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School of Medicine

The role of elastase as an inflammatory stimulus in chronic obstructive pulmonary disease

By

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ABSTRACT

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The Role of Elastase as an Inflammatory Stimulus in Chronic Obstructive Pulmonary Disease

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Chronic obstructive pulmonary disease (COPD) is a debilitating disease that as of yet has no cure and therapy is limited to symptomatic relief. A major risk factor for the development of COPD is smoking, although the disease does have some component of genetic predisposition. In excess of £500 million funding per year is required to accommodate the needs of COPD patients and approximately 27, 000 deaths per year in the UK can be attributed to COPD. COPD is comprised of three conditions-chronic bronchitis, bronchiolitis and emphysema and these will be present in the COPD patient to varying degrees.

It is well accepted that COPD is a disease characterised by increases in inflammation and as such inflammatory stimuli, such as cigarette smoke and lipopolysaccharide (LPS) from bacterial cell walls, have been associated with disease development and progression. Consequently, there are associated increases in inflammatory cytokines such as TNFα in the COPD patient. Although inflammation plays a major role in the pathogenesis of COPD there are other factors to consider such as proteolytic damage caused by disturbances in the proteinase/anti-proteinase balance. This is of particular importance in emphysema where increases in neutrophil elastase concentration results in the destruction of elastin fibres which results in a decrease in lung function. Elastase is also known to contribute to the mucus hypersecretion associated with chronic bronchitis. The proteolytic actions of elastase are well characterised but there is gathering evidence to suggest that it may also be able to act as an inflammatory stimulus, thereby increasing its role in the pathogenesis of COPD.

This study has utilised a human lung explant model to investigate whether elastase can initiate an inflammatory response; concentrations of the pro-inflammatory cytokine TNFα and the anti-inflammatory cytokine IL-10 in the culture supernatant have been investigated as part of this. Data from this model (n=36) has shown that elastase can significantly increase both TNFα and IL-10 compared to control. Elastase stimulation, for 24hrs, caused the release of 30.1±8.0pg TNFα/mg tissue and 3.1±0.5pg IL-10/mg tissue. This response is comparable to that produced by LPS. We have also found that elastase can induce a Th2 type response from the parenchymal explants, with increases in IL-4, IL-5 and IL-13.

The inflammatory response detailed in this study appears to be unique to elastase and cannot be reproduced with other serine proteinases, such as trypsin and chymotrypsin, or a cysteine proteinase, papain. Our data has also shown that the proteolytic activity of elastase can be inhibited by an elastase-specific inhibitor, elastatinal, and by doing so attenuates the TNFα response; elastase stimulation alone produced 127.5±72.1pg/mg tissue, whereas with the inhibitor this production dropped to 40.4±9.0pg/mg tissue.

As of yet, the exact mechanism by which elastase induces inflammation is unknown but we have investigated the relationship between elastase and two candidate receptors-proteinase-activated receptor (PAR)-2 and Toll-like receptor (TLR)-4. Although elastase stimulation does not appear to alter the gross amount of these receptors present in the parenchyma, we have found that those patients with mild to moderate COPD tend to have greater levels of both PAR-2 and TLR-4. We have also utilised synthetic activating peptides for PAR-2 and in comparison to elastase stimulation it is suggested that elastase may cause its inflammatory and Th2 effects via distinct pathways.

Preface

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Abbreviations

A1AT	α_1-antitrypsin
AAA	abdominal aortic aneurysm
ADAM33	a disintegrin and metalloprotease 33
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
ALI	acute lung injury
ANOVA	analysis of variance
ARDS	acute respiratory distress syndrome
ATS	American Thoracic Society
BAL	bronchoalveolar lavage
BCA	bicinchoninic acid
BSA	bovine serum albumin
COPD	chronic obstructive pulmonary disease
CHX	cycloheximide
CR3	complement receptor 3
DAG	diacylglycerol
EC	endothelial cells
ECL	electrochemiluminesence
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
FEV_1	forced expiratory volume in 1 second
FcR	Fc receptor
FCS	foetal calf serum
FVC	forced vital capacity
GEF	guanine exchange factor
GMA	glycol methacrylate
GOLD	Global Initiative for Chronic Obstructive Lung Disease
HBD2	human beta-defensin 2
HRP	horseradish peroxidise
HUVEC	human umbilical vein endothelial cells
IFN	interferon
IgE	immunoglobulin E
IκB	inhibitor of NFκB
IL	interleukin
$InsP_3$	inositol triphosphate
LBP	lipopolysaccharide-binding protein
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MCP	monocyte chemoattractant protein
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase
MSD	Meso-scale discovery
NFκB	nuclear factor κB
NLR	Nod-like receptor
PAMP	pathogen-associated molecular pattern
PAR	proteinase-activated receptor

PBS	phosphate buffered saline
PIC	proteinase-inibitor cocktail
PKC	protein kinase C
PLCβ	phospholipase β
PMN	polymorphonuclear leukocyte
PMSF	phenyl methyl sulphonyl fluoride
PPE	porcine pancreas elastase
PRR	pattern recognition receptor
ROK	Rho kinase
RPMI-1640	Roswell Park Memorial Institute-1640
RT-PCR	reverse transcription polymerase chain reaction
RV	rhinovirus
SBTI	soybean trypsin inhibtor
SEM	standard error of the mean
SGH	Southampton General Hospital
SIRS	systemic inflammatory response syndrome
SLPI	secretory leukoprotease inhibitor
SNP	single-nucleotide polymorphism
TBS	tris buffered saline
Tc	cytotoxic T-cell
Th	T-helper cell
TIMP	tissue inhibitor of metalloproteinases
TLR	Toll-like receptor
TMB	3,3,5,5-Tetramethylbenzidine
TNF	tumour necrosis factor

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Chapter 1: Introduction

1. Introduction

1.1 Chronic Obstructive Pulmonary Disease (COPD)

COPD is the fourth leading cause of death worldwide and it is set to rise in the coming years (Murray & Lopez, 1996). It is a debilitating disease that has no cure and therefore represents an unmet therapeutic need. Due to this, COPD is a huge burden on health services across the globe and it is estimated that in the UK alone £500million per annum is spent on COPD management (British Lung Foundation, 2000). These factors highlight the need for further investigation into the pathogenesis of the disease, with a view to determining possible therapeutic targets.

1.2 Definition and diagnosis of COPD

COPD is defined by the American Thoracic Society (ATS) as "a disease state characterised by the presence of airflow limitation due to chronic bronchitis or emphysema" (ATS, 1995). Although this gives an overview of the disease it is the guidelines set out by the Global Initiative for Obstructive Lung Disease (GOLD) that are used when diagnosing COPD as they take into consideration the lung function data, as determined by spirometry, along with concurrent symptoms. These guidelines are detailed in table 1 (GOLD, 2005).

From examining the set of parameters laid down by GOLD it would appear that the diagnosis of COPD would be relatively simple but the reality is far more complex. COPD is a disease comprising three separate conditions, emphysema, chronic bronchitis and bronchiolitis and each of these components have to be considered before a diagnosis may be made. The diagnosis of COPD is also complicated by the possible presence of asthma, which occurs in

approximately 10% of all COPD sufferers leading to a high incidence of misdiagnosis (Jeffery, 1998 and O'Byrne & Postma, 1999). Also, patients with COPD are likely to be reluctant to visit their GP due to the strong link between smoking and the development of the disease and hence diagnosis is likely to occur later in the disease progression.

GOLD Status	COPD status	Spirometry	Symptoms
0	At risk	Normal ($FEV_1/FVC > 70\%$)	Chronic cough, sputum production
I	Mild	$FEV_1/FVC < 70\%$ $FEV_1 \geq 80\%$ predicted	With or without chronic symptoms- cough, sputum production
II	Moderate	$FEV_1/FVC < 70\%$ $50\% \leq FEV_1 < 80\%$ predicted	With or without chronic symptoms- cough, sputum production
III	Severe	$FEV_1/FVC < 70\%$ $30\% \leq FEV_1 < 50\%$ predicted	With or without chronic symptoms- cough, sputum production
IV	Very severe	$FEV_1/FVC < 70\%$ $FEV_1 < 30\%$ predicted or < 50% with chronic respiratory failure	Chronic respiratory failure

Table 1.1: Disease severity as defined by GOLD. COPD severity is characterised by changes in lung function with FEV_1 (forced expiratory volume in 1 second) and FVC (forced vital capacity) decreasing as the disease progresses. The ratio of these two read-outs is particularly important in staging the disease. Adapted from Global Strategy for the diagnosis, management and prevention of chronic obstructive pulmonary disease, Executive Summary, 2005 (GOLD, 2005).

1.3 Epidemiology of COPD

COPD is the fourth leading cause of death in the world and this is set to rise over the coming years (Murray & Lopez, 1996). In the UK alone, it is thought that 1.5% of the population have been diagnosed with COPD but this is likely to be underestimated due to problems with

diagnosis. It is estimated that 2% of males between the ages of 45 and 65 and up to 7% of males over the age of 75 are diagnosed with COPD (British Lung Foundation, 2000). The incidence of COPD in females has also risen from 0.8% in 1990 to 1.36% in 1997 (Soriano *et al.*, 2000). This latter data is derived from the prevalence of physician diagnosed COPD but, due to a high incidence of misdiagnosis, this figure is likely to be significantly higher.

Although COPD is a leading cause of death, a thirty year trend has shown an overall decrease in the number of male deaths from the disease (from 143 per 100 000 people in 1971 to 66 per 100 000 people in 1999), whereas female deaths over the same period have slowly risen from 125 to 140 (again per 100 000 people) (Office for National Statistics**,** 1999). While it is important to assess the mortality attributed to COPD it is also imperative to acknowledge that it is the associated morbidity that incurs great cost to the NHS with approximately £800million per annum being spent on the treatment and care of COPD patients (Guest, 1999) along with further economic cost due to factors such as lost work days by both patients and carers.

1.4 COPD risk factors

There are several risk factors associated with COPD, but the most prominent is smoking with over 90% of COPD patients having a smoking history and approximately half of all smokers developing the disease (Snider, 1989). This is not only confined to those who smoke tobacco but also substances such as cannabis and there is some evidence to suggest that the latter may be of greater risk due to the way in which the smoke is inhaled (Tan *et al.* 2009); smoke is inhaled deeper and held within the lungs for a greater length of time thus allowing any irritants or particulates increased opportunity to cause damage to the airways and parenchyma.

However, the same study suggests that cannabis smoking alone does not increase the risk of developing COPD but it is increased if conventional tobacco cigarettes are also smoked.

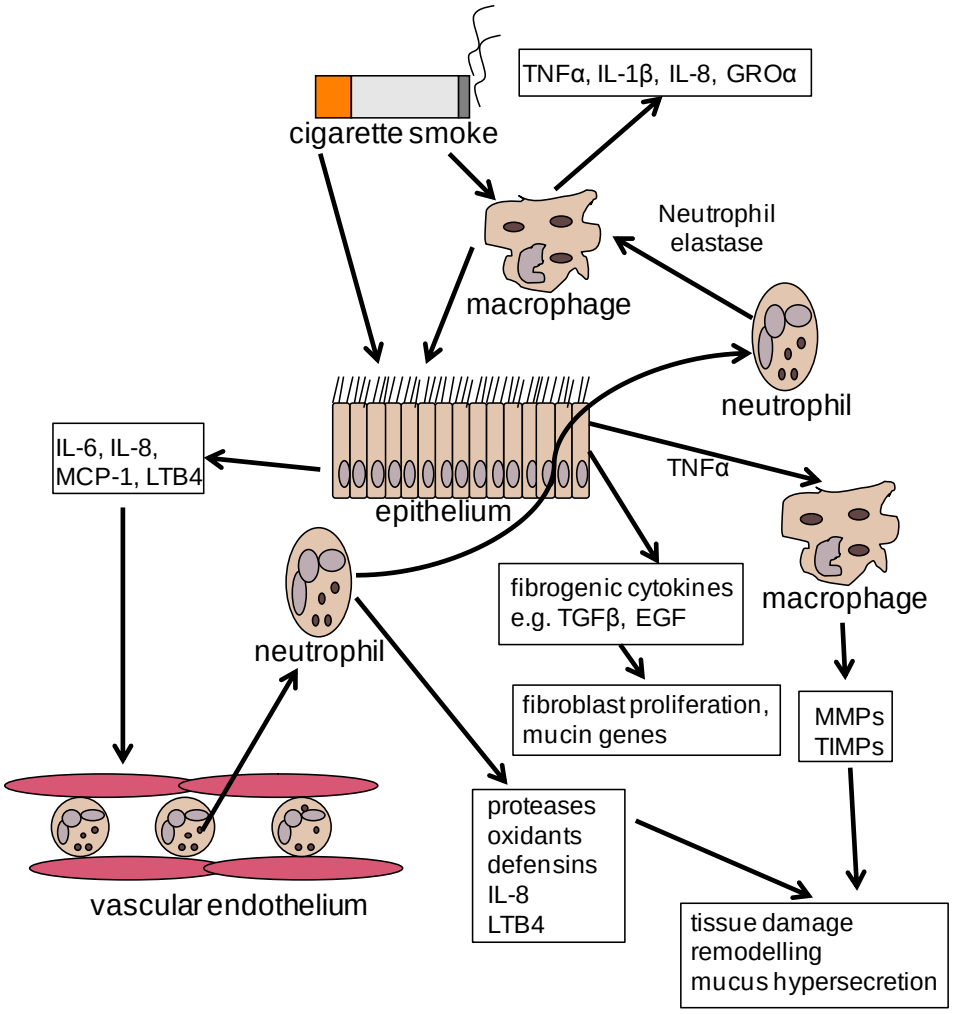

Figure 1.1: Cigarette smoke causes inflammation in the airways. Cigarette smoke causes the release of pro-inflammatory cytokines, proteases and oxidants from epithelial cells, macrophages and neutrophils. These mediators in turn cause tissue damage, remodelling and mucus hypersecretion thereby exacerbating the inflammatory environment. Adapted from Chung (2001).

Cigarette smoke contains many noxious chemicals, such as tar, benzene, nitrosamines and heavy metals such as lead and cadmium that can act as irritants to the epithelial cells within the airways thus provoking an inflammatory response (Barsanti *et al.*, 2007 and Chang *et al.*, 2005). After exposure to irritants, epithelial cells, along with macrophages, release a range of

chemotactic mediators for neutrophils such as the chemokine interleukin (IL)-8 and leukotriene B_4. Neutrophils become activated and ultimately undergo respiratory burst, a process which results in the release of proteases, oxidants and defensins, which can then contribute to the inflammatory milieu. These neutrophil products, along with mediators released directly from macrophages, play a role in the tissue destruction, remodelling and mucus hypersecretion that is characteristic of COPD. This process is summarised in figure 1.1.

Although smoking plays a substantial role in the causation of COPD it is not the sole cause and other factors such as diet and environment must also be considered. For example, a diet low in anti-oxidants can contribute to an already altered oxidant/anti-oxidant balance initiated by cigarette smoke inhalation, which can then increase the diseased state (Rahman *et al.*, 2006). Air pollution may also contribute to the initiation of COPD although evidence for this is limited and it is difficult to determine which components of air pollution may be responsible as data considering the atmosphere composition is insufficient to draw firm conclusions. However, although air pollution is comprised of particulates of many sizes, it is only those with a diameter of less than 10μm that are small enough to be inhaled into the lungs and therefore it is the proportion of these in the atmosphere that are important when considering this factor as a causative agent for COPD. Zanobetti and colleagues (2008) have shown that those with COPD have increased mortality risks as the levels of small particulate air pollution increases. Another source of small airborne particulates is those generated from cooking with biomass fuels in enclosed spaces; this presents particular problems in developing countries and it is likely to be an important risk factor in the development of COPD within the female

populations of these areas (Dennis *et al.,* 1996). Once inhaled, these particulates have much the same effect as the irritants found in cigarette smoke as detailed in figure 1.1.

1.5 Genetics of COPD

While environmental factors play a predominant role in the development of COPD it is important not to overlook genetic predispositions, in particular that of α1-antitrypsin (A1AT) deficiency. A1AT is an anti-proteinase that is usually found in sufficient concentrations to balance the effects of the proteinase neutrophil elastase, however, in those with A1AT deficiency this anti-proteinase is severely reduced thus allowing an increase in elastase-mediated destruction of the parenchyma. This balance will be discussed further in section 1.11.4**.** The gene coding for A1AT has three main alleles with the M allele giving normal levels of the anti-proteinase, the S allele giving a slight decrease and the Z allele resulting in a much greater decrease. This latter allele has been shown to have links with the development and progression of emphysema (Dowson *et al.,* 2001). Although this is the most common genetic cause of COPD, A1AT deficiency only accounts for 1-2% of all COPD cases (Lomas and Silverman, 2001).

More recently there has been gathering evidence to suggest that defects in other genes may also contribute to the development of COPD. Gingo *et al.* (2008) have shown that there is an association between single nucleotide polymorphisms (SNPs) in the promoter region of the TNFα gene and the development of COPD and in particular it is the –308 minor allele of this gene that shows the greatest link to COPD. Other genes that have been shown to have a link with COPD include those coding for matrix metalloproteinases (MMPs) and tissue inhibitors

of metalloproteinases (TIMPs) (Sampsonas *et al.*, 2006) and Pillai *et al.* (2009) have identified two SNPs at the α-nicotinic acetylcholine receptor at locus CHRNA 3/5 that may be linked to susceptibility to COPD.

Although these studies have shown potential genetic targets for the development of COPD, further study is required to investigate the complex relationships between these genetic variations and environmental risk factors.

1.6 Pathology of COPD

COPD is a complex disease comprising of three separate conditions (chronic bronchitis, bronchiolitis and emphysema) and therefore it is important to consider both the individual effects of the different components and the cumulative actions contributing to the overall disease state. Also, it is vital to note that the different pathologies of COPD are on a sliding scale with every patient having varying degrees of disease.

1.6.1 Chronic bronchitis

Chronic bronchitis presents as chronic inflammation of the central airways and is defined by the British Medical Research Council (1965) as having a productive cough present on the majority of the days, in a three-month period, in each year, for a minimum of two successive years. Associated with this is the hypersecretion of mucus and sub-mucosal gland hyperplasia, which affects the large conducting airways. Hypersecretion of mucus may arise due to the stimulatory actions of neutrophil elastase and chymase on the goblet cells as well as the activation of sensory nerve endings in the airways. Takeyama *et al.* (1999) have also

shown that epidermal growth factor (EGF) can stimulate the expression of the gene MUC5AC which ultimately leads to the production and secretion of mucus from epithelial cells both *in vitro* and *in vivo.* This effect was potentiated with the addition of TNFα and thus suggests that EGF-stimulated mucus production could play a role in the pathogenesis of chronic bronchitis and, therefore, COPD. EGF, along with other growth factors, also induces hyperplasia of submucosal cells as well as the proliferation of goblet cells (Casalino-Matsuda *et al.,* 2004), all of which progress the development of chronic bronchitis and exacerbate COPD.

1.6.2 Bronchiolitis

As with many of the components of COPD, bronchiolitis is characterised by increases in inflammatory cells such as neutrophils and CD8+ T cells in the small, peripheral airways (Lams *et al.,* 1998). It is thought that these changes in the inflammatory environment contribute to the airways obstruction found in COPD with lung function decreasing as the signs of bronchiolitis increases (Saetta *et al.,* 1998). Along with increased inflammation, the small peripheral airways are also affected by fibrosis mediated by an influx of fibroblasts and myofibroblasts. Continued injury and repair of these airways eventually results in the formation of scar tissue along with remodelling of the airway walls with increased deposition of collagen. This leads to narrowing of the lumen and the establishment of permanent airways obstruction and a decrease in FEV_1.

Other definitive characteristics of bronchiolitis include increased smooth muscle cell mass (Hogg *et al.,* 2004) and the loss of alveolar attachments, the latter of which contribute to the loss of elastic recoil found in COPD patients and ultimately leads to the early closure of

bronchioles upon expiration. Changes to the elastic recoil are also characteristic of emphysema as detailed in section 1.6.3 below.

1.6.3 Emphysema

Emphysema is defined as 'a condition of the lung characterised by abnormal, permanent enlargement of airspaces distal to the terminal bronchiole accompanied by destruction of their walls and without obvious fibrosis' (Lang *et al.,* 1994). The increase in size of these airspaces leads to poor efficiency of gas exchange and therefore a decrease in lung function. This change in structure is detailed in figure 1.2.

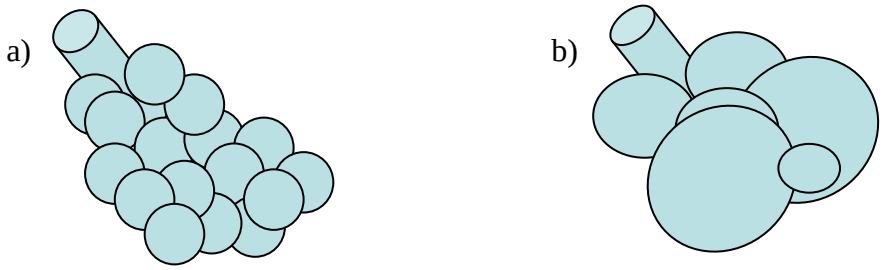

Figure 1.2: Alveolar destruction in emphysema. a) Alveolar structure within normal healthy lungs-distinct air sacs can be seen which provides a large surface area for efficient gas exchange. In contrast b) shows the altered structure in an emphysematous lung-alveolar walls have been degraded, resulting in larger air spaces and decreased surface area and increased chances of air trapping within the lung.

Associated with the destruction of the alveolar walls is a loss of elastic recoil (Hogg *et al.,* 1994) due to the degradation of elastin by proteinases such as neutrophil elastase. This causes the airways to collapse earlier during expiration leading to air trapping within the lungs, which ultimately leads to decreased lung function. Although the symptoms of emphysema are broadly the same in each patient there are three separate patterns of distribution of alveolar destruction: panacinar, centriacinar and paraseptal emphysema (Snider *et al.,* 1991).

Emphysema can develop due to the ageing process (Verbeken *et al.,* 1992) and A1AT deficiency (Needham and Stockley, 2004) and in these cases it is more likely that the patient will have panacinar emphysema whereas if smoking primarily causes the damage then it is more likely that centriacinar emphysema will develop (Hansel & Barnes, 2004).

1.7 COPD: An inflammatory disease

As suggested in the previous sections, COPD is a disease driven by inflammatory processes and there have been many studies showing an increase in the number of inflammatory cells within the sputum and bronchoalveolar lavage (BAL) fluid from COPD patients (Pesci *et al.* 1998, Keatings *et al.*, 1996). The principal inflammatory cells found in these patients are macrophages, neutrophils and mast cells, unlike in asthma where the eosinophil predominates (Matsumoto *et al.,* 2008). COPD is also associated with an infiltration of CD8+ T cells into the airway lumen (Tetley, 2005) and there is evidence to suggest that there is a correlation between the number of CD8+ cells in the lumen and the disease severity (Gadgil *et al.,* 2006). The inflammatory cell infiltrate, which is an integral part of the pathogenesis of this disease, also contributes to the obstruction of the airways.

Although COPD is predominantly a disease of the lungs it is important to acknowledge the systemic inflammatory effects that accompany this condition. Patients suffering from chronic hypoxia, caused by inefficient gas exchange at the level of the alveoli due to the emphysematous component of COPD, are at risk of developing pulmonary vasoconstriction and pulmonary hypertension (Kessler *et al.,* 2001). This hypertension is coupled with remodelling of the arteriole walls and the infiltration of inflammatory cells such as

macrophages and CD8+ T cells. The ultimate effect of these processes is increased smooth muscle surrounding the vessels, as well as increases in proteoglycans and collagen within the walls of the vessels; the resulting fibrosis causes the obstruction of the arterioles and exacerbates the hypertensive situation.

As well as vascular effects, COPD also presents with more generalised signs such as changes in metabolism with gross weight loss and respiratory and skeletal muscle fatigue. It has been shown that weight loss may be associated with increased levels of TNFα as well as soluble TNFα receptors with Schols *et al.* (1996) showing a link between increased resting energy expenditure in COPD patients and an increase in sTNF-R75. This study also showed increases in the inflammatory chemokine IL-8, C-reactive protein (an indicator of inflammation) and lipopolysaccharide binding protein (LBP) in the COPD group compared to the control group; all of which indicate systemic inflammation. Having established that markers of systemic inflammation are increased in those with COPD, it is prudent to assess where these markers are generated and how a local inflammatory response can result in a systemic effect. Local production of inflammatory cytokines such as TNFα, IL-6 and IL-1 feedback to the liver and adrenal glands thus resulting in the production of acute phase proteins and glucocorticoids respectively. The up-regulation of acute phase proteins such as C-reactive protein (CRP) results in an increase in the local host defence and consequentially an increase in the local inflammation. This potentiates a self-amplifying cycle of inflammation both at the local site of infection or invasion and systemically. On the other hand, the presence of glucocorticoids can have both up- and down-regulatory effects on the level of inflammation; directly glucocorticoids increase the hepatic production of acute phase

proteins but indirectly they decrease this production via a suppression of the cytokines that are likely to induce an acute phase response such as TNFα, IL-1 and IL-6.

1.8 COPD immunology

1.8.1 The innate immune system

The innate immune system plays a vital role in the recognition of self from non-self and, as such, is important in the removal of harmful pathogens from the host in the healthy individual. However, in the diseased state, as with COPD, this system can become compromised which leads to a decrease in host defences and an ultimate increase in infection and disease severity. In the normal situation pathogens are recognised as non-self via pathogen-associated molecular patterns (PAMPs) on their surface and these PAMPs are detected by pattern recognition receptors (PRRs) on phagocytic cells such as neutrophils and macrophages.

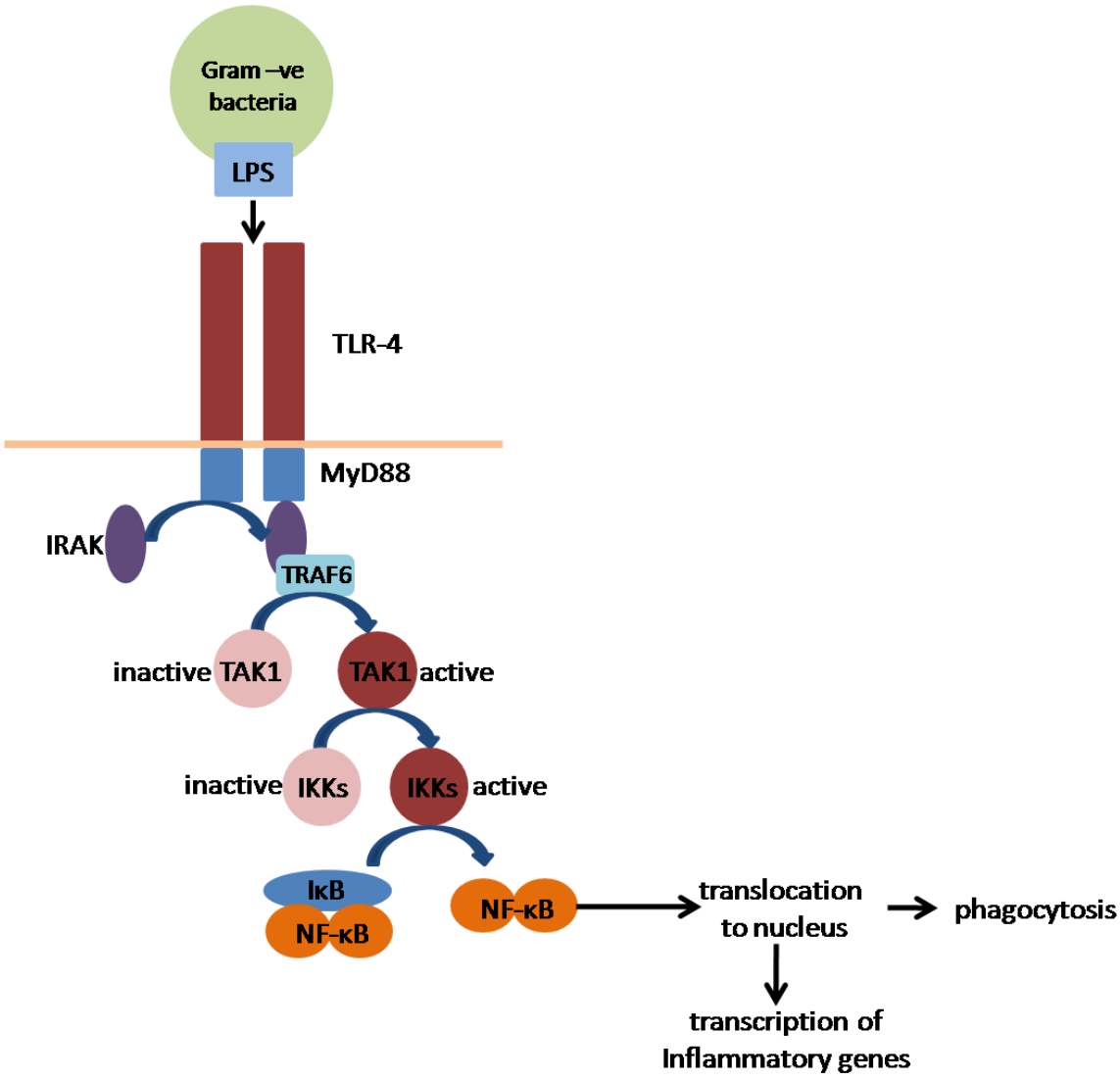

Figure 1.3: Pathogen recognition by the innate immune system via TLR-4. LPS on the bacterial cell wall is recognised by TLR-4 and the binding of this PAMP (LPS) to the PRR on TLR-4 results in the recruitment of the adaptor protein MyD88. MyD88 then interacts with the serine/threonine kinase IRAK, thereby recruiting it to the receptor complex. This situation is repeated with the activation and addition of TAK1 and IKK. Ultimately, NF-κB becomes phosphorylated and can dissociate from its inhibitor IκB in order to translocate to the nucleus to initiate the transcription of inflammatory genes. This translocation can also result in the initiation of phagocytosis. Adapted from Medzhitov & Janeway, 2000.

An example of this is when Toll like receptor (TLR)-4 recognises LPS from the cell wall of Gram-negative bacteria; the lipid A moiety of LPS is recognised and bound by plasma

lipopolysaccharide-binding protein (LBP) and the resulting complex binds to the CD14 scavenger receptor found on the cell membrane of the phagocytic cells. Following this, TLR-4 is activated which results in the initiation of the NFκB pathway and the dissociation of NFκB from its inhibitor IκB, thus allowing the transcription factor to be translocated to the nucleus to facilitate the expression of pro-inflammatory genes, such as those for TNFα, and to initiate the process of phagocytosis (Zhang and Ghosh, 2000). The pathogen is endocytosed into the cell where it fuses with granules containing microbicidal agents such as lysozyme and lactoferrin and antibiotic peptides such as α-defensins, and these act to destabilise the microbial membranes. Neutrophils and macrophages also release proteinases such as elastase and cathepsin G, which act to degrade invading pathogens.

As well as recognising invading pathogens, the innate immune system also plays an important role in the clearance of particulate matter as well as dead and dying host cells in order to maintain healthy airways. The removal of these innocuous particles involves their internalisation into phagocytic cells in a similar fashion to the removal harmful pathogens. However, unlike pathogen removal this process does not result in further activation of the phagocytic cells and the release of pro-inflammatory mediators. Whereas pathogens are recognised by receptors such as the TLRs and the Nod-like receptors (NLRs) (Ratner *et al.*, 2007 and reviewed in Le Bourhis & Werts 2007 and Carneiro *et al.,* 2008), non-pathogenic material, such as the particulates found in cigarette smoke, are firstly recognised by opsonins such as complement and the collectins and as such Warheit *et al* (1985) have shown that particulate asbestos can activate complement to form C5a in rat lungs. C5a then acts as a chemoattractant for macrophages that can then phagocytose the particles; it is possible that other particulates may also have this ability. The resultant complexes of opsonin and foreign

particles are then bound by the either the CR3 or FcR receptor on the cell surface and internalised via the phagocytic process (reviewed in Aderem & Underhill, 1999). Other receptors that are important in the innate immune system include the scavenger receptors that are found primarily on macrophages and play a major role in the removal of apoptotic host cells as well as invading bacteria (Peiser & Gordon, 2001).

Bacterial infections play a major role in COPD exacerbations and so it is clear that the innate immune system in these patients is imperfect. As mentioned, LPS from Gram-negative bacteria is recognised by TLR-4 and MacRedmond *et al.* (2007) have shown that TLR-4 is up-regulated in the nasal epithelium of those with mild to moderate COPD and it is significantly increased in those with severe COPD. Considering the relationship between COPD exacerbations and bacterial load it is sensible to assume that TLR-4 is up-regulated in response to this increase, but as the disease progresses with greater exacerbation frequency this defence system fails to overcome infection. TLR-4 gene expression was also shown to be decreased after exposure of airway epithelial cells (cell line A549) to cigarette smoke extract, thus providing some evidence to suggest that increases in cigarette smoke inhalation is associated with increased severity of disease. MacRedmond *et al.* (2007) have also shown an associated increase in human β-defensin-2 (HBD2) in mild to moderate COPD patients with a significant decrease in those with more severe disease compared to the former group. In support of these findings, Herr *et al.* (2009) have shown that both current and former smokers have significantly decreased levels of HBD-2 in response to infection with community acquired pneumonia, a common infection for COPD patients. These data suggest possible reasons as to

why those with COPD are affected to a greater extent by infections that a healthy individual will find minor.

