

Defective Innate Immunity in Chronic Obstructive Pulmonary Disease

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By

Dr Catherine MR Thomas

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Airway Disease Section
National Heart and Lung Institute
Imperial College London
Dovehouse Street
London SW3 6LY

Abstract

Chronic obstructive pulmonary disease (COPD) is a chronic respiratory disease, comprising chronic bronchitis, small airways fibrosis and emphysema. The primary risk factor for developing COPD, in industrialised nations is cigarette smoking. Exacerbations of COPD, of which approximately half are due to bacterial infection, are associated with worsening quality of life, more rapid decline in FEV₁ and increased mortality. In healthy individuals, alveolar macrophages (AM) clear inhaled bacterial pathogens from the lung by phagocytosis, resulting in sterility of the lower respiratory tract. However, COPD patients have increased bacterial colonisation of the lower airways compared to healthy smokers and non-smokers. The increased rates of bacterial colonisation in COPD, suggests that there may be a mechanistic defect in the clearance of bacteria by phagocytic cells.

The main aim of this thesis was to investigate the hypothesis that defective innate immunity in COPD patients results in reduction in bacterial phagocytosis, with increased frequency of acute exacerbation, and that this defective phagocytosis may be explained by instability of microtubules. Phagocytic ability of both macrophages and neutrophils from COPD patients was compared to age-matched, non-smokers and smokers. Monocyte-derived-macrophages (MDMs) were used as a model of AM. Both MDMs and neutrophils from all subject groups displayed equivalent phagocytosis of inert beads, showing that cells from all subject groups are capable of phagocytosis. However, both MDMs and neutrophils from smokers and COPD patients showed reduced uptake of both *Haemophilus influenzae* (by 48%, $p < 0.001$ and 28%, $p < 0.01$ respectively) and *Streptococcus pneumoniae* (by 52%, $p < 0.001$ and 32%, $p < 0.05$ respectively). Whilst MDMs showed defective phagocytosis of bacteria, intracellular killing remained intact. When COPD patients were divided into those with a history of frequent ($\geq 2/y$) or infrequent exacerbations ($< 1/y$), frequent exacerbators had significantly reduced phagocytosis of bacteria compared to infrequent exacerbators. No differences were seen when phagocytosis at baseline was compared to phagocytosis at times of exacerbation.

As COPD patients appear to have defective phagocytosis, and with recent meta-analyses showing an increased risk of pneumonia with fluticasone propionate (FP), the

effects of both budesonide (BUD) and FP on phagocytosis by MDMs and neutrophils from COPD patients was assessed. No differences were found in phagocytosis of bacteria by MDMs in the presence of either steroid, or in the ability of these cells to perform functions of intracellular killing. In the presence of BUD, neutrophils showed significantly improved uptake of *H. influenzae* (with a maximal effect of 67%, $p < 0.05$), but neither FP nor BUD had any impact on phagocytosis of beads or *S. pneumoniae*.

Further investigation into the mechanisms underlying defective phagocytosis revealed increased susceptibility of COPD MDMs to microtubule disruption. Associated with this finding was a reduced level of acetylated tubulin in COPD MDMs. Addition of a microtubule stabiliser increased acetylated tubulin and significantly increased bacterial phagocytosis (maximal increase of 20% and 40% in phagocytosis of *Haemophilus influenzae* and *Streptococcus pneumoniae* respectively). In contrast, neutrophils displayed no differences in acetylated tubulin and showed no improvement in phagocytosis after exposure to microtubule stabilisers, suggesting an alternative mechanistic defect in neutrophils compared to MDMs. Acetylated microtubules are deacetylated by the enzymes HDAC6 and SIRT2. Exposure to the deacetylase inhibitors, tubacin (HDAC6 inhibitor) or AGK2 (SIRT2 inhibitor), led to increases in levels of acetylation of tubulin but no improvements in phagocytosis, whilst knockdown of either HDAC6 or SIRT2 revealed a similar picture, with increased acetylation but no improvements in phagocytosis. These findings suggest that increased acetylation of tubulin alone is not sufficient to improve phagocytosis, but rather that the defect in phagocytosis is related to microtubule instability. Knockdown of C6orf134, a newly discovered tubulin acetyl-transferase, in healthy MDMs led to reductions in levels of acetylated tubulin and reduced bacterial, but not inert bead, phagocytosis, mimicking the defect seen in COPD MDMs. This suggests that alterations in activity or expression of this protein may account for the defective phagocytosis seen in COPD MDMs. Improving phagocytosis by stabilisation of microtubules may therefore lead to reduced levels of bacterial colonisation and improved exacerbation frequency in COPD patients.

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I confirm that I have performed all experiments and analyses reported in this thesis.
Catherine MR Thomas

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List of Abbreviations

ACD	Acid citrate dextran
AM	Alveolar macrophage
ANOVA	Analysis of variance
BAL	Bronchoalveolar lavage
BHI	Brain heart infusion
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Copy deoxyribonucleic acid
CFU	Colony forming units
CCL	Chemokine (C-C) motif ligand
COPD	Chronic obstructive pulmonary disease
CR	Complement receptor
CXCL	Chemokine (C-X-C motif) ligand
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
D-PBS	Dulbecco's phosphate buffered saline
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FcR	Fc receptor
FCS	Foetal calf serum
FEV ₁	Forced expiratory volume in 1 second
FITC	Fluorescein isothiocyanate

FSC	Forward scatter
FVC	Forced vital capacity
GM-CSF	Granulocyte macrophage-colony stimulating factor
GOLD	Global initiative for Obstructive Lung Diseases
GRO α	Growth-related oncogene protein α
HBSS	Hanks' balanced salt solution
HDAC	Histone deacetylase
HI	<i>Haemophilus influenzae</i>
HLA-DR	Human leukocyte antigen DR-1
HRP	Horseradish peroxidase
IFN- γ	Interferon- γ
Ig	Immunoglobulin
ICAM	Intercellular adhesion molecule
ICS	Inhaled glucocorticosteroids
IL	Interleukin
L-glu	L-glutamine
LPS	Lipopolysaccharide
LTB ₄	Leukotriene B ₄
MAPK	Mitogen activated protein kinase
MCP	Monocyte chemotactic protein
MDMs	Monocyte-derived macrophages
MFI	Median fluorescent intensity
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MOPS	3-(N-morpholino) propane sulphonic acid
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
MR	Mannose receptor
MTT	Methylthiazolyldiphenyl-tetrazolium bromide

NAD ⁺	Nicotinamide adenine dinucleotide ⁺
NE	Neutrophil elastase
<i>N</i> -fMLP	<i>N</i> -formyl-methionyl-leucyl-phenyl-alanine
NF-κB	Nuclear factor κB
NHS	National Health Service
NS	Never smoker
NTHI	Non-typeable <i>Haemophilus influenzae</i>
O ₂ ⁻	Superoxide ion
OD	Optical density
PAB	PBS supplemented with BSA and azide
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PE	Phycoerythrin
PI3K	Phosphatidyl inositol-3 kinase
PMN	Polymorphonucleocyte (granulocyte)/neutrophil
PRR	Pattern-recognition receptors
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Room temperature
Rt-PCR	Real time polymerase chain reaction
S	Smoker
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SiRNA	Small interfering RNA
SIRT	Sirtuin
SP	<i>Streptococcus pneumoniae</i>
SR	Scavenger receptor
SSC	Side scatter

α TAT	α -Tubulin acetyl-transferase
TGF β	Transforming growth factor β
THY	Todd Hewitt broth with yeast extract
TLR	Toll like receptor
TNF α	Tumour necrosis factor- α
Tris	Trizma base
Tris-HCL	Tris hydrochloride
v/v	Volume by volume
w/v	Weight by volume
λ	Wavelength

Chapter 1

INTRODUCTION

1.1 Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is an increasing global health problem, predicted to become the third commonest cause of death and the fifth most common burden of disease worldwide by 2020 (Lopez and Murray, 1998).

1.1.1 Definition of COPD

The Global Initiative for Obstructive Lung Disease (GOLD) definition states that *“Chronic Obstructive Pulmonary Disease (COPD) is a common, preventable and treatable disease, characterized by persistent airflow limitation that is not fully reversible, is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases. Exacerbations and co-morbidities contribute to the overall severity in individual patients.”* (GOLD, 2011).

This condition is diagnosed using spirometry, whereby a ratio of $FEV_1:FVC$ of $<70\%$, in patients with symptoms of dyspnoea, chronic cough and/or sputum production and a history of exposure to risk factors is indicative of COPD. Reduced lung function parameters correlate with disease severity, with GOLD stratifying COPD into four stages according to FEV_1 percent predicted (Table 1.1) (GOLD, 2011).

GOLD stage	FEV_1 % predicted values
I (Mild)	$>80\%$
II (Moderate)	50-80%
III (Severe)	30-50%
IV (Very severe)	$<30\%$

Table 1.1. Classification of severity of COPD

1.1.2 Epidemiology of COPD

COPD is currently the fifth commonest cause of death in England, resulting in more than 25,000 deaths per year (Halpin, 2011). Despite advances in the management of COPD over the last few decades, COPD mortality is increasing, which is in direct contrast to other chronic diseases including cancer, heart disease and stroke (Jemal *et al.*, 2005). The overall 5-year survival from diagnosis in mild COPD is 78% in men and 72% in women, although these survival figures drop in severe disease to 30% and 24% respectively (Soriano *et al.*, 2000). Not only is COPD associated with significant mortality, but the burden of the disease to both the patient and to society in general is high. In the 'Confronting COPD' survey, 80% of patients reported two or more symptoms (breathlessness, cough and sputum production) on all or most days (Rennard *et al.*, 2002). In the UK, COPD imposes a substantial financial burden on both the NHS and society, with direct costs, *i.e.* the provision of healthcare and medication, and indirect costs, *i.e.* loss of productivity and social security costs, estimated to be in excess of £1.2 billion per year, with costs correlating with severity of disease (Hilleman *et al.*, 2000; Fletcher *et al.*, 2011).

The global prevalence of COPD is estimated to be approximately 10% in adults aged ≥ 40 years; however, it is likely to be under-diagnosed, as an estimated 45-65% of patients with COPD do not seek medical attention as they perceive their symptoms to be "normal" (Halbert *et al.*, 2006; Halpin and Miravittles, 2006). In developed countries, approximately 95% of cases of COPD are cigarette-smoking related, with the remainder being secondary to occupational and environmental exposure (Barnes *et al.*, 2003). In parts of the developing world, the use of biomass fuel in home cooking is a major risk factor for the development of COPD (Zhang and Smith, 2007). However, even after 25 years of smoking, only ~20% of smokers develop COPD, suggesting an underlying genetic susceptibility (Rennard and Vestbo, 2006; Lundback *et al.*, 2003). COPD is also a risk factor for the development of systemic co-morbidities, including cardiovascular disease, depression, osteoporosis, muscle wasting and lung cancer (Barnes and Celli, 2009; Decramer *et al.*, 2008). Individuals with COPD who also have elevated circulating C-reactive protein (CRP), have been shown to have an increased risk of death from arrhythmias, acute myocardial infarction, stroke and pulmonary

embolism (Agusti *et al.*, 2003). Taken together, the evidence suggests that the contribution of COPD to the global health burden is enormous.

1.1.3 Pathophysiology of COPD

COPD can be described as comprising three distinct disease processes; (i) chronic bronchitis, defined by a productive cough for greater than three months on two consecutive years and characterised by mucus hypersecretion; (ii) small airways inflammation and remodelling; and (iii) emphysema with destruction of lung parenchyma, loss of lung elasticity and closure of small airways (GOLD, 2011). These processes contribute to the chronic airflow obstruction, as measured by a reduction in FEV₁ and in the ratio of FEV₁ to FVC. These distinct pathophysiological processes usually occur in combination in patients with COPD, however, the proportion of emphysema to obstructive bronchiolitis may differ between individuals with COPD, giving rise to the different phenotypes of COPD seen in clinical practice.

1.1.3.1 Mucus Hypersecretion

Accumulation of mucus in the airways results from increased production and secretion of mucus together with decreased mucociliary clearance (Burgel and Nadel, 2008; Randell and Boucher, 2006). Mucus hypersecretion occurs as a result of hyperplasia of epithelial goblet cells and hypertrophy of mucus glands (Innes *et al.*, 2006). This is predominant in the larger airways, with the volume of stored mucin correlating with reductions in the FEV₁/FVC ratio in smokers (Innes *et al.*, 2006). However, in contrast to healthy airways, mucus also accumulates in the lumen of small conducting airways, with the degree of mucus accumulation closely associated with severity of airflow obstruction in COPD (Hogg *et al.*, 2004). This accumulation of airway mucus originally led to the British hypothesis of the 1950-60s, whereby recurrent airway infection and mucus hyper-secretion was thought to cause COPD (FLETCHER, 1959). However, a subsequent study found insufficient evidence to support a role for bacterial infection in chronic bronchitis (Tager and Speizer, 1975). The British hypothesis was further refuted when tobacco smoking was shown to be the predominant cause of COPD (Fletcher and Peto, 1977). More recently, the presence of cough and sputum production in smokers with normal lung function has shown no

relationship to an increased risk of developing COPD, compared to the risk in healthy smokers (Vestbo and Lange, 2002). However, there is now increasing evidence to suggest that excessive mucus production and impaired mucociliary clearance do contribute to airway obstruction in COPD (Fahy and Dickey, 2010). Studies have shown that mucus hypersecretion is a risk factor for accelerated decline in lung function (Vestbo *et al.*, 1996), with more recent evidence suggesting exacerbations of COPD play a direct role in accelerating the decline in FEV₁ (Donaldson *et al.*, 2002). Therefore, the evidence now suggests that recurrent infection and mucus hypersecretion does indeed have a significant role in the progression of COPD.

1.1.3.2 Chronic obstructive bronchiolitis

Chronic inflammation and remodelling of the terminal bronchioles, leads to narrowing of the small airways (airways <2mm in diameter) resulting in airflow limitation (Hogg *et al.*, 2004). There is increased thickness of the wall of small airways due to epithelial metaplasia, goblet cell hyperplasia and submucosal gland hypertrophy (Hogg *et al.*, 2004). The degree of small airway wall thickness is related to the progression of disease from GOLD stage 1 to 4 (Hogg *et al.*, 2004). The degree to which the airway lumen is filled with an inflammatory exudate, and the number of airways that contain acute inflammatory cells organised into follicles, as well as the absolute numbers of B and CD8⁺ T lymphocytes, were all more weakly associated with disease progression, suggesting that airway remodelling is the major cause of small airway obstruction (Hogg *et al.*, 2004). The mechanisms of fibrosis in COPD are not well understood, though may relate to increased peripheral airway expression of transforming growth factor (TGF)- β , which in turn, induces release of connective tissue growth factor (CTGF) stimulating collagen deposition in the airways (Barnes *et al.*, 2003; Takizawa *et al.*, 2001; Ihn 2002).

1.1.3.3 Emphysema

Emphysema is defined as permanent destructive enlargement of airspaces distal to the terminal bronchioles, leading to airflow limitation resulting from the loss of elastic recoil of the lung (Barnes *et al.*, 2003). Both panacinar and centrilobular emphysema may occur in smokers (Kim *et al.*, 1991). However, the degree of emphysema is poorly

reflected by measurement of spirometry, with emphysematous changes often present prior to any reduction in FEV₁. Changes in gas transfer are associated with emphysema and may precede changes in FEV₁ by many years (Gould *et al.*, 1991). The destruction of the parenchyma is thought to be due to excessive proteolytic activity. This is associated with increased inflammatory cell infiltrate, with activated neutrophils and macrophages releasing elastolytic enzymes, including neutrophil elastase and matrix-metalloproteinase-9 (MMP-9), resulting in destruction of lung tissue, leading to emphysema (Stockley, 1999; Russell *et al.*, 2002).

1.1.4 Pharmacotherapy for COPD

Given the global health burden of COPD, there is the need for therapies directed at the underlying pathophysiology of the disease, aimed at slowing disease progression and improving mortality. However, smoking cessation in early disease and home oxygen therapy in those with persistent hypoxaemia are the only specific therapeutic interventions in COPD that have shown a reduction in mortality (Anthonisen *et al.*, 2005;MRC, 1981), whilst concomitant use of statins has also been shown to reduce the risk of COPD death in a matched cohort study (Frost *et al.*, 2007). The mainstay of current treatment in COPD, the use of long-acting bronchodilators and inhaled glucocorticosteroids (Barnes *et al.*, 2003), have not been shown to significantly alter mortality (Calverley *et al.*, 2007). Several large trials examining the impact of inhaled therapy (long-acting bronchodilators and inhaled glucocorticosteroids) on lung function, mortality and health status, have shown improvements in health status and a reduction in the annual decline in FEV₁ (Calverley *et al.*, 2003b;Calverley *et al.*, 2003a;Pauwels *et al.*, 1999;Calverley *et al.*, 2007;Wedzicha *et al.*, 2008). Combination therapy, using a long-acting β_2 -adrenoceptor agonist in combination with an inhaled glucocorticosteroid, has shown greater benefits in reduction of FEV₁ (by approximately 70-90ml) over the use of individual drugs (Calverley *et al.*, 2003b;Calverley *et al.*, 2003a). This may simply be related to improved patient compliance, although, there is also evidence of synergy, as glucocorticosteroids overcome desensitisation of β_2 -adrenoceptors by increasing gene transcription and receptor expression, whilst β_2 agonists increase nuclear translocation and prime the glucocorticoid receptor (Sarir *et al.*, 2008).

Two large multi-centre studies have shown that combination therapy has a role in reducing exacerbation frequency. The 'Towards a revolution in COPD health' (TORCH) study demonstrated a 25% reduction in the rate of exacerbations of COPD, in those patients taking combination therapy (salmeterol and fluticasone propionate) compared to placebo (Calverley *et al.*, 2007). The INSPIRE study, which examined the effects of adding tiotropium to combination therapy, confirmed a reduction in exacerbation rates compared to both tiotropium alone, and in combination (Wedzicha *et al.*, 2008).

The mainstays of treatment for exacerbations of COPD are antibiotics and oral glucocorticosteroids (Rabe and Wedzicha, 2011). Systemic glucocorticosteroids given in exacerbations of COPD, reduce the length of hospital admission and post-bronchodilator FEV₁ (Davies *et al.*, 1999), however, they have significant side effects, including weight gain, elevated blood glucose, hypertension and in the longer term cataracts and osteoporosis. Treatment of exacerbations with glucocorticosteroids, in combination with antibiotics, increased the length of time to next exacerbation and was associated with reduced mortality (Roede *et al.*, 2008). The antibiotic study by Anthonisen *et al.*, showed that giving antibiotics for exacerbations of COPD gave rise to significant benefits, with resolution of exacerbation by 21 days in ~70% of COPD patients who received antibiotics compared to 55% taking placebo, together with a more rapid improvement in lung function (Anthonisen *et al.*, 1987). Using antibiotics at exacerbation to eradicate bacteria in the airways relates to exacerbation recovery and reduction in inflammatory markers (myeloperoxidase and LTB₄) in the sputum (White *et al.*, 2003). This suggests that antibiotics are most effective in exacerbations associated with increased dyspnoea, sputum volume and purulence in more severe COPD (Anthonisen *et al.*, 1987; Daniels *et al.*, 2010). Therefore, in order to optimise the treatment of, and improve outcomes after, exacerbations of COPD, this requires these events to be well defined and characterised.

1.2 Exacerbations of COPD

Exacerbations of COPD are described as “*an acute event characterized by a worsening of the patient’s respiratory symptoms that is beyond normal day to day variations, and leads to a change in underlying medication*” (GOLD, 2011).

Acute exacerbations of COPD are the commonest cause of acute medical admission to hospital in the UK (Wedzicha and Seemungal, 2007). Exacerbations are associated with significant mortality and are a major determinant of COPD costs, ranging from 40-63% of the total direct costs associated with COPD, with this increasing with disease severity (Hilleman *et al.*, 2000;Halpin and Miravittles, 2006). In the UK, the in-hospital mortality associated with an acute exacerbation of COPD is 8%, with a further third of patients with an acute exacerbation readmitted within 30 days, of which 15% die within 90 days (COPD National Audit, 2008). Recovery from exacerbations is slow, with approximately 14% of patients remaining symptomatic at 35 days after the onset of exacerbation, whilst in a small proportion of patients (~7%), symptoms never return to baseline level after an exacerbation (Seemungal *et al.*, 2000).

Defining exacerbation frequency is complicated, and there are many reasons for this. Firstly, the definition of an exacerbation is unclear, secondly, many exacerbations are not reported to health-care professionals and are either self-treated or untreated (Seemungal *et al.*, 1998). Nevertheless, on average, COPD patients experience one to two exacerbations annually (Sethi and Murphy, 2008). Exacerbations increase in frequency and severity with progression of the disease, as demonstrated by the fact that GOLD stage 2 patients have ~0.85 exacerbations/year compared to ~2/year for stage 4 patients (Hurst *et al.*, 2010). Despite the problems in defining and reporting of exacerbations, frequent exacerbators are generally characterised as those who develop two or more exacerbations per year that require physician intervention. These patients display a more rapid decline in FEV₁, more frequent hospitalisations and worsening quality of life, demonstrating the role exacerbations play in the progression of COPD and further supporting the ‘British hypothesis’ (Donaldson *et al.*, 2002;Seemungal *et al.*, 1998). More recently the ECLIPSE study showed that, in the cohort of COPD patients followed, the mean decline in FEV₁ was only 33ml/year,

however, there was a large standard deviation and exacerbations were weakly associated with an accelerated decline of ~2ml/year (Vestbo *et al.*, 2011).

1.2.1 Defining an exacerbation - Anthonisen's criteria

Anthonisen's criteria were originally designed to analyse the effects of antibiotics on exacerbations, as opposed to defining an exacerbation and guiding patient management for which they are often now used (Anthonisen *et al.*, 1987). The criteria divide patients into three groups according to their symptoms:

- Type 1 exacerbations were defined as increased or new onset dyspnoea, sputum volume and sputum purulence.
- Type 2 exacerbations require 2 of the above symptoms.
- Type 3 exacerbations require 1 of the above symptoms, in addition to one of the following:
 - Upper respiratory tract infection in last 5 days
 - Increased wheeze
 - Increased cough
 - Fever

These criteria were used to define the occurrence of an acute exacerbation in the COPD patients followed in the present study.

1.2.2 Causes of COPD exacerbations

The causes of exacerbations are complex, with the host, viral and bacterial infections, and environmental pollution all possibly interacting (Fig. 1.1).

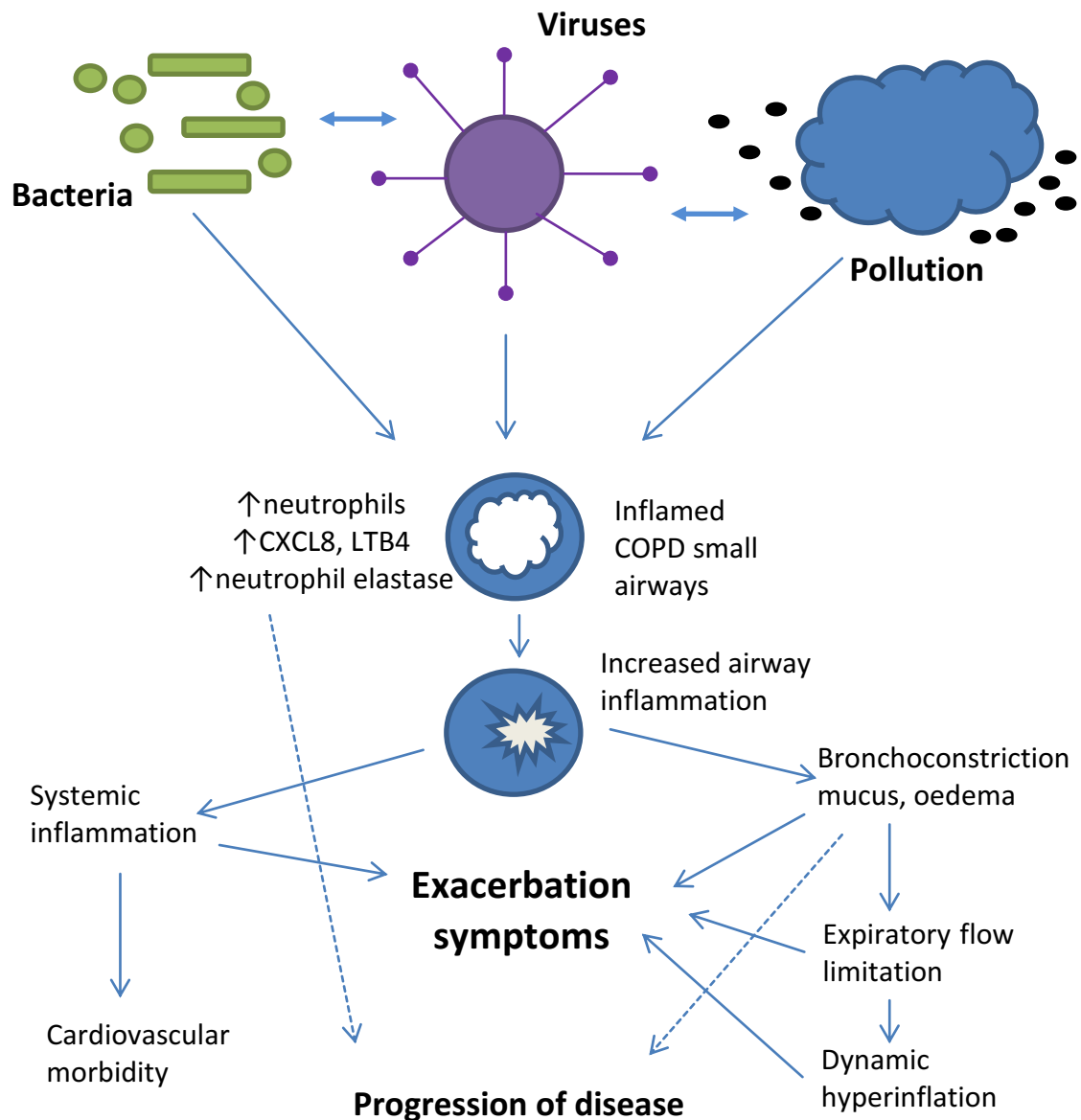


Figure 1.1. Causes and effect of exacerbations of COPD

Bacteria, viruses and pollution are causative factors in exacerbations of COPD, either individually or in conjunction with one another. Exacerbations of COPD are associated with increased airway inflammation which drives symptomatology and disease progression. Adapted from Wedzicha and Seemungal, 2007.

The commonest causes of exacerbation are shown in Table 1.2. The majority of exacerbations of COPD are caused by viral and/or bacterial infections, with bacteria present in half of all exacerbations (Sethi and Murphy, 2008).

Bacterial	Viral	Environmental (Pollution)
<i>Haemophilus influenzae</i> <i>Streptococcus pneumoniae</i> <i>Moraxella catarrhalis</i> <i>Pseudomonas aeruginosa</i>	Rhinovirus Influenza Parainfluenza Coronavirus Adenovirus Respiratory Syncytial Virus (RSV)	Ozone Particulate matter Sulphur dioxide Nitrogen Oxides Biomass fuels

Table 1.2. Commonest causes of exacerbation in COPD patients

These represent the commonest bacterial, viral and environmental causes of acute exacerbations of COPD, adapted from Wedzicha and Seemungal, 2007.

Exacerbations caused by co-infection with virus and bacteria are associated with higher levels of inflammatory markers in sputum, including interleukin (IL)-6, CXCL-8 and myeloperoxidase, compared with those generated by infection caused by either bacteria or virus alone (Wilkinson *et al.*, 2006). The reason for this is unclear, however, *in vitro* studies have shown that infection of bronchial epithelial cells with respiratory syncytial virus (RSV) increases expression of intracellular adhesion molecule (ICAM)-1 and platelet activating-factor (PAF), which promotes increased adherence and invasion of non-typeable *Haemophilus influenzae* (NTHI) and *Streptococcus pneumoniae* (Avadhanula *et al.*, 2006). Rhinovirus has also been shown to induce ICAM-1 expression on bronchial epithelial cells (Papi and Johnston, 1999). ICAM is important in the recruitment of neutrophils, which are increased in number in patients with COPD during an exacerbation. Rhinovirus infection of BEAS2b cells activates the transcription factor, Nuclear Factor- κ B (NF- κ B), which is important in the regulation of a number of pro-inflammatory genes, including CXCL8 production, which is a potent neutrophil chemoattractant (Biagioli *et al.*, 1999). In addition, rhinovirus infection of macrophages reduces their ability to phagocytose bacterial pathogens (Oliver *et al.*, 2008), which could predispose to co-infection with bacteria.

1.2.3 Pathophysiology of COPD exacerbations

During exacerbations of COPD, neutrophils increase in number in the bronchial wall and in broncho-alveolar lavage (BAL) (Balbi *et al.*, 1997; Saetta *et al.*, 1994). This is associated with increased levels of neutrophil chemoattractants, such as CXCL8 and leukotriene B₄ (LTB₄) (Gompertz *et al.*, 2001). Additionally, levels of neutrophil elastase are significantly increased in sputum during exacerbations, and correspond to the increased neutrophil count (Fujimoto *et al.*, 2005). COPD exacerbations are also associated with increased serum levels of CXCL8, soluble intercellular adhesion molecule (sICAM-1) and E-selectin, the latter two required for neutrophil migration from the circulation (Gerritsen *et al.*, 2005). Therefore, inflammation appears to be amplified during exacerbation and is associated with increased neutrophil recruitment with resultant increased proteinase secretion. This potentially causes further tissue damage and contributes to further progression of COPD (Wedzicha and Seemungal, 2007; Sapey and Stockley, 2006). Patients with more frequent exacerbations have greater levels of inflammation at both steady state and at exacerbation, with higher levels of sputum IL-6 and CXCL8, compared to COPD patients with infrequent exacerbations.

The levels of pro-inflammatory mediators (myeloperoxidase, CXCL8, LTB₄ and neutrophil elastase) present in the sputum of COPD patients with an exacerbation correlate with bacterial load, irrespective of the pathogen present (Hill *et al.*, 2000). Isolation of pathogenic bacteria from bronchoalveolar lavage of COPD patients is also associated with increased levels of tumour necrosis factor (TNF)- α and higher neutrophil counts compared to those from the BAL of culture negative patients (Soler *et al.*, 1999). This suggests that the presence of bacteria in the airways is associated with increased inflammation, which, given that some COPD patients display bacterial colonisation in the stable state (Rosell *et al.*, 2005; Wilkinson *et al.*, 2006), may explain the increased inflammation in COPD airways even at stable state, increasing further during exacerbations.

1.2.4 Bacterial colonisation in COPD

Patients with COPD appear to be more prone to respiratory infections, with patients exhibiting bacterial colonization of the lower airways in the stable state (Rosell *et al.*, 2005; Wilkinson *et al.*, 2006). Protected specimen brush sampling of the lower airways showed that approximately 4% of healthy adults had greater or equal to 10^2 colony-forming units (CFU)/ml of potentially pathogenic bacteria, compared to 29% of COPD patients with stable disease and 54% of COPD patients with an exacerbation (Rosell *et al.*, 2005). Another study of patients with moderate to severe COPD, showed that bacteria could be isolated from the lower airways in 48% of stable COPD patients, rising to 70% of patients at exacerbation, with an associated increase in bacterial load (Wilkinson *et al.*, 2006). Taken together, these studies imply that bacterial colonisation of the lower airway is prevalent in COPD. Levels of TNF- α and CXCL8, as well as neutrophil degranulation products, including myeloperoxidase and neutrophil elastase, are elevated in the sputum of COPD patients with bacterial colonisation, compared to those without (Bresser *et al.*, 2000). Numbers of neutrophils and levels of CXCL8 and matrix-metalloproteinase (MMP) 9 in BAL have also been shown to be elevated in association with bacterial colonisation in COPD (Sethi *et al.*, 2006).

Despite the presence of colonising bacteria in the airways, it is suggested that acquisition of a new strain of bacteria appears to be associated with development of an exacerbation (Sethi *et al.*, 2002). However, bacterial colonisation of the lower airway has been shown to be positively correlated with exacerbation frequency and concentrations of CXCL8 in sputum (Patel *et al.*, 2002), suggesting that bacterial load may drive both exacerbation frequency and neutrophilic inflammation. Although a number of bacterial species have been isolated in the COPD airway (Table 1.2), one of the commonest bacteria to be isolated from COPD patients in both the stable state or at exacerbation is *Haemophilus influenzae*.

1.2.4.1 *Haemophilus influenzae*

Haemophilus influenzae is a gram-negative coccobacillus from the family *Pasteurellaceae*. It is the most common pulmonary pathogen isolated from the airways in COPD patients, and is associated with 20-30% of all exacerbations of COPD

(Sethi and Murphy, 2008;Sethi *et al.*, 2002). The presence of *H. influenzae* within the respiratory tract may be underestimated outside of exacerbations, as the presence of bacteria is usually derived from positive sputum cultures, however, bacteria at concentrations of less than 10^2 CFU/ml will yield negative cultures. Furthermore, *H. influenzae* can form biofilms, densely packed communities of cells surrounded by a self-produced matrix of extracellular polymeric substance, (Murphy and Kirkham, 2002), which also reduce the sensitivity of cultures and *H. influenzae* may also remain viable inside host cells thereby evading detection (Forsgren *et al.*, 1994;Murphy *et al.*, 2004;Craig *et al.*, 2001). Therefore, *H. influenzae* may be present in the respiratory tract despite a negative culture.

H. influenzae has been found in bronchial mucosal biopsy specimens from 87% of exacerbating COPD patients who required intubation, compared to 33% of stable COPD patients, but not in healthy subjects (Bandi *et al.*, 2001). Non-culture-based detection techniques, such as quantitative real time polymerase chain reaction (qrt-PCR) using primers specific for particular bacterial species, identified *H. influenzae* in sputum, bronchial epithelium and inside subepithelial macrophages in COPD patients, suggesting that this is a colonising pathogen (Murphy *et al.*, 2004). Using an *in vitro* model of airway epithelium, this bacterium induced mucus hyper-secretion, reduced ciliary beat frequency, caused epithelial damage and enhanced release of IL-6, CXCL8 and TNF- α (Adler *et al.*, 1986;Wilson *et al.*, 1985;Read *et al.*, 1991;Khair *et al.*, 1994), suggesting that this pathogen can cause many features associated with both acute exacerbations and the pathogenesis of COPD. The outer-membrane protein P6 of *H. influenzae* can induce production of both CXCL8 and TNF- α from human monocyte-derived macrophages (Berenson *et al.*, 2005). Strains of *H. influenzae*, isolated from COPD patients at exacerbation, have shown increased epithelial cell adherence, increased induction of CXCL8 and increased neutrophil recruitment compared to colonising strains (Chin *et al.*, 2005). Therefore, *H. influenzae* is associated with the ability to induce mucus hypersecretion, neutrophil recruitment and release of inflammatory cytokines, all of which may drive progression of COPD. However, *H. influenzae* is not the only bacterial species that is commonly isolated from the COPD airway.

1.2.4.2 *Streptococcus pneumoniae*

Streptococcus pneumoniae is found in the upper airways of healthy individuals and is considered a commensal bacterium; however, it is the commonest cause of community acquired pneumonia in adults and also causes otitis media and meningitis (Sethi, 2001). Along with *H. influenzae*, it is amongst the commonest bacteria isolated from COPD patients and accounts for up to 10% of bacteria isolated from exacerbating COPD patients (Sapey and Stockley, 2006). It is a Gram-positive, α -haemolytic diplococcus, enveloped within a polysaccharide capsule. This capsule enhances the virulence of *S. pneumoniae* by evading phagocytic killing by leukocytes, as it prevents binding of C3b (complement) to the bacterium (Sethi and Murphy, 2001). *In vitro* studies using epithelial cells have shown that *S. pneumoniae* induces mucus hypersecretion and that the cell wall polysaccharide activates complement and induces cytokines, including IL-1 β , IL-6 and TNF- α (Adler *et al.*, 1986; Catterall, 1999). *S. pneumoniae*, in association with *Haemophilus influenzae* and *Pseudomonas aeruginosa*, can form a biofilm which increases antibiotic resistance and reduces culture sensitivity (Domenech *et al.*, 2011), hence, its prevalence may be underestimated.

Why patients with COPD appear to be more susceptible to colonisation with these particular bacterial species is unclear, but it may be that the high occurrence of bacterial colonisation results from defects in the host innate immune response.

1.3 The immune system and the lung

The immune system comprises the innate and adaptive immune systems which cooperate to protect the host from infection and invading organisms. Any failure in the normal immune function can have detrimental consequences to the host, and may contribute to susceptibility to infection and disease.

1.3.1 Barrier immunity

Mechanical barriers are the first line of defence against pathogens entering the airways in humans. Despite continuous exposure to inhaled particles, pathogenic bacteria and viruses, typically, the lung is maintained in a healthy state without any

overt inflammatory response, suggesting that most pathogens are neutralised prior to activation of the adaptive or innate immune system (Chaudhuri and Sabroe, 2008). The upper airway is colonised with commensal flora, creating an environment in which pathogens are unable to establish (Chaudhuri and Sabroe, 2008). The mechanics of sneezing and coughing are in place to expel inhaled particles and pathogens and the lung lining is constantly 'swept' clean by the mucociliary system which removes debris and pathogens, leading to clearance via the digestive tract (Sabroe *et al.*, 2007). Mucus lining the airways, and the fluid lining the alveoli, contain multiple soluble constituents which can bind to and by various mechanisms aid in elimination of bacteria, as outlined in Table 1.3.

Soluble constituents	Role
Lysozyme	Lytic to bacterial membranes
Lactoferrin	Excludes iron from bacterial metabolism
Defensins	Released from leukocytes and epithelial cells – bind and kill bacteria via pore formation or prevention of protein synthesis
Cathelicidins	Released from neutrophils and macrophages
Collectins: Mannose-binding protein Conglutinin Lung surfactant proteins - SP-A, SP-D.	Serve as opsonins for bacterial products (mannose or LPS) to promote phagocytosis, Can trigger the complement cascade. Also directly inhibit bacterial growth
Secretory IgA	Prevents adhesion of pathogens to mucosal surfaces Serves as opsonin for bacterial products
Complement and IgG	Serve as opsonins for bacterial products

Table 1.3. Soluble constituents of airway and alveolar fluid and their role in innate immunity

In addition to mechanical and soluble factors, an effective inflammatory response requires specialist cells to neutralise and clear the insult (Chaudhuri and Sabroe, 2008). The cellular responses are divided into the innate and adaptive immune systems; innate immunity is nonspecific and is activated immediately on host exposure to a

pathogen, whilst the adaptive immune system is antigen-specific, designed to eliminate a specific antigen, and takes hours to days to initiate protection (Alberts *et al.*, 2002).

1.3.2 Adaptive immunity

The adaptive immune system consists of the humoral response, mounted by B lymphocytes which produce antibodies (IgG, IgM, IgA and IgE), and the cell mediated response, consisting of T lymphocytes (Alberts *et al.*, 2002) (Fig. 1.2). The adaptive immune response is outside the focus of this thesis and, therefore, is outlined only in brief below (Fig. 1.2). The key feature of the adaptive immune response is memory, so that when the host is challenged with a repeat infection, the adaptive immune response is strong and specific to that pathogen (Alberts *et al.*, 2002). B and T cells are produced in the bone marrow, with T cells travelling to and developing in the thymus (Alberts *et al.*, 2002). B cells, exposed to antigen, become activated, proliferate and differentiate into plasma cells, whilst synthesising and secreting antibody. T cells differentiate into either T helper cells, after antigen presentation by MHC class II molecules on the surface of antigen-presenting cells, which then produce cytokines to regulate either humoral and cell-mediated immunity, or cytotoxic T cells, which bind and kill infected cells by inducing apoptosis (Alberts *et al.*, 2002).

In the COPD lung, cytotoxic T cells are the predominant T cell, with the numbers of T cells relating to worsening airflow obstruction and emphysema (Saetta *et al.*, 1998). Cytotoxic T cells release proteolytic enzymes such as perforin and granzymes which, when activated, cause cell death through apoptosis and necrosis (Cosio *et al.*, 2009). B cells are increased in the large airways of patients with COPD, as well as being found organised into lymphoid follicles around smaller airways in severe disease (Hogg *et al.*, 2004), suggesting that the adaptive immune system plays a role in more severe disease.

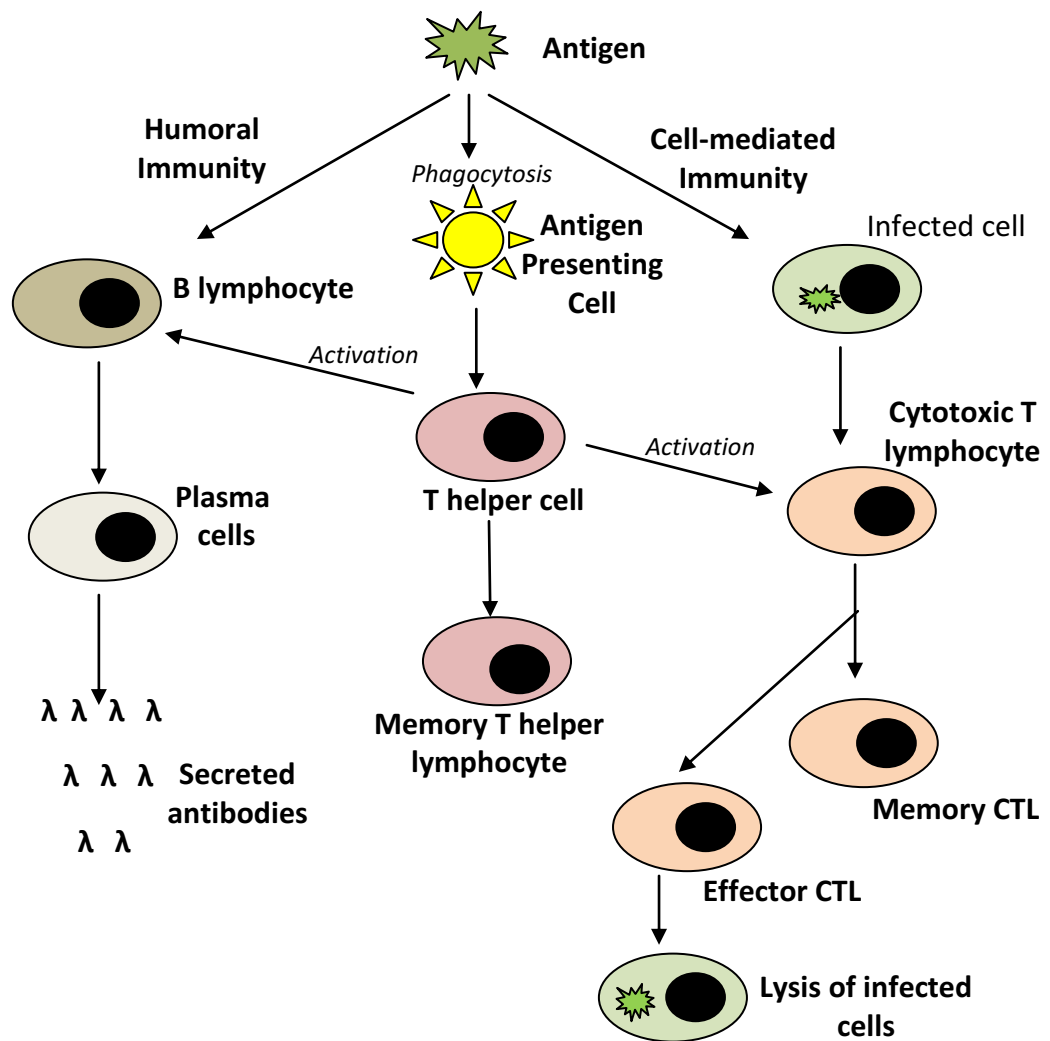


Figure 1.2. Adaptive immunity

Schematic outline of the differentiation, activity and interaction between B and T lymphocytes of the humoral and cell mediated adaptive immune system.

1.3.3 Innate immunity

Whilst physical defences and soluble constituents play a role in clearance of pathogens from the airways, the cellular components of the innate immune system are responsible for the majority of bacterial clearance, with macrophages and neutrophils representing the main phagocytic cells of the innate immune system (Janeway and Medzhitov, 2002).

1.3.3.1 Macrophages in the lung

Alveolar macrophages (AM) account for approximately 95% of airspace leukocytes, with lymphocytes (1-4%) and neutrophils (1%) comprising the remainder (Martin and Frevert, 2005). Therefore, the macrophage is the sentinel phagocytic cell of the innate immune system in the lung (Martin and Frevert, 2005). AM are highly differentiated cells that mature locally from blood-borne, bone marrow-derived monocytes (Droemann *et al.*, 2005). AM are long-lived, with evidence that they may survive for several months, or even years (Thomas *et al.*, 1976). AM are avidly phagocytic and are responsible for clearance of inhaled particulates and pathogens that reach the alveolar space (Martin and Frevert, 2005). They are also responsible for the generation of mediators involved in inflammation, as well as orchestrating the resolution of inflammation including the removal of apoptotic cells (efferocytosis), and have a role in antigen presentation in the initiation of an immune response, although this role is more effectively served by dendritic cells (Droemann *et al.*, 2005; Aderem and Underhill, 1999; Chaudhuri and Sabroe, 2008). Interstitial macrophages (IM) are monocyte-derived cells that reside between the pulmonary vasculature and the airway. They are postulated to be precursors of AM as they are smaller in size, display less proficient phagocytic ability and oxidant production, but exhibit improved antigen-presentation compared to AM (Fathi *et al.*, 2001). At present, it is not clear whether IM and AM exist as different populations of macrophages or whether they are purely a spectrum of macrophage differentiation.

There is increasing evidence that macrophages exist as different phenotypes, which may respond differently upon exposure to local environmental conditions, similar to the Th1:Th2 paradigm in lymphocytes. Interferon gamma (IFN γ) and cytokines such as granulocyte macrophage-colony stimulating factor (GM-CSF) and TNF- α , as well as bacterial products, such as lipopolysaccharide (LPS), may elicit 'classical' activation that is associated with a pro-inflammatory macrophage described as 'M1'. (Gordon, 2003; Mantovani, 2008). M1 macrophages are characteristically less phagocytic whilst releasing increased levels of inflammatory cytokines and chemokines, such as TNF- α , IL-1 β , IL-6, IL-12 and CXCL8 and reactive oxygen species (Mantovani, 2008; Chen *et al.*, 2003). Alternatively activated macrophages are driven by IL-4 and IL-13 and behave

like 'M2' macrophages as described by Mantovani (Gordon, 2003; Mantovani 2008). Recently the M2 phenotype has enlarged and encompasses several macrophage phenotypes, with those activated by IL-4 and IL-13 designated M2a, those activated by IL-1 β and LPS designated M2b and those activated by IL-10, glucocorticosteroid hormones or immune complexes designated M2c (Mantovani, 2008). In general, M2 macrophages secrete IL-10 and express high levels of mannose and scavenger receptors, and consequently are more phagocytic (Mantovani, 2008). The key properties of M1 and M2 (including all M2 subsets (M2s)) macrophages are shown in Figure 1.3. In the lung, AM are considered to display an M2 phenotype, however, in COPD these cells are highly pro-inflammatory and may be skewed towards an M1 phenotype, due to the presence of TNF- α and LPS. Latterly, it has been suggested that macrophages exist as a spectrum, rather than distinct phenotypes, and may display polarity dependent upon the local environment (Mosser and Edwards, 2008).

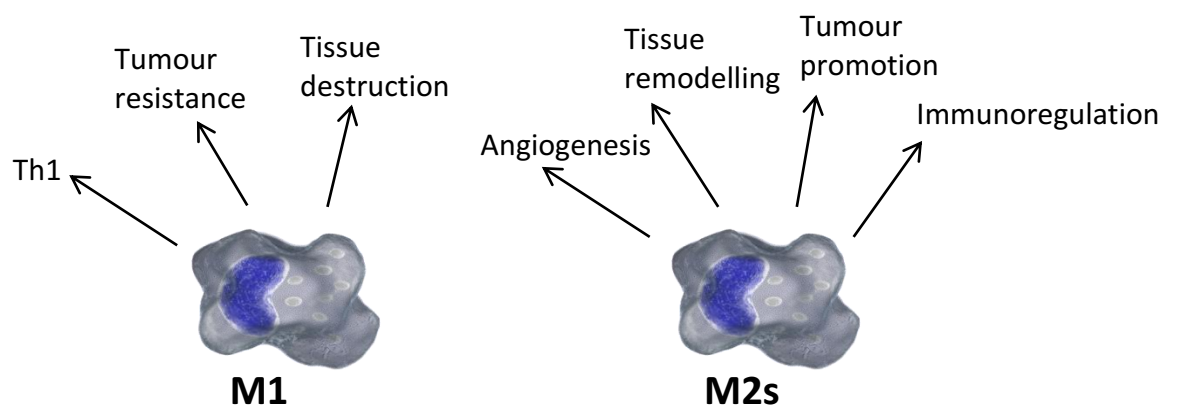


Figure 1.3. Macrophage polarisation.

Key properties and functions of polarised macrophages, with M2 subsets (M2s) encompassing the diverse forms of M2 activation. Adapted from Mantovani A; Macrophage diversity and polarisation. *Blood*, 2006

1.3.3.2 Macrophages in COPD

Macrophages are increased in number by 5-10 fold in the airways, lung parenchyma, sputum and BAL of smokers and patients with COPD, with numbers relating to disease severity (Finkelstein *et al.*, 1995; Di *et al.*, 1998). This increase may arise secondary to increased recruitment of monocytes, or due to increased longevity of resident

macrophages (Barnes, 2004). There are increased levels of CCL2 and CXCL1, both monocyte chemoattractants, in sputum and BAL of COPD patients, as well as evidence of increased migration of COPD monocytes towards CXCL1 (Traves *et al.*, 2002a;Traves *et al.*, 2004). Macrophage longevity is difficult to determine, though there are reports of cigarette particulates being present in alveolar macrophages more than 2 years after smoking cessation (Marques *et al.*, 1997). Macrophages activated by cigarette smoke release chemokines (Barnes *et al.*, 2003), leading to the recruitment of neutrophils and T lymphocytes. *In vitro* studies have shown that macrophages from COPD patients release higher levels of CXCL8 at baseline, and after stimulation with cigarette smoke media, than macrophages from control subjects (Culpitt *et al.*, 2003). Inflammatory cytokines, such as TNF- α , are also found in higher levels in the sputum of COPD patients (Keatings *et al.*, 1996). Therefore, there is evidence that alveolar macrophages, present in increased numbers in COPD patients, appear to have a more pro-inflammatory phenotype, suggesting they may drive inflammation in COPD.

Macrophages secrete several elastolytic enzymes including MMP-2, MMP-9, MMP-12 and cathepsins, which may contribute to airway damage, emphysema and mucus hyper-secretion (Barnes *et al.*, 2003) (see Fig. 1.4). *In vitro* studies have shown that macrophages from COPD patients have greater elastolytic activity at baseline compared to those from healthy smokers, with levels increased further by cigarette smoke (Di *et al.*, 1998;Russell *et al.*, 2002;Lim *et al.*, 2000). The predominant elastolytic enzyme released by human macrophages appears to be MMP-9 (Russell *et al.*, 2002). AM from COPD patients have been shown to have increased levels and activity of MMP-9 compared to controls, with levels further increased by both cigarette smoke and LPS (Russell *et al.*, 2002). MMP-9 is strongly evident in emphysematous lung at sites of macrophage accumulation (Ohnishi *et al.*, 1998), suggesting that MMP-9 released from macrophages contributes to emphysema with evidence that levels and activity are elevated in COPD.

