EXAMINATION OF VIRAL AND BACTERIAL EXACERBATIONS OF AIRWAYS INFLAMMATION AND FUNCTION

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DOCTOR OF PHILOSOPHY

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Diolch yn fawr iawn i bob un ohonoch!

Summary

Chronic obstructive pulmonary disease (COPD) is an umbrella term that encompasses chronic bronchitis, emphysema and airway obstruction. COPD patients are also prone to acute exacerbations (AECOPD) caused primarily by viral and bacterial infection, which leads to an increase in inflammation, a worsening of symptoms and can lead to death. There is an unmet clinical to better understand and treat AECOPD as well as COPD in general, but this is hindered by unreliable animal models of COPD and AECOPD. The aim of this thesis was to establish an animal model of COPD that could be exacerbated by an infectious agent.

Firstly an LPS model of COPD was established in the guinea pig, which resulted in a macrophage and neutrophil inflammatory profile, emphysematous changes, a decrease in lung function and partial steroid insensitivity that could be partially reversed with low dose theophylline. Human parainfluenza 3 virus failed to cause any infection in the guinea pig, so a model of AECOPD could not be established in this model.

A chronic cigarette smoke model in the mouse was established, which again demonstrated a similar phenotype to COPD. This model was able to be exacerbated by the bacteria non-typeable *Haemophilus influenza* (NTHi) with increases in neutrophils and the neutrophil chemoattractant CXCL1. However, it was also observed that while NTHi could exacerbate the model, responses to NTHi in cigarette smoke challenged mice compared to sham challenged animals were impaired, with significant decreases in CXCL8, TNF-α, IFN-γ and IL-10. This impairment was also observed in monocyte derived macrophages (MDMs) challenged with cigarette smoke extract (CSE) with significant impairment of Il-1β, while chronic LPS challenge also impaired Il-6 and phagocytosis.

The data in this thesis highlights a possible increase in steroid responses by low dose theophylline in an LPS model in the guinea pig. It has also demonstrated chronic cigarette smoke exposure in the mouse can be exacerbated by NTHi, however the inflammatory response is impaired compared to sham challenged animals suggesting that cigarette smoke impairs the innate immune response. MDMs also demonstrated an impaired response to NTHi after CSE or LPS challenge.

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Abbreviations

AECOPD	Acute Exacerbations of COPD
AHR	Airway Hyperresponsivness
AM	Alveolar Macrophages
AP-1	Activator of Protein 1
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
b.i.d	Twice a Day
BALF	Bronchoalveolar Lavage Fluid
BHi	Brain Heart Infusion
BPI	Bactericidal/Permeability Protein
BSA	Bovine Serum Albumin
BSC-1	African Green Monkey Renal Epithelial Cells
Ca	Calcium
cAMP	Cyclic Adenosine Monophosphate
CAT	COPD Assessment Test
CD	Cluster of Differentiation
CFU	Colony Forming Unit
cGMP	Cyclic Guanosine Monophosphate
CO	Carbon Monoxide
CO_2	Carbon Dioxide
COPD	Chronic Obstructive Lung Disease
CREB	cAMP Response Element-Binding Protein
CRP	C Reactive Protein
CS	Cigarette Smoke
CSE	Cigarette Smoke Extract
Cstat	Static Compliance
CXCL	Chemokine (c-x-c motif)Ligand
CytoD	Cytochalasin D
DAG	Diacylglycerol
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FEV	Forced Expiratory Volume
FVC	Forced Vital Capacity
G_{aw}	Airway Conductance
GC	Glucocorticoid
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GOLD	Global Initiative on Chronic Obstructive Lung Disease
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase

HPIV	Human Parainfluenza Virus
ICAM	Intracellular Adhesion Molecule
ICS	Inhaled Corticosteroid
IgA	Immunoglobulin A
IKK	IκB Kinase
IL	Interleukin
IMS	Industrial Methylated Spirit
i.n	Intra nasal
IP	Inositol Phosphate
IRAK	Interleukin-1 Receptor Associated Kinase
IRF	Interferon Regulatory Factor
LA	Long Acting
LAL	Limulus Amebocyte Lysate
LBP	Lipopolysaccharide Binding Protein
Log	Logarithmic
LPS	Lipopolysaccharide
LRR	Leucine Rich Repeats
LTB	Leukotriene B
LTC	Leukotriene C
M_3	Muscarinic Acetylcholine Receptor 3
MAPK	Mitogen Activated Protein Kinase
MCP	Monocyte Chemoattractant Protein
M-CSF	Macrophage Colony Stimulating Factor
MDM	Monocyte Derived Macrophage
MHC	Major Histocompatability Complex
MLCK	Myosin Light Chain Kinase
MLI	Mean Linear Intercept
MMP	Matric Metalloproteinase
mMRC	Modified British Medical Research Council
MPO	Myeloperoxidase
NAD	Nicotinamide Adenine Dinucleotide
NBF	Neutral Buffered Formalin
NET	Neutrophil Extracellular Traps
NF-κB	Nuclear Factor Kappa Light Chain Enhancer of Activated B Cells
NICE	National Institute for Health and Care Excellence
NKK	Nitrosamine 4-(Methyl-Nitrosamino)-1-(3-Pyridyl)-1-Butanone
NOD	Nucleotide-Binding Oligomerization Domain
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
NTHi	Non-Typeable Haemophilus influenza
OD	Optical Density
P50/65	Transcription Factor P50/65
PAMPs	Pathogen Associated Molecular Patterns
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline

PBST	PBS plus Tween 20
PCR	Polymerase Chain Reaction
PDE	Phospodiesterase
PECAM	Platlet Endothelial Cell Ashesion Molecule
PEEP	Positive End Expirtory Pressure
PI3K	Phosphatidylinositide 3-kinase
PIV	Parainfluenza Virus
PKA	Protein Kinase A
PM	Particulate Matter
Poly I:C	Polyinosinic:polycytidylic acid
PPE	Pancreatic Procine Elastase
Pplat	Plateau Pressure
PRR	Pattern Recognition Receptor
R_{aw}	Airway Resistance
RD	Reagent Diluent
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPM	Revolutions Per Minute
RT-PCR	Reverse Transcriptase PCR
s.i.d	Once a Day
SA	Short Acting
SEM	Standard Error of the Mean
sG_{aw}	Specific Airway Conductance
sR_{aw}	Specific Airway Resistance
TANK	TRAF family member-associated NF-κB activator
TIMP	Tissue Inhibitor of metalloproteinase
TIR	Toll-Interleukin 1 Receptor
TLC	Total Lung Capacity
TLR	Toll-Like Receptor
TNF-α	Tumour Necrosis Factor Alpha
TPM	Total Particulate Matter
TRAF	TNF Receptor Associated Factor
TRIF	TIR-domain-containing adapter-inducing interferon-β
UBC	Ubiquitin Conjugating Enzyme
UEV	Ubiquitin Conjugating Enzyme Varient
VCAM	Vascular Cell Adhesion Molecule
VT	Tidal Volume
WHO	World Health Organisation

CHAPTER 1

Introduction

1.1 Chronic Obstructive Pulmonary Disease

Chronic Obstructive Pulmonary Disease (COPD) is defined by the Global Initiative on Chronic Obstructive Lung Disease (GOLD, 2011) as:

> *"A common preventable and treatable disease, characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases"*.

COPD is an umbrella term that encompasses a wide range of chronic lung diseases. It includes:

- Chronic bronchitis with increased inflammatory cells and mucous production.

- Emphysema with destruction of functional alveoli tissue, resulting in increased airspaces, a decrease in elasticity of the lung and complete closure of small airways.

- Chronic obstructive bronchiolitis with fibrous tissue deposition and small airway obstruction (Barnes, 2003).

Different individuals have one, two or all of these conditions, in varying degrees of severity and ratios; therefore each COPD patient is unique. Some COPD patients also exhibit airway hyperresponsiveness (AHR), whereby an individual responds to an innocuous dose of a bronchoconstrictive agent such as histamine or methacholine. AHR is a controversial symptom in COPD, with no definitive consensus (Vestbo and Hansen, 2001)); however some studies have linked AHR to increased mortality in COPD patients (Hospers *et al.,* 2000).

It is difficult to accurately determine the numbers of people suffering from COPD due to difficulties in recognising and diagnosing the disease (van den Boom *et*

al.,1998). However, the World Health Organisations (2010) most recent estimates predict that 210 million people worldwide currently suffer from COPD, 3 million people died from COPD in 2005 and COPD is predicted to be the third most common cause of mortality by 2020.

1.2 Causes of COPD: The Risk Factors

The most important risk factor for COPD is the relationship between genes and the environment. There are many different environmental factors that can increase the risk of developing COPD, but the individual must be genetically susceptible for these environmental factors to have an effect (Sandford and Silverman, 2002).

The main risk factor for COPD is cigarette smoke. This accounts for approximately 73% of COPD related deaths in developed countries and 40% in less-developed countries (WHO, 2010). Studies have shown a direct relationship between smoking and a decrease in lung function. This cause shows how important genes are in COPD, as not all smokers will develop COPD (Mannino & Buist, 2007), with only 15-20% of smokers developing the disease. However, this could be a slightly conservative estimate (Celli *et al.*, 2004) with the actual figures ranging from 20-40% (Daheshia, 2005). Maternal smoking during pregnancy (Svanes *et al.*, 2009) is also a risk, as is passive smoking, with one study showing COPD as a result of passive smoking is responsible for 1.9 million deaths a year in china (Yin *et al.*, 2007). The effect of cigarette smoke is discussed in more detail in chapter 5.

Other risk factors include genetics, pollutants, infections and age. The only proven genetic risk factor for COPD is α_1 anti-trypsin deficiency (Barnes *et al.*, 2002). Polymorphisms in many genes have increased prevalence in COPD patients including matrix metalloproteinases (MMPs) (Joos *et al*, 2002) and MMP regulators

such as TIMP-2 (Hirano *et al*, 2001). Polymorphisms in TNF-α (Sakao *et al.*, 2001) and IL-13 (van der Pouw Krann *et al*,. 2002) also have increased prevalence, as do polymorphisms in anti-oxidant enzymes such as glutathione S-transferase, hemeoxygenase and microsomal epoxide hydrolase (He *et al*, 2002). Exposure to pollutants have been linked to COPD (Lopez *et al*, 2006), as have infections (Svanes *et al.* 2009) and asthma (Silvia *et al.*, 2004).

1.3 The Symptoms and Clinical Diagnosis of COPD

COPD should be suspected if a patient presents with a cough that has sputum production, dyspnoea (shortness of breath) or a history of exposure to risk factors. COPD is diagnosed when the forced expiratory volume (FEV_1): forced vital capacity (FVC) ratio is less than or equal to 0.7 (Celli *et al*, 2004). In more severe cases of COPD, fatigue and weight loss can also occur (Schols *et al.* 1993)

The global initiative for chronic obstructive lung disease (GOLD, 2011) has issued guidelines to determine how a patient is to be treated. These guidelines take into account the symptoms, degree of airflow limitation and frequency of exacerbations.

The symptoms are determined via one of two approved questionnaires, the COPD Assessment Test (CAT) or the Modified British Medical Research Council (mMRC) breathlessness scale (appendix I). A CAT test score of <10 or a mMRC score of 0-1 signifies a less symptomatic patient, while a CAT score ≥10 and an mMRC score ≥ 2 is a more symptomatic patient. Airflow Limitation is classified as mild, moderate, severe or very severe (GOLD 1, 2, 3 or 4 respectively), depending on the percentage of FEV_1 predicted for the patient, as shown in table 1.1 (Celli *et al*, 2004).

Severity	Post bronchodilator FEV_1/FVC	FEV_1 % Predicted
At risk	> 0.7	≥ 80
GOLD 1	≤ 0.7	≥ 80
GOLD 2	≤ 0.7	50-80
GOLD 3	≤ 0.7	30-50
GOLD 4	≤ 0.7	< 30

Table 1.1: Differentiation between at risk, mild, moderate and severe COPD (adapted from Celli *et al*, 2004).

Patient	Characteristic	GOLD Score	Exacerbations/Year	mMRC Score	CAT Score
A	Low Risk Less Symptoms	1-2	≤ 1	0-1	< 10
B	Low Risk More Symptoms	1-2	≤ 1	≥ 2	≥ 10
C	High Risk Less Symptoms	3-4	≥ 2	0-1	< 10
D	High Risk More Symptoms	3-4	≥ 2	≥ 2	≥ 10

Table 1.2: Classification of COPD patients depending on their symptoms, airflow limitation and exacerbation frequency (adapted from GOLD, 2011)

Exacerbation risk is determined by the frequency of exacerbations that take place in one year and is usually assessed by previously treated episodes. One or less exacerbations a year is low risk while two or more are classed as high risk. Patients are classified as high risk or low risk depending on the highest score from airflow limitation or exacerbation tests. This is summarised in table 1.2.

1.4 Treatment of COPD

COPD is a very difficult condition to treat. There is no cure for COPD, with all treatments being used to relieve or reduce symptoms, so an effective treatment is desperately needed. There are two main drug groups used to treat COPD, which are bronchodilators and corticosteroids. Single drug therapy is not an advised course of treatment, with several drug combinations used, dependent on the severity and risk of the patient as summarised in table 1.3 (GOLD, 2011).

1.4.1 Bronchodilators

The purpose of bronchodilators is to relieve the symptoms of COPD sufferers. There are several types used such as the β_2-agonists, anticholinergics and phosphodiesterase inhibitors, which all act on airway smooth muscle, causing a relaxation and making it easier to get air into the lungs (Celli *et al.*, 2004). There are short acting bronchodilators, such as the short acting β_2 agonist salbutamol or the short acting anticholinergic ipratropium bromide, which are able to provide fast relief of symptoms but only last for between 1 and 4 hours, or there are longer acting bronchodilators, such as the long acting β_2 agonist formoterol or the long acting anticholinergic tiotropium, which take longer to have an effect but can relieve symptoms for up to 12 hours (Barnes, 2003). Ultra-long acting β_2 agonists are also available, such as indacaterol, which can relieve symptoms for approximately 24 hours, making it a one dose a day product, and is as effective as tiotropium in clinical

Patient	Characteristic	First Choice Treatment	Second Choice Treatment	Alternative Treatment
A	Low Risk Less Symptoms	SA anticholinergic Or SA β_2-agonist	LA anticholinergic or LA β_2-agonist or SA β_2-agonist and short acting anticholinergic	Theophylline
B	Low Risk More Symptoms	LA anticholinergic or LA β_2-agonist	LA anticholinergic and long acting β_2-agonist	SA β_2-agonist and/or SA anticholinergic
C	High Risk Less Symptoms	ICS and LA β_2-agonist or ICS and LA anticholinergic	LA anticholinergic and LA β_2-agonist	Phosphodiesterase 4 inhibitor, SA β_2-agonist and/or SA anticholinergic Theophylline
D	High Risk More Symptoms	ICS and LA β_2-agonist or ICS and LA anticholinergic	ICS and LA anticholinergic or ICS, LA β_2-agonist LA anticholinergic Or ICS, LA β_2-agonist and phosphodiesterase 4 inhibitor or LA anticholinergic and LA β_2-agonist Or LA anticholinergic and phosphodiesterases 4 inhibitor	Carbocysteine SA β_2-agonist and/or SA anticholinergic Theophylline

Table 1.3: Recommended treatment programmes for GOLD A, B, C and D COPD sub- categories (GOLD, 2011). ICS=Inhaled Corticosteroids, SA= Short Acting, LA=Long Acting. Written order is alphabetical and does not signify order of preference for treatment

trials (Donohue *et al.*, 2010). Longer acting drugs are the preferred treatment over the shorter acting ones as they provide a more stable improvement in symptomatic relief and are more convenient in the required dosing regimen (GOLD, 2011).

1.4.1.1 Beta$_2$-agonists

The β_2 agonists are a class of drugs, such as salbutamol, formoterol and indacaterol, which exhibit an affect via the β_2 adrenergic receptor. These β_2 agonists are selective for the β_2 adrenergic receptor, which is located on smooth muscle, without causing an effect on the β_1 adrenergic receptors found on the heart. Stimulation of the β_2 adrenergic receptor (figure 1.1(A)) causes a relaxation of smooth muscle, which in the airways is witnessed as a bronchodilation. β_2 adrenergic receptors are G-protein coupled receptors which upon stimulation by an agonist increases the production of cyclic adenosine monophosphate (cAMP) via adenylate cyclase. An increase in cAMP causes the activation of protein kinase A (PKA) which in turn acts on myosin-light chain kinase (MLCK) by phosphorylating it and inhibiting its action. This inhibition of MLCK prevents the phosphorylation of myosin, thereby inhibiting smooth muscle contraction (Rang *et al.*, 2002).

1.4.1.2 Anticholinergics

Anticholinergics are antagonists of acetylcholine receptors. There are two types of acetylcholine receptors, muscarinic acetylcholine receptor and nicotinic acetylcholine receptor, which are named depending on the response to either the agonist muscarine or nicotine. Muscarinic receptors are G-protein coupled receptors and are the most important acetylcholine receptor in the treatment of COPD, with the muscarinic acetylcholine receptor 3 (M_3) being the main target for treatment. The M_3 receptor is

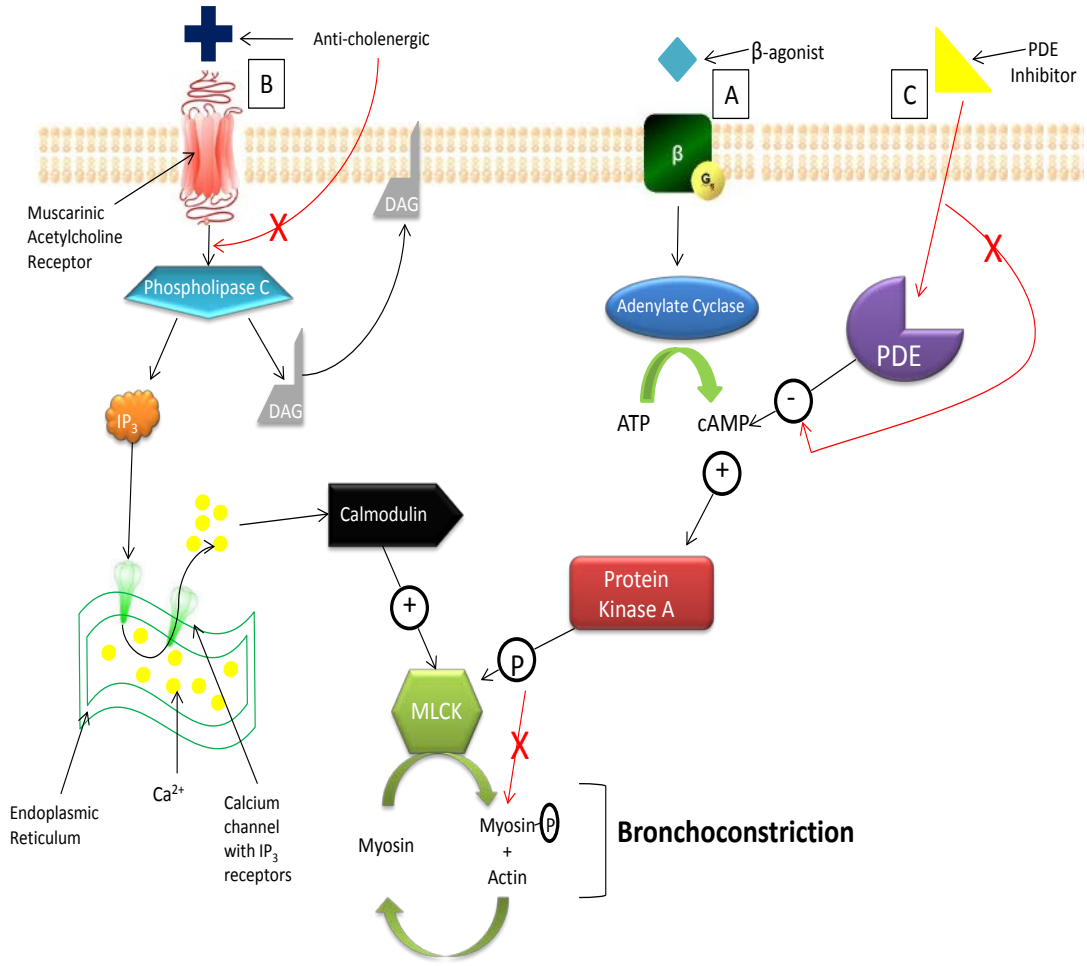


Fig 1.1: Control of bronchoconstriction via the β-agonist receptor, muscarinic acetylcholine receptor and phosphodiesterases (PDE). (A) Stimulation of the β-agonist receptor increases cAMP vie adenylate cyclase. Increased cAMP levels activate protein kinase A, phosphorylating myosin light-chain kinase (MLCK) preventing the phosphorylation of myosin and its interaction with actin, resulting in a bronchoconstriction. (B) Stimulation of the muscarinic acetylcholine receptor by acetylcholine activates phospholipase C which forms diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 binds to receptors on calcium channels located on the endoplasmic reticulum increasing intracellular calcium concentrations. Calcium binds to calmodulin causing it to up-regulate MLCK, resulting in phosphorylated myosin interacting with actin and causing a bronchoconstriction. Anti-cholenergics bind to muscarinic acetylcholine receptors blocking this pathway and preventing bronchoconstriction. (C) Phosphodiesterases degrade cAMP causing lower concentrations within the cell. This prevents the activation of protein kinase C and the phosphorylation of MLCK allowing for the phosphorylation of myosin to interact with actin and cause bronchoconstriction. Inhibition of PDEs does not decrease intracellular cAMP so allows for easier activation of protein kinase A.

