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Abnormalities in bacterial sensing and inflammatory response to pathogens in the lung parenchyma of chronic obstructive pulmonary disease patients

by

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ABSTRACT

Abnormalities in bacterial sensing and inflammatory response to pathogens in the lung parenchyma of chronic obstructive pulmonary disease patients

Chronic obstructive pulmonary disease (COPD) is a progressive disease that results in loss of lung function. COPD patients are prone to periods of increased systemic and localised inflammation and elevation of symptom, termed exacerbations, increased morbidity and mortality. Smoking is the main risk factor. Most exacerbations are triggered following infection with viruses and bacteria such as non-typeable *Haemophilus influenza*, *Moraxella cattarhalis* and Streptococcus pneumonia. Colonisation by new bacterial species and a shift in existing colonising strains can also induce exacerbation.

This study investigated inflammatory responses to bacterial exposure in lung parenchyma explants from surgical patients (COPD and controls) in relation to their disease status. Stimuli included *Escherischia coli* endotoxin (lipopolysaccharide, LPS) and heat killed *H. influenza* intended to mimic pulmonary response to the multiple ligands present during bacterial colonisation. Potential contribution of the pre-existing cellular inflammatory environment to the excessive inflammation seen in COPD was examined by compared inflammatory cell load (control/COPD lung parenchyma) using immunohistochemistry (IHC) and flow cytometry (FC). Little difference was observed in the inflammatory cell load between control and COPD lungs; although a modest reduction in lung macrophages was observed in the COPD lungs by FC ($P = 0.09$). Since COPD is characterised by excessive inflammatory responses to smoke particulates, we hypothesised that similar responses would be observed following exposure to bacterial ligands; providing an explanation why COPD patients suffer exacerbations often requiring hospitalisation, whereas control individuals do not.

An *ex vivo* stimulation model was optimised to determine the peak time-point for mediator secretion and the optimum ligand dose using LPS and *H. influenzae* as stimuli. Time course study showed that TNF-α peaked at 24 h for both stimuli. Using optimised conditions, the inflammatory mediator response to both stimuli was compared between lung explants (control/COPD). Stimulation with *H. influenzae* and LPS led to significant release of the majority of cytokines/chemokines (TNF-α, IL-1β, -6, -8, -10, -17, MCP-1, MIP-1β) measured. There was a significantly elevated release of IL-6 and MCP-1 in the COPD group. IL-8 secretion appeared to be depressed by exposure to bacterial ligands in the controls, but unaltered in the COPD group. Since the inflammatory cell load in lung tissues from control/COPD subjects did not give us an answer to account for the excessive inflammatory response observed in COPD patients. We, therefore, measured the expression of two bacterial sensing ligands (TLR 2 and 4) on the surface of epithelial and inflammatory cells resident in the lung tissues at baseline in these two cohorts using FC. A decrease in TLR 4 expression on $CD8^+$ T-lymphocytes in lung tissues from the COPD group was observed. This difference was suggestive of abnormal bacterial sensing in the COPD lung, but the observed reduction is contrary to our prediction of increased levels in COPD. It may be that suppressed bacterial recognition prevents bacterial clearance through cell-mediated immunity, pre-disposing COPD patients to bacterial colonisation, while the overall mediator responses are only modestly elevated.

In term of exacerbation treatment, we looked at the efficacy of two novels inhibitors of an inflammatory cascade namely p38MAPkinase pathway and compared them to corticosteroids (CS). Inhaled (PF-0371545) and oral (PHA-797804) inhibitors were applied to our bacterial ligand stimulation model in dose-response studies; Fluticasone propionate (FP) was the positive control. TNF-α at 24 h was used as the primary outcome. PHA-797804 was more effective than PF-0371545 as an anti-inflammatory agent in our model, possibly reflecting its solubility characteristics. Despite requiring a higher dose than FP, this inhibitor may be useful as an adjunct to existing corticosteroid therapy, enabling a reduction in dose of both inhibitors to limit side-

effects. In summary, this model was able to cause an inflammatory reaction in lung tissue and lung tissue from COPD patients was found to be more inflammatory.

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clinician, “never” denotes never smoked. Non parametric Mann Whitney U test showed no statistical difference between the group in age and pack years.169

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Definitions and Abbreviations

AAT	Alpha-1 antitrypsin
ABC	Avidin biotin complex
AEC	Aminoethylcarbazole
APC	Allophycocyanin
ATS	American Thoracic Society
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
COPD	Chronic obstructive pulmonary disease
CXCL	Chemokine ligand
DAB	Diaminobenzidine
DMEM	Dulbecco's Modified Eagle's Medium
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ENA	Epithelial cell-derived neutrophil-activating peptide
FACS	Fluorescence activated cell sorter
FC	Flow cytometry
FEV1	Forced expiratory volume
FITC	Fluorescein isothiocyanate
FVC	Force vital capacity
GMA	Glycol methacrylate
H	*Heamophilus*
H infl	*Heamophilus influenzae*

HCL	Hydrochloride
HEPES	2-hydroxyethyl-1-piperazineethanesulfonic acid
HKB	Heat killed bacteria
HLA	Human leukocytes antigen
HRP	Horseradish peroxidase
HSP	Heat shock protein
IFN	Interferon
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IKK	IkappaB kinases
IL	Interleukin
IRAK1	Interleukin-1 receptor-associated kinase 1
I-κB	Inhibitor of κB
LTB 4	Leukotriene B 4
LPS	Lipopolysaccharide
LRP	Leucine-rich repeats
LTA	Lipoteichoic acid
MAPK	Mitogen-Activated-Protein-Kinase
MCP	Monocyte Chemotactic Protein
MFI	Mean fluorescence intensity
MK	Mevalonate kinase
MMP	Matrix metalloproteinase
MyD88	Differentiation factor 88
MAL	MyD88 Adapter Like Protein

NE Neutrophil elastase

NF- κB Nuclear factor κB

NHLBI National Heart, Lung, and Blood Institute

NICE National Institute for Health and Clinical Excellence

NTHI Nontypable *Haemophilus influenzae*

PAMP Pathogen associated molecular pattern

PBMC Peripheral blood mononuclear cells

PBS Phosphate buffered saline

PE Phycoerythrin

PerCP Peridinin chlorophyll protein

PGN Peptidoglycan

PMSF Phenyl methyl sulphonyl fluoride

Poly (I-C) Polyinosinic:polycytidylic acid

PRR Pattern Recognition Receptor

PSG Penicillin, Streptomycin and Gentamycin

RPMI Roswell Park Memorial Institute

SEM Standard error of the mean

SNP Single nucleotide polymorphisms

TAK 1 Transforming growth factor β–activated kinase 1

TBS Tris Buffered Saline

TGF Transforming growth factor

TIR Toll-interleukin-1 receptor

TIRAP Toll-Interleukin 1 Receptor Domain Containing Adaptor Protein

TIRIF Toll-Interleukin 1 Receptor Domain Containing Adaptor Inducing Interferon B

TLR Toll like receptor

TMB Tetramethyl benzidine

TNF Tumor necrosis factor

WHO World Health Organisation

DECLARATION OF AUTHORSHIP

I, ACHIKA MOREAU, declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

Abnormalities in bacterial sensing and inflammatory response to pathogens in the lung parenchyma of chronic obstructive pulmonary disease patients .....

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission.

Signed: .....

Date: .....

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Chapter 1: Introduction

1.1 Chronic obstructive pulmonary disease definition and epidemiology

Chronic Obstructive Pulmonary Disease (COPD) is defined by the American Thoracic Society (ATS) as "a disease process involving progressive chronic airflow obstruction because of chronic bronchitis, emphysema or both" (ATS, 1995). It is thought to happen as a consequence of long-term exposure to toxic gases and particles; in the West, this generally occurs as a result of long-term cigarette smoking. COPD is a major cause of morbidity worldwide. It is anticipated to become the third leading cause of death and the fourth most common cause of disability in the world by 2020 (Rabe et al., 2007) and affects 14 million patients in the United States alone, with the National Heart, Lung, and Blood Institute (NHLBI) estimating the annual cost for the US at $38.8 billion in 2005 (Foster et al., 2006). In the UK, approximately 3 million people have COPD; about 900,000 have been identified as COPD patients and an estimated 2 million people are yet to be diagnosed with COPD National Institute for Health and Clinical excellence NICE (2010).

1.1.1 Risk factors

In developed countries, cigarette smoking continues to be the main cause of COPD and is believed to account for approximately 90% of the cases. In developing countries the exposure to combustion products of biomass fuel can cause COPD from inhaled biomass smoke with the same clinical characteristics and mortality as tobacco smoke (Ramirez-Venegas et al., 2006, Regalado et al., 2006). Other factors, specifically occupational exposures which involve exposure to toxic particulates, may also participate in the development of COPD (Pauwels et al., 1999). COPD becomes more prevalent with increasing age (Fletcher and Peto, 1977). There is a well-defined genetic component for the development of the disease in a subset of individuals, which is alpha-1 antitrypsin (AAT) deficiency, but this is responsible for only 1% of the cases (Lomas and Silverman, 2001). Other studies have implicated genetic risk factors in the development of COPD but show conflicting reports of these genes identities. An association between single nucleotide polymorphisms (SNPs) in the promoter region of the tumour necrosis factor-alpha (TNF-α) gene and the development of COPD has been shown (Gingo et al., 2008). Other genes that have been implicated are genes encoding for matrix metalloproteinase and tissue inhibitors of metalloproteinase (Sampsonas et al., 2006, Pillai et al., 2009). Also the expression of hedgehog interacting protein (HHIP) at both mRNA and protein levels was found to be reduced in COPD lung tissues (Zhou et al., 2012). Others such as Ezzie et al. (2012) have implicated small non-coding RNA molecules (MicroRNAs) that modulate the levels of specific

genes and proteins. Another study (Cho et al., 2012) reported the identification of a susceptibility locus on chromosome 19q13. These studies, however, have not been consistently replicated, possibly due to limitations arising from the heterogeneity in smoking behaviours and comorbidities in the cohorts analysed in these studies. Co-morbidities associated with COPD include cardiovascular disease, depression, hypertension, diabetes and obesity and are usually associated with worse outcome (Almagro et al., 2002, Groenewegen et al., 2003, Gudmundsson et al., 2006, Wedzicha et al., 2000).

1.1.2 Diagnosis

Patients who have dyspnea, produce sputum on a regular basis or/and have chronic cough and have a history of exposure to the risk factors mentioned above are considered for COPD diagnosis. Spirometry is then used to make the diagnosis.

A reduced FEV_1/FVC ratio such that a post bronchodilator FEV_1/FVC ratio is less than 0.7, where FEV_1 represent the volume of air that can be forcibly exhaled in one second and FVC the forced vital capacity, indicate airflow limitation and therefore COPD (NICE, 2010) and The Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines to staging the disease (GOLD, 2016). The assessment of airflow limitation is done using FEV_1 to stage COPD severity into 4 stages (Table 1.1). Disease staging is important for determining the optimum treatment regime for the patient. However, the severity of airflow limitation is not the only factor that is taken into account in COPD assessment. Exacerbation risk and the presence of co-morbidities are also considered to guide therapy NICE (2010, GOLD, 2016). Furthermore, co-morbidities may also significantly affect outcome, even after long-term smoking cessation.

Table 1.1 Assessment of airflow limitation in COPD (Taken from Global Initiative for Chronic Obstructive Lung Disease GOLD 2016).

In patients with FEV_1/FVC < 0.70:		
GOLD 1	Mild	$FEV_1 \geq 80\%$ predicted
GOLD 2	Moderate	$50\% \leq FEV_1 < 80\%$
GOLD 3	Severe	$30\% \leq FEV_1 < 50\%$ predicted
GOLD 4	Very severe	$FEV_1 < 30\%$ predicted

1.2 Pathophysiology of the disease

COPD is a heterogeneous complex disease and pathologically three distinct disease processes can be observed, chronic bronchitis, bronchiolitis and emphysema.

1.2.1 Chronic bronchitis

The ATS defines chronic bronchitis as chronic cough and sputum production lasting at least three months for at least two years. Associated with this is the hyper secretion of mucus and sub-mucosal gland hyperplasia, which affects the large, conducting airway. Vestbo et al. (1996) reported that the production of sputum, as well as chronic cough, were associated with decline in FEV_1. Bronchitis is characterized by goblet cell metaplasia occurring as a result of local inflammation of the large airways, and several studies have provided evidence for an inflammatory process in the large airways (Di Stefano et al., 1996, Fournier et al., 1989). An inflammatory response in the mucus-secreting apparatus of the bronchi between 2 and 4 mm in diameter is associated with the symptoms of chronic bronchitis (Mullen et al., 1985, Saetta et al., 1997). Mucus hyper secretion is a direct result of goblet cell hyperplasia in COPD, and it is thought that overproduction and hyper secretion by goblet cells and the diminished removal of mucus are the main mechanisms responsible for excessive mucus in chronic bronchitis (Vestbo et al., 2013, Kim and Criner, 2013).

1.2.2 The small airways (bronchiolitis)

A major location of airway obstruction in COPD is the small conducting airway that includes smaller bronchi and bronchioles that are less than 2 mm in diameter (Yanai et al., 1992, Hogg et al., 1968).

Structural abnormalities in small airways in smokers with and without COPD involve accumulation of mucus exudates in the lumen and infiltration of the walls by inflammatory cells (Hogg et al., 2004). This obstruction occurs by blocking of the small airways lumen through mucus plugging or narrowing of the lumen due to the accumulation of inflammatory cells in the bronchiolar sub-epithelial tissue and also deposition of connective tissue in a way that thickens the walls and restrict the airway lumen (Matsuba and Thurlbeck, 1972). These lesions are responsible for the way bronchiolitis contributes to airflow limitation that defines COPD, (Verbeken et al., 1992, Hogg et al., 1968, Hogg et al., 2004). Also among the cellular events that occur is replacement of surfactant secreting Clara cells with mucus secreting cells (Shapiro and Ingenito, 2005)

1.2.3 Emphysema

Emphysema is defined by permanent destruction of alveolar wall. The size of the airspaces enlarges as a result of the destruction of the alveolar wall and that leads to poor gas exchange. It is believed that emphysematous lung destruction reduces maximal expiratory airflow and contributes to airflow limitation by loss of lung elasticity through destruction of alveolar attachment and parenchymal destruction and closure of small airways, all of these lead to a decline in lung function. Disability and premature death usually happens because of the slow progressive airflow limitation. There are two major types of emphysema mainly caused by smoking: Panacinar and centrilobular (Saetta et al., 1994). Ageing (Verbeken et al., 1992) and genetics, (A1AT) deficiency (Needham and Stockley, 2004) can also be a cause Emphysema is diagnosed mainly by computerized tomography (CT) scans or X-rays (Coxson et al., 2013), through low attenuation area (Kitaguchi et al., 2016, Goddard et al., 1982) but what happens in lung parenchyma at the cellular level is not well characterized in living patients due to the difficulties in accessing lung parenchyma tissue as it can only be obtain from post-mortem or patients that are being operated on (Segura-Valdez et al., 2000). Thus, much of our understanding comes from bronchoalveolar lavage (BAL) and peripheral airway specimens collected post-mortem. Leopold and Gough (1957) showed an infiltration of the terminal and pre-terminal bronchioles with lymphocytes and plasma cells and that led to centrilobular destruction. This was later confirmed by Hogg et al. (2004) who showed that an inflammatory process in the small airway was an important component of emphysematous destruction in COPD.

1.3 Effect of cigarette smoke

Pryor and Stone (1993) reported the use of a filter to divide cigarette smoke into two phases, tar and gas-phase smoke. Gas-phase smoke comprises both carbon-centred and oxygen-centred radicals. Tar has high concentrations of radicals especially semiquinone that can lead to the formation of superoxide and hydrogen peroxide. These exogenous oxidants further contribute to an increase in endogenous oxidants released from activated phagocytes that are recruited and activated by cigarette smoke (Pryor and Stone, 1993), which leads to an imbalance between oxidant and anti-oxidants. As previously described, smoking appears to affect the tissue pathology in lung compartments in different ways. Smoke induces an inflammatory response in both animals (Bhalla et al., 2009) and human lungs (Di Stefano et al., 1996). In humans, the inflammation persists long after smoking cessation (Hogg et al., 2004, Rutgers et al., 2000a, Willemse et al., 2005, Lapperre et al., 2007).

1.3.1 Evidence from animal models

A number of smoking animal models have been developed in order to elucidate the pathophysiological mechanisms that occur in response to acute and chronic smoke exposure. These include guinea pigs, dogs, rabbits, and also rats and mice. Guinea pigs were reported to be susceptible species to emphysematous changes, while susceptibility in mice varies from strain to strain and rat strains were more resistant to the induction of emphysema-like lesions (Wright and Churg, 1990). The suitability of such models is open to question, however, particularly in chronic models where smoke exposure levels equivalent to long term smokers cannot easily be achieved, and resultant pathology is affected by the dissimilarity of rodent lungs to those of humans (Churg and Wright, 2007). It is not easy to reproduce small airway pathology especially when using small animals such as the mouse and rat as they only have few levels of airway branching and there are challenges in measuring lung function considering that COPD definition relies on lung function measures. This indicates a potential advantage for developing models in larger animals such as the pig or sheep. However, animal models do not reflect human genetics or environment with regard to COPD.

In animal models, smoking leads to an increased inflammation in the pulmonary compartment, and some morphological changes similar to those in humans. However, important differences remain, particularly in the distribution of the pathology. While COPD is heterogeneous in humans, in animals, emphysema tends to be widespread and homogeneous. The reasons for these differences are unclear. Also, animal models often do not mimics the irreversible airflow obstruction with associated cough and sputum production (Mercer et al., 2015). There are also different disease stages within COPD, and only some of them may be mimicked in animal models. Furthermore the exact mechanisms behind these structural and inflammatory changes have also not been elucidated, but could reflect physiological or behavioural differences between animals and humans (Aaron et al., 2001). In addition, mice also differ considerably in respiratory tract function and anatomy from man (Mercer et al., 2015).

1.3.2 Parenchyma, central and peripheral airway changes in COPD

Cigarette smoke contains many oxidant and free radicals that affect lung parenchyma indirectly by activating inflammatory cells and therefore inducing inflammation that potentially leads to the mechanisms that are thought to be responsible for the development and progression of COPD/emphysema, which include the protease-antiproteases imbalance, epithelial cell apoptosis, oxidative stress, and extracellular matrix remodeling. These effects result in alveolar wall destruction, leading to an increase in size of the airspaces which then leads to poor

efficiency of gas exchange, air trapping and airflow obstruction (Churg and Wright, 2007, Moretto et al., 2009, Barnes, 2000, Rahman, 2005, Barnes et al., 2003, Demedts et al., 2006, Vlahovic et al., 1999).

Chronic bronchitis and bronchiolitis are two diseases that result from an inflammation in the central and peripheral airway. In the large airway of COPD patient, smoking results in infiltration by inflammatory cells, enlarged bronchial mucous glands and an increased number of goblet cells that can lead to mucus hyper secretion, contributing to airflow obstruction (Nagai et al., 1985).

In the peripheral airways, smoking induces an inflammation that is then followed by fibrosis and goblet cell metaplasia. This leads to structural changes in the bronchioles and the loss of elastin. Furthermore many studies have showed an increase in the number of inflammatory cells within the sputum and BAL fluid from COPD patients (Pesci et al., 1998, Vaitkus et al., 2013, Keatings et al., 1996). Narrowing of the lumen of bronchioles by inflammatory cell infiltrate is also thought to contribute to airflow obstruction.

1.4 Exacerbation in COPD: Definition and costs

Patients with COPD are not always in stable state but are prone to periods of exacerbations of the disease, and therefore assessment of COPD includes exacerbation risk as to establish the severity of the disease.

Exacerbation often occurs in COPD patients and describes the event of sudden worsening in airway function and respiratory symptoms. An episode usually lasts 7 days. However, returning to baseline function status can take a few months (Mannino et al., 2002, Rabe et al., 2007). The classical symptoms include: dyspnea, wheezing, cough and sputum production. It is an integral manifestation of COPD for most patients, with a subset of patients suffering frequent exacerbations, and high frequency is associated with worse prognosis (Donaldson et al., 2002). An exacerbation is diagnosed when a patient with COPD experiences sustained increase in cough, sputum production, and/or dyspnea that is more than normal everyday variation that is to say worsening in respiratory symptoms and change in medication (Wedzicha and Donaldson, 2003). Therefore, patients with COPD who exacerbate frequently represent a population requiring significant clinical intervention, as the consequences of an exacerbation (ECOPD) range from a self-limited illness to progressive respiratory failure and death with increasing health care cost (Donaldson et al., 2002, Quint et al., 2008). Price et al. (2006) reported in a UK National COPD Audit 2003 that 1.5 in ten patients with COPD admitted to hospital are reported

dead within 90 days of admission, and over 1 in 3 are readmitted to hospital during that time. Inhaled steroids were not found to reduce exacerbation frequency, however, inhaled corticosteroid with long acting β agonist (combined therapy) were more protective against exacerbations (Dransfield et al., 2013).

1.4.1 COPD exacerbation and microbiology

Exacerbations represent inflammatory events with increased airway and systemic inflammatory markers (Perera et al., 2007). Additionally, its clinical course (acute or sub-acute onset followed by slow recovery over days or even weeks) suggests an infectious cause. Bacteria and viruses are among the principal causes, with bacteria being associated with 50% of exacerbations, although it is thought that many of these cases may be secondary to initial viral triggers. This is supported by the many reports that have shown increased susceptibility of COPD patients to respiratory pathogens (Pang et al., 2008). It is reported that exacerbation can indicate an inappropriate excessive inflammatory response to colonizing bacteria, brought about by a current viral infection (Clancy et al., 1995, Perera et al., 2007) (Figure 1.1).

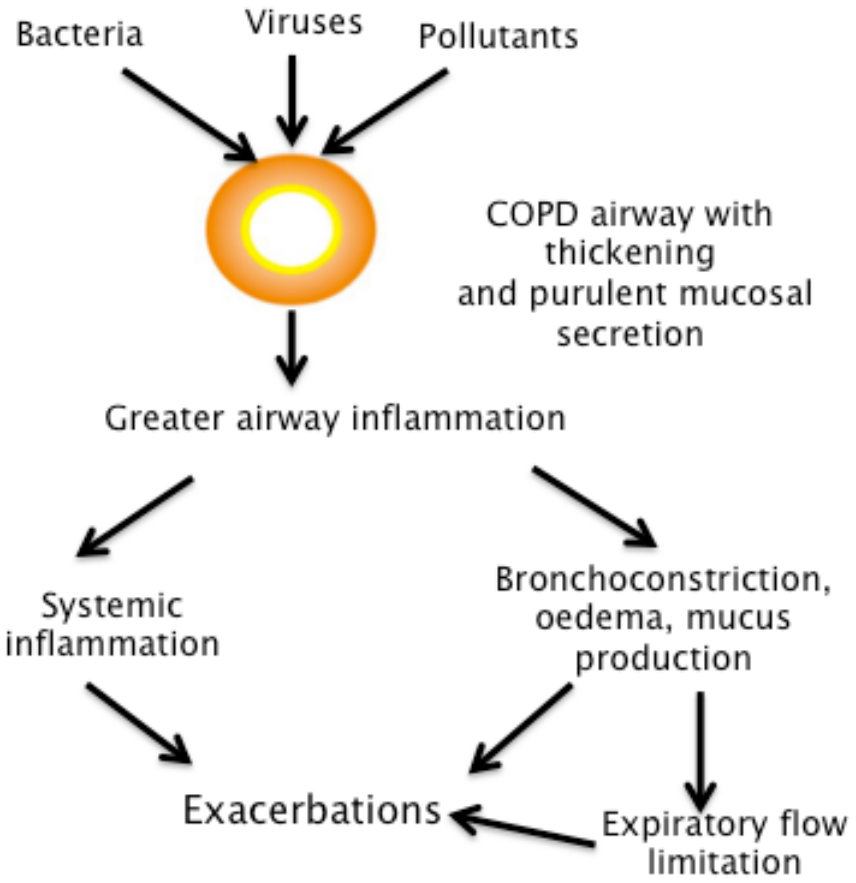

Figure 1.1 Triggers of COPD exacerbations. Exposure of the airways to bacteria, viruses and pollutants can lead to mucus hyper-secretion and airway inflammation, resulting in systemic inflammation and bronchoconstriction. The consequences of flow limitation and greater inflammation is exacerbation. Adapted from (Chung and Adcock, 2008).

1.4.1.1 Viruses

It is believed that 50% of exacerbations are viral and that antecedent viral infection is necessary for the development of bacterial exacerbations of COPD. In support of this theory, Soler et al. (1998) reported that patients who suffered from severe exacerbations, showed infection at the same time with influenza virus and a bacterial infection in 60% of cases. Similar finding were described by Murphy et al. (2005) who reported that *M. catarrhalis* exacerbations show a seasonal pattern similar to rhinovirus infections, which suggests an association between these two pathogens.

1.4.1.2 Bacteria

Bronchoscopic sampling of the tracheo-bronchial tree with either protected specimen brushings and/or BAL and sputum analysis has been used to look more closely to airway bacterial flora during exacerbations and frequently *H. influenzae* was isolated from COPD patients (Wedzicha and Donaldson, 2003). It is commonly present in the airways of adult with COPD (Monso et al., 1995, Murphy and Sethi, 1992, Chin et al., 2005). When present, this organism is the most dominant of the bacterial species (Butt et al., 1990, Bandi et al., 2001).

Bacteria have also been reported to mediate acute infective episodes (Murphy, 2006). Much evidence about the involvement of bacteria in exacerbations comes from antibiotics studies, since exacerbations are generally associated with increased sputum purulence and volume, and patients show benefit from antibiotics therapy as clinical trials found antibiotics to clinically improve patients with acute exacerbated COPD (Sethi, 2000b, Adams et al., 2000). Furthermore, oral immunotherapy with inactivated non-typable *H. influenzae* lessens severity of acute exacerbations in severe COPD (Tandon et al., 2010).

It is estimated that as many as 50% of COPD patients suffer from lower airway colonization by three strains of bacteria: noncapsulated *H. influenzae*, *Streptococcus Pneumoniae*, and *M. catarrhalis*. *H. influenzae* colonization was shown to relate to the degree of airflow obstruction and, in addition to colonizing during clinically stable periods, is associated with occurrence of exacerbations (Veeramachaneni and Sethi, 2006). Furthermore, Sethi et al. (2002) reported a critical role for colonizing non-typable *H. influenzae* in the pathogenesis of acute exacerbations of COPD.

Studies have demonstrated that smoking was a predictor of airway colonization (Monso et al., 1999). Another study of tracheobronchial colonization (Irwin et al., 1982) found evidence of colonisation in 50% of smokers. This effect is believed to be related to the impairment of lower airway clearance seen in COPD as smoking leads to the reduction in mucociliary clearance through secondary ciliary dyskinesia. Coupled with greater mucus secretion due to submucosal gland hypertrophy and hyperplasia in chronic bronchitis, mucociliary impairment can lead to a reduction in the ability to clear bacterial colonies and therefore creation of a local microenvironment, suitable to certain bacterial strains (Sethi, 2000b, Patel et al., 2002, Chung and Adcock, 2008). This in itself may make ideal conditions for biofilm formation. This in turn results in persistent, chronic bacterial colonization of the lower airway (Murphy and Sethi, 1992), which can in return stimulate secondary host defence and lead to airway inflammation even in stable state and therefore chronic inflammatory response with lung damage (Stockley, 1998).

Indeed this persistent inflammation causes a chronic cytokine milieu that in turn leads to increased inflammation. Studies have shown that airway inflammation in COPD both in the stable and during exacerbation is related to airway colonization (Hill et al., 2000) and levels of some cytokines were increased in COPD patients with airway colonization (Bresser et al., 2000). It is possible that host recognition of pathogens through pattern recognition receptors is altered, and thus results in skewed immunity to be tolerant of bacterial colonization.

1.4.2 Effect of exacerbations

Exacerbation cause changes in lung function, and it is reported that it accelerates the rate of decline in FEV_1. The accelerated decline in FEV_1 with age has been linked to the severity of airway inflammation (Di Stefano et al., 1998). Donaldson et al. (2002) have shown that patients who experienced frequent exacerbations (> 2.92 per year) suffered a significantly greater decline in FEV_1 than patients who had infrequent exacerbation (Figure 1.2) and were prone to readmission to the hospital; exacerbations frequency is strongly related to the airway inflammatory markers. Additionally, patients who experience an abnormal number of exacerbations have higher inflammatory markers when stable, and more likely to have increased bacterial colonization (Patel et al., 2002). Furthermore, Sethi (2000b) reported that exacerbations that are linked to the two gram-negative bacteria *H. influenzae* and *M. catarrhalis*, are associated with significantly higher levels of inflammatory markers compared to the stable state. Such increases in inflammation instigate airway mucosal edema, bronchospasm and airway mucus hypersecretion, which in turn can be related to the typical symptoms of exacerbations of dyspnea, wheezing, cough and sputum production (Aaron et al., 2001, White et al., 2003).

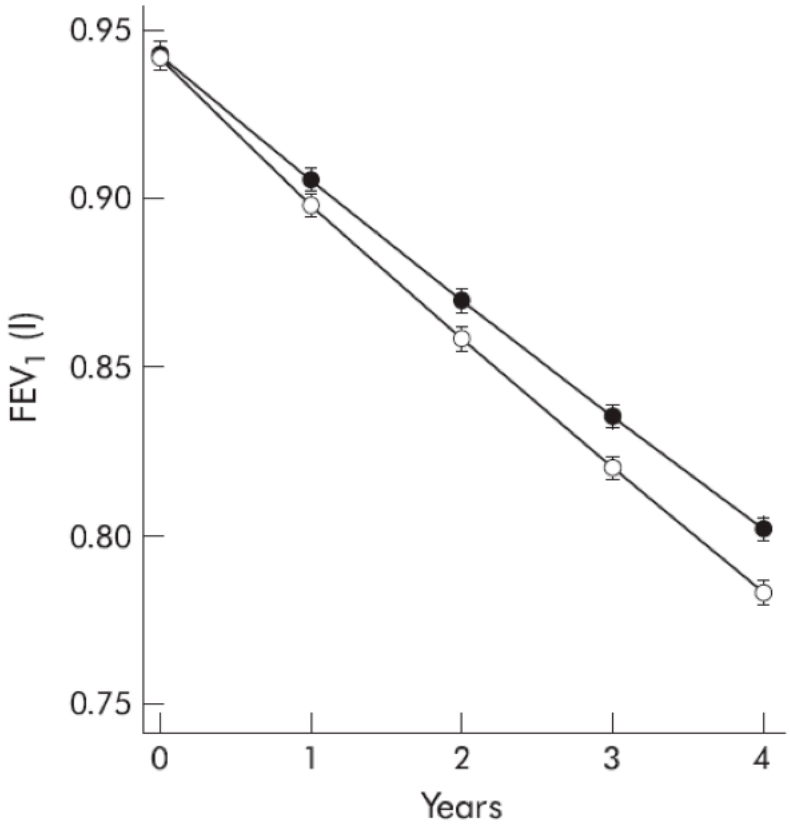

Figure 1.2 Relationship between FEV_1 and exacerbation over the course of several years. Greater loss of lung function in frequent exacerbators compared to infrequent exacerbators. Filled circles represent frequent exacerbators and hollow circles represent infrequent exacerbators. Adapted from (Donaldson et al., 2002).

1.4.3 Causes of bacterial exacerbations

Bacteria protect themselves from antibiotics and from innate respiratory epithelial clearance mechanisms by forming biofilms. *H. influenzae* biofilms were reported to be resistant/tolerant to a variety of antibiotics (Slinger et al., 2006). Acquisition of new strains of certain bacterial species (Figure 1.3) was discovered to be associated with a greater than two-fold increased risk of exacerbations of COPD (Sethi et al., 2002). Also pre-existing strains can cause exacerbations by modification of their antigenic structure as the modification allows them to escape the host immune response and multiply in the airways leading to a greater inflammation and therefore symptoms (Duim et al., 1994).

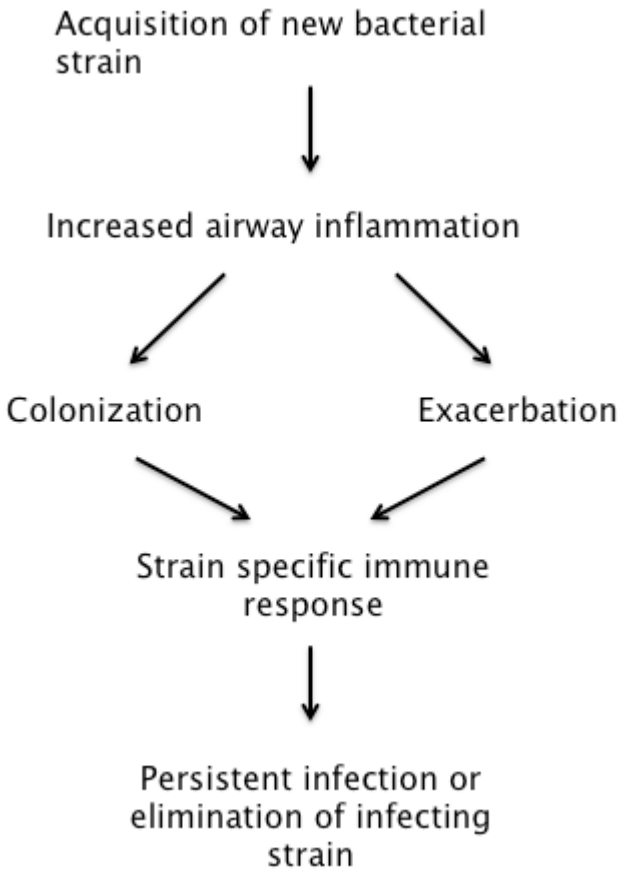

Figure 1.3 Association between acquisition of new bacterial strains and exacerbation. Acquisition of new strains can lead to changes in airway inflammation, resulting in either colonization or exacerbation. The new strain is then either eliminated, or leads to persistent infection. Adapted from (Sethi et al., 2002).

1.4.4 Bacterial adherence and localization

H. Influenzae is an extracellular bacteria, and so the first interaction between *H. influenzae* and the host is the adherence of bacteria to the mucus that is secreted by the goblet cells distributed throughout the pseudostratified epithelium of the trachea and bronchi, with additional secretory components provided by the sub mucosal glands. Bacteria can also adhere to the upper airway. *H. influenzae*'s outer membrane proteins P2 and P5 facilitate binding of the organism to this mucus (Reddy et al., 1997). The subsequent steps involve invasion of respiratory epithelial cells, transcytosis to the subepithelial compartment, or formation of microcolonies, that leads to a biofilm (Erwin and Smith, 2007, Ikeda et al., 2015). Some isolates of NTHI express pili, which are polymeric helical structures of a 5 nm diameter and up to 450 nm long. Pili encourage interaction with heparin binding extra-cellular matrix protein and facilitate bacterial binding to fibronectin (St Geme, 2002). *H. Influenzae* also expresses several adhesion molecules. This bacteria was found in the bronchial submucosa of patients with chronic

bronchitis and also between epithelial cells (van Schilfgaarde et al., 1999). It is also believed that bacteria can get tangled in the mucus and escape the innate immune clearance. Additionally, *H. influenzae* has been identified in macrophage-like cells (Forsgren et al., 1994).

1.5 Inflammation in COPD

Increased inflammation is a characteristic for many respiratory conditions including influenza infection, asthma and, as suggested in the previous sections, COPD. However, the patterns of inflammation differ. COPD is known for abnormal inflammatory response to tobacco smoking, and displays characteristics indicative of repetitive tissue injury that result from cigarette smoke insult in that it shows persistent inflammation. Lung repair mechanisms start immediately following each insult and involve an acute inflammatory response followed by an attempt at resolution.

Part of the inflammatory response is associated with vascular changes and tissue remodelling (Hogg et al., 2004, Kim et al., 2013). The tissue remodelling in emphysema starts with a process of connective tissue deposition that thickens the terminal bronchioles in moderate COPD, and removal of the bronchiole in severe COPD (Hogg et al., 2004). In the bronchus, tissue remodelling appears to be characterised by moderately increased sub-epithelial collagen deposition and patchy conversion of the normal pseudostratified epithelium by goblet cell metaplasia and squamous metaplasia.

The other part involves a cellular component. In the cellular component of this inflammation, there is movement and recruitment of inflammatory cells to the site of insult, and release of cytokines. Not only the innate immune response with epithelial activation and infiltration of neutrophils and macrophages, but also the adaptive immune response with influx of cytotoxic $CD8^+$ T cells, $CD4^+$ T helper cells and B cells, is included in the inflammatory process. Furthermore increased numbers of lymphoid follicles are found in the lungs of patients with COPD (Chung and Adcock, 2008). Remarkably, inflammation in the lungs persists for years after smoking cessation and evidence is now pointing towards the importance of the adaptive immune system due to the increasing presence of T lymphocytes in the airway and lung tissue (van der Strate et al., 2006, Hogg et al., 2004). This leads to the hypothesis that autoimmunity may be the process that drives the disease, and components of the extracellular matrix were put forward as potential antigens. Lee et al. (2007) reported that emphysema could be an autoimmune disease characterised by anti-elastin antibodies and T-helper type-1 (Th1) responses, which correlates with emphysema severity. However, the role of anti-elastin

antibodies is still debated. Another study by Rinaldi et al. (2012) showed that collagen V-mediated autoimmunity was increased in smokers but in variation to the work of Lee et al. (2007) did not identify a systemic immune response against elastin. There is also evidence of increased titres of antinuclear antibodies in COPD according to Bonarius et al. (2011).

In addition to pulmonary inflammation, COPD patients also suffer from a systemic inflammation that is present in some patients with stable disease and all patients during exacerbations. The increased presence systemically of inflammatory mediators such as acute phase proteins and markers of oxidative stress were regarded as evidence for the presence of systemic component to COPD. The ATS/ERS guidelines indicate that even though COPD affects the lungs, it also create significant systemic consequences (Celli and MacNee, 2004) as it is thought to be associated with the co-morbidities that are linked to the disease, and certainly reduced quality of life.

1.5.1 Epithelium in COPD

Airway epithelial cells act as the primary line of host defence against various environmental insults, such as cigarette smoke and bacteria (Murphy et al., 2005).

The epithelial lining of the airways provides a barrier against microorganisms and harmful molecules through mechanical clearance of the mucus where bacteria and inhaled particles are trapped (Adler and Li, 2001).

Endogenous and exogenous oxidants such as superoxides and H_2O_2 may impair mucociliary clearance by decreasing respiratory epithelial cilia beat frequency. The initial site of exposure to tobacco smoke is the epithelial mucosa of the lung and repeated damage with smoke can result in bronchial epithelial squamous metaplasia, a feature of COPD, especially in moderate to severe disease (Kim et al., 2008, Lapperre et al., 2007). Both COPD and lung cancer are associated with epithelial changes such as squamous metaplasia (Herfs et al., 2012).

1.5.2 Epithelium and bacteria in COPD

Bacteria have the potential to disrupt host defence mechanisms and evade the host immune system by forming biofilms, and their presence in the lower airways of COPD patients indicate an infringement of these mechanisms. That leads to a vicious cycle of epithelial cell damage, degrading complement and immunoglobulins (Moller et al., 1998), impaired mucociliary clearance, mucus hypersecretion, increased sub-mucosal vascular leakage and impaired phagocytosis, which can lead to increased bacterial load, increased cytoxicity (Taylor et al.,

2010), and inflammation (Figure 1.4). Moreover, airway bacterial load was found to promote airway inflammation (Toews et al., 1979). In line with that, Hill et al. (2000) reported that there were strong association between airway inflammation and the presence of bacteria at 10^6 or greater colony-forming units per millilitre. This inflammation is associated with loss of epithelial integrity and results in leakage of serum proteins into the airways.

Lung explants from patients with end-stage pulmonary disease showed epithelial and sub-epithelial localization of *H. influenzae* that had penetrated into the sub-epithelial layer by passing between epithelial cells (Moller et al., 1998). That makes airway bacterial infection a major challenge in healthcare for COPD.

These innate and adaptive immune responses of the airway epithelium can be impaired by different agents, and that leads to susceptibility to infection. Bronchial epithelial cells from patients with COPD were found to express higher levels of MUC18 that functions as an adhesion molecule to keep bacteria on epithelial cells (Schulz et al., 2003, Simon et al., 2011).

This damage to the epithelium/lung is thought to be the major reason for recruitment of inflammatory cells in airway.

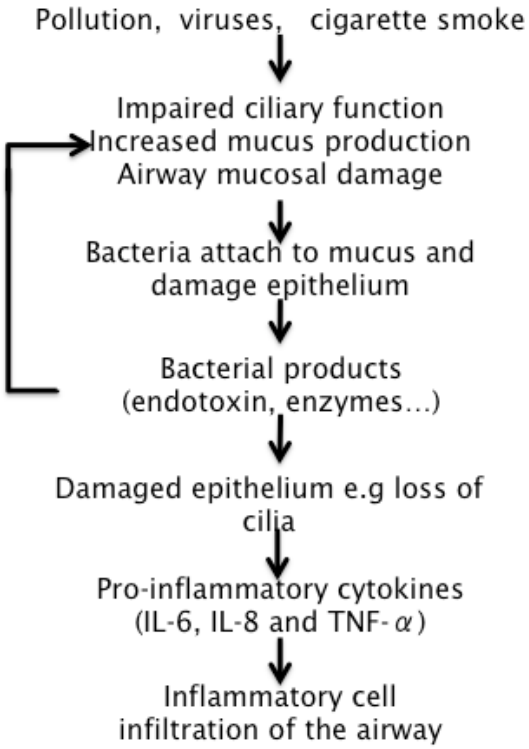

Figure 1.4 Pathways by which bacteria cause damage to the airways. Smoke, viruses and pollution can cause damage to the airway mucosal cells. Bacteria can then more easily attach to the epithelium. The bacteria release various products such as

endotoxin, resulting in the release of mediators such as pro-inflammatory cytokines and chemokines, which leads to inflammatory cell infiltration (Khair et al., 1996).

1.5.3 Types of inflammatory cells infiltrate in COPD

1.5.3.1 Neutrophils

Neutrophils are important in the pathogenesis of chronic lung diseases because of their ability to release a variety of oxidants and proteolytic enzymes; they also have the ability to synthesize pro-inflammatory cytokines, chemokines and growth factors, in response to a variety of stimuli (Cassatella, 1999).

The COPD disease process demonstrates a predominantly neutrophilic pattern in sputum, which is inversely correlated with lung function FEV_1 (Di Stefano et al., 1998), indicating that neutrophilic inflammation is closely linked to increased airway obstruction. This idea is supported by Hogg et al. (2004) who, has shown that sputum neutrophilia is high in advanced COPD, and is linked with the presence of greater airflow obstruction, together with an accelerated decline in lung function. Exacerbations associated with bacterial pathogens, specifically *H. Influenzae*, exhibit significantly more neutrophilic inflammation than non-bacterial episodes. Once activated, neutrophils release proteins such as neutrophil elastase, matrix metalloproteinase MMPs, and oxygen radicals such as super-oxide anion, hydrogen peroxide and hypohalides (Henricks and Nijkamp, 2001) all of which can cause tissue damage and are essential to the pathogenesis of COPD. The phagocytic ability of neutrophils is impaired by cigarette smoke extract through suppression of caspase-3-like activity in the neutrophils. As caspase activation was found to be required for neutrophile phagocytosis as well as apoptosis and cigarette smoke suppress its activity at a level that is sufficient to impair neutrophil phagocytic activity (Stringer et al., 2007). Studies of pulmonary infection have shown a close relationship between bacterial load and neutrophil recruitment (Toews et al., 1979).

Many neutrophil chemoattractants were found in lung secretions during infections. It is believed that bacteria themselves may generate some of these compounds (Wilson et al., 1985). However most are probably the result of activation of neutrophils and/or macrophages in the COPD lung. Clearance of neutrophils from the lung after an insult involves apoptosis of these cells, followed by their phagocytosis by macrophages.

Perturbation of this cycle, either by prolonged activation of neutrophils or delayed apoptosis would lead to further insult to the lung (Filep and El Kebir, 2009). In COPD, impaired phagocytosis of neutrophils has been reported. The latter could lead to secondary necrosis of

neutrophils and release of neutrophil elastases and MMP (Richens et al., 2009). This impaired phagocytosis can also lead to impaired bacterial clearance, and therefore colonization of the lung.

1.5.3.2 Monocytes/macrophages

Blood monocytes differentiate into macrophages when they migrate through the blood vessel walls into different organs. Macrophages are important in the defence of the respiratory tract.

Normally, alveolar macrophages are located adjacent to the alveolar spaces and make nearly 95% of airspace leukocytes, with 1 to 4% lymphocytes and only 1% neutrophils and are highly phagocytic, producing multiple inflammatory mediators (Johansson et al., 1997). In the alveolar walls and in airway walls, interstitial macrophages are located and they account for half of the total numbers of macrophages in the lung (Barnes, 2004).

Macrophages are cells of the innate immune system and are heterogeneous and complex. Different subsets of macrophages with distinct function have been described. Those that mediate defence of the host against bacteria and viruses called M1 macrophages or classically activated macrophages. And those that have anti-inflammatory function and participate in wound healing called M2 or alternatively activated. Macrophages contribute to the resolution of inflammation by releasing anti-inflammatory mediators and removing apoptotic cells by efferocytosis and they can also present antigen. M1 and M2 phenotypes are characterised by plasticity indicating the existence of intermediate phenotypes and cell surface markers are used differentiate these phenotypes. In a recent study, Desch et al. (2016) detected five distinct mononuclear phagocytes in the lung using flow cytometry. The mannose receptor CD206 was used to discriminate lung mononuclear phagocytic cells and was present in four with the exception of the pulmonary dendritic cells. One of the fours that were $CD206^{+}$ was alveolar macrophages and was distinguished using CD16 that differentiate tissue monocytes from alveolar macrophages. The last two subsets that the group identified were monocytes derived cells.

The role of macrophages in removal of potentially pathogenic microorganisms via phagocytosis is crucial in maintaining the normally sterile environment within the lung. They are effectors cells in COPD through their potential release of extracellular matrix proteins and lipid mediators such leukotrienes, prostaglandins, cytokines, chemokines and MMPs which can attract neutrophils, monocytes and T lymphocytes. Their phagocytic capacity is compromised in COPD. This could be due to the effect of smoking (Kirkham et al., 2004, Hodge et al., 2007). Alveolar macrophages from COPD patients phagocytose a small number of apoptotic airway epithelial

cells (Barnes, 2004), and less *H. influenzae* compared with smokers without COPD (Berenson et al., 2006). This also affects the rate of clearance of apoptotic neutrophils (Kirkham et al., 2004, Hodge et al., 2007).

Their increased number that was seen in some of the lung compartments can be the result of recruitment of monocytes from the circulation in response to monocyte-selective chemokines produced in the lungs (Banerjee et al., 2004). The elevated numbers of macrophages are associated with the severity of COPD (Di Stefano et al., 1998).

1.5.3.3 T cells in COPD

T cells differentiate into two subpopulations expressing CD4 (helper cells) or CD8 (cytotoxic T cells) after being presented with antigen that is taken up by dendritic cells that highly express class I major histocompatibility complex (MHC) molecule. Naive T cells can enter lung parenchyma (outside blood vessels) once activated by antigen-bearing dendritic cells. In COPD their numbers in lung parenchyma and small airways correlate inversely with lung function (Saetta et al., 1998, Saetta et al., 1999). Although they are prominent in COPD airways, their role still remains speculative. In contrast to asthma, which is normally associated with a Th2 inflammatory profile, COPD patients show a more Th1-driven T-lymphocyte phenotype, with the helper T cell population principally secreting INF-γ. The latter was found to predominate in the peripheral circulation of COPD patients (Paats et al., 2012). Furthermore, Freeman et al. (2010) reported enhanced cytotoxic activity of these cells in COPD by higher concentrations of perforin in sputum and lung parenchyma.

1.5.4 Systemic inflammatory cell changes in COPD

There are two facets to the potential for inflammatory cells to cause damage in the COPD lung – namely, an increase in cell numbers or an alteration in cell activation status, which could make the cells more likely to damage tissues in which they are resident. For example, neutrophils harvested from blood circulation of COPD patients showed high level of reactive oxygen species (ROS) and also showed enhanced expression of some adhesion molecules like Mac-1 compared to smokers with normal lung functions and never smokers (Noguera et al., 2001). Neutrophils from COPD patients are also less likely to die by apoptosis and demonstrate raised markers of activation status (Taggart et al., 2005, Anthony et al., 2013). They also demonstrate increased binding capacity to the endothelium compared to those from healthy subjects (Noguera et al., 2001).

In a similar vein, circulating monocytes are also partially activated and show abnormal morphology in COPD, and additionally show defects in IL-10 inhibitory effect on IL-8 (Castellucci et al., 2015). They also express disease specific genes and show a profile of released pro inflammatory molecules resulting from LPS stimulation distinct form control (Aldonyte et al., 2003, Poliska et al., 2011).

COPD is typically associated with a Th1 response due to increased circulatory levels of IFN-γ, however the primary lymphocyte dysfunction is an elevation of $CD8^+$ T lymphocytes in the circulation, and their increased activation status (Hodge et al., 2006, Wang et al., 2013).

Another study also demonstrated an increase in the number of $CD8^+$ T cells infiltrating the pulmonary arteries, as well as a relationship between leucocytes infiltration and endothelial dysfunction in COPD patients (Peinado et al., 1999). The bronchial wall is more vascularised in COPD compared to healthy control, which may contribute to or facilitate leukocyte recruitment in COPD (Kranenburg et al., 2005).

1.5.5 Inflammation in the different compartments of the lung

COPD is a disease that broadly affects the pulmonary system, from the conducting to the respiratory airways. Whilst the primary trigger for the disease is thought to be the insult of the bronchial and alveolar compartments by the components of smoke, the downstream effects of the inflammation that is caused by such insult, such as an increased inflammatory cell load, and the products thereof, is thought to then contribute to the disease pathogenesis. Many studies have reported an increase in inflammatory cells in the different compartments of the lung of COPD patients (Maestrelli et al., 1995, Keatings et al., 1996, O'Donnell et al., 2004).

1.5.5.1 Inflammatory cells in bronchial biopsies and sub-mucosa

Several groups have looked to bronchial biopsy (Di Stefano et al., 1996, Saetta et al., 1993, Turato et al., 1995, O'Shaughnessy et al., 1997). Immunohistochemical examination of bronchial biopsies has revealed higher numbers of neutrophils infiltrating the bronchial epithelium in COPD (Pesci et al., 1998). Their number has also been found to be elevated in bronchial lavage of patient with chronic bronchitis (Thompson et al., 1989). Neutrophils may not be the most significant cells contributing to airways pathology in the bronchial compartment in COPD however Results have shown that patients with COPD have an elevated number of macrophages in the bronchial compartment compared to healthy non-smokers (Wilson et al., 1985). Macrophages were increased in number in the bronchial sub-mucosa of COPD, and their number increased with increasing disease severity. In addition to the increased number of

macrophages, an increase in the number of infiltrating $CD8^+$ T cells has been reported using immunohistochemical markers in the airway submucosa in samples taken from COPD patients compared to normal non-smoking subjects and subjects with chronic bronchitis without airflow limitation (Fournier et al., 1989, Di Stefano et al., 1996, Saetta et al., 1993, O'Shaughnessy et al., 1997). An increase in neutrophils, macrophages and $CD8^+$ T cells in the bronchial mucosa, has been reported (Saetta et al., 1993). Similar increases in neutrophils and $CD8^+$ T cells, as well as airway eosinophilia, were reported using bronchial biopsy from a cohort study at exacerbation (Saetta et al., 1994, Zhu et al., 2001). A separate COPD phenotype characterised by eosinophilia is now recognised, however represents a very small proportion of COPD patients and may represent those with overlap syndrome.

1.5.5.2 Inflammatory cells in sputum

In studies of induced sputum (a biofluid that allows evaluation of cell infiltrate in the airway lumen) neutrophils were found to make up a significant percentage of inflammatory cells in sputum and have been shown to be present in elevated percentage in COPD (Keatings et al., 1996, Rutgers et al., 2000b). O'Donnell et al. (2004) reported that sputum neutrophil counts were significantly related to COPD severity. In line with these findings, Papi et al. (2006) reported that sputum neutrophils were raised during COPD exacerbation. Sputum from current smokers showed increased mast cell numbers compared to ex-smokers who instead had increased neutrophils (Wen et al., 2010). A lower percentage of macrophages and T lymphocytes in sputum than in BAL in COPD have also been reported in chronic bronchitis patients (Maestrelli et al., 1995).

1.5.5.3 Inflammatory cells in the peripheral airways

Saetta et al. (1998), using immunohistochemistry in a study done on airways from surgical specimens, observed increased T-lymphocyte infiltration in peripheral airways of smokers with airway obstruction in comparison to smokers without airway obstruction, whilst the number of neutrophils, macrophages were not different between the two groups. In contrast, the results of Bosken and co-workers (1992) reported no differences in $CD8^+$ T cells in the peripheral airway; however the number of submucosal polymorphonuclear leukocytes was related to the smoking pack years. This inflammatory cell infiltrate contributes to the obstruction of the airways (Hogg et al., 2004) reported that progression of COPD is associated with increasing infiltration of the small airways (bronchioles, which is those that are smaller than 2 mm in diameter) by neutrophils, macrophages, $CD4^+$ T cells, and other lymphocyte subtypes and recently, Utokaparch et al. (2014) reported increased macrophages and neutrophils in the small airway.

1.5.6 Immunohistochemistry finding about cell infiltration

In extension to these findings, Saetta et al. (1999) using immunohistochemistry showed that smokers with COPD have an infiltration of $CD8^+$T cells in lung parenchyma and pulmonary arteries but not macrophages. These findings were in contrast to Finkelstein and co-workers who demonstrated an increase in macrophages and T cells in lung parenchyma (Finkelstein et al., 1995). They, however, only examined the alveolar walls, which may not have provided a complete picture of the parenchymal infiltration by these cells; also, the lung was not washed to remove blood vessels and was fixed in formalin without removal of the small airways.

Using lung parenchyma from patients undergoing thoracotomy and from those who died suddenly from non-respiratory reasons, Eidelman et al. (1990) found an increased number of inflammatory cells localized within the alveolar wall of smokers.

1.5.7 Cytokines and inflammation in COPD

Tumour necrosis factor-α (TNF-α) is an important cytokine in most inflammatory reactions (Barnes, 2008). It is a pro-inflammatory cytokine, produced by macrophages, T cells, B cells, monocytes, neutrophils, eosinophils, fibroblasts, keratinocytes, epithelial, mast cells and endothelial cells under inflammatory conditions (Suzuki et al., 2008).

TNF-α is thought to up-regulate the transcription factor NF-KB which then switches on the transcription of a number of genes, leading to up-regulation of the inflammatory response (Chung et al., 2001). Macrophages and bronchial epithelial cells are stimulated by TNF-α and IL-1β to produce matrix metalloproteinase-9 (MMP-9), and extracellular matrix glycoproteins such as tenascin respectively.

Cigarette smoke enhances CXCL8 (IL-8) gene expression and release by bronchial epithelial cells, and also the release of TNF-α and IL-6 by alveolar macrophages through activation of NF-KB (Chung et al., 2001). Furthermore a variety of inflammatory mediators and proteases, such as TNF-α, TGF-β, IL-1β, and IL-8 are secreted by cigarette smoke activated epithelial cells (Hellermann et al., 2002, Mio et al., 1997, Kawasaki et al., 2001).

Inhibitors of TNF-α were shown to be effective therapies in several chronic inflammatory conditions, including rheumatoid arthritis (Lipsky et al., 2000), however treatment of COPD patients with anti-TNF-α antibody infliximab did not lead to a beneficial results (Rennard et al., 2007).

IL-8, a chemo-attractant of neutrophils that is secreted by macrophages, neutrophils, and airway epithelial cells, is present at high concentrations in induced sputum and BAL fluid of patients with COPD (Beeh et al., 2003).

Additionally, bacterial products can initiate and perpetuate lung inflammation indirectly by inducing the local secretion of IL-8 (Khair et al., 1996). During bacterial infection, there is an acute phase response where there is release of TNF-α and then IL-6 followed by neutrophil degranulation.

Moreover, exacerbations that are linked with *H. influenzae* demonstrated higher levels of IL-8, TNF-α, epithelial-derived neutrophil attractant (ENA-78) and neutrophil elastase (NE), stimuli that will lead to the activation of NF-Kβ (Sethi, 2000a, Gompertz et al., 2001, Mallia and Johnston, 2006).

COPD patients have significantly higher levels of monocyte chemo-attractant protein-1 (MCP-1) in their sputum when compared with healthy volunteers and smokers. Also, alveolar macrophages from smokers and COPD patients release high amount of MMP-9, IL-6 and MCP-1 (Aldonyte et al., 2003).

1.6 Innate immune system and pathogens recognition receptors in COPD

Pattern-recognition receptors (PRRs) activate innate immune cells after pathogen encounter. There is a variety of pattern recognition receptors that are used by the innate immune system and these receptors can be expressed on the cell surface, in intracellular compartments, or secreted into the bloodstream and tissue fluids (Akira et al., 2006). Their main functions include opsonization, activation of complement and coagulation cascades, and phagocytosis. A class of PRR that have been described in mammalian cells is that of Nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs). It is composed of 23 members in humans (Inohara and Nunez, 2003). NOD-1 and NOD-2 are the most-characterised NLRs and are involved in the detection of intracellular bacteria (Chamaillard et al., 2003, Girardin et al., 2003). Retinoic acid inducible gene-I (RIG)-like receptors (RLRs) are also another class of PRRs that have been identified. These comprise two cytosolic PRRs named RIG-I and melanoma differentiation-associated gen 5 (MDA5). These receptors detect intracellular RNA species related to virus infection (Mogensen and Paludan, 2005).

Amid the best-documented PRRs are Toll-like receptors (TLRs) (Akira et al., 2001). TLRs were reported to initiate recognition of pathogen-associated molecular patterns (PAMP), which are preserved molecular structures that exist in microbes as a part of the innate immune system. TLRs recognize bacterial ligands and some autoantigens and consequently resulting in the activation of proinflammatory cytokines and chemokines (Akira et al., 2001). To date, nine have been identified within this family (TLR 1-9), and reported to be expressed by a variety of cells.

1.6.1 TLR structure and signalling

Structurally TLRs consist of two domains: an extracellular (ectodomain) and a cytoplasmic domain (Figure 1.5). TLRs extracellular domain has a horseshoe structure and contains leucine-rich repeats (LRR). The concave surface of the LRR domains is implicated directly in the recognition of many pathogens (Akira et al., 2001). After ligand binding, TLRs dimerize and undertake the conformational change necessary for the recruitment of downstream signalling molecules (Underhill and Ozinsky, 2002). TLRs signals through two pathways: Differentiation factor 88 (MyD88)-mediated pathway and the Toll-interleukin-1 receptor (TIR)-domain-containing adaptor mediated pathway. The MyD88 pathway leads to the activation of the transcription factor NF-KB. The latter activates several genes that are implicated in the inflammatory reactions. While the Toll-interleukin-1 receptor (TIR)-domain is responsible for the induction of IFNs, whose stimulation brings about an antiviral state in the cells. TLR 2, which is the main PRR recognizing gram-positive bacteria, triggers only the activation of MyD88-mediated pathway while TLR 4 activates both pathways, so its agonists activate NF-KB and induce IFN production.

Four TIR domain adaptors are involved in the function of TLRs. These domains can act in pairs: MyD88 and TIRAP/MAL, and also TRIF/TICAM-1 and TRAM1. Whereas TLR 4 uses all four, TLR 9 signals via MyD88, and TLR 3 mainly via TRIF. TLR signalling through MyD88 cause the activation of mitogen- activated protein kinases (MAPKs) and (NF-KB). Most of the TLRs induce a Th1-type response on activation, although TLR 2 is believed to be involved in the induction of Th2 responses (Redecke et al., 2004) and TLR 4 can have anti-inflammatory role when activated via the MyD88-independent pathway (Chaplin, 2006). While both TLR 2/4 recognize bacterial component, TLR 3 recognizes poly (I-C) and double stranded viral RNA (Alexopoulou et al., 2001).

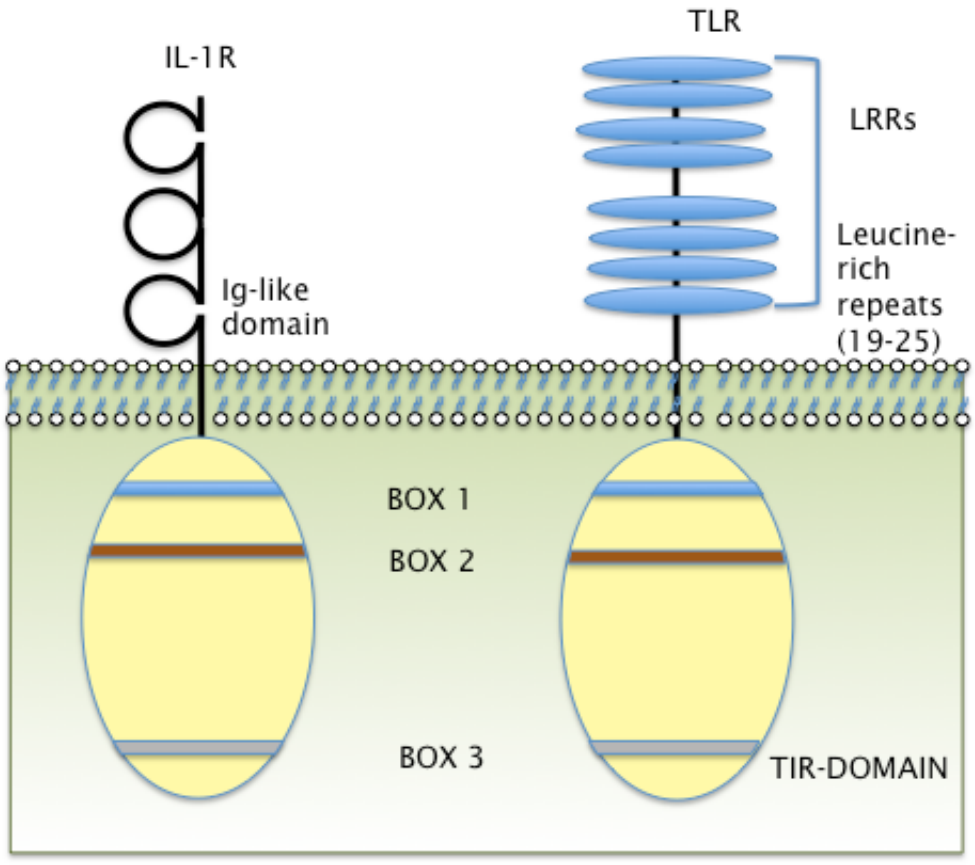

Figure 1.5 Structure of a typical toll-like receptor (TLR). This consists of TLR extracellular domain containing leucine-rich repeats which is involved in protein-ligand binding, and the cytoplasmic TIR domain, which is involved in adaptor protein binding. TIR = the Toll/Interleukin-1 receptor. Adapted from (Akira and Takeda, 2004).

As mentioned before, activation of TLR 4 can be mediated through both MyD88 dependent or independent pathways. Association of MyD88 (in the dependent pathway) recruits and encourages the interaction with Interleukin-1 receptor-associated kinase IRAK4 this leads to the phosphorylation of IRAK1 and subsequent phosphorylation of TRAF6. TRAF6 stimulate activation of transforming growth factor B-activated kinase 1 TAK1 causing the activation of I kappa B kinase IKK complex and at the end phosphorylation of I-KB. Phosphorylated I-KB undertakes ubiquitination and degradation. Freed NF-KB moves into the nucleus and induce the expression of inflammatory cytokine genes. TAK1 simultaneously activates MAP kinase cascade, causing the activation of AP-1 and induction of cytokine genes. In the MyD88-independent pathway, TRIF is recruited to the TIR domain, which further convey the signal. This leads to phosphorylation of IRF3 and also TRAF6 causing the induction of pro-inflammatory cytokine genes and type interferon genes (Figure 1.6).

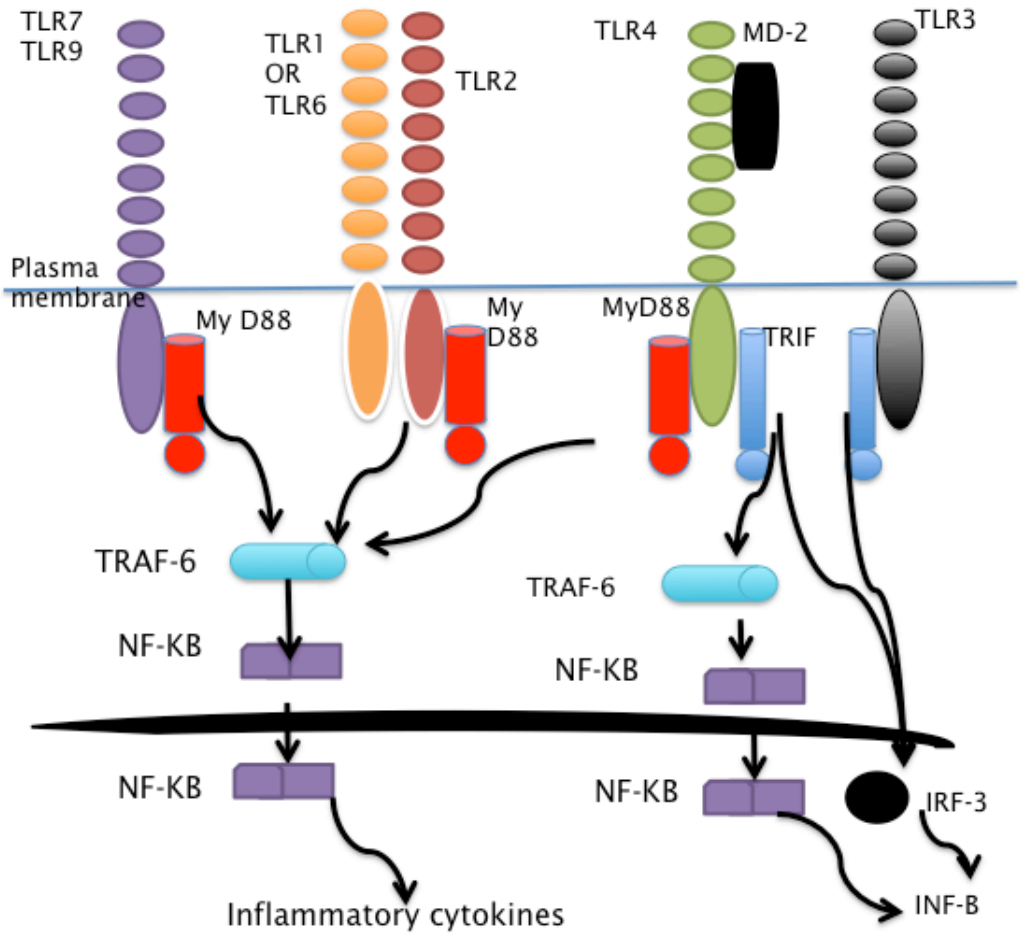

Figure 1.6 Toll-like receptors (TLR) signal transduction pathways. All TLRs other than TLR 3 proteins use the adapter protein MyD88 to initiate a signaling pathway, resulting in the activation of the transcription factor (NF-KB) in a TRAF-6-dependent manner. TLR 4 uses an additional pathway called the MyD88-independent pathway. These signaling events lead into the expression of the pro-inflammatory cytokines, such as IL-1β and TNF-α. MyD88 = Myeloid differentiation primary response gene 88, TRAF-6 = TNF receptor associated factor-6. NF-KB = Nuclear factor kappa light chain enhancer of activated B cells. IRF = Interferon regulatory factors. TRIF = TIR-domain-containing- adapter-inducing interferon-β. Taken from (Takeda and Akira, 2005).

1.6.2 TLR and bacterial component

1.6.2.1 TLR 4 ligand: LPS

LPS, a major component of the outer membranes of Gram-negative bacteria, is the main ligand for TLR 4, other ligands include respiratory syncytial virus protein F, and some endogenous ligands like heat shock protein and lipoteichoic acid (Palsson-McDermott and O'Neill, 2004). LPS

can provoke a life-threatening condition called endotoxic shock (Palsson-McDermott and O'Neill, 2004).

The cell wall of *H. influenzae* contains a lipopolysaccharide that lacks the O-side chains of LPS, and is termed lipooligosaccharide (LOS). It has been reported that LOS can inhibit ciliary function and cause death of ciliary mucosal cells (Denny, 1974).

LPS is glycolipid composed of a hydrophilic polysaccharide and a hydrophobic domain, called lipid A, which account for the biological activity of LPS. The latter is a powerful stimulus for several inflammatory mediators and immunoregulatory cytokines from resident and inflammatory cells in the lung. Activation of the immune system by LPS is the first step in a series of events thought to lead to the manifestation of Gram-negative sepsis, a condition that results in as many as 20,000 deaths annually in the United States (Palsson-McDermott and O'Neill, 2004).

