The p38 MAPK pathway in human airway smooth muscle: roles in asthma.

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Abstract

The mitogen activated protein kinase (MAPK) signaling pathways play key roles in mediating inflammatory responses. Asthma is a disease characterized by excessive inflammation and the mainstay of treatment is the use of glucocorticoids which, among other effects, control the activity of p38 MAPK. Human airway smooth muscle (HASM) cells contribute to the pathology of asthma as they regulate bronchomotor tone, proliferate, migrate and secrete inflammatory cytokines. I investigated these processes in HASM using MAPK inhibitors and the glucocorticoid dexamethasone. Following TNFα stimulation, HASM cell production of GM-CSF, IL-1β, IL-33 and CXCL8 were ERK1/ERK2 dependent; all but CXCL8 were diminished by dexamethasone. Neutrophil migration in response to conditioned media from HASM cells was also ERK1/ERK2 dependent. CXCL12 displayed chemotactic activity for HASM which was shown to express the CXCR4 receptor. HASM migration was partially p38 MAPK dependent. These results highlight the potential for important and divergent roles for the MAPKs in HASM in asthma.

Abrégé

L’asthme est une maladie inflammatoire dont les glucocorticoïdes constituent le principal traitement via le contrôle de la voie p38 MAPK. Les cellules musculaires lisses bronchiques (CLM) jouent un rôle clé dans la physiopathologie de l'asthme notamment dans le remodelage des voies aériennes via leur capacité à proliférer, migrer et sécréter des médiateurs inflammatoires. La stimulation des CLM avec du TNFα entraine une activation des voies MAPK ERK et p38, induisant l’expression des gènes GM-CSF, IL-1β, IL-33 et CXCL8. L’activation de la voie MAPK ERK est importante dans la migration des neutrophiles exposée à du milieu conditionné provenant de CLM stimulées par TNFα via son rôle sur l’expression de CXCL8. En contrepartie, la voie p38 MAPK joue un rôle important dans la migration des CLM en réponse à CXCL12, un chimiokine élevée dans les bronches de patients asthmatiques. Ces résultats ont mis en évidence un rôle important et divergeant des MAPKs dans les CLM dans la pathophysiologie de l’asthme.

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Abbreviations

α-SMA	Alpha smooth muscle actin
AHR	Airway hyperresponsiveness
ATF	Activating transcription factor
BAL	Broncho-alveolar lavage
bFGF	Basic fibroblast growth factor
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
CREB	Cyclic AMP response element-binding
CS	Corticosteroid
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine receptor
DKO	Double knock out
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DUSP1	Dual specificity protein phosphatase 1
ERK 1	Extra cellular signal regulated kinase 1
FFIR	Force fluctuation induced relengthening
G	Acceleration due to gravity
GC	Glucocorticoid
GR	Glucocorticoid receptor
GRE	Glucocorticoid receptor element
HASM	Human airway smooth muscle
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HSP27	Heat shock protein 27
IL-1,2..	Interleukin-1 through 33
IL-8	Interleukin -8 also CXCL8
I□□□	Inhibitor of nuclear factor kappa B alpha
□	Micro (10^{-6})

M	Milli (10^{-3})
M	Molar
MAPK	Mitogen activated protein kinase
MAPKAP-K2	MAPK activated protein kinase 2
MKP1	MAPK phosphatase 1
MMP	matrix metalloproteinase
MSK1	Mitogen and stress activated kinase 1
MSK2	Mitogen and stress activated kinase 2
mRNA	Messenger ribonucleic acid
N	Nano (10^{-9})
NFκB	Nuclear factor kabba B
PDGF-BB	Platelet derived growth factor (homodimer BB)
qRT-PCR	Quantitative real time polymerase chain reaction
RNA	Ribo nucleic acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SM22	Transgelin
TIMP	Tissue inhibitor of metalloproteinases
Th2	T-helper cell type 2
TGFß	Transforming growth factor beta
TNFα	Tumour necrosis factor alpha
TNFR1	Tumour necrosis factor alpha type 1 receptor
VCAM	Vascular cell adhesion molecule

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Chapter 1: Introduction

1.1 Asthma: prevalence and defining features

The definition of asthma is controversial due to its heterogeneous presentation of symptoms. Classically, asthma is said to bring about the following defining features of the airways: inflammation, hyperresponsiveness (AHR) and remodelling which lead to obstruction (Hargreave and Nair 2009). These physiologic changes manifest to a greater or lesser extent depending on the severity of the disease, and are temporally expressed as an 'attack' (wheezing, coughing, dyspnoea) in response to various stimuli. Diagnosis is made based on clinical symptoms as well as pulmonary function tests that indicate a significant reduction of flow expiratory volume over 1 second (FEV_1). Rescue from an asthma attack usually necessitates the use of inhaled Beta 2 agonists to relax smooth muscle followed by anti-inflammatory medications as well as removal of the stimuli if one is present. In severe cases, hospitalization is required to restore normal breathing; in fact, such acute cases are most commonly seen in children of all ages (Braman 2006).

Asthma is a chronic inflammatory disease of the airways that affects approximately 300 million people worldwide. In North America 10% of the population suffers from asthma, and of these, between 5 and 10% are classified as severe asthmatics, indicating a disease state of heightened morbidity that is refractory to normal pharmacologic treatment options (Soler, Imhof et al. 1990).

Intensive study continues into the aetiology of asthma, revealing clear contributions from genetic factors as well as environmental and lifestyle triggers that contribute to this dysregulated immune response (Holgate 1997). In parallel, ongoing investigations have already shed light into how the disease progresses or retreats depending on processes that occur at the genetic, cellular and tissue level. This research has contributed greatly to the understanding of how exacerbations occur as well as how to manage recovery from attacks.

1.1.1 Airway Inflammation

Inflammation of the airways is the main underlying pathological feature of asthma, which in turn leads to both remodelling of the airways as well as airway hyperresponsiveness. This inflammation can be of an acute or chronic nature.

In acute inflammatory asthmatic reactions, the body is responding to exposure to an allergen, virus, pollutant, cold or psychological stress. Approximately 80% of these are allergic reactions, in which an early phase response is characterized by the activation of immunoglobulin E (IgE) sensitized mast cells and macrophages. This brings about the release of reactive oxygen species, vasodilators, histamines and eicosanoids that cause important increases in blood flow and permeability of the microvasculature, resulting in fluid accumulation in the airways (Persson, Erjefalt et al. 1991). With an increase in mucus and plasma secretion the airway smooth muscle contracts, leading to airway obstruction.

Cell recruitment dominates the processes underlying the late phase reaction, which begins approximately 6 to 9 hours later. Macrophages secrete interleukin-1 (IL-1), tumour necrosis factor alpha (TNFα) and interferon gamma (IFN-γ) that are responsible for selective adhesion molecules such as intra-cellular adhesion molecule 1 (ICAM-1) being up-regulated, increasing adhesion between leukocytes and endothelial cells that line the micro-vasculature (Holgate 1993). Macrophages, epithelial cells, as well as smooth muscle cells secrete the chemokines chemokine ligand 8 (CXCL8) and eotaxin which bring about neutrophil and then eosinophil migration into the epithelium and submucosa (Lampinen, Carlson et al. 2004; John, Zhu et al. 2009). The activation of T-cells results in T-helper cell Type 2 (Th2) cytokine release of IL-4, IL-5, IL-13 and granulocyte macrophage colony stimulating factor (GM-CSF) which act on inflammatory cells (Kay, Ying et al. 1991; Durham, Ying et al. 1992; Till, Durham et al. 1997). IL-4 and IL-13 induce vascular cell adhesion molecule 1 (VCAM-1) expression, which is integral to the adherence of basophils and eosinophils to the endothelium (Bochner and Schleimer 1994; Bochner, Klunk et al. 1995). Eosinophils become activated upon binding to adhesion molecules, causing release of granule proteins and superoxides (Munoz, Hamann et al. 1999). Ample evidence implicates IL-5 in the priming of eosinophils that affect adhesion, diapedesis, migration and the release of inflammatory mediators (Rothenberg, Petersen et al. 1989; Carlson, Hakansson et al. 1991; Moser, Fehr et al. 1992). GM-CSF also primes eosinophils and neutrophils and has been identified as the most important cytokine in enhancing eosinophil survival (Park, Choi et al. 1998).

Chronic inflammation has a much more complex pathology, where all cells of the airway are involved and become activated. Epithelial cells, the usual first line of defense, are shed as a result of reduced viability, possibly due to increased levels of TNFα, an inflammatory cytokine found to be elevated in asthmatics (Kips and Pauwels 1996; Mueller, Chanez et al. 1996). Goblet cell numbers are increased, resulting in greater mucus secretion. Importantly, eosinophils continue to release inflammatory and cytotoxic mediators and cytokines that result in processes such as vascular leakage and smooth muscle contraction (Laitinen and Laitinen 1994). Neutrophils are also found in greater number in the airways in the late phase reaction. These cells are seen in many different patients including those suffering from nocturnal and long-standing asthma (Martin, Cicutto et al. 1991). Their contribution to the inflammatory process includes the release of MMP-9, a matrix metalloproteinase implicated in ECM breakdown as well as smooth muscle hyperplasia and it correlates with asthmatic disease severity (Macdowell and Peters 2007). Myofibroblasts contribute to inflammation via the release of extra cellular matrix proteins (ECM), providing the important matrix in which airway smooth muscle attaches and proliferates. While these events are reversible, asthmatics show permanent changes of the airways that reflect chronic inflammation, a process referred to as remodelling.

1.1.2 Airway Hyperresponsiveness (AHR)

Airway hyperresponsiveness is a characteristic feature of asthma, and can be defined as the exaggerated response of the airways to constrictor agonists. Typically this includes a steeper dose response curve and higher maximum constriction. The level of AHR (bronchoconstriction occurring in response to a measured dose of inhaled agonist) is directly correlated with disease severity in asthmatics. Asthmatics lose 20% of their FEV_1 with a dose of less than 8 mg/ml agonist, while non-asthmatics need more than 16 mg/ml of constrictor agonist to have the same loss of lung function (O'Byrne and Inman 2003).

The mechanisms that drive airway hyperresponsiveness in asthmatics are not fully understood and continue to be debated. Inflammatory processes are thought to contribute to the initial onset. For example, epithelial shedding may allow more bronchoconstrictors to reach the muscle layer, resulting in contraction of airway smooth muscle (Jeffery, Wardlaw et al. 1989). Alternatively, Brightling et al. demonstrated that mast cell infiltrates of airway smooth muscle are increased in asthmatics. They propose these resident mast cells contribute to AHR via their production of histamine, tryptase or cysteinyl leukotrienes: potent spasmogens of smooth muscle

(Brightling, Bradding et al. 2002). The mechanics of the airway smooth muscle alone has been the focus of investigation in two important areas: velocity of shortening and force fluctuation induced re-lengthening (FFIR). Asthmatics experience greater airway muscle shortening compared to non asthmatics, and the normal protective aspect of re-lengthening in response to deep inspiration (FFIR) is impaired (Fish, Ankin et al. 1981; Mitchell, Ruhlmann et al. 1994; Seow, Schellenberg et al. 1998). The molecular mechanisms behind these differences are in part due to the p38 mitogen-activated protein kinase (p38 MAPK) pathway discussed in greater detail below. However, AHR can also occur following exposure to cold, psychological stress and exercise, which cannot be explained by an allergic model.

1.1.3 Airway remodelling

It was previously believed that asthma was a completely reversible disease. However, this did not explain the subset of asthmatics that experienced only partial reversibility in response to inhaled beta2-agonists and prolonged corticosteroid therapy. In cases of fatal asthma, pathologic investigations revealed structural alterations that have since led to a new paradigm that includes a permanent component of the disease, referred to as airway remodelling (Holgate).

Remodelling is a collective term used to describe the structural changes that occur in mild to severe asthmatic airways compared to non-asthmatic airways. These include an overall thickening of the airway wall, subepithelial fibrosis, mucus metaplasia, myofibroblast hyperplasia, airway smooth muscle hypertrophy and hyperplasia, and epithelial hypertrophy. Thickened air walls contain inflammatory infiltrates, increased muscle mass and connective tissue deposition. On pathologic findings, fatal asthma shows a 50 to 300% increase of thickness, while milder asthmatics show increases from 10 to 100% (Elias, Zhu et al. 1999). This increase is thought to cause significant narrowing during bronchospasm (James and Wenzel 2007). Subepithelial fibrosis, a thickening of the *lamina reticularis*, is characterized by deposition of fibronectin as well as type I, III and V collagen and has been positively correlated with disease severity (Cohn, Elias et al. 2004). A striking change in airway morphology is the increase of smooth muscle mass. Debate continues as to whether this increase is due to myocyte hyperplasia and/or hypertrophy, or whether muscle cells migrate towards inflammatory mediators (Woodruff and Fahy 2002; Woodruff, Dolganov et al. 2004). This increased mass contributes to AHR and bronchospasm. As well, increased numbers of goblet cells in an already amplified epithelial layer cause increased mucus production and bronchial mucus plugs (Aikawa, Shimura et al. 1992).

The accumulation of liquid on the luminal face contributes a greater surface tension against which smooth muscle must contract.

The stimuli that bring about remodelling are not completely understood. In the 1980s, the advance in techniques of flexible bronchoscopy revealed that asymptomatic asthmatics had inflammation of the airways, leading to the hypothesis that chronic inflammation could contribute to remodelling (Jarjour, Peters et al. 1998). Biomarkers of this inflammation considered to be important in remodelling are numerous. For example, many cytokines are found in elevated quantities in the bronchoalveolar lavage (BAL) or tissue of asthmatics and include: transforming growth factor beta (TGF-β), whose expression positively correlates with basement membrane thickness and fibroblast number, GM-CSF, known to cause pulmonary eosinophilic inflammation and fibrosis, IL-6, a pleitropic cytokine and CXCL12 a chemokine implicated in angionesis (Miadonna, Gibelli et al. 1997; Minshall, Leung et al. 1997; Hoshino, Aoike et al. 2003). As well, the dynamic processes of fibrosis necessitate a balance of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs). Both MMP-9 and TIMP-1 are found in exaggerated quantities in sputum and biopsies from patients with asthma, correlating with ongoing airway fibrosis (Vignola, Riccobono et al. 1998; Vermeer, Denker et al. 2009). Overall, the current dogma is that these structural changes are linked to functional changes that are reflected by increased hyperresponsiveness and bronchoconstriction.

1.1.4 Severe Asthma; refractory asthma

The American Thoracic Society (ATS) in 2000 held a workshop to address the definition of a subset of asthmatics that suffer from a particularly difficult disease state, also referred to as severe asthma. The term *refractory* was chosen to encompass what had previously been labeled ‘brittle, steroid-dependent, steroid-resistant, chronic, difficult to control, near-fatal, fatal, irreversible and severe’ asthma (ATS 2000). Refractory asthma is defined on the basis of medications required to control symptoms, as well as the frequency of exacerbations and airflow limitation. There are two major and seven minor criteria, and in order to qualify the patient must have at least one major criterion and two minor criteria (Table 1). Additionally, other medical conditions must have been excluded from the diagnosis, with optimal treatment of symptoms already underway, with full compliance from the patient.

Table 1

Refractory Asthma: workshop consensus for clinical features

Major characteristics	*In order to achieve control to a level of mild-moderate persistent asthma:* *Treatment with continuous or near continuous oral corticosteroids* *Requirement for treatment with high dose inhaled corticosteroids*
Minor characteristics	Requirement for daily treatment with a controller medication in addition to inhaled corticosteroids (long acting Beta-agonists, theophylline, or leukotriene antagonist) Asthma symptoms requiring short acting beta-agonist use on a daily or near daily basis Persistent airway obstruction (FEV1 <80% predicted; diurnal PEF variability > 20%) One or more urgent care visits for asthma per year Three or more steroid 'bursts' per year Prompt deterioration with a >25% reduction in oral or inhaled corticosteroids. Near fatal asthma event in the past

Source: American Thoracic Society (ATS 2000)

The causes of refractory asthma remain unclear, and debate is ongoing as to whether or not refractory asthma is an extension of milder forms of asthma, or a separate disease entirely. Support exists for both ideas. As an extension of milder asthma, refractory asthma has presented with evidence of the Th2 phenotype including increased eosinophils and lymphocytes on autopsy and endobronchial samples. In the BAL fluids of corticosteroid (CS) resistant patients, eosinophils, IL-4 and IL-5 were elevated compared to steroid–responsive asthmatics (ATS 2000). The alternative explanation for CS resistance is that the kind of inflammation seen in these patients is unique and not affected by steroids. The main pathological difference between the two is the presence of the neutrophil. Multiple studies have demonstrated neutrophils have been found in greater numbers in the airways of patients with refractory asthma compared to milder forms (Wenzel, Szefler et al. 1997; Humbert, Holgate et al. 2007). As well, fatal and near fatal asthma attacks have shown the presence of increased neutrophils, indicating their

involvement with the most serious forms of the disease (Sur, Crotty et al. 1993). Neutrophils are poorly responsive to steroid treatment, and studies show that some forms of corticosteroid (CS) treatment results in their increase, as it renders these cells resistant to apoptosis (Macdowell and Peters 2007). Exactly how neutrophils are activated and what role they play is still being investigated.

1.1.5 Treatment of Asthma

Over the last 75 years highly effective medications have been developed that have reduced the morbidity and mortality of asthma significantly. Indeed, most asthmatics can now effectively control their symptoms with few side effects. Despite this, in refractory and especially corticosteroid resistant asthma, the need exists for novel therapies. The goal of treatment in asthma is to control the manifestations of the disease. The focus of this control is twofold: to stop and reverse bronchoconstriction and control inflammatory processes.

1.1.5.1 Non steroidal medications that control inflammation and bronchoconstriction

In mammals, adrenergic receptors bind adrenalin and mediate important responses to extreme stress. This includes the liberation of metabolic products such as glucose and fatty acids, restriction of blood flow to peripheral organs, increase in heart rate, and importantly for asthmatics- relaxation of smooth muscle. In cases of extreme allergic reactions, intramuscular doses of epinephrine (adrenaline) are life-saving (Shapiro 2002).

Beta2-adrenergic receptor agonists are medications that selectively target the adrenergic receptors responsible for muscle relaxation, which reopen the lumen of the airways and subsequently provide a drop in airway resistance. Short acting inhaled beta2-agonists are used as the first line of treatment in rescue from bronchoconstriction, although longer acting beta2-agonists have now been designed to prevent exacerbations over as much as 24 hours. In fact, the most effective anti-asthma medications designed to date are those that combine long term beta2-agonists and corticosteroids. These two classes of medications work separately to regulate different processes, but also show synergism (Barnes 2002; Kuna and Kuprys 2002).

Novel medications to reduce inflammation are the focus of much research; however, none have been as successful as corticosteroids. Cysteinyl-leukotrienes (cys-LTs) are

eicosanoids; inflammatory mediators produced from arachidonic acid metabolism that act in bronchoconstriction. Potent cys-LT receptor antagonists (such as montelukast^{TM}) have provided important add-on therapy for refractory asthma (Namenyi, Puha et al. 1999). Other bronchodilators are the anti-cholinergic drugs that block the muscarinic (M_2 and/or M_3) receptors on smooth muscle cells. However, their action only reverses cholinergic bronchoconstriction, considered to be a minor component of the overall bronchoconstriction seen in asthma (Barnes 1993). Theophylline is a methyl xanthine whose bronchodilator effect is attributed to inhibition of phosphodiesterases in airway smooth muscle. Side effects from this drug at higher doses limit its use, but recently treatment with lower doses has been associated with anti-inflammatory effects similar to that of corticosteroids, namely the regulation of histone deacetylases that regulate inflammatory genes (Ito, Lim et al. 2002).

TNFα is a cytokine that has been shown to be increased in cases of severe asthmatics compared to mild or non-asthmatics (Ying, Robinson et al. 1991). It is produced by a variety of cell types, including mast cells and leukocytes, and contributes to the asthmatic phenotype by indirectly recruiting eosinophils and neutrophils into the airways. Preliminary studies on anti-TNF therapy in asthmatics have demonstrated an improvement in lung function, quality of life, AHR and exacerbation rate (Brightling, Berry et al. 2008). However, anti-TNF therapy is associated with a higher risk of malignancies and infection, making its widespread use in asthma untenable.

1.1.5.2 Glucocorticoids

The use of inhaled glucocorticoids, a class of corticosteroids (CS) has been the leading reason for decreased morbidity and mortality is asthma (Allen 2006; Ito, Chung et al. 2006). Its introduction into mainstay asthma treatment followed promising tests by H. Brown and colleagues, who, in 1972 showed that a group of patients on inhaled doses of beclomethasone dipropionate (BDP) had better control of their asthma and reduced need for oral corticosteroids (Brown, Storey et al. 1972).

The mechanism of action of glucocorticoids is well described (reviewed in (Barnes). Glucocorticoids diffuse across cell membranes and bind to cytoplasmic glucocorticoid receptors (GR). These activated units dimerize and translocate to the nucleus. The main function of the activated GR is to downregulate (*transrepress*) genes that encode inflammatory cytokines,

adhesion molecules, enzymes and receptors. This occurs as GRs interact with coactivator molecules such as CREB-binding protein, which have intrinsic histone acetyltransferase activity (HAT) (Janknecht and Hunter 1996; Urnov and Wolffe 2001). Acetylation of the core histone 4 on chromatin opens DNA for transcription. GCs reverse this process by recruiting histone deactylase-2 (HDAC2), which effectively rewinds the DNA and stops transcription (de Ruijter, van Gennip et al. 2003).

Additionally, glucocorticoids bind to the glucocorticoid receptor element (GRE) in promoter regions of GC responsive genes. They then associate with co-activators with HAT activity. This GRE binding causes *transactivation* of anti-inflammatory genes such as annexin-1, IκBα, Beta2-adrenoceptor and MKP-1. Annexin-1 has important roles in regulating prostaglandins and leukotrienes in alveolar macrophages (De Caterina, Sicari et al. 1993). MKP1, also known as DUSP1 or dual specificity protein phosphatase 1, is the main negative regulator of p38 MAPK, a kinase involved in the regulation of many inflammatory genes. DUSP1 effectively reduces or abrogates these processes by deactivating p38 MAPK. In a 2007 study using murine macrophages with a GR knock out, the GC dexamethasone was able to increase DUSP1 expression 18 fold in normal murine macrophages but completely unable to in the KO cells. The KO mice experienced a mortality rate of 88% when treated with lipopolysaccharide (LPS), compared with mice treated with LPS and an inhibitor of p38 MAPK (SB203580). In accord with the improved survival were lower plasma levels of TNFα and IL-6. These findings proved that GCs act by upregulating DUSP1, which in turn is responsible for reducing p38 MAPK, mediated proinflammatory cytokine production (Bhattacharyya, Brown et al. 2007). In a directly related phenomenon, phosphorylation of the glucocorticoid receptor by p38 MAPK also occurs (Irusen, Matthews et al. 2002; Weigel and Moore 2007). This can affect its stability, DNA binding, translocation to the nucleus or interaction with other proteins such as molecular chaperons. Indeed, this mechanism has been postulated as a possible contribution in glucocorticoid resistance. DUSP1 deactivation of p38 MAPK thus has direct consequences for overall GC activity.

Unfortunately, glucocorticoid use comes with side effects such as growth retardation in children, insulin resistance, skin fragility and osteoporosis. While not all of the mechanisms behind these processes are well understood, one way this occurs is when the GR binds to genes with a negative GRE, leading to a *transrepression*. Downregulation of important genes involved

in the synthesis of proteins such as keratin and osteocalcin (importantly involved in bone synthesis) follows (Dostert and Heinzel 2004).

Alternatively, there are a sub-set of patients who become resistant to the effects of GCs: many with refractory asthma, or asthmatics who smoke. The mechanisms behind this resistance continue to be identified. Genetic investigations have revealed that morphogenetic protein receptor type II is a GC sensitivity determining gene in normal patients, although there are 11 different genes that separate GC sensitive from GC resistant patients (Hakonarson, Bjornsdottir et al. 2005; Donn, Berry et al. 2007). In GC resistant asthmatics, DUSP1 deficiency and increased p38 MAPK activity have been seen in alveolar macrophages after glucocorticoid exposure (Bhavsar, Hew et al. 2008). Other factors may include defective IL-10 secretion by T-cells and abnormal histone deacetylase 2 activity (Hew, Bhavsar et al. 2006; Xystrakis, Kusumakar et al. 2006). Lack of relief from GC treatment poses serious medical management problems. While lifestyle changes such as cessation of smoking can help a percentage of this population, new drug therapies that address this problem would provide relief not only for asthmatics but also other chronic inflammatory conditions.

1.2 Human Airway Smooth Muscle (HASM)

Huber and Koessler first described airway wall thickening in the bronchi of asthmatics in the 1920's (Huber and Koessler 1922). Since that time, these structural changes, now termed remodelling, have been well described and are directly correlated with severity of the disease (Wiggs, Moreno et al. 1990). One of the most striking changes seen in remodelling is the increase in smooth muscle mass (or volume). Airway smooth muscle (ASM) was previously viewed as a passive player in airway inflammation, contracting and relaxing as directed by pro-inflammatory mediators and bronchodilators respectively. It has now become clear that airway smooth muscle is not solely a contractile apparatus. A plethora of research has revealed ASM to be responsive to both exogenous and endogenous mediators, producing extra-cellular matrix (ECM) proteins as well as cytokines, growth factors and prostanoids (Singer, Salinthone et al. 2004). Additionally, ASM's interaction with inflammatory cells includes their recruitment via the release of chemokines and surface adhesion molecules as well as providing an ideal microenvironment for mast cells (Howarth, Knox et al. 2004). While the contractile function of ASM underlies its main role in AHR, its synthetic responses are gaining more attention.

1.2.1 Mechanisms that increase Airway Smooth Muscle

Several studies have investigated whether or not the increase of smooth muscle in the airways is due to an increase in cell size (hypertrophy) or number (hyperplasia). In fatal asthma, pathological investigations shows HASM cell volume is increased due to both of these conditions (Ebina, Takahashi et al. 1993). In mild to moderate cases of asthma, Woodruff et al. demonstrated that hyperplasia was responsible for the two-fold increase seen in asthmatic airway smooth muscle volume compared to control subjects. However, smooth muscle cell hypertrophy was absent (Woodruff, Dolganov et al. 2004)**.**

Mediators leading to regulation of hypertrophy and hyperplasia of ASM proliferation include peptide growth factors such as platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF). Alternatively, TGF-β has been shown to increase ASM cell size, along with IL- 1β and IL-6 (De, Zelazny et al. 1995; Goldsmith, Bentley et al. 2006). The muscle of asthmatics is often subjected to contractile agonists, which on their own are considered weak stimulants of proliferation. However, some studies reveal that constricting agents synergize with growth factors and elicit growth responses (Panettieri, Goldie et al. 1996; Krymskaya, Orsini et al. 2000). Since increased muscle volume has been associated with increased hyperresponsiveness, this circular process may perpetuate proliferation of ASM.