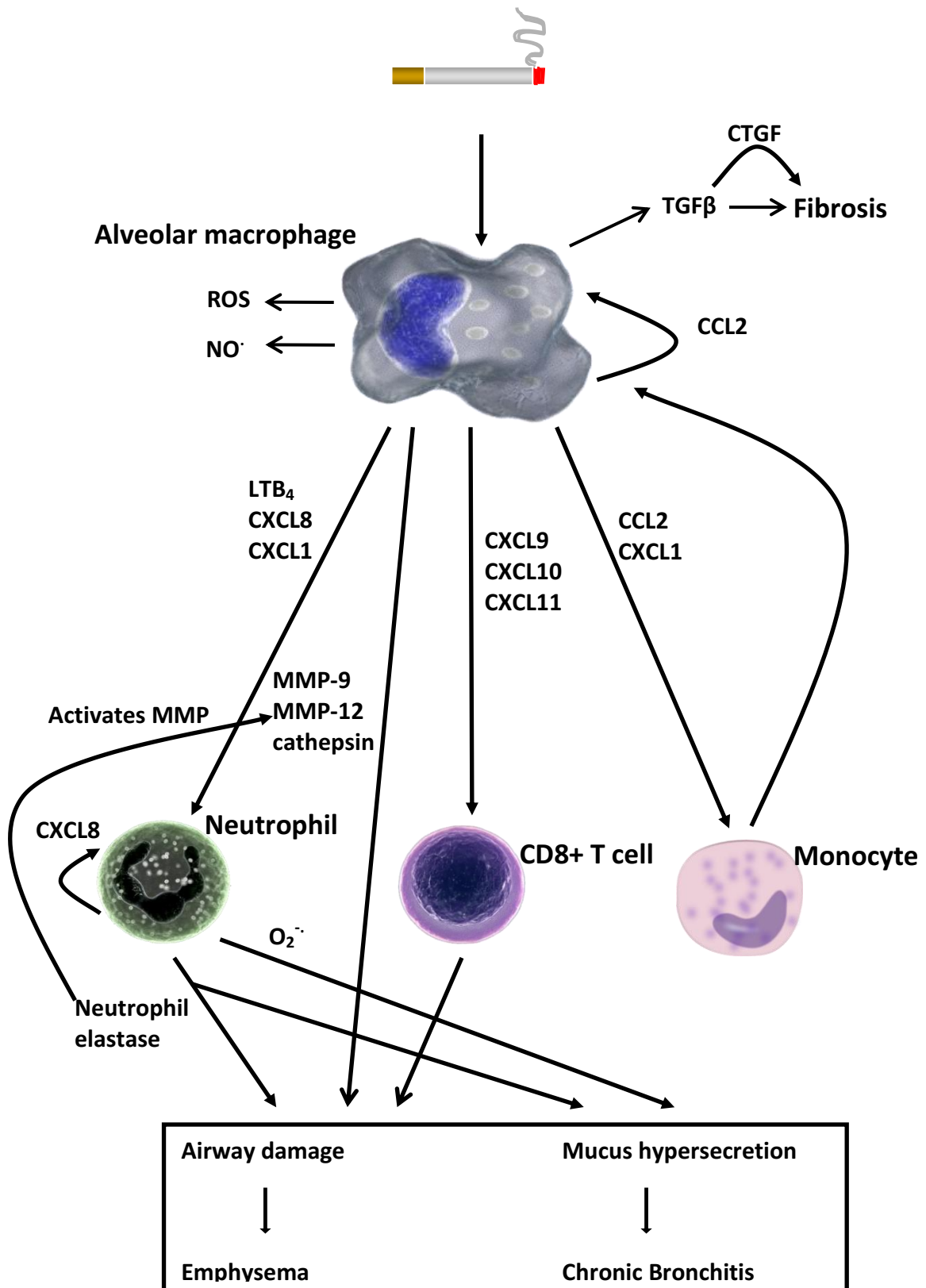


Figure 1.4. Role of macrophages and neutrophils in the pathogenesis of COPD

Stimuli, including cigarette smoke, leads to activation of macrophages and release of chemokines, recruiting neutrophils, monocytes and CD8+ T cells, and cytokines, reactive oxygen species (ROS), nitric oxide (NO[•]) and superoxide anion (O₂^{•-}) and proteases, particularly matrix metalloproteinases (MMP), that contribute to the pathogenesis of COPD. (Transforming growth factor (TGF)-β, Connective tissue growth factor (CTGF), CCL2 (monocyte-chemoattractant peptide (MCP-1)), CXCL1 (growth-regulated oncogene (GRO)-α)

1.3.3.3 Neutrophils in the lung

Neutrophils are produced in the bone marrow and are the predominant granulocyte in the circulation (Chaudhuri and Sabroe, 2008). At sites of infection, neutrophils are the first phagocyte to be rapidly recruited, migrating into tissue along a gradient of chemotactic factors, such as CXCL8, LTB₄ and CXCL1, that are released from epithelial cells, macrophages and, in the case of CXCL8, neutrophils themselves (Traves *et al.*, 2002b; Bazzoni *et al.*, 1991) (Fig. 1.4). Neutrophil migration from the vascular space into the airway lumen occurs by a process of rolling, margination and diapedesis (Wagner and Roth, 2000). Cell rolling is mediated by selectins and ICAM-1 expressed on endothelial cells, followed by β_2 integrin-mediated margination (LFA-1 (CD11a/CD18) and macrophage-antigen (MAC)-1 (CD11b/CD18)) and diapedesis between endothelial cells (Wagner and Roth, 2000).

Neutrophils exposed to bacterial products (such as *N*-formyl-methionyl-leucyl-phenyl-alanine (fMLP) or pro-inflammatory mediators (such as TNF- α and IFN γ) become primed and initiate potent anti-microbial responses (Parker *et al.*, 2005). Pathogens are taken up and killed by release of reactive oxygen species and proteases into the phagosome. Neutrophils are short lived, with resolution of neutrophilic inflammation occurring by initiation of neutrophil apoptosis, followed by clearance through efferocytosis by macrophages (Rossi *et al.*, 2007). Failure of this mechanism, leads to neutrophil necrosis and release of reactive oxygen species and proteases, such as neutrophil elastase and cathepsin G, into the airway, with significant associated proteolytic damage to the surrounding tissues, contributing to the pathogenesis of COPD (Parker *et al.*, 2005).

1.3.3.4 Neutrophils in COPD

Sputum and BAL of patients with COPD contains higher numbers of neutrophils compared to smoking and non-smoking controls (70% neutrophils in COPD vs. 36% and 25% in smokers and non-smoking controls respectively), with smaller, but significant, increases in the numbers of neutrophils seen in airways and lung parenchyma (Keatings *et al.*, 1996; Finkelstein *et al.*, 1995). Indeed, sputum neutrophilia is a key finding in COPD patients (Keatings *et al.*, 1996). The numbers of both circulating neutrophils and neutrophils in induced sputum relates to the decline in FEV₁ (Keatings

et al., 1996; Stanescu *et al.*, 1996). Circulating neutrophils from COPD patients display enhanced chemotaxis, extracellular proteolysis and produce more reactive oxygen species when compared to controls (Burnett *et al.*, 1987; Culpitt *et al.*, 2002). Levels of neutrophilic chemotactic factors, such as LTB₄, CXCL8 and CXCL1, are also increased in COPD airways (Hill *et al.*, 1999; Traves *et al.*, 2002b; Tanino *et al.*, 2002). In addition, E-selectin is also upregulated on endothelial cells from COPD patients and this, coupled with increased expression of the adhesion molecule MAC-1 (CD11b/CD18) on circulating neutrophils, leads to slowing down neutrophils in the circulation, allowing for increased migration into, and retention of neutrophils in, airway spaces (Di *et al.*, 1994b; Yamagata *et al.*, 2007a). Therefore, there is substantial evidence that there is increased recruitment of neutrophils into the lungs in COPD, with numbers of neutrophils relating to disease severity, with these cells associated with increased proteolytic activity, with further tissue damage and mucus hypersecretion, which in turn drives the progression of COPD. Furthermore, the numbers of circulating neutrophils are increased during, and relate to, the severity of exacerbations of COPD (Papi *et al.*, 2006). Levels of CXCL8, LTB₄ and neutrophil elastase in the airway are also further increased during exacerbations of COPD, and relate to bacterial load (Fujimoto *et al.*, 2005; Hill *et al.*, 2000).

A major role for both macrophages and neutrophils in the innate immune system in the lung is the phagocytosis and clearance of inhaled bacterial pathogens. As both of these professional phagocytes are increased in numbers in the lungs of COPD patients, with further increases seen at exacerbation, the increased rate of bacterial colonisation is suggestive of a defect in the phagocytic process.

1.4 Phagocytosis

Phagocytosis is a complex process by which a cell engulfs and removes a foreign particle or pathogen. Many cells are capable of phagocytosis but macrophages and neutrophils are deemed 'professional' phagocytes (Rabinovitch, 1995). Phagocytosis describes the uptake of particles, larger than 0.5µm in diameter, into cells by an actin-dependent mechanism (Aderem and Underhill, 1999). The process of phagocytosis can be divided into a series of steps, namely particle recognition, re-arrangement of the actin cytoskeleton, engulfment and formation of the phagosome (Fig. 1.5)

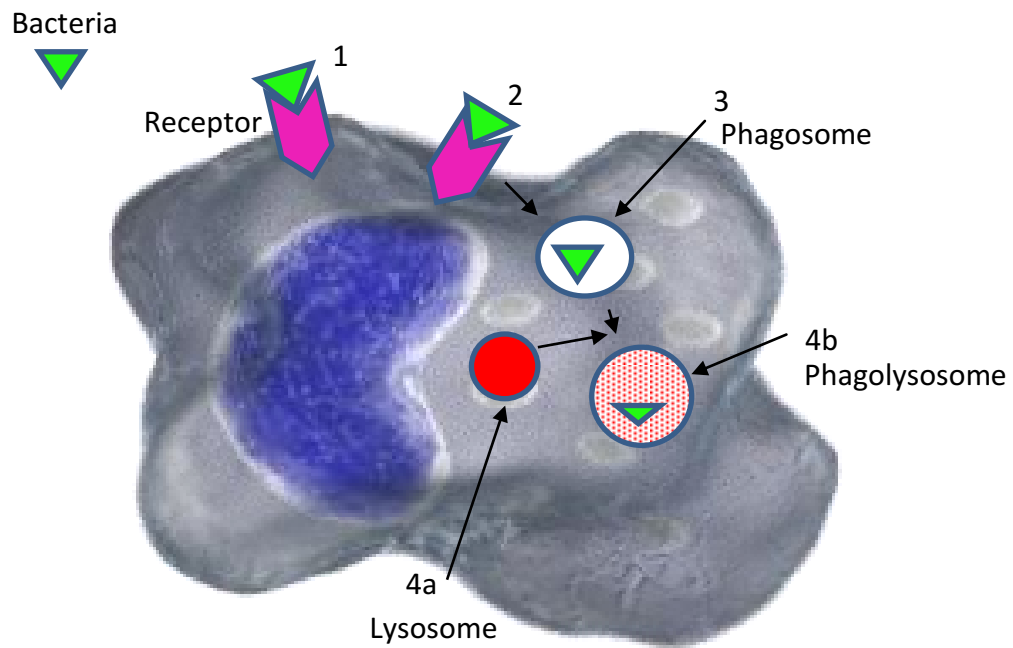


Figure 1.5. The process of phagocytosis

(1) Cell surface receptors recognise and bind particles; (2) induction of actin polymerisation; (3) actin-rich membrane extensions reach around the particle, pulling it into the cell, generating a phagosome; (4) this undergoes maturation and acidification prior to (a) fusion with a lysosome to (b) form a phagolysosome (Aderem, 2003; Aderem and Underhill, 1999). Ingested particles are then destroyed by exposure to reactive oxygen intermediates and degradative enzymes (Droemann *et al.*, 2005).

1.4.1 Particle recognition

One of the complexities of phagocytosis is the diversity of receptors capable of driving this response, with many particles being recognised by more than one receptor, receptors with dual functions, such as adhesion and internalisation, and receptor synergy (Aderem and Underhill, 1999) (Fig. 1.6). It is essential that the immune system can differentiate pathogens from self and this requires specific recognition of conserved pathogen motifs by phagocytic receptors. These pathogen-associated molecular patterns (PAMPs) are recognised by pattern-recognition receptors (PRRs) (Janeway, Jr., 1992) leading to phagocytosis. This can be either pattern based, or occur as a result of opsonisation, or as a combination of the two (Aderem, 2003). PAMPs include mannans in yeast walls, formylated peptides in bacteria, LPS on gram-negative bacteria and lipoteichoic acid (LTA) on gram-positive bacteria. Cellular receptors

recognising these motifs include the mannose receptor (MR) and DEC 205, that recognize mannans, and β_2 -integrins, such as CD11b/CD18, and scavenger receptors that recognise bacterial surface components (Aderem and Underhill, 1999). Components that opsonise the infectious agent before recognition via surface receptors include mannose binding protein, surfactant protein A, complement and immunoglobulins, which opsonise and are recognised through C1q, surfactant-associated protein A receptor 210, the complement receptor C3b or C3bi or the Fc receptor (FcR) family respectively (Aderem and Underhill, 1999).

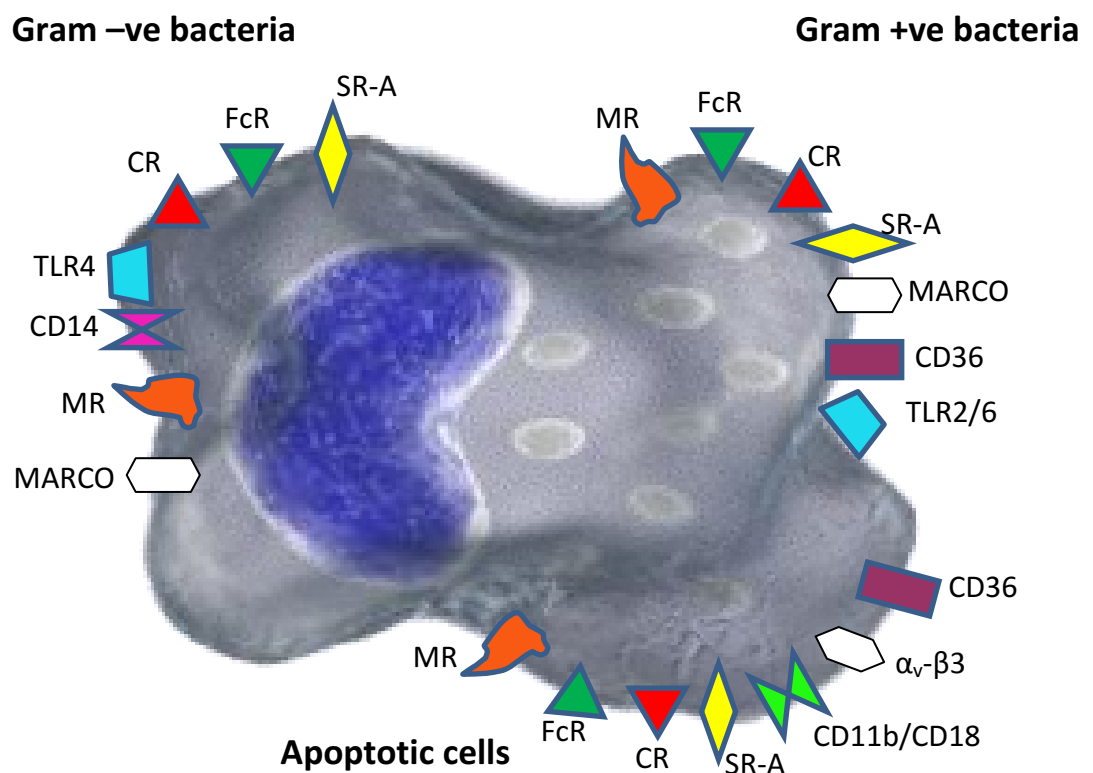


Figure 1.6. Receptors involved in particle recognition and phagocytosis

Phagocytic receptor expression on macrophages, with many receptors involved in recognition of more than one particle or pathogen. Receptors involved in direct recognition of particles include mannose receptor (MR), scavenger receptors (SR-A, CD36, macrophage receptor with collagenous structure (MARCO)), CD14 and Toll like receptors (TLR), which are pattern recognition receptors but do not have a role in phagocytosis. Receptors involved in indirect recognition include Fc receptors (FcR) and complement receptors (CR).

1.4.1.1 Fc-Receptor and complement receptor mediated phagocytosis

Current knowledge of macrophage and neutrophil phagocytosis is mostly derived from studying FcR-mediated phagocytosis (Lee *et al.*, 2003). Therefore, FcR-mediated phagocytosis will be briefly examined, although the phagocytic prey used in this thesis is unopsonised and, thus more likely to be internalised via scavenger receptors; this is in order to mimic inhaled particles and pathogens, which are thought less likely to be opsonised due to the lack of serum within the lung. However, in those patients with sputum production it is possible that opsonisation may take place in the lower respiratory tract as sputum contains opsonins including IgA, IgG and to a lesser degree complement (Lever *et al.*, 1985). In humans, Fc-receptors include FcγRI (CD64), FcγRIIA (CD32) and FcγRIII (CD16), with CD16 and CD32 being the predominant receptors on resting neutrophils (Lee *et al.*, 2003). FcRs have cytoplasmic tails containing immunoreceptor tyrosine activation motifs (ITAMs). Ligand binding results in receptor cross-linking and tyrosine phosphorylation of ITAMs by src family kinases. A second protein tyrosine kinase (Syk) is then recruited to the phosphorylated ITAM domain and activated, which in turn triggers particle internalisation by activating phosphoinositide (PI)3-kinase and phospholipase C (Aderem and Underhill, 1999).

Electron microscopy studies have shown differences in internalisation of particles opsonised by either IgG or complement (Allen and Aderem, 1996). During FcR-mediated phagocytosis, pseudopods extend from the cell, reaching out around the particle, before fusing and drawing the particle into the cell (Allen and Aderem, 1996). In contrast, in complement receptor (CR)-mediated phagocytosis particles appear to sink into the cell, with minimal, if any, pseudopodia formation (Allen and Aderem, 1996). Formation of the phagosome in FcR-mediated phagocytosis is blocked by both protein kinase (PK)-C and tyrosine kinase inhibitors, whilst CR-mediated phagocytosis is blocked by PKC but not tyrosine kinase inhibition (Allen and Aderem, 1996). In contrast to FcR-mediated phagocytosis, CR-mediated phagocytosis requires intact microtubules, with nocodazole, a microtubule destabilising agent, inhibiting internalisation of complement-opsonised erythrocytes but not those opsonised with IgG (Newman *et al.*, 1991). FcR-mediated phagocytosis appears to trigger release of

pro-inflammatory molecules such as reactive oxygen species, again contrasting with CR-mediated phagocytosis (Aderem *et al.*, 1985; Wright and Silverstein, 1983).

1.4.1.2 Scavenger receptor mediated phagocytosis

Scavenger receptors (SR) are classified, according to their structure, into eight classes (A-H) and have been shown to act as PRR, recognising LPS, LTA and yeast zymosan (Areschoug and Gordon, 2009). SRs are phagocytic receptors mediating non-opsonised phagocytosis of pathogens by macrophages, and act as co-receptors for the Toll-like receptors (TLR) resulting in pro-inflammatory cytokine responses to various PAMPs (Hoebe *et al.*, 2005; Jeannin *et al.*, 2005). MARCO (macrophage receptor with collagenous structure) is a member of the class A SR family, which is the dominant phagocytic receptor for unopsonised inert particles and gram-positive and gram-negative bacteria on human AM (Arredouani *et al.*, 2005). MARCO knockout mice are significantly more susceptible to pneumococcal infection than wild-type mice, suggesting a role for this receptor in phagocytosis of *S. pneumoniae* (Arredouani *et al.*, 2004). Several other SR have also been identified on macrophages, including CD36 (class B SR), that recognises LTA and acts as a co-receptor for TLR2, with CD36 knockout mice susceptible to infection with *S. aureus* (Hoebe *et al.*, 2005). Treatment of monocyte-derived macrophages (MDMs) with nocodazole reduced internalisation of non-opsonised latex particles by ~50%, suggesting that phagocytosis via scavenger receptors requires intact microtubules, similar to CR-mediated phagocytosis (Sulahian *et al.*, 2008). Inhibition of tyrosine kinases, PKC and PI-3 kinase, all reduced internalisation of beads, whilst inhibition of PLC had no effect on internalisation (Sulahian *et al.*, 2008), confirming that the signalling pathways that are involved in the phagocytic process vary depending on the phagocytic receptor involved.

1.4.2 Particle internalisation

Internalisation of particles and pathogens requires activation of signalling pathways which in turn leads to rearrangement of the actin cytoskeleton, mobilisation of the microtubule network, and fusion of the phagosome with a lysosome to form a phagolysosome. Microtubules appear to play a role in particle internalisation but

there is also the requirement for an intact microtubule network for the movement of the phagosome (Allen and Aderem, 1996).

1.4.2.1 Microtubules

Microtubules are the tracks for the transport of organelles, membrane vesicles and secretory granules (Hirokawa, 1998). In cells in interphase, microtubules are found radiating out from the microtubule-organising centre (MTOC) and are required for cell division (Araki, 2006). The function of microtubules is dependent on dyneins and kinesins, which are motor proteins that use energy derived from ATP hydrolysis for movement (Hirokawa, 1998). The microtubule system controls vesicular trafficking, providing tracks through the cell along which components move (Araki, 2006). In the process of phagocytosis, microtubules appear to play a role in particle internalisation via both complement and scavenger receptors, with an additional role of microtubules in regulating β_2 integrin (part of the complement receptor) mobility and clustering in macrophages (Newman *et al.*, 1991). Furthermore, the movement of the phagosome requires an intact microtubule network (Allen and Aderem, 1996). After internalisation, the phagosome is transported through the cell, from the periphery toward the cell centre and towards the MTOC, fusing with endocytic organelles prior to fusion with a lysosome to form a phagolysosome (Allen and Aderem, 1996).

Microtubules are thought to be required for transporting the phagosome and lysosome into close proximity to enable fusion, rather than driving membrane fusion directly (Desjardins *et al.*, 1994). Nocodazole, a microtubule disruptor, inhibited movement of the late phagosomes in macrophage phagocytosis, confirming their importance in this process (Desjardins *et al.*, 1994). In neutrophils, the phagosome acquires anti-microbial activity through fusion with preformed vesicles and granules (Lee *et al.*, 2003). These include primary (azurophilic) granules characterised by myeloperoxidase and secondary (or specific) granules, that are thought to be the main source of phagosomal nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, generating reactive oxygen species which are antimicrobial (Lee *et al.*, 2003). Microtubules have been shown to contribute to the localised delivery of primary granules to the phagosome, as this can be disrupted by colchicine, a microtubule

disruptor, but may not be involved in transport of secondary granules (Tapper and Grinstein, 1997).

1.5 Phagocytosis in COPD

Studies have shown defective phagocytosis and killing of *Candida spp.* by alveolar macrophages (AM) from COPD patients compared to healthy controls (Ferrara *et al.*, 1996; Vecchiarelli *et al.*, 1991). AM from COPD patients also show defective phagocytosis of *E. coli* (Finney-Hayward *et al.*, 2005; Taylor *et al.*, 2010). Other studies, concentrating on commonly isolated respiratory pathogens, have shown significantly reduced phagocytosis of non-typeable *H. influenzae* (NTHI) by AM from COPD patients and smokers compared to AM from ex-smokers and healthy controls (Berenson *et al.*, 2006a; Marti-Llitas *et al.*, 2009). Previous work in our laboratory has shown that MDMs from COPD subjects also display defective phagocytosis of *H. influenzae*, as well as *S. pneumoniae*, compared to smokers and healthy controls (Taylor *et al.*, 2010), which indicates that this may be an inherent aberration of macrophages from susceptible individuals, rather than the result of an environmental exposure.

Neutrophils from patients with chronic bronchitis have also shown a reduced phagocytic index and killing of *Candida spp.* by approximately 50% in comparison to healthy subjects (Fietta *et al.*, 1988). Cigarette smoking may exacerbate this response, since neutrophils from healthy subjects incubated in the presence of cigarette smoke extract (CSE) showed significantly reduced phagocytosis of *Escherichia coli* when compared to untreated neutrophils (Prieto *et al.*, 2001). Similarly, CSE treated neutrophils exposed to two different strains of *Staphylococcus aureus* showed significantly reduced phagocytosis (~15%) compared to >60% of control cells (Guzik *et al.*, 2011). This is further supported by data from COPD patients, whereby neutrophils show reduced phagocytosis of *E. coli* compared with cells from healthy controls (Stringer *et al.*, 2007). Taken together these data suggest that the phagocytic mechanism regulating bacterial removal in COPD may not only be defective in macrophages, but may also be defective in neutrophils.

The mechanisms underlying defective phagocytosis have been suggested to be related to reduced expression of phagocytic receptors, with both MARCO and the mannose

receptor implicated (Baqir *et al.*, 2008; Hodge *et al.*, 2008). However, prior work on monocyte-derived macrophages showed no alterations in receptor expression when comparing cells from COPD patients to smoking and healthy controls (Taylor *et al.*, 2010). An alternative mechanism involving alterations in PI-3 kinase signalling has also been postulated, with the association of reduced phagocytosis of NTHI with reduced phosphorylation of Akt, a downstream effector of PI3K (Marti-Llitas *et al.*, 2009). PI3K signalling is required for F-actin accumulation in the periphagosomal region at the cell membrane (Greenberg and Grinstein, 2002). Alterations in PI-3 kinase signalling have been demonstrated to play a role in the altered chemotactic ability displayed by COPD neutrophils in comparison to healthy and smoker cells (Sapey *et al.*, 2011), and this may represent a common defect between chemotaxis and phagocytosis in these cells. The involvement of microtubules in the phagocytic defect present in COPD has not previously been investigated; therefore, the present study will focus on the role microtubules play in the mechanisms underlying defective phagocytosis in COPD.

1.6 Hypothesis

There is evidence of increased rates of bacterial colonisation in COPD patients in the stable state, despite increased numbers of phagocytic cells, namely macrophages and neutrophils, in the lower airways. This suggests that increased levels of bacterial colonisation in COPD may be due to defective phagocytosis.

The hypothesis for this thesis states that defective innate immunity in COPD patients leads to a reduction in bacterial phagocytosis and that this defective phagocytosis may relate to microtubule instability.

1.7 Aims

1. Assess macrophage and neutrophil phagocytosis in COPD, compared to controls matched for age and smoking history
2. Assess the effect of acute exacerbations on the phagocytic ability of macrophages
3. Establish a model of sputum macrophage phagocytosis
4. Investigate the effects of commonly prescribed inhaled corticosteroids on phagocytosis in COPD
5. Investigate underlying mechanisms of defective phagocytosis

Chapter 2

MATERIALS AND METHODS

2.1. Materials

Materials used in this thesis are listed below.

AGK2	Enzo Life Sciences, Exeter, UK
Alexa-fluor 488 carboxylic acid	Molecular Probes, Invitrogen, Paisley, UK
Alexa-fluor 488-labelled Goat anti-mouse IgG	Molecular Probes, Invitrogen, Paisley, UK
Alexa-fluor 488-labelled Goat anti-rabbit IgG	Molecular Probes, Invitrogen, Paisley, UK
All stars negative control siRNA	Qiagen, Crawley, UK
Annexin V apoptosis kit	Becton Dickinson, Oxford, UK
Bio-Rad protein assay	Biorad, Munich, Germany
Brain heart infusion broth	Fisher Scientific, Loughborough, UK
β -mercaptoethanol	Sigma-Aldrich, Dorset, UK
Budesonide	A gift from Astra Zeneca, Lund, Sweden
C6orf134 (pool of 4) siRNA	Qiagen, Crawley, UK
Cell dissociation solution	Sigma-Aldrich, Dorset, UK
Cell Lights Tubulin-GFP	Molecular Probes, Invitrogen, Paisley, UK
Cell Lights Actin-RFP	Molecular Probes, Invitrogen, Paisley, UK
Cell tracker red CMPTX	Molecular Probes, Invitrogen, Paisley, UK
Citifluor	Sigma-Aldrich, Dorset, UK
Columbia agar	Fisher Scientific, Loughborough, UK
Costar 96-well black tissue culture plates	Fisher Scientific, Loughborough, UK
Costar 24- and 96-well tissue culture plates	Fisher Scientific, Loughborough, UK

Coverslips	Raymond A Lamb, Eastbourne, UK
Cytochalasin D	Sigma-Aldrich, Dorset, UK
Di-sodium hydrogen citrate 1.5 hydrate	VWR International Ltd, Poole, UK
4',6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI)	Sigma-Aldrich, Dorset, UK
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich, Dorset, UK
Dulbecco's phosphate buffered saline (D-PBS)	Sigma-Aldrich, Dorset, UK
10x D-PBS	Gibco, Invitrogen, Paisley, UK
ECL detection solution	GE healthcare, Buckinghamshire, UK
ELISA antibodies	R&D systems
Epothilone B	Sigma-Aldrich, Dorset, UK
<i>E.coli</i> (K-12 strain) BioParticles (labelled with Alexa-fluor 488)	Molecular Probes, Invitrogen, Paisley, UK
FACSflow	Becton Dickinson, Oxford, UK
FACSclean	Becton Dickinson, Oxford, UK
Fluorescently labelled carboxylate modified polystyrene microspheres (2.0 µm diameter)	Molecular Probes, Invitrogen, Paisley, UK
Fluor-de-Lys HDAC activity assay	Enzo Life Sciences, Exeter, UK
Fluticasone propionate	A gift from Astra Zeneca, Lund, Sweden
Foetal bovine serum	Gibco, Invitrogen, Paisley, UK
Formaldehyde	Sigma-Aldrich, Dorset, UK
Full range rainbow molecular weight marker	GE healthcare, Buckinghamshire, UK

5% (^w / _v) Glucose	Baxter Healthcare Ltd, Norfolk, UK
Glycerol	Sigma-Aldrich, Dorset, UK
<i>Haemophilus influenzae</i>	Dr Paul Langford (Paediatric Infectious Disease, Imperial College, London)
Haemophilus supplement	Fisher Scientific, Loughborough, UK
Hank's balanced salt solution	Sigma-Aldrich, Dorset, UK
HDAC6 (pool of 4) siRNA	Qiagen, Crawley, UK
Hemohes 6% (^w / _v)	Braun, Melsungen, Germany
High Capacity cDNA Reverse Transcription kit.	Applied Biosystems, Warrington, UK
HiPerfect	Qiagen, Crawley, UK
Horse blood (defibrinated)	Fisher Scientific, Loughborough, UK
HRP-labelled Goat anti-rabbit IgG	New England Biolabs, Herts, UK
HRP-labelled Goat anti-mouse IgG	New England Biolabs, Herts, UK
Human recombinant GM-CSF	R&D systems
Human recombinant TNF α	R&D systems
Hybond ECL	Amersham Pharmacia Biotech, GE healthcare, Buckinghamshire, UK
Hyperfilm ECL	Amersham Pharmacia Biotech, GE healthcare, Buckinghamshire, UK
L-glutamine	Gibco, Invitrogen, Paisley, U
Lab-tek Permanox chamber slides	Fisher Scientific, Loughborough, UK
Light green	Sigma-Aldrich, Dorset, UK
LS columns and collection tubes	Miltenyi Biotec, Surrey, UK

MACS Monocyte isolation kit	Miltenyi Biotec, Surrey, UK
Methanol	BDH, Poole, Dorset, UK
Methylthiazoyldiphenyl-tetrazolium bromide (MTT)	Sigma-Aldrich, Dorset, UK
Microscope slides	Raymond A Lamb, Eastbourne, UK
Mini complete protease inhibitor cocktail tablets	Roche, Mannheim, Germany
Mouse anti-human TNF α antibody	R&D systems
Mouse monoclonal anti-acetylated tubulin antibody	Sigma-Aldrich, Dorset, UK
Mouse monoclonal anti-SIRT2 antibody	Sigma-Aldrich, Dorset, UK
Mouse monoclonal anti-HDAC6 antibody	Sigma-Aldrich, Dorset, UK
Nocodazole	Sigma-Aldrich, Dorset, UK
NuPage MOPS SDS running buffer	Invitrogen, Paisley, UK
Nylon mesh filters	Falcon, Oxford, UK
Paclitaxel	Sigma-Aldrich, Dorset, UK
Paraformaldehyde	Sigma-Aldrich, Dorset, UK
PE mouse anti-human TLR4 antibody	eBioscience, Hatfield, UK
PE mouse anti-human TLR2 antibody	eBioscience, Hatfield, UK
PE mouse anti-human CD11b antibody	eBioscience, Hatfield, UK
Penicillin	Gibco, Invitrogen, Paisley, UK
Percoll	GE Healthcare, Buckinghamshire, UK
pHrodo dye, succinimidyl ester	Molecular Probes, Invitrogen, Paisley, UK

Phosphate buffered saline tablets	Sigma-Aldrich, Dorset, UK
Phosphatase inhibitor cocktail 2	Sigma-Aldrich, Dorset, UK
Polyoxyethylene-sorbitan monolaurate (Tween-80)	Sigma-Aldrich, Dorset, UK
Polystyrene FACS tubes	Becton Dickinson, Oxford, UK
Protease inhibitor cocktail 1	Sigma-Aldrich, Dorset, UK
QIAshredder spin column	Qiagen, Crawley, UK
Quick-Diff kit	Gentaur, Belgium
Rabbit monoclonal actin antibody	Cell signalling, New England Biolabs, Hertfordshire, UK
Rabbit monoclonal alpha-tubulin antibody	New England Biolabs, Herts, UK
Rabbit polyclonal anti-SIRT2 antibody	Sigma-Aldrich, Dorset, UK
Rabbit polyclonal anti-HDAC6 antibody	Sigma-Aldrich, Dorset, UK
RIPA buffer	Cell signalling, New England Biolabs, Hertfordshire, UK
RNase-free DNase	Qiagen, Crawley, UK
RNeasy mini RNA extraction kit	Qiagen, Crawley, UK
Roswell Park Memorial Institute media (RPMI)-1640	Sigma-Aldrich, Dorset, UK
0.9% (^w / _v) saline	Baxter Healthcare Ltd, Norfolk, UK
Sample buffer, Laemmli, 2x concentrate	Sigma-Aldrich, Dorset, UK
Saponin	Sigma-Aldrich, Dorset, UK
SIRT2 (pool of 4) siRNA	Qiagen, Crawley, UK
4-12% SDS-PAGE gel	Invitrogen, Paisley, UK

Sphingosine-1-Phosphate Elisa kit	Tebu-Bio, UK
Sodium azide	Sigma-Aldrich, Dorset, UK
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich, Dorset, UK
Sterile needles	BDH, Poole, Dorset, UK
Sterile water	Sigma-Aldrich, Dorset, UK
<i>Streptococcus pneumoniae</i>	Dr Paul Langford (Paediatric Infectious Disease, Imperial College, London)
Streptomycin	Gibco, Invitrogen, Paisley, UK
Sulphuric acid (H ₂ SO ₄)	Sigma-Aldrich, Dorset, UK
Syringes	Becton Dickinson, Oxford, UK
TaqMan probes (C6orf134, SIRT2, HDAC6 and HPRT1)	Applied Biosystems, Warrington, UK
TaqMan universal mastermix	Applied Biosystems, Warrington, UK
Todd-Hewitt broth	Fisher Scientific, Loughborough, UK
Toluidine blue	Sigma-Aldrich, Dorset, UK
Trypan blue	BDH, Poole, Dorset, UK
Triton-X-100	Sigma-Aldrich, Dorset, UK
Trizma base (TRIS)	Sigma-Aldrich, Dorset, UK
Tubacin	Enzo Life Sciences, Exeter, UK
Tubulin Tracker	Molecular Probes, Invitrogen, Paisley, UK
Vacutainer 6ml K2 EDTA	Becton Dickinson, Oxford, UK
Vectastain ABC elite kit – Mouse IgG	Vector laboratories, California, USA
Venofundin	B.Braun, Sheffield, UK

Yeast supplement	Fisher Scientific, Loughborough, UK
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2.2 Methods

2.2.1 Subject selection

Healthy volunteers and healthy smokers were recruited by the National Heart and Lung Institute (NHLI), Imperial College, London. COPD patients were recruited either through the Royal Free Hospital, Pond Lane, London, Wexham Park Hospital, Slough or by the National Heart and Lung Institute (NHLI), Royal Brompton Hospital, London. All subjects gave written informed consent as approved by the London Chelsea Ethics Committee (Ethics number 08/H0706/08).

2.2.1.1. Healthy volunteers

The inclusion criteria for healthy volunteers were: age 40-80 years, no history of respiratory disease or co-morbidity, never smokers, with normal lung function as predicted for sex, age and height and no evidence of atopy on skin prick tests to common aero-allergens.

2.2.1.2. Smokers

The inclusion criteria for healthy smoking volunteers were: age 40-80 years, no history of respiratory disease or co-morbidity, current smokers with a smoking history of >10 pack years (where one pack year is defined as smoking one pack of cigarettes per day for one year) with normal lung function as predicted for sex, age and height, no reversibility and no evidence of atopy on skin prick tests to common aero-allergens.

2.2.1.3. COPD patients

The inclusion criteria for COPD patients were: aged 40-80 years, no history of other respiratory disease or major co-morbidity, smoking history of >10 pack years and no evidence of atopy on skin prick tests to common aero-allergens. Lung function: FEV₁% predicted <80%, FEV₁:FVC ratio <0.7 and reversibility of <200ml in FEV₁ (<15% FEV₁% predicted) to inhaled β_2 agonist. In addition, for baseline samples no history of exacerbation or oral steroid or antibiotic usage in the preceding 6 weeks.

2.2.2 Clinical measurements

Clinical measurements were either performed by either myself, nursing staff or lung function technicians at the NHLI, Royal Brompton Hospital, Royal Free or Wexham Park Hospital.

2.2.2.1. Spirometry

Forced expiratory volume in 1 second (FEV₁) and forced vital capacity (FVC) were measured using a spirometer (Vitalograph, Buckingham, Bucks, UK). Values were taken as the best of three reproducible attempts. Values are expressed either as absolute volume or as percentage predicted based on sex, weight and height and calculated by software (based on Crapo tables; Crapo *et al.*, 1981).

2.2.2.2. Reversibility

Reversibility testing was performed according to BTS guidelines. Subjects performed spirometry as outlined above (section 2.2.2.1), then were given a nebulised solution of salbutamol 2.5mg. After 20 min, spirometry was performed once again. A significant response was defined as a change of over 15% from the baseline FEV₁ that also exceeded 200ml in absolute terms (1997).

2.2.3 Blood Separation

Human subjects gave 60ml of whole blood by venepuncture. Whole blood was mixed (1:1) with 20ml 6% (^w/_v) Hemohes and 5ml 0.9% (^w/_v) saline and incubated at room temperature for 45 min to sediment red blood cells. The top, leukocyte-rich, layer was aspirated and washed twice with Dulbecco's phosphate buffered saline (D-PBS) by centrifugation (400 x *g* for 10 min).

The resulting cell pellet contains a mixture of leukocytes, therefore, Percoll gradients, were used to separate the polymorphonuclear (PMN) cell fraction from the peripheral blood mononuclear cell (PBMC) fraction. A 100% (^v/_v) Percoll solution was prepared using 10% (^w/_v) 10x PBS. Using this solution, Percoll fractions of 81% (^v/_v), 68% (^v/_v) and 55% (^v/_v) in 1x D-PBS were prepared. To prepare the gradients, 4ml of 81% (^v/_v) solution was transferred into a 15ml Falcon tube, followed by 4ml of 68% (^v/_v) solution. The cell pellet was resuspended in 3ml of the 55% (^v/_v) solution and overlaid onto the

gradient. The cell fractions are then separated according to density by centrifugation at $750 \times g$ for 25 min, at room temperature. The PBMC fraction was harvested from the 55% (v/v) /68% (v/v) Percoll interface and the granulocytes from the 68% (v/v) /81% (v/v) Percoll interface. Any red blood cells in the PMN fraction were lysed using 1ml ice cold sterile water; cells were resuspended in water for 10 sec before adding 49ml D-PBS. Both cell fractions were washed with D-PBS and resuspended in 1ml of RPMI 1640 medium. Cell counts were performed using a Neubauer haemocytometer, after staining with 1:100 Kimura stain (0.05% (v/v) toluidine blue, 0.03% (v/v) light green, 10% (v/v) saponin, 0.7M phosphate buffer).

The granulocyte fraction was resuspended at 1×10^6 cells/ml in RPMI 1640 medium and allowed to rest for 1h at 4°C to reduce neutrophil activation. In some experiments, the PBMC fraction was divided into two equal parts to allow for comparison of different techniques for the isolation of monocytes as indicated (see sections 2.2.3.1 and 2.2.3.2).

2.2.3.1 Separation of monocytes by adherence

PBMC were resuspended at 1×10^6 monocytes/ml in complete medium (RPMI 1640, 10% (v/v) FBS, 10mg/ml (1% (v/v)) penicillin/streptomycin, 2mM (1% (v/v)) L-glutamine), transferred to 96 well black plates at 100 μ l/well and incubated at 37°C , 5% (v/v) CO_2 for 2h to allow adherence of monocytes. Media and non-adherent cells were aspirated from the plate and fresh media containing GM-CSF (2ng/ml) was added. Cells were incubated at 37°C , 5% (v/v) CO_2 for 12d, with fresh media containing GM-CSF added on d4 and d8.

2.2.3.2 Separation of monocytes by negative selection

Monocytes were isolated from the PBMC fraction by negative selection using the Miltenyi Monocyte Isolation Kit II as per manufacturer's instructions. The resulting cell suspension was centrifuged and resuspended in 1ml complete medium (containing GM-CSF at 2ng/ml) and cells counted. The cells were then resuspended at 1×10^6 cells/ml in complete medium containing GM-CSF (2ng/ml) and transferred to 96 well black plates at 100 μ l/well and incubated for 12d as described in section 2.2.4.1.

2.2.3.3 Protein assay

Cells were lysed using a freeze thaw technique and then supernatants (1 μ l) were added in triplicate to a 96 well plate. To produce a standard curve, 1mg/ml BSA was diluted in D-PBS (0.125-1mg/ml). 200 μ l of Bradford protein assay solution (diluted 1:5 in distilled water (dH₂O)) was added to each well and the absorbance read at λ 595nm. A standard curve was plotted and unknown protein concentrations interpolated (level of sensitivity given by the standard curve was 0.0625mg/ml protein). Interpolated x values were then multiplied by 5 to account for the dilution factor and protein concentrations derived.

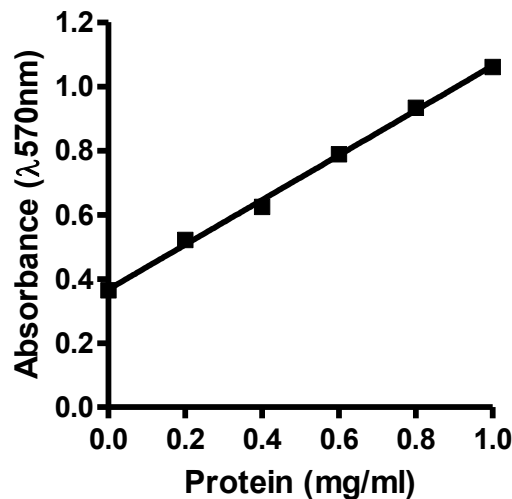


Figure 2.1. Protein standard curve used to derive unknown protein concentrations.

BSA was diluted in D-PBS to given concentrations from 0.2-1mg/ml. Bradford protein assay reagent was added and the absorbance read at λ 595nm. Values were plotted to give a standard curve, from which unknown protein concentrations can be calculated.

2.2.3.4 Viability assay

Cell media was aspirated and 100 μ l of methylthiazoyldiphenyl-tetrazolium bromide (MTT) solution (1mg/ml) added to each well. The plates were incubated at 37°C for 30 min, then, after aspirating the MTT solution, 100 μ l of DMSO was added per well. Absorbance was then measured on the spectrophotometer at λ 570nm. Data was normalised to the viability of control cells (given as 100%), to give a measure of viability compared to untreated cells.

2.2.4 Sputum induction and processing

Sputum induction was carried out as described by Pavord *et al* (Pavord *et al.*, 1997). Subjects performed spirometry, followed by administration of 7ml 3% (v/v) saline using an ultrasonic nebuliser. Spirometry was re-measured after each saline nebuliser – if FEV_1 fell by $<10\%$ the process was continued using 4% (v/v) and 5% (v/v) saline. However, if the FEV_1 fell $>10\%$ but $<20\%$, nebulisation with 3% (v/v) saline was repeated. If the FEV_1 fell by $>20\%$ the process was discontinued. Patients were allowed to expectorate sputum at any point during induction, with the recommendation that prior to expectoration, the subject blew their nose, rinsed their mouth and then expectorated sputum into a sterile container.

Sputum was stored and processed on ice within 2 h of obtaining sample. Sputum plugs were selected from the whole sample of sputum obtained, weighed and an equal volume of D-PBS was added and mixed by inversion. Dithiothreitol (DTT) was added to give a final concentration of 0.05% (w/v). The sample was rocked on a bench rocker at room temperature for 30 min, prior to filtering through 100 μm nylon gauze. The resulting filtrate was then centrifuged at 400 $\times g$ for 10 min. Supernatants were aspirated and stored at -80°C . The cell pellet was washed in D-PBS, resuspended in 1ml complete media containing amphotericin (1% v/v) and differential cell count performed. Viability was confirmed using trypan blue, with samples discarded if $<70\%$ viability. To confirm that the sample was from the lower respiratory tract, squamous cells should represent $<10\%$ of total cell count; those with $>10\%$ squamous cells were discarded. Cell suspensions were adjusted to 1×10^6 macrophages/ml with RPMI and 100 μl added to FACS tubes for use in phagocytosis assays. Cytospins were performed with 50 μl of cell suspension, air dried and stored at -80°C .

2.2.5 Bacterial Culture

2.2.5.1 *Streptococcus pneumoniae*

Frozen cultures of *S. pneumoniae* serotype 9V, strain 10692 (strain taken from a COPD patient in exacerbation) were streaked onto a Columbia blood agar plate and incubated at 37°C and 5% (v/v) CO_2 overnight. The following morning, 20ml aliquots of Todd-Hewitt broth containing 5% (v/v) yeast extract (THY broth) were inoculated with

single colonies of bacteria from the plate. The inoculated broth was incubated in a shaker incubator at 37°C, 200 rpm; each hour 1ml aliquots were taken for optical density (OD) readings at $\lambda 600\text{nm}$. Additionally, serial dilutions of the bacteria were plated on blood agar plates and incubated overnight at 37°C. The following day colonies were counted and colony forming units (CFU)/ml calculated:

$$\text{CFU/ml} = \frac{(\text{Number of colonies on plate}) \times \text{dilution factor}}{(\text{Amount plated (ml)})}$$

The turbid broth was divided into fresh universal tubes (2ml of inoculated broth added to 18ml fresh THY broth) and bacteria incubated at 37°C, 200 rpm. Once the cultures reached an optical density of 1.2 (Fig. 2.2) the bacteria were harvested by centrifugation at 1600xg for 15 min, then resuspended in D-PBS. The bacteria were then heat killed in a water bath at 70°C for 2 h, followed by washing twice. To determine the efficiency of heat killing, 100 μl of bacterial suspension was plated for overnight incubation at 37°C; a colony count of zero was taken as adequate heat killing.

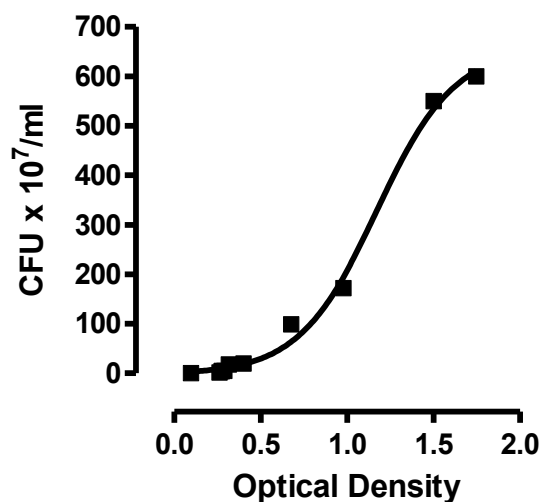


Figure 2.2. Growth curve of *Streptococcus pneumoniae* with optical density plotted against colony forming units at each time point

Bacteria were inoculated into THY broth and incubated at 37°C. At hourly time points, 1ml aliquots were taken for optical density reading ($\lambda 600\text{nm}$), serial dilution, plating and then overnight incubation, before colony counting and calculation of CFU/ml. CFU/ml was plotted against OD. The linear portion of the graph (where growth is exponential) was used to derive a CFU/ml for a given OD for subsequent bacterial cultures.

2.2.5.2 *Haemophilus influenzae*

H. influenzae was grown using the a similar method as described for *S. pneumoniae* (section 2.2.5.1); briefly, a frozen stock of Non-typeable *H. influenzae* strain 1479 (taken from a COPD patient during exacerbation) was plated on Columbia agar supplemented with bovine hemin and NAD⁺ (10µg/µl) and incubated overnight before being inoculated into brain heart infusion (BHI) broth with bovine hemin and NAD⁺ (10µg/µl). Standard growth curves were derived and bacteria were subsequently cultured to a given OD (Fig. 2.3) before heat killing as described in section 2.2.5.1.

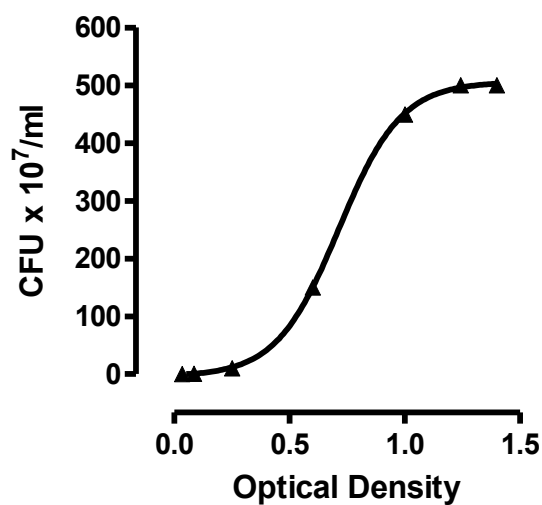


Figure 2.3. Growth curve of *Haemophilus influenzae* with optical density plotted against colony forming units at each time point

Bacteria were inoculated into BHI broth and incubated at 37°C. At hourly time points, 1ml aliquots were taken for optical density reading at λ600nm and serial dilutions followed by plating and incubation overnight, before colony counting and calculation of CFU. CFU/ml was plotted against OD. The linear portion of the graph (where growth is exponential) was used to derive a CFU/ml for a given OD for subsequent bacterial cultures.

2.2.6 Fluorescent labelling of bacteria

2.2.6.1 Labelling of bacteria with Alexa-fluor 488 dye

Heat-killed bacteria were resuspended in 1ml sodium bicarbonate buffer (8.4g NaHCO₃ in 100ml distilled water) before adding 10µl Alexa-fluor 488 dye ((ex λ488nm/em λ520nm) reconstituted in DMSO (1mg/ml)). Bacteria were then rotated overnight, in

the dark, at room temperature. Bacteria were then washed repeatedly in D-PBS to remove unbound dye, resuspended in D-PBS and stored at -20°C.

2.2.6.2 Labelling bacteria with pHrodo™ dye

Bacteria were resuspended in 750µl of freshly prepared 100mM sodium bicarbonate solution. Stock solution of amine-reactive pHrodo SE (10mM in DMSO) was then added to give a final concentration of 0.5mM. Bacteria were incubated for 45 min in the dark, at room temperature. The bacteria were then washed in HBSS by centrifugation (15000xg, 60 s), the supernatant aspirated and bacteria resuspended in 1ml of 100% methanol. The bacteria were once again centrifuged (15000 x g, 60 s), the supernatant aspirated and resuspended in 1ml HBSS for a further two washes. Finally the bacteria were resuspended in 1ml of HBSS and stored at 4°C in the dark.

2.2.6.3 Labelling of live bacteria

Bacteria were harvested by centrifugation (1600xg, 15 min), then resuspended in a working solution of broth containing CellTracker Red CMPTX dye (ex λ577nm/em λ602nm) to give a final concentration of 12.5µM, followed by incubation at 37°C for 45 min. The working solution was then replaced with pre-warmed fresh broth for 30 min. Bacteria were washed repeatedly in D-PBS to remove free dye and stored at -20°C.

2.2.7 Phagocytosis Assays

2.2.7.1 Phagocytosis of Beads

Fluorescently labelled carboxylate-modified polystyrene microspheres (2.0µm diameter, yellow-green fluorescence (ex λ505nm/em λ515nm)) were diluted in RPMI media to concentrations indicated.

MDMs: Bead solutions (100µl) were added to wells containing 1×10^5 macrophages and incubated at 37°C, 5% (v/v) CO₂ for the times indicated. After incubation, unbound beads were washed off with D-PBS. Fluorescence of extracellular particles was quenched by adding 100µl/well Trypan Blue (1% (v/v)) for 1 min. Excess fluid was removed and fluorescence determined by fluorimetry (BMG Fluostar plate reader) at excitation λ480nm and emission λ520nm.

Neutrophils: Bead solutions (900µl) were added to FACS tubes containing 1×10^5 neutrophils and incubated at 37°C, 5% (v/v) CO₂ for the times indicated. After incubation, tubes were placed on ice to quench phagocytosis, then washed twice (400xg, 5 min, 4°C) with ice cold D-PBS. Cell pellets were resuspended and fixed in 300µl FACSfix (0.5% (v/v) paraformaldehyde in FACSflow) ready for analysis. Samples were analysed using a Becton Dickinson FACScan machine using CellQuest software. Cells were gated according to forward and side scatter values correlating to neutrophils (Fig. 2.4) and for each analysis 10,000 events were collected.

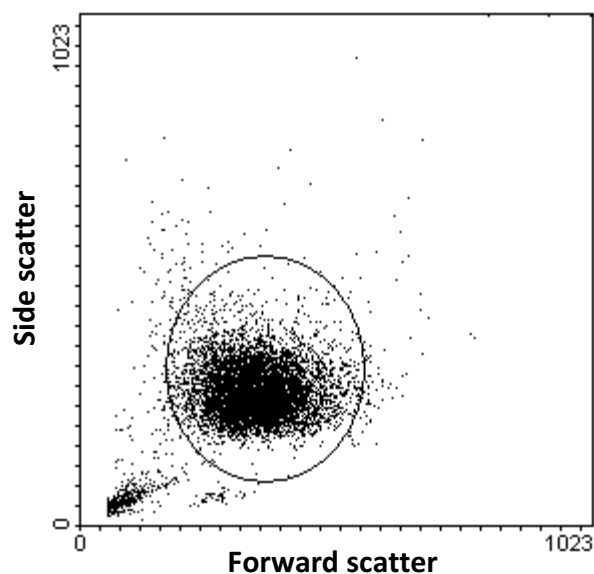


Figure 2.4. FACS dot plot showing a neutrophil population

Cell populations were gated according to properties of forward scatter (cell size) and side scatter (cell density). 10,000 gated events were collected for analysis.

FACS analysis of neutrophil phagocytosis shows a shift in the cell population to the right; as beads are taken up into cells, each cell becomes more fluorescent, the average fluorescence of the cell population increases (denoted as the median fluorescent intensity (MFI)) and the histogram moves to the right along the x-axis (Fig. 2.5). Percentage phagocytosis is calculated as the percentage of cells that have taken up ≥ 1 bead or bacteria and moved out of the control population. Further analysis of neutrophil phagocytosis of beads by FACS can discriminate between the numbers of beads taken up into cells (Fig. 2.5).

