UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Medicine

Investigation of the role of IgE-dependent cytokine release from human peripheral lung tissue in allergic lung inflammation

by

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Thesis for the degree of Doctor of Philosophy

March 2011

UNIVERSITY OF SOUTHAMPTON
ABSTRACT
FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES
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INVESTIGATION OF THE ROLE OF IgE-DEPENDENT CYTOKINE RELEASE FROM HUMAN PERIPHERAL LUNG TISSUE IN ALLERGIC LUNG INFLAMMATION
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Abstract

Little is known about the contribution of inflammation in distal lung tissue compared to the proximal airways. We have investigated the inflammatory response to anti-IgE and LPS in matched proximal airway and distal lung tissue explants and have shown that both compartments of the lung release cytokine in a concentration-dependent manner to these stimuli. The majority of cytokines were released in a traditional fashion with increasing concentrations as stimulus concentration increased. The release of IL-5 however followed a different pattern and produced a bell-shaped dose response curve. We found that both tissues respond in a similar fashion to these stimuli but that distal lung tissue releases substantially more of the majority of cytokines. IL-6 was the only cytokine to go against this trend with IL-6 release from proximal airway tissue explants being equivalent to release from distal lung tissue. We also investigated the kinetics of cytokine release and determined that early release of TNFα and IL-1β was followed by a cascade of other cytokines, with all cytokine release in both tissue types reaching significance by 24hrs. Immunohistochemical analysis of inflammatory cell populations revealed there is a similar distribution within each compartment but distal lung tissue contains significantly more mast cells, macrophages and neutrophils compared to the proximal airways. This provides evidence that distal lung tissue has the potential to contribute substantially to lung inflammation and may highlight the need for therapeutic interventions to target this compartment of the lung.

This study has utilised a human lung explant model to investigate the early asthmatic response (EAR) in passively sensitised distal lung tissue after exposure to *Dermatophagoides pteronyssinus* extract. Passive sensitisation of bronchial strips successfully passed on sensitivity to HDM extract, as seen in smooth muscle contraction. The release of cytokines such as TNFα; IL-10; the Th_2 cytokines IL-5 and IL-13; and histamine, an early phase mediator released by mast cells; in passively sensitised proximal airway and distal lung tissue were investigated. Stimulation with HDM extract failed to cause release of these mediators however.

Supernatants generated using the human lung explant model contain a complex mixture of inflammatory and anti-inflammatory mediators. We adapted a whole blood neutrophil shape change assay to determine how functional the neutrophil chemokine IL-8 is within generated supernatants. We found that IL-8 in supernatant was functional and that supernatant caused significantly more shape change than expected when compared to the concentration of IL-8 present in them. Upon neutralisation of IL-8 within supernatant, we found that IL-8 is fundamental for neutrophil shape change. We investigated possible synergy between the two chemokines IL-8 and Groα and showed that a greater than additive effect on shape change occurs in the presence of these chemokines at low concentrations. Upon antagonism of the CXCR2 receptor this synergism was abrogated.

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DECLARATION OF AUTHORSHIP

I, Gregory Rankin declare that the thesis entitled

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and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

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GD Rankin, JA Warner, DA Hall. 2010. Comparison of allergic peripheral lung and airway tissue inflammation. *J. Allergy Clin. Immunol.;* **125**, S1:AB48

GD Rankin, D Hall, JA Warner. 2010. Comparison of peripheral lung and airway tissue inflammation. Online abstract: ERS, Barcelona, Spain http://www.ersnet.org/learning_resources_player/abstract_print_10/main_frameset.htm P2406

R Spruce, **G Rankin**, J Ward, SJ Wilson, C Pickard, JA Warner. 2010. Characterisation of T cell populations in the lung. *Thorax*; **65**: A67

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GD Rankin, D Hall, SJ Wilson, JA Warner. 2011. Comparison of Allergic Inflammation in the Distal and Proximal Airways. *J. Allergy Clin. Immunol.;* **127**, S:AB61

GD Rankin, SJ Wilson, DA Hall and JA Warner. 2011. Inflammatory cells in the proximal and distal airways: Effects of statins. Accepted at ATS, Denver

GD Rankin, SJ Wilson, DA Hall and JA Warner. 2011. Inflammatory cells in the proximal and distal airways: Effect of steroids. Accepted at ERS, Amsterdam

R Spruce, **G Rankin**, C Pickard, JA Warner. 2011. Characterisation of T cell subsets in proximal and distal airways. Accepted at ERS, Amsterdam

Signed: ……..

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Acknowledgements

Firstly, I would like to thank my supervisors Dr Jane Warner and Dr David Hall for all their help and advice throughout my PhD. Thank you for supporting me and teaching me how to become a good scientist. My thanks also go to the MRC and GlaxoSmithKline who funded this study.

This study could not have been completed without the help and co-operation of a number of people. My thanks go to all the surgical consultants we have worked with and their surgical teams at Southampton General Hospital for providing us with our specimens. Also the staff of Theatre 4, in particular Anya, Bonnie, Anca and Izzie for making collection much easier and all the staff on ward D2. Thanks also go to the Pathology department at Southampton General Hospital for providing us with airway tissue and the Immunology department for carrying out IgE titres on our serum pools. I would also like to thank the Immunohistochemistry department, especially Dr Susan Wilson, Jon Ward and Helen Rigby for all their technical expertise.

I would also like to thank everyone I have worked with in and around the lab in particular Rebecca, Kelly, Hilary and Claire. Your advice and friendship has helped me greatly and made working and social life throughout this thesis more fun than it probably should have been. This goes for all the other students that have passed through while I have been here in particular Dave, Jen, Charlie, Becki, Chris, Sarah J and Sarah W. I would also like to thank Justine, Satchi and Krupa for making my placement at GSK run a lot smoother with many tea/chocomilk and cake tea breaks. My thanks also go to Val, Vicki, and Sally and anyone else at GSK I may have missed who have helped me with my project.

There are several others who have had to put up with my moaning and groaning throughout these years that need my thanks. My housemates Katrina, Matt, Emma and Katherine for putting up with me and taking the time to read my work and force me to sit at my desk and write and my old housemate Jake who encouraged me to go forward with this PhD. Special thanks to my parents for all their love and support; hopefully if they get round to reading this thesis they might remember what I actually have been doing. Also Mum thanks for the delicious brownies that I have my housemates addicted to, which comes in handy when I'm too lazy to make a cup of tea.

List of Abbreviations

α_1-AT	Alpha$_1$-anti-trypsin
AIgE	Anti-IgE
AP-1	Activator protein-1
APC	Antigen-presenting cell
ARDS	Acute respiratory distress syndrome
ATII	Alveolar type II cells
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BCR	B cell receptor
BLTR	Leukotriene B$_4$ receptor
BSA	Bovine serum albumin
CHO	Chinese hamster ovary
COPD	Chronic obstructive pulmonary disorder
COX	Cyclooxygenase
CXCR	CXC receptor
EAR	Early asthmatic reaction
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ELR	Glutamic acid-leucine-arginine
FACS	Fluorescence-activated cell sorter
FcεRI	High affinity IgE receptor
FEV$_1$	Forced expiratory volume in 1 second
FLIPR	Fluorescent laser imaging plate reader
FS	Forward scatter
FVC	Forced vital capacity
GINA	Global Initiative for Asthma
GMA	Glycol methacrylate
GM-CSF	Granulocyte colony-stimulating factor
GSK	GlaxoSmithKline
Groα	Growth-related oncogene α
HDM	House dust mite
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IgE	Immunoglobulin E
IL-	Interleukin-
IL-1RAcP	IL-1 receptor accessory protein
ITAM	Immunoreceptor tyrosine-based activation motif
iu/ml	International units per ml
Jak	Janus kinase
kUA/L	kilo units of allergen-specific antibody
LAR	Late asthmatic reaction
LAT	Linker for activation of T cells
LDH	Lactate dehydrogenase
LT	Leukotriene
MC$_T$	Mast cell containing tryptase
MC$_{TC}$	Mast cell containing tryptase and chymase
MCP-1	Monocyte chemotactic protein-1
MHC	Major histocompatability complex
MMP	Matrix metalloproteinase
NAP-2	Neutrophil activating peptide-2

NHLBI	National Heart, Lung and Blood Institute
NF-κB	Nulcear factor of κB
PBMC	Peripheral blood mononuclear cell
PMN	Polymorphonuclear leukocyte
RAST	Radioallergosorbent test
RPMI	Roswell Park Memorial Institute
LPS	Lipopolysaccharide
PAR	Protease-activated receptor
PBS	Phosphate buffered saline
PG	Prostaglandin
PLA_2	Phospholipase A_2
RAST	Radioallergosorbent test
RIP-1	Receptor-interacting protein-1
RT-PCR	Reverse transcriptase-polymerase chain reaction
SCF	Stem cell factor
SH2	Src-homology 2
SOCS	Suppressor of cytokine signalling
SODD	Silencer of death domain
SPT	Skin prick test
STAT	Signal Transducers and Activators of Transcription
Tc	Cytotoxic T cell
TCR	T cell receptor
Th	T-helper cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNF-R1	TNF-receptor 1
TNF-R2	TNF-receptor 2
TMB	3,3,5,5-tetramethylbenzidine
TRADD	TNF receptor-associated death domain
TRAF2	TNF receptor-associated factor 2
TSLP	Thymic stromal lymphopoietin
SQ-U	Standardised allergen extract unit (ALK-Abelló)
VCAM-1	Vascular cell adhesion molecule-1
WHO	World Health Organisation

Chapter 1
Introduction

1.1 Introduction

1.1.1 The Lung

The lung is a specialised organ of the respiratory system designed to enable the transport of oxygen from the atmosphere into the blood stream and carbon dioxide from the blood stream into the atmosphere. Essentially, lung tissue is composed of a network of branching tubes that end in millions of tiny sacs called alveoli that make up the bulk of lung tissue and provide a huge surface area for gas exchange (Young and Heath, 2000). These thin-walled structures give the lung its spongy, soft appearance and provide the largest surface area in the body in contact with the external environment, with an estimated area of 100m^2 that comes into contact with approximately 10,000L of air each day (Wang *et al*, 2008).

1.2.1 Anatomy of the lung

The lower respiratory system is made of two functional components: the conducting zone and the respiratory zone. A schematic of the respiratory tree and these zones are shown in figure 1.1. The conducting zone extends from the trachea where it divides into the two main bronchi. These then divide into the secondary bronchi, supplying the lobes of each lung before dividing again into the tertiary bronchi that supply the segments of each lobe (Young and Heath, 2000). This branching continues deeper into each lung, with the formation of the bronchioles and ending in the terminal bronchioles and the beginning of the respiratory zone. This zone becomes increasingly involved with gas exchange as you move down the small airways and is composed of the respiratory bronchioles, alveolar ducts and terminates with the alveoli (Seeley *et al*, 2003).

Figure 1.1

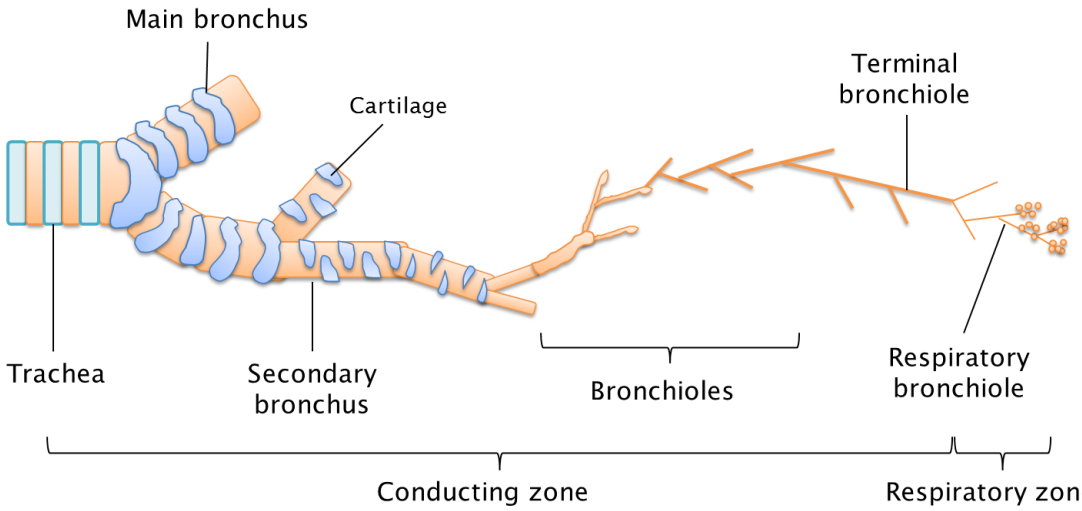


Figure 1.1 The respiratory tree. Schematic diagram of the respiratory tree. Progressive transition from large proximal airways to peripheral small airways and alveoli in distal lung tissue.

As the airways become smaller there is a progressive transition in their physiological structure, with changes in supportive and mucosal elements. One of the most prominent features is the supporting cartilage, which is present as C-shaped rings connected by smooth muscle in the trachea, and gradually decreases and is absent beyond the tertiary bronchi, along with the number of mucous glands. Conversely, the layer of smooth muscle, which controls the diameter of the airways and resistance to airflow within the respiratory tree, becomes increasingly prominent as the airways decrease in size (Young and Heath, 2000). The epithelium also changes between the transition between the conducting and respiratory zones. In the conducting zone the epithelium consists of pseudostratified ciliated columnar cells that change to ciliated simple columnar epithelium in the larger bronchioles and finally ciliated simple cuboidal epithelium in the terminal bronchioles (Seeley *et al*, 2003). The mucus producing goblet cells and ciliated epithelium present in this compartment of the lung function as a mucus-cilia elevator, trapping particles and debris in the air and removing them from the respiratory system.

1.2.2 Proximal airways

The proximal airways consist of the large airways (>2mm in diameter) of the conducting zone. As mentioned earlier, these airways are lined with predominantly ciliated columnar epithelial cells, interspersed with mucus producing goblet cells that both constitute about 30% of the cell populations present in the epithelium (Kierszenbaum, 2002). These cells extend into the airway lumen and sit upon the basal lamina, the top layer of the basement membrane composed of structural glycoproteins (Kierszenbaum, 2002). Another 30% of the cells present in the airway epithelium are basal cells. These cells rest on top of the basal lamina but do not extend to the lumen and function as a stem cell population to replace damaged cells in the epithelium (Stevens and Lowe, 1997). Scattered throughout the proximal airway epithelium are neuroendocrine cells called Kulchitsky cells that form around 8% of the cell population and are thought to function in regulating smooth muscle tone by secreting serotonin and calcitonin (Young and Heath, 2000, Kierszenbaum, 2002). Beneath the epithelium lies a thin layer of fibroelastic tissue termed the lamina propria. Within this layer and the submucosa, there are layers of discontinuous smooth muscle and seromucous glands, which secrete lysozyme and glycoprotein into the airway lumen via secretory ducts. The lamina propria is also part of the mucosa-associated lymphoid tissue (MALT) and often contains diffusely scattered lymphocytes and plasma cells that aid in defence against microorganisms and are more commonly associated with bronchial glands (Young and Heath, 2000, Stevens and Lowe, 1997). Larger lymphoid aggregates may also be present in proximal airways and function in a similar manner to lymph nodes (Stevens and Lowe, 1997). Surrounding the outermost layer of cartilage plates or

smooth muscle is the adventitia; fibroelastic tissue that merges with the surrounding lung parenchyma (Young and Heath, 2000).

1.2.3 Distal lung tissue

Distal lung tissue consists of the very small airways at the end of the bronchial tree and the surrounding lung parenchyma they supply. Within this compartment are the small airways (<2mm in diameter) of the terminal bronchioles along with the respiratory subunits of the lung. This consists of the alveoli and alveolar ducts, supplied by a respiratory bronchiole, which is termed an acini.

Progressing into the respiratory zone, the walls and epithelium of the small airways become thinner and more specialised for roles in respiration. The respiratory bronchioles consist of collagenous and elastic connective tissue and bundles of smooth muscle present beneath a ciliated cuboidal epithelium containing sparse numbers of Clara cells, which become the prominent cell type in more distal regions of the bronchiole (Young and Heath, 2000). These specialised cells produce one of the components of lung surfactant, contain cytochrome P450 enzymes to detoxify noxious substances and are able to divide and differentiate to replace damaged cells, repairing the epithelium (Young and Heath, 2000). The respiratory bronchioles divide further into the alveolar ducts and end with the alveolar sac, which in turn opens into several alveoli. The walls of these specialised structures are extremely thin, consisting of simple squamous epithelial cells in close proximity to a network of capillaries, supplied by pulmonary vessels that follow the general course of the airways (Young and Heath, 2000). Collagen and elastic fibres provide a 3-D supporting meshwork around these structures that allow the tissue to expand and recoil during inspiration and expiration respectively (Seeley *et al*, 2000). Small aggregations of smooth muscle cells are also present surrounding the alveolar ducts and openings of the alveolar sac and alveoli, and enable regulation of alveolar air movements (Young and Heath, 2000).

The alveoli themselves are made of 2 types of specialised epithelial cell, type I and II pneumocytes. The type I pneumocyte is a large thin squamous cell that forms 90% of the alveolar surface and is where gas exchange predominantly takes place (Seeley *et al*, 2003). Type II pneumocytes on the other hand are small round or cube-shaped cells that represent 60% of the cells in the surface epithelium and 10% of the surface area. (Kierszenbaum, 2002). The main function of these cells is to secrete surfactant, an important surface-active lipoprotein complex that functions to reduce surface tension and prevent alveolar collapse during expiration. Unlike the type I pneumocyte, these cells also retain the ability for cell division and can differentiate into type I pneumocytes to replace damaged cells and repair the alveolar lining (Young and Heath, 2000).

1.3 Lung Pathology

The lung is a highly specialised and important organ essential for life. Unsurprisingly therefore, inflammatory diseases of the lung can be life threatening and severely affect quality of life. Chronic conditions affecting the lungs such as asthma and chronic obstructive pulmonary disease (COPD) are complex and involve inflammatory processes that result in airflow limitation and obstruction (Jeffery, 1992).

1.4 Asthma

Asthma is one of the most common chronic diseases affecting 300 million people worldwide (WHO, accessed on 14/07/09). It is estimated asthma accounts for 1 in 250 deaths worldwide and its economic cost is considerable (GINA, accessed on 28/01/10). In the UK, asthma affects around 20% of the population, with over one fifth of children and around 15% of adults being diagnosed with asthma (BTS Burden of lung disease report). Allergic asthma represents the largest group of asthma patients requiring treatment, and the group in which the most epidemiology studies have been focused on (WHO, accessed on 14/07/09, Holgate *et al*, 2006). From these studies the prevalence of allergy-related diseases appears to be on the increase (Bartra *et al*, 2007, Pearce *et al*, 2007). The cause of this increase is largely unknown leading to many possible theories, such as the 'hygiene hypothesis', pollution, smoking, increased allergen exposure and genetic predispositions. It is likely a combination of these factors is responsible for the noted increase in allergic disease, in addition to an increase in clinical recognition and diagnosis.

Asthma can be classified as allergic (extrinsic) or non-allergic (intrinsic) (Rackeman, 1947). Allergic asthma commonly manifests early in life and is clearly defined as an atopic disorder characterised by high levels of circulating specific immunoglobulin E (IgE) and positive skin prick tests to common aeroallergens (Humbert *et al*, 1999). The opposite can be said for non-allergic asthma. Non-allergic asthma is typically late-onset; patients lack circulating allergen-specific IgE and do not respond to skin prick tests (Barnes, 2009). The pathology of allergic and non-allergic asthma is very similar and both result in chronic inflammation of the airways characterised by reversible airways obstruction and bronchial hyper-responsiveness and inflammation (Humbert *et al*, 1999, NHLBI, accessed on 20/01/10). In cases with ongoing constant chronic inflammation a degree of fixed airways obstruction can also occur as a result of tissue remodelling.

1.5 Allergy

Allergy is an abnormal inflammatory immune response to an environmental allergen that manifests itself with different symptoms, ranging from runny nose and sneezing to anaphylaxis and death, depending on the severity of the immune response (Galli *et*

al, 2008). It is a complex disease that involves many different effector cells that release a wide range of inflammatory mediators. These lead to other conditions depending on the site of inflammation, such as allergic (atopic) asthma, atopic dermatitis, allergic rhinitis and food allergy.

1.6 Allergens and Der p1

Inhalation of allergens in sensitised individuals can trigger allergic asthma exacerbation and inflammation of the airways. An allergen is an environmental antigen, usually proteinacious in nature (Holgate *et al*, 2006), which causes an abnormal cascade of immunological responses in a sensitised individual. Inhaled allergens result in an inflammatory reaction that consists of smooth muscle contraction (i.e. narrowing of the airways), oedema, mucus secretion and goblet cell hyperplasia, influx of inflammatory cells, especially eosinophils and T cells, and a significant increase in allergen-specific IgE (Kaliner, 1987).

Major allergens that cause allergic disease in sensitised individuals originate from cats and dogs, tree and grass pollens, moulds and house dust mite (HDM) (Kay, 2000). The major cat allergen, Fel d1, is found in the skin and hair follicles and produced by sebaceous, anal and salivary glands. In dogs, the major allergen is Can f1 found in hair, dander and saliva. These allergens can take months to degrade and are easily scattered through the air as small airborne particles (Baxi and Phipatanakul, 2010). Deciduous trees such as the birch family and grasses such as Timothy grass are potent producers of airborne pollens. Pollination usually takes place in seasons in short durations with tree pollen counts peaking in the spring (April-May) and grass pollen counts throughout the summer (June-August) (Myygind et al, 1996), causing allergic exacerbations during these seasons. Fungal spores on the other hand can cause seasonal and perennial allergy symptoms, with spore release peaking in mid-summer and indoor exposure depending on mould growth in humid environments (Baxi and Phipatanakul, 2010).

A common trigger for allergic asthma is the perennial allergen, Der p1, found in the faeces of the HDM *Dermatophagoides pteronyssinus* (WHO, accessed on 14/07/09). House dust mites feed off organic detritus, such as shed flakes of human skin, and thrive in warm, humid environments. They are therefore found in most households, present especially in bedding, pillows and carpets. Der p1 is a major trigger for allergic disease due to the large frequency of individuals sensitive to it and its high concentration within HDM extract (Thomas *et al*, 2002, Chapman *et al*, 1980, Takai *et al*, 2005). cDNA cloning and protein sequencing have shown that it has a degree of homology with a group of cysteine proteases e.g. papain, actinidin, cathepsin B and cathepsin H, with residues in the active site, based on those determined for papain,

being highly conserved (Chua *et al*, 1988). It is also thought that the enzymatic nature of Der p1 may facilitate its entry across epithelial layers and thus increase its exposure as an allergen (Wan *et al*, 1999).

1.7 Allergy and IgE

Allergic disease is characterised by high concentrations of circulating IgE and allergen-specific IgE within allergic individuals. IgE is the least abundant immunoglobulin class (Rauter *et al*, 2007); it has a role in host defence against parasites and is also important in allergy. It is a glycoprotein composed of four chains, two heavy chains and two light chains, connected together by disulphide bridges to form the characteristic 'Y' shape of antibodies. These chains can be separated into two domains, the constant domain 'ε' and the variable domain that contains the antigen binding complementarity determining regions (see figure 1.2). It is these regions that enable immunoglobulins to be specific for particular antigens.

Figure 1.2

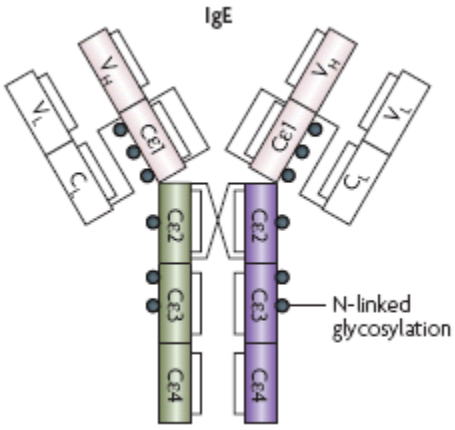


Figure 1.2 Schematic representation of the domain structures of IgE. Constant domains are depicted by a C, variable domains by a V and these are split into the inner heavy chains ($_H$) or outer light chains ($_L$). Image taken from Gould, 2008.

IgE binds to cells that express IgE receptors, such as mast cells, with such high affinity that these cells can be permanently coated with IgE and thus sensitised for rapid activation in the event of allergen binding (Holgate *et al*, 2006). In the case of atopic individuals, their mast cells can be permanently saturated with allergen-specific IgE and therefore primed to elicit an inflammatory response upon allergen exposure.

1.8 Mast cells

It is the exposure of allergen to primed immune cells that initiates allergic inflammation. One of the most important cells involved in this initial phase is the mast cell. These cells are derived from $CD34^+$ haematopoetic progenitor cells and are released in the blood as precursors, to finally mature in vascularised tissues. Here they

express their cytoplasmic granules and high affinity receptors for IgE (FcεRI), which makes them distinguishable from other immune cells (Holgate *et al*, 2006, Wedemeyer and Galli, 2000). They are richly distributed in the skin and mucosal surfaces of the respiratory and gastrointestinal (GI) tracts, beneath epithelial surfaces that are exposed to the external environment, which aids their exposure to allergens (Wedemeyer and Galli, 2000, Kaliner, 1987).

Immunohistochemical studies have revealed that there are two mast cell subtypes, MC_T and MC_{TC}, distinguishable by their protease content (Irani *et al*, 1989). MC_T is abundant in the protease tryptase whereas MC_{TC} contains both serine proteases tryptase and chymase. Both subtypes are present within tissue but appear to change in relative abundance depending on disease process i.e. MC_T appears to be increased in allergy while MC_{TC} is increased in fibrosis. This is suggestive that MC_T has an 'immune system related' role driven towards host defence, whereas MC_{TC} has a more 'non-immune system related' role with links to tissue remodelling and angiogenesis (Holgate *et al*, 2006). Both subtypes however express FcεRI and therefore may participate fully in IgE-mediated allergic and parasitic reactions. Within the lung the principal mast cell subtype is MC_T, found predominantly in the alveolar wall, but MC_{TC} is also found situated around venules and arterioles (Irani *et al*, 1989).

1.9 FcεRI-mediated mast cell activation and signalling

FcεRI is a heterotetramer composed of four polypeptide chains, α, β and a disulphide-linked homodimer of γ chains. The α chain is the only subunit involved in IgE binding with the other predominantly cytoplasmic receptor subunits being involved in signal transduction (Kalesnikoff and Galli, 2008). Mast cell FcεRI-signalling involves a complex series of intracellular signalling molecules that integrate multiple pathways resulting in degranulation, release of preformed mediators and production of the lipid mediators, the eicosanoids, and multiple cytokines (Bradding *et al*, 2006). For the purpose of this project the 'principal' signalling cascade for activated mast cells will be discussed and summarised in figure 1.3.

The FcεRI-mediated activation of mast cells is initiated upon aggregation of these receptors caused by cross linkage of bound IgE. A proportion of the β chain is associated with specialised microdomains of the cell membrane called lipid rafts which contain the protein tyrosine kinase Lyn (Gilfillan and Tkaczyk, 2006). Aggregation of receptors activates Lyn initiating transphosphorylation of tyrosine residues present in the immunoreceptor tyrosine-based activation motifs (ITAMs) of the β and γ domains of the receptors (Galli *et al*, 2008). Phosphorylation of these residues provides high affinity docking sites for SH2 domains resulting in an increased association of Lyn and

another tyrosine kinase Syk, which binds to the γ chain (Benhamou *et al*, 1993). Interaction of both of these tyrosine kinases allows for trans- and autophosporylation of their catalytic domains, increasing their catalytic activity and amplifying the signal. Lyn and Syk also phosphorylate residues of the transmembrane adaptor molecule LAT (linker for activation of T cells), which is also associated with lipid rafts and is crucial for downstream signalling. This allows for the assembly of a macromolecular signalling complex termed a 'signalosome' (Holgate *et al*, 2006). The phosphorylation of LAT results in the recruitment of several types of downstream molecules including signalling enzymes, cytosolic adaptor molecules and guanine-nucleotide exchange factors, involving multiple pathways that result in the different effector functions of the mast cell. This is summarised in figure 1.3.

Figure 1.3

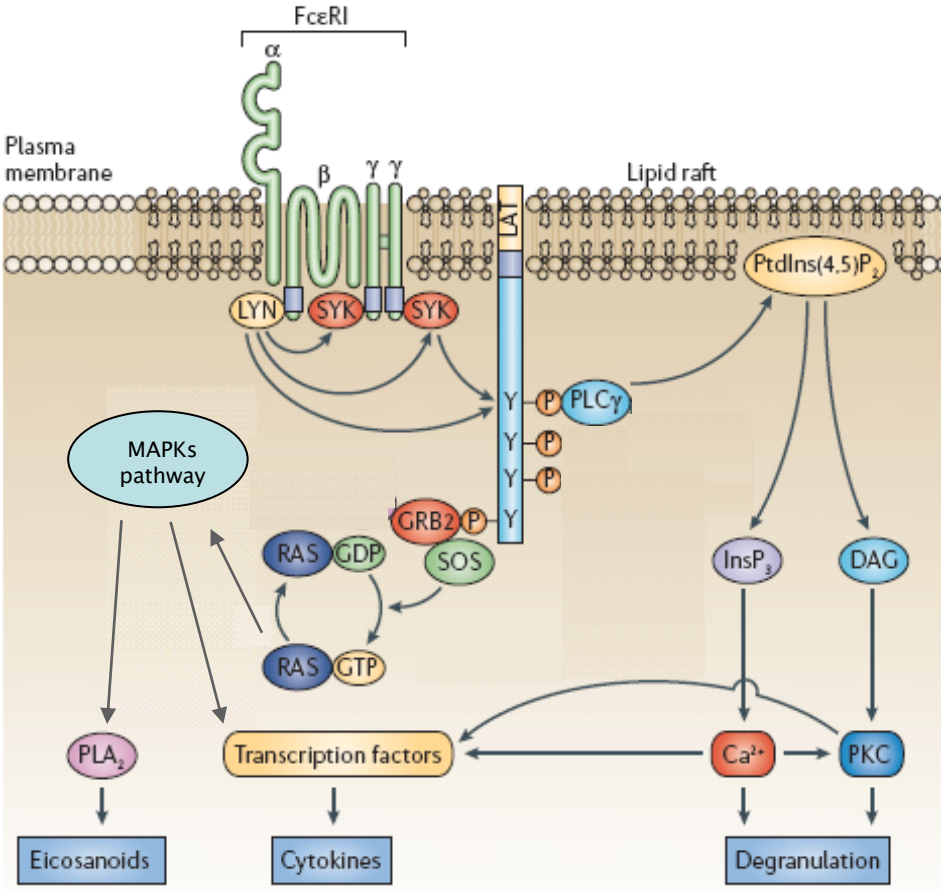


Figure 1.3 'Principal' signalling cascade in activated mast cells. For clarity, only one high affinity IgE receptor (FcεRI) is shown. FcεRI aggregation results in phosphorylation and activation of the tyrosine kinases Lyn and Syk, which go on to phosphorylate the adaptor molecule, LAT. The LAT signalosome results in direct and indirect activation of phospholipase Cγ, which goes on to convert the membrane lipid phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$) to the second messengers inositol-1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). $InsP_3$ causes the release of intracellular Ca^{2+} stores, which along with DAG activates protein kinase C (PKC). The mobilisation of Ca^{2+} and activation of PKC results in mast cell degranulation and activation of cytokine transcription factors. LAT also acts as a docking site for growth-factor-receptor-bound protein 2 (GRB_2) which facilitates binding of the guanine nucleotide exchange factor (GEF), son of sevenless homologue (SOS). This complex shifts the equilibrium of Ras from the inactive (GDP-bound) to the active (GTP-bound) state. Ras leads to the activation of the mitogen-activated protein kinases (MAPKs) pathway. This culminates in production of eicosanoids via activation of the enzyme phospholipase A_2 (PLA_2) and production of cytokines via activation of transcription factors. Figure adapted from Gilfillan and Tkaczyk, 2006.

1.10 Sensitisation Phase

Before the allergic phenotype can take effect the allergic individual must first be exposed to and sensitised to the allergen. This is summarised in figure 1.4 below. This primary exposure to the allergen occurs at external epithelial surfaces of the body, where the allergen comes into contact with antigen-presenting cells (APCs), for example dendritic cells, which are scanning the area for possible threats. The APC ingests and processes the allergen and presents it to and activates naïve $CD4^+$ T cells via its MHC II complex within local lymphoid tissue (Bharadwaj *et al*, 2007). An individual predisposed to allergy is skewed towards a type 2 T cell cytokine profile, thus upon activation and development, a T helper cell (Th2) cytokine profile is initiated (Galli *et al*, 2008). B cells are subsequently activated by Th2 cells upon presentation of the antigen and engagement of CD40 on B cells with CD40 ligand on the T cell (Lanzavecchia *et al*, 1985). This results in B cell differentiation to allergen-specific immunoglobulin (Ig) producing plasma cells. In an atopic individual, the release of interleukin (IL-) 4 and 13 from activated Th2 cells causes an isotype switch from IgM production in B cells to IgE production (Lebman and Coffman, 1988, Punnonen and de Vries, 1994). IgE is subsequently bound to inflammatory cells such as resident tissue mast cells and circulating basophils via FcεRI. The binding of IgE to these receptors not only primes the inflammatory cell for activation upon re-exposure to the allergen, but also increases expression of new FcεRI. Thus, mast cells and basophils in subjects with high levels of IgE, typical of patients with allergic disorders, can have significantly more 'open' FcεRI than nonallergic individuals (Wedemeyer and Galli, 2000). This increases the ability of these cells to bind more allergen-specific IgE and thus their ability to express IgE-dependent effector functions (Wedemeyer and Galli, 2000).

Figure 1.4

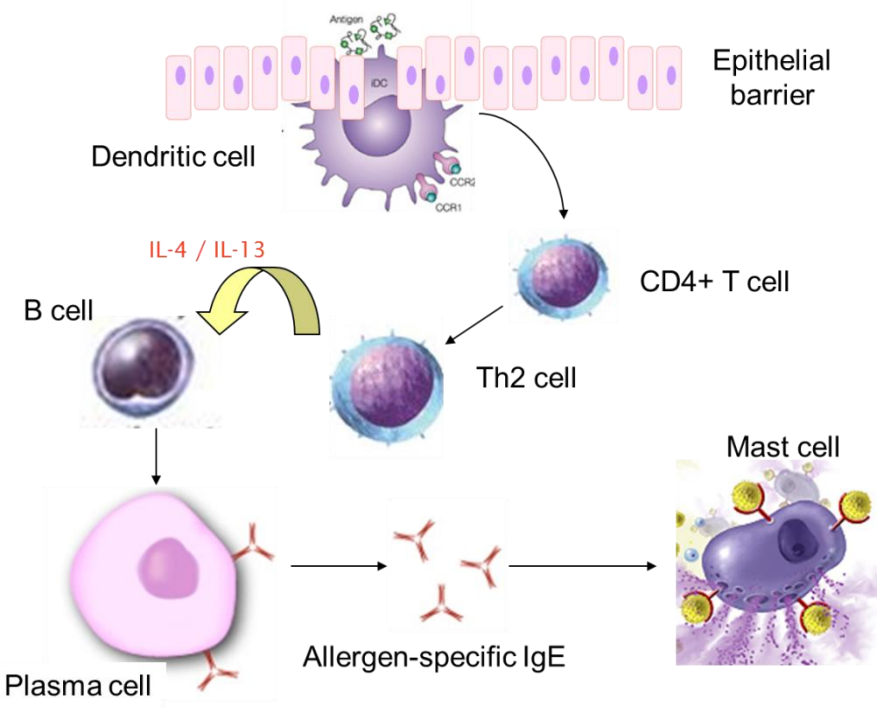


Figure 1.4 Sensitisation phase. Dendritic cells ingest allergen and present it to naïve T cells via MHCII within local lymphoid tissue. The T cell is activated and in an allergic individual is skewed towards a Th_2 type cell. The Th_2 cell presents the peptide complex to B cells and secretes IL-4 and -13 which act on the B cell causing an isotype switch from IgM to IgE production. The activated B cell differentiates into an antibody producing plasma cell, which releases allergen-specific IgE. This IgE binds to $Fc_\varepsilon RI$ on mast cells, priming these cells for activation upon encountering the specific allergen. When this occurs, allergen crosslinks IgE bound to these receptors, initiating clustering of the receptor and initiating downstream signalling that culminates in activation of the cell and degranulation.

1.11 Effector Phase

Re-exposure of the sensitised individual to the allergen results in the allergic reaction, which can be split into two distinct phases: the early and late phase reactions. The early phase reaction occurs within seconds to minutes (2-30 minutes) of allergen exposure and is predominantly mast cell mediated. Allergen binds to and cross links IgE-bound FcεRI on the surface of mast cells and basophils, and upon clustering of these receptors into trimers or more, a signal cascade is initiated that results in the activation and degranulation of the inflammatory cell (Gould and Sutton, 2008). A mixture of preformed inflammatory mediators are released in this process such as histamine, protease-proteoglycan complexes and TNFα, as well as *de novo* synthesis of leukotrienes, prostaglandins and other pro-inflammatory cytokines and chemokines (Steffen *et al*, 1989, Bloeman *et al*, 2007). In asthma, this results in inflammation, oedema, vasodilation, bronchoconstriction, increased mucus production, tissue damage and leukocyte recruitment, all of which leading to impaired lung function.

The late phase reaction can develop within hours of allergen exposure, often after the early phase has subsided and is characterised by the recruitment and activation of

predominantly T cells and eosinophils (Wedemeyer and Galli, 2000). This adds to the already established Th2 cytokine profile, as well as increasing the number of inflammatory effector cells present that continue to release many of the mediators mentioned above. These cells also release new inflammatory mediators that contribute to exacerbating the inflammatory reaction, such as reactive oxygen species, matrix metalloproteinases (MMPs) and other proteases that damage surrounding cells, adding to the inflammatory response and promoting tissue remodelling. This phase is a lot slower and long-lived than the early phase reaction and may contribute to a chronic inflammatory process due to the overlapping of late phase reactions, which can persist from days to years (Wedemeyer and Galli, 2000).

Due to the large number of inflammatory cells involved in the atopic phenotype, a highly complex mixture of inflammatory mediators is released over the time course of an allergic reaction (re-exposure to allergen in a sensitised individual). As described above, these can be separated into early and late phase mediators due to the type of cell activated during these periods.

1.12.1 Early Phase Reaction

Upon allergen exposure mast cells are one of the first inflammatory cells to be activated and initiate the allergic reaction. The mediators released on activation can be separated into preformed mediators, stored within the cytoplasmic granules, and those that are synthesised upon activation.

1.12.2 Preformed Mediators

Upon allergen exposure, an allergic phenotypic response can be seen almost instantaneously due to the release of preformed inflammatory mediators from mast cell granules. Within these storage granules is a crystalline complex of mediators ionically bound to a matrix of proteoglycans, such as heparin and chondroitin sulphates. These stored mediators are released rapidly upon cell degranulation, and account for the early phase response seen within seconds or minutes after allergen exposure (Holgate *et al*, 2006).

One of the major mediators stored within these granules, which is mainly found stored in mast cells and basophils, is the biogenic amine, histamine. Around 1-4pg/cell is present and accounts for vasodilation; increased permeability of post-capillary venules, resulting in oedema or swelling; contraction of smooth muscle; and increased mucus secretion; most of these resulting through the actions of H_1 receptors (Wedemeyer and Galli, 2000, Bloemen *et al*, 2007, Averbeck *et al*, 2007). These actions are relatively short-lived due to the rapid breakdown and short half-life of histamine (Beall and Vanarsdel, 1960, Averbeck *et al*, 2007).

Also contained within the mast cell granules is a range of proteases. The serine protease tryptase is the major protease present regardless of mast cell subtype. Tryptase is a 130kDa tetrameric protein that is stored in a fully active form within the granules (Schwartz and Bradford, 1986). There are however two subtypes of the protease, α and β, with β-tryptase predominating in allergic reactions (Holgate *et al*, 2006). β-tryptase has been linked to the production and activation of growth factors and thus is associated with increased airway smooth muscle cell hyperplasia (Tatler *et al*, 2008). Studies have also shown that mast cell tryptase stimulates the production of type I collagen from human lung fibroblasts, along with increasing proliferation of this cell type (Cairns and Walls, 1997). All of which may contribute to airway remodelling.

Depending on the subtype of mast cell, another serine protease, chymase, may also be released upon degranulation. Chymase has also been associated with airway remodelling by initiating collagen fibril formation by cleaving procollagen (Kofford *et al*, 1997), as well as activating matrix metalloproteinases (MMPs), which contribute to matrix destruction and turnover (Tchougounova *et al*, 2005). There is also interest in the role of chymase in anaphylaxis and its ability to convert angiotensin I to II (Reilly *et al*, 1982). More recently mast cell granules have also been shown to contain the proteases cathepsin G and carboxypeptidase, however these proteases have been studied less.

The proteoglycan matrix of the granules also has a role to play after degranulation. In particular, heparin, which is a potent anti-coagulant, has been shown to stabilise the active tetramer of β-tryptase (Schwartz and Bradford, 1986, Hallgren *et al*, 2005); enhance collagen binding to fibronectin (Johansson and Höök, 1980); and interestingly provide a protective role against the cleavage of eotaxin, a potent eosinophil chemokine (Ellyard *et al*, 2007).

1.12.3.1 Newly synthesised mediators

The newly generated mast cell mediators consist of the lipid mediators, the eicosanoids, and a wide range of cytokines, chemokines and growth factors.

1.12.3.2 Eicosanoids

The eicosanoids are generated from the liberation of arachidonic acid from the cell membrane by phospholipase A_2. Depending on which metabolic pathway utilised, they can be separated into two groups, the cysteinyl leukotrienes and the prostaglandins (see figure 1.5).

Figure 1.5

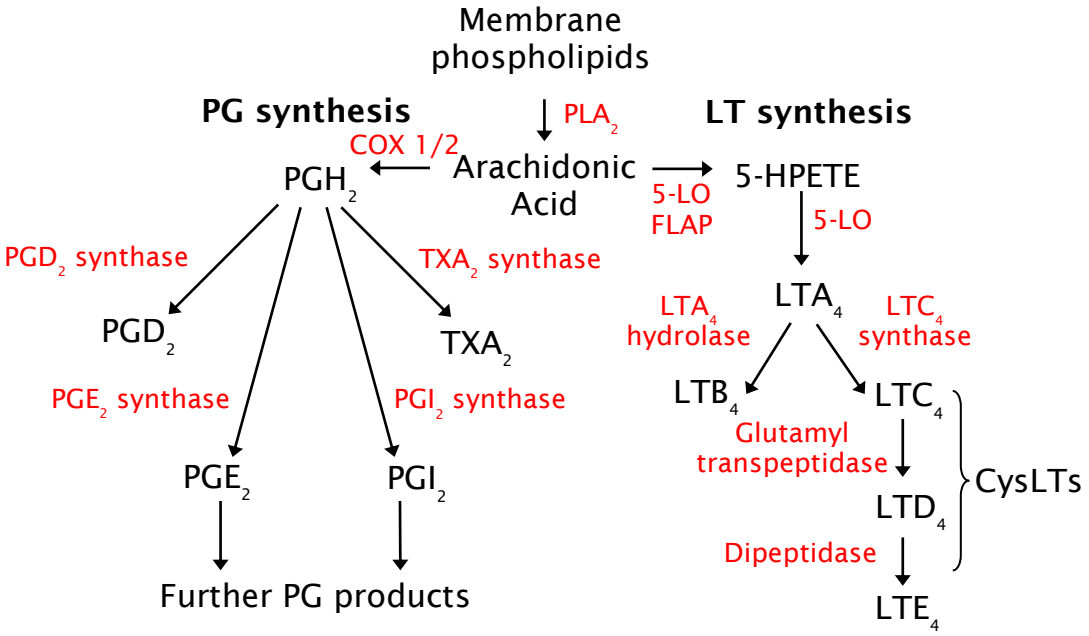


Figure 1.5 Synthetic pathways for the eicosanoids. Within the activated mast cell, phospholipase A_2 (PLA_2) hydrolyses phospholipid to produce arachidonic acid, the precursor metabolite for the lipid mediators the leukotrienes (LTs) and the prostaglandins (PGs). For LT synthesis, arachidonic acid is converted to 5-hydroperoxyeicosatetraenoic acid (5-HPETE) by the enzymes 5-lipoxygenase (5-LO) and 5-lipoxygenase-activating protein (FLAP) and is in turn converted to LTA_4. LTA_4 can then either be hydrolysed to LTB_4 by LTA_4 hydrolase or be conjugated to reduced glutathione to form the first of the cysteinyl-leukotrienes (CysLTs) LTC_4 by LTC_4 synthase. The other CysLTs, LTD_4 and LTE_4, are produced sequentially first by the cleavage of a glutamic acid residue from the glutathione moiety and finally the cleavage of a glycine residue. For PG synthesis arachidonic acid is converted to the intermediate PGH_2 by the cyclo-oxygenase enzymes I or II. PGH_2 is then converted by a number of enzymes to form the different PGs. Within mast cells the predominant PG produced is PGD_2 by the enzyme PGD_2 synthase.

The cysteinyl leukotrienes are considered important mediators in asthma and allergy and have proven to be good targets for drug therapy (Claesson and Dahlén, 1999). They are derived from the oxidation of liberated arachidonic acid by the enzymes 5-lipoxygenase (5-LO) and its coenzyme 5-lipoxygenase-activating protein (FLAP) at the perinuclear membrane, yielding the precursor 5-hydroperoxyeicosatetraenoic acid (5-HPETE) (Malaviya *et al*, 1993, Dixon *et al*, 1990). This in turn is converted by the same enzymes to leukotriene A_4 (LTA_4), which can then be converted to the first of the cysteinyl leukotrienes, leukotriene C_4 (LTC_4), in the presence of LTC_4 synthase (Boyce, 2007). Cleavage of LTC_4 by extracellular enzymes ubiquitous to the lung and blood results in the production of the other cysteinyl leukotrienes LTD_4 and its excretory metabolite LTE_4. The cysteinyl leukotrienes have a wide range of actions important to the pathophysiology of asthma. They are potent bronchoconstrictors able to cause prolonged bronchial and vascular smooth muscle contraction; increase mucus production and secretion; increase vascular permeability leading to oedema; and they are potent eosinophil (LTC_4) and neutrophil (LTB_4) chemoattractants, all of which resulting in airways obstruction and chronic inflammation by the recruitment of new inflammatory cells (Claesson and Dahlén, 1999).

The prostaglandins (PGs) form the other arm of arachidonic acid metabolism. In this case, arachidonic acid is converted into prostaglandin G_2 (PGG_2) and then PGH_2 by the cyclo-oxygenase enzyme (COX). There are two isoforms of COX however, one that is present in nearly every cell, that is considered to be used for homeostatic and house-keeping duties called COX I, and the other being the induced isozyme that is considered to be used in the incidence of inflammation called COX II (Smith *et al*, 1996). Upon formation of the PGH_2 precursor, the final products of this metabolic process depend on the cell specificity and the presence of certain cofactors (Dubois *et al*, 1998). The main early phase PG is PGD_2, which is also able to cause bronchoconstriction and neutrophil chemotaxis (Boyce, 2007).

1.12.3.3 Mast cell cytokines

A wide array of cytokines are released by activated mast cells contributing to inflammation. The main classic pro-inflammatory cytokine TNFα is stored within mast cell granules for immediate release upon degranulation but is also newly synthesised (Steffen *et al*, 1989, Baumgartner *et al*, 1994). Other newly synthesised cytokines include IL-4, -5, -8 and -13, granulocyte macrophage colony stimulating factor (GM-CSF) and monocyte chemotactic protein-1 (MCP-1) (Bloemen *et al*, 2007, Galli *et al*, 2008). The importance and function of some of these cytokines will be discussed later in this chapter.

1.13.1 Late Phase Reaction

The late phase reaction is caused by the influx of new inflammatory cells such as eosinophils, neutrophils and T cells, brought about by the early phase reaction; all of which become activated and release a cocktail of inflammatory mediators.

1.13.2 Eosinophils

Eosinophils are haematopoetic granulocytes about 8μM in diameter and are distinguishable due to their distinctive bi-lobed nucleus and their eosinophilic granules (Holgate *et al*, 2006). Mediators released during inflammation recruit these cells from the circulatory system and target them towards the site of inflammation. Once in the tissue, mediators such as GM-CSF and IL-5 are able to prime and activate the cell but also prolong its survival (Yamaguchi *et al*, 1988, Park *et al*, 1998). Eosinophils themselves once primed also produce these survival-inducing cytokines that act in an autocrine fashion but also recruit and prime more eosinophils (Bloeman *et al*, 2007). Once activated the cell is able to degranulate, releasing the contents of its granules, synthesise other inflammatory cytokines and metabolise arachidonic acid to produce lipid mediators. The granules contain pre-synthesised basic proteins including major basic protein, eosinophil cationic protein and eosinophil peroxidase. These proteins

are toxic to parasites but also to host tissue, damaging structural cells of the lung and ultimately increasing the inflammatory burden (Hamid and Minshall, 2000). Eosinophils have also been shown to store and produce other inflammatory cytokines such as IL-6, IL-8, TNFα and RANTES, although production of these cytokines is low compared to other cell types (Holgate *et al*, 2006). On the other hand, they are potent producers of leukotrienes, in particular LTC_4, which has shown to be over expressed in eosinophils from patients with aspirin-intolerant asthma (Cowburn *et al*, 1998)

1.13.3 Neutrophils

It is widely accepted that the late asthmatic phase is largely mediated by the infiltration of T cells and eosinophils, nevertheless, other inflammatory cells attracted to the site of inflammation such as neutrophils will also contribute to the inflammatory milieu and tissue damage. Evidence of neutrophil influx within the airways of asthmatics has been demonstrated by a number of groups along with increased levels of the potent neutrophil chemoattractant IL-8 (Gibson *et al*, 2001, Fahy *et al*, 1995, Kikuchi *et al*, 2009). *Per se*, the presence of increased neutrophils is likely to contribute to tissue damage within the lung and has been well documented in patients with chronic obstructive pulmonary disease (COPD), a largely neutrophil associated disease. Thus neutrophil activity may still be an important component in the pathology of asthma.