Proliferation may also have a genetic component. Indeed, the increased level of AHR seen in the Fisher rat compared to the Lewis rat correlates with the greater volume of airway smooth muscle in the former (Eidelman, DiMaria et al. 1991). As well, studies have shown that ASM that is isolated from these two strains and grown in culture maintains a differential growth rate, as do ASM cells isolated from asthmatics compared to controls (Eidelman, DiMaria et al. 1991; Johnson, Roth et al. 2001).

New research has focused on the ability of smooth muscle cells to migrate as an explanation of increased ASM volume. While there is no evidence yet that migration of smooth muscle occurs *in vivo*, in chemotaxis assays *in vitro*, HASM has been shown to migrate in response to CCL19 (Kaur, Saunders et al. 2006), epidermal growth factor (EGF), platelet derived growth factor (PDGF-BB) and various chemokines. Very recent work has demonstrated that HASM cells possess primary cilia that enable them to migrate (Wu, Du et al. 2009). This study revealed the cilia contained EGF receptors and intact cells were better able to migrate than de-ciliated or non-ciliated cells in response to EGF. Finally, HASM has also been shown to migrate towards conditioned media produced from stimulated epithelial cells. This may explain why the

airway smooth muscle layer is located closer to the epithelium in asthmatic patients (Takeda, Sumi et al. 2009).

1.2.2 HASM contribution to inflammatory milieu via secretion of ECM

The interstitial extracellular matrix surrounds ASM, providing vital connective tissue to which ASM attaches. The relationship between ASM and the ECM is bidirectional: ASM can contribute to the composition of the ECM, and its composition has important implications for ASM. Cultured human airway smooth muscle cells exposed to 10 % serum from an asthmatic has been shown to generate more fibronectin, laminin-γ, perlecan and chondroitin sulphate compared to serum from a healthy individual (Johnson, Black et al. 2000). This same study showed ASM can also produce elastin, various laminins, collagen I, II and V and decorin. A growth factor of importance in this process is TGF-β, shown to increase the expression of connective tissue growth factor (CTGF) to greater levels in asthmatics versus controls (Burgess, Johnson et al. 2003). Conversely, ASM can affect the degradation of the ECM since it has been shown to generate progelatinase A (a precursor to MMP-2) and gelatinase B (MMP-9) (Foda, George et al. 1999).

The ECM can affect how smooth muscle responds to stimuli in relation to proliferation and migration. In vitro experiments show that ASM will not migrate without an extracellular matrix (Parameswaran, Radford et al. 2004). The asthmatic airway favours increased deposition of collagen I and fibronectin, whereas the healthy airway has a greater composition of laminin (Arm and Lee 1992). In cell culture experiments, Hirst et al. revealed that both collagen and fibronectin coated culture plates had greater proliferative responses compared to cells grown on plastic alone, whereas laminin reduced the proliferative response (Hirst, Twort et al. 2000). Further studies show that the components of the ECM influence the expression of contractile and structural proteins within HASM (Freyer, Johnson et al. 2001). This reveals a contributing role the ECM is likely to have on asthmatic ASM behaviour.

1.2.3 HASM contribution to inflammatory milieu via secretion of Cytokines.

Evidence suggests that ASM is an important source of inflammatory mediators in asthma. Indeed, the downregulation of a vast array of pro-asthmatic genes is seen in ASM treated with

the glucocorticoid dexamethasone (Hakonarson, Halapi et al. 2001). A variety of triggers including pathogen products, TNFα and IL-1β, interleukins from Th2 cells and growth factors bring about the synthesis of these mediators.

ASM *in vivo* produces eotaxin, a potent chemoattractant for eosinophils. A marked increase of eotaxin staining has been seen in immunohistochemical analysis of bronchoscopic biopsies from asthmatic patients compared to non-asthmatics (Ghaffar, Hamid et al. 1999). Other immunohistochemistry and *in situ* hybridization studies have revealed regulated on activation normal T cell expressed and secreted (RANTES) and monocyte chemotactic protein 1 (MCP-1) are expressed in smooth muscle of subjects with asthma (Sousa, Lane et al. 1994; Berkman, Krishnan et al. 1996). Chemokine ligand 10, a potent attractant of activated T cells and natural killer cells is expressed along with its ligand, chemokine receptor 3 (CCR3) by ASM in asthmatic patients (Shan, Redhu et al. ; Hardaker, Bacon et al. 2004; Brightling, Ammit et al. 2005).

Airway smooth muscle cells have also been shown *in vitro* to produce inflammatory mediators. Recent work shows HASM produces markedly increased levels of CXCL8, IL-6 and eotaxin, both at the mRNA and protein level in response to thymic stromal lymphopoietin (TSLP), an IL-7 type cytokine implicated in the allergic response (Shan, Redhu et al.). CXCL8 is a potent chemokine for neutrophils, the dominant inflammatory cells seen in refractory asthma, and has been shown to be upregulated by ASM preferentially in response to TNFα and IL-1β and not to Th2 cytokines such as IL-4 or IL-13 (John, Au et al. 1998; Watson, Grix et al. 1998). A recent study has also found that HASM cells isolated from asthmatic subjects in culture released higher basal levels of CXCL8 than non asthmatic HASM cells. These same cell populations, when stimulated with TNFα showed CXCL8 production was approximately 7 times greater in the asthmatic-derived cells (John, Zhu et al. 2009). These cytokine expression results are similar to those obtained by Oliver et al. who investigated rhinovirus infection of asthmatic versus non-asthmatic ASM. Here IL-6, considered pro-inflammatory due to its role in remodelling, was also measured (Ammit, Moir et al. 2007). Infection of the asthmatic-derived ASM cells produced more IL-6 than those from non-asthmatic cells. Interestingly, only the asthmatic cells produced CXCL8 in response to the viral infection (Oliver, Johnston et al. 2006). IL-33, the newest cytokine to be discovered in the IL-1 family, is expressed at higher levels in asthmatic than healthy individuals (Prefontaine, Nadigel et al.). ASM has been shown to produce IL-33, both at the mRNA and protein level. Its role in asthma includes binding to its receptor (ST2) on the

surface of Th2 cells, a process that polarizes them to produce IL-5 and IL-13 (Schmitz, Owyang et al. 2005). Additionally, cultured ASM cells express GM-CSF which stimulates the maturation, survival and activation of eosinophils (Oltmanns, Issa et al. 2003). HASM also produces the prostaglandin E2 (PGE2), in response to IL-1β stimulation (Pascual, Carr et al. 2006). PGE2 is an eicosanoid produced from the metabolism of arachidonic acid. The functions of PGE2 are both pro and anti-inflammatory and include smooth muscle contraction and relaxation, vasodilatation and hyperalgesia. PGE2 has been shown to desensitize H1 histamine and beta adrenergic receptors in smooth muscle (Laporte, Moore et al. 1998). These PGE2 mediated processes are effectively inhibited by glucocorticoid action.

1.3 Cell signaling

Cells respond and adapt to changes in their environment by activating signaling pathways. These pathways set in motion many important cellular functions including: interactions with other cells, the regulation of genes that control stress responses, metabolism, motility, proliferation, differentiation, survival and apoptosis. Many of these processes are key to maintaining homeostasis of the organism. The environmental signals to which cells respond comprise: alterations in osmolarity or concentrations of growth factors/nutrients, cell damaging agents, cytokines and chemokines, molecules that are recognized as belonging to pathogens and UV radiation. Chief signaling pathways include nuclear factor kappa B (NFκB) and mitogen-activated protein kinases (MAPKs).

1.3.1 Mitogen-activated protein kinases (MAPKs) overview

The mitogen-activated protein kinases are a group of highly conserved protein kinases that respond to extracellular stimuli through a three tiered cascade of phosphorylation. Two well characterized sub-families of MAPKs include the ERK1/ERK2 and p38 MAPKs (Figure 1). A number of features are shared between these pathways, including how they are sequentially activated. Both have a family of MAPK kinase kinases (MAPKKK) which activate MAPK kinases (MAPKK) which then act upon MAPKs. The MAPK, once phosphorylated on its threonine and tyrosine residues (in the activation loop of kinase subdomain VIII) becomes

activated and able to act on a wide array of downstream substrates (Hanks and Hunter 1995; Enslen, Brancho et al. 2000).

MAPKs are subjected to spatial and temporal control by complex mechanisms that include crosstalk and negative feedback regulation. Dephosphorylation of these kinases is the most energy efficient mode of deactivation, and to date at least ten protein phosphatases have been identified that serve this function (Liu, Shepherd et al. 2007). Dual specificity protein phosphatase 1 (DUSP1), previously named MKP1, is the archetype of the MAPK phosphatase family. This phosphatase has been extensively studied *in vitro* and *in vivo* revealing it preferentially deactivates p38 MAPK and JNK over ERK1/ERK2 (Sun, Charles et al. 1993; Zhao, Shepherd et al. 2005). Glucocorticoids exert their anti-inflammatory properties partially through the induction of DUSP1, which makes its role in the pathology of asthma a subject of intense study (Kassel, Sancono et al. 2001; Lasa, Abraham et al. 2002).

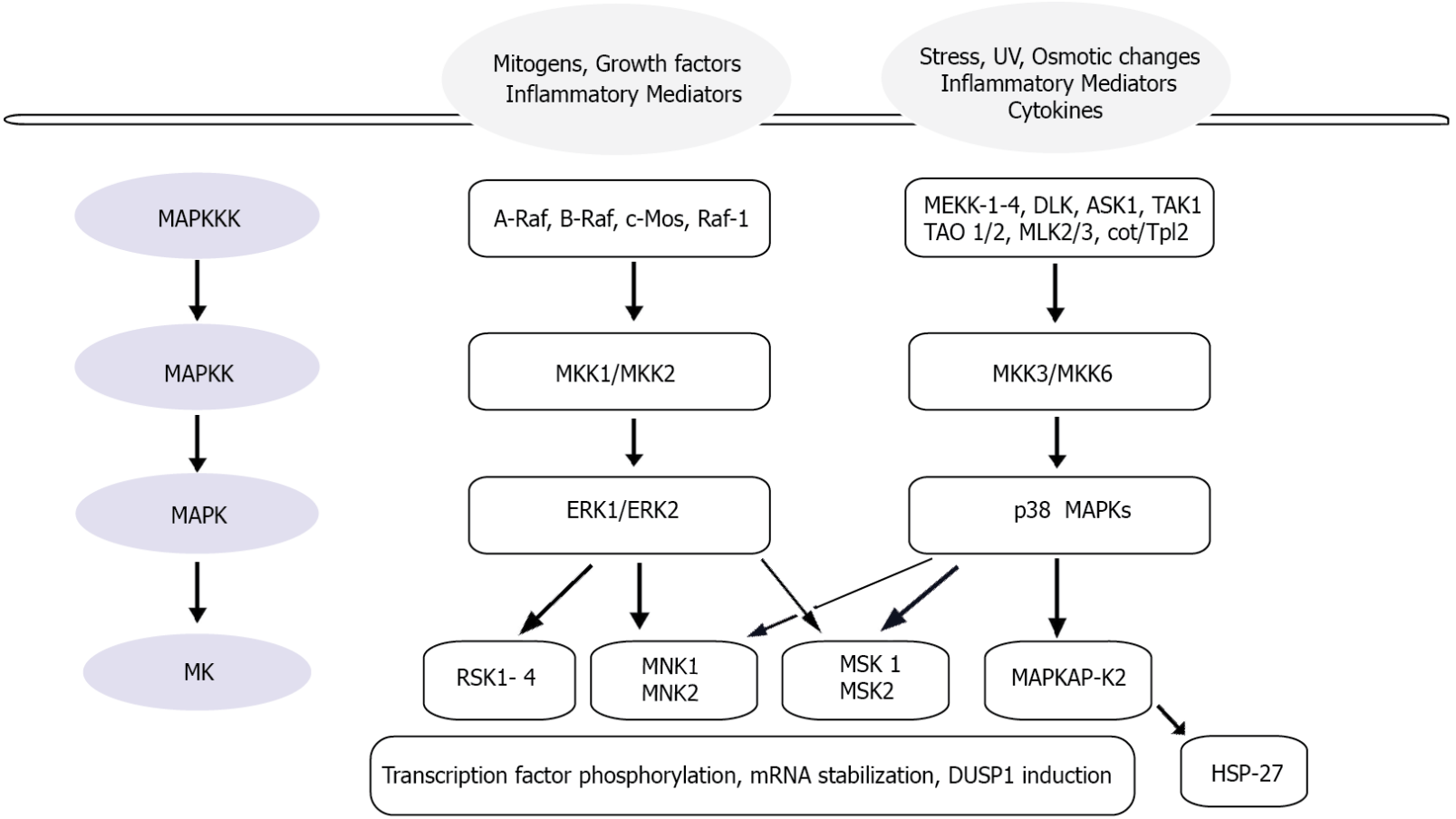

Figure 1: ERK1/ERK2 and p38 MAPK signaling pathways

1.3.1.1 p38 MAPK

There are four isoforms of p38 MAPK: α, β, γ, and δ. Of these, p38α is the best characterized. Han et al. first described p38α in 1994 as being a 38 kilodalton protein that became phosphorylated in response to the endotoxin lipopolysaccharide (LPS) and hyperosmolarity. This study revealed its activation was necessary for the production of TNFα and IL-1, setting its first known role as one of mediating inflammation (Han, Lee et al. 1994). In parallel, other researchers found p38α MAPK to be activated in response to heat shock, osmotic stress, IL-1 and ultra violet radiation (Freshney, Rawlinson et al. 1994; Raingeaud, Gupta et al. 1995). It is now known that the p38 MAPKs are activated preferentially by inflammatory cytokines and environmental stress, and along with c-JUN terminal kinases (JNKs), are sometimes referred to as stress-activated protein kinases (SAPKs).

The p38 MAPK pathway is illustrated in figure 1. Activation of this pathway can occur through numerous classes of receptors including cytokine receptors, Toll-like receptors, G-protein-coupled receptors and growth factor receptors (Lee, Mira-Arbibe et al. 2000; Kim, Kim et al. 2004; Matsuzawa, Saegusa et al. 2005). The ensuing phosphorylation of MAPKKK enzymes may be cell specific but include MEKK1-4, MLKs, ASK1 and TAK1. These in turn activate MKK3 and MKK6 which are responsible for the selective activation of p38 MAPK, as they do not activate either the JNKs or ERK1/ERK2.

The consequences of p38 MAPK activity are vast and include many normal immune responses. p38 MAPK is activated in neutrophils, macrophages and T-cells and participates in functional responses including chemotaxis, granular exocytosis, adherence and apoptosis (Ono and Han 2000). p38 MAPK is also responsible for the regulation of many different genes implicated in inflammation including IL-1, TNF, CXCL8, Cyclooxygenase 2 (COX-2) and VCAM 1 (Lee, Laydon et al. 1994; Ridley, Dean et al. 1998; Kotlyarov, Neininger et al. 1999; Loitsch, von Mallinckrodt et al. 2000; Fuste, Escolar et al. 2004). Other cellular processes, such as transcription regulation, chromatin remodelling, and cytoskeleton reorganization are also regulated by p38 MAPK activity. How this kinase mediates inflammatory and anti-inflammatory processes is relevant to the pathology of asthma.

One important downstream target of p38 MAPK that was identified early on is the protein kinase MAPKAP-K2, which is believed to mediate inflammation through post-transcriptional regulation of mRNA, although the exact mechanism is still unknown. The

presence of an adenine/uridine rich element (ARE) on the 3' untranslated region of messenger RNA (mRNA) transcripts is associated with a shorter half life. MAPKAP-K2 has been shown to phosphorylate and thus stabilize proteins that are involved in ARE binding and mRNA stabilization (Kotlyarov, Neininger et al. 1999). In another study, knock out murine models of MAPKAP-K2 have been shown to have less endothelial cell permeability due to less adherens junction breakdown and actin polymerization, resulting in reduced plasma infiltration of the airways. In this same model, overall Th2 cytokine expression and vascular cell adhesion molecule 1 (VCAM 1) production were significantly decreased (Gorska, Liang et al. 2007). Indeed, one of the suggested mechanisms of glucocorticoid anti-inflammatory action is via the reduction of MAPKAP-K2 activity via DUSP1 downregulation of p38 MAPK (Barnes and Adcock 2009).

It is also well established that MAPKAP-K2 leads to phosphorylation of heat shock protein 27 (HSP27) (Cuenda, Rouse et al. 1995). HSP27 is a chaperone protein that functions in filamentous actin cap binding, and when phosphorylated leads to actin polymerization (Landry and Huot 1995). This results in stiffer and longer actin fibers. Force fluctuation induced re-lengthening (FFIR) is a broncho-protective element of deep inspiration that is impaired in asthmatics following bronchoconstriction. It has been shown in a canine model that a reduction of HSP27 phosphorylation correlated with the use of GCs, and was associated with increased FFIR (Lakser, Dowell et al. 2008). Due to this role in AHR, p38 MAPK targets continue to be investigated for their role in cytoskeletal remodelling leading to impaired relengthening.

In addition to promoting inflammation, p38 MAPK is involved in key negative feedback loops that have anti-inflammatory functions and regulate its own expression (figure 2). DUSP1 is one of those mechanisms already discussed. Two of the kinases downstream of p38 MAPK are mitogen and stress activated kinase 1 and 2 (MSK1/2), usually present in the nuclei of quiescent cells. MSK1/2 phosphorylate the transcription factors CREB and ATF1, which are involved in DUSP1 transcription, the main phosphatase that deactivates p38 MAPK (Wiggin, Soloaga et al. 2002). A recent study shows that MSK1/2 double knock-out mice (DKO) have lower DUSP1 expression, indicating that this kinase participates in the self-regulation of p38 MAPK (Ananieva, Darragh et al. 2008). As well, this same study revealed MSK1/2 DKO mice had greater production of TNFα, IL-6 and IL-12 and significantly lower production of IL-10, a potent anti-inflammatory cytokine. These studies reveal a role for downstream kinases of p38 MAPK in preventing or controlling excessive inflammation. Overall, while p38 MAPK has an

inflammatory arm it also has anti-inflammatory and intrinsic self-regulating mechanisms. Indeed, it may be one of the reasons why pharmacologic p38 MAPK inhibitors to date have had the anticipated success in preventing inflammation clinically.

Figure 2

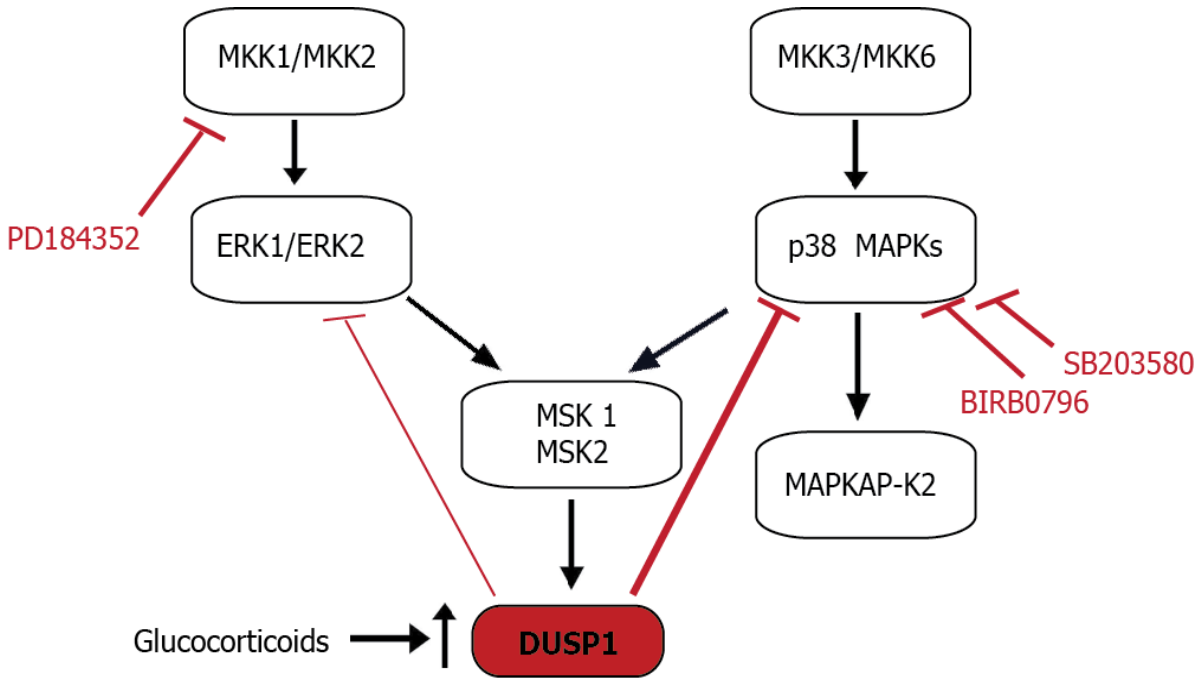

Figure 2: negative regulators and inhibitors of MAPK signalling

1.3.1.2 Inhibitors of the p38 MAPK pathway

A class of pyridinyl imidazole chemical compounds developed in the early 1990's were found to limit the production of the inflammatory cytokines/proteins IL-6, IL-8, Cox-2, NOS, TNFα, and IL-1β (Perregaux, Dean et al. 1995; Boehm, Smietana et al. 1996; Blanque, Cottereaux et al. 1997). Termed CSAIDs for 'cytokine-suppressive anti-inflammatory drugs',

these compounds were proven to be effective by binding to a small protein identified as p38 MAPK, which lead to its discovery (Lee, Laydon et al. 1994). The crystalline structure of p38 MAPK was determined in 1996, revealing the kinase to have two domains divided by a deep channel that houses a hydrophobic ATP and substrate binding pocket (Wilson, Fitzgibbon et al. 1996). Binding of ATP causes a conformational change of p38, and is necessary for phosphate transfer. The pyridinyl imidazoles bind within this ATP pocket, locking it in an activated state but unable to phosphorylate other substrates (Young, McLaughlin et al. 1997). While effective, these compounds used in pre-clinical trials suffered from 'off target effects' attributed to their lack of specificity. SB203580, an example of this first class of inhibitors, has been found to inhibit not only p38MAPK but also receptor interacting protein 2 (Rip2) at the same concentrations as p38 MAPK (Godl, Wissing et al. 2003; Argast, Fausto et al. 2005). RIP2 is an essential mediator of Nod 1 and Nod 2 signaling and has been shown to mediate both innate and adaptive immune responses by activating the NFκB pathway (Inohara, Koseki et al. 1999; Inohara, Koseki et al. 2000; Chin, Dempsey et al. 2002). As such, SB203580 cannot be interpreted exclusively as an inhibitor of p38 MAPK.

A second generation of p38 MAPK inhibitors improved the original design wherein the central imidazole ring is replaced by a pyrrole, pyrazole or pyrazoline, allowing better access to the ATP pocket (Fitzgerald, Patel et al. 2003). Many of these compounds have exhibited significant efficacy in disease models of chronic inflammation, arthritis, airway disease and pain, such that several have advanced to clinical trials (reviewed in (Lee and Dominguez 2005). AMG 548 (Amgen) was the first to be tested in human trials and reached phase I for rheumatoid arthritis (RA), while VX-745 and later VX-702 (Vertex) moved on to phase II RA trials (Damjanov, Kauffman et al. 2009). SCIO 469 (Scios, Johnson and Johnson) has been intensely investigated for the treatment of multiple myeloma and pain. In both murine and rat models of myeloma this compound has shown reduced tumor burden and osteolytic bone disease as well as an overall increase in survival rate. (Navas, Nguyen et al. 2006; Vanderkerken, Medicherla et al. 2007; Cottrell, Meyenhofer et al. 2009).

BIRB0796 belongs to the third class of p38 MAPK inhibitors that, unlike the previous compounds, attach to an allosteric binding site on p38 MAPK (Pargellis, Tong et al. 2002). This causes a disruption in the activation loop and blocks phosphorylation by MKK6 (Sullivan, Holdgate et al. 2005). This inhibitor has shown a high level of specificity for p38 MAPK and is effective at nanomolar concentrations in cell culture. BIRB0796 has been tested in clinical trials

for the treatment of Chron's disease and RA (Schreiber, Feagan et al. 2006; Cohen, Cheng et al. 2009). To date there is no proof of clinical efficacy of BIRB0796 from either study and adverse effects include dizziness, skin reactions and GI upset. Indeed, most p38 MAPK inhibitors do not pass to clinical utility as they exhibit dose-limiting toxicities that primarily affect the liver, skin or CNS.

Work continues into more selective inhibitors of p38 MAPK with reduced toxicity for the treatment of neurodegenerative disorders (Munoz, Ranaivo et al. 2007), stroke (Barone, Irving et al. 2001), arthritis (Wu, Wang et al.) and airway inflammation (Munoz, Ramsay et al. ; Nath, Leung et al. 2006) among others. In this project I used both BIRB0796 (preferentially) but also SB203580 to demonstrate the effects of p38 MAPK blockade.

1.3.1.3 ERK1/ERK2 MAPK

Extra cellular signal regulated kinase 1 and 2 belong to a family of extra cellular regulated kinases (ERK1 through 5). ERK1/ERK2 are expressed in almost all tissues and respond strongly to growth factors, phorbol esters and serum. They also respond, although less so, to cytokines, osmotic stress and microtubule disorganization (reviewed in (Pearson, Robinson et al. 2001). ERK1/ERK2 signaling has been shown to be a key process in cellular proliferation and as such has received much attention as a possible therapeutic target in the treatment of cancer. Within the context of asthma, the proliferative consequences of ERK signaling have made it of interest regarding airway smooth muscle hyperplasia (Lee, Johnson et al. 2001). Additionally, a study of ERK1/ERK2 has shown this pathway to have a role in rat ASM hyperresponsiveness and cytokine release (Wuyts, Vanaudenaerde et al. 2003).