LPS-binding protein (LBP) is the first protein involved in LPS recognition (Schumann et al., 1990), which transfers the LPS to the co-stimulatory molecule CD14, which is known to have a role in activation of many of the TLRs. This allows LPS to be transferred to the LPS receptor complex made of TLR 4 and sensing protein MD-2 (Hailman et al., 1994, Tobias et al., 1995) (Figure 1.7). Beutler (2004) demonstrated that TLR 4 operate as the membrane-spanning component of the mammalian LPS receptor complex and is the only TLR that requires extracellular chaperones and a sensing protein for activation.

TLR 4 works as a cell-surface co-receptor for CD14, leading to LPS mediated NF-KB activation and eventually causing the cellular events. The LPS-resistant C57BL/10ScCr mouse appears not to have a TLR 4 locus (Poltorak et al., 1998). These observations strongly support the concept that TLR 4 is the dominant LPS receptor in mammals. Functional polymorphisms in TLR 4 have been described and many studies have paid a lot of attention the Asp299Gly polymorphism, with the infrequent allele (Gly299) causing LPS hypo-responsiveness. This has been associated with a low risk of atherosclerosis (Sabroe et al., 2004).

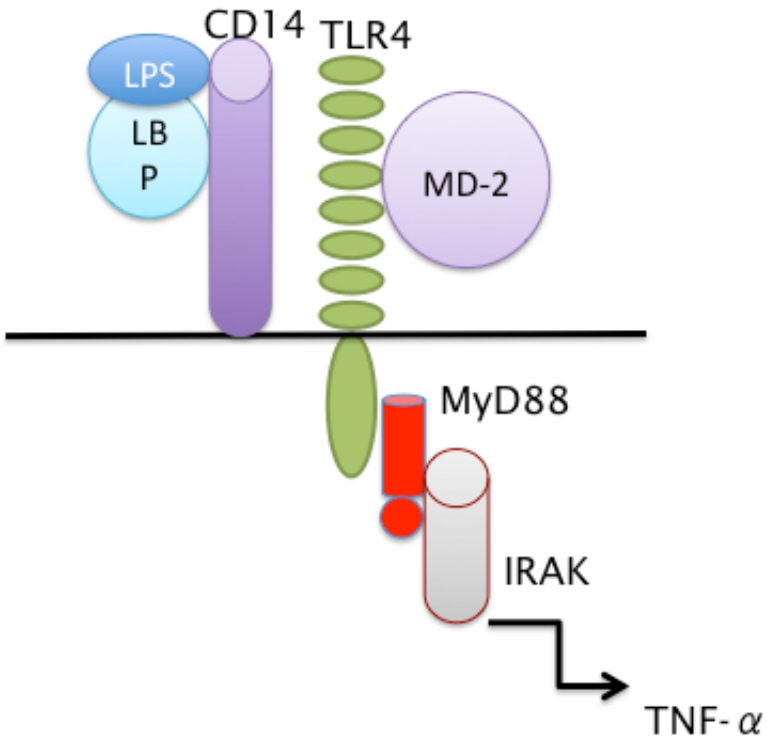

Figure 1.7 Diagrammatic representation of the LPS-receptor complex (TLR 4). First, LPS binds to LBP, and then subsequently binds to CD14. Binding to CD14 enables it to be transferred to the TLR 4-MD2 complex. The consequence of this sequence of events is LPS-mediated NF-KB activation and subsequent cellular events including TNF-α release. IRAK = Interleukin-1 receptor- associated kinase. Taken from (Jimenez-Dalmaroni et al., 2009).

1.6.2.2 TLR 2 ligands

TLR 2 recognizes cell wall constituents of extracellular bacteria, and its cell surface protein. TLR 2 recognizes a wide array of ligands that include bacterial lipoteichoic acid, lipopeptides and peptidoglycan, as well as yeast cell wall particle zymosan. In addition, TLR 2 mediates cellular response to a wide variety of microorganisms; endogenous ligands have also been reported (Curtin et al., 2009). TLR 2 ligand recognition and signalling activity is determined by heterodimeric interaction with either TLR 1 or TLR 6 and new studies report on TLR 2/TLR 10 dimers (Guan et al., 2010). It has been proposed that a ligand complex forms where CD36 binds the ligand and transfers them to CD14 which then loads the ligand onto the heterodimers (Figure 1.8) (Jimenez-Dalmaroni et al., 2009). However, CD14 and CD36 are not necessary for TLR 2 signalling and their role is to magnify responses. The main TLR 2 ligands that have been characterized are lipoproteins that are ubiquitous to all bacteria. After ligand binding the

heterodimers initiate a MyD88-dependent pathway that lead to the activation and translocation of NF-KB to the nucleus and eventually gene transcription of cytokines.

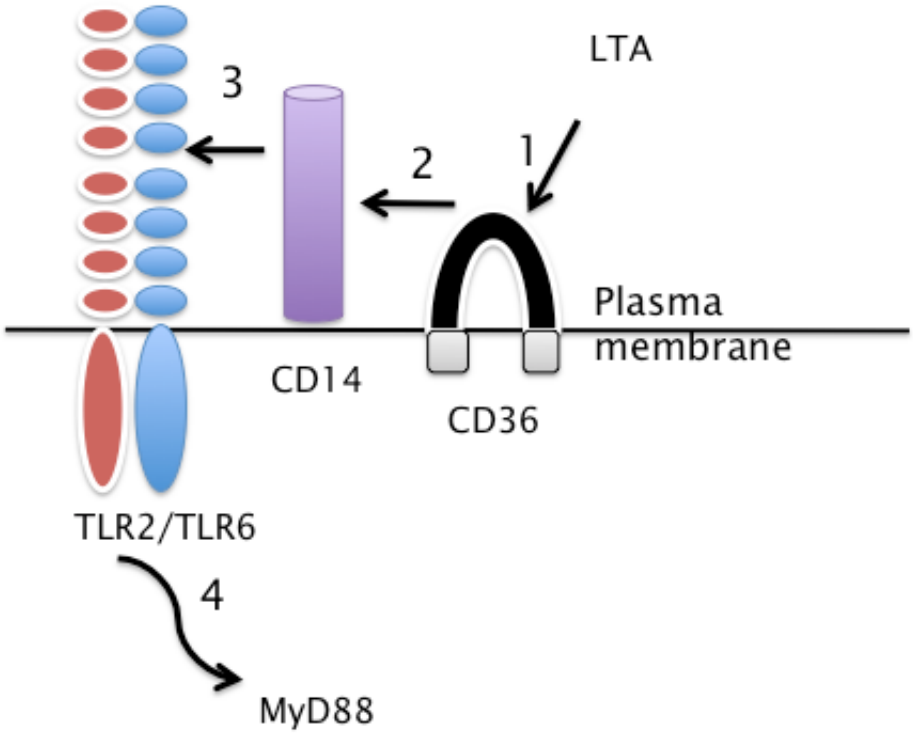

Figure 1.8 Diagrammatic representation of Lipoteichoic acid (LTA) binding to TLR 2. Model of CD36-dependent activation of the TLR 2 signaling pathway. CD36 binds LTA (step 1) which is transferred to CD14 (step 2). CD14 transfers LTA to TLR 2/TLR 6 (step 3) and the MyD88 pathway is activated (step 4). Taken from (Jimenez-Dalmaroni et al., 2009).

1.6.2.3 TLR 2 and 4 expressions

TLR 2/4 show different expression patterns in different cell types. Immunohistochemistry has been used to localize TLR 2 to alveolar macrophages and alveolar epithelial cells (Droemann et al., 2003). The regulation of TLR 2 expression shows conflicting results. In blood cells, the receptors are predominantly expressed by monocytes with the lymphocytes being TLR 2 negative (Muzio et al., 2000). In addition, Flo et al. (2001) showed increased levels of TLR 2 protein after LPS stimulation in human monocytes. In contrast, Muzio et al. (2000) showed no increased TLR 2 mRNA after LPS stimulation. This discrepancy could be due to the lack of correlation between mRNA and surface protein expression. It has been reported that IFNγ up-regulated TLR 4 mRNA and its surface expression, also similar results were observed with MD2, which associates with TLR 4 (Bosisio et al., 2002, Juarez et al., 2010). The team also noticed that LPS has opposite effects on TLR 4 at the mRNA and surface protein levels, with up-regulation of

the former and down-regulation of the latter after LPS is internalized and transported to the Golgi apparatus. It has also been shown that airway smooth muscle cells (ASMCs) express TLR 1 to TLR 10 mRNA at basal level (Sukkar et al., 2006). The same group also found that the expression of TLR 2/4/3 is controlled by cytokines and TLR ligands, and their activation leads to chemokine release in ASMCs. Ritter has also reported that stimulation of primary small airway epithelial cells with TLR 3 ligand leads to an increase in cytokine and chemokines production (Ritter et al., 2005). However, they observed no inflammatory response of the cells to LPS stimulation. Likewise, Tsutsumi-Ishii and Nagaoka (2003) found that A549 cells do not express TLR 4. At the same time, Monick et al. (2003) found that infection by respiratory syncytial virus augmented TLR 4 expression and membrane localization on airway epithelial cells (A549 cells, primary human airway epithelial cells (HAE), human tracheobronchial epithelial cells and HeLa cells) suggesting that a priming event is needed to induce LPS responsiveness to those epithelial cells that are in frequent contact with environmental stimuli (intestine and lung) and are hyporesponsive to LPS at baseline. However, von Scheele et al. (2010) reported increased expression of TLR 2 after stimulation of primary bronchial epithelial cells with LPS and TNF-α. Stimulation of both alveolar macrophages and monocytes with LPS increased their TLR 4 expression.

In an acute model of inflammation by exposing mice to cigarette smoke, Doz et al. (2008) found that acute lung inflammation generated by 3 days of exposure to cigarette smoke is TLR 4, IL-1R1, and MyD88 dependent. Lack of TLR 4, MyD88, or IL-1R1 signalling abrogated neutrophil recruitment to the lung parenchyma and BAL. Moreover, NTHI infection up-regulated TLR 3 expression in a human bronchial epithelial cell line (Sajjan et al., 2006). In addition, other work also mention that TLRs influence apoptosis and cell proliferation (Sabroe et al., 2005).

1.6.2.4 TLR 2/4 in COPD

The lower respiratory tract represents a primary site for the introduction and deposition of pathogenic microorganisms, and as mentioned before, it is now clear that bacterial infection could be a key driver of COPD exacerbation. However, the relationship between bacterial colonization and pulmonary inflammatory response is not well characterized. Furthermore, much of the evidence discussed here has arisen from cells harvested from blood or from studies on cell lines.

So there is a question into what exactly happens in the lung parenchyma. In a study using flow cytometry on monocytes (Pons et al., 2006), TLR 2 was found to be increased in monocytes from COPD patients compared to control subjects, and that these cells upon challenge with

preparations containing TLR 2 ligands secreted high levels of inflammatory mediators. They did not see any changes in TLR 4 between the groups and similar findings were reported by Droemann et al. (2005). However, the latter found altered alveolar macrophages phenotype with reduced expression of TLR 2 in smokers and COPD patients. TLR 2 and TLR 4 have been implicated in the pathogenesis of inflammation and their ligands were found to augment cytokine and chemokine production by certain cells.

Having investigated the conflicting literature regarding TLR and bacterial infection it is clear that more research is needed to investigate the role of these receptors in bacterial infection in COPD. Considering the relationship between COPD exacerbations and bacterial load we can assume that TLR play a role in the response of the lungs to the presence of bacteria, and it may be the case that, as the disease progresses with greater exacerbation frequency this defence system may fail to overcome infection. It is possible that TLR may be chronically elevated or reduced in patients with COPD.

1.7 Human and animal models in COPD

1.7.1 Mice and rat models

In general mice are the animal models of choice for the *in vivo* study of COPD. Many reports on mice and rats describe alveolar duct enlargements when the animals are exposed to mainstream cigarette smoke. Emphysema starts to develop after 3-4 months of smoke exposure and pulmonary air space expansion and tissue destruction were shown (March et al., 1999). Using rat trachea, Wang et al. (2003) demonstrated that cigarette smoke could directly cause airway remodelling specifically airway wall fibrosis. The latter represent pathological structures that correlate with abnormal pulmonary function test (Hogg et al., 2004). However, mice lack respiratory bronchioles and cannot develop centrilobular emphysema.

More recently, models that use a combination of cigarette smoke CS plus LPS were reported, Hardaker et al. (2010) constructed an *in vivo* rat model imitating characteristic trait of acute exacerbations of COPD including lung function decline and increased lung inflammation. Also, Zhou et al. (2012) reported on COPD rat model that was constructed after intratracheally administrated LPS twice (on days 1 and 14) and frequently exposing the animal to CS for 5 weeks.

Moreover, models of elastase and LPS induced lung injury were established that show structural and functional features typical of human COPD, including pulmonary emphysema, and markedly

increased numbers of inflammatory cells (Sajjan et al., 2009). Also exposure of mice to inhaled LPS, 4 h a day for 4 week, was shown to result in emphysema-like changes that persisted up to 4 weeks (Brass et al., 2008). Furthermore, LPS or *H. influenzae* inhalation in mice models resulted in neutrophils, macrophages and T lymphocyte recruitment, as well as increased inflammatory mediators like TNF-α, IL-1β, MMP-9 and fibrinogen. In addition, cigarette smoke exposed mice also had an increase in CD8:CD4 ratio in their lung (Motz et al., 2008). Harrison et al. (2008) in addition reported significant increase of both $CD8^+$ and $CD4^+$ T cells in BAL from cigarette smoke exposed mice.

1.7.2 Guinea pig model

A guinea pig model of cigarette smoke-induced lung disease, in which chronic exposure to cigarette smoke resulted in increases in lung volumes and decreased airflow has been established (Wright et al., 2007). The same group (Wright, 2001) also reported an elevation in the number of alveolar pores in guinea pigs subjected to cigarette smoke, a process thought to represent ultramicroscopic emphysema. Smoking was reported to increase neutrophil and parenchymal macrophage infiltration, while Budesonide improved cell infiltrate (Wang et al., 2015). And smoking cessation lowered neutrophil count to control level (Jobse et al., 2014). Also recently, Dominguez-Fandos et al. (2012) established a guinea pig model that shows where the cigarette smoke on the guinea pig mimics those seen in COPD patients.

However, there are several problems associated with animal models, for example bronchial glands are sparse in mice, rats, and guinea pigs. There are only few models of acute exacerbation in animal studies, as in most cases the disease is mildly reproduced. GOLD III/IV physiologic changes and corresponding severely destructive anatomic lesions were never produced in animal models of cigarette smoke induced COPD no matter how long the animals are smoked (Churg and Wright, 2007, Wright and Churg, 1990).

1.7.3 Models of COPD in humans

It is not easy to model COPD as it is a complex disease that includes lung parenchyma destruction, airway changes and chronic inflammation. Additionally, a number of pharmaceutical interventions when used cross-species can be ineffective. However, many models have been developed to gain more knowledge into the mechanism of the disease and to provide opportunities to develop new treatments.

Ex vivo models investigate the effects of cigarette smoke exposure on cell lines in culture. Using an *ex vivo* model of lung parenchyma, Saetta et al. (1985) examined the lungs of smokers and non-smokers by looking at the relationship of the number of alveolar attachments with airways inflammation and lung function. The three indices of alveolar attachments were measured which included the number of attachments, distance between attachments and percentage of abnormal attachments, and these correlated significantly with the score for airways inflammation and with the elastic recoil pressure in smokers and that led them to conclude that smokers have fewer alveolar attachments. The loss of attachments represented the beginning in the destruction of the lung parenchyma. In addition, different culture models using cell lines or primary cells have been used to look at the contribution of bronchial epithelium and alveolar macrophages in the acute inflammation of COPD. Exposure of bronchial epithelial spheroid cells to LPS (this is 3-D culture system that keeps the airway epithelium in a well-differentiated and polarised state) resulted in an elevated production of IL-8 in a time and LPS dose-dependent manner and a peak was noted at 24 h culture at a dose of 10 µg/ml LPS (Deslee et al., 2007). While stimulation of alveolar macrophages by 1µg/ml of LPS (10 fold lower concentration than the previous model) resulted in an elevation of MCP-1 and IL-6 in COPD compared to control (Armstrong et al., 2009). Similar results using similar LPS concentration was reported by Aldonyte et al. (2003) in a macrophage/monocyte cell model.

Ex vivo models have been useful for bridging the gap between animals and humans in other tissues, like skin, (Stojadinovic and Tomic-Canic, 2013, Phillips et al., 2013). And they may also prove to be very useful in lung.

1.8 Treatment in COPD

As mentioned before, COPD is a global health problem and a major cause of morbidity and mortality in the UK. Still, current therapies, whilst alleviating symptoms, fail to prevent disease progression or mortality. The driving force in COPD is the abnormal chronic inflammation in the lung of patients with COPD. Yet, no existing anti-inflammatory treatments significantly alter the chronic inflammatory response or resolve the progressive loss of lung function. COPD continues to result in significant mortality.

1.8.1 General treatment in COPD

Current therapy includes prescribed inhaled corticosteroids ICS. Inhaled glucocorticoids are potent at controlling the inflammation in some lung disease like asthma. However, in COPD only

patients with severe and very severe disease derive some benefit from them (Calverley et al., 2003, Spencer et al., 2004). Corticosteroids interfere with the ability of transcription factors like (NF-KB) to attach to the promoter regions of inflammatory genes, bringing into play a wide range of anti-inflammatory effects (Adcock et al., 2004). Despite that, cytokine production from COPD alveolar macrophages is reported to be GC-resistant (Armstrong et al., 2009). In addition, long-term treatment with ICS has not been found to modify the rate of decline of FEV_1 (Burge et al., 2000). Thus, steroids were proven to be of limited effect for COPD (Barnes, 2000). And many studies have concluded that inhaled glucocorticoids lack impact on the progressive decline in FEV_1 (Vestbo et al., 1999, Burge et al., 2000).

Some steroid side effects have been reported that include: increased risk of oropharyngeal candidiasis (Burge et al., 2000). Also, hoarseness or dysphonia and skin bruising (Calverley et al., 2008). Long-term use of ICS has also been linked to increased risk of pneumonia (Kew and Seniukovich, 2014). In addition to steroids, long-acting β2-adrenoreceptor agonists, and long-acting anticholinergics are used. However they do not have any proven disease-modify effects, being concerned mainly with control of symptoms of breathlessness.

COPD patients suffer from exacerbations, and to date prevention and treatment of exacerbations has been challenging. However, some studies report that combination of inhalers of corticosteroids, long acting beta agonists, and long acting anticholinergics, reduce exacerbation burden and hospital admissions (Calverley et al., 2007, Tashkin et al., 2008). While not all exacerbations have a bacterial origin, antibiotics like macrolides are usually prescribed and while some clinical studies have shown benefits of long-term macrolides in COPD and demonstrated a reduction in exacerbation frequency (Seemungal et al., 2008), others did not find any significant benefit on health status, exacerbation rate or sputum bacterial load (Banerjee et al., 2005). Smoking cessation remains the most effective intervention in terms of cost for COPD and while oxygen therapy is associated with high costs it is the only intervention that reduces mortality rate in advanced COPD (Law et al., 2014, Party, 1981).

1.8.2 Emerging therapies

As previously mentioned, COPD is partially steroid resistant and therefore steroids do not halt the inflammation fully (Culpitt et al., 2003, Bourbeau et al., 2007, Suissa and Barnes, 2009). Alternative anti-inflammatory pathways have been explored as potential targets for COPD therapy (Babu and Morjaria, 2015). Use of humanised monoclonal antibodies directed against individual pro-inflammatory mediators such as TNF-α has been disappointing in humans despite showing promise in animal trials (Rennard et al., 2013). This may be due to functional

redundancy of many mediators: many are activated through similar pathways and have positive feedback mechanisms serving to promote inflammation as in the case of TNF-α (Berkow et al., 1987). Interleukin-8 (CXCL8) antibody have also been used (Kaur and Singh, 2013), but without the hoped-for clinical benefits.

Promoting anti-inflammatory mediator production such as IL-10 has also not yielded positive results in humans because of the reported haematological side effect (Barnes et al., 2003); so, further studies in IL-10 as a target treatment for COPD are needed (Ogawa et al., 2008). Inhibition of matrix metalloproteinases, in principle to reduce proteolytic destruction in the lung arising from inflammatory cell activation, has also been explored using AZD1236 a selective MMP9 and MMP12 inhibitor but had no clinical efficacy (Dahl et al., 2012).

This has resulted in a widespread belief that pathway inhibitors rather than individual mediators are most likely to be effective anti-inflammatory agents. NSAID treatment is sometimes avoided in COPD due to the risk of inducing bronchospasm in patients with overlap syndrome, although ibuprofen use is widespread in COPD due to its anti-inflammatory effects. Inhibitors of other inflammatory mediators such as Leukotriene B4 (LTB4), a powerful chemoattractant of neutrophils (Crooks et al., 2000, Beeh et al., 2003), and phosphodiesterase-4 (PDE-4), which is an enzyme that modulates the formation of cAMP in airway smooth muscle, reducing bronchospasm (Compton et al., 2001, Gamble et al., 2003), also Prostaglandin inhibitors (Snell et al., 2013), have been investigated in COPD but have not become established therapies, principally due to their side effects , which include weight loss and emesis.

P38 MAPK has been targeted for various chronic inflammatory diseases, such as rheumatoid arthritis, Crohn's disease, COPD, asthma, and psoriasis. The availability of so many structurally diverse inhibitors of p38 MAPK has encouraged the efforts to identify more potent, safe, and efficacious p38 MAPK inhibitors.

1.9 p38 Mitogen-Activated-Protein-Kinase

P38 MAPK exists in four different isoforms: p38α, p38β, p38γ, and p38δ; each isoform is encoded by a separate gene (Hale et al., 1999).

1.9.1 P38 MAPK biology

While p38α and p38β are ubiquitously expressed, the expression of p38γ, and p38δ is more restricted (Lee et al., 2000). The expression of p38α and related kinases is variable in different

cells with p38α being predominant kinase in monocytes and macrophages also its activation has been observed in many hematopoietic and non heamatopoietic cell types upon activation (Lee et al., 1999).

A lot of this work was started because of the finding that p38 plays a central key role in the regulation of biosynthesis and actions of pro-inflammatory mediators such as IL1β and TNF-α (Lee et al., 1994). Stress, inflammatory cytokines such as TNF-α, endotoxin and cigarette smoke are among the variety of stimuli that activate p38 MAPK. Several substrate for p38 Map kinase have been identified and that include, among other kinases, MAPKAP k2/3, p38-regulated/activated protein kinase (PRAK) (New et al., 1998), and MAP kinase-interacting kinase 1and 2 (MNK1/2) (Fukunaga and Hunter, 1997). The most important substrate is MK2 and once phosphorylated it can unmask a nuclear localization signal that permits the translocation of the MK2-p38 MAPK complex to the nucleus.

1.9.2 Mechanism of action of p38 MAP kinase

P38 is activated via a signalling cascade that is conserved from yeast to mammals, in which stimulation of MAPKs requires the upstream activation of MAPK kinase and MAPK kinase kinase (Johnson and Lapadat, 2002). This leads to the phosphorylation and activation of other kinases or transcription factors that then lead to stabilized mRNA and an increase or decrease in the expression of certain target genes (Salituro et al., 1999).

P38 Map kinase is one of the three established serine-threonine protein kinases that are characterized by a common activation motif composed of Thr-X-Tyr sequence, where X is a Gly in p38, located on a flexible loop termed the phosphorylation lip (Figure 1.9) proximal to the ATP and substrate sites (Robbins et al., 1993). In the latter, the Lip consists of 13 residues. P38 is activated in response to stimulus by phosphorylation on both threonine and tyrosine in the motif. Activation is achieved by dual-specificity serine/threonine MAPK kinases, MKK3 and MKK6.

p38 MAPK has a conserved activation loop made of Asp168-Phe169- Gly170 (DFG). The loop can change conformation that the Phe side chain is located in a hydrophobic pocket called (DFG-in conformation). However, the Phe side chain can also move to a different position (DFG-out conformation).

Selective inhibitors of p38α kinase were reported to stop the production and activity of inflammatory cytokines and have shown efficacy in animal models of acute inflammation and arthritis (Salituro et al., 1999, Badger et al., 2000). P38 MAPK was activated in alveolar

macrophages (Renda et al., 2008), and its inhibition reduced cytokine production from these cells (Kent et al., 2009). There are many inhibitors in clinical development for the treatment of COPD (Singh et al., 2010) and it is possible that by halting the P38 Map kinase pathway the inflammation can be stopped.

Some of the first compounds that have reached clinical studies were discontinued for safety reasons principal side effects included hepatotoxicity and potential carcinogenic effect owing to their potent induction of some cytochrome P450 isoenzyme (Laufer et al., 2003), as well as vast kinase selectivity patterns and inhibiting off target kinases (Dominguez et al., 2005). This led to the discovery of the new, highly selective generation of small molecule kinase inhibitors (over 20 compounds published) that have few side effects. This selectivity is considered impressive especially as the human genome contains over 500 protein kinases sharing a high degree of sequence homology (Manning et al., 2002).

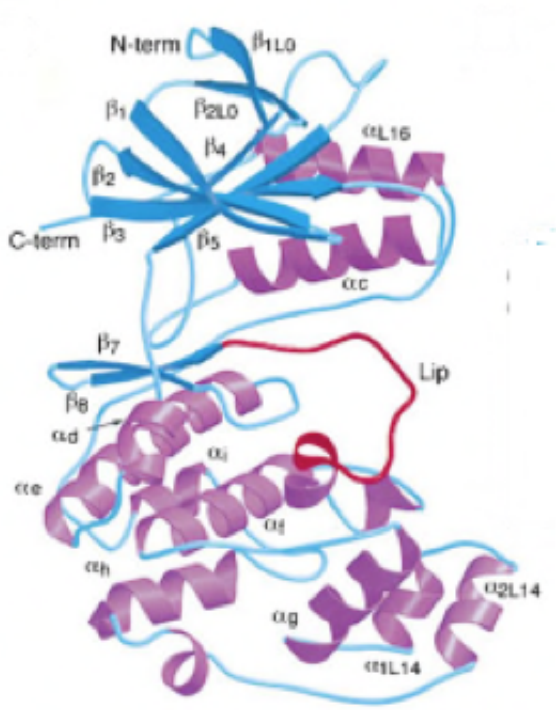

Figure 1.9 Molecular structure of p38α. Ribbon diagram showing a-helices in purple and blue strands are cyan; the Lip (red) is a flexible loop where the activation motif is located. The motif gets phosphorylated upon stimulus and leads to p38 activation. Adapted from (Wang et al., 1997).

1.9.3 Role of p38 MAPK in cytokine production and function

Although p38 MAPK regulates the production of TNF-α and IL-1β, p38 MAPK inhibitors are also thought to inhibit not only the production of pro-inflammatory cytokines but also their actions. P38 MAPK regulates gene expression of various key inflammatory genes by four different mechanisms (Kumar et al., 2003). First mechanism is phosphorylation of transcription factors. It also regulates mRNA stability by the effect of downstream kinases such as MK2. Third, p38 MAPK regulates mRNA translation into protein via the action of downstream kinases such as MK2, which leads to the phosphorylation of the AU binding proteins that control translation; this is the main mechanism by which p38 MAPK regulates production of TNF-α (Kumar et al., 2003). Finally, p38 MAPK regulates the phosphorylation of histone H3 in chromatin at NF-KB binding sites of certain genes. This event is crucial for successful transcriptional induction of IL-8 and MCP-1 by NF-KB (Saccani et al., 2002, Soloaga et al., 2003). As p38 MAPK has been shown to mediate the release and signalling of many of inflammatory key mediators, it is believed to be a key target for various pulmonary diseases including COPD.

1.9.4 P38 MAP kinase inhibitors

The pyridinylimidazole compounds, called CSBP, were found to be selective inhibitors to p38 MAP kinase soon after their first preparation as inflammatory cytokines synthesis inhibitors (Lee et al., 1994). Later on CSBP were used as the template for the majority of p38 inhibitors (Figure 1.10), namely SKF 86002 (Lee et al., 1994) and SB 203580 (Young et al., 1997). In 1999, another class of p38 inhibitors represented by VX-745, that showed high selectivity to p38 MAP kinase was reported. This class binds the active site in p38 MAP kinase.

These kinase inhibitors utilise the ATP binding site, and inhibit the kinase by competing with the binding of ATP. They do this by forming one or more key hydrogen bonds within the conserved hinge region existing between the N- and C-terminal lobes of the kinase. Other compounds also use the DFG-out conformation as it shows a large hydrophobic domain in the kinase (Regan et al., 2002). These specific classes of inhibitor molecules attach to the conserved Asp-Phe-Gly (DFG) motif at the beginning of the ATP binding site generating a new pocket that is not accessible in the active kinase conformation (Figure 1.10).

By making use of the induced pocket, a sub-micromolar range of p38 Map kinase inhibitors were achieved, sparing the conventional hinge interaction (Gill et al., 2005). Several p38 kinase inhibitors were discontinued at the clinical trials stage due to their unacceptable safety profiles, such as high liver enzymes with significant incidence of skin rash (Dominguez et al., 2005). A

more recent example is BIRB-796 that has been progressed to a phase II study in Crohn's disease but did not show clinical benefit and is now discontinued (Schreiber et al., 2006).

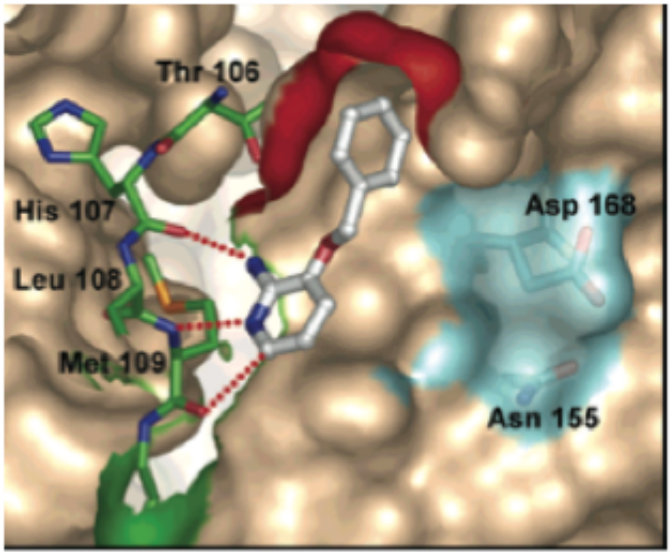

Figure 1.10 Close-up view of the keybinding pocket of p38MapKinase. The hydrophobic region 1 (red), which is not occupied by ATP, is a known key specificity pocket. Region 2 (green) is also hydrophobic and accommodates aliphatic moieties on inhibitors, which contain terminal polar groups, to modulate potency and physicochemical properties. The ribose-binding region (blue) contains the amino acids that interact with the hydroxyl groups of the ribose of ATP. A compound is outlined in grey and is shown making key hydrogen bond contacts (red dotted lines) to hinge residues. Taken from Gill et al. (2005).

1.9.5 P38 MAPK in chronic obstructive pulmonary disease

The activation of p38 MAPK pathway is involved in the pathogenesis of COPD, as phosphorylated p38 was specifically increased in alveolar macrophages and alveolar walls from patients with COPD compared with smoking and non-smoking controls (Renda et al., 2008). In addition, phospho-p38 expression was associated with the degree of lung function impairment and with the number of $CD8^+$ T-lymphocytes penetrating the alveolar walls, suggesting an opposite correlation between the activation of p38 MAPK and the decrease in FEV_1 and FEV_1/FVC.

Selective inhibitors decrease the expression of inflammatory markers in the blood of patients with COPD (Singh et al., 2010), and are powerful at suppressing the release of pro-inflammatory

mediators *in vitro*, such as TNF-α and CXCL8 (Tudhope et al., 2008, Smith et al., 2006). They also suppressed oxidant-mediated lung inflammation in a murine tobacco smoke exposure model, where glucocorticoids were not effective (Medicherla et al., 2008, Williams et al., 2008). Also, Marwick et al. (2004) looked at rat lungs that had been subjected to cigarette smoke and detected enhanced p38 phosphorylation, that was associated with elevated activation of the transcription factors NF-KB and AP-1, which led them to conclude that selective targeting of p38 MAPK in COPD may potentially be an effective alternative therapeutic strategy to glucocorticoids. P38 MAPK is expressed in many inflammatory cells, including macrophages, neutrophils, mast cells, eosinophils, CD4 cells, bronchial epithelial cells, and fibroblasts.

1.10 Summary

Although a number of COPD therapies seek to modulate inflammatory cell recruitment and activation pathways, none have yet served to significantly halt progression of the disease and while smoking cessation in animal can reduce the inflammation this is not the case in human. This reduction in lung inflammation in animals following smoking cessation that contrasts what happens in humans where there is an on-going inflammation after smoking has been stopped (Turato et al., 1995, Rutgers et al., 2000a) further confirm the need for human models that would closely represent *in vivo* COPD lung.

Whilst the majority of recent research into inflammatory responses in lung parenchyma in COPD has been carried out using animal models, post-mortem tissue or cell lines, it is theoretically better to maintain the ability of different cells to interact with each other and with their surrounding matrix as they would *in vivo*. Furthermore, it is important to investigate inflammatory responses in humans rather than using animal models or post-mortem tissue to ensure physiological relevance and applicability of the disease phenotype. Therefore the lung explant model was developed to study inflammation in human lung explants from control subjects and those with COPD both at baseline, and following *ex-vivo* treatment with TLR ligands and p38 MAPK inhibitors. In order to understand the inflammatory mechanisms of COPD during bacterial exacerbation, a controlled but complex stimulus in the form of a heat killed bacterial preparation was used alongside LPS, which is a well-characterised TLR agonist, as stimuli.

Heat killed bacteria was used as a stimulus as it is challenging to control bacterial growth in co-cultures. This study used LPS, a very well characterized TLR 4 agonist used in many *in vitro* studies, as a comparator. There are, however, drawbacks to using killed bacteria such as that

there may be epitopes exposed in killed bacteria, which are not normally exposed in live bacteria. The relevant doses for stimulation were calculated empirically for each preparation, and so LPS was necessary to use as a more standardised comparator stimulus.

This project describes the optimization of this model and its use to elucidate the mechanisms underlying bacterial-induced exacerbation, and to predict the efficacy of p38MAPK inhibition as a therapy for exacerbations in COPD patients.

Hypothesis

The hypothesis of this study was that lung tissues from control subjects, and subjects with COPD have different inflammatory profiles at baseline (cellular and mediator), and respond differently to stimulation with bacterial ligands as a result of this difference.

Aims

Aim 1: To identify and quantify inflammatory and structural cell populations in human resected lung tissue, and to compare these populations between control subjects and subjects with COPD.

Aim 2: To compare the inflammatory response to bacterial ligands in lung tissue explants from control and COPD subjects.

Aim 3: To assess the utility of the explant model in examining novel therapeutic interventions to inflammation in COPD, in particular to examine the efficacy of p38MAPK inhibition in reducing the production of inflammatory markers associated with COPD exacerbation symptoms using the lung tissue explant model.

Chapter 2: Materials and methods

2.1 Materials

Lipopolysacharides (LPS, L2630) this serotype was chosen because it was highly purified and contamination with protein or nucleic acid represent less < 1%.

Bovine serum albumin (BSA), tetramethylbenzidine (TMB) and Dulbecco's phosphate buffered saline (DPBS) were all purchased from Sigma-Aldrich (Dorset, UK). Streptomycin/penicillin/gentamycin and Roswell Park Memorial Institute-1640 (RPMI) 1640 were obtained from PAA-the cell culture company (Somerset, UK). Lymphopreb, aminoethylcarbozole liquid substrate system, (AEC); Mayer's haemotoxylin; from (Abcam, Cambridge, UK). Methyl benzoate; tris; hydrochloric acid and acetone from (Fisher Scientific Ltd., Loughborough, UK); GMA solutions containing solution A, solution B and benzoyl peroxide from Park Scientific Northampton UK. Sodium azide, hydrogen peroxide and avidin-biotin complex (ABC) from Dako (Denmark). All ELISA kits and reagents for individual mediator quantifications were obtained from Invitrogen (Paisley, UK). Multiplexed ELISA kits prepared bespoke to measure eight mediators (TNF-α, IL-8, IL-6, IL-17, IL-1β, MCP-1, MIP-1β, IFN-γ) and cytokines sourced from human. P38 MAP kinase inhibitors (PHA-00797804 and PF-03715455 (Table 2.1) Pfizer, Sandwich, UK)

Other reagents included: multi-spot plate, detection antibody mix. Cytokine standards, diluent 1 (RPMI) for dilution of standards, diluent 100 and Read buffer. Permwash from Biosciences and lastly fluticasone propionate and dimethyl sulfoxide were from Sigma Aldrich.

Table 2.1 Weight and molecular weight of the p38 Map kinase inhibitors.

Inhibitors	Weight	Molecular Weight
PHA-00797804,	10.1 mg	477.31
PF-03715455	10.0 mg	700.285

2.2 Human lung explant model

Human lung parenchyma tissue was obtained according to ethical guidelines for the Southampton University Hospitals NHS Trust, (LREC number: 08/H0502/32 and LREC number: 09/H0504/109) from patients undergoing lobectomy for carcinoma resection, bullectomy for bullae repair, or lung volume reduction surgery. Tissue from the lobectomy was dissected by a clinical pathologist and was as distant to the tumour margin as possible, and from tissue showing no gross signs of abnormality. Clinical and demographic data was collected from patient notes, anonymised, and stored in a password-secured electronic database. Dissected tissue was collected from the operating room in physiological saline solution before being transferred into room temperature Dulbecco's PBS for processing. Tissue was dissected free from airways, pleura and visible blood vessels, and was cut using dissection scissors into 2-3 mm^2 fragments, and was periodically washed with saline buffer to remove red blood cells. Tissue was stored in a flask with RPMI-1640 medium containing HEPES but without L-glutamine, supplemented with 1% penicillin, 1% streptomycin and 1% gentamycin (RPMI-PSG) at 37°C in 5% carbon dioxide/air overnight.

The following day tissue fragments were rinsed with DPBS and placed in a petri-dish with 10 ml of RPMI with PSG, and then transferred into separate wells in a 24-well tissue culture plate in 0.9 ml of RPMI-PSG, ready to be stimulated according to the experiment. Appropriate stimulus was added in a 0.1 ml volume, and left for different lengths of time depending on the experiment: 1, 2, 4, 6, 24 and 48 h post-addition of LPS/heat killed bacteria (HKB) for time-course experiments, and 24 h for LPS/HKB dose-responses. Six Eppendorf tubes (0.6 ml) were labelled for each well and one of these was weighed. The tissue fragments were collected at each appropriate time point and blotted dry on blue roll and put into the weighed tubes. It was then re-weighed to allow for the wet tissue weight to be calculated. The tissue was frozen at -70°C and 180 μl of the supernatant were placed in each of the other labelled tubes and stored at -70°C until analysis.

2.3 Killed bacteria and LPS experiments

Clinically isolated non-typeable *H. influenzae* (ATCC number 35039) were grown in agar plates, quantified on the basis of turbidity measurements, then heat killed and stored in aliquots at -70°C (gift from Dr Iain Kilty, Pfizer, Sandwich, UK).

2.3.1 Dose response curves

The tissue was prepared as previously described. RPMI (0.9 ml) was placed in each well of 24 plates. Three-five tissue explants were added to each well to give approximately 30 mg wet weight of tissue, and the tissue was stimulated with various concentrations of *H. influenzae* ($2x10^8$, $2x10^7$, $2x10^6$, $2x10^5$, $2x10^4$, heat-killed colony-forming units (CFU)/ml) or 100, 10, 1, 0.1, 0.01 ng/ml LPS for 24 h before being analysed for IL-10 and TNF-α.

2.3.2 Time-course

The model (section 2.2) was used to analyse the time-course of cytokine release by the tissue using both LPS (100 ng/ml) and HKB ($2x10^8$ CFU/ml) as stimulants. Tissue fragments were stimulated with the appropriate stimulus for various time points over a 48 h period: 1, 2, 4, 6, 24 and 48 h. At each time point the tissue and supernatant were harvested as described in section 2.2.

2.3.3 Coefficient of variation

In order to measure the internal variability of the model, replicate tissues were used to calculate the coefficient of variation (CV). Six different patients were recruited for this analysis where four replicates for each control or stimulant (LPS 100 ng/ml and killed bacteria 10^6 cfu/ml) of every sample was used. Tissue was incubated for 24 h before being analysed for a range of cytokines.

2.4 Analysis by ELISA

The standard ELISA kit was first used to measure the level of TNF-α and IL-10 in the supernatant and later multiplex ELSA was used to measure as well as TNF-α and IL-10 the level of several other cytokines in the supernatant that include: IL-6, IL-8, IL-10, IL-17, TNF-α, IL-1β, MCP-1 and MIP-1β. After measurement, the concentration was corrected for tissue weight.

2.4.1 Tumour necrosis factor α and Interleukin-10 analysis by standard ELISA kit

PBS-Tween contained 0.1% (v/v) Tween 20 in PBS (wash buffer), assay buffer contained 0.5% (w/v) BSA in PBS, ELISA coating buffer contained 0.1 mM $NaHCO_3$ and 0.1 mM Na_2CO_3 at pH 9.4. Human TNF-α and IL-10 specific ELISA kits that have sensitivity in the low pg/ml, were purchased from Invitrogen Ltd. (Paisley, UK) and performed according to the manufacturer's

recommendations. In brief, 96-well microtitre plates (Greiner Bio-One, UK) were coated with the capture antibody diluted in the coating buffer (TNF-α 2 mg/ml and IL-10 1mg/ml) and incubated overnight at 4ºC.

The following day the plates were washed three times with 0.1% (v/v) PBS Tween and unspecific binding was blocked for 2 h with 0.5% (w/v) BSA in PBS. The plates were then washed as before and 100 µl of sample or standard, with a top standard for TNF-α of 1000 pg/ml and for IL-10 500 pg/ml, was added to each well, along with 50 µl of the appropriate mouse anti-human detection antibody (0.16 mg/ml for IL-10 and 0.32 mg/ml for TNF-α). This was incubated for 2 h with continual agitation. After the incubation time the plate were washed and 100 µl of the streptavidin-HRP was added and incubated for 30 min after which the plates were washed and 100 ml of TMB working solution was added to visualize the signal. Then, 50 µl of 1 M HCl was used to terminate the reaction, and plates were read at an optical density of 450 nm using a microplate reader (ELx808, Bio Tek, Leeds, UK). To get the data a graph was constructed using linear plots of the standard curve and then interpolated the unknown concentrations from the equation of the curve.

2.4.2 IL-6, IL-8, IL-10, IL-17, TNF-α, IL-1β, MCP-1 and MIP-1β analysis by multiplex ELISA

Meso Scale Discovery (MSD) is a molecular technique able to measure up to 10 cytokines in an MSD 96-well multi-spot plate where capture antibodies are coated in a single spot on the bottom of the wells. Several MSD plates were customized, a kind gift from Pfizer.

As mentioned above customized plates were made to measure 8 cytokines. IL-10, IL-8 IL-6, TNF-α, IL-1β, MCP-1, IL-17 and MIP-1β. ELISA was carried out as per the manufacturer's instructions. In brief, the cytokines standards were provided at 50 µg/ml in cell culture media with 10% fetal bovine serum and a combined working solution was made at 1 µg/ml using RPMI based medium as dilutent 1. A serial dilution was made ranging from (10 000 pg/ml to 2.4 pg/ml). Samples or standards were added at appropriate dilutions to the wells and left for 2 h with continual agitation at room temperature before, the detection antibody mix, labelled with MSD Sulfo-Tag reagent and pre-mixed to provide a 50X stock solution and prepared at 1X in diluent 100, was added and incubated for 2 h. Finally, the plate were washed 3 times with PBS + 0.05% tween 20 before read buffer, supplied with the kit and diluted 2-fold in deionized water, was added. The plates were analysed using a SECTOR Imager and cytokine concentrations were determined by comparison to the standard curve and interpolation of unknown values from 4-point logistical regression analysis of the curves.

2.5 Tissue preparation for immunohistochemistry staining

Lung tissue was processed for embedding in glycol methacrylate (GMA) as previously described by Britten et al. (1993). In brief, lung tissue was dissected and immediately washed with PBS prior to fixation in chilled acetone -20^{o}C containing 20 mM of iodoacetamide and 2 mM phenyl methyl sulphonyl fluoride (PMSF) at -20°C for 16–20 h. The tissue was then transferred to fresh acetone and left at room temperature for 15 min and then immersed in methyl benzoate at room temperature for further 15 min. The samples were then immersed in GMA monomer (JB4 kit) containing 5% methyl benzoate for 6 h during which the GMA was changed 3 times. JB-4 is a water-soluble, glycol methacrylate, embedding kit that contains solution A (monomer) and solution B (Accelerator) and benzoyl peroxide.

The samples were then embedded by mixing JB4 solution A and B in the presence of benzoyl peroxide (GMA embedding resin), and left at 4^{o}C for 48 h in order to polymerise. Blocks were then stored in an airtight container at -20°C. Two-micron sections of lung tissue were cut from the GMA blocks using (Leica 2065 SUPERCUT Microtome Lab vista USA) and placed onto clean poly-L-Lysine coated slides and air-dried. Three or four explants from each lung sample were cut and stained with toluidine to choose those with the best morphology for the immunohistochemistry staining. Sections that had airways were not used. Once immunohistochemistry had been performed on the chosen sections, the numbers of positively stained nucleated cells were observed using a light microscope. Region of the slide where it has good parenchymal morphology were counted. Human nasal polyp and tonsil tissue were used as a positive control for staining.

2.5.1 Detection of inflammatory cells

The tissue sections were processed for immunohistochemical localization of using specific antibodies as previously described (Britten et al., 1993). Briefly, sections were initially incubated with 0.3% (v/v) hydrogen peroxide and 0.1% (w/v) sodium azide for 30 min to inhibit endogenous peroxidases. The tissue was then washed with TBS 3x5 min. Slides were drained and culture medium (DMEM containing 20% (v/v) foetal calf serum and 1 % (w/v) bovine serum albumin) was applied to block the sections for 30 min. Mouse monoclonal anti CD68 antibody was used to detect macrophages, a mouse anti neutrophil elastase for neutrophils a mouse anti tryptase for mast cells, a mouse anti CD3 for T cells and a mouse anti eosinophils cationic protein for eosinophils.

The following day, sections were washed with TBS and a rabbit anti-mouse biotinylated secondary antibody was added for 2 h at room temperature at 1:1000 for 2 h. The slides were further washed with TBS and drained for the application of Avidin biotin peroxidase complex for 2 h (2 µl A + 2 µl B in 150 µl TBS per slide) where A is the Avidin and B the biotinylated enzyme. They were then washed and drained and AEC substrate was applied for 20 min at room temperature. A final rinse with TBS was performed on the slides before being washed under running tap water for 5 min. Slides were drained and aqua mount (AbD Seroec UK) was applied, and the slides were baked at 80°C for 30 min in an incubator with fan (Gallenkamp, UK) before mounting coverslips to the finished slides with DPX.

Antibodies were selected to identify each cell type of interest, based upon surface antigen expression of specific markers for each cell type. In brief, neutrophils were identified using anti-neutrophil elastase (HNE) monoclonal antibody, macrophages using an anti-CD68 monoclonal antibody, T lymphocytes using anti-CD3, mast cells using tryptase antibody and eosinophils using eosinophil cationic protein (ECP) antibody. Whilst every attempt was made to identify fully specific cell markers, some of these monoclonal antibodies are not entirely specific because many different proteins in addition to the protein of interest may in fact share the single epitope toward which a monoclonal is directed. Therefore, such a monoclonal would not be specific even though it recognizes only a single epitope.

While CD68 antibodies are very useful and standard markers of macrophages, small amount of the antigen is found on some lymphoic and non-haemopoietic cells (Pulford et al., 1990). Tryptase on the other hand is mast cell specific (Payne and Kam, 2004). Anti-neutrophil elastase is specific for neutrophil elastase, but again small amounts can also be secreted from macrophages. Lastly monoclonal antibodies to CD3 complex are highly specific as CD3 is a complex known as a cluster of differention 3 found on T lymphocytes (Yanagi et al., 1984).

Details of the source, clone, cellular location and target for each antibody tested are shown in Table 2.2.

Each antibody that has already been titrated across a dilution series using the standardised staining procedure was first used to stain nasal polyp and tonsil tissue as a target using the method specified in the methods section. The optimised dilution for each antibody was chosen by visual observation of staining intensity and background staining, to determine the most specific staining dilution using our standardised conditions. The optimised dilutions are also indicated in Table 2.2.

2.5.2 TLR 2/4 titration and detection

A serial dilution was made for the titration of TLR 2/4. The initial concentration of the antibodies was 1 mg/ml and serial dilutions of 1:200, 1:500, 1:1000, 1:2000 and 1:3000 were made. The isotype dilution factor was calculated by dividing the isotype's neat concentration (20 g/l) by the antibody's working concentration (calculated by dividing the antibody's neat concentration by the dilution factors). A matched rabbit IgG control for each concentration was included.

For TLR detection tissue was processed as in section 2.5.1. The antigen was faintly detected at 1:2000; saturation with high background staining was seen at 1:200. Swine anti-rabbit was used as secondary antibody at 1:1200 and DAB substrate was used for TLR instead of AEC.

A dilution of 1:500 was subsequently chosen as it gave the optimal staining with minimum background interference.

Table 2.2 Description and details of antibodies used in IHC. Lung parenchyma from human lung was embedded in GMA, sectioned and the sections stained for macrophages, neutrophils, mast cells, eosinophils, T cells, TLR 2 and TLR 4 by indirect immunohistochemistry. This table shows the source, dilution, subtype, isotype of the antibodies and also the epitope stained.

Antibody	Source	Subtype	Isotype	Epitope stained	Dilution
Macrophages	Mouse	Monoclonal	IgG3	CD68	1:200
Neutrophils	Mouse	Monoclonal	IgG1	Neutrophil Elastase	1:1000
Mast cells	Mouse	Monoclonal	IgG1	Mast cell tryptase	1:1600
Eosinophils	Mouse	Monoclonal	IgG1	ECP	1:1000
T cells	Mouse	Monoclonal	IgG1	CD3	1:300

TLR	Rabbit	Polyclonal	IgG	TLR2	1:500
TLR	Rabbit	polyclonal	IgG	TLR4	1:500

2.5.3 Area of GMA-embedded tissue determination

Explants were fixed for GMA processing and then immunohistochemistry was used to detect inflammatory cells as described earlier. The advantage of GMA embedding over other procedures is that antigen-revealing steps are generally not required, since epitopes are well conserved using the combination of acetone fixation and resin embedding employed using this technique. The resin method is particularly suitable for small biopsy sections where paraffin is less suited, and we found using generalised protein stains (e.g. toluidine blue) on preliminary sections that the structure of lung parenchyma was well preserved, despite the lack of inflation of the alveolar space with air or other supports such as gelatin, which are often used in larger tissue pieces (Figure 2.1).

As the inflammatory cells could potentially be distributed throughout the tissue, and some contaminating lymphocytes from the circulation, arising from excision of the tissue during surgery, could be present within the lumen of the alveolar space, a bespoke programme to exclude “airspaces”, i.e. non parenchymal tissue, was employed to calculate the area of actual tissue that was occupied by each cell type. An example image of how the software excluded airspaces is shown in Figure 2.1. The software was able to calculate the number of each cell type per unit area of parenchyma tissue from each patient giving a value of cells/mm^2. This analysis was performed from 3 sections per tissue block for each patient, and data were expressed as the mean value from the replicates.

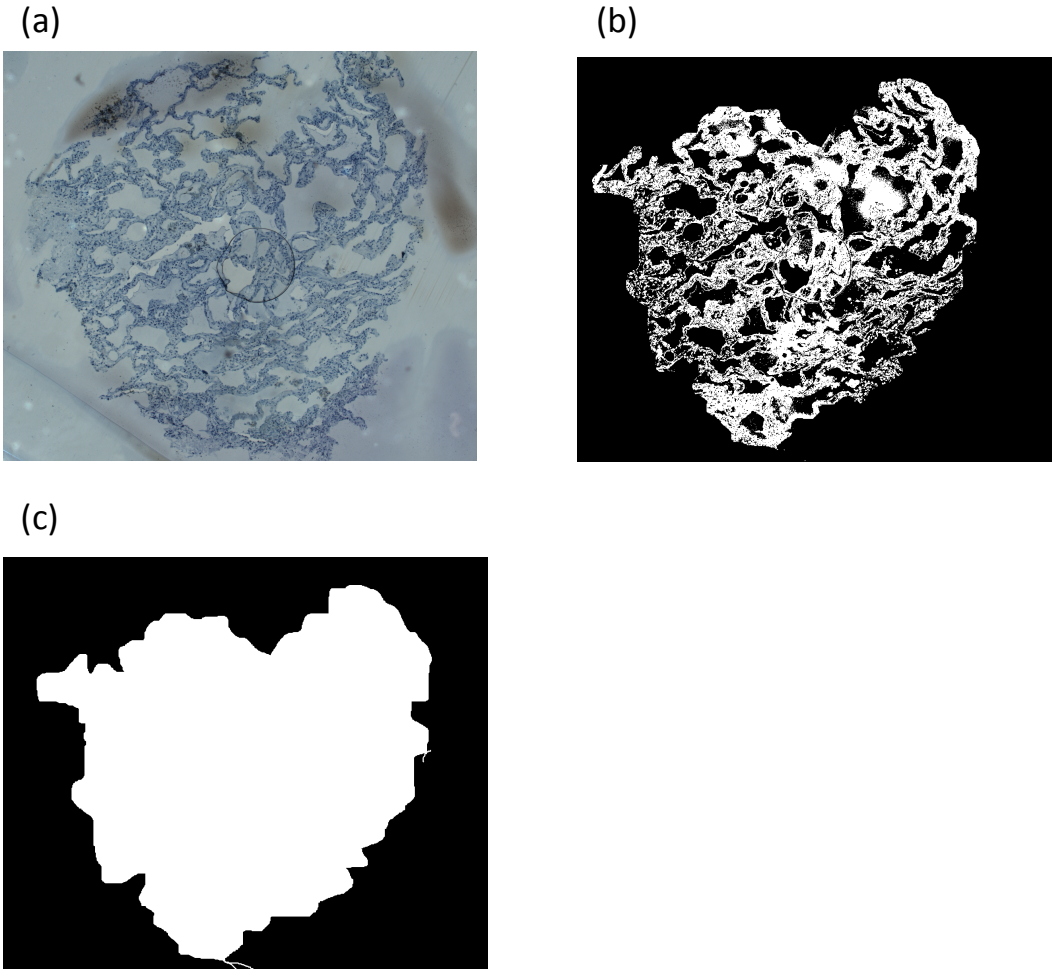

Figure 2.1 Determination of the appropriate area of GMA-embedded lung parenchyma tissue suitable for quantification by image analysis. An image of the total area of tissue to be quantified was taken using light microscopy, with visualisation of the counterstained tissue seen in blue coloration (a). The software converted the positive staining to a white coloration, and negative staining shown in black, to differentiate the staining area from "airspaces" (b). The total area of staining measured could then also be determined (c), in order to calculate the area of staining excluding airspaces.

2.5.4 Quantification of inflammatory cells

Two stained sections from the same block were analysed per patient. Slides were examined using a Leica DMLB microscope with a magnification of 40x. Inflammatory cells were counted across the whole section from two independent sections, and the average count calculated. Then, the unit area of cell quantification was determined by capturing an image of the stained

section using a Zeiss axiocam digital camera, and then the stained area was determined using specific software (Zeiss KS400, v, Zeiss, Germany), where airspaces were excluded from the area measured (mm^2) following contrast adjustment to account for staining intensity. The inflammatory cell count per unit area was then calculated by dividing the number of inflammatory cells by the calculated unit area, to give cells/mm^2.

2.6 Flow cytometric analysis

Flow cytometry was used to measure inflammatory cells and TLR.

2.6.1 Isolation of PBMC/neutrophils for inflammatory cell analysis

Antibodies to be used for the inflammatory cell analysis were first optimised using PBMC from a healthy volunteer.

2.6.1.1 Isolation of PBMC

First, blood was drawn into lithium heparin and mixed with an equal volume of PBS. Blood was then laid gently into an equal volume of lymphoprep and centrifuged at 800 g for 30 min at 20°C before the cells harvested at interface using sterile Pasteur pipette into new 50 ml Falcon. The cells were centrifuged at 400 g for 10 min at 20°C, supernatant discarded and cell pellet re-suspended in 10 μl PBS. Then, 40 μl of PBS were added and 10 μl was taken for cell counting using a haemocytometer. After counting, the cells were centrifuged as before, the supernatant discarded, and the cells resuspended to a concentration of 1×10^6 cells/ml using FACS buffer. Finally, 100 μl of this cell slurry was added to FACS tubes prior to staining, resulting in 1×10^5 cells/tube.

2.6.1.2 Isolation of neutrophils

First 9 ml of blood was drawn into a container and mixed with 4.5 ml of dextran in 50 ml Falcon tube. Blood was then rested at room temperature in a culture hood for 90 min until there is a clear interface between the blood and plasma. Plasma then removed with a 25 ml sterile Pasteur pipette and red blood cells discarded. The plasma is then centrifuged at 250 g, followed by removal of the supernatant and re-suspension of the pellet by flicking the tube. Then 10 ml of hypotonic lysis buffer was added to the tube and mixed carefully followed by 10 ml of lysis recovery buffer. The cells were centrifuged at 400 g for 5 min at 20°C, supernatant discarded and cell pellet re-suspended in 5 ml FACs buffer. Then the lymphoprep with the cell slurry was

overlaid and centrifuged at 800 g for 30 min that led to several layers including blood mononuclear cells. The cells were then re-suspended in 3 ml FACS buffer before being counted using haemocytometer. Finally, 100 μl of the cell was added to FACS tubes prior to staining, resulting in 1×10^5 cells/tube. The blood mononuclear cells layer used as negative population that does not bind CD16b.

2.6.2 Digestion of tissue

Using the model described in section 2.2, flow cytometry experiments were conducted on cells dispersed from tissue samples following treatment and 24 h incubation. Tissue was prepared as previously described, and the following day the culture medium was completely removed and replaced with 1 ml of basal phenol red free RPMI. The tissue was then transferred to 4 ml of digestion medium, made of 1 mg/ml collagenase from *Clostriduim histolyticum* (Sigma, 268 units/mg) diluted in phenol red-free RPMI in 25 well universal tubes containing a magnetic stirrer bar. The tubes were then placed in an incubator on a magnetic stirrer at 37°C for 90 min with rapid agitation. The digested cell slurry was then passed through a 100 μm cell strainer into a polypropylene FACS tube ready for antibody staining. The FACS tubes were centrifuged at 400 g for 6 min at 4°C. The supernatant was then carefully discarded, the cells collected in one tube and 2 ml FACS buffer added (DPBS containing 0.5% (w/v) BSA and 2 mM EDTA) before another centrifugation.

2.6.3 Identification of different cell types

Supernatant was discarded and 50 μl of FACS buffer containing 10% (v/v) human IgG (Fcγ blocker) per tube were added. After 10 min incubation in ice, 50 μl from the mixture was added to each of the labelled tubes. The primary antibodies or equal volumes of isotypes (isotype control) or FACS buffer (unstained control) were added, and tubes topped up to 100 μl with FACS buffer and incubated for 30 min on ice, in the dark. Two ml of FACS buffer per tube were then added and the cells centrifuged to form a pellet as before. If intracellular staining is required the cells get re-suspended in 100μl Permwash, the intracellular antibody also gets added. After 30 min incubation on ice in the dark 2 ml of Permwash was added and the tube centrifuged at 400 g for 5 min.

Finally the supernatant was discarded and resuspended in 500 μl FACS buffer before being analysed using a FACS Aria (BD, Bioscences, San Jose, CA 95131 USA); equipped with red blue

and green lasers as previously described (Vijayanand et al., 2007). Cells were filtered through 100 μm strainer caps just prior to analysis.

Table 2.3 Description and details of antibodies used flow cytometry. Lung parenchyma from human lung was digested and then stained for leukocytes, epithelial cells, lineage and T cells. This table shows the isotype and subtype of the antibodies. It also shows the epitope stained.

Antibody	Subtype	Isotype	Clone	Epitope stained	Fluorochrome	Quantity (μ/test)
Leukocytes	Monoclonal	IgG1	HI30	CD45	PE-Alexa Fluor 610	10
Epithelial cells	Monoclonal	IgG2ak	MOPC-173	Epcam	PerCPCy5	10
HLA-DR	Monoclonal	IgG2ak	L243	MHC	APC-Cy7	5
Lineage	Monoclonal	IgG1k IgG2bk	SK7, L27 3G8, SJ25C1	CD3, CD14,CD16, CD20, CD56	FITC	10 10
T cells	Monoclonal	IgG1	SK7	CD3	PE-Cy7	5
T cells	Monoclonal	IgG1k	RPA-TA	CD8	APC	5

2.6.4 Using Flow cytometry to quantify TLR 2/4

Flow cytometry was used to detect TLR 2 and TLR 4. Antibodies were first titrated against peripheral blood mononuclear cells due to the scarcity of tissue samples.

2.6.4.1 Isolation for PBMCs for TLR 2/4

Antibodies to be used for TLR2/4 analysis were first optimised using PBMC from a healthy volunteer as described above (Section 2.6.1). The antibodies were titrated by adding varying

concentration of antibody to a fixed volume of FACS buffer and analysing the resultant FACS spectra for specific fluorescence shifts into the gate determined from isotype. Details of the antibodies and dilutions used are described below.

Table 2.4 A range of dilutions was used to titrate TLR 2/4 antibody in human monocytes to subsequently use in lung parenchyma and stained using flow cytometry. TLR 2/4 antibody were conjugated to PE.

Isotype	20 µ l	10 µ l	5 µ l	1 µ l	0.5 µ l	0.1 µ l
TLR 2/4	20 µ l	10 µ l	5 µ l	1 µ l	0.5 µ l	0.1 µ l

Phycoerythrin-conjugated anti-human TLR 2 (clone TL2.1), TLR 4 (clone HTA125), or the relevant phycoerythrin-conjugated isotype control antibodies (mouse IgG2a, K) were used in a dilution. We first tried to detect TLR 2 and 4 while fixating the tissue with formaldehyde. Intracellular staining allows for the detection of intracellular antigen like CD68. However, permeabilization led to the loss of TLRs, so the staining was done with live cells.

2.6.5 Description and details of antibodies used flow cytometry

Lung parenchyma from human lung was digested and then stained for leukocytes, HLA-DR ephithelial cells, lineage and T cells. Table 2.3 shows the isotype and subtype of the antibodies. It also shows the epitope stained.

The antibodies were used with corresponding isotypes as detailed in Table 2.3. The pan-leukocyte antibody CD45 was labelled with PE-Alexa Fluor 610. T cell antibodies were labelled with PE-Cy 7 for CD3 and with APC for CD8. Epithelial cell antibody EpCAM was labelled with PerCP-Cy5.5. For macrophages detection: HLA-DR was labelled with APC-Cy7 and lineage labelled with FITC.

2.6.6 Quantification of inflammatory cells using flow cytometry

A FACS ARIA flow cytometer (Becton Dickinson Immunocytometry Systems (BDIS), Oxford, UK) was used to record cell events using compensation settings and instrument configurations optimized previously. Data were analysed using FACS DIVA software to set gates according to isotype control antibody signals. Cell numbers were calculated as the geometric specific mean fluorescence intensity (sMFI, mean value of test antibody from which the fluorescence value of the isotype antibody had been subtracted) of the gated cell population.

2.7 Incubation with P38 MAP kinase inhibitors

The inhibitors were first made into an original stock solution of 10 mM using DMSO as diluent. It was then stored as 10 µl aliquots. Solutions were made to give concentrations in the range 500 to 1 nM that contained the IC50, which is between 50-10 nM for both drugs. The recorded aqueous solubility of PF-03715455 inhibitors was 1.2 µM and that of PHA-797804 was 4.3 µM (data supplied by Pfizer).

Serial dilutions of the 10 mM stock of each drug were prepared in DMSO. These dilutions were then diluted in the appropriate volume of RPMI to achieve the desired final concentration of the drug (500, 100, 50, 10, 5, 1 and 0 nM), which in all cases contained a final concentration of 0.1% (v/v) DMSO carrier.

The tissue was then prepared as previously described. One ml from the pre made RPMI with the various relevant concentrations of either drug PHA and PF was placed in each well of 24 plates. Three-five tissue explants were added to each well and incubated at 37^{o}C in a tissue incubator for 2 h before 100 ng/ml of LPS was added. Negative controls containing no LPS or drug but including carrier where appropriate were prepared in parallel. Positive controls for drug efficacy included fluticasone propionate at 10 nM. Supernatant was collected after 24 h and stored at -80^{o}C prior to analysis. Tissue was also stored at -80^{o}C for possible use in future work.

2.8 Statistical analysis of results

Statistical analysis was performed using StatView for Mac (GraphPad software inc, La Jolla, CA, USA) and GraphPad Prism (v.3.0) for PC. Data distribution was determined as normal or skewed via the D'Agostino normality test. Data was not normally distributed and was analysed via non-parametric tests (Dunn's Multiple Comparison test, non parametric Mann Whitney U test, Wilcoxon signed-rank test and ANOVA as appropriate) and depicted as median values.

Chapter 3: Comparison of inflammatory cell load in the lung parenchyma of Control and COPD subjects

3.1 Introduction

As mentioned before the full range of inflammatory cells recruited to the lung tissue itself during COPD exacerbations is not clear, as it is difficult to carry out bronchial biopsies at exacerbations, as it can be an overly invasive procedure for acutely ill patients, so information scarce and further work is needed. Most of the information available about lung parenchyma has been extrapolated from alveolar lavage and also sputum, however these compartments may not be representative of the cell population in alveolar tissue which could reasonably be assumed to contribute to alveolar cell destruction in the emphysematic patient (Martin et al., 1985, Keatings et al., 1996, O'Donnell et al., 2004). There are only a few studies examining the inflammatory process in the lung parenchyma of COPD patients due to the difficulty of access to such tissues.

Since there is conflicting evidence of the inflammatory cell infiltrate in the lung parenchyma, the aim of this chapter is to further clarify the changes in inflammatory cell numbers in the lung parenchyma of COPD patients.

To do this, two different quantitative techniques, namely immunohistochemistry and flow cytometry were employed. Immunohistochemistry is the standard technique for this kind of analysis but is laborious and has limited throughput; conversely, flow cytometry used for this purpose has not previously been reported in lung parenchymal tissue but offers several potential benefits in terms of quantitation and speed of analysis.

In order to do this, inflammatory cell loads in two separate cohorts of Control/COPD patients were analysed firstly by immunohistochemistry using monoclonal antibodies directed against key inflammatory cell types, and secondly a flow cytometry method was developed in order to analyse inflammatory cell types in cells enzymatically dispersed from lung parenchymal tissue.

3.2 Part I: Cell quantification using immunohistochemistry

First cells were quantified using IHC.

3.2.1 Method: Characterization of inflammatory cells by IHC

In order to characterize the inflammatory cell populations in lung parenchyma, immunohistochemistry was initially chosen as the gold standard method as it has been used previously (Saetta et al., 2003).

Human lung tissue was obtained, with written informed consent, from patients who were undergoing carcinoma resection at Guy's Hospital in London or at Southampton General Hospital (n = 31). Patients were undergoing either carcinoma resection or bullectomy. The patients' age, gender, lung function and smoking history were recorded (Table 3.1).

Table 3.1 Patient characteristics for IHC: Human lung tissue was embedded in GMA and then stained. Patients with a FEV_1/FVC ratio of < 0.7 were classified as COPD. Those with FEV_1/FVC ratio of ≥ 0.7 were defined as Control patients. The patient characteristics, including smoking status were recorded. Age, FEV_1/FVC and FEV_1% predicted are shown as median and quartile range. For gender, M = male and F = female. Under smoking status, current denotes current smokers, ex denotes ex-smokers as determined by the patients clinician. Never means never-smokers.

Age	61 (49-69)	71 (69.5-75)
Gender	7F/5M	8F/11M
FEV_1/FVC	0.76 (0.71-0.83)	0.62 (0.56-0.65)
FEV_1% predicted	100 (89-100)	75 (63.25-82.5)
Smoking	10 ex/1 Never/1 current	15 ex/4 current
Pack years	30 (24.75-45)	40 (30-50)

As mentioned in chapter 2 human lung parenchyma samples that had been embedded in GMA was stain for different cell types. Examples of the staining patterns observed are shown in Figure 3.1. These demonstrate that the different antibodies, despite optimisation, gave different staining intensities, dependent upon the antigen being detected, and therefore

presumably the abundance of each antigen, the antigen preservation, or the affinity of the antibody for its intended target. For example, CD3 staining was generally faint, whereas neutrophil elastase and tryptase were easily detected.

The majority of inflammatory cell types were found located in the tissue interstitium or sub-epithelium, with the exception of macrophages, which were found within the tissue but also attached to the wall (Figure 3.1 (a) to (g)).