found on glandular tissue, smooth muscle and vascular endothelium. Stimulation of the M_3 receptor (figure 1.1(B)) causes activation of phospholipase C. This results in the formation of inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 is soluble and diffuses through the membrane, binding to IP_3 receptors on calcium channels in the endoplasmic reticulum, increasing receptors on calcium channels in the endoplasmic reticulum and increasing intracellular calcium. This calcium is able to bind to calmodulin, altering its structure and allowing it to regulate MLCK, causing phosphorylation of myosin. This phosphorylation of myosin allows it to interact with actin causing a constriction of the smooth muscle, which causes a bronchoconstriction. Anticholinergics are antagonists for the muscarinic receptor. Drugs such as tiotropium and ipatropium bind to the M_3 receptor blocking acetylcholine from binding, and preventing the contraction of the smooth muscle via this inositol pathway (Rang *et al.*, 2002). However, anticholinergics have a large number of side effects, from mild symptoms such as drowsiness and decreases in concentration, to more severe symptoms such as hallucinations and heart failure. This highlights that anticholinergics must be carefully monitored and managed in patients (Mintzer and Burns, 2000)

1.4.1.3 Phosphodiesterase Inhibitors

Phosphodiesterases are a family of enzymes, with 11 known isoforms, that break phosphodiester bonds. They are important mediators of cell signalling via their action on second messengers such as cAMP and cGMP. Phosphodiesterases (PDE) are important in COPD and asthma therapies due to their effect on cAMP, which affects bronchoconstriction and inflammation. The different isoforms have different tissue distributions, with the PDE5 isoform being abundant in airway smooth muscle (Moncada and Martin 1993), while the PDE4 isoform is found mainly in

inflammatory cells (Currie *et al.*, 2008). PDE's are responsible for the degradation of cAMP, an important messenger in bronchodilator and inflammatory cascades, to its inactive nucleotide form (Chung, 2006; Giembycz and Field, 2010). Inhibition of PDE, via non-selective methylxanthine drugs such as theophylline, can have an effect on bronchoconstriction, similar to, but not as potent as, that of a β_2 agonist. This is via a very similar mechanism as that of the β_2 agonists, but not acting via the β_2 adrenergic receptor. Inhibition of PDE (Fig 1.1(C)) causes a decrease in the degradation of cAMP, so increases its concentration within airway smooth muscle. This results in the activation of Protein Kinase A (PKA) which phosphorylates MLCK, preventing the phosphorylation of myosin. This pathway blocks myosin and actin from interacting, which is responsible for the bronchoconstriction of smooth muscle (Rang *et al.*, 2002). However, the toxic and therapeutic doses almost overlap; meaning theophylline has a large number of side effects, including nausea, headaches and drowsiness. Due to these side effects, where theophylline used to be a first line drug for the treatment of asthma, it has now been phased out, only used in extreme cases or where patients find inhalation of β_2-agonists difficult (Currie *et al*, 2008). The anti-inflammatory effects of theophylline are described in more detail in chapter 3

1.4.1.4 Selective Phosphodiesterase Inhibitors

PDE5 is concentrated in the airway smooth muscle, so can affect bronchodilation (Moncada and Martin 1993). Phosphodiesterases are also present in all immune and pro-inflammatory cells, so are also important targets for anti-inflammatory therapies. PDE4 is the most widely targeted and researched phosphodiesterase, with the PDE4 selective inhibitor, roflumilast, just being the first approved for COPD therapy (Giembycz and Field, 2010). Theophylline is a non-selective PDE inhibitor, but

methylxanthine derivatives have been synthesised targeting PDE4 or PDE5 isoforms. The first generation of PDE4 selective inhibitors included rolipram, which showed promising effects in animals, but had too many side effects e.g. nausea. Second generation PDE4 inhibitors, such as roflumilast and cilomilast, partially overcame these side effects and still exhibited anti-inflammatory effects (Currie *et al.,* 2008). PDE4 is present in neutrophils, macrophages, lymphocytes, eosinophils, mast cells, goblet cells, endothelial and epithelial cells. The decrease in concentration of cAMP, via the action of PDE4, within inflammatory cells causes a release of pro-inflammatory cytokines. Inhibition of PDE4 decreases these cytokines, decreasing inflammation. PDE4 inhibitors decrease the release of CXCL8, LTB_4, reactive oxygen species and MMP-9 from neutrophils, important mediators in COPD pathogenesis. They also decrease TNF-α, LTC_4 LTB_4, while increasing the release of the anti-inflammatory cytokine IL-10, from macrophages. T-lymphocyte cytokines are also reduced including IL-2, IL-4 IL-5 and interferon, with epithelial cells also decreasing the release of TNF-α and IL-6. Endothelial cells have decreased expression of adhesion molecules, such as ICAM, VCAM and PECAM, all contributing to a decrease in inflammation (Currie *et al.*, 2008).

1.4.2 Corticosteroids

COPD is still treated with corticosteroids, even though it has been widely reported to have little effect on the underlying physiological inflammation (Williamson *et al*., 2011) or slow down disease progression (Vestbo *et al.*, 1999). Corticosteroids do however have a limited ability to improve lung function and decrease the frequency of exacerbations (Spencer *et al.,* 2004).

1.4.2.1 Mechanism of Action

Steroids exhibit their effect by interaction with glucocorticoid receptors (GR), which are intracellular receptors with a high affinity for glucocorticoids. Steroids are lipophilic molecules derived from cholesterol, so easily diffuse through the cell membrane into the cell and bind to GRs. Binding of a glucocorticoid to a GR induces a conformational change in the receptor resulting in the presentation of DNA-binding domains. Two 'activated' glucocorticoid receptors form a homodimer and move into the nucleus. The GR and steroid complex also interacts with co-activator molecules such as cAMP response element-binding protein (CREB) and histone deacetylase 2 (HDAC2), depending on steroid dose (Rang *et al*, 2002; Barnes, 2006).

1.4.2.2 Transactivation

High doses of steroid result in the transactivation of anti-inflammatory cytokines, by the up regulation of anti-inflammatory gene transcription; such as the gene encoding Il-10. The GR interacts with CREB-binding protein, which has the intrinsic enzyme histone acetyltransferase (HAT). This enzyme is responsible for the addition of acetyl groups to histone proteins, causing the DNA to unwind. Less compact DNA allows for the GR to bind to glucocorticoid response elements (GRE) present on the promoter or enhancer regions of the genes (Dombrowsky & Ulig, 2007), controlling gene transcription. Via this process, steroids are able to increase the release of anti-inflammatory cytokines. (Rang *et al*, 2002; Barnes, 2006). This is summarised in figure 1.2

1.4.2.3 Transrepression

During inflammation activation of NF-κB results in its associated proteins, P50 and P65, translocating into the nucleus and interacting with CREB-binding protein. In

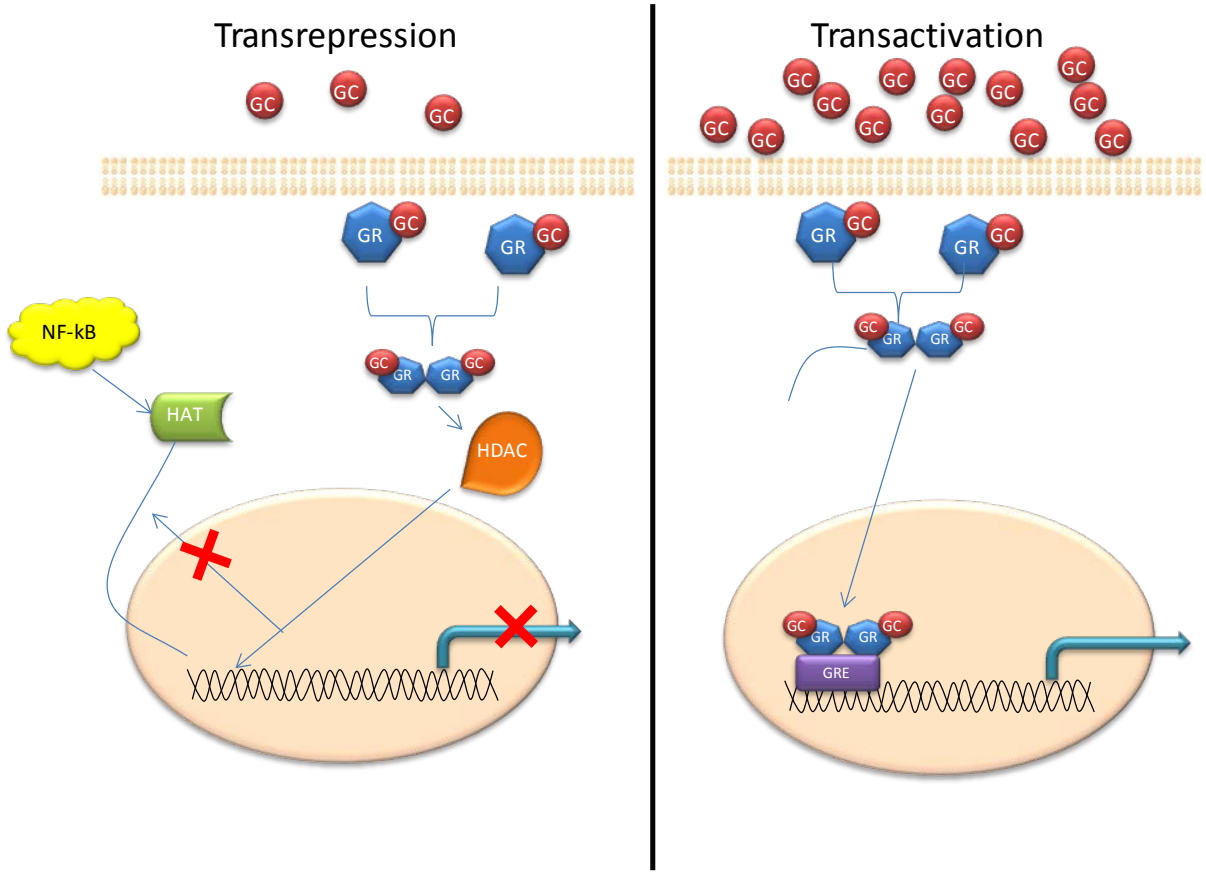


Fig 1.2: The glucocorticoid receptor (GR): glucocorticoids (GC) bind to the glucocorticoid receptor, form a homodimer and enter the nucleus. The glucocorticoid-GR homodimer binds to glucocorticoid response elements (GRE) altering transcription.

turn the proteins intrinsic HAT adds acetyl groups to histone proteins, unwinding the DNA and subsequently up regulating pro-inflammatory cytokines. Low dose steroid treatment inhibits this process via transrepression. The GR dimerises and translocates into the nucleus as in transactivation, however the GR complex interacts with histone deacetylase (HDAC) 2 instead of HAT. HDAC2 is responsible for the removal of acetyl groups from histone proteins, so is therefore the reciprocal enzyme to HAT.

The removal of acetyl groups from the histone proteins causes the DNA to bind more tightly to the histone proteins, shrouding the promoter/enhancer regions of pro-inflammatory cytokines and decreasing the transcription (Rang *et al,* 2002; Barnes, 2006). This is summarised in figure 1.2

1.4.3 Non-Drug Based Management of COPD

There are additional ways to manage COPD symptoms as well as drug management programmes. A change in diet, to a healthier, more nutritious one, can help COPD symptoms; COPD patients can also rehabilitate their lungs and slow down FEV_1 decline through exercise programmes. Many patients, particularly in more severe COPD, are given long-term oxygen therapy, where they breathe highly oxygenated air to counteract the reduction in oxygen intake. There are also surgical options for COPD, with a bullectomy, removal of a portion of lung, to aid in exhalation, and lung transplants being a few options (Celli *et al.*, 2004).

1.5 Steroids in Stable COPD

Steroids are used in the control of stable COPD and in the treatment of exacerbations. ICS are the advised treatment, only in combination with a long acting bronchodilator. Long term oral steroid therapy is not advised (GOLD, 2011). The

effectiveness however, particularly in respect of the effect on stable disease, is widely debated (Barnes, 2000b; Calverley, 2000). In asthma corticosteroid treatment is highly effective at reducing inflammation, however in COPD this is not the case.

Stable COPD shows an active resistance to inhaled corticosteroids, such as prednisolone, fluticasone and budesonide, with long-term, high doses of corticosteroid failing to slow the progression of the disease (Alsaeedei *et al,* 2002; Culpitt *et al,* 1999). Clinical trials have shown improvement in lung function with ICS, however the benefit only lasted for 6 months and did not affect the rate of lung function decline (Soriano *et al.*, 2007). Addition of inhaled budesonide to long acting bronchodilators did show significant improvement in lung function in one clinical trial (Bölukbas *et al.,* 2011), while another clinical trial showed no change in lung function with budesonide compared to placebo (Vestbo *et al.,* 1999). Brightling *et al.* (2004) demonstrated an eosinophillic subtype of COPD that responds well to ICS, with significant improvements in lung function.

Inhaled corticosteroids also have mixed results on inflammation. Qi Gan *et al.* (2005) showed prolonged ICS therapy (greater than 6 weeks) was effective at reducing some inflammatory parameters in sputum, predominantly neutrophil numbers. Macrophage numbers were unaffected. Many studies have found similar results, with Ozol *et al.* (2005), Reid *et al.* (2008) and Thompson *et al.* (1992) demonstrating reduced neutrophil numbers in the broncholavage fluid of COPD patients after ICS treatment, while macrophage numbers remain unchanged. Culpitt *et al.* (1999) however show high dose inhaled corticosteroids failed to supress any cell type but did reduce IL8 in the sputum, while Ozol *et al.* (2005) also demonstrated reduced IL8 levels in the BALF after ICS.

Other studies highlight the possible detriment of ICS in COPD with those on steroid treatment being more likely to acquire pneumonia (Crim *et al.,* 2009; Singh *et al.,* 2009), which has been shown to have increased mortality in COPD patients (Rello *et al.*, 2006), as well as tuberculosis (Jick *et al.*, 2006).

It is reported that one benefit of continued ICS therapy is the reduction in exacerbation frequency in COPD patients (GOLD, 2011). Frequent exacerbators and moderate to severe COPD patients demonstrate a significant decrease in the number of exacerbations after fluticasone treatment (Jones *et al.,* 2003). The addition of fluticasone to salmeterol was also exacerbation protective in a study by Calverly *et al.,* (2007) compared to salmeterol alone. Wedzicha *et al.,* (2008) demonstrated fluticasone in combination with salmeterol was no more protective than tiotropium alone, but did reduce the need of oral corticosteroid treatment. Agarwal *et al,* (2010) however describe only modest reductions in exacerbation frequency and conclude the benefit of ICS in this regard is 'overstated'. Rice *et al.* (2000) also describe limited benefits of chronic oral corticosteroid treatment in reducing exacerbation frequency.

The use of ICS in COPD is not clear cut, with some patients responding better than others. It is important therefore for clinicians to treat each individual on a case by case basis, weighing up the benefits and risks and the particular COPD phenotype present.

1.5.1 Steroid Insensitive Mechanisms in COPD

The reasons for steroid insensitivity in COPD still remains unclear, while studies have shown that macrophages and neutrophils, the predominant inflammatory cell

types in COPD, play a very important role in this steroid insensitivity (Culpitt *et al*, 2003).

Alveolar macrophage cytokine suppression occurs in non-smokers treated with steroid, but steroids are much less effective in smokers and patients with COPD, who exhibit partial responses, where some cytokines are supressed, while others are unaffected. Steroid insensitivity in alveolar macrophages is believed to be due to a reduction in histone deacetylase (HDAC)-2 activity. HDAC is important in the regulation of inflammation due to its recruitment by steroids, resulting in the removal of acetyl groups from histone proteins, decreasing pro-inflammatory transcription. Barnes *et al* (2004) propose that this reduction in HDAC is due to the increased oxidative stress burden in COPD. It has previously been shown that oxidative stress alone is able to decrease the activity of HDAC and increase the acetylation of histones (Tomita *et al,* 2003).This decreases the activity of HDAC, therefore decreasing the effectiveness of the steroid, while at the same time up-regulating pro-inflammatory cytokines due to the over expression of the HDAC reciprocal enzyme histone acetyltransferase (HAT).

Neutrophils have also been implicated in the steroid resistance observed in COPD patients. Many studies have shown that neutrophils and their associated cytokines are not suppressed in COPD by inhaled corticosteroids (Keatings *et al.*,1997; Culpitt *et al.*, 1999), while some studies show that corticosteroids may have some beneficial anti-inflammatory effect (Ozol *et al.*, 2005). A possible reason for this is described by Strickland *et al.* (2001) where they describe differences in glucocorticoid receptor subtypes in neutrophils, with neutrophils exhibiting the β subtype of glucocorticoid receptor not responding to the steroid, while the α subtype do. They conclude that most neutrophils have the β subtype and are therefore intrinsically unresponsive, and

can have this unresponsiveness increased in an inflammatory environment by up-regulation of the β glucocorticoid receptor. Corticosteroid action on the β glucocorticoid receptor can actually have a detrimental effect, increasing the longevity of neutrophils significantly. Plumb *et al*, (2012) also implicate the glucocorticoid receptor in neutrophils in decreased sensitivity to steroids. They demonstrate a reduction in total glucocorticoid receptor in airway neutrophils from control and COPD subjects compared to neutrophils isolated from the blood. This led to decreased sensitivity to dexamethasone on TNF-α and CXCL8 release.

The transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) has also been implicated in steroid insensitivity due to its role in decreasing oxidative stress. Nrf2 up-regulates antioxidants in times of high oxidative stress by binding to the promoter region and up-regulating transcription. However, Nrf2 levels have been shown to be decreased in chronic oxidative stress environments, resulting in an increase in oxidative stress. This increase has been shown to decrease HDAC levels *in vivo* (Adenuga *et al.*, 2010), and Nrf2 deficient mice have been shown to more susceptible to emphysema induced by cigarette smoke (Iizuka *et al.*, 2005).

Since steroids and bronchodilators are the main treatment of COPD, with less than satisfactory results, an effective treatment for the disease is urgently required. To achieve this, an animal model of COPD that allows the intricate pathophysiology of the disease to be studied as well as being used to screen new drugs is desperately needed.

1.6 The Pathophysiology of COPD

COPD is an abnormal inflammatory response of the lungs to noxious particles or gas. There are many mechanisms by which this process progresses, with many

inflammatory and other cells involved. COPD is mainly associated with an elevated level of neutrophils, macrophages and CD8+ T cells (Barnes *et al.*, 2002). The exact pathophysiology that takes place in COPD is poorly understood, but the general theories from the literature are described.

A toxin, such as cigarette smoke, activates monocytes and epithelial cells in the lungs. Activated macrophages release a large number of chemokines, specifically CXCL8, TNF-α and leukotriene B_4 (LTB_4), which are all strong neutrophillic chemotactic factors, attracting neutrophils into the lungs. Activated mucosal epithelium in the lungs also up regulate the presentation of neutrophil adhesion molecules, such as E- and P-selectin, ICAM-1 and 2, VCAM-1 and PECAM-1, as well as increasing the production of mucous secreted from goblet cells and attracting monocytes via monocyte chemoattractant protein (MCP-1) release. Granulocyte-macrophage colony stimulating factor (GM-CSF) is also released, and this chemokine increases neutrophil survival in the lung as well as stimulating macrophage differentiation. Once in the lungs neutrophils undergo degranulation, releasing serine protease such as neutrophil elastase. They also release matrix metalloproteinases, defensins and reactive oxygen species, which are partly responsible for the emphysema witnessed in COPD patients. Macrophages are long living cells, once in the lung they also release inflammatory proteins responsible for the loss in lung elasticity and pro-inflammatory cytokines such as TNF-α. Macrophages are prolonged even further in COPD due to an increase in the anti-apoptotic protein Bcl-X_l. T-lymphocyte numbers are also elevated during COPD, in particular CD8+ T-lymphocytes. These are cytotoxic cells able to induce apoptosis of other cells. In COPD the elevated numbers of these cells cause apoptosis of lung parenchyma cells by release of granzymes and perforins. T cells also release many

different cytokines adding to the cytokine cloud inducing further inflammation. The T- lymphocytes are particularly localised around areas of cell death, adding to the emphysema (Barnes and Rennard., 2009; Daheshia, 2005; Barnes, 2000a). This process is summarised in figure 1.3.

1.7 Airway Inflammation

As mentioned, COPD is a chronic inflammatory disease with a number of cell types playing a vital role. The predominant cell types are neutrophils and macrophages, which are the first responders to toxins, infections and injury. The vast number of cytokines secreted from cells, in turn attract more inflammatory cells, creating a perpetual inflammatory cycle, unable to be adequately controlled, involving both the innate and adaptive immune response.

1.7.1 The Innate Immune Response

Innate immunity is a response to pathogens and/or toxins, whereby the response is no different whether the immune system has come into contact with it before or not. It is a generic immunity, whereby bacteria, for example, are treated similarly, irrespective of species or strain (Delves and Roitt, 2006). The innate immunity involves activation of neutrophils, monocytes/macrophages, eosinophils, basophils, dendritic cells, mast cells and natural killer cells (Delves *et al.*, 2011).