Neutrophils form the majority, around 70%, of circulating leukocytes with a primary role in dealing with infection and injury within the innate immune system. They are granulocytes derived from the bone marrow but have a much shorter life span than other leukocytes. As such, they express a variety of different receptors for chemotactic agents in order to move rapidly to sites of inflammation, tissue injury and infection (Holgate *et al*, 2006). Neutrophil granules contain many different enzymes including proteinases, elastase, gelatinase, collagenase and lysozyme, all designed to digest ingested bacteria. These enzymes can also be released extracellularly during inflammation adding to tissue damage (Bloeman *et al*, 2007). Like eosinophils, neutrophils must be primed and activated in order to release the contents of their granules. Upon activation, neutrophils are able to undergo a respiratory burst within seconds (Wymann *et al*, 1987). This process leads to the production of oxygen radicals, hydrogen peroxide (H_2O_2) and superoxide (O^{2-}). These can be converted into even more toxic products designed to be bactericidal. Neutrophils are also a source of lipid mediators with their major product being LTB_4. Like eosinophils, neutrophils also release products such as LTB_4 and IL-8 that can act back on the cell itself but are also potent neutrophil chemoattractants. This results in the recruitment of more neutrophils to the site of inflammation (Ford-Hutchinson *et al*, 1980, Bazzoni *et al*, 1991). Neutrophils have also been shown to produce IL-1, IL-6, IL-12, TNFα, M-CSF and

GM-CSF, however due to their short life span, the ability to generate these cytokines *de novo* is limited (Holgate *et al*, 2006).

1.13.4 Monocytes, macrophages and dendritic cells

Monocytes are also derived from pluripotent bone marrow stem cells and comprise around 2-8% of the total leukocyte count in healthy individuals. Monocytes circulate in the blood with a half-life of around 2-3 days and either move into tissues in response to an inflammatory stimulus or migrate randomly into tissues (Holgate *et al*, 2006). Once within a tissue or interstitial space, depending on its environment i.e. presence of specific maturation cytokines, monocytes differentiate into macrophages or immature dendritic cells, where they can exist for months.

Principally, macrophages are phagocytes designed to ingest and kill invading bacteria and remove tissue debris aiding in wound repair. In order to carry out this role, macrophages contain phagocytic granules, granules containing enzymes and have the capability of producing reactive oxygen and nitrogen species termed respiratory burst. Macrophages also are able to function as APCs and thus are able to participate in the acquired immune response. In this regard, they are able to process and present antigen to and activate T lymphocytes (Ziegler and Unanue, 1981). Regardless as to which side of the immune system is triggered, macrophages are an abundant source of inflammatory mediators producing toxic products, eicosanoids, and cytokines and chemokines; playing an important role in inflammatory processes (Nathan, 1987).

Dendritic cells play a key role in antigen presentation to the acquired immune system and form the principal, most efficient APC present in the lung (Holgate *et al*, 2006). Immature dendritic cells constantly scan their surrounding area for noxious stimuli, using a variety of receptors called pattern recognition receptors or PRRs. Upon encountering and uptake of a presentable antigen, the cell is activated becoming a mature dendritic cell and migrates to local lymphoid tissue (Bharadwaj *et al*, 2007). The antigen is degraded into fragments in lysosomal compartments and subsequently these fragments are expressed at the cell surface within the major histocompatability complex II (MHCII). The MHCII-peptide complex is then presented to naïve T cells via their T cell receptor, which along with interaction of co-stimulatory molecules activates the cell causing it to divide rapidly and secrete an array of cytokines that augment and promote the immune response (Masten and Lipscomb, 1999). Although dendritic cells are extremely important in activating the adaptive immune system, they also are a potential source of pro and anti-inflammatory cytokines such as TNFα, IL-6 and IL-10, thus contributing to the inflammatory milieu. (Faith *et al*, 2009).

1.13.5 T cells

T cells are derived from bone marrow progenitor cells that migrate to the thymus where they undergo selection to become mature T cells (Holgate *et al*, 2006). These cells are distinguishable from other lymphocytes due to the expression of the T cell receptor (TCR). They can also be divided into helper T cells (Th cells) that express CD4 and have an immunomodulatory role, or cytotoxic T cells, that express CD8 and destroy tumour and virally infected cells. Th cells can be further subdivided by the profile of cytokines they release i.e. Th_0 release IL-2, -4 -5 and IFNγ, Th_1 release IL-2 and IFNγ and Th_2 cells release IL-4, -5 and -13 (Del Prete, 1992, Venkayya *et al*, 2002). In regards to atopy and asthma, it is believed that a Th_2 response predominates, resulting in B cell isotype switch to IgE production, an aspect of allergic disease (Mckenzie *et al*, 1993, Punnonen and de Vries, 1994, Lebman and Coffman, 1988). They also support the activity of other inflammatory cells such as the mast cell and eosinophil and release a variety of chemoattractants resulting in an inflammatory infiltrate.

1.13.6 B cells

B cells are a different form of lymphocyte generated in the bone marrow. Like T cells, B cells are initially produced as immature cells that migrate to secondary lymphoid tissues such as the lymph nodes and the spleen, where they encounter antigen and other immune cells. The first step in B cell activation involves the binding of antigen to the B cell receptor (BCR). Antigen is then internalised through the BCR, processed in endosomal compartments and presented to specific Th cells, which have been previously primed to this antigen by APCs, via the MHCII complex (Lanzavecchia, 1985). Interaction between the MHCII-peptide complex and the TCR, along with interaction of other co-stimulatory molecules, results in Th cell activation and production of immunoregulatory cytokines, which subsequently cause activation and proliferation of the B cell into antibody-producing plasma B cells and long-lived memory B cells (McHeyzer-Williams and Ahmed, 1999). As discussed previously, atopic individuals have predominately Th_2 cells that, when stimulated, produce the cytokines IL-4 and IL-13. These cytokines have been shown to cause antibody class switch recombination in activated B cells to IgE production by binding to their relevant receptor on the B cell, in coordination with direct interaction via the T cell CD40 ligand with the B cell CD40 receptor (Pène *et al*, 1988, Lebman and Coffman, 1988, Punnonen and de Vries, 1994).

Although their main function is to produce antibodies, B cells also have the ability to present antigen to T cells and contribute to the pathogenesis of allergic disease by acting as APCs (Lindell *et al*, 2008). Other studies have shown however that the capacity of B cells to act as APCs is diminished compared to professional APCs in the lung, such as dendritic cells (Masten and Lipscomb, 1999). The study by Lindell *et al*

was demonstrated in the context of chronic allergic inflammation where a continuing supply of allergen and influx of B cells to the site of inflammation may help favour B cell antigen presentation. Thus B cells may also be important effector cells in chronic inflammation of the lung in diseases such as allergic asthma.

1.13.7 Lung structural cells – Epithelial cells, fibroblasts and smooth muscle

Chronic inflammation of the airways in pulmonary diseases such as asthma and COPD results in abnormal structural changes that undoubtedly involve airway structural cells. These cells are now also widely accepted as being able to contribute to inflammatory processes themselves by releasing multiple mediators that act in an autocrine and paracrine fashion (Denburg *et al*, 1991).

The airway epithelium primarily functions as a barrier, as the first line of defence in the innate immune system against insult to the lung. In this regard the epithelium has a number of protective mechanisms including mucus production, mucociliary clearance and the production of oxygen/nitrogen reactive species (Martin *et al*, 1997). Epithelial cells have also been shown to express pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and protease activated receptors (PARs), which upon activation initiates the production of inflammatory cytokines (Hammad and Lambrecht, 2008). These in turn attract inflammatory cells and activate surrounding tissue and neighbouring cells. Epithelial cells have been shown to release a multitude of mediators including lipid mediators, reactive oxygen species and inflammatory cytokines such as TNFα, IL-6 and IL-8 (Martin *et al*, 1997).

In close proximity to epithelial cells lie fibroblasts. These cells are primarily involved in the synthesis of ECM and collagen and are involved in wound healing. The close interaction of epithelial cells and subepithelial fibroblasts has sparked interest, particularly in asthma pathogenesis, where a prominent feature is marked airway wall thickening caused during aberrant wound-healing in chronic inflammation. This is seen in asthmatics particularly in the subepithelial reticular layer, with increased deposition of type I, III and V collagen and fibronectin, attributed to subepithelial fibroblasts (Brewster *et al*, 1990). Fibroblasts have also been shown to contribute to asthma pathogenesis by secreting eotaxin in a Th_2 environment (Wenzel *et al*, 2002) and produce increased levels of IL-6 when in contact and activated by T cells, which may contribute to fibrogenesis in the airway (Loubaki *et al*, 2010).

The importance of smooth muscle in asthma pathogenesis is mostly attributed to airflow obstruction upon contraction. Smooth muscle hypertrophy and hyperplasia also contribute to this (Damera *et al*, 2009). More recently, more focus is being placed on these cells in also playing a role in inflammatory responses in the lung. Airway smooth

muscle has been shown to release an array of cytokines and chemokines that have an immunomodulatory role and attract inflammatory cells to the site of inflammation (Damera *et al*, 2009). Mast cells are often found within smooth muscle bundles and smooth muscle has also been shown to release mast cell chemokines, survival and proliferative factors (Hollins *et al*, 2008), which may have particular relevance in asthma and airway hyper-responsiveness.

In chronic inflammatory diseases, multiple cell types are likely to all play a role in the pathogenesis of the disease and the formation of an inflammatory microenvironment within the tissue. As such it is widely accepted that inflammatory diseases of the lung involve a complex interplay between inflammatory and structural cells, the mediators they release and the ECM.

1.14 Models investigating human lung inflammation

Rodent models of asthma are widely used to investigate allergic mechanisms and identify new therapeutic targets (Shin et al, 2009). This approach is limited by the fundamental differences between rodent and human airways and the need to sensitise all of the animals in a relatively short period of time (Kumar and Foster, 2002). Studying human cells and tissues presents more challenges but may provide the insight into allergic mechanisms required to develop therapeutic approaches. *In vivo* sampling in humans to study allergic lung inflammation is commonly conducted using techniques such as bronchoscopy, bronchoalveolar lavage (BAL) and induced sputum. These techniques however are limited by the difficulty in obtaining samples and to sampling of predominantly the proximal airways and the airway lumen. This may not reflect inflammatory processes throughout the lung and sampling of the airway lumen may not reflect inflammatory processes within the tissue (O'Donnell *et al*, 2006). Tissue obtained from lung resection surgery has been used extensively over the last 40 years to study allergic airways inflammation with studies ranging from intact tissue (Bochner *et al*, 1987, Schleimer *et al*, 1983) to isolated and purified cells (Ballard *et al*, 1978, Holgate *et al*, 1985). We have chosen to concentrate on a lung tissue explant model of allergic inflammation but the source of the lung tissue cannot be ignored. Patients undergoing lung resection rarely have asthma, are typically older, have a smoking history and often have a range of co-morbidities. We have found that 48% of our patients have evidence of airways obstruction which cannot be ignored as a confounding factor. Although the main focus of this thesis is allergic inflammation and asthma, we have also taken into consideration inflammatory processes involved in patients with COPD.

1.15 Chronic Obstructive Pulmonary Disease (COPD)

COPD is a major cause of mortality and morbidity and is estimated to become the 3rd leading cause of death worldwide by 2020 (Murray and Lopez, 1997). An estimated 210 million people have the disease with 80 million of these suffering from moderate to severe degrees (WHO, accessed on 08/02/11). It is an incurable, progressive condition and is usually not recognised, diagnosed or treated in its early stages (Rabe *et al*, 2007). In the 'Confronting COPD survey' the estimated direct and indirect cost on a patient with COPD in the UK was £819 each with a total societal cost of £1640 per patient per year (Britton, 2003). Taking into account undiagnosed patients, the total societal cost was estimated at £2.1 billion per annum (Britton, 2003) making COPD one of the highest ranking economic burdens in the UK.

COPD is classified by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) as 'airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases' (Rabe *et al*, 2007). Symptoms include chronic and progressive dyspnea, cough and excessive mucus production which result in increasing lower quality of life. It is now widely accepted that COPD is a multicomponent disease involving lung and systemic inflammation, with links to not only airflow obstruction but also structural changes, mucociliary dysfunction and systemic effects such as muscle wasting and reduced body mass index (Stockley, 2009).

As the classification of COPD involves airflow limitation, spirometry is the most widely used and accepted way of diagnosing and determining severity of disease. Following guidelines created by GOLD, disease status can be classified using spirometric cut points, which have been summarised in table 1.1 for clarification.

Table 1.1

GOLD Status	Spirometry	Symptoms
GOLD I Mild COPD	$FEV_1/FVC<0.7$ $FEV_1 \geq 80\%$ predicted	Chronic cough, sputum production may be present
GOLD II Moderate COPD	$FEV_1/FVC<0.7$ $50\% \leq FEV_1 < 80\%$ predicted	Dyspnea on exertion, cough and sputum production sometimes present
GOLD III Severe COPD	$FEV_1/FVC<0.7$ $30\% \leq FEV_1 < 50\%$ predicted	Dyspnea, reduced exercise capacity, fatigue, repeated exacerbations
GOLD IV Very severe COPD	$FEV_1/FVC<0.7$ $FEV_1 <30\%$ predicted or $FEV_1 <50\%$ predicted plus chronic respiratory failure	Possible respiratory failure, cor pulmonale. Quality of life appreciably impaired and life threatening exacerbations

Table 1.1 Spirometric classification of COPD severity based on post bronchodilator FEV_1. Respiratory failure defined as arterial partial pressure of O_2 <8kPa (60mmHg) with or without arterial partial pressure of CO_2 >6.7kPa (50mmHg) while breathing air at sea level. Table adapted from Rabe *et al*, 2007).

1.16 Risk Factors

It is well documented that the single most commonly encountered and major risk factor for COPD in high- and middle-income countries is tobacco smoke. In 2005 WHO estimated 5.4 million people died due to tobacco use and this death toll is expected to rise to 8.3 million deaths by 2030 (WHO, accessed on 14/02/11). As such it is unsurprising that with longer-living populations and increased tobacco usage, mortality and morbidity from COPD is set to increase.

Not all smokers will go on to develop COPD however, with the risk of developing this disease depending on gene-environment interactions. One of best documented genetic risk factors for COPD is α_1-anti-trypsin (A1-AT) deficiency. This is a rare recessive trait that is most common in Northern European origin (Rabe *et al*, 2007) and often results in early-onset emphysema in patients, with increased risk in smokers. Deficiency in this protease inhibitor results in a protease-anti-protease mismatch that allows excessive protease activity and tissue inflammation and destruction. Other risk factors include occupational dusts and chemicals and indoor and outdoor air pollution (WHO, 14/02/11).

1.17.1 Pathology of COPD

A chronic inflammatory infiltrate of macrophages, neutrophils and $CD8^+$ T cells, as a result of smoking, has long been accepted as the major cause in the development of COPD (Cosio *et al*, 2002, O'Donnell *et al*, 2005). Increased levels of the chemokine IL-8 and neutrophil-derived protease, elastase, have also been heavily implicated (Yamamoto *et al*, 1997). The accumulation of other inflammatory mediators, proteolytic enzymes and oxidative stress also contribute to the development of airway abnormalities, structural changes and disease progression (O'Donnell *et al*, 2005).

COPD encompasses a set of conditions that develop as a result of various disease processes and are referred to as chronic bronchitis and emphysema.

1.17.2 Chronic bronchitis

Chronic bronchitis is diagnosed as a chronic productive cough for 3 months in each of 2 successive years, provided other causes have been ruled out (Mannino, 2003). It consists of inflammation of the epithelium of the central airways, which extends into the mucus-producing glands. This results in increased mucus production, a deficiency in mucociliary clearance, disruption of the epithelial barrier and thickening of the bronchial walls (Hogg, 2004). A degree of small airways (<2mm in diameter) obstruction also falls under this term. This is characterised by an inflammatory exudate present in mucus plugs and increased connective tissue deposition in the

adventitia compartment, which restricts the opening of these airways during inhalation, resulting in the majority of airflow limitation (Hogg, 2004).

1.17.3 Emphysema

Emphysema comprises the destruction of the alveolar walls and permanent enlargement of the airspaces distal to the terminal bronchioles (Mannino, 2003). This results in the loss of elastic recoil and intraluminal pressure, reducing maximum expiratory flow and resulting in the collapse of the small airways (Mannino, 2003, Hogg, 2004). Two types of emphysema can occur and predominate depending on patient phenotype. Centriacinar emphysema mainly occurs in smokers and results in the destruction and dilation of the respiratory bronchioles, with the more distal alveolar duct and alveoli left intact (Hogg, 2004). On the other hand, panacinar emphysema is more common in patients with A1-AT deficiency and is characterised by destruction and dilation of the entire acinus: respiratory bronchiole, alveolar duct and alveoli (Kierszenbaum, 2002). This genetic defect results in decreased production of a major serine protease inhibitor, A1-AT, which has a particular role in protecting lung tissue from neutrophil elastase. Neutrophils and neutrophil elastase have been heavily implicated in the pathophysiology of COPD and resulting emphysema, as the main substrate for this protease is elastin, a major constituent of distal lung tissue.

1.18 COPD Exacerbation

COPD is often associated with exacerbations of disease that have a profound and long-lasting effect on patients (Celli and Barnes, 2007). They can be fatal in severe cases and account for the majority of healthcare costs for COPD, representing 35-40% of the overall burden (Stockley *et al*, 2009). An exacerbation is defined as 'an event in the natural course of the disease characterised by a change in the patient's baseline dyspnea, cough and or sputum that is beyond normal day to day variation, is acute, and may warrant a change in regular medication' (Rabe *et al*, 2007). Patients with acute exacerbation of chronic bronchitis have increased neutrophilic inflammation and increased levels of IL-8, TNFα and neutrophil elastase in sputum, compared to stable patients (Sethi *et al*, 2000). Over time, exacerbation frequency and severity increases, with inflammatory processes accelerating the progression of disease, which is associated with increased airflow impairment (Celli and Barnes, 2007). The most common triggers of COPD exacerbation are bacterial or viral infection and air pollution (White *et al*, 2003), with 50% of exacerbations attributed to bacterial infection (Murphy, 2006).

1.19 Pharmacotherapy of inflammatory lung conditions

The main aim of treatment of incurable inflammatory conditions of the lung, such as asthma and COPD, is management of the disease and reduction in its progression. This

is a balance between the benefits of using a drug and its possible side effects. In mild intermittent disease where inflammation is low, reliever medication is often sufficient in order to alleviate occasional symptoms. In situations where disease is more chronic and uncontrolled, inflammation persists and can lead to the progression of more severe disease. In these situations anti-inflammatory drugs are used to reduce and prevent inflammatory processes.

Common bronchodilators used in the treatment of asthma and COPD include β_2–agonists, xanthines and anti-cholinergics. The β_2-agonists such as the short-acting drugs salbutamol and terbutaline, or the longer-acting agents salmeterol and formoterol, are the most commonly used bronchodilator for asthma treatment, and most effective when inhaled (Barnes, 1997). The main target of this class of drug is the β_2-adrenergic receptor, which is highly expressed by human airway smooth muscle. Upon binding to the receptor, β_2-agonists act as functional antagonists able to prevent and reverse the effects of bronchoconstrictor substances by causing smooth muscle relaxation (Barnes, 1997). Stimulation of the G-protein coupled receptor results in activation of adenylate cyclase and subsequent increase in intracellular cAMP, resulting in smooth muscle relaxation. There is also growing evidence for the role of β_2-agonists as anti-inflammatory agents. β_2-agonists have been shown to inhibit the IgE-dependent generation of histamine, cys-LTs, PGD_2 (Scola et al, 2009) and cytokines such as IL-5 and TNFα from mast cells (Johnson, 2002). Evidence has also been shown for inhibitory effects on macrophages, eosinophils, T cells and neutrophils (Johnson, 2002, Barnes, 1999) as well as potentially aiding in mucociliary clearance by increasing ciliary beating on epithelial cells (Barnes, 1999, Rang et al, 2003). Intensive usage of β_2-agonists however, particularly long-acting agonists, often results in the development of tolerance to inflammatory cell stabilisation (Scola et al, 2004, Peachell, 2006).

Another bronchodilator used in the treatment of asthma and COPD is the xanthine drug theophylline. The mechanism of action of this drug is less well understood, with its bronchodilator actions being attributed to inhibition of PDE isozymes resulting in increased cAMP, and/or competitive antagonism of adenosine at its receptor (Rang et al, 2003). Theophylline is a relatively weak relaxant of airway smooth muscle, with high doses needed, however it has also been shown to have anti-inflammatory effects and inhibit the late phase response to allergen (Barnes, 1997). Due to its toxicity and narrow therapeutic range, the benefits of theophylline's actions must be weighed against its side-effects

The anti-cholinergics include the shorter-acting ipatropium bromide and long-acting tiotropium bromide. These drugs relax bronchoconstriction caused by parasympathetic

stimulation by blocking muscarinic receptors, specifically M_3 receptors and may also reduce hypersecretion (Rang et al, 2003). This class of bronchodilator have proven to be more effective in COPD patients than patients with severe asthma (Barnes, 1997, Gross, 2006).

A more novel class of bronchodilator are the cys-LT receptor antagonists such as montelukast and zafirlukast. These drugs selectively block cys-LT_1 receptors that mediate a number of effects that contribute to inflammation in asthma, discussed above in section 1.12.3.2. Some of these effects result in reducing inflammation and have been shown to improve symptoms, lung function and reduce exacerbations and early and late phase asthmatic reactions (Montuschi and Peters-Golden, 2010). Other inhibitors of the leukotriene pathway are also in development such as 5-LO and FLAP inhibitors.

In asthma and COPD treatment, bronchodilators are central to symptomatic relief. With more severe asthma, the use of inhaled corticosteroids is the first choice and most effective preventer therapy (BTS 2009 Asthma Guidelines) and is often used in combination with a short-acting or long-acting inhaled β_2-agonist. The introduction of inhaled corticosteroids revolutionised asthma therapy, proving to be effective in reversing specific airway inflammation by decreasing the numbers of infiltrating inflammatory cells and suppressing the release of inflammatory mediators. The effects of corticosteroids have proven to be much less effective in COPD (Chung *et al*, 2009), with little long-term effect in the decline in lung function (Stockley *et al*, 2009). Although steroids in this case do not prevent disease progression, they have been proven to decrease exacerbation frequency and are therefore often prescribed to symptomatic patients with COPD that are at risk of repeated exacerbations (Rabe *et al*, 2007). In severe cases of asthma, where inhaled corticosteroid therapy fails to control the disease, oral steroids are prescribed to deliver a higher concentration of the drug systemically. This is generally avoided in COPD therapy due to unfavourable benefit-to-risk ratio with high doses of steroid (Rabe *et al*, 2007).

Classically, corticosteroids work by diffusing across cell membranes, binding to the cytosolic glucocorticoid receptor and activating it by releasing it from chaperone proteins. This enables translocation of the receptor to the cell nucleus where it can interact to switch off inflammatory genes and switch on anti-inflammatory genes (Barnes, 2010). Steroids have also been shown to increase the expression of proteins that destabilise the mRNA of inflammatory proteins preventing their transcription (Barnes, 2010). Together, these actions prevent the production and secretion of inflammatory cytokines and favour anti-inflammatory processes.

Another class of anti-inflammatory used in the treatment of asthma are the cromones, including cromolyn sodium and nedocromil sodium. These agents reduce the early and late asthmatic reaction and decrease airway hyper-reactivity, but are less effective than inhaled glucocorticoids and therefore more suited for some patients with mild asthma (Barnes, 1997). The cromones are thought to act as 'mast cell stabilisers' however their mechanism of action is not fully understood. Some evidence has also shown that they can reduce exaggerated neuron reflexes triggered by irritant receptors, suppress the response of sensory C fibres to irritant and inhibit the release T cell cytokines (Rang et al, 2003).

In patients with more severe persistant allergic asthma that is uncontrolled even with glucocorticoid treatment, the addition of omalizumab as an add-on therapy has proven to significantly reduce exacerbations, use of steroid and improve quality of life (Humbert et al, 2005). Omalizumab is a humanised monoclonal anti-IgE antibody that binds to soluble IgE and prevents it binding to FcεRI. This reduces IgE binding to cells that express this receptor, such as mast cells and basophils and therefore reduces the number and extent to which these cells are 'primed' for activation upon encountering allergen, ultimately reducing the release of inflammatory mediators during an allergic response (Humbert et al, 2005).

1.20.1 Cytokines

Cytokines are low weight soluble glycoproteins, about 8-80KDa in size that act as potent extracellular signalling molecules. They have a range of effects involved in inflammation, defence against viral infection and influence in the proliferation, differentiation and survival of cells (Roitt *et al*, 2001). Over 200 different human cytokines have been identified, highlighting the complexity of this system, which also shows redundancy with different cytokines exhibiting similar functions and several cell types responding to and generating the same cytokine. Ultimately, cytokines bind to their receptors expressed on target cells initiating intracellular signalling in the form of kinase cascades. This results in the phosphorylation of cellular substrates and the activation of transcription factors that bind to promoter regions of a gene and up regulate its transcription (Holgate *et al*, 2006).

Upon exacerbation of an allergic response, early phase mediators such as histamine, prostaglandins and leukotrienes result in the initial clinical manifestation of bronchoconstriction and mucus production. During this early phase, cytokines also play an important role in the development and pathogenesis of asthma, augmenting the microenvironment and recruiting inflammatory cells to the lungs. A Th_2 type response characteristically dominates in allergic inflammatory reactions that influence

a wide range of actions. The release of IL-4 and IL-13 stimulate the production of IgE increasing the sensitisation of an individual to particular allergens. These cytokines also up regulate vascular cell adhesion molecule-1 (VCAM-1) aiding in the specific recruitment of eosinophils and basophils, inflammatory cells characteristically involved in asthma and allergic inflammation (Kay, 2000). The production, maturation and prolonged survival of eosinophils are also increased by other cytokines linked to asthma pathogenesis including IL-5, IL-9 and GM-CSF. The release of chemokines and up regulation of adhesion molecules facilitates the recruitment of inflammatory cells. These cells go on to cause the delayed second wave of inflammatory responses characteristic of allergic inflammation and the late asthmatic reaction. Typically in asthma, eosinophils and T cells are selectively recruited by the release of specific chemokines such as the eotaxins, RANTES, MCP-3 and MCP-4, which are promoted in a Th_2 environment (Kay, 2000).

The promotion of allergic airway disease in asthmatic patients has also been associated to a number of novel cytokines such as IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) (Finkelman et al, 2010). These cytokines stimulate the production of Th_2 cytokines from a number of cell types including Th_2 cells themselves, mast cells, basophils and eosinophils and also increase the differentiation and proliferation of Th_2 cells directly and indirectly via actions through dendritic cells (Finkelman et al, 2010, Oboki et al, 2011, Liu, 2006). Other inflammatory cytokines, such as the IL-17 family, have also been linked to airway inflammation in diseases such as asthma and COPD Mosely et al, 2003). IL-17A and F have been shown to be released from T cells, eosinophils, mast cells and basophils and have been associated with increased tissue eosinophilia, neutrophilia and airway remodelling (Hizawa et al, 2006).

We have chosen to investigate a panel of inflammatory cytokines which will be discussed in further detail below.

1.20.2 TNFα and IL-10

TNFα is a ubiquitous pro-inflammatory cytokine produced by a vast array of cells. Within the lung the main cell types to produce and release this cytokine are macrophages, mast cells and epithelial cells (Ohkawara *et al*, 1992). During an allergic inflammatory response TNFα functions to activate inflammatory cells (Thomas, 2001) and increase endothelial adhesion molecule expression, aiding in the infiltration of leukocytes to the site of inflammation (Pober *et al*, 1986). TNFα is released throughout the inflammatory process including the initial response, as a preformed store of TNFα is present within the granules of mast cells and thus can contribute to the early phase reaction (Walsh *et al*, 1991).

TNFα is able to exert its effects through ligation with two distinct transmembrane receptors, TNF receptor-1 (TNF-R1) and TNF-R2 (Li and Lin, 2008). TNF-R1 is the key receptor involved in TNF signalling and is ubiquitously expressed whereas TNF-R2 is highly regulated and expressed mainly in cells of the lymphoid system (Parameswaran and Patial, 2010). TNFα binding to TNF-R1 triggers the trimerisation of receptors and release of the inhibitory protein, silencer of death domains (SODD) from the intracellular domain of the receptor (Parameswaran and Patial, 2010). This activation enables the recruitment of the adaptor protein, TNF receptor-associated death domain (TRADD), which recruits a series of other adaptor proteins including receptor-interacting protein-1 (RIP-1) and TNFR-associated factor 2 (TRAF2) (Wajant and Scheurich, 2011). The assembled signalling complex leads to the activation of the transcription factors nuclear factor of κB (NF-κB) and activator protein-1 (AP-1) that induce the expression of genes involved in a range of inflammatory processes (Li and Lin, 2008).

IL-10 can be described as a classic anti-inflammatory cytokine and is mainly produced by T cells, B cells, monocytes and macrophages with a role in regulating and dampening the inflammatory response (Conti *et al*, 2003). In fact, it has been shown by Wang and colleagues that IL-10 is able to inhibit the transcription of inflammatory cytokine genes in peripheral blood mononuclear cells (PBMCs) stimulated with lipopolysaccharide (LPS), a membrane component of gram negative bacteria (Wang *et al*, 1994). This group went on to demonstrate that IL-10 inhibits the activation of NF-κB, a transcription factor constitutively expressed in the cytoplasm of most cells, which may explain the mechanism behind IL-10 induced cytokine suppression (Wang *et al*, 1995).

Signal initiation occurs when IL-10 binds to the extracellular domains of the IL-10 receptor complex composed of four transmembrane polypeptide chains: two IL-10R1 chains that bind ligand and two IL-10R2 chains that initiate signal transduction (Petska et al, 2004). Upon ligand binding dimerization of the receptor chains leads to activation of constitutively bound Janus kinase (Jak)-1 and initiation of the Jak/Signal Transducers and Activators of Transcription (STAT) pathways (Zdanov, 2010). Phosphorylation of STAT proteins enables translocation to the nucleus where they drive transcription of STAT-3 responsive genes, such as the suppressor of cytokine signalling (SOCS) family, which go on to cause some of the anti-inflammatory effects of IL-10 (Petska et al, 2004).

1.20.3 IL-5 and IL-13

IL-5 is a Th_2 cytokine released by Th_2 cells, eosinophils and mast cells (Del Prete, 1992, Broide *et al*, 1992, Bradding *et al*, 1994). Experiments carried out using cultures of normal human bone marrow have verified a role for IL-5 in the selective production of eosinophils (Clutterbuck *et al*, 1989). Along with cell culture experiments IL-5 has been proven to prolong eosinophil survival and induce eosinophil superoxide anion production and migration (Yamaguchi *et al*, 1988). The ability of IL-5 to selectively cause eosinophil proliferation has also been demonstrated *in vivo*, using irradiated mice that were transplanted with bone marrow infected with recombinant virus bearing the IL-5 coding sequence (Vaux *et al*, 1990). The resulting chronic secretion of IL-5 in these mice produced persistent eosinophilia. This is reflected in humans in a study by Hamid *et al*, 1991, where the expression of IL-5 mRNA in asthmatic bronchial mucosa correlated significantly with the number of activated T cells and eosinophils, whereas healthy controls were negative for IL-5 mRNA and contained very few eosinophils. As mentioned previously, eosinophils and T cells are considered to be a key inflammatory infiltrate in asthma. As such IL-5 has strong links to this disease and other allergic disorders.

IL-5 shares a common signalling pathway to other haemopoietic cytokines such as IL-3 and GM-CSF, which have similar biological activities (Woodcock et al, 1999). Each cytokine receptor is composed of two transmembrane subunits, a ligand binding cytokine-specific α subunit and a common shared signalling β subunit (βc) (Martinez-Moczygemba et al, 2007). Upon cytokine binding to the α subunit, the receptor becomes activated upon dimerization of βc to the α subunit, which induces transactivation and phosphorylation of βc-associated Jak-1 and 2 (Woodcock et al, 1999). Activation of this heteromeric complex results in initiation of the JAK/STAT, MAPK and PI3-K pathways, which act in conjunction to promote growth, differentiation, proliferation, effector function and survival of the target cell (Martinez-Moczygemba and Huston, 2003).

IL-13 is predominantly released by Th_2 cells (Mckenzie *et al*, 1993) but also mast cells (Burd *et al*, 1995, Jaffe *et al*, 1996), basophils (Shimizu *et al*, 1998) and eosinophils (Schmid-Grendelmeier *et al*, 2002). It is also strongly implicated in the development of allergy and asthma due to its role in B cell differentiation and immunoglobulin isotype switch to IgE production. Evidence for this has been gathered by Punnonen and colleagues, who showed that IL-13 induced IL-4 independent CD23 expression, IgG_4 and IgE production in purified human B cells and induction of germ-line ε mRNA (Punnonen *et al*, 1993). However the presence of co-stimulatory signals i.e. CD40 interaction, provided by activated T cells, was also shown to be essential for

immunoglobulin isotype switch. Interestingly, it has also been shown that B cells already committed to IgE synthesis are able to produce IL-13, which may act in an autocrine fashion (Hajoui *et al*, 2004).

It has been shown that the IL-13 receptor signalling complex shares components with the IL-4 receptor, which explains why these cytokines have similar biological roles (Zurawski et al, 1995). The IL-4 receptor α chain is fundamental for IL-13 signalling when associated with the IL-13 receptor α1 subunit, which together form the functional heterodimeric IL-13 receptor (Wills-Karp, 2004). Upon binding of IL-13 to this complex and subsequent dimerization and activation of the receptor, constitutively associated Jak1 and Tyk-2 kinases are phosphorylated and in turn trigger a number of intracellular signalling pathways. A consequence of this is phosphorylation of STAT6, which has been linked with B cell isotype switching, airway hyperresponsiveness and mucus production (Wills-Karp, 2004).

1.20.4 IL-1

IL-1 consists of two forms IL-1α and IL-1β that are synthesised as large precursors and cleaved into their active smaller forms (March *et al*, 1985). Both isoforms are considered to induce pro-inflammatory effects in a vast number of effector cells with similar biological activities to TNFα, including activation of leukocytes and endothelial cells (Le and Vilcek, 1987). Pro-IL-1α is fully active but remains mainly intracellularly and released only upon cell death or when cleaved by calcium-dependent, membrane-associated cysteine proteases called calpains (Dinarello, 1997). On the other hand release of mature IL-1β occurs upon activation of IL-1β–secreting cells for example via bacterial stimulation of Toll-like receptors that induce inflammasone complexes and result in activation of caspase-1 (Netea et al, 2010). Caspase-1 then cleaves pro-IL-1β into its biologically active form, which is subsequently released by the cell. Like TNFα, IL-1 is also produced by a vast array of cells including neutrophils (Malyak *et al*, 1994), macrophages and epithelial cells (Thorley *et al*, 2007).

IL-1α and IL-1β both bind to the same receptors, IL-1R1 and IL-1R2, however only IL-R1 induces signal transduction, with IL-1R2 acting as a 'decoy' receptor, particularly for IL-1β (Dinarello, 1997). Upon binding of IL-1 to IL-1R1, a conformational change occurs in the receptor which allows for the binding of the IL-1R accessory protein (IL-1RAcP) resulting in activation of intrinsic GTPase activity in the cytoplasmic domains of the receptor and a GTPase activating protein (Dinarello, 1997). Subsequent signalling events depend on the cells involved however generally activation of MAP kinases and transcription factors result in the biological actions of these cytokines.

1.20.5 IL-6

IL-6 is considered to be a pleiotropic cytokine, with a broad range of actions in inflammation and immunity but also homeostatic and neuroendocrine functions (Barton, 1997). It was originally discovered as a B cell stimulatory factor that enhanced immunoglobulin secretion in B cells (Kishimoto, 2006) and caused their differentiation into plasma cells (Barton, 1997). It has also been shown to promote T cell growth and differentiation into cytotoxic T cells (Holgate *et al*, 2006), have effects in bone biology (Barton, 1997) and is one of the main inducers of C-reactive protein and serum amyloid A production (Kishimoto, 2006), acute phase proteins produced by the liver during inflammation. IL-6 is predominantly produced by macrophages, monocytes and epithelial cells (Thorley *et al*, 2007) but also mast cells, fibroblasts, eosinophils and smooth muscle (Holgate *et al*, 2006, Hollins *et al*, 2008).

IL-6 elicits its actions via two receptors, a conventional receptor-bound pathway by binding to the transmembrane IL-6R and via a trans-signalling pathway by binding to soluble IL-6R (Ding et al, 2009). The conventional pathway results in dimerization of the IL-6R with gp130 upon ligand binding, whereas in the alternative pathway the IL-6/soluble IL-6R complex is able to associate with gp130 on the cell membrane to instigate intracellular signalling (Ding et al, 2009). Both pathways result in the activation of Jak proteins associated with gp130, which lead to the induction of STAT-3 and MAPK pathways (Ding and Jones, 2006).

1.21.1 Chemokines

A distinct superfamily of cytokines that attract leukocytes to sites of inflammation and infection are the chemokines. These peptides are typically around 68-120 amino acids with 20-70% homology and are structurally similar containing a backbone of at least three β-pleated sheets and a C-terminal α-helix (Rollins, 1997). They can be subdivided into 4 families on the basis of the relative position of their amino-terminal cysteine residues (Premack *et al*, 1996, Luster, 1998). The chemokine families are thus characterised upon their cysteine motif and named accordingly i.e. C, CC, CXC or CXXXC, the X denoting any amino acid. Chemokine receptors are also distinguished based on the chemokine family that binds to them i.e. CXC receptors (CXCRs) only bind CXC chemokines.

The CXC and CC subfamilies represent the largest groups of chemokines and have been extensively studied. The CXC chemokines can be further subdivided by the presence of the amino acid sequence, glutamic acid-leucine-arginine, ELR, which immediately precedes the CXC motif near the N-terminal region of the peptide. This ELR motif has been shown to be fundamental in neutrophil chemotaxis (Hérbert *et al*,

1991) whereas CXC chemokines lacking this motif act on lymphocytes (Clark-Lewis *et al*, 1993, Luster, 1998).

1.21.2 IL-8

The most well characterised ELR-CXC chemokine is the well-established neutrophil chemoattractant IL-8. IL-8 is produced by a wide variety of cell types including monocytes/macrophages, neutrophils, T cells, mast cells, fibroblasts, epithelial cells and endothelial cells (Rollins, 1997). IL-8 has been implicated as the major neutrophil chemotactic factor in the lung (Kunkel *et al*, 1991) with strong links to pulmonary disorders such as adult respiratory distress syndrome (ARDS) (Goodman *et al*, 1996), cystic fibrosis (Dean *et al*, 1993), COPD (Yamamoto *et al*, 1997) and severe asthma (Gibson *et al*, 2001, Kikuchi *et al*, 2009).

1.21.3 Groα

Groα is another ELR-CXC neutrophil attracting chemokine, similar to IL-8. There are considerably less studies investigating Groα production in inflammation, although some studies have documented the release of both chemokines together from macrophages, neutrophils and epithelial cells, for example in patients with Helicobacter *pylori* gastritis (Eck, 2000). Like IL-8, Groα has also been linked to COPD, with sputum of COPD patients containing significantly higher levels of Groα compared to healthy smokers and non-smokers (Traves, 2002).

1.22 IL-8 and chemokine receptors

IL-8, also known as CXCL8, elicits its actions through CXCRs. To date 5 human CXCRs have been identified and 2 of these, CXCR1 and CXCR2, are predominantly restricted to neutrophils (Luster, 1998). Moser *et al*, 1991, demonstrated using competition binding and desensitisation studies that IL-8 was able to bind with high affinity to two binding sites on neutrophils, whereas other structurally similar CXC chemokines, such as Groα, were able to bind to only one of these sites with high affinity (CXCR2). It is now widely accepted that the chemokine system exhibits redundancy with multiple chemokines able to bind to the same receptor and some chemokines being able to bind to more than one receptor (Lee *et al*, 1992, Moser *et al*, 1991, Ludwig *et al*, 1997). In any case, chemokine receptors, whether they belong to the CXC, CC or other subfamilies are structurally related, belonging to the 7 transmembrane G protein coupled receptor superfamily.

1.23 Chemokine signalling pathways

Thelen *et al*, 1988, have shown that the initial activation of neutrophils by a range of chemoattractants including IL-8 were all inhibited by pre-treatment of the cells with *pertussis* toxin, confirming that the IL-8 receptors are dependent on G-protein

signalling. By co-transfecting COS-7 cells with cDNAs encoding CXCR1 or CXCR2 and different G proteins, Wu *et al*, 1993, demonstrated that these receptors couple to $G_{\alpha i2}$, $G_{\alpha i3}$, $G_{\alpha 14}$, and $G_{\alpha 16}$ subunits of the G_q family which activates phospholipase C (PLC). Further co-transfection studies indicated that the β_2-isoform of PLC was activated upon ligand-receptor binding and that it was actually the $\beta\gamma$ subunits of the G-protein, which were specifically required for PLC-β_2 activation (Katz *et al*, 1992). Upon IL-8 binding to its receptors, a conformational change in the receptor causes the dissociation of the now GTP-bound α subunit of the G protein and the $\beta\gamma$ subunit complex. This free complex then activates PLC-β_2, which in turn converts the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into the second messenger molecules diacylglycerol (DAG) and inositol trisphosphate (IP_3). The action of these second messenger molecules is widely accepted with IP_3 acting to mobilise Ca^{2+} by causing calcium channels on the endoplasmic reticulum to open, and DAG acting in conjunction with Ca^{2+} to activate protein kinase C (PKC). The activation of PKC results in the phosphorylation of further downstream signalling molecules and the increase in cytosolic Ca^{2+} concentration from intracellular and extracellular sources (Von Tscharner *et al*, 1986) (refer to figure 1.6). This signalling cascade is believed to account for the activation of various neutrophil functions including shape change/migration, exocytosis of granules and the respiratory burst and is summarised in figure 1.6.

Figure 1.6

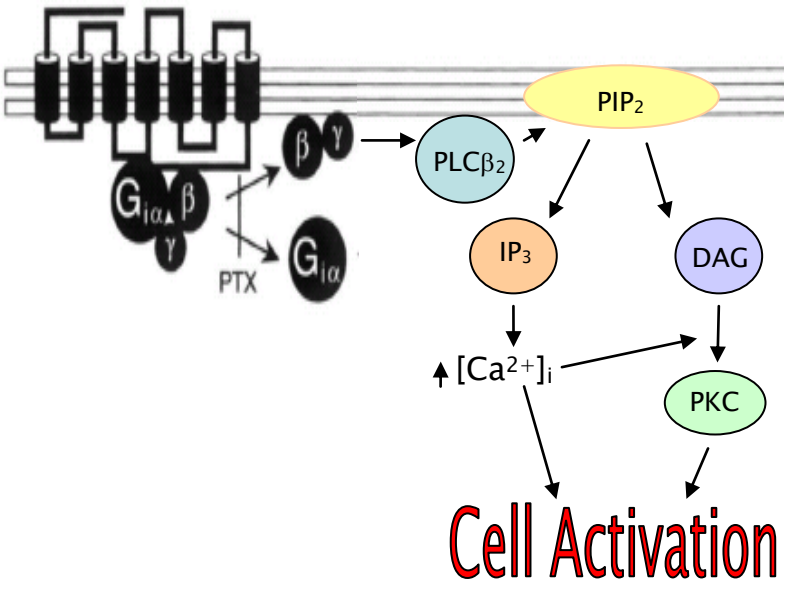


Figure 1.6 Schematic representation of the 'classical' chemoattractant receptor signalling pathway. Upon chemoattractant-receptor binding, the G-protein dissociates and the $\beta\gamma$ subunits activate the enzyme phospholipase C-β_2 (PLCβ_2). This enzyme converts phosphatidylinositol-4,5-bisphosphate (PIP_2) to the second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 causes Ca^{2+} mobilisation and in conjunction with DAG activates the enzyme protein kinase C (PKC). The increase in intracellular Ca^{2+} concentration and activation of PKC lead to cell activation and effector function. Adapted from Bokoch, 1995.

1.24 Neutrophil Shape Change

The ability of neutrophils to change shape is key to their capability to function as motile cells and migrate to sites of inflammation. The kinetics of this response to stimulation with several chemoattractants, including IL-8, has been studied and like the respiratory burst has been found to be virtually instantaneous (Baggiolini and Clark-Lewis, 1992). Scanning electron microscopy has shown that upon stimulation, neutrophils extend large thin cytoplasmic lamellae within seconds, as shown in figure 1.7, changing the shape of the cell from a round-smooth shape to a flattened bipolar, elliptical shape by 300 seconds (Watts *et al*, 1991, Baggiolini and Kernen, 1992, Zigmond *et al*, 1981).

Figure 1.7

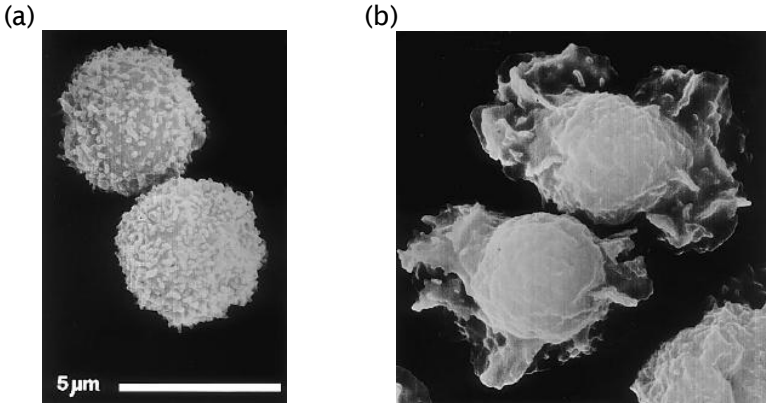


Figure 1.7 Neutrophil shape change. Electron scanning microscopy of neutrophils (a) at rest in buffered saline and (b) 5 seconds after stimulation with a chemoattractant. Images taken from Baggiolini, 1998.

It is widely believed that the shape change response within neutrophils results in restructuring of the cell cytoskeleton, mainly accomplished by the modulation of actin, resulting in the activation of the contractile system (Howard and Meyer, 1984). G-actin is a globular monomeric protein containing an ATP binding site at its centre. Upon binding of ATP to this site, G-actin monomers are able to bind together to form a trimer, which is then able to act as a nucleation site for the addition of further monomers and the growth of the protofilament (Widnell and Pfenninger, 1990). The created polymer now forms a filament consisting of F-actin. These filaments form microfilaments, a major constituent of the cell cytoskeleton. Changes in cell motility and shape are associated with actin polymerisation which is seen in an experimental setting as an increase in F-actin and decrease in G-actin. Within neutrophils this modulation of actin status has been investigated in regard to stimulation with the chemoattractant fMLP by Howard and Oresajo, 1985. At resting state, neutrophils are spherical with F-actin diffuse throughout the cell, however after 10 minutes the cells become polarised and the F-actin is concentrated in lamellipodia and other focal submembranous sites (Howard and Oresajo, 1985). During this time course F-actin

reaches a maximum between 45-60 seconds and gradually decreases during the next 10 minutes. Even at this later time point however, the relative F-actin content is elevated above control levels and persists for as long as 20 minutes after stimulation. The importance of the contribution of actin polymerisation to neutrophil shape change was confirmed by the utilisation of the mycotoxin cytochalasin D, which prevents monomer addition at the positive fast growing end of F-actin and inhibited shape change in a dose-dependent manner.

The robust shape change response in neutrophils after stimulation with a chemoattractant can be used as a measure of the activation of the cell. It has been proven that changes in cell shape are reflected in changes in forward angle light scatter, as determined by flow cytometry, and this correlates with microscopically evaluated neutrophil shape change and measurements of chemotaxis using Boyden chambers (Cole *et al*, 1995). This presents a rapid, objective approach of assessing neutrophil activation and has been utilised by other groups in the literature (Nicholson *et al*, 2007).

1.25 Aims

In vivo studies of allergic lung inflammation in the literature mainly involve murine models. These give us an insight into the mechanisms of allergic disease but are inefficient in explaining these processes in humans, where genetic and environmental factors play key roles. Asthma classically was thought to involve mainly the large central airways. It is now believed that inflammation can extend throughout the airways and involve more distal tissue. Allergic lung inflammation of peripheral human lung tissue has not been studied extensively and we hypothesise that lung parenchyma may have a role in allergic inflammatory processes of the lung. We aim to compare the release of inflammatory cytokines from matched proximal airways and distal lung tissue to assess their contributions to inflammation.

The transfer of sensitivity to an allergen from an atopic to a non-atopic individual via serum was discovered by Prausnitz and Küstner and termed the Prausnitz-Küstner reaction (Prausnitz and Küstner, 1921). This methodology has been used in human bronchial tissue *ex vivo* whereby allergen-specific IgE in atopic serum binds to effector cells in a process called passive sensitisation. In these studies the contractile response of smooth muscle within the bronchial tissue in response to allergen and other spasmogens were investigated (Tunon de Lara *et al*, 1995, Watson *et al*, 1998). The aim of this study is to develop a human *ex vivo* model to mimic the early phase allergic reaction by passively sensitising human distal lung tissue and to investigate IgE-mediated cytokine release in response to the common environmental allergen, Der p1.

Supernatants generated using the lung explant model (Bochner *et al*, 1987) contain a complex mixture of inflammatory mediators that can be quantified. However, little is known about how functional these mediators may be within this mixture. As a result, we also aim to develop an assay using neutrophil shape change to investigate the functionality of the neutrophil chemokine IL-8.