In contrast to the suppressive effect cigarette smoke has on the ability of the innate immune system to combat bacterial infections, Kang *et al.* (2008) have shown that airway and parenchymal inflammatory and remodelling responses to viral ligands, such as poly I:C, after exposure to cigarette smoke in mice are upregulated. Hewson *et al.* (2005) have shown that in humans TLR-3 mRNA and protein is upregulated by rhinovirus (RV), a common infection for COPD patients, in human bronchial epithelial cells. This study also showed that inhibition of TLR-3 results in an upregulation of the inflammatory cytokines IL-6 and IL-8 and it is thought that this may be due to a down-regulation of anti-viral effects resultant from the blocking of the receptor, which ultimately leads to increased viral replication and an upregulation of non-TLR-3 receptors such as protein kinase R. These data highlight the need for effective viral control and also the effects of defective immune responses as are likely in COPD.

Having determined that host defences in COPD are impaired it is clear to see how the airways of these patients easily become colonised by bacterial and viral infections, and considering the increases in receptors that recognise these pathogens found in those with mild to moderate disease it would be assumed that these infections could be cleared via an increase in phagocytosis. However, Lundborg *et al.* (2004) have shown that alveolar macrophages, when exposed to aggregates of ultrafine carbon particles or diesel exhaust particles, have impaired phagocytic properties. This indicates that both exposure to air pollutants and also the

particulates found in cigarette smoke hinders the ability of the inflammatory cells to perform their inherent task of pathogen removal, thus perpetuating disease progression.

1.8.2 Autoimmunity in COPD

Recent thoughts regarding the role of inflammation in the pathogenesis of COPD have revolved around a possible autoimmune component to the disease. This has stemmed from the fact that even when smoking cessation has occurred, those with COPD still have increased levels of inflammation in comparison to healthy ex-smoker controls (Rutgers *et al.*, 2000). This study has shown increased numbers of macrophages in lung biopsies and increased neutrophils in sputum samples from COPD patients compared to control patients. This ongoing inflammatory situation is reminiscent of unresolved inflammation in autoimmune conditions such as rheumatoid arthritis and so it has been postulated that a similar autoimmune mode of disease may be applicable to COPD. Following on from this idea, Taraseviciene-Stewart *et al.* (2004) developed an animal model of autoimmune emphysema. This model involves the immunisation of Sprague-Dawley rats with human umbilical vein endothelial cells (HUVECs), resulting in the production of anti-endothelial cell (EC) antibodies by the host. The ultimate effect of this was the development of centrilobular emphysema with associated alveolar cell apoptosis and activation of MMPs. Although this model initiates an autoimmune version of emphysema in rats, anti-EC antibodies have yet to be detected in human subjects with COPD and thus greater investigation into this area is required before firm conclusions can be made.

1.9 Inflammatory cells: the neutrophil and macrophage in COPD

COPD, as an inflammatory disease, is characterised by the presence of inflammatory immune cells such as macrophages and neutrophils. As mentioned previously, both of these cell types play important roles in the innate immune system however the disease state is accompanied by an influx of these inflammatory cells. These cells become activated to release chemoattractant cytokines, such as IL-8, therefore perpetuating the inflammatory milieu. Many groups have investigated the presence of both macrophages and neutrophils in COPD and Pesci *et al.* (1998) have shown that macrophages in bronchoalveolar lavage (BAL) fluid from those with COPD are significantly increased compared to control. Finkelstein *et al.* (1995) have also shown an increase in macrophages in those with more severe emphysema than those with milder disease as determined by alveolar wall density in sections taken from resected parenchymal tissue. An increase in macrophage number contributes to an overall increase in inflammation in the airways and parenchyma especially in smokers where activation of these cells occurs more frequently. The ultimate effect of this situation is an increase in inflammatory and proteolytic stimuli such as IL-8 and macrophage elastase (MMP-12) thus propagating the destructive environment associated with COPD.

Although macrophages are important in the pathogenesis of COPD it is vital to consider the role of other inflammatory cells such as neutrophils. Traditionally, COPD is associated with neutrophil influx and these cells are thought of as having a pivotal role in the development of COPD, especially with regard to the release of proteinases, such as elastase, known to have elastolytic actions on the parenchyma. Finkelstein *et al.* (1995), in the same study that showed a correlation between increased macrophage number and increased inflammation in the

parenchyma, have shown in comparison that there is a decrease in the number of neutrophils in those with severe emphysema compared to those with mild to moderate disease. This suggests that the number of neutrophils present does not play as important a role in the pathogenesis of emphysema as previously thought even though neutrophil elastase is implicated in the development of emphysema. Conversely, this finding may not present the full scenario as neutrophils move rapidly through the airway and as such are difficult to quantify. It is possible that instead of the gross number of neutrophils increasing it is an increase in the number of cells undergoing degranulation that is the important factor. In support of this, Ilumets *et al.* (2008) have shown an increase in neutrophil proteinases in the sputum of patients with COPD exacerbations. It is also possible that due to the increased macrophage presence in the COPD lung a greater amount of phagocytosis of dead and dying neutrophils may occur; Makris *et al.* (2009) have shown increased levels of neutrophil apoptosis in those with COPD compared to those without.

1.10 Cytokines and their role in inflammation and COPD

The inflammatory characteristics of COPD are well reflected by the cytokine profile of these patients and as such the pro-inflammatory cytokine TNFα is a prominent feature along with other cytokines such as IL-6 and IL-8. Studies have shown an associated increase of these cytokines in BAL and sputum from patients with COPD compared to healthy donors (Kuschner *et al.*, 1996 and Keatings *et al.*, 1996). In support of this, Hackett *et al.* (2008) have shown the localisation of TNFα to macrophages and mast cells in the parenchyma and that this cytokine plays a major role in the initiation of cytokine cascades involving IL-6 and IL-8; neutralisation of TNFα prevents the associated increases in these cytokines. This study

also shows that the TNFα response itself can be augmented via the blockade of IL-10, thus suggesting that this anti-inflammatory cytokine plays a regulatory role in the inflammatory response. Armstrong *et al.* (1996) have shown that LPS-induced TNFα production from human alveolar macrophages and peripheral blood monocytes can be attenuated, at both the mRNA and protein levels, via the addition of IL-10. These data are supported by a study by Cassatella *et al.* (1993) who have shown that IL-10 can inhibit the LPS-induced TNFα response from human polymorphonuclear leukocytes (PMNs) as well as the cytokine production from those cells undergoing phagocytosis. This study has also shown that this anti-inflammatory cytokine is capable of inhibiting other inflammatory cytokines in this system such as IL-1β and IL-8. Having assessed the inhibitory role of IL-10 on TNFα it is interesting to note that TNFα itself can up-regulate IL-10 thus providing a source for its own regulation (Wanidworanun and Strober, 1993); purified human monocytes were incubated for 24hrs with recombinant TNFα and IL-10 mRNA and protein levels assessed by RT-PCR and ELISA respectively and at both levels the pro-inflammatory cytokine increased the expression of IL-10. This effect could not be replicated by the use of other pro-inflammatory cytokines such as IL-1β and IL-6 thus highlighting the close relationship between TNFα and IL-10 in the regulation of inflammation.

Considering the inflammatory properties of TNFα, it would be sensible to assume that this cytokine plays a major role in the pathogenesis of COPD and as such would be up-regulated in those with more severe disease. This idea is supported by a study by Hacievliyagil *et al.* (2005), who have shown that TNFα is increased in induced sputum from patients with severe to very severe COPD compared to those with mild to moderate disease. Associated with this

finding was a significant increase in IL-6 and IL-8 in the more severe group of patients. This study also showed a negative correlation between the IL-6 concentration in the sputum and FEV_1/FVC, thus suggesting that IL-6 is important in disease progression. Finally from this study, it has been shown that the concentration of all three cytokines is positively correlated to the smoking load (pack years) of the subjects and so this adds extra weight to the theory that smoking leads to an increased disease status. Mio *et al.* (1997) and Dubar *et al.* (1993) have also shown an association between cigarette smoke and the activation of bronchial epithelial cells and the subsequent increased release of IL-8 into the surrounding tissue. Following on from this, these studies have also shown that after cigarette smoke exposure alveolar macrophages release IL-6 and TNFα therefore potentiating the inflammatory milieu and exacerbating the disease status.

As well as having a strong inflammatory cytokine profile, COPD is traditionally thought as being defined by T-helper cell (Th)1 cytokines (Majori *et al.*, 1998 and Grumelli *et al.*, 2004), whereas other lung conditions such as asthma are defined more by the presence of Th2 cytokines such as IL-4 and IL-13 (as reviewed in Kuipers *et al.*, 2004). However, more recently there is some evidence to suggest that Th2 cytokines may play a greater role in the pathogenesis of COPD than first thought. Barceló *et al.* (2006) have investigated the intracellular cytokine profile of T lymphocytes in COPD patients and concluded that these subjects were characterised by increased Th2 cytokines and that these cytokines correlated negatively to the percent predicted FEV_1 values. This study suggests that the cytokine involvement in COPD is far more complex than originally thought; Barceló and colleagues

have only investigated those with stable COPD and not those with exacerbations and so it is clear that this area is in need of further investigation.

1.11 Pathology Hypotheses

Over the years there have been many theories as to the main cause of COPD and therefore it is important to investigate these differing findings in order to assess where the current understanding lies.

1.11.1 The British Hypothesis

This hypothesis lays emphasis on recurrent lung infections as the root cause of COPD in smokers and in particular the loss of lung function associated with these infections (Fletcher, 1959). It was postulated that the disease process is initiated by mucus hypersecretion and that this would lead to decreased defence of the bronchial tree thus leaving the airways susceptible to recurrent bacterial and viral infections. These infections ultimately lead to a decrease in the patient's lung function and, whereas a healthy subject would recover this loss, a COPD patient would be unable to, thus perpetuating the disease progression. This means that although lung function decreases over time even in the healthy lung, COPD causes this loss to come quicker and to a greater extent (figure 1.4).

This hypothesis for the pathogenesis of COPD remained unchallenged for several years until a study by Fletcher *et al.* (1976) showed that there was no relationship between loss of lung function (shown as a decrease in FEV_1) and the frequency of respiratory infections and the associated sputum quantity and quality. This work has been largely unopposed until recently

where Banerjee *et al.* (2004) have shown that those with stable moderate to severe COPD, and pathogenic microorgnisms in their sputum, have increased airways inflammation and a poorer health status.

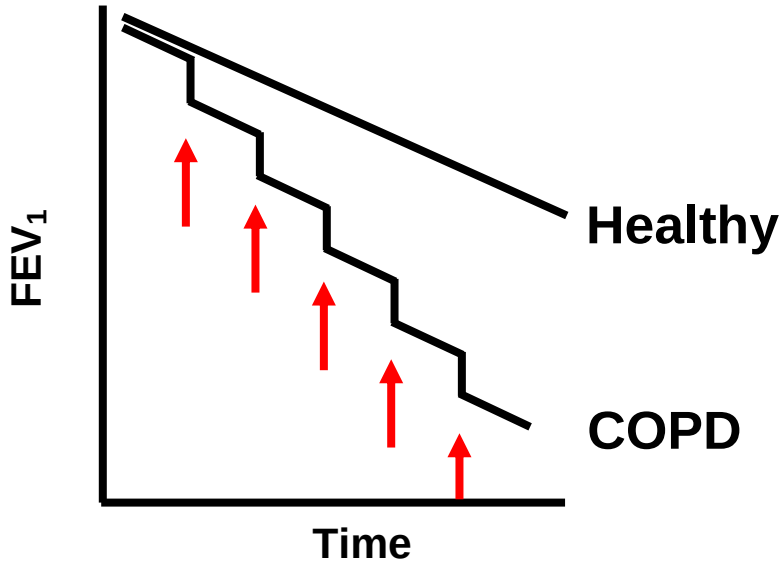

Figure 1.4: Loss of lung function associated with recurrent bacterial and viral infections. Lung function declines in the healthy lung over time (top line) but this loss occurs at a greater rate and extent in those with COPD (bottom line) especially following exacerbations mediated by bacterial and viral infections (red arrows).

Although there is evidence both for and against this hypothesis it is clear that exacerbations mediated by bacterial and viral infections do play a role in the pathogenesis of COPD. However, the idea that they are the sole method by which the disease progresses is unlikely considering that there must be some level of damage to the normal innate immune response to pathogens as well as the mechanisms behind mucuciliary clearance before these infections can take hold and cause the irreversible loss of lung function found in these patients.

1.11.2 The Dutch Hypothesis

In opposition to the British hypothesis, the Dutch hypothesis places emphasis on the loss of lung function found in COPD being attributable to an underlying airway hyper-reactivity, such as that found in asthma (Orie *et al.*, 1961). The hypothesis suggests that a patient can progress from having acute bronchitis, which leads to chronic asthma and ultimately ends in COPD- all with common causal mechanisms. However, asthma and COPD present in very different ways in the patient and also progress in a different manner; asthma is characterised by reversible airflow obstruction whereas the obstruction in COPD is largely irreversible. Also, asthma is usually episodic unlike COPD, which is progressive and the response to bronchodilators and corticosteroids is far better in asthmatics compared to those with COPD. From assessing these differences it would appear that the Dutch hypothesis is unfounded and that these two diseases cannot have a common origin. However, it is possible for some asthmatics to show a more progressive phenotype, as in COPD and also for some COPD patients to have an element of airflow obstruction that is indeed reversible thus providing support for this hypothesis. Although there appears to be some overlap in the characteristics of these two airways diseases it is possible that they are not part of the same disease but are in fact found concurrently in the same patient; approximately 10% of COPD patients also have asthma and are usually defined as having wheezy bronchitis (Barnes, 2000).

This theory would also suggest that COPD and asthma share a genetic predisposition and that the differences between the two diseases would come about due to environmental factors. In asthma, there is an association with genes coding for Th2 cytokines and their receptors (Loza & Chang, 2007 and Hunninghake *et al.*, 2007)**,** but little evidence suggests a link between

these polymorphisms and the development of COPD. On the other hand, the strongest genetic link to COPD is a deficiency in the anti-proteinase A1AT but this accounts only for approximately 1% of all COPD cases (Lomas and Silverman, 2001) and so far there are no links between this deficiency and the development of asthma. Recent evidence, however, suggests that there may be a link between polymorphisms in the gene ADAM33 (a disintegrin and metalloprotease 33) and both asthma (van Eerdewegh *et al.*, 2002) and COPD (van Diemen *et al.*, 2005).

Although so far there is little evidence to suggest that asthma and COPD belong to the same continuous spectra of airways disease there is some indication that the presence of asthma may in fact be a significant risk factor for the development of COPD. Silva *et al.* (2004) have shown in their longitudinal study that those with active asthma are significantly more likely to develop COPD compared to those either with non-active asthma or no asthma. The active asthma group also had a significantly higher risk of developing symptoms of chronic bronchitis or being diagnosed with emphysema, both of which are components of COPD.

Taking the evidence into consideration it is likely that asthma and COPD, although pathologically different, do share some similar characteristics in some patients but this connection is not substantial enough to support the Dutch hypothesis fully. As such, it is more likely that both the Dutch and the British hypotheses play a combined role (along with other factors that will be discussed shortly) in the development and progression of COPD.

1.11.3 Oxidative stress: changes in the oxidant/anti-oxidant balance

There are many arms to the pathogenesis of COPD, of which increases in the oxidative stress of the lungs plays a vital role. The delicate balance between oxidants and anti-oxidants can be altered by increases in oxidants, both endogenous and exogenous, and also decreases in the inherent anti-oxidant defence mechanisms.

In COPD, exogenous oxidants primarily take the form of those generated by cigarette smoke with 10^{14} free radicals generated per "puff" (Pryor and Stone, 1993). The presence of cigarette smoke in the airways also contributes to an increase in endogenous oxidants produced as a result of activation of inflammatory cells such as macrophages and neutrophils. COPD patients have been shown to have an increase in oxidant levels in breath condensate; Dekhuijzen *et al.* (1996) have shown increased levels of H_2O_2 in COPD patients compared to both ex-smokers and non-smokers and that these levels increase further during an exacerbation. In the healthy lung, increases in oxidative stress would be counterbalanced by an associated increase in anti-oxidant measures. However, in the COPD lung substandard defences mar this equilibrium and it has been shown that COPD patients have decreased levels of important anti-oxidants such as glutathione peroxidase (Duthie *et al.*, 1991).

Ultimately, imbalances between oxidants and anti-oxidants result in an increased inflammatory environment due to increased activation of inflammatory cells and the associated release of inflammatory mediators such as TNFα. Coupled with this is the ability of oxidants to inactivate anti-proteinases such as A1AT (Carp and Janoff, 1980) and secretory

leukoprotease inhibitor (SLPI), thus allowing unopposed proteolysis to occur resulting in increased damage to the parenchyma and further disease progression.

1.11.4 Changes in the proteinase/anti-proteinase balance

The proteinase/anti-proteinase theory of COPD pathogenesis hinges upon disruptions to the normal homeostatic balance between proteinases such as neutrophil elastase and anti-proteinases such as A1AT. When this balance tips in favour of the proteinases the resultant unopposed proteolysis leads to the degradation of the extracellular matrix, which ultimately gives rise to the characteristic increased alveolar airspaces associated with emphysema.

When considering the proteinase/anti-proteinase balance it is important to assess the two different ways in which this balance may be altered: (1) the proteinase burden may be increased and (2) the anti-proteinase defences may be compromised. Cigarette smoke is well known to activate inflammatory cells to release both inflammatory and proteolytic mediators (Moretto *et al.,* 2009 and Churg *et al.,* 2007) and this situation is exacerbated by the influx of macrophages and neutrophils associated with airways inflammation. The activation of these cells not only results in an increase in the proteinase burden by the release of mediators such as elastase from neutrophils and MMP-12 (matrix metalloproteinase-12) from macrophages but also contributes to the decrease in functional anti-proteinase defences; both macrophages and neutrophils release oxidants that act to decrease the activity of A1AT and TIMPs (tissue inhibitor of metalloproteinases). The level of oxidants in the airways can also be increased by those found in cigarette smoke itself. The changes in the proteinase/anti-proteinase balance are summarised in figure 1.5 (adapted from Abboud & Vimalanathan, 2008).

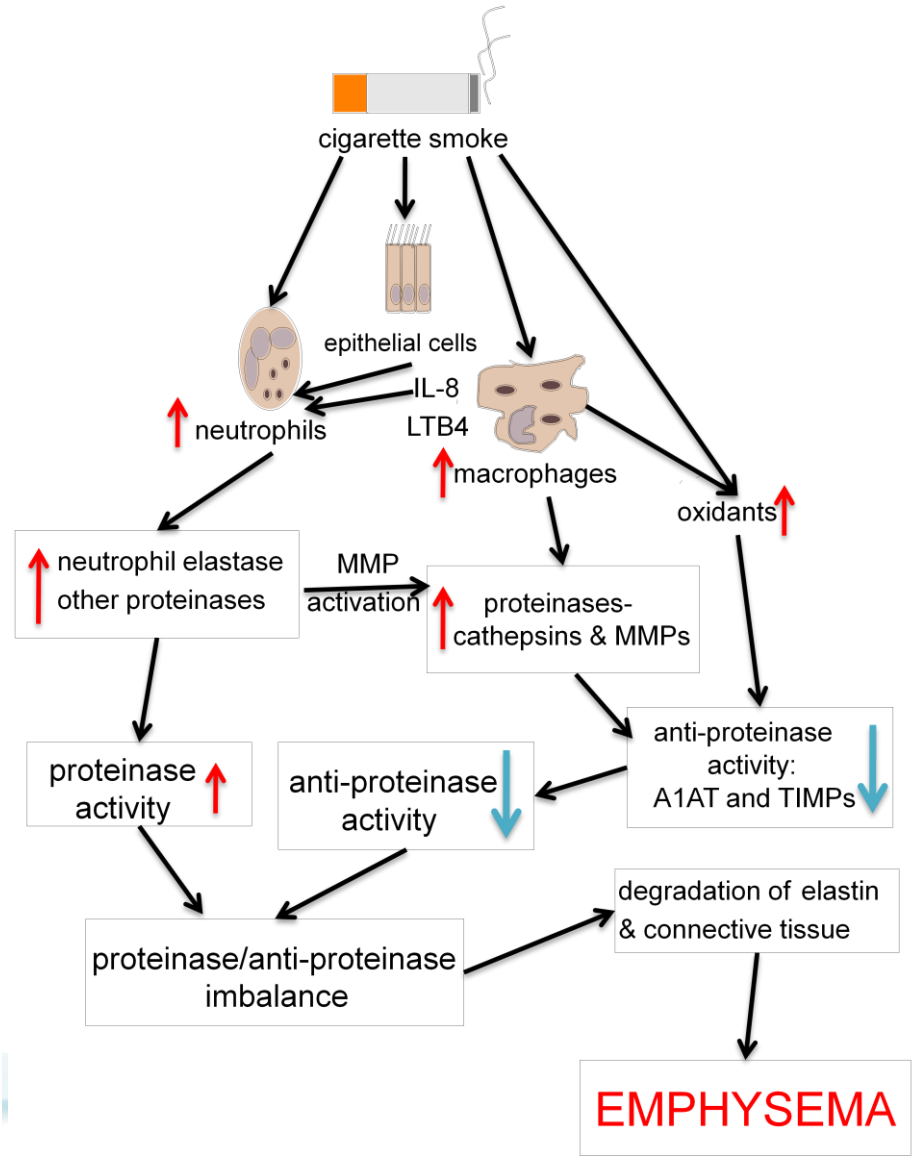

Figure 1.5: Proteinase/anti-proteinase balance. Cigarette smoke activates epithelial cells, neutrophils and macrophages, resulting in the release of oxidants and proteinases. Oxidants degrade anti-proteinases such as A1AT leading to decreased anti-proteinase defences. The consequence of increased proteinases and decreased anti-proteinases is overall destruction of the parenchyma and ultimately emphysema. Adapted from Abboud & Vimalanathan (2008).

The link between increased proteolytic burden and the development of emphysema is well supported by animal models of emphysema and COPD; Birrell *et al.* (2005) have utilised a porcine-pancreas elastase (PPE) model of emphysema in rats and have shown that not only do

the rats show evidence of emphysematous changes to their airspaces but also increase in inflammatory cell burden. This suggests that not only does elastase contribute to the structural changes to the parenchyma but also to the inflammatory features of the disease. Shapiro *et al.* (2003) also support the role of elastase in the development of emphysema and COPD by showing that neutrophil elastase knockout mice are significantly protected from cigarette smoke-induced emphysema compared to their wild-type littermates.

After considering all current theories of the pathogenesis of COPD, it is clear that one hypothesis cannot totally explain the development and progression of the disease but in fact it is a combination of all that is required. However, for the purpose of this study, the proteinase/anti-proteinase hypothesis will be concentrated on while assessing the role of elastase and the generation of an inflammatory response.

1.12 Elastase

Elastase is well known for its proteolytic actions in the development of emphysema (for review see Churg & Wright, 2005) but more recently there has been interest in elastase as more than just a proteinase and therefore, the purpose of this study is to investigate the role of elastase with particular emphasis on its ability to induce an inflammatory response.

1.12.1 Sources of elastase

Elastase is released from two cellular sources in the lung; neutrophils release neutrophil elastase and macrophages release macrophage elastase, also known as MMP-12. Traditionally, neutrophil elastase has been thought to play a more dominant role in COPD

compared to macrophage elastase but this idea is being challenged. Considering both neutrophils and macrophages are increased in COPD and as such are considered to be important mediators of the inflammation associated with the disease (Peleman *et al.*, 1999 and Pesci *et al.*, 1998), it is sensible to assume that both forms of elastase would play a role. In fact, Hautamaki *et al.* (1997) have shown that macrophage elastase knockout mice do not develop emphysema after cigarette smoke exposure unlike the wild type. These mice also fail to show macrophage accumulation in the lungs, again unlike the wild types. Shapiro *et al.* (2003) have shown a similar situation with neutrophil elastase knockout mice and in fact this study has shown that neutrophil elastase is necessary for the presence of macrophage elastase as well as the activation of pro-MMP-12 to the fully active form of MMP-12. Shapiro and colleagues have also established that neutrophil elastase degrades TIMPs, the inhibitors of MMP-12, and macrophage elastase degrades A1AT, the inhibitor of neutrophil elastase. This highlights the close relationship between the two forms of elastase which is supported by a study by Nénan *et al.* (2005) who have shown that macrophage elastase is integral to the release of neutrophil elastase and vice versa.

Although macrophage elastase plays an important role in COPD, this study concentrates on the role of neutrophil elastase and will utilise PPE as a surrogate for neutrophil elastase.

1.12.2 Neutrophil elastase

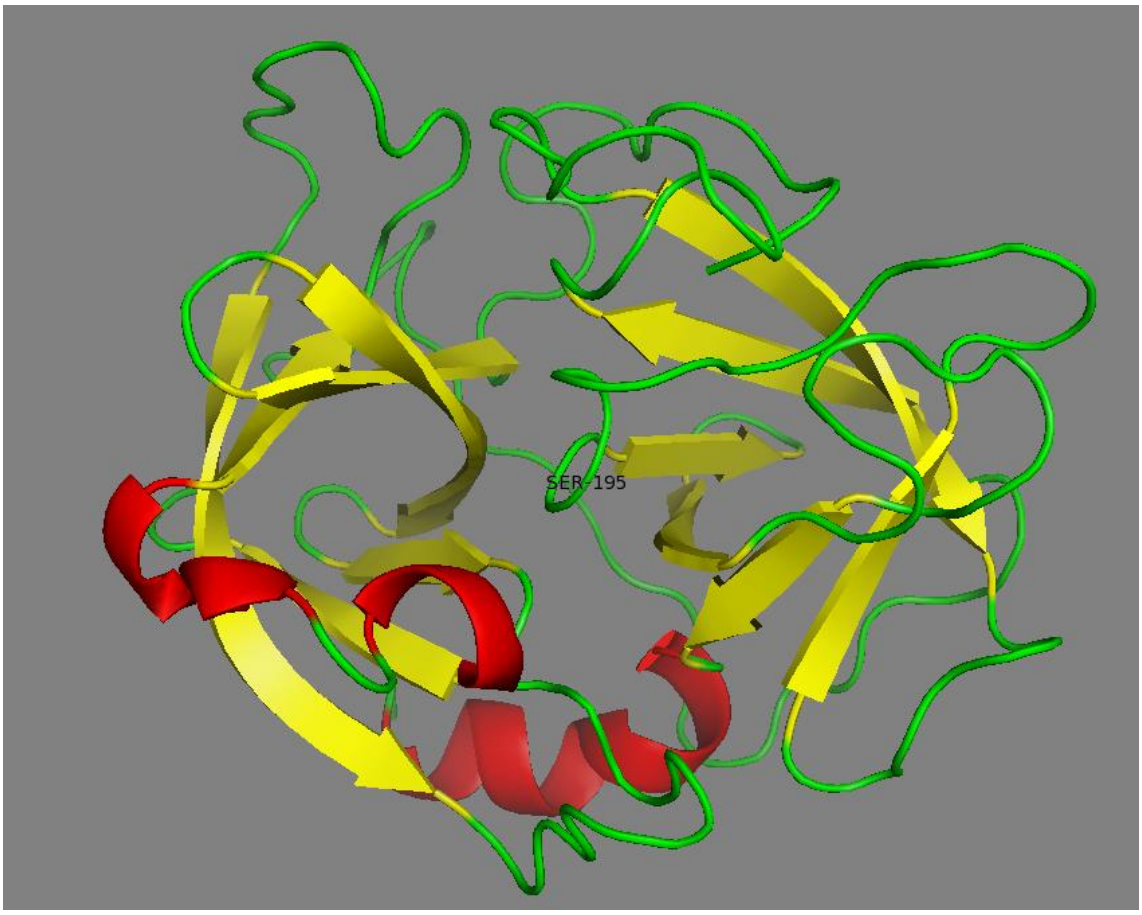

Figure 1.6: Structure of human neutrophil elastase. Structure of human neutrophil elastase as determined by x-ray crystallography (Cregge *et al.,* 1998). Red represents α-helices and yellow indicates β-pleated sheets with serine 195 shown at the active site of the proteinase, thus making elastase a serine proteinase.

Neutrophil elastase is a member of the serine proteinase family, so-called due to the serine found at the active site of the enzyme, and is synthesised and stored in the azurophilic granules of neutrophils (Lominadze *et al.,* 2005). Within these granules are approximately 67,000–100,000 neutrophil elastase molecules, giving a cellular concentration of neutrophil elastase of 1-2pg (Vender, 1996). Elastase is released from the neutrophil when the cell undergoes respiratory burst in response to activation by either invading pathogens or other stimuli such as cigarette smoke, and it is at this point that the enzyme becomes proteolytically active. During

release it is possible for elastase to become bound to the neutrophil membrane and by doing so remains active but endogenous inhibitors, such as A1AT are unable to inactivate it, possibly due to steric hindrance. However, these inhibitors can bind elastase once it has become detached from the cell membrane and this inhibition usually protects the lung from excessive degradation of the extracellular matrix.

1.12.3 Elastase: proteolysis and inflammation

Elastase is classically thought of as a proteinase with its preferred substrate being the parenchymal matrix protein elastin (Lucey *et al.*, 1998). Elastin is required to preserve the structural integrity of the parenchyma and its presence is also an important factor in maintaining elastic recoil of the lungs. However, the adult lung is unable to re-synthesise functional elastin and thus an increase in elastolytic activity within the lung ultimately results in decreased elastin, parenchymal destruction and increased alveolar spaces. As a consequence, it has been shown by Gottlieb (1996) that COPD patients have increased levels of desmosine, an elastin breakdown product, in their urine, indicating parenchymal destruction. Although the relationship between elastase and elastin is well characterised, it is important to note that elastase is capable of degrading most major matrix proteins thus compounding its destructive effects and increasing disease severity.

Although elastase is traditionally thought of as a proteinase, there is gathering interest in this enzyme as a mediator of inflammation and as such it has been implicated in the pathogenesis of inflammatory lung conditions such as acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). Kodama *et al.* (2007) have highlighted the importance of elastase in these

conditions by investigating the plasma neutrophil elastase levels of patients with systemic inflammatory response syndrome (SIRS) and associated ALI/ARDS. This study showed that those with SIRS and ALI/ARDS had significantly higher plasma elastase levels than those with SIRS alone, thus indicating that high levels of elastase may be a sensible indicator for the development of ALI/ARDS.

Having investigated the literature regarding elastase and inflammation it is clear that this proteinase does have a role to play in both the development and propagation of inflammation but the precise mechanisms by which this is attained are still unclear and require further elucidation.

1.13 Proteinase-activated receptors (PARs)

PARs are G-protein coupled receptors consisting of seven transmembrane domains and to date there have been four distinct receptors identified within this family, PARs 1-4 (Kawabata & Kawao, 2005). Activation of theses receptors initiates signalling cascades in various tissues, namely those in the respiratory, gastrointestinal and nervous systems (Kawabata & Kawao, 2005). Of the four members of this family it is PARs-1 and -2 that are found in the respiratory system and so this section will focus on the involvement of these receptors in the lung and in particular in COPD.

1.13.1 Activation of PARs

Activation of these receptors is caused via cleavage of specific peptide bonds within the amino terminus of the protein by various serine proteinases (Brass and Molino, 1997). For PARs-1, -3 and -4

the endogenous agonist for this activation is thrombin (Ishihara *et al.* 1997 and Khan *et al.,* 1999) whereas PAR-2 is preferentially cleaved by trypsin, tryptase and the coagulation factors VIIa and Xa (Kawabata & Kawao, 2005). This cleavage acts to reveal a new amino terminus, which can act as a tethered ligand and via intramolecular binding activates the receptor (figure 1.7).

It is also possible for these receptors to become activated via synthetic peptides that correspond to the sequence of the tethered ligand, which can be applied exogenously to the system. This is found to work successfully with PARs-1, -2 and -4, however PAR-3 does not appear to be activated in this way. The sequences of the tethered ligands are as follows (Ossovskaya and Bunnett, 2004).

Although PARs may be activated by proteinases, it is also possible for them to be disabled via cleavage at sites other than those required for activation; there is some evidence to suggest that both neutrophil elastase (Dulon *et al.*, 2003) and elastase from bacteria, such as *Pseudomonas aeruginosa*, (Dulon *et al.*, 2005) may act to disarm PAR-2 in lung epithelial cells.

