University of Glasgow

Effects of IL-33 on the Interaction between HMC-1 Cells and Human Airway Smooth Muscle Cells ©

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This dissertation is submitted for the fulfilment of the requirements for the degree of Masters of Science (Med Sci) by Research

M.Sc. (Med Sci) Research
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Abbreviations

15-HETE	15-hydroxy-eicosatetraenoic acid
ADA	adalimumab
ADAM33	a disintegrin and metalloproteinase 33
AHR	airways hyper-responsiveness
ANOVA	analysis of variance
ASM	airway smooth muscle
BAL	broncholalveolar lavage
BEC	bronchial epithelial cell
bFGF-2	basic fibroblast growth factor 2
C5	complement factor 5
CCL	chemokine (C-C motif) ligand
CD	cluster differentiation
COSHH	Control of substances hazardous to health
CTGF	connective tissue growth factor
CTLA	cytotoxic T-lymphocyte antigen
CXCL	chemokine (C-X-C motif) ligand
CXCR	chemokine, (C-X-C) motif receptor
DC	dendritic cells
DPP10	dipeptidyl peptidase 10
EAR	early-phase asthmatic reactions
ECM	extracellular matrix
ECP	eosinophil cationic protein
EDN	eosinophil-derived neurotoxin

EMTU	epithelial-mesenchymal trophic unit
ENT	etanercept
EPO	eosinophil peroxidase
FACS	fluorescent activated cell sorting
FCS	foetal calf serum
FGF	fibroblast growth factor
GBRC	Glasgow biomedical research centre
GDNF	glial-derived neurotrophic factor
GERD	gastroesophageal reflux
GM-CSF	granulocyte macrophage colony stimulating factor
HASM	human airway smooth muscle
HB-EGF	heparin-binding epidermal growth factor
HLMC	human lung mast cell
HMC	human mast cell
HMC-1	human mastocytosis cell line-1
HUCBMC	human umbilical cord blood-derived mast cell
ICAM	intracellular adhesion molecule
IDMEM	Iscove's modified Dulbecco modified eagle medium
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL-1RAcP	IL-1 receptor accessory protein
LAR	late-phase asthmatic reactions
LFA	lymphocyte function associated antigen
LT	leukotriene

MAP kinase	mitogen-activated protein kinase
MBP	major basic protein
MCP	monocyte chemotactic protein
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor *kappa*-light-chain-enhancer of activated B cells
NGF	nerve growth factor
NO	nitric oxide
PAF	platelet activating factor
PAMP	pathogen associated molecular patterns
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PE	phycoerytherin
PG	prostaglandin
PHF11	plant homeodomain finger protein-11
RANTES	regulated on activation normal T-cell expressed and secreted
rpm	revolutions per minute
SCF	stem cell factor
SNP	single nucleotide polymorphism

TGF	transforming growth factor
Th	T helper cells
TIM1	T-cell immunoglobulin mucin-domain
TLR	toll like receptor
TNF	tumour necrosis factor
TSLC	tumour suppressor in lung cancer
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VLA	Very late activating antigen
β_2.agonists	β_2-adrenoceptors agonists
β-agonists	beta adrenoceptor agonists

Abstract

Asthma is one of the most common chronic diseases in the world, affecting approximately 300 million people worldwide. Asthma has many phenotypes, allergic asthma being the most common form. Most cases are initiated by IgE antibodies, generally referred to as IgE-mediated allergic asthma. Treatments include β_2-agonists and inhaled corticosteroids, although there is no definitive cure for the disease. Asthma results in a plethora of pathological processes, present in many types of asthma. This can make differential diagnosis difficult, although several processes are characteristic of the disease pathology including; airway remodelling, airways hyperreactivity and airways inflammation.

The interaction of mast cells and human airway smooth muscle (HASM) cells play an important role in the characteristic pathological processes involved with asthma.

The recently discovered cytokine, interleukin 33 (IL-33), is thought to play an important role in a variety of autoimmune diseases; including asthma and atopic dermatitis.

The aims of this study were to investigate the interaction of mast cells and HASM cells *in vitro*, analysing the adhesion molecules involved and the potential presence of chemokines relevant to the adhesion process. In addition, the effects of IL-33 on the above processes were also investigated.

HMC-1 cells were analysed for the presence of several adhesion molecules, using a primary and secondary antibody, which were quantified using a fluorescently activated cell sorting machine (FACS). Once the presence of the adhesion molecules were ascertained, the effects of IL-33 on the adhesion molecule expression was performed. Anti-TNF-α therapies were also used to

assess whether a change in adhesion molecule expression, on the HMC-1 cells, was apparent.

The results confirm the presence of the adhesion molecules intracellular adhesion molecule 1 (ICAM-1), and Integrins α4 and β1. The presence of IL-33 (21 hours exposure) resulted in an up regulation of ICAM-1 on HMC-1 cells, being statistically different to the HMC-1 cells left untreated (99% confidence level). IL-33 seemed to have little effect on Integrin α4 or β1 expression on the HMC-1 cells. The anti-TNF-α therapies lead to a decrease in ICAM-1 expression on HMC-1 cells, after being exposed to both TNF-α and IL-33. This reduction in ICAM-1 expression was statistically different to the positive controls (HMC-1 cells treated with TNF-α alone) using both anti-TNF-α therapies with TNF-α and IL-33, at the 95% confidence level.

The effects of IL-33 on HMC-1 cell/HASM cells could lead to differences in adhesion, via ICAM-1. These effects may also lead to HMC-1 cells releasing TNF-α, having an autocrine effect on the HMC-1 cells.

Recommendations for future work include; analysing other adhesion molecules relevant to mast cell/HASM cell interactions, including tumour suppressor in lung cancer-1 (TSLC-1) and vascular cell adhesion molecule-1 (VCAM-1). Using mast cells derived from human umbilical cord blood stem cells would represent an improvement to using HMC-1 cells. *In vivo* research would also prove invaluable, quantifying and localising mast cells in asthma induced mice for example.

1. Introduction

1.1 Overview of lung anatomy and physiology

The lungs are located in the thoracic cavity, composed of a spongy texture and are honeycombed with a moist epithelium that serves as the respiratory surface (Campbell *et al.,* 1999). During respiration, air passes into the trachea, which is made up of rings of cartilage helping it to maintain its structure. The trachea then branches into two bronchi, leading to each of the lungs. Within the lung, these bronchi divide further into finer tubes called bronchioles (Campbell *et al.,* 1999). The epithelium lining the major branches of the trachea and subsequent bronchi are covered by cilia and a thin film of mucous. The mucous traps contaminants (i.e. pollen and dust), which is transported by the cilia towards the pharynx, where it is swallowed into the oesophagus (Campbell *et al.,* 1999). This process helps cleanse the respiratory system.

1.2 Aetiology of asthma

The definition of asthma has changed repeatedly over the past several decades. In particular asthma is no longer regarded as a condition defined by a physiological, functional parameter. It is now has cellular and biochemical components, increasing the complexity of the diseases pathology (Johanssen *et al.,* 2004).

Asthma has many phenotypes, which are summarised below:

1.2.1 Allergic asthma

The most common form of asthma is allergic asthma (Lung 2008). Most cases are initiated by immunoglobulin E (IgE) antibodies, generally referred to as IgE-mediated allergic asthma (Johanssen *et al.,* 2004). The causes of allergic

asthma include: pet dander, pollen and dust mites, wood dust, smoke, irritants, chemicals, viral/bacterial infections, stress and the emotional state of the patient (Lung 2008).

Most childhood asthma is considered an allergic type of asthma, occurring more often in young boys than girls. Research has shown that maternal smoking can contribute to asthma or other impairment of infant lung function, even before the child is born. Continued exposure to cigarette smoking can irritate the respiratory tract and make infants and children particularly vulnerable to allergic asthma (Floreani *et al.,* 1999).

1.2.2 Intrinsic asthma/Non-atopic asthma

Intrinsic asthma is not likely to develop in children; its typical onset occurs after the age of 40. Possible causes of intrinsic asthma include respiratory irritants such as; perfumes, cleaning agents, fumes, smoke and cold air, upper respiratory infections, and gastroesophageal reflux (GERD) (Braman, 2003).

1.2.3 Exercise induced asthma

At least eleven percent of the non-asthmatic population in the UK experience exercise-induced asthma (Lung, 2008). Many of these people have allergies or a family history of allergies.

Exercise-induced asthma can affect anyone at any age and can be attributed to the loss of heat and moisture in the lungs that occurs with strenuous exercise. Frequent coughing during exercise may be the only symptom of exercise-induced asthma, although during cold and dry conditions, exercise-induced asthma symptoms can become more severe (Lung, 2008).

1.2.4 Nocturnal Asthma

Nocturnal asthma normally affects people when they are sleeping. Asthmatic symptoms can occur regardless of the time of day a person is sleeping, although they tend to be quite severe between midnight and 4am. Nocturnal asthma can be triggered by allergens in bedding or the bedroom, a decrease in room temperature and GERD (Lung, 2008). Approximately 75% of asthmatic patients in the UK are awakened by asthmatic symptoms at least once per week, with approximately 40% experiencing nocturnal symptoms on a nightly basis (Sunderland, 2005).

1.2.5 Occupational asthma

Occupational asthma occurs directly as a result of breathing chemical fumes, wood dust, or other irritants over long periods of time. A total of 200 agents have been implicated in causing occupational asthma in the workplace (Chan-Yeung *et al.,* 1994).

1.2.6 Steroid resistant asthma

Overuse of asthma medications, such as steroidal based drugs, can lead to status asthmaticus, a severe asthma attack that doesn't responds to medication and may require mechanical ventilation to reverse (Lung, 2008).

1.2.7 Hereditary predisposition

Ober *et al*., conducted a review investigating genetic factors that can predispose individuals to asthma and atopy. Their review identified 54 genes across 6 populations studied, with 25 being associated with

2-5 population-based studies. Genome wide-linkage analysis revealed several genes associated with asthma and atopy, in particular; chromosomes 5q31–33, 6p21, and 12q13–q24 (Ober *et al.,* 2006). At least 14 genes in the 5q region have been associated with an asthmatic or atopic phenotype, several of which are responsible for interleukin 4 (IL-4) and IL-13 synthesis, cytokines important in the pathophysiolgy of the disease (Wills-Karp and Ewat, *2004*).

ADAM33 is a member of a family of genes that encode membrane-anchored zinc-dependent metalloproteinases that are implicated in cell–cell interactions, cell fusion and cell signalling (Eerdewegh *et al.,* 2002). One of two known ADAM33 transcripts are expressed in human airway smooth muscle and airway fibroblasts, which may indicate that it is involved in the remodelling of airway tissues (Eerdewegh *et al.,* 2002). So far, at least 54 single nucleotide polymorphisms (SNPs) have been identified (Eerdewegh *et al.,* 2002). These results indicate that SNPs in ADAM33 could result in variations of protein levels, although further research is needed to fully elucidate ADAM33s role in asthma (Wills-Karp and Ewat, 2004).

Allergic asthma is most relevant to the work presented in this dissertation and therefore most of the following discussion will relate to it.

1.3 Pathophysiology

Asthma results in a plethora of pathological processes, present in many types of asthma. This can make differential diagnosis difficult, although several processes are characteristic of the disease pathology:

1.3.1 Airways Inflammation

Once an individual becomes sensitized to a certain allergen, subsequent exposure can result in an inflammatory cascade, resulting in inflammatory cells becoming activated (National Jewish Health, 2006). Inhaled allergen challenge in allergic patients leads to an early phase allergic inflammatory reaction and in some cases this may be followed by a late-phase reaction (Bousquet *et al.,* 2000). During the early phase inflammatory process, the airway lumen becomes filled with mucous composed of plasma proteins exuded by the airway cilia, and mucous glycoproteins secreted from epithelial cells lining the airways (Barnes, 1996). The airway wall becomes infiltrated with inflammatory cells, eosinophils and lymphocytes in particular. This leads to shedding of the airway epithelium resulting in clumps of epithelial cells dispersed throughout the airway cavity (Barnes, 1996). Bronchoscopy of the airways reveals a swollen and reddened state in the asthmatic patient. Additional diagnostic tests include analysis of the bronchoalveolar lavage (BAL) fluid and biopsies taken from the airways. Both tests confirm the presence of macrophages, mast cells and T-cells all of which contribute to the inflammatory processes involved with asthma (Barnes, 1996).

1.3.2 Airways hyper-responsiveness (AHR)

AHR can result in breathing difficulties and resistance to normal airflow. Changes in airway function are accompanied by inflammatory cell infiltrates composed of eosinophils, T cells, monocytes and neutrophils, as well as cytokines associated with a Th_2 response (Brusasco *et al.,* 1990). This suggests that AHR is partially dependent on inflammatory events.

Airway remodelling may also contribute to AHR since the architecture of the lung is being remodelled i.e. an increased level of airways smooth muscle mass could lead to bronchoconstriction and partial occlusion of the trachea, which can contribute to an increase in breathing difficulties (Lloyd *et al.*, 2007).

As the disease progresses, a decline in lung function is apparent, leading to an increase in breathing difficulties.

1.3.3 Airway remodelling and subepithelial fibrosis

Airway remodelling is an ongoing process in reaction to persistent inflammation, as the body activates a variety of mechanisms in an attempt to repair the lungs. This leads to permanent structural and functional changes. Airway remodelling results in different airway characteristics, for example: epithelial cells that line the airway are damaged and the basement membrane underneath becomes thicker. An increase in the number and size of bronchial blood vessels is also present, suggesting that angiogenesis, the formation of new blood vessels from pre-existing endothelium, is an essential component of tissue growth and remodelling in asthma (McDonald, 2001). Additionally, the airways loose elasticity and the walls thicken, leading to excess muscle and eventually bronchoconstriction. This can result in long-term loss of lung function and an increase in the severity of asthma (Lloyd *et al.,* 2007).

Other theories oppose the idea that chronic inflammation is required for the remodelling process to occur, in particular Holgate *et al.,* have proposed that reactivation of the epithelial-mesenchymal trophic unit (EMTU) contributes to airway remodelling. The EMTU collectively refers to epithelial and mesenchymal cell mediated bidirectional growth control. Reactivation of the

EMTU can lead to excessive matrix deposition, as well as the production of mediators that can potentiate and intensify the remodelling responses throughout the airway wall (Holgate *et al.,* 2000). The increased matrix deposition seen in asthma is also likely to be caused by increased secretion from mesenchymal cells, myofibroblasts for example, in concert with an imbalance between matrix-degrading enzymes and inhibitors of these proteases (Lazaar and Panetierri, 2005).

1.4 Prevalence

Asthma is one of the most common chronic diseases in the world, affecting approximately 300 million people worldwide (Massoli *et al.,* 2004). It has become common in both children and adults in recent decades. Increasing prevalence of asthma has been associated with increases in atopic sensitization, along with similar increases in other allergic disorders such as eczema and rhinitis (Massoli *et al.,* 2004).

Over 5 million people in the UK have asthma, with approximately 1.4 million of them being children aged less than 16 years old (British Lung Foundation, 2008). In the UK, it is an important cause of impaired quality of life, consumption of prescribed drugs, use of primary care, hospital admission and mortality across the whole spectrum of age, sex, ethnicity, socioeconomic status and geographical location (Anderson *et al.,* 2007).

1.5 Treatments

Asthma aetiology and pathophysiology can vary, coupled with the fact that differential diagnosis can be difficult. Nevertheless, treatments are available that can ease symptoms as well as help individuals lead healthier lives.

Beta adrenoceptor agonists (β-agonists) were traditionally based on adrenaline, helping to relieve bronchoconstriction. These non-specific treatments had unpleasant side effects, leading to the development of specific β_2-adrenoceptors agonists (β_2-agonists), such as salbutamol, terbutaline and fenoterol (Sears and Lotvall, 2004). These fast acting treatments target the β_2-adrenoceptors located within bronchial smooth muscle, offering short term relief. Other β_2-agonists, such as Formoterol and salmeterol, have longer durations of action and can also be combined with inhaled corticosteroids (Sears and Lotvall, 2004). This combination improves lung function, and reduces symptoms and exacerbations of the asthmatic patient, compared to an increased dose of corticosteroids alone (Sears and Lotvall, 2004).

The long term effects of using long-acting β_2-agonists can result in the development of tolerance to the bronchoprotective role. Other research groups have reported an increase in patient morbidity and mortality from long-term usage of β_2-agonists, especially fenoterol (Spitzer *et al.,* 1992).

Inhaled corticosteroids are widely used to treat cases of moderate to severe cases of asthma. Their anti-inflammatory effects help to reduce bronchial inflammation as well as improving pulmonary function (Boushy, 2005).

The combined usage of long-acting β_2-agonists and inhaled corticosteroids has lead to an improvement in clinical effectiveness, possibly through complementary modes of action (Remington *et al.,* 2005).

1.6 Immune responses and asthma pathogenesis

T cell responses to irritants, such as viral infections or allergens, are thought to trigger asthmatic symptoms in genetically predisposed individuals. T-cell activation is initiated through the interaction of the T-cell receptor on T cells and major histocompatibility complex II (MHC class II) molecules on the surface of dendritic cells (DC). Further interaction of CD80 and CD86 on DCs with their respective receptors on T cells (CTLA4 and CD28) enables T-cell activation to proceed (Willis-Karp and Ewat, 2004). In asthmatic individuals, antigen presentation is thought to result in the polarization of T cell differentiation towards a Th_2 pattern; whereas T cells from non-atopic, non-asthmatic individuals show the opposing Th_1 (IFN-γ and IL-2) pattern of cytokine secretion (Figure 1) (Karp *et al.,* 2000). Activated Th_2 cells secrete a panel of cytokines, such as IL-4 (essential for T-cell survival and expansion, as well as B cell differentiation), IL-13 and IL-5 (Figure 1). IL-13 activates a receptor complex that is composed of the IL-4Rα and IL-13Rα1 on many cell types (figure 1) in the airway wall and is thought to mediate many processes that are relevant to asthma pathology (Willis-Karp and Ewat, 2004). In particular, IL-5 and IL-13 secretion results in mass eosinophilic infiltration of the airways (Figure 1) (Saelta and Tevato, 2001). The small airways become saturated with eosinophils in the "outer" section of the airway wall (between the smooth muscle and alveolar attachments), whereas the greatest density of eosinophils in the large airways is in the "inner" section of the airway wall (between the smooth muscle bundles and the basement membrane) (Saelta and Tevato, 2001).

1.6.1 Early-phase asthmatic reaction.

1.6.1.1 Mast cells

The most important cell type in the early-phase asthmatic reactions (EAR) is the mast cell (Venkatesha *et al.,* 2007*).* Mast cell activation in asthma is driven by exposure of the airways to inhaled aeroallergens, as well as in response to cytokines released from Th_2 cells. This causes cross-linking of allergen-specific IgE bound to the mast cell high affinity IgE receptor FcεRI (Cruse et al., 2005). After immunological activation via the IgE receptor, the mast cell releases a variety of cytokines, lipid-derived mediators, amines, proteases and proteoglycans. These substances have the propensity to regulate adjacent cells and the metabolism of the extracellular matrix of connective tissues (Stevens and Austen, 1989). The cytokines released from mast cells also have an important role with the late-phase asthmatic reaction (LAR), discussed later on. Alternative mast cell stimuli include: proteases, adenosine and stem cell factor (SCF) (Cruse *et al.,* 2005).

