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The role of tumour necrosis factor alpha in acute inflammation of human lung tissue

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ABSTRACT

DEPARTMENT OF CELLULAR AND DEVELOPMENTAL SCIENCES
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Doctor of Philosophy

THE ROLE OF TUMOUR NECROSIS FACTOR ALPHA IN ACUTE INFLAMMATION OF HUMAN LUNG TISSUE.

By Tillie-Louise Hackett

Chronic obstructive pulmonary disease (COPD) is a progressive and irreversible disease of the airways. It has been widely hypothesised that the persistent exposure to cigarette smoke initiates, the chronic inflammation, responsible for the parenchymal destruction and extensive remodelling of the bronchiolar walls that is characteristic of COPD. Patients with COPD also suffer from acute episodes of inflammation termed exacerbations, which are an important cause of morbidity and mortality in COPD patients. Exacerbations are particularly important, as there is evidence that exacerbation frequency predicts the accelerated decline in lung function observed in COPD patients.

This thesis has assessed the role of TNFα in acute inflammation in human lung tissue. TNFα has previously been shown to play an important role in many chronic inflammatory diseases, and is now accepted as an important therapeutic target in rheumatoid arthritis, inflammatory bowl disease and severe asthma. This thesis aims to investigate the contribution of cytokines, in particular TNFα, in the acute inflammatory response and how they may be affected by disease. This thesis illustrates that TNFα is the key cytokine in the acute inflammatory response, initiated by LPS, in human lung tissue. In addition we have demonstrated that the main cellular sources of TNFα in acute inflammation are alveolar macrophages and mast cells. An important clinical finding of this study is that patients with COPD and current smokers have an exaggerated TNFα response, which would lead to the elevated release of pro-inflammatory mediators and extracellular matrix destruction during inflammation. We have also verified that IL-10 can modulate TNFα expression in human lung tissue, and we have also demonstrated that patients with COPD produce lower levels of IL-10 in response to an LPS challenge. This study therefore highlights the role of TNFα and IL-10 in the progression of COPD and provides important future therapeutic targets for the disease.

We have also demonstrated that the decline in protein expression with disease could result from the elevated metabolic turnover due to the enhanced oxidation of proteins in COPD patients. In particular we have shown that oxidised HSA, an important antioxidant, is turned over more rapidly in human lung tissue. We have also begun to develop the technique iTRAQ to analyse the changes in protein expression in diseased and naïve human lung tissue. While this approach requires further development iTRAQ potentially provides an important proteomic tool for identifying markers of disease.

Preface

Parts of this thesis have been published, or are in preparation for publication, in the following:

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T-L Hackett L Barnicott and JA Warner. The role of IL-13 in allergic inflammation. *In preparation for the AAAAI*

T-L Hackett and JA Warner. The effect of lipopolysaccharide on cytokine production in human lung tissue. *Am J Respir Crit Care MED. 169:A556 2004*

T-L Hackett and JA Warner. The role of Tumour necrosis factor-α in airways inflammation in human lung. Am J Respir Crit Care MED. 170:A100 2005

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Abbreviations

ACE	Aminoethyl carbozole
BAL	Bronchoalveolar lavage
CD	Cluster designation
COPD	Chronic obstructive pulmonary disease
DNA	Deoxyribose nucleic acid
DNPH	Dinitrophenyl hydroziene
DNTP	Dinucleotide phophate
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbant assay
FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
GOLD	Global initiative for chronic obstructive pulmonary disease
GMA	Glyco methacrylate
GRO-α	Growth related oncogene-α
HRP	Horse radish peroxidase
HSA	Human serum albumin
IA	Immunosorbent assay
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IPF	Interstitial pulmonary fibrosis
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MAB	Monoclonal antibody
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MudPIT	Multiple dimensional protein identification technologies
nIL-10 Ab	Neutralising IL-10 antibody
nTNFα Ab	Neutralising TNFα antibody
OD	Optical density
PIC	Protease inhibitor complex
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethyl-sulphonyl fluoride
RNA	Ribonucleic acid
RNase	Ribonucleic acidase
ROS	Reactive oxygen species
RT	Reverse transcription
SDS	Sodium dodecyl sulphate
SNP	Single nucleotide polymorphism
Taq	*Thermus aquaticus* (DNA polymerase)
TBS	Tris buffered saline
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TMB	3, 3', 5, 5' tetramethyl benzedine
TNF	Tumour necrosis factor
WB	Western blot

Contents

Chapter 1.

Introduction

1.1 Overview of chronic obstructive pulmonary disease (COPD)

1.1.1 Definition of COPD

Chronic obstructive pulmonary disease (COPD) is a leading cause of morbidity and mortality worldwide. COPD is defined by the American Thoracic society, as a disease state characterised by the presence of airflow obstruction due to either or both of the conditions chronic bronchitis and emphysema. The airflow obstruction is generally progressive, may be accompanied by airway hyper reactivity, and may be partially reversible (ATS, 1995). A more recent definition comes from the Global strategy for the diagnosis, management and prevention of COPD (GOLD). COPD is defined by GOLD (2001) as a disease state characterised by airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with abnormal inflammatory response of the lungs to noxious particles or gases. The GOLD guidelines particularly take into account the importance of cigarette smoking and airway inflammation.

The term COPD encompasses chronic obstructive bronchitis characterised by the obstruction of small airways. Emphysema is characterised by the enlargement of small air spaces and destruction of lung parenchyma, loss of lung elasticity, and closure of small airways. Lastly, chronic bronchitis is defined by the presence of a productive cough for more than three months and up to two years. Most COPD patients suffer with all three pathological conditions but the extent of each disease varies between individuals. The varying degrees of overlap of these conditions present in COPD patients leads to the wide heterogeneity seen in the disease. Diagnosis of COPD can be further complicated by the presence of asthma, often termed "wheezy" COPD, which is present in approximately 10% of COPD patients (Jeffrey, 1998, O'Byrne and Postma, 1999, Barnes, 1999). The problem with COPD is that pulmonary damage often occurs years before the patient is aware of their symptoms. Physician-diagnosed COPD using spirometric airflow limitation as defined by GOLD (FEV_1/ FVC less than 0.7 and FEV_1 less than 80% predicted) only

diagnoses patients who already have airflow obstruction. Research is therefore required to diagnose patients most at risk early on in the development of the disease.

1.1.2 Epidemiology of COPD

COPD is the fourth leading cause of death in the western world and is set to rise to the third by 2020 (Murray *et al.*, 1996). This increasing trend in mortality is seen in females over 55 years and males over 75 years. The vast difference in age may well be due to the increasing proportion of young females who smoke. Studies from the last decade indicate 4-6% of the adult population suffer from clinically relevant COPD (Loddenkemper *et al.*, 2003). This equates to an estimated 3.0 million people suffering from COPD in the UK. The prevalence of COPD increases greatly with age and recent surveys show only small differences between sexes. (Loddenkemper *et al.*, 2003). In the UK the disease is responsible for 9% of hospital admissions imposing a financial burden on the National Heath Service currently estimated at £818 million per annum (Barnes, 1999). The global burden of disease study had estimated the worldwide prevalence of COPD as 834 per 100,000 people, which yields approximately 44 million cases of COPD worldwide. COPD is the only leading cause of death that is increasing in prevalence worldwide. Therefore the need for research into the potential risk factors and causative mechanisms of the disease is more prominent than ever.

1.1.3 Risk factors associated with COPD

Risk factors predisposing individuals to COPD are under continual review. Smoking (both active and passive) is accepted as the most important predictor of airflow obstruction and development of COPD (O'Byrne and Postma, 1999). Evidence suggests that active cigarette smoking accounts for between 85 and 90% of COPD cases (Snider, 1989).

Cigarette smoking accelerates age related decline in FEV_1 from approximately 30mls per year to 45mls per year. However while some smokers remain unaffected, the annual decline can be as great as 70-120mls FEV_1/year. These susceptible smokers, with continued smoking will develop clinically significant airflow obstruction (Fletcher *et al.*, 1977). There is also evidence to suggest cessation of smoking reduces the rate of lung function decline to approximately that of a non-smoker (Willemse *et al.*, 2004). Cigarette smoke is thought to initiate proteolytic destruction of the lung parenchyma leading to enlarged airspaces and hence emphysema. A second potential effect of smoking is inflammatory narrowing of the peripheral airways, characterised by oedema, mucus hypersecretion and obliteration of the peripheral airways (Sandford *et al.*, 1997). However only 15-20% of smokers actually develop COPD (Fletcher *et al.*, 1977) but observations from pathological studies suggests the prevalence of clinically silent COPD is underestimated (Muller *et al.*, 1988, Gurney *et al.*, 1992). Thus while smoking is an important risk factor it is likely that there are also other causal mechanisms, which interact with smoking and influence disease susceptibility and severity.

There is evidence that genetics plays a role in susceptibility of the disease, as COPD tends to cluster in families (Cosio *et al.*, 1979, *Sandford et al.*, 2000). The most important genetic defect linked to the development of COPD is α_1-antitrypsin deficiency (α_1-AT) (Laurel and Eriksson, 1963). Although α_1-AT deficiency is hereditary and the second most important risk factor in susceptibility to COPD, it is a relatively rare condition accounting for less than 1% of the total cases of COPD (ATS, 1995). α_1-AT is an acute phase protease inhibitor that protects the lungs from degradation by proteases such as neutrophil elastase. Homozygotes for the Z variant defective gene therefore have an increased risk of developing COPD. Genetic studies have also shown weak associations with other anti-proteinases such as α_1-antichymotrypsin, α_2-macroglogulin and secretory leukoproteinase

inhibitor (SLPI) and the development of COPD. It is probable in time many more candidate genes will be identified in the development of COPD.

Environmental factors may also contribute to the development of COPD. There is evidence for an association between exposure to occupational dust, gases, fumes (Becklake, 1989, Kauffmann *et al.*, 1979) and particulate air pollution with the development of COPD. Passive smoking is also an important factor in the development of the disease. Studies have shown that children of smoking parents have a 1-5% reduction in FEV1 by the age of 14 compared to children of non-smoking parents, and therefore show a higher risk of developing the disease (Tager *et al.*, 1983).

Lung infections may also play a part in the pathogenesis of the disease. Bacterial infections cause inflammation and neutrophil influx, both of which are implicated in COPD. For example, patients with a history of pneumonia or recurrent chest infections are found to be particularly at risk. Evidence suggests that latent adenoviral infections may also play a role in the pathogenesis of COPD by amplifying the inflammatory response to smoking and also increasing steroid resistance (Matsuse *et al.*, 1992, Seemungal *et al.*, 2000).

Bronchial hyper-responsiveness is common in COPD, but its severity tends not to be as significant as that found in asthmatics. Studies have shown bronchial hyper-responsiveness to be a predictor of accelerated decline of lung function in COPD patients (Taylor *et al.*, 1985, Yan *et al.*, 1985, Ramsdale et al., 1984). However there is no firm evidence for bronchial hyper-responsiveness being a key risk factor in the pathogenesis of COPD.

1.1.4 Pathology of COPD

COPD is a mixture of pathological changes in the large airways (chronic bronchitis), small airways (bronchiolitis) and alveoli (pulmonary emphysema). The extent of each pathology is very heterogeneous between individuals and is difficult to estimate.

Chronic Bronchitis

This is defined as a productive cough on most days for at least three months over at least two consecutive years, which cannot be attributed to other pulmonary or cardiac causes (West et al., 1995, ATS, 1995). In cigarette smokers this usually manifests itself as chronic mucus hypersecretion, which is a result of hypertrophy of the mucus glands in the central conducting airways (MRC, 1965, Fletcher *et al.*, 1984, West, 1995). Oxidative stress caused by smoking damages cilia and the epithelial cells lining the airways, which tend to take on a more squamous appearance and produce lysozyme, possibly as a defence mechanism. Agents in cigarette smoke are thought to irritate the lung epithelium, increasing mucus secretion from goblet cells by stimulating local sensory nerves (See figure 1.1).

Figure 1.1

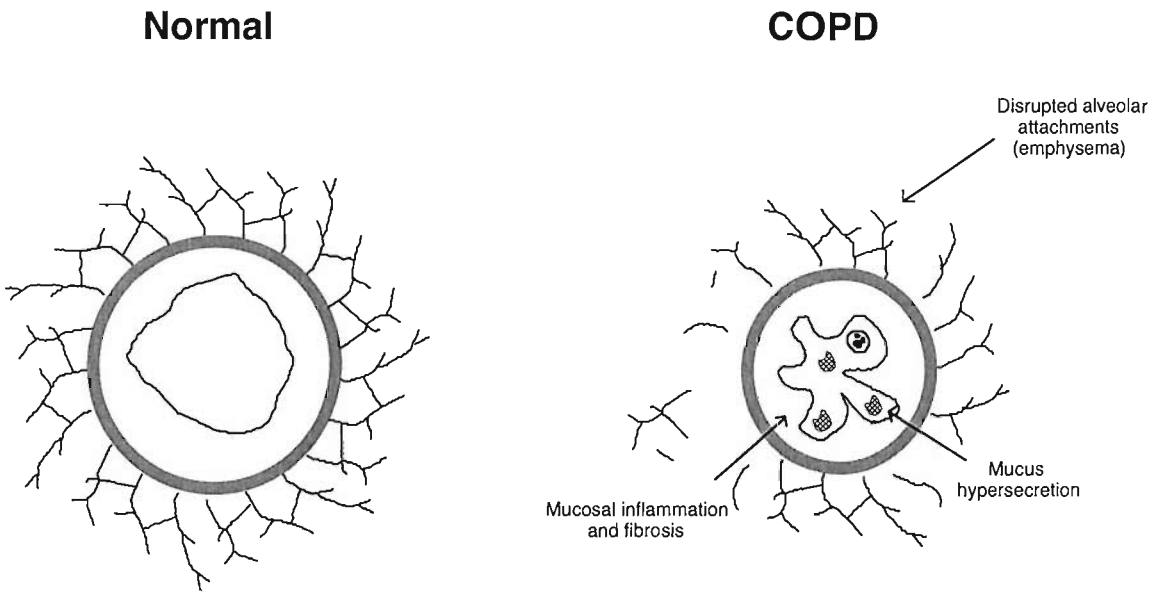


Figure 1.1 The cumulative features of COPD. Airway integrity in normal individuals is maintained by alveolar attachments. In COPD these attachments are disrupted, possibly by proteolytic damage, contributing to airway closure. Peripheral airways are also obstructed by damage resulting from inflammation and mucus secretion.

With the continual insult of cigarette smoke submucosal glands undergo hyperplasia and proliferation, leading to chronically high levels of mucus production. The cessation of smoking results in a rapid resolution of mucus hypersecretion. The small airways have been shown to become narrowed and show evidence of inflammatory cell infiltration (West, 1995). In addition to inflammatory cytokines, activated neutrophils also release neutrophil elastase, a potent stimulant of mucus secretion, perpetuating chronic mucus secretion.

Chronic bronchiolitis

Bronchiolitis causes a productive cough and airflow limitation, but in contrast to chronic bronchitis the obstruction occurs in the peripheral airways. Obstruction is caused by an aberrant inflammatory response thought to involve macrophages, CD8+ T-lymphocytes and neutrophils, with resultant fibrosis (Bruist *et al.*, 1984). Bronchiolitis is often found to

Figure 1.2

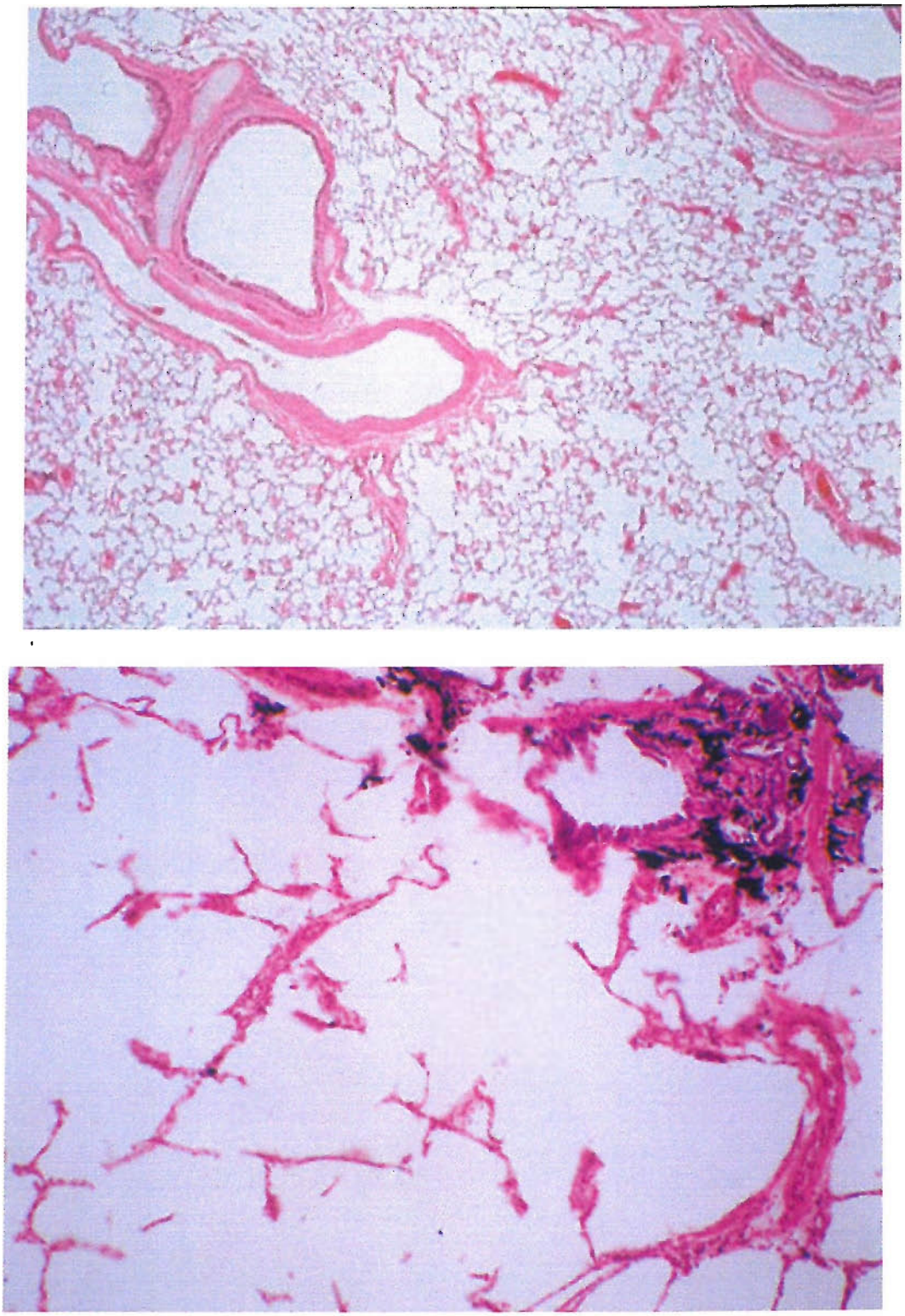


Figure1.2 upper panel. Normal human lung tissue stained with H&E. This section demonstrates the defined alveolar spaces for gas exchange in a normal individual. Magnification x40. Sourced form Wheaters functional histology 5th edition. **Figure 1.2 lower panel. Lung tissue from an emphysema patientstained with H&E.** This section shows the ptrteolytic destruction of the extracellular matrix and fibrosis of the basement membrane, which is characteristic of emphysema.Magnification x100. Sourced from Wheaters functional histology 5th edition.

and neutrophils, with resultant fibrosis (Bruist *et al.*, 1984). Bronchiolitis is often found to co-exist with destruction of the alveolar walls (see figure 1.1); emphysema (Fletcher *et al.*, 1984, Snider *et al.*, 1985)

Emphysema

Emphysema is defined pathologically as the presence of permanent enlargement of the airspaces distal to the terminal bronchioles, accompanied by destruction of their walls and their capillary networks without obvious fibrosis, resulting in non-functional air spaces (see figure 1.2, previous page) (Snider *et al.*, 1985). This results in the reduction of driving pressure and obstructive collapse of peripheral airways causing a reduction in FEV_1 (Fletcher *et al.*, 1984, Snider *et al.*, 1985).). Acinar wall destruction can be characterised as two specific types. In *Centrilobular* emphysema, by far the most common form, destruction is limited to the central part of the acinar lobule with the alveoli remaining unscathed. By contrast *panacinar* emphysema is characterised by widespread destruction of the entire respiratory unit (see figure 1.3), and is associated with α_1-AT deficiency (Thurlbeck, 1963).

Figure 1.3

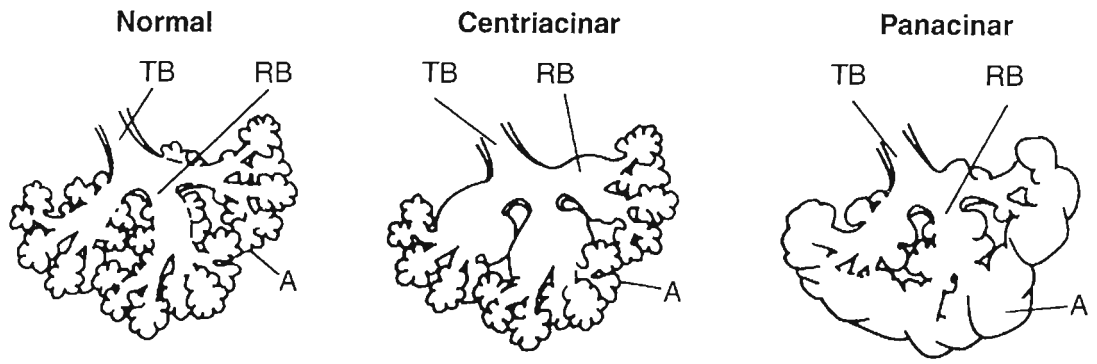


Figure 1.3 Centriacinar and panacinar emphysema. Destruction in centriacinar emphysema is confined to the terminal (TB) and respiratory bronchioles (RB). In panacinar emphysema the peripheral alveoli (A) are also involved. (From West, 1995)

These two types of emphysematous destruction can be distinguished topographically. *Centrilobular* emphysema is found to affect the upper regions of the lungs, moving downwards through disease progression, where as *panacinar* emphysema is found to have no regional preference (West, 1995). There is debate about the differences between bronchiolitis and emphysema and which of the two is the primary contributor to lung obstruction (Gelb *et al.*, 1996), however the important underlying feature of each is the aberrant inflammatory response (Jeffrey, 1998).

1.2 Chronic inflammation

COPD is characterised by chronic airway inflammation leading to airway remodelling and parenchymal destruction (Jeffery *et al.*, 1998). These processes are linked to the exposure of the airways to continual cigarette smoke resulting in an inflammatory phenotype. However the precise mechanisms by which smoking causes destruction and remodelling are unclear. Investigations have shown that the principal cells involved in COPD are neutrophils, macrophages and CD8+ve T lymphocytes and these play a key role in the pathogenesis of COPD. These cells are able to release pro-inflammatory cytokines and chemokines, proteolytic enzymes and generate reactive oxygen species (ROS). All of these mediators induce a chronic sustained inflammatory response leading to destruction of the lung parenchyma and airway remodelling.

1.2.1 The Neutrophil

Neutrophils form the front line defence of the immune system and are distinguished as cells with a lobed nucleus, and extensive granular cytoplasm. The granules of neutrophils represent a very heterogeneous but highly regulated packaging system of storage granules.

The storage granules contain a variety of secretory proteinases including neutrophil elastase and matrix metalloproteinases, antimicrobial agents such as myeloperoxidase and membrane associated proteins (vitronectin receptors). These densely packed granules enable the neutrophil to release a variety of mediators in an appropriate fashion depending on the stimulus. The organisation of these granules therefore fundamentally affects how neutrophils interact with the external environment. There is abundant evidence from *in vivo* studies that neutrophils play a key role in COPD. Studies employing induced sputum (Keatings *et al.*, 1997a, Peleman *et al.*, 1999) and bronchoalveolar lavage (BAL) (Thompson *et al.*, 1989, Lacoste *et al.*, 1993) have consistently demonstrated increased numbers of neutrophils, in the lungs of smokers with COPD compared to controls. Importantly as well as neutrophils, elevated levels of neutrophil derived enzymes have also been widely demonstrated in sputum and BAL fluid from subjects with COPD (Keatings *et al.*, 1997b, Betsuyaku *et al.*, 1999 and Hill *et al.*, 1999).

1.2.2 The macrophage

Macrophages are the primary phagocytic cell in the immune system; they are found ubiquitously throughout the body and differentiate according to defensive needs. Macrophages are the predominant defence cell both in normal human lung and during chronic inflammatory conditions such as COPD where they are found to comprise 95-98% of the total cell content recovered by BAL (Shapiro, 1999). Studies have shown smokers not only have more alveolar macrophages than non-smokers but they also have a different morphology (Schaberg *et al.*, 1995). Significantly macrophages are found to concentrate in the respiratory bronchioles where emphysematous lesions first develop, therefore suggesting a major role for them in the pathogenesis of the disease (Cosio and Guerassimov, 1999, Finkelstein *et al.*, 1995). Studies of emphysematous lung tissue from

human subjects have demonstrated a direct relationship between alveolar macrophage density in the parenchyma and the severity of lung destruction (Finkelstein *et al.*, 1995).

Macrophages are a source of pro inflammatory cytokines such as IL-1β and TNFα, chemotactic factors such as IL-8, reactive oxygen species, mucus gland activators and an array of matrix metalloproteinases. Most studies have focussed on macrophage release of MMPs, which are capable of degrading the ECM. Shapiro and colleagues (1999) have demonstrated macrophage metalloproteinase (MMP-12) knockout mice do not develop emphysema when exposed to cigarette smoke, while normal mice do. MMP-12 has been demonstrated by immunohistochemistry to be present in the lungs of COPD patients but not in the lungs of healthy controls. *In vitro* cultured alveolar macrophages taken from COPD subjects also express elevated amounts of both MMP-9 and MMP-1 compared to normal subjects. Macrophages are also important in neutrophil recruitment and clearance. It is therefore possible that tissue damage in COPD may arise due to the macrophage population being overwhelmed by large numbers of infiltrating neutrophils (Haslett *et al.*, 1999).

1.2.3 T-lymphocytes

T-lymphocytes play a pivotal role in controlling inflammatory responses. T-lymphocytes are distinguished by the expression of CD3 antigens on their surface and are further divided by either the expression of CD4+ or CD8+ markers. CD4+ cells have a pro-inflammatory function; on activation they secrete pro-inflammatory lymphokines such as IFNγ, TNFα, and IL-8 all implicated in COPD. CD4+ T cells are activated by presentation of antigens by the major histocompatibility complex class II, found on monocytes, macrophages and B-lymphocytes. CD8+ T cells kill malignant or infected cells by recognising antigens presented with major histocompatibility complex class I, which is found on all types of nucleated cells in the body.

Studies have found increased numbers of macrophages and activated T-cells in lung biopsies from chronic bronchitis patients (Saetta *et al.*, 1993, Finkelstein *et al.*, 1995). Excessive recruitment of CD8+ T cells may occur in response to repeated viral infections, which is a feature of smokers with COPD (Seemungal *et al.*, 1998). It is unknown whether tissue damage occurs as a direct or indirect consequence of CD8+ T cells apoptotic effect on infected cells.

1.2.4 The Eosinophil

Eosinophils are found to predominate in airway and mucosal tissues in asthma, allergic rhinitis, eosinophilic pneumonia and chronic cough (Erjefalt and Persson *et al.*, 2000). Evidence for the role of eosinophils in COPD is conflicting. Studies have demonstrated elevated numbers of eosinophils in sputum (Fujimoto *et al.*, 1999), BAL (Linden *et al.*, 1993) and the airway wall (Lacoste *et al.*, 1993, Betsuyaku *et al.*, 1999) of COPD patients. Other investigators, however, have found no increase in eosinophil numbers in COPD (Lacoste *et al.*, 1993, Saetta *et al* 1993, Peleman *et al.*, 1999). It is conceivable that this discrepancy is the result of overlap between COPD and asthma. One study in particular identified sputum eosinophilia in COPD patients, clinically benefited with corticosteroid treatment (Pizzichini et al., 1998). Another hypothesis suggests the influx of eosinophils is a by-product of ongoing smoking induced inflammation. In particular IL-8 is able to exert a chemotactic effect on primed eosinophils (Warringa *et al.*, 1993) in addition to neutrophils. Although the importance of eosinophils in COPD remains uncertain, patients exhibiting eosinophilia may represent a sub population in COPD, who would benefit from steroid treatment.

1.3 Role of exacerbations

COPD patients also suffer from acute inflammatory episodes termed exacerbations. Exacerbations are an important feature of COPD and are an important cause of morbidity and mortality in COPD patients (Seemungal *et al.*, 1998). Patients who suffer with frequent exacerbations have significantly lower health status (Seemungal *et al.*, 1998) and there is also evidence that exacerbation frequency predicts accelerated decline in lung function (Kanner *et al.*, 1979, Kanner *et al.*, 2001). Connors *et al*, (1996), found that approximately half of patients admitted to hospital for an exacerbation of COPD died within the following 2 years. The variability of this prognosis is due to the sheer heterogeneity of the disease. Acute exacerbations of COPD are defined as a sustained worsening of the patient's condition, from the stable state and beyond normal day to day variations that is acute in onset and necessitates a change in regular medication in a patient with underlying COPD (Rodriguez-Roisin, 2000). However, some symptoms of exacerbations are more important than others, in the description by Anthonisen *et al* (1987) exacerbations were further defined into three types. In Type 1 exacerbations patients have all the major symptoms, increased dyspnoea, sputum volume and purulence; type 2 exacerbations only involve two of the latter symptoms; and type 3 exacerbations only involve one symptom combined with cough and wheeze.

Dyspnoea is a key symptom of exacerbations and is often associated with a bacteria and viral infection prior to the exacerbation. It is thought bacterial or viral infections increase the inflammatory burden within the lung (Seemungal *et al.*, 2000) but the cause of exacerbations is not entirely clear. There is also wide variability in the frequency with which patients experience exacerbations (Patel *et al.*, 2002). Distinguishing among subtypes may lead to better guidelines for treatment of COPD.

Predictors for frequent exacerbations include clinical factors such as daily cough, wheeze and sputum production as well as the frequency of exacerbations in the previous year (Seemungal et al., 1998). COPD exacerbations are associated with considerable physiological deterioration and increased airway inflammatory changes (Bhowmik *et al.*, 2000). A recent study in which patients were defined as frequent and infrequent exacerbators, patients with frequent exacerbations had a faster decline in lung function (FEV_1) than infrequent exacerbators (Donaldson *et al*, 2002). It has been calculated that the contribution of exacerbations to lung function decline is approximately 25% (Wedicha *et al*, 2003). The schematic diagram in figure 1.4 represents the decline in lung function observed in COPD patients during exacerbations.

Figure 1.4

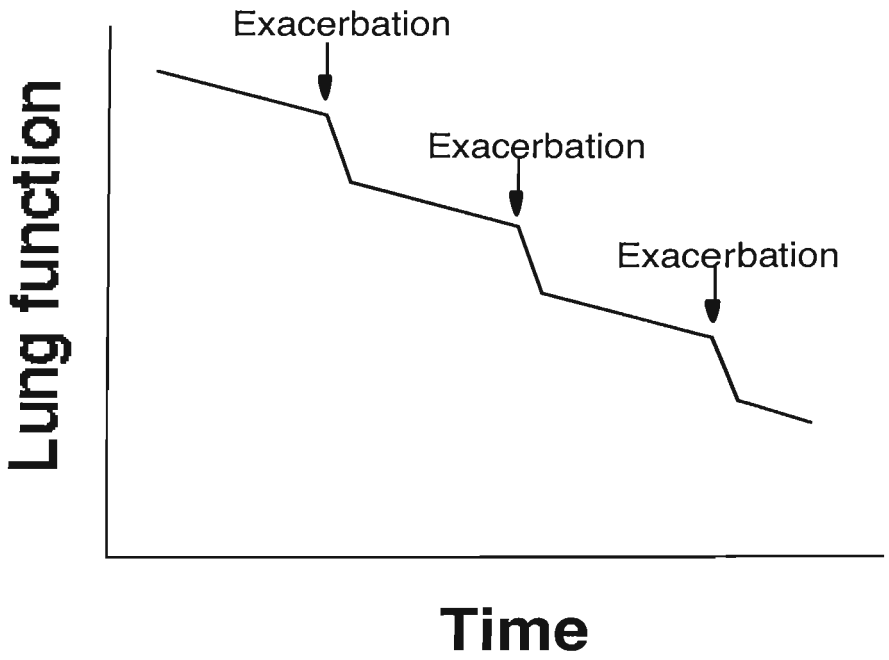


Figure 1.4 The effect of exacerbation on lung function. The schematic diagram represents the accelerated decline in lung function observed in COPD patients during acute exacerbations. As indicated by the diagram the lung function lost during an exacerbation is never fully regained.

The incomplete recovery of lung function after an exacerbation means that the patient may not regain their stable lung function, which will contribute to the decline in lung function with time. Further evidence of incomplete exacerbation recovery was recently provided by a study of patients with chronic bronchitis; recovery of quality-of-life scores was incomplete after infective exacerbations, especially when the exacerbation recurred during the study follow up (Spencer & Jones, 2003)

Cigarette smoking is therefore still the most important factor in COPD progression. A study of 5,887 patients in the Lung Health Study showed that COPD smokers with chronic bronchitis had exacerbations 1.6-1.9 times more frequently than those without (Kanner *et al.*, 2001). This suggests factors associated with smoking and chronic bronchitis predispose patients to exacerbation, possibly due to reduced mucociliary clearance (Mossberg *et al.*, 1986), which facilitates bacterial invasion of the mucosa (Wilson *et al.*, 1985). In addition it has been shown that lower respiratory infection promoted FEV_1 decline in current smokers but not ex-smokers with mild COPD (Kanner *et al*, 2001). These studies emphasise the importance of targeting COPD exacerbations to reduce disease progression. The findings also indicate that smoking is an important factor in exacerbations and therefore COPD progression.

1.3.2 Airway inflammation at exacerbation

COPD patients are characterised by varying degrees of inflammation affecting both the large and small airways as well as the alveoli, resulting in mucus hypersecretion, airway narrowing and alveolar destruction. This chronic 'background' inflammation is thought to be heightened during exacerbations. Increases in various inflammatory cells and inflammatory markers have been found during COPD exacerbation, and include inflammatory cytokines interleukin-6 (IL-6), IL-8, the neutrophil chemoattractant IL-8, leukotriene b_4 (LTb_4) and neutrophil elastase (Bhowmik et al., 2000, Roland et al., 2001,

Hill et al., 2000). However the mechanisms involved in exacerbations and their effects on the disease are poorly understood.

COPD exacerbations have been associated with a number of etiological factors, including infection and pollution. COPD exacerbations are frequently triggered by upper respiratory tract infections, which are more common in the winter months. It is also possible that patients are more susceptible to exacerbations in the winter, as lung function in COPD patients shows small but significant decreases with reduction in outdoor temperature (Donaldson et al., 1999). COPD patients have also been found to have more hospital admissions during times of increased environmental pollution (Anderson et al., 1995).

1.3.3 Role of bacteria in exacerbations of COPD

The association between bacterial colonisation and COPD has been studied for many years (May, 1953). Bacterial colonisation of the distal airways with pathogenic and non-pathogenic agents has been observed in numerous studies (Marcy *et al.*, 1987, Irwin *et al.*, 1982, Riise et al., 1994). However the role of bacteria in disease progression remains relatively unclear. Bacteria are frequently found in sputum and lower airways samples using protected brush techniques from stable COPD patients (Soler *et al*, 1999, Cabello *et al.*, 1997). Evidence suggests that airway inflammation increases with higher airway bacteria loads. In particular, Monso *et al.*, (1995) showed elevated airway bacterial load in quantitative sputum cultures from patients with COPD. Other studies indicate that up to one third of COPD patients are colonised at any one time (Zalcain et al., 1999, Murphy et al., 1992). H. *Influenzae* and S. *pneumoniae* are the organisms most commonly detected in the lower airways. Lower airway bacterial colonisation can result in activation of the host defences leading to the release of inflammatory cytokines and subsequent neutrophil recruitment, mucus hyper secretion, impaired mucociliary clearance and respiratory

epithelial cell damage (Adler *et al.*, 1986, Wilson *et al.*, 1985). Studies of stable chronic bronchitis patients have shown airway bacterial load to correlate with neutrophils, markers of neutrophilic inflammation and TNFα (Hill *et al.*, 2000, Soler *et al.*, 1999). Further, Monso *et al* (1995) compared patients during exacerbation matched to patients in a stable clinical state and showed marked increases in pathogen recovery and bacterial load, which has also been shown in other bronchoscopy studies (Fagon *et al.*, 1990, Soler *et al.*, 1998). Most microbiological studies find the same organisms within COPD patients when they are clinical stable or suffering an exacerbation. Background colonisation and inflammation in the clinically stable state of COPD patients has led to uncertainty concerning the role of bacteria during exacerbations (Hirschman *et al.*, 2000). Inconclusive results from placebo-controlled trials of antibiotics for exacerbations raise further concerns regarding the importance of bacteria in exacerbations (Nicotra *et al.*, 1982, Sachs *et al.*, 1995). However, new molecular techniques have recently shown that bacterial colonisation is not stable and frequent turnover of discrete strains occurs (Murphy *et al.*, 1999). It has been suggested that changes in strains, rather than continual bacterial colonisation, could be responsible for the symptoms of exacerbations (Aaron *et al.*, 2001).

1.3.4 Role of viruses in exacerbations

Lower respiratory tract viral infections in COPD patients can also cause direct damage to airway epithelium. Hegele *et al* (1995) showed viral infections can result in loss of ciliated epithelium and increased mucus production, leading to airway narrowing and hyperresponsiveness. This altered environment may also promote secondary bacterial infections. Viral infections are found to be associated with increased isolations of H. *influenzae* and S. *pneumoniae* (Poole *et al.*, 2000)

A recent study by Seemungal and colleagues (2000), found rhinovirus in sputum from the lower airways was associated with 23% of COPD exacerbations. The presence of

rhinovirus was also associated with elevated levels of IL-6 in the lower airways (Seemungal *et al.*, 2000), while other studies have found no difference between healthy individuals and COPD patients (Smith *et al.*, 1980, Greenberg *et al.*, 2000). The contribution of viruses to acute exacerbations of COPD still remains to be determined although vaccination, especially against influenza has been shown to be of clinical benefit in COPD patients (Poole *et al.*, 2000).

1.3.5 Role of pollution in exacerbations

Although there is considerable epidemiologic data that increased pollutants are associated with COPD hospital admissions, the mechanisms involved are largely unknown. As COPD exacerbations are closely linked to respiratory infections, the hypothesis that pollutants can increase susceptibility to viral infections has been proposed. One study has investigated the effects of personal exposure to NO_2 on the risk of airflow obstruction in asthmatic children with respiratory infections (Lineaker *et al.*, 2000). This study suggested that with higher personal pollutant exposure, there was a greater risk of an asthmatic exacerbation following a respiratory infection. Thus, similar mechanisms may be operating in patients with COPD, and further studies are required on the association of pollution and infection.

1.4 The innate immune response of the respiratory epithelium

The airway epithelium represents a primary site for the introduction and deposition of potentially pathogenic microorganisms into the body. The innate immune response of the respiratory epithelium is therefore vital for the resolution of bacterial infections. The initial line of defence by the epithelium is to form a barrier, which separates the luminal surface of the airways from the basolateral surface. Secondly, the columnar epithelial cells, which form this protective barrier, are also ciliated and the co-ordinated beating of the cilia clears much of the material inhaled into the lungs. Thirdly, sub mucosal glands within the

epithelia contain secretory cells including mucus and serous cells, which constitutively express a wide range of peptides, mucin glycoproteins and organic molecules. The secretory products form a mucus that provides the airways with a constantly present antimicrobial milieu. In response to bacterial infection the airway epithelium can increase the production of antimicrobial agents and also induce a signalling network to recruit phagocytic cells (Kaliner *et al.*, 1991).

1.4.1 Acute infections

Initiation of acute infections occurs when the primary lung defence is overcome and the secondary host defence system is activated leading to neutrophil infiltration and inflammation within the lung. Bacterial factors such as lipopolysaccharide (Wilson *et al.*, 1985) and PhoE (Kilian *et al.*, 1990) facilitate bacterial adherence to the epithelium. Evidence has shown that bacterial adherence to bronchial epithelial cells stimulates the release of pro-inflammatory cytokines such as IL-8 and TNFα. However in some individuals bacterial clearance is impaired and bacterial colonisation ensues. Colonisation is generally associated with a defect in mucociliary clearance related to ciliary dysfunction, epithelial destruction or abnormal mucus production. Failure to remove bacteria from the lung delays bacterial clearance and provides a suitable environment for bacterial replication. In COPD, patients often have altered or a reduced ciliated epithelium and increased mucus production (MRC, 1965, Snider *et al.*, 1985). Low-grade inflammatory processes, probably associated bacterial colonisation, are therefore often observed in patients with chronic bronchitis and emphysema in the stable clinical state.

1.4.2 The Acute inflammatory response

It is generally recognised that the rate of removal of particles from alveolar surfaces by mechanical means is very slow, in contrast to the more rapid tracheobronchial mucociliary

system. It is therefore the role of the alveolar macrophage that determines the effectiveness of the innate immune response. The macrophage constitutes ~85% of all alveolar inflammatory cells retrieved by bronchoalveolar lavage (Sibille & Reynolds, 1990). Macrophages possess various receptors on the cell surface such as the toll-like receptors (TLR), and scavenger receptors, which can recognise bacterial products. Alveolar macrophages are avidly phagocytic and engulfed microorganisms are subjected to a wide range of toxic intracellular molecules, including superoxide anion, hydroxyl radicals, peptides and lysozyme. Alveolar macrophages respond to the usual daily challenges of bacteria entering the terminal airways. Initiation of acute infections occurs when the primary lung defence is overcome and the secondary host defence system is activated leading to neutrophil infiltration and inflammation within the lung. Under these conditions the alveolar macrophages release pro-inflammatory cytokines, which leads to the recruitment of neutrophils. These cytokines include IL-8 a major neutrophil chemoattractant, and TNFα, which further enhances neutrophil migration by up-regulating the expression of endothelial cell adhesion molecules, such as P- selectin.

Neutrophils move rapidly from the marginating pool in the pulmonary vasculature into the alveolar spaces in response to inflammatory stimuli. Emigration of the neutrophil begins with the cell rolling along the endothelial surface of the blood vessel. This is facilitated by interactions between L-selectin on the neutrophil surface with E- and P-selectins on endothelial cells (Ley *et al.*, 1995, Simon *et al.*, 1995). Signalling via these selectins or stimulation by inflammatory mediators such as LTB_4 and IL-8 (Simon *et al.*, 1995, Borregaard *et al.*, 1994) cause the neutrophil to rapidly change from an L-selectin presenting cell to a CD11b/CD18 (β2 integrin) expressing cell. CD11b/CD18 presentation causes the neutrophil to firmly adhere to the endothelium (Huges *et al.*, 1992). Activation of neutrophils also makes them more responsive to chemotactic agents and under the influence of LTB_4 they exit the circulation via diapedesis and up the chemotactic gradient

to the inflammatory site. Neutrophils also have receptors for opsonins allowing enhanced phagocytosis and can participate in the inflammatory response via secretion of pro-inflammatory cytokines such as TNFα.

It has also been demonstrated that the alveolar epithelial cells actively participate in the inflammatory response during bacterial infection (Crestani & Aubier, 1998). Indeed these cells are able to synthesis cytokines and acute phase proteins. These cells are also activated by alveolar macrophage products such as TNFα, which increase their membrane fluidity allowing soluble surface scavenger receptor, CD14 to the membrane, which is important for TLR signalling.

1.5 Inflammatory factors involved in the in innate defence

All cells present in the alveoli participate to some extent in the inflammation of the innate immune response and therefore need to communicate with each other if an effective host defence is to be mounted. Mechanisms are therefore required to initiate this inflammatory response, but also to localise, reinforce, and ultimately resolve it. Some of the essential components of the immune system that play a critical role in these processes are the soluble factors of innate defence.

Complement activation

The early events of complement activation can be triggered by one of three pathways. The classical pathway is activated by antigen-antibody complexes, the alternative pathway by microbial-cell walls, and the lectin pathway by the interaction of microbial carbohydrates with manose-binding protein in the plasma. The outcome is the generation of opsonins (C3b) chemoattractant (C5a) or membrane attack complex (C5b, C6, C7, C8 and C9), which proliferates cell membranes leading to cell death.

Acute phase proteins

The molecules collectively referred to as acute-phase proteins enhance resistance to infection and promote repair of damaged tissue. In addition to some complement proteins, the acute phase proteins include C-reactive protein, serum amyloid A protein, proteinase inhibitors, and coagulation proteins.

Cytokines

Cytokines are extracellular signalling proteins, usually of <80 kDa in mass and are produced in different cell types involved in cell-cell interactions. They affect closely adjacent cells, and therefore function in a predominantly paracrine fashion, but may also act on distant cells. Cytokines are rarely produced individually; rather, they are produced along with other cytokines in patterns characteristic of particular diseases. The effects of individual cytokines can also be influenced by other cytokines released simultaneously from the same cell or from target cells following activation by the cytokine, inducing synergistic or antagonistic effects (Chung, 2001). The effects of cytokines are mediated by their binding to high affinity cell surface receptors. Depending on the functional response initiated, cytokines are classified as pro-inflammatory cytokines, chemoattractant cytokines (chemokines), anti-inflammatory cytokines or growth factors.

Pro-inflammatory cytokines are predominantly produced by activated immune cells and are involved in the amplification of inflammatory reactions by up-regulating the expression of various cytokines, adhesion molecules, proliferation and recruitment of inflammatory cells. Two of the most important pro-inflammatory cytokines are TNFα and IL-β.

Chemokines are chemotactic cytokines of 8-10 kDa involved in attracting leukocytes into tissues. The chemokine families are named according to the structure of conserved

cysteine-containing motifs. The two major families are CXC chemokines, in particular IL-8 is important in attracting and activating neutrophils, and CC chemokines involved in attraction of eosinophils and monocytes.

Anti-inflammatory cytokines such as IL-10 and IL-1 receptor antagonist are involved in the regulation and resolution of inflammatory reactions once the inflammatory agent has been cleared, by inhibition of cytokine production, antigen presentation and macrophage phagocytosis.

Growth factors such as transforming growth factor β (TGFβ) and epidermal growth factor (EGF) influence the proliferation of many structural cells, such as fibroblasts and airway smooth muscle cells, and the turnover of matrix proteins. They may be involved in airway remodelling processes (Border et al., 1994, Brewster et al., 1990, Takeyama et al., 1999).

Since the pathology of COPD is that of a chronic inflammatory process with tissue damage and repair processes, it is not surprising that many cytokines may play a role in this condition.

1.5.1 Tumour necrosis factor α

TNFα has long been regarded as a pro-immune cytokine involved in antimicrobial type-1 immunity. However, the precise role of TNFα in bacterial infections remains poorly understood. TNFα was originally investigated for its anti tumour activity (Carswell *et al.*, 1975). It is now known that TNFα has a far wider range of actions including tissue remodelling, activation of macrophages and up-regulation of adhesion molecules (Beutler, 1995, Brasier, 1996). TNFα is primarily produced by activated macrophages but is also secreted by other cell types including T cells, dendritic cells and epithelial cells (Vassalli, 1992). The TNFα gene is tightly regulated at both the transcriptional and translational

level. TNFα production typically starts with the binding of a ligand, commonly microbial products, to cell surface TLRs. Activation of TLRs stimulates a signal transduction pathway that activates transcription factors such as nuclear factor-κB (NF-κB). Epithelial cells, macrophages and dendritic cells are integral participants in the innate defence and therefore have a high proportion of TLRs for ligand binding.

Human TNFα is synthesised as a 26 kDa membrane bound protein composed of 233 amino acids. The TNFα ectodomain is cleaved at the cell surface by a specific metalloproteinase TNFα converting enzyme (TACE/ADAM17) to yield a monomeric soluble form of 17 kDa (Black *et al.*, 1997). After separating from the cell membrane, soluble TNFα aggregates into tri-molecular complexes (51 kDa homodimers) that subsequently bind to the receptors. Both soluble and membrane bound forms of TNFα are biologically active and exert their effects via binding to TNFα receptors (TNFR) 1 and 2. TNFR1 is widely expressed whereas TNFR2 is expressed predominantly on leukocytes and endothelial cells (Smith *et al.*, 1990). TACE also cleaves the extracellular domain of the TNFα receptors forming soluble TNFRs that are free to bind to tri-molecular TNFα rendering it biologically inactive.

Activation of the TNFα receptors can lead to a multitude of responses. On one hand TNFα can regulate cell survival or apoptosis via activation of caspases by TNFR1 and on the other TNFα can also activate inflammation and cell survival by activation of NF-κB dependent genes via TNFR2 (see figure 1.5) (Liu *et al.*, 1996). The exact timing and extent of TNFα activation and receptor expression must therefore be important in the varied responses evoked by TNFα.

In terms of inflammation, TNFα is one of the most potent physiological inducers of NF-κB, which in turn co-ordinates numerous genes in response to pathogens and pro-inflammatory cytokines (Javelaud *et al.*, 2000). NF-κB is a multi-protein kinase complex

Figure 1.5

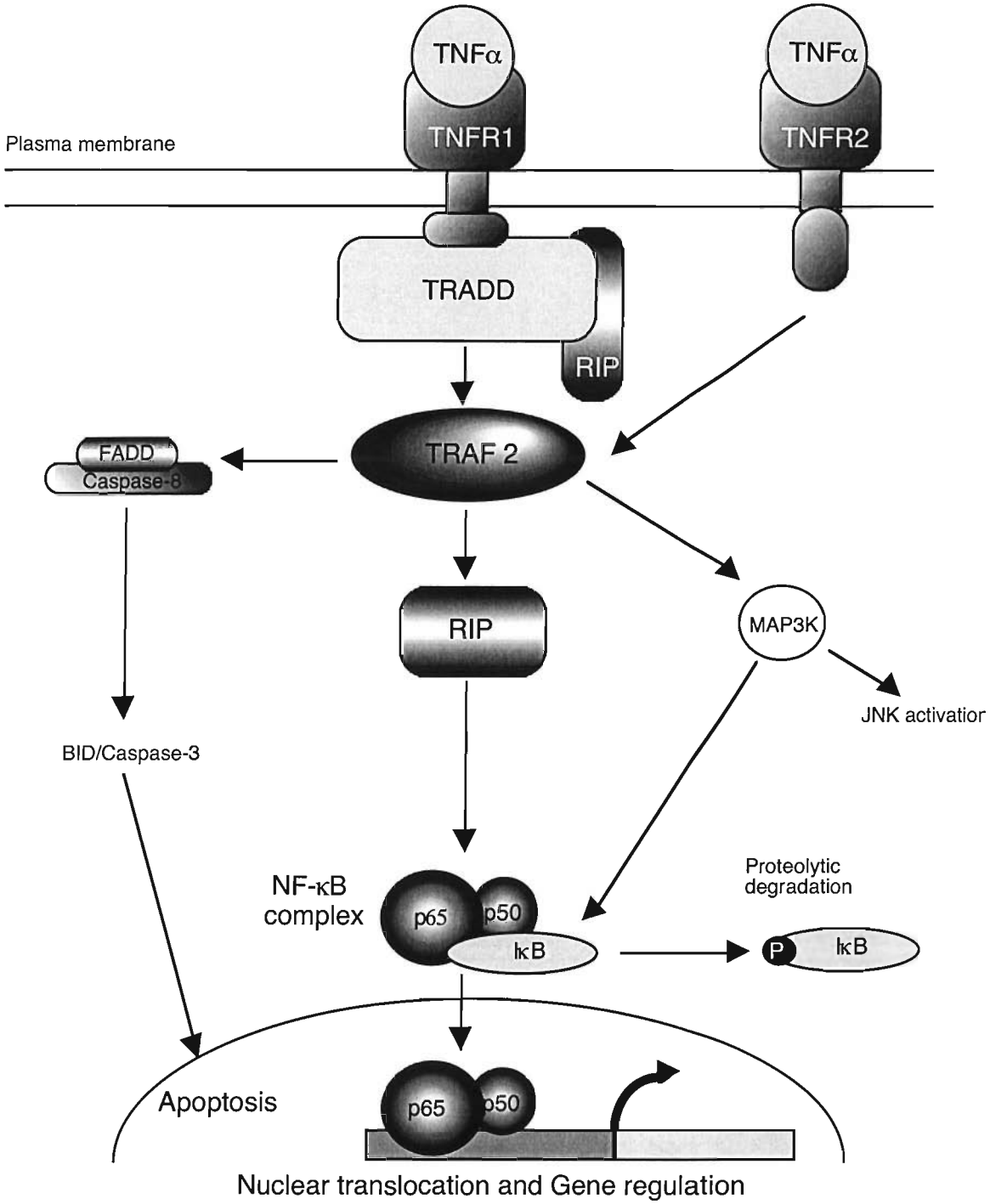


Figure 1.5 Intracellular signalling pathways downstream of TNFα. Binding of TNFα to TNFR1 results in the configuration of TRADD (TNFR-associated death domain) and FADD (Fas-associated death domain). TRADD complex recruits the adaptor protein TRAF-2 (TNFR-associated factor 2) whereas FADD stimulates the caspase cascade. Known downstream signalling molecules that interact with TRAF-2 are NIK (NF-κB-inducing kinase), RIP (receptor-interacting protein) and ASK1 (apoptosis signalling-related kinase 1) and these are capable of channelling signals towards cell death and inflammation. Binding of TNFα to TNFR2 recruits the adaptor protein TRAF-2, which directly activates the inflammatory cascade via the generation of NF-κB or p38 MAPK (mitogen-activated protein kinase).

consisting of two subunits with kinase activity, I-κB kinase 1 and 2 (p65 and p50) and a NF-κB modulator NEMO, required for activation of the kinase subunits. NF-κB is kept inactive in the cytosol through interaction with its inhibitor I-κB which masks the nuclear localisation sequence of NF-κB. Following the binding of TNFα to its receptor via the signalling mechanisms shown in figure 1.5, NF-κB is activated by the phosphorylation of IκB and its subsequent degradation. Once activated NF-κB is able to translocate to the nucleus and activate its target genes. In addition to TNFα, these target genes include interleukin-1 (IL-1) and interleukin-6 (IL-6), which are cytokines with some overlapping functions with TNFα. TNFα signalling is particularly important in the immune sysytem, as TNFRs mediate many of the apoptotic and survival signals in lymphocytes and other hemopoitic cells (Baud et al., 2001).

1.5.2 Bacterial lipopolysaccharide

Bacterial lipopolysaccharide (LPS) is the major structural component of the outer wall of gram-negative bacteria and is a potent initiator of the acute inflammatory response. Alveolar macrophages have long been considered to be the major cell type in the lung involved in LPS recognition (Koay *et al.*, 2002). However, epithelial cells have also been shown to express receptors necessary for the recognition of LPS (Becker *et al.*, 2000). It is therefore unresolved whether the airway epithelium plays an active or passive role in the response to endotoxins such as LPS.

The recognition of pathogen is mediated by a set of receptors referred to as pattern-recognition receptors. The three functional classes of pathogen-recognition receptors are signalling receptors, endocytic receptors, and secreted proteins. Studies over the years have demonstrated that a family of signalling receptors, known as the Toll-like receptors (TLRs), play a crucial role in mammalian host defence. In particular TLR4 recognises LPS

from gram-negative bacteria. Recent genetic and biochemical experiments have highlighted the critical role of TLRs in LPS-induced NF-κB activation (Chow *et al.*, 1999, Jiang *et al.*, 2000, Nomura *et al.*, 2000).

LPS recognition is mediated, in part, by CD14 (Pugin *et al.*, 1993, Wright *et al.*, 1990). CD14 is expressed as a 55 kDa protein in two forms; the soluble form (sCD14) is found in serum, and the membrane-bound form (mCD14) is tethered to the cell surface by a glycosylphosphatidylinositol linkage. Originally characterised on phagocytic cells, respiratory epithelial cells have also been demonstrated to express CD14. Neither of the forms of CD14 have intrinsic signalling properties as they lack a transmembrane domain (Pugin *et al.*, 1993). Although mCD14 requires TLR-4, sCD14 requires both LPS-binding protein (LBP) and TLR4 to induce downstream signalling cascades (Fenton *et al.*, 1998). It is widely believed that mCD14 transfers LPS to its high-affinity receptor, TLR4 (da Silva *et al.*, 2002, Hoshino *et al.*, 1999). On binding of LPS to TLR4 via CD14, a number of molecules are recruited to the receptor to mediate NF-κB activation (see figure 1.6, over the page). First the adaptor molecule myeloid differentiation factor 88 (MyD88) binds to the receptor and interacts through its death with the protein kinase IL-1 receptor-associated kinase (IRAK), which in turn, recruits the adaptor TRAF-6 to the receptor complex (Hatada *et al.*, 2000). Evolutionarily conserved signalling intermediate in Toll pathways (ECSIT) bridges TRAF-6 to MEKK1. Downstream signalling components MEKK1, MEKK2, and MEKK3 or NIK, which are activated by TAK1, link the receptor complex and phosphorylation of IKK-α and IKK-β in the IKK complex. The IKK complex then phosphorylates IkB-α, IkB-β, and IkBε at amino-terminal serines and results in degradation of the inhibitors by the ubiquitin-proteasome pathway and the subsequent release of NF-κB. Translocation of the activated transcription NF-κB into the nucleus leads to gene expression, in particular expression of the cytokines TNFα and IL-1β,

Figure 1.6

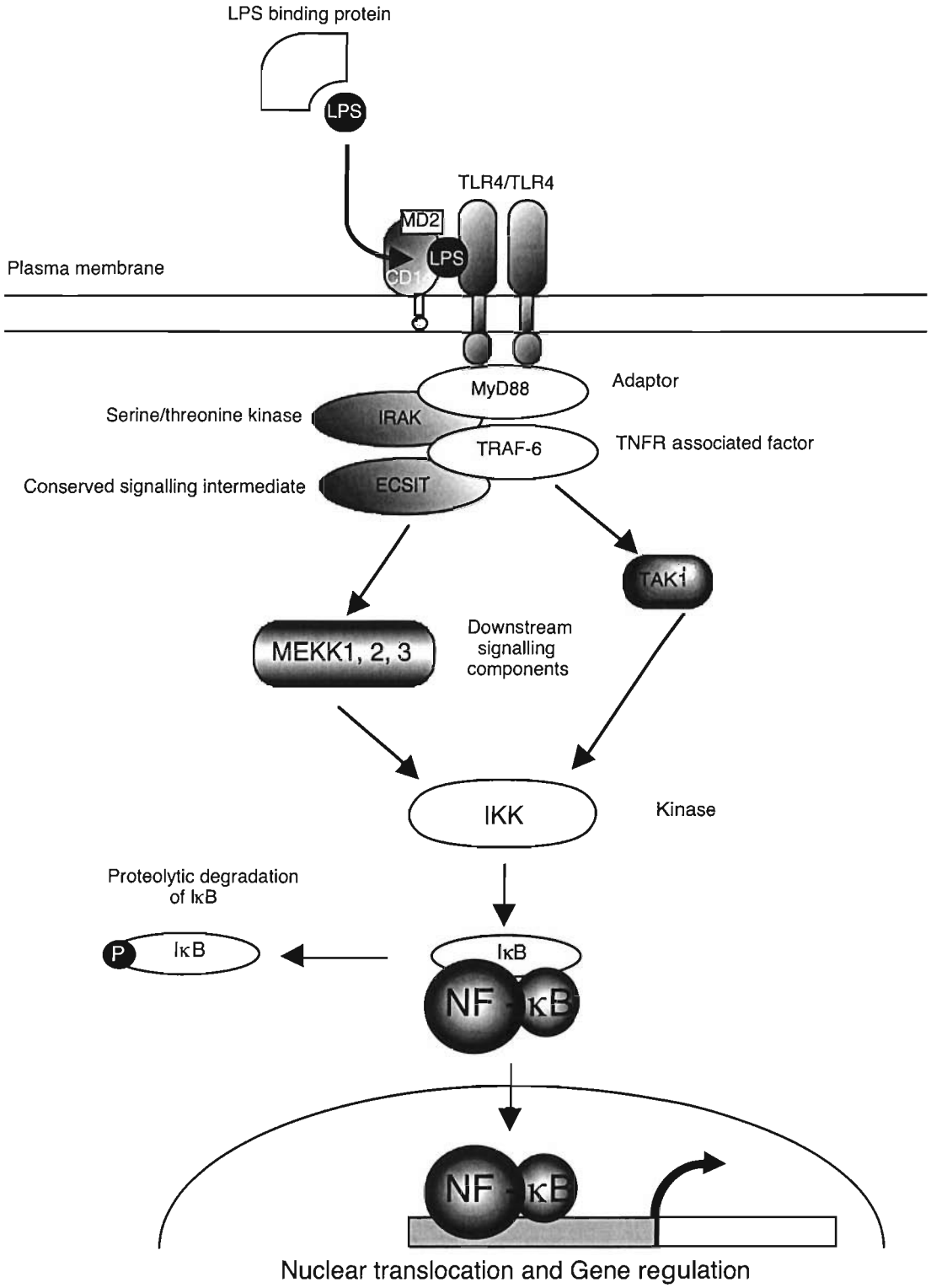


Figure 1.6 Signalling pathway linking Toll-like receptor-4 (TLR-4) to NF-κB. On binding of LPS/LPS binding protein to TLR-4 via CD14, the adaptor molecule myeloid differentiation factor 88 (MyD88) binds to the receptor and interacts through its death domain with the IL-1 receptor-associated kinase (IRAK), which, in turn, recruits the adaptor TRAF-6 and the evolutionarily conserved signalling intermediate in Toll pathways (ECSIT) to the receptor complex. Downstream components MEKK1, MEKK2, and MEKK3 or NIK, which are activated by TAK1, link the receptor complex and phosphorylation of K-α and IKK-β in IKK complex. IKK complex phosphorylates IκB-α, IκB-β, and IκBε at amino-terminal serines and results in degradation of the inhibitors by the ubiqutin-proteasome pathway and the subsequent release of NF-κB.

chemokines and adhesion molecules (Hirano *et al.*, 1997). However the cascade of cytokines released in response to LPS in the human lung are unknown.

1.8 Inflammation and oxidative stress

There is overwhelming evidence that COPD is associated with airway and airspace inflammation. Numerous markers of inflammation have been shown to be elevated in the sputum of patients with COPD, such as IL-8 and TNFα (Keating *et al.*, 1996). Oxidative stress within the lungs can also activate oxidant sensitive transcription factors such as nuclear factor-κB and activator protein-1 (AP-1) that are important for gene transcription of inflammatory cytokines such as TNFα, IL-8 and IL-6 in COPD. Therefore oxidative stress is thought to be an important component of inflammation and may contribute to disease progression. The presence of oxidative stress also has other important consequences for the pathogenesis of COPD; these include oxidative inactivation of antiproteinases, airspace epithelial injury and increased sequestration of neutrophils in the pulmonary microvasculature.

Elevated oxidative stress in COPD patients is derived from the increased burden of oxidants present in cigarette smoke. Cigarette smoke is a complex mixture of chemical compounds with the majority consisting of free radicals and other oxidants (Pryor *et al.*, 1993). The epithelial lining fluid and mucus are the first line of defence in the lungs against inhaled oxidants; they act by quenching many of the short-lived radicals in the gas phase of cigarette smoke. The major anti-oxidants in the respiratory tract lining fluid include mucin, glutathione, uric acid, protein (largely albumin) and ascorbic acid (vitamin C) (Cross et al., 1994). The direct increase in the oxidative burden produced by inhaling cigarette smoke is further enhanced in the lungs of smokers by the release of reactive oxygen species released from inflammatory leucocytes recruited into the lungs. In fact studies have shown elevated levels of oxidants such as O_2^- and hydrogen peroxide are

released from the leukocytes of smokers, compared to those of non-smokers (Mac Nee *et al.*, 1989, Morrison *et al.*, 1998).

Oxidative stress also plays a role in the proteinase anti-proteinase balance as the release of proteinases such as elastase from recruited leukocytes elevates the proteolytic burden within the lung. This proteolytic burden is further enhanced as the cognate innate inhibitor of elastase, α_1-AT, is inactivated by oxidation of the Met residue 353 at its active site by oxidants in cigarette smoke (Hubbard *et al.*, 1987). Another major inhibitor of neutrophil elastase in secretory leukoproteinase inhibitor, which is also inactivated by oxidants (Kramps *et al.*, 1991). However it is clear that the imbalances between increased elastase burden in the lungs and a functional deficiency of α_1-AT due to its inactivation by oxidants is an over simplification, not least because other proteinases and antiproteinases such as the MMPs and TIMPs are likely also to have an important role.

1.9 COPD management

Smoking cessation is the only treatment in patients with COPD that is effective in slowing down disease progression. The lung health study found that patients who stopped smoking successfully had significantly lower rates of decline in FEV_1 than patients who continue to smoke (Willemse *et al.*, 2004). One of the reasons smoking cessation is so important is that no pharmacological treatment is available to reverse the deterioration of lung function found in COPD patients.

Current therapies to limit lung damage during COPD exacerbations include oral corticosteroids and bronchodilators to reduce dyspnea and antibiotics. Antibiotics have traditionally been the frontline defence in COPD exacerbations with studies showing greater treatment success with antibiotics than placebo (Anthonisen *et al*, 1987). A study by Seemungal *et al* (2000) showed treatment with prednisolone during exacerbation prolonged the time to the next exacerbation, reducing exacerbation frequency. Prevention

of COPD exacerbations would not only reduce airway inflammatory changes but would also have important social economic benefits. For frequent exacerbators, vaccinations and long-term antibiotic treatment are therefore used as preventative measures. There has also been much research into the use of macrolide antibiotics (Suzuki *et al*, 2002), mucolytic agents (Poole *et al*, 2000) and inhaled steroids (Burge *et al*, 2000). All of these studies have shown some benefit to the prevention of exacerbations although further study is required. Therefore any future preventative measure against exacerbations or a reduction in their frequency would have a considerable benefit to patients with COPD.