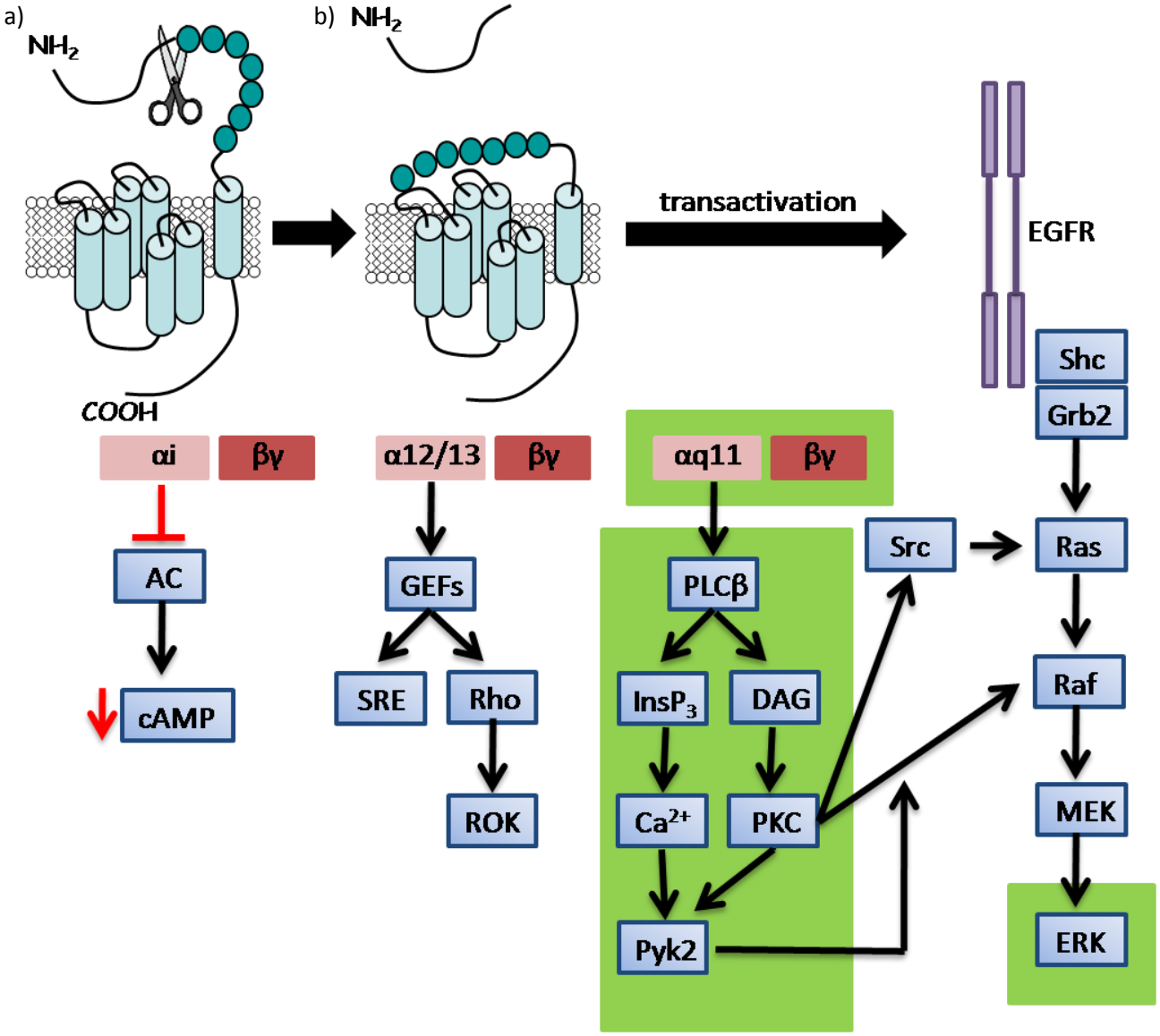

Figure 1.7: PAR-1 and -2 activation and downstream signalling. The amino terminal of the PARs contains a site prone to cleavage by proteinases as shown in a). This cleavage acts to reveal a tethered ligand, which can then bind to another part of the receptor to initiate downstream signalling pathways as shown in b). PAR-1 can couple to $G_i\alpha$ to inhibit adenylyl cyclase which in turn decreases cAMP. Coupling to $G_{12/13}\alpha$ by PAR-1 and subsequent binding to guanine exchange factors (GEFs) causes the activation of Rho, Rho kinase (ROK) and serum response elements (SRE). PAR-1 can also act through $G_{q11}\alpha$ to initiate calcium mobilisation via phospholipase Cβ (PLCβ) and inositol triphosphate ($InsP_3$). Activation of this G-protein also causes the production of diacylglycerol (DAG) which then activates protein kinase C (PKC). PAR-1 is also capable of initiating the MAP kinase pathway via the transactivation of the EGF receptor (EGFR). PAR-2 signalling is not fully elucidated but it is thought that it acts through those pathways highlighted in green in a similar manner to PAR-1. Adapted from Ossovskaya & Bunnett, 2004.

PAR	Synthetic peptide (Mouse/Human)
1	TFRIRD/SFLLRN
2	SLIGKVD/SLIGRL
4	GYPGQV

Table 1.2: PARs and their synthetic peptides. PARs-1, -2 and -4 can be activated by synthetic short amino acid chains, which are detailed here. Peptides are stated firstly as those for mouse studies and secondly for human studies for PARs-1 and -2. The activating peptide for PAR-4 is for human studies. PAR-3 cannot be activated by a synthetic peptide.

1.13.2 PARs and inflammation in the lung

Although all four PARs have roles within the body it is PARs-1 and -2 that are found predominantly in the respiratory system (Cocks and Moffatt, 2001) and so it is thought that they, and in particular PAR-2, may play a role in the inflammatory process in diseases such as COPD and asthma. However, the roles of these receptors in the lung are yet to be fully characterised and understood and so further investigation into this area is necessary.

Vliagoftis *et al.* (2000) have shown that with the activation of PAR-2 in the human alveolar epithelial cell line A549 there is an associated increase in the expression or the release of MMP-9 and granulocyte macrophage-colony stimulating factor, which are important pro-inflammatory mediators. In the same cell line, Asokananthan *et al.* (2002) and Kawao *et al.* (2005) have evidence for the production of IL-6, IL-8 and prostaglandin E_2 after stimulation with activated PAR-2 and/or PAR-1 and they have replicated this with human bronchial epithelial cells.

Although PAR-2 is thought to predominantly mediate pro-inflammatory actions there is some evidence to support the idea that it may also have some anti-inflammatory properties (Kawabata & Kawao, 2005); PAR-2 agonists are able to induce epithelial prostanoid-

dependent bronchodilation (Kawabata *et al.,* 2004), which would act to oppose constriction caused by inflammation. PAR-2 activation has also been shown in animal models to negate inflammatory responses such as the recruitment of polymorphonuclear leukocytes caused by stimulation with bacterial LPS and the enhancement of vascular permeability seen after challenge with histamine (Moffatt *et al.,* 2002, Cicala *et al.,* 2001 and Cocks & Moffatt, 2001). The activation of PAR-2 is known to induce an endothelium dependent nitric oxide relaxation of isolated vascular tissue (Magazine *et al.*, 1996).

As discussed, the involvement of PARs in lung inflammation is controversial, with no definitive answer available. This stresses the need for further investigation into these receptors and candidate stimuli for their activation, such as elastase.

1.13.3 PARs and COPD

Much of the evidence discussed here has arisen from studies on cell lines and animal models, so there is a question as to how these conclusions fit in with clinical findings and especially COPD. Immunohistochemistry has been used to localise PARs to bronchial smooth muscle, epithelium and mast cells in normal human tissue (D'Andrea *et al.,* 1998). In a study by Miotto *et al.* (2002) an increase in PAR-2 expression was seen in the bronchial vessels of patients with bronchitis compared to those with COPD and there was also an associated increase found in bronchial smooth muscle and bronchial glands in the former subset of patients. This study also showed that there was no significant difference in PAR-2 expression in the central airways of smokers compared to non-smokers, which may seem unexpected due to the increase in inflammation seen in smokers and the possible link between PAR-2 and

inflammation. Thus, if the expression of the receptor is unchanged then its link with inflammation could be due to an increase in proteinases such as trypsin or other activating mediators such as LPS rather than an increase in receptor number; neutrophil tryptase, for example, is found to be increased in inflammatory disease in the lungs which could increase receptor activation (Ossovskaya & Bunnett, 2004).

1.13.4 PARs and elastase

The involvement of both elastase and PARs has been established in COPD and so it is a sensible idea to suggest that elastase may play a role in either the activation or disarmament of these receptors.

Dulon *et al.* (2005) suggest that elastase may cleave PAR-2 at a site located downstream of that utilised by trypsin, its endogenous activator, in the extracellular domain of the amino terminal, thus rendering the receptor inactive. Following on from this idea, elastase has been shown by Suzuki *et al.* (2005) to be involved in apoptosis of distal lung epithelial cells via actions of PAR-1. It is possible that this could contribute to the degradation of tissue seen in emphysema along with the direct proteolytic effects of elastase on the elastin fibres.

There have also been links made with the activation of the PARs by elastase and the ultimate up-regulation of both IL-6 and IL-8 (Wang *et al.,* 2006 and Uehara *et al.,* 2003), both of which are increased in COPD. However, there seems to be no link as of yet between other inflammatory cytokines, such as TNFα and IL-1β, and the activation or deactivation of PARs

by elastase. Therefore, there is a niche for further investigation into the role played by elastase and PARs in inflammation with a particular interest in emphysema and COPD.

1.14 Aims and objectives

This study aims to investigate the role of elastase in the pathogenesis of COPD with particular reference to the initiation of inflammation by this proteinase. Specifically, the aims of this study are:

- To investigate whether elastase can initiate an inflammatory response in human lung parenchymal explants.
- To establish whether elastase needs to remain proteolytically active to cause an inflammatory response in the explants.
- To determine whether there is a link between the actions of elastase and PAR-2.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Buffers

RPMI-1640 medium was supplemented with 1% penicillin, 1% streptomycin and 1% gentamycin (5000units/ml, 5mg/ml and 10mg/ml respectively); **Tyrode's salts** contained 1.8mM $CaCl_2$, 1nM $MgCl_2$, 2.7mM KCl, 137mM NaCl, 0.42mM NaH_2PO_4, 5.5mM D-glucose with 12mM $NaHCO_3$; **phosphate buffered saline (PBS)** contained 0.1M NaCl, 2.7mM KCl, 1.8mM KH_2PO_4 and 10mM Na_2HPO_4; **coating buffer for α_1-antitrypsin ELISA** contained 0.015mM Na_2CO_3 and 0.035mM $NaHCO_3$, pH 9.6; **PBS Tween** contained 0.1% Tween 20 in PBS; **coating buffer for cytokine ELISAs** contained 0.05mM Na_2CO_3 and 0.05mM $NaHCO_3$, pH 9.4; **reagent diluent for cytokine ELISAs** contained 0.14mM NaCl, 0.01mM Na_2HPO4, 1.47mM KH_2PO_4, 2.68mM KCl and 5.0g/l bovine serum albumin (BSA); **Towbins buffer** contained 20% methanol, 25mM tris and 190mM glycine; **0.1M sodium phosphate buffer**; **culture medium for immunohistochemistry** contained DMEM supplemented with 1% BSA and 20% foetal calf serum (FCS); **tris buffered saline (TBS)** contained 5mM tris, 4mM HCl and 130mM NaCl.

2.1.2 Other reagents

Porcine pancreatic elastase; papain; lipopolysaccharide (LPS); elastatinal; soybean trypsin inhibitor (SBTI); proteinase inhibitor cocktail containing 2mM AEBSF, 1mM EDTA, 130µM bestatin, 14µM E-64, 1µM leupeptin and 0.3µM aprotinin; neutralising TNFα antibody and isotype control antibody from R&D Systems (Abingdon, UK); goat anti-human α_1-antitrypsin antibody from Cambridge Bioscience Ltd; rabbit anti-human α_1-antitrypsin antibody from Dako (Denmark); goat anti-rabbit immunoglobulins HRP from Dako (Denmark); ELISA

Duoset kits for IL-6, IL-8, IL-10 and TNFα from Biosource (Europe, SA); 3,3',5,5' tetramethylbenzidine (TMB); 1M H_2SO_4; multi-spot cytokine assay from MesoScale Discovery (Maryland, USA); BCA protein assay reagent A containing sodium carbonate, sodium bicarbonate, BCA detection reagent and sodium tartrate in 0.1M sodium hydroxide from Pierce (Rockford, IL); BCA protein assay reagent B from Pierce (Rockford, IL); N-succinyl-Ala-Ala-Ala-p-nitroanilide; lactate dehydrogenase (LDH) assay kit and LDH standard from Roche Diagnostics (Germany); acetone; phenyl methyl sulphonyl fluoride (PMSF); iodoacetamide; methyl benzoate from Fisher (Loughborough, UK); GMA solutions A and B and benzoyl peroxide from Park Scientific; sodium azide; hydrogen peroxide; streptavidin biotin complex from Dako (Denmark); AEC substrate; Mayer's haemotoxylin; rabbit anti-human PAR-2 antibody and rabbit anti-human TLR-4 antibody from Abcam (Cambridge, UK); swine anti-rabbit biotinylated antibody from Dako (Denmark); electrochemiluminescence detection system (ECL) containing a stable peroxide buffer and a luminol/enhancer solution from Pierce (Rockford, IL); x-ray film from Kodak; developer and fixer from Photosol RG (Essex, UK); PAR-1 activating peptide (TFRIFD-NH2), PAR-1 scrambled peptide (FTRIFD-NH2), PAR-2 activating peptide (SLIGKVD-NH2) and PAR-2 scrambled peptide (LSIGKVD-NH2) from Peptide Protein Research Ltd (Fareham, UK); dexamethasone; cycloheximide.

All reagents purchased from Sigma in Poole, Dorset unless otherwise stated.

2.2 Human lung explant model

Human lung parenchyma was obtained from two sites - Guy's Hospital, London and Southampton General Hospital (SGH). At both sites, tissue was obtained with informed consent and the study had been reviewed by the local ethics committees. Tissue from Guy's hospital was from patients undergoing lobectomy for carcinoma resection. Tissue from SGH was mainly from patients either undergoing lobectomy for carcinoma resection or bullectomy for the repair of bullae. Tissue received from lobectomy was from the normal margin as far from the tumour as possible. Clinical and demographic data was collected at the same time as tissue collection. This data is summarised in table 2.1 below.

Site: Procedure	**Gender (M/F)**	**Age (years)**	**FEV_1/FVC**	**Smoking status Current/Ex/Never/Unknown**
Guy's: Lobectomy	27/11	65.4 ± 1.2	0.67 ± 0.02	16/17/1/4
SGH: Lobectomy	10/14	64.5 ± 1.9	0.71 ± 0.02	10/14/0/0
SGH: Bullectomy	8/1	46.7 ± 6.9	N/A	4/4/1/0
SGH: Other	5/2	59.7 ± 8.6	0.58 ± 0.12	2/2/3/0

Table 2.1: Patient characteristics. Demographic and clinical data were collected with each sample. Age and FEV1/FVC are shown as the mean±sem. N/A indicates not available, as bullectomy patients did not undergo lung function tests.

Following surgery, tissue was placed straight into saline before being transferred to PBS for dissection. Tissue was cut into small fragments (approximately 2-3mm^2) and was periodically washed with fresh PBS to remove any blood from the preparation. Tissue was left overnight in RPMI-1640, supplemented with penicillin/streptomycin and gentamycin, at 37ºC and in a 5% CO_2 environment.

The following day, approximately 25mg of tissue was placed into fresh RPMI-1640 and the appropriate stimulus was added and left for varying lengths of time depending on the experiment: 1, 2, 4, 6, 24 and 48hrs for kinetics and 24hrs for dose responses and inhibitor studies (Bochner *et al.*, 1987). At each appropriate time point the tissue was removed, weighed and stored at -70ºC until required for analysis. Supernatants were also aliquotted and stored at -70ºC until analysed.

2.2.1 Dose response

Using the model, as described in 2.2, elastase dose response experiments were conducted. The concentrations of elastase used were 100, 30, 10, 3 and 1µg/ml. Dose responses to PAR-1 and PAR-2 activating peptides were also carried out. Scrambled versions of these peptides were used as negative controls. Concentrations of these peptides ranged from 12.5µM to 200µM. Tissue was incubated with each of these stimuli for 24hrs before the tissue was removed, weighed and stored at -70°C. Supernatants were collected and also stored at -70°C until required for analysis.

2.2.2 Kinetics

The model (section 2.2) was used for kinetics studies of elastase (100µg/ml), LPS (100ng/ml) and papain. The papain dose was chosen to match the number of proteolytic units in the elastase stimuli in order to directly compare the two proteinases; both elastase and papain were used at 0.84 proteolytic units/ml. Tissue fragments were stimulated with the appropriate stimulus for various time points over a 48hr period: 1, 2, 4, 6, 24 and 48hrs. At each time point the tissue and supernatant were harvested as described in section 2.3. Some kinetics

were run in duplicate with one set being stored as normal and the other being stored in the presence of a proteinase inhibitor cocktail (PIC), both at -70ºC.

2.2.3 1 hour stimulation

Tissue fragments were stimulated with elastase (100µg/ml), LPS (100ng/ml) or papain (0.84 proteolytic units) for one hour and then transferred to fresh media. The kinetics time points and harvesting took place as stated in section 2.4.

2.2.4 Elastase inhibition

Elastatinal and SBTI were used to inhibit elastase and LPS within the model system. Elastase (100µg/ml) and LPS (100ng/ml) were incubated with elastatinal (100, 10 or 1µg/ml) or SBTI (1, 0.1 or 0.01µg/ml) prior to being added to the tissue fragments. Fragments were cultured for 24hrs before being harvested as previously stated (section 2.3).

2.2.5 Steroid sensitivity

Tissue was incubated overnight with various doses of dexamethasone (0.1-100nM) on the day of receipt of the specimen. The following day, this pre-incubated tissue was transferred to fresh media before stimulation with fresh dexamethasone at the corresponding concentrations and either elastase or LPS. Tissue and supernatants were recovered after 24hrs and stored as per all other explant experiments.

2.3 Cytokine degradation by elastase

Recombinant human cytokines (TNFα, IL-6, IL-8 and IL-10) or α_1-anti-trypsin (A1AT) were incubated with 100μg/ml elastase, in RPMI-1640 at 37°C, for the kinetics time periods. Concentrations of cytokines/A1AT used were as follows: TNFα, IL-6 and IL-8-500pg/ml, IL-10-250pg/ml and A1AT-1000ng/ml. The concentrations of these markers after incubation with elastase were measured via ELISA.

2.4 Analysis by ELISA

2.4.1 α_1-anti-trypsin (A1AT)

A1AT was detected in the supernatants by the use of a sandwich ELISA. The capture antibody, goat anti-human A1AT, was diluted in A1AT coating buffer to a concentration of 1μg/ml and incubated on a 96-well microtitre plate for 4hrs at 4ºC. The plate was then washed thoroughly and blocked overnight with 30% soya milk in 0.1% PBS Tween, again at 4ºC. Samples and standards, with a top standard of 2000ng/ml, were loaded at the appropriate dilution and left for 2hrs at 4ºC. Again, the plate was washed thoroughly before the detection antibody, rabbit anti-human A1AT, was added at a concentration of 0.55μg/ml for 2hrs at 4ºC. Goat anti-rabbit immunoglobulins-HRP was then added, after washing, for 2hrs at 4ºC. A1AT concentrations were visualised by the addition of TMB and the reaction stopped with 1M HCl. The optical density was obtained at 450nm. A typical standard curve is shown in figure 2.1.

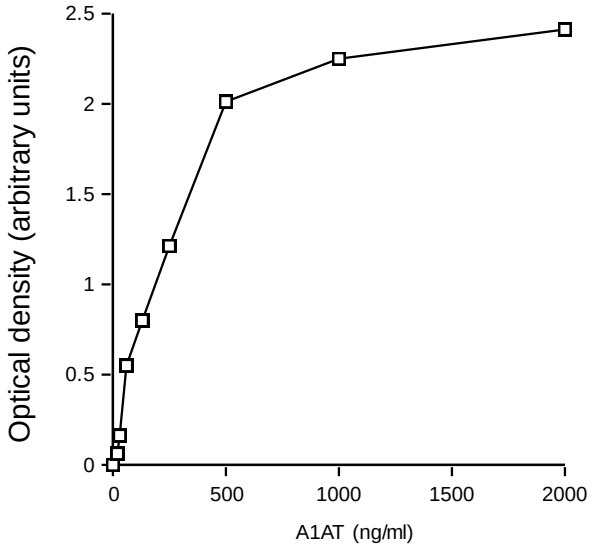

Figure 2.1: A1AT ELISA. A sandwich ELISA was used to determine the concentration of A1AT in samples generated by the human lung explant model by comparison to a standard curve of recombinant A1AT. Optical densities were measured at 450nm.

2.4.2 IL-5, IL-6, IL-8, IL-10, TNFα and MIP-1β

ELISA kits for the analysis of these cytokines were purchased from Invitrogen (previously known as Biosource) and carried out as per the manufacturer's instructions (antibody concentrations are detailed in table 2.2). 96-well microtitre plates were coated with the appropriate capture antibodies diluted in coating buffer, and left overnight at 4°C. The following day after thorough washing with 0.1% PBS Tween, unspecific binding was blocked by reagent diluent for 2hrs at room temperature with continual agitation. The plate was then washed again and samples and standards added at the appropriate dilutions. The detection antibody was also added at this point. For all cytokines except IL-5, this was left for 2hrs at room temperature before being washed and the streptavidin-HRP conjugate added and left for 30mins at room temperature. For IL-5, samples were left for 2hrs at room temperature, washed with 0.1% PBS Tween before the detection antibody was added for 1hr at

room temperature. The streptavidin-HRP conjugate was then added, after washing as before, for 45 minutes at room temperature. Cytokine concentration was detected via the addition of TMB and stopped with 1M HCl and the optical density determined at 450nm.

Cytokine	Capture antibody (μg/ml)	Detection antibody (μg/ml)
IL-5	1.25	0.25
IL-6	1	0.16
IL-8	1	0.04
IL-10	1	0.16
TNFα	2	0.32
MIP-1β	2	0.4

Table 2.2: Antibody concentrations for cytokine ELISAs. Concentrations for capture and detection antibodies for ELISAs purchased from Invitrogen are given as μg/ml.

2.4.3 IL-1β and IL-13

ELISA kits for the detection of IL-1β and IL-13 were purchased from R&D Systems and were carried out as per the manufacturer's instructions. Briefly, 96-well microtitre plates were coated with the appropriate capture antibody at a concentration of 4μg/ml for IL-1β and 2μg/ml for IL-13 and left overnight at room temperature with constant agitation (as for all incubations). Plates were subsequently washed as for the ELISAs detailed in the previous section and blocked with 1% BSA in PBS for 2hrs. Samples and standards were added at appropriate dilutions after washing and left for 2hrs before being washed again and the detection antibodies added for 2hrs (300ng/ml for IL-1β and 150ng/ml for IL-13). Finally, after further washing the streptavidin-HRP conjugate was added for 20 minutes before adding TMB as for the ELISAs previously stated.

2.5 Analysis by multi-spot cytokine assay

24hr samples from the kinetics experiments were analysed via the Meso-scale discovery (MSD) multi-spot cytokine assay for ten Th1/Th2 cytokines. Firstly, the multi-spot plate was blocked with the blocking solution provided in the kit for 1hr at room temperature with continuous agitation. After washing with 0.05% PBS Tween, samples were added and left for a further 2hrs at room temperature, again with continual agitation. The detection antibody solution was then added to each well at a concentration of 1μg/ml. This was left for 2hrs at room temperature with agitation. Finally, the plate was washed with 0.05% PBS Tween before read buffer (as supplied with the kit) was added. The plate was analysed using a SECTOR Imager and cytokine concentrations were determined by comparison to the standard curve. Miss Kelly Lowings carried out this procedure.

2.6 Preparation of lung homogenate

Lung tissue was sonicated using a Soniprep 150 sonicator for 12 cycles of 10s sonication followed by 20s rest at an amplitude of 10microns. Each sample was covered with 100μl ice-cold PIC in 1% PBS Tween and after sonication was centrifuged for 15mins at 14000rpm, 4°C. The supernatants were then removed and stored at -70°C until required for analysis.

2.7 Total protein assay

Supernatant from sonicated tissue (as described in section 2.6) was analysed for the total protein concentration. The sample was added at a 1:50 dilution, with distilled water, to a 96-well microtitre plate and to this 100μl of the BCA protein assay substrate was added. Optical density was determined at 540nm and compared to a standard curve of human serum albumin.

2.8 Elastase activity assay

Tissue was sonicated as per the method set out in section 2.6 with the modification of having no PIC present in the 1% PBS Tween. Samples were added at a dilution of 1:10 with 0.1M sodium phosphate buffer. The elastase substrate, N-succinyl-Ala-Ala-Ala-p-nitroanilide, was then added at a concentration of 100μg/ml and the optical density determined at 405nm. A typical standard curve is shown in figure 2.2.

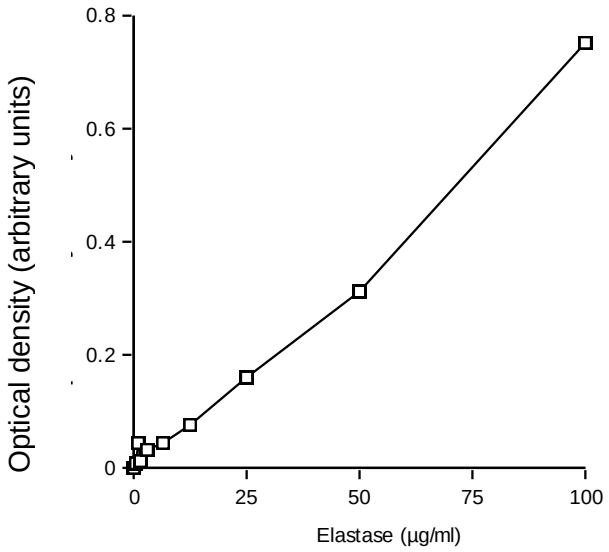

Figure 2.2: Elastase activity standard curve. The elastase substrate N-succinyl-Ala-Ala-Ala-p-nitroanilide was used to determine the amount of elastase activity within the samples. Each sample was compared to a standard curve of porcine pancreas elastase after optical densities were determined at 405nm.

2.9 Lactate dehydrogenase (LDH) assay

The LDH assay was used to measure cell death. Sonicating tissue as described in section 2.5 generated a positive control giving 100% cell death. Supernatants from the human lung explant experiments were analysed via this assay at a dilution of 1:10 with 0.1% PBS Tween along with the positive control. The assay was carried out as per the manufacturer's

instructions. The optical density at 490nm was measured and cell death was expressed as a percentage of the positive control.

2.10 Glycol methacrylate (GMA) processing of explants

GMA processing of parenchyma was carried out as per the method set out by Britten *et al.* (1993). Briefly, explants were fixed in ice-cold acetone supplemented with 2mM PMSF and 20mM iodoacetamide overnight. The following day the fixative was replaced with fresh acetone and left at room temperature for 15mins before being placed in methyl benzoate for a further 15mins. Tissue was then infiltrated with processing solution (5% methyl benzoate in glycol methacrylate) for 2hrs at 4°C. This was repeated for a total of 3 times before embedding the samples in embedding solution (GMA solution A, GMA solution B and benzoyl peroxidase). Blocks were left for 2 days at 4°C in order to polymerise before being stored at -20°C until analysis.

2.11 Detection of PAR-2 and TLR-4 by immunohistochemistry

Explants were processed as described in section 2.10 and 2μm sections were cut for immunohistochemistry. Initially, endogenous peroxidases were inhibited with 0.1% sodium azide and 0.3% hydrogen peroxide for 30mins and then after washing with TBS the sections were blocked with culture medium (DMEM supplemented with 1% BSA and 20% FCS) for a further 30mins. A rabbit anti-human PAR-2 or rabbit anti-human TLR-4 antibody was added at a concentration of 4μg/ml for PAR-2 and 2μg/ml for TLR-4 and left overnight at room temperature. The following day, sections were washed with TBS and a swine anti-rabbit biotinylated secondary antibody was added for 2hrs at room temperature. Further washing and

the addition of a streptavidin-biotin peroxidase complex at a 1:200 dilution for another 2hrs followed this before washing and applying the AEC substrate for 20mins at room temperature. Sections were rinsed in TBS and tap water before being counterstained with Mayer's haematoxylin.

2.12 Dot blots for PAR-2 and TLR-4

Dot blots for PAR-2 and TLR-4 were performed with supernatant from sonicated tissue (section 2.6). Each sample contained 10ng protein as determined by the total protein assay as described in section 2.7. Nitrocellulose membranes were soaked in Towbins buffer before being placed into the dot blot apparatus and a vacuum applied. Supernatants were applied to the membranes which were then blocked overnight at 4°C in 5% BSA in PBS Tween. The following day after washing with 0.1% PBS Tween the membranes were exposed to a rabbit anti-human PAR-2 antibody (0.1μg/ml) or rabbit anti-human TLR-4 **(**0.5μg/ml**)** for 2hrs. The membranes were then washed again (3 x 15mins) before a biotinylated $F(ab')_2$ swine anti-rabbit antibody was added for a further 2hrs. An avidin-biotin complex was added after further washing (3 x 15mins) and left for 1hr. All incubations were carried out at room temperature with continual agitation on a rocker. The membranes were then washed again with 0.1% PBS Tween before being left overnight at 4°C in fresh PBS Tween. The following day the membranes were developed using an autorad ECL system (section 2.13).

2.13 Development of dot blot membranes

An ECL development system was used to visualise the PAR-2 content of the samples. ECL was added to the membranes before exposing x-ray film to them. The film was then placed in

developer until dots became apparent, washed in water and then placed in fixer before being washed again, all under red light. Dots were then analysed via densitometry (ScanAnalysis) to determine relative amounts of PAR-2 and TLR-4 in the different samples.

2.14 Statistical analysis

Statistical analysis was performed using StatView for Mac and Prism for PC. Data distribution was determined as normal or skewed via the D'Agostino normality test. Data deemed not normally distributed was analysed via non-parametric tests (Wilcoxon signed rank test and ANOVA as appropriate) and depicted as median values. Conversely, data determined to be normally distributed are shown as mean values.

Chapter 3: Elastase and the inflammatory response

3.1 Introduction

COPD is characterised as an inflammatory disease and the importance of inflammatory mediators such as TNFα and the associated anti-inflammatory cytokine IL-10 is well known. There are many inflammatory stimuli associated with COPD such as particulates in cigarette smoke and air pollution as well as viral and bacterial pathogens, which are particularly important when considering acute inflammatory exacerbations of the disease (as reviewed in White *et al.,* 2003). Although the acute inflammation associated with exacerbations is an important factor in COPD pathogenesis and progression it cannot be forgotten that an underlying chronic inflammatory situation is also present. Recently there has been interest in the serine proteinase elastase and its possible role in this inflammation.

Elastase is a serine proteinase that is well known for its proteolytic actions on elastin fibres within the lung (Barroso *et al.,* 2006), which ultimately leads to the characteristic increased airspaces associated with emphysema as well as the loss of elastic recoil. The destruction of elastin fibres in emphysema patients is highlighted by the presence of the elastin breakdown product desmosine, an elastin precursor in their urine (Fiorenza *et al.,* 2002 and Gottlieb, 1996). Desmosine cross-linking is required for the production of functional tropoelastin molecules which go on to form elastic fibres and Laurent *et al.,* (1983) have shown that cigarette smoke can inhibit the formation of this cross-linking thus inhibiting the downstream formation of new elastic fibres and ultimately worsening the degrading effects on the parenchyma. However, there is some evidence to suggest that elastic fibres can be repaired by rat lung interstitial fibroblast cultures (Morris *et al.,* 1998) although there is little evidence to suggest that the same would happen in humans.

In the healthy lung the proteolytic effects of elastase are usually opposed by the actions of anti-proteinases such as A1AT, which maintains a balance between degradation and fibrosis of the tissue. However, in the COPD lung the oxidant/anti-oxidant balance is shifted in favour of oxidants, which in turn compromises the anti-proteinase defences of the lung thus allowing the effects of elastase to continue unfettered. As well as having decreased anti-proteolytic protection, the presence of elastase itself is also increased in those with COPD due to the influx of inflammatory cells such as neutrophils (O'Donnell *et al.,* 2004 and Hogg *et al.*, 2005). These cells become activated by inflammatory stimuli and consequently undergo respiratory burst which releases elastase, amongst other mediators, into the surrounding milieu causing proteolytic destruction.

Although elastase is particularly known for its proteolytic actions there has recently been increased interest in the idea that elastase can act as an inflammatory stimulus. The aim of this chapter is to assess whether elastase can induce an inflammatory response in human lung parenchyma explants and if so whether this is comparable to a known inflammatory stimulus, LPS.

3.2 Methods

Tissue from 36 donors was used in the human lung explant model for the following experiments. Patient characteristics such as age, gender, smoking history and lung function test results were collected at the time of surgery and are summarised in table 3.1. Where subsets of patients have been used for individual experiments the clinical data has been matched as far as possible.

Age	64.5 ± 2.1 years
Gender	26M 10F
FEV_1/FVC	0.68 ±0.02
Smoking status	14 current, 18 ex, 1 non, 3 unknown

Table 3.1: Patient characteristics. Age and FEV_1/FVC are shown as mean ± sem. For gender, M=male and F=female. Under smoking status, current denotes current smokers, ex denotes ex-smokers as determined by the patient's clinician, non denotes non-smokers and unknown is for those patients for whom smoking status could not be obtained.

The majority of the data in this chapter is not normally distributed (as determined by D'Agostino normality test) and so are depicted as median values and tested via non-parametric means described as appropriate in figure legends. An exception to this is section 3.3.6, the effect of dexamethasone on the elastase response, which was deemed to be normally distributed and so is shown as mean values.