The innate immunity, unlike the adaptive immunity, is unable to differentiate strains of pathogen from the next, but seeks out common denominators to varying types of pathogen (i.e. bacteria, viruses, fungi) to initiate a response. The innate immunity involves a wide range of cell surface receptors called pattern recognition receptors (PRRs). These receptors are able to recognise a small number of molecules present on pathogens, called pathogen associated molecular patterns (PAMPs). There are

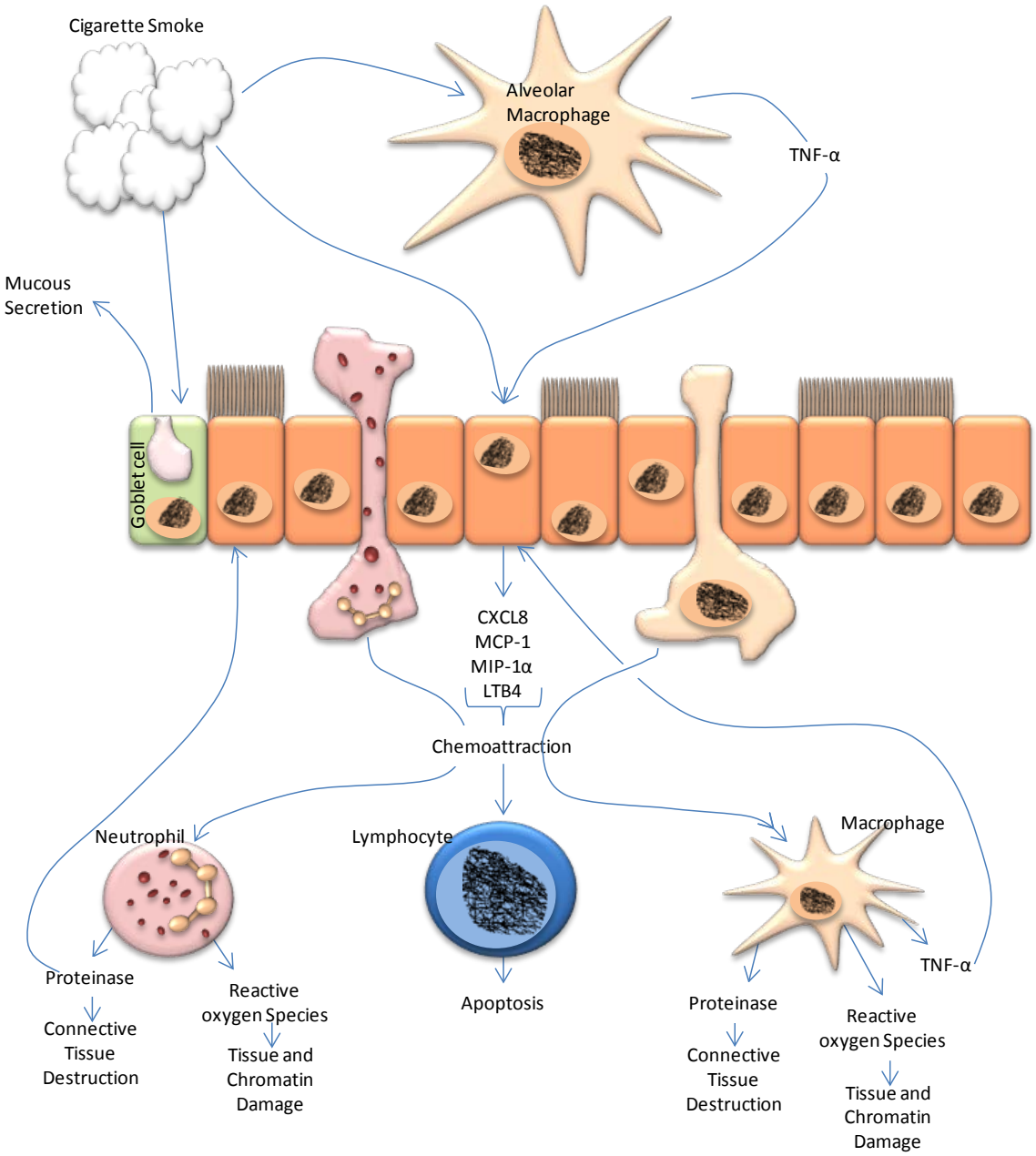


Fig 1.3: The effect of cigarette smoke on cells and cytokines in the lung (adapted from Daheshia, 2005)

many different PRRs on the cell surface, with each one being responsible for recognising particular PAMPs. Recognition of a PAMP induces cytokine release, as well as phagocytosis, via C-type lectins and scavenger receptors, of the pathogen/toxin. (Delves & Roitt, 2006).

1.7.2 Toll-like receptors

Toll-like receptors are one type of PRR and are important receptors of the immune system. The first toll-protein was discovered in *Drosophila,* where it is responsible for dorsal-ventral patterning during embryonic development (Anderson *et al.* 1985). There are many different toll-like receptors in man, with each responsible for recognising a small number of PAMPs. This is summarized in table 1.4. Recognition of a PAMP induces cytokine release, which is appropriate for the type of response required to deal with the pathogen recognised (Delves & Roitt, 2006; Lu *et al.*, 2008). Toll like receptors consist of two functional units. There is an extra cellular leucine-rich repeat (LRR) region, which is 'horseshoe' in shape and is responsible for recognising PAMPs (Bell *et al.*, 2003), and an intracellular region, the Toll-Il-1 receptor (TIR) responsible for signalling within the cell (Fig 1.4) (Playfair & Chain, 2009). Specific agonists of TLRs can be administered *in vivo* to mimic smoke induced inflammation in COPD. The effect of TLR stimulation is discussed in more detail in chapter 3.

1.7.3 Neutrophils

Neutrophils are by far the most numerous leukocytes in the circulation accounting for approximately 70-80% of white blood cells. They are polymorphonuclear cells, consisting of a nucleus organised into several lobes making it ideal for the neutrophils to perform their function efficiently of phagocytising pathogens and toxins.

In the lungs, an infection or toxin results in the release of cytokines which up-regulate adhesion molecules on the vascular endothelium and causes secretion of chemokines, such as the neutrophil chemoattractant protein CXCL8. Neutrophils bind to the adhesion molecules, are activated and migrate through the vascular wall into the tissues where they phagocytose the toxin as well as secreting cytokines, reactive oxygen species and proteases (Smith, 1994). Neutrophils play a role in COPD due to this basic response to toxins, infection and injury. Smokers have been shown to have a large increase in neutrophil numbers isolated from bronchoalveolar lavage fluid (BALF) (Hunninghake & Crystal, 1983), with COPD patients exhibiting an even greater increase again (Confalonieri *et al,* 1998). The degree of severity of COPD is directly associated with neutrophil numbers, with high neutrophil numbers signifying a greater severity and faster progression of the disease (Di Stefano *et al,* 1998*).* The chemoattractant CXCL8 has also been shown to be increased in the BALF of COPD patients (McCrea *et al*, 1994). The secretion of the protease neutrophil elastase, has been widely reported as a contributory factor towards emphysema in COPD. Janoff *et al* (1977) demonstrated that instillation of neutrophil elastase alone into the lungs of dogs could cause histological changes in the lung similar to those witnessed in humans with emphysema.

Neutrophils are short lived cells, only surviving for 4-6 hours; however, they are able to survive longer with steroid treatment due to the inhibition of their apoptosis (Meagher *et al,* 1996). This could partly explain the reason steroids are ineffective in treating the pathophysiology of COPD.

Toll-Like Receptor	Pattern Associated Molecular Pattern (PAMP)
TLR1	Gram +ve Peptidoglycan , Lipoproteins
TLR2	Lipoproteins, Mycobacterial Lipoarabinomannan, Yeast Zymasan
TLR3	Viral Double-Stranded RNA
TLR4	Gram –ve LPS
TLR5	Flagellin
TLR6	Mycobacterial Lipoarabinomannan, Yeast Zymasan
TLR7	Viral Single-Stranded RNA
TLR8	
TLR9	Viral and Bacterial CpG DNA Sites
TLR10	PAMP and Function Unknown

Table 1.4: Human Toll-like receptors and their pathogen associated molecular patterns (PAMP) (adapted from Delves & Roitt, 2006).

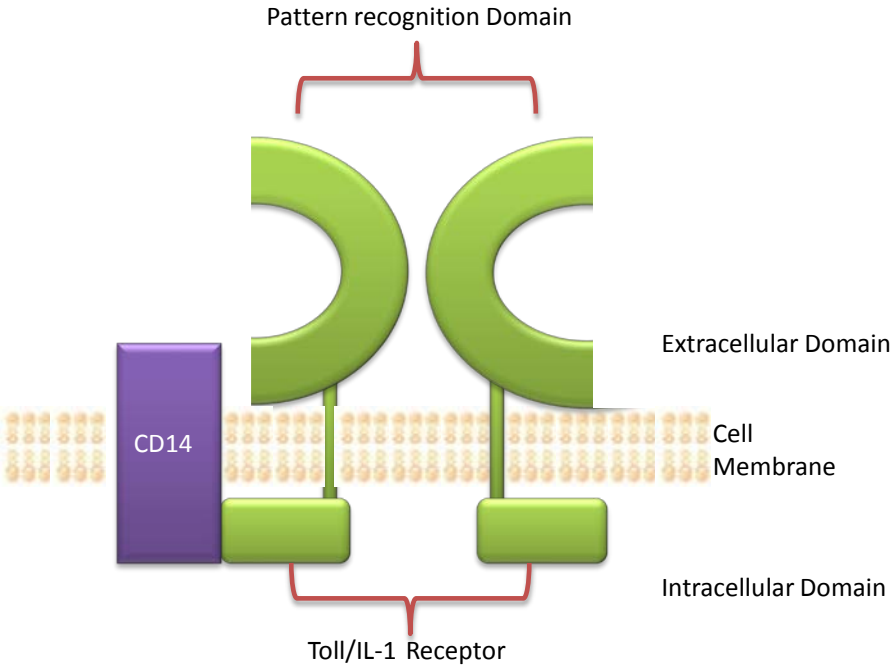


Fig 1.4: Structure of Toll-like receptors: extracellular pattern recognition receptor and intracellular Toll-Il-1 receptor (TIR) (Adapted from Murphy *et al.*, 2008)

1.7.4 Monocytes/Macrophages

Monocytes are white blood cells that differentiate into tissue specific macrophages after migration into those tissues. Alveolar macrophages are monocytes that have travelled into the lungs, and are important cells in both the innate and adaptive immune response. Just like neutrophils, macrophages are phagocytic cells, but can also act as antigen presenting cells and are believed to be the key cell type in orchestrating the immune response in COPD (Barnes, 2004a). Macrophages are long lived cells, surviving for several months after differentiation under normal conditions; however, studies have shown that alveolar macrophages of smokers and patients with COPD can survive for up to two years (Tetley, 2002). Upon activation, macrophages secrete many cytokines and chemoattractants, with the expression of monocyte chemoattractant protein (MCP-1) and the neutrophil chemoattractant protein CXCL8 being up regulated (Barnes 2004a). As well as further cell recruitment and phagocytosis, macrophages have been shown to play a pivotal role in structural remodelling of the lungs by secretion of matrix metalloproteinases (MMP). These are zinc- and calcium-dependant proteases secreted to alter the extracellular matrix within tissues. In COPD, studies have shown over expression of MMPs from macrophages, which have been implicated in the emphysematous remodelling observed in the disease (Demedts *et al,* 2006). Macrophages and their role in COPD will be discussed in much more detail in chapter 8.

1.7.5 Eosinophils

Eosinophils are granulocytes with a polymorphonucleus. They have a well-defined role in the pathophysiology of asthma but have a less defined role in COPD. Eosinophil levels are very low in most COPD patients, with very little increase compared to healthy samples (Lacoste *et al,* 1993). However, a subset of COPD

patients exhibit higher levels of eosinophilia in sputum, but as yet their role are not identified (Brightling *et al.,* 2005). Eosinophils are increased during an exacerbation of COPD (Fujimoto K. e*t al.* 2005), while the eosinophilic subtype of COPD have more protection against exacerbations after treatment with ICS (Burge *et al.,* 2000).

1.7.6 The Adaptive Immune Response and T-Lymphocytes

Unlike the innate immune system, the adaptive immune system is pathogen specific due to the recognition of antigens, specific to the type of pathogen that presents it. The adaptive immune system involves two types of lymphocyte, the T-lymphocyte and the B-lymphocyte, which differentiate in the thymus and bone marrow respectively. The B-lymphocyte has two main functions. They are responsible for producing antibodies (with the help of the T helper cell) to the specific antigen that was recognised, as well as forming memory B-cells to remember the pathogen long after it has been encountered.

T-lymphocytes comprise of five different subtypes, helper (CD4+), cytotoxic (CD8+), memory, regulatory and natural killer T cells, with each performing a specific role in the immune response. CD4+ T cells recognise antigens presented by antigen presenting cells on the major histocompatibility complex (MHC) type II, and aid in maturation and function of other immune cells. CD8+ T cells respond to antigens presented on the MHC type I and induce apoptosis of the cells presenting them. Memory T-cells are cells that are specific to an antigen and survive long after the antigen has cleared. They are able to differentiate quickly in response to the antigen in the future, speeding up the response time. Regulatory T-cells are responsible for controlling the immune response of other T-cell types and are important in autoimmunity. Finally, natural killer cells are cells that have both CD4+

and CD8+ T-cell properties and are important in destroying tumour and cancer cells (Delves *et al.,* 2011).

The adaptive immune system is very important in asthma but plays a smaller role in COPD. T-cells have been shown to be increased in COPD, but are predominantly CD8+ T cells, and are particularly concentrated around areas of parenchymal destruction (Cosio *et al.,* 2002). Saetta *et al.* (1998) demonstrate an increased CD8+ T-cell population in the lungs of smokers is indicative of COPD development, while smokers with lower CD8+ T-cells were asymptomatic. This means CD8+ T-cells may play a role in COPD pathogenesis. Regulatory T cells have also been shown to be increased, in an attempt to control the inflammation and possibly suppress CD8+ T-cells (Smyth *et al.,* 2007; Plumb *et al.,* 2009).

Lymphoid follicles are a structure containing high numbers of T-cells, B-cells and dendritic cells (van der Strate *et al.,* 2006). They have been found in increased numbers in the lungs of COPD patients (Hogg *et al.,* 2004) and are believed to be sites of antigen presentation and lymphocyte maturation, multiplication and storage (van der Strate *et al.,* 2006). Their exact role in COPD is currently unknown but several hypotheses have been put forward. Plumb *et al.* (2009) found elevated regulatory T-cells in lymphoid follicles in COPD which they surmise are an attempt to alter immune regulation and suppress CD8+ T-cells and auto-immunity. The role of autoimmunity in COPD is controversial and not well understood, but Feghali-Bostwick *et al.* (2008) observed increased auto-antibodies to epithelial cells in COPD, possibly due to B-cell antibody production within lymphoid follicles.

1.8 Exacerbations of COPD

As well as underlying inflammation in COPD, patients suffer from exacerbations, increasing inflammation, reducing lung function and decreasing their quality of life.

Exacerbations are defined as:

> "*An acute event characterised by a worsening of the patient's respiratory symptoms that are beyond normal day-to-day variations and leads to a change in medication"* (GOLD, 2011)

There are many sources of exacerbations: pollutants in the atmosphere, a change in temperature (particularly a decrease) and bacterial and/or viral infections of the respiratory tract (Barnes, 2003).

1.8.1 Bacterial Infections

Bacteria play a complex role in COPD, with COPD patients exhibiting increased bacterial colonization compared to controls during the stable disease state. Bacterial colonization has been linked to increased disease severity, rate of lung function decline and frequency of exacerbations (Patel *et al.*, 2002). Cigarette smoke is shown to be a risk factor for increased colonization, with individuals with COPD who continue to smoke having increased bacterial colonization compared to ex-smokers with COPD (Zalacain *et al.*, 1999).

The role of bacteria during exacerbations of COPD is poorly understood due to them also being present in stable COPD. However, the numbers of bacteria are elevated in exacerbations when compared to stable COPD and antibiotic treatment during an exacerbation shortens the time and severity. Organisms such as *Haemophilus influenza*, *Streptococcus pneumonia*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* have been implicated in exacerbations, as well as less common

organisms such as Chlamydia, Mycoplasma and Legionella (Wedzicha & Seemungal, 2007). A study performed by Wilkinson *et al* (2006) showed that bacterial colonisation of the airways is increased in COPD patients in general, and those presenting with an exacerbation show a higher level of bacterial colonisation (48.2 and 69.6% respectively).

The theory of lung sterility in healthy individuals is a controversial subject. Many studies were unable to culture bacteria using standard microbiological techniques, leading to the theory that the lung was sterile. However, more recent studies using more advanced genetic identification techniques have highlighted that bacteria are present in the lungs during health. This has led to the hypothesis that the type of bacteria present is important when comparing health and disease (Erb-Downward *et al.*, 2011).

1.8.1.1 Non-typeable *Haemophilus influenza*

Of all bacteria implicated in COPD, NTHi may be the most important in both stable and exacerbated states. NTHi is an opportunistic pathogen (King, 2012) and is the most frequently isolated bacterium in COPD, accounting for between 30% and 60% of all stable COPD samples (Sethi *et al.*, 2006) and up to 87% of samples during acute exacerbations (Bandi *et al.*, 2001). Rossell *et al.* (2005) also showed NTHi was the most frequently sampled bacteria in healthy smokers.

1.8.2 Viral Infections

Viral infections, by such viruses as rhinovirus, influenza virus, parainfluenza virus and adenovirus, among others, are a frequent cause of COPD exacerbations. Rhinovirus, responsible for the common cold, is the most common cause of viral exacerbations (McManus *et al.*, 2008), with viral exacerbations accounting for

approximately half of all exacerbations. Viruses infect the upper respiratory tract and cause a decrease in lung function. Of all exacerbations, viral exacerbations are by far the most severe, also showing longer recovery times (Wedzicha & Seemungal, 2007). A study by Hutchinson *et al.* (2007) showed moderate to severe COPD patients were 11 times more likely to catch a viral infection than healthy individuals. Mallia *et al.* (2012) also showed that a significant proportion of virally exacerbated COPD patients go on to develop a secondary bacterial exacerbation. Viral exacerbations are discussed in more detail in chapter 4.

This increase in susceptibility to bacterial and viral infections may be due to an impaired immune response and will be discussed in more detail in chapters 7 and 8.

1.8.3 Pollutants

An increase in environmental pollution has been linked to an increase in hospital admissions due to exacerbations of COPD (Anderson *et al*, 1997). An increase in common environmental pollutants that can trigger exacerbations, such as nitrogen oxides, sulphur dioxide, ozone and small particulates (<PM_{10} and $PM_{2.5}$) have been shown to increase hospital admissions for patients with COPD (Ko *et al*, 2007). A study has also shown that an increase in air pollution significantly increases the death rate of COPD patients (Sunyer *et al*, 2000).

1.9 Treatments of Exacerbations

GOLD (2011) recommend three pharmacological treatments for exacerbations, with the main aims to improve symptoms and decrease the frequency of exacerbations. Treatments of exacerbations are continuous, with many treatments for stable COPD being used to reduce the frequency of exacerbations and, therefore, improve the

patient's quality of life, without actually affecting the underlying disease. However, these treatments are inadequate, with more effective treatments urgently required.

1.9.1 Bronchodilators

Studies have shown that bronchodilators are able to reduce the frequency of exacerbations (Niewoehner D *et al.,* 2005; Vincken W *et al.,* 2002), but have a limited effect on lung function. This applies to all bronchodilator subtypes including β_2 agonists (Caverly *et al*, 2007), anticholinergics (Vincken *et al*, 2002; Casaburi *et al,* 2002) and PDE inhibitors, such as theophylline (Rossi *et al* 2002). Although GOLD (2011) recommends β_2 agonists with or without anticholinergics as a treatment for exacerbations, there is evidence that only anticholinergics are effective during severe exacerbations, while β_2 agonists could increase the risk of mortality (Salpeter *et al.*, 2006).

1.9.2 Corticosteroids

GOLD (2011) recommends systemic/oral corticosteroids as a treatment for exacerbations, while inhaled corticosteroids are predominantly used to decrease exacerbation frequency. Many studies have shown that systemic steroids are beneficial in reducing the morbidity, mortality and the duration of hospital stays (Niewoehner *et al*, 1999; Davies *et al*, 1999; Thompson *et al.*, 1996; Alia *et al.,* 2011), with Lindenhaur *et al.* (2010) showing that low dose oral steroids are as equally effective as high dose intravenous steroids.

1.9.3 Antibiotics/Antivirals

The use of antibiotics, such as amoxicillin and clarithromycin, in treating exacerbations is controversial, as problems with sampling of mucous during an exacerbation and recognition of the causative agent, makes it difficult to determine when antibiotics are a beneficial treatment. Antibiotics could even compound the

problem by increasing the stock of antibiotic-resistant strains (Wedzicha & Seemungal, 2007). However, several studies have shown antibiotics to be effective in improving the time taken to recover from an exacerbation (Wilson *et al.*, 2012), as well as reducing the frequency of exacerbations with prolonged treatment (Albert *et al.*, 2011). However, the study by Albert *et al.*, (2011) used prolonged treatment with the macrolide antibiotic azithromycin, which has been shown to have additional anti-inflammatory effects in COPD (Parnham *et al.*, 2005). This makes it difficult to differentiate whether it is the anti-bacterial or anti-inflammatory properties of the antibiotic that is beneficial in this instance.

NICE (2009) guidelines also support the use of antivirals in COPD. COPD patients are deemed an at risk group who can be prescribed antivirals if they present with flu like symptoms. However the guidelines stress that annual influenza vaccination programmes for COPD are the preferred and most effective treatment against influenza.

1.9.4 Non-Pharmacological Treatment

The degree of non-pharmacological intervention required is dependent on the severity of the exacerbation. Supplemental oxygen during an exacerbation has been shown to significantly decrease mortality and is recommended to all patients who are suffering from an acute exacerbation of COPD (Austin *et al.*, 2010). For more severe exacerbations, admission to hospital for ventilatory support or mechanical ventilation (non-invasive or invasive) may be required. GOLD (2011) suggests that hospital admission is required if a patient has severe dyspnoea that fails to respond to normal treatment, mental deterioration, worsening hypoxaemia and/or respiratory acidosis or a need for invasive mechanical ventilation.

1.10 Pathophysiology of Exacerbations

The pathophysiology of acute exacerbations of COPD is poorly understood, due to the difficulties in sampling. However, it is known that there is an increase in inflammatory cells and cytokines in sputum and blood (Wedzicha & Seemungal, 2007), increased protease levels (Mercer *et al.,* 2005) and oxidative stress (Drost *et al.,* 2005).

