercelli D. Advances in asthma and allergy genetics in 2007. *J Allergy Clin Immunol* 2008; 122(2):267-271.

- (262) Moffatt MF. Genes in asthma: new genes and new ways. *Curr Opin Allergy Clin Immunol* 2008; 8(5):411-417.
- (263) Grimm PC, Nickerson P, Jeffery J, Savani RC, Gough J, McKenna RM et al. Neointimal and tubulointerstitial infiltration by recipient mesenchymal cells in chronic renal-allograft rejection. *N Engl J Med* 2001; 345(2):93-97.
- (264) Abe R, Donnelly SC, Peng T, Bucala R, Metz CN. Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. *J Immunol* 2001; 166(12):7556-7562.
- (265) British Thoracic Society guidelines on diagnostic flexible bronchoscopy. *Thorax* 2001; 56 Suppl 1:i1-21.
- (266) Juniper EF, Cockcroft DW, Hargreave FE. Histamine and Methacholine Inhalation Tests: A Laboratory Tidal Breathing Protocol. Second ed. Lund, Sweden: Astra Draco AB, 1994.
- (267) Pavord ID, Pizzichini MM, Pizzichini E, Hargreave FE. The use of induced sputum to investigate airway inflammation. *Thorax* 1997; 52(6):498-501.
- (268) Duffy SM, Leyland ML, Conley EC, Bradding P. Voltage-dependent and calcium-activated ion channels in the human mast cell line HMC-1. *J Leukoc Biol* 2001; 70(2):233-240.
- (269) Bradding P, Okayama Y, Kambe N, Saito H. Ion channel gene expression in human lung, skin, and cord blood-derived mast cells. *J Leukoc Biol* 2003; 73(5):614-620.
- (270) Brightling CE, Kaur D, Berger P, Morgan AJ, Wardlaw AJ, Bradding P. Differential expression of CCR3 and CXCR3 by human lung and bone marrow-derived mast cells: implications for tissue mast cell migration. *J Leukoc Biol* 2005; 77(5):759-766.
- (271) Olsson N, Rak S, Nilsson G. Demonstration of mast cell chemotactic activity in bronchoalveolar lavage fluid collected from asthmatic patients before and during pollen season. *J Allergy Clin Immunol* 2000; 105(3):455-461.
- (272) Komiya A, Nagase H, Yamada H, Sekiya T, Yamaguchi M, Sano Y et al. Concerted expression of eotaxin-1, eotaxin-2, and eotaxin-3 in human bronchial epithelial cells. *Cell Immunol* 2003; 225(2):91-100.
- (273) Marini M, Vittori E, Hollemborg J, Mattoli S. Expression of the potent inflammatory cytokines, granulocyte-macrophage-colony-stimulating factor and interleukin-6 and interleukin-8, in bronchial epithelial cells of patients with asthma. *J Allergy Clin Immunol* 1992; 89(5):1001-1009.
- (274) Sauty A, Dziejman M, Taha RA, Iarossi AS, Neote K, Garcia-Zepeda EA et al. The T cell-specific CXC chemokines IP-10, Mig, and I-TAC are expressed by activated human bronchial epithelial cells. *J Immunol* 1999; 162(6):3549-3558.
- (275) Lamkhieoued B, Renzi PM, Abi-Younes S, Garcia-Zepeda EA, Allakhverdi Z, Ghaffar O et al. Increased expression of eotaxin in bronchoalveolar lavage and airways of asthmatics contributes to the chemotaxis of eosinophils to the site of inflammation. *J Immunol* 1997; 159(9):4593-4601.
- (276) Lilly CM, Nakamura H, Belostotsky OI, Haley KJ, Garcia-Zepeda EA, Luster AD et al. Eotaxin expression after segmental allergen challenge in subjects with atopic asthma. *Am J Respir Crit Care Med* 2001; 163(7):1669-1675.
- (277) Dent G, Hadjicharalambous C, Yoshikawa T, Handy RL, Powell J, Anderson IK et al. Contribution of eotaxin-1 to eosinophil chemotactic activity of moderate and severe asthmatic sputum. *Am J Respir Crit Care Med* 2004; 169(10):1110-1117.