3.3 Results

3.3.1 Elastase dose response

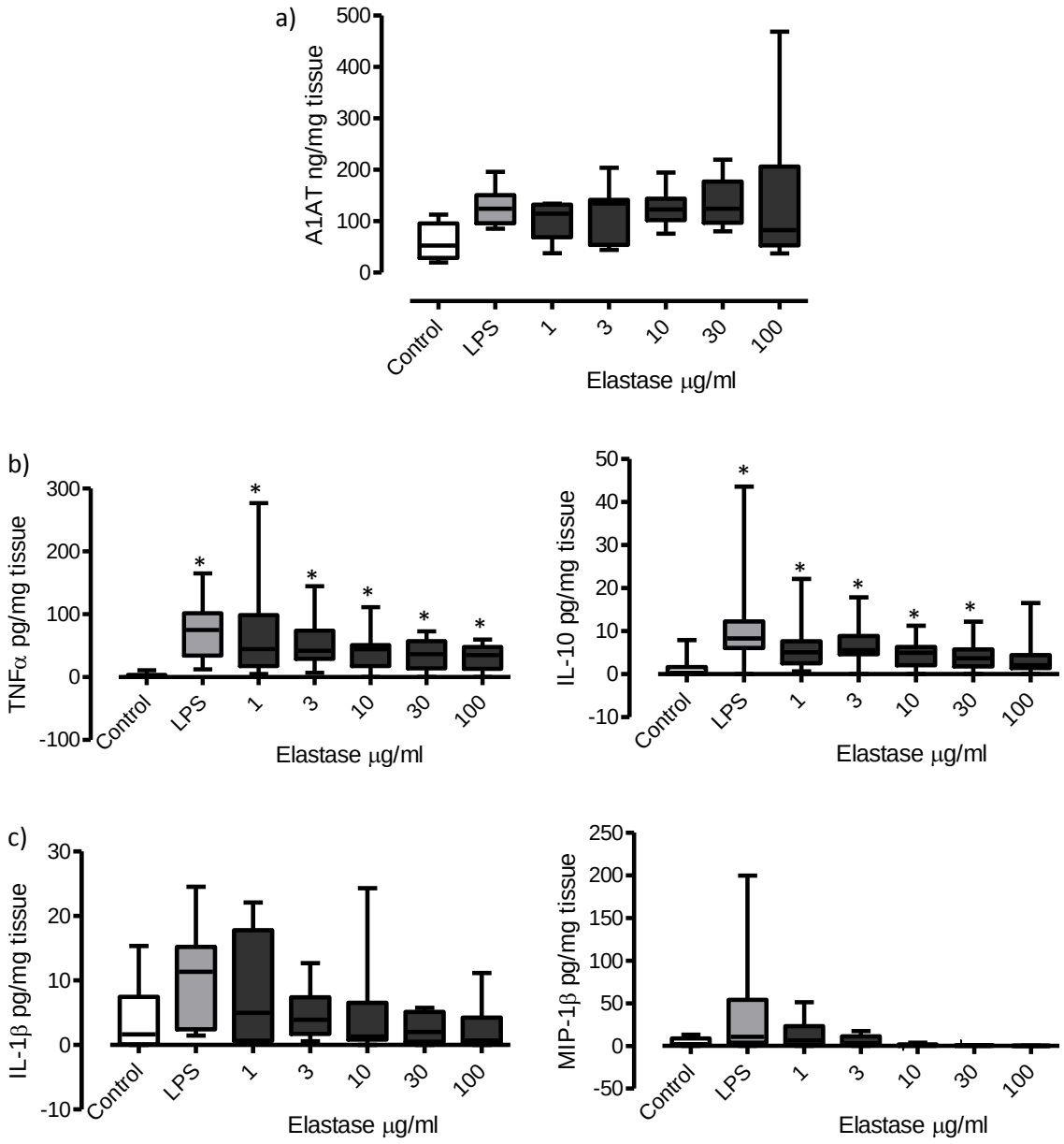

Figure 3.1: Elastase dose response. a) shows the A1AT response (n=8), b) shows the TNFα and IL-10 response (n=15) and c) shows the IL-1β and MIP-1β production (n=15) after stimulation with various concentrations of elastase for 24hrs. All values are the median±interquartile range and * denotes a significant increase when compared to control ($p<0.05$) as determined via ANOVA. Concentrations are expressed as ng or pg/mg tissue.

Previous work in our lab has shown that exposure of human lung tissue to elastase increases the production of the anti-proteinase A1AT (Seth, 2004). These studies had suggested that the optimum concentration of elastase was 100µg/ml and so because of this many early experiments were carried out using this concentration. However, this study could not replicate the previously seen increase in A1AT with increasing elastase concentrations and so full dose responses to the proteinase were carried out to ascertain the effect of elastase on the cytokines of interest.

Tissue from 15 donors was incubated with 100, 30, 10, 3 or 1µg/ml porcine pancreas elastase for 24hrs. Buffer controls were also generated. Supernatants were analysed for various cytokines (TNFα, IL-10, MIP-1β and IL-1β) and the anti-proteinase A1AT via ELISA.

Figure 3.1a, the elastase dose response for A1AT, shows that there is a high basal level of A1AT and that the response after stimulation with elastase does not exceed this for any dose used. However, data for both the inflammatory cytokine TNFα, and the anti-inflammatory cytokine IL-10 shows a different story (figure 3.1b). For all concentrations of elastase used, the TNFα concentration increased significantly compared to the control in a dose-dependent manner. Similarly, IL-10 also increased significantly with all doses except for the highest dose of 100µg/ml, and again this was in a dose-dependent manner.

Another inflammatory cytokine, IL-1β, was also measured and although a dose response curve is evident an increase in this cytokine is only significant when a concentration of 1µg/ml elastase is applied to the tissue (figure 3.1c). The final mediator investigated was MIP-1β, a

cytokine chemotactic for neutrophils and released from macrophages, both of which are present in the parenchymal explants. This chemokine shows no significant increase in concentration with any dose of elastase used, although it is significantly decreased by the two highest doses (30 and 100μg/ml). For TNFα, IL-10 and IL-1β the elastase-induced response was of a similar nature to that induced by LPS stimulation, thus suggesting that elastase can induce an inflammatory response.

Having established that elastase can cause a dose dependent release of both TNFα and IL-10 at the concentrations used it was important to establish the lowest concentration of the proteinase that could elicit this response. Dose responses were repeated using concentrations of elastase between 0.001 and 100μg/ml and TNFα and IL-10 concentrations were measured via ELISA.

Figure 3.2 shows the TNFα and IL-10 response from explants from 20 donors after stimulation with elastase (0.001-100μg/ml), LPS (100ng/ml) or buffer alone for 24hrs. It can be seen in a) that a significant increase in TNFα can be elicited by elastase at concentrations of 10 and 100μg/ml and that this effect is diminished once the concentration drops below this range. TNFα levels are analogous to the control at elastase concentrations of 0.001 and 0.01μg/ml. Similarly, elastase at a concentration of 10μg/ml induces a significant release of IL-10 but this effect is dampened slightly when a concentration of 100μg/ml is used. IL-10 concentrations after stimulation with either 0.001 or 0.01μg/ml are equivalent to control levels as with the TNFα response.

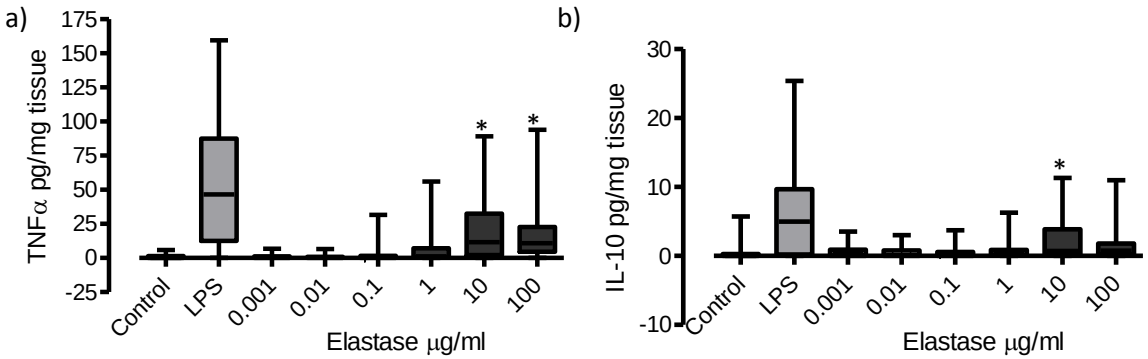

Figure 3.2: Extended elastase dose response. Tissue (n=22) was incubated for 24hrs with a range of concentrations of elastase (0.001-100μg/ml), LPS (100ng/ml) or buffer alone. Supernatants concentrations of TNFα and IL-10 were determined via ELISA and are expressed as pg/mg tissue. * indicates $P<0.05$ when compared to the control as determined by ANOVA.

It is important to point-out at this point that there is a difference between the concentrations of the cytokines in response to elastase stimulation in this set of experiments compared to the previous figure. This difference can be explained by the fact that different patient samples were used in the two sets of experiments highlighting the heterogenic nature of these responses, which will be discussed in a later section (section 3.3.4). We have ruled out batch variability as the reason for these differences as we have tested the batch-to-batch elastase activity as well as using different batches on the same tissue and results have been consistent (data not shown).

3.3.2 Kinetics

Tissue from 32 donors was incubated with elastase (100μg/ml), LPS (100ng/ml) or left un-stimulated as a buffer control. Incubation times were 1, 2, 4, 6, 24 and 48hrs and at each time-point tissue was harvested and weighed and the supernatant collected for storage at -70°C until analysis via ELISA.

Figure 3.3 shows the kinetics profiles for a) TNFα and b) IL-10 comparing the responses from elastase and LPS. For both cytokines there is a very low level of constitutive release, which does not change over the entire 48hrs. However, when the tissue is stimulated with LPS there is a significant increase in TNFα from 4hrs which remains significant up to and including the 48hr time-point; the increase peaks at 24hrs and then plateaus out to 48hrs. This was expected as LPS is an inflammatory stimulus and TNFα is classically thought of as a pro-inflammatory cytokine. Interestingly, though, when elastase stimulation occurs a similar pattern is seen. Again, the increase in cytokine release is significant from 4 to 48hrs, albeit at lower values than with the LPS stimulation (elastase 22.1 pg/mg tissue and LPS 40.2 pg/mg tissue, 24hrs).

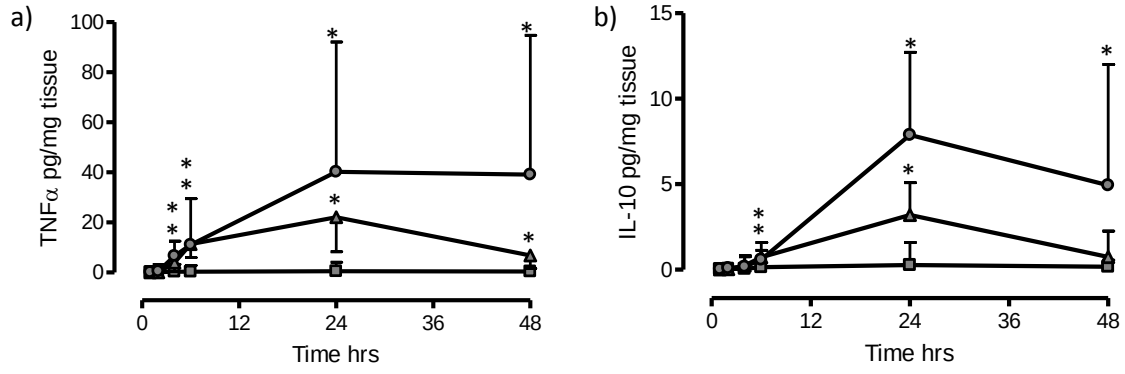

Figure 3.3: Kinetics profiles of TNFα and IL-10. a) shows the TNFα response over 48hrs stimulation with buffer control (open squares), elastase (open circles) or LPS (closed circles). The same set up is seen for the IL-10 response in b). All values are the median±interquartile range and expressed as pg/mg tissue (n=36). * denotes statistical significance when compared to control ($p<0.05$) as determined by Wilcoxon signed rank test.

When looking at the data generated for IL-10, an important anti-inflammatory cytokine, a similar pattern to the TNFα trends is seen. When stimulated with LPS, IL-10 increases greatly over the first 24hrs, peaking at 7.9 pg/mg tissue and then declines over the next 24hrs to 4.9 pg/mg tissue. This data becomes significant at 4hrs and remains so for the remainder of the time course. When the tissue was stimulated with elastase, there was a significant increase in

IL-10 at 6 and 24hrs (0.7 pg/mg tissue and 3.2 pg/mg tissue respectively). However, unlike the LPS stimulation of IL-10 release or the effect of elastase on TNFα, the increase in IL-10 drops back to basal levels by the 48hr time-point; 0.7 pg/mg tissue when stimulated with elastase compared to 0.2 pg/mg tissue when left unstimulated. It is worth noting at this point that a decrease in IL-10 concentration past the 24hr time-point is also observed when other stimuli are used, therefore suggesting that this cytokine is prone to metabolism within the system. This idea is further expanded upon later in the chapter.

As well as TNFα and IL-10, A1AT and other cytokines (IL-6, IL-8, IL-1β and MIP-1β) have also been investigated for their kinetics profiles in response to elastase and LPS stimulation. Cytokine data is summarised in table 3.2. However, data for these mediators shows less distinct patterns than for the previous cytokines already mentioned. In contrast to previous data obtained in our group (Seth, 2004) we failed to see an elastase-specific increase in A1AT over 24hrs (data not shown). This pattern is seen regardless of gender or disease severity.

Cytokine	Control	Elastase	LPS
IL-6	1484 (468-3497)	28.0 (0.79-425)	3661 (1897-7366)
IL-8	1132 (707-3091)	118 (14-1588)	4938 (2485-11383)
MIP-1β	3.1 (0.6-12.1)	0.3 (0.02-1.1)	31.3 (7.4-110)
IL-1β	0.5 (0.06-4.7)	1.3 (0.7-3.7)	11.4 (2.4-15.2)

Table 3.2: Effect of elastase and LPS on other cytokines. This shows the change in cytokine production after stimulation with either elastase or LPS as measured by ELISA. Values shown are median (interquartile range) and are pg cytokine/mg tissue.

3.3.3 Heterogeneity in the inflammatory response

The TNFα and IL-10 responses both show a great deal of inter-patient variability after elastase and LPS stimulation and one factor that may be a cause of this is disease severity. Most of the

patients in this investigation are either at risk of COPD or already have some level of airways obstruction and we have used the GOLD classifications to split patients into those with no evidence of airways obstruction (GOLD 0) and those with mild to moderate airways obstruction (GOLD I and II). This is summarised in table 3.3.

GOLD	Gender	Age	FEV_1/FVC
0	8M 4F	63.8±2.8	0.76±0.02
I & II	12M 5F	66.9±1.6	0.61±0.01

Table 3.3: Patient characteristics dependent on disease status. Patients were reclassified according to their disease status. GOLD indicates the disease severity with 0 being no disease and I/II being mild to moderate disease. For gender M denotes male and F denotes female. Ages are expressed as mean±sem in years and FEV_1/FVC is also shown as mean±sem.

When TNFα production, after elastase stimulation, is split by disease severity it can be seen that both sets of patients have a similar pattern of production for the first 6hrs (figure 3.4a). However, after this point the patients with GOLD I & II level disease have an increase in production of TNFα compared to those with GOLD 0 airways obstruction. This production peaks at 24hrs and diminishes by 48hrs and at this point both those with and without disease show the same concentration of this pro-inflammatory cytokine. Although there is an increase in production at 24hrs for the mild to moderate group this interesting trend does not reach statistical significance due to continued inter-patient variability.

A similar situation is seen with the IL-10 data (figure 3.4b) with both sets of patients following the same pattern of release; however those with mild or moderate disease have greater levels of IL-10 throughout the whole time course. Again, although this data shows a note-worthy trend it does not reach significance.

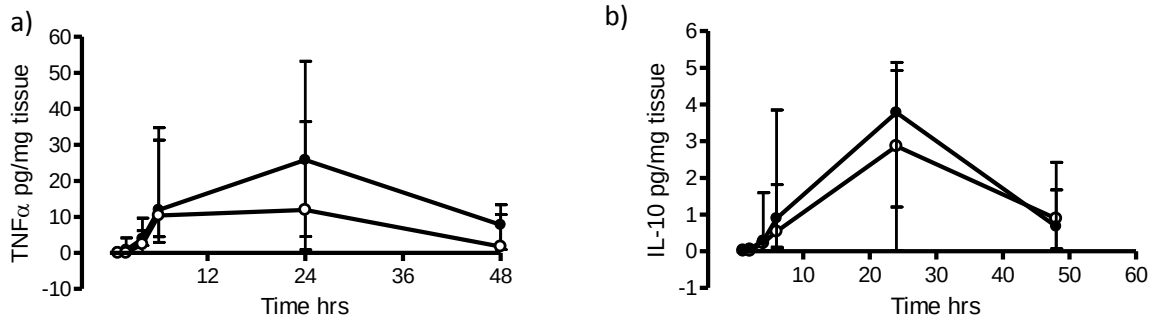

Figure 3.4: TNFα and IL-10 split by disease status. TNFα and IL-10 responses after elastase stimulation were reclassified according to disease severity; GOLD 0 (n=12) denotes no disease (open circles) and GOLD I & II (n=17) denotes mild to moderate airways obstruction (filled circles). Values are median±interquartile range and expressed as pg/mg tissue.

As neither TNFα nor IL-10 show significant differences with disease severity, and considering there is still a high level of variability it is obvious that there are other factors that contribute to the differences generated by different patients. Other parameters such as age, gender and smoking status have also been investigated and no trends are apparent (data not shown). It is therefore possible that these differences could be due to changes in the expression or activation of a receptor(s) that elastase may stimulate. This idea will be investigated further in Chapter 5.

3.3.4 Cytokine degradation by elastase

The kinetics studies have shown a decrease in cytokine production after 24hrs of elastase stimulation, whereas the control samples appear to have a stable concentration. A possible reason for this decline is that elastase, or other proteinases up-regulated by inflammation, may be able to degrade the cytokines as well as stimulating their release; after 24hrs degradation

may outweigh production. To investigate this, recombinant human cytokines and A1AT were incubated with elastase in RPMI-1640 or left in buffer alone to mimic the lung explant model.

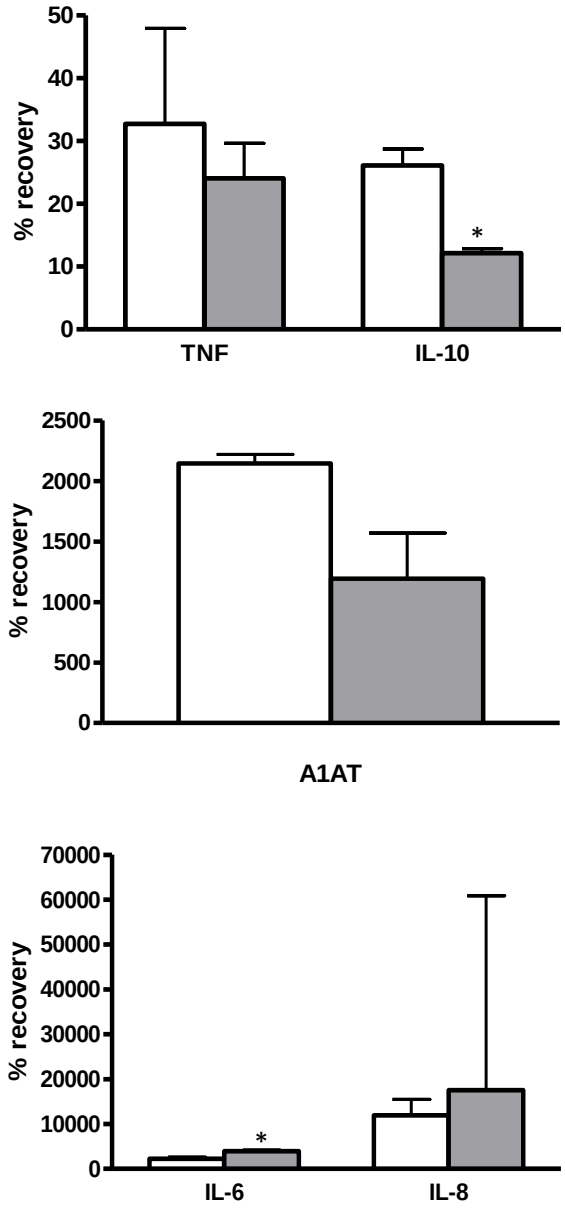

Figure 3.5: Cytokine degradation by elastase. Recombinant human cytokines/A1AT were incubated with (closed bars) or without (open bars) elastase (100μg/ml) for 24hrs and recovery was determined by ELISA (n=5). Values are shown as median+interquartile range and expressed as percent recovery. * denotes $P<0.05$ when compared to incubation without elastase as determined by Wilcoxon signed rank test.

Figure 3.5 shows the percentage recovery of the recombinant cytokines after incubation with or without elastase for 24hrs. It can be seen with TNFα and IL-10 that there is some cytokine degradation even in the absence of the stimulus, highlighting the fragile nature of these mediators. In the presence of elastase, recovery of TNFα and IL-10 is markedly decreased suggesting elastase has the ability to degrade these cytokines and thereby modulate the inflammatory response both to elastase itself as well as other stimuli such as LPS. When investigating other cytokines it is interesting to note that a recovery far greater than 100% is seen. Considering there are no other sources of these cytokines in this system these data suggest that the cytokines are being degraded as with TNFα and IL-10 and it is the constituent parts that are being detected by the ELISA. Although this data suggests that elastase can degrade cytokines it is important to recognise that in the *in vivo* situation anti-proteolytic defences such as A1AT would temper the proteolytic actions of elastase. Figure 3.5 also shows data for the recovery of A1AT with and without elastase and it can be seen that the proteolytic effects of elastase are less drastic than with the cytokines, thus suggesting that A1AT may be more resilient to proteolytic attack. However, although the scenario of elastase degrading cytokines is possible, it has to be considered whether it is probable considering the role of A1AT and other endogenous anti-proteinases.

3.3.5 Multi-cytokine analysis

Thus far the elastase-induced response has centred on the inflammatory and anti-inflammatory cytokines TNFα and IL-10. However, there is the potential that other cytokines may also be affected and this idea requires investigation. A range of Th1/Tc1 and Th2 cytokines were

measured via a multi-cytokine assay after 24hrs incubation with elastase, LPS or media alone (n=12).

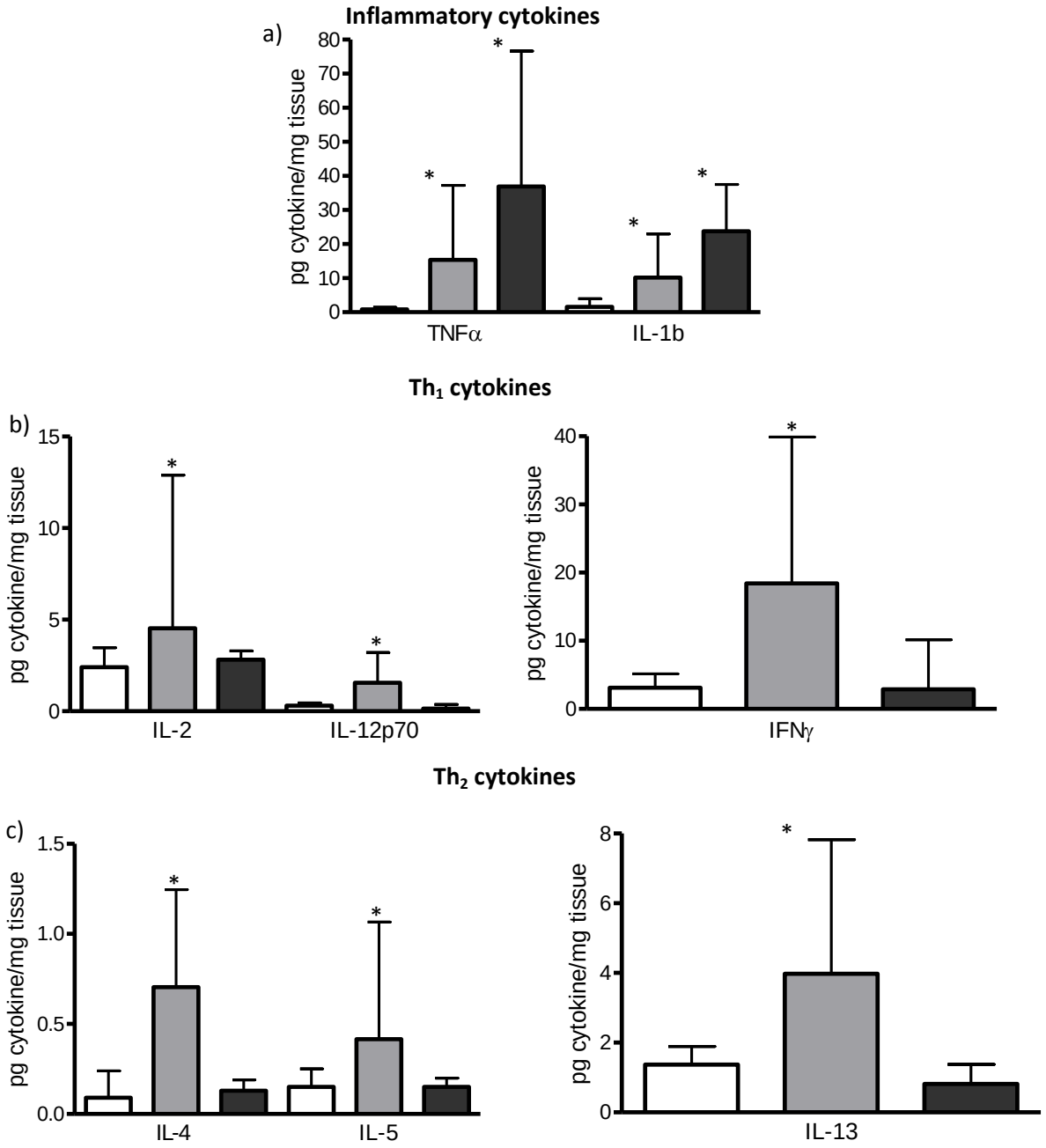

Figure 3.6: Multi-cytokine analysis. Explants were incubated with elastase, LPS or buffer control for 24hrs and a range of cytokine responses were measured (n=12). a) shows the inflammatory cytokines, b) shows the Th1/Tc1 type cytokines and c) shows the Th2 type cytokines. For each, the clear bars are the buffer controls, the light grey bars are stimulated with elastase and the dark grey bars are stimulated with LPS. All values are median+upper quartile and are expressed as pg/mg tissue. * denotes statistical significance when compared to the control, $p<0.05$ as determined by Wilcoxon signed rank test.

Figure 3.6a shows the concentrations of the inflammatory cytokines TNFα and IL-1β. Data for TNFα is comparable to that determined via ELISA and shows a significant increase with both elastase and LPS stimulation (15.4 and 36.9 pg/mg tissue respectively) compared to control samples (0.9 pg/mg tissue). There is also a significant increase in IL-1β with both stimuli using this technique (elastase=10.1 and LPS=23.8 pg/mg tissue compared to 1.6 pg/mg tissue, $p<0.05$) however this was not apparent when ELISA analysis was performed.

Th1/Tc1 cytokines were also investigated (figure 3.6b) and it is apparent that elastase can induce a significant increase in IL-2, IL-12p70 and IFNγ (4.5, 1.6 and 18.4 pg/mg tissue respectively). In comparison, LPS had no significant effect above the control levels. A similar increase can also be observed with the Th2 cytokines IL-4, IL-5 and IL-13 (figure 3.6c). All three cytokines show a significant increase after stimulation with elastase (IL-4: 0.7 vs. 0.1, IL-5: 0.4 vs. 0.2, IL-13: 3.4 vs. 1.4, all pg/mg tissue) whereas there is no effect after incubation with LPS.

3.3.6 Effect of dexamethasone on the elastase response

We have previously shown that the LPS-induced inflammatory response can be attenuated by pre-treatment with the steroid dexamethasone (Sword *et al.*, 2007) and as steroid treatment is common in COPD patients it was important to investigate the effect of dexamethasone on the elastase-induced inflammatory response.

To investigate the effect of dexamethasone on the elastase response parenchymal explants were incubated with a range of concentrations of dexamethasone (0.1-1000μM) overnight.

The following day explants were moved to fresh media and re-stimulated with the same concentrations of dexamethasone before being stimulated with elastase (100µg/ml) for a further 24hrs. LPS stimulation was also conducted at a concentration of 100ng/ml. Tissue and supernatants were harvested as previously described. Supernatants were analysed for TNFα and IL-10 content via ELISA.

In agreement with our previous studies (Sword *et al.,* 2007) figure 3.7a and b show that the LPS-induced release of TNFα and IL-10 can indeed be inhibited after pre-treatment with dexamethasone. For TNFα this effect is dose-dependent with the greatest effects seen with 100 and 1000µM dexamethasone with decreases of 75.4% and 74.4% respectively in TNFα production compared to LPS stimulation alone and in fact this inhibition is statistically significant for 1000, 100 and 10µM dexamethasone. With IL-10, this effect is less pronounced; although an inhibition of 47.6% is seen with the highest dose of dexamethasone used this fails to reach significance. This suggests that the anti-inflammatory response induced by LPS stimulation is somewhat less resistant to steroid pre-treatment than the pro-inflammatory response.

When considering the effect of dexamethasone on the elastase-induced response it can be seen that for TNFα the trend is similar to that for the LPS response (figure 3.7c). However, with elastase there is a considerable decrease (46.0%) in the amount of TNFα present in the supernatant after incubation with even the lowest concentration of dexamethasone, unlike with LPS. This inhibition increases to 90.7% with 1000µM dexamethasone showing that elastase-induced production of TNFα is highly susceptible to steroid treatment; all doses of

dexamethasone used produced significant inhibition. Although the inflammatory effects of elastase can be inhibited with steroid treatment, figure 3.7d shows that the anti-inflammatory effects are more resistant whereby the production of IL-10 is only affected by concentrations of dexamethasone greater than 1µM and the greatest inhibition is seen with a concentration of 100µM (65.6%).

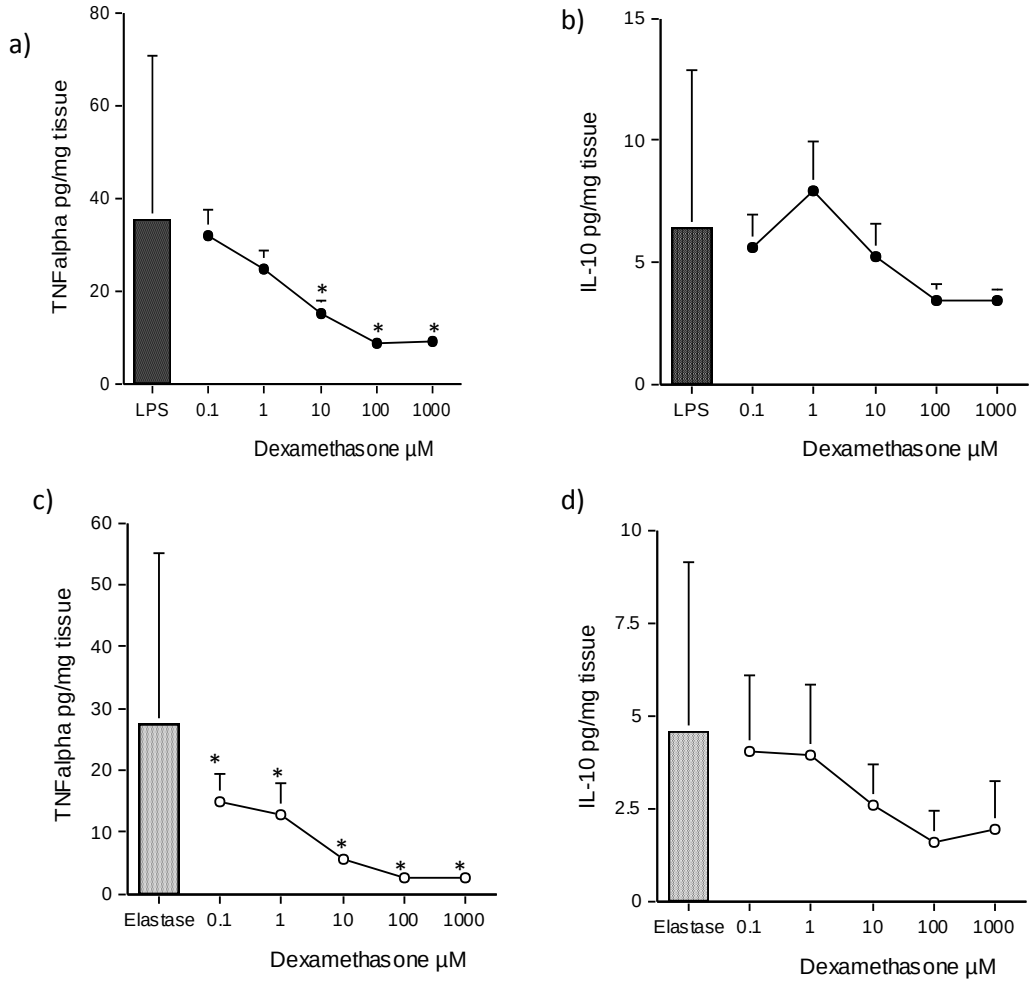

Figure 3.7: Effect of dexamethasone pre-treatment on elastase- and LPS-induced inflammatory responses. Parenchymal explants (n=10) were pre-incubated with the steroid dexamethasone overnight before being stimulated with either elastase (100µg/ml) or LPS (100ng/ml) for 24hrs. TNFα and IL-10 concentrations in the supernatant were measured via ELISA and are expressed as pg/mg tissue. Data were determined to be normally distributed according to D'Agostino normality test and so are expressed as mean±sem and * indicates $P<0.05$ when compared to stimulus alone.