1.10 Aims

It has been highlighted in this chapter that COPD is a progressive inflammatory disease of the lung, characterised by a chronic and irreversible decrease in lung function. In addition to the chronic inflammatory nature of the disease many studies have described acute episodes of inflammation during exacerbations. However it remains unclear exactly how exacerbations lead to accelerated decline in lung function. This thesis aims to investigate the contribution of cytokines, in particular TNFα, in the acute inflammatory response and how they are affected by disease status. In addition we aim to assess if the mediators involved in acute inflammation are stimuli specific and uniform between different species. Finally, moving away from acute inflammation, we also aim to look at differences in protein expression in individuals with a range of lung functions using the extremely powerful proteomic approach iTRAQ.

Chapter 2.

Materials and methods

2.1 Chemicals and Reagents

2.1.1 Materials

Thermoprime plus DNA polymerase was purchased from **ABgene** (Epsom, UK). Full range molecular weight marker, streptavidin horseradish peroxidase conjugate and ECL Western blotting detection agents were purchased from **Amersham** (Amersham, UK). Chromogenes for immunohistochemistry; aminoethylcarbozole (AEC) liquid system and diaminobenzedine (DAB) liquid system were purchased from **BioGenex** (San Raman, USA). Trans-blot nitrocellulose was purchased from **BIO-RAD** (Hertfordshire, UK). Butanol, Ethanol, glacial acetic acid, methanol and sodium chloride were purchased from **Fisher Scientific UK Ltd**, (Leicestershire, UK). Methyl benzoate, tris, hydrochloric acid and acetone, were purchased from **Merck** (West Drayton, UK). For fixing samples for immunohistochemistry, a JB-4 embedding kit comprising of solution A: butoxyethanol monomer, solution B: NN-dimethylanaline monomer in polyethylene glycol and catalyst: benzoyl peroxide was purchased from **Park Scientific Ltd** (Northampton, UK). Super signal-chemiluminescent substrate was purchased from **Pierce** (Rockford, USA). Human β-actin, IL-10 and TNFα PCR primer pairs from **R&D Systems Europe Ltd** (Minneapolis, USA). DNA extraction was preformed with DNeasy tissue kit purchased from **Qiagen** (Germany). 30% Acrylamide/bis acrylamide solution, agarose, bovine serum albumin, bromophenol blue, Coomassie brilliant blue, ethidium bromide, Enhanced avian RT first strand synthesis kit, gentamycin, human serum albumin, PCR core kit with *Taq* DNA polymerase, Penicillin, RPMI 1640 medium, protease inhibitor cocktail containing 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), transepoxysuccinyl-L-leucyl-amido (4-guanidino) butane (E-64), bestatin, leupeptin, aprotinin, and sodium EDTA, N,N,N'N' tetramethyl ethylenediamine (TEMED), 3,3',5,5' tetramethylbenzidine (TMB) liquid substrate system, and all other chemicals not listed here were purchased from **Sigma-Aldrich Inc**, (USA).

Table 2.1 Table of antibodies used

Epitope	Clone	Procedure	Source
TNFα	MAB	WB	R&D Systems
IL-6	MAB	WB	R&D Systems
IL-10	MAB	WB	R&D Systems
IL-8	MAB	WB	R&D Systems
IgG isotype control	MAB	WB	R&D Systems
Human serum albumin	Polyclonal	IA (capture)	DAKO
Human serum albumin	MAB	IA	DAKO
Human serum albumin- HRP	Polyclonal	IA	DAKO
Dinitrophenyl	Polyclonal	IA	Sigma

Table 2.1 Proceedures denoted by abbreviations; WB= Western blot, IA= ELISA.

2.1.2 Buffers and Solutions

The following buffers were used for Western blotting: **Running buffer** for the SDS-PAGE gel (0.375M Tris and 1% SDS at pH 8.8). **Stacking buffer** (0.125M Tris and 1% SDS at pH 6.8). **5X Sample buffer** (0.6M Tris, 1% SDS and 50% glycerol). **Electrode buffer** (0.025M Tris, 0.192M Glycine and 0.1% SDS at pH 8.3). **Coomassie blue stain** (0.5% Coomassie brilliant blue in 10% glacial acetic acid and 45% methanol); Gels were destained in 10% glacial acetic acid and 10% methanol. **Towbins buffer** for electrophoretic transfer (25mM Tris, 190mM glycine and 20% methanol). The following buffers were used for tissue culture: **RPMI 1640** supplemented with 1% penicillin, 1% streptomycin and 1% gentamycin;. **Dulbecco's Phosphate buffered saline (PBS)** contained 0.1M NaCl, 2.7mM KCL, 1.8mM KH_2PO_4 and 10mM Na_2HPO_4. **Tyrodes buffer** contained. The following buffers were used for ELISA: **0.1% PBS-Tween** (0.1% Tween in PBS/, **2% PBS-Tween** (2% Tween in PBS). The following buffers were used for ELISAs: **Tris buffered saline** (0.13M NaCl, 50mM Tris and 40mM HCl, pH 7.6); **TBST wash buffer** (50mM Tris, 40mM HCl, 150mM NaCl, and 0.05% Tween 20, pH 7.6); **Coating buffer** (0.1mM $NaHCO_3$ 0.1mM Na_2CO_3 at pH 9.6). **Blocking solution** contained 30% Soya milk in PBS.

2.2 Preparation of clinical samples

2.2.1 Characterisation of human lung tissue

Human lung tissue was collected with ethical consent from patients undergoing resection for carcinoma; tissue used was from the normal margin from around the tumour site. Data were collected regarding the patient's lung function, smoking history, asthmatic state, age, gender, and medication where possible.

2.2.2 Preparation of human lung tissue for primary cell culture

Human lung tissue was finely chopped into fragments using dissection scissors during several washes with Tyrode's buffer containing 0.1% sodium bicarbonate. Fragments were incubated in RPMI medium supplemented with 1% penicillin, streptamycin and gentamycin at 37°C in 5% CO_2/air for 16 hours and experiments set up the following day. Methods for tissue culture experiments can be found in the appropriate chapters.

2.2.3 Preparation of lung tissue homogenates for analysis

Fragments of characterised human lung were finely chopped using dissection scissors and weighed. An ice-cold solution of PBS containing 1% Tween and commercially available protease inhibitor cocktail (100µl PBS-Tween/PIC) was added to 30mg of tissue (1ml of inhibitor cocktail inhibits the equivalent of 1mg of USP pancreatin). The samples were homogenised on ice using a XL-2020 sonicator set at a amplitude of 3 microns; 12 cycles for 10 seconds sonication followed by 20 a second rest period. Following sonication, samples were centrifuged at 15,000 g for 15 minutes at 4°C, and supernatants removed for storage at –70°C prior to analysis.

2.2.4 Oxidation and derivatisation of the HSA and human tissue

A stock solution of 30mg/ml of HSA was oxidised with equal volumes of 9% hydrogen peroxide and incubated at room temperature for 30mins. Oxidised HSA was then derivatised using an adapted method previously described by Buss et al (1997), briefly, 100µl of the oxidised HSA was then derivatised with 100µl of 10mM DNPH in trifluroacetic acid and100µl of H_2O. Samples were then incubated at room temperature for 45 mins, with vortexing every 10-15 mins. Derivatised protein was then precipitated on ice with 10% trichloroacetic acid for 30 mins. Following which the sample was centrifuged at 15,000 g for 5 mins and the supernatant removed. The pellet was then washed 3 times with 100µl of ethanol/ethyl acetate (1:1) and then allowed to dry. Finally the pellet was broken up with sonication and re-suspended in 0.5mls of 6M guanidine hydrochloride in 0.5M potassium phosphate (pH 2.5). The A_{375} was then measured and the carbonyl content of the oxidised HSA standard was then determined using ε_{375} 22,000M^{-1} cm^{-1} (Levine *et al.*, 1990). For baseline human tissue all samples were derivatised using the derivatisation method above.

2.3 Analysis of cytokines levels.

2.3.1 Tumour necrosis factor α, interleukin 1β and interleukin-13 ELISAs

TNFα, IL-1β and IL-13 levels were measured in human lung supernatant using commercially available ELISAs from RnD Systems (Minneapolis, MN). The limit of detection for the TNFα assay was 0.31 pg/ml, 0.07 pg/ml for IL-1β and 0.5 pg/ml for IL-13. The assays were carried out as per manufacturers instructions. Briefly, a 96 microtitre plate was coated with coating antibody for either human TNFα/IL-1β in PBS for 16 hours at 4°C. 100µl of sample or standard was pipetted into the appropriate wells. Following incubation for 2 hours at 25°C, the plate was washed with 0.1% PBS-Tween, and blotted before 100µl of anti-TNFα/IL-1β/IL-13 was added to each well. Following further

incubation of 2 hours at 25°C the plate was again washed blotted and 100µl of anti-mouse IgG/ HRP conjugate added to each well and incubated for 35 minutes at 25°C. Following the final wash and blot 100µl of HRP substrate 3, 3', 5, 5' tetramethyl benzedine (TMB) was added to each well. The reaction between HRP and TMB was terminated by the addition of 1M H_2SO_4 and the optical density of each well was read at 450 nm.

2.3.2 Interleukin 5, 6, 8, and 10 ELISAs

IL-5, IL-6, IL-8, and IL-10 were all measured in human lung supernatant using commercially available ELISA Duosets from Biosource (Europe, SA). The limits of detection for IL-5, IL-6, IL-8, and IL-10 were 0.26 pg/ml, 0.28 ng/ml, 0.26pg/ml, and 0.25 pg/ml respectively. The protocol followed was identical to that in the accompanying literature. Briefly, a 96 microtitre plate was coated with the relevant coating antibody for each ELISA, in coating buffer (50 mM NaCl, 50 mM $NaHCO_3$), for 16 hours at 4°C. The plate was then washed thoroughly and blocked for 2 hours with 5% BSA in 0.1% PBS-Tween at 25°C. The plates were then washed and blotted and 100µl of sample or standard was pipetted into the appropriate wells, along with 50µl of mouse anti-human IL-5/IL-6, IL-8 or IL-10 antibody. Following incubation for 2 hours at 25°C, the plate was washed, blotted and 100µl of anti-mouse IgG/ HRP conjugate added to each well and incubated for 35 minutes at 25°C. Following the final washes and blotting, 100µl of HRP substrate TMB was added to each well. The reaction between HRP and TMB was terminated by the addition of 1M H_2SO_4 and the optical density of each well was read at 450nm.

2.3.3 Matrix metalloproteinase-9 and Tissue inhibitor of matrix metalloproteinases-1 ELISAs

MMP-9 and TIMP-1 were measured in lung supernatant using commercially available ELISA kits from Amersham Pharmacia Biotech (Buckinhamshire, UK). The limit of

detection for MMP-9 and TIMP-1 was 0.2 ng/ml and 0.5 ng/ml respectively. Briefly, 100µl of sample or standard was pipetted into the appropriate wells. Following incubation for 2 hours at 25°C the plate was washed thoroughly and blotted before 100µl of anti-MMP-9/anti-TIMP-1-peroxidase conjugate was added to each well. Following further incubation of 2 hours at 25°C the plate was again washed, blotted and 100µl of HRP substrate, TMB was added to each well. The reaction between HRP and TMB was terminated by the addition of 50µl H_2SO_4 (1M) and the optical density of each well was read at 450nm.

2.3.4 Rodent tumour necrosis factor alpha, interleukin 1β, 6 and 10 ELISAs.

TNFα, IL-1β, IL-6 and IL-10 levels were measured in rodent lung supernatant using commercially available ELISAs from R&D Systems (Minneapolis, MN). The assays were carried out as per manufacturers instructions, as previously described for the human-TNFα/IL-1β R&D Systems ELISAs. The limit of detection for the TNFα, IL-1β, IL-6 and IL-10 assay s was 0.03pg/ml, 0.05pg/ml, 0.07pg/ml and 0.02pg/ml respectively.

2.3.5 Human Serum Albumin ELISA

For measuring total levels of human serum albumin (HSA) in samples we used a HSA ELISA assay. Briefly, a 96 well plate was incubated with 14mg/ml of rabbit HSA antibody in coating buffer at 4°C for 6 hours. Following incubation, the plate was washed as described previously and incubated overnight with PBS-Tween containing 30% milk. The following day the plate was washed again and an HSA standard curve (1.5-1000ng/ml) and samples were added and incubated at 4C for 2 hours. Following incubation, the plate was washed and a rabbit anti HSA antibody conjugated to HRP was added at a concentration of 130 ng/ml for 2 hours before a final wash. The plate was developed with the HRP substrate

system (TMB), the reaction stopped with 1M H_2SO_4 and optical density read at 450nm. The limit of detection for this protocol was 0.3ng/ml.

2.3.6 Oxidised human serum albumin ELISA

To measure total levels of oxidised human serum albumin a 96 well plate was incubated with 10 ng/ml of mouse anti HSA antibody in coating buffer at 4°C for 6 hours. Following incubation, the plate was washed and incubated overnight with 0.1 % PBS-Tween containing 30% soya milk. Following the overnight block, plates were washed and a derivatised HSA standard curve (45.4 - 0.02 ng/ml) and derivatised samples added and incubated at 4°C for 2 hours. For derivatisation method for both standards and samples see method 2.2.4. Following the incubation with samples, the plate was washed and incubated with 1:5000 rabbit anti dinitrophenyl (DNP) antibody, which had a specific antibody concentration of 1.0 – 1.7 μg/μl, for 2 hours at 4°C. Finally, after washing the plate was coated with 60 ng/ml of anti rabbit HRP conjugate for 2 hours at 4°C. The plate was developed with TMB, the reaction stopped with 1M H_2SO_4 and optical density read at 450nm. The limit of detection for this was 0.02ng/ml.

2.3.7 Lactate dehydrogenase assay

LDH levels were measured in lung supernatant using a commercially available assay and LDH standard (2000-0.9 pg/ml) from Roche (Indianapolis IN, USA). To obtain total LDH levels within the tissue. Tissue was homogenised on ice using a sonicator set at amplitude of 2 microns; for 12 cycles of 10 seconds sonication followed by 20 seconds rest. Following sonication samples were centrifuged at 15,000g for 15 minutes at 4°C, and supernatant removed for storage. The limit of detection of the assay was 0.5pg/ml.

2.3.8 Bicinchonic acid (BCA) assay

Total protein levels of lung homogenates were measured using a commercially available BCA assay from BioRad using a Human Serum Albumin (HSA) standard curve. Limit of detection for HSA was 4ng/ml.

2.3.9 Endotoxin assay

Contamination of anti-IgE serum was analysed using an endotoxin assay previously described by Keler and Nowotny (1986). Briefly the method uses 1,9-dimethhylmethylene blue (DMB) which binds to lipid A, causing metachromasia. For the assay 50µl of serum was added to 1ml of dye solution (0.05mM DMB, 0.02M glycine, 0.06M NaCl, 0.05M NaOH, and 0.4% ethanol) and optical density read at 535nm. The sensitivity of the assay was 1µg of LPS.

2.3.10 Western blot analyses

Samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) in a gradient Tris-glycine gel purchased from Bio-Rad (Herts, UK). The proteins were transferred to a nitrocellulose membrane (Bio-Rad) and blocked overnight with 20% soya milk. Mouse monoclonal antibodies against TNFα, IL-6 and IL-10 were purchased from RnD Systems (Minneapolis, MN) and used at a 1:1000 dilution following the manufactures instructions. HRP Mouse monoclonal antibodies were purchased from DAKO (Denmark) and also used at a 1:1000 dilution. Proteins were visualised by Super signal west from Pierce.

2.4 Immunohistochemistry

2.4.1 Acetone fixation and GMA embedding of lung tissue.

Biopsies of human lung tissue were placed in ice cold acetone containing protease inhibitors 2mM phenylmethyl-sulphonyl fluoride (PMSF) and 20mM iodoacetamide- and

fixed overnight at –20°C. The following morning the fixative was replaced with acetone at room temperature for 15 mins, followed by methyl benzoate for a further 15 mins. The sample was then infiltrated with processing solution: 5% methyl benzoate in glycol methacrylate (JB4 solution A) at 4°C for 6 hours, during which the processing solution was changed 3 times. The sample was finally embedded by mixing JB4 solutions A and B (see 2.1.1) in the presence of the catalyst benzoyl peroxide, and polymerised at 4°C for 48 hours. Embedded samples were stored at -20°C in the presence of dessicant. Two micron sections were cut using a Leica Supercut 2065 microtome, floated out onto ammonia water (1:500), and mounted on poly-L-Lysine coated slides. To assess tissue morphology slides were stained with Toluidine blue (1% Toluidine and 1% borax).

2.4.2 Staining of GMA embedded lung tissue

Following cutting and mounting, the sections were treated with a solution of 0.1% sodium azide and 0.3% hydrogen peroxide; to inhibit any endogenous peroxidase activity. Slides were then washed and treated with blocking medium (DMEM containing 20% foetal calf serum and 1% bovine serum albumin) to inhibit non-specific binding for 30 mins. Following blocking, primary antibodies were applied at the appropriate dilutions in TBS and incubated at room temperature overnight. Following the overnight incubation the slides were washed and secondary antibodies applied and incubated for 2 hours at room temperature before being washed again. Streptavidin biotin-peroxidase complexes were added for a further 2 hour incubation. All of the antibodies used for staining of GMA embedded tissue are listed in table 2.2. Following washing, the chromogen aminoethyl carbozole (ACE) was added as per manufacturers instructions and incubated for 20 minutes at room temperature. The slides were then immersed and washed in running tap water, counter stained in Mayer's haematoxylin stain for 90 seconds and then blued in tap

water for 5 minutes. The slides were then treated with crystal mount and baked at 80°C for 20 minutes. Slides were allowed to cool and coverslips applied with DPX.

Table 2.2 Antibodies used for staining of GMA embedded lung tissue

Antibody	Clone	Epitope stained	Source
Mast cell tyrptase	AA1	Mast cell tyrptase	DAKO
CD68	PGM1	Activated macrophages	DAKO
Neutrophil elastase	NP57	Neutrophil elastase	DAKO
IL-10	23738.11	IL-10	R&D Systems
TNFα	2B3	TNFα	Biosource

Table 2.2 Lists the antibodies used for IHC and the sources they were obtained form.

2.5 Molecular Biology

2.5.1 Preparation of mRNA from human lung tissue.

Following primary cell culture, lung tissue fragments were frozen immediately and stored at -80°C prior to use. For extraction of mRNA from human lung tissue RNeasy mini kits from Qiagen were used as per manufacturers instructions. Briefly human lung tissue was homogenised in a 1.5 micro tube using a pellet pestle in the presence of a highly denaturing guanidine isothiocynate-containing buffer, which immediately inactivates RNases to ensure isolation of intact RNA. Ethanol is then added to the lysate, creating conditions that promote selective binding of the RNA to the RNeasy silica-gel-based membrane. The sample was then applied to the RNeasy mini column. Total RNA binds to the membrane and contaminants are efficiently washed away, and high quality RNA is then eluted in 30µl of RNase-free water.

2.5.2 Identification of TNFα and IL-10 by RT-PCR

TNFα and IL-10 expression in human lung after LPS stimulus was investigated using commercially available primers from R&D Systems. Primers for TNFα were designed to amplify a 414 bp product and primers for IL-10 were designed to amplify a 427 bp product. β-actin expression was examined to standardise the PCR results over the 24 hour experiment. Under normal conditions β-actin is constitutively expressed and therefore its expression should remain constant throughout the primary cell culture experiment. Commercially available β-actin primers were also sourced from R&D Systems. To ensure that there was no contamination of mRNA in samples by cellular DNA, PCR was preformed on extracted RNA, which had not been subjected to reverse transcription.

For reverse transcription of RNA into cDNA an enhanced Avain first strand synthesis kit was used and the manufacturers instructions were followed. The optimised reaction conditions for reverse transciptase have been summarised in table 2.3.

Table 2.3 Reaction conditions for reverse transcription

Reaction component	Concentration
RNA template	0.2 µg/µl
Deoxynucleotide mix	500 µM each Dntp
Oligo $(dT)_{23}$	3.5 µM
10X PCR buffer for eAMV-RT	1X
Enhanced avian RT	1 unit/µl
Nuclease free water	

Table 2.3 Lists the concentration of reaction components used for reverse transcription of RNA to cDNA.

Thermal cycling parameters for reverse transcription were as follows:

1. Incubation at 70°C for 10 minutes
2. Reverse transcription at 42°C for 50 minutes

For the optimal PCR conditions the R&D Systems' PCR primer pairs protocol was followed and is summarised below in table 2.4.

Table 2.4 Reaction conditions for PCR

Reaction component	Concentration
Taq DNA Polymerase	0.05 units/µl
10X PCR buffer with 25 mM $MgCl_2$	1X
Deoxynuclotide mix	200 µM each dNTP
Primer pairs	7.5 µM
Nuclease free water	

Table 2.4 Lists the concentrations of reaction components used for PCR

Thermal cycling parameters for `PCR were as follows:

1. Denaturing 94°C 45 seconds
2. Annealing 55°C 45 seconds } 30 cycles were preformed
3. Elongation 72°C 45 seconds

4. Final elongation 72°C 10 minutes

Following completion of the cycles, the PCR products were separated on a 1% agarose gel impregnated with ethidium bromide (0.5µg/ml).

2.6 Preparation of genomic DNA from human lung tissue

Genomic DNA was isolated from human lung tissue using DNeasy spin columns from Qiagen as per the manufacturers instructions. Briefly, 25mg of human lung tissue lysed using proteinase K for 16 hours at 55°C. The lysate was then loaded onto the DNeasy spin column and centrifuged for 1min at 6,000 g to remove contaminants. The column was then centrifuged for 3mins at 20,000 g to dry the DNeasy membrane. DNA selectively bound to the DNeasy membrane was incubated with elution buffer at room temperature for 1 min, and then centrifuged for 1 min at 6,000 x g to elute of the DNA. Purified DNA from human lung tissue using the DNeasy method typically had an A260/A280 ratio between 1.7 and 1.9.

2.6.1 Typing of DNA samples G/A SNP at position -308 in the promoter region of the TNFα gene.

The primers used for Typing of DNA samples G/A SNP at position -308 in the promoter region of the TNFα gene, were kindly donated by Professor R. Grimble from the University of Southampton.

Table 2.5 Primers used for identification of G/A SNP at position -308 in the promoter region of the TNFα

Primer	Sequence 5' to 3'
C1 primer (common primer)	TCT CGG TTT CTT CTC CAT CG
C2 primer (G specific primer, TNF1 allele)	ATA GGT TTT GAG GGG CAT GG
C2 primer (A specific primer, TNF2 allele)	ATA GGT TTT GAG GGG CAT GA
63 control primer	TGC CAA GTG GAG CAC CCA A
64 control primer	GCA TCT TGC TCT GTG CAG AT

Table 2.5 The primers used for competitive-PCR are shown above from 5' to 3'.

The optimised reaction conditions for competitive-PCR are shown in table 2.3.

Table 2.6 Reaction conditions for competitive-PCR

Reagent	Concentration
10x Reaction buffer	1x
dNTP solution	2 mM
$MgCl_2$ solution	25 mM
Thermoprime plus DNA polymerase	
Sucrose/cresol red solution	60 %
Forward specific primers	1 μM
Reverse control primers	0.3 μM
Ultra pure water	

Table 2.6 The optimised reaction conditions for competitive PCR.

Each sample required two separate reactions in order to be typed for the –308 polymorphism. The two primer mixes required for the typing of the TNFα -308 polymorphism are shown below in table 2.4.

Table 2.7 Primer mixes for G/A allele typing of the TNFα promoter.

TNF1 primer mix		**TNF2 primer mix**	
Reagent	Concentration used	Reagent	Concentration used
C1 primer	10 μM	C1 primer	10 μM
C2 primer	10 μM	C3 primer	10 μM
63 control primer stock	15 μM	63 control primer stock	15 μM
64 control primer stock	15 μM	64 control primer stock	15 μM
Ultra pure water		Ultra pure water	

Table 2.7 The TNF1 primer mix detects the more common TNF1 (G) allele at position –308 of the TNFα gene. TNF2 primer mix detects the less common TNF2 (A) allele.

Table 2.8 Thermal cycling parameters for the competitive PCR reactions.

Temperature (°C)	**Time (s)**	**Number of cycles**
96	60	1
96 65 72	15 50 40	10
96 60 72	10 50 40	20
4	Soak	1

Following completion of the cycles, the PCR products were separated on a 1% agarose gel impregnated with ethidium bromide (0.5μg/ml) for DNA visualisation.

2.6.2 Typing of DNA samples G/A SNP at position -1082 in the promoter region of the IL-10 gene.

The primers used for typing of DNA samples G/A SNP at position -1082 in the promoter region of the IL-10 gene, were kindly donated by Professor R. Grimble, University of Southampton.

Table 2.9 Primers used for identification of G/A SNP at position -1082 in the promoter region of the IL-10.

Primer	Sequence 5' to 3'
C1 primer (common primer)	CTT GGA TTA AAT TGG CCT TAG A
C2 primer (G specific primer)	CTA CTA AGG CTT CTT TGG GAG
C3 primer (A specific primer)	ACT ACT AAG GCT TCT TTG GGA A
63 control primer	TGC CAA GTG GAG CAC CCA A
64 control primer	GCA TCT TGC TCT GTG CAG AT

Table 2.9 The primers used for competitive-PCR are shown above from 5' to 3'. The optimised reaction conditions for competitive-PCR are shown in table 2.3.

For reaction conditions refer to the previous table 2.5.2. As for TNFα each sample requires two separate reactions in order to type the –1082 polymorphism. The two primer mixes required for the typing of the IL-10 -1082 polymorphism are shown below in table 2.6.

Table 2.10 Primer mixes for G/A allele typing of the IL-10 promoter.

IL-10 G primer mix		**IL-10 A primer mix**	
Reagent	Concentration used	Reagent	Concentration used
C1 primer	10 µM	C1 primer	10 µM
C2 primer	10 µM	C3 primer	10 µM
63 control primer stock	15 µM	63 control primer stock	15 µM
64 control primer stock	15 µM	64 control primer stock	15 µM
Ultra pure water		Ultra pure water	

The IL-10A primer mix detects the more common IL-10 (A) allele at position –1082 of the IL-10 gene. IL-10G primer mix detects the less common IL-10 (G) allele.

Table 2.11 Thermal cycling parameters for the competitive PCR reactions.

Temperature (°C)	Time (secs)	Number of cycles
96	120	1
96 65 72	20 50 40	10
96 60 72	20 50 40	20
4	Soak	1

Following completion of the cycles, the PCR products were separated on a 1% agarose gel impregnated with ethidium bromide (0.5μg/ml) for DNA visualisation.

2.7 Proteomics

2.7.1 Cation exchange chromatography

The combined peptide mixture was separated by strong cation exchange (SCX) chromatography on a Dionex nano-LC system using a Polysulfoethyl A column (i.d 4.6 mm x 150 mm, 5μm, 30nm) (Phenomenex). Sample was dissolved in 500 μL loading buffer (25% v/v acetonitrile, 10 mM phosphoric acid and loaded and washed isocratically for 20 min at 200 μL/min to remove excess reagent. Peptides were eluted with a linear gradient of 0 – 500 mM KCl (25% v/v acetonitrile, 10 mM phosphoric acid) at 200 μL/min, with fractions collected at 1 min intervals.

Fractions were lyophilised and resuspended in 50 μL of analar™ water. Each fraction was analysed by nano- LC – MS/MS performed on a CapLC liquid chromatography system using a Dionex 75 μm x 150 mm C_{18} reverse phase column coupled to a tandem mass spectrometer. Peptides were eluted using a linear gradient of 5 – 80%/ 162 mins (Buffer A: 5% acetonitrile, 95% H_2O + 0.1 % HCOOH; Buffer B: 95% acetonitrile, 5% H_2O + 0.1% HCOOH)

2.7.2 Mass Spectrometry and data processing

All data were acquired using a Q-TOF Global Ultima (Waters Ltd) fitted with a nanoLockSpray™ source to achieve better than 10ppm mass accuracy. A survey scan was acquired from *m/z* 375 to 1800 with the switching criteria for MS to MS/MS including (i) ion intensity and (ii) charge state. The collision energy used to perform MS/MS was varied according to the mass and charge state of the eluting peptide. RF lens voltages were

adjusted to 0.6 V to enable detection of low mass reporter ions generated from the fragmentation of iTRAQ labelled peptides to enable quantification.

All MS/MS spectra were automatically processed using MassLynx 4.0 (Waters Ltd) and searched against the NCBI non-redundant database (June, 2005 versions), using ProteinLynx Global Server 2.2. Proteins were only assigned if, for each peptide ion, greater or equal to three experimentally derived y ions could be matched to the predicted spectra.

2.8 Statistical analysis

Statistical analyses of results were carried out using Statview software™. Non-parametric Wilcoxon Signed Rank analysis was carried out on paired data. The gross outcomes of bivariate associations were analysed by Spearman Rank correlations and the net effects of multiple variables were analysed using multiply regression where appropriate. $P<0.05$ was generally considered as significant.

Chapter 3.

The acute inflammatory response in human lung tissue

3.1 Introduction

The lung forms a unique interface between the body and its environment. Although the structure of the lung is ideal for gas exchange, it is also vulnerable to potentially infectious microorganisms and noxious substances. The pulmonary tissue must therefore be able to generate a rapid host defence to resolve infection. The response is termed acute inflammation and is characterised by the release of pro-inflammatory mediators and the recruitment of leukocytes from the blood (Delclaux & Azoulay, 2003). Once the infection is controlled the lung must then switch to an anti-inflammatory repair phase in which inflammation should resolve with normal repair to the surrounding tissue.

Inflammation within the lung, thus involves a delicate balance between pro-inflammatory and anti-inflammatory mediators. In diseases such as COPD this balance is altered and a chronic state of inflammation ensues. The underlying inflammation caused in smoking associated COPD is thought to be due to the recruitment of inflammatory cells in to the lung induced by the exposure to cigarette smoke (Jeffrey, 1998, Rahman, 2005). In addition COPD patients may also suffer acute episodes of inflammation termed exacerbations.

Acute exacerbations are an important cause of morbidity and mortality, with severe COPD patients suffering from 3-8 exacerbations per year (Seemungal *et al.*, 1998). These exacerbations are defined by profound symptomatic deterioration often requiring hospitalisation, however little is known about their underlying pathology (Barnes, 1999). Exacerbations tend to occur during the winter months due to the increased prevalence of colds and flu. COPD patients also report dyspnea and common cold symptoms immediately prior to onset of an exacerbation. It is therefore likely that either bacterial or viral infection initiates a chain of events, culminating in increased inflammatory burden within the lung (Seemungal *et al.*, 2000). Early data from the cohort studies by Fletcher *et*

al (1977) suggested that exacerbations had no effect on lung function decline in COPD. However recent evidence suggests that exacerbations have an important effect on COPD progression. Work by Kanner *et al.*, (2001) has shown that among smokers exacerbations are associated with rapid decline in lung function. The East London study also reported that COPD patients with histories of frequent exacerbations had faster declines in FEV_1 than patients who had infrequent exacerbations (Donaldson *et al.*, 2005). The permanent loss of lung function is thought to be the result of extracellular matrix remodelling and loss of functional alveoli during this heightened inflammatory period.

Members of the cytokine family are some of the most potent inflammatory mediators. Cytokines are produced in the lung by local resident cells such as alveolar macrophages, lung epithelial cells and fibroblasts. Cytokines are rarely produced individually, but as a network of cytokines, which are characteristic of particular conditions. Within an inflammatory cascade cytokines are able to regulate the amplitude and duration of an inflammatory response, due to their transient production. The temporal relationship between cytokines is therefore important; however little is known of the cascade of downstream mediators produced in response to inflammatory stimuli.

Another important family of remodelling mediators are the matrix metalloproteinases (MMPs). The matrix metalloproteinases (MMPs) are a family of 24 homologous zinc dependent endopeptidases involved in extracellular matrix turnover and remodelling in both normal and diseased tissues. The catalytic activity of MMPs is regulated at multiple levels but in particular by pro-inflammatory cytokines such as TNFα and IL-1β, which have been shown to regulate MMP transcription and release (Lacraz et al., 1995; Saren et al., 1996). The progressive structural damage associated with emphysema and other forms of COPD is due to degradation of selective extracellular matrix components (ECM). Collectively MMPs are capable of degrading all components

of the ECM. It is therefore not surprising MMPs are the subject of much research in COPD (Culpitt *et al.*, 2004).

Research to date has proposed many mediators, which may have roles to play in acute exacerbations of COPD. However the mechanisms involved in exacerbations still remain unclear, largely due to problems associated with obtaining clinical samples. It is unethical to obtain bronchoalveolar lavage samples from COPD patients during an exacerbation. Clinical studies of exacerbations have therefore only measured inflammatory markers in sputum and blood (Bhowmik *et al.*, 2000, Hill *et al.*, 2000). However due to the nature of sputum samples it is not possible to define the location or the kinetic release of mediators measured. Sputum samples can also be contaminated with mediators found in saliva such as MMPs. Although blood samples can be obtained over the time course of an exacerbation they only give a systemic view of the inflammatory response from within the lung. We therefore wanted to understand the kinetics of cytokines involved in acute inflammation of human lung tissue. This chapter aims to address this problem by setting up a model of inflammation in human lung tissue to analyse the kinetic profile of mediators involved in inflammation.

3.2 Methods

3.2.1 Patient characteristics for human lung tissue experiments

Lung tissue was removed from 54 patients (24F/30M) undergoing resection for carcinoma; tissue used was from the non-tumours normal margin surrounding the tumour site. Data relating to age, gender, lung function and smoking history was obtained for patients (see table 3.1). Where different groups of patients were used for individual experiments, the patient characteristics were controlled to ensure the clinical details of the whole group were represented.

Table 3.1 Patient characteristics of subjects prior to removal of lung tissue

No. of Subjects	54
Age (Yr.)	63.4 ±1.1
Gender	24 Female, 30 Male
Lung Function (FEV_1/FVC)	0.69 ±0.02
Smoking Status	8 Non smokers 21 Ex smokers 25 Current smokers
Pack years	47.9 ±4.2

Table 3.1 Patient characteristics of subjects prior to removal of lung tissue. Tissue samples were taken from a group of 54 patients. Patient details including age, gender, lung function given as the ratio of FEV_1/FVC, smoking status and pack years are listed as the mean ±SEM.

3.2.2 Primary cell culture of human lung tissue

Tissue samples were processed as previously described in preparation of human lung tissue for primary cell culture page chapter 2.2.2. Tissue was then either stimulated with (100ng/ml) lipopolysaccharide or buffer control. For cyclohexamide experiments the tissue

was pre-treated for one hour with 10μg/ml cyclohexamide prior to LPS stimulus. Human lung fragments and supernatant were harvested at 1, 2, 4, 6, 24, and 48 hour time points and the tissue weighed. Both the human lung fragments and supernatant collected were stored at -80°C.

3.2.3 Cytokine analysis

The levels of human TNFα, IL-1β, IL-5, IL-6, IL-8, IL-10, MMP-1, MMP-7, TIMP-1 and $TGF\beta_1$ were measured in the supernatant using commercially available ELISAs (See sections 2.3.2 to 2.3.6). The cytokines measured in the supernatant were corrected for tissue weight and are expressed as ng/mg of tissue. LDH levels were measured in lung supernatant using commercially available assay and LDH standard from Roche, See section 2.3.7. Finally Western blot analysis was performed using methodology in section 2.3.10.

3.3 Results

3.3.1 Cytokine release in response to varying concentrations of LPS

Our initial experiments looked at the response of human lung tissue to stimulation with a wide range of concentrations of LPS. We initially focused on a range of pro-inflammatory cytokines that have previously been shown in the literature to be elevated in response to LPS from various cell types (Beutler, 1995 Brasier & Li, 1996).

Figure 3.1 shows that the levels of TNFα, IL-6, and IL-8 continued to rise with increasing concentrations of LPS and the dose response curves did plateau within the range of concentrations used. Generally, statistically significant increases were observed for all cytokines following LPS stimulus compared to buffer control ($P \leq 0.05$). Having established 100ng/ml of LPS initiated the release of a range of pro-inflammatory cytokines we turned our attention to a more detailed kinetic analysis of the response.

Figure 3.1

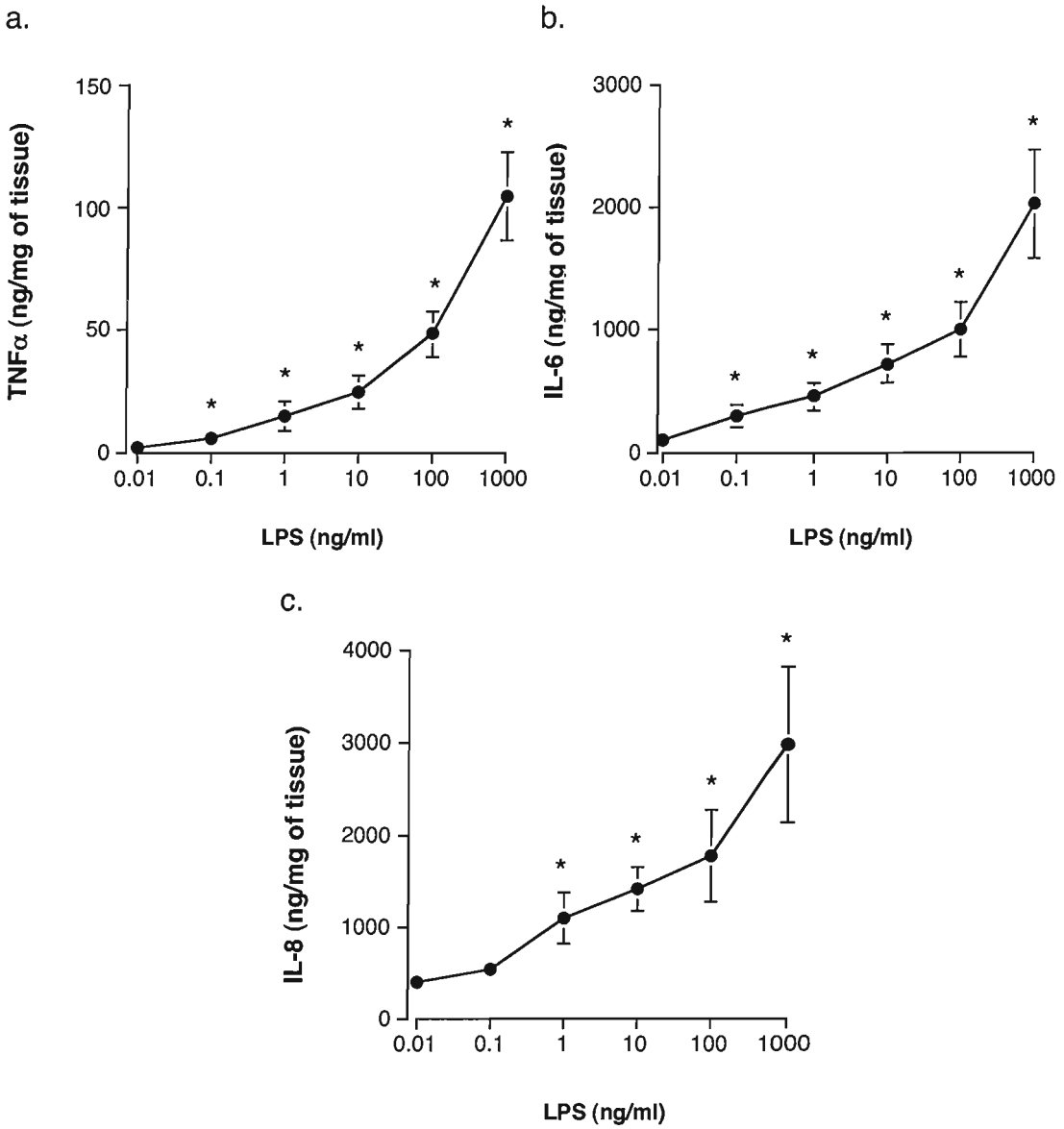


Figure 3.1 LPS dose response curves of cytokine release. Human lung tissue (n=12) was stimulated with a range of concentrations of LPS and incubated for 24hrs. Release of the cytokines TNFα (3.1a), IL-6 (3.1b), and IL-8 (3.1c) into the supernatant were measured by ELISA. The values given are the mean ±SEM and are expressed as ng/mg of tissue, * indicates $P<0.05$ compared to control.

3.3.2 TNFα in human lung supernatant

We analysed the kinetic release of TNFα using ELISA. As figure 3.2 indicates the release of the pro-inflammatory cytokine TNFα, was statistically elevated in the LPS stimulated tissue as early as 1hr. The level of TNFα release continued to rise at 2 and 4hrs with release peaking at 6hrs (mean=16.5ng/mg of tissue) compared to buffer control (mean=0.5ng/mg of tissue, $P<0.05$). At 6hrs the TNFα release was seen to plateau with the elevated release of TNFα being maintained until 48hrs.

Figure 3.2

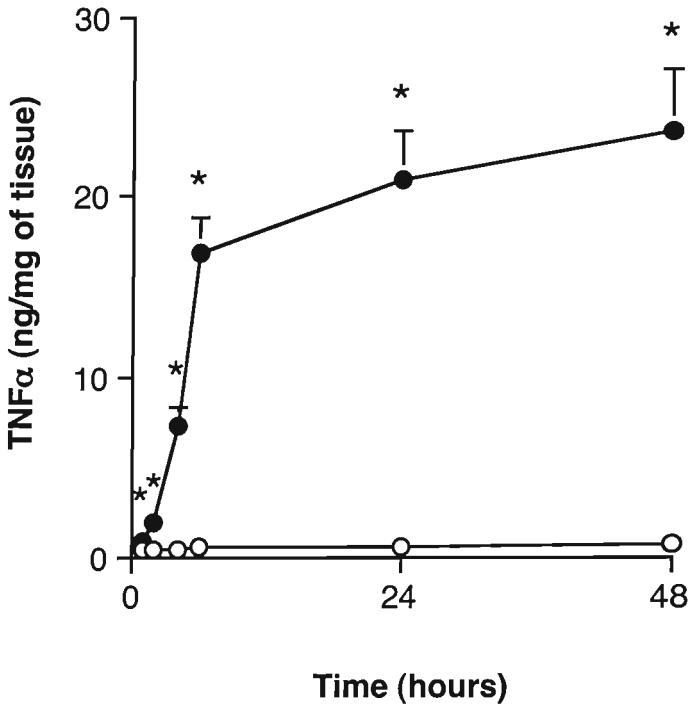


Figure 3.2 TNFα levels in supernatant from human lung fragments. Human lung tissue (n=54) was stimulated with 100ng/ml LPS (filled circles) or buffer control (open circles). The release of TNFα into the supernatant was measured by ELISA. Values given are the mean ± SEM and are expressed as ng/mg of tissue. The data was statistically analysed using the Wilcoxon-Signed Rank test, * indicates a P value <0.05 compared to control.

3.3.3 Western blot for TNFα in human lung supernatant

The supernatants described in figure 3.2 were also used to analyse the molecular profile of TNFα using western blot. Supernatant electrophorised on a 12% SDS-PAGE gel indicated only the free molecular form of TNFα was present at all of the time points analysed (see figure 3.3). The blot is representative of the molecular profile observed for all individuals in the study.

Figure 3.3

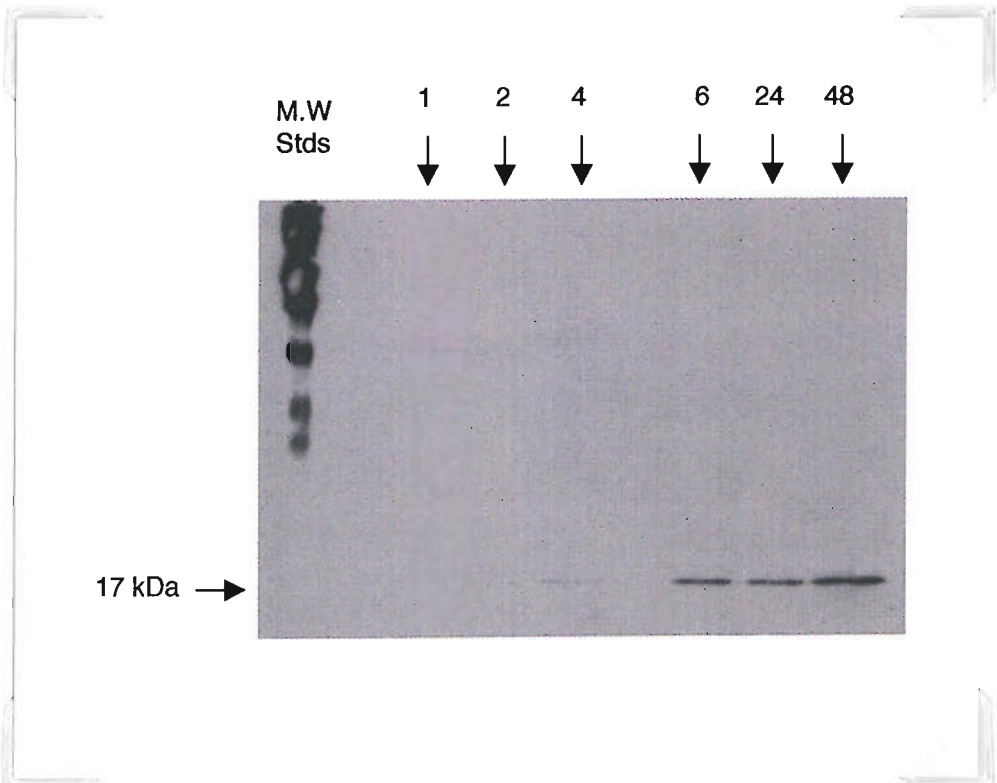


Figure 3.3 Western blot analysis of TNFα in supernatant from human lung tissue stimulated with LPS. Following tissue culture with LPS, supernatants were separated on a 12% SDS-polyacrylamide gel and then transferred on to nitrocellulose. Lane one corresponds to incubation with LPS for 1hr, lane 2 for 2hrs, lane 3 for 4hrs, lane 4 for 6hrs, lane 5 for 24hrs and lane 6 for 48hrs. The blot depicted is a typical example of the molecular profile of TNFα released by all individuals in the study.

3.3.4 Lactate dehydrogenase in human lung tissue

We wanted to ensure the TNFα response observed was not the result of cell death. LDH, a marker of tissue necrosis, was therefore analysed in the tissue supernatant to determine the degree of cell lysis. For all time points between 1 and 48hrs we found no difference in the levels of LDH released between the LPS and control groups (data not shown). More importantly, as shown in figure 3.4, the absolute levels of LDH release for all of the time points were 5% of total tissue LDH, which is an expected value for viable tissue.

Figure 3.4

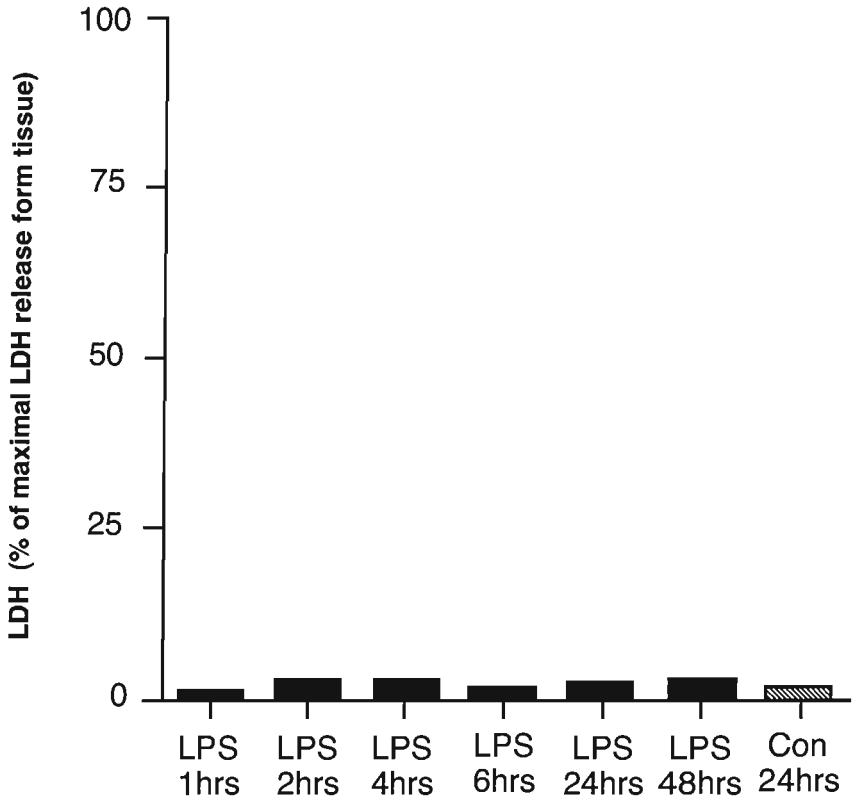


Figure 3.4 LDH release from human lung tissue. Human lung tissue (n=15) was stimulated with 100ng/ml LPS or buffer control. The release of LDH into the supernatant was measured using a commercial LDH assay. Values shown are expressed as a percentage of cell death, with total cell death being estimated from the maximum LDH release from homogenised lung tissue.

3.3.5 IL-6 in human lung supernatant

A second pro-inflammatory cytokine IL-6 was also statistically increased in the LPS stimulated supernatant at 6hrs. However, as shown in figure 3.5 the maximum response of IL-6 occurred later than TNFα, at 24hrs (mean=575.7ng/mg of tissue) compared to control tissue (mean= 204.8ng/mg of tissue, $P<0.05$). As with TNFα, the release of IL-6 reached a plateau at 24hrs with levels remaining elevated until 48hrs. Western blot analysis showed only the free molecular form of IL-6 to be present (data not shown).

Figure 3.5

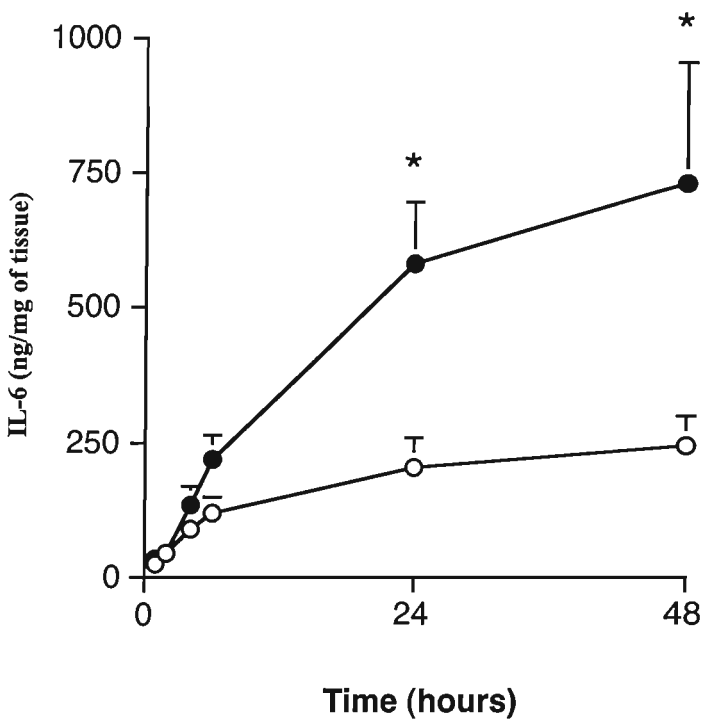


Figure 3.5 IL-6 release from human lung tissue. Supernatants from figure 3.2 were analysed for IL-6 using ELISA. Values given are the mean ± SEM and are expressed as ng/mg of tissue. The data was statistically analysed using the Wilcoxon-Signed Rank test, * indicates a P value <0.05 compared to control.

3.3.6 TNFα correlation with IL-6 released from human lung supernatant

The data illustrated in figures 3.2 and 3.5 was used to correlate the levels of TNFα released at 6hrs with the levels of IL-6 released at 24hrs for each individual patient. As shown in figure 3.6 the correlation indicates that the level of TNFα released at 6hrs actually predicts the magnitude of the IL-6 response at 24hrs (Rho=0.69, $P<0.05$).

Figure 3.6

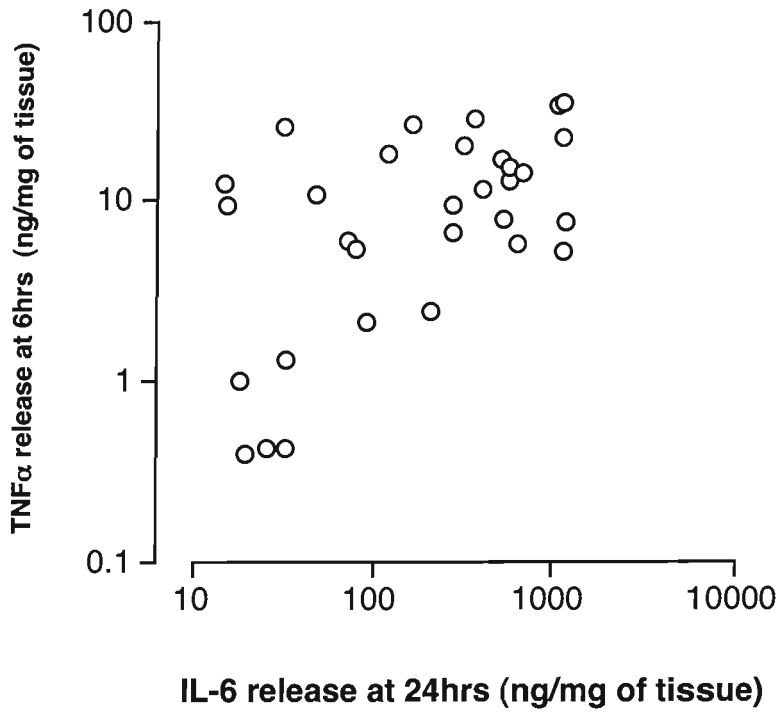


Figure 3.6 Correlation of TNFα and IL-6 released from human lung supernatant. Data from figures 3.2 and 3.5 was re-plotted to show the relationship between TNFα released at 6hrs and IL-6 released at 24hrs. Data was analysed using the non-parametric Spearman rank correlation, values given are expressed as ng/mg of tissue.

3.3.7 IL-8 in human lung supernatant

As shown in figure 3.7 the release of the pro-inflammatory chemokine IL-8 followed a similar pattern to IL-6 release with a maximum response at 24hrs (mean=1435.3ng/mg of tissue verses mean=623.8ng/mg of tissue in the control, $P<0.05$) and remained elevated at 48hrs. Western blot analysis showed only the free molecular form of IL-8 to be present (data not shown).

Figure 3.7

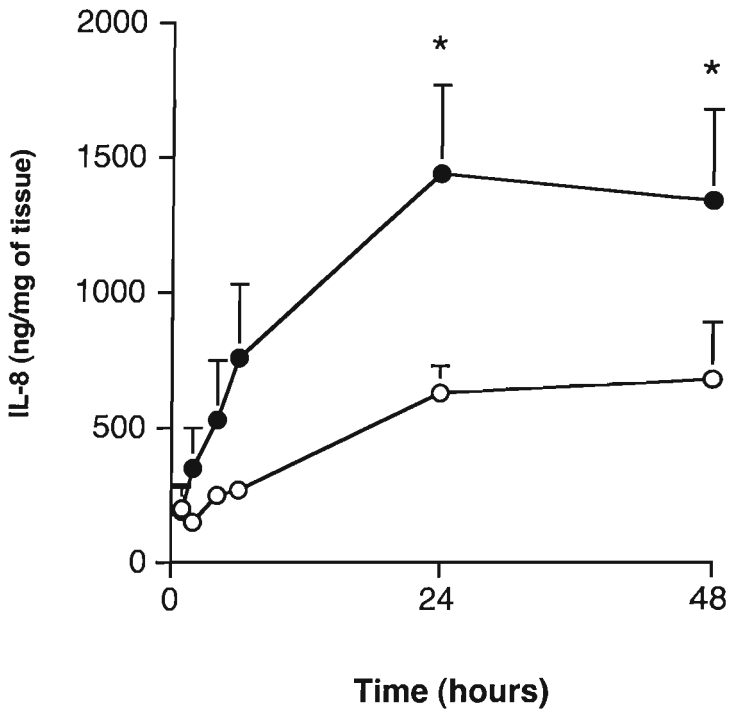


Figure 3.7 IL-8 release from human lung tissue. Supernatants from figure 3.2 were analysed for IL-8 using ELISA. Values given are the mean ± SEM and are expressed as ng/mg of tissue. The data was statistically analysed using the Wilcoxon-Signed Rank test, * indicates a P value <0.05compared to control.

3.3.8 TNFα correlation with IL-8 released from human lung supernatant

The data from figures 3.2 and 3.7 was re-plotted to show the relationship between TNFα released at 6hrs and IL-8 released at 24hrs. As previously seen with the IL-6 data the correlation again indicates the level of TNFα released at 6hrs predicts the level of IL-8 released at 24hrs (Rho=0.49, P<0.05) as shown in figure 3.8.

Figure 3.8

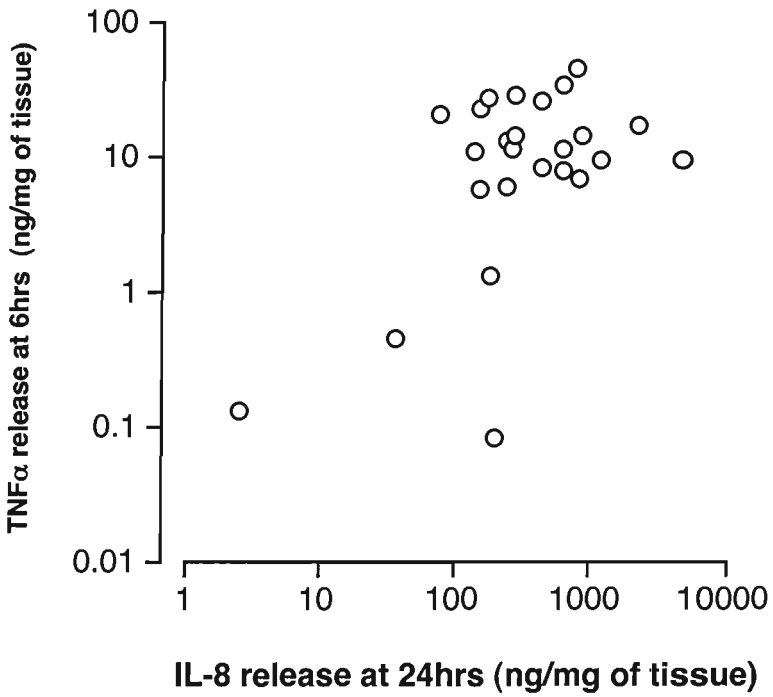


Figure 3.8 Correlation of TNFα and IL-8 released from human lung supernatant. The correlation used data on TNFα released at 6hrs and IL-8 released at 24hrs from human lung. Data was analysed using the non-parametric Spearman correlation, values given are expressed as ng/mg of tissue.

3.3.9 Growth related oncogene-α in human lung supernatant

GRO-α is also a member of the CXC chemokine family and shares significant sequence homology with IL-8. In the presence of LPS the release of GRO-α was statistically elevated at 24hrs and starting to reach a plateau in release by 48hrs (mean

=245.1ng/mg of tissue verses mean =84.4 ng/mg of tissue in the control, P=<0.05) (see figure 3.9).

Figure 3.9

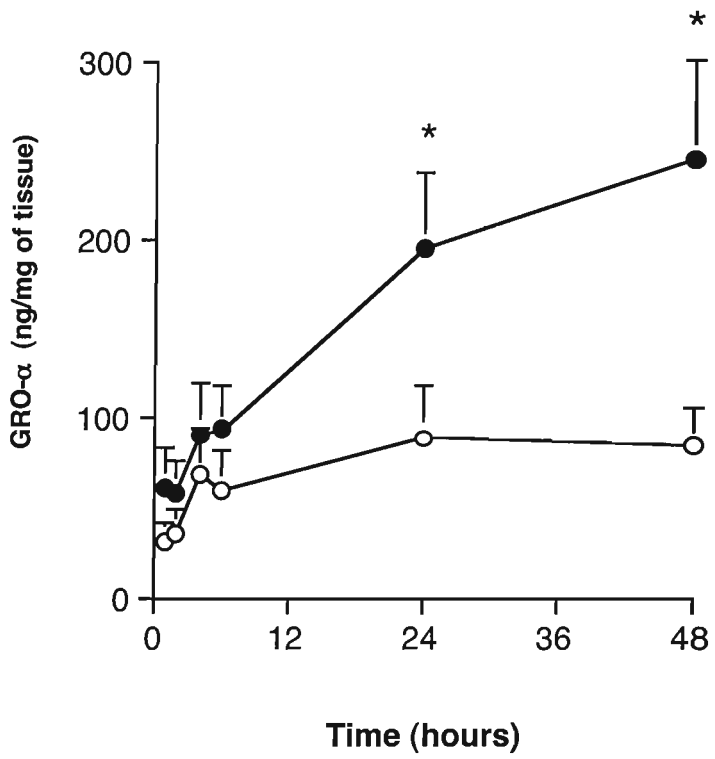


Figure 3.9 GRO-α release from human lung tissue. A subset of 19 samples from the 54 in figure 3.2 were analysed for GRO-α using ELISA. Values given are the mean ± SEM and are expressed as ng/mg of tissue. The data was statistically analysed using the Wilcoxon-Signed Rank test, * indicates a P value <0.05.

The levels of GRO-α release from the tissue followed a similar pattern and Western blot analysis showed only the free molecular form to be present (data not shown). There was a general trend for the levels of TNFα at 24hrs to predict the release of GRO-α at 48hrs Rho=0.48, however this was just outside statistical significance (P=0.055, data not shown).

3.3.10 IL-1β in human lung supernatant

Interestingly, not all pro-inflammatory cytokines are released in response to LPS. As figure 3.10a indicates no IL-1β was evident above the limit of detection, when the tissue was stimulated with LPS or buffer control.

Figure 3.10

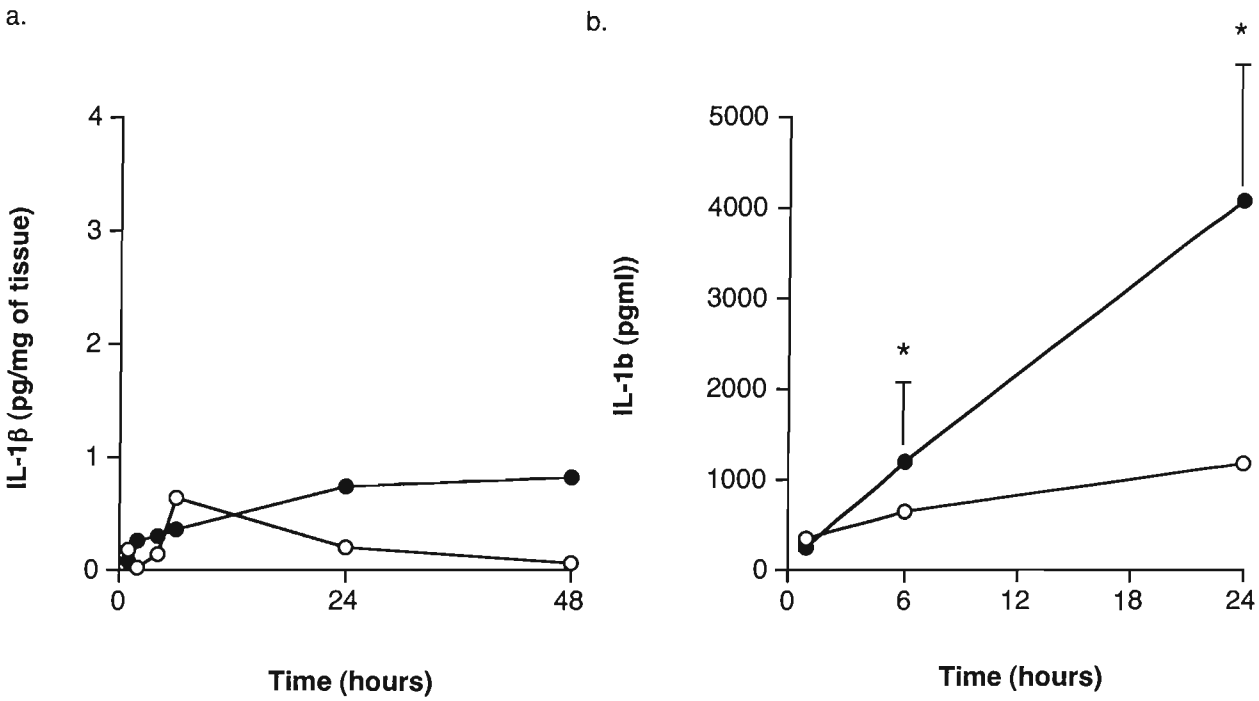


Figure 3.10 IL-1β release from human lung tissue and macrophages. In figure 3.10a supernatants from figure 3.2 were analysed for IL-1β using ELISA. In figure 3.10b, macrophages (n=10) were stimulated with 100ng/ml LPS (filled circles) or buffer control (open circles) and the release of IL-1β again measured by ELISA. Values given are the mean ± SEM and are expressed as ng/mg of tissue. The data was statistically analysed using the Wilcoxon-Signed Rank test, * indicates $P<0.05$ compared to control.

During tissue processing a substantial number of alveolar macrophages and monocytes are removed from the tissue. The macrophage is known to be one the primary sources of IL-1β within the lung (Kelk *et al.*, 2005). In a small set of experiments we stimulated the recovered macrophages with (100ng/ml) LPS or buffer control and measured the release of IL-1β in the supernatant (see methods 2.2.3). As shown in figure 3.10b LPS stimulated IL-1β release was elevated at 1 and 6hrs with levels still increasing at 24hrs (mean= 4062.6ng/mg of tissue) compared to control (mean= 1160.9ng/mg of tissue, $P<0.05$).

3.3.11 IL-5 in human lung supernatant

IL-5 is a pro-inflammatory cytokine associated with a Th_2 response. As shown in figure 3.11 we observed no difference in the release of IL-5 in the LPS stimulated or control tissue.