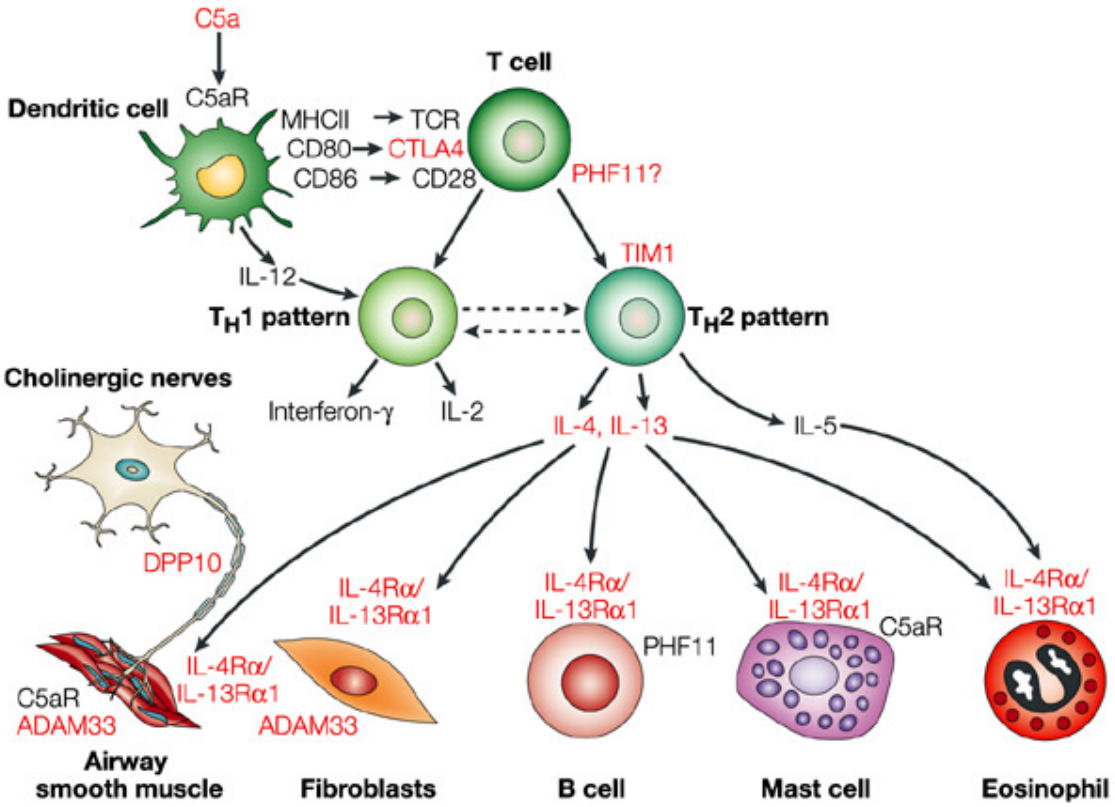

Figure 1. Innate and adaptive immune responses involved with asthma. Words in red in the figure represent candidate genes, with evidence for linkage to asthma. ADAM33 (a disintegrin and metalloproteinase 33), DPP10 (dipeptidyl peptidase 10), PHF11 (plant homeodomain (PHD) finger protein-11), C5 (complement factor 5), TIM1 (T-cell immunoglobulin and mucin-domain containing molecules). Adapted from Willis-Karp and Ewat (Willis-Karp and Ewat, 2004).

1.6.1.2 Basophils

Basophils also express the IgE receptor FcεRI and degranulate after re-exposure to allergen. CC-chemokines such as; CCL11, CCL24 (eotaxin-2), CCL26 (eotaxin-3), CCL7, CCL13 and CCL5, IL-3, IL-5, granulocyte macrophage colony stimulating factor (GM-CSF) and histamine-releasing factor can induce basophil mediator release (Prussin and Metcalfe, 2006). As well as stimulating the release of histamine and leukotrienes, CCL11 and CCL24 are also chemoattractant for basophils (Forssmann *et al.,* 1997).

Basophils produce a spectrum of mediators, including histamine, leukotrienes, IL-4, and IL-13 (Schroeder *et al.,* 2001)

1.6.2 Late-phase asthmatic reactions

The LAR involves excessive inflammation of the airways resulting in several structural changes, airways remodelling for example. These changes are induced by various mediators derived from inflammatory cells, such as; eosinophils, neutrophils, mast cells, T cells, macrophages, dendritic cells (DCs), endothelial cells, airway smooth muscle cells (ASM) and bronchial epithelial cells (BECs).

1.6.2.1 Eosinophils

In most asthma phenotypes, there are increases of eosinophils in the tissues, blood and bone marrow and, in general, raised numbers correlate with disease severity. Eosinophils are able to produce a variety of inflammatory mediators that have the potential to participate in mucosal damage (Figure 2).

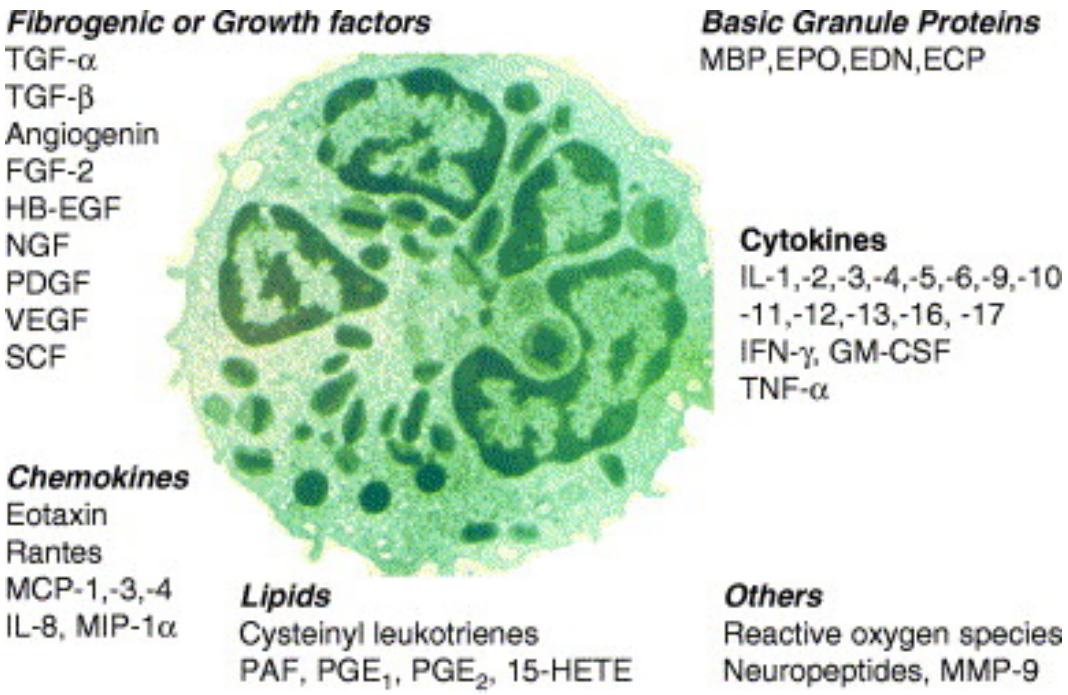

Figure 2. Inflammatory mediators derived from eosinophils. Abbreviations: ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; EPO, eosinophil peroxidase; FGF, fibroblast growth factor; GM-CSF, granulocyte/macrophage-colony stimulating factor; HB-EGF, heparin-binding epidermal growth factor; 15-HETE, 15-hydroxy-eicosatetraenoic acid; IFN, interferon; IL, interleukin; MBP, major basic protein; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinase; NGF, nerve growth factor; PAF, platelet activating factor; PDGF, platelet-derived growth factor; PG, prostaglandin; RANTES, regulated on activation normal T-cell expressed and secreted; SCF, stem cell factor; TGF, transforming growth factor; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor. Adapted from Kay, 2005 (Kay, 2005).

They include basic granular proteins, membrane-derived lipids, chemokines, cytokines, fibrogenic and growth factors, in addition to reactive oxygen species and neuropeptides (Figure 2) (Kay, 2005). Other fibrogenic and growth factors released from eosinophils are likely to be involved with the repair and remodelling processes associated with asthma as well. Eosinophils involved in the inflammatory response also produce large amounts of free radicals, such as superoxide and peroxynitrite (Maddox and Shwartz, 2002). These free radicals have the potential to cause further damage to neighbouring cells and tissues.

1.6.2.2 Neutrophils

Neutrophils produce a variety substances, including leukotrienes, cytokines (IL-1β, IL-6, TNF-α, TGF-β, CXCL8), proteases (elastase, collagenase, matrix metalloproteinase (MMP) 9), microbiocidal products (lactoferrin, myeloperoxidase, lysozyme), reactive oxygen intermediates (superoxide, hydrogen peroxide, OH^-) and nitric oxide (NO) (Wenzel, 2001)
Secretion of these products can cause airway narrowing, increased mucous secretion and increased ASM responsiveness (Gibbon *et al.,* 2001).

1.6.2.3 T cells

Cytokines secreted from Th_1 and Th_2 cells are heavily involved in the pathogenesis of asthma. Th_2 cells secrete cytokines, such as IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13 and GM-CSF, exerting a plethora of inflammatory effects. During the early stages of asthma, Th_2 cytokines are responsible for the production of IgE (Kuipers and Lambrecht, 2004). In later stages of asthma, these cytokines are responsible for the ongoing inflammation. Th_1 cells secrete

cytokines, such as IFN-γ, IL-2, IL-12, IL-18, TNF-α, and TNF-β (Mosman and Sad, 1996).

Chemokine production is also slightly different in Th_1 and Th_2 cells. Th_1 cells produce CCL5, and Th_2 cells produce CCL1 and CCL22 (macrophage-derived chemokine (MDC) (Smit and Lukacs, 2006).

1.6.2.4 Dendritic cells

Dendritic cells (DCs) are involved in both antigen presentation and the LAR. Increased numbers of DCs in asthmatic airways could be responsible for the persistence of chronic T cell-mediated allergic inflammation. Once allergen-specific Th_2 cells are recruited into the lung, repeated allergen presentation by lung DCs may drive the persistent stimulation of allergen-specific memory Th_2 cells. Repeated Th_2 cell stimulation exacerbates acute asthma episodes and consequently perpetuates the state of chronic inflammation that contributes to the airways remodelling and AHR, consistent with chronic asthma (Lipscomb and Masten, 2002). Depending on the stimulus, human DCs are able to produce CCL2, CCL3, CCL4, CCL17, CCL22 and CXCL8 (Penna *et al.,* 2002).

1.6.2.5 Macrophages

Macrophages are recruited to the airways of asthmatic individuals following allergen challenge. They secrete cytokines and chemokines, including IL-1, IFN-γ, TNF-α, IL-6, CCL2, CCL3, and CXCL8 (Gosett *et al.,* 1999), leading to the recruitment and activation of other inflammatory cells. Macrophages also inhibit allergic inflammation through the secretion of various inhibitory

mediators, including prostaglandins and IL-10. The secretion of these inhibitory mediators could potentially be impaired in asthmatic patients (Barnes, 2002).

1.6.2.6 Airway smooth muscle cells

Human airway smooth muscle (HASM) cells behave differently in asthmatic patients. They tend to proliferate faster due to altered patterns of matrix deposition and secrete greater amounts of connective tissue derived growth factor (stimulated by transforming growth factor beta (TGF-β)) (Bradding, 2007).

HASM cells are also able to produce a variety of cytokines and chemokines that promote the recruitment of inflammatory cells, leading to the activation of other lung-resident cells contributing to airway remodelling (Lloyd *et al.,* 2007). The airway remodelling process involves progressive occlusion of the trachea leading to AHR. Increased muscle mass is also thought to contribute to the development of AHR

1.6.2.7 Modulation of the immune response in asthma

There are several other molecules and cell surface receptors that can modulate the immune response involved with asthma, these are mentioned below:

TIM1 (T-cell immunoglobulin and mucin-domain containing molecules) is thought to be involved with Th_2 differentiation processes, through an as yet undefined pathway (Umetsu *et al.,* 2005). Specifically, TIM-1 is expressed on $CD4^+$ T cells after activation, and its expression is preferentially retained in Th_2 but not Th_1 cells. *In vitro* stimulation of $CD4^+$ T cells with a TIM-1-specific

monoclonal antibody and T cell receptor ligation enhances T cell proliferation in Th_2 cells, leading to the synthesis of IL-4 but not IFN-γ (Umetsu *et al.*, 2005). There is also evidence to suggest that the newly discovered asthma gene C5 (complement factor 5) binds to its receptor (C5aR) on DCs, human monocytes and macrophages to induce the production of the Th_1-promoting cytokine, IL-12 (Karp *et al.*, 2000).

DPP10 (dipeptidyl peptidase 10) is thought to modulate the activity of many chemokines and cytokines that regulate the inflammatory process. DPP10 is also expressed on central neurons, indicating that it might be important in the neuronal control of airway smooth-muscle tone (Kere and Laitinan, 2004). Furthermore, PHF11 (plant homeodomain (PHD) finger protein-11) is expressed on unactivated (Th_0) cells and might be involved in T-cell activation and differentiation, as well as immunoglobulin synthesis (Kere and Laitinan, 2004).

Bradykinin is also an important mediator involved with the pathogenesis of asthma. Its biosynthesis involves the action of kininogenase on vasoactive peptides formed during the inflammatory response from the α2 globulins; kininogens (Barnes, 1992). Kininogenases include plasma kallikrein and tissue kallikrein. There is also evidence for kinin activity in the BAL fluid from asthmatic patients, where bradykinin is likely to be formed from plasma that has exuded from the inflamed airways, by the action of plasma and tissue kallikreins (Barnes, 1992).

Bradykin has several effects on airway function in the asthmatic lung including; airway smooth muscle contraction, release of epithelial bronchodilators and increased mucous secretion (Barnes, 1992).

1.7 Mast cells

1.7.1 Development and subtypes

Mast cells develop from $CD34^+$ bone marrow progenitors and maturation occurs throughout human tissues, circulating as committed immature precursor forms (Austen and Boyle, 2001). During mast cell development, SCF plays an important role interacting via the mast cells c-Kit receptor (Williams and Galli, 2000). This is confirmed by evidence from Kitamura *et al.,* that mouse strains deficient in the c-Kit receptor (W/Wv) or the membrane-bound form of SCF, the Sl/SLd strain, were deficient in mast cells (Kitamura *et al,.* 1978). Reconstituted mast cells were then reintroduced into W/Wv mice, later giving rise to immature mast cells when cultured in the presence of SCF (Kitamura *et al,.* 1978). Other cytokines are also important for mast cell development and survival, including interleukin-3 (IL-3) and Th_2 associated cytokines such as IL-4 and IL-9 (Metcalf et *al.,* 1997).

Human mast cells contain measurable levels of tryptase, chymase, a cathepsin G-like protease, and carboxypeptidase. Mast cells containing tryptase and chymase are denoted as MC_{TC}, whereas the MC_T subtype contains only tryptase. MC_T predominate in the alveolar septa of the lung and in the small intestinal mucosa, and the MC_{TC} are mainly found in the skin and in the small intestinal submucosa (Metcalf et *al.,* 1997).

In the normal human lung, 90% of the mast cells present consist of the MC_T subtype; the remainder are MC_{TC} (Severine *et al.,* 2001). Recent studies on asthmatic patients indicate that this balance shifts, resulting in an increase of the MC_{TC} subtype (Bradding *et al.,* 2006). This shift in mast cell subtype may contribute to several of the pathological features associated with asthma.

MC_{TC} cell activation/degranulation usually results in chymase secretion. Once secreted, chymase is able to cleave SCF at one of its amino acid sites, Phenylanine^{159} (the 159 corresponds to the specific amino acid number), resulting in two other metabolites: SCF^{1-159} and $SCF^{160-166}$. The larger fragment, SCF^{1-159}, has an altered biological effect on MC_{TC} cells resulting in increased histamine release (Severine *et al.,* 2001). Increases of histamine in the local microenvironment can lead to bronchoconstriction and smooth muscle cell activation.

1.7.2 Location

Mast cells are widely distributed throughout vascularized tissues, epithelia, and in close proximity to blood vessels, nerves, smooth muscle cells, mucous-producing glands and hair follicles. Mast cells are also present near surfaces exposed to the outside environment, including the skin, airways and gastrointestinal tract. It is no coincidence that pathogens, allergens and other environmental agents are frequently encountered at these sites as well (Metcalf et *al.,* 1997).

1.7.3 Receptors and activation

Apart from mast cell activation via the FcεRI receptors, there are several other important stimuli that can lead to mast cell activation and subsequent degranulation (Figure 3). Mast cell effector functions can also be stimulated by IgE binding to IgG receptors (FCγR1 and III activate, whereas FcγRII is inhibitory), complement receptors, histamine-releasing factors, and toll like receptors (TLRs) (Robinson, 2004). These alternative receptors such as; TLR4,

TLR2, CD48 and complement receptor 1 recognize microbes and their products through direct pattern recognition or pathogen associated molecular patterns (PAMPs). Upon stimulation, these receptors can activate mast cells absent of antibody binding (Boyce, 2003).

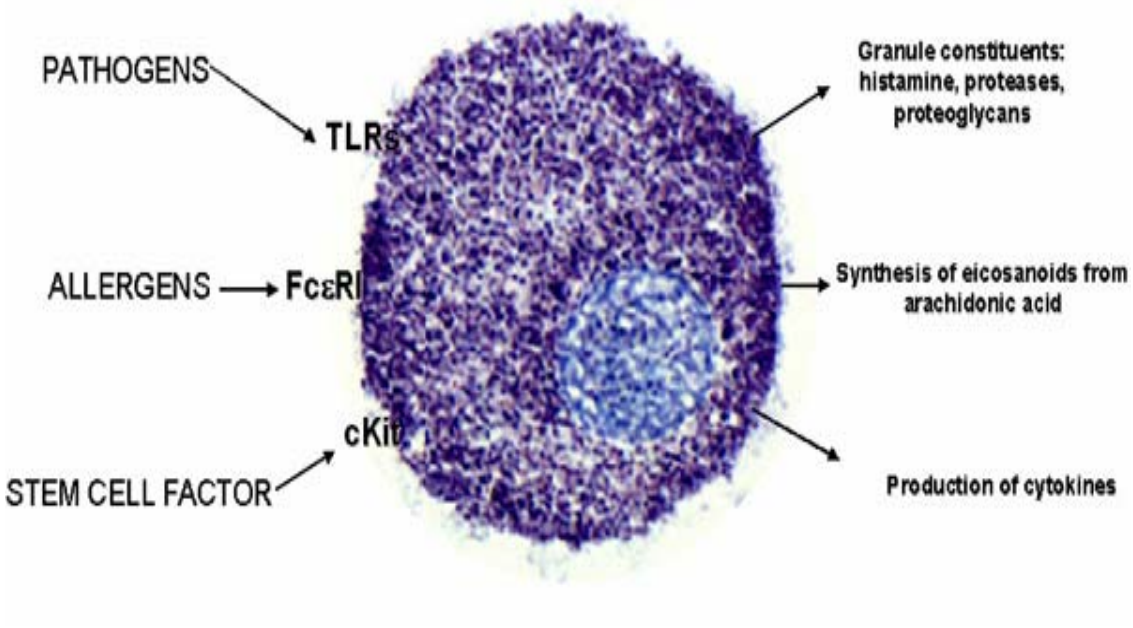

Figure 3. Mast cell receptors with their associated ligands, and the products released after mast cell activation. Adapted from Galli *et al.,* (Galli *et al.,* 2005).

1.7.4 Mediators and their effects

Mediators produced by mast cells can be divided into 3 groups; preformed mediators (e.g. histamine), newly synthesized lipid mediators (e.g. prostaglandins and leukotrienes), and cytokines and growth factors (e.g. tumour necrosis factor alpha (TNF-α) and vascular endothelial growth factor (VEGF)) (Prussin and Metcalfe, 2006). These categories are not mutually exclusive since at least one cytokine, TNF-α, occurs as a preformed mediator and as a newly synthesized molecule (Prussin and Metcalfe, 2006).

1.7.4.1 Preformed mediators

Preformed mediators are packaged within secretory granules in the mast cell and upon activation are released into the extracellular environment within minutes. Granules released include histamine, proteases (tryptase, chymase, and carboxypeptidase) and proteoglycans (heparin and chondroitin sulphate E) (Galli *et al.,* 2001). Histamine secretion can result in smooth muscle contraction (leading to bronchoconstriction), nerve ending stimulation leading to vasodilation and bronchoconstriction along with mucous secretion (Figure 4.) (Galli *et al.,* 2001). The i*n vitro* effects of tryptase include; cleavage of complement molecules C3 and C3a, activation of fibroblasts, accumulation of inflammatory cells, as well as enhancing histamine-induced smooth muscle contraction (Johnson and Knox, 1997). Tryptase secretions can also lead to tissue degradation, as well as airway remodelling and the build up of tissue fluid (oedema) leading to tissue swelling (Figure 4). Tryptase has also been

used as a marker of mast cell degranulation, where increased concentrations of this protease have been found in the BAL fluid of asthmatic patients (Barnes, 2002). Apart from the effects of the chymase on SCF mentioned above, it can also lead to increased mucous secretions from goblet cells, as well as tissue degradation (Figure 4) (Bousquet *et al.,* 2000).

1.7.4.2 Lipid mediators

Lipid mediators include prostaglandins, leukotrienes and inflammatory metabolites derived from the peroxidation of arachidonic acid. Several of these lipid mediators, such as prostaglandin D2 (PGD2) and leukotriene C4 (LTC4), may be newly produced after mast cell activation.

Prostaglandins have several effects on the airways, including bronchoconstriction, plasma exudation, sensitization of nerve endings, and effects on inflammatory cells (Figure 4). PGD2 is involved in the recruitment of inflammatory cells, stimulating the chemotaxis of Th_2 cells, eosinophils, neutrophils and basophils *in vitro* and *in vivo* (Hirai *et al.,* 2001). Additionally, overproduction of PGD2 in mice results in enhanced pulmonary inflammation following allergen challenge (Fujitani *et al.,* 2002), confirming its role as a pro-inflammatory lipid mediator. Higher levels of PGD2 have also been detected in asthmatic patients BAL fluid, upon allergen challenge (Kosteins and Ulven, 2006).

Leukotriene B4 (LTB4) is a potent neutrophil chemoattractant that enhances neutrophil-endothelial interactions and stimulates neutrophil activation. This leads to degranulation and the release of mediators, enzymes and superoxides.