Chapter 2
Materials and Methods

2.1 Materials and Methods

2.1.1 Buffers and solutions

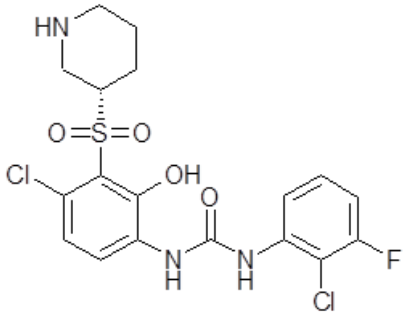
RPMI-1640 medium with 25mM HEPES was supplemented with 50 units/ml penicillin, 0.05mg/ml streptomycin and 0.1mg/ml gentamycin; **phosphate buffered saline (PBS)** contained 0.1M NaCl, 2.7mM KCl, 1.8mM KH_2PO_4; **Tris buffered saline** contained 4.96mM Tris and 137.9mM NaCl; **Krebs buffer** contained 118mM NaCl, 25mM $NaHCO_3$, 4.7mM KCl, 1.22mM $MgSO_4.H_2O$, KH_2PO_4, 11.1mM D-glucose, 2.6mM $CaCl_2$; **PBS Tween** contained 0.1% Tween 20 in PBS; **coating buffer for TNFα, IL-6, IL-8, and IL-10 ELISAs** contained 0.05mM Na_2CO_3 and 0.05mM $NaHCO_3$, pH 9.4; **reagent diluent for TNFα, IL-6, IL-8 and IL-10 ELISAs** contained 0.14mM NaCl, 0.01mM Na_2HPO_4, 1.47mM KH_2PO_4, 2.68mM KCl and 5g/l bovine serum albumin (BSA); **coating buffer for IL-1β, IL-5 and IL-13 ELISAs** was PBS; **reagent diluent for IL-5 and IL-13 ELISAs** contained 1% BSA in PBS; **CHO-H_1 culturing media** was MEM Alpha medium supplemented with 10% dialysed fetal bovine serum and 2mM L-glutamine; **FLIPR buffer** contained 145mM NaCl, 5mM KCl, 10mM HEPES, 10mM D-glucose, 8mM $CaCl_2$, 2.5mM probenecid, 0.5mM Brilliant Black; **immunohistochemistry blocking medium** was Dulbecco's modified Eagles medium containing 20% fetal calf serum (Fisher Scientific, Loughborough, UK) and 1% BSA; **Mayer's haematoxylin** contained 0.1% haematoxylin, 5% potassium alum, 0.1% citric acid, 5% chloral hydrate (all from Fisher Scientific, Loughborough, UK) and 0.02% sodium iodate; **anti-coagulant solution** contained 3.8% tri-sodium citrate; **shape change assay buffer** contained 0.02% D-glucose, 10mM HEPES and 1% BSA in PBS; **fixation buffer** contained 2.5% CellFIX 10X concentrate (BD Biosciences), 22.5% sterile water in PBS; **lysis buffer** contained 155mM NH_4Cl and 10mM $KHCO_3$.

2.1.2 Other reagents

Goat anti-human IgE (ε-chain specific) antibody; IgG from goat serum; lipopolysaccharide (LPS); Brefeldin A; phenyl methyl sulphonyl fluoride; iodoacetamide; methyl benzoate from Fisher Scientific (Loughborough, UK); GMA solution A (2-hydroxyethyl methacrylate), GMA solution B (N,N-dimethylaniline Poly (ethylene oxide)) and benzoyl peroxide plasticized catalyst (benzoyl peroxide dicyclohexyl phthalate) all from Polysciences Inc (Northampton, UK); monoclonal mouse anti-human CD1a, CD3, CD4, CD8, CD20, CD68, mast cell tryptase (AA1) and neutrophil elastase (NOE) antibodies all from Dako (Ely, UK); monoclonal mouse anti-human eosinophil cationic protein (EG2) from Diagnostic Development; polyclonal F(ab')2 biotinylated rabbit anti-mouse secondary antibody from Dako (Ely, UK); streptavidin-biotin complex reagents from Vector Laboratories (CA, USA); AEC substrate kit (BioGenex, CA, USA); permanent aqueous mounting medium from AbD Serotech (Kidlington, UK); histamine; probenecid; brilliant black; house dust mite D. *pteronyssinus* allergen (Der p1) and

skin prick test kit from ALK-Abello; mixed nut extract was a gift from Dr Andrew Walls' group, University of Southampton; ELISA cytoset kits for TNFα, IL-6, IL-8 and IL-10 from Biosource (Europe, SA); ELISA duoset kits for IL-5, IL-13 and IL-1β from R&D Systems (Abingdon, UK); lactate dehydrogenase (LDH) assay kit and LDH standard from Roche Diagnostics (Germany); $CaCl_2$; recombinant human IL-8 and Groα from Peprotech (London, UK); neutralising IL-8 antibody and isotype control antibody from R&D Systems (Abingdon, UK); Versene (Gibco); Fluo-4 Ca^{2+} indicator dye from Invitrogen Molecular Probes (Paisley, UK); anti-CXCR1 antibody and isotype control antibodies; CXCR2 small molecule receptor antagonist compound A, BLT receptor antagonist SB209247 and CHO-H_1 cells were donated as a gift from Dr David Hall, GlaxoSmithKline (Stevenage, UK).

Compound A structure:



All reagents purchased from Sigma (Poole, UK) unless otherwise stated.

2.1.3 Antibodies used in immunohistochemistry

Epitope	Clone	Source
CD68	PG-M1	Dako
Mast cell tryptase	AA1	Abcam
Neutrophil elastase	NP57	Dako
CD3	UCHT1	Dako
CD4	4B12	Dako
CD8	DK25	Dako
Eosinophil cationic protein	EG2	Diagnostics Development
CD1a	010	Dako
CD20	L26	Dako

2.2.1 Human lung explant model

Tissue was obtained with informed consent and ethical approval (SREC 08/H0502/32) from patients undergoing lung resection surgery from Southampton General Hospital. In the case of patients with carcinoma undergoing lobectomy, peripheral lung tissue (parenchyma or distal airways) received was from the normal margin away from the tumour and bronchial (proximal airway) tissue was obtained from just below the resection margin. Patient data such as age, gender, prescribed medication, smoking history, lung function and history of asthma or allergy were obtained at the same time as consent. This data is summarised in the appropriate sections. Tissue was placed into normal saline immediately following surgical removal.

Parenchyma and cartilage was dissected off airway tissue. Parenchyma and airway tissue was transferred to PBS and cut into fragments whilst being periodically washed with PBS to remove any blood. Cutting was halted once desired explant size of approximately 2mm^3 was achieved. Tissue was then washed once more and placed in culture flasks with enough supplemented RPMI-1640 to completely cover the fragments. Tissue was incubated overnight at 37°C with 5% CO_2.

The following day tissue was washed for a final time before approximately 30mg of tissue was plated out in tissue culture plates in RPMI-1640. The required stimulus was then added to the tissue, which was then incubated at 37ºC, 5% CO_2 for a set length of time; 30 minutes, 2 hours, 6 hours, 24 hours and 48 hours. At each time point, tissue was removed, weighed and then stored at −70°C, along with aliquoted supernatant for later analysis (see figure 2.1).

Figure 2.1

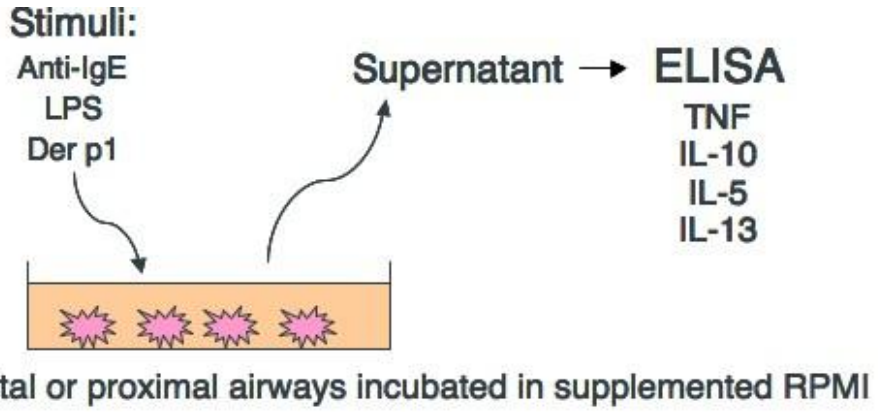


Figure 2.1 Lung Explant Model. Tissue is incubated for 30 minutes, 2, 6, 24 and 48hrs in RPMI-1640 with required concentration of stimulus. At set time point tissue is removed along with supernatant and both are stored at −70ºC until needed. Cytokines within the supernatant are quantified by ELISA.

2.2.2 Proximal airway Vs distal lung tissue - Patient characteristics

Tissue was obtained from 79 donors and used in the lung explant model. Patient characteristics are summarised below in table 2.1.

Table 2.1

Age	67.1 ± 1.1
Gender	39 Male 40 Female
Lung function (FEV1/FVC)	0.67 ± 0.01
COPD status	34 No COPD 38 COPD 7 unknown
Smoking status	27 Current 42 Ex 8 Non 2 unknown
Years Ex	9.6 ± 1.6
Pack years	40.4 ± 3.3
History of asthma	67 No asthma 12 asthma
History of allergy	56 No allergy 23 allergy
Steroid treatment	55 No steroid 21 Steroid

Table 2.1 Proximal airway vs distal lung tissue patient characteristics. Patient characteristics of matched proximal airway and distal lung tissue utilised in chapter 3. Age and FEV_1/FVC are shown as mean ± SEM. Asthma diagnosis was also determined by the patient's clinician.

History of asthma was obtained from patient notes at time of consent. There was no data on asthma severity. History of allergy was also recorded at this time from patient notes however this data is extremely tenuous; only 3 patients had convincing allergic history with the majority of allergy being reported to penicillin, Elastoplast and various drugs.

Patient characteristics for dose response and kinetics data and where patients have been subdivided into groups are shown in the relevant results sections.

2.2.3 Anti-IgE and LPS dose responses

Matched proximal and distal airways were prepared as in section 2.2 and plated out in a 24 well tissue culture plate in supplemented RPMI. 1 in 10 serial dilutions of anti-IgE (0.1-1000μg/ml) and LPS (0.1-1000ng/ml) were then added to relevant wells to establish dose responses to these stimuli in both tissue types. 1000μg/ml goat IgG was used as an isotype control for the anti-IgE antibody and a control well was left unstimulated to establish baseline release of inflammatory mediators from the tissue.

Plates were then incubated for 24 hours at 37°C, 5% CO_2 before tissue was harvested and supernatant removed as described in section 2.2.

2.2.4 Anti-IgE and LPS kinetics

Tissue from both lung compartments was treated and plated out as in section 2.2. Matched tissue was stimulated with 100μg/ml and 1μg/ml anti-IgE, 100ng/ml LPS and an unstimulated control, and incubated at 37°C, 5% CO_2. Tissue was then harvested and supernatant removed at 2, 6, 24 and 48 hour time points and stored as summarised in figure 2.1.

2.3 Human Serum

Plasma from venous blood from healthy individuals around the department who underwent venepuncture was obtained from Ficoll-Paque separation or centrifugation. 20mM $CaCl_2$ was added where appropriate to allow coagulation. Serum was collected from these samples under sterile conditions and frozen. Donors were assessed for atopy to the house dust mite *Dermatophagoides pteronyssinus* and grass allergen, which was determined by skin prick test (SPT). A positive reaction was confirmed with a weal diameter of at least 2mm. Serum was thawed and pooled to form an atopic and non-atopic serum pool. Total IgE and allergen-specific IgE were obtained for each serum pool courtesy of the department of Immunology at Southampton General Hospital and summarised below in table 2.2. Serum pools were aliquoted and stored at -20°C.

Table 2.2

Serum Pool I

Serum Pool	Total IgE (iu/ml)	Der p1 specific (kUA/L)	RAST Class
Atopic	137	16.1	3
Non-atopic	33	2.10	2

Serum Pool II

Serum Pool	Total IgE (iu/ml)	Der p1 specific (kUA/L)	RAST Class
Atopic	389	54	4
Non-atopic	14	<0.35	0

Interpretation of allergic disease

RAST Class	kUA/L	Interpretation
0	<0.35	Undetectable
1	0.4 - 0.7	low
2	0.7 - 3.5	moderate
3	3.5 - 17.5	high
4	17.5 - >100	very high

Table 2.2 Serum IgE titres. Serum from non-atopic and atopic individuals were pooled together and total and Der p1-specific IgE titres were obtained for serum pool I and II.

2.4.1 Passive Sensitisation

Following the protocol in section 2.2, on day 1 after cutting the tissue into explants and washing, tissue was divided into 3 and placed into culture flasks with RPMI-1640 or culture medium containing atopic or non-atopic serum. Sensitised tissue was incubated either at 4ºC or room temperature, 5% CO_2 and unsensitised control tissue at room temperature or 37ºC, 5% CO_2. After sensitising overnight, tissue was treated as in section 2.2. After plating out the tissue, relevant wells had 100μg/ml anti-IgE added as a positive control for mast cell activation, 100ng/ml LPS as a positive control for an inflammatory response and 1:10 serial dilutions to the allergen Der p1 (1-1000SQ-U/ml). After optimisation, tissue was sensitised using 10% serum at room temperature, 5% CO_2.

2.4.2 Bronchial smooth muscle contraction

This procedure was performed at GlaxoSmithKline Stevenage research site. Bronchi were dissected from donated lung and cut into spiral strips of approximately 2cm in length. Tissue was passively sensitised as in section 2.4.1 in atopic and non-atopic serum (serum pool II) overnight. The following day tissue was washed in Krebs buffer and strung up in tissue organ baths in oxygenated Krebs buffer and tension was adjusted to approximately 1g. Tissue viability was assessed upon methacholine

stimulation and contraction of bronchial smooth muscle. Maximum change in tension was recorded at plateauing of contraction. Tissue was then thoroughly washed with Krebs buffer while in the tissue baths until baseline tension was restored and allowed to rest. Bronchial strips were then stimulated with increasing concentrations of Der p1 allergen (1-100SQ-U/ml) and change in tension recorded. Change in tension was then also recorded after stimulation with anti-IgE and methacholine.

2.5 Analysis by ELISA

2.5.1 TNFα, IL-10, IL-6 and IL-8

ELISA kits for these cytokines were purchased from Biosource and were carried out as per the manufacturer's instructions. 96 well ELISA plates were coated with the appropriate capture antibody in coating buffer at a concentration of 2µg/ml for TNFα and 1µg/ml for IL-6, -8 and -10 and left over night at 4ºC. Plates were washed the following morning using PBS Tween and unspecific binding was prevented by adding reagent diluent and leaving for 2 hours at room temperature with continual agitation. Plates were washed again and standards and samples were added at the desired dilutions. Detection antibody in reagent diluent was immediately added at concentrations of 0.32µg/ml for TNFα, 0.16µg/ml for IL-6 and IL-10 and 0.04µg/ml for IL-8. After incubating for 2 hours with continual agitation at room temperature, plates were washed and the streptavidin-HRP conjugate made up in reagent diluent was added. This was then left for 30 minutes as before. Plates were then washed for a final time and TMB substrate was added. 1M HCl was then added when required to halt the colorimetric reaction. Optical density was determined at 450nm and after analysis cytokine concentration was calculated and corrected for tissue weight. The limits of detection for TNFα, IL-6 and IL-8 ELISAs were 0.26pg/mg and IL-10 ELISA was 0.13pg/mg.

2.5.2 IL-5, IL-13 and IL-1β

ELISA kits for these cytokines were purchased from R&D Systems and were carried out as per the manufacturer's instructions. Capture antibody in PBS was applied to 96 well plates at a concentration of 2µg/ml for both IL-5 and IL-13 and 4µg/ml for IL-1β. Plates were left over night at room temperature with continual agitation. After washing with PBS Tween, plates were blocked using reagent diluent and left for 2 hours at room temperature with continual agitation. Again, plates were washed and standards and samples were applied at desired dilutions. Plates were then left at room temperature for 2 hours with continual agitation before being washed again. Detection antibody made up in reagent diluent to a concentration of 125ng/ml, 150ng/ml, 300ng/ml, and 400ng/ml for IL-5, IL-13 and IL-1β respectively was added and plates were left for a further 2 hours with continual agitation before being washed again. The streptavidin-

HRP conjugate was then applied and the plates left for 20 minutes with continual agitation before being washed for a final time. IL-5, IL-13 and IL-1β concentrations were then visualised as with the other cytokines upon the application of TMB and 1M HCl. The limits of detection for IL-1β, IL-5 and IL-13 ELISAs were 0.07, 0.39 and 0.78pg/mg respectively.

2.6 Lactate dehydrogenase (LDH) assay

The LDH assay was used as a measure of cell viability. The assay was carried out as per the manufacturer's instructions (Roche Diagnostics, Germany). Samples generated using the lung explant model described in section 2.2.1 were run on a 96 well plate at 1:10 dilutions in PBS Tween with an LDH standard curve. Optical density was measured at 490nm and cell death was expressed as fold over control.

2.7 Histamine bioassay

Chinese hamster ovary (CHO) cells stably expressing the histamine (H_1) receptor were cultured in cell culture media at 37°C, 5% CO_2. Cells were removed from flasks using Versene and seeded onto 96 well clear bottomed, black sided plates at required cell density and incubated overnight. The following day media was aspirated off and replaced with fluorescent laser imaging plate reader (FLIPR) buffer containing 4μM Fluo-4 and incubated for at least 45 minutes to allow uptake of the indicator dye into the cells before being replaced with fresh FLIPR buffer. Agonist plates were created on separate 96 well plates with dilutions of generated lung supernatant (see section 2.2) and histamine standard curves containing 0.5mM Brilliant Black.

Basal fluorescence of cells was measured using a FLIPR machine every 2 seconds for 10 seconds, then every second for 70 seconds upon addition of agonist using an excitation laser of 488nM and a 510-570nM bandpass filter to measure emission. Fold increase in fluorescence was calculated by dividing the peak fluorescence by the basal fluorescence.

2.8.1 Glycol methacrylate (GMA) processing of tissue

Tissue was placed into ice-cold acetone containing 2mM phenyl methyl sulphonyl fluoride and 20mM iodoacetamide and left to fix overnight at −20ºC. The following day tissue was placed in fresh acetone and left for 15 minutes at room temperature followed by methyl benzoate for a further 15 minutes. Tissue was then infiltrated with processing solution (5% methyl benzoate in glycol methacrylate, GMA solution A) and incubated for 2 hours at 4ºC. This process was repeated twice more before embedding the samples in embedding solution (0.7% benzoyl peroxide and 2.5% GMA solution B in GMA solution A). The specimens were then left to polymerise at 4ºC for 48 hours before being moved for storage at −20ºC.

2.8.2 GMA immunohistochemistry

2µm tissue sections were cut and allowed to dry on glass microscope slides. A solution of 0.1% NaN_3 and 0.3% H_2O_2 was then added for 30 minutes to inhibit endogenous peroxidase activity. Sections were washed three times every 5 minutes with TBS after which blocking medium was applied for 30 minutes to prevent unspecific binding of antibodies. Slides were then drained and primary antibodies at appropriate dilutions were added. Coverslips were applied and slides were incubated overnight at room temperature. The following day slides were washed 3 times for 5 minutes, drained and a biotinylated secondary antibody was applied at a concentration of 0.72µg/ml and incubated for 2 hours at room temperature. The washing procedure was then repeated, slides drained and the streptavidin biotin-peroxidase complex was added to the sections and left for 2 hours. Slides were washed again, drained and AEC substrate added for 20 minutes after which slides were rinsed with TBS and washed in running tap water for 5 minutes. Sections were counterstained with Mayer's haematoxylin and blue in running tap water. Slides were then drained, Crystal Mount applied and baked at 80°C until dry. After cooling, slides were mounted in DPX and coverslipped.

Tissue section areas were calculated using the image analysis program KS400.

2.8.3 Cell count – Patient characteristics

Table 2.3 below summarises the patient characteristics of tissue used in GMA immunohistochemistry.

Table 2.3

	Lobectomy n=41
Age	65.6 ± 1.8
Gender	19 Male 22 Female
Lung function (FEV1/FVC)	0.66 ± 0.02
COPD status	18 No COPD 22 COPD 1 unknown
Smoking status	13 Current 23 Ex 5 Non
Years Ex	9.6 ± 1.9
Pack years	40.5 ± 4.2
History of asthma	32 No asthma 9 asthma
History of allergy	29 No allergy 13 allergy
Steroid treatment	26 No steroid 15 Steroid

Table 2.3 Patient characteristics for tissue used in GMA immunohistochemistry. Age and FEV_1/FVC are shown as mean ± SEM. Asthma diagnosis was also determined by the patient's clinician.

Again only 5 patients from this group had a convincing history of allergy.

2.8.4 Inflammatory cell staining

Following the protocols in sections 2.8 matched proximal and distal lung tissue sections were stained for a panel of inflammatory cells using antibodies summarised below in table 2.4.

Table 2.4

Cell stained	Epitope stained	Conc used (μg/ml)
Macrophage	CD68	0.8
Mast Cell	Mast cell tryptase	0.075
Neutrophil	Neutrophil elastase	0.07
T cell	CD3	1
T helper cell	CD4	2
Cytotoxic T cell	CD8	0.5
Eosinophil	Eosinophil cationic protein	0.1
Dendritic cell	CD1a	4.95
B cell	CD20	40.8

Table 2.4 Antibodies used for inflammatory cell staining. List of monoclonal mouse anti-human primary antibodies used in GMA immunohistochemistry to stain a panel of inflammatory cells.

Staining protocol was completed as in section 2.8.2. Positively stained nucleated cells were counted and counts were corrected for tissue section area and expressed as cell count/mm^2.

2.9.1 Whole blood neutrophil shape change assay

Whole blood neutrophil shape change assay was adapted from a method by Nicholson et al, 2007. FACS tubes were blocked with shape change assay buffer containing BSA for 30 minutes before emptying tubes. Blood from healthy volunteers (Ethics approval: SOMSEC0015.07) was taken by venepuncture using a butterfly needle and syringe, and added to a Falcon tube containing 3.8% sodium citrate (9 parts blood to 1 part citrate). Blood was used within 30 minutes of collection. 100μl of blood was transferred into pre-BSA coated FACS tubes containing 50μl of either stimulus in assay buffer or assay buffer and incubated at 37ºC for 5 minutes. Volume of stimuli and assay buffer was adjusted to obtain desired agonist concentrations. Ice-cold fixation buffer was added and tubes were left on ice for a further 5 minutes. Erythrocytes were lysed upon the addition of 2ml lysis buffer and samples left on ice. Samples were read using the FACScalibur flow cytometer from BD Biosciences and a total of 10000 events were counted. Due to the higher autofluorescence of eosinophils, neutrophils were distinguishable and gated for analysis. Neutrophil shape change was determined by calculating mean neutrophil forward scatter using the CellQuest Pro program version 5.2.1 from BD Biosciences.

2.9.2 Agonist dose response curves

Chemokines were diluted with assay buffer and added to blocked FACS tubes at a volume to give a final agonist concentration of 0.039-10nM IL-8 and 0.002-1000nM Groα upon addition of blood. Similarly, dose response curves to distal airways supernatant generated, as in section 2.2, were achieved by diluting supernatant with assay buffer and adding to FACS tubes to give a final dilution of 1:1000-1:3 upon the addition of blood. Protocol outlined in section 2.9.1 was then followed.

2.9.3 IL-8 neutralisation

Recombinant IL-8, Groα and supernatant were incubated with neutralising antibody in eppendorfs for 30 minutes before being added to blocked FACS tubes. Initially dose responses to the neutralising antibody were conducted using a concentration of 0.31-10μg/ml when incubated with agonist, ending with a final concentration of 0.031-1μg/ml upon the addition of blood. In subsequent experiments the final concentration of 1μg/ml neutralising antibody was used. The protocol in section 2.9.1 was then followed.

2.9.4 IL-8 and Groα co-stimulation

IL-8 and Groα were added together in blocked FACS tubes to give a final concentration of 0.313nM IL-8 and 0.625nM Groα upon the addition of blood. Protocol outlined in section 2.9.1 was then followed.

2.9.5 Neutrophil receptor antagonism

Blood was incubated with anti-CXCR1 antibody, CXCR2 small molecule antagonist or leukotriene receptor (BLTR) antagonist in pre-BSA coated FACS tubes for 15 minutes before addition of agonist and incubation at 37°C for 5 minutes. Fixation and lysis buffer was then added and samples analysed as described in section 2.9.1. Initially, dose responses of anti-CXCR1 antibody (0.01-100μM) and CXCR2 antagonist (0.1-10000nM) were conducted. In subsequent experiments 1μg/ml anti-CXCR1 antibody, 1000nM CXCR2 antagonist and 20nM BLTR antagonist were used.

2.10 Statistical analysis

Statistical analysis was performed using InStat3 and Statview 5.0 using non-parametric statistical tests. Friedman tests with Dunn's multiple comparison post test and Wilcoxon matched pairs test were used as appropriate for paired data. Spearman's Rank Correlation tests were used to assess the relationship between two variables. Mann-Whitney tests were performed to compare two groups of unpaired samples and Kruskal-Wallis test with Dunn's multiple comparison test where more than two groups were compared.

Chapter 3

Proximal airway vs distal lung tissue

3.1 Introduction

Asthma is a chronic inflammatory disorder of the airways that causes recurrent episodes of wheezing, breathlessness, chest tightness and coughing caused by inflammatory processes that result in generalised but reversible airways obstruction, bronchial hyper-responsiveness and inflammation (WHO, accessed on 14/07/09). Allergens or other irritants can trigger exacerbations of allergic asthma, initiating inflammatory processes that cause smooth muscle contraction and mucus hyper secretion in an already inflamed sensitive tissue. It is a complex disease involving a wide variety of effector cells; however it is generally thought that mast cells, eosinophils and T lymphocytes play a key role in inflammation.

Traditionally, allergic diseases of the airway have been separated into upper (e.g. allergic rhinitis) or lower (e.g. asthma) airway disorders that have been diagnosed and treated by clinicians as such (Grossman, 1997). A concept that has arisen moving away from classifying airway inflammation as upper or lower is the 'one airway, one disease' hypothesis (Leynaert *et al*, 2000). This suggests that upper and lower airway inflammation is linked, acting as one common airway, and is able to instigate inflammatory processes throughout. Epidemiological studies have shown that asthma and rhinitis and other inflammatory conditions of nasal origin often exist as co-morbidities, indicating a possible link between inflammation of the upper and lower airways (Bachert *et al*, 2004, Grossman, 1997, Serano *et al*, 2005). It is still unclear as to the interrelationship between upper and lower airway disorders, with different theories postulated to try and explain the association. A study by Braunstahl *et al*, 2000, demonstrated using segmental bronchial provocation with allergen in patients with nonasthmatic allergic rhinitis, increased nasal obstruction, symptom score, peripheral blood eosinophilia and increased eosinophil infiltration of the nasal mucosa; along with inflammation at the site of allergen administration, characterised by increased eosinophil number and decrease in FEV_1. In a later study they did the reverse using a nasal challenge of nonasthmatics with allergic rhinitis (Braunstahl *et al*, 2001). Here they showed an increase in expression of endothelial adhesion molecules and eosinophilic allergic inflammation of the nasal and bronchial mucosa. They concluded that a possible common interrelating mechanism for inflammation in both tissues results from systemic dissemination of mediators, giving rise to generalised airway inflammation.

The vast majority of the literature has focussed on disease processes that occur at what are thought to be the main sites of disease pathology, for example the large central

airways in asthma. More current thinking is that inflammatory processes may extend throughout the respiratory tree placing more emphasis on the small airways and distal lung parenchyma and their contribution to disease and lung function. There are limited studies assessing inflammatory processes occurring within these two compartments, mainly due to accessibility of distal lung tissue. Less invasive techniques such as induced sputum and bronchoalveolar lavage (BAL) have been used to sample proximal airway and more distal lung tissue respectively. This sampling is of the exudate within the airway lumen and may not reflect events and cellular infiltrate within the tissue however. Endobronchial and more recently transbronchial bronchoscopies address this problem by allowing biopsies of the proximal and distal lung compartments respectively (Kraft *et al*, 1996). These studies are rare though and there is limited data on the release of inflammatory mediators; as these are invasive procedures only limited amounts of tissue can be taken and is mainly used for immunohistochemistry. Surgical resections have been used in previous studies to investigate inflammatory processes from distal lung tissue and have shown that this compartment is able to release a complex mixture of inflammatory cytokines (Hackett *et al*, 2008). Inflammatory processes occurring in proximal airway and distal lung tissue from the same patient however has not been investigated.

To date, research has mainly focussed on comparing inflammation of the upper and lower airways. Distal lung tissue however encompasses a vast array of inflammatory cells that would provide a pool of inflammatory mediators that could also have systemic effects on other tissues of the respiratory system. This leaves a gap in the literature to investigate the inflammatory processes of the proximal airways (>2mm diameter) compared to the small airways (<2mm diameter) and more distal lung tissue (parenchyma).

From patients undergoing lobectomy surgical procedure for lung carcinoma, it was possible to obtain proximal airway and distal lung tissue from normal sections of the lung, away from the tumour. A substantial number of these patients also had some degree of lung obstruction as classified by lung function tests and therefore had underlying COPD. COPD is characterised by an abnormal inflammatory infiltrate (Cosio *et al*, 2002, O'Donnell *et al*, 2005) that results in structural changes and tissue destruction caused by chronic inflammation that results in airway collapse, predominantly in the small airways.

The aim of this chapter is to examine the inflammatory process in proximal airway and distal lung tissue allowing us to make a direct comparison and investigate whether there are differences in the inflammatory profiles of these tissues. We hypothesise that distal

lung tissue plays an important role in inflammation providing a pool of inflammatory mediators and involving an array of cell populations.

We also aim to compare the two compartments in patients with COPD and patients with normal lung function to assess whether there are any differences caused during chronic inflammation.

3.2 Methods

Tissue from 79 donors was used in the lung explant model for the following experiments (see materials and methods chapter, table 2.1). A number of these patients were prescribed systemic or inhaled corticosteroid at the time of surgery and have been removed from the results shown in this and subsequent chapters, unless otherwise stated, as steroid treatment may have an effect on the inflammatory response and cellular infiltrate.

3.2.1 Patient characteristics

Patient characteristics such as age, gender, lung function, smoking history and history of asthma are summarised below and were obtained at time of consent before surgery. As mentioned in section 2.2.2 table 2.1, allergic history was very weak and therefore has been removed from subsequent patient characteristics. Patient characteristics for dose response experiments are summarised in table 3.1. All patients used in immunohistochemical analysis are summarised in table 3.2 and split into those with normal lung function and those with COPD in table 3.3.

Table 3.1

	Anti-IgE n=30	LPS n=36
Age	65.5 ± 1.7	66.5 ± 1.6
Gender	13 Male 17 Female	18 Male 18 Female
Lung function (FEV1/FVC)	0.68 ± 0.02	0.68 ± 0.02
COPD status	13 No COPD 14 COPD 3 unknown	16 No COPD 16 COPD 4 unknown
Smoking status	10 Current 16 Ex 4 Non	12 Current 20 Ex 4 Non
Years Ex	9.7 ± 2.5	10.5 ± 2.3
Pack years	44.0 ± 4.8	45.4 ± 4.6
History of asthma	28 No asthma 2 asthma	34 No asthma 2 asthma

Table 3.1 Characteristics of patients without corticosteroid treatment for anti-IgE and LPS dose responses. Age and FEV_1/FVC are shown as mean ± SEM. Asthma diagnosis was also determined by the patient's clinician.

Table 3.2

Age	65.9 ± 1.9
Gender	11 Male 15 Female
Lung function (FEV1/FVC)	0.69 ± 0.02
COPD Status	14 No COPD 11 COPD 1 unknown
Smoking status	8 Current 15 Ex 3 Non
Years Ex	11.5 ± 2.7
Pack Years	41.5 ± 5.1
History of asthma	24 No asthma 2 asthma

Table 3.2 Characteristics of patients without corticosteroid treatment used in immunohistochemistry. Age and FEV_1/FVC are shown as mean ± SEM. Asthma diagnosis was also determined by the patient's clinician.

Table 3.3

	No COPD n=14	COPD n=11
Age	66.1 ± 3.1	65.6 ± 2.3
Gender	5 Male 9 Female	6 Male 5 Female
Lung function (FEV1/FVC)	0.76 ± 0.01	0.60 ± 0.02 *
Smoking status	3 Current 8 Ex 3 Non	5 Current 6 Ex 0 Non
Years Ex	14.9 ± 4.5	7.8 ± 3.3
Pack Years	25.1 ± 3.8	54.1 ± 7.1 *
History of asthma	13 No asthma 1 asthma	10 No asthma 1 asthma

Table 3.3 Characteristics of patients without steroid treatment split by COPD status used in immunohistochemistry. Age and FEV_1/FVC are shown as mean ± SEM. Asthma diagnosis was also determined by the patient's clinician. * indicates $p<0.05$ comparing the two groups using the non-parametric Mann-Whitney test.

3.2.1 Anti-IgE and LPS Stimulation

Initial dose response experiments were conducted using anti-IgE and LPS. Anti-IgE is an antibody that binds to and crosslinks IgE bound to FcεRI on mast cells. This causes mast cell degranulation and activation and thus mimics an allergic response, whereas LPS is a component of the cell wall of gram-negative bacteria and used to mimic an inflammatory response to bacterial infection.

Proximal airway and distal lung tissue explants were stimulated for 24 hours with 0.1-1000μg/ml anti-IgE and 0.1-1000ng/ml LPS for dose response experiments (see section 2.2.3). For kinetics experiments matched tissue was stimulated with 1 and 100μg/ml anti-IgE and 100ng/ml LPS for 2, 6, 24 and 48 hours. Tissue was subsequently harvested and weighed and supernatant aliquoted before being stored at -80ºC. When required, supernatant was thawed and analysed for an array of cytokines using ELISA. Cytokines were quantified and corrected for tissue weight.

3.2.2 GMA immunohistochemistry

Matched distal lung tissue and proximal airway explants were fixed after dissection and processed for GMA immunohistochemistry (see section 2.8). Sections of tissue were cut and stained for a panel of inflammatory cells. Cell populations were counted and counts were corrected for tissue section area.

3.3 Results

3.3.1 Dose response cytokine release

Dose response concentrations used in these experiments ranged from 0.1-1000μg/ml anti-IgE and 0.1-1000ng/ml LPS. An isotype control (IgG from goat serum) at 1000μg/ml was also used as a control for the anti-IgE antibody and buffer controls were also generated. Patient characteristics are summarised in table 3.1.

Supernatants were analysed for a panel of inflammatory and anti-inflammatory cytokines using ELISA (see section 2.4). These included the classical pro-inflammatory and anti-inflammatory cytokines TNFα and IL-10 and the Th_2 cytokines IL-5 and IL-13. Other inflammatory cytokines measured were IL-1β, IL-6 and IL-8.

Figure 3.1

a) Stimulation with anti-IgE b) Stimulation with LPS

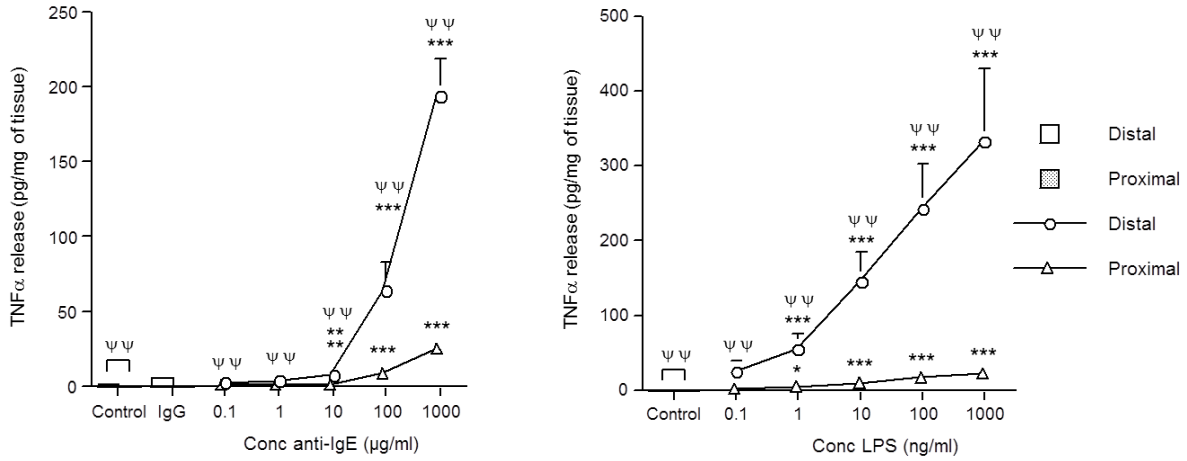


Figure 3.1 TNFα release from matched distal lung and proximal airway tissue explants in response to anti-IgE and LPS. Matched distal lung (open bars and open circles) and proximal airway tissue (shaded bars and open triangles) explants were stimulated with a) anti-IgE (0.1-1000μg/ml) (n=30), IgG isotype control (1000μg/ml) (n=7), b) LPS (0.1-1000ng/ml) (n=36) or buffer control for 24 hours and supernatant analysed for TNFα. All values are expressed as mean±SEM and expressed as pg/mg tissue. * indicates $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared to control values. ψψ indicates $p<0.01$ comparing same dose concentrations between the two tissue types.

Both proximal airways (triangles) and distal lung tissue (circles) responded in a concentration dependent manner to anti-IgE and LPS stimulation. Figure 3.1 shows both compartments of the lung released significant amounts of the pro-inflammatory cytokine TNFα above control levels to concentrations of 10-1000μg/ml anti-IgE and 1-1000ng/ml LPS. There was virtually no TNFα release from unstimulated control explants and explants incubated in the presence of 1000μg/ml IgG, the isotype control for the anti-IgE antibody.

Strikingly, distal lung tissue (circles) releases substantially more cytokine compared to proximal airway tissue (triangles), releasing 5 to 10 fold more TNFα. This pattern is also seen for the release of IL-10, which mimics the TNFα response closely (data not shown).

Interestingly, the shape of the TNFα LPS dose response curve, particularly with distal lung tissue, appears to be different from the anti-IgE response, appearing more linear and the anti-IgE curve rising dramatically at the highest concentrations giving the curve an exponential shape. A different pattern of release is seen when examining release of the Th_2 cytokine IL-5.

Figure 3.2

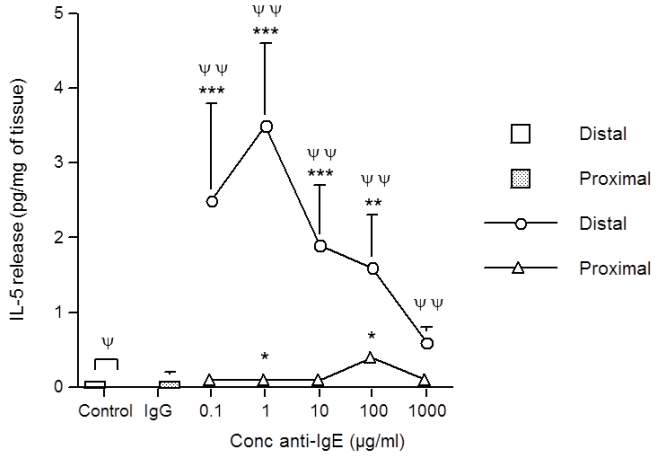


Figure 3.2 IL-5 release from matched distal lung and proximal airway explants after anti-IgE stimulation. Matched distal lung (open bars and open circles) and proximal airway tissue (shaded bars and open triangles) explants were stimulated with anti-IgE (0.1-1000µg/ml) (n=30), IgG isotype control (1000µg/ml) (n=7) or buffer control for 24 hours and supernatant analysed for IL-5. All values are expressed as mean±SEM and expressed as pg/mg tissue. * indicates $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared to control values. ψ indicates $p<0.05$, ψψ $p<0.01$ comparing same dose concentrations between the two tissue types.

In regards to IL-5, distal lung tissue produces a bell-shaped dose response curve in response to anti-IgE stimulation, with significant release above control at 0.1-100µg/ml and maximum release occurring at 1µg/ml. The amount of IL-5 released is very low however which creates a large signal to noise ratio and large error bars. There was no distinct response or pattern of IL-5 release from proximal airway tissue after anti-IgE stimulation as there was virtually no cytokine measured. There was also no release of IL-5 in response to LPS stimulation.

Release of other inflammatory cytokines quantified from lung supernatants are shown below in table 3.4.

Table 3.4

Cytokine (pg/mg)	100μg/ml anti-IgE		100ng/ml LPS	
	Distal	Proximal	Distal	Proximal
IL-1β	9.1 ± 2.7 ** ψ	2.6 ± 0.8 ***	19.3 ± 4.8 *** ψψ	3.6 ± 1.1 ***
IL-8	5610 ± 971 *	3978 ± 914 **	5106 ± 1050	3299 ± 556 **
IL-10	14.6 ± 4.1 *** ψψ	5.2 ± 1.5 ***	19.8 ± 2.4 *** ψψ	3.3 ± 0.8 ***
IL-13	0.6 ± 0.3	1 ± 0.5	1 ± 0.4	1.2 ± 0.6

Table 3.4 Cytokine release from distal lung and proximal airway explants. Distal lung and proximal airway explants were stimulated with 100μg/ml anti-IgE and 100ng/ml LPS and buffer control. Supernatant removed was analysed for IL-1β (anti-IgE n=20, LPS n=26), IL-8 (anti-IgE n=26, LPS n=32), IL-10 and IL-13 (anti-IgE n=30, LPS n=36). Cytokine release was corrected for tissue weight. All values are expressed as mean±SEM and expressed as pg/mg tissue. * indicates $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared to control and ψ indicates $p<0.05$ and ψψ $p<0.01$ compared to same dose concentration between the two tissue types.

All the cytokines measured in table 3.4 were released in a concentration-dependent fashion, similar to the TNFα curves in figure 3.1, and at levels significantly higher than unstimulated control, with the exception of IL-13 where there was no release measured. There was also no significant release of any cytokine in response to 1000μg/ml IgG antibody used as an isotype control. There was no release of other cytokines such as IL-4, IL-12 and IL-17 found in previous preliminary experiments conducted.

While both proximal airway and distal lung tissue explants initiated concentration dependent cytokine release in response to both anti-IgE and LPS, cytokine release was significantly lower (between 5-10 fold lower) from the proximal airway explants as shown in figures 3.1 and 3.2 and table 3.4. IL-6 was the only cytokine to go against this trend.

Figure 3.3

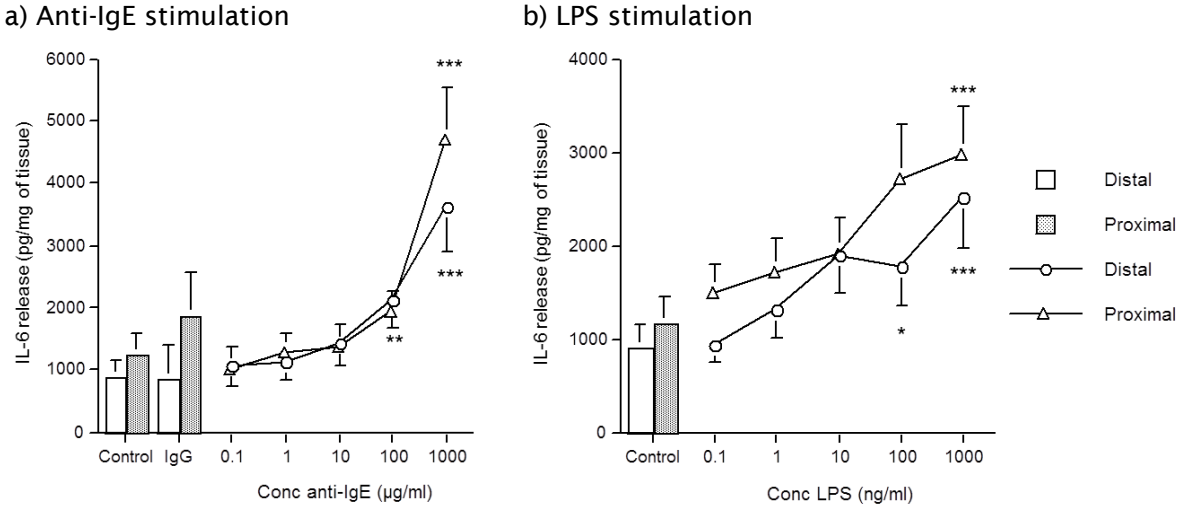


Figure 3.3 IL-6 release from distal lung and proximal airway tissue in response to anti-IgE and LPS. Distal lung (open bars and open circles) and proximal airway (shaded bars and open triangles) explants were stimulated with anti-IgE (0.1-1000µg/ml) (n=26), IgG isotype control (1000µg/ml) (n=5), LPS (0.1-1000ng/ml) (n=32) or buffer control for 24 hours and supernatant analysed for IL-6. All values are expressed as mean±SEM and expressed as pg/mg tissue. * indicates $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared to control values.

There was significant release of IL-6 from both compartments at the highest concentrations of anti-IgE and LPS. Interestingly however, in this case, there is no difference in the amounts of IL-6 release from distal and proximal airway tissue.

IL-6 and IL-8 also differ from the other cytokines with much higher levels being released in both compartments. Even unstimulated control tissue released around 1ng/mg IL-6 and 2ng/mg IL-8, whereas there is very little release at control levels of the other cytokines. This spontaneous release may be due to a latent tissue injury response caused during the dissection of tissue into explants.

3.3.2 Cytokine release as percentage of the maximum

To compare patterns of release in more detail we decided to plot this data as a percentage of the maximum amount of cytokine released. The data from figure 3.1 a) has been reworked in this way and is shown in figure 3.4.

Figure 3.4

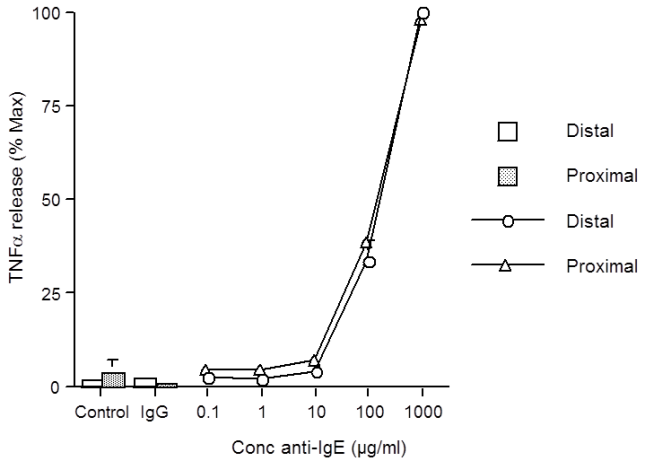


Figure 3.4 TNFα release from distal lung and proximal airway explants in response to anti-IgE plotted as percentage max. Data from figure 3.2 a) was reworked and presented as percentage of the maximum release of TNFα for proximal airway (shaded bars and triangles) and distal lung tissue (open bars and circles). All values are expressed as mean±SEM and expressed as a percentage of the maximum response.

Figure 3.4 shows when comparing TNFα release from the two tissues, after anti-IgE stimulation, as a percentage of the maximum released, the dose response curves become super-imposable. This trend is representative of all the cytokines measured and demonstrates that there is a similar pattern of release of cytokine from both compartments of the lung, but that distal lung tissue is able to release significantly more of these cytokines by 24 hours. We went on to investigate whether there was a correlation between cytokine release from distal and proximal lung tissue.

Figure 3.5

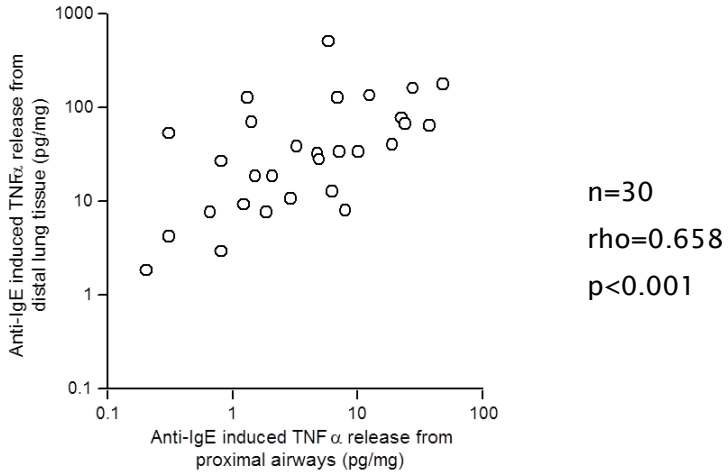


Figure 3.5 Scatterplot of anti-IgE induced TNFα release from proximal and distal lung tissue. Proximal airway tissue (x axis) and distal lung tissue (y axis) were stimulated with 100µg/ml anti-IgE, supernatant was analysed for TNFα and release expressed as pg/mg of tissue. Individual matched pairs (n=30) were plotted and correlation determined using the non-parametric Spearman's Rank correlation test.

Figure 3.5 shows that by plotting TNFα release induced by stimulation with 100μg/ml anti-IgE from matched distal lung and proximal airway explants, a relationship between the amounts of cytokine released from both lung compartments can be seen. So if there is a high degree of TNFα release from proximal airway tissue (x axis), you would also expect to see a high level of TNFα release from distal lung tissue. This trend was seen in all the cytokines measured after anti-IgE stimulation but was less evident in LPS stimulated explants, with significant correlation occurring for only TNFα ($p<0.001$, rho=0.579) and IL-10 release ($p<0.01$, r=0.461).

3.3.3 Kinetics of the inflammatory response

We were also interested in the order cytokines were released after an inflammatory insult and whether there were differences between the magnitude of response from distal and proximal lung tissue at different time points. Matched tissue was thus stimulated at time 0 with either 100μg/ml anti-IgE, 1μg/ml anti-IgE, 100ng/ml LPS or left unstimulated in RPMI as a control and incubated for 2hrs, 6hrs, 24hrs and 48hrs. Tissue was removed at these time points and the supernatants were analysed for cytokine release by ELISA. The patients in these groups were similar to patients used in the dose response experiments and are comparable. Again any patients on corticosteroid treatment have been omitted.