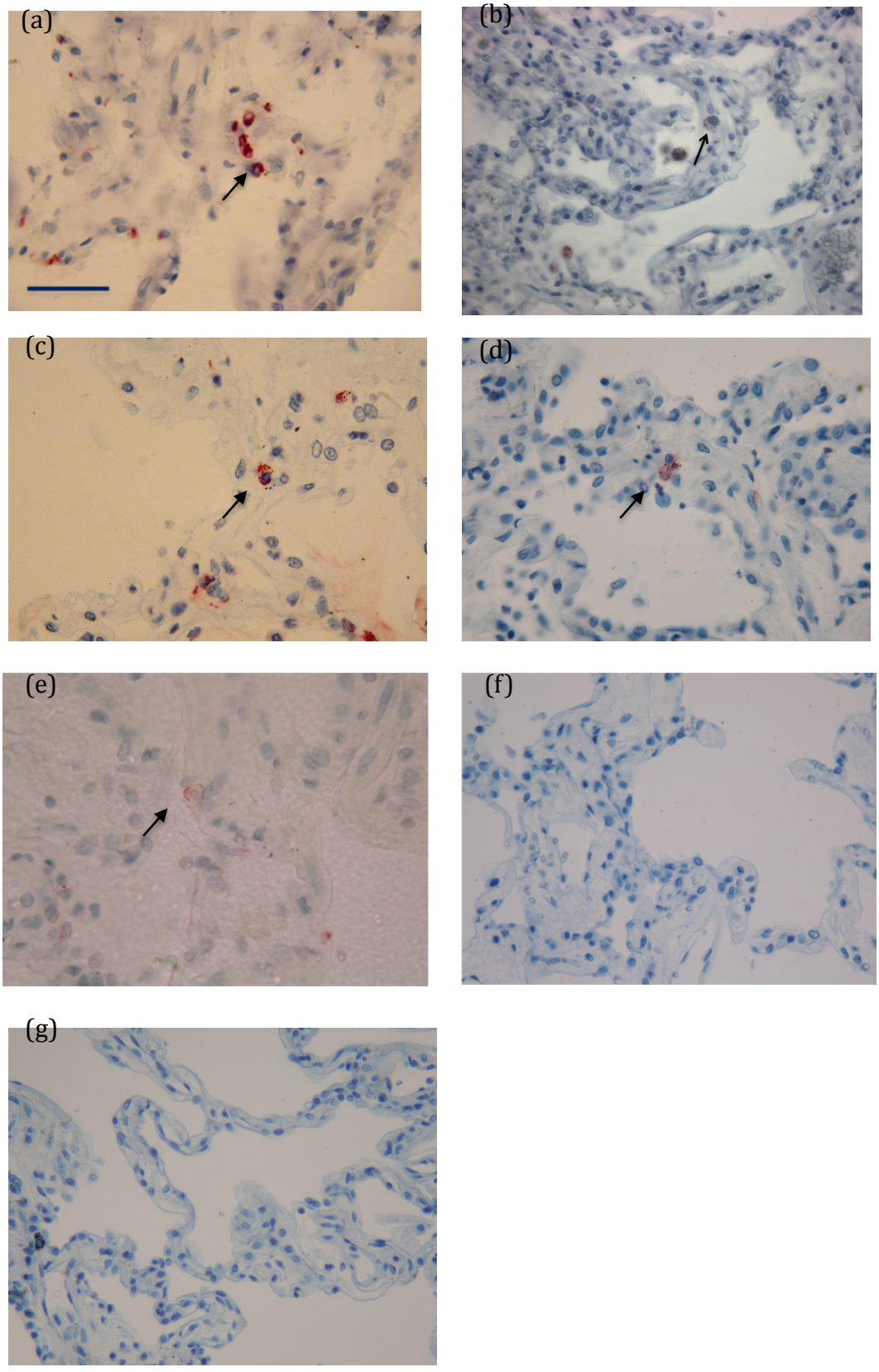

Figure 3.1 Immuno-histochemical staining for various inflammatory cell types in GMA-embedded lung parenchyma samples viewed at 40 magnifications. Immuno-

histochemistry is used to identify different inflammatory cell types using cell-specific markers. Positive staining is seen in red (arrow), with blue haematoxylin counterstain. Typical staining patterns are shown for neutrophils (neutrophil elastase, a), macrophages (CD68, b), mast cells (mast cell chymase, c), eosinophils (eosinophil cationic protein, d) and T cells (CD3, e) staining in parenchymal tissue. Control slides, incubated with TBS buffer and negative control IgG as opposed to specific monoclonal antibodies show no staining (f and g). Scale bar represents 100 µm. Images are representative of n = 31. Arrows indicate examples of positive staining for each cell type.

3.2.2 Immunohistochemisty result

3.2.2.1 Cell numbers quantification

Immunohistochemistry did not reveal a difference in inflammatory cell load between Control and COPD subjects for Neutrophils, Macrophages, Eosinophils and Mast cells.

In lung parenchyma, we did not find any difference in the number of inflammatory cells/mm^2 between the groups regarding neutrophils, macrophages, eosinophils and mast cells except for T cells where an apparent significant increase in the control group was seen (see Figure 3.2). Part of the reason for this lack of difference could be due to the scarcity of certain cell types, giving rise to significant amounts of null data.

Substantial numbers of neutrophils could be detected in some patients from all groups (Figure 3.2 (a)) also a great deal of inter-patient variability was seen regarding the number of these cells however no over all difference between the groups. Similarly a number of macrophages were noted in both groups and few patients did not have any at all but there was not any significant difference between the groups (Figure 3.2(b)). One factor that may be responsible for this variability is disease heterogeneity, however our cohort was not large enough to associate sub-phenotypes of COPD with inflammatory cell indices. Mast cell number differed between individuals, and there was no overall difference between the groups (Figure 3.2 (c)). Regarding T cells, where significant increase in the Control group was seen ($p = 0.028$), the staining revealed very few $CD3^+$ cells. A couple of COPD patients had few T cells compared to the rest that show zero T cells. However, for the Control a sub group had few T cells compared to another sub group that show zero T cells (Figure 3.2 (d)). Finally, eosinophils were investigated and there was no significant difference in the number of eosinophils between COPD and control group and many individuals had a zero or low number of these cells (Figure 3.2 (e)).

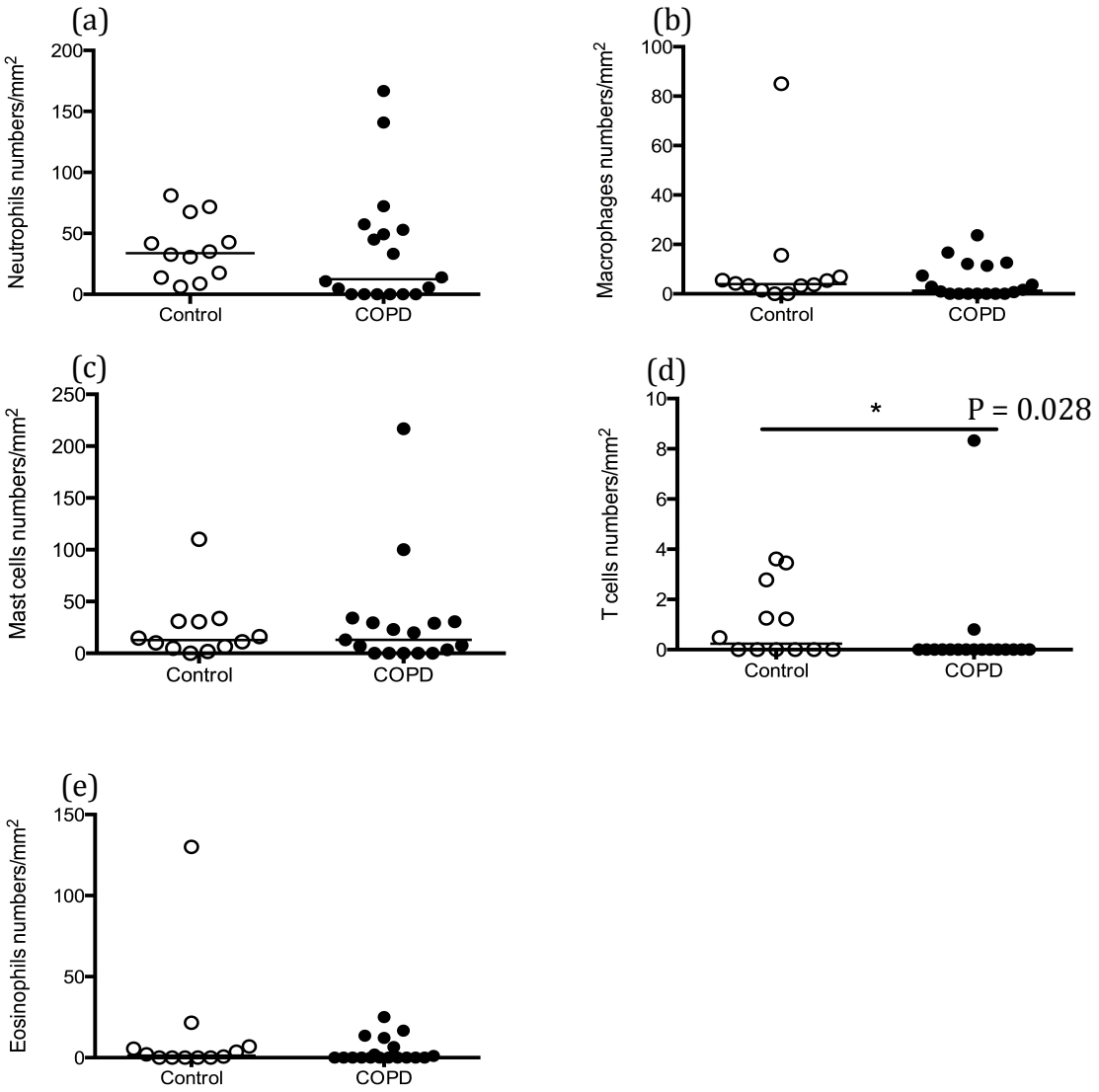

Figure 3.2: Quantification of inflammatory cells in lung parenchyma tissue samples of controls and subjects with COPD. Tissue samples were assessed using immunohistochemistry. Individual counts for neutrophils (a), macrophages (b), mast cells (c), T cells (d) and eosinophils (e) in the lung parenchyma, adjusted for airspaces to give the numbers of cells/mm^2. Open circles represent control subjects (n = 12), and closed circles represent individuals with COPD (n = 19). Horizontal bars represent median values. Non parametric Mann-Whitney U tests were applied to compare the two groups. P = 0.028 for T cells.

Probably the key reason for a lack of difference between Control and COPD in the different cell types other than T cells (where there was a large number of 0 value), using this method was the low density of inflammatory cells of any type within tissue samples processed in this manner. Although GMA sections had the advantage of good structural and antigen preservation, the

total surface area to be calculated for each tissue was relatively small, compared to previous studies performed in larger tissue blocks where paraffin sections would normally be used.

3.2.2.2 Correlation of inflammatory cell load with clinical measures of COPD severity

A further correlation analysis was performed to further clarify the finding of inflammatory cells quantification. Therefore, the quantity of inflammatory cells was compared with lung function measurements such as FEV_1/FVC ratio and FEV_1% predicted. The correlation analysis was performed separately in the Control and COPD groups to prevent skewing of the data by the already known significant difference in certain cell types in sputum from these two patient groups.

As the data were not normally distributed, spearman's correlation analysis was performed, which showed no correlation between the number of inflammatory cells and FEV_1/FVC ratio (Figure 3.3 (a)-(b)-(c)-(e)-(d)).

No statistically significant correlation was also observed when comparing macrophages, mast cells and eosinophils with FEV_1% (Figure 3.4 (b)-(c)-(e)). As for neutrophils a very modest possible positive correlation $r = 0.50$ was likely in COPD as p value was significant $p = 0.038$, (Figure 3.4 (a)), however, because the r value is far from 1 it is unlikely that there is a correlation. In respect to T cells, the r value was virtually 0 indicating no association between T cells number and FEV_1% predicted.

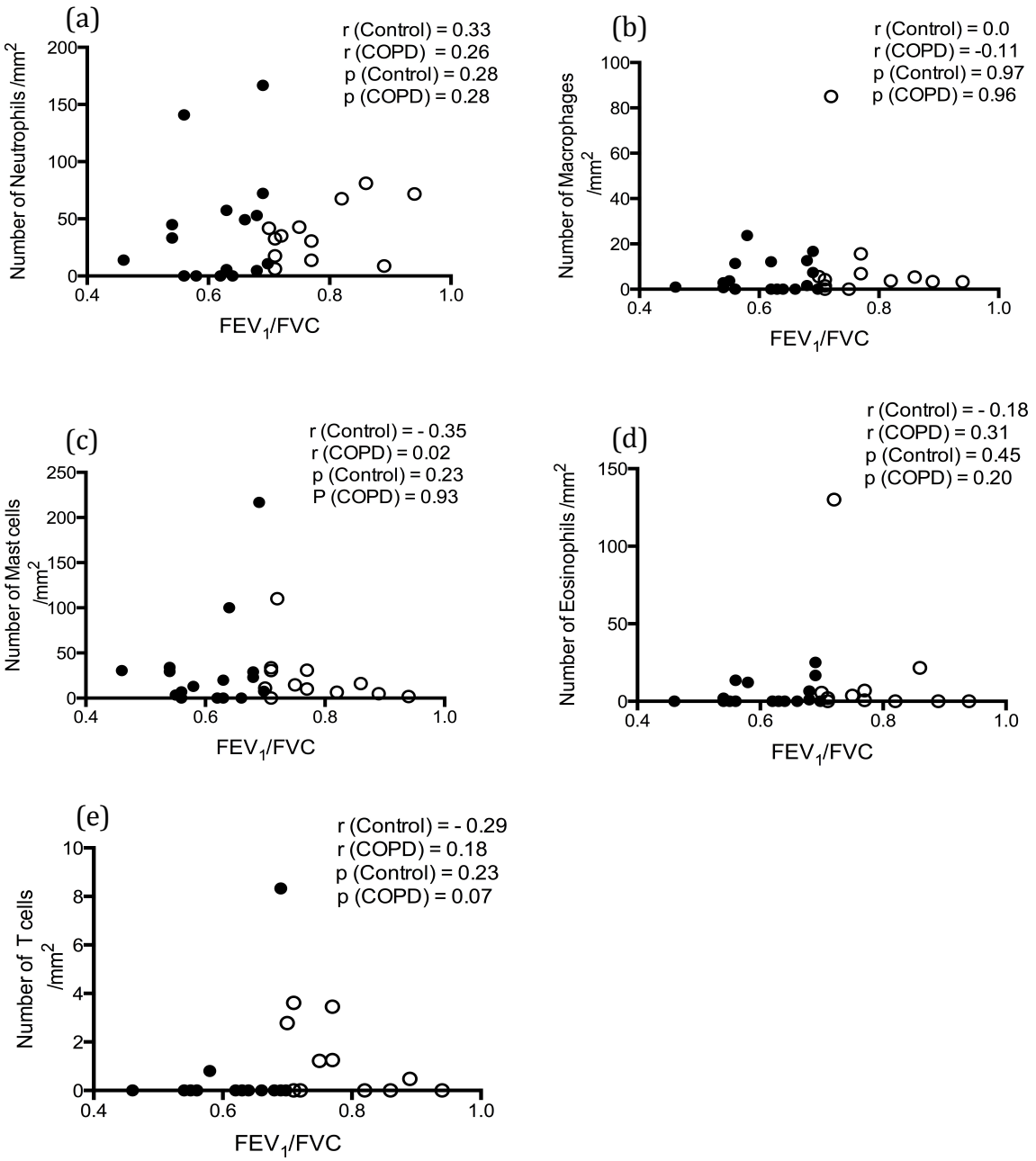

Figure 3.3: Correlation analysis to determine the relationship between inflammatory cell number and lung function (FEV_1/FVC) in lung parenchyma of control subjects and individuals with COPD. Inflammatory cell numbers in lung parenchyma tissue immunohistochemically stained for inflammatory cells was plotted against lung function data (FEV_1/FVC). Dot plots are shown for: Neutrophils (a), macrophages (b), mast cells (c), T cells (d) and eosinophils (e). Data for controls and individuals were compared separately using Spearman's Rank correlation analysis, and the summary of the statistical analysis is shown as r-values on each graph. Closed circles show values for individuals with COPD (n = 19); open circles indicate data

values for control subjects (n=12). No statistically significant correlations were observed.

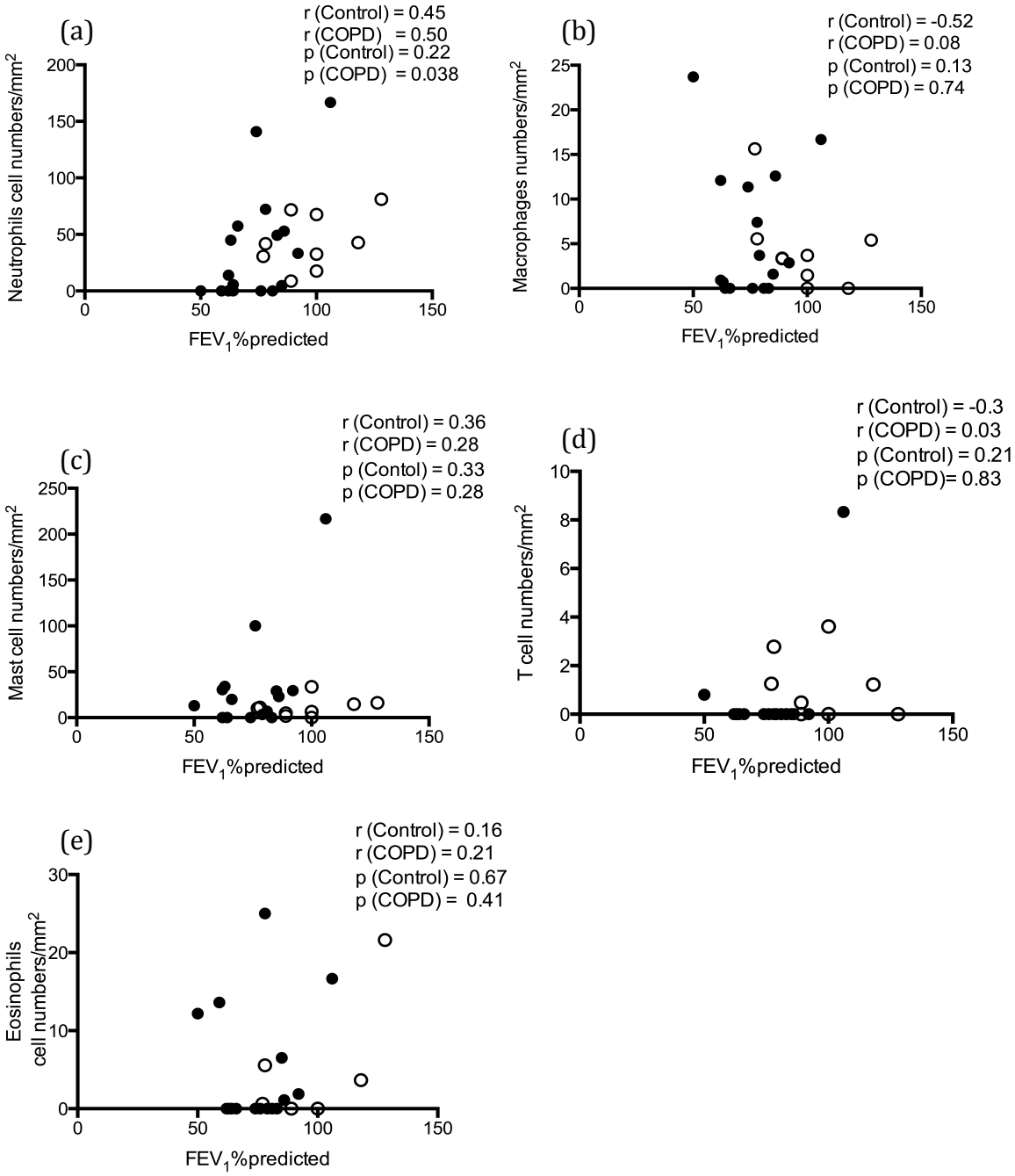

Figure 3.4 Spearman's rank correlation analysis between FEV_1% predicted and the numbers of inflammatory cells in lung parenchyma: Neutrophils (a), macrophages (b), mast cells (c), T cells (d) and eosinophils (e). Result for Control and COPD patients is shown as r-values on each graph. Closed circles indicate COPD (n = 19); open circles indicate control (n = 12). The p values are shown.

3.3 Part II: Quantification of inflammatory cells using flow cytometry

Flow cytometry was used next to quantify inflammatory cells.

3.3.1 Method Optimisation: Characterization of inflammatory cells by flow cytometry

As there was a perceived limitation to IHC method due to the scarcity of some cell types, which resulted in significant amounts of zero value data, we decided to attempt to characterize the inflammatory cell numbers using flow cytometry, as it could theoretically allow better recovery of cells and more unbiased quantification. Flow cytometry has previously been used to analyse inflammatory cell numbers and activation status in gut tissue samples (Connor et al., 2004). It has also been used to analyse cells in the bronchial compartment of asthmatic subjects (Hidi et al., 2000, Vijayanand et al., 2007). Flow cytometry theoretically is more quantitative and allows higher throughout, but is complex to optimise due to the multiplexed nature of the analysis system, and to the pre-processing of samples, including enzymatic digestion of the tissue using collagenase, which could potentially destroy cell surface antigens essential for cell identification. Our aim was to optimize this system for cell quantitation in parenchymal tissue, in order to provide an invaluable tool for cell analysis in lung tissue.

Antibodies to be used for the inflammatory cell analysis were firstly optimised using peripheral blood mononuclear cells (PBMCs a kind gift of Dr Karl Staples) and purified neutrophils from a healthy volunteer. The method of isolation of PBMCs using density centrifugation, and neutrophils by pre-treatment with dextran is described in the methods section.

All the antibodies were titrated by adding varying concentrations of antibody (20, 10, 5, 1, 0.5, 0.1 µl) to a fixed volume of FACS buffer and analysing the resultant FACS spectra for specific fluorescence shifts into the control gate determined from isotype staining. Details of the antibody, fluorochrome, isotype, dilution, clone and source of each of the antibodies is shown in Table 2.3.

We developed a multistage staining process based on standard flow cytometry staining procedures in our laboratory, where PBMCs/neutrophils were firstly stained using antibodies directed against extracellular markers. The cells were then fixed and permeabilised to allow intracellular staining as CD68 was intracellular. Neutrophils were stained using CD16b in FITC, CD45 in PE-AF10 and HLA-DR.

As the antibody that had been used for immunohistochemical detection of macrophages (CD68) was only available conjugated to the PE fluorochrome, which was not readily available colour

for our analysis, we decided to use HLA-DR-APCCy7 and lineage-1 in FITC for the staining of macrophages as this has been shown to identify cells with morphology consistent with macrophage cells (Staples et al., 2012). Figure 3.5 shows an example of the gating strategy to identify these cells.

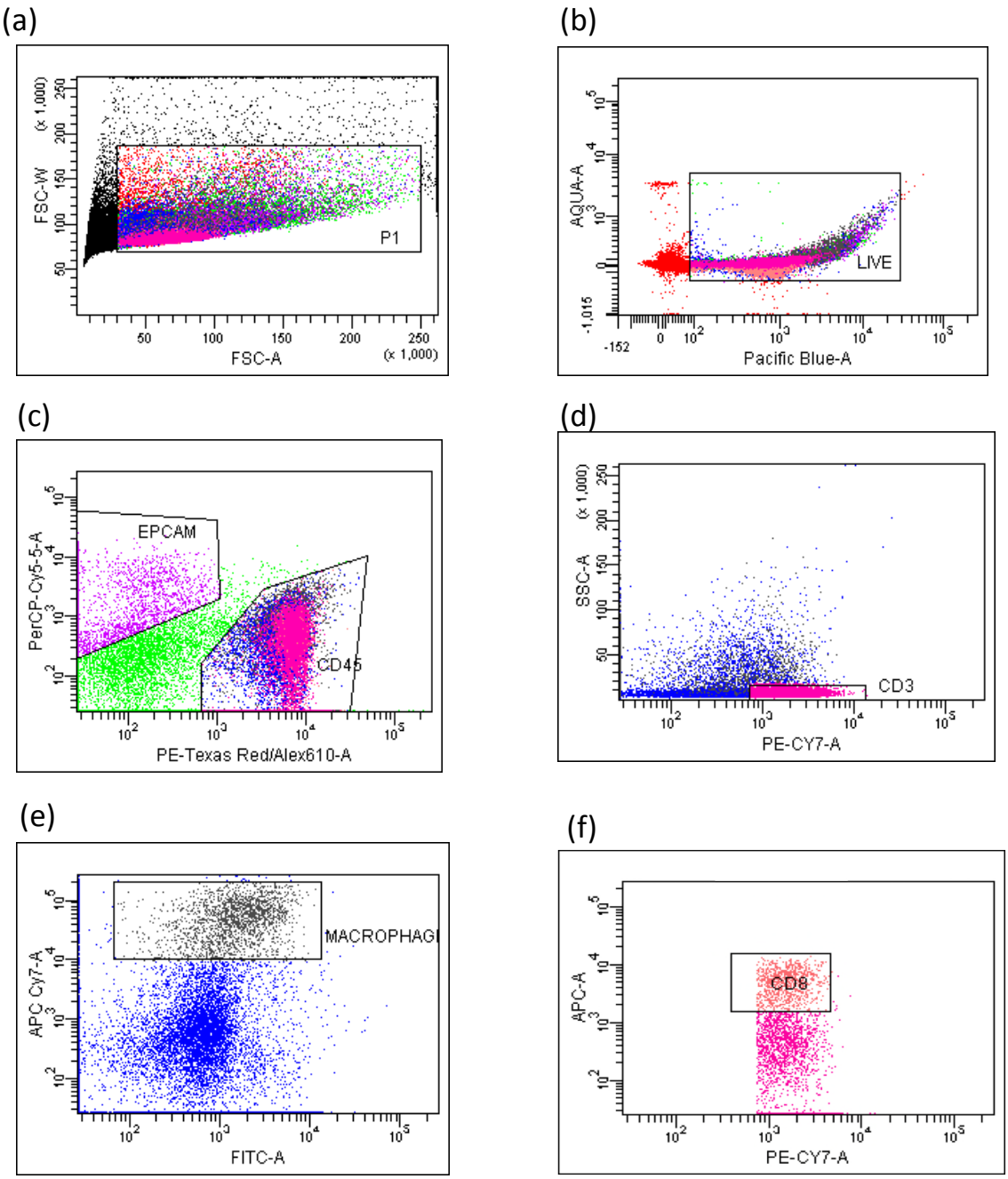

Figure 3.5 Gating strategy for identification of different cell types in collagenase-dispersed lung parenchyma tissue using flow cytometry. Enzymatically dispersed cells were identified by staining for cell-specific surface markers using separate fluorochromes, and then analyzed using a flow cytometer. Flow cytometry dot-plots are shown for

each stage of the cell discrimination, demonstrating the gating strategy used to identify different cell types, discriminating positive from negative staining using isotype and fluorochrome-matched control antibodies for each specific antibody. Firstly, cells were gated on size using forward scatter properties gate (a). Then, viability stains were used to differentiate live cells from dead cells, dead cells were excluded using LIVE/DEAD aqua dead cell stain gate (b). Using CD326 and CD45 specific antibodies respectively, epithelial cells and inflammatory cell populations could be identified (c). Then, T-lymphocytes could be identified using a gate for CD3-positive cells (d). Excluding T-lymphocytes, macrophages could be identified by high positive staining for a combination of HLA-DR and Lineage-1 antibody cocktail. Finally, cytotoxic T-lymphocytes could be positively identified using antibodies directed against CD8 (f). The gating strategy is described in detail in the methods section.

Having optimised the staining strategy using isolated PBMCs and neutrophils, similar stains were performed in resected tissue samples. Preliminary analysis found that CD16b-positive cells were not identifiable in lung tissue samples. This could be because they have a very limited half-life (maximum 3 days) in the circulation, and the duration and stresses of tissue digestion, coupled with overnight incubation in cell culture post-surgery, could have led to significant apoptosis of these cells. Furthermore, if they had become activated, neutrophils would probably have lost their viability following these treatments. Therefore, neutrophils were excluded from subsequent analyses.

Figure 3.5 shows the optimised gating strategy in resected tissue, firstly to select singlet cells from purified cell preparations using forward scatter characteristics (FSC-W/FSC-A) (Figure 3.5 (a)). Viable cells were then selected using the viability marker calcein-violet in the pacific blue channel. Dead cells (in the AQUA channel) were excluded on the same axes (Figure 3.5 (b)). Then, cell-specific antibodies to identify leukocytes (CD45), macrophages ($CD45^+/CD3^-/HLA\text{-}DR^+$ and $Lineage^+$) lymphocytes ($CD45^+/CD3^+$), and epithelial cells ($CD326^+/CD45^-$) were used. As it was previously known that collagenase digestion destroyed the CD4 epitope, CD8 alone was used to identify cytotoxic T lymphocytes, and the CD4 population had to be inferred from the CD8 negative population. Whilst this was not ideal, one would expect the majority of $CD3^+$ T lympyhocytes to be either $CD4^+$ or $CD8^+$ ($CD3^+/CD8^+$), and so was considered an acceptable, if not ideal gating strategy. Indeed, the live population was then gated for two different cell types (epithelial cells and $CD45^+$ inflammatory cells) (Figure 3.5 (c)). Inflammatory cells $CD45^+$ were then gated for $CD3^+$ T lymphocytes (Figure 3.5 (d)), and sub-gated for $CD8^+$ T lymphocytes and

$CD8^-$ T lymphocytes (Figure 3.5 (f)). An inverse gate was used to exclude T cells from the leukocytes population and then macrophages were identified as $CD45^+$, HLA-DR^+, $Lineage^+$ cells (Figure 3.5 (e)).

3.3.1.1 Comparison of inflammatory cell profiles in lung parenchyma tissue samples control and COPD individuals using multicolour flow cytometry

Human lung tissue dissected distantly from the resection margin and showing normal gross morphology was obtained from 41 patients who were undergoing carcinoma resection (n = 41). Patients' age, gender, lung function and smoking history were recorded (Table 3.2). Tissue was processed by collagenase digestion from freshly dissected material as previously described in section 2.2.

Table 3.2 Patient characteristics for Flow Cytometry: Human lung tissue was collected and then stained. The patient characteristics, including smoking status were recorded. Patients with a FEV_1/FVC ratio of < 0.7 were classified as COPD. Those with FEV_1/FVC ratio of ≥ 0.7 were defined as Control patients. FEV_1/FVC, FEV_1 % predicted, age and pack years are shown as median and quartile range. For gender, M = male and F = female. Under smoking status, current denotes current smokers, ex denotes ex-smokers as determined by the patients clinician. Non parametric Mann Whitney U test showed no statistical difference between the group in age and pack years.

	Control	COPD
Gender	18 (8F/10M)	23 (10F/13M)
Age	66 (55.25-70)	64 (63.5-72)
FEV_1 % predicted	92.18 (77.81-101.85)	72.78 (55.89-89.24)
FEV_1/FVC	0.79 (0.75-0.81)	0.61 (0.55-0.64)
Smoking status	14 Ex/4 Current	18 Ex/5 current
Pack years	30 (20.06-38.75)	39 (12.75 – 56.25)

3.3.2 Flow cytometry result: Quantification of inflammatory cell numbers in lung parenchyma tissue

In order to validate the use of the flow cytometer for quantitative cell measures and to measure the validity of using tissue weights for adjustment of cytokine analyses, the number of inflammatory cell numbers recovered post-digestion was compared with the starting wet tissue weight (Figure 3.7) in both groups (4 never smokers and a pipe smoker were included in patients cohort for Figures 3.6 and 3.7).

A reasonably small positive association was observed between the two variables for both group in the case of epithelial cells r = 0.30 (control) and r = 0.23 (COPD), for CD45 r = 0.44 (Control) and r = 0.20 (COPD). A vey modest positive association was observed in the control group for CD3 cells, r = 0.50 and for $CD3^{+}/CD8^{-}$ cells r = 0.51, indicating that, despite some outliers (high weight could be caused by variability in tissue hydration during the weighing process), the weight of the tissue was reasonably correlated with cell recovery. However, for macrophages r was very close to 0 (r = -0.18) in COPD group, which indicates that there is no relation between the tissue weight and the number of macrophages.

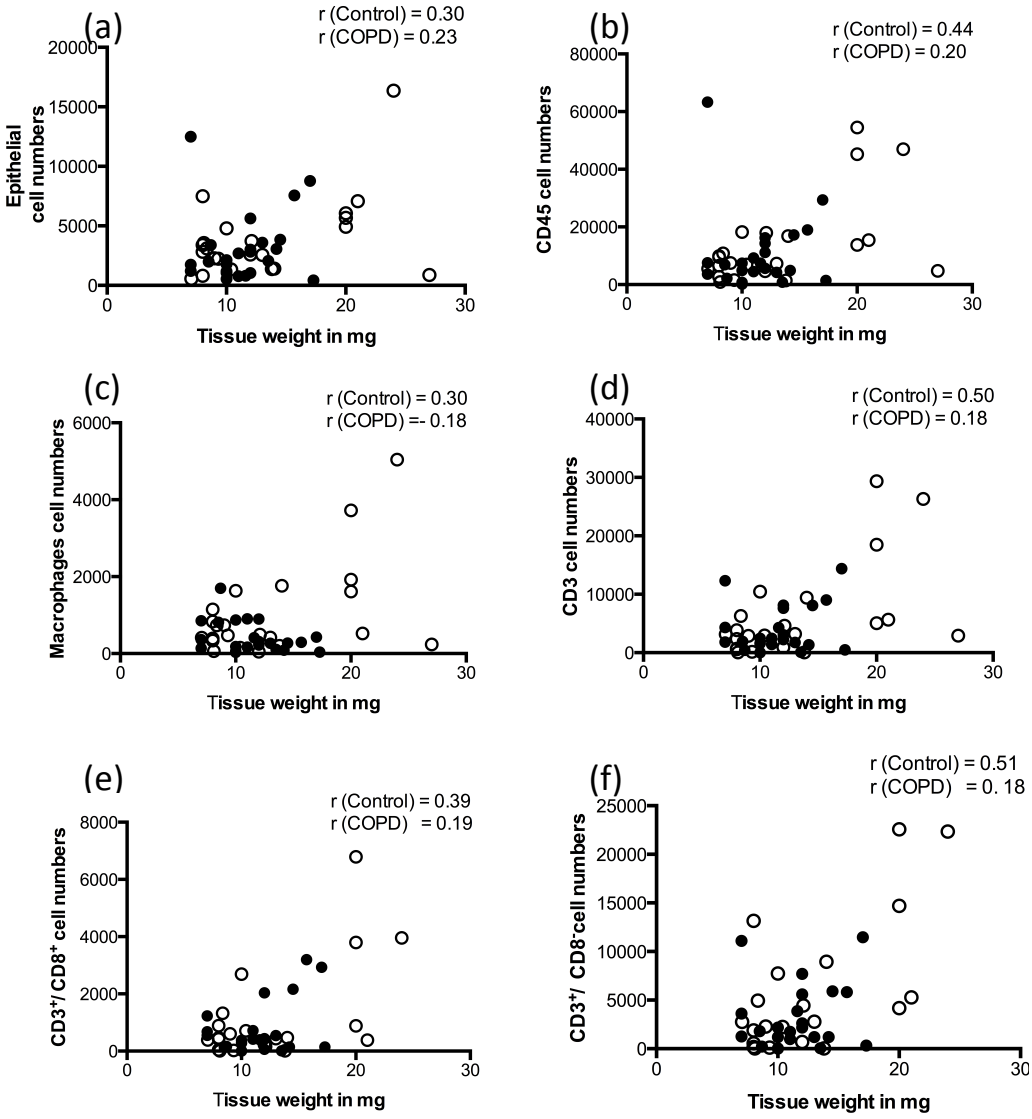

Figure 3.6 Correlation analysis to determine the relationship between inflammatory cell number and tissue weight in lung parenchyma of control subjects and individuals with COPD. Inflammatory cell numbers in lung parenchyma tissue calculated from flow cytometry data was plotted against tissue wet weight values (mg). Dot plots are shown for: Epithelial cells (a), $CD45^+$ leukocytes (b), macrophages (c), $CD3^+$ T cells (d), $CD3^+/CD8^+$ T-lymphocytes (e) and $CD3^+/CD8^-$ T-lymphocytes (f). Data for controls and individuals were compared separately using Spearman's Rank correlation analysis, and the summary of the statistical analysis is shown as r-values on each graph. Closed circles show values for individuals with COPD (n = 24); open circles indicate data values for control subjects (n = 22). No statistically significant correlations were observed.

Comparison of tissue weights between the control and COPD groups indicated that a slightly more tissue weight and more variability between patients in the amount of tissue had been analysed in the control group (Figure 3.7).

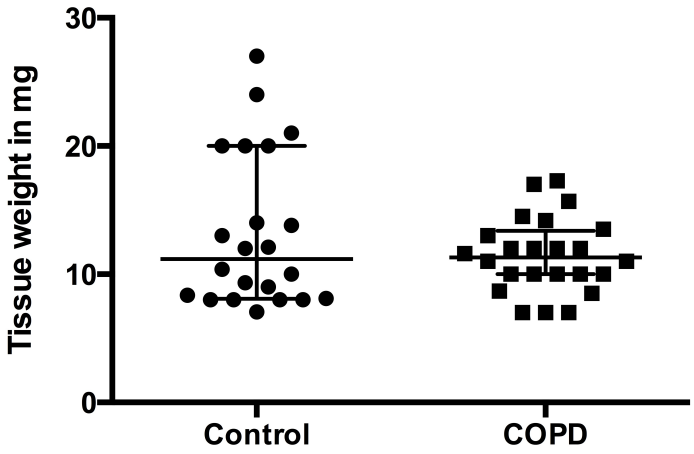

Figure 3.7 Individual wet tissue weight for lung parenchyma tissue samples from control and COPD donors. N = 22 control and 24 COPD donors. Data were compared using Non parametric Mann-Whitney U test. Bars represent median and interquartile range. No difference was observed.

The optimum method for analysing the inflammatory cell component of the tissue was then investigated by plotting the cell numbers obtained in different formats. We first looked at the absolute cell number for each cell (data not shown) and as % of live cell number (Appendix A 1), this data with no adjustment was regarded as not useful because it just reflected the initial tissue weight difference between the control and COPD population. Then data was displayed as cell numbers adjusted for tissue weight (Figure 3.8). Correction for tissue weight resulted in an apparent normalization of the majority of inflammatory cell types. Another way of plotting data was to display epithelial cells and CD45 as % of live cells (Figure 3.9 (a) and (b)) and express macrophages and $CD3^+$ cells as % of $CD45^+$cells, $CD3^+/CD8^+$ and $CD3^+/ CD8^-$ cells as % of $CD3^+$ cell numbers (Figure 3.9 (c-d-e-f)).

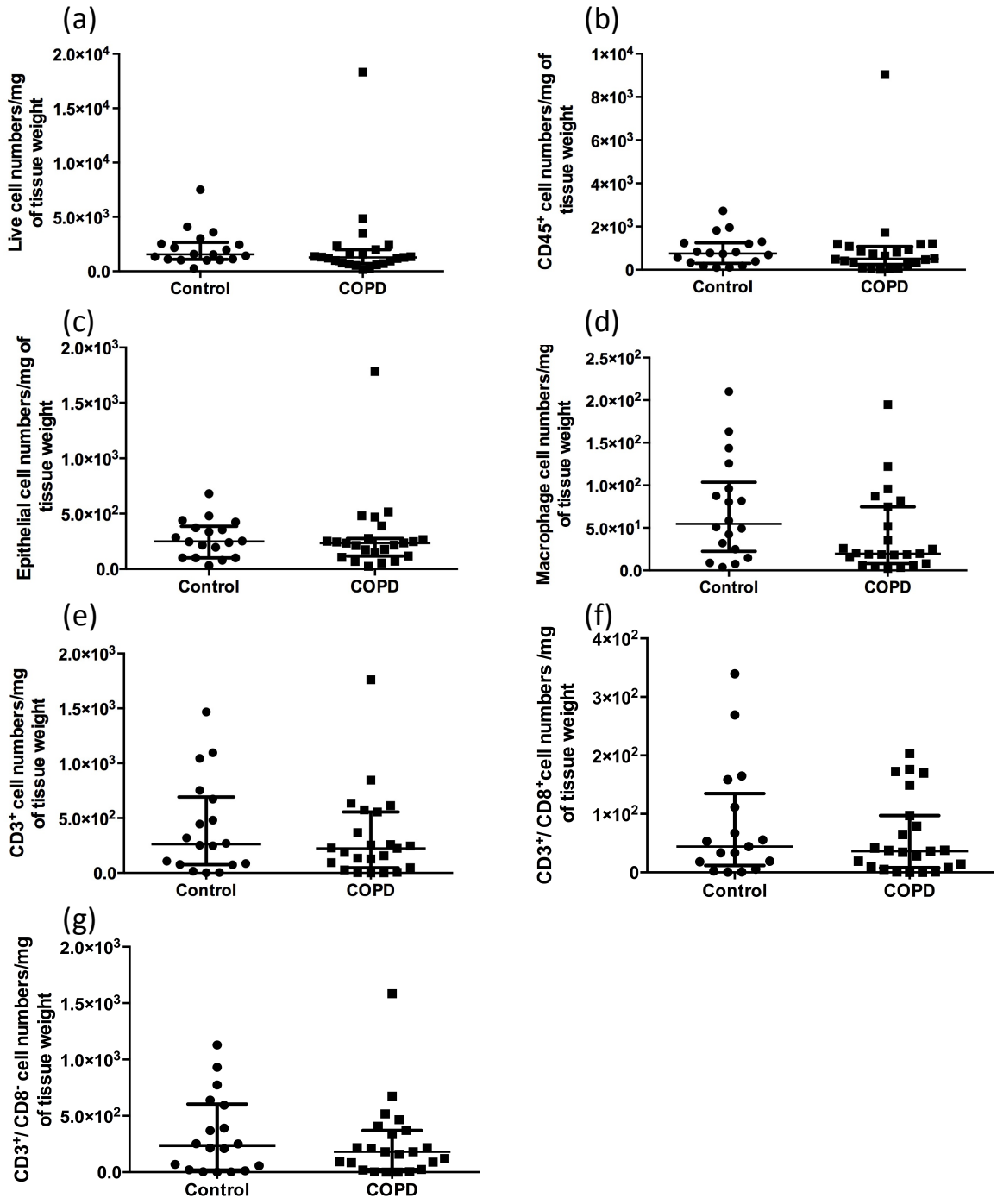

Figure 3.8 Quantification of inflammatory cell numbers in lung parenchyma tissue samples from control and COPD patients using flow cytometry. Comparisons of inflammatory cells in parenchyma from control individuals and donors with COPD. Cells were quantified by flow cytometry as previously described, and cell numbers were adjusted for tissue weight. Bars represent median and interquartile range. Non parametric Mann-Whitney U tests were applied to compare between controls (n=18) and individuals with COPD (n= 23).

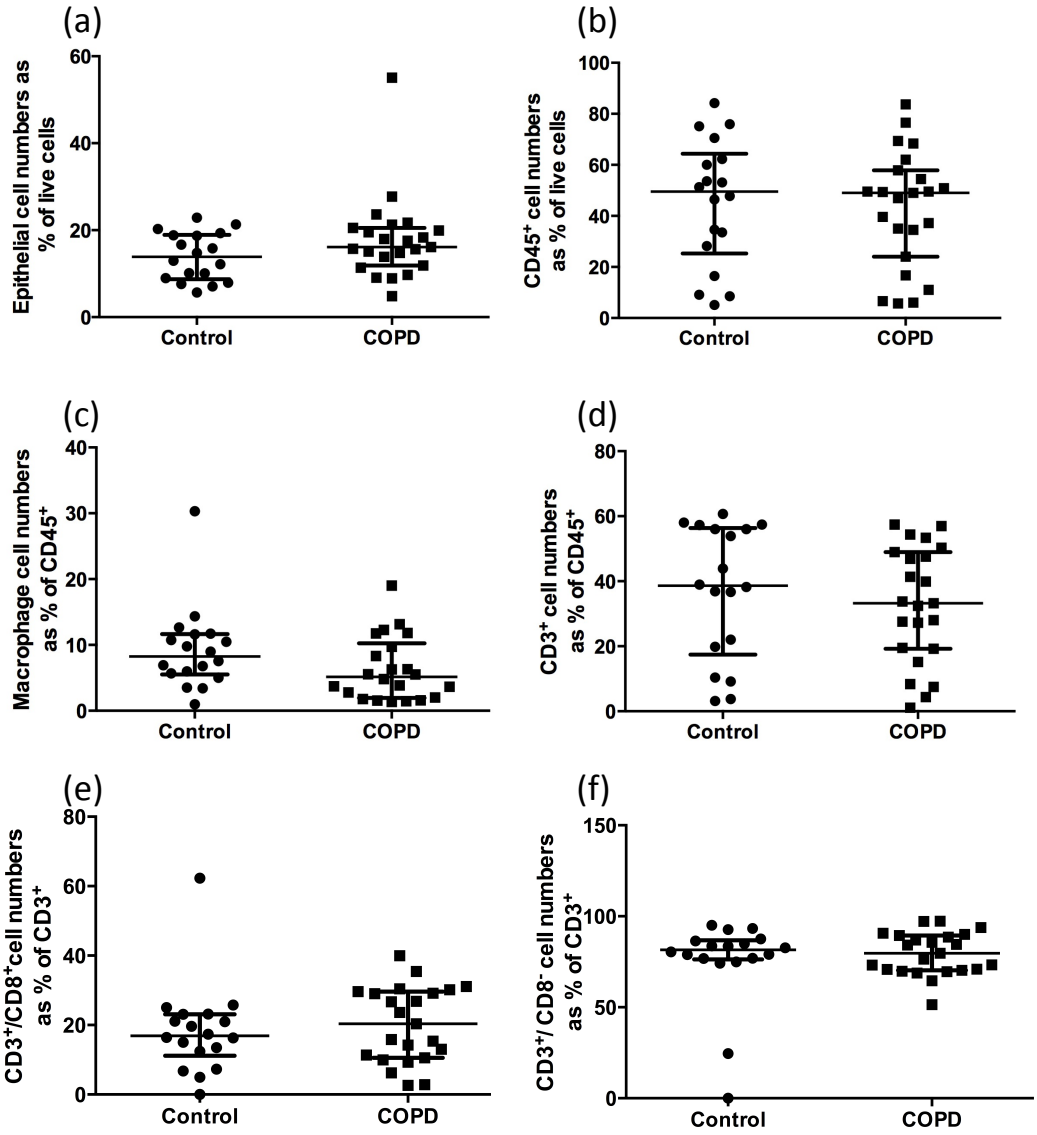

Figure 3.9 Quantification of inflammatory cell numbers in lung parenchyma tissue samples from control and COPD patients using flow cytometry. Comparisons of inflammatory cells in parenchyma from control individuals and donors with COPD Cells were quantified by flow cytometry as previously described, and cell numbers are shown as the percentage of parent cell populations. Bars represent median and interquartile range. Non parametric Mann-Whitney U tests were applied to compare controls (n = 18) and individuals with COPD (n = 23).

3.4 Discussion

In general, we found using immunohistochemistry that there were low numbers of cells per field of all cell types. This is partly due to the nature of the tissue analysed, as parenchyma contains large regions unoccupied by tissue, reducing the cell count in the field of view.

Furthermore, a number of the cell types would be expected to be present in low numbers (such as T cells and eosinophils). Indeed, we found that both of these cell types were absent in a number of the sections analysed, leading to large amounts of zero data points data, making statistical analysis challenging. It is possible that this could be overcome by counting larger areas of tissue, or increased tissue numbers. Also using paraffin may produce a better result because of the larger amount of tissue that could be used. Different cell markers may also yield different results, although we do not have evidence that this would have changed our results.

Flow cytometry data has shown that more cells can be recovered with this method than we did with IHC. Figure 3.6 shows that we can recover up to 20,000 epithelial cells, 80,000 CD45 that (include up to 6,000 macrophages and up to 30,000 CD3) per 30 mg of tissue providing many more data points, faster and with greater accuracy than the traditional immunohistochemical methods. It was noticed that more tissue weight seemed to have gone into the control group (Figure 3.7). The reasons for this are not clear, since tissue pieces were not pre-weighed prior to analysis, however, possible causes include: accidental bias due to restricted quantities of tissue in the COPD group, it is possible that a significant part of pulmonary parenchyma in some of the COPD patient has been destroyed by the pathogenic process like alveolar wall destruction, and also lower wet tissue weight per unit volume due to increased alveolar spaces as a result of emphysema. When looking at analysing the data generated by flow cytometry, the most useful comparisons was by adjusting epithelial cells and $CD45^+$ for live cell number and expressing macrophages and $CD3^+$ cells as % of $CD45^+$ (Figure 3.9 (c) and (d)) and expressing $CD8^+$ and not $CD8^+$ as % of $CD3^+$cells (Figure 3.9 (e) and (f)) suggested a better normalization due to reduced numbers of outliers.

However, using both methods we did not find any significant difference between the two groups for epithelial cells, neutrophils, mast cells and eosinophil. The increased number of T cell in the control group is highly the result of the high number of 0 values in most COPD subjects.

We observed a tendency towards fewer macrophages in the COPD group. This pattern was in line with, (Saetta et al., 1999) who showed no change in macrophages in alveolar walls and, (Hodge et al., 2004) who found no significant change in the percentage of alveolar macrophages or absolute alveolar macrophage numbers in the BAL of the groups tested. In contrast to this, others demonstrated an increase in macrophages in the bronchial compartment (bronchial biopsies) from COPD, showing the location-specific regulation of inflammatory cell recruitment in COPD (O'Shaughnessy et al., 1997, Saetta et al., 1993).

The non-uniformity and compartmentalisation of inflammatory cells across the airway has been demonstrated by Maestrelli et al. (1995) and inflammatory marker differences in different compartment have also been reported (Ji et al., 2014). It is possible that macrophages in COPD are less adherent and can move quickly to bronchioles. More recently, Isajevs et al. (2011) using airways (large and small) reported significant increase in macrophage numbers in COPD compared to control non-smokers. Most studies that reported an increase in macrophages were BAL studies, where, as previously mentioned, macrophages are the predominant cell type. However, not all BAL studies report an increase. And while BAL studies only report on the number of macrophages found in the alveolar spaces, in our study we also included macrophages adherent to the alveolar surface. In summary, calculations involving macrophage numbers in biofluids may reflect cell recruitment into the lumen, whereas those, such as ours, that also include analysis of cells within the tissue may show no change because of cells resident in the alveolar lumen or tissues. The lack of change in macrophage numbers in alveolar compartments of COPD patients may mean that macrophages do not play an important role at this stage of diseases severity in the COPD patient group we have investigated, which includes mild to moderate patients (i.e. those suitable for surgery). However, many of these patients are on COPD therapies including corticosteroid treatment and other medication such as Lamzoprasole, Symbicort, Brinzolamide, Simvastatin, Omiprazole, Mebeverine and Sprodinosol. Which might be expected to modulate inflammatory cell recruitment. It is possible that macrophages may play an important role at the later stages of COPD/severe emphysema, as some studies supported their role and that of their secreted metalloproteinases in emphysema (Hautamaki et al., 1997). However, their increase in numbers in the larger airway compared to the small airway has been reported (Saetta et al., 1993, Lams et al., 1998, Isajevs et al., 2011); it is believed that cigarette smoke causes oxidative damage with subsequent sequestration and accumulation of macrophages in the large airway (Nishikawa et al., 1999).

In this study, it is possible that the tissue processing method led to bias in the recovery of different cell types. During dissection of the tissue, there were a number of washing steps, which could results in the release of macrophages into the washing solution**.** This may have removed macrophages from the luminal surface. Whilst this bias would normally affect both control and COPD groups equally, we cannot rule out the possibility that macrophages in COPD are less adherent (ie due to translocation through the tissue stroma) resulting in reduced numbers of macrophages resident in the tissue post-processing and digestion. We do know for example that COPD is associated with the elevation of several MMPs such as MMP-12 and -9, which are known to be products of macrophages, that aid tissue migration (Babusyte et al., 2007, Wallace et al., 2008). MMP-1 staining was significantly increased in smokers (Wallace et

al., 2014). Also in the lung parenchyma of patient with emphysema, increase of MMP-9 activity has been reported, (Ohnishi et al., 1998), as well as greater number of alveolar macrophages from COPD patients that have increased elastolytic activity

No increase in neutrophils in the alveolar tissue of COPD patients was found; this is in alignment with, Saetta et al. (1998) who did not find an increase in neutrophils in lung parenchyma. Others have found an increase in neutrophils in other lung compartments, such as large and small airway (Isajevs et al., 2011). Isajevs reported significant higher number of neutrophils using large and small airways. In addition, Battaglia et al. (2007) also reported that neutrophils and mast cells were higher in the small airways of smokers with and without COPD. Also, Hodge et al. (2004), Thompson et al. (1989) reported increased neutrophils using bronchoalveolar lavage in COPD patients. Sampling from different compartment of the lung does lead to different results. Rutgers et al. (2000b) found that differential cell count of neutrophils in sputum did not correlate with those in BAL.

In the current study, one possible explanation for the lack of increase of neutrophil in the lung parenchyma is that neutrophils might migrate rapidly from the tissue into the lumen. Thus, it may be possible that at any given time there are few neutrophils in tissue. In term of correlation result between neutrophils and FEV_1 % predicted in COPD, the p value was 0.03, however, r = 0.5 (not 1). This could be due to the low number and big spread of data. It is also possible that there are more neutrophil in mild COPD in lung parenchyma compared to moderate and that as the disease progress neutrophil accumulate elsewhere like in the epithelium in the small airway.

Earlier studies by Eidelman et al. (1990) and Finkelstein et al. (1995) suggested that in the peripheral lung neutrophil are not the primary participant in the lung parenchyma destruction and even inversely related.

Other inflammatory cell numbers did not correlate with either lung function: FEV_1 % predicted or FEV_1/FVC. Abboud et al. (1998) reported no correlation between FEV_1/FVC and macrophages counts in BAL, however, they found a negative correlation between macrophages and the diffusion of carbon monoxide DCO indicating that even though we did not find correlation in relation to FEV_1/FVC and %FEV_1 there may be a correlation with other lung function parameters.

No difference in the number of epithelial cells between control and COPD patients was observed in this study, consistent with other reports which have also not shown a difference. In asthma the epithelium is known to be lost in the bronchial compartment, whereas COPD is associated with epithelial change but not loss. Changes in the alveolar compartment in both chronic conditions are not well understood. Since a proportion of COPD patients will have

emphysema, it might be predicted that some alveolar epithelium might be lost, however, flow cytometry might not be the most appropriate method to analyse these changes as it cannot make allowance for increase alveolar spaces and adjustments for wet tissue weight may not usefully normalise the data for epithelial counts.

COPD is normally associated with a mixture of goblet cell hyperplasia/hypertrophy and squamous cell metaplasia in the bronchial compartment, and it is possible that there are also changes in the proportions of type I and type II pneumocytes. Bolton et al. (2009) reported an increase in the numbers of cells staining for surfactant protein SP-D in type II pneumocyte in a model of spontaneous hypertensive rats that were exposed to tobacco smoke. Type II pneumocytes are the cells that are responsible for the production and secretion of surfactant: they are thought to play a role in the development of COPD. Also, it was reported that the surfactant protein SP-A levels were significantly lower in patients with COPD and smokers when compared with non-smokers (Vlachaki et al., 2010). Moreover, smokers with COPD had significantly lower SP-D levels in BAL than healthy smokers in a cross-sectional study (Sims et al., 2008). While granzyme A that stimulates the IL-6 and IL-8 was found to be significantly higher in type II pneumocytes of patients with COPD compared to the control group (Sower et al., 1996).

These reported changes are suggestive of dysfunction/depletion of type II pneumocytes in COPD, and are backed up by proteomic analyses of sputum, which suggest deficiencies in type II pneumocyte-derived proteins in COPD (Nicholas et al., 2009). Flow cytometry could be used to measure pneumocyte populations if cell surface markers specific for type I and II pneumocytes could be found.

Based on animal models (Maeno et al., 2007, Borchers et al., 2007), $CD8^+$ T cells may cause considerable damage to lung parenchyma. We looked at T cells first using IHC and found a very low number with large number of samples with zero data point. Using flow cytometry, we measured both $CD8^+$ and non-CD8 positive T cells. However, our result showed using flow cytometry no differences in $CD8^+$ T cells in COPD compared to control (Figure 3.8 (e)). This finding is consistent with others, Hodge et al. (2004) who found no change in T cells in BAL in the groups tested. In contrast, previous studies, such as those by Saetta et al. (1999) have reported an increased infiltration of $CD8^+$ T cell in lung parenchyma of COPD patients. At variance with our study, these investigators only looked at the alveolar walls and did not include alveolar spaces, which may account for these differences.

Another explanation is the small number of patients used in their study (10 COPD and 8 non smokers and 6 normal control). These seemingly incongruent findings may also be due to the

definition of COPD and controls used in different studies, hence the way they recruited their patients. In their study, Majo et al. (2001) have implicated T-lymphocytes in emphysema and it is possible that the lack of separation into bronchitis and emphysema group obscured a difference that we might otherwise have seen. CT evidence of emphysema for our patients could not be obtained, as they were not recruited specifically to a study involving expert analysis of high- resolution computed tomography HR-CT images. Therefore the degree of obstruction in lung function was used to categorise the patients, which could arise from bronchitis or emphysema or a combination of both. In term of lung function measurement we adhered to current guidelines taking both FEV_1 and FEV_1/FVC into account, whereas some other studies, conducted before these guidelines were introduced, selected groups solely based on FEV_1 predicted < 80% and not based on the ratio FEV_1/FVC. Therefore, they were not classified according to the current standard GOLD criteria for COPD (where FEV_1/FVC must be equal or less to 0.7) and so may have included individuals with other overlapping inflammatory pathologies.

More recently, a study by Hou et al. (2013) reported a positive correlation between regulatory T cells (Tregs), and FEV_1 % predicted in BAL indicating a relationship between T cells and lung function. Tregs are cells that suppress the proliferation and activation of CD8 (Lan et al., 2005). In addition, Brozyna et al. (2009) showed that patients with COPD had significant increase in the percentage and absolute numbers of $CD8^+$ T cells compared to control in blood and BAL. Also, in bronchial mucosa an inverse relationship between CD8 T cells and FEV_1 % predicted has been reported in subject with chronic bronchitis (O'Shaughnessy et al., 1997). Using immunohistochemistry, Isajevs et al. (2011) showed significant increase in $CD8^+$ T cells in small and large airways. Similar finding were reported by Lancas et al. (2011) using lung strips that are mainly lung parenchyma but also comprises small proportion of small airways and vessels. It is possible that many of the T cells they found were from blood vessels or even small airways (Kim et al., 2013, Stewart and Criner, 2013). Furthermore, Olloquequi et al. (2010) reported increased number of CD8 in small airway of COPD patient and lung parenchyma.

The lack of increased number of $CD8^+$ T cells in the COPD group that was observed in this study does not exclude the possibility of functional difference. Indeed, these cells could have a cytotoxic effect in the lung parenchyma of COPD patients without any increase in numbers as recently reported by Freeman et al. (2010), where lung $CD8^+$ T cells from COPD subject released more granzyme and perforin which would increase their cytotoxicity. Specific memory T cell recall (Th1 and Th17) to elastin fragment in smokers with emphysema has been also described (Lee et al., 2007). Isolated from the blood of smokers these cells showed increase release of IL-

17A and IFN-γ that promote pro-elastolytic lung environment as they act to simulate MMP12 secretion by macrophages (Shan et al., 2012, Kheradmand et al., 2012). Similar finding were reported by Zhu et al. (2009) who found a negative correlation between activated CD8, measured by the amount of IFN-γ released and the expression of MHC-II, and FEV_1% predicted in peripheral blood.

It has also been reported that T lymphocytes prefer to accumulate in the lamina propria of the bronchi rather than the alveolar region. It has been reported that human lymphocytes adhere less to the alveolar epithelium compared to the bronchial epithelium (Ainslie et al., 2002). The same team characterized the adhesion receptors that are involved in T cells adhesion to bronchial epithelium and that include P-selectin, PSGL-1, L-selectin, LFA-1, ICAM-1 and 2. This was later supported by Battaglia et al. (2007), who reported in a comparison study of inflammatory cells between large and small airways in smokers, that the lamina propria of the large airways is infiltrated by lymphocytes as these may prefer to reach lung region catered for by bronchial blood vessels, while that of the small airways is infiltrated with neutrophils and mast cells, as these cells tend to move into the capillaries of the pulmonary system. They also characterised the adhesion pathways involved in T cell adhesion to bronchial epithelium. We did not look for T cell adhesion molecules in this study as the model is not yet optimised to look for adhesion molecules.

The small number of eosinophils in lung parenchyma that we observed was expected. Eosinophils are not generally increased in COPD except in some cases of exacerbations. However, some studies reported that both the peripheral eosinophil counts and sputum eosinophil counts were found to be significantly higher in the COPD with asthma group (Kitaguchi et al., 2012).

On the other hand, there is a controversy about mast cell findings. While we did not see a difference between the groups, which is in alignment with some previous findings (Lancas et al., 2011). Others reported an increase in their numbers in COPD (Grashoff et al., 1997, Wen et al., 2010, Ballarin et al., 2012, Soltani et al., 2012).

A role of these cells in the pathogenesis of COPD cannot be ruled out since some like, Grashoff et al. (1997) reported an increase in their number in the epithelium of the airway wall of the peripheral airways. Ballarin et al. (2012) reported increased number in the smooth muscle layer of the small airways and lung parenchyma in COPD with centrilobular emphysema. Amin et al. (2003) showed increased number in the central airway. Moreover, Andersson et al. (2010) reported phenotype alteration and increased expression of C5a receptor CD88 and found a

positive correlation between reduced densities of their total number and reduced lung function in all anatomical compartments of the lung. Other data showed that the sputum levels of mast cell tryptase in the severe and moderate patients were greater than that in mild patients with COPD (Zhang et al., 2004).

One might expect to see elevated numbers of mast cells in COPD but this phenomenon was not observed in the current study. Reports have suggested that mast cells reside mainly close to blood vessel, smooth muscle cells, and mucus-producing glands (Brinkman, 1968) so it is possible that, because of the extensive washing steps involved in processing the lung tissue samples for *ex vivo* flow cytometry, that mast cell numbers were low in the lung parenchyma. It is also possible that mast cells may be activated instead of increased; indeed some reports have suggested that these cells have receptors that can be activated in disease, such as toll-like receptors (Varadaradjalou et al., 2003, Marshall et al., 2003).

3.5 Conclusion

Little difference was observed in inflammatory cell load between lung tissue from control and COPD patients using either immunohistochemistry or flow cytometry. We suspect that immunohistochemistry did not allow for accurate observation of the cells in lung parenchyma due to limitations in the number of obtainable data points.

In previous studies, others Saetta et al. (1998) have shown an increased number of $CD8^+$ T cells in samples which were fixed in 4% formaldehyde in phosphate-buffered saline at pH 7.2 and, after dehydration, embedded in paraffin wax. In our case, the tissue was embedded in GMA, and the difference between the ways the tissue was processed could have resulted in a difference in our observations, although normally GMA preserves cell epitopes better than paraformaldehyde and paraffin embedding methods. GMA embedding is normally done on very small tissue samples, whereas paraffin embedded tissue is normally done on greater amounts of tissue, allowing for larger fields for cell quantification which might have been beneficial for this study and would be a useful avenue for further work.

The novel flow cytometric assay described in this study, used for identification of various cell types from lung parenchyma, is certainly more rapid than other methods, and due to a higher cell throughput, could be more quantitative, and additionally has the potential to allow further investigation of cell characteristics, such as cytokine production and receptor expression, especially if the cells can be sorted for PCR analysis. It is possible that manual counting as opposed to flow cytometry has underestimated the number of lymphocytes compared with

flow cytometry. The development of such a model, where the identification of multiple cells types in multiplex from collagenase-dispersed resected lung tissue, is a novel development, which will open up new avenues for lung tissue analysis. Similar studies in bronchial tissues from lung biopsies have been performed in asthmatic subjects and have revealed differences in T cell receptor profiles in asthma (Vijayanand et al., 2010), but have not previously analysed the structural cell component of lung tissues. Flow cytometry also has some disadvantages: tissue must be disturbed to analyse the cells and that can affect the cellularity of the tissue. Also certain antigen may be weakly present. Furthermore, the distribution of the cells within the tissue remains uncertain when compared with IHC.

Despite highly conflicting evidence, most publications on COPD suggest inflammation consisting of an increase in macrophages, neutrophils and $CD8^+$ T cells in the lung. This information was mainly derived from (BAL), bronchial biopsy and sputum studies and not ex *vivo* explants which all measure different lung compartments, so it is difficult to directly compare these findings. Also, previous studies have shown no correlation between inflammatory cell numbers in BAL and biopsy (Maestrelli et al., 1995, Rutgers et al., 2000a). It is possible that lung parenchyma does not correlate with either; biopsy because it is in the bronchial compartment, BAL because the sampling method is notoriously difficult to normalise for sample recovery of the saline following application to the lobe.

There is almost certainly an anatomic compartmentalization of the inflammatory cells in COPD. Macrophages are implicated in emphysema and it is believed that in response to cigarette smoke constitutive macrophages produce macrophage metalloelastase this in return generate a chemotactic gradient that attract monocytes into the lung parenchyma (Hunninghake et al., 1981). Furthermore, macrophages from emphysematous lung express and have higher level of MMPs that have anti-elastin activity and therefore suggesting implication in the disease process (Finlay et al., 1997, Imai et al., 2001). It is also possible that these cells may contribute to progression of COPD disease either directly via cytokine production or indirectly via cytolysis as in the case of $CD8^+$.

Directly analysing the inflammatory cell component of lung tissues may be a more effective approach than examining the blood/tissue mediator profiles in COPD because whilst there is widespread redundancy in inflammatory mediator production (as evidenced by the lack of efficacy of monoclonal therapies in chronic lung diseases), this is less likely to be the case in the examination of effector cells in the lung compartment. In the next chapter we examine the tissue responses to stimulation in control and COPD patients in order to determine whether,

although inflammatory cell numbers are unchanged, whether cell-mediated responses are defective in COPD, contributing to disease pathogenesis.

Chapter 4: Comparison of lung parenchymal tissue responses to LPS and bacterial stimulation in control and COPD subjects

4.1 Introduction

COPD is an inflammatory condition where colonization by pathogenic organisms is frequently found, both in stable disease (Cabello et al., 1997, Patel et al., 2002) and also during periods of exacerbation (Aguirre et al., 2015, Monso et al., 1995, Bhowmik et al., 2000). The increased susceptibility of COPD patients to microbial infections is a concern because exacerbations are the principal cause of hospitalisation and morbidity/mortality in COPD, and they contribute to an accelerated lung function decline (Kanner et al., 2001, Donaldson et al., 2002).

The response of individual cell types to microbial stimulations such as endotoxin (LPS) have been modelled extensively *in vitro* (Denning et al., 1998, Deslee et al., 2007, Nichols et al., 2013) as mentioned in chapter 1. However, since the innate and adaptive response to microbial stimulation is multifactorial involving both classical and so-called "semi-professional" cell types in tissue responses *in vivo*, these models may not fully represent the lung tissue responses as it is difficult to mimic *in vivo* situations using many of these models due to a number of concerns that include: Transformation of the cells for immortalised culture, the degree of carry-over of the starting patient phenotype in primary cell lines, and the effects of proteolytic dissociation of primary cells during the culture process.

This chapter examines the possibility that preserving the tissue architecture by using explanted lung tissue with *ex vivo* exposure to bacterial components could prove useful as a model of bacterial exacerbation in COPD.

This chapter looks at two parts: The first part focuses on developing an *ex vivo* model of human lung explant stimulation with bacterial-type stimulants such as LPS (from *E. coli*) and heat killed bacteria (*H. influenzae*) relevant to COPD exacerbations. LPS is widely used to mimic inflammatory responses arising as a result of microbial-induced COPD exacerbation, both *in vitro* and *in vivo* (Chen et al., 2013). LPS-induced lung inflammation in cell lines is well described (de Godoy et al., 1996, Aldonyte et al., 2003, Dean et al., 2008, Smit et al., 2009). However, whether the inflammatory response closely reflects what happens in human lung tissue is not known, and how the responses compare between LPS from an irrelevant bacterial source (*E. coli*), and a relevant but heat killed bacterial species (*H. influenzae*) has not been fully explored.

As mentioned in Chapter 1, the immune system uses a variety of pathogen recognition receptors (PRRs) upon pathogen encounter therefore these receptors may be responsible for any differential inflammatory responses to bacterial pathogens in chronic lung diseases with an exacerbation component. TLRs are of importance as they recognise a variety of endotoxins especially TLR 2 and 4. While the cellular location of these receptors is well documented as mentioned in chapter 1 their expression in the lung parenchyma has not been well explored. In addition, the effect of disease status on their expression is also relatively unknown, but could explain the abnormal inflammatory responses seen in COPD where the total inflammatory cell load is not dramatically different from that of smokers without COPD.

Therefore, the second part of this chapter investigates the importance of TLRs in inflammation, to see if there are differences between control and COPD groups. Having looked at the inflammatory cells in the lung parenchyma explants and found no differences between the groups using both IHC and flow cytometry except for the increased T cells in control that it is highly likely the result of high number of 0 cells. It was necessary to investigate any other changes in these cell populations. As differences in activation status or response to stimuli due to dissimilarity in expression of bacterial receptors may well be present. While others have shown that TLR expression is up regulated in cell lines stimulated with microbial ligands (Groskreutz et al., 2006, Xu et al., 2008), it is possible that TLR may be chronically elevated or reduced in lung parenchyma in patients with COPD. Our explant model allows us to examine the TLR expression status of lung parenchymal cells. This includes structural (epithelial) and leukocyte cells (T-lymphocytes and macrophages), both at baseline and following stimulation *ex vivo* with bacterial ligands.