It also has weak effects on smooth muscle, but may contribute to airway narrowing by producing local oedema and increased mucous secretion (Figure 4) (Black *et al.,* 1989). LTB4 levels are also higher in asthmatic patients compared to controls (Montuschi and Barnes, 2002, Cap *et al.,* 2004).

1.7.4.3 Cytokines and growth factors

Cytokines released from activated mast cells will have an effect on the EAR as well as the LAR, potentially leading to persistent as well as chronic respiratory distress.

Mast cell derived TNF-α is a major pro-inflammatory cytokine, strongly involved in the pathogenesis of asthma (Bradding *et al.,* 1994). In addition, it up-regulates endothelial and epithelial adhesion molecules, increases AHR and also leads to bronchoconstriction (Figure 4). Two recent studies have shown that TNF-α expression is increased in severe asthma, reflected by an increase in the BAL as well as increased protein expression on peripheral blood mononuclear cells (PBMCs) (Bradding *et al.,* 2006).

Other cytokines produced by mast cells include IL-4; associated with Th_2 cell differentiation and IgE synthesis, IL-3, GM-CSF, and IL-5; critical for eosinophil development and survival, IL-6, IL-8, and IL-16 (Bradding *et al.,* 2006). Mast cells also secrete the pro-fibrogenic cytokines; TGF-β and basic fibroblast growth factor 2 (bFGF-2). They also produce chemokines, such as CC-chemokine ligand-3 (CCL3). These chemokines have the ability to stimulate histamine release from mast cells and basophils (Prussin and Metcalfe, 2006). CCL2 and CCL11 (eotaxin-1) are also secreted from mast cells, involved in leukocyte attraction during inflammation. Increased levels of

CCL2 and CCL3 have also been found in the BAL fluid of asthmatics from several research groups (Cruikshank *et al.,* 1995, Holgate *et al.,* 1997) . This links mast cell degranulation with the LAR in asthma.

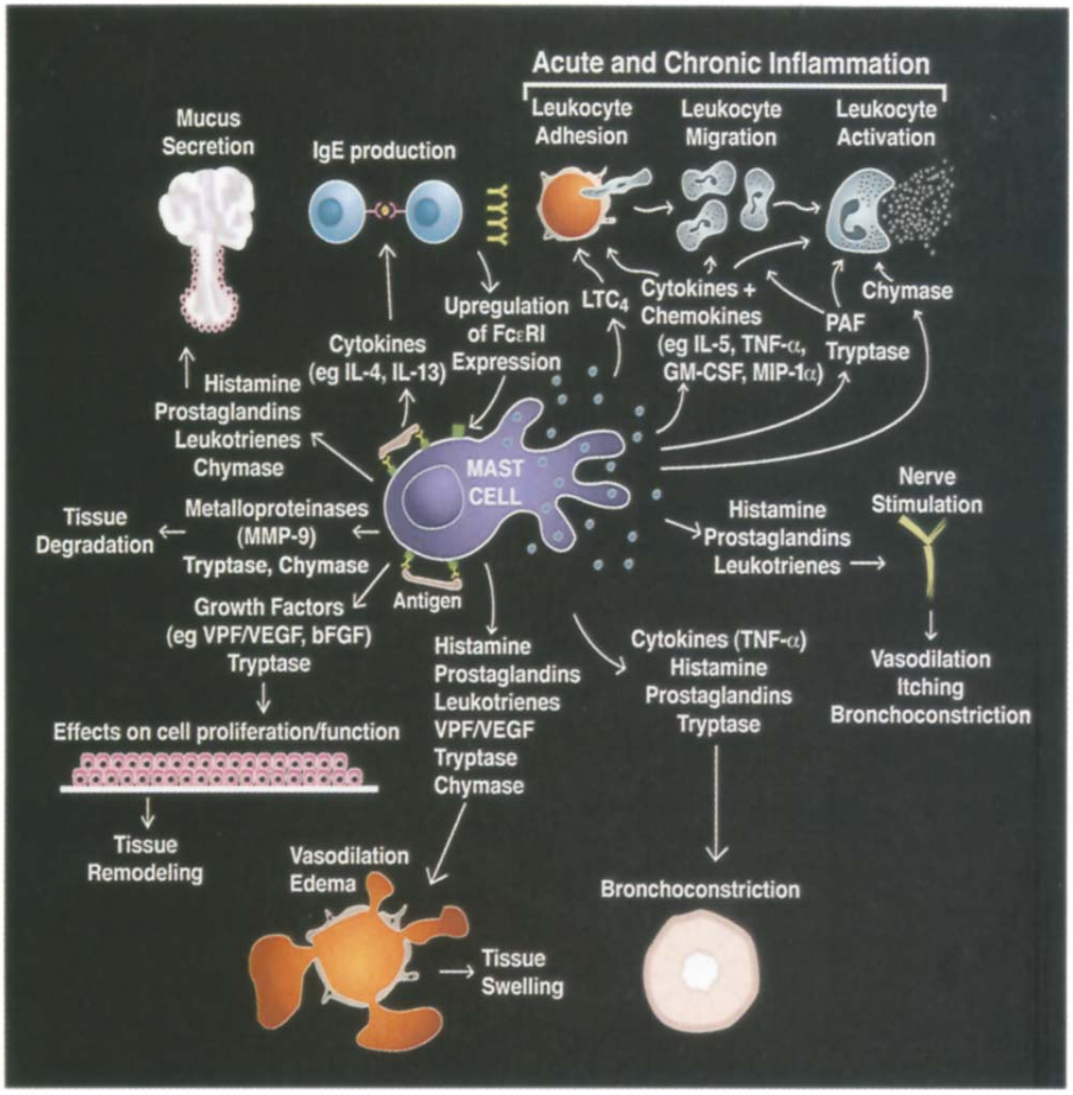

Figure 4. Major mediators, cytokines, and growth factors produced by IgE- and antigen-dependent activation of mast cells. Adapted from Williams *et al.*, (Williams *et al.*, 2000).

1.8 Airway smooth muscle cells

1.8.1 Development and differentiation

Airways smooth muscle cells (ASM) arise from pluripotent mesodermal cells that, under the appropriate chemical signals, become myoblasts. Myoblasts then fuse together, again with the appropriate growth factors and cytokines present, to form muscle fibers that later develop into non-striated smooth muscle cells or mature ASM cells (McDonald, 1997).

ASM cell differentiation begins by the formation of a ring of mesenchymal cells immediately underlying the epithelium of the epithelial buds (McHugh, 1995). These rapidly form interlocking bundles that become wider and increasingly compact along the bronchial tree to the trachea (McHugh, 1995). Their orientation is perpendicular to the long axis of the airway. The ASM exhibits rhythmic contractility (i.e. it is a phasic-type smooth muscle - rapid contraction and relaxation) soon after formation and along with the spontaneous airway narrowing, cause' expansion of the tubule walls (Sparrow *et al.,* 1995). This stretching is the mechanical stimulus to smooth muscle formation and lung growth (Sparrow *et al.,* 1995).

Confocal microscopy studies of foetal pig lung show that cells localised at the base of the lung bud stain for α-actin and smooth muscle-myosin (an indication that ASM differentiation is taking place), where a new basement membrane is being assembled (Weichselbaum and Sparrow, 1999). The basement membrane plays a critical role in ASM development by facilitating mesenchymal cell spreading and elongation (Weichselbaum and Sparrow, 1999).

Neural tissue, i.e. precursor ganglia interconnected by nerve trunks and smaller bundles, forms a sheath over ASM layers in foetal mouse lung explants. These are guided by glial-derived neurotrophic factor (GDNF) that appears to be produced by ASM cells (Tollet e*t al.,* 2002). This may indicate that nerve tissue development associated with ASM cells is, in part, dependent upon ASM production of the neuronal growth and survival inducing protein GDNF.

1.8.2 Receptors and activation

Several chemokines are produced by ASM cells *in vitro,* after stimulation with several cytokines (IL-4, IL-13, IL-α, IL-1β, TNF-α and TGF-β) (Watson *et al.,* 1998, Moore *et al.,* 2002). Since ASM cells are stimulated with the above cytokines, it follows that the associated receptors are present on their cell surfaces, i.e. IL-4Rα and IL-13Rα1 (Figure 1).

ASM cells from asthmatic patients tend to behave differently to ASM cells from normal patients. For example; in response to TGF- β, ASM cells of asthmatic patients produce more connective tissue growth factor (CTGF) as well as increased synthesis of collagen. CTGF can lead to increased deposition of extracellular matrix, potentially contributing to the airway remodelling process (Borger *et al.,* 2006).

Bradykinin can also increase IL-6, CXCL8 and PGE2 release (Pang and Knox, 1998). IL-6 is a pleiotropic cytokine that has both pro-inflammatory and anti-inflammatory properties in asthma.

Activated ASM cells from asthmatic patients can also generate extracellular matrix (ECM) proteins. The deposition of these matrix proteins have the

potential to cause cell proliferation and contribute to airway remodelling (Lazaar and Panettieri, 2005).

1.8.3 Mediators and their effects

After stimulation and subsequent activation, ASM cells release a plethora of chemokines, including; monocyte chemotactic protein-1 (MCP-1), CCL5, CCL7, CCL11, CCL13, and CXCL8 (Watson *et al.,* 1998, Moore *et al.,* 2002). These chemokines are involved with the recruitment of inflammatory cells into the lung and promote the upregulation of adhesion molecules on the cell surface. Furthermore, GM-CSF (an important stimulator of maturation, activation and survival of several inflammatory cells), is produced by ASM cells when stimulated by a combination of IL-1β and TNF-α (Saunders *et al.,* 1997). In particular, GM-CSF promotes stem cell differentiation into granulocytes and monocytes.

ASM cells secrete a wide variety of matrix proteins, including fibronectin, collagen, and laminin. These proteins are increased in asthmatic patients (Lazaar and Panettieri, 2005). Fibronectin is an important glycoprotein that helps stabilize the attachment of the ECM to cells lining the airway, whereas laminin is the major non-collagenous component of the basal lamina of epithelial cells. Recent studies have demonstrated that ASM cells also secrete VEGF, which is an efficacious endothelial mitogen that increases fibronectin expression by ASM cells (Lazaar and Panettieri, 2005). Johnson *et al.* have also shown that ECM proteins secreted by ASM cells from asthmatic patients differ from non-asthmatic ASM cells (Johnson *et al.,* 2005). Cells plated on the ECM derived from asthmatic ASM cells displayed greater rates of proliferation

(Johnson *et al.,* 2005). In addition to altering ASM cell proliferation, ECM composition also effect ASM cell survival, for example; Fibronectin, laminin, and collagens I and IV have anti-apoptotic properties (Lazaar and Panettieri, 2005).

1.9 Interaction of mast cells and HASM cells

Although human mast cells (HMCs) and HASM cells interact with numerous other inflammatory cells, contributing to the pathogenesis of asthma, the remainder of this dissertation will focus on how they interact with each other and how these interactions contribute to the characteristic changes seen in asthma.

1.9.1 HMC mediators: their effects on HASM cells

Roughly three quarters of the mediators released by activated HMCs have an effect on HASM cells. Their effects can be divided into several broad categories related to the functions of the HASM and its altered characteristics in asthma.

The release of histamines, tryptase, LTD4, via matrix metalloproteinase (MMP-1), low concentrations of TNF-α, low concentrations of PGE2 and TGF-β all have a proliferative effect on HASM cells.

TNF-α, at concentrations ranging from 0.3 to 30pM, can stimulate proliferation via the TNF-α p55 receptor present on HASM cells (Amrani *et al.,* 1996). LTD4 on the other hand, indirectly induces proliferation by stimulating an increase in MMP-1, which leads to the proteolysis of insulin growth factor binding protein allowing more insulin growth factor to bind to its receptor and

induce proliferation (Rajah *et al.,* 1996, Reynolds *et al.,* 1998). While TGF-β1 is capable of converting human lung fibroblasts into myofibroblasts (Broekelmann *et al.,* 1991). This conversion could possibly be an explanation for the presence of fibrosis and increased bulk of muscle present in the airways of asthma patients (Broekelmann *et al.,* 1991, Hashimoto *et al.,* 2001).

The release of heparin, IL-4, high concentrations of TNF-α and high concentrations of PGE2 all have an anti-proliferative effect on HASM cells. At a concentration of 300pM, TNF-α has indirect anti-proliferative effects by; reducing the proliferative effects of mitogens acting on G protein-coupled receptors, such as thrombin, and on receptors having intrinsic tyrosine kinase activity such as epidermal growth factor (Stewart *et al.,* 1995). IL-4 inhibits HASM cell proliferation by inhibiting cyclin D1, an important protein for cell cycle progression into G_1 as well as inhibiting proliferation induced by mitogens such as thrombin (GTP-coupled receptor) or epidermal growth factor (intrinsic tyrosine kinase-coupled receptor) (Hawker *et al.,* 1998).

1.9.2 Chemotaxis and adherence of HMCs to HASM cells

HASM cell production of IL-6, IL-8, RANTES, GM-CSF, eotaxin, and MCP-1 results in the recruitment and activation of several key inflammatory cells in asthma. In particular, stimulated HASM cells are capable of interacting with HMCs through the release of cytokines and surface protein expression (Ammit *et al.,* 2000).

SCF exists in two forms: a membrane-bound form and a smaller soluble form (sSCF), which is thought to be the cleaved extracellular portion of the membrane-bound form (Broudy, 1997). SCF is chemotactic and the primary

regulating factor for HMC growth, function, and survival (Bischoff and Dahinden, 1992, Broudy, 1997). Therefore, HASM cells releasing SCF will have the ability to recruit, activate, and sustain HMCs within its microenvironment, subsequently promoting inflammation. IL-8 is also chemotactic for HMCs, possessing both CXCR1 and CXCR2 receptors specific to IL-8 (Lippert *et al.,* 1998). The most abundantly expressed chemokine receptor on HMCs is actually CXCR3. This chemokine can induce HMC migration towards HASM cells since they express the CXCR3 ligand, CXCR10. In particular, HASM cells from asthmatic patients show increased levels of CXCR10 compared to healthy controls. This could explain the increased levels of HMCs in the HASM layers of asthmatic patients (Brightling *et al.,* 2005). The HASM cell expression of CXCR10 is also inducible by TNF-α, released by HMCs, and IL-4, one of the characteristic cytokines released during a Th_2 response (Brightling *et al.,* 2005).

PGE2, IL-6, and GM-CSF are thought to be involved with negative feedback mechanisms to suppress activated HMCs in the HASM layer. Although PGE2 and IL-6 induce *in vitro* differentiation of cord blood mononuclear cells into fully functional mature HMCs (Saito *et al.,* 1995).

Once HMCs migrate towards the smooth muscle layers, they begin to attach to HASM cells through a variety of adhesion molecules. In particular; Intercellular adhesion molecule-1 (ICAM-1), Vascular cell adhesion molecule-1 (VCAM-1) and Integrin $\alpha_4\beta_1$ (Tsang *et al.,* 2005, Yang *et al.,* 2006). These adhesion molecules enable avid binding between HASM cells and HMCs *in vitro*, present on both cell types. The ligands associated with the above adhesion molecules (ICAM-1, VCAM-1 and Integrin $\alpha_4\beta_1$) include;

Lymphocyte function associated antigen-1 (LFA-1), Very late activating antigen-4 (VLA-4) and CD14, in that order. The adhesion molecule ligands are not mutually exclusive with respect to the adhesion molecule they can bind to, for example, Integrin $\alpha_4\beta_1$ can also bind to VLA-4.

The tumour suppressor in lung cancer-1 (TSLC-1), present on HMCs, is also involved with HMC/HASM cell adhesion and binds, in part, through an as yet undefined Ca^{2+} dependent pathway (Yang *et al.,* 2006).

Binding of HMC to HASM cells results in sustained activation of both cell types. This interaction could potentially lead to a prolonged inflammatory response as well as contribute to further airway remodelling and AHR. The adhesion molecules involved in this process are of particular interest, since their cell surface expression is likely to be inducible by external substances released in the local microenvironment. Tsang *et al.,* have reported the synergistic effects of TNF-α and SCF on the cell surface expression of ICAM-1 on human mastocytosis cell line-1 (HMC-1) cells (structurally and functionally similar to HMCs), after 48 hours of exposure (Tsang *et al.,* 2005). TNF-α alone is also able to up-regulate ICAM-1 expression on HMC-1 cells (Tsang *et al.,* 2005). HMCs are therefore producing TNF-α which could have an autocrine effect, possibly leading to a modulation of cell surface adhesion molecule expression. In addition, the synergistic effects of both TNF-α and SCF in up-regulating ICAM-1 expression on HMC-1 cells is a concomitant product of the cytokines released from both HMC and HASM cells. The ability to modulate cell surface adhesion molecules present on HMC-1 cells makes it an attractive target for investigation, especially in light of HMC/HASM cell interactions and subsequent contribution to the pathogenesis of asthma.

1.10 Interleukin-33

HMC/HASM cell interactions can be influenced by a variety of cytokines, released from other immune molecules involved in asthma. One such cytokine, Interleukin-33 (IL-33), is thought to play an active role in a variety of inflammatory disorders including asthma (Schmitz *et al.,* 2005, Allakhverdi *et al.,* 2007). In particular, there is a growing body of evidence that implicates IL-33 and it's ability to activate as well as promote the survival of HMCs both *in vitro* and *in vivo* (Iikura *et al.,* 2007, Ali *et al.,* 2007, Ho *et al.,* 2007). In addition, IL-33 mRNA has been shown to be expressed on a variety of immune cells, including HASM cells (Schmitz *et al.,* 2005). These findings make the study of IL-33 and it's involvement with HMC/HASM cell interactions an interesting avenue for further investigation.

1.10.1 Structure and location

IL-33 is a recently identified member of the IL-1 family of molecules (IL-1 and IL-18 for example) (Iikura *et al.,* 2007). IL-33 consists of 270 amino acids with an atomic mass of 30.7kDa. It is synthesized as a 31-kDa protein that lacks a clear signal peptide (Schmitz *et al.,* 2005). This IL-33 precursor has been shown to be cleaved by caspase-1 into its mature form, *in vitro,* although there is a lack of *in vivo* evidence to support this statement (Carriere *et al.,* 2006).

HASM cells, as well as epithelial cells, forming the bronchus or small airways demonstrate a constitutive expression of IL-33 mRNA. Primary lung or dermal fibroblasts and keratinocytes stimulated with TNF-α and IL-1β also induce

IL-33 gene expression (Schmitz *et al.,* 2005). In addition, activated dendritic cells and macrophages express low levels of human IL-33 mRNA (Schmitz *et al.,* 2005).

1.10.2 Co-receptors and location

IL-33 is the natural ligand of ST2, an orphan member of the IL-1 receptor superfamily (Allakhverdi *et al.,* 2007). The gene encoding the IL-33 receptor also encodes a soluble molecule (sST2) by alternative splicing as well as a variant form expressed in humans, ST2v, the functions of which are unclear (Xu *et al.,* 1998).

The ST2 receptor consists of an IL-33Rα-chain and a β-chain. These transmembrane proteins form a heterodimer, resulting in the close association of the cytosolic Toll-like IL-1R (TIR) homology domains (Martin and Wesche, 2002). The recently identified β-chain of ST2, the IL-1 receptor accessory protein (IL-1RAcP), has been proposed to be essential for IL-33 signalling, and the TIR domains of both chains are required to facilitate this signalling (Ali *et al.,* 2007). IL-1RAcP represents the β-chain necessary for IL-33 to induce responses in Th_2 cells and murine bone marrow-derived mast cells (Ali *et al.,* 2007).

The ST2 receptor is preferentially expressed on Th_2 but not Th_1 cells, mature mouse mast cells (MCs) and also on cultured bone marrow-derived immature mast cells (Mortiz *et al.,* 1998).

1.10.3 IL-33 functions

IL-33 mediates its biological effects via the IL-1 receptor ST2, activates NF-κB and MAP kinases, and drives production of Th_2-associated cytokines from *in vitro* polarized Th_2 cells. *In vivo*, IL-33 induces the expression of IL-4, IL-5, and IL-13 and leads to severe pathological changes in mucosal organs (Xu *et al.,* 1998). In mice, IL-33 also induces eosinophilia, splenomegaly, and increased levels of serum IgE (Schmitz *et al.,* 2005).

In addition, macrophage-associated ST2L as well as sST2 have been reported to display anti-inflammatory properties (Brint *et al.,* 2004).

Apart from its effects as a pro-inflammatory cytokine, Carriere *et al.,* report that IL-33 is an endothelium-derived, chromatin-associated nuclear factor with transcriptional repressor properties (Carriere *et al.,* 2007).

IL-33 also acts both alone and in concert with thymic stromal lymphopoietin to expedite the *in vitro* maturation of $CD34^+$ mast cell precursors, as well as inducing the secretion of Th_2 cytokines and Th_2-attracting chemokines (Allakhverdi *et al.,* 2007). IL-33 has also been shown to promote the survival and adhesion of human umbilical cord blood-derived mast cells (HUCBMCs) to fibronectin (released from HASM cells), as well as inducing secretions of IL-8 and IL-13 from HUCBMCs (Iikura *et al.,* 2007). IL-33 may therefore play an important role in mast cell-mediated inflammation, as well as modulation of HMC/HASM cell interactions.