Figure 3.11

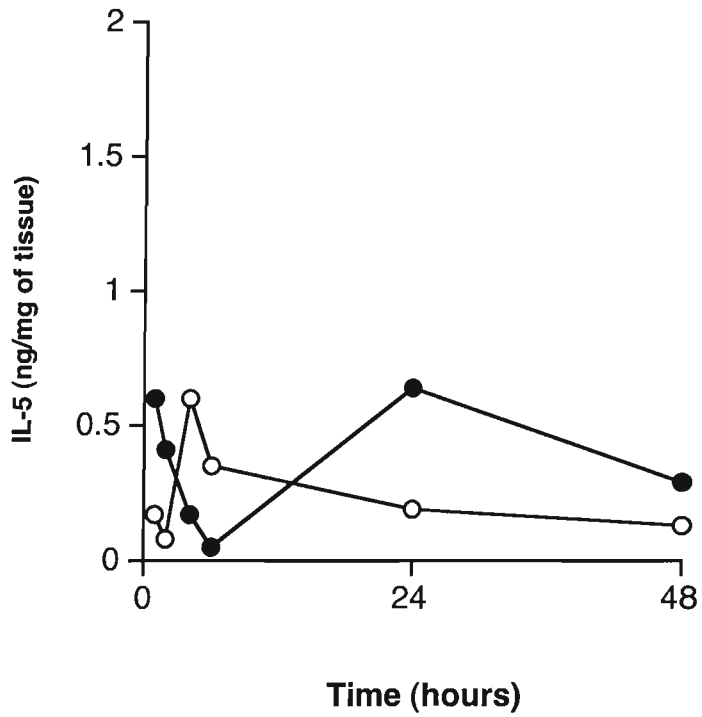


Figure 3.11 IL-5 release from human lung tissue. Supernatants from figure 3.2 were analysed for IL-5 using ELISA. Values given are the mean and are expressed as ng/mg of tissue, error bars were omitted for clarity. The data was statistically analysed using the Wilcoxon-Signed Rank test.

3.3.12 IL-10 in human lung supernatant

We were interested to observe if there was any evidence of resolution and repair following the inflammatory stimulus. Intriguingly, the anti-inflammatory cytokine, IL-10 was elevated at 24hrs with levels still increasing at 48hrs (mean=16.9ng/mg of tissue) compared to undetectable levels in the control, $P<0.05$ (see figure 3.11). Further analysis of the supernatant by Western blot indicated only the free molecular form of IL-10 to be present.

Figure 3.12

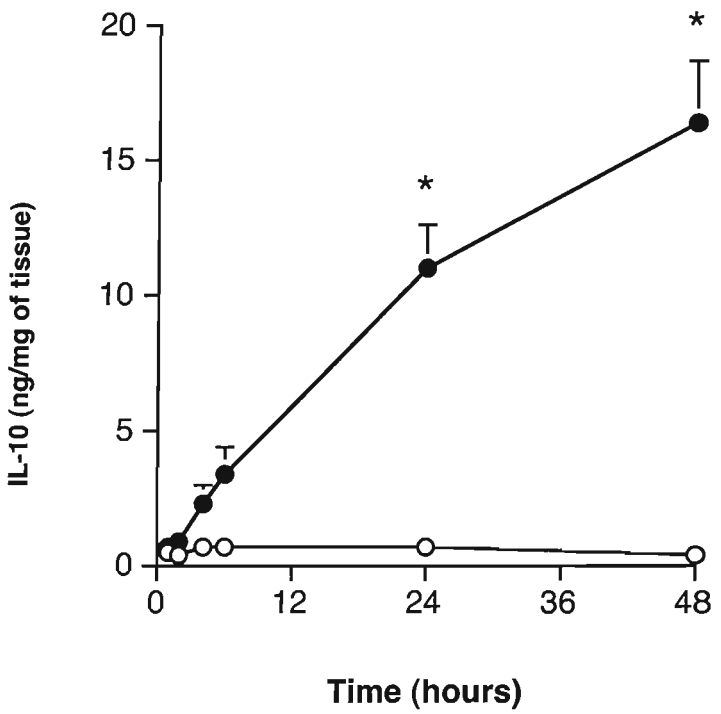


Figure 3.12 IL-10 release from human lung tissue. Supernatants from figure 3.2 were analysed for IL-10 using ELISA. Values given are the mean ± SEM and are expressed as ng/mg of tissue. The data was statistically analysed using the Wilcoxon-Signed Rank test, * indicates a P value <0.05 compared to control.

3.3.13 TNFα correlation with IL-10 release from human lung supernatant

The data illustrated in figures 3.2 and 3.12 was used to correlate the levels of TNFα released at 6hrs with the levels of IL-10 released at 48hrs for each individual patient. The correlation indicates that the level of TNFα released at 6hrs predicts the levels of IL-10 released at 48hrs (Rho =0.58,P<0.05).

Figure 3.13

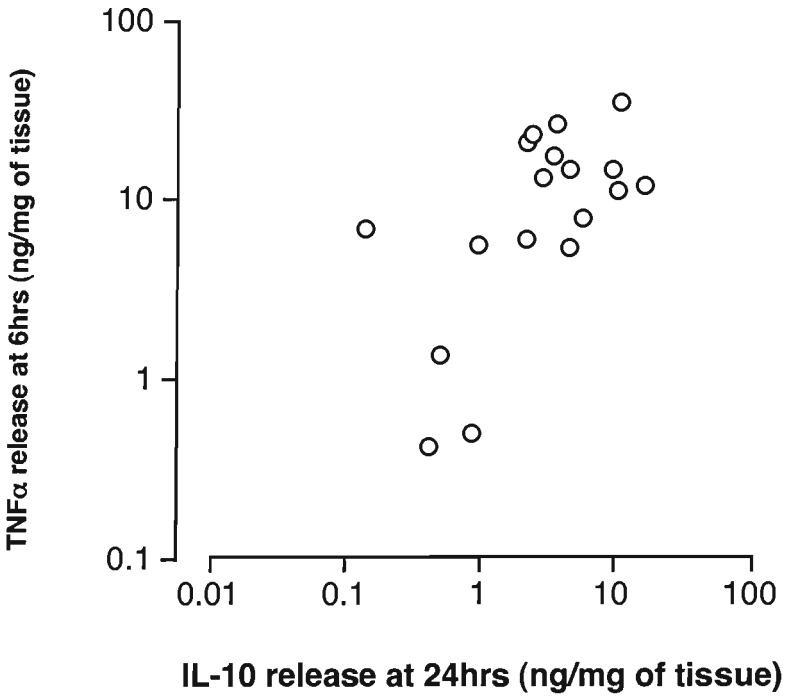


Figure 3.13 Correlation of TNFα and IL-10 released from human lung supernatant. The correlation used data on TNFα released at 6hrs and IL-10 released at 48hrs from human lung. Data was analysed using the non-parametric Spearman correlation, values given are expressed as ng/mg of tissue.

3.3.14 Transforming growth factor β_1 in human lung tissue

Transforming growth factor β_1 is one of the key cytokines associated with repair and remodelling. A subset of 21 samples from the 54 in figure 3.2 were used to analyse TGFβ_1

release. However we were barely able to detect levels of $TGF\beta_1$ in the supernatant above the limit of detection of the assay. As $TGF\beta_1$ is usually tightly bound to the extracellular matrix we decided to look for $TGF\beta_1$ within the lung tissue itself. After culture with LPS or buffer control, lung fragments were homogenised (see method 2.2.3) and the supernatant analysed using ELISA. Although we could detect up to 5ng/mg of tissue of $TGF\beta_1$ there was no statistical difference between the amount of $TGF\beta_1$ produced in the LPS stimulated samples and in the controls. However, a more interesting pattern was observed when TNFα production was correlated with $TGF\beta_1$ production. Figure 3.14 shows there is a positive correlation between TNFα at 24hrs and $TGF\beta_1$ at 48hrs, and therefore TNFα is predicting $TGF\beta_1$ release.

Figure 3.14

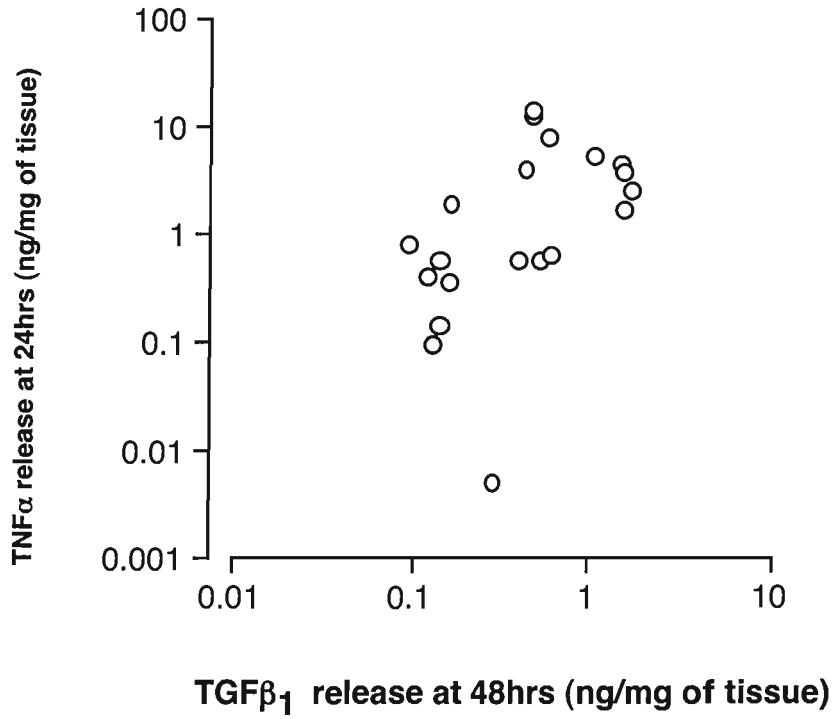


Figure 3.14 Relationship between TNFα production at 24hrs and $TGF\beta_1$ production at 48hrs in LPS stimulated lung tissue. Lung tissue from 21 patients was incubated in 100ng/ml of LPS for 24 and 48 hours. The lung tissue was collected and homogenised (see method 2.2.3) and TNFα and $TGF\beta_1$ ELISAs were performed on the homogenised lung tissue.

3.3.15 MMP-1 in human lung supernatant

Collectively matrix metalloproteinases are responsible for the turnover and degradation of the extracellular matrix. As proteinases MMPs can also modulate the activity of a variety of non-matrix proteins, for example MMP-1 can also directly modulate the activity of TNF-α (Parks & Shapiro, 2001). As shown in figure 3.15 the release of MMP-1 was statistically elevated at 6hrs, reaching a maximum response at 24hrs (mean=12.4ng/mg of tissue verses mean=2.2ng/mg of tissue in the control, P=<0.05), which remained elevated at 48hrs. However, we found no correlation between TNFα release and the MMP-1 response to LPS (data not shown).

Figure 3.15

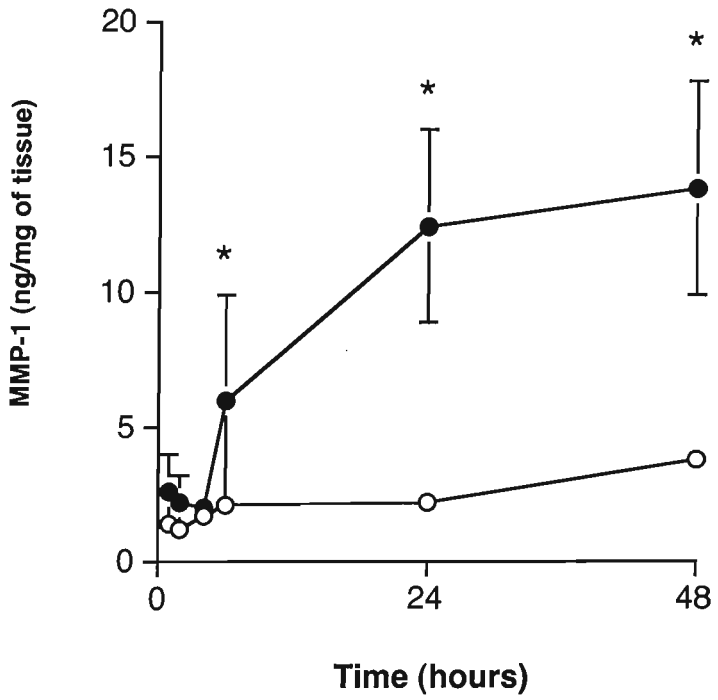


Figure 3.15 MMP-1 release from human lung tissue. Supernatants from figure 3.2 were analysed for MMP-1 using ELISA. Values given are the mean ± SEM and are expressed as ng/mg of tissue. The data was statistically analysed using the Wilcoxon-Signed Rank test, * indicates a P value <0.05 compared to control.

3.3.16 MMP-7 in human lung supernatant

As well as degrading components of the ECM, MMP-7 is also capable of cleaving cell surface bound TNFα to produce its soluble form (Haro *et al.*, 2000). The release of MMP-7 was found to be statistically elevated at 6hrs, reaching a maximum response at 24hrs (mean= 25.5ng/mg of tissue verses undetectable levels in the control, P=<0.05), which remained elevated until 48hrs (see figure 3.16a). Interestingly, as shown in figure 3.16b we observed a strong correlation between the release of TNFα and MMP-7 from as early as 4 hours right up until the 48 hour time point (Rho=0.964, P=0.003).

Figure 3.16

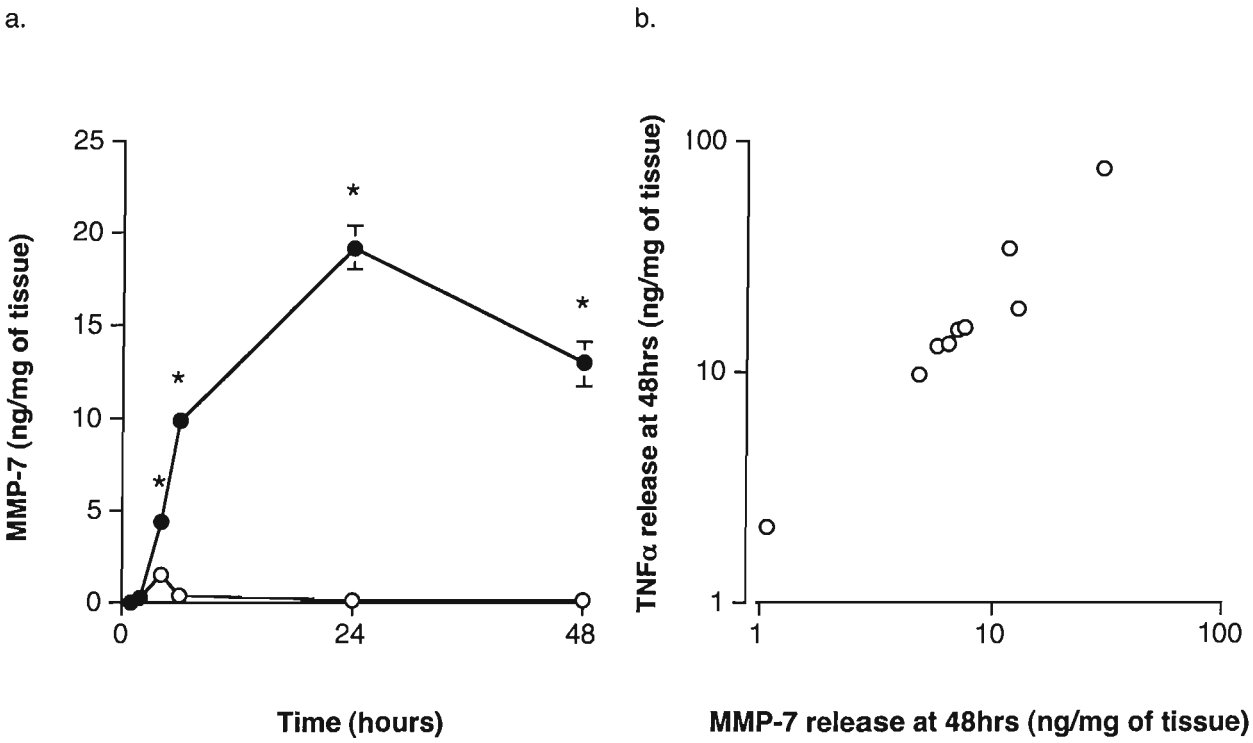


Figure 3.16a MMP-7 release from human lung tissue. Supernatants from figure 3.2 were analysed for MMP-1 using ELISA. Values given are the mean ± SEM and are expressed as ng/mg of tissue. The data was statistically analysed using the Wilcoxon-Signed Rank test. * indicates a P value <0.05.
Figure 3.16b Correlation of TNFα and MMP-7 release from human lung supernatant. The correlation used data on TNFα released at 48hrs and MMP-7 released at 48hrs from human lung. Data was analysed using the non-parametric Spearman correlation, values given are expressed as ng/mg of tissue.

3.3.17 MMP-9/TIMP-1 in human lung supernatant

MMP-9 has been reported to be elevated in current smokers with COPD (Segura-Valdez *et al.*, 2000, Mercer, 2002), however we found no overall change in the levels of MMP-9 in response to LPS (data not shown). There was a general trend for the cognate inhibitor of MMP-9, TIMP-1, to be elevated at all time points in the LPS stimulated tissue but this trend for elevated levels of TIMP-1 did not reach statistical significance (data not shown). However, when the mean levels of TNFα at 6hrs were correlated with the mean levels of TIMP-1 at 24hrs shown in figure 3.12. TNFα levels at 6hrs did predict the release of TIMP-1 at 24hrs (Rho=0.65, P<0.05).

Figure 3.17

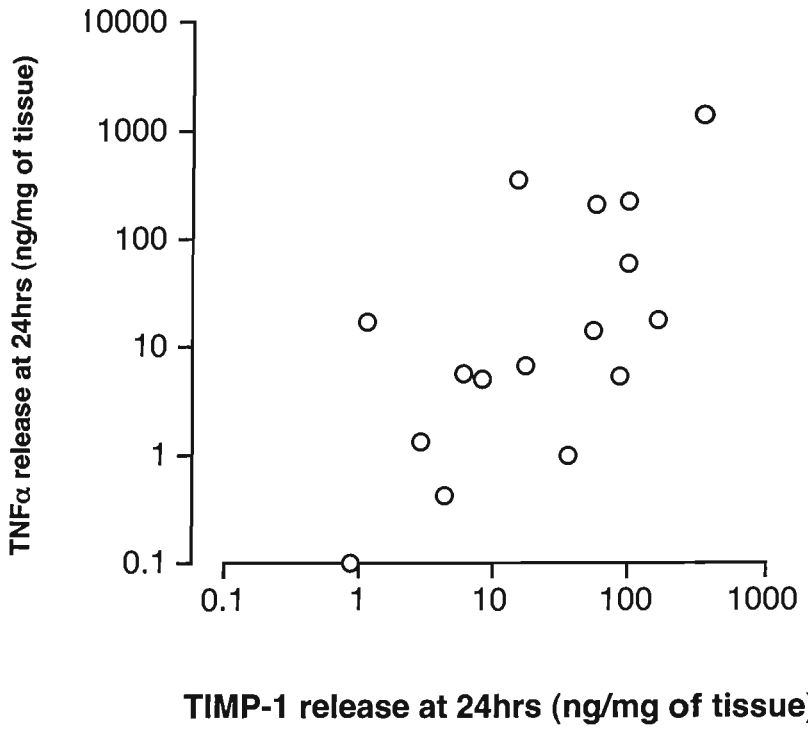


Figure 3.17 Correlation of TNFα and TIMP-1 released from human lung supernatant. The correlation used data on TNFα released at 6hrs and TIMP-1 released at 24hrs from human lung. Data was analysed using the non-parametric Spearman correlation, values are expressed as ng/mg of tissue.

3.3.18 Effect of cyclohexamide treatment on cytokine release

We were interested to know if LPS stimulated *de novo* protein synthesis of the cytokines released or if they were from previously stored sources. To investigate LPS stimulated cytokine production tissue fragments were pre-treated with cyclohexamide a protein synthesis inhibitor, for 1 hour before stimulus with LPS or buffer control (see method 3.2). We chose to concentrate on four cytokines released at different time points in the response TNFα, IL-6, IL-8 and IL-10. Cyclohexamide abolished the release of TNFα in response to LPS stimulation at all time points including 48hrs (mean=54.5 ng/mg of tissue compared to LPS alone mean=0.4ng/mg of tissue, P=<0.05), shown figure 3.18a. Cyclohexamide treatment also inhibited the LPS stimulated production of both IL-6 and IL-8 back to the baseline levels observed in the previous kinetic data (see figures 3.18 b and c). Like TNFα, IL-10 release was completely abolished with cyclohexamide treatment (see figure 3.18d). This data indicates the cytokines released are newly synthesised in response to LPS. The supernatants from tissue treated with and without cyclohexamide were also assayed for LDH. We found no increase in the level of LDH release from the cyclohexamide treated tissue compared to the controls (data not shown).

Figure 3.8

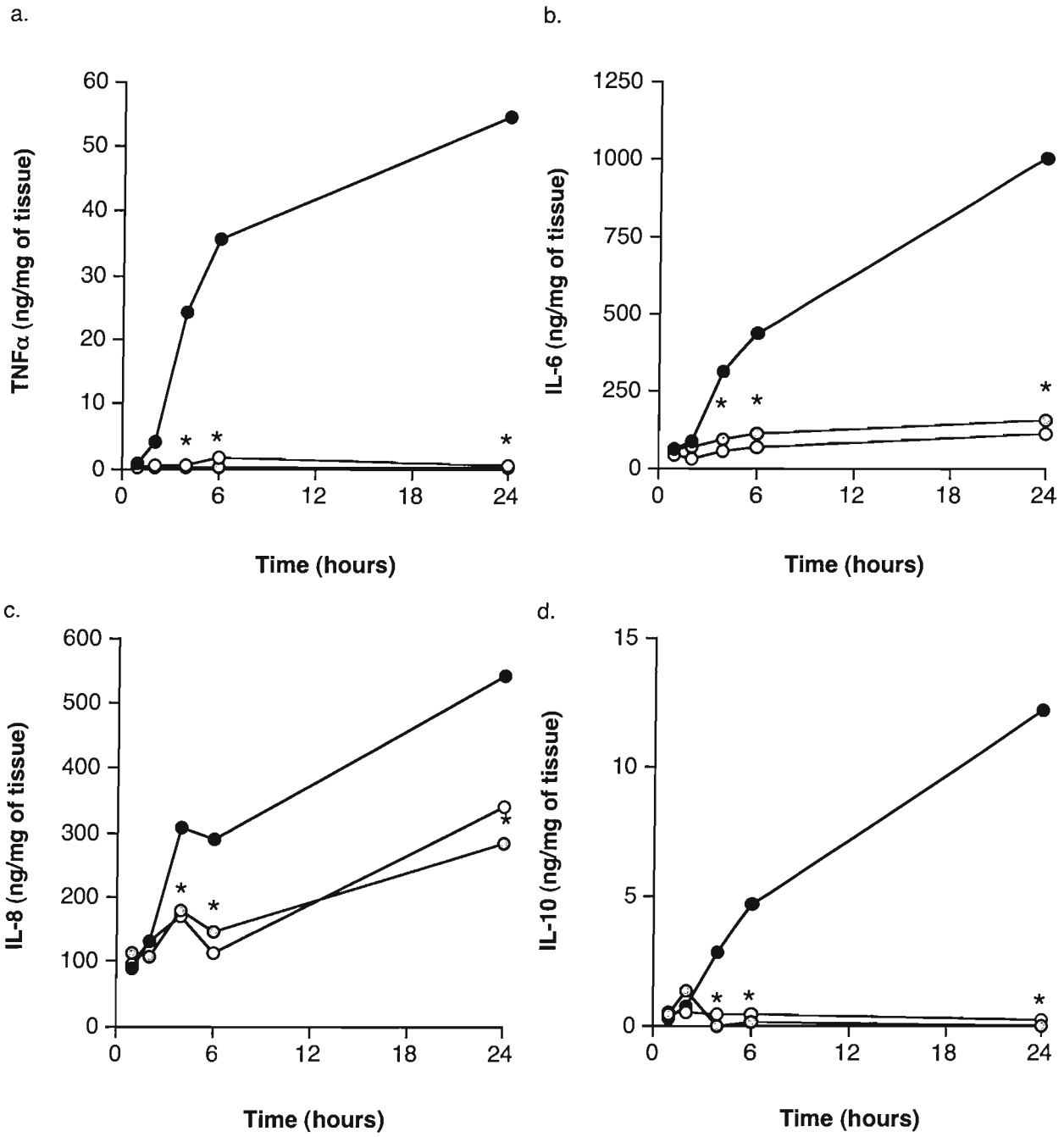


Figure 3.18 Effect of cyclohexamide treatment on cytokine release. Human lung tissue (n=15) was pre-treated with cyclohexamide for 1hr before addition of LPS (grey circles) or buffer control (open circles). The cyclohexamide data is compared with LPS stimulated cytokine release (filled circles). The release of TNFα shown in figure 3.18a, IL-6 see figure 3.11b, IL-8 see figure 3.11c and finally IL-10 as shown in figure 3.11d were all measured using specific ELISAs. Values shown are the mean and are expressed as ng/mg of tissue and the error bars have been removed for clarity. The data was statistically analysed using the Wilcoxon-Signed Rank test, * indicates a P value <0.05.

3.3.19 RNA extraction from human lung tissue

In our study pre-treatment with cyclohexamide inhibited LPS stimulated cytokine release. If LPS is indeed stimulating de novo protein synthesis in the tissue we would expect to find increased mRNA expression. After extensive work up we were able to extract relatively good quality RNA from the cultured human lung tissue (see method 2.5.1). As shown by the gel in figure 3.19 we could detect ribosomal RNA bands at 1.9 kb (18S) and 5.0 kb (28S).

Figure 3.19

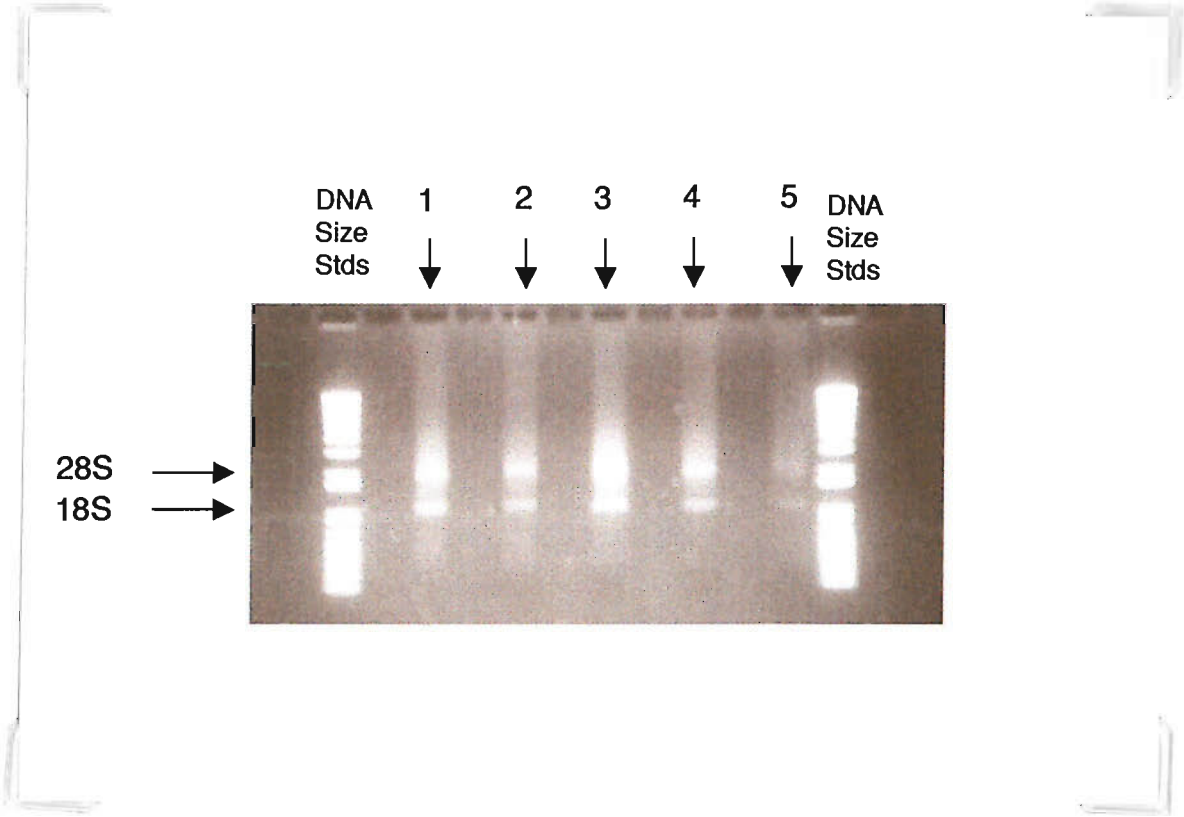


Figure 3.19 RNA integrity in human lung tissue. Lung tissue was cultured with LPS for 1 (lane 1), 2 (lane 2), 4 (lane 3), 6 (lane 4), and 24 hours (lane 5) and RNA extracted. The results were visualised on a 1% agarose gel impregnated with ethidium bromide (0.5µg/ml).

3.3.20 mRNA expression from human lung tissue

To confirm our hypothesis that LPS was stimulating de novo protein synthesis, we analysed the mRNA expression of TNFα and IL-10 in human lung tissue. The extracted RNA shown in figure 3.19 was used to produce cDNA using reverse transcription, and the presence of cytokine transcripts were probed for using specific primers for TNFα and IL-10 (see method 2.5.2). To standardise the PCR results over the different time points we chose to look at β-actin expression, which under normal conditions, is constitutively expressed. For the primers and methods used see Method 2.5.2. As shown by the gel in figure 3.20a the expression of β-actin was relatively constant at each time point in the LPS stimulated tissue. The constitutive expression of β-actin was also observed in tissue cultured with buffer control (data not shown). In contrast we only observed TNFα mRNA expression at the 1 and 2 hour time points (see figure 3.20b). This data corresponds to the early release of TNFα shown in figure 3.2. In the case of IL-10, we observed LPS stimulated mRNA expression between 4 and 6 hours in the response (see figure 3.20c). This would explain the later release of IL-10 seen in figure 3.10. No expression of TNFα or IL-10 was observed in tissue cultured with buffer alone (data not shown).

Figure 3.20

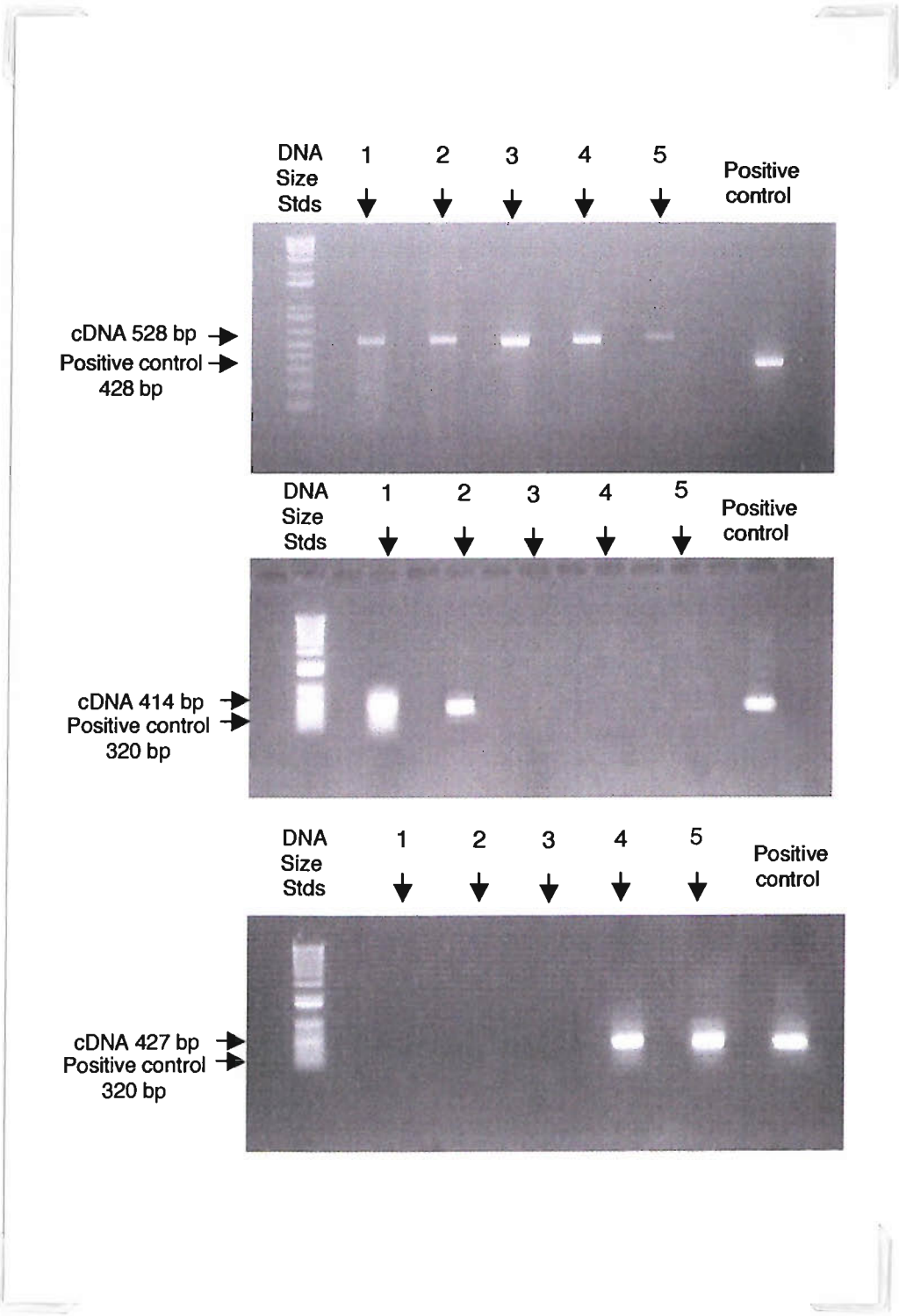


Figure 3.20 TNFα and IL-10 mRNA expression in human lung tissue. RNA extracted from human lung tissue cultured with LPS or buffer control for 1, 2, 4, 6, and 24 hours and was used to produce cDNA. Using PCR the cDNA was probed for the mRNA expression of β-actin (gel 3.20a), TNFα (gel 3.20b) and IL-10 (gel 3.20c). For all the gels in the composite lane 1 shows mRNA levels in tissue cultured with LPS for 1 hour, lane 2 for 2 hours, lane 3 for 4 hours, lane 4 for 6 hours and lane 5 for 24 hours. The results were visualised on a 1% agarose gel impregnated with ethidium bromide (0.5μg/ml).

3.4 Discussion

During a bacterial infection patients with COPD are prone to exacerbations of their illness. Infections are associated with increased airway inflammation (Bhowmik *et al.*, 2000), which may play an important role in the pathogenesis of the disease. There is little information available on the nature and sequence of inflammatory markers involved in these exacerbations. Clinical samples such as bronchial biopsies are rarely taken during these episodes due to the severe deterioration of lung function in COPD patients. As invasive measures to observe the kinetics of exacerbations in situ are not possible, a model of inflammation within human lung tissue could aid our understanding of these events. In fact, characterisation of the cytokines involved in acute inflammation and their temporal relationships may well help to define key events or cytokines involved in the progression of COPD.

Our acute inflammatory model shows TNFα is produced following LPS stimulation. This is not too surprising, as the endotoxins of both Gram +ve and Gram -ve bacteria have been shown to stimulate TNFα production in macrophages (Simpson et al, 1999), and epithelial cells (Read et al., 1993, Khair et al., 1996). LPS in particular, has been shown to induce TNFα release from adult rat alveolar epithelial cells (McRitchie et al., 2000). In our model TNFα release was rapid and statistically elevated from 1hr post challenge, reaching a maximum release at 6hrs, which was maintained until 48hrs. TNFα release occurred earlier than the other cytokines measured, indicating TNFα plays a key role in regulating the response to LPS. *In vivo* studies have shown patients with chronic bronchitis and COPD with a positive bacterial culture for H. *influenzae* have higher concentrations of TNFα in their sputum (Bresser *et al.*, 2000). Similarly, the presence of potentially pathogenic microbes in BAL from COPD patients is strongly associated with neutrophilia and elevated levels of TNFα (Soler *et al.*, 1999, Nash *et al*, 1991).

Our model, along with the literature therefore supports a role for TNFα in COPD progression. While TNFα is the first cytokine to be released, levels of TNFα recorded are typically much lower than the other cytokines measured. Concentrations of IL-6 were up to 60 fold greater than TNFα, suggesting the temporal relationships between cytokines might be more important than the concentrations of cytokines released. The subtlety of the TNFα response is therefore an important consideration especially when looking at the role of TNFα in different conditions. TNFα has previously been shown to play a key role in many inflammatory diseases. For example TNFα is now accepted as an important target in rheumatoid arthritis (Hurlimann *et al.*, 2002). The role of TNFα in rheumatoid arthritis is biased towards a pro-inflammatory, destructive role. In comparison TNFα has also been shown to have a role in the inflammatory processes affecting the distal airways of systemic scleroderma patients, which culminates in irreversible fibrosis (Silver *et al.*, 1995). In scleroderma it would appear TNFα plays quite a different role promoting fibrosis rather than destruction of the ECM. When defining the role of TNFα it would therefore be prudent to examine the context of the inflammatory milieu before defining the role of this pleotrophic cytokine. Analysis of the cytokine cascade lead by TNFα in our model has indicated TNFα can promote the expression of both pro and anti-inflammatory mediators. It may therefore be hard to characterise the role of individual pleotrophic cytokines such as TNFα in disease progression.

The acute release of TNFα was followed by elevated levels of IL-6, which were maximal at 24hrs and maintained up to 48hrs later. TNFα release at 6hrs predicted the release of IL-6 both at 24 and 48hrs. TNFα has been shown to increase IL-6 and IL-8 specific mRNA from bronchial epithelial cells (Cromwell *et al.*, 1992). IL-6 is released by epithelial cells and macrophages in the airways and exerts pleiotropic pro-inflammatory effects on both the immune and hematopoietic systems (Levine SJ, 1993). In the East

London study Bhowmik *et al* (2000), showed the levels of IL-6 were elevated at 24 and 48hrs in induced sputum from COPD patients during an exacerbation compared to stable conditions. These time points correspond with the temporal release of IL-6 we observe in our model. Other studies have previously shown high baseline levels in COPD patients compared to healthy smokers and controls (Chung KF, 2001). In our LPS model we also observed high basal release of IL-6 from the human lung tissue. Previous research and our own therefore suggests a role for IL-6 in bacterial derived inflammation.

During an innate immune response leukocytes are recruited to inflammatory foci by chemokines, which are a family of cytokines with strong chemoattractant properties. Investigators have shown the principle cells recruited and involved in the pathogenesis of COPD are neutrophils, macrophages, and CD8+ve T lymphocytes. The source and relative contribution of chemokines involved in the recruitment of theses leukocytes during exacerbations in COPD patients is however unresolved. In our model TNFα release at 6hrs also predicted the release of chemokines IL-8 and GROα. IL-8 is the main mediator of neutrophil chemotaxis (Solar *et al.*, 1999). In line with our cytokine cascade, TNFα has previously been shown to stimulate greater release of IL-8 from bronchial epithelial cells obtained from patients with moderate COPD compared to healthy smokers and control subjects (Schultz *et al.*, 2004). Elevated levels of IL-8 in COPD patients has previously been reported in both induced sputum and (Keatings *et al.*, 1996) and bronchoalveolar lavage (Riise *et al.*, 1995) during exacerbations. In the east London study they also reported the elevated release of IL-8 in induced sputum from COPD patients during an exacerbation at 24 and 48 hours (Seemungal et al., 2000). In regards of temporal release in our study the levels of IL-8 were maximal at 24hrs and maintained up to 48hrs later, which fits with the *in vivo* data. The elevated release of IL-8 in response to LPS would lead to the recruitment of neutrophils and up-regulation of the inflammatory burden within the lung. It

is therefore not surprising much research has revolved around the role of IL-8 in the pathogenesis of COPD.

Previous work by Witherden and co-workers (2003) in primary human alveolar type II epithelial cells also demonstrated elevated levels of IL-8 and GRO-α are released following an LPS stimulus. The study also observed high basal levels of both cytokines with IL-8 release being 20-fold greater than GRO-α. TNFα induced mRNA and protein release of GROα has also been demonstrated to be elevated in primary bronchial epithelial cells from COPD patients compared to controls (Schulz et al., 2004). Although there is evidence for elevated levels of GRO-α in BAL from COPD patients (Traves *et al.*, 2004) its role in disease progression is still to be determined. Like IL-8, GRO-α is also a neutrophil chemoattractant, however its functional role is not entirely confined to the attraction and activation of inflammatory cells. GRO-α also has strong angiogenic and growth promoting properties, which are important in repair (Vanderbilt et al., 2003). In our study the maximal release of GRO-α peaked later than IL-8 at 48hrs in the LPS response. This later release may support the role of GROα in repair and resolution following the initial inflammatory response, rather than just simply a chemoattractant. Although we have shown GRO-α to be elevated in response to LPS, more research is required to determine its role in COPD exacerbations.

Interestingly, not all pro-inflammatory cytokines were up-regulated in response to LPS. Although LPS is a blunt stimulus and is able to activate many cell types no detectable changes in the pro-inflammatory cytokines IL-1β and IL-5 were found in our model. In the literature IL-1β is referred to as a key cytokine in the innate inflammatory response. Endotoxins from bacteria, in particular leukotoxin, have been shown to trigger abundant production and secretion of bioactive IL-1β by human macrophages (Kelk *et al.*, 2005). When we cultured alveolar macrophages from the tissue we found LPS also stimulated IL-1β release and in large quantities. The fact that we do not observe IL-1β release in the

tissue is therefore interesting and may indicate TNFα plays a more dominant role in LPS induced inflammation.

IL-5 has been acknowledged for many years to be an important mediator of Th_2 driven chronic inflammatory conditions such as allergic asthma. IL-5 has been shown to activate (Clutterbuck *et al.*, 1987) and mediate eosinophil integrin-dependent adhesion (Walsh *et al.*, 1990). Basophils, another major effector cell in asthma, are also primed for enhanced histamine release by IL-5 (Bischoff *et al.*, 1990). As we found no IL-5 was released following LPS stimulation this supports our hypothesis that LPS is invoking a Th_1 rather than a Th_2 response.

It is tempting to speculate how the release of TNFα, IL-6, IL-8 and GRO-α during an exacerbation would lead to the recruitment of neutrophils and further release of inflammatory cytokines. During emigration inflammatory cells release proteases such as MMP-9 to degrade the basement membrane and thus gain passage through the endothelial layer. MMPs are also released from secretory vesicles by inflammatory cells in situ and the surrounding alveolar epithelium in response to inflammatory stimuli. Collectively MMPs are capable of degrading all components of the ECM. The expression of MMP-1 and MMP-7 has been demonstrated in airway and alveolar epithelial cells at sites of overt damage in emphysema patients (Imai *et al.*, 2001, Sheppard, 2002). Indeed a number of studies have examined the expression of MMPs in COPD and have shown elevated levels of MMP-2, MMP-8, MMP-9 and MMP-12 in both sputum and BAL in addition to MMP-1 and MMP-7 (Culpitt *et al.*, 2005, Vernooy *et al.*, 2004). Elevated levels of MMPs would lead to a protease-antiprotease imbalance and an enhanced proteolytic environment. It is therefore easy to see how this elevated inflammatory burden within the lung due to bacterial infection would perpetuate and lead to the destruction of surrounding ECM in diseases such as COPD.

In recent years it has become evident that MMPs may have greater roles to play in inflammation than just simply degrading the ECM. MMP-1 is also able to cleave cell surface molecules and non-matrix substrates of which TNFα is one (Parks & Shapiro, 2001). MMP-1 is expressed during physiological and pathological tissue remodelling and is produced by a variety of normal cells such as fibroblasts, macrophages, endothelial and epithelial cells (Nagase & Woessner, 1999). Although MMP-1 was elevated over time with LPS stimulus we found no correlation between the release of TNFα and MMP-1 in our study. It has also been shown that MMP-7 is able to activate latent TNFα on the surface of macrophages (Haro *et al.*, 2000). In adult human lung, whether normal or diseased, MMP-7 is expressed by epithelial cells lining the peri-bronchial glands and conducting airways and is up regulated in response to bacterial load (Dunsmore *et al.*, 1998, Lopez-Brado *et al.*, 2000). In our model we observed a strong correlation between the release of TNFα and MMP-7 from 4 hours in the LPS response. As the levels of TNFα peak before the maximal release of MMP-7 it may indicate TNFα is driving the release of MMP-7 to activate latent TNFα in the response. The elevated levels of MMP-7 in our inflammatory model may therefore play a more essential role in the inflammatory response than just simply remodelling the ECM. In fact studies using metalloproteinase inhibitor GI 129471 in humans have shown inhibition of TNFα processing suggesting that MMPs represent a novel target for therapeutic intervention in TNFα associated inflammation (McGeehan *et al.*, 1994).

Numerous papers have been published demonstrating the up-regulated expression of matrilysin in tumours (Wielockx et al., 2004). The tissue in our model was obtained from patients undergoing resection for cancer, however we found no baseline levels of MMP-7 in the normal margin tissue used in the experiments. Although not conclusive this data gives an indication the tissue used in the experiments is as 'normal' as can be expected.

Particular attention has focused on the role of MMP-9 in COPD, which has the capacity to degrade type IV collagen, a major component of the basement membrane within the lung. Increased levels of MMP-9 have been found in the BAL fluid from patients with emphysema (Finlay et al., 1997), and the sputum from chronic bronchitics (Vignola et al., 1998) compared to healthy individuals. However we did not find any significant rise in the levels of MMP-9 up to 48hrs with LPS stimulus. TIMP-1 the innate inhibitor of MMP-9, has been shown to be elevated in sputum and BAL from COPD patients compared to controls (Higashimoto et al., 2005, Mercer 2001). We did find elevated levels of TIMP-1 in response to LPS, however these changes did not reach statistical significance. Interestingly, the release of TNFα at 6hrs did predict the release of TIMP-1 at 24hrs and 48hrs in the study. TNFα may therefore also modulate anti-inflammatory remodelling factors up to 48hrs later in the acute inflammatory response. Chronic inflammation leads to remodeling and narrowing of small airways in COPD patients (Musil *et al.*, 2004). Fibrosis and connective tissue deposition are also observed in progressive inflammation (Cosio *et al.*, 1978). It is temping to speculate the fibrotic foci observed in COPD patients could be associated with elevated levels of TIMP-1 in response to acute inflammation mediated by TNFα.

In normal lung following the release of pro-inflammatory cytokines and mediators in response to stimuli, there would be a switch towards resolution and repair. We were therefore interested to investigate if any mediators associated with resolution and repair were released in our model. IL-10 is a potent anti-inflammatory cytokine, which resolves pro-inflammatory responses by inhibiting cytokine production in many inflammatory cell types (Takanashi *et al.*, 1999, Cassatella *et al.*, 1993). Elevated levels of the anti-inflammatory cytokine IL-10 were detected at 24hrs and were still increasing at 48hrs. Again levels of TNFα at 6hrs predicted the levels of IL-10 released at both 24 and 48hrs, suggesting that TNFα may modulate IL-10 release. Indeed TNFα has been shown

previously to mediate the biosynthesis of IL-10 in foetal rat alveolar cells (Haddad, 2002). IL-10 has been referred to as a counter inflammatory cytokine due to its anti-inflammatory effects and temporal association with TNFα. IL-10 is also able to regulate the release of remodelling mediators such as TIMPs (Lim *et al.*, 2000). Release of the anti-inflammatory cytokine IL-10 may therefore indicate that the initial inflammatory response to LPS in the lung is turning towards resolution of the inflammation and repair. Levels of IL-10 have previously been reported to be decreased in sputum from patients with COPD and healthy smokers compared to healthy non-smokers (Takanashi *et al.*, 1999). In other conditions involving airway inflammation such as cystic fibrosis, IL-10 is also reduced in the epithelial lining fluid compared to healthy controls (Bonfield *et al.*, 1995). The decline in IL-10 in diseases such as COPD and cystic fibrosis would lead to unregulated inflammation, which is a characteristic of both diseases. More research is therefore required as to the potential role or indeed the reason for the decline of IL-10 in these diseases.

The resolution and repair, which follows an inflammatory response involves a complicated interplay of various cytokines, such as transforming growth factor β_1. TGFβ_1 is a multifunctional cytokine, which promotes the growth of fibroblasts and smooth muscle cells and induces the synthesis of matrix components such as collagens I, IV, ECM associated proteins and proteoglycans. In this study, although TGFβ_1 did not increase directly in response to LPS, TNFα at 24hrs predicted TGFβ_1 production at 48hrs in the tissue. This further indicates the role of TNFα in the repair response following inflammation. TNFα has been demonstrated to induce TGFβ_1 expression in fibroblasts through the extracellular signal-related kinase (ERK) pathway (Sullivan *et al.*, 2005). To date there is little evidence for the role of TGFβ_1 in COPD. One study has shown decreased levels of TGFβ_1 in alveolar macrophages from patients with COPD compared to normals (Pons *et al.*, 2005). In contrast the increased expression of TGFβ_1 mRNA and

protein has been demonstrated in the small airway epithelium from tobacco smokers and patients with COPD (Takizawa *et al.*, 2001, de Boer at al., 1998). The epithelial expression of $TGF\beta_1$ mRNA and protein has also been shown to correlate with the number of intraepithelial macrophages and mast cells in COPD patients compared to controls (de Boer at al., 1998). The role of $TGF\beta_1$ as a chemotactic factor for the recruitment of macrophages and mast cells in COPD patients may indicate $TGF\beta_1$ also has pro-inflammatory roles .

$TGF\beta_1$ is generally associated with fibrotic lung diseases such as systemic scleroderma and interstitial pulmonary fibrosis (IPF). However the modulation of $TGF\beta_1$ by TNFα could account for the fibrotic foci observed histologically in COPD patients. Indeed the increased expression of TNFα in alveolar macrophages and proliferating type II pneumocytes in individuals with acute fibrotic changes in the lung (Pan *et al.*, 1996) would seem to support this hypothesis. Together these reports and the data shown here supports the idea that TNFα may have a key role in the development of both inflammation and fibrosis.

Following the characterisation of the inflammatory cascade stimulated by LPS and tissue viability assessed using the LDH assay. We investigated if the cytokines released in response to LPS were produced by *de novo* protein synthesis or released from previously stored granules. Treatment with cyclohexamide, a protein synthesis inhibitor, completely obliterated LPS stimulated release of TNFα, and the other cytokines measured. This preliminary data indicates LPS induces *de novo* protein synthesis of the cytokines released. Previous work by McRitchie and co-workers (2000) has also shown treatment with cyclohexamide blocks TNFα-mediated release of cytokines from bronchial epithelial rat cells. For *de novo* protein synthesis we would therefore expect to find elevated levels of mRNA. Despite substantial technical difficulties we were able to obtain good quality RNA from the cultured lung tissue. Following reverse transcriptase-PCR to produce cDNA

primers for TNFα and IL-10 were used to amplify the cDNA of interest. In addition we also looked at the expression of β-actin, which is constitutively expressed in normal tissue. In our cultured tissue we found the level of β–actin expression was the same at all the time points measured up to the 24 hour time point. This data indicates the viability of the tissue as mRNA is still produced after 24 hours of incubation. We chose to look at the expression of TNFα, a pro-inflammatory cytokine, released early on in the inflammatory cascade. We found TNFα mRNA expression was indeed elevated at the 1 and 2 hour time points, which fits with the temporal pattern of protein release previously measured. The early expression of TNFα mRNA further supports the hypothesis that TNFα is an early key cytokine in the inflammatory cascade. In the case of IL-10 we observed mRNA expression at the later time points of 4 and 6 hours. Again the expression of IL-10 mRNA fits with temporal release of protein observed in the inflammatory cascade and supports its later anti-inflammatory role in the response. The expression of TNFα and IL-10 mRNA in the tissue therefore indicates LPS is stimulating de novo protein synthesis in our model. However, further research would be required to look at the mRNA expression of the other mediators expressed in our model. This finding is inline with previous research that has shown LPS induces transcription of TNFα and IL-10 (Cassatella *et al.*, 2005, Biragyn *et al.*, 1995).

In conclusion our data suggests the lung tissue may have two phases of response when stimulated with LPS. Firstly, the acute inflammatory phase driven by TNFα, followed by the release of pro-inflammatory cytokines IL-6, IL-8 and GRO-α. Following the initial inflammation the later release of the anti-inflammatory cytokine IL-10 and remodelling mediators such as TIMP-1 and $TGF\beta_1$ may indicate the initiation of a resolution and repair phase. If as this work suggests TNFα is the key early response cytokine in the kinetics of an acute inflammatory response it may represent an important future therapeutic target in pulmonary inflammation.

Chapter 4.

The role of TNFα and IL-10 in acute inflammation

4.1 Introduction

TNFα has been previously been shown to play an important role in many chronic inflammatory diseases and is now accepted as an important therapeutic target in rheumatoid arthritis and sepsis (Feldmann *et al.*, 2001, Stuber *et a*l., 1995). Following recognition of the importance of TNFα in this respect several humanised monoclonal antibodies have been developed to neutralise its biological activity. Clinical trials of these humanised antibodies (such as infliximab) have been successful and they are now used clinically in a range of chronic inflammatory diseases including rheumatoid arthritis, Crohns disease, and sepsis (Hurlimann *et al.*, 2002, Sandborn, 2005 and Panacek *et al.*, 2004). In our acute inflammatory model TNFα release occurred earlier than the other cytokines measured, indicating TNFα may play a key role in regulating the response to LPS. We were therefore interested to further investigate the role of TNFα in our model using a neutralising TNFα antibody.

We believe that TNFα plays a critical role in regulating the inflammatory cascade in human lung tissue. It is therefore important to identify which cells are responsible for its synthesis. We have used immunohistochemistry firstly to characterise the cell populations within human lung fragments and secondly to identify cells producing different cytokines. Identification of the cells responsible for cytokine production during lung inflammation may help to therapeutically target cytokine release from these specific cells.

Having identified cytokine cascades as a central feature in our model of lung inflammation, we were interested to know if any of the clinical parameters collected on the tissue donors correlated with *in vitro* responses. Data available included the patients age, gender, lung function and smoking history. Due to the nature of their disease the majority of our patients have a smoking history and approximately 65% have some degree of airway obstruction. Stratifying the disease COPD has not been easy but in 2001 the global initiative for chronic obstructive lung disease (GOLD) guidelines were introduced (GOLD,

2001). The initiative provides a simple objective method of classifying the severity of disease by measuring the degree of lung function abnormality in COPD patients. Using the GOLD guidelines we hope to assess if disease severity affects LPS stimulated cytokine release in our patient cohort. Understanding which cytokines are modulated in disease conditions such as COPD could provide important therapeutic targets.

COPD is generally regarded as a progressive disease but patients responses are heterogeneous. In particular some patients show a rapid decline in lung function, which has led some authors to speculate that there may be a genetic component to COPD. The production of TNFα is complex and appears to be regulated by a wide range of factors including other cytokines. Previous studies have shown that polymorphisms in the TNFα promoter can influence the severity of rheumatic disease (Rood *et al.*, 2000, Ozen *et al.*, 2002). In the TNFα promoter a G⇒A transition at position –308 relative to the transcriptional start site results in increased TNFα production (Knight *et al.*, 1999, Wilson *et al.*, 1997). Individuals with TNF-308 A/A genotype are described as being high producers of TNFα, TNF-308 A/G genotypes as moderate producers and TNF-308 G/G genotypes as normal producers (Rood *et al.*, 2000). Studies have shown that both the TNF-308 A/A and the TNF-308 A/G genotypes occur at a higher frequency in patients with rheumatological diseases (Rood *et al.*, 2000, Ozen *et al.*, 2002). As TNFα appears to play an important key role in our inflammatory model we speculate that polymorphisms in cytokines such as TNFα may contribute to the development of COPD. In part this chapter aims to further characterise the role of TNFα in inflammation and investigate the relationship between TNFα and the severity of the underlying COPD.

4.2 Methods

4.2.1 Patient characteristics for human lung tissue experiments

Lung tissue was removed from 96 patients (36F/60M) undergoing resection for carcinoma; tissue used was from the non-tumours normal margin surrounding the tumour site. Data relating to age, gender, lung function, smoking history, was obtained for patients (see table 4.1). Where different groups of patients were used for individual experiments, the patient characteristics were controlled to ensure the clinical details of the whole group were represented.

Table 4.1 Patient characteristics prior to removal of lung tissue

No. of Subjects	96
Age (Yr.)	64.5 ±1.1
Gender	36 Female, 60 Male
Lung Function (FEV_1/FVC)	0.68 ±0.01
Smoking Status	9 Non smokers 51 Ex smokers 36 Current smokers
Pack years	45.2 ±3.1

Table 4.1Tissue samples were taken from a group of 96 patients. Patient details including age, gender, lung function given as the ratio of FEV_1/FVC, smoking status and pack years are listed as the mean ±SEM.

4.2.2 Primary cell culture of human lung tissue

Tissue samples were processed as previously described in chapter 2.2.2 preparation of human lung tissue for primary cell culture. Tissue was then either pre-incubated with 100ng/ml neutralising TNFα/IL-10 antibody or isotype matched control for one hour before stimulation with 100ng/ml of lipopolysaccharide or buffer control. Human lung fragments and supernatant were harvested at 1, 2, 4, 6, 24, and 48 hour time points and the tissue fragments weighed. Both the human lung fragments and supernatant collected were stored at -80°C. The levels of human TNFα, IL-6, IL-8, and IL-10 were measured in the supernatant using commercially available ELISAs (See sections 2.3.1 to 2.3.2).

4.2.3 Immunohistochemistry of human lung tissue

Human lung tissue from 18 individuals (9F/9M) consisting of 7 current, 7 ex and 4 non-smokers, (FEV_1/FVC 0.7), was embedded in glycol methacrylate (GMA) (see method 2.4.1). Immunohistochemical staining of the samples, using the streptavidin biotin peroxidase detection system, was used to analyse the expression of TNFα and IL-10, and also to determine their cellular source in the tissue (see methods 2.4.2). The antibodies used for the GMA staining are listed in the methods table 2.4.

4.2.4 Classification of COPD by severity using the GOLD guidelines

The GOLD guidelines were used to stratify patients by disease severity. The guidelines used are from the GOLD guide to COPD diagnosis, management and prevention (2004) and are listed below (see table 4.2).

Table 4.2 The GOLD guidelines for COPD diagnosis

Stage 0: At risk	Lung function is normal ($FEV_1/FVC > 70\%$).
Stage I: Mild COPD	Mild airflow limitation ($FEV_1/FVC < 70\%$ but $FEV_1 \geq 80\%$ predicted). At this stage individuals are often unaware that they may have abnormal lung function.
Stage II: Moderate COPD	Worsening airflow limitation ($50\% \geq FEV_1 < 80\%$ predicted)
Stage III: Severe COPD	Further worsening of airflow limitation ($30\% \geq FEV_1 < 50\%$ predicted). Exacerbations of symptoms are especially common in this group of patients.
Stage IV: Very severe COPD	Severe airflow limitation ($FEV_1 < 30\%$ predicted) or $FEV_1 < 50\%$ predicted plus chronic respiratory failure.

Table 4.2 Describes the criteria used to define GOLD status

4.2.5 Genotyping for TNFα and IL-10 alleles

For genotyping of the TNFα and IL-10 alleles the data includes 42 of the individuals previously described in chapter 4.1 and frozen tissue from an additional 54 individuals. As shown in table 4.1 the characteristics of the 96 individuals were broadly similar to the characteristics given in table 3.1. Genomic DNA was extracted from frozen human lung tissue using DNeasy spin columns from Quiagen as per manufacturers instructions (see method 2.6.1). TNFα -308 and IL-10 – 1082 single nucleotide polymorphisms (SNPs) were selected for genotyping due to their documented, but variable association with cytokine production and diseases that have an inflammatory basis (Waldron-Lynch *et al.*, 2001, Ozen *et al.*, 2002 and Tagore *et al.*, 1999). SNP genotyping was preformed using a two- reaction amplification refractory mutation system polymerase chain reaction (ARMS-PCR) approach. The methods used have been previously published (Howell et al., 2002, Smith et al., 2004), but briefly in this approach two separate PCR reactions are performed per SNP. Each PCR reaction also contained an additional pair of PCR primers, amplifying a sequence from the third intron of the human leukocyte antigen

DRB1 gene to act as an internal control for successful PCR. The primers used and full PCR cycling parameters are given in the methods 2.6.2 –3.

4.3 Results

4.3.1 The effect of neutralising TNFα antibody on IL-6 release

The kinetic data presented in chapter 3 demonstrated that a succession of cytokines are released in response to LPS, with TNFα at the head of the cascade. If TNFα is indeed initiating the inflammatory cascade of cytokines, removing TNFα should arrest or

attenuate the cytokine cascade. In our model a neutralising TNFα antibody was used to block the biological activity of TNFα.

Figure 4.1

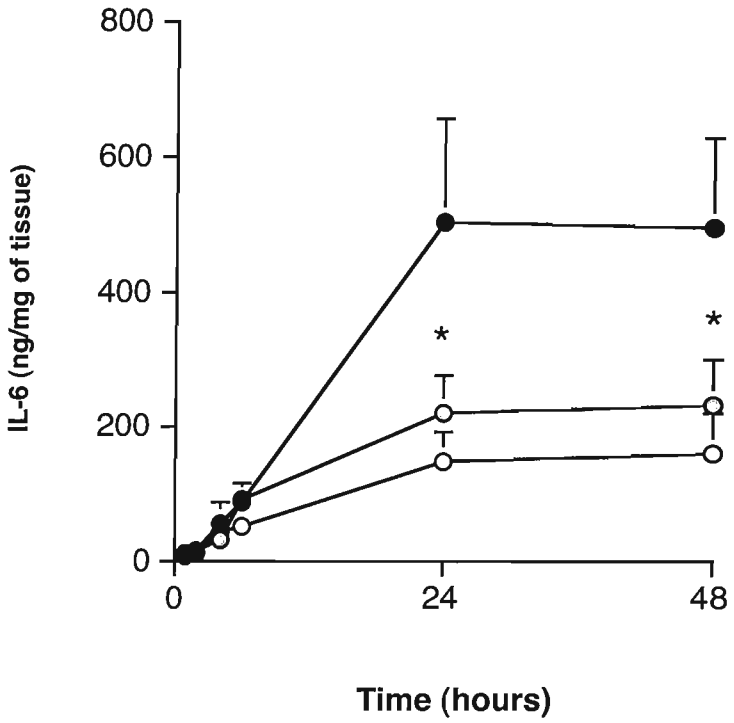


Figure 4.1 Effect of pre-treatment with nTNFαAb on IL-6 release. Human lung tissue (n=15) was incubated with nTNFαAb (grey circles) or an isotype control (filled circles) for 1hr and then stimulated with 100ng/ml LPS. The baseline levels of IL-6 from tissue incubated with only the isotype control are also indicated (open circles). The release of IL-6 into the supernatant was measured by ELISA. Values given are the mean ± SEM and are expressed as ng/mg of tissue. The data was statistically analysed using the Wilcoxon-Signed Rank test, * indicates that LPS stimulated IL-6 release is significantly decreased in the presence of nTNFα antibody ($P<0.05$).

As shown in figure 4.1 pre-treatment with neutralising TNFα antibody (nTNFα Ab) for 1hour before LPS stimulation reduced the release of IL-6 at 24hrs (mean= 216 ng/mg of tissue) compared to LPS and isotype control (mean= 501 ng/mg of tissue, $P<0.05$). We can also see in figure 4.1 that neutralisation of TNFα reduces the release of IL-6 back to baseline levels seen in the isotype control even at 48hrs.

4.3.2 The effect of neutralising TNFα antibody on IL-8 release

Neutralisation of TNFα also resulted in a reduction in IL-8 release. As shown in figure 4.2, the levels of IL-8 released were reduced at 24hrs with nTNFαAb treatment (mean= 319 ng/mg of tissue) compared to LPS and isotype control (mean= 767 ng/mg of tissue, $P<0.05$). As previously shown with IL-6, nTNFα Ab also inhibited IL-8 release back to the baseline levels seen with the isotype control up to the 48 hour time point (see figure 4.2).

Figure 4.2

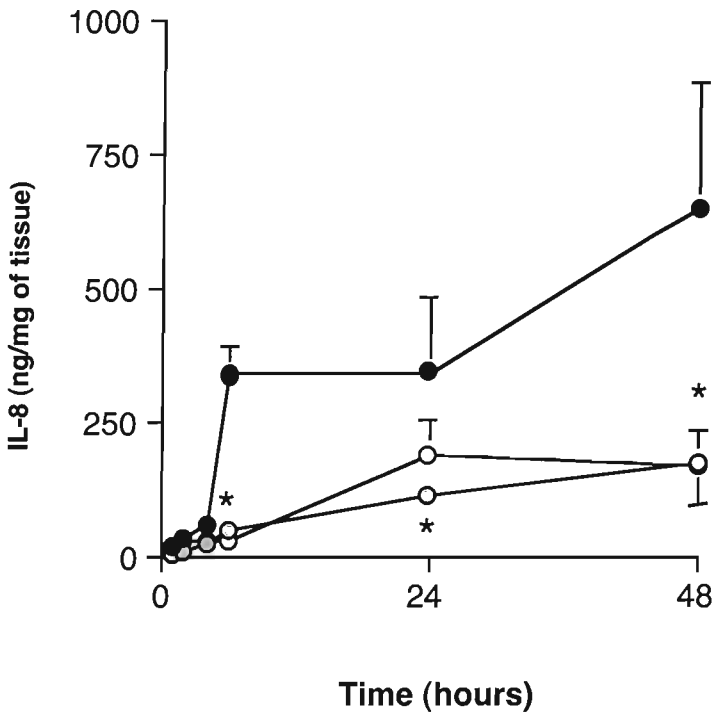


Figure 4.2 Effect of pre-treatment with nTNFαAb on IL-8 release. Supernatants from figure 4.2 were analysed for IL-8 using ELISA. As before the tissue was incubated with either nTNFαAb (grey circles) or an isotype control (filled circles) for 1hr and then stimulated with 100ng/ml LPS. The baseline levels of IL-6 are also indicated (open circles). Values given are the mean ± SEM and are expressed as ng/mg of tissue. The data was statistically analysed using the Wilcoxon-Signed Rank test, * indicates that LPS stimulated IL-8 release is significantly decreased in the presence of nTNFα antibody ($P<0.05$).