- (278) Birring SS, Parker D, Brightling CE, Bradding P, Wardlaw AJ, Pavord ID. Induced sputum inflammatory mediator concentrations in chronic cough. *Am J Respir Crit Care Med* 2004; 169(1):15-19.
- (279) Gibson PG. Cough is an airway itch? *Am J Respir Crit Care Med* 2004; 169(1):1-2.

- (280) Salvi SS, Babu KS, Holgate ST. Is asthma really due to a polarized T cell response toward a helper T cell type 2 phenotype? *Am J Respir Crit Care Med* 2001; 164(8 Pt 1):1343-1346.
- (281) Bradding P, Roberts JA, Britten KM, Montefort S, Djukanovic R, Mueller R et al. Interleukin-4, -5, and -6 and tumor necrosis factor-alpha in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines. *Am J Respir Cell Mol Biol* 1994; 10(5):471-480.
- (282) Dahl ME, Dabbagh K, Liggitt D, Kim S, Lewis DB. Viral-induced T helper type 1 responses enhance allergic disease by effects on lung dendritic cells. *Nat Immunol* 2004; 5(3):337-343.
- (283) Miotto D, Christodoulouopoulos P, Olivenstein R, Taha R, Cameron L, Tsicopoulos A et al. Expression of IFN-gamma-inducible protein; monocyte chemotactic proteins 1, 3, and 4; and eotaxin in TH1- and TH2-mediated lung diseases. *J Allergy Clin Immunol* 2001; 107(4):664-670.
- (284) Bochner BS, Hudson SA, Xiao HQ, Liu MC. Release of both CCR4-active and CXCR3-active chemokines during human allergic pulmonary late-phase reactions. *J Allergy Clin Immunol* 2003; 112(5):930-934.
- (285) Liu L, Jarjour NN, Busse WW, Kelly EA. Enhanced generation of helper T type 1 and 2 chemokines in allergen-induced asthma. *Am J Respir Crit Care Med* 2004; 169(10):1118-1124.
- (286) Pilette C, Francis JN, Till SJ, Durham SR. CCR4 ligands are up-regulated in the airways of atopic asthmatics after segmental allergen challenge. *Eur Respir J* 2004; 23(6):876-884.
- (287) Wardlaw AJ, Brightling CE, Green R, Woltmann G, Bradding P, Pavord ID. New insights into the relationship between airway inflammation and asthma. *Clin Sci (Lond)* 2002; 103(2):201-211.
- (288) El Shazly A, Berger P, Girodet PO, Ousova O, Fayon M, Vernejoux JM et al. Fractalkine produced by airway smooth muscle cells contributes to mast cell recruitment in asthma. *J Immunol* 2006; 176(3):1860-1868.
- (289) Trian T, Benard G, Begueret H, Rossignol R, Girodet PO, Ghosh D et al. Bronchial smooth muscle remodeling involves calcium-dependent enhanced mitochondrial biogenesis in asthma. *J Exp Med* 2007; 204(13):3173-3181.
- (290) Hirst SJ, Hallsworth MP, Peng Q, Lee TH. Selective induction of eotaxin release by interleukin-13 or interleukin-4 in human airway smooth muscle cells is synergistic with interleukin-1beta and is mediated by the interleukin-4 receptor alpha-chain. *Am J Respir Crit Care Med* 2002; 165(8):1161-1171.
- (291) Morgan AJ, Symon FA, Berry MA, Pavord ID, Corrigan CJ, Wardlaw AJ. IL-4-expressing bronchoalveolar T cells from asthmatic and healthy subjects preferentially express CCR 3 and CCR 4. *J Allergy Clin Immunol* 2005; 116(3):594-600.
- (292) Uguccioni M, Mackay CR, Ochensberger B, Loetscher P, Rhee S, LaRosa GJ et al. High expression of the chemokine receptor CCR3 in human blood basophils. Role in activation by eotaxin, MCP-4, and other chemokines. *J Clin Invest* 1997; 100(5):1137-1143.
- (293) Ying S, Meng Q, Zeibecoglou K, Robinson DS, Macfarlane A, Humbert M et al. Eosinophil chemotactic chemokines (eotaxin, eotaxin-2, RANTES, monocyte chemoattractant protein-3 (MCP-3), and MCP-4), and C-C chemokine receptor 3 expression in bronchial biopsies from atopic and nonatopic (Intrinsic) asthmatics. *J Immunol* 1999; 163(11):6321-6329.