3.3.7 Protein synthesis inhibition by cycloheximide

So far, this chapter has shown that elastase can induce an inflammatory response from the parenchymal explants however it is unknown whether the cytokines released in response to this stimulation are pre-formed or newly synthesised. To elucidate this, the protein synthesis inhibitor cycloheximide (CHX) was used. Explants were incubated with CHX for an hour before being stimulated with either elastase (100µg/ml), LPS (100ng/ml) or buffer alone for 24hrs. Concentrations of TNFα and IL-10 in the supernatant were determined by ELISA and compared to incubation with these stimuli without the CHX pre-treatment.

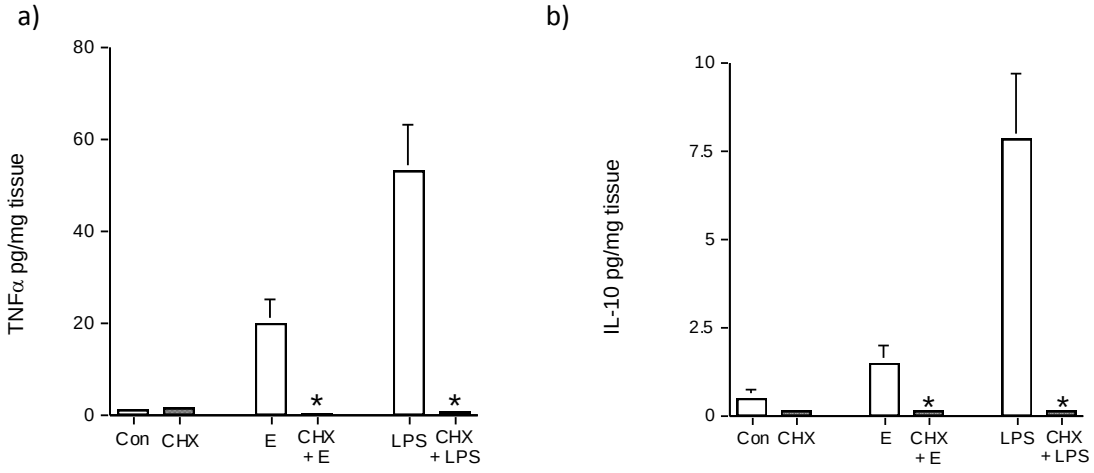

Figure 3.8: Protein synthesis inhibition with cycloheximide. Explants from 19 donors were incubated with CHX for 1hr before being stimulated with either elastase (100µg/ml), LPS (100ng/ml) or buffer alone for 24hrs (closed bars). Explants from the same donors were also incubated with these stimuli but without pre-treatment with CHX (open bars). Cytokine concentrations were determined via ELISA; a) shows the TNFα response and b) shows the IL-10 response. Values are shown as mean±sem and are expressed as pg/mg tissue. * indicates $P<0.05$ when compared to the same stimuli without pre-treatment with CHX.

Figure 3.8a shows that pre-treatment with the protein synthesis inhibitor CHX can diminish both the elastase and LPS-induced TNFα response back to control levels; 18.9±5.0 pg/mg tissue down to 0.12±0.1 pg/mg tissue for the elastase response and 53.1±10.0 pg/mg tissue

reduced to 0.7±0.2 pg/mg tissue for the LPS-induced response. Similarly, CHX pre-treatment also inhibits the elastase and LPS-induced IL-10 responses (figure 3.8b). In this case the elastase-induced response is decreased from 1.5±0.5 pg/mg tissue without CHX to 0.1±0.1 pg/mg tissue with the inhibitor. Likewise, the LPS-induced response is diminished from 7.9±1.8 pg/mg tissue to 0.1±0.1 pg/mg tissue when the explants were incubated with CHX. Taking into consideration the data it is sensible to assume that any increase in either TNFα or IL-10 in response to elastase or LPS stimulation is due to the release of newly synthesised cytokines and not those already pre-formed and stored within the cells.

3.4 Discussion

Neutrophil elastase is well known for its proteolytic actions on elastin and other matrix proteins and the balance of this proteinase with its anti-proteinases such as A1AT play an important role in conditions such as emphysema. Although this disease is predominantly characterised by the degradation of the alveolar spaces there is some degree of residual inflammation that accompanies this and the degree to which elastase contributes to this is an area not fully explored. This study first aimed to replicate data from a previous study (Seth, 2004), which suggested that an increase in elastase stimulation would increase the amount of A1AT found in the supernatant. Figure 3.1 shows that this is not the case; elastase stimulation does not increase the A1AT response above that of the control. However, there is high basal production of this proteinase and so therefore it is possible that, considering many of the subjects used in this study have some degree of inflammatory airways obstruction, A1AT may already be being produced to its maximal concentration and therefore cannot respond to the elastase stimulation. It is also possible that the high concentrations of elastase used in this study may both cause an increase in A1AT but also the degradation of such an increase, figure 3.5, and therefore it appears there is little net change in the amount of A1AT being produced. It is also important to note that unlike in the *in vivo* situation the explants are not supplied with a circulation that would provide further A1AT and so the role of elastase and any associated up-regulation of the anti-proteinase defences would ideally need to be studied *in vivo*.

The link between elastase, the proteinase-anti-proteinase balance and the development of emphysema has been well investigated (Hayes *et al.*, 1975, Nakajoh *et al.*, 2003 and reviewed in Abboud *et al.*, 2008) but it is necessary to note that emphysema is usually accompanied by

chronic bronchitis and bronchiolitis and as such is classed as COPD. COPD is characterised by both low-level continual inflammation as well as acute inflammation associated with bacterial and viral-induced exacerbations. The inflammatory response to LPS has been well defined within this system by Hackett *et al.* (2008) and a similar method has been employed by this study to assess the role that elastase may play in the continual background inflammation. TNFα is classically thought of as an inflammatory cytokine and stimulation with LPS significantly increases its production by the parenchymal explants (Hackett *et al.*, 2008). The current study has confirmed this effect and as such has used LPS as a positive control when investigating the inflammatory properties of elastase. Figure 3.1 shows that elastase can induce a significant increase in both TNFα and the anti-inflammatory cytokine IL-10. This effect is dose-dependent and kinetics studies have shown that increases in these cytokines are also time-dependent with the greatest increases occurring after 24hrs stimulation with the proteinase (figure 3.2). This is the same trend as is seen with LPS stimulation of the explants. However, LPS is also capable of inducing an increase in other pro-inflammatory cytokines such as IL-1β and IL-6, whereas elastase stimulation does not cause a significant increase in IL-1β and in fact decreases the IL-6 response from the explants (table 3.1). When investigating the effect of elastase on the IL-8 response we found high levels of heterogeneity with no distinct patterns. This is contradictive to work by Ho *et al.*, (2009) which suggests that neutrophil elastase suppresses the IL-8 response from human airway smooth muscle cells but induces this cytokine in A549 alveolar epithelial cells via differential actions on the NFκB signalling pathway.

Hackett *et al.* (2008) have also shown that the LPS-induced TNFα response from the parenchymal explants is different dependent on the baseline disease state of the individual donor. When splitting the donor responses by the stage of disease (figure 3.4) it is noted that although there is no significant difference between those with no disease (GOLD 0) and those with mild to moderate disease (GOLD I and II) there is a tendency for those in the latter group to have a slightly increased TNFα and IL-10 response after 24hrs of elastase stimulation. Many of the donors used in this study do not have a formal diagnosis of COPD and as such do not receive treatment for this and therefore drug effects cannot explain the lack of a significant difference compared to that seen after stimulation with LPS. Although formal diagnoses are rare, many of the subjects do have decreased lung function thus suggesting a degree of airways obstruction-indicative of COPD. As such, patients have been divided primarily on their FEV_1/FVC ratio with those having a reading above 0.7 being classified as having no disease and those falling below this cut-off having either GOLD I or II disease, as defined by the guidelines set out by GOLD (2005). This separation has shown that these groups do in fact share many similarities, as summarised in table 3.2, and the difference between their lung function data is not well defined; the GOLD 0 group have an average FEV_1/FVC ratio of 0.76 and the GOLD I and II group have an average reading of 0.61. In order to fully understand the effect of baseline disease status on the subsequent inflammatory response to elastase a greater study with a wider spread of disease is required. This needs to include subjects defined as having normal lung function with no risk of COPD as well as those classified as having COPD of greater severity, i.e. those classified as having GOLD III or IV disease; however due to the nature of severe COPD and the associated increased frequency of exacerbations

(Tsoumakidou *et al.,* 2004) it is difficult, and somewhat unethical, to obtain research samples from this cohort of subjects.

Having shown that differences in the disease status of these subjects is not the cause for the great inter-patient variability it is important to investigate possible other sources. Gender, age and smoking history have been assessed but show no correlation with the inflammatory response. One avenue that could be explored is the expression and activation of receptors that elastase may act through; it is thought that the up regulation of PAR-2 via luminal intestinal proteinases (such as trypsin and tryptase) increases inflammation in the gastrointestinal tract and may be relevant in inflammatory bowel disorders such as Crohn's disease (Cenac *et al.,* 2002). PAR-2 is also implicated in other inflammatory conditions such as rheumatoid arthritis and Busso *et al.,* (2007) have shown that PAR-2 is increased in the synovium of rheumatoid arthritis patients compared to those with osteoarthritis. Considering the role of PAR-2 in the aforementioned inflammatory diseases it is therefore wise to consider this receptor in the pathogenesis of COPD and its role as a candidate to explain the heterogeneity seen in this patient group. Another candidate receptor for which elastase could mediate its inflammatory properties could be TLR-4, considering the similarity between the elastase-induced and LPS-induced responses with regard to TNFα and IL-10. There is also some evidence to suggest that elastase may activate TLR-4 in macrophages thus initiating an inflammatory response (Geraghty *et al.,* 2007). The similarities between these two stimuli were further investigated by the pre-treatment of the explants with dexamethasone and data generated from this set of experiments goes someway to elucidate which receptor elastase may act through. The relationship between both these candidate receptors will be discussed in chapter 5.

It has been suggested that PAR-2-mediated anti-inflammatory and muscle relaxant responses within mouse airways are resistant to pre-treatment with dexamethasone (Saleh *et al.*, 2008). However, this study also gives evidence for the opposite to occur in A549 carcinomic human alveolar basal epithelial cells. Taking this into consideration it is sensible to suggest that if elastase does in fact activate PAR-2 then pre-treatment of the parenchyma with dexamethasone should have no inhibitory effect on the elastase-induced cytokine release. However, this study has shown that elastase-induced TNFα production can be inhibited by dexamethasone thus suggesting that this response is not mediated by PAR-2 and in fact could be mediated by TLR-4 as with the LPS response, which is susceptible to steroid treatment. Conversely, the IL-10 response to elastase stimulation is more resistant to steroid treatment and so could suggest that this response is regulated via a different route, possible via PAR-2. However, further investigation into the receptors and pathways activated by elastase is required before firm conclusions can be made.

Another possible reason for the heterogeneity expressed by these patients is underlying co-morbidities. The average ages of the GOLD 0 and GOLD I and II groups are 63.8 and 66.9 years respectively and so it is likely that these patients will also have conditions such as hypertension and hypercholesterolemia and as such may be receiving the appropriate treatment which may ultimately have indirect effects on the inflammation initiated in response to elastase stimulation. Due to the nature of the subjects used in this study it is difficult to elucidate the role that these co-morbidities may play but it is a factor that is to be considered.

So far, this chapter has discussed the production of cytokines in response to elastase stimulation but it is important to remember that this model represents a dynamic system where mediators are metabolised as well as synthesised. Stimulation with elastase for greater than 24hrs, as shown in figure 3.2, yields an apparent decrease in cytokine production but in fact it is more likely that at this point metabolism and degradation of the cytokine is occurring at a greater rate than production. Figure 3.5 details the effects of elastase stimulation on recombinant human cytokines and from this it is clear that TNFα and IL-10 are susceptible to degradation by elastase; it is also possible that IL-6 and IL-8 may be prone to proteolytic degradation. van Kessel *et al.,* (1991) have previously documented the degradation of human recombinant TNFα and Bank *et al.,* (1999) have shown that elastase can degrade human recombinant IL-6 from as early as 15 minutes after initial stimulation. Data from the current study fits with these previous studies. Degradation of IL-8 and the abolition of its chemotactic effects have also been found previously (Leavell *et al.*, 1997); recombinant human IL-8 was incubated with 10μg/ml human neutrophil elastase and it was found that this completely abolished the chemotactic effects of this cytokine due to proteolysis at specific sites within the protein by elastase. Although this current study cannot comment on the chemotactic activity of IL-8 after incubation with elastase, we have shown that IL-8 detection by ELISA is increased. However, the conditions under which these experiments were carried out did not include any external sources of IL-8 and so therefore it is possible that elastase degrades IL-8 in such a way that the breakdown products are detected by the ELISA thus giving a false increase in this cytokine. This phenomenon could explain why we have seen variable effects on the IL-8 response in human explants stimulated with elastase; dependent on the disease status of the patient their IL-8 may be more or less susceptible to elastolytic attack.

Although we have shown that elastase can degrade these cytokines after just 24hrs it is important to realise that the parenchymal environment is far more complex than that represented by the degradation studies. As the integrity of the explants is maintained exposure of the cytokines to the proteinase will be substantially decreased and thus the degradation effects of elastase will be shielded. This is apparent when looking at the decrease in cytokine production in the kinetics studies after 24hrs where cytokine concentration is not reduced completely.

Having investigated the degradation effects of elastase on the cytokine response it was imperative to explore cytokine synthesis after elastase stimulation. Pre-treatment with the protein synthesis inhibitor CHX followed by 24hrs stimulation with elastase showed a significant decrease in both the TNFα and IL-10 response. This suggests that these cytokines are newly synthesised upon stimulation with the proteinase. It was possible that elastase caused this inflammatory response via its proteolytic actions, by cleaving bound cytokines from the matrix, rather than inducing the synthesis, and subsequent release, of inflammatory cytokines from inflammatory cells such as macrophages and neutrophils. It appears, however that elastase induces new synthesis of these cytokines rather than the release of pre-formed proteins in a manner similar to that performed by LPS. This adds weight to the proposal that elastase can initiate an inflammatory response without accompanying proteolysis. This idea will be expanded upon in the next chapter.

So far this chapter has focussed on the inflammatory and anti-inflammatory response in the parenchymal explants in response to elastase but we have also utilised multi-cytokine analysis

to determine the effects of elastase and LPS on a range of Th1/Tc1 and Th2 cytokines. Figure 3.6 shows that elastase can elicit a significant increase in both Th1/Tc1 and Th2 cytokines as well as the inflammatory mediators. In contrast, LPS only causes a significant response from TNFα and IL-1β. This shows that as well as having inflammatory effects, elastase also has a wider range of properties that LPS does not possess. This is a surprising finding considering COPD is traditionally considered to be a Th1/Tc1 type disease and both Hodge *et al.,* (2007) and Majori *et al.,* (1999) have shown that COPD patients have increased Th1 type cytokines in peripheral blood, bronchoalveolar lavage and intraepithelial T cells. However, asthma is associated more with the expression of Th2 cytokines (as reviewed in Kuipers *et al.,*2004) and Humbert *et al.,* (1997) have shown increases in the Th2 cytokine IL-13, which contributes to eosinophilia-a characteristic feature of asthma. We have shown that elastase can increase both Th1/Tc1 cytokines as well as Th2 cytokines and therefore, these findings suggest that COPD either has characteristics of both Th1/Tc1 and Th2 disease and/or that elastase may be involved in the pathogenesis of asthma. Barceló *et al.* (2006) have shown some evidence to suggest that the intracellular cytokine production of T cells from those with COPD is skewed toward a Th2 profile. Traditionally, elastase has more association with COPD than asthma and as such this study has focussed on the role this proteinase plays in the former condition. As LPS was unable to initiate a response from the Th1/Tc1 or Th2 cytokines it can be concluded that LPS stimulation does not play a role in defining the Th1/Th2 skew of the disease unlike elastase. Another factor that also needs to be considered with this data is the fact that smokers are known to have increased IgE levels, which is associated with asthma and allergy (Borish *et al.,* 2005) and thus supports the Th2 skewing of the elastase-induced response.

In conclusion, this chapter has found that elastase can induce an inflammatory response in the form of TNFα and an anti-inflammatory response with increases in IL-10 production. We have also shown that this response is due to the new synthesis of these proteins and not the expression of pre-formed cytokines or the cleavage of cytokines bound to the extracellular matrix. Lastly, we have shown that elastase, unlike LPS, can cause significant increases in both Th1/Tc1 and Th2 cytokines, which may contribute to the overall disease pathogenesis.

Chapter 4: Proteolytic Activity and Inflammation

4.1 Introduction

Having established that elastase can induce an inflammatory response it was important to investigate whether this response is specific to this proteinase or whether it can be reproduced with a proteinase from either the same proteinase family, for example trypsin or chymotrypsin (both serine proteinases), or a different family, for example a cysteine proteinase such as papain. With elastase, the serine residue present within the active site is integral to its enzymatic activity whereas with papain this is due to a cysteine residue in the active site instead; papain consists of 212 amino acid residues and its structure is held together by three disulphide bridges that form a cleft containing the active site for proteolysis to occur (Drenth *et al.,* 1968). As with elastase, papain has also been used as a research tool to induce emphysema-like changes in animal models (Fló *et al.,* 2006, Hoffman *et al.,* 2005 and Hubmayr *et al.,* 1993) but there has been little investigation into whether papain can induce an inflammatory response as well as structural changes.

Although we have shown that elastase can induce an inflammatory response it is, as of yet, unknown whether its proteolytic actions are responsible for this. In the physiological system, elastase is predominantly inhibited by A1AT in a 1:1 molar ratio (Pannell *et al.,* 1974) but, as shown in the previous chapter, elastase does have the ability to degrade this anti-proteinase. Also, in COPD anti-proteinases are prime targets for oxidant damage thus rendering them inactive and therefore exerting no inhibitory effect on elastase and other proteinases. In order to study whether elastase is required to maintain its proteolytic properties to induce inflammation we have utilised an inhibitor of elastase, elastatinal, which is not prone to proteolytic attack. Elastatinal has its inhibitory effects by forming a hemicetal adduct between

its aldehyde group and the serine residue in the active site of elastase which is usually required for proteolysis (Williams *et al.*, 1987). This inhibitor does not have any effect on other proteinases such as chymotrypsin (Feinstein *et al.*, 1976).

This chapter aims to determine whether the inflammatory effect already discussed is specific to elastase and whether this effect can be abolished via the inhibition of the proteolytic activity of the proteinase.

4.2 Methods

The human lung explant model was used to investigate the need for proteolytic activity in inducing the inflammatory response. Tissue from 32 patients undergoing carcinoma resection was obtained from Guy's Hospital, London and cultured for up to 48hrs. Tissue from 13 patients undergoing carcinoma resection or bullus repair was obtained from Southampton General Hospital and cultured for 24hrs. Patient characteristics are detailed in table 4.1. Subsets of patients for separate experiments were matched according to their clinical and demographic data as far as possible.

a)

Age	65.9 ± 1.2 years
Gender	22M 10F
FEV_1/FVC	0.66 ±0.02
Smoking status	13 current, 14 ex, 2 non, 3 unknown

b)

Age	61.2± 4.4 years
Gender	5M 8F
FEV_1/FVC	0.66 ±0.02
Smoking status	4 current, 8 ex, 0 non, 1 unknown

Table 4.1: Patient characteristics. a) data from Guy's Hospital, London. b) data from Southampton General Hospital. Age and FEV_1/FVC are shown as mean ± sem. For gender, M=male and F=female. Under smoking status, current denotes current smokers, ex denotes ex-smokers as determined by the patient's clinician, non denotes non-smokers and unknown is for those patients for whom smoking status could not be obtained.

4.3 Results

4.3.1 Specificity of elastase-induced inflammation

The previous chapter has shown that elastase can initiate an inflammatory response however it is unknown whether this response is specific to this proteinase or whether other proteinases from the same family could give the same effect. Elastase is a serine proteinase and so we have compared its inflammatory effects to those initiated by other proteinases from the same family, namely trypsin and chymotrypsin. Parenchymal explants (n=13) were incubated for 24hrs with LPS (100ng/ml), elastase, trypsin or chymotrypsin. All proteinases were used at a concentration equivalent to 100µg/ml elastase: 0.84 proteolytic units/ml. Supernatants were collected and analysed via ELISA for TNFα and IL-10.

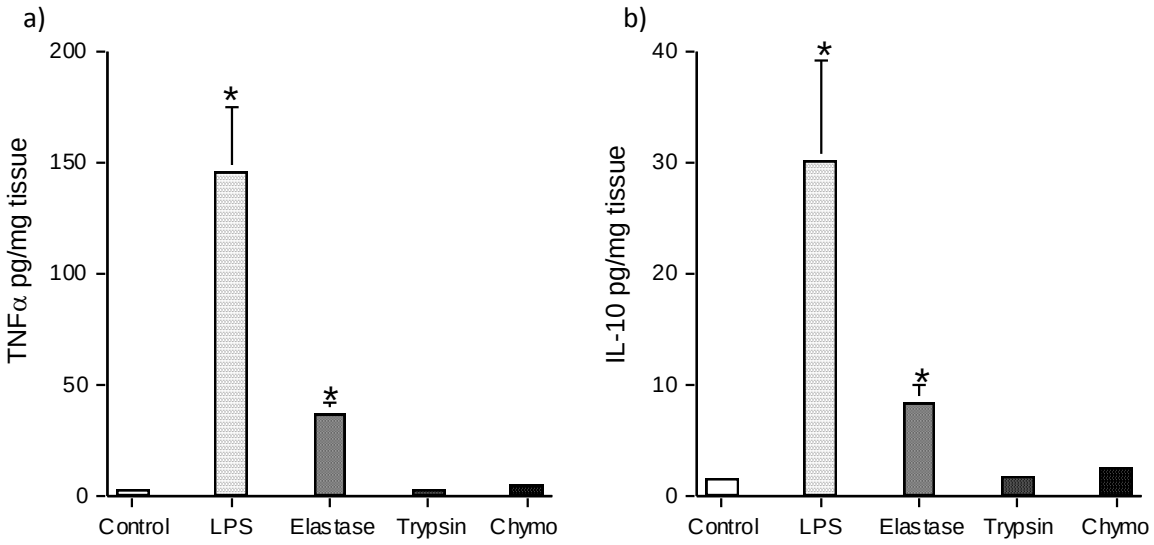

Figure 4.1: Specificity of elastase-induced inflammation. Explants from 13 donors were incubated with LPS (100ng/ml), elastase (0.84 proteolytic units/100µg/ml), trypsin (0.84 proteolytic units) or chymotrypsin (chymo, 0.84 proteolytic units) for 24hrs and supernatant concentrations of TNFα and IL-10 was determined by ELISA. Values shown are the mean±sem and are expressed as pg cytokine/mg tissue. * denotes $P<0.05$ when compared to the control.

As seen previously, figure 4.1 shows that elastase induces significant increases in the concentrations of TNFα and IL-10 released from the parenchymal explants. However, stimulation with trypsin or chymotrypsin has no effect above control levels on either of these cytokines, thus suggesting that the effect seen with elastase is specific to elastase in particular and is not a characteristic of all serine proteinases.

4.3.2 Papain stimulation

Having established that the inflammatory response elicited by elastase cannot be replicated by other serine proteinases, we investigated whether a proteinase from another family could induce an inflammatory response. Papain is a cysteine proteinase derived from papaya and thus a novel stimulus for the parenchymal explants. However, papain has previously been used in animal models of emphysema (Fló *et al.,* 2006, Hoffman *et al.,* 2005 and Hubmayr *et al.,* 1993) and thus was deemed a suitable comparison for elastase stimulation.

Explants (n=14) were incubated with papain, at an equivalent concentration to elastase (0.84 proteolytic units/ml) for up to 48hrs, after which TNFα and IL-10 concentrations were ascertained by ELISA. Figure 4.2a shows that papain stimulation does not increase TNFα synthesis and in fact the concentration of this cytokine is consistently lower than that released constitutively, although not significantly. A similar story can be seen when IL-10 is investigated (figure 4.2b). Again, papain failed to have any effect over control levels. This data establishes that papain is unable to evoke an inflammatory response, unlike elastase, but it is imperative to assess the effect of this proteinase on other cytokines.

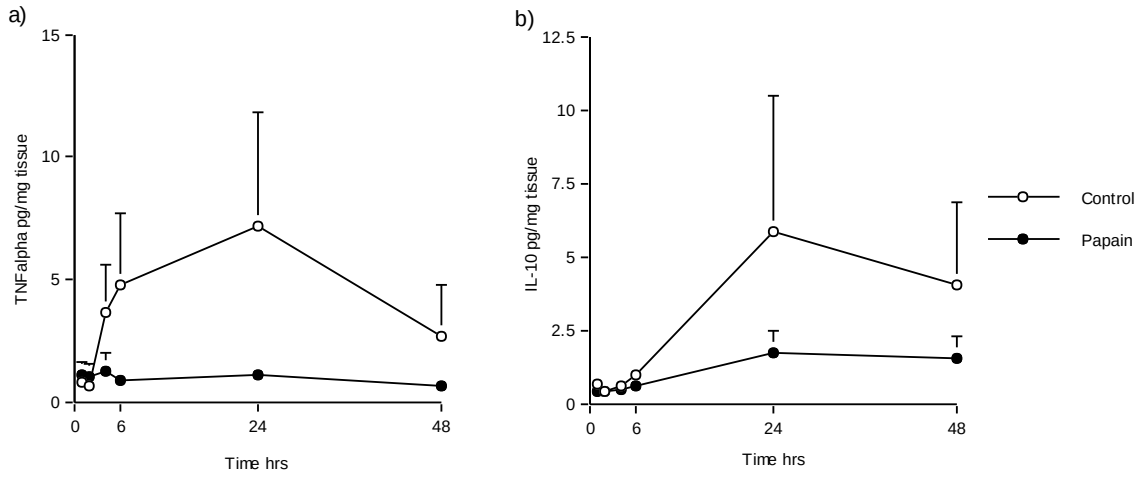

Figure 4.2: Stimulation with papain. Explants (n=15) were stimulated with papain, 0.84 proteolytic units/ml, (closed circles) or buffer control (open circles) for a range of time points up to 48hrs. a) shows the effect on TNFα release after stimulation with papain and b) shows the effect this stimulation has on the IL-10 response. All values are mean±sem and are expressed as pg/mg tissue.

MSD multi-cytokine analysis was used to determine whether papain could increase Th1/Tc1 or Th2 cytokines in a manner comparable to elastase. This data is summarised in table 4.1 below. For all cytokines measured, papain caused no effect over the control samples at 24hrs.

Cytokine	Control	Papain
IFNγ	3.51 ± 0.57	3.74 ± 0.73
IL-1β	3.67 ± 1.43	2.62 ± 0.94
IL-2	2.38 ± 0.33	1.24 ± 0.34
IL-4	0.12 ± 0.04	0.11 ± 0.02
IL-5	0.20 ± 0.06	0.12 ± 0.02
IL-12p70	0.33 ± 0.09	0.32 ± 0.07
IL-13	1.35 ± 0.22	1.51 ± 0.33

Table 4.2: Effect of papain stimulation on a range of Th1/Tc1 and Th2 cytokines. The release of a range of Th1/Tc1 and Th2 cytokines from parenchymal explants after 24hrs stimulation with papain were assessed by the MSD multi-cytokine analysis. All values are mean±sem and are pg cytokine/mg tissue.

4.3.3 Elastase inhibition

Having established that not all proteinases can initiate an inflammatory response we next examined the requirement for proteolytic activity. We investigated the effects of the reversible elastase-specific inhibitor elastatinal (Umezawa & Aoyagi, 1977) on elastase dependent release of TNFα and IL-10. These effects were compared to the actions of SBTI, a trypsin specific inhibitor, which should have no effect on elastase activity.

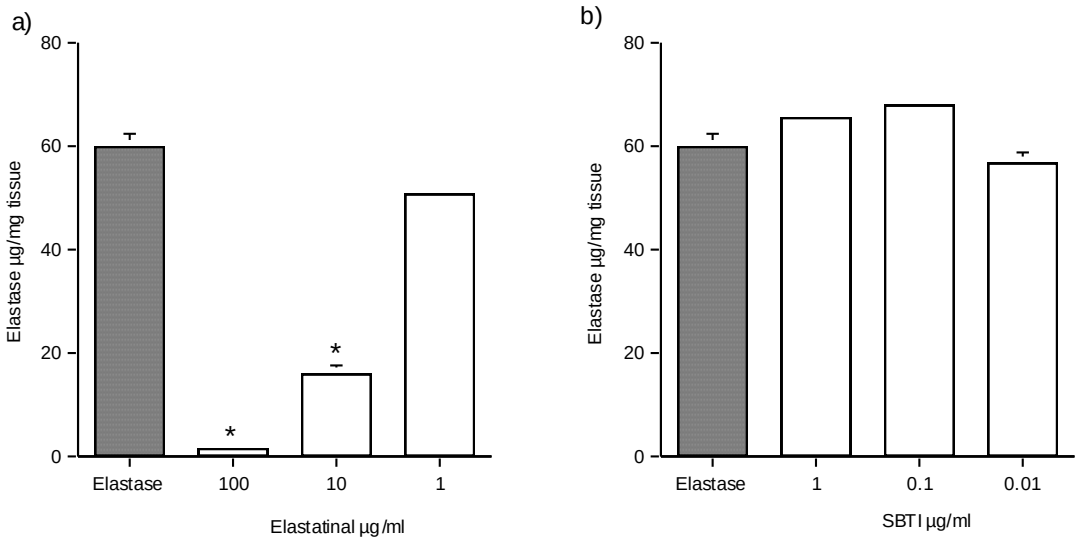

Figure 4.3: Elastase inhibition by elastatinal and SBTI. Elastase was inhibited by elastatinal (a) and SBTI (b), n=8. The closed bars show the activity for elastase on its own and the open bars show the activity after inhibition. Values are the mean±sem and are expressed as µg/mg tissue. * denotes statistical significance ($p<0.05$)

Initially, explants were incubated with elastase and the inhibitors for 6 and 24hrs however, preliminary data suggested that 24hrs alone would be a more informative time point to study and so subsequent samples were treated in this way (n=13). An elastase activity assay was used to confirm that the enzyme had been inhibited by elastatinal and SBTI, n=8 (figure 4.3); SBTI had no inhibitory effects on elastase and elastatinal inhibited elastase in a dose dependent manner with 100 and 10µg/ml causing a significant decrease in elastase activity ($p<0.001$).