Figure 3.6

a) TNFα release

b) TNFα release as % Max

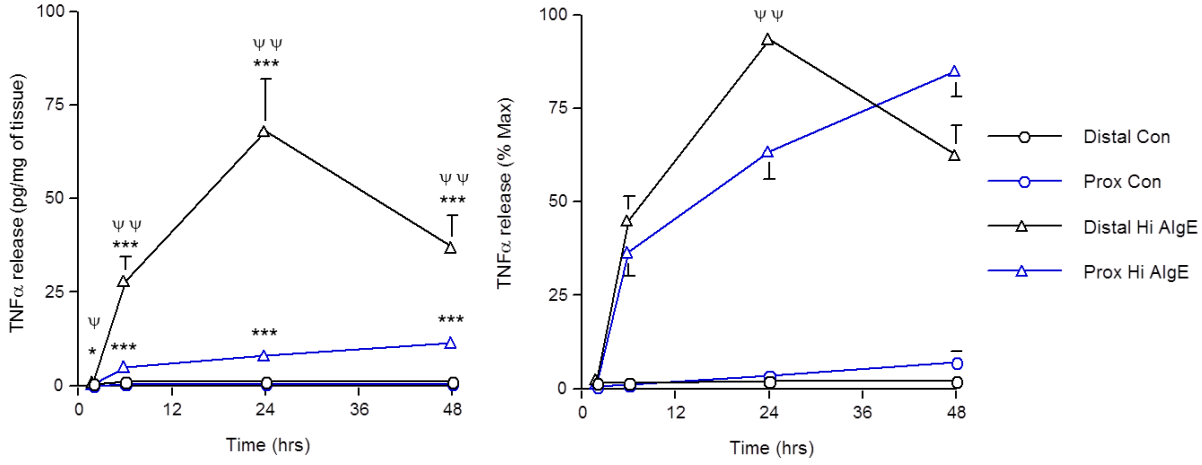


Figure 3.6 TNFα kinetics data from matched distal lung and proximal airway explants in response to anti-IgE. Distal lung (black) and proximal airway (blue) tissue was either stimulated with anti-IgE (triangles) or control tissue was left unstimulated in RPMI (circles). Tissue and supernatant was removed at 2, 6, 24 and 48hrs. (a) For TNFα release tissue was stimulated with 100μg/ml anti-IgE (n=17). (b) TNFα release data from (a) was reworked as percentage of the maximum released. * indicates $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared to relevant time point control and ψ indicates $p<0.05$ and ψψ $p<0.01$ comparing cytokine release between the two tissue types.

Figure 3.6 a) shows the kinetics of release for TNFα at relevant points over 48 hours of stimulation with anti-IgE. Over this time period there was very little release of this cytokine from unstimulated control tissue (circles). Distal lung tissue stimulated with a high concentration of anti-IgE (black triangles) on the other hand releases significant amounts of TNFα by 2hrs, peaking at 24hrs and beginning to decline by 48hrs. Significant release from proximal airway tissue (blue triangles) above control levels occurred slightly later by 6hrs and steadily increases. Again it is clearly evident that distal lung tissue releases substantially more TNFα than proximal airway tissue, with this being statistically significant even by the earliest time point.

When expressing this data as a percentage of the maximum release of cytokine in figure 3.6 b), the curves are superimposable at 6hrs however by 24hrs there is a significant difference between the pattern of TNFα released from distal lung tissue, which peaks at this point, whereas proximal airway tissue has not reached maximum release yet. By 48hrs TNFα levels fall in the distal compartment, whereas in the proximal airways they are still increasing.

A similar pattern in the kinetics of TNFα release is seen with stimulation of distal lung tissue with 100ng/ml LPS (data not shown). Proximal airway tissue however also released significant amounts of TNFα above control levels by 2hrs with LPS stimulation and when data was reworked as percentage maximum of release the curves for both tissue types remained superimposable at all the time points (data not shown). This may suggest a difference in the kinetics of cytokine release in response to the different stimuli.

Significant release of the anti-inflammatory cytokine IL-10 on the other hand occurs after TNFα at 6hrs, peaking at 24hrs and plateauing in response to both anti-IgE and LPS in both compartments of the lung (see table 3.5). Graphs of percentage of maximum release of cytokine resulted in superimposable curves for both stimuli (data not shown).

For IL-5 kinetics a concentration of 1μg/ml anti-IgE was chosen as this was the optimum concentration at causing IL-5 release determined from dose response data (see figure 3.2).

Figure 3.7

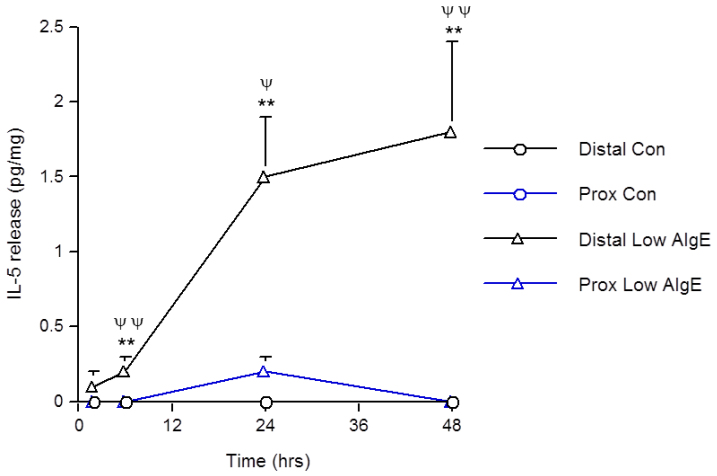


Figure 3.7 IL-5 kinetics data from matched distal lung and proximal airway explants in response to anti-IgE. Distal lung (black) and proximal airway (blue) tissue was either stimulated with anti-IgE (triangles) or control tissue was left unstimulated in RPMI (circles). Tissue and supernatant was removed at 2, 6, 24 and 48hrs. Tissue was stimulated with 1μg/ml anti-IgE (n=10) and IL-5 release quantified by ELISA. ** indicates $p<0.01$ compared to relevant time point control and ψ indicates $p<0.05$, ψψ $p<0.01$ comparing cytokine release between the two tissue types.

There was no significant release of IL-5 from proximal airway tissue in response to 1μg/ml anti-IgE stimulation, whereas there was significant IL-5 release from distal lung tissue by 6hrs that continued to rise by 48hrs, as shown in figure 3.7. This pattern of release is generally seen with the other cytokines measured, IL-1β, IL-6 and IL-8, for both anti-IgE and LPS stimulation and is summarised below in table 3.5. There was no release of IL-5 with LPS stimulation and no release of IL-13 after anti-IgE or LPS stimulation (data not shown).

Table 3.5
a) Stimulation with 100μg/ml anti-IgE

Cytokine (pg/mg)	Distal lung tissue Time (hrs)			
	2	6	24	48
IL-1b	0.7 ± 0.4	1.6 ± 0.8	7.5 ± 3.4 *	9.3 ± 3.9 *
IL-6	23 ± 13	98 ± 46 ψ	2988 ± 864 ** ψψ	5051 ± 1251 ** ψψ
IL-8	109 ± 86	272 ± 120 ψ	7498 ± 1243 **	13506 ± 2269 ** ψ
IL-10	0.0 0.0	0.8 0.2 ** ψ	6.9 1.7 *** ψψ	5.6 1.3 *** ψψ
Cytokine (pg/mg)	Proximal airway tissue Time (hrs)			
	2	6	24	48
IL-1b	0.3 ± 0.2	0.6 ± 0.3	2.3 ± 0.5 *	4.6 ± 1.7 *
IL-6	20 ± 11	80 ± 23	3018 ± 693 **	4507 ± 622
IL-8	134 ± 86	167 ± 95 *	5011 ± 1240 *	7469 ± 1004
IL-10	0.0 ± 0.0	0.1 ± 0.0	1.7 ± 0.5 **	1.5 ± 0.4 **

b) Stimulation with 100ng/ml LPS

Cytokine (pg/mg)	Distal lung tissue Time (hrs)			
	2	6	24	48
IL-1b	1.9 ± 1	4.4 ± 2 *	18.5 ± 5.9 ** ψ	16.9 ± 3.6 ** ψψ
IL-6	10 ± 7 *	410 ± 194	4272 ± 1100 **	7354 ± 1793 **
IL-8	59 ± 46 *	780 ± 254	9243 ± 1443 ** ψ	14842 ± 2515 ** ψ
IL-10	0.0 ± 0.0	2.4 ± 0.6 *** ψψ	22.5 ± 3.6 *** ψψ	23.2 ± 4.4 *** ψψ
Cytokine (pg/mg)	Proximal airway tissue Time (hrs)			
	2	6	24	48
IL-1b	0.5 ± 0.2 *	1.4 ± 0.9 *	3.1 ± 0.9 **	4.2 ± 1.2 **
IL-6	33 ± 18.5	249 ± 74	3668 ± 1077 **	5865 ± 1403 *
IL-8	158 ± 133	785 ± 340	4918 ± 763 **	7349 ± 1304
IL-10	0.0 ± 0.0	0.4 ± 0.1 **	3.0 ± 0.8 ***	2.2 ± 0.5 **

Table 3.5 Kinetics of cytokine release in response to anti-IgE and LPS stimulation. Matched distal lung and proximal airway tissue was treated with a) 100μg/ml anti-IgE, b) 100ng/ml LPS or buffer control. Tissue and supernatant was removed at 2, 6, 24 and 48hrs. Supernatants were analysed for IL-1β (anti-IgE, n=7; LPS n=10), IL-6, IL-8 (anti-IgE n=11; LPS n=13) and IL-10 (anti-IgE n=17; LPS n=17). All values are expressed as mean±SEM. * indicates $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared to relevant time point control. ψ indicates $p<0.05$ and ψψ indicates $p<0.01$ comparing cytokine release between the two tissue types.

Table 3.5 a) shows that distal lung tissue stimulated with anti-IgE, released significant amounts of IL-1β, IL-6 and IL-8 by 24hrs that continued to increase by 48hrs. In proximal airway tissue, significant release of all these cytokines over control occurred by 24hrs. A similar pattern of release was seen after LPS stimulation as shown in table 3.5 b). Again

distal lung tissue released substantially more of these cytokines than proximal airway tissue with the exception of IL-6. Data for IL-13 release is not shown as there was very little of this cytokine measured.

By plotting the kinetics data as a percentage of maximum release for all the cytokines measured it is possible to visualise the sequence in which they are released.

Figure 3.8

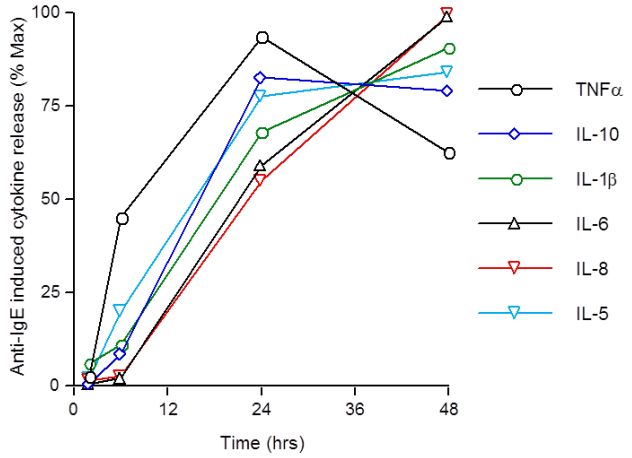


Figure 3.8 Kinetics of cytokine release from distal lung tissue in response to anti-IgE. Data was reworked from figures 3.6 and 3.7 and table 3.4 and expressed as a percentage of maximum release. Distal lung tissue explants were stimulated with anti-IgE and supernatant analysed for TNFα (black circles), IL-10 (blue diamonds), IL-1β (green circles), IL-6 (black triangles), IL-8 (red triangles) and IL-5 (light blue triangles).

Figure 3.8 shows that upon stimulation with anti-IgE, TNFα (black circles) is highlighted as the lead cytokine that is released initially and is followed by release of the other cytokines. This pattern is duplicated in proximal airway tissue for anti-IgE release and also for LPS stimulated explants from both compartments (data not shown). Interestingly, IL-1β levels expressed as a percentage of maximum release consistently appears slightly higher than all other cytokines at 2hrs, which also may implicate it as an early mediator in this cytokine cascade. Patterns of cytokine release are similar regardless of inflammatory insult or tissue type, with quick release of TNFα that peaks and tails off by 48hrs, and more steady longer lasting release of the other cytokines.

3.3.4 Tissue Viability

LDH assays were conducted to assess tissue viability after the addition of anti-IgE and LPS, to ensure they were not causing excessive cell death.

There is a trend of increased LDH release in both lung compartments with increasing concentrations of anti-IgE and LPS. Anti-IgE induced LDH release is slightly higher than LPS however only low levels of LDH were measured. There was no statistical difference in LDH release between proximal airway and distal lung tissue.

We also utilised the LDH assay to check whether tissue viability decreased dramatically at later time points. Unsurprisingly LDH levels did rise slightly on average at this later time point however they were still low and were not significantly higher than control levels for the same time point.

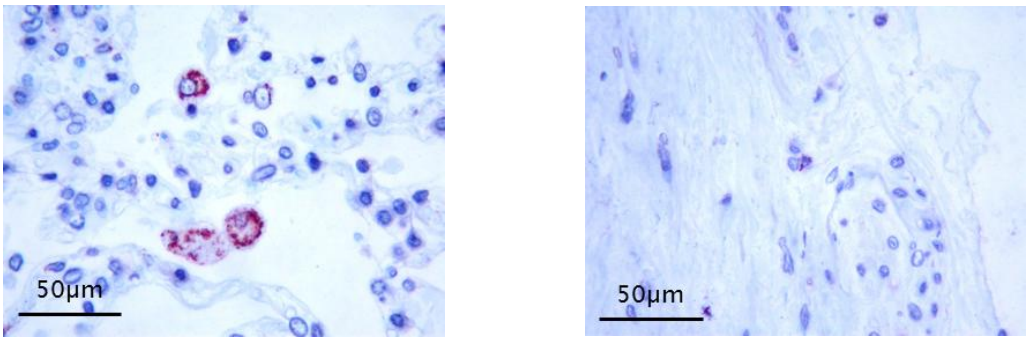
3.3.5 Immunohistochemistry of proximal airway and distal lung tissue

Due to the considerable difference in the amount of cytokines being released from proximal and distal lung tissue we were interested in the resident and inflammatory cells present within these lung compartments and their relative cell densities. Tissue was processed and embedded in GMA and sections were stained for a panel of inflammatory cells (see section 2.8.3). Cells were stained using to detect: Macrophages (CD68); mast cells (tryptase (AA1)); neutrophils (neutrophil elastase (NOE)); T cells (CD3); T helper cells (CD4); cytotoxic T cells (CD8); eosinophils (eosinophil cationic protein (EG2)); dendritic cells (CD1a); B cells (CD20). Examples of sections stained for macrophages, mast cells, neutrophils and T cells are shown in figure 3.9.

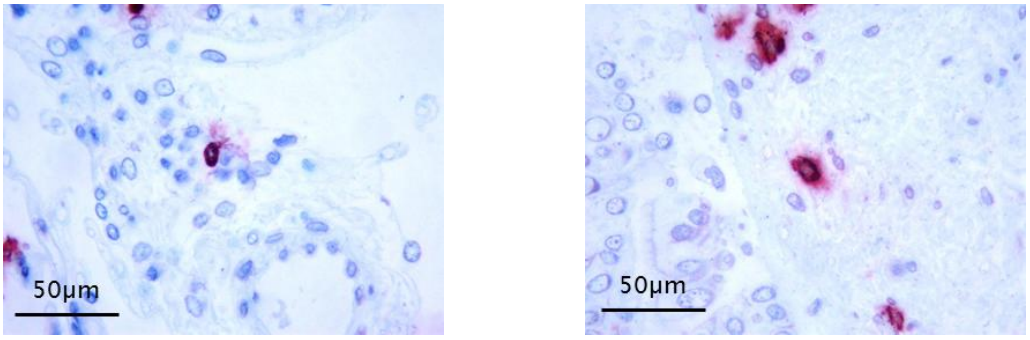
Figure 3.9

Distal Lung tissue | Proximal airway tissue

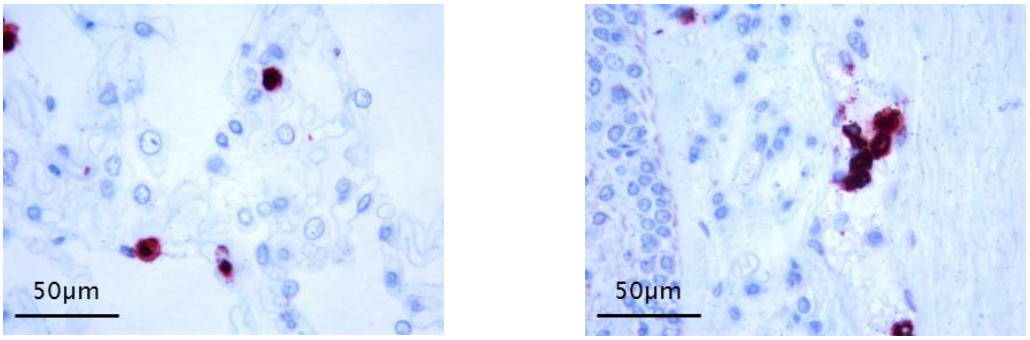
a) Macrophages – CD68



b) Mast cells – AA1



c) Neutrophils – NOE



d) T cells – CD3

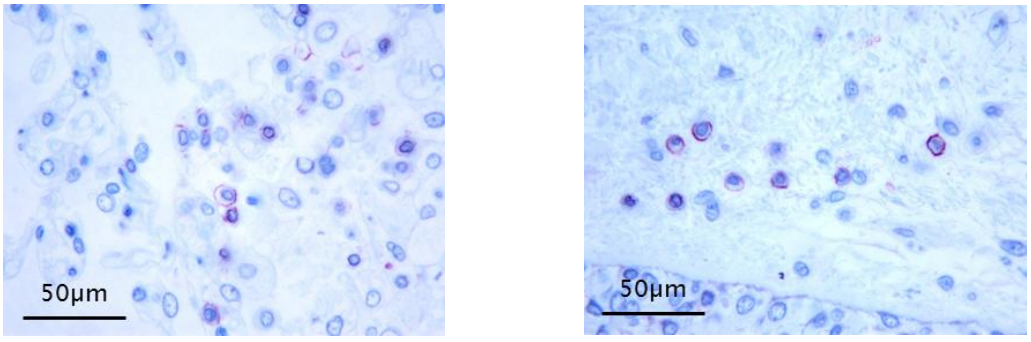


Figure 3.9 Images of immunohistochemical staining of lung tissue. Cells stained with AEC chromagen (red) and counterstained with Mayer's haematoxylin. Scale bar indicates 50µm.

Matched sections of distal lung and proximal airway tissue were stained, positive cells counted and cell counts were corrected for the area of the tissue section (see section 2.8). Patient characteristics are summarised in table 3.2.

Figure 3.10

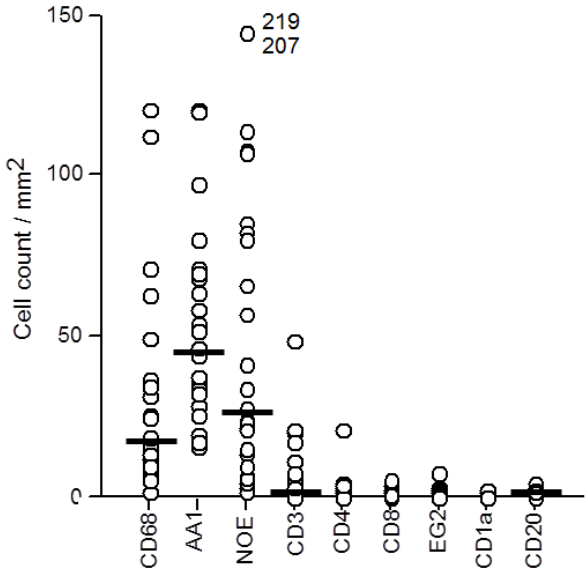


Figure 3.10 Numbers of inflammatory cells in distal lung tissue. Distal lung tissue (n=25) stained for macrophages (CD68), mast cells (AA1), neutrophils (NOE), T cells (CD3), T helper cells (CD4), cytotoxic T cells (CD8), eosinophils (EG2), dendritic cells (CD1a) and B cells (CD20). Cell counts were corrected for tissue section area and expressed as cell count/mm^2. Bar represents the median.

It is important to be careful when comparing different cell types within tissue sections as due to stereology you are more likely to detect large cells such as macrophages compared to smaller cells such as T cells in thin tissue sections. As cell nuclei are fairly consistent in size, we have compensated for bias caused by stereology by only counting positively stained nucleated cells.

Figure 3.10 shows the individual cell counts/mm^2 of different populations of inflammatory cells in distal lung tissue. The vast majority of cells stained and the predominant cell types in distal lung tissue were mast cells (AA1) and neutrophils (NOE), followed by macrophages (CD68). There were also slightly more T cells (CD3) present than the rest of the cell types investigated. The same distribution can be seen in the proximal airways with the exception that macrophage number decreases dramatically. In both compartments there are only very low numbers of T cells, eosinophils, dendritic cells and B cells. Figure 3.10 also demonstrates there is a large degree of variation in cell number between individuals.

Figure 3.11

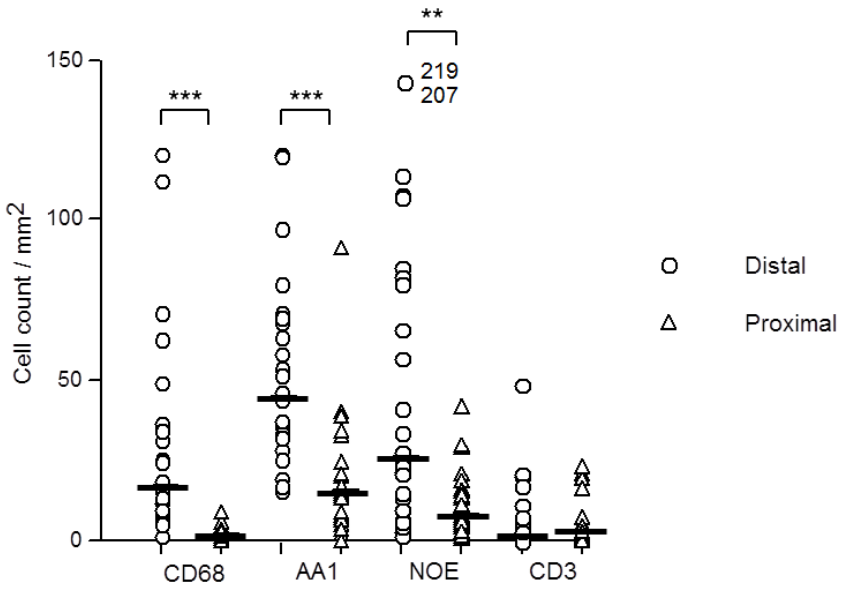


Figure 3.11 Comparison of inflammatory cells in distal lung and proximal airway tissue. Comparison of numbers of inflammatory cells in matched distal lung and proximal airway tissue (n=25) stained for macrophages (CD68), mast cells (AA1), neutrophils (NOE) and T cells (CD3). Cell counts were corrected for tissue section area and expressed as cell count/mm^2. Bar represents the median and * indicates $p<0.05$, ** $p<0.01$ and *** $p<0.001$ comparing numbers of cell types. Non-parametric Wilcoxon Signed Rank test was used to determine significance.

Figure 3.11 shows the comparison of the main cell types between distal lung tissue and the proximal airways. Distal lung tissue had significantly more macrophages, mast cells and neutrophils than the proximal airways. There was no difference in T cell, eosinophil, dendritic cell or B cell number however.

3.3.6 Effect of COPD on inflammatory markers

This thesis mainly focuses on asthma and allergic inflammation in the lung. Ideally we would have liked to compare inflammatory responses in allergic asthmatics and non-asthmatics. Unfortunately, we only received tissue from a very small number of diagnosed asthmatics and patients that only had an unconvincing history of allergy reported in their notes, such as penicillin and Elastoplast (see materials and methods section 2.2.2).

We cannot ignore the fact that a considerable number of patients within this study have some degree of COPD. This is determined by spirometry data obtained from patient notes at time of consent. This lung function data is used clinically to diagnose COPD, as set out by GOLD guidelines, and enables us to categorise patients into those with normal lung function and those with mild/moderate COPD. We thus are able to compare these two groups to investigate whether COPD status has an effect on the numbers of resident inflammatory cells and the release of inflammatory cytokines. Within the literature, COPD classically involves chronic inflammation characterised by

increased numbers of neutrophils, macrophages and $CD8^+$ T cells, as well as abnormal release of inflammatory mediators such as the neutrophil chemokine IL-8. Clinical data of patients used in section 3.3.5 for immunohistochemical analysis (table 3.2) were thus divided into those with or without COPD and characterised in table 3.3.

There was no difference in age, gender split or smoking status between the two groups. The COPD group had significantly lower lung function and significantly higher pack year history.

Figure 3.12

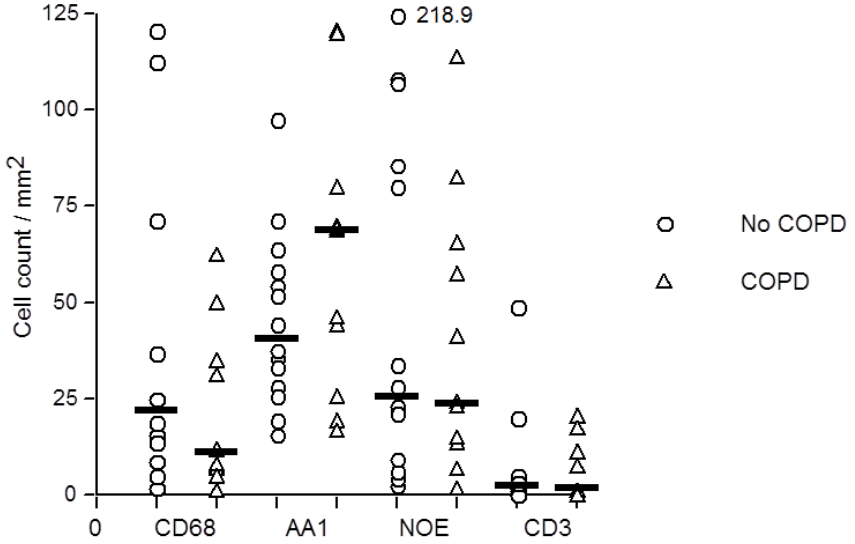


Figure 3.12 Comparison of inflammatory cells in distal lung tissue of patients with or without lung obstruction. Distal lung tissue of patients with no COPD (n=14) and patients with COPD (n=11) were stained for macrophages (CD68), mast cells (AA1), neutrophils (NOE) and T cells (CD3). Cells were counted, corrected for tissue section area and expressed as cell count/mm^2. Bar represents median. COPD status was determined using spirometry data following GOLD guidelines.

Figure 3.12 shows cell count data for the comparison of patients with normal lung function (No COPD, circles) and patients with some degree of lung obstruction (COPD, triangles). We found that there was no difference between the two groups for any of the panel of inflammatory cells stained in distal lung tissue or in the proximal airways. We also compared cytokine release between these two groups and found no statistical difference between them for anti-IgE or LPS stimulation (data not shown). There was also no correlation between cell count data and lung function (FEV_1/FVC) (data not shown).

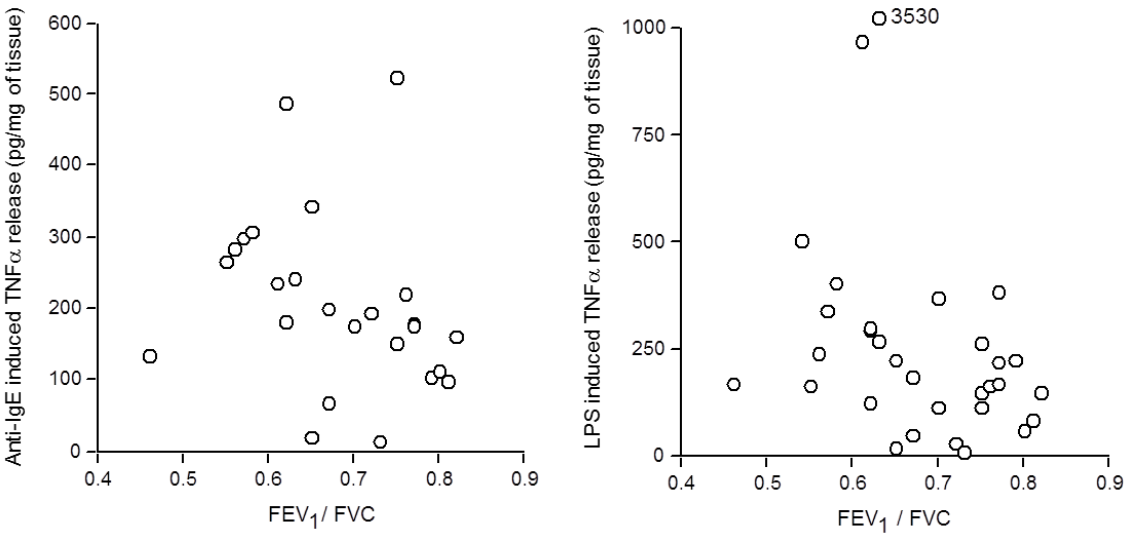
As we had cell count data and quantified cytokine release from patients we were also interested to see whether there was a correlation between the two and use cell numbers to predict cytokine release. We found however there was no correlation between any of the cell populations with any of the cytokines measured (data not

shown), with the exception of a weak correlation of mast cell numbers in distal lung tissue with anti-IgE induced IL-8 release (r=0.432, p<0.05).

We also found a relationship between anti-IgE and LPS induced TNFα release and lung function.

Figure 3.13

a) Stimulation with 1000μg/ml anti-IgE b) Stimulation with 1000ng/ml LPS



n=25 rho=-0.474 p<0.05 n=31 rho=-0.419 p<0.05

Figure 3.13 Correlation between lung function and TNFα release from distal lung tissue. Distal lung tissue was stimulated with 1000μg/ml anti-IgE (n=25) and 1000ng/ml LPS (n=31) and incubated for 24hrs at 37°C, 5% CO_2. Tissue was then harvested and supernatant analysed for TNFα release and expressed as pg/mg tissue. Individual patient lung function (FEV_1/FVC) (x axis) and TNFα release (y axis) were plotted and correlation determined using the non-parametric Spearman's Rank correlation test.

We found no significant difference when comparing cytokine release from patients with normal lung function and those with mild/moderate COPD. There was however a relationship between lung function and anti-IgE and LPS induced TNFα release, as shown in figure 3.13. This relationship indicates that as lung function decreases i.e. increasing severity of COPD, the release of TNFα caused by inflammatory insult increases. No significant relationship between lung function and cytokine release was seen with any of the other cytokines investigated (data not shown).

We also investigated other parameters that may have an effect on cell numbers or cytokine release such as gender, allergic status and smoking history and found that these had no effect. We did however notice a trend in the patients on corticosteroid treatment that warranted further investigation.

3.3.7 Effect of corticosteroids on inflammatory cell numbers

Inhaled and systemic (in more severe disease) corticosteroid is a frontline treatment for inflammatory conditions of the lung such as asthma and COPD. As we previously mentioned, corticosteroid treatment may have an effect on the numbers of inflammatory cells in lung tissue. As such, we decided to compare cell count and cytokine data from patients prescribed with inhaled or oral corticosteroid at the time of surgery with patients that were not prescribed any form of steroid. As steroid is used in the treatment of COPD, the majority of patients prescribed this treatment in this study had some degree of lung obstruction. Only 4 patients on steroid treatment had normal lung function. In order to assess the effect of corticosteroid treatment on lung tissue we compared COPD patients with or without steroid treatment. Patient characteristics are summarised in table 3.6.

Table 3.6

	COPD no steroid n=11	COPD steroid n=11
Age	65.6 ± 2.3	68.3 ± 2.7
Gender	6 Male 5 Female	6 Male 5 Female
Lung function (FEV1/FVC)	0.60 ± 0.02	0.56 ± 0.03
Smoking status	5 Current 6 Ex	5 Current 6 Ex
Years Ex	7.8 ± 3.3	4.6 ± 2.5
Pack yrs	54.1 ± 7.1	42.1 ± 8.9
History of asthma	10 No asthma 1 asthma	6 No asthma 5 asthma

Table 3.6 Patient characteristics of cell count data for comparison of COPD patients with or without steroid treatment. Age and FEV_1/FVC are shown as mean ± SEM. Asthma diagnosis was also determined by the patient's clinician. No significant difference was seen in age, gender, lung function, smoking status, years ex or pack years.

There was no significant difference in age, gender, lung function, smoking status or smoking history between the two groups. Non-parametric Mann-Whitney tests were used to compare cell numbers for the panel of inflammatory cells stained as in section 3.2.6. There was no statistical difference between macrophage, neutrophil, T cell (including $CD4^+$ and $CD8^+$ subtypes), eosinophil, dendritic cell or B cell numbers, although there was a trend of lower macrophages in distal lung tissue and increased T cells in both compartments in the steroid group (data not shown).

Figure 3.14

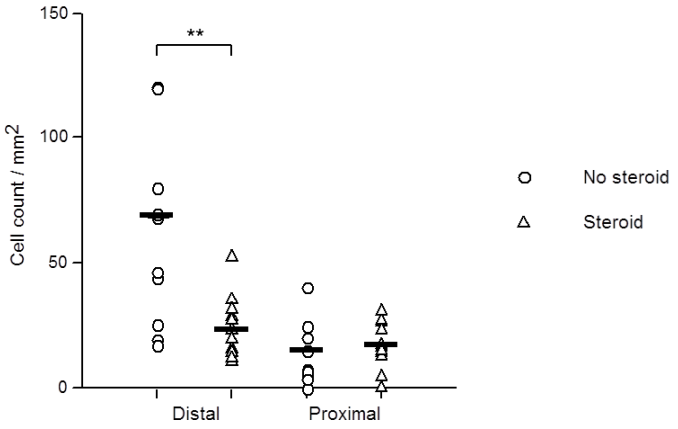


Figure 3.14 Comparison of mast cell numbers in COPD patients with or without steroid treatment. Matched distal lung and proximal airway tissue explants from COPD patients not on corticosteroid (n=11) and on current corticosteroid treatment (n=11) were stained for mast cells (AA1). Positively stained cells were enumerated and cell counts were corrected for section area and expressed as cell count/mm^2. Bar represents median and ** indicates p<0.01. Non-parametric Mann-Whitney tests were used to compare the two groups.

Figure 3.14 shows that corticosteroid treatment significantly reduces mast cell numbers in distal lung tissue. Of these patients, two were taking the systemic corticosteroid prednisolone whereas the other nine patients were on inhaled corticosteroid treatment. Only one of the patients on inhaled corticosteroid was not also using bronchodilators. Surprisingly there was no effect of steroid treatment on the numbers of mast cells in the proximal airways.

3.3.8 Effect of steroid on cytokine release

Using the dose response data obtained in section 3.3.1 and including cytokine release from patients on corticosteroid treatment, we can compare the effect of steroid on cytokine release in COPD patients (as carried out in section 3.3.7 for cell count data). The patients in these two groups had similar age, lung function, years ex and pack yrs.

As corticosteroids are known anti-inflammatory agents we hypothesised that steroid would decrease the release of inflammatory cytokines. We thus investigated its effects on the classical inflammatory cytokine TNFα and anti-inflammatory cytokine IL-10 on anti-IgE (figure 3.15) and LPS (figure 3.16) induced inflammation.

Figure 3.15

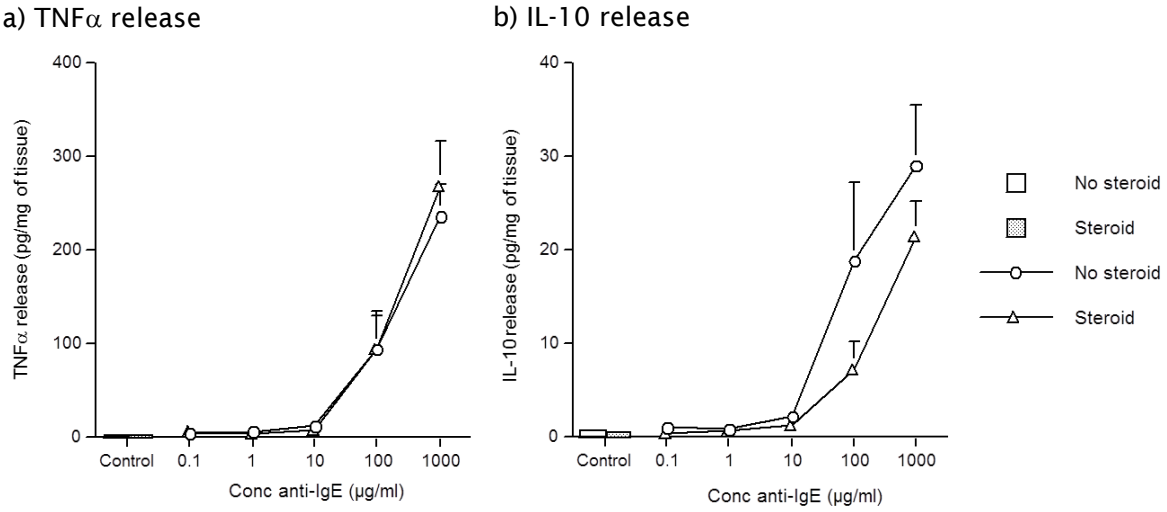


Figure 3.15 Anti-IgE induced distal lung tissue TNFα and IL-10 release from patients with or without steroid treatment. Distal lung tissue from COPD patients without corticosteroid treatment (open bars and open circles) (n=14) and COPD patients on corticosteroid treatment (shaded bars and open triangles) (n=14) after stimulation with 0.1-1000µg/ml anti-IgE. a) TNFα and b) IL-10 release expressed as mean±SEM.

Figure 3.15 shows that steroid-naïve and patients prescribed steroids still released TNFα and IL-10 in a concentration-dependent manner to anti-IgE stimulation. Surprisingly however, there was no difference in TNFα release between the two groups with anti-IgE stimulation, as shown by the superimposable dose response curves in figure 3.15 a). This was unexpected as we previously have shown in figure 3.14 that mast cells are decreased in the distal lung tissue of patients on corticosteroid treatment. There was also no difference in IL-10 release between the two groups.

Figure 3.16

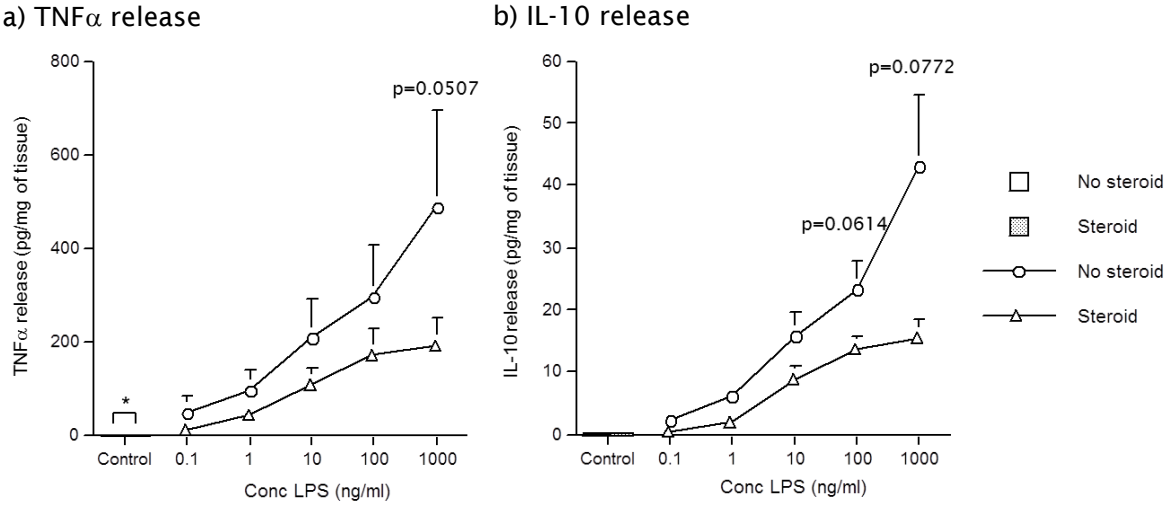


Figure 3.16 LPS induced distal lung tissue TNFα and IL-10 release from patients with or without steroid treatment. Distal lung tissue from COPD patients without corticosteroid treatment (open bars and open circles) (n=16) and COPD patients on corticosteroid treatment (shaded bars and open triangles) (n=14) after stimulation with 0.1-1000ng/ml LPS. a) TNFα and b) IL-10 release expressed as mean±SEM.

On the other hand, figure 3.16 shows there is a trend for COPD patients on steroid treatment (triangles) to release less TNFα and IL-10 than COPD patients without treatment (circles). Although there was no statistical difference between the release of cytokine induced by LPS stimulation, the highest concentrations of LPS were very close to reaching significance. Intriguingly, this virtually identical pattern is seen for both these closely related but opposing cytokines. There was also no difference in release seen in the proximal airways.

As we previously have shown that corticosteroid treatment affects mast cell numbers in distal lung tissue we also investigated the release of the Th_2 cytokine IL-5, which has been shown to be produced by mast cells.

Figure 3.17

a) Stimulation with anti-IgE b) Release as percentage of maximum release

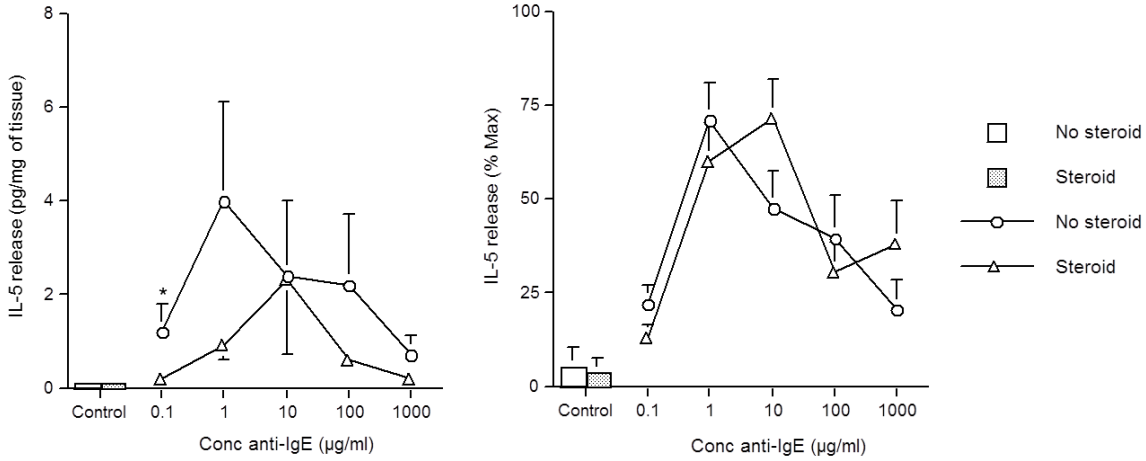


Figure 3.17 Distal lung tissue IL-5 release from patients with or without steroid treatment. Distal lung tissue from COPD patients without corticosteroid treatment (open bars and open circles) (n=14) and COPD patients on corticosteroid treatment (shaded bars and open triangles) (n=14) after stimulation with 0.1-1000µg/ml anti-IgE. a) IL-5 release expressed as mean±SEM. b) Data from a) reworked as percentage of maximum release. * indicates $p<0.05$ when comparing same dose concentration between groups. Non-parametric Mann-Whitney test was used to determine significance.

As seen in figure 3.2, IL-5 release occurs in a bell-shaped pattern in both groups with anti-IgE stimulation. Again, the steroid group generally releases less cytokine with a significant difference between the two groups at the lowest concentration of anti-IgE used. Intriguingly, IL-5 release in figure 3.17 a) and the same data expressed as a percentage of the maximum release in figure 3.17 b) indicate there may be a right hand shift in the steroid group IL-5 dose response curve. As very little of this cytokine is released however and as there is a high degree of error due to the large signal to noise ratio, this makes it difficult to interpret this data.

Of note, the only other cytokine where a statistically significant difference in cytokine release between the two groups was seen was with IL-6 release. This effect was most noticeable with anti-IgE stimulation with a trend in reduced response in the steroid group (100µg/ml anti-IgE induced IL-6 release: Non steroid group - 1545±386pg/mg IL-6; steroid group - 401±18, $p<0.05$). This trend was also seen for basal release of the cytokine however this did not reach significance.

3.4 Discussion

Airway inflammation is central to the pathogenesis of many pulmonary diseases such as asthma and COPD, involving a plethora of inflammatory and structural cells and the mediators they release. Undoubtedly, animal model studies and *in vitro* work have provided extensive insight into inflammatory mechanisms; however the key limitation of these studies is how closely they reflect *in vivo* mechanisms in humans. Bronchoscopy and sputum induction enable *in vivo* sampling in humans however this is mainly restricted to the central proximal airways. Bronchoalveolar lavage (BAL) is used to sample at sites more distal to this however all these sampling techniques may not reflect the environment within the tissue (O'Donnell et al, 2006). More recently, inflammation in lung disease is thought to encompass the whole lung, bringing the contribution of distal lung tissue and the small airways to the forefront (Hyde *et al*, 2009). Studies involving lung resection tissue enables investigation of the distal airways and lung parenchyma and their contribution to disease pathology. We have utilised the human lung explant model to investigate inflammatory processes *ex vivo* in intact tissue explants that maintain the tissue microenvironment. We also show novel data, to our knowledge, in the direct comparison of inflammatory cell composition and mechanisms in matched tissue from the proximal airways and distal lung tissue.

In this chapter we utilised an anti-IgE antibody to mimic allergic inflammation and the endotoxin LPS to mimic inflammation caused during bacterial infection. We have demonstrated that both proximal airway and distal lung tissue respond to these stimuli, releasing inflammatory cytokines in a concentration dependent manner, but that distal lung tissue and the small airways release substantially more of the majority of cytokines measured. To our knowledge, the comparison of cytokine release from matched tissue from these compartments of the lung is novel and therefore it is difficult to compare our findings with other studies. Conversely, techniques such as induced sputum, which is used to sample the proximal airways, and BAL, which samples more distal tissue, in the same patients, has shown similar cytokine concentrations between the two (Moodley *et al*, 2000 and McGarvey *et al*, 2002). As mentioned earlier, induced sputum and BAL may not reflect mechanisms within tissue however, and dilute BAL supernatant may decrease the precision of cytokine analysis making comparisons difficult.

This is the first study to investigate direct comparisons of cytokine release from proximal and distal lung tissue. Within the literature, there are however studies that scrutinize these sites individually. We have shown that proximal airway and distal lung tissue respond in a concentration dependent manner to anti-IgE and LPS, releasing a panel of inflammatory cytokines including TNFα, IL-1β, IL-5, IL-6 and IL-8 and the anti-

inflammatory cytokine IL-10. In support of this, Barnicott *et al* also showed a similar trend in release from parenchymal fragments to anti-IgE (Barnicott *et al*, 2006). TNFα, IL-1β and IL-8 have also been shown to be released in response to IgE-mediated mechanisms in other studies. Calderón *et al* have shown that cultured human epithelial cells from nasal biopsies from non-atopics and atopics with and without rhinitis constitutively produce these cytokines (Calderón *et al*, 1997). They went on to show the production of TNFα and IL-1β was significantly increased in epithelial cell cultures from atopic rhinitis patients biopsied in the pollen season, demonstrating the increased production of these cytokines in an IgE-mediated response, although release of IL-8 did not reach significance. Increased release of IL-6 and the Th_2 cytokine IL-5 have also been shown in BAL (Tonnel *et al*, 2001, Broide *et al*, 1992) and exhaled breath condensate (Matsunaga *et al*, 2006) of asthmatics, adding to the evidence that these cytokines are important in allergic inflammation of the lung.

In our study, distal lung tissue stimulated with anti-IgE released IL-5 with a distinct bell-shaped dose response curve, reproducing similar findings by Barnicott *et al*, 2006. Purified human lung mast cells have also been shown to be a potential source of IL-5 by RT-PCR experiments, *in situ* hybridisation and detection of the protein, showing a very similar bell-shaped dose response curve (Okayama *et al*, 1995). Other investigators have shown increased IL-5 production from BAL cells and PBMCs from HDM allergic asthmatics when stimulated with HDM extract (Tang *et al*, 1997) and production of IL-5 and IL-13 in response to anti-IgE treatment in cultured human bronchial smooth muscle cells (Gounni *et al*, 2005). Human skin stimulated with anti-IgE also showed production of IL-8 in mast cells using immunoelectron microscopy, although a mast cell line used in the same study did not. The authors argued this may in part be due to immaturity of the cell line however (Möller *et al*, 1993).

Ex vivo quantification of IL-5 and 13 is difficult due to the very low amounts released by tissue. This is reflected by the number of studies having to use RT-PCR to investigate induction of these cytokines at the mRNA level. The very low release makes interpretation of results more difficult due to a large signal to noise ratio and large degree of patient variability is likely to confound the data. As such, it is not surprising we did not detect any IL-13 using our model, or any strong evidence of IL-5 release from the proximal airways, which we have shown to produce substantially less cytokine than distal lung tissue.

In regards to LPS stimulation, our results support data published by Hackett *et al*, 2008, for cytokine release from distal lung tissue, although they reported very little release of IL-1β that did not reach significance. In a more clinical study investigating

inflammatory markers from COPD patients during bacterial exacerbation, induced sputum was shown to have elevated TNFα and IL-8 (Aaron *et al*, 2001), which supports our data on the release of these cytokines from the proximal airways. Studies using primary human cell cultures and immunolocalisation have been employed to pinpoint the cell populations producing these cytokines. Matched isolated alveolar macrophages and alveolar type II (ATII) cells were shown to produce TNFα, IL-1β, IL-6 and IL-8 in a concentration dependent manner to the same concentrations of LPS used in our study (Thorley *et al*, 2007). Interestingly this study also highlighted differences between the degree of cytokine release from these two cell types. Macrophages released significantly more TNFα and IL-1β, whereas epithelial cells released significantly more IL-6, IL-8, MCP-1 and Groα.