4.3.3 The effect of neutralising TNFα antibody on IL-10 release

The release of IL-10 was also completely obliterated with pre-treatment of nTNFαAb. As shown in figure 4.3 the most marked difference with nTNFα Ab treatment is at 48hrs (mean= 0.3 ng/mg of tissue) compared to the LPS stimulated isotype control (mean= 18.3 ng/mg of tissue, $P<0.05$).

Figure 4.3

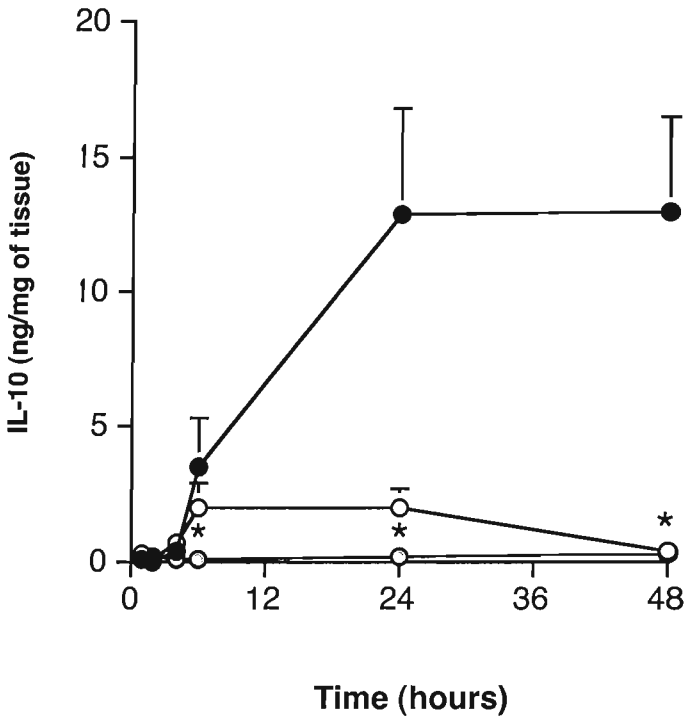


Figure 4.3 Effect of pre-treatment with nTNFαAb on IL-10 release. Supernatants from figure 4.3 were analysed for IL-10 using ELISA. The tissue was incubated with either nTNFαAb (grey circles) or an isotype control (filled circles) for 1hr and then stimulated with 100ng/ml LPS. The baseline levels of IL-6 are also indicated (open circles). Values given are the mean ± SEM and are expressed as ng/mg of tissue. The data was statistically analysed using the Wilcoxon-Signed Rank test, * indicates that LPS stimulated IL-10 is significantly reduced by nTNFα antibody ($P<0.05$).

4.3.4 The effect of neutralising IL-10 antibody on TNFα release

Within the literature there is evidence that IL-10 can regulate TNFα release via a negative feedback mechanism (Armstrong *et al.*, 1996, Gazzinelli *et al.*, 1996). As TNFα is the key cytokine in our inflammatory response we were therefore interested if IL-10 was able to

regulate the release of TNFα in our model. To block the biological activity of IL-10 in these experiments we used a neutralising IL-10 antibody (nIL-10 Ab). As shown in figure 4.4 pre-treatment with nIL-10Ab for 1hour before LPS stimulation actually augmented the release of TNFα, especially at the time points where we previously started to see IL-10 release (see figure 3.10). The effect of neutralisation of IL-10 is most marked when we look at the 48hour time point in figure 4.4, where the levels of TNFα released are still dramatically increasing unchecked (mean= 28.9 ng/mg of tissue) compared to the isotype and LPS control (mean= 8.5 ng/mg of tissue, $P<0.05$).

Figure 4.4

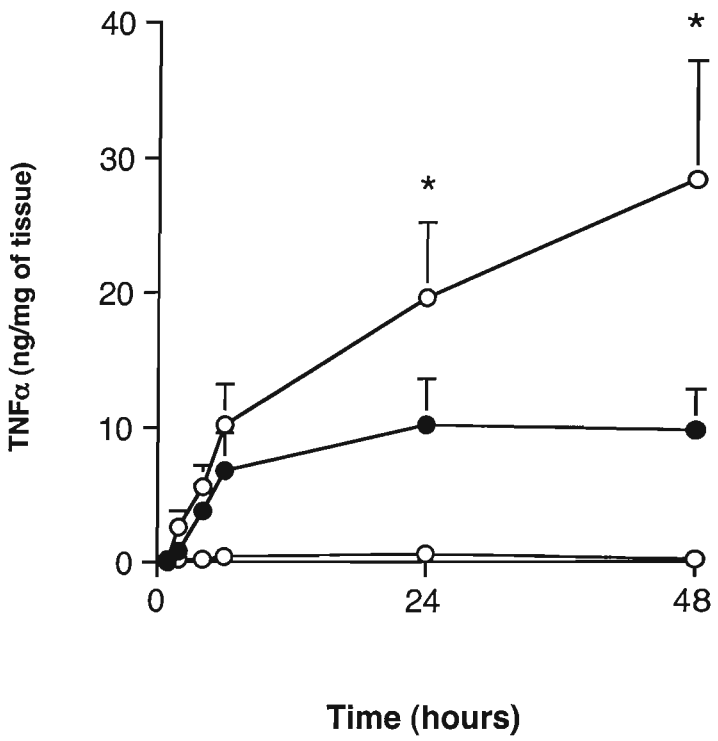


Figure 4.4 Effect of pre-treatment with nIL-10Ab on TNFα release. Human lung tissue (n=11) was incubated with nIL-10Ab (grey circles) or an isotype control (filled circles) for 1hr and then stimulated with 100ng/ml LPS. The baseline levels of TNFα from tissue incubated with only the isotype control are also indicated (open circles). The release of TNFα into the supernatant was measured by ELISA. Values given are the mean ± SEM and are expressed as ng/mg of tissue. The data was statistically analysed using the Wilcoxon-Signed Rank test, * indicates that LPS stimulated release of TNFα is significantly increased in the presence of nIL-10 antibody ($P<0.05$).

4.3.5 The effect of neutralising IL-10 antibody on IL-6 release

As neutralising the activity of IL-10 resulted in augmented release of TNFα, we were interested if neutralising IL-10 elevated the release of any other cytokines involved in the inflammatorycascade. As shown in figure 4.5 pre-treatment with nIL-10Ab for 1hour before LPS stimulation also resulted in augmented release of IL-6 at 24hrs, which was again maintained until 48hrs (mean= 810 ng/mg of tissue) compared to isotype control and LPS stimulation (mean= 565 ng/mg of tissue, $P<0.05$).

Figure 4.5

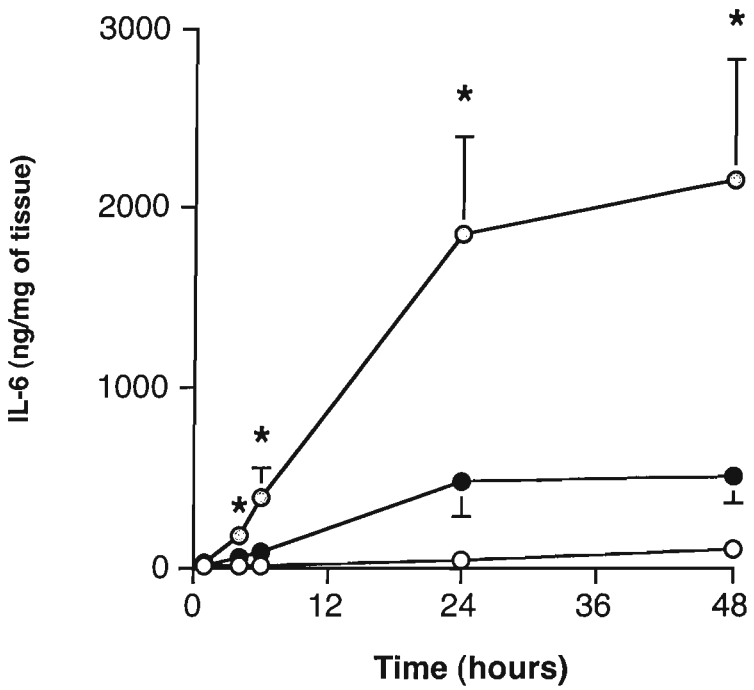


Figure 4.5 Effect of pre-treatment with nIL-10Ab on IL-6 release. Supernatants from figure 4.4 were analysed for IL-6 using ELISA. As before the tissue was incubated with either nTNFαAb (grey circles) or an isotype control (filled circles) for 1hr and then stimulated with 100ng/ml LPS. The baseline levels of IL-6 are also indicated (open circles). Values given are the mean ± SEM and are expressed as ng/mg of tissue. The data was statistically analysed using the Wilcoxon-Signed Rank test, * indicates that LPS stimulated release of IL-6 is significantly increased in the presence of nIL-10 antibody ($P<0.05$).

4.3.6 The effect of neutralising IL-10 antibody on IL-8 release

As shown in figure 4.6 pre-treatment with nIL-10Ab for 1hour before LPS stimulation also resulted in the elevated release of IL-8 at 24hrs. The release of IL-8 remained elevated until 48hrs (mean= 2156 ng/mg of tissue) compared to isotype control and LPS stimulation (mean= 507 ng/mg of tissue, $P<0.05$).

Figure 4.6

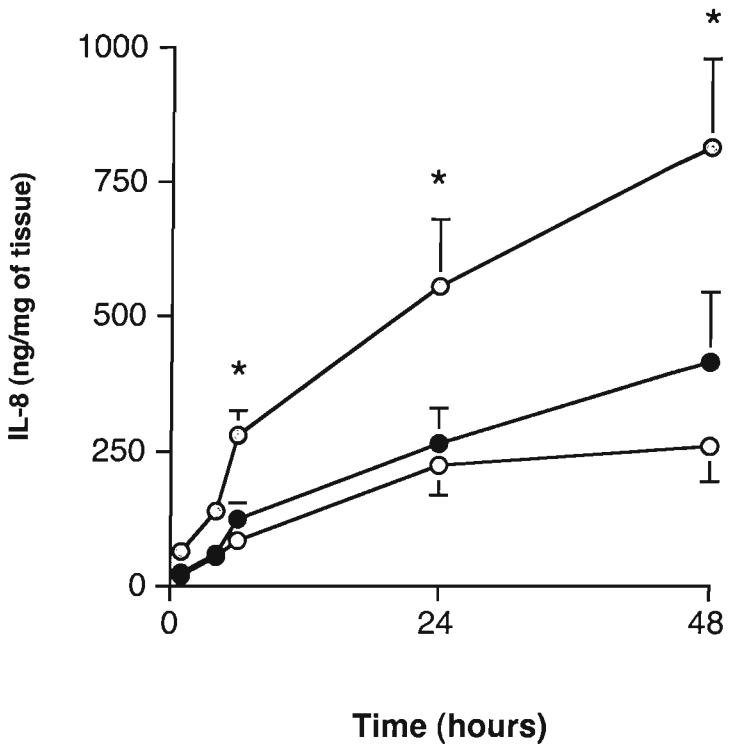


Figure 4.6 Effect of pre-treatment with nIL-10Ab on IL-8 release. Supernatants from figure 4.4 were analysed for IL-8 using ELISA. The tissue was incubated with either nTNFαAb (grey circles) or an isotype control (filled circles) for 1hr and then stimulated with 100ng/ml LPS. The baseline levels of IL-6 are also indicated (open circles). Values given are the mean ± SEM and are expressed as ng/mg of tissue. The data was statistically analysed using the Wilcoxon-Signed Rank test, * indicates that LPS stimulated release of IL-8 is significantly increased in the presence of nIL-10 antibody ($P<0.05$).

4.3.7 Immunohistochemistry of human lung tissue

We then turned our attention to the cellular sources of cytokines in the model. We focused on the cellular sources of TNFα and IL-10, as they appear to play key roles in the cytokine cascade. Immunohistochemistry is an extremely powerful technique allowing the

visualisation of proteins in their native tissue. In this study the tissue used in the model is mainly lung parenchyma. Following primary culture the tissue samples were fixed in acetone and embedded in GMA for immunohistochemical analysis.

4.3.8 TNFα in lung parenchyma

TNFα expression was analysed in a cohort of 18 patients consisting of 7 current, 7 ex and 4 non-smokers with a range of lung functions (FEV_1/FVC). The staining presented here is from two individuals, to illustrate the differences observed between current smokers with and without obstructed lung disease. Both were embedded and stained with a monoclonal antibody for TNFα (see method 2.4.2) after 1hr of LPS stimulation in culture. Figure 4.7 (upper panel, over the page) shows TNFα staining in a sample obtained from a male current smoker, 60 years of age. This individual presented with an FEV_1/FVC of 45% indicating obstructive lung disease. The picture depicts intense TNFα staining associated within cell bodies (indicated by the arrows). The second sample (figure 4.7, lower panel) was from a male current smoker, 59 years of age. This individual however had an FEV_1/FVC ratio of 84%, indicating normal lung function. As can be seen there is less TNFα immunoreactivity in this sample compared to the current smoker with poor lung function.

After 6hrs of LPS stimulation the staining for TNFα within the parenchyma is much more diffuse in both samples (see figure 4.8). TNFα is mainly associated with the ECM and endothelial cells, with little immunoreactivity found within cells. However TNFα staining is more intense in the impaired lung tissue (upper panel) compared to the 'normal' lung (lower panel).

Figure 4.7

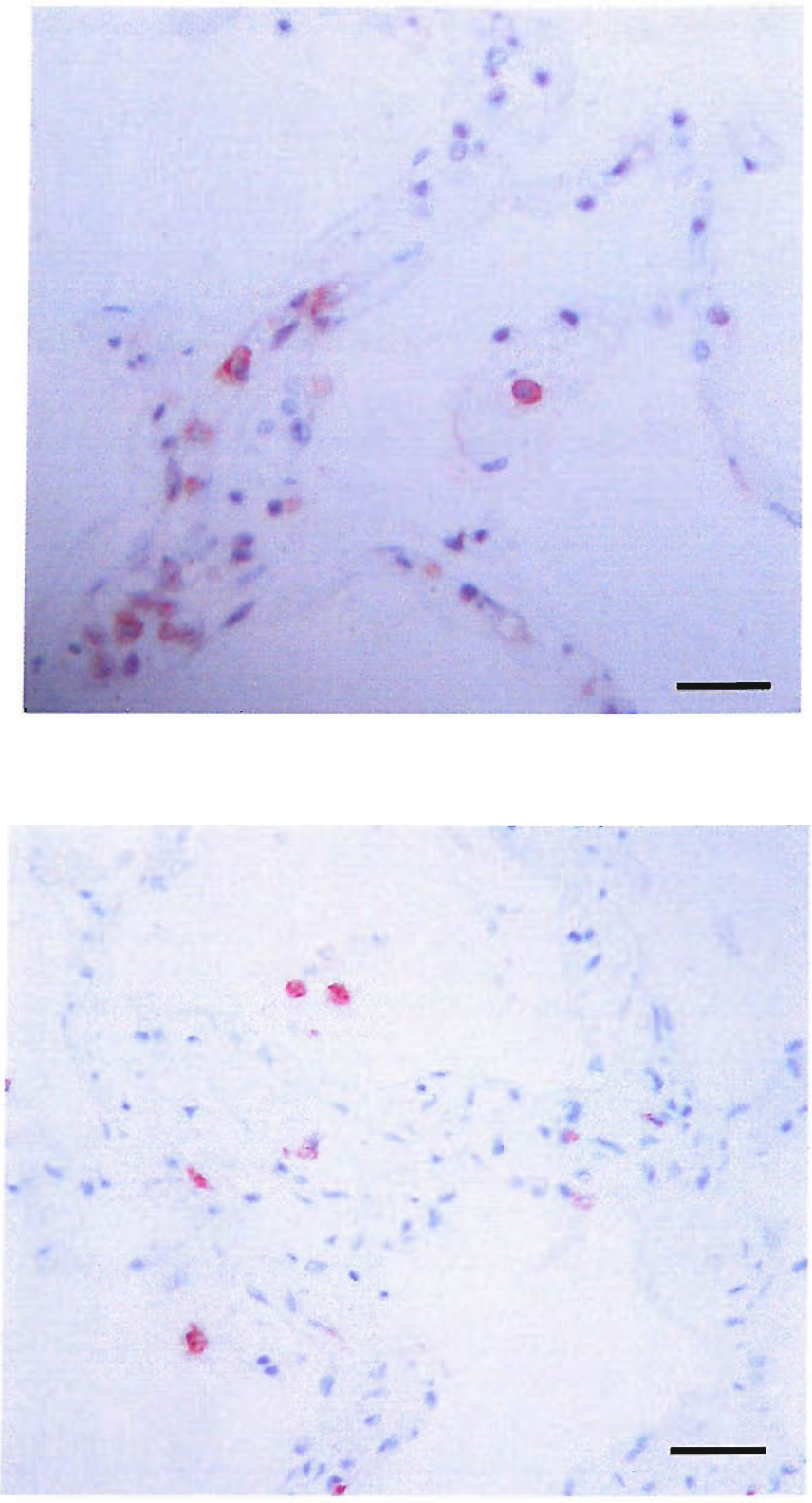


Figure 4.7. TNFα staining post 1 hour LPS stimulation in lung parenchyma. Staining for TNFαin the lung parenchyma from a 60-year-old current smoker with obstructive lung disease, following stimulation of the tissue with LPS for 1 hour (upper panel). Compared to TNFα staining in lung parenchyma from a 59-year-old current smoker with normal lung function, again following LPS stimulus for 1 hour. Bar represents 50μm, positive cells are stained red.

Figure 4.8

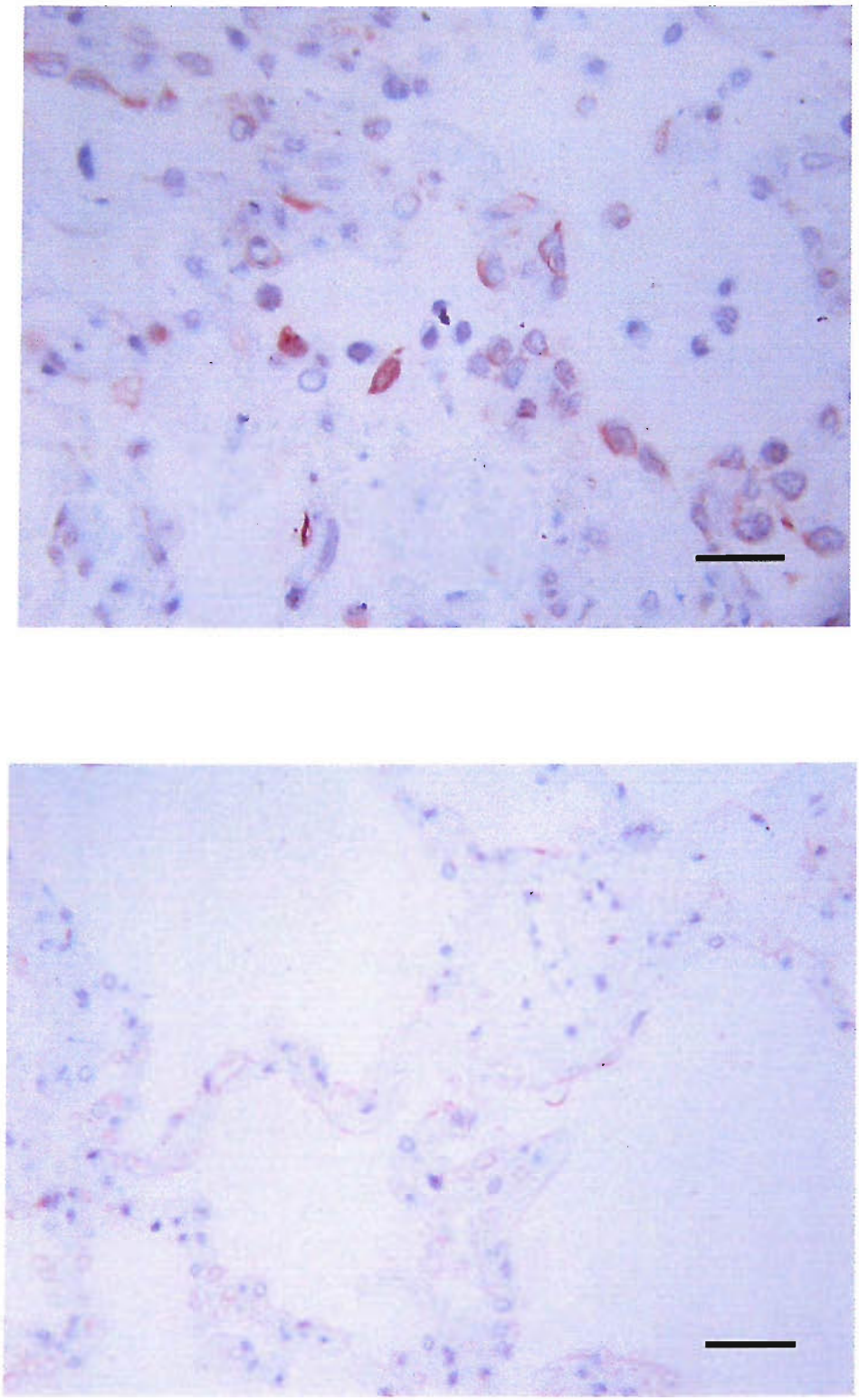


Figure 4.8. TNFα staining post 6 hour LPS stimulation in lung parenchyma. Staining for TNFαin the lung parenchyma from a 60-year-old current smoker with obstructive lung disease, following stimulation of the tissue with LPS for 6 hours (upper panel). Compared to TNFα staining in lung parenchyma from a 59-year-old current smoker with normal lung function, again following LPS stimulus for 6 hours. Bar represents 50μm, positive cells are stained red.

4.3.9 Co-localisation of TNFα

Co-localisation analysis of sequential sections was conducted to identify inflammatory cells contributing to TNFα release during the inflammatory response. The markers used were neutrophil elastase (neutrophils), CD68 (activated macrophages), and AA1 (mast cells). All sections stained positive for varying amounts of neutrophil elastase, CD68 and AA1. Figures 4.9, 4.10 and 4.11 (over the page) show sequential sections of lung parenchyma from a 65 year old female smoker, with a FEV_1/FVC of 0.68 stained with monoclonal antibodies for TNFα, CD68, AA1 and neutrophil elastase. The staining in figure 4.9 illustrates the co-localisation of TNFα with activated macrophages, TNFα was also found to co-localise with mast cells as shown in figure 4.10, which was consistently found for all individuals in our study. The co-localisation of TNFα release with neutrophils was not observed.

Mast cells are capable of both newly synthesising and storing TNFα in secretory granules. However in the staining undertaken it is impossible to assess if the TNFα observed in the mast cells at one hour is being newly synthesised or is present due to being previously stored in granules. A further study would therefore be required to establish whether macrophages or mast cells contribute to the majority of the TNFα released in our model. Finally empirical analysis indicated that the numbers and distribution of inflammatory cells was similar in all tissue sections.

Figure 4.9

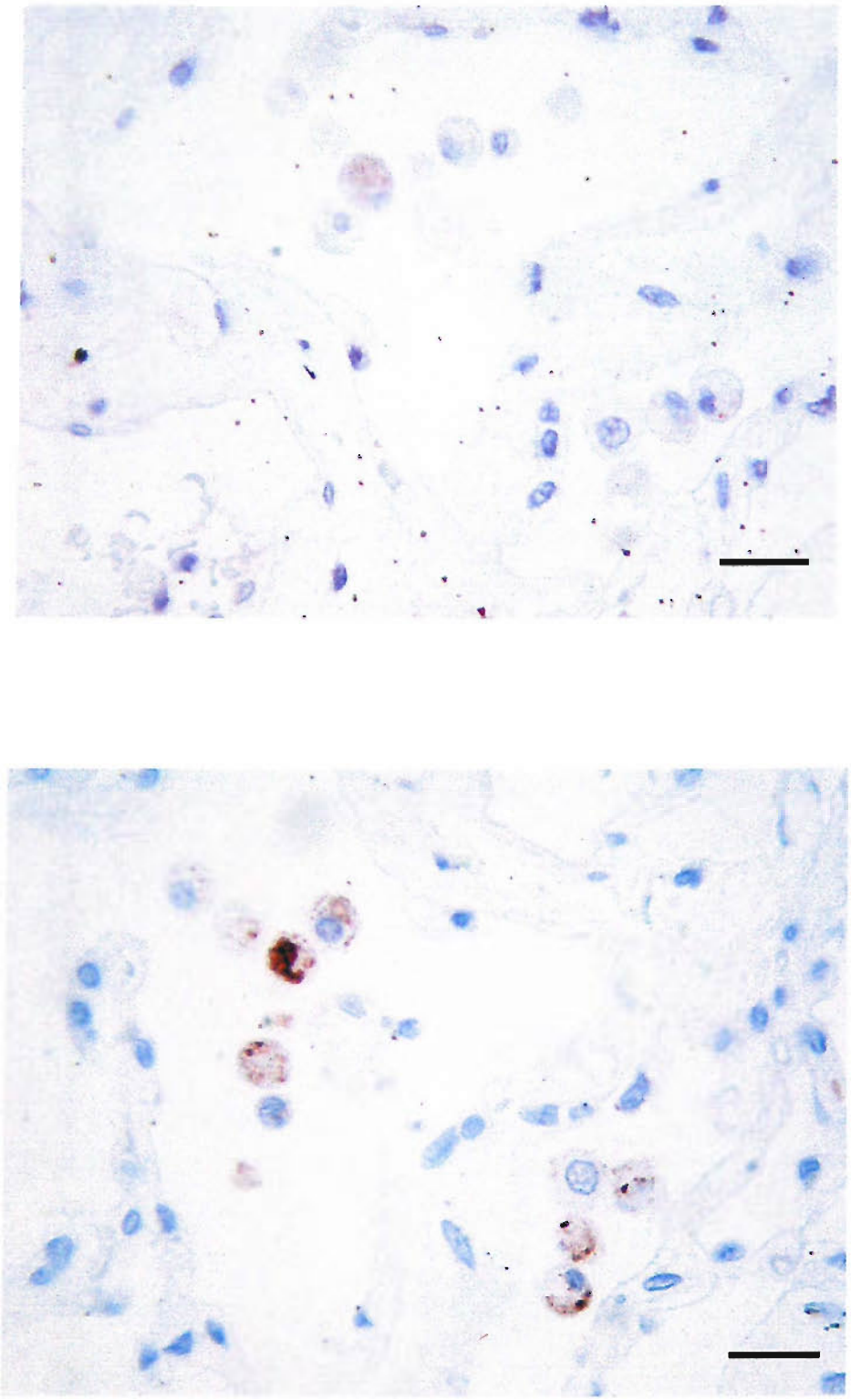


Figure 4.9. Co-localisation of TNFα with macrophages in the lung parenchyma. Lung was obtained from a 65-year-old female smoker, with normal lung function and stimulated with LPS for 1 hour. The tissue was then embedded and sequential sections of lung parenchyma obtained and stained with monoclonal antibodies for TNF α (upper panel) and CD68 (lower panel). Bar represents 4μm, positive cells are stained red.

Figure 4.10

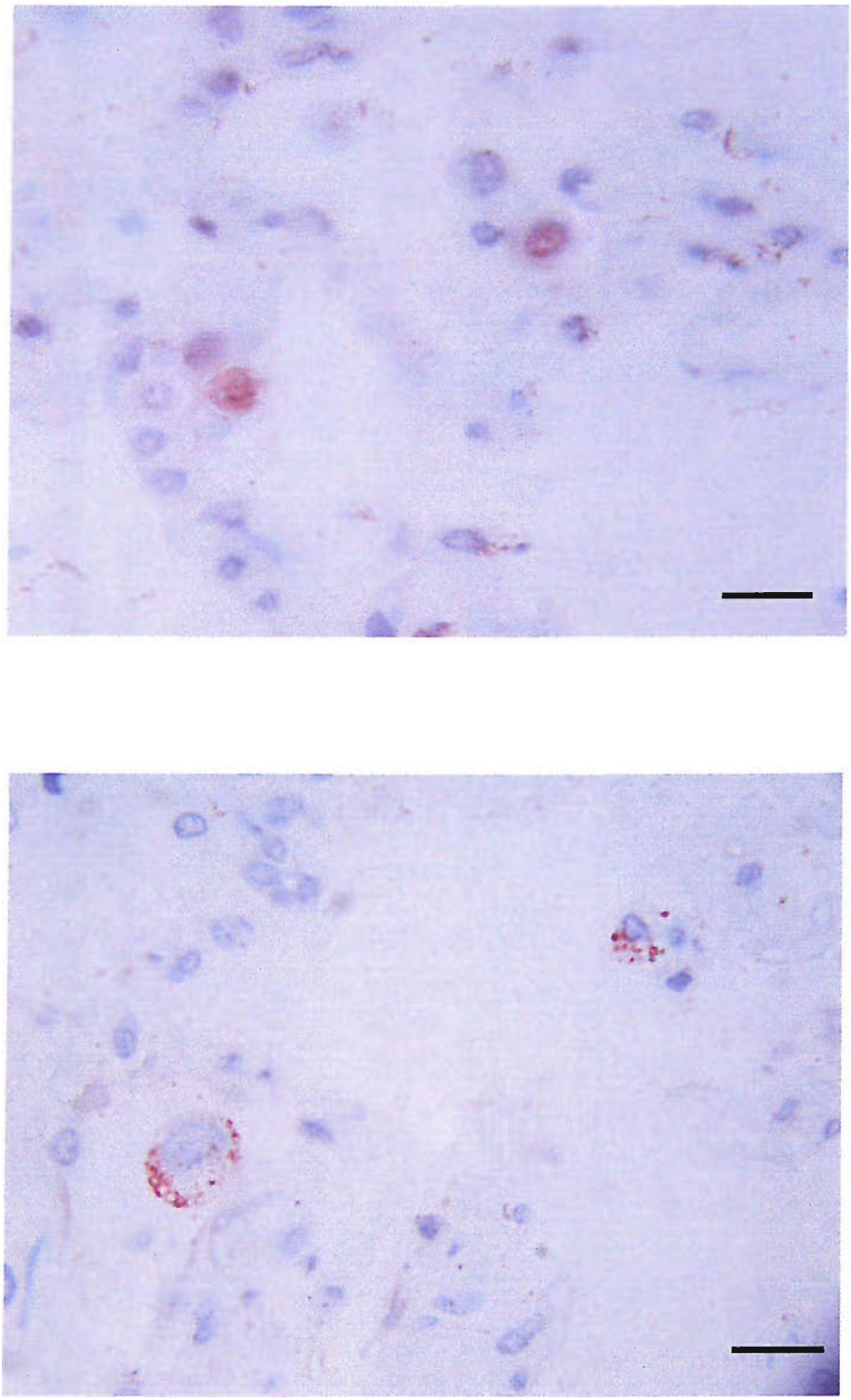


Figure 4.10. Co-localisation of TNFα with mast cells in the lung parenchyma. Lung was obtained from a 65-year-old female smoker, with normal lung function and stimulated with LPS for 1 hour. The tissue was then embedded and sequential sections of lung parenchyma obtained and stained with monoclonal antibodies for TNF α (upper panel) and AA1 (lower panel). Bar represents 4μm, positive cells are stained red.

Figure 4.11

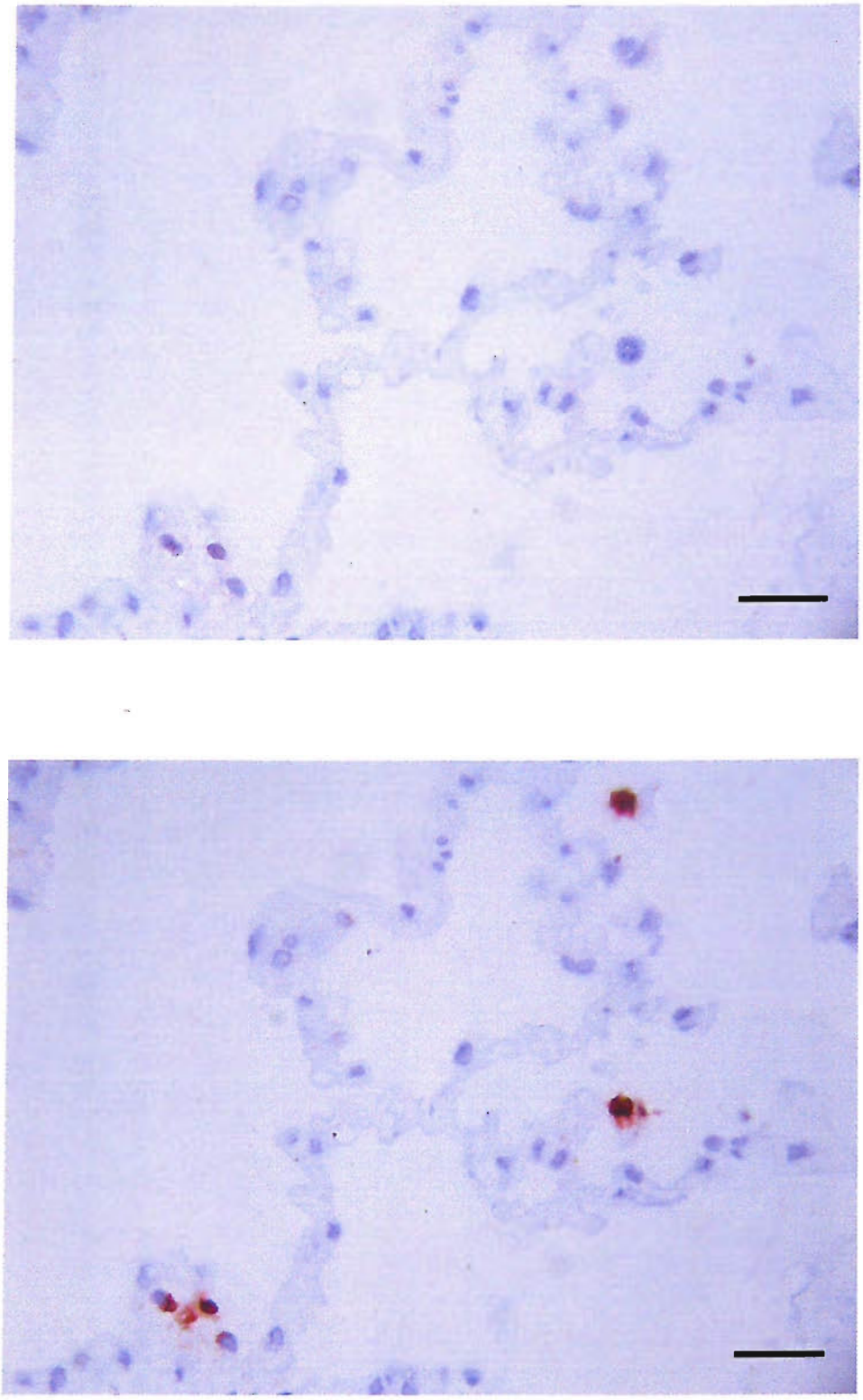


Figure 4.11. Co-localisation of TNFα with neutrophils in the lung parenchyma. Lung was obtained from a 65-year-old female smoker, with normal lung function and stimulated with LPS for 1 hour. The tissue was then embedded and sequential sections of lung parenchyma obtained and stained with monoclonal antibodies for TNF α (upper panel) and neutrophil elastase (lower panel). Bar represents 20μm, positive cells are stained red.

4.3.10 IL-10 staining in lung parenchyma

The same tissue used in to co-localise TNFα with cells in the lung parenchyma was also used to analyse IL-10 release in tissue stimulated with LPS for 6hrs. In comparison to TNFα we observed practically no IL-10 release at 6hrs. From the kinetic data shown in figure 3.10 (over the page) we previously observed IL-10 release peaked at 48hrs in response to LPS. Unfortunately we did not embed tissue from the 48hr time point due to concerns of tissue antigenicity. However, as shown in figure 4.12 the IL-10 that was observed was found to be co-localised with activated macrophages in the lung parenchyma.

Figure 4.12

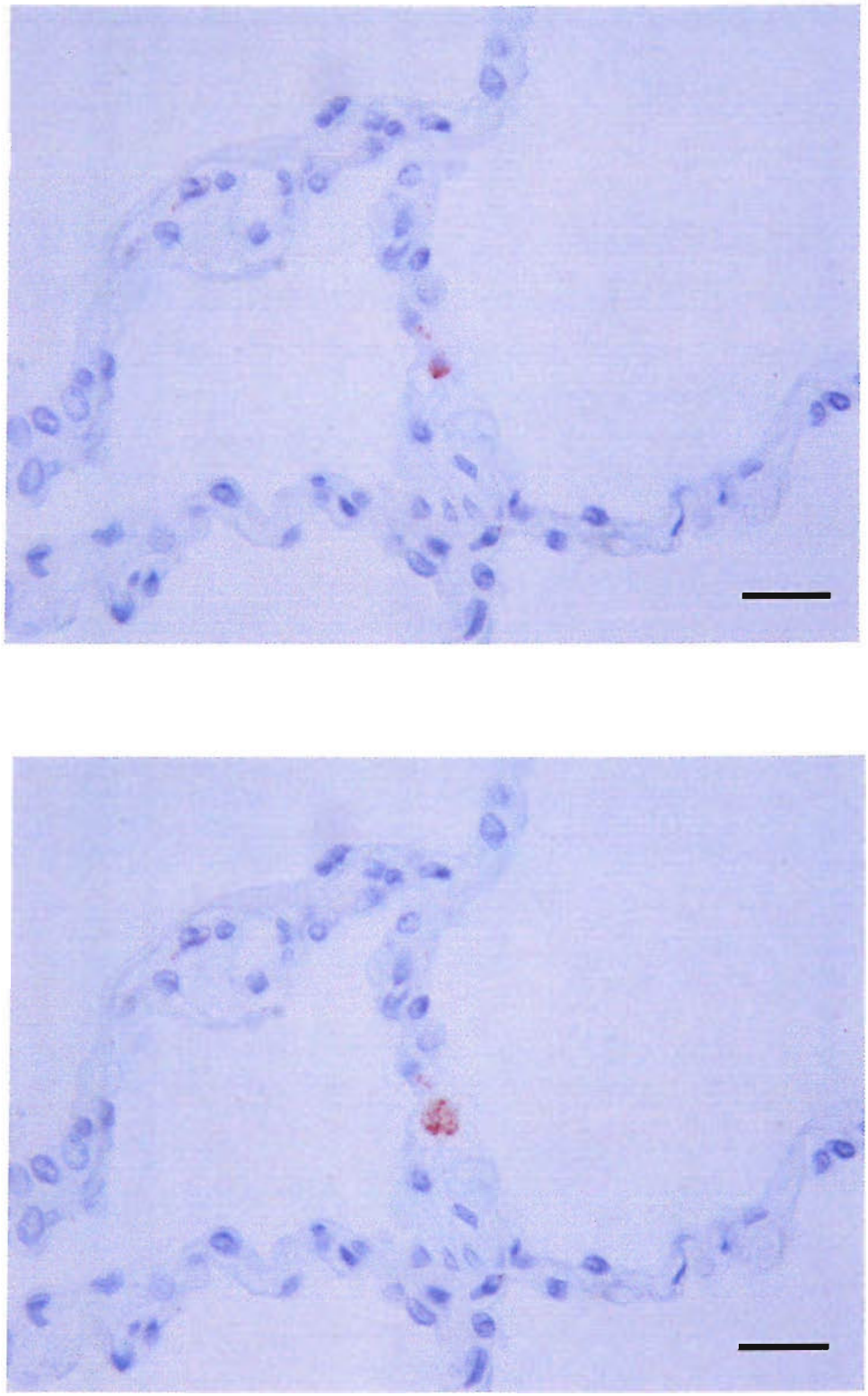


Figure 4.12. Co-localisation of IL-10 with macrophaes in the lung parenchyma. Lung was obtained from a 65-year-old female smoker, with normal lung function and stimulated with LPS for 1 hour. The tissue was then embedded and sequential sections of lung parenchyma obtained and stained with monoclonal antibodies for IL-10 (upper panel) and CD68 (lower panel). Bar represents 5μm, positive cells are stained red.

4.3.11 Heterogeneity in the TNFα response

We have shown evidence using neutralising TNFα antibodies and immunohistochemistry that supports the hypothesis that TNFα is a key cytokine in the acute inflammatory response. Interestingly, over the course of the kinetic experiments we noted substantial variation in the amounts of TNFα released in response to LPS between individuals. Figure 4.13 shows the heterogeneity in the LPS stimulated release of TNFα at 6 hours, from 54 patients.

Figure 4.13

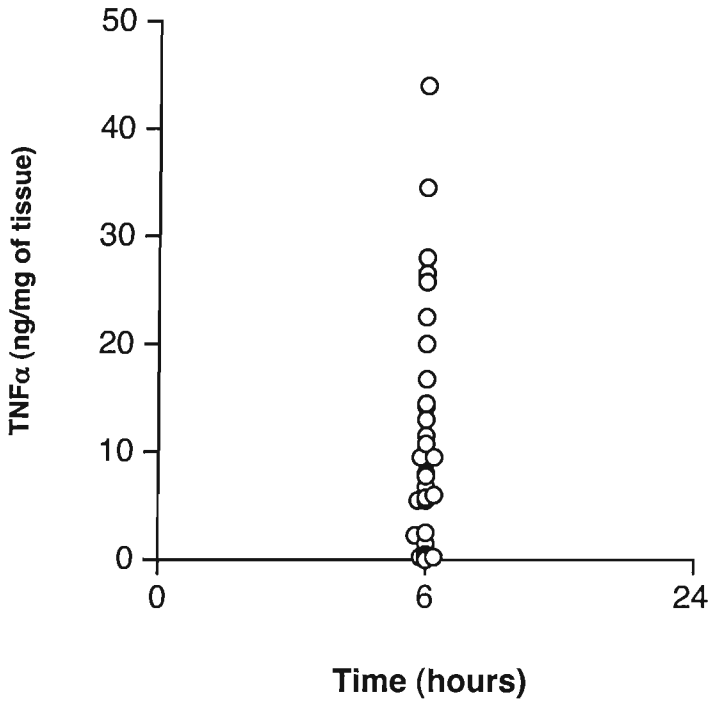


Figure 4.13 The heterogeneity of TNFα release from human lung tissue. The data is reworked from figure 3.1, human lung tissue was stimulated with LPS for 6 hrs and release of TNFα measured by ELISA, value are expressed as ng/mg of tissue.

4.3.12 Re-classification of TNFα data using GOLD guidelines

Having noted the heterogeneity in TNFα release in response to LPS. We were interested to investigate if disease severity was influencing TNFα release in our acute inflammatory model. Patients were classified using the GOLD guidelines for COPD. For full definitions

see methods 4.2.4 but briefly, GOLD 0 indicates patients, which are at risk of developing COPD. GOLD 1 classifies patients with mild COPD and GOLD 2 indicates patients with moderate COPD. The TNFα kinetic data from figure 3.1 was re-analysed using the GOLD guidelines. Unfortunately, full spirometric analysis for all 54 individuals in the study was not available and the data shown therefore only includes 36 of the patients. As shown by figure 4.14 all individuals followed a similar release of TNFα up to the 6 hour time point. However, at 24hrs TNFα release continued to increase in individuals classified as GOLD 1 (mean= 26.5 ng/mg of tissue) and GOLD 2 (mean= 27.6 ng/mg of tissue, $P<0.05$) compared to GOLD 0 individuals (mean= 12.3 ng/mg of tissue, $P<0.05$). By 48hrs TNFα release is shown to plateau in all groups.

Figure 4.14

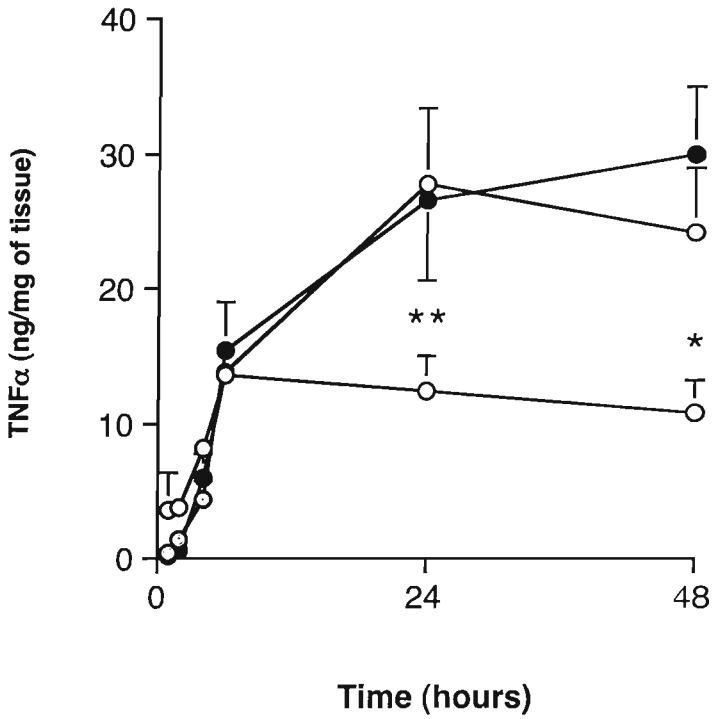


Figure 4.14 Re-classification of TNFα release using GOLD guidelines. Data from figure 3.2 was re-classified as GOLD 0 (open circles), GOLD 1 (grey circles) and GOLD 2 (filled circles) individuals using the GOLD guidelines. Values given are the mean ±SEM and are expressed as ng/mg of tissue. The data was statistically analysed using the Wilcoxon-Signed Rank test, ** indicates $P<0.05$ for both GOLD 1 and 2 compared to GOLD 0, while * indicates $P<0.05$ for GOLD 2 compared to GOLD 0.

4.3.13 Re-classification of IL-6 data using GOLD guidelines

As disease severity influenced TNFα release in our model we interested to know if severity of disease directed the release of other cytokines in the inflammatory cascade. The IL-6 kinetic data from figure 3.4 was re-classified again using the GOLD guidelines and is shown in figure 4.15. Again as we saw in the TNFα data, all individuals followed a similar release of IL-6 up to 6 hours. By 24hrs we can observe a clear difference in IL-6 release with GOLD 2 patients releasing more IL-6 (mean= 773 ng/mg of tissue) compared to GOLD 1 (mean= 511 ng/mg of tissue, $P<0.05$) and GOLD 0 patients (mean= 217 ng/mg of tissue, $P<0.05$). The elevated release of IL-6 with disease severity was still maintained at 48hrs.

Figure 4.15

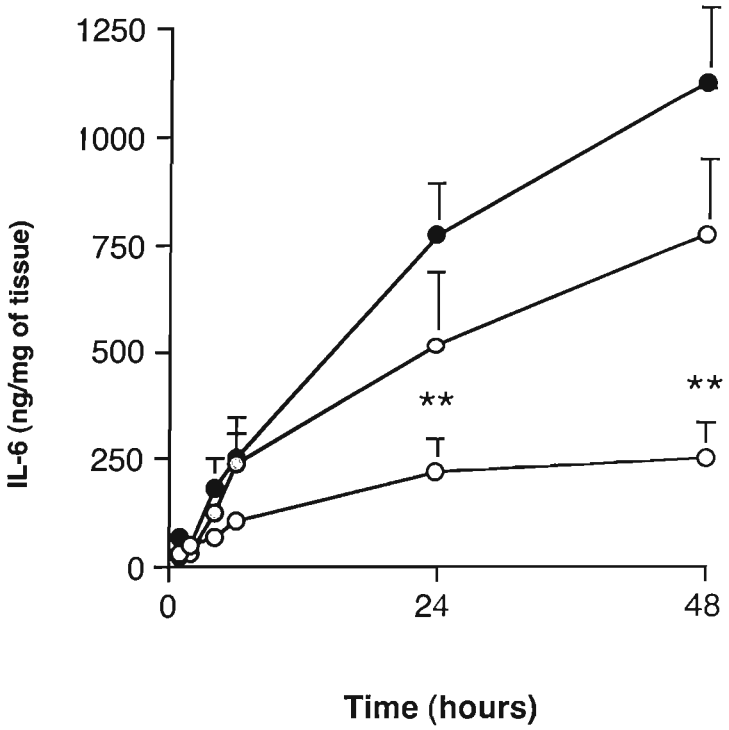


Figure 4.15 Re-classification of IL-6 release using GOLD guidelines. Data from figure 3.5 was re-classified as GOLD 0 (open circles), GOLD 1 (grey circles) and GOLD 2 (filled circles) individuals using the GOLD guidelines. Values given are the mean ±SEM and are expressed as ng/mg of tissue. The data was statistically analysed using the Wilcoxon-Signed Rank test, ** indicates $P<0.05$ for both GOLD 1 and 2 compared to GOLD 0.

4.3.14 Re-classification of IL-8 data using GOLD guidelines

Patients classified as GOLD 0 and GOLD 1 followed a similar pattern of IL-8 release over all of the time points. Although GOLD 2 patients followed a similar pattern of release as shown by figure 4.16, GOLD 2 patients also released more IL-8 at all of the time points. The difference being greatest at 48hrs with GOLD 2 patients releasing (mean= 2041 ng/mg of tissue) compared to a mean value of IL-8 release of 1147 and 1046 ng/mg of tissue respectively from GOLD 0 and 1 patients ($P<0.05$).

Figure 4.16

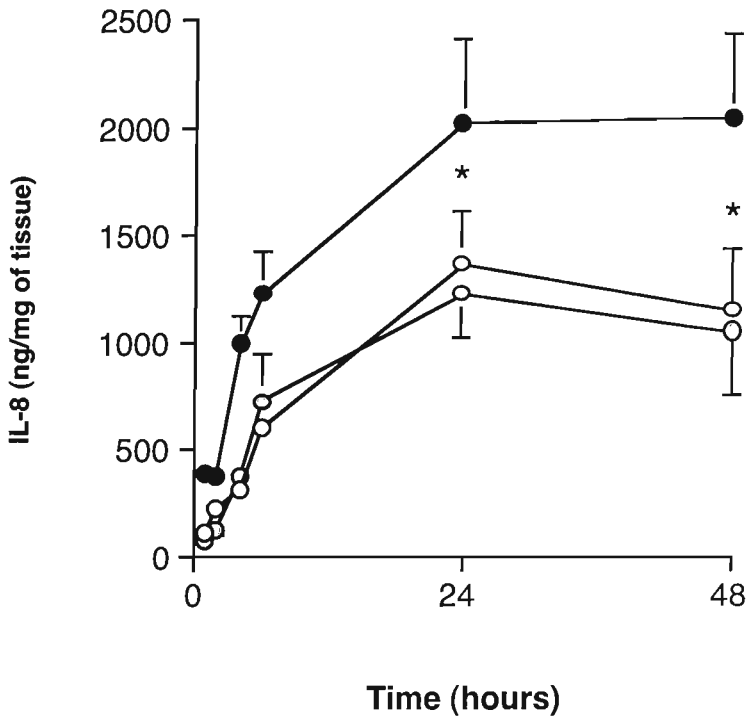


Figure 4.16 Re-classification of IL-8 release using GOLD guidelines. Data from figure 3.7 was re-classified as GOLD 0 (open circles), GOLD 1 (grey circles) and GOLD 2 (filled circles) individuals using the GOLD guidelines. Values given are the mean ±SEM and are expressed as ng/mg of tissue, error bars have been removed for clarity. The data was statistically analysed using the Wilcoxon-Signed Rank test, * indicates $P<0.05$ for GOLD 2 compared to GOLD 0 and 1.

4.3.15 Re-classification of IL-10 data using GOLD guidelines

Finally, the kinetic IL-10 data from figure 3.8 was also re-analysed using the GOLD guidelines as is shown in figure 4.17. All individuals followed a similar pattern of release for IL-10 up to 4 hours. By 24hrs we can observe a clear difference in IL-10 release with GOLD 0 patients releasing more IL-10 (mean= 17.9 ng/mg of tissue) in comparison to GOLD 1 (mean= 8.5 ng/mg of tissue, $P<0.05$) and GOLD 2 patients (mean= 7.8 ng/mg of tissue, $P<0.05$).

Figure 4.17

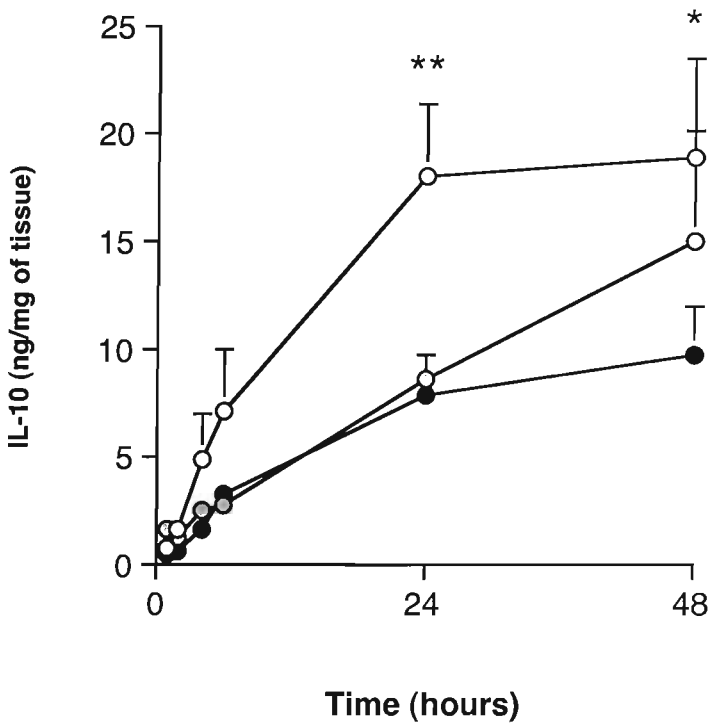


Figure 4.17 Re-classification of IL-10 release using GOLD guidelines. Data from figure 3.12 was re-classified as GOLD 0 (open circles), GOLD 1 (grey circles) and GOLD 2 (filled circles) individuals using the GOLD guidelines. Values given are the mean ±SEM and are expressed as ng/mg of tissue. The data was statistically analysed using the Wilcoxon-Signed Rank test, ** indicates $P<0.05$ for GOLD 2 compared to GOLD 0 and 1, while * indicates $P<0.05$ for GOLD 2 compared to GOLD 0.

4.3.16 Cytokine release from current and ex-smokers

Given the above data we were therefore interested to see if any other clinical parameters of the patients were predictors of cytokine release. The data illustrated in figure 3.1 was used to analyse the differences in TNFα release from current and ex-smokers. The release of TNFα was statistically elevated in the current smokers from 6 hours (mean=12.3ng/mg of tissue) compared ex-smokers (mean= 6.1ng/mg of tissue, $P<0.05$) and remained elevated as shown in figure 4.18a. As with TNFα we found the release of IL-6 was statistically elevated in current smokers from 6hrs (mean=510.2ng/mg of tissue) verses the ex smokers (mean= 220.0ng/mg of tissue, $P<0.05$) and remained elevated at 48hrs as shown in figure 4.18b. Chi squared analysis of the data indicated there was no relationship between GOLD status and smoking history (Chi squared=0.23, P=0.59). Finally, in contrast to TNFα and IL-6 we found no difference between current and ex-smokers for the cytokines IL-8 and IL-10 (data not shown).

Figure 4.18

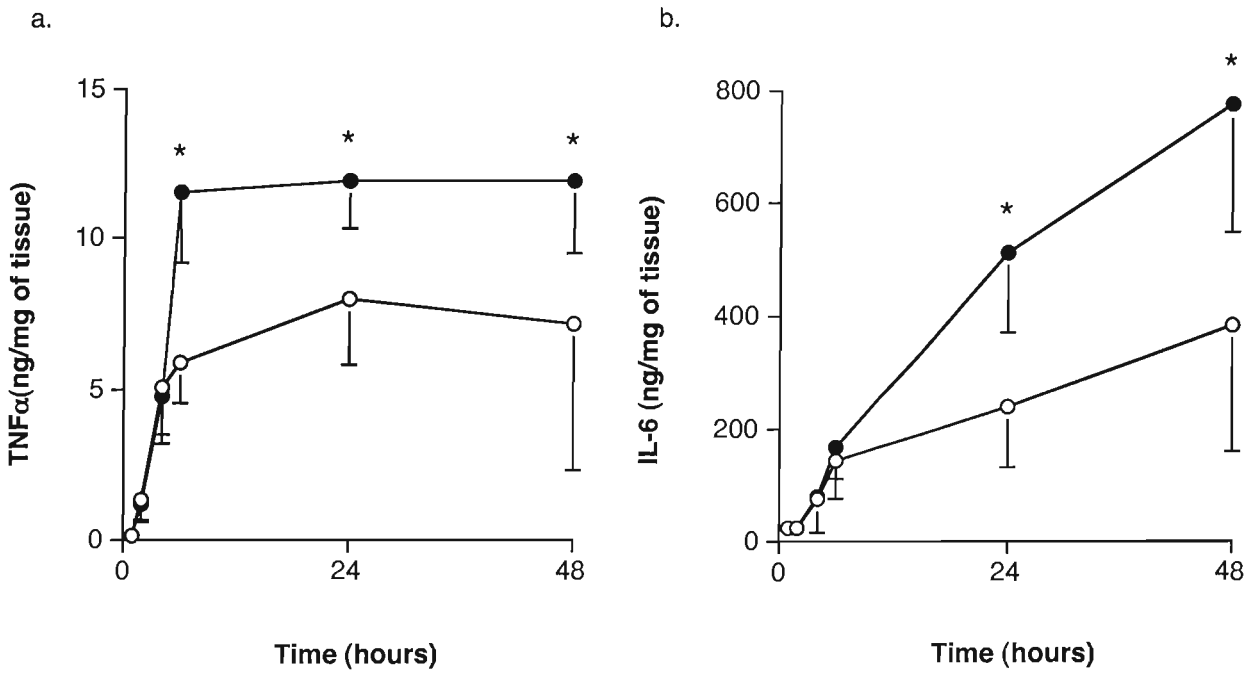


Figure 4.18 Cytokine levels in supernatants from current and ex-smokers. For figure 4.18a data from figure 3.1 was re-classified into current smokers (filled circles) and ex-smokers (open circles). Data from figure 3.7 was also reclassified into current smokers (filled circles) and ex-smokers (open circles) see figure 4.18b. Values given are the mean ± SEM and are expressed as ng/mg of tissue. The data was statistically analysed using the Wilcoxon-Signed Rank test, * indicates $P<0.05$ for current smokers compared to ex smokers.

4.3.17 Polymorphisms in the TNFα promoter

Our data has indicated there is a relationship between the release of TNFα from human lung tissue and the presence of COPD. In diseases associated with chronic inflammation such as rheumatoid arthritis, polymorphisms in the TNFα promoter at base -308 have been shown to modify TNFα production (Knight *et al.*, 1999, Wilson *et al.*, 1997). In these studies individuals were classified as normal, moderate or high producers of TNFα depending on their alleles for the TNFα promoter. Analysis of the TNFα promoter in lung tissue of current and ex smokers could therefore potentially provide important information on the genetic basis of acute inflammation mediated by TNFα. In our study we typed the DNA of 96 individuals for the G/A single nucleotide polymorphism at position –308 in the promoter region of the TNFα gene using competitive PCR. The agarose gel in figure 4.19 illustrates the PCR products from six randomly selected lung tissue samples and genotype controls.

Figure 4.19

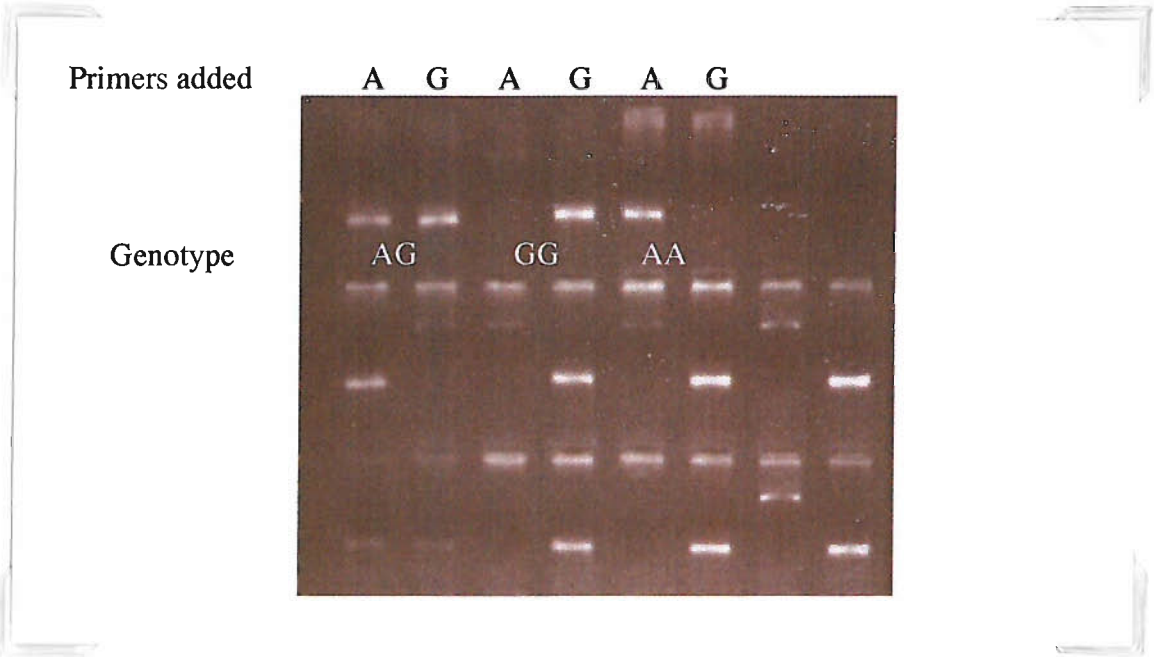


Figure 4.19 Example of typical TNFα –308 ARMs-PCR gel. Human lung tissue was removed from (n=96) individuals during lung resection procedures, and DNA extracted using DNA spin columns. Competitive ARMs-PCR was performed on each of the DNA samples, with forward specific primers for SNP A/G at base -308 and reverse common primer (primers shown in method 2.6.2). Genotype controls were included on each gel, from top left Con1 shows the PCR products for a AG genotype, Con2 a GG genotype and Con3 a AA genotype. As an example of the typical PCR products obtained from the patients in our study, in the second and third row are a total of 6 patients, which are representative of the group as a whole. The results were visualised on a 1% agarose gel, impregnated with ethidium bromide (0.5μg/ml).

We found the percentage of each promoter type was the same as the general population (Howel, 2002). The promoter data indicates our population of patients are not pre-disposed towards a greater inflammatory response even though they are being treated for cancer. The table in figure 4.2 shows the percentage of each promoter type found in the general population and our cohort of 96 patients.

Table 4.2 Percentage of each SNP in the TNFα promoter at base -308

Genotype	Promoter phenotype	% SNP in the general population Howel *et al.*, 2004 (n=214)	% SNP in the lung study population (n=96)
AA	High producer	5.6	4.1
AG	Moderate producer	26.2	28.1
GG	Normal producer	68.2	67.7

Table 4.2 Frequency of TNFα promoter –308 SNPs. The promoter types of each individual in the study (n=96) were used to calculate the frequency of each promoter type in the population of lung samples collected. The frequency of each promoter in the general population is taken from a study by Howel, 2004.

4.3.18 Polymorphisms in the TNFα promoter and TNFα release

Genetically based differences in the inflammatory response due to polymorphisms in the TNFα could contribute to the initiation and maintenance of airway inflammation. However, when we analysed the relationship between promoter type and the release of TNFα by individuals in the inflammatory model, we found no association. In fact with further detailed analysis we found no relationship between the TNFα promoter and any of the clinical parameters such as lung function and disease status as shown in figure 4.3.

Table 4.3 Relationship between the TNFα promoter and clinical parameters

TNFα Promoter –308 SNP Vs	TNFα release	P = 0.39
	Lung function (FEV_1/FVC)	P = 0.14
	Age	P = 0.85
	Gender	P = 0.48

Table 4.3 Relationship between the TNFα promoter and clinical parameters. The TNFα -308 promoter types of each individual in the study (n=96) were used to analyse the relationship between TNFα release, lung function, age and gender. The data was statistically analysed using the Kruskal Wallis test, a P value <0.05 was considered significant.

4.3.19 Polymorphisms in the IL-10 promoter

In our model we have shown IL-10 can affect the production of TNFα in acute inflammation, which is in line with previous findings (Armstrong *et al.*, 1996, Gazzinelli *et al.*, 1996). As we found no correlation between SNPs in the TNFα promoter and TNFα production in the response, we were interested to investigate if IL-10 was driving the heterogeneity in the response of TNFα. The IL-10 -1082_A allele is known to correlate with reduced IL-10 production. Previously, patients with 'low producer allele' IL-10-1082_A have been shown to develop immunological inflammatory diseases, such as inflammatory bowel disease (Tagore *et al.*,1999), severe forms of rheumatoid arthritis (Hajeer et al., 1998), and systemic lupus erythematosus (Lazarus et al., 1997). In these studies individuals were classified as high, normal, or low producers of IL-10 depending on their alleles for the IL-10 promoter. Analysis of the IL-10 promoter in human lung tissue could therefore potentially provide important information on the genetic basis of the

regulation of inflammation by IL-10. The DNA of the 96 individuals in figure 4.20 was also used to type the promoter region of the IL-10 gene using competitive PCR. The agarose gel in figure 4.18 illustrates the PCR products from randomly selected lung tissue samples and genotype controls.