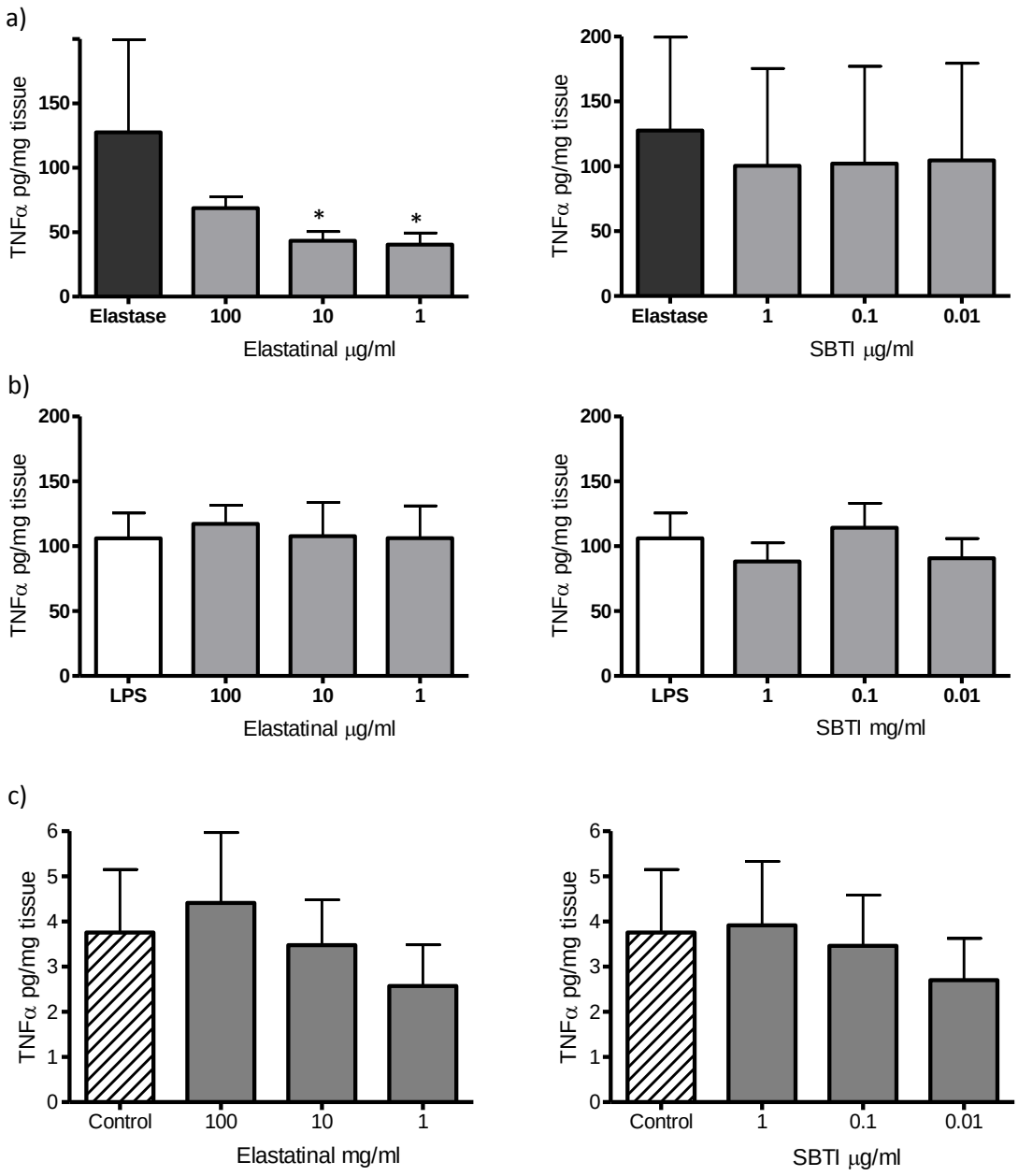

Figure 4.4: TNFα response after elastase inhibition. Elastase or LPS was incubated with either elastatinal or SBTI before being added to the explants. At 24hrs the TNFα response was measured (n=13). a) shows the response after inhibition with elastatinal, b) shows the response after inhibition with SBTI and c) shows the effect of the inhibitors alone on TNFαrelease. The dark grey bars show the response when elastase was added alone, the clear bars when LPS was added alone, the hashed bars when control media was used alone and the light grey bars are when the inhibitors were used. All values are mean±sem and expressed as pg/mg tissue. * denotes statistical significance when compared to elastase alone ($p<0.05$).

Firstly, the TNFα response to elastase stimulation was investigated. Figure 4.4a shows that 100µg/ml elastase alone can produce 127.5±72.1 pg/mg tissue but when the inhibitor is added this drops considerably to 40.4±9.0pg/mg tissue. This effect is seen with both 10 and 1µg/ml of elastatinal causing inhibition of the elastase-induced response, albeit not complete inhibition. When looking at the highest dose of elastatinal it can be seen that although the TNFα response is decreased, this decrease does not reach statistical significance. This could be due to the inhibitor itself showing some degree of TNFα induction at a high dose (figure 4.4c), albeit not significantly. In comparison, elastatinal had no inhibitory effect on the LPS-induced TNFα response at all concentrations, including the higher concentration of 100µg/ml. SBTI was unable to inhibit either the elastase or LPS response (figure 4.4b).

In comparison to the TNFα response, IL-10 production in response to elastase plus the inhibitors showed a different effect; the highest dose of elastatinal had no inhibitory effects upon IL-10 production and the lower doses only had a limited effect on reducing the elastase initiated response (figure 4.5a). However, LPS-induced IL-10 production is slightly increased by the presence of elastatinal, which then diminishes in a dose-dependent fashion (figure 4.5a). Elastatinal alone also induces some IL-10 production but although it is slightly greater than control levels it is small compared to either the elastase or LPS induced response and does not reach statistical significance. SBTI, however, showed that it is capable of reducing IL-10 production induced by elastase but not that induced by LPS (figure 4.5b). This suggests that this IL-10 response is not completely mediated by elastase but partly due to trypsin. The possibility of trypsin contamination of the elastase used in this study has been investigated via a trypsin activity assay and no contamination has been found (data not shown). This therefore

suggests that any trypsin-induced response is due to the endogenous presence of this proteinase and not from the exogenous application of the stimuli.

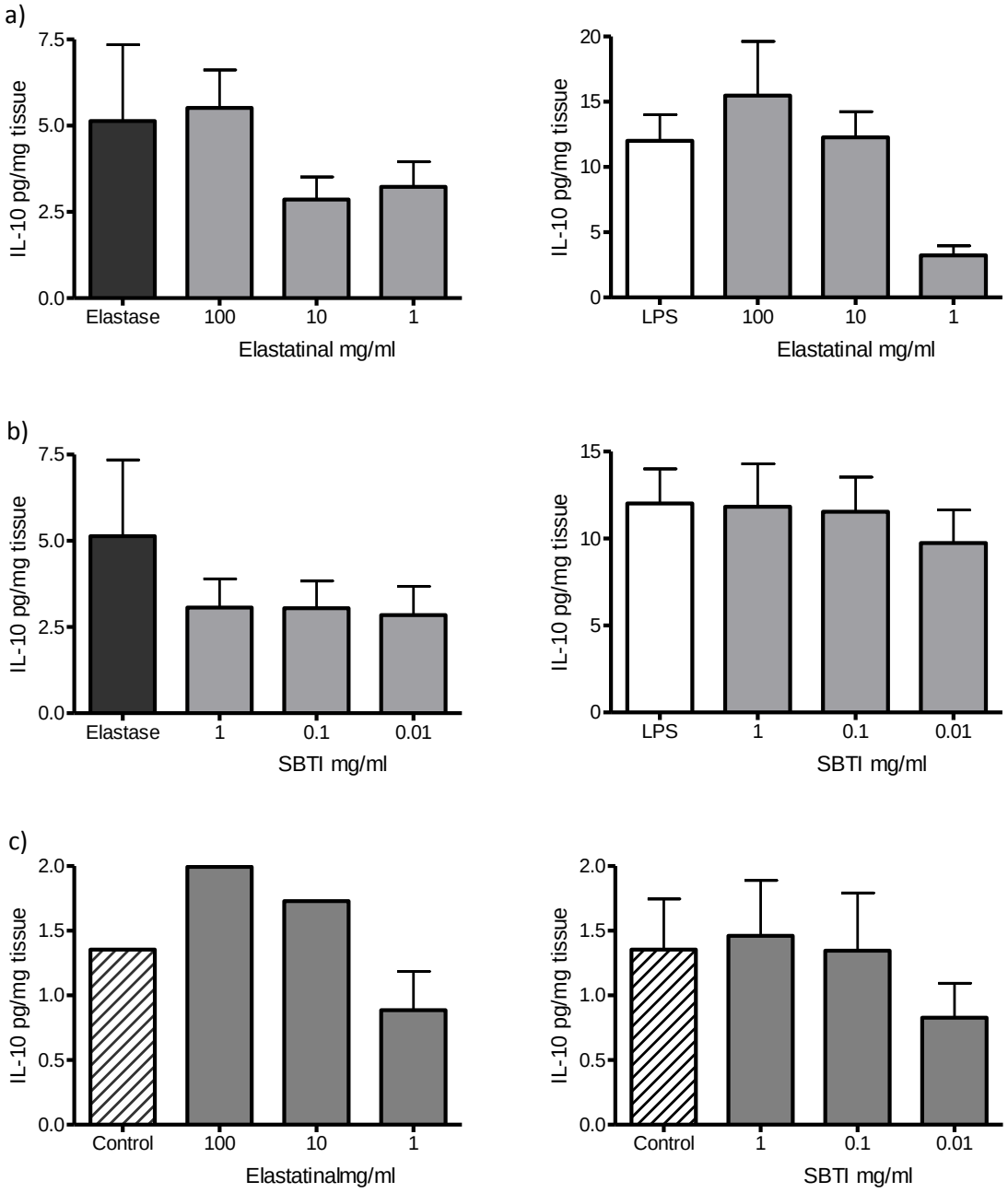

Figure 4.5: IL-10 response after elastase inhibition. Elastase or LPS was incubated with either elastatinal or SBTI before being added to the explants. At 24hrs the IL-10 response was measured (n=13). a) shows the response after inhibition with elastatinal and b) shows the response after inhibition with SBTI. The dark grey bars show the response when elastase was added alone, the clear bars when LPS was added alone and the light grey bars are when the inhibitors were used. All values are mean±sem and expressed as pg/mg tissue.

4.3.4 Elastase inhibition in storage

As shown in section 3.3.5 elastase is capable of degrading cytokines and so it was important to investigate whether the proteolytic activity could be inhibited whilst the supernatants were in storage. A proteinase inhibitor cocktail (PIC) was added to the supernatants after stimulation with elastase for a range of time-points, which were then stored at -70°C until analysis. TNFα and IL-10 concentrations were determined for supernatants stored with and without PIC (n=9). The elastase activity assay showed that PIC could significantly decrease the activity of the proteinase (data not shown).

Figure 4.6a shows the effect PIC has on TNFα production after elastase stimulation. There is a decrease in cytokine detection when PIC is added to the supernatant compared to when it is not and this is particularly marked at the 24hr time-point (162.3±80.9pg/mg tissue without PIC and 93.9±44.5pg/mg tissue with PIC, $p<0.05$). The discrepancy between those samples stored with and without PIC is still apparent at 48hrs although it is no longer significant.

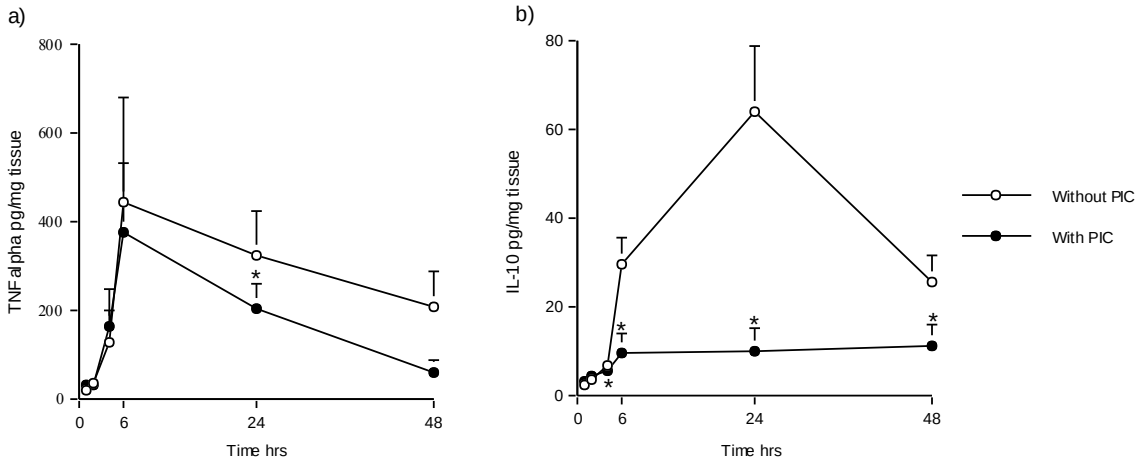

Figure 4.6: Storage of supernatants with and without PIC. Supernatants were stored either with or without PIC and the TNFα (a) and IL-10 (b) responses were determined (n=9). Open circles are those stored without PIC and closed circles are those stored with PIC. All values are mean±sem and are expressed as pg/mg tissue. * denotes a significant decrease when compared to samples stored without PIC ($p<0.05$).

IL-10 concentrations were also determined and these data show a more extreme difference between the two conditions (stored with and without PIC) than for TNFα (figure 4.6b). There is a significant decrease in concentration of IL-10 measured from 4hrs, which continues for the remainder of the 48hr time course. This is most evident at the 24hr time-point where the concentration of IL-10 decreases from 58.2±10.1pg/mg tissue without PIC to 0.9±0.3 pg/mg tissue with PIC. This suggests that the addition of PIC may be altering the structure of the IL-10 protein, thus rendering it undetectable by the ELISA system and as such it was deemed appropriate to discontinue the use of PIC in the storage of these samples.

4.3.5 One hour stimulation with elastase

Data so far has established that continuous stimulation with elastase for up to 48hrs induces both a TNFα and an IL-10 response. However, it is unknown whether the stimulus is continually required or whether the inflammatory response could be initiated after a short incubation with elastase. To investigate this, explants were exposed to elastase for one hour only and then transferred to fresh media with no stimuli for up to 48hrs (n=6). As with other experiments, TNFα and IL-10 concentrations were determined via ELISA.

Figure 4.7a shows that for TNFα there is no difference in the response to either continuous stimulation or stimulation for just an hour. This is also the case for IL-10 with both conditions reaching maximum release at 24hrs (21.1±5.8pg/mg tissue with continuous stimulation compared to 25.5±7.2 with stimulation for an hour) and then dropping nearer to baseline at 48hrs.

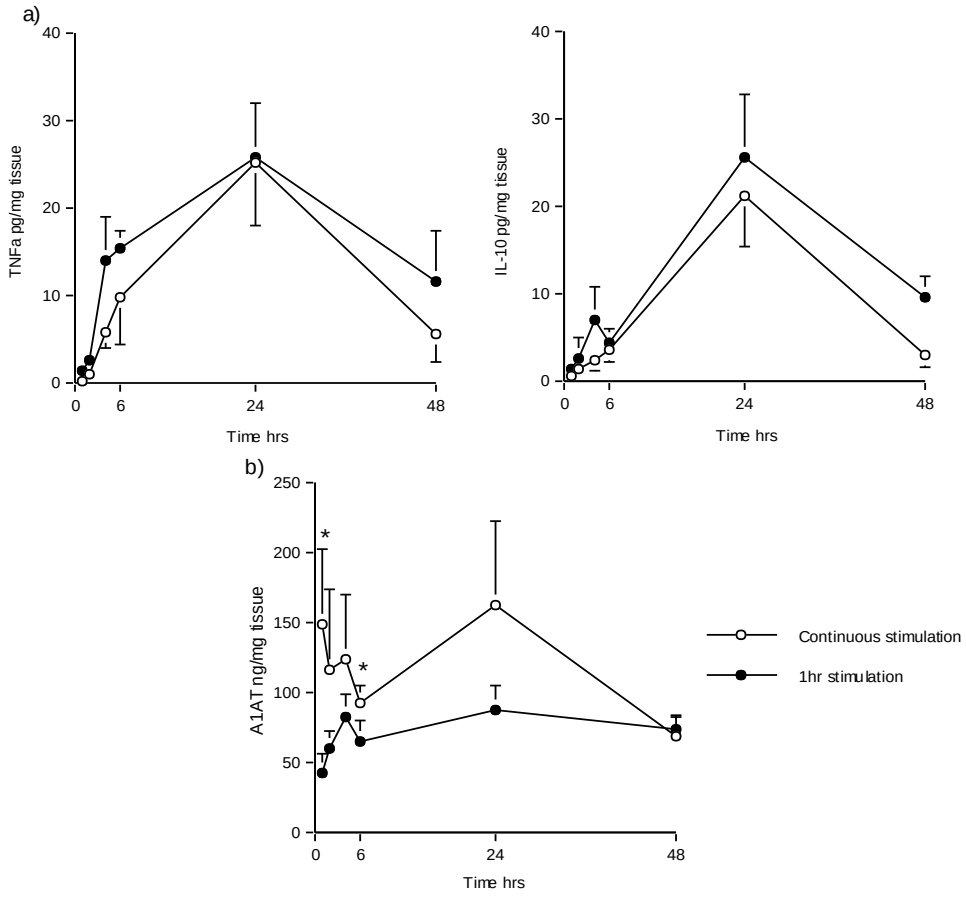

Figure 4.7: 1hr stimulation with elastase. Explants were stimulated either continuously for up to 48hrs or for 1hr only and then transferred to fresh buffer for up to 48hrs (n=6). a) shows the TNFα and IL-10 response and b) shows the A1AT response. Open circles are continuous stimulation and closed circles are 1hr stimulation. All values are mean±sem and are expressed as pg or ng/mg tissue. * denotes significance, $p<0.05$.

However, when supernatants were analysed for A1AT a different pattern is seen (figure 4.7b). When the explants are stimulated continuously for 48hrs, the concentration of A1AT peaks at 24hrs (162.2±60.2ng/mg tissue) whereas when the tissue is stimulated for a short amount of time there is a decrease in production. There are also significant differences between the two forms of stimulation at both the 1hr and 6hr time points ($p<0.05$). Despite these differences both sets of data have the same concentration of A1AT by 48hrs.

4.3.6 Cell death in response to elastase stimulation

Although we have provided evidence for the induction of an inflammatory response after stimulation with elastase it is important to clarify whether the responses seen are in fact due to elastase stimulation alone or whether cell death could contribute. In order to assess the level of cell death within the supernatants a lactate dehydrogenase (LDH) assay was used, n=8. LDH is an intracellular enzyme that is released into the supernatant when the cell undergoes necrosis and so therefore the greater amount of LDH present, the greater the number of dead cells present.

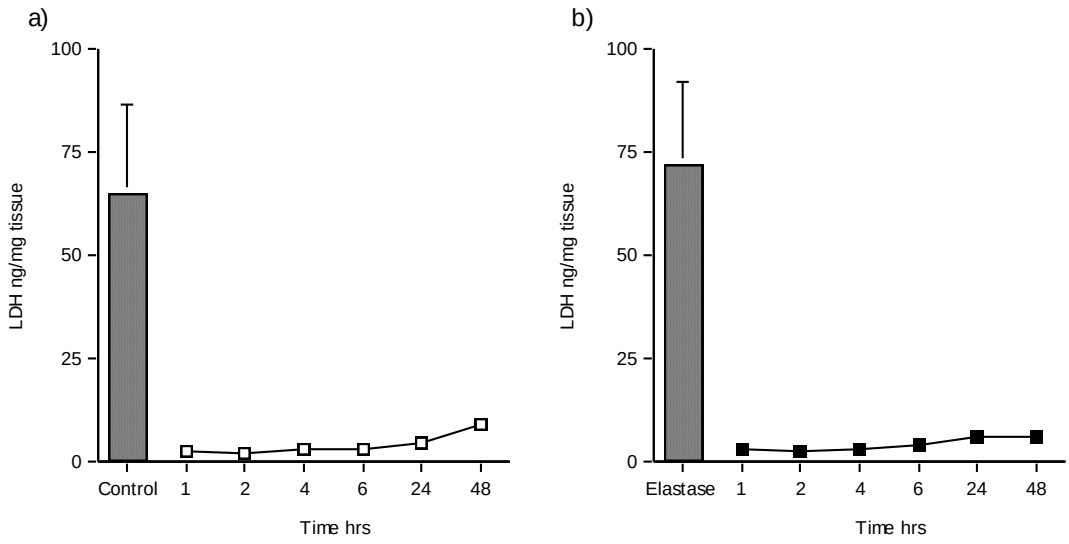

Figure 4.8: Cell death in response to elastase stimulation. a) shows levels of cell death for the control samples and b) shows cell death for the elastase stimulated samples (n=8). In both cases the bars represent the amount of LDH released from homogenised tissue and the lines are the amount of LDH within the supernatants after culture for varying time spans. Values are mean±sem and are expressed as ng/mg tissue.

Figure 4.8 shows the concentration of LDH present when control tissue was sonicated to simulate total cell death (64.7±21.8ng/mg tissue) and also the amount present within the supernatant over the 48hr incubation. It can be seen that the greatest release of LDH occurs at

48hrs but this is only 9.0±2.8 ng/mg tissue. When the same approach is taken to the elastase stimulated samples there is 71.6±20.3ng/mg tissue LDH released by the sonicated tissue compared to a maximum of 5.8±2.0 ng/mg tissue from the supernatant at 48hrs.

4.4 Discussion

This chapter aimed to determine whether the inflammatory response detected after stimulation with elastase is specific to this proteinase or whether it could be replicated either by proteinases from the same family or from another family. Figure 4.1 highlights that stimulation with trypsin or chymotrypsin, both serine proteinases, at a concentration equivalent to that used for elastase, does not cause an increase in either TNFα or IL-10. In comparison to this study, Lundberg *et al.,* (2000) have shown that trypsin stimulation of rat peritoneal macrophages can induce TNFα release both *in vitro* and *in vivo* using a model of acute pancreatitis. Systemic application of this proteinase resulted in pulmonary inflammation indicated by oedema, congestion of the lung tissue and infiltration of leukocytes into the pulmonary tissue as determined by histological means. Trypsin is also an endogenous activator of PAR-2 and as such many studies have shown that the activation of PAR-2 via this proteinase can induce the up-regulation of inflammatory cytokines such as IL-8 which has been found in respiratory epithelial cells as well as bronchial fibroblasts (Asokananthan *et al.,* 2002 and Page *et al.,* 2003). The evidence suggests that trypsin should be able to induce an inflammatory response from the parenchymal explants, however previous studies have concentrated on single cell lines and not *ex vivo* explants and so it is difficult to directly compare these systems. It is possible that due to the maintained matrix architecture of the explants trypsin cannot reach its target cells and so no inflammatory effects could be induced. Alternatively, endogenous elastase released via the respiratory burst of neutrophils could act upon PAR-2 to render it inactive in a similar way to its actions on PAR-1 where it cleaves a site downstream of the tethered ligand (Renesto *et al.,* 1997) or as with an elastolytic metalloproteinase released from *Pseudomonas aeruginosa* which cleaves the N-terminal

domain of PAR-2 on respiratory epithelial cells without causing downstream signalling, thus rendering the receptor redundant and unable to respond to trypsin stimulation (Dulon *et al.*, 2005).

This current study has shown that other serine proteinases are unable to induce an increase in either TNFα or IL-10 production and so it was interesting to investigate the actions of a non-serine proteinase. Figure 4.2 depicts the effects of papain, a cysteine proteinase, on the production of the aforementioned cytokines from the parenchymal explants. Papain is a non-human proteinase and thus should have no biological effect on the parenchymal explants apart from general unspecific proteolysis and as such if papain were to increase either of these cytokines this would suggest that the effects so far seen are not unique characteristics of elastase stimulation. General unspecific cleavage could possibly facilitate the release of TNFα and IL-10 from the extracellular matrix where they may be bound, but would not induce new synthesis of these mediators. McCluskey (1958) applied papain to the ears of rabbits and found that the proteinase can degrade cartilage and so it is possible that it may also be able to degrade other matrix proteins thus disrupting the integrity of the tissue and causing the release of cytokines. We have applied papain directly to the explants and measured the TNFα and IL-10 response up to 48hrs post stimulation; papain does not induce an increase in either mediator across all time-points and in fact the amount measured is consistently lower than that released by the control tissue, although not significantly so. This data suggests that the effects seen after elastase stimulation are in fact specific to the actions of this serine proteinase and not due to unspecific proteolysis.

Having determined that the inflammatory effects initiated by elastase are likely to be specific to that proteinase, we investigated whether these effects could be attenuated by the use of an elastase-specific inhibitor. Elastatinal binds to elastase forming a hemicetal adduct with the active site of elastase thus inhibiting its proteolytic actions (Williams *et al.*, 1987). Figure 4.3 shows the elastase activity after incubation with elastatinal as determined by the elastase activity assay as detailed in section 2.8. Elastatinal diminishes the activity of the proteinase in a dose dependent manner with 10 and 100µg/ml elastatinal causing a significant decrease. Having found that elastatinal significantly inhibits elastase it was important to determine whether this effect is selective for elastase. Figures 4.4b and 4.5b show the TNFα and IL-10 responses after stimulation with LPS that had been pre-incubated with elastatinal. Neither cytokine responses are adversely affected by the addition of elastatinal, thus suggesting that the inhibitor has no effect on the inflammatory properties of LPS. Turning our attention to the effects of elastatinal on the inflammation-inducing characteristics of elastase we can see that the TNFα response is significantly decreased by the use of 1 and 10µg/ml elastatinal (figure 4.4a) but not by the highest dose of the inhibitor. This could be due to the inhibitor itself having an effect on the TNFα response.

In a similar fashion to the TNFα response, elastatinal also caused a decrease in the IL-10 response albeit not a significant reduction. The lack of a significant decrease in IL-10 suggests that proteolytic activity from elastase may not be needed to induce the previously seen induction of this anti-inflammatory cytokine. However, considering elastase can degrade IL-10 via proteolysis as detailed in section 3.5 it is also possible that the insignificant decrease is due to IL-10 not being degraded after release by the elastatinal-inhibited elastase. On the

other hand, the significant reduction in the TNFα response suggests that for this cytokine to be released from the parenchymal explants elastase is required to be proteolytically active. It is also important to remember that the proteolytic actions of elastase may contribute to the overall inflammatory atmosphere via more than one route; elastase may activate cell membrane receptors such as PAR-2 (Uehara *et al.,* 2003) or TLR-4 (Hietaranta *et al.,* 2004) as well as generating protein fragments that may act as inflammatory mediators. As such, Senior *et al.* (1980) have shown that collagen fragments can act as chemoattractants for inflammatory cells such as neutrophils and monocytes and a similar action has been found for elastin fragments (Riley *et al.,* 1988). Therefore, if the elastolytic activity of the enzyme is decreased, degradation of the matrix is subsequently decreased, the presence of breakdown products is reduced and the inflammatory response may be tempered.

Having successfully decreased the elastase-induced inflammatory response by the use of an elastase-specific inhibitor it is rational to suggest that the clinical use of an elastase inhibitor would be advantageous in the treatment of COPD. Traditionally clinical trials with inhibitors of elastase have been largely unsuccessful; MR889 is a small molecule inhibitor of elastase and after four weeks of administration showed no change in plasma elastin-derived peptides or urinary desmosine (a marker of elastin degradation) (Luisetti *et al.,* 1996). However, the trial did not measure levels of inflammation and so other effects of elastase inhibition may have been missed. More recently there has been greater success with Sivelestat, a synthetic inhibitor of elastase, both in animal models (Yasui *et al.,* 1995) and in humans (Okayama *et al.,* 2006). These studies have used markers of inflammation as their read-outs thus supporting the role of elastase as an inflammatory stimulus. This suggests also that although

previous inhibitors, such as MR889, appeared to have no effect on the classical effects of elastase *in vivo* they may have had actions on the inflammatory properties of this proteinase; this is an area of research in need of wider study.

The previous chapter showed that elastase degrades cytokines in storage and so inhibition of this degradation was investigated. Supernatants were stored either with or without PIC and assayed for TNFα and IL-10 via ELISA. After storage in PIC, TNFα release was decreased significantly after 24hrs elastase stimulation and IL-10 release was decreased significantly from 4hrs. This effect is contrary to what would have been expected; if elastase degrades cytokines in storage then inhibiting this breakdown should increase the amount of cytokine detected by the ELISA system. It is possible that the expected result did not occur due to the interaction between the PIC and the released cytokines thus causing the proteins to be less detectable; PIC may either bind the cytokines causing the complex not to be recognised or any free PIC in the samples could directly affect the antibodies used in the ELISA thereby rendering them unable to bind IL-10. All subsequent experiments did not involve PIC due to this reason.

It has been discussed how elastase can induce the release and degradation of cytokines and other mediators and a balance between these properties must be met. So far, we have shown that the continual presence of elastase in the model causes an inflammatory response but it was unknown as to whether this inflammation could be triggered by a shorter stimulation with elastase. We hypothesised that a shorter stimulation with elastase could induce an inflammatory response and degradation of the cytokines would be limited, thus showing an

overall increase in cytokine production. Tissue was stimulated with elastase for one hour only before being transferred to fresh media with no stimulus for up to 48hrs. For both TNFα and IL-10 the responses after continuous stimulation and one hour exposure are similar with no significant differences. However, the decrease in both TNFα and IL-10 at the 48hr time-point is greater with the tissue stimulated for one hour only compared to that constantly in the presence of elastase. Conversely, when investigating the concentration of A1AT after one hour stimulation with elastase it can be seen that no peaks of production occur, unlike with the cytokines previously mentioned. However, when the tissue is continually stimulated with elastase a peak production of A1AT occurs at 24hrs, but decreases again by 48hrs in line with the amount of A1AT produced by the short stimulation. These data suggest that for the cytokines only a short burst of an inflammatory stimulus is required to initiate a response however, for the anti-proteinase a continual stimulus is required. This also shows that any degradation of the cytokines by elastase must be occurring within the first hour of stimulation or that later degradation is caused by the consequential release of other proteolytic agents such as endogenous elastase from neutrophils or tryptase from mast cells. This has implications for the pathogenesis of COPD in that intervention against the proteolytic and inflammatory effects of elastase must occur at an early stage in the progression of the disease for any advantageous effect to be seen, which would prove difficult due to poor early diagnosis of COPD (Bednarek *et al.,* 2008).

In summary, this chapter has shown that the inflammatory effects induced by elastase stimulation of parenchymal explants are likely to be specific to this proteinase and that by inhibiting the proteolytic properties of elastase these effects can be diminished. This gives

some promise to the use of inhibitors of elastase in the clinical setting, although this is an area in need of further investigation. We have also shown that only a short burst of elastase stimulation is required to initiate an inflammatory response equivalent to that seen with the continual presence of the proteinase, thus highlighting the importance of early intervention in the disease progression of COPD and also the potency of the effects of elastase itself.

Chapter 5: Elastase, receptors and inflammation

5.1 Introduction

This study has so far shown that elastase can initiate an inflammatory response in human lung parenchymal explants and that this response can be attenuated by elastatinal, a specific elastase inhibitor. However, it is unclear exactly how this proteinase brings about this inflammatory effect. We propose that elastase may act through a membrane receptor to initiate downstream signalling for an inflammatory response. Possible candidates that could mediate this response are proteinase-activated receptor (PAR)-2 and Toll-like receptor (TLR)-4.

PARs are a family of four G-protein coupled receptors consisting of seven transmembrane domains and of the four known PARs it is PAR-1 and PAR-2 that are found in the lung. However, all four receptors are activated via the same mechanism; cleavage of specific peptide bonds in the amino terminus of the receptor reveals a new tethered ligand which can act back on itself to initiate intramolecular binding (reviewed in Macfarlane *et al.*, 2001), resulting in the initiation of downstream signalling effects such as the up-regulation of cytokine production (Zhang *et al.*, 2008, Ostrowska *et al.*, 2007). In the *in vivo* environment, it is thrombin and trypsin that preferentially cleave the amino terminals of PAR-1 (Vu *et al.*, 1991) and PAR-2 (Nystedt *et al.*, 1994), respectively. However, it is possible to simulate the activation of these receptors *in vitro* via the use of synthetic activating peptides: TFRIFD-NH_2 for PAR-1 and SLIGKVD-NH_2 for PAR-2 (Al-Ani *et al.*, 1999). These synthetic ligands allow the effects of receptor activation to be investigated without the added influence of proteolysis by endogenous activators, therefore providing a cleaner experimental system.

In the lung, PARs have been localised to several cell types including bronchial smooth muscle cells, epithelial cells (D'Andrea *et al.*, 1998) and macrophages (Roche *et al.*, 2003) and there is some evidence to suggest that PAR-2 expression is increased in the bronchial vessels, smooth muscle and glands of patients with bronchitis alone compared to those with COPD (Miotto *et al.*, 2002). Roche and colleagues (2003) have also shown that PAR-1 mRNA and protein is increased in alveolar macrophages from smokers compared to non-smokers, whereas PAR-2 mRNA is decreased with no change to protein expression. As smoking is indicated as a major risk factor for the development of COPD it is possible that these receptors also play a role in mediating the associated inflammation. In support of this idea, Asokananthan *et al.* (2002) have shown an increase in the production of the pro-inflammatory mediators IL-6, IL-8 and prostaglandin E_2 by human bronchial epithelial cells after activation of PAR-1 and/or PAR-2. However, in contrast, Miotto *et al.* (2002) showed no difference in receptor expression in the central airways of smokers compared to non-smokers thus suggesting that this area requires greater investigation in order for a definitive relationship between smoking and PAR expression to be determined.

Having established in the previous chapters that elastase can induce an inflammatory response, and considering the literature suggests that PARs may also play a role in inflammation, it is important to assess the current knowledge on the relationship between elastase and the receptors. It has been shown by Dulon *et al.* (2005) that elastase cleaves the extracellular domain of PAR-2 downstream of the site cleaved by trypsin, its endogenous activator, thus inactivating the receptor. However, Suzuki *et al*. (2005) have demonstrated that activation of

PAR-1 by elastase contributes to apoptosis of distal lung epithelial cells, therefore possibly contributing to the emphysematous changes seen in the COPD lung.

We primarily suggest that elastase acts via PAR-2 but it is important to recognise that the inflammatory response induced by elastase shows some similar features to that induced by LPS which mediates its inflammatory effects through TLR-4 (Su *et al.,* 2000, Faure *et al.*, 2000 and Baumgarten *et al.*, 2001). Therefore, it is possible that elastase may also act through this receptor to initiate an inflammatory response. In support of this hypothesis, it has been shown by Hieteranta and colleagues (2004) that pancreatic elastase can induce a pro-inflammatory response via TLR-4 and Tokairin *et al.,* (2008) have shown that elastase-induced emphysema in mice results in the upregulation of TLR-4 on alveolar macrophages.

It is clear that the role of elastase as an inflammatory mediator is yet to be fully understood, especially in relation to which receptor(s) it may work through. This chapter aims to investigate the relationship between elastase and the proposed receptors (PAR-2 and TLR-4) as well as the effect that disease status has on the expression of receptor protein in lung parenchyma.