Structural cells such as epithelial cells, are the predominant cell type within our tissue explants, therefore this may explain why in our study we saw no difference in IL-6 production between the two lung compartments. There was however a significant difference in IL-8 production, albeit less prominent than other cytokines such as TNFα, however the contribution of IL-8 release from inflammatory cells may account for this. In support of this hypothesis, Thorley and colleagues also showed that basal release of IL-8 was significantly higher in macrophages, whereas basal release of IL-6 was higher in epithelial cells. In our model we also see high concentrations of these cytokines in control tissue, which has also been shown by Barnicott and Hackett. The importance of this constitutive release is unknown but may be explained due to a tissue injury effect. Of note, increasing evidence within the literature has brought attention to IL-6 and IL-13 in proximal airway inflammation, albeit in murine models i.e. airway hyperreactivity (Walter *et al*, 2001, Venkayya *et al*, 2002, Taube *et al*, 2004), and mucus production (Whittaker *et al*, 2002, Neveu *et al*, 2009). Thus the presence of specific cells secreting these cytokines may also be increased in airway tissue compared to the lung parenchyma.

Immunohistochemical analysis has indicated that TNFα is predominantly expressed in macrophages and mast cells in distal lung explants stimulated with LPS (Hackett *et al*, 2008). Other studies have also shown significant release of these cytokines in response to LPS stimulation using different models. An allergic mouse model has been utilised and showed increased production of IL-5 and IL-13 in lung homogenates and mouse mast cell lines stimulated with LPS (Nakae *et al*, 2007). The release of these cytokines was abolished in bone marrow derived mast cells from mice with a non-functional mutation in toll-like receptor 4 (Masuda *et al*, 2002). Although mast cells are not classically thought to respond to LPS stimulation, it is possible that inflammatory mediators released in response to infection could activate these cells.

In summary, there is evidence that all these inflammatory cytokines are released during allergic and bacterial inflammation, which explains why similar patterns of cytokine release were seen in our model regardless of stimulatory agonist. This highlights the importance of cytokine kinetics and cytokine cascades in the immune response. We have shown that significant release of all the cytokines investigated, with the exception of IL-13, occurs by 24 hours, and in the majority increases till 48 hours. Expressing kinetics data together clearly highlights TNFα as a key early mediator in initiating inflammation. IL-1β at early time points (particularly by 2hrs) was also consistently slightly increased compared with other cytokines, providing evidence for its involvement in the early stages of the inflammatory cascade of cytokines.

The importance of TNFα and IL-1β has also been shown by Thorley and colleagues using blocking antibodies. They showed that IL-6 release from macrophages was most effectively inhibited when both cytokines were blocked, whereas Il-6 release from ATII cells depended on the effects of TNFα. They do not show any data or comment on the effect on IL-8 or Groα in these blocking studies, but do show that ATII cells and not macrophages depended on the autocrine effect of TNFα and IL-1β for MCP-1 release. Similar results were shown using a neutralising TNFα antibody that reduced IL-6, 8 and 10 levels back to baseline after LPS stimulation in lung parenchymal explants (Hackett *et al*, 2008). Mast cell lines and human primary cord blood-derived mast cells have also been shown to induce IL-8 mRNA and protein synthesis via an autocrine effect involving LTB_4, after stimulation with IL-1β and TNFα (Kim *et al*, 2010). The concentrations of these cytokines (10ng/ml) used in this study to stimulate cells however is very high and may not be physiologically relevant. To put this into context, on average a maximum of around 500pg/ml IL-1β and 6600pg/ml TNFα is released from distal lung tissue explants in our study.

In vivo mouse and rodent models have also been used to investigate the importance of TNFα and IL-1β in initiating inflammatory cascades and in disease progression. In one such study, LPS was given via intratracheal injection, causing the induction of IL-1β and TNFα mRNA by 1 hour and an inflammatory exudate, detected in BAL, consisting of neutrophils by 6 hours, macrophages by 24 hours and lymphocytes by 48 hours (Ulich *et al*, 1991). Injection of IL-1β or TNFα recapitulated these results; however TNFα was much less potent.

In order to compare the patterns of cytokine release between proximal airway and distal lung tissue in more detail, we expressed this data as a percentage of the maximum amount of cytokine released. In doing so we obtained superimposable

curves for all the cytokines measured. There was also strong correlation between the release of individual cytokines between the two compartments. This data suggests that distal lung tissue and proximal airway tissue respond in the same way to these stimuli, but the magnitude of response differs for the majority of the cytokines measured. This may indicate that similar cells in each tissue type are releasing the relevant cytokine, with the different magnitude of release between the tissues being due to the relative cell density of the cytokine secreting cells.

Using immunohistochemistry we have shown that in both proximal airway and distal lung tissue the predominant inflammatory cells present are mast cells and neutrophils, followed by macrophages in the distal lung compartment. We have also shown that distal lung tissue contains substantially more of these cells. There were similar numbers of T cells, eosinophils, dendritic cells and B cells in the two tissue types; however numbers of these cells were very low. There is evidence in the literature to support these findings. Within rat lung, Renzi *et al*, quantified the different cellular populations within the airways and parenchyma. They found that the macrophage was the predominant cellular infiltrate in distal tissue followed by similar amounts of lymphocytes and neutrophils, with eosinophils being the least abundant leukocyte present (Renzi *et al*, 1993). Their data also shows there are around 10 times more of these cells present in distal lung tissue compared to the proximal airways. Within humans, eosinophils and macrophages were shown to be the majority leukocyte present from histological analysis of endobronchial and transbronchial biopsies from nocturnal and non-nocturnal asthmatics, with the majority of cells present in the alveolar tissue (Kraft *et al*, 1996). The increase in eosinophil count is likely to reflect the asthmatic phenotype and may explain the very low numbers of eosinophils in our study, as asthmatics were rare and severity of disease was unknown.

Other studies have also shown increased cell density within the small airways and distal lung tissue (Hamid *et al*, 1997, Balzar *et al*, 2002) however they do show some differences in the quantities of cell populations present. In the study by Hamid and colleagues, T cell numbers were much higher than we saw (around 150 cells/mm^2) and were the predominant inflammatory cell population, whereas the numbers of T cells in our study were very similar to those found by Kraft. In Hamid's study frozen sections of tissue were used, which may account for some discrepancies. The inflammatory cell composition found in distal lung tissue by Balzar *et al*, with macrophages, mast cells and neutrophils as the predominant inflammatory cells also supports our findings, although mast cell numbers were quite low. In the same study however they found a different distribution in the proximal airways with similar numbers of mast cells, neutrophils and T cells. The patients used in this study were severe steroid-dependent

asthmatics however and therefore the increase in T cells may reflect disease severity. The effect of steroid may also explain why mast cell numbers were lower but this will be discussed later. In contradiction to these studies, the proximal airways have also been shown to contain a higher cell density than the distal airways (Faul *et al*, 1997). This was in a study of sudden asphyxic asthma death however. Here T cells were raised in the proximal airway and there were also an equal number of macrophages present. This study and the findings in the proximal airways of severe asthmatics by Balzar and colleagues add to the evidence that T cells play an important role in severe asthma pathogenesis.

Ideally, we would have liked to compare inflammatory processes in the proximal airway and distal lung tissue of asthmatic patients. Patients with a history of asthma were rare however and therefore we could not make any direct comparisons between asthmatics and non-asthmatics. A considerable number of patients consented to our study had some degree of lung obstruction though, as determined by lung function tests. Within the literature there is evidence of increased T cells, macrophages, neutrophils, eosinophils, NK cells and B cells within the lung tissue of COPD patients (Di Stefano *et al*, 1996, Di Stefano *et al*, 1998, Rutgers *et al*, 2000, Hogg *et al*, 2004), and more recently evidence for a decrease in total mast cell density, with a change in phenotype from MC_T cells to MC_{TC} (Andersson *et al*, 2010). There is also evidence of elevated inflammatory cytokines such as TNFα and IL-8 in the sputum of COPD patients (Keatings *et al*, 1996). We however found no statistical difference in any of the cell populations and cytokine release between patients with normal lung function and those with mild/moderate COPD. In the studies mentioned where differences were found, the COPD groups consisted of patients with severe disease. In fact, in the study by Stefano *et al*, 1998, severe COPD smokers had significantly more neutrophils, macrophages and NK cells compared to healthy smokers, whereas in mild/moderate COPD smokers there was only elevated neutrophil numbers. The studies by Hogg and colleagues and Andersson's group also split their COPD patients by severity of disease and showed changes in inflammatory cell numbers is particularly associated with severe stages of COPD. The majority of the COPD patients in our study had only mild/moderate COPD (GOLD I and II) and therefore it is unlikely that we would see a difference in the inflammatory infiltrate or cytokine profiles of this group. In contradiction to this, distal lung tissue explants from COPD patients classified as GOLD II (moderate disease) have been shown to have an exaggerated TNFα, IL-6 and IL-8 and diminished IL-10 response after LPS stimulation (Hackett *et al*, 2008). The higher numbers of COPD patients in this study allowed for the stratification by GOLD status, however numbers within our study are too low to do this. On the other hand, we have

shown that there is a relationship between lung function and TNFα release induced by anti-IgE or LPS stimulation which supports Hackett's data.

Cigarette smoking is known to induce an inflammatory response and is the major risk factor for the development of COPD, characterised by an abnormal cellular infiltrate and structural changes resulting in airflow obstruction. We found no statistical difference in the inflammatory cellular composition or cytokine release from current smokers and ex-smokers with normal lung function. Unfortunately as we only had 4 lifelong non-smokers we could not make a comparison with this group. A study specifically investigating the effect of cigarette smoking found that smokers compared to non-smokers, a fifth of which were ex-smokers, had increased neutrophils, macrophages, IL-1β, IL6, IL-8 and MCP-1 in BAL, providing evidence that smoking can cause infiltrating cells and an inflammatory response (Kuschener *et al*, 1996). This is supported by other groups who show increased neutrophils and $CD8^+$ T cells that correlated with smoking history (Costabel *et al*, 1992). On the other hand smoking has also been shown to inhibit the production of inflammatory cytokines, such as IL-1β, produced by alveolar macrophages (Yamaguchi *et al*, 1989). Other studies have reported no difference between non-smokers and smokers in regards to inflammatory infiltrate in bronchial biopsies and again only reported significant increases of macrophages and $CD8^+$ T cells in COPD groups with severe disease (O'Shaughnessy *et al*, 1997, Lams *et al*, 2000).

Inhaled corticosteroids are the most effective anti-inflammatory treatment for asthma control (Chung *et al*, 2009) and have more recently been applied to treat more severe COPD, where they have been shown to reduce exacerbations (Rabe *et al*, 2007). Corticosteroids have therefore been studied extensively and have been shown to elicit a range of effects that culminate in an anti-inflammatory response. These include reducing inflammatory cells and their ability to produce and release inflammatory mediators (Rabe *et al*, 2007).

A subpopulation of patients within our study was prescribed inhaled or oral corticosteroids at time of surgery. Only four of these patients did not have COPD and therefore in order to assess the effect of steroid we compared COPD patients prescribed steroid treatment, with COPD patients who were not. Of note, there are a number of limitations to consider: patient compliance, inhaler technique, duration of treatment and unknown dose of steroid taken. Also different types of steroid were used such as the oral steroid prednisolone, and different inhaled steroids such as fluticasone, budesonide and beclomethasone, all of which have different pharmacokinetic properties. In our study the majority of patients were taking an

inhaled corticosteroid, two were on oral prednisolone and one patient was prescribed a fluticasone nasal spray.

We have shown that patients on steroid treatment had significantly fewer mast cells in distal lung tissue compared to COPD patients without treatment. Surprisingly we saw no difference in the proximal airways, which we hypothesised to be the main site of action for inhaled corticosteroid. The reduction in mast cell number by corticosteroids has been reported in a number of studies. In a murine model, glucocorticoid applied to the skin reduced cutaneous mast cell numbers and was attributed to an increase in the number of mast cells undergoing apoptosis (Finotto *et al*, 1997). Also in humans, treatment with prednisolone has been shown to reduce mucosal mast cells in the gut in patients with inflammatory bowel disease (Goldsmith *et al*, 1990) and in proximal airway tissue (Djukanovic *et al*, 1997). A study using more comparable steroid treatment i.e. inhaled corticosteroid, also showed a significant decrease in mucosal mast cells in the proximal airways of COPD patients, but also a significant increase in neutrophils (Gizycki *et al*, 2002). In this study patients were given 3 months treatment with 500μg fluticasone proprionate twice daily or placebo, and biopsies were taken at baseline and after treatment. This drug has a relatively high receptor binding affinity and the dosage used in this study was high (Chung *et al*, 2009). In contrast, in our study, 2 patients out of 11 were prescribed an inhaler containing fluticasone and 1 patient was prescribed a fluticasone nasal spray. An equal number of patients were prescribed beclomethasone and the majority were prescribed budesonide, which are 43 times and twice less potent in terms of binding affinity than fluticasone (Chung *et al*, 2009). Although we do not know the doses these patients were taking, it is unlikely they were receiving an equipotent dose compared to Gizycki's study. High doses of corticosteroid may therefore be needed in order to affect mast cell numbers in the proximal airways. This will need to be clarified in a study comparing different drugs at different potencies and their effects in the proximal airways and distal lung tissue.

Mast cells are traditionally regarded as steroid resistant and have been shown to be able to release mediators, such as histamine and the eicosanoids, in an IgE-mediated mechanism, which was not inhibited by pre-treatment with steroid (Schleimer *et al*, 1983). The effect of corticosteroid in reducing inflammation in asthma has partly been attributed to the reduction in mast cells and thus the mechanisms behind this have sparked particular interest. In a mouse model, dexamethasone had no effect on mast cells cultured in the mast cell survival factors, IL-3 or stem cell factor (SCF) (Finotto *et al*, 1997). When these cells were cultured with SCF producing fibroblasts, dexamethasone dose dependently reduced SCF mRNA and protein synthesis, which resulted in increased apoptosis of mast cells. Going back to their *in vivo* model, the

depletion of cutaneous mast cells by topical corticosteroid was completely reversed upon local administration of SCF via injection (Finotto *et al*, 1997). Dexamethasone has also been implicated in the inhibition of mast cell migration in rat peritoneal mast cells (Jeong *et al*, 2003) supplying more evidence for the mast cell reducing effects of corticosteroid.

In *in vitro* experiments using mast cells cultured from human cord blood mononuclear cells, dexamethasone dose-dependently inhibited the maturation of mast cells, but had no effect on histamine release or synthesis and release of leukotriene (Smith *et al*, 2002), an effect that has also been shown using purified human lung mast cells and lung explants (Schleimer *et al*, 1983). This may explain steroid ineffectiveness in acute allergic reactions, such as anaphylaxis, or potentially steroid-resistant asthma. Other perplexing data on the action of corticosteroid was the effect on regulation of SCF production from human primary lung fibroblasts. In this study short treatment (2.5-10hrs) destabilised SCF mRNA and decreased protein synthesis, whereas longer treatment (24-48hrs) stimulated SCF gene transcription (Kassel *et al*, 1998). Therefore corticosteroids could have different actions depending on dose and time.

IL-6 has also been associated as a mast cell survival factor. In a human mast cell line, IgE was shown to induce survival of mast cells upon removal of SCF, IL-6 and IL-10, with elimination of this effect upon addition of a neutralising IL-6 antibody (Cruse *et al*, 2008). They went on to show IL-6 mRNA upregulation and increased release of IL-6 from mast cells after IgE sensitisation, providing evidence for the autocrine production of this cytokine. This is of particular interest as we have shown that proximal airway and distal lung tissue release similar amounts of IL-6, which may reflect actions to do with immune homeostasis. The interaction of structural and inflammatory cells may have a particular role in this. Co-culture of human lung fibroblasts with a mast cell line was shown to significantly increase IL-6 production, specifically in fibroblasts (Matthew Fitzgerald, 2004). Treatment with dexamethasone decreased the production of IL-6 and has also been shown to decrease IL-6 secretion from airway smooth muscle cells (Quante *et al*, 2008). We have shown that the effect of steroid treatment can dampen down the IL-6 response, in particular to anti-IgE stimulation in distal lung tissue, which may be due to effects on structural cells themselves; the reduction in mast cell numbers; or, a combination of both. It is also possible the direct action of corticosteroid on structural cells to reduce IL-6 and SCF production could cause the decrease in mast cell numbers.

Our data has also revealed a trend of reduced TNFα and IL-10 release from distal lung tissue stimulated with LPS, although there was no significant difference between the

COPD patients on prescribed steroid and those not on steroids. Corticosteroids are well documented anti-inflammatories with a principal mechanism of action in suppressing the transcription of inflammatory genes, and so decreased cytokine release was expected. The decrease in IL-10 production is likely to be attributed to the fall in TNFα release. Our data shows a clear stimulus-specific effect however, with this marked difference in cytokine release after LPS stimulation, whereas there was no effect of steroid on anti-IgE induced TNFα and IL-10 release. This was unexpected as we have shown that mast cell numbers are significantly lower in the distal tissue of patients prescribed steroid. It is feasible however that there are still enough mast cells present to mount a full inflammatory response, especially as the release of inflammatory cytokines such as TNFα and IL-1β, which have been shown to be released by mast cells, are able to stimulate other inflammatory cells initiating the cytokine cascade described earlier.

It is difficult to explain why our data has shown no difference in mast cell number or cytokine release in the proximal airway and a marked decrease in mast cell numbers in distal lung tissue. Potentially, inhaled steroid deposited in the central airways may be absorbed into the circulation and taken straight to the distal lung compartment where it is eliciting its actions, before moving on around the body and being broken down in the liver on first-pass metabolism. Alternatively, advances in drug delivery and the combination of prescribed bronchodilators for the majority of patients in this study may allow deeper penetration of the corticosteroid into the lung periphery.

3.4.1 Summary

This chapter has shown that distal lung tissue releases substantially more inflammatory cytokines and contains considerably more inflammatory cells, particularly mast cells, neutrophils and macrophages compared to the proximal airways. This is potentially a novel finding and indicates distal lung tissue may have a more active phenotype, adding to the argument that this compartment of the lung is able to contribute to disease pathogenesis. This may be of particular relevance to diseases that have a systemic component, such as COPD, where inflammatory processes are known to occur in the distal lung and the release of inflammatory mediators could spill out and into the circulation.

Although there is a difference in magnitude of response between the two compartments, we have also shown that there is a great deal of similarity in their cytokine profiles and relative framework of inflammatory cells.

This chapter also adds to the growing evidence of the effects of corticosteroid in reducing mast cell numbers in lung tissue and dampening down the inflammatory response.

Ideally we would have liked to have identified the cellular source of inflammatory cytokines in this system, potentially using double staining immunohistochemistry. Other immunohistochemical studies in the literature have determined the source of cytokines implicated in lung inflammation. A study by Ohkawara using this technique and immunoelectron microscopy, observed staining for TNFα in lung tissue. After stimulation with anti-IgE, they showed TNFα present in mast cells in the submucosal layer, macrophages in alveolar spaces and in bronchial epithelial cells (Ohkawara *et al*, 1992). This has also been confirmed by Bradding *et al*, who also showed staining for IL-5 present in mast cells and eosinophils in the submucosa and IL-6-positive cells, particularly around submucosal glands, which were predominantly mast cells, in bronchial biopsies of asthmatics (Bradding *et al*, 1994).

Chapter 4
Passive Sensitisation

4.1 Introduction

Allergy is characterised as an abnormal immune reaction, mediated predominantly by leukocytes, to a common environmental allergen. Allergy manifests itself in a number of pathological phenotypes, such as allergic rhinitis, atopic dermatitis and atopic asthma (Galli *et al*, 2008). These diseases often affect quality of life and present an increasingly expensive burden to the health system, with their prevalence appearing to be increasing.

Atopic asthma is a complex chronic disease characterised by generalised but reversible airways obstruction, bronchial hyper-responsiveness and airways inflammation (NHLBI, accessed on 20/01/10). An important component of the initial inflammatory process that leads to this phenotype is attributed to an IgE-mediated inflammatory response. Increased IgE production is a feature of allergic disease and is linked to Th_2 polarisation of the immune system (Del Prete, 1992). Consequently, upon sensitisation to an allergen, B cells from atopic individuals synthesise allergen-specific IgE. This subsequently binds to inflammatory cells expressing the high affinity IgE receptor, FcεRI. One of the most important cells in the lung that expresses this receptor is the mast cell. Due to the high affinity binding of FcεRI to IgE, mast cells can be permanently coated with these immunoglobulins and thus primed for activation (Holgate *et al*, 2006).

Inhaled allergens are a common cause of asthma exacerbation and chronic inflammation in the allergic lung. One of the most common triggers for allergic asthma is the perennial allergen Der p1, found in the faeces of the house dust mite (HDM) *Dermatophagoides pteronyssinus*.

Upon allergen exposure, allergen is able to bind to allergen-specific IgE on the mast cell surface and form crosslinks with neighbouring allergen-specific IgE-receptor complexes. This enables clustering of the receptors into trimers or more, resulting in a signalling cascade that activates the mast cell causing it to degranulate (Wedemeyer and Galli, 2000). Degranulation results in the release of preformed inflammatory mediators such as histamine, proteases and cytokines including TNFα, and induces the production of newly formed inflammatory mediators such as the eicosanoids and cytokines (Steffen *et al*, 1989, Bloeman *et al*, 2007). These mediators are known as the early phase mediators.

There are two distinct inflammatory phases involved in the asthmatic phenotype designated the early phase (EAR) and late phase (LAR) reactions. The EAR occurs immediately, lasting between 30-60 minutes and encompasses the release of early

phase mediators from mast cells. Within the EAR, chemokines and chemoattractants such as IL-8, leukotriene (LT) B_4, monocyte chemoattractant protein (MCP)-1 and eotaxin are released into the inflammatory cocktail and result in the recruitment of other leukocytes such as neutrophils, T cells and eosinophils to the lung (Bloeman *et al*, 2007, Galli *et al*, 2008). This influx of new inflammatory cells culminates in the LAR 4-6 hours after the EAR. Symptoms and inflammation last as long as 24-48 hours due to release of inflammatory mediators from these recruited cells (Wedemeyer and Galli, 2000).

Traditionally, asthma is thought of as a disease of the central airways. More recently, it has become widely accepted that inflammatory processes extend throughout the whole lung and therefore both the small airways and distal lung tissue are thought to be able to contribute to disease pathology and progression. This has not been researched extensively and therefore there is a gap in the scientific literature to study allergic mechanisms in distal lung tissue and the effect of allergen-induced inflammation.

It has been demonstrated that by incubating tissue with atopic serum containing a high titre of allergen-specific IgE, it is possible to prime the resident tissue mast cells for degranulation upon exposure to the relevant antigen. This process, known as passive sensitisation, has been used by various groups to study the allergic process in human proximal airways (Tunon de Lara *et al*, 1995, Watson *et al*, 1998). Little is known however about this process in distal lung tissue.

We hypothesise that 'primed' distal lung tissue has the ability to release substantial amounts of inflammatory mediators upon mast cell degranulation and activation. The aim of this chapter is to investigate IgE-mediated allergic inflammation in peripheral lung tissue passively sensitised to the common perennial allergen Der p1 contained in HDM extract (ALK-Abéllo) and to quantify the release of different inflammatory cytokines.

4.2 Methods

4.2.1 Passive sensitisation of lung tissue

Tissue was incubated in 10% serum at room temperature in 5% CO_2 overnight. Serum used was from serum pool II, donated from healthy volunteers with positive skin prick tests to *Dermatophagoides pteronyssinus* HDM extract and healthy non-atopic volunteers with no response to skin prick testing. Serum IgE titres are summarised in materials and methods, table 2.2. Explants from the same lung were treated with atopic and non-atopic serum and control explants incubated in supplemented RPMI. Lung tissue explants were stimulated with 100μg/ml anti-IgE, as a positive control for an allergic reaction, 100ng/ml LPS, as a positive control for an inflammatory response, and 1-1000 SQ-U/ml purified HDM extract. Tissue was harvested at 30 minutes, to investigate release of early phase mediators, and 24hrs to investigate late phase mediators. ELISA was utilised to measure cytokine release and a histamine bioassay to quantify histamine release.

4.2.2 Histamine bioassay

Chinese hamster ovary (CHO) cells, stably transfected with the histamine H_1 receptor, were cultured and seeded onto 96-well clear bottomed, black sided plates. Cells were incubated with fluorescent calcium indicator dye (see materials and methods section 2.6) for 45 minutes before administration of agonist (histamine or supernatant). Calcium influx after histamine binding and subsequent increase in fluorescence in cells was measured using a fluorometric imaging plate reader.

4.2.3 Smooth muscle contraction experiments

Bronchial airway strips were dissected from donated lung at GlaxoSmithKline, Stevenage and passively sensitised as in section 4.2.1. Tissue was strung up in organ baths in oxygenated Krebs buffer and change in tension recorded upon smooth muscle contraction. Viability of tissue was assessed using 10μM methacholine and tissue was stimulated with 1-100SQ-U/ml HDM extract. After allergen challenge tissue was stimulated with anti-IgE to assess maximal contractile response to allergic stimuli, followed by a final methacholine challenge.

4.3 Results

4.3.1 Effect of allergen extract on control tissue

Allergen extracts can often be contaminated by other environmental constituents, such as LPS, and can contain multiple proteases. Initial experiments were conducted to investigate the effect of our HDM extract to determine whether any potential LPS contamination or protease activity had an inflammatory effect on lung tissue. Distal lung tissue explants were stimulated with either 1-1000µg/ml anti-IgE or 1-1000 SQ-U/ml HDM extract for 24 hours and supernatants analysed for TNFα content. 100ng/ml LPS was used as a positive control and 1000µg/ml goat-IgG as an isotype control for the anti-IgE antibody.

Figure 4.1

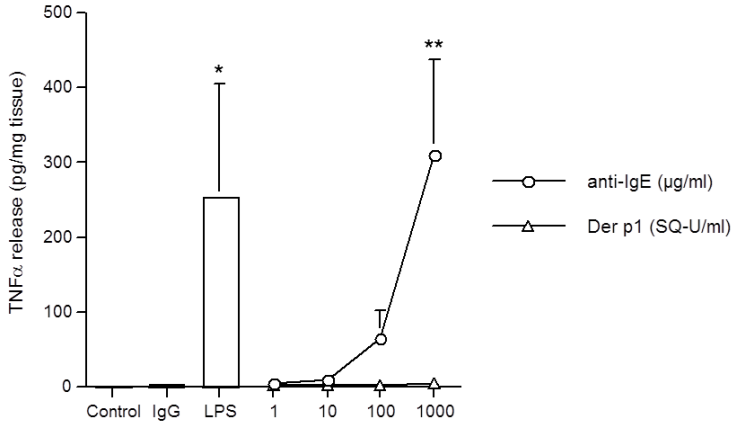


Figure 4.1 Dose responses to anti-IgE and Der p1 from control distal lung tissue. Distal lung tissue (n=5) was incubated in supplemented RPMI-1640 and stimulated with 1-1000µg/ml anti-IgE (circles) and 1-1000SQ-U/ml Der p1 (triangles) for 24 hours. 100ng/ml LPS was used as a positive control and 1000µg/ml goat-IgG was used as the isotype control. All values are expressed as mean±SEM * indicates $p<0.05$ and ** $p<0.01$.

Significant quantities of TNFα were released from tissue stimulated with 1000µg/ml anti-IgE and 100ng/ml LPS at 24 hours when compared to control (figure 4.1). TNFα release from tissue incubated with HDM extract remained at control levels indicating that the HDM extract has no inflammatory effect on unsensitised lung tissue. We concluded that if an inflammatory response occurs in tissue incubated in HDM-allergic serum it will be due to an IgE-mediated mechanism caused by the allergenic components of the HDM extract, in particular the protein Der p1.

4.3.2 Effect of sensitising serum on tissue

Atopic serum contains many more constituents than just IgE. It was therefore important to assess whether incubating lung tissue explants with human serum used for sensitisation had any effect on the tissue and the release of inflammatory mediators. Control lung tissue and lung tissue incubated in atopic serum pooled from

HDM-allergic volunteers was stimulated with 1-1000µg/ml anti-IgE, 100ng/ml LPS and 1000µg/ml IgG.

Figure 4.2

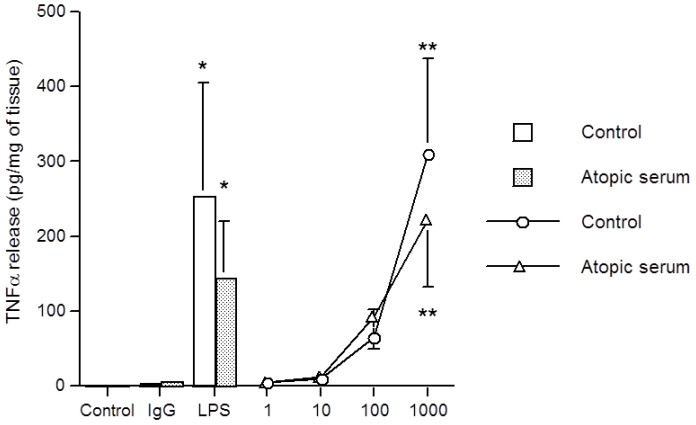


Figure 4.2 Dose responses to anti-IgE from control tissue and tissue incubated in atopic serum. Distal lung tissue (n=5) was incubated in RPMI-1640 (open bars and circles) or RPMI-1640 supplemented with 10% atopic serum (serum pool 1) (shaded bars and triangles) with dose responses to anti-IgE for 24 hours. 100ng/ml LPS was used as a positive control and 1000µg/ml IgG was used as the isotype control. All values are expressed as mean±SEM and expressed as pg/mg tissue. * indicates $p<0.05$ ** indicates $p<0.01$ compared to control.

The response to anti-IgE in sensitised and unsensitised tissue was assessed to determine whether factors within human serum were having an effect on the release of inflammatory mediators. In both control and tissue incubated with 10% atopic serum, a significant amount of TNFα was released when tissue was stimulated with 1000µg/ml anti-IgE and 100ng/ml LPS (figure 4.2). There was a trend for tissue incubated with serum to release slightly less TNFα, however this difference is not significant. This was also duplicated using a non-atopic serum pool. We concluded that the serum was not having an effect on the anti-IgE and LPS responses.

4.3.3 Tissue Viability

Lactate dehydrogenase (LDH) assays were utilised to determine if any treatment of the tissue, such as addition of stimuli or incubation in serum, was affecting cell viability. LDH release showed little difference when compared to unstimulated control tissue indicating that the tissue under all conditions is remaining viable with minimal cell death occurring.

From these preliminary experiments we concluded that incubation with 10% serum and stimulation with HDM extract were having no negative effects on the lung explants.

4.3.4 Cytokine release from an allergic donor

In order to determine whether the passive sensitisation was successful we investigated the release of different cytokines that may be involved in an allergic inflammatory response.

Lung tissue obtained from a nut allergic donor (HL042, see table 4.1) enabled us to identify cytokines being released in a known allergic reaction by stimulating the tissue with mixed nut extract, in order to screen for appropriate mediators in passive sensitisation experiments. Of note, this patient was on prescribed corticosteroids at time of surgery.

Table 4.1

HL042	
Age	20
Gender	Female
History of asthma	Since childhood, well controlled
History of allergy	Aspirin, NSAIDs, Nuts
Reason for surgery	Bullectomy, spontaneous pneumothorax

Table 4.1 Donor HL042 patient data - history of nut allergy. Data from nut allergic patient undergoing surgery for bullus repair.

Figure 4.3

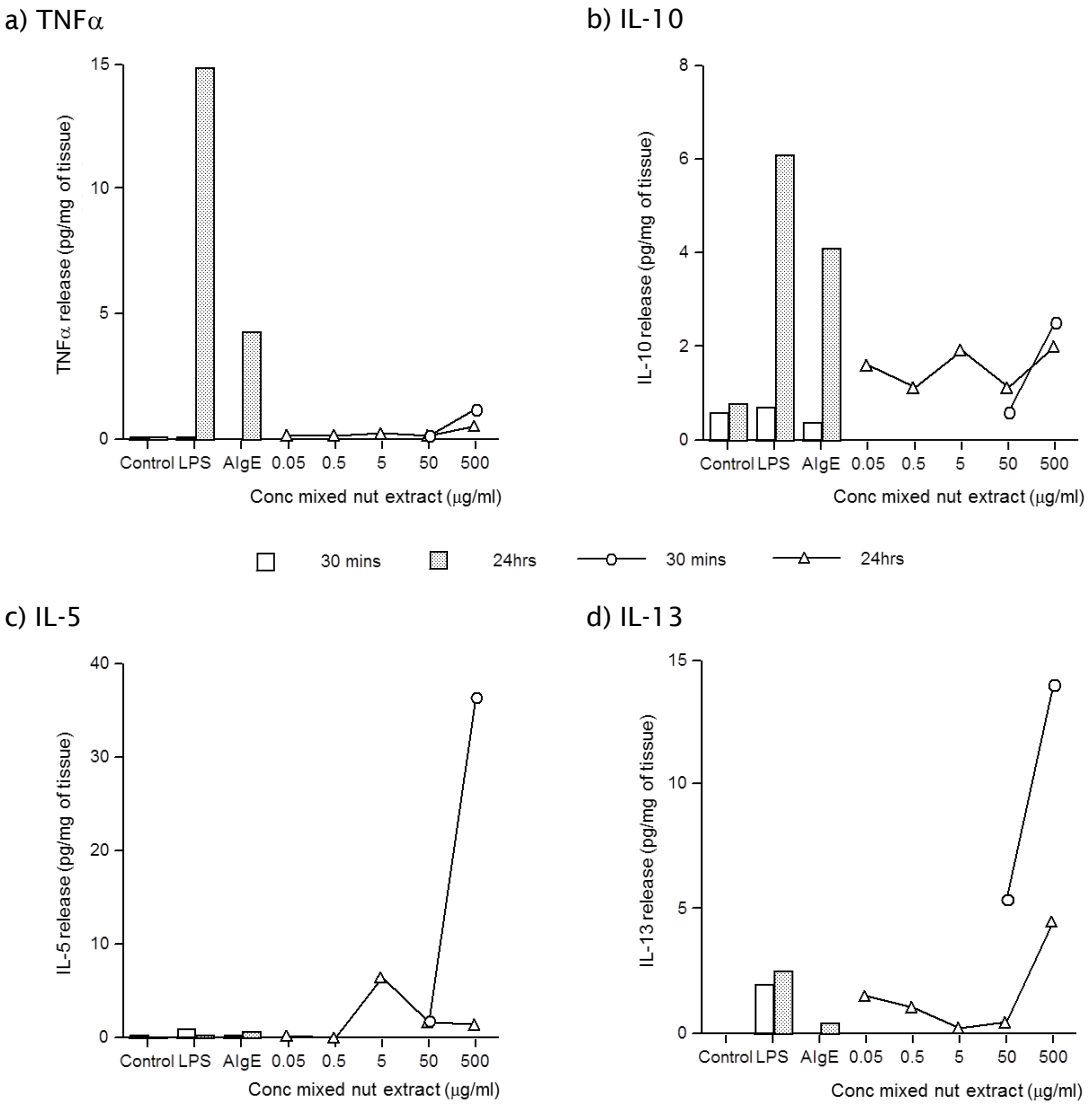


Figure 4.3 Stimulation of distal lung tissue from a nut allergic patient with mixed nut extract. HL042 explants were incubated in RPMI-1640 and stimulated with 100μg/ml anti-IgE, 100ng/ml LPS or 0.05-500μg/ml mixed nut extract. Tissue was harvested and supernatant aliquoted at 30 minutes (open bars and circles) and 24 hours (shaded columns and triangles). Concentrations of (a) TNFα, (b) IL-10, (c) IL-5 and (d) IL-13 were determined by ELISA and corrected for tissue weight.

Figure 4.3 shows that there was negligible release of the classical pro- and anti-inflammatory cytokines a) TNFα and b) IL-10 and the Th_2 cytokines c) IL-5 and d) IL-13 from unstimulated distal lung tissue explants. At 30 minutes (open bars and circles) there was little release of TNFα and IL-10 in tissue stimulated with LPS, anti-IgE or nut extract. On the other hand, at 30 minutes there was marked release of IL-5 and IL-13 in response to the nut extract (figure 4.3c and d). At 24 hours (shaded bars and triangles) a clear TNFα and IL-10 response is seen following LPS and anti-IgE stimulation when compared to control, however no apparent pattern is seen in response to the nut extract dose response. In the case of IL-5 and IL-13 there is little response to LPS and anti-IgE whereas there does appear to be some release of these

cytokines in response to the nut extract, albeit at lower concentrations than seen at the earlier time point. The results obtained suggest that IL-5 and IL-13, particularly at the 30 minute time point, would give a good indication as to whether the lung explants were being successfully passively sensitised and responding to allergen stimulation.

4.3.5 Passive Sensitisation of distal lung tissue explants

A number of preliminary experiments were also conducted in order to optimise passive sensitisation of lung tissue. Consequently, we compared incubating tissue with 10% and 20% serum at 4ºC and room temperature. We concluded that the optimum conditions for the passive sensitisation protocol were to incubate tissue in 10% serum at room temperature overnight before stimulating the explants the following day, similar to protocols used by Tunon de Lara *et al*, 1995 and Watson *et al*, 1998 for the passive sensitisation of bronchial strips. We also created a new serum pool (serum pool II) to increase our Der p1-specific IgE titre to a RAST class of 4 (see materials and methods chapter, table 2.2).

Passive sensitisation experiments were conducted using the optimised protocol and supernatants analysed for cytokine release. As in chapter 3, patients on prescribed corticosteroids were omitted.

Figure 4.4

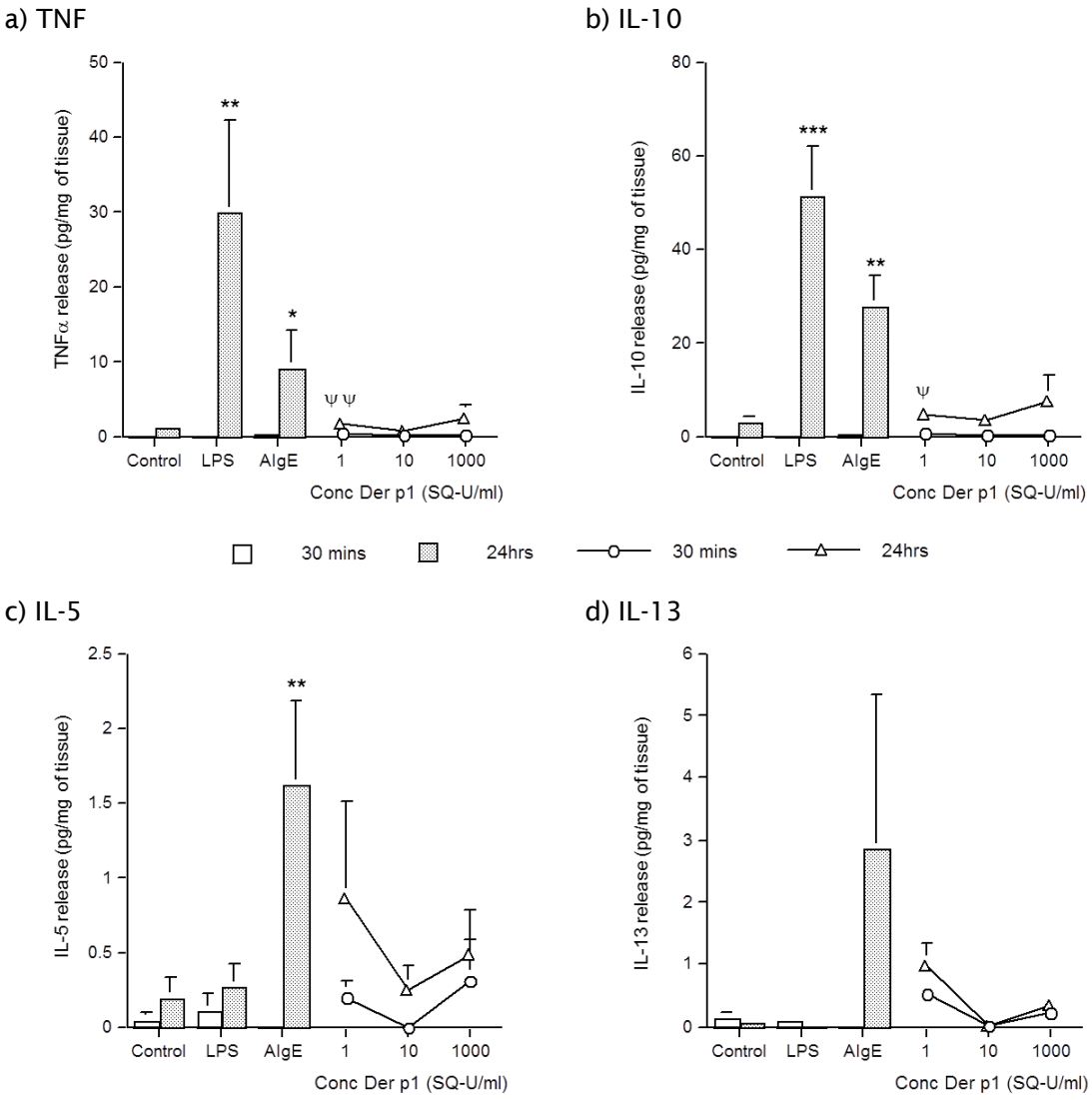


Figure 4.4 Cytokine release from sensitised tissue after stimulation with allergen. Distal lung tissue (n=13) was incubated with 10% atopic serum (serum pool II) at room temperature overnight and then stimulated with 100ng/ml LPS, 100μg/ml anti-IgE and 1-1000SQ-U/ml Der p1. Explants were harvested and supernatants aliquoted at 30 minutes (open bars and circles) and 24 hours (shaded bars and triangles). Concentrations of (a) TNFα, (b) IL-10, (c) IL-5 and (d) IL-13 were determined by ELISA and corrected for tissue weight. ψ indicates $p<0.05$ and ψψ $p<0.01$ for 30 minute time points compared to 30 minute control. * indicates $p<0.05$, ** $p<0.01$ and *** $p<0.001$ for 24 hour time points compared to 24 hour control.

From figure 4.4, where significant cytokine release was determined from lung parenchyma passively sensitised with atopic serum and then stimulated with Der p1, we have shown the corresponding cytokine release from control tissue in table 4.2 below, in order to compare the responses.

Table 4.2

1 SQ-U/ml Der p1	Atopic (pg/mg)	Control (pg/mg)
TNF 30mins	0.31 ± 0.1	0.65 ± 0.2
IL-10 30mins	0.54 ± 0.2	1.10 ± 0.4

Table 4.2 Cytokine release from distal lung tissue explants stimulated with 1SQ-U/ml Der p1. Data from figure 4.4 has been reworked to compare the significant cytokine release induced by stimulation of tissue passively sensitised with atopic serum with a low dose of Der p1, compared to the equivalent response in control tissue incubated in RPMI-1640.

Figure 4.4 shows that stimulation of passively-sensitised distal lung tissue explants with 100μg/ml anti-IgE failed to cause any significant release of any of the cytokines measured at the 30 minute time point but did cause significant release of the cytokines IL-5, TNFα and IL-10 at 24 hours. A similar pattern is seen with LPS stimulation (100ng/ml) except no significant release of IL-5 was measured at either time point. There was also no significant release of IL-13 measured at either time point. Stimulation with the lowest concentration of HDM extract (1SQ-U/ml) at only the 30 minute time point for TNFα and IL-10 release was found to be significant using the non-parametric Friedman test with Dunn's multiple comparison post hoc test. This very low level of release is unlikely to be biologically significant as release was close to the assay limits of detection and therefore there is likely to be a large signal to noise ratio. Also when taking into account data presented in table 4.2, comparing these significant points with unsensitised control tissue stimulated with 1SQ-U/ml HDM extract, it can be seen that the control tissue released more of each cytokine. This suggests that the passive sensitisation of distal lung tissue did not work as we did not see an increase in cytokine release from sensitised tissue compared to control tissue.

4.3.6 Passive sensitisation of bronchial strips

The results shown so far are unable to confirm whether or not lung explants were being successfully passively sensitised. In order to validate our serum pool we utilised a bronchial smooth muscle contraction model as previously described by Tunon de Lara *et al*, 1995 and Watson *et al*, 1998. Thus bronchial strips from donated lung were incubated overnight in atopic serum, non-atopic serum (serum pool II) and RPMI control, as described in section 2.4.1. Tissue was strung up into tissue baths and stimulated with methacholine, a non-selective muscarinic receptor agonist that causes bronchial smooth muscle contraction, increasing concentrations of the allergen Der p1, and 100μg/ml anti-IgE. Change in tension in the bronchial strip was recorded and measured throughout, as shown below in figure 4.6, a read out from a representative experiment.

Figure 4.5

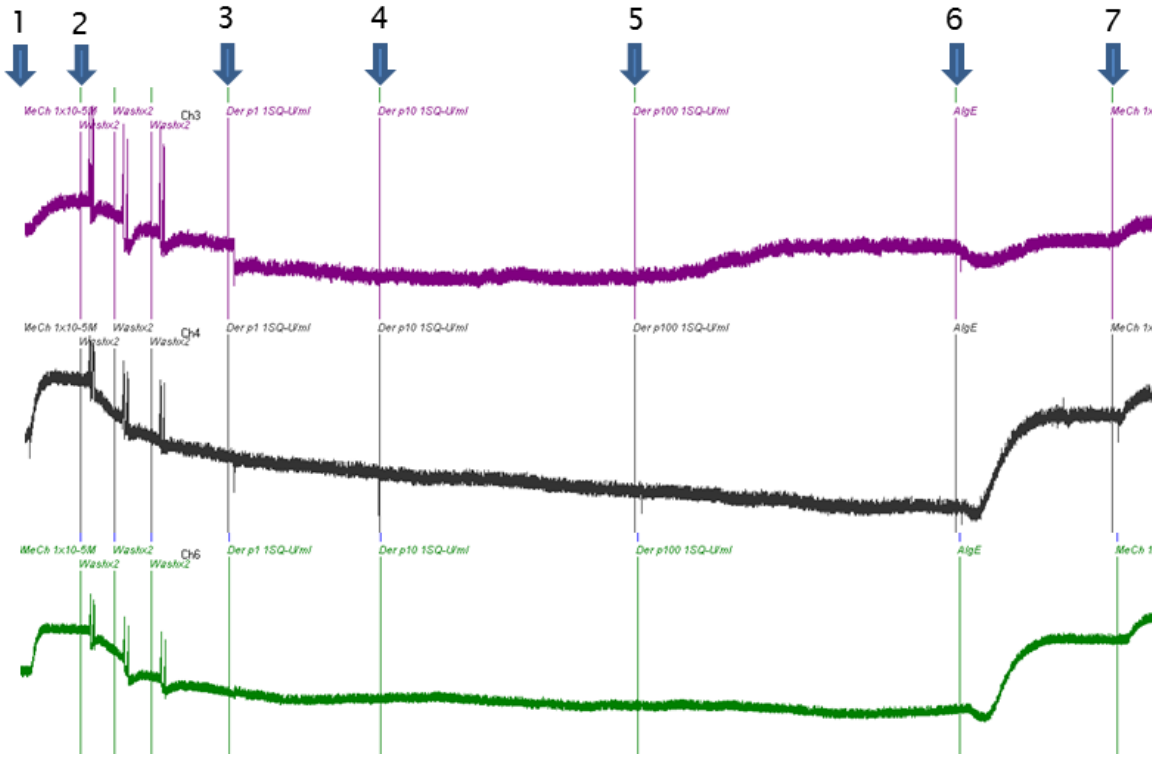


Figure 4.5 Representative traces from passive sensitisation of proximal airway strips. Bronchial strips were incubated in 10% atopic serum (top, purple), non-atopic serum (middle, black) and in RPMI (bottom, green) and treated with (1) 10μM methacholine, (2) washed x3 with Kreb's buffer, then exposed to (3) 1, (4) 10 and (5) 100SQ-U/ml Der p1, (6) anti-IgE and (7) 10 μM methacholine. Figure is representative of 5 experiments.

Change in tension from baseline was recorded and final results are expressed in figure 4.7 as a percentage of the initial methacholine contraction.

Figure 4.6

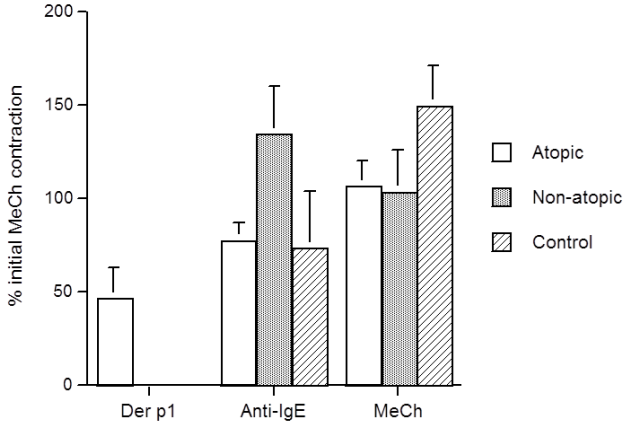


Figure 4.6 Smooth muscle contraction of passively sensitised bronchial tissue. Bronchial strips were incubated overnight in 10% atopic serum (open bars), 10% non-atopic serum (shaded bars) and RPMI containing no serum (striped bars) at room temperature (n=5). Tissue viability was assessed with initial treatment with 10μM methacholine and then washed thoroughly 3 times and allowed to return to basal tension. Tissue was then stimulated with 100SQ-U/ml Der p1, followed by anti-IgE and then a final dose of methacholine. Change in tension was recorded and results expressed as a percentage of the smooth muscle contractile response to the initial dose of methacholine.

Figure 4.6 shows that only bronchial tissue incubated in the atopic serum pool contracted upon stimulation with 100SQ-U/ml Der p1. There was no effect of Der p1 in the control tissue incubated in RPMI and tissue incubated with the non-atopic serum pool. This suggests the Der p1-specific IgE present in the atopic serum pool was able to bind to mast cells present in the tissue and allow mast cell activation and degranulation upon allergen provocation, resulting in contraction of the smooth muscle. We can conclude that the atopic serum (serum pool II) was able to successfully passively sensitise bronchial tissue. This data may indicate that the serum was able to passively sensitise the tissue sufficiently to induce a visible result but was not potent enough to fully sensitise the tissue.