Figure 4.20

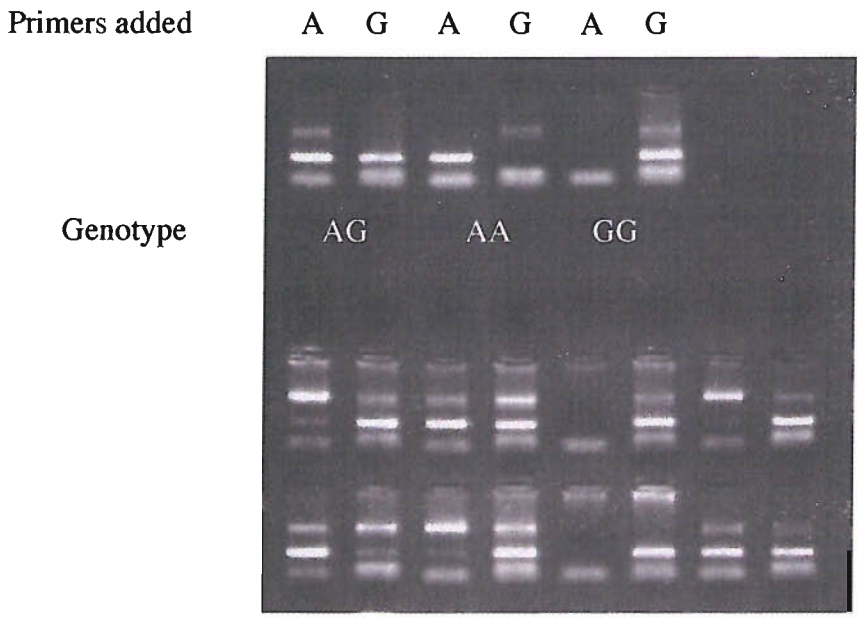


Figure 4.20 Example of typical IL-10 –1082 ARMs-PCR gel. Human lung tissue was removed from (n=96) individuals during lung resection procedures, and DNA extracted using DNA spin columns. Competitive ARMs-PCR was performed on each of the DNA samples, with forward specific primers for SNP A/G at base -1082 and reverse common primer (primers shown in method 2.6.3). Genotype controls were included on each gel, from top left Con1 shows the PCR products for a AG genotype, Con2 a AA genotype and Con3 a GG genotype. As an example of the typical PCR products obtained from the patients in our study, in the second and third row are a total of 8 patients, which are representative of the group as a whole. The results were visualised on a 1% agarose gel, impregnated with ethidium bromide (0.5μg/ml

We found that the percentage of each promoter type was the same as the general population previously described by Tagore *et al* (1999). However we did observe differences when comparing our study to the general population genotyped in a smaller study by Seifart *et al* (2005). The table in figure 4.4 shows the percentage of each promoter

type found in our study of 96 patients compared to the general population previously reported Tagore *et al* (1999) and Seifart *et al* (2005).

Table 4.4 Percentage of SNPs in the IL-10 promoter at base -1082

Genotype	Promoter phenotype	% SNP in the general population by Seifart *et al.*, 2005 (n=243)	% SNP in the general population by Tagore *et al.*, 1999 (n=330)	% SNP in the current lung study (n=96)
AA	Low producer	35.4	28.0	25
AG	Normal producer	47.3	42.0	44.8
GG	High producer	17.3	30.0	31.2

Table 4.4 Frequency of IL-10 promoter types. The promoter types of each individual in the study (n=96) were used to calculate the frequency of each promoter type in the population of lung samples collected. The frequency of each promoter in the general population are taken from studies by Seifart *et al.*, 2004 and Tagore *et al.*, 1999.

4.3.20 Polymorphisms in the IL-10 promoter and TNFα release

However when we analysed the relationship between IL-10 promoter –1082 SNPs and the release of TNFα or IL-10 by individuals in the inflammatory model, we found no relationship. As with the TNFα promoter we also found no correlation between the IL-10 promoter and any of the clinical parameters shown in figure 4.5.

Table 4.5 Correlations between the IL-10 promoter and clinical parameters

IL-10 Promoter –1082 SNP Vs	TNFα release	P = 0.89
	IL-10 release	P = 0.69
	Lung function (FEV_1/FVC)	P = 0.41
	Age	P = 0.75
	Gender	P = 0.43

Table 4.5 Relationship between the TNFα promoter and clinical parameters. The TNFα -308 promoter types of each individual in the study (n=96) were used to analyse the relationship between TNFα release, lung function, age and gender. The data was statistically analysed using the Kruskal Wallis test, a P value <0.05 was considered significant.

4.4 Discussion

TNFα has previously been recognised as an important cytokine in many chronic inflammatory diseases and is now accepted as an important target in rheumatoid arthritis, inflammatory bowel disease and sepsis (Hurlimann *et al.*, 2002, Sandborn, 2005 and Panacek *et al.*, 2004). In our acute inflammatory model TNFα release occurred earlier than the other cytokines measured, indicating TNFα may play a key role in regulating the response to LPS. We were therefore interested to further investigate the role of TNFα in our model.

TNFα production was first described in macrophages and monocytes. Since then other cells including mast cells, eosinophils, and epithelial cells have been shown to be able to synthesise TNFα (Vassalli *et al.*, 1992, Skerrett *et al.*, 2004, Thomas, 2001). Our data suggests both macrophages and mast cells may contribute to TNFα production in our model. It has previously been demonstrated that cell-surface TLR-4 mediated activation of macrophages and mast cells by LPS results in the production of inflammatory cytokines including TNFα (Tackeuchi *et al.*, 2001). Mast cells are capable of both releasing newly synthesised TNFα and storing TNFα within secretory granules. However with the co-localisation technique used it is not possible to specify if TNFα is being released in response to LPS or if the staining simply depicts TNFα stored in secretory granules within mast cells. It is therefore difficult to determine which cell populations make the greatest contribution to TNFα release in the pulmonary response. However macrophages do not store TNFα within secretory granules (Kaartinen *et al.*, 1996) and this suggests that the co-localised TNFα must be newly synthesised in response to LPS. In the previous chapter we have shown evidence that suggests TNFα is newly synthesised in response to LPS stimulus. The range of cytokines released within the response are also associated more

with a Th_1 response. Therefore the alveolar macrophage appears the most likely candidate for TNFα release in the LPS response.

Immunohistochemistry also showed the TNFα staining to be very diffuse and bound to endothelial cells within the parenchyma by 6 hours (see figure 4.8). However it is not clear if this is newly synthesised TNFα from the endothelial cells or bound TNFα from other sources. Epithelial and endothelial cells form the first line of defence against bacterial pathogens and studies have shown these cells can synthesis TNFα in response to LPS (Skerrett *et al.*, 2004). Due to the diffuse staining in the samples we were not able to assess if epithelial and endothelial cells were involved in the TNFα response. It must be noted that the small numbers in the immunohistochemistry precludes us from making broad statements about the amounts of TNFα released and disease severity.

As well as determining the cellular sources of TNFα we were also interested to investigate if TNFα was the key cytokine in the LPS driven cytokine cascade. We found neutralisation of the low levels of TNFα obliterated the LPS stimulated cytokine release up to 48hrs later in the response. The antibody data therefore further indicates the key role of TNFα in the inflammatory cascade. The levels of TNFα released in response to LPS were much lower than the levels of IL-6 and IL-8. The data therefore indicates that the temporal release of cytokines in the inflammatory cascade is more important than just simply the concentration of cytokines released. Blockade of TNFα activity by use of monoclonal antibodies, such as Infliximab is used clinically in a range of chronic inflammatory diseases, including rheumatoid arthritis, Crohns disease, and sepsis (Hurlimann *et al.*, 2002, Sandborn, 2005, Paneck *et al.*, 2004). Clinical trials in rheumatoid arthritis patients, on combinational therapy with methotrexate and Infliximab have shown better clinical and functional benefits compared to treatment with methotrexate alone (St Clair *et al.*, 2004). These studies are complemented by *in vitro* studies with rheumatoid arthritis synoviocytes, which have shown treatment with Infliximab inhibits TNFα bioactivity (Marotte *et al.*,

2005). In our study we have shown TNFα is a key cytokine in acute inflammation and therefore the use of monoclonal antibodies such as Infliximab could have a therapeutic role in bacterial derived inflammation. To date the therapeutic use of Infliximab in COPD has only been studied in one small clinical trial containing 14 patients treated with Infliximab and 8 with placebo. Over the 8 weeks of the study no significant differences in baseline characteristic we observed between the Infliximab and placebo treatment groups (Van der Vaart *et al.*, 2005). However there are important considerations to take from this study; firstly the study contained no patients with severely restricted lung function; secondly none of the patients suffered an exacerbation during the study. Our study has alluded to the role of TNFα in acute inflammation and therefore its possible role in COPD exacerbations. To obtain a clearer picture of the therapeutic use of antibodies such as infliximab in COPD larger clinical trials incorporating exacerbations of the disease are required.

In the event that individuals with COPD respond differently to therapeutic treatments we were interested to know what factors might influence TNFα release in the LPS driven cytokine cascade. In our acute inflammatory model we observed a significant heterogeneity in TNFα release following LPS stimulus between individuals. Determining the factors that affect the release of TNFα may predict which individuals may have inappropriate responses during acute inflammation and may benefit from treatment. To allow us to analyse TNFα release with disease severity the individuals in the study were reclassified using the GOLD guidelines for COPD diagnosis. Interestingly we found individuals classified as having some form of lung function impairment (GOLD groups 1 and 2) had an elevated TNFα response compared to individuals with normal lung function (GOLD 0). The relationship between TNFα and disease severity is an important clinical finding in our study. Previous research has demonstrated elevated levels of TNFα in peripheral blood and sputum of patients with stable COPD (Di Francia *et al.*, 1994, Keatings *et al.*, 1996). Polymorphisms in the promoter region of TNFα gene have been

implicated in the chronic inflammatory condition rheumatoid arthritis (Rood *et al.*, 2000, Ozen *et al.*, 2002). We were therefore interested to determine if SNPs in TNFα promoter were influencing TNFα released in response to LPS.

Polymorphisms in the promoter region of the TNFα gene have previously been shown to predict the severity of disease in rheumatoid arthritis (Rood *et al.*, 2000, Ozen *et al.*, 2002). Our investigations found no relationship between the SNP at base –308 in the TNFα promoter and any clinical parameters measured in this study. In fact the percentage of each promoter type in the study was the same as in the general population. Therefore this data for the TNFα promoter indicates that individuals included in our study are not predisposed to a pro-inflammatory state. With regards to COPD the SNP at base -308 in the TNFα promoter has not been shown to predict the severity of COPD (Sandford *et al.*, 2000). However there appears to be some correlation with the promoter and COPD in the Japanese population (Sakao *et al.*, 2001). Interestingly, in our study the TNFα promoter SNPs also failed to predict the amount or rate of TNFα released. Therefore, although this is a relatively small study the data suggests that SNP at base –308 in the TNFα promoter is not a predictor of an individual's innate inflammatory response.

The GOLD data also showed elevated IL-6 and IL-8 release with lung function impairment. IL-6 and IL-8 have both been shown to be elevated in COPD patients in several studies (Bhowmik *et al.*, 2000, Keatings *et al.*, 1997). IL-6 in particular has been shown to be elevated in exacerbations of COPD and to correlate with inflammatory cell influx during exacerbation (Seemugal *et al.*, 2000). With increased disease severity it is easy to understand how elevated levels of IL-6 would lead to heightened acute phase responses during exacerbation. In the case of IL-8, release was only elevated in individuals with the most severe lung function impairment in this study. Elevated levels of IL-8 would lead to the recruitment of neutrophils into the lung and destruction of the lung parenchyma. Neutrophilia is common in severe COPD patients it is therefore not too surprising that the

patients with the greatest restriction in lung function also have elevated levels of IL-8. For both the IL-6 and IL-8 promoter polymorphisms have been found to be associated with elevated cytokine release. SNPs in the IL-6 and IL-8 promoters were not analysed in this study, as they did not appear to play such an important role as TNFα and IL-10 in our model. In addition no association between the development of COPD and either the IL-6 (–174 G/C) or IL-8 (–251 A/T) SNPs has previously been shown (Seifart *et al.*, 2005, Arinir *et al.*, 2005). The data therefore indicates the disease COPD is the cause of these elevated levels of cytokines.

In contrast to the other cytokines analysed in this chapter the GOLD data indicates IL-10 release declines with disease severity. IL-10 is a potent anti-inflammatory cytokine important in the resolution of inflammation. The finding that the levels of IL-10 are decreased in the chronic inflammatory condition COPD is therefore extremely interesting. In support of this finding the levels of IL-10 in induced sputum from COPD patients has previously been reported to be significantly lower in comparison to healthy non-smokers (Takanashi *et al.*, 1999). IL-10 was first identified in Th_2 lymphocytes, and is also produced by monocytes and macrophages (Moore *et al.*, 2001). In our study immunohistochemistry of the lung tissue found IL-10 to be co-localised with macrophages, which is consistent with the literature. We were only able to find low levels of IL-10 at 6hrs but this is consistent with the previous levels of protein expression found in chapter 3.

IL-10 is able to ameliorate inflammation by its ability to inhibit cytokine release and the expression of surface antigens. In the literature there is strong evidence for IL-10 modulating the production of TNFα in many inflammatory cell types (Armstrong *et al.*, 1996). Again using a neutralising antibody we were able to show IL-10 was indeed able to attenuate TNFα release in the LPS model. When the biological activity of IL-10 was inhibited the release of TNFα was augmented in the LPS model. The release of IL-6 and IL-8 were also shown to be elevated probably as a direct result of the elevated levels of

TNFα release in the inflammatory cascade. Previous studies have shown IL-10 inhibits TNFα production by down-regulating cytokine mRNA expression in activated macrophages (Armstrong *et al.*, 1996, Fiorentino *et al.*, 1991). The study by Fiorentino and colleagues also reported neutralisation of IL-10 using a monoclonal antibody increased the production of IL-6 by LPS. This data supports the hypothesis that pro and anti-inflammatory mediators are in a perpetual cycle within the lung to maintain tissue homeostasis. However in diseases associated with chronic inflammation such as COPD this balance has been disrupted. We have shown TNFα to be the key cytokine in our inflammatory cascade and its release is influenced by disease severity. As IL-10 is able to affect the production of TNFα in our model, we were interested to determine if SNPs in the IL-10 promoter were influencing TNFα release in the response.

The IL-10 -1082-A allele is known to correlate with reduced IL-10 production. Patients with the postulated 'low producer allele' IL-10-1082_A have been shown to develop chronic inflammatory diseases, such as inflammatory bowel disease (Tagore *et al.*, 1999), and severe forms of rheumatoid arthritis (Hajeer *et al.*, 1998). In terms of COPD there is a certain amount of controversy in the literature as to the role of polymorphisms in the IL-10 promoter. A recent study by Seifart and co-workers (2005) has demonstrated that the IL-10-1082_A allele is more common in patients with COPD compared to aged matched smokers. However, when compared to the general population also genotyped in this study they found no correlation. This is in line with a second larger study which found no association with the -1082 SNP and patients with COPD compared to the normal population. In our study we also found no association with SNPs in the IL-10 promoter and the release of IL-10 or indeed TNFα in the model. In addition, we also found no correlation with disease severity and the IL-10 promoter. These findings and supporting literature may indicate the differences in expression of cytokines with disease severity are only seen in established disease.

When analysing the rest of the clinical data we noted smokers produced more TNFα and IL-6 compared to ex-smokers in response to LPS. This data emphasises the importance of smoking in acute inflammation of the lung. Evidence in support of this finding includes, smokers have been shown to have higher levels of bronchial alveolar lavage TNFα compared to non-smokers (Kuschner *et al.*, 1996). The east London study also found IL-6 correlated with smoking history (Wedzicha *et al.*, 2003). Exacerbations in current smokers are associated with increased lung function decline compared to non-smokers (Kanner *et al.*, 2001). Airflow limitation (FEV_1 as % predicted) has also been shown to correlate with pack years of cigarette smoking (Martin *et al.*, 1985 and Thompson *et al.*, 1989). Enhanced cytokine production in acute inflammation in current smokers may explain the accelerated lung function decline seen in persistent smokers with COPD. However it is important to note further statistical analyses found no correlation between smoking status and disease severity. Both may therefore have important roles in disease progression. In comparison the levels of IL-8 and IL-10 were no different between current and ex-smokers.

Nevertheless, differences in the level of expression of both TNFα and IL-10 may have influences on the pathogenesis of COPD especially during exacerbations and therefore represent important therapeutic targets. Therapeutic approaches aimed at stalling the inflammatory march are currently targeted at pro-inflammatory mediators. Numerous studies have suggested some of the beneficial actions of steroids may be due to mediation of TNFα, however steroids lack specificity. Current anti-TNFα therapies focus on the use of monoclonal antibodies to block either TNFα or its receptors. In terms of therapeutic treatment of acute inflammation our study indicates neutralisation of TNFα with humanised monoclonal antibodies such as infliximab, could help to resolve inflammation. As previously mentioned Infliximab has been tested in a small cohort of COPD patients, which resulted in no clinical benefit though further studies are needed. In this chapter we

have shown in our acute inflammatory model elevated levels of TNFα is not the result of SNPs –308 or –1082 in the TNFα and IL-10 promoters and therefore requires further investigation. The results from the infliximab trial are therefore not too surprising. However the use of infliximab during exacerbations where TNFα maybe involved in acute inflammation maybe of therapeutic use and requires further study. An important consideration with monoclonal antibodies is the expensive and high incidence of side effects. Rheumatoid arthritis patients in Infliximab trials have been shown to be at increased risk of contracting diseases such as tuberculosis due to a reduced innate immune system (Giles and Bathon, 2004). If IL-10 is able to modulate the release of TNFα in acute inflammation; a more ideal therapy would be the development of small molecule inhibitors, which could augment the release of IL-10 and temper the TNFα response without compromising the immune system.

Chapter 5.

Acute inflammation – stimulus specific?

5.1 Introduction

Inflammation can occur in many different contexts therefore identifying common elements in inflammation could provide useful multifaceted therapies. Conversely identifying key mediators involved in particular inflammatory conditions could be exploited to develop drugs against specific therapeutic targets. In the previous two chapters we have characterised the cytokine cascade released in response to LPS in human lung tissue. We have shown TNFα plays a key role at the head of the cytokine cascade, and coordinates the release of various cytokines such as IL-6, IL-8 and IL-10. LPS itself is a blunt stimulus, as a number of different cells can respond to its presentation. However LPS does stimulate a specific cytokine cascade, which does not involve every pro-inflammatory mediator, as we have previously shown in our model. This is in line with the general literature, which characterises LPS as a stimulus that evokes a Th_1 cytokine associated immune response (Stout *et al.*, 2005, Henricson *et al.*, 1993).

In contrast, allergic inflammation is an example of a Th_2 cytokine driven response. We were therefore interested to investigate if we could stimulate the lung to produce a different cytokine cascade more in line with a Th_2 response. Allergic inflammation is increasingly prevalent in the Western world; in the UK alone the European respiratory health survey estimated that 25% of the adult population suffer from allergic wheeze (Upton et al., 2000, McDonald *et al.*, 2005). Allergies are associated with elevated levels of IgE, which binds to high affinity IgE receptors (FcεR1) on mast cells in tissues, and basophils in blood. Cross-linking of the IgE-FcεR1 complex by allergen stimulates mast cells to degranulate (Galli *et al.*, 1993). During this process mast cells release large numbers of secretory granules, which contain a variety of immunoregulatory cytokines, chemotactic factors, and pro-inflammatory mediators. Mast cells are also capable of synthesising additional cytokines in response to various stimuli. Mast cells are therefore

generally regarded as important effector cells in inflammation and allergic reactions (Burd *et al.*, 1995). From the immunohistochemistry in chapter four we know there are a substantial number of mast cells within our lung tissue fragments. In this chapter we therefore aim to stimulate a Th_2 associated cytokine cascade by cross-linking the IgE-FcεR1 surface receptors.

In addition, we were also interested to ascertain if there are any similarities or differences in acute inflammation between species. Many previous experimental studies of acute inflammation have used *in vitro* and *in vivo* rodent models to study human lung disease. Between rodents and humans there are many similarities but also differences in the cytokines which are expressed in each species. Species differentiation has been highlighted by the Th_1/Th_2 paradigm in mice and humans. In mice the cytokines released in response to stimuli are either Th_1 or Th_2 derived. However this concept does not strictly apply in humans where in both Th_1 and Th_2 responses there is a much greater overlap in the cytokines released. Therefore, in this chapter we also aim to compare the inflammatory response to LPS in humans with that in rodent lung tissue.

5.2 Methods

5.2.1 Patient characteristics for human lung tissue experiments

Lung tissue was removed from 19 patients (7F/12M) undergoing resection for carcinoma; tissue used was from the normal margin surrounding the tumour site. Data relating to age, gender, lung function, and smoking history, was obtained for patients (see table 3.1).

Table 5.1 Patient characteristics of subjects prior to removal of lung tissue

No. Of Subjects	19
Age (Yr.)	67.6 ±11.5
Gender	7 Female, 12 Male
Lung Function (FEV_1/FVC)	0.70 ±0.1
Smoking Status	3 Non smokers 10 Ex smokers 6 Current smokers
Asthmatics	1
Allergy	3

Tissue samples were taken from a group of 19 patients. Patient details including age, gender, lung function given as the ratio of FEV_1/FVC, smoking status and pack years are listed.

5.2.2 Primary cell culture of human lung tissue

Tissue samples were processed as previously described in method 2.2.2 preparation of human lung tissue for primary cell culture. Tissue was stimulated with either anti-IgE, LPS or isotype buffer control as outlined in methods 5.2.3-5.3.4 below. The levels of TNFα, IL-5, IL-6, IL-8, IL-10, and IL-13 were measured in the supernatant using

commercially available ELISAs (See methods 2.3.1-2). LDH levels were measured in lung supernatant using commercially available assay and LDH standard from Roche, See method 2.3.6.

5.2.3 Dose response experiments

Tissue was stimulated with either 1000, 100, 10, 1 or 0.1 μg/ml of anti-IgE or isotype buffer control and incubated at 37°C for 24hrs. Human lung fragments and supernatant were harvested, the tissue weighed and both were stored at -80°C.

5.2.4 Kinetic experiments

Lung fragments were prepared as outlined in 2.2.2. Tissue was stimulated with either 100 or1μg/ml of anti-IgE or 100ng/ml of LPS or isotype buffer control for 1, 2, 4, 6, 24, or 48 hours at 37°C. Following incubation tissue and supernatant were harvested, the tissue weighed and samples stored at -80°C.

5.2.5 Cyclohexamide treatment

For cyclohexamide experiments the tissue was pre-treated for one hour with 10μg/ml cyclohexamide prior to LPS stimulus. Human lung fragments and supernatant were harvested at 1, 2, 4, 6, 24, and 48 hour time points and the tissue weighed. Both the human lung fragments and supernatant collected were stored at -80°C.

5.2.6 Primary cell culture of rodent tissue

Rodent lung tissue was removed from 6 female Wistar rats, aged 6 months. Tissue fragments were processed as previously described in chapter 2.2.2 preparation of human lung tissue for primary cell culture. Tissue was then stimulated with either (100ng/ml) LPS

or buffer control. The levels of TNFα, IL-6, IL-10 and IL-1β were measured in the supernatant using commercially available rodent ELISAs (See method 2.3.4).

5.3 Results

5.3.1 Cytokine levels in response to varying concentrations of anti-IgE

Our initial experiments looked at the response of human lung tissue to stimulation with a wide range of concentrations of anti-IgE. Figure 5.1 (over the page) shows that the maximal release of IL-5 and IL-13 (see panels a and b) occurred in response to stimulation with 1μg/ml anti-IgE, whilst higher concentrations of anti-IgE were actually associated with less cytokine release. These bell shaped curves are a typical feature of anti-IgE stimulation.

In contrast, the levels of TNFα, IL-10, IL-6 and IL-8 (see panels c, d, e, and f respectively) continued to rise with increasing concentrations of anti-IgE. For these cytokines, the dose response curves did not turn over within the range of concentrations of anti-IgE used. Generally statistical significant increases ($P<0.05$) were observed for all cytokines with anti-IgE stimulus compared to buffer control.

Figure 5.1

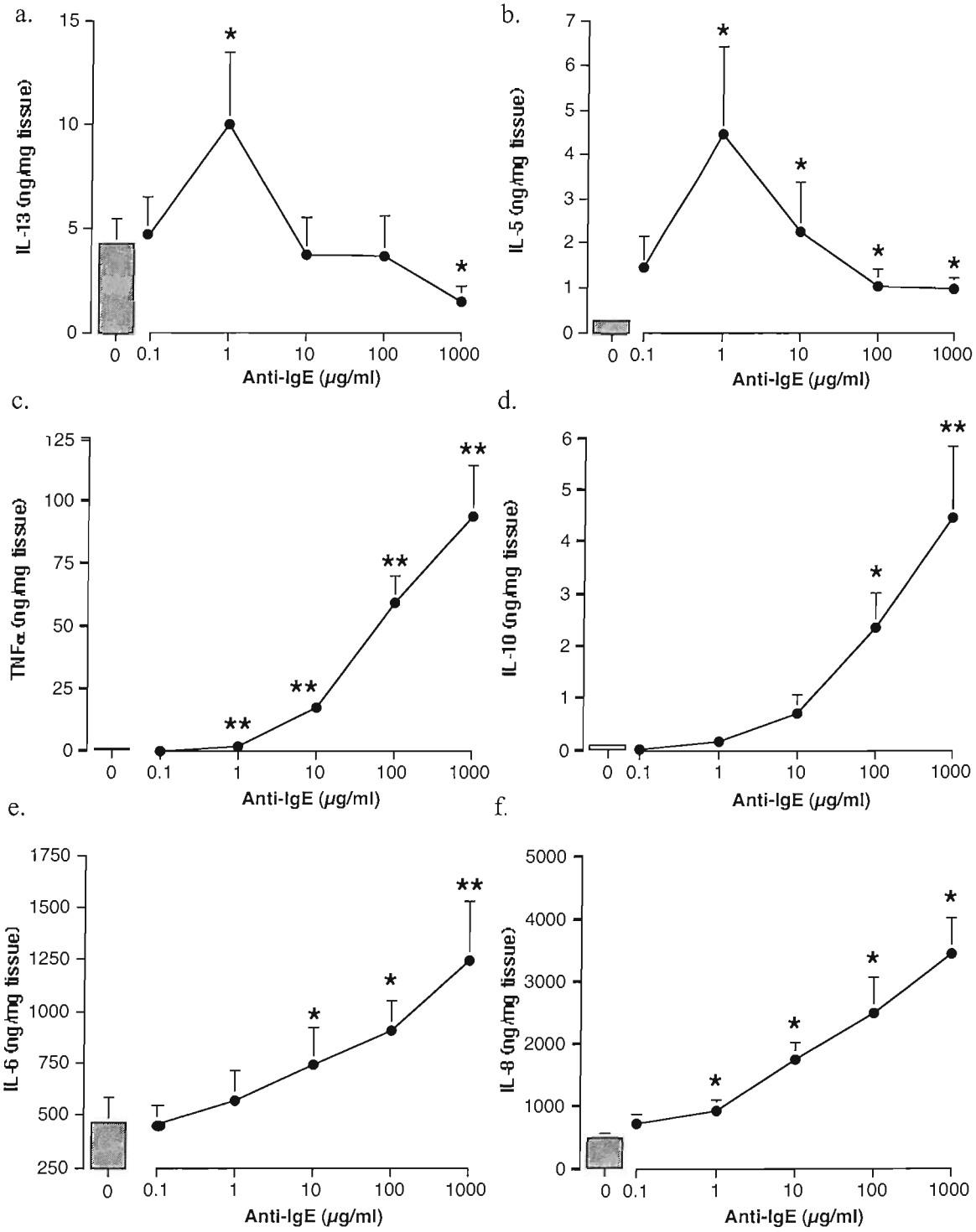


Figure 5.1 a – f. Anti-IgE dose response curves. Human lung tissue (n=19) was stimulated with a range of concentrations of anti-IgE (filled circles) or buffer control (▨) and incubated for 24hrs. Cytokine levels in the supernatant were measured by ELISA, values are expressed as mean ±SEM and are expressed as ng/mg of tissue. The data was statistically analysed using the Wilcoxon-Signed Rank test, * indicates $P<0.05$; ** indicates $P<0.001$.

5.3.2 LDH release from human lung tissue

With the observation that maximal TNFα release occurred with the greatest concentration of anti-IgE. We wanted to be sure the cytokine response we were seeing was not the result of cell death. LDH, a marker of tissue necrosis was again measured as in method 2.3.6. As shown in figure 5.2 for all time points there was no difference in LDH release between all stimuli and buffer control. The anti-IgE serum was also assayed for contamination (see method 2.2.8), no LPS was detected by the assay (data not shown).

Figure 5.2

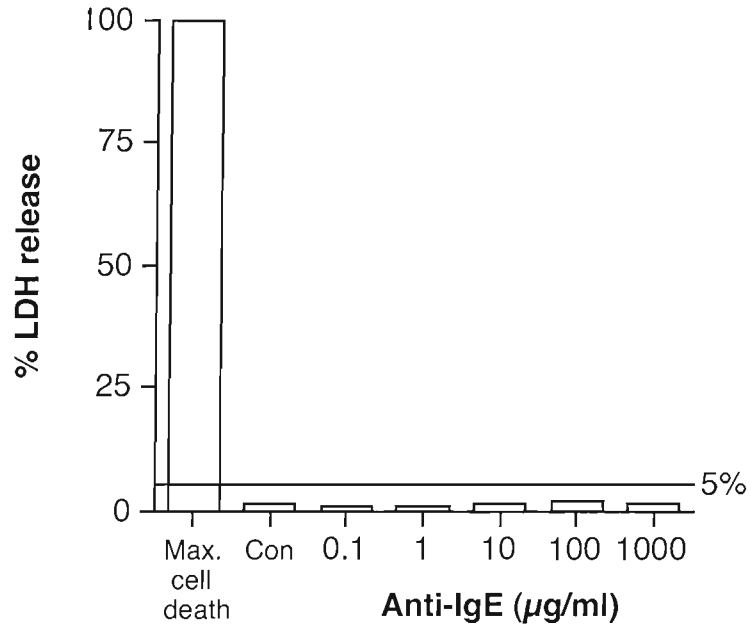


Figure 5.2 LDH release from human lung tissue. LDH release was measured in human lung tissue supernatant (n=8) using an LDH assay. Values shown are expressed as a percentage of cell death, with total cell death being estimated from the maximal LDH release from homogenised lung tissue.

5.3.3 The temporal pattern of IL-13 and IL-5 release in response to anti-IgE compared to LPS

Having confirmed cytokine release in the response was not the result of cell death. We were keen to explore the temporal patterns of cytokine release in response to anti-IgE. We were also interested to compare the cascade of cytokines previously observed in our LPS acute inflammation model with the IgE response. With the data from our previous dose

response curves we chose to investigate IL-13 and IL-5 release with 1μg/ml anti-IgE, and the other cytokines with 100μg/ml anti-IgE.

Figure 5.3

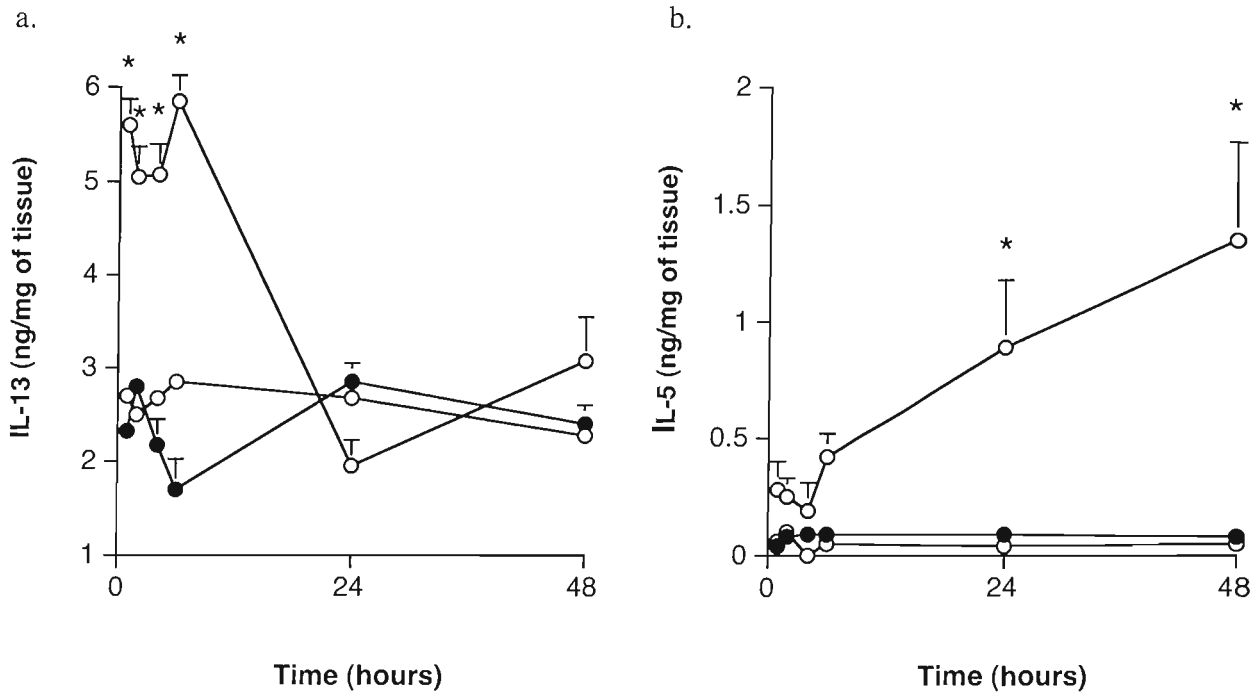


Figure 5.3a Comparison of anti-IgE and LPS mediated IL-13 release. Human lung tissue (n=19) was stimulated with either 1μg/ml anti-IgE (grey circles), 100ng/ml LPS (filled circles) or buffer control (open circles). Supernatants were collected at 1, 2, 4, 6, 24, and 48hrs and cytokine levels measured using ELISA. **Figure 5.3b Comparison of anti-IgE and LPS mediated IL-5 release.** The supernatants previously described in figure 5.3a were analysed for IL-5 using ELISA. For both figures the values given are the mean ± SEM and are expressed as ng/mg of tissue. The data was statistically analysed using the Wilcoxon-Signed Rank test, * indicates a P value of <0.05 compared to LPS stimulated tissue and control. Error bars have been omitted for clarity.

Firstly as shown in figure 5.3a the levels of IL-13 are elevated from the outset at 1hr with levels peaking at 6hrs in the IgE stimulated tissue (mean=5.8ng/mg of tissue) compared to control (mean=2.8ng/mg of tissue, $P<0.05$) and LPS stimulated tissue (mean=1.7ng/mg of tissue, $P<0.05$). Following the 6hr time point the levels of IgE stimulated IL-13 decrease to a sub maximal plateau by 24hrs, which is maintained until 24hrs.

As shown in figure 5.3b the levels of IL-5 were relatively low but increased with time reaching a maximum at 24hrs (mean=1.6 ng/mg of tissue) compared to control (mean=0.1ng/mg of tissue, $P<0.05$) see figure 5.3b. With LPS stimulation we observed a

similar levels of IL-5 release from the tissue (mean=0.2ng/mg of tissue) compared to the tissue incubated with buffer control (mean=0.1ng/mg of tissue).

5.3.4 The temporal pattern of TNFα and IL-6 release in response to anti-IgE compared to LPS

When we compare the temporal pattern of TNFα and IL-6 release from tissue stimulated with either anti-IgE (100μg/ml) or LPS (100ng/ml), the response is relatively similar. As shown in figure 3.4a we could not detect TNFα release in the control tissue. However when the tissue was stimulated we did observe a TNFα response, which was broadly similar for both stimuli. However TNFα release peaked later at 24hrs (mean= 61.2ng/mg of tissue) with anti-IgE stimulus compared LPS stimulus (mean=41.7ng/mg of tissue, $P<0.05$).

Figure 5.4

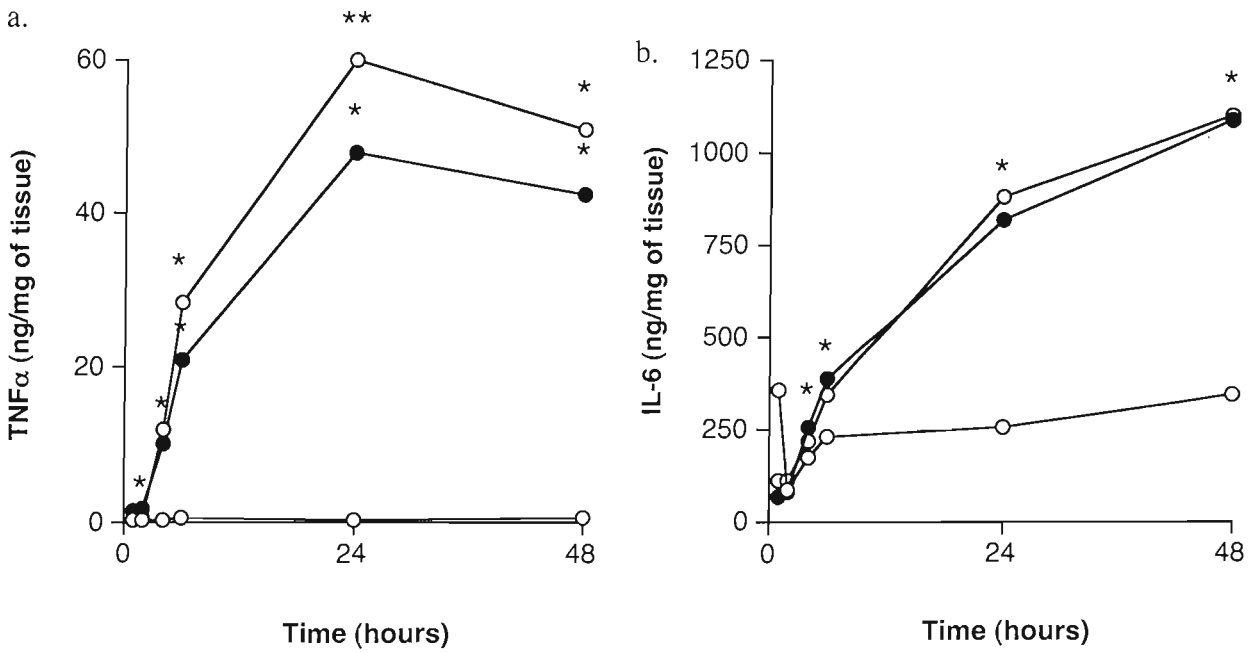


Figure 5.4 Comparison of anti-IgE and LPS mediated TNFα and IL-6 release. Supernatants from figure 3.3 were analysed for TNFα and IL-6 using ELISA. Values given are the mean ± SEM and are expressed as ng/mg of tissue. The data was statistically analysed using the Wilcoxon-Signed Rank test, * indicates $P<0.05$ compared to control and ** indicates $P<0.05$ compared to control and LPS stimulated tissue. Error bars have been omitted for clarity.

When we look at the release of IL-6 we observed basal release in the control tissue. When the tissue was stimulated the release of IL-6 was elevated with both stimuli and as shown in figure 5.4b the anti-IgE response exactly mimicked the IL-6 response seen with LPS stimulation.

5.3.5 The temporal pattern of IL-8 and IL-10 release in response to anti-IgE compared to LPS

When we compare the release of IL-8 and IL-10 with the two stimuli we observe a difference in the temporal release of these cytokines.

Figure 5.5

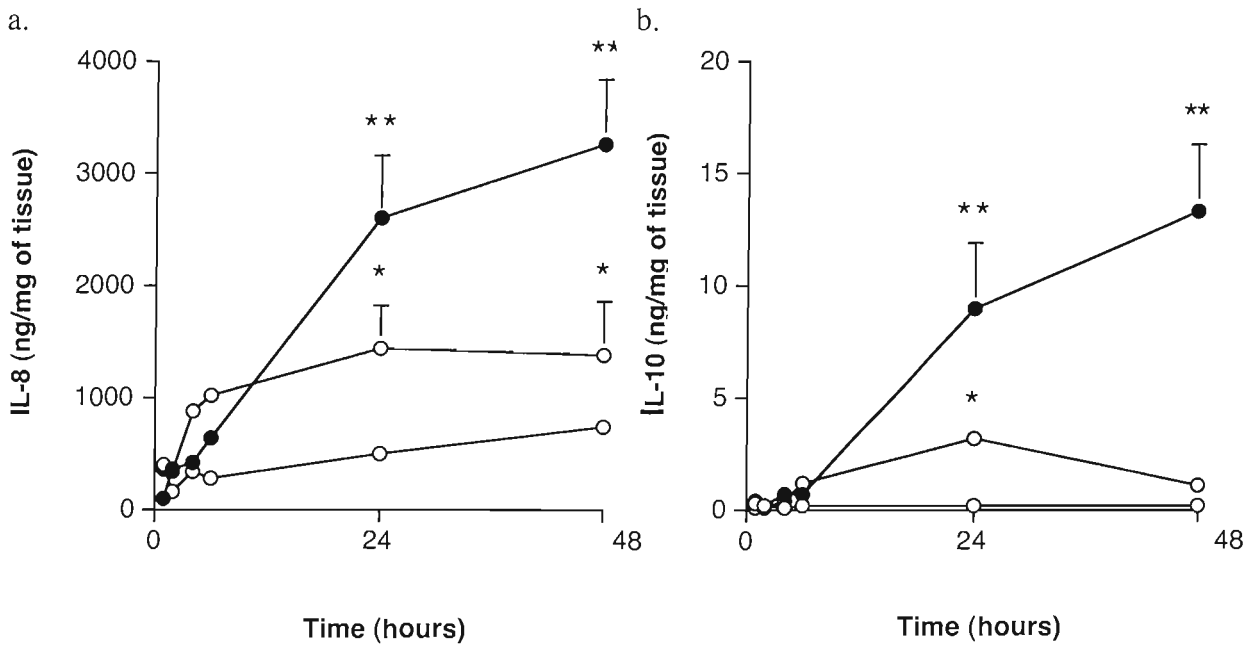


Figure 5.5 Comparison of anti-IgE and LPS mediated cytokine release. Supernatants from figure 3.3 were analysed for IL-8 and IL-10 using ELISA. Values given are the mean ± SEM and are expressed as ng/mg of tissue. The data was statistically analysed using the Wilcoxon-Signed Rank test, * indicates $P<0.05$ compared to control, and ** indicates $P<0.05$ compared to control and LPS stimulated tissue.

As shown in figure 3.5a IL-8 release is much greater following exposure to LPS with a maximal response at 24hrs (mean=2581ng/mg of tissue) compared to control (mean=563ng/mg of tissue). However for the anti-IgE response the release of IL-8 is maximal by 6hrs (mean=1423ng/mg of tissue) and then reaches a plateau. In the case of

IL-10 LPS stimulates a relatively high IL-10 response with release still increasing at 48hrs (mean=13.5ng/mg of tissue) compared to undetectable levels in the control. In comparison IL-10 release is much lower in response to anti-IgE with a maximal response of mean=3.2ng/mg of tissue (see figure 3.5b).

5.3.6 Does an individual's response to LPS predict their anti-IgE response

We were interested to see if an individuals TNFα response to LPS predicted their TNFα response to anti-IgE. When we compared each individuals TNFα release at 24hrs to both stimuli we found no correlation (see figure 5.6a). We also found no-correlation between the release of IL-10 and IL-8 with the two stimuli (data not shown). However in the case of IL-6 individuals who were good responders to the LPS stimulus were also good anti-IgE responders, P<0.001, Rho=0.68, (see figure 5.6b).

Figure 5.6

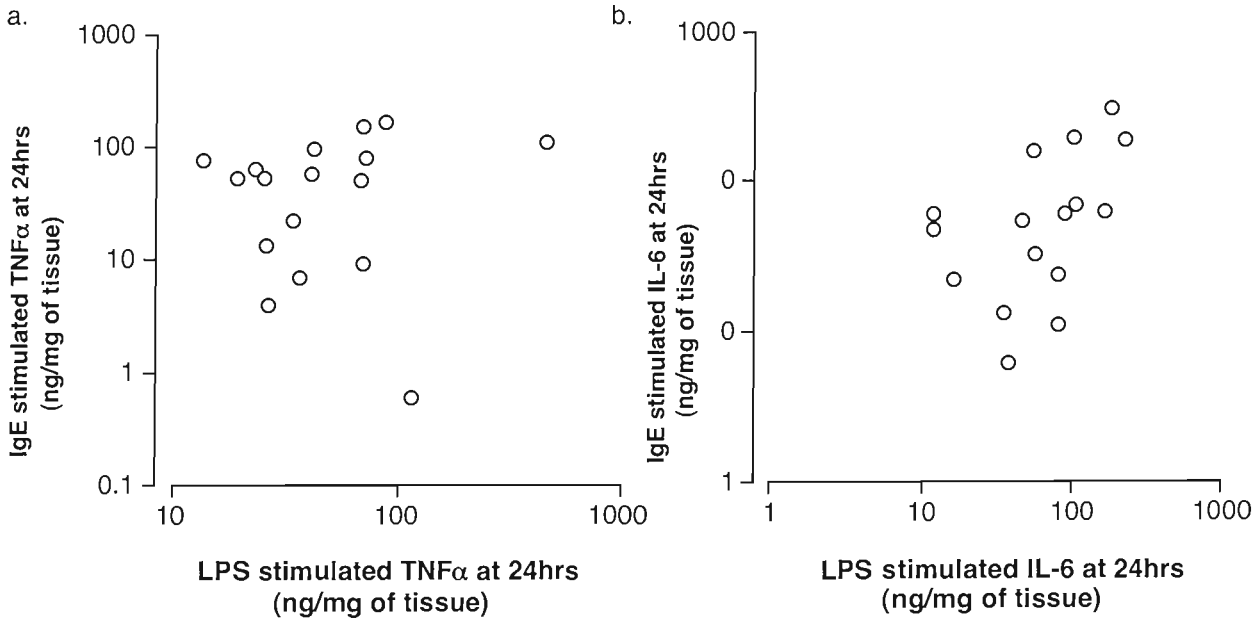


Figure 5.6 Comparison of anti-IgE and LPS mediated TNFα and IL-6 release. The correlations used data on LPS stimulated TNFα and IL-6 release at 24hrs and anti-IgE mediated TNFα and IL-6 release at 24hrs from human lung. Data was analysed using the non-parametric Spearman correlation, values given are expressed as ng/mg of tissue.

Having previously shown GOLD status influences an individuals response to LPS, we re-classified the 19 patients in this chapter using the GOLD guidelines. However we found no difference in the levels of cytokines released with disease status (data not shown). On further analysis of the patient data collected four individuals were clinically described as having an allergy or allergic asthma. The response of these individuals was however no different to the group as a whole (data not shown). Finally smoking status also did not influence an individuals response to anti-IgE (data not shown).

5.3.7 Effect of cyclohexamide treatment on cytokine release

We have previously shown in chapter 3 that the cytokines released in response to LPS are newly synthesised. We were therefore interested to know if cross-linking anti-IgE also stimulated de novo protein synthesis or stimulated previously stored sources of cytokines. Tissue was pre-treated with cyclohexamide a protein synthesis inhibitor for 1hr before stimulus with anti-IgE or buffer control (see method 5.2.5). Cyclohexamide treatment inhibited the anti-IgE stimulated production of IL-13, IL-5, IL-6 and IL-8 back to the baseline levels previously observed in the anti-IgE kinetic data See figures 5.7a, 5.7b, 5.7d and 5.7e respectively. In the case of TNFα and IL-10, which are not released in the basal tissue, cyclohexamide abolished the release of these cytokines, see figures 5.7c and 5.7f.

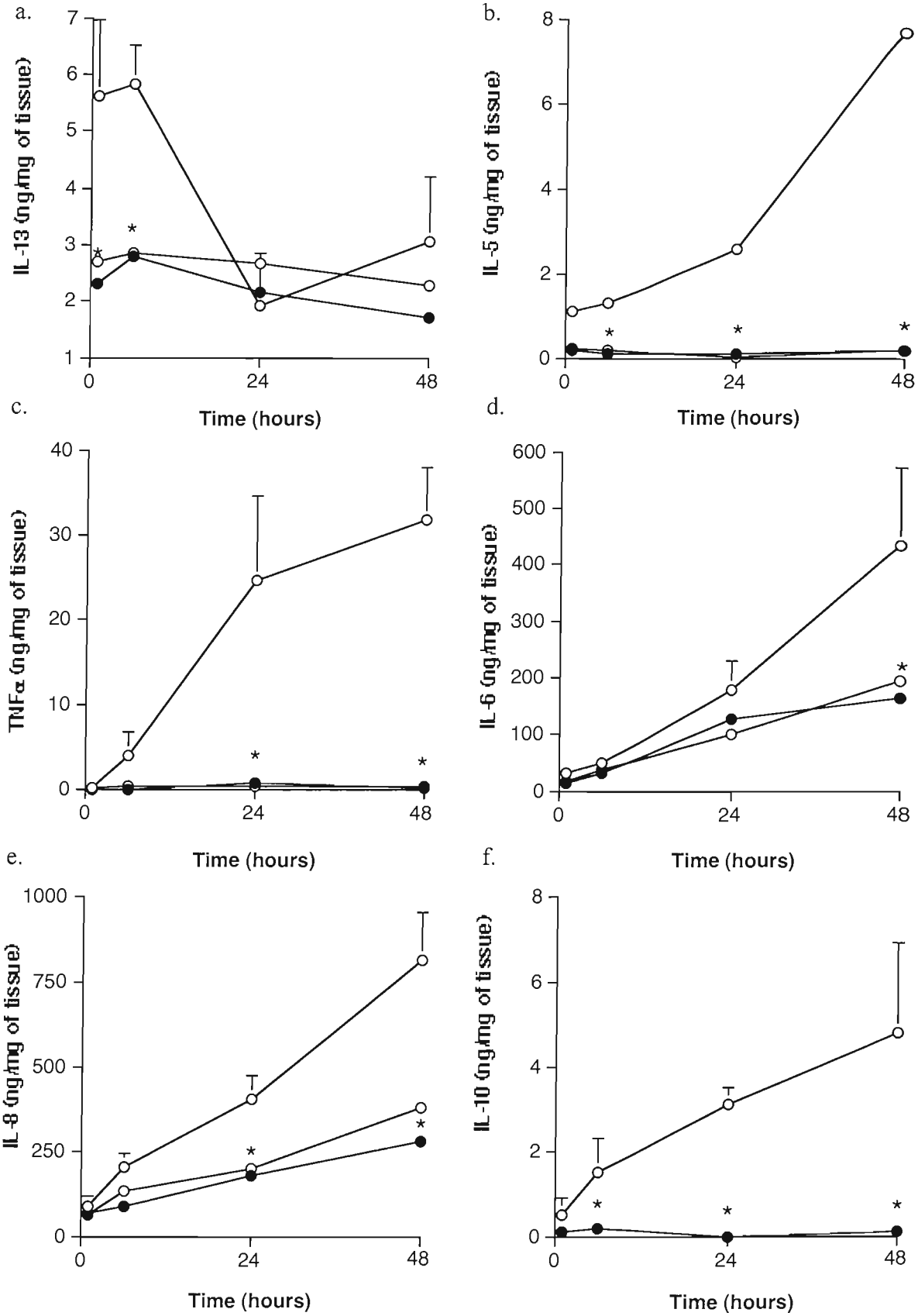


Figure 5.7 Effect of cyclohexamide treatment on cytokine release. Human lung tissue (n=8) was pre-treated with cyclohexamide for 1hr before addition of anti-IgE (filled circles) or buffer control (open circles). The cyclohexamide data is compared with anti-IgE stimulated cytokine release (grey circles). The release of IL-13 shown in figure 5.6a, Il-5 see figure 5.6b, TNFα see figure 5.6c, Il-6 see figure 5.6d, IL-8 see figure 5.6e and finally IL-10 as shown in figure 5.6f were all measured using specific ELISAs. Values shown are the mean ±SEM and are expressed as ng/mg of tissue. The data was statistically analysed using the Wilcoxon-Signed Rank test, * indicates a P value <0.05.

5.3.8 Cytokine release from rodent lung

Obtaining human lung tissue for pharmaceutical research is both time consuming due to irregular supply of tissue and ethically difficult. Rodent models are therefore often used to look at the effects of drugs and mediators in lung tissue, but is their inflammatory response to LPS the same as humans? For these set of experiments the rodent lung tissue was prepared as in method 5.2.6.

5.3.9 TNFα in rodent lung supernatant

As TNFα was the key cytokine in the human response to LPS we started by investigating the release of TNFα in rats using ELISA. As shown in figure 3.8 the release of TNFα from rodent lung tissue in response to LPS was elevated at 1, 2, and 4 hours reaching a maximal release at 6 hours (mean=20.8ng/mg of tissue) compared to buffer control

Figure 5.8

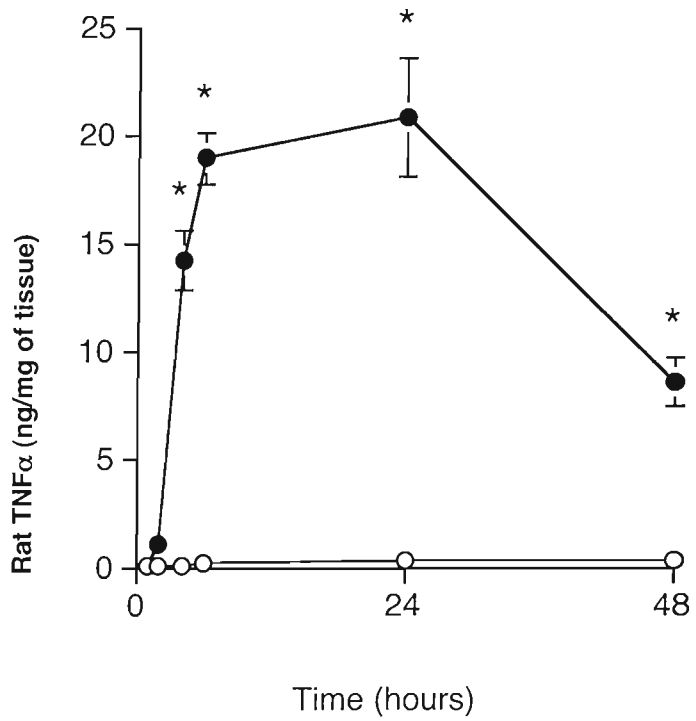


Figure 5.8 Levels of TNFα in supernatant from rodent lung tissue fragments. Rodent lung tissue (n=6) was stimulated with 100ng/ml LPS (filled circles) or buffer control (open circles). The release of TNFα into the supernatant was measured by ELISA. Values shown are the mean ± SEM and are expressed as ng/mg of tissue. The data was statistically analysed using the Wilcoxon-Signed Rank test, * indicates $P<0.05$.

(mean=0.3ng/mg of tissue, P=<0.05). The release of TNFα from LPS stimulated rodent tissue up to the 24 hour time point is therefore similar to the human TNFα response previously shown in figure 3.2. However in the rodent tissue TNFα release tended to fall by 48hrs in comparison to the maintained release in the human response (see figure 3.2).

5.3.10 IL-6 in rodent lung supernatant

We observed the release of pro-inflammatory cytokine IL-6 peaked at 24 hours in the human model (see figure 3.5). In striking contrast to the human LPS model we found no difference between LPS stimulated release and baseline levels of rodent IL-6 (see figure 3.9). The data indicates IL-6 is not released in response to LPS in rats, which is dissimilar to the elevated IL-6 response observed in humans.

Figure 5.9

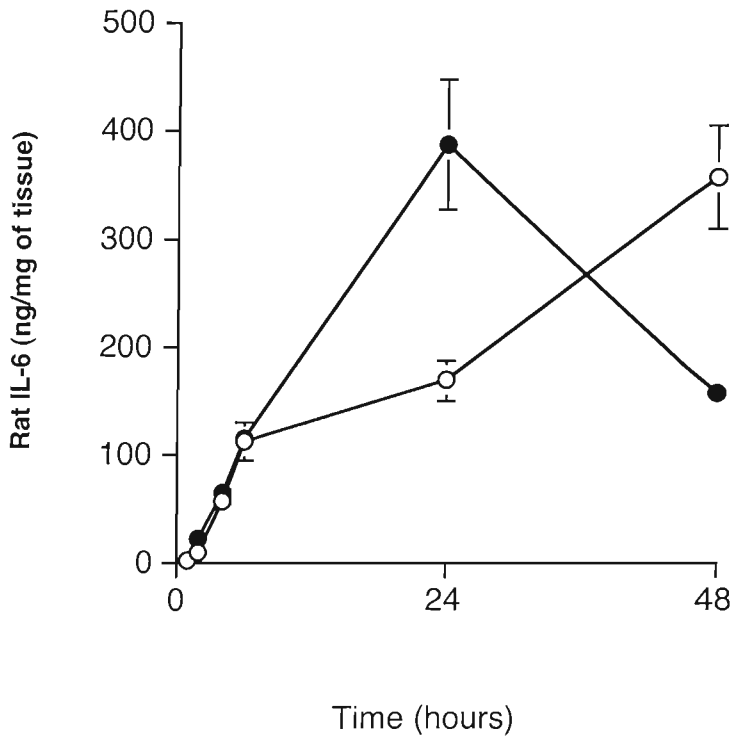


Figure 5.9 IL-6 release from rodent lung tissue. Rodent lung tissue (n=6) was stimulated with 100ng/ml LPS (filled circles) or buffer control (open circles). The release of IL-6 into the supernatant was measured by ELISA. Values shown are the mean ± SEM and are expressed as ng/mg of tissue.

5.3.11 IL-10 in rodent lung supernatant

We have previously shown the interplay between IL-10 and TNFα in the LPS stimulated inflammatory response in the human model (see figure 4.4). As we observed TNFα release in response to LPS in the rodent model, we were interested to know if IL-10 was also able to modulate the inflammatory response in the rat. Surprisingly, as shown in figure 3.10 we found no detectable levels of IL-10 in the rat supernatant with LPS stimulus. The data therefore, again indicates the response to LPS in rat is distinct to humans.

Figure 5.10

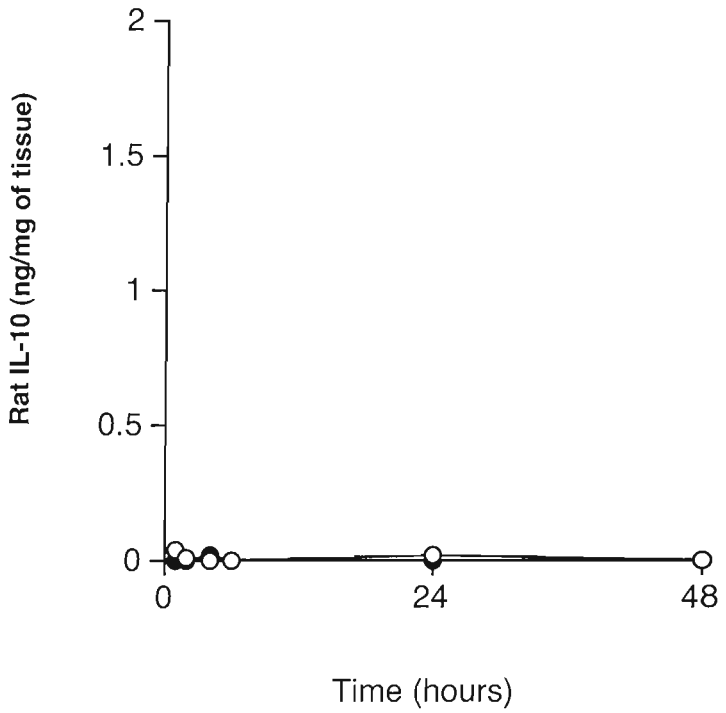


Figure 5.10 IL-10 release from rodent lung tissue. Rodent lung tissue (n=6) was stimulated with 100ng/ml LPS (filled circles) or buffer control (open circles). The release of IL-10 into the supernatant was measured by ELISA. Values shown are the mean ± SEM and are expressed as ng/mg of tissue.

5.3.12 IL-1β in rodent lung supernatant

There is no equivalent to human IL-8 in the rat. As there are differences in the cytokines released in response to LPS in the rat we decided to investigate if IL-1β was involved in the response. As shown in figure 5.11 in the rat supernatant we found IL-1β was statistically elevated at 24 hours (mean=1.2ng/mg of tissue) compared to control (mean=0.8 ng/mg of tissue). These levels of IL-1β are relatively low however previous studies have shown IL-1β levels of 300pg/ml to have physiological effect in the rat (Haddad *et al.*, 2002).

Figure 5.11

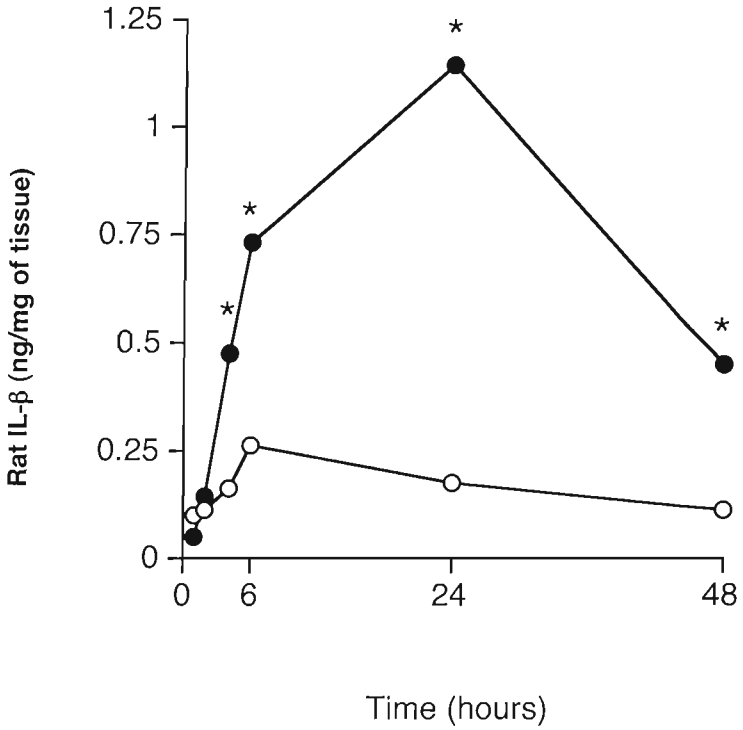


Figure 5.11 IL-1β release from rodent lung tissue. Rodent lung tissue (n=6) was stimulated with 100ng/ml LPS (filled circles) or buffer control (open circles). The release of IL-1β into the supernatant was measured by ELISA. Values given are the mean ± SEM and are expressed as ng/mg of tissue. The data was statistically analysed using the Wilcoxon-Signed Rank test, * indicates $P<0.05$ compared to control.

5.4 Discussion

In our initial study we investigated the response of human lung tissue to LPS stimulation. We established that a distinctive cytokine cascade is released in response to LPS. The response included various mediators such as TNFα, IL-6 and IL-10, which are all cytokines typically associated with a Th_1 response. We were therefore interested to investigate if we could stimulate a different cytokine cascade in the human lung tissue. Cross-linking of the high affinity FcεR1 receptors on the surface of mast cells by the allergen-IgE complex is a central event in the induction of allergic inflammation. To initiate a Th_2 associated cytokine cascade we therefore used an anti-IgE antibody to cross-link the high affinity FcεR1 receptors.

In response to cross linking of IgE we were able to measure the release of IL-13 and IL-5, which were not released in the LPS model. The results show the maximal release of IL-13 and IL-5 occurred at 1μg/ml of anti-IgE, with characteristic bell shaped curves. The profile of IL-13 and IL-5 release is characteristic of the response previously shown in the literature (Galli *et al.*, 1993, Burd *et al.*, 1995), the reasons for this pattern of release however remain unclear.

In the literature both IL-13 and IL-5 have attracted much attention for their possible role in allergic inflammation (Grunig *et al.*, 1998, Stuart et al., 1993). IL-13 is released from T cells, basophils and mast cells, and most notably promotes B cell differentiation, proliferation, and importantly the class switch to IgE production (Zhu *et al.*, 1999). In terms of synthesis the cyclohexamide data from a small subset of patients indicated that the IL-13 released in response to IgE cross-linking was newly synthesised. It is well documented that mast cells increase the synthesis of cytokines and release stored mediators in response to stimuli. In particular purified human lung mast cells have previously been shown to transcribe, synthesise and release IL-13 after IgE-dependent activation

(Kobayashi *et al.*, 1998). The cyclohexamide data along with the work reported elsewhere (Royer *et al.*, 2001) indicates IL-13 is newly synthesised by mast cells in response to IgE cross-linking. However to confirm this hypothesis further studies would be required.

In the previous two chapters we have shown that TNFα is the key cytokine in response to LPS and is released early in the response. As shown by the kinetic data IL-13 release peaked early in the response with levels remaining high until 4 hours when release fell back to baseline levels. This early temporal release may therefore indicate a key role for IL-13 in the cytokine cascade. Recent studies by Walter et al (2001) and Kumar et al (2004) have used mouse models of chronic allergic inflammation to demonstrate IL-13 knock out mice and neutralisation of IL-13 with a IL-13 specific antibody in naïve mice, yields a number of beneficial anti-inflammatory effects. These beneficial effects included reduced accumulation of eosinophils and other chronic inflammatory cells. These findings, considered with our own observations of early increases in IL-13, suggest that IL-13 may play a more central role in allergic inflammation than previously thought. However without further studies involving the inhibition of IL-13, we can only speculate that IL-13 could act as a lead cytokine in the allergic inflammatory cascade.

IL-5 is a classical Th_2 associated cytokine and is produced by T cells, mast cells and the bronchial epithelium (Bentley *et al.*, 1993). IL-5 plays an important role in the activation and survival of eosinophils (Sur *et al.*, 1995). With cross-linking of the IgE we found the levels of IL-5 were elevated at 2 hours and continued to rise up to the 48hour time point. Previous studies with human lung mast cells have shown IL-5 expression is only induced following IgE receptor cross-linking (Okayama *et al.*, 1995). This is inline with our data as LPS did not stimulate the release of IL-5. Preliminary cyclohexamide data also indicated IL-5 release in response to anti-IgE was newly synthesised. IgE dependent expression of mRNA for IL-5 has previous been shown in human lung mast cells by Okayama and colleagues (1995), and therefore supports our findings.