5.2 Methods

Human lung tissue was obtained, with written informed consent, from patients undergoing carcinoma resection at Guy's Hospital London or either carcinoma resection or bullectomy at Southampton General Hospital; patient characteristics can be found in table 5.1 below. Explants were fixed for GMA processing and immunohistochemistry was used to detect the PAR-2 and TLR-4 as described in section 2.11. Relative amounts of PAR-2 and TLR-4 were quantified by dot blot and densitometry (sections 2.12 and 2.13 respectively).

Age	62.6±2.34
Gender	18M 10F
FEV_1/FVC	0.70±0.02
Smoking status	13 current, 11 ex, 3 non, 1 unknown

Table 5.1a: Patient characteristics for tissue from Guy's Hospital, London. Age and FEV_1/FVC are shown as mean ± sem. For gender, M=male and F=female. Under smoking status, current denotes current smokers, ex denotes ex-smokers and non denotes non-smokers as determined by the patient's clinician.

Age	61.6±3.9
Gender	9M 7F
FEV_1/FVC	0.65±0.06
Smoking status	6 current, 8 ex, 2 non

Table 5.1b: Patient characteristics for tissue from Southampton General Hospital-carcinoma resection. Age and FEV_1/FVC are shown as mean ± sem. For gender, M=male and F=female. Under smoking status, current denotes current smokers and ex denotes ex-smokers as determined by the patient's clinician.

Age	48.2±9.6
Gender	5M 1F
Smoking status	4 current, 1 ex, 1 non

Table 5.1c: Patient characteristics for tissue from Southampton General Hospital-bullectomy. For gender, M=male and F=female. Under smoking status, current denotes current smokers and ex denotes ex-smokers as determined by the patient's clinician. These patients did not undergo lung function tests.

The effect of a PAR-2 activating peptide (SLIGKVD-NH_2) was also investigated using the explant model (section 2.2). Explants were incubated for 24hrs with the synthetic peptide before the tissue was recovered, weighed and stored at -70°C and the supernatant aliquotted and also stored at the same temperature. Supernatants were then analysed for cytokine production via ELISA.

5.3 Results

5.3.1 Localisation of PAR-2 in lung parenchyma by immunohistochemistry

Many of the cellular locations of PAR-2 have been well documented (D'Andrea *et al.*, 1998) but it was necessary to determine whether this receptor is present within the lung parenchyma used in this study. Explants were fixed and stained via GMA immunohistochemistry for PAR-2 and figure 5.1 shows PAR-2 is clearly present within these explants. Staining appears to be localised to the cell membrane which is as expected for PAR-2 considering it is a transmembranous receptor. The morphology of the cells to which PAR-2 staining has been found is consistent with inflammatory cells such as neutrophils and macrophages. This concurs with previous studies by Roche *et al.* (2002) and Howells *et al.* (1997).

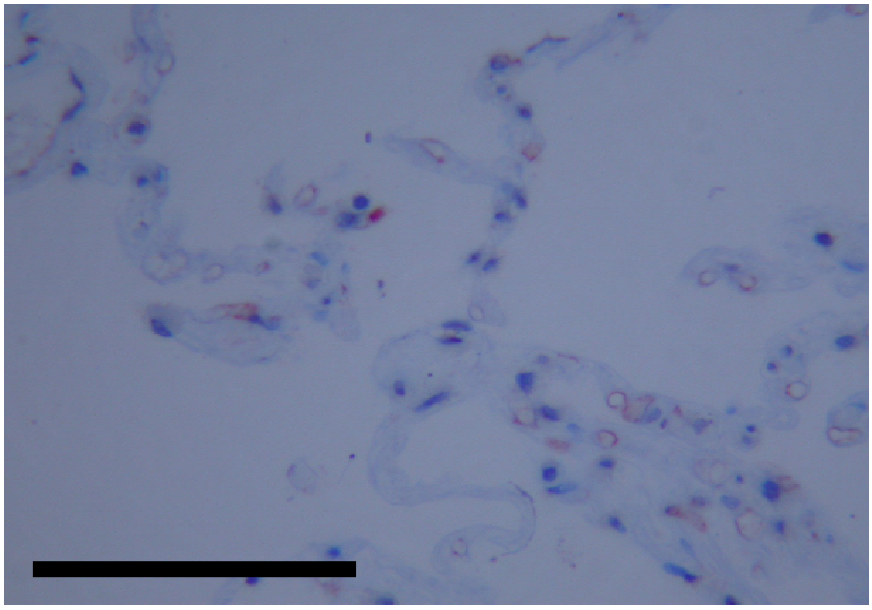

Figure 5.1: PAR-2 staining in parenchymal tissue. GMA immunohistochemistry was used to identify PAR-2 within the parenchymal explants. PAR-2 is stained in red. Isotype control antibody showed no staining (not shown). Scale bar represents 100µm. Immunohistochemistry courtesy of Dr. Jane Warner.

5.3.2 PAR-2 detection by dot blot

Having shown that PAR-2 can be detected by immunohistochemistry we went on to investigate the expression of this receptor by dot blot. Unstimulated parenchymal explants were sonicated and the resulting supernatants were loaded onto nitrocellulose, blocked overnight with 5% BSA in 0.1% PBS Tween and PAR-2 was detected using a rabbit polyclonal anti-human PAR-2 antibody. The use of an isotype control antibody showed no detection on the dot blot.

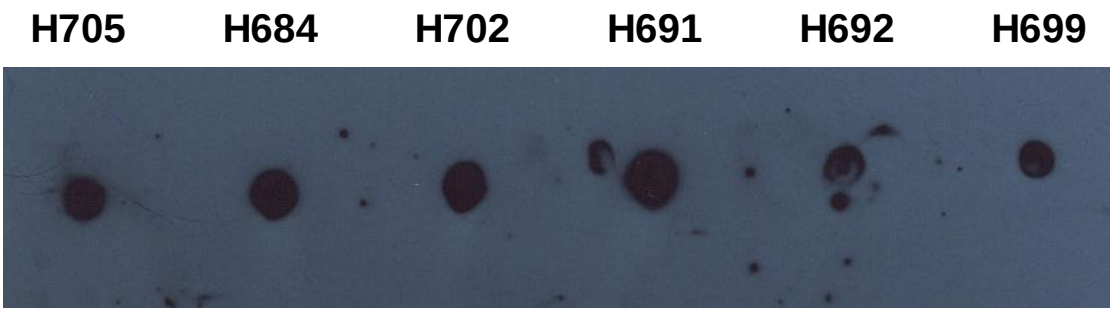

Figure 5.2: PAR-2 detection by dot blot. Unstimulated parenchyma was sonicated and the supernatant run on dot blots for PAR-2. Numbers above the dots refer to the patient identity numbers.

5.3.3 PAR-2 expression and disease status

Knowing that PAR-2 is indeed present in the parenchyma, as determined by the aforementioned techniques, we went on to investigate the effect disease status has on the expression of PAR-2. Tissue from 11 subjects with no evidence of COPD (GOLD 0) and 12 subjects with mild to moderate COPD (GOLD I & II), as determined by lung function data in line with criteria set down by GOLD (2005), was sonicated and dot blots performed. All samples were corrected for protein content and a rabbit anti-human PAR-2 antibody was used

to detect this receptor. Densitometry on the blots was used to establish the relative amount of PAR-2 in the samples.

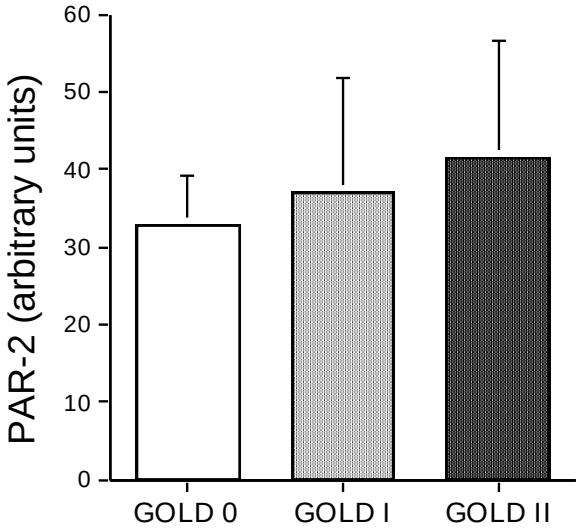

Figure 5.3: PAR-2 in different stages of disease. The amount of PAR-2 in sonicated tissue was determined by dot blot and densitometry. The clear bar denotes those with no disease (GOLD 0), the light grey bar shows those with mild disease (GOLD I) and the dark grey bar shows those with moderate disease (GOLD II). n=11, 5 and 6 for GOLD 0, I and II respectively. Values are mean±sem and are expressed in arbitrary units.

Figure 5.3 shows that there is a trend, although not statistically significant, for increased amounts of PAR-2 in parenchyma from those subjects with mild to moderate COPD compared to those that show no evidence of the disease (GOLD 0: 33.0±6.1 arbitrary units, GOLD I: 37.1±14.6 arbitrary units and GOLD II: 41.6±15.0 arbitrary units).

5.3.4 Elastase stimulation and PAR-2 expression

Having detected a trend for increased levels of PAR-2 in those with evidence of mild to moderate COPD we investigated the effect of elastase stimulation on the amount of PAR-2 in the parenchyma. Explants from 9 subjects with a range of disease severities were either

stimulated with elastase (100µg/ml) or incubated in media alone for 24hrs. After recovery, tissue was homogenised and dot blots for PAR-2 were performed.

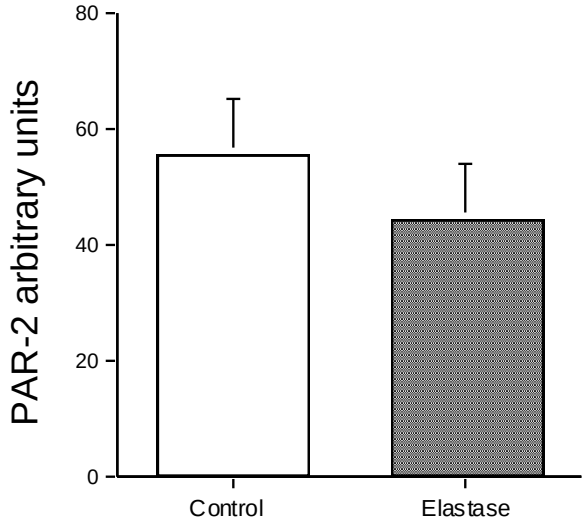

Figure 5.4: PAR-2 expression after elastase stimulation. Explants, n=9, were stimulated with elastase (filled bar) for 24hrs or left unstimulated (clear bar) for the same length of time. Relative amounts of PAR-2 were determined by dot blot and densitometry. All values are mean±sem and are expressed as arbitrary units.

It can be seen in figure 5.4 that there is a trend for parenchyma stimulated with elastase to show a lower amount of PAR-2 when assessed by dot blot compared to unstimulated tissue (41.3±9.0 arbitrary units compared to 54.3±8.7 arbitrary units respectively). As COPD, and in particular emphysema, is associated with an increase in elastase it is surprising that those with greater levels of disease tend to have higher amounts of PAR-2 (figure 5.3) considering we have shown that the addition of elastase appears to decrease the presence of PAR-2 (figure 5.4). It must be taken into consideration, however, that this data includes subjects with a range of disease severity from those with no disease (GOLD 0) to those with moderate disease (GOLD II) and therefore it is important to reassess the data taking the disease status into consideration as shown in figure 5.5.

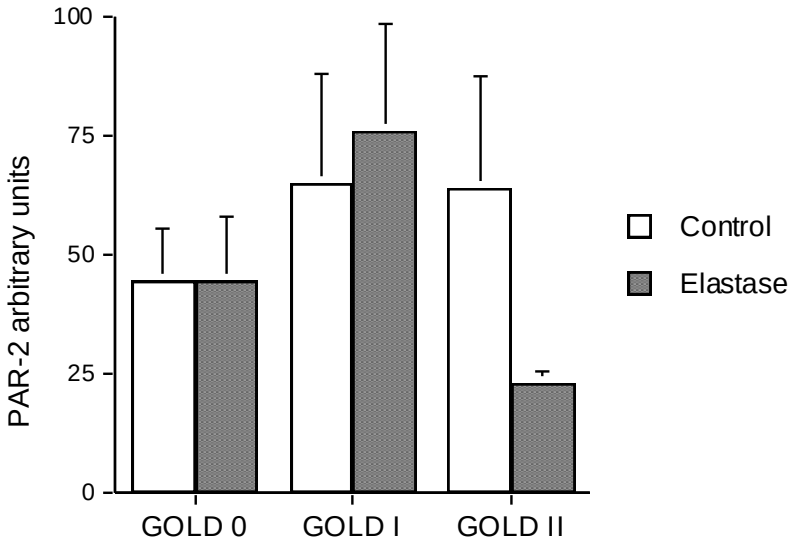

Figure 5.5: PAR-2 expression after elastase stimulation-split by GOLD status. Explants from patients with no disease, GOLD 0 (n=4), mild disease, GOLD I (n=2) and moderate disease, GOLD II (n=3) were stimulated with 100µg/ml elastase for 24hrs before being homogenised. PAR-2 presence was determined via dot blot and assessed by densitometry. All values are the mean±sem and are expressed in arbitrary units.

In figure 5.5 we can see that in those patients with no evidence of airways obstruction (GOLD 0) elastase stimulation has no effect on the amount of PAR-2 present in the parenchyma. However, although both the GOLD I and GOLD II patient groups have similar amounts of PAR-2 in the control samples elastase stimulation causes two distinct effects; PAR-2 is increased after elastase stimulation in those with mild airways obstruction (GOLD I) (64.6±23.3 arbitrary units for the control samples compared to 75.9±22.4 arbitrary units for the elastase stimulated samples) whereas in those with moderate disease (GOLD II) elastase stimulation causes a substantial decrease in PAR-2 presence within the parenchyma (63.8±23.3 arbitrary units for the control samples compared to 22.9±2.4 for the elastase stimulated samples). Even though this trend is interesting it is not statistically significant, although this may be due to the groups being underpowered with low n values.

5.3.5 Activation of PAR-2 via a synthetic activating peptide: SLIGKVD-NH_2

It has been shown previously that PAR-2 can be activated by a synthetic activating peptide, SLIGKVD-NH_2 (Al-Ani *et al.*, 1999). We have stimulated parenchymal explants with this peptide to assess the cytokine profile induced by PAR-2 activation; a similar profile to that shown by elastase stimulation would suggest that elastase activates PAR-2. Explants were stimulated with the synthetic PAR-2 activating peptide SLIGKVD-NH_2, the scrambled PAR-2 peptide as a control or 100µg/ml elastase for 24hrs before tissue and supernatant were recovered and stored at -70°C until required for analysis via ELISA.

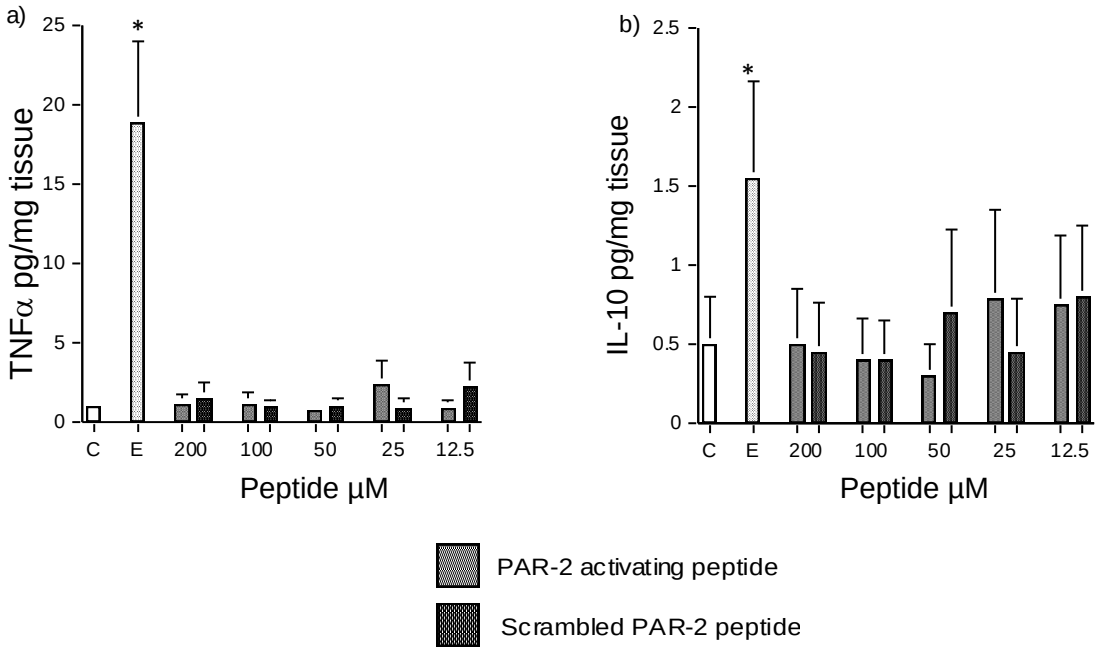

Figure 5.6: TNFα and IL-10 response after stimulation with SLIGKVD-NH_2. Parenchymal explants (n=8) were incubated for 24hrs with various concentrations of the synthetic PAR-2 activating peptide SLIGKVD-NH_2, a scrambled version of this peptide, 100µg/ml elastase or media alone. Tissue was harvested, weighed and stored along with supernatants at -70°C until analysed via ELISA for TNFα (a) and IL-10 (b). C denotes control and E denotes elastase stimulation. * denotes $P<0.05$ when compared to control.

Figure 5.6a shows the TNFα release in response to stimulation with elastase, PAR-2 activating peptide and the scrambled peptide. As seen previously, elastase causes a significant release of

this inflammatory cytokine compared to control. However, in contrast to this, the PAR-2 activating peptide failed to cause any increase in TNFα with levels remaining close to the control levels as well as those for the scrambled peptide for all concentrations used. A similar scenario is also seen with the IL-10 response to PAR-2 activating peptide stimulation (figure 5.6b). Again, elastase produces a robust IL-10 response but the peptide does not alter the amount of IL-10 compared to control levels.

Although this study predominantly focuses on the TNFα and IL-10 responses from the parenchyma, we have also shown that elastase can alter the release of other inflammatory cytokines such as IL-6 and IL-8 albeit with great inter-patient variability. With this in mind, the effects of the activating peptide on IL-6 and IL-8 release from parenchyma were assessed (figure 5.7). It has been previously stated in table 3.2 that elastase has a variable effect on IL-8 release, even though patient groups are matched as far as possible with regards to clinical and demographic characteristics; the subgroup of patients used for the current experiments show a high basal level of IL-8 which decreases after stimulation with elastase. In contrast to the data for TNFα and IL-10, the PAR-2 activating peptide does appear to have some effect on the release of IL-8 especially at a dose of 50μM although this is not statistically significant. However, this effect is not reproduced with the other doses and there is a great level of variability across all data in this figure.

Similarly, when analysing the data for IL-6 release in response to stimulation with the PAR-2 activating peptide (figure 5.7b) there is high constitutive release of the cytokine with a decrease after elastase stimulation. Also, as with IL-8, there is a slight increase, though not

significant, with 50µM PAR-2 activating peptide. However there is little difference in the amount of IL-6 released after stimulation with the other concentrations of the peptide. In addition, the amount of IL-6 released after stimulation with the PAR-2 activating peptide is not dissimilar to the concentration measured after stimulation with the scrambled peptide.

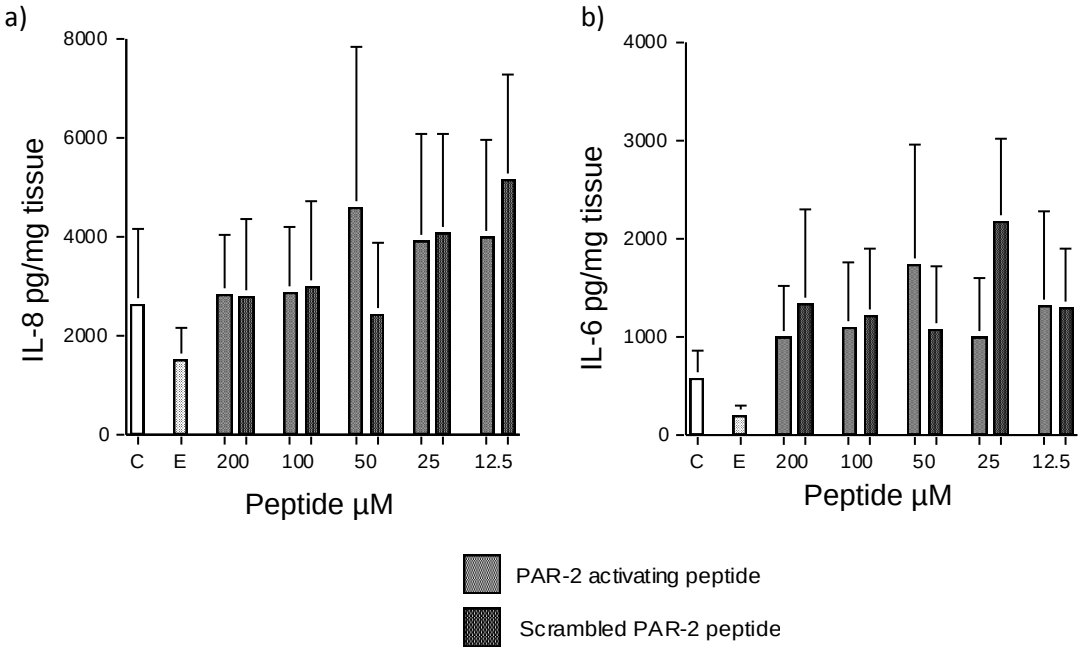

Figure 5.7: IL-8 and IL-6 response after stimulation with SLIGKVD-NH_2. Parenchymal explants (n=5) were incubated for 24hrs with various concentrations of the synthetic PAR-2 activating peptide SLIGKVD-NH_2, a scrambled version of this peptide, 100µg/ml elastase or media alone. Tissue was harvested, weighed and stored along with supernatants at -70°C until analysed via ELISA for IL-8 (a) and IL-6 (b). C denotes control and E denotes elastase stimulation.

COPD is traditionally associated with an inflammatory cytokine response and this study has shown that elastase is capable of causing a significant increase in pro-inflammatory cytokines. However, we have also shown that elastase can increase the production of cytokines that are usually linked with more of an allergic phenotype, namely IL-5 and IL-13 (figure 3.6c). Although this data is replicated for IL-5 in the current sub-set of patients, elastase failed to cause the release of any IL-13 (figure 5.8) and it is likely that this is due to the high level of

inter-patient variability continually seen with the subjects used in this study. Conversely, for both IL-5 and IL-13 the PAR-2 activating peptide induces an increase when used at concentrations in the range of 25-200µM (figure 5.8). However, the scrambled peptide also follows the same pattern suggesting that this version of the peptide is still able to bind to the receptor to cause activation. This could be addressed by using a peptide still with the same amino acid composition as PAR-2 but with much less sequence homology.

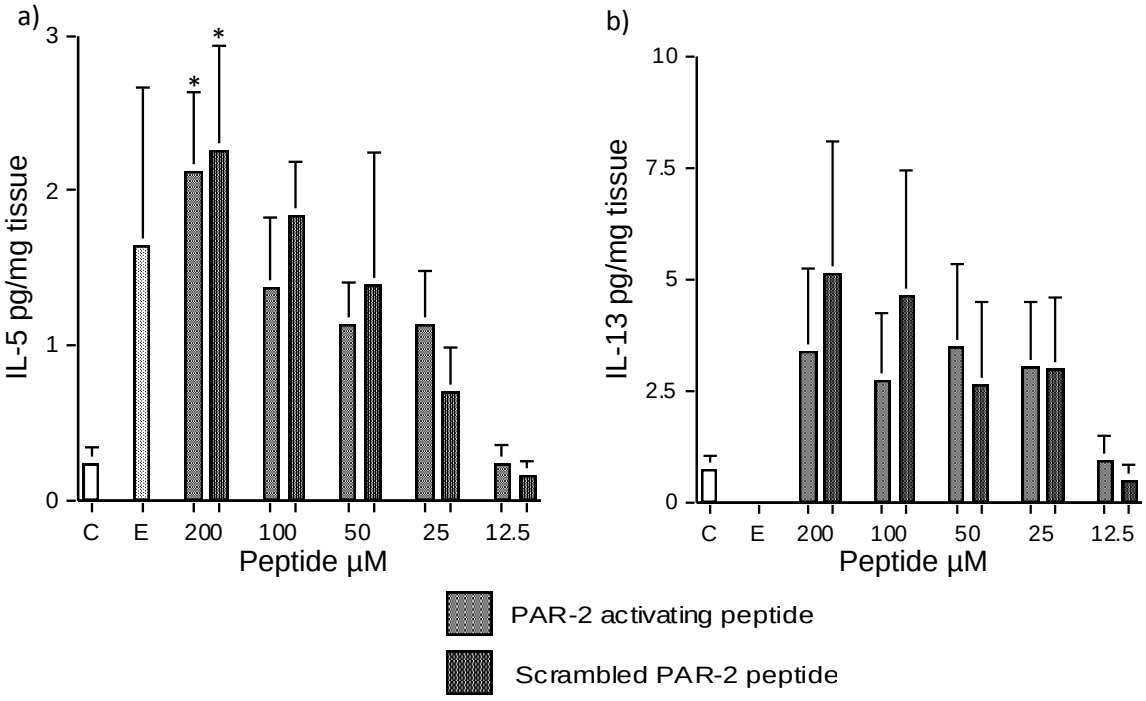

Figure 5.8: IL-5 and IL-13 response after stimulation with SLIGKVD-NH_2. Parenchymal explants (n=5) were incubated for 24hrs with various concentrations of the synthetic PAR-2 activating peptide SLIGKVD-NH_2, a scrambled version of this peptide, 100µg/ml elastase or media alone. Tissue was harvested, weighed and stored along with supernatants at -70°C until analysed via ELISA for IL-5 (a) and IL-13 (b). C denotes control and E denotes elastase stimulation. * denotes $P<0.05$ when compared to control.

When comparing the effect of elastase stimulation with the PAR-2 activating peptide on the release of IL-5 it can be seen that the proteinase, at a concentration of 100µg/ml, has a similar effect to 100µM of the peptide (figure 5.8a). After this point the peptide causes a dose-dependent decrease in the concentration of IL-5 detected in the supernatant. In comparison,

IL-13 is increased with the addition of the activating peptide but the response appears to be less dependent on the concentration of the peptide, until it is decreased to 12.5μM where the IL-13 response is comparable to the control (0.92±0.6 pg/mg tissue after stimulation with the peptide compared to 0.71±0.3 pg/mg tissue for the control samples). However, as with the other cytokines measured, there is an IL-5 and IL-13 response generated by the scrambled peptide as well as the activating peptide. This further highlights the need for a more appropriate control sequence.

Having shown no similarities between the effects of stimulation with elastase and the PAR-2 activating peptide on the release of any of the pro-inflammatory cytokines measured (TNFα, IL-10, IL-8 and IL-6) it can be surmised that elastase may not be acting via PAR-2 in order to elicit its inflammatory effects, but in fact may utilise another receptor(s). However, when considering the data for IL-5 in this group of patients and IL-13 for previous groups of patients it is fair to say that elastase and the PAR-2 activating peptide can induce similar effects and therefore it is possible that both of these stimuli may cause their effects via the same route i.e. the activation of PAR-2 either by cleaving the tethered ligand or by assuming the role of such a ligand.

5.3.6 Elastase and TLR-4

The previous section has alluded to the idea that elastase may cause its effects via more than one route; PAR-2 for the induction of Th2 type cytokines and an unknown route for the initiation of the inflammatory response. Considering the similarities between the elastase-induced and LPS-induced inflammatory responses as shown in chapter 3 it is possible that

elastase may act through the LPS receptor, TLR-4. There is also some evidence in the literature that pancreatic elastase can activate TLR-4 in human myeloid cells to initiate an inflammatory response associated with severe acute pancreatitis (Hietaranta *et al*., 2004), thus supporting the idea that the same route may occur within the parenchyma and COPD.

5.3.7 Localisation of TLR-4 in lung parenchyma by immunohistochemistry

As with PAR-2 in section 5.3.1, it was necessary to determine whether TLR-4 is present within the lung tissue and so immunohistochemistry was utilised to assess this (figure 5.9).

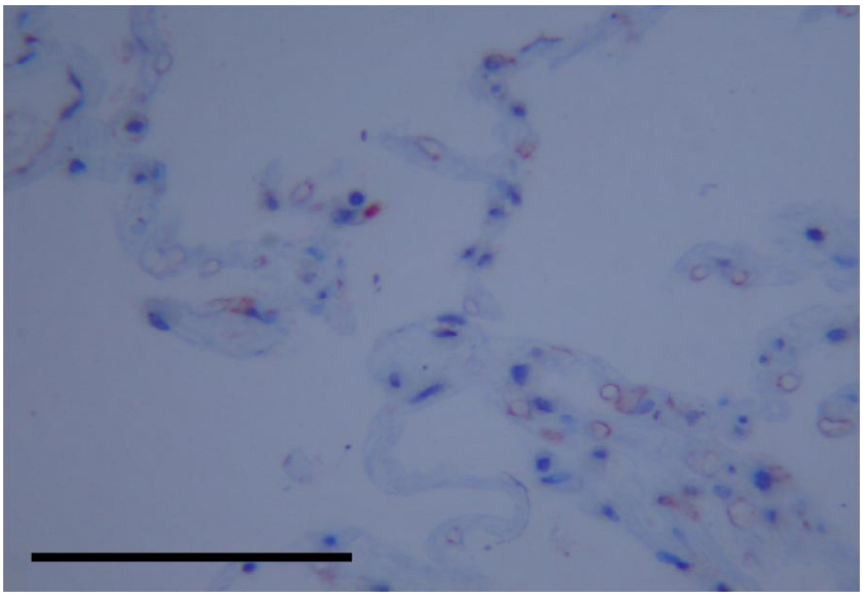

Figure 5.9: TLR-4 staining in parenchymal tissue. GMA immunohistochemistry was used to identify TLR-4 within the parenchymal explants. TLR-4 is stained in red. The use of an isotype control showed no staining. Scale bar represents 100µm. Immunohistochemistry courtesy of Dr. Jane Warner.

5.3.8 TLR-4 detection by dot blots

Dot blots were used to assess the relative amounts of TLR-4 in parenchyma that had been stimulated with 100µg/ml elastase or media alone for 24hrs. Tissue was sonicated as detailed in section 2.6 and dot blots performed for TLR-4 as in section 2.12 and 2.13. A rabbit polyclonal anti-human TLR-4 antibody was used at a concentration of 0.5µg/ml to detect the

receptor and samples were corrected for protein concentration with 10µg of protein loaded for each. As with the PAR-2 dot blot the use of an isotype control antibody showed no detection on the TLR-4 dot blot.

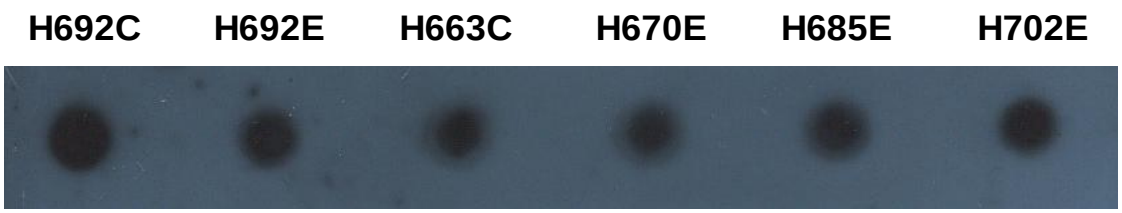

Figure 5.10: Detection of TLR-4 by dot blot. Parenchymal explants were sonicated and the resultant supernatants (10µg protein) loaded onto nitrocellulose in order for dot blots to be performed. Numbers above the dots refer to the patient identity numbers; C denotes control samples and E denotes samples stimulated with 100µg/ml elastase for 24hrs.

5.3.9 Elastase and TLR-4 expression in lung parenchyma

Having shown by immunohistochemistry and dot blots that TLR-4 is present in the parenchyma, we went on to investigate the effect of elastase stimulation of the explants on the relative amounts of TLR-4 found in the tissue. Figure 5.11 shows that elastase has no effect, compared to the control samples, on the amount of this receptor found in the lung tissue (52.7±10.6 arbitrary units for control samples compared to 53.4±9.6 arbitrary units for elastase-stimulated samples). Although this data shows that elastase does not change the amount of TLR-4 in the tissue it cannot be ruled out that elastase may activate the receptor.

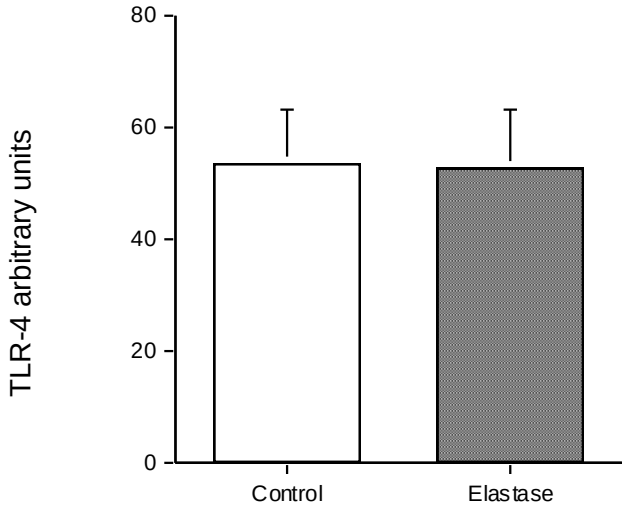

Figure 5.11: TLR-4 expression after elastase stimulation. Explants, n=9, were stimulated with elastase (filled bar) for 24hrs or left unstimulated (clear bar) for the same length of time. Relative amounts of TLR-4 were determined by dot blot. All values are mean±sem and are expressed as arbitrary units.