Many different cell surface receptors initiate ERK1/ERK2 signaling through the membrane bound protein Ras and include both receptor tyrosine kinases, non-receptor tyrosine kinases and G-protein coupled transmembrane receptors (Campbell, Khosravi-Far et al. 1998). Ras activation leads to activation of the MAPKKKs A-Raf, B-Raf, Raf-1 and c-Mos. Interestingly, 30% of all human cancers have a mutation in Ras while 60% of malignant melanoma is associated with a mutation in B-Raf (Downward 2003; Mercer and Pritchard 2003). These MAPKKKs activate in turn MEK1 and MEK2, which phosphorylate ERK1/ERK2. Activated ERK1/ERK2 phosphorylate numerous substrates including membrane proteins, cytoskeletal proteins and other signaling molecules.

In conjunction with p38 MAPK, ERKs also phosphorylate MSK1/2, leading to transcription of the phosphatase DUSP1, as mentioned above (Deak, Clifton et al. 1998). Another little known function of ERK1/ERK2 is that it phosphorylates the phosphatases involved in its own regulation. Phosphorylation of MAPK phosphatase 3 (which is specific to ERK) leads to its degradation- sustaining ERK activity. Alternatively, ERK phosphorylation of DUSP1 stabilizes it, increasing its half life by two or three fold (Brondello, Pouyssegur et al. 1999; Marchetti, Gimond et al. 2005). Since DUSP1 preferentially deactivates p38 MAPK, this may represent an element of cross talk between the ERKs and p38 MAPKs whereby increased ERK signaling translates into reduced p38 MAPK activity and vice versa.

1.3.1.4 Inhibitors of the ERK MAPK pathway

There has been extensive interest in the ERK/ERK2 pathway as a possible drug target for the treatment of cancer since a study in 2002 reported a high incidence of B-Raf (a MAPKKK) mutations in many human cancers (Davies, Bignell et al. 2002). One B-Raf inhibitor, PLX4032, is selective for mutated forms of the B-Raf protein and has shown potent anti-tumor effects in a murine model (Yang, Higgins et al.). Although many Raf inhibitors are in clinical trials, the only drug to yet make regulatory approval is the C-Raf inhibitor BAY 43-9006 (Sorafenib™) (Wilhelm, Carter et al. 2004; Adnane, Trail et al. 2006). While C-Raf mutations are not as common as those of B-Raf, this drug shows clinical efficacy against renal and hepatic tumors.

The high degree of selectivity of MEK1/MEK2 for ERK1/ERK2 phosphorylation has also made the MEK kinases a much investigated drug target. The first inhibitor of MEK1/MEK2 was PD98059, described by Dudley et al in 1995 (Dudley, Pang et al. 1995). A more potent but equally specific compound U0126 was created not long after (Favata, Horiuchi et al. 1998), but both compounds had pharmaceutical limitations for use in human trials. The development of Cl-1040 and MEK inhibitors in this class permitted the three dimensional structure of MEK1/MEK2 to be solved, revealing these kinases to have a unique inhibitor binding pocket adjacent to the ATP binding site (Ohren, Chen et al. 2004) . Like p38 MAPK, the binding of inhibitors within this hydrophobic pocket causes a conformational change that locks MEK into a closed and inactive form, rendering it unable to activate ERK1/ERK2. CL-1040 was the first to advance to human trials and showed promise in the treatment of colon, breast, and pancreatic cancers (LoRusso, Krishnamurthi et al. ; Sebolt-Leopold, Dudley et al. 1999; Rinehart, Adjei et al.

2004). However, ocular side effects pushed the further development of structural analogues PD 0325901 and AZD 6244, which are now in clinical trials.

The MEK1/MEK2 inhibitor PD184352 is a non-ATP-competitive specific inhibitor that blocks MEK activity by binding to an activation domain (Delaney, Printen et al. 2002). PD184352 is effective at low concentrations (2 μM) and was used in this project to demonstrate the effect of ERK1/ERK2 inhibition.

1.3.2 NFKappaB Signaling pathway

The NFκB family of transcription factor proteins have been the focus of much study since their discovery approximately 20 years ago (Sen and Baltimore 1986). Activation of this pathway is a hallmark of most infections, and has thus been considered a central mediator in the innate immune response. Lately there has also been increasing evidence that NFκB responds to physical, oxidative and chemical stress. The roles of NFκB outside of immunity are beyond the scope of this paper but include important contributions to the stress response, development and oncogenesis.

The NFκB proteins are highly conserved and well described. In unstimulated cells, homodimers and heterodimers of these proteins sit sequestered in the cytosol due to their association with members of the inhibitor of NFκB (IκB) family (Bours, Burd et al. 1992). IκB contact with the p50-p65 heterodimer masks the nuclear localization signal required for its export from the cytoplasm to the nucleus. Phosphorylation of IκB occurs from upstream kinase IKK, which has two catalytic subunits (α and β) and a regulatory subunit γ (Zandi, Rothwarf et al. 1997; Rothwarf, Zandi et al. 1998). The two catalytic subunits are responsible for the phosphorylation of critical serine residues Ser32 and Ser36 on IκBα, a process that leads to its ubiquitination and subsequent degradation (Baldi, Brown et al. 1996). This effectively unmasks the nuclear localization signal, liberating NFκB and resulting in nuclear translocation and DNA binding. A second level of regulation occurs in the nucleus as further phosphorylation of NFκB is required for transcriptional activity to begin (Wang and Baldwin 1998).

The NFκB pathway is primarily activated when pattern recognition receptors identify invading bacterial and viral pathogens on the cell surface. An intracellular cascade leads to NFκB activation that results in the transcriptional activation of many inflammatory genes, including those that encode surface adhesion molecules, inflammatory cytokines and chemokines (Baeuerle and Henkel 1994). Many of these cytokines and chemokines go on to create further

signaling cascades that lead to a second wave of NFκB activation. This second wave leads to upregulation of a variety of anti-microbial molecules.

Despite NFκB being regarded as a master switch in mediating immune responses, one study has shown that this pathway does not contribute to airway hyperresponsiveness. In a murine KO model for IκBα, both control and KO mice were subjected to allergic challenge via the inhalation of ovalbumin. Compared to wild type mice, KO mice had decreased inflammation associated with asthma including: BAL fluids containing reduced numbers of eosinophils and lymphocytes, and decreased epithelial gene expression of RANTES, MCP-1 and eotaxin. The mice were mechanically ventilated and airway resistance and elastance were measured in response to methacholine challenge. There was no difference between the two groups despite the changes observed in inflammatory parameters, suggesting hyperresponsiveness may be uncoupled from NFcB driven inflammation. (Poynter, Cloots et al. 2004)

TNFα is one of the anti-microbial molecules released in the second wave of NFκB activation. TNFα binds to cell surface receptors and is not internalized but rather causes trimerization of the TNFα-type 1 receptor (TNFR1). This binding recruits the death domain containing molecule TRADD which then interacts with RIP and TRAF2. This signaling cascade leads to TAK1 and TAB1 activation and eventual IκBα degradation, resulting in NFκB activation. Interestingly, TAK1 is a MAPKKK that leads to p38 MAPK activation as well (Goeddel 1996) (Figure 1).

1.4 Signaling in HASM in context of asthma

The MAPKs discussed above regulate a vast array of cellular processes from gene transcription to cell migration. In airway smooth muscle, the characterization and impact of this signaling has been made possible through the use of relatively specific inhibitors of the p38 MAPK and ERK1/ERK2 pathways, as well as through overexpression assays, knock down and knock out models. While initial work focused on animal models, more recently human airway cell culture and biopsy have provided excellent tools for determining the outcome of MAPK inhibition. Some of these results are reviewed here.

1.4.1 Gene expression in HASM regulated by p38 MAPK and ERK1/ERK2

As per its known role, p38 MAPK regulates important inflammatory mediators in HASM. Both IL-6 and IL-8 mRNA production and cytokine secretion are dependent on p38 MAPK following TNFα stimulation or stimulation with a cytokine cocktail (Munoz, Ramsay et al. ; Hedges, Singer et al. 2000). Interestingly, the Munoz study revealed that p38α was the primary isoform responsible for this expression, with a partial contribution by p38β but none from p38δ or p38γ MAPK. Cyclooxygenase 2, (COX-2) is an inducible enzyme that increases the expression of PGE2. As mentioned earlier, the effects of PGE2 can be bronchoprotective. The expression of COX-2 and PGE2 has been found to be sensitive to p38 MAPK blockade (Laporte, Moore et al. 2000; Pype, Xu et al. 2001) and recent work has shown the mechanisms of COX-2 regulation may depend on MAPK activation of the NFKB pathways in HASM (Lin, Lee et al.).

Eotaxin production in response to IL-1β, IL-4 or IL-17A is sensitive to both p38 MAPK and ERK1/ERK2 blockade (Hallsworth, Moir et al. 2001; Moore, Church et al. 2002; Rahman, Yamasaki et al. 2006). GM-CSF is positively regulated by ERK1/ERK2 but negatively regulated by p38 MAPK in response to IL-1 β (Hallsworth, Moir et al. 2001). The same pattern is true for the production of IL-1β in response to a cytokine cocktail of TNFα/IL-1β /INF-γ (Hedges, Singer et al. 2000). The regulation of cytokine production in HASM can also be stimulus dependent. RANTES synthesis is mediated by ERK1/ERK2 if the stimulus is IL-1β (Hallsworth, Moir et al. 2001) and both p38 MAPK and ERK are involved if platelet activating factor (PAF) is the stimulus (Maruoka, Hashimoto et al. 2000).

HASM express the adhesion molecules ICAM-1 and VCAM-1. The control of ICAM-1 synthesis mirrors GM-CSF: held in check by p38 MAPK and dependent on ERK1/ERK2. On the other hand, VCAM-1 expression depends on concerted signaling between the MAPKs and NFκB pathways (Lin, Luo et al. 2009).

1.4.2 HASM migration and p38 MAPK

One of the earliest studies of HASM cell migration implicated p38 MAPK and its downstream targets MAPKAP-K2 and HSP27 in the process of migration (Hedges, Dechert et al. 1999), as has been shown for other cell lines (Roussel, Houle et al. ; Rousseau, Houle et al.

1997). The study used canine tracheal smooth muscle cells, an HSP27 phosphorylation mutant and p38 MAPK and ERK1/ERK2 inhibitors. Cells that expressed the mutated HSP27 protein and cells that were pretreated with the p38 MAPK inhibitor SB203580 did not migrate compared to controls. As mentioned earlier, HSP27 is an actin cap binding protein, which, when phosphorylated, dissociates from actin and promotes dynamic changes in vitro. When cells migrate their actin fibers are assembled at the leading edge of the plasma membrane and disassembled at the trailing edge (Gerthoffer 2008). The above study speaks to the importance of HSP27 in regulating this process. Moreover, p38 MAPK is known to phosphorylate l-caldesmon, a protein involved in actin remodelling. Caldesmon is an actin cross linking protein that, when bound to actin filaments, prevents disassembly and thus inhibits motility of vascular cells; phosphorylation of l-caldesmon causes dissociation from the filaments (Jiang, Huang et al. ; Yamakita, Yamashiro et al. 1992). In HASM cells, p38 MAPK activation following urokinase stimulation has been shown to be required for phosphorylation of l-caldesmon (Goncharova, Vorotnikov et al. 2002). This same study revealed that p38 MAPK activation was essential for urokinase mediated HASM migration, associating the release and disassembly of actin to p38 MAPK phosphorylation of caldesmon.

Other work has shown that p38 MAPK may mediate p21 activated kinase 1 (PAK1) signaling in relation to migration. PAKs are serine/threonine kinases that have been studied for their role in regulating the cytoskeleton and cell migration (Sells, Knaus et al. 1997). An investigation by Dechert et al. linked this activation to downstream activation of p38 MAPK following PDGF stimulation. Here, a catalytically inactive PAK1 mutant reduced p38 MAPK activation and completely abrogated both spontaneous and PDGF induced migration in canine ASM cells compared to cells infected with wild type PAK1. (Dechert, Holder et al. 2001). These studies underscore the essential functions of p38 MAPK signaling in relation to actin dynamics and smooth muscle cell migration.

The ERK1/ERK2 pathway may be indirectly involved in HASM migration. Like p38 MAPK, ERK1/ERK2 has been proven to phosphorylate l-caldesmon leading to migration; however, this work was done in vascular smooth muscle cells and has not yet been shown in airway smooth muscle (Jiang, Huang et al. ; Yamboliev and Gerthoffer 2001). Another more recent study has shown that ERK1/ERK2 may mediate migration via MMP production (Ito, Fixman et al. 2009). MMPs are matrix metalloproteinases, proteases that regulate extra-cellular matrix composition. Tissue inhibitors of MMPs (TIMPs) effectively inhibit these enzymes by

binding to their catalytic domain (Massova, Kotra et al. 1998). In this investigation, HASM cells treated with PDGF and TGF-β showed significant increases in MMP-1, MMP-3 and TIMP-1 mRNA that was ERK1/ERK2 dependent. HASM supernatant in which MMP-3 secretion was knocked down via the use of siRNA interference was less effective in causing HASM migration compared to controls. The processes underlying MMP-3 mediated migration have yet to be elucidated.

1.4.3 Air Smooth Muscle Mechanics: MAPK regulation

Since the discovery that MAPKAP-K2 phosphorylates and activates HSP27, much attention has turned to how p38 MAPK affects cytoskeletal remodelling. In the context of asthma, this remodelling is of interest as it pertains to the 'plasticity-elasticity' balance. Normal airway smooth muscle is described as 'plastic', indicating a normal response to being stretched following bronchoconstriction. Asthmatic smooth muscle is defined as 'elastic', as deep inspiration does not return the airway to a larger diameter, but rather to its original narrowed size, much as an elastic band would. The response to mechanical stretch in ASM has focused on the length of actin filaments. Shorter filaments are suggested to favour a more plastic behavior as they would have to dissemble from a parallel arrangement to one in series in order to stretch, resulting in loss of rigidity and strength. Longer filaments would be able to remain in parallel, resulting in greater force and a less flexible phenotype in response to stretch (reviewed in (Kotlikoff, Kannan et al. 2004) .

One of the earliest studies to support this idea came from Lakser et al. who demonstrated that bovine muscle strips treated with SB203580 increased the degree to which they lengthened following induced load fluctuations (Lakser, Lindeman et al. 2002). This work was confirmed in another study that measured the ability of canine muscle strips to re-lengthen in response to load fluctuations prior to and following treatment with latrunculin-B (which prevents the formation of actin filaments), or jasplankinolide (which stabilizes them) or both. Latrunculin-B treatment resulted in a significant increase in FFIR whereas jasplankinolide alone or in conjunction with Latrunculin-B did not affect FFIR (Mitchell, Dowell et al. 2008). This experiment confirmed the importance of actin length in the FFIR response. As mentioned in the section on p38 MAPK, the

role of HSP27 in its phosphorylated state has been directly connected to canine muscle strip FFIR (Lakser, Dowell et al. 2008).

In a brand new twist, however, the ERK pathway has been found to produce similar results. A study of canine tracheal muscle strips indicated they respond to the ERK1/ERK2 inhibitor U0126 by re-lengthening in response to load fluctuations (Dowell, Lavoie et al.). This change was negatively correlated to h-caldesmon phosphorylation. ERK1/ERK2 has been shown to phosphorylate tropomyosin, an actin filament side binding protein (Houle, Rousseau et al. 2003). Caldesmon enhances actin/tropomyosin binding, contributing to filament stability by protecting actin from the severing protein gelsolin (Ishikawa, Yamashiro et al. 1989). The disassociation of these two proteins from actin following phosphorylation by ERK, leading to shorter filaments, may explain the re-lengthening observed by Dowell et al., however, these mechanisms remain to be confirmed.

1.4.4 Regulation of proliferation in HASM by MAPKs

The proliferative response in HASM has been extensively examined due to its role in airway remodelling. A number of inflammatory mediators and growth factors expressed in asthmatic airways are known mitogens for ASM when examined *in vitro*. As expected, these studies have also established the requirement of the ERK1/ERK2 pathway in cell proliferation by controlling the expression of cyclin D1, an enzyme required for cell cycle progression though the G1/S phase. (Ramakrishnan, Musa et al. 1998; Page, Li et al. 1999; Ravenhall, Guida et al. 2000; Lee, Johnson et al. 2001)

However, an examination of p38 MAPK mitogenic signaling provides conflicting results. One study shows a hypo-proliferative role for p38 MAPK shown by decreased expression of PDGF-induced cyclin D1 in bovine ASM (Page, Li et al. 2001). In contrast, a murine *in vivo* model used a specific p38 MAPK inhibitor (SD282) in ovalbumin challenged mice, and cell proliferation was assessed at necropsy. The ASM cell count was decreased in animals provided with the p38 MAPK inhibitor under ovalbumin challenge compared to mice treated with ovalbumin and vehicle , suggesting proliferation was p38 MAPK dependent (Nath, Leung et al. 2006).

In human airway smooth muscle research shows p38 MAPK can modulate proliferation and may depend on the mitogen(s). A recent study has shown that a naturally occurring mixture

of mitogens and growth factors, as excreted by epithelial cells in co-culture with HASM, significantly increase proliferation. This proliferation is significantly reduced upon incubation with SB203580 (10 μM) (Malavia, Raub et al. 2009). In other work, cells stimulated with basic fibroblast growth factor (bFGF) demonstrated a significant reduction when treated with either SB203580 or SB202190 (10 μM). Interestingly, these changes to proliferation occurred independently of cyclin D1 protein and mRNA levels, indicating regulatory processes exist that still need to be determined (Fernandes, Ravenhall et al. 2004). This work contrasts with a study that shows TGF-β- induced proliferation was potentiated when SB203580 (10 μM) was used in cultured HASM (Xie, Sukkar et al. 2007). Proliferation was also examined by Salinthone et al. in a novel approach. Here the p38 MAPK pathway was blocked by SB239063 in serum-treated HASM cells with no change to levels of proliferation. However, overexpressing wild type HSP27 decreased the levels of proliferation but increased cell survival by upregulating the anti-oxidant glutathione, shedding light on the possible changes that may be occurring during the determination of cell counts (Salinthone, Ba et al. 2007).

Many studies have confirmed that dexamethasone decreases HASM cell proliferation without affecting the ERK1/ERK2 pathway (Stewart, Fernandes et al. 1995; Fernandes, Guida et al. 1999). Since it is well established that dexamethasone increases DUSP1 levels, and DUSP1 preferentially deactivates p38 MAPK, this suggests that DUSP1 controls the proliferative role of p38 MAPK.

1.5 Rationale, Objectives

There is a strong link between the p38 MAPK pathway and the treatment of asthma. This has been evidenced by the upregulation of DUSP1 with corticosteroid treatment, the mainstay of asthma management. The main role of DUSP1 is to deactivate p38 MAPK, which activates downstream kinases that have both inflammatory and anti-inflammatory arms. The processes under the control of these two arms may be dysregulated in asthma. Alternatively, it is possible the runaway inflammation seen in the asthmatic airway is a result of impaired DUSP1 activity, a topic that continues to be intensely investigated.

Previous work in our own laboratory confirmed the activation of p38 MAPK in epithelial and muscle cells of asthmatic bronchial biopsies. Therefore I investigated p38 MAPK activation and its roles in airway smooth muscle cell function, within the context of asthma. Smooth muscle

was chosen as it provides insight into the processes of cytokine production, migration of inflammatory cells, remodelling and airway hyperresponsiveness in asthma.

The first objective of this project was to characterize the activation of p38 MAPK and its two downstream arms within airway smooth muscle in response to stimulation with TNFα. I decided to monitor the parallel signaling pathways of ERK1/ERK2 and NFκB in order to obtain a more complete view of inflammatory signaling. A second objective was to measure the expression of genes involved in both the positive and negative regulation of inflammation that were mediated by p38 MAPK, ERK1/ERK2 or treatment with the GC dexamethasone. The final objective was to understand which functional processes such as migration, proliferation, velocity of shortening or FFIR might be affected by p38 MAPK. With this information I wanted to interpret how the inhibition of MAPKs might be of therapeutic benefit in treating refractory asthma.

Chapter 2: Materials and Methods

2.1 Materials

The following were obtained from Invitrogen, (Carlsbad CA): 10,000 Units/ml Penicillin + 10,000 μg/ml Streptomycin, Amphotericin B 250 μg/ml, Gentamicin, DMEM (Dulbecco's Modified Eagle Medium: 4.5 g/L D-glucose, (+) L-glutamine, 110 mg/L sodium pyruvate), HAM's F-12, HBSS (Hanks Buffered Saline Solution), 0.25% trypsin-0.04% EDTA, qualified foetal bovine serum (FBS), Ultra-Pure water, Trizol, DNase I Amplification Grade, EDTA, Random Primers, Deoxynucleotides, Super-script II, DTT, RNAse out.

Agonists were obtained as follows: TNFα from Enzo Life Sciences (Plymouth Meeting, PA), CXCL12, TGFß and PDGF- BB from R&D Systems (Minneapolis, MN), IL-17 A from Biovision (Mountain View, CA) and tert-butyl hydroperoxide from Sigma-Aldrich (Oakville, Ontario).

Inhibitors were obtained as follows: BIRB0796 was kindly provided by Professor Sir Philip Cohen (Medical Research Council Protein Phosphorylation Unit, University of Dundee, UK). SB203580 was purchased from InvivoGen (San Diego, CA). PD184352 was bought from US Biological (Swampscott, MA). Dexamethasone was kindly provided by Dr. Q. Hamid (Meakins Christie Research Institute, McGill University, Montreal,QC).

2.2 Methods

2.2.1 Human Airway Smooth Muscle Cell Culture

The local Institutional Review Board approved use of these cells for research purposes. Primary human airway smooth muscle cells were obtained from donors of human surgical and lung transplant specimens. Tracheal-bronchial sections measuring 5 x 5 mm were incubated under gentle agitation in Hanks buffered saline solution containing (HBSS) collagenase type IV (0.4 mg/ml), soybean trypsin inhibitor (1 mg/ml), and elastase type IV (0.38 mg/ml) (all from Sigma). The digested tissue was then filtered through a 125-μm Nytex mesh, and the resulting cell suspension was centrifuged at 200 *g* for 5 minutes. The pellet was suspended in DMEM: F-

12 medium containing 10% FBS (v/v), 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B and 50μg/ml gentamicin, and then plated in 25-cm^2 culture flasks (Sarstedt, Newton, NJ). Cells were cultured at 37º C in 5 % CO_2. The medium was changed daily for 4 days and then substituted for DMEM: F12 containing 10% FBS (v/v), 100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B which was changed every 48 hours until cells became confluent. Cells (passage 1) were identified as smooth muscle cells by positive immunohistochemical staining for smooth muscle α-actin followed by positive identification of calponin and smooth muscle myosin heavy chain by immunoblotting.

Cells from passage 1 through 6 were then subcultured by discarding the media, rinsing twice with HBSS and detaching with 800 μL 0.25% trypsin-0.04% EDTA solution per flask. The trypsin was blocked using medium with 10% FBS (v/v) followed by centrifugation for 5 minutes at 200 *g*. The pellet was re-suspended and cells were counted using a standard haemocytometer with trypan blue exclusion and plated at a density of 20,000 cells/well in six well tissue culture plates (Sarstedt, Newton, NJ) in fresh 10% FBS DMEM: F12 medium. Cells were left to grow to 100% confluence for protein extraction and quantitative real time PCR. For immunofluorescent staining, cells were plated at a density of 3000 cells per micro chamber on Labteck II 8-well microchamber permanox slides (Nalge Nunc International, Rochester, NY) and left to grow to 80% confluence. For all experiments the media was changed for DMEM: F-12 with 0.1% FBS (v/v) over a minimum of 48 hours. Serum-deprived cells were treated with the concentrations of agonists as described. All experiments included a control containing the vehicle Dimethyl Sulfoxide (DMSO), 1:1000 (v/v) (Fisher Scientific, Fairlawn, NJ).

2.2.2 Proliferation assay

Cell proliferation was determined via immunofluorescent staining of incorporated bromodeoxyuridine (BrdU), a thymidine analog, using a commercially available kit (FITC BrdU Flowkit, BD Sciences, San Diego, CA). Human airway smooth muscle cells were grown to confluence, detached using 0.25% trypsin-EDTA, centrifuged and re-suspended. Cells were then counted and seeded in 6 well tissue culture plates at a density of 1 x 10^4 cells per well and allowed to grow for three days or until 40% confluent. The medium was then changed to DMEM: F-1 with 0.1 % FBS for 24 hours to synchronize the cell cycle. All experiments included a 10% FBS condition as a positive control, and a 10 % FBS stained with isotype as a

negative control. Baseline staining was assessed from a 0.1% FBS control. Inhibitors used were BIRB0796 at 0.1μM or PD184352 at 2.0 μM one hour prior to the addition of agonists. PDGF-BB 15 ng/ml, TGFß 1 ng/ml or CXCL 12 80 ng/ml were then added to each well in duplicate and left to incubate for six hours at which time 10 μg/ml BrdU was added to the cell medium and left for an additional 18-hour incubation period. Cells were then harvested by trypsinization and frozen overnight at -80ºC in freezing media containing 10% DMSO and 90% FBS (v/v). The samples were thawed no sooner than 12 hours later, washed to remove the freezing media and treated with 30 μg/ml DNase to expose the BrdU epitopes. Samples were stained in darkness with a FITC-conjugated anti-BrdU mouse monoclonal antibody diluted 1:50 as per manufacturer's instructions for 30 minutes at room temperature. The cell samples were then washed, and acquisition of fluorescent signal was measured on a FACS Calibur flow cytometer using Cell Quest Pro software (BD Biosciences, San Diego, CA) on 10,000 cells per condition in duplicate. Results were expressed as percentage of BrdU positive cells.

2.2.3 Migration Assays

2.2.3.1 Neutrophil chemotaxis Assay

Neutrophils migration assays were performed using a modified Boyden chamber (Transwell, Fisher Scientific) with 5 μm pores. HASM cells were pre-treated for 1 hour with 0.1 μM BIRB0796 or 2.0 μM PD1843522 before being stimulated with TNFα 10 ng/ml for 2 hours. Additionally, cells were treated with each inhibitor alone or vehicle DMSO 1:1,000 (v/v). The cells were then washed and the medium was replaced by serum free DMEM-F12. The cells were then left for an additional 18 hour incubation period at 37°C. Supernatants were collected and the cells discarded. The conditioned media of each well was added to the lower chamber of each transwell. Fresh human neutrophils (2×10^5) that were concurrently isolated from peripheral blood as described previously (Powell, Gravel et al. 1993) were deposited in the upper well. The chamber was placed in a humidified incubator under 5% CO_2 for 5 hrs at 37°C. Cells located on the upper surface of the membrane were scraped off with a cotton swab, and the cells that had crossed the polycarbonate filter were collected from the lower compartment and counted with a haemocytometer. Results are represented as a percentage of migrated cells.