4.3.7 Passive sensitisation of parenchymal and bronchial tissue explants

The serum pool has been validated and we have shown that it can successfully sensitise tissue and cause bronchial smooth muscle contraction upon allergen stimulation, which confirms that the failure of the passive sensitisation in section 4.3.5 was not due to an inability of the serum to sensitise mast cells. We therefore carried out parallel experiments using parenchymal and bronchial tissue explants from the same lung sample used for validation of the serum by bronchial smooth muscle contraction in section 4.3.6.

When generated supernatant from matched distal and proximal airway explants was analysed, again there was no significant release of TNFα, IL-10, IL-5 or IL-13 from either compartment in response to the HDM extract (n=6, data not shown).

As there was no detectable increase in the production of inflammatory cytokines from passively sensitised tissue we decided to investigate the release of a known preformed and stored mast cell mediator, histamine.

4.3.8 Histamine bioassay

We utilised a histamine bioassay developed at GlaxoSmithKline, Stevenage, to assess whether histamine was a more suitable marker for passive sensitisation in our model. This bioassay involved using CHO cells stably transfected with the histamine H_1 receptor. A Ca^{2+} influx occurs upon histamine binding to its receptor on these cells, which can be quantified using a fluorescent calcium indicator dye as described in section 2.7.

Initially, experiments were conducted to establish the optimum number of cells needed per well of the assay plate. Plates were therefore seeded with different numbers of cells and these stimulated with a dose response of histamine.

Figure 4.7

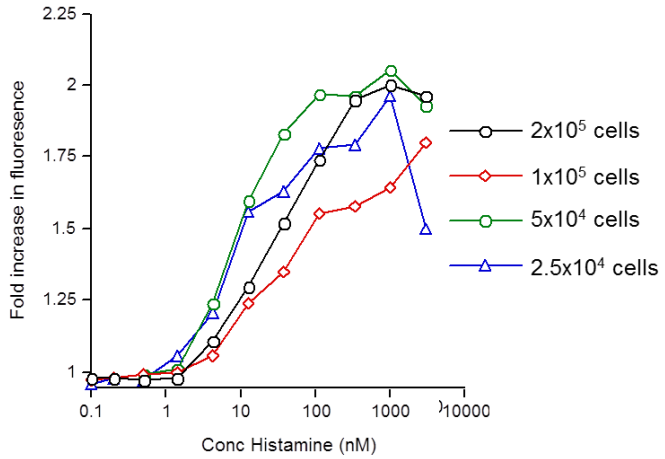


Figure 4.7 Establishing optimum CHO-H_1 cell density. Clear bottomed black-sided plates were seeded with $2.5x10^4$ (blue triangles), $5x10^4$ (green circles), $1x10^5$ (red diamonds) and $2x10^5$ CHO-H_1 cells (black circles) and stimulated with 0.1-3000nM histamine (n=4). Results expressed as mean fold increase in fluorescence.

In subsequent experiments a cell density of $5x10^4$ cells per well was selected as this number of cells seemed to be slightly more sensitive than other cell densities and produced a full dose response curve.

We also needed to confirm that the calcium influx was being caused by the activation of the H_1 receptor expressed on the CHO cells. We therefore utilised the H_1 receptor antagonist mepyramine to confirm the specificity of the response.

Figure 4.8

a) Stimulation with histamine

b) Stimulation with supernatant

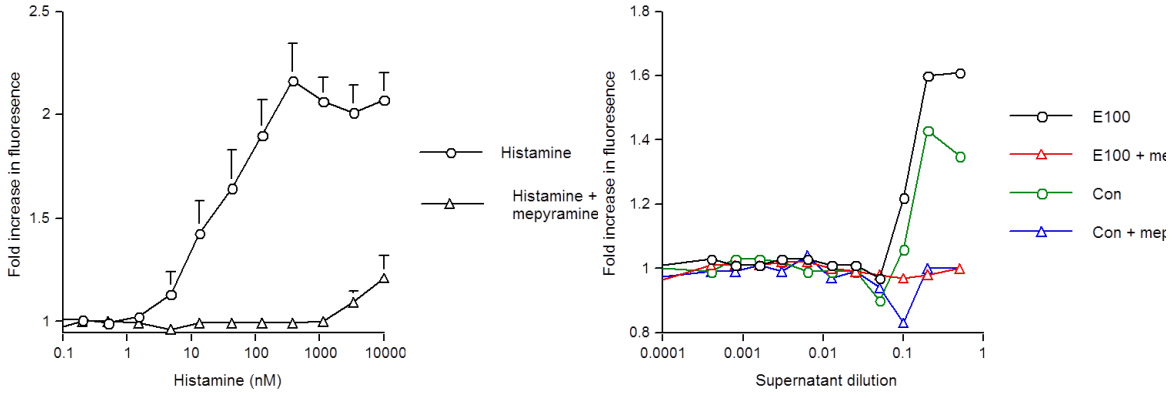


Figure 4.8 Effect of mepyramine on CHO-H_1 cells. 5x10^4 CHO-H_1 cells were stimulated with a) 0.2-10000nM histamine in the absence (circles) or presence (triangles) of 1μM mepyramine (n=12) and b) supernatant generated from unstimulated control distal lung tissue explants (green circles and blue triangles) and from explants stimulated with 100μg/ml anti-IgE (E100) (black circles and red triangles) in the absence (circles) and presence (triangles) of 1μM mepyramine (n=1).

Figure 4.8 a) shows that upon the pre-incubation of cells with mepyramine (triangles) the histamine-induced Ca^{2+} mobilisation in CHO-H_1 cells was antagonised indicating that the histamine receptors are functional and the Ca^{2+} mobilisation response is being mediated through them. In 4.8 b) supernatant from unstimulated (green and blue) and anti-IgE stimulated (black and red) distal lung tissue explants were also able to cause calcium mobilisation as seen by the increase in fluorescence. This was also effectively blocked by mepyramine indicating that only histamine within the supernatants was inducing this response. This data also shows that there is basal release of histamine from unstimulated distal lung tissue, as this also caused an increase in fluorescence. This may be due to an injury response during the preparation of the explants.

The histamine bioassay was utilised to analyse the release of histamine from the nut allergic patient in section 4.2.4, to assess whether this was truly an allergic response to the nut extract.

Figure 4.9

a) Histamine release

b) Histamine release (fold over control)

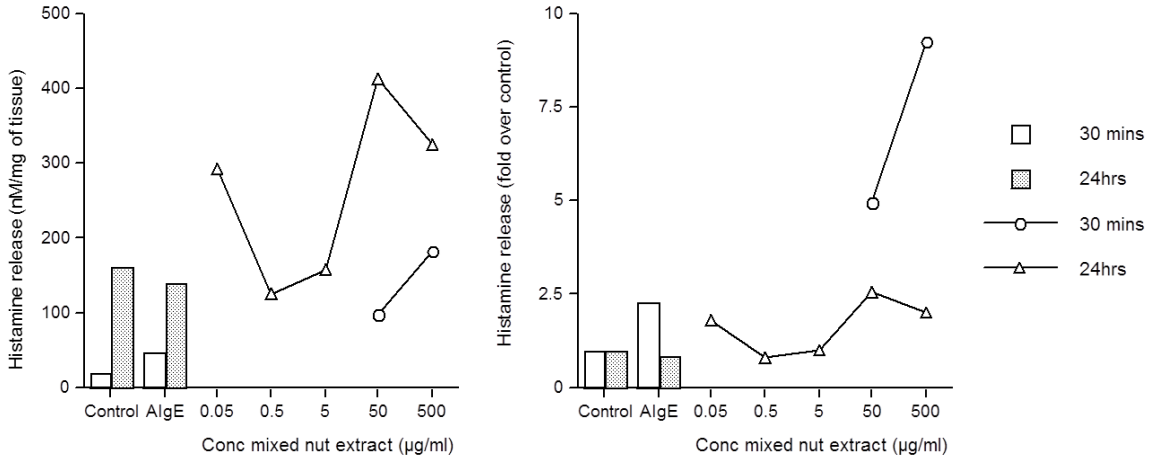


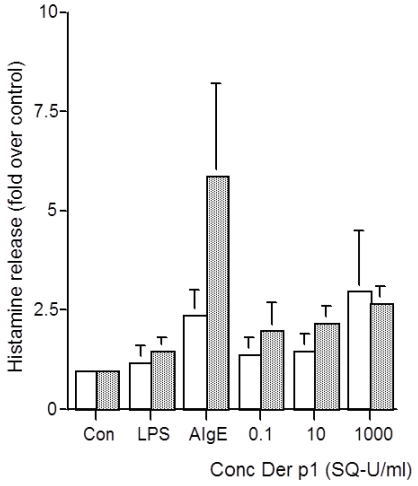
Figure 4.9 Histamine release from lung tissue stimulated with a mixed nut extract. Distal lung tissue explants from patient HL042 were incubated in RPMI-1640 and stimulated with 100µg/ml anti-IgE and 0.05-500µg/ml mixed nut extract. Tissue was harvested and supernatant aliquotted at 30 minutes (open bars and circles) and 24 hours (shaded bars and triangles). a) Histamine concentration was obtained using the histamine bioassay, corrected for tissue weight and expressed as nM/mg tissue. b) Data from a) was reworked and expressed as fold over control for the same time point.

Figure 4.9 shows that the mixed nut extract caused a substantial amount of histamine release compared to control levels and the anti-IgE response by 30 minutes and still by 24 hours. There was more histamine present in the supernatant at 24 hours compared to 30 minutes for all the conditions (figure 4.9 a), however when expressing the same data as fold over control for each time point, in figure 4.9 b), it is evident that the main response occurred by 30 minutes, with an almost 10-fold increase in histamine release (circles) induced by the highest concentrations of mixed nut extract, whereas this drops to only around a 2.5-fold increase at 24 hours (triangles). The high levels of histamine detected support the clinical data and allergic phenotype obtained for patient HL042 (see table 4.1) and strengthen the hypothesis that the cytokine data shown in figure 4.3 was released due to an allergic mast cell response.

We went on to utilise the histamine bioassay to assess histamine release from control lung parenchyma and airway and lung tissue that had been incubated with atopic serum. Data for the 30 minute time point is shown, as at this time point the greatest difference from control was observed in HL042.

Figure 4.10

a) Distal lung tissue



b) Proximal airway

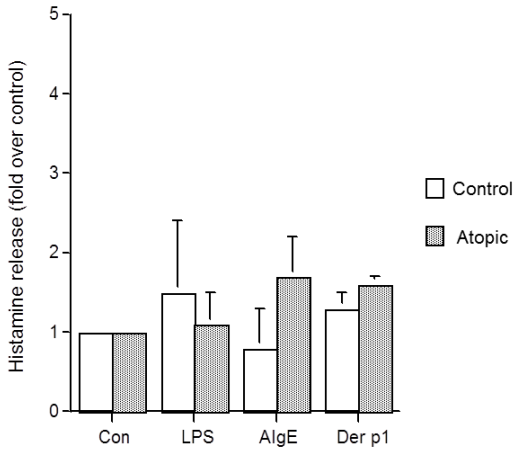


Figure 4.10 Histamine release from distal lung tissue and proximal airway explants. a) Distal lung tissue (n=4) and proximal airway (n=3) explants were incubated with 10% atopic serum (serum pool II) (shaded bars) or in RPMI without serum (open bars) at room temperature overnight and then stimulated with 100ng/ml LPS, 100μg/ml anti-IgE or 0.1-1000 SQ-U/ml Der p1. Proximal airway explants were stimulated with 1000SQ-U/ml Der p1. Explants were harvested and supernatants aliquotted at 30 minutes. Histamine concentration was obtained using a histamine bioassay and corrected for tissue weight. Data is expressed as fold over control.

In both lung compartments control tissue released more histamine than tissue that had been incubated with atopic serum for all stimuli (data not shown). Expressing this data as fold over relevant control in figure 4.10 showed that there was no significant difference between control and sensitised tissue.

4.4 Discussion

We have utilised the lung explant model (Bochner *et al*, 1987) to investigate the effect of passive sensitisation of distal lung tissue, by incubating tissue with pooled serum from atopic individuals sensitised to a common environmental allergen, Der p1. Supernatants were then analysed for inflammatory cytokines.

Initial experiments were conducted to assess whether *Dermatophagoides pteronyssinus* allergen extract, the main allergenic constituent of which being the protein Der p1, could have an intrinsic inflammatory effect on lung tissue. It has previously been shown that Der p1 has cysteine protease activity (Chua *et al*, 1988) and may cause an inflammatory effect by activating protease-activated receptors (PARs); however there is conflicting data within the literature (Asokananthan *et al*, 2002, Adam *et al*, 2006).

Asokananthan and his group showed that Der p1 induced release of the cytokines IL-6 and IL-8 in a human alveolar type II pneumocyte cell line, A549, and PAR1/2 transfected HeLa cells. Using Ca^{2+} influx and the cysteine protease inhibitor E64 they associated this cytokine release with PAR2 activation (Asokananthan *et al*, 2002). Conversely, Adam *et al*, using the same cell lines, showed conflicting data where IL-8 release was independent of PAR2 activation (Adam *et al*, 2006). Both studies isolated Der p1 from house dust mite faecal extracts, however only Adam *et al* conducted experiments to ensure the isolated Der p1 was free of Der p3 and other serine protease contaminants. In this study they showed that the serine protease Der p3 triggered IL-8 release from A549 cells via PAR2 activation. This suggests that the cytokine release proposed by Askokananthan to be attributed to PAR2 may be due to contamination of their isolated Der p1 with other serine proteases.

The protease activity of Der p1 has also been linked to cleavage of tight junction proteins (Wan *et al*, 1999) and epithelial cell damage in the form of desquamation and shrinking that cause the release of inflammatory cytokines in the A549 epithelial cell line (Kauffman *et al*, 2006). In the study by Wan *et al*, 1999, 8-10ng of Der p1 protein was sufficient to reduce tight junction continuity and occludin (a constituent protein of tight junctions) content by 4 hours. In the study by Kauffman *et al*, 2006, a concentration of 10μg/ml purified Der p1 caused cell shrinking and desquamation. These morphological changes were reversed upon the addition of chymostatin, a non-specific protease inhibitor, and antipain, a serine protease-specific inhibitor. This may indicate that the purified Der p1 used by this group may be contaminated with serine proteases, as with Asokananthan's work. Surprisingly, Kauffman did not investigate the effect of the cysteine-protease inhibitor, E64, on the morphological changes in the

A549 cultures, but did utilise this inhibitor when investigating cytokine secretion. In this case E64 significantly reduced the secretion of IL-6 and IL-8 produced by A549 cells stimulated for 24 hours with 20μg/ml purified Der p1.

All these studies used purified Der p1 from HDM extract however, which may contain differing allergenic constituents and thus varying concentrations of active Der p1. This makes interpreting and comparing concentrations of Der p1 used difficult. Other contaminants could also be present and the activity of Der p1 in these studies may not occur *in vivo*. In fact, an *in vivo* model using mice deficient in toll-like receptor 4 (TLR4) demonstrated that house dust mite (HDM) extract elicits allergic inflammation via TLR4-mediated activation of airway structural cells, due to contamination with LPS and thus the activity of Der p1 is inconsequential (Hammad *et al* 2009). This may not be the case however in humans.

In our study we used standardised allergen extract provided by ALK-Abelló. In preliminary experiments we assessed whether our Der p1 had an inflammatory effect on peripheral lung tissue by stimulating unsensitised explants with 1-1000 SQ-U/ml *Dermatophagoides pteronyssinus* allergen extract (SQ-U/ml indicates a standardised concentration of allergen extract that has been enzymatically and immunologically tested to enable comparable studies). Similar concentrations of standardised HDM extract have been used in other studies. For example, Tunon de Lara *et al*, 1995, used 300SQ-U/ml HDM extract, to induce contractile responses in passively-sensitised isolated human bronchi. This indicates that the concentration range of HDM used in our study was appropriate. Our experiments show that there was no release of the classic inflammatory cytokine TNFα and no effect on cell viability of unsensitised distal lung tissue fragments incubated with our HDM extract within the concentration range used. We concluded that, in our model, this particular HDM extract has no inflammatory effect and thus if an inflammatory response was measured in sensitised tissue, it is due to an IgE receptor-mediated effect caused by the binding of Der p1 and subsequent cross linkage of receptor-bound IgE on mast cells.

It was also important to assess the effect of incubating tissue in human serum, which contains many different constituents that may influence either the tissue directly or the mediators being measured. For example, in the literature it has been reported that an immunoglobulin pool in human serum can bind to some cytokine receptors inhibiting the binding of the cytokine, which may preclude subsequent inflammatory events (Mosedale and Grainger, 1999). Our normal culture conditions occur in serum free media reflecting the low levels of protein in the alveoli. Proteins present in serum could potentially bind cytokine; however tissue was washed extensively with PBS before

stimulation so it is unlikely that high concentrations of serum constituents will remain within the tissue. We did however see a trend of decreased cytokine levels quantified in the supernatants of passively-sensitised lung tissue, although this was not significantly different from tissue incubated without serum.

As previously mentioned, Tunon de Lara *et al* successfully passively sensitised isolated bronchial tissue as demonstrated by isometric contraction upon the addition of Der p1 (Tunon de Lara *et al*, 1995). In this study tissue was incubated in an undiluted atopic serum pool, obtained from asthmatic subjects whose concentration of both total and specific IgE to *D. pteronyssinus* was above 1000 IU/ml and 17.5 radioallergosorbent test (RAST) units/ml (i.e. 4+ RAST titre) respectively. A non-atopic serum pool with a total IgE concentration of less than 10 IU/ml was also used as a control. In a similar study conducted by Watson *et al*, similar concentrations of IgE within the serum was used however they incubated tissue in diluted serum (10% serum) and still obtained contraction of bronchial tissue, albeit from a different species of HDM, *D. farinae* (Watson *et al*, 1998). Both of these studies passively sensitised tissue by incubating it in serum overnight at room temperature. Within our study we adopted a similar protocol, incubating overnight at room temperature and in 10% serum for passive sensitisation, as utilised by Watson *et al*. This would reduce the amount of serum pool used and also reduce any effect serum may have on the tissue as discussed earlier. The serum pool used in our study also has a 4+ RAST titre for Der p1-specific IgE however the concentration of total IgE is less than 1000 IU/ml, being measured at 389 IU/ml (see table 2.2 in materials and methods). The lower concentration of total IgE and therefore allergen-specific IgE may mean that serum pool II may be less efficient at passively sensitising tissue compared to the serum used by other groups.

In preliminary experiments serum had no effect on cell viability and had no significant effect on cytokine release, although tissue incubated in serum on average released slightly less TNFα. Significant release of TNFα however was still achieved in response to anti-IgE and LPS stimulation in distal lung tissue, both incubated in 10% serum and control tissue. This coincides with data obtained in chapter 3 and data obtained by Barnicott and Hackett, who also showed comparable TNFα release from distal lung tissue in response to anti-IgE and LPS respectively (Barnicott *et al*, 2006, Hackett *et al*, 2008).

The main aim of this chapter was to develop a model to investigate allergic inflammation in peripheral lung tissue in response to stimulation with allergen. At time of consent, details of allergic history were obtained and thus it was possible to stimulate tissue obtained from a donor with a history of nut allergy (table 4.1). Distal

lung tissue from this donor was stimulated with a mixed nut extract (0.05-500μg/ml) and inflammatory cytokines were measured at 30 minute and 24 hour time points. The cytokine profile obtained enabled us to pinpoint likely cytokines that would give us a good indication of whether lung tissue passively sensitises and whether stimulation with Der p1 initiates an allergic inflammatory response. Due to the substantial release of the Th_2 cytokines IL-5 and IL-13 at the 30 minute time point and to a lesser extent at 24 hours, we concluded that release of these cytokines, especially at 30 minutes, may give a good indication as to whether the passive sensitisation was successful. At both time points however, stimulation with 100μg/ml anti-IgE, a concentration which elicited around a 4-fold increase in TNFα and IL-10 release above control at 24 hours, failed to cause notable release of IL-5 or IL-13 at either time point. This may be due to the effect of the bell-shaped dose response of IL-5 release in response to anti-IgE as discussed in chapter 3. We have also shown previously that anti-IgE does not cause IL-13 release in distal lung tissue. Alternatively, the anti-IgE antibody is not functioning as expected or stimulation with the mixed nut extract may have stimulated cells other than mast cells, for example a T cell and/or eosinophil infiltrate. Patient HL042 had a history of asthma since childhood therefore this cellular infiltrate may likely be present in asthmatic lung tissue (see table 4.1).

In subsequent attempts to passively sensitise distal lung tissue with atopic serum, no clear release of any of the cytokines measured above indicated that an allergic inflammatory response was occurring to the allergen. At both time points there was no significant release of IL-5 above control to LPS or Der p1 and there is only a small, but significant nonetheless, increase in TNFα and IL-10 release in response to 1SQ-U/ml Der p1. In a study by Mitsuta *et al*, 2001, significant release of IL-5 and TNFα was measured at 24 hours from lung tissue passively sensitised to *D. farinae* stimulated with 1.5μg/ml of the mite allergen. In this study however the serum utilised had a RAST of greater than 5, tissue was only sensitised for 2 hours and around 10 times more tissue (300mg) was challenged, whereas in our study the average tissue weight is 20-30mg. Increasing the amount of tissue will increase the number of effector cells present that could participate in an anti-IgE mediated fashion. This would allow for a greater degree of cytokine release, which would be easier to detect by ELISA. Unfortunately in Mitsuta's study they do not mention where they obtained their HDM allergen from, however they did test the endotoxin level of their culture medium containing mite extract and found it to be less than 50pg/ml. It is also unclear as to whether Mitsuta's group corrected cytokine release for tissue weight.

As bronchial hyper-responsiveness is an important feature of asthma many groups have used passive sensitisation to investigate the early phase reaction and airway

contractility. As previously mentioned, this model was used by Tunon de Lara *et al* who showed that only tissue incubated with an allergen-specific atopic serum would contract to allergen stimulation, whereas control tissue and tissue incubated in non-atopic serum did not; results that we have duplicated. The atopic serum contains substantially more allergen-specific IgE than the non-atopic serum and thus these results suggest that the tissue response is caused by an IgE-mediated effect. Evidence for this hypothesis is strengthened by results published by Watson *et al* who used the same model but were able to prevent passive sensitisation of bronchial tissue by depleting atopic serum of IgE. An immunohistochemical approach has also been conducted by Berger *et al* to determine which cells bound IgE from the serum. They found that there were significantly more IgE-bearing cells in passively-sensitised bronchial tissue compared to non-sensitised tissue, and the main cell binding this IgE was the mast cell (Berger *et al*, 1998). These mast cells were found in all bronchial compartments but mainly localised to the submucosa and smooth muscle layers of the bronchial wall. This provides evidence that IgE in the serum is able to penetrate deep within the dense bronchial tissue and bind to effector cells and thus would have no problem penetrating much less dense peripheral lung tissue.

It is feasible that IgE could bind to the airway smooth muscle to cause a direct effect, however the study by Berger *et al* also highlighted that there was no IgE-binding directly to the smooth muscle and therefore contractility was caused by the release of mediators from inflammatory effector cells upon activation by allergen. There is however conflicting data within the literature. A study by Gounni *et al* demonstrated high affinity IgE receptor expression on human airway smooth muscle cells, which upon IgE binding and crosslinking with an anti-IgE antibody, induced mobilisation of free calcium critical for smooth muscle contraction and the release of Th_2 cytokines (Gounni *et al*, 2005). To date, this is the only group to have reported these findings however and in contradiction to their work, it has more recently been shown *in vitro* that smooth muscle cell contraction was dependent on mast cell activation and not via a direct IgE-dependent response (Margulis *et al*, 2009).

Passive sensitisation of airway tissue and subsequent smooth muscle contraction therefore is a proven successful model that we could utilise to assess the viability of our atopic serum pool to passively sensitise tissue. Using this model our atopic serum (serum pool II) successfully passively sensitised bronchial tissue, in each case causing smooth muscle contraction upon stimulation with 100 SQ-U/ml Der p1. There was however no definitive release of IL-5, IL-13, IL-10 or TNFα by 30 minutes or 24 hours compared to matched control distal lung tissue and proximal airway tissue fragments in response to allergen stimulation. These fragments were from some of the same

donors that were used in the bronchial smooth muscle contraction experiments, which were successfully passively sensitised and contracted upon allergen stimulation.

As mentioned earlier, the levels of cytokine released from passively sensitised tissue may not be feasible for interpretation and perhaps smooth muscle contraction is more sensitive and therefore easier to obtain a meaningful read out from. On the other hand for some reason distal lung tissue may not passively sensitise. Contrary to this point however, some studies have investigated the contribution of the small airways (<2mm in diameter) to the EAR and airway hyper-responsiveness using passive sensitisation, albeit using contractility as a measurable parameter. In fact, Ellis *et al* showed that passively sensitised peripheral bronchi (0.5-2mm) were around 20-fold more potent at contracting than central bronchi (5-12mm) (Ellis *et al*, 1994). This was also supported by work using precision-cut lung slices, which enable examination of the contractile response of the small airways right down to the terminal bronchioles (<1.5mm) using quantitative videomicroscopy (Wohlsen *et al*, 2003). Both these studies showed that the EAR became stronger with decreasing airway size, with increased contractility and release of bronchoconstricting mediators such as histamine, leukotrienes and prostanoids. The release of these mediators from sensitised mast cells within and in close proximity to smooth muscle may account for the sensitivity of the airway contraction experiments. This is consistent with data we presented in chapter 3, where we have shown that distal lung tissue contains substantially more mast cells than the proximal airways. On the other hand, immunohistochemical analysis of biopsies from severe and mild asthmatics and normal non-atopic controls found a pattern of IgE-bound cells distributed along mucosal surfaces, extending from the proximal (>2mm) to the distal airways (<2mm) but not in the lung parenchyma (alveolar tissue), despite the presence of mast cells (Balzar *et al*, 2007). This was the case in all subjects regardless of levels of serum IgE. The authors concluded that the IgE process may be regulated by local mucosal responses rather than systemic, and that elevated serum IgE may be insufficient to induce high IgE binding to mast cells located in peripheral lung tissue, away from mucosal surfaces (Balzar *et al*, 2007). This may also indicate preferential binding of IgE to mast cells located within the mucosa, i.e. within airway tissue, over distal lung tissue mast cells and may explain why our atopic serum pool was able to sensitise bronchial tissue but was ineffective with distal lung tissue. However there was no detectable cytokine release from either proximal airway or distal lung tissue explants. In support of this hypothesis, it has been shown that expression of the high affinity IgE receptor on mast cells is highest in the central bronchi and gradually decreases with progression down the respiratory tree to the peripheral lung tissue, to become virtually absent in the alveolar region (Andersson *et al*, 2009).

As there was little cytokine release from sensitised lung fragments in response to allergen in our system, we decided to investigate the release of other preformed mast cell mediators. Histamine is a well known mast cell mediator, stored in granules and released upon degranulation. It plays an important role in the EAR contributing to bronchoconstriction, vasodilation and vascular permeability, mainly through interactions with the H_1 receptor (Lundequist and Pejler, 2010). We utilised a histamine bioassay developed by GlaxoSmithKline (Stevenage) using CHO cells stably transfected with the H_1 receptor (see materials and methods). Initial experiments were conducted showing that these cells responded in a concentration-dependent manner to histamine and that only histamine within the supernatant caused calcium mobilisation into the cells, indicating a high level of assay specificity.

Using this histamine bioassay we analysed supernatant generated from lung tissue from a nut-allergic individual (see table 4.1). Substantially more histamine was released by 30 minutes and 24 hours above control after stimulation with a mixed nut extract, indicative of mast cell degranulation. This strengthens the evidence that the cytokine and histamine response from this donor was a true allergic response. We also measured more histamine release at 24 hours compared to 30 minutes although the difference between control levels was less. Kinetically, maximum histamine release from human lung parenchyma fragments has been shown to occur by 15 minutes (Shulman *et al*, 1981). In this study they only went up to a 20 minute time point investigating the contribution of mast cells to the EAR however. More recently it has been shown that mast cells are able to rebuild their granular stores after degranulation and are able to undergo several cycles of degranulation and re-granulation, a process that takes less than 24 hours (Lundequist and Pejler, 2010). In fact, Xiang *et al* used time-lapse photography to show that mast cells are capable of recovering by 15 minutes, although these experiments were conducted using mouse mast cells (Xiang *et al*, 2001). This may explain why net histamine release is greater at 24 hours, especially as increased spontaneous release is likely to be occurring, as suggested by the observed increase in control histamine levels.

Histamine release from sensitised distal lung tissue and proximal airway fragments in response to Der p1 did not, however, exceed control levels. We can conclude that our atopic serum was not sufficient to passively sensitise lung fragments for measurable mediator release. Of interest nonetheless is the amount of histamine release between the different lung compartments, with distal lung tissue releasing 2-4 times more histamine than proximal airway tissue (data not shown). Other studies by Schulman *et al* went on to successfully passively sensitise distal lung tissue and bronchial airway fragments to measure histamine and cyclooxygenase (COX) metabolite release. In

agreement with our findings, they showed that distal lung tissue releases substantially more histamine than bronchial tissue along with COX metabolites, particularly prostaglandin D_2 (PGD_2) (Schulman *et al*, 1982). In this study they used substantially more tissue (around 50 times the weight) and a serum pool that contained substantially higher levels of IgE (around 6 times more) with a high specificity to their allergen of choice, ragweed antigen E. As mentioned earlier, increasing tissue weight may make it possible to measure the release of mediators. However, when doubling the amount of tissue utilised we still did not measure mediator release above control levels (data not shown).

It is more likely that our atopic serum pool (serum pool II) did not contain sufficient IgE to sensitise mast cells in distal lung tissue. It is also possible that the ratio of irrelevant IgE and Der p1-specific IgE in our atopic serum pool may have been too great. Consequently, binding of non-Der p1-specific IgE to mast cells could provide steric hindrance preventing IgE crosslinkage and FcεRI aggregation upon addition of the allergen. This inhibition of passive sensitisation has been demonstrated using basophils, leukocytes within peripheral blood that also express the high affinity IgE receptor. In the study by Van Toorenenbergen *et al*, they showed that in order to sensitise basophils only a small amount of allergen-specific IgE was needed to bind to free FcεRI, but pre-incubation of basophils with excess irrelevant IgE resulted in inhibition of subsequent passive sensitisation (Van Toorenenbergen *et al*, 1983).

4.4.1 Summary

In conclusion, we successfully passively sensitised proximal airway tissue that contracted upon stimulation with Der p1. No conclusive cytokine or histamine release from proximal airway or distal lung tissue fragments was measured however. This may reflect the sensitivity of the smooth muscle contraction experiments as only very small amounts of potent bronchoconstrictors from activated mast cells, such as leukotrienes, are needed to induce contraction. Mast cells have also been shown to be located in and close to smooth muscle layers (Berger *et al*, 1998) and express high levels of the high affinity IgE receptor, whereas distal lung mast cells have lower expression (Andersson *et al*, 2009). Only a small amount of allergen-specific IgE is required to bind to free FcεRI to sensitise cells (Van Toorenenbergen *et al*, 1983), however they must be in close proximity in order for allergen to crosslink the molecules; this is more likely to occur in bronchial mast cells that express more FcεRI (Andersson *et al*, 2009). In addition, the atopic serum pool used in our study probably contained too little Der p1-specific IgE and too much irrelevant IgE that would make sensitising peripheral lung mast cells even more challenging. Ideally, serum from atopic donors with severe

allergy to a specific allergen would have been used to create an allergic serum with a very high total IgE and very high allergen specific-IgE titre.

Chapter 5
Neutrophil Shape Change

5.1 Introduction

The human lung explant model has been used extensively to investigate the inflammatory process in lung tissue *ex vivo* (Bochner *et al*, 1987). Utilising this model, we have generated supernatants from lung tissue stimulated with inflammatory stimuli, such as anti-IgE and lipopolysaccharide (LPS), and quantified the release of a range of inflammatory cytokines and chemokines using ELISA. This data, however, does not give an indication as to how functional these inflammatory mediators are.

It has been shown that cytokines are susceptible to proteolytic degradation (Zhao *et al*, 2005); bind to receptors, including soluble receptors; form complexes with autoantibodies and other cytokine-binding proteins (Shute *et al*, 1997, Kurdowska *et al*, 1996, Marshall *et al*, 2001); or may be trapped within the extracellular matrix (ECM) bound to proteoglycans (Witt and Lander, 1994). This means that a number of factors may influence the ability of these inflammatory mediators to elicit their physiological role within this complex environment.

We have shown in chapter 3 that lung tissue produces substantial amounts of IL-8, which has also been reported in other studies (Hacket *et al*, 2008). It is well established that IL-8 is a potent neutrophil chemoattractant and causes a robust shape change response in neutrophils. Neutrophils are also an abundant cell population present in peripheral blood and are therefore an ideal target for developing a shape change bioassay. Upon stimulation by IL-8, neutrophils rapidly change from a resting spherical shape to become more polar and form extending pseudopodia that facilitate the extravasation of the migrating cell (Zigmond *et al*, 1981, Watts *et al*, 1991, Baggliolini and Kernon, 1992). Using flow cytometry, this change in neutrophil shape is seen as an increase in forward scatter and can be used to investigate neutrophil response to different stimuli (Cole *et al*, 1995).

This chapter focuses on the development of a whole blood assay to assess the functionality of the chemokine IL-8 within our generated supernatants. The assay assesses neutrophil shape change in a system that closely mimics the chemotactic process and the *in vivo* environment, making the assay physiologically relevant.

We hypothesise that the shape change response to IL-8 within generated supernatant may be inhibited by other constituents present in the supernatant or tissue that may prevent it from binding to receptors and eliciting a response in neutrophils.

5.2 Methods

5.2.1 Generation of lung explant supernatant

Distal lung tissue supernatants were generated using the lung explant model as described in materials and methods, section 2.2.1. Distal lung tissue was stimulated with a high (100μg/ml) and low (1μg/ml) concentration of anti-IgE (AIgE), the standard positive control concentration (100ng/ml) of LPS and tissue was also incubated in RPMI as an unstimulated control. These concentrations were chosen as we have previously shown in chapter 3 that a high concentration of anti-IgE and LPS induces the release of a significant amount of IL-8. We chose to investigate a low concentration of anti-IgE as we have also shown that anti-IgE stimulation can produce bell-shaped cytokine dose response curves, as shown in figure 3.2.

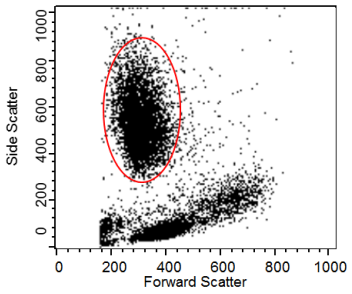
As we have shown in chapter 3 that steroid treatment can alter distribution of inflammatory cells and effect cytokine release from tissue, we have omitted patients prescribed corticosteroid at time of surgery from subsequent analysis.

5.2.2 Whole blood neutrophil change assay

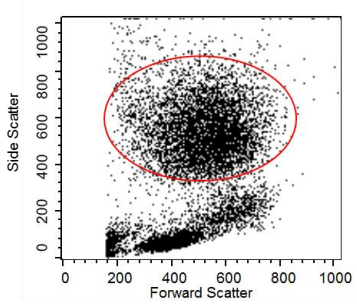
9ml blood was taken from healthy volunteers using a syringe and mixed with 1ml 3.8% sodium citrate to prevent clotting and used within 30 minutes of venepuncture. 100μl of whole blood was stimulated with 0.039-10nM IL-8, 0.002-1000nM Groα, or dilutions of supernatant generated using the lung explant model (described in section 2.2.1) and incubated at 37°C for 5 minutes. Cells were then fixed using ice-cold fixation buffer and left on ice for a further 5 minutes. Red blood cells were then gently lysed using an ammonium chloride lysis buffer (see section 2.9.1). Samples were then analysed by flow cytometry based on their forward scatter / side scatter profile as shown in figure 5.1 a) and b).

Figure 5.1

a) Buffer control



b) Stimulation with IL-8



c) Cell Autofluorescence

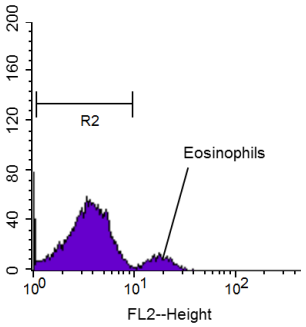


Figure 5.1 Representative analysis of stimulated whole blood using flow cytometry. a) Unstimulated whole blood. b) Whole blood stimulated with IL-8. Granulocyte cell population is circled in red. c) Autofluorescence of gated granulocytes. Eosinophils identified as the smaller cell population with a higher autofluorescence. Neutrophils gated within R2 region.

For neutrophil shape change analysis, granulocytes were gated and neutrophils selected as the cell population with low fluorescence through the FL-2 gate (R2 region), as shown in figure 5.1 c). Neutrophil shape change was determined by calculating the difference in mean neutrophil forward scatter between stimulated and unstimulated samples.

5.2.3 Neutralisation or antagonism experiments

For neutralisation experiments 1μg/ml neutralising IL-8 antibody was mixed with the agonist solution and incubated at room temperature for a minimum of 30 minutes.

For receptor antagonism experiments, whole blood was incubated with the relevant inhibitor at room temperature for 15 minutes before the addition of agonist. Concentrations of 1μM CXCR2 antagonist (compound A donated by GlaxoSmithKline), 1μg/ml anti-CXCR1 antibody and 20μM leukotriene B_4 receptor (BLTR) antagonist (SB209247 donated by GlaxoSmithKline) were used in these experiments.

Upon stimulation of whole blood with agonist the protocol was followed as described above in section 5.2.1 or section 2.9.

5.3 Results

5.3.1 IL-8 Dose Response

Initial experiments involved developing an assay using neutrophil shape change to assess the functionality of chemokines within whole blood. Firstly, we chose to investigate the well-known neutrophil chemoattractant IL-8 (CXCL8) and look at its effect on neutrophil shape change.

Figure 5.2

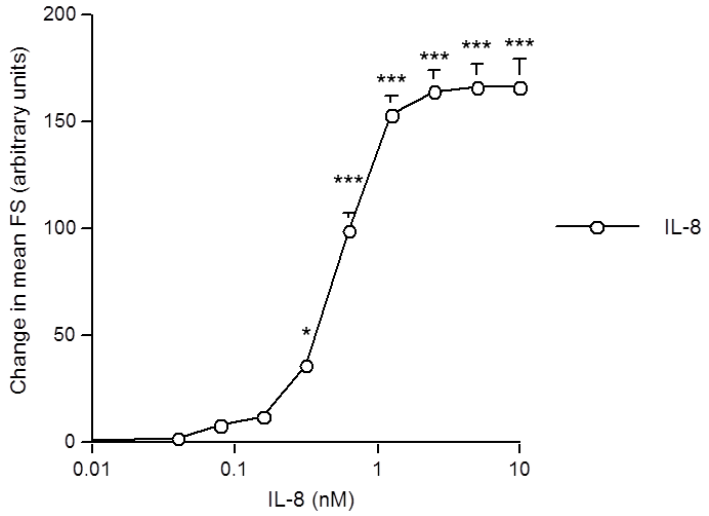


Figure 5.2 Dose response to recombinant human IL-8. Human whole blood (n=15) was incubated for 5 minutes with recombinant human IL-8 (0.039-10nM) at 37ºC. Cells were then fixed for 5 minutes and erythrocytes lysed before analysis using FACScalibur. Results are expressed as mean±SEM. * indicates $p<0.05$, *** indicates $p<0.001$

The change in neutrophil shape in response to IL-8 consistently resulted in a sigmoidal dose response curve. Between 0.3-1nM IL-8, an extremely steep slope occurs, before maximal change in mean forward scatter is reached at concentrations above 2.5nM IL-8, as shown in figure 5.2. Statistically significant amounts of shape change occurs at concentrations greater than or equal to 0.313nM IL-8 with the EC_{50} equalling approximately 0.55nM IL-8.

The assay appears to demonstrate a clear response to recombinant human IL-8. However, to investigate the reproducibility of the assay, blood was taken from the same donor over the course of two weeks and stimulated with IL-8, as shown in figure 5.3.

Figure 5.3

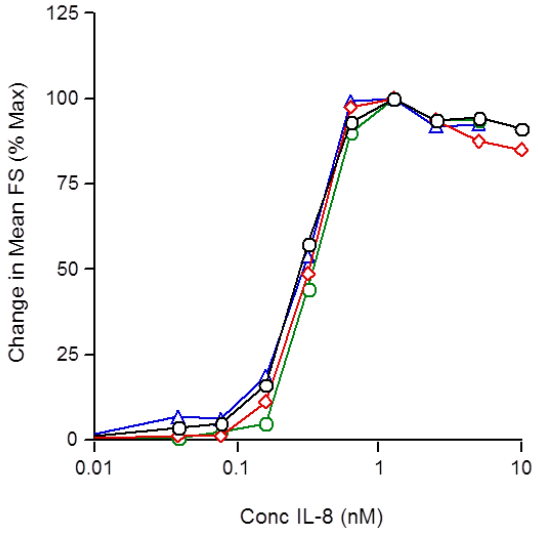


Figure 5.3 Response to recombinant human IL-8 in the same blood donor over the course of 2 weeks. Blood was stimulated as in materials and methods with a dose response to IL-8 (0.039-10nM). Results expressed as change in mean forward scatter as a percentage of the maximum change in mean forward scatter.

Figure 5.3 demonstrates that when treating the blood from the same donor with IL-8 on different occasions the neutrophil shape change response is superimposable, indicating that the assay is extremely reproducible.

5.3.2 Supernatant Dose Responses

We then applied the shape change assay to supernatants from the lung explant model, as described previously in section 5.2.1.

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Figure 5.4

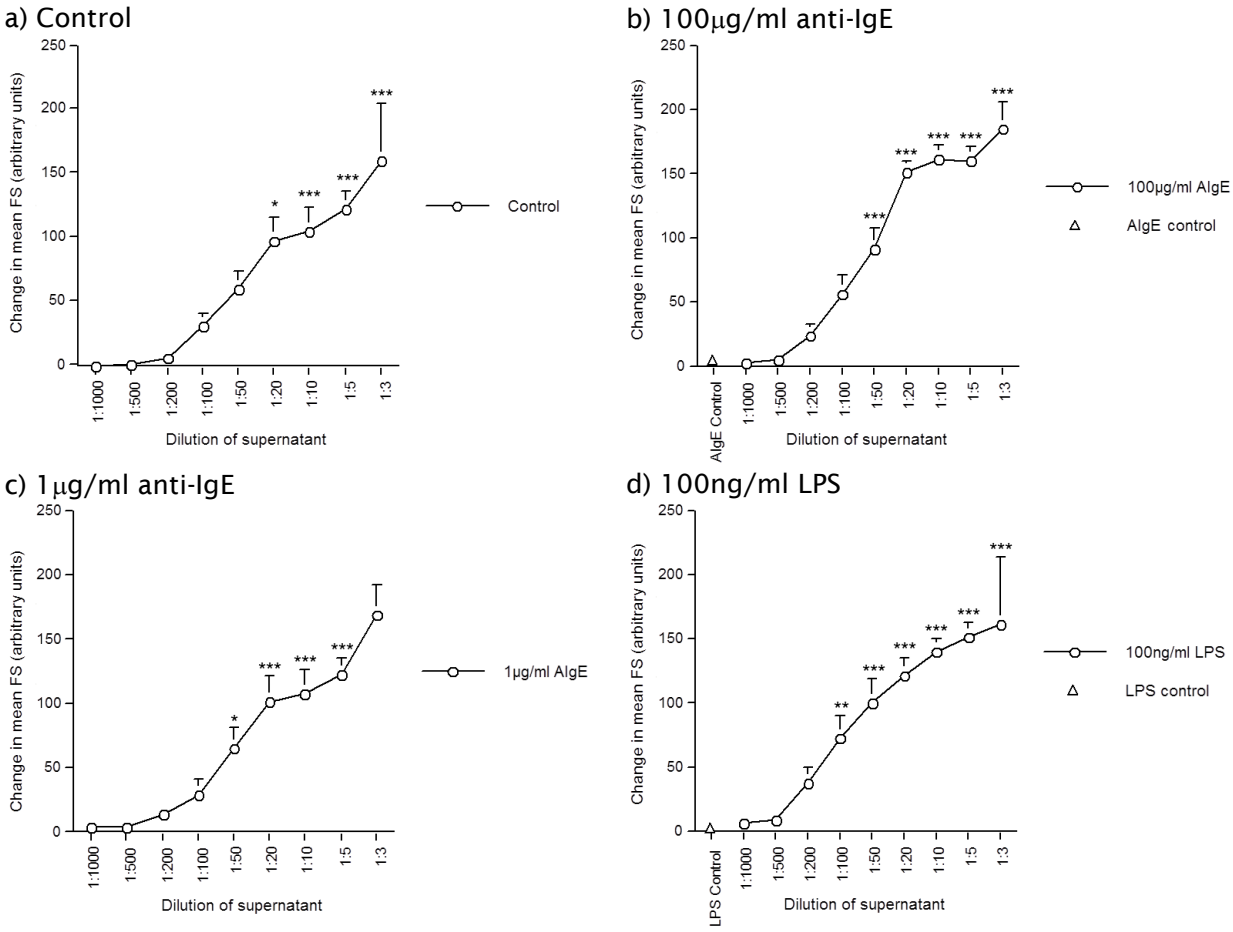


Figure 5.4 Dose responses to supernatant obtained from a) control lung tissue and lung explants stimulated with b) 100μg/ml anti-IgE, c) 1μg/ml anti-IgE and d) 100ng/ml LPS. Whole blood was stimulated with supernatant (n=13) generated using the lung explant model (see section 2.1). Blood was also stimulated with 10μg/ml anti-IgE and 10ng/ml LPS as controls (n=12) (see (b), (d)). Results expressed as mean±SEM. * indicates $p<0.05$, ** indicates $p<0.01$ and *** $p<0.001$ when compared to unstimulated cells.

In each case a clear dose-dependent response was achieved with each supernatant able to cause neutrophil shape change, equivalent to the maximum shape change response to recombinant IL-8, which occurs at around a change in mean forward scatter of 150 (figure 5.2). Whole blood was also stimulated with 10μg/ml anti-IgE (figure 5.4 b) and 10ng/ml LPS (figure 5.4 d) as controls (triangles) to determine whether these stimuli already present within the supernatants was causing neutrophil activation, either directly or by a secondary reaction i.e. activation and degranulation of basophils. As shown in figure 5.3 b) and d) these stimuli had no effect on neutrophil shape change.

By obtaining dose responses to each supernatant category a comparison between the different supernatants could be made.

Figure 5.5

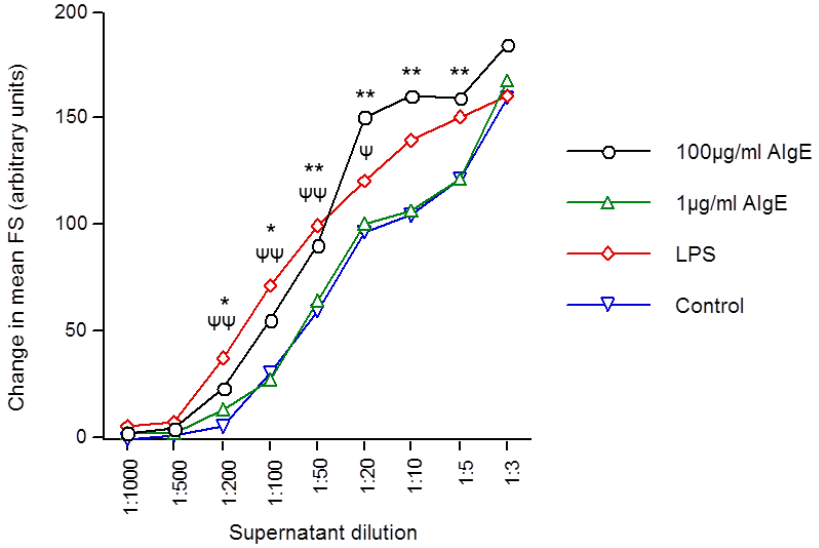


Figure 5.5 Comparison of neutrophil shape change from supernatants. Data from figure 4.3 was reworked to compare the different stimuli (n=13). Error bars have been omitted for clarity. * indicates p<0.05 and ** p<0.01 comparing 100µg/ml anti-IgE supernatant and Ψ indicates p<0.05 and ΨΨ p<0.01 comparing LPS supernatant with control at same dilutions. Non-parametric Wilcoxon-signed rank statistical test was used.

Supernatant generated from distal lung tissue stimulated with the high concentration of anti-IgE (circles) and LPS (diamonds) causes a greater response in neutrophils compared to low anti-IgE (green triangles) and control (blue traingles) supernatant. This is suggested by their leftward shift in their dose response curves and significantly higher change in mean forward scatter particularly at 1:200-1:20 dilutions, shown in figure 5.5. Interestingly, supernatant generated from unstimulated distal lung tissue and tissue stimulated with the low concentration of anti-IgE (1µg/ml) caused identical responses in neutrophils.

By comparing the dose-dependent change in neutrophil shape in response to recombinant IL-8 with shape change induced by supernatant, it is possible to calculate a theoretical 'equivalent' value of IL-8 which would cause the same degree of shape change at a certain dilution of supernatant. This calculated 'equivalent' concentration of IL-8 in the supernatant can then be compared to the actual measured IL-8 concentration determined by ELISA.