4.1.1 Hypothesis and objectives

We hypothesised that explants from COPD patients would have increased baseline expression of TLRs because they have a greater likelihood of exposure to microbial pathogens, and from *in vitro* evidence in the literature, stimulation leads to a greater expression.

The first objective of this chapter was to further refine and develop an *ex vivo* model of human lung explant stimulation with bacterial products to mimic the processes that occur during infective bacterial exacerbations of COPD in order to investigate changes in our cell populations in terms of the activation status, by looking at the cytokine release after stimulation with LPS or *H. influenza* (we used killed bacteria so as not to have uncontrolled growth).

The second objective was to look at TLR expression on inflammatory and structural cells in parenchymal lung tissue of control and COPD subjects at baseline, and to investigate if this expression was limited to cell types relevant to such a response, namely the epithelial cells and macrophages, which form the front-line of defence against bacterial pathogens in the alveolar spaces.

The third objective was to measure TLRs response to bacterial stimulation using flow cytometry and to investigate if stimulation may lead to an increased expression, with the aim of dissemination of mechanisms of exacerbation and potential therapeutic intervention pathways.

4.2 Method: Optimisation of a lung tissue explant model for bacterial stimulation

4.2.1 Patients characteristics

Human lung tissue was obtained, with written informed consent, from 17 patients who were undergoing carcinoma resection or bullous repair at Southampton General Hospital. The patients' age, gender, lung function (when available) and smoking history were recorded. This information can be found in (Table 4.1). Explants were prepared as in section 2.2 and stimulated as in Section 2.3.1 for dose response curve and Section 2.3.2 for time course. Figure 4.1 is a diagrammatic representation of the human lung tissue explant model that shows the way the tissue and supernatant were prepared. Heat killed bacteria was chosen to prevent uncontrolled growth.

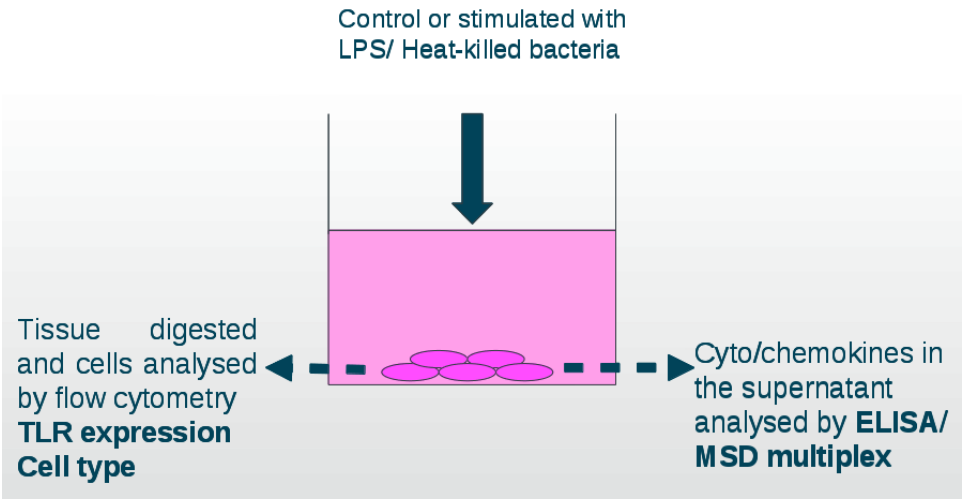

Figure 4.1 Diagramatic representation of the human lung explant tissue model described in the optimisation of a lung tissue explant model for bacterial stimulation section.

Human lung parenchyma tissues obtained from patients undergoing resection for lung cancer was dissected into approximately 2 mm^2 pieces, and then rested overnight in culture medium. Tissue was then treated with bacterial lipopolysaccharide (LPS) 100 ng/ml, or heat-killed *H. influenza* bacteria $0.02x10^8$ (cfu/ml), or carrier control for 24 h. Post-stimulation, the cell-free supernatant was collected for cytokine analysis using multiplexed ELISA, and stimulated tissues were enzymatically disaggregated and analyzed by flow cytometry.

Table 4.1 Patient characteristics for dose response and time course studies: clinical data was collected for each sample and shown as median and quartile range. Patients with a FEV_1/FVC ratio of < 0.7 were classified as COPD. Those with FEV_1/FVC ratio of ≥ 0.7 were defined as Control patients. * indicates that some patients did not have lung function taken as bullectomy patients did not undergo lung function test.

Gender	69 (62-74.25)
Age	9F/8M
FEV_1% predicted	75.67 (68.63-98.79)*
FEV_1/FVC	0.69 (0.47-0.74)*
Smoking Status	12 EX/3 Current/ 2 Never
Pack years	47(28.4-55)

4.3 Result

4.3.1 LPS and heat killed *H. influenzae* dose response

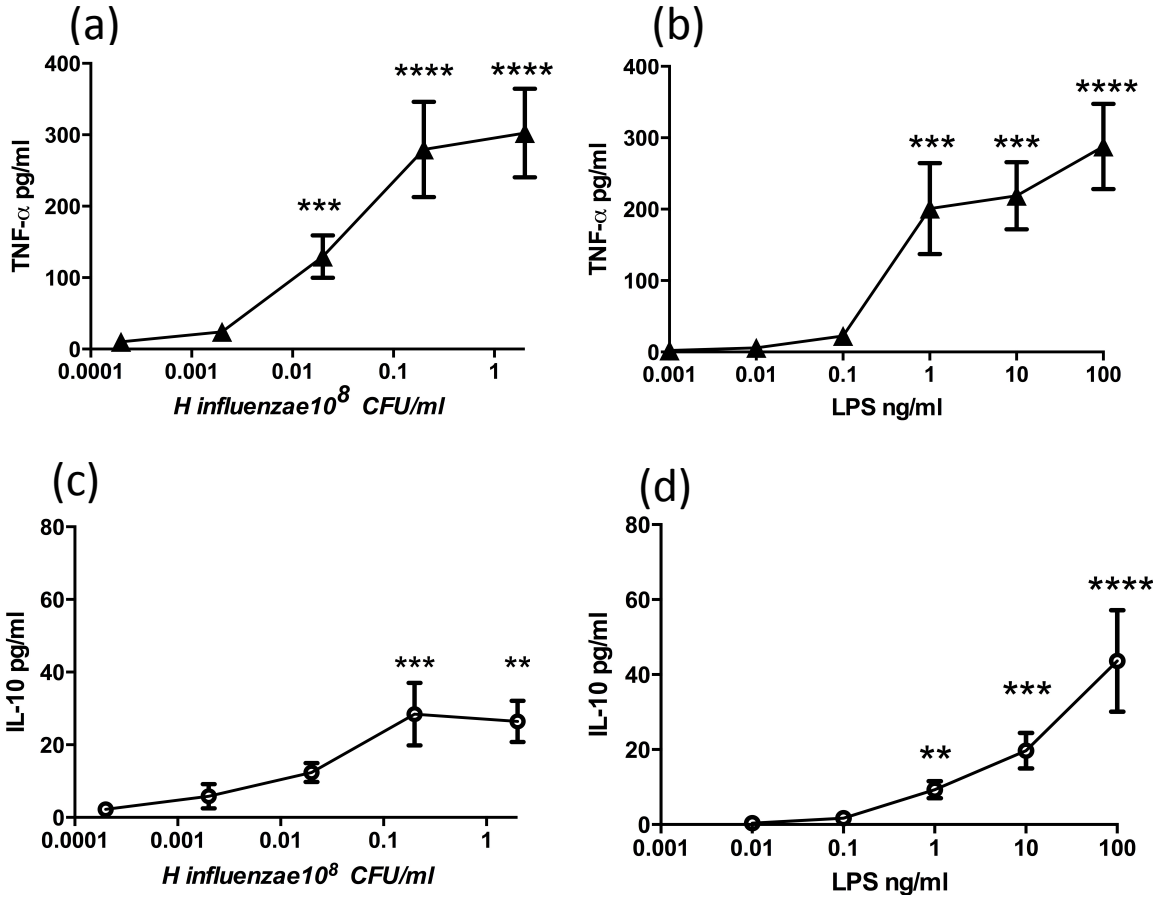

Figure 4.2 Dose-response curves of lung explants following bacterial ligand stimulation for 24h. Conditioned culture media samples from lung tissue explants cultured as described in the Method section and Figure (4.1) were analysed by ELISA for TNF- α and IL-10. The dose range was 2×10^8, 2×10^7, 2×10^6 or 2×10^4, colony-forming units/ml heat-killed *H. influenzae* (a and c) or 100, 10, 1, 0.1 and 0.01 ng/ml LPS (b and d). TNF-α (a and b) (filled triangles) and IL-10 (c and d) (hollow circles), release were measured by ELISA. Results are expressed as median +/- inter-quartile range (n = 10). Values between treatments were compared using One Way ANOVA, comparing each treatment to the control (ie unstimulated) value. **= $p \leq 0.01$, ***= $p \leq 0.001$, ****= $p \leq 0.0001$.

Dose-response experiments were performed to determine the ability of heat killed *H. influenzae* to cause release of cytokines from the lung tissue Heat-killed *H. influenzae* stimulated the

release of TNF-α, and IL-10 in a dose-dependent manner. Figure (4.2) shows the release of TNF-α (subfigure a) and IL-10 (subfigure c).

Stimulation of tissue with *H. influenzae* induced significant levels of TNF-α release from 0.02 × 10^8 cfu/ml, which reached a plateau at 2×10^8 cfu/ml and the starting point for increase was 0.002×10^8 cfu/ml. IL-10 was seen to be less sensitive, however, its starting point for increase was 0.0002×10^8 cfu/ml and significant amount was released at 0.2×10^8 cfu/ml.

Stimulation of lung explants with LPS induced a linear dose-dependent increase in TNF-α. The starting point for increase was 0.1 ng/ml and significant amounts of TNF-α and IL-10 were released from a concentration of 1 ng/ml. The amount of TNF-α and IL-10 kept rising and stayed significant at 100 ng/ml (Figure 4.2(b) and (d)). The apparent lower sensitivity in fold increase of IL-10 could be related to the IL-10 ELISA kit properties.

There was a good response for both cytokines for both stimulus even though the response did not fully peak at maximum LPS dose. Previous work on lung parenchyma (Hackett et al., 2008) showed that release of TNF-α from LPS-stimulated tissue was maximal at 1000 ng/ml therefore, we ran a dose response within the range of 0.001-100 ng/ml, so LPS top concentration of 100 ng/ml was used, as there was still continued release of TNF-α. Concentrations of *H. influenzae* that generated sub-maximal release of cytokines (0.02×10^8 cfu/ml) was selected for subsequent experiments other than for the time course where $2x10^8$ has been used as it was run at the same time as time course. We hoped to use these optimised conditions to give reproducible stimulation experiments with which we could abrogate responses using prospective pharmaceutical agents.

4.3.2 LPS and H. influenzae time course

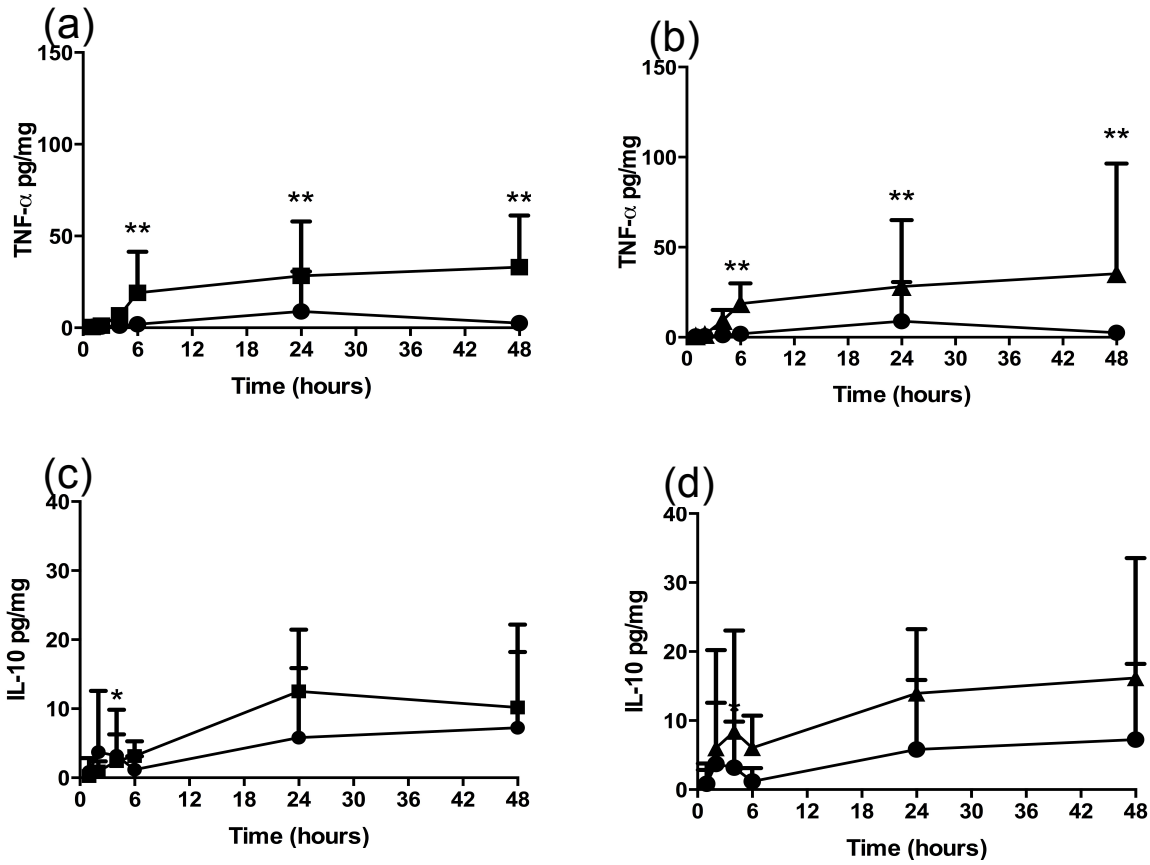

Figure 4.3 Time-course response curves of lung tissue explant responses to bacterial ligand stimulation. Responses were measured by quantifying cytokines, TNF-α (a) and IL-10 (b), in the tissue-conditioned culture medium following 24 h of stimulation. Explants were treated with 2×10^8 colony-forming units/ml heat-killed *H. influenzae* (filled triangles) or 100 ng/ml LPS (filled squares) for 2, 4, 6, 24 or 48 h. Control (unstimulated) wells (filled circles) were treated with carrier alone. Results are expressed as median +/- inter-quartile range (n = 15). Values between treatments were compared using one way ANOVA, comparing each treatment to the control (ie unstimulated) value. *= $p \leq 0.05$ and **= $p \leq 0.01$.

It has been previously reported that TNF-α stimulation in *ex vivo* tissue reaches a peak at 24 h post-stimulation with LPS (Hackett et al., 2008). We wished to confirm this within our own experiments. Tissues from 15 donors (details of the cohort shown in Table 4.1) were stimulated

with a fixed concentration of LPS (100 ng/ml) or killed bacteria (2×10^8 cfu/ml) for a range of time-points (1, 2, 4, 6, 24 and 48 h). (2×10^8 cfu/ml) was chosen as initial work in our lab suggested this to be the optimum concentration and so it was used in this early work. However, the dose response result suggested that the optimum concentration was (2×10^6 cfu/ml) and so that concentration was used in later experiments. Tissue-conditioned medium was analysed for cytokine content by ELISA, normalising for tissue weight (Figure 4.3 (a), (b), (c) and (d)).

For both cytokine there was very low level of constitutive release from human lung parenchyma. This was significantly increased by stimulation with LPS or *H. influenzae*. LPS induces around 50-fold increase in TNF-α (Figure 4.3 (a)). There was very little increase of TNF-α at 4 h and significant increase at 6 h. It was found that, in accordance with other published data (Hackett et al., 2008), peak TNF-α values were observed between the 12 and 24 h time-points. The peak at 24 h stayed stable, indicating no loss of data quantity at 48 h. The maximum may have been reached by 12 h but there was no indication that collecting it at 24 h would be less useful. More TNF-α was released from explants that were stimulated with *H. influenzae*.

In assessing the kinetics of TNF-α and IL-10 release after stimulation of lung explants with LPS or *H. influenzae*, we found that the stimuli displayed slightly different kinetics, and the release IL-10 followed biphasic pattern (Figure 4.3) and as far as we know this is the first time that it has been shown in lung parenchyma (Figure 4.3 (b)), with an early peak at 2 h with a low point at 6 h then followed by a second increase that peaks and plateau at 24 h.

4.3.3 Examination of lung tissue cytokine responses of the lung tissue model – power calculation and replicate requirements for the extended cytokine array

As we were interested in extending the mediator analysis profile in human lung tissue responses to bacterial and LPS stimulation, and intended to use the model to examine the inhibition of such responses (see chapter 5), we needed first to calculate the coefficient of variation of such stimulations in an optimisation group by running 4 replicates of each treatment (unstimulated, LPS-stimulated, bacterial-stimulated) in tissue samples from 6 donors, using the secretion of 8 different mediators analysed in multiplex as a read-out.

By looking at the CV values of the different cytokines analysed, we can see a high variance between individual wells (intra subject) in Table 4. 2: (a) tissue from control and (b) tissue from COPD. Looking at the inter subject CV values (Table 4.3) similar wide variation is seen across all

the cytokines from both the control (b) and COPD (c). Table 4.3 (a) represents the values of the two groups combined (Table 4.3).

Due to the variability in responses and the limitation of CV for reproducibility as the samples are from different patients who may or may not have the same medications also have different health issue probably meaning that the tissues have been subjected to different conditions, also measurement are almost always prone to various sorts of errors, that may lead to a different value than the true one. Another issue here with reproducibility is that, even with the same subject, the same piece of tissue cannot be stimulated twice.

A pragmatic approach to replicates for the full cohort analysis was taken, in which stimulations were performed in triplicate for each patient, balancing cost/time with the need to minimise variability in the results. In all conditions, 3-5 tissue pieces were incubated per well, to further minimise variability. Previous results using bronchial explants in singlet wells have demonstrated good stimulus responses measured using inflammatory mediator outputs, indicating that our approach was highly conservative.

Table 4.2 Coefficient of variation is defined as ratio of the standard deviation to the mean and has no units: Six different samples were used and four replicate for each sample. Human lung fragments were treated with LPS 100 ng/ml, or *H*. *influenzae* 2 $\times 10^6$ or unstimulated control. Shown are the within the subject CV values of quadruplicates assays of LPS or heat-killed bacteria or control from six different patients. TNF-α, IL10, IL-6, IL-8, IL-1β, IL17, MCP-1 and MIP-1β release were measured by meso scale discovery MSD plates (a) Control values and (b) COPD value

(a)

	TNF- α	IL-10	IL-6	IL-8	IL-10	IL-17	MCP-1	MIP-1 β
Control	34.01	33.38	9.74	43.14	28.22	68.46	39.87	55.27
LPS	23.04	21.38		23.08	32.68	80.46	49.52	22.47
Bacteria	24.67	12.73		43.34	58.45	21.03	30.15	22.00
Control	54.49	30.40	95.29	23.17	60.36	40.38	18.33	18.64
LPS	12.20	19.21		71.39	34.93	67.10	18.39	16.13
Bacteria	34.99	31.37		42.32	50.51	21.85	32.39	33.12

(b)

	TNF- α	IL-10	IL-6	IL-8	IL-10	IL-17	MCP-1	MIP-1 β
Control	22.15	41.24	62.35	24.30	53.81	22.98	6.22	6.71

LPS	66.66	54.01	47.20	117.94	56.45	63.37	66.99	47.87
Bacteria	46.60	34.59	34.59	107.62	33.89	31.06	49.10	47.07
Control	34.38	23.74	72.03	120.40	32.64	22.94	40.80	26.76
LPS	28.23	22.63	129.07	155.85	31.14	31.01	19.66	55.65
Bacteria	42.63	53.92	70.51	59.28	22.47	41.60	65.73	63.10
Control	28.30	58.15	36.78	30.15	29.55	13.86	35.53	20.76
LPS	33.75	43.42		54.79	26.44	35.15	34.56	52.82
Bacteria	58.06	31.48	15.30	115.69	35.43	71.99	25.54	27.46
Control	47.67	35.21	65.38	62.64	37.03	56.83	48.18	40.69
LPS	68.31	70.28	52.38	87.31	94.94	58.54	71.89	45.65
Bacteria	56.18	45.55	23.44	57.17	48.52	27.48	41.31	54.13

Table 4.3 Coefficient of variation is defined as ratio of the standard deviation to the mean and has no units: Six different samples were used and four replicate for each sample. Human lung fragments were treated with LPS 100 ng/ml, *H. influenzae* 2× 10^6 or unstimulated control. Shown are the inter subjects CV values of six different patients. Assays of LPS or heat-killed bacteria or control were done in quadruplicates. TNF-α, IL10, IL-6, IL-8, IL-1β, IL17, MCP-1 and MIP-1β release were measured by meso scale discovery MSD plates. (a) Combined, (b) Control values and (c) COPD value.

(a) All

	TNF- α	IL-10	IL-6	IL-8	IL-1β	IL-17	MCP-1	MIP-1 β
Control	62.75	64.04	82.39	178.35	50.31	42.32	69.33	72.46
LPS	43.97	53.50	187.88	182.63	21.31	51.88	48.20	85.57
Bacteria	48.26	59.31	68.90	101.72	78.78	68.62	56.40	95.03

(b) Control

	TNF- α	IL-10	IL-6	IL-8	IL-1β	IL-17	MCP-1	MIP-1 β
Control	5.29	33.00	18.29	29.5	11.25	33.66	23.67	53.31
LPS	23.77	33.30		23.62	25. 90	39.28	26.50	80.07
Bacteria	2.81	19.92		20.03	53.40	1.52	5.33	75.87

(c) COPD

	TNF- α	IL-10	IL-6	IL-8	IL-1β	IL-17	MCP-1	MIP-1 β
Control	47.46	63.87	92.49	140.03	34.68	43.88	70.81	75.50
LPS	32.60	60.07	165.84	138.60	18.06	33.86	34.93	70.82
Bacteria	49.72	74.96	68.90	61.97	8.06	35.28	35.78	84.08

4.3.4 Time-course analysis of mediator responses to bacterial and LPS stimulation of human lung tissue samples

As the mediator analysis was extended to a larger cohort, the method of cytokine/chemokine analysis was altered to include multiplexed analysis of 8 individual mediators. Because the response of individual mediators was likely to be variable with time dependent on the individual cytokine/chemokine, a time-course analysis of these 8 mediators was initially performed using the multiplexed cytokine analysis as a read-out. Details of the cohort are shown in Table 4.4 (5 control and 6 COPD).

Table 4.4 Patient characteristics for time course studies of TNF-α, IL-10, IL- 6, IL-8, IL-1β, IL17, MCP-1 and MIP-1β: clinical data was collected for each sample and shown as median and quartile range. Patients with a FEV_1/FVC ratio of < 0.7 were classified as COPD. Those with FEV_1/FVC ratio of ≥ 0.7 were defined as Control patients Ex denote ex-smoker, current denote current smoking and never denote never smoking. Non parametric Mann Whitney U test showed no statistical difference between the group in age and pack years.

	Control	COPD
Gender	3F/2M	3M/3F
Age	70 (62-70)	68 (63.5-72.5)
FEV_1%predicted	96.47 (73-119.04)	70.38 (42.80-72.76)
FEV_1/FVC	0.77 (0.74-0.80)	0.45 (0.42-0.49)

Smoking status	3 EX/1 Current/1Never	5 Ex/1Current
Pack year	45 (32.9-52.5)	49 (39-57.5)

4.3.4.1 Time course analysis of all cytokines

Tissue from 11 donors was used in the human lung explant model for the following experiments Table 4.4 above. We replicated the result for TNF-α and IL-10 as before but we gained valuable information about the time-course of responses in the other mediators.

TNF-α (Figure 4.4) (LPS) and (Figure 4.5) (*H. influenzae*) was hardly detectable at the basal level. It showed a different pattern compared to other cytokines. Release of the pro-inflammatory cytokine TNF-α was significantly higher in the LPS and bacteria stimulated tissue as early as 4 h, continued to rise and peaked at 6 h, measuring 20 pg/mg, compared to undetectable level in the non-stimulated control. In the earlier cohort (Figure 4.3) TNF-α while was rising at 4 h it was not significant until 6 h. It is possible that this difference is due to the kit used to measure the cytokine as it was measured using ELISA kit and for this experiment we used MSD plate. Both LPS and *H. influenzae* caused TNF-α to reach significant early release at 4 h compared to other cytokines (except for MIP-1β and IL-10 when stimulated with *H. influenzae*). This is in line with the work of Hackett et al. (2008) and this also confirms that TNF-α is a key initiator in the inflammatory cascade.

The biphasic pattern of IL-10 (Figure 4.3 (b)) was seen again with a peak at around 2 h that was significant for the heat killed bacteria treated samples. This rapid peak was also seen earlier in the optimisation part of the time course (Figure 4.3 (b)) at 4 h in response to stimulation with *H. influenzae*. The peak was followed by a fall that reached a low level at 6 h. Next, a rise that reached a peak at 24 h. But although this data show a note worthy trend it does not reach significance.

IL-8 on the other hand (Figure 4.4 (d)) and (Figure 4.5 (d)) was detected in the supernatants very early after 1 to 2 h reaching a peak at 6 h. The release decreased thereafter dropping dramatically at 24 h below the basal level for both stimuli.

IL-1β (Figure 4.4 (e)) and (Figure 4.5 (e)) was hardly detectable at the basal level. It showed different pattern for each stimulus. After incubation with LPS, the amount of IL-1β increased significantly at 6 h, however unlike the effect of LPS on TNF-α, the release of IL-1 β drops back

to basal level by 24 h. When the tissue was stimulated with killed bacteria, there was a steady increase of the release IL-1β that was significant at 48 h (25 pg/ml).

The release of MIP-1β, a pro-inflammatory chemokine mainly involved in the recruitment of mononuclear cells, (Figure 4.4 (f)) and (Figure 4.5(f)) was significantly higher as early as 2 h for heat-killed bacteria and 4 h for LPS, it continued to rise and peaked at 6 h (300 pg/mg) compared to undetectable level in the non-stimulated control.

When looking at the data generated for IL-6, MCP-1, (Figure 4.4 (c and g)) and (Figure 4.5 (c and g) stimulation resulted in noticeable release and steady increase for both cytokines albeit not significant.

Lastly, IL-17 was detectable at basal level at 6 h and remained low until 48 h. Stimulation did not cause any significant release (Figure 4.4 (h)) and (Figure 4.5 (h)).

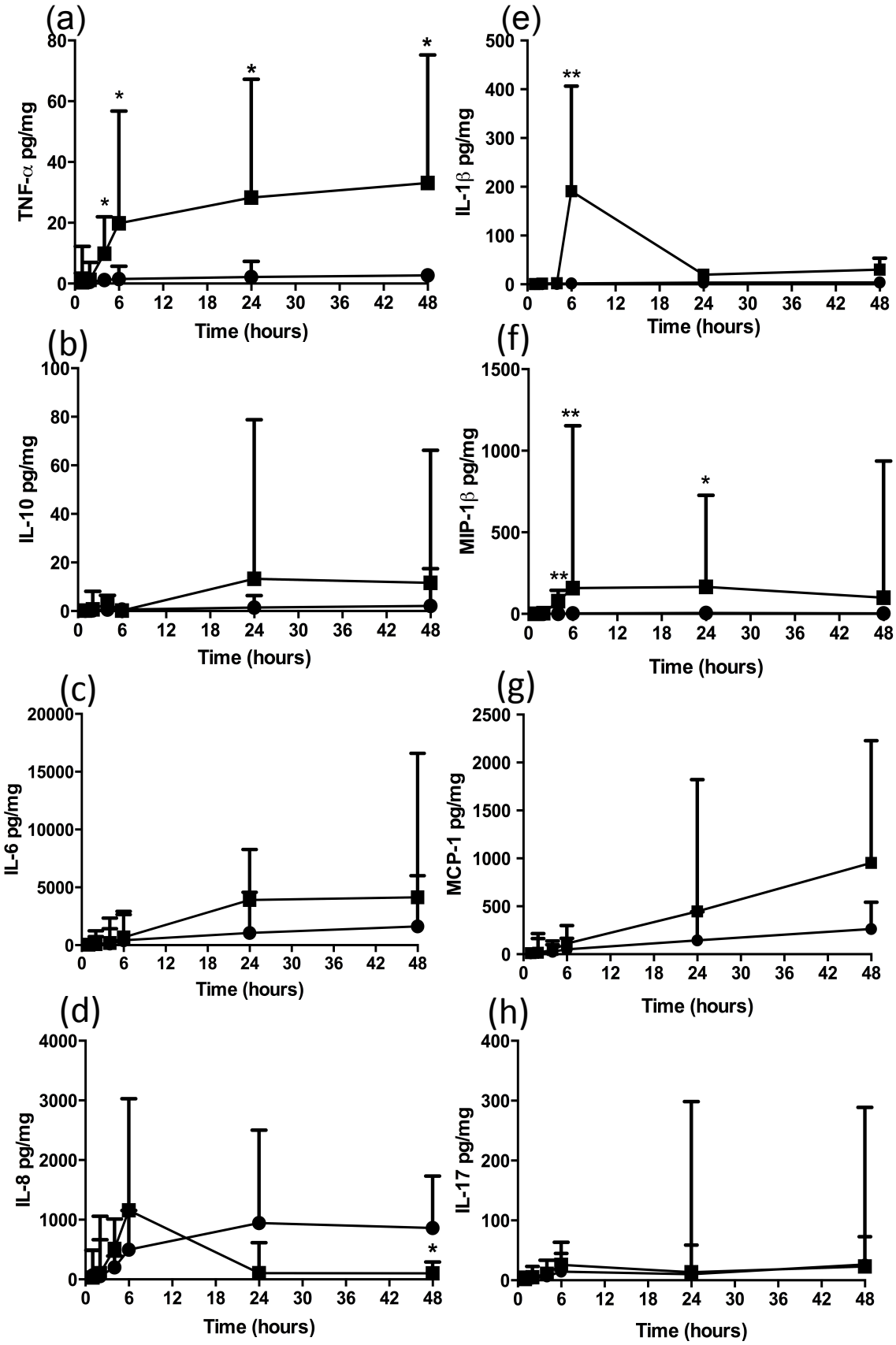
(a)
TNF-α pg/mg
Time (hours)
(b)
IL-10 pg/mg
Time (hours)
(c)
IL-6 pg/mg
Time (hours)
(d)
IL-8 pg/mg
Time (hours)
(e)
IL-1β pg/mg
Time (hours)
(f)
MIP-1β pg/mg
Time (hours)
(g)
MCP-1 pg/mg
Time (hours)
(h)
IL-17 pg/mg
Time (hours)

Figure 4.4 Time-course response curves of lung tissue explant responses to bacterial LPS stimulation (filled square) or carrier controls (filled circles). Responses were measured by quantifying cytokines by multiplexed ELISA. TNF-α (a), IL-10 (b), IL-6 (c), IL-8 (d), IL-1 β (e), MIP-1β (f), MCP-1(g), and IL-17 (h), were measured in the tissue-conditioned culture medium following 24 h of stimulation and adjusted for tissue weight. Explants were treated with 100 ng/ml LPS (filled squares) for 2, 4, 6, 24 or 48 h. Control (unstimulated) wells (filled circles) were treated with carrier alone. Results are expressed as median +/- inter-quartile range (n = 11). Values between treatments were compared using one way ANOVA, comparing each treatment to the control (ie unstimulated) value. *= $p \leq 0.05$ and **= $p \leq 0.01$.

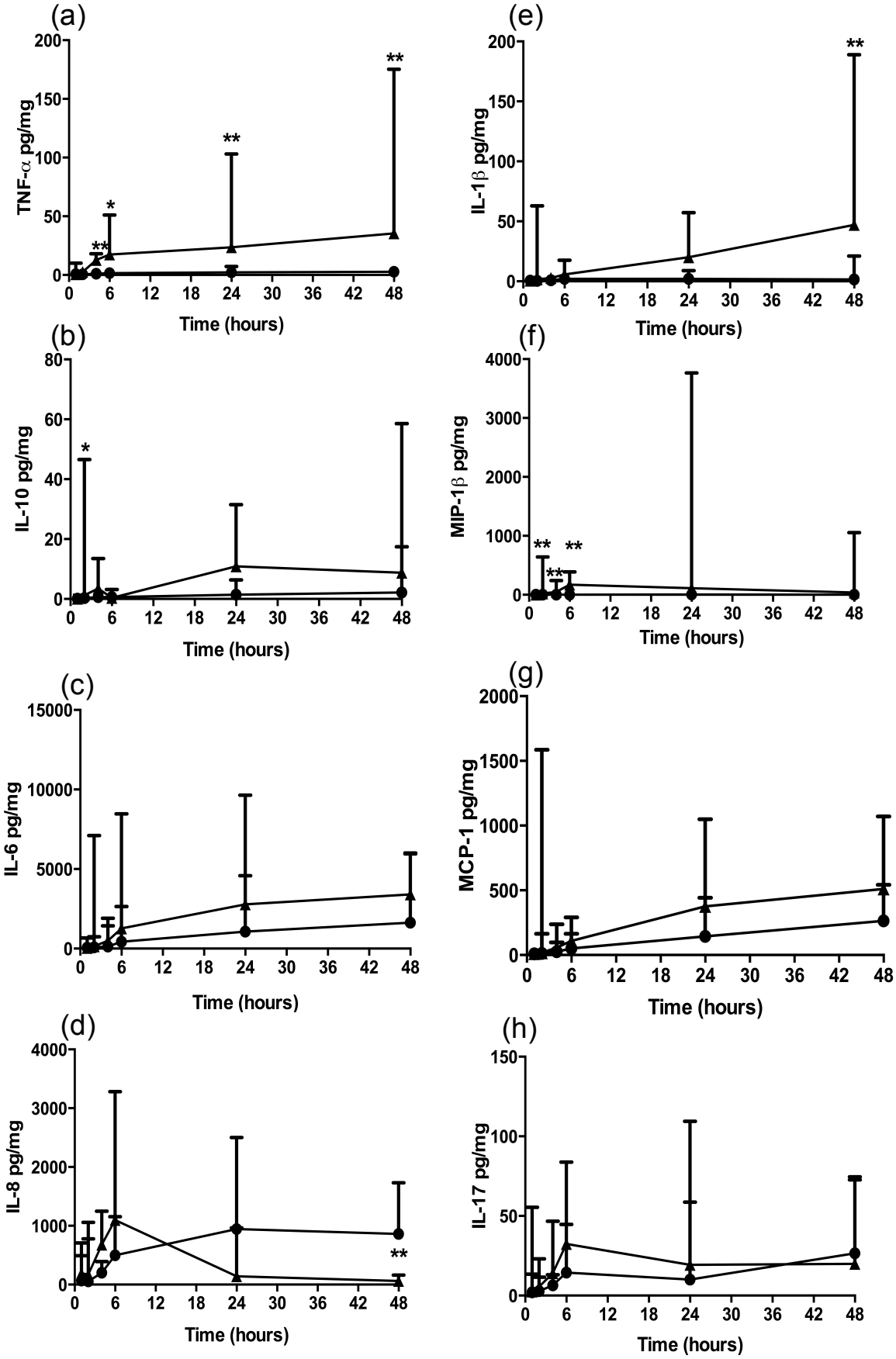
(a)
TNF-α pg/mg
Time (hours)
(b)
IL-10 pg/mg
Time (hours)
(c)
IL-6 pg/mg
Time (hours)
(d)
IL-8 pg/mg
Time (hours)
(e)
IL-1β pg/mg
Time (hours)
(f)
MIP-1β pg/mg
Time (hours)
(g)
MCP-1 pg/mg
Time (hours)
(h)
IL-17 pg/mg
Time (hours)

Figure 4.5 Time-course response curves of lung tissue explant responses to heat-killed bacteria stimulation (filled triangles) or carrier controls (filled circles). Responses were measured by quantifying cytokines by multiplexed ELISA. TNF-α (a), IL-10 (b), IL-6 (c), IL-8 (d), IL-1 β (e), MIP-1β (f), MCP-1(g), and IL-17 (h), were measured in the tissue-conditioned culture medium following 24 h of stimulation and adjusted for tissue weight. Explants were treated with 2×10^6 colony-forming units/ml heat-killed *H. influenzae* or carrier control for 2, 4, 6, 24 or 48 h. Results are expressed as median +/- inter-quartile range (n=11). Values between treatments were compared using one way ANOVA, comparing each treatment to the control (ie unstimulated) value. *= $p \leq 0.05$ and **= $p \leq 0.01$.

4.3.4.2 Detailed examination of cytokine/chemokine responses to LPS and heat killed *H. influenzae* in human lung parenchyma explants from Control and COPD patients

As the time course suggested that 24 h was the optimum time to measure cytokine release for the majority of mediators, this time-point was used to ascertain the effect of bacterial products on cytokine release using a larger cohort of patients. Tissue from 45 donors was used in the human lung explant model for 24 h stimulation experiments. Patient characteristics such as age, gender, smoking history and lung function test results were collected at the time of surgery. Results in (Table 4.5) show that both controls were either smokers or ex-smokers and while controls had normal lung function and a median of 30 pack years smoking history. The COPD subjects had a median of 40 pack years smoking history. Tissue was incubated with 100/ml LPS or 2×10^6 CFU/ml heat killed bacteria for 24 h. Carrier controls were also generated. Supernatants were analysed for various cytokines/chemokines (TNF-α, IL-10, IL-8, IL-6, IL-17, MCP-1 MIP-1β and IL-1β) via multiplex ELISA. The majority of the data in this chapter was not normally distributed as determined by D'Agostino normality test. Therefore, the data are shown as median values and tested via non-parametric means as appropriate (described in figure legends).

Table 4.5 Patient characteristics for 24 h stimulation studies: clinical data was collected for each sample and shown as median and inter-quartile range. Patients with a FEV_1/FVC ratio of < 0.7 were classified as COPD. Those with FEV_1/FVC ratio of ≥ 0.7 were defined as Control patients Ex denotes ex-smoker, current denotes current smoking. Non parametric Mann Whitney U test showed no statistical difference between the group in age and pack years.

	Control	COPD
Gender	10F/9M	14M/12F
Age	66 (55.5-70.5)	67 (63.25-73.5)
FEV_1%predicted	85.19 (75.46-98.96)	74.16 (61.97-98.43)
FEV_1/FVC	0.77 (0.73-0.80)	0.61 (0.50-0.64)
Smoking status	16 Ex/3 current	19 Ex/7current
Pack year	30 (19.06-45)	40 (14.25-60)

Both ex- and current smokers were included in the analysis, as we had no *a priori* evidence of an effect of smoking on cytokine release *ex vivo*. Pilot comparisons using just ex-smokers were performed and similar result was obtained (see appendix A.2) other than for IL-8. Very few samples were from never smokers however, so they were excluded from this section of the study. The ex-smoking period was set to 6 months, which defined a truly ex-smoker phenotype as opposed to those patients who ceased smoking upon cancer diagnosis (maximum 1 month prior to resection), who would have been regarded as current smokers in our study.

TNF-α, IL-10, IL- 1β and MIP-1β (CCL-4) IL-6 and MCP-1 release

Both control and COPD groups showed a low basal level for the cytokines measured and no significant difference was seen between the two groups.

The response after stimulation either with LPS or *H. influenzae* was a significant increase compared to non-treated for TNF-α (a), IL-10 (b), MIP-1β (CCL-4) (c) and IL-1β (e) and for both groups (Figure 4.6). We had already observed an early response of MIP-1b, a monocyte attractant, to bacterial ligand exposure of the tissues.

On The other hand IL-6 and MCP-1, stimulation caused a significant increase in mediator expression only in the COPD group (Figure 4.6 (f) and (g)).

IL-8 release

IL-8, distinct to the other mediators measured, showed completely different pattern. After stimulation, IL-8 was down-regulated in the control group following stimulation with LPS and heat killed bacteria. This is a surprising finding, since animal models suggest elevation of IL-8 in

exacerbation models. We do not know if this represents a true tissue response to stimulus, a feature of the explant model or a dilution issue would require future investigation. Samples were diluted enough and values were within the curve. However a higher fold dilution might have been required. Time-course analysis showed that initial IL-8 responses to LPS were elevated, followed by a period of suppression in which the secretion fell below baseline. This could be a feature of the elevated IL-10 which occurs at later time-points, and which is known to suppress inflammatory cytokine secretion. It is possible that the immediate response is sufficient to initiate downstream cytokine and chemokine events that are capable of neutrophil recruitment in themselves, however it is also possible that the IL-8 secreted *ex vivo* was simply degraded over time, giving the impression of a reduction due to the increase in IL-8-degrading enzymes. The overall effect was an elevated IL-8 levels in the tissue supernatants from COPD patients post-stimulation (Figure 4.6 (d)). Also this results need to be validated independently by other researchers.

IL-17 release

IL-17 on the other hand did not show any significant difference after stimulation (Figure 4.6 (h)). It is a cytokine that is mainly secreted by Th 17 cells and depends on the presence of TGF-β1 and IL-6 at the optimum concentrations (Mangan et al., 2006).

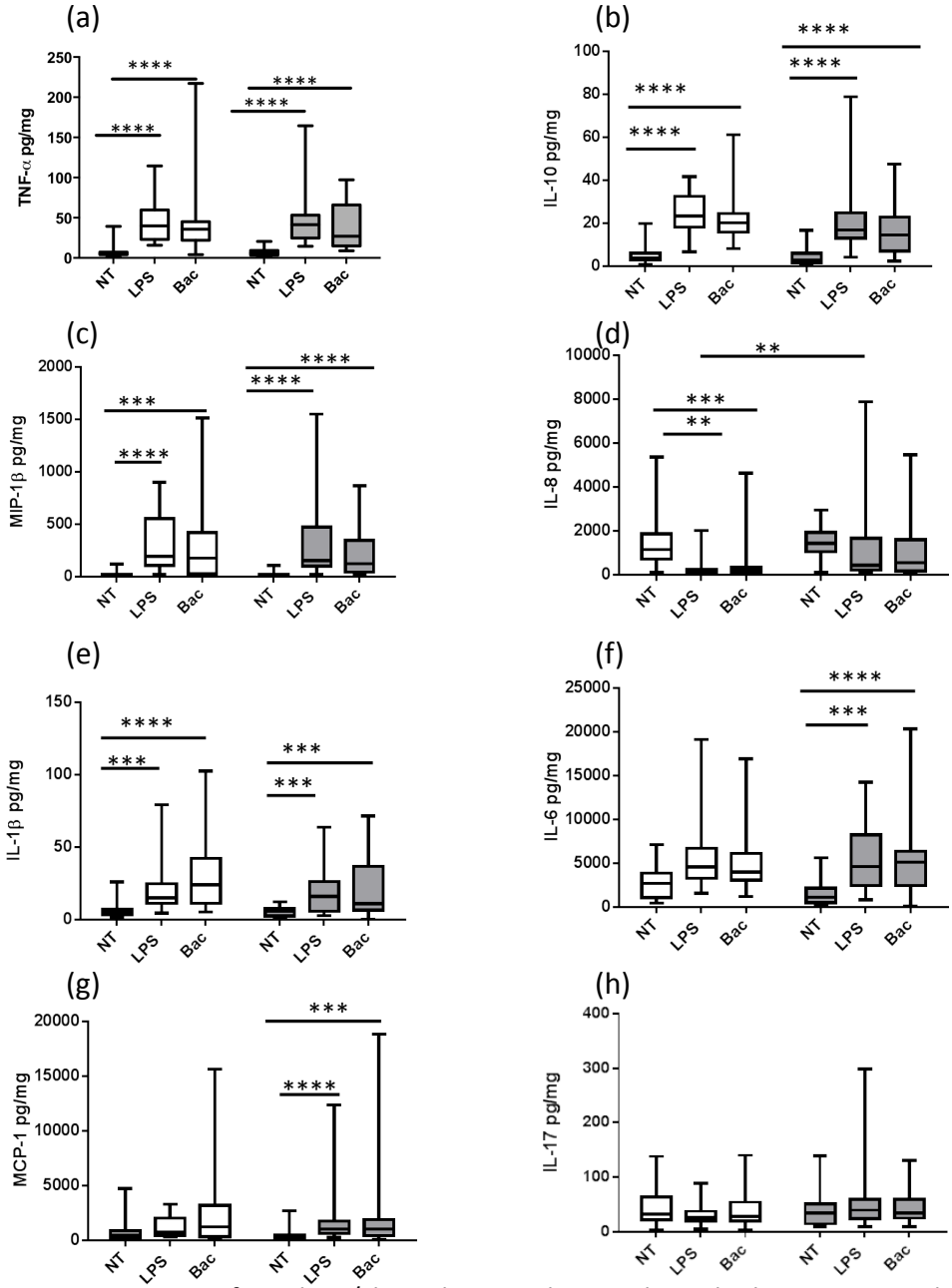

Figure 4.6 Comparison of cytokine/chemokine mediator release by lung tissue explants from healthy and COPD patients following bacterial ligand stimulation. Lung tissue explants were stimulated for 24 h with either 100 ng/ml LPS (LPS), or $2x10^6$ cfu/ml heat-killed *H. influenzae* (Bac), or carrier control (NT) and then mediator release into the tissue-conditioned medium was quantified using multiplexed ELISA (TNF-α, IL-10, IL-6, IL-8, IL-1β, MIP-1β, MCP-1, and IL-17) (see Method section for details). Filled whiskers represent data from subjects with COPD (n = 26) and hollow whiskers represent the control subjects (n = 19). Box-and whiskers represent medians +/- interquartile range. Intra-group analysis was by paired one-way ANOVA, and inter-group analysis was by non parametric Mann-Whitney U test.

4.3.5 Are there defects in bacterial detection in COPD that could account for the inflammatory differences and bacterial susceptibility seen in these patients?

Although a difference in the number of inflammatory cells between the groups was not seen in Chapter 3: we did observe differences in inflammatory responses to bacterial stimulation in healthy and COPD patients. One possibility is that a difference in the cell phenotype in the form of altered TLR expression exists. We therefore decided to quantify the expression of the bacterial specific TLR 2 and 4 receptors on the surface of both structural (epithelial) and immune cells (T lymphocytes, monocytes).

4.3.5.1 Characterization of TLR expression in human lung tissue explants by immunohistochemistry

First IHC was used to characterise TLR on human lung tissue explant.

4.3.5.1.1 Choice of antibodies used for staining TLR and optimisation

Explants were embedded in GMA and sections of tissues prepared for immunohistochemistry as described in Methods section. Sections were stained for TLR 2 and 4 as described in Chapter 2. The detection reagents used were rabbit polyclonal antibodies from Abcam, which had been previously used on similar tissue samples (Kelly Lowing PhD, personal communication). The source/clone of these antibodies, and the range of concentrations investigated are outlined in Section 2.5.2 and. the optimum concentration was chosen where there was greatest differential between specific and background staining when the control and specific antibodies were compared (data not shown).

4.3.5.1.2 Localization and quantification of TLR 2/4 using immunohistochemistry

Several studies have previously demonstrated expression of TLR 2 and 4 in monocyte and macrophages at baseline and upon stimulation. Expression of TLRs in the lung has been described in the work of Droemann et al. (2003) where they found TLR 2 in alveolar macrophages and alveolar epithelial cells. Guillot et al. (2004), von Scheele et al. (2010) have demonstrated that TLR 4 is expressed in human alveolar and bronchial epithelial cells and alveolar macrophages. Since the lower respiratory airway was found to be colonised with microbial pathogens it was expected to find TLR receptors expressed in the cells resident and infiltrating the lung parenchyma. Table 4.6 shows patients characteristics.

Table 4.6 Patient characteristics for TLR 2/4 staining using immunohistochemistry: . Patients with a FEV_1/FVC ratio of < 0.7 were classified as COPD. Those with FEV_1/FVC ratio of ≥ 0.7 were defined as Control patients clinical data was collected for each sample and shown as median and quartile range. Ex denotes ex-smoker, current denotes current smoking and never denotes never smoking.

	Non COPD	COPD
Gender	5M/8F	6M/3F
Age	67 (50-71)	71 (61-75)
FEV1% predicted	82 (76.75 -94.75)	74 (66-81)
FEV1/FVC	0.77 (0.73-0.85)	0.56 (0.54-0.63)
Smoking status	7 Ex/ 3 current/4 Never/	6 Ex/ 3 Current
Pack years	50 (22.5-50)	30 (30-45)

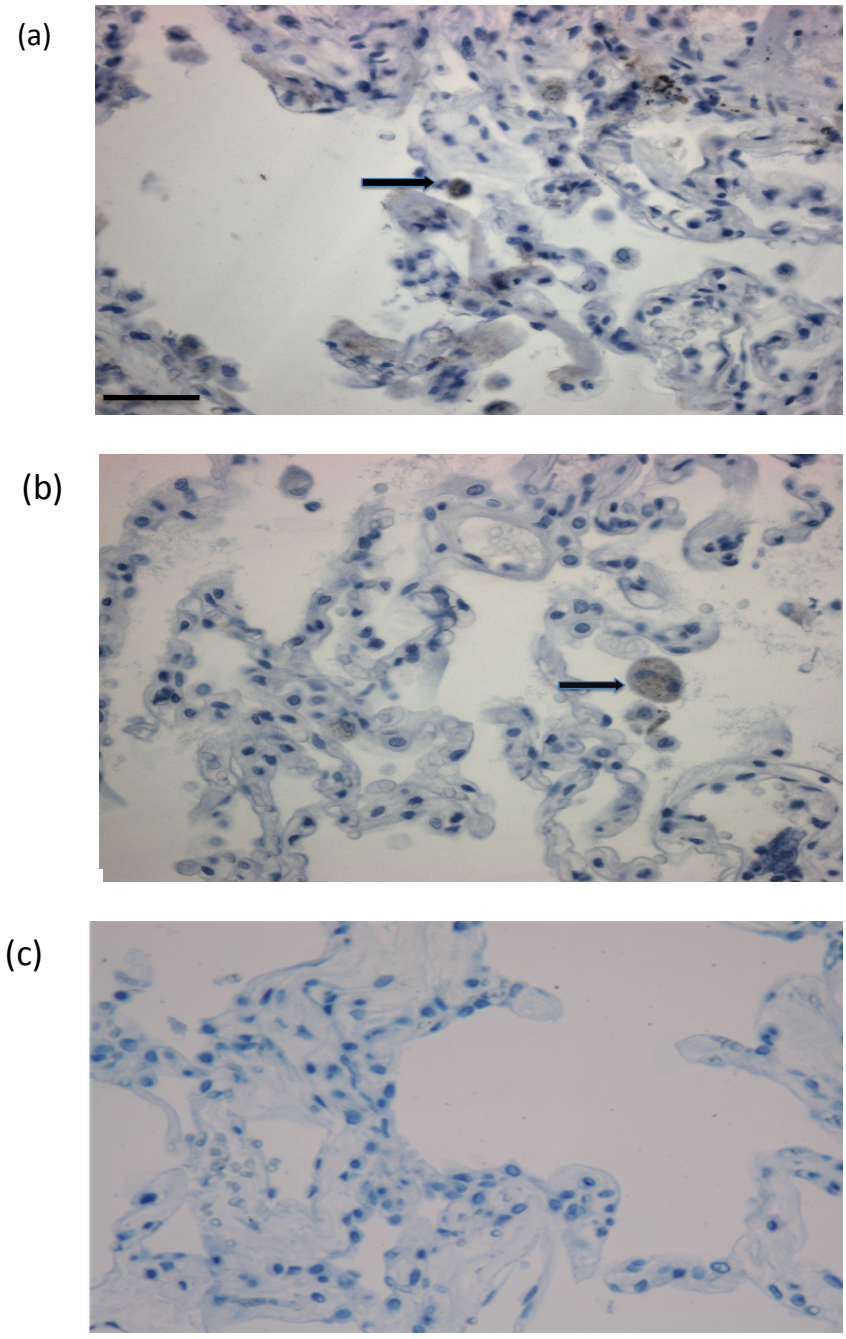

Figure 4.7 Immuno-histochemical staining pattern of human lung parenchymal tissues for TLR 2 and 4. Lung tissues embedded in GMA were sectioned and stained using polyclonal antibodies specific for a) TLR 2 and b) TLR 4 (see Method section 4.4.2 for details). Positive staining is shown in brown, counterstain is Mayer's haematoxylin (blue). Isotype antibody was used to control for background staining (c). Micrographs shown are typical of 22 tissues stained. Scale bar represents 100 µm.

Figure 4.7 (a) and (b) shows TLR 2 and 4 expressed in cells that look like macrophages from their morphology using bright field microscopy. Furthermore, the staining for TLR 2 and TLR 4 as indicated by the arrow (Figure 4.7 (a) and (b)) was not very clear (faint brown). It also appeared to be granular and not localized to the cell membrane, which was an unexpected cellular distribution since these receptors are normally found in the cell membrane, unlike other TLRs such as TLR 3, which reside in intracellular vesicles.

Because of the low level of the TLR staining in our experiments, we chose to compare the groups looking at the percentage of cells positive for TLR rather than quantifying the amount expressed in each cell (Figure 4.8). A big variation was seen between individuals including several individuals who did not show positive TLR staining for either receptor, despite the staining being performed simultaneously on all samples. This resulted in wide variance in the results, probably contributing to a lack of measured difference between the groups.

In our samples processed in GMA, we did not observe TLR 2 or 4 staining in the epithelial cells at all.

Due to the low intensity of staining of TLRs observed in our preliminary study, and considering the published evidence of TLR expression in epithelial cells *in vitro*, we decided to try and optimise the detection of TLRs using flow cytometry of cells dispersed from lung parenchyma samples, as this is a method which might improve sensitivity of detection of the TLRs and would certainly provide greater throughput of tissue samples due to the ease of processing. This method also has the advantage of a collection of significant numbers of data points to improve statistical analysis of the samples where patient numbers are limited.

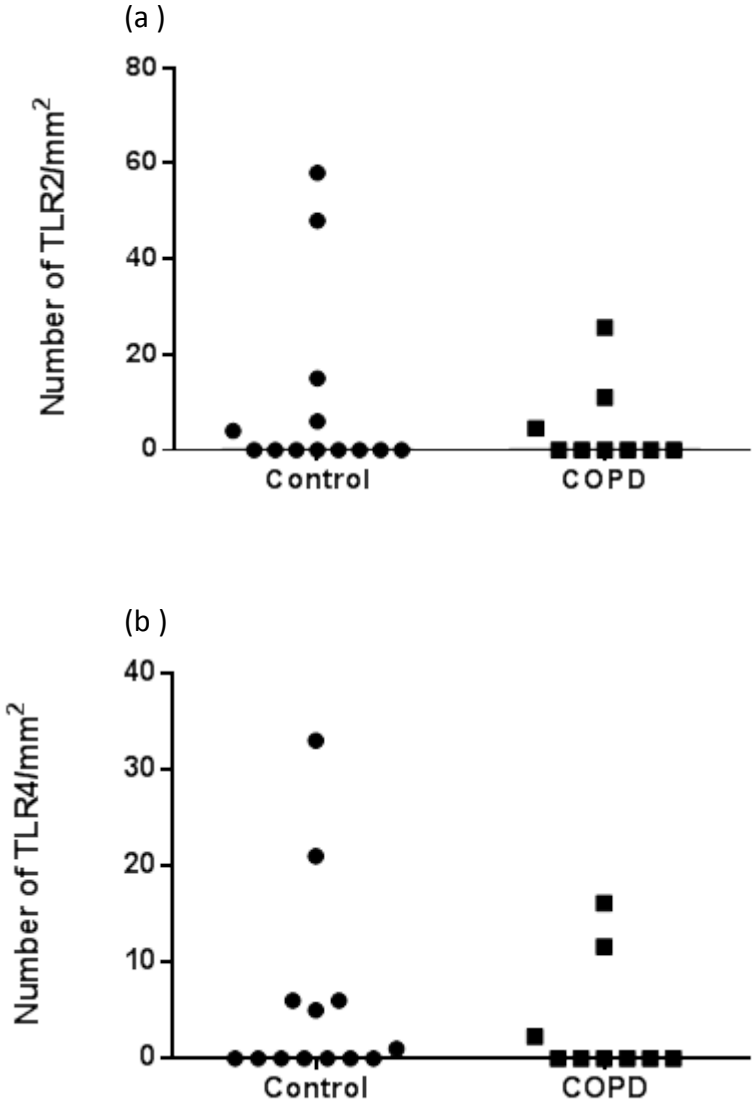

Figure 4.8 Quantification of the area of TLR 2 and 4 staining of lung parenchymal tissue samples visualised by immunohistochemistry. Tissues were processed and stained as shown in Figure 4.7. Staining of the tissues was quantified as described in the Methods section, excluding airspaces. Data shows the number of cells/mm^2 of tissue that stained positively for TLR 2 (a) and TLR 4 (b), in the control (13) and COPD (9) groups. N = 22, Individual data points were plotted, data were compared between groups using non parametric Mann Whitney U test.

4.3.5.2 Characterization of TLR by flow cytometry

Flow cytometry was used next to characterise TLR in human lung parenchyma.

4.3.5.2.1 Titration of TLR antibodies and patient characterisation

Initially, peripheral blood mononuclear cells (PBMCs) were used to titrate TLR antibodies as lymphocytes and monocytes are known to express TLR 2 and 4 at baseline, and sufficient cells could be collected each time to perform a full titration (Section 2.6.1) and Table 2.7. Additionally, tissue samples were not used because of the potential for heterogeneous expression between samples from the surgical source available at Southampton.

The samples were stained as previously described for other cell markers, with a range of different TLR antibodies dilutions (details of the titration are shown in Table 2.7). The optimum dilution for each antibody was determined by selecting the maximum specific mean fluorescence intensity (the antibody concentration, which gave the greatest specific signal when compared to the non-specific signal obtained from the isotype antibody dilution. The sMFI was calculated by subtracting the MFI of the isotype antibody from the MFI of the specific antibody for each dilution. If the background staining level was too high where the sMFI was optimal, then the next dilution down was selected. This was to prevent problems with compensation between other colours multiplexed on the flow cytometer, as PE is a highly fluorescent fluorochrome and can affect other colours if the stain is too intense on an individual cell type. For TLR 2 the point that had a maximum specific fluorescence was obtained using 20 μl per reaction, while 5 μl was optimal for TLR 4 (representative histogram plots of the flow cytometry data used to optimise the antibodies is shown in Figure 4.9.

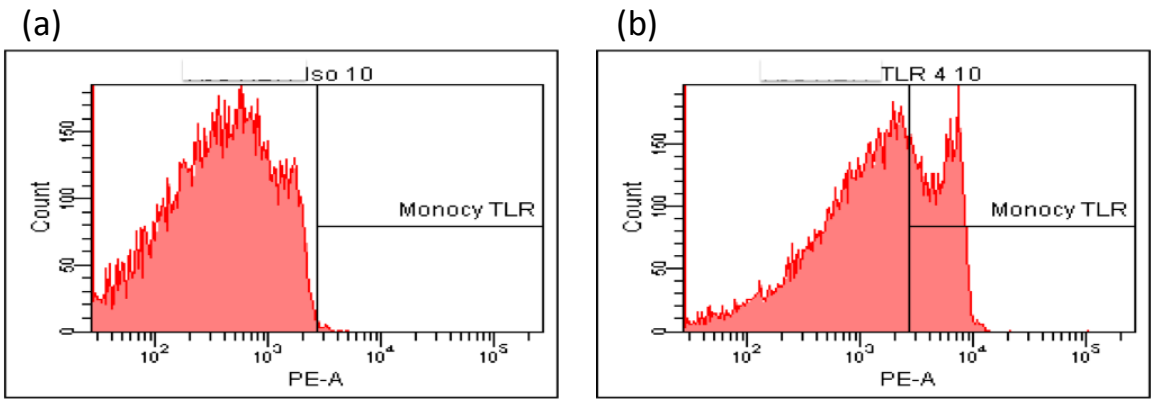

Figure 4.9 Gating strategy for flow-cytometric analysis of TLR 4 cell surface expression on monocytes. Peripheral blood mononuclear cells (PBMC) were isolated from a healthy donor, and the monocytes isolated by positive magnetic selection using $CD14^+$ beads (see Method (2.6.4.1) for details). Monocytes were then stained and analysed by flow cytometry, comparing the monocyte populations (gated against

any contaminating cells on size), using either isotype control antibodies conjugated to PE (a) or monoclonal antibodies specific for TLR 4 also conjugated to PE (b). Positive staining gates (Monocyte TLR) were set using a 1% overlap with the isotype control.

4.3.5.3 Patient characterisation

Human lung tissue from the normal margin was obtained from patients who were undergoing carcinoma resection (n = 40). The patients' age, gender, lung function (when available) and smoking history were recorded. This information can be found in (Table 4.9). Tissue was processed as previously described in section 2.2.

Table 4.7 Patient characteristics for the lung tissue samples used to quantify TLR expression by flow cytometry. . Patients with a FEV_1/FVC ratio of < 0.7 were classified as COPD. Those with FEV_1/FVC ratio of ≥ 0.7 were defined as Control patients. Clinical data was collected for each sample and shown as median and quartile range. Same cohort (missing one sample from the control group) used for cell number analysis by FACS. For gender, M = male and F = female. Under smoking status, current denotes current smokers, ex denotes ex smokers. Non parametric Mann Whitney U test showed no statistical difference between the group in age and pack years.

	Control	COPD
Gender	17 (8F/9M)	23 (10F/13M)
Age	66 (55.25-67)	64 (63.5-72)
FEV_1%predicted	88.8 (76.40-101.19)	72.78 (55.89-89.24)
FEV_1/FVC	0.79 (0.76-0.81)	0.61 (0.55-0.64)
Smoking status	13 EX/4 current	18 EX/5 current
Pack year	30 (18.75-40)	39 (12.75-56.25)

4.3.5.4 TLR 2/4 expression and quantification using flow cytometry

Macrophages, epithelial cells and $CD3^+/CD8^+$ and $CD3^+/CD8^-$ T lymphocytes dispersed from lung parenchyma samples using collagenase were identified by flow cytometry using specific antibodies for each cell types, gating according to the same principles outlined in chapter 3 (Figure 3.6). The expression of TLR 2 and 4 was demonstrated on each cell type using specific monoclonal antibodies for each TLR in addition to the other cell markers (Figure 4.10).

Specific TLR expression was demonstrated on live rather than fixed cells, as initial optimisation results suggested that permeabilization and fixation of the cells using proprietary reagents (BD Fix/perm solution, (see chapter 2) resulted in complete loss of TLR staining. This finding was surprising, as previous studies had shown that fixation does not affect TLR staining on airway smooth muscle cells using flow cytometry (Sukkar et al., 2006). However, it is possible that TLR expression in smooth muscle cells is greater than in the epithelium, overcoming any reduction in detection due to fixation of the cells.

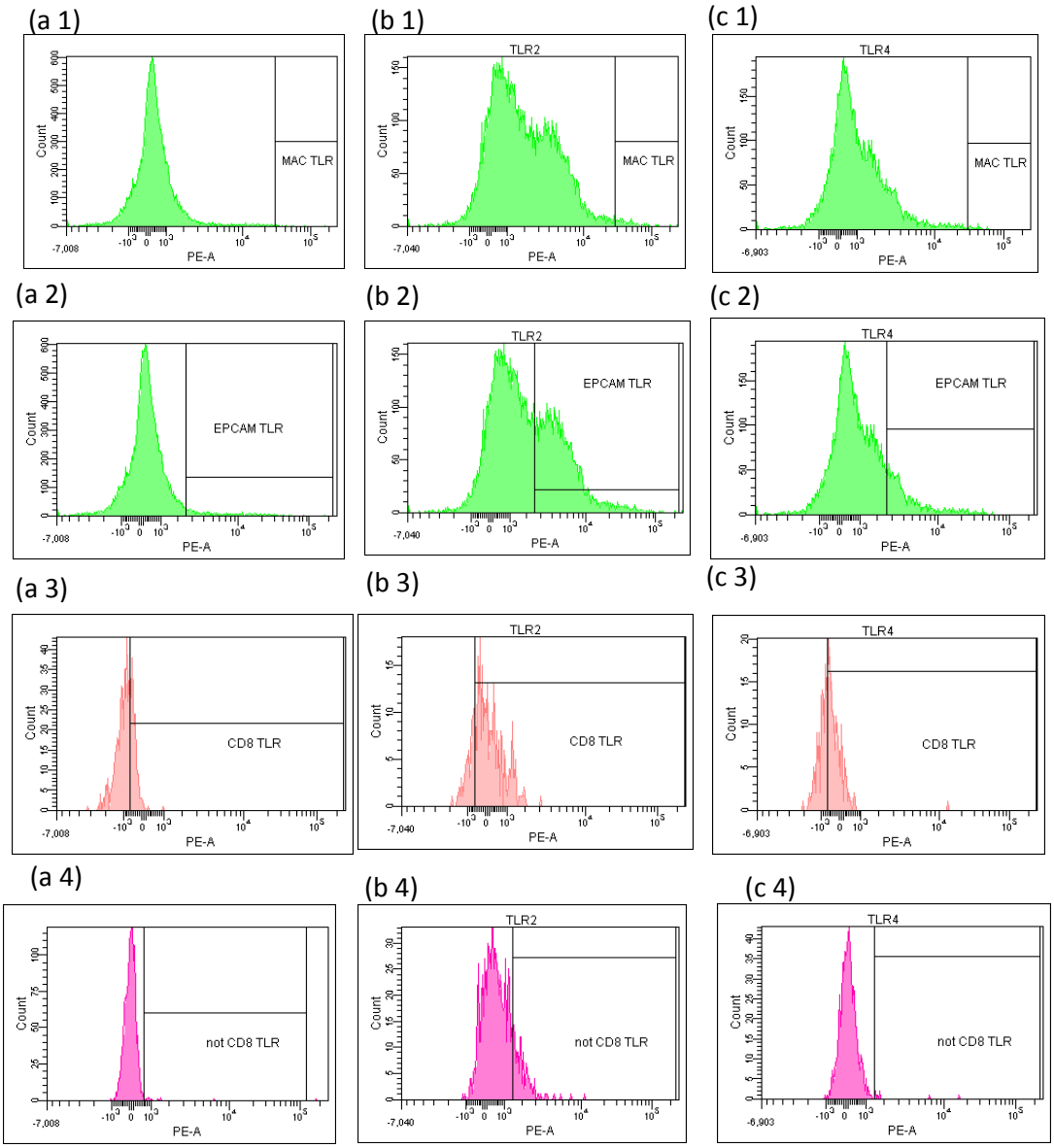

Figure 4.10 Detection of specific TLR 2 and TLR 4 staining in different cell populations dispersed from parenchymal lung tissue explants. Each cell type (macrophages, epithelial cells, $CD8^+$ T lymphocytes and $CD8^-$ T lymphocytes respectively, was identified by a specific gating strategy using cell-specific surface markers as shown in Figure 3.6. Histograms of these cell populations were then gated against the isotype with a 1% overlap. Staining in each cell population: For the **isotype**: macrophages (a 1), epithelial cells (a 2), CD8 T cells ($CD3^+/CD8^+$) (a 3), non CD8 T cells ($CD3^+/CD8^-$) (a 4); For specific **TLR 2**: macrophages (b 1), epithelial cells (b 2), CD8 T cells ($CD3^+/CD8^+$) (b 3), not CD8 T cells ($CD3^+/CD8^-$) (b 4) and finally specific **TLR 4**: macrophages (c 1), epithelial cells (c 2), CD8 T cells ($CD3^+/CD8^+$) (c 3), not CD8 T cells ($CD3^+/CD8^-$) (c 4). The specific mean fluorescence intensity (sMFI) for each TLR was calculated by

subtracting the intensity of the PE staining for the isotype control from the MFI value obtained using the specific TLR 2/4 antibodies.

4.3.5.4.1 Pattern of TLR 2 and 4 staining using flow cytometry

We observed that TLR 2 and 4 positive cells did not form distinct populations when stained using these antibodies, but instead, a whole-population shift was observed upon application of the TLR-specific antibodies. This is not unusual for cell surface receptors, especially where the cells have not been specifically stimulated to increase expression above baseline. As a result of this expression pattern, data were subsequently examined as sMFI rather than percentage expression of the parent population.

Figure 4.10 shows representative examples of the specific whole population shift in the population stained for TLRs 2 and 4 in the cells isolated from resected tissue. In macrophages: Isotype (a 1), TLR 2 (b 1) and TLR 4 (c 1). In epithelial cells: isotype (a 2), TLR 2 (b 2) and TLR 4 (c 2). In T cell ($CD3^+/CD8^+$): Isotype (a 3), TLR 2 (b 3) and TLR 4 (c 3). Finally in ($CD3^+/CD8^-$) T cells: Isotype (a 4), TLR 2 (b 4) and TLR 4 (c 4). Similar challenges to the specific gating for macrophages were observed as those seen in Chapter 3.