2.2.3.2 HASM Migration Assay

HASM cell migration was evaluated using a 48-Well Micro Chemotaxis Chamber (Neuro Probe, Gaithersburg, MD). In this system, chemo-attractants placed in lower wells differentially attract cells in a suspension on the other side of a membrane in the upper wells. Cells that migrate through the membrane are stained and counted. Primary human airway smooth muscle cells, passages 1 though 6, were grown to confluence and serum starved as described above. Cells were then detached using 0.05% trypsin, re-suspended in 10% FBS medium (v/v) to neutralize the trypsin, centrifuged for 10 minutes at 180 *g* and re-suspended in serum free medium supplemented with 0.5% bovine serum albumin (BSA). Cells were then counted, centrifuged as before, and resuspended in serum free medium containing 0.5% BSA and either DMSO (1:1000 v/v) or 0.1μM BIRB0796. Cells were then left to agitate gently at room temperature for 30 minutes in order for the p38 MAPK inhibitor to permeate the cells.

A 25 mm x 80 mm polycarbonate membrane (Neuro Probe) with 8 micron pores was coated with 10 ml of 0.01% Type I collagen (Inamed, Fremont CA) solution and left to air dry for 15 minutes at room temperature. The bottom wells of the chamber were filled with 28 μl of medium containing CXCL 12 or PDGF-BB which also had either DMSO or BIRB0796. Each condition was loaded in triplicate to ensure replication. Three wells with medium containing only DMSO 1:1000 (v/v) were used to assess baseline migration. The chamber was then assembled with the membrane serving to separate the bottom wells with their media from the top wells. Upper wells were then loaded with cell suspensions either containing BIRB0796 or DMSO at a density of 50,000 cells per well in 50 μl total volume of media or 6,000 cells/mm^2. The chamber was then incubated for 4 hours at 37ºC in 5% CO_2.

The chamber was then disassembled and the membrane was removed. The upper side was scraped clean of all non-migrated cells and left to dry for 10 minutes as room temperature before being fixed in 4% paraformaldehyde (Sigma- Aldrich) for 10 minutes. The membrane was then rinsed in phosphate buffered solution (PBS) and let dry. To stain the cytoplasm of the HASM cells the membrane was immersed in an eosin stain solution for 15 seconds, followed immediately by 15 seconds staining in a methylene blue solution which primarily colours the nucleus (Protocol Hema 3)(Fisher Scientific). The stained membrane was thoroughly rinsed in distilled water to remove background staining, allowed to dry and then mounted on a 75 mm x 50 mm microscope slide with 35 mm x 50 mm coverslip (Fisher Scientific) using Cryoseal mounting medium (Thermo Scientific, Nepean Ontario). The slide was then visualized by

microscopy under 400X magnification, and cells in each well were counted manually in 5 separate fields to obtain an average value of migrated cells per well.

2.2.4 RNA isolation

HASM cells were disrupted using 1 ml Trizol per well. The RNA was then extracted by the addition of 200 µl chloroform (Fisher) followed by centrifugation for 15 minutes at 12,000 X g at 4 º C. The aqueous phase was then carefully pipetted off the organic phase, transferred to a new micro-centrifuge tube and 500 µl isopropanol (Fisher) was added to precipitate the RNA using centrifugation for 10 minutes at 12,000 X g at 4 º C. The supernatant was decanted and the RNA pellet was washed with 1 ml 75% ethanol (Commercial Ethanols, Brantford, Ontario), vortexed briefly and then centrifuged for 5 minutes at 12,000 X g at 4 º C. The supernatant was again decanted with traces of ethanol being removed via pipette, the sample was then left to dry for 10 minutes at room temperature before being rehydrated in 10 µl sterile water. The diluted RNA was then cooled on ice for 10 minutes, heated for 10 minutes at 65 º C to dissolve and quantified using a nano-drop (Thermo Scientific) system.

2.2.5 Reverse Transcription and Quantitative real-time PCR

1 µg total RNA was treated with 1 µl DNase I Amplification Grade to remove endogenous DNA. The reaction was stopped using EDTA and a 10 minute incubation at 65ºC. To create double stranded template sections on the single strand of RNA, random primers as well as all four deoxynucleotides were added and left to bind for 10 minutes at 65º C. 1 µl Super-script II reverse transcriptase was then added to reverse transcribe cDNA from the RNA, as well as 2 uL of the reducing agent DTT and 1 µl RNAse out to remove possible contaminant ribonucleases. The samples were left to incubate for 10 minutes at 25 º C, heated for 50 minutes at 42 º C for the polymerase to extend the double strand and then heated further to 70 º C to inactivate the enzyme in a BioRad My Cycler thermal cycle (BioRad, Hercules, CA). The resulting cDNA was then quantified and normalized to 1 µg/µl.

Alternatively, tissue samples of RNA was extracted using a RNeasy micro kit (Qiagen, cat #74004, Mississauga, Ontario) from patient samples of adult endobronchial tissue specimens

obtained from the Fonds de recherche en santé du Quebec (FRSQ) Respiratory Health Network Tissue bank (MCI/Meakins-Christie Laboratories Tissue bank, McGill University, Montreal, Québec). Written consent was obtained from all subjects as was approval for the use of these tissues by the hospital ethics committee. Respiratory physicians made the clinical diagnosis of asthma (mild or severe) based on the American Thoracic workshop for Refractory Asthma(ATS 2000).

For quantitative real-time PCR samples were tested in a 96 well plate with each condition containing 200 ng cDNA in a total volume of 2.5 µL sterile water with 0.3 µM of each forward and reverse primer (Integrated DNA Technologies, Coralville, IA- see Table 2), 5 µl iTAQ SYBR Green Supermix with Rox (BioRad) as well as 1.9 µl sterile water. The plate was sealed and cycled as follows using a Step-One-Plus machine (Applied Biosystems, Foster City, CA): 95 º C for 10 min, then 50 cycles of 95 º C for 10 seconds and 60 º C for 45 seconds. Each condition was tested in duplicate and plated in duplicate using the housekeeping gene GAPDH for normalization. Relative fluorescence and therefore gene amplification was interpreted as fold induction from cycle threshold values using the Pfaffl mathematical model. Primer efficiencies were determined using a standard curve generated from a 3 fold serial dilution of HASM cDNA.

Table 2

Gene	Forward primer (5 prime -3 prime)	Reverse primer (5 prime -3 prime)
Alpha SMA	GAAGGAATAGCCACGCTCAG	ACCCACAATGTCCCCATCTA
Actin B	CTCTTCCAGCCTTCCTTCCT	AGCACTGTGTTGGCGTACAG
ATF 3	TGGAGAAGCTGGAAAGTGT	TCTGGAGTCCTCCCATTCTG
GAPDH	AGCAATGCCTCCTGCACCACC	TCGGAGAAGCTGGAAAGTGT
GMCSF	ACTACAAGCAGCACTGCCCT	AAGGGGATGACAAGCAGAAA
Gro-alpha	AGGGAATTCACCCCAAGAAC	TAACTATGGGGGATGCAGGA
IkB alpha	GCTGATGTCAATGCTCAGGA	CCCCACACT TCAACAGGAGT
IL-6	GTGTGAAAGCAGCAAAGAGG	TGCAGGAACTGGATCAGG
IL-8	GTGCAGTTTTGCCAAGGAGT	CTCTGCACCCAGTTTTCCTT
IL-33	CAAAGAAGTTTGCCCCATGT	AAGGCAAAGCACTCCACAGT
IL-1 beta	GGACAAGCTGAGGAAGATGC	TCGTTATCCCATGTGTCGAA
MKP1	CTGCCTTGATCAACGTCTCA	ACCCTTCCTCCAGCATTCTT
SM 22 (transgelin)	ATGACATGCTTTCCCTCCTG	AAGAATGATGGGCACTACCG
TNFAIP3 (A 20)	ATGCGGAAAGCTGTGAAGAT	TCCAGTGTGTATCGGTGCAT
Tubulin	AATTCCAGACCAACCTGGTG	CTCAAAGCAAGCATTGGTGA

2.2.6 Immunofluorescence

After stimulation, HASM cells in each well of a Labteck II microchamber slide were washed gently with PBS 1X to remove all traces of media, and then fixed by the addition of approximately 400 µL per chamber of 3.7% paraformaldehyde in PBS for 20 minutes. The paraformaldehyde was removed, the cells washed twice with PBS and then permeabilized to allow for greater anti-body penetration using a solution of 0.1% saponin in PBS for 15 minutes. To block non specific binding sites and reduce background staining this was followed by 30 minutes of incubation with a 5% milk 0.1% saponin solution. The primary monoclonal mouse antibody α–SMA (Sigma, Catalogue # A2547) was diluted in a 1% milk 0.1% saponin PBS

solution at a concentration of 1:400 and applied for an incubation period of 60 minutes. The cells were then washed 6 times with PBS before a second staining with Alexa568 Phalloidin 0.67 µg/ml (Invitrogen, Cat #A12380) and goat anti-mouse Alex Fluor488 40 µg/ml (Invitrogen) fluorescent anti-body was added to each chamber and left to incubate in the dark for 60 minutes. The slide was then washed rapidly 4 times with PBS and incubated with a nuclear staining of 5 µg/ml Hoescht (bis-benzamide) (Sigma cat# B2883) for 5 minutes in darkness. The slide was then washed again, the chamber walls removed and cells were mounted using Permafluor aqueous mounting media (Thermo scientific). Cells were visualised using 200X magnification through Olympus BX51 filters. Images were captured with Image pro software 6.0.

2.2.7 Immunohistochemistry

Five micron thick sections of bronchoscopic biopsies from asthmatic patients were obtained from **t**he tissue bank of the Respiratory Health Network of the FRSQ. Slides were first de-paraffinated in xylene for 20 minutes before being rehydrated in decreasing concentrations of alcohol. The slides were rinsed with PBS and then subjected to 12 minutes of heat induced epitope retrieval using 10 mM citrate buffer, pH 6.0, in order to break cross linking that is induced by formalin fixing and maximally expose possible anti-body binding sites. The sections were then permeabilized to improve antibody penetration by incubating in a solution of 0.2 % Triton/PBS for 30 minutes. Removal of endogenous peroxidases was achieved by incubating samples in 5% H_20_2. This was followed by blocking of non specific binding sites with Universal Blocking Solution for 30 minutes (Dakocytomation, Mississauga, Ontario). Primary antibodies were then added for overnight incubation at 4 ° C diluted in Dako antibody diluent (Dakocytomation) as follows: 0.01µg/ml Phospho- MAPKAPK2 (Thr 334) (Polyclonal Rabbit Cat # 3007 Cell Signaling Technology, Danvers, MA), 1:1500 ATF3 (Polyclonal Rabbit, Cat # 600-401-493 Rockland, Gilbertsville, PA), 0.126 µg/ml Phospho- ERK (Monoclonal Rabbit, Cat # 4370, Cell Signaling Technology), and 0.5µg/ml MKP-1 (Polyclonal rabbit Cat # 07535 Upstate, Lake Placid, NY), 1:50 Phospho- MSK1 (Thr 581) (Polyclonal Rabbit Cat # 9595, Cell Signaling), 2.5 µg/ml CXCR4 (Monoclonal mouse, Cat # MAB172, R&D Systems). Isotype controls were prepared for each sample by replacing the primary antibody with a non specific immunoglobulin from rabbit or mouse at the same concentration. The slides were rinsed of their primary anti-bodies and a biotinylated anti-rabbit or anti-mouse antibody was applied at a dilution of 1:100 (Dakocytomation). This second layer was left to incubate for 45 minutes at

room temperature. The slides were again rinsed before a third layer of 1:100 streptavidin horseradish peroxidase complex was added (Dakocytomation). Immunoreactivity was developed with diaminobenzidine chromogen (Dakocytomation). The slides were counterstained with haematoxylin and saturated lithium carbonate for 3 seconds to stain the nuclei of cells. The slides were dehydrated in increasing concentrations of alcohol with the final step in xylene prior to being mounted for imaging.

2.2.8 Cell Lysis and Immunoblotting

Immediately following stimulation, HASM cells were put on ice and supernatants were collected and frozen at -20 º C. The cells were then rinsed with ice-cold PBS and each well was lysed in 80 µL buffer containing 50 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (v/v) Triton x-100, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.27 M sucrose, 5mM sodium pyrophosphate decahydrate, one complete miniprotease inhibitor mixture and 2 mM dithiothreitol. Extracts were clarified for 5 minutes at 4 º C at 12,000 X *g* to pellet the cell debris and leave soluble proteins in the supernatant. Supernatants were then added to loading buffer (0.24 mM Tris-HC, 8% SDS, 40% glycerol and 36% distilled water) containing 1X TCEP (Thermo Scientific). A portion of the supernatant was retained for quantification using the Bradford method.

Quantified and normalized proteins were boiled for 5 minutes at 95 º C. For each gel 30 µgs of protein were deposited per well and separated by SDS-PAGE on a 10 % Pro-pure Next Gel with Pro-Pure Running Buffer (Amresco, Solon, OH) using BioRad Powerpac HC (150 V/3.0 A/300W) at 150 volts for 90 minutes. The proteins were then transferred from the gel to a nitrocellulose membrane using ice cold transfer buffer for 35 minutes at 100 Volts. The membranes were rinsed in Ponceau (Sigma) to reveal successful protein transfer, rinsed in PBS and then blocked for one hour in Odyssey Blocking Buffer (Li-Cor Biosciences, Lincoln, NE) for one hour at room temperature to block non specific binding sites on the membrane. Primary antibodies were applied as listed in Table 3 and left overnight to incubate at 4 º C or alternatively at room temperature for 90 minutes. The primary antibody was then washed off using PBS with 0.05% Tween, and secondary antibodies of goat anti-rabbit IgG (DyLight^{TM}800 , Cat #35571 Thermo Scientific) and goat anti-mouse IgG DyLight^{TM}680 (Cat #35518, Thermo Scientific) were applied for 45 minutes in the dark at room temperature at a dilution of 1:15,000. The membranes were washed as before and the signal was detected and quantified using a Licor

Odyssey imaging system. All protein signals detected were normalized to those proteins listed in results.

Table 3

Antibody	Details	Catalogue number	Company
p-38/SAPK2	Monoclonal mouse dilution 0.4 ng/ml	05–454	Millipore(Temecula, CA)
Phospho-p38 MAPK (Thr^{180}/Tyr^{182})	Polyclonal rabbit dilution 1:1000	09-272	Millipore
Phospho-MAPKAPK2 (Thr^{334})	Polyclonal rabbit dilution 2 ng/ml	3007	Cell Signaling Technology
Phospho-MSK1 (Thr^{581})	Polyclonal rabbit dilution 1:1000	9595	Cell Signaling Technology
MKP1	Polyclonal rabbit dilution 0.5 µg/ml	07535	Upstate (Lake Placid, NY)
IKB α	Polyclonal rabbit dilution 1 µg/ml	06-494	Millipore
CXCR4	Mouse Monoclonal dilution 5 µg/ml	MAB172	R&D systems
GAPDH	Monoclonal mouse dilution 0.25µg/ml	MAB374	Millipore
p44/42 ERK MAPK	Monoclonal mouse dilution 0.3 ng/ml	9107	Cell Signaling Technology
Phospho-ERK Thr^{202}/Tyr^{204}	Monoclonal rabbit 25 ng/ml	4370	Cell Signaling Technology
Normal Rabbit IgG	0.4 µg/µl diluted as per experimental sample	2027	Santa Cruz

2.2.9 Enzyme Linked Immunosorbent Assay (ELISA)

Human IL-8 DuoSet ELISA kits were purchased from R&D Systems (Minneapolis, MN) for IL-8 detection. High affinity 96-well binding plates were coated with 100 µL per well of IL-8 anti-body at 4.0 µg/ml (NUNC-Immuno, Thomas Scientific, Swedesboro, NJ) and left to incubate for 12 hours. After incubation the plate was washed three times in PBS 1X with 0.05% Tween 20 and non-specific binding sites were blocked using 300 uL reagent diluent containing 1% BSA for one hour. Supernatants collected during TNF alpha stimulations were centrifuged for 3 minutes at 10,000 g before 100 µL per well was used in duplicate for each condition and left to bind for two hours along with a standard dilution of provided recombinant IL-8. The plates were again washed and treated with 100 µL/well of IL-8 detection antibody (20 ng/ml) and let bind for two hours at room temperature. This anti-body was washed off as before and the plate was then treated for 20 minutes with 50 µL/well horse radish peroxidase solution (1:200) that binds to the detection antibody. After the excess is washed off, 100 µL of substrate containing H_20_2 for the peroxidase to cleave was added to each well. The substrate was left on for 20 minutes which, once cleaved, resulted in a color change that was stopped by the addition of 50 µL 2N sulfuric acid/well. The plate was then read at 450 nm in a Tecan Infinite M1000 plate reader using icontrol software.

2.2.10 Rat tracheal muscle strip velocity and re-lengthening (FFIR).

Ten week old male Lewis rats (Harlan, Indianapolis, IN) were sacrificed and the trachea was dissected out and placed on ice in a calcium free Krebs solution (composition in nM: NaCl: 118, KCl: 4.5, KH_2PO_4 : 1.2, $MgSO_4$: 2.5, $NaHCO_3$: 25.5 and Glucose: 10.0) at a pH of 7.4; aerated with 95% O_2 / 5% CO_2 for a minimum of 30 minutes. Entire muscle strips on adjacent rings were then removed and cleaned, although the epithelium was left intact. In situ lengths of each strip were measured before being attached using aluminium foil clips between a force transducer (model 404A, Aurora Scientific, Ontario) and a length controller (model 322C-I, Aurora Scientific, Ontario) within a bath of ciruculating and aerated Krebs solution maintained at 37 º C. Strips were then stretched to their reference length as follows:

unstretched length x 1.25 = L0

and clamped at this length for a period of 30 minutes during which time each strip was equilibrated using a protocol of electric field stimulation (50 Volts, 5ms pulse, 30Hz) for 10 seconds every 4 minutes. During the set up and equilibration, strips were bathed in a Krebs solution containing calcium (2.5mM $CaCl_2$) as well as either 0.1 µM BIRB0796 or DMSO 1: 10,000 (v/v) such that the muscle was exposed to the inhibitor or vehicle for at least one hour prior to challenge. Healthy muscle contracted normally during this time and was considered viable for continuation with the experiment. The muscle was thus considered in its optimal state to respond to challenge.

The circulating Krebs solution was then changed for one containing $5x10^{-4}$M Methacholine (Sigma) with and without 0.1 µM BIRB0796. The muscle force was measured over 60 seconds until maximum force was obtained. Quick releases of 0.2 FMax (20 % of maximum force) were then allowed for 110 ms and stretch back to original length was allowed for 1 second. This was repeated every minute for 30 minutes. Velocity measurements taken during this time are represented as change in L0 per second.

To measure re-lengthening the muscle was then clamped at 32% FMax and allowed to shorten for 20 minutes in a circulating bath containing Krebs solution and either inhibitor or vehicle as before. The strip was then subjected to a FFIR (force fluctuation induced relengthening) protocol (Fredberg, Inouye et al. 1999) of sinusoidal oscillations over 10 minutes of 2Hz +/- 0.25 FMax. Measurements of muscle length were continuously recorded during this time and expressed as length over reference length (L0) changing over time.

2.2.11 Statistical Analysis

Statistical analysis was performed using Graph Pad software. One way analysis of variance followed by a multiple comparison test (Bonferroni) was used to test differences between groups. Any p value < 0.05 was considered significant.

Chapter 3: Results

3.1 Introduction

Previous work from our laboratory revealed strong activation of the p38 MAPK pathway in bronchoscopic biopsies from asthmatic patients (Lucie Roussel, personal communication). Specifically, immunohistochemical analysis revealed the phosphorylation of MAPKAP-K2 and its downstream target HSP-27 in both epithelial and human airway smooth muscle cells were increased in severe asthmatic subjects versus non asthmatic individuals (Figure 3).

Thus a primary line of investigation was undertaken on bronchial biopsies from asthmatic and non asthmatic patients in order to assess the expression of p38 MAPK and its downstream targets in these populations. These findings, at both the mRNA and protein level were supported by further study in primary cultures of human airway smooth muscle cells. This work better characterized the response of p38 MAPK, ERK1/ERK2 and the NFκB pathway to TNFα, a potent pro-inflammatory cytokine implicated in the pathology of refractory asthma (Berry, Brightling et al. 2007).

3.2 Results

3.2.1 Immunohistochemistry: evaluation of non, mild and severe asthmatic biopsies.

Bronchoscopic biopsies from non, mild and severe asthmatics were probed for ERK1/ERK2, targets of the p38 MAPK pathway, their common downstream target MSK1 and the phosphatase DUSP1 (Figure 3A through D). Positive staining was assessed as increased brown coloration of either the cytoplasmic contents or nulei and is indicated by arrows in each figure.

Severe asthmatics had the most overall staining of phospho-MAPKAP-K2, followed by mild asthmatics and then non-asthmatics. Positive staining was observed in airway smooth muscle cells as well as the epithelium, and this staining was strongest in the nucleus.

The activation of ERK1/ERK2 was only witnessed in a few muscle cells of non-asthmatics and overall severe asthmatics had more staining than either mild or non-asthmatics.

This staining was mostly nuclear. The epithelium showed both cytoplasmic and nuclear staining in non and mild asthmatics, but was less pronounced in the epithelium of severe asthmatics. Endothelial cells were evident in the biopsy from the severe asthmatic biopsy and showed strong nuclear and cytoplasmic staining of activated ERK1/ERK2.

P-MSK1 had predominantly nuclear staining of the epithelium in all three samples, and there appears to be stronger epithelial staining in the severe asthmatic. However, the muscle cells showed very little staining in non asthmatics, while mild and severe asthmatics appeared to have an overall increase in coloration which was mostly seen in the nucleus.

Finally, DUSP1 staining was strongest in the epithelium of mild asthmatics, both in the nuclear and cytoplasmic compartments, and less evident in the epithelium of severe or non-asthmatics. DUSP1 was strongly evident in the cytoplasm of smooth muscle cells from all samples; however, it appeared to be most pronounced in the nuclei of the severe asthmatic biopsies. Isotype controls confirmed the anti-body specificity (Figure 3E).

The overall staining of these samples indicated that the p38 MAPK pathway was active and that its main negative regulator DUSP1 was expressed more in the epithelium and muscle of mild and severe asthmatics respectively. It was thus necessary to confirm whether or not this staining was indicative of DUSP1 expression at the mRNA level.

3.2.2 DUSP1 mRNA levels are increased in mild and severe asthmatics

Asthmatics patients are often prescribed glucocorticoids which have been shown to enhance the expression of DUSP1 (Bhattacharyya, Brown et al. 2007). In conjunction with findings in the immunohistochemical analyses that indicated higher protein expression of DUSP1 in asthmatics, we decided to verify DUSP1 transcript levels in bronchoscopic biopsies from mild and severe asthmatic patients compared to non-asthmatics. Figure 4 shows these results as analyzed via quantitative real time PCR: both mild and severe asthmatics had significantly increased levels of DUSP1 compared to non-asthmatics. While the mild asthmatics had the highest overall levels, this group was not significantly different from severe asthmatics. These results confirmed our findings that DUSP1 is up-regulated in these patients, suggesting this phosphatase is attempting to dampen the inflammatory signal of p38 MAPK and ERK1/ERK2. Our focus therefore turned to an *in vitro* cell model where we could better understand the regulatory functions of these MAPKs.

3.2.3 TNFα drives CXCL8 synthesis in HASM more potently than IL-17A

Both the IL-17 and TNFα axes have been proposed to contribute to some of the pathological features of asthma (Molet, Hamid et al. 2001; Berry, Hargadon et al. 2006), including the synthesis of CXCL8 which results in the chemotactic migration of neutrophils (Roussel, Houle et al.). In order to find an appropriate *in vitro* model with which to explore our preliminary findings, primary cultures of HASM cells were incubated for 4 hours in the presence of either IL-17A (20ng/ml) or TNFα (10 ng/ml) and the expression of CXCL8 was measured both at the gene and protein level.

Figure 5 shows that incubation with IL-17A resulted in no real change of CXCL8 gene expression, while incubation with TNFα resulted in a two hundred fold increase. Secreted protein measured from the supernatants of these cells confirmed that CXCL8 is not secreted via stimulation with IL-17A, whereas CXCL8 is secreted four fold over control with TNFα stimulation. These findings determined that it was better to stimulate HASM cells with TNFα than IL-17 in order to provoke an inflammatory reaction. This cytokine was used to stimulate cells for all experiments involving characterization of the p38 MAPK, ERK1/ERK2 and NFκB pathways.

3.2.4 TNFα activates MAPKs and NFκB in HASM cells

3.2.4.1 Activation of MAPKs and NFκB over time following TNFα stimulation

TNFα (10 ng/ml) was used to stimulate HASM cells in order to confirm that p38 MAPK and ERK1/ERK2 were activated in these cells in response this inflammatory cytokine. Serum-deprived cells from three different donors were stimulated for increasing amounts of time over a period of 180 minutes. The cell lysates were separated by SDS-PAGE and probed for the activated forms of p38 MAPK and its downstream targets MAPKAP-K2 and MSK1, as well as its negative regulator DUSP1. Additionally, further immunoblotting experiments were performed to follow the activation of ERK1/ERK2 and then NFκB, which was assessed via the degradation of its inhibitor IκBα.

Figure 6 shows that the p38 MAPK pathway is strongly stimulated in a single wave like fashion by TNFα with a peak in phosphorylation for all proteins between 15 and 30 minutes.

Phospho-p38 MAPK itself peaks near 15 minutes. MAPKAP-K2 is phosphorylated immediately by p38 MAPK, as its peak of activation is at the same time point. ERK1/ERK2 is maximally activated between 15 and 30 minutes as well, although the results of phosphorylation from one patient's cells was much lower at 15 minutes than 30, resulting in larger error when analyzing the data between these two time points. MSK1 is phosphorylated by both ERK1/ERK2 and p38MAPK, so its highest state of activation near 15 minutes correlates nicely with both upstream catalysts. Of interest, DUSP1 levels do not change significantly over time, although there is a slight increase at 15 minutes followed by a gradual decline to its lowest level by 90 minutes.