Figure 5.6

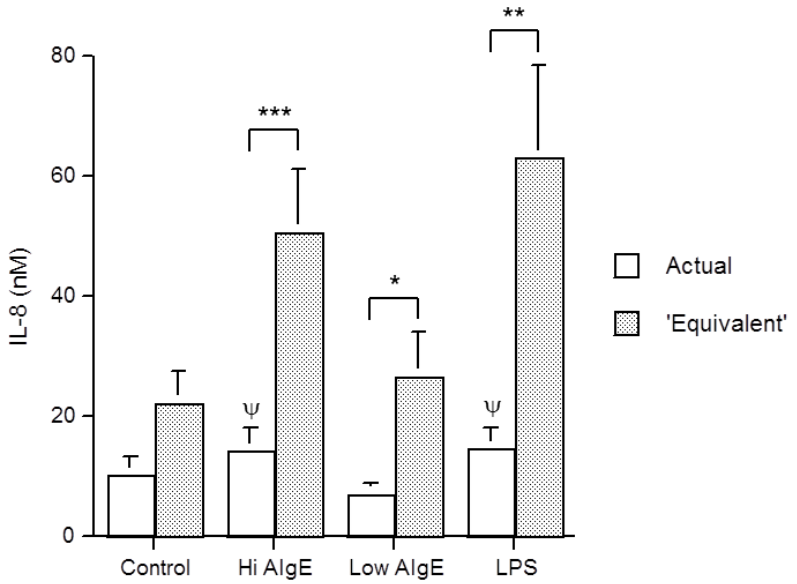


Figure 5.6 Comparison between actual supernatant IL-8 and calculated 'equivalent' IL-8 for different stimuli. IL-8 concentration in distal lung tissue supernatant (actual, open bars) was determined by ELISA and 'equivalent' IL-8 in supernatant (shaded bars) was calculated by comparing change in neutrophil forward scatter caused by supernatant with a recombinant human IL-8 standard. Hi AIgE denotes supernatants obtained from lung tissue stimulated with 100μg/ml anti-IgE, low AIgE denotes 1μg/ml anti-IgE and LPS denotes stimulation with 100ng/ml LPS (n=13). Control supernatants were obtained from unstimulated tissue incubated in RPMI-1640. Results are expressed as mean±SEM. * indicates $p<0.05$, ** $p<0.01$ and *** $p<0.001$. ψ indicates $p<0.05$ for actual supernatant IL-8 compared to control.

Figure 5.6 shows there is significantly more IL-8 in supernatants from lung tissue stimulated with 100μg/ml anti-IgE and 100ng/ml LPS compared to unstimulated control tissue. This may account for the earlier observation that these supernatants cause a greater response in neutrophils (figure 5.5). Figure 5.6 also shows that in each case the amount of shape change occurring exceeds what we would have expected from the concentration of IL-8 measured in the supernatants by ELISA. This is shown by the significantly higher 'equivalent' concentrations of IL-8 (shaded bars) in each supernatant category compared to their actual IL-8 content (open bars), with the exception of control supernatant.

It is possible this finding may be due to inaccuracy of the ELISA used to quantify IL-8 release in the supernatant. In order to check that IL-8 calculated by ELISA was accurate, we tested a concentration of recombinant IL-8 that was close to the EC_{50} for the IL-8 dose response curve for neutrophil shape change (figure 5.2), in triplicate in an ELISA. The concentration determined by ELISA was extremely close to the concentration of IL-8 run on the plate and so we were confident that the IL-8 concentrations within the supernatants determined by ELISA were correct.

At this stage we decided to create a pooled supernatant for each stimulated category in order to reduce variability between supernatant used to stimulate neutrophils from different individuals. All the results described above in section 5.3.2 were reproduced using pooled supernatant (data not shown).

5.3.3 Neutralisation of IL-8

Lung tissue has the capacity to release a multitude of mediators that can recruit inflammatory cells. We hypothesised that stimulated distal lung tissue was releasing other chemokines or chemoattractants in addition to IL-8, which was causing this excess shape change seen in figure 5.6. In order to investigate this a neutralising IL-8 antibody was utilised to abrogate IL-8 induced activation of neutrophils.

Preliminary experiments were conducted to assess the optimum working concentration for the neutralising IL-8 antibody. Dose responses of the antibody was incubated with set concentrations of recombinant IL-8 and set concentrations of the antibody with IL-8 dose responses (data not shown). From these experiments we concluded that a concentration of 1µg/ml neutralising IL-8 antibody was sufficient to neutralise all IL-8 within the supernatants, which fell in accordance with the manufacturer antibody data sheet.

It was also important to assess the specificity of the neutralising antibody. Subsequently, we incubated the antibody with another similar chemokine known to cause shape change in neutrophils, Groα. Groα has a similar structure to IL-8, both being CXC chemokines containing the ELR motif.

Figure 5.7

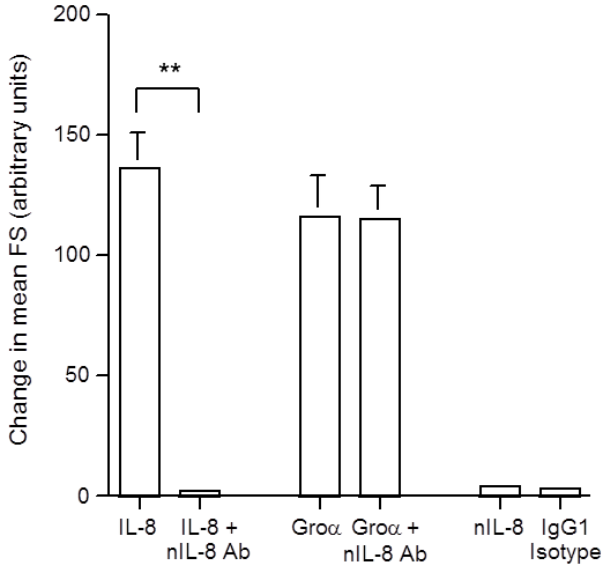


Figure 5.7 Neutrophil shape change in response to IL-8 and Groα incubated with or without neutralising IL-8 antibody. 1.25nM IL-8 (n=12) and 20nM Groα (n=6) were incubated with or without 1µg/ml neutralising IL-8 antibody for 30 minutes before whole blood stimulation. Blood was also stimulated with 1µg/ml neutralising IL-8 antibody (n=12) and 1µg/ml IgG_1 isotype control (n=6). Results expressed as mean±SEM. ** indicates $p<0.01$.

We concluded that the neutralising IL-8 antibody was highly specific as the antibody had no effect on the neutrophil shape change response caused by Groα. Figure 5.7 also shows that the working concentration of the antibody and the isotype control also have no direct effect on neutrophil shape change.

Our next step was to use the neutralising IL-8 antibody with our pooled supernatants. From our dose response curves shown in figure 5.4 we decided on using a 1:20 dilution of our supernatants, as this concentration of supernatant gives a significant and near maximal shape change response.

Figure 5.8

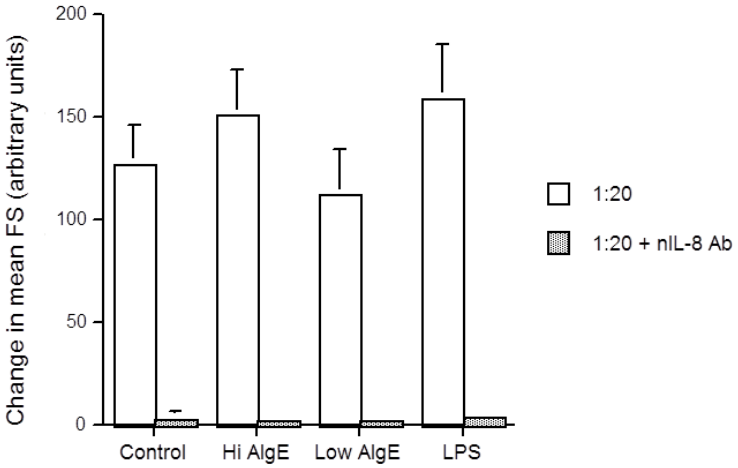


Figure 5.8 Neutrophil shape change in response to supernatant and supernatant incubated with neutralising IL-8 antibody. Data shown as change in mean forward scatter for each supernatant category at a 1:20 dilution with (shaded bars) or without (open bars) pre-incubation with 1μg/ml neutralising IL-8 antibody (n=5 for both). Hi AlgE denotes supernatants obtained from lung tissue stimulated with 100μg/ml anti-IgE, low AlgE denotes 1μg/ml anti-IgE and LPS denotes stimulation with 100ng/ml LPS. Control supernatants were obtained from unstimulated tissue incubated in RPMI-1640. Results expressed as mean±standard error of the mean.

Interestingly, in all the supernatant categories, upon IL-8 neutralisation (shaded bars), neutrophil shape change was completely inhibited. These results still did not explain why excess neutrophil shape change was being observed as demonstrated in figure 5.6 however.

5.3.4 Groα Dose Response

The effect of Groα itself on neutrophil shape change was then examined.

Figure 5.9

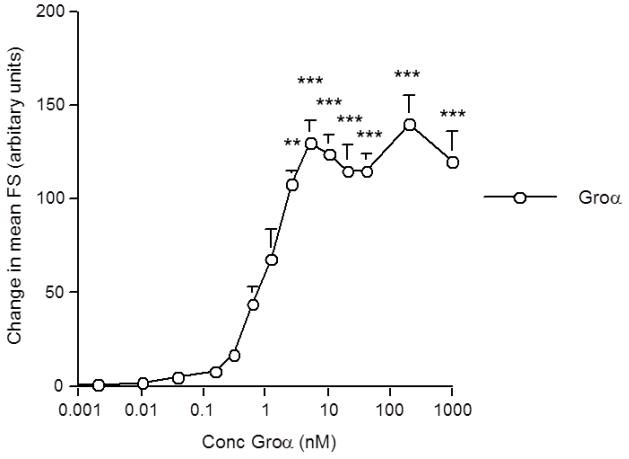


Figure 5.9 Neutrophil shape change in response to Groα. Whole blood was stimulated with 0.002-1000nM recombinant human Groα (n=15). Results are expressed as mean±SEM. *** indicates p<0.001.

Figure 5.9 shows that the dose response curve to Groα has a shallower slope and appears to cause less shape change in comparison to IL-8, with a lower maximum change in mean forward scatter. At the higher concentrations of Groα there is also some fluctuation that may indicate a biphasic response with shape change peaking at 5nM and 200nM.

5.3.5 Co-stimulation with IL-8 and Groα

Having established dose responses for IL-8 and Groα, we went on to investigate the possibility of synergy between these two chemokines. By stimulating neutrophils in whole blood with a mixture of IL-8 and Groα we can compare the amount of shape change caused by this mixture with the predicted amount of shape change caused by adding the two together, as shown in figure 5.10.

Figure 5.10

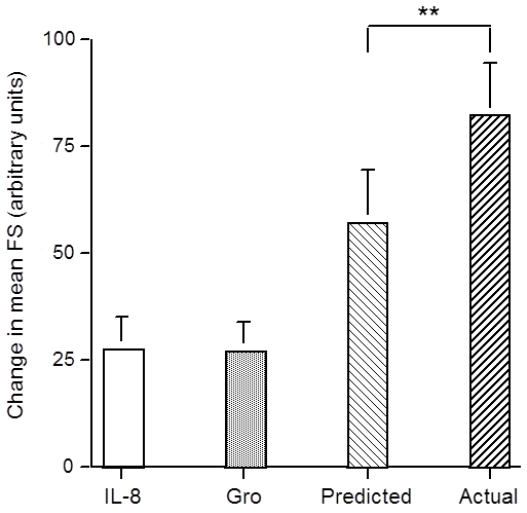


Figure 5.10 Stimulation of whole blood with the chemokines IL-8, Groα and a mixture of the two. Whole blood was stimulated with 0.313nM IL-8 (open bar), 0.625nM Groα (shaded bar) and a combination of both (actual, dark diagonal striped bar). Predicted neutrophil shape change was calculated by combining the amount of shape change caused by both stimuli together (predicted, light diagonal striped bar) (n=8). Results are expressed as mean±SEM. ** indicates $p<0.01$.

Figure 5.10 shows that neutrophil shape change is increased if a mixture of the chemokines IL-8 and Groα are used to stimulate whole blood. 0.313nM IL-8 caused a change in neutrophil mean forward scatter of 28±7 and 0.625nM Groα caused 29±7 (arbitrary units). A predicted amount of shape change was calculated by adding the respective individual responses of the chemokines together, giving a value of 57±12. Stimulation of whole blood with a combination of these chemokines at these concentrations however caused significantly more shape change than predicted, with a value of 82±12. This suggests that a degree of synergy is occurring between the two

chemokines and may be a possible explanation for the excess amount of shape change occurring within the supernatants previously shown in figure 5.6.

5.3.6 Antagonism of CXCR1 and 2

IL-8 and Groα are both CXC chemokines and therefore act through CXC receptors. Neutrophils predominantly express CXCR1 and 2 and both IL-8 and Groα have been shown to act through these receptors. In order to further investigate the action of IL-8 and Groα, we utilised a CXCR2 small molecule antagonist (compound A donated by GlaxoSmithKline) and an anti-CXCR1 antibody to inhibit these receptors and observe the effect of this inhibition on neutrophil shape change.

Initially we carried out experiments to establish the desired working concentrations of antagonist and inhibiting antibody on a range of concentrations of IL-8. Both the CXCR2 antagonist and anti-CXCR1 antibody were effective at reducing the neutrophil shape change response to a near maximal (1.25nM) and mid-range (0.625nM) concentration of IL-8 (data not shown). 10μM CXCR2 antagonist reduced the neutrophil response the most; however the DMSO vehicle control for this concentration also reduced neutrophil shape change. It has been suggested that DMSO could potentially cause membrane stabilisation which could account for this observation (Yamahita *et al*, 2000). At midrange concentrations of IL-8, a 10-fold lower concentration of the CXCR2 antagonist caused a similar amount of inhibition as the highest concentration, and at this concentration the DMSO control had no effect on neutrophil shape change. In regard to the anti-CXCR1 antibody the highest concentration used caused neutrophil activation and an increase in neutrophil shape change (data not shown). In contrast, 1μg/ml of the antibody caused a modest decrease in shape change and appeared to have little effect on neutrophils by itself. As a result, for subsequent experiments we used 1000nM CXCR2 antagonist and 1μg/ml anti-CXCR1 antibody.

Initially the role of the CXCR1 and CXCR2 receptors on neutrophil shape change in response to IL-8 and Groα was investigated.

Figure 5.11

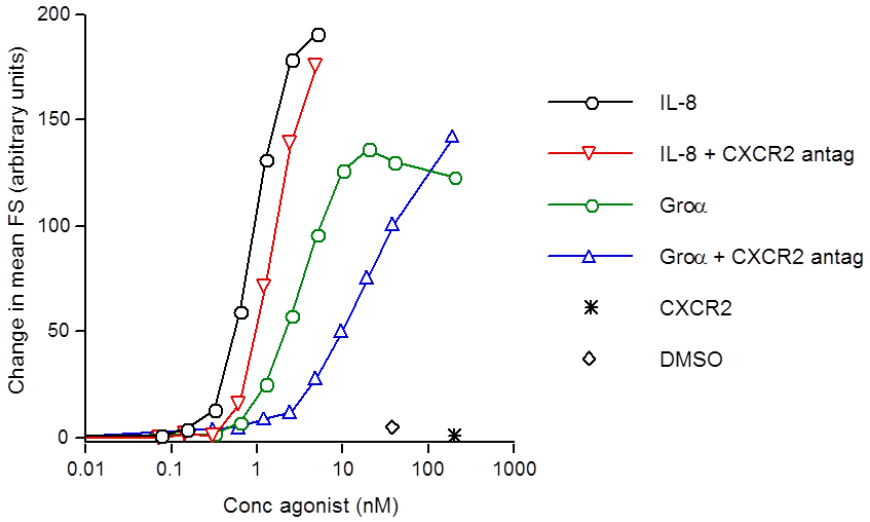


Figure 5.11 Effect of a CXCR2 antagonist on neutrophil shape change in response to IL-8 and Groα. Whole blood was stimulated with IL-8 and Groα (n=9) in the presence of 1000nM CXCR2 antagonist. Whole blood was also incubated with CXCR2 antagonist and DMSO vehicle as controls (n=5). Results are expressed as mean. Error bars have been omitted for clarity.

As shown previously in figures 5.2 and 5.9, similar shape change responses were obtained for both IL-8 (black circles) and Groα (green circles) in the absence of the antagonist, with IL-8 causing a greater maximal increase in neutrophil mean forward scatter than Groα. Figure 5.11 shows the effect on neutrophil shape change to chemokine upon antagonism of CXCR2. Upon stimulation of whole blood with IL-8 in the presence of the CXCR2 antagonist, there is just under a two-fold right hand shift of the IL-8 dose response curve (red triangles). In the case of Groα, a more noticeable right hand shift of just over four-fold in the dose response curve is seen upon antagonism of the CXCR2 receptor (blue triangles). Again the DMSO (diamond) and antagonist (asterisk) controls had no effect on neutrophil shape change.

Similar experiments were carried out using the anti-CXCR1 antibody to determine the effect of inhibiting activation of neutrophils via CXCR1. Results are shown in figure 5.12.

Figure 5.12

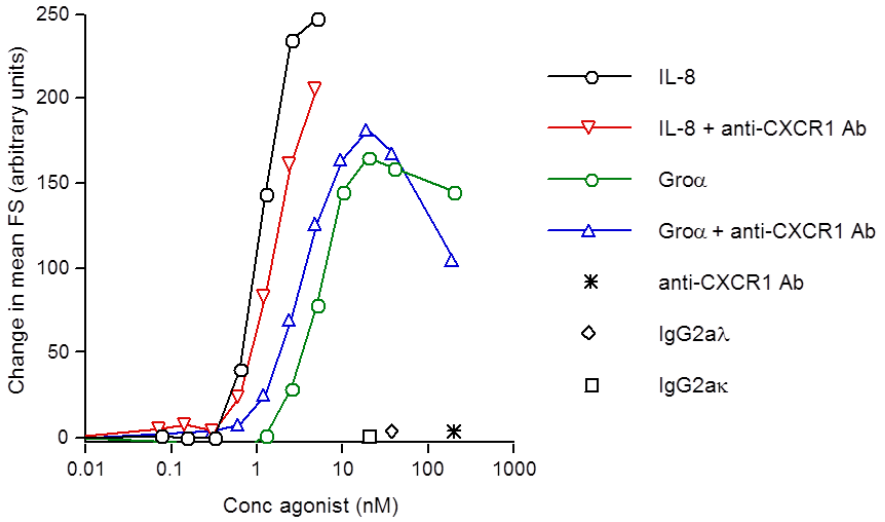


Figure 5.12 Effect of an anti-CXCR1 antibody on neutrophil shape change response to IL-8 and Groα. Whole blood was stimulated with IL-8 and Groα in the presence of 1μg/ml anti-CXCR1 antibody (n=4). Whole blood was also incubated with anti-CXCR1 antibody and antibody isotype controls (n=3), IgG2aλ and IgG2aκ. Results are expressed as mean. Error bars have been omitted for clarity.

Figure 5.12 shows that upon the inhibition of CXCR1 there is between just under a 1.5-2-fold right hand shift at the higher concentrations of the IL-8 dose response curve, with less effect at the lower concentrations (red triangles compared to black circles). Surprisingly there is around a two-fold left hand shift in the Groα dose response curve in the presence of the anti-CXCR1 antibody (blue triangles compared to green circles), whereas the antibody on its own did not cause any increase in neutrophil mean forward scatter (asterisk). The isotype controls used for this antibody also did not cause any neutrophil shape change (diamond and square). In preliminary experiments the anti-CXCR1 antibody did cause neutrophil activation at higher concentrations and so may be causing a very low activation at the concentration used here, which is only noticeable in the presence of the CXCR2 agonist Groα. This effect is reversed at the highest concentration of Groα however.

In case the left hand shift of the Groα dose response curve caused by the anti-CXCR1 antibody was being mediated through increased activation of CXCR2, we decided to see if this effect was prevented by the CXCR2 antagonist. We therefore incubated whole blood with 0.01-100μg/ml of the CXCR1 antibody in the presence and absence of the CXCR2 antagonist.

Figure 5.13

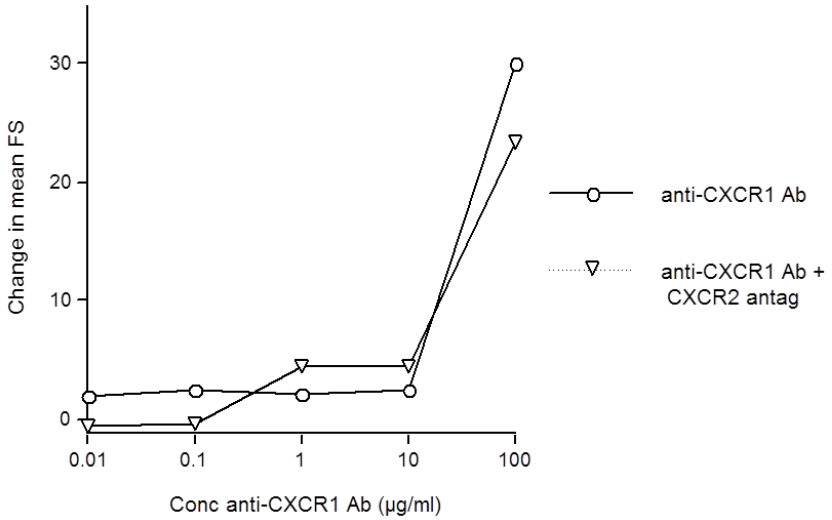


Figure 5.13 Effect of an anti-CXCR1 antibody on neutrophil shape change. Whole blood was incubated in the presence of anti-CXCR1 antibody (0.01-100μg/ml) with or without 1000nM CXCR2 antagonist (n=5). Results expressed as mean. Error bars have been omitted for clarity.

Figure 5.13 shows that the CXCR2 antagonist had little to no effect on preventing neutrophil activation by the anti-CXCR1 antibody. This suggests this activation is being caused by means independent of CXCR2 or potentially the antibody is affecting the receptor at a different binding site to the site blocked by the antagonist.

In any case we went on to investigate the neutrophil shape change response to both agonists in the presence of both the CXCR2 antagonist (1000nM) and the anti-CXCR1 antibody (1μg/ml).

Figure 5.14

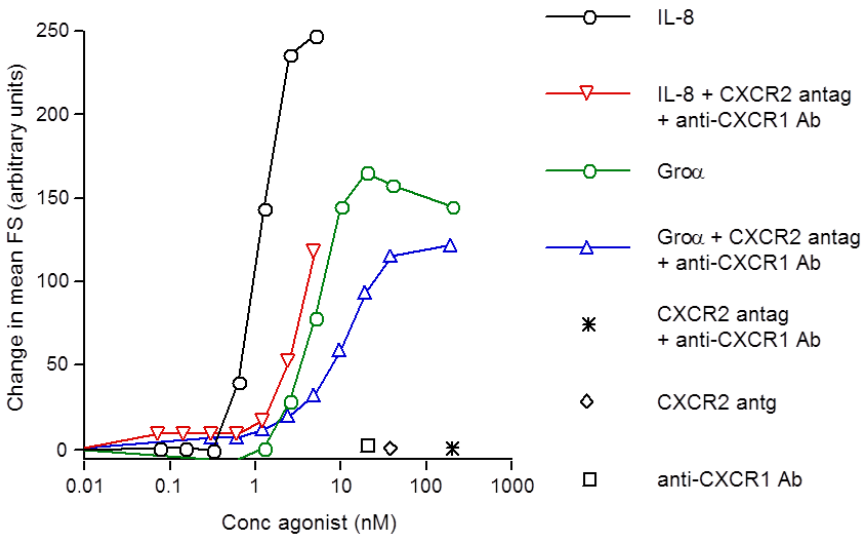


Figure 5.14 Effect of a CXCR2 antagonist and anti-CXCR1 antibody on the neutrophil shape change response to IL-8 and Groα. Whole blood was stimulated with IL-8 and Groα in the presence of 1000nM CXCR2 antagonist and 1µg/ml anti-CXCR1 antibody (n=4). Whole blood was also incubated with anti-CXCR1 antibody and CXCR2 antagonist individually and together (n=4). Results are expressed as mean. Error bars have been omitted for clarity.

Figure 5.14 shows that when both CXCR1 and CXCR2 chemokine receptors are blocked, there is a marked effect on the IL-8 dose response curve (black circles) causing an increase in the right hand shift to around 6-8-fold (red triangles) compared to the shift seen previously in figures 5.11 and 5.12, when the receptors were targeted individually. In regards to the effect on the Groα dose response curve (green circles), a similar right hand shift is seen in comparison to the effect caused by the CXCR2 antagonist in figure 5.11 of about 4 fold. Interestingly, when both receptors are targeted however, the maximal response is decreased (blue triangles). Again there was no effect caused by any of the inhibitors by themselves or together (diamond, square and asterisk).

5.3.7 Effect of receptor blockade on supernatant and synergy

We next investigated the effect of knocking out chemokine receptor function and its effect on the neutrophil shape change response induced by distal lung supernatant. As we have previously shown in figure 5.8 that neutrophil shape change is completely abolished upon neutralisation of IL-8, we hypothesised that inhibiting CXCR1 and CXCR2 would give similar results.

Figure 5.15

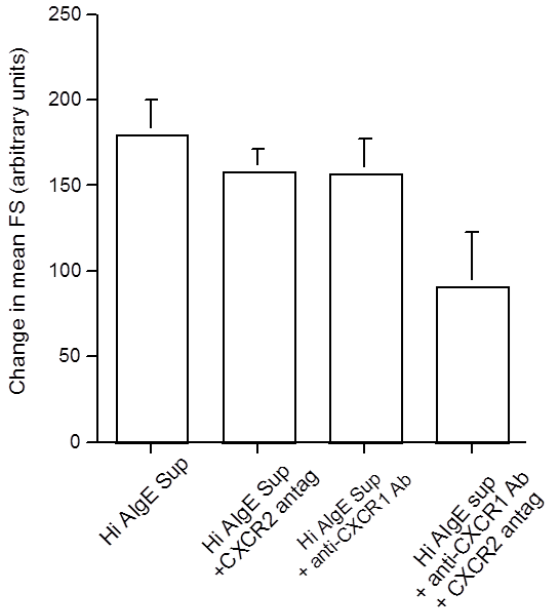


Figure 5.15 Effect of receptor antagonism on neutrophil shape change induced by supernatant. Whole blood was incubated with 1μg/ml anti-CXCR1 antibody, 1000nM CXCR2 antagonist or both for 15 minutes before stimulation with a 1:20 dilution of supernatant generated from distal lung tissue stimulated with 100μg/ml anti-IgE (n=3). Results are expressed as mean±SEM.

Figure 5.15 shows that the CXCR2 antagonist and the anti-CXCR1 antibody by themselves did not have much of an effect on neutrophil shape change. However when the two were used together the neutrophil response was decreased by just under 50%. This was reproduced for all the supernatant categories. Total elimination of the neutrophil response was not expected as the supernatant contains a very high concentration of IL-8, as determined by ELISA, that are greater than or equivalent to the highest concentration of IL-8 used in figure 5.14 (5nM). Taking this into account, receptor antagonism in neutrophils stimulated with supernatant had a very similar effect on those stimulated with recombinant IL-8 on its own.

We also used CXCR blockade to investigate the synergy we saw in figure 5.10.

Figure 5.16

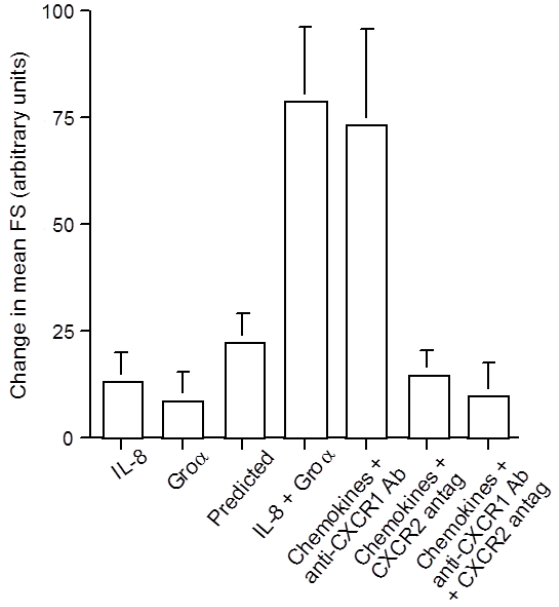


Figure 5.16 Effect of CXCR blockade on synergy between IL-8 and Groα. Whole blood was stimulated with 0.313nM IL-8, 0.625nM Groα and both chemokines together. Whole blood was also preincubated with 1μg/ml anti-CXCR1 antibody, 1000nM CXCR2 antagonist or both for 15 minutes before stimulation with the mixture of both chemokines (n=3). Results are expressed as mean±SEM.

Figure 5.16 again shows a clear synergistic response between IL-8 and Groα. There is a marked increase in neutrophil shape change when whole blood was stimulated with both chemokines together over the predicted amount of shape change calculated by adding their individual responses together. The anti-CXCR1 antibody had little effect on the shape change response to the mixture of both chemokines, whereas interestingly the CXCR2 antagonist had a striking effect, bringing the shape change response back down to levels similar to stimulation with the individual chemokines by themselves. A similar effect was seen when both receptors were blocked, which was surprising as we would have expected the response to be brought right back to baseline levels, as only very low concentrations of chemokine were being used. The stimulatory effect of the anti-CXCR1 antibody may account for this however.

5.3.8 Targeting other possible candidate receptors

As our generated lung supernatants contain a large concentration of IL-8, concentrations that we have shown to cause a significant degree of neutrophil shape change (figures 5.4 and 5.5), it is likely that the CXCR1 and CXCR2 receptors would be completely saturated. We also showed that there was a degree of synergy between these receptors but only at sub-saturation levels (figure 5.10). As such we decided to investigate other possible reasons why an excess amount of neutrophil shape change was seen to be caused by

distal lung supernatant (figure 5.6). Activation of other receptors involved in neutrophil chemoattraction could explain this observation. We initially chose another known potent neutrophil chemoattractant that has links with respiratory diseases such as asthma, the lipid mediator leukotriene B_4 (LTB_4).

Preliminary experiments were conducted to investigate the neutrophil shape change response to LTB_4 in this system and to test whether we could successfully knock out its effects using an LTB_4 receptor (BLTR) antagonist, SB209247 donated from GlaxoSmithKline. We showed that 20nM LTB_4 is able to cause a maximal shape change response in neutrophils as compared to the IL-8 dose response curves (figure 5.2) (data not shown). 20μM BLTR antagonist was also effective at reducing LTB_4 induced neutrophil shape change effectively and the antagonist itself and DMSO vehicle control had no effect on neutrophil activation (data not shown).

We went on to investigate antagonising BLTR using this antagonist and the subsequent effect on neutrophil shape change in response to IL-8 and our supernatants.

Figure 5.17

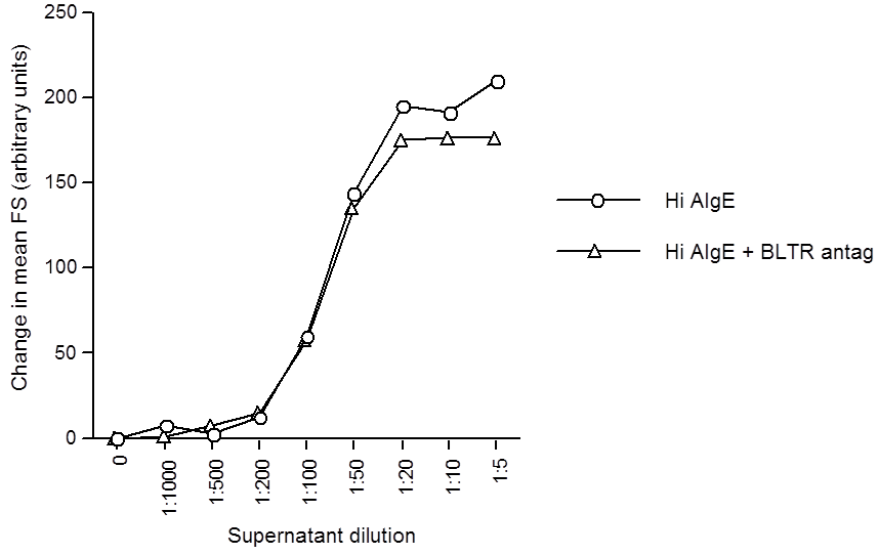


Figure 5.17 Effect of leukotriene receptor antagonist on neutrophil shape change. Whole blood was preincubated with 20μM BLTR antagonist for 15 minutes before stimulation with supernatant generated from distal lung tissue stimulated with 100μg/ml anti-IgE (n=3). Results are expressed as mean. Error bars have been removed for clarity.

The BLTR antagonist had little effect on the IL-8 response (data not shown). Interestingly, at the lower dilutions of all the supernatant categories (as shown in figure 5.17 for stimulation with high anti-IgE supernatant) where the concentration of mediators including

IL-8 will be greater, a trend of decreased maximal change in neutrophil forward scatter occurs, a trend that may also be occurring at the highest concentrations of the IL-8 dose response curve (data not shown), although this is less evident. Figure 5.16 shows that blocking the leukotriene receptors has only a small effect on the maximal degree of neutrophil shape change and little effect on the IL-8 EC_{50}. This suggests that LTB_4 may augment the neutrophil response at high levels of activation, perhaps through an autocrine effect, and is unlikely to explain the degree of excess shape change seen in our supernatant categories, as shown in figure 5.6. The numbers of repeats for these experiments are however low, rendering these observations inconclusive.

5.4 Discussion

The chemokine IL-8 is a potent neutrophil chemoattractant. We have shown in chapter 3 that substantial amounts of IL-8 are released from distal lung tissue using the lung explant model, which has also been shown in other studies (Hackett et al, 2008). The functionality of this chemokine within generated supernatants however has not been addressed. It is well known that IL-8 induces shape change in neutrophils and thus can be used in a bioassay to determine IL-8 function. In order to assess how functional IL-8 was within generated supernatants, we adapted a whole blood neutrophil shape change assay (Nicholson et al, 2007), using flow cytometry as a proven method for rapid and objective determination of change in neutrophil shape, determined by an increase in mean forward scatter (Cole et al, 1995).

Figures 5.2 and 5.3 show that we are able to stimulate neutrophils in whole blood with recombinant IL-8 and effectively and reproducibly obtain concentration-dependent curves. A dramatic change in neutrophil shape occurred between 0.3-1nM IL-8 reaching maximal shape change at 2.5nM, with an EC_{50} of 0.55nM IL-8. This data coincides with neutrophil shape change data obtained by Nicholson et al, 2007, who obtained similar concentration-dependent responses with a substantial degree of neutrophil shape change between 0.1-1nM and reaching a maximum at 2-3nM IL-8. Interestingly, Ludwig et al showed similar dose response curves using a chemotaxis assay. In this study a rapid increase in the number of migrating neutrophils occurred between 0.2-2nM, with maximum migration occurring between 4-120nM IL-8 (Ludwig et al, 1997). This supports the hypothesis that change in neutrophil shape may correlate strongly with chemotaxis.

In our study, stimulation of whole blood with supernatants generated from distal lung explants incubated with 1 and 100μg/ml anti-IgE, 100ng/ml LPS and untreated control, all caused neutrophil shape change in a dose-dependent fashion. Stimulation of whole blood with anti-IgE (10μg/ml) and LPS (10ng/ml), as controls for the presence of these compounds within the supernatants, failed to elicit any shape change response in neutrophils, confirming that the stimuli themselves did not cause neutrophil shape change in this system. Other investigators have also shown that supernatant from anti-IgE stimulated distal lung tissue activates neutrophils (Erger and Casale, 1998). In the study by Erger and Casale, neutrophil activation was determined using a chemotaxis assay and tissue was incubated with 450μg/ml anti-IgE for 30 minutes. Controls for this concentration of anti-IgE were not conducted however, which does not rule out migration of neutrophils due to activation by the stimulus itself.

In vivo and *in vitro* studies have shown that a number of factors can influence the ability of cytokines and chemokines to perform their physiological functions. IL-8 itself has been shown to be bound by autoantibodies in bronchial biopsies and serum (Shute et al, 1997) and in lung fluid of acute respiratory distress syndrome (ARDS) patients. This was shown to inhibit the interaction of IL-8 with its receptors (Kurdowska et al, 1996). We hypothesised that IL-8 generated using the lung explant model may not be fully functional and therefore we anticipated that neutrophil activation, determined using the shape change assay would be less than expected. In our study supernatant from stimulated distal lung tissue explants caused a significantly higher degree of shape change than was expected as determined by the ELISA-measured concentration of IL-8 within them. This provides evidence that IL-8 is functional within our supernatants and indicates that other mediators within the supernatant may also be contributing or causing neutrophil activation.

We went on to use a neutralising IL-8 antibody, which we proved to be specific for IL-8, to investigate the neutrophil response to our supernatants in the absence of functional IL-8. Surprisingly, neutrophil shape change caused by all the supernatant categories treated with the neutralising IL-8 antibody was completely abolished. This contrasts with the data from Erger and Casale who showed that a neutralising antibody to IL-8 reduced anti-IgE induced chemotactic activity by approximately 40% (Erger and Casale, 1998). There are however some differences between our studies; supernatants from this study were collected at a 30 minute time point whereas in our study we have used a 24 hour time point. This may suggest that at an earlier time point other neutrophil chemoattractants, such as the eicosanoids, released from mast cells may have a role in neutrophil attraction, whereas by 24 hours, IL-8 is the key chemotactic mediator. In chapter 3, we showed that significant release of IL-8 from distal lung tissue stimulated with anti-IgE occurred by 24 hours and not earlier time points, which supports this hypothesis. However, in the study by Erger and Casale, the effect of the anti-IgE itself cannot be ruled out as no control was performed.

A neutralising IL-8 antibody has been used in other models to discern the importance of IL-8 within different inflammatory diseases. For example, Goodman et al, 1996, investigated the effect of neutrophil chemotaxis to bronchoalveolar lavage (BAL) from patients with ARDS and normal controls, in the presence and absence of a neutralising IL-8 antibody. They found that the chemotactic activity of BAL from patients with ARDS was reduced to control levels when incubated with neutralising antibody. These findings are in agreement with our data and suggest that the presence of IL-8 at later time points is

required for neutrophil chemotaxis and may be important in an environment where chronic inflammation is present.

We have shown that the presence of functional IL-8 in our supernatants is critical for neutrophil shape change; this does not however explain the excess shape change that they caused. It is likely other neutrophil chemokines are also released by distal lung tissue and may contribute to cause this excess. Thus we also investigated the effect of the similar ELR-CXC chemokine Groα on neutrophils in whole blood. We established a concentration-dependent response of neutrophils to recombinant human Groα (figure 5.9), which concurs with data obtained from Nicholson et al, 2007 and Ludwig et al, 1997. Intriguingly, at the higher concentrations of Groα there is some fluctuation that may indicate a biphasic response with shape change peaking at 5nM and 200nM. This phenomenon was also noticed and investigated by Ludwig's group, although they focused on another CXCR2 chemokine, neutrophil-activating peptide-2 (NAP-2). They showed that NAP-2 exhibits a very similar response curve to Groα and thus the mechanism is likely to be the same. By desensitising the CXCR2 receptor on neutrophils and inhibiting the CXC receptors selectively using receptor-specific antibodies, they showed the first optimum of the NAP-2 response curve was mediated through CXCR2 and the second optimum at the higher concentrations, acted through CXCR1. Furthermore, competition binding studies have shown that NAP-2 and Groα bind with high affinity to CXCR2 and have a much lower affinity for CXCR1, whereas IL-8 binds with high affinity to both receptors (Ahuja and Murphy, 1996).

By stimulating whole blood with low concentrations of IL-8 (0.313nM) and Groα (0.625nM) we discovered a synergistic relationship between these two chemokines. In the same experiments when CXCR2 was blocked using a specific antagonist for the receptor, this synergistic response was abrogated. NAP-2 is an ELR-CXCR2 chemokine, similar to Groα, and has been shown to cause a very similar chemotactic response in neutrophils, as discussed above (Ludwig et al, 1997). Neutrophil response to NAP-2 therefore may be comparable to Groα. In contradiction to our data, Gouwy et al, 2004, have shown that there was no synergistic chemotactic response in neutrophils stimulated with IL-8 and NAP-2. The concentration of IL-8 (0.6nM) and NAP-2 (13.2nM) used by Gouwy et al, however, is slightly higher than the concentrations used in our study and at concentrations above our calculated EC_{50} values for IL-8 and Groα. As such, competition between IL-8 and NAP-2 and saturation of CXCR1 and 2 at these higher concentrations may overshadow any possible synergy that may be seen at suboptimal concentrations. In

our study the synergy seen between IL-8 and Groα occurred at concentrations at the lower end of their dose response curves (0.313nM IL-8 and 0.625nM Groα) that caused minimal neutrophil shape change by themselves. However, upon co-stimulation a synergistic response to neutrophil shape change is seen. Conversely, it is possible Groα may interact in a different way to NAP-2 with CXCR2 to cause the synergy we have observed.

Studies investigating neutrophil activation by measuring intracellular calcium flux provide some evidence supporting our observation of synergy between IL-8 and Groα. Neutrophils briefly exposed to Groα, before subsequent stimulation with IL-8 caused significant enhancement of $[Ca^{2+}]_i$ flux, part of the intracellular cascade involved with neutrophil activation and shape change (Hauser et al, 1999). This group also showed that the increased $[Ca^{2+}]_i$ response observed after the priming of neutrophils in autologous plasma and subsequent stimulation with IL-8, was inhibited when the neutrophils were treated with a CXCR2 blocking antibody.

The accumulation and activation of neutrophils has been associated with many diseases and therefore the method of neutrophil attraction and antimicrobial function has been the focus of considerable interest. It is well documented that many neutrophil functions e.g. shape change, exocytosis and respiratory burst, can be elicited through the activation of the chemokine receptors CXCR1 and 2 (Bagglioni et al, 1992). We therefore wanted to investigate the effects of blocking the action of these receptors and the subsequent effect on the neutrophil shape change response to IL-8 and Groα. We hypothesised that blocking activation of CXCR2 would decrease neutrophil shape change to both chemokines with the greatest effect on Groα.

The small molecule CXCR2 antagonist was successful in preventing the effect of Groα on the neutrophil shape change response shown by the right hand shift in its dose response curve. In contrast, the antagonist had a small effect on IL-8 activation of neutrophils causing a slight right hand shift in the IL-8 dose response curve. 1μg/ml anti-CXCR-1 antibody also had a modest effect on the IL-8 dose response curve, giving a slight shift to the right. This antibody did cause activation of neutrophils in the presence of chemokine however, shown by the slight left hand shift of the Groα dose response curve. When both receptors were blocked a more distinct inhibitory response was induced in neutrophils stimulated with IL-8, seen by the more pronounced right hand shift in the dose response curve. The Groα dose response when both receptors were blocked and when just CXCR2 was blocked appeared very similar suggesting that Groα at the concentrations used was

predominantly acting through CXCR2, which is in agreement with the literature. In this system IL-8 was able to act through both receptors, which corresponds with data shown by other groups for IL-8 receptor binding (Moser et al, 1991).

Signalling through the CXCRs and the biological implications of this are complex. Studies have been conducted to try and elucidate differential functional roles between CXCR1 and CXCR2 with often conflicting results (Hammond et al, 1995, Chuntharapai and Kim, 1995. This is made more difficult by the ability of these receptors to regulate their expression, the large degree of chemokine redundancy, especially for CXCR2, and the ability of some agonists such as IL-8 to bind to both receptors, and to have different effects at different concentrations. This is especially evident with regards to neutrophil chemotaxis. In one study, although both CXCR1 and 2 were shown to induce chemotaxis after IL-8 binding, utilisation of receptor-specific antibodies revealed CXCR1 to be the predominant mediator of the chemotactic response to IL-8 (Hammond et al, 1995). In contrast to this, CXCR2 has been implicated as the main receptor involved in neutrophil migration by other studies. Using a chemotaxis assay, neutrophils migrating to concentrations of less than 1nM IL-8 had significantly downmodulated CXCR2 but not CXCR1 (Chuntharapai and Kim, 1995). It has been previously shown that upon ligand binding to these receptors they are internalised, degraded and recycled back to the cell surface (Samanta et al, 1990). This indicates that in the study by Chuntharapai and Kim, IL-8 at these concentrations preferentially binds to CXCR2 causing a migratory response. The authors hypothesised that CXCR2 may be important in neutrophil chemotaxis at sites distant from the inflammatory insult, whereas CXCR1 comes more into play at the site of inflammation where chemokine concentrations will be higher.

Although these receptors are similar, differences in the activation of signalling cascades and resulting effector functions have been shown between the two. In one such study, Groα, NAP-2 and IL-8 all activated neutrophils as measured by calcium mobilisation, whereas only IL-8 was shown to activate the signalling enzyme phospholipase D (L'Heureux et al, 1995). IL-8 was also significantly more potent at producing superoxide and upregulating CD11b expression when neutrophils were primed with TNFα or granulocyte-macrophage colony-stimulating factor (GM-CSF). These findings indicate that CXCR1 may be more important in formation of the respiratory burst and adhesion to endothelial cells. In support of this, IL-8 has been shown to upregulate neutrophil bactericidal capacity, which was prevented by blocking CXCR1, whereas blocking of CXCR2 had no effect (Hartl et al, 2007). This was linked to increased oxidative burst and the

production of α-defensins. The authors went on to investigate the loss of CXCR1 receptor *in vivo* in cystic fibrosis patients where a high proteolytic environment was shown to cleave the receptor. In this case, upon administration of the anti-protease α_1-anti-trypsin to patients, the formation of CXCR1 cleavage products reduced and bacterial killing by airway neutrophils improved (Hartl et al, 2007). This strengthens the theory that CXCR1 is important at the site of inflammation where the ability to fight infection is needed.

Other cytokines with neutrophil-activating and chemotactic properties are also thought to act in a synergistic fashion with IL-8, such as GM-CSF (Shen et al, 2004), monocyte chemotactic protein-1 (MCP), MCP-2 and -3, stromal cell-derived factor-1α (SDF-1α) (Gouwy et al, 2004) and TNFα (L'Heureux et al, 1995, Bauldry et al 1990). This indicates that a wide array of inflammatory mediators could influence and expand neutrophil function and activation by acting through alternative receptors. On the other hand, priming of neutrophils by some of these cytokines such as TNFα, required preincubation with neutrophils for 30 minutes in these studies. This is not reflected in our study where neutrophils are exposed to a mixture of cytokines and chemokines present in supernatant at the time of stimulation. It is unclear however whether the priming event requires this amount of time. In any case, substantial amounts of IL-8 have been measured in our supernatants by ELISA. As such, IL-8 is likely to saturate CXCR1 and 2; therefore the excess shape change induced by supernatants is unlikely to be due to a CXCR2-mediated event.

Supernatant generated from stimulated distal lung tissue contains a multitude of inflammatory mediators that could have effects on neutrophils, which may explain the excess shape change response we saw. In order to try and elucidate possible mediator candidates we decided to block relevant neutrophil receptors that may be involved in chemotaxis and shape change. We already ruled out the effects of CXCR1 or CXCR2 and so moved on to investigate the effect on blocking the LTB_4 receptor, BLTR. LTB_4 is known within the literature to be a neutrophil chemoattractant and upon stimulation with 20nM LTB_4 a robust neutrophil shape change response was seen, which was effectively inhibited using a BLTR antagonist. The utilisation of the antagonist had little effect on lung supernatant dose responses; however there was a trend in a slight decrease in maximal shape change. Ideally, the amount of LTB_4 in these supernatants would have been determined. It is unlikely however that they would have contained a substantial amount of this mediator, as we showed that IL-8 was essential for neutrophil shape change using the neutralising IL-8 antibody and therefore if LTB_4 was present a response would still have

been observed. Alternatively, this may indicate that within generated supernatants LTB_4, or other mediators, are not fully functional.

At the highest concentrations of supernatant (smallest dilutions) it is possible that the LTB_4 concentration may have been sufficient to increase the maximal shape change response. This trend was also associated with the recombinant IL-8 dose response however. It is well documented that neutrophils are able to release LTB_4 upon activation (Ford-Hutchinson et al, 1980). It is therefore more likely that at high concentrations of IL-8, neutrophils release LTB_4 that may act back on the cell in an autocrine fashion to augment the shape change response. In any case, the effects of LTB_4 are not likely to explain the excess shape change caused by distal lung supernatant.

5.4.1 Summary

In this chapter our main aim was to assess the functionality of the chemokine IL-8 as determined by change in neutrophil mean forward scatter. We have shown that IL-8 in distal lung supernatant is fully functional and critical for neutrophil shape change.

By comparing neutrophil shape change caused by supernatant and shape change caused by recombinant IL-8 we calculated an 'equivalent' concentration of IL-8 that would elicit the same response caused by supernatant. When comparing this value with actual IL-8 concentrations in supernatant determined by ELISA, it was apparent that the supernatant was causing excess shape change, which was not explained by their IL-8 concentration.

The contribution of CXCR1 or CXCR2 is unlikely to explain this excess shape change, as there was little impact of blocking these receptors on shape change. However, we have shown that at low concentrations there is a synergistic effect between IL-8 and the CXCR2 chemokine Groα, which may be involved in the shape change response.

Other factors present in the complex mixture of cytokines and chemokines in supernatant is also able to rapidly augment neutrophil response and will be difficult to pinpoint a single factor or combination of factors that may be acting in concert with IL-8 to cause excess neutrophil shape change. Targeting other neutrophil receptors is likely to provide further insight into this possibility.

Chapter 6
General Discussion

6.1 General Discussion

6.1.1 Models of allergic lung inflammation

The pathogenesis of asthma has been studied extensively using animal models to investigate allergic mechanisms and potential therapies. Studies involving mouse models have been particularly effective in highlighting key aspects of the disease such as the contribution of T cells, eosinophils and the Th_2 environment (Hogan *et al*, 1998, Wills-Karp *et al*, 1998). Murine models enable the study of disease mechanisms *in vivo* that is not possible in human tissue. The wide availability of inbred and transgenic strains, along with immunological reagents to knock-out or induce targets believed to be important in asthma pathogenesis, has helped unravel some of the complexities of this disease (Shin *et al*, 2009). There are major limitations to these studies however due to the considerable physiological differences between mice and humans. Mouse models do not completely reproduce asthma pathology and remodelling mechanisms, nor do they exhibit spontaneous airway hyperresponsiveness (Kumar and Foster, 2002). The anatomical structure of the lung is different (Hyde *et al*, 2009) and mouse models do not mimic human exposure where low level allergen provocation occurs over many months to years (Shin *et al*, 2009, Kumar and Foster, 2002). These disadvantages therefore limit the translation of disease mechanisms between species. In conclusion, mouse models are excellent tools for disentangling allergic mechanisms in a controlled system, which is more difficult in human tissue; however clinical relevance can only be determined in human studies.