Macrophages

Tissue macrophages were generally higher in background PE staining than other cell types, and we observed that the background staining of macrophages was variable between patients. However, we were able to satisfactorily obtain PE histograms for all of the patient samples, and (Figure 4.10 (b 1)) shows a representative plot which demonstrates the increase in the fluorescence intensity in PE seen as a population shift, not distinct two peaks, for TLR 2 when compared to the isotype antibody (Figure 4.10 (a 1)).

When staining for TLR 4 in macrophages (Figure 4.10 (1c)), lower intensity staining than TLR 2 (Figure 4.10 (1b)), was noted, even at the optimized dose, and the data histogram was more complex.

Epithelial cells

A similar pattern was also noted with epithelial cells for each of the TLRs, as a population shift for TLR 2 was again not two distinct peaks but a whole population shift, and there was lower staining for TLR 4 (Figure 4.10: c 2) than TLR 2 (Figure 4.10: b 2).

T cells

T cells (both ($CD3^+/CD8^+$) and ($CD3^+/CD8^-$)) maintained the same pattern, but showed a lower staining intensity than the other cell types (Figure 4.10 (3b and 3c)) and (Figure 4.10 (4b and 4c)).

The ease with which we detected TLRs on all of the cell types using flow cytometry suggests that this is a superior method to immunohistochemistry for this purpose, however there are also drawbacks of flow cytometry, notably that the cellular distribution within the tissue cannot be determined on cells dispersed from tissues, so that pattern of staining cannot be determined. The advantages of high throughput, large numbers of positive data points and increased sensitivity were felt to outweigh any disadvantages however, and therefore we used this methodology to quantify TLR expression on the difference cell types in the context of health and disease (COPD).

4.3.5.4.2 Comparison of TLR 2 and 4 in control and disease

Both ex-and current smokers were included in the analysis (Table 4.9), as we had no *a priori* evidence of an effect of smoking on TLR expression *ex vivo*. Pilot comparisons using just ex-smokers were performed and similar result was obtained (see appendix A.3 for TLR2 and Appendix A.4 for TLR4).

Epithelial cells

When we compared the TLR 2 and 4 expression on the cell surface of epithelial cells (Figure 4.11 (a) and 4.12 (a)), between control and COPD subjects, we did not observe any significant differences despite a modest decrease in median sMFI of TLR 4 (Figure 4. 12 (a)).

This may indicate that epithelial cells do not contribute differently to bacterial detection in the lung parenchyma of COPD patients when compared to control subjects. There is little published evidence in this cell type, since differentiated alveolar epithelial cells are difficult to replicate in *in vitro* culture.

Macrophages

We found that the median of TLR 2 (Figure 4.11 (c)) expression was slightly higher on macrophages in COPD subjects than in controls while there was a modest decrease for that of TLR 4 in COPD (Figure 4.12 (c)) but no significance between the groups for both TLRs.

T lymphocytes

Median of TLR 2 expression was also slightly higher in T cells $CD8^+$ (Figure 4.11 (e)) but no significant difference between the groups. Freeman et al. (2013) have previously reported that the percentage of $CD8^+$ cells that expressed TLR 2 was higher in COPD than the control, and that the percentage of lung $CD8^+$ T cells expressing TLR 2 increased with worsening emphysema, suggestive of a specific COPD phenotype. As we did not find a separate high-expressing population of these cells, we did not quantify the percentage of positive cells for TLRs in this way. However, it was reassuring to find a similar pattern of increased expression in COPD in T-lymphocytes. A highly significant decrease of TLR4 staining on the surface of ($CD3^+$/$CD8^+$) T cells in the COPD patients compared to control subjects (Figure 4.12 (e)).

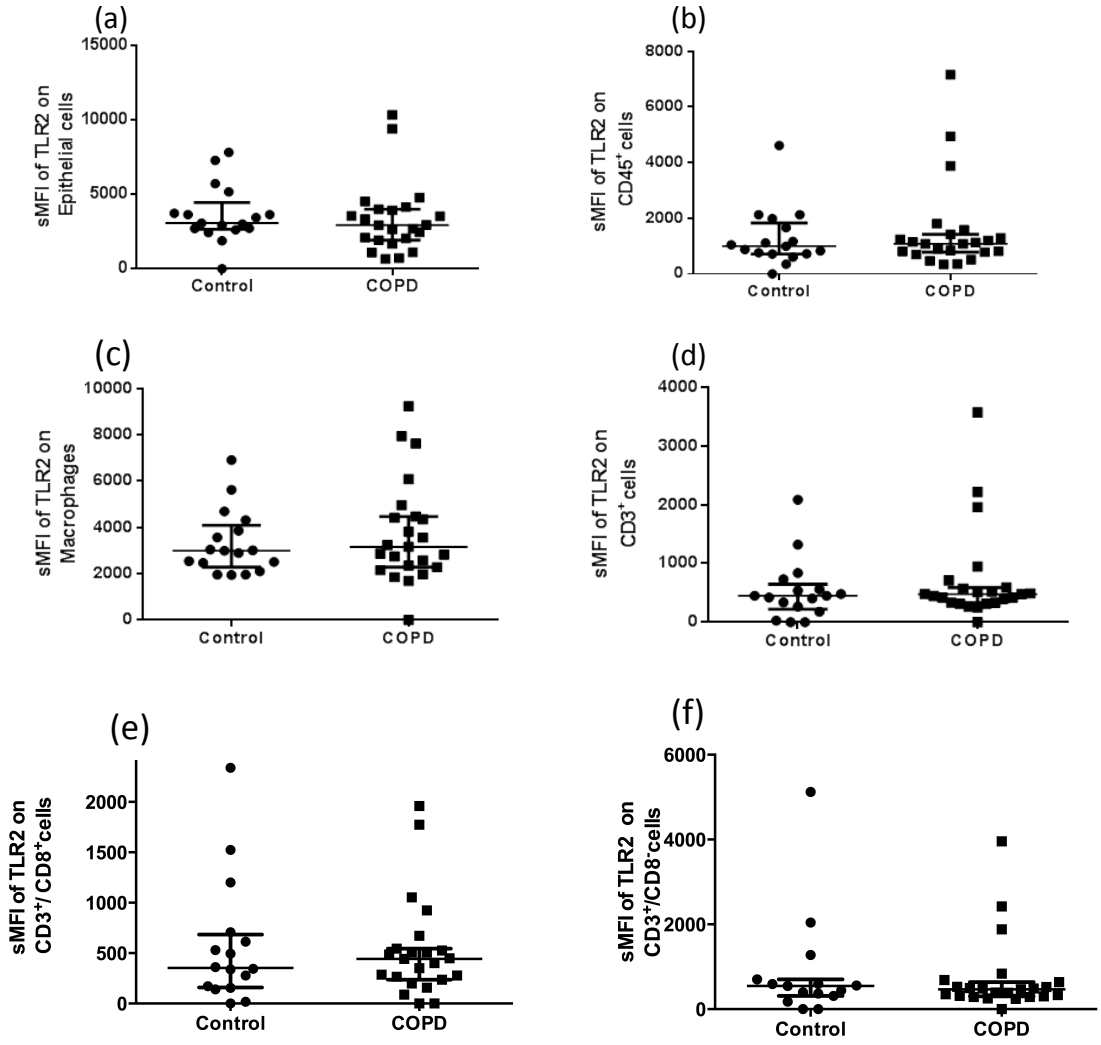

Figure 4.11 Quantification of TLR 2 staining intensity on the surface of cells dispersed from lung tissues measured at baseline, and quantified using flow cytometry as described in Figure 4.10. Cells dispersed from the tissues were stained using monoclonal anti-

TLR 2 antibody conjugated to PE. Data shows specific mean fluorescent intensity (sMFI) of anti-TLR 2 staining in epithelial cells (a), CD45 (b), macrophages (c), CD3 (d), ($CD3^+/CD8^+$) T lymphocytes (e) and ($CD3^+/CD^-$) T-lymphocytes (f). Individual data plots are shown, bars = median value +/- interquartile range. Groups were compared using non parametric Mann Whitney U test. Control = Non COPD subjects n = 17. COPD = patients with COPD (GOLD stages 1-2) n = 23.

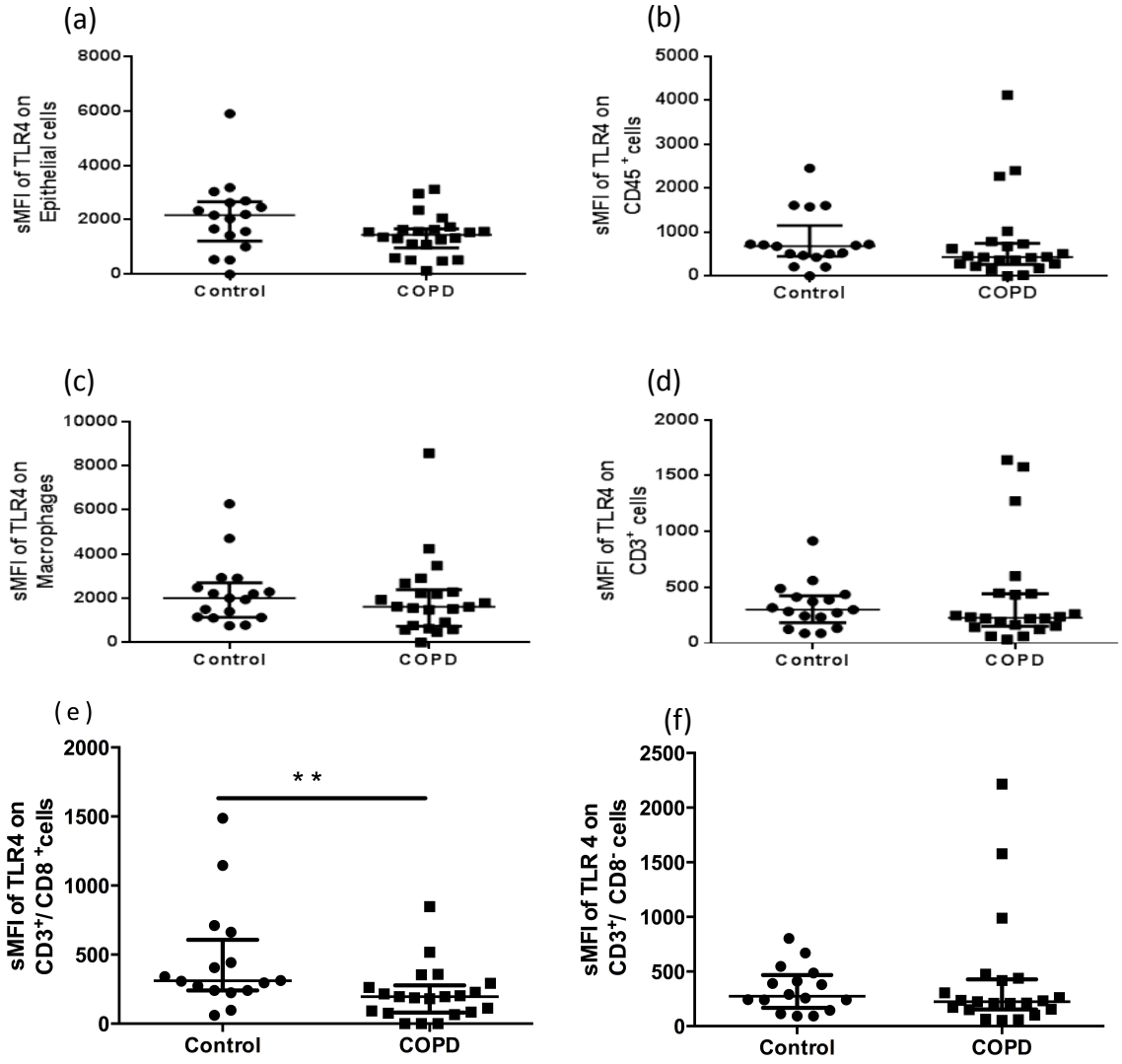

Figure 4.12 Quantification of TLR 4 staining intensity on cells dispersed from lung tissues measured at baseline, and quantified using flow cytometry as described in Figure 4.10. Cells dispersed from the tissues were stained using monoclonal anti-TLR 4 antibody conjugated to PE. Data shows specific mean fluorescent intensity (sMFI) of anti-TLR 4 staining in epithelial cells (a), $CD45^+$ leukocytes (b) macrophages (c), $CD3^+$ T lymphocytes (d), ($CD3^+/CD8^-$) T lymphocytes (e) and ($CD3^+/CD8^-$) T-lymphocytes (f). Individual data plots are shown, bars = median value +/- interquartile range. Groups were compared using non parametric Mann Whitney U

test. Control = Non-COPD subjects n= 17, COPD = patients with COPD (GOLD stages 1-2) n = 23. **= $p < 0.01$.

4.3.5.5 TLR 2 and 4 Stimulation

Human lung tissue from the normal margin was obtained from 11 patients who were undergoing carcinoma resection. The patients' age, gender, and smoking history were recorded. This information can be found in (Table 4.9). Tissue was processed as previously described in Section 2.2.

Table 4.8 Patient characteristics for TLR 2/4 profile after tissue stimulation with either LPS or *H. influenzae*. Patients with a FEV_1/FVC ratio of < 0.7 were classified as COPD. Those with FEV_1/FVC ratio of ≥ 0.7 were defined as Control patients. Clinical data was collected for each sample and shown as median and quartile range. For gender, M=male and F=female. Under smoking status, current denotes current smokers, ex denotes ex smokers.

Gender	5F/6M
Age	66.5 (58-73.5)
FEV_1% predicted	73.13 (58.70 -81.07)
FEV_1/FVC	0.67 (0.61-0.69)
Smoking status	10 EX/1 current
Pack years	26.5 (12.75-43.5)

4.3.5.5.1 TLR 2 and 4 expressions in different cell types after stimulation

Exposure to the ligands was examined by a time-course experiment in which tissues were stimulated with LPS or heat-killed *H. influenzae* bacteria for 0, 6, 24 and 48 h. Following each time-point, the tissues were immediately enzymatically dispersed, stained for TLR expression and examined by flow cytometry. Due to the complex nature of these experiments, only 11 patient samples were examined, and thus, due to the low numbers, this data can only be considered as a preliminary examination of the time-course of TLR expression following TLR ligand stimulation *ex vivo.*

TLR 2 and 4 expressions on T lymphocyte

We did not observe any significant effect of the treatments, with the exception of an observed decrease in TLR 4 expression with both bacterial and LPS stimulation at 24 h on the ($CD3^+/CD8^-$) lymphocytes (Figure 4.14 (e1 and e2)). Surprisingly, we did not observe a similar pattern with the ($CD3^+/CD8^+$) T lymphocytes (Figure 4.14 (d1 and d2), despite the reduced expression of TLR 4 observed in these cells in COPD patients.

TLR 2 and 4 expressions on epithelial cells

We found that there was an initial reduction in TLR expression within the first 6 h of ex *vivo* culture of lung tissues, see Figure 4.13 (a1 and a2) and Figure 4.14 (a1 and a2). This may have been due to the trauma to the tissue of being excised during surgery, despite the tissue having been rested overnight prior to the onset of the time-course experiment

Subsequent to this initial reduction, we did not see an effect of stimulation on TLR expression on epithelial cells.

TLR 2 and 4 expressions on macrophages

As for epithelial cells, there was no significant release or decrease, see Figure 4.13 (b1 and b2) and Figure 4.14 (b1 and b2).

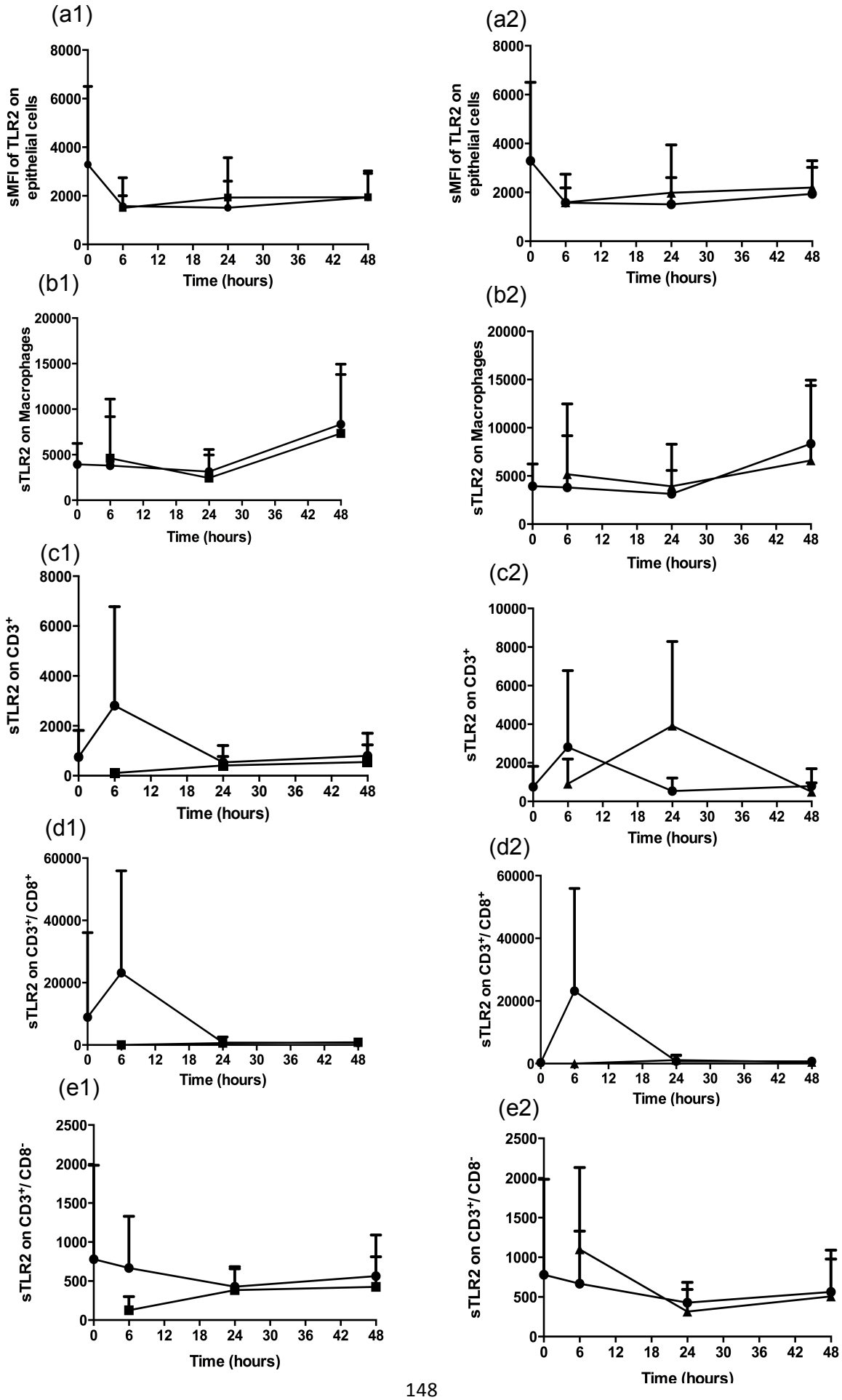
(a1)
sMFI of TLR2 on epithelial cells
Time (hours)
(a2)
sMFI of TLR2 on epithelial cells
Time (hours)
(b1)
sTLR2 on Macrophages
Time (hours)
(b2)
sTLR2 on Macrophages
Time (hours)
(c1)
sTLR2 on CD3+
Time (hours)
(c2)
sTLR2 on CD3+
Time (hours)
(d1)
sTLR2 on CD3+/ CD8+
Time (hours)
(d2)
sTLR2 on CD3+/ CD8+
Time (hours)
(e1)
sTLR2 on CD3+/ CD8-
Time (hours)
(e2)
sTLR2 on CD3+/ CD8-
Time (hours)

Figure 4.13 Time-course experiment to measure the effect of bacterial ligand stimulation on TLR 2 cell surface expression, on different cell types dispersed from lung tissue explants. Lung tissues were stimulated as described in Figure 4.1 for time-points extending from 0-48 h. At each time-point, triplicate tissue samples were enzymatically dispersed, and the resident cells quantified by flow cytometry. Each cell population was stained for TLR 2 as described in Figure 4.10. Data are expressed as specific mean fluorescence intensity (sMFI) for each TLR. LPS = filled squares, heat killed *H. influenzae* bacteria = filled triangles. Control = filled circles. (n = 11) except for 6 h time point, where (n = 2). *= $p < 0.05$. Kruskal-Wallis test was used to look for statistical significance.

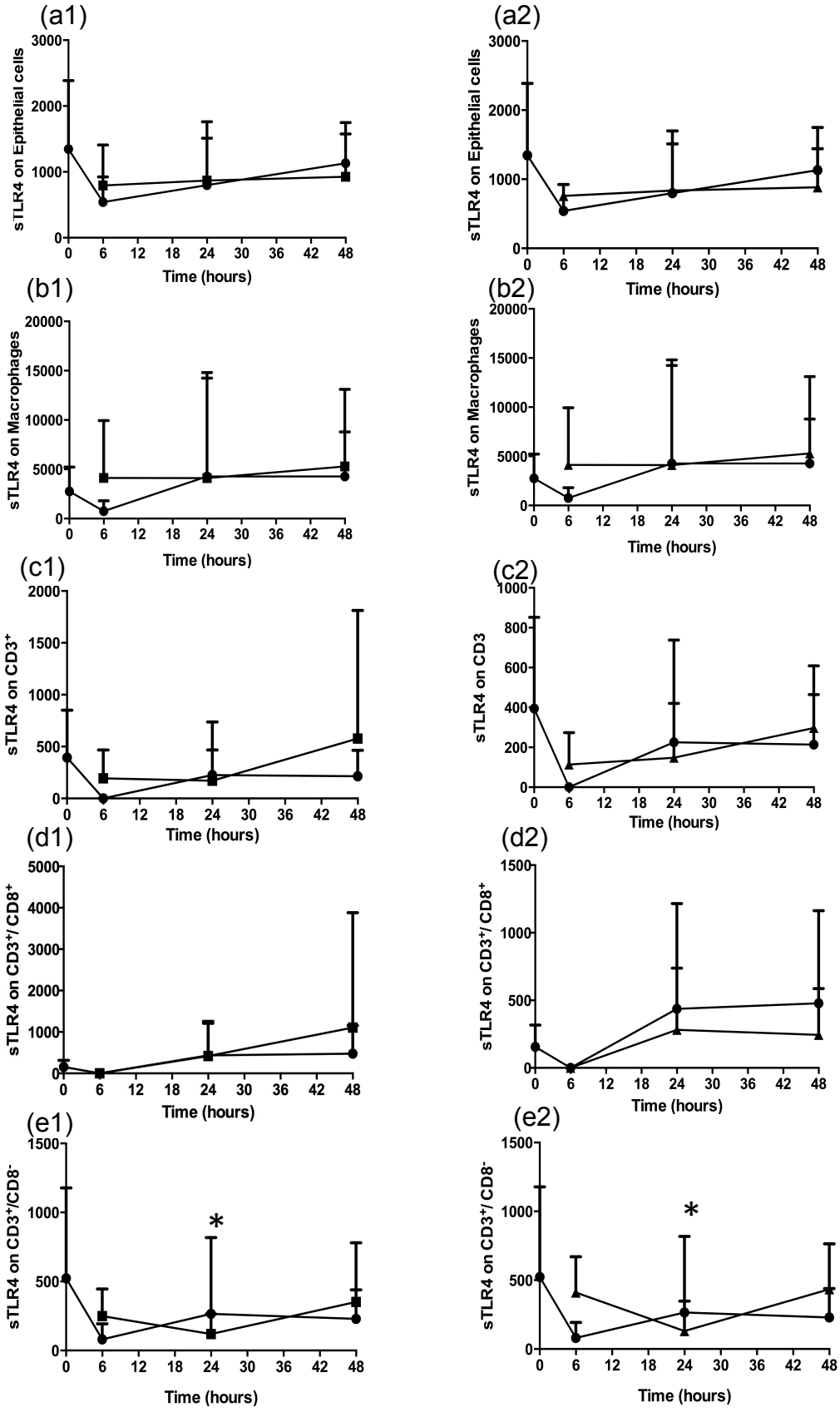
(a1)
sTLR4 on Epithelial cells
3000
2000
1000
0
0 6 12 18 24 30 36 42 48
Time (hours)
(a2)
sTLR4 on Epithelial cells
3000
2000
1000
0
0 6 12 18 24 30 36 42 48
Time (hours)
(b1)
sTLR4 on Macrophages
20000
15000
10000
5000
0
0 6 12 18 24 30 36 42 48
Time (hours)
(b2)
sTLR4 on Macrophages
20000
15000
10000
5000
0
0 6 12 18 24 30 36 42 48
Time (hours)
(c1)
sTLR4 on CD3+
2000
1500
1000
500
0
0 6 12 18 24 30 36 42 48
Time (hours)
(c2)
sTLR4 on CD3
1000
800
600
400
200
0
0 6 12 18 24 30 36 42 48
Time (hours)
(d1)
sTLR4 on CD3+/CD8+
5000
4000
3000
2000
1000
0
0 6 12 18 24 30 36 42 48
Time (hours)
(d2)
sTLR4 on CD3+/CD8+
1500
1000
500
0
0 6 12 18 24 30 36 42 48
Time (hours)
(e1)
sTLR4 on CD3+/CD8-
1500
1000
500
0
*
0 6 12 18 24 30 36 42 48
Time (hours)
(e2)
sTLR4 on CD3+/CD8-
1500
1000
500
0
*
0 6 12 18 24 30 36 42 48
Time (hours)

Figure 4.14 Time-course experiment to measure the effect of bacterial ligand stimulation on TLR 4 cell surface expression, on different cell types dispersed from lung tissue explants. Lung tissues were stimulated as described in Figure 4.1 for time-points extending from 0-48 h. At each time-point, triplicate tissue samples were enzymatically dispersed, and the resident cells quantified by flow cytometry. Each cell population was stained for TLR 4 as described in Figure 4.10. Data are expressed as specific mean fluorescence intensity (sMFI) for each TLR. LPS = filled squares, heat killed *H. influenzae* bacteria = filled triangles. Control = filled circles. (n = 11) except for 6 h time point, where (n = 2). *= $p < 0.05$. Kruskal-Wallis test was used to look for statistical significance.

4.4 Discussion

This chapter first looked at refining and developing an *ex vivo* model of human lung explant stimulation with bacterial products and part of this refining was to do a coefficient of variation to look at the variability in cytokine secretion.

In our human explants model of lung parenchyma infection by Heat-killed *H. influenzae*, dose response experiments were designed to establish if the bacterial preparation was able to generate a response in a dose dependent manner (Figure 4.2 (a) and (c)). TNF-α has been named as an important cytokine that is able to initiate inflammatory cascades during exacerbations of chronic inflammatory conditions (Patel et al., 2003) and so was used to assess the capacity of *H. influenzae* to induce inflammation. The release of TNF-α cytokine from lung parenchyma explants and the amount released (200 pg/ml) was significantly higher than control 0 pg/ml demonstrating its reproducibility as a pro-inflammatory response to bacterial exposure in lung parenchyma. Khair et al. (1994) and Khair et al. (1996) demonstrated similar results, where normal human epithelial cells released several cytokines including significant amount of TNF-α 0.04 pg/ug protein after *H. influenzae* incubation compared 0.01 pg/ug protein for the cells incubated with medium alone. However due to the difficulty of isolation of pneumocytes from human tissue, little data exists on human lung pneumocytes responses to LPS. Most of the existing data has been generated from bronchial epithelial cell lines or immortalised pneumocyte-derived cell lines in which the responsiveness to LPS may be abnormal. The amount of TNF-α released from explants that were stimulated with *H. influenzae* was slightly higher than that released from LPS. It is important to remember that *H. influenza* has more endotoxin components that may contribute to the overall inflammation via several routes. For

instance through TLR 4 for monophosphoryl lipid A (MPL) and LPS (Hirano et al., 2011), and through TLR 2 for P6, a 16 kDa outer membrane lipoprotein that is well conserved in *H. influenzae* (Chen et al., 2004). This higher response could have the result of potential stimulation of other TLRs, or even PAMPS, which were not investigated in this study. However, because this is a heat-killed bacterium, it cannot produce the responses that would be generated by replicating bacteria, which may be significantly more potent.

The lack of a significant release of IL-10 (Figure 4.4 (b)) after LPS stimulation could be due to various things like the large variation in cytokine release between individuals. IL-10 has been shown to simultaneously increase the expression and release of anti-inflammatory molecules (Moore et al., 2001). Human experiments demonstrated that IL-10-mediated inhibition of LPS-induced pro-inflammatory cytokine mRNA expression in human phagocytes happened in two phases. The first phase is rapid, independent of protein synthesis and a delayed phase where the *novo* protein synthesis is required to allow for longer activation of signal transducer and activator of transcription 3 STAT3 (that is considered to be crucial for delivering the downstream IL-10 mediated anti-inflammatory signals) by IL-10, which is necessary for triggering the anti-inflammatory response. However, as mentioned earlier there is the possibility of an assay variation.

TNF-α was not the only cytokine that picked early, MIP-1β, a chemokine that induce chemotaxis of monocytes and natural killer cells, was released even earlier indicating its importance in COPD exacerbation. Chemokines production is influenced by pro inflammatory cytokines like TNF-α, and since MIP-1β peaked before TNF-α, it is possible that its secretion is induced by other cytokines that we didn't measure, such as IL-2 or IFN-γ.

Conversely to the previous cytokines, IL-8 showed a significant decrease in the control group after stimulation compared to COPD group. We were surprised by this finding, since animal models suggest elevation of IL-8 in exacerbation models, and whether this represents a true tissue response to stimulus, a feature of the explant model or a dilution issue (even though the sample was diluted enough and values were within the curve, a higher dilution might have been needed) would require future investigation. Time-course analysis showed that initial IL-8 responses to LPS were elevated, followed by a period of suppression in which the secretion fell below baseline. This could be a feature of the elevated IL-10 which occurs at later time-points, and which is known to suppress inflammatory cytokine secretion. It is possible that the immediate response is sufficient to initiate downstream cytokine and chemokine events that are capable of neutrophil recruitment in themselves, however it is also possible that the IL-8 secreted *ex vivo* was simply degraded over time, giving the impression of a reduction due to the

increase in IL-8 degrading enzymes. The overall effect was an elevated IL-8 levels in the tissue supernatants from COPD patients post-stimulation (Figure 4.6 (d)). IL-8 is a chemokine involved in recruitment of neutrophils to infected tissues. IL-8 contributes to COPD airway inflammation by being involved in the recruitment and activation of neutrophils, particularly in the setting of infection-mediated COPD exacerbations. MMP-2 and MMP-9 can also attract neutrophil to the site of inflammation. We have not measured these MMPs but it is possible that they may have acted synergistically to increase the level of IL-8 in the COPD group. Further work is needed to clarify this situation.

The current study showed a significant increase in IL-6 and MCP-1 after both stimuli in the COPD group compared to the control group. Interestingly the time course for these two cytokines while it showed noticeable release after stimulation that did not reach significance compared to the control. While this could be the result of not enough cells in our tissue to release significant measureable quantities of IL-6 or fewer samples number (n = 11) for the time course compared to other study that had bigger number n = 37 in the case of IL-6. This lack of significance also suggest that may be a subset of patients did not release significant amount of IL-6 and MCP-1, so looking at the 24 h cytokine release, with a larger patient cohort that was split into control and COPD, we saw a significant release in the COPD group but not control. Again, there is also the possibility that there might have been an assay issue related to dilution. Excess mucus production could possibly facilitate the release of IL-6 in the COPD group also polymorphism of this gene have linked to an increased COPD risk. And its transcription is regulated by bacterial endotoxin (LPS) and pro-inflammatory cytokines like IL-1β and TNF-α. It is possible that the production of IL-6 was suppressed in the control group and only high LPS concentrations could lead to a significant increase. This is in line with the work of Eickmeier et al. (2010) who found a reduced release of IL-6 in their control group. The increase in the COPD group may also indicate that COPD patients are primed to resolve acute airway inflammation after an infectious assault (Jones, 2005). As for MCP-1, it is possible that bacterial stimuli are not an effective chemoattractant signal for monocytes and macrophages. Classically, extracellular bacterial infections are associated with neutrophilic responses, and whilst these may be co-ordinated by macrophages, there are normally large numbers of these cells already resident within the lung (Di Stefano et al., 2004). *De novo* recruitment might not be required, and our evidence from chapter 3 suggests no difference in macrophage numbers in COPD compared to control subjects. Another explanation for the lack of up-regulation of MCP-1 in control is the fact that it is a cytokine that is up-regulated by TNF-α (Rosseau et al., 2000, Eghtesad et al., 2001) and IL-1β (Witherden et al., 2004), and there may not have been enough of these cytokines to stimulate

significant release in the control subjects. It is also possible that longer time-points would be needed to see a difference. Furthermore it is possible that in diseased tissue some proteins, such as Resistin that was found to increase MCP-1 in COPD, are highly expressed (Kumor-Kisielewska et al., 2013, Pirvulescu et al., 2014). LPS and resistin may have worked together to lead to a higher production of MCP-1 in COPD tissue compared to control. In addition a polymorphism in MCP-1 gene has been associated with COPD (Bai et al., 2012). And affect the expression of MCP-1 in response to an inflammatory stimulus (Rovin et al., 1999). Finally, stimulation also resulted in noticeable release of IL-17 albeit not significant.

LPS inhalation led to an increase of IL-17 in peripheral blood CD4 and CD8 (Gupta et al., 2015), stimulation of human PBMC after a long culture period with heat-killed *Candida albicans* (Rogers et al., 2013) as well as lung parenchyma exposure to cigarette smoke (Chang et al., 2014) all resulted in an increase in IL-17 indicating the possibility that we might have had a different result have we had a subset of active smokers or a long incubation period. However, the involvement of IL-17 in COPD is still debated.

Having found that lung parenchyma from COPD is more inflammatory decided by the increase release of IL-6 and MCP-1 compared to control, it was important to determine if TLR expression is also up-regulated. First we looked the pattern of expression of these receptors in different cells using both IHC and flow cytometry (Figure 4.7 and Figure 4.10).

IHC showed an intracellular expression of TLR that could be an artefact due to the method used for embedding, as the tissue was first immersed in acetone overnight as part of the normal fixation process, and this could have affected the membrane localization of the proteins, especially since acetone have been reported to have the ability to cause a complete loss of plasma membrane (Hoetelmans et al., 2001). Endogenous peroxidases were inhibited using H_2O_2, however there is a possibility that this inhibition may not have been complete (Bussolati and Radulescu, 2011). Interestingly, others have reported intracellular localisation of both TLR 2 and 4, Ueta et al. (2004) in the ocular mucosal epithelium, and Sukkar et al. (2006) demonstrated intracellular localisation of TLR 2 and 4 in airway smooth muscle, and Guillot et al. (2004) reported intracellular location of TLR 4 in pulmonary epithelial cells leading us to believe that our findings could represent a true intracellular staining. We have also considered double staining with CD68 antibodies to confirm if the cells were indeed macrophage cells, however we found out in preparatory work (not shown) that CD68 is not fully specific for macrophages, but instead also stains a number of cell types including neutrophils, basophils, B-cells (Strobl et al., 1995), dendritic cells, epithelial cells (Travaglione et al., 2002), and

osteoblasts (Heinemann et al., 2000) reducing its utility for co-localisation studies. CD68 is a glycoprotein that binds to low density lipoproteins, and is a marker for phagocytes in general, which may explain its lack of specificity.

Other groups have localized TLR 2 and TLR 4 in alveolar macrophages and epithelial cells in lung parenchyma (Droemann et al., 2003, Go et al., 2014). Droemann et al. (2003) used mouse monoclonal antibodies to detect TLR 2, which may have been more sensitive than the rabbit polyclonal antibodies used in this study, although this is probably also a specific phenomenon of the affinity of any antibodies used in any one study. Also they performed detection by an alkaline phosphatase-labelled streptavidin-biotin LSAB technique while Avidin-Biotin Complex (ABC) was used in this work. However, the ABC system has been used by others and did not affect the expression of TLR 2 on human lymphoid tissue (Ochoa et al., 2003) and TLR 2 on human epithelial cells (Hertz et al., 2003).

Similar pattern of staining was noticed using both methods which was that TLR2 stains more than TLR 4 (Figure 4.10). This may simply reflect a lower affinity of the TLR 4 antibody for its receptor, although it is tempting to conclude that TLR 4 expression may be significantly lower in these cells than TLR 2, since similar patterns were observed by immunohistochemistry using completely separate (and polyclonal) antibodies.

In agreement with our immunohistochemistry data, we observed the greatest sMFIs on airway macrophages from the lung tissue, suggesting that the low intensity staining we observed by immunohistochemistry in the epithelium reflected a true expression of TLRs in this cell type, and not an artefact of non-specific staining Figure 4.11 and Figure 4.12. While there was a modest but non-significant decrease in median sMFI of TLR 4, on ($CD3^+/CD8^-$) T cells in COPD group compared to control group (Figure 4.12 (f)), there was a highly significant decrease of TLR 4 staining on the surface of ($CD3^+/CD8^+$) T cells in the COPD patients compared to control subjects (Figure 4.12 (e)). Freeman et al have previously demonstrated an elevated percentage of TLR 4 on $CD8^+$ T lymphocytes in COPD compared to healthy subjects. There were differences in our experimental approaches, which could account for the differences observed between our study and theirs, most notably in the origin of the cells examined. This difference in result between our study and Freemans in term of TLR 4 on CD8 could be due to a number of things. In our study, the T lymphocytes were resident within the lung tissue, and we were careful to examine only live cells to reduce the possibility of false positives due to exposure of intracellular TLR ligands in dead or dying cells, whereas such safeguards were not used in the Freeman study. We did not examine percentage positivity due to the lack of a high-expression sub-population. However, we know that it is possible to observe an overall increase in percentage positivity,

whilst showing a median reduction in sMFI in receptor expression. It is possible that examination of mRNA might elucidate the true expression status of the TLR ligands, although in the case of receptors, membrane movements can be more significant than mRNA changes post-stimulation.

The significance of a defective TLR 4 expression on $CD8^+$ T lymphocytes has yet to be examined, however, a faulty detection of bacterial ligands by these cells might lead to either increased or decreased mediator responses coordinated by this cell type, dependent upon whether the dominant response was suppressive or pro-inflammatory.

In vitro evidence suggests that stimulation of lymphocytes and monocytes with bacterial ligands can increase TLR expression, at least transiently (Skinner et al., 2005, Bao et al., 2017), and that some of this stimulation is under the control of cytokines (Homma et al., 2004, Winder et al., 2009). Considering the relationship between COPD exacerbations and bacterial load, we might assume therefore that TLR expression should be higher in COPD subjects at baseline. However, we observed the opposite for TLR 4. It may be the case that chronic exposure to TLR 4 ligands leads to downregulation or consumption of the TLR 4 receptor, and that this may result in abnormal cytokine responses to bacterial presence in COPD patients. Earlier in this chapter we observed differences in cytokine responses to LPS (a TLR 4 ligand) between control and COPD subjects. These abnormal responses were observed in both pro-and anti-inflammatory cytokines as in the case of IL-8 where it was seemingly down-regulated in the control group but not in the COPD patients. Another abnormal response was seen in the case of IL-6 and MCP-1 where stimulation lead to increased release of these cytokines in COPD but control. Finally, we did not see a significant release of IL-10, anti-inflammatory cytokine, at 24 h after stimulation. Our results may in fact demonstrate that abnormal TLR 4 expression in COPD is either a cause or a reflection of altered cytokine expression localised in the lung tissue. This is said, of course bearing in mind the limitations and possible issues in this work.

It is tempting to think that abnormal TLR and cytokine expression in COPD patient lungs could lead to the increased susceptibility to bacterial infections observed in this patient group. In this study we measured the TLR and cytokine proteins directly, rather than using mRNA. Although it would be desirable also to quantify RNA, this would be a technical challenge in lung tissue due to the small number of cells recovered from lung tissue explants. Furthermore, there is not a strong relationship between mRNA and protein expression across the whole transcriptome, suggesting that directly measuring protein may be more useful than quantifying mRNA levels between patients.

TLR expression has been previously observed to be up-regulated by bacterial ligand exposure *in vitro* in monocytes (Paolillo et al., 2011). Patients with COPD show evidence of bacterial colonisation even in stable disease. If constant exposure to bacterial ligands modulates bacterial sensing mechanisms in lung tissue, this might lead to abnormal inflammatory responses, potentially pre-disposing lung tissue in COPD patients to further or more prolonged bacterial infections. We therefore wished to examine TLR expression following *ex vivo* exposure of lung tissue to LPS or heat killed bacteria to see if similar patterns could be observed *ex vivo*.

While looking at TLR expression at the baseline was useful in away that it allowed us to see that TLR expression at baseline may be abnormal in COPD at least in T cell CD8 positive cells. We wanted to further explore this notion so we turn our attention to the effect of stimulation on the expression of these receptors.

Our preliminary data in T lymphocytes suggest that TLR stimulation *ex vivo* does not lead to the same patterns of TLR 4 reduction on T lymphocytes observed in COPD patient lung samples, however these data did not come from large numbers of patients, and it is possible that an extended period of chronic exposure to bacterial ligands would be required to result in the patterns we observed *in vivo* (in freshly collected lung tissue samples from patients).

In macrophages and epithelial cell, we found that stimulation did not lead to a significant difference form the baseline for TLR 2 and TLR 4.

As for alveolar macrophage, it is not clear that stimulation lead to change in expression. While von Scheele et al. (2010) showed that stimulation of both alveolar macrophages and monocytes with LPS increased their TLR 4 expression. Sahlander et al. (2010) reported that the expression of TLR 2 in blood monocytes was not altered by exposure to LPS and (Southworth et al., 2012) have also reported that LPS stimulation alone did not lead to increase TLR 2/4 in alveolar macrophages but increase was seen after addition of INF-γ. It is possible that the washing step of our tissue removed the IFN-γ present in the tissue conditioned culture medium supernatant, leading to the transient reduction in TLR expression we observed *ex vivo*. Further experiments with additional exogenous IFN-g could help to further elucidate the mechanisms behind TLR monitoring of bacterial infections. Conflicting results regarding the expression TLR 2/4 after LPS stimulation of human blood monocytes has also been reported.

In term of epithelial cells, our findings are in contrast to those of Shuto et al. (2002) who reported that even though TLR 2 is expressed at low level in epithelial cells, stimulation with *H. influenzae* led to significant up-regulation in primary human airway epithelial cells (NHBE) and von Scheele et al. (2010) also reported its increased expression after stimulation of primary

bronchial epithelial cells with LPS and TNF-α. Monick et al. (2003) found that infection by respiratory syncytial virus augmented TLR 4 expression and membrane localization on airway epithelial cells (A549 cells, primary human airway epithelial cells (HAE), human tracheobronchial epithelial cells and HeLa cells) suggesting that a priming event is needed to induce LPS responsiveness to those epithelial cells that are in frequent contact with environmental stimuli (intestine and lung) and are hyporesponsive to LPS at baseline. However, it may well be that human lung parenchyma has a different pattern of response to stimulus in TLR regulation. Type I and II pneumocytes are functionally very different from bronchial epithelial cells, which although more freely available, are not representative of the respiratory part of the lung, but instead of the conducting airways.

In addition, single cell cultures, especially of immortalised cell lines with little phenotypic relationship to *in vivo* cell phenotypes, may quite reasonably show different patterns when compared to the physiologically tissue explant where the networking between cells is preserved, but where the cytokine interplay is likely to be more complex. In addition to cytokines, cigarette smoke exposure may also affect TLR expression. (MacRedmond et al., 2007) reported down-regulation of TLR 4 mRNA and protein expression in airway epithelial cell after cigarette smoke extract. They also reported a dose dependent down-regulation of TLR 4 mRNA and protein after fluticasone incubation and impaired IL-8 response to LPS in COPD subjects with subsequent increase in susceptibility to gram-negative bacteria.

Since cigarette smoke and COPD therapies such as fluticasone were not present *ex vivo* in our study, it is possible that the withdrawal of these stimuli in culture also affected TLR expression over the duration of the study.

We do not always have full clinical data about the subjects recruited into this study and some of them have been taking steroids as part of their normal medication for COPD. It is possible that this would have had an effect on TLR expression and might have been responsible of the down-regulation of TLR 4 on ($CD3^+/CD8^-$) that we reported.

It is possible that TLR 4 expression is abnormal in COPD patients with different stages and different exposure to cigarette smoke. This theory is supported by the work of Lee et al. (2012) who showed that TLR 4 was down-regulated in severe COPD patients with emphysema. However, in the current study it was not possible to find out if the patients had emphysema or not as CT scans were not routinely taken or available for the majority of patients and also we do not have cases of severe COPD.

Most of our subjects were either smokers or ex-smokers, and it is possible that chronic cigarette smoke exposure has blunted TLR 4 responses in tissue from these patients, and led to the lack of difference between the groups we have seen. It is possible that we would have had a different result, had we had a non-smoking control group, however non-smoking subjects were rarely undergoing the kind of lung resections for which we could obtain tissues from the surgical unit.

It is also possible that removal from the normal homeostatic tissue environment modulated TLR expression through unknown mechanisms**,** but furthermore, during the resting phase, the tissues were incubated in culture medium containing antibiotics to ensure sterility of the tissue cultures, a necessary step for downstream analysis, however this may have reduced or increased exposure to bacterial ligands, depending upon the fate of the affected bacteria (if indeed any viable bacteria were present). If exposure to bacterial ligands truly modulates TLR expression, then this in itself could lead to the reduction in TLR expression we observed at the early time-points.

This work has demonstrated that the human lung explant model is useful in modelling responses to bacterial stimulation, providing an alternative viewpoint to monolayer and *in vitro* differentiated cell cultures. It was demonstrated in Chapter 3: that the tissue used in this model contains multiple cell types, and, therefore, is more likely to reflect the complexity of responses observed in human infection than simple cell lines. Furthermore, these are not cells propagated in culture, but are primary material cells that are kept in their native intact states and are unlikely to undergo de-differentiation and/or proliferation as occurs in most models. As a result this may produce more relevant responses than propagated cell lines. Certainly the array and quantity of inflammatory mediators produced in response to stimuli such as LPS appears to be more complex and robust than in most *in vitro* models. In our model of acute bacterial exposure in human lung tissue, we showed that several cytokines were released following stimulation. Several groups have also shown the release of the same cytokines in COPD patients with bacterial infection (King et al., 2012, Moghaddam et al., 2008) providing accessory evidence of the relevance of our model. The significant release of IL-6 and MCP-1 show that COPD patients were more inflammatory following LPS stimulation compared to the control group.

Some disadvantages of our model may be that the tissue could lose some *in vivo* characteristics after being cultured. Also, some elements of the adaptive immune response are probably not active within the tissue as there is no possibility of specific migration of immune cells into the

tissue from circulatory sources. The absence of blood circulation may significantly alter several cell processes, including endothelial cell function and associated responses, however, within the confines of *ex vivo* culture, we believe that this may represent an advance in our understanding of bacterial induced inflammatory responses in human lung tissue.

This study shows a clear difference between cytokine production in cultured cells and explants in patients with COPD and control subjects, and while many reported increased cytokine release in the COPD groups compared to the control after LPS stimulation, that result was not replicated completely in this model. Similar results were reported in a study that compared TNF-α release between sputum cells and blood cells where sputum cells produced constitutively elevated levels of TNF-α compared to blood cells, but the amount of TNF-α produced was significantly lower in COPD patients than in controls. These sputum cells did not respond to LPS by increasing TNF-α production. In contrast, blood cells did not produced TNF-α spontaneously, but a significant increase in its release was seen in response to LPS, which did not differ between the study groups (Dentener et al., 2006).

Our data using flow cytometry showed that epithelial cells, macrophages and T cells express TLR 2/4 (Figure 4.10). When comparatively evaluated, the expression of TLR 2 and TLR 4 expressed as specific mean fluorescence intensity (sMFI) in macrophages, epithelial cells and T cells showed an emerging pattern in the form of high TLR 2 expression and only moderate staining for TLR 4 (Figure 4.11) and (Figure 4.12), which is in line with our findings using immunohistochemistry. TLR expression was equivalent in macrophages and epithelial cells, and lower in T cells in COPD, providing exciting initial evidence that TLR responses may be inadequate in COPD, and could lead to the blunted cytokine responses we observed in the COPD patients. This faulty monitoring and response to bacterial presence could be part of the mechanism by which COPD patients are more susceptible to bacterial infections than control.

4.5 General conclusion

To conclude, quantification of inflammatory cells using flow cytometry did not show a difference between disease and control and while we adhered to the GOLD guidelines for COPD diagnostic there is still the possibility of diagnostic uncertainty as COPD patients at best have moderate severity while some of the smokers probably had COPD.

This work shows that LPS is capable of inducing an inflammatory response that mimics that generated by *H. influenzae* using human lung parenchyma tissue in our *ex vivo* model.

There were differences in tissue responses to LPS and *Haemophilus influenza* between control and COPD subjects. Lung tissues from COPD patients responded to these stimuli by producing IL-6 and MCP-1 where tissues from controls did not produce significant amount, and demonstrated maintained IL-8 secretion, where in control subjects, the IL-8 response appeared suppressed. These abnormally regulated mediators may be responsible for monocyte and neutrophil accumulation amongst other functions, and could account for excessive inflammatory cell accumulation during bacterial exacerbation observed from *in vivo* studies. However as mentioned before the possibility of an assay issue cannot be ruled out.

We were also able to assess TLR 2/4 expression in lung parenchyma using flow cytometry. This methodology demonstrated that cells in the lung parenchyma explants express TLR 2/4 receptors that play an important role in immune response to pathogens. TLR 4 was down-regulated on $CD3^+/CD8^+$ T lymphocytes in COPD lung tissue. This TLR is critical to LPS response and its downregulation could well affect bacterial sensing by cytotoxic T cells, affecting their activation and consequently their effector functions.

This is a model that compares very favourably with that of other models of COPD Table (4.9).

Metcalfe et al. (2014) developed a model involving human alveolar macrophage cells from COPD and Control where these cells were exposed to cigarette smoke followed TLR ligands (NHHi and LPS) to mimic real life situation for COPD patients. CS exposure resulted in suppression of TLR ligands induced TNF-α IL-10, IL-6 and Rantes release while it did not have an effect on IL-8 release suggesting it to be the tool by which alveolar macrophages can cause neutrophilic inflammation. Different finding were reported by similar model using primary bronchial epithelial cells (PBEC) from COPD, smokers without COPD and non smokers (Comer et al., 2013). Where CSE followed by LPS reduced IL-8 release in the COPD group but not other two groups. Suggesting an immunosuppressive effect of CSE on epithelial cells. Treatment of PBEC with LPS alone led to an increased release of IL-6 and IL-8. The reduction of IL-8 seen using PBEC was not consistent with the work of Pace et al. (2008) who reported that combined CSE and LPS increase IL_8 release. Alveolar macrophages have also been used by Gerlach et al. (2015) to investigate LPS stimulation as a model for bacterial infection and measured CCL2, MMP-9 and IL-6. Strong release of IL-6 by the COPD group compared to the other groups was reported. However different finding were reported by Knobloch et al. (2011) using similar model were there was a strong IL-6 from alveolar macrophages from non-smokers compared to COPD. IL-8 and MMP-9 was increased in COPD compared to smokers and non-smokers. These differences as well as highlighting the difficulty of obtaining similar reproducible results because of the heterogeneity of COPD patients also highlight the effect methodological differences like serum

concentration in the media and cell number density. Earlier human cell model looking at the effect of LPS on alveolar macrophages (Goodman et al., 1998) found that LPS stimulated an increase release of ENA-78, Gro subfamily members as well as release of IL-8.

The difficulty of generating cultures of type I and II pneumocytes is well known, and there are drawbacks to cell culture models in general, in terms of their degree of transformation and/or differentiation, and the influence of this on cell function. When removed from their host tissue, primary bronchial epithelial cells are dissociated (e.g., trypsinisation) during which process they may also undergo dedifferentiation, proliferation and lose some of their specific functions when grown as monolayers.

Table 4.9 Mediators measured in different cell types like macrophages, in mice and human, epithelial cells and monocytes. Work done by different researchers.

Name	Cell type	Mediators
Gerlach et al., 2015	Alveolar macrophages	CCL2, IL-6 and MMP-9
Metcalfe et al., 2014	Alveolar macrophages	TNF-α, IL-6, IL-10, RANTES and CXCL8
Comer et al., 2013	Primary bronchial epithelial cells	IL-8, IL-6
Knobloch et al., 2011	Alveolar macrophages	GM-CSF, MMP-9, MCP-1, IL-6, IL8
Pace et al., 2008	Human bronchial epithelial cell line (16-HBE)	IL-8 and IP-10
Goodman et al., 1998	Human alveolar macrophages	IL-8, ENA-78, GRO α, GRO β, and GROγ

As a consequence extrapolation of data resulting from these studies to *in vivo* situations may not be enough. This is because COPD is a complex disease where there is a lot of cross- cell networking. Although monolayer cells can provide useful evidence of pathways and networks of cell activation in response to bacterial ligands, we feel that lung tissue, removed from patients with established healthy or COPD phenotypes, probably represents a more accurate

model of bacterial response than simpler models, although the complexity of response, due to the wide array of cell types present, can present challenges in itself.

There is no doubt that each model has its strengths and weaknesses. However, *ex vivo* lung tissue model has the potential to allow for more accurate assessments that could mirror the *in vivo* lung tissue since it maintains the architecture of the tissue. Therefore, our model may be a suitable model for the testing of pharmaceutical agents that may be useful in the treatment of bacterial exacerbation, and the next chapter provides preliminary evidence that our model is useful in pre-clinical testing of inflammatory pathway therapeutics such as corticosteroids and p38MAPK.

Chapter 5: P38 MAP kinase inhibitors

5.1 Introduction

5.1.1 Novel therapeutic strategies for COPD

As mentioned in chapter 1 P38 Map kinases inhibitors have emerged as a potential new therapeutic strategy for COPD and other inflammatory diseases as they have the potential to augment the effects of steroids, whilst working through an independent pathway. It is also hoped that these drugs might be not only an effective anti-inflammatory therapy, but also could modify disease progression, for example by slowing lung function decline in these patients (MacNee et al., 2013).

In this study two compounds were investigated. These are currently formulated and in phase II clinical development for potential delivery via the inhaled route (PF-0715455), and via the oral route (PH-796804) (Selness et al., 2011).

5.1.2 PF-0715455 Compound structure

PF-0715455 is a novel, potent and selective inhaled inhibitor for the p38 α enzyme, which was identified after subsequent optimization of various properties of several advanced lead compounds. It is a DFG-out binding p38 inhibitor (Figure 5.1) and is reported to have high potency with a low inhaled dose, a good duration of action for therapeutically relevant period, and also has several ways of clearance.

The pharmacokinetics of this compound has been assessed both *in vitro* and *in vivo.* It is metabolized quickly in rat, dog, and human liver microsomes (Millan et al., 2011). The compound showed a good pre-clinical safety profile and is reported to be in Phase I clinical trials (Millan et al., 2011). Its selective p38 inhibition was demonstrated first by its potent inhibition of pro-inflammatory cytokine release measured by inhibition of TNF-α release after LPS stimulation in human isolated PBMCs (Millan et al., 2011). Secondly, inhibition of LPS- induced lung neutrophilia in rats has been reported (Millan et al., 2011).

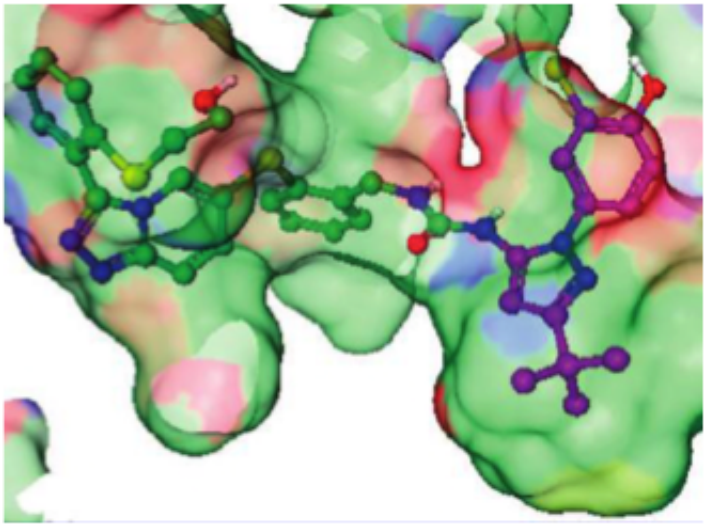

Figure 5.1 Binding site surface of p38 map kinase inhibitor PF-0715455 in p38α co-crystal structure. The region of PF-0715455 (highlighted in purple) is binding into the pocket created by the DFG loop movement. Taken from Millan et al. (2011).

5.1.3 PHA structure and Properties

PH-796804 was identified as a potent, highly selective, ATP-competitive inhibitor of human p38α kinase that has high safety profile pre-clinically and clinically. Binding of PH-796804 to p38α kinase inhibited both its phosphorylation and activity (Xing et al., 2009) (Figure 5.2).

The compound is reported to have completed phase II clinical trials in COPD and it is still in Phase II development for the treatment of other inflammation-mediated diseases such as neuropathic pain and rheumatoid arthritis (Xing et al., 2012, Goldstein et al., 2010). It was demonstrated to inhibit both p38 phosphorylation and the phosphorylation of the downstream substrate hsp-27. *In vitro* experiments in the human pre-monocytic cell line U937, primary human monocytes and human whole blood monocytes demonstrated a selective inhibition of LPS-stimulated production of both TNF-α and IL-1β in a concentration-dependent manner. *In vivo* animal models showed the oral efficacy and potency of PH-797804 as an inhibiter of cytokine production. This was demonstrated by the compound's ability to inhibit LPS-induced TNF-α production in rats and cynomolgus monkeys with comparable efficacy (Hope et al., 2009).

In human trials, PH-797804 suppressed LPS-induced cytokine production in a double blind, placebo controlled, single-dose study. This was demonstrated by decreased TNF-α and IL-6 production in a dose dependent manner in healthy human subjects. More recently PHA-797804 reduced airway neutrophilic inflammation after LPS challenge in healthy subject (Singh et al.,

2015). Furthermore, the compound showed encouraging results in suppressing chronic inflammation in rat streptococcal cell wall-induced arthritis models (Hope et al., 2009).

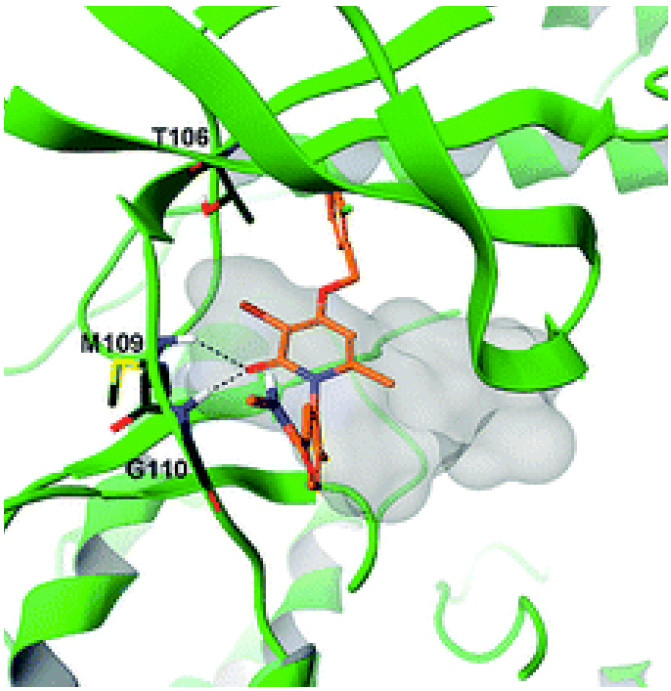

Figure 5.2 Molecular structure of p38 inhibitor PH-797804. The binding interaction is illustrated by crystal co-structure with the p38 kinase domain. Hydrogen bonds are drawn as dotted lines. Taken from Xing et al. (2009).

There is, however, a limitation to the value of human trials utilising LPS as a stimulus. For example, LPS induced cytokine release *in vivo* in human subjects means firstly that dose has to be carefully monitored for safety reasons, and secondly there is the potentiality of cross reactivity of cytokines masking the true response. Resulting in only a limited numbers of systemic cytokines could be measured.

The degree of agreement between animal and human mediator profiles following LPS stimulation has not been systematically studied by transcript studies, but any observed differences could explain the discord between animal models and human trials as far as proof of efficacy of other novel therapies. As previously mentioned, p38 MAPK was shown to mediate the release of many key mediators like TNF-α, IL-8, IL-6 and IL-1β, all of which are components of airway inflammation, and capable of inducing further inflammation. Therefore, the induced inflammation is clearly complex and requires careful analysis to determine the key mediators in this process.

An *ex vivo* lung model such as ours could be useful in further evaluating the mediator profile following LPS stimulation of intact lung tissues, and examining the activity of novel inhibitors

such as p38MAPK inhibitors in a localised and controlled *ex-vivo* exposure to LPS which presents no risk to patients. This allows the evaluation of efficacy of novel inhibitors prior to progression in clinical trials, and also enables the localised measurement of a vast array of cytokines in tissues. Such a model may allow us to elucidate the cellular mechanism that may be involved using this kind of therapy.

We hypothesised that an *ex vivo* lung tissue model has the potential to further characterise the pharmacokinetics of p38 mapkinase inhibitors in a fully humanised testing system, and we therefore aimed to use our human lung explant model to enhance information and the likely effectiveness of P38 Map kinase inhibitors.

The aims of this chapter are:

- To assess the pharmacological activity of the two different P38 Map kinase inhibitors described above.
- To determine whether they block LPS- induced cytokine production equally in tissue from control patients and individuals with COPD.

The objectives of this chapter are:

- Find out whether the PF and PHA can inhibit LPS-induced TNF-α production in human lung tissue.
- Determine the inhibition profile of both inhibitors in human lung parenchyma explants using this model
- To compare the efficacy of the compounds in human lung tissue with monolayer cell cultures.
- Compare the results obtained with the two novel inhibitors to that of Fluticasone propionate, the one of the current frontline therapies for COPD.

5.2 Methods

Our human lung explant model was used to investigate the efficacy of two novel p38 Map kinase inhibitors in inhibiting TLR-driven inflammatory cytokine release. As previously mentioned, the inhibitors were formulated for oral (PF-0371545) and systemic (PHA-797804) application. The corticosteroid fluticasone propionate, a steroid commonly used in topical formulations in COPD and asthma, was included as a positive control. Novel compounds were applied prophylactically 2 h prior to stimulation of the tissues with LPS, and were present throughout the culture period. The drug efficacy was measured using resected human

parenchymal tissue samples from 12 patients undergoing carcinoma resection at Southampton General Hospital (patient characteristics are described in (Table 5.1).

Table 5.1 Patients characteristics for resected parenchymal tissue samples used in drug efficacy analysis. Basic clinical data was collected for each patient. . Patients with a FEV_1/FVC ratio of < 0.7 were classified as COPD. Those with FEV_1/FVC ratio of ≥ 0.7 were defined as Control patients. Age, FEV_1/FVC, FEV_1% predicted, smoking status, pack years and years ex are shown as the median and quartile range. For gender, M = male and F = female. Under smoking status, "current" denotes current smokers, "ex" denotes ex-smokers as determined by the patient's clinician, "never" denotes never smoked. Non parametric Mann Whitney U test showed no statistical difference between the group in age and pack years.

	Control	COPD
Age (years)	69 (63.5, 75.25)	63.54 (60, 70)
Gender	3M/3F	4M/2F
FEV_1/FVC	0.74 (0.73, 0.76)	0.59 (0.49, 0.66)
FEV_1% predicted	104.8 (95.67, 111.2)	54.4 (42.04, 73.9)
Smoking status	3 EX/1 Current/2 Never	4EX/2 Current
Pack years	18 (10.5, 31.5)	35 (30, 43.7)
Years EX	28 (19, 37)	6 (3.2, 11)

5.2.1 Inhibitor preparations

The recorded aqueous solubility of PF-03715455 inhibitors was 1.2 µM and that of PHA-797804 was 4.3 µM (data supplied by Pfizer). Thus they had to be prepared in a way that reduced the chance of precipitation when added to aqueous solutions such as the tissue culture medium used for explant culture, where the final concentration of DMSO had to be at a maximum of 0.1% (v/v) to minimise cell toxicity. In order to achieve this, the inhibitors were both stored as 10 mM stock solutions in DMSO at -20^{o}C and then pre-diluted just before use in DMSO. The final dilution was made in culture medium such that the final DMSO concentration was 0.1%.

Where dilution series were made (0, 1, 5, 10, 50, 100 and 500 nM for both drugs), dilutions were made in DMSO prior to a final dilution in culture medium so that the concentration of 0.1% (v/v) DMSO was constant. Carrier controls contained DMSO at the same concentration without additional drugs. Once diluted, inhibitors were kept for the minimum time possible on ice prior to application to explant cultures to reduce the chance of precipitation. Similarly, fluticasone propionate stocks were prepared in DMSO at a concentration of 10 mM and prepared so that the final DMSO concentration was also 0.1% (v/v).

5.2.2 Human lung parenchyma explant assays

Resected lung tissue obtained from patients undergoing surgery for lung cancer was prepared as previously described in section 2.2. Two h prior to LPS stimulation, explants were exposed prophylactically to a dilution series of each drug in 1 ml of culture medium for 2 h in normal culture conditions. 10 nM of fluticasone propionate in 0.1% (v/v) DMSO was used as positive control. The inhibitors were added 2 h before the addition of LPS to allow for their interaction with tissue.

After 2 h of prophylactic exposure to the drugs/steroid, tissues were stimulated by the addition of 100 ng/ml LPS per well, and incubated for a further 24 h in the presence of the inhibitors. Control wells were exposed to carrier control (RPMI culture medium) rather than LPS. At the end of the experiment, the tissues were then weighed and the supernatants were collected and stored at -80°C for later analysis as previously described in section 2.2. Figure 5.3 shows diagrammatic representation of the experiment.

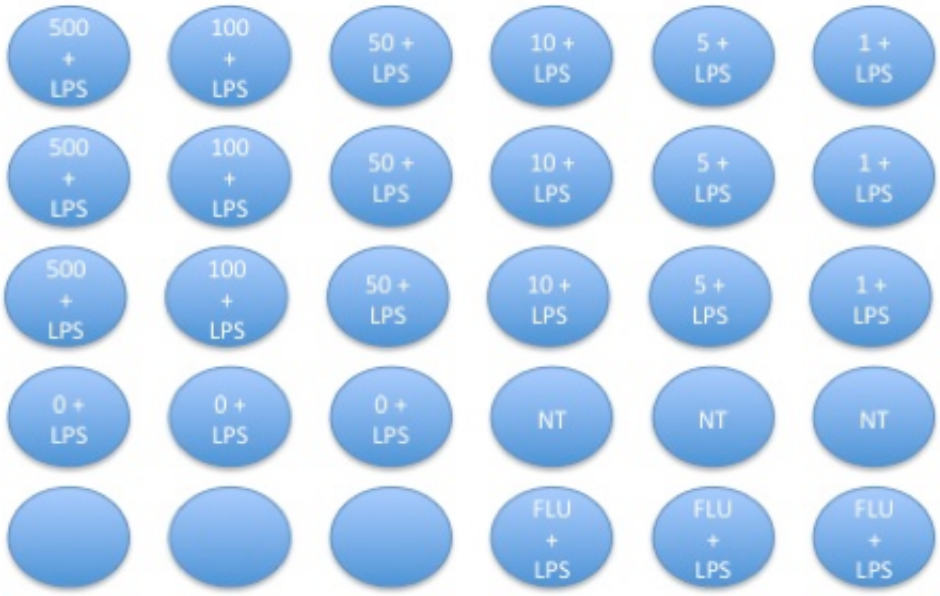

Figure 5.3 Experimental plan of inhibitor dose-response experiments. One ml of pre-made RPMI with various relevant concentrations 500, 100, 50, 10, 5, 1 and 0 nM of either drug (PHA and PF) was placed in each well of a 24-well culture plate in triplicate. 3-5 tissue explants were added to each well and then left for 2 h before the addition of 100 ng/ml of LPS. A negative control (no LPS, no drug) and a positive control (LPS + 10 nM Fluticasone propionate) were also added. Tissue-conditioned culture medium was collected after 24 h centrifuged to remove cells as described in the Methods section, and stored for subsequent analysis by ELISA.

5.2.3 Quantification of cytokine section by LPS-exposed explants in the presence of inhibitors

Cytokines secreted over 24 h by the lung parenchyma tissue explants treated as previously described were quantified in tissue-conditioned media by multiplexed ELISA using pre-coated ELISA plates (MSD) as previously described in section 2.4.

5.2.4 Data analysis

Six tissues that were found to be unresponsive to LPS stimulation (measured by whether TNF-α and IL-10 were elevated following LPS-exposure of the tissues) were excluded from the analysis. Dose dependent inhibition of cytokine release was then determined in LPS-responsive tissues by plotting the mean (of duplicate) weight-normalised cytokine concentrations (pg/mg/ml) against drug concentration for each individual. In the unresponsive tissue the baseline values of TNF-α

in the supernatants were higher than those treated with the inhibitor and therefore excluded from the calculations.