NFκB activation was confirmed by the degradation of its inhibitor IκBα which was reduced to its lowest level between 15 and 30 minutes. These preliminary experiments confirmed that TNFα was an appropriate agonist to bring about p38 MAPK, ERK1/ERK2 and NFκB signaling that could be tracked over time.

3.2.4.2 Glucocorticoids reduce p38 MAPK activity but not ERK1/ERK2 or NFκB

In order to judge the ability of glucocorticoids to increase DUSP1 levels and prevent MAPK activity in both p38 MAPK and ERK1/ERK2 signaling, it was necessary to stimulate HASM cells in the presence of the anti-inflammatory dexamethasone. Due to the uniformity of activation near the 30 minute mark from the results shown in figure 6, the same three cell lines were stimulated with TNFα (10 ng/ml) for 30 minutes after pre-treatment for one hour with 100nM dexamethasone, 0.1 μM BIRB0796 , 2.0 μM PD184352 or vehicle.

Figure 7A illustrates that the phosphorylation of p38 MAPK is reduced by its inhibitor and not by PD184352 as previously reported (Kuma, Sabio et al. 2005). The phosphorylation of p38 MAPK is also reduced by dexamethasone, which was the expected outcome due to the suspected up-regulation of DUSP1. Figure 7C shows that MAPKAP-K2 is reduced in the presence of BIRB0796 and dexamethasone, which suggests the diminished function of its upstream catalyst p38 MAPK. Due to a low number of experiments, statistical significance was not achieved. ERK1/ERK2 phosphorylation is only slightly reduced by pre-treatment with dexamethasone and increased in the presence of p38 MAPK inhibition (Figure 7B). MSK1 phosphorylation by both ERK1/ERK2 and p38 MAPK is confirmed in figure 7D where phosphorylated MSK1 is returned to basal levels when inhibitors of either pathway are used. Interestingly, dexamethasone does not reduce the activation of MSK1, in accordance with its

known anti-inflammatory role (Ananieva, Darragh et al. 2008). DUSP1 was levels were unchanged (Figure 7E).

IκBα was completely degraded by stimulation with TNFα, indicating significant NFκB activation, although this was not reduced or increased by pre-treatment with dexamethasone or inhibition of p38 MAPK or ERK1/ERK2 (Figure 7F).

Taken together these results demonstrated the ability of glucocorticoids to limit p38 MAPK activity by reducing both p38 MAPK and MAPKAP-K2 phosphorylation. Dexamethasone was, however, unable to dampen either ERK1/ERK2 or NFκB activation. In order to better understand how p38 MAPK activation may be regulating inflammatory processes we decided to examine which genes may be under the control of p38MAPK or ERK1/ERK2, and how these genes were affected by treatment with a glucocorticoid.

3.2.5 TNFα induces inflammatory gene expression in HASM

3.2.5.1 HASM optimally respond within 8 hours to TNFα stimulation

HASM cells were treated with TNFα (1 ng/ml) for 4, 6, 8 and 24 hours in order to determine the ideal length of time for stimulation when evaluating gene expression. The results of this analysis is represented in Figure 8, which revealed that the 8 hour time point had strong activation of genes of interest, including IL-6, CXCL8, DUSP1 and IKBα. All gene expression analysis was therefore done using 8 hours of stimulation. As well, since we had achieved good results with 10 ng/ml of the agonist TNFα in immunoblotting, this concentration was employed for all further testing.

3.2.5.2 TNFα induces ERK1/ERK2 dependent CXCL8 and IL-33

In order to assess the impact of the glucocorticoid dexamethasone as well as the importance of each MAPK pathway on gene regulation, cells were pre-treated with 100 nM dexamethasone, 0.1 μM BIRB0796, 5.0 μM SB203580 or 2.0μM PD184352 for one hour prior to stimulation.

Figure 9 portrays genes involved in the recruitment or stabilization of leukocytes (CXCL8 and GM-CSF) as well those involved in cytokine production and asthma severity (IL-33 and Il-1β) and remodelling (IL-6) (Park, Choi et al. 1998; Ammit, Moir et al. 2007; John, Zhu et al. 2009; Prefontaine, Lajoie-Kadoch et al. 2009).

There was stimulation by TNFα of all genes, although only CXCL8 and IL-33 showed statistically significant increases over unstimulated cells. Overall, this increase was not dependent on p38 MAPK as evidenced by the lack of reduction in gene expression when cells were pre-incubated with BIRB0796. On the other hand, both GM-CSF and IL-1β were significantly increased when p38 MAPK was blocked; suggesting this kinase in fact blocks their expression.

Conversely, CXCL8, and IL-33 were reduced when the ERK1/ERK2 pathway was inhibited; indicating this signaling pathway is necessary for their up regulation by TNFα. This was statistically significant for both IL-33 and CXCL8.

With the exception of CXCL8, all gene expression appeared to be diminished by the pre-incubation with dexamethasone although this was only statistically significant for IL-33. To better understand the inflammatory balance at the level of gene expression, the next experiment assayed the expression of genes known to be engaged in negative feedback loops that re-establish homeostasis.

3.2.5.3 TNFα and dexamethasone upregulate DUSP1 and reduce ATF3 expression

DUSP1, A20, IκBα and ATF3 have all been implicated in the regulation of either p38MAPK, NFκB or TNFα signaling (Baeuerle and Baltimore 1988; Heyninck, De Valck et al. 1999; Gilchrist, Henderson et al. 2008). Figure 9 shows their response to TNFα treatment in the absence or presence of one hour pre-incubation with 100 nM dexamethasone, 0.1 μM BIRB0796, 5.0 μM SB203580 or 2.0μM PD184352.

DUSP1 was significantly increased following TNFα stimulation when pre-treated with dexamethasone as expected, but not by TNFα stimulation alone, and this expression was unaffected by inhibition of either MAPK pathway. Both A20 and IκBα were significantly increased following TNFα stimulation, but their induction was not diminished by the use of dexamethasone, or via the inhibition of either MAPK pathway. Interestingly, ATF3 responded with a significant (four fold) increase following TNFα stimulation which was sensitive to pre-

treatment with dexamethasone. As well, blocking the ERK1/ERK2 pathway resulted in another increase in its expression to near eight fold that of basal levels. This suggested a braking mechanism inherent between the ERK1/ERK2 pathway and ATF3 expression. Overall, both the inflammatory genes and their negative regulators were not found to be p38 MAPK dependent, whereas several, including the cytokines CXCL8 and IL-33 were ERK1/ERK2 dependent.

3.2.6 CXCL8 ELISA following TNFα 8 stimulation

In the above gene analysis a striking result was CXCL8 transcriptional up-regulation following TNFα stimulation (figure 9). Moreover, this increase was shown to be strongly ERK1/ERK2 dependent as transcript levels were reduced by approximately 75% in the presence of PD184352. To confirm whether or not this trend was mirrored in secreted levels of this chemokine, cell supernatants taken from the stimulated cells in the three independent experiments were analyzed by ELISA for their CXCL8 protein concentrations in response to TNFα stimulation for 8 hours.

TNFα brought about a significant increase in CXCL8, but this increase was not reduced by pre-treatment with p38 MAPK inhibitors or dexamethasone. Although 75% of CXCL8 mRNA synthesis was blocked by pre-treatment with PD184352, the secreted protein levels were unchanged, indicating that post-transcriptional regulation of this chemokine's mRNA may partially restore some of the inhibition (Figure 11).

3.3 Summary of results

Initial work focused on the expression of both MAPKs in samples obtained from non through mild to severe asthmatics. These represented a snapshot in time of the *in vivo* asthmatic condition with regards to MAPK activation.

All immunohistochemical analyses revealed that p38 MAPK, ERK1/ERK2 and their mutual downstream targets were indeed most active in severe asthmatics. This included the negative regulator DUSP1, which was notably present in all samples but has stronger nuclear staining in the severe asthmatics. These results corroborate immunofluorescent staining of bronchial biopsies in work done by Liu et al., where the phosphorylation of ERK and p38 MAPK

in both epithelial and airways smooth muscle closely mirrored disease severity in asthmatic subjects (Liu, Liang et al. 2008).

DUSP1 was markedly increased in mild and severe asthmatic airway mRNA, a result consistent with the understanding that asthmatics control their disease by taking glucocorticoids. Of interest was the wide spread in mRNA expression of DUSP1 in the severe asthmatic population, which could reflect medication interactions or glucocorticoid resistance. Indeed, these patients suffer from airway inflammation suggesting that GCs are not effective in controlling the disease.

HASM primary cell culture provided further insight into how an inflammatory reaction would manifest, in both temporal and quantifiable terms. Immunoblot analysis revealed p38 MAPK, ERK1/ERK2 and NFκB all become activated within 15 minutes of contact with the inflammatory cytokine TNFα, and returned to basal levels within approximately 90 minutes. Unexpectedly, while the downstream kinases MAPKAP-K2 and MSK1 were activated, DUSP1 levels remained remarkably stable. This was confirmed in three different patients, all of which showed robust levels of this phosphatase at basal and stimulated levels. This is in contrast to HASM work by others where DUPS1 basal levels are only faintly expressed and TNFα (10 ng/ml) brings about strong activation within 60 minutes (Moutzouris, Che et al. ; Quante, Ng et al. 2008) .

The effect of glucocorticoids was evaluated by incubating HASM cells with 100 nM dexamethasone for one hour prior to stimulation. Notable reductions were seen in the p38 MAPK pathway both in p38 MAPK phosphorylation and that of MAPKAP-K2. However, phosphorylated forms of MSK1 were augmented, which fit well with the idea that glucocorticoids shift the activity from the inflammatory MAPKAP-K2 to the anti-inflammatory MSK1/MSK2. Glucocorticoid pre-treatment did not significantly affect ERK1/ERK2 phosphorylation, which may point to ERK1/ERK2 being responsible for MSK1 phosphorylation. GCs also did not increase levels of IκBα. These results cumulatively point toward the p38 MAPK pathways being attenuated the most by GCs following inflammatory stimulus.

The expression of genes that regulate inflammation was investigated to provide further insight into how p38 MAPK may exert its effect. TNFα elicited significant increases in CXCL8 and IL-33. Of these two, only IL-33 showed a reduction in expression with SB203580, a relatively specific p38 MAPK inhibitor (Kuma, Y., G. Sabio, et al. 2005). However, expression was unchanged with BIRB0796, a more specific inhibitor of p38 MAPK, indicating that changes

in IL-33 expression were independent of p38 MAPK. SB203580 is also known to affect the protein kinase RIP2, therefore these results may suggest a role for this pathway in regulating IL-33 expression in responses to TNFα. In agreement with other work, p38 MAPK inhibition increased the expression of GM-CSF in ASM (Hallsworth, Moir et al. 2001). IL-1 β was also increased with p38 MAPK blockade, in contrast to its historical function seen in monocytes (Lee, Laydon et al. 1994). However, our work is perhaps more in line with Hedges et al. who treated ASM cells with a mixture of cytokines including TNFα and saw no real change in IL-1B expression with (SB203580) p38 MAPK inhibition (Hedges, Singer et al. 2000). In this same study, partial reductions of IL-6 and CXCL8 expression were seen with inhibition of p38 MAPK whereas in our assay, IL-6 and CXCL8 were unchanged by pre-treatment with either BIRB0796 or SB203580. These results indicate p38 MAPK modulates the expression of GM-CSF and IL-1 β, but does not regulate CXCL8, IL-6 or IL-33.

In contrast, inhibiting ERK1/ERK2 significantly reduced expression of CXCL8 and IL-33. Although statistical significance was not achieved for IL-1β and GM-CSF when all inhibitors are considered in the ANOVA analysis, comparison of TNFα stimulation to TNFα with PD184352 alone shows statistical significance, and supports the notion that ERK1/ERK2 mediates the expression of all four genes. Indeed, GM-CSF, IL-1β and CXCL8 have been shown to be dependent of the ERK1/ERK2 pathway in ASM (Hedges, Singer et al. 2000; Hallsworth, Moir et al. 2001). Since IL-33 regulation by MAPKs has not been reported, our findings of ERK1/ERK2 regulation of this cytokine are novel.

Dexamethasone treatment did not produce a pattern of gene expression that mirrored either of the MAPKs as it reduced the expression of all the cytokines except CXCL8. Although GM-CSF and IL-1B are known to be reduced by glucocorticoids, our other results were at times inconsistent with work done by others (Sousa, Poston et al. 1993). Investigators within this institute have studied IL-33 in HASM with no change to gene expression in the presence of dexamethasone (Prefontaine, Lajoie-Kadoch et al. 2009). As well, CXCL8 induction has been shown to be partially reduced by dexamethasone by other researchers although at doses 100 fold over what we used in our study (John, Au et al. 1998). Otherwise there is a paucity of data on CXCL8 gene expression in response to glucocorticoid use in HASM. IL-6 is a complex cytokine that has been shown to be unchanged or decreased with the use of glucocorticoids (McKay, Hirst et al. 2000; Ammit, Lazaar et al. 2002). Since glucocorticoids increase DUSP1 and thus reduce p38 MAPK activation we had expected to see a similar profile between dexamethasone and

BIRB0796 usage. However, there was a disconnect between GC treatment and p38 MAPK inhibition: indeed in genes such as IL-1β, inhibiting p38 MAPK with BIRB0796 resulted in an increase in expression, and yet treatment with dexamethasone resulted in a decrease.

We thus turned our attention to negative regulators of inflammation: DUSP1, IκBα, A20 and ATF3. With the exception of DUSP1, all genes were upregulated in response to TNFα. Of these, none was regulated by either MAPK, except ATF3. ATF3 has been recently shown to control allergic pulmonary inflammation in a knock out mouse mode, and our data showed it was significantly increased when ERK1/ERK2 was inhibited (Gilchrist, Henderson et al. 2008). This suggests ERK1/ERK2 attenuates ATF3 and indicates how ERK1/ERK2 activation may be causing inflammation in asthma. Two of the genes investigated responded to pre-treatment with dexamethasone. First, as expected, there was a significant increase in DUSP1, a result we had not seen at the protein level. Secondly, ATF3 was downregulated by dexamethasone, suggesting a possible deleterious effect of steroid treatment. These results confirmed part of our original hypothesis in regards to DUSP1 but also revealed that the negative regulation of NFκB and TNF signaling (via IκBα and A20), occur independently of p38 MAPK, ERK1/ERK2 and GCs studied here.

Overall these preliminary experiments revealed that MAPKs were indeed active in both an *in vitro* and *ex vivo* model. While we confirmed that steroid treatment upregulated DUSP1 at the gene expression level, we could not marry the effect of dexamethasone to an inflammatory gene also regulated by p38 MAPK. Despite this, the strong control of the ERK1/ERK2 pathway in airway smooth muscle cells became clear. The focus of further investigations was on the possible mitigating effects of p38 MAPK on processes involved in refractory asthma including: the migration of neutrophils, smooth muscle proliferation and migration, and airway hyperresponsiveness.

Chapter 4: results

4.1 Introduction

The results obtained in our preliminary stimulations with TNFα revealed p38 MAPK, ERK1/ERK2 and NFκB were all strongly activated in a time dependent manner in HASM cells. TNFα also enhanced the expression of many genes and elucidated several that were ERK1/ERK2 MAPK dependent. Yet no obvious function for p38 MAPK activation was seen at the CXCL-8 protein expression level. Connecting the increased activation of p38 MAPK in asthmatics to a biological function in human airway smooth muscle is the focus of this chapter. The functional relevance of this activation was investigated using proliferation and migration assays. Additionally, contractile proteins were evaluated at the level of gene expression and their cytoskeleton protein transformations were seen through immunofluorescent staining. Finally, using a rat model to assess FFIR and velocity of shortening, airway smooth muscle strip mechanics were evaluated in the presence of a p38 MAPK inhibitor.

4.2 Results

4.2.1 Neutrophil migration is ERK1/ERK2 and not p38 MAPK dependent

Neutrophil recruitment from the circulatory system has previously been shown to be p38 MAPK dependent (Roussel, Houle et al.). However, our previous findings indicated that CXCL8 mRNA transcription in HASM cells was ERK1/ERK2 dependent (Figure 9A), and the secreted protein is known to strongly promote neutrophil migration.

In order to confirm which pathway was essential to this process, HASM were treated with TNFα in absence or presence of ERK1/ERK2 and p38 MAPK inhibitors. The resulting conditioned media was then tested for its capacity to recruit freshly isolated human neutrophils. Figure 12 shows that TNFα-stimulation led to the recruitment of more than half of the neutrophils added to the upper chamber. Blocking the activation of ERK1/ERK2 returned the number of migrating neutrophils recruited to near basal levels whereas blocking p38 MAPK activity had no impact. CXCL8 synthesis measured in the conditioned media was in accordance

with the trends observed in neutrophil migration. However, in this assay, when ERK1/ERK2 was inhibited there were lower levels of CXCL8 than was seen in the previous measures of secreted CXCL8 (Fig11). These differences are possibly due to the different techniques used in these two assays. In a neutrophil migration assay the agonists and inhibitors are left in cell culture for two hours and then the cells are rinsed prior to leaving the cells in new media for neutrophil migration. Thus the supernatants collected post-migration contain only the secreted proteins from two hours post stimulation.

This result confirmed a surprising role for the ERK1/ERK2 MAPK and not the p38 MAPK pathway in promoting neutrophil recruitment to sites of inflammation by HASM cells. However, it left the role of p38MAPK activation in HASM cells unanswered. The remaining experiments thus focused primarily on p38 MAPK.

4.2.2 HASM Proliferation is ERK1/ERK2 but not p38 MAPK dependent.

As outlined in the introduction, HASM cell proliferation contributes to airway bronchoconstriction in asthma. Thus I used a flow cytometry cell proliferation assay using BrdU incorporation into DNA synthesis to assess whether or not p38 MAPK modulated the proliferation of smooth muscle cells *in vitro*. Soluble mediators, growth factors and cytokines serve as mitogens that initiate signal transduction in HASM that can lead to cell proliferation (Stewart 2001). As such, cells were stimulated with CXCL 12 (80 ng/ml), a chemokine found to be elevated in asthmatics as mentioned above. As well, transforming growth factor beta (TGFß) (1 ng/ml) was employed as it has previously been shown to modulate airway smooth muscle proliferation, but downstream of MAPK activation (Cohen, Ciocca et al. 1997). Platelet derived growth factor (PDGF- BB) at 15 ng/ml served as a positive control. All three conditions were tested in the presence or absence of 0.1 μM BIRB0796 or 2.0μM PD 184352.

Figure 14A shows that treating the cells with CXCL 12 did not affect positive staining for BrdU when compared to the 0.1% FBS control. CXCL 12 used in conjunction with BIRB0796 resulted in an increase of 7% over CXCL 12 alone, whereas CXCL 12 used in conjunction with PD184352 resulted in a reduction of 31% compared to CXCL 12 alone. In panel B, PDGF-BB treatment resulted in an increase of 20% in positive BrdU staining, with an additional 12% increase with p38 MAPK inhibition compared to PDGF alone, and a decrease of

48% in staining with ERK1/ERK2 inhibition. These results did not reach statistical significance as they were performed twice only. In panel C, the positive control was significantly increased from the baseline vehicle (control), and TGFß treatment of cells resulted in an 18% reduction of staining compared to control, also significant. Here pre-treatment with BIRB0796 resulted in a minor increase (4.5%) compared to TGFß alone while treatment with PD184352 resulted in a significant (12%) reduction in staining compared to TGFß alone. These results reached statistical significance as they were reproduced three times.

In all three conditions inhibiting the p38 MAPK pathway resulted in a small increase in BrdU incorporation but did not reveal an important role for p38 MAPK in HASM cells, whereas inhibiting the ERK1/ERK2 pathway resulted in a significant decrease in BrdU incorporation compared to control as evidenced by the assay with TGFß. Indeed, blocking the ERK1/ERK2 pathway resulted in a reduction to below baseline levels of proliferation. This assay confirmed the role of ERK1/ERK2 in HASM, but left unanswered the question why p38MAPK is active in asthmatic airway smooth muscle. Our focus turned to HASM migration, as p38 MAPK is well known to play a role in chemotactic migration (Rousseau, Houle et al. 1997; Rousseau, Dolado et al. 2006) .

4.2.3 HASM Migration in response to CXCL12 and PDGF is p38 MAPK dependent

In order to test the hypothesis that p38 MAPK is involved in HASM migration *in vitro* in response to pro-migratory agents, a migration assay using a Boyden chamber (chemotaxis) was chosen with an incubation period of 4 hours. The conditions tested included CXCL 12 (80 ng/ml) and PDGF-BB (15 ng/ml), in the presence or absence of 0.1 μM BIRB0796, compared to control (DMSO).

CXCL12 is a potent chemoattractant of lymphocytes and has recently been shown to be increased significantly in the BAL of asthmatics where it correlated with increased numbers of macrophages, lymphocytes and eosinophils (Negrete-Garcia, Velazquez et al. ; Bleul, Fuhlbrigge et al. 1996). However, prior to using CXCL12 as a chemotactic agent, it was necessary to confirm the presence of its receptor CXCR4 on HASM cells, a finding which had not been previously reported. Bronchoscopic biopsies from mild asthmatics were probed for CXCR4 and visualized via immunohistochemistry. Additionally, this receptor was visualized on HASM cells using immunofluorescence.

Immunohistochemical analysis revealed positive cytoplasmic staining in ASM of CXCR4, additionally, some nuclear staining was observed (Figure 13 A). An isotype control confirms specificity of staining (panel B). In Fig 13C and D the receptor was visualized via immunofluorescence. Briefly, cells grown on microchamber Labteck slides were left untreated (C) or were treated with 80 ng/ml CXCL 12 for 15 minutes (D). Basal levels of the receptor are seen, mostly on the cell membrane and less in the nuclear compartment. After treatment with CXCL12 there were increased circular structures on the HASM cells, as if internalization of the bound receptor was occurring. This marking confirmed the receptor was present in unstimulated cells and are likely functional upon ligand binding. Negative controls for these experiments were void of any fluorescence signal.

CXCL12 has not previously been used as a pro-migratory agent for HASM cells, therefore a dose response curve was established using a range of 3 ng/ml to 240 ng/ml CXCL 12 which was added to serum free media in the bottom well of the chemotaxis chamber. HASM cells migrated best at a dose of 80 ng/ml (figure 13C), which agreed with data published on circulating fibrocytes (Phillips, Burdick et al. 2004). As such, this dose was used in further assays for evaluating p38 MAPK implication in migration.

The migration assay was also performed in the presence of PDGF-BB as a positive control. In order to assess how inhibiting p38 MAPK would affect migration, cells were incubated for 30 minutes with either BIRB0796 or DMSO immediately prior to being loaded in the upper portion of the chamber for migration. Cells were gently rocked during this time at room temperature to ensure they did not attach- a technique that has been described previously (Takeda, Sumi et al. 2009). The bottom well contained serum free media with one of the two agonists as well as inhibitor or vehicle.

As figure 13D shows, the addition of CXCL 12 to media in the bottom well of the chamber resulted in a 1.6 fold increase in migration compared to baseline. In comparison, adding PDGF-BB to the media of the lower chamber resulted in 6.3 fold increase in migration, as expected. Of interest, the results show that inhibiting p38 MAPK resulted in a decrease to 1.1 fold over control for treatment with CXCL 12 and a decrease to 4.3 fold with PDGF-BB. If analyzed separately, both CXCL 12 and PDGF-BB were statistically different from the baseline vehicle DMSO in their ability to cause chemotaxis and were significantly inhibited by BIRB 0796. However, since all agonists were assayed at the same time, they were included in the

Anova analysis, and proved only PDGF-BB to be statistically significant overall. However, this suggested that HASM migration was at least partially p38 MAPK dependent.

4.2.4 Contractile proteins are unchanged with dexamethasone, p38 MAPK or ERK1/ERK2 inhibition.

Unlike other muscle cells, smooth muscle does not terminally differentiate. Instead, fully mature smooth muscle cells have the ability to adapt to their environment and modulate their phenotype (Halayko, Salari et al. 1996; Halayko, Rector et al. 1997). This plasticity of phenotype produces both a synthetic or 'proliferative' phenotype as well as what is considered a more differentiated or 'contractile' phenotype. It is possible to monitor this evolution by monitoring the abundance of contractile proteins.

In order to verify whether or not the activation of p38 MAPK was involved in the process of differentiation in HASM, gene expression analysis using quantitative real-time PCR evaluated the expression of four different proteins. Smooth muscle alpha-actin (α-SMA) was measured since it is a major constituent of the contractile apparatus and greater immunoreactivity of α-SMA has been seen in asthmatics (Benayoun, Druilhe et al. 2003). Transgelin, also known as SM22, is a cross linking protein and while its function is not fully understood its relative expression has been associated with contractile and synthetic phenotypes in airway smooth muscle cells (Halayko, Salari et al. 1996). Additionally, beta actin was evaluated as it is known to have a role in cell motility, and tubulin was measured since it is essential for cell division and microtubule assembly (Badley, Couchman et al. 1980; Bhattacharya, Frankfurter et al. 2008). Serum-deprived HASM cells were stimulated with 10 ng/ml TNFα for 8 hours in the presence and absence of both p38 MAPK and ERK1/ERK2 inhibitors. Figure 15 shows that TNFα stimulation does not significantly change the proteins investigated, nor was their expression dependent on p38 MAPK or ERK1/ERK2.

In order to investigate contractile protein expression in a more qualitative fashion and to assess the possibility of p38MAPK involvement, immunofluorescent staining of actin within cultured HASM cells was performed.

4.2.5 α-SMA and total actin respond to CXCL12, PDGF-BB and H_20_2 but not p38 MAPK inhibition.

The results from qRT-PCR showed total amounts of contractile proteins were not significantly different during stimulation with TNFα. We thus investigated whether or not the contractile protein α-SMA as well as total actin changed in morphology when exposed to growth factors, oxidative stress or migratory cytokine stimulus, and whether these changes were p38 MAPK dependent via immunofluorescent staining of cultured HASM cells.

Cells were incubated for one hour with 0.1 μM BIRB0796 before being stimulated for 15 minutes with CXCL12 (80 ng/ml), PDGF-BB (15 ng/ml) or tert-butyl hydrogen peroxide (500 μM). Baseline controls included DMSO and BIRB0796. After stimulation cells were stained for α-SMA as well as total actin using a monoclonal α-SMA anti-body and Phalloidin.