Clinical studies performed on patients suffering from an acute COPD exacerbation have shown increases in systemic levels of cytokines responsible for inflammation. Increases in IL-6 and 8 were observed during the start of an exacerbation as well as increases in soluble tumour necrosis factor, C-reactive protein (CRP) and bactericidal/permeability increasing protein (BPI) (Groenewegen *et al.,* 2007; Perera *et al.,* 2007).

Systemic neutrophil numbers have been shown to increase by up to 80% when compared to baseline levels (Pinto-Plata *et al.,* 2007). Increases in sputum and blood neutrophils have been linked to an increase in exacerbation severity (Papi *et al.,* 2006). The cytokine levels witnessed in the systemic circulation have also been linked to number and severity of exacerbations. An increase in CRP 14 days post exacerbation has signified a recurrent exacerbation is approximately 25% more likely to occur within 50 days when compared to patients with normal CRP levels. However, it is still unknown whether the increase in systemic cytokines are due to increases in baseline levels during stable COPD, or are due to an unresolved exacerbation (Perera *et al.,* 2007). Increases in clotting factors, such as fibrinogen, are also witnessed during an exacerbation, which can increase secondary cardiac issues for exacerbated COPD patients (Groenewegen *et al.,* 2007; Wedzicha & Seemungal, 2007).

Different exacerbation phenotypes have been described by Bafadhel *et al.* (2011) who show differential responses between the phenotypes. The bacteria-predominant phenotype was found to have higher IL-1β levels in the sputum and a pro-inflammatory response; the virus-predominant had higher serum CXCL10 and a Th1 response and the eosinophil-predominant phenotype had elevated blood eosinophilia and a Th2 response. They propose these markers could be used to identify the type of exacerbation taking place and highlight the appropriate treatment. Papi *et al.* (2006) however show increased eosinophilia is related to virus exacerbations. TNF-α is increased at exacerbation and has been shown to cause an increase in RANTES (Wong *et al.*, 2006), a chemotactic factor for eosinophils, which may be responsible for increased eosinophila at exacerbation (Hacievliyagil *et al.*, 2006).

1.10.1 Frequent and Infrequent Exacerbators

Frequent exacerbators are described as those patients who experience 2 or more exacerbations per year (GOLD, 2011). The ECLIPSE study highlighted the best prediction of exacerbations was a previous history of exacerbations with those who had two or more exacerbations in year one and two, being much more likely to have 2 or more in year three. Interestingly they also highlight a subset of patients who appear to not suffer from exacerbations, at least over the three years in which they were studied (Hurst *et al.*, 2010). This means that the frequent exacerbators are a particular phenotype who will have increased disease progression and a reduced quality of life.

It is as yet unknown what differences exist between frequent and infrequent exacerbators. An increase in viral susceptibility has been proposed (Wedzicha, 2004), which may be due to increased ICAM expression in COPD (Di Stefano *et al.*,

1994). COPD patients who have a higher level of bacterial colonisation have been shown to be more susceptible to exacerbations (Patel *et al.,* 2002) and a genetic predisposition to exacerbations has also been raised with Takabatake *et al.* (2006) demonstrating increased CCL1 gene polymorphisms in frequent exacerbators. However, it has also been proposed that frequent exacerbators are those patients who fail to adequately adhere to their treatment regimen (Bender, 2012). A direct link between COPD severity and exacerbation has been shown, with those in GOLD stage 4 having more frequent exacerbations than those in GOLD stage 2 (Hurst *et al.,* 2010). An animal model that exhibits similar responses to AECOPD would help elucidate the pathophysiology and improve treatments.

1.11 Animal Models of COPD

Due to the poor treatment of COPD and AECOPD, many different animal models of COPD, with differences in species and triggers of inflammation have been developed. However, each model has its use and every model has its limitations. An effective animal model of COPD and AECOPD would help elucidate possible mechanisms that can be targeted for treatment. Animal models of COPD are primarily rodents, including mice, rats, guinea pigs and hamsters. The LPS-induced animal model of COPD will be discussed in more detail in chapter 3, and the tobacco smoke animal model will be discussed in more detail in chapter 5, with an overview of types of animal models discussed in this chapter.

1.11.1 Choice of animal species.

Many animal species are used in respiratory research, each with advantages and disadvantages of their own. Guinea pigs have very similar lung structure and responses when compared to humans (Canning & Chou, 2008). They are however more expensive and have fewer biochemical tools available when compared to other

animals, such as mice and rats. Mice and rats do not closely resemble human lung function or structure, however mice have a vast biochemical assay arsenal, as well as having the advantage of genetically manipulated strains allowing for detailed molecular analysis of a disease process (Wright *et al.,* 2008). Rats develop minimal disease, so are not a particularly good choice of species for COPD models (Wright *et al.,* 2008). Larger animals, such as dogs and primates, could also be used, for example if longer studies are required. However, larger species are much more expensive and are considered 'less ethical' than rodents. The choice of animal model therefore, relates to the desired outcomes of the investigation.

1.11.2 Choice of COPD Inflammatory Trigger

1.11.3 Cigarette Smoke Models

Cigarette smoke models are used in a number of species, including mice, rats, dogs and guinea pigs. Seventy-three percent of COPD sufferers are/were smokers making these models clinically relevant. Cigarette smoke exposure has numerous advantages and disadvantages with regard to the modelling of COPD. The primary advantage is clinical relevance in terms of the cause of COPD, however, just like in COPD the model takes a long time to develop and is difficult to standardise from laboratory to laboratory (Wright *et al,* 2008). Cigarette smoke exposure has been shown to increase neutrophils, associated MMPs and, after chronic exposure, cause emphysema in mice (Churg *et al,* 2004), rats (Lee *et al,* 2005) and guinea pigs (Wright *et al.* 2002; Dominguez-Fandos *et al.* 2012).

Acute and chronic cigarette smoke models have been developed in order to better understand COPD. Overall, acute models show a predominantly neutrophilic response, while more chronic models have neutrophil and macrophage involvement.

Cytokine profiles are similar between the different models, with increases in TNF-α and CXCL8 (reviewed in table 1.5).

Acute models show mixed results in response to steroids, with some studies showing the models to be steroid sensitive (Wan *et al.*, 2010) while others show insensitivity at lower doses (Lecerlc *et al.*, 2006; Marwick *et al.*, 2009). The chronic models are less sensitive to steroid than the acute models (Wan *et al.*, 2010). Wan *et al.* (2010) also describe a reduction in roflumilast sensitivity in the chronic model compared to the acute model. They surmise that the acute model could be used to screen for drug efficacy, with those showing promise then being tested in the chronic model.

Chronic models also demonstrate similar changes in lung function as COPD, including increased total lung capacity, static compliance and airway hyperresponsiveness, while acute models show no change in lung function (Table1.5). However, most importantly, acute cigarette smoke models of COPD do not show any histological changes that are observed in COPD, while more chronic models have been shown to exhibit emphysema, goblet cell metaplasia and airway smooth muscle hyperplasia (Table 1.5).

To try to overcome the length of time required for inflammatory and histological changes to appear, alterations to the smoke model have been developed, including intranasal administration of tobacco–infused media. This decreases the time to less than 40 days, while increasing neutrophil and lymphocyte numbers within the BALF, causing mucin hyper-secretion and airway hyperresponsiveness (Miller *et al*, 2002).

Acute cigarette smoke models have their use in drug screening but chronic cigarette smoke models are more representative of the disease, with increased inflammation

						Drug Responses	
Reference	**Species**	**Agent**	**Inflammation**	**Lung function**	**Histology**	**Steroids**	**Other**
Wright et al., 2002	Mice	Acute Cigarette Smoke (4 cigarettes)	Increased MCP-1, TNF-α and MIP-2 gene expression				
Churg et al., 2003	Mice	Acute Cigarette Smoke (4 cigarettes)	NF-κB, MIP-2, MCP-1 and TNF-α upregulated. TNF-α levels increased in lung				
Leclerc et al., 2006	Mice	Acute Cigarette Smoke (3 days)	Increased total cell influx, predominantly neutrophilia			Dexamethasone sensitive at higher dose, with reduced neutrophils	
Marwick et al., 2009	Mice	Acute Cigarette Smoke (3 days)	Increased total cell influx, predominantly neutrophilia, increased TNF-α and CXCL1			Budesonide (1mg/kg) did not suppress inflammation	
Wright et al., 2002	Guinea Pig	Acute Cigarette Smoke (3 day)	Increased total cell influx, predominantly neutrophilia				
Hsiao et al., 2013	Mice	Acute Cigarette Smoke (3 day)	Increased total cell influx, predominantly neutrophilia				
Wan et al., 2010	Mice	Acute Cigarette Smoke (3 day)	Increased total cell influx, predominantly neutrophilia			Budesonide (1mg/kg) suppressed neutrophils	Roflumilast significantly reduced neutrophils
Lee et al., 2007	Mice	Sub-chronic cigarette smoke (3 weeks)	Increased cell influx into BALF, increase in a large range of cytokines including TNF-α, IL-8 IL-1β, IL-6 and IL-10	Reduction in minute volume	Macrophage infiltration into the lungs		
Miller et al., 2002	Guinea Pig	Tobacco-Infused Media (4 weeks)	Neutrophilia, Lymphocytes	Airway hyperresponsivness	Mucous		
Dominguez-Fandos et al., 2012	Guinea Pig	Chronic Cigarette Smoke (3 Months)	Increased macrophage number in the alveolar septum only, increased eosinophils				
Wan et al., 2010	Mice	Chronic Cigarette Smoke (12 weeks)	Increased Neutrophil, Macrophage and lymphocyte numbers			Budesonide (1mg/kg) did not reduce neutrophils or macrophages but did reduce lymphocytes	Roflumilast has no effect on any cell type
Lee et al., 2005	Rats	Cigarette Smoke (16 weeks)	Cell influx, MMP-9		Emphysema		
Wright et al., 2002	Guinea Pig	Chronic Cigarette Smoke (6 months)	No change in total cells. Neutrophilia, increased TNF-α		Emphysema		
Churg et al., 2004	Mice	Chronic Cigarette Smoke (6 months)	Neutrophilia, macrophage influx, increase in MMP 2, 9 and 12		Emphysema		
Dominguez-Fandos et al., 2013	Guinea Pig	Chronic Cigarette Smoke (6 Months)	Increase in neutrophils, macrophages and eosinophils		Lymphoid Follicles, airway smooth muscle hyperplasia, increased collagen deposition		
Wright and Churg, 1990	Guinea Pig	Chronic Cigarette Smoke (12 Months)		Increased vital capacity, total lung capacity and static compliance. Decrease in FEV1/FVC ratio	Emphysema		

Table 1.5: A review of cigarette smoke animal models

and macrophage involvement, a decrease in lung function and histological changes. Drug responses between the two models are also different, signifying differences in pathways between the models.

1.11.4 Lipopolysaccharide (LPS) Models

LPS has a scientific basis for its use in models of COPD, with cigarette smoke containing large quantities of LPS as well as being shown to activate similar inflammatory pathways (Hasday *et al.*, 1999).

Many animal models have been used to test the effects of LPS induced inflammation in the lungs, both acutely and chronically (reviewed in table 1.6). Acute LPS challenge shows inflammatory cell influx and increased pro-inflammatory cytokine expression. No acute models describe any changes in lung function or lung histology. Chronic LPS models however have been described showing prolonged bronchoconstriction and airway hyperresponsiveness. Many also describe histological changes, with increased emphysema, goblet cell metaplasia and airway smooth muscle hyperplasia (Table 1.6).

The corticosteroid dexamethasone had mixed results in the chronic model, with descriptions of lung function improving and worsening with dexamethasone treatment (Toward and Broadley, 2001; 2002; Kaneko et al., 2007). There were also partial responses in inflammation, with a reduction in neutrophils in the BALF but no effect on macrophages (Toward and Broadley, 2001), and reductions in tissue leucocytes but not BALF cells (Kaneko *et al.*, 2007). Goblet cell hyperplasia was also improved with dexamethasone (Toward and Broadley, 2002). Toward and Broadley (2001) demonstrated steroid insensitivity to dexamethasone after a single LPS exposure, however data in this thesis shows a single exposure to LPS is sensitive to dexamethasone, all be it at a higher dose.

						Drug Responses	
Reference	Species	Agent	Inflammation	Lung function	Histology	Steroids	Other
Toward and Broadley, 2001	Guinea Pig	LPS (single exposure)	Macrophage and neutrophil influx			Dexamethasone is ineffective at reducing inflammation	Rolipram increases inflammation
Birrell et al., 2006	Rat	LPS (single exposure)	Neutrophilia and Eosinophilia. Increases in TNF-α, CXCL1, IL-1β and MMP-9				
Lee et al., 2007	Mice	LPS (5 exposures)	Cell influx in BALF and a large range of cytokines increased including TNF-α, CXCL1, IL-1β, IL-6 and IL-10				
Toward and Broadley, 2001	Guinea Pig	LPS (18 days)	Larger neutrophil and macrophage influx.	Prolonged bronchoconstriction		Dexamethasone had no effect on lung function but ablates neutrophils with no effect on macrophage number	Rolipram reduces bronchoconstriction, neutrophilia and macrophage numbers.
Toward and Broadley, 2002	Guinea Pig	LPS (18 days)	Neutrophil and Macrophage infiltration into the lungs	Prolonged bronchoconstriction	Goblet cell and Clara cell metaplasia	Dexamethasone worsened lung function by prolonging bronchoconstriction, but reduced goblet cell hyperplasia and leucocyte infiltration	Rolipram worsened lung function but reduced goblet cell hyperplasia
Lee et al., 2007	Mice	LPS (3 weeks)	Cell influx in BALF and a large range of cytokines increased including TNF-α, CXCL1, IL-1β and IL-6	No effect on minute volume			
Brass et al., 2008	Mice	LPS (4 Weeks)	Mainly macrophage involvement, increased MMP-9		Emphysema, Collagen deposition		
Kaneko et al., 2007	Guinea Pig	LPS (4 weeks)	Increased leucocytes and neutrophils in the BALF and tissue	Airway hyperresponsiveness	Emphysema, goblet cell metaplasia	Dexamethasone had no effect on cell number in the BALF but decreased numbers in the tissue. It also decreased AHR .	High dose theophylline reduced cell influx into the tissue and improved airway hyperresponsiveness. Low and high dose theophylline reduced goblet cell hyperplasia
Vernooy et al.,2002	Mice	LPS (12 weeks, Intratracheal)	Increased macrophage and CD8+ lymphocyte infiltration. Increased TNF-α and IFN-γ mRNA expression		Emphysema, goblet cell metaplasia and airway smooth muscle cell hyperplasia		
Pera et al., 2011	Guinea Pig	LPS (12 weeks, Intranasal)	Neutrophilia		Emphysema, goblet cell metaplasia and collagen deposition		Tiotropium improves all parameters except emphysema

Table 1.6: A review of lipopolysaccharide animal models

The PDE4 inhibitor rolipram also showed mixed results in the models, with the same chronic LPS model exhibiting improved lung function and inflammation in one study and showing reduced lung function in a different study (Toward and Broadley 2001; 2002). Theophylline and tiotropium has also been shown to be beneficial in chronic LPS models (Kaneko *et al.*, 2007; Pera *et al.*, 2011)

Importantly, as with the cigarette smoke models, no acute model described histological changes. The chronic models did however describe differences in histology including emphysema, goblet and clara cell metaplasia and increased collagen deposition (Table 1.6), which were found in models from 18 days to 12 weeks. The chronic LPS model is therefore a better representation of COPD as it has similar inflammation, but is superior over the acute model, with changes in lung function and histology.

1.11.5 Emphysema Models

Many animal models of emphysema have been created that cause extensive damage to the lungs, independent of inflammation. Kasahara *et al* (2000) showed that inhibition of the vascular endothelial growth factor receptor, responsible for the vasculogenesis and angiogenesis if endothelial cells, was able to quickly cause emphysematous changes without inflammation. There are also numerous studies involving the direct instillation of various elastases directly into the lungs of animals, including human neutrophil elastase (Janoff *et al*, 1977; Lucey *et al*, 1988) and pancreatic porcine elastase (PPE) (Lai & Diamond, 1990). These again result in the rapid development of emphysema, without the COPD-associated inflammation. Therefore, none of these models are sufficient for studying the pathophysiology of COPD, but are only suitable for examining treatment of emphysema.

1.11.6 Genetic Models

Genetically altered animal models allow for the analysis of a specific gene knockout/polymorphism on the cause and progression of the disease. Pallid mice are a model of α_1 anti-trypsin deficiency, which, as mentioned, is the only proven genetic risk factor. Cavarra *et al* (2001) showed the importance of α_1 anti-trypsin in the formation of emphysema, with pallid mice exhibiting rapid acceleration of histological change compared to wild-type controls. Studies with MMP-12 knockout mice have also demonstrated the importance of MMPs in the development of emphysema, with MMP-12 deficient mice not developing emphysema when wild type controls do (Hautamaki *et al*, 1997). However, genetic models as a model of COPD exhibit a too simplistic view with regards to the disease as a whole. COPD is a result of many genetic and environmental factors, with a knockout of one gene alone not causing the disease.

All animal models of human disease have limitations. There is a need to develop a model that exhibits a large number of features associated with the disease; inflammation, a decrease in lung function and histological changes; however the model must also exhibit aspects of steroid resistance and have the potential to be exacerbated. A model exhibiting these properties would allow the complex pathways involved in the stable and exacerbated phase of COPD to be investigated, improving the treatment of the disease.

1.11.7 Animal Models of Acute Exacerbation of COPD

1.11.7.1 Viral Exacerbations

Animal models of AECOPD have been developed to try and better understand the pathophysiology taking place. Many different species of animal have been used, with a wide variety of viruses being given.

Meshi *et al* (2002) demonstrated that adenovirus on top of chronic smoke challenge in the guinea pig increased inflammation and increased emphysema compared to smoke challenge alone. Gualano *et al.* (2008) investigated the effect of influenza in mice exposed to smoke for up to 3 days and found increased inflammation and viral titre in smoke challenged mice compared to the control. Robbins *et al.* (2006) also showed different responses to influenza in mice, this time after chronic smoke exposure. The smoke challenged animals had an increase in inflammation after virus inoculation and had a greater mortality rate than virus only challenged animals.

Parainfluenza virus has been used in many studies as it is a much safer virus to work with than influenza, which requires high containment and a vaccination program. It is mainly used in guinea pigs, with studies demonstrating an infection in guinea pigs with parainfluenza virus (Blomqvist *et al.*, 2002; Adamko *et al.*, 1999). Para influenza in the guinea pig increases cell influx into the lungs as well as increasing airway hyperresponsivness (Toward *et al.*, 2005; Broadley *et al.*, 2010a) and has been demonstrated as a good model of asthma exacerbations (Riedel *et al.*, 1996). The effect of parainfluenza inoculation in a guinea pig model of COPD is not described in the literature and may be a useful model to develop.

Viral mimetics, such as the TLR3 agonist Poly I:C, have also been used as exacerbation agents. Kang *et al.* (2008) observed increased inflammation, emphysema and fibrosis in mice challenged with cigarette smoke and poly I:C compared to smoke only challenged animals.

1.11.7.2 Bacterial Exacerbations

Animal models have been developed to better understand the pathophysiology of bacterial exacerbations of COPD. These have been in many different species, using

many different stimuli to induce COPD-like inflammation/histology, as well as using different bacteria. The most frequently isolated bacteria in both stable and exacerbated disease is non-typeable *Haemophilus influenza* (Sethi *et al.,* 2006; Bandi *et al.,* 2001), meaning most animal models of exacerbations use this bacterium for clinical relevance, even though it is a human only pathogen (King, 2012). However, Slater (1990) demonstrated an infection in rats with NTHi, after damaging the mucosal lining, meaning NTHi may infect animal models with epithelial damage.

Drannik *et al.,* (2004) showed *Pseudomonas aeroginosa* on top of a 6-8 week smoke exposure in the mouse, significantly increased inflammatory cell and cytokine production compared to bacteria only challenged mice. Cigarette smoke also decreased the bacterial clearance of *Pseudamonas* from the lungs. Gaschler *et al.* (2009; 2010) demonstrated, also in a murine chronic cigarette smoke model, that NTHi increased inflammation compared to smoke only challenged animals. However, it was also shown that TNF-α levels were impaired by smoke in response to NTHi, and bacterial clearance was increased with smoke. Treatment with dexamethasone decreased bacterial clearance in both the sham and smoke challenged mice (Gaschler *et al.,* 2009).

Ganesan *et al.* (2012) demonstrated mice challenged with elastase and LPS prior to NTHi inoculation had increased inflammation compared to elastase/LPS only challenged mice. The elastase/LPS and NTHi mice also demonstrated prolonged inflammation, bacterial colonisation and increased emphysema compared to sham/NTHi challenged mice. Wang *et al.* (2010) also showed porcine pancreatic elastase exposed hamsters had decreased NTHi clearance compared to NTHi only challenged animals.

Most studies show an increase in inflammation after bacterial challenge compared to the model of stable COPD. Some models also highlight that animal models that mimic the chronic inflammation and/or the histological changes in COPD have different responses to bacteria compared to naïve/sham animals.