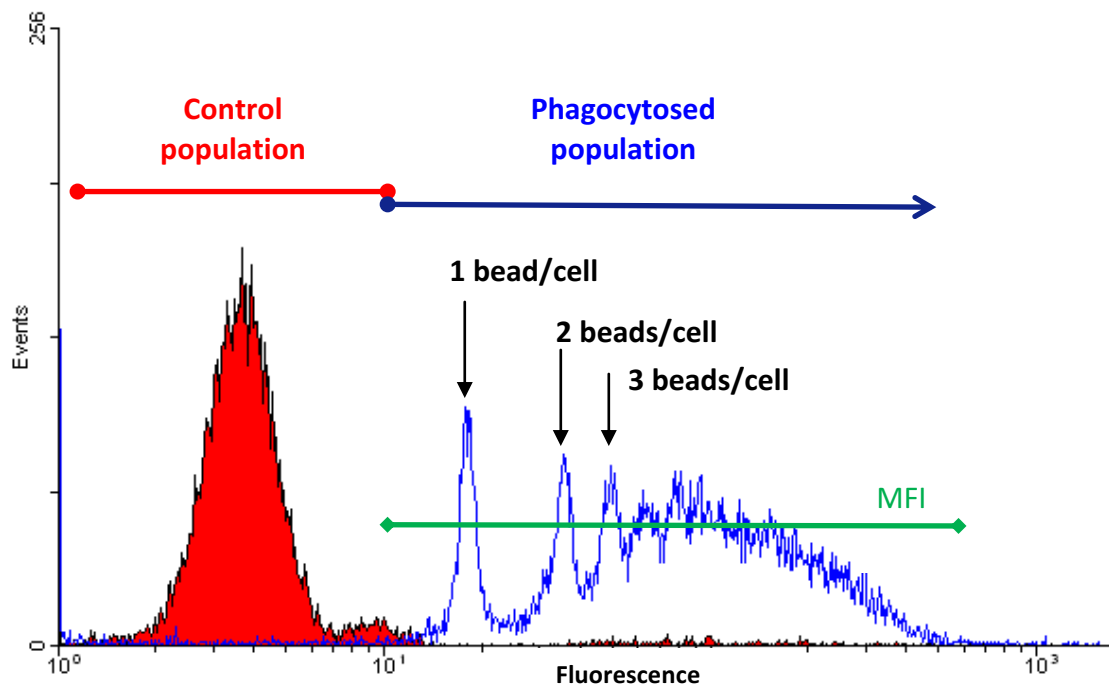


Figure 2.5. Neutrophil phagocytosis of polystyrene beads – quantified by flow cytometry.

Neutrophils were incubated with 900 μ l of 50x10⁶beads/ml for 15 minutes. Fluorescence of internalised beads was detected by FACS analysis. ■ denotes control population of neutrophils, ■ denotes neutrophils that have undergone phagocytosis of beads. Neutrophils that ingest fluorescently-labelled beads increase in fluorescence, therefore the population shifts to the right along the x-axis. Different peaks on the histogram plot represent increasing numbers of beads phagocytosed/cell. The average fluorescence of the population that has undergone phagocytosis is denoted as the median fluorescent intensity (MFI) - this gives a measure of the amount of prey taken up. Data shown are representative of n=4.

2.2.7.2 Phagocytosis of Bacteria

Bacteria were sonicated for 1 min prior to use to prevent aggregation. Bacteria were diluted to the appropriate concentrations in D-PBS. For MDMs phagocytosis assays, 100 μ l of bacterial suspension was transferred to wells, whilst for neutrophil assays 900 μ l of bacterial suspension was added to FACS tubes and incubated, washed and analysed as described above.

2.2.8 Measurement of TNF- α from cell supernatant

Measurement of TNF- α was performed using ELISA according to manufacturer's guidelines. Briefly, 96 well NUNC Maxisorp plates were coated with 1 μ g/ml (100 μ l/well) mouse anti-human TNF α antibody (capture) diluted in sterile PBS. The plates were incubated overnight at room temperature. The capture antibody was removed and the plates were blocked with blocking buffer containing 1% ($^w/v$) BSA, 5% ($^w/v$) sucrose and 0.05% ($^w/v$) sodium azide (150 μ l/well), for 2 h at room temperature.

Blocking buffer was removed and standards of human recombinant TNF α were added to appropriate wells. Cell supernatants from MDMs after 4h phagocytosis of beads/bacteria were diluted; 1:2 (control and beads); 1:5 (*S.pneumoniae*); 1:40 (*H.influenzae*). The plates were incubated for 2 h at room temperature. Plates were washed three times in wash buffer (PBS containing 0.05% ($^v/v$) Tween20) then detection antibody was added to all wells at a concentration of 0.1 μ g/ml before a further 2 h incubation at room temperature. Plates were washed and 100 μ l streptavidin-horseradish peroxidase (HRP) added to each well, diluted 1:200 from stock solution. The plates were incubated for 20 min at room temperature. The plates were washed and 100 μ l substrate solution was added to each well (equal volumes of Colour Reagent A (H₂O₂) and Colour Reagent B (Tetramethylbenzidine) mixed directly before addition to plates). The reaction was stopped by the addition of 50 μ l/well 1M sulphuric acid.

The optical density of each well was determined in a spectrophotometer, read at λ 450nm, while subtracting values at λ 650nm to account for imperfections in the plate, and concentrations of cytokines were derived from extrapolation from standard curves (level of sensitivity of standard curve 31.25pg/ml TNF- α).

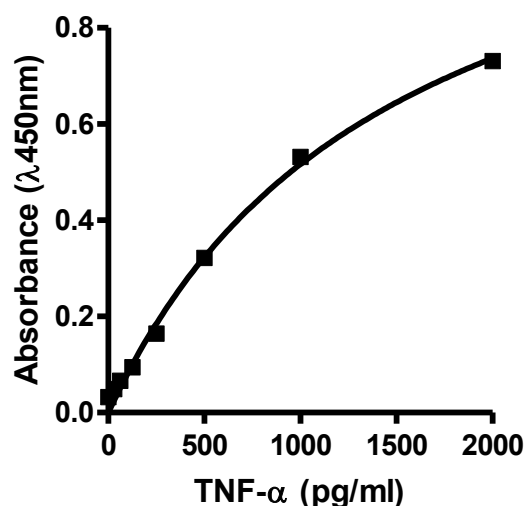


Figure 2.6. Standard curve used to derive unknown concentrations of TNF-α

TNF-α standard was diluted in D-PBS to given concentrations from 31.25-2000pg/ml. Plates were incubated with detection antibody (0.1μg/ml) for 2 h prior to addition of HRP for 20 min. Substrate solution was then added to each well and the reaction stopped by addition of 50 μl/well 1M sulphuric acid. The absorbance was read at λ450nm. Values were plotted to give a standard curve, from which unknown TNF-α concentrations can be calculated.

2.2.9 Western blotting

Neutrophil cell pellets, containing 10^7 cells, were snap frozen in liquid nitrogen, before lysis directly into sample buffer (4% (^{w/v}) SDS, 20% (^{v/v}) glycerol, 10% (^{v/v}) 2-mercaptoethanol, 0.004% (^{w/v}) bromphenol blue, 0.125M Tris-HCl) followed by boiling for 5 min, snap freezing and storage at -80°C. MDMs cell pellets were washed in ice-cold D-PBS prior to lysis in 25μl/well RIPA buffer (20mM Tris-HCl, 150 mM NaCl, 1mM Na₂-EDTA, 1mM EGTA, 1% (^{v/v}) Triton-X, 1% (^{w/v}) sodium deoxycholate, 1% (^{w/v}) NP-40, 2.5mM sodium pyrophosphate, 1μg/ml leupeptin) with added protease inhibitor cocktail and phosphatase inhibitors, before storage at -80°C.

2.2.9.1 Gel Electrophoresis

MDMs lysates were centrifuged at 1600 x *g* for 3 min and the resulting supernatants used to measure protein levels (see section 2.2.3.3). Equal protein concentrations of

each sample (20µg made up to 30µl of sample buffer), were then boiled for 3 min prior to loading onto the gel.

SDS-PAGE gels (4-12%) were used for separation of proteins according to molecular size. Gels were secured in a gel tank along with MOPS running buffer (250mM MOPS, 250mM Tris, 5mM EDTA, 1% (^w/_v) SDS). 7.5µl of a molecular weight rainbow marker was added to the gel. 25µl of each sample was loaded into individual wells of the gel. The gel tank was then connected to a power source and proteins were resolved at 120 mA and 120V for 2 h.

2.2.9.2 Transfer of protein to nitrocellulose

Transfer cassettes were assembled containing two sponge pads, between which were placed four sheets of filter paper, all soaked in transfer buffer (0.191M glycine, 0.02M Tris-HCl, 20% (^v/_v) methanol). The gel and Hybond nitrocellulose membrane were placed between the filter paper and the cassette closed. The cassettes were placed into the transfer tank, along with an ice block, and transfer buffer was added. Proteins were transferred from gel to nitrocellulose at 200mA, 200V for 1 h.

2.2.9.3 Immunoblotting

In order to minimise non-specific binding of the antibodies to the membrane, the membrane was blocked by washing in 5% (^w/_v) milk dissolved in TBS-Tween 20 (0.5M Tris base, 9% (^w/_v) NaCl, 0.5% (^v/_v) Tween 20) for 1 h at RT. Blocking solution was then replaced with fresh 5% milk/TBS-Tween 20 containing a primary antibody against the protein of interest, prior to incubation for 16 h at 4°C.

The membranes were washed repeatedly in TBS-Tween 20 then incubated with fresh 5% (^w/_v) milk/TBS-Tween 20 containing an appropriate HRP-conjugated secondary antibody and incubated for 1 h at RT. The membranes were washed repeatedly in TBS-Tween 20, blotted, and then incubated with 10ml enhanced chemiluminescence (ECL) detection solution for 1 min at RT. After blotting, the membranes were wrapped in clingfilm and exposed to Hyperfilm for appropriate times, prior to the films being developed using an AFP imaging developer.

2.2.10 Measurement of expression of cell surface markers

For flow cytometric analysis of surface markers, neutrophils were resuspended at 1×10^6 cells/ml in D-PBS, with 100 μ l added to each FACS tubes. Antibodies were then added at the dilutions specified in Table 2.1, according to manufacturer's recommendations:

Antibody	Dilution
Isotype control (0.2mg/ml)	1:10
Anti-CD11b (0.2mg/ml)	1:10
Anti- TLR2 (0.2mg/ml)	1:10
Anti- TLR4 (0.4mg/ml)	1:20

Table 2.1. Antibody dilutions used for cell surface marker quantification

Cells were then incubated for 30 min at 4°C in the dark, prior to washing with ice-cold PAB (D-PBS with 5% ($^w/v$) BSA and 0.1% ($^w/v$) sodium azide) then fixed for immediate analysis.

2.2.11 Confocal Microscopy

2.2.11.1 Confirmation of phagocytosis by MDMs

Monocytes (2×10^5) were cultured directly on Lab-tek Permanox chamber slides and differentiated into MDMs by incubation for 12 d in media containing GM-CSF (2ng/ml) (see section 2.2.3). Fluorescently labelled beads or bacteria were added to chambers and incubated for 4 h. Particles or bacteria not internalised were removed by aspiration and chambers washed repeatedly with D-PBS.

Cells were then incubated at 37°C for 45 min in a working solution of media containing CellTracker Red CMPTX dye (12.5 μ M). The working solution was then replaced with appropriate pre-warmed media for a further 30 min. Cells were then fixed by incubating with 4% ($^w/v$) paraformaldehyde for 15 min. Cells were then washed with

D-PBS and the nuclei stained by incubation with DAPI (2 μ M) for 3 min. Following repeated washing in D-PBS, the chambers were removed from the microscope slides and a coverslip mounted using Citifluo. Slides were stored at 4°C in the dark.

2.2.11.2 Staining with Tubulin Tracker™

MDMs were cultured directly on Lab-tek Permanox chamber slides (see section 2.2.3) and fluorescently labelled beads or bacteria were added to chambers and incubated for 4 h. Particles or bacteria not internalised were removed by aspiration and chambers washed repeatedly with D-PBS. Cells were then incubated with Tubulin Tracker (Invitrogen, UK), 250nM in RPMI media, for 30 min at 37°C, prior to fixation with 4% (^w/_v) paraformaldehyde in D-PBS. After washing with D-PBS, slides were incubated with diaminidino phenylindole (DAPI) at 2 μ M in PBS for 3 min. After further washing, slides were air dried, then mounted with citifluo and stored at 4°C in the dark, or visualised immediately.

2.2.11.3 Visualisation

Slides were viewed on a Leica SP2 upright confocal microscope with images of the three stains overlaid using Volocity™ software. In order to visualise internalisation of the particles, images were taken at sequential slices then remodelled into orthogonal views using Volocity™ software.

2.2.12 Live imaging

MDMs were cultured as described in section 2.2.3. MDMs in black microplates with clear bottoms were viewed on a heated stage. Fluorescent phagocytic prey (diluted in media) was then added to wells of interest and the cells imaged with a Zeiss LSM-510 inverted confocal microscope. Images and videos were analysed and edited with Volocity™ software.

2.2.13 Knockdown

MDMs were transiently transfected with siRNA using the HiPerfect method (Qiagen, Crawley, UK). In all experiments two controls were used; untransfected cells and cells transiently transfected with equal concentrations of a negative control (scramble) siRNA.

MDMs were cultured in both 24 and 96 well plates as described in section 2.2.3. Supernatants were aspirated and 30 μ l or 100 μ l of RPMI media added per well to 96 and 24 well plates respectively. siRNA (see Appendix A) was reconstituted in RPMI media as follows; for 24 well plates, 750ng of each siRNA was added, giving a total of 3 μ g siRNA, in a volume of 100 μ l per well, whilst for 96 well plates, 250ng of each siRNA (total 1 μ g) was added in a volume of 30 μ l per well. Hiperfect (1 μ l/well for 96well plates and 6 μ l/well for 24 well plates) was then added and the samples briefly vortexed, then rested for 10 min to form complexes. In a dropwise fashion, 30 μ l or 100 μ l was added per well of a 96 or 24 well plate respectively. The plates were then returned to the incubator and after 4h, each well was topped up with 140 μ l or 400 μ l of complete media (96 or 24 well plate respectively). Plates were then incubated for a further 48h, prior to either phagocytosis assays (96-well plates, see section 2.2.7), lysis for western blotting (24 well plates, see section 2.2.9) or real time-PCR (24 well plates, see section 2.2.14) for analysis of the degree of knockdown achieved.

2.2.14 TaqMan Real time polymerase chain reaction (rt-PCR)

2.2.14.1 RNA isolation

MDMs were cultured in 24 well plates (see section 2.2.3), then the protein of interest was knocked down using siRNA (see section 2.2.13). Total RNA was isolated using the Qiagen RNeasy miniprep extraction kit following the manufacturer's protocol. In brief, cells were lysed in RLT buffer containing 1% (v/v) β -mercaptoethanol. The resulting lysate was homogenised, using a QiashredderTM column, by centrifugation at $\geq 8000 \times g$ for 2 min. The effluent was then diluted with an equal volume of 70% (v/v) ethanol, mixed well and up to 700 μ l transferred to a spin column. The spin column was centrifuged at $\geq 8000 \times g$ for 15 s, the effluent discarded, and then washed by addition of 350 μ l RW1 buffer, followed by repeat centrifugation at $\geq 8000 \times g$ for 15 s.

After discarding the effluent, any contaminating DNA was removed by 15 min incubation, at room temperature, with RNase-free DNase. Samples were then washed, first in 350 μ l RW1 buffer, then twice in RPE buffer, with the final spin for 2 min at $\geq 8000 \times g$. In a new spin column, after dry centrifugation at $\geq 8000 \times g$ for 1 min, the

bound RNA was eluted from the column by addition of 30µl RNase-free water and a final centrifugation performed at $\geq 8000 \times g$ for 1 min.

2.2.14.2 RNA analysis

Each RNA sample was analysed by a Nanodrop ND-1000 UV-Vis spectrophotometer. First a zero reading was obtained with RNase-free water, then 1µl of each sample was analysed to give a measure of the RNA concentration in ng/µl. Once the RNA concentrations was known (in ng/µl), the volume of each sample required to give 1µg of RNA was calculated and then made up with nuclease-free water to give a final amount of 1µg of RNA in 10µl nuclease-free water.

2.2.14.3 cDNA

Primers and probe sets for C6orf134, HDAC6 and SIRT2 were purchased for use with the TaqMan system. First the RNA isolated from MDMs (see section 2.2.14.1) was converted to cDNA, using the High Capacity cDNA Reverse Transcription kit. Following the manufacturer's protocol a mastermix was prepared as follows: for each reaction, RT buffer (2µl), dNTP mix (0.8µl), random primers (2µl), reverse transcriptase (1µl) and nuclease-free water (4.2µl). In PCR tubes, 10µl of mastermix solution was mixed with 10µl of each RNA sample (containing 1µg of RNA) and these were then loaded into a thermal cycler and heated for 10 min at 25°C followed by 120 min at 37°C then 5 min at 85°C.

The PCR reaction was then performed in triplicate. First the master mix was prepared: For each sample, 20µl TaqMan Universal PCR Master Mix, 2µl TaqMan probe (either C6orf134, HDAC6, SIRT2 or control, HPRT1) and 10µl nuclease-free water. For each of the samples, 24µl of the above master mix was added to 9.8µl of cDNA, and from this total amount, 10µl was pipetted in triplicate into a 96 well PCR plate. The plate was loaded into the 7500 Real Time PCR system (Applied Biosystems (Life Technologies), Paisley, UK) for analysis. Analysis of Taqman data was performed using SDS software (Applied Biosystems, California, USA). Graphs were obtained with data for each well of the plate and from this data the $\delta\delta CT$ method was used to give a level of gene expression of the gene of interest (see appendix B). This involves, firstly, taking an average of the triplicate values for each of the samples, then, for each averaged

sample, subtracting the values for gene expression of the gene of interest from those of the house-keeping gene (HPRT1), to normalise for variability in RNA concentrations. Finally, the values of the knockdown samples are divided by the control values for each set of samples (the standard) to give a percentage change in gene expression compared to the control cells (see Appendix B).

2.2.15 HDAC activity assay

2.2.15.1 Immunoprecipitation

MDMs were lysed as for Western blots (section 2.2.9). Protein assays were performed on samples and cell lysate, containing 200µg of protein, were reconstituted in 100 µl of RIPA buffer (150 mM NaCl, 1% Triton-X (^v/_v), 0.5% sodium deoxycholate (^w/_v), 0.1% SDS (^w/_v), 50mM Tris-HCl) and transferred to a fresh tube. 5µl of rabbit polyclonal anti-HDAC6 antibody (1mg/ml) was added and the eppendorfs rotated overnight at 4°C.

Agarose beads (protein-A agarose) were gently pipetted to mix and 20µl added to each eppendorf, prior to rotation for a further 2 h at 4°C. The eppendorfs were centrifuged at 3000 x g, 4°C for 20 s, the supernatant aspirated and 400µl of wash buffer added (0.5% RIPA buffer with ½ tablet of complete protease inhibitor). The eppendorfs were centrifuged at 3000 x g, 4°C for 20 s and the wash repeated a further 2 times.

2.2.15.2 HDAC activity assay

After aspirating the supernatant, 110µl of HDAC assay buffer (50mM TRIS, pH 8.0, 137mM sodium chloride, 2.7mM potassium chloride, 1mM magnesium chloride) was added to each tube, and gently pipetted to mix. 50µl of each sample was added to each well of a white 96 well plate and the volume adjusted to 80µl with HDAC assay buffer, followed by incubation at 37°C for 1 h. 20µl of *Fluor de Lys*[™] developer concentrate was then added to each well and the plate incubated for a further 30 min at 37°C, prior to being analysed by fluorimetry (BMG Fluostar plate reader) at excitation λ480nm and emission λ520nm. In order to assess the amount of HDAC6 protein in each of the samples, the remaining 60µl of each sample was spun down (3000 x g, 30 sec), and a protein assay was performed (see section 2.2.3.3). Following this, the supernatants were aspirated and then each sample was resuspended in

sample buffer. The samples were then boiled for 3 min prior to Western blotting (see section 2.2.9). Results were presented as the level of HDAC6 activity in a given sample, as a measure of relative fluorescence, relative to the protein level of HDAC6 in the sample.

2.2.16 Intracellular killing assay

Monocytes were cultured for 12 d in the presence of GM-CSF (2ng/ml) to generate MDMs, as described in section 2.2.3. Media was removed and 100µl of live bacterial solutions, either *H. influenzae* or *S. pneumoniae*, were added and incubated at 37°C and 5% (v/v) CO₂ for 4 h. Supernatants were aspirated and serially diluted in D-PBS (100µl of aspirate in 900µl D-PBS, repeated 6 times to give a final dilution of 10⁻⁶); 10µl of each dilution was then plated onto agar plates for colony counts after 24 h (to give a count of extra-cellular bacteria). The MDMs were then lysed with 100µl of dH₂O, supernatants aspirated and serially diluted, as before, in D-PBS (100µl of aspirate in 900µl D-PBS, repeated 4 times to give a final dilution of 10⁻⁴), with 10µl of each dilution plated onto agar plates, to give a count of viable intracellular bacteria. In addition, 100µl of live bacterial solutions, either *H. influenzae* or *S. pneumoniae*, was plated into empty wells and incubated at 37°C and 5% (v/v) CO₂ for 4 h, after which the solutions were aspirated, diluted to 10⁻⁶ in D-PBS and 10µl of each dilution was then plated onto agar plates for colony counts after 24 h (to give a control count of total number of bacteria available to cells within the 4 h). After 24 h, colony counts were performed on all plates and CFU/ml calculated (see section 2.2.5.1). The following formula was then used to calculate intracellular killing;

$$\% \text{ Intracellular Killing} = (1 - (IC/Control - EC)) \times 100$$

Where IC represents the CFU/ml of intracellular bacteria, EC represents CFU/ml of extracellular bacteria (in the supernatant) and control is the CFU/ml of bacteria incubated in the well without cells.

To calculate the ability of cells to phagocytose and kill ingested bacteria, the following formula can be used;

$$\% \text{ total killing} = 100 - (((EC + IC)/Control) \times 100)$$

Where IC represents the CFU/ml of intracellular bacteria, EC represents CFU/ml of extracellular bacteria (in the supernatant) and control is the CFU/ml of bacteria incubated in the well without cells.

2.2.17 Statistics

Comparisons between groups (either subject groups or cell type) were carried out using Kruskal-Wallis (non-parametric multiple comparison) tests, followed by Dunn's post correctional tests. Comparisons between varying concentrations of drug were carried out using Wilcoxon signed rank tests, comparing sample medians to the control. Correlation coefficients were carried out using Spearman's rank correlation. Direct comparison of two groups was carried out using the Mann-Witney test. A p value of $p < 0.05$ was taken to be significant. All statistical analyses were carried out using GraphPad Prism (version 5) software (San Diego, USA). Each 'n' represents the experiment carried out on one patient.

Chapter 3

RELATIONSHIP BETWEEN DISEASE AND PHAGOCYTOSIS IN MACROPHAGES

3.1 Introduction

Alveolar macrophages (AM) account for approximately 95% of airspace leukocytes and are the sentinel phagocyte of the innate immune system within the lung (Martin and Frevert, 2005). They express high levels of immunoglobulin, complement and mannose receptors on their cell surface, as well as numerous scavenger receptors, including MARCO, that facilitate phagocytosis of both opsonised and unopsonised particles (Gordon and Read, 2002). In addition, receptors, such as toll-like receptors (TLR), are also present on the cell surface that recognise pathogens from self (Gordon and Read, 2002). Macrophages are increased 5-10 fold in the airways, lung parenchyma, sputum and bronchoalveolar lavage (BAL) of smokers and patients with COPD compared with healthy individuals, and their increased number correlates with disease severity (Finkelstein *et al.*, 1995; Di *et al.*, 1998). However, in COPD patients, despite increased numbers of macrophages within the lung, increased bacterial colonisation is apparent in the lower airways, suggesting reduced bacterial clearance mechanisms.

Studies have shown defective phagocytosis and killing of *Candida spp.* by AM from COPD patients compared to healthy controls (Ferrara *et al.*, 1996; Vecchiarelli *et al.*, 1991). AM from COPD patients also show defective phagocytosis of *E. coli* (Finney-Hayward *et al.*, 2005; Taylor *et al.*, 2010). Other studies have concentrated on commonly isolated respiratory pathogens. AM from COPD patients and smokers have significantly reduced phagocytosis of non-typeable *H. influenzae* (NTHI) strains compared to AM from ex-smokers and healthy controls (Berenson *et al.*, 2006a; Marti-Llitas *et al.*, 2009). The reasons for these observations are unclear, however, rhinovirus exposure of macrophages from resected lung tissue impairs the ability of cells to phagocytose *E. coli* but not latex beads (Oliver *et al.*, 2008), suggesting that viral exposure may alter the innate immune response. Previous work in our laboratory has shown that MDMs from COPD subjects also display defective phagocytosis of *H. influenzae*, as well as *S. pneumoniae*, compared to healthy smokers without COPD and non-smoking controls (Taylor *et al.*, 2010), which indicates that this may be an inherent aberration of macrophages from susceptible individuals, rather than the result of an environmental exposure.

In normal lung, airway epithelial cells may be damaged following infection or exposure to environmental pollution and subsequently become apoptotic (Hodge et al., 2003). These apoptotic cells are removed via efferocytosis. AM from COPD patients also have a reduced ability to efferocytose apoptosed airway epithelial cells, leading to secondary necrosis of the epithelial cells, perpetuating an inflammatory response (Hodge et al., 2003). Likewise, efferocytosis of apoptotic neutrophils is required to curtail neutrophilic inflammation within the lung. However, Kirkham et al (2004) showed that, *in vitro*, MDMs interacting with cigarette smoke-modified extracellular matrix proteins down-regulated their ability to remove apoptotic neutrophils (Kirkham et al., 2004). This suggests that cigarette smoke may modulate macrophage function indirectly. Failure of macrophages to efferocytose neutrophils, leads to necrosis of neutrophils with release of reactive oxygen species and proteases with the potential for subsequent airway damage.

In order to investigate macrophage function in COPD, alveolar macrophages would be the cell of choice; however, isolation of AM requires subjects to undergo bronchoscopy and lavage. This is an invasive procedure with inherent risks, which are increased in subjects with COPD (Peacock M. et al., 1994). This, coupled with the problems of low cell yield from bronchoalveolar lavage (BAL) in healthy subjects and low recovery of BAL in COPD patients, makes an *in vitro* derived model of AM advantageous (Lofdahl et al., 2005). MDMs have, therefore, been developed as a functionally equivalent model for AM. Monocytes can be differentiated in the presence of growth factors, namely macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Akagawa, 2002). However, MDMs differentiated in the presence of GM-CSF have been described as being more akin to AM in terms of surface molecular expression, resistance to oxidative stress, phagocytic capacity and susceptibility to pathogens (such as human immunodeficiency virus (HIV) and *M. tuberculosis*) and have a consistent and stable phenotype (Winkler et al., 2008). Given the increased cellular yield available with the use of MDMs, and the increased risks of bronchoscopy, MDMs will be used as a model of AM for this study.

Therefore, the aim of this chapter is to first identify the most appropriate technique for the isolation of monocytes and then examine the ability of the derived MDMs to

phagocytose beads and bacteria. The ability of MDMs from different subject groups to phagocytose beads and bacteria will then be investigated. Having assessed phagocytosis by MDMs, their ability to kill bacteria once they are ingested will also be examined. In addition, sputum macrophages will be isolated and their phagocytic ability compared to that of MDMs. Sputum macrophages can be obtained from spontaneous or induced sputum, a procedure that is less invasive than bronchoscopy, but may represent an alternative model for AM.

Having established baseline phagocytic capabilities, the ability of macrophages from COPD subjects to phagocytose bacteria during exacerbation will be investigated. Exacerbations are described as an acute worsening of symptoms often requiring a change in treatment in a patient with COPD (adapted from GOLD definition, 2011). Frequent exacerbations, defined as 2 or more exacerbations per year requiring treatment, are associated with a more rapid decline in lung function, worsened quality of life, increased likelihood of hospital admission and increased mortality (Wedzicha and Seemungal, 2007). COPD patients are often colonised with bacteria in the stable state; however, during exacerbations up to 70% of patients have bacterial colonisation in the lower airways (Wilkinson *et al.*, 2006; Monso *et al.*, 2005; Zalacain *et al.*, 1999). In addition, isolation of a new strain of bacteria is associated with increased risk of exacerbation in COPD patients (Sethi *et al.*, 2002). Therefore, if macrophages display reduced phagocytosis at baseline, this may worsen at times of exacerbation, with increased bacterial load or infection with new strains, in turn, leading to further increases in bacteria with poorer clinical outcomes. Therefore, this chapter also aims to establish the capacity of MDMs, both from frequent and infrequent exacerbators to phagocytose both at baseline and during an exacerbation.

3.2 Methods

3.2.1 Subject selection

Healthy, smoking and COPD subjects were recruited as described in section 2.2.1.

3.2.2 Study timeline

Blood (60ml) and induced sputum was taken from COPD patients and age-matched healthy smoking and non-smoking volunteers at baseline. COPD patients were required to have been free from exacerbation or antibiotic or steroid treatment for the preceding 6 weeks for a baseline sample. COPD patients were followed up, and at the point of exacerbation had a further blood and sputum sample taken (Fig. 3.1).



Figure 3.1. Timeline for recruitment of subjects into study.

3.2.3 Blood separation

Two different techniques were initially assessed and compared for the optimal isolation of monocytes from blood as described in section 2.2.3. Monocytes were then cultured for 12 d in the presence of GM-CSF (2ng/ml) to generate monocyte-derived macrophages (MDMs).

3.2.4 Phagocytosis Assays

Phagocytosis assays were performed using fluorescently-labelled beads or bacteria exposed to MDMs for time points up to 4 h, as described in section 2.2.7.

3.2.5 Confocal microscopy

After phagocytosis assays were complete, MDMs were stained using Cell Tracker Red (Invitrogen, UK) then fixed with 4% (^w/_v) paraformaldehyde and imaged on a Leica SP2 upright confocal microscope as described in section 2.2.11.

3.2.6 Intracellular killing

Monocytes were cultured for 12 d in the presence of GM-CSF (2ng/ml) to generate MDMs. MDMs were incubated with live bacteria for 4 h and then lysed, diluted in D-PBS and plated onto agar plates, to give a count of viable intracellular bacteria, as described in section 2.2.16. The following formula was then used to calculate intracellular killing;

$$\% \text{ Intracellular Killing} = (1 - (\text{IC} / \text{Control} - \text{EC})) \times 100$$

Where IC represents the CFU/ml of intracellular bacteria, EC represents CFU/ml of extracellular bacteria (in the supernatant) and control is the CFU/ml of bacteria incubated in the well without cells.

3.2.7 Sputum processing

Sputum was processed as described in section 2.2.4 and phagocytosis assays were then performed as described in section 2.2.7.

3.2.7.1 Preparation of cytopins and Quick-Diff staining

Sputum was processed as described in section 2.2.4. Macrophages (5×10^4 /50µl) were transferred into cytopin funnels attached, with a filter, to microscope slides. Cells were centrifuged for 5 min at 600 x g. Slides were allowed to air-dry, then stored at -80°C for subsequent staining using the REASTAIN Quick-Diff kit. This allows for differential staining of cellular components of leukocytes.

Staining was performed as described in the manufacturer's handbook. In brief, each slide was immersed for 4 s in fixative (100 %_v methanol), 6 s in Red solution (12 %_w/v Eosine Yellow, 0.1 %_w/v sodium azide), 10 s in Blue solution (0.09%_w/v Azur II , 5%_v/v Glycerol, 0.1%_w/v sodium azide) and washed repeatedly in distilled water until excess stain was removed. Slides were allowed to air dry, before a cover slip was added and cells viewed under a light microscope.

3.3 Results

3.3.1 Subject Demographics

The characteristics of healthy, smoking and COPD subjects used in this study are shown in Table 3.1. Healthy and smoking volunteers were matched for age and lung function, while smokers and COPD subjects were matched for smoking history. There were significant differences in age and lung function between COPD and control groups (healthy and smoker subjects).

	Healthy	Smoker	COPD
Age (years)	58 ± 2	58 ± 1	66 ± 2 *
Gender (M:F)	11 : 12	11 : 11	16 : 8
FEV₁ (L)	2.6 ± 0.2	2.5 ± 0.1	1.2 ± 0.1 ***
FEV₁ % predicted	95 ± 2.4	86 ± 2.2	49 ± 4.1 ***
FVC (L)	3.8 ± 0.4	3.6 ± 0.3	2.5 ± 0.2 **
FEV₁/FVC	78 ± 1.2	74 ± 0.9	44 ± 2.5 ***
Smoking history (pack years)	0	40 ± 3.4	54 ± 5.6
Current smokers	0	22	10
Treatment	nil	Statin n=5	Seretide n=20 Becotide n=2 Tiotropium n=14 Theophylline n=4 Statin n=6

Table 3.1. Subject demographics

Where * represents $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to both healthy and smoking controls. Data is presented as mean ± SEM.

3.3.2 Effect of monocyte isolation on generation of MDMs

To determine the most appropriate method for monocyte isolation that would yield MDMs suitable for future experiments, a comparison of negative selection of monocytes using Miltenyi beads and an adherence method was performed. Monocytes isolated by each of these techniques were adhered to the plates and were

then cultured for 12 d to generate MDMs; any non-adherent cells were removed at D4 and d8 during media changes, therefore a population of adherent monocytes derived from tow techniques was studied. MDMs were lysed and protein assays performed, to allow comparison of the amount of protein in each well, as a surrogate for cell numbers. There was no significant difference in protein levels when comparing plates prepared from either adherence or negative selection (Fig. 3.2).

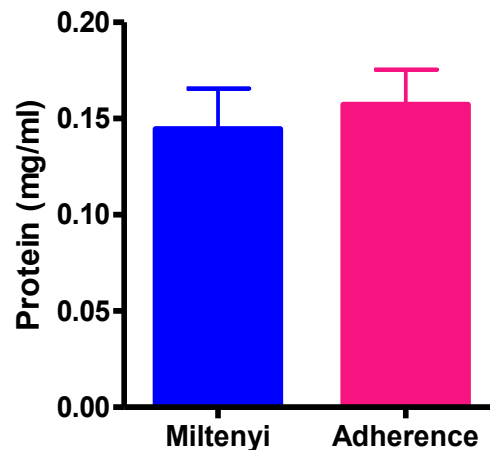


Figure 3.2. Comparison of average protein derived from two techniques for separating monocytes

Monocytes from healthy volunteers were isolated using either negative selection or adherence and cultured for 12d in the presence of GM-CSF (2ng/ml). The average amount of protein was ascertained as described in the methods section (2.2.3.3). Data are presented as mean $\pm$ SEM, n=5.

3.3.3 Effect of monocyte isolation on MDMs phagocytosis

Having established that both monocyte isolation techniques yielded similar cell numbers, the next step was to confirm that the MDMs derived from the different methods were functionally similar. Phagocytosis of beads was used as an indicator of macrophage function. There were no significant differences observed in the ability of the MDMs, derived from either negative selection or adherence, to phagocytose beads at concentrations of 10^7 beads/ml or 5×10^7 beads/ml (Fig. 3.3). From these results, given the higher yield of cells with equivalent functionality of the derived

macrophages, adherence was selected as the optimum technique for separating monocytes from the PBMC fraction.

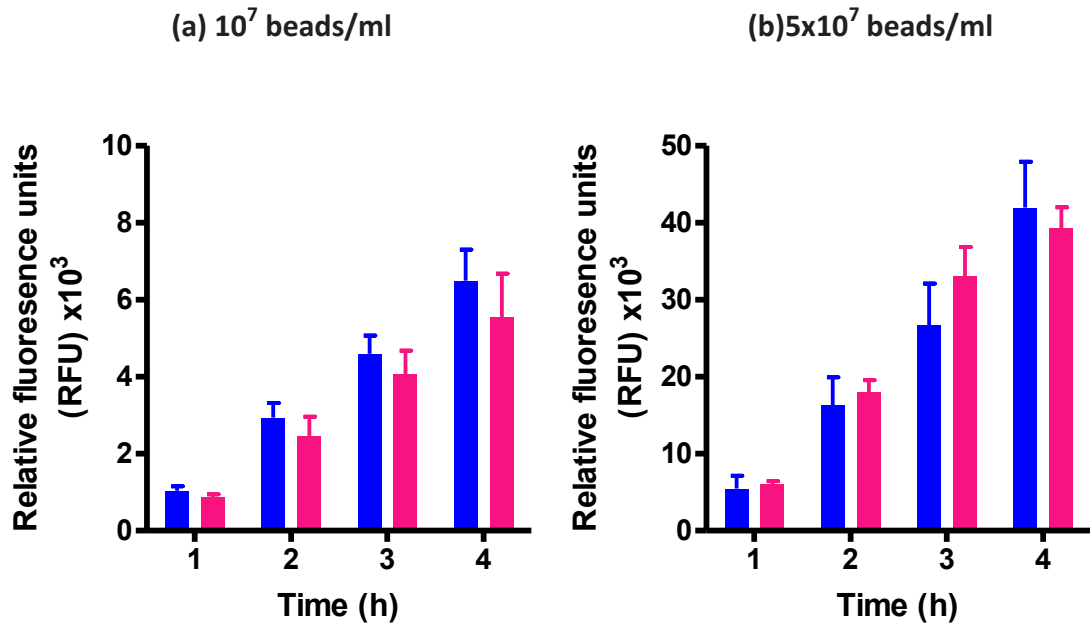


Figure 3.3. Comparison of phagocytosis of beads by MDMs derived from two monocyte isolation techniques

Monocytes from healthy volunteers were separated using either the Miltenyi technique (■) or adherence (■). Cells were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. Cells were incubated with 100 μ l of beads at either (a) 10^7 beads/ml or (b) 5×10^7 beads/ml for the times indicated and phagocytosis quantified by fluorimetry. Data are presented as mean $\pm$ SEM, n=4.

3.3.4 Establishment of optimum conditions for MDMs phagocytosis of beads

Having selected the adherence technique for isolating monocytes, it was important to establish the optimum incubation time and concentration of fluorescently-labelled beads to be used for future phagocytosis assays. MDMs were incubated for up to 4 h with either 5×10^6 beads/ml, 10×10^7 beads/ml or 5×10^7 beads/ml and phagocytosis quantified by fluorimetry. Bead phagocytosis by MDMs increased in both a time and concentration-dependent manner (Fig. 3.4). From these data, an incubation time of 4 h and a bead concentration of 5×10^7 beads/ml were selected to give optimum measurement of bead phagocytosis for future experiments.

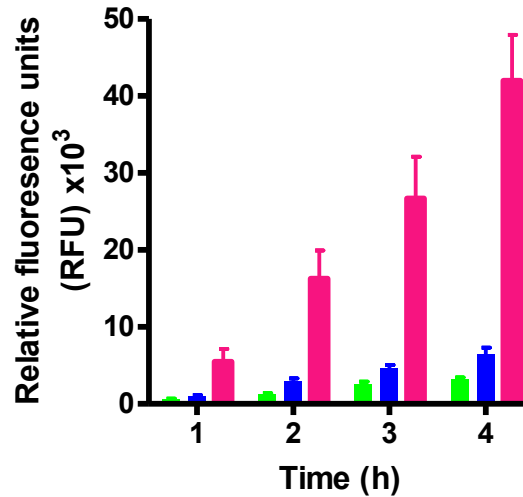


Figure 3.4. Effect of incubation time and bead concentration on MDMs phagocytosis of beads

Monocytes from healthy volunteers were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. Cells were incubated with 100µl of beads at varying concentrations; (■) 5×10^6 beads/ml, (■) 10^7 beads/ml or (■) 5×10^7 beads/ml for the times indicated. Fluorescence was then quantified by fluorimetry. Data are presented as mean $\pm$ SEM, n=4.

To confirm that the fluorescence measured in the phagocytosis assays was related to internalisation of beads, and not simply due to non-specific binding of beads to cells, further experiments were carried out using cytochalasin D (2µM). Cytochalasin D is an actin microfilament inhibitor which inhibits phagocytosis (Parod and Brain, 1986). In the presence of cytochalasin D, phagocytosis of beads was significantly reduced in all subjects groups, by a maximum of ~60% (Fig. 3.5), suggesting that non-specific binding accounts for approximately 40% of bead phagocytosis observed. No differences were observed in the response of cells from healthy subjects, smokers or patients with COPD, suggesting that non-specific binding is similar across all groups, allowing for direct comparison of phagocytic responses (Fig. 3.5).

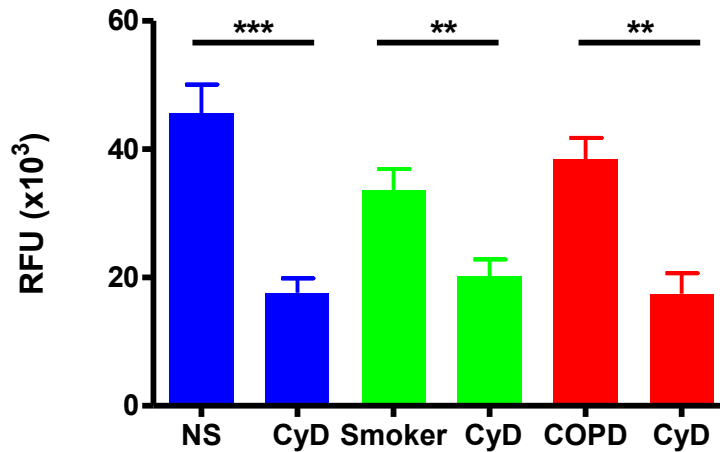


Figure 3.5. Effect of Cytochalasin D (CyD) on inhibiting phagocytosis of beads across subject groups

MDMs from healthy controls (■), healthy smokers (■) and COPD patients (■) were incubated for 4 h with 100µl of 50x10⁶ beads/ml at 37°C with or without pre-incubation for 1h with Cytochalasin D (2µM). Fluorescence of internalised beads was quantified by fluorimetry. Data shown are mean ± SEM, Healthy n=11, smoker n=8, COPD n=9, where **represents p<0.01, ***p<0.001.

3.3.5 Confocal microscopy of MDMs bead phagocytosis

To further confirm that beads were being internalised, and not adhering to the cell surface, confocal microscopy was performed. MDMs were cultured on chamber slides and incubated with 50x10⁶ beads/ml for 4 h, prior to staining of the cytoplasm and nucleus. Cells were viewed on a confocal microscope, and sequential images taken through the cell (Fig. 3.6a). These images were then overlaid to examine the cell in the x, y and z planes (Fig. 3.6b). This confirmed internalisation of beads within the cell, where the bead as shown by the white arrow can be seen surrounded by cytoplasm in the x, y and z planes.

(a) MDMs with fluorescently labelled beads (b) Orthogonal view

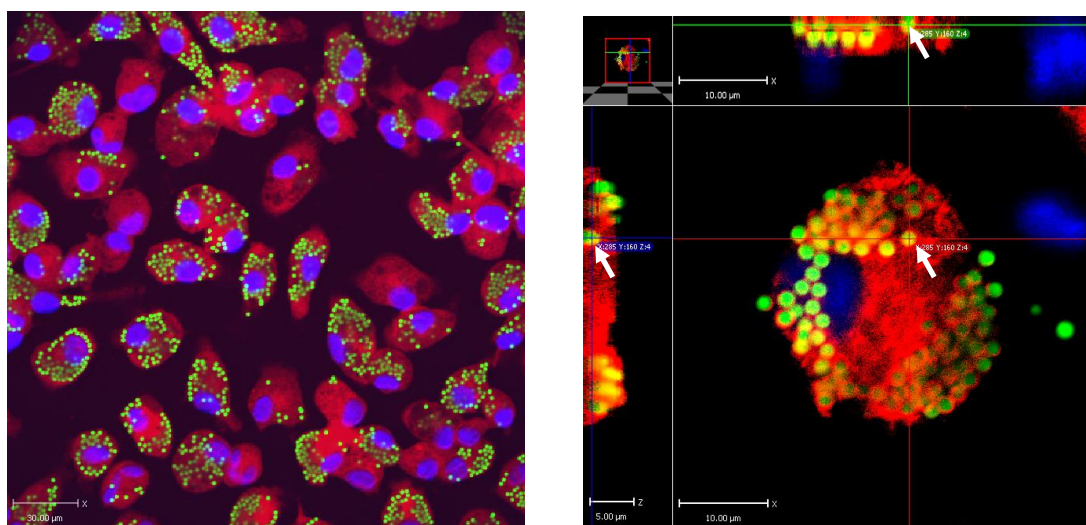


Figure 3.6. Confocal microscopy images of MDMs having undergone phagocytosis of beads

MDMs were cultured on chamber slides, prior to incubation for 4 h with 100μl of 50×10^6 beads/ml at 37°C. Cytoplasm was stained with Cell Tracker Red ((12.5μM) Invitrogen, UK) for 45 min. Cells were fixed and nuclei stained with DAPI (2μM, blue). Cells were imaged on a Leica SP2 upright confocal microscope (x63 objective lens, with 5x zoom and 50 sections for z stacks) and images viewed with Volocity™ software. Bead shown in x, y and z planes as marked by the white arrow. Representative image of n=3.

3.3.6 Establishment of optimum conditions for MDMs phagocytosis of bacteria

Having confirmed the ability of MDMs to phagocytose fluorescently-labelled beads, the ability of MDMs to ingest clinically relevant bacterial pathogens was investigated. MDMs were incubated for up to 6 h with increasing concentrations of either gram-positive (*Streptococcus pneumoniae*) or gram-negative (*Haemophilus influenzae*) heat-killed bacteria (Fig 3.7). The concentrations of bacteria were chosen to be equivalent to the levels of bacteria present in the airway in clinically relevant infection (where clinically relevant infection is given as $>10^7$ CFU/ml). MDMs phagocytosis of bacteria increased in both a time and concentration-dependent manner (Fig. 3.7).

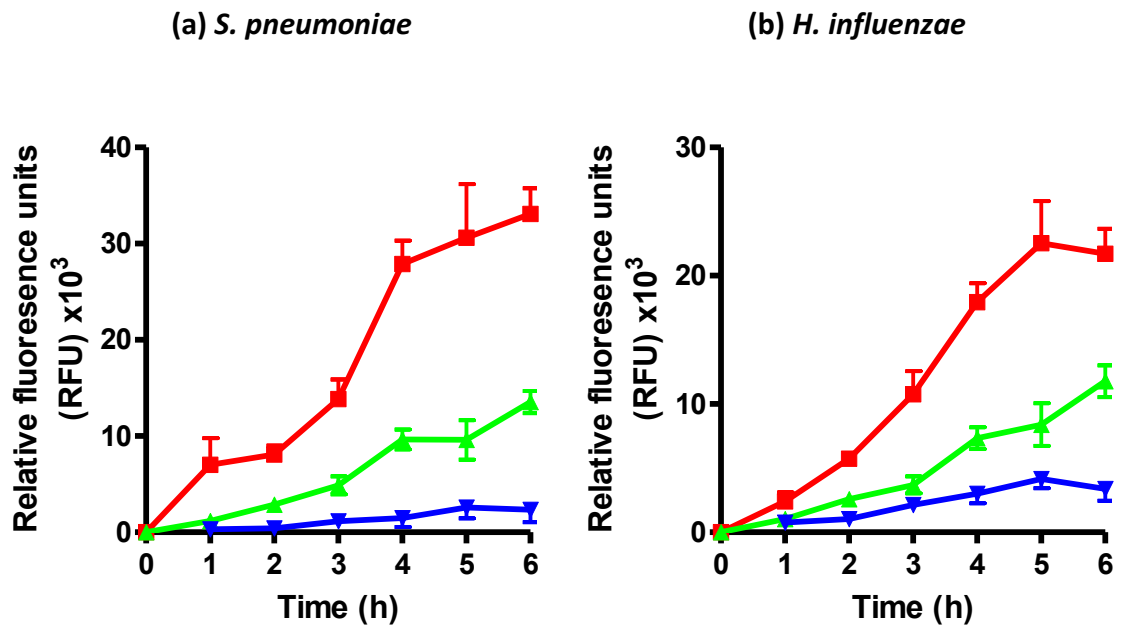


Figure 3.7. Effect of incubation time and concentration of bacteria on phagocytosis by MDMs

Monocytes from healthy volunteers were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. Cells were incubated with 100 μ l of either *S. pneumoniae* or *H. influenzae* solution at varying concentrations (given by CFU/ml); 4x10⁷/ml (▼), 1x10⁸/ml (▲) or 4x10⁸/ml (■), for the times indicated, then phagocytosis was quantified by fluorimetry. Data are presented as mean $\pm$ SEM, n=4.

From these data, the highest concentration of bacteria was chosen for future experiments to give the maximal window in which to measure alterations in phagocytosis. At this concentration (4x10⁸CFU/ml), the rate of uptake of bacteria appeared to slow from the 4-5 h time point with both strains of bacteria. Therefore, 4 h was chosen as the optimal incubation time for future bacterial phagocytosis assays.

Once again, to confirm that the measurement of fluorescence was related to internalisation of bacteria and not non-specific binding of bacteria to cells, further experiments were performed in the presence of cytochalasin D. MDMs incubated with cytochalasin D (2 μ M) had a reduction in phagocytosis of bacteria by approximately 90% for both bacteria (Fig. 3.8), suggesting that non-specific binding accounts for

approximately 10% of phagocytosis observed, irrespective of the bacterial species assessed.

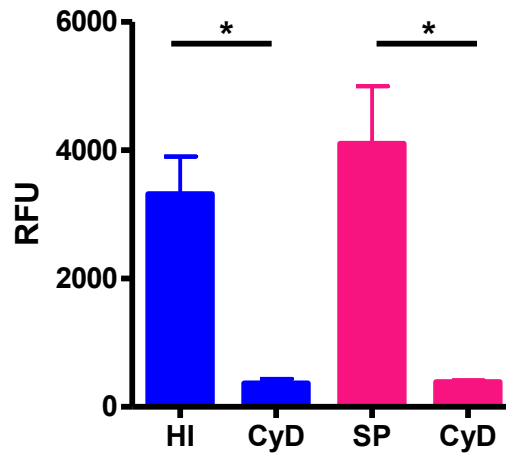


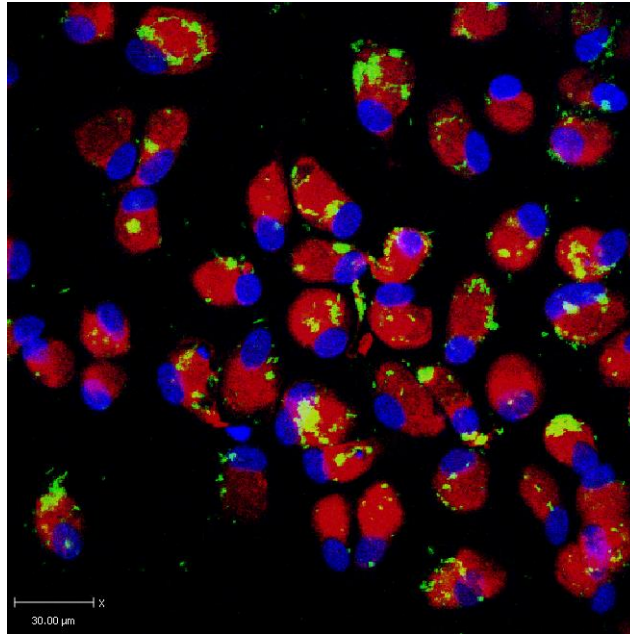
Figure 3.8. Effect of Cytochalasin D (CyD) on inhibition of bacterial phagocytosis

Healthy MDMs were incubated for 4 h with 100µl of either *H. influenzae* (HI■) or *S. pneumoniae* (SP■) at 37°C with or without pre-incubation for 1h with Cytochalasin D (2µM). Fluorescence of internalised bacteria was quantified by fluorimetry and % non-specific binding calculated relative to control. Data shown are mean ± SEM, n=5.

3.3.7 Confocal microscopy confirming bacterial phagocytosis by MDMs

To further confirm that bacteria were internalised during phagocytosis assays, confocal microscopy was performed. MDMs were incubated with bacterial solutions (*H. influenzae* and *S. pneumoniae*) for 4 h, prior to washing and staining of the cytoplasm and nucleus and fixation. Cells were then viewed on a confocal microscope, and orthogonal views taken of the cells. These show that the bacteria are within the cell, surrounded by cytoplasm (Fig. 3.9 and 3.10).