Figure 16 reveals α-SMA stained in green and total actin stained in red. The control for BIRB0796 was DMSO, however, no difference in actin formation can be seen between these two conditions (Fig. 16A). Treating the cells with CXCL12 brought about lamellipodial extensions that were especially visible through F-actin staining, indicating focal contacts were being created between the cell membrane and the Labteck slide (Fig 16B). Treatment with PDGF-BB resulted in distinct elongation of the cells indicative of lamellipodia and cell movement (Fig 16C). In both conditions there was no visible difference between those cells treated with BIRB0796 and those treated with DMSO. In Figure 16D, hydrogen peroxide treatment resulted in thicker and shorter actin fibers, indicative of a stress fiber bundle, but there was no change when the cells were exposed to BIRB0796. Overall these images provided evidence that HASM transforms when exposed to mitogens and reactive oxygen species like H_20_2, however, pre-treating the cells with a p38 MAPK inhibitor did not alter these responses. As such, we investigated one final avenue of possible p38 MAPK influence- that of airway smooth muscle mechanics.

4.2.6 Muscle strip mechanics: velocity of shortening and FFIR

Airway hyper-responsiveness, a major component of asthma, has previously been shown to be dependent on p38 MAPK (Lakser, Dowell et al. 2008; Williams, Issa et al. 2008). Both the velocity of shortening of airway smooth muscle as well as its ability to re-lengthen was assessed using entire tracheal muscle strips from Lewis rats. These experiments were done in

collaboration with Dr. Anne-Marie Lauzon's laboratory, (Meakins-Christie Laboratories, McGill University, Montreal, QC). All experiments were performed using 0.1 μM BIRB0796 or vehicle (DMSO 1:10,000 v/v) to assess the importance of p38 MAPK in regulating velocity of shortening during methacholine challenge as well as re-lengthening of each strip under oscillations that replicate normal breathing (FFIR or force fluctuation induced re-lengthening).

Prior to these experiments it was crucial to confirm that p38 MAPK was able to become activated in response to methacholine in these tissues, and that this activation could be reduced by pre-treatment with p38 MAPK inhibitors. For this, individual muscle strips were dissected from sacrificed rats as described in materials and methods. Several strips per condition were incubated for one hour with 0.1 μM BIRB0796, 5.0 μM SB203580 or DMSO 1:10,000 before being stimulated for 15 minutes with 5×10^{-4} M methacholine. The strips were then homogenized and proteins were extracted from the homogenate. Immunoblots of these proteins were probed with anti-bodies for total as well as phosphorylated p38 MAPK. The tissue responded to methacholine challenge by activating p38 MAPK, and this activation was reduced via pre-treatment with both 5.0μM SB203580 and 0.1 μM BIRB0796 (Figure 17).

Figure 18 shows data from two strips taken from adjacent section of trachea and subjected to challenge with 5×10^{-4} M methacholine. Velocity of shortening was measured during each quick release. The y axis shows the shortening velocity in units of lengths per second. The x axis shows the trend over time. Both strips initially shortened at 7 to 8 % of their reference length per second (L0/s) but slowed to 4% per second after 30 minutes. The results for the two strips were similar.

Following this velocity measurement, figure 19 shows three tracheal strips: test (0.1 μM BIRB0796), control (DMSO) and one test strip without calcium (as a control to measure the necessity of calcium), which were subjected to FFIR. The y axis measures actual length over reference length. The x axis shows events over time: at time zero muscle force has been clamped and the muscle is allowed to shorten for 20 minutes. At this point the length controller simulates breathing by applying an oscillating force to the muscle strip. Again the strips lengthened almost identically- indicating that blocking the p38 MAPK pathway using BIRB0796 does not affect rat airway muscle mechanics under these conditions. The strip incubated without calcium was unable to re-lengthen, as expected.

4.3 Summary of results

Refractory asthma has been associated with neutrophilia and hypersecretion of CXCL8 (Barnes 2007; John, Zhu et al. 2009). Neutrophil migration and concomitant CXCL8 expression was thus assayed in response to conditioned media from TNFα stimulation of HASM cells (Figure 12). This media had strong biological activity demonstrated by its chemotactic powers, producing a three-fold increase in freshly isolated human neutrophil migration compared to media alone. Cells that had been previously treated with BIRB0796 did not show any difference in migration, but cells that had been treated with PD184352 showed a substantial reduction (50%) in migrated neutrophils. The corresponding CXCL8 levels of these supernatants revealed that blocking p38 MAPK increased secreted protein levels, while inhibiting ERK1/ERK2 reduced them. This experiment demonstrated that neutrophil migration was not solely dependent on CXCL8 levels, and was independent of p38 MAPK activity but ERK1/ERK2 dependent. This study highlights a potential role of targeting the ERK1/ERK2 MAPKs in order to decrease neutrophilia in refractory asthma.

We also assayed HASM cell migration in response to CXCL12 and PDGF-BB, in the presence of BIRB0796 (Figure 13). The results brought forth two noteworthy findings. The first was that HASM did indeed migrate in the presence of CXCL12, a novel finding. The second was that migration towards both chemotactic agents used here was partially dependent on p38 MAPK. However, since Boyden chambers cannot be disassembled during an assay, cells must be placed in contact with their inhibitors prior to being deposited on the membrane to which they adhere and which they migrate through. This confounds the results as inhibitors and their vehicle DMSO, may differentially interfere with essential cellular processes involved in attachment to ECMs. The reduction in cell numbers seen might therefore reflect either a reduction in attachment or migration. Nevertheless, this was the first indication of what p38 MAPK might be controlling in airway smooth muscle. While the increase in migration provoked by CXCL12 was modest (2 fold), it compares well to work done by others where PDGF induced 3 fold HASM migration that was partially p38 MAPK dependent (Parameswaran, Cox et al. 2002).

Since our migration assay had pointed to a role for p38 MAPK in the remodelling process we also decided to verify its relevance to proliferation of HASM cells, especially in response to the novel cytokine CXCL12. PDGF-BB was again used as a positive control and TGFβ as a negative control. Our work revealed CXCL12 did not induce proliferation although both of the

positive control conditions (PDGF-BB) produced the expected response (Figure 14). In all three experiments blockade of the ERK1/ERK2 pathway resulted in a significant drop in proliferation, while p38 MAPK inhibition either produced no change or, in the case of PDGF-BB, it potentiated the increase of proliferation. Indeed, activation of the p38 MAPK pathway has been shown to antagonize proliferation by decreasing cyclin D1 expression in airway smooth muscle (Page, Li et al. 2001).

Our last focus of study turned to HASM in its known contractile role. Here we evaluated contractile proteins at the cellular and tissue level (Figure 15). Myocytes that differentiate into a contractile phenotype have a higher abundance of contractile proteins. HSP27 is known to cap the barbed end of actin, and this process is abrogated upon phosphorylation by MAPKAP-K2, leading to actin polymerization. As a logical extension of this, we investigated whether alpha or beta actin gene levels were increased in ASM with p38 MAPK inhibition, along with SM22, and tubulin. None of these proteins was significantly increased in the presence of TNFα, nor reduced in the presence of MAPK inhibitors or the GC dexamethasone. This concurs with work done by Woodruff et al. on asthmatic bronchial biopsies analyzed for a series of contractile proteins including αSMA, that were found to be similar to healthy subjects (Woodruff, Dolganov et al. 2004). While not significant in these preliminary experiments, our analysis of SM22 showed it was reduced by half its basal level with dexamethasone, warranting repeated experiments. Given that this protein's function is poorly understood and its reduction is associated with differentiation to a more synthetic phenotype, there may be a connection between the use of glucocorticoids and a loss of contractile phenotype of ASM (Halayko, Rector et al. 1997).

Since it remains possible that structural changes occur in contractile proteins at any given point in time without changes in gene expression, we next looked at the morphology of total actin and αSMA upon stimulation with CXCL12, hydrogen peroxide and PDGF-BB in the presence of BIRB0796 (Figure 16). There were no striking differences between the cells treated with BIRB0796 and those treated with its vehicle DMSO. However, the different agonists used brought about obvious changes in cell shape, especially at the level of F-Actin. Treatment with PDGF-BB brought about distinct elongation of the cell fibers with membrane ruffling indicative of newly synthesized lamellipodia. These F-actin projections were also apparent when the HASM was treated with CXCL12, and suggested that the changes seen in morphology were related to the cells ability to migrate, which complemented the results we had obtained in HASM

migration assays. Treatment with hydrogen peroxide resulted in thicker and shorter actin fibers, indicative of a stress fiber bundle, but was unchanged in the presence of BIRB0796.

With only one p38 MAPK dependent function elucidated by our findings we made one final investigation of p38 MAPK in airway smooth muscle at the tissue level. Here intact tracheal strips from Lewis rats were used to assess two elements of hyperresponsiveness: velocity of shortening and force fluctuation induced re-lengthening (Figures 17 through 19). Work from Lakser et al. has shown bovine airway smooth muscle will re-lengthen to a greater extent when p38 MAPK is inhibited (Lakser, Lindeman et al. 2002). Following this same protocol, rat tracheal muscle strips pre-treated with BIRB0796 showed no difference in their velocity of shortening or their ability to re-lengthen compared to strips that were treated with vehicle only. Despite careful control of all conditions, we concluded there were two differences in our experimental design- we used a rat model instead of a bovine model, and BIRB0796 instead of SB203580. These differences may explain the different results obtained, and may warrant further investigation.

Chapter 5: Tables and Figures

Figure 3

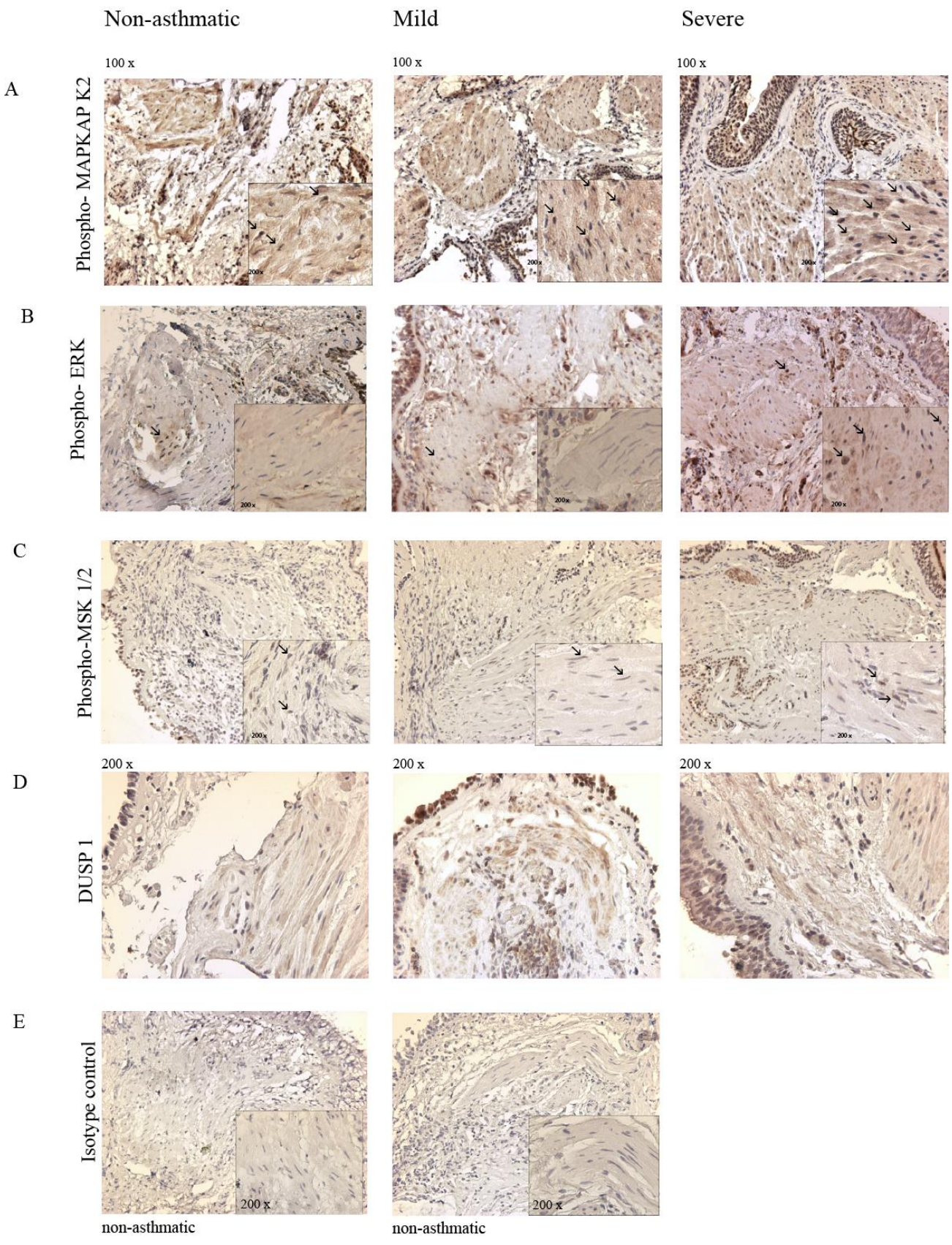
Non-asthmatic
Mild
Severe
A
Phospho- MAPKAP K2
100 x
100 x
100 x
B
Phospho- ERK
C
Phospho-MSK 1/2
D
DUSP 1
200 x
200 x
200 x
E
Isotype control
200 x
200 x
non-asthmatic
non-asthmatic

Figure 3) Increased activation of MAPKs in airway smooth muscle cells of severe asthmatics. Panels are 100 X magnification while inserts are 200 X magnification except for DUSP1. Nuclear staining is indicated by black arrows. A) Phospho-MAPKAPK2: localization of phosphorylation at Thr334 B) Phospho-ERK: localization of phosphorylation on both Thr 202 and Tyr 204 C) Phospho-MSK1 phosphorylated at Thr581 D) DUSP1 localization includes cytoplasmic and nuclear staining E) Normal Rabbit IgG Isotype controls -panels indicate doses at equal or greater concentration than all primary anti-bodies.

Figure 4

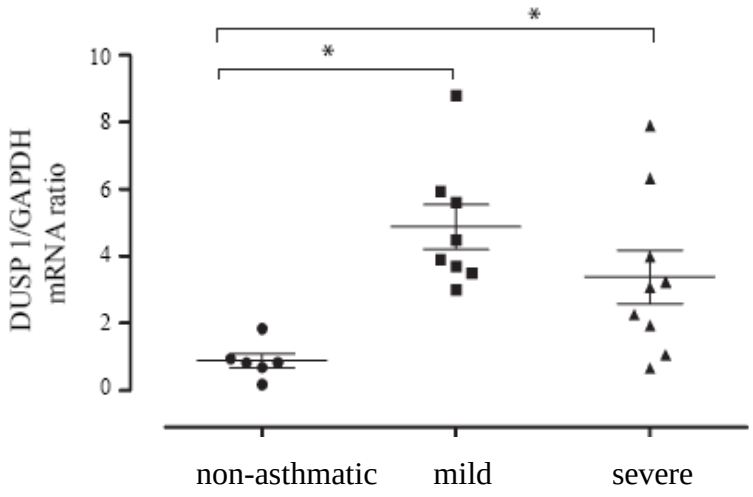

Figure 4) DUSP1 expression is elevated in mild and severe asthmatics. DUSP1 transcript levels in tissues of bronchoscopic biopsies of non-asthmatic individuals (n=6) compared to mild (n=8) and severe asthmatic patients (n=9) as analyzed by quantitative real-time PCR. The data is presented for severe and mild asthmatics compared to controls. * = $p < 0.05$ asthmatic compared to non-asthmatics.

Figure 5

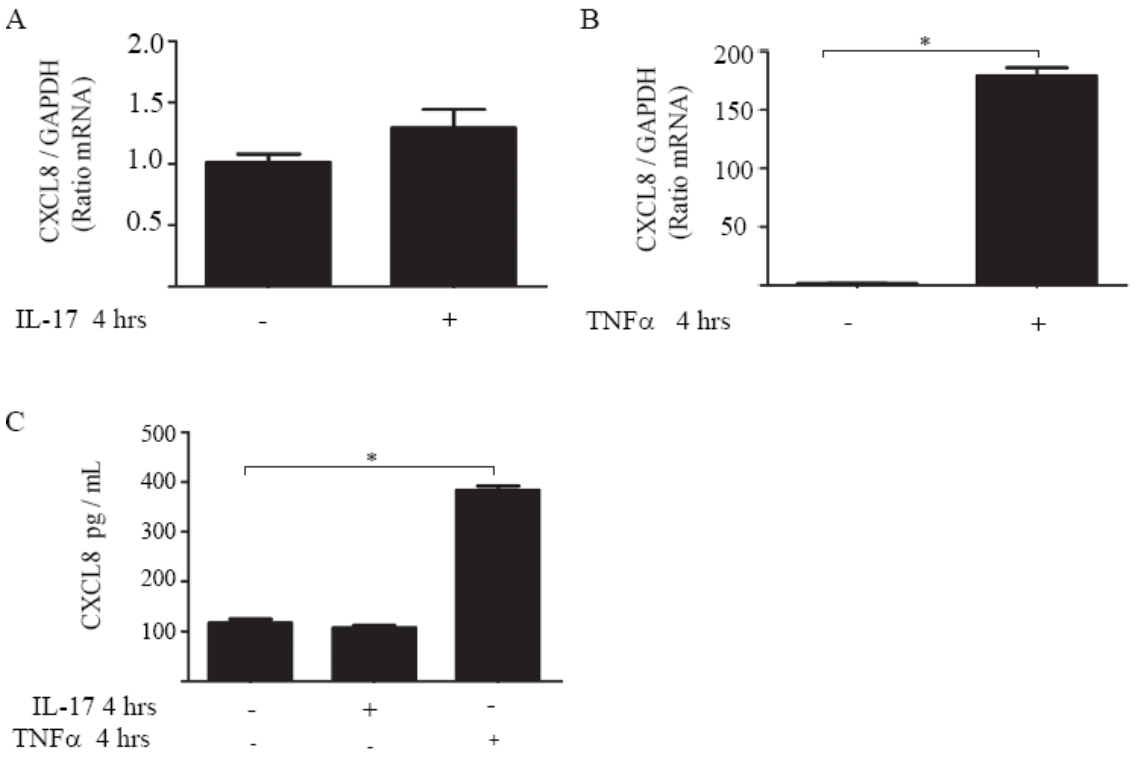

Figure 5) TNFα more potently induces CXCL8 in HASM than IL-17A. HASM cells were left untreated (-) or exposed (+) to 20 ng/ml IL-17A or 10 ng/ml TNFα for 4 hours. After stimulation total RNA was extracted and supernatants were collected. The amount of CXCL8 mRNA as analyzed via quantitative real-time PCR is shown in panels A and B. CXCL8 protein secretion as analyzed by ELISA is shown in panel C. *n* = 4 from two different donors * = p <0.05, stimulated compared to untreated.

Figure 6

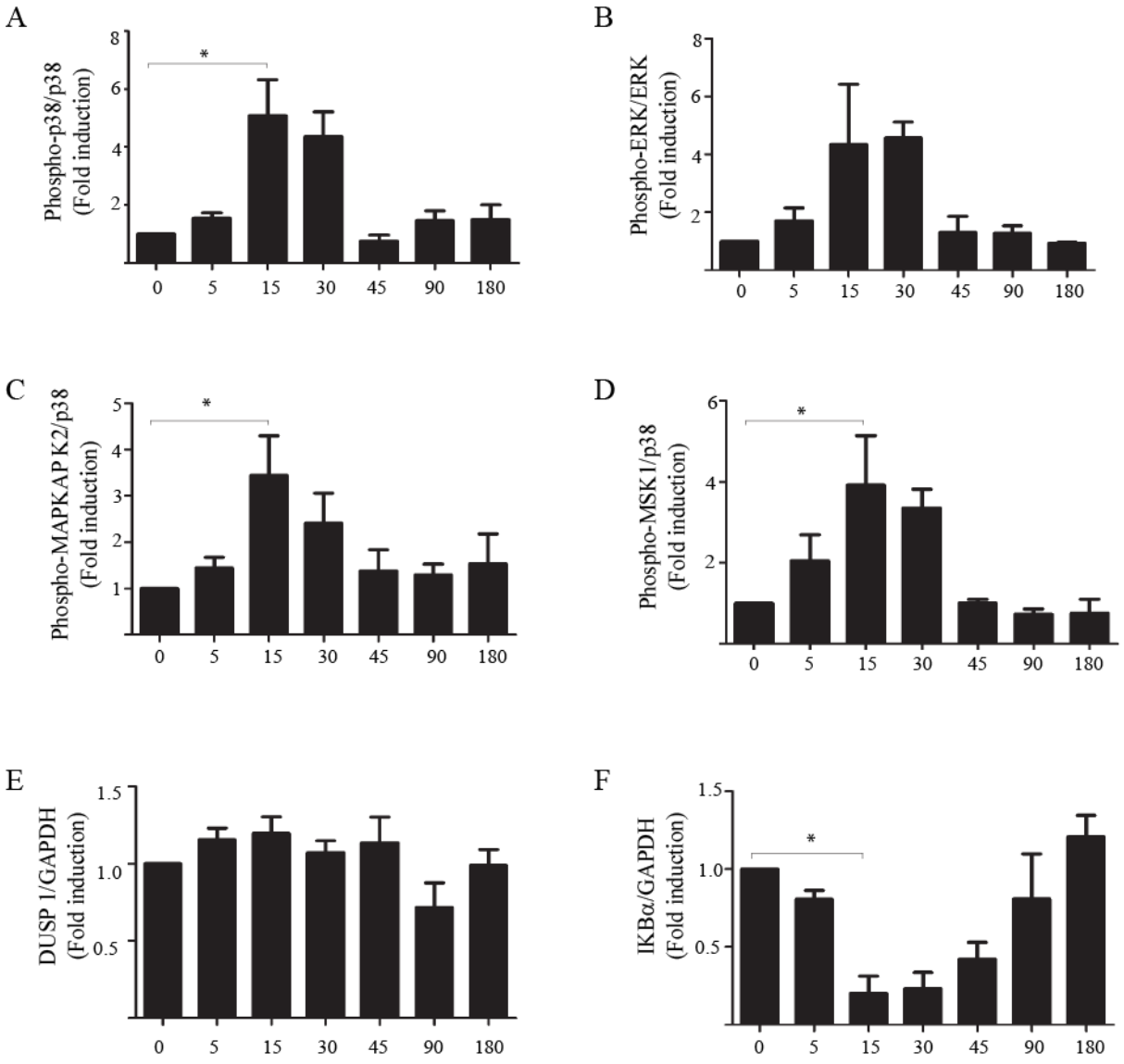

Figure 6) TNFα activates the MAPKs and NFκB in a time dependent manner. HASM cells were treated for increasing times over 180 minutes as indicated with 10 ng/ml TNFα or not (time zero). Cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with antibodies that recognize the proteins quantified in panels above: A) phosphorylated and all forms of p38 MAPK, B) phosphorylated and all forms of ERK C) phosphorylated forms of MAPKAPK2 and total p38 MAPK D) phosphorylated forms of MSK1 and total p38MAPK E) all forms of DUSP1 and GAPDH F) all forms of IκBα and GAPDH. Data is expressed as the phosphorylated kinase (or protein) over all forms of the protein or over the housekeeping gene glyceraldehyde-3-phosphatase (GAPDH) as acquired by fluorescent detection on a LiCor

Odyssey imager. *n* = 3 from three different donors * = p <0.05, stimulated compared to untreated.

Figure 7

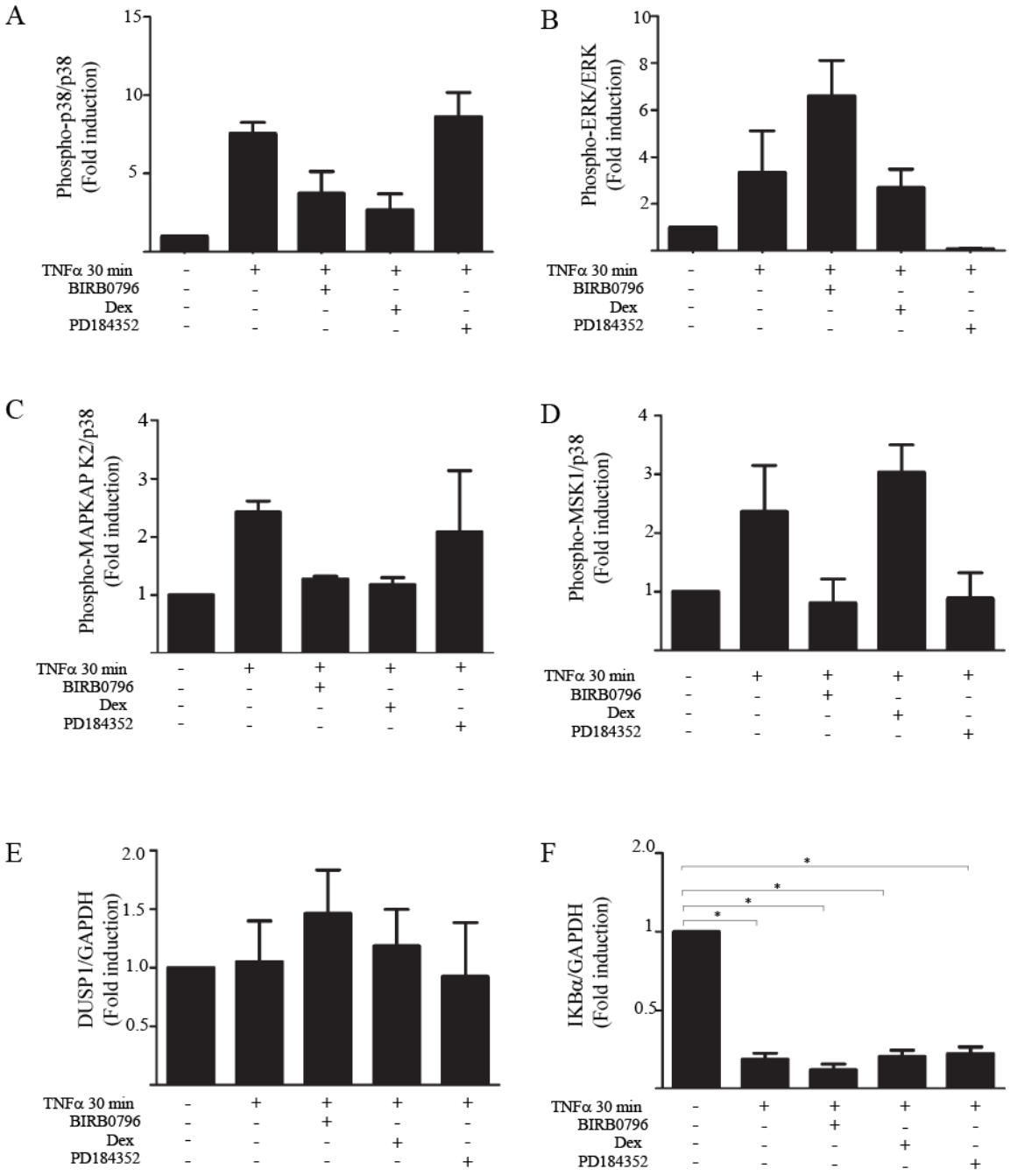

Figure 7) Dexamethasone attenuates p38 MAPK activity but not ERK1/ERK2 or NFκB. HASM

cells were left untreated (-) or treated (+) for one hour with 0.1 μM BIRB0796, 2.0 μM PD 184352 or 100nM Dexamethasone, then exposed for 30 minutes to 10 ng/ml TNFα (+) or not (-). Cell lysates were processed as in figure 4 and probed as follows: A) phosphorylated and all forms of p38MAPK, B) phosphorylated and all forms of ERK, C) phosphorylated forms of MAPKAPK2 and total p38MAPK, D) phosphorylated forms of MSK12 and total p38MAPK, E) all forms of DUSP1 and GAPDH, F) all forms of IκBα and GAPDH. Data is expressed as the phosphorylated kinase (or protein) over all forms of the protein or as a ratio to the housekeeping gene glyceraldehyde-3-phosphatase (GAPDH) as acquired by fluorescent detection on a LiCor Odyssey imager. *n* = 2 or 3 from two different donors * = $p < 0.05$, stimulated compared to untreated.