1.12 Scope of thesis

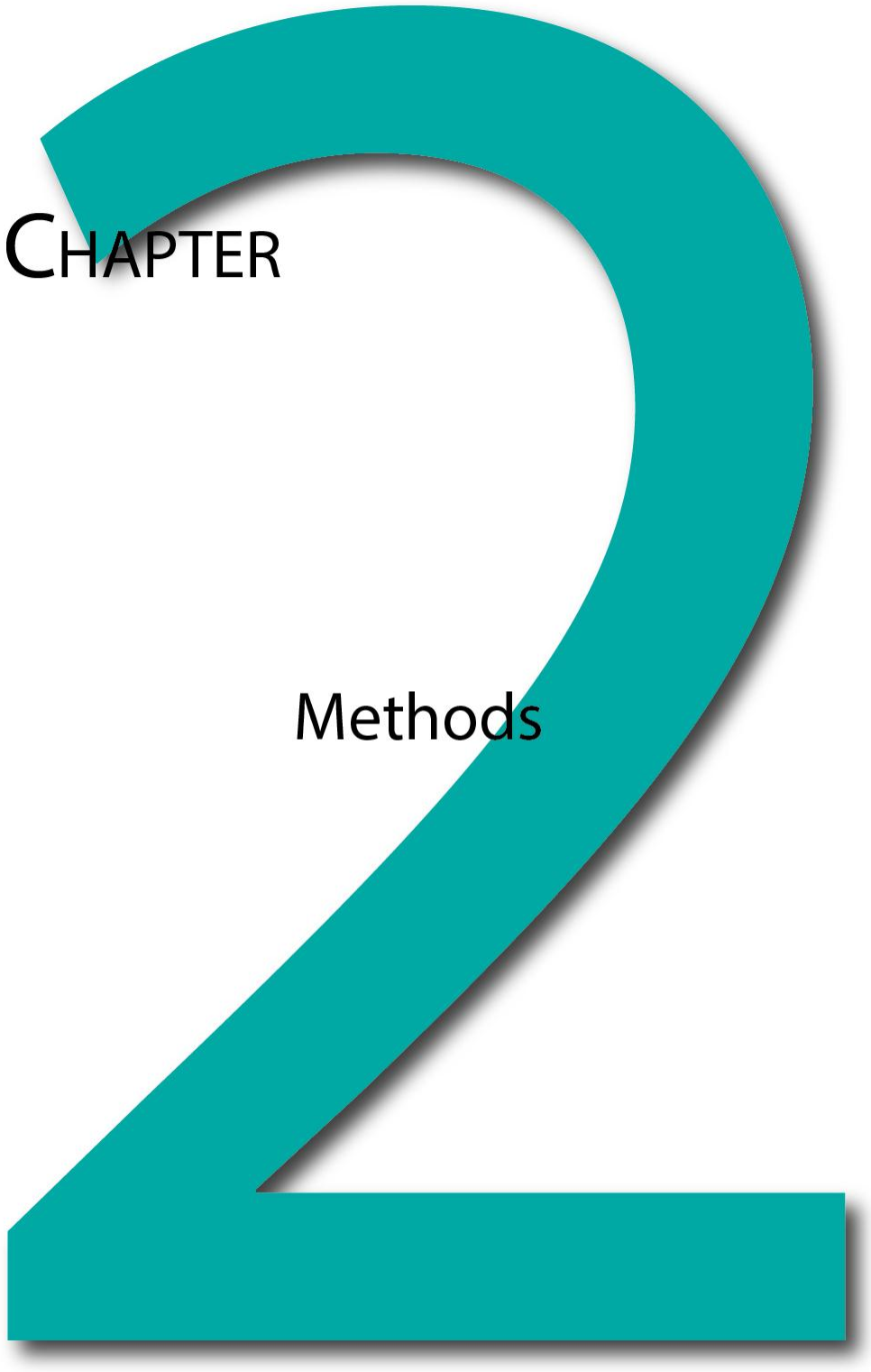
There remain many unknowns in COPD. The literature identifies several aspects of the disease that needs further investigation. The reason for altered bacterial colonisation and clearance, increased severity and susceptibility to viral infections and the pathways involved in exacerbations of COPD are as yet unknown. This thesis aims to develop an animal model that closely resembles COPD and has the potential to be exacerbated. The model could then be used to help elucidate these mechanisms and improve treatments for COPD and AECOPD.

1.12.1 Aims

The aims of this thesis are:

- to develop a chronic LPS-induced model of inflammation in the guinea pig and investigate the steroid sensitivity, the effect of low dose theophylline on the steroid sensitivity and exacerbation potential of the model.
- to investigate the effect of human parainflunza 3 virus and the viral mimetic poly I:C on the guinea pig as potential exacerbation agents.
- to develop chronic cigarette smoke induced inflammation in the mouse exhibiting a similar phenotype to COPD.
- to determine the dose of non-typeable *Haemophilus influenzae* required to cause an infection and inflammation in Balb/C mice so it can be used as an exacerbating agent in the chronic cigarette smoke model

- to investigate the effect of chronic, low level cigarette smoke or LPS on monocyte derived macrophage (MDM) cytokine release and phagocytic ability in response to non-typeable *Haemophilus influenza*e.


CHAPTER
2
Methods

2.1 Animal Experiments

2.1.1 Animal Husbandry

All animals were allowed a minimum of seven days to acclimatise after delivery before any experiments were performed. All experiments were conducted in accordance with the Animals (Scientific Procedures) Act, 1986.

2.1.2 Guinea Pig

Groups of six, male, Dunkin Hartley guinea pigs (200-250g) were obtained from Charles River (Germany). They were housed in plastic bottom cages with Alpha-dri bedding (Lillico, Surrey) in a room with a 12 hour dark/light cycle, maintained at 18°C ± 2°C, 50% ± 10% humidity and were supplied with dried guinea pig food (Harlan, UK) and water with ascorbic acid enrichment *ad libitum*. Hay, wooden blocks and cardboard tubing was supplied for environmental enrichment.

2.1.3 Mouse

Groups of ten Balb/C mice were obtained from Charles River (UK). They were housed in plastic bottom cages with saw dust and shredded paper bedding in a room with a 12 hour dark/light cycle, maintained at 18°C ± 2°C, 50% ± 10% humidity and were supplied with dried mouse food (Harlan, UK) and water *ad libitum*. Cardboard tubing and wooden blocks were supplied for environmental enrichment.

2.2 Challenges

2.2.1 Lipopolysaccharide Exposures

Guinea pigs received 30µg/ml of lipopolysaccharide (LPS) (*E.Coli* 026:B6, Sigma), or a control solution of saline, nebulised using a Wright nebuliser at 0.3mls/min at a constant pressure of 20 psi in a sealed Perspex chamber (15x15x32cm) for 1 hour.

2.2.2 Acute Lipopolysaccharide Exposures

A single 30µg/ml LPS or saline exposure was performed before the guinea pigs were culled 24 hours after the exposure. A bronchoalveolar lavage was performed (as described in 2.6) to determine total and differential leucocyte numbers, as well as cytokine levels. Drug treated animals received drugs as described in Table 2.1 for 6 consecutive days prior to the lavage.

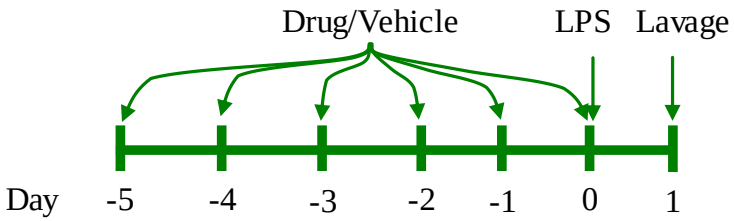


Figure 2.1: A schematic representation of the acute LPS exposure protocol with six day drug treatment

2.2.3 Chronic Lipopolysaccharide Exposures

Exposures were performed using the method described by Toward and Broadley (2001). Guinea pigs received a sub threshold dose of 0.3mM histamine 24 hours before the first exposure to 30µg/ml of LPS or saline, as explained in 2.2.7. Guinea pigs then received 30µg/ml of LPS or saline every other day until a total of nine exposures were reached. Airway function was recorded using whole body plethysmography (Buxco, UK) after the first, fifth and ninth exposure using specific airway conductance (sG_{aw}) as the measured parameter. sG_{aw} was measured at 0 minutes, 1, 2, 3 and 4 hours after exposure and compared to measurements performed immediately prior to the corresponding LPS or saline exposure. This allowed for percentage change in sG_{aw} to be calculated. Animals were exposed to another histamine exposure 24 hours after the ninth exposure then killed with a

sodium pentobarbital overdose (400mg/kg), and a bronchoalveolar lavage was performed to determine total and differential cell counts and cytokine levels.

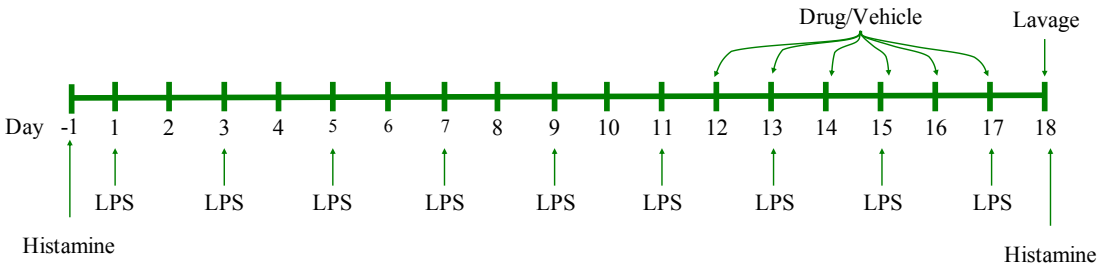


Figure 2.2: A schematic representation of the chronic LPS exposure protocol with six day drug treatment

2.2.4 Cigarette Smoke Challenge

Balb/C Mice received cigarette smoke (1R3F Kentucky research cigarettes,) or air (sham) into a sealed perspex chamber (7000cm^3) at a flow rate of 0.6 l/min for 30 minutes. Smoke was produced by peristaltic suction with 4 seconds of smoke per minute. The average particulate matter within the chamber was recorded, giving the number of small particles in the air. This was used to analyse the densitiy of smoke in the chamber and adjustments to the amount of smoke entering the chamber altered accordingly to maintain consistant readings across all groups. The average total particulate matter was 444.1 ± 1.9 tpm/M^3

2.2.5 Acute Cigarette Smoke Challenge

Mice were exposed to cigarette smoke or air for 30 mintes, twice a day, for 3 consecutive days. A minimum of 5 hours was allowed between exposures. 24 hours after the final exposure the mice were culled and a bronchalveolar lavage was perfromed to determine total and differential leucocyte numbers and cytokines.

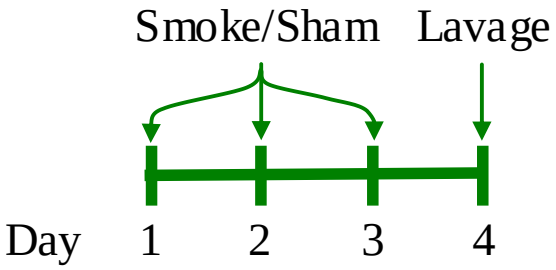


Figure 2.3: A schematic representation of the acute cigarette smoke protocol.

2.2.6 Chronic Cigarette Smoke Challenge

Mice were exposed to cigarette smoke or air for 30 minutes, twice a day, 5 days a week, for 5 weeks. On the sixth week, mice received twice daily smoke or air for 3 days. Animals were killed 24 hours after the final smoke or air challenge and a bronchoalveolar lavage was performed to determine total and differential leucocyte numbers.

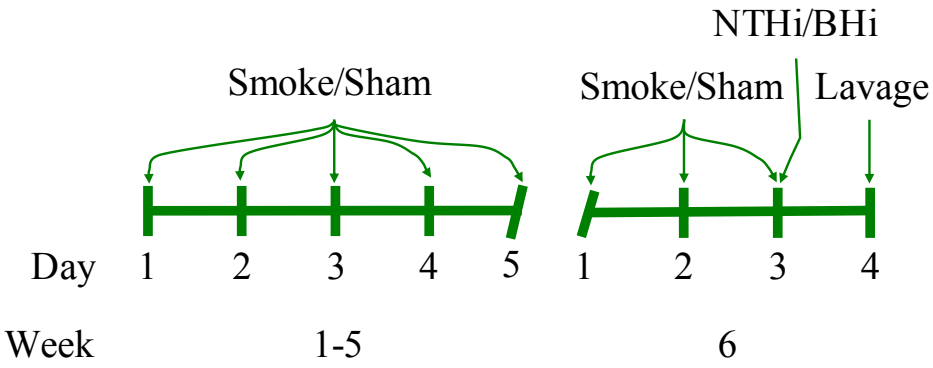


Figure 2.4: A schematic representation of the chronic cigarette smoke protocol with NTHi/BHi challenge

2.2.7 Histamine Exposures

A sub threshold dose of histamine needed to be determined to evaluate airway hyperresponsivness (AHR). Histamine is a spasmogen released primarily from mast cells upon activation (Riley and West, 1953). Histamine acts as an agonist for histamine receptors, with the H_1 receptor found to be more associated with smooth muscle in humans (Hardy *et al.*, 1996) and guinea pigs (Hill *et al.*, 1977). Histamine is therefore a bronchodilator in the airways by activation of the H_1 receptor. The

response to histamine can increase in some disease states, with some COPD patients exhibiting AHR (Hospers *et al.*, 2000).

AHR was performed by administering 0.3, 0.5 and 0.7mM histamine delivered for 2 minutes, at 20% duty per chamber and a constant flow rate of 2 litres per minute per chamber using the whole body plethysmography set up. Figure 2.5 showed 0.3mM histamine was a sub threshold dose that caused no response in naive guinea pigs. A bronchoconstrictive dose of histamine was also determined in order to evaluate the effectiveness of bronchodilator drugs, such as theophylline. 0.7mM of histamine delivered for 2 minutes, at 20% duty per chamber and a constant flow rate of 2 litres per minute per chamber, exhibited significant bronchoconstriction in naive animals.

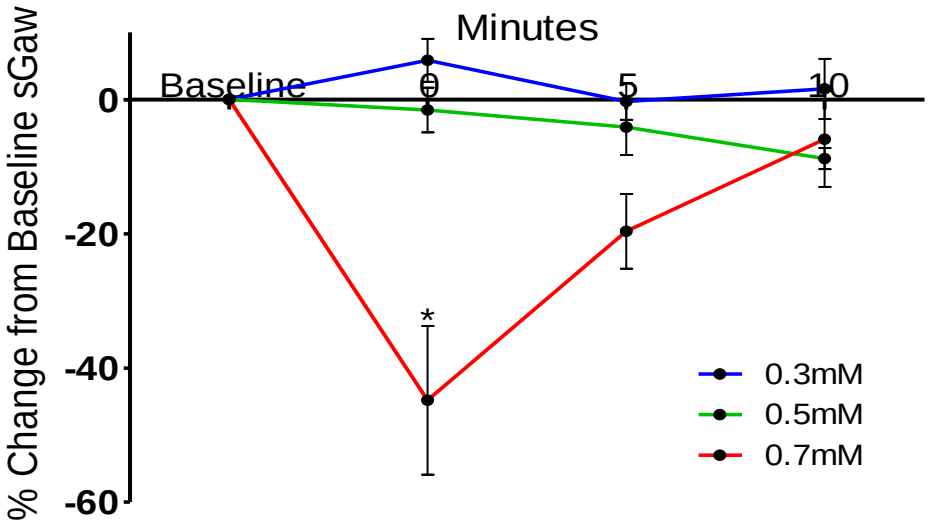


Figure 2.5: Response of the airways to a histamine exposure (0.3, 0.5 or 0.7mM) in naive guinea pigs. Mean changes in sG_{aw} are expressed as mean percentage change from baseline ± S.E.M. at 0, 5 and 10 minutes after exposure, with a negative value denoting a bronchoconstriction. * significantly different from baseline sG_{aw} values. One-way Analysis of Variance (*P<0.05; n=6).

2.3 Airway Function

The parameters analysed in the experiments were specific airway conductance (sGaw), total lung volume and static compliance of the lung. sGaw is a parameter that is used to measure bronchoconstriction in the lung by assessing the conductance of the airways. Static compliance measurements analyse the elasticity of the lung, with increased compliance signifying a loss of elasticity, while total lung volume measures the volume of air the lungs can hold. These measurements were performed one of two ways; whole body plethysmography or using a resistance and compliance set-up.

2.3.1 Whole Body Plethysmography

A whole body plethysmograph was used to measure changes in lung volume and airway resistance. Airway resistance (R_{aw}) is affected by the length, diameter and number of airways present in the lungs and is inversely proportional to the airway conductance (G_{aw}). Specific airway conductance (sG_{aw}) takes into account G_{aw} and variations in total lung volume between individuals to give a specific change in airway conductance for individuals irrespective of total lung volumes (Briscoe & Dubois, 1958).During a bronchoconstriction, the smooth muscle of the airway contracts, decreasing the diameter of the airways. This increases the resistance (R_{aw}) and thus decreases the conductance (G_{aw}).

Guinea pigs were placed in a restraint with an elastomeric rubber seal being placed over their head and around their neck. They were placed in a double chamber plethysmograph, consisting of a thoracic chamber and a nasal chamber, which was separated by the rubber seal (Figure 2.6). Each chamber contained a pneumotachograph and flow transducer, allowing the measurement of nasal and thoracic flows independently. The changes in flow were analyzed by Biosystem XA

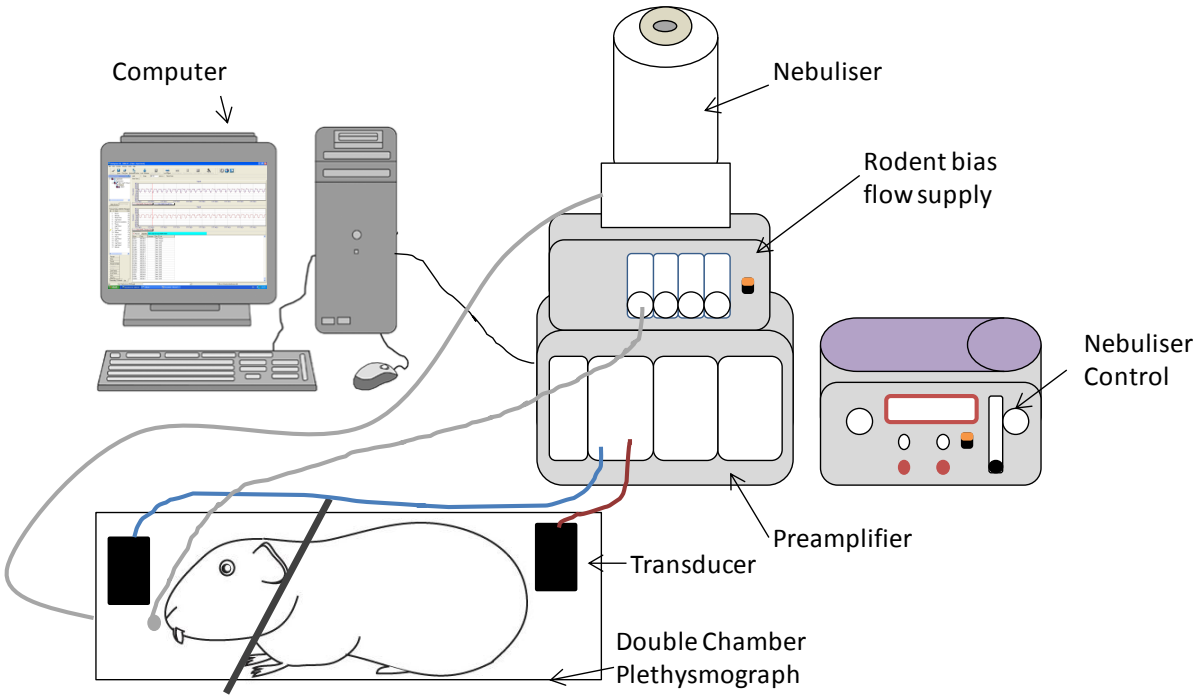


Fig 2.6: A schematic diagram of the Buxco, double-chamber plethysmograph setup.

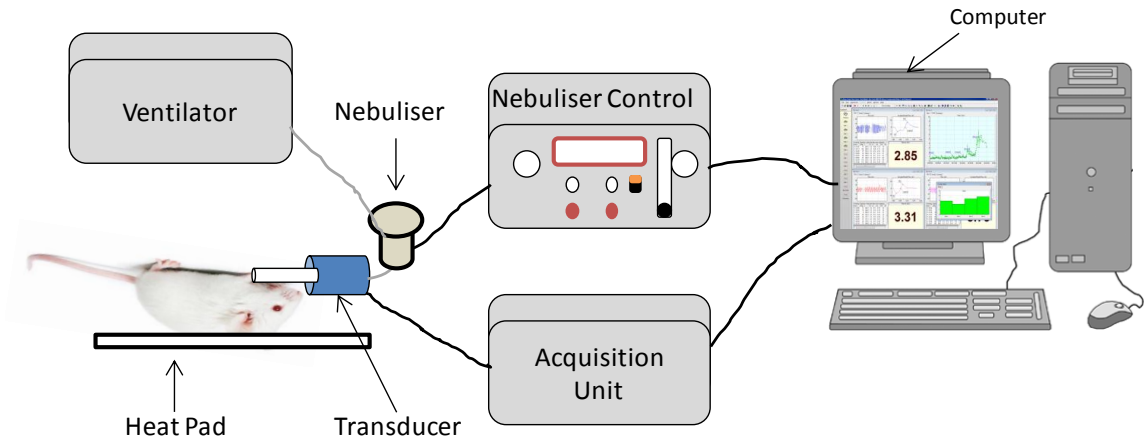


Fig 2.7: A schematic diagram of the EMMS, resistance and compliance setup.

software (Buxco®, Wilmington, North Carolina, USA) and analyzed using Non-invasive Airway Mechanics (NAM) to give values for sG_{aw}.