1.11 Aims of the project

The aims of the project are to:

1. Investigate the interaction of mast cells and HASM cells *in vitro*, firstly using a human mast cell line. This will involve analysing the adhesion molecules involved and the potential presence of chemokines relevant to the adhesion process.
2. Elucidate the role of IL-33 in the above process.

2. Methods

2. Materials and Methods

Several experimental protocols were used during this study including; Cell culture of HMC-1 cells and flow cytometry. All relevant COSHH forms can be located in the GBRC Level 4 laboratory COSHH handbook.

2.1 Reagents

All cell culture media used during the project were obtained from Sigma Aldrich UK. Anti-β1 integrin, α4 integrin and ICAM-1 functional blocking antibodies (all mouse IgG_1 subtypes) were obtained from R&D systems, Abingdon, UK. Isotype immunoglobulins were obtained from R&D systems and the phycoerytherin (PE) anti mouse IgG1 secondary antibody was obtained from Abcam, Cambridge, UK. The cytokine TNF-α was donated as a kind gift from Lucy Balantyne, University of Glasgow, while the anti-TNF-α therapies; Enterecept and Adalimunab were a kind gift from Dr Axel Hueber, University of Glasgow.

2.2 Cell Culture

HMC-1 cells were a kind gift of Prof Butterfield, Mayo Clinic. HMC-1 cells were maintained in Iscove's modified Dulbecco modified eagle medium (IDMEM) supplemented with heat inactivated bovine serum 10% and alpha thioglycerol.

HMC-1 cells were kept in an incubator with 5% CO_2 at 37^{o}C, when not being used during an experiment.

2.3 Analysis of ICAM-1 expression on HMC-1 cells using Fluorescent-activated cell sorting (FACS)

In order to accurately measure ICAM-1 expression on HMC-1 cells using FACS, an ICAM-1 adhesion cell blocker was used. As well as an isotype control, a secondary antibody was also needed to specifically bind to the ICAM-1.

The following antibodies were used:

Anti human ICAM-1 (10μg/ml)

PE anti mouse IgG_1 (secondary antibody)

Isotype control (10μg/ml)

A total of 6 tubes were used:

Tube number and contents (all tubes contained 100 000 HMC-1 cells without IDMEM medium)

1: Unstained (i.e. just HMC-1 cells)

2. Isotype control – mouse IgG_1

3. PE anti mouse IgG_1

4. Isotype control – mouse IgG_1 and PE anti mouse IgG_1

5. Anti human ICAM-1

6. Anti human ICAM-1 and PE anti mouse IgG_1

10µl of each reagent was added to 90µl of staining buffer (consisting of Phosphate buffered saline (PBS), Foetal calf serum (FCS) 0.5% and NaN_3 0.01%) and pipetted into each tube (at the concentrations listed above, not including the secondary antibody).

The tubes were then left for 30 minutes to enable binding to take place, and then centrifuged (1400 revolutions per minute (rpm) for 5 minutes) and resuspended in FACS flow (except for tubes 3 and 6). After that, tubes 1/2/4 and 5 were left for 30 minutes (on ice).

The secondary antibody was then added at a second stage (2µl in 100µl of staining buffer) to tubes 3 and 6, and subsequently left for 30 minutes. After this, they were centrifuged a second time (1400rpm for 5 minutes) and resuspended in FACS flow eventually being put on ice with the other tubes. All tubes were then put through the FACS machine for analysis. Cells were analysed on a FACS Calibur machine (BD Bioscience, Oxford, UK). 10,000 events were collected in the gated region and further analysis was performed using Flojo software (Treestar Inc, Ashland Oregeon).

2.4 Analysis of ICAM-1 expression on HMC-1 cells pre-treated with IL-33, using FACS

This experiment consisted of duplicate results for each time point, except for the positive and negative control groups.

Tubes contained 200 000 HMC-1 cells with IDMEM and IL-33 was also added at different time points (concentration of 100ng/ml, 5µl per tube). A total of six time points were used with 3 groups of negative and positive

controls. TNF-α was used as the positive control since previous experiments carried out by Tsang *et al.,* demonstrated an up-regulation of ICAM-1 expression when HMC-1 cells were exposed to TNF- α at a concentration of 20ng/ml (Tsang *et al*., 2005). The following time points were used, with IL-33 added at these times so the tubes could be run through the FACS machine at the same end point: 1, 18, 20, 24, 26 and 48 hours. A total of 48-hours exposure was used; since previous experiments conducted analysing ICAM-1 expression (using TNF-α) on HMC-1 cells used the same time frame (Tsang *et al*., 2005).

During the IL-33 time course, HMC-1 cells were kept in 24 well NUNC-MAXISORB Immuno plates (Fisher Scientific, UK). After the final IL-33 sample was added and left for the appropriate length of time, the contents from the Immuno plates were transferred to Falcon tubes and subsequently treated/labelled as follows:

Tubes 1-12 were used for the different time points and tubes 13-15 were the positive controls (TNF-α added on day one until the end of the experiment) and tubes 16-18 were the negative controls. Tubes 19-21 were used to assess the expression of ICAM-1 on HMC-1 cells that have had no prior treatments. An extra tube was initially used to identify the location of the HMC-1 population during flow cytometry analysis. This tube contained HMC-1 cells only that have had no prior treatments. This tube yielded no usable result; therefore I have not assigned it a tube number.

After the final IL-33 sample was added and left for the appropriate length of time, tubes were centrifuged (1400 rpm for 5 minutes) and resuspended in an

Fcε receptor blocker (50µl) to prevent non-specific binding by the primary and secondary antibodies. The tubes were then left for 15 minutes.

The following antibodies/reagents were used to assess ICAM-1 expression on HMC-1 cells:

Anti human ICAM-1 (10µg/ml)

PE anti mouse IgG_1 (secondary antibody)

Isotype control (10µg/ml)

TNF-α (20ng/ml)

10µl of each reagent was added to 90µl of staining buffer (consisting of PBS, FCS 0.5% and NaN_3 0.01%) at the concentrations listed above, not including the secondary antibody.

After the tubes were exposed to the receptor blocker, the following were added (100µl) to each tube along with more of the Fc epsilon receptor blocker (50µl) and left for 30 minutes:

Tube number and content:

Tubes 1-12: (IL-33 time course, at this point the IL-33 had been removed):

Anti human ICAM-1 (10µg/ml)

Tubes 13-15: (positive control): Anti human ICAM-1 (10µg/ml)

Tubes 16-18 (negative control): Isotype control (10µg/ml)

Tubes 19-21 (control): Anti human ICAM-1 (10µg/ml) no IL-33

All tubes were then centrifuged (1400rpm for 5 minutes) and respuspended with the secondary antibody (2µl in 100µl of staining buffer), being subsequently left for 30 minutes. After this, they were centrifuged again (1400rpm for 5 minutes) and resuspended in FACS flow.

All tubes were then put through the FACS machine for analysis. 10,000 events were collected in the gated region and further analysis was performed using Flojo software (Treestar Inc, Ashland Oregeon).

2.5 Analysis of ICAM-1 expression on HMC-1 cells pre-treated with IL-33 and anti-TNF-α therapies (Etanercept and Adalimumab), using FACS

The same protocol from 2.4 was used to assess whether using anti-TNF-α therapies would alter ICAM-1 expression on HMC-1 cells. All tubes were left for 20 hours (except the isotype control) and subsequently processed in the same fashion as in protocol 2.4. The flowing reagents were used and assigned to the following tubes, subsequently being treated and analysed using flow cytometry as in protocol 2.4:

The concentrations of IL-33, TNF-α and the isotype control used during this experiment were the same as in protocol 2.4. The anti-TNF-α therapies used were Etanercept and Adalimumab. Initially, concentrations of 100µg/ml for both therapies were used to ensure any TNF-α present was either inactivated (Adalimumab) or prevented from binding to its cell surface receptors on the HMC-1 cells (Etanercept). Lower concentrations of 100ng/ml were subsequently used, since the higher concentration yielded low numbers of HMC-1 cells in the gated region, potentially being toxic to the HMC-1 cells.

The combinations of reagents used during this protocol were:

Tube number 1: Unstained HMC-1 cells (no treatment)

2: Isotype control

3: TNF-α (positive control)

4: No IL-33

5: IL-33

6, 7: IL-33 and isotype control (IgG1)

8, 9: TNF-α and isotype control (IgG1)

10, 11: TNF-α and Etanercept

12, 13: TNF-α and Adalimumab

14, 15: IL-33 and Etanercept

16, 17: IL-33 and Adalimumab

18, 19: Etanercept

20, 21: Adalimumab

2.6 Analysis of Integrins α_4 and β_1 expression on HMC-1 cells treated with IL-33, using FACS

The same protocol from 2.4 was used to assess whether using IL-33 would alter integrins α_4 and β_1 expression on HMC-1 cells. The flowing reagents were used and assigned to the following tubes, subsequently being treated and analysed using flow cytometry as in protocol 2.4 (FACS Calibur machine (BD Bioscience, Oxford, UK):

The concentrations of IL-33, TNF-α and the isotype control used during this experiment were the same as in protocol 2.4. The following time points were used, with IL-33 added at these times so the tubes could be run through the FACS machine at the same end point: 1, 18, 21, 24 and 48 hours. The combinations of reagents used during this protocol were:

Tube 1: unstained HMC-1 cells

Tubes 2: (negative control) Isotype control (10μg/ml)

Tube 3: positive control - Anti human integrin α_4 and β_1 (10μg/ml) and TNF-α (20ng/ml)

Tubes 4: (control) Anti human integrin α_4 and β_1 (10μg/ml) no IL-33

Tubes 5-24: Time course - Anti human integrin α_4 and β_1 (10μg/ml) and IL-33 (100ng/ml)

10,000 events were collected in the gated region and further analysis was performed using Flojo software (Treestar Inc, Ashland Oregeon).

3. Results

3. Results

The results from the FACS machine were obtained in a similar pattern each time. Firstly a preliminary experiment was set up to assess whether an adhesion molecules expression could be measured accurately, using unconjugated antibodies with a secondary antibody added at a later stage. The results for ICAM-1 expression indicated a shift away from the isotype control, indicating that HMC-1 cells express the ICAM-1 adhesion molecule at basal levels. These results have been omitted from this section. The Mean Fluorescence Intensity (MFI) values were used throughout the results section, since the investigation was aimed at elucidating the effects of IL-33 on the expression of the adhesion molecules per HMC-1 cell. Therefore the MFI is an average of adhesion molecule expression per HMC-1 cell, which was then calculated as an average from all gated HMC-1 cells expressing the adhesion molecule of interest begin studied at the time.

The raw data collected is included in an appendix (Appendix 6.1).

3.1 ICAM-1 expression on HMC-1 cells exposed to IL-33 (time course)

MFI values obtained from the FloJo software package, used to analyse the FACS data, were recalculated as a percentage of the positive control (TNF-α) value. This was performed in duplicate for each separate time course carried out on different days, enabling direct comparison of all data points collected. Additionally, this will enable parametric statistical analysis of the data sets presented here, in the results section.

The MFI percentages of ICAM-1 expression compared to TNF-α over the time course; 0, 1, 18, 20, 24, 26, 48 hours exposure to IL-33 were; 59.21%, 45.64%,

65.74%, 95.32%, 76.69%, 71.18% and 67.52% respectively (Figure 5). The general trend from the graph shows a slight decrease of ICAM-1 expression on the HMC-1 cells from 0 to 1 hour, with a sharp increase up to 20 hours of exposure to IL-33. This expression then sharply decreases from 20 to 26 hours, with little variation in ICAM-1 expression from 26 to 48 hours exposure to IL-33 (Figure 5).

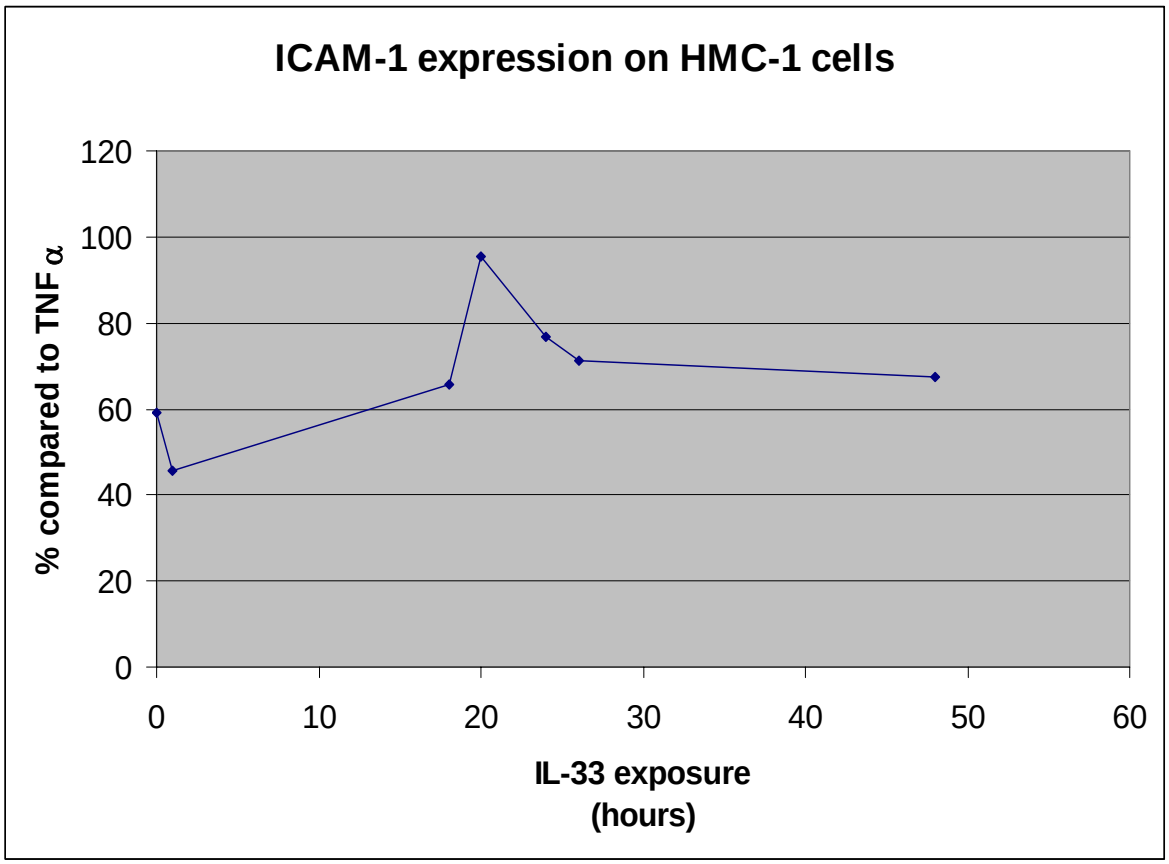

Figure 5. Time course of ICAM-1 expression on HMC-1 cells, after treatment with IL-33 (100ng/ml).

3.2 ICAM-1 expression on HMC-1 cells exposed to IL-33 and TNF-α

HMC-1 cells exposed to IL-33 for 20 hours were shown to promote the greatest increase in up-regulating ICAM-1 expression over the time course carried out (Figure 5). This up-regulation is represented graphically in the form of FACS plots, where a definitive shift away from the isotype control is evident (Figure 6). The positive control (TNF-α) revealed the greatest increase in up-regulating ICAM-1 expression on HMC-1 cells of all the treatment groups, which is supported by the journal literature (Tsang *et al.,* 2005). Again this up-regulation is represented graphically in the form of a FACS plot, with a clear shift away from the isotype control (Figure 7).

ICAM-1 expression on HMC-1 cells exposed to IL-33 (100ng/ml) for 20 hours and receiving no treatment compared to TNF-α treated HMC-1 cells were; 88.46%, 53.82% and 100% respectively (Figure 8). The IL-33 (HMC-1 cells treated for 20 hours), untreated and TNF-α results represent mean values taken from 7 repeated experiments. The error bars for ICAM-1 expression on HMC-1 cells in Figure 8 do not overlap, although it cannot be assumed that the results are significantly different. Consequently, further statistical analyses are required for clarification.

Using the students' unpaired t-test to compare ICAM-1 expression of HMC-1 cells treated with IL-33 for 20 hours and TNF-α with the untreated group, revealed statistical significance in both cases ($P<0.01$) (Figure 8).

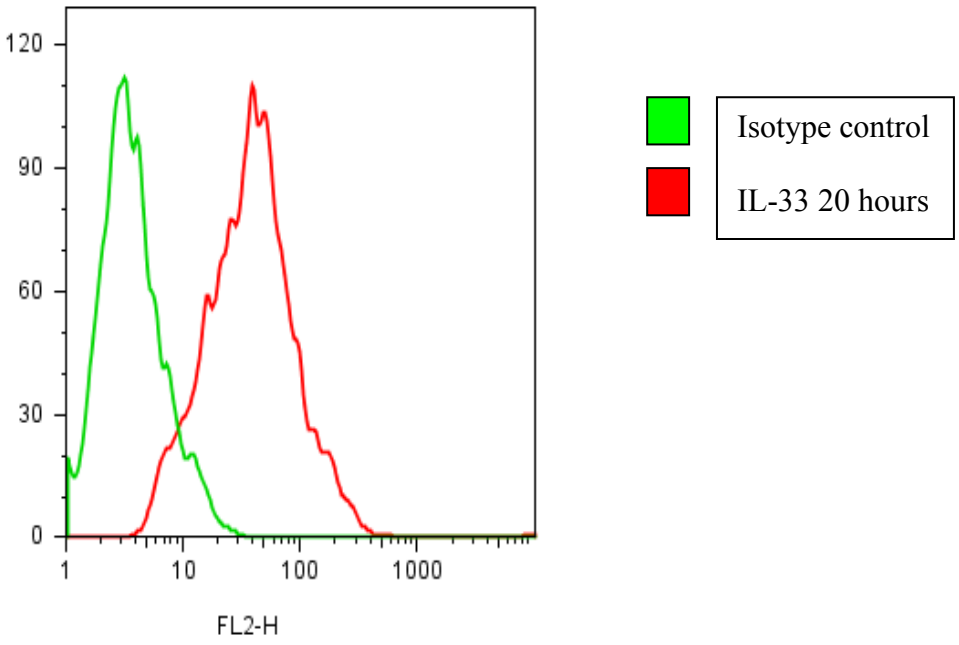

Figure 6. FACS plot of HMC-1 cells expressing ICAM-1 after 20 hour exposure to IL-33 versus the isotype control.

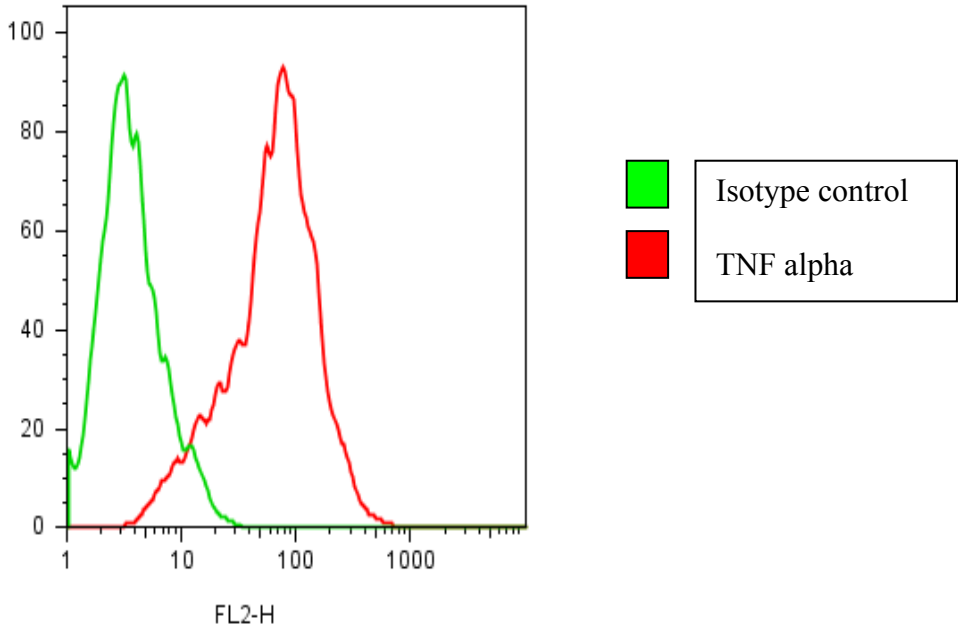

Figure 7. FACS plot of HMC-1 cells expressing ICAM-1 after exposure to TNF-α versus the isotype control.