- (294) De Lucca GV. Recent developments in CCR3 antagonists. *Curr Opin Drug Discov Devel* 2006; 9(4):516-524.
- (295) Stellato C, Brummet ME, Plitt JR, Shahabuddin S, Baroody FM, Liu MC et al. Expression of the C-C chemokine receptor CCR3 in human airway epithelial cells. *J Immunol* 2001; 166(3):1457-1461.

- (296) Haley KJ, Lilly CM, Yang JH, Feng Y, Kennedy SP, Turi TG et al. Overexpression of eotaxin and the CCR3 receptor in human atherosclerosis: using genomic technology to identify a potential novel pathway of vascular inflammation. *Circulation* 2000; 102(18):2185-2189.
- (297) Roque M, Kim WJ, Gazdoin M, Malik A, Reis ED, Fallon JT et al. CCR2 deficiency decreases intimal hyperplasia after arterial injury. *Arterioscler Thromb Vasc Biol* 2002; 22(4):554-559.
- (298) Chandrasekar B, Bysani S, Mummidi S. CXCL16 signals via Gi, phosphatidylinositol 3-kinase, Akt, I kappa B kinase, and nuclear factor-kappa B and induces cell-cell adhesion and aortic smooth muscle cell proliferation. *J Biol Chem* 2004; 279(5):3188-3196.
- (299) Chandrasekar B, Mummidi S, Perla RP, Bysani S, Dulin NO, Liu F et al. Fractalkine (CX3CL1) stimulated by nuclear factor kappaB (NF-kappaB)-dependent inflammatory signals induces aortic smooth muscle cell proliferation through an autocrine pathway. *Biochem J* 2003; 373(Pt 2):547-558.
- (300) Kodali RB, Kim WJ, Galaria II, Miller C, Schecter AD, Lira SA et al. CCL11 (Eotaxin) induces CCR3-dependent smooth muscle cell migration. *Arterioscler Thromb Vasc Biol* 2004; 24(7):1211-1216.
- (301) Chan V, Burgess JK, Ratoff JC, O'Connor BJ, Greenough A, Lee TH et al. Extracellular matrix regulates enhanced eotaxin expression in asthmatic airway smooth muscle cells. *Am J Respir Crit Care Med* 2006; 174(4):379-385.
- (302) Wardlaw AJ, Brightling C, Green R, Woltmann G, Pavord I. Eosinophils in asthma and other allergic diseases. *Br Med Bull* 2000; 56(4):985-1003.
- (303) Kay AB. Pathology of mild, severe, and fatal asthma. *Am J Respir Crit Care Med* 1996; 154(2 Pt 2):S66-S69.
- (304) Bradding P, Holgate ST. The mast cell as a source of cytokines in asthma. *Ann N Y Acad Sci* 1996; 796:272-281.
- (305) Redington AE, Madden J, Frew AJ, Djukanovic R, Roche WR, Holgate ST et al. Transforming growth factor-beta 1 in asthma. Measurement in bronchoalveolar lavage fluid. *Am J Respir Crit Care Med* 1997; 156(2 Pt 1):642-647.
- (306) Silveira PS, Fredberg JJ. Smooth muscle length adaptation and actin filament length: a network model of the cytoskeletal dysregulation. *Can J Physiol Pharmacol* 2005; 83(10):923-931.
- (307) Meyer-ter-Vehn T, Gebhardt S, Sebald W, Buttmann M, Grehn F, Schlunck G et al. p38 inhibitors prevent TGF-beta-induced myofibroblast transdifferentiation in human tenon fibroblasts. *Invest Ophthalmol Vis Sci* 2006; 47(4):1500-1509.
- (308) He SH, Chen P, Chen HQ. Modulation of enzymatic activity of human mast cell tryptase and chymase by protease inhibitors. *Acta Pharmacol Sin* 2003; 24(9):923-929.