2.3.2 Resistance and Compliance Measurements

Resistance and compliance measurements can be used to measure a change in airway resistance, compliance and conductance, tidal volume, total lung capacity, inspiratory and expiratory flow, pressure and breathing frequency. The important parameters in COPD are compliance and total lung capacity, with both increasing due to emphysema and the loss of elasticity of the lung (O'Donnell and Lavaeneziana, 2006). Static compliance was used in the experiments, which takes into account the pressure and volume of the lung during a stable phase of breathing, i.e. on peak inspiration when

there is no variation in airflow. Static compliance is calculated using the following equation:

$$C_{stat} = \frac{V_T}{P_{plat} - PEEP}$$

Cstat = Static compliance, V_T = Tidal volume, Pplat = Plateau pressure, PEEP= positive end expiratory pressure

Static compliance is beneficial over other measurements of compliance as it is able to identify not only changes in compliance, but also over-inflation in the lung (Nikischin *et al.* 1998). Total lung capacity, as described by Lundsgaard and van Slyke (1918), is the total volume of air the lung can hold and is the vital capacity plus the residual volume. The vital capacity is the maximum amount of air a person can expire after a full inspiration. The residual volume is the amount of air left in the lungs after a full expiration.

Unlike whole body plethysmography, it is an invasive way to measure pulmonary function, with mice being anaesthetised with hypnorm/hypnoval, before the trachea was cannulated. Mice were placed on a heat mat and the cannula attached to a plethysmograph. The plethysmograph was attached to an acquisition unit, which was in turn attached to a ventilator, with the whole system being controlled by data acquisition software (EMMS, England) (fig 2.7). The software controlled the animals breathing and was able to perform forced manoeuvres tests at frequent intervals to determine total lung capacity and static compliance.

2.4 Exacerbating Agents

2.4.1 Human Parainfluenza 3 Inoculations

Human parainfluenza 3 virus was cultured in an African green monkey, renal epithelial (BSC-1) cell line to achieve a minimum viral titre of at least 10^8 virus particles per ml. The virus particles were quantified by RT-PCR following RNA extraction as described in 2.6.8.

Animals received 0.3mM histamine on day 1 to determine airway responsiveness prior to any viral inoculations taking place. Guinea pigs received either 125µl of virus or a control of virus-free media, twice in each nostril on days 2 and 3. All viral inoculations were performed in a laminar flow cupboard to prevent viral spread. As well as this, after the first inoculation with virus, animals were kept in an isolator with an independent air supply to prevent viral spread. They were housed in twos or threes, dependent on size, in plastic cages with steel cage tops. Each cage contained hay, wooden blocks and cardboard tubes for environmental enrichment. Animals inoculated with medium were returned to the main animal house facility. Animals received their second dose of 0.3mM histamine on day 7 to test for airways

responsiveness, and were then killed by a sodium pentobarbital overdose for a bronchoalveolar lavage to be performed.

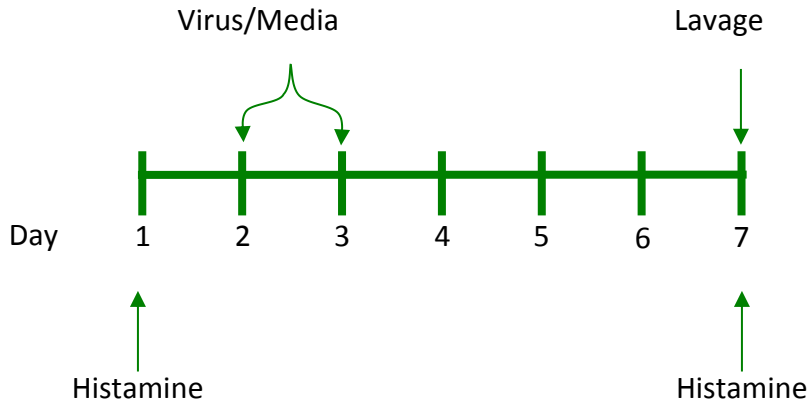


Figure 2.8: A schematic diagram of the 7 day virus/media inoculation protocol.

2.4.2 Poly I:C Inoculations

Guinea pigs received 0.3mM histamine on day 1 to determine airway responsiveness. Guinea pigs received 125µl of poly I:C (2mg/ml) or a saline control per nostril per day, on days 2, 3 and 4. Airway responsiveness to 0.3mM histamine was measured on day 5, 24 hours after the final poly I:C instillation. The animals were then killed by an overdose of sodium pentobarbital and a bronchoalveolar lavage was performed.

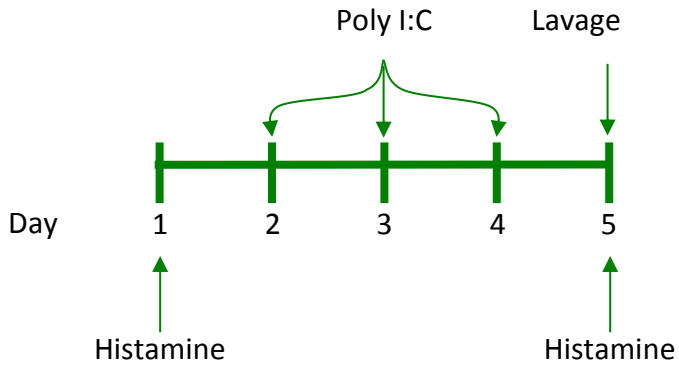


Figure 2.9: A schematic diagram of the 7 day virus/media inoculation protocol.

2.4.3 Non-typeable *Haemophilus influenzae* (NTHi) Inoculations

NTHi was grown on chocolate agar plates (Becton Dickinson, UK) before being inoculated into supplemented brain-heart infusion broth (Becton Dickinson, UK) at an optical density reading of 600nm (OD_{600}) greater than 1.1. A 1:10 dilution of the infusion was adjusted accordingly to give an OD_{600} reading of 0.4, which corresponded to $6x10^9$ CFU/ml.

Mice were anaesthetised with an intraperitoneal injection of hypnorm (0.1mg/kg fentanyl citrate/3.15mg/kg fluanisone) /hypnovel (1.55mg/kg midazolam) before they received 50µl of supplemented brain-heart infusion broth or NTHi at a concentration of $6x10^9$ CFU/ml intra nasally. Animals were killed 24 hours after intranasal administration (unless otherwise stated). All bacterial inoculations took place in a laminar flow cupboard to prevent bacterial spread. After bacterial inoculation animals were housed in groups of 5 in an OptiMice bio-containment cage system (Animal Care Systems, USA) with an independent air supply.

2.5 *In Vivo* Drug Administration

2.5.1 Corticosteroids

The effect of the corticosteroids budesonide and dexamethasone (Sigma Aldrich, UK) were evaluated against the LPS model in chapter 4. All drugs were given once a day for 6 consecutive days before the end of an experiment and were received thirty minutes before an LPS challenge. Doses, vehicles and administration can be found in table 2.1.

2.5.2 Theophylline

The effect of theophylline was evaluated in the chronic LPS model in chapter 4. Theophylline was administered orally via a gavage needle, twice a day for 6

consecutive days before the end of an experiment. Animals receiving theophylline alone did so 30 minutes before any challenge. Animals receiving both theophylline and a corticosteroid, firstly received theophylline 15 minutes before the steroid was administered. Details relating to doses, vehicles and administration can be found in table 2.2

To evaluate the bronchodilator effects of theophylline against a bronchoconstrictive dose of histamine (Chapter 4), theophylline was administered 30 minutes before a histamine challenge to allow for adequate absorption.

Drug	Dose	Vehicle	Route of Administration
Budesonide	0.6 mg/ml *s.i.d*	30% DMSO 30% Ethanol 40% Saline	Nebulised/Inhaled (15Mins)
Dexamethasone	10mg/kg *s.i.d*	30% DMSO 30% Ethanol 40% Saline	Intra-peritoneal

Table 2.1: Doses, vehicles and administration of corticosteroids

Drug	Dose	Vehicle	Route of Administration
Theophylline	• 5mg/kg • 50mg/kg *b.i.d* (~ 8 hours between doses)	0.5% Methyl cellulose and 0.1% Tween®20 in Saline	Oral (Gavage Needle)

Table 2.2: A table showing the dosage, vehicle and route of administration for drugs

2.6 Posthumous Analysis

2.6.1 Bronchoalveolar Lavage of the Guinea Pig

At the end of all experiments, animals were killed by an overdose of sodium pentobarbital (Euthatal (400mg/kg)) by bilateral intraperitoneal injection. An incision was made into the neck and the trachea was cannulated using polypropylene cannulas (7-9FG dependent on trachea size). The lungs were removed, with the right lobes being clamped at the bronchi. 0.5ml/100g of saline was instilled into the left lobes where it was left for three minutes before removing the bronchoalveolar lavage fluid (BALF) with a syringe. This process was repeated one more time to maximise BALF return. All lobes were tied off apart from the largest lobe of right lung. The clamp was then removed and the large right lobe insuflated with 10% formaldehyde at a constant pressure of 25mm/H_2O. The largest lobe of right lung was stored in 10% formaldehyde for further histology explained in 2.8, the remaining right lobes were stored at - 80°C for further analysis.

2.6.2 Cellular Influx

Total and differential cell counts were determined in the BALF. Total cell counts were performed using a Neubauer haemocytometer to determine total cells per ml. 100µl of BALF was placed under the cover slip which was evenly distributed by capillary action and viewed at 100X magnification. The haemocytometer has a 25 square grid, with cells present within five of these squares (top left, top right, middle, bottom left and bottom right) being counted. These numbers were multiplied by 5 and averaged to determine the number of cells in the 25 square grid. Each square has an area of 0.04mm^2, meaning the 25 squares have an area of 1mm^2. The depth of the haemocytometer is 0.1mm meaning the volume is only 0.1mm^3, therefore the value must be multiplied by 10,000 to achieve cells per 1cm^3 or cells per ml.

To determine differential cell counts, slides of the BALF were made by placing 100µl of each BALF sample into a funnel of a slide maker which was then spun at 1000rpm for 7 minutes using a Shandon cytospin (Thermo Scientific, UK). The slides were allowed to air dry for 5 minutes before being stained using Leishman's stain (1.5% in 100% methanol)(Sigma Aldrich UK) for 5 minutes, then rinsed and allowed to dry overnight. Microscopy was then carried out at 1000X, counting 200 cells, differentiating between neutrophils, monocytes/macrophages, eosinophils and lymphocytes.

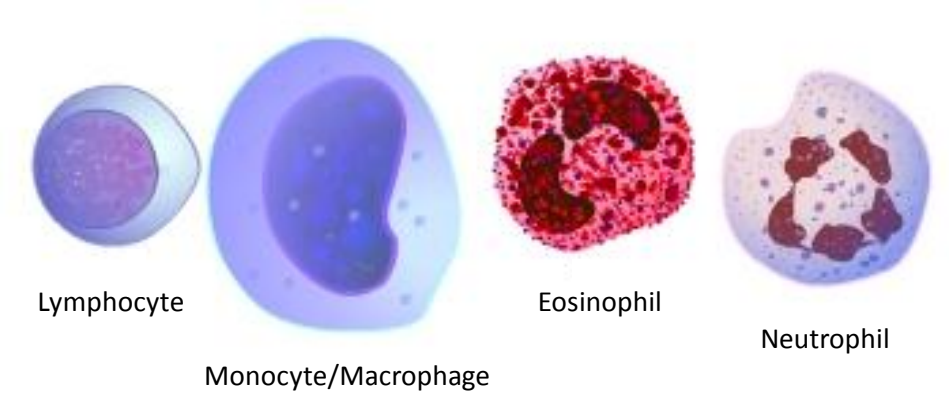


Figure 2.10 Different leucocytes identified in the BALF after Leishmans stain (Adapted from www.rnceus.com).

These were used to calculate the percentage of each cell, and along with the total cell counts, the number of each cell per ml. The remaining BALF was spun at 3800rpm for 6 minutes and the supernatant stored at -80°C for biochemical analysis. The pellet was discarded.

2.6.3 Guinea Pig Lung Homogenisation

The lung was homogenised using a Precellys tissue homogeniser (Precellys, France) at 100mg of lung per 1ml of sterile phosphate buffered saline (PBS). The tissue homogeniser was run on a 3 x 5second cycle with 15 second gaps. The homogenised

lung was then spun at 1300rpm for 1 min to pellet the remaining tissue, with the supernatant being stored at -80°C for further analysis.

2.6.4 Quantification of Guinea Pig Total Protein

Total protein was determined using a protein assay kit (Fisher, UK). 10µl of standard protein dilutions of bovine serum albumin (BSA) ranging from 2mg/ml to 0.01mg/ml, as well as a blank were placed in duplicate in a 96 well microtitre plate. 0.5 µl of each sample was placed in the plate in duplicate before 200 µl of working reagent was added to each well. The plate was sealed with a plastic lid and placed in an incubator at 37°C for 30 minutes. The plate was then removed and allowed to cool at room temperature for 5 minutes before being read on a spectrophotometer at 540nm. A normalised curve of standards was produced and the equation of the line used to determine the unknown protein concentration of the samples.

2.6.5 Guinea Pig Cytokine Analysis

Cytokine analysis was performed on bronchoalveolar lavage fluid and homogenised lung tissue by ELISA (R&D duoset kits, R&D, UK). BALF was placed in the plate neat while a 1:5 dilution was required for the lung tissue.

2.6.6 TNF-α

TNF-α ELISAs were performed as per the instructions. Briefly, high affinity 96 well plates were coated with 100 µl of the capture antibody, mouse anti guinea pig TNF-α at a concentration of 4 µg/ml per well overnight at room temperature. Excess capture antibody was then washed off with phosphate buffered saline with Tween 20 (PBST) 3 times before being blotted dry. 300µl of Reagent Diluent (RD) (1% BSA in PBS, pH 7.2-7.4 (0.2µm filtered)) was added to each well to block unbound sites and prevent non specific binding and incubated for 1 hour at room temperature. Wells

were then washed 3 times with PBST before 100µl of sample, standards or blank was added and incubated for 2 hours at room temperature. Standards were a seven point standard curve using 2-fold serial dilutions with the highest concentration being 2ng/ml. Plates were the washed again three times with PBST. 100µl of detection antibody at 4µg/ml was then added to each well and incubated for 2 hours at room temperature. Plates were again aspirated and washed 3 times with PBST before being blotted dry.

100µl of enzyme-labelled (horseradish peroxidase) Streptavidin, 1/200 dilution was added to each well and incubated for 20 minutes at room temperature, avoiding direct light. Unbound label was washed off 3 times with PBST and blotted dry. 100µl of the enzyme substrate, o-Phenylenediamine, was added to each well and incubated at room temperature for approximately 20 minutes, or until the colour develops sufficiently. 50µl of the stop solution (2.5M sulphuric acid) was added to each well and the plate read at 490nm on a spectrophotometer.

2.6.7 CXCL-8

CXCL-8 duosets were performed exactly the same as the TNF-α duosets with the only difference being the working concentration of the detection antibody in the CXCL-8 kit at 20ng/ml. However, unlike the TNF-α duoset which is guinea pig specific, a specific CXCL-8 kit did not exist for guinea pigs resulting in a human kit being used.

2.6.8 Quantification of Virions

2.6.8.1 RNA Extraction

RNA extraction was performed on leukocyte-free BALF and on a sample of homogenised lung for all groups that received virus or media. RNA extraction was performed using a high pure viral nucleic acid kit (Roche, UK) according to the

method supplied. Briefly 200µl of BALF or homogenised lung was added to 200µl of working solution (30µl of poly A and 1.5ml of binding buffer) and 50µl of proteinase K and incubated at 72°C for 10 minutes. Binding buffer was then added before the entire sample is transferred to a high filter tube, placed in a collection tube and centrifuged (13,000RPM for 1 minute). Inhibitor removal buffer was added and the sample was centrifuged again. Wash buffer was added, the sample centrifuged, and then the wash buffer step was repeated. Elution buffer was then added, the sample was centrifuged for a final time discarding the filter tube and the fluid containing viral RNA was aliquoted into cryotubes for storage at -80°C.

2.6.8.2 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed on extracted RNA (from 2.6.8.1) using a SuperScript first-strand synthesis kit (Invitrogen, UK) according to the method supplied. Briefly 8µl of sample was added to 1µl of dNTP mix and 1µl of RNA/primer mix, which along with a negative and positive control was incubated at 65°C for 5 minutes, before being placed on ice for 1 minute. 10X RT buffer, 25mM MgCl2, 0.1M DTT and RNase OUT was then added to each and then incubated for 2 minutes at 25°C. Superscript II RT was then added to each tube, except the negative control, and incubated at 25°C for 10 minutes, 42°C for 50 minutes and 70°C for 15 minutes before being chilled to 4°C. A LightCycler TaqMan Master Kit (Roche, Germany) was used to quantify the DNA produced by the thermal cycle step, according to the method supplied. 5µl of each sample was placed in a capillary tube with PCR grade water, PIV3 primers and the master mix solution. The capillaries were centrifuged at 700G for 5 seconds, before being placed in the light cycler, programmed for 1 cycle of 95°C for 10 minutes, 50 cycles of 95°C for10 seconds then 60°C for 1 minute and finally 1 cycle of 40°C for 30 seconds. The lightcycler software then supplies

number of virus particles per µl, which was converted into number of virus particles/ml.

2.7 Bronchoalveolar Lavage of the Mouse

At the end of all experiments, animals were killed with an overdose of a 1 in 10 dilution sodium pentobarbital (Euthatal (400mg/kg)) by intraperitoneal injection. The abdomen was cut open and blood was removed from the inferior *vena cava* by a needle and syringe containing 50µl of EDTA before the animal was exsanguinated by cutting the descending aorta. An incision was then made into the neck and the trachea was cannulated. 0.4mls of saline was instilled into the lungs and immediately removed three times using a syringe to obtain BALF samples. The lungs were then removed from the chest and the right lung tied and removed. The top right lung lobe was removed for analysis of tissue cytokine and chemokine levels. Two of the right lung lobes were removed for bacterial viability analysis if applicable. The remaining right lung lobe was stored at -80°C for later gene expression analysis if required. The left lung was then inflated with 10% Neutral Buffered Formalin (10% NBF) at a constant pressure of 25cm/H_2O. Once the lungs were fully inflated the trachea was tied off, the lungs removed and placed in a pot of 10% NBF and sent to the histology department for histological processing and staining.

2.7.1 Cellular Influx

Total and differential cell counts were determined by examining the BALF.

2.7.2 Total Cell Counts of the Mouse

The BALF was centrifuged at 1400rpm for 10 minutes at 4°C. The supernatant was aliquoted into tubes and stored at -80°C for further analysis. The pellet was resuspended in 0.5ml of methyl violet fixative and was thoroughly vortexed. 15µls

was then pipetted into the haemocytometer, with 3 large squares being counted. Samples containing large numbers of cells were further diluted to increase accuracy. The total numbers of cells was multiplied by 10 and then multiplied by the total volume the cells were suspended in to give the number of cells per ml.

2.7.3 Differential Cell Counts of the Mouse

100µl of uncentrifuged BALF was placed into a cytofunnel of a slide maker and spun at 700rpm for 5 minutes using a Shandon cytospin (Thermo Scientific, UK). Slides were allowed to air dry for 5 minutes before being stained using a Bayer Hematek slide stainer (Bayer, UK). Slides were stained with an accustain automated Wright-Giemsa stain (Sigma Aldrich, UK). Slides were then allowed to air dry for several minutes before being mounted and cover slipped. Microscopy was then carried out at 1000X, counting 200 cells, differentiating between neutrophils, monocytes/macrophages, eosinophils and lymphocytes.

2.7.4 Mouse Blood Processing

Blood samples were spun at 3500rpm for 10 minutes before the plasma was removed, aliquoted and stored at -80°C for further analysis

2.7.5 Mouse Lung Homogenisation

2.7.6 Homogenisation for Cytokine Analysis

A lobe of right lung was placed into 1 ml of phosphate buffered saline containing a protease inhibitor cocktail (Roche, UK). The lobes were homogenised for 30 seconds using a handheld tissue homogeniser (Fisher, UK).

2.7.7 Homogenisation for Bacterial Viability Analysis

Two right lobes of lung were placed in 1 ml of sterile brain-heart infusion broth (Becton Dickinson, UK). The lobes were homogenised for 30 seconds using a handheld tissue homogeniser (Fisher, UK).

2.7.8 Quantification of Mouse Total Protein

The quantification of mouse total protein was performed in the same way as previously described for the guinea pig (2.6.4)

2.7.9 Mouse Cytokine Analysis

Cytokines were analysed using the MesoScale Discovery system (MesoScale Discovery, USA) as per the instructions. Briefly, a mouse pro-inflammatory 7-plex base kit was used, which contained 96-well pre-coated plates with Interferon-ɣ, IL-1β, IL-10, IL12p70, IL6, CXCL1 and TNF-α capture antibodies. A 7 point standard curve was prepared by 1 in 4 serial dilutions in PBS; with the highest standard being 10000pg/ml. 25µl of standard/sample or blank was placed into the wells of a pre-coated plate, covered and incubated at room temperature with vigorous shaking (300-1000rpm) for 1 hour. The detection antibody was prepared by performing a 1 in 50 dilution of the provided detection antibody in Diluent 100 (MSD, USA) and adding 25µl to each well. Again, the plates were sealed and incubated with vigorous shaking for 1 hour at room temperature. The plate was then washed 3 times using PBST and blotted dry. The read buffer was then prepared by diluting 1 in 2 in dH_20 and adding 150µl to each well. Plates were then analysed in an MSD SECTOR plate reading instrument (MesoScale Discovery, USA).