Figure 8

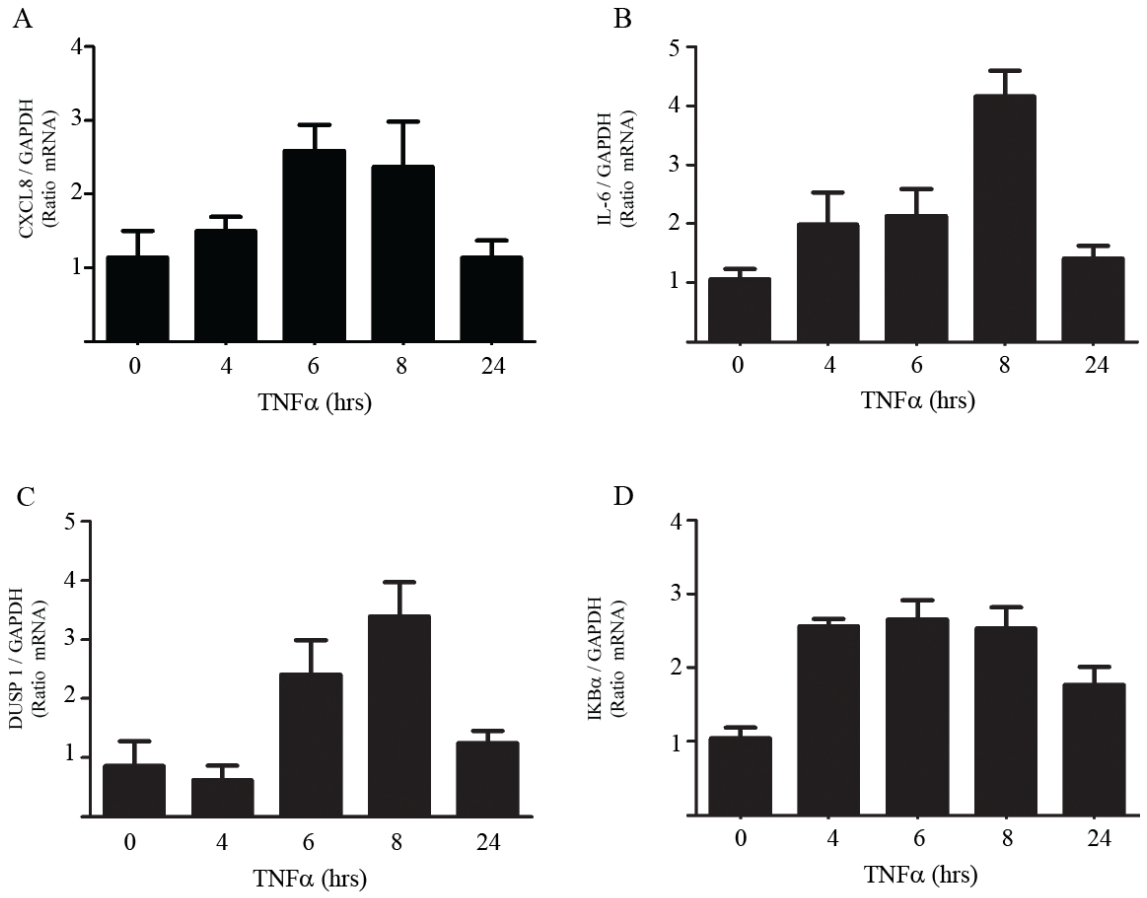

Figure 8) 8 hours of TNFα stimulation maximally induces gene expression. HASM cells were treated with 1 ng/ml TNFα for 4,6,8 or 24 hours, or not (0) in order to assess maximal gene expression by quantitative real-time PCR for CXCL8 (A) IL-6 (B) DUSP1 (C) or IKBα (D). *n =2* from one donor.

Figure 9

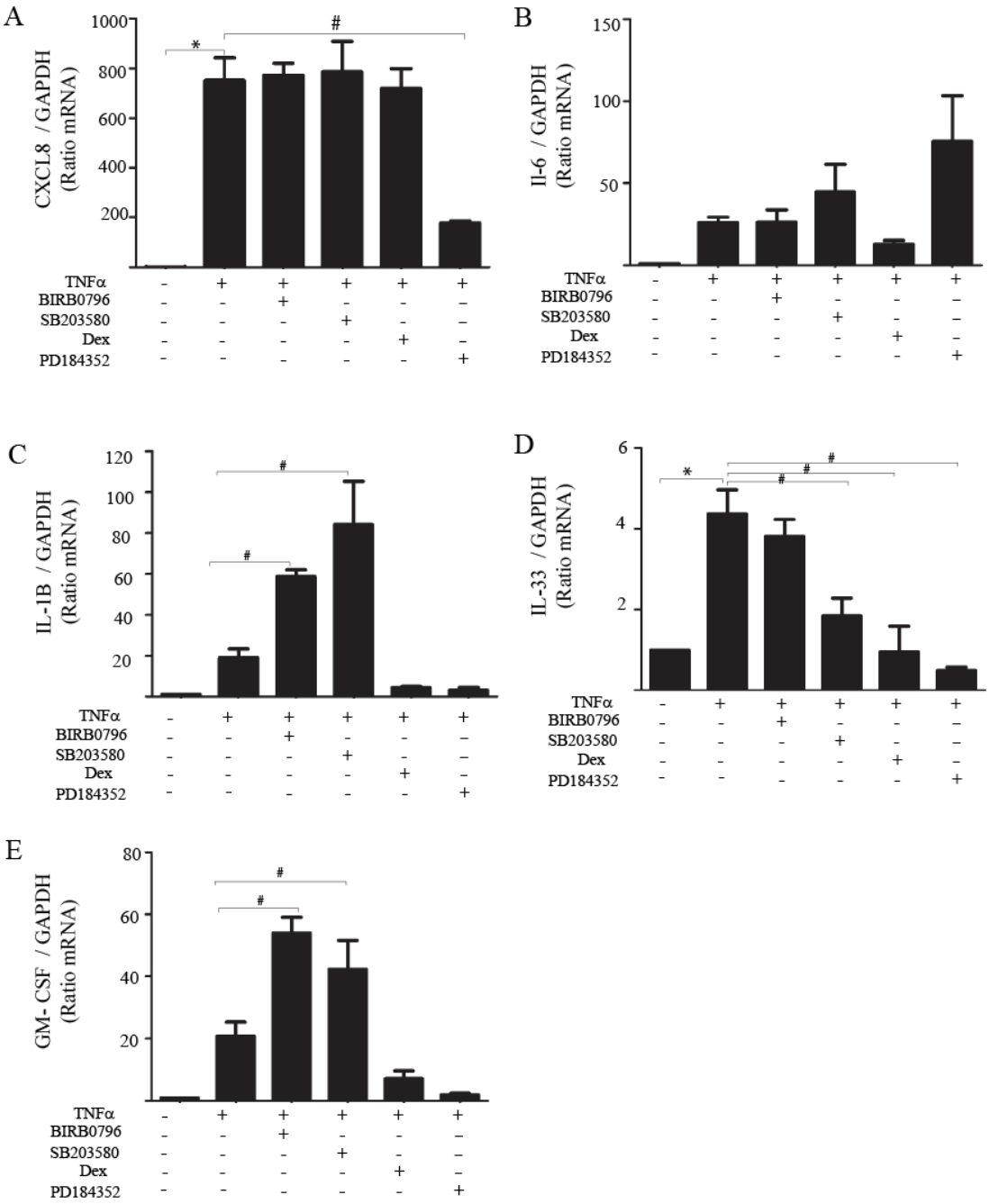

Figure 9) TNFα induces CXCL8 and IL-33 expression that is ERK1/ERK2 but not p38 MAPK dependent. HASM cells were left untreated (-) or treated (+) for one hour with 0.1 μM BIRB0796, 2.0 μM PD184352, 5.0 μM SB203580 or 100nM Dexamethasone prior to exposure to 10 ng/ml TNFα (+) or not (-) for 8 hours. After stimulation total RNA was extracted and different gene's mRNA abundance was determined via qRT-PCR. Panel A) CXCL8, B) IL-6, C)

IL-1B , D) IL-33, E) GM-CSF. *n* = 4 from two different donors. * = p <0.05 compared to unstimulated, # = p <0.05 compared to TNFα.

Figure 10

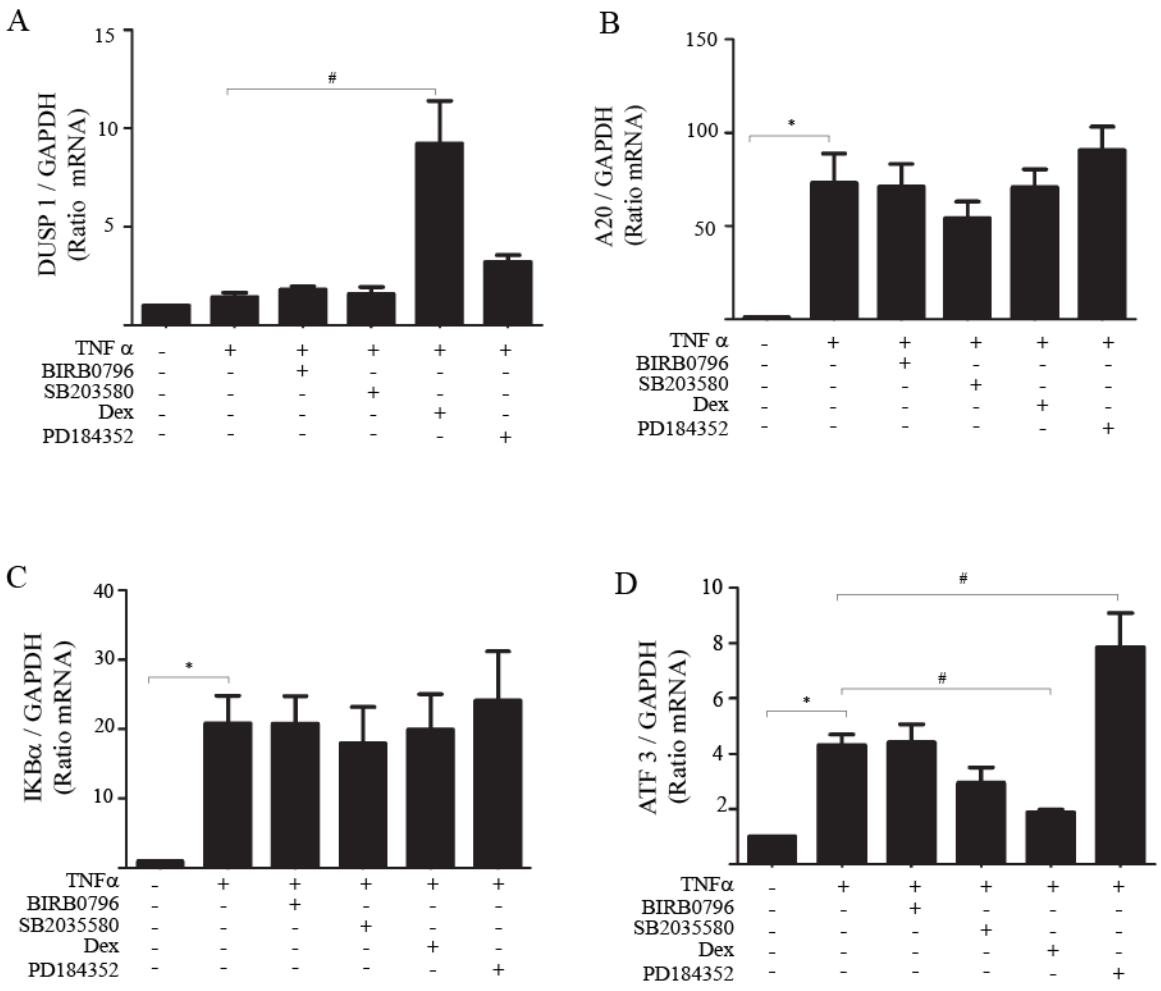

Figure 10) Dexamethasone potently induces DUSP1 but does not prevent IκBα degradation or A20 expression. HASM cells were left untreated (-) or treated (+) for one hour with 0.1 μM BIRB0796, 2.0 μM PD184352, 5.0 μM SB203580 or 100nM Dexamethasone prior to exposure to 10 ng/ml TNFα (+) or not (-) for 8 hours. After stimulation total RNA was extracted and different gene's mRNA abundance was determined via qRT-PCR. Panel A) DUSP1, B) A20, C) IκBα, D) ATF 3. *n* = 4 from two different donors. * = p <0.05 compared to unstimulated, # = p <0.05 compared to TNFα.

Figure 11

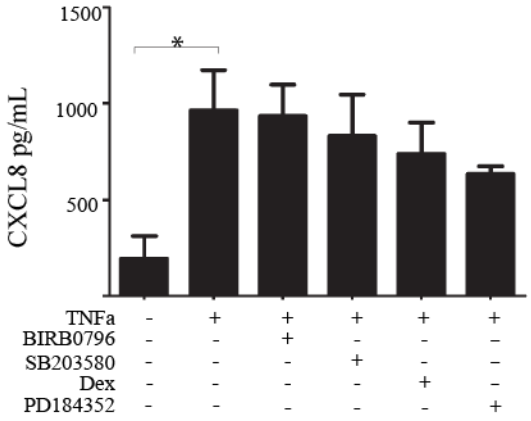

Fig 11) CXCL8 synthesis following TNFα stimulation. HASM cells were left untreated (-) or treated (+) for one hour with 0.1 μM BIRB0796, 2.0 μM PD184352, 5.0 μM SB203580 or 100nM Dexamethasone prior to exposure to 10 ng/ml TNFα (+) or not (-) for 8 hours. Supernatants were collected and analyzed for protein levels of CXCL8 by ELISA n = 6 from three separate donors. * = $p < 0.05$ compared to unstimulated.

Figure 12

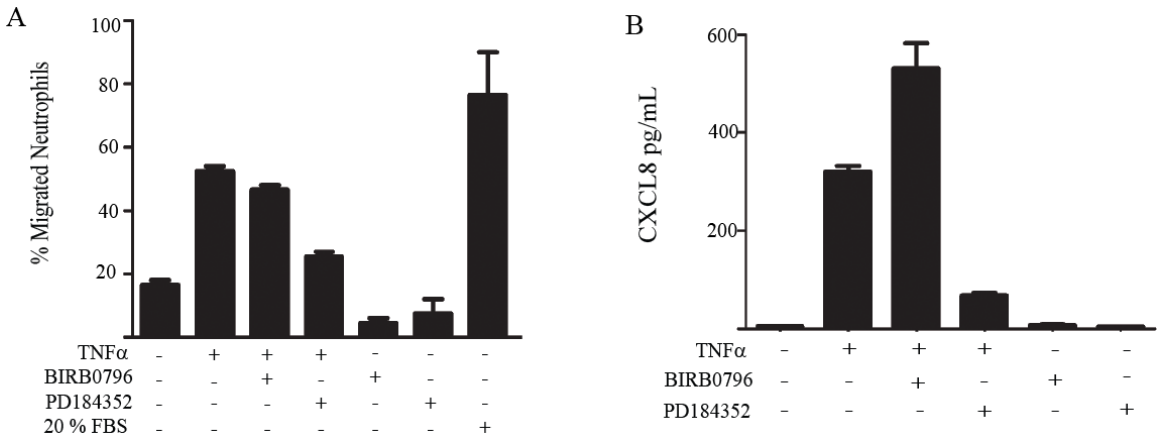

Fig 12) A) Neutrophil migration is ERK1/ERK2 but not p38 MAPK dependent. HASM cells were left untreated (-) or treated (+) for one hour with 0.1 μM BIRB0796 or 2.0 μM PD184352 prior to exposure to 10 ng/ml TNFα (+) or not (-) for 2 hours. Cells were then washed and medium was replaced by serum free medium for 18 hours. This conditioned media was then used for migration of freshly isolated human neutrophils over 5 hours. B) CXCL8 secretion is ERK1/ERK2 dependent. Supernatants collected from panel A were used to assay secreted CXCL8 via ELISA. *N=2* from one donor.

Figure 13

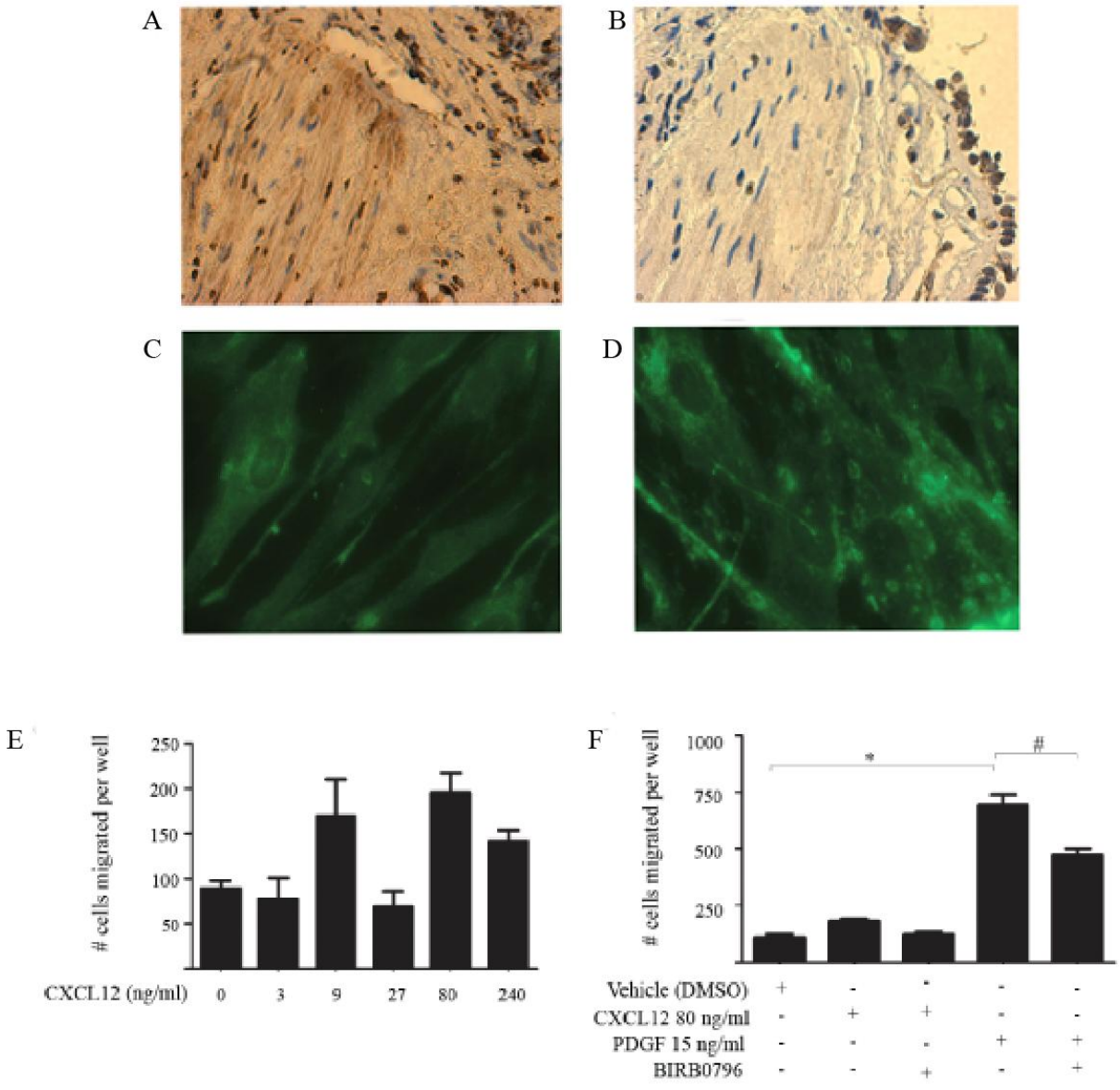

Fig 13) The receptor CXCR4 is confirmed on HASM biopsies from mild asthmatics A) The localization of CXCR4 was determined using immunohistochemistry on bronchoscopic biopsies of mild asthmatics. B) Isotype (mouse) control. Panels are 400 X magnification. C)

Immunofluorescent staining of CXCR4 receptor. HASM cells grown on Labteck microchamber slides were left untreated (C) or treated (D) with 80 ng/ml CXCL12 for 15 minutes. Panels are 400 X magnification. **HASM migrates in the presence of CXCL12 and PDGF and is p38 MAPK dependent**. E) 4 x 10^5 HASM cells were allowed to migrate in a Boyden chamber towards media containing increasing doses of CXCL12. Migration reached a plateau near 80 ng/ml, and this dose was used in further experiments. *n* = 2 from one donor**.** F) Chemotaxis assay towards CXCL12 or PDGF-BB: HASM cells were detached and gently agitated in the absence (-) or presence (+) of 0.1 μM BIRB0796 or 2.0 μM PD184352 before being added to a Boyden chamber where bottom wells contained CXCL12 (80 ng/ml) or PDGF-BB (15 ng/ml). Cells were allowed to migrate for four hours and then counted under 200X magnification. *n* = 4 from two different donors**.** * = $p < 0.05$ compared to vehicle (DMSO) alone, # = $p < 0.05$ compared to agonist alone.

Figure 14

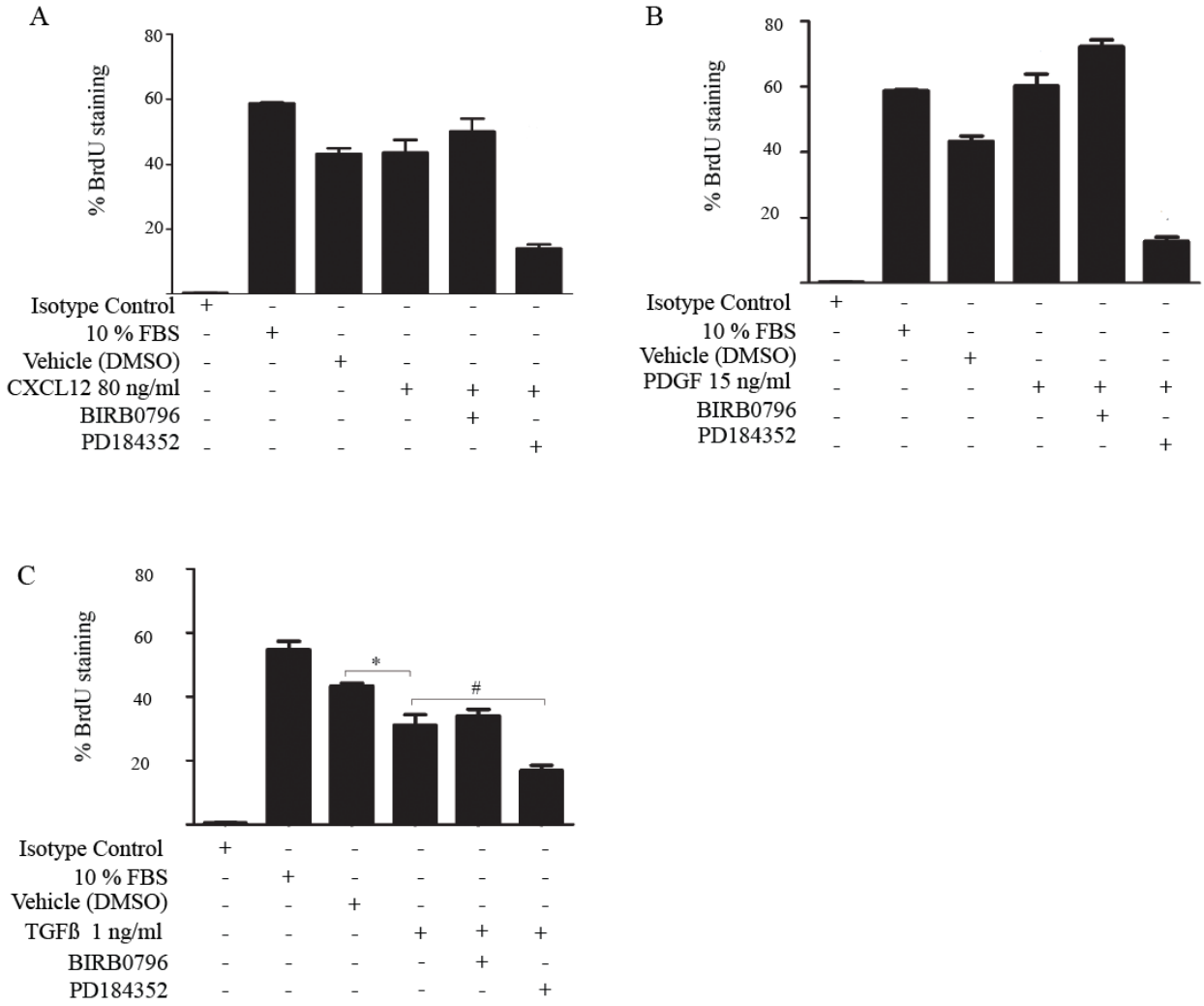

Figure 14) ERK1/ERK2 is pro-proliferative while p38 MAPK antagonizes proliferation. Responses show positive staining for BrdU in 10,000 cells per condition. HASM cells were treated for one hour with 0.1 μM BIRB0796 or 2.0μM PD184352 prior to being exposed to CXCL 12 (80 ng/ml), PDGF-BB (15 ng/ml) or TGFß (1 ng/ml) and allowed to proliferate for 18 hours. Cells were evaluated by flow cytometry for FITC-conjugated BrdU incorporation. Panel A) CXCL12, B) PDGF-BB, C) TGFß. Panel A and B: *n* = 2, Panel C) n=3 from two different donors * = $p < 0.05$ compared to DMSO alone, # = $p < 0.05$ compared to agonist alone.