- (309) Crivellato E, Finato N, Isola M, Pandolfi M, Ribatti D, Beltrami CA. Number of pericryptal fibroblasts correlates with density of distinct mast cell phenotypes in the crypt lamina propria of human duodenum: implications for the homeostasis of villous architecture. *Anat Rec A Discov Mol Cell Evol Biol* 2006; 288(6):593-600.
- (310) Gailit J, Marchese MJ, Kew RR, Gruber BL. The differentiation and function of myofibroblasts is regulated by mast cell mediators. *J Invest Dermatol* 2001; 117(5):1113-1119.
- (311) Chen FH, Samson KT, Miura K, Ueno K, Odajima Y, Shougo T et al. Airway remodeling: a comparison between fatal and nonfatal asthma. *J Asthma* 2004; 41(6):631-638.
- (312) Costanzo MJ, Yabut SC, Almond HR, Jr., Andrade-Gordon P, Corcoran TW, De Garavilla L et al. Potent, small-molecule inhibitors of human mast cell tryptase. Antiasthmatic action of a dipeptide-based transition-state analogue containing a benzothiazole ketone. *J Med Chem* 2003; 46(18):3865-3876.

- (313) Krishna MT, Chauhan A, Little L, Sampson K, Hawksworth R, Mant T et al. Inhibition of mast cell tryptase by inhaled APC 366 attenuates allergen-induced late-phase airway obstruction in asthma. *J Allergy Clin Immunol* 2001; 107(6):1039-1045.
- (314) Boulet LP, Chapman KR, Cote J, Kalra S, Bhagat R, Swystun VA et al. Inhibitory effects of an anti-IgE antibody E25 on allergen-induced early asthmatic response. *Am J Respir Crit Care Med* 1997; 155(6):1835-1840.
- (315) Djukanovic R, Wilson SJ, Kraft M, Jarjour NN, Steel M, Chung KF et al. Effects of treatment with anti-immunoglobulin E antibody omalizumab on airway inflammation in allergic asthma. *Am J Respir Crit Care Med* 2004; 170(6):583-593.
- (316) Fahy JV, Fleming HE, Wong HH, Liu JT, Su JQ, Reimann J et al. The effect of an anti-IgE monoclonal antibody on the early- and late-phase responses to allergen inhalation in asthmatic subjects. *Am J Respir Crit Care Med* 1997; 155(6):1828-1834.
- (317) Noga O, Hanf G, Kunkel G. Immunological and clinical changes in allergic asthmatics following treatment with omalizumab. *Int Arch Allergy Immunol* 2003; 131(1):46-52.
- (318) Wang J, Zohar R, McCulloch CA. Multiple roles of alpha-smooth muscle actin in mechanotransduction. *Exp Cell Res* 2006; 312(3):205-214.
- (319) Sugiura H, Liu X, Duan F, Kawasaki S, Togo S, Kamio K et al. Cultured lung fibroblasts from ovalbumin-challenged "asthmatic" mice differ functionally from normal. *Am J Respir Cell Mol Biol* 2007; 37(4):424-430.
- (320) Schildmeyer LA, Braun R, Taffet G, Debiase M, Burns AE, Bradley A et al. Impaired vascular contractility and blood pressure homeostasis in the smooth muscle alpha-actin null mouse. *FASEB J* 2000; 14(14):2213-2220.
- (321) Zimmerman RA, Tomasek JJ, McRae J, Haaksma CJ, Schwartz RJ, Lin HK et al. Decreased expression of smooth muscle alpha-actin results in decreased contractile function of the mouse bladder. *J Urol* 2004; 172(4 Pt 2):1667-1672.
- (322) Kinner B, Zaleskas JM, Spector M. Regulation of smooth muscle actin expression and contraction in adult human mesenchymal stem cells. *Exp Cell Res* 2002; 278(1):72-83.
- (323) Johnson PR, Ammit AJ, Carlin SM, Armour CL, Caughey GH, Black JL. Mast cell tryptase potentiates histamine-induced contraction in human sensitized bronchus. *Eur Respir J* 1997; 10(1):38-43.
- (324) Krishna MT, Chauhan A, Little L, Sampson K, Hawksworth R, Mant T et al. Inhibition of mast cell tryptase by inhaled APC 366 attenuates allergen-induced late-phase airway obstruction in asthma. *J Allergy Clin Immunol* 2001; 107(6):1039-1045.