(a) MDMs with fluorescently labelled *H. influenzae*



(b) Orthogonal view

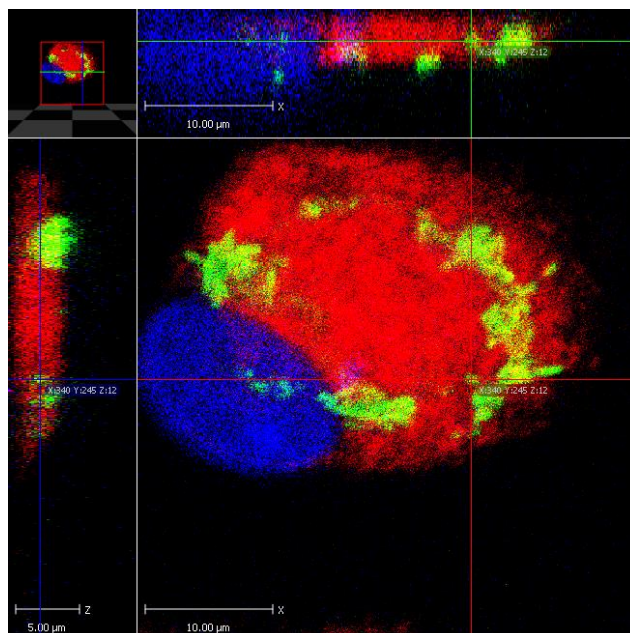
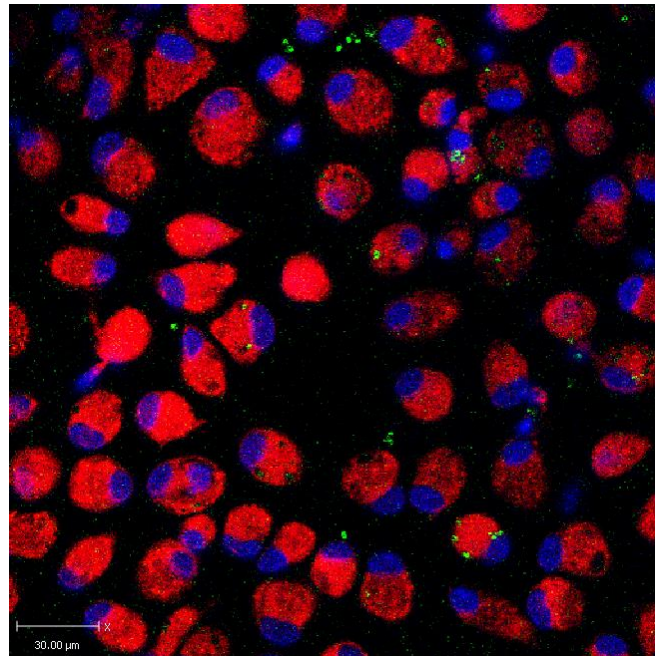


Figure 3.9. Confocal microscopy images of MDMs having undergone phagocytosis of *H. influenzae*

MDMs were cultured on chamber slides, prior to incubation for 4 h with 100µl of *H. influenzae* solution at 37°C. Cytoplasm was stained with Cell Tracker Red ((12.5µM)

Invitrogen, UK) for 45 min. Cells were fixed and nuclei stained with DAPI (2 μ M, blue). Cells were imaged on a Leica SP2 upright confocal microscope (x63 objective lens, with 5x zoom and 50 sections for z stacks) and images viewed with VolocityTM software. Representative image of n=3.

(a) MDMs with fluorescently labelled *S. pneumoniae*



(b) Orthogonal view

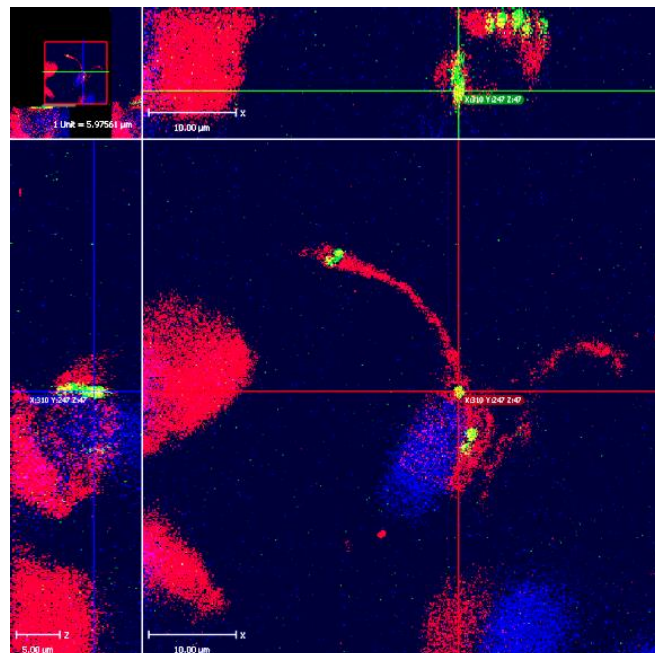


Figure 3.10. Confocal microscopy images of MDMs having undergone phagocytosis of *S. pneumoniae*

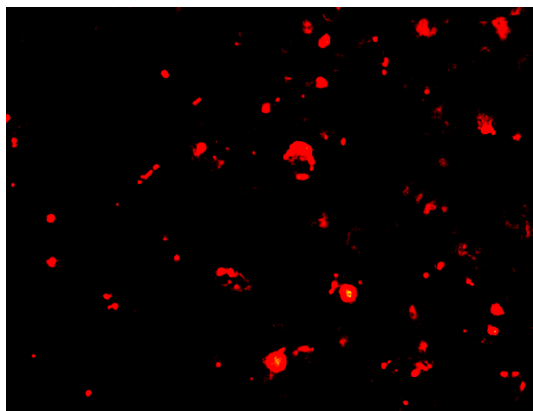
MDMs were cultured on chamber slides, prior to incubation for 4 h with 100 μ l of *S. pneumoniae* solution at 37°C. Cytoplasm was stained with Cell Tracker Red ((12.5 μ M)

Invitrogen, UK) for 45 min. Cells were fixed and nuclei stained with DAPI (2 μ M, blue). Cells were imaged on a Leica SP2 upright confocal microscope (x63 objective lens, with 5x zoom and 50 sections for z stacks) and images viewed with VolocityTM software. Representative image of n=3.

3.3.8 Confirmation of internalisation of bacteria by MDMs, using pHrodoTM

It remained a possibility that although bacteria are entering the cell, the process of phagocytosis was aberrant. To confirm that the phagosome was fusing correctly with the lysosome during the phagocytic process, bacteria were labelled with an alternative dye. pHrodoTM (Invitrogen, UK) is a pH sensitive dye that will only fluoresce at low pH which occurs under the acidic conditions found inside the phagolysosome. MDMs incubated with pHrodo-labelled *H. influenzae* or *S. pneumoniae* show low levels of fluorescence at 1 h (Fig. 3.11a and 3.12a), in comparison to high fluorescence at 4 h (Fig. 3.11b and 3.12b), confirming internalisation of bacteria by MDMs.

(a) MDMs at 1 h



(b) MDMs at 4 h

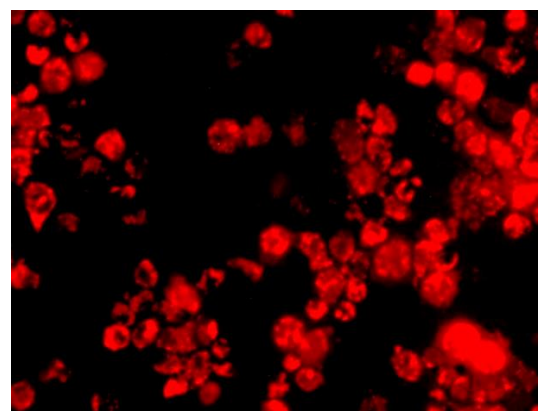
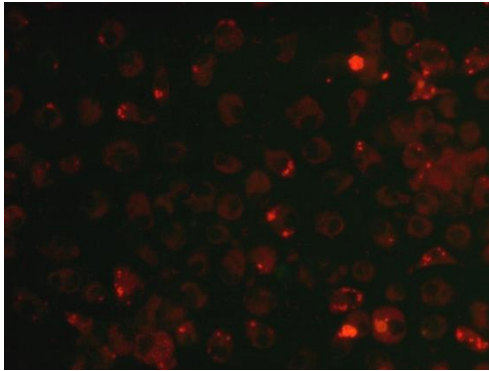


Figure 3.11. Fluorescent microscopy images of MDMs having undergone phagocytosis of pHrodo-labelled *H. influenzae*

Monocytes from healthy volunteers were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. Cells were incubated with 100 μ l of pHrodo labelled *H. Influenzae* solution for 4h, with images taken every 30 min. Cells viewed on an inverted fluorescent microscope, x40 objective lens. Representative image of n=4 experiments.

(a) MDMs at 1 h



(b) MDMs at 4 h

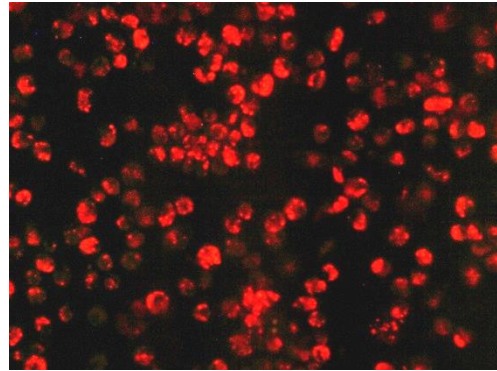


Figure 3.12. Fluorescent microscopy images of MDMs having undergone phagocytosis of pHrodo-labelled *S. pneumoniae*

Monocytes from healthy volunteers were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. Cells were incubated with 100µl of pHrodo labelled *S. pneumoniae* solution for 4h, with images taken every 30 min. Cells viewed on an inverted fluorescent microscope, x40 objective lens. Representative image of n=4 experiments.

3.3.9 Effect of disease on phagocytosis of beads and bacteria by MDMs

Having established optimum conditions for phagocytosis experiments, the effect of disease on phagocytic function was assessed. Monocytes were isolated from healthy, smoker and COPD subjects, MDMs were derived and their ability to phagocytose beads was compared. Over a time course of 1-4 h, there were no significant differences in the ability of MDMs derived from COPD patients, healthy or smoking subjects to phagocytose beads (Fig. 3.13). This confirms that MDMs from all subject groups are capable of phagocytosis.

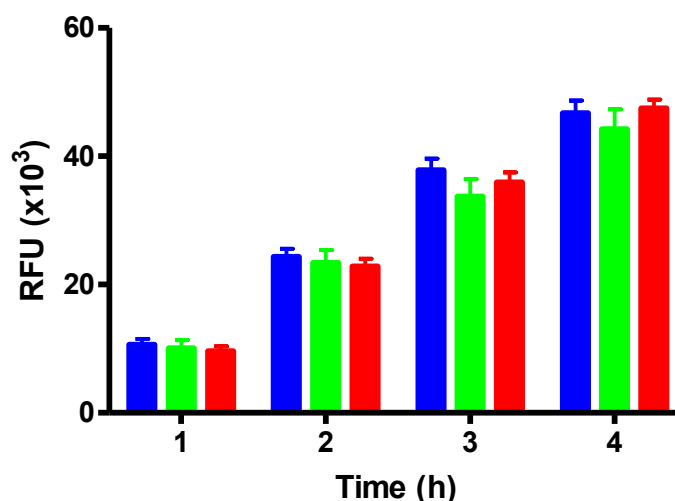


Figure 3.13. Effect of disease on phagocytosis of beads by MDMs

Monocytes from healthy controls (■), healthy smokers (■) and COPD patients (■) were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. Cells were incubated with 100 μ l of 50 $\times 10^6$ beads/ml for the times indicated, then quantified by fluorimetry. Data are presented as mean $\pm$ SEM, n=20 for healthy, n=22 for smokers, n=23 for COPD.

Having confirmed that MDMs from all subject groups were capable of equivalent uptake of inert particles, the next step was to assess whether disease state had any effect on the phagocytosis of bacteria. Fluorescence microscopy images of MDMs isolated from COPD subjects show qualitatively reduced uptake of fluorescently labelled *H. influenzae* compared to healthy controls (Fig. 3.14a-b). When all subject groups were compared, smoker MDMs showed significantly reduced uptake of *H. influenzae* at all time points (Fig 3.15), whilst COPD MDMs showed significantly reduced phagocytosis of *H. influenzae* at 2-4 h (Fig. 3.15). At 4 h, phagocytosis of *H. influenzae* was reduced by 28% and 58% in COPD and smoker MDMs respectively, compared to healthy MDMs.

(a) Healthy MDMs incubated with HI (b) COPD MDMs incubated with HI

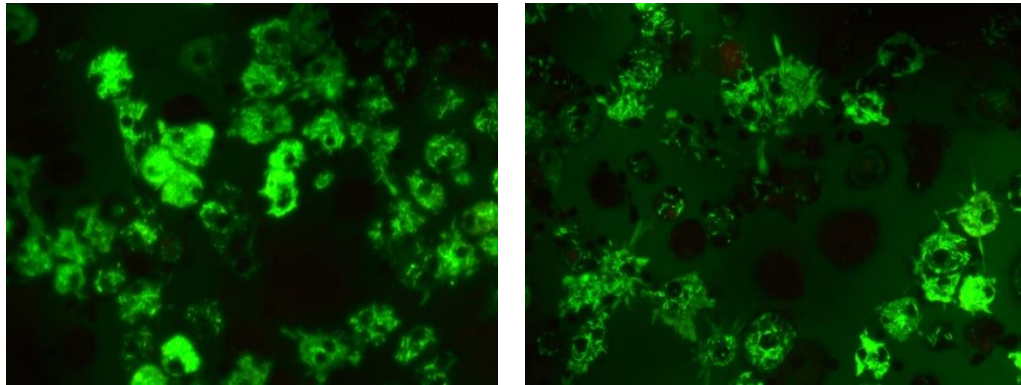


Figure 3.14. Fluorescent microscopy images of (a) healthy MDMs and (b) COPD MDMs having undergone phagocytosis of *H. influenzae*

Monocytes were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. Cells were incubated with 100 μ l of Alex-fluor 488 (Invitrogen, UK) labelled *H. influenzae* solution for 4h. Cells were washed then viewed on an inverted fluorescent microscope, x40 objective lens. Images are representative of 4 experiments.

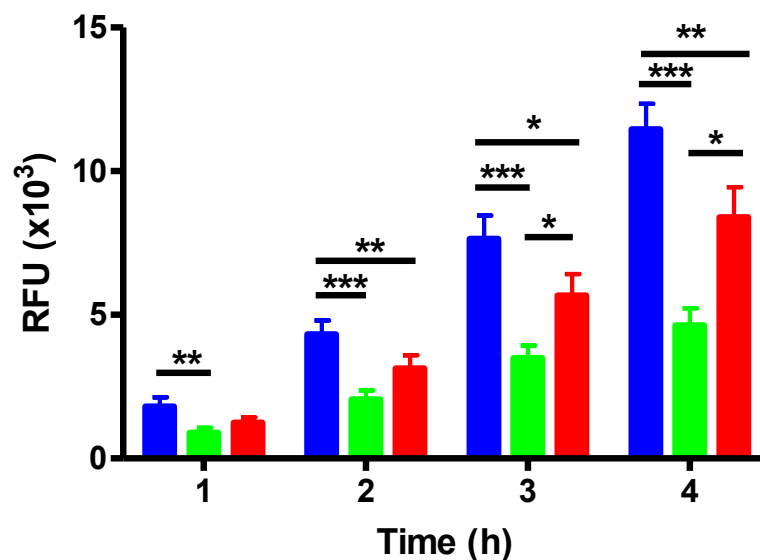
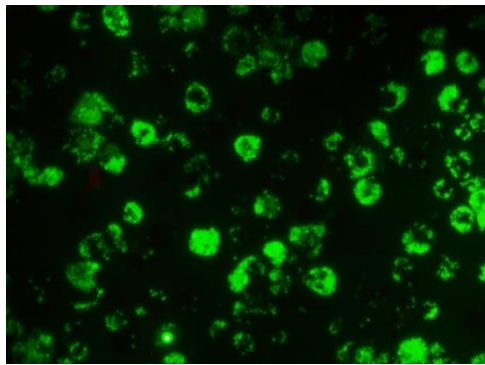


Figure 3.15. Effect of disease on phagocytosis of *H. influenzae* by MDMs.

Monocytes from healthy controls (■), healthy smokers (■) and COPD patients (■) were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. Cells were incubated with 100 μ l of *H. influenzae* solutions for the times indicated, then quantified by fluorimetry. Data are presented as mean $\pm$ SEM, n=20 for healthy and smoker subjects, n=24 for COPD subjects, where * represents p<0.05, ** p<0.01 and *** p<0.001.

MDMs phagocytosis of *S. pneumoniae* showed a similar pattern to *H. influenzae*, with fluorescence microscopy images suggestive of reduced uptake of *S. pneumoniae* by COPD subjects compared to healthy controls (Fig. 3.16a-b). Smoker MDMs showed significantly reduced uptake at all time points (Fig. 3.17), whilst COPD MDMs showed reduced phagocytosis at all time points, reaching significance at 4 h (Fig. 3.17). At 4 h, phagocytosis of *S. pneumoniae* was reduced by 32% and 52% in COPD and smoker MDMs respectively, compared to healthy MDMs.

(a) Healthy MDMs incubated with SP



(b) COPD MDMs incubated with SP

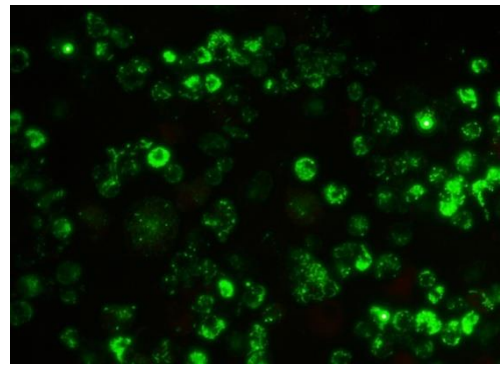


Figure 3.16. Fluorescent microscopy images of (a) healthy MDMs and (b) COPD MDMs having undergone phagocytosis of *S. pneumoniae*

Monocytes were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. Cells were incubated with 100 μ l of Alexa-fluor 488 (Invitrogen, UK) labelled SP solution for 4h. Cells were washed then viewed on an inverted fluorescent microscope, with a x40 objective lens. Images representative of 4 experiments.

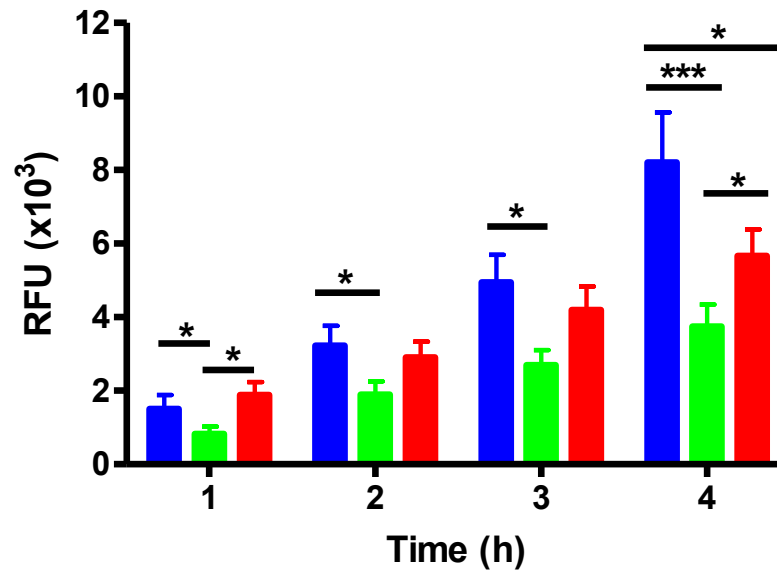


Figure 3.17. Effect of disease on phagocytosis of *S. pneumoniae* by MDMs

Monocytes from healthy controls (■), healthy smokers (■) and COPD patients (■) were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. Cells were incubated with 100μl of bacterial solutions for the times indicated, then quantified by fluorimetry. Data are presented as mean ± SEM, n=21 for healthy subjects, n=20 for smoker subjects and n=24 for COPD, where * represents p<0.05 and *** p<0.001.

3.3.10 Phagocytosis assays with live bacteria

The bacterial phagocytosis assays performed above used heat-killed bacteria. Therefore, to ensure that the altered bacterial phagocytosis seen with smoker and COPD MDMs was not related to the use of heat-killed bacteria, experiments were repeated using live bacteria of the same strains. These experiments were performed at a 1 h timepoint to minimise bacterial growth during the phagocytosis assay. Experiments with live bacteria confirmed the previous findings, with COPD MDMs showing significantly reduced phagocytosis of both *H. influenzae* and *S. pneumoniae* in comparison to healthy controls (Fig. 3.18a and b).

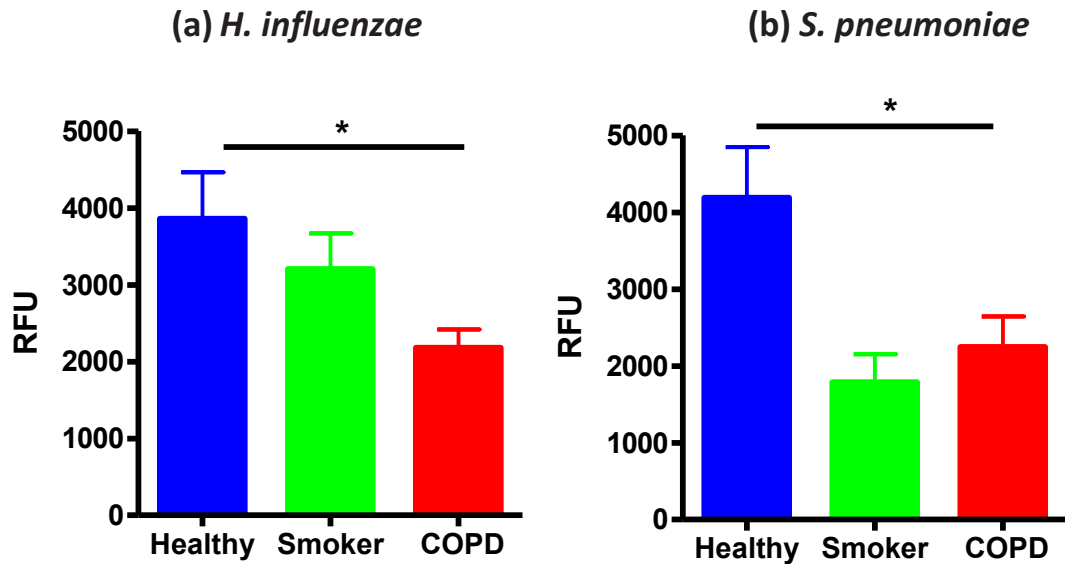


Figure 3.18. Effect of live bacteria on phagocytosis of (a) *H. influenzae* and (b) *S. pneumoniae* by MDMs from different subject groups

Monocytes from healthy controls (■), healthy smokers (■) and COPD patients (■) were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. Cells were incubated with 100μl of live bacterial solutions for 1 h, and then quantified by fluorimetry. Data are presented as mean ± SEM, n=10 for healthy and COPD subjects, n=8 for smoker subjects, where *represents p<0.05 COPD vs healthy controls.

3.3.11 Cell viability

To confirm that the alterations in phagocytosis observed with both smoker and COPD MDMs were not simply a consequence of reduced viability in these cells, in comparison to healthy cells, MTT assays were performed. After phagocytosis assays were complete, cells were incubated with MTT solution for 30 min and absorbance read on the spectrophotometer. No differences were seen in cell viability between MDMs from healthy, smoker and COPD subjects (Fig. 3.19).

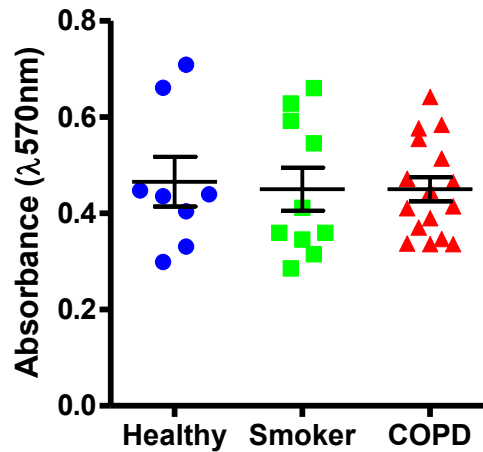


Figure 3.19. Viability of MDMs from different subject groups

Monocytes from healthy controls (●), healthy smokers (■) and COPD patients (▲) were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. Cells were used in phagocytosis assays prior to incubation with 100μl of MTT solution for 30 min at 37°C. Supernatants were aspirated and 100μl of DMSO added. Plates were read at λ570nm on a spectrophotometer. Data presented here are values from control cells (without prey) but are representative of those cells with prey. Data are presented as mean ± SEM, n=8 for healthy, n=10 for smoker subjects and n=16 for COPD subjects.

3.3.12 Intracellular killing

Having established that MDMs from smoker and COPD subjects showed reduced uptake of bacteria compared to healthy controls, it was important to determine whether these cells also had altered ability to kill intracellular bacteria. Therefore, MDMs were incubated for 4 h with live bacteria, after which cells were lysed and the supernatants plated onto agar for colony counting after 24 h. The number of bacteria ingested by cells was calculated as shown in section 3.2.5. There were no significant differences seen in the ability of MDMs from healthy, smoker or COPD subjects to kill intracellular bacteria (Fig. 3.20).

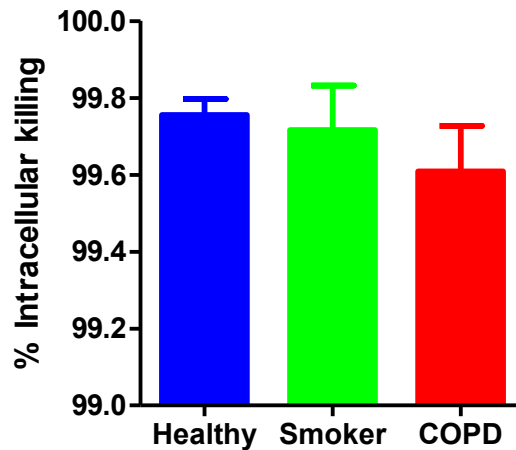


Figure 3.20. Ability of MDMs from different subject groups to kill intracellular bacteria

Monocytes from healthy controls (■), smokers (■) and COPD patients (■) were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. Cells were incubated with live *H. influenzae* for 4 h at 37°C. Supernatants were aspirated, diluted and plated on agar for 24 h colony counts. MDMs were lysed, supernatants diluted and plated for 24 h colony counts. Intracellular killing was calculated using the equation described in section 3.2.5. Data are presented as mean ± SEM, n=4 for all groups.

3.3.13 Relationship between phagocytosis of gram negative and gram positive bacteria

When the ability of MDMs from all subject groups to phagocytose *H. influenzae* (HI) was compared to the ability of MDMs from all subject groups to phagocytose *S. pneumoniae* (SP), a strong positive correlation was found (Fig. 3.21), suggesting that any defect in uptake of bacterial pathogens is non-specific.

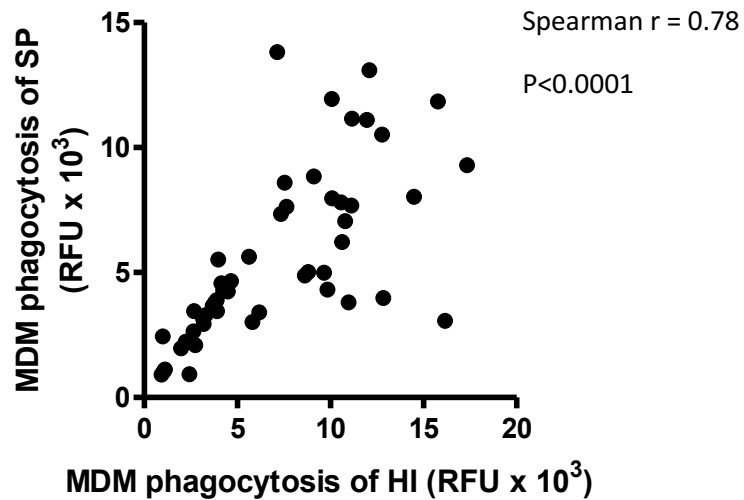


Figure 3.21. Correlation between the ability of MDMs from different subject groups to phagocytose *H. influenzae* and *S. pneumoniae*

To assess whether reduced phagocytic ability was related to age, lung function or smoking history, correlations between phagocytosis of either gram positive or gram negative bacteria, and these parameters were performed. No correlations were seen between the ability of MDMs to phagocytose either *H. influenzae* or *S. pneumoniae* and age, lung function parameters or smoking history, neither when performed within subject groups nor when subjects from all disease group were analysed as a whole (Fig. 3.22-25).

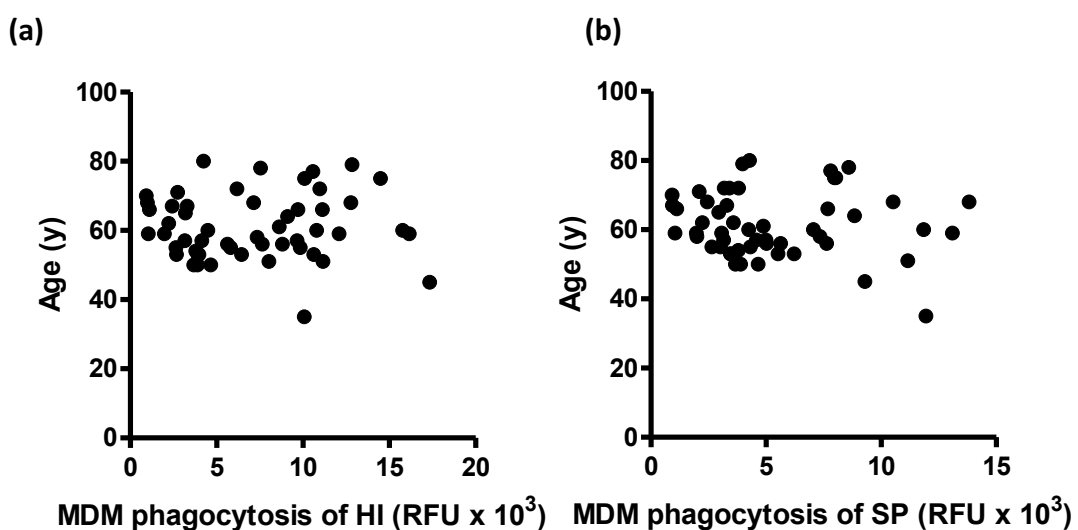


Figure 3.22. Correlation between the age of subjects from all disease groups and ability of their MDMs to phagocytose (a) *H. influenzae* or (b) *S. pneumoniae*

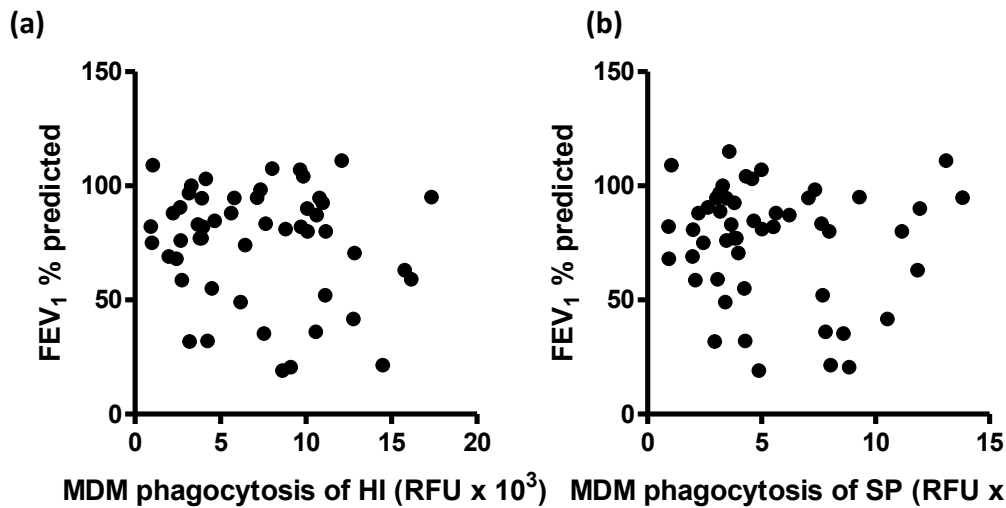


Figure 3.23. Correlation between the FEV₁ % predicted of subjects from all disease groups and ability of their MDMs to phagocytose (a) *H. influenzae* or (b) *S. pneumoniae*

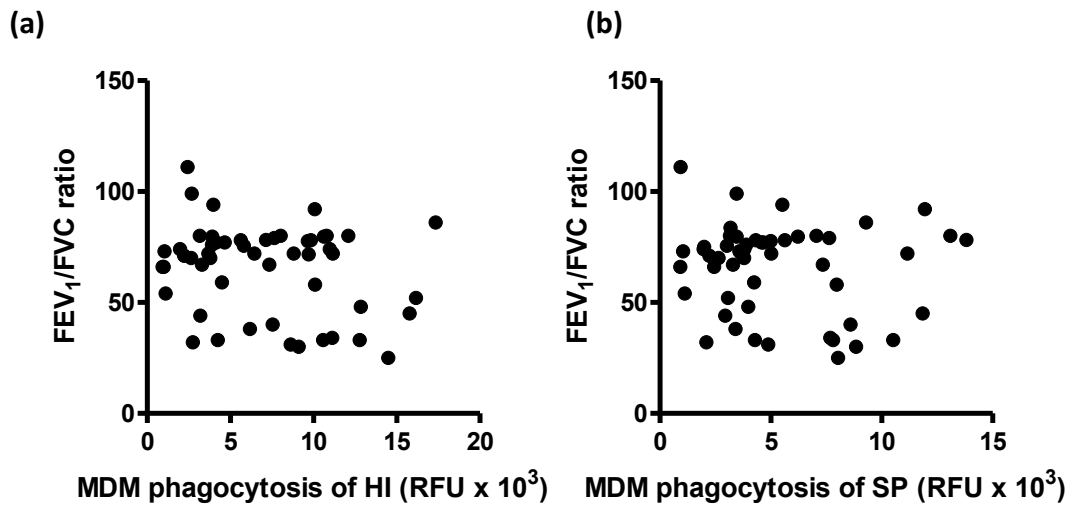


Figure 3.24. Correlation between the FEV₁/FVC ratio of subjects from all disease groups and the ability of their MDMs to phagocytose (a) *H. influenzae* or (b) *S. pneumoniae*

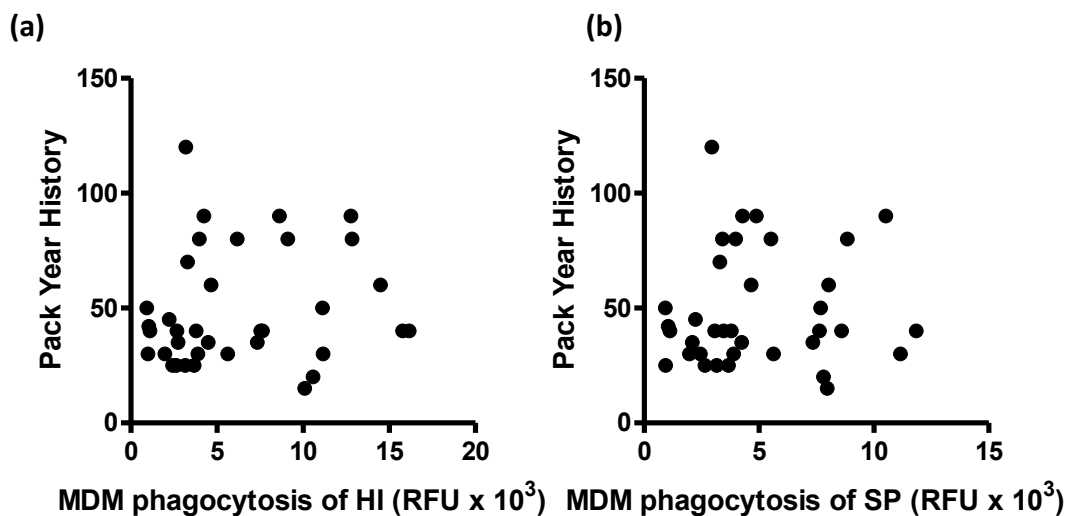


Figure 3.25. Correlation between the smoking history from smoking and COPD subjects and the ability of their MDMs to phagocytose (a) *H. influenzae* or (b) *S. pneumoniae*

3.3.14 Effect of disease on cytokine release from MDMs

Having shown that MDMs from smokers and COPD patients had altered phagocytic responses compared with healthy subjects, another functional output was investigated to assess whether there were other similar alterations. The release of TNF α from MDMs, after 4 h incubation with prey, was analysed by ELISA. There was no significant difference in baseline release of TNF α in untreated MDMs from healthy, smoker or COPD subjects (Fig. 3.26a-c). TNF- α release from MDMs incubated with beads and bacteria (gram-positive and gram-negative) was significantly increased from baseline values in all subject groups ($p < 0.001$) (Fig. 3.26a-c). COPD MDMs, incubated with beads, released significantly more TNF- α compared to healthy or smoker MDMs. In comparison, smoker MDMs released higher levels of TNF- α after incubation with *S. pneumoniae*, ($p < 0.01$ smoker vs. COPD) whilst healthy MDMs released significantly more TNF- α compared to COPD and smoker MDMs when incubated with *H. influenzae* ($p < 0.05$ healthy vs. smokers). There was an approximate 10 fold greater TNF- α release after incubation with *S. pneumoniae* compared to beads, with a further 5 fold increase after incubation with *H. influenzae* compared to *S. pneumoniae* (Fig. 3.26a-c).

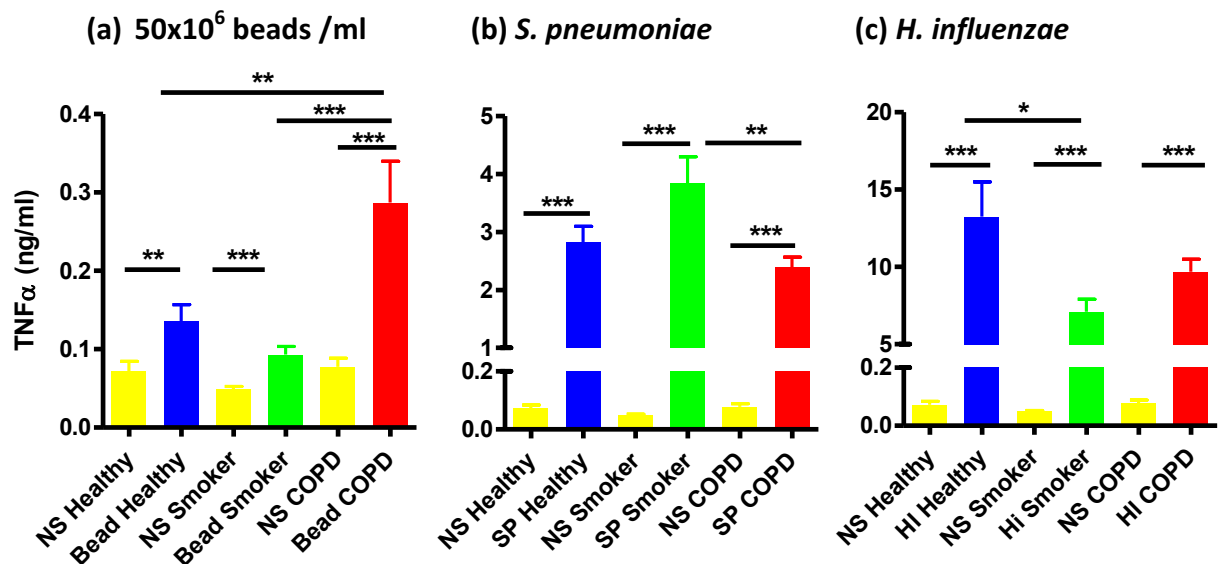


Figure 3.26. TNF- α release from MDMs after 4h incubation with phagocytic prey

Supernatants, taken from MDMs post 4 h phagocytosis assays with either beads or bacteria, were diluted as appropriate and an ELISA performed as per manufacturers' protocol. Non-treated MDMs (NS $\square$) $n=4$ all groups. Treated MDMs, healthy ($\blacksquare$) $n=8$, smokers ($\blacksquare$) $n=5$, COPD ($\blacksquare$) $n=15$, where * represents $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3.15 Effect of Exacerbation frequency on the ability of MDMs to phagocytose bacteria

Frequent exacerbators are defined as those experiencing 2 or more exacerbations per year, requiring treatment. To assess whether exacerbation frequency had any effect on phagocytosis, the COPD subjects were divided into frequent and infrequent exacerbators on the basis of their exacerbation history over the previous 2 years. The demographics of frequent and infrequent exacerbators are shown in Table 3.27. The only significant difference between the two groups is the smoking history with frequent exacerbators having a significantly lower pack year history than infrequent exacerbators.

	Infrequent (n=12)	Frequent (n=12)
Age (years)	69 ± 2	69 ± 2
Gender (M:F)	7:5	8:4
FEV₁ (l)	1.1 ± 0.14	1.4 ± 0.14
FEV₁ % predicted	46 ± 5.5	52 ± 5.6
FEV₁/FVC (%)	42 ± 2.5	47 ± 4.4
Smoking history (pack years)	64 ± 8	44 ± 6**
Current smokers	6	4
Exacerbations in prior yr	0.8 ± 0.11	2.4 ± 0.15
Treatment	Seretide n=8 Becotide n=2 Tiotropium n= 6	Seretide n=12 Tiotropium n=8

Table 3.27. Demographics of the frequent vs. infrequent exacerbators

Where * represents $p < 0.01$ frequent vs. infrequent exacerbators. Data is presented as mean ± SEM.

Comparison of phagocytosis of either *H. influenzae* or *S. pneumoniae* between frequent and infrequent exacerbators showed that frequent exacerbators have significantly lower uptake of both bacteria compared to infrequent exacerbators (Fig. 3.28).

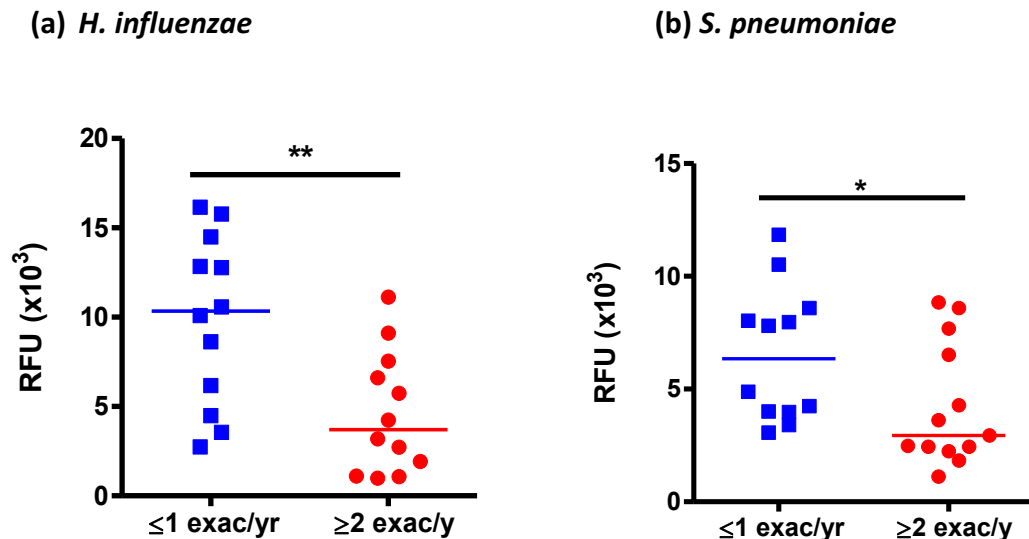


Figure 3.28. Effect of exacerbation frequency on ability of MDMs to phagocytose bacteria

Monocytes from COPD patients were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. Phagocytosis assays were performed with either *H. influenzae* or *S. pneumoniae* and phagocytosis analysed by fluorimetry. Patients were divided on exacerbation history into infrequent (≤ 1 exacerbation/y (mean exacerbations 0.8 ± 0.11), $n=12$ (■)) or frequent (≥ 2 exacerbations/y (mean exacerbations 2.4 ± 0.15), $n=12$ (●)). Data are presented with line at median, where * represents $p<0.05$, ** represents $p<0.01$.

3.3.16 Effect of Acute Exacerbations of COPD on MDMs phagocytosis of beads and bacteria

Given that MDMs from all COPD patients display reduced phagocytosis at baseline, the next step was to determine whether there was any further reduction in phagocytosis of bacteria at times of acute exacerbation. Acute exacerbations were defined on the basis of Anthonisen's criteria; ≥ 2 major symptoms of increased or new dyspnoea, sputum purulence or volume, or 1 major and minor symptoms including cough, fever, wheeze and cold. The demographics of those COPD patients who developed an acute

exacerbation over the course of the study are shown below (Table 3.29). There were no differences between these patients in terms of age, lung function or smoking history when compared to the COPD cohort as a whole (Table 3.1).

Age (years)	67 ± 1
Gender (M:F)	5:5
FEV₁ (l)	1.1 ± 0.1
FEV₁ % predicted	47 ± 4.0
FVC (l)	2.3 ± 0.1
FEV₁/FVC (%)	44 ± 2.6
Smoking history (pack years)	62 ± 6
Current smokers	3
Exacerbations in prior yr	1.8 ± 0.33
Treatment	Seretide n=9 Becotide n=1 Tiotropium n=8

Table 3.29. Demographics of the COPD subjects exacerbating during the study

Data is presented as mean ± SEM.

Blood was sampled from the patients on day 1 of an exacerbation, prior to starting antibiotics or oral glucocorticosteroids. Monocytes were then isolated and MDMs derived. These MDMs were then used in phagocytosis assays and the results compared to baseline values for each patient. No differences were seen in the ability of COPD MDMs to phagocytose either beads or gram negative or positive bacteria at exacerbation compared to baseline (Fig. 3.30).

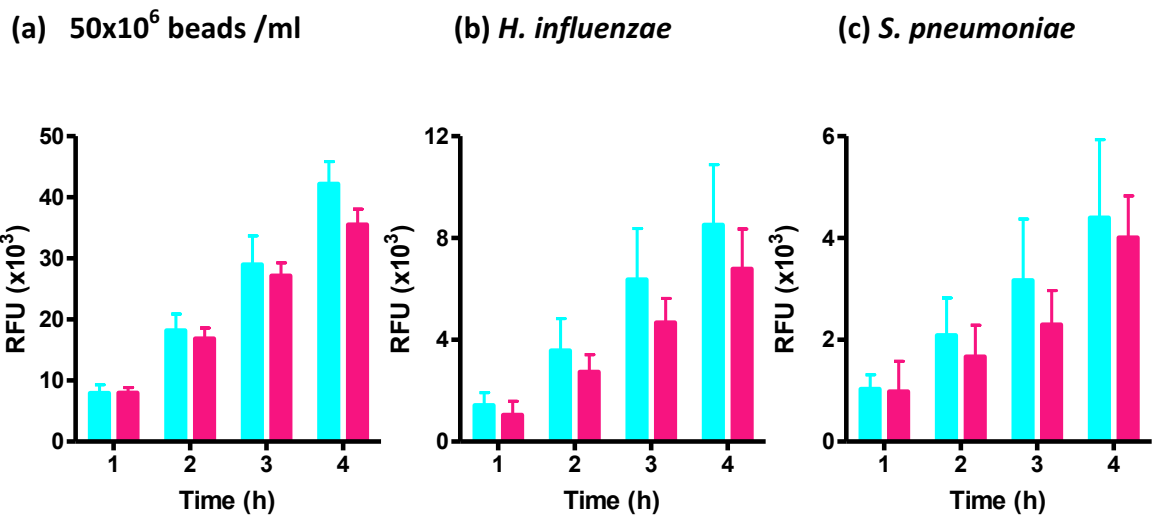


Figure 3.30. Effect of acute exacerbation on phagocytosis of (a) beads (b) *H. influenzae* and (c) *S. pneumoniae* by COPD MDMs

Monocytes from COPD patients taken at baseline (■) and at exacerbation (■) were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. Cells were incubated with 100µl of bead or bacterial solutions for the times indicated, then quantified by fluorimetry. Data are presented as mean ± SEM, n=10.

3.3.17 Phagocytosis by Sputum macrophages

Having shown that MDMs could be used to assess phagocytosis in different subject groups, sputum macrophages were examined to see if they showed similar defects in phagocytosis. Sputum samples were discarded if the squamous cell count was >10% (suggesting oropharyngeal contamination of the sample) or if viability, as assessed by trypan blue, was <70%. Differential cell counts were performed using Kimura staining. Of all the sputum samples obtained 6 samples from each of healthy and smoker subjects were discarded due to squamous cell contamination or poor cellularity, whilst 5 samples from COPD patients were discarded due to poor viability. The differential count of sputum samples that were used in phagocytosis assays are shown in Table 3.31. Overall, COPD patients had the highest total cell count, but the majority of these cells were neutrophils (Table 3.31)

	Healthy (n=8)	Smokers (n=9)	COPD (n=14)
Total non-squamous cell count (x10⁶/ml)	2.6±0.5	3.1±0.8	3.9 ±1.1
Macrophages (% of total cell count)	41.1±5.3	42.6±5	20.3±3
Neutrophils (% of total cell count)	29.1±2.8	31.8±5.5	64.7±4
Other (primary epithelial cells, lymphocytes) (% of total cell count)	16.1±2.7	16.5±3.3	10.2±2

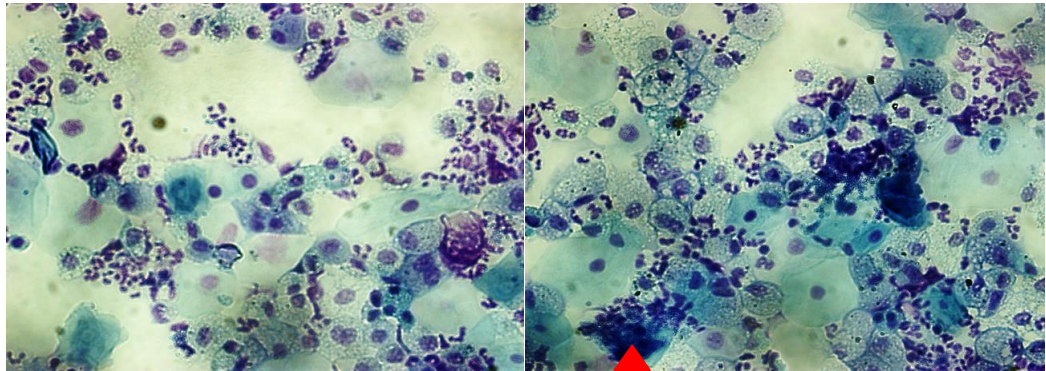
Table 3.31. Total cell counts in sputum from different subject groups, with differential as a percentage of total count

Sputum was obtained from healthy controls, healthy smokers and COPD patients and cell counts were performed using Kimura staining. Macrophages were resuspended at 1×10^5 macrophages per FACS tube in RPMI media for phagocytosis assays. Data are presented as mean $\pm$ SEM.

Cytospins were performed of each sputum sample and stained with Quick-Diff™ (Gentaur, Belgium). Comparing a sample from a healthy and COPD subject below (Fig. 3.32a and b) it can be seen that the COPD sample already contains bacterial contamination. The bacterial contamination can be more clearly seen when the same image is viewed at higher magnification (Fig. 3.32c).

(a) Healthy subject

(b) COPD patient



(c) COPD patient

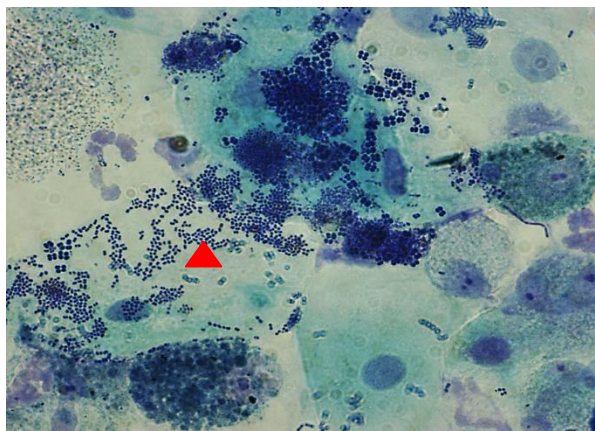


Figure 3.32a-c. Cytospins of healthy and COPD sputum samples

Cytospins were performed on 50 μ l of each sputum sample, allowed to air dry and then stained with Diff Quik™, as per the manufacturer's protocol. Slides were then viewed on a Zeiss microscope with (a, b) an x40 objective lens and (c) an x100 objective lens. Bacteria are shown by the red arrowhead.

Experiments were then performed to investigate the phagocytic responses of sputum macrophages using flow cytometry. Sputum macrophages isolated from smokers showed increased phagocytosis compared to both healthy controls and COPD subjects (Fig. 3.33a) and increased uptake of beads in comparison to COPD macrophages (Fig. 3.33 b).

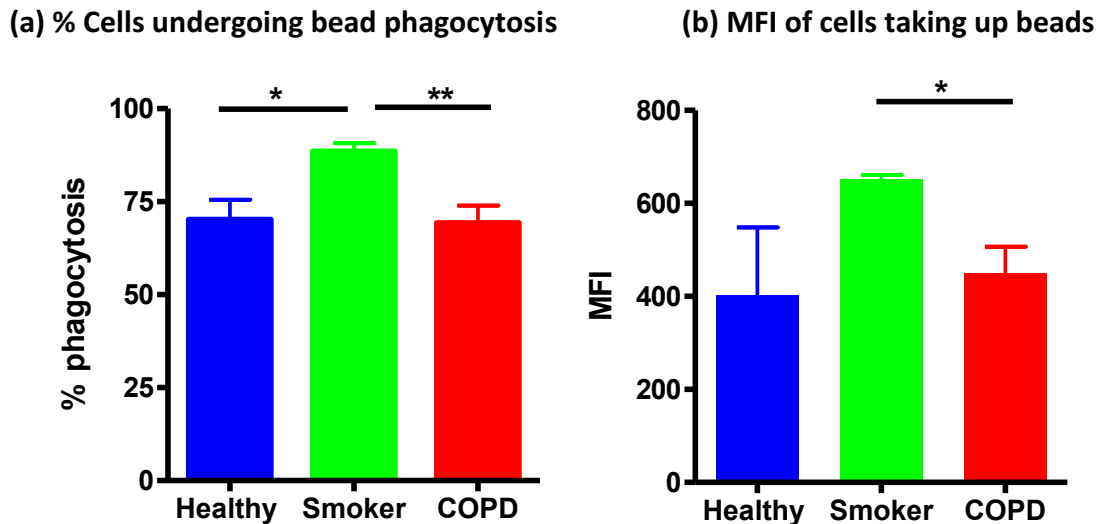


Figure 3.33. Ability of sputum macrophages from all subject groups to phagocytose beads

Sputum of healthy controls (■), healthy smokers (■) and COPD patients (■) was processed and macrophages were resuspended at 1×10^5 macrophages per FACS tube in RPMI media. Cells were incubated with 900 μ l of 50×10^6 beads/ml for 4 h, and then quantified by flow cytometry. Macrophages were gated according to properties of size and granularity. Data are presented as mean $\pm$ SEM, n=6 for healthy, n=6 for smokers, n=9 for COPD, where * represents $p < 0.05$ and ** $p < 0.01$.