Again the early temporal release of IL-5 may support a key role for the cytokine in allergic inflammation. In support of this, studies in IL-5 gene knock-out mice and naïve mice treated with blocking antibodies to IL-5 have shown suppressed eosinophilic activation and airway hyperresponsiveness during allergen challenge (Foster *et al.*, 2002. However clinical trials of humanised IL-5 monoclonal antibodies such as Mepolizulab in asthmatic patients have produced disappointing results as therapeutic treatments for asthma. This suggests that blocking IL-5 is not likely to be a useful approach for asthma therapy or it may highlight the multifaceted therapies, which may be required to treat heterogeneous diseases such as allergic asthma (Floodpage *et al.*, 2003).

The levels of IL-13 and IL-5 are relatively low compared to the other cytokines measured. However, both IL-13 and IL-5 are released early in the anti-IgE stimulated cytokine cascade. We have previously shown in chapter 4 that the early temporal release of TNFα is more important then than the magnitude of release in the LPS response. Therefore the rapid release of IL-13 and IL-5 may indicate a key role for these cytokines in the allergic inflammatory cascade. In fact previous experimental data only supports the role of IL-5 and IL-13 as key mediators in allergic inflammation (Grunig *et al.*, 1998, Stuart *et al.*, 1993).

Moving on to the other cytokines released in the cascade, cross-linking of IgE also stimulated the release of TNFα, IL-6, IL-8 and IL-10. The release of these cytokines occurred in a dose dependent fashion with 1000μg/ml of anti-IgE eliciting the maximum release of all four cytokines. The dose response curves for TNFα, IL-6, IL-8 and IL-10 are quite distinct from bell shaped response curves observed for IL-13 and IL-5. This dose dependent response for TNFα, IL-6, IL-8 and IL-10 is however in line with previous findings in the literature (Lorentz *et al.*, 2000).

As we found no LPS contamination of the anti-IgE serum and the lung releases TNFα, IL-6, IL-8 and IL-10 in response to both LPS and anti-IgE. We were very interested

to investigate if the temporal release of these cytokines was the same in both responses. The temporal release of TNFα in response to both stimuli up to 6 hours was broadly similar. However TNFα release in response to LPS appears to plateau after 6hrs, in comparison TNFα release continues to rise and is doubled by 24 hours in the IgE mediated response. When we analysed if individuals with a good TNFα response to LPS were also good anti-IgE responders we found no correlation. The heterogeneity of the IgE response could also not be explained by the patients GOLD status or history of atopy. This suggests that the release of TNFα is not an innate characteristic of the patient but more stimulus dependent. TNFα is usually associated with cell-mediated immunological Th_1 responses, which have been categorised using mouse models. However it is becoming apparent that inbred murine models of immunology may be limited by species differences, and do not reflect diseases experienced in man. Allergic inflammation is perceived as a Th_2 response, however increasing evidence indicates that other cytokines, which in mice are classically considered to belong to Th_1 type profiles, are also associated with allergic inflammation. One such mediator is TNFα, which has been shown to increased in asthmatic airways in proportion to disease severity, and inhalation of recombinant TNFα is enhances bronchial hyperresponsiveness and sputum neutrophillia in normal subjects (Shah *et al.*, 1995, Thomas *et al.*, 1995). Genetic studies have also revealed that the polymorphism of the TNFα promoter is associated with asthma and its severity (Witte *et al.*, 2002).

The sensitized mast cell is known to store a number of cytokines such as TNFα along with histamine within its granules. When mast cells are activated via IgE-dependent mechanisms these cytokines along with histamine are released during degranulation (Xiang *et al.*, 2001). One function of the pleotrophic cytokine TNFα, is a chemoattractant for granulocytes including eosinophils and neutrophils, probably by up-regulating cellular adhesion molecules to facilitate inflammatory cell migration. The chemoattractant activity of TNFα is further amplified in the presence of IL-5. The importance of TNFα has further

been highlighted by a clinical trial of a humanised monoclonal antibody (etanercept) in severe asthmatics (Howarth *et al.*, 2005). Trials have shown significant clinical response with patients gaining improvements in lung function and quality of life. The clinical benefit of etanercept in severe asthmatics mirrors that achieved with therapy in other chronic inflammatory diseases such as rheumatoid arthritis, inflammatory bowl disease and psoriasis. When looking at pleotrophic cytokines such as TNFα it is therefore important to look at the cytokine milieu into which they are released to understand its multiple actions.

In the case of IL-6 the responses to both stimuli can be practically superimposed upon one another. For IL-6 alone we observed individuals with a good IL-6 response to LPS at 24 hours also had a good anti-IgE response. IL-6 has previously has been reported to be up-regulated at both the gene transcriptional and protein level in mast cells after IgE mediated activation (Kempuraj *et al.*, 2003, Xiang *et al.*, 2001). IL-6 is a pleotrophic cytokine with both pro and anti-inflammatory roles in the acute phase response and immune responses (Heinrich *et al.*, 2003). The acute phase response enhances host resistance, minimises tissue injury and promotes the resolution and repair of inflammation. Elevated levels of IL-6 have been found in induced sputum from both atopic asthmatics and COPD patients post exacerbation (Tillie-Leblond *et al.*, 2005, Bhowmik *et al.*, 2000) The similarity in the temporal release of IL-6 to both stimuli and its up regulation in various inflammatory conditions indicates IL-6 may well play a more general role in the resolution of many types of inflammation. This is in line with previous literature reviewed by Chung and Barnes (1999).

The temporal release of IL-8 is similar in response to both stimuli however the magnitude of the anti-IgE stimulated release of IL-8 is much greater than the LPS response. IL-8 expression has previously been demonstrated in un-stimulated and stimulated mast cells with anti-IgE at both the mRNA and protein level (Gibbs *et al.*, 2001). The constitutive expression of IL-8 in mast cells may therefore account for the high

basal release observed with buffer control. IL-8 is a potent neutrophil recruiting and activating factor. Neutrophils have been implicated in the pathogenesis of many inflammatory lung diseases, including the acute respiratory distress syndrome, COPD and asthma (Pease & Sabroe, 2002, Keatings *et al.*, 1996). Asthmatics in particular have elevated levels of IL-8 and neutrophils in sputum samples taken during exacerbations of the disease (Fahy *et al.*, 1995). In addition mast cells are also capable of responding to IL-8 induced signals. Mast cells accumulate during inflammation, at least in part, due to the redistribution and recruitment of neighbouring mast cells. The release of IL-8 in the anti-IgE response may therefore be important in the recruitment of further mast cells to the inflammatory foci to resolve inflammation. Hence the detection of IL-8 in clinical samples from patients with these diseases has led clinicians to believe that antagonism of IL-8 may be a practical therapeutic strategy for disease management.

Of particular interest is the difference observed in the overall magnitude of IL-10 released in response to each stimulus. Consistently individuals produce much less IL-10 in response to anti-IgE compared to LPS stimulus. Indeed an individual's IL-10 response to LPS was not a predictor of their anti-IgE response and this may well represent a fundamental difference in the two types of inflammation being modelled. Royer and colleagues (2001) have previously reported human mast cells have the ability to synthesise and release IL-10 spontaneously, as well as after FcεR1 activation. In addition the same group also demonstrated that inhibition of IL-10 using a neutralising IL-10 antibody removed the inhibitory activity of IL-10 on the release of IL-5, IL-8 and TNFα. Previous findings by Borish *et al* (1996) showed that levels of IL-10 are lower in BAL fluid of asthmatic patients compared to normal individuals. There is also evidence, which suggests IL-10 polymorphisms are associated with total serum IgE levels in atopic dermatitis patients (Shin *et al.*, 2005). Low IL-10 levels would permit less regulated TNFα activity and a stronger pro-inflammatory response. The elevated levels of TNFα seen in the anti-

IgE response compared to LPS may therefore be a result of low levels of IL-10. This data fits with observations reported by Ordonez and colleagues (2000) and Thomas *et al*, (1995) that there are abundant levels of TNFα and IL-8 in the asthmatic airway. Unfortunately, we did not have sufficient time to investigate the effect of neutralising IL-10 in our IgE mediated response, so we cannot elaborate on the potential autocrine production of IL-10 in the IgE response. However there is research, which suggests IL-10 could be a potential therapeutic target in allergic asthma. In particular the effects of leukotriene modifiers such as monteleukast have been shown to increase IL-10 production in sensitised cultured peripheral blood mononuclear cells from asthmatic patients sensitized by allergen (Stelmach *et al.*, 2005). Inhaled corticosteroids are regarded as the first-line controller for anti-inflammatory treatment in the management of asthma. However, there is an increasing awareness of the risk of long-term adverse effects of inhaled corticosteroids. Therefore modifiers of IL-10 production such as monteleukast may well provide new therapeutic strategies for the management of allergic asthma without the associated long-term side effects of steroids.

In summary this chapter has shown different stimuli stimulate distinct cytokine cascades in human lung tissue. The data has also revealed some inflammatory mediators are more stimulus specific than others, which are common to different inflammatory conditions. IL-5 and IL-13 are examples of specific mediators involved in allergic inflammation and would be good therapeutic targets in diseases such as asthma. In comparison, IL-6 appears to play a more general role in inflammation as the IL-6 response is very similar to both stimuli. IL-6 would therefore be a good broad-spectrum target in inflammation rather than a therapeutic target for specific diseases. The Th_1/Th_2 paradigm has previously been used to divide inflammatory diseases based on the profile of cytokines released in different conditions. Whilst this paradigm provides a good framework for considering how events may vary in different types of inflammation, the divisions are not

exclusive and recent work suggests a much greater overlap between each phenotype (Liu *et al.*, 2004). To some extent our data suggests TNFα would be a good general inflammatory target. Indeed in inflammatory conditions from rheumatoid arthritis to severe asthma neutralisation of TNFα has provided clinical benefit to patients. However this chapter has indicated that the role of TNFα is different depending on the inflammatory condition. It may therefore be more prudent to see inflammatory conditions as neither rigidly constrained Th_1 or Th_2 responses, but rather that inflammatory cells can potentially produce a whole spectrum of cytokine profiles; and skewing of the response to either Th_1 or Th_2 cytokine profiles may therefore depend on the nature of the antigen present.

As we have discussed previously in this chapter there are several cytokines, which have a general role in inflammation independent of the nature of the stimulus. We were therefore interested to assess if the cytokines involved in inflammation are the same in all species. Many drug screens and models of COPD use rodents as model systems (Wright & Chung, 2002, Tigani *et al.*, 2002). In response to LPS rat lung fragments also showed early release of TNFα with levels peaking at 6hrs. LPS has previously been shown to induce TNFα release from adult rat alveolar epithelial cells, with a maximum stimulation of release at 6hrs (McRitchie *et al.*, 2000). In the rat model LPS did not stimulate the release of IL-6 or IL-10, which is different to the human response. This is in contrast with previous observations in foetal rat alveolar epithelial cells which when stimulated with recombinant TNFα did release IL-6 and IL-10 (Haddad *et al.*, 2002). However no studies have previously investigated the response of rodent alveolar cells to LPS. Interestingly, LPS did stimulate the release of IL-1β in the rat model, which was not detected in the human model. The release of IL-1β is consistent with previous studies which have used recombinant TNFα and LPS to induce the release of IL-1β in both foetal and adult rat alveolar epithelial cells (Haddad *et al.*, 2002). Together these observations indicate that the rat immune response to LPS is different to cytokine cascade released by humans. There are

many reasons why the cytokine cascade in rodents is different to man. Firstly within the cytokine family there is much redundancy it is therefore possible in rodents other cytokines maybe involved in the inflammatory response to LPS. In particular IL-1β appears to play a more important role in the rodent inflammatory response to LPS compared to the human cytokine cascade. Another important consideration to take in to account between the two models is the environmental conditions, of which smoking is an important factor. It is extremely difficult to represent the array of environmental insults that barrage the human lung on a daily basis. As a result individuals respond very differently to stimuli due to previous insults that have occurred over one persons lifetime. In addition diseases of an inflammatory nature such as COPD may even prime the lungs of individuals to respond differently to environmental stimuli. In comparison experimental animals are kept in very clean and controlled environmental conditions. It is therefore very unlikely that the rodent lungs used in this chapter have ever been exposed to the range of environmental insults our patients encounter. It is therefore understandable how the rodent immune response differs to the individuals in our study. When developing model systems immunological variation between species is therefore of important consideration. The very fact that our model of inflammation incorporates human lung tissue with varying degrees of lung function, smoking history, age and gender enables us to characterise the immune response in individuals with COPD. However rodent models have important roles to play in research and drug development as human lung tissue is a limited and time consuming resource. In comparison rodent tissue is plentiful and easily obtainable and for specific questions such as the effect of drugs on the release of individual cytokines like TNFα, rodent lung tissue is a useful model. However, when interpreting data from any model one should always keep in mind the limitations of the model.

Chapter 6.

Factors affecting protein expression

6.1 Introduction

The work in this thesis has demonstrated that many factors such as disease severity and smoking status can affect the expression of mediators in COPD. The mechanisms by which these mediators are altered are however unknown. Protein expression can be modified at various levels from transcription of the protein mRNA, to translation and during post- translational modification. Proteins are also targeted for destruction and differ markedly in their half-lives. The metabolic turnover of extracellular proteins is affected by a variety of factors, which include oxidation, proteolysis and loss of secondary and tertiary protein structure. However, the precise mechanisms of protein turn over have not been fully elucidated. They are thought to involve the uptake of damaged proteins by scavenger receptor type A found on macrophages and sinusoidal liver epithelial cells (Swart *et al.*, 1999, Duryee *et al.*, 2005). In contrast, cytosolic proteins are targeted for destruction via the well characterised ubiquitin pathway.

Previous studies have shown current smokers with COPD have elevated levels of oxidative stress due to persistent chronic inflammation compared to healthy smokers (MacNee, 2005, Rahman, 2005). The elevated oxidative stress in these individuals is thought to be the result of an increased burden of inhaled oxidants, as well as the addition of reactive oxygen species (ROS) generated by various inflammatory, immune and epithelial cells of the airways (Pryor & Stone, 1993). As well as ROS activated inflammatory cells also release an array of proteases and cytokines such as TNFα and IL-1β. These cytokines act to induce further protease production from both recruited inflammatory cells and the surrounding connective tissue (Aaron et al., 2001). The result is an elevated proteolytic burden within the lung of COPD patients. Both elevated oxidative stress and proteolytic activity within COPD patients could therefore lead to heightened protein turnover. We were therefore interested to investigate changes in protein expression with disease and the relationship with protein turnover.

In chapter three we investigated a range of mediators that were involved in the acute inflammatory model, however it is not feasible to analyse every protein expressed in the lung in this fashion. We were therefore interested in developing a technique to allow broad screening of clinical samples to identify proteins, which may be changed with disease. The interest in proteomic analysis of human clinical samples has been greatly increased over the years as mass throughput liquid chromatography and mass spectrometry technologies have evolved. Proteomic analysis of clinical samples represents an extreme challenge due to the sheer size of the human proteome. A means of addressing the complexity of clinical samples is the application of multi-dimensional protein identification technologies (MudPIT) such as iTRAQ. To date the technique has only been used successfully on bacterial lysates. However if adapted for clinical samples iTRAQ could provide a valuable proteomic tool. In this chapter we report attempts to develop the iTRAQ technique for proteomic analysis of the human lung from a range of individuals with lung function impairment.

6.2 Methods

6.2.1 Patient characteristics for human lung tissue experiments

Lung tissue was removed from 57 patients (30F/27M) undergoing resection for carcinoma; tissue used was from the non-tumourous normal margin surrounding the tumour site. Data relating to age, gender, lung function and smoking history was obtained for patients (see table 6.1). When defining patients smoking status ex-smokers were defined as having given up smoking for 3 years of more. Where different groups of patients were used for individual experiments, the patient characteristics were controlled to ensure the clinical details of the whole group were represented.

Table 6.1 Patient characteristics of subjects prior to removal of lung tissue

No. Of Subjects	57
Age (Yr.)	64.0 ±1.6
Gender	30 Female, 27 Male
Lung Function (FEV_1/FVC)	0.69 ±0.02
Smoking Status	6 Non smokers 22 Ex smokers 25 Current smokers 4 Unknown smoking status
Pack years	45.9 ±4.6

Table 6.1 Patient characteristics of subjects prior to removal of lung tissue. Tissue samples were taken from a group of 57 patients. Patient details including age, gender, lung function given as the ratio of FEV_1/FVC, smoking status and pack years are listed.

6.2.2 Patient classification and sample preparation for proteomic analysis

From the 57 individuals included in this study eight current smokers were selected classified by their lung function in to two groups; healthy current smokers with a

$FEV_1/FVC>0.70$ and unhealthy smokers with a $FEV_1/FVC>0.55$. The clinical parameters for each group are listed below see table 6.2 The baseline lung tissue of these individuals was homogenised using a fastPrep® instrument using the following methodology. Briefly, 50mg of lung tissue was homogenised in 0.5mls of 0.5% SDS triethylammonium bicarbonate buffer (pH 8.5) in pre-chilled lysing matrix D tubes at a speed of 6.0 for 45 seconds. Following homogenisation tubes were spun at 12,000 g for 1 minute at 4°C. Total protein was measured in each sample using the BCA protein assay (see method 2.3.8). The supernatant was then used for electrophoresis or proteomic analysis.

Table 6.2 Patient characteristics of subjects classified as healthy or unhealthy current smokers

Lung number	FEV_1/FVC	Age	Gender	Classification
H602	0.92	69	Female	Healthy
H615	0.84	59	Female	"
H625	0.74	50	Male	"
H628	0.70	70	Female	"
Average	**0.80**	**62**	**3F/1M**	
H567	0.55	54	Female	Unhealthy
H611	0.54	68	Male	"
H636	0.53	73	Male	"
H642	0.50	68	Male	"
Average	**0.53**	**65**	**1F/3M**	

Table 6.2 Patients were defined using their lung function as healthy (FEV1/FVC>0.70) or unhealthy (FEV1/FVC>0.55) current smokers. The patient characteristics FEV1/FVC, age and gender are also listed for each individual.

6.2.3 Primary cell culture of human lung tissue

Tissue samples were processed as previously described in preparation of human lung tissue for primary cell culture page chapter 2.2.2. Tissue was then either incubated with 200μg/ml HSA or oxidised HSA for 1 hour and then stimulated with either (100ng/ml) lipopolysaccharide or buffer control. Human lung fragments and supernatant were

harvested at 1, 2, 4, 6, 24, and 48 hour time points and the tissue weighed. Both the human lung fragments and supernatant collected were stored at -80°C.

6.2.3 Human serum albumin and carbonyl human serum albumin ELISAs

The number of HSA carbonyl residues were measured in the supernatants using an in house ELISA. For the full methodology for this assay see method 2.3.6. Briefly, a 96 well plate was coated with a monoclonal HSA antibody in coating buffer (pH 9.4). Following blocking for non-specific binding, a DNPHderivatised carbonyl-HSA standard curve and derivatised samples (see method 2.2.4) were added to the plate and incubated for 2 hours. Following this the plates were washed removing unbound derivatised protein. Bound DNP could then be detected using a DNP polyclonal antibody, which itself could be detected by a HRP conjugated secondary antibody. Addition of chromogen TMB caused a colour change, which when terminated with acid was quantifiable at an optical density of 450nm. This method was run in parallel with an in house ELISA measuring total immuno-competent HSA. The protocol for the measurement of total HSA was identical to the HSA carbonyl ELISA, except a polyclonal HSA antibody was used instead of the polyclonal DNP antibody, see method 2.3.5. With both the number of carbonyl residues and total HSA we were able to calculated the number of carbonyl residues per HSA molecule. Finally, Western blot analysis was performed using the HSA ELISA antibodies for the methodology see method 2.3.9.

6.2.4 Proteomics - iTRAQ

The pooled homogenised samples (see method 6.2.2) from each group termed healthy and unhealthy current smokers were labelled with the iTRAQ reagents as per manufacturers instructions. Briefly 150μg of pooled protein sample from each group was treated with the supplied denaturant and reducing reagents for 1 hour at 60°C. Following the addition of a

cysteine-blocking reagent the sample was treated with trypsin for 16 hours at 37°C to digest the sample. The following day each sample was then incubated with specific iTRAQ reporter reagents for 1 hour at room temperature. Finally the samples were pooled and lyophilised.

6.2.5 Cation exchange chromatography

The combined peptide sample was separated by strong cation exchange chromatography on a Dionex nano-LC system using a Polysulfoethyl A column (i.d 4.6 mm x 150 mm, 5μm, 300Å) (Phenomenex). The sample was dissolved in 500 μL loading buffer (25% v/v acetonitrile, 10 mM phosphoric acid) and loaded and washed isocratically for 20 min at 200 μL/min to remove excess reagents. The peptides were eluted with a linear gradient of 0 – 500 mM KCl (25% v/v acetonitrile, 10 mM phosphoric acid) at 200 μL/min, and fractions were collected at 1 min intervals and lyophilised.

6.2.6 Cation/anion exchange chromatography

The protein samples were separated by strong cation exchange (SCX) and strong anion exchange (SAX) chromatography on a Dionex nano-LC system using a Polysulfoethyl A column (i.d cation 2 mm x 50 mm, cation 2 mm x 250 mm) (Phenomenex). Chromatography was carried out as in method 6.2.5.

6.2.7 Mass Spectrometry and data processing

Fractions were resuspended in 50 μL of analar™ water. Each fraction was then analysed by nano- LC – MS/MS performed on a CapLC liquid chromatography system using a Dionex 75 μm x 150 mm C_{18} reverse phase column coupled to a tandem mass spectrometer. Peptides were eluted using a linear gradient of 5 – 80%/ 162 mins (Buffer A:

5% acetonitrile, 95% H_2O + 0.1 % formic acid; Buffer B: 95% acetonitrile, 5% H_2O + 0.1% formic acid)

All data were acquired using a Q-tof Global Ultima (Waters Ltd) fitted with a nanoLockSpray™ source to achieve better than 10ppm mass accuracy. A survey scan was acquired from *m/z* 375 to 1800 with the switching criteria for MS to MS/MS including (i) ion intensity and (ii) charge state. The collision energy used to perform MS/MS was varied according to the mass and charge state of the eluting peptide. RF lens voltages were adjusted to 0.6 V to enable detection of low mass reporter ions generated from the fragmentation of iTRAQ labelled peptides to enable quantification.

All MS/MS spectra were automatically processed using MassLynx 4.0 (Waters Ltd) and searched against the NCBI non-redundant database (June, 2005 versions), using ProteinLynx Global Server 2.2. Proteins were only assigned if, for each peptide ion, greater or equal to three experimentally derived y ions could be matched to the predicted spectra.

6.3 Results

6.3.1 Gross changes in protein expression with lung function

Firstly, we wanted to investigate if there were any gross differences in protein expression in patients with different lung function (FEV_1/FVC). As there are many clinical differences between the patient cohort used in this study, our initial experiments focused on current smokers. Baseline tissue from 8 current smokers defined either as healthy smokers with a $FEV_1/FVC > 0.8$ or unhealthy smokers with a $FEV_1/FVC < 0.7$ was homogenised. The samples in each group were then pooled and adjusted for total protein. As shown in figure 5.6 we observed differences in the baseline levels of protein expression

Figure 6.1

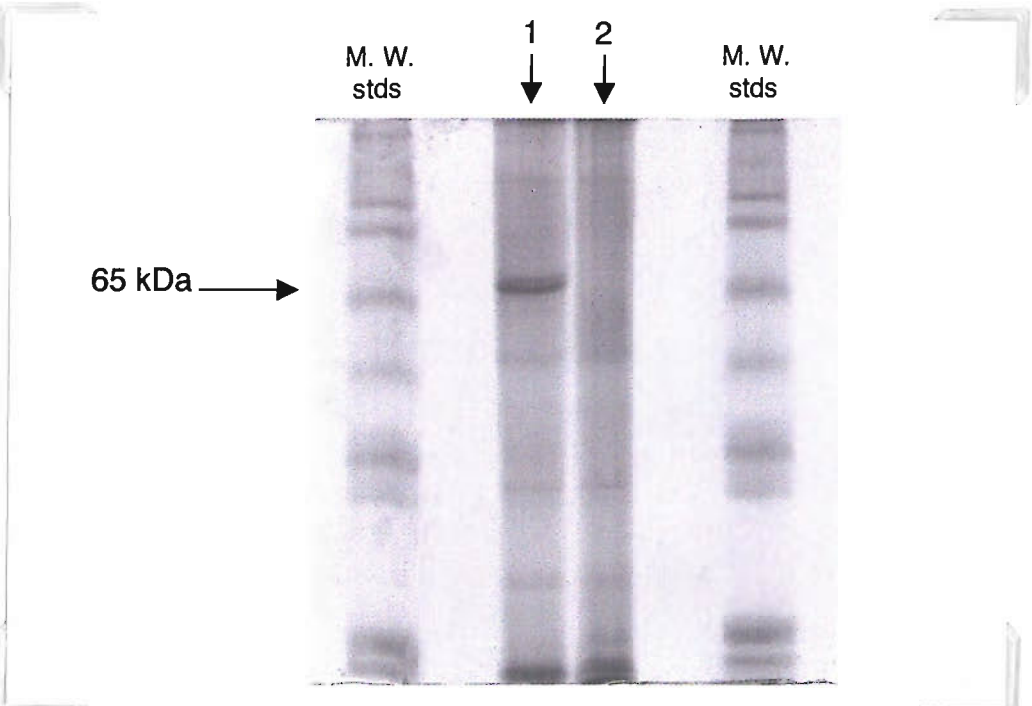


Figure 6.1 Changes in protein expression in human lung tissue with lung function. Human lung tissue from healthy current smokers ($FEV_1/FVC>0.8$, n=3) and unhealthy smokers ($FEV_1/FVC>0.8$, n=3) was homogenised (see method 2.2.3), adjusted for total protein, pooled and separated on a 12% SDS-polyacrylamide gel. From left: pooled group of healthy smokers (lane 1), pooled group of unhealthy smokers (lane 2).

in homogenised lung tissue, between healthy and unhealthy current smokers. In particular we found a band at 65kDa, which was present in the healthy current smoker group but not the unhealthy smoker group. Trypsin digest of the 65kDa band and mass spectrometer analysis confirmed our initial observations that the 65kDa band was human serum albumin.

6.3.2 Relationship between baseline levels of human serum albumin and disease severity

On further investigation of baseline levels of HSA in a larger group of 57 individuals we could not confirm our initial observation of decreased levels of HSA and poor lung function (FEV_1/FVC), (Rho 0.3, P=0.07). We have previously shown that the severity of COPD influences cytokine release in our acute inflammatory model. We were interested if disease severity or any of the other clinical parameters influenced the baseline levels of HSA. On further analysis we did find a relationship between disease severity and baseline levels of HSA. As shown in figure 6.2 patients defined as GOLD 2 were found to have lower levels of HSA (median= 24.4 μg/ml) compared to GOLD 1 (median= 48.4 μg/ml) and GOLD 0 (median= 71.9 μg/ml) individuals (P<0.05).

Figure 6.2

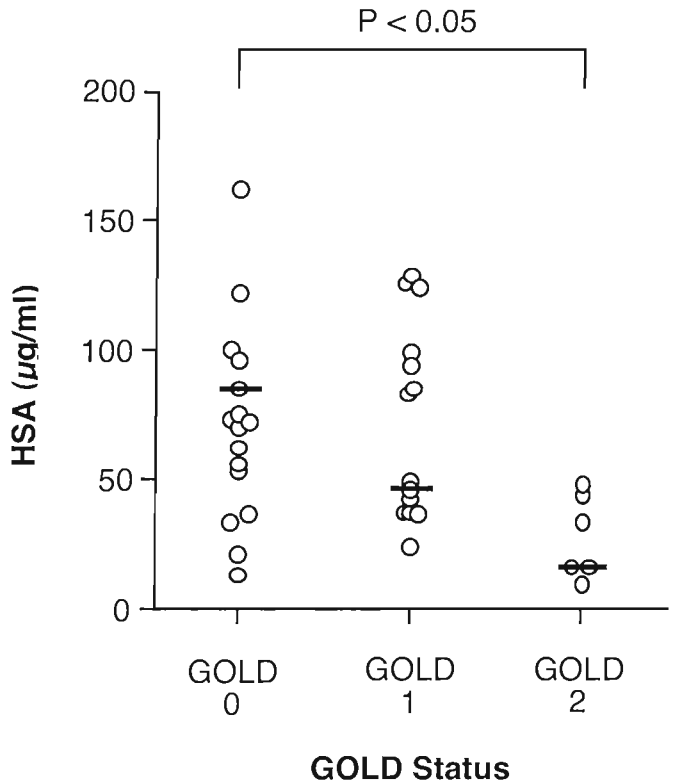


Figure 6.2 Relationship between disease severity and baseline levels of HSA. Human lung tissue from 36 individuals classified by the GOLD guidelines was homogenised (see method 2.2.3) and adjusted for total protein. HSA levels were measured in lung homogenates using ELISA. The median is marked as a solid bar and expressed as μg/ml. Data was analysed using the non-parametric Kruskal Wallis test, P<0.05 was considered to be statistically significant.

For this analysis patients were again defined by the GOLD guidelines given in method 4.2.4. The data shown includes 36 individuals, as complete lung function data was not available for all 57 patients.

6.3.3 Relationship between baseline levels of human serum albumin and smoking status

Having found a relationship between disease status and baseline levels of HSA we turned our attention to the other clinical parameters collected in the study. Analysis including all 53 individuals with identifiable smoking histories indicated that current smokers had lower levels of HSA (median= 47.5 μg/ml) compared to ex smokers (median= 69.7 μg/ml) and non smokers (median= 65.0 μg/ml, $P<0.05$), as shown in figure 6.2.

Figure 6.3

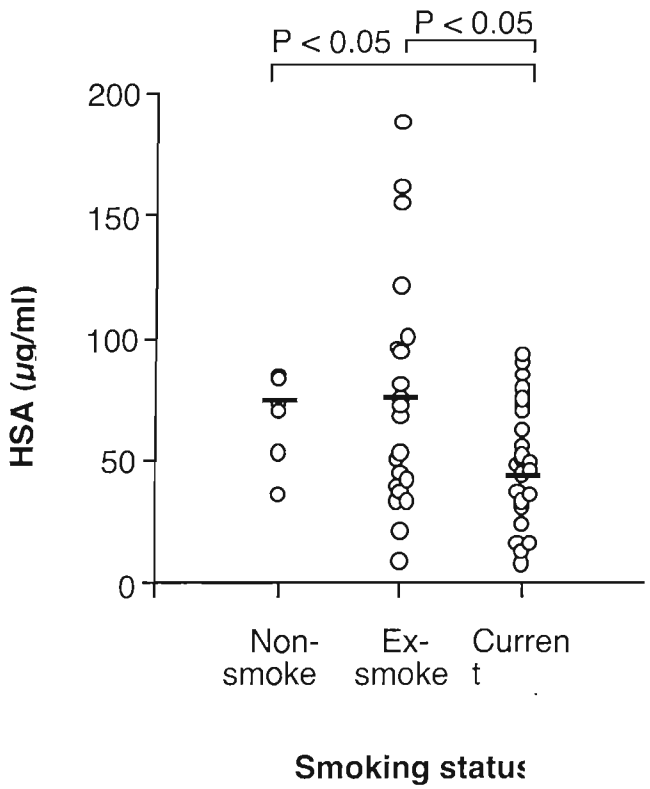


Figure 6.3 Relationship between smoking status and baseline levels of HSA. Human lung tissue from current smokers (n=25), ex smokers (n=21) and non-smokers (n=7) was homogenised (see method 2.2.3) and adjusted for total protein. HSA levels were measured in lung homogenates using ELISA. The median is marked as a solid bar and expressed as μg/ml. Data was analysed using the non-parametric Kruskal Wallis test, $P<0.05$ was considered to be statistically significant.

Chi squared analysis of the data indicated there was no relationship between GOLD status and smoking history (Chi-squared=0.216, P=0.51). On further analysis none of the other clinical parameters such as age or gender influenced the baseline levels of HSA. Determining which factors are most important in influencing HSA levels therefore requires further study.

Figure 6.3.4 Relationship between disease severity and levels of carbonylated human serum albumin

COPD patients and current smokers could be genetically pre-disposed to decreased levels of HSA due to polymorphisms in the HSA gene. However to date the known genetic conditions involving HSA are extremely rare (Murray *et al.*, 1983, Koot *et al.*, 2004). It therefore begs the question is HSA turned over more rapidly in these individuals? Oxidation is an important factor in the metabolic turnover of protein; we therefore wanted to investigate the levels of oxidised HSA in our patient cohort. The formation of carbonyl groups on amino acid residues as a result of free radical-initiated reactions is well documented. We therefore designed an ELISA to measure carbonyl HSA molecules in clinical samples (see methods 2.3.6).

Baseline tissue homogenates from the 36 individuals shown in figure 6.2 were derivatised and the level of carbonyl residues measured using an 'in house' ELISA. The numbers of carbonyl residues along with the values for total HSA shown in figure 6.2 were then used to calculate the number of carbonyl residues per HSA molecule. We found patients with no airway obstruction, defined as GOLD 0, had very little carbonylated HSA (median= 0.45 carbonyl residues/ HSA molecule). However with disease severity the number of carbonyl residues per molecule of HSA increased to 2.3 carbonyl residues/HSA molecule with mild COPD (GOLD 1) and further increased to 4.9 carbonyl residues/HSA molecule in moderate COPD patients (GOLD 2), $P<0.05$.

Figure 6.4

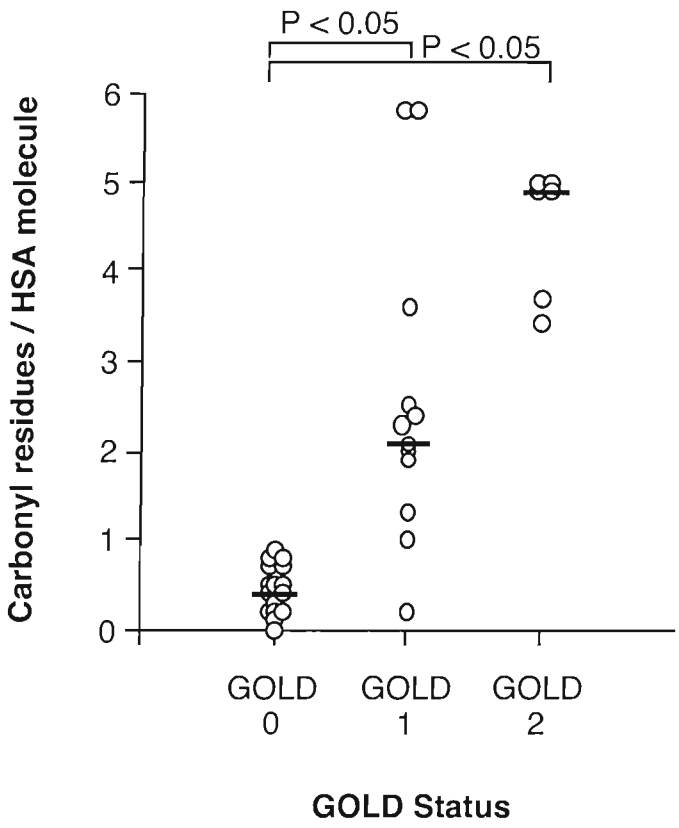


Figure 6.4 Relationship between disease severity and baseline levels of carbonylated HSA. Human lung tissue from 36 individuals classified using the GOLD guidelines was homogenised (see method 2.2.3), derivatised and the number of carbonyl residues measured using ELISA (see method 2.3.6). The median is marked as a solid bar and expressed as carbonyl residues/HSA molecule. Data was analysed using the non-parametric Kruskal Wallis test, P<0.05 was considered to be statistically significant.

Figure 6.3.5 Relationship between smoking status and levels of carbonylated human serum albumin

As we have previously shown smoking status influenced baseline levels of HSA we were interested if the levels of carbonylated HSA were also affected. Interestingly as shown in figure 6.3 we found no difference between the number of carbonylated HSA molecules in ex-smokers (median= 0.86 carbonyl residues/HSA molecule) and the non-smoker group (median= 0.6 carbonyl residues/HSA molecule). However when we look at the current

smokers we found a greater number of carbonyl residues per HSA molecule (median= 3.2 carbonyl residues/ HSA molecule) compared to both the ex and non-smokers (P<0.05).

Figure 6.5

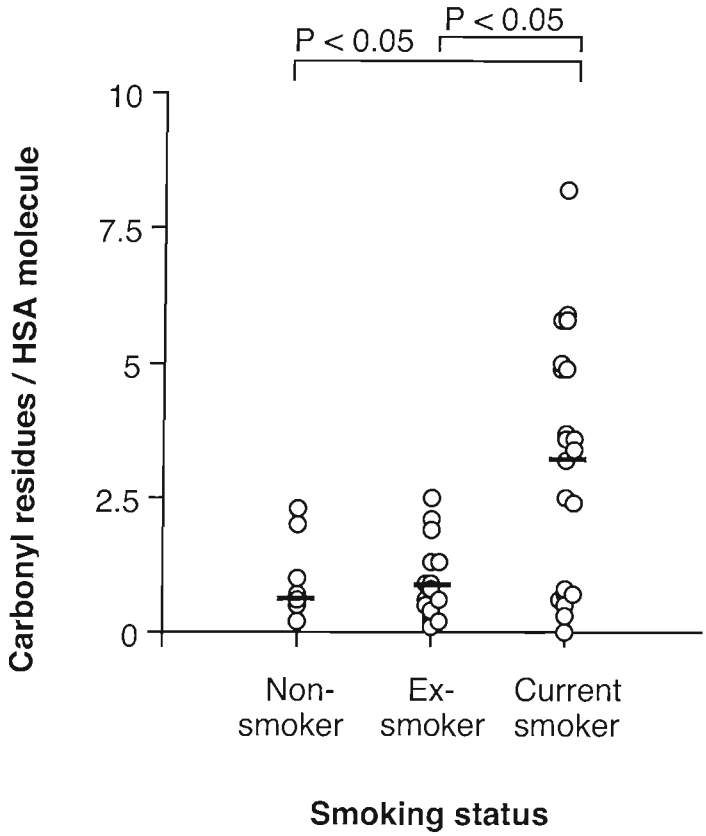


Figure 6.5 Relationship between levels of oxidised HSA and smoking status. Human lung tissue from current smokers (n=25), ex smokers (n=21) and non-smokers (n=7) was homogenised (see method 2.2.3). Samples were derivatised and the number of carbonyl residues measured using ELISA (see method 2.3.6). The median is marked as a solid bar and expressed as carbonyl residues/HSA molecule. Data was analysed using the non-parametric Kruskal Wallis test, P<0.05 was considered to be statistically significant.

6.3.6 Turnover of HSA in human lung tissue

As the levels of carbonylated HSA are increased with the severity of disease and smoking (see figures 6.4 and 6.5). We were interested to know if oxidised HSA is turned over more rapidly in the lung. Increased turn over of oxidised HSA could account for the decline in baseline levels of HSA observed in COPD patients and current smokers. To investigate HSA turnover, human lung tissue from 12 individuals (6 ex, 5 current and 1 non-smoker,

5F/7M, average $FEV_1/FVC=0.64$, average age=68) was cultured with 200µg/ml HSA or oxidised HSA for 1, 2, 4, 6 and 24 hours (see method 6.2.3). The supernatants were then analysed using an 'in house' HSA ELISA see method 2.3.5 for full details. As shown in figure 6.4 when the tissue was cultured with 200µg/ml HSA the levels of HSA in the supernatant remained relatively constant over the 24 hour experiment. In striking contrast when the tissue was cultured with 200µg/ml oxidised HSA we observed a decrease in the detectable levels of HSA after 4 hours of culture. In fact by the 24 hour time point the levels of detectable oxidised HSA had decreased to 105.7 µg/ml compared to 213.5 µg/ml unmodified HSA, $P<0.05$. Due to the small number of individuals in this study we could not analyse the relationship between HSA turnover and disease severity or smoking status.

Figure 6.6

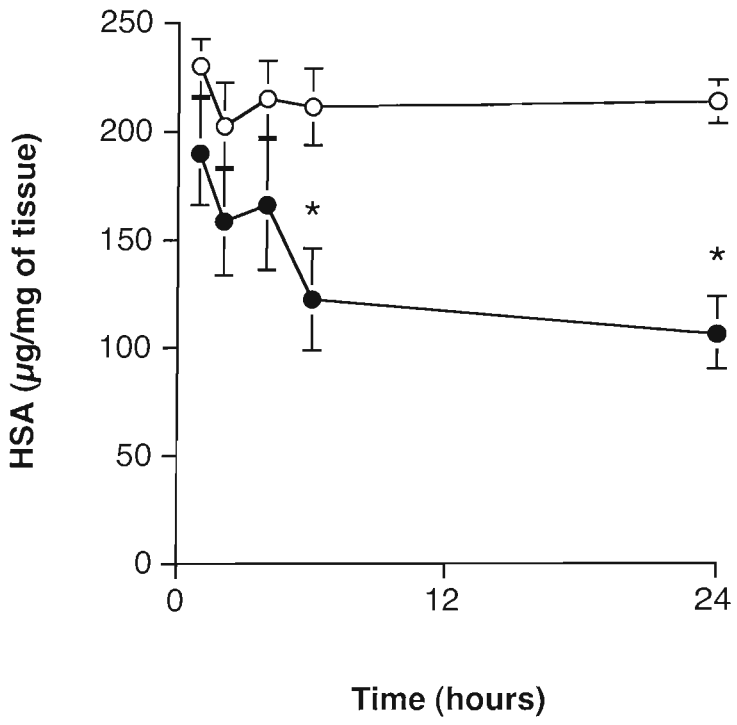


Figure 6.6 Turn over of HSA and oxidised HSA in human lung tissue. Human lung tissue (n=12) was incubated with 200µg/ml HSA (open circles) or 200µg/ml oxidised HSA (filled circles) for 1, 2, 4, 6, and 24 hours (see method 6.2.3). Samples were analysed for the levels of HSA using ELISA (see method 2.3.5). Values given are the mean ± SEM and are expressed as µg/ml. The data was statistically analysed using the Wilcoxon-Signed rank test, * indicates a P value <0.05.

6.3.7 Western blot analysis of HSA turn over in human lung tissue

The supernatants used in figure 6.6 were also analysed using Western blots probed with antibodies for HSA. As shown in figure 6.7a we can see when the tissue was cultured with 200μg/ml HSA the levels remained relatively constant over the 24 hour experiment, which is in line with the previous ELISA data shown in figure 6.6. When the tissue was cultured with oxidised HSA we again observed a decline in the detectable levels of HSA from the 4 hour time point (see figure 6.7b). As shown by both blots in figure 6.7 we also ran a HSA standard which indicated only the free molecular form of HSA was present in the supernatant. The blot is representative of the molecular profile of HSA observed for all individuals in the study.

Figure 6.7

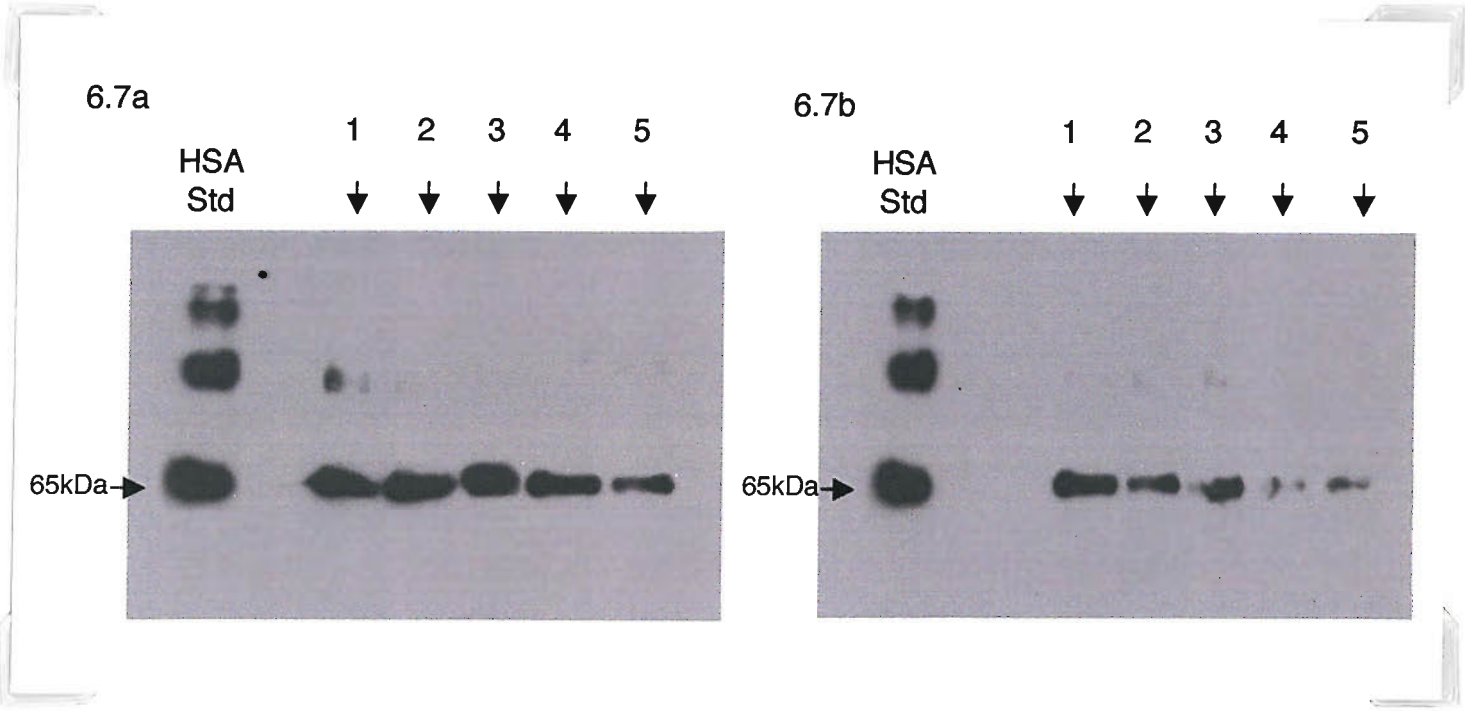


Figure 6.7 Western blot analysis of HSA and oxidised HSA turn over in human lung tissue. Human lung tissue (n=12) was cultured with 200μg/ml HSA or oxidised HSA and incubated for 1, 2, 4, 6, or 24 hours (see method 6.2.3). Supernatants were separated on a 12% SDS-polyacrylamide gel and then transferred on to nitrocellulose. The supernatants cultured with HSA are depicted in Figure 6.7a and the supernatants cultured with oxidised HSA are shown in figure 6.7b. For each figure lane one corresponds to incubation of the tissue for 1hr, lane 2 for 2hrs, lane 3 for 4hrs, lane 4 for 6hrs and lane 5 for 24hrs finally, lane 6 is a 200μg/ml HSA standard. The blot depicted is a typical example of the molecular profile of HSA observed for all individuals in the study.

6.3.8 The effect of inflammation on HSA turnover in tissue culture

Chronic inflammation and oxidative stress are important features in the pathogenesis of COPD (MacNee, 2005). In this thesis we have developed and characterised a model of acute inflammation. We were therefore interested to use this model to investigate HSA turnover during inflammation of the lung. As shown in figure 6.8 following LPS stimulation we observed a reduction in the detectable levels of HSA at 4 hours, which reached a maximal decline to 157.0 μg/ml at 24 hours compared to HSA and buffer control (mean= 213.5 μg/ml, $P<0.05$). Interestingly when we look at oxidised HSA, LPS actually augments HSA turnover with detectable levels declining to 73.4 μg/ml compared to HSA and LPS (mean= 157.0 μg/ml, $P<0.05$).

Figure 6.8

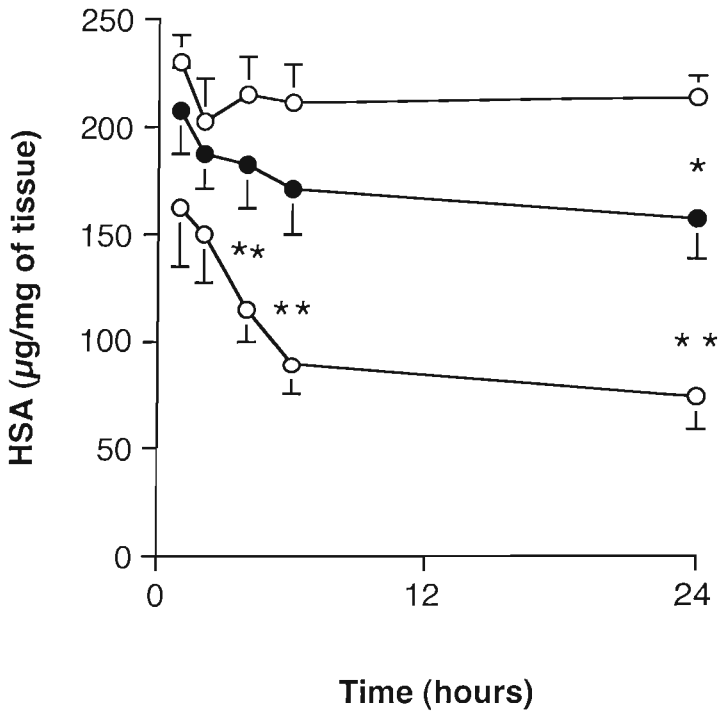


Figure 6.8 The effect of inflammation on HSA turnover in tissue culture. Human lung tissue from 12 individuals was cultured with 200μg/ml HSA or oxidised HSA for 1 hour. Tissue was then either stimulated with 100ng/ml LPS or buffer control and harvested at 1, 2, 4, 6, and 24 hours (see method 6.2.3). Tissue incubated with HSA and buffer control is represented by the open circles, HSA and LPS by the filled circles and oxidised HSA and LPS by the grey circles. The supernatants were analysed using 'in house' HSA ELISAs see methods 2.3.5 for full details. Values given are the mean ± SEM and are expressed as μg/ml. The data was statistically analysed using the Wilcoxon-Signed rank test, * indicates a P value <0.05 compared to HSA control, and ** indicates a P value <0.05 compared to HSA control and HSA stimulated with LPS.

6.3.9 Western blot analysis of HSA turn over and inflammation in human lung tissue

The supernatants used in figure 6.8 were also analysed using Western blots probed with antibodies for HSA. As shown in figure 6.9a we can see when the tissue was cultured with unmodified HSA and LPS we observed a decline in the detectable levels of HSA from the 4 hour time point, which is inline with the previous ELISA data shown in figure 6.8. When the tissue was cultured with oxidised HSA and LPS we could only detect HSA up to the 4 hour time point (see figure 6.9b). This indicates oxidised HSA turnover is augmented in the presence of inflammation. As shown by both blots in figure 6.9 we also ran a HSA standard which indicated only the free molecular form of HSA was present in the supernatant. The blot is representative of the molecular profile of HSA observed for all individuals in the study.

Figure 6.9

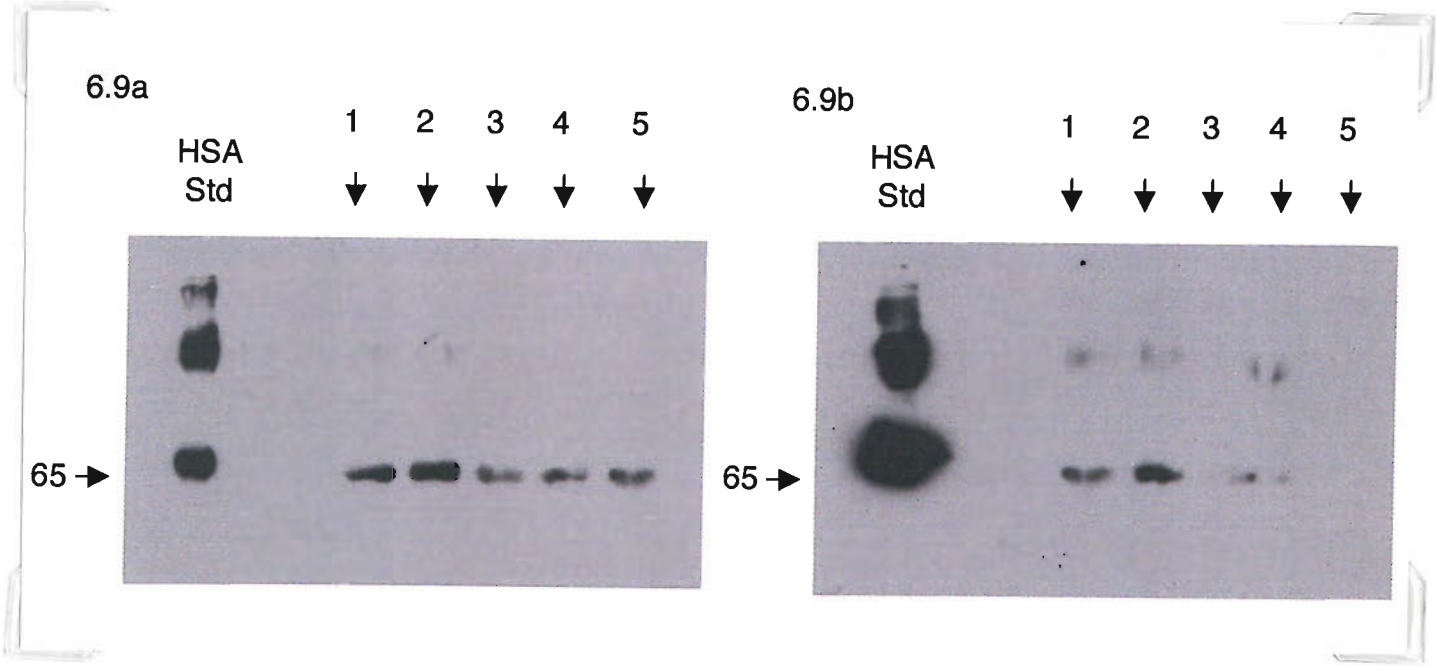
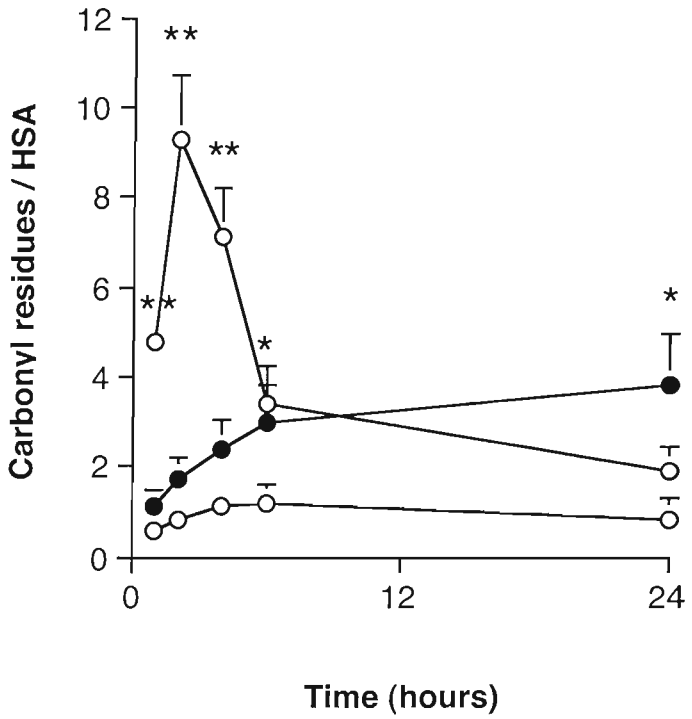


Figure 6.9 Western blot analysis of HSA and oxidised HSA turn over in human lung tissue. Human lung tissue (n=12) was cultured with 100μg/ml HSA or oxidised HSA and then stimulated with either 100ng/ml LPS or buffer control and incubated for 1, 2, 4, 6, or 24 hours (see method 6.2.3). Supernatants were separated on a 12% SDS-polyacrylamide gel and then transferred on to nitrocellulose. The supernatants cultured with HSA and LPS are depicted in Figure 6.9a and the supernatants cultured with oxidised HSA and LPS are shown in figure 6.9b. For each figure lane one corresponds to incubation of the tissue for 1hr, lane 2 for 2hrs, lane 3 for 4hrs, lane 4 for 6hrs and lane 5 for 24hrs finally, lane 6 is a 100μg/ml HSA standard. The blot depicted is a typical example of the molecular profile of HSA observed for all individuals in the study.

6.3.10 The effect of inflammation on HSA oxidation in tissue culture

If the rate of HSA turnover correlates with the oxidation of HSA residues we would expect the levels of carbonylated HSA to increase following LPS challenge. Indeed we found following incubation of HSA with LPS for 24 hours the number of carbonyl residues increased to 3.8 carbonyl residues/HSA molecule compared to HSA and buffer control (mean= 0.8 carbonyl residues/HSA molecule, $P<0.05$). In the case of oxidised HSA already modified with 4.8 carbonyl residues/HSA molecule, LPS stimulation actually lead to further carbonylation of the HSA present to 9.3 carbonyl residues/HSA molecule. However we then observed a sharp decline in the number of carbonyl residues for both the oxidised and unmodified HSA at the 4 hour time point which may indicate substantial turn over of these highly oxidised molecules of HSA.

Figure 6.10



6.10 The effect of inflammation on HSA oxidation in tissue culture. Human lung tissue from 12 individuals was cultured with 200µg/ml HSA or oxidised HSA for 1 hour. Tissue was then either stimulated with 100ng/ml LPS or buffer control and harvested at 1, 2, 4, 6, and 24 hours (see method 6.2.3). Tissue incubated with HSA and buffer control is represented by the open circles, HSA and LPS by the filled circles and oxidised HSA and LPS by the grey circles. The supernatants were analysed using an 'in house' carbonylated HSA ELISAs see methods 2.3.6 for full details. Values given are the mean ± SEM and are expressed as µg/ml. The data was statistically analysed using the Wilcoxon-Signed rank test, * indicates a P value <0.05 compared to HSA control, and indicates a P value <0.05 compared to HAS control and HAS stimulated with LPS.

6.11 Proteomic analysis of clinical samples

We have previously shown in this chapter that analysis of clinical samples using SDS-PAGE can identify mediators, which are altered in disease. However further investigation of large numbers of samples using a high throughput ELISA, failed to validate our initial observations. In addition SDS-PAGE is not sensitive enough as it can only provide information on gross changes in protein expression and further analysis is required to quantify and validate the protein of interest. To further characterise changes in protein expression in disease we therefore required a sensitive, high throughput technique. We chose to study the proteome of human lung tissue using multiple dimensional protein identification technology. Applied biosystems amine-specific labelling reagents for multiplexed relative and absolute protein quantification (iTRAQ) is an extremely powerful technique. The methodology uses amine-specific labelling reagents to label multiple peptides in a protein digest. The technique also allows multiplexing of experiments so naïve and diseased states can be run in the same experiment in duplicate. The basic principle behind the technique iTRAQ is shown in figure 6.9 (over the page). Briefly, following trypsin digest, the control and test samples are labelled with two different iTRAQ tags. The tags bind to the amino terminus of the trypsin fragments and lysine and are designed to fragment to produce specific reporter tags when ionised in the mass spectrometer (MS). The specific reporter tags have different molecular weights to enable the MS to provide quantification within different samples. Following labelling the two samples are loaded together onto a tandem mass spectrometer (MS/MS). Samples are firstly separated by nano reverse phase chromatography then the MS measures the relative intensity and charge state of the peptides, which is used to generate a peptide candidate list for fragmentation using MS/MS. Fragmentation data enables the identification of peptides and hence the protein from which it is derived. All of the MS/MS spectra are automatically processed using a MassLynx 4.0 and searched against the NCBI non-redundant database.

Proteins are only assigned for each peptide ion if three or more experimentally derived y ions could be matched to the predicted spectra. Finally, the relative abundance of each peptide is then used to analyse the difference in expression of each protein in the control and test samples.

Figure 6.11

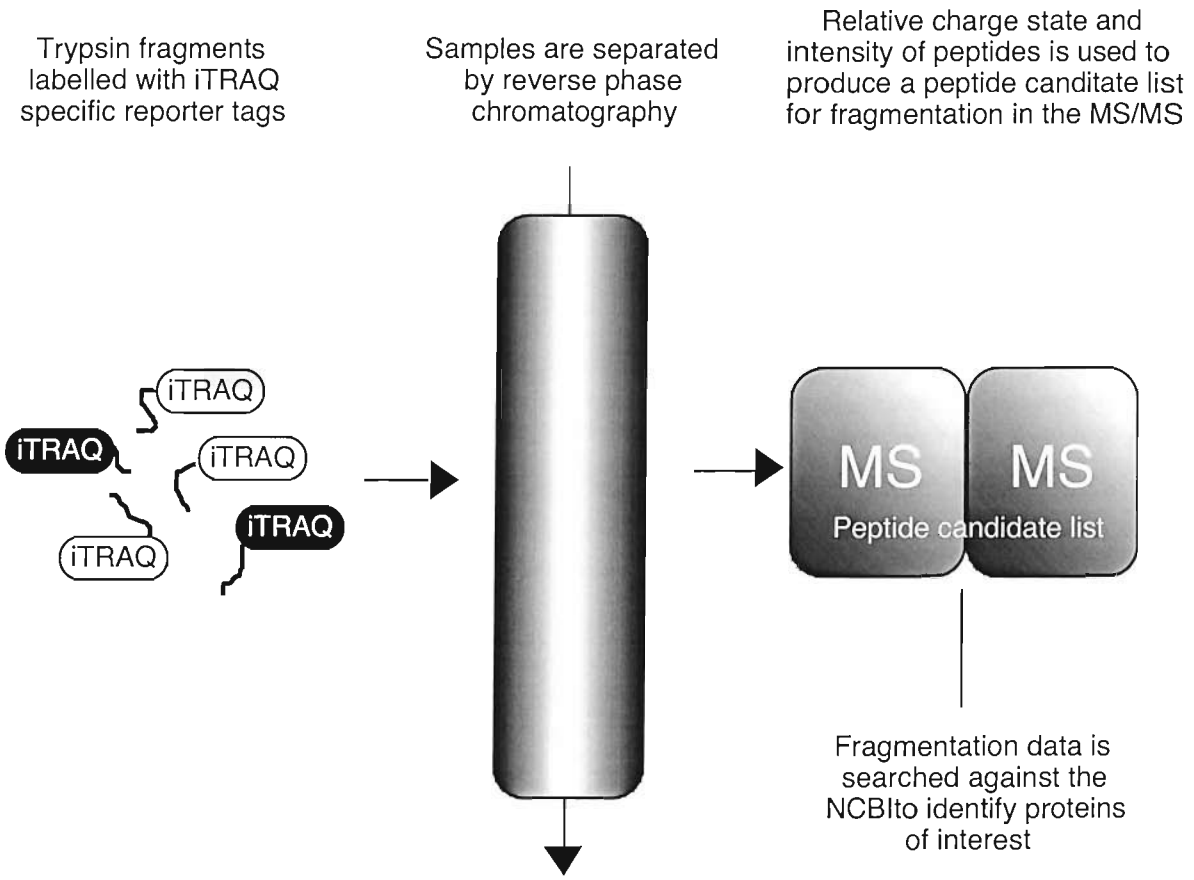


Figure 6.11 Schematic diagram of the technique iTRAQ. Following trypsin digest and labelling with iTRAQ reagents samples were loaded onto a tandem mass spectrometer (MS/MS). Firstly samples are separated by nano reverse phase chromatography and the relative intensity and charge state of the peptides is used to generate a peptide candidate list for fragmentation by MS/MS. Fragmentation data enables the identification of peptides and hence the protein from which it is derived. All of the MS/MS spectra were processed using a MassLynx 4.0 and searched against the NCBI non-redundant database. The relative abundance of each peptide is then used to analyse the difference in expression of each protein in the control and test samples.

To date iTRAQ has only been used successfully to analyse the proteome of bacterial cell lysates. If modified for clinical samples the technique could provide a

valuable proteomic tool for the analysis of altered protein expression in disease. The iTRAQ technology is sensitive and is based on comparing differences between two samples for example diseased and naïve tissue. Our initial experiments therefore looked at the differences between current smokers with different lung functions (FEV_1/FVC). As previously described individuals were defined as either 'healthy' or 'unhealthy' smokers (see method 6.2.2). As we were starting with human tissue our first task was to develop a methodology compatible with the iTRAQ labelling technology (see method 6.2.2). Following homogenisation, samples were pooled, trypsin digested and the fragments of the two groups labelled with two different iTRAQ tags (see method 6.2.4). The samples were then processed using MS/MS as described in methods 6.2.7.

However from our preliminary experiments we were only able to detect a small number of peptides at very low abundance. The relative abundance and the m/z ratio for each peptide detected in our initial experiment is shown in Figure 6.12.