2.7.10 Quantification of Colony Forming Units

100µl of lung homogenised in brain heart infusion broth (as described in 2.7.7) was streaked on chocolate agar plates (Becton Dickinson, UK) with a large dilution range

using 1 in 10 serial dilutions in BHi broth. The plates were incubated at 37°C in 5% CO_2 for 16 hours. Plates were read on a protoCOL 2 colony counter (Synbiosis, UK) with the limit of detection ranging from a minimum of 30 to a maximum of 300 colonies per plate. Plates falling below 30 were considered to have no growth while plates exceeding 300 were considered too confluent for accurate counting. The relevant dilution factors were input into the programme to give colony forming units (CFU)/ml

2.8 Histological Analysis

2.8.1 Tissue Processing

Histology was performed on the lobe of unlavaged, insuflated lung that had been stored in 10% formaldehyde. Two 3-5mm slices of lung were cut 1mm below the bronchus and placed in a histology cassette for processing using the following protocol:

50% industrial methylated spirit (IMS) – 1 hour

70% IMS – 1 hour

90% IMS – 1 hour

100% IMS – 1 hour 30 minutes

100% IMS – 1 hour 30 minutes

100% IMS – 1 hour 30 minutes

50% IMS:50% chloroform – overnight

Chloroform – 1 hour 30 minutes

Chloroform – 1 hour 30 minutes

Paraffin wax – 6 hours

Once the lungs had been processed, they were embedded in paraffin wax blocks. This was performed by placing the lung sections in a metal mount, covering them with molten paraffin wax and allowing them to harden on a cold plate. The embedded samples were cut into 3-5µm sections using a microtome and mounted onto polysine coated glass slides (Thermo Scientific, UK). The slides were allowed to dry overnight at 37°C before being stained.

2.8.2 Tissue Rehydration/Dehydration

Tissue section were rehydrated using the following procedure:

Histoclear – 5 Minutes

Histoclear – 5 Minutes

100% IMS - 5 Minutes

100% IMS - 5 Minutes

90% IMS - 5 Minutes

70% IMS -5 Minutes

50% IMS - 5 Minutes

Distilled Water –5 Minutes

Dehydration of the tissues was performed after staining by following this procedure in reverse

2.8.3 Haematoxylin and Eosin Stain - General Morphology

Mayer's Haematoxylin – 3 minutes

Rinse in distilled water

Running tap water – 5 minutes

Acid ethanol – dip 8-12 times to destain

Running tap water – 2 minutes

Distilled water – 2 minutes

Eosin – 20 seconds

2.8.4 Alcian Blue/Periodic Acid Schiff Stain - Mucous

1% Alcian blue dissolved in 3% aqueous acetic acid (pH 2.5) – 5 minutes

Running tap water – 5 minutes

Periodic acid (0.5%) – 5 minutes

Running tap water – 5 minutes

Distilled water – 5 minutes

Schiff's reagent – 10 minutes

Running tap water – 10 minutes

Mayer's haematoxylin – 20 seconds

Running tap water – 5 minutes

2.8.5 Mean Linear Intercept

Mean linear intercept was calculated by taking a photograph of the haematoxylin and eosin stained slides at X100 magnification. Two lines were drawn across the photograph, one in the centre horizontally and one in the centre vertically. The number of times the line intercepted an alveolar wall was recorded and the values averaged. If the line intercepted an airway or a blood vessel the line was moved up or right appropriately until it was no longer intercepting the artefact.

2.9 Monocyte Derived Macrophages

Blood was acquired from Novartis's on site donor panel with all relevant consent acquired and donors remaining anonymous. Briefly, 20mls of blood was added to 2mls of EDTA in sterile falcon tubes before 10mls of PBS and 10mls of 4% PBS-Dextran was added to each falcon tube, mixed by gentle inversion and then left on ice for 30 minutes to allow for erythrocyte sedimentation. Following sedimentation, 30mls of the supernatant was slowly layered on top of 15mls of Ficoll-Paque plus (GE Healthcare, UK) and centrifuged at 1800rpm for 20 minutes at 4°C.

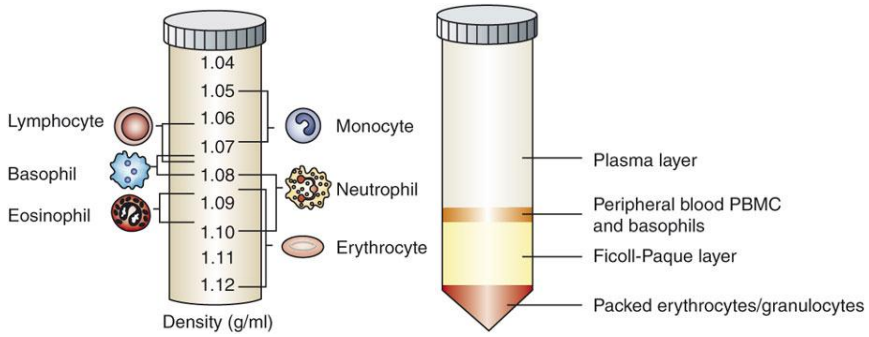


Figure 2.11 The density separation of blood using Ficoll (Munoz and Leff, 2007)

The peripheral blood mononuclear cell (PBMC) layer was then removed into sterile falcon tubes and topped up to 50mls with PBS before being centrifuged again at 1400rpm for 10mins. The supernatant was discarded and the pellet was re-suspended in 1ml of PBS. The cells from the same donor were pooled by re-suspending the pellets in the same 1ml of PBS with cell counts then being performed. 10-15mls of PBS was then added and the tubes centrifuged at 1300 rpm for 10mins at 4°C. The supernatant was discarded and the pellet re-suspended in 80µl of MACS buffer (PBS, pH 7.2, 2 mM EDTA and 0.5 % bovine serum albumin) per 10^7 cells before 20µl of $CD14^+$ microbeads (Miltenyibiotec, Germany) per 10^7 cells was added, mixed and incubated at 4°C for 15minutes. A MACS separating column

(Miltenyibiotec, Germany) was placed in a magnetic MACS separator (Miltenyibiotec, Germany) and prepared by rinsing 3 times with 3mls of MACS buffer before the cell suspension was added into the column. The column was then washed 3 times with 3mls of MACS buffer to collect unlabelled cells. The column was then placed in a falcon tube with 5mls of MACS buffer being flushed through the column to remove the labelled cells. The cells were centrifuged at 1300rpm for 10mins before being re-suspended in 1ml of RPMI 1640 (Invitrogen, UK). Total cell counts were performed before the cells were diluted in the relevant volume of RPMI 1640 with 10% foetal calf serum (Invitrogen, UK), 1% glutamine, 1% penicillin/streptomycin and 2µg/ml GM-CSF to acquire a concentration of $1x10^6$ cells per ml. Cells were seeded into wells at a concentration of 100,000 cells per well in the 96 well format and 30,000 cells per well in the 384 well format. Cells were allowed to adhere for 4 days before the media was changed

2.9.1 Cigarette Smoke Extract/LPS Challenge of Monocyte Derived Macrophages (MDM)

Cigarette smoke extract was produced by passing five cigarettes (1R3F Kentucky research cigarettes) through 100mls of RPMI 1640 media at a flow rate of 0.6l/min. This solution was considered 100% cigarette smoke extract (CSE). 1 in 10 serial dilutions were performed ranging from 3% to 0.03% CSE, 1 in 10 serial dilutions of LPS (E. Coli O26:B6) were also performed ranging from 1ng/ml to 0.01ng/ml in GM-CSF enriched RPMI 1640. MDMs were treated with the dilutions of CSE, LPS or GM-CSF RPMI control on the fifth day of the experiment. Media was changed on days 8 and 10 of the experiment and replaced with GM-CSF enriched media containing CSE, LPS or GM-CSF alone. Cells were challenged continuously for a total of 1 week before parameters were analysed.

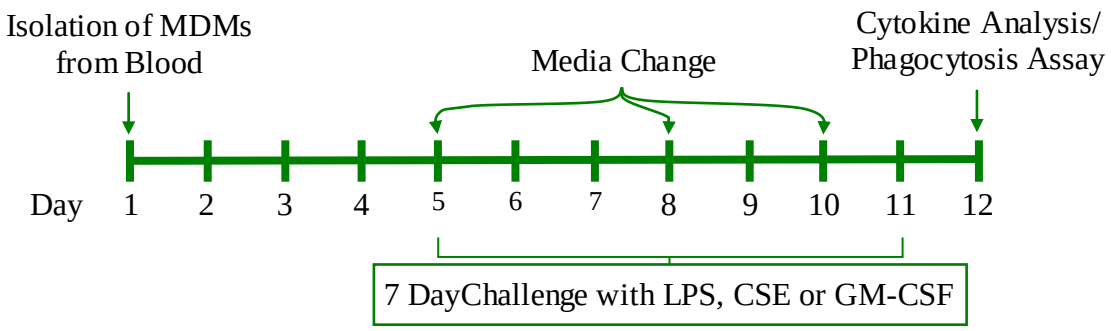


Figure 2.12 A schematic diagram of the monocyte-derived macrophage protocol.

2.9.2 Cytokine Analysis

On the twelfth day of the experiment the supernatant was removed and stored at -80°C for further cytokine analysis. After defrosting, cytokines were analysed using the MesoScale Discovery system previously described in 2.9.9. The Human 10 Plex base kit was used, which contained plates pre-coated in antibodies to detect GM-CSF, IL-1β, IL-10, IL-12p70, IL-2, IL-4, IL-5, IL-6, CXCL-8 and TNF-α. Cytokine analysis was performed on cells seeded in the 96 well format.

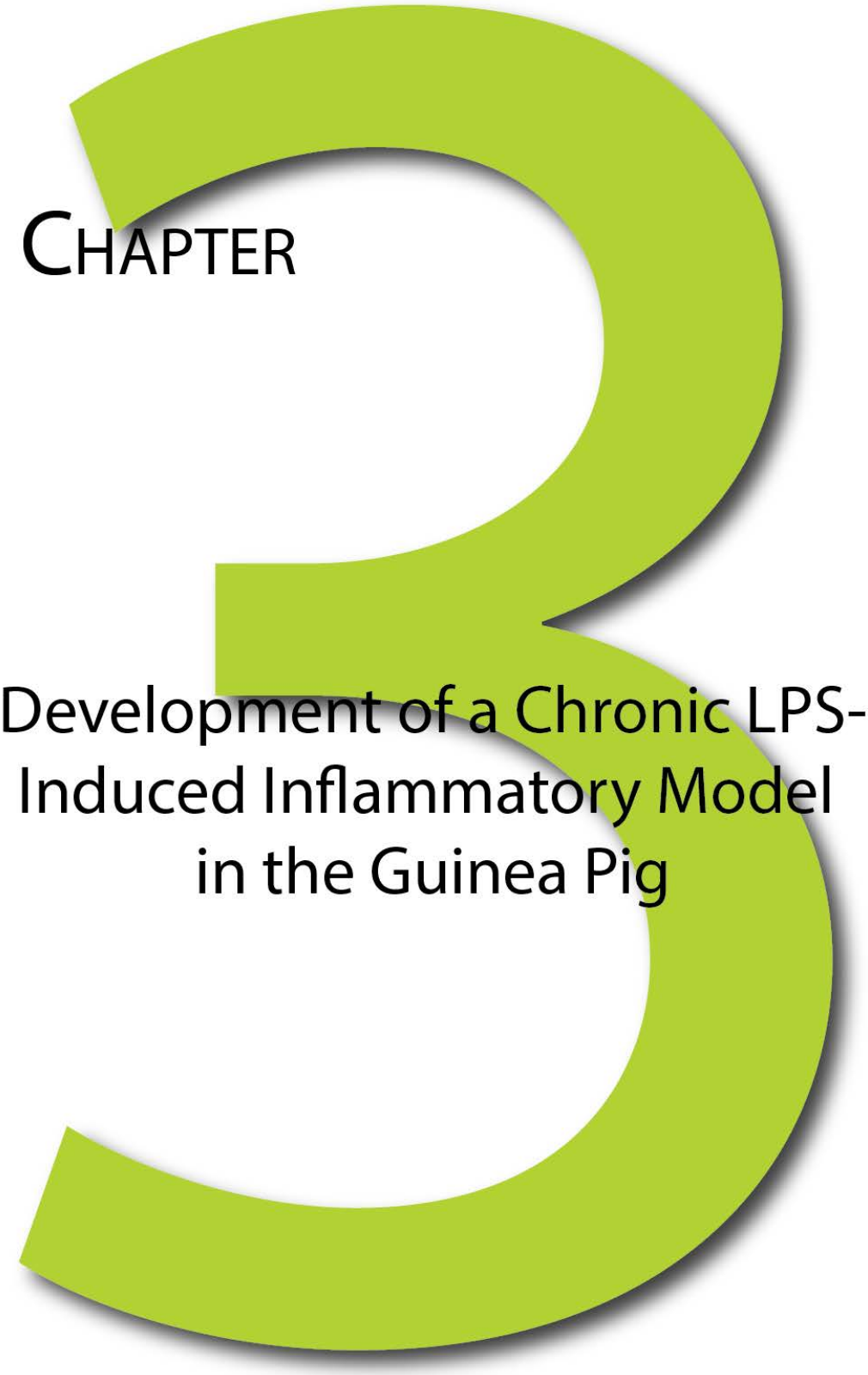
2.9.3 Phagocytosis Assay

On the twelfth day of the experiment a phagocytosis assay was performed on cells seeded in the 384 well format. Briefly, pHrodo™ BioParticles® fluorescent particles (Invitrogen, UK) were defrosted before 2ml of Uptake Buffer (Hank's balanced salt solution containing 20mM hydroxyethyl piperazineethanesulfonic acid, pH7.4) was pipetted into the vial. 100µls of opsonising reagent (Invitrogen, UK) was added to the bioparticles and the solution was thoroughly vortexed to completely re-suspend the particles and incubated for 1 hour at 37°C. 13mls of uptake buffer was then added and the tube centrifuged at 1000g for 15 minutes at 20°C to wash the particles. The supernatant was discarded and the pellet re-suspended in 800µl of uptake buffer. The media was then aspirated from the cells and 15µl of uptake buffer was added to

each well. 2.5µl of 50µg/ml cytochalasin D was added to the relevant wells as a negative control to inhibit phagocytosis with 2.5µl of uptake buffer added to all other wells and incubated for 30 minutes at 37°C. 2.5µl of bioparticles was then added to all wells and the plate centrifuged at 300g for 1 minute to settle the particles. The plate was immediately read at an excitation wavelength of 544nm and read at an emission wavelength of 612nm in a Spectramax florescence plate reader. The plate was read every 30 minutes up to 4 hours with incubation at 37°C between readings.

2.10 Statistical Analysis

All results were plotted as mean ± standard error of the mean (SEM). sGaw measurements were standardised to percentage change from baseline, to take calibration differences of the plethysmograph into account and was used to determine area under the curve falling below baseline. Cytokines measured from monocyte derived macrophages were standardised to percentage of controls to take into count large variation in baseline values between samples. Graphs were drawn and results statistically analysed using GraphPad Prism 5. In all studies where two points from different groups were compared a Mann-Whitney test was performed. In studies where two points from the same group were compared a paired student's t-test was used. If three or more groups were being compared a Kruskal-Wallis with a *post hoc* Dunn's test was used.



CHAPTER 3

Development of a Chronic LPS-Induced Inflammatory Model in the Guinea Pig

3.1 Lipopolysaccharide Exposure as a Model of COPD

3.1.1 Lipopolysaccharide

Lipopolysaccharide (LPS) is the outer component of gram negative bacterial cell walls. It confers resistance of the bacteria to host defence mechanisms and decreases the uptake of antibiotics. It is also referred to as endotoxin, and can cause septic shock in patients who have a systemic infection (Greenwood *et al.,* 2007). LPS is comprised of lipids and carbohydrates, and is split into four main units, lipid A, disaccharide diphosphate, core oligosaccharide and a repeating O-antigen side chain. The immunologically active component of LPS is lipid A, due to its interaction with the innate immune system (Lu *et al.,* 2008) (Figure 3.1). There is a scientific rationale in using LPS as a stimulus for COPD models. Studies have demonstrated LPS is present in large quantities in cigarette smoke (Hasday *et al.,* 1999), with both activating TLR4, inducing similar inflammation (Doz *et al.,* 2008).

3.1.2 LPS and Toll-like receptor 4

LPS is unable to activate an immune response on its own. A transporter protein called lipopolysaccharide binding protein (LBP) is needed to transport the LPS to TLR-4. LPS binds to LBP forming an LPS-LBP complex, which then travels to TLR-4 (Fig 3.2). Binding to TLR 4, and its associated co-receptor CD14, initiates the transcription of inflammatory mediators and cytokines. This is done via two pathways, the MyD88-dependent and independent pathways (Lu *et al.,* 2008). The pathways are summarized in figure 3.3, and result in the release of pro-inflammatory cytokines, such as TNF-α and CXCL8, via activation of NF-kB and AP-1 (Lu *et al,* 2008; Zughaier *et al,* 2004; Bagchi *et al,* 2007). Lipopolysaccharide exposure, although acting via a singular pathway and mechanism, results in a similar influx of cell types into the lungs, as observed in COPD (Aul *et al.,* 2012).

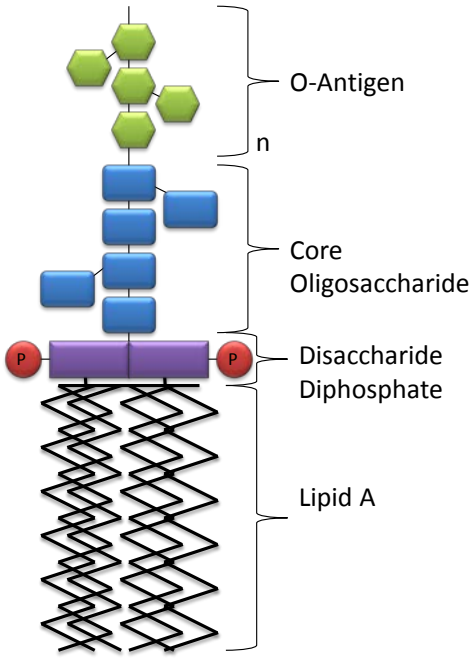


Fig 3.1: The structure of lipopolysaccharide. Lipopolysaccharide consists of lipid A, disaccharide diphosphate, core oligosaccharide and a repeating O-antigen side chain

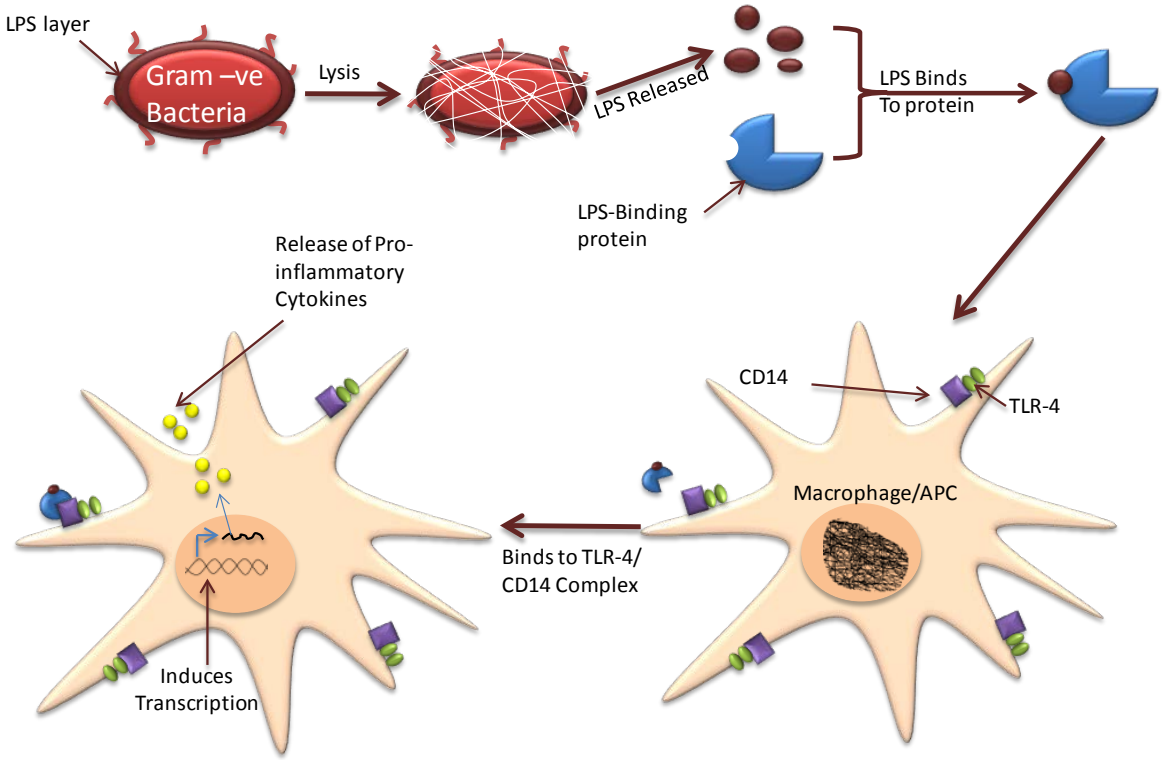


Fig 3.2: Transport of LPS to TLR4/CD14 via LPS-binding protein. Lysis of gram negative bacteria results in the release of free lipopolysaccharide which is scavenged by lipopolysaccharide binding protein. This LPS-LBP complex travels to Toll-like receptors on the cell surface, which along with CD14, induces transcription of pro-inflammatory cytokines via the MyD88 dependent and independent pathways. (Adapted from student.ccbcmd. edu).