In contrast to MDMs, sputum macrophages from all subject groups showed equivalent phagocytosis and uptake of *H. influenzae* (Fig. 3.34a and b). Uptake of *S. pneumoniae* by sputum macrophages was similar to that seen with beads. Smoker macrophages showing increased percentage phagocytosis compared to both healthy and COPD macrophages (Fig. 3.35a), with increased uptake compared to COPD macrophages only (Fig. 3.35b).

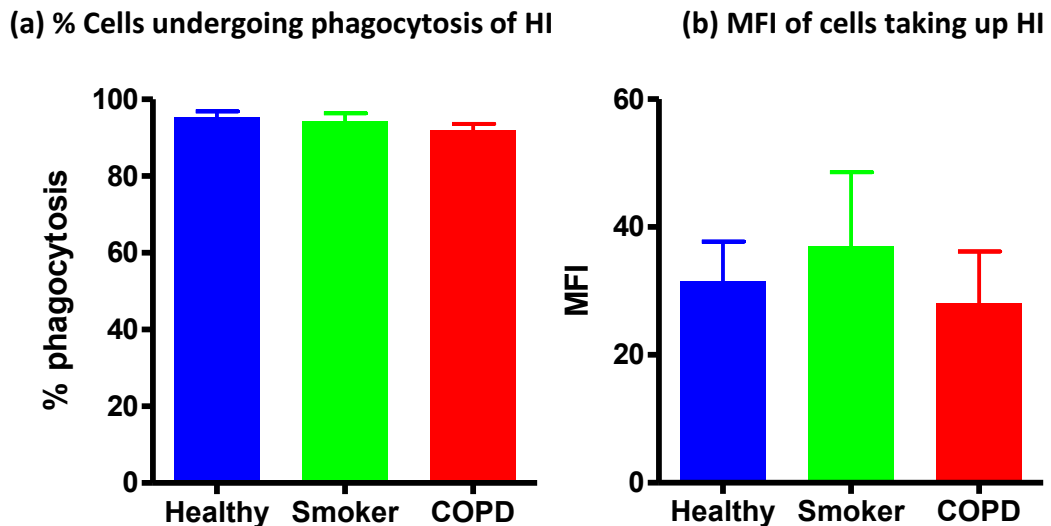


Figure 3.34. Ability of sputum macrophages from all subject groups to phagocytose *H. influenzae*

Sputum of healthy controls (■), healthy smokers (■) and COPD patients (■) was processed and macrophages were resuspended at 1×10^5 macrophages per FACS tube in RPMI media. Cells were incubated with 900 μ l of bacterial solution for 4 h, and then quantified by flow cytometry. Macrophages were gated according to properties of size and granularity. Data are presented as mean $\pm$ SEM, n=6 for healthy, n=7 for smokers, n=9 for COPD, where * represents $p < 0.05$ and ** $p < 0.01$.

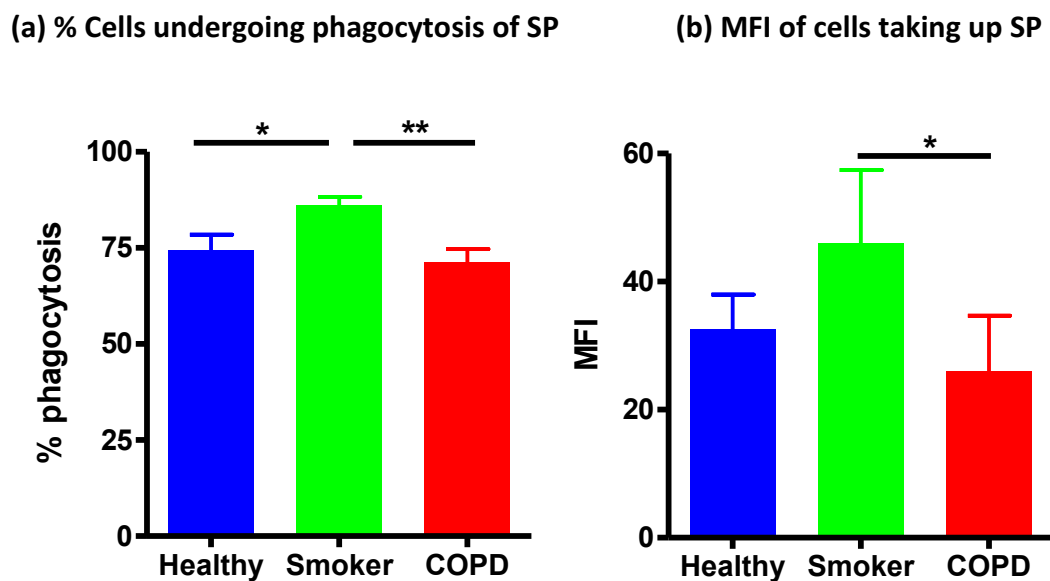


Figure 3.35. Ability of sputum macrophages from all subject groups to phagocytose *S. pneumoniae*

Sputum of healthy controls (■), healthy smokers (■) and COPD patients (■) was processed and macrophages were resuspended at 1×10^5 macrophages per FACS tube in RPMI media. Cells were incubated with 900 μ l of bacterial solution for 4 h, and then quantified by flow cytometry. Macrophages were gated according to properties of size and granularity. Data are presented as mean $\pm$ SEM, n=6 for healthy, n=6 for smokers, n=8 for COPD, where * represents $p < 0.05$ and ** $p < 0.01$.

3.4 Discussion

Examining two techniques for the isolation of monocytes showed that the final yield of MDMs was similar whether Miltenyi negative selection or the adherence technique was used. The technique of Miltenyi negative selection employs a column of magnetic beads to bind cells that are not required (including CD3, CD7, CD19, CD56, CD123 and Glycophorin A positive beads), allowing monocytes to pass through the column. Included in the magnetic beads are those to bind CD16+ cells. There are several monocyte subsets, which are divided into classical (CD14++, CD16-), intermediate (CD14++ CD16+) and non-classical (CD14+CD16++) (Ziegler-Heitbrock, 2007). CD16+ monocytes have been postulated to be more mature, pro-inflammatory (with increased cytokine release and chemokine receptor expression) and have increased HLA-DR expression with associated increased antigen presenting capacity (Ziegler-Heitbrock, 2007;Merino *et al.*, 2011). They have been shown to express similar patterns of surface antigens to tissue macrophages, which are reported to be less phagocytic compared to alveolar macrophages (Ziegler-Heitbrock, 2007). In addition, CD16+ monocytes have also been found to have increased senescence (Merino *et al.*, 2011). One could therefore postulate that the isolation of monocytes by negative selection may exclude a subset of monocytes, whilst adherence would include all monocyte subsets. MDMs derived from the CD16+ subset of monocytes may therefore differ in their phagocytic ability; however, experiments presented herein showed that the MDMs derived from monocytes isolated by Miltenyi or adherence had equivalent ability to phagocytose beads. Additionally, Taylor *et al* showed defective bacterial phagocytosis in COPD MDMs similar to that presented in this study, using MDMs derived from monocytes isolated using negative selection, as compared to adherence used in this study (Taylor *et al.*, 2010).

MDMs phagocytosis of both inert particles and bacteria increased in a time and concentration dependent manner, with maximal uptake around 4 h, similar to current evidence on macrophage phagocytosis in the literature (Takizawa *et al.*, 1996;Cannon and Swanson, 1992). Examining phagocytosis at 4 h was selected, as the level of fluorescence obtained at 4 h enables small alterations in phagocytosis to be clearly evident. In addition, using multiple time points in future assays would give a true level

of the phagocytic defect. Macrophages appear to have an immense capacity for uptake of particles or pathogens; it has been estimated that, when necessary, macrophages can internalize the equivalent of >100% of their surface area within 30 min (Greenberg and Grinstein, 2002). The ability of macrophages to ingest significant numbers of particles is further exemplified by their capability to ingest particles 3-times their volume (Cannon and Swanson, 1992).

Treatment of MDMs with cytochalasin D revealed that 40-50% of measurable bead phagocytosis and 10-15% of measurable bacterial phagocytosis were due to non-specific binding. Therefore, particles were adhering to the cell surface, increasing measurable cellular fluorescence, without being internalised. Macrophages have been shown to exhibit similar levels of non-specific binding in the presence of cytochalasin, with one study showing ~30% non-specific binding after 30 min incubation with latex beads, whilst another showed ~60% reduction in bead internalisation after 20 min incubation with beads in the presence of cytochalasin D (15 μ M) (Winkler *et al.*, 2008; Sulahian *et al.*, 2008). The increase in non-specific binding with latex beads compared to bacteria may be related to size; the beads used herein are 2 μ M in diameter, whilst the bacteria measure 1-2 μ M in maximum length. Therefore it may be related to speed of uptake, with evidence in the literature of slower internalisation of larger particles (Lee *et al.*, 2011). In addition, the receptor involved in phagocytosis alters the rate of internalisation of particles and pathogens, therefore it may be that particles and pathogens are being taken up by differing receptors resulting in differences in non-specific binding (Lee *et al.*, 2011).

MDMs from all subject groups showed equivalent phagocytosis of beads, showing that all were capable of phagocytosis. However, MDMs from smoker and COPD subjects showed significantly reduced phagocytosis of both gram positive and gram negative bacteria. Both MDMs and AM have been shown in previous studies to have equivalent bead phagocytosis, whilst phagocytosis of alternative prey has again been shown to be defective across different subject groups (Taylor *et al.*, 2010; Hodge *et al.*, 2003). Importantly the defect in phagocytosis of bacteria by MDMs was not related to the use of heat-killed bacteria, as similar reductions in phagocytosis of bacteria were seen with the use of live bacteria. The alterations in phagocytosis showed no correlation with

age, smoking history or lung function, suggesting that defective phagocytosis is not simply a phenomenon of ageing, nor is it simply associated with disease severity. This suggests that the defect leading to reduced uptake of bacteria by MDMs is inherent within the cells of the patient and is not caused by worsening disease or increased exposure to cigarette smoke. There was, however, a correlation between the ability of MDMs to phagocytose gram positive bacteria and their ability to phagocytose gram negative bacteria, with the correlation strongest at lower levels of phagocytosis, suggesting that the defect underlying reduced bacterial uptake is not specific to type or strain of bacteria.

In the study by Taylor *et al*, (Taylor *et al.*, 2010), whilst there was a trend to reduced phagocytosis of bacteria in smokers, this was not significant, in comparison to findings in the present study. The smoker subjects used herein are older (mean 58y vs. 53y) with an increased pack year history (mean 40 vs. 33); however, the evidence from the correlation data suggests that these parameters should have no effect on phagocytosis. Therefore, it may be related to the experimental design; a single time point of 1 h was used by Taylor *et al*, whereas examination of phagocytosis at longer time points may have shown a more marked reduction in phagocytosis by smoker MDMs. The different time points used may also explain the discrepancy between heat killed and live bacteria, where smoker cells have higher levels of phagocytosis compared to COPD with *H. influenzae* in live bacteria compared to heat killed. The longer time points used in this study were to ensure that the reduction seen in phagocytosis in COPD MDMs, (Taylor *et al.*, 2010), was due to an inherent defect in phagocytosis in these cells and not simply due to slower rate of uptake of bacteria by cells from COPD patients compared to healthy subjects.

This findings of reduced phagocytosis in the smoker population appears to suggest an effect of current smoking on phagocytosis, however, in the study by Taylor *et al*, the smoker subjects were all current smokers yet had improved levels of phagocytosis compared to COPD and this is also seen in this study when phagocytosis was performed using live *H. influenzae*. In addition, within the COPD cohort nearly half the subjects were current smokers, with no discernible differences found in levels of phagocytosis between smoking and non-smoking COPD subjects. It has previously

been shown that MDMs interacting with cigarette smoke-modified extracellular matrix proteins displayed reduced efferocytosis (Kirkham *et al.*, 2004) suggesting active smoking would lead to reduced phagocytosis, however, it is not clear why the effect was so marked in this study and differed to previous results and this requires further investigation.

Whilst there are clear alterations in the ability to ingest bacteria by smoker and COPD MDMs, this study showed no differences in the ability of cells from different subject groups to kill bacteria once internalised. This suggests that once bacteria are internalised into the phagosome, the functions of lysosomal fusion and the anti-microbial activity within the formed phagolysosome remains intact. This is supported by the literature, where no differences were found in intracellular bacterial killing between macrophages from healthy, smokers and COPD subjects (Berenson *et al.*, 2006a), whilst murine AM exposed to CSE have been shown to have increased intracellular killing ability (Marti-Llitas *et al.*, 2009).

The release of TNF- α from MDMs does, however, show further discrepancies in macrophage function between different subject groups. COPD MDMs released significantly higher levels of TNF- α in response to phagocytosis of inert particles compared to healthy and smoker cells, suggesting an abnormal inflammatory response. TNF- α is reported to be increased in blood, sputum and BAL of patients with COPD (Keatings *et al.*, 1996; Di *et al.*, 1994a); therefore, this heightened inflammatory response to inert particles may be expected in COPD. However, in response to *H. influenzae*, COPD MDMs produced significantly less TNF- α , suggesting a blunted response. Mouse AM exposed to CSE have been shown to produce attenuated levels of TNF- α compared to non-exposed controls (Gaschler *et al.*, 2008). AM from COPD patients have shown reduced release of TNF- α in response to NTHI, compared to smokers and healthy controls (Berenson *et al.*, 2006b). Rhinovirus exposure of AM has also been shown to significantly impair the LPS-induced secretion of TNF- α (Oliver *et al.*, 2008). Given that the attenuated levels of TNF- α are seen predominantly in response to gram negative bacteria, it is suggestive of altered recognition via or signalling from TLR4 in COPD cells. However, whilst expression of TLR2 appears to be variable in COPD, with both increased and reduced expression reported on peripheral

blood monocytes and AM, respectively, compared to healthy controls (Droemann *et al.*, 2005(Pons *et al.*, 2006), in both studies, TLR4 expression was unaltered. Work carried out previously by our group has also shown no alterations in either TLR2 or TLR4 expression on MDMs from COPD patients compared to controls (Taylor *et al.*, 2010). It may be that COPD and smoker MDMs release reduced levels of cytokines at an individual cellular level, however, due to the increased numbers of cells present in the lungs of smokers and COPD patients, the overall levels of cytokine are increased. Alternatively, it may be that COPD MDMs release cytokines at a different rate to healthy MDMs, as a limitation of the present study was that only a single time point was measured.

When COPD patients were classified based on their exacerbation history over the prior 2 y, frequent exacerbators were shown to have significantly reduced phagocytosis, of both gram positive and gram negative bacteria, compared to infrequent exacerbators. Interestingly, the frequent exacerbator group had a significantly lower pack year history and overall slightly better lung function compared to the infrequent exacerbators, once again confirming that this defect in phagocytosis is not related to disease severity or smoking history. Previous work by Patel *et al* has shown that frequent exacerbators have higher rates of bacterial colonisation compared to infrequent exacerbators, (Patel *et al.*, 2002), and this reduced ability to phagocytose bacteria may, in part, explain this observation. Therefore, a reduction in phagocytosis in COPD may lead to increased bacterial load within the lung and increased susceptibility to infection, driving more frequent exacerbations. Frequent exacerbations of COPD are associated with more rapid decline in FEV₁, increased hospitalisations, increased mortality and increased costs (Wedzicha and Seemungal, 2007). In addition, frequent exacerbations have a marked effect on patient quality of life, with increased dyspnoea, reduced exercise capacity and a greater likelihood of becoming housebound (Sapey and Stockley, 2006). Examining the ability of macrophages from COPD patients to phagocytose bacteria may then highlight a group of patients in whom targeted anti-microbial therapy may have significant benefits. Whether the defect exists within phagocytic cells and drives exacerbation frequency or *vice versa* is not entirely clear. However, given that the cells used in this study are circulating cells and have not been exposed to the lung environment, it suggests that

the defect leading to reduced bacterial phagocytosis is inherent within the cell. Furthermore, acute exacerbations of COPD did not significantly alter phagocytosis beyond baseline levels. This adds further weight to the suggestion that the defect underlying reduced phagocytosis is inherent within the cell and is not particularly susceptible to environmental changes. A limitation of the present study is the small numbers of paired baseline and exacerbation phagocytosis data, thus only allowing for limited conclusions to be drawn on this data. A further limitation is the paucity of data on whether the exacerbations were related to a viral or bacterial exacerbation, and, with regards to the latter, whether this involved a strain change from the colonising bacteria, as this may have variable impact on the phagocytic defect.

In contrast to the defective phagocytosis of bacteria in MDMs from smokers and COPD patients compared to healthy controls, sputum macrophages showed reduced phagocytosis of beads and *S. pneumoniae* by COPD macrophages only in contrast to smoker cells. Berenson *et al* found similar data, with COPD AM showing reduced phagocytosis of *H. influenzae* only when compared to smoker and not healthy controls (Berenson *et al.*, 2006a). It has been shown that induced sputum predominantly obtains cells from the surface of central but not peripheral airways, (Alexis *et al.*, 2001), and therefore sputum macrophages may not be fully representative of alveolar macrophages. Sputum from COPD patients has been shown to have an increased percentage of neutrophils and lower percentage of macrophages when compared to smokers, whilst smokers have been shown to have an increased percentage of macrophages compare to healthy controls (Rufino *et al.*, 2007; Lensmar *et al.*, 1998). In the present study, in sputum from COPD patients, neutrophils represented the majority of the cells with a reduced percentage of macrophages, compared to both smoker and healthy subjects where macrophages were the predominant phagocytic cell. This differing ratio of phagocytic cells within each sample represents one of the main problems with this phagocytosis assay in sputum. In COPD sputum samples, where there is a significantly increased proportion of neutrophils, the phagocytic prey used in the assay is likely to be taken up predominantly by the neutrophils, given the rapidity with which neutrophils phagocytose prey, potentially thus appearing to alter macrophage phagocytosis. In addition, pre-existing bacterial contamination was often seen in the COPD samples, but not healthy or smoker samples, which may also have an

effect on the subsequent phagocytosis assays. To truly measure macrophage phagocytosis in sputum, the macrophages would need to be isolated.

This study has shown that smoker and COPD MDMs have defective uptake of bacteria, but show no alterations in bead uptake in comparison to healthy controls. The discrepancy between uptake of particles versus pathogens is suggestive of a defect at the level of the pathogen recognition receptors, resulting either in down-regulation or reduced expression of receptors leading to reduced uptake. Rat bone marrow derived macrophages incubated with apoptotic neutrophils for 30 min, then re-exposed to identical prey after 48 h, showed ~50% reduction in uptake of apoptotic neutrophils on the second exposure (Erwig *et al.*, 1999). However, their ability to engulf apoptotic neutrophils, after an initial exposure to opsonised erythrocytes, (uptake via a different receptor) was unaffected (Erwig *et al.*, 1999), suggesting either down regulation of receptor expression or an element of satiety to the same exposure. It was subsequently shown that ligation of cell surface integrins reduced uptake of apoptotic neutrophils, but not of other particles, similar to effects of prior exposure, indicating desensitisation to the same prey. The monocytes used in this thesis are circulating cells; therefore, are unlikely to have been exposed to prey. Therefore, it is unlikely that altered receptor expression due to a desensitisation mechanism could account for the defects in bacterial phagocytosis seen in these cells.

Therefore, it could be that the differences seen in phagocytic capacity for beads and bacteria could be explained by reduced receptor availability, with different receptors involved in uptake of particles versus pathogens. Exposure of THP-1 cell-derived macrophages to cigarette smoke extract resulted in significantly reduced MARCO expression, with an associated reduction in bacterial clearance of *Mycoplasma pneumonia* (Baqir *et al.*, 2008). MARCO has been shown to be one of the main phagocytic receptors for unopsonised pathogenic bacteria, including *S. pneumoniae* (Arredouani *et al.*, 2004). In a murine model, influenza infection led to release of interferon- γ , which inhibited bacterial clearance by AM and correlated with downregulation of MARCO on AM (Sun and Metzger, 2008). However, previous work in our group has shown no differences in expression of MARCO, nor altered expression of other pathogen recognition receptors including CD206, CD163 and CD36 on MDMs

from COPD patients compared to controls (Taylor *et al.*, 2010). Therefore, whilst reduced MARCO expression has been shown to lead to reduced phagocytosis, this latter study suggests that altered receptor expression may not account for the reduction in phagocytosis of bacteria seen in the present study suggesting the defect may be related to signalling pathways beyond the receptors and the subsequent cytoskeletal rearrangements required for effective phagocytosis, which will be investigated further in Chapter 6.

In conclusion, MDMs from smokers and COPD patients show defective phagocytosis of pathogenic bacteria, but retain the ability to perform intracellular killing. It is unlikely that the use of ICS or LABA were related to reductions in phagocytosis as healthy smokers had lower levels of phagocytosis with no exposure to treatment. Previous work in the laboratory has shown that phagocytosis by MDMs from COPD subjects was unaltered by exposure to tiotropium, LABA or theophylline, with improvements in phagocytosis shown with budesonide (Taylor *et al.*, 2010). The effects of ICS on phagocytosis in COPD have been explored in more detail in Chapter 5 of this thesis.

This altered uptake of bacteria is not associated with increasing age or disease progression, nor with smoking history, suggesting the defect is inherent within the cell and not caused by worsening disease. Furthermore, there was a distinct association between the phagocytic ability of MDMs and the frequency of exacerbations. This may allow for the ability of MDMs to ingest bacteria to be used as a predictor for those patients in whom targeted and aggressive anti-microbial therapy may have increased benefits.

As neutrophils are the other main phagocyte in the lung at times of infection, the next chapter will examine whether neutrophil phagocytosis is in anyway altered and the mechanisms underlying defective phagocytosis will subsequently be examined.

3.4.1 Limitations

Experiments should be repeated in stable state with alveolar macrophages to ensure the defect is seen in alveolar macrophages as well as MDMs. Increased numbers of paired baseline and exacerbation samples would be needed to ensure that there was truly no differences seen in phagocytosis. A further limitation was that the type of

exacerbation, viral or bacterial, was not known in this study, as the type of exacerbation may lead to altered effects on phagocytosis at time of exacerbation. The sputum data was limited as there was a mix of phagocytic cells contributing to the phagocytosis measured, therefore, this should be repeated with separation of macrophages prior to phagocytosis assays.

Chapter 4

COMPARISON OF PHAGOCYTOSIS BY NEUTROPHILS FROM HEALTHY, SMOKER AND COPD SUBJECTS

4.1 Introduction

Neutrophils are the first phagocyte to be recruited to sites of infection, thus dominating the early immune response to foreign material (Lee *et al.*, 2003). The role of neutrophils in the lung is to clear bacterial pathogens to prevent colonisation, hence a pulmonary bacterial infection often leads to massive neutrophil influx from the circulation into the lung (Lee *et al.*, 2003).

Neutrophils are increased in number in the lungs of patients with COPD compared to those of smokers without respiratory disease (Stanescu *et al.*, 1996). In addition, the numbers of pulmonary neutrophils correlates with the degree of airway obstruction and an increased rate of decline in FEV₁ (Stanescu *et al.*, 1996;Donaldson *et al.*, 2002). This may be related to either increased neutrophil recruitment as a result of the disease, with chemotaxis towards increased levels of inflammatory chemokines, or may result from delayed apoptosis. Studies in patients with neutrophilic asthma have shown increased neutrophil longevity in the lung (Ryttila *et al.*, 2006). This contrasts with COPD and smokers, where significantly reduced numbers of neutrophils undergo spontaneous apoptosis compared to healthy controls (Ryttila *et al.*, 2006;Pletz *et al.*, 2004). Nonetheless, neutrophil numbers are increased in the lungs of smokers compared to healthy controls, yet despite these increased numbers, smokers show increased susceptibility to pulmonary infections, which has been suggested to be due to an impaired neutrophil anti-bacterial capability (Bagaitkar *et al.*, 2008).

An important anti-bacterial function of neutrophils is the ability to phagocytose bacterial pathogens. Impairment of this function could lead to an increased propensity for bacterial colonisation. Evidence for this possibility includes the observation that neutrophils from patients with chronic bronchitis have a reduced phagocytic index and killing of *Candida spp.* by approximately 50% in comparison to healthy subjects (Fietta *et al.*, 1988). Cigarette smoking may exacerbate this response, since neutrophils from healthy subjects incubated in the presence of cigarette smoke media (CSE) showed significantly reduced phagocytosis of *Escherichia coli* when compared to untreated neutrophils (Prieto *et al.*, 2001). Similarly, CSE treated neutrophils exposed to two different strains of *Staphylococcus aureus* showed significantly reduced phagocytosis (~15%) compared to >60% of control cells (Guzik *et al.*, 2011). This is further

supported by data from COPD patients, whereby neutrophils show reduced phagocytosis of *E. coli* compared with cells from healthy controls (Stringer *et al.*, 2007). Taken together these data suggest that the phagocytic mechanism regulating bacterial removal in COPD may not be defective in macrophages alone, but may also be defective in neutrophils.

Therefore, this chapter aims to investigate the ability of neutrophils to phagocytose both inert particles and pathogenic bacteria, and compare the responses of neutrophils from non-smokers, smokers and COPD subjects.

4.2 Methods

4.2.1 Subject selection

Healthy, smoking and COPD subjects were recruited as described in section 2.2.1.

4.2.2 Blood separation

Neutrophils were isolated from blood as described in section 2.2.3. Neutrophils were rested for 1 h prior to use in experiments.

4.2.3 Phagocytosis Assays

Phagocytosis assays were performed using fluorescently labelled beads or bacteria that were incubated with neutrophils for time points up until 1 h, at the concentrations indicated, as described in section 2.2.7.

4.2.4 Measurement of cell surface receptor expression

Receptor expression was analysed by flow cytometry (see section 2.2.10). The following antibodies were used at the dilutions specified in table 4.1.

Antibody	Dilution
Isotype control	1:10
Anti-CD11b	1:10
Anti- TLR2	1:10
Anti- TLR4	1:20

Table 4.1. Antibody dilutions used for quantification of cell surface receptor expression.

Receptor expression was described as the median fluorescent intensity (MFI) of neutrophils incubated with the antibody of interest, divided by the MFI of neutrophils incubated with isotype control (to account for non-specific binding).

4.2.5 Assessment of viability of neutrophils

Viability was assessed using the Annexin apoptosis kit (BD, Oxford, UK), following the protocol as described by the manufacturer. In brief, 1×10^5 neutrophils were re-suspended in binding buffer (supplied in the kit; 0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 25 mM CaCl_2). Annexin-V (a marker of apoptosis) and 7-AAD (a marker of cellular necrosis) were added to the tube, at a 1:20 dilution, then incubated in the dark for 15 min at RT. Neutrophil viability was analysed by flow cytometry. Cells were gated (see section 2.2.7) and a quadrant marker set up round the control population. Cells displaying apoptosis will increase in fluorescence along the x-axis, while those displaying necrosis will increase in fluorescence on the y-axis. The percentage of cells falling into different quadrants can then be analysed (Fig. 4.1).

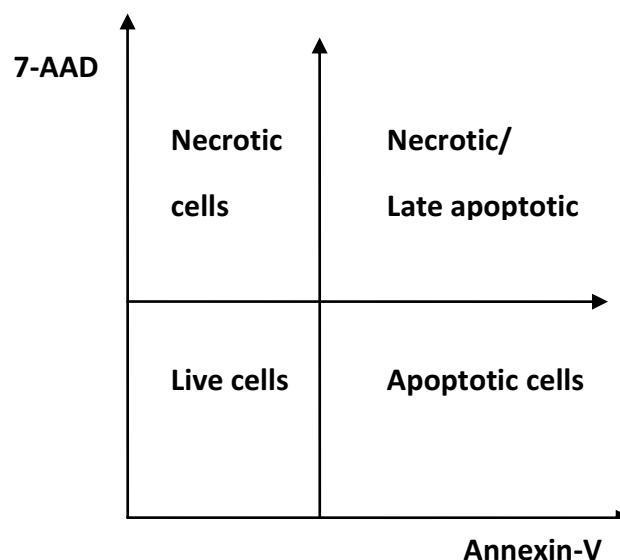


Figure 4.1. Analysis of neutrophil viability

Annexin and 7-AAD were added to the tube containing 1×10^5 neutrophils, at a 1:20 dilution, then incubated in the dark for 15 min at RT. Neutrophil viability was analysed by flow cytometry. The percentage of cells falling in each of the four quadrants could then be analysed.

4.3 Results

Firstly, experiments were performed to establish the ability of neutrophils to phagocytose beads and bacteria.

4.3.1 Subject Demographics

The characteristics of healthy, smoking and COPD subjects used in this study are shown in Table 4.2. There were significant differences in age and lung function between COPD and control groups (healthy and smoker subjects).

	Healthy	Smoker	COPD
Age (years)	58 ± 2	58 ± 1	66 ± 2 *
Gender (M:F)	11 : 12	11 : 11	16 : 8
FEV ₁ (l)	2.6 ± 0.2	2.5 ± 0.1	1.2 ± 0.1 ***
FEV ₁ % predicted	95 ± 2.4	86 ± 2.2	49 ± 4.1 ***
FVC (l)	3.8 ± 0.4	3.6 ± 0.3	2.5 ± 0.2 **
FEV ₁ /FVC (%)	78 ± 1.2	74 ± 0.9	44 ± 2.5 ***
Smoking history (pack years)	0	40 ± 3.4	54 ± 5.6

Table 4.2. Subject demographics

Where * represents $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to both healthy and smoking controls. Data is shown as mean ± SEM.

4.3.2 Neutrophil phagocytosis of beads

Initial experiments were performed to establish the optimum time point and concentration of beads at which to measure neutrophil phagocytosis. Cells were incubated with beads for increasing times and phagocytosis measured by FACS. These experiments revealed rapid uptake of beads by neutrophils, with the maximum

number of cells phagocytosing beads within 5 min (Fig. 4.3). This did not change over the following hour, indicating that phagocytosis was dependent on the concentration of beads (Fig. 4.3).

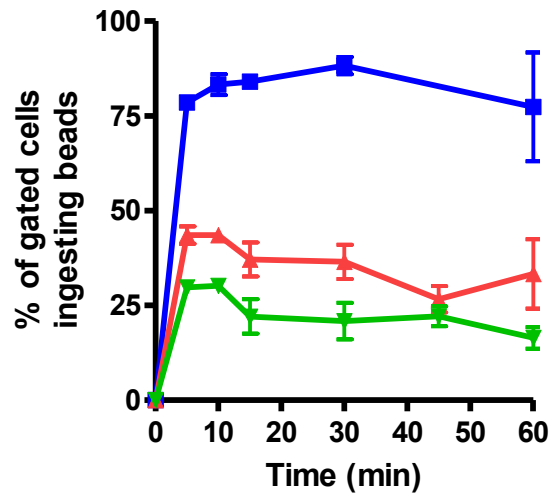


Figure 4.3. Effect of incubation time and bead concentration on phagocytosis by neutrophils

Neutrophils from healthy volunteers were isolated using Percoll gradients. Polystyrene bead solutions (900µl) (▼ 5x10⁶/ml, ▲ 10x10⁶/ml or ■ 50x10⁶/ml) were added to 1x10⁵ neutrophils in FACS tubes, and incubated at 37°C for the times indicated. Phagocytosis was stopped with ice cold PBS, cells washed, resuspended in FACSfix and analysed on FACScan with WinMDI software. Data shown are mean+/-SEM, n=4.

From these data, a sub-maximal concentration of 10x10⁶beads/ml was chosen for use in future experiments. Given that maximal neutrophil phagocytosis had been reached by the first time point of 5 min, further experiments were carried out at shorter time points. Neutrophils showed a rapid uptake of beads, with maximal phagocytosis reached at 2 min, with no change in percentage phagocytosis up to 30 min (Fig. 4.4).

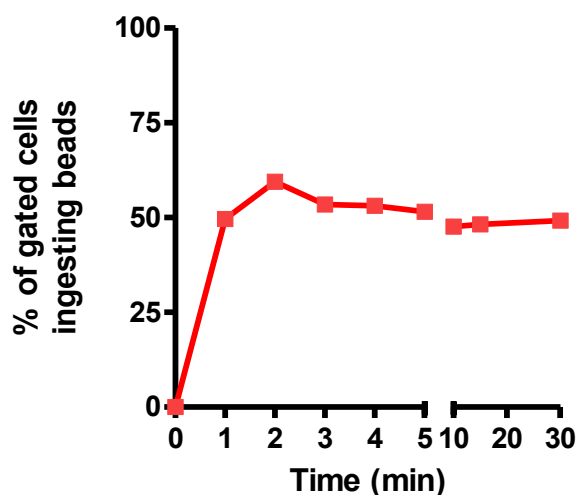


Figure 4.4. Time course of neutrophil phagocytosis of polystyrene beads

Neutrophils from healthy volunteers were isolated using Percoll gradients. Polystyrene beads ($10 \times 10^6/\text{ml}$) were added to 1×10^5 neutrophils in FACS tubes, and incubated at 37°C for the times indicated. Phagocytosis was stopped with ice cold D-PBS, cells washed, resuspended in FACSfix and analysed on FACScanto using FACSDiva software. Data shown are mean $\pm$ SEM, n=5.

4.3.3 Determination of non-specific binding

Given the rapidity of bead uptake by neutrophils, further experiments were carried out to confirm that the measurement of fluorescence was related to phagocytosis and not due to non-specific binding of beads to cells. Experiments were performed either at 4°C or in the presence of cytochalasin D ($2\mu\text{M}$), an actin microfilament inhibitor, both of which inhibit active phagocytosis (Parod and Brain, 1986).

Both cytochalasin D and reducing the temperature to 4°C caused an approximate 60% reduction in phagocytosis (Fig. 4.5). This suggests that non-specific binding accounts for up to 40% of bead phagocytosis measured.

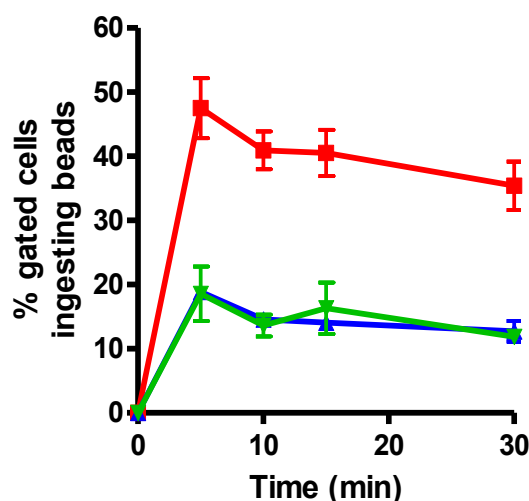


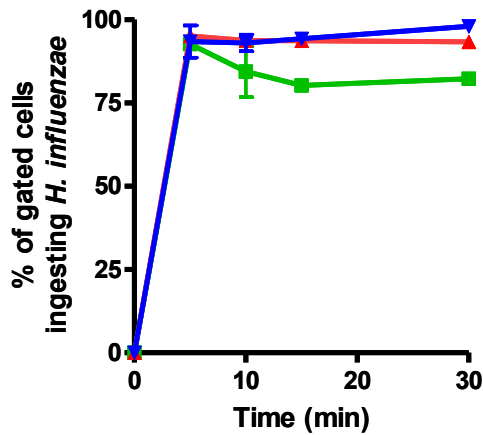
Figure 4.5. Effect of 4°C or Cytochalasin D on neutrophil phagocytosis of beads

Neutrophils from healthy volunteers were isolated using Percoll gradients. Neutrophils (1×10^5) were incubated with 900 μ l of 10×10^6 beads/ml at 4°C (▲) or 37°C (■), or at 37°C after 30 min pre-treatment with cytochalasin D (2 μ M) (▼). Phagocytosis was stopped with ice cold PBS, cells washed, resuspended in FACSfix and analysed on FACScan with WinMDI software. Data shown are mean $\pm$ SEM, n=6 for 37°C, n=4 for 4°C and cytochalasin D.

4.3.4 Neutrophil phagocytosis of bacteria

Having established the ability of neutrophils to ingest inert particles, their ability to ingest clinically relevant pathogens was examined. Neutrophils phagocytosed both Gram-positive and Gram-negative bacteria rapidly, within 5 min, in a concentration-dependent manner (Fig. 4.6). The highest concentration of bacterial solution and a time point of 15 min were selected to give optimal measurement of phagocytosis for future experiments.

(a) % phagocytosis of HI



(b) % phagocytosis of SP

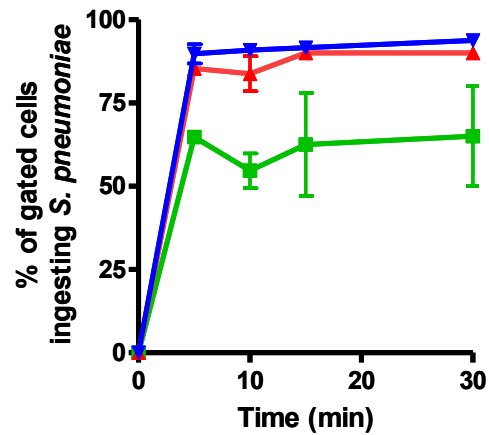


Figure 4.6a and 4.6b. Effect of incubation time and bacterial concentration on phagocytosis of (a) *H. influenzae* and (b) *S. pneumoniae* by neutrophils

Neutrophils from healthy volunteers were isolated using Percoll gradients. Bacterial solutions, given by CFU/ml, ($\blacksquare$ 4×10^7 /ml, $\blacktriangle$ 1×10^8 /ml or $\blacktriangledown$ 4×10^8 /ml) were added to 1×10^5 neutrophils in FACS tubes and incubated at 37°C for the times indicated. Phagocytosis was stopped with ice cold PBS, cells washed, resuspended in FACSfix and analysed on FACScanto with FACSDiva software. Data shown are mean $\pm$ SEM, n=4.

4.3.5 Assessing internalisation of bacteria - pHrodo™ labelled bacteria

To further investigate the non-specific binding of neutrophils with bacteria, *H. influenzae* was labelled with pHrodo dye™ (Invitrogen, UK), a pH specific fluorescent dye that only fluoresces at acidic pH (*i.e.* within the phagolysosome). This revealed that 100% of cells phagocytosed the bacteria from 5 min onwards, as shown by the phagocytic (blue) population having shifted in entirety to the right from the control (red) population (Fig. 4.7a).

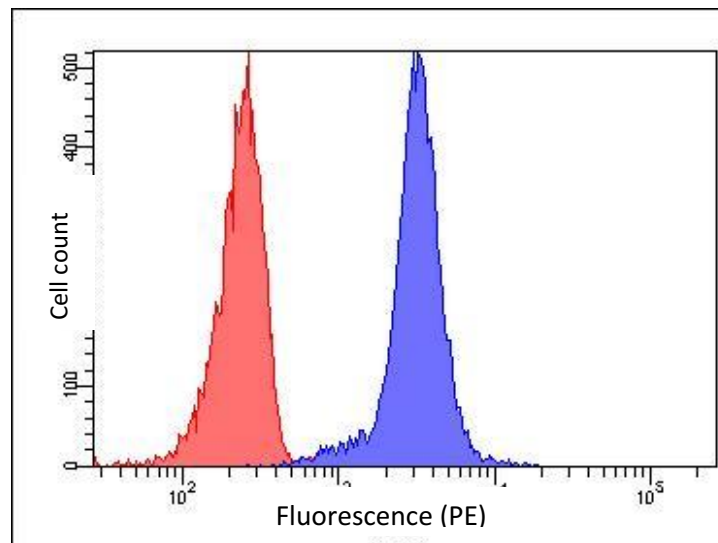


Figure 4.7a. Neutrophil phagocytosis of pHrodo labelled *H. influenzae*

Neutrophils from healthy volunteers were isolated using Percoll gradients. pHrodo labelled *H. influenzae* was added to 1×10^5 neutrophils in FACS tubes, and incubated at 37°C for 5 min. Phagocytosis was stopped with ice cold PBS and analysed on FACScanto with FACSDiva software. The population to the left of the x-axis is the **control population**, whilst neutrophils taking up bacteria become fluorescent – **phagocytic population**. Data shown is representative of $n=4$.

Analysis of the median fluorescent intensity of the phagocytosing neutrophils population showed that the amount of bacteria ingested increased to the 15 min time point, with no further subsequent increase (Fig. 4.7b).

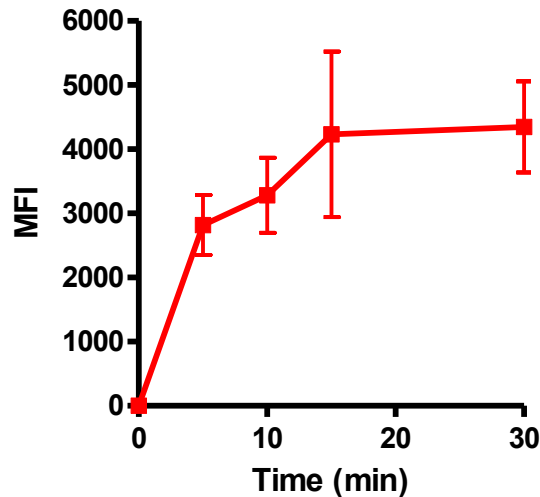


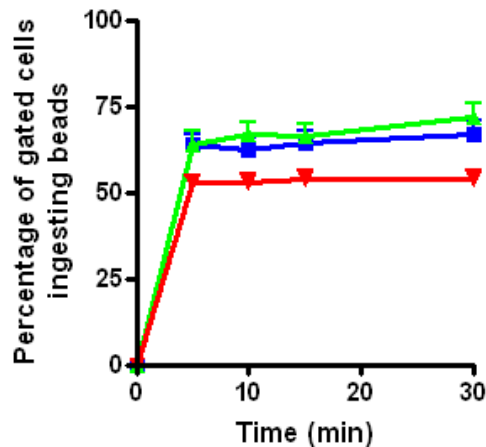
Figure 4.7b. Time course of neutrophil phagocytosis of pHrodo labelled *H. influenzae*

Neutrophils from healthy volunteers were isolated using Percoll gradients. pHrodo labelled *H. influenzae* was added to 1×10^5 neutrophils in FACS tubes, and incubated at 37°C for the times indicated. Phagocytosis was stopped with ice cold PBS and analysed on FACScanto using FACSDiva software. Data shown are mean $\pm$ SEM, n=4.

4.3.6 Effect of disease on phagocytosis of beads and bacteria by neutrophils

Data presented in Chapter 3 showed that MDMs from smoking and COPD subjects, whilst capable of phagocytosis, displayed defective uptake of bacteria in comparison to healthy controls. Whether this phenomenon was also apparent in neutrophils was not known. Therefore, having established an assay for neutrophil phagocytosis of both inert particles and bacteria, a comparison of phagocytosis by neutrophils from non-smokers, smokers and COPD patients was performed. Neutrophils from COPD patients showed an equivalent ability to phagocytose beads when compared to non-smoking and smoking controls, with no significant differences in the amount of beads taken up (MFI) between the three subject groups (Fig. 4.8).

(a) Percentage phagocytosis



(b) Median fluorescent intensity

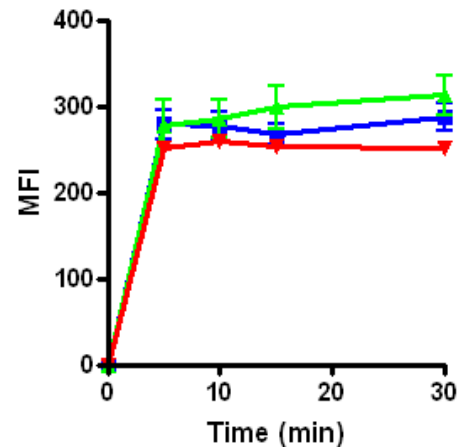


Figure 4.8. Effect of disease state on (a) percentage phagocytosis of beads by neutrophils and (b) median fluorescent intensity of beads

Neutrophils from healthy (■), smoking (▲) volunteers and COPD (▼) patients were isolated using Percoll gradients. Polystyrene beads (10×10^6 /ml) were added to 1×10^5 neutrophils in FACS tubes, and incubated at 37°C for the times indicated. Phagocytosis was stopped with ice cold PBS, cells washed, resuspended in FACSfix and analysed on FACScanto with FACSDiva software. Data shown are mean $\pm$ SEM, $n=20$ for all groups.

Approximately half of the neutrophils within the gated population, from both COPD and control subjects, had taken up beads at 5 min (Fig. 4.8a). With increasing time there was no further increase in either the percentage of cells ingesting beads (Fig. 4.8a), or in the number of beads ingested by each cell (Fig. 4.8b).

4.3.7 Neutrophil phagocytosis of *Streptococcus pneumoniae*

Having confirmed that all neutrophils were capable of phagocytosing beads in a similar manner, the capacity of neutrophils to remove bacterial pathogens was also investigated. Neutrophils from all subjects displayed equivalent ability to phagocytose *S. pneumoniae* (Fig. 4.9). However, differences were seen in the amount of *S. pneumoniae* taken up by neutrophils, with those cells isolated from smoker and COPD patients ingesting significantly less *S. pneumoniae* when compared to neutrophils

isolated from control subjects (Fig 4.9). Neutrophils from COPD patients showed significantly reduced uptake at 5 min compared to healthy controls (Fig. 4.9b).

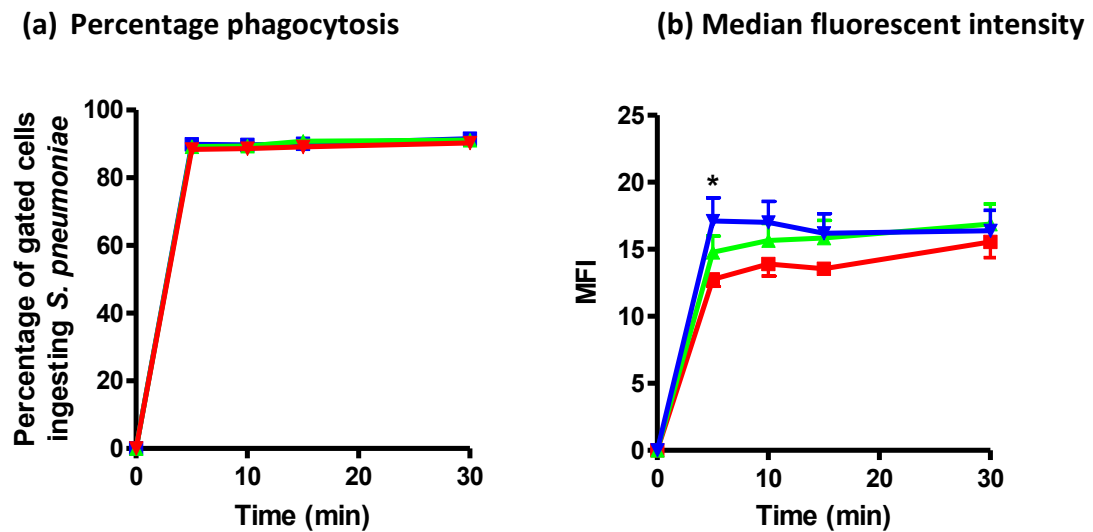


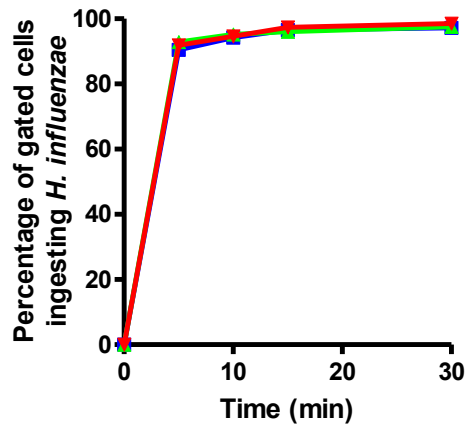
Figure 4.9. Effect of disease state on (a) percentage phagocytosis of *S. pneumoniae* by neutrophils and (b) median fluorescent intensity of *S. pneumoniae*

Neutrophils from healthy (▼), smoking (▲) volunteers and COPD (■) patients were isolated using Percoll gradients. Bacterial solutions (900µl) were added to 1×10^5 neutrophils in FACS tubes, and incubated at 37°C for the times indicated. Phagocytosis was stopped with ice cold PBS, cells washed, resuspended in FACSfix and analysed on FACScanto with FACSDiva software. Data shown are mean+/-SEM, n=20 for non-smoker and smoker volunteers, n=21 for COPD, where * represents $p < 0.05$ COPD vs. healthy.

4.3.8 Neutrophil phagocytosis of *Haemophilus influenzae*

Having shown a small but significant difference between COPD and healthy neutrophils in the uptake of gram positive bacteria, the uptake of *H. influenzae* was then investigated. Neutrophils incubated with *H. influenzae* (gram negative bacterium) showed no differences in percentage phagocytosis across the three disease groups (Fig. 4.10a). However, whilst neutrophils from healthy volunteers show increasing ingestion of *H. influenzae* with time, neutrophils from both smoking and COPD subjects showed a significantly reduced capacity to ingest equivalent amounts of *H. influenzae* when compared to healthy controls (Fig. 4.10b). At 30 min, neutrophils from smokers and COPD subjects show a 28% and 48% reduction in uptake of *H. influenzae*, respectively, in comparison to neutrophils from healthy subjects (Fig. 4.10b)

(a) Percentage phagocytosis



(b) Median fluorescent intensity

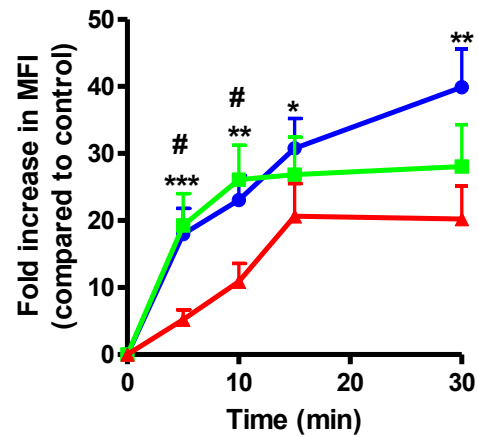


Figure 4.10. Effect of disease state on (a) percentage phagocytosis of *H. influenzae* by neutrophils and (b) median fluorescent intensity of *H. influenzae*

Neutrophils from healthy (●), smoking (■) volunteers and COPD (▲) patients were isolated using Percoll gradients. Bacterial solutions (900μl) were added to 1×10^5 neutrophils in FACS tubes, and incubated at 37°C for the times indicated. Phagocytosis was stopped with ice cold PBS, cells washed, resuspended in FACSfix and analysed on FACScanto with FACSDiva software. Data shown are mean+/-SEM, n=21 for all groups, where * represents $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ COPD vs. healthy and # represents $p < 0.05$ COPD vs. smoker.

This data is depicted below in an overlaid histogram, clearly showing the differences in the ability of neutrophils from different subject groups to ingest *H. influenzae*. The population of COPD cells is least fluorescent (lowest uptake), with healthy cells taking up the most bacteria and smokers in the middle (Fig. 4.11).

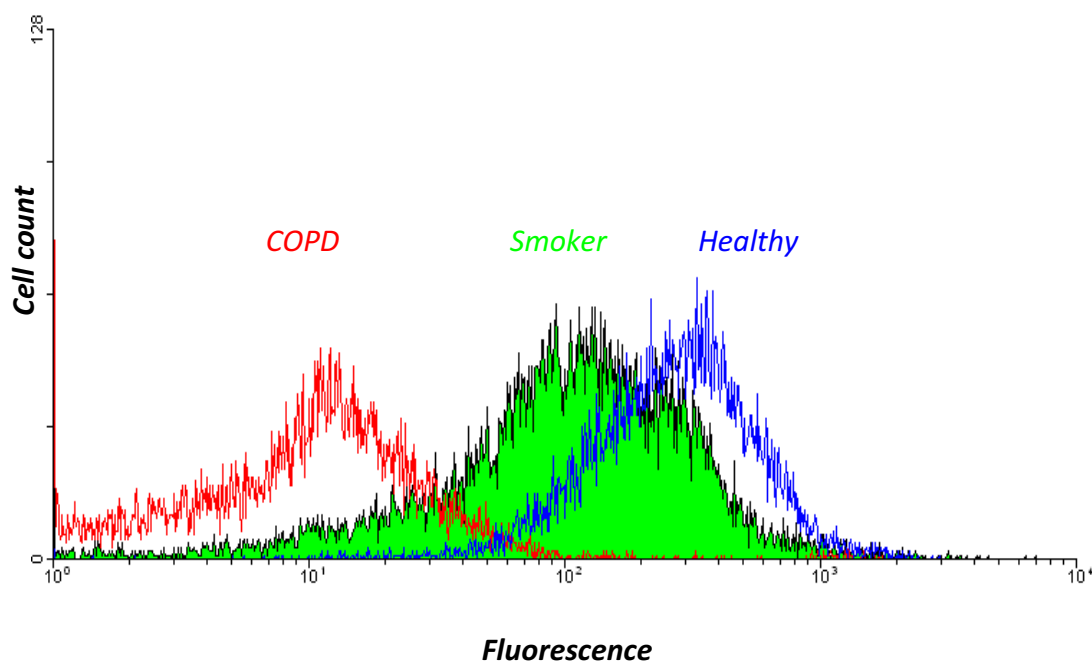


Figure 4.11. Effect of disease state on the uptake of *H. influenzae*

Neutrophils from healthy (■), smoking (■) volunteers and COPD (■) patients were isolated using Percoll gradients. Bacterial solutions (900μl) were added to 1×10^5 neutrophils in FACS tubes, and incubated at 37°C for the times indicated. Phagocytosis was stopped with ice cold PBS, cells washed, resuspended in FACSfix and analysed on FACScanto with FACSDiva software.