Curves were fitted using nonlinear four–point logistical regression. IC50 values were calculated using GraphPad prism software version 5 and those values were used to calculate the mean IC50. The IC50 is the drug concentration at which a 50% inhibition of the maximum mediator concentration can be observed, and in this case, computer software was used to calculate the IC50 values. To calculate the IC50 for each compound, dose-response curves were constructed to incorporate a range of concentrations that incorporated the IC50s calculated using monoculture systems (data supplied by Pfizer), which was known to be in the range of 10-50 nM for both inhibitors when tested on LPS-treated PBMCs. The dilution series values ranged from a concentration that was 10 fold lower than the lowest predicted concentration to a concentration that was 10 fold higher than the highest predicted IC50 value (1-500 nM). The amount of TNF-α released was plotted against the log of the drug concentration and nonlinear curve fit was then applied (see section 5.2.3)

5.3 Results

As p38 regulates the production of cytokines such as TNF-α, the compounds used in this study were profiled by assessing their efficacy in the inhibition of LPS-stimulated TNF-α production using human lung parenchyma explants cultured *ex vivo*. Efficacy is defined as the maximum effect that a given drug will produce regardless of the dose. Potency is defined as the amount of a given drug that is required to give a given effect. IL-10 production was also quantified as a measure of anti-inflammatory mediator production, however it did not give good result to draw graphs.

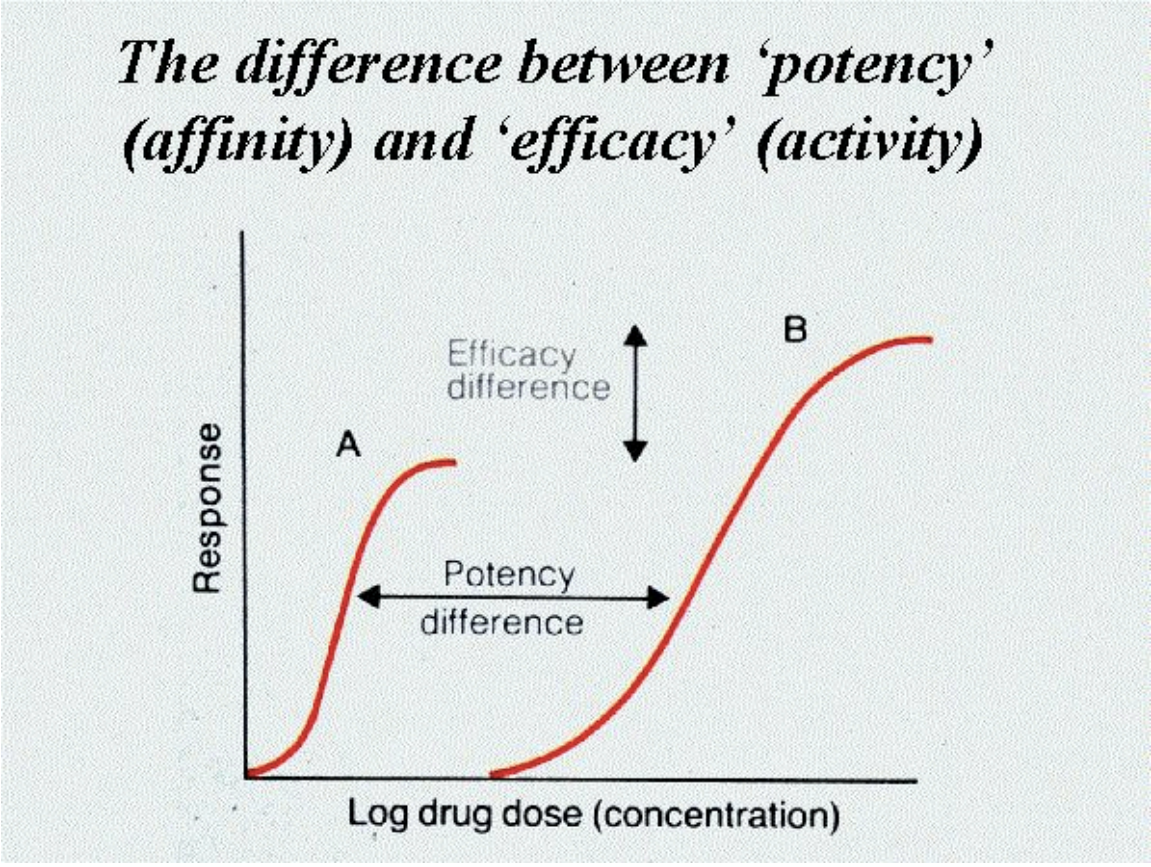

Figure 5.3 Difference between efficacy and potency (taken from: http://www.medbullets.com/step1-pharmacology/7007/efficacy-vs-potency).

5.3.1 Individual dose response

An example of a dose-response curve for the tissue samples from one patient can be seen in Figure 5.4.

5.3.1.1 Dose response for PF-03715455

The dose response for PF-03715455 was examined using lung tissue samples from 12 donors. However, Seven samples did not produce measurable IC50 values using the analysis software and consequently were not included in the calculation of the mean IC50 value. Figure (5.4) is a representative graph of a good dose response curve. The rest of the individual graphs are shown in (Appendix A6).

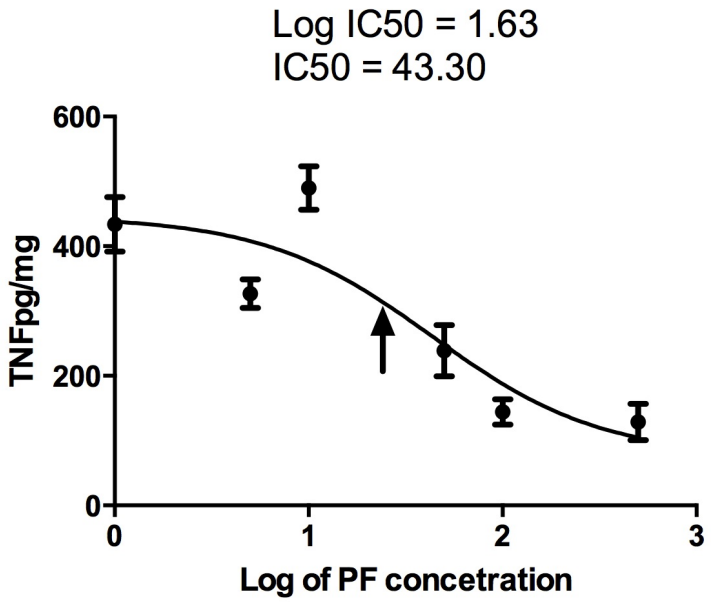

Figure 5.4 Dose response of the PF compound to examine inhibition of LPS-induced TNF-α release. Graph shows a typical inhibition curve for one donor after 2 h prophylactic treatment with the compound at various concentrations: 500, 100, 50, 10, 5, 1 and 0 nM and subsequent stimulation with LPS in the presence of inhibitor for a further 24 h. Concentrations are expressed as mean (+/- standard deviation) pg/mg tissue. Curve was interpolated by 4-point logistical regression to obtain the IC50 value (GraphPad Prism softward, v 5.2), see Methods for details.

5.3.1.2 Dose response for PHA

The efficacy of PHA on inhibiting LPS-induced cytokine release from tissue explants was also measured. Figure 5.5 shows a representative dose-inhibition profile from one individual. Five samples produced graphs from which IC50 values could be quantified. Some of data sets were excluded as the tissue lacked an LPS mediated increase in TNF-α production. The rest of the individual graphs is shown in (Appendix A5).

The table below shows the individual IC50 the mean for both inhibitors and the standard error of the mean SEM.

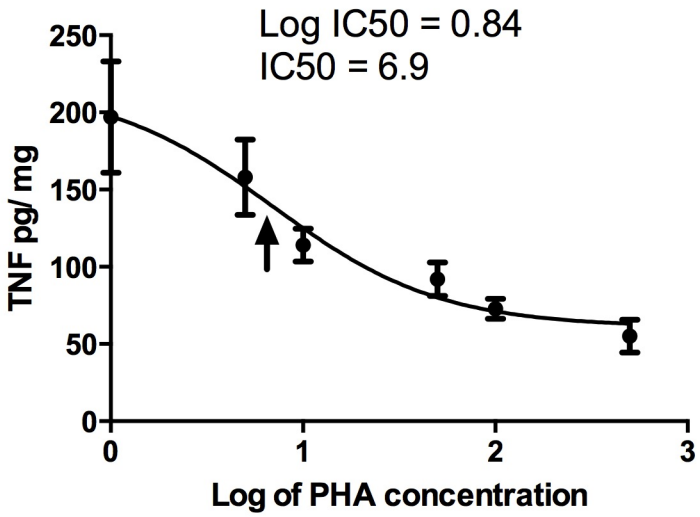

Figure 5.5 Dose response of the PHA compound to examine inhibition of LPS-induced TNF-a release. Graph shows a typical inhibition curve for one donor after 2 h prophylactic treatment with the compound at various concentrations 500, 100, 50, 10, 5, 1 and 0 nM and subsequent stimulation with LPS in the presence of inhibitor for a further 24 h. Concentrations are expressed as mean (+/- standard deviation) pg/mg tissue. Curve was interpolated by 4-point logistical regression to obtain the IC50 value (GraphPad Prism softward, v 5.2), see Methods for details.

Table 5.2 Individual IC50 the mean for both inhibitors and the standard error of the mean SEM.

	PHA	PF
	16.88	43.5
	155	5.11
	4.07	10.63
	14.21	11.1
	6.9	148.3
Mean	39.4	43.7
SEM	29	27

5.3.2 Absolute and % maximum TNF-α inhibition by both inhibitors

5.3.2.1 Absolute TNF-α inhibition by PHA and PF

PHA and PF inhibition of TNF-α was tested at six different concentrations in triplicate plus the control. Figure (5.6 (a) and (b)) shows absolute TNF-α at (1 nM, 5 nM, 10 nM, 50 nM, 100 nM and 500 nM) fluticasone concentration was 10 nM.

The effect of these concentrations on TNF-α inhibition was very different between the two inhibitors. PHA inhibited LPS-induced TNF-α secretion significantly at and above 5 nM (P = 0.0156) (Figure 5.6 (a)). The mean IC50 was 39.4 nM. As the concentration of PHA increased to 50 nM even higher significant inhibition of TNF-α with P = 0.0078 was observed. A further increase of PHA to 100 nM and 500 nM also resulted in significant TNF-α inhibition P = 0.078, consistent with the IC50 measurements.

PF-03715455, however, did not significantly inhibit TNF-α production even at high concentrations. This lack of inhibition is consistent with PF-03715455 being formulated for use as an inhaled inhibitor and a much higher concentration may be needed to see significant effect. PF-03715455 had an IC50 of 43.7.

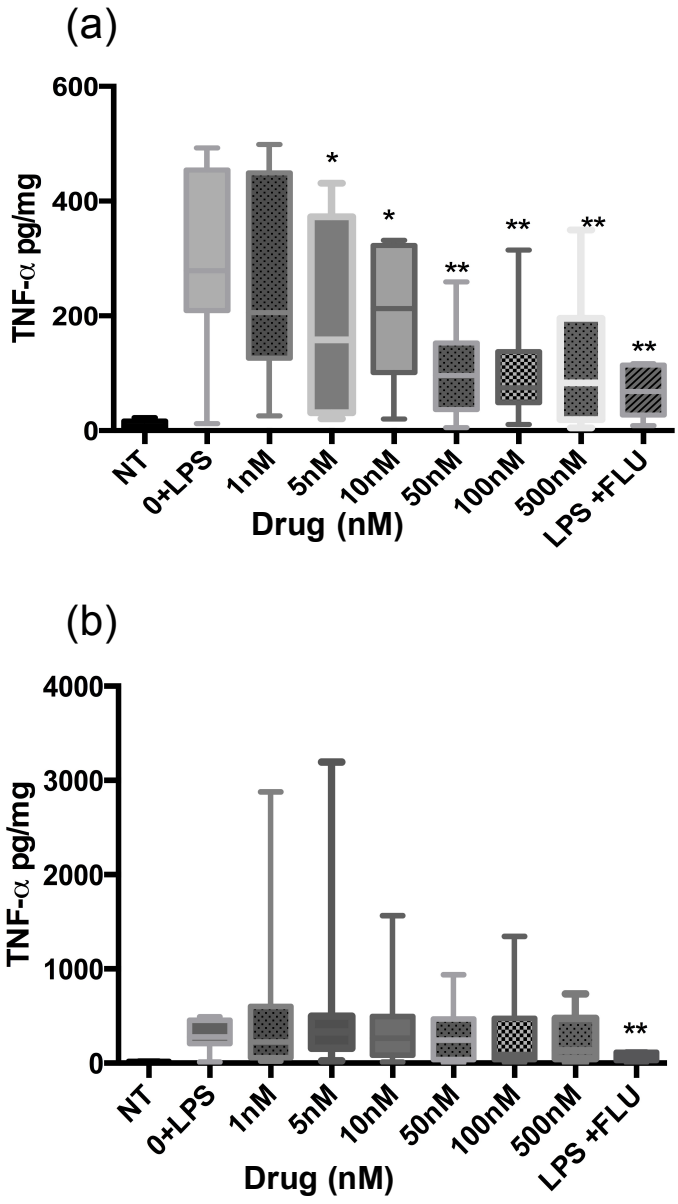

Figure 5.6 Inhibition of pro-inflammatory cytokine release from lung tissue explants stimulation with LPS. The quantity of TNF-α secreted into the tissue-conditioned medium following stimulation of lung tissue explants as described in Figures 5.6 and 5.7. Positive inhibitor control was 100 ng/ml LPS + 10 nM fluticasone propionate (LPS + FLU), negative inhibitor control was LPS alone (0 + LPS). Specific dose-inhibition response (1 nM, 5 nM, 10 nM, 50 nM, 100 nM and 500 nM for 2 h followed by LPS stimulation then incubated for 24 h) after PHA (a) and PF (b) treatment are shown. (n = 6), Box and whisker plots denote median TNF-α concentrations (pg/mg tissue), interquartiles and range. * denotes $p < 0.05$ when compared to LPS-stimulated containing carrier control (0 + LPS). ** denotes $p < 0.01$ when inter-treatment comparisons were made using Wilcoxon signed-rank test.

5.3.2.2 Percentage of maximum % of TNF-α inhibition by both inhibitors

As well as demonstrating inhibition in terms of absolute concentration of the inhibitors, data were also analysed as a percentage of the maximum inhibition to reduce the effect of variability in absolute values on the data. Figures 5.7 (a) and (b) show the amount of TNF-α released expressed as % of the maximum inhibited compared to the maximum of TNF-α released when the tissue was stimulated with LPS alone. Expressing the result this way confirmed the result shown in the previous sections. Significant TNF-α inhibition was noted using PHA-797804 from 50 nM with $P = 0.015$ (Figure 5.7 (a)). However, we did not see any significant inhibition of LPS-induced TNF-α release using PF-03715455.

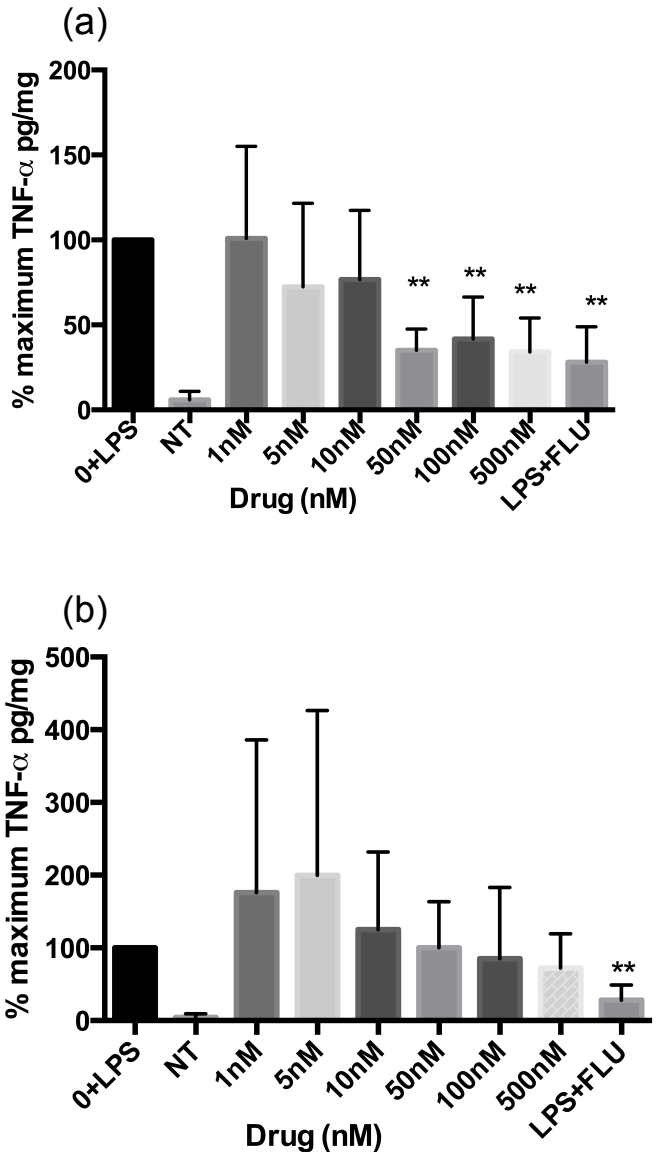

Figure 5.7 % maximum of TNF-α release from lung tissue explants stimulation with LPS. The quantity of TNF-α secreted into the tissue-conditioned medium following stimulation of lung tissue explants as described in Figures 5.6 and 5.7. Positive inhibitor control was 100 ng/ml LPS + 10 nM fluticasone propionate (LPS + FLU), negative inhibitor control was LPS alone (0 + LPS). Specific dose-inhibition response (1 nM, 5 nM, 10 nM, 50 nM, 100 nM and 500 nM for 2 h followed by LPS stimulation then incubated for 24h) after PHA (a) and PF (b) treatment are shown. (n = 6), Box and whisker plots denote mean percentage of maximum (negative inhibitor control, 0+LPS) TNF-α concentrations (pg/mg tissue), interquartiles and range. *denotes $p < 0.05$ when compared to LPS-stimulated containing carrier

control (0 + LPS). **denotes $p < 0.01$ when inter-treatment comparisons were made using Wilcoxon signed-rank test.

5.3.3 Effect of fluticasone

Parenchymal explants were also incubated with fluticasone as a positive control (10 nM) for 2 h before LPS was added and tissue left for 24 h. Fluticasone inhibited LPS-induced TNF-α production significantly ($p = 0.0078$). This is consistent with previous results from our lab where the same concentration inhibited virus-stimulated TNF-α release (Nicholas et al., 2015). Fluticasone has been reported to have high lipophilicity and therefore has larger volume distribution (Latorre et al., 2015). That could have contributed to the better results observed with FP when compared with the inhibitors examined in this chapter.

5.4 Discussion

Although corticosteroids, alongside bronchodilator therapy, are a mainstay treatment for COPD, there is much evidence to suggest that there is steroid resistance in this disease group (Burge et al., 2000, Calverley et al., 2003, Pauwels et al., 2001). Long term oral steroid use has considerable side-effects, that include suppression of the adrenal response (Henzen et al., 2000) increased fracture risk, hypertension and fluid retention (Vestergaard et al., 2007, Walters et al., 2014) and whilst these are reduced in topical applications using inhalers, are still thought to have some deleterious side-effects like increased risk of pneumonia (Singh et al., 2009), providing a rationale for finding alternatives to corticosteroids which could be used either separately or in conjunction with the current therapies. Among the many different novel approaches to COPD therapy, the use of p38MAPK inhibitors as a class of immunosuppressant is well developed as a concept, but not yet in use as a frontline therapy for COPD.

This chapter has sought to find out if the lung parenchyma explant model can be used to study the effect of inhibitors such as p38MAPK inhibitors *ex vivo*, which would proof of efficacy prior to *in-vivo* trials. This study firstly aimed to establish the effective inhibitory concentrations of two different p38 inhibitors intended for different application routes, one topical and one systemic. In the human lung parenchyma explant model LPS stimulated production of TNF-α was inhibited by PHA-797804 in concentration dependent manner for most subjects, with an IC50 value of 39.4 nM.

Data from a previous study (Hope et al., 2009) showed that the IC50 for the PHA-797804 in LPS stimulated human monocytes and U937 cells was in the low nM range (3.4 and 5.9 nM). The

difference between these values may reflect the complexity of cell interactions and also drug:protein interactions in *ex vivo* cultures as opposed to cell monocultures, as it probably better reflects the physiological *in vivo* milieu compared to standard culture systems. Whilst the culture medium used for *ex vivo* culture was serum-free, and therefore mostly excluded the binding effects of serum albumin, the tissue may still contain considerable quantities of tissue fluid even after the extensive rinsing necessary for inhibitor analyses, giving potential for significant quantities of proteins with high drug binding capacities (such as serum albumin) to be present. These "contaminating" proteins could interact with test therapeutic agents and either inhibit or increase their observed potency.

The use of culture medium with defined protein content (e.g. human serum albumin or alternatives) could be used to standardise protein concentration in the cultures, and could theoretically improve secreted mediator quantification by reducing binding to the culture plastic. The use of carrier protein in tissue cultures is well established, but was omitted from our study in order to minimise unwanted interactions between LPS, mediators and the novel therapeutics applied to the tissues.

Similarly, for the compound PF-03715455 (formulated for topical application as an inhalant) reduced potency in terms of IC50 was observed in lung tissues when compared to PBMC experiments (< 10 nM) (Millan et al., 2011). Similar effects of the culture conditions on the bioavailability of the inhibitor could have caused this effect, although it is likely that the poor solubility of this compound contributed to its lack of efficacy in our model, despite efforts to maintain the compound in solution

In addition, the high IC50 value in tissue for both inhibitors could also reflect the complexity of cell populations capable of secreting such mediators in donor tissues. Recently, Gaffey et al. (2013) reported that lung neutrophils did not have p38 Map kinase immunoreactivity and their stimulation with LPS did not activate p38 while it led to cytokine release. This suggests that p38 inhibitors are unlikely to have an effect on cytokines released from neutrophils. However, in the same study p38 inhibition in isolated COPD lung CD8 cells and epithelial cells reduced cytokine production.

We noted significant variability in tissue response to LPS stimulation (see also chapter 4). It is not currently clear whether this reflects biological differences in terms of inflammatory cell load, cell activation status or therapeutic status (for example, some patients may be taking oral steroids for co-morbidities as well as for COPD), but there were insufficient numbers of tissue samples for us to make safe conclusions as to whether CS use carried over into explant cultures.

Recent evidence from bronchial biopsy explants suggests that inhaled corticosteroids can have carry-over effects in tissues collected by bronchoscopy when cultured *ex vivo* for 48 h (Nicholas, B personal communication), but further investigation of the time needed for "washout" of steroids from tissue explants is warranted.

Furthermore, the time between surgical excision and laboratory processing of the tissues, whilst minimised where possible, was quite variable between patients. It is possible that, in the time between when the tissue was removed and when it was used, that the cells within the tissue may have suffered from the hypoxic environment. Our standardised method was to store the tissue in room temperature saline for transit prior to processing. Cold buffer was not used, so as to minimise the potential for induction of cold-shock proteins and other antigen presentation effects which could affect lung-resident cells analysed by flow cytometry, however the lack of cold storage could have altered our results in terms of cell viability.

The lack of adequate buffering capacity in saline, when compared to many commonly used fluids for organ transport could also have affected tissue viability and responses. Alternative buffers for transportation could have included tissue culture medium or even PBS. We did not use these because PBS can be toxic to tissues, especially when re-mixed with buffers containing calcium such as tissue culture media. Culture media were not used due to the potential for contamination with microbial organisms for which these media are ideally suited, however the best tissue storage medium would have to be determined empirically in future studies.

Lung epithelium in the alveolar compartment receives oxygen direct from the external environment, and also from tissue capillaries supplied by the bronchial circulation. There is no blood supply to the tissue when the lungs are removed, and there are also temperature changes and others changes in, for example, pH when tissue is surgically removed. Thus cell homeostasis may have been slowed down or impaired following tissue excision. Cells with short life such as neutrophils may die more quickly than other cell types, or could suffer delayed cell death (Scheel-Toellner et al., 2004). Also epithelial cells get blood supply from blood capillaries and are more vulnerable to blood deficiency, therefore, would suffer from a lack of oxygen thus part of the tissue may die from hypoxia.

Aside from tissue viability, another possible reason for low tissue responsiveness to LPS stimulation is that the tissue may not have the right cells for it to respond. Macrophages co-ordinate inflammation in the lung through antigen presentation and also inflammatory mediator release, and observed no significant reduction in these cells in the COPD group (see chapter 3). However, from the samples that responded to LPS only two were from the COPD

group and the rest from the control group, and due to the low representation of identifiably control or COPD individuals, it was not possible to examine the effect of disease status on inhibitor responses.

The relationship between oxidant stress and cytokine response has been eluded to previously. Smoking (either historical or current) could impact on LPS-induced responses. The disadvantage of using lung tissues from smokers is that potentially the by-products of smoking could still be present within the lungs and affect downstream experimental results. Indeed we have often observed that lung tissue from current smokers contains tar, and purified alveolar macrophages from lung tissues can be heavily loaded to smoke particles. Such particulate-loaded macrophages are thought to represent the M2 macrophage phenotype, demonstrating reduced responsiveness to pro-inflammatory stimuli (Shaykhiev et al., 2009, Hiemstra, 2013).

We observed that the demographics of the control and COPD patients included in our study were different, even though not significantly, in terms of smoking habit and/or years since stopping smoking. The COPD group seemed to have smoked more while control group had an average of 18 pack years the COPD group had an average of 35. It is possible that smoking caused some erratic LPS response in some subjects.

We did not do a separate test for tissue or cell viability for the work in this chapter, as flow cytometry was not performed for every tissue sample tested. Although our initial intention had been to quantify lactate dehydrogenase (LDH) activity in the culture supernatants from the tissues as a measure of cytotoxicity as this enzyme is released by necrotic cells, this methodology has not been validated for use with lung tissue previously and is subject to several drawbacks, principally that tissue is a complex collection of multiple cell types. Each individual cell type may contain very different intracellular concentrations of LDH, meaning that any results would have to be adjusted in some way to account for the contribution of individual cell types. LDH activity is generally measured as a ratio of “test” sample to “total” sample generated by lysing the whole cell population at the end of the test. Whilst it would be possible to generate a positive sample by lysing tissue cells using sonication, preliminary data from our laboratory has indicated that different cell types within the tissue contain widely variable amounts of LDH, meaning that the total LDH value would be influenced by a sub-group of the cells present in the tissue, and would not necessarily reflect overall toxicitiy or the effects on individual cell types.

In addition to this, several cells resident within tissue produce oxidant molecules that can affect the LDH assay, in particular granulocytes and mature macrophages, both of which are present in

lung parenchyma, and produce oxidative intermediates that interfere with LDH activity and invalidate the test for use in this culture system. Therefore, we do not have any information about the toxicology of the inhibitors when applied to lung tissues. In future studies, we would aim to perform specific flow cytometry studies to determine cell viability in each individual tissue sample, which might indicate a relationship between LPS-induced cytokine production and tissue viability, bearing in mind that tissue processing for flow cytometry itself could affect cell viability.

Whilst it is theoretically possible to measure cell viability using flow cytometry, our initial attempts have shown that whilst it is relatively straightforward to determine "live" cells from digested tissue as a positive population, dead cells are harder to distinguish from non-cellular tissue material using the available reagents.

The anti-inflammatory effect of PH-797804 was stronger than that of PF-03715455. Figure (5.6) (a), (b)) and, figure (5.7 (a), (b)) show that PHA inhibited TNF-α significantly from 50 nM while PF did not inhibit TNF-α significantly. It is possible that due to the maintained matrix of tissue and presumed low intrinsic permeability as reported by Millan et al. (2011), PF-03715455 could not penetrate the tissues, and so very little anti-inflammatory effect was observed, especially when compared with PHA-797804 which showed a much lower IC50 value.

Alternatively the lower potency of the IC50 value for PF-03715455 of 43.6 nM and the lack of a significant TNF-α inhibition that was observed at the concentration used compared to that of PH possibly suggest a solubility issue. Both inhibitors were kept in DMSO to try and keep them as soluble as possible until the onset of the experiment. However, it has been reported that the PF-03715455 compound had no detectable solubility in water or in simulated lung fluid (SLF). And later with improved properties it demonstrated in whole cell PBMC experiment a solubility level that was above that of fluticasone (Millan et al., 2011). However, because of its low solubility we may assume that it has low level of intrinsic cellular permeability.

PF-03715455 was shown to have slow dissociation kinetics against the p38 α enzyme, which translated into a relatively long duration of action in *in vivo* experiments. This would lead to a decrease in the IC50. Furthermore, tissue has tissue fluid that has protein and inhibitors bind strongly to albumin making it unavailable for cell penetration. Hope et al. (2009) reported that PHA is an inhibitor that has a strong plasma binding properties and only unbound compound is available to penetrate the cell. They further reported that while TNF-α production was inhibited in the whole blood with an IC50 value of 85 nM, this IC50 value was only 2.8 nM based on the

free fraction of the compound. As mentioned before PF-03715455 has a low intrinsic permeability.

Finally, the pharmacological characteristics of these inhibitors could have influenced these results. Oral inhibitors are often selected on the basis of high potency to account for losses in local concentrations associated with systemic delivery. Systemic delivery can however lead to enhanced side-effects in non-target organs such as the liver or kidneys. Some side effects reported in regards to PHA in a phase II clinical trial include nasopharangitis and a case of gastrointestinal haemorrhages (MacNee et al., 2013). There are also reports of a lack of sustained efficacy in disease reported for some of drugs used in RA (Cohen et al., 2009, Alten et al., 2010) with significant decrease in inflammatory biomarkers including CRP in the first weeks, but this was not sustained during the whole trial period of 12 weeks. However, for PHA (MacNee et al., 2013) reported significant reduction in CRP that was maintained for the duration of the six weeks trials.

Inhaled inhibitors are more localised, therefore the gastrointestinal, liver and central nervous system are less exposed. However, local concentrations are not easy to control, and specialised formulations have to be developed to allow delivery of such therapeutics via aerosol routes, such development is costly (Brocklebank et al., 2001, Virchow, 2004, Friebel et al., 2012). Often this route is chose for inhibitors with poor solvency characteristics, rather than out of choice for delivery method. Recent studies by Singh et al. (2015), Friebel et al. (2012) using both p38 map kinase inhibitor PF-03715455 and PHA-797804 confirmed the potency of the latter over the inhaled inhibitor. PHA had a greater impact on airway neutrophilic inflammation compared to PF after healthy subjects were challenged with LPS.

Steroids are used in the treatment in COPD, and fluticasone propionate (FP) was used in this study as a positive control. Fluticasone is a common corticosteroid receptor agonist given to COPD patients in inhaler form. The dose of 10 nM applied to lung tissue explants to inhibit LPS-induced TNF-α has been demonstrated to be efficacious in other lung explant models without evidence of toxicity (Nicholas, 2015). Figure 5.6 and Figure 5.7 show TNF-α inhibition by fluticasone (10 nM) after LPS stimulation in the parenchymal tissue of 6 subjects.

Fluticasone significantly inhibited TNF-α ($p = 0.0078$). It has been shown previously that COPD is steroid resistant in a wider population. Our finding that it was effective in our explant model in all subjects may reflect the high bioavailability of the CS in this system, but also might be due to the fact that 10 nM is on the upper limit of predicted local concentrations in lung tissue (Brandsma et al., 2013). This dose was used due to empirical calculation of CS efficacy using the

lung model with LPS stimulation (Warner, J personal communication). CS would also be predicted to act principally within the bronchial compartment *in vivo* as it is designed to relieve bronchial inflammation, dependent upon the efficiency of inhaler delivery. Thus the localised concentration of FP determined in the alveolar compartment may not be a reliable measure of efficacy or optimal FP concentration in this compartment and warrants further investigation.

It would also be interesting to investigate steroid resistance using lung tissue explants, however we recognise that it would require a larger cohort than the one studied here, and would require better understanding of co-morbidities and therapies in the cohort to account for systemic corticosteroid usage. It is possible that, if in this study we had had enough patients to split into two groups, i.e. control and COPD, or using multiple doses of CS, we would have seen a difference between the groups in terms of CS efficacy. Because of the wide range of COPD severities in our cohort, it is possible that disease severity may have had an effect on the result. Therefore, there may also be more substantial difference in the amount of inhibition if there were enough subjects to split according to severity of the disease. We also cannot rule out the possibility that *ex vivo* culture might restore CS sensitivity through removal of systemic pathways leading to suppression.

It would also be useful to analyse a combined treatment of steroid and p38 inhibitors. This can be achieved by culturing the tissue with or without steroid or p38 mapkinase inhibitors. Each steroid concentration can also be used with different concentration of inhibitors and a baseline control. This would allow for the evaluation of additive and synergistic interactions. The main analysis would be analysing cytokine release like TNF-α and IL-8.

All patient samples produced similar basal level of TNF-α. It was interesting to note that there is a detectable background level of secretion of TNF-α from lung tissues without additional stimulus. This may have been due to the trauma to the tissue associated with removal from the patient, processing, or culture conditions. However, the maximum level of LPS-stimulated TNF-α production was highly variable between the patients. Only those samples that responded to LPS were chosen, as we chose to use this as a surrogate marker for tissue viability. It could have reflected some kind of other effects that we do not understand, such as the overall expression of pro-inflammatory mediators, the presence of cell or humoural immune components, co-expression molecules on T-lymphocytes, total lung injury score, or an impairment of heamodynamics etc.

We do not know how long it took before the tissue was placed in the saline container after being removed from the donor. It is also possible that the time of stimulation after tissue

removal from the body is crucial to the activation state and LPS responsiveness in tissue. Higham et al. (2014) reported that long or short isolation procedures affect the corticoid inhibition of LPS stimulated cytokines from alveolar macrophages. In this study, tissues were "rested" overnight following dissection and prior to stimulation. This resting period was generally between 15-24 h, although stimulations and culture period post-stimulation were always for the same duration. If culture time before stimulation can indeed affect LPS responsiveness, we currently do not know if this is the case. Further work is needed to elucidate this effect.

The overall conclusion of this chapter is that of the two novel inhibitors tested one, PHA, was effective at reducing LPS-induced inflammation in the lung, whereas the other PF was not at all effective. The difference between these compounds may reflect different solubility levels known to be properties of these molecules. Neither was as effective as FP in reducing inflammation, however this work demonstrates for the first time that p38MAPK inhibition can control and COPD patients, with inhibitor profiles similar to, but not identical to, in vitro values. The lack of similarity between *in vitro* and *ex vivo* studies could reflect simple differences in experimental conditions, but may also reflect important biological differences between cultured cells and fully differentiated human tissues, and demonstrates the potential utility of the lung parenchyma explant model to add to the lexicon of pharmaceutical inhibitors testing platforms, as it provides a biologically relevant, fully human tissue model reflective of real disease pathology, with no increased risk to the participants of exposure to prospective novel therapeutics.

In rheumatoid arthritis, only modest clinical efficacy has been seen with oral p38 inhibitors. Data from clinical trial studies showed that prolonged p38 inhibition lead to an up-regulation of compensatory inflammatory pathway. This was supported by the fact that while initially there was a drop in an inflammatory marker C-reactive protein (CRP) observed that was not sustained after two weeks of dosing with the treatment (Goldstein et al., 2010) and therefore only a transient or no clinical efficacy (Cohen et al., 2009). Anti-inflammatory treatments could also potentially reduce protection from microbial infections, example steroid use can predispose to certain infections including fungal (Giannella et al., 2013, Cucchetto et al., 2015).

Despite these risks PHA-797804 has completed phase II trial for COPD and it was reported that the compound showed significant efficacy as measured by FEV_1 in the treated group when compared to the placebo group, as well as a meaningful increase in dyspnoea index (MacNee et al., 2013). As mentioned before several p38 kinase inhibitors were stopped because of poor safety profiles (Dominguez et al., 2005). Initial short-term efficacy was observed in some of the

compounds that advanced to testing in RA patients, but was later lost with further treatment possibly because of feedback control of the p38 kinase network (Hammaker and Firestein, 2010). Therefore it is possible that other upstream or downstream targets in the p38 kinase pathway may avoid this feedback loop while showing better safety.

5.5 Conclusion

Inhaled compound are probably a better approach to a disease of the lung as they can enhance the therapeutic window between efficacy and some of the common adverse events seen with oral p38 inhibitors. However, we did not observe effective inhibition of LPS-induced inflammation in lung tissues using the p38MAPK inhibitor formulated for inhaled delivery. This may have been due to the application of this formulation in a culture model, where the compound solubility may have been compromised. We have not yet developed methods of application of therapeutic compounds to lung tissue explants in the gas phase, however this would be an interesting addition to our model if it proved feasible.

A good level of inhibition of inflammation was observed using the compound formulated for oral delivery, which has a more favourable solubility profile. Inhibition levels nearly comparable to those of fluticasone propionate were observed. Significantly, this inhibition is via a mechanism independent of corticosteroid inhibition, and paves the way for further studies in which LPS responses are treated with p38MAPK and FP in combination, to see if the immunosuppression is additive or synergistic. Combination therapies are desirable as they might help to reduce the required corticosteroid dose, and thus the concomitant side-effects of long-term CS use.

Chapter 6: Conclusion

COPD is characterised by inflammation of the lung during the stable state and also during the periods of exacerbation. Bacteria have been found to play a major role during stable and exacerbation state, and *H. influenzae* has been frequently isolated and shown to produce inflammatory responses. This study has modelled events that take place during bacterial exacerbation in the lung parenchyma. To do that, an *ex vivo* explant model of human lung parenchyma using LPS and killed *H. influenzae* was developed. Live bacteria were not used as it would have been difficult to control growth.

Using both stimuli, we have shown that the model was able to cause an acute inflammation in the lung parenchyma. Along with the pro-inflammatory TNF-α increased release, we showed the increased release of the anti-inflammatory cytokine IL-10 in a biphasic manner in human lung parenchyma. This is a model that is reliable, more complex and physiologically relevant than simple monolayer cell cultures as it maintains both cell-to-cell and cell-to-matrix interactions. An *in vivo* model may have been superior, however it is unethical to perform such work *in vivo* in patients with obstructive lung disease. A disadvantage of this *ex vivo* model is the lack of blood circulation so it would not have been possible to look at cells that migrate after stimulation for example (*do novo* recruitment).

In Chapter 3 we quantified the inflammatory cell number using immunohistochemistry first and we did not find any difference between the groups in: Macrophages, eosinophils, mast cells, and neutrophils. There was an increase in T cell in the control group, however, a significant amount of zero value data was found with immunohistochemistry making that increase seem unlikely to be true. Using flow cytometry, we found that both the COPD and non COPD group have unaltered ratios of the structural and inflammatory cell types analysed, a similar pattern to that observed using immunohistochemistry. Flow cytometry is a method that allows for higher numbers of data points in cell analysis and therefore enables a better quantification of cell numbers and surface marker expression. A difference between the two groups using this method was in the tendency towards a lower number of macrophages in the COPD group that did not reach significance ($p = 0.09$).

Macrophages are very important cells in the pathogenesis of COPD as they release MMPs that can degrade collagen and elastin which are components of the extra cellular matrix and therefore causing tissue injury in COPD. They are also important in cigarette smoke derived particles phagocytosis and clearance of apoptotic neutrophils. Flow cytometry cannot distinguish between tissue compartments however, and so our lack of difference in cell

populations between non COPD and COPD patients may simply reflect the net overall effect of leukocyte trafficking from the circulation to the airway lumen, rather than those specifically lodged within the tissue itself. However, there are no other studies in lung parenchyma with which to compare our findings to date, as this tissue is difficult to obtain and analyse in non COPD and COPD patients.

While we quantified neutrophil using IHC, our early experiments using flow cytometry showed that they were sparse so we did not quantify them. This is probably due to the short life of neutrophils and possibly the smoking status of our patients. Active smoking can lead to an increase in neutrophils in sputum; however, in this study, the majority of the patients were ex smokers. More current smokers in the COPD group may have led to an increase in T cells.

The development of this model has also allowed us to investigate a whole range of cytokines (TNF-α, IL-10, IL-6, IL-8, Il-17, MCP-1, MIP-1β and IL-1β) in the supernatant from the explant after stimulation with both LPS and *H. influenzae*. These cytokines were chosen because of their importance in the COPD inflammatory cascade, and many have not been previously analysed in these tissues. Stimulation responses of these mediators were differential for each cytokine/chemokine.

LPS and bacterial stimulation caused significant increase compared to non treated for TNF-α, IL-10, IL-1β and MIP-1β for both groups. However, it only caused significant increase in the COPD group for IL-6 and MCP-1. IL-17 on the other hand did not show any significant increase probably because it needs longer incubation time as it is a cytokine that defends against bacteria and mediate cross talk between the adaptive and immune system (Kramer and Gaffen, 2007), but may have been present in low amounts simply due to the scarcity of TH17 cells.

One of the main differences seen between the two groups in term of cytokine measurement is the level of IL-8 that was significantly reduced in the control group, which is composed of a mixture of smokers and ex smokers. This is a significant finding considering that IL-8 is the main chemokine that recruits neutrophils to the site of inflammation: while the control group suppressed IL-8 production after LPS stimulation, the COPD group did not. Importantly, it is unlikely that macrophages are the only cells responsible for IL-8 release, as their number tended to be reduced in COPD group and other studies have reported diminished IL-8, TNF-α and IL-1β responses of COPD (Berenson et al., 2006). However, there is a possibility that the IL-8 result may not have been true result and just an assay variation, while the sample was diluted enough a higher fold dilution may still be required. We also have followed the GOLD guideline for COPD

diagnosis. Though, there may be an overlap as COPD patients mainly mild and at best moderate severity COPD while some of the control smokers probably had COPD.

TLRs have been named as one of the main pathogen receptors that are associated with inflammation in the COPD. As LPS and killed *H. influenzae* were shown to induce inflammation in this model of lung parenchyma explant, we looked to see their expression both at baseline and post-stimulation. Using IHC, TLR 2 and 4 were seen in cells that morphologically looked like macrophages on the alveolar surface, and when looked at using flow cytometry, there was higher intensity staining for TLR 2 compared to TLR 4. There was no difference between the groups in regards to TLR 2 expression on any cell type, whereas for TLR 4, a significant decrease was noted in $CD8^+$ T lymphocytes in the COPD group, which may indicate an immune dysfunction in COPD patients. This is the first systematic study of cell surface expression of TLR proteins on multiple cells types in the alveolar compartment of lung tissues from control and COPD patients, and paves the way for further in-depth analysis of TLR 4 deficiency in lung tissue-resident $CD8^+$ T lymphocytes in COPD.

Although we have not seen any changes in the expression of TLR 2/4 on epithelial cells and macrophages after LPS and *H. influenzae* stimulation, it cannot be ruled out that these stimuli have activated them at protein synthesis level, which in the short time-period analysed (24 h) might not have results in changes to the cell surface expression. A further investigation into the expression of these receptors, particularly in relation to time-course, in this population is required. The scarcity of lung tissues for analysis, and the stringency of our clinical parameters to phenotype patients as either non COPD or COPD, meant that smaller than ideal cohort sizes were analysed for this study. A bigger number would allow for investigation of patients at different stages of the disease, or maybe with specific phenotypes such as predominant bronchitis or emphysema, may have clarified any observed differences in relation to COPD subtypes.

As well as using stimuli, this model permitted the use of modifying substances like inhibitors. Since steroids do not halt the inflammatory process in COPD, p38 MAP kinase inhibitors have recently emerged after laboratory studies showed that their activation contributes to inflammation in COPD. These molecules were found to reduce neutrophil infiltration induced by inhaled endotoxin, reduce the level of certain cytokines and therefore represents a novel anti-inflammatory based on MAPK inhibition.

Because of their potential efficacy, p38 MAPK inhibitors could be very useful as they reduce the function of transcription factors like NF-KB, which are responsible for a wide range of

inflammatory diseases. Our *ex vivo* model allows the application of novel therapeutics prior to phase III studies, without risk to patients. We tested two novel p38 MAK kinase inhibitors in this *ex vivo* model, PF-0371545 formulated for inhaled application and PHA-797804 formulated for oral application. Fluticasone was used as a positive control as it is a current frontline therapeutic corticosteroid prescribed for COPD patients.

The oral inhibitor was found to be more potent than the inhaled PF-0371545. PF had a lower potency of the IC50 value and did not inhibit TNF-α at the concentrations used compared to that PHA. Inhaled inhibitors are however more desirable for a disease of the lung, as the resultant more localised exposure allows for a greater therapeutic window by increasing localised drug concentrations whilst avoiding systemic organotoxicity.

To conclude, we have shown in this study that LPS and *H. influenzae* are capable of inducing an inflammatory response from human parenchyma using an *ex vivo* explant model. Having established that those stimuli can cause the aforementioned response we can assume that these effects are happening because of the bacterial product activating TLRs, among other PRRs, on different cells in the lung parenchyma. However, exactly which cells are activated by TLR stimulation, and which cells are responsible for the different cytokine released require more in-depth investigations that are beyond the scope of this study. This could be done by sorting the different cell types from within the cultured lung tissue samples and analysing them.

We have established a model that will further contribute to our understanding of the mechanisms of inflammation in lung parenchyma during exacerbation. The model also allowed us to test two molecules that represent a novel anti-inflammatory therapy based on p 38 MAPK inhibition. These novel molecules may be able to attenuate disease severity as it has been proved to improve FEV_1 in COPD patients (MacNee et al., 2013), and therefore the testing of these novel compounds within our culture system provides an exciting new avenue of research for effective COPD therapeutics.

6.1 Future Work

Several questions have risen from this study, which require further investigation:

With regard to cell count, many of our patients were ex-smokers and it would be interesting to have sufficient number of current and ex smokers to compare with each other directly, as current smoking may affect a number of the parameters measured. 78% of the patients were ex smokers, many of whom had only been ex smokers for 6 months to 1 year. It is uncertain

whether this is long enough to affect the inflammatory processes quantified in our explant model

While we have shown that all inflammatory cells express TLR 4 on lung parenchyma, it will be of interest to identify the cells that play the main role in TLR 4 mediated LPS mediated inflammation in lung parenchyma, as they may be responsible for many of the cytokine abnormalities observed in chronic lung diseases driven by bacterial infections. A study by Berenson et al. (2014) established an association between NTHI induced IL-8 and the expression of both TLR 2 and 4 in alveolar macrophages, and it is possible that this may be true for other cells. We could investigate this by sorting the cells dispersed from lung tissues and stimulating them with LPS *ex vivo*, and then measuring the cytokine that have been released. Alternatively, intracellular staining of LPS stimulated lung tissues from explants for various cytokines/chemokines is technically possibly although it would require a significant amount of lung tissue.

A longitudinal study to look at the changes in the expression of TLR 2/4 throughout disease progression would also provide a wealth of information, as it may explain the more severe inflammation seen in the severe cases of COPD. Previous studies have reported impaired TLR 2 and 4 signalling in alveolar macrophages in exacerbation prone COPD patients. However, our lung tissues were obtained from patients undergoing lobectomies, mainly for lung cancer, and in order to qualify for surgery, these patients could not have such severe COPD that the surgery posed an immediate risk to their health, so our phenotype in the COPD group was, by necessity, representative only of mild-moderate status.

Another aspect that might be useful to look at in the context of COPD exacerbation is co-infections with viruses and bacteria, since antecedent viral infection can precede the development of bacterial exacerbation. There is much to be learned from whether viral infection can directly pre-dispose cells to bacterial invasion, or whether the subsequent increased susceptibility in COPD is due to aberrant inflammatory responses or other, as yet undefined mechanisms. Live bacteria could be also used if the right culture conditions could be established. This would give a better model of real-life bacterial infections, since killed bacteria, whilst being a useful model, may not induce all of the changes observed in *in vivo* bacterial infections.

This study has investigated two novel anti-inflammatory drugs in the form of p38 map kinase inhibitors. It is important to investigate this further by combining them with steroid. The latter are the most widely used anti-inflammatory therapy for COPD patients. However many patients

do not report improvement despite high-dose therapy (Soriano et al., 2007). Since both steroid and p38 MAP kinase inhibitors reduce inflammatory gene transcription (Kent et al., 2009), using different mechanisms, it is possible that combining the two treatments would result in an additive if not synergistic effect. Our development of the model also paves the way for testing of other, even more experimental compounds, where the model can act as a bridge between simple monolayer culture platforms and either animal or human studies, especially where a fully humanised testing platform is desirable.

Appendix A Supplementary Material

A.1 Chapter 3 Appendix

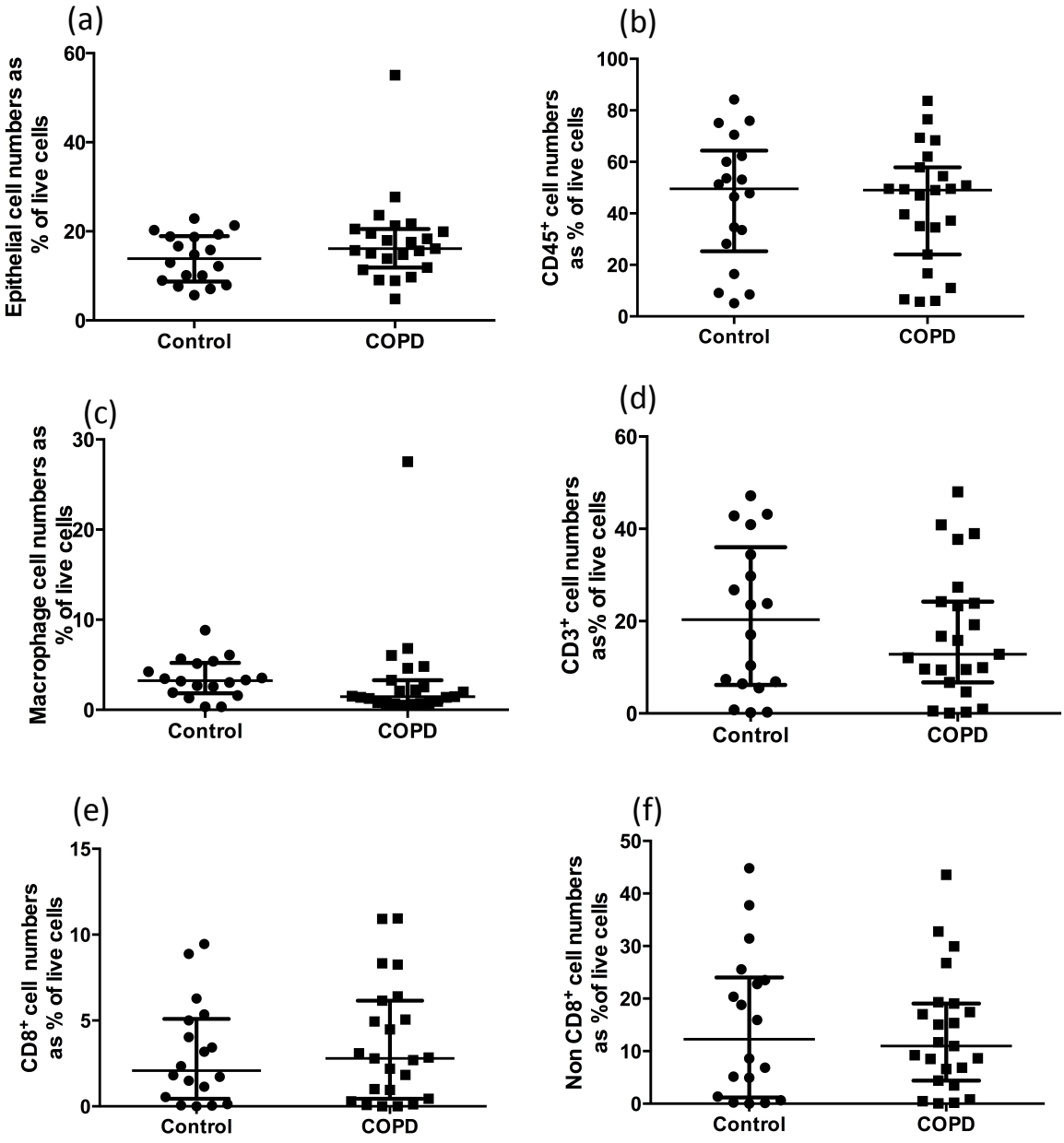

Figure A.1 Quantification of inflammatory cell numbers in lung parenchyma tissue samples from control and COPD patients using flow cytometry. Comparisons of inflammatory cells in parenchyma from control individuals and donors with COPD were quantified by flow cytometry as previously described, and cell numbers are shown as the percentage live cell number. Bars represent median and interquartile range. Non parametric Mann-Whitney U tests were applied to compare between controls (n = 18) and individuals with COPD (n = 23).

A.2 Chapter 4 Appendix

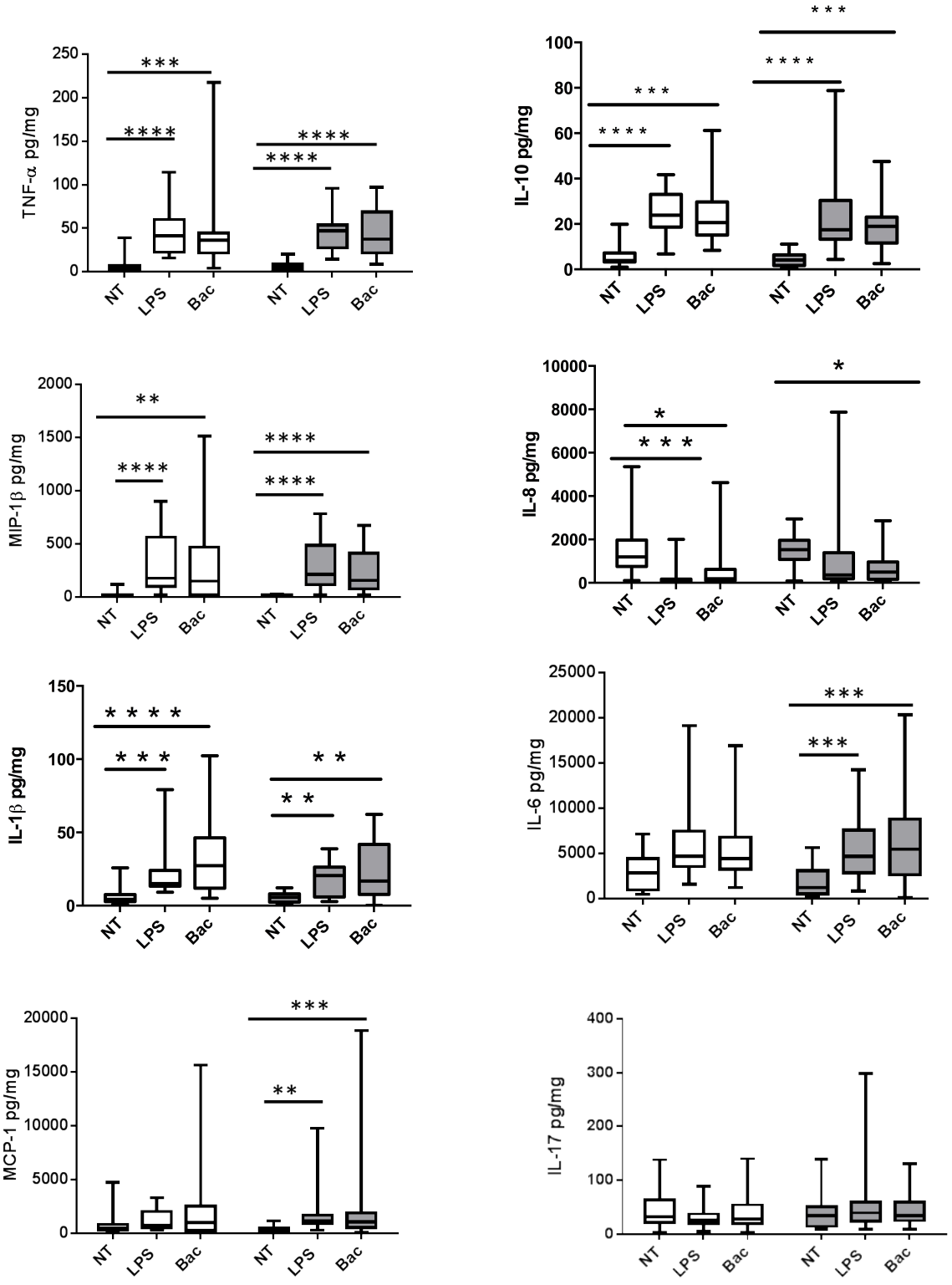

Figure A.2 Profile of cytokine release at 24 h. Results of cytokine release from human explanted lung tissue following stimulation with *H. influenzae* and LPS. Human lung fragments were treated with 26 colony-forming units/ml heat-killed *H.influenzae* (Bac), or 100ng/ml LPS 24 h, or non treated control (NT). TNF, IL-10, IL-6, IL-8, IL-1β, MIP-1β,

MCP-1, and IL-17 release were measured by MSD. Filled whiskers represent COPD patient and hollow whiskers represent the control (Non-COPD). Results were expressed as median and quartile. (Non-COPD = 16 and COPD = 19).

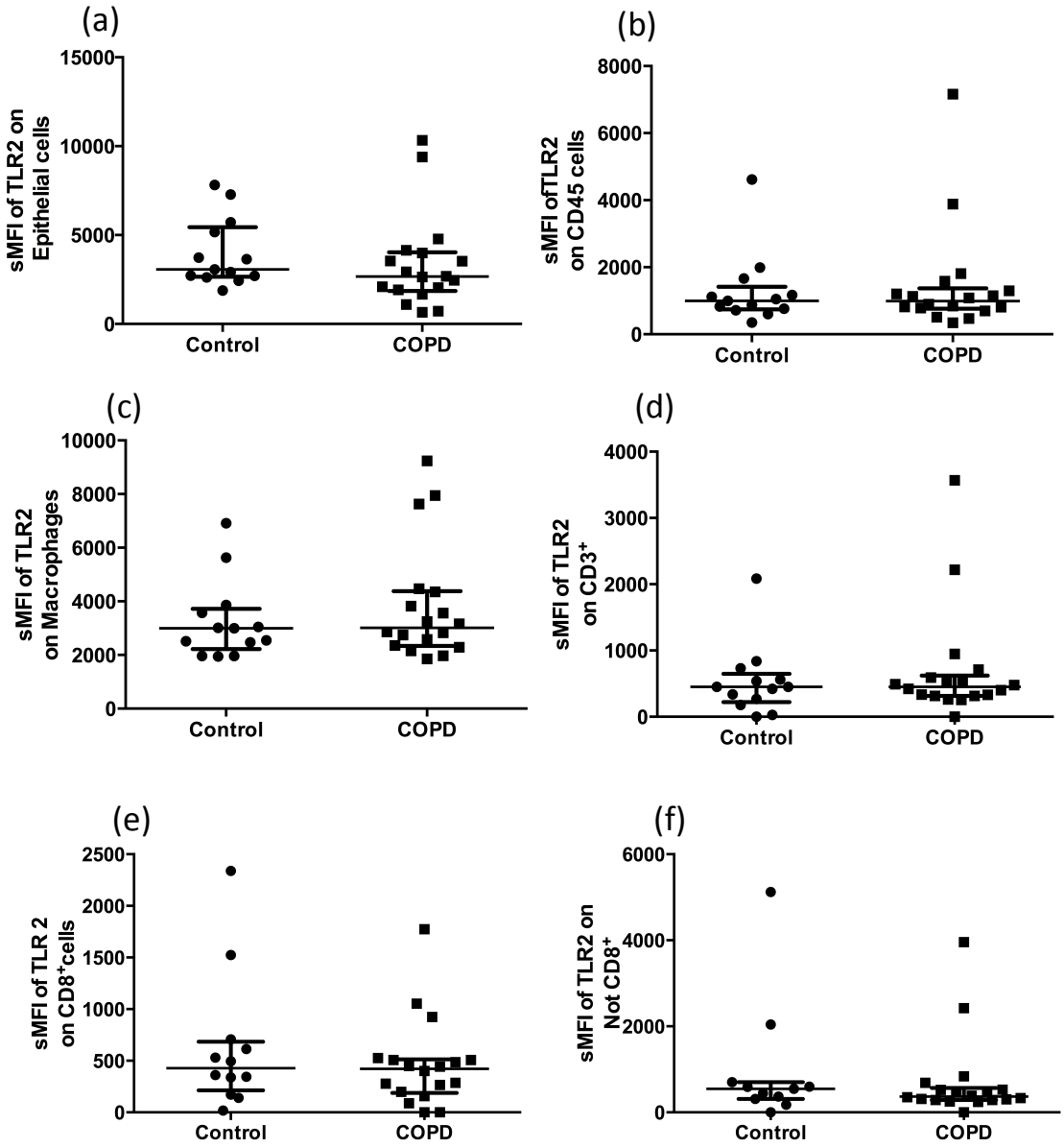

Figure A.3 Quantification of TLR 2 staining intensity on the surface of cells dispersed from lung tissues measured at baseline, and quantified using flow cytometry as described in Figure 4.10. Cells dispersed from the tissues were stained using monoclonal anti-TLR 2 antibody conjugated to PE. Data shows specific mean fluorescent intensity (sMFI) of anti-TLR 2 staining in epithelial cells (a), CD45 (b), macrophages (c), CD3 (d), $CD8^+$ T lymphocytes (e) and $CD8^-$ T-lymphocytes (f). Individual data plots are shown, bars = median value +/- interquartile range. Groups were compared using Non parametric Mann Whitney U test. Control subjects n = 13, COPD patients (GOLD stages 1-2) n = 18

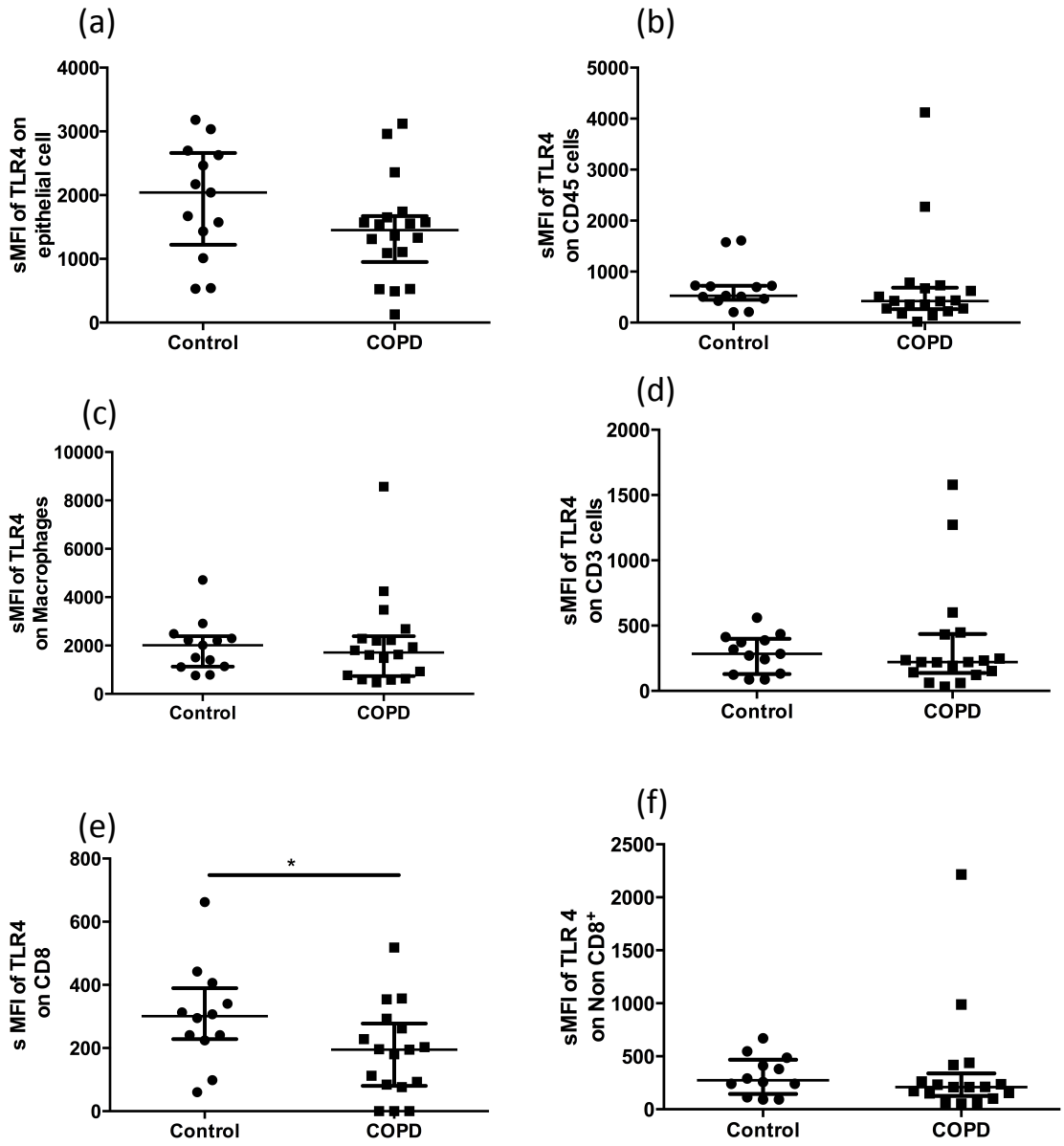

Figure A.4 Quantification of TLR 4 staining intensity on cells dispersed from lung tissues measured at baseline, and quantified using flow cytometry as described in Figure 4.10. Cells dispersed from the tissues were stained using monoclonal anti-TLR 4 antibody conjugated to PE. Data shows specific mean fluorescent intensity (sMFI) of anti-TLR 4 staining in epithelial cells (a), $CD45^+$ leukocytes (b) macrophages (c), $CD3^+$ T lymphocytes (d), $CD8^+$ T lymphocytes (e) and $CD8^-$ T-lymphocytes (f). Individual data plots are shown, bars = median value +/- interquartile range. Groups were compared using Non parametric Mann Whitney U test. Control = control subjects n=13, COPD = patients with COPD (GOLD stages 1-2) n = 18. *=$p<0.05$.

A.3 Chapter 5 Appendix

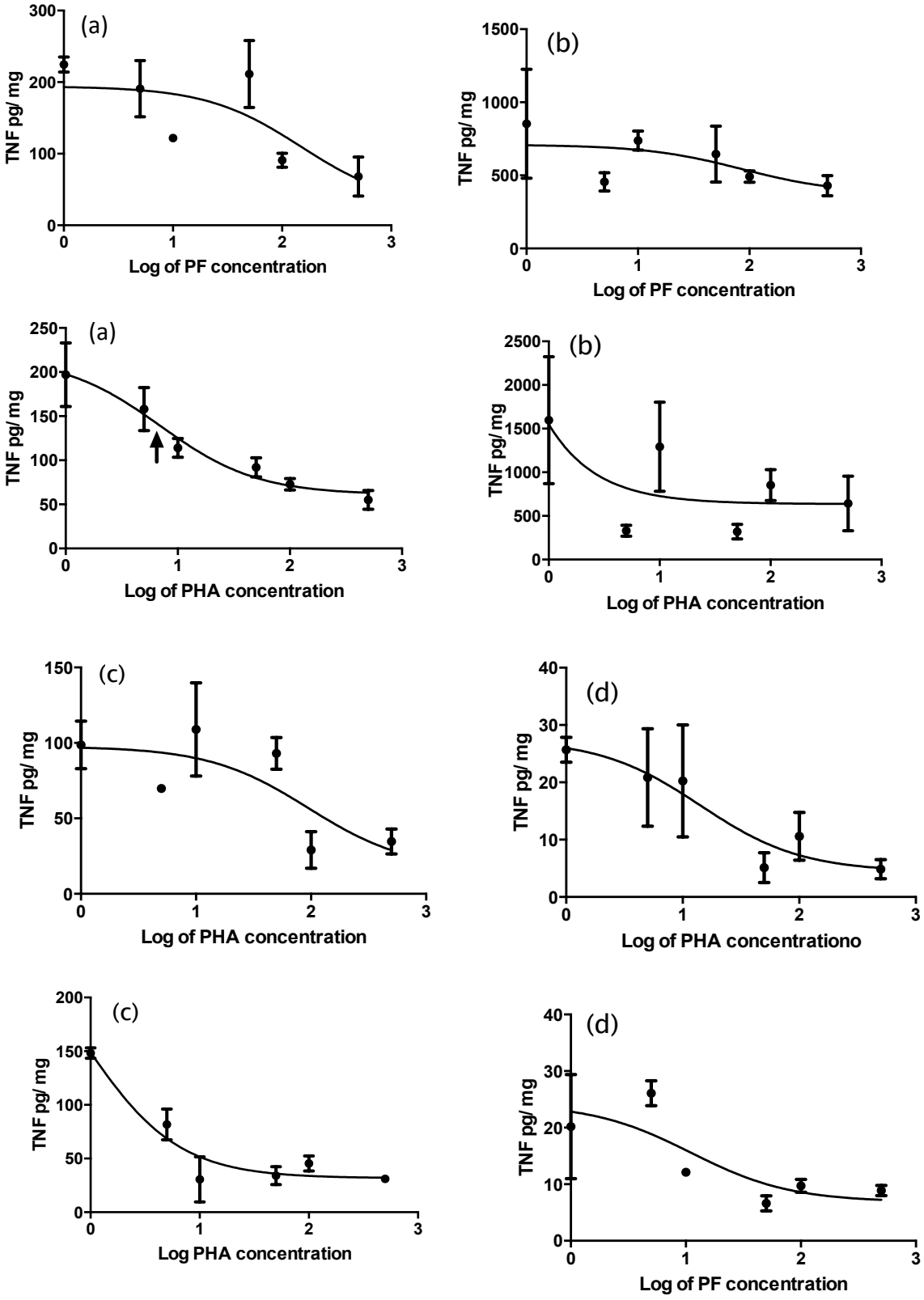

Figure A.5 PHA and PF dose responses: Shows TNF-α responses for n= 12 donors after treatment with various concentrations of PHA and PF for 2 h and stimulation with LPS for 24 h. Concentrations are expressed as pg/mg tissue.