5.3.10 TLR-4 expression and disease severity

Although we have found that elastase does not cause any change in the expression of TLR-4, it cannot be dismissed that this proteinase may still act through this receptor and as sputum concentrations of elastase are increased as COPD becomes more severe (Bizeto *et al.*, 2008) we wanted to investigate the effect of disease severity on the expression of TLR-4.

It can be seen in figure 5.12 that patients with mild to moderate airways obstruction (GOLD I and II) have an increased amount of TLR-4 in the parenchyma compared to those with no evidence of disease (GOLD 0); 73.4±16.2 arbitrary units for GOLD I and II compared to 34.9±12.1 arbitrary units for GOLD 0. At present this trend is not statistically significant but this is likely to be due to a low number of subjects in each group and therefore with greater power it is possible that it may in fact become significant.

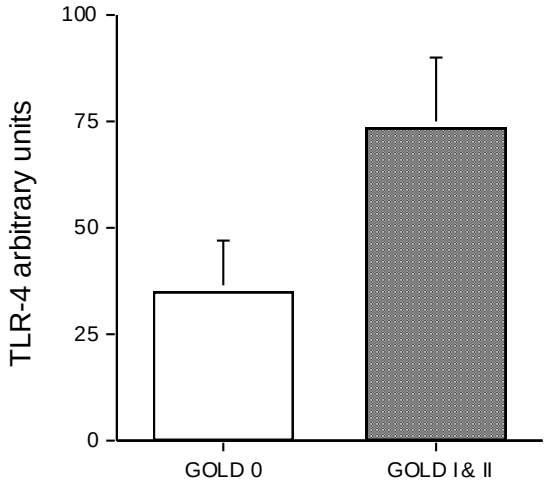

Figure 5.12: TLR-4 expression and GOLD status. Unstimulated explants from patients with no evidence of airways obstruction (GOLD 0, n=4) and mild to moderate airways obstruction (GOLD I & II, n=5) were sonicated and the resultant supernatants probed for TLR-4 via dot blots. The presence of TLR-4 is expressed as mean±sem and are arbitrary units.

5.4 Discussion

This chapter has sought to investigate possible mechanisms for how elastase may elicit its inflammatory effects. It is well known that PAR-2 is activated by serine proteinases such as trypsin and mast cell tryptase (Corvera *et al.*, 1997, Molino *et al.*, 1997 and Bolton *et al.*, 2003) and so it is logical to assume that elastase may also be able to act through this receptor. There is also evidence to suggest that neutrophil elastase can activate PAR-1 and by doing so initiate apoptosis in lung epithelial cells (Suzuki *et al.*, 2005). It is also possible for proteinases to be able to activate more than one member of the PAR family with thrombin activating PAR-1 (Chambers *et al.*, 1998), PAR-3 (Ostrowska and Reiser, 2008) and PAR-4 (Xu *et al.*, 1998), therefore elastase could activate PAR-2 as well as PAR-1 in the pulmonary system.

In order to investigate the possible relationship between elastase and PAR-2, this study first determined the presence of PAR-2 within the parenchymal explants via immunohistochemistry (figure 5.1). From this, it is suggested that PAR-2 is localised to inflammatory cells such as neutrophils and macrophages and this is supported by several studies (Howells *et al*. (1997) and Roche *et al*. (2003)). This localisation also fits with the idea that PAR-2 is involved in inflammation within the lung and its close proximity to the cellular sources of elastase suggests that, logistically, this proteinase could activate the receptor.

Having established the presence of PAR-2 in the parenchyma this study aimed to investigate the relationship between PAR-2 expression and the severity of airways obstruction demonstrated by the subjects as determined by their lung function values.

Figure 5.3 shows the amount of PAR-2 detected in the parenchyma of 22 subjects via dot blot, split by COPD severity as detailed by the GOLD guidelines (2005). It can be seen that although there is no significant differences between the amount of PAR-2 in the parenchyma and disease severity there is indeed a trend for there to be increased amounts of the receptor in those with more severe disease. It is possible that the effect of disease severity on the expression of PAR-2 is not that clear cut in this case due to the fact that those classed as having GOLD II COPD are predominantly at the less severe end of the spectrum, and those defined as having no COPD have lung functions close to the GOLD I cut-off value of FEV_1/FVC of 0.7 (GOLD, 2005). Hence, if a wider spread of disease were to be analysed then there may be a more substantial difference in the amount of PAR-2 detected in the parenchyma. PAR-2 expression has been linked with disease progression in other systems such as atherosclerosis where increased levels of PAR-2 were detected in atherosclerotic lesions via immunohistochemistry as vascular inflammation also increased (Napoli *et al.*, 2004). This study ultimately showed that PAR-2 was increased significantly in class III disease compared to class I or II. Conversely, there was no difference with class IV disease and the authors surmise that this is due to other inflammatory mechanisms playing a role when the disease reaches this level of severity. Chin *et al.* (2008) have also shown that PAR-2 expression is increased in colonic mucosa from patients with Crohn's disease compared to that from patients with non-inflammatory bowel disease and it is well documented that PAR-2

expression is greater in rheumatoid arthritis compared to other forms of the disease. (Kelso *et al.*, 2007, Nakano *et al.*, 2007 and Busso *et al.*, 2007).

Having established that PAR-2 is increased in those with more severe disease the study went on to investigate the effect of elastase stimulation of the parenchymal explants on PAR-2 expression in this tissue (figures 5.4 and 5.5). When divided by disease status it is clear there are differences in the response to elastase stimulation. Those with no airways obstruction show no difference in PAR-2 expression after elastase stimulation, whereas although both the GOLD I and II groups have a similar level of PAR-2 in the control samples, this is increased slightly in the GOLD I group but decreased quite substantially in the GOLD II group after stimulation with elastase. This suggests that the involvement of elastase and PAR-2 in the pathogenesis of COPD is more complex than originally thought and it is possible that other mechanisms are involved in this system once the patient develops a moderate level of disease. This area clearly needs further investigation to establish the mechanisms involved in this phenomenon.

Although there is a trend between the amount of PAR-2 in the parenchyma and elastase stimulation, further investigation was required to establish whether elastase actually activates this receptor. A PAR-2 activating peptide SLIGKVD-NH_2 was utilised as a comparison for stimulation with elastase with the rationale that should this peptide initiate an inflammatory response similar to that found with elastase then it is likely that elastase activates PAR-2. There is some evidence to suggest that this peptide is capable of producing an inflammatory response in the form of TNFα release from astrocytoma cells (Kim *et al.*, 2002). The elastase-

stimulated response is supported by a study by Uehara *et al.* (2003) who have shown that neutrophil serine proteinases such as elastase can activate human non-epithelial cells to produce inflammatory cytokines such as IL-8 and MCP-1. This group have also shown in the same study that elastase can cleave PAR-2, with particular preference for the tethered ligand, in a similar fashion to the known PAR-2 activator trypsin. This evidence, albeit not in the pulmonary system, suggests that not only can elastase cleave PAR-2 at the tethered ligand site, but by doing so will elicit an inflammatory response. However, the current study does not concur with this idea. Figure 5.7a shows that after incubation with the activating peptide for 24hrs, the parenchymal explants failed to release TNFα above that of control levels. This is also the case when looking at the concentrations of IL-6 and IL-8 in the supernatants (figure 5.8). When stimulated with elastase, the explants release an increased amount of TNFα as well as an increase in the anti-inflammatory cytokine, IL-10; the PAR-2 activating peptide also had no effect on IL-10. This contradiction between the literature and the current study suggests that the effects seen are system specific and as such, elastase may cause varying effects via PAR-2 depending on which cells are present. This also suggests that the inflammatory effect of elastase documented with the parenchymal explants is not due to the involvement of PAR-2.

So far we have compared the elastase-induced inflammatory effects, and the possible relationship with PAR-2, from this study to systems other than the lungs due to there being little literature on the relationship between inflammation in human parenchyma and PAR-2. Although not completely comparable, animal models provide useful insights into possible effects that could happen in humans; Ebeling *et al.* (2005) have used a murine model to

investigate the role of PAR-2 in allergic airways inflammation and hyper-responsiveness. In their study they sensitised mice to ovalbumin then re-exposed the animals to the allergen along with a PAR-2 activating peptide. Protein levels in BAL fluid and mRNA in lung tissue for several cytokines and chemokines were measured after administration of ovalbumin alone, PAR-2 activating peptide alone, or the two in combination. Of the cytokines and chemokines measured it was TNFα that had increased mRNA and protein levels with IL-5 having increased mRNA and IL-13 having increased protein levels. This data suggests an important role for PAR-2 in allergen-induced inflammation in the lungs. We have also investigated protein levels of IL-5 and IL-13 in the supernatant from the explants after stimulation with elastase or the PAR-2 activating peptide. Figure 5.8 shows that elastase can increase IL-5 protein levels significantly above those for the control; however for IL-13, elastase shows a decrease in the amount of cytokine present. This could be due to degradation of the cytokine by elastase, or another proteinase, or a down-regulation mechanism that has not been investigated. When looking at the effects of the PAR-2 activating peptide it is obvious that it increases IL-5 protein levels in a dose-dependent manner with the lowest dose (12.5nM) having the same effect as the control. Also, the PAR-2 activating peptide increases IL-13 protein levels albeit not in a dose-dependent way as for IL-5, although again the lowest dose has no effect over the control.

The predominant cellular source for IL-5 and IL-13 is the mast cell and it has been shown that these cytokines are released from mast cells after activation to undergo degranulation (Lorentz *et al.,* 1999 and Toru *et al.,*1998). The data suggests that the PAR-2 activating peptide can also cause the degranulation of mast cells and the subsequent release of IL-5 and IL-13. To

investigate this idea further histamine levels could be measured after stimulation. This would prove a useful read-out, as histamine is also a mast cell degranulation product and increases in histamine levels can be detected at early time-points such as one hour (Wang and Lau, 2007). The response to the PAR-2 activating peptide could also be compared to anti-IgE, which cross-links IgE on the mast cell to initiate degranulation and calcium influx into the mast cell after stimulation would also suggest activation by the peptide.

The studies mentioned above have predominantly detected PAR-2 via immunostaining methods on the cell surface, whereas this study has used homogenised tissue and dot blots to assess the presence of this receptor throughout the entire cell. Although it is imperative to investigate cell surface expression it is also important to remember that intracellular stores of PAR-2 exist that are protected against proteolytic activation. Böhm *et al.* (1996) have shown that before activation with trypsin PAR-2 can be found, via immunofluorescence, in the Golgi apparatus as well as in post-Golgi vesicles. Once the cell surface receptors have been activated by trypsin they become internalised into early endosomes and then ultimately trafficked to lysosomes for degradation. Böhm and colleagues have also shown that pre-treatment with brefeldin A, which disrupts Golgi stores, and cycloheximide, which inhibits protein synthesis, decreases receptor translocation to the cell surface and ultimately decreases calcium influx and signalling. This further implies the importance of intracellular stores as well as cell surface expression. This information highlights the dynamic nature of receptor activation, desensitisation and resensitisation within this system and therefore indicates the need for further in depth studies of the kinetics and cellular locations of receptor expression within the parenchyma.

This chapter has so far discussed the role of PAR-2 and its potential relationship with elastase but it is obvious that the effects seen after stimulation with elastase cannot be completely explained by PAR-2 involvement. In particular, the differences in cytokine release after stimulation with elastase or the activating peptide for PAR-2 suggests that there may be another receptor involved in mediating the inflammatory effects of elastase. Considering the similarities between inflammation induced by elastase and LPS, as shown in chapter three, this study suggests that interactions between elastase and TLR-4 could explain the responses seen considering LPS is well known to activate this receptor (Su *et al.,* 2000, Faure *et al.,* 2000 and Baumgarten *et al.,* 2001). After confirming the presence of TLR-4 in the parenchyma via immunohistochemistry and dot blot, the amount of TLR-4 in the parenchyma after elastase stimulation was established and compared to unstimulated samples. Figure 5.11 details that elastase has no effect on the expression of the receptor but this does not rule out the idea that elastase may activate the receptor. Johnson *et al.,* (2004) have shown that elastase can initiate systemic inflammatory response syndrome (SIRS) in mice and that this response is dependent on the presence of functional TLR-4, as mutant mice lacking this receptor failed to develop SIRS after elastase stimulation. Hietaranta *et al.,* (2004) have also shown that pancreatic elastase can induce the release of TNFα from human myeloid cells via a TLR-4 pathway, thus further supporting the notion that elastase may cause some of its inflammatory actions in the lung by activating this receptor.

Having investigated the effect of elastase stimulation on the expression of TLR-4 in the parenchyma we went on to establish the relationship between TLR-4 expression and disease

severity as defined by the GOLD guidelines (2005). We have shown that an increase in disease severity is associated with an increase in TLR-4 expression in the parenchyma (figure 5.12) and as such could explain why those with more severe disease show a greater inflammatory response to both traditional inflammatory stimuli, such as LPS, as well as stimuli not usually known for their inflammatory properties such as elastase. Hauber *et al.* (2005) have shown that the number of TLR-4 positive neutrophils and macrophages is upregulated in the submucosa of those with cystic fibrosis compared to control subjects but this study does not investigate the level of expression of this receptor. Our group have previously shown that the numbers of inflammatory cells in COPD and non-COPD parenchyma are no different (Hackett *et al.,* 2008 and Woods, 2008). We therefore propose that any increase in TLR-4 would be due to increased receptor expression and not increases in inflammatory cells. However, whether the increase in TLR-4 is a cause or an effect of increased inflammation in these patients is yet to be determined and requires further investigation.

In summary, this chapter has shown that PAR-2 expression tends to increase in the parenchyma of those with mild to moderate disease compared to those with no disease and that elastase stimulation causes differing effects on this expression depending on the disease status of the subject. We have also shown that elastase may in fact cause different responses via different pathways and it is proposed that a Th2 allergic response is mediated via PAR-2 and an inflammatory response is mediated via TLR-4. However, before firm conclusions can be made with regard to this hypothesis further study is required.

Chapter 6:
General Discussion

6.1 General Discussion

COPD is defined by inflammation of the lungs, both chronic and acute, and this study has sought to investigate the role played by elastase in this inflammation. An *ex vivo* explant model has been utilised to determine the effects of this proteinase which has enabled us to measure the temporal release of cytokines from human parenchyma from a range of subjects with varying degrees of airways obstruction; it would be both unethical and impractical to perform this type of investigation via the more traditional method of bronchoalveolar lavage. As well as being able to mimic an acute inflammatory response in the tissue, the explants retain both cell to cell and cell to matrix interactions (with more than one cell type) thus providing a more physiologically relevant situation compared to cell lines that are also used as a model. However, although this current model provides a physiologically relevant situation it is not without its pitfalls. The major disadvantage is that the explants have no accompanying circulatory system, as would be found in the *in vivo* situation or if using an animal model, thus the role of other cells and mediators that would usually migrate to the site of inflammation cannot be assessed. Also, due to the range of disease phenotypes found in the subjects there is a high level of inter-patient variability, therefore calling for large numbers of patients to be studied. Many of the subjects used in this study underwent carcinoma resection and also usually have a number of co-morbidities, such as hypertension, which could complicate the model. However, the study has more recently recruited younger patients undergoing bullectomy who have relatively little co-morbidity, therefore providing a greater insight into how normal tissue responds to an inflammatory episode.

The explant model utilised in this study allows the addition of many stimuli including inflammatory mediators, such as LPS, as well as modifying substances such as drugs and antibodies. For the purpose of the current study we have used porcine pancreatic elastase as a model for neutrophil elastase, which has been shown to be a suitable stimulus for experimental emphysema and pulmonary inflammation in several studies (Lucey *et al.,* 1998, Birrell *et al.,* 2005 and Suzuki *et al.,* 2009). The traditional link between elastase (neutrophil-, macrophage- and bacterial-derived) and emphysema/COPD has been accredited to the proteolytic actions of elastase on matrix components such as elastin (Reilly and Travis, 1980 and Chapman and Stone, 1984) and collagen (Heck *et al.,* 1986 and Zhu *et al.,* 2001). However there has been recent interest in the inflammatory properties of elastase and how these could contribute to disease initiation and progression (for reviews see Lungarella *et al.,* 2008 and Taggart *et al.,* 2005). It is well known that elastase plays a pro-inflammatory role in other diseases such as systemic inflammatory response syndrome (SIRS) (Johnson *et al.,* 2004) and acute lung injury (ALI) (Kodama *et al.,* 2007). It has also been shown that plasma levels of neutrophil elastase can predict the severity of acute pancreatitis and as such can be used as a clinical marker for the disease (Dominguez-Munoz *et al.,* 2005). In support of the inflammatory role elastase plays in these systems we have shown in this study that elastase can also induce an inflammatory response, in the form of increased TNFα release, in human parenchymal explants in a manner comparable to the inflammatory stimulus LPS. As well as showing an increase in this pro-inflammatory cytokine we have also shown that release of the anti-inflammatory cytokine IL-10 is also increased after elastase stimulation. The release of these two cytokines supports the idea that elastase can propagate an inflammatory milieu with TNFα which can be tempered by the anti-inflammatory IL-10. Peak release of both these cytokines

occurs after 24hrs of stimulation with elastase, but the TNFα response is initiated 2hrs earlier than the IL-10 response, which concurs with data shown by Hackett *et al.* (2008) whereby TNFα was shown to be a key primary mediator in the initiation of an inflammatory cytokine cascade.

The general protocol for the addition of elastase to the explants in this study was to administer one dose of the proteinase and collect the supernatant and tissue after a specified time period with the stimulus therefore simulating the more chronic inflammatory situation associated with COPD. However, it is important to remember that COPD is also characterised by bouts of acute inflammation and as such we have shown that incubating explants with elastase for one hour and then transferring the tissue to fresh media without stimulus also initiates an inflammatory response comparable to continuous stimulation. This suggests that elastase is capable of initiating both chronic and acute inflammation and also highlights the need for swift intervention of this response with regard to patient recovery.

Considering the ability of elastase to induce an inflammatory response it would be sensible to assume that inhibiting elastase in the lung would decrease the inflammation in the system, thereby decreasing the disease progression of COPD. We have shown in this study that the use of a specific elastase inhibitor, elastatinal, significantly decreases the TNFα response to elastase stimulation but has less of an effect on the IL-10 response. However, the IL-10 response is decreased, albeit not significantly, by the trypsin inhibitor SBTI suggesting that the anti-inflammatory response in this model is not mediated completely by the exogenous elastase but possibly also by endogenously released trypsin-like enzymes. Although we have

shown that elastase-induced inflammation can be inhibited in the *ex vivo* situation the *in vivo* situation is somewhat different. To date several synthetic elastase inhibitors have been developed but there has been little success in using these in a clinical set-up. Kuraki *et al.* (2002) have shown that the specific neutrophil elastase inhibitor ONO-6818 is capable of inhibiting neutrophil elastase-induced emphysema in rats but this compound has proved unsuccessful in clinical trials due to safety concerns (Ono Pharmaceutical Co., Ltd, 2002). Another elastase inhibitor, MR889, has progressed to clinical trial and Luisetti *et al*. (1996) have shown that this compound is well-tolerated in humans up to 4 weeks administration; however the advantageous clinical effects of MR889 appear to be limited to those patients with short disease duration, which showed a decrease in urinary desmosine levels suggesting a decrease in elastin breakdown. Even though many of the elastase inhibitors available fail to improve the inflammation associated with COPD in a safe manner, Sivelestat (ONO-5046) has been shown by Okayama *et al.* (2006) to significantly increase the lung function of patients with acute respiratory distress syndrome thus suggesting that the inhibition of elastase does contribute to a decrease in pathology. Sivelestat has also been shown by Yasui *et al.* (1995) to be able to decrease LPS-induced acute lung inflammation in the hamster. This finding suggests that elastase is involved to a greater extent in the pathogenesis of inflammation than first thought. In our explant model we have shown that elastatinal has no effect on LPS-induced inflammation, however our system does not include a circulation unlike the hamster *in vivo* model utilised by Yasui and colleagues. It could therefore be concluded that LPS initiates an inflammatory cascade causing an influx of neutrophils from the surrounding circulation to the site of inflammation, where these inflammatory cells degranulate, thus increasing the elastase concentration in the tissue rather than causing the already present

neutrophils to degranulate. The elastase inhibitor can then act upon the newly present proteinase to decrease the inflammatory response.

We have so far suggested that inhibiting elastase would result in a decrease in the inflammatory response associated with COPD, but the systemic effects of this inhibition must be considered. Although elastase is implicated in disease due to its proteolytic and inflammatory properties, it is unknown what the long-term effects of elastase inhibition would be on its homeostatic role. Therefore, it has to be considered whether it would prove to be of greater advantage to inhibit the downstream effects of elastase stimulation, such as TNFα release, rather than elastase itself; anti-TNFα therapy, such as Etanercept, has already been shown to have advantageous effects in the treatment of rheumatoid arthritis (Bathon *et al.*, 2000 and Genovese *et al.*, 2002) and psoriasis (Gottlieb *et al.*, 2003). Although anti-TNFα therapy has its advantages, its route of administration is problematic with the drug having to be subcutaneously injected at least on a weekly basis. Also, the blanket inhibition of TNFα leaves the patient susceptible to other infections such as tuberculosis (Gómez-Reino *et al.*, 2003 and Tubach *et al.*, 2009) and therefore attenuating the inflammatory response may be of greater advantage rather than a blanket inhibition. Although anti-TNFα therapy could be utilised to inhibit the downstream inflammatory effects of elastase it has to be noted that the proteolytic actions remain unchallenged and so the emphysematous changes to the parenchyma would continue to develop. This conundrum of whether to target the proteinase itself or just the consequences of its activation requires further investigation before a firm solution can be decided upon.

This discussion has so far focussed on the inflammatory actions of elastase but we have also shown, in chapter 3, that this proteinase also induces the release of other cytokines and not just pro- and anti-inflammatory mediators. The traditionally accepted dogma for the pathogenesis of COPD is that it is a Th/Tc1-driven disease with little involvement of Th2 cytokines (Hodge *et al.,* 2007 and Majori *et al.,* 1999). In contrast, asthma is usually thought of as having a Th2 rather than a Th/Tc1 phenotype (Kuipers *et al.,* 2004). In support of the idea that COPD is mediated by a Th/Tc1 response we have shown that stimulation of parenchyma from patients with some evidence of COPD results in the induction of Th/Tc1 cytokines. However, we have also shown that the same stimulation also induces the release of Th2 cytokines, thus opposing the conventional view-point. From this data two conclusions could be decided upon: (1) COPD is defined by both a Th/Tc1 and Th2 phenotype and is still a separate condition to asthma or (2) COPD is defined by both a Th/Tc1 and Th2 phenotype and is a development of asthma as suggested by the Dutch Hypothesis (Orie *et al.,* 1961). It is possible that considering the subjects in this study predominantly have only mild to moderate COPD that it is this early stage of the disease that is characterised by a Th2 phenotype, which changes to a Th1/Tc1 phenotype as the disease progresses. An extensive longitudinal study would be required to ascertain whether this is the case. Although both of these hypotheses could be true it is unlikely that COPD is a continuation of asthma due to the differing genetic predispositions (Lomas and Silverman, 2001, Loza & Chang, 2007 and Hunninghake *et al.*, 2007) as well as the different presentations of the disease with asthma predominantly affecting the airways with little parenchymal involvement, whereas COPD does cause parenchymal destruction. Therefore, considering the evidence it is more likely that COPD does have some component of a Th2 phenotype whilst remaining a distinct condition.

Having established that elastase stimulation can evoke a Th2 response it is vital to assess the role of elastase in Th2 type diseases, such as asthma, as well as its known role in COPD. Whereas COPD is characterised by the presence of neutrophils and macrophages, an eosinophilic environment better defines asthma (Bousquet *et al.,* 1990). However, there is some evidence to suggest that neutrophilic inflammation can also contribute to the pathogenesis of asthma, especially severe asthma, (Jatakanon *et al.,* 1999) and also that neutrophilic products, such as neutrophil elastase, can activate eosinophils therefore propagating the inflammatory milieu (Hiraguchi *et al.,* 2008). In their study, Hiraguchi *et al.,* (2008) have also shown that neutrophil elastase activation of eosinophils induces the release of neutrophilic chemokines such as IL-8 thus suggesting a positive feedback relationship between the two inflammatory cells and a greater role for neutrophil elastase in severe asthma. It has also been shown by Marguet *et al.* (1999) that childhood asthma is associated with BAL neutrophilia. Taking these studies into account it can be suggested that neutrophils, and their products, are important early in the pathogenesis of asthma, but may undergo apoptosis as the disease progresses and the eosinophil takes over as the predominant inflammatory cell. However, as the disease becomes more severe, the presence of neutrophils is again increased.

Although asthma may be the obvious Th2-type disease to compare to COPD with regards to the role elastase plays it is important to acknowledge that elastase is also implicated in the development of other conditions that are characterised by either a solely Th2 environment or one that contains both Th2 and Th1/Tc1 cytokines, such as abdominal aortic aneurysms (AAA). AAA are characterised by a Th2 profile (Shimizu *et al.,* 2004) and also by the degradation of matrix macromolecules such as collagen and elastin (Huffman *et al.,* 2000 and

Satta *et al.*, 1995), both of which can be degraded by elastase. Cohen *et al.*, (1991) have also shown that neutrophils from those with AAA have a greater affinity for elastin-breakdown products, which increases with disease severity. The resulting neutrophil chemotaxis to the site of destruction and inflammation results in an increase in elastase thereby exacerbating the disease state. Having assessed these examples, it is clear that the relationship between elastase and inflammation (with particular reference to the associated cytokine profile) is a complex situation yet to be fully understood and an area of research which requires greater investigation.

Although this study has predominantly focussed on the inflammatory aspects of elastase stimulation on human lung parenchyma it cannot be overlooked that challenge with this proteinase is also capable of generating a cytokine response usually associated with allergy e.g. IL-5 and IL-13. Considering the two distinct consequences of elastase stimulation detailed in this study (inflammation and allergy) it is unlikely that a common mechanism links these effects. Previous data (Woods, 2008) has shown that the cellular composition of parenchyma from subjects included in the current study is characterised by higher numbers of neutrophils, macrophages and mast cells compared to the number of eosinophils. Woods also showed that there were no differences in cell counts between patient groups. Taking into account this cellular distribution it could be argued that the inflammatory response elicited by elastase occurs via activation of the neutrophils, macrophages and mast cells due to their predominance. On the other hand, cellular density is not necessarily correlated to the magnitude of the response from such cells; a lower prevalence of eosinophils in the tissue does not mean that these cells are less important in the generation of an inflammatory, or more

likely an allergic, response. The model utilised in this study allows a physiologically relevant situation to be examined but in order to determine the overall relationship between elastase and the cytokine response it would be necessary to investigate cell lines comprising of the individual cell types and combinations thereof. Another technique that could be employed is co-localisation via immunohistochemistry which would determine which cells produce which cytokines in response to stimulation with elastase.

Even though cellular ratios are important in assessing the initiation and propagation of the inflammatory/allergic response it is also important to realise that the type of response elicited could be due to the receptor(s) activated by the stimulus. It is also possible that receptor(s) may mediate differing responses dependant on which cell type they are resident on, as well as whether the surrounding milieu favours a Th1/Tc1 or Th2 environment. For example, it has been shown that both macrophages and eosinophils possess the G-protein coupled receptor PAR-2 (Roche *et al.,* 2003 and Bolton *et al.,* 2003) and the former is associated predominantly with an inflammatory role whereas the latter is known for its allergic properties. As this receptor is known to be activated by serine proteinases such as trypsin it is possible that elastase, also a serine proteinase, could act through PAR-2 on both macrophages and eosinophils to elicit its distinct effect; activation of macrophages via this route could explain the TNFα and IL-10 inflammatory response whereas activation of eosinophils could explain the presence of IL-5 and IL-13. It has also been suggested that PAR-2 may play a role in the inflammation associated with COPD (Miotto *et al.,* 2002) and as elastase has been shown in this study to induce an inflammatory response from parenchymal tissue from those with COPD, it is possible that elastase may mediate this response via activation of PAR-2.

Although it is possible that activation of the same receptor on different cell types may cause different responses we have shown that when a PAR-2 synthetic activating peptide is added to the parenchyma, which contains several cell types including macrophages and eosinophils, we are able to detect an IL-5 and IL-13 response but not an inflammatory response. Therefore, in this system we propose that the distinct actions of elastase work not through the same receptor but through two separate receptors, namely PAR-2 for the allergic type response and TLR-4 for the inflammatory response. Links between TLR-4 and inflammation are already well established concerning bacterial LPS stimulation and pancreatic elastase has also been shown to activate this receptor in human myeloid cells (Hietaranta *et al.*, 2004).

Having chosen PAR-2 and TLR-4 as our candidate receptors we investigated the effect of elastase stimulation on the expression of these receptors via dot blot. Although we have not shown any change in the expression of either of these receptors after elastase challenge it cannot be ruled out that elastase may activate them. Further investigation regarding the downstream signalling events after receptor activation would be required to fully elucidate the mechanism by which elastase causes its actions. After further investigation into the expression of these receptors within our patient population we did, however, detect a trend for those with a greater disease severity to have increased amounts of both receptors in question; this could go some way to explain the more intense inflammatory responses seen in those with progressed COPD compared to the 'normal' response. However, it is beyond the realms of this study to speculate as to whether the increases seen with these receptors is the cause or effect and as such it would be desirable to conduct a longitudinal study on the changes in receptor expression in the parenchyma as the disease progresses. However, this approach

poses many ethical considerations in the human concerning sample collection and so could be restricted to animal models of emphysema and COPD.

6.2 Conclusions

To conclude, we have shown in this study that elastase is capable of inducing an inflammatory response from human lung parenchyma using an *ex vivo* explant model. We have also shown that this response can be modified by an elastase-specific inhibitor, elastatinal, and that a similar response cannot be obtained by other serine proteinases. As well as inducing an inflammatory response, this study has given evidence for the induction of an allergic/Th2 type response in the form of IL-5 and IL-13 release after stimulation with elastase. Having established that elastase can indeed cause the aforementioned responses we surmise that these distinct effects may occur due to elastase either activating the same receptor on different cell types i.e. PAR-2 on macrophages and eosinophils or activating different receptors on the same cells i.e. PAR-2 and TLR-4 on the same cells. However, the exact mechanism by which elastase causes its effects requires greater in-depth investigation beyond the scope of this study.

6.3 Future work

Many questions have arisen from this study which require further investigation and the area that predominates is the mechanism by which elastase induces its responses. This study has assumed a role for PAR-2 and possibly TLR-4 in mediating the elastase response but this has yet to be proved conclusively; if elastase were to activate either receptor there would be an accompanying calcium influx into the cell and so this read-out could be examined. To further

investigate the role of either of these receptors in this system cell lines known to express the specific receptors in question could be used in place of the parenchymal tissue that contains many cell types. However, this approach assumes that the elastase response is generated by the actions of one cell-type and not consequential activation of another cell-type e.g. elastase may activate neutrophils, which then undergo degranulation releasing pro-inflammatory mediators into the surrounding environment that may go on to activate other cells such as macrophages.

Another approach to this subject could be to inhibit either the receptors themselves, or their downstream signalling pathways, before stimulation with elastase; if the elastase response is ablated by the inhibition then it could be assumed that that receptor is vital in processing the downstream effects of elastase stimulation. This could be investigated using either the model utilised in this study, cell lines or on a larger scale with a knockout animal system. Takizawa *et al.* (2005) have previously investigated allergic airways inflammation in PAR-2 knockout mice in response to ovalbumin sensitisation and challenge and have shown significant decreases in both eosinophils and lymphocytes in the PAR-2 deficient mice compared to the wild type. Similarly, Nigo *et al.* (2006) have utilised a TLR-4 knockout mouse to assess LPS-mediated inflammation in the airways and concluded that TLR-4 molecules on mast cells in this system play a vital role in the mediation of this response due to a lack of eosinophilic infiltration after LPS stimulation in TLR-4 knockout mice compared to the wild type.

This study has utilised porcine pancreatic elastase as a surrogate for neutrophil elastase and so another area of investigation would be to compare the effects of this proteinase to elastases

from other sources such as inherent neutrophils and macrophages from the host as well as those from invading pathogens such as the bacteria *Pseudomonas aeruginosa*. It has been shown by Dulon *et al.*, (2005) that elastase from this bacterium is able to disable PAR-2 in respiratory epithelial cells and so it would be interesting to assess whether intrinsic and extrinsic elastases would have the same affect or whether the disarmament of this receptor is specific to the pathogen.

Finally, it is clear from the findings of this study that this area of research would benefit from long-term studies into the development and progression of COPD and the mechanisms behind this. However, although this is the ideal situation it is important to realise there are many prohibitive factors in conducting studies such as these including funding, ethical approval, subject participation and utilising the most appropriate methods for sample/data analysis.

Chapter 7: References

7. References

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