4.3.9 Neutrophil viability

Having shown that neutrophils from different subject groups showed altered uptake of bacteria, it was important to ensure that cells from smoker and COPD subjects did not have altered viability that could account for differences seen in phagocytosis. Cells from all subject groups (both control cells and cells incubated with prey) were incubated with Annexin-V, a marker of apoptosis, and 7-AAD, a marker of cell necrosis, and then analysed by flow cytometry (see section 4.2.5). An example of the dot plot obtained is shown in Fig. 4.12, with healthy neutrophils showing 94.1% viable cells.

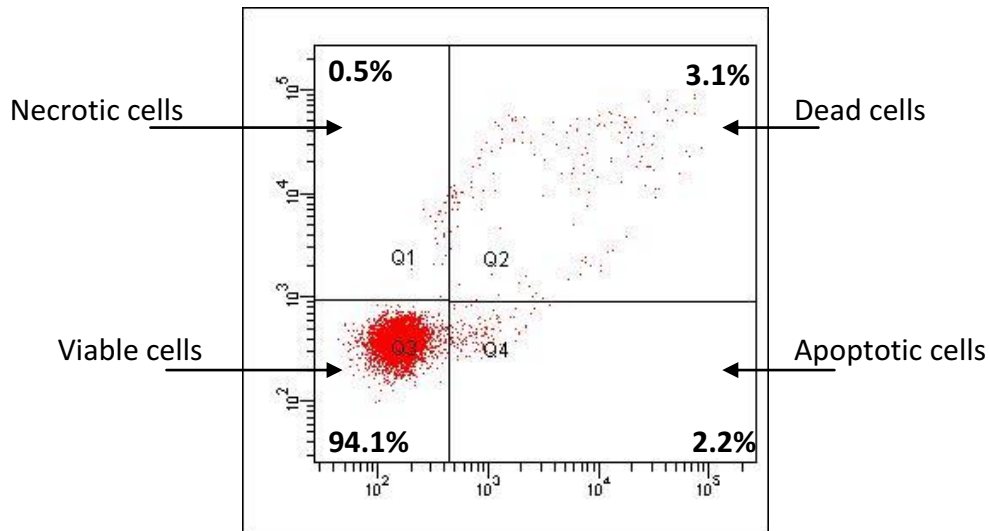


Figure 4.12. Analysis of neutrophil viability

Annexin and 7-AAD were added to 100 μ l of 1×10^5 neutrophils, at a 1:20 dilution, then incubated in the dark for 15 min at RT. Neutrophil viability was analysed by flow cytometry. The percentage of cells falling in each quadrant could then be analysed.

Assessment of neutrophil viability indicated that there were no differences were seen in the percentage of viable cells across the three subject groups (Fig. 4.13).

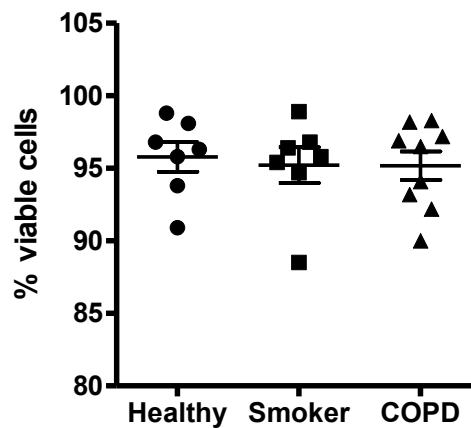


Figure 4.13. Effect of disease state on cell viability of neutrophils

Neutrophils from healthy (●), smoking (■) volunteers and COPD (▲) patients were isolated using Percoll gradients. Neutrophils at 1×10^5 in FACS tubes were incubated with 5 μ l of Annexin PE (marker of apoptosis) and 5 μ l of 7-AAD (marker of necrosis) at RT in the dark for 15 min. Neutrophils were analysed on FACScanto with FACSDiva software. Data presented is control cells and is representative of cells exposed to prey. Data shown are mean $\pm$ SEM, n=7 for non-smoker and smoker volunteers, n=8 for COPD.

4.3.10 Neutrophil cell surface receptor expression

In order to investigate whether decreased uptake of bacteria could be associated with reduced cell surface receptor expression of CD11b (a cell adhesion molecule and part of the CR3 receptor), toll-like receptor 2 (TLR2, involved in recognition of Gram positive bacteria) and toll-like receptor 4 (TLR4, involved in recognition of Gram negative bacteria). There were no significant differences seen in neither the percentage of neutrophils expressing CD11b, TLR2 or TLR4 (Fig. 4.14a-c), nor the level of expression of CD11b, TLR2 or TLR4 on neutrophils from healthy or smoking volunteers or COPD patients (Fig. 4.15a-c).

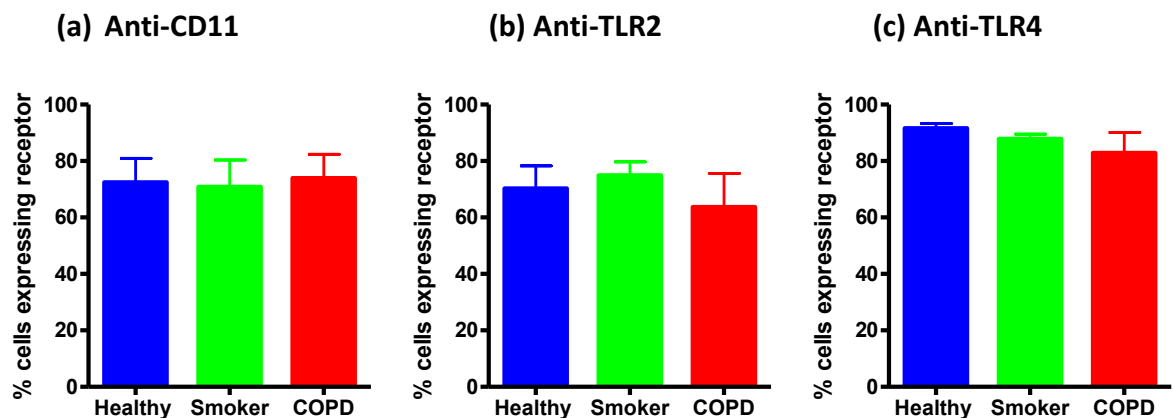


Figure 4.14. Effect of disease on percentage of neutrophils expressing the receptors: (a) anti-CD11 (b) anti-TLR2 and (c) anti-TLR4

Neutrophils from healthy (■), smoking (■) volunteers and COPD (■) patients were isolated using Percoll gradients. Neutrophils (1×10^5) were added to FACS tubes. Fluorescently labelled antibodies (1:10 dilution for CD11 and TLR2, 1:20 dilution for TLR4) or isotype control (1:10 dilution) were added to individual tubes, then incubated at 4°C for 30 min, washed with ice cold PBS and resuspended in FACSfix prior to analysis on FACScanto using FACSDiva software. Data shown are mean $\pm$ SEM, n=10.

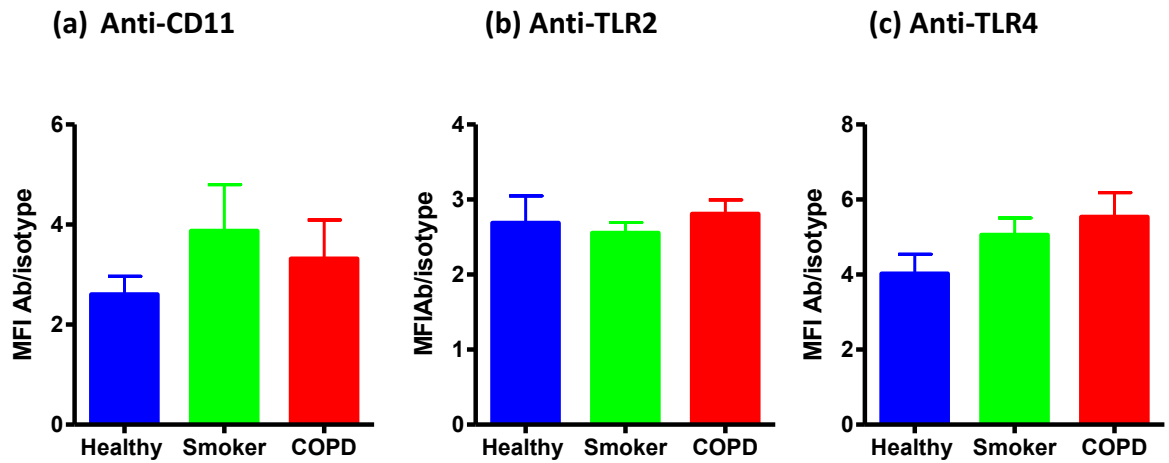


Figure 4.15. Effect of disease state on neutrophil receptor expression; (a) anti-CD11 (b) anti-TLR2 and (c) anti-TLR4

Neutrophils from healthy (■), smoking (■) volunteers and COPD (■) patients were isolated using Percoll gradients. Neutrophils (1×10^5) were added to FACS tubes. Fluorescently labelled antibodies (1:10 dilution for CD11 and TLR2, 1:20 dilution for TLR4) or isotype control (1:10 dilution) were added to individual tubes, then incubated at 4°C for 30 min, washed with ice cold PBS and resuspended in FACSfix prior to analysis on FACScanto with FACSDiva software. Data shown are mean $\pm$ SEM, n=10.

4.4 Discussion

Initial experiments on neutrophil phagocytosis showed that the neutrophils ingested particles extremely rapidly, with phagocytosis of fluorescently-labelled beads plateauing from around 2 min, suggesting a degree of activation. However this speed of uptake is confirmed by previous studies, which have shown that neutrophils ingesting latex beads showed rapid uptake that was largely complete by 90 s, with particle uptake and vacuole closure completed within 20 s (Segal *et al.*, 1980). As seen with phagocytosis of beads, neutrophil phagocytosis of both Gram positive and Gram negative bacteria is maximal at 5 min. It is likely that, as with beads, the maximal phagocytosis of bacteria is reached already at an even shorter time point. This is in direct contrast to macrophages that take several minutes to engulf particles (Henry *et al.*, 2004). A comparison of the uptake of zymosan versus antibody-coated latex beads (3 μ M) by neutrophils revealed that the time taken for engulfment (particle uptake and vacuole closure) was on average 167 s for zymosan compared to 66 s for opsonised latex beads (Lee *et al.*, 2011). Increasing the size of the beads (to 5 μ M) reduced speed of engulfment to ~90 s, suggesting that the longer engulfment time for zymosan is not simply target-size related, but rather a combination of size and different receptors involved in the phagocytic process (Lee *et al.*, 2011). The engulfment time of the particles used in the present study is likely to be more rapid than that given for zymosan, reflecting their smaller particle size (polystyrene beads and *H. influenzae* ~2 μ M and *S. pneumoniae* ~1 μ M).

Treatment of neutrophils with cytochalasin D or incubation at 4°C revealed that ~40% of measurable phagocytosis of beads was due to non-specific binding. Therefore, particles were bound to the surface of the cell increasing cellular fluorescence but not internalised. This is similar to studies of macrophages where non-specific binding of approximately 30%, after incubation with latex beads for 30 min (Winkler *et al.*, 2008). Neutrophil phagocytosis of latex beads showed that even at 0 s, ~14% of cells had associated particles; this is unlikely to represent phagocytosis, and therefore is considered to be a measure of non-specific binding (Segal *et al.*, 1980).

As there appears to be a relatively high level of non-specific binding, it was important to determine that internalisation could be confirmed and measured. Therefore, bacteria labelled with pHrodo™ dye (Invitrogen, UK) were used to determine the degree of internalisation of bacteria incubated with neutrophils; this particular dye has low level fluorescence at neutral pH, with marked fluorescence at acidic pH, *i.e.* within the phagolysosome. At the earliest time point of 5 min, all cells had internalised bacteria. The fluorescence emitted from neutrophils was reduced in comparison to that of macrophages (chapter 3). This may be due to the increased acidic nature of the phagolysosome of the macrophage (pH<5) compared with neutrophils (Lee *et al.*, 2003). In neutrophils, activation of the oxidative burst, after fusion of granules with the neutrophil phagosome, leads to consumption of H⁺ ions, which increases pH transiently, leading to alkalinisation of the phagolysosome, which subsides after minutes (Nordenfelt and Tapper, 2011). It is then unclear whether the pH remains neutral or becomes more acidic and it has been speculated that the phagosomal pH may be lower than that measured during *in vitro* studies (Lee *et al.*, 2003;Nordenfelt and Tapper, 2011). Therefore, measuring earlier time points of phagocytosis with pHrodo™ dye, may not generate fluorescence due to a more alkaline environment within the phagosome. However, from 5 min it is likely that the pH of the phagosome returns to neutral or even as speculated becomes more acidic, allowing the dye to fluoresce (Lee *et al.*, 2003;Nordenfelt and Tapper, 2011). Nonetheless, these studies indicate that bacteria are engulfed and processed rapidly.

When the effect of disease on neutrophil phagocytosis was examined, it was clear that neutrophils from all subject groups were capable of phagocytosis. However, smoker and COPD neutrophils showed defective uptake of bacteria. This defect was more marked following exposure to *H. influenzae* than *S. pneumoniae*, with COPD neutrophils showing reduced uptake at all time points and smoker neutrophils showing reduced uptake after 10 min. Whilst neutrophils from healthy subjects continued to ingest bacteria with increasing time, both COPD and smoker neutrophils displayed 'early satiety'.

Exactly why this is the case is unclear as there is little data in the literature regarding the 'stop' signals regulating phagocytosis. One possibility would be that available receptors on the cell surface become depleted and are no longer available to bind the particle of interest. As both the inert particles and bacteria used in these experiments were unopsonised, phagocytosis of the different prey may be triggered by the same receptors. However, neutrophils from all subject groups were capable of ingesting similar amounts of beads, suggesting there was sufficient receptor expression in neutrophils from all subjects. There is little in the literature regarding expression of scavenger or mannose receptors (these being the likely receptors involved in phagocytosis of non-opsonised prey) on neutrophils in smokers or COPD subjects. Analysis of the distinct receptors involved in recognition of gram positive and negative bacteria (TLR2 and TLR4 respectively) and CD11b (part of the complement receptor) showed no differences in expression across the disease groups. As discussed in the previous chapter, TLR2 has been found to be both up- and down-regulated on monocytes and AM respectively in COPD (Droemann *et al.*, 2005; Pons *et al.*, 2006). Blood neutrophils from COPD patients have been shown to have no differences in TLR2 or TLR4 expression compared to healthy controls (von, I *et al.*, 2011). Another study has shown increased TLR4 expression on BAL cells from COPD compared to healthy and smoking controls, with the majority of these TLR4+ cells being neutrophils (Pace *et al.*, 2011). In contrast, levels of TLR2 expression were found to be similar across the three groups (Pace *et al.*, 2011). This latter study recruited patients from intensive care with respiratory failure, which may account for some of the differences seen in expression of TLR. Neutrophils in the submucosa of bronchial biopsies have been shown to have increased CD11b and CD44 co-expression in COPD compared to smokers (Di *et al.*, 2009). Expression of CD11b on circulating neutrophils has also been shown to be higher in COPD subjects compared to healthy controls (Noguera *et al.*, 2001; Yamagata *et al.*, 2007b) in contrast to the findings in this present study. These differences may be due to different study populations, including severity of disease and concomitant infection.

Stringer *et al* showed defective phagocytosis of *E. coli* in CSE-exposed neutrophils compared to healthy controls was associated with suppression of caspase-3 activity (Stringer *et al.*, 2007). Caspase-3 is required for spontaneous neutrophil apoptosis and

phagocytosis-induced neutrophil death (Zhang *et al.*, 2003), although there is no evidence of a causal link between suppression of caspase-3 and reduced phagocytosis. Prieto *et al* also showed reduced phagocytic function in neutrophils from COPD patients compared to healthy controls (Prieto *et al.*, 2001). Glycophosphopeptical, an immunomodulator, improved the percentage of COPD neutrophils undergoing phagocytosis, but did not improve uptake of bacteria (Prieto *et al.*, 2001). Glycophosphopeptical has been shown to improve natural killer cell activity in mice (Prieto *et al.*, 2001). The underlying mechanism behind the effect of glycophosphopeptical was not examined. However, it may prime neutrophils, so altering composition of cell surface receptors and activating actin polymerisation (Nordenfelt and Tapper, 2011). It has been shown that neutrophils primed with TNF- α have been shown to have increased killing of opsonised *S. aureus*, increased phagocytosis of complement-opsonised NTHI (associated with elevated levels of CR3 and CR4), whilst neutrophils primed with hydrogen peroxide show increased phagocytosis of opsonised (IgG) erythrocytes (Ferrante *et al.*, 1993; Pricop *et al.*, 1999; Tan *et al.*, 1995).

In order to investigate the mechanism underlying the defective phagocytosis of bacteria displayed by neutrophils from smokers and COPD, it is appropriate to examine the ability of neutrophils from the same subject groups to undergo chemotaxis. The processes of chemotaxis and phagocytosis appear to converge during signalling to the cytoskeleton during the formation of protrusions (Heinrich and Lee, 2011). Chemotaxis has also been shown to be altered in COPD patients compared to healthy and smoking subjects. COPD patients show increased neutrophil migration toward fMLP compared to healthy controls (Burnett *et al.*, 1987) and display increased rolling, tethering and migration, compared to healthy and smoking controls (Woolhouse *et al.*, 2005). Neutrophils from COPD patients display increased random speed but reduced directed speed and reduced accuracy to chemoattractants including CXCL8, CXCL1, fMLP and sputum, compared to neutrophils from healthy and smoking subjects (Sapey *et al.*, 2011).

No differences were found in receptor expression (CXCR1, CXCR2 and FPR1), however, COPD neutrophils produced less pseudopods compared to healthy and smoker cells

(Sapey *et al.*, 2011). Activation of the small GTPase, rac drives both protrusions both in cell migration and during phagocytosis (Castellano *et al.*, 2000; Heinrich and Lee, 2011; Levskaya *et al.*, 2009). Therefore, it may be that defects in cytoskeletal rearrangement affecting chemotaxis in neutrophils may also lead to altered phagocytosis. Sapey *et al* showed that COPD neutrophils incubated with PI3-kinase inhibitors had reduced distance travelled, whilst improved directed speed and accuracy towards chemoattractants, whereas the inhibitors had minimal effect in healthy and smokers cells (Sapey *et al.*, 2011). Activation of the PI3K pathway is required for phagocytosis, resulting in the localisation of F-actin to specific points on the cell membrane for phagocytic cup formation (Greenberg and Grinstein, 2002). Therefore, it may be that there is a common defect in the signalling pathway accounting for both defects in chemotaxis and phagocytosis in neutrophils in COPD.

In conclusion, these data show significantly reduced uptake of pathogenic bacteria by neutrophils from both COPD and smoking subjects in comparison to healthy controls. The possible mechanisms underlying this defect will be examined further in the subsequent chapter.

4.4.1 Limitations

Assays should be performed to ensure neutrophils from all groups are not differentially activated. Receptor studies were limited and should be broadened to include a variety of different receptors including scavenger receptors.

Chapter 5

A COMPARISON OF THE EFFECTS OF FLUTICASONE PROPIONATE AND BUDESONIDE ON THE PHAGOCYtic ABILITY OF MDMs AND NEUTROPHILS

5.1 Introduction

Inhaled corticosteroids (ICS) are now recommended for COPD patients with an $FEV_1 \leq 50\%$ predicted, who experience frequent exacerbations or persistent breathlessness over a 12-month period (NICE guidelines, 2010), and as such, ICS are now prescribed to approximately 70% of COPD patients (Singanayagam *et al.*, 2011). ICS have been shown to improve symptoms, health status and reduce decline in FEV_1 , whilst decreasing exacerbation rates (Postma and Calverley, 2009). However, the TORCH study, designed to look at all cause mortality in COPD, showed a non-significant (6%) increase in mortality in patients taking fluticasone propionate (Suissa and Barnes, 2009). A post-hoc analysis of these data revealed an increased risk of pneumonia of ~50% in those patients taking inhaled corticosteroids (Crim *et al.*, 2009). This finding is of increased importance as patients with COPD that are hospitalised with pneumonia have been shown to have increased mortality compared to those without COPD (Restrepo *et al.*, 2006; Holguin *et al.*, 2005).

This phenomenon has been studied further and two studies that compared the combination of fluticasone propionate and salmeterol with either salmeterol alone, or tiotropium bromide alone, have also shown an increased frequency of pneumonia in the fluticasone-containing treatment arm (200% and 100% increase respectively) (Kardos *et al.*, 2007; Wedzicha *et al.*, 2008). Similarly, a Canadian population-based observational study reported a 70% increase in the risk of hospital admission with pneumonia in those COPD patients taking ICS, with the greatest increase in hospitalisation observed with highest doses of ICS (Ernst and Suissa, 2008). Taken together, these studies indicate that ICS increase the risk of pneumonia in COPD patients, however, a recent meta-analysis examining treatment of COPD patients with budesonide showed no increased risk of pneumonia (Sin *et al.*, 2009), with the lower risk of pneumonia associated with budesonide confirmed in a further comparison of randomised controlled trials of combination inhalers (Halpin *et al.*, 2011). This would suggest that the effect on pneumonia risk in COPD patients is not drug class dependent, but may be specific to fluticasone propionate.

It remains unclear why different ICS appear to have differential effects on the increased risk of pneumonia. Whilst all glucocorticoids act by binding to the

glucocorticoid receptor, each drug exhibits differential pharmacokinetic properties which may account for the clinical differences seen. Fluticasone propionate is more lipophilic than budesonide, and as a result is more likely to be retained in mucus in the lumen of the airway and cleared via mucociliary clearance and cough (Edsbacker *et al.*, 2008). Fluticasone propionate has been shown to have considerably lower plasma concentrations, possibly due to increased deposition of inhaled drug in central airways following airways constriction (Mortimer *et al.*, 2007). This contrasts with budesonide where there is no reduction in plasma concentrations with bronchoconstriction (Mortimer *et al.*, 2007). There is now evidence that, after inhalation, fluticasone propionate resides for a prolonged time (hours) in the airway mucus, most likely in the form of undissolved particles. This contrasts with budesonide which dissolves completely and is absorbed into the airway tissue within a few minutes (Dalby *et al.*, 2009). Whether the prolonged persistence of fluticasone propionate within the airway lumen may affect host defenses and account for the increased risks of pneumonia is as yet unknown.

One hypothesis is that clearance of particulate fluticasone by phagocytic cells of the innate immune system results in high concentrations of the drug residing within the phagolysosomes. Phagocytosis of pathogenic bacteria by macrophages has already been shown to be impaired in COPD patients (Taylor *et al.*, 2010); therefore, any further impairment would lead to further reduction in clearance of bacteria from the lung, potentially increasing the risk of pneumonia.

Therefore, the aim of this study is to compare the effects of two glucocorticosteroids, budesonide and fluticasone propionate, over a broad range of concentrations, on the ability of both macrophages and neutrophils to ingest and kill pathogenic bacteria.

5.2 Methods

5.2.1 Subject recruitment

The inclusion criteria for COPD patients were as follows: age 40-85 years, no history of other respiratory disease or major co-morbidity, smoking history of >10 pack years and no evidence of atopy on skin prick tests to common aero-allergens. Lung function: FEV₁% predicted <80%, FEV₁: FVC ratio <0.7 and reversibility of <200ml in FEV₁ (<15% FEV₁% predicted) to inhaled β_2 agonist. In addition, patients had to be free from acute exacerbation or oral steroid or antibiotic usage in the preceding 6 weeks.

5.2.2 Blood Separation

Whole blood was taken by venepuncture after informed consent. Granulocytes were separated from the PBMC fraction using Percoll gradients (see section 2.2.3). Monocytes from the PBMC fraction were adhered and cultured in the presence of GM-CSF (2ng/ml) for 12d to differentiate into MDMs. The granulocyte fraction was resuspended at 1×10^6 cells/ml in D-PBS and rested at 4°C for 1 h, to reduce neutrophil activation.

5.2.3 Phagocytosis Assays

Particulate suspensions of both steroids were prepared by resuspension in 0.2% (^w/_w) Tween 80 in D-PBS. The suspension was then sonicated for 5 min to ensure uniformity of particulates in solution. Both MDMs and neutrophils were incubated with either budesonide or fluticasone (at concentrations 10^{-12} to 10^{-5} made up in RPMI) or vehicle control for 1h prior to addition of beads or bacteria.

5.2.3.1 Phagocytosis of Beads

Fluorescently labelled polystyrene microspheres (2.0 μ m diameter, yellow-green fluorescence (ex λ 505nm/em λ 515nm) were diluted in complete media to concentrations indicated (as described in Section 2.2.7).

MDMs: Bead solutions (10 μ l of 5×10^7 /100 μ l) were added to wells containing 1×10^5 MDMs and incubated at 37°C, 5% (^v/_v) CO₂ for 4 h. After incubation, unbound beads were washed off with D-PBS. Fluorescence of extracellular particles was quenched by

adding 100µl/well of Trypan Blue (2% (v/v)) for 1min. Excess fluid was removed and fluorescence determined by fluorimetry (BMG Fluostar plate reader) at excitation λ 480nm and emission λ 520nm.

Neutrophils: Bead solutions (11µl of $10^7/100\mu\text{l}$) were added to FACS tubes containing 1×10^5 neutrophils and incubated at 37°C, 5% (v/v) CO₂ for 15 min. After incubation, tubes were placed on ice to quench phagocytosis, then centrifuged (400xg, 5 min, 4°C) with ice cold D-PBS. Cell pellets were resuspended and fixed in 300µl FACSfix (0.5% (v/v) paraformaldehyde in FACSflow) ready for analysis. Samples were analysed using a Becton Dickinson FACSCanto machine using FACS Diva software. Cells were gated according to forward and side scatter values correlating to neutrophils and for each analysis 10,000 events were collected (See section 2.2.7).

5.2.3.2 Phagocytosis of Bacteria

Stock solutions of *H. influenzae* and *S. pneumoniae* were sonicated for 1 min prior to use to prevent aggregation. Bacteria were diluted to the appropriate concentrations in D-PBS. For MDMs phagocytosis assays, 10µl of stock solution was transferred to wells, whilst for neutrophil assays 11µl of stock solution was added to FACS tubes and incubated, washed and analysed as described in section 2.2.7.

5.2.4 Intracellular killing assays

MDMs were incubated with either fluticasone or budesonide (10^{-5}M), or media alone, for 1 h, prior to addition of 11µl of live bacterial stock solution and incubation at 37°C for 4 h. Supernatants were removed and serial dilutions performed in D-PBS (to 10^{-6}) and multiple dilutions (10 µl of each) were plated onto iso-sensitest agar plates for overnight incubation. This gives a count of bacteria not taken up by the cells. Cells were then lysed with dH₂O for 3 min, the supernatants removed, diluted to 10^{-4} , and multiple dilutions plated as before for overnight incubation. This gives a count of viable intracellular bacteria. Colonies were counted the following morning and a measure of CFU/ml calculated. Intracellular killing was then calculated as described in section 2.2.16.

5.2.5 Viability assays

MDMs were incubated with MTT solution for 30 min, before aspiration and solubilisation with DMSO. Plates were read on a spectrophotometer at absorbance λ 570nm. The data was normalised to cell viability of the vehicle control (given as 100% viable).

5.2.6 Confocal Microscopy

Monocytes (2×10^5) were cultured directly on Lab-tek Permanox chamber slides and differentiated into MDMs by incubation for 12 d in media containing GM-CSF (2ng/ml). Particulate suspensions of fluticasone propionate or budesonide were added to the chambers and incubated for 1-4 h. Particles not internalised were removed by aspiration and chambers washed repeatedly with D-PBS.

5.2.6.1 Staining of cells

Cells were stained with Cell Tracker Red CMPTX dye (12.5 μ M), followed by fixation with 4% ($^w/v$) paraformaldehyde and nuclear staining with DAPI (2 μ M), as described in section 2.2.13.1.

5.2.6.2 Visualisation

Slides were viewed on a Leica TCS 4D Confocal microscope with images of the three stains overlaid using VolocityTM software. In order to visualise internalisation of the particles, images were taken at sequential slices then remodelled into orthogonal views using VolocityTM software.

5.2.7 Statistical Analyses

Data points, and values in the text and figure legends, represent the mean $\pm$ s.e.m. of 'n' independent donors. Statistical differences were determined using the Wilcoxon signed rank test. Values of $p < 0.05$ were considered significant

5.3 Results

5.3.1 Subject demographics

Eleven patients with COPD (GOLD stage 2-4) were recruited into the study (Table 5.1).

Age (y)	63 +/- 3
M:F	9:2
Pack year history (y)	48 +/- 9
Current smokers	7
FEV₁ (l)	1.7 +/- 0.2
% FEV₁ predicted	57.5 +/- 7
FEV₁/FVC ratio (%)	54 +/- 4

Table 5.1. Subject demographics

Data presented as mean +/- SEM.

5.3.2 Effect of steroids on MDMs phagocytic responses

No significant differences were seen in the ability of MDMs to phagocytose beads in the presence of either fluticasone propionate or budesonide (Fig. 5.1).

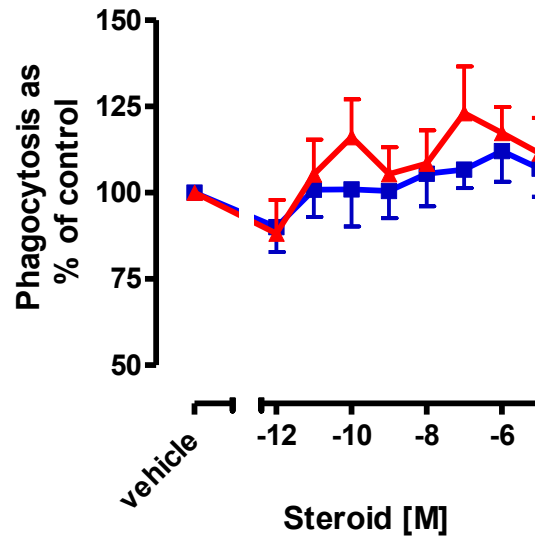


Figure 5.1. Effect of budesonide or fluticasone propionate on phagocytosis of fluorescently labelled beads by MDMs.

MDMs were isolated from COPD subjects, pre-incubated for 1h with steroid (budesonide (▲) or fluticasone propionate (■)) and then incubated with 100µl of 50x10⁶beads/ml containing steroid for 4h. Analysis was then performed using a fluorescent plate reader. Data shown are mean+/- SEM, n=10, where 100% is defined as the phagocytic response of MDMs in the presence of vehicle

Neither fluticasone propionate nor budesonide led to significantly increased phagocytosis of *H. influenzae* in MDMs, nor where there any differences between the two steroids (Fig. 5.2).

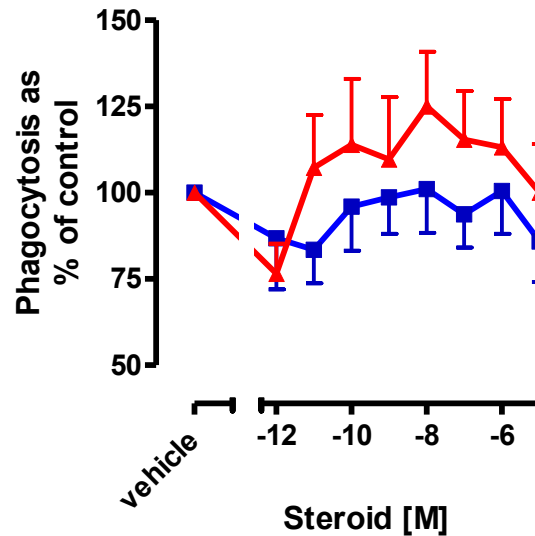


Figure 5.2. Effect of budesonide or fluticasone propionate on phagocytosis of fluorescently labelled *H. influenzae* by MDMs.

MDMs were isolated from COPD subjects, pre-incubated for 1h with steroid (budesonide (▲) or fluticasone propionate (■)) and then incubated with 100µl of *H. influenzae* solution containing steroid for 4h. Analysis was then performed using a fluorescent plate reader. Data shown are mean+/- SEM, n=10, where 100% is defined as the phagocytic response of MDMs in the presence of vehicle.

Neither budesonide nor fluticasone altered phagocytosis of *S. pneumoniae* by MDMs from COPD patients (Fig. 5.3).

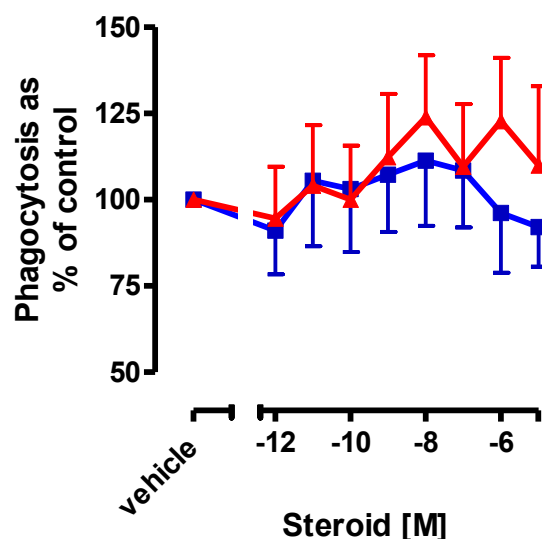


Figure 5.3. Effect of budesonide or fluticasone propionate on phagocytosis of fluorescently labelled *S. pneumoniae* by MDMs.

MDMs were isolated from COPD subjects, pre-incubated for 1h with steroid (budesonide (▲) or fluticasone propionate (■)) and then incubated with 100µl of *S. pneumoniae* solution containing steroid for 4h. Analysis was then performed using a fluorescent plate reader. Data shown are mean $\pm$ SEM, n=10, where 100% is defined as the phagocytic response of MDMs in the presence of vehicle.

5.3.3 Cell viability

It was possible that some of the differences seen in phagocytosis between the steroids and at varying steroid doses may have resulted from altered MDMs viability. Therefore, cell viability assays were performed. However, viability of MDMs was unaffected by increasing concentrations of either steroid when compared to vehicle control (Fig. 5.4 and 5.5), nor was there any difference seen in viability with the different phagocytic prey (Fig. 5.4-5.5).

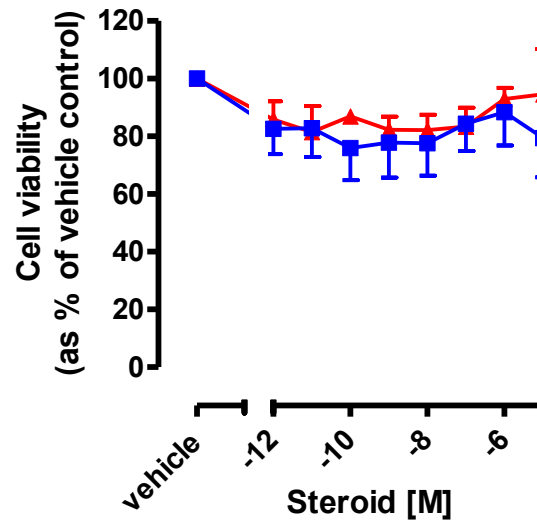


Figure 5.4. Effect of budesonide or fluticasone propionate on viability of MDMs incubated with fluorescently labelled beads.

MDMs were isolated from COPD subjects, pre-incubated for 1h with steroid (budesonide (▲) or fluticasone propionate (■)), then incubated with 100µl of 50×10^6 beads/ml containing steroid for 4h. After phagocytosis assays, MTT was added at 50µl/well, incubated for 30 min, aspirated and DMSO added at 50µl/well. Analysis was performed using a spectrophotometer ($\lambda 570\text{nm}$). Data are mean $\pm$ SEM, n=5.

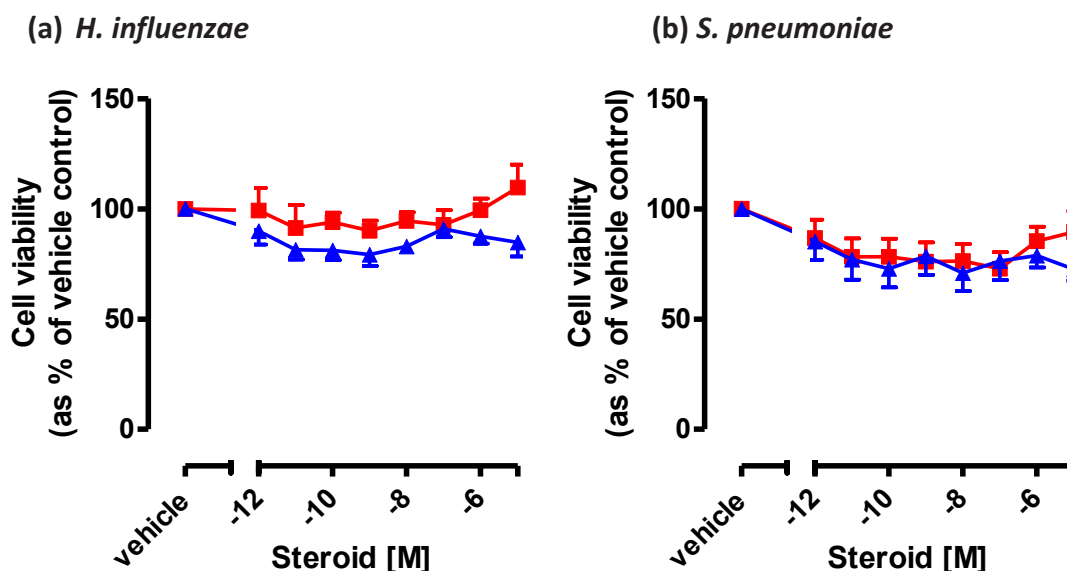


Figure 5.5. Effect of budesonide or fluticasone propionate on viability of MDMs incubated with fluorescently labelled (a) *H. influenzae* and (b) *S. pneumoniae*.

MDMs were isolated from COPD subjects, pre-incubated for 1h with steroid (budesonide (▲) or fluticasone propionate (■)), then incubated with 100µl of bacterial solution containing steroid for 4h. After phagocytosis assays, MTT was added at 50µl/well, incubated for 30 min, aspirated and DMSO added at 50µl/well. Analysis was performed using a spectrophotometer (λ570nm). Data are mean+/- SEM, n=5.

5.3.4 Confocal Microscopy

In order to evaluate whether steroid particles were internalised by MDMs, confocal microscopy was performed. Fluticasone propionate particles were thought to be of a similar size to the polystyrene microspheres used (2µM; personal communication from Dr Anna Miller-Larsson, Astra-Zeneca R&D, Sweden). MDMs incubated with polystyrene beads show internalised particles clearly evident within the cells (Fig. 5.6; evident as black dots). In contrast, MDMs incubated with fluticasone propionate show no clear evidence of internalised particulate matter (Fig. 5.7).

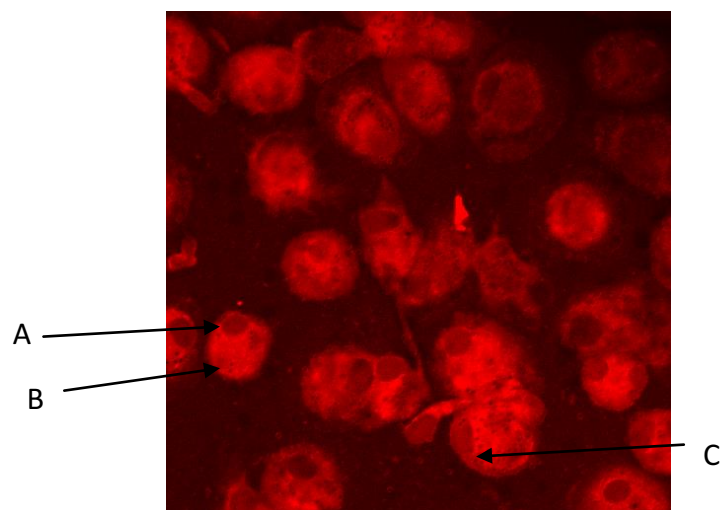


Figure 5.6. MDMs incubated with polystyrene beads for 4 h.

MDMs were incubated polystyrene beads for 4 h, then labelled with Cell Tracker Red CMPTX dye ($12.5\mu\text{M}$) for 45 min, followed by washing, fixation and mounting with Citifluo. Slides were viewed on a Leica TCS 4D Confocal microscope at 40 x magnification.

A = nucleus, B = cytoplasm, C = black dots where no staining of cytoplasm, suggestive of internalised particulate matter.

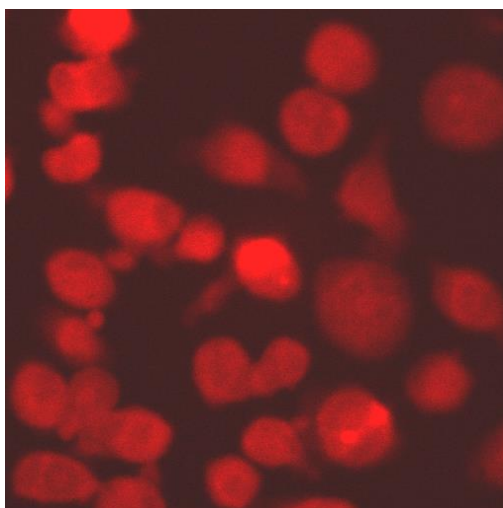


Figure 5.7. MDMs incubated with particulate fluticasone propionate for 4 h.

MDMs were pre-incubated with fluticasone propionate ($10\mu\text{M}$) for 4 h, followed by aspiration of media, then incubation with CellTracker Red CMPTX dye ($12.5\mu\text{M}$) for 45 min. Cells were washed, fixed and mounted with Citifluo. Slides were viewed on a Leica TCS 4D Confocal microscope at 40 x magnification.

5.3.5 Intracellular killing

Whilst steroids did not significantly alter the phagocytic capacity of MDMs for bacteria, it remained possible that these drugs could impair the bacterial killing process. In order to investigate this possibility, MDMs were incubated with live bacteria (either *H. influenzae* or *S. pneumoniae*) and killing assessed. However, incubation of MDMs with either fluticasone propionate or budesonide had no significant effect on bacterial killing (Figs. 5.8 and 5.9). Whilst the data suggests that fluticasone impaired intracellular killing of *H. influenzae* compared to budesonide, with the percentage of live intracellular bacteria increased by ~50% in the presence of fluticasone propionate, this finding is not significant and is likely to be related to the low n number and experimental variability (Fig. 5.8).

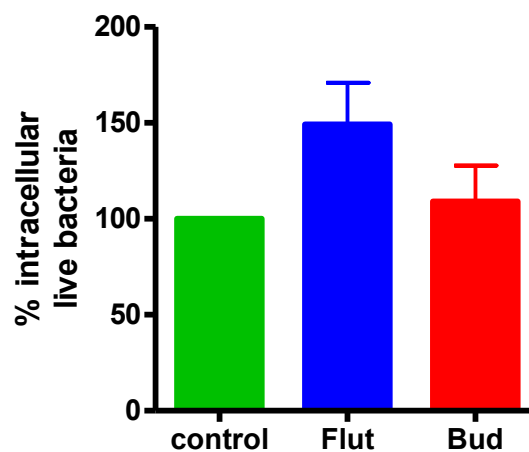


Figure 5.8. Effect of fluticasone propionate or budesonide on intracellular killing ability of MDMs incubated with *H. influenzae*

MDMs were isolated from COPD subjects, pre-incubated for 1h with steroid (budesonide (■) or fluticasone propionate (■)) and then incubated with 11µl of stock bacterial solution. Supernatant was removed, cells were lysed with dH₂O, then this was diluted to 10⁻⁴, plated and colonies counted after overnight incubation. CFU/ml were calculated for cells incubated with and without steroid and % of intracellular live bacteria calculated (where 100% is taken as CFU/ml from control cells). Data shown are mean±/- SEM, n=4.

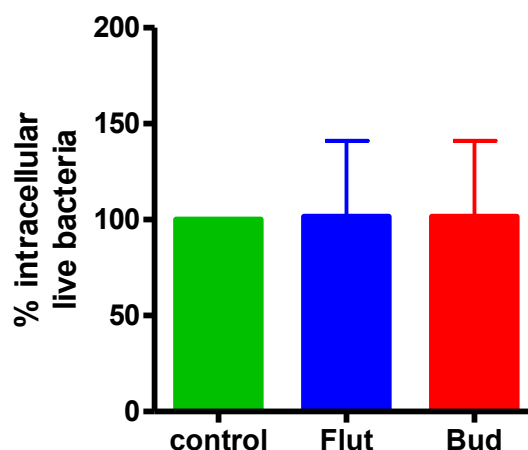


Figure 5.9. Effect of fluticasone propionate or budesonide on intracellular killing ability of MDMs incubated with *S. pneumoniae*

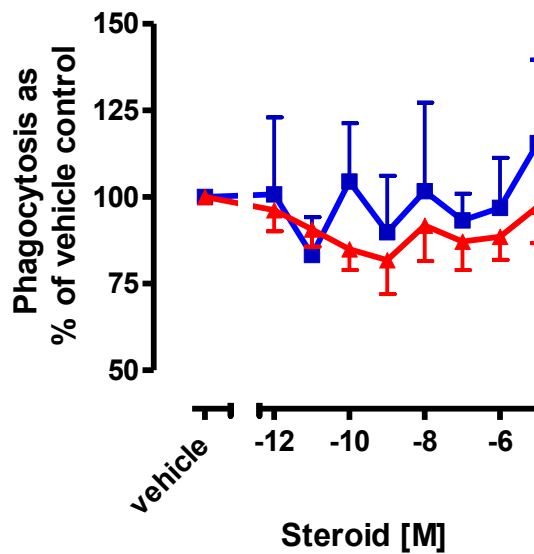
MDMs were isolated from COPD subjects, pre-incubated for 1h with steroid (budesonide (■) or fluticasone propionate (■)) and then incubated with 11μl of stock bacterial solution. Supernatant was removed, cells were lysed with dH₂O, then this was diluted to 10⁻⁴, plated and colonies counted after overnight incubation. CFU/ml were calculated for cells incubated with and without steroid and % of intracellular live bacteria calculated (where 100% is taken as CFU/ml from control cells). Data shown are mean± SEM, n=4.

5.3.6 Effect of steroids on neutrophil phagocytic responses

Since steroids had little effect on MDMs phagocytosis or bacterial killing, it was unlikely that steroids were altering macrophage innate functions. Therefore, the effect of both steroids on neutrophil phagocytosis was evaluated. Three subjects were excluded either due to very high neutrophil counts suggestive of acute infection or due to insufficient cells for FACS analysis. Data are presented as the percentage phagocytosis compared to vehicle control (*i.e.* the percentage of cells ingesting phagocytic prey) and the median fluorescent intensity (MFI) compared to vehicle control (*i.e.* the amount of phagocytic prey taken up by the neutrophils) for each phagocytic assay (see section 2.2.7).

Neither budesonide nor fluticasone propionate altered uptake of beads by neutrophils from COPD patients (Fig. 5.10), indicating that neither steroid alters the mechanism of phagocytosis in neutrophils.

(a) Percentage phagocytosis



(b) MFI

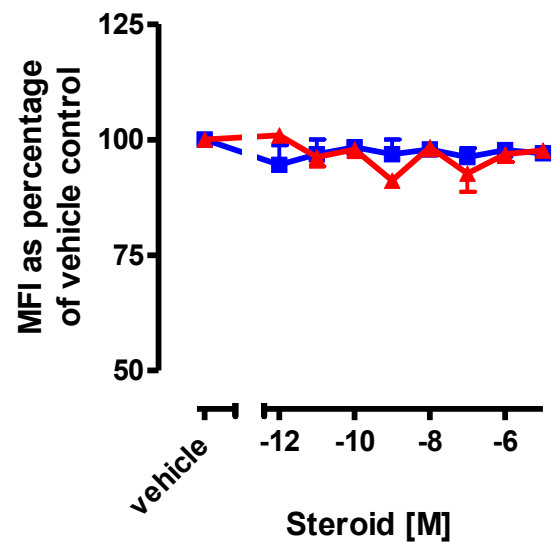
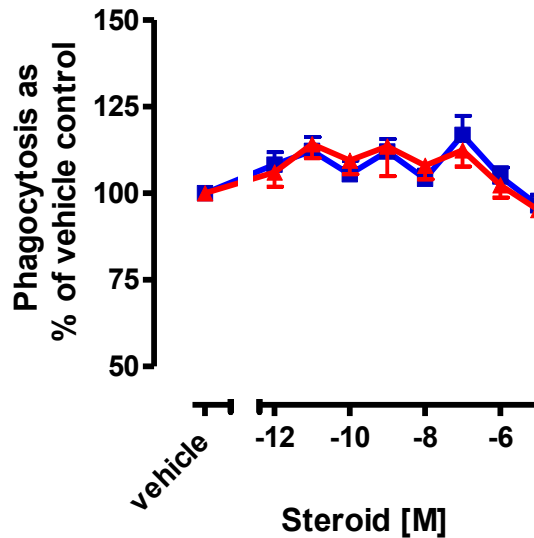


Figure 5.10. Effect of budesonide or fluticasone propionate on phagocytosis of fluorescently labelled beads by neutrophils.

Neutrophils were isolated from COPD subjects, pre-incubated for 1h with steroid (budesonide (▲) or fluticasone propionate (■)) and then incubated with 900μl of 10×10^6 beads/ml containing steroid for 15 min. Neutrophils were washed and fixed. Analysis was performed using flow cytometry. Data shown are mean $\pm$ SEM, n=7.

When *H. influenzae* were used as phagocytic prey, the presence of either steroid again had no effect on the phagocytic ability of neutrophils (Fig. 5.11a). However, both budesonide and fluticasone propionate increased the amount of *H. influenzae* ingested by the neutrophils at all concentrations except the highest dose used (Fig. 5.11b), with this increase reaching statistical significance at budesonide concentrations of 100pM, 10nM and 1μM and 1μM of fluticasone propionate ($p < 0.05$). Whilst both budesonide and fluticasone propionate led to increased uptake of *H. influenzae* compared to control, no differences were seen between the two steroids (Fig. 11b).

(a) Percentage phagocytosis



(b) MFI

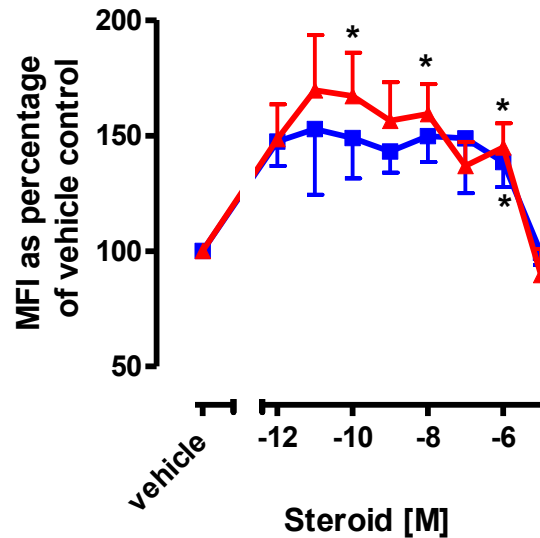
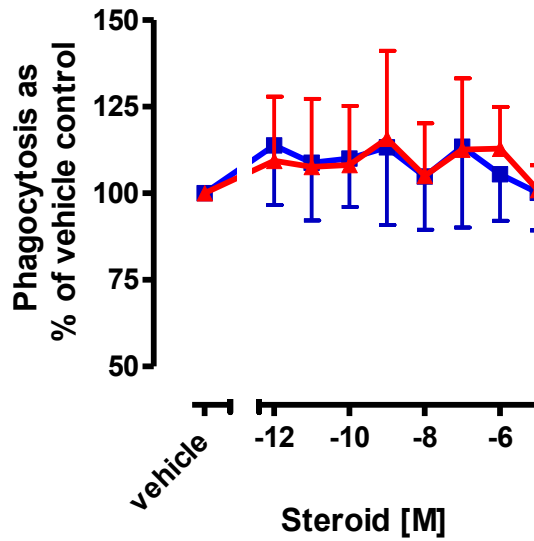


Figure 5.11. Effect of budesonide or fluticasone propionate on phagocytosis of fluorescently labelled *H. influenzae* by neutrophils.

Neutrophils were isolated from COPD subjects, pre-incubated for 1h with steroid (budesonide (▲) or fluticasone propionate (■)), then incubated with 900µl of *H. influenzae* solution containing steroid for 15 min. Cells were washed, fixed and analysed by flow cytometry. Data shown are mean+/- SEM, n=7 (where * represents $p < 0.05$).

In contrast to the results with *H. influenzae*, when neutrophils were incubated with *S. pneumoniae*, neither budesonide nor fluticasone propionate had any effects on either the phagocytic ability of neutrophils (Fig. 5.12a), nor the amount of bacteria ingested by the neutrophils (Fig. 5.12b).

(a) Percentage phagocytosis



(b) MFI

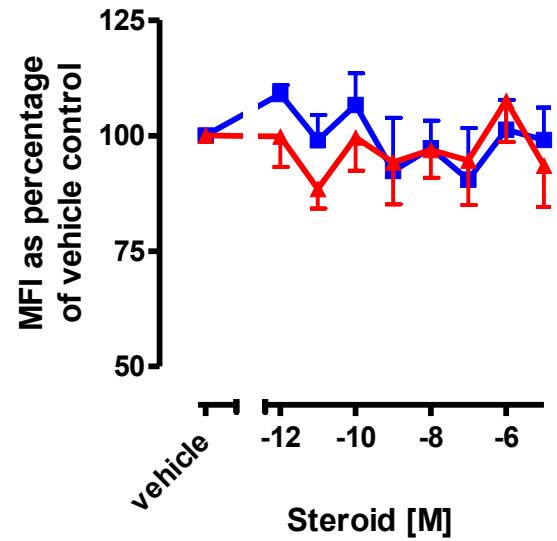


Figure 5.12. Effect of budesonide or fluticasone propionate on phagocytosis of fluorescently labelled *S. pneumoniae* by neutrophils.