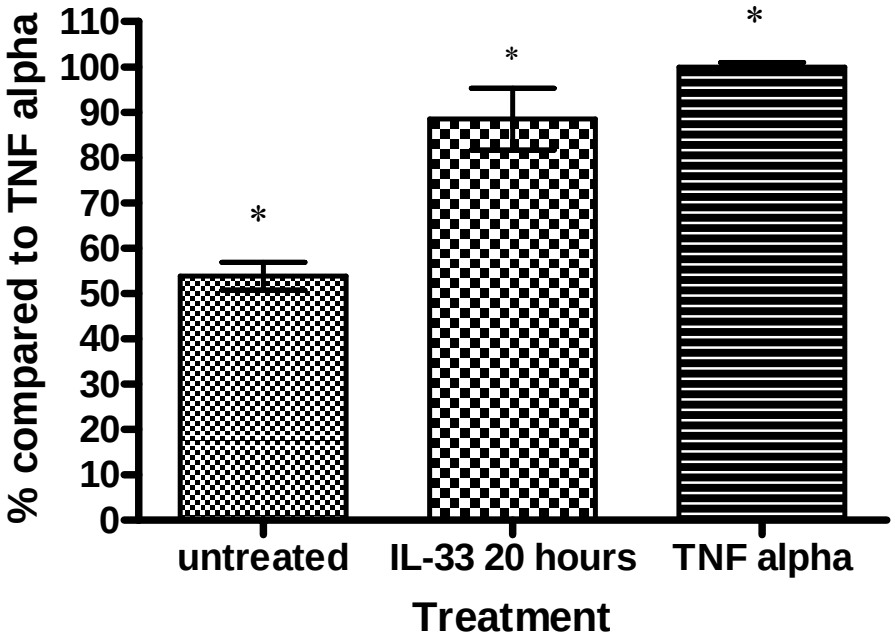

Figure 8. Histogram of ICAM-1 expression on HMC-1 cells, after being treated with IL-33 (100ng/ml) for 20 hours, TNF-α (20ng/ml) and being left untreated. The ICAM-1 expression of HMC-1 cells treated with IL-33 for 20 hours and TNF-α are both statistically different to the untreated group (* $P < 0.01$ using the students unpaired t-test).

3.3 ICAM-1 expression on HMC-1 cells exposed to anti-TNF-α therapies

ICAM-1 expression on HMC-1 cells exposed for 20 hours to Adalimumab (100ng/ml) with TNF-α, Etanercept (100ng/ml) with TNF-α and receiving no treatment compared to TNF-α treated HMC-1 cells were; 58.91%, 62.62%, 53.82% and 100% respectively (Figure 9). The anti-TNF-α therapy results at the concentrations stated (HMC-1 cells treated for 20 hours) represent mean values taken from 4 repeated experiments. The error bars for ICAM-1 expression on HMC-1 cells treated with the anti-TNF-α therapies, TNF-α and untreated HMC-1 cells do not overlap (Figure 9), although it cannot be assumed that the results between the HMC-1 cells treated with anti-TNF-α therapies, are significantly different to the HMC-1 cells treated with TNF-α and being left untreated. Consequently, further statistical analyses are required for clarification. Using a one way ANOVA statistical test to compare ICAM-1 expression of HMC-1 cells treated with Adalimumab and Etanercept for 20 hours (with TNF-α) and the untreated group with HMC-1 cells treated with TNF-α, revealed statistical significance in all 3 cases ($P<0.05$) (Figure 9). Post hoc analysis of the results (Tukey test with a Bonferroni adjustment) specifically identified the HMC-1 cells treated with Adalimumab and Etanercept for 20 hours (with TNF-α) and the untreated group as being statistically different to the HMC-1 cells treated with TNF-α, at the 99% confidence level.

Results from the HMC-1 cells treated with anti-TNF-α therapies at higher concentrations (100μg/ml) revealed a dramatic reduction in ICAM-1 expression, along with a reduction in gated HMC-1 cells in general. This suggests the higher concentrations originally used were too high and may have

been toxic to the HMC-1 cells, therefore these results have been omitted from this section.

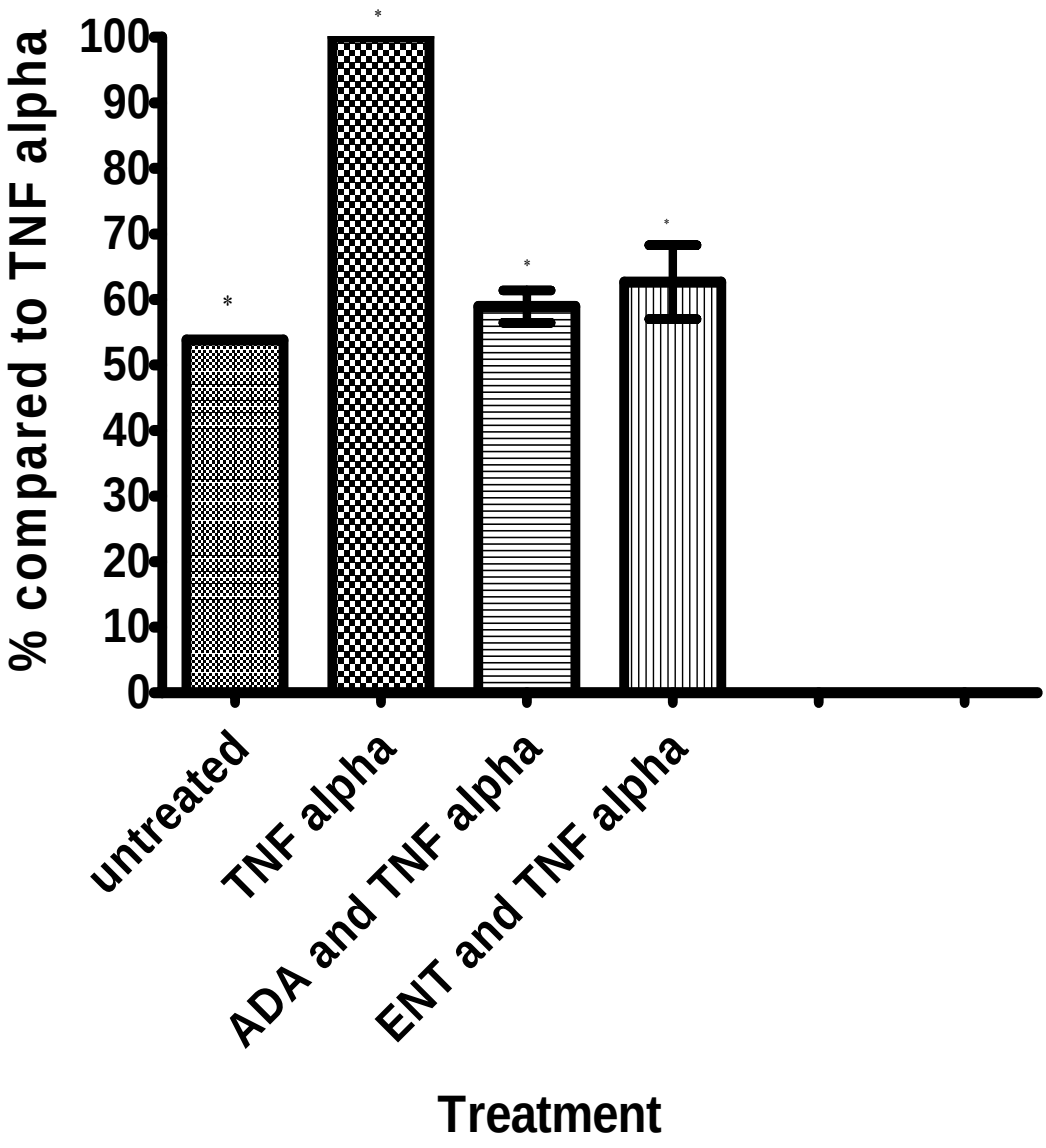

Figure 9. Histogram of ICAM-1 expression on HMC-1 cells, after being treated for 20 hours with; TNF-α (20ng/ml), Adalimumab (ADA – 100ng/ml) with TNF-α and Etanercept (ENT – 100ng/ml) with TNF-α. The ICAM-1 expression of HMC-1 cells treated with TNF-α is statistically different to the untreated, Adalimumab with TNF-α and Etanercept with TNF-α groups (* $P < 0.01$ using an ANOVA and Tukey test statistical analysis).

3.4 ICAM-1 expression on HMC-1 cells exposed to anti-TNF-α therapies and IL-33

HMC-1 cells exposed to IL-33 for 20 hours were shown to promote the greatest increase in up-regulating ICAM-1 expression versus the other treatment groups. This up-regulation is represented graphically in the form of FACS plots, where a definitive shift away from the isotype control is evident (Figures 10 and 11). HMC-1 cells treated with Adalimumab with IL-33 and Etanercept with IL-33 lead to a reduction in ICAM-1 expression, comparable with untreated HMC-1 cell ICAM-1 expression (Figures 10 and 11). This clear shift of ICAM-1 expression, away from the HMC-1 cells treated with IL-33 for 20 hours, is evident in both cases although the Adalimumab seemed to have more of an effect in reducing ICAM-1 expression (Figure10) compared to Etanercept (Figure 11).

ICAM-1 expression on HMC-1 cells exposed for 20 hours to Adalimumab (100ng/ml) with IL-33, Etanercept (100ng/ml) with IL-33 and receiving no treatment compared to IL-33 treated HMC-1 cells were; 57.47%, 60.76%, 53.82% and 88.46% respectively (Figure 12). The anti-TNF-α therapy results at the concentrations stated (HMC-1 cells treated for 20 hours) represents mean values taken from 4 repeated experiments. The error bars for ICAM-1 expression on HMC-1 cells treated with the anti-TNF-α therapies, TNF-α and untreated HMC-1 cells do not overlap (Figure 12), although it cannot be assumed that the results between the HMC-1 cells treated with anti-TNF-α therapies are significantly different to the HMC-1 cells treated with TNF-α and being left untreated.

Consequently, further statistical analyses are required for clarification.

Using a one way ANOVA statistical test to compare ICAM-1 expression of HMC-1 cells treated with Adalimumab and Etanercept for 20 hours (with IL-33) and the untreated group with HMC-1 cells treated with IL-33, revealed statistical significance in all 3 cases ($P<0.05$) (Figure 12). Post hoc analysis of the results (Tukey test with a Bonferroni adjustment) specifically identified the HMC-1 cells treated with Adalimumab and Etanercept for 20 hours (with IL-33) and the untreated group as being statistically different to the HMC-1 cells treated with IL-33, at the 95% confidence level.

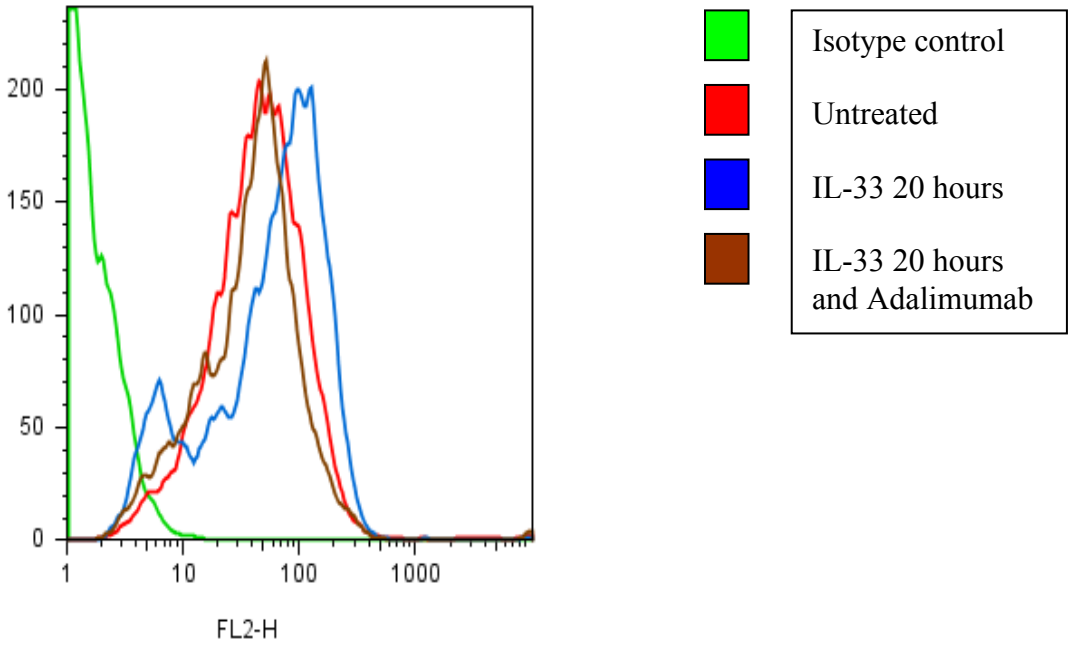

Figure 10. FACS plot of HMC-1 cells expressing ICAM-1 after; 20 hours exposure to IL-33, treatment with IL-33 and Adalimumab for 20 hours, untreated and the isotype control.

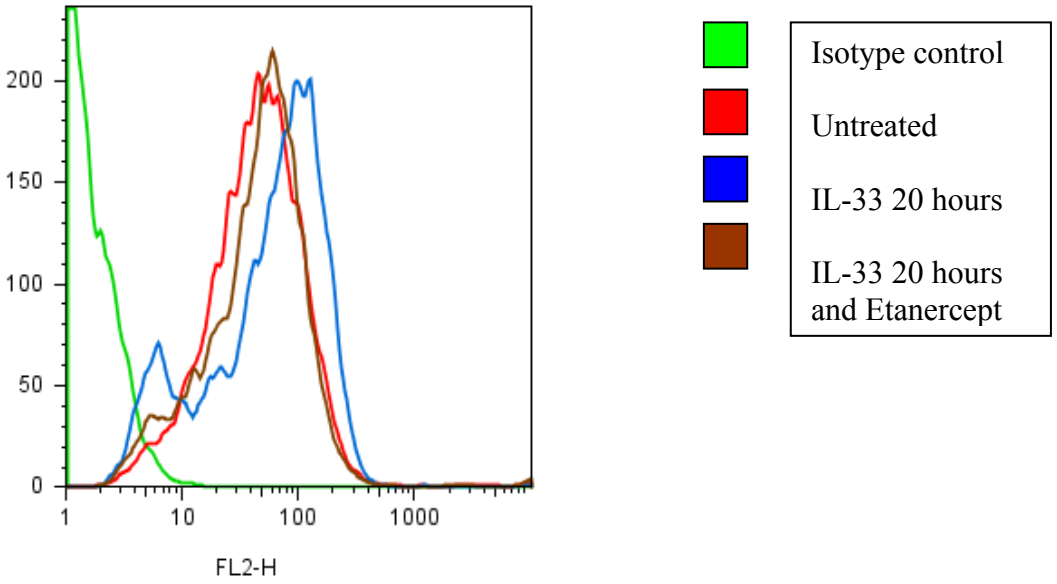

Figure 11. FACS plot of HMC-1 cells expressing ICAM-1 after; 20 hour exposure to IL-33, treatment with IL-33 and Etanercept for 20 hours, untreated and the isotype control.

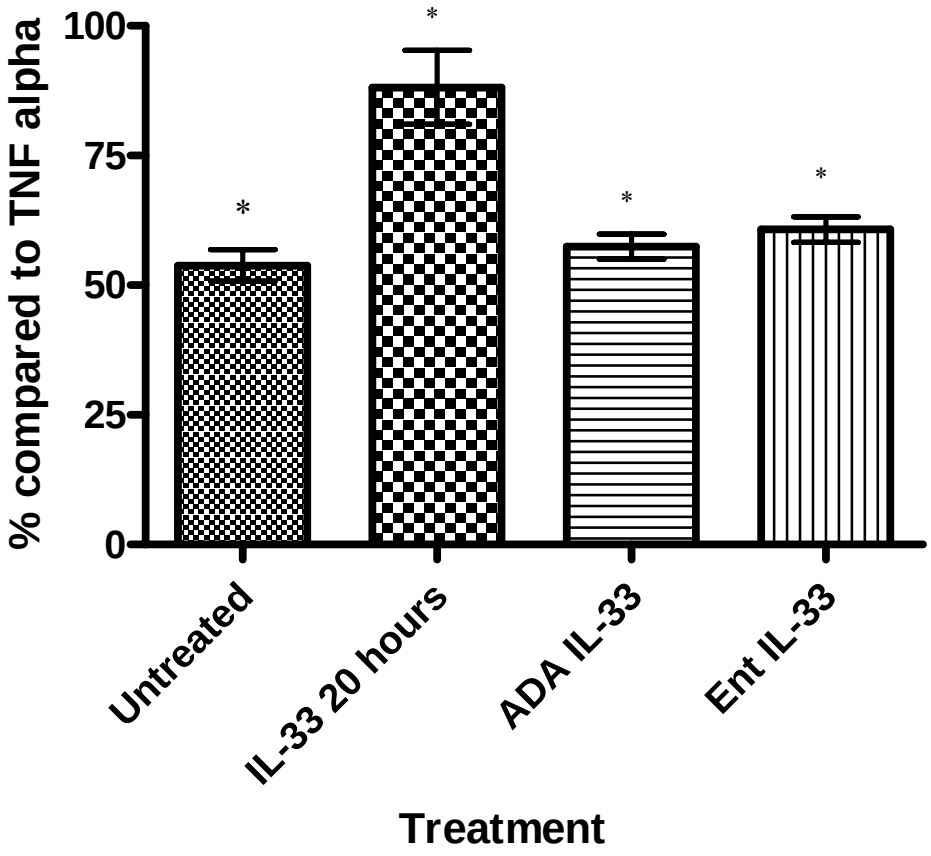

Figure 12. Histogram of ICAM-1 expression on HMC-1 cells, after being treated for 20 hours with; IL-33 (100ng/ml), Adalimumab (ADA – 100ng/ml) with IL-33 and Etanercept (ENT – 100ng/ml) with IL-33. The ICAM-1 expression of HMC-1 cells left untreated, treated with Adalimumab with IL-33 and Etanercept with IL-33 were all statistically different to the HMC-1 cells treated for 20 hours with IL-33 (100ng/ml) (* $P < 0.05$ using an ANOVA and Tukey test statistical analysis).

3.5 Integrin-α4 expression on HMC-1 cells exposed to IL-33 (time course)

MFI values obtained from the FloJo software package, used to analyse the FACS data, were recalculated as a percentage of the positive control (TNF-α) value. This was performed four times for each separate time course carried out on different days, enabling direct comparison of all data points collected.

The MFI percentages of integrin-α4 expression compared to TNF-α over the time course; 0, 1, 18, 21, 24, and 48 hours exposure to IL-33 were; 28.57%, 45.93%, 57.49%, 42.70%, 66.91% and 44.77% respectively (Figure 13). The general trend from the graph shows an increase of integrin-α4 expression on the HMC-1 cells from 0 to 1 hour, with a gradual increase up to 18 hours of exposure to IL-33. This expression then decreases from 18 to 21 hours, increases from 21 to 24 hours exposure then gradually decreases from 24 to 48 hours exposure to IL-33 (Figure 13).

3.6 Integrin-α4 expression on HMC-1 cells exposed to IL-33 and TNF-α

HMC-1 cells exposed to IL-33 for 24 hours were shown to promote the greatest increase in up-regulating integrin-α4 expression over the time course carried out (Figure 13). This up-regulation is represented graphically in the form of FACS plots, where a slight shift away from the isotype control is evident (Figure 14). Integrin-α4 expression on untreated HMC-1 cells reveals only a slight shift away from the isotype control, with TNF-α making somewhat of a modest shift away from the isotype control as well.

Integrin-α4 expression on HMC-1 cells exposed to IL-33 (100ng/ml) for 24 hours and receiving no treatment compared to TNF-α treated HMC-1 cells were; 66.91%, 44.77% and 100% respectively (Figure 15). The IL-33 (HMC-1

cells treated for 24 hours), untreated and TNF-α results represent mean values taken from 4 repeated experiments. The error bars for integrin-α4 expression on HMC-1 cells in Figure 15 do not overlap, although it cannot be assumed that the results are significantly different. Consequently, further statistical analyses are required for clarification.

Using the students' unpaired t-test to compare integrin-α4 expression of HMC-1 cells treated with IL-33 for 24 hours with the untreated group revealed no statistical difference ($P>0.05$). Conversely, integrin-α4 expression of HMC-1 cells treated with TNF-α compared to the untreated group were statistically different at the 95% confidence level (Figure 15).