Studying disease mechanisms in human tissue, although ideal, has its own limiting factors. Over the last century, post mortem tissue has been used to study the pathology of chronic lung disease and provided the first evidence of inflammation and tissue remodelling in the lung. In cases of fatal severe asthma histopathologic changes included mucus plugging, epithelial damage, smooth muscle hypertrophy, mucus gland hyperplasia, thickening of the basement membrane, emphysema and an inflammatory infiltrate (Houston *et al*, 1953, Shapiro and Tate, 1965, Dunnill *et al*, 1969). A major advantage to these studies is the ability to examine the effect of disease throughout the whole lung, and highlighted the involvement of distal lung tissue in asthma (Carroll *et al*, 1996, Haley *et al*, 1998, Mauad *et al*, 2004). As this tissue is obtained from fatal cases of asthma, these studies represent findings in severe disease which may not reflect disease pathogenesis in mild and moderate asthma. Also the clinical data on post mortem studies is often limited and it is difficult to characterise the patients.

Obtaining fresh samples of human tissue is more difficult and involves invasive procedures. Bronchoscopy has been used to obtain biopsies of proximal airway tissue and induced sputum and BAL allow sampling of the airway lumen centrally and in more distal regions respectively. These techniques along with human cell lines and primary cell culture have provided evidence for inflammatory mechanisms in different stages of chronic lung disease and identified key inflammatory cell types in asthma and COPD (Holgate *et al*, 2006, Cosio *et al*, 2002, O'Donnell *et al*, 2005). These techniques are limited however by the difficulty in sampling and the relatively small amount of tissue or cells obtained in which to conduct experiments. They also may not reflect mechanisms throughout the lung and within intact tissue. These studies are often expensive resulting in small study populations, however the patients are well characterised and confounding factors such as co-morbidities or effects of drugs or smoking can be more easily controlled. A major advantage of these studies is that patients can be sampled multiple times, enabling the examination of changes in inflammatory processes after treatments or as the disease progresses. More recently, less invasive techniques such as exhaled breath analysis have been developed. This technique may provide a good tool for monitoring inflammatory diseases and the effects of different therapies, by measuring inflammatory biomarkers such as nitric oxide (Kharitonov and Barnes, 2004).

Lung resection surgery is another source of human tissue that has been utilised over the last 40 years. This source allows proper examination of distal lung tissue and provides substantially more tissue in which cells can be isolated and purified (Ballard *et al*, 1978, Holgate *et al*, 1985) or intact tissue explants can be used to study inflammatory mechanisms (Bochner *et al*, 1987, Schleimer *et al*, 1983). Tissue from lung resection surgery also enables the investigation of disease as it is occurring and at different stages or severity. This source of tissue also has its limitations however that must be kept in mind. Lung resection surgery is often conducted for the removal of a tumour. Although the tissue used in these models is from macroscopically normal tissue away from the tumour, the effect of cancer on this tissue is still unknown. Also patients are often older, may have a range of co-morbidities and may be taking a number of different drugs at the time of surgery. The majority of these patients also have a history of smoking and in this thesis 48% of patients had some degree of lung obstruction as determined by spirometry data. A small proportion of patients were also previously diagnosed with asthma. Ideally, we would have liked to compare inflammatory mechanisms in asthmatic and non-asthmatic tissue, however the number of asthmatic patients observed in this study was too low. Also the clinical data obtained from patient notes is very limited and therefore characterising these asthmatic patients would not have been possible.

Ex vivo explant models can be used to investigate inflammation in different tissues, providing intact tissue structures and extracellular matrix interactions. This presents a more physiologically relevant model in which to study human inflammatory processes. Using this type of model, there is little data in the literature investigating the production of inflammatory mediators from different compartments of the lung from the same patient. This thesis has encompassed the use of a lung tissue explant model from surgically resected tissue to investigate aspects of allergic inflammation. We have utilised an anti-IgE antibody to mimic allergic inflammation and investigate the early phase response in proximal airway and distal lung tissue from the same patients. We have also utilised the endotoxin LPS to mimic bacterial infection and as a positive control to compare with IgE-mediated responses. To our knowledge this is the first study to compare cytokine release from different compartments of the lung in matched tissue.

6.1.2 Asthma vs COPD

Asthma and COPD are both chronic inflammatory conditions that are typically thought of as distinct diseases. Asthma is predominantly an allergic disease (Holgate *et al*, 2006) that usually presents in childhood and is characterised by reversible airflow obstruction, airway hyperresponsiveness and inflammation (Humbert *et al*, 1999, Kim and Rhee, 2010). COPD on the other hand is predominantly caused due to cigarette smoking, develops later in life and is characterised by a progressive decline in lung function with airflow limitation that is not fully reversible (Kim and Rhee, 2010, Rabe *et al*, 2007). They do share some similarities that have caused much debate about an overlap between the two conditions (Jeffery, 1998).

The sites of inflammation and remodelling in asthma and COPD were classically thought of as being distinct, with the proximal airways being affected in asthma, and distal lung tissue in COPD. In regards to asthma, studies have now shown that both the proximal and distal airways are inflamed with evidence of remodelling mechanisms in a wide range of disease severity (Carroll *et al*, 1993, Mauad *et al*, 2004). In COPD both proximal and distal compartments of the lung have also been implicated (Di Stefano *et al*, 1996, O'Shaughnessy *et al*, 1997, Hogg *et al*, 2004). COPD is typically used as an umbrella term and is clinically characterised as chronic bronchitis and emphysema (shaded area in figure 6.1), conditions that effect proximal and distal compartments of the lung respectively (Rabe *et al*, 2007). These overlapping features (see figure 6.1) make differential diagnosis, especially in an older population, difficult for primary care physicians that is further complicated when patients present with multiple conditions (Soriano *et al*, 2003).

Figure 6.1

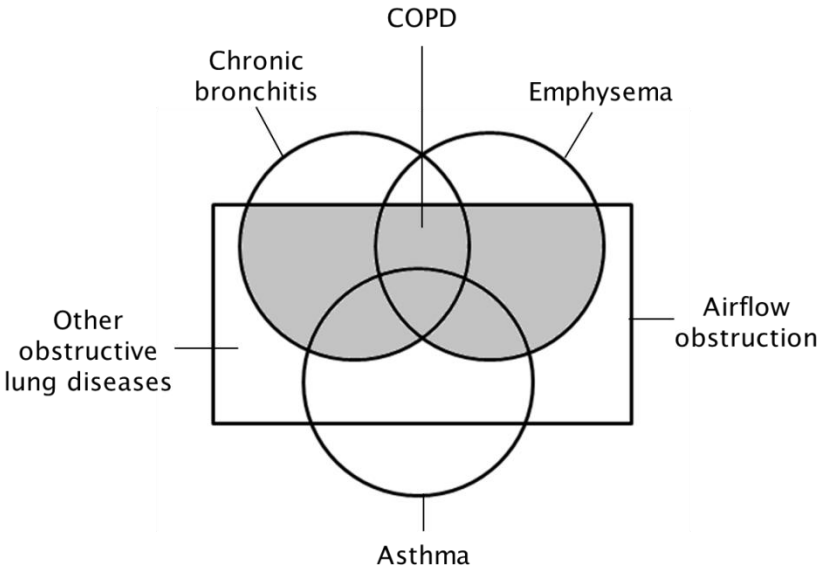


Figure 6.1 Non-proportional Venn diagram of obstructive lung diseases. Subsets of patients comprising COPD are shaded. In many cases it is difficult to differentiate patients with asthma whose airflow obstruction does not remit completely, from patients with chronic bronchitis or emphysema who have partially reversible airflow obstruction with airway hyperreactivity; therefore they can be classified as having COPD. Also patients with asthma may have chronic bronchitis and/or emphysema resulting in irreversible airflow obstruction and therefore can be characterised as having COPD. Patients with asthma with completely reversible airflow obstruction and patients with chronic bronchitis and/or emphysema without airflow obstruction are not considered to have COPD. Other obstructive lung diseases with known etiology and pathology such as cystic fibrosis are not included as a COPD subset. Figure adapted from Soriano *et al*, 2003.

Figure 6.1 shows the overlap in asthma and COPD as determined by airflow obstruction, which results from remodelling mechanisms caused by inflammation. These structural changes include airway smooth muscle and mucous gland hypertrophy and hyperplasia and subepithelial fibrosis in the proximal airways (Dunnill *et al*, 1969, Kim and Rhee, 2010). Also a characteristic feature of COPD is the destruction of the alveolar walls (Mannino, 2003), which has also been shown to occur in severe asthma (Mauad, 2004, Shapiro and Tate, 1965).

Although structural remodelling in asthma and COPD can overlap, the inflammatory profiles behind these changes appear to be distinct. Inflammatory mechanisms in asthma are classically thought to involve eosinophils and $CD4^+$ T cells as well as a Th_2 environment, involving cytokines such as IL-4, -5 and -13 (Holgate *et al*, 2006, Chung, 2001). In contrast COPD characteristically involves neutrophils, macrophages and $CD8^+$ T cells, with the main pro-inflammatory mediators involved being IL-8, IL-1 and TNFα (Keatings *et al*, 1996, Chung, 2001, Cosio *et al*, 2002, O'Donnell *et al*, 2006).

We have investigated the release of inflammatory mediators induced by IgE-dependent and LPS-mediated mechanisms in matched proximal airway and distal lung tissue. This simulates inflammation in allergic asthma and mimics bacterial infection, which is

considered to be important in COPD exacerbations (Murphy, 2006). We have shown that both proximal airway and distal lung tissue release significant amounts of TNFα, IL-10, IL-1β, IL-6 and IL-8 in a dose-dependent manner to both these stimuli. Other studies have also documented the release of these cytokines during allergic inflammation. IL-6, IL-8 and IL-1β have been shown to be released *in vitro* by asthmatic bronchial epithelial cells (Mattoli *et al*, 1992, Calderón *et al*, 1997) and isolated human mast cells have been shown to release TNFα and IL-8 after stimulation with anti-IgE (Cruse *et al*, 2005, Coward *et al*, 2002). IL-6 and IL-5 have also been shown to be increased in the BAL of asthmatics (Tonnel *et al*, 2001, Broide *et al*, 1992) and similar findings have been reported from *ex vivo* distal lung fragments (Barnicott *et al*, 2006). Macrophages, although not classically thought to be activated via IgE-dependent mechanisms have been shown to express the low affinity IgE receptor and release TNFα, IL-1β, IL-8 and IL-10 after stimulation with anti-IgE (Gosset *et al*, 1999). Macrophages therefore could also contribute to IgE-dependent cytokine release in our model.

IL-5 was the only cytokine to show a different pattern of release from distal lung tissue, with a bell-shaped dose response curve to anti-IgE stimulation. Release of IL-5 was generally very low and release was not detected from proximal airway explants. Other studies have shown increased IL-5 release from cultured BAL and $CD4^+$ T cells of allergic asthmatics stimulated with HDM (Tang *et al*, 1997). A similar bell-shaped dose response profile was also obtained in human lung mast cells stimulated with comparable concentrations of anti-IgE (Okayama *et al*, 1995). This pattern of release has also been seen with other mast cell mediators such as histamine and prostaglandin D_2 (Schulman *et al*, 1983) and in basophils, another cell population expressing FcεRI (Conroy *et al*, 1977). It is therefore likely that other cells, such as macrophages, neutrophils and structural cells contribute to the dose-dependent release of TNFα, IL-10, IL-1β, IL-6 and IL-8, which may explain the similar dose response curves seen between anti-IgE and LPS.

In regards to LPS stimulation, several studies have also shown the dose-dependent release of these mediators from different cell types. Isolated macrophages and alveolar type II cells have been shown to release TNFα, IL-1β, IL-6 and IL-8 *in vitro* (Thorley *et al*, 2007). The increased release of these mediators has also been shown in human nasal lavage and BAL after LPS was administered intranasally (Sigsgaard *et al*, 2000) or via bronchoscope (Hoogerwerf *et al*, 2008). TNFα and IL-8 have also been shown in disease with increased concentrations found in the sputum of COPD patients (Aaron *et al*, 2001). We also saw no release of IL-5 after LPS stimulation.

Our data shows that a wide range of pro-inflammatory cytokines are released during allergic and bacterial inflammation. This could give rise to generalised airway inflammation but shows that allergic or asthmatic IgE-dependent inflammatory processes also instigate a distinct pattern of Th_2 cytokines, which is not seen with LPS stimulation.

An elegant study conducted by Fabbri and colleagues comparing age matched patients with a history of asthma or COPD and similar fixed airflow obstruction, indicated that the characteristic inflammatory profiles of these diseases remained distinct (Fabbri *et al*, 2003). The techniques utilised in this study were limited to the proximal airways however and therefore specific analysis of the distal lung compartment was not achieved. Other studies however have shown increased macrophages, neutrophils and $CD8^+$ T cells in the proximal airways and distal lung tissue of COPD patients (Di Stefano *et al*, 1996, Di Stefano *et al*, 1998, Rutgers *et al*, 2000, Hogg *et al*, 2004).

Ideally we would have liked to investigate allergic inflammation in asthmatic or allergic lung tissue however this tissue was rare. A weakness of our study was the limited clinical data we could obtain and therefore it was difficult to completely characterise patients. Also patients undergoing lung resection surgery often have some degree of undiagnosed obstructive lung disease. We therefore utilised spirometry data and the GOLD guidelines to distinguish patients with lung obstruction and those with normal lung function. A considerable number of patients within our study had mild/moderate COPD, as determined by lung function data. We however found no significant difference in the number of macrophages, neutrophils or $CD8^+$ T cells in the proximal airways or distal lung tissue of mild/moderate COPD patients compared to patients with no evidence of obstructive lung disease. The studies by Hogg and Di Stefano have shown that as COPD progresses into more severe disease, the characteristic infiltrate becomes more pronounced (Hogg *et al*, 2004, Di Stefano *et al*, 1998). This may explain why no differences were seen in our study, where the patients analysed had mild/moderate COPD. It is also worth mentioning that in the studies by Hogg and Di Stefano paraffin or frozen section immunohistochemistry was used, which samples a larger and thicker section of tissue. We utilised GMA immunohistochemistry which samples a much thinner section of tissue and therefore a smaller window. Due to this limitation we only counted positively-stained nucleated cells so as to not bias our results when comparing cell populations due to stereology. Hogg and Di Stefano do not mention whether they have taken stereology into account and therefore this makes comparing findings between studies more difficult.

There are numerous studies that also document increased release of inflammatory cytokines such as TNFα and IL-8 from COPD patients compared to smoking and non-smoking control groups (Keatings *et al*, 1996, Aaron *et al*, 2001). Although we did not find any statistically significant difference between the magnitude of cytokine release between our COPD and non-COPD groups, there was a significant relationship between decreasing lung function and increasing TNFα release. A similar relationship has been shown previously in distal lung tissue fragments where COPD patients with moderate disease had a significantly exaggerated TNFα response, compared to patients with normal lung function (Hackett *et al*, 2008). There was no difference observed between patients with mild COPD, again highlighting changes in pathology as severity increases. In contrast to these findings however, cultured epithelial cells from COPD patients released less IL-6 and -8 compared to normal smokers and ex-smokers (Patel *et al*, 2003).

Differences in airway inflammation as severity of disease increases have also been observed in asthma, with similarities to inflammatory mechanisms in COPD. Severe asthmatics have increased neutrophils in induced sputum, BAL and in endobronchial and transbronchiol biopsy specimens compared to mild/moderate asthmatics (Jatakanon *et al*, 1999, Wenzel *et al*, 1997). This neutrophilia may explain the increase in emphysematous tissue described by Shapiro and Tate, 1965 and Mauad *et al*, 2004 in severe asthma and adds evidence to the overlap between COPD and asthma. Conversely, in exacerbations of COPD, a marked increase in eosinophils has been observed (Saetta *et al*, 1996). Also a subset of COPD patients with eosinophilia has been shown to improve when eosinophil numbers were reduced after treatment with oral corticosteroid (Chanez *et al*, 1997). This eosinophilic inflammation seen in a subset of COPD patients may be indicative of eosinophilic bronchitis (Fabbri *et al*, 2003) and adds to the complexity of the disease.

Smokers have been shown to have an increased neutrophilic infiltrate with increased IL-8 in BAL (Morrison *et al*, 1998) and production by bronchial epithelial cells (Mio *et al*, 1997). Alveolar macrophages from smokers have also been shown to produce increased levels of TNFα and IL-10 (Lim *et al*, 2000), however when macrophages have been exposed to cigarette smoke this has been shown to decrease the production of these mediators (Dubar *et al*, 1993). As smoking is a major risk factor for the development of COPD, we also evaluated whether smoking status had any effect on the inflammatory infiltrate or cytokine profile in our tissue. Again, we found no significant difference in these parameters when comparing current smokers with ex-smokers. Due to the nature of the source of tissue and the strong links of smoking to lung cancer, the majority of patients had a smoking history; only four patients had no smoking

history. This is another limitation to the study where the non-smoking group is too small to compare with current and ex-smokers. The data on smoking history is also relatively weak as it is obtained from the general history in patient notes. In some cases the calculation of pack years was not possible and as these patients are having surgery for smoking-related conditions they may have underrated their smoking history.

We also investigated the kinetics of cytokine release from the proximal and distal airways and found that a similar pattern of release occurs in both compartments. We have shown that anti-IgE and LPS induced significant release of TNFα, IL-10, IL-1β, IL-5, IL-6 and IL-8 that occurs by 24 hours and increases till 48 hours, with the exception of TNFα, which levels off. Similar data has been previously shown by Barnicott *et al*, 2006 and Hackett *et al*, 2008, from distal lung tissue explants. The release of these cytokines occurred in sequence with significant release of TNFα and IL-1β occurring first by 2-6hrs. This data highlights these cytokines in particular as early initiators of inflammatory processes that begin a cascade of subsequent inflammatory and anti-inflammatory cytokines. *In vitro* studies using isolated macrophages and epithelial cell cultures have effectively demonstrated the ability of TNFα and IL-1β, which were released after one hour, to induce inflammatory cytokine release from these cell types (Thorley *et al*, 2007, Sharma *et al*, 2007). *In vivo* studies in mice have also shown administration of these cytokines initiates inflammatory cascades resulting in inflammation and an inflammatory infiltrate in tissue (Ulich *et al*, 1991). After stimulation with anti-IgE, mast cells have been shown to release TNFα as early as 15 minutes (Coward *et al*, 2002), and release other inflammatory mediators after activation by TNFα or IL-1β (Kim *et al*, 2010). These cytokine cascades provide crosstalk between multiple cell types and may explain the similar patterns of cytokine release after anti-IgE or LPS stimulation. This may also explain some of the similarities in inflammatory processes seen between asthma and COPD.

This data highlights the complex inflammatory network of cytokines and mediators that is released during inflammation. As a vast array of cytokines and chemokines are likely to be involved in disease pathogenesis, it is difficult to identify particular targets for possible therapies, especially from quantifiable data such as ELISAs. An aspect of this thesis was therefore to develop a bioassay, which could be used to identify novel chemokines or chemoattractants that may be important in inflammation.

We have shown that these distinct compartments of the lung have a similar underlying inflammatory mechanism in response to anti-IgE and LPS stimulation; however distal lung tissue is able to secrete significantly more of the majority of cytokines measured,

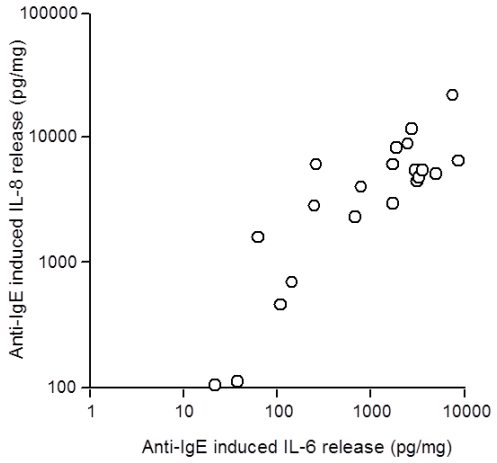
with the exception of IL-6. Immunohistochemical analysis of cell populations present in the tissue revealed that distal lung tissue contains substantially more mast cells, macrophages and neutrophils than proximal airway tissue. The distribution of inflammatory cells within each compartment was also similar and may explain the difference in magnitude and similar patterns of cytokine release observed between the two. This difference in distribution of inflammatory cells has also been reported by other groups (Kraft *et al*, 1996, Hamid *et al*, 1997, Balzar *et al*, 2002).

The difference in magnitude of TNFα, IL-10, IL-1β, IL-5 and IL-8 release may be explained by the increased numbers of macrophages, mast cells and neutrophils in distal lung tissue. TNFα is produced by many cells including mast cells (Coward *et al*, 2002), T cells (Chung, 2001) and epithelial cells (Thorley *et al*, 2007) however its principal source is the macrophage (Chung, 2001, Sharma *et al*, 2007). Macrophages are also potent producers of IL-1β, IL-6, IL-8 and IL-10 (Chung, 2001, Thorley 2007, Conti *et al*, 2003). IL-1β has also been shown to be produced by an array of cells including neutrophils (Malyak *et al*, 1994), epithelial cells (Thorley *et al*, 2007), fibroblasts, B cells and T cells (Chung, 2001). IL-5 is released by Th_2 cells, eosinophils and mast cells (Del Prete, 1992, Broide *et al*, 1992, Bradding *et al*, 1994) and IL-8 is produced by neutrophils, T cells, mast cells, fibroblasts, epithelial cells and endothelial cells (Rollins, 1997, Chung, 2001, Cruse *et al*, 2005). We also have shown a relationship between mast cell numbers in distal lung tissue and anti-IgE induced IL-8 release. Mast cells may therefore be important producers of IL-8 in allergic inflammation by themselves (Cruse *et al*, 2005) but also may induce release from other cell types from the release of mast cell specific products such as tryptase (Cairns and Walls, 1996).

Similar amounts of IL-6 however were released by the proximal airways and distal lung tissue. IL-6 has been shown to be released by macrophages, T cells and B cells however fibroblasts and airway epithelial cells are more potent producers of this cytokine (Thorley *et al*, 2007, Crestani *et al*, 1994, Matthew Fitzgerald, 2004, Holgate *et al*, 2006). These structural cells are present throughout the lung and therefore may contribute to inflammatory processes equally in each compartment. These cells also release IL-8 upon activation (Rollins, 1997, Chung, 2001) and we saw a strong relationship between anti-IgE- and LPS-induced IL-6 and IL-8 release in both lung compartments, as shown below in figure 6.2 for distal lung tissue.

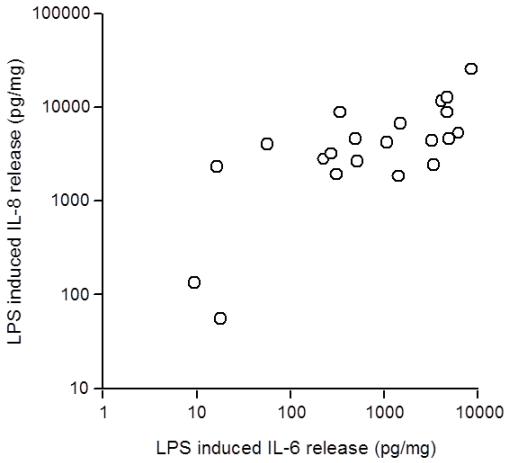
Figure 6.2

a) Stimulation with 100μg/ml anti-IgE



n=22 rho=0.783 p<0.001

b) Stimulation with 100ng/ml LPS



n=22 rho=0.762 p<0.001

Figure 6.2 Correlation between IL-6 and IL-8 release from distal lung tissue. Distal lung tissue was stimulated with a) 100μg/ml anti-IgE and b) 100ng/ml LPS. Lung tissue supernatants were analysed for IL-6 and IL-8 release by ELISA and corrected for tissue weight. Individual patient cytokine release (n=22) is plotted and correlation determined using the non-parametric Spearman's Rank test.

Figure 6.2 shows there is a strong relationship between the release of IL-6 and IL-8. This may indicate they are released by the same cell populations such as fibroblasts and epithelial cells. This is only speculation however and would need to be confirmed within the model perhaps by utilising double-staining techniques. Also, there was a significant difference in IL-8 production between proximal and distal airways, which may be explained by the ability of macrophages, mast cells and neutrophils to also release this cytokine. Also the difference in IL-8 production between the proximal and distal airways was less pronounced than the other cytokines investigated.

As asthma and COPD are obstructive lung conditions the pharmacotherapy for these diseases is often similar, with bronchodilators central to symptomatic relief. The introduction of corticosteroid has been effective in asthma therapy but has proven to be much less effective in COPD (Chung *et al*, 2009), with little long-term effect in the decline in lung function (Stockley *et al*, 2009). A small proportion of asthmatics also fail to respond to high dose corticosteroid treatment, reaffirming the overlap between asthma and COPD, as COPD is regarded as a largely steroid-resistant disease (Barnes, 2004). Nevertheless steroid treatment in COPD patients has been linked to a decrease in exacerbation frequency in patients at risk of repeated exacerbations (Rabe *et al*, 2007). Increased awareness of the importance of COPD and exacerbations in recent years, especially in healthcare physicians, may mean patients are monitored more closely and treated more rapidly with antibiotics however. Therefore the link with inhaled corticosteroid therapy may be tenuous. In any case, as COPD awareness has increased over the last few years many more patients are being treated with inhaled

corticosteroid, which enables the study of the effect of these drugs in disease progression.

We were able to collect tissue samples from 15 patients who were prescribed corticosteroid at the time of surgery. There are some major limitations to take into consideration when comparing responses in this steroid group with patients who were not taking steroid however. These include compliance, relative dosage, inhaler technique, the duration of treatment and the different pharmacokinetics and dynamics of different corticosteroid therapies.

As there were only 4 patients without any degree of lung obstruction we compared COPD patients who were prescribed corticosteroid at the time of surgery with COPD patients who were not prescribed any steroid treatment. These groups were matched for age, lung function and smoking history. Most strikingly, when we investigated differences between these groups we observed a significant decrease in mast cell numbers in the distal lung tissue of steroid patients. Corticosteroids have previously been implicated in the reduction of mast cells in other models (Finotto *et al*, 1997, Goldsmith *et al*, 1990). Surprisingly we saw no mast cell reduction in the proximal airways, where the action of steroid has been shown to decrease mast cell numbers in asthma and COPD patients in studies utilising bronchoscopy biopsies (Djukanovic *et al*, 1997, Gizycki *et al*, 2002). The effect of corticosteroid in distal lung tissue compared to proximal airway tissue has not been studied extensively and therefore these drugs may have site-specific effects.

Classically, corticosteroids elicit their anti-inflammatory actions by decreasing the transcription of pro-inflammatory genes (Barnes, 2010). We therefore also examined the effect of steroid on the release of pro-inflammatory mediators in both compartments of the lung. We have shown that corticosteroid had no effect on IgE-dependent TNFα and IL-10 release but did cause a significant reduction in IL-5 and IL-6 release from distal lung tissue. Mast cells are typically considered to be steroid-resistant cells as corticosteroid has been shown to have no effect on IgE-mediated production and release of lipid mediators such as histamine, LTC_4 and PGD_2 (Cohan *et al*, 1989, Schleimer *et al*, 1983). However other studies have now shown that corticosteroid can suppress induction of pro-inflammatory cytokine mRNA in a mast cell line (Nakamura *et al*, 2005). Cytokine release was not investigated in the studies by Cohan and Schleimer and therefore inhibition may have occurred in their models. Also *in vivo* studies in mouse models have reported inhibition of IgE-dependent TNFα (Wershil *et al*, 1995) and IL-5 (Eum, *et al*, 2003) and in a model of chronic asthma the reduction in mRNA expression of TNFα, IL-5, IL-6 and IL-10 by dexamethasone (Herbert

et al, 2008). Dexamethasone has also been shown to inhibit IL-5 mRNA expression and release of protein in human lung tissue fragments stimulated with anti-IgE (Glaum *et al*, 1995). It is also commonly used in experimental settings, however its effects may not be comparable to commonly used inhaled corticosteroid drugs used in the treatment of asthma or COPD. IL-5 and a reduction in tissue eosinophilia have been shown however in allergic rhinitis patients treated with the inhaled corticosteroid fluticasone (Masuyama *et al*, 1998), indicating that the effects between the drugs may be similar.

We saw no significant difference in LPS-induced cytokine release, however there was a trend for patients prescribed steroid to have a lower TNFα and IL-10 response. Corticosteroids are effective in the management of asthma and less effective in COPD, therefore we were surprised that the release of these cytokines was inhibited more so in LPS compared to anti-IgE stimulation.

There is evidence of a strong link between allergic asthma and rhinitis, with these inflammatory conditions of the lower and upper airways often presenting as co-morbidities (Bachert *et al*, 2004, Grossman, 1997, Serano *et al*, 2005). It has been hypothesised that a common interrelating mechanism for inflammation may link these two diseases, resulting in generalised airway inflammation (Braunstahl *et al*, 2001). This concept is known as the 'one airway, one disease' hypothesis (Leynaert *et al*, 2000). It is also more widely accepted that the small airways and distal lung tissue may play an important role in the pathogenesis of chronic inflammatory diseases such as asthma and COPD. Due to the difficulty in obtaining this tissue in humans, distal lung tissue has been studied in much less depth. Our initial hypothesis was that distal lung tissue may have an important role in inflammation, which could contribute to disorders of the lung.

We hypothesised and have proven that distal lung tissue plays an important role in producing inflammatory mediators. If the 'one airway, one disease' hypothesis is correct, then this large pool of inflammatory mediators produced in distal lung tissue is likely to contribute to inflammatory processes in the proximal airways. The substantial release of inflammatory cytokines and subsequent cytokine cascades could give rise to generalised airway inflammation that may also have systemic effects, such as increased production and survival of leukocytes. This also may have particular relevance to COPD which is considered to have a systemic component, due to the overflow of cytokines into the circulation (Wouters, 2005). The effect of lung inflammation on systemic effects has also been investigated using a mouse model (Markovic *et al* 2009). In this study, mice were administered with LPS intranasally and

lung perfusate containing inflammatory mediators was collected and used to stimulate mouse liver vascular endothelial cells and neutrophils *in vitro*. These cells were activated, inducing ROS production, expression of adhesion molecules and upregulation of NF-κB. This study has shown that lung-derived mediators released systemically, activated liver endothelial cells and circulating neutrophils. Human distal lung tissue could therefore also contribute to inflammation in other organs. We have shown that mediators released from distal lung tissue into culture supernatant are able to induce a robust shape change response in neutrophils. It would be interesting to extend the model to see the effect of lung supernatant on leukocyte recruitment to other organs and the effects on these cells themselves. A limitation to this however is that it is unknown how far these mediators are able to travel to elicit effects at distant sites.

In any case, we have contributed data to the evidence that distal lung tissue has the ability to release substantial amounts of inflammatory mediators. This could warrant the development of future therapies to target inflammation in distal lung tissue in order to dampen inflammatory processes throughout the lung and systemically. As the small airways of asthmatics are considered to be inflamed, new inhaled corticosteroid drugs are in development to penetrate deeper into the lung to target the lung periphery. Over the last 15 years drug delivery systems have been modified to minimise deposition of the drugs in the oropharynx and increase delivery to the airways (Leach, 1998). More recently fine-particle aerosols with a median diameter of 1μm have become available to increase drug delivery to distal lung tissue (Lahzami and King, 2008). One such drug is ciclesonide and has already been shown to reduce inflammation in the small airways of mild/moderate asthmatics, determined by decreased exhaled alveolar nitric oxide and alveolar trapping (Cohen *et al*, 2008). These new therapies will enable direct targeting to the lung, minimising unwanted systemic effects of steroid therapy and may be beneficial in the treatment of COPD.

6.1.3 Passive sensitisation

We have shown that distal lung tissue is able to release inflammatory cytokines in an IgE-dependent fashion. Stimulation of lung tissue with anti-IgE and LPS is useful in providing an insight into inflammatory mechanisms in the lung but other factors could also affect inflammation caused by allergens or bacteria. Anti-IgE is able to crosslink any IgE bound to receptors on mast cells activating the cell, whereas allergen will only bind to allergen-specific IgE molecules. Many allergens also contain intrinsic protease activity that could instigate inflammatory responses. We therefore wanted to develop a model to assess the effect of a common allergen Der p1, found in HDM extract, in sensitised lung tissue.

By injecting serum from an atopic patient intracutaneously into a non-atopic subject, Prausnitz and Küstner showed that sensitivity to an allergen could be transferred (Prausnitz and Küstner, 1921). The factor within serum that caused this sensitivity to an allergen was IgE (Watson *et al*, 1998, Tunon de Lara *et al*, 1995). Incubation of tissue in atopic serum containing a high concentration of allergen-specific IgE allows effector cells expressing the high affinity IgE receptor, FcεRI, to become saturated. This process mimics the sensitisation phase that occurs *in vivo* and thus has been termed 'passive sensitisation'. The potential of this would be useful in studying allergic inflammation in humans and give a more relevant insight over murine studies into the inflammatory mechanisms taking place, particularly during the early phase reaction. It could also be used to examine the effects of different allergens, discerning if the resulting inflammation is due to their ability to bind to IgE and activate mast cells, or whether they contain other inflammatory properties e.g. enzymatic activity.

Allergic inflammation has not been studied extensively in distal lung tissue, with the main focus in the literature being on inflammatory mechanisms in the conducting airways. Within chapter 4, the methodology of passive sensitisation was utilised to attempt to transfer the allergic phenotype to non-atopic lung tissue. The production of inflammatory cytokines, after stimulation of tissue with HDM extract, was investigated. Unfortunately, in this study we have shown that passively sensitising human distal lung tissue has proven to be difficult, as evaluated by cytokine and histamine release.

Passive sensitisation has been proven to work in lower airway tissue using bronchial strip contraction experiments (Tunon de Lara *et al*, 1995, Watson *et al*, 1998), although cytokine release has not been investigated. Measuring cytokines in organ bath setups will be extremely difficult due to the volume of buffers used, resulting in the subsequent massive dilution of the mediators released. Using this model we also successfully sensitised bronchial strips that contracted upon stimulation with HDM extract. When applying the same passive sensitisation protocol to distal lung tissue and proximal airway explants, we obtained no measurable response in cytokine or histamine release however.

Mitsuta and colleagues have utilised passive sensitisation to study the early phase reaction in distal lung tissue. In this case, stimulation of tissue with allergen resulted in substantial release of IL-5 and TNFα, almost 10 times more than we saw with anti-IgE stimulation (Mitsuta *et al*, 2001). It is unclear whether they have corrected these values for tissue weight however but if they have not, then the values obtained are comparable, as our model uses around 10 times less tissue. Also in an *ex vivo* study, BAL cells and peripheral blood mononuclear cells (PBMCs) of atopic asthmatics and

atopic non-asthmatics showed increased IL-5 production after stimulation with allergen (Tang *et al*, 1997). This highlights the importance of IL-5 in allergic responses. We were also able to obtain distal lung tissue from a nut allergic patient, which we stimulated with a mixed nut extract. In this case, there was substantial release of the Th_2 cytokines IL-5 and IL-13 by 30 minutes that reduced by 24hrs but was still high. There was also a small amount of TNFα and IL-10 release at these time points that was slightly lower than the anti-IgE-induced release of these cytokines. Anti-IgE stimulation did not cause any IL-5 or IL-13 release in this patient; however the concentration used was at a point on the IL-5 bell-shaped dose response curve that caused minimal IL-5 release. Taken together, these data suggest that anti-IgE is able to cause a more robust TNFα and IL-10 response compared to allergen, whereas allergen produces a robust Th_2 cytokine response. This may be aided by the activation of T cells however.

There are problems with interpreting too much from the experiment conducted with the nut-allergic patient that must be taken into consideration. This was the response from only one patient, who also had previously been diagnosed with asthma and therefore is likely to have an exaggerated Th_2 type response. The nut allergen used in this case is also not physiologically relevant as it is not an inhaled allergen. We also have no data on the severity of the patient's allergy and therefore the mediators measured may be more indicative of an anaphylactic response.

This leaves the question open as to whether some unknown aspect of distal lung tissue inhibits passive sensitisation, or whether the atopic serum used in our study was ineffective. Immunohistochemical studies have shown that mast cells in distal lung tissue express much lower levels of FcεRI (Andersson *et al*, 2009) and have less IgE bound to them (Balzar *et al*, 2007). Therefore upon incubation with atopic serum, only a limited number of free IgE receptors will bind the Der p1-specific IgE. On the other hand, mast cells are well regarded to highly express high affinity IgE receptors and have been shown to have on average 7500 molecules of IgE present on their surface (Schulman *et al*, 1983) leaving a substantial amount of unoccupied receptors (Metzger et al, 1986). Therefore distal lung tissue mast cells do have a large number of 'open' receptors available to bind IgE. The binding of irrelevant IgE molecules can provide steric hindrance however, preventing allergen binding and crosslinkage (Van Toorenenbergen *et al*, 1983). Unfortunately, the atopic pool of serum we utilised had a relatively low total IgE content and allergen-specific IgE titre when compared to other models of passive sensitisation. This may explain why this model of passive sensitisation did not work.

Nevertheless, a potential limitation to this model is whether allergen is able to penetrate deep enough into the lung to reach distal tissue. In a group of cat-allergic asthmatics exposed to cat, evidence of worsening small airways obstruction and increased air trapping at 6 and 23 hours after exposure was measured using high resolution tomography and closing volume spirometry (Zeidler *et al*, 2006). Also in a separate study, BAL from HDM-allergic asthmatics and non-asthmatics contained significantly more IL-5 after HDM inhalation, which correlated with BAL eosinophil numbers in the asthmatic group (Tang *et al*, 1997). This provides some evidence that the small airways are involved in allergic inflammation of the lung. This may be directly via IgE-dependent mechanisms caused by allergen binding to effector cells in the small airways, or indirectly caused by mediator release further up the respiratory tract that sends cascades of cytokines down to cause inflammation in more distal tissue. Either way, inflammation is occurring in distal lung tissue as a result of allergen provocation.

6.1.4 Neutrophil shape change assay

In vivo (e.g. BAL) and *ex vivo* (e.g. tissue explants) studies modelling inflammation generate supernatants that contain a wide range of inflammatory and anti-inflammatory mediators released from multiple cell types. We have generated supernatants from an *ex vivo* lung explant model which contains a complex mixture of inflammatory mediators that have been quantified by ELISA. However, little is known about the functionality of chemokines within these supernatants. Also they potentially contain novel chemoattractants that would not be detected using specific ELISA techniques. We therefore developed a leukocyte shape change bioassay in order to investigate these questions.

We have shown that distal lung tissue produces substantial amounts of IL-8. IL-8 is an important neutrophil chemokine that has been shown to be upregulated in asthma (Gibson *et al*, 2001, Fahy *et al*, 1995, Kikuchi *et al*, 2009) and in COPD (Yamamoto *et al*, 1997, Keatings *et al*, 1996). It is also known to induce a robust shape change response in neutrophils (Watts *et al*, 1991, Baggiolini and Kernen, 1992). Neutrophils themselves are easily obtainable from the peripheral blood of volunteers, in which they are the largest leukocyte population and are easily distinguishable by their size and granularity. Neutrophils were therefore an ideal cell type to utilise when developing this bioassay, using their shape change response as a biomarker.

Cytokines are vulnerable to a number of events that can affect their ability to carry out their function. They can be degraded (Zhao *et al*, 2005); bind to receptors, including soluble receptors; form complexes with autoantibodies and other cytokine-binding proteins (Shute *et al*, 1997, Kurdowska *et al*, 1996, Marshall *et al*, 2001); or may be trapped within the extracellular matrix bound to proteoglycans (Witt and Lander,

1994). We therefore hypothesised that we would see a deficit in neutrophil shape change due to the decreased functionality of chemokines within generated supernatants.

Supernatant generated from unstimulated distal lung tissue and tissue stimulated with anti-IgE and LPS all caused significant degrees of neutrophil shape change. This was comparable to the shape change response induced by recombinant IL-8. When comparing these responses, supernatant from stimulated lung tissue caused excess shape change that was not explained by the presence of IL-8 alone within them. The IL-8 was subsequently shown to be essential for neutrophil shape change upon utilisation of a neutralising IL-8 antibody. In support of this finding, IL-8 in BAL from ARDS patients was also shown to be essential for neutrophil chemotaxis (Goodman *et al*, 1996). During the early phase reaction in allergic inflammation, other chemoattractants may also play a role however as after IL-8 neutralisation, neutrophil chemotaxis was still evident (Erger and Casale, 1998). We concluded in our model, at the 24 hour time point, IL-8 is fully functional and essential for neutrophil shape change.

These findings may highlight IL-8 as a potential target to reduce inflammation of the lung, with particular relevance to COPD, which is considered to have an important neutrophilic component, but also in severe asthma and ARDS. However, due to the ubiquitous production of IL-8 by many cells and its importance in leukocyte recruitment, knocking out the effect of this chemokine may impair the immune response, leaving the body open to infection. As a result, the IL-8 receptors may prove to be a wiser target.

We have also shown using this assay that there is a synergistic relationship between low concentrations of IL-8 and Groα to cause neutrophil shape change. Upon CXCR2 antagonism this synergistic response was abrogated, highlighting the importance of CXCR2 activation. This supports data presented by other groups that CXCR2 activation primes neutrophils for increased responses (Hauser *et al*, 1999). This assay could therefore be used to investigate other mixtures of chemokines as has been done by Shen *et al*, 2004 and Gouwy *et al*, 2004. Synergy between these chemokines may be important in leukocyte attraction at sites distant from the site of inflammation, where the concentrations on the edge of a chemotactic gradient are lower.

The main unanswered question was in regard to the excess neutrophil shape change response observed when whole blood was stimulated with lung supernatant. Supernatant is likely to contain a complex mixture of mediators that could influence

neutrophil function and thus pinpointing the mediator(s) responsible for this increased response will be difficult. Although we have shown that there is a synergistic relationship between the two CXC chemokines, IL-8 and Groα, this was shown at low concentrations. Lung supernatant contains high concentrations of IL-8, which is likely to saturate both CXC receptors (CXCR1 and 2). Therefore the excess neutrophil shape change is unlikely to be caused by activation of these receptors. Using a BLTR antagonist we also showed that LTB_4 does not explain the excess shape change observed, but did lower the maximal shape change response. In any case, our data suggests that at physiological levels, the presence of IL-8 is fundamental for neutrophil shape change.

This assay has potential to be used to identify other chemokines or mediators important for leukocyte activation/attraction and identify possible drug targets. It could also be used to discern possible differences in the inflammatory profiles of diseases and pinpoint which leukocyte population may be more central in that disease. Also due to the inexpensive and easy access of human blood and the relative simplicity and effectiveness of the assay, it could prove to be a powerful tool for screening different drugs for their effects on leukocyte activation and chemoattraction.

6.2 Summary

We have shown that proximal airway and distal lung tissue have similar patterns of anti-IgE- and LPS-induced cytokine release but that IgE-dependent inflammation also causes the release of IL-5, with a distinct bell-shaped response. Release of TNFα and IL-1β precedes the release of other mediators and is likely to initiate a cascade of inflammatory cytokines. This communication between different cells may cause a degree of generalised airway inflammation, which may explain inflammatory events throughout the lung in chronic lung conditions such as asthma and COPD. Distal lung tissue releases substantially more of the majority of cytokines investigated and contains significantly more macrophages, mast cells and neutrophils than the proximal airways. Corticosteroid treatment decreases mast cell number in the distal airways of COPD patients and was most effective at inhibiting anti-IgE induced IL-5 and IL-6 production. The distal airways may therefore be a more effective target for drug therapy.

We have also shown that IL-8 released into supernatant is functional and fundamental for neutrophil shape change. There is also a synergistic relationship between suboptimal concentrations of IL-8 and Groα that is mediated through CXCR2 however an unknown factor within generated supernatant acts with IL-8 to cause excess neutrophil shape change.

6.3 Future Work

- Immunohistochemistry to determine cellular source of cytokines.

The release of inflammatory cytokines was compared between matched proximal airway and distal lung tissue; however this data does not give an exact indication of which cells are releasing these cytokines. This is particularly important as we have shown that for the majority of the cytokines measured, distal lung tissue was able to release significantly more. A possible explanation for this finding may be that there is a distinct difference in the cell density of cytokine secreting cells between the two compartments of the lung. As such, future work using immunohistochemical techniques, such as double-staining, could be used to identify and quantify these cytokine releasing cells.

- Increase n numbers – Is there differences in tissue from patients with different diseases?

We found no statistical difference in cytokine release or cell distribution when comparing patients with or without COPD or current or ex-smokers, which contradicts many studies in the literature (Di Stefano *et al*, 1998, Rutgers *et al*, 2000, Hogg *et al*, 2004). This may be because group sizes were relatively small and disease severity mostly mild to moderate. Increasing numbers in our groups and obtaining more non-smokers and patients with severe COPD would increase the power of the study and provide a more comprehensive view of disease progression and the pathological changes that occur.

Increasing numbers of patients prescribed steroid will enable stratification of this group and clarify what types of treatment, such as inhaled corticosteroid on its own or in combination with β-agonist, or oral corticosteroids, cause the effects we have seen on mast cell numbers and cytokine release.

- Isolation of cells from tissue.

The explant model enables the stimulation of cells within their physiological environment, keeping cell-cell and cell-matrix interactions intact. In order to better understand cell communication and the cytokine cascades initiated in inflammation, the isolation of cells could be used to unravel which cytokines are released by certain cell types and under what conditions. The shape change bioassay could also be utilised

to investigate which cell populations or microenvironment is best at recruiting certain cell populations. Also some aspect of this intricate environment may effect the passive sensitisation of effector cells. Mechanical dispersion of cells from lung parenchyma could enable the passive sensitisation of these cells outside the tissue environment.

- Use receptor antagonists and neutralising antibodies.

Hackett *et al*, 2008 showed that the cytokine cascade was abrogated upon neutralisation of TNFα. The utilisation of neutralising antibodies could clarify the importance of different cytokines in the induction of the cytokine cascade such as IL-1β or the resolution of inflammation such as IL-10, within tissue and isolated cell cultures.

A potential strategy to help isolate mediators acting with IL-8 to cause shape change may be to target neutrophil receptors, such as the prostaglandin receptors. Other mediators that could also be investigated are the inflammatory cytokines such as TNFα and GM-CSF and adenosine and other nucleotides that are known to activate and alter neutrophil function.

- Immunohistochemistry of sensitised tissue to investigate whether IgE is binding to effector cells.

IgE is a large molecule and may not be able to penetrate into the tissue in order to bind to mast cells. Also, other investigators have shown an absence of cells with IgE bound to their surface in the lung parenchyma of asthmatics (Balzar *et al*, 2007) and that mast cells present in the parenchyma have a low expression of FcεRI (Andersson *et al*, 2009). By staining passively sensitised tissue with an anti-IgE antibody conjugated to a fluorochrome or the enzyme horseradish peroxidase, we may be able to visualise if the serum IgE is able to bind to receptors on cells in the tissue. It would also be interesting to investigate mast cell heterogeneity in FcεRI and mast cell protease expression within our explants between the proximal airways and distal lung tissue.

- Use RT-PCR to investigate upregulation of genes for inflammatory cytokines.

RT-PCR provides a more sensitive and alternative method of investigating the upregulation of cytokines. Although upregulation of cytokine mRNA may not correspond with cytokine production, RT-PCR may provide evidence that passive sensitisation was successful.

- Stimulate explants with Der p1 and LPS.

There is gathering evidence within the literature that allergic inflammation and asthma is linked to TLR4 activation. As discussed in section 4.3, Hammad and colleagues showed that LPS present in HDM extract elicits an allergic phenotype in a murine model (Hammad *et al*, 2009). Also, in humans, asthma severity has been linked to the concentration of endotoxin present in house dust (Michel *et al*, 1996). We have shown that our HDM allergen extract does not cause the production of the pro-inflammatory cytokine TNFα and thus deduced any endotoxin present to be very low. The use of an endotoxin assay will be worthwhile in confirming this. It will be interesting however to investigate whether there may be a synergistic effect when co-stimulating passively sensitised explants with Der p1 and LPS.

- Quantify release of other neutrophil chemotactic mediators present in lung supernatant

As an alternative approach it may be useful to determine what other mediators are present in lung supernatant. This may also reveal other avenues of interest, for example, studies have shown similar amounts of Groα and IL-8 to be released *ex vivo* (Sachse *et al*, 2005), which opens up the question why we saw no neutrophil shape change response when IL-8 was neutralised in supernatant.

- Investigate activation of other cell populations.

This shape change bioassay was developed using whole blood and as such data can be acquired for the other cell populations present i.e. eosinophils, which are considered to be important in the pathophysiology of asthma.

- Investigate synergy between IL-8 and other CXCR2 chemokines

We have shown a synergistic shape change response in neutrophils stimulated with suboptimal concentrations of IL-8 and Groα and have shown through CXCR2 blockage that activation through this receptor was essential. It would be worth investigating whether other CXCR2 chemokines also have this synergistic effect, strengthening the importance of CXCR2 and ruling out the effect of Groα itself.

- Investigate leukocyte response to supernatants generated from different tissues with different disease states.

Another interesting avenue of research the shape change bioassay could be used for would be to investigate whether supernatants generated from different tissues or from different disease states, such as asthma, COPD, ARDS or cystic fibrosis, have distinct effects on leukocytes in whole blood. This could also be expanded to explore whether leukocytes from these patients behave differently compared to healthy controls. Evidence of this has already been shown by a group at Imperial College who have shown peripheral blood mononuclear cells from COPD patients demonstrate increased chemotactic responses to certain chemokines when compared to controls (Traves *et al*, 2004). The shape change assay may provide a quick and objective way of identifying important mediators, in different diseases or tissues, based on the activation profiles of leukocytes in whole blood.

Chapter 7
References

7.1 References

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