Neutrophils were isolated from COPD subjects, pre-incubated for 1h with steroid (budesonide (▲) or fluticasone propionate (■)) and then incubated with 900µl of *S. pneumoniae* solution containing steroid for 15 min. Neutrophils were washed and fixed. Analysis was performed using flow cytometry. Data shown are mean+/- SEM, n=7.

5.4 Discussion

The hypothesis under investigation was that clearance of particulate fluticasone by phagocytic cells of the innate immune system results in impairment of phagocytosis/killing of bacteria, in turn resulting in impaired/delayed bacterial clearance and increased risk of pneumonia. This may be due to accumulation of particles within the phagolysosome. This hypothesis was developed from recent evidence suggesting that rates of pneumonia were higher in those COPD patients taking fluticasone propionate compared with budesonide (Crim *et al.*, 2009; Sin *et al.*, 2009; Kardos *et al.*, 2007; Wedzicha *et al.*, 2008).

Analysis of the phagocytic capacity of MDMs showed that budesonide marginally increased phagocytosis of beads and uptake of *H. influenzae* compared to control, as well as increasing the uptake of *H. influenzae* by neutrophils. This improvement in phagocytosis of *H. influenzae* was seen at concentrations of budesonide that would be expected in the human airway during regular treatment with budesonide (Van den Bosch *et al.*, 1993). In contrast, fluticasone propionate only showed increased uptake of *H. influenzae* by neutrophils, with MDMs showing no alterations in phagocytosis with any phagocytic prey.

Previously, budesonide has been shown to improve phagocytosis of *H. influenzae* and *S. pneumoniae* in MDMs (Taylor *et al.*, 2010), however, it has also been shown to reduce phagocytosis of *E. coli* by alveolar macrophages (Zetterlund *et al.*, 1998). In both of the reported studies, solubilised budesonide was used, whereas in the present study both steroids were solubilised in Tween 80 and are assumed to remain particulate. Alternatively, the differential effects on phagocytic ability seen with budesonide may be related to the different incubation times, for which cells are exposed to phagocytic prey, used in each of the studies. Zetterlund *et al* incubated macrophages with bacteria for only 10 min, in comparison to the 1 h time point used by Taylor *et al* and the 4 h time point used in this study. There is little in the literature on fluticasone and its effects on phagocytosis.

A mechanism by which steroids may improve phagocytosis may be related to alterations in receptor expression. Dexamethasone has previously been shown to improve phagocytosis of latex beads and *E. coli* by MDMs, with an associated increase in expression of scavenger receptors (MARCO, mannose receptors and CD163) (Gratchev *et al.*, 2005). Similarly, in monocytes and MDMs, increased efferocytosis in the presence of dexamethasone has been associated with increased expression of CD16 and CD163 (Giles *et al.*, 2001). Furthermore, dexamethasone treated cells showed a reduction in paxillin present in podosomes, associated with reduced tyrosine phosphorylation, and a marked increase in levels of Rac (Giles *et al.*, 2001). Rac proteins are critical regulators of phagocytic function, involved in actin assembly, pseudopod extension and generation of reactive oxygen species (ROS) for pathogen elimination, and, in addition, are important in neutrophil migration (Costa *et al.*, 2010). Therefore, it could be that different steroids have differential effects on receptor expression or on cytoskeletal rearrangement, in turn leading to differential bacterial clearance within the lung.

In contrast to the phagocytosis data, the intracellular killing data is suggestive of fluticasone impairing the intracellular killing of *H. influenzae* compared to budesonide however, this alteration in intracellular killing may simply be related to the low numbers of this experiment that were performed, with wide variability and therefore it is hard to draw firm conclusions from this data. One could postulate that if fluticasone were to impair intracellular killing this may in turn lead to cell necrosis with release viable bacteria back into the lower airway, however, the relevance of this finding to the increased risk of pneumonia would have increased weight if fluticasone propionate had shown similar impairment of MDMs killing of *S. pneumoniae*, as this is the bacteria more commonly isolated in pneumonia.

More recently, two clinical trials have been published addressing the question of increased pneumonia risk in those with COPD taking ICS. Singanayagam *et al* conducted a prospective observational study on COPD patients hospitalized with a primary diagnosis of pneumonia (Singanayagam *et al.*, 2011). They found approximately 78% of patients were taking ICS, with 67.9% of those taking ICS containing fluticasone and 21% containing budesonide (Singanayagam *et al.*, 2011).

No differences were seen in the severity of pneumonia between the ICS and non-ICS arms, nor were there any differences in 30 day or 6 month mortality or length of hospital stay (Singanayagam *et al.*, 2011). These findings were confirmed by Chen *et al* in a large retrospective cohort study in the USA, which showed a reduction in mortality from pneumonia at 30 and 90 days in those COPD patients taking ICS compared to those that were not (Chen *et al.*, 2011). These studies examined pneumonia as the primary diagnosis and therefore did not consider the incidence of developing pneumonia in the ICS compared to non-ICS arms. In addition, these studies did not attempt to investigate the impact of different ICS on the outcome of pneumonia. However, these studies suggest that, if ICS do increase the risk of developing pneumonia, there is no associated increase in mortality or morbidity.

It is difficult, however, to explain the discrepancy between fluticasone propionate appearing to increase the risk of pneumonia whilst both fluticasone propionate and budesonide have been shown to reduce rates of exacerbation in COPD, up to 50% of which are associated with bacteria. Analysis of the INSPIRE study again showed that treatment with the combination of salmeterol and fluticasone propionate was associated with an increased risk of pneumonia compared to use of tiotropium alone (Calverley *et al.*, 2011). However, no differences were seen in the duration of the pneumonia or in mortality from pneumonia (Calverley *et al.*, 2011). Interestingly, one half of the pneumonias in patients on the combination inhaler were associated with an ongoing or unresolved exacerbation (Calverley *et al.*, 2011). Therefore, it would appear that there may be an effect of ICS on either the host immune system or the pathogen which delays or prevents resolution of infection. This study would suggest that the mechanism by which fluticasone causes increased rates of pneumonia is likely to be more complicated than simply impairment of host defences by altered phagocytic ability in cells of the innate immune system.

5.4.1 Limitations

Due to the small sample size used in this study, no significant differences were seen between steroids due to variability in the data. This sample size would have to be increased to assess whether there were any true differences between steroids. A further limitation was the use of particulate steroids in comparison to the literature

using solubilised steroids, therefore comparison should be made between these two forms of both steroids to assess whether further differences can be seen.

Chapter 6

MECHANISM UNDERLYING DEFECTIVE PHAGOCYTOSIS IN SMOKER AND COPD MDMs AND NEUTROPHILS

6.1 Introduction

Phagocytosis is a complex series of events by which particles are internalised within the cell. Essentially, cell surface receptors recognise and bind to a particle, in turn generating signals which induce actin polymerisation under the membrane, at the point of contact (Aderem, 2003). Actin rich membrane extensions reach out to enfold the particle, fusing and forming the phagosome which is pulled into the centre of the cell (Aderem, 2003). The phagosome matures by a series of fusion events to form the phagolysosome which kills the pathogen (Aderem, 2003). Microtubules are involved in membrane trafficking during phagocytosis, and have important roles in the early stages of phagosome formation and in the migration of the phagosome from cell edge to the centre, along the microtubule network (Niedergang and Chavrier, 2004).

Defects in phagocytosis may arise due to mechanistic changes at any point along the pathway. Alterations in receptor expression, of both the mannose receptor and MARCO, have been shown to be related to reduced phagocytosis of bacteria by macrophages (Hodge *et al.*, 2008; Baqir *et al.*, 2008; Sun and Metzger, 2008). Similarly, alterations in PI3K signalling have also been shown to be associated with reduced phagocytosis of NTHI in macrophages (Marti-Llitas *et al.*, 2009). The data presented in the previous chapters, along with previous work in this laboratory, has not found any differences in receptor expression in phagocytic cells from different subject groups (Taylor *et al.*, 2010), nor any differential sensitivity to cytochalasin (an actin microfilament inhibitor) in cells from different subject groups. However, previous work in the laboratory has suggested increased sensitivity of COPD cells to colchicine, a microtubule-destabilising agent (Finney-Hayward *et al.*, 2005).

Microtubules are formed from α - and β -tubulin heterodimers (see Figure 6.1). Microtubules are dynamic organelles that constantly polymerise and depolymerise depending on the cellular requirements (Peachman *et al.*, 2004). Depolymerised (or dynamic) microtubules are important in cell motility and cell mitosis, whilst polymerised or stable microtubules are required for phagocytosis. Colchicine acts by binding to tubulin dimers and preventing polymerisation (Peachman *et al.*, 2004). On the other hand, microtubules can be stabilised, using agents such as taxol, which reduces the dissociation of tubulin dimers from the microtubule polymer (Peachman *et al.*

al., 2004). Taxol has been shown to inhibit mitosis with no associated effect on phagocytosis (Peachman *et al.*, 2004).

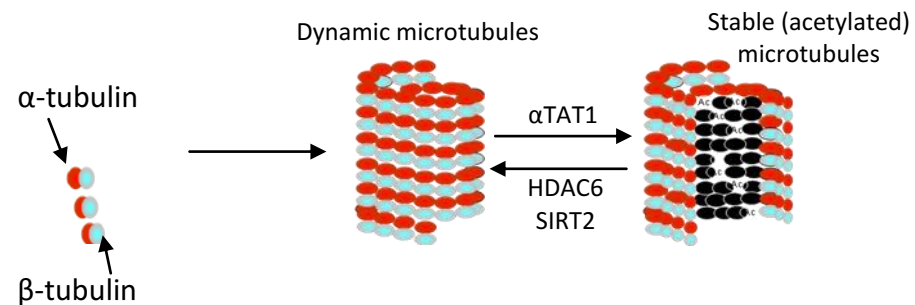


Figure 6.1. Diagram of microtubule polymerisation and depolymerisation.

Microtubules are formed from α - and β -tubulin heterodimers. Microtubules can exist in the dynamic state or in the polymerised (stable) state. Acetylation of polymerised microtubules is carried out by α -tubulin acetyl-transferase, whilst deacetylation is carried out by HDAC6 and SIRT2.

Reversible acetylation of microtubules has been implicated in the regulation of microtubule stability and function (Piperno *et al.*, 1987). Deacetylation of microtubules is dependent on the histone deacetylases, HDAC6 and SIRT2. HDAC6 is located exclusively in the cytoplasm and associates with microtubules (Hubbert *et al.*, 2002), and has been shown, *in vitro*, to potently deacetylate α -tubulin in assembled microtubules, with overexpression promoting chemotactic cell movement (Hubbert *et al.*, 2002). SIRT2 (sirtuin type 2) colocalizes with microtubules and HDAC6 (North *et al.*, 2003). It deacetylates lysine K-40 of α -tubulin both *in vitro* and *in vivo* (North *et al.*, 2003), whilst knockdown of either SIRT2 or HDAC6, using siRNA in 293T cells, caused hyperacetylation of α -tubulin similarly (North *et al.*, 2003).

Recently discovered is the acetyl transferase for α -tubulin (Akella *et al.*, 2010; Shida *et al.*, 2010). Akella *et al* have shown that, *in vitro*, MEC-17, a protein required for the function of touch receptor neurons in *C. elegans*, exclusively acetylates K-40 of α -tubulin (Akella *et al.*, 2010). Disruption of MEC-17 in *Tetrahymena* caused microtubule lability whilst overexpression led to greatly increased acetylation of microtubules (Akella *et al.*, 2010). In zebrafish, a model used to assess function in vertebrates, disruption of MEC-17 produced phenotypes consistent with neuromuscular defects

(Akella *et al.*, 2010). Shida *et al* also showed that α TAT1 (C6orf134) specifically acetylates K40 of α -tubulin, with a preference for polymerised microtubules over monomeric tubulin (Shida *et al.*, 2010), with knockdown of α TAT1 in mammalian cells leading to an 80% reduction in acetylation at K40 (Shida *et al.*, 2010).

The aim of this chapter will be to investigate whether alterations in acetylated tubulin in MDMs and neutrophils may be related to the phagocytic defect seen in cells from COPD patients. Using Epopthilone B, a microtubule stabilising agent derived from the mycobacterium *Sorangium cellulosum*, currently involved in oncology trials, the effects of stabilising microtubules, on both phagocytosis and levels of acetylated tubulin, will be examined. Inhibition of both the acetyl transferase and the deacetylases of microtubules will also be examined in terms of the effects on phagocytosis and on levels of acetylated tubulin.

6.2 Methods

6.2.1 Subject selection

Healthy, smoking and COPD subjects were recruited as described in section 2.2.1.

6.2.2 Blood separation

PBMC and neutrophils were isolated from whole blood using discontinuous Percoll gradients as described in section 2.2.3. Monocytes were isolated from PBMC using adherence and cultured for 12 d in the presence of GM-CSF (2ng/ml) to derive monocyte-derived macrophages (MDMs).

6.2.3 Phagocytosis Assays

Phagocytosis assays were carried out on using fluorescently labelled beads or bacteria incubated with MDMs for 4 h and neutrophils for 15 min, as described in section 2.2.7.

6.2.4 Western Blot

Western blot was carried out as described in section 2.2.9. Primary monoclonal antibodies to the protein of interest were used in the dilutions shown below;

Anti-acetylated tubulin	1:1000
Anti-actin	1:10000
Anti- α -tubulin	1:1000
Anti-HDAC6	1:1000

Table 6.1. Dilutions of primary monoclonal anti-bodies used in Western Blot

6.2.5 Immunoprecipitation

Immunoprecipitation of HDAC6 was carried out as described in section 2.2.15. Polyclonal anti-HDAC6 antibody was used at 1:20 dilution.

6.2.6 HDAC activity assay

The Fluor-de-lys activity assay was carried out as per the manufacturers' protocol, as described in section 2.2.15.

6.2.7 Transfection

Transfection with the appropriate SiRNA was carried out as described in section 2.2.13. In each well of a 24 well plate, 3µg of SiRNA was used, whilst 1µg of SiRNA was used per well in a 96 well plate.

6.2.8 rt-PCR

Rt-PCR was carried out as described in section 2.2.14.

6.2.9 Confocal microscopy

After phagocytosis assays were complete, MDMs were stained with Tubulin Tracker (Invitrogen, UK), (250nM), diluted in RPMI media, for 30 min at 37°C, then fixed with 4% paraformaldehyde and imaged on a Leica SP2 upright confocal microscope as described in section 2.2.11.2.

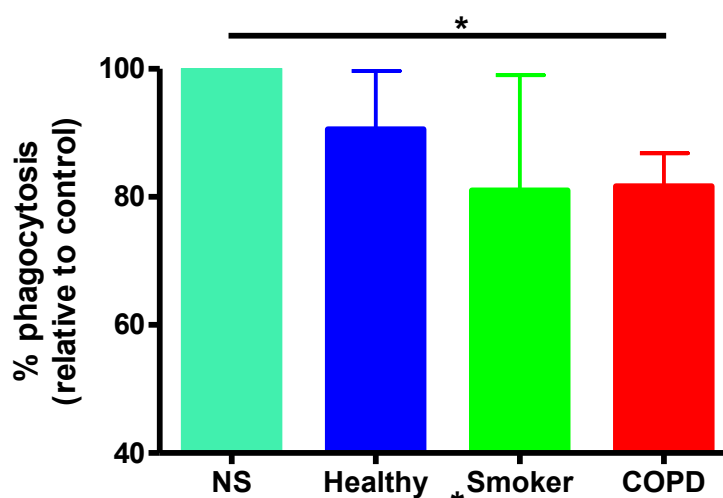
6.3 Results

The previous chapters have shown that both MDMs and neutrophils from smokers and COPD patients show defective uptake of bacteria when compared to healthy controls. All cells have been shown to be capable of phagocytosis, given their equivalent ability to phagocytose beads, yet cells from smokers and COPD subjects show reduced uptake of bacteria.

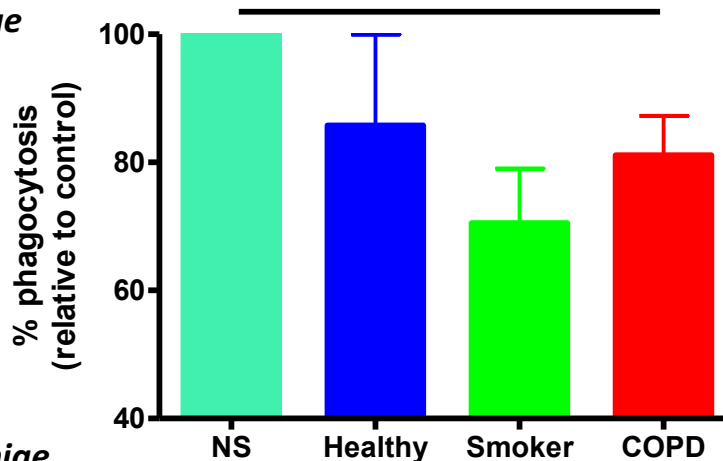
6.3.1 Effect of microtubule disruptors on phagocytosis by MDMs from different subject groups

Previously, phagocytosis of *E. coli* had been shown to be significantly reduced in colchicine exposed MDMs from COPD patients, with no effect on phagocytosis by MDMs from either healthy or smoker volunteers (Finney-Hayward *et al.*, 2005). This suggests an enhanced susceptibility to microtubule disruptors in cells from COPD patients. Therefore, this potential susceptibility was investigated further. In the first instance, phagocytosis experiments were repeated with *H. influenzae* and *S. pneumoniae* in the presence of nocodazole, an alternative microtubule disruptor. The concentration of nocodazole selected (10 μ M) was equivalent to that used previously in the laboratory and by others in the investigation of phagocytosis (Cannon and Swanson, 1992). Phagocytosis of beads, *H. influenzae* and *S. pneumoniae* was significantly reduced in COPD MDMs, compared to healthy and smoker MDMs (Fig. 6.2).

(a) Beads



(b) *H. influenzae*



(c) *S. pneumoniae*

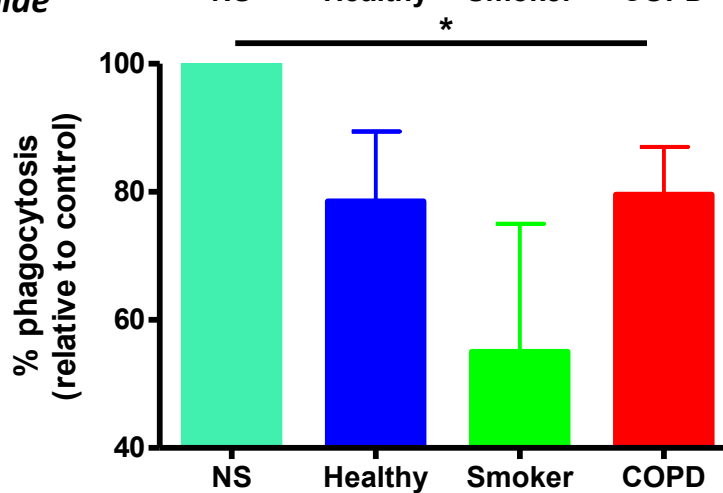


Figure 6.2. Effects of nocodazole on phagocytosis by MDMs from different subject groups

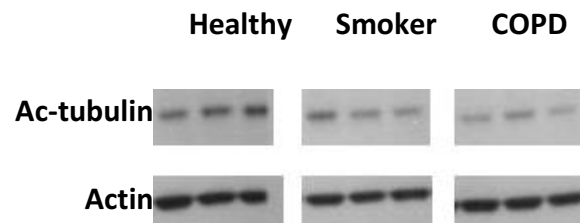
MDMs from healthy controls (■), healthy smokers (■) and COPD patients (■) were pre-treated for 1 h with nocodazole (10μM), followed by 4 h incubation with (a) beads, (b) *H. influenzae* (HI) or (c) *S. pneumoniae* (SP). Phagocytosis was analysed using

fluorimetry. Data are presented as percentage phagocytosis relative to control (NS), mean $\pm$ SEM, healthy n=5, smoker n=3, COPD n=10, where * represents $p < 0.05$.

6.3.2. Levels of acetylated tubulin in cells from different subject groups

Having shown that, in COPD MDMs, phagocytosis appears to be reduced in the presence of microtubule disruptors, the next step was to investigate whether levels of stable (or acetylated) microtubules were altered in COPD MDMs. Using Western blotting, MDMs were lysed and levels of acetylated tubulin analysed (Fig. 6.3a). COPD MDMs had significantly less acetylated tubulin in comparison to healthy MDMs (Fig. 6.3b). The reduction in levels of acetylated tubulin in smoker MDMs compared to healthy controls did not reach statistical significance, likely due to the lower 'n' number used (Fig. 6.3b).

(a)



(b)

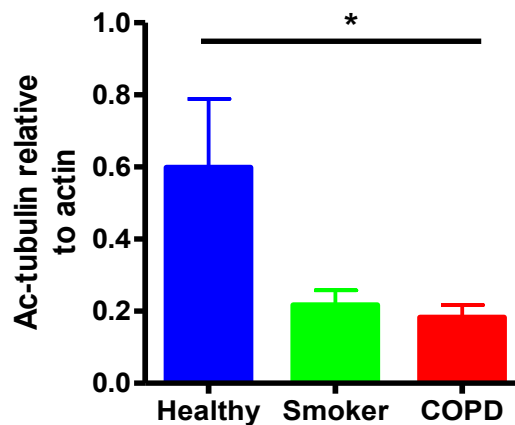
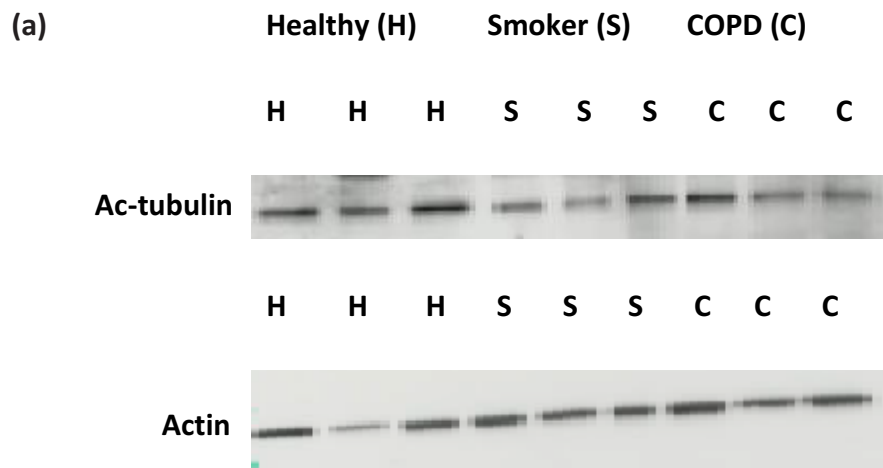


Figure 6.3a and b. Comparison of levels of acetylated tubulin in MDMs from different subject groups

Monocytes from healthy controls (■), healthy smokers (■) and COPD patients (■) were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. Cells were lysed in RIPA buffer and proteins separated by Western blot, followed by immunoblotting with primary

anti-acetylated tubulin antibody. Blots were stripped and re-probed with primary anti-actin antibody as a loading control. Data are presented as mean $\pm$ SEM, n=5-7 for all groups.

To assess whether neutrophils displayed similar discrepancies in levels of acetylated tubulin, acetylated tubulin was measured in neutrophils from different subject groups. There were no differences seen in levels of acetylated tubulin in neutrophils from any subject group (Fig. 6.4).



(b)

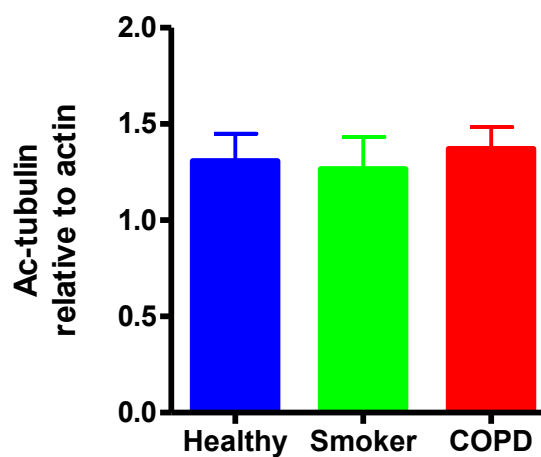


Figure 6.4a and b. Comparison of levels of acetylated tubulin in neutrophils from different subject groups

Neutrophils from healthy controls (■), healthy smokers (■) and COPD patients (■) were snap frozen, then lysed in 2xSDS buffer and proteins separated by Western blot, followed by immunoblotting with primary anti-acetylated tubulin antibody. Blots were stripped and re-probed with primary actin antibody as a loading control. Western blot

(a) shows n=3 for each group. (b) Data are presented as mean $\pm$ SEM, n=6 for all groups.

6.3.3. Staining of microtubules in MDMs with tubulin tracker™

Tubulin tracker™ (Molecular Probes, Invitrogen, UK) stains polymerised microtubules within cells, and therefore was used to visualise the arrangement of microtubules within MDMs. Resting microtubules reside in the MTOC (microtubule organising centre) which is located in the peri-nuclear region of the cell (Fig. 6.5a). In contrast, MDMs exposed to prey, in this case *H. influenzae*, display diffuse staining of the cells as the microtubule network spreads out in the cytoplasm towards the cell periphery during the process of phagocytosis (fig. 6.5b).

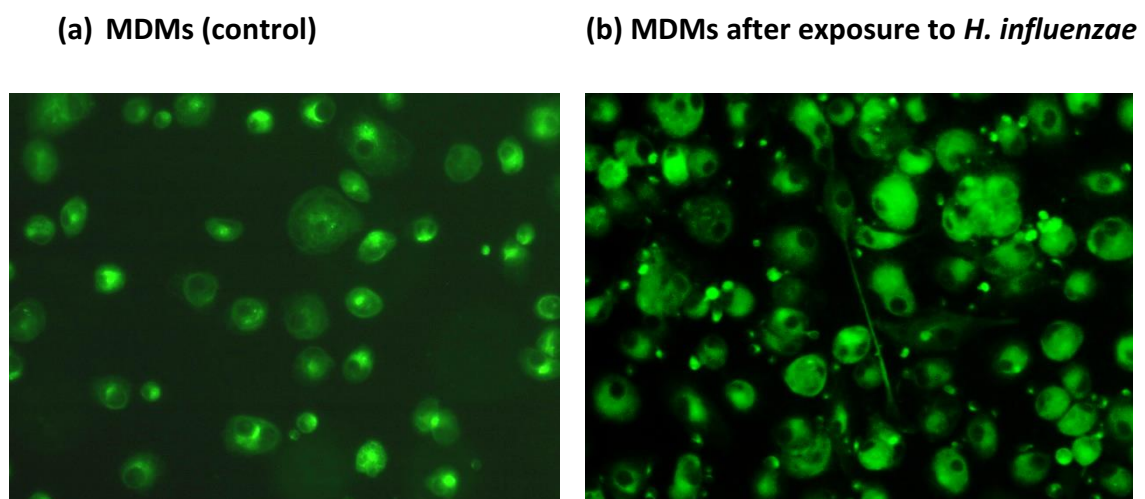


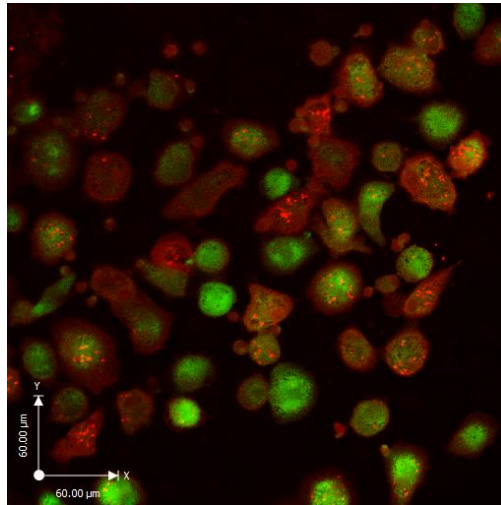
Figure 6.5. MDMs stained with tubulin tracker showing the cellular location of polymerised microtubules.

MDMs exposed to *H. influenzae* for 4 h and MDMs (unstimulated) were stained with Tubulin Tracker (250nM) in RPMI media for 30 min at 37°C, washed with D-PBS and viewed on a fluorescent inverted microscope with a x40 objective lens.

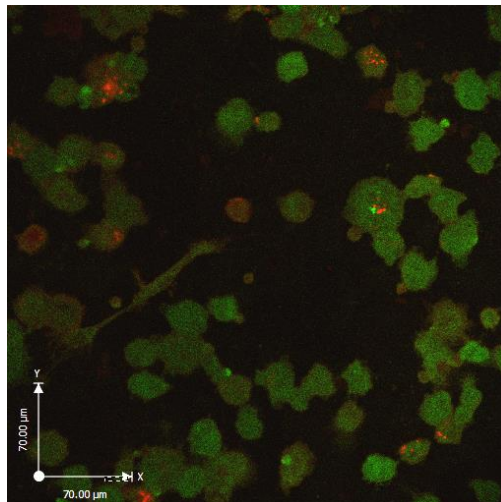
Repeat experiments were then performed with pre-incubation of MDMs with either nocodazole (microtubule disruptor) or Etoposide B (microtubule stabiliser) in the presence of labelled *S. pneumoniae* to assess whether alterations in polymerised microtubules could be examined (Fig. 6.6a-c). In the presence of nocodazole there appear to be increased MTOC present in the cells, whilst in the presence of Etoposide

B there appears to be increased phagocytosis and increased microtubular staining, compared to control MDMs (Fig. 6.6a-c).

(a) MDMs



(b) MDMs + Nocodazole



(c) MDMs + Epopthilone B

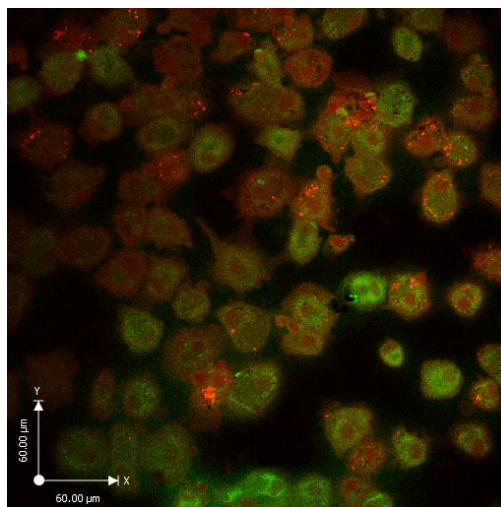


Figure 6.6. MDMs stained with tubulin tracker after phagocytosis of pHrodo™ labelled *S. pneumoniae* in the presence of nocodazole or Epopthilone B

MDMs (unstimulated, pre-incubated for 1 h with Nocodazole (10 μ M) or Etoposide B (10nM)) were exposed to pHrodo labelled (red) *S. pneumoniae* for 4h, then stained with Tubulin Tracker (250nM) (green) and viewed on a Leica upright Sp2 confocal microscope with a x40 objective lens.

6.3.4. Effects of a microtubule stabiliser (Etoposide B) on phagocytosis by MDMs

Given that COPD MDMs had reduced phagocytosis in the presence of nocodazole, suggesting increased sensitivity to microtubule disruption, the effects of Etoposide B, a microtubule stabiliser, were investigated. Cells were pre-incubated with Etoposide B for 1 h prior to phagocytosis assays. In both COPD and smoker MDMs, Etoposide B had little effect on the phagocytosis of beads (Fig. 6.7a and b). However, in COPD MDMs, Etoposide B significantly improved phagocytosis of *H. influenzae* (by ~20% at 10nM), with an improvement of ~10% in phagocytosis of *S. pneumoniae* (Fig. 6.7a). In smoker MDMs, Etoposide B again showed markedly improved bacterial phagocytosis (by ~30-40%) though this did not reach statistical significance (Fig. 6.7b). In contrast, in healthy MDMs, Etoposide B had no effect on the phagocytosis of any prey (Fig. 6.7c)

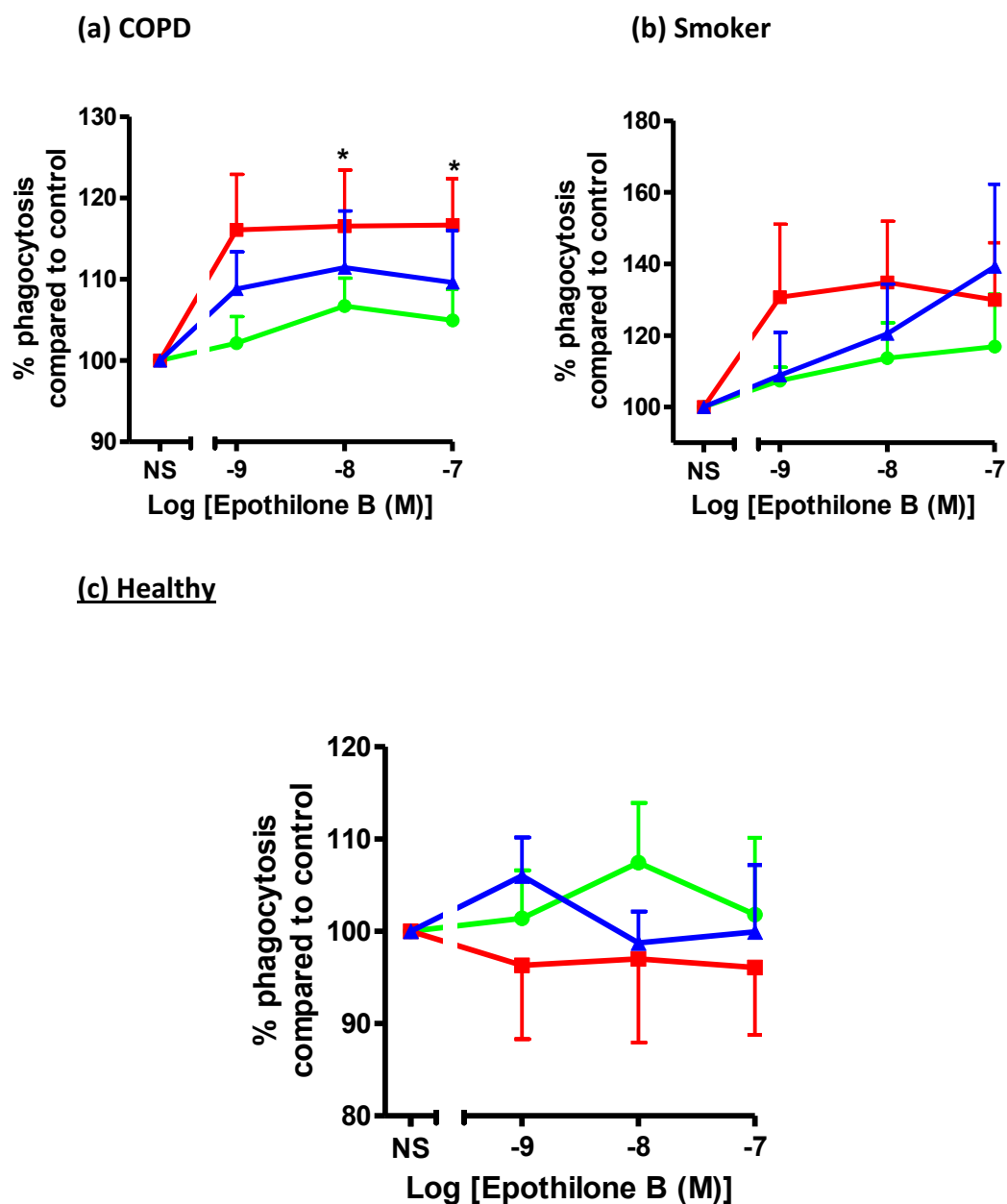


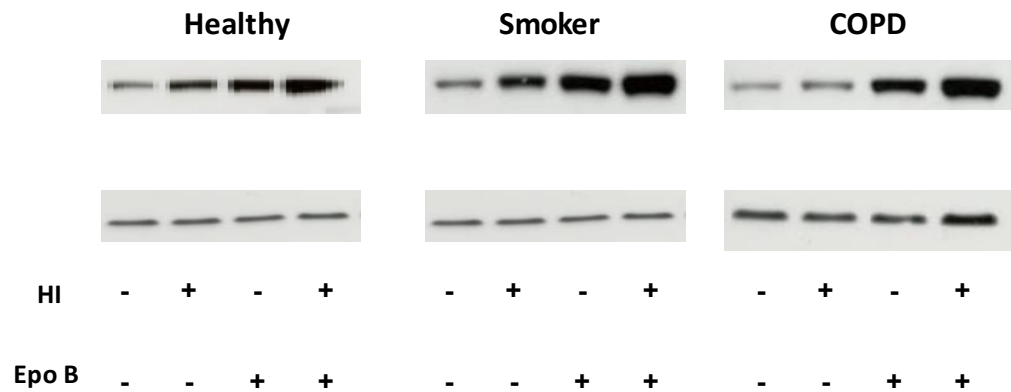
Figure 6.7(a-c). Comparison of phagocytosis by MDMs from different subject groups after incubation with Epothilone B

Monocytes were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. MDMs were pre-treated for 1 h with Epothilone B at varying concentrations, followed by 4 h incubation with beads (●), *H. influenzae* (■) or *S. pneumoniae* (▲). Phagocytosis was analysed using fluorimetry. Data are presented as mean $\pm$ SEM, COPD n=10, Smoker n=6, Healthy n=4.

6.3.5. Effect of Epothilone B on levels of acetylated tubulin within MDMs

Having shown that stabilising microtubules appeared to lead to small improvements in phagocytosis, the next step was to assess whether pre-incubation with Epothilone B would alter acetylated tubulin within the cells. Epothilone B was used at a concentration of 10nM in these experiments, as this concentration was associated with significant increases in bacterial phagocytosis (Fig. 6.7). Experiments were performed and showed that incubation with *H. influenzae* alone leads to an increase in levels of acetylated tubulin within the cell (Fig. 6.8a and b). This effect of *H. influenzae* was increased in cells from smokers and healthy subjects compared with MDMs from COPD patients (Fig. 6.8a and b), suggesting that acetylation of tubulin in response to phagocytic prey is reduced in COPD macrophages. In contrast, incubation with Epothilone B prior to the addition of HI leads to a significant increase in levels of acetylated tubulin in MDMs from all subject groups (Fig. 6.8a and b).

(a)



(b)

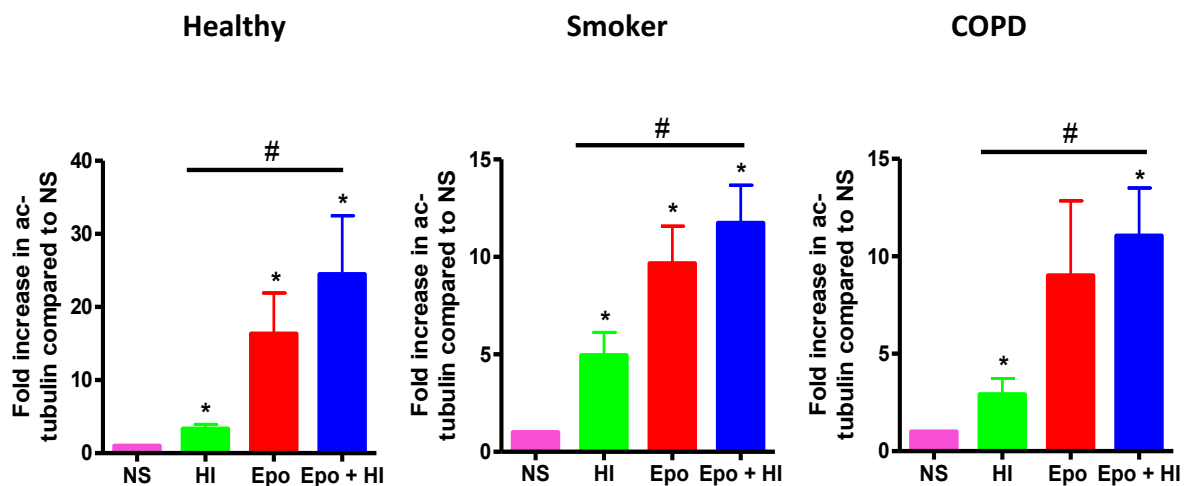


Figure 6.8a and b. Comparison of levels of acetylated tubulin in MDMs from different subject groups after incubation with Epothilone B vs. prey alone

MDMs were pre-treated for 1 h with Epothilone B (10nM), followed by 4 h incubation with *H. influenzae*. Cells were lysed in RIPA buffer and proteins separated by Western blot, followed by immunoblotting with primary anti-acetylated tubulin antibody. NS (non-stimulated), HI (cells exposed to *H. influenzae*), Epo (Epothilone B pre-treatment), Epo+HI (Epothilone B pre-treatment and exposure to prey). Data are presented as mean $\pm$ SEM, n=6 for all groups. *represents p<0.05 compared to NS, # represents p<0.05 HI vs. Epothilone B plus HI.

6.3.6. Effects of Epothilone B on neutrophil phagocytosis

Exposure of MDMs to Epothilone B led to improvements in phagocytosis of bacteria, therefore, whether similar effects would be seen with neutrophils was determined.

Pre-incubation of neutrophils with Epothilone B had no effect on phagocytosis, with no improvements seen in phagocytosis of either *H. influenzae* (Fig. 6.9a) or *S. pneumoniae* (Fig. 6.9b) in COPD or smoker neutrophils.

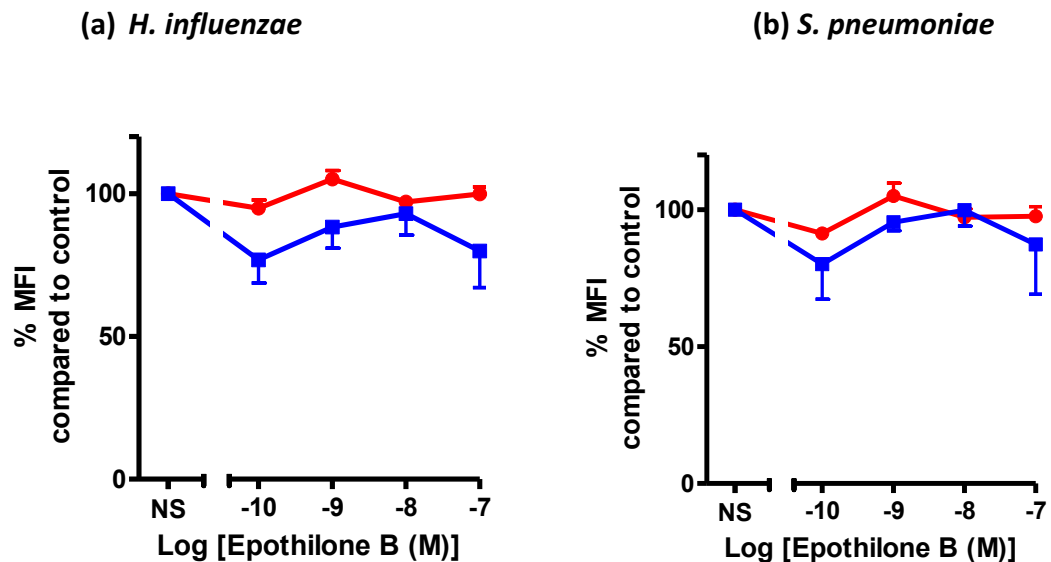


Figure 6.9. Comparison of phagocytosis by neutrophils from COPD and smokers after incubation with Epothilone B

Neutrophils were isolated from whole blood of smoker (■) and COPD (●) subjects, pre-treated for 1 h with Epothilone B at varying concentrations, followed by 15 min incubation with *H. influenzae* (a) or *S. pneumoniae* (b). Phagocytosis was analysed by flow cytometry. Data are presented as mean ± SEM, COPD n=6-8, Smoker n=4.

This data suggests that while microtubule stability plays a part in the defective phagocytosis seen in MDMs, there may be an alternative defect in neutrophils. Therefore, further investigation of the microtubule abnormality was confined to MDMs work.

6.3.7. HDAC6 activity within MDMs derived from different subject groups

As microtubules switch between dynamic and stable states their acetylation status also changes, with the more stable microtubules becoming acetylated (Shida *et al.*, 2010). The tubulin deacetylases present in cells are HDAC6 and SIRT2 (Hubbert *et al.*, 2002;North *et al.*, 2003). The data presented in this chapter has shown that the levels of acetylated tubulin are lower in COPD and smoker MDMs, which could in turn be

related to increased expression or activity of deacetylases. Therefore, an HDAC6 activity assay was performed by immunoprecipitating HDAC6 from MDMs from all subject groups and performing a fluor-de-lys activity assay for HDAC6 (Enzo Lifesciences, UK). HDAC6 protein from the same samples was quantified by western blot to give a final measure of HDAC6 activity relative to protein in each sample. There was no significant difference in the HDAC6 activity of healthy, smoker or COPD MDMs (Fig. 6.10).

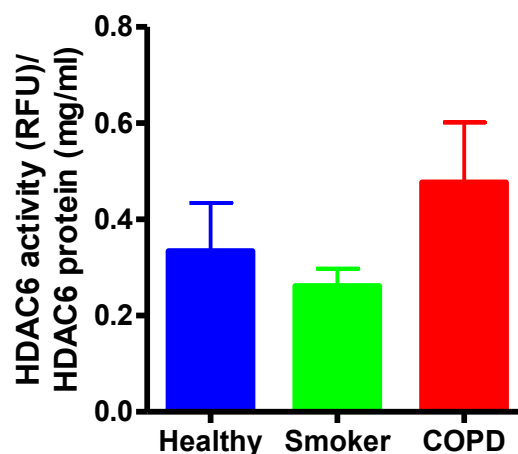


Figure 6.10. Investigation of HDAC6 activity in MDMs from different subject groups

Monocytes from healthy controls (■), healthy smokers (■) and COPD patients (■) were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. Cells were lysed in RIPA buffer and HDAC6 immunoprecipitated. A *Fluor-de-Lys™* HDAC6 activity assay, analysed by fluorimetry, was performed along with a protein assay to determine HDAC6 protein concentrations in the samples. Data are presented as mean $\pm$ SEM, n=6 for healthy and COPD, n=4 for smokers.

6.3.8. Effects of HDAC6 inhibitor (Tubacin) on phagocytosis and levels of acetylated tubulin in COPD MDMs

To further investigate the role of the deacetylases on phagocytosis by MDMs, specific inhibitors to either HDAC6 or SIRT2 were used. Firstly, MDMs were exposed to Tubacin, a specific HDAC6 inhibitor, prior to phagocytosis assays. Tubacin significantly improved bead phagocytosis at 10 μ M, whilst having little effect on phagocytosis of *S.*

pneumoniae and causing a significant reduction, of approximately 25%, in phagocytosis of *H. influenzae* (Fig. 6.11)

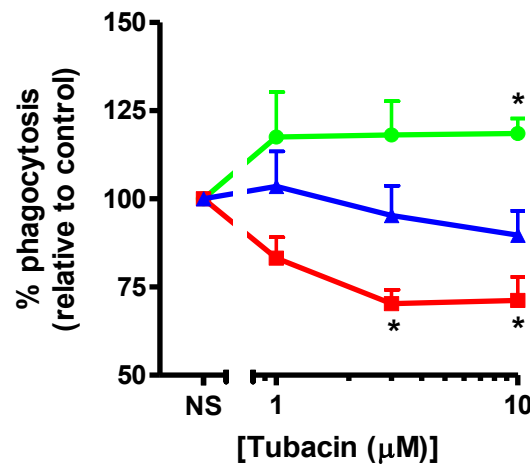


Figure 6.11. Comparison of phagocytosis by COPD MDMs after pre-incubation with Tubacin

Monocytes were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. MDMs were pre-treated for 1 h with Tubacin at varying concentrations, followed by 4 h incubation with beads (●), *H. influenzae* (■) or *S. pneumoniae* (▲). Phagocytosis was analysed using fluorimetry. Data are presented as mean $\pm$ SEM, COPD n=7.

Since tubacin did not improve bacterial phagocytosis, the effect of this drug on the acetylation of microtubules was assessed. Addition of tubacin to COPD MDMs with and without incubation with *H. influenzae* led to a significant increase in levels of acetylated tubulin (Fig. 6.12).

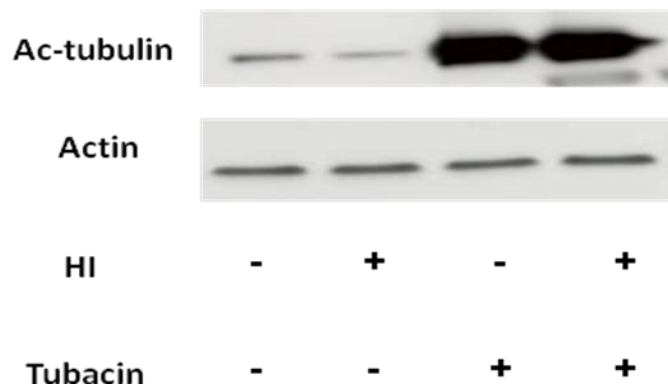


Figure 6.12. Effect of HDAC6 inhibitor (tubacin) on levels of acetylated tubulin within MDMs

MDMs from COPD patients were pre-treated for 1 h with tubacin (10 μ M), followed by 4 h incubation with *H. influenzae* (HI). Cells were lysed in RIPA buffer and proteins separated by Western blot, followed by immunoblotting with primary anti-acetylated tubulin antibody. Blots were then stripped and re-probed with primary anti-actin antibody. Representative of n=4.

6.3.9. Effects of SIRT2 inhibitor (AGK2) on phagocytosis and levels of acetylated tubulin in COPD MDMs

Although an HDAC6 inhibitor increased acetylated tubulin, it did not alter bacterial phagocytosis. Therefore a SIRT2 inhibitor was investigated. Addition of AGK2, a SIRT2 inhibitor, showed a significant improvement in bead phagocytosis at 1 μ M, had little effect on phagocytosis of *S. pneumoniae* and significantly reduced phagocytosis of *H. influenzae* by approximately 20% (Fig. 6.13).

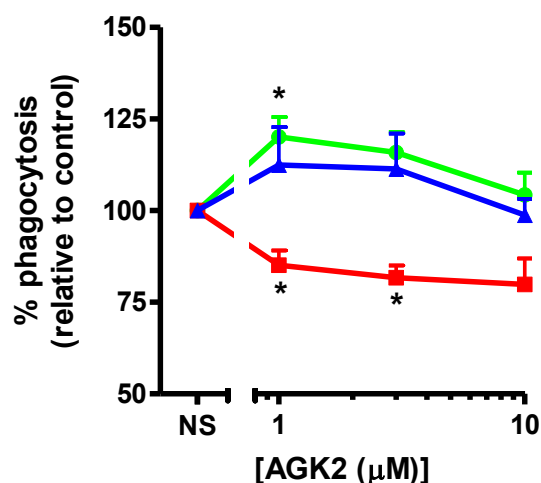


Figure 6.13. Comparison of phagocytosis by COPD MDMs after incubation with AGK2

Monocytes were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. MDMs were pre-treated for 1 h with AGK2 at varying concentrations, followed by 4h incubation with beads (●), *H. influenzae* (■) or *S. pneumoniae* (▲). Phagocytosis was analysed using fluorimetry. Data are presented as mean $\pm$ SEM, COPD n=7.

Since AGK2 did not improve bacterial phagocytosis, the ability of this drug to increase levels of acetylated tubulin was examined. In contrast to the increase in levels of acetylated tubulin seen with tubacin, addition of AGK2 to COPD MDMs with or without incubation with *H. influenzae* did not increase levels of acetylated tubulin compared to addition of prey alone (Fig. 6.14).

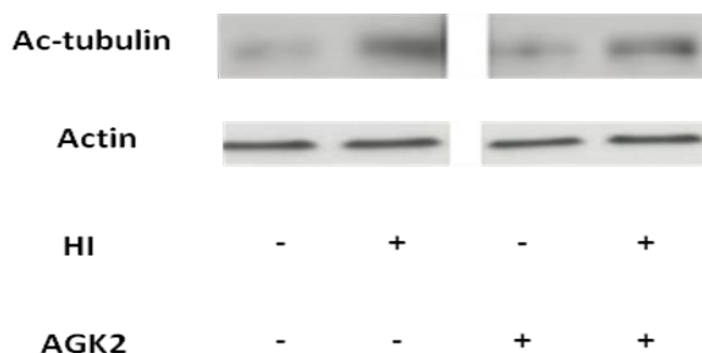


Figure 6.14. Effect of SIRT2 inhibitor (AGK2) on levels of acetylated tubulin within COPD MDMs

MDMs were pre-treated for 1 h with AGK2 (10μM), followed by 4 h incubation with *H. influenzae* (HI). Cells were lysed in RIPA buffer and proteins separated by Western blot, followed by immunoblotting with primary anti-acetylated tubulin antibody. Blots were then stripped and re-probed with primary anti-actin antibody. Representative of n=2.

6.3.10. Transfection of COPD MDMs with siRNA to knockdown SIRT2

Since the SIRT2 inhibitor, AGK2, had no effect on bacterial phagocytosis nor on levels of acetylated tubulin, it may simply be that this drug is ineffective in this system. AGK2 is thought to be the most selective SIRT2 inhibitor, 15-fold more selective for SIRT2 than SIRT1 and SIRT 3 ($IC_{50} = 3.5\mu M$). Therefore, COPD MDMs were transfected with siRNA to knockdown SIRT2 and phagocytosis assays performed. Transfection with SIRT2 siRNA led to approximately an 80% knockdown of SIRT2 as analysed by rt-qPCR (Fig. 6.15), with no effect on viability of the MDMs (Fig. 6.16).