Figure 6.12

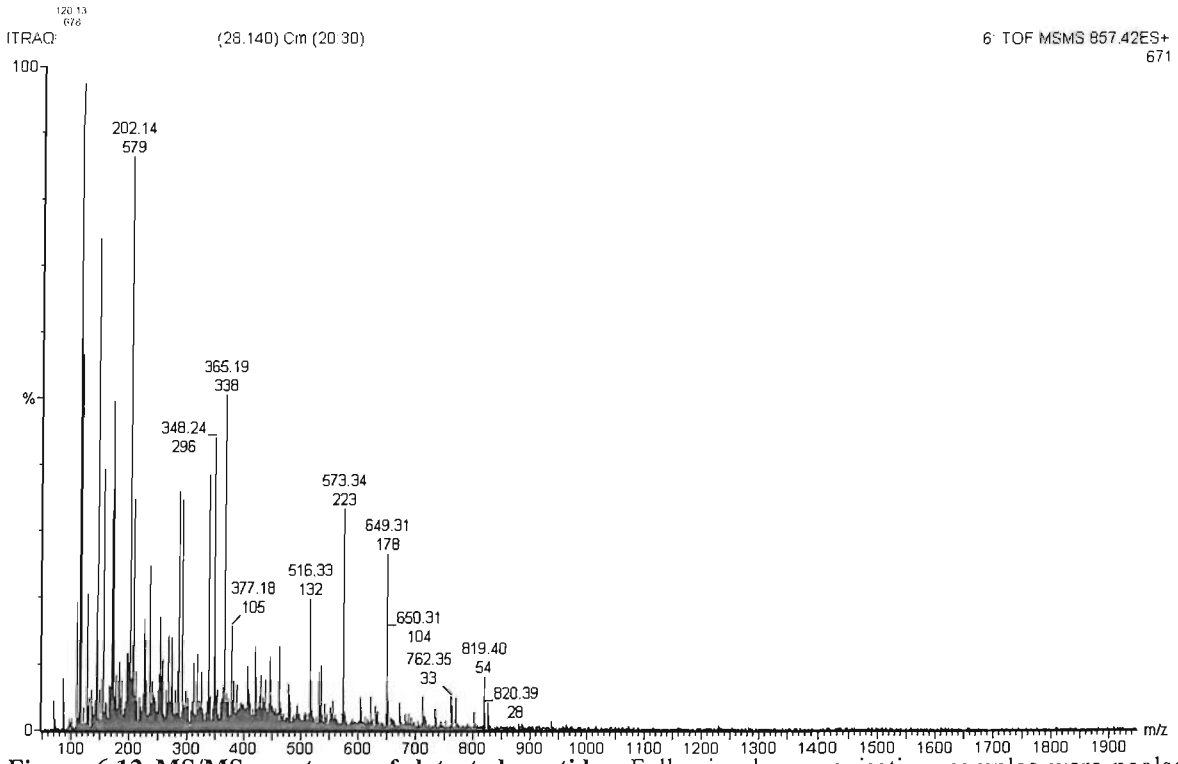


Figure 6.12 MS/MS spectrum of detected peptides. Following homogenisation, samples were pooled, trypsin digested and the fragments of the two groups labelled with two different iTRAQ tags (see method 6.2.4). The samples were then processed using MS/MS as described in methods 6.2.7. The m/z ratio MS/MS spectrum showing the relative abundances of each peptides detected is shown in figure 6.12.

With further investigation this was attributed to the large quantities of abundant peptides in the sample, which simply mask the lower abundant peptides. To solve this problem we used cation exchange to fractionate the sample post labelling so fractions containing fewer peptides could be loaded on to the MS/MS separately (see method 6.2.5). Following fractionation of the sample we still detected a large number of peptides associated with a few highly abundant proteins. The highly abundant proteins found in our lung tissue and their functions are listed below in table 6.3

Table 6.3 Abundant proteins and their function

Actin	One of the contractile elements in skeletal, cardiac, and smooth muscle fibres.
Annexin	A family of proteins that share the property of binding calcium and phospholipids.
Betaglobulin	A globulin in blood plasma that carries iron -transferrin, and siderophilin
Haemoglobulin &Macroglobulin	A plasma globulin of high molecular weight.
Ca^{2+} binding protein	Proteins which bind Ca^{2+} such as calmodulin
Histone H3 & H4	A class of protein molecules found in the chromosomes of eukaryotic cells. They complex with the DNA and pack the DNA into tight masses of chromatin,
Serum albumin	Albumin normally constitutes about 55% of the plasma proteins and serves to maintain the osmotic pressure of the blood. They also adhere chemically to various substances in the blood, e.g., amino acids, and thus play a role in their transport.
Vimentin	Vimentin is a protein that forms vimentin filaments, which in turn forms part of the cytoskeleton of living cells.

Table 6.3 Abundant proteins and their function. The highly abundant proteins detected using iTRAQ in human lung tissue are listed above with their function.

Further analysis of the fractionated samples indicated the highly abundant proteins constituted up to 70% of the total protein in each fraction. Although, serum albumin is listed within table 6.3 and we have previously shown it to be altered in disease. However we wanted to be able to use iTRAQ to analyse a wide range of proteins with disease. We therefore needed to refine the technique to deplete the abundant proteins to enable the analysis of the low abundant proteins in the sample fractions. We choose to remove the

abundant proteins using pre-protein fractionation of the sample using cation/anion exchange chromatography (see method 6.2.6). Briefly, before trypsin digestion and labelling of the sample each pooled sample was fractionated using an cation/anion exchange chromatography. A small volume of the fraction was then separated by electrophoresis on a 12% polyacrylamide gel to identify fractionations with abundant proteins. An example of one of the gels is shown in Figure 6.13, lane 2 contains one of the protein fractions containing the majority of one of the abundant proteins, HSA. In the other lanes are protein fractions in which less abundant protein are no longer masked by abundant proteins such as HSA.

Figure 6.13

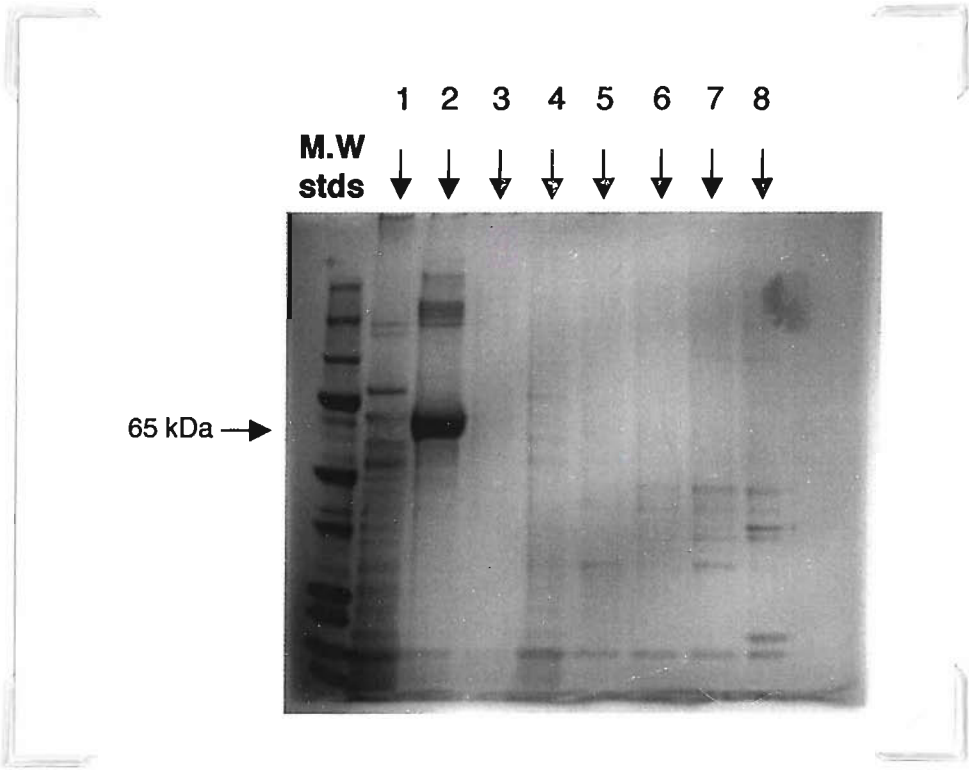


Figure 6.13 12% SDS-PAGE of proteins separated by cation exchange. Homogenised samples from each group were pooled and then separated by cation/anion exchange (see method 6.2.6). The fractions were then separated by electrophoresis on a 12% polyacrylamide gel to identify fractions with abundant proteins. As shown in figure 6.13 lane one corresponds to fraction 5, lane 2 fraction 6, lane 3 fraction 31, lane 4 fraction 12, lane 5 fraction 14, lane 6 fraction 20, lane 7 fraction 24 and lane 8 fraction 28. The blot depicted is a typical example of the fractionations collected for each group.

Following protein fractionation the fractions containing the least abundant proteins were again pooled and labelled using the iTRAQ reagents (See method 6.2.4). Following labelling the samples were again fractionated using cation exchange chromatography. However although we had removed the majority of the abundant proteins in the sample due to the pre-protein fractionation we had also elevated the salt concentrations due to processing of the sample. Therefore we were unable to obtain efficient labelling and in addition problems with chromatography occurred due to sample content. Following this work companies such as Agilent have developed high affinity depletion columns to remove abundant proteins in serum, however this technology was not available to us at the time. These methodologies offer a great advance in the analysis of clinical serum samples. However sufficient work would be required to develop methodologies for efficient use of agilent columns for the analysis of human tissue.

6.4 Discussion

We have shown many factors such as disease severity and smoking status can affect the expression of mediators in the disease COPD. The mechanisms by which these mediators are altered are however unknown. Previous studies have shown that current smokers with COPD have elevated levels of oxidative stress. This is thought to result from the increased burden of inhaled oxidants in smoke and ROS generated by activated inflammatory cells. When activated inflammatory cells also release an array of proteolytic enzymes, which enhances the proteolytic burden within the lung. The elevated oxidative stress and proteolytic activity within COPD patients could consequently affect the metabolic turnover of extracellular proteins within the lung. We were therefore interested to investigate protein expression with disease and the relationship with protein turnover.

Our initial investigations with a small number of individuals indicated lower levels of HSA in individuals with poor lung function ($FEV_1/FVC < 0.55$) compared to individuals with normal lung function. However this initial observation between the baseline levels of HSA and lung function was lost with a larger sample size. Interestingly when the data was re-classified by GOLD status we found individuals with COPD had lower levels of HSA. The GOLD classification criteria uses lung function to stratify COPD patients; therefore our initial findings may have been related to disease rather than an individuals FEV_1/FVC.

A number of studies have previously shown decreased levels of the antioxidants ascorbic acid and Vitamin E in COPD patients during exacerbations compared to stable period levels (Tug *et al.*, 2004), but to date there is no evidence of reduced levels of HSA. However studies of patients hospitalised with acute exacerbations of COPD have found that the level of serum albumin measured in the first 24 hours after admission is a good predictor of mortality for COPD patients (Connors *et al.*, 1996, Khilnani *et al.*, 2004).

Human serum albumin is used clinically to maintain colloid osmotic pressure and is also viewed to serve an antioxidant role in patients with damaged vascular endothelium and patients with acute lung injury (Lang *et al.*, 2002, Quinlan *et al.*, 2004). In fact a direct protective effect of albumin is indicated from many epidemiological studies (Bourdon *et al.*, 1999). HSA is known to reflect the underlying nutritional status of individuals and is as these studies suggest affected by the severity of chronic illness. Theses factors may therefore indicate that the decreased levels of HSA in COPD patients are a result of the disease.

HSA is one of the major antioxidants in the respiratory tract lining fluid, which also includes mucin, superoxide dismutase, glutathione, uric acid and ascorbic acid. Since the extracellular fluid contains only small amounts of antioxidant enzymes, it has been proposed that HSA may represent the major and predominant circulating antioxidant in plasma (Bourdon *et al.*, 1999). A current hypothesis in the pathogenesis of COPD is that increased oxidant burden both directly as a result of smoking or indirectly by the release of increased amounts of ROS from airspace leukocytes- may not be adequately counterbalanced by the lung antioxidant systems, resulting in oxidative stress. Decreased levels of the antioxidant HSA in COPD patients could therefore lead to an excess of oxidants leading to; enhanced pro-inflammatory gene expression and protein release, inactivation of antiproteinases, and oxidative tissue injury. It is unlikely that COPD patients are genetically predisposed to produce lower levels of HSA as although SNPs can occur in the gene it is extremely rare that they affect the synthesis of HSA (Murray *et al.*, 1983, Koot *et al.*, 2004). It therefore begs the question is HSA turned over more rapidly in COPD patients?

We found that with disease severity the number of carbonyl residues per HSA molecule increased compared to individuals with no airways obstruction. Oxidised HSA has previously been reported in BAL from COPD patients (Foreman *et al.*, 1999). The

formation of carbonyl groups on amino acid residues as a result of free radical-initiated reactions is well documented and is a marker of protein degradation and turnover (Stadtman, 1990, Davies , 1987). In fact the oxidative modification of proteins and lipids has been implicated in the etiology of a number of diseases including atherogenesis and diabetes (Njorge *et al.*, 1987, Baynes *et al.*, 1989). In particular oxidised HSA is a reliable marker of oxidative stress in patients with chronic renal failure and individuals on hemodialysis therapy (Himmelfarb *et al.*, 2001). As the severity of disease correlates with the number of carbonyl HSA residues in our study. The extent of oxidised HSA may represent an antioxidant disturbance within the lungs of COPD patients. Oxidised HSA could therefore possibly be used as a marker of disease as well as oxidative stress in COPD patients.

We also found a relationship between the smoking history of an individual and baseline levels of HSA, with current smokers having lower baseline levels of HSA compared to ex smokers and non-smokers. Cigarette smoking has been associated with decreased levels of the anti-oxidants ascorbate and vitamin C (Duthie *et al.*, 1993, Anderson et al., 1988 and Pelletier, 1975), however to date there is no evidence of reduced levels of HSA in current smokers. There is evidence, which suggests that cigarette smoking induces albuminuria in normal individuals compared to non smokers (Pinto-Sietsma *et al.*, 2000). However it is difficult to determine the biological significance of protein secretion from the body and levels of HSA within the lung, due to the abundance of HSA within plasma and the respiratory lining fluid. The main causal factor of smoking related COPD is thought to be chronic oxidative stress caused by long-term cigarette smoking (Catin *et al.*, 1985, MacNee & Rahman, 1999). We were therefore interested if smoking status affected the levels of HSA oxidation, and as a result its turnover.

Indeed we found current smokers had elevated numbers of carbonyl residues per HSA molecule compared to ex and non-smokers. Cigarette smoke has been shown to

modify human plasma proteins, producing carbonyl proteins with lost sulfhydryl groups (Reznick *et al.*, 1992, O'Neil *et al.*, 1994). One particular study, has also shown the content of oxidised proteins recovered in BAL is greater in smokers compared with non-smoking control subjects (Lenz *et al.*, 1996). Another has shown plasma anti-oxidant activity is decreased acutely in cigarette smokers, following acute exacerbations in COPD patients (Rahman and MacNee, 1996). The presence of cigarette smoke also increases the local concentration of neutrophils and ultimately leads to the release of an array of ROS and proteolytic enzymes (MacNee *et al.*, 1989). Following multivariate analysis we found no relationship between COPD and smoking status. It is therefore possible that cigarette smoke both directly and indirectly could lead to the oxidation of proteins such as HSA in addition to the disease.

As previously mentioned oxidation of residues results in the metabolic turn over of proteins. It is therefore not too surprising that the levels of carbonylated HSA are greater in the current smokers compared to ex and non-smokers. It is particularly interesting that the ex smokers defined as having given up smoking for 3 years or more had the same number of carbonyl residues as the non-smokers. When we look as the levels of total HSA individuals defined as ex smokers also had the same level of total HSA as the non-smoker's. This data suggests that if you cease smoking you prevent the elevated oxidation of HSA and therefore its rapid turn over. The data however can not indicate the time scale required for the resolution of smoking related oxidative stress, however within 3 years the levels of total HSA had returned to normal. It is well document that smoking cessation is the only effective treatment in slowing down of the accelerated decline in FEV_1 and thus progression of the disease COPD. If as our data suggests the oxidant/anti-oxidant imbalance is resolved with smoking cessation this furthers the role of antioxidant disturbances in the progression of COPD.

HSA is also able to serve as a depot and transport protein in the circulation, because it can bind reversibly a large number of endogenous and exogenous compounds. HSA has two major primary drug binding sites, termed site I and site II, which can bind a variety of compounds such as warfarin, and non steroid anti-inflammatory drugs (NSAIDs) (Chuang *et al.*, 1999, Rahman *et al.*, 1993). A resent study has shown that oxidation of HSA leads to diminished binding affinities for these drugs (Anraku *et al.*, 2001). Oxidation of HSA may therefore have important implications in drug delivery and also the transport of endogenous proteins within the body.

We have shown that the levels of HSA are decreased in current smokers with COPD, and the HSA present is in a more highly carbonylated form in these individuals. We also found that oxidised HSA is turned over in human lung tissue compared to unmodified HSA and the turnover of protein by the lung was also confirmed by Western blot. The formation of carbonyl groups on amino acid residues as a result of free radical-initiated reactions is well documented and is a marker of protein degradation and turnover (Stadtman, 1990, Davies, 1987). HSA is a single non-glycosylated polypeptide containing 35 cysteine residues all involved in the formation of stabilising disulphide bonds except ^{34}cysteine. In plasma this free thiol group is quantitatively the most important scavenger of oxidants. The formation of carbonyl groups on HSA also occurs on basic residues of amino acids such as histidine, lysine and arginine. A recent study has shown oxidation of HSA decreases its denaturation enthalpy, suggesting that oxidised forms of HSA are more easily denatured (Anraku *et al.*, 2001). There is also evidence that oxidation of HSA alters its ligand binding properties probably due to conformational changes (Anraku *et al.*, 2001). These data and supporting literature therefore suggests that oxidised HSA would be turned over more rapidly within human lung tissue.

The precise mechanisms involved in the metabolic turnover of extracellular proteins have not been fully elucidated. They are thought to involve the uptake of damaged

proteins by type A scavenger receptors found on macrophages and the sinusoidal liver epithelial cells (Swart *et al.*, 1999, Duryee *et al.*, 2005). Oxidised proteins can also be taken up by most cells including epithelial cells, either through receptor mediated endocytosis or pinocytosis, and is catabolised through lysosomal degradation (Hastings *et al.*, 1995, Grune *et al.*, 1997). The main turnover of both normal and abnormal intracellular cytosolic and nuclear proteins involves the proteasome complex (Rock *et al.*, 1994).

In particular studies have shown macrophages adhere to oxidant modified BSA through the class A scavenger receptor (SRA) (Zhang *et al.*, 1993). The SRA is principally expressed on macrophages has very broad ligand binding capacity and is thought to play a role in innate immunity. Binding to the SRA triggers macrophage activation and the release of ROS and inflammatory cytokines such as TNFα (Haworth *et al.*, 1997). The SRA can also act as a tissue adhesion receptor for the macrophage resulting in tissue retention, and has therefore been implicated in atherosclerosis and Alzheimer's disease (el Khoury *et al.*, 1994). The SRA could therefore provide a potential mechanism for the elevated turnover of modified HSA in smokers with COPD. In addition the retention of macrophages within the lung tissue and activated release of ROS may have important consequences in the development of COPD.

The data also indicated that inflammation increases the rate of oxidised HSA turnover and also affected the turnover of unmodified HSA. Inflammation is a prominent feature of COPD as shown by the presence in the airways of activated neutrophils and macrophages and increased numbers of inflammatory mediators (Keatings *et al.*, 1997, Bhowmik *et al.*, 2000). It is well documented that HSA is vulnerable to ROS (Davies, 1987). It is therefore not too surprising the data indicates that during inflammation the number of carbonyls on HSA increases and it is turned over more rapidly by the tissue.

If ROS species released from leukocytes in COPD patients are not counter balanced by the lung antioxidant systems, the result is oxidative stress. An excess of

oxidants can potentially lead to enhanced pro-inflammatory gene expression and protein release, inactivation of antiproteinases, and oxidative tissue injury leading to disease progression (Drost *et al.*, 2005). During exacerbations of the disease the inflammatory burden within the lung is heightened and patients experience a significant decline in lung function, which is never fully regained. During these episodes of elevated oxidative stress antioxidants such as HSA are more susceptible to oxidation and potentially are turned over more rapidly. It may therefore be important to consider the potential therapeutic role of HSA in the oxidant/antioxidant balance during exacerbations in these patients.

Finally, to further characterise changes in protein expression with disease we therefore required a sensitive, high throughput technique. Two-dimensional gel-based approaches have been moderately successful in characterising the proteomes of different organisms, tissues and organelles, but are generally insufficient to resolve the very complex proteome of human tissue. Gel based methods are also prone to biases against lower abundance, hydrophobic and lower molecular weight proteins. The need for broad screening techniques of multiple proteins has therefore lead to developments in mass spectrometry and techniques such as iTRAQ, which now allows for the identification of proteins to be carried out rapidly and accurately (Lin *et al.*, 2003).

However we were unsuccessful in further developing iTRAQ to further characterise changes in protein expression in individuals with lung function impairment. In terms of human samples iTRAQ to date has only been used successfully for the analysis of human endometrial tissue (DeSouza *et al.*, 2005), saliva (Hardt et al., 2005) and plasma (Ramstrom *et al.*, 2005). Even for these successful studies the iTRAQ experiments only led to the identification and quantification of 7% of the expected proteins (DeSouza *et al.*, 2005). The major difficulty in sample analysis for all of these studies was the level of abundant proteins for example HSA is present in concentrations corresponding to 50% of the total protein content in plasma. For serum and saliva the use of high affinity depletion

columns for the removal of abundant proteins such as immunoglobulin G and HSA significantly enhances the detection of low abundant proteins. Unfortunately this technology was not available to us and there are also important technical considerations, as depletion columns cannot be used with detergents such as lauryl sulphate (SDS), which is used for homogenising tissues. In addition HSA is able to bind a multitude of endogenous compounds, which could be altered with disease. Removal of HSA would therefore also remove these proteins from the sample being analysed.

Since iTRAQ reagents derivatise primary amine groups they tag virtually all peptides except those lacking both lysine and reactive N-terminal amino acids. In theory iTRAQ should therefore provide far greater proteome coverage, allowing the relative abundance of proteins to be determined, compared to 2D gel electrophoresis-MALDI MS separation. The technique iTRAQ therefore provides great potential for the analysis of protein expression in diseased and naïve tissue, however the proteins elucidated still have to be validated by larger studies using more "traditional" techniques. Multiple marker techniques such as iTRAQ may therefore turn out to be better suited for initial marker discovery.

In summary we have shown that the levels of HSA are decreased in individuals with COPD and current smokers and these individuals also have elevated levels of carbonylated HSA. In addition we have also shown that oxidised HSA is turned over more rapidly in human lung tissue especially in the presence of inflammation. We therefore postulate that the decreased levels of HSA in current smokers and individuals with COPD is due to the elevated metabolic turnover of oxidised HSA, which could play an important role in the oxidant/anti-oxidant balance in these individuals.

Chapter 7.

General discussion

7.0 General discussion

TNFα has previously been shown to play an important role in many chronic inflammatory diseases and is now accepted as an important therapeutic target in rheumatoid arthritis, inflammatory bowel disease and psoriasis (Feldmann *et al.*, 1999, Stuber *et al.*, 1996). COPD is characterised by chronic airway inflammation and it has therefore been suggested that TNFα may well play a role in the disease. Indeed there is literature, which supports the involvement of TNFα in COPD. TNFα has been shown to be elevated in the sputum of patients (Keatings *et al.*, 1996), and TNFα immunoreactivity is increased in the airways of patients with COPD (Muller *et al.*, 1996).

COPD patients also suffer from acute episodes of inflammation termed exacerbations, which are an important cause of morbidity and mortality in COPD patients. It is thought bacterial or viral infections are important factors in COPD exacerbations as they increase the inflammatory burden within the lung (Seemungal *et al*, 2000). The association between bacterial colonisation and COPD has been studied for many years (May, 1953, Marcy *et al.*, 1982, Irwin *et al*, 1982, Rise *et al.*, 1994 and Donaldson *et al.*, 2005). In particular TNFα is found to be associated with bacterial inflammation in COPD, *in vivo* studies have shown chronic bronchitis and COPD patients with a positive bacterial culture for H. *influenzae* have higher concentrations of TNFα in their sputum (Bresser *et al.*, 2000). Similarly, the presence of potentially pathogenic microbes in BAL from COPD patients is strongly associated with elevated levels of TNFα and neutrophilia (Soler *et al.*, 1999, Nash *et al.*, 1991). It is also worth mentioning that TNFα may not only amplify inflammatory events within the airways during acute exacerbations. The relationship between high levels of circulating TNFα (Di Francia *et al.*, 1994) or LPS stimulated increases in TNFα production by blood monocytes (De Gody *et al.*, 1996) and weight

losing COPD patients has been repeatedly reported. Therefore TNFα may well play a role in the systemic inflammation, which is also observed in these severe COPD patients.

However the role of TNFα during episodes of acute inflammation is unknown. This line of research is hindered by the fact that clinical samples such as BAL are rarely taken during these episodes due to severe deterioration of lung function in COPD patients. Therefore it is not possible to study the mediators, which are released during the inflammatory response *in vivo*. In addition, by the time patients present with exacerbations at hospital the initiation of the inflammatory cascade has already occurred. Work in this thesis supports published literature and extends data on LPS stimulated TNFα and its role in acute inflammation. This utilises a model of acute inflammation involving primary culture of human lung tissue from smokers and ex-smokers with a range of lung functions. The advantage of this model is that it allows the analysis of the temporal release of cytokines during acute inflammation, which would be unethical to determine using current bronchial alveolar lavage techniques in COPD patients.

An important finding of this thesis is that LPS stimulated TNFα plays a key role in regulating the acute inflammatory response. TNFα is a potent inflammatory and immunomodulatory cytokine capable of initiating apoptosis, activation of inflammatory cells, tissue remodelling and activation of NF-κB, which in turn co-ordinates transcription of numerous pro-inflammatory cytokines and adhesion molecules (Blackwell *et al.*, 1997). Numerous studies have shown that LPS stimulates TNFα synthesis, however there is little information on the inflammatory events, which follow TNFα release. The immunohistochemistry data in this thesis suggests that both macrophages and mast cells may contribute to TNFα production in our acute inflammatory model. This work is inline with previous studies, which have shown that LPS stimulation leads to the expression of TNFα in both macrophages and mast cells (Vassalli *et al.*, 1992, Skerrett *et al.*, 2004,

Thomas *et al.*, 2001). The expression of TNFα from both of these cells has been attributed to the finding that TLR-4 is expressed on the cell surface of macrophages and mast cells and are therefore able to respond to the presence of LPS (Tackeuchi *et al.*, 1999).

We have demonstrated that TNFα is the first cytokine released in the inflammatory cascade in response to LPS. In addition, neutralisation of the biological activity of TNFα leads to the blockade of the following cytokine cascade. It is therefore important to understand how TNFα could affect the temporal release of mediators in the distinct cytokine cascade. The intracellular signalling pathways leading to NF-κB activation in response to TNFα are well understood (see figure 1.5). However, surprisingly little is known about the downstream gene targets of NF-κB and the kinetics of their induction. Previous work has shown NF-κB controls distinct groups of target genes whose pattern of expression appear to be an orchestrated cascade of early and late phase target gene responses. One study has shown that in macrophages LPS-stimulated recruitment of NF-κB to target genes occurs in two temporally distinct phases (Saccani *et al.*, 2001). A subset of genes whose promoter is already heavily acetylated before stimulation is constitutively and immediately accessible to NF-κB and is transcribed immediately after NF-κB recruitment. In contrast, other target genes are not immediately accessible to NF-κB, and recruitment requires the formation of additional transcription factor complexes. These modifications in chromatin structure could therefore allow the temporal activation of selective genes in response to a single cytokine such as TNFα (Saccani *et al.*, 2001).

Figure 7.1

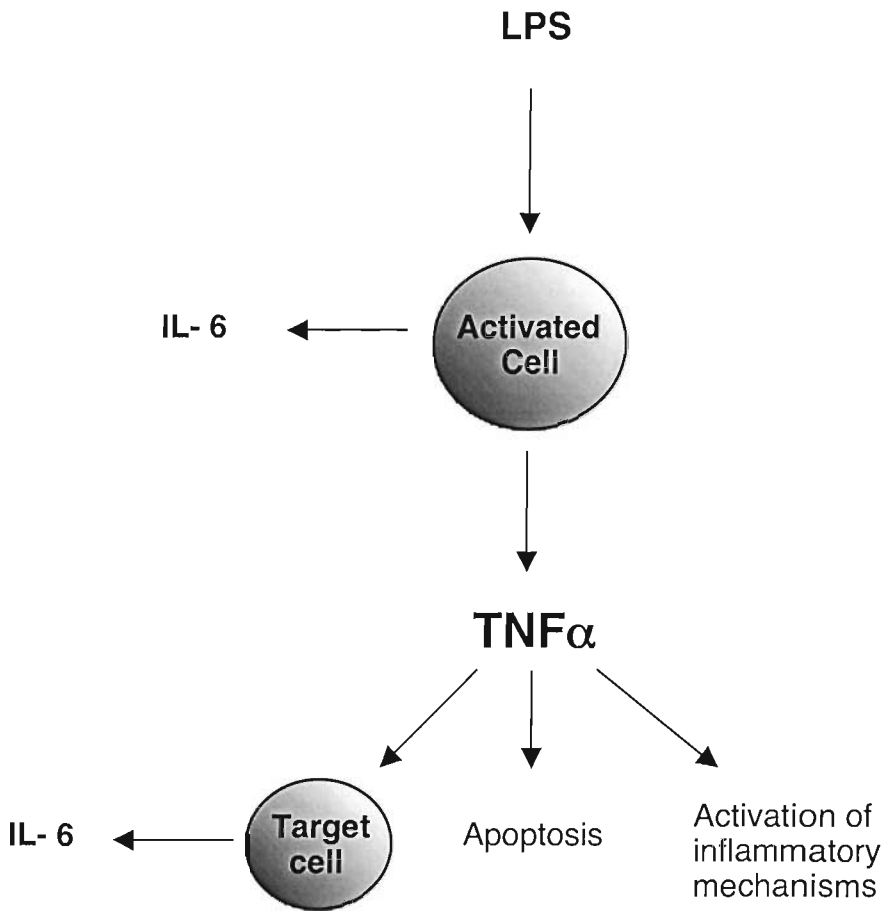


Figure 7.1 TNFα stimulated cytokine release. TNFα released from macrophages and mast cells could therefore stimulate the cell in a paracrine fashion, leading to the release of the other cytokines detected in the inflammatory response. In contrast, a host-generated stimulus, such as TNFα or IL-1, is required for the production of fibroblast and epithelial derived cytokines.

As shown in figure 7.1 TNFα released from macrophages and mast cells could therefore stimulate the cell in a paracrine fashion, leading to the release of the other cytokines detected in the inflammatory response. This is inline with previous gene array studies which indicated that an activating signal such as LPS can induce expression of hundreds of genes in macrophages and that the expression of these genes is differentially regulated by different cytokines (Wells *et al.*, 2003, Williams *et al.*, 2002, Lang *et al.*, 2002).

Cells such as alveolar macrophages and mast cells have the ability to release multiple cytokines in response to an exogenous stimulus such as LPS. In contrast, a host-generated stimulus, such as TNFα or IL-1, is required for the production of fibroblast and epithelial derived cytokines. The latter interaction is demonstrative of cytokine-networking as shown in figure 7.1, whereby one cell population is dependent upon mediators synthesised by a neighbouring cell. Alveolar macrophages are strategically situated at the air tissue interface in the alveoli and alveolar ducts and are therefore the first cells encountered by inhaled organisms and antigens in the lower respiratory tract. This interaction may be extremely important in the lung where the alveolar space is in contact with the ambient environment, which may provide the exogenous stimulus (LPS) for alveolar macrophage-derived cytokines. By its very nature an inflammatory cascade ensures that a single stimulus leads to an amplification of the response to resolve infection. The release of TNFα in response to LPS could potentially provide an important amplification step in the inflammatory response as TNFα receptors are expressed on nearly all cell types. As we have previously demonstrated by the immunohistochemistry in this thesis once LPS stimulated TNFα was released, TNFα staining was found to be associated with multiple cell types. It is therefore likely that multiple cells are recruited in the inflammatory response in either an autocrine or paracrine fashion through TNFα signalling.

The mast cell is not traditionally thought of as having a role in COPD. Mast cells tend to be localised between the epithelium and parenchyma of the respiratory tract, gut and skin. They are therefore among the first of the inflammatory cells to come into contact with invading pathogens. Tissue mast cells have traditionally been defined as key effector cells of immediate-type allergic reactions. They are able to exert their biological effects by releasing preformed and *de novo* synthesised mediators such as histamine, proteases, leukotrienes, prostaglandin and cytokines upon cell activation by antigen-induced IgE

receptor cross-linking (Okayama *et al.*, 2005, Metcalf *et al.*, 1997). There is now increasing evidence that mast cells are also involved in IgE-independent inflammatory and repair processes, which has been illustrated by their ability to be activated by bacterial products and in particular LPS (Metcalfe *et al.*, 1997, Nygen & Dahlen, 1981). Studies have shown that mature mast cells can express Th_1 type cytokines such as TNFα, IL-1β, IL-6 and IL-8. In addition stimulation with IgE-dependant and IgE-independent agonists (LPS) has also been demonstrated to enhance the expression of TNFα (Kempuraj *et al.*, 2003, Xiang *et al.*, 2001, Gibbs *et al.*, 2001). The Dutch hypothesis refers to the idea that COPD is a form of asthma. Smokers have been shown to have elevated levels of serum IgE and many COPD patients show some degree of reversible airway obstruction particularly in the early stages of the disease (Jensen *et al.*, 1992, Burrows *et al.*, 1981). However smoking is not a risk factor for the development of asthma and COPD is typically associated with increased numbers of activated macrophages, neutrophils and CD8+ve T cells (Cosio & Gurassimou, 1999, Keatings *et al.*, 1997, Finkelstein *et al.*, 1995). Therefore in recent years there has been much less emphasis on the Dutch hypothesis, although most clinical research indicates there is some overlap between severe asthma and COPD. Our work therefore indicates that mast cell derived cytokines released in response to IgE-independent stimuli could play an important role in COPD exacerbations.

We have also shown that TNFα is released following cross-linking of the FceR1 with anti-IgE. Mast cells are typically associated with the production of Th_2 type cytokines in allergy and parasite immunity (Okayama *et al.*, 2005, Bentley *et al.*, 1993). It is therefore not too surprising therefore that we also observed the release of Th_2 associated cytokines IL-5 and IL-13 with FcεR1 cross-linking. The capability of mast cells to release both Th_1 and Th_2 cytokines via IgE-dependent mechanisms has only recently come to light (Gibbs *et al.*, 2001, Lorentz *et al.*, 2000). As mast cells are generally thought of as important effector cells in allergic asthma their ability to release Th_1 and Th_2 cytokines

may have important implications in the disease. Allergic asthma is characterised by bronchial hyperresponsiveness (BHR) and variable airflow obstruction, which in moderate disease can be controlled using, inhaled corticosteroids (British Thoracic Society, 2003). However in persistent or severe asthma, which accounts for approximately 10% of patients, continuous oral corticosteroids are only partially effective (Howel *et al.*, 2005). This severe spectrum of the disease exhibits an altered inflammatory cell profile involving neutrophils and represents an important unmet clinical need (Busse *et al.*, 2000, Anon, 2003). Although asthma is considered an eosinophilic disorder associated with Th_2 cytokines the expression of TNFα classically a Th_1 type cytokine is increased in asthmatic airways in proportion to disease severity. Genetic association studies have also shown a strong association between TNFα gene polymorphism and BHR and asthma (Li Kam Wa *et al.*, 1999, Noguchi *et al.*, 2002). In addition inhalation of recombinant TNFα in both rodents and normal or asthmatic humans enhances BHR and sputum neutrophilia. TNFα is therefore considered a new therapeutic target in chronic asthma. A recent study by Howarth and colleagues (2005) has shown in a small open label study, treatment of severe asthmatics with etanercept, a neutralising TNFα antibody, was associated with improvement of asthma symptoms, lung function, and BHR. Our observations therefore provide further evidence for the role of TNFα in severe asthma, and further indicates a need for larger placebo controlled trials of anti-TNFα therapies.

An important clinical finding in this thesis is that the levels of TNFα release were significantly higher in LPS stimulated tissue from patients with COPD (GOLD 1 and 2) compared to individuals with normal lung function (GOLD 0). The exaggerated release of TNFα would ultimately lead to a pro-inflammatory imbalance within the lungs of these patients culminating in the further release of pro-inflammatory cytokines such as IL-6 and IL-8. We have previously shown in the inflammatory cascade TNFα release predicted the

magnitude of the IL-6 and IL-8 response. The finding that the levels of IL-6 and IL-8 are also exaggerated in patients with COPD classified by the GOLD criteria is therefore not too surprising. The release of these cytokines would lead to the recruitment of inflammatory cells and culminate in the destruction of the ECM. As COPD is characterised by chronic inflammation, inflammatory cell recruitment and tissue destruction, resulting in the loss of lung function. Differences in the expression of TNFα may therefore have important implications in the progression of COPD especially during exacerbations.

It is therefore important to elucidate factors, which could be influencing the release of TNFα in COPD. It is possible that the differences in TNFα expression in patients with COPD are genetic or they could be a result of the disease itself. Polymorphisms in the promoter region of the TNFα gene have been implicated in the chronic inflammatory condition rheumatoid arthritis (Rood *et al.*, 2000, Ozen *et al.*, 2002). However in our study we found no relationship between the SNP at base –308 in the TNFα promoter and any of the clinical parameters measured in this study. We have however shown in this study that IL-10 influences LPS stimulated TNFα release, as neutralisation of IL-10 augments the TNFα response. This finding is inline with previous literature, which demonstrates the ability of IL-10 to influence TNFα release (Armstrong *et al.*, 1996, Fiorentino *et al.*, 1991). Therefore the finding of this study that the levels of IL-10 are decreased in COPD patients defined by the GOLD criteria is extremely interesting. IL-10 has recently emerged as an anti-inflammatory cytokine that inhibits the secretion of pro-inflammatory cytokines by monocytes and or macrophages and the release of free oxygen radicals (Bogdan *et al.*, 1991, Gazzinelli *et al.*, 1992, Fiorention *et al.*, 1991). IL-10 can inhibit the synthesis of pro-inflammatory cytokines such as TNFα by inhibiting NF-κB activation and promoting the degradation of cytokine mRNA. Further studies have shown IL-10 inhibits the *in vivo* secretion of TNFα and protects against lethality of endotoxins in a murine model of septic shock if administered before challenging the mice with LPS (Howard *et al.*, 1993, Gerard

et al., 1993). Decreased levels of IL-10 in COPD patients could potentially therefore lead to the elevated TNFα release observed in COPD and ultimately unresolved inflammatory responses to bacteria in exacerbations of the disease. We were therefore interested in the role of the IL-10 promoter in disease progression. Previous work has shown SNP at base -1082 in IL-10 promoter can affect LPS-induced secretion (Tagore *et al.*, 1999, Hajeer *et al.*, 1998). However in our study we found no relationship between SNP -1082 and IL-10 production or any of the clinical parameters. It is possible that other polymorphisms in the IL-10 promoter or IL-10 receptors could be important in IL-10 expression and thus requires further investigation.

Any investigation of protein levels in disease tends to focus on the production of proteins however their metabolism is also important. We have shown that oxidised protein is turned over more rapidly in human lung tissue compared to unmodified protein, and LPS derived inflammation enhances this turnover. In particular we have focused on the decrease of the anti-oxidant HSA and the resultant oxidative damage that could ensue within the airways in COPD. If oxidative stress renders proteins more susceptible to proteolytic degradation by modifying amino acid chains and cleavage of peptide bonds leading to the formation of protein aggregates; the effect of oxidation may well have important consequences for the turnover of other proteins within the disease. In particular we have shown lower levels of IL-10 release in patients with COPD. Numerous studies have shown that oxidative stress is increased in the lungs of patients with COPD compared with healthy smokers (Lenz *et al.*, 1996, Rahman & MacNee, 1996). As we have shown polymorphisms in the IL-10 promoter did not affect IL-10 production, enhanced turnover of IL-10 may be responsible for the decrease seen with disease.

Another factor that influenced TNFα release was smoking status, with current smokers releasing more TNFα compared to ex-smokers. A large body of evidence now exists demonstrating increased oxidative stress in smokers with and without COPD and has

lead to the proposed role of oxidant-antioxidant imbalance in disease progression. Oxidative stress produces not only direct injurious effects in the lungs leading to increased airway epithelial permeability, but also activates molecular mechanisms that initiate lung inflammation. Oxidative stress may therefore play a fundamental role in enhancing inflammation through the up-regulation of redox-sensitive transcription factors, such as NF-κB, activating protein-1 and c-Jun-N-terminal kinase (JNK) to name a few. Cigarette smoke has also been shown to activate all of these signalling systems (Rahman & MacNee, 1998, Di Stefano *et al.*, 2002, MacNee & Rahman, 2000). In particular NF-κB regulates the transcription of the genes for TNFα and many of the inflammatory cytokines such as IL-6 and IL-8. NF-κB in its inactive form is present in the cytosol bound to IκB. Phosphorylation of IκB leading to its subsequent degradation and the release of the activated free form of NF-κB is a redox sensitive reaction (Flohe *et al.*, 1997). Studies in macrophage cell lines and alveolar and bronchial epithelial cells have shown oxidants cause the release of inflammatory mediators such as IL-8, IL-1 and nitric oxide, and these events are associated with increased activation of NF-κB (Parmentier *et al.*, 2000, Jimenez *et al.*, 2000). The enhanced transcription of TNFα with smoking induced oxidative stress could therefore lead to the elevated levels previously observed in this study. However the presence of oxidative stress and its relationship with airflow limitation may well be an epiphenomena, as oxidative stress also occurs as a result of inflammation, a characteristic feature of COPD. Multi variant analysis of our data indicated that smoking status and disease severity are both important independent variables, which can modify the disease. As there are currently no therapeutic treatments to control the decline of lung function in COPD patients, smoking cessation in these individuals is as important as ever.

It is clear the innate immune response during an exacerbation represents an important stage of disease progression. Damage to the airways caused by exacerbations generally occurs in the advanced stages of the disease and is mostly irreversible.

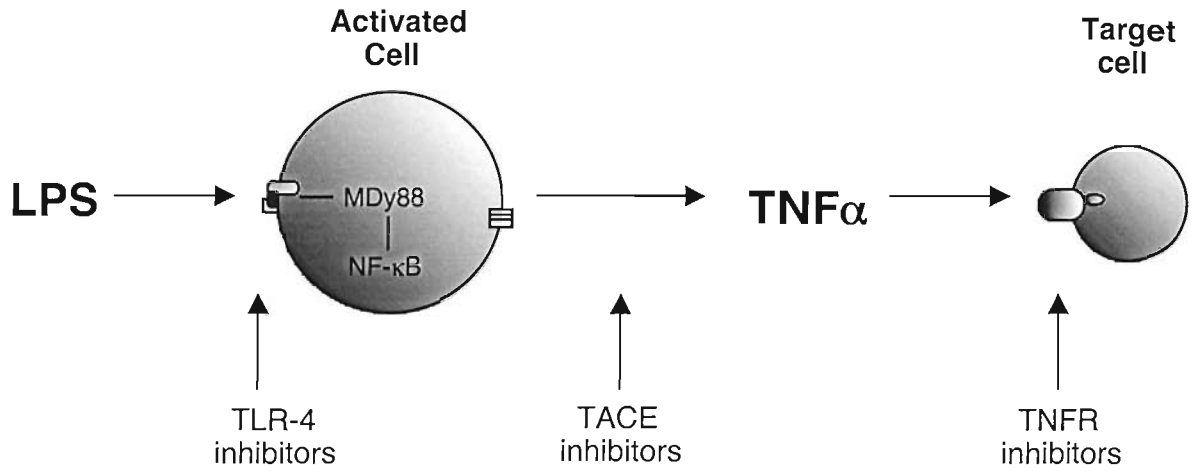
Preventative treatment with flu jabs and steroids has shown some clinical benefits in COPD patients. Ease of diagnosis and awareness of COPD in primary care has also lead to effective recognition of pre-exacerbations symptoms such as cold-like symptoms, and ensuring antibiotics are prescribed rapidly. However there are currently no therapeutic treatments that effectively target airway damage once an exacerbation has developed. An import finding in this study is neutralisation of TNFα leads to cessation of the inflammatory cascade stimulated by LPS. TNFα may therefore represent an important therapeutic target in the treatment of COPD exacerbations where currently no therapies exist. While humanised antibodies such as infliximab have proven efficacious in TNFα driven inflammatory conditions such as rheumatoid arthritis (St Clair *et al.*, 2004) the use of neutralising TNFα in COPD is still to be properly investigated. To date the therapeutic use of infliximab has only been investigated in one small clinical study by Van der Vaart and colleagues (2005) in the Netherlands, which showed no clinical benefits over the 8-week trial. However the Van der Vaart study only included patients classified with mild to moderate COPD.

The use of humanised TNFα antibodies such as infliximab in patients with RA and recently etanercept in severe asthmatics appears to have the greatest clinical effect on a small subset of patients, which tend to be the most severe cases of the respective disease (Howel *et al.*, 2005, Hurlimann *et al.*, 2002). We have shown that the TNFα response to LPS is increased with severity of the COPD classified by the GOLD criteria. In addition patients with severe COPD are noted to have high systemic levels of TNFα, which usually results in cachexia and a rapid decline in health of these patients. The use of anti TNFα antibodies therefore requires much larger clinical trials including patients with a range of disease severity. Also of important note the therapeutic effect of infliximab during exacerbations was also not investigated within the Van der Vaart study. We have shown that TNFα is the key cytokine in LPS derived acute inflammation and without stimulation

we could not detect baseline release of TNFα from the tissue. This data indicates in future clinical trials of humanised monoclonal antibodies it may be more prudent to focus on their therapeutic role in exacerbations of the disease rather than in stable state patients.

However humanised antibodies are associated with a range of side effects from headaches and respiratory distress to increased risk of infections and cancers (Breedveld *et al.*, 2000). The side effects of the humanised antibodies are due mainly to their systemic action on the body as they cannot be specifically targeted to sites of inflammation. Systemic immunosuppression is therefore often an important problem in patients receiving these antibodies, especially with latent tuberculosis. In particular infliximab appears to be associated with greater risks of infection and therefore patients require careful monitoring (Giles & Bathon, 2004). There is also the issue of cost as humanised monoclonal antibodies are expensive to produce. For a disease such as COPD, which is set to rise in prevalence by 2020 worldwide, there is a need for therapeutic options, which are affordable to all. There is therefore a need for well tolerated small molecule inhibitors to modulate TNFα, which need to be targeted to the site of inflammation to minimise systemic effects.

Figure 7.2



We therefore need to consider the sites at which TNFα production can be targeted. Using the diagram in figure 7.2 we can identify the possible points of modulation in TNFα production. LPS initiates the inflammatory response by binding to TLR-4 with LPS binding protein. The development of decoy molecules for the TLR-4 receptor or modulators of the resultant signalling cascade which leads to the activation of transcription factors, could therefore specifically target the LPS-initiated signalling cascade. In particular glucan phosphate has been shown to improve survival in experimental models of sepsis. It is now understood that glucan phosphate treatment affects the TLR mediated MyD88-dependant NF-κB pathway (Li *et al.*, 2004). Further experimental studies with glucan phosphate may therefore lead to the identification of TLR signalling inhibitors.

Once synthesised TNFα is exported to the cell as membrane bound pro-TNFα (26 kDa) which when cleaved by TNFα converting enzyme (TACE) leads to the free soluble form of TNFα (17 kDa). A recent study has successfully converted broad spectrum, sultam-based MMP inhibitors into potent TACE inhibitors, lacking MMP activity (Cherney et al., 2005). The development of TACE inhibitors could therefore represent

another therapeutic avenue for the depletion of biologically active TNFα. Finally TNFα is known to exert its biological effects by binding to TNF-RI and II on the surface of target cells. Soluble TNFα decoy receptors could therefore attenuate TNFα signalling by preventing soluble TNFα binding to target cells. Recent work by Murali and colleagues (2005) have shown targeted conformational perturbation using small molecules that lodge in surface cavities that reside distal to protein-protein interaction sites is an alternative way to modify TNFα receptor functions. This study therefore opens an innovative approach to structure-based drug design of small molecule inhibitors of TNFα

All of the above therapeutic targets would result in the inhibition of the biological activity of TNFα. We therefore have a dichotomy as elevated levels of TNFα may lead to chronic inflammation but inflammation is also required for the resolution of infection. Many existing drugs blanket the effects of proteins by either inhibiting the biological activity or affecting the expression of target proteins. However as we have indicated TNFα has important roles in tissue homeostasis. Therefore in COPD it would appear modulation of TNFα release would provide more therapeutic benefit.

An important clinical finding of this study is the decline in the levels of IL-10 with the severity of disease. We have also shown that neutralisation of the levels of IL-10 actually lead to the augmented release of TNFα in the acute inflammatory response. In support of this finding there is substantial evidence that IL-10 can modulate the release of TNFα via a negative feedback loop, however the mechanism is not completely understood (Armstrong *et al.*, 1996, Fiorentino *et al.*, 1991). As previously mentioned in this discussion it therefore appears that the resolution of the inflammatory response by the release of IL-10 is disturbed in COPD. The therapeutic effect of recombinant human IL-10 has been studied in several inflammatory diseases such as psoriasis, Crohn's disease and rheumatoid arthritis. Overall these studies have shown that IL-10 administered by intravenous or subcutaneous route is well tolerated without serious side effects (Moore et

al., 2001). IL-10 in induced sputum from COPD patients has previously been reported to be significantly lower in comparison to healthy non-smokers (Takanashi et al., 1999). Therapeutic administration of rIL-10 to enhance basal levels in COPD may therefore help to modulate and resolve the chronic inflammation observed in COPD.

Administering rIL-10 specifically to the airways would however be difficult. The administration of aerosolised rIL-10 into the lung would face many challenges before reaching its site of action in the small airways. The majority of drugs administered by inhalers up in the stomach rather than in the airways. Secondly the lung itself is a hostile environment, mucus and proteases within the lung ensure that inhaled particles are degraded and removed as part of the innate defence of the lung. However treatment of COPD patients with rIL-10 administered systemically would probably lead to immunosuppression and fibrosis in tissues where inflammation is not present. Recent evidence has shown that activators and inhibitors of protein kinase C (PKC) and inhibitors of phosphatases PP1 and PP2A allow LPS induced TNFα secretion to be dissociated from IL-10 secretion (Boehringer et al., 1999). Based on this data agents inhibiting PKC and or activating PP1 and PP2A may help to reduce the inflammatory state by increasing the level of IL-10 secretion, while maintaining TNFα release, however further studies are required.

In terms of therapeutic targets we have also demonstrated that the levels of HSA are decreased in both patients with COPD and current smokers. A current hypothesis in the pathogenesis of COPD is that increased oxidant burden both directly as a result of smoking and indirectly by the release of ROS from airspace leukocytes may not be adequately counterbalanced by the lungs antioxidant systems (MacNee 2005). During exacerbations of the disease the oxidant burden is also elevated by heightened inflammation. During these episodes of elevated oxidative stress antioxidants such as HSA are more susceptible to oxidation and are potentially turned over more rapidly. It may therefore be important to consider the potential therapeutic role of HSA in the oxidant/anti-oxidant balance during

exacerbations of these patients. HSA is used clinically to maintain colloid osmotic pressure and is also viewed to serve an antioxidant role in patients with damaged vascular endothelium and patients with acute lung injury (Lang *et al.*, 2002, Quinlan *et al.*, 2004). In fact a direct protective effect of albumin is indicated from many epidemiological studies (Bourdon *et al.*, 1999). As HSA is one of the major antioxidants in the respiratory tract lining fluid, therapeutically enhancing the levels of HSA in the lungs of COPD patients could therefore decrease the oxidative burden reportedly observed in these patients (Bourdan *et al.*, 1999).

In addition we have also demonstrated that HSA from current smokers and patients with COPD is present in a more carbonylated form. The formation of carbonyl groups on amino acid residues as a result of free radical-initiated reactions is well documented as a marker of protein degradation and turnover (Stadman, 1990, Davies, 1987). We therefore hypothesised that the decrease in HSA is the result of enhanced metabolic turnover due to the enhanced oxidation of the protein in COPD patients. A possible mechanism for the accelerated decline in HSA turnover in the type A scavenger receptor (SR-A). One study in particular has shown macrophages adhere to oxidant modified HSA by the SR-A resulting in macrophage retention in the tissue (Haworth, 1997). The SR-A could therefore provide a potential mechanism for the elevated turnover of modified HSA in smokers and COPD patients. In addition binding of oxidised proteins to SR-A triggers macrophage activation and the release of ROS and inflammatory cytokines such as TNFα (Haworth, 1997). Therefore, in addition to the oxidant burden that would ensue with decreased levels of HSA, oxidised HSA may also result in the retention of alveolar macrophages and the elevated release of inflammatory cytokines and ROS. As we have indicated in this thesis the alveolar macrophage is an important source of TNFα in the acute inflammatory response. Elevated levels of the oxidised HSA may therefore have important consequences in the development of COPD.

In an attempt to further identify therapeutic targets in COPD we chose to characterise protein expression in current smokers with and without COPD using iTRAQ. However we were unsuccessful in developing a suitable methodology to analyse human lung tissue using this proteomic approach. Following our research recent technological advances have provided tools such as depletion columns, which may enable the analysis of human tissues with iTRAQ. The technique iTRAQ therefore potentially provides an important proteomic tool for the analysis of protein expression in diseased and naïve tissue, especially for identifying markers of disease.

7.1 Conclusion

It is clear that TNFα has an important role to play in the progression of COPD. Our work confirms this as we have shown that LPS stimulated TNFα is the key cytokine in the acute inflammatory response in human lung tissue. In addition we have demonstrated that the main cellular sources of TNFα in acute inflammation is the alveolar macrophage and mast cell. An important clinical finding of this study is that patients with COPD and current smokers have an exaggerated TNFα response, which would lead to the elevated release of pro-inflammatory mediators and ECM destruction during inflammation. We have also verified that IL-10 can modulate TNFα expression in human lung tissue. The fact that we have demonstrated that patients with COPD express lower levels of IL-10 in response to LPS stimulus is therefore extremely interesting. We have also demonstrated that the decline in protein expression with disease could result from the elevated metabolic turnover due to enhanced oxidation of proteins in COPD patients. In particular we have shown that oxidised HSA, an important antioxidant, is turned over more rapidly in human lung tissue. We have also begun to develop the technique iTRAQ to analyse the changes in protein expression in diseased and naïve human lung tissue. While this approach requires further development iTRAQ potentially provides an important proteomic tool for

identifying markers of disease. This study is therefore important as it has highlighted the role of TNFα and IL-10 in the progression of COPD and therefore provides important future therapeutic targets, which are required to control disease progression.

7.2 Future work

This thesis suggests a number of different directions, which could be potentially pursued and three of those avenues are listed below.

A) We have utilised a human lung tissue model to investigate the release of cytokines in inflammation. This study has shown that TNFα is the key pro-inflammatory cytokine in acute inflammation and is elevated in COPD. It would therefore be important to further define the role of TNFα in acute inflammation. We could disperse the human lung tissue to obtain isolated cell populations in particular macrophages and mast cells. Cell populations would allow us to investigate further the signalling pathways involved in the LPS response. In addition to bacterial infection viruses such as Rhinovirus are also associated with COPD exacerbations. With cell populations we could also study the inflammatory response initiated by rhinovirus. Defining the key signalling events in acute inflammation could help to develop future therapeutic avenues for small molecule inhibitors.

B) During this study we have also shown that IL-10 may have a key anti-inflammatory role in the acute inflammatory response and levels are decreased in COPD. It is therefore important to elucidate the mechanisms behind IL-10 release within diseased and normal lungs. A number of pharmacological agents have been reported to modulate the production of IL-10 in the airways. In particular a number of studies have shown that PDE inhibitors can increase IL-10 production. Using human lung tissue fragments or primary cells obtained from bronchial brushings of stable COPD patients we could investigate IL-10 production in naïve and diseased tissue/cells. Using small molecule

inhibitors such as diacerein, PDE inhibitors and rIL-10 we could also investigate the effect of potential therapeutic treatments in exacerbations.

C) Finally, we have indicated that TNFα and IL-10 release could be important in the resolution of exacerbations. However analysing the release of TNFα and IL-10 in clinical samples such as BAL and bronchial biopsies during exacerbations is unethical in COPD patients. Humanised monoclonal antibodies such as infliximab and etanercept have been used in clinical trials in rheumatoid arthritis, asthma and stable COPD patients with limited side effects. The role of TNFα in exacerbations could therefore be unequivocally answered with a large placebo controlled trial of COPD patients during exacerbation. The study would require rigorous sputum sampling and following patients with diary cards such as in the East London study to accurately diagnose exacerbations and therapeutically treat patients. In addition to the clinical data collected from the study the well characterised sputum samples could also be used analyse the production of TNFα and IL-10 during exacerbations.

Chapter 8.

References

7.0 References

Aaron SD, Angel JB, Lunau M, Wright K, Fex C, Le Saux N, Dales RE. 2001. Granulocyte inflammatory markers and airway infection during acute exacerbation of chronic obstructive pulmonary disease.
Am J Respir Crit Care Med. **163**(2):349-55.

Adler KB, Hendley DD, Davis GS. 1986. Bacteria associated with obstructive pulmonary disease elaborate extracellular products that stimulate mucin secretion by explants of guinea pig airways.
Am J Pathol. **125**(3):501-14.

Ajuebor MN, Das AM, Virag L, Flower RJ, Szabo C, Perretti M. 1999. Role of resident peritoneal macrophages and mast cells in chemokine production and neutrophil migration in acute inflammation: evidence for an inhibitory loop involving endogenous IL-10.
J Immunol. **162**(3):1685-91.

Anderson HR, Limb ES, Bland JM, Ponce de Leon A, Strachan DP, Bower JS. 1991. Health effects of an air pollution episode in London, December 1991.
Thorax. **50**(11):1188-93.

Anderson R, Theron AJ, Ras GJ. 1988. Ascorbic acid neutralizes reactive oxidants released by hyperactive phagocytes from cigarette smokers.
Lung. **166**(3):149-59.

Anraku M, Yamasaki K, Maruyama T, Kragh-Hansen U, Otagiri M. 2001. Effect of oxidative stress on the structure and function of human serum albumin.
Pharm Res. **18**(5):632-9.

Anthonisen NR, Manfreda J, Warren CP, Hershfield ES, Harding GK, Nelson NA. 1987. Antibiotic therapy in exacerbations of chronic obstructive pulmonary disease.
Ann Intern Med. **106**(2):196-204.

Armstrong L, Jordan N, Millar A 1996. Interleukin 10 (IL-10) regulation of tumour necrosis factor alpha (TNF-alpha) from human alveolar macrophages and peripheral blood monocytes.
Thorax. **51**(2):143-9.

Arinir U, Klein W, Rohde G, Stemmler S, Epplen JT, Schultze-Werninghaus G. 2005. Polymorphisms in the interleukin-8 gene in patients with chronic obstructive pulmonary disease.
Electrophoresis. **26**(15):2888-91

ATS Standards for the diagnosis and care of patients with COPD. 1995. Definitions, epideminology, pathophysiology and staging.
Am. J. Resp. Care Med. **152**: s78-s83.

Barnes P.J. 1998. Chronic obstructive pulmonary disease: New opportunities for drug development.
Trends in pharmacol. Sci. **19**:415-423

Barnes P.J. 1999. Epidemiology and mechanisms. *Managing chronic obstructive disease pulmonary disease* **Ch 1**: Science Press: 1-18

Barnes PJ. 2001. Cytokine modulators as novel therapies for airway disease. *Eur Respir J Suppl.* **34**:67s-77s

Baud V, Karin M. 2001. Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol.* **11**(9):372-7

Becker MN, Diamond G, Verghese MW, Randell SH 2000. CD14-dependent lipopolysaccharide-induced beta-defensin-2 expression in human tracheobronchial epithelium.
J Biol Chem. **22**;275(38):29731-6

Becklake MR. 1989. Occupational exposures: evidence for a causal association with chronic obstructive pulmonary disease.
Am Rev Respir Dis. **140**(3 Pt 2):S85-91

Bentley AM, Meng Q, Robinson DS, Hamid Q, Kay AB, Durham SR. 1993. Increases in activated T lymphocytes, eosinophils, and cytokine mRNA expression for interleukin-5 and granulocyte/macrophage colony-stimulating factor in bronchial biopsies after allergen inhalation challenge in atopic asthmatics.
Am J Respir Cell Mol Biol. **8**(1):35-42

Bentley AM, Hamid Q, Robinson DS, Schotman E, Meng Q, Assoufi B, Kay AB, Durham SR. 1993. Prednisolone treatment in asthma. Reduction in the numbers of eosinophils, T cells, tryptase-only positive mast cells, and modulation of IL-4, IL-5, and interferon-gamma cytokine gene expression within the bronchial mucosa
Am J Respir Cell Mol Biol. **8**(1):35-42.

Betsuyaku T, Shipley JM, Liu Z, Senior RM. 1999. Neutrophil emigration in the lungs, peritoneum, and skin does not require gelatinase B.
Am J Respir Cell Mol Biol. **20**(6):1303-9

Beutler B. 1995. TNF, immunity and inflammatory disease: Lessons of the past decade. *J Invest Med.* **43**:227–235.

Bischoff SC, Brunner T, De Weck AL, Dahinden CA. 1990. Interleukin 5 modifies histamine release and leukotriene generation by human basophils in response to diverse agonists.
J Exp Med. **1**;172(6):1577-82

Bhowmik A, Seemungal TA, Sapsford RJ, Devalia JL, Wedzicha JA. 1998. Comparison of spontaneous and induced sputum for investigation of airway inflammation in chronic obstructive pulmonary disease.
Thorax. **53**(11):953-6.

Bhowmik A, Seemungal TA, Sapsford RJ, Wedzicha JA. 2000. Relation of sputum inflammatory markers to symptoms and lung function changes in COPD exacerbations.
Thorax. **55**(2):114-20

Biragyn A, Nedospasov SA. 1995. Lipopolysaccharide-induced expression of TNF-alpha gene in the macrophage cell line ANA-1 is regulated at the level of transcription processivity.
J Immunol. **155**(2):674-83

Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, Stocking KL, Reddy P, Srinivasan S, Nelson N, Boiani N, Schooley KA, Gerhart M, Davis R, Fitzner JN, Johnson RS, Paxton RJ, March CJ, Cerretti DP 1997. A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells.
Nature **20**;385(6618):729-33.

Blackwell TS, Christman JW. 1997. The role of nuclear factor-kappa B in cytokine gene regulation.
Am J Respir Cell Mol Biol. **17**(1):3-9

Blake GJ, Ridker PM. 2001. Novel clinical markers of vascular wall inflammation.
Circ Res. **89**(9):763-71

Bogdan C, Vodovotz Y, Nathan C. 1991. Macrophage deactivation by interleukin 10.
J Exp Med. **174**(6):1549-55.

Bonfield TL, Konstan MW, Burfeind P, Panuska JR, Hilliard JB, Berger M. 1995. Normal bronchial epithelial cells constitutively produce the anti-inflammatory cytokine interleukin-10, which is downregulated in cystic fibrosis.
Am J Respir Cell Mol Biol. **13**(3):257-61

Bourdon E, Loreau N, Blache D. 1999. Glucose and free radicals impair the antioxidant properties of serum albumin.
FASEB J. **13**(2):233-44.

Border WA, Noble NA. 1999. Transforming growth factor beta in tissue fibrosis.
N Engl J Med. **331**(19):1286-92

Borish L, Aarons A, Rumbyrt J, Cvietusa P, Negri J, Wenzel S. Interleukin-10 regulation in normal subjects and patients with asthma.
J Allergy Clin Immunol. 1996 Jun;97(6):1288-96.

Borregaard N, Kjeldsen L, Sengelov H, Diamond MS, Springer TA, Anderson HC, Kishimoto TK, Bainton DF. 1994. Changes in subcellular localization and surface expression of L-selectin, alkaline phosphatase, and Mac-1 in human neutrophils during stimulation with inflammatory mediators.
J Leukoc Biol. **56**(1):80-7.

Bosken C.H., Hards J., Gatter K., Hogg J.C. 1992. Characterisation of the inflammatory response in peripheral airways of current smokers using immunohistochemistry.
Am. Rev. Resp. Dis. **145**:911-917.

Brasier AR, Li J. 1996. Mechanisms for inducible control of angiotensinogen gene transcription.
J Hypertens. **27**(2):465–475.

Breedveld FC. 2000. Therapeutic monoclonal antibodies.
Lancet. **355**(9205):735-40.

Bresser P, Out TA, van Alphen L, Jansen HM, Lutter R. 2000. Airway inflammation in nonobstructive and obstructive chronic bronchitis with chronic haemophilus influenzae airway infection. Comparison with noninfected patients with chronic obstructive pulmonary disease.
Am J Respir Crit Care Med. **162**(3 Pt 1):947-52.

Brewster CE, Howarth PH, Djukanovic R, Wilson J, Holgate ST, Roche WR. 1990. Myofibroblasts and subepithelial fibrosis in bronchial asthma.
Am J Respir Cell Mol Biol. **3**(5):507-11.

Buist A.S. 1984. Current status of small airways disease. *Chest*. **86**:100-105

Burd PR, Thompson WC, Max EE, Mills FC. 1995. Activated mast cells produce interleukin 13.
J Exp Med. **181**(4):1373-80.

Burge PS, Calverley PM, Jones PW, Spencer S, Anderson JA, Maslen TK. 2000. Randomised, double blind, placebo controlled study of fluticasone propionate in patients with moderate to severe chronic obstructive pulmonary disease: the ISOLDE trial.
BMJ. ;**320**(7245):1297-303

Burrows B, Halonen M, Barbee RA, Lebowitz MD. 1981. The relationship of serum immunoglobulin E to cigarette smoking.
Am Rev Respir Dis. **124**(5):523-5.

Buss H, Chan TP, Sluis KB, Domigan NM, Winterbourn CC. 1997. Protein carbonyl measurement by a sensitive ELISA method.
Free Radic Biol Med. **23**(3):361-6.

Cabello H, Torres A, Celis R, El-Ebiary M, Puig de la Bellacasa J, Xaubet A, Gonzalez J, Agusti C, Soler N. 1997. Bacterial colonization of distal airways in healthy subjects and chronic lung disease: a bronchoscopic study.
Eur Respir J. **10**(5):1137-44.

Carilli AD, Gohd RS, Gordon W. 1964. A virological study of chronic bronchitis.
N Engl J Med. **16**;270:123-7

Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B. 1975. An endotoxin-induced serum factor that causes necrosis of tumors.
Proc Natl Acad Sci U S A. **72**(9):3666-70.

Cassatella MA, Meda L, Bonora S, Ceska M, Constantin G. 1993. Interleukin 10 (IL-10) inhibits the release of proinflammatory cytokines from human polymorphonuclear leukocytes. Evidence for an autocrine role of tumor necrosis factor and IL-1 beta in mediating the production of IL-8 triggered by lipopolysaccharide.
J Exp Med. **1**;178(6):2207-11.