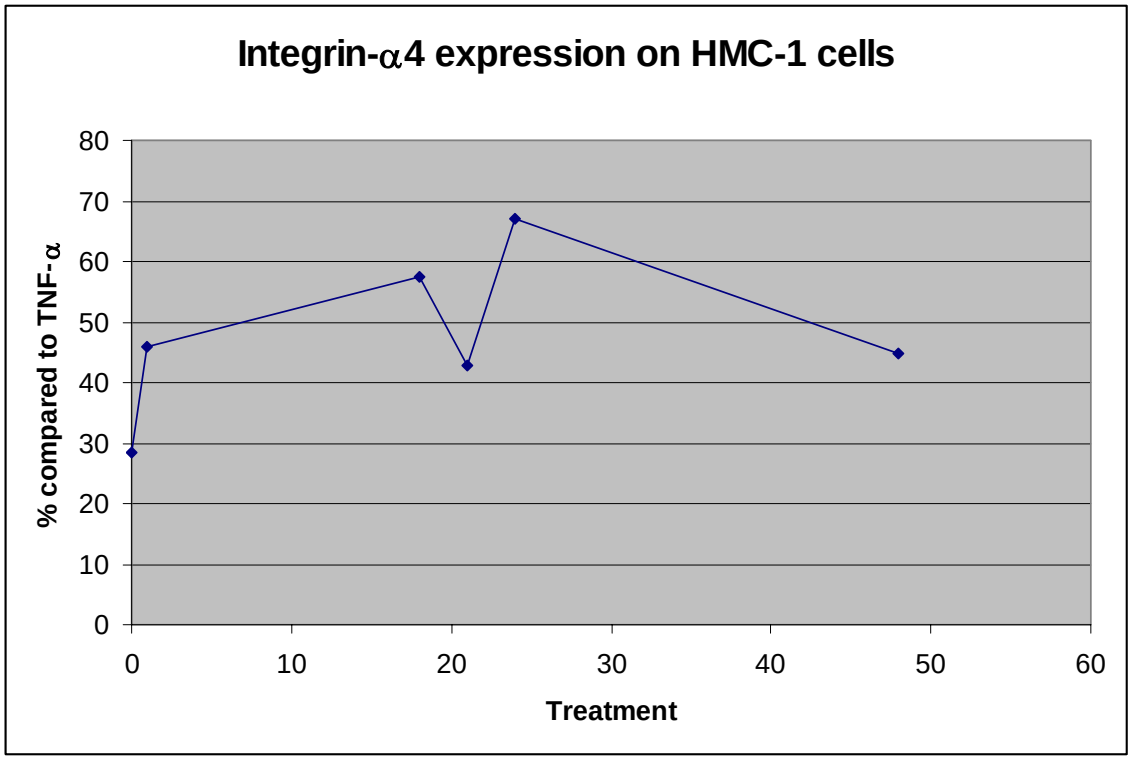

Figure 13. Time course of integrin-α4 expression on HMC-1 cells, after treatment with IL-33 (100ng/ml).

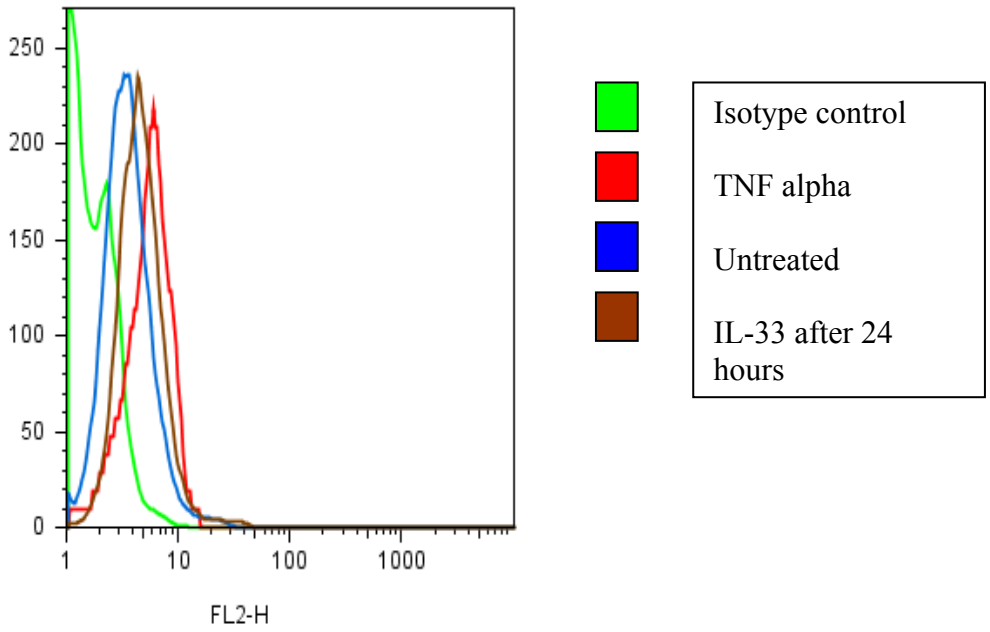

Figure 14. FACS plot of HMC-1 cells expressing integrin-α4 after; 21 hour exposure to IL-33, treatment with TNF-α, untreated and the isotype control.

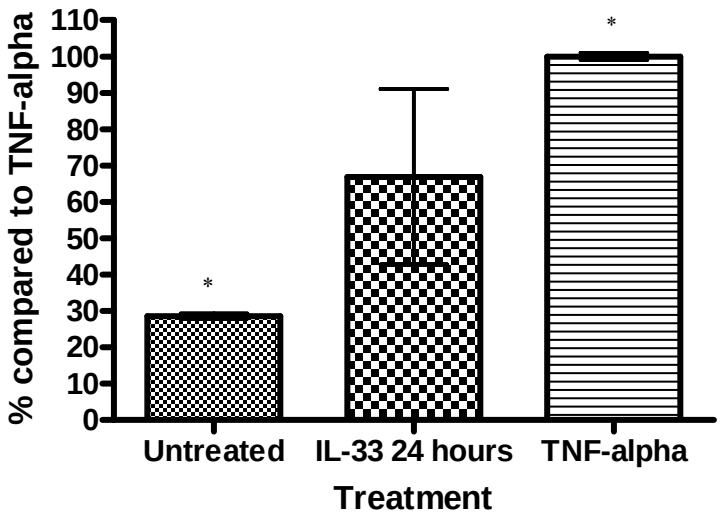

Figure 15. Histogram of integrin-α4 expression on HMC-1 cells, after being treated with IL-33 (100ng/ml) for 24 hours, TNF-α (20ng/ml) and being left untreated. The expression of integrin-α4 on HMC-1 cells treated with IL-33 for 24 hours revealed no statistical difference to the untreated group at the 95% confidence level. Integrin-α4 expression on HMC-1 cells treated with TNF-α was statistically different to the untreated group (* $P < 0.01$ using the students unpaired t-test).

3.7 Integrin-β1 expression on HMC-1 cells exposed to IL-33 (time course)

MFI values obtained from the FloJo software package, used to analyse the FACS data, were recalculated as a percentage of the positive control (TNF-α) value. This was performed four times for each separate time course carried out on different days, enabling direct comparison of all data points collected.

The MFI percentages of integrin-β1 expression compared to TNF-α over the time course; 0, 1, 18, 21, 24, and 48 hours exposure to IL-33 were; 72.80%, 103.63%, 110.79%, 116.55%, 98.17% and 114.62% respectively (Figure 16). The general trend from the graph shows an increase of integrin-β1 expression on the HMC-1 cells from 0 to 1 hour, with a gradual increase up to 21 hours of exposure to IL-33. This expression then decreases from 21 to 24 hours then gradually increases from 24 to 48 hours exposure to IL-33 (Figure 16).

3.8 Integrin-β1 expression on HMC-1 cells exposed to IL-33 and TNF-α

HMC-1 cells exposed to IL-33 for 21 hours were shown to promote the greatest increase in up-regulating integrin-β1 expression over the time course carried out (Figure 16). This up-regulation is represented graphically in the form of FACS plots, where a shift away from the isotype control is evident (Figure 17). Integrin-β1 expression on untreated and TNF-α treated HMC-1 cells also reveal a shift away from the isotype control. HMC-1 cells treated with IL-33 for 21 hours produced a greater increase in the expression of integrin-β1 compared to TNF-α treated HMC-1 cells (Figure 17).

Integrin-β1 expression on HMC-1 cells exposed to IL-33 (100ng/ml) for 21 hours and receiving no treatment compared to TNF-α treated HMC-1 cells were; 116.55%, 72.80% and 100% respectively (Figure 18). The IL-33

(HMC-1 cells treated for 21 hours), untreated and TNF-α results represent mean values taken from 4 repeated experiments. The error bars for integrin-β1 expression on HMC-1 cells in Figure 18 do not overlap, although it cannot be assumed that the results are significantly different. Consequently, further statistical analyses are required for clarification.

Using the students' unpaired t-test to compare integrin-β1 expression of HMC-1 cells treated with IL-33 for 21 hours and TNF-α with the untreated group, revealed no statistical difference in both cases ($P>0.05$).

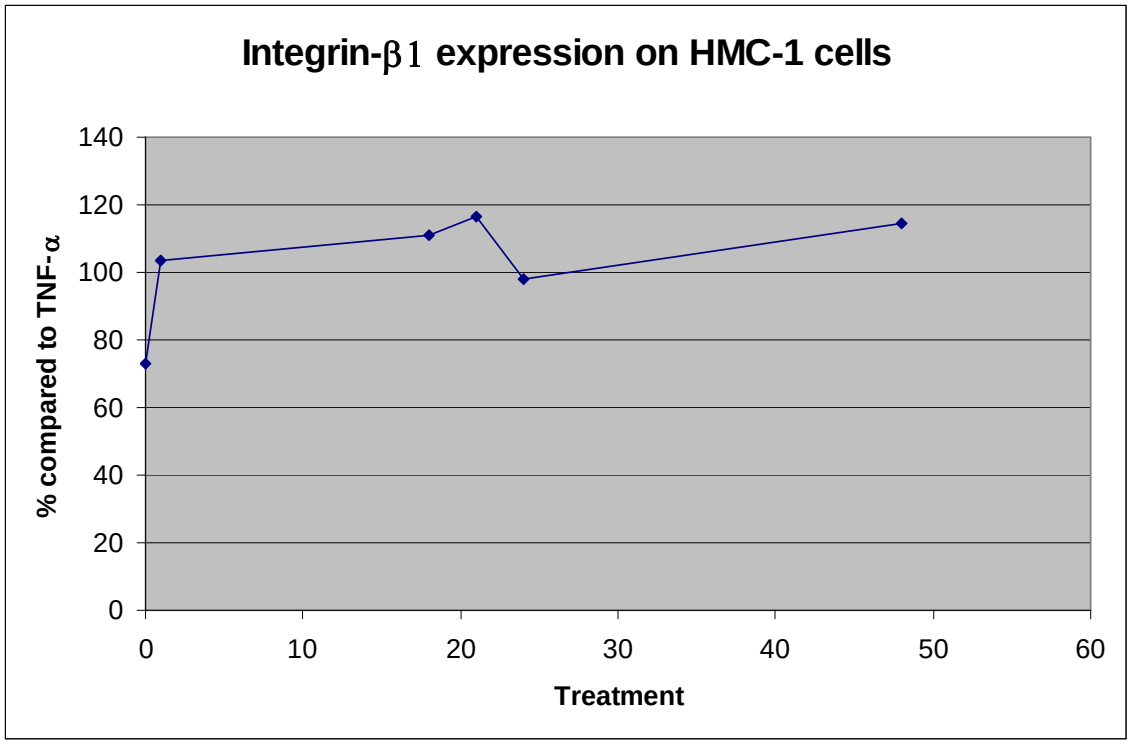

Figure 16. Time course of integrin-β1 expression on HMC-1 cells, after treatment with IL-33 (100ng/ml).

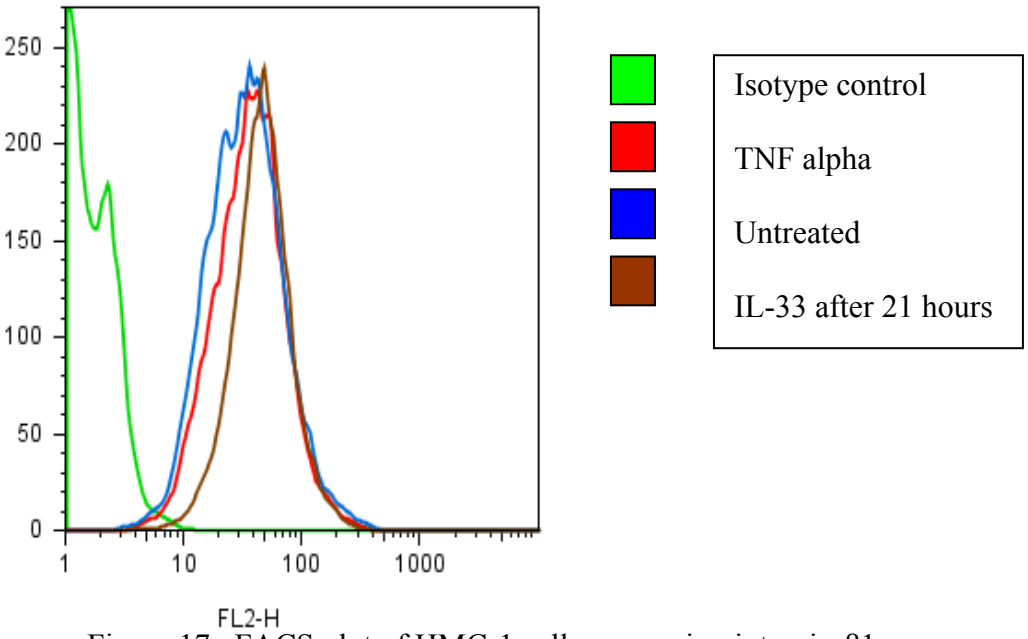

Figure 17. FACS plot of HMC-1 cells expressing integrin-β1 after; 21 hour exposure to IL-33, treatment with TNF-α, untreated and the isotype control.

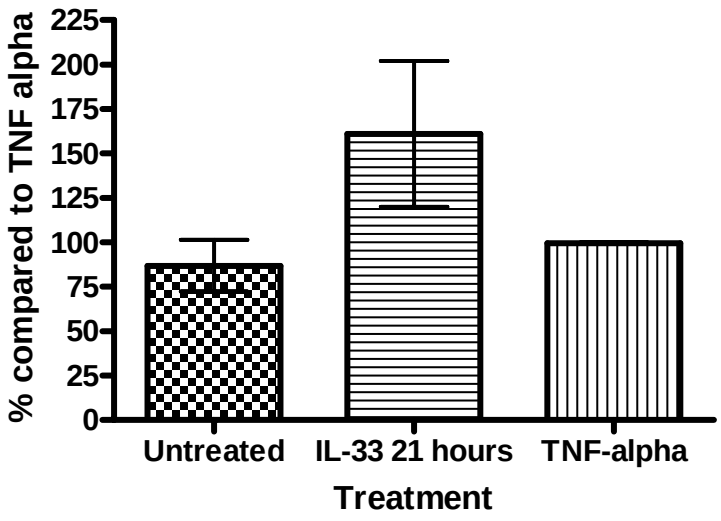

Figure 18. Histogram of integrin-β1 expression on HMC-1 cells, after being treated with IL-33 (100ng/ml) for 21 hours, TNF-α (20ng/ml) and being left untreated. The expression of integrin-β1 on HMC-1 cells treated with IL-33 for 21 hours and TNF-α revealed no statistical difference to the untreated group at the 95% confidence level (students' unpaired t-test).

4. Discussion

4. Discussion

The results presented in this dissertation represent data involving the expression of various adhesion molecules on HMC-1 cells. It is thought that variations in adhesion molecule expression on HMC-1 cells could lead to altered patterns of HMC-1 and HASM cell interactions. The effects of IL-33 on adhesion molecule expression of HMC-1 cells were also investigated, revealing some interesting results.

ICAM-1 expression on IL-33 treated HMC-1 cells revealed a clear pattern over the 48 hours of exposure. The highest expression of ICAM-1 on the IL-33 treated HMC-1 cells was at 20 hours, which was 95.32% compared to the ICAM-1 expression of TNF-α treated HMC-1 cells. As a consequence of this result, it was repeated 7 times to ascertain whether this pattern was reproducible. The mean value from the 7 results of ICAM-1 expression on IL-33 treated HMC-1 cells after 20 hours was 88.46%, compared to the ICAM-1 expression of TNF-α treated HMC-1 cells. Using the students' unpaired t-test to compare ICAM-1 expression of HMC-1 cells treated with IL-33 for 20 hours and TNF-α with the untreated group, revealed statistical significance in both cases ($P<0.01$).

IL-33 mRNA has been shown to be expressed on HASM cells, so theoretically these cells should be able to release IL-33 when stimulated (Schmitz *et al.,* 2005). HMCs present in the early phase reactions of asthma could provide this stimulation. Since the IL-33 receptor, ST2, is present on cultured bone marrow-derived immature mast cells, human peripheral and cord blood-derived $CD34^+$ progenitor mast cells and mature mouse mast cells (Mortiz *et al.,* 1998, Ali *et al.,* 2007, Allakhverdi *et al.,* 2007), HMCs should also become

stimulated by IL-33. Stimulated HMCs would result in an increase in ICAM-1 expression (as suggested in the present study), potentially leading to increased interactions between HASM cells and HMCs.

Events that follow the interactions mentioned above could be cyclic in nature. For example; HMCs present in early phase asthmatic reactions stimulate HASM cells. They then release IL-33, which in turn increases ICAM-1 expression on HMCs leading to increased HASM interactions/adhesion as well as potentially increasing HMC mediator release. At first, HMC mediator release would involve low levels of TNF-α, heparin, histamine and PGE2. These mediators are thought to have a proliferative effect on HASM cells and could be linked to the several pathological features associated with asthma; airway remodelling for example (Amrani *et al.,* 1996). In particular, TNF-α is thought to induce HASM cell proliferation via the TNF-α p55 receptor (Amrani *et al.,* 1996). When IL-33 levels begin to rise, the mediators released from HMCs begin to increase as well, potentially having an anti-proliferative effect on HASM cells (Stewart *et al.,* 1995). The mediators released may dissipate in a transient fashion, leading to another round of HASM stimulation by activated HMCs during the early phase asthmatic reaction.

Previous studies implicated TNF-α as a positive inducer of ICAM-1 expression on HMC-1 cells, supporting the use of TNF-α as a positive control in the present study (Tsang *et al.,* 2005). Several other studies have also identified the IL-33 receptor complex (ST2) present on a variety of mast cell sources, including; differentiated murine-derived mast cells, HUCBMCs and $CD34^+$ progenitor-derived mast cells

(Ali *et al.,* 2007, Allakhverdi *et al.,* 2007, Iikura *et al.,* 2007). Since TNF-α is one of the primary mediators released from mast cells, its release could be partly due to IL-33 stimulation. Once stimulated and subsequently activated, TNF-α release would have an autocrine effect on the activated mast cell resulting in an up-regulation of ICAM-1 expression. This would consequently influence mast cell/HASM cell interactions/adhesion.

HMC-1 cells exposed to IL-33 for 20 hours were used for the remainder of the ICAM-1 experiments, since this time-frame resulted in optimal ICAM-1 expression. Using anti-TNF-α therapies also helped to test the above hypothesis. ICAM-1 expression on HMC-1 cells exposed for 20 hours to Adalimumab (100ng/ml) with TNF-α, Etanercept (100ng/ml) with TNF-α and receiving no treatment compared to TNF-α treated HMC-1 cells were; 58.91%, 62.62%, 53.82% and 100% respectively (Figure 9). The concentration of anti-TNF-α therapies used (100ng/ml) resulted in adequate numbers of gated HMC-1 cells; therefore these concentrations were deemed unlikely to be toxic. ICAM-1 expression on the HMC-1 cells treated with TNF-α and Adalimumab resulted in a 42.09% decrease compared to the HMC-1 cells treated with only TNF-α. For the HMC-1 cells treated with Etanercept and TNF-α, there was a 38.38% decrease in ICAM-1 expression. These results are relatively comparable with the untreated HMC-1 cells. This evidence further supports previous studies (Tsang *et al.,* 2005) that TNF-α is a potent inducer of ICAM- expression on HMC-1 cells, since the anti-TNF-α therapies lead to an average 40.24% decrease in ICAM-1 expression.

Using a one way ANOVA statistical test to compare ICAM-1 expression of HMC-1 cells treated with Adalimumab and Etanercept for 20 hours

(with TNF-α) and the untreated group with HMC-1 cells treated with TNF-α, revealed statistical significance in all 3 cases ($P<0.05$) (Figure 9). Post hoc analysis of the results (Tukey test with a Bonferroni adjustment) specifically identified the HMC-1 cells treated with Adalimumab and Etanercept for 20 hours (with TNF-α) and the untreated group as being statistically different to the HMC-1 cells treated with TNF-α, at the 99% confidence level. The statistical tests were based on 4 repeated experiments, revealing statistical significance at the 95% confidence level for the ANOVA test, and for the post hoc analysis, the confidence level increased to 99%.