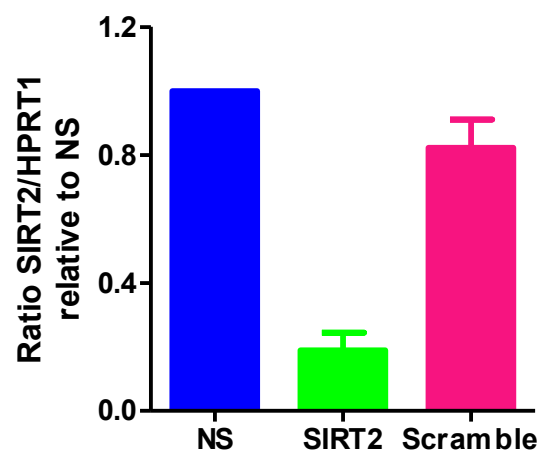


Figure 6.15. Rt-PCR analysis of degree of SIRT2 knockdown after siRNA transfection

Monocytes from COPD subjects were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. MDMs were transfected using HiPerfect (Qiagen, UK) with either 12 μM of SIRT2 siRNA or scramble siRNA (All stars negative control, Qiagen, UK) for 48 h. MDMs were lysed in RLT buffer and RNA extracted using RNA mini-prep kit (Qiagen, UK) prior to rt-PCR using Taqman. Data are presented as mean $\pm$ SEM, n=4.

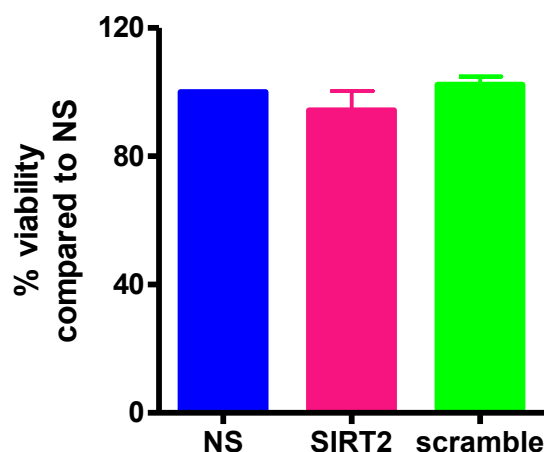


Figure 6.16. Viability of MDMs after SIRT2 knockdown by siRNA transfection

Monocytes from COPD subjects were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. MDMs were transfected using HiPerfect (Qiagen, UK) with either 12 μ M of SIRT2 siRNA or scramble siRNA (All stars negative control, Qiagen, UK) for 48 h. Viability of MDMs was assessed by MTT assay and analysed by spectrophotometry at λ 570nm. Data are presented as mean $\pm$ SEM, COPD n=4.

6.3.11 Effect of knockdown of SIRT2 on the phagocytic ability of COPD MDMs

Having shown that transfection of COPD MDMs achieved an approximate 80% knockdown of SIRT2 with no loss of viability, these transfected cells were then used in phagocytosis assays. Transfected cells were exposed to beads, *H. influenzae* or *S. pneumoniae* for 4 h, however, no significant differences were seen in the ability of transfected COPD MDMs to phagocytose either beads or bacteria (Fig. 6.17).

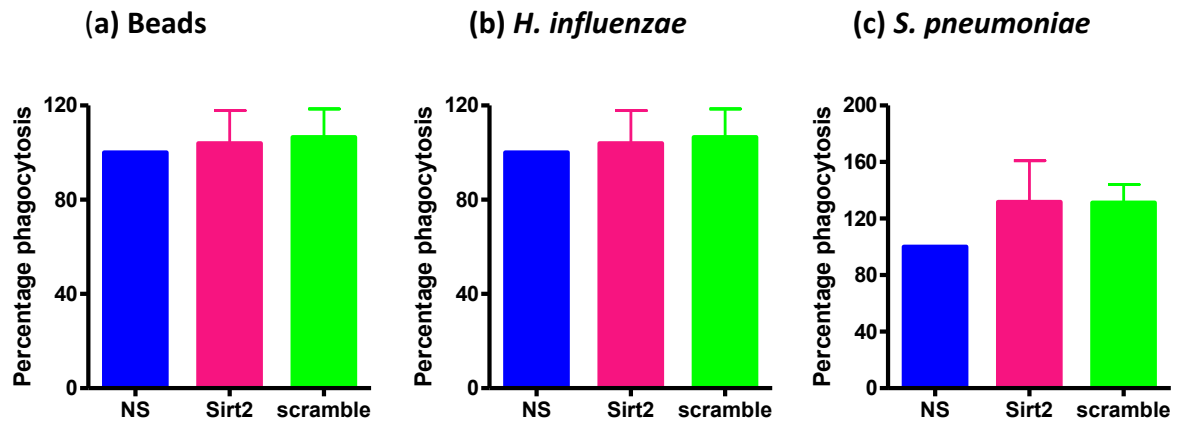


Figure 6.17. Phagocytosis by transfected COPD MDMs after SIRT2 knockdown

Monocytes from COPD subjects were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. MDMs were transfected using HiPerfect (Qiagen, UK) with either 12 μ M of SIRT2 siRNA or scramble siRNA (All stars negative control, Qiagen, UK) for 48 h. Phagocytosis assays were performed with incubation with prey for 4 h, prior to analysis by fluorimetry. Data are presented as mean $\pm$ SEM, n=4.

6.3.12. Transfection of COPD MDMs with siRNA to knockdown HDAC6

Since knockdown of SIRT2 in COPD MDMs had no significant effect on phagocytosis, the next step was to investigate whether HDAC6 knockdown would have any effect on phagocytosis. Transfection with HDAC6 siRNA led to approximately 70% knockdown of HDAC6 as analysed by rt-PCR (Fig. 6.18), with no effect on viability of the MDMs (Fig. 6.19).

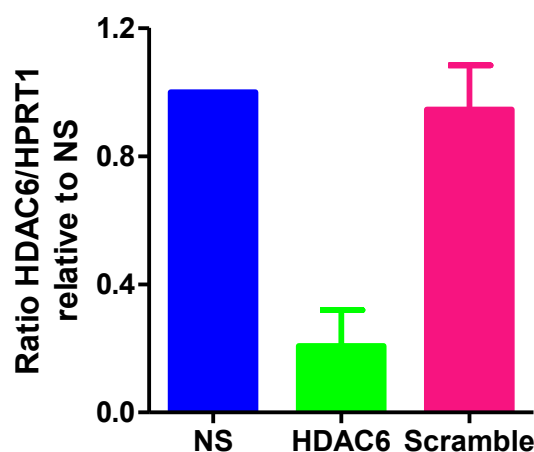


Figure 6.18. Rt-PCR analysis of degree of HDAC6 knockdown after siRNA transfection

Monocytes from COPD subjects were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. MDMs were transfected using HiPerfect (Qiagen, UK) with either 12 μ M of HDAC6 siRNA or scramble siRNA (All stars negative control, Qiagen, UK) for 48 h. MDMs were lysed in RLT buffer and RNA extracted using RNA mini-prep kit (Qiagen, UK) prior to rt-PCR using Taqman. Data are presented as mean $\pm$ SEM, n=4.

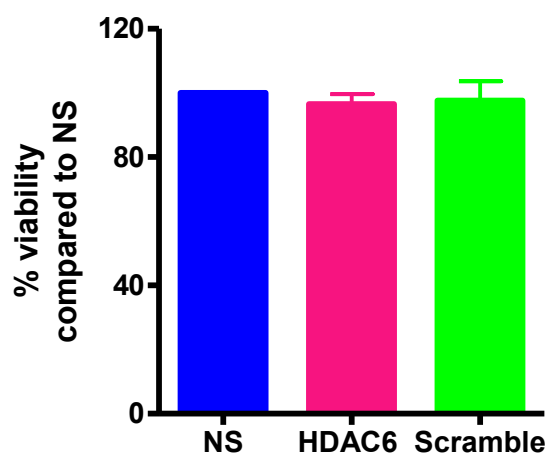


Figure 6.19. Viability of MDMs after HDAC6 knockdown by siRNA transfection

Monocytes from COPD subjects were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. MDMs were transfected using HiPerfect (Qiagen, UK) with either 12 μ M of HDAC6 siRNA or scramble siRNA (All stars negative control, Qiagen, UK) for 48 h. Viability of MDMs was assessed by MTT assay and analysed by spectrophotometry at λ 570nm. Data are presented as mean $\pm$ SEM, COPD n=5.

6.3.13 Effect of knockdown of HDAC6 on the level of acetylated tubulin in COPD MDMs

Having shown that HDAC6 could be knocked down by transfection with siRNA, western blots were performed to investigate whether knock down of HDAC6 led to increased acetylation of tubulin. Increased levels of acetylated tubulin can be seen in cells with HDAC6 knockdown, both alone and after exposure to *H. influenzae*, in comparison to control and negative siRNA control (Fig. 6.20).

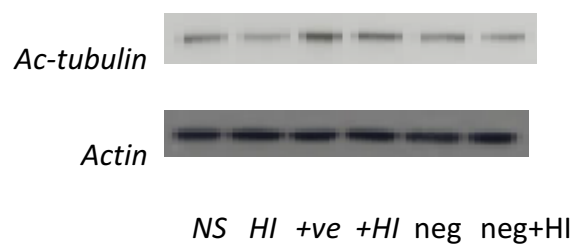


Figure 6.20. Western blot of acetylated tubulin levels in COPD MDMs transfected with HDAC6 siRNA

Monocytes from COPD subjects were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. MDMs were transfected using HiPerfect (Qiagen, UK) with either 12μM of HDAC6 siRNA or scramble siRNA (All stars negative control, Qiagen, UK) for 48 h. HI was then added for 4 h. MDMs were lysed in RIPA buffer and proteins separated by Western blot, followed by immunoblotting with primary anti-acetylated tubulin antibody. Blots were then stripped and re-probed with primary anti-actin antibody. Representative of n=2, where NS = control cells, HI = control cells exposed to *H. influenzae*, +ve = HDAC6 knockdown, +HI = HDAC6 knockdown exposed to *H. influenzae*, neg = scramble siRNA and neg + HI = scramble plus *H. influenzae*.

6.3.14 Effect of knockdown of HDAC6 on the phagocytic ability of COPD MDMs

Having shown that transfection of COPD MDMs achieved an approximate 70% knockdown of HDAC6 with no loss of viability, and furthermore, that knockdown results in increased acetylation of tubulin, these cells were then used in phagocytosis assays. Transfected cells were exposed to beads, *H. influenzae* or *S. pneumoniae* for 4 h, however, no significant differences were seen in the ability of transfected COPD MDMs to phagocytose either beads or bacteria (Fig. 6.21).

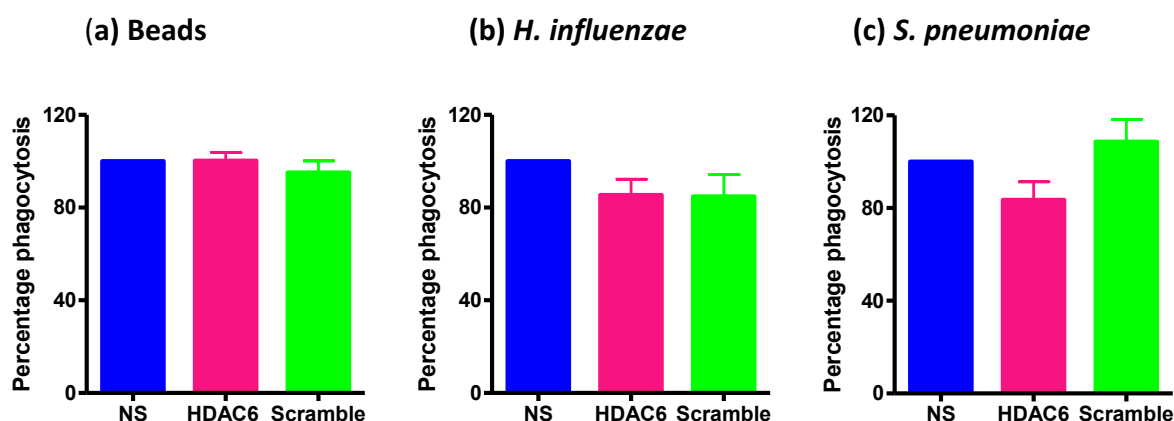


Figure 6.21. Phagocytosis by transfected COPD MDMs after HDAC6 knockdown

Monocytes from COPD subjects were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. MDMs were transfected using HiPerfect (Qiagen, UK) with either 12 μ M of HDAC6 siRNA or scramble siRNA (All stars negative control, Qiagen, UK) for 48 h. Phagocytosis assays were performed with incubation with prey for 4 h, prior to analysis by fluorimetry. Data are presented as mean $\pm$ SEM, n=6.

6.3.15. Transfection of Healthy MDMs with siRNA to knockdown C6orf134

Inhibition of the deacetylases by either inhibitors or knockdown of the genes failed to lead to improvements in bacterial phagocytosis. Therefore, the low levels of acetylated tubulin in COPD and smoker cells may be as a result of reduced expression or activity of the acetyl-transferase involved in acetylation of microtubules. The next step in the investigation into this defect was to examine the effects of knocking down C6orf134 (α -tubulin acetyl transferase 1) in healthy MDMs, to see if the defect observed in COPD MDMs could be replicated. Transfection with C6orf134 siRNA led to approximately a 60% knockdown of C6orf134 as analysed by rt-PCR (Fig. 6.22), with no effect on viability of the MDMs (Fig. 6.23).

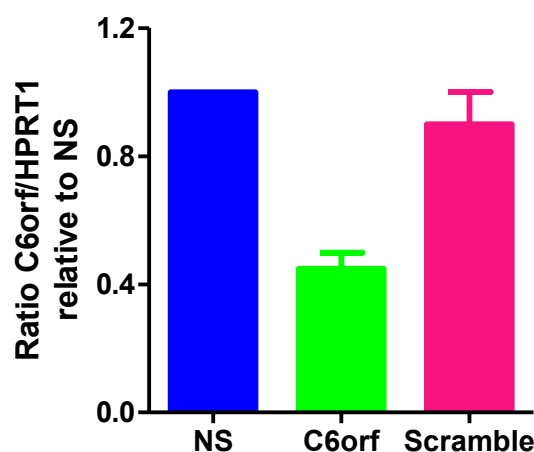


Figure 6.22. Rt-PCR analysis of degree of C6orf134 knockdown after siRNA transfection

Monocytes from non-smoker subjects were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. MDMs were transfected using HiPerfect (Qiagen, UK) with either 12 μ M of C6orf134 siRNA or scramble siRNA (All stars negative control, Qiagen, UK) for 48 h. MDMs were lysed in RLT buffer and RNA extracted using RNA mini-prep kit (Qiagen, UK) prior to rt-PCR using Taqman. Data are presented as mean $\pm$ SEM, n=6.

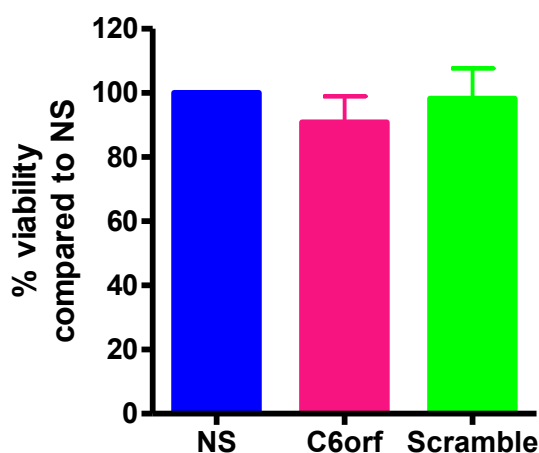


Figure 6.23. Viability of MDMs after HDAC6 knockdown by siRNA transfection

Monocytes from non-smoker subjects were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. MDMs were transfected using HiPerfect (Qiagen, UK) with either 12 μ M of C6orf134 siRNA or scramble siRNA (All stars negative control, Qiagen, UK) for 48 h. Viability of MDMs was assessed by MTT assay and analysed by spectrophotometry at λ 570nm. Data are presented as mean $\pm$ SEM, COPD n=4.

6.3.16. Effect of knockdown of C6orf134 on the phagocytic ability of non-smoker MDMs

Having shown that transfection of non-smoker MDMs with C6orf134 could be achieved, with an approximate 60% knockdown and no loss of viability, these cells were then used in phagocytosis assays. Transfected cells were exposed to beads, *H. influenzae* or *S. pneumoniae* for 4 h. Transfected cells showed no differences in ability to phagocytose beads (Fig. 6.24a), however, they showed significantly reduced phagocytosis of both *H. influenzae* and *S. pneumoniae*, with reductions in bacterial phagocytosis of ~20% (Fig. 6.24b and c).

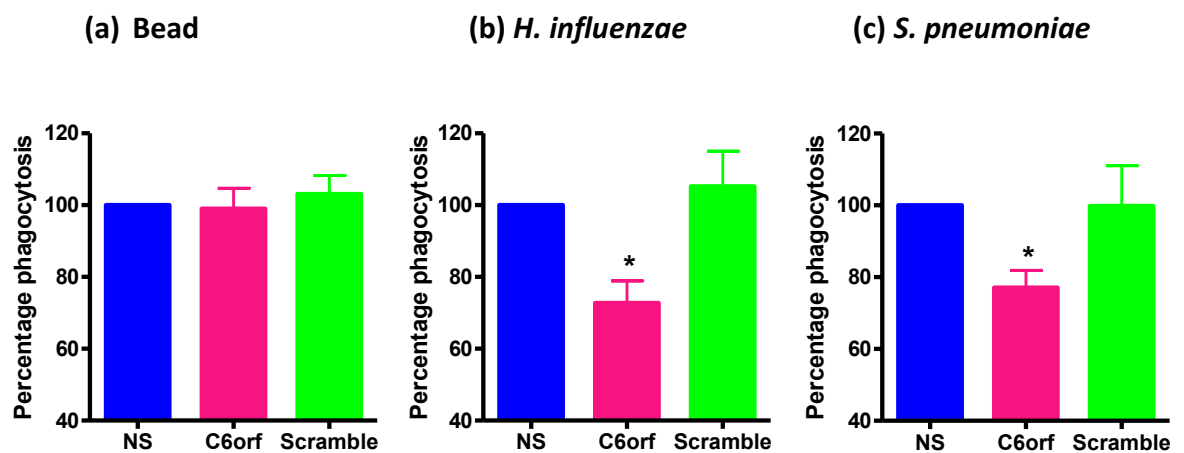


Figure 6.24. Phagocytosis by transfected healthy MDMs after C6orf134 knockdown

Monocytes from healthy (non-smoker) subjects were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. MDMs were transfected using HiPerfect (Qiagen, UK) with either 12 μ M of C6orf134 SiRNA or scramble SiRNA (All stars negative control, Qiagen, UK) for 48 h. Phagocytosis assays were performed with incubation with prey for 4 h, prior to analysis by fluorimetry. Data are presented as mean $\pm$ SEM, n=6, where * represents p<0.05 knockdown vs. NS.

6.3.17. Investigation of levels of C6orf134 in COPD subjects compared to healthy subjects

This data suggests that knocking down C6orf134 in healthy MDMs leads to reduced phagocytosis, mimicking the defect seen in COPD subjects. Therefore, to investigate this phenomenon, the next step was to examine whether C6orf134 was expressed at lower levels in MDMs from COPD patients compared to healthy subjects. Quantitative rt-PCR was performed, showing equivalent expression of C6orf134 in both healthy and

COPD MDMs (Fig. 6.25). Whilst the level of expression of C6orf134 appeared to be significantly reduced in COPD compared to healthy MDMs, (Fig. 6.26a), this reduction in gene expression was mimicked in the housekeeping gene (HPRT1), which was also expressed at lower levels in COPD MDMs compared to healthy (Fig. 6.26b).

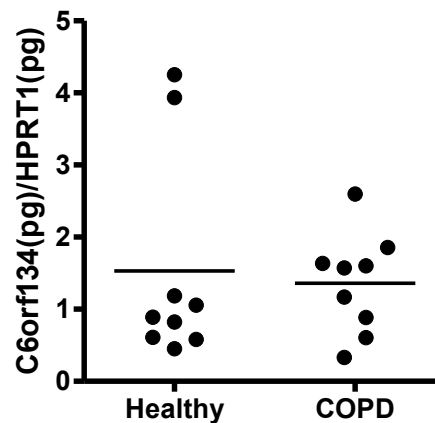


Figure 6.25. Quantitative rt- PCR of C6orf134 in healthy vs. COPD MDMs

MDMs were lysed in RLT buffer and RNA extracted using RNA mini-prep kit (Qiagen, UK) prior to conversion to cDNA. Equivalent amounts of cDNA were then used for rt-PCR using Taqman. Amount of gene (pg) could be extrapolated from a standard curve of known quantities of cDNA run on the same plate. Data are expressed as expression of C6orf134 (pg) relative to HPRT1 expression, mean $\pm$ SEM, n=9 for each group.

(a) C6orf134

(b) HPRT1

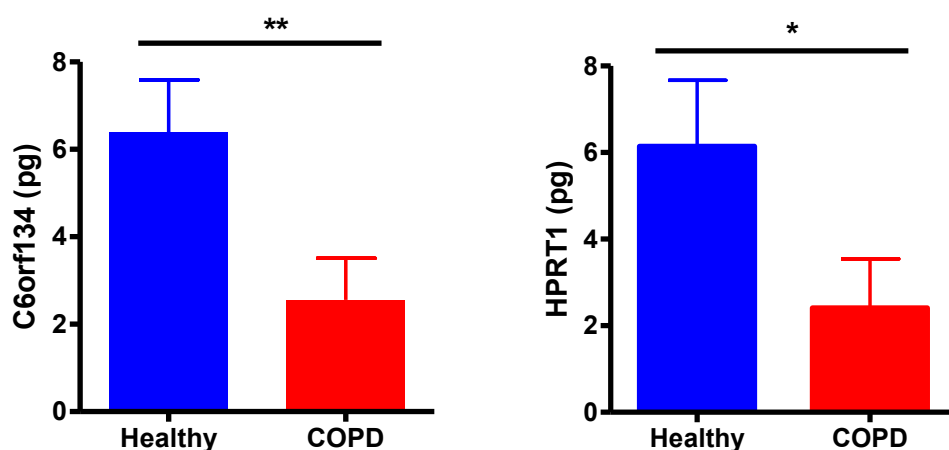


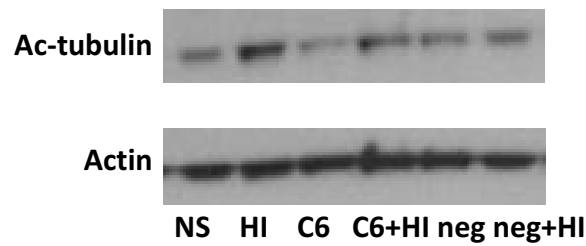
Figure 6.26a and b. Quantitative rt- PCR of C6 orf134 and HPRT1 in healthy vs. COPD MDMs

MDMs were lysed in RLT buffer and RNA extracted using RNA mini-prep kit (Qiagen, UK) prior to conversion to cDNA. Equivalent amounts of cDNA were then used for rt-PCR using Taqman. Data are presented as mean $\pm$ SEM, n=9 for each group.

6.3.18. Effect of knockdown of C6orf134 on the levels of acetylated tubulin within healthy MDMs

As COPD MDMs have altered bacterial phagocytosis associated with reduced levels of acetylated tubulin, and having shown that knockdown of C6orf134 in healthy MDMs models the reduced bacterial phagocytosis, levels of acetylated tubulin within the cells were then examined. Healthy MDMs were transfected with C6orf134, prior to exposure to *H. influenzae* for 4 h and analysis of levels of acetylated tubulin by Western blot (Fig 6.27a). Levels of acetylated tubulin increased after addition of *H. influenzae* in all cells (NS, knockdown and negative control) compared to baseline (Fig. 6.27a and b). Importantly, however, MDMs transfected with C6orf134 clearly show reduced levels of acetylated tubulin both at baseline and after exposure to *H. influenzae*, when compared to NS and negative controls (Fig. 6.27a and b).

(a)



(b)

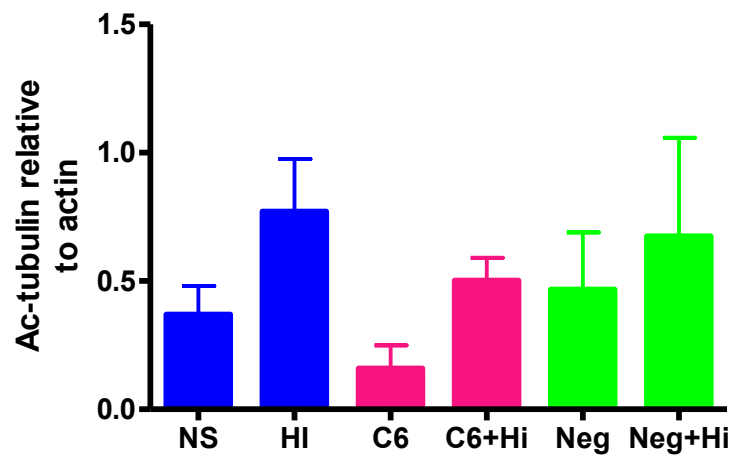


Figure 6.27a and b. Effect of C6orf134 knockdown on levels of acetylated tubulin within non-smoker MDMs

Monocytes from healthy subjects were cultured in complete media containing GM-CSF (2ng/ml) for 12 d to give MDMs. MDMs were treated with HiPerfect alone (NS (■)), or transfected with either 12μM of C6orf134 siRNA (■) or scramble siRNA (All stars negative control, Qiagen, UK) (■) for 48h, prior to 4 h incubation with *H. influenzae*. Cells were lysed in RIPA buffer and proteins separated by Western blot, followed by immunoblotting with primary anti-acetylated tubulin antibody. Blots were then stripped and re-probed with primary anti-actin antibody. Representative of n=2, where NS = control cells, HI = control cells exposed to *H. influenzae*, C6 = C6orf134 knockdown, C6+HI = C6orf134 knockdown exposed to *H. influenzae*, neg = scramble siRNA and neg + *H. influenzae* = scramble plus *H. influenzae*.

6.4 Discussion

Previous work in our laboratory has shown an increased sensitivity of COPD macrophages to the presence of colchicine, a microtubule disruptor, with significant reductions in phagocytosis (Finney-Hayward *et al.*, 2005). In the present study, a similar observation, though less marked, was observed in the presence of nocodazole, a microtubule disruptor with similar activity to colchicine. COPD MDMs had significantly reduced phagocytosis of beads and bacteria in the presence of nocodazole, compared to healthy MDMs. Similarly, smoker MDMs showed increased sensitivity to microtubule disruptors, with equivalent reductions in phagocytosis compared to COPD MDMs; the statistical significance of this reduction, compared to healthy cells, was not analysed due to the low 'n' numbers. Previous studies using human MDMs have shown that FcγR-mediated phagocytosis was unresponsive to nocodazole, whilst complement receptor mediated phagocytosis was inhibited (Newman *et al.*, 1991). A further study has shown that non-primed peritoneal macrophages were susceptible to the effects of colchicine on phagocytosis of IgG-opsonised erythrocytes, an effect that was abolished on activation of macrophages (Khandani *et al.*, 2007). However, the macrophages used herein are all non-primed and the prey used is unopsonised, therefore neither of these mechanisms should account for the differences observed in phagocytosis, suggesting that disease may account for the differences seen.

Reversible acetylation of microtubules has been implicated in the regulation of microtubule stability and function (Piperno *et al.*, 1987), with acetylation used as a marker of stable microtubules. In the present study, levels of acetylated tubulin were found to be significantly lower in COPD patients compared to healthy subjects, suggesting that this reduction in microtubule acetylation may relate to microtubule instability and to the alterations seen in phagocytosis. Similarly, smoker MDMs showed reduced levels of acetylated tubulin, similar to COPD patients, compared to healthy subjects, though this reduction did not reach statistical significance due to the low 'n' number. In contrast, there were no differences seen in acetylated tubulin in neutrophils from different subject groups.

The addition of a microtubule stabiliser, Epothilone B, led to significant improvements in levels of acetylated tubulin and in bacterial phagocytosis in both smoker and COPD MDMs, increasing phagocytosis by up to 20% in COPD and up to 30-40% in smoker MDMs. In healthy MDMs, despite increasing levels of acetylated tubulin, Epothilone B did not increase phagocytosis, which may suggest that phagocytosis in healthy cells is already taking place at or near maximal capacity. Epothilone B had no effect on neutrophil phagocytosis of bacteria. This suggests that, while alterations in microtubule stability appear to be related to defective phagocytosis in macrophages, the defect is different in neutrophils. Given the rapidity of neutrophil phagocytosis, with a maximal response at 2 min, compared to hours for macrophages, it would appear that an alternative mechanism underlies the defect seen in phagocytosis in neutrophils, particularly as microtubule turnover has a reported half-life of 5-10 minutes (McNally, 1996). The defect underlying alterations in neutrophil phagocytosis requires further investigation; expression of scavenger receptors and the effect of PI3K inhibitors, shown to improve chemotaxis in COPD neutrophils, (Sapey *et al.*, 2011) have not previously been examined in phagocytosis by neutrophils from different subject groups.

Given that COPD MDMs showed lower levels of acetylated tubulin, levels of the tubulin deacetylases were investigated; if levels of these enzymes were elevated it may account for the reduced acetylation of tubulin. However, reduction in levels of acetylated tubulin could occur as a result of either reduced acetyl-transferase activity (α TAT1) or increased activity of one or both of the deacetylases, HDAC6 and SIRT2.

There was a trend showing increased HDAC6 activity in COPD MDMs. Treatment of COPD cells with tubacin, a selective inhibitor of HDAC6 (IC₅₀=4nM), led to hyperacetylation of tubulin, however, despite an increase in levels of acetylated tubulin, no associated improvement in bacterial phagocytosis was seen. This hyperacetylation of tubulin after tubacin treatment corresponds with the current literature, where A549 cells treated with tubacin (10 μ M) showed a three-fold increase in levels of acetylated α -tubulin (Haggarty *et al.*, 2003). Rather than an improvement in phagocytosis, in fact a reduction in phagocytosis of *H. influenzae* was seen with both tubacin and AGK2, which was not seen to the same extent with knockdown of either

HDAC6 or SIRT2. Both tubacin and AGK2 are highly selective for deacetylation of HDAC6 and SIRT2 respectively, with the IC₅₀ for other HDACs or sirtuins 1000 fold and >15 fold greater respectively. This suggests that both tubacin and AGK2 may be having an off target effect leading to the reduction in *H. influenzae* phagocytosis. Knockdown of COPD MDMs with either HDAC6 or SIRT2 siRNA confirmed the lack of improvement in phagocytosis, with knockdown of HDAC6 also showing increased acetylation of tubulin. Contrasting this with the findings with Etoposide B (increased acetylation and improved phagocytosis) suggests that it may be microtubule stability and not simply microtubule acetylation that is altered in COPD.

Previously, HDAC6 was thought to control both stability and acetylation of microtubules. It has been shown that the acetylation site on α -tubulin is located on the interior of microtubules (Nogales *et al.*, 1999). Therefore, it is suggested that acetylation occurs on microtubule polymers, whilst deacetylation preferentially occurs on tubulin dimers and is linked to depolymerisation (Black *et al.*, 1989). Cells exposed to the HDAC6 inhibitor, trichostatin A, were shown to be highly acetylated and more resistant to de-polymerisation by colchicine, compared to control cells (Matsuyama *et al.*, 2002). In addition, over-expression of HDAC6 led to accelerated depolymerisation by colchicine (Matsuyama *et al.*, 2002). In contrast, Haggarty *et al.* have shown that whilst tubacin treatment always increased levels of acetylated α -tubulin, no stabilisation of microtubules was observed in the presence of nocodazole or exposure to cold (0°C) (Haggarty *et al.*, 2003). These findings suggest that increasing tubulin acetylation by inhibition of deacetylases is not sufficient to stabilise microtubules. This supports the findings in this chapter whereby increasing tubulin acetylation by inhibition of deacetylases has no effect on phagocytosis, whilst stabilising microtubules in the presence of Etoposide B leads to an improvement in phagocytosis. Furthermore, this suggests that the defect leading to altered phagocytosis in COPD is one of microtubule instability as opposed to altered acetylation.

The recent discovery of the α -tubulin acetyl-transferase (α TAT1, also known as C6orf134 or MEC-17) allowed investigation of whether alterations in this enzyme could account for the altered phagocytosis seen. Knockdown of C6orf134 in healthy MDMs was suggestive of not only reduced levels of acetylated α -tubulin in the cells, but more

significantly, of a reduction in bacterial phagocytosis, by ~20%, with unchanged bead phagocytosis. Therefore, knocking down C6orf134 appears to mimic the defect seen in COPD cells. One would therefore expect that levels of C6orf134 would be lower in COPD patients compared to healthy to account for the defects seen in COPD macrophages. Quantitative rt-PCR initially appeared to confirm this, however, COPD MDMs also showed a reduction in HPRT1 (the housekeeping gene) compared to healthy cells, therefore, expression of C6orf134 was not altered in COPD. Western blotting also did not allow for differentiation of levels of C6orf134 in MDMs from different subject groups due to poor specificity of the anti-bodies available. Therefore, an activity assay will be established to examine whether COPD MDMs have reduced ability to acetylate tubulin compared to healthy cells.

In conclusion, this chapter has shown that the mechanistic defect underlying the alterations in phagocytosis seen in MDMs and neutrophils from COPD patients and smokers is not the same, and further work is required to elucidate the defect in neutrophils. In COPD MDMs, improving levels of tubulin acetylation was only associated with an increase in phagocytosis by the use of microtubule stabilisers, suggesting that the defect leading to altered phagocytosis in COPD is one of microtubule instability as opposed to altered acetylation. Finally, knockdown of C6orf134 in healthy MDMs appears to mimic the defect seen in COPD cells, with reduced acetylation of tubulin and reduced phagocytosis. This would suggest that C6orf134 must be acting not only as an acetyl-transferase but may also act as a microtubule stabilising protein.

6.4.1 Limitations

The sample size used in the mechanistic part of the study needs to be increased throughout, as in some of the latter experiments, results are suggestive of a difference but this would need to be confirmed with increased numbers. Work into the role of C6orf134 was limited due to its recent identification and lack of available antibodies or assays to investigate its protein levels or activity.

Chapter 7

DISCUSSION

7.1 Discussion

The hypothesis for this thesis states that defective innate immunity in COPD patients leads to a reduction in bacterial phagocytosis and that this defective phagocytosis may relate to microtubule instability. The data presented herein has shown reduced uptake of both gram positive and gram negative bacteria by MDMs from smokers and COPD patients compared to controls. This is on a background of MDMs from all subject groups ingesting equivalent amounts of latex beads, confirming that MDMs from all subject groups are capable of phagocytosis. Furthermore, neutrophils from smokers and COPD patients have also been shown to have defective phagocytosis, with significantly reduced uptake of bacteria, but again not of latex beads. Studies to date in the literature have shown either reduced phagocytosis of *E. coli* by neutrophils from COPD patients, compared to healthy controls, or reduced phagocytosis of bacteria by healthy neutrophils exposed to cigarette smoke extract (Stringer *et al.*, 2007; Prieto *et al.*, 2001; Guzik *et al.*, 2011). The finding of reduced uptake of common respiratory pathogens by neutrophils from COPD patients compared to controls has not previously been shown. Finally, the work presented herein has shown that, for the first time, it appears to be a defect of microtubule instability that is associated with altered bacterial phagocytosis in COPD MDMs. This may suggest a novel mechanism for defective phagocytosis in COPD MDMs and identifies a new target for the development of therapies for COPD.

This study confirms the findings of defective bacterial phagocytosis by COPD MDMs, as previously shown by Taylor *et al.*, 2010, whilst showing that COPD patients also have reduced uptake of bacteria by neutrophils. No relationship was seen between the severity of the phagocytic defect in macrophages and either increasing age, smoking history or lung function (Chapter 3). This suggests that the defect in phagocytosis is inherent in the cell and is not altered by ageing, disease severity or the length of exposure to cigarette smoke. Of particular note, these are circulating cells, therefore, these cells do not display an altered phenotype after exposure to the lung environment. One might postulate that in COPD patients, cells with an inherent defect in uptake of bacteria will migrate into the lung, and there be exposed to oxidative stress, cigarette smoke and bacteria, which may in turn cause further reductions in the

ability of these cells to take up bacteria. One of the limitations of this study is the use of MDMs as a model of alveolar macrophages. Whilst this has had its advantages, in that it displays the defect is inherent within circulating cells, to consolidate the findings in this study, one needs to confirm the defect in alveolar macrophages from smokers and COPD patients, comparing these to alveolar macrophages from healthy individuals.

Whilst MDMs from smokers appeared to show the greatest reduction in ability to take up bacteria, suggesting active smoking may have an additional direct effect on reducing uptake of bacteria, this is not the case for neutrophils, with cells from COPD patients showing a more significant defect in uptake of bacteria in comparison to smokers. Whether, the differences in the ability of neutrophils to phagocytose bacteria is the reason that smokers do not appear to suffer with recurrent exacerbations, in contrast to COPD patients, is not known (Bagaitkar *et al.*, 2008). Though both smoker and COPD neutrophils and macrophages have reduced ability to take up bacteria, this defect may deteriorate further in the lung environment, reaching a critical point at which the rate of bacterial growth exceeds that of clearance allowing bacterial colonisation and infection. If this were not the case, smokers and COPD patients should have increased susceptibility to infections outside the lung, however, there is no evidence for such risks.

One of the interesting questions that arose from this study was whether the defect in bacterial phagocytosis in COPD patients could have an impact on exacerbation frequency. Previously, it has been shown that patients who exhibit frequent exacerbations have higher rates of bacterial colonisation when compared to infrequent exacerbators (Patel *et al.*, 2002). Therefore, reduced uptake of bacteria by both macrophages and neutrophils could predispose the patient to bacterial colonisation and increased bacterial load, which in turn, may predispose to more frequent exacerbations. This is important because frequent exacerbations of COPD are associated with more rapid decline in FEV₁, increased hospitalisations, poorer performance status, increased mortality and increased costs (Wedzicha and Seemungal, 2007). Moreover, frequent exacerbations have a marked effect on patient quality of life, with increased dyspnoea, reduced exercise capacity and a greater

likelihood of becoming housebound (Sapey and Stockley, 2006). Therefore, the COPD cohort studied herein, was classified on the basis of their exacerbation history over the preceding year, as exacerbation frequency remains stable over a period of several years (Wedzicha and Seemungal, 2007). When baseline MDMs phagocytosis values were compared for those COPD patients with a history of infrequent exacerbations versus those with frequent exacerbations, phagocytosis of both gram positive and gram negative bacteria was significantly reduced in those COPD patients with more frequent exacerbations. Therefore, examining the ability of macrophages from COPD patients to take up bacteria could be used as a simple, and cost-effective, test to highlight a group of patients in whom targeted, aggressive, anti-microbial therapy at time of exacerbation, or the use of maintenance anti-microbial therapy, may have significant benefits. Given that the cells used in this study are circulating cells and have not been exposed to the lung environment; and given that acute exacerbations of COPD did not significantly alter phagocytosis beyond baseline levels, it adds support to the evidence that the defect leading to reduced bacterial phagocytosis is inherent. Furthermore, given that the frequent exacerbators were not those with more severe COPD, nor those with an increased smoking history, it suggests that it may be the degree of the defect in phagocytosis that drives the frequent exacerbation phenotype. A limitation of the exacerbation data presented is the relatively small sample size, and in order to confirm that acute exacerbations truly have no additional effect on phagocytosis, a larger sample size (n of >30) should be investigated. Furthermore, to add strength to the finding that the defects seen in phagocytosis increase as exacerbation frequency increases, one would need to include COPD patients with a greater range of exacerbation frequencies in the preceding year. If this gave rise to a strong correlation between phagocytosis and exacerbation frequency, this would provide more robust evidence for the defect in phagocytosis to be driving the frequent exacerbation phenotype. In addition, investigating whether defective phagocytosis is present in other phenotypes of COPD, where chronic bronchitis and bacterial colonisation are less prominent in the course of disease, or alternatively in conditions where chronic bacterial colonisation is a major feature of disease, such as bronchiectasis, would give further information regarding whether the phagocytic defect is unique to COPD and, furthermore, whether it drives disease phenotype.

Reductions in the rate of exacerbations of COPD have been shown with the use of inhaled glucocorticosteroids, with several large randomised controlled trials showing ICS reduce exacerbation frequency by up to 25% and reduce decline in FEV₁ (Calverley *et al.*, 2003a; Calverley *et al.*, 2003b; Qaseem *et al.*, 2011). Given that COPD patients have been shown to have relative corticosteroid resistance, with reduced expression of histone deacetylase (HDAC) 2 and HDAC activity (Ito *et al.*, 2005), the beneficial effects of inhaled corticosteroids on exacerbation frequency and lung function decline may be suggested to be related to improvements in phagocytosis rather than the direct anti-inflammatory effects of inhaled corticosteroids. The data presented in this thesis (Chapter 5) lends support to this conclusion, with the finding that the greatest improvements in phagocytosis with ICS are seen in neutrophil uptake of *H. influenzae*, the bacteria more likely to be involved in COPD exacerbations. The present study has also shown that frequent exacerbators have significantly reduced phagocytic ability compared to infrequent exacerbators, therefore, it may be that ICS have a more pronounced effect in frequent exacerbators, with improvements in phagocytosis caused by the ICS sufficient to lead to improvements in exacerbation frequency in these patients.

How fluticasone propionate can simultaneously reduce the rate of exacerbations in COPD, up to 50% of which are associated with bacteria, whilst being shown to increase the risk of pneumonia (Calverley *et al.*, 2007) is difficult to explain. However, it may be that all ICS give a slight increased risk of pneumonia, possibly related to impaired resolution of exacerbations (Calverley *et al.*, 2011), with the lesser risk seen with budesonide being more related to its ability to improve the underlying impairment of phagocytosis seen in COPD to a greater extent than fluticasone propionate, rather than fluticasone propionate further impairing bacterial clearance. The greatest improvements in phagocytosis by both MDMs and neutrophils, pre-incubated with either steroid, were seen with *H. influenzae*. This perhaps explains the greater effect of ICS on improving a proportion of exacerbations of COPD, as opposed to the effect of ICS on pneumonia or those exacerbations that go on to cause pneumonia, which may be caused by *S. pneumoniae*, as there was little effect on phagocytosis of *S. pneumoniae*.

It is important to understand the mechanism driving defective phagocytosis and MDMs from COPD patients appeared to have increased sensitivity to microtubule disruptors. In addition, COPD MDMs were found to have reduced levels of acetylated tubulin compared to controls. Acetylated tubulin is used as a marker of microtubule stability. The use of Etoposide, a microtubule stabilizer, gave clear improvements in bacterial phagocytosis in COPD MDMs with an associated increase in acetylated tubulin. In contrast, knockdown of either of the tubulin deacetylases, HDAC6 or SIRT2, in COPD MDMs, led to an increase in tubulin acetylation but no concomitant improvement in bacterial phagocytosis. Therefore, altered microtubule stability, and not simply altered acetylation of microtubules, appears to be the defect underlying altered phagocytosis in COPD. Strikingly, knockdown of α TAT1 in healthy MDMs appeared to mimic the defect seen in COPD MDMs, with reduced acetylation of tubulin and reduced bacterial phagocytosis, whilst bead phagocytosis remained unaltered. This suggests that COPD patients may have reduced activity of tubulin acetyl-transferase (C6orf134), as no differences were seen in gene expression, which may relate to reduced bacterial uptake by macrophages.

How defective microtubule stability impacts on bacterial but not bead phagocytosis is not entirely clear. It may be related to differential recognition and internalization, dependent upon specific cell surface receptors. Microtubules are required in phagocytosis via complement and scavenger receptors A, but not via the Fc receptor (Newman, 1991). Whilst the prey used in this study are not opsonised, and therefore are likely to be taken up by scavenger receptors, it may be that, like the FcR, there may be some classes of scavenger receptor where microtubules do not play as prominent a role in internalisation, thereby allowing phagocytosis to take place as normal. Alternatively, it may be related to the involvement of microtubules in conformational changes of receptors (Newman, 1991), with again differential involvement of microtubules in receptors involved in bacterial as opposed to bead phagocytosis. Finally, with the predominant role of microtubules in phagocytosis being their role in trafficking of the phagosome through the cell (Allen, 1996), it may be that microtubules are differentially invoked to phagosomes containing beads as opposed to bacteria.

Given that knockdown of C6orf134 in healthy MDMs mimics exactly the phagocytic defect seen in COPD, it may suggest altered activity of C6orf134 in COPD patients. As previously discussed, this defect is seen within circulating cells which suggests either a genetic predisposition or a systemic effect of cigarette smoking, perhaps on the bone marrow itself. Large genome wide studies of COPD have shown no alterations on chromosome 6 where C6orf134 is located (Bakke *et al.*, 2011). Further genome studies should concentrate on this chromosome, investigating whether there is any alteration in this gene in large COPD populations in comparison to healthy controls. It may be, however, that the defect in these cells arises through the systemic effects of cigarette smoking, perhaps through epigenetic modifications rather than a specific genetic defect.

Neutrophils also displayed defective phagocytosis of bacteria, however, in contrast to MDMs, there was no altered acetylation of tubulin nor was there any improvement in phagocytosis with Epopthilone B. Therefore, it would appear that the defect in neutrophils is not related to microtubule instability; given the speed of uptake of prey in neutrophils is faster than that of the turnover of microtubules it points to an alternative defect. COPD neutrophils have also been shown to have altered chemotaxis. The use of PI3-kinase inhibitors led to a reduction in the distance travelled by COPD neutrophils, with improved directed speed and accuracy towards chemoattractants; in contrast the inhibitors had minimal effect in healthy and smoker cells (Sapey *et al.*, 2011). In mouse macrophages, inhibition of PI3K with the PI3K inhibitor LY294002, directly resulted in reduced phagocytosis of NTHI (Marti-Llitas *et al.*, 2009). Activation of the PI3K pathway is required for phagocytosis, resulting in the localisation of F-actin to the peri-phagosomal region at the cell membrane (Greenberg and Grinstein, 2002). Therefore, it may be that there is a common defect in the signalling pathway accounting for both defects in chemotaxis and phagocytosis in neutrophils in COPD and this requires further investigation.

In conclusion, this thesis has shown defective uptake of bacteria by both MDMs and neutrophils in both smokers and COPD patients, which in MDMs is related to altered microtubule stability. Given that smokers also show altered microtubule stability and altered levels of acetylated tubulin compared to healthy controls, it may be that this

could be used as a susceptibility test for those smokers who are likely to go on to develop COPD. In turn, this would allow targeted smoking cessation therapy, an extremely cost effective intervention if it will prevent disease progression. Furthermore, this thesis has shown that COPD patients with a frequent exacerbator phenotype have significantly worse phagocytosis than those with infrequent exacerbations. Again, detection of this defect in COPD patients may allow earlier phenotyping of these patients, identifying those patients who are likely to develop frequent exacerbations as disease progresses, enabling earlier intervention with aggressive anti-microbial therapy and inhaled therapy to try and reduce exacerbation frequency, with considerable cost benefit implications and improvements in patient quality of life. The use of microtubule stabilisers has been shown to improve phagocytosis in the region of 20%; currently these drugs are licensed as anti-cancer drugs, as stabilising microtubules reduces cell division, and are therefore associated with significant systemic side effects. It may be that there is a role for these drugs as inhaled therapy so reducing systemic effects. One may also have to review the risk to benefit ratio of using these drugs, as to whether even systemic usage is worth trialling. Studies have quoted a 5 y mortality of ~25% in those frequent exacerbators who incur recurrent hospital admissions (Soriano *et al.*, 2000), a survival statistic worse than many common cancers, therefore, it may be that the increased side effects of a treatment may be acceptable if there was evidence of significant improvements in patient health and quality of life. More importantly, the finding of altered microtubule stability allows for the focused development of new therapies which could be targeted to this defect.

Currently, the mainstay of exacerbation treatment is antibiotics and steroids, both of which have variable effectiveness due to the heterogeneity of exacerbations. This thesis has shown evidence suggestive of altered activity of tubulin acetyl-transferase (α TAT) in COPD cells compared to those from healthy controls, causing reduced microtubule stability and reduced phagocytosis. Therefore, targeting tubulin acetyl-transferase, by stimulating α TAT to increase activity may allow for a novel therapy directed at reducing bacterial colonisation and exacerbation frequency. A novel therapy targeting improvement in phagocytosis may have significant benefits for

patients, in terms of quality of life, disease progression and mortality, with resultant significant improvements in costs for the NHS.

7.2 Future work

There are a number of questions that remain following the completion of this thesis, and a number of experiments could be performed to address many of these questions.

To define in greater detail the phagocytic defect in MDMs, two populations of COPD patients should be used; those with and those without evidence of stable state bacterial colonisation. The premise would be that those with bacterial colonisation would show a greater reduction in phagocytic ability than those without. This would lend weight to the hypothesis that defective phagocytosis leads to increased bacterial colonisation and in turn increased exacerbation frequency.

Using these two groups as the starting point, phagocytosis experiments would be repeated as well as looking at exacerbation frequency within the two groups (dividing each group again into frequent and infrequent exacerbators) and comparing phagocytic ability across the groups. Where possible at stable state, experiments should be repeated using alveolar macrophages to confirm the defect.

If differences were seen in phagocytosis between the two groups then further experiments on the mechanistic defect underlying this could be performed on those MDMs displaying reduced phagocytosis (to further investigate the relevance of C6orf134 to the reductions seen in phagocytosis, including setting up an activity assay to assess whether there is reduced activity in COPD)

Both as part of the MDMs work and the exacerbation work, it would be important to know the colonising strain and then one could assess whether cells show a greater defect in phagocytosis of this particular pathogen over and above other pathogens. Similarly, at times of exacerbation, the type of exacerbation should be analysed, and then it can be ascertained whether there are alterations in phagocytosis based on the type of exacerbation (viral or bacterial) and in the case of bacterial exacerbations, whether change in bacterial strain has a greater or lesser effect on phagocytic ability. Furthermore, at times of exacerbation the length of time to recovery and the time to next exacerbation should be investigated, along with any associate relationship to differences in phagocytic ability.

In parallel with the MDMs work, neutrophils should be investigated from the same subjects and phagocytic defects analysed along the same group divisions.

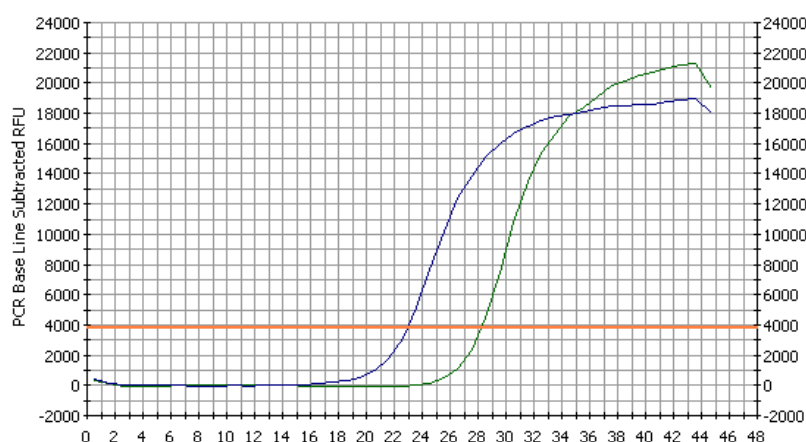
Appendix A

SiRNA target sequences

Hs_HDAC6_5	CACCGTCAACGTGGCATGGAA
Hs_HDAC6_6	CACTTCGAAGCGAAATATTAA
Hs_HDAC6_10	CCGGAGGGTCCTTATCGTAGA
Hs_HDAC6_2	CCGCATTATCCTTATCCTAGA
Hs_C6orf134_7	GAGGCTCATAATGAGGTAGAA
Hs_C6orf134_9	ACCGCACCAACTGGCAATTGA
Hs_C6orf134_5	AACCGCCATGTTGTTTATATT
Hs_C6orf134_6	CCCGCCGACCCGGAACCACAA
Hs_SIRT2_6	AATCTCCACATCCGCAGGCAT
Hs_SIRT2_1	ACCGGCCTCTATGACAACCTA
Hs_SIRT2_3	CCCGCTAAGCTGGATGAAAGA
Hs_SIRT2_2	CAGCGCGTTTCTTCTCCTGTA

Appendix B

Example of an Rt-PCR graph



The graph (amplification plot) shows the number of PCR cycles plotted against increasing fluorescence. The horizontal line is the threshold (set on each experiment) – the point at which the amplification plot crosses the threshold is given as the Ct value (cross threshold value), with the lower the Ct value, the greater the starting amount of DNA in the sample.

Example of $\delta\delta\text{CT}$ calculation

This is an approximation method to give the fold change in the target and reference (control) genes, thereby allowing the calculation of a fold change corrected for any variations in the reference gene

Using the example below, in the top table are the rt-PCR Ct values for the reference gene, with the target gene, C6orf134, in the lower table (where NS = control sample, K'down = HDAC6 siRNA, scramble = negative control siRNA)

Reference gene			
NS	K'down	Scramble	
21.7719	21.7269	23.0287	
21.7721	21.8967	21.8633	
22.6617	21.337	21.0856	
Average			
22.06857	21.65353	21.99253	

Target gene			
NS	K'down		Scramble
	24.9472	25.4794	24.0794
	24.1687	27.0698	24.0092
	23.3142	23.3273	23.4077
Average			
	24.14337	25.29217	23.8321

Fold changes are then calculated in two steps:

Taking the control sample as an example, first the target gene is subtracted from the house keeping gene (*eg.* $22.06857 - 24.14337 = -2.0748$)

Then, from the values generated in step 1, the control value is subtracted from the knockdown values (*eg.* $-3.63863 - (-2.0748) = -1.56383$)

sample	NS	-2.0748	1	1
48+		-3.63863	-1.56383	0.338251
48-		-1.83957	0.235233	1.177097

Finally the degree of change in gene expression is calculated by 2 to the power of the number generated, for *eg.* $2^{-1.56383} = 0.338251$. This value, subtracted from 1 and then multiplied by 100 gives a percentage knockdown of the target gene.

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