Cassatella MA, Tamassia N, Crepaldi L, McDonald PP, Ear T, Calzetti F, Gasperini S, Zanderigo F, Bazzoni F. 2005. Lipopolysaccharide primes neutrophils for a rapid response to IL-10.
Eur J Immunol. **35**(6):1877-85.

Chanez P, Vignola AM, O'Shaugnessy T, Enander I, Li D, Jeffery PK, Bousquet J. 1997. Corticosteroid reversibility in COPD is related to features of asthma.
Am J Respir Crit Care Med. **155**(5):1529-34.

Chow JC, Young DW, Golenbock DT, Christ WJ, Gusovsky F. 1999. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction.
J Biol Chem. **274**(16):10689-92.

Chung KF. 2001. Cytokines in chronic obstructive pulmonary disease.
Eur Respir J **34**:50s-59s

Clutterbuck E, Shields JG, Gordon J, Smith SH, Boyd A, Callard RE, Campbell HD, Young IG, Sanderson CJ. 1987. Recombinant human interleukin 5 is an eosinophil differentiation factor but has no activity in standard human B cell growth factor assays.
Eur J Immunol. **17**(12):1743-50

Connors AF Jr, Dawson NV, Thomas C, Harrell FE Jr, Desbiens N, Fulkerson WJ, Kussin P, Bellamy P, Goldman L, Knaus WA. 1996. Outcomes following acute exacerbation of severe chronic obstructive lung disease. The SUPPORT investigators (Study to Understand Prognoses and Preferences for Outcomes and Risks of Treatments)
Am J Respir Crit Care Med. **154**(4 Pt 1):959-67. *Erratum in: Am J Respir Crit Care Med* **1997 155**(1):386

Cosio M, Ghezzo H, Hogg JC, Corbin R, Loveland M, Dosman J, Macklem PT. 1978. The relations between structural changes in small airways and pulmonary-function tests.
N Engl J Med. **8**;298(23):1277-81.

Crestani B, Aubier M. 1998. Inflammatory role of alveolar epithelial cells.
Kidney Int Suppl. **65**:S88-93

Cromwell O, Hamid Q, Corrigan CJ, Barkans J, Meng Q, Collins PD, Kay AB. 1992. Expression and generation of interleukin-8, IL-6 and granulocyte-macrophage colony-stimulating factor by bronchial epithelial cells and enhancement by IL-1 beta and tumour necrosis factor-alpha.
Immunology. **77**(3):330-7

Culpitt SV, Rogers DF, Traves SL, Barnes PJ, Donnelly LE. 2005. Sputum matrix metalloproteases: comparison between chronic obstructive pulmonary disease and asthma.
Respir Med. **99**(6):703-10. Epub 2004 Dec 13.

Davies KJ, Delsignore ME. Protein damage and degradation by oxygen radicals. III. Modification of secondary and tertiary structure.
J Biol Chem. **262**(20):9908-13.

de Boer WI, van Schadewijk A, Sont JK, Sharma HS, Stolk J, Hiemstra PS, van Krieken JH. 1998. Transforming growth factor beta1 and recruitment of macrophages and mast cells in airways in chronic obstructive pulmonary disease.
Am J Respir Crit Care Med. **158**(6):1951-7.

da Silva Correia J, Ulevitch RJ. 2002 MD-2 and TLR4 N-linked glycosylations are important for a functional lipopolysaccharide receptor.
J Biol Chem. **277**(3):1845-54

Debord P, Louchahi K, Tod M, Cournot A, Perret G, Petitjean O. 1994. Influence of renal function on the pharmacokinetics of diacerein after a single oral dose.
Eur J Drug Metab Pharmacokinet. **19**(1):13-9.

Delclaux C, Azoulay E. 2003. Inflammatory response to infectious pulmonary injury.
Eur Respir J Suppl. **42**:10s-14s.

DeSouza L, Diehl G, Rodrigues MJ, Guo J, Romaschin AD, Colgan TJ, Siu KW. 2005. Search for cancer markers from endometrial tissues using differentially labeled tags iTRAQ and cICAT with multidimensional liquid chromatography and tandem mass spectrometry.
J Proteome Res. **4**(2):377-86

Di Francia M, Barbier D, Mege JL, Orehek J. 1994. Tumor necrosis factor-alpha levels and weight loss in chronic obstructive pulmonary disease.
Am J Respir Crit Care Med. **150**(5 Pt 1):1453-5.

Di Stefano A, Capelli A, Lusuardi M, Balbo P, Vecchio C, Maestrelli P, Mapp CE, Fabbri LM, Donner CF, Saetta M. 1998. Severity of airflow limitation is associated with severity of airway inflammation in smokers.
Am J Respir Crit Care Med. **158**(4):1277-85

Donaldson GC, Seemungal T, Jeffries DJ, Wedzicha JA. 1999. Effect of temperature on lung function and symptoms in chronic obstructive pulmonary disease.
Eur Respir J. **13**(4):844-9.

Donaldson GC, Seemungal TA, Bhowmik A, Wedzicha JA. 2002. Relationship between exacerbation frequency and lung function decline in chronic obstructive pulmonary disease
Thorax. **57**(10):847-52.

Donaldson GC, Seemungal TA, Patel IS, Bhowmik A, Wilkinson TM, Hurst JR, Maccallum PK, Wedzicha JA. 2005. Airway and systemic inflammation and decline in lung function in patients with COPD.
Chest. **128**(4):1995-2004.

Dougados M, Nguyen M, Berdah L, Mazieres B, Vignon E, Lequesne M. 2001. Evaluation of the structure-modifying effects of diacerein in hip osteoarthritis: ECHODIAH, a three-year, placebo-controlled trial. Evaluation of the Chondromodulating Effect of Diacerein in OA of the Hip.
Arthritis Rheum. **44**(11):2539-47.

Douni E, Sfikakis PP, Haralambous S, Fernandes P, Kollias G. 2004. Attenuation of inflammatory polyarthritis in TNF transgenic mice by diacerein: comparative analysis with dexamethasone, methotrexate and anti-TNF protocols.
Arthritis Res Ther. **6**(1):R65-R72.

Drannik AG, Pouladi MA, Robbins CS, Goncharova SI, Kianpour S, Stampfli MR. 2004. Impact of cigarette smoke on clearance and inflammation after Pseudomonas aeruginosa infection.
Am J Respir Crit Care Med. **170**(11):1164-71

Drost EM, Skwarski KM, Sauleda J, Soler N, Roca J, Agusti A, MacNee W. 2005. Oxidative stress and airway inflammation in severe exacerbations of COPD.
Thorax. **60**(4):293-300.

Dunsmore SE, Saarialho-Kere UK, Roby JD, Wilson CL, Matrisian LM, Welgus HG, Parks WC. 1998. Matrilysin expression and function in airway epithelium.
J Clin Invest. 1998 Oct 1;102(7):1321-31.

Duryee MJ, Freeman TL, Willis MS, Hunter CD, Hamilton BC 3rd, Suzuki H, Tuma DJ, Klassen LW, Thiele GM. 2005. Scavenger receptors on sinusoidal liver endothelial cells are involved in the uptake of aldehyde-modified proteins.
Mol Pharmacol. **68**(5):1423-30

Duryee MJ, Freeman TL, Willis MS, Hunter CD, Hamilton BC 3rd, Suzuki H, Tuma DJ, Klassen LW, Thiele GM. 2005. Scavenger receptors on sinusoidal liver endothelial cells are involved in the uptake of aldehyde-modified proteins.
Mol Pharmacol. **68**(5):1423-30

Duthie GG, Arthur JR, Beattie JA, Brown KM, Morrice PC, Robertson JD, Shortt CT, Walker KA, James WP. 1993. Cigarette smoking, antioxidants, lipid peroxidation, and coronary heart disease.
Ann N Y Acad Sci. **686**:120-9

el Khoury J, Thomas CA, Loike JD, Hickman SE, Cao L, Silverstein SC. 1994. Macrophages adhere to glucose-modified basement membrane collagen IV via their scavenger receptors.
J Biol Chem. **269**(14):10197-200.

Evans MD, Pryor WA. 1994. Cigarette smoking, emphysema, and damage to alpha 1-proteinase inhibitor.
Am J Physiol. **266**(6 Pt 1):L593-611

Fagon JY, Chastre J, Trouillet JL, Domart Y, Dombret MC, Bornet M, Gibert C. 1990. Characterization of distal bronchial microflora during acute exacerbation of chronic bronchitis. Use of the protected specimen brush technique in 54 mechanically ventilated patients.
Am Rev Respir Dis. **142**(5):1004-8.

Fahy JV, Kim KW, Liu J, Boushey HA. 1995. Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation.
J Allergy Clin Immunol. **95**(4):843-52.

Falgarone G, Dougados M. 2001. Diacerein as a disease-modulating agent in osteoarthritis.
Curr Rheumatol Rep. **3**(6):479-83.

Feldmann M, Brennan FM, Foxwell BM, Maini RN. 2001. The role of TNF alpha and IL-1 in rheumatoid arthritis.
Curr Dir Autoimmun. **3**:188-99

Fenton MJ, Golenbock DT. 1998. LPS-binding proteins and receptors.
J Leukoc Biol. **64**(1):25-32

Finlay GA, O'Driscoll LR, Russell KJ, D'Arcy EM, Masterson JB, FitzGerald MX, O'Connor CM. 1997. Matrix metalloproteinase expression and production by alveolar macrophages in emphysema.
Am J Respir Crit Care Med. **156**(1):240-7

Fletcher C.M., Pride M.B. 1984. Definition of emphysema, chronic bronchitis, asthma and airflow obstrction: twenty five years from the CIBRA symposium.
Thorax. **39**:81-85

Fletcher C, Peto R. 1977. The natural history of chronic airflow obstruction.
Br Med J. **25**;1(6077):1645-8.

Fletcher CM, Pride NB. 1984. Definitions of emphysema, chronic bronchitis, asthma, and airflow obstruction: 25 years on from the Ciba symposium.
Thorax. **39**(2):81-5

Flood-Page P, Menzies-Gow A, Phipps S, Ying S, Wangoo A, Ludwig MS, Barnes N, Robinson D, Kay AB. 2003. Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics.
J Clin Invest. **112**(7):1029-36.

Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A. 1991. IL-10 inhibits cytokine production by activated macrophages.
J Immunol. **147**(11):3815-22.

Ford-Hutchinson AW, Bray MA, Doig MV, Shipley ME, Smith MJ. 1980. Leukotriene B, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes.
Nature. **17**;286(5770):264-5

Foreman RC, Mercer PF, Kroegel C, Warner JA. 1999. Role of the eosinophil in protein oxidation in asthma: possible effects on proteinase/antiproteinase balance.
Int Arch Allergy Immunol. **118**(2-4):183-6.

Foster PS, Hogan SP, Yang M, Mattes J, Young IG, Matthaei KI, Kumar RK, Mahalingam S, Webb DC. 2002. Interleukin-5 and eosinophils as therapeutic targets for asthma.
Trends Mol Med. **8**(4):162-7.

Galli SJ, Gordon JR, Wershil BK. 1993. Mast cell cytokines in allergy and inflammation.
Agents Actions Suppl. **43**:209-20

Gazzinelli RT, Oswald IP, James SL, Sher A. 1992. IL-10 inhibits parasite killing and nitrogen oxide production by IFN-gamma-activated macrophages.
J Immunol. **148**(6):1792-6

Gazzinelli RT, Wysocka M, Hieny S, Scharton-Kersten T, Cheever A, Kuhn R, Muller W, Trinchieri G, Sher A. 1996. In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha.
J Immunol. 157(2):798-805

Geiger T, Andus T, Klapproth J, Hirano T, Kishimoto T, Heinrich PC 1988. Induction of rat acute-phase proteins by interleukin 6 in vivo.
Eur J Immunology **18**: 717-721

Gelb AF, Hogg JC, Muller NL, Schein MJ, Kuei J, Tashkin DP, Epstein JD, Kollin J, Green RH, Zamel N, Elliott WM, Hadjiaghai L. 1996. Contribution of emphysema and small airways in COPD.
Chest. **109**(2):353-9.

Gerard C, Bruyns C, Marchant A, Abramowicz D, Vandenabeele P, Delvaux A, Fiers W, Goldman M, Velu T. 1993. Interleukin 10 reduces the release of tumor necrosis factor and prevents lethality in experimental endotoxemia.
J Exp Med. **177**(2):547-50

Gerritsen WB, Asin J, Zanen P, van den Bosch JM, Haas FJ. 2005. Markers of inflammation and oxidative stress in exacerbated chronic obstructive pulmonary disease patients.
Respir Med. **99**(1):84-90.

Gibbs BF, Wierecky J, Welker P, Henz BM, Wolff HH, Grabbe J. 2001. Human skin mast cells rapidly release preformed and newly generated TNF-alpha and IL-8 following stimulation with anti-IgE and other secretagogues.
Exp Dermatol. **10**(5):312-20.

Giles JT, Bathon JM. 2004. Serious infections associated with anticytokine therapies in the rheumatic diseases.
J Intensive Care Med. **19**(6):320-34

Gomez FP, Rodriguez-Roisin R. 2002. Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines for chronic obstructive pulmonary disease.
Current Opinion in Pulmonary Medicine. 8(2):81-86

GOLD Global stratergy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease 2001: National Heart, Lung, and Blood Institue and World Health Organisation Global Initiative for Chronic obstructive pulmonary disease: executive summary.
Respiratory care 46(8):798-825

GOLD guidelines. 2004: GOLD guide to COPD diagnosis, management and prevention . http://www.goldcopd.org

Greenberg SB, Allen M, Wilson J, Atmar RL. 2000. Respiratory viral infections in adults with and without chronic obstructive pulmonary disease.
Am J Respir Crit Care Med. **162**(1):167-73

Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, Sheppard D, Mohrs M, Donaldson DD, Locksley RM, Corry DB. 1998. Requirement for IL-13 independently of IL-4 in experimental asthma.
Science. **282**(5397):2261-3

Grune T, Davies KJ. 1997. Breakdown of oxidized proteins as a part of secondary antioxidant defenses in mammalian cells.
Biofactors. **6**(2):165-72

Gurney JW, Jones KK, Robbins RA, Gossman GL, Nelson KJ, Daughton D, Spurzem JR, Rennard SI. 1992. Regional distribution of emphysema: correlation of high-resolution CT with pulmonary function tests in unselected smokers.
Radiology. **183**(2):457-63.

Gump DW, Phillips CA, Forsyth BR, McIntosh K, Lamborn KR, Stouch WH. 1976. Role of infection in chronic bronchitis.
Am Rev Respir Dis. **113**(4):465-74

Haddad JJ. 2002. Recombinant TNF-alpha mediated regulation of the I kappa B-alpha/NF-kappa B signaling pathway: evidence for the enhancement of pro- and anti-inflammatory cytokines in alveolar epithelial cells.
Cytokine. **21**;17(6):301-10.

Hajeer AH, Lazarus M, Turner D, Mageed RA, Vencovsky J, Sinnott P, Hutchinson IV, Ollier WE. 1998. IL-10 gene promoter polymorphisms in rheumatoid arthritis.
Scand J Rheumatol. **27**(2):142-5

Hastings R.H., Folkesson H.G.,Petersen V., Ciriales R., Matthay M.A. 1995. Cellular uptake of albumin from lungs of anesthetized rabbits.
Am. J. Physiol. **269**:L453-L462.

Halliwell B. 1988. Albumin- an important extracellular antioxidant?
Biochem. Pharmacol. **37**:569-571.

Hanano T, Adachi K, Aoki Y, Morimoto H, Naka Y, Hisadome M, Fukuda T, Sumichika H. 2000. Novel phenylpiperazine derivatives as dual cytokine regulators with TNF-alpha suppressing and IL-10 augmenting activity.
Bioorg Med Chem Lett. **1**;10(9):875-9.

Hardt M, Witkowska HE, Webb S, Thomas LR, Dixon SE, Hall SC, Fisher SJ. 2005. Assessing the effects of diurnal variation on the composition of human parotid saliva: quantitative analysis of native peptides using iTRAQ reagents.
Anal Chem. **77**(15):4947-54

Haro H, Crawford HC, Fingleton B, Shinomiya K, Spengler DM, Matrisian LM. 2000. Matrix metalloproteinase-7-dependent release of tumor necrosis factor-alpha in a model of herniated disc resorption.
J Clin Invest. **105**(2):143-50.

Hatada EN, Krappmann D, Scheidereit C. 2000. NF-kappaB and the innate immune response.
Curr Opin Immunol. **12**(1):52-8.

Haworth R, Platt N, Keshav S, Hughes D, Darley E, Suzuki H, Kurihara Y, Kodama T, Gordon S. 1997. The macrophage scavenger receptor type A is expressed by activated macrophages and protects the host against lethal endotoxic shock.
J Exp Med. **186**(9):1431-9.

Hegele RG, Hayashi S, Hogg JC, Pare PD. 1995. Mechanisms of airway narrowing and hyperresponsiveness in viral respiratory tract infections.
Am J Respir Crit Care Med. **151**(5):1659-64; discussion 1664-5. Review

Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F. 2003. Principles of interleukin (IL)-6-type cytokine signalling and its regulation.
Biochem J. **374**(Pt 1):1-20

Henricson BE, Manthey CL, Perera PY, Hamilton TA, Vogel SN. 1993. Dissociation of lipopolysaccharide (LPS)-inducible gene expression in murine macrophages pretreated with smooth LPS versus monophosphoryl lipid A.
Infect Immun. **61**(6):2325-33.

Higashimoto Y, Yamagata Y, Iwata T, Okada M, Ishiguchi T, Sato H, Masuda M, Itoh H. 2005. Increased serum concentrations of tissue inhibitor of metalloproteinase-1 in COPD patients.
Eur Respir J. **25**(5):885-90.

Himmelfarb J, McMonagle E. 2001. Albumin is the major plasma protein target of oxidant stress in uremia.
Kidney Int. **60**(1):358-63.

Hirano S. 1997. Quantitative time-course profiles of bronchoalveolar lavage cells following intratracheal instillation of lipopolysaccharide in mice.
Ind Health. **35**(3):353-8.

Hill AT, Campbell EJ, Hill SL, Bayley DL, Stockley RA. 2000. Association between airway bacterial load and markers of airway inflammation in patients with stable chronic bronchitis.
Am J Med. **109**(4):288-95.

Hirschman JV. 2000. Do bacteria cause exacerbations of COPD.
Chest **118**:193–203

Hogg JC 2004. Pathophysiology of airflow limitation in chronic obstructive pulmonary disease.
Lancet. 364(9435);709-721.

Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S. 1999. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product.
J Immunol. **162**(7):3749-52.

Howard M, Muchamuel T, Andrade S, Menon S. 1993. Interleukin 10 protects mice from lethal endotoxemia.
J Exp Med. **177**(4):1205-8

Howarth PH, Babu KS, Arshad HS, Lau L, Buckley M, McConnell W, Beckett P, Al Ali M, Chauhan A, Wilson SJ, Reynolds A, Davies DE, Holgate ST. 2005. Tumour necrosis factor (TNFalpha) as a novel therapeutic target in symptomatic corticosteroid dependent asthma.
Thorax. **60**(12):1012-8

Howell WM, Turner SJ, Collins A, Bateman AC, Theaker JM. 2002. Influence of TNFalpha and LTalpha single nucleotide polymorphisms on susceptibility to and prognosis in cutaneous malignant melanoma in the British population.
*Eur J Immunogenet.*29(1):17-23.

Hughes BJ, Hollers JC, Crockett-Torabi E, Smith CW. Recruitment of CD11b/CD18 to the neutrophil surface and adherence-dependent cell locomotion.
J Clin Invest. **90**(5):1687-96.

Hurlimann D, Forster A, Noll G, Enseleit F, Chenevard R, Distler O, Bechir M, Spieker LE, Neidhart M, Michel BA, Gay RE, Luscher TF, Gay S, Ruschitzka F. 2002. Anti-tumor necrosis factor-alpha treatment improves endothelial function in patients with rheumatoid arthritis.
Circulation. **22**;106(17):2184-7.

Irwin RS, Erickson AD, Pratter MR, Corrao WM, Garrity FL, Myers JR, Kaemmerlen JT. 1982. Prediction of tracheobronchial colonization in current cigarette smokers with chronic obstructive bronchitis.
J Infect Dis. **145**(2):234-41.

Imai K, Dalal SS, Chen ES, Downey R, Schulman LL, Ginsburg M, D'Armiento J. 2001. Human collagenase (matrix metalloproteinase-1) expression in the lungs of patients with emphysema.
Am J Respir Crit Care Med. **163**(3 Pt 1):786-91.

Javelaud D, Wietzerbin J, Delattre O, Besancon F 2000.
Induction of p21Waf1/Cip1 by TNFalpha requires NF-kappaB activity and antagonizes apoptosis in Ewing tumor cells.
Oncogene. **6**;19(1):61-8.

Jeffery PK. 1998. Structural and inflammatory changes of COPD: a comparison with asthma.
Thorax. **53**: 129-136 Chest.

Jensen EW, Espersen K, Kanstrup IL, Christensen NJ. 1995. Basal and exercise-induced changes in plasma catecholamines: relationship to age, smoking habits and sampling times.
Clin Physiol. **15**(4):299-305.

Jiang Q, Akashi S, Miyake K, Petty HR. 2000. Lipopolysaccharide induces physical proximity between CD14 and toll-like receptor 4 (TLR4) prior to nuclear translocation of NF-kappa B.
J Immunol. **165**(7):3541-4.

Kaartinen M, Penttila A, Kovanen PT. 1996. Mast cells in rupture-prone areas of human coronary atheromas produce and store TNF-alpha.
Circulation. **94**(11):2787-92

Kaliner MA 1991. Human nasal respiratory secretions and host defense.
Am Rev Respir Dis. 144(3 Pt 2):S52-6.

Kanner R.E., Renzetti A.D. Jr., Klauber M.R. 1979. Variables associated with changes in spirometry in patients with obstructive lung diseases.
Am J Med;**67**:44-50.

Kanner R.E., Anthonisen N.R., Connett J.E. 2001. Lower respiratory illnesses promote FEV_1 decline in current smokers but not ex-smokers with mild chronic obstructive pulmonary disease. Results from the lung health study.
Am J Respir Crit Care Med;**164**:358-64.

Kauffmann F, Drouet D, Lellouch J, Brille D. 1979. Twelve years spirometric changes among Paris area workers.
Int J Epidemiol. **8**(3):201-12

Keatings VM, Collins PD, Scott DM, Barnes PJ. 1996. Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma.
Am J Respir Crit Care Med. **153**(2):530-4

Kelk P, Claesson R, Hanstrom L, Lerner UH, Kalfas S, Johansson A. 2005. Abundant secretion of bioactive interleukin-1beta by human macrophages induced by Actinobacillus actinomycetemcomitans leukotoxin.
Infect Immun. **73**(1):453-8.

Kempuraj D, Huang M, Kandere-Grzybowska K, Basu S, Boucher W, Letourneau R, Athanassiou A, Theoharides TC. 2003. Azelastine inhibits secretion of IL-6, TNF-alpha and IL-8 as well as NF-kappaB activation and intracellular calcium ion levels in normal human mast cells.
Int Arch Allergy Immunol. **132**(3):231-9.

Khair OA, Davies RJ, Devalia JL. 1996. Bacterial-induced release of inflammatory mediators by bronchial epithelial cells.
Eur Respir J. **9**(9):1913-22

Khilnani GC, Banga A, Sharma SK. 2004. Predictors of mortality of patients with acute respiratory failure secondary to chronic obstructive pulmonary disease admitted to an intensive care unit: a one year study.
BMC Pulm Med. **27**;4:12.

Kilian M, Mestecky J, Kulhavy R, Tomana M, Butler WT. 1980. IgA1 proteases from Haemophilus influenzae, Streptococcus pneumoniae, Neisseria meningitidis, and Streptococcus sanguis: comparative immunochemical studies.
J Immunol. (**6**):2596-600

Knight JC, Kwiatkowski D. 1999. Inherited variability of tumor necrosis factor production and susceptibility to infectious disease.
Proc Assoc Am Physicians. **111**(4):290-8

Koay MA, Gao X, Washington MK, Parman KS, Sadikot RT, Blackwell TS, Christman JW 2002. Macrophages are necessary for maximal nuclear factor-kappa B activation in response to endotoxin.
Am J Respir Cell Mol Biol. **26**(5):572-8

Kobayasi T, Asboe-Hansen G. 1969. Degranulation and regranulation of human mast cells. An electron microscopic study of the whealing reaction in urticaria pigmentosa.
*Acta Derm Venereol.***49**(4):369-81

Koot BG, Houwen R, Pot DJ, Nauta J. 2004. Congenital analbuminaemia: biochemical and clinical implications. A case report and literature review.
Eur J Pediatr. **163**(11):664-70

Krutzik SR, Sieling PA, Modlin RL 2001. The role of Toll-like receptors in host defense against microbial infection.
Curr Opin Immunol. **13**(1):104-8

Kumar RK, Herbert C, Webb DC, Li L, Foster PS. 2004. Effects of anticytokine therapy in a mouse model of chronic asthma.
Am J Respir Crit Care Med. **170**(10):1043-8

Kuschner WG, D'Alessandro A, Wong H, Blanc PD. 1996. Dose-dependent cigarette smoking-related inflammatory responses in healthy adults.
Eur Respir J. **9**(10):1989-94

Lacraz S, Nicod LP, Chicheportiche R, Welgus HG, Dayer JM. 1995. IL-10 inhibits metalloproteinase and stimulates TIMP-1 production in human mononuclear phagocytes.
J Clin Invest. **96**(5):2304-10.

Laurell C.B., Eriksson. 1963. The electrophoretic alpha-1-globulin pattern of serum in α_1 antitrypsin deficiency.
Scand. J. Clin. Lab. Invest. **15**: 132-140

Lazarus M, Hajeer AH, Turner D, Sinnott P, Worthington J, Ollier WE, Hutchinson IV. 1997. Genetic variation in the interleukin 10 gene promoter and systemic lupus erythematosus.
J Rheumatol. **24**(12):2314-7

Lang JD, McArdle PJ, O'Reilly PJ, Matalon S. 2002. Oxidant-antioxidant balance in acute lung injury.
Chest. **122**(6 Suppl):314S-320S.

Lang R, Patel D, Morris JJ, Rutschman RL, Murray PJ. 2002. Shaping gene expression in activated and resting primary macrophages by IL-10.
J Immunol. **169**(5):2253-63.

Lenz AG, Costabel U, Maier KL. 1996. Oxidized BAL fluid proteins in patients with interstitial lung diseases.
Eur Respir J. **9**(2):307-12.

Levine SJ, Larivee P, Logun C, Angus CW, Shelhamer JH. 1993. Corticosteroids differentially regulate secretion of IL-6, IL-8, and G-CSF by a human bronchial epithelial cell line.
Am J Physiol. **265**(4 Pt 1):L360-8.

Ley K, Bullard DC, Arbones ML, Bosse R, Vestweber D, Tedder TF, Beaudet AL. 1995. Sequential contribution of L- and P-selectin to leukocyte rolling in vivo.
J Exp Med. **181**(2):669-75.

Lipsky PE, van der Heijde DM, St Clair EW, Furst DE, Breedveld FC, Kalden JR, Smolen JS, Weisman M, Emery P, Feldmann M, Harriman GR, Maini RN; Anti-Tumor Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy Study Group. 2000. Infliximab and methotrexate in the treatment of rheumatoid arthritis. Anti-Tumor Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy Study Group.
N Engl J Med. **343**(22):1594-602.

Li Kam Wa TC, Mansur AH, Britton J, Williams G, Pavord I, Richards K, Campbell DA, Morton N, Holgate ST, Morrison JF. 1999. Association between - 308 tumour necrosis factor promoter polymorphism and bronchial hyperreactivity in asthma.
Clin Exp Allergy. **29**(9):1204-8.

Lim S, Roche N, Oliver BG, Mattos W, Barnes PJ, Chung KF. 2000. Balance of matrix metalloprotease-9 and tissue inhibitor of metalloprotease-1 from alveolar macrophages in cigarette smokers. Regulation by interleukin-10.
Am J Respir Crit Care Med. **162**(4 Pt 1):1355-60

Lin Z, Crockett DK, Lim MS, Elenitoba-Johnson KS. 2003. High-throughput analysis of protein/peptide complexes by immunoprecipitation and automated LC-MS/MS.
J Biomol Tech. **14**(2):149-55.

Linaker CH, Coggon D, Holgate ST, Clough J, Josephs L, Chauhan AJ, Inskip HM. 2000. Personal exposure to nitrogen dioxide and risk of airflow obstruction in asthmatic children with upper respiratory infection.
Thorax. **55**(11):930-3.

Linden M, Rasmussen JB, Piitulainen E, Tunek A, Larson M, Tegner H, Venge P, Laitinen LA, Brattsand R. 1993. Airway inflammation in smokers with nonobstructive and obstructive chronic bronchitis.
Am Rev Respir Dis. **148**(5):1226-32.

Liu L, Jarjour NN, Busse WW, Kelly EA. 2004. Enhanced generation of helper T type 1 and 2 chemokines in allergen-induced asthma.
Am J Respir Crit Care Med. **169**(10):1118-24

Liu ZG, Hsu H, Goeddel DV, Karin M 1996. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death.
Cell. **1**;87(3):565-76.

Loddenkemper R, Gibson GJ, Sibille Y 2003 The burden of lung disease in Europe: why a European White Book on lung disease?
Eur Respir J. **22**(6):869

Lopez-Boado YS, Wilson CL, Hooper LV, Gordon JI, Hultgren SJ, Parks WC. 2000. Bacterial exposure induces and activates matrilysin in mucosal epithelial cells.
J Cell Biol. **148**(6):1305-15.

Lorentz A, Schwengberg S, Sellge G, Manns MP, Bischoff SC. 2000. Human intestinal mast cells are capable of producing different cytokine profiles: role of IgE receptor cross-linking and IL-4.
J Immunol. **164**(1):43-8.

MacNee W, Wiggs B, Belzberg AS, Hogg JC. 1989. The effect of cigarette smoking on neutrophil kinetics in human lungs.
N Engl J Med. **321**(14):924-8

Macnee W, Rahman I. 1999. Oxidants and antioxidants as therapeutic targets in chronic obstructive pulmonary disease.
Am J Respir Crit Care Med. **160**(5 Pt 2):S58-65

MacNee W. 2005. Pulmonary and systemic oxidant/antioxidant imbalance in chronic obstructive pulmonary disease.
Proc Am Thorac Soc. **2**(1):50-60

Marotte H, Maslinski W, Miossec P. 2005. Circulating tumour necrosis factor-alpha bioactivity in rheumatoid arthritis patients treated with infliximab: link to clinical response.
Arthritis Res Ther. **7**(1):R149-55

May J.R. 1953. The bacteriology of chronic bronchitis.
Lancet. **39**:663-7

Marcy TW, Merrill WW. 1987. Cigarette smoking and respiratory tract infection.
Clin Chest Med. **8**(3):381-91

Margarson M.P., and Soni. N. 1998. Serum albumin: touchstone or totem?
Anaesthesia. **53**:789-803.

Marotte H, Maslinski W, Miossec P. 2005. Circulating tumour necrosis factor-alpha bioactivity in rheumatoid arthritis patients treated with infliximab: link to clinical response.
Arthritis Res Ther. **7**(1):R149-55.

Martin TR, Raghu G, Maunder RJ, Springmeyer SC. 1985. The effects of chronic bronchitis and chronic air-flow obstruction on lung cell populations recovered by bronchoalveolar lavage.
Am Rev Respir Dis. **132**(2):254-60.

Matsuse T, Hayashi S, Kuwano K, Keunecke H, Jefferies WA, Hogg JC. 1992. Latent adenoviral infection in the pathogenesis of chronic airways obstruction.
Am Rev Respir Dis. **146**(1):177-84.

McDonald JC, Chen Y, Zekveld C, Cherry NM. 2005. Incidence by occupation and industry of acute work related respiratory diseases in the UK, 1992-2001.
Occup Environ Med. **62**(12):836-42.

McGeehan GM, Becherer JD, Bast RC Jr, Boyer CM, Champion B, Connolly KM, Conway JG, Furdon P, Karp S, Kidao S, et al. 1994. Regulation of tumour necrosis factor-alpha processing by a metalloproteinase inhibitor.
Nature. **370**(6490):558-61

McRitchie DI, Isowa N, Edelson JD, Xavier AM, Cai L, Man HY, Wang YT, Keshavjee SH, Slutsky AS, Liu M.2000. Production of tumour necrosis factor alpha by primary cultured rat alveolar epithelial cells.
Cytokine. **12**(6):644-54.

Mercer P, 2001. The role of matrix metalloproteinases in chronic obstructive pulmonary disease.
PhD thesis

Mio T, Romberger DJ, Thompson AB, Robbins RA, Heires A, Rennard SI. 1997. Cigarette smoke induces interleukin-8 release from human bronchial epithelial cells.
Am J Respir Crit Care Med. **155**(5):1770-6.

Nomura F, Akashi S, Sakao Y, Sato S, Kawai T, Matsumoto M, Nakanishi K, Kimoto M, Miyake K, Takeda K, Akira S. 2000. Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression.
J Immunol. **164**(7):3476-9

Monso E., Rosell A., Manterola, Mossberg B, Afzelius B, Camner P. 1986. Mucociliary clearance in obstructive lung diseases. Correlations to the immotile cilia syndrome.
*Eur J Respir Dis Suppl.***146**:295-301

Monso E, Ruiz J, Rosell A, Manterola J, Fiz J, Morera J, Ausina V. 1995. Bacterial infection in chronic obstructive pulmonary disease. A study of stable and exacerbated outpatients using the protected specimen brush.
Am J Respir Crit Care Med. **152**(4 Pt 1):1316-20

Monso E, Rosell A, Bonet G, Manterola J, Cardona PJ, Ruiz J, Morera J. 1999. Risk factors for lower airway bacterial colonization in chronic bronchitis.
Eur Respir J. **13**(2):338-42.

Monto AS, Bryan ER. 1978. Susceptibility to rhinovirus infection in chronic bronchitis.
Am Rev Respir Dis. **118**(6):1101-3

Mossberg B, Afzelius B, Camner P. 1986. Mucociliary clearance in obstructive lung diseases. Correlations to the immotile cilia syndrome.
Eur J Respir Dis Suppl. **146**:295-301

Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. 2001. Interleukin-10 and the interleukin-10 receptor.
Annu Rev Immunol. **19**:683-765.

Muller NL, Staples CA, Miller RR, Abboud RT. 1988. "Density mask". An objective method to quantitate emphysema using computed tomography.
Chest. **94**(4):782-7.

Musil J. 2004. Pathogenesis of the chronic obstructive pulmonary disease (COPD)]
Vnitr Lek. **50**(9):663-7

Murphy TF, Sethi S, Klingman KL, Brueggemann AB, Doern GV. 1999. Simultaneous respiratory tract colonization by multiple strains of nontypeable haemophilus influenzae in chronic obstructive pulmonary disease: implications for antibiotic therapy.
J Infect Dis. **180**(2):404-9

Murphy TF, Sethi S. 1992. Bacterial infection in chronic obstructive pulmonary disease.
Am. Rev. Respir. Dis. **146**:1067-1083.

Murray CJ, Lopez AD. 1996. Evidence-based health policy--lessons from the Global Burden of Disease Study.
Science. **1;**274(5288):740-

Murray JC, Demopulos CM, Lawn RM, Motulsky AG. 1983. Molecular genetics of human serum albumin: restriction enzyme fragment length polymorphisms and analbuminemia.
Proc Natl Acad Sci U S A. 80(19):5951-5

MRC. 1965. Definition and classification of chronic bronchitis for clinical and epidiological purposes.
Lancet.775-780

Nagase H, Woessner JF Jr. 1999. Matrix metalloproteinases.
J Biol Chem. **274**(31):21491-4

Nakamura H, Yoshimura K, McElvaney NG, Crystal RG. 1992. Neutrophil elastase in respiratory epithelial lining fluid of individuals with cystic fibrosis induces interleukin-8 gene expression in a human bronchial epithelial cell line.
J Clin Invest. **89**(5):1478-84.

Nakayama T, Kodama M, Nagata C. 1984. Generation of hydrogen peroxide and superoxide anion radical from cigarette smoke.
Gann. **75**(2):95-8.

Nash JR, McLaughlin PJ, Hoyle C, Roberts D. 1991. Immunolocalization of tumour necrosis factor alpha in lung tissue from patients dying with adult respiratory distress syndrome.
Histopathology. **19**(5):395-402.

Nicotra MB, Rivera M, Awe RJ. 1982. Antibiotic therapy of acute exacerbations of chronic bronchitis. A controlled study using tetracycline.
Ann Intern Med. **97**(1):18-21

Noguchi E, Yokouchi Y, Shibasaki M, Inudou M, Nakahara S, Nogami T, Kamioka M, Yamakawa-Kobayashi K, Ichikawa K, Matsui A, Arinami T. 2002. Association between TNFA polymorphism and the development of asthma in the Japanese population.
Am J Respir Crit Care Med. **166**(1):43-6.

O'byrne PM, Postma DS. 1999. The many faces of airway inflammation. Asthma and chronic obstructive pulmonary disease. Asthma Research Group.
Am J Respir Crit Care Med. **159**(5 Pt 2):S41-63

Okayama Y, Petit-Frere C, Kassel O, Semper A, Quint D, Tunon-de-Lara MJ, Bradding P, Holgate ST, Church MK. 1995. IgE-dependent expression of mRNA for IL-4 and IL-5 in human lung mast cells.
J Immunol. **155**(4):1796-808

Okayama Y. 2005. Mast cell-derived cytokine expression induced via Fc receptors and Toll-like receptors.
Chem Immunol Allergy. **87**:101-10

O'Neill CA, Halliwell B, van der Vliet A, Davis PA, Packer L, Tritschler H, Strohman WJ, Rieland T, Cross CE, Reznick AZ. 1994. Aldehyde-induced protein modifications in human plasma: protection by glutathione and dihydrolipoic acid.
J Lab Clin Med. **124**(3):359-70.

Ordonez CL, Shaughnessy TE, Matthay MA, Fahy JV. 2000. Increased neutrophil numbers and IL-8 levels in airway secretions in acute severe asthma: Clinical and biologic significance.
Am J Respir Crit Care Med. **161**(4 Pt 1):1185-90

Ozen S, Alikasifoglu M, Bakkaloglu A, Duzova A, Jarosova K, Nemcova D, Besbas N, Vencovsky J, Tuncbilek E. 2002. Tumour necrosis factor alpha G-->A -238 and G-->A -308 polymorphisms in juvenile idiopathic arthritis.
Rheumatology (Oxford). **41**(2):223-7.

Parks WC, Shapiro SD. 2001. Matrix metalloproteinases in lung biology.
Respir Res. **2**(1):10-9

Pan LH, Ohtani H, Yamauchi K, Nagura H. 1996. Co-expression of TNF alpha and IL-1 beta in human acute pulmonary fibrotic diseases: an immunohistochemical analysis.
Pathol Int. 46(2):91-9.

Panacek EA, Marshall JC, Albertson TE, Johnson DH, Johnson S, MacArthur RD, Miller M, Barchuk WT, Fischkoff S, Kaul M, Teoh L, Van Meter L, Daum L, Lemeshow S, Hicklin G, Doig C; 2004. Monoclonal Anti-TNF: a Randomized Controlled Sepsis Study Investigators. Related Articles, Links [Abstract] Efficacy and safety of the monoclonal anti-tumor necrosis factor antibody F(ab')2 fragment afelimomab in patients with severe sepsis and elevated interleukin-6 levels.
Crit Care Med. **32**(11):2173-82

Patel IS, Seemungal TA, Wilks M, Lloyd-Owen SJ, Donaldson GC, Wedzicha JA 2002**.** Relationship between bacterial colonisation and the frequency, character, and severity of COPD exacerbations.
*Thorax.***57**(9):759-64.

Pauwels RA, Rabe KF. Burden and clinical features of chronic obstructive pulmonary disease (COPD).
Lancet. **364**(9434):613-20

Pease JE, Sabroe I. 2002. The role of interleukin-8 and its receptors in inflammatory lung disease: implications for therapy.
Am J Respir Med. **1**(1):19-25

Pelletier O.1975. Vitamin C and cigarette smokers.
Ann N Y Acad Sci. **258**:156-68

Pesci A, Balbi B, Majori M, Cacciani G, Bertacco S, Alciato P, Donner CF. 1998 Inflammatory cells and mediators in bronchial lavage of patients with chronic obstructive pulmonary disease.
Eur Respir J. 12(2):380-6

Peters T. 1985. Serum albumin.
Adv. Protein chem. **37**:161-245

Pinto-Sietsma SJ, Mulder J, Janssen WM, Hillege HL, de Zeeuw D, de Jong PE. 2000. Smoking is related to albuminuria and abnormal renal function in nondiabetic persons.
Ann Intern Med. **133**(8):585-91.

Pizzichini E, Pizzichini MM, Gibson P, Parameswaran K, Gleich GJ, Berman L, Dolovich J, Hargreave FE. 1998. Sputum eosinophilia predicts benefit from prednisone in smokers with chronic obstructive bronchitis.
Am J Respir Crit Care Med. **158**(5 Pt 1):1511-7

Poole PJ, Chacko E, Wood-Baker RW, Cates CJ. 2000. Influenza vaccine for patients with chronic obstructive pulmonary disease.
Cochrane Database Syst Rev. (**4**):CD002733

Pons AR, Sauleda J, Noguera A, Pons J, Barcelo B, Fuster A, Agusti AG. 2005. Decreased macrophage release of TGF-beta and TIMP-1 in chronic obstructive pulmonary disease.
Eur Respir J. **26**(1):60-6.

Pryor WA, Stone K. 1993. Oxidants in cigarette smoke. Radicals, hydrogen peroxide, peroxynitrate, and peroxynitrite.
Ann N Y Acad Sci. **686**:12-27; discussion 27-8

Pugin J, Schurer-Maly CC, Leturcq D, Moriarty A, Ulevitch RJ, Tobias PS. 1993. Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14.
Proc Natl Acad Sci U S A. **90**(7):2744-8.

Quinlan GJ, Mumby S, Martin GS, Bernard GR, Gutteridge JM, Evans TW. 2004. Albumin influences total plasma antioxidant capacity favorably in patients with acute lung injury.
Crit Care Med. **32**(3):755-9.

Rahman I, MacNee W. 1996. Oxidant/antioxidant imbalance in smokers and chronic obstructive pulmonary disease.
Thorax. **51**(4):348-50

Rahman I. 2005. Oxidative stress in pathogenesis of chronic obstructive pulmonary disease: cellular and molecular mechanisms.
Cell Biochem Biophys. **43**(1):167-88

Ramsdale EH, Morris MM, Roberts RS, Hargreave FE. 1984. Bronchial responsiveness to methacholine in chronic bronchitis: relationship to airflow obstruction and cold air responsiveness.
Thorax. **39**(12):912-8.

Ramstrom M, Hagman C, Mitchell JK, Derrick PJ, Hakansson P, Bergquist J. 2005. Depletion of high-abundant proteins in body fluids prior to liquid chromatography fourier transform ion cyclotron resonance mass spectrometry.
J Proteome Res. **4**(2):410-6.

Read TE, Harris HW, Grunfeld C, Feingold KR, Kane JP, Rapp JH. 1993. The protective effect of serum lipoproteins against bacterial lipopolysaccharide.
Eur Heart J. **14** Suppl K:125-9

Reznick AZ, Cross CE, Hu ML, Suzuki YJ, Khwaja S, Safadi A, Motchnik PA, Packer L, Halliwell B. 1992. Modification of plasma proteins by cigarette smoke as measured by protein carbonyl formation.
Biochem J. **286** (Pt 2):607-11.

Riise GC, Larsson S, Larsson P, Jeansson S, Andersson BA. 1994. The intrabronchial microbial flora in chronic bronchitis patients: a target for N-acetylcysteine therapy?
Eur Respir J. 7(1):94-101

Riise GC, Ahlstedt S, Larsson S, Enander I, Jones I, Larsson P, Andersson B. 1995. Bronchial inflammation in chronic bronchitis assessed by measurement of cell products in bronchial lavage fluid.
Thorax. **50**(4):360-5

Rock KL, Gramm C, Rothstein L, Clark K, Stein R, Dick L, Hwang D, Goldberg AL. 1994. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules.
Cell. **78**(5):761-71.

Rodriguez-Roisin R. 2000. Towards a concensus definition for COPD exacerbations.
Chest; **117**:398-401S.

Roland M, Bhowmik A, Sapsford RJ, Seemungal TA, Jeffries DJ, Warner TD, Wedzicha JA. 2001. Sputum and plasma endothelin-1 levels in exacerbations of chronic obstructive pulmonary disease.
Thorax. **56**(1):30-5.

Rood MJ, van Krugten MV, Zanelli E, van der Linden MW, Keijsers V, Schreuder GM, Verduyn W, Westendorp RG, de Vries RR, Breedveld FC, Verweij CL, Huizinga TW. 2000. TNF-308A and HLA-DR3 alleles contribute independently to susceptibility to systemic lupus erythematosus.
Arthritis Rheum. **43**(1):129-34

Royer B, Varadaradjalou S, Saas P, Gabiot AC, Kantelip B, Feger F, Guillosson JJ, Kantelip JP, Arock M. 2001. Autocrine regulation of cord blood-derived human mast cell activation by IL-10.
J Allergy Clin Immunol. **108**(1):80-6.

Saccani S, Pantano S, Natoli G. 2001. Two waves of nuclear factor kappaB recruitment to target promoters.
J Exp Med. **193**(12):1351-9.

Sachs AP, Koeter GH, Groenier KH, van der Waaij D, Schiphuis J, Meyboom-de Jong B. 1995. Changes in symptoms, peak expiratory flow, and sputum flora during treatment with antibiotics of exacerbations in patients with chronic obstructive pulmonary disease in general practice.
Thorax. **50**(7):758-63

Sandborn WJ. 2005. New concepts in anti-tumor necrosis factor therapy for inflammatory bowel disease.
Rev Gastroenterol Disord. **5**(1):10-8

Sandford AJ, Pare PD. 2000. Genetic risk factors for chronic obstructive pulmonary disease.
Clin Chest Med. **21**(4):633-43

Sakao S, Tatsumi K, Igari H, Shino Y, Shirasawa H, Kuriyama T.2001. Association of tumor necrosis factor alpha gene promoter polymorphism with the presence of chronic obstructive pulmonary disease.
Am J Respir Crit Care Med. **163**(2):420-2.

Saren P, Welgus HG, Kovanen PT. 1996. TNF-alpha and IL-1beta selectively induce expression of 92-kDa gelatinase by human macrophages.
J Immunol. **1**;157(9):4159-65.

Scanlon PD, Connett JE, Waller LA, Altose MD, Bailey WC, Buist AS. 2000. Smoking cessation and lung function in mild-to-moderate chronic obstructive pulmonary disease. The Lung Health Study.
Am J Respir Crit Care Med. **161**(2 Pt 1):381-90

Schaberg T., Klein U., Rau M., Eller J., Lode H. 1995. Subpopulations of alveolar macrophages in smokers and non-smokers: Relation to the expression of CD11/CD18 molecules and superoxide anion production.
Am J Respir Crit Care Med. **151**(5):1551-8.

Schulz C, Kratzel K, Wolf K, Schroll S, Kohler M, Pfeifer M. 2004. Activation of bronchial epithelial cells in smokers without airway obstruction and patients with COPD.
Chest. **125**(5):1706-13

Scott KW. 1973. Bacteriology of the sputum in exacerbation and remission of chronic bronchitis.
Br J Dis Chest. **67**(1):43-8

Seifart C, Dempfle A, Plagens A, Seifart U, Clostermann U, Muller B, Vogelmeier C, von Wichert P. 2005. TNF-alpha-, TNF-beta-, IL-6-, and IL-10-promoter polymorphisms in patients with chronic obstructive pulmonary disease.
Tissue Antigens **65**(1):93-100

Seemungal TA, Donaldson GC, Paul EA, Bestall JC, Jeffries DJ, Wedzicha JA. 1998 Effect of exacerbation on quality of life in patients with chronic obstructive pulmonary disease.
Am J Respir Crit Care Med. **157**(5 Pt 1):1418-22.

Seemungal TA, Donaldson GC, Bhowmik A, Jeffries DJ, Wedzicha JA. 2000. Time course and recovery of exacerbations in patients with chronic obstructive pulmonary disease.
Am J Respir Crit Care Med. **161**(5):1608-13.

Seemungal TA, Harper-Owen R, Bhowmik A, Jeffries DJ, Wedzicha JA. 2000b. Detection of rhinovirus in induced sputum at exacerbation of chronic obstructive pulmonary disease.
Eur Respir J. **16**(4):677-83

Sethi S., Murphy T.F. 2001. Bacterial infection in chronic obstructive pulmonary disease in 2000: a state-of-the-art review.
Clin. Microbiol. Rev. **14**:336-363

Shah A, Church MK, Holgate ST. 1995. Tumour necrosis factor alpha: a potential mediator of asthma.
Clin Exp Allergy. 1995 Nov;25(11):1038-44.

Shapiro S.D. 1994. Elastolytic metalloproteinases produced by human mononuclear phagocytes: potential role in destructive lung disease.
Am. J. Crit. Care Med. **150**:s160-s164.

Shapiro S.D. 1999. The macrophage in chronic obstructive pulmonary disease.
Am. J. Resp. Cell Mol. Biol. **20**:1100-1102.

Shapiro S.D., Senior R.M. 1999. Matrix metalloproteinases- Degradation and more.
Am. J. Crit. Care Med. **160**

Sheppard D. 2002. Roger S. Mitchell lecture. Uses of expression microarrays in studies of pulmonary fibrosis, asthma, acute lung injury, and emphysema.
Chest. **121**(3 Suppl):21S-25S

Shin HD, Park BL, Kim LH, Kim JS, Kim JW. 2005. Interleukin-10 haplotype associated with total serum IgE in atopic dermatitis patients.
Allergy. **60**(9):1146-51

Sibille Y, Reynolds HY. 1990. Macrophages and polymorphonuclear neutrophils in lung defense and injury.
Am Rev Respir Dis. **141**(2):471-501

Silver RM. 1995. Interstitial lung disease of systemic sclerosis.
Int Rev Immunol. **12**(2-4):281-9

Simon SI, Burns AR, Taylor AD, Gopalan PK, Lynam EB, Sklar LA, Smith CW. 1995. L-selectin (CD62L) cross-linking signals neutrophil adhesive functions via the Mac-1 (CD11b/CD18) beta 2-integrin.
J Immunol. **155**(3):1502-14.

Simpson SD, Ho Y, Rice PA, Wetzler LM. 1999. T lymphocyte response to Neisseria gonorrhoeae porin in individuals with mucosal gonococcal infections.
J Infect Dis. **180**(3):762-73.

Skerrett SJ, Liggitt HD, Hajjar AM, Ernst RK, Miller SI, Wilson CB. 2004. Respiratory epithelial cells regulate lung inflammation in response to inhaled endotoxin.
Am J Physiol Lung Cell Mol Physiol. **287**(1):L143-52

Smith CB, Golden CA, Kanner RE, Renzetti AD Jr. 1980. Association of viral and Mycoplasma pneumoniae infections with acute respiratory illness in patients with chronic obstructive pulmonary diseases.
Am Rev Respir Dis. **121**(2):225-32.

Snider G.L., Kleinerman J., Thurlbeck W.M. 1985. The definition of emphysema. Report of a National Heart and Blood Institute, devision of lung diseases, workshop.
Am. Rev. Resp. Dis. **132**:182-185.

Snider GL. 1989. Chronic obstructive pulmonary disease: risk factors, pathophysiology and pathogenesis.
*Annu Rev Med.***40**:411-29.

Smith CA, Davis T, Anderson D, Solam L, Beckmann MP, Jerzy R, Dower SK, Cosman D, Goodwin RG 1990. A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins.
Science. 25;248(4958):1019-23.

Smith KC, Bateman AC, Fussell HM, Howell WM. 2004. Cytokine gene polymorphisms and breast cancer susceptibility and prognosis.
Eur J Immunogenet. 31(4):167-73.

Soler N, Torres A, Ewig S, Gonzalez J, Celis R, El-Ebiary M, Hernandez C, Rodriguez-Roisin R. 1998. Bronchial microbial patterns in severe exacerbations of chronic obstructive pulmonary disease (COPD) requiring mechanical ventilation.
Am J Respir Crit Care Med. **157**(5 Pt 1):1498-505.

Soler N, Ewig S, Torres A, Filella X, Gonzalez J, Zaubet A. 1999. Airway inflammation and bronchial microbial patterns in patients with stable chronic obstructive pulmonary disease.
Eur Respir J. **14**(5):1015-22.

Spencer S, Jones PW; GLOBE Study Group. 2003. Time course of recovery of health status following an infective exacerbation of chronic bronchitis.
Thorax. **58**(7):589-93.

Stadtman ER. 1990. Metal ion-catalyzed oxidation of proteins: biochemical mechanism and biological consequences.
Free Radic Biol Med. **9**(4):315-25

Stanescu D, Sanna A, Veriter C, Kostianev S, Calcagni PG, Fabbri LM, Maestrelli P. 1996. Airways obstruction, chronic expectoration, and rapid decline of FEV1 in smokers are associated with increased levels of sputum neutrophils.
Thorax. **51**(3):267-71

St Clair EW, van der Heijde DM, Smolen JS, Maini RN, Bathon JM, Emery P, Keystone E, Schiff M, Kalden JR, Wang B, Dewoody K, Weiss R, Baker D. 2004. Combination of infliximab and methotrexate therapy for early rheumatoid arthritis: a randomized, controlled trial.
Arthritis Rheum. **50**(11):3432-43.

Stelmach I, Majak P, Jerzynska J, Kuna P. 2005. The effect of treatment with montelukast on in vitro interleukin-10 production of mononuclear cells of children with asthma.
Clin Exp Allergy. **35**(2):213-20.

Stockley R.A. 1994. The role of proteinases in the pathogenesis of chronic bronchitis.
Am J Respir Crit Care Med. **150**:s109-s113.

Stout RD, Jiang C, Matta B, Tietzel I, Watkins SK, Suttles J. 2005. Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences.
J Immunol. **175**(1):342-9

Stuber F, Udalova IA, Book M, Drutskaya LN, Kuprash DV, Turetskaya RL, Schade FU, Nedospasov SA. 1995. -308 tumor necrosis factor (TNF) polymorphism is not associated with survival in severe sepsis and is unrelated to lipopolysaccharide inducibility of the human TNF promoter.
J Inflamm. **46**(1):42-50

Sullivan DE, Ferris M, Pociask D, Brody AR. 2005. Tumor necrosis factor-alpha induces transforming growth factor-beta1 expression in lung fibroblasts through the extracellular signal-regulated kinase pathway.
Am J Respir Cell Mol Biol. **32**(4):342-9.

Suzuki H, Ikeda K. 2002. Mode of action of long-term low-dose macrolide therapy for chronic sinusitis in the light of neutrophil recruitment.
Curr Drug Targets Inflamm Allergy. **1**(1):117-26

Sur S, Gleich GJ, Offord KP, Swanson MC, Ohnishi T, Martin LB, Wagner JM, Weiler DA, Hunt LW. 1995. Allergen challenge in asthma: association of eosinophils and lymphocytes with interleukin-5.
Allergy. **50**(11):891-8.

Swart PJ, Beljaars L, Kuipers ME, Smit C, Nieuwenhuis P, Meijer DK. 1999. Homing of negatively charged albumins to the lymphatic system: general implications for drug targeting to peripheral tissues and viral reservoirs.
Biochem Pharmacol. **58**(9):1425-35.

Tagore A, Gonsalkorale WM, Pravica V, Hajeer AH, McMahon R, Whorwell PJ, Sinnott PJ, Hutchinson IV. 1999. Interleukin-10 (IL-10) genotypes in inflammatory bowel disease.
Tissue Antigens. **54**(4):386-90.

Tager IB, Weiss ST, Munoz A, Rosner B, Speizer FE. 1983. Longitudinal study of the effects of maternal smoking on pulmonary function in children.
N Engl J Med. **22**;309(12):699-703.

Takeuchi O, Akira S. 2001. Toll-like receptors; their physiological role and signal transduction system.
Int Immunopharmacol. **1**(4):625-35.

Takeyama K, Dabbagh K, Lee HM, Agusti C, Lausier JA, Ueki IF, Grattan KM, Nadel JA. 1999. Epidermal growth factor system regulates mucin production in airways.
Proc Natl Acad Sci U S A. **96**(6):3081-6.

Takanashi S, Hasegawa Y, Kanehira Y, Yamamoto K, Fujimoto K, Satoh K, Okamura K. 1999. Interleukin-10 level in sputum is reduced in bronchial asthma, COPD and in smokers.
Eur Respir J. **14**(2):309-14

Takizawa H, Tanaka M, Takami K, Ohtoshi T, Ito K, Satoh M, Okada Y, Yamasawa F, Nakahara K, Umeda A. 2001. Increased expression of transforming growth factor-beta1 in small airway epithelium from tobacco smokers and patients with chronic obstructive pulmonary disease (COPD).
Am J Respir Crit Care Med. **163**(6):1476-83.

Tamura T, Ohmori K. 2001. Rhein, an active metabolite of diacerein, suppresses the interleukin-1alpha-induced proteoglycan degradation in cultured rabbit articular chondrocytes.
Jpn J Pharmacol. **85**(1):101-4

Taylor RG, Joyce H, Gross E, Holland F, Pride NB. 1985. Bronchial reactivity to inhaled histamine and annual rate of decline in FEV1 in male smokers and ex-smokers.
Thorax. **40**(1):9-16.

Tigani B, Schaeublin E, Sugar R, Jackson AD, Fozard JR, Beckmann N. 2002. Pulmonary inflammation monitored noninvasively by MRI in freely breathing rats.
Biochem Biophys Res Commun. **22**;292(1):216-21

The European lung white book. Chronic obstructive pulmonary disease
Part 2 major respiratory diseases, **Ch** 2: 34-43

Thomas PS. 2001. Tumour necrosis factor-alpha: the role of this multifunctional cytokine in asthma.
Immunol Cell Biol. **79**(2):132-40

Thomas PS, Yates DH, Barnes PJ. 1995. Tumor necrosis factor-alpha increases airway responsiveness and sputum neutrophilia in normal human subjects.
Am J Respir Crit Care Med. **152**(1):76-80.

Thompson AB, Daughton D, Robbins RA, Ghafouri MA, Oehlerking M, Rennard SI. 1989. Intraluminal airway inflammation in chronic bronchitis. Characterization and correlation with clinical parameters.
Am Rev Respir Dis. **140**(6):1527-

Thompson WH, Nielson CP, Carvalho P, Charan NB, Crowley JJ. 1996. Controlled trial of oral prednisone in outpatients with acute COPD exacerbation.
Am J Respir Crit Care Med. **154**(2 Pt 1):407-12

Thurlbeck WM. 1963. The incidence of pulmonary emphysema.
Am Rev of Respir Disease **87**:206

Tillie-Leblond I, Gosset P, Tonnel AB. 2005. Inflammatory events in severe acute asthma.
Allergy. **60**(1):23-9.

Traves SL, Smith SJ, Barnes PJ, Donnelly LE. 2004. Specific CXC but not CC chemokines cause elevated monocyte migration in COPD: a role for CXCR2.
J Leukoc Biol. **76**(2):441-50.

Tug T, Karatas F, Terzi SM. 2004. Antioxidant vitamins (A, C and E) and malondialdehyde levels in acute exacerbation and stable periods of patients with chronic obstructive pulmonary disease.
Clin Invest Med. **27**(3):123-8.

Upton MN, McConnachie A, McSharry C. 2000. Intergenerational 20 year trends in the prevalence of asthma and hay fever in adults: the Midspan family study surveys of parents and offspring.
BMJ **321**: 88-92.

Vanderbilt JN, Mager EM, Allen L, Sawa T, Wiener-Kronish J, Gonzalez R, Dobbs LG. 2003. CXC chemokines and their receptors are expressed in type II cells and upregulated following lung injury.
Am J Respir Cell Mol Biol. **29**(6):661-8. Epub 2003 Jun 26.

van der Vaart H, Koeter GH, Postma DS, Kauffman HF, ten Hacken NH. 2005. First study of infliximab treatment in patients with chronic obstructive pulmonary disease.
Am J Respir Crit Care Med. **172**(4):465-9

Vassalli P, Grau GE, Piguet PF. 1992. TNF in autoimmune diseases, graft-versus-host reactions, and pulmonary fibrosis.
Immunol Ser. **56**:409-30

Vernooy JH, Lindeman JH, Jacobs JA, Hanemaaijer R, Wouters EF. 2004. Increased activity of matrix metalloproteinase-8 and matrix metalloproteinase-9 in induced sputum from patients with COPD.
Chest. **126**(6):1802-10

Vignola AM, Riccobono L, Mirabella A, Profita M, Chanez P, Bellia V, Mautino G, D'accardi P, Bousquet J, Bonsignore G. 1998. Sputum metalloproteinase-9/tissue inhibitor of metalloproteinase-1 ratio correlates with airflow obstruction in asthma and chronic bronchitis.
*Am J Respir Crit Care Med.***158**(6):1945-50.

Walter DM, McIntire JJ, Berry G, McKenzie AN, Donaldson DD, DeKruyff RH, Umetsu DT. 2001. Critical role for IL-13 in the development of allergen-induced airway hyperreactivity.
J Immunol. **167**(8):4668-75

Waldron-Lynch F, Adams C, Amos C, Zhu DK, McDermott MF, Shanahan F, Molloy MG, O'Gara F. 2001. Tumour necrosis factor 5' promoter single nucleotide polymorphisms influence susceptibility to rheumatoid arthritis (RA) in immunogenetically defined multiplex RA families.
Genes Immun. **2**(2):82-7

Walsh GM, Hartnell A, Wardlaw AJ, Kurihara K, Sanderson CJ, Kay AB. 1990. IL-5 enhances the in vitro adhesion of human eosinophils, but not neutrophils, in a leucocyte integrin (CD11/18)-dependent manner.
Immunology. **71**(2):258-65.

Wanidworanun C, Strober W. 1993. Predominant role of tumor necrosis factor-alpha in human monocyte IL-10 synthesis.
J Immunol. **15**;151(12):6853-61.

Wedzicha JA, Donaldson GC. 2003. Exacerbations of chronic obstructive pulmonary disease.
Respir Care. **48**(12):1204-13; discussion 1213-5

Wells CA, Ravasi T, Faulkner GJ, Carninci P, Okazaki Y, Hayashizaki Y, Sweet M, Wainwright BJ, Hume DA. 2003. Genetic control of the innate immune response.
BMC Immunol. **26**;4:5.

Wertheim WA, Kunkel SL, Standiford TJ, Burdick MD, Becker FS, Wilke CA, Gilbert AR, Strieter RM. 1993. Regulation of neutrophil-derived IL-8: the role of prostaglandin E2, dexamethasone, and IL-4.
J Immunol. **15**;151(4):2166-75.

West, J.B. 1995. Pulmonary pathophysiology- the essentials. 5th Ed. Williams and Wilkins.

Wielockx B, Libert C, Wilson C. 2004. Matrilysin (matrix metalloproteinase-7): a new promising drug target in cancer and inflammation?
Cytokine Growth Factor Rev. **15**(2-3):111-5

Willemse BW, Postma DS, Timens W, ten Hacken NH. 2004. The impact of smoking cessation on respiratory symptoms, lung function, airway hyperresponsiveness and inflammation.
Eur Respir J. **23**(3):464-76.

Williams L, Jarai G, Smith A, Finan P. 2002. IL-10 expression profiling in human monocytes.
J Leukoc Biol. **72**(4):800-9.

Wilson R, Roberts D, Cole P. 1985. Effect of bacterial products on human ciliary function in vitro.
Thorax. **40**(2):125-3

Wilson AG, Symons JA, McDowell TL, McDevitt HO, Duff GW. 1997. Effects of a polymorphism in the human tumor necrosis factor alpha promoter on transcriptional activation.
Proc Natl Acad Sci U S A. **94**(7):3195-9.

Witherden IR, Vanden Bon EJ, Goldstraw P, Ratcliffe C, Pastorino U, Tetley TD. Primary human alveolar type II epithelial cell chemokine release: effects of cigarette smoke and neutrophil elastase.
Am J Respir Cell Mol Biol. **30**(4):500-9.

Witte JS, Palmer LJ, O'Connor RD, Hopkins PJ, Hall JM. 2002. Relation between tumour necrosis factor polymorphism TNFalpha-308 and risk of asthma.
Eur J Hum Genet. **10**(1):82-5.

Wright JL, Churg A. 2002. Animal models of cigarette smoke-induced COPD.
Chest. **122**(6 Suppl):301S-306S

Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein.
Science. **249**(4975):1431-3.

Xiang Z, Block M, Lofman C, Nilsson G. IgE-mediated mast cell degranulation and recovery monitored by time-lapse photography.
J Allergy Clin Immunol. **108**(1):116-21.

Yan K, Salome CM, Woolcock AJ. 1985. Prevalence and nature of bronchial hyperresponsiveness in subjects with chronic obstructive pulmonary disease.
Am Rev Respir Dis. **132**(1):25-9.

Yaron M, Shirazi I, Yaron I. 1999. Anti-interleukin-1 effects of diacerein and rhein in human osteoarthritic synovial tissue and cartilage cultures.
Osteoarthritis Cartilage. **7**(3):272-80

Zalcain R, Sobradillo V, Amilibia J, Barron J, Achotegui V, Pijoan JI, Llorente JL 1999. Predisposing factors to bacterial colonization in chronic obstructive pulmonary disease.
Eur Respir J. **13**(2):210-348.

Zhang H, Yang Y, Steinbrecher UP. 1993. Structural requirements for the binding of modified proteins to the scavenger receptor of macrophages.
J Biol Chem. **268**(8):5535-42.

Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, Zhang Y, Elias JA. 1999. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production.
J Clin Invest. **103**(6):779-88