ICAM-1 expression on HMC-1 cells exposed for 20 hours to Adalimumab (100ng/ml) with IL-33, Etanercept (100ng/ml) with IL-33 and receiving no treatment compared to IL-33 treated HMC-1 cells were; 57.47%, 60.76%, 53.82% and 88.46% respectively (Figure 12). The anti-TNF-α therapy results at the concentrations stated (HMC-1 cells treated for 20 hours) represent mean values taken from 4 repeated experiments. ICAM-1 expression on the HMC-1 cells treated with IL-33 for 20 hours and Adalimumab resulted in a 30.99% decrease compared to the HMC-1 cells treated with IL-33 for 20 hours alone. For the HMC-1 cells treated with Etanercept and IL-33 for 20 hours, there was a 27.70% decrease in ICAM-1 expression. The ability of the anti-TNF-α therapies to down-regulate ICAM-1 expression on the HMC-1 cells was significantly high, using both TNF-α and IL-33 (20 hour exposure) treated HMC-1 cells. The decrease in ICAM-1 expression using the anti-TNF-α therapies with TNF-α treated HMC-1 cells lead to a greater reduction compared to the IL-33 treated HMC-1 cells (20 hours exposure). This could be because IL-33 stimulated HMC-1 cells produce less TNF-α compared to adding TNF-α

to the HMC-1 cells. This may also indicate that IL-33 could have other effects on the HMC-1 cells that lead to an up-regulation of ICAM-1 expression, independent of the autocrine effects of TNF-α.

Interestingly, the Adalimumab seemed to have a greater effect on HMC-1 cell reduction of ICAM-1 expression, treated with both IL-33 for 20 hours and TNF-α. Adalimumab's mode of action binds to the TNF-α, preventing it from binding to its cell surface receptor. Adalimumab seemed to be more effective than Etanercept, since Adalimumab binds to the TNF-α receptor preventing TNF-α from binding to the HMC-1 cell.

Using a one way ANOVA statistical test to compare ICAM-1 expression of HMC-1 cells treated with Adalimumab and Etanercept for 20 hours (with IL-33) and the untreated group with HMC-1 cells treated with IL-33, revealed statistical significance in all 3 cases ($P<0.05$) (Figure 12). Post hoc analysis of the results (Tukey test with a Bonferroni adjustment) specifically identified the HMC-1 cells treated with Adalimumab and Etanercept for 20 hours (with IL-33) and the untreated group as being statistically different to the HMC-1 cells treated with IL-33, at the 95% confidence level. Statistical differences were obtained between all groups, using only 4 repeated experiments.

Several other adhesion molecules have also been implicated with the adhesion of HLMC to HASM cells, including integrins α4 and β1 (Yang *et al.*, 2006). Since IL-33 revealed interesting results in relation to ICAM-1 expression on HMC-1 cells, its influence on the expression of other adhesion molecules present on HMC-1 and HASM cells were also investigated.

The MFI percentages of integrin-α4 expression compared to TNF-α over the time course; 0, 1, 18, 21, 24, and 48 hours exposure to IL-33 were; 28.57%,

45.93%, 57.49%, 42.70%, 66.91% and 44.77% respectively (Figure 13). The expression of integrin-α4 on HMC-1 cells revealed a somewhat erratic pattern, after exposure to IL-33 (100ng/ml) over the time periods studied. However, HMC-1 cells exposed to IL-33 for 24 hours were shown to promote the greatest increase in up-regulating integrin-α4 expression over the time course carried out (Figure 13). This up-regulation is represented graphically in the form of FACS plots, where a slight shift away from the isotype control is evident (Figure 14). It is interesting to note that integrin-α4 expression on untreated HMC-1 cells reveals only a slight shift away from the isotype control, with TNF-α making somewhat of a modest shift away from the isotype control as well. Taking these results together with the time-course results, IL-33 and TNF-α seemed to have little effect on integrin-α4 expression on HMC-1 cells.

Integrin-α4 expression on HMC-1 cells exposed to IL-33 (100ng/ml) for 24 hours and receiving no treatment compared to TNF-α treated HMC-1 cells were; 66.91%, 44.77% and 100% respectively (Figure 15).

Using the students' unpaired t-test to compare integrin-α4 expression of HMC-1 cells treated with IL-33 for 24 hours with the untreated group revealed no statistical difference ($P>0.05$). Conversely, integrin-α4 expression of HMC-1 cells treated with TNF-α compared to the untreated group were statistically different at the 95% confidence level (Figure 15). Again, these results further support the notion that HMC-1 cells treated with IL-33 had little effect on integrin-α4 expression.

The MFI percentages of integrin-β1 expression compared to TNF-α over the time course; 0, 1, 18, 21, 24, and 48 hours exposure to IL-33 were; 72.80%, 103.63%, 110.79%, 116.55%, 98.17% and 114.62% respectively (Figure 16).

The expression of integrin-β4 on HMC-1 cells exposed to IL-33 (100ng/ml) revealed little variation over the time frame studied. However, HMC-1 cells exposed to IL-33 for 21 hours were shown to promote the greatest increase in up-regulating integrin-β1 expression over the time course carried out (Figure 16). This up-regulation is represented graphically in the form of FACS plots, where a shift away from the isotype control is evident (Figure 17). Integrin-β1 expression on untreated and TNF-α treated HMC-1 cells also reveal a shift away from the isotype control. It is interesting to note that HMC-1 cells treated with IL-33 for 21 hours produced a greater increase in the expression of integrin-β1 compared to TNF-α treated HMC-1 cells (Figure 17). Taking these results together with the time-course results, IL-33 and TNF-α seemed to have little effect on integrin-β1 expression on HMC-1 cells.

Integrin-β1 expression on HMC-1 cells exposed to IL-33 (100ng/ml) for 21 hours and receiving no treatment compared to TNF-α treated HMC-1 cells were; 116.55%, 72.80% and 100% respectively (Figure 18).

Using the students' unpaired t-test to compare integrin-β1 expression of HMC-1 cells treated with IL-33 for 21 hours and TNF-α with the untreated group, revealed no statistical difference in both cases ($P>0.05$). Again, these results further support the notion that HMC-1 cells treated with IL-33 had little effect on integrin-β1 expression.

Both IL-33 (100ng/ml) and TNF-α (20ng/ml) seemed to have little effect on integrin-α4 and integrin-β1 expression on HMC-1 cells. Even exposure to the above cytokines over 48 hours seemed to have little effect on integrin-α4 and integrin-β1 on the HMC-1 cells. Bone marrow cells induced to differentiate

into mast cells via IL-3 express the murine integrin alpha 4 and beta 1 proteins. Surface staining analysis indicates that these cells also express an alpha 4-containing integrin complex throughout the differentiation process (Gurish *et al.,* 1992). Since the experiments conducted in this investigation involved analysing each integrin subunit individually, IL-33 and TNF-α might have an effect on the integrin complex α4β1, as apposed to having an effect on the individual subunits.

The current investigation has revealed several interesting results to add to the body of research aimed at elucidating the molecular as well as immunological mechanisms involved with asthma. In particular, the role of IL-33 has been implicated in a variety of immunological pathways that contribute not only to the pathogenesis of asthma, but to other autoimmune diseases as well, atopic dermatitis for example (Allakhverdi *et al.,* 2007). A role for ST2/IL-33 in human allergic diseases is supported by the recent finding that an SNP in the ST2 promoter results in enhanced ST2 expression and is associated with an increased risk of developing atopic dermatitis (Shimizu *et al.,* 2005).

Recommendations for future work include; using human umbilical cord blood stem cells exposed to IL-3 and SCF to enable maturation of $CD34^{+}$ progenitor cells into HMCs. This would represent an improvement on using HMC-1 cells, since induced HMCs from human umbilical cord blood stem cells represent a more realistic representation of the cells involved within a human asthmatic patient (Allakhverdi *et al.,* 2007). Using human lung mast cells (HLMCs) from donor lung tissue would also represent an improvement to using HMC-1 cells, for the same reasons mentioned above (Yang *et al.,* 2006). Using alternative sources of HMCs would require additional applications to an ethics committee,

as well as obtaining consent from the patients involved in any potential future studies.

In addition to using alternative mast cell sources, analysing other cell surface adhesion molecules present on HMC-1 cells would also help to further expand the current methodology developed. TSLC-1 would be an interesting adhesion molecule to investigate, particularly since Yang *et al.,* implicated TSLC-1 as being important in the recruitment and retention of HLMCs by HASM in asthma (Yang *et al.,* 2006).

Research into positive inducers of adhesion molecules present on HMC-1 cells (mast cells in general) would also be useful, especially since the results for integrin α4 and integrin β1 subunits revealed little change in expression after exposure to IL-33 (100ng/ml) and TNF-α (20ng/ml). Using a primary antibody that targets the whole integrin α4β1 subunit on the HMC-1 cells would also be beneficial. Further analysis using Flow cytometry after exposure to IL-33 and TNF-α etc could then be performed.

Additional *in vitro* work to further elucidate IL-33s role in the interaction of mast cells and HASM cells could involve setting up a time course using IL-33 on HMC-1 cells, investigating ICAM-1 expression again but also checking the tissue culture supernatant for TNF-α production to see if there is a correlation e.g. is IL-33 stimulating the mast cells to produce TNF-α production, having an autocrine effect resulting in an up-regulation of ICAM-1 expression. Setting up an adhesion assay would also help to investigate the chemotactic role of HASM cells on mast cells, and whether IL-33 has any influence on the resultant adhesion involved in the retention of mast cells. The CXC10/CXCR3 axis has been shown to mediate HLMC migration towards HASM cells from asthmatic

patients (Brightling *et al.,* 2005), so this area of research might reveal some interesting results. For example; mast cells and HASM cells could be fluorescently labelled, counted and left in culture medium, exposed to IL-33 and TNF-α over a time-course, and then analysed using FACS to see if the numbers of mast cells present remain the same after the non-adherent mast cells are washed off.

The next logical step from *in vitro* experiments would be looking at *in vivo* models. In particular, asthma induced mice could be exposed to IL-33 at different concentrations and then processed to confirm if asthma has been induced. For example, assessment of the BAL fluid for various immune molecules, analysis of the blood and tissue sections taken for histological analysis. In particular, localisation of the murine mast cells would be interesting to investigate with the asthma phenotype.

It is hoped that the work presented here will provide the basis for future investigations into a highly interesting area of immunological research. Improved understanding of the molecular and immunological mechanisms that contribute to the aetiology and pathogenesis of the asthma phenotype will hopefully one day lead to improvements in the diagnosis and treatment of the disease.

5. References

Ali, S., Huber, M., Kollewe, C., Bischoff, S.C., Falk, W., Martin, M.V. (2007). IL-1 receptor accessory protein is essential for IL-33-induced activation of T lymphocytes and mast cells. Proc Nat Acad of Sci; 104(47):18660-8665.

Allakherdi, Z., Smith, D.E., Comeau, M.R., Delespesse, G. (2007). Cutting Edge: The ST2 Ligand IL-33 Potently Activated and Drives Maturation of Human Mast Cells. J Immunol; 179:2051-054.

Ammit, A.J., Hoffman, R.K., Amrani, Y., Lazaar, A.L., Hay, D.W.P., Torphy, T.J., Penn, R.B., Panettieri Jr, R.R. (2000). Tumour necrosis factor-α-induced secretion of RANTES and interleukin-6 from human airway smooth muscle cells modulation by cyclic adenosine monophosphate. Am J Resp Cell Mol Bio; 23:794-802.

Amrani, Y., Panettieri Jr, R.A., Frossard, N., Bromner, C. (1996). Activation of the TNF alpha-p55 receptor induces monocyte proliferation and modulates agonist-evoked calcium transients in human tracheal smooth muscle cells. Am J Resp Cell Mol Bio; 15(1):55-63.

Anderson, H.R., Gupta, R., Strachen, D.P., Limb, E.S. (2007). 50 years of asthma: UK Trends from1955-2004. Thorax; 62:85-90.

Austen, K.F., Boyle, S.A. (2001). Mast cell lineage development and phenotypic regulation. Leuk Resp; 25:551-18.

Barnes, P.J. (1992). Bradykinin and asthma. Thorax; 47:979-83.

Barnes, P.J. (1996). Pathophysiology of asthma. Br J Clin Pharmacol; 42:3-10.

Barnes, P.J. (2002). The role of inflammation and anti-inflammatory medication in asthma. Resp Med; 96(suppl):S9-S15.

Bischoff, S.C., Dahinden, C.A. (1992). C-kit ligand: a unique potentiator of mediator release by human lung mast cells. J of Exp Med; 175:237-244.

Black, P.N., Fuller, R.W., Taylor, G.W., Barnes, P.J., Dollery, C.T. (1989). Effect of inhaled leukotriene B4 alone and in combination with prostaglandin D2 on bronchial responsiveness to histamine in normal subjects. Thorax; 44(6):491-95.

Borger, P., Tamm, M., Black, J.L., Roth, M. (2006). Asthma: Is it due to an abnormal airway smooth muscle cell? Am J of Resp and Crit Care Medicine; 174:364-72.

Boushey, H.A. (2005). Daily Inhaled Corticosteroid Treatment should not be prescribed for mild persistent asthma. Am J Resp Crit Care Med; 172:412-14.

Bousquet, J., Jeffery P.K., Brusse, W.W., Johnson, M., Vignola, A.M. (2000). Asthma: From bronchoconstriction to airways inflammation and remodelling. Am J Resp Crit Care Med; 161(5):1720-745.

Boyce, J.A. (2003). Mast cells; beyond IgE. J of Allergy and Clin Immunol; 111(1):24-32.

Bradding, P. (2007). Mast cell regulation of airway smooth muscle function in asthma. Eur Respir J; 29:827-30.

Bradding, P., Roberts, J.A., Britten, X.M., Montefort, S., Djukanovic, R., Mueller, R. (1994). Interleukin-4, -5 and -6 and tumour necrosis factor-alpha in normal and asthmatic airways; evidence for the lumen mast cell as a source of these cytokines. Am J Resp Cell Mol Bio; 10:471-80.

Bradding, P., Walls, A.F., Holgate, S.T. (2006). The role of the mast cell in the pathophysiology of asthma. J of Allergy and Clin Immunol; 117(6):1277-284.

Braman, S. (2003). Asthma in the elderly. Clin in Ger Med; 19(1):54-75.

Brightling, C.E., Ammit, A.J., Kaur, D., Black, J.L., Wardlaw, A.J., Hughes, J.M., Bradding, P. (2005). The CXCL10/CXCR3 Axis Mediates Human Lung Cell Migration to Asthmatic Airway Smooth Muscle. Am J of Resp and Crit Care Med; 171:1103-108.

Brint, E.K., Xu, D., Lui, H., Dunne, A., McKenzie, A.N., O'Neil, L.A., Liew, F.Y. (2004). ST2 is an inhibitor of interleukin-1 receptor and toll-like receptor 4 signalling and maintains endotoxin tolerance. Nat Immunol; 5(4):373-79.

British lung Foundation (2008). Accessed on: 17/09/08. Available from: http://www.lunguk.or/.

Broekelmann, T.J., Limper, A.H., Colby, T.V., McDonald, J.A. (1991). Transforming growth factor β1 is present at site of extracellular matrix gene expression in human pulmonary fibrosis. Proc Natl Acad Sci USA; 88:6642-646.

Broudy, V.C. (1997). Stem cell factor and haematopoiesis. Blood; 90(4):1345-364.

Brusasco, V., Crimi, E., Gianiorio, P., Lantero, S., Rossi, G.A. (1990). Allergen-induced increase in airway responsiveness and inflammation in mild asthma. J Appl Physiol; 69:2209-214.

Campbell, N.A., Reece, J.B., Mitchell, L.G. (1999). Biology, Fifth edition. Benjamin Cummings, an imprint of Addison Wesley Longman Inc.

Cap, P., Chladek, J., Pelial, F., Maly, M., Petru, V., Barnes, P.J. (2004). Gas chromatography/mass spectroscopy analysis of exhaled leukotrienes in asthmatic patients. Thorax; 59(6):465-70.

Carriere, V., Roussel, L., Ortega, N., Lacorre, D.A., Americh, L., Aguilar, L., Bouche, G., Girard, J.D. (2006). IL-33, the IL-1-like cytokine ligand for ST2

receptor, is a chromatin-associated nuclear factor *in-vivo*. Proc Nat Acad Sci; 104:282-87.

Chan-Yeung, M., Malo, J.L. (1994). Aetiological agents in occupational asthma. Eur Resp J; 7:346-71.

Cruikshank, W.W., Long, A., Tarpy, R.E., Kornfield, H., Caroll, M.P., Teran, L. (1995). Early identification of interleukin-16 (lymphocyte chemo-attractant factor) and macrophage inflammatory protein 1 alpha (MIP/alpha) in bronchoalveolar lavage fluid of antigen-challenged asthmatics. Am J Resp Cell Mol Bio; 13(6):738-47.

Cruse, G., Kaur, D., Young, W., Duffy, S.M., Brightling, C.E., Bradding, P. (2005). Activation of human lung mast cell by monomeric immunoglobulin E. Eur Respir J; 25:858-63.

Eerdewegh, P.V., Little, R.D., Dupuis, C., Del Mastro, R.G., Falls, K., Simon, J., Torrey, D., Pandit, S. *et al.,* (2002). Association of the ADAM33 gene with asthma and bronchial hyperresponsiveness. Nature; 418:426-30.

Florean, A.A., Rennard, S.I. (1999). The role of cigarette smoke in the pathogenesis of asthma and as a trigger for acute symptoms. Cur Opinion in Pulm Med; 5(1):38-45.

Forssmann, V., Uguccioni, M., Loetscher, P., Dalinden, C.A., Langen, H., Thelen, M. (1997). Eotaxin-2, a novel cc chemokine receptor CCR3, and acts like eotaxin on human eosinophils and basophil leukocytes. J Exp Med; 185(12): 2171-176.

Fujitani, Y., Kanaoke, Y., Antake, K., Vodome, N., Okazaki-Hatake, K., Urade, Y. (2002). Pronounced eosinophils lung inflammation and Th_2 cytokine release in human lipocalin-type prostaglandin D synthase transgenic mice. J Immunol; 168(1):443-49.

Galli, S.J., Metcalf, D.D., Dvorak, A.M. (2001). Basophils and mast cells and their disorders. McGraw Hill publishers, New York.

Galli, S.J., Nakae, S., Tsai, M. (2005). Mast cells in the development of adaptive immune responses. Nature Immunol; 6:135-142.

Gibbon, P.G., Simpson, J.L., Saltos, N. (2001). Heterogenecity of airway inflammation in persistent asthma: evidence of neutrophilic inflammation and increased sputum IL-8. Chest; 119:1329-336.

Gosset, P., Tulie-Leblond, I., Oudin, S., Paramentier, O., Wallert, B., Joseph, M., Tonnel, A.B. (1999). Production of Chemokines and proinflammatory and anti-inflammatory Cytokines by human alveolar macrophages activated by IgE receptors. J of Allergy and Clin Immunol; 130(2):289-97.

Gurish, M.F., Bell, A.F., Smith, T.J., Ducharme, L.A., Wang, R.., Weis, J.H. (1992). Expression of murine beta 7, and beta 1 integrin genes by rodent mast cells. J Immunol; 149(6):1964-972.

Hashimoto, S., Gon, Y., Takeshita, I., Matsumoto, K., Marouka, S., Horie, T. (2001). Transforming Growth Factor-B1 Induces Phenotypic modulation of Human Lung Fibroblasts to Myofibroblasts through a C-Jun-NH2-Terminal Kinase-Dependant Pathway. Am J Resp Crit Care Med; 163(1):152-57.

Hawker, K.M., Johnson, P.R.A., Hughes, J.M., Black, J.L. (1998). Interleukin-4 inhibits mitogen-induced proliferation of human airway smooth muscle cells in culture. Am J Physiol Lung Cell Mol Physiol; 275:469-77.

Hirai, H., Tanaka, K., Yoshie, D., Ogawa, K., Kenmotsu, K., Takamori, Y. (2001). Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. J Exp Med; 193(2):255-61.

Ho, L.H., Ohno, T., Oboki, K., Kajuwara, N., Suto, H., Iikura, M., Okayama, Y., Akira, S., Saito, H., Galli, S.J., Nakae, S. (2007). IL-13 production by mouse mast cells independently of IgE-FcεRI signals. J Leuk Biol; 82:1481-490.

Holgate, S.T., Bodey, K.S., Janezic, A., Frew, A.J., Kaplan, A.P., Teran, L.M. (1997). Release of RANTES, MIP-1 alpha and MCP-1 in asthmatic airways

following endobronchial allergen challenge. Am J Respir Crit Care Med; 156(5):1377-383.

Holgate, S.T., Davies, P.E., Cackie, P.M., Wilson, S.T., Puddiescombe, S.M., Corden, J.L. (2000). Epithelial-mesenchymal interaction in the pathogenesis of asthma. J Allergy Clin Immunol; 105(2 pt 1):193-204.

Iikura, M., Suto, H., Kajuwara, N., Oboki, K., Ohno, T., Okayama, Y., Saito, H., Galli, S.J., Nakae, S. (2007). Tli-33 can promote survival, adhesion and cytokine production in human mast cells. Lab Invest; 87:971-78.