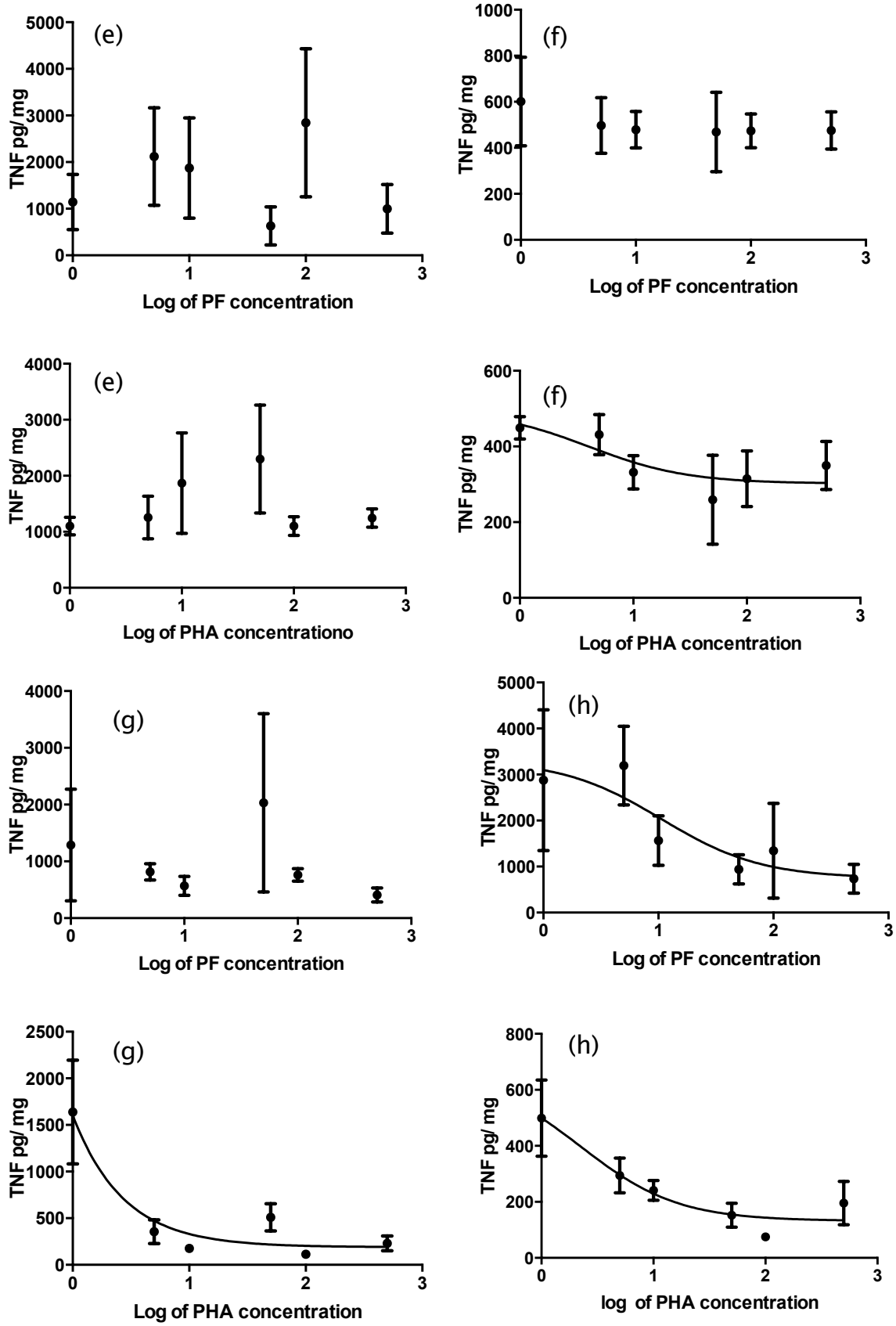

Figure A.6 PHA and PF dose responses: Shows TNF-α responses for n = 12 donors after treatment with various concentrations of PHA and PF for 2 h and stimulation with LPS for 24 h. Concentrations are expressed as pg/mg tissue.

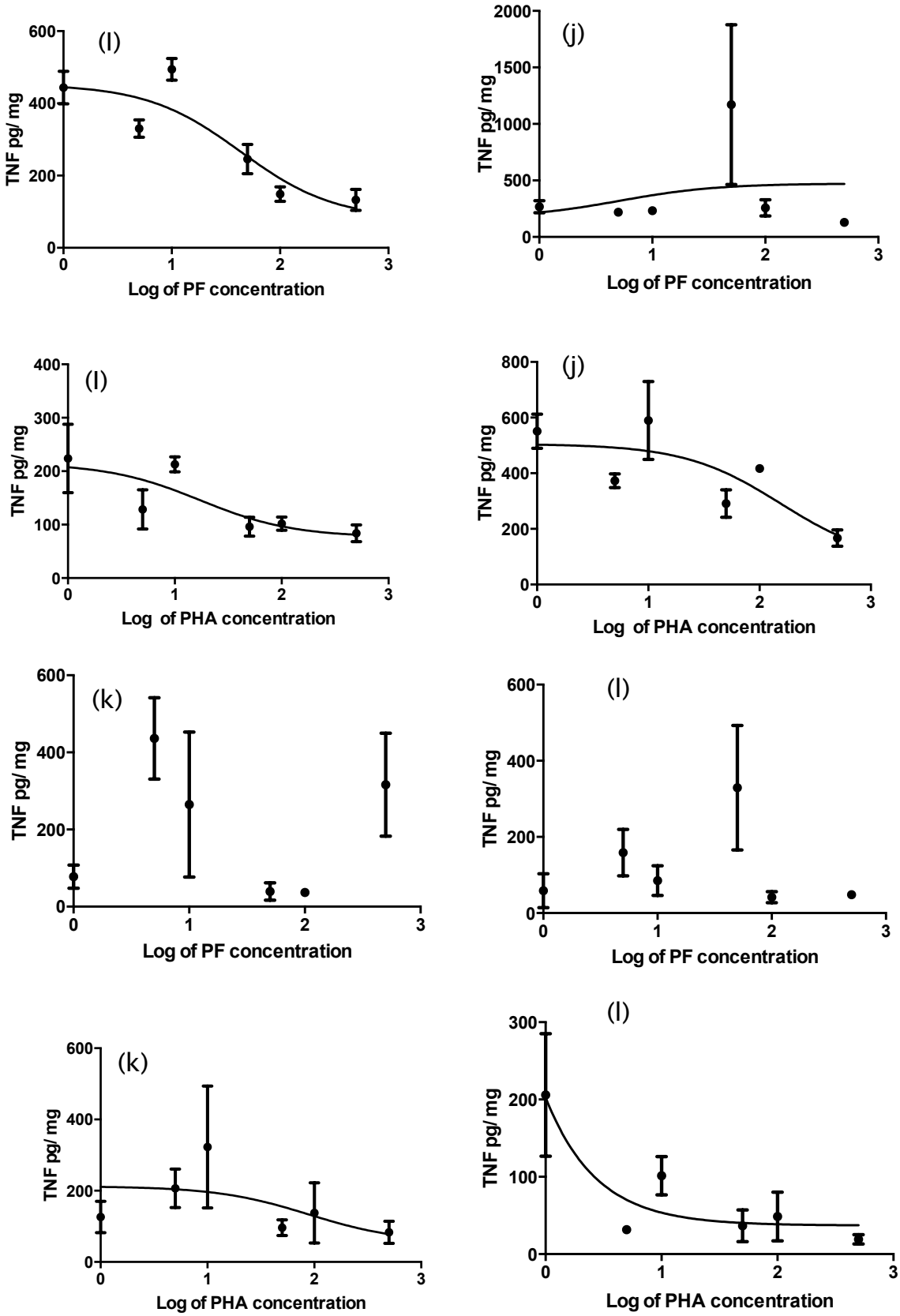

Figure A.7 PHA and PF dose responses: Shows TNF-α responses for n= 12 donors after treatment with various concentrations of PHA and PF for 2 h and stimulation with LPS for 24 h. Concentrations are expressed as pg/mg tissue.

Bibliography

Aaron, S. D., Angel, J. B., Lunau, M., Wright, K., Fex, C., Le Saux, N. and Dales, R. E. (2001) 'Granulocyte inflammatory markers and airway infection during acute exacerbation of chronic obstructive pulmonary disease', *Am J Respir Crit Care Med,* 163(2), pp. 349-55.

Abboud, R. T., Ofulue, A. F., Sansores, R. H. and Muller, N. L. (1998) 'Relationship of alveolar macrophage plasminogen activator and elastase activities to lung function and CT evidence of emphysema', *Chest,* 113(5), pp. 1257-63.

Adams, S. G., Melo, J., Luther, M. and Anzueto, A. (2000) 'Antibiotics are associated with lower relapse rates in outpatients with acute exacerbations of COPD', *Chest,* 117(5), pp. 1345-1352.

Adcock, I. M., Ito, K. and Barnes, P. J. (2004) 'Glucocorticoids: effects on gene transcription', *Proc Am Thorac Soc,* 1(3), pp. 247-54.

Adler, K. B. and Li, Y. (2001) 'Airway epithelium and mucus: intracellular signaling pathways for gene expression and secretion', *Am J Respir Cell Mol Biol,* 25(4), pp. 397-400.

Aguirre, E., Galiana, A., Mira, A., Guardiola, R., Sanchez-Guillen, L., Garcia-Pachon, E., Santibanez, M., Royo, G. and Rodriguez, J. C. (2015) 'Analysis of microbiota in stable patients with chronic obstructive pulmonary disease', *APMIS,* 123(5), pp. 427-32.

Ainslie, M. P., McNulty, C. A., Huynh, T., Symon, F. A. and Wardlaw, A. J. (2002) 'Characterisation of adhesion receptors mediating lymphocyte adhesion to bronchial endothelium provides evidence for a distinct lung homing pathway', *Thorax,* 57(12), pp. 1054-9.

Akira, S. and Takeda, K. (2004) 'Toll-like receptor signalling', *Nat Rev Immunol,* 4(7), pp. 499-511.

Akira, S., Takeda, K. and Kaisho, T. (2001) 'Toll-like receptors: critical proteins linking innate and acquired immunity', *Nat Immunol,* 2(8), pp. 675-80.

Bibliography

Akira, S., Uematsu, S. and Takeuchi, O. (2006) 'Pathogen recognition and innate immunity', *Cell,* 124(4), pp. 783-801.

Aldonyte, R., Jansson, L., Piitulainen, E. and Janciauskiene, S. (2003) 'Circulating monocytes from healthy individuals and COPD patients', *Respir Res,* 4, pp. 11.

Alexopoulou, L., Holt, A. C., Medzhitov, R. and Flavell, R. A. (2001) 'Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3', *Nature,* 413(6857), pp. 732-8.

Almagro, P., Calbo, E., Ochoa de Echaguen, A., Barreiro, B., Quintana, S., Heredia, J. L. and Garau, J. (2002) 'Mortality after hospitalization for COPD', *Chest,* 121(5), pp. 1441-1448.

Alten, R. E., Zerbini, C., Jeka, S., Irazoque, F., Khatib, F., Emery, P., Bertasso, A., Rabbia, M. and Caulfield, J. P. (2010) 'Efficacy and safety of pamapimod in patients with active rheumatoid arthritis receiving stable methotrexate therapy', *Ann Rheum Dis,* 69(2), pp. 364-7.

Amin, K., Ekberg-Jansson, A., Lofdahl, C. G. and Venge, P. (2003) 'Relationship between inflammatory cells and structural changes in the lungs of asymptomatic and never smokers: a biopsy study', *Thorax,* 58(2), pp. 135-42.

Andersson, C. K., Mori, M., Bjermer, L., Lofdahl, C. G. and Erjefalt, J. S. (2010) 'Alterations in lung mast cell populations in patients with chronic obstructive pulmonary disease', *Am J Respir Crit Care Med,* 181(3), pp. 206-17.

Anthony, D., Seow, H. J., Uddin, M., Thompson, M., Dousha, L., Vlahos, R., Irving, L. B., Levy, B. D., Anderson, G. P. and Bozinovski, S. (2013) 'Serum amyloid A promotes lung neutrophilia by increasing IL-17A levels in the mucosa and gammadelta T cells', *Am J Respir Crit Care Med,* 188(2), pp. 179-86.

Armstrong, J., Sargent, C. and Singh, D. (2009) 'Glucocorticoid sensitivity of lipopolysaccharide-stimulated chronic obstructive pulmonary disease alveolar macrophages', *Clin Exp Immunol,* 158(1), pp. 74-83.

ATS (1995) 'Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. American Thoracic Society', *Am J Respir Crit Care Med,* 152(5 Pt 2), pp. S77-121.

Babu, K. S. and Morjaria, J. B. (2015) 'Emerging therapeutic strategies in COPD', *Drug Discov Today,* 20(3), pp. 371-9.

Babusyte, A., Stravinskaite, K., Jeroch, J., Lotvall, J., Sakalauskas, R. and Sitkauskiene, B. (2007) 'Patterns of airway inflammation and MMP-12 expression in smokers and ex-smokers with COPD', *Respir Res,* 8, pp. 81.

Badger, A. M., Griswold, D. E., Kapadia, R., Blake, S., Swift, B. A., Hoffman, S. J., Stroup, G. B., Webb, E., Rieman, D. J., Gowen, M., Boehm, J. C., Adams, J. L. and Lee, J. C. (2000) 'Disease-modifying activity of SB 242235, a selective inhibitor of p38 mitogen-activated protein kinase, in rat adjuvant-induced arthritis', *Arthritis Rheum,* 43(1), pp. 175-83.

Bai, J., Song, H., Cai, C., Zhang, M., Xu, S. and Tan, J. (2012) 'The association of monocyte chemotactic protein-1 and CC chemokine receptor 2 gene variants with chronic obstructive pulmonary disease', *DNA Cell Biol,* 31(6), pp. 1058-63.

Ballarin, A., Bazzan, E., Zenteno, R. H., Turato, G., Baraldo, S., Zanovello, D., Mutti, E., Hogg, J. C., Saetta, M. and Cosio, M. G. (2012) 'Mast cell infiltration discriminates between histopathological phenotypes of chronic obstructive pulmonary disease', *Am J Respir Crit Care Med,* 186(3), pp. 233-9.

Bandi, V., Apicella, M. A., Mason, E., Murphy, T. F., Siddiqi, A., Atmar, R. L. and Greenberg, S. B. (2001) 'Nontypeable Haemophilus influenzae in the lower respiratory tract of patients with chronic bronchitis', *Am J Respir Crit Care Med,* 164(11), pp. 2114-9.

Banerjee, D., Khair, O. A. and Honeybourne, D. (2004) 'Impact of sputum bacteria on airway inflammation and health status in clinical stable COPD', *Eur Respir J,* 23(5), pp. 685-91.

Banerjee, D., Khair, O. A. and Honeybourne, D. (2005) 'The effect of oral clarithromycin on health status and sputum bacteriology in stable COPD', *Respir Med,* 99(2), pp. 208-15.

Bibliography

Bao, L., Yu, J., Zhong, H., Huang, D. and Lu, Q. (2017) 'Expression of toll-like receptors in T lymphocytes stimulated with N-(3-oxododecanoyl)-L-homoserine lactone from Pseudomonas aeruginosa', *APMIS*.

Barnes, P. J. (2000) 'Chronic obstructive pulmonary disease', *N Engl J Med,* 343(4), pp. 269-80.

Barnes, P. J. (2004) 'Alveolar macrophages as orchestrators of COPD', *COPD,* 1(1), pp. 59-70.

Barnes, P. J. (2008) 'Immunology of asthma and chronic obstructive pulmonary disease', *Nat Rev Immunol,* 8(3), pp. 183-92.

Barnes, P. J., Shapiro, S. D. and Pauwels, R. A. (2003) 'Chronic obstructive pulmonary disease: molecular and cellular mechanisms', *Eur Respir J,* 22(4), pp. 672-88.

Battaglia, S., Mauad, T., van Schadewijk, A. M., Vignola, A. M., Rabe, K. F., Bellia, V., Sterk, P. J. and Hiemstra, P. S. (2007) 'Differential distribution of inflammatory cells in large and small airways in smokers', *J Clin Pathol,* 60(8), pp. 907-11.

Beeh, K. M., Kornmann, O., Buhl, R., Culpitt, S. V., Giembycz, M. A. and Barnes, P. J. (2003) 'Neutrophil chemotactic activity of sputum from patients with COPD: role of interleukin 8 and leukotriene B4', *Chest,* 123(4), pp. 1240-7.

Berenson, C. S., Garlipp, M. A., Grove, L. J., Maloney, J. and Sethi, S. (2006) 'Impaired phagocytosis of nontypeable Haemophilus influenzae by human alveolar macrophages in chronic obstructive pulmonary disease', *J Infect Dis,* 194(10), pp. 1375-84.

Berenson, C. S., Kruzel, R. L., Eberhardt, E., Dolnick, R., Minderman, H., Wallace, P. K. and Sethi, S. (2014) 'Impaired innate immune alveolar macrophage response and the predilection for COPD exacerbations', *Thorax,* 69(9), pp. 811-8.

Berkow, R. L., Wang, D., Larrick, J. W., Dodson, R. W. and Howard, T. H. (1987) 'Enhancement of neutrophil superoxide production by preincubation with recombinant human tumor necrosis factor', *J Immunol,* 139(11), pp. 3783-91.

Beutler, B. (2004) 'Inferences, questions and possibilities in Toll-like receptor signalling', *Nature,* 430(6996), pp. 257-63.

Bhalla, D. K., Hirata, F., Rishi, A. K. and Gairola, C. G. (2009) 'Cigarette smoke, inflammation, and lung injury: a mechanistic perspective', *J Toxicol Environ Health B Crit Rev,* 12(1), pp. 45-64.

Bhowmik, A., Seemungal, T. A., Sapsford, R. J. and Wedzicha, J. A. (2000) 'Relation of sputum inflammatory markers to symptoms and lung function changes in COPD exacerbations', *Thorax,* 55(2), pp. 114-20.

Bolton, S. J., Pinnion, K., Oreffo, V., Foster, M. and Pinkerton, K. E. (2009) 'Characterisation of the proximal airway squamous metaplasia induced by chronic tobacco smoke exposure in spontaneously hypertensive rats', *Respir Res,* 10, pp. 118.

Bonarius, H. P., Brandsma, C. A., Kerstjens, H. A., Koerts, J. A., Kerkhof, M., Nizankowska-Mogilnicka, E., Roozendaal, C., Postma, D. S. and Timens, W. (2011) 'Antinuclear autoantibodies are more prevalent in COPD in association with low body mass index but not with smoking history', *Thorax,* 66(2), pp. 101-107.

Borchers, M. T., Wesselkamper, S. C., Harris, N. L., Deshmukh, H., Beckman, E., Vitucci, M., Tichelaar, J. W. and Leikauf, G. D. (2007) 'CD8+ T cells contribute to macrophage accumulation and airspace enlargement following repeated irritant exposure', *Exp Mol Pathol,* 83(3), pp. 301-10.

Bosisio, D., Polentarutti, N., Sironi, M., Bernasconi, S., Miyake, K., Webb, G. R., Martin, M. U., Mantovani, A. and Muzio, M. (2002) 'Stimulation of toll-like receptor 4 expression in human mononuclear phagocytes by interferon-gamma: a molecular basis for priming and synergism with bacterial lipopolysaccharide', *Blood,* 99(9), pp. 3427-31.

Bosken, C. H., Hards, J., Gatter, K. and Hogg, J. C. (1992) 'Characterization of the inflammatory reaction in the peripheral airways of cigarette smokers using immunocytochemistry', *Am Rev Respir Dis,* 145(4 Pt 1), pp. 911-7.

Bourbeau, J., Christodoulopoulos, P., Maltais, F., Yamauchi, Y., Olivenstein, R. and Hamid, Q. (2007) 'Effect of salmeterol/fluticasone propionate on airway inflammation in COPD: a randomised controlled trial', *Thorax,* 62(11), pp. 938-43.

Bibliography

Brandsma, C. A., Timens, W., Jonker, M. R., Rutgers, B., Noordhoek, J. A. and Postma, D. S. (2013) 'Differential effects of fluticasone on extracellular matrix production by airway and parenchymal fibroblasts in severe COPD', *Am J Physiol Lung Cell Mol Physiol,* 305(8), pp. L582-9.

Brass, D. M., Hollingsworth, J. W., Cinque, M., Li, Z., Potts, E., Toloza, E., Foster, W. M. and Schwartz, D. A. (2008) 'Chronic LPS inhalation causes emphysema-like changes in mouse lung that are associated with apoptosis', *Am J Respir Cell Mol Biol,* 39(5), pp. 584-90.

Bresser, P., Out, T. A., van Alphen, L., Jansen, H. M. and 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), pp. 947-952.

Brinkman, G. L. (1968) 'The mast cell in normal human bronchus and lung', *J Ultrastruct Res,* 23(1), pp. 115-23.

Britten, K. M., Howarth, P. H. and Roche, W. R. (1993) 'Immunohistochemistry on resin sections: a comparison of resin embedding techniques for small mucosal biopsies', *Biotech Histochem,* 68(5), pp. 271-80.

Brocklebank, D., Ram, F., Wright, J., Barry, P., Cates, C., Davies, L., Douglas, G., Muers, M., Smith, D. and White, J. (2001) 'Comparison of the effectiveness of inhaler devices in asthma and chronic obstructive airways disease: a systematic review of the literature', *Health Technol Assess,* 5(26), pp. 1-149.

Brozyna, S., Ahern, J., Hodge, G., Nairn, J., Holmes, M., Reynolds, P. N. and Hodge, S. (2009) 'Chemotactic mediators of Th1 T-cell trafficking in smokers and COPD patients', *COPD,* 6(1), pp. 4-16.

Burge, P. S., Calverley, P. M., Jones, P. W., Spencer, S., Anderson, J. A. and Maslen, T. K. (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), pp. 1297-303.

Bussolati, G. and Radulescu, R. T. (2011) 'Blocking endogenous peroxidases in immunohistochemistry: a mandatory, yet also subtle measure', *Appl Immunohistochem Mol Morphol,* 19(5), pp. 484.

Butt, H. L., Clancy, R. L., Cripps, A. W., Murree-Allen, K., Saunders, N. A., Sutherland, D. C. and Hensley, M. J. (1990) 'Bacterial colonisation of the respiratory tract in chronic bronchitis', *Aust N Z J Med,* 20(1), pp. 35-8.

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

Calverley, P., Pauwels, R., Vestbo, J., Jones, P., Pride, N., Gulsvik, A., Anderson, J. and Maden, C. (2003) 'Combined salmeterol and fluticasone in the treatment of chronic obstructive pulmonary disease: a randomised controlled trial', *Lancet,* 361(9356), pp. 449-56.

Calverley, P., Rennard, S., Nelson, H., Karpel, J., Abbate, E., Stryszak, P. and Staudinger, H. (2008) 'One-year treatment with mometasone furoate in chronic obstructive pulmonary disease', *Respiratory Research,* 9(1), pp. 73.

Calverley, P. M., Anderson, J. A., Celli, B., Ferguson, G. T., Jenkins, C., Jones, P. W., Yates, J. C. and Vestbo, J. (2007) 'Salmeterol and fluticasone propionate and survival in chronic obstructive pulmonary disease', *N Engl J Med,* 356(8), pp. 775-89.

Cassatella, M. A. (1999) 'Neutrophil-derived proteins: selling cytokines by the pound', *Adv Immunol,* 73, pp. 369-509.

Castellucci, M., Rossato, M., Calzetti, F., Tamassia, N., Zeminian, S., Cassatella, M. A. and Bazzoni, F. (2015) 'IL-10 disrupts the Brd4-docking sites to inhibit LPS-induced CXCL8 and TNF-alpha expression in monocytes: Implications for chronic obstructive pulmonary disease', *J Allergy Clin Immunol,* 136(3), pp. 781-791 e9.

Celli, B. R. and MacNee, W. (2004) 'Standards for the diagnosis and treatment of patients with COPD: a summary of the ATS/ERS position paper', *Eur. Respir. J.,* 23(6), pp. 932-946.

Chamaillard, M., Girardin, S. E., Viala, J. and Philpott, D. J. (2003) 'Nods, Nalps and Naip: intracellular regulators of bacterial-induced inflammation', *Cell. Microbiol.,* 5(9), pp. 581-592.

Chang, Y., Al-Alwan, L., Alshakfa, S., Audusseau, S., Mogas, A. K., Chouiali, F., Nair, P., Baglole, C. J., Hamid, Q. and Eidelman, D. H. (2014) 'Upregulation of IL-17A/F from human lung tissue explants with cigarette smoke exposure: implications for COPD', *Respir Res,* 15, pp. 145.

Chaplin, D. D. (2006) '1. Overview of the human immune response', *J Allergy Clin Immunol,* 117(2 Suppl Mini-Primer), pp. S430-5.

Chen, P., Hou, J., Ding, D., Hua, X., Yang, Z. and Cui, L. (2013) 'Lipopolysaccharide-induced inflammation of bronchi and emphysematous changes of pulmonary parenchyma in miniature pigs (Sus scrofa domestica)', *Lab Anim (NY),* 42(3), pp. 86-91.

Chen, R., Lim, J. H., Jono, H., Gu, X. X., Kim, Y. S., Basbaum, C. B., Murphy, T. F. and Li, J. D. (2004) 'Nontypeable Haemophilus influenzae lipoprotein P6 induces MUC5AC mucin transcription via TLR2-TAK1-dependent p38 MAPK-AP1 and IKKbeta-IkappaBalpha-NF-kappaB signaling pathways', *Biochem Biophys Res Commun,* 324(3), pp. 1087-94.

Chin, C. L., Manzel, L. J., Lehman, E. E., Humlicek, A. L., Shi, L., Starner, T. D., Denning, G. M., Murphy, T. F., Sethi, S. and Look, D. C. (2005) 'Haemophilus influenzae from patients with chronic obstructive pulmonary disease exacerbation induce more inflammation than colonizers', *Am J Respir Crit Care Med,* 172(1), pp. 85-91.

Cho, M. H. and Castaldi, P. J. and Wan, E. S. and Siedlinski, M. and Hersh, C. P. and Demeo, D. L. and Himes, B. E. and Sylvia, J. S. and Klanderman, B. J. and Ziniti, J. P. and Lange, C. and Litonjua, A. A. and Sparrow, D. and Regan, E. A. and Make, B. J. and Hokanson, J. E. and Murray, T. and Hetmanski, J. B. and Pillai, S. G. and Kong, X. and Anderson, W. H. and Tal-Singer, R. and Lomas, D. A. and Coxson, H. O. and Edwards, L. D. and MacNee, W. and Vestbo, J. and Yates, J. C. and Agusti, A. and Calverley, P. M. and Celli, B. and Crim, C. and Rennard, S. and Wouters, E. and Bakke, P. and Gulsvik, A. and Crapo, J. D. and Beaty, T. H. and Silverman, E. K. and Curtis, J. and Kazerooni, E. and Hanania, N. and Alapat, P. and Bandi, V. and Guntupalli, K. and Guy, E. and Mallampalli, A. and Trinh, C. and Atik, M. and DeMeo, D. and Hersh, C. and Washko, G. and Jacobson, F. and Barr, R. G. and

Thomashow, B. and Austin, J. and MacIntyre, N. and Washington, L. and McAdams, H. P. and Rosiello, R. and Bresnahan, T. and McEvoy, C. and Tashjian, J. and Wise, R. and Hansel, N. and Brown, R. and Diette, G. and Casaburi, R. and Porszasz, J. and Fischer, H. and Budoff, M. and DeBakey, M. and Sharafkhaneh, A. and Trinh, C. and Kamal, H. and Darvishi, R. and Niewoehner, D. and Allen, T. and Anderson, Q. and Rice, K. and Foreman, M. and Westney, G. and Berkowitz, E. and Bowler, R. and Friedlander, A. and Lynch, D. and Schroeder, J. and Newell, J. and Criner, G. and Kim, V. and Marchetti, N. and Satti, A. and Mamary, A. J. and Steiner, R. and Dass, C. and Bailey, W. and Dransfield, M. and Nath, H. and Ramsdell, J. and Friedman, P. and McLennan, G. and van Beek, E. J. and Thompson, B. and Look, D. and Martinez, F. and Han, M. and Kazerooni, E. and Wendt, C. and Allen, T. and Sciurba, F. and Weissfeld, J. and Fuhrman, C. and Bon, J. and Anzueto, A. and Adams, S. and Orozco, C. and Ruiz, M. and Crapo, J. and Silverman, E. and Make, B. and Regan, E. and Moyle, S. and Stinson, D. and Beaty, T. and Klanderman, B. and Laird, N. and Lange, C. and Cho, M. and Santorico, S. and Hokanson, J. and DeMeo, D. and Hansel, N. and Hersh, C. and Hetmanski, J. and Murray, T. and Lynch, D. and Schroeder, J. and Newell, J. and Reilly, J. and Coxson, H. and Judy, P. and Hoffman, E. and Washko, G. and Estepar, R. S. and Ross, J. and Leek, R. and Zach, J. and Kluiber, A. and Sieren, J. and Baumhauer, H. and McArthur, V. and Kazlouski, D. and Allen, A. and Mann, T. and Rodionova, A. and Jensen, R. and Farzadegan, H. and Meyerer, S. and Chandan, S. and Bragan, S. and Murphy, J. and Everett, D. and Wilson, C. and Knowles, R. and Powell, A. and Piccoli, J. and Robinson, M. and Forbes, M. and Wamboldt, M. and Hokanson, J. and Sontag, M. and Black-Shinn, J. and Kinney, G. and Ivanov, Y. and Kostov, K. and Bourbeau, J. and Fitzgerald, M. and Hernandez, P. and Killian, K. and Levy, R. and Maltais, F. and O'Donnell, D. and Krepelka, J. and Vestbo, J. and Wouters, E. and Quinn, D. and Bakke, P. and Kosnik, M. and Agusti, A. and Sauleda, J. and de Mallorca, P. and Feschenko, Y. and Gavrisyuk, V. and Yashina, L. and Yashina, L. and MacNee, W. and Singh, D. and Wedzicha, J. and Anzueto, A. and Braman, S. and Casaburi, R. and Celli, B. and Giessel, G. and Gotfried, M. and Greenwald, G. and Hanania, N. and Mahler, D. and Make, B. and Rennard, S. and Rochester, C. and Scanlon, P. and Schuller, D. and Sciurba, F. and Sharafkhaneh, A. and Siler, T. and Silverman, E. and Wanner, A. and Wise, R. and ZuWallack, R. and Coxson, H. and Crim, C. and Edwards, L. and Lomas, D. and MacNee, W. and Silverman, E. and Tal Singer, R. and Vestbo, J. and Yates, J. and Agusti, A. and Calverley, P. and Celli, B. and Crim, C. and Miller, B. and MacNee, W. and Rennard, S. and Tal-Singer, R. and Wouters, E. and Yates, J. and Benditt, J. and Criner, G. and DeCamp, M. and Diaz, P. and Ginsburg, M. and Kaiser, L. and Katz, M. and Krasna, M.

and MacIntyre, N. and McKenna, R. and Martinez, F. and Mosenifar, Z. and Reilly, J. and Ries, A. and Scanlon, P. and Sciurba, F. and Utz, J. and Silverman, E. K. and Lomas, D. A. and Make, B. J. and Agusti, A. and Sauleda, J. and Calverley, P. M. and Donner, C. F. and Levy, R. D. and Pare, P. D. and Rennard, S. and Vestbo, J. and Wouters, E. F. (2012) 'A genome-wide association study of COPD identifies a susceptibility locus on chromosome 19q13', *Hum. Mol. Genet.,* 21(4), pp. 947-957.

Chung, K. F. and Adcock, I. M. (2008) 'Multifaceted mechanisms in COPD: inflammation, immunity, and tissue repair and destruction', *Eur Respir J,* 31(6), pp. 1334-56.

Chung, Y. M., Park, K. J., Choi, S. Y., Hwang, S. B. and Lee, S. Y. (2001) 'Hepatitis C virus core protein potentiates TNF-alpha-induced NF-kappaB activation through TRAF2-IKKbeta-dependent pathway', *Biochem Biophys Res Commun,* 284(1), pp. 15-9.

Churg, A. and Wright, J. L. (2007) 'Animal models of cigarette smoke-induced chronic obstructive lung disease', *Contrib Microbiol,* 14, pp. 113-25.

Clancy, R., Pang, G., Dunkley, M., Taylor, D. and Cripps, A. (1995) 'Acute on chronic bronchitis: A model of mucosal immunology', *Immunol Cell Biol,* 73(5), pp. 414-7.

Cohen, S. B., Cheng, T. T., Chindalore, V., Damjanov, N., Burgos-Vargas, R., Delora, P., Zimany, K., Travers, H. and Caulfield, J. P. (2009) 'Evaluation of the efficacy and safety of pamapimod, a p38 MAP kinase inhibitor, in a double-blind, methotrexate-controlled study of patients with active rheumatoid arthritis', *Arthritis Rheum,* 60(2), pp. 335-44.

Comer, D. M., Kidney, J. C., Ennis, M. and Elborn, J. S. (2013) 'Airway epithelial cell apoptosis and inflammation in COPD, smokers and nonsmokers', *Eur Respir J,* 41(5), pp. 1058-67.

Compton, C. H., Gubb, J., Nieman, R., Edelson, J., Amit, O., Bakst, A., Ayres, J. G., Creemers, J. P., Schultze-Werninghaus, G., Brambilla, C., Barnes, N. C. and International Study, G. (2001) 'Cilomilast, a selective phosphodiesterase-4 inhibitor for treatment of patients with chronic obstructive pulmonary disease: a randomised, dose-ranging study', *Lancet,* 358(9278), pp. 265-70.

Connor, S. J., Paraskevopoulos, N., Newman, R., Cuan, N., Hampartzoumian, T., Lloyd, A. R. and Grimm, M. C. (2004) 'CCR2 expressing CD4+ T lymphocytes are preferentially recruited to the ileum in Crohn's disease', *Gut,* 53(9), pp. 1287-94.

Coxson, H. O., Dirksen, A., Edwards, L. D., Yates, J. C., Agusti, A., Bakke, P., Calverley, P. M., Celli, B., Crim, C., Duvoix, A., Fauerbach, P. N., Lomas, D. A., Macnee, W., Mayer, R. J., Miller, B. E., Muller, N. L., Rennard, S. I., Silverman, E. K., Tal-Singer, R., Wouters, E. F. and Vestbo, J. (2013) 'The presence and progression of emphysema in COPD as determined by CT scanning and biomarker expression: a prospective analysis from the ECLIPSE study', *Lancet Respir Med,* 1(2), pp. 129-136.

Crooks, S. W., Bayley, D. L., Hill, S. L. and Stockley, R. A. (2000) 'Bronchial inflammation in acute bacterial exacerbations of chronic bronchitis: the role of leukotriene B4', *Eur Respir J,* 15(2), pp. 274-80.

Cucchetto, G., Cazzadori, A., Conti, M., Cascio, G. L., Braggio, P. and Concia, E. (2015) 'Treatment of chronic pulmonary aspergillosis with voriconazole: review of a case series', *Infection,* 43(3), pp. 277-86.

Culpitt, S. V., Rogers, D. F., Shah, P., De Matos, C., Russell, R. E., Donnelly, L. E. and Barnes, P. J. (2003) 'Impaired inhibition by dexamethasone of cytokine release by alveolar macrophages from patients with chronic obstructive pulmonary disease', *Am J Respir Crit Care Med,* 167(1), pp. 24-31.

Curtin, J. F., Liu, N., Candolfi, M., Xiong, W., Assi, H., Yagiz, K., Edwards, M. R., Michelsen, K. S., Kroeger, K. M., Liu, C., Muhammad, A. K., Clark, M. C., Arditi, M., Comin-Anduix, B., Ribas, A., Lowenstein, P. R. and Castro, M. G. (2009) 'HMGB1 mediates endogenous TLR2 activation and brain tumor regression', *PLoS Med,* 6(1), pp. e10.

Dahl, R., Titlestad, I., Lindqvist, A., Wielders, P., Wray, H., Wang, M., Samuelsson, V., Mo, J. and Holt, A. (2012) 'Effects of an oral MMP-9 and -12 inhibitor, AZD1236, on biomarkers in moderate/severe COPD: a randomised controlled trial', *Pulm Pharmacol Ther,* 25(2), pp. 169-77.

de Godoy, I., Donahoe, M., Calhoun, W. J., Mancino, J. and Rogers, R. M. (1996) 'Elevated TNF-alpha production by peripheral blood monocytes of weight-losing COPD patients', *Am J Respir Crit Care Med,* 153(2), pp. 633-7.

Dean, R. A., Cox, J. H., Bellac, C. L., Doucet, A., Starr, A. E. and Overall, C. M. (2008) 'Macrophage-specific metalloelastase (MMP-12) truncates and inactivates ELR+ CXC chemokines and generates CCL2, -7, -8, and -13 antagonists: potential role of the macrophage in terminating polymorphonuclear leukocyte influx', *Blood,* 112(8), pp. 3455-64.

Demedts, I. K., Demoor, T., Bracke, K. R., Joos, G. F. and Brusselle, G. G. (2006) 'Role of apoptosis in the pathogenesis of COPD and pulmonary emphysema', *Respir Res,* 7, pp. 53.

Denning, G. M., Wollenweber, L. A., Railsback, M. A., Cox, C. D., Stoll, L. L. and Britigan, B. E. (1998) 'Pseudomonas pyocyanin increases interleukin-8 expression by human airway epithelial cells', *Infect Immun,* 66(12), pp. 5777-84.

Denny, F. W. (1974) 'Effect of a toxin produced by Haemophilus influenzae on ciliated respiratory epithelium', *J Infect Dis,* 129(2), pp. 93-100.

Dentener, M. A., Louis, R., Cloots, R. H., Henket, M. and Wouters, E. F. (2006) 'Differences in local versus systemic TNFalpha production in COPD: inhibitory effect of hyaluronan on LPS induced blood cell TNFalpha release', *Thorax,* 61(6), pp. 478-84.

Desch, A. N., Gibbings, S. L., Goyal, R., Kolde, R., Bednarek, J., Bruno, T., Slansky, J. E., Jacobelli, J., Mason, R., Ito, Y., Messier, E., Randolph, G. J., Prabagar, M., Atif, S. M., Segura, E., Xavier, R. J., Bratton, D. L., Janssen, W. J., Henson, P. M. and Jakubzick, C. V. (2016) 'Flow Cytometric Analysis of Mononuclear Phagocytes in Nondiseased Human Lung and Lung-Draining Lymph Nodes', *Am J Respir Crit Care Med,* 193(6), pp. 614-26.

Deslee, G., Dury, S., Perotin, J. M., Al Alam, D., Vitry, F., Boxio, R., Gangloff, S. C., Guenounou, M., Lebargy, F. and Belaaouaj, A. (2007) 'Bronchial epithelial spheroids: an alternative culture model to investigate epithelium inflammation-mediated COPD', *Respir Res,* 8, pp. 86.

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

Di Stefano, A., Caramori, G., Ricciardolo, F. L., Capelli, A., Adcock, I. M. and Donner, C. F. (2004) 'Cellular and molecular mechanisms in chronic obstructive pulmonary disease: an overview', *Clin Exp Allergy,* 34(8), pp. 1156-67.

Di Stefano, A., Turato, G., Maestrelli, P., Mapp, C. E., Ruggieri, M. P., Roggeri, A., Boschetto, P., Fabbri, L. M. and Saetta, M. (1996) 'Airflow limitation in chronic bronchitis is associated with T-lymphocyte and macrophage infiltration of the bronchial mucosa', *Am J Respir Crit Care Med,* 153(2), pp. 629-32.

Dominguez, C., Powers, D. A. and Tamayo, N. (2005) 'p38 MAP kinase inhibitors: many are made, but few are chosen', *Curr Opin Drug Discov Devel,* 8(4), pp. 421-30.

Dominguez-Fandos, D., Peinado, V. I., Puig-Pey, R., Ferrer, E., Musri, M. M., Ramirez, J. and Barbera, J. A. (2012) 'Pulmonary inflammatory reaction and structural changes induced by cigarette smoke exposure in the Guinea pig', *COPD,* 9(5), pp. 473-84.

Donaldson, G. C., Seemungal, T. A., Bhowmik, A. and Wedzicha, J. A. (2002) 'Relationship between exacerbation frequency and lung function decline in chronic obstructive pulmonary disease', *Thorax,* 57(10), pp. 847-52.

Doz, E., Noulin, N., Boichot, E., Guenon, I., Fick, L., Le Bert, M., Lagente, V., Ryffel, B., Schnyder, B., Quesniaux, V. F. and Couillin, I. (2008) 'Cigarette smoke-induced pulmonary inflammation is TLR4/MyD88 and IL-1R1/MyD88 signaling dependent', *J Immunol,* 180(2), pp. 1169-78.

Dransfield, M. T., Bourbeau, J., Jones, P. W., Hanania, N. A., Mahler, D. A., Vestbo, J., Wachtel, A., Martinez, F. J., Barnhart, F., Sanford, L., Lettis, S., Crim, C. and Calverley, P. M. (2013) 'Once-daily inhaled fluticasone furoate and vilanterol versus vilanterol only for prevention of exacerbations of COPD: two replicate double-blind, parallel-group, randomised controlled trials', *Lancet Respir Med,* 1(3), pp. 210-223.

Droemann, D., Goldmann, T., Branscheid, D., Clark, R., Dalhoff, K., Zabel, P. and Vollmer, E. (2003) 'Toll-like receptor 2 is expressed by alveolar epithelial cells type II and macrophages in the human lung', *Histochem Cell Biol,* 119(2), pp. 103-8.

Droemann, D., Goldmann, T., Tiedje, T., Zabel, P., Dalhoff, K. and Schaaf, B. (2005) 'Toll-like receptor 2 expression is decreased on alveolar macrophages in cigarette smokers and COPD patients', *Respir Res,* 6, pp. 68.

Duim, B., van Alphen, L., Eijk, P., Jansen, H. M. and Dankert, J. (1994) 'Antigenic drift of non-encapsulated Haemophilus influenzae major outer membrane protein P2 in patients with chronic bronchitis is caused by point mutations', *Mol Microbiol,* 11(6), pp. 1181-9.

Eghtesad, M., Jackson, H. E. and Cunningham, A. C. (2001) 'Primary human alveolar epithelial cells can elicit the transendothelial migration of CD14+ monocytes and CD3+ lymphocytes', *Immunology,* 102(2), pp. 157-64.

Eickmeier, O., Huebner, M., Herrmann, E., Zissler, U., Rosewich, M., Baer, P. C., Buhl, R., Schmitt-Grohe, S., Zielen, S. and Schubert, R. (2010) 'Sputum biomarker profiles in cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) and association between pulmonary function', *Cytokine,* 50(2), pp. 152-7.

Eidelman, D., Saetta, M. P., Ghezzo, H., Wang, N. S., Hoidal, J. R., King, M. and Cosio, M. G. (1990) 'Cellularity of the alveolar walls in smokers and its relation to alveolar destruction. Functional implications', *Am Rev Respir Dis,* 141(6), pp. 1547-52.

Erwin, A. L. and Smith, A. L. (2007) 'Nontypeable Haemophilus influenzae: understanding virulence and commensal behavior', *Trends Microbiol,* 15(8), pp. 355-62.

Ezzie, M. E., Crawford, M., Cho, J. H., Orellana, R., Zhang, S., Gelinas, R., Batte, K., Yu, L., Nuovo, G., Galas, D., Diaz, P., Wang, K. and Nana-Sinkam, S. P. (2012) 'Gene expression networks in COPD: microRNA and mRNA regulation', *Thorax,* 67(2), pp. 122-131.

Filep, J. G. and El Kebir, D. (2009) 'Neutrophil apoptosis: a target for enhancing the resolution of inflammation', *J Cell Biochem,* 108(5), pp. 1039-46.

Finkelstein, R., Fraser, R. S., Ghezzo, H. and Cosio, M. G. (1995) 'Alveolar inflammation and its relation to emphysema in smokers', *Am J Respir Crit Care Med,* 152(5 Pt 1), pp. 1666-72.

Finlay, G. A., O'Driscoll, L. R., Russell, K. J., D'Arcy, E. M., Masterson, J. B., FitzGerald, M. X. and O'Connor, C. M. (1997) 'Matrix metalloproteinase expression and production by alveolar macrophages in emphysema', *Am J Respir Crit Care Med,* 156(1), pp. 240-7.

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

Flo, T. H., Halaas, O., Torp, S., Ryan, L., Lien, E., Dybdahl, B., Sundan, A. and Espevik, T. (2001) 'Differential expression of Toll-like receptor 2 in human cells', *J Leukoc Biol,* 69(3), pp. 474-81.

Forsgren, J., Samuelson, A., Ahlin, A., Jonasson, J., Rynnel-Dagoo, B. and Lindberg, A. (1994) 'Haemophilus influenzae resides and multiplies intracellularly in human adenoid tissue as demonstrated by in situ hybridization and bacterial viability assay', *Infect Immun,* 62(2), pp. 673-9.

Foster, T. S., Miller, J. D., Marton, J. P., Caloyeras, J. P., Russell, M. W. and Menzin, J. (2006) 'Assessment of the economic burden of COPD in the U.S.: a review and synthesis of the literature', *COPD,* 3(4), pp. 211-8.

Fournier, M., Lebargy, F., Le Roy Ladurie, F., Lenormand, E. and Pariente, R. (1989) 'Intraepithelial T-lymphocyte subsets in the airways of normal subjects and of patients with chronic bronchitis', *Am Rev Respir Dis,* 140(3), pp. 737-42.

Freeman, C. M., Han, M. K., Martinez, F. J., Murray, S., Liu, L. X., Chensue, S. W., Polak, T. J., Sonstein, J., Todt, J. C., Ames, T. M., Arenberg, D. A., Meldrum, C. A., Getty, C., McCloskey, L. and Curtis, J. L. (2010) 'Cytotoxic potential of lung CD8(+) T cells increases with chronic obstructive pulmonary disease severity and with in vitro stimulation by IL-18 or IL-15', *J Immunol,* 184(11), pp. 6504-13.

Freeman, C. M., Martinez, F. J., Han, M. K., Washko, G. R., Jr., McCubbrey, A. L., Chensue, S. W., Arenberg, D. A., Meldrum, C. A., McCloskey, L. and Curtis, J. L. (2013) 'Lung CD8+ T cells in COPD have increased expression of bacterial TLRs', *Respir Res,* 14, pp. 13.

Friebel, C., Steckel, H. and Muller, B. W. (2012) 'Rational design of a dry powder inhaler: device design and optimisation', *J Pharm Pharmacol,* 64(9), pp. 1303-15.

Fukunaga, R. and Hunter, T. (1997) 'MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates', *EMBO J,* 16(8), pp. 1921-33.

Gaffey, K., Reynolds, S., Plumb, J., Kaur, M. and Singh, D. (2013) 'Increased phosphorylated p38 mitogen-activated protein kinase in COPD lungs', *Eur Respir J,* 42(1), pp. 28-41.

Gamble, E., Grootendorst, D. C., Brightling, C. E., Troy, S., Qiu, Y., Zhu, J., Parker, D., Matin, D., Majumdar, S., Vignola, A. M., Kroegel, C., Morell, F., Hansel, T. T., Rennard, S. I., Compton, C., Amit, O., Tat, T., Edelson, J., Pavord, I. D., Rabe, K. F., Barnes, N. C. and Jeffery, P. K. (2003) 'Antiinflammatory effects of the phosphodiesterase-4 inhibitor cilomilast (Ariflo) in chronic obstructive pulmonary disease', *Am J Respir Crit Care Med,* 168(8), pp. 976-82.

Gerlach, K., Kohler-Bachmann, S., Jungck, D., Korber, S., Yanik, S., Knoop, H., Wehde, D., Rheinlander, S., Walther, J. W., Kronsbein, J., Knobloch, J. and Koch, A. (2015) 'Endothelin receptor-antagonists suppress lipopolysaccharide-induced cytokine release from alveolar macrophages of non-smokers, smokers and COPD subjects', *Eur J Pharmacol,* 768, pp. 123-30.

Giannella, M., Munoz, P., Guinea, J., Escribano, P., Rodriguez-Creixems, M. and Bouza, E. (2013) 'Growth of Aspergillus in blood cultures: proof of invasive aspergillosis in patients with chronic obstructive pulmonary disease?', *Mycoses,* 56(4), pp. 488-90.

Gill, A. L., Frederickson, M., Cleasby, A., Woodhead, S. J., Carr, M. G., Woodhead, A. J., Walker, M. T., Congreve, M. S., Devine, L. A., Tisi, D., O'Reilly, M., Seavers, L. C., Davis, D. J., Curry, J., Anthony, R., Padova, A., Murray, C. W., Carr, R. A. and Jhoti, H. (2005) 'Identification of novel p38alpha MAP kinase inhibitors using fragment-based lead generation', *J Med Chem,* 48(2), pp. 414-26.

Gingo, M. R., Silveira, L. J., Miller, Y. E., Friedlander, A. L., Cosgrove, G. P., Chan, E. D., Maier, L. A. and Bowler, R. P. (2008) 'Tumour necrosis factor gene polymorphisms are associated with COPD', *Eur Respir J,* 31(5), pp. 1005-12.

Girardin, S. E., Boneca, I. G., Carneiro, L. A., Antignac, A., Jehanno, M., Viala, J., Tedin, K., Taha, M. K., Labigne, A., Zahringer, U., Coyle, A. J., DiStefano, P. S., Bertin, J., Sansonetti, P. J. and Philpott, D. J. (2003) 'Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan', *Science,* 300(5625), pp. 1584-1587.

Go, H., Koh, J., Kim, H. S., Jeon, Y. K. and Chung, D. H. (2014) 'Expression of toll-like receptor 2 and 4 is increased in the respiratory epithelial cells of chronic idiopathic interstitial pneumonia patients', *Respir Med,* 108(5), pp. 783-92.

Goddard, P. R., Nicholson, E. M., Laszlo, G. and Watt, I. (1982) 'Computed tomography in pulmonary emphysema', *Clin Radiol,* 33(4), pp. 379-87.

GOLD (2016) *GlobalL Srategy for the Diagnosis, ManagementT, and Prevention of Chronic Obstructive Pulmonary Disease (Updated 2016)*: Global Initiative for Chronic Obstructive Lung Disease, Inc. Available at: http://goldcopd.org/global-strategy-diagnosis-management-prevention-copd-2016/.

Goldstein, D. M., Kuglstatter, A., Lou, Y. and Soth, M. J. (2010) 'Selective p38alpha inhibitors clinically evaluated for the treatment of chronic inflammatory disorders', *J Med Chem,* 53(6), pp. 2345-53.

Gompertz, S., O'Brien, C., Bayley, D. L., Hill, S. L. and Stockley, R. A. (2001) 'Changes in bronchial inflammation during acute exacerbations of chronic bronchitis', *Eur Respir J,* 17(6), pp. 1112-9.

Goodman, R. B., Strieter, R. M., Frevert, C. W., Cummings, C. J., Tekamp-Olson, P., Kunkel, S. L., Walz, A. and Martin, T. R. (1998) 'Quantitative comparison of C-X-C chemokines produced by endotoxin-stimulated human alveolar macrophages', *Am J Physiol,* 275(1 Pt 1), pp. L87-95.

Grashoff, W. F., Sont, J. K., Sterk, P. J., Hiemstra, P. S., de Boer, W. I., Stolk, J., Han, J. and van Krieken, J. M. (1997) 'Chronic obstructive pulmonary disease: role of bronchiolar mast cells and macrophages', *Am J Pathol,* 151(6), pp. 1785-90.

Groenewegen, K. H., Schols, A. M. and Wouters, E. F. (2003) 'Mortality and mortality-related factors after hospitalization for acute exacerbation of COPD', *Chest,* 124(2), pp. 459-467.

Groskreutz, D. J., Monick, M. M., Powers, L. S., Yarovinsky, T. O., Look, D. C. and Hunninghake, G. W. (2006) 'Respiratory syncytial virus induces TLR3 protein and protein kinase R, leading to increased double-stranded RNA responsiveness in airway epithelial cells', *J Immunol,* 176(3), pp. 1733-40.

Guan, Y., Ranoa, D. R., Jiang, S., Mutha, S. K., Li, X., Baudry, J. and Tapping, R. I. (2010) 'Human TLRs 10 and 1 share common mechanisms of innate immune sensing but not signaling', *J Immunol,* 184(9), pp. 5094-103.

Gudmundsson, G., Gislason, T., Lindberg, E., Hallin, R., Ulrik, C. S., Br?ndum, E., Nieminen, M. M., Aine, T., Bakke, P. and Janson, C. (2006) 'Mortality in COPD patients discharged from hospital: the role of treatment and co-morbidity', *Respir. Res.,* 7, pp. 109.

Guillot, L., Medjane, S., Le-Barillec, K., Balloy, V., Danel, C., Chignard, M. and Si-Tahar, M. (2004) 'Response of human pulmonary epithelial cells to lipopolysaccharide involves Toll-like receptor 4 (TLR4)-dependent signaling pathways: evidence for an intracellular compartmentalization of TLR4', *J Biol Chem,* 279(4), pp. 2712-8.

Gupta, V., Banyard, A., Mullan, A., Sriskantharajah, S., Southworth, T. and Singh, D. (2015) 'Characterization of the inflammatory response to inhaled lipopolysaccharide in mild to moderate chronic obstructive pulmonary disease', *Br J Clin Pharmacol,* 79(5), pp. 767-76.

Hackett, T. L., Holloway, R., Holgate, S. T. and Warner, J. A. (2008) 'Dynamics of pro-inflammatory and anti-inflammatory cytokine release during acute inflammation in chronic obstructive pulmonary disease: an ex vivo study', *Respir Res,* 9, pp. 47.

Hailman, E., Lichenstein, H. S., Wurfel, M. M., Miller, D. S., Johnson, D. A., Kelley, M., Busse, L. A., Zukowski, M. M. and Wright, S. D. (1994) 'Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14', *J Exp Med,* 179(1), pp. 269-77.

Hale, K. K., Trollinger, D., Rihanek, M. and Manthey, C. L. (1999) 'Differential expression and activation of p38 mitogen-activated protein kinase alpha, beta, gamma, and delta in inflammatory cell lineages', *J Immunol,* 162(7), pp. 4246-52.

Hammaker, D. and Firestein, G. S. (2010) '"Go upstream, young man": lessons learned from the p38 saga', *Ann Rheum Dis,* 69 Suppl 1, pp. i77-82.

Hardaker, E. L., Freeman, M. S., Dale, N., Bahra, P., Raza, F., Banner, K. H. and Poll, C. (2010) 'Exposing rodents to a combination of tobacco smoke and lipopolysaccharide results in an exaggerated inflammatory response in the lung', *Br. J. Pharmacol.,* 160(8), pp. 1985-1996.

Harrison, O. J., Foley, J., Bolognese, B. J., Long, E., 3rd, Podolin, P. L. and Walsh, P. T. (2008) 'Airway infiltration of CD4+ CCR6+ Th17 type cells associated with chronic cigarette smoke induced airspace enlargement', *Immunol Lett,* 121(1), pp. 13-21.

Hautamaki, R. D., Kobayashi, D. K., Senior, R. M. and Shapiro, S. D. (1997) 'Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice', *Science,* 277(5334), pp. 2002-4.

Heinemann, D. E., Lohmann, C., Siggelkow, H., Alves, F., Engel, I. and Koster, G. (2000) 'Human osteoblast-like cells phagocytose metal particles and express the macrophage marker CD68 in vitro', *J Bone Joint Surg Br,* 82(2), pp. 283-9.

Hellermann, G. R., Nagy, S. B., Kong, X., Lockey, R. F. and Mohapatra, S. S. (2002) 'Mechanism of cigarette smoke condensate-induced acute inflammatory response in human bronchial epithelial cells', *Respir Res,* 3, pp. 22.

Henricks, P. A. and Nijkamp, F. P. (2001) 'Reactive oxygen species as mediators in asthma', *Pulm Pharmacol Ther,* 14(6), pp. 409-20.

Henzen, C., Suter, A., Lerch, E., Urbinelli, R., Schorno, X. H. and Briner, V. A. (2000) 'Suppression and recovery of adrenal response after short-term, high-dose glucocorticoid treatment', *Lancet,* 355(9203), pp. 542-5.

Herfs, M., Hubert, P., Poirrier, A. L., Vandevenne, P., Renoux, V., Habraken, Y., Cataldo, D., Boniver, J. and Delvenne, P. (2012) 'Proinflammatory cytokines induce bronchial hyperplasia and squamous metaplasia in smokers: implications for chronic obstructive pulmonary disease therapy', *Am. J. Respir. Cell Mol. Biol.,* 47(1), pp. 67-79.

Hertz, C. J., Wu, Q., Porter, E. M., Zhang, Y. J., Weismuller, K. H., Godowski, P. J., Ganz, T., Randell, S. H. and Modlin, R. L. (2003) 'Activation of Toll-like receptor 2 on human

tracheobronchial epithelial cells induces the antimicrobial peptide human beta defensin-2', *J Immunol,* 171(12), pp. 6820-6.

Hidi, R., Timmermans, S., Liu, E., Schudt, C., Dent, G., Holgate, S. T. and Djukanovic, R. (2000) 'Phosphodiesterase and cyclic adenosine monophosphate-dependent inhibition of T-lymphocyte chemotaxis', *Eur Respir J,* 15(2), pp. 342-9.

Hiemstra, P. S. (2013) 'Altered macrophage function in chronic obstructive pulmonary disease', *Ann Am Thorac Soc,* 10 Suppl, pp. S180-5.

Higham, A., Lea, S., Ray, D. and Singh, D. (2014) 'Corticosteroid effects on COPD alveolar macrophages: dependency on cell culture methodology', *J Immunol Methods,* 405, pp. 144-53.

Hill, A. T., Campbell, E. J., Hill, S. L., Bayley, D. L. and Stockley, R. A. (2000) 'Association between airway bacterial load and markers of airway inflammation in patients with stable chronic bronchitis', *Am J Med,* 109(4), pp. 288-95.

Hirano, T., Kodama, S., Kawano, T., Maeda, K. and Suzuki, M. (2011) 'Monophosphoryl lipid A induced innate immune responses via TLR4 to enhance clearance of nontypeable Haemophilus influenzae and Moraxella catarrhalis from the nasopharynx in mice', *FEMS Immunol Med Microbiol,* 63(3), pp. 407-17.

Hodge, S., Hodge, G., Ahern, J., Jersmann, H., Holmes, M. and Reynolds, P. N. (2007) 'Smoking alters alveolar macrophage recognition and phagocytic ability: implications in chronic obstructive pulmonary disease', *Am J Respir Cell Mol Biol,* 37(6), pp. 748-55.

Hodge, S., Hodge, G., Nairn, J., Holmes, M. and Reynolds, P. N. (2006) 'Increased airway granzyme b and perforin in current and ex-smoking COPD subjects', *COPD,* 3(4), pp. 179-87.

Hodge, S. J., Hodge, G. L., Holmes, M. and Reynolds, P. N. (2004) 'Flow cytometric characterization of cell populations in bronchoalveolar lavage and bronchial brushings from patients with chronic obstructive pulmonary disease', *Cytometry B Clin Cytom,* 61(1), pp. 27-34.

Hoetelmans, R. W., Prins, F. A., Cornelese-ten Velde, I., van der Meer, J., van de Velde, C. J. and van Dierendonck, J. H. (2001) 'Effects of acetone, methanol, or paraformaldehyde on cellular structure, visualized by reflection contrast microscopy and transmission and scanning electron microscopy', *Appl Immunohistochem Mol Morphol,* 9(4), pp. 346-51.

Hogg, J. C., Chu, F., Utokaparch, S., Woods, R., Elliott, W. M., Buzatu, L., Cherniack, R. M., Rogers, R. M., Sciurba, F. C., Coxson, H. O. and Pare, P. D. (2004) 'The nature of small-airway obstruction in chronic obstructive pulmonary disease', *N Engl J Med,* 350(26), pp. 2645-53.

Hogg, J. C., Macklem, P. T. and Thurlbeck, W. M. (1968) 'Site and nature of airway obstruction in chronic obstructive lung disease', *N Engl J Med,* 278(25), pp. 1355-60.

Homma, T., Kato, A., Hashimoto, N., Batchelor, J., Yoshikawa, M., Imai, S., Wakiguchi, H., Saito, H. and Matsumoto, K. (2004) 'Corticosteroid and cytokines synergistically enhance toll-like receptor 2 expression in respiratory epithelial cells', *Am J Respir Cell Mol Biol,* 31(4), pp. 463-9.

Hope, H. R., Anderson, G. D., Burnette, B. L., Compton, R. P., Devraj, R. V., Hirsch, J. L., Keith, R. H., Li, X., Mbalaviele, G., Messing, D. M., Saabye, M. J., Schindler, J. F., Selness, S. R., Stillwell, L. I., Webb, E. G., Zhang, J. and Monahan, J. B. (2009) 'Anti-inflammatory properties of a novel N-phenyl pyridinone inhibitor of p38 mitogen-activated protein kinase: preclinical-to-clinical translation', *J Pharmacol Exp Ther,* 331(3), pp. 882-95.

Hou, J., Sun, Y., Hao, Y., Zhuo, J., Liu, X., Bai, P., Han, J., Zheng, X. and Zeng, H. (2013) 'Imbalance between subpopulations of regulatory T cells in COPD', *Thorax,* 68(12), pp. 1131-9.

Hunninghake, G. W., Davidson, J. M., Rennard, S., Szapiel, S., Gadek, J. E. and Crystal, R. G. (1981) 'Elastin fragments attract macrophage precursors to diseased sites in pulmonary emphysema', *Science,* 212(4497), pp. 925-7.

Ikeda, M., Enomoto, N., Hashimoto, D., Fujisawa, T., Inui, N., Nakamura, Y., Suda, T. and Nagata, T. (2015) 'Nontypeable Haemophilus influenzae exploits the interaction between protein-E and vitronectin for the adherence and invasion to bronchial epithelial cells', *BMC Microbiol,* 15, pp. 263.

Imai, K., Dalal, S. S., Chen, E. S., Downey, R., Schulman, L. L., Ginsburg, M. and 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), pp. 786-91.

Inohara, N. and Nunez, G. (2003) 'NODs: intracellular proteins involved in inflammation and apoptosis', *Nat. Rev. Immunol.,* 3(5), pp. 371-382.

Irwin, R. S., Erickson, A. D., Pratter, M. R., Corrao, W. M., Garrity, F. L., Myers, J. R. and Kaemmerlen, J. T. (1982) 'Prediction of tracheobronchial colonization in current cigarette smokers with chronic obstructive bronchitis', *J. Infect. Dis.,* 145(2), pp. 234-241.

Isajevs, S., Taivans, I., Svirina, D., Strazda, G. and Kopeika, U. (2011) 'Patterns of inflammatory responses in large and small airways in smokers with and without chronic obstructive pulmonary disease', *Respiration,* 81(5), pp. 362-71.

Ji, J., von Scheele, I., Bergstrom, J., Billing, B., Dahlen, B., Lantz, A. S., Larsson, K. and Palmberg, L. (2014) 'Compartment differences of inflammatory activity in chronic obstructive pulmonary disease', *Respir Res,* 15, pp. 104.

Jimenez-Dalmaroni, M. J., Xiao, N., Corper, A. L., Verdino, P., Ainge, G. D., Larsen, D. S., Painter, G. F., Rudd, P. M., Dwek, R. A., Hoebe, K., Beutler, B. and Wilson, I. A. (2009) 'Soluble CD36 ectodomain binds negatively charged diacylglycerol ligands and acts as a co-receptor for TLR2', *PLoS One,* 4(10), pp. e7411.

Jobse, B. N., McCurry, C. A., Morissette, M. C., Rhem, R. G., Stampfli, M. R. and Labiris, N. R. (2014) 'Impact of inflammation, emphysema, and smoking cessation on V/Q in mouse models of lung obstruction', *Respir Res,* 15, pp. 42.

Johansson, A., Lundborg, M., Skold, C. M., Lundahl, J., Tornling, G., Eklund, A. and Camner, P. (1997) 'Functional, morphological, and phenotypical differences between rat alveolar and interstitial macrophages', *Am J Respir Cell Mol Biol,* 16(5), pp. 582-8.

Johnson, G. L. and Lapadat, R. (2002) 'Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases', *Science,* 298(5600), pp. 1911-2.

Jones, S. A. (2005) 'Directing transition from innate to acquired immunity: defining a role for IL-6', *J Immunol,* 175(6), pp. 3463-8.

Juarez, E., Nunez, C., Sada, E., Ellner, J. J., Schwander, S. K. and Torres, M. (2010) 'Differential expression of Toll-like receptors on human alveolar macrophages and autologous peripheral monocytes', *Respir Res,* 11, pp. 2.

Kanner, R. E., Anthonisen, N. R., Connett, J. E. and Lung Health Study Research, G. (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(3), pp. 358-64.

Kaur, M. and Singh, D. (2013) 'Neutrophil chemotaxis caused by chronic obstructive pulmonary disease alveolar macrophages: the role of CXCL8 and the receptors CXCR1/CXCR2', *J Pharmacol Exp Ther,* 347(1), pp. 173-80.

Kawasaki, S., Takizawa, H., Takami, K., Desaki, M., Okazaki, H., Kasama, T., Kobayashi, K., Yamamoto, K., Nakahara, K., Tanaka, M., Sagai, M. and Ohtoshi, T. (2001) 'Benzene-extracted components are important for the major activity of diesel exhaust particles: effect on interleukin-8 gene expression in human bronchial epithelial cells', *Am J Respir Cell Mol Biol,* 24(4), pp. 419-26.

Keatings, V. M., Collins, P. D., Scott, D. M. and Barnes, P. J. (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), pp. 530-4.

Kent, L. M., Smyth, L. J., Plumb, J., Clayton, C. L., Fox, S. M., Ray, D. W., Farrow, S. N. and Singh, D. (2009) 'Inhibition of lipopolysaccharide-stimulated chronic obstructive pulmonary disease macrophage inflammatory gene expression by dexamethasone and the p38 mitogen-activated protein kinase inhibitor N-cyano-N'-(2-[8-(2,6-difluorophenyl)-4-(4-fluoro-2-methylphenyl)-7-oxo-7,8-dihy dropyrido[2,3-d] pyrimidin-2-yl]aminoethyl)guanidine (SB706504)', *J Pharmacol Exp Ther,* 328(2), pp. 458-68.

Kew, K. M. and Seniukovich, A. (2014) 'Inhaled steroids and risk of pneumonia for chronic obstructive pulmonary disease', *Cochrane Database Syst Rev,* 3, pp. CD010115.

Khair, O. A., Davies, R. J. and Devalia, J. L. (1996) 'Bacterial-induced release of inflammatory mediators by bronchial epithelial cells', *Eur Respir J,* 9(9), pp. 1913-22.

Khair, O. A., Devalia, J. L., Abdelaziz, M. M., Sapsford, R. J., Tarraf, H. and Davies, R. J. (1994) 'Effect of Haemophilus influenzae endotoxin on the synthesis of IL-6, IL-8, TNF-alpha and expression of ICAM-1 in cultured human bronchial epithelial cells', *Eur Respir J,* 7(12), pp. 2109-16.

Kheradmand, F., Shan, M., Xu, C. and Corry, D. B. (2012) 'Autoimmunity in chronic obstructive pulmonary disease: clinical and experimental evidence', *Expert Rev Clin Immunol,* 8(3), pp. 285-92.

Kim, V. and Criner, G. J. (2013) 'Chronic bronchitis and chronic obstructive pulmonary disease', *Am. J. Respir. Crit. Care Med.,* 187(3), pp. 228-237.

Kim, V., Rogers, T. J. and Criner, G. J. (2008) 'New concepts in the pathobiology of chronic obstructive pulmonary disease', *Proc Am Thorac Soc,* 5(4), pp. 478-85.

Kim, W. D., Chi, H. S., Choe, K. H., Oh, Y. M., Lee, S. D., Kim, K. R., Yoo, K. H., Ngan, D. A., Elliott, W. M., Granville, D. J., Sin, D. D. and Hogg, J. C. (2013) 'A possible role for CD8+ and non-CD8+ cell granzyme B in early small airway wall remodelling in centrilobular emphysema', *Respirology,* 18(4), pp. 688-96.

King, P. T., Lim, S., Pick, A., Ngui, J., Prodanovic, Z., Downey, W., Choong, C., Kelman, A., Baranyai, E., Francis, M., Moshinsky, R., Bardin, P. G., Holmes, P. W. and Holdsworth, S. R. (2012) 'Lung T-cell responses to nontypeable Haemophilus influenzae in patients with chronic obstructive pulmonary disease', *J Allergy Clin Immunol,* 131(5), pp. 1314-1321.