Figure 15

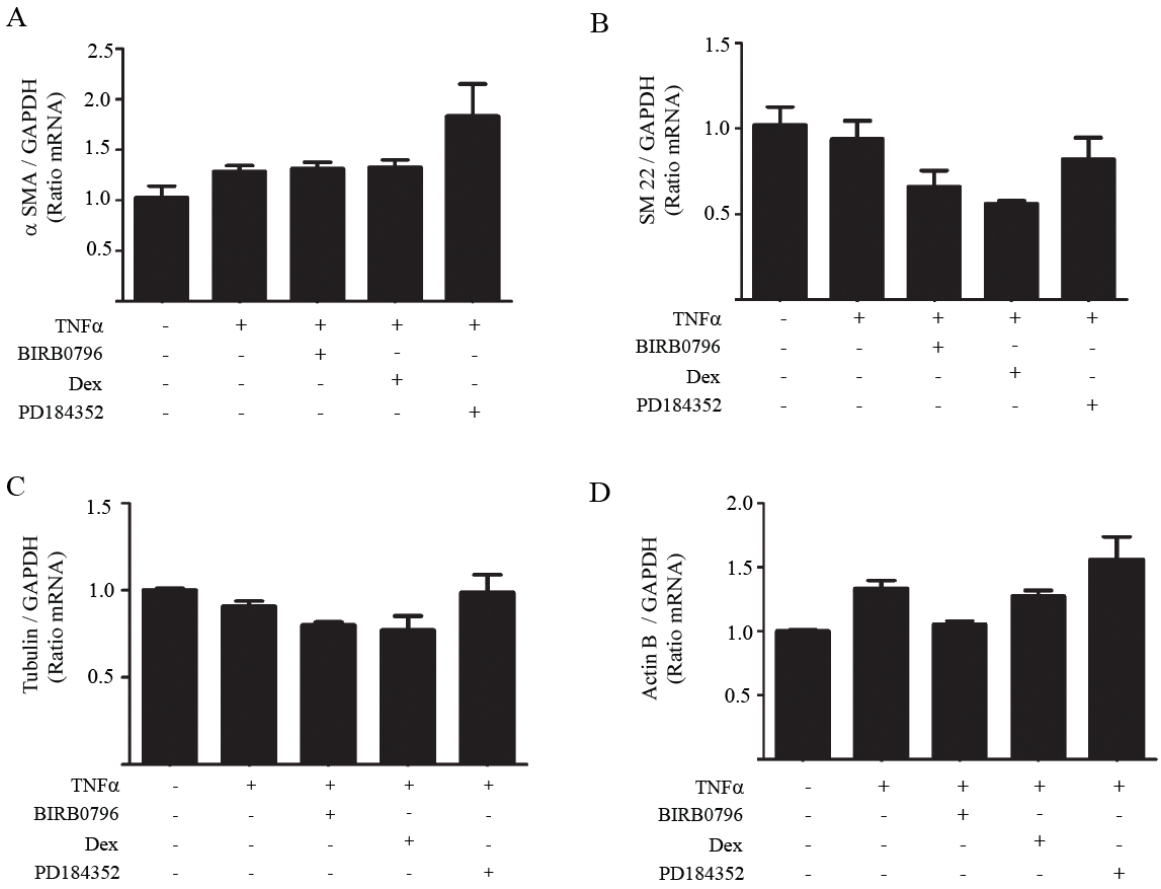

Figure 15) Contractile protein gene expression is unaffected by dexamethasone or MAPK inhibition. HASM cells were left untreated (-) or treated (+) for one hour with 0.1 μM BIRB0796, 2.0 μM PD184352 or 100nM Dexamethasone prior to exposure to 10 ng/ml TNFα (+) or not (-) for 8 hours. After stimulation total RNA was extracted and mRNA abundance was determined via qRT-PCR. A) αSMA, B) SM22 (transgelin), C) Tubulin, D) Actin B. *n* = 4 from two different donors.

Figure 16

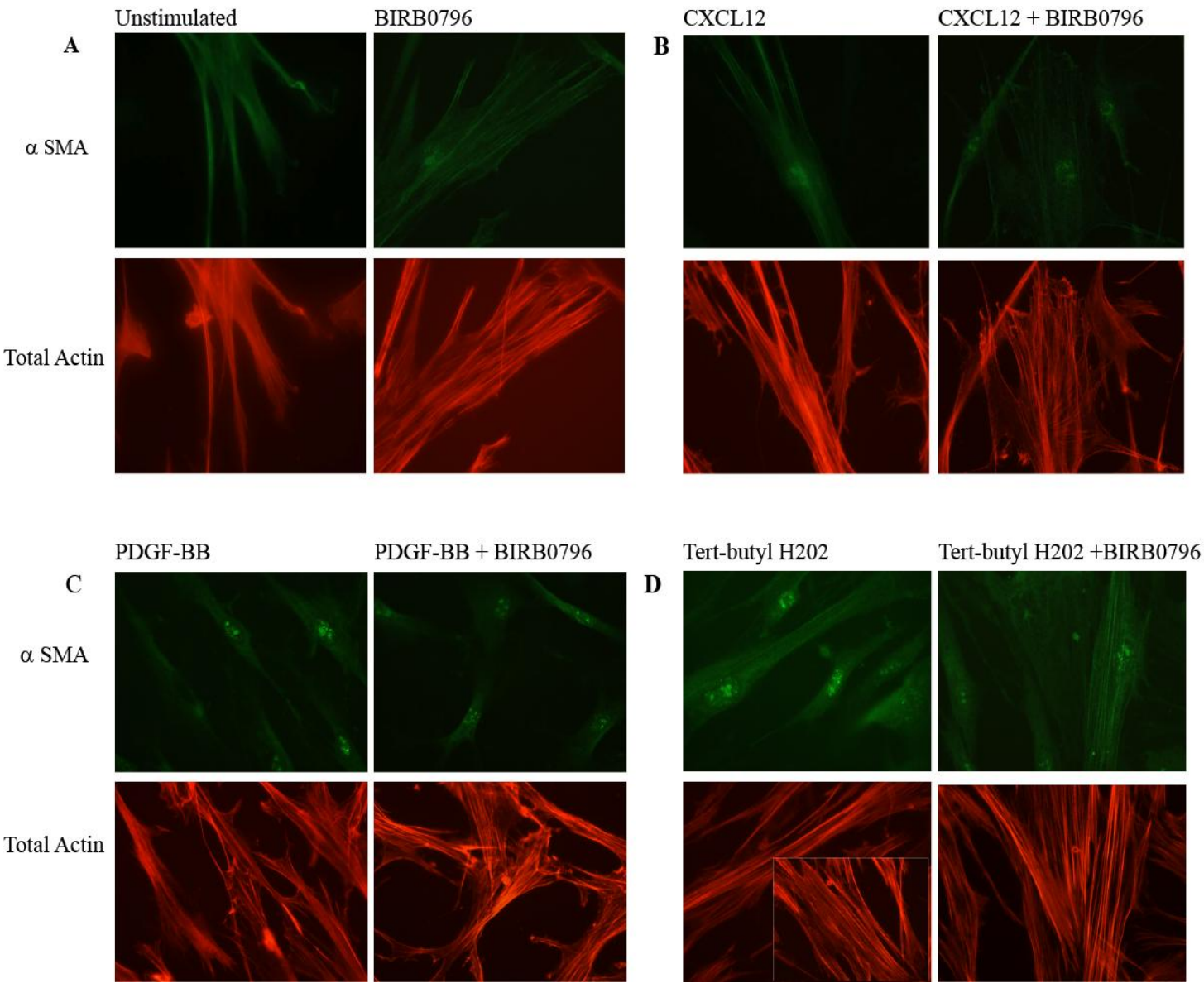

Fig 16) αSMA and total filamentous actin responses to chemokines and reactive oxygen species are not p38 MAPK dependent. HASM cells were grown on microchamber Labteck slides and serum-deprived cells were either left untreated or treated for one hour with 0.1 μM BIRB0796. Cells were then exposed to CXCL12 (80 ng/ml), PDGF-BB (15 ng/ml) or 500 μM tert-butyl hydrogen peroxide for 15 minutes. Fixed cells were then probed for αSMA and total filamentous actin and visualized under 200X microscopy. Control conditions contain DMSO 1 in 1,000 (v/v). Images are situated at identical locations on slide and could be merged. Photos are representative of 4 photos per well.

Figure 17

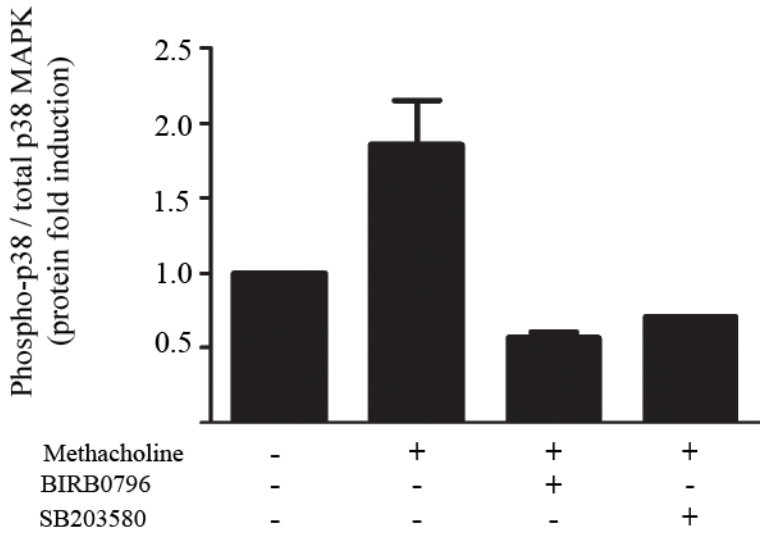

Fig 17) p38 MAPK becomes activated in rat tracheal muscle strips following methacholine stimulation. Tracheal rings dissected from Lewis rats were untreated (-) or treated (+) for one hour with 0.1 μM BIRB0796, or 5.0 μM SB203580 before being treated with $4x10^{-4}$ M methacholine. Proteins extracted from these strips were probed for phosphorylated and total p38 MAPK. *n* = 2 from two different rats.

Figure 18

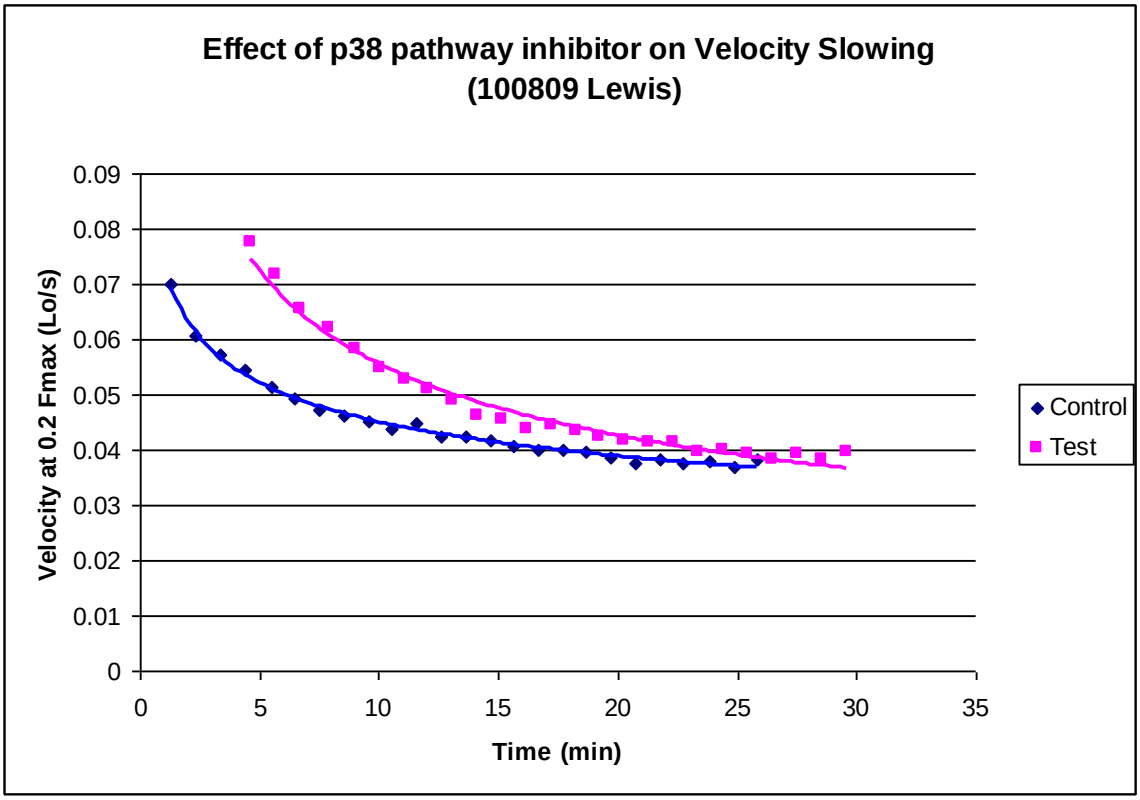

Figure 18) Smooth muscle velocity of shortening is unaffected by p38 MAPK blockade. Lewis rat tracheal muscle strips were incubated (test) or not (control) with 0.1 μM BIRB0796 for one hour before being subjected to $5x10^{-4}$M methacholine challenge. Velocity of shortening measurements are recorded by a force transducer at 20% of maximal force capacity of each strip. Measures are taken at quick release every minute and represented as velocity of optimal length (L0) per second.

Figure 19

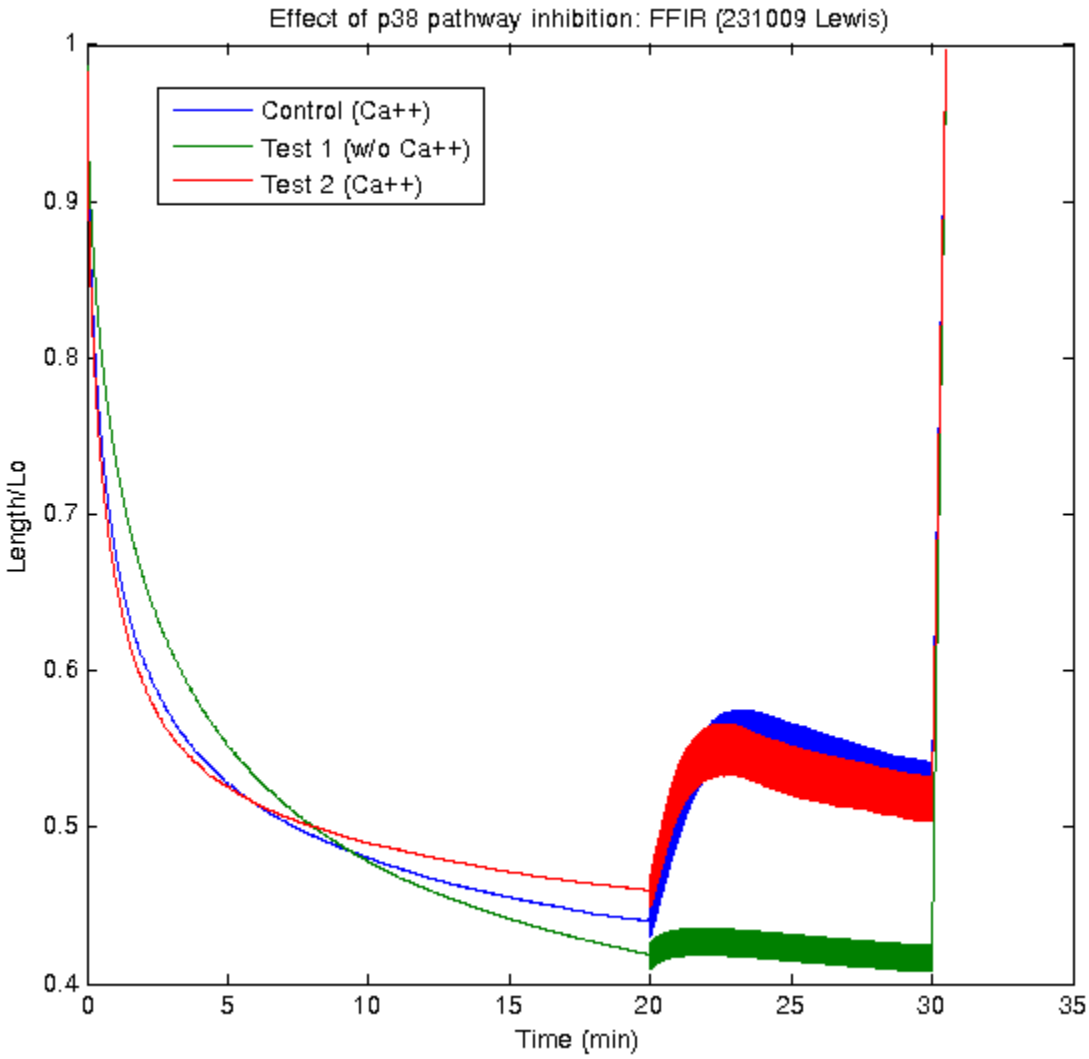

Figure 19) Force fluctuation induced re-lengthening is similar in vehicle or BIRB0796 treated tracheal muscle strips. . Lewis rat tracheal muscle strips were incubated (test 2) or not (control) with 0.1 μM BIRB0796 for one hour before being subjected to $5x10^{-4}$ M methacholine challenge. A negative control strip without calcium is represented by 'test 1'. Strips are clamped for 20 minutes at 32% FMax before being subjected to oscillations that simulate normal breathing. Oscillations cause re-lengthening that is measured instantaneously. Re-lengthening is expressed as a measure of new length/optimal length over time.

Chapter 6: Conclusion

6.1 Role for p38 MAPK in refractory asthma

The focus of this study was to understand the role played by p38 MAPK in refractory asthma. Some of the work completed here has elucidated novel aspects of p38 MAPK activity in human airway smooth muscle as it relates to the pathology of asthma, but obviously important questions remain.

I have shown in chapter three that p38 MAPK is increased in asthmatic airway smooth muscle and that this can be modeled *in vitro* in cells that are stimulated by TNFα. The downstream targets of p38 MAPK were differentially affected by treatment with dexamethasone. On the one hand, MAPKAP-K2 phosphorylation was attenuated while MSK1 phosphorylation was increased. Additionally, DUSP1 levels were increased. These results point to a possible tipping of the signaling balance away from the inflammatory MAPKAP-K2 and towards the anti-inflammatory MSK1 by GCs, perhaps via DUSP1.

Support for the idea that MSKs and GC derived DUSP1 control the inflammatory milieu exists. *In vitro* models have shown that cells that fail to express DUSP1 cannot down-regulate p38 MAPK activation with dexamethasone treatment (Lasa, Abraham et al. 2002; Zhao, Wang et al. 2006). In a positive feedback loop, MSK1/MSK2 are responsible for transcriptional induction of DUSP1. *In vivo* evidence of this is provided by Ananieva et al, who show DUSP1 mRNA levels to be significantly lower in MSK1/MSK2 knockout murine macrophages compared to wild type mice (Ananieva, Darragh et al. 2008). Additionally they show that these cells produce significantly less IL-10 than DUSP1 double knockout mice, which, in turn, produce more than wild type mice (Zhao, Wang et al. 2006). This proves that MSKs are responsible for producing IL-10 when p38 MAPK activation is unchecked. In an additive role, IL-10 contributes to a more robust expression of DUSP1 in macrophages, and has been shown to synergize with dexamethasone in this regard (Hammer, Mages et al. 2005). IL-10 is known to inhibit eosinophilia, thus this difference reveals a therapeutic benefit of MSK1/MSK2. Thus, MSKs and DUSP1 are intricately involved and co-dependent in regulating the inflammatory milieu in macrophages. In HASM, our data shows that both DUSP1 and pMSK1 are increased in cells exposed to dexamethasone and TNFα (Figure 7D, 8A), while pMAPKAP-K2 gets reduced to

basal levels by the same treatment (Figure 7C). This supports the hypothesis that dexamethasone treatment targets the inflammatory arm of the p38 MAPK pathway without affecting the anti-inflammatory functions mediated through MSK1. Moreover, MSK1 is phosphorylated by the ERK1/ERK2 MAPK, which may maintain MSK1 activation in the absence of p38 MAPK signaling. In contrast to the earlier proposed roles for ERK1/ERK2 in driving inflammation through CXCL8 up-regulation, this would suggest that the ERK1/ERK2 MAPKs can contribute to anti-inflammatory effects as well. This highlights the complexity of cell signaling systems regulating inflammation. Moreover, IL-10 was unmeasurable in HASM cells, and the ability of DUSP1 activity linking p38 MAPK and an inflammatory cytokine or process was not revealed in our investigation.

In chapter four I demonstrate that HASM cells express the CXCR4 receptor for the chemokine CXCL12, a finding not yet described. Additionally, migration of HASM towards CXCL12 was partially p38 MAPK dependent. While it is necessary to concede that a mixture of cytokines, growth factors and inflammatory mediators permeate the extracellular fluids in which smooth muscle resides, the fact that CXCL12 is elevated in asthmatics and mediates fibrosis provides an interesting link between CXCL12, HASM migration and the remodelling process (Negrete-Garcia, Velazquez et al. ; Phillips, Burdick et al. 2004). Certainly this information, coupled with the cytoskeletal changes seen at the cellular level, is worthy of further investigation such that CXCL12 might be added to the list of known chemotactic agents for HASM. Additionally, p38 MAPK mediated migration should be re-tested under conditions where adherence and migration of cells are not confounded.

Much investigation has been concentrated on p38 MAPK as a drug target since its discovery as a mediator of inflammation. In the context of airway inflammation, one p38 MAPK inhibitor, SB239063, reduced IL-6, MMP-9 and neutrophil infiltration in the BAL of guinea pigs following inhaled endotoxin (Underwood, Osborn et al. 2000). More recently, and specifically in the field of airway smooth muscle, an *in vitro* study demonstrated a new p38 MAPK inhibitor ML340 has low activity against hepatic cytochrome P450 enzymes, and has been as effective as SB203580 in reducing IL-6 and TNFα production following TNFα stimulation (Munoz, Ramsay et al.). In light of the results presented here, it may be of interest to suppress only MAPKAP-K2 in order to prevent disruption of beneficial signal to MSK1. Further research into this selective inhibition may result in effective treatment of the more inflammatory effects of p38 MAPK.

6.2 Roles for ERK1/ERK2 in refractory asthma

The ERK1/ERK2 pathway was also found to be an important mediator of inflammation in this study. This pathway was seen activated in severe asthmatics via immunohistochemical staining, which led us to investigate it further.

In vitro experiments suggested that while TNFα strongly activated ERK1/ERK2, dexamethasone treatment of HASM cells did not significantly reduce its phosphorylation, confirming GC induced DUSP1 deactivates p38 MAPK preferentially. In gene expression assays, the inhibition of ERK1/ERK2 resulted in a substantial reduction of all cytokines except IL-6 and caused an increase in ATF3, a transcription factor shown to control allergic pulmonary inflammation. With effective control over inflammatory cytokine expression and an anti-inflammatory transcription factor, these results point towards a powerful role for ERK1/ERK2 in contributing to inflammation in asthma.

Of these, a novel finding was that the ERK1/ERK2 pathway regulated chemokine secretion from HASM cells that potently controlled neutrophil migration. CXCL8 secretion, which is known to mediate neutrophil migration, was amongst the genes strongly down-regulated by ERK1/ERK2 inhibition, primarily evident at the transcript level. The importance of the ERK pathway, neutrophils and how they migrate has been previously investigated in airway disease (Fuhler, Knol et al. 2005; Li, Liao et al. 2007). To my knowledge, ERK1/ERK2 dependent neutrophil migration from airway smooth muscle conditioned media has not been demonstrated previously, and represents an significant advance in understanding the signaling responsible for generating leukocyte chemotactic mediators. This migration assay warrants further investigation and should include pre-treatment with dexamethasone such that the impact of p38 MAPK with increased DUSP1 activity could be assessed within this picture.

In light of these findings a novel target for treatment of refractory asthma may be the ERK1/ERK2 pathway. It is possible that glucocorticoids reach the limit of their effectiveness due to the fact that ERK1/ERK2 may be resistant to its effects, at least in HASM cells. As outlined in the introduction, inhibitors of the ERK1/ERK2 pathway have been the focus of extensive investigation for therapeutic interventions options, particularly in the field of oncology. The success story of Sorafenib, a Raf kinase inhibitor used in cancer treatment, may encourage future testing of MEK pathway inhibitors for the treatment of refractory asthma.

In conclusion, HASM contributes to the inflammatory milieu seen in the asthmatic airway and has been shown here to migrate, proliferate and produce key inflammatory cytokines

and transcription factors. Importantly, it is a source of chemotactic mediators that encourage neutrophil migration, a hallmark of refractory asthma. The MAPK pathways are significant signaling mediators of these effects in HASM, and as such these protein kinases and more importantly their downstream targets represent attractive therapeutic targets.

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Appendix:

Table 4. Subject demographics

Groups*	Controls	Mild asthma	Severe asthma
Sex (F:M)	04:04	05:03	04:05
Age (y)	39 (20-81)	37 (25-54)	42 (26-61)
Disease duration (y)	N/A	16 (4-57)	28 (7-44)
FEV1 % predicted	105 (7,2)	90 (5,9)	71 (8,9)
FEV1 (L)	2 (0,6)	3 (0,3)	3 (0,3)
FVC % predicted	108 (6,5)	101 (5,3)	84 (5,6)
FVC (L)	3 (0,6)	4 (0,4)	4 (0,3)
FEV1/FVC (%)	82 (3,1)	78 (1,7)	72 (6,2)
Atopy (F:M)	N/A	03:03	03:05

M. Male, F. Female

* Age and disease duration presented as medians (range).

All other data presented as means ± (SEM).