Johansson, S.G.O., Bieber, T., Dahl, R., Friedman, P.S., Lanier, B.Q., Lockey, R.F., Motala, C., Martell, J.A.O., Platts-Mills, T.A.E., Ring, J., Thien, F., Cauwenberg, P.V., Williams, H.C. (2004). Revised nomenclature for allergy for global use: Report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. J of Allergy and Clin Immunol; 113(5):832-36.

Johnson, P.R., Burgess, J.K., Underwood, P.A., Au, W., Poniris, M.H., Tamm, M., Roth, M.R., Ge, Q., Black, J.L. (2005). Extracellular matrix proteins modulate asthmatic airway smooth muscle cell proliferation via an autocrine mechanism. J Allergy Clin Immunol; 13(4):690-96.

Johnson, S.R., Knox, A.J. (1997). Synthetic functions of airway smooth muscle in asthma. Trends in Pharmacol Sci; 18(8):288-92.

Karp, C.L., Grupe, A., Schadt, E., Ewart, S.L., Keane-Moore, M., Cuomo, P.J., Kohl, J., Wahl, L., Kuperman, D., Germer, S., And, D., Peltz, G., Willis-Karp, W. (2000). Identification of complement factor 5 as a susceptibility loci for experimental allergic asthma. Nature Immunol; 1:221-26.

Kay, A.B. (2005). The role of eosinophils in the pathogenesis of asthma. Trends Mol Med; 11(4):148-52.

Kere, J., Laitnan, T. (2004). Positionally cloned susceptibility genes in allergy and asthma. Cur Opinion in Immunol; 16(6):689-94.

Kitamira, Y., Go, S., Hatanaka, K. (1978). Decrease of mast cells in W/Wv mice and their increase by bone marrow transplantation. Blood; 52:447-52.

Kosteins, E., Ulven, T. (2006). Emerging roles of DP and CRTH2 in allergic inflammation. Trends Mol Med; 12(4):148-58.

Kuipers, H., Lambrecht, B.N. (2004). The interplay of dendritic cells, Th_2 cells and regulatory T cells in asthma. Curr Opinion in Immunol; 16(6):702-08.

Lazaar, A.L., Panettieri, Jr, R.A. (2005). Airway smooth muscle as a regulator of immune responses and bronchomotor type. Clin in Chest Med; 27(1):53-69.

Lippert, V., Artuc, M., Grutzkan, A., Moller, A., Kenderessy-Szabo, A., Schadendorf, D., Norgaver, J., Hartmann, K., Schweitzer-Stenner, R., Zuberbiar, T., Henz, B.M., Kriger-Krasagakes, S. (1998). Expression and Functional Activity of the IL-8 Receptor Type CXCR1 and CACR2 on Human Mast Cells. J Immunol; 161:2600-608.

Lipscombe. M.F., Masten, B.J.P.R. (2002). Dendritic cells: Immune regulators in health and disease. J Physiol Rev; 82(1):97-130.

Lloyd, C., Robinson, D.S. (2007). Allergen-induced airway remodelling. Eur Respir; 29:1020-032.

Lung (2008). Accessed on: 06/08/08. Available from: lung.com.

Maddix, L., Shwartz, D.A. (2002). The pathophysiology of asthma. Annual Rev Med; 53:477-98.

Martin, M.V., Wesche, H. (2002). Summary and comparison of the signalling mechanisms of the Toll/interleukin-1 receptor family. Biochim Biophys Acta; 1592(3):265-80.

Massoli, M., Fabian, D., Holt, S., Beasley, R. (2004). The global burden of asthma; executive summary of the GINA Dissemination Committee Report. Allergy; 59(5):469-78.

Mcdonald, D.M., (2001). Angiogenesis and remodelling of airway vasculature in chronic inflammation. Am J Resp Crit Care Med; 164(10-pt2):s39-s45.

McDonald, J.A. (1997). Lung Growth and Development. Informa Health Care Publishings.

McHugh, K.M. (1995). Molecular analysis of Smooth muscle development in the mouse. Am J of Anat; 204(3):278-90.

Metcalf, D.D., Baram, D., Mekuri, Y.A. (1997). Mast cells. Phys Rev; 77(4):1033-079.

Montuschi, P., Barnes, P.J. (2002). Exhaled leukotrienes and prostaglandins in asthma. J Allergy Clin Immunol; 109(4):615-20.

Moore, P.E., Church, T.L., Chism, D.D., Panettieri Jr, R.A., Shore, S.A. (2002). IL-13 and IL-4 cause eotaxin release in human airway smooth muscle cells: a role for ERH. Am J Phys Lung Cell Mol Phys; 282(4):L847-52.

Moritz, D.R., Rodewald, H.R., Gheyselirck, J., Klemenz, R. (1998). The IL-1 Receptor Related T1 Antigen is Expressed on Immature and Mature Mast Cells on Fetal Blood Mast Cell Progenitors; J Immunol; 161:4747-886.

Mosmann, T.R., Sad, S. (1996). The expanding Universe of T-cell subsets: Th_1, Th_2 and more. Immunol Today; 17(3):138-46.

National Jewish Health (2008). Accessed on: 07/08/08. Available from: National Jewish health.com.

Ober, C., Hoffjan, S. (2006). Asthma genetics 2006: the long and winding road to gene discovery. Gen and Immun; 7:95-100.

Pang, L., Knox, A.J. (1998). Bradykinin stimulates IL-8 production in cultured human airway smooth muscle cells: Role of cyclooxygenase products. J Immunol; 161(5):2509-515.

Penna, G., Vulcano, M., Sozzani, G., Adorini, L. (2002). Differential migration behaviour and chemokine production by myeloid and plasmocytoid dendritic cells. Hum Immunol; 63(12):1164-171.

Prussin, C., Metcalfe, D.D. (2006). IgE, mast cells, basophils, and eosinophils. J Allergy Clin Immunol; 17(2 suppl mini primer):s450-s456.

Rajah, R., Nunn, S.E., Harrick, D.J., Grunstein, M.M., Cohan, P. (1996). Leukotriene D4 induces MMP-1, which functions as an IGFBP protease in human airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol; 271(6):L1014-L022.

Remington, T., Digiovine, B.B. (2005). Long-acting (beta) agonists: anti-inflammatory properties and synergy with corticosteroids in asthma. Cur Opinion in Pulm Med; 11(1):74-78.

Reynolds, A., Panettieri Jr, R.A., Tan, E.M.L., Ciocca, V., Littman, M.A., Leonard, T.B., Hay, D.W.P. (1998). Effects of LTD4 on Human Airway smooth muscle cell proliferation, matrix expression, and contraction *in vitro*: Differential sensitivity to cysteinyl leukotriene receptor Agonists. Am J Resp Cell and Mol Bio; 19(3):453-61.

Robinson, D.S. (2004). The role of mast cells in asthma: induction of airway hyperresponsivness by interaction with smooth muscles? J Allergy and Clin Immunol; 114(1):58-65.

Saelta, M., Tevato, G. (2001). Airway pathology in asthma. Eur Res J; 18(Suppl 34):18s-23s.

Saito, H., Ebisawa, M., Sakaguchi, N., Onela, T., Iikura, Y., Yanagida, M., Uzemaki, H., Nakahata, T. (1995). Characterisation of cord blood-derived human mast cells cultured in the presence of steel factor and interleukin-6. Int Arch Allergy Immunol; 107(1-3):63-5.

Saunders, M.A., Mitchell, J.A., Seldon, P.M., Yacoub, M.H., Barnes, J., Giembycz, M.A., Belvisi, M.G. (1997). Release of granulocyte-macrophage

colony stimulating factor by human cultured airway smooth muscle cells: suppression by dexamethasone. Brit J of Pharm; 120:545-46.

Schmitz, J., Owyang, A., Oldham, E., Song, Y., Murphy, E., McClanaham, T.K., Zurawki, G., Moshrefi, M., Qin, J., Li, X., Gorman, D.M., Bazan, J.F., Kastelein, R.A. (2005). IL-33, an Interleukin-1-like Cytokine that Signals via the IL-1 Receptor-Related Protein ST2 and Induces T Helper Type-2-Associated Cytokines. Immunity; 23(5):479-90.

Sears, M.R., Lotvall, J. (2004). Past present and future β2-adrenoreceptor agonists in asthma management. Resp Med; 99(2):152-70.

Severine, P., Ammit, A.J., Black, J.L., Amour, C.L. (2001). Human mast cell and airway smooth muscle interactions: implications for asthma. Am J Phys Cell Mol Physiol; 281:1313-323.

Shimuzu, M.A., Matsuda, K., Yanagisawa, T., Hirota, M., Akahoshi, N., Inomata, K., Ebe, K., Tanaka, H., Suguira, K. (2005). Functional SNPs in the distal promoter of ST2 gene are associated with atopic dermatitis. Human Mol Gen; 14:2919-927.

Shroeder, J.T., MacGlashan Jr, D.W., Lickenstein, L.M. (2001). Human basophils: mediator release and cytokine production. Adv Immunol; 77:93-122.

Sidney, S., Braman, S. (2003). Asthma in the elderly. Clin in Ger Med; 19(1):57-75.

Smit, J.J., Lukacs, N.W. (2006). A closer look at chemokines and their role in asthmatic responses. Eur J Pharm; 553:277-88.

Sparrow, M.P., Warwick, S.P., Everette, A.W. (1995). Innervation and function of the distal airways in the developing bronchial tree of fetal pig lung. Am J Resp Cell and Mol Bio; 13(5):518-25.

Spitzer, W.O., Suissa, S., Ernst, P., Horwitz, R.I., Habbica, D., Cockcroft, D., Bowin, S.F., Mcnuff, H., Bruist, A.S., Rebuill, A.S. (1992). The use of beta-agonists and the risk of death and near death from asthma. New Eng J of Med; 326:501-06.

Stevens, L.R., Austen, K.E. (1989). Recent advances in the cellular and molecular biology of mast cells. Immunol Today; 11(10):381-86.

Stewart, A.G., Tomlinson, P.R., Fernandes, D.J., Wilson, J.W., Harris, T. (1995). Tumour necrosis factor alpha modulates mitogenic responses of human cultered airway smooth muscle. Am J Resp Cell and Mol Bio; 12(1):110-19.

Sunderland, E.R. (2005). Nocturnal asthma. J of Allergy and Clin Immunol; 116(6):1179-86

Tollet, J., Everett, A.W., Sparrow, M.P. (2002). Development of neural tissue and airway smooth muscle in fetal mouse lung explants: A role for glial-derived neutrophilic factor in lung innervation. Am J Resp Cell and Mol Bio; 26(4):420-29.

Tsang, C., Wong, C., Ip, W., Lam, C.W. (2005). Synergistic effect of SCF and TNF-α on the up-regulation of cell-surface expression of ICAM-1 on human leukemic mast cell line (HMC-1) cells. J Leuk Biol; 78:239-47.

Umetsu, S.E., Lee, W.L., McIntire, J.J., Downey, L., Sanjanwala, B., Aubari, O., Berry, G.J., Nagumo, H., Freeman, G.J., Umetsu, D.T., Dekruff, R.H. (2006). TIM-1 induces T cell activation and inhibits the development of peripheral tolerance. Nature Immunol; 6:447-54.

Venkatesha, R.T., Thangam, E.B., Zaida, A.U., Ali, H. (2005). Distinct regulation of C3a-induced MCP-1/CC12 and RANTES/CC15 production in human mast cells by extracellular signal regulated kinase and P13 kinase. Mol Immunol; 42(5):581-87.

Watson, M.L., Grix, S.P., Jordan, N.J., Place, G.A., Dodd, S., Leithead, J. (1998). Interleukin 8 and monocyte chemoattractant protein 1 production by cultured human airway smoot5h muscle cells. Cytokine; 10(5):346-52.

Weichselsaum, M., Sparrow, M.P. (1999). A confocal microscopic study of the formation of Ganglia in the Airways of Fetal Pig Lung. Am J Resp Cell and Mol Bio, 21(5):607-20.

Wenzel, S.E. (2001). The significance of the neutrophil. Clin and Exp Allergy Rev; 1(2):89-92.

Williams, C.M., Galli, S.J. (2000). The diverse potential effector and immunoregulatory roles of mast cells in allergic disease. J Allergy and Clin Immunol; 105:847-59.

Willis-karp, M., Ewat, S.L. (2004). Time to draw breath: asthma susceptibility genes are identified. Nature Rev Gen; 5:376-87.

Xu,D., Chan, W.L., Leung, B.D., Huang, F.P., Wheeler, R., Piedrafita, D., Robinson, J.H., Liew, F.Y. (1998). Selective expression of a stable cell surface molecule on type-2 but not type-1 helper cells. J Exp Med; 187(5):787-94.

Yang, W., Kaur, D., Okayama, Y., Ito, A., Wardlaw, A.J., Brightling, C.E., Bradding, P. (2006). Human Lung Mast Cells adhere to Human Airway Smooth Muscle, in part, via Tumour Suppressor in Lung Cancer-1. J Immunol; 176:1238-243.

6. Appendices

6.1 Raw data

	Mean immunofluorescence intensity (all curves are roughly gaussian)						ICAM-1 expression		
	IL-33 48 hours	26	24	20	18	1	no IL-33	TNF alpha	isotype
A	3713.62		3151.32		2997.52	2216.06		4778.82	38.88
	2844.03		4366.66		3387.5			5884.97	
								3904.093	
mean	3278.825		3758.99		3192.51	2216.06		4855.961	38.88
% of TNF	67.52165019		77.40980622		65.74414	45.63587		100	0.800665
B		288.53	379.03	433.98			254.24	429.35	6.9
		322.65	273.27	384.57					
mean		305.59	326.15	409.275			254.24	429.35	6.9
% of TNF		71.17503203	75.96366601	95.3243275			59.21509	100	1.60708
	48	26	24	20	18	1			
mean of % TNF	67.52165019	71.17503203	76.68673611	95.3243275	65.74414	45.63587	59.21509	100	1.752711

	Mean immunofluorescence intensity (all curves are roughly gaussian)					
	Ent IL-33	ENT TNF	ENT	ADA IL-33	ADA TNF	ADA
290708	100.86	117.62	79.59	96.49	111.24	113.54
100ug/ml	114.93	123.77	76.58	74.17	110.28	82.1
	107.895	120.695	78.085	85.33	110.76	97.82
mean % of TNF	58.33108072	65.2511218	42.21495378	46.13181	59.87998	52.88425
030708	146.65	223.28	120.38	198.31	211.47	112.67
100ng/ml	283.46	164.67	172.9	176.58	172.59	130.24
100ug/ml						
	215.055	193.975	146.64	187.445	192.03	121.455
mean % of TNF	63.19570967	57.00117543	43.09138995	55.08228	56.42962	35.69057
070808	93.98	115.17	52.5	101.83	112.39	89.7
100ng/ml	93.4	104.13	65.45	90.5	84.86	71.76
	93.69	109.65	58.975	96.165	98.625	80.73
mean % of TNF	58.30843913	68.24122479	36.70338561	59.84877	61.37976	50.24272

		IL-33 all 20 hours		ICAM-1 expression						
		IL-33 IgG1	TNF IgG1		IL-33 IgG1		no IL-33	IL-33 20	TNF	isotype
			50ng/ml	100ng/ml	50ng/ml	100ng/ml				
	290708	56.28					94.82	82.5	184.97	2.01
ENT/ADA	100ug/ml	61.77					76.35	108.46		
		59.025					85.585	95.48	184.97	2.01
	mean % of TNF	31.91058					46.26967	51.61918	100	1.086663
	030708	118.37					229.51	270.3	340.3	3.65
ENT/ADA	100ng/ml	101.85								
IgG1	100ug/ml									
		110.11					229.51	270.3	340.3	3.65
	mean % of TNF	32.35674					67.44343	79.42991	100	1.072583
			IL-33 IgG1							
		100ng/ml	50ng/ml	100ng/ml						
ENT/ADA	070808	54.28	60.94	57.9			81.26	122.72	160.68	4.58
	100ng/ml	113.71	93.57	65.63				88.64		
		83.995	77.255	61.765			81.26	105.68	160.68	4.58
	mean % of TNF	52.27471	48.08003	38.43976			50.57257	65.77048	100	2.850386

	Mean immunofluorescence intensity (all curves are roughly gaussian)						
	alpha 4						
	IL-33 48	24	21	18	1	no IL-33	TNF
110708	3.45	10.89	3.86	4.83	4.07	2.56	9.18
	5.9	5.83	4.75	9.73	5.52		
	4.675	8.36	4.305	7.28	4.795	2.56	9.18
mean % of TNF	50.92592593	91.06754	46.89542	79.30283	52.23312	27.88671	100
10808	6.17	7.66	5.93	6.47	5.53	4.3	23.76
	5.18	4.91	5.39	4.02	6.12		5.64
	5.675	6.285	5.66	5.245	5.825	4.3	14.7
mean % of TNF	38.60544218	42.7551	38.5034	35.68027	39.62585	29.2517	100

	Integrin alpha 4 beta 1 expression							
	beta 1							
	IL-33 48	24	21	18	1	no IL-33	TNF	isotype
110708	29.25	23.95	46.36	43.76	37.97	12.32	17.04	13.81
	35.15	27.17	22.46	18.86				
mean	32.2	25.56	34.41	31.31	37.97	12.32	17.04	13.81
% of TNF	188.9671	150	201.9366	183.7441	222.8286	72.30047	100	81.0446
10808	61.01	58.13	58.54	53.81	55.41	48.6	47.99	12.28
	48.55	57.21	56.53	56.15	6			
mean	54.78	57.67	57.535	54.98	30.705	48.6	47.99	12.28
% of TNF	114.1488	120.1709	119.8896	114.5655	63.98208	101.2711	100	25.58866
090808	40.92	15.27	26.4	35.22	22.35	32.22	71.86	3.37
	17.62	19.73	13.59	13.75	12.27			
	29.27	17.5	19.995	24.485	17.31	32.22	71.86	3.37
mean % of TNF-α	40.73198	24.35291	27.82494	34.0732	24.08851	44.83718	100	4.689674

	Proportion of cells expressing ICAM-1(%)								
	IL-33 48 hours	26	24	20	18	1	no IL-33	TNF alpha	isotype
A									
gated from isotype = 2.2%	91.21		93.73		95.01	97.46		84.21	2.2
	96.5		90.13		92.31			67.73	
								87.93	
B									
gated from isotype = 1.91%		99.54	99.95	99.65			99.45	99.77	1.91
		100	99.74	99.62					
				97.38			97.24	94.92	2
				93.46			96.16		
				97.86			86	89.64	2.29
				90.29					
				83.4			99.16	98.92	2.48
				92.478			98.345	84.91	2.036667
				99.635			93.77333	94.49333	2.385

		Proportion of cells expressing ICAM-1				IL-33 all 20 hours	
		Ent IL-33	ENT TNF	ENT	ADA IL-33	ADA TNF	ADA
	290708	95.49	94.15	94.56	95.21	94.5	97.01
	100ug/ml	96.6	97.04	94.67	96.07	93.89	95.24
	gated from isotype = 2%						
	030708	96.67	97.96	98.51	97.5	99.37	96.59
ENT/ADA	100ng/ml	97.5	97.83	99.07	99.43	99.26	99.82
IgG1	100ug/ml						
	gated from isotype = 1.83%						
	070808	92.3	87.29	81.96	92.49	89.05	78.67
ENT/ADA	100ng/ml	91.3	85.51	84.21	91.4	82.51	87.67
	gated from isotype = 2.29%						

	ICAM-1 expression							
	TNF IgG1	IL-33 IgG1			no IL-33	IL-33 20	TNF	isotype
	92.17	94.92			97.24	97.38	94.92	2
	85.83	95.27			96.16	93.46		
ENT/ADA	98.93	98.79			99.16	97.86	98.92	2.48
IgG1	99.04	98.72						
	TNF IgG1		IL-33 IgG1					
	50ng/ml	100ng/ml	50ng/ml	100ng/ml				
ENT/ADA	84.34	64.85	78.32	83.47	86	90.29	89.64	2.29
	80.62	84.44	89.32	77.17		83.4		

	Proportion of cells expressing Integrin alpha 4						
	alpha 4						
	IL-33 48	24	21	18	1	no IL-33	TNF
110708	11.54	15.7	10.38	17.65	12.14	3.61	47.67
gated from isotype = 2.21%	40.85	22.94	27.37	21.92	8.12		
10808	20.03	32.54	11.87	23.47	7.06	11.67	41.91
gated from isotype = 2.32%	10.44	8.41	11.75	8.89	26.94		14.56

		Proportion of cells expressing Integrin beta 1						
	beta 1							
	IL-33 48	24	21	18	1	no IL-33	TNF	isotype
110708	96.48	97.61	99.74	99.04	97.11	69.9	31.09	2.21
gated from isotype = 2.21%	91.22	91.91	92.31	94.4				
10808	99.37	99.53	99.43	99.47	99.62	98.84	27	2.32
gated from isotype = 2.32%	99.32	99.57	99.47	99.58	22.68		99.03	
090808	93.03	44.42	77.65	92.13	66.11	89.16	98.1	2.12
gated from isotype = 2.12%	55.46	56.9	39.65	34.21	28.04			