Kirkham, P. A., Spooner, G., Rahman, I. and Rossi, A. G. (2004) 'Macrophage phagocytosis of apoptotic neutrophils is compromised by matrix proteins modified by cigarette smoke and lipid peroxidation products', *Biochem Biophys Res Commun,* 318(1), pp. 32-7.

Kitaguchi, Y., Komatsu, Y., Fujimoto, K., Hanaoka, M. and Kubo, K. (2012) 'Sputum eosinophilia can predict responsiveness to inhaled corticosteroid treatment in patients with overlap syndrome of COPD and asthma', *Int J Chron Obstruct Pulmon Dis,* 7, pp. 283-9.

Kitaguchi, Y., Yasuo, M. and Hanaoka, M. (2016) 'Comparison of pulmonary function in patients with COPD, asthma-COPD overlap syndrome, and asthma with airflow limitation', *Int J Chron Obstruct Pulmon Dis,* 11, pp. 991-7.

Knobloch, J., Hag, H., Jungck, D., Urban, K. and Koch, A. (2011) 'Resveratrol impairs the release of steroid-resistant cytokines from bacterial endotoxin-exposed alveolar macrophages in chronic obstructive pulmonary disease', *Basic Clin Pharmacol Toxicol,* 109(2), pp. 138-43.

Kramer, J. M. and Gaffen, S. L. (2007) 'Interleukin-17: a new paradigm in inflammation, autoimmunity, and therapy', *J Periodontol,* 78(6), pp. 1083-93.

Kranenburg, A. R., de Boer, W. I., Alagappan, V. K., Sterk, P. J. and Sharma, H. S. (2005) 'Enhanced bronchial expression of vascular endothelial growth factor and receptors (Flk-1 and Flt-1) in patients with chronic obstructive pulmonary disease', *Thorax,* 60(2), pp. 106-13.

Kumar, S., Boehm, J. and Lee, J. C. (2003) 'p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases', *Nat Rev Drug Discov,* 2(9), pp. 717-26.

Kumor-Kisielewska, A., Kierszniewska-Stepien, D., Pietras, T., Kroczynska-Bednarek, J., Kurmanowska, Z., Antczak, A. and Gorski, P. (2013) 'Assessment of leptin and resistin levels in patients with chronic obstructive pulmonary disease', *Pol Arch Med Wewn,* 123(5), pp. 215-20.

Lams, B. E., Sousa, A. R., Rees, P. J. and Lee, T. H. (1998) 'Immunopathology of the small-airway submucosa in smokers with and without chronic obstructive pulmonary disease', *Am J Respir Crit Care Med,* 158(5 Pt 1), pp. 1518-23.

Lan, R. Y., Ansari, A. A., Lian, Z. X. and Gershwin, M. E. (2005) 'Regulatory T cells: development, function and role in autoimmunity', *Autoimmun Rev,* 4(6), pp. 351-63.

Lancas, T., Kasahara, D. I., Gross, J. L., Pires-Neto, R. C., Deheinzelin, D., Mauad, T., Negri, E. M. and Dolhnikoff, M. (2011) 'Cholinergic hyperresponsiveness of peripheral lung parenchyma in chronic obstructive pulmonary disease', *Respiration,* 82(2), pp. 177-84.

Lapperre, T. S., Sont, J. K., van Schadewijk, A., Gosman, M. M., Postma, D. S., Bajema, I. M., Timens, W., Mauad, T. and Hiemstra, P. S. (2007) 'Smoking cessation and bronchial epithelial remodelling in COPD: a cross-sectional study', *Respir Res,* 8, pp. 85.

Latorre, M., Novelli, F., Vagaggini, B., Braido, F., Papi, A., Sanduzzi, A., Santus, P., Scichilone, N. and Paggiaro, P. (2015) 'Differences in the efficacy and safety among inhaled corticosteroids (ICS)/long-acting beta2-agonists (LABA) combinations in the treatment of chronic obstructive pulmonary disease (COPD): Role of ICS', *Pulm Pharmacol Ther,* 30, pp. 44-50.

Laufer, S. A., Wagner, G. K., Kotschenreuther, D. A. and Albrecht, W. (2003) 'Novel substituted pyridinyl imidazoles as potent anticytokine agents with low activity against hepatic cytochrome P450 enzymes', *J Med Chem,* 46(15), pp. 3230-44.

Law, S., Boyd, S., Macdonald, J., Raeside, D. and Anderson, D. (2014) 'Predictors of survival in patients with chronic obstructive pulmonary disease receiving long-term oxygen therapy', *BMJ Support Palliat Care*.

Lee, J. C., Kassis, S., Kumar, S., Badger, A. and Adams, J. L. (1999) 'p38 mitogen-activated protein kinase inhibitors--mechanisms and therapeutic potentials', *Pharmacol. Ther.,* 82(2-3), pp. 389-397.

Lee, J. C., Kumar, S., Griswold, D. E., Underwood, D. C., Votta, B. J. and Adams, J. L. (2000) 'Inhibition of p38 MAP kinase as a therapeutic strategy', *Immunopharmacology,* 47(2-3), pp. 185-201.

Lee, J. C., Laydon, J. T., McDonnell, P. C., Gallagher, T. F., Kumar, S., Green, D., McNulty, D., Blumenthal, M. J., Heys, J. R., Landvatter, S. W. and al., e. (1994) 'A protein kinase involved in the regulation of inflammatory cytokine biosynthesis', *Nature,* 372(6508), pp. 739-46.

Lee, S. H., Goswami, S., Grudo, A., Song, L. Z., Bandi, V., Goodnight-White, S., Green, L., Hacken-Bitar, J., Huh, J., Bakaeen, F., Coxson, H. O., Cogswell, S., Storness-Bliss, C., Corry, D. B. and Kheradmand, F. (2007) 'Antielastin autoimmunity in tobacco smoking-induced emphysema', *Nat. Med.,* 13(5), pp. 567-569.

Lee, S. W., Kim, D. R., Kim, T. J., Paik, J. H., Chung, J. H., Jheon, S., Huh, J. W., Lee, J. H. and Lee, C. T. (2012) 'The association of down-regulated toll-like receptor 4 expression with airflow limitation and emphysema in smokers', *Respir Res,* 13, pp. 106.

Leopold, J. G. and Gough, J. (1957) 'The centrilobular form of hypertrophic emphysema and its relation to chronic bronchitis', *Thorax,* 12(3), pp. 219-35.

Lipsky, P. E., van der Heijde, D. M., St Clair, E. W., Furst, D. E., Breedveld, F. C., Kalden, J. R., Smolen, J. S., Weisman, M., Emery, P., Feldmann, M., Harriman, G. R. and Maini, R. N. (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), pp. 1594-1602.

Lomas, D. A. and Silverman, E. K. (2001) 'The genetics of chronic obstructive pulmonary disease', *Respir Res,* 2(1), pp. 20-6.

MacNee, W., Allan, R. J., Jones, I., De Salvo, M. C. and Tan, L. F. (2013) 'Efficacy and safety of the oral p38 inhibitor PH-797804 in chronic obstructive pulmonary disease: a randomised clinical trial', *Thorax,* 68(8), pp. 738-45.

MacRedmond, R. E., Greene, C. M., Dorscheid, D. R., McElvaney, N. G. and O'Neill, S. J. (2007) 'Epithelial expression of TLR4 is modulated in COPD and by steroids, salmeterol and cigarette smoke', *Respir Res,* 8, pp. 84.

Maeno, T., Houghton, A. M., Quintero, P. A., Grumelli, S., Owen, C. A. and Shapiro, S. D. (2007) 'CD8+ T Cells are required for inflammation and destruction in cigarette smoke-induced emphysema in mice', *J Immunol,* 178(12), pp. 8090-6.

Maestrelli, P., Saetta, M., Di Stefano, A., Calcagni, P. G., Turato, G., Ruggieri, M. P., Roggeri, A., Mapp, C. E. and Fabbri, L. M. (1995) 'Comparison of leukocyte counts in sputum, bronchial biopsies, and bronchoalveolar lavage', *Am J Respir Crit Care Med,* 152(6 Pt 1), pp. 1926-31.

Majo, J., Ghezzo, H. and Cosio, M. G. (2001) 'Lymphocyte population and apoptosis in the lungs of smokers and their relation to emphysema', *Eur Respir J,* 17(5), pp. 946-53.

Mallia, P. and Johnston, S. L. (2006) 'How viral infections cause exacerbation of airway diseases', *Chest,* 130(4), pp. 1203-10.

Mangan, P. R., Harrington, L. E., O'Quinn, D. B., Helms, W. S., Bullard, D. C., Elson, C. O., Hatton, R. D., Wahl, S. M., Schoeb, T. R. and Weaver, C. T. (2006) 'Transforming growth factor-beta induces development of the T(H)17 lineage', *Nature,* 441(7090), pp. 231-4.

Manning, G., Whyte, D. B., Martinez, R., Hunter, T. and Sudarsanam, S. (2002) 'The protein kinase complement of the human genome', *Science,* 298(5600), pp. 1912-34.

Mannino, D. M., Homa, D. M., Akinbami, L. J., Ford, E. S. and Redd, S. C. (2002) 'Chronic obstructive pulmonary disease surveillance--United States, 1971-2000', *Respir Care,* 47(10), pp. 1184-99.

March, T. H., Barr, E. B., Finch, G. L., Hahn, F. F., Hobbs, C. H., Menache, M. G. and Nikula, K. J. (1999) 'Cigarette smoke exposure produces more evidence of emphysema in B6C3F1 mice than in F344 rats', *Toxicol Sci,* 51(2), pp. 289-99.

Marshall, J. S., McCurdy, J. D. and Olynych, T. (2003) 'Toll-like receptor-mediated activation of mast cells: implications for allergic disease?', *Int Arch Allergy Immunol,* 132(2), pp. 87-97.

Martin, T. R., Raghu, G., Maunder, R. J. and Springmeyer, S. C. (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), pp. 254-60.

Marwick, J. A., Kirkham, P. A., Stevenson, C. S., Danahay, H., Giddings, J., Butler, K., Donaldson, K., Macnee, W. and Rahman, I. (2004) 'Cigarette smoke alters chromatin remodeling and induces proinflammatory genes in rat lungs', *Am J Respir Cell Mol Biol,* 31(6), pp. 633-42.

Matsuba, K. and Thurlbeck, W. M. (1972) 'The number and dimensions of small airways in emphysematous lungs', *Am J Pathol,* 67(2), pp. 265-75.

Medicherla, S., Fitzgerald, M. F., Spicer, D., Woodman, P., Ma, J. Y., Kapoun, A. M., Chakravarty, S., Dugar, S., Protter, A. A. and Higgins, L. S. (2008) 'p38alpha-selective mitogen-activated protein kinase inhibitor SD-282 reduces inflammation in a subchronic model

of tobacco smoke-induced airway inflammation', *J Pharmacol Exp Ther,* 324(3), pp. 921-9.

Mercer, P. F., Abbott-Banner, K., Adcock, I. M. and Knowles, R. G. (2015) 'Translational models of lung disease', *Clin Sci (Lond),* 128(4), pp. 235-56.

Metcalfe, H. J., Lea, S., Hughes, D., Khalaf, R., Abbott-Banner, K. and Singh, D. (2014) 'Effects of cigarette smoke on Toll-like receptor (TLR) activation of chronic obstructive pulmonary disease (COPD) macrophages', *Clin Exp Immunol,* 176(3), pp. 461-72.

Millan, D. S., Bunnage, M. E., Burrows, J. L., Butcher, K. J., Dodd, P. G., Evans, T. J., Fairman, D. A., Hughes, S. J., Kilty, I. C., Lemaitre, A., Lewthwaite, R. A., Mahnke, A., Mathias, J. P., Philip, J., Smith, R. T., Stefaniak, M. H., Yeadon, M. and Phillips, C. (2011) 'Design and synthesis of inhaled p38 inhibitors for the treatment of chronic obstructive pulmonary disease', *J Med Chem,* 54(22), pp. 7797-814.

Mio, T., Romberger, D. J., Thompson, A. B., Robbins, R. A., Heires, A. and Rennard, S. I. (1997) 'Cigarette smoke induces interleukin-8 release from human bronchial epithelial cells', *Am J Respir Crit Care Med,* 155(5), pp. 1770-6.

Mogensen, T. H. and Paludan, S. R. (2005) 'Reading the viral signature by Toll-like receptors and other pattern recognition receptors', *J. Mol. Med.,* 83(3), pp. 180-192.

Moghaddam, S. J., Clement, C. G., De la Garza, M. M., Zou, X., Travis, E. L., Young, H. W., Evans, C. M., Tuvim, M. J. and Dickey, B. F. (2008) 'Haemophilus influenzae lysate induces aspects of the chronic obstructive pulmonary disease phenotype', *Am J Respir Cell Mol Biol,* 38(6), pp. 629-38.

Moller, L. V., Timens, W., van der Bij, W., Kooi, K., de Wever, B., Dankert, J. and van Alphen, L. (1998) 'Haemophilus influenzae in lung explants of patients with end-stage pulmonary disease', *Am J Respir Crit Care Med,* 157(3 Pt 1), pp. 950-6.

Monick, M. M., Yarovinsky, T. O., Powers, L. S., Butler, N. S., Carter, A. B., Gudmundsson, G. and Hunninghake, G. W. (2003) 'Respiratory syncytial virus up-regulates TLR4 and sensitizes airway epithelial cells to endotoxin', *J Biol Chem,* 278(52), pp. 53035-44.

Monso, E., Rosell, A., Bonet, G., Manterola, J., Cardona, P. J., Ruiz, J. and Morera, J. (1999) 'Risk factors for lower airway bacterial colonization in chronic bronchitis', *Eur. Respir. J.,* 13(2), pp. 338-342.

Monso, E., Ruiz, J., Rosell, A., Manterola, J., Fiz, J., Morera, J. and 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), pp. 1316-20.

Moore, K. W., de Waal Malefyt, R., Coffman, R. L. and O'Garra, A. (2001) 'Interleukin-10 and the interleukin-10 receptor', *Annu Rev Immunol,* 19, pp. 683-765.

Moretto, N., Facchinetti, F., Southworth, T., Civelli, M., Singh, D. and Patacchini, R. (2009) 'alpha,beta-Unsaturated aldehydes contained in cigarette smoke elicit IL-8 release in pulmonary cells through mitogen-activated protein kinases', *Am J Physiol Lung Cell Mol Physiol,* 296(5), pp. L839-48.

Motz, G. T., Eppert, B. L., Sun, G., Wesselkamper, S. C., Linke, M. J., Deka, R. and Borchers, M. T. (2008) 'Persistence of lung CD8 T cell oligoclonal expansions upon smoking cessation in a mouse model of cigarette smoke-induced emphysema', *J Immunol,* 181(11), pp. 8036-43.

Mullen, J. B., Wright, J. L., Wiggs, B. R., Pare, P. D. and Hogg, J. C. (1985) 'Reassessment of inflammation of airways in chronic bronchitis', *Br Med J (Clin Res Ed),* 291(6504), pp. 1235-9.

Murphy, T. F. (2006) 'The role of bacteria in airway inflammation in exacerbations of chronic obstructive pulmonary disease', *Curr Opin Infect Dis,* 19(3), pp. 225-30.

Murphy, T. F., Brauer, A. L., Grant, B. J. and Sethi, S. (2005) 'Moraxella catarrhalis in chronic obstructive pulmonary disease: burden of disease and immune response', *Am J Respir Crit Care Med,* 172(2), pp. 195-9.

Murphy, T. F. and Sethi, S. (1992) 'Bacterial infection in chronic obstructive pulmonary disease', *Am Rev Respir Dis,* 146(4), pp. 1067-83.

Muzio, M., Bosisio, D., Polentarutti, N., D'Amico, G., Stoppacciaro, A., Mancinelli, R., van't Veer, C., Penton-Rol, G., Ruco, L. P., Allavena, P. and Mantovani, A. (2000) 'Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells', *J Immunol,* 164(11), pp. 5998-6004.

Nagai, A., West, W. W., Paul, J. L. and Thurlbeck, W. M. (1985) 'The National Institutes of Health Intermittent Positive-Pressure Breathing trial: pathology studies. I. Interrelationship between morphologic lesions', *Am Rev Respir Dis,* 132(5), pp. 937-45.

Needham, M. and Stockley, R. A. (2004) 'Alpha 1-antitrypsin deficiency. 3: Clinical manifestations and natural history', *Thorax,* 59(5), pp. 441-5.

New, L., Jiang, Y., Zhao, M., Liu, K., Zhu, W., Flood, L. J., Kato, Y., Parry, G. C. and Han, J. (1998) 'PRAK, a novel protein kinase regulated by the p38 MAP kinase', *EMBO J,* 17(12), pp. 3372-84.

NICE (2010) *Chronic Obstructive Pulmonary Disease in over 16s: Diagnosis and Management* London: National Institute for Health and Clear Excellenceguidance/cg101). Available at: https://http://www.nice.org.uk/guidance/cg101/resources/chronic-obstructive-pulmonary-disease-in-over-16s-diagnosis-and-management-35109323931589.

Nicholas, B., *ERS abstract*

Nicholas, B., Staples, K. J., Moese, S., Meldrum, E., Ward, J., Dennison, P., Havelock, T., Hinks, T. S., Amer, K., Woo, E., Chamberlain, M., Singh, N., North, M., Pink, S., Wilkinson, T. M. and Djukanovic, R. (2015) 'A novel lung explant model for the ex vivo study of efficacy and mechanisms of anti-influenza drugs', *J Immunol,* 194(12), pp. 6144-54.

Nicholas, B. L., O'Connor, C. D. and Djukanovic, R. (2009) 'From proteomics to prescription-the search for COPD biomarkers', *COPD,* 6(4), pp. 298-303.

Nichols, J. E., Niles, J. A., Vega, S. P. and Cortiella, J. (2013) 'Novel in vitro respiratory models to study lung development, physiology, pathology and toxicology', *Stem Cell Res Ther,* 4 Suppl 1, pp. S7.

Nishikawa, M., Kakemizu, N., Ito, T., Kudo, M., Kaneko, T., Suzuki, M., Udaka, N., Ikeda, H. and Okubo, T. (1999) 'Superoxide mediates cigarette smoke-induced infiltration of neutrophils into the airways through nuclear factor-kappaB activation and IL-8 mRNA expression in guinea pigs in vivo', *Am J Respir Cell Mol Biol,* 20(2), pp. 189-98.

Noguera, A., Batle, S., Miralles, C., Iglesias, J., Busquets, X., MacNee, W. and Agusti, A. G. (2001) 'Enhanced neutrophil response in chronic obstructive pulmonary disease', *Thorax,* 56(6), pp. 432-7.

O'Donnell, R. A., Peebles, C., Ward, J. A., Daraker, A., Angco, G., Broberg, P., Pierrou, S., Lund, J., Holgate, S. T., Davies, D. E., Delany, D. J., Wilson, S. J. and Djukanovic, R. (2004) 'Relationship between peripheral airway dysfunction, airway obstruction, and neutrophilic inflammation in COPD', *Thorax,* 59(10), pp. 837-42.

O'Shaughnessy, T. C., Ansari, T. W., Barnes, N. C. and Jeffery, P. K. (1997) 'Inflammation in bronchial biopsies of subjects with chronic bronchitis: inverse relationship of CD8+ T lymphocytes with FEV1', *Am J Respir Crit Care Med,* 155(3), pp. 852-7.

Ochoa, M. T., Legaspi, A. J., Hatziris, Z., Godowski, P. J., Modlin, R. L. and Sieling, P. A. (2003) 'Distribution of Toll-like receptor 1 and Toll-like receptor 2 in human lymphoid tissue', *Immunology,* 108(1), pp. 10-5.

Ogawa, Y., Duru, E. A. and Ameredes, B. T. (2008) 'Role of IL-10 in the resolution of airway inflammation', *Curr Mol Med,* 8(5), pp. 437-45.

Ohnishi, K., Takagi, M., Kurokawa, Y., Satomi, S. and Konttinen, Y. T. (1998) 'Matrix metalloproteinase-mediated extracellular matrix protein degradation in human pulmonary emphysema', *Lab Invest,* 78(9), pp. 1077-87.

Olloquequi, J., Ferrer, J., Montes, J. F., Rodriguez, E., Montero, M. A. and Garcia-Valero, J. (2010) 'Differential lymphocyte infiltration in small airways and lung parenchyma in COPD patients', *Respir Med,* 104(9), pp. 1310-8.

Paats, M. S., Bergen, I. M., Hoogsteden, H. C., van der Eerden, M. M. and Hendriks, R. W. (2012) 'Systemic CD4+ and CD8+ T-cell cytokine profiles correlate with GOLD stage in stable COPD', *Eur Respir J,* 40(2), pp. 330-7.

Pace, E., Ferraro, M., Siena, L., Melis, M., Montalbano, A. M., Johnson, M., Bonsignore, M. R., Bonsignore, G. and Gjomarkaj, M. (2008) 'Cigarette smoke increases Toll-like receptor 4 and modifies lipopolysaccharide-mediated responses in airway epithelial cells', *Immunology,* 124(3), pp. 401-11.

Palsson-McDermott, E. M. and O'Neill, L. A. (2004) 'Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4', *Immunology,* 113(2), pp. 153-62.

Pang, B., Hong, W., West-Barnette, S. L., Kock, N. D. and Swords, W. E. (2008) 'Diminished ICAM-1 expression and impaired pulmonary clearance of nontypeable Haemophilus influenzae in a mouse model of chronic obstructive pulmonary disease/emphysema', *Infect Immun,* 76(11), pp. 4959-67.

Paolillo, R., Romano Carratelli, C., Sorrentino, S., Mazzola, N., Mita, L. and Rizzo, A. (2011) 'Expression of IL-23, VEGF and TLR2/TLR4 on mononuclear cells after exposure to Pseudomonas aeruginosa', *Int J Immunopathol Pharmacol,* 24(4), pp. 961-73.

Papi, A., Bellettato, C. M., Braccioni, F., Romagnoli, M., Casolari, P., Caramori, G., Fabbri, L. M. and Johnston, S. L. (2006) 'Infections and airway inflammation in chronic obstructive pulmonary disease severe exacerbations', *Am J Respir Crit Care Med,* 173(10), pp. 1114-21.

Party, M. R. C. W. (1981) 'Long term domiciliary oxygen therapy in chronic hypoxic cor pulmonale complicating chronic bronchitis and emphysema. Report of the Medical Research Council Working Party', *Lancet,* 1(8222), pp. 681-6.

Patel, I. S., Roberts, N. J., Lloyd-Owen, S. J., Sapsford, R. J. and Wedzicha, J. A. (2003) 'Airway epithelial inflammatory responses and clinical parameters in COPD', *Eur Respir J,* 22(1), pp. 94-9.

Patel, I. S., Seemungal, T. A., Wilks, M., Lloyd-Owen, S. J., Donaldson, G. C. and Wedzicha, J. A. (2002) 'Relationship between bacterial colonisation and the frequency, character, and severity of COPD exacerbations', *Thorax,* 57(9), pp. 759-64.

Pauwels, R. A., Buist, A. S., Ma, P., Jenkins, C. R., Hurd, S. S. and Committee, G. S. (2001) 'Global strategy for the diagnosis, management, and prevention of chronic obstructive

pulmonary disease: National Heart, Lung, and Blood Institute and World Health Organization Global Initiative for Chronic Obstructive Lung Disease (GOLD): executive summary', *Respir Care,* 46(8), pp. 798-825.

Pauwels, R. A., Lofdahl, C. G., Laitinen, L. A., Schouten, J. P., Postma, D. S., Pride, N. B. and Ohlsson, S. V. (1999) 'Long-term treatment with inhaled budesonide in persons with mild chronic obstructive pulmonary disease who continue smoking. European Respiratory Society Study on Chronic Obstructive Pulmonary Disease', *N Engl J Med,* 340(25), pp. 1948-53.

Payne, V. and Kam, P. C. (2004) 'Mast cell tryptase: a review of its physiology and clinical significance', *Anaesthesia,* 59(7), pp. 695-703.

Peinado, V. I., Barbera, J. A., Abate, P., Ramirez, J., Roca, J., Santos, S. and Rodriguez-Roisin, R. (1999) 'Inflammatory reaction in pulmonary muscular arteries of patients with mild chronic obstructive pulmonary disease', *Am J Respir Crit Care Med,* 159(5 Pt 1), pp. 1605-11.

Perera, W. R., Hurst, J. R., Wilkinson, T. M., Sapsford, R. J., Mullerova, H., Donaldson, G. C. and Wedzicha, J. A. (2007) 'Inflammatory changes, recovery and recurrence at COPD exacerbation', *Eur Respir J,* 29(3), pp. 527-34.

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

Phillips, P. L., Yang, Q., Davis, S., Sampson, E. M., Azeke, J. I., Hamad, A. and Schultz, G. S. (2013) 'Antimicrobial dressing efficacy against mature Pseudomonas aeruginosa biofilm on porcine skin explants', *Int Wound J*.

Pillai, S. G., Ge, D., Zhu, G., Kong, X., Shianna, K. V., Need, A. C., Feng, S., Hersh, C. P., Bakke, P., Gulsvik, A., Ruppert, A., Lodrup Carlsen, K. C., Roses, A., Anderson, W., Rennard, S. I., Lomas, D. A., Silverman, E. K. and Goldstein, D. B. (2009) 'A genome-wide association study in chronic obstructive pulmonary disease (COPD): identification of two major susceptibility loci', *PLoS Genet,* 5(3), pp. e1000421.

Pirvulescu, M. M., Gan, A. M., Stan, D., Simion, V., Calin, M., Butoi, E. and Manduteanu, I. (2014) 'Subendothelial resistin enhances monocyte transmigration in a co-culture of human endothelial and smooth muscle cells by mechanisms involving fractalkine, MCP-1 and activation of TLR4 and Gi/o proteins signaling', *Int J Biochem Cell Biol,* 50, pp. 29-37.

Poliska, S., Csanky, E., Szanto, A., Szatmari, I., Mesko, B., Szeles, L., Dezso, B., Scholtz, B., Podani, J., Kilty, I., Takacs, L. and Nagy, L. (2011) 'Chronic obstructive pulmonary disease-specific gene expression signatures of alveolar macrophages as well as peripheral blood monocytes overlap and correlate with lung function', *Respiration,* 81(6), pp. 499-510.

Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B. and Beutler, B. (1998) 'Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene', *Science,* 282(5396), pp. 2085-8.

Pons, J., Sauleda, J., Regueiro, V., Santos, C., Lopez, M., Ferrer, J., Agusti, A. G. and Bengoechea, J. A. (2006) 'Expression of Toll-like receptor 2 is up-regulated in monocytes from patients with chronic obstructive pulmonary disease', *Respir Res,* 7, pp. 64.

Price, L. C., Lowe, D., Hosker, H. S., Anstey, K., Pearson, M. G. and Roberts, C. M. (2006) 'UK National COPD Audit 2003: Impact of hospital resources and organisation of care on patient outcome following admission for acute COPD exacerbation', *Thorax,* 61(10), pp. 837-842.

Pryor, W. A. and Stone, K. (1993) 'Oxidants in cigarette smoke. Radicals, hydrogen peroxide, peroxynitrate, and peroxynitrite', *Ann N Y Acad Sci,* 686, pp. 12-27; discussion 27-8.

Pulford, K. A., Sipos, A., Cordell, J. L., Stross, W. P. and Mason, D. Y. (1990) 'Distribution of the CD68 macrophage/myeloid associated antigen', *Int Immunol,* 2(10), pp. 973-80.

Quint, J. K., Baghai-Ravary, R., Donaldson, G. C. and Wedzicha, J. A. (2008) 'Relationship between depression and exacerbations in COPD', *Eur Respir J,* 32(1), pp. 53-60.

Rabe, K. F., Hurd, S., Anzueto, A., Barnes, P. J., Buist, S. A., Calverley, P., Fukuchi, Y., Jenkins, C., Rodriguez-Roisin, R., van Weel, C. and Zielinski, J. (2007) 'Global strategy for the

diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary', *Am J Respir Crit Care Med,* 176(6), pp. 532-55.

Rahman, I. (2005) 'Oxidative stress in pathogenesis of chronic obstructive pulmonary disease: cellular and molecular mechanisms', *Cell Biochem Biophys,* 43(1), pp. 167-88.

Ramirez-Venegas, A., Sansores, R. H., Perez-Padilla, R., Regalado, J., Velazquez, A., Sanchez, C. and Mayar, M. E. (2006) 'Survival of patients with chronic obstructive pulmonary disease due to biomass smoke and tobacco', *Am J Respir Crit Care Med,* 173(4), pp. 393-7.

Reddy, M. S., Murphy, T. F., Faden, H. S. and Bernstein, J. M. (1997) 'Middle ear mucin glycoprotein: purification and interaction with nontypable Haemophilus influenzae and Moraxella catarrhalis', *Otolaryngol Head Neck Surg,* 116(2), pp. 175-180.

Redecke, V., Hacker, H., Datta, S. K., Fermin, A., Pitha, P. M., Broide, D. H. and Raz, E. (2004) 'Cutting edge: activation of Toll-like receptor 2 induces a Th2 immune response and promotes experimental asthma', *J Immunol,* 172(5), pp. 2739-43.

Regalado, J., Perez-Padilla, R., Sansores, R., Paramo Ramirez, J. I., Brauer, M., Pare, P. and Vedal, S. (2006) 'The effect of biomass burning on respiratory symptoms and lung function in rural Mexican women', *Am J Respir Crit Care Med,* 174(8), pp. 901-5.

Regan, J., Breitfelder, S., Cirillo, P., Gilmore, T., Graham, A. G., Hickey, E., Klaus, B., Madwed, J., Moriak, M., Moss, N., Pargellis, C., Pav, S., Proto, A., Swinamer, A., Tong, L. and Torcellini, C. (2002) 'Pyrazole urea-based inhibitors of p38 MAP kinase: from lead compound to clinical candidate', *J Med Chem,* 45(14), pp. 2994-3008.

Renda, T., Baraldo, S., Pelaia, G., Bazzan, E., Turato, G., Papi, A., Maestrelli, P., Maselli, R., Vatrella, A., Fabbri, L. M., Zuin, R., Marsico, S. A. and Saetta, M. (2008) 'Increased activation of p38 MAPK in COPD', *Eur Respir J,* 31(1), pp. 62-9.

Rennard, S. I., Flavin, S. K., Agarwal, P. K., Lo, K. H. and Barnathan, E. S. (2013) 'Long-term safety study of infliximab in moderate-to-severe chronic obstructive pulmonary disease', *Respir Med,* 107(3), pp. 424-32.

Rennard, S. I., Fogarty, C., Kelsen, S., Long, W., Ramsdell, J., Allison, J., Mahler, D., Saadeh, C., Siler, T., Snell, P., Korenblat, P., Smith, W., Kaye, M., Mandel, M., Andrews, C., Prabhu, R., Donohue, J. F., Watt, R., Lo, K. H., Schlenker-Herceg, R., Barnathan, E. S. and Murray, J. (2007) 'The safety and efficacy of infliximab in moderate to severe chronic obstructive pulmonary disease', *Am. J. Respir. Crit. Care Med.,* 175(9), pp. 926-934.

Richens, T. R., Linderman, D. J., Horstmann, S. A., Lambert, C., Xiao, Y. Q., Keith, R. L., Boe, D. M., Morimoto, K., Bowler, R. P., Day, B. J., Janssen, W. J., Henson, P. M. and Vandivier, R. W. (2009) 'Cigarette smoke impairs clearance of apoptotic cells through oxidant-dependent activation of RhoA', *Am. J. Respir. Crit. Care Med.,* 179(11), pp. 1011-1021.

Rinaldi, M., Lehouck, A., Heulens, N., Lavend'homme, R., Carlier, V., Saint-Remy, J. M., Decramer, M., Gayan-Ramirez, G. and Janssens, W. (2012) 'Antielastin B-cell and T-cell immunity in patients with chronic obstructive pulmonary disease', *Thorax,* 67(8), pp. 694-700.

Ritter, M., Mennerich, D., Weith, A. and Seither, P. (2005) 'Characterization of Toll-like receptors in primary lung epithelial cells: strong impact of the TLR3 ligand poly(I:C) on the regulation of Toll-like receptors, adaptor proteins and inflammatory response', *J Inflamm (Lond),* 2, pp. 16.

Robbins, D. J., Zhen, E., Owaki, H., Vanderbilt, C. A., Ebert, D., Geppert, T. D. and Cobb, M. H. (1993) 'Regulation and properties of extracellular signal-regulated protein kinases 1 and 2 in vitro', *J Biol Chem,* 268(7), pp. 5097-106.

Rosseau, S., Selhorst, J., Wiechmann, K., Leissner, K., Maus, U., Mayer, K., Grimminger, F., Seeger, W. and Lohmeyer, J. (2000) 'Monocyte migration through the alveolar epithelial barrier: adhesion molecule mechanisms and impact of chemokines', *J Immunol,* 164(1), pp. 427-35.

Rovin, B. H., Lu, L. and Saxena, R. (1999) 'A novel polymorphism in the MCP-1 gene regulatory region that influences MCP-1 expression', *Biochem Biophys Res Commun,* 259(2), pp. 344-8.

Rutgers, S. R., Postma, D. S., ten Hacken, N. H., Kauffman, H. F., van Der Mark, T. W., Koeter, G. H. and Timens, W. (2000a) 'Ongoing airway inflammation in patients with COPD who do not currently smoke', *Thorax,* 55(1), pp. 12-8.

Rutgers, S. R., Timens, W., Kaufmann, H. F., van der Mark, T. W., Koeter, G. H. and Postma, D. S. (2000b) 'Comparison of induced sputum with bronchial wash, bronchoalveolar lavage and bronchial biopsies in COPD', *Eur Respir J,* 15(1), pp. 109-15.

Sabroe, I., Dower, S. K. and Whyte, M. K. (2005) 'The role of Toll-like receptors in the regulation of neutrophil migration, activation, and apoptosis', *Clin Infect Dis,* 41 Suppl 7, pp. S421-6.

Sabroe, I., Whyte, M. K., Wilson, A. G., Dower, S. K., Hubbard, R. and Hall, I. (2004) 'Toll-like receptor (TLR) 4 polymorphisms and COPD', *Thorax,* 59(1), pp. 81.

Saccani, S., Pantano, S. and Natoli, G. (2002) 'p38-Dependent marking of inflammatory genes for increased NF-kappa B recruitment', *Nat Immunol,* 3(1), pp. 69-75.

Saetta, M., Baraldo, S., Corbino, L., Turato, G., Braccioni, F., Rea, F., Cavallesco, G., Tropeano, G., Mapp, C. E., Maestrelli, P., Ciaccia, A. and Fabbri, L. M. (1999) 'CD8+ve cells in the lungs of smokers with chronic obstructive pulmonary disease', *Am J Respir Crit Care Med,* 160(2), pp. 711-7.

Saetta, M., Baraldo, S., Turato, G., Beghe, B., Casoni, G. L., Bellettato, C. M., Rea, F., Zuin, R., Fabbri, L. M. and Papi, A. (2003) 'Increased proportion of CD8+ T-lymphocytes in the paratracheal lymph nodes of smokers with mild COPD', *Sarcoidosis Vasc Diffuse Lung Dis,* 20(1), pp. 28-32.

Saetta, M., Di Stefano, A., Maestrelli, P., Ferraresso, A., Drigo, R., Potena, A., Ciaccia, A. and Fabbri, L. M. (1993) 'Activated T-lymphocytes and macrophages in bronchial mucosa of subjects with chronic bronchitis', *Am Rev Respir Dis,* 147(2), pp. 301-6.

Saetta, M., Di Stefano, A., Maestrelli, P., Turato, G., Ruggieri, M. P., Roggeri, A., Calcagni, P., Mapp, C. E., Ciaccia, A. and Fabbri, L. M. (1994) 'Airway eosinophilia in chronic bronchitis during exacerbations', *Am J Respir Crit Care Med,* 150(6 Pt 1), pp. 1646-52.

Saetta, M., Di Stefano, A., Turato, G., Facchini, F. M., Corbino, L., Mapp, C. E., Maestrelli, P., Ciaccia, A. and Fabbri, L. M. (1998) 'CD8+ T-lymphocytes in peripheral airways of smokers with chronic obstructive pulmonary disease', *Am J Respir Crit Care Med,* 157(3 Pt 1), pp. 822-6.

Saetta, M., Ghezzo, H., Kim, W. D., King, M., Angus, G. E., Wang, N. S. and Cosio, M. G. (1985) 'Loss of alveolar attachments in smokers. A morphometric correlate of lung function impairment', *Am Rev Respir Dis,* 132(4), pp. 894-900.

Saetta, M., Turato, G., Facchini, F. M., Corbino, L., Lucchini, R. E., Casoni, G., Maestrelli, P., Mapp, C. E., Ciaccia, A. and Fabbri, L. M. (1997) 'Inflammatory cells in the bronchial glands of smokers with chronic bronchitis', *Am J Respir Crit Care Med,* 156(5), pp. 1633-9.

Sahlander, K., Larsson, K. and Palmberg, L. (2010) 'Altered innate immune response in farmers and smokers', *Innate Immun,* 16(1), pp. 27-38.

Sajjan, U., Ganesan, S., Comstock, A. T., Shim, J., Wang, Q., Nagarkar, D. R., Zhao, Y., Goldsmith, A. M., Sonstein, J., Linn, M. J., Curtis, J. L. and Hershenson, M. B. (2009) 'Elastase- and LPS-exposed mice display altered responses to rhinovirus infection', *Am J Physiol Lung Cell Mol Physiol,* 297(5), pp. L931-44.

Sajjan, U. S., Jia, Y., Newcomb, D. C., Bentley, J. K., Lukacs, N. W., LiPuma, J. J. and Hershenson, M. B. (2006) 'H. influenzae potentiates airway epithelial cell responses to rhinovirus by increasing ICAM-1 and TLR3 expression', *FASEB J,* 20(12), pp. 2121-3.

Salituro, F. G., Germann, U. A., Wilson, K. P., Bemis, G. W., Fox, T. and Su, M. S. (1999) 'Inhibitors of p38 MAP kinase: therapeutic intervention in cytokine-mediated diseases', *Curr Med Chem,* 6(9), pp. 807-23.

Sampsonas, F., Karkoulias, K., Kaparianos, A. and Spiropoulos, K. (2006) 'Genetics of chronic obstructive pulmonary disease, beyond a1-antitrypsin deficiency', *Curr Med Chem,* 13(24), pp. 2857-73.

Scheel-Toellner, D., Wang, K., Assi, L. K., Webb, P. R., Craddock, R. M., Salmon, M. and Lord, J. M. (2004) 'Clustering of death receptors in lipid rafts initiates neutrophil spontaneous apoptosis', *Biochem Soc Trans,* 32(Pt 5), pp. 679-81.

Schreiber, S., Feagan, B., D'Haens, G., Colombel, J. F., Geboes, K., Yurcov, M., Isakov, V., Golovenko, O., Bernstein, C. N., Ludwig, D., Winter, T., Meier, U., Yong, C. and Steffgen, J. (2006) 'Oral p38 mitogen-activated protein kinase inhibition with BIRB 796 for active

Crohn's disease: a randomized, double-blind, placebo-controlled trial', *Clin Gastroenterol Hepatol,* 4(3), pp. 325-34.

Schulz, C., Petrig, V., Wolf, K., Kratzel, K., Kohler, M., Becker, B. and Pfeifer, M. (2003) 'Upregulation of MCAM in primary bronchial epithelial cells from patients with COPD', *Eur Respir J,* 22(3), pp. 450-6.

Schumann, R. R., Leong, S. R., Flaggs, G. W., Gray, P. W., Wright, S. D., Mathison, J. C., Tobias, P. S. and Ulevitch, R. J. (1990) 'Structure and function of lipopolysaccharide binding protein', *Science,* 249(4975), pp. 1429-31.

Seemungal, T. A., Wilkinson, T. M., Hurst, J. R., Perera, W. R., Sapsford, R. J. and Wedzicha, J. A. (2008) 'Long-term erythromycin therapy is associated with decreased chronic obstructive pulmonary disease exacerbations', *Am J Respir Crit Care Med,* 178(11), pp. 1139-47.

Segura-Valdez, L., Pardo, A., Gaxiola, M., Uhal, B. D., Becerril, C. and Selman, M. (2000) 'Upregulation of gelatinases A and B, collagenases 1 and 2, and increased parenchymal cell death in COPD', *Chest,* 117(3), pp. 684-694.

Selness, S. R., Devraj, R. V., Devadas, B., Walker, J. K., Boehm, T. L., Durley, R. C., Shieh, H., Xing, L., Rucker, P. V., Jerome, K. D., Benson, A. G., Marrufo, L. D., Madsen, H. M., Hitchcock, J., Owen, T. J., Christie, L., Promo, M. A., Hickory, B. S., Alvira, E., Naing, W., Blevis-Bal, R., Messing, D., Yang, J., Mao, M. K., Yalamanchili, G., Vonder Embse, R., Hirsch, J., Saabye, M., Bonar, S., Webb, E., Anderson, G. and Monahan, J. B. (2011) 'Discovery of PH-797804, a highly selective and potent inhibitor of p38 MAP kinase', *Bioorg Med Chem Lett,* 21(13), pp. 4066-71.

Sethi, S. (2000a) 'Bacterial infection and the pathogenesis of COPD', *Chest,* 117(5 Suppl 1), pp. 286S-91S.

Sethi, S. (2000b) 'Infectious etiology of acute exacerbations of chronic bronchitis', *Chest,* 117(5 Suppl 2), pp. 380S-5S.

Sethi, S., Evans, N., Grant, B. J. and Murphy, T. F. (2002) 'New strains of bacteria and exacerbations of chronic obstructive pulmonary disease', *N Engl J Med,* 347(7), pp. 465-71.

Shan, M., Yuan, X., Song, L. Z., Roberts, L., Zarinkamar, N., Seryshev, A., Zhang, Y., Hilsenbeck, S., Chang, S. H., Dong, C., Corry, D. B. and Kheradmand, F. (2012) 'Cigarette smoke induction of osteopontin (SPP1) mediates T(H)17 inflammation in human and experimental emphysema', *Sci Transl Med,* 4(117), pp. 117ra9.

Shapiro, S. D. and Ingenito, E. P. (2005) 'The pathogenesis of chronic obstructive pulmonary disease: advances in the past 100 years', *Am. J. Respir. Cell Mol. Biol.,* 32(5), pp. 367-372.

Shaykhiev, R., Krause, A., Salit, J., Strulovici-Barel, Y., Harvey, B. G., O'Connor, T. P. and Crystal, R. G. (2009) 'Smoking-dependent reprogramming of alveolar macrophage polarization: implication for pathogenesis of chronic obstructive pulmonary disease', *J Immunol,* 183(4), pp. 2867-83.

Shuto, T., Imasato, A., Jono, H., Sakai, A., Xu, H., Watanabe, T., Rixter, D. D., Kai, H., Andalibi, A., Linthicum, F., Guan, Y. L., Han, J., Cato, A. C., Lim, D. J., Akira, S. and Li, J. D. (2002) 'Glucocorticoids synergistically enhance nontypeable Haemophilus influenzae-induced Toll-like receptor 2 expression via a negative cross-talk with p38 MAP kinase', *J Biol Chem,* 277(19), pp. 17263-70.

Simon, G. C., Martin, R. J., Smith, S., Thaikoottathil, J., Bowler, R. P., Barenkamp, S. J. and Chu, H. W. (2011) 'Up-regulation of MUC18 in airway epithelial cells by IL-13: implications in bacterial adherence', *Am J Respir Cell Mol Biol,* 44(5), pp. 606-13.

Sims, M. W., Tal-Singer, R. M., Kierstein, S., Musani, A. I., Beers, M. F., Panettieri, R. A. and Haczku, A. (2008) 'Chronic obstructive pulmonary disease and inhaled steroids alter surfactant protein D (SP-D) levels: a cross-sectional study', *Respir Res,* 9, pp. 13.

Singh, D., Siew, L., Christensen, J., Plumb, J., Clarke, G. W., Greenaway, S., Perros-Huguet, C., Clarke, N., Kilty, I. and Tan, L. (2015) 'Oral and inhaled p38 MAPK inhibitors: effects on inhaled LPS challenge in healthy subjects', *Eur J Clin Pharmacol,* 71(10), pp. 1175-84.

Singh, D., Smyth, L., Borrill, Z., Sweeney, L. and Tal-Singer, R. (2010) 'A randomized, placebo-controlled study of the effects of the p38 MAPK inhibitor SB-681323 on blood biomarkers of inflammation in COPD patients', *J Clin Pharmacol,* 50(1), pp. 94-100.

Singh, S., Amin, A. V. and Loke, Y. K. (2009) 'Long-term use of inhaled corticosteroids and the risk of pneumonia in chronic obstructive pulmonary disease: a meta-analysis', *Arch Intern Med,* 169(3), pp. 219-29.

Skinner, N. A., MacIsaac, C. M., Hamilton, J. A. and Visvanathan, K. (2005) 'Regulation of Toll-like receptor (TLR)2 and TLR4 on CD14dimCD16+ monocytes in response to sepsis-related antigens', *Clin Exp Immunol,* 141(2), pp. 270-8.

Slinger, R., Chan, F., Ferris, W., Yeung, S. W., St Denis, M., Gaboury, I. and Aaron, S. D. (2006) 'Multiple combination antibiotic susceptibility testing of nontypeable Haemophilus influenzae biofilms', *Diagn. Microbiol. Infect. Dis.,* 56(3), pp. 247-253.

Smit, L. A., Heederik, D., Doekes, G., Krop, E. J., Rijkers, G. T. and Wouters, I. M. (2009) 'Ex vivo cytokine release reflects sensitivity to occupational endotoxin exposure', *Eur Respir J,* 34(4), pp. 795-802.

Smith, S. J., Fenwick, P. S., Nicholson, A. G., Kirschenbaum, F., Finney-Hayward, T. K., Higgins, L. S., Giembycz, M. A., Barnes, P. J. and Donnelly, L. E. (2006) 'Inhibitory effect of p38 mitogen-activated protein kinase inhibitors on cytokine release from human macrophages', *Br J Pharmacol,* 149(4), pp. 393-404.

Snell, N., Foster, M. and Vestbo, J. (2013) 'Efficacy and safety of AZD1981, a CRTH2 receptor antagonist, in patients with moderate to severe COPD', *Respir Med,* 107(11), pp. 1722-30.

Soler, N., Torres, A., Ewig, S., Gonzalez, J., Celis, R., El-Ebiary, M., Hernandez, C. and 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), pp. 1498-505.

Soloaga, A., Thomson, S., Wiggin, G. R., Rampersaud, N., Dyson, M. H., Hazzalin, C. A., Mahadevan, L. C. and Arthur, J. S. (2003) 'MSK2 and MSK1 mediate the mitogen- and

stress-induced phosphorylation of histone H3 and HMG-14', *EMBO J,* 22(11), pp. 2788-97.

Soltani, A., Ewe, Y. P., Lim, Z. S., Sohal, S. S., Reid, D., Weston, S., Wood-Baker, R. and Walters, E. H. (2012) 'Mast cells in COPD airways: relationship to bronchodilator responsiveness and angiogenesis', *Eur Respir J,* 39(6), pp. 1361-7.

Soriano, J. B., Sin, D. D., Zhang, X., Camp, P. G., Anderson, J. A., Anthonisen, N. R., Buist, A. S., Burge, P. S., Calverley, P. M., Connett, J. E., Petersson, S., Postma, D. S., Szafranski, W. and Vestbo, J. (2007) 'A pooled analysis of FEV1 decline in COPD patients randomized to inhaled corticosteroids or placebo', *Chest,* 131(3), pp. 682-9.

Southworth, T., Metryka, A., Lea, S., Farrow, S., Plumb, J. and Singh, D. (2012) 'IFN-Î³ synergistically enhances LPS signalling in alveolar macrophages from COPD patients and controls by corticosteroid-resistant STAT1 activation', *Br. J. Pharmacol.,* 166(7), pp. 2070-2083.

Sower, L. E., Klimpel, G. R., Hanna, W. and Froelich, C. J. (1996) 'Extracellular activities of human granzymes. I. Granzyme A induces IL6 and IL8 production in fibroblast and epithelial cell lines', *Cell Immunol,* 171(1), pp. 159-63.

Spencer, S., Calverley, P. M., Burge, P. S. and Jones, P. W. (2004) 'Impact of preventing exacerbations on deterioration of health status in COPD', *Eur Respir J,* 23(5), pp. 698-702.

St Geme, J. W., 3rd (2002) 'Molecular and cellular determinants of non-typeable Haemophilus influenzae adherence and invasion', *Cell Microbiol,* 4(4), pp. 191-200.

Staples, K. J., Hinks, T. S., Ward, J. A., Gunn, V., Smith, C. and Djukanovic, R. (2012) 'Phenotypic characterization of lung macrophages in asthmatic patients: overexpression of CCL17', *J Allergy Clin Immunol,* 130(6), pp. 1404-12 e7.

Stewart, J. I. and Criner, G. J. (2013) 'The small airways in chronic obstructive pulmonary disease: pathology and effects on disease progression and survival', *Curr Opin Pulm Med,* 19(2), pp. 109-15.

Stockley, R. A. (1998) 'Lung infections. 1. Role of bacteria in the pathogenesis and progression of acute and chronic lung infection', *Thorax,* 53(1), pp. 58-62.

Stojadinovic, O. and Tomic-Canic, M. (2013) 'Human ex vivo wound healing model', *Methods Mol. Biol.,* 1037, pp. 255-264.

Stringer, K. A., Tobias, M., O'Neill, H. C. and Franklin, C. C. (2007) 'Cigarette smoke extract-induced suppression of caspase-3-like activity impairs human neutrophil phagocytosis', *Am J Physiol Lung Cell Mol Physiol,* 292(6), pp. L1572-9.

Strobl, H., Scheinecker, C., Csmarits, B., Majdic, O. and Knapp, W. (1995) 'Flow cytometric analysis of intracellular CD68 molecule expression in normal and malignant haemopoiesis', *Br J Haematol,* 90(4), pp. 774-82.

Suissa, S. and Barnes, P. J. (2009) 'Inhaled corticosteroids in COPD: the case against', *Eur Respir J,* 34(1), pp. 13-6.

Sukkar, M. B., Xie, S., Khorasani, N. M., Kon, O. M., Stanbridge, R., Issa, R. and Chung, K. F. (2006) 'Toll-like receptor 2, 3, and 4 expression and function in human airway smooth muscle', *J Allergy Clin Immunol,* 118(3), pp. 641-8.

Suzuki, M., Betsuyaku, T., Nagai, K., Fuke, S., Nasuhara, Y., Kaga, K., Kondo, S., Hamamura, I., Hata, J., Takahashi, H. and Nishimura, M. (2008) 'Decreased airway expression of vascular endothelial growth factor in cigarette smoke-induced emphysema in mice and COPD patients', *Inhal Toxicol,* 20(3), pp. 349-59.

Taggart, C. C., Greene, C. M., Carroll, T. P., O'Neill, S. J. and McElvaney, N. G. (2005) 'Elastolytic proteases: inflammation resolution and dysregulation in chronic infective lung disease', *Am J Respir Crit Care Med,* 171(10), pp. 1070-6.

Takeda, K. and Akira, S. (2005) 'Toll-like receptors in innate immunity', *Int Immunol,* 17(1), pp. 1-14.

Tandon, M. K., Phillips, M., Waterer, G., Dunkley, M., Comans, P. and Clancy, R. (2010) 'Oral immunotherapy with inactivated nontypeable Haemophilus influenzae reduces severity of acute exacerbations in severe COPD', *Chest,* 137(4), pp. 805-11.

Tashkin, D. P., Littner, M., Andrews, C. P., Tomlinson, L., Rinehart, M. and Denis-Mize, K. (2008) 'Concomitant treatment with nebulized formoterol and tiotropium in subjects with COPD: a placebo-controlled trial', *Respir Med,* 102(4), pp. 479-87.

Taylor, A. E., Finney-Hayward, T. K., Quint, J. K., Thomas, C. M., Tudhope, S. J., Wedzicha, J. A., Barnes, P. J. and Donnelly, L. E. (2010) 'Defective macrophage phagocytosis of bacteria in COPD', *Eur Respir J,* 35(5), pp. 1039-47.

Thompson, A. B., Daughton, D., Robbins, R. A., Ghafouri, M. A., Oehlerking, M. and Rennard, S. I. (1989) 'Intraluminal airway inflammation in chronic bronchitis. Characterization and correlation with clinical parameters', *Am Rev Respir Dis,* 140(6), pp. 1527-37.

Tobias, P. S., Soldau, K., Gegner, J. A., Mintz, D. and Ulevitch, R. J. (1995) 'Lipopolysaccharide binding protein-mediated complexation of lipopolysaccharide with soluble CD14', *J Biol Chem,* 270(18), pp. 10482-8.

Toews, G. B., Gross, G. N. and Pierce, A. K. (1979) 'The relationship of inoculum size to lung bacterial clearance and phagocytic cell response in mice', *Am Rev Respir Dis,* 120(3), pp. 559-66.

Travaglione, S., Falzano, L., Fabbri, A., Stringaro, A., Fais, S. and Fiorentini, C. (2002) 'Epithelial cells and expression of the phagocytic marker CD68: scavenging of apoptotic bodies following Rho activation', *Toxicol In Vitro,* 16(4), pp. 405-11.

Tsutsumi-Ishii, Y. and Nagaoka, I. (2003) 'Modulation of human beta-defensin-2 transcription in pulmonary epithelial cells by lipopolysaccharide-stimulated mononuclear phagocytes via proinflammatory cytokine production', *J Immunol,* 170(8), pp. 4226-36.

Tudhope, S. J., Finney-Hayward, T. K., Nicholson, A. G., Mayer, R. J., Barnette, M. S., Barnes, P. J. and Donnelly, L. E. (2008) 'Different mitogen-activated protein kinase-dependent cytokine responses in cells of the monocyte lineage', *J Pharmacol Exp Ther,* 324(1), pp. 306-12.

Turato, G., Di Stefano, A., Maestrelli, P., Mapp, C. E., Ruggieri, M. P., Roggeri, A., Fabbri, L. M. and Saetta, M. (1995) 'Effect of smoking cessation on airway inflammation in chronic bronchitis', *Am J Respir Crit Care Med,* 152(4 Pt 1), pp. 1262-7.

Ueta, M., Nochi, T., Jang, M. H., Park, E. J., Igarashi, O., Hino, A., Kawasaki, S., Shikina, T., Hiroi, T., Kinoshita, S. and Kiyono, H. (2004) 'Intracellularly expressed TLR2s and TLR4s contribution to an immunosilent environment at the ocular mucosal epithelium', *J Immunol,* 173(5), pp. 3337-47.

Underhill, D. M. and Ozinsky, A. (2002) 'Toll-like receptors: key mediators of microbe detection', *Curr Opin Immunol,* 14(1), pp. 103-10.

Utokaparch, S., Sze, M. A., Gosselink, J. V., McDonough, J. E., Elliott, W. M., Hogg, J. C. and Hegele, R. G. (2014) 'Respiratory viral detection and small airway inflammation in lung tissue of patients with stable, mild COPD', *COPD,* 11(2), pp. 197-203.

Vaitkus, M., Lavinskiene, S., Barkauskiene, D., Bieksiene, K., Jeroch, J. and Sakalauskas, R. (2013) 'Reactive oxygen species in peripheral blood and sputum neutrophils during bacterial and nonbacterial acute exacerbation of chronic obstructive pulmonary disease', *Inflammation,* 36(6), pp. 1485-93.

van der Strate, B. W., Postma, D. S., Brandsma, C. A., Melgert, B. N., Luinge, M. A., Geerlings, M., Hylkema, M. N., van den Berg, A., Timens, W. and Kerstjens, H. A. (2006) 'Cigarette smoke-induced emphysema: A role for the B cell?', *Am. J. Respir. Crit. Care Med.,* 173(7), pp. 751-758.

van Schilfgaarde, M., Eijk, P., Regelink, A., van Ulsen, P., Everts, V., Dankert, J. and van Alphen, L. (1999) 'Haemophilus influenzae localized in epithelial cell layers is shielded from antibiotics and antibody-mediated bactericidal activity', *Microb Pathog,* 26(5), pp. 249-62.

Varadaradjalou, S., Feger, F., Thieblemont, N., Hamouda, N. B., Pleau, J. M., Dy, M. and Arock, M. (2003) 'Toll-like receptor 2 (TLR2) and TLR4 differentially activate human mast cells', *Eur J Immunol,* 33(4), pp. 899-906.

Veeramachaneni, S. B. and Sethi, S. (2006) 'Pathogenesis of bacterial exacerbations of COPD', *COPD,* 3(2), pp. 109-15.

Verbeken, E. K., Cauberghs, M., Mertens, I., Lauweryns, J. M. and Van de Woestijne, K. P. (1992) 'Tissue and airway impedance of excised normal, senile, and emphysematous lungs', *J Appl Physiol,* 72(6), pp. 2343-53.

Vestbo, J., Hurd, S. S., Agusti, A. G., Jones, P. W., Vogelmeier, C., Anzueto, A., Barnes, P. J., Fabbri, L. M., Martinez, F. J., Nishimura, M., Stockley, R. A., Sin, D. D. and Rodriguez-Roisin, R. (2013) 'Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary', *Am. J. Respir. Crit. Care Med.,* 187(4), pp. 347-365.

Vestbo, J., Prescott, E. and Lange, P. (1996) 'Association of chronic mucus hypersecretion with FEV1 decline and chronic obstructive pulmonary disease morbidity. Copenhagen City Heart Study Group', *Am J Respir Crit Care Med,* 153(5), pp. 1530-5.

Vestbo, J., Sorensen, T., Lange, P., Brix, A., Torre, P. and Viskum, K. (1999) 'Long-term effect of inhaled budesonide in mild and moderate chronic obstructive pulmonary disease: a randomised controlled trial', *Lancet,* 353(9167), pp. 1819-23.

Vestergaard, P., Rejnmark, L. and Mosekilde, L. (2007) 'Fracture risk in patients with chronic lung diseases treated with bronchodilator drugs and inhaled and oral corticosteroids', *Chest,* 132(5), pp. 1599-607.

Vijayanand, P., Durkin, K., Hartmann, G., Morjaria, J., Seumois, G., Staples, K. J., Hall, D., Bessant, C., Bartholomew, M., Howarth, P. H., Friedmann, P. S. and Djukanovic, R. (2010) 'Chemokine receptor 4 plays a key role in T cell recruitment into the airways of asthmatic patients', *J Immunol,* 184(8), pp. 4568-74.

Vijayanand, P., Seumois, G., Pickard, C., Powell, R. M., Angco, G., Sammut, D., Gadola, S. D., Friedmann, P. S. and Djukanovic, R. (2007) 'Invariant natural killer T cells in asthma and chronic obstructive pulmonary disease', *N Engl J Med,* 356(14), pp. 1410-22.

Virchow, J. C. (2004) 'Guidelines versus clinical practice--which therapy and which device?', *Respir Med,* 98 Suppl B, pp. S28-34.

Vlachaki, E. M., Koutsopoulos, A. V., Tzanakis, N., Neofytou, E., Siganaki, M., Drositis, I., Moniakis, A., Schiza, S., Siafakas, N. M. and Tzortzaki, E. G. (2010) 'Altered surfactant protein-A expression in type II pneumocytes in COPD', *Chest,* 137(1), pp. 37-45.

Vlahovic, G., Russell, M. L., Mercer, R. R. and Crapo, J. D. (1999) 'Cellular and connective tissue changes in alveolar septal walls in emphysema', *Am J Respir Crit Care Med,* 160(6), pp. 2086-92.

von Scheele, I., Larsson, K. and Palmberg, L. (2010) 'Budesonide enhances Toll-like receptor 2 expression in activated bronchial epithelial cells', *Inhal Toxicol,* 22(6), pp. 493-9.

Wallace, A. M., Loy, L. B., Abboud, R. T., D'Armiento, J. M., Coxson, H. O., Muller, N. L., Kalloger, S., Li, X., Mark Elliott, W., English, J. C., Finley, R. J. and Pare, P. D. (2014) 'Expression of matrix metalloproteinase-1 in alveolar macrophages, type II pneumocytes, and airways in smokers: relationship to lung function and emphysema', *Lung,* 192(4), pp. 467-72.

Wallace, A. M., Sandford, A. J., English, J. C., Burkett, K. M., Li, H., Finley, R. J., Muller, N. L., Coxson, H. O., Pare, P. D. and Abboud, R. T. (2008) 'Matrix metalloproteinase expression by human alveolar macrophages in relation to emphysema', *COPD,* 5(1), pp. 13-23.

Walters, J. A., Tan, D. J., White, C. J., Gibson, P. G., Wood-Baker, R. and Walters, E. H. (2014) 'Systemic corticosteroids for acute exacerbations of chronic obstructive pulmonary disease', *Cochrane Database Syst Rev,* 9, pp. CD001288.

Wang, J., Urbanowicz, R. A., Tighe, P. J., Todd, I., Corne, J. M. and Fairclough, L. C. (2013) 'Differential activation of killer cells in the circulation and the lung: a study of current smoking status and chronic obstructive pulmonary disease (COPD)', *PLoS One,* 8(3), pp. e58556.

Wang, L., Zhang, B., Li, Z., Li, J., Liu, Q. and Sun, W. (2015) 'Budesonide mitigates pathological changes in animal model Of COPD through reducing neutrophil elastase expression', *Int J Clin Exp Med,* 8(4), pp. 5227-35.

Wang, R. D., Tai, H., Xie, C., Wang, X., Wright, J. L. and Churg, A. (2003) 'Cigarette smoke produces airway wall remodeling in rat tracheal explants', *Am J Respir Crit Care Med,* 168(10), pp. 1232-6.

Wang, Z., Harkins, P. C., Ulevitch, R. J., Han, J., Cobb, M. H. and Goldsmith, E. J. (1997) 'The structure of mitogen-activated protein kinase p38 at 2.1-A resolution', *Proc. Natl. Acad. Sci. U.S.A.,* 94(6), pp. 2327-2332.

Wedzicha, J. A. and Donaldson, G. C. (2003) 'Exacerbations of chronic obstructive pulmonary disease', *Respir Care,* 48(12), pp. 1204-13; discussion 1213-5.

Wedzicha, J. A., Seemungal, T. A., MacCallum, P. K., Paul, E. A., Donaldson, G. C., Bhowmik, A., Jeffries, D. J. and Meade, T. W. (2000) 'Acute exacerbations of chronic obstructive pulmonary disease are accompanied by elevations of plasma fibrinogen and serum IL-6 levels', *Thromb. Haemost.,* 84(2), pp. 210-215.

Wen, Y., Reid, D. W., Zhang, D., Ward, C., Wood-Baker, R. and Walters, E. H. (2010) 'Assessment of airway inflammation using sputum, BAL, and endobronchial biopsies in current and ex-smokers with established COPD', *Int J Chron Obstruct Pulmon Dis,* 5, pp. 327-334.

White, A. J., Gompertz, S., Bayley, D. L., Hill, S. L., O'Brien, C., Unsal, I. and Stockley, R. A. (2003) 'Resolution of bronchial inflammation is related to bacterial eradication following treatment of exacerbations of chronic bronchitis', *Thorax,* 58(8), pp. 680-5.

Willemse, B. W., ten Hacken, N. H., Rutgers, B., Lesman-Leegte, I. G., Postma, D. S. and Timens, W. (2005) 'Effect of 1-year smoking cessation on airway inflammation in COPD and asymptomatic smokers', *Eur Respir J,* 26(5), pp. 835-45.

Williams, A. S., Issa, R., Durham, A., Leung, S. Y., Kapoun, A., Medicherla, S., Higgins, L. S., Adcock, I. M. and Chung, K. F. (2008) 'Role of p38 mitogen-activated protein kinase in ozone-induced airway hyperresponsiveness and inflammation', *Eur J Pharmacol,* 600(1-3), pp. 117-22.

Wilson, R., Roberts, D. and Cole, P. (1985) 'Effect of bacterial products on human ciliary function in vitro', *Thorax,* 40(2), pp. 125-31.

Winder, A. A., Wohlford-Lenane, C., Scheetz, T. E., Nardy, B. N., Manzel, L. J., Look, D. C. and McCray, P. B., Jr. (2009) 'Differential effects of cytokines and corticosteroids on toll-like receptor 2 expression and activity in human airway epithelia', *Respir Res,* 10, pp. 96.

Witherden, I. R., Vanden Bon, E. J., Goldstraw, P., Ratcliffe, C., Pastorino, U. and Tetley, T. D. (2004) 'Primary human alveolar type II epithelial cell chemokine release: effects of cigarette smoke and neutrophil elastase', *Am J Respir Cell Mol Biol,* 30(4), pp. 500-9.

Wright, J. L. (2001) 'The importance of ultramicroscopic emphysema in cigarette smoke-induced lung disease', *Lung,* 179(2), pp. 71-81.

Wright, J. L. and Churg, A. (1990) 'Cigarette smoke causes physiologic and morphologic changes of emphysema in the guinea pig', *Am. Rev. Respir. Dis.,* 142(6 Pt 1), pp. 1422-1428.

Wright, J. L., Postma, D. S., Kerstjens, H. A., Timens, W., Whittaker, P. and Churg, A. (2007) 'Airway remodeling in the smoke exposed guinea pig model', *Inhal Toxicol,* 19(11), pp. 915-923.

Xing, L., Devadas, B., Devraj, R. V., Selness, S. R., Shieh, H., Walker, J. K., Mao, M., Messing, D., Samas, B., Yang, J. Z., Anderson, G. D., Webb, E. G. and Monahan, J. B. (2012) 'Discovery and characterization of atropisomer PH-797804, a p38 MAP kinase inhibitor, as a clinical drug candidate', *ChemMedChem,* 7(2), pp. 273-80.

Xing, L., Shieh, H. S., Selness, S. R., Devraj, R. V., Walker, J. K., Devadas, B., Hope, H. R., Compton, R. P., Schindler, J. F., Hirsch, J. L., Benson, A. G., Kurumbail, R. G., Stegeman, R. A., Williams, J. M., Broadus, R. M., Walden, Z. and Monahan, J. B. (2009) 'Structural Bioinformatics-Based Prediction of Exceptional Selectivity of p38 MAP Kinase Inhibitor PH-797804', *Biochemistry,* 48(27), pp. 6402-6411.

Xu, F., Xu, Z., Zhang, R., Wu, Z., Lim, J. H., Koga, T., Li, J. D. and Shen, H. (2008) 'Nontypeable Haemophilus influenzae induces COX-2 and PGE2 expression in lung epithelial cells via activation of p38 MAPK and NF-kappa B', *Respir Res,* 9, pp. 16.

Yanagi, Y., Yoshikai, Y., Leggett, K., Clark, S. P., Aleksander, I. and Mak, T. W. (1984) 'A human T cell-specific cDNA clone encodes a protein having extensive homology to immunoglobulin chains', *Nature,* 308(5955), pp. 145-9.

Yanai, M., Sekizawa, K., Ohrui, T., Sasaki, H. and Takishima, T. (1992) 'Site of airway obstruction in pulmonary disease: direct measurement of intrabronchial pressure', *J Appl Physiol (1985),* 72(3), pp. 1016-23.

Young, P. R., McLaughlin, M. M., Kumar, S., Kassis, S., Doyle, M. L., McNulty, D., Gallagher, T. F., Fisher, S., McDonnell, P. C., Carr, S. A., Huddleston, M. J., Seibel, G., Porter, T. G., Livi, G. P., Adams, J. L. and Lee, J. C. (1997) 'Pyridinyl imidazole inhibitors of p38 mitogen-activated protein kinase bind in the ATP site', *J Biol Chem,* 272(18), pp. 12116-21.

Zhang, X. J., He, S. H., Xie, H., Wei, J. F. and Liu, C. T. (2004) 'Correlations between chymase activity, levels of tryptase, and eotaxin in the sputum of patients with chronic obstructive pulmonary disease and their clinical implications', *Zhonghua Jie He He Hu Xi Za Zhi,* 27(10), pp. 649-53.

Zhou, X., Baron, R. M., Hardin, M., Cho, M. H., Zielinski, J., Hawrylkiewicz, I., Sliwinski, P., Hersh, C. P., Mancini, J. D., Lu, K., Thibault, D., Donahue, A. L., Klanderman, B. J., Rosner, B., Raby, B. A., Lu, Q., Geldart, A. M., Layne, M. D., Perrella, M. A., Weiss, S. T., Choi, A. M. and Silverman, E. K. (2012) 'Identification of a chronic obstructive pulmonary disease genetic determinant that regulates HHIP', *Hum. Mol. Genet.,* 21(6), pp. 1325-1335.

Zhu, J., Qiu, Y. S., Majumdar, S., Gamble, E., Matin, D., Turato, G., Fabbri, L. M., Barnes, N., Saetta, M. and Jeffery, P. K. (2001) 'Exacerbations of Bronchitis: bronchial eosinophilia and gene expression for interleukin-4, interleukin-5, and eosinophil chemoattractants', *Am J Respir Crit Care Med,* 164(1), pp. 109-16.

Zhu, X., Gadgil, A. S., Givelber, R., George, M. P., Stoner, M. W., Sciurba, F. C. and Duncan, S. R. (2009) 'Peripheral T cell functions correlate with the severity of chronic obstructive pulmonary disease', *J Immunol,* 182(5), pp. 3270-7.