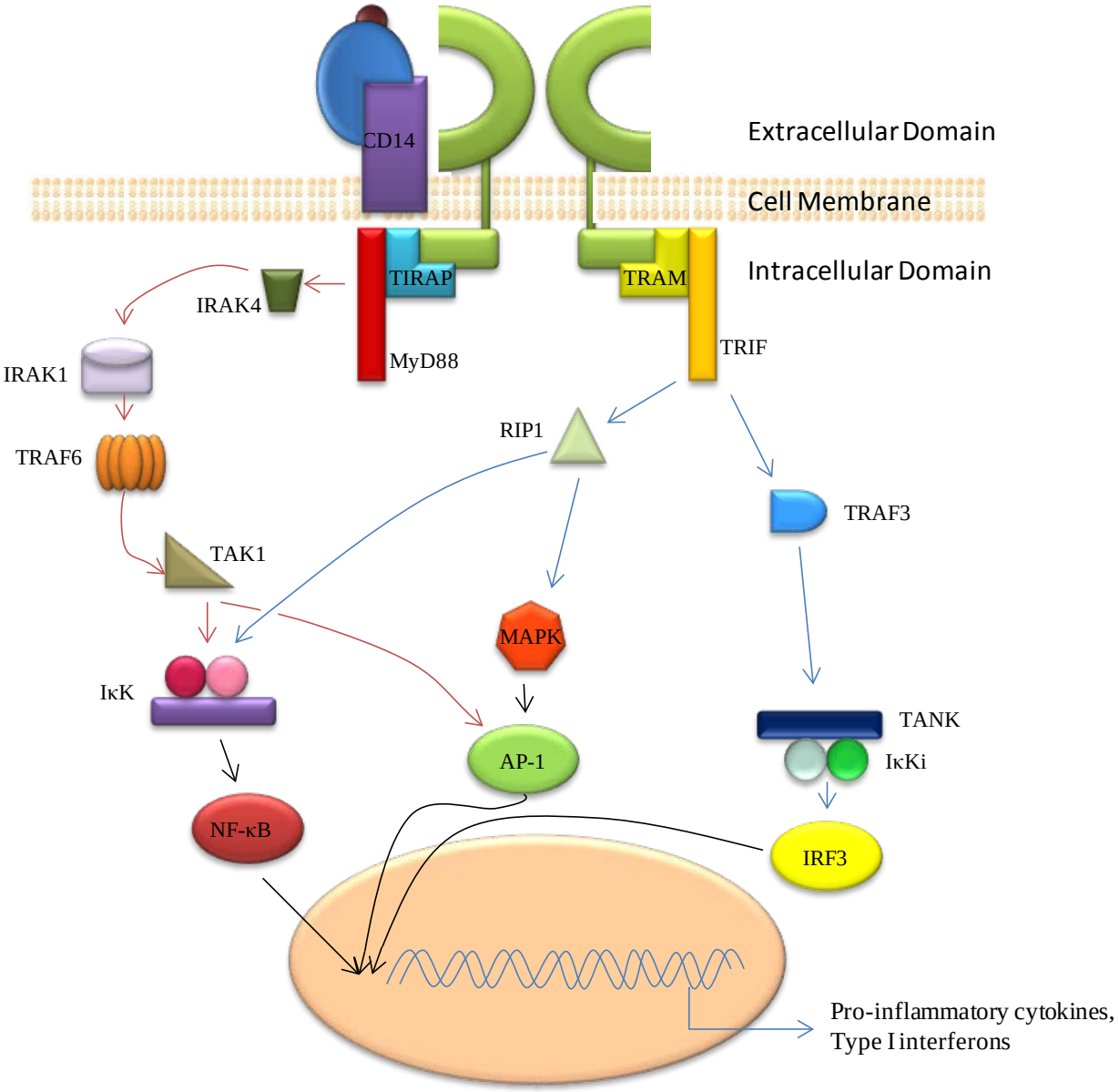


Fig 3.3: The MyD88 dependent (red arrows) and independent (blue arrows) pathways. Upon activation, the MyD88 dependant pathway activates IRAK-4, which in turn activates IRAK-1, causing the formation of a complex between TRAF-6 and ubiquitin-conjugating enzymes (UBC13 and UEV1A). These complexes activate TAK1, which then activates IKK and MAPK. Phosphorylation of IKKβ causes the transcription of pro-inflammatory cytokines by translocating NF-κB. MAPK also activates AP-1, which also causes expression of pro-inflammatory cytokines. The MyD88 independent pathway uses TIR-domain-containing adapter-inducing interferon-β (TRIF), instead of the MyD88 adaptor protein. This activates RIP1 causing the translocation of NF-κB and AP-1 by the same mechanisms as the MyD88 dependant pathway. TRIF also recruits TRAF3 which associates with TANK and IKKi to cause the translocation of IRF3, which causes transcription of type 1 Interferons. (Adapted from Lu *et al*, 2008).

3.1.3 Acute or Chronic LPS Exposure

Acute LPS exposure has been shown to differ from chronic LPS exposure in several ways. It is important for a model of COPD to exhibit numerous aspects of the disease, including similar inflammation, histological changes, with increased emphysema, and decreases in lung function. It was previously mentioned that LPS has been shown to exhibit similar inflammation to COPD (Aul *et al.,* 2012), with increases in neutrophils and macrophages in the lung. However, no acute model has been shown to have emphysematous changes or a decline in lung function. Kaneko *et al* (2007) demonstrated histological changes in a chronic LPS exposure model in the guinea pig after 15 exposures to LPS; however, this is a long protocol and takes approximately one month to achieve these changes. A model that demonstrates the phenotype of COPD in a shorter time frame could speed up research into the disease.

3.2 Aim

The aim of this chapter is to develop a chronic LPS-induced model of inflammation in the guinea pig.

3.2.1 Objectives

Investigate the response to a single challenge of LPS as well as LPS administered every 24 hours or 48 hours up to 9 exposures by:

- Measuring inflammatory cell influx, TNF-α and CXCL8 release in the BALF and lung tissue
- Investigating the effect of LPS exposure on lung function, histology and airway hyperresponsiveness.

3.3 Methods

3.3.1 Animal Husbandry

Animals were housed as described in 2.1

3.3.2 Challenges

3.3.2.1 Lipopolysaccharide Exposures

Guinea pigs received 30µg/ml of lipopolysaccharide (LPS) (unless otherwise stated) (*E. Coli* 026:B6 in saline, Sigma), or a control solution of saline, nebulized using a Wright nebulizer at 0.3mls/min at a constant pressure of 20 psi in a sealed Perspex chamber (15x15x32cm) for 1 hour.

3.3.2.2 Acute Lipopolysaccharide Exposures

A single 30µg/ml LPS or saline exposure was performed before the guinea pigs were culled 24 hours after the exposure. A bronchoalveolar lavage was performed (as described in 2.6) to determine total and differential leucocyte numbers, as well as cytokine levels.

3.3.2.3 Chronic Lipopolysaccharide Exposures

Exposures were performed using the method described by Toward and Broadley (2001). Guinea pigs were exposed to **30µg/ml** of LPS or saline **every other day until** a total of nine exposures were reached, or **15µg/ml every day** for nine days. Airway function was recorded using whole body plethysmography (Buxco) using specific airway conductance (sG_{aw}) as the measured parameter. sG_{aw} was measured at 0 minutes, 1, 2, 3 and 4 hours after exposure and compared to measurements performed immediately prior to the corresponding LPS or saline exposure. This allowed for percentage change in sG_{aw} to be calculated. Area under the curve (AUC) analysis was also performed on data that fell below baseline after exposure 1, 5 and 9, chosen as they were the first, middle and last exposure. Animals were killed 24

hours after the ninth exposure with a sodium pentobarbital overdose (400mg/kg), and a bronchoalveolar lavage was performed to determine total and differential cell counts and cytokine levels.

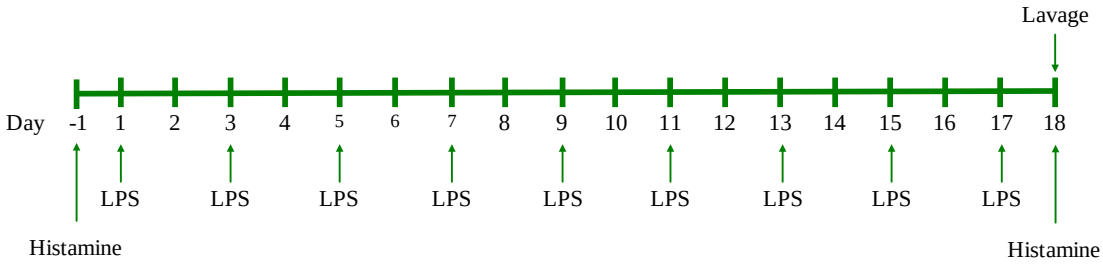


Figure 3.4 A diagram representing the chronic protocol for saline/LPS challenge in the guinea pig.

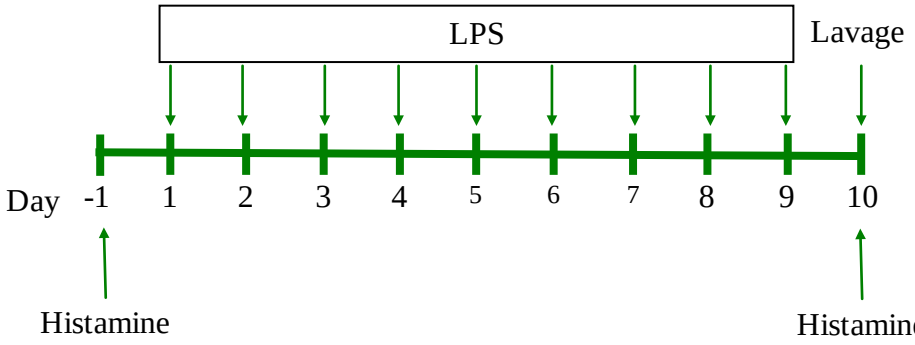


Figure 3.5. A diagram representing the consecutive 15µg/ml LPS protocol in the guinea pig

3.3.3 Measurement of Airway Responsiveness

Airway hyperresponsivness was determined via whole body plethysmography. A dose of 0.3mM histamine was used, as this was a sub threshold dose causing no response in animals before a challenge but was able to cause a response after a challenge. Histamine was nebulized for 2 minutes at a duty of 20% per chamber and a constant flow rate of 2 litres per minute per chamber. A baseline measurement was taken immediately prior to the histamine exposure. Animals then received histamine directly into the nasal chamber of the whole body plethysmograph, where their response was measured for 10 minutes after the 2 minute histamine nebulization. The percentage deviation from baseline at 0, 5 and 10 minutes was calculated, where

a negative value denoted a bronchoconstriction, while a positive value signified a bronchodilation.

3.3.4 Histological Analysis

Samples were treated and stained as described in 2.8

3.3.4.1 Mean Linear Intercept

Mean linear intercept was calculated by taking a photograph of the haematoxylin and eosin stained slides at X100 magnification. Two lines were drawn across the photograph, one in the centre horizontally and one in the centre vertically. The number of times the line intercepted an alveolar wall was recorded and the values averaged. If the line intercepted an airway or a blood vessel the line was moved up or right appropriately until it was no longer intercepting the artifact. This measurement allowed for a rough calculation of the density of alveolar walls and indicated the presence of emphysema if the density was reduced.

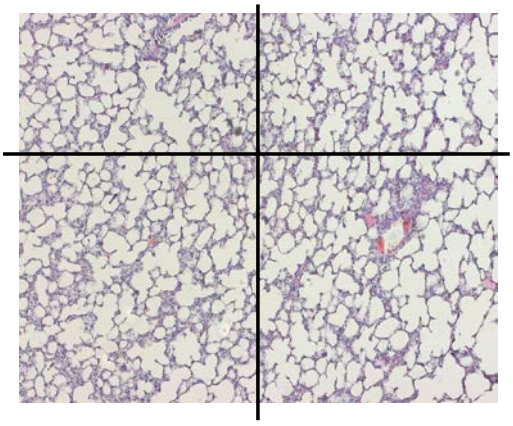


Figure 3.6 An example of mean linear intercept. Horizontal lines were placed on a stained section and the number of times the line intercepted an alveolar wall counted

3.4 Results

The Effect of Acute Saline or Acute LPS Challenge on Cell Influx, TNF-α and CXCL8 Levels

Acute LPS challenged animals showed a significantly greater number of total leucocytes, macrophages ($P<0.01$) and neutrophils ($P<0.001$)) in the BALF compared to acute saline challenged animals (fig 3.7). There was also a significant increase in TNF-α (521 ± 24 and 404 ± 34 pg/mg respectively) and CXCL8 (168 ± 14 and 26 ± 20 pg/mg correspondingly) levels in the lung tissue when acute LPS and acute saline were compared, but no change in levels in the BALF for either cytokine (fig 3.8).

The Effect of Nine Consecutive 15µg/ml LPS Exposures on Cell Influx and Lung Function.

In the nine consecutive 15 µg/ml LPS challenged animals there was a significant increase in total leucocyte, macrophage, and neutrophil numbers compared to the chronic saline challenged animals ($P<0.01$) (fig 3.9).

Lung function for saline exposed animals over the nine exposures peaked at 9.7±1% at exposure 2 to a low of -4.6 ± 2.6% at exposure 6. LPS challenged animals showed a peak of 10.9 ± 3.9% after the first exposure before a drop to -15.0 ± 6.8% at exposure 4. Similar responses were observed after exposures 5, 6 and 7 before a peak bronchoconstriction occurred at exposure 8 of -21.8±3.2%. However exposure 9 showed a much smaller bronchoconstriction of only 7.1 ± 7% (fig 3.10).

When the first, middle and last exposure trace was plotted, saline showed very little deviation from baseline, except after exposure 9 where there was a peak bronchodilation of 8.8 ± 4.1%. LPS however, showed a peak bronchodilation after

the first exposure of 10.9 ± 3.9%, before a peak bronchoconstriction of -10.9 ± 4.9% 1 hour after the fifth exposure. This was not compounded with a peak of -7.1 ± 7% 2 hours after the ninth exposure (fig 3.10).

When the first, middle and last exposure traces were analyzed using area under the curve, nine consecutive 15µg/ml LPS exposures (fig 3.10) showed no significant difference in lung function compared to the saline challenged animals (fig 3.10).

When airway hyperresponsiveness was analyzed saline showed no significant increase in pre- and post- AHR at 0, 5 or 10 minutes after histamine challenge. Similarly LPS challenged animals also showed no increased AHR at 0, or 5 minutes but did show a significant increase 10 minutes after exposure to 0.3mM histamine compared to pre LPS responses (-0.6 ± 2.7 and -17.2 ± 4.9% correspondingly).

The Effect of Chronic Saline or Chronic LPS Challenge on Cell Influx, TNF-α and CXCL8 Levels and Lung Function

Chronic LPS showed a significant increase in total leucocytes numbers compared to chronic saline challenged animals (2.2 ± 0.12 and 0.22 ± 0.024 $x10^7$cells/ml respectively). Chronic LPS also significantly increased macrophages and neutrophils compared to chronic saline challenged animals ($P<0.01$) (fig 3.12).

Chronic LPS significantly increased TNF-α levels in the BALF compared to chronic saline challenged animals (1469 ± 369 and 115 ± 25pg/ml respectively) (fig 3.13). However, TNF-α levels in the lung tissue were quite similar, with no significant difference between chronic saline and chronic LPS challenged animals. With CXCL8, this trend was reversed, with no significant difference between chronic saline and chronic LPS in the BALF, but a significant increase in CXCL8 levels in

the lung tissue of chronic LPS (123 ± 28pg/mg) compared to chronic saline (29 + 13pg/mg) challenged animals.

Lung function for saline exposed animals over the nine exposures peaked at 9.7±1% at exposure 2 to a low of -4.6 ± 2.6% at exposure 6. LPS challenged animals showed a peak of 6.8 ± 3.2% after the first exposure before a drop to -37.7 ± 3.7% at exposure 5. There was a similar response after exposures7, 8 and 9, where exposure 9 showed a bronchoconstriction of -30.8 ± 3.1% (Fig 3.14).

When the first, middle and last exposure traces were analyzed using area under the curve, chronic 30µg/ml LPS exposures showed a significantly increased area under the curve, signifying a worsening of lung function compared to chronic saline challenged animals ($P<0.01$) (fig 3.14).

There was no significant difference between histamine responses when before and after saline challenge responses, or before and after LPS challenge responses were compared at 0, 5 or 10 minutes after histamine exposure (fig 3.15).

The Mean Linear Intercept for Chronic Saline and Chronic LPS Challenged Guinea Pigs

Chronic LPS challenge significantly decreases the mean linear intercept compared to chronic saline challenged animals, signifying an increase in emphysema ($P<0.05$) (fig 3.16). Figure 3.17 shows chronic LPS challenged lungs exhibit larger emphysematous spaces compared to the chronic saline exposed animals.

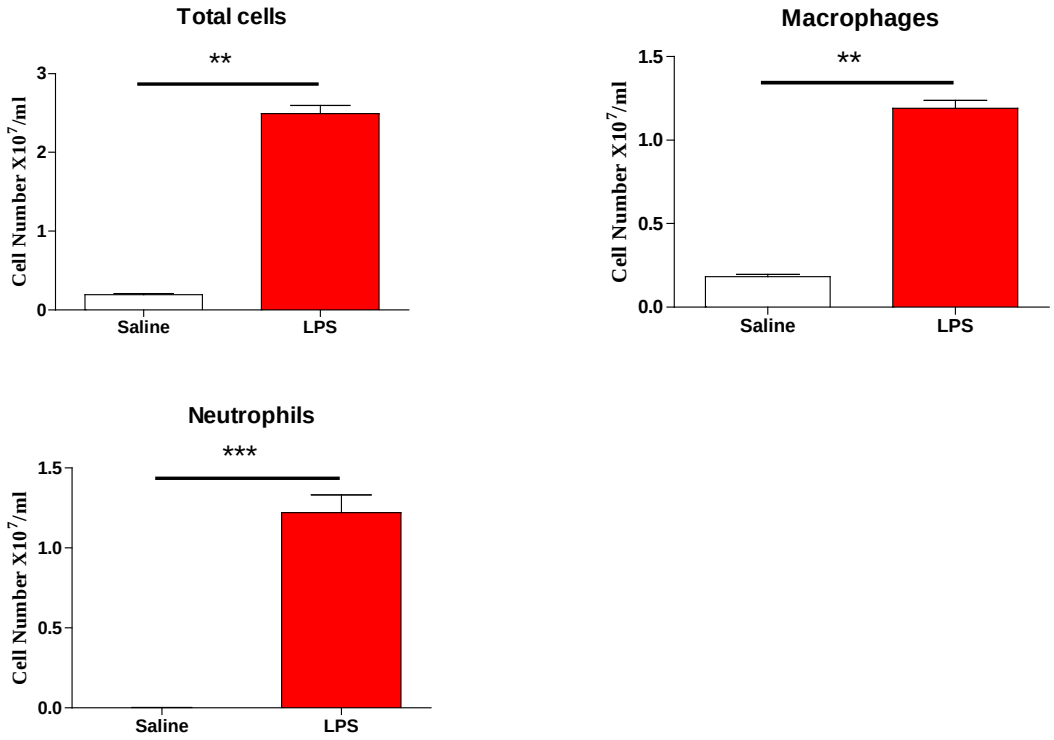


Figure 3.7 The Effect of Acute Saline or Acute LPS Challenge on Cell Influx into the Lung of Guinea-pigs. Total cell, macrophage and neutrophil numbers for acute saline and acute LPS challenged guinea-pigs. Total cells, macrophage and neutrophils were all significantly increased after acute LPS challenge compared to acute saline challenged animals. Mean ± SEM, N=6, **P< 0.01,***P<0.001. Mann-Whitney Test.

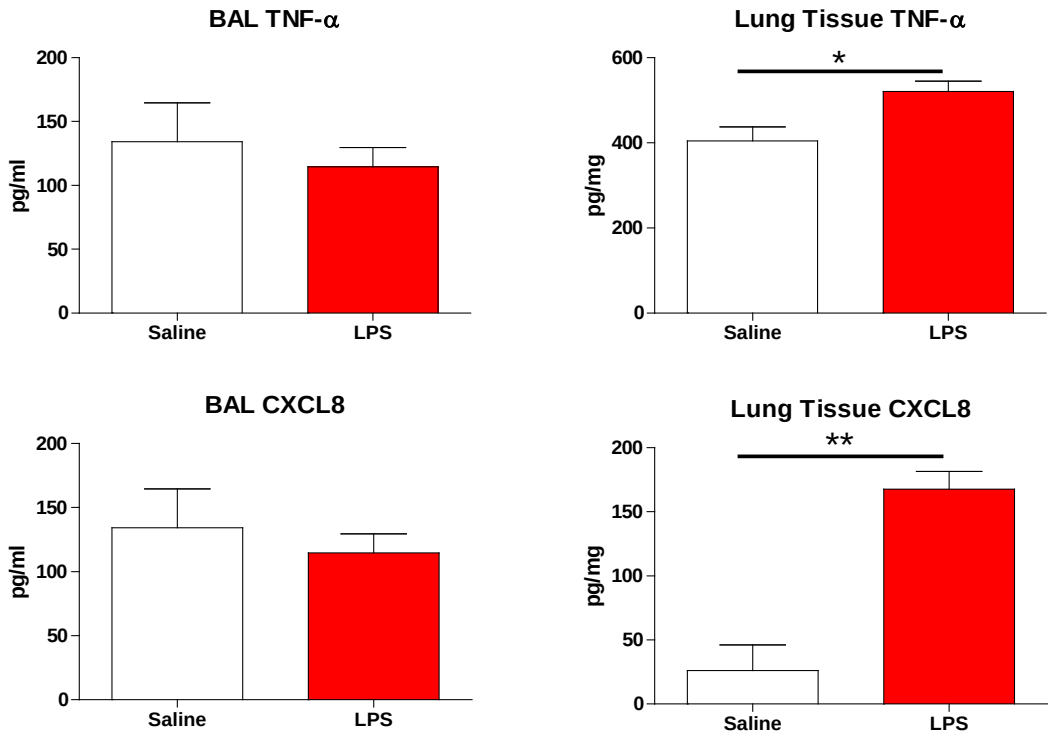


Figure 3.8 The Effect of Acute Saline or Acute LPS Challenge on TNF-α and CXCL8 Levels in the BALF and Lung Tissue of Guinea-pigs. TNF-α and CXCL8 levels in both BALF and lung tissue for acute saline and acute LPS challenged guinea-pigs. There was no significant difference between the groups when TNF-α and CXCL8 are compared in the BALF. However, lung tissue showed a significant increase in CXCL8 and TNF-α when acute LPS was compared to acute saline. Mean ± SEM, N=6, *P<0.05, **P<0.01 Mann-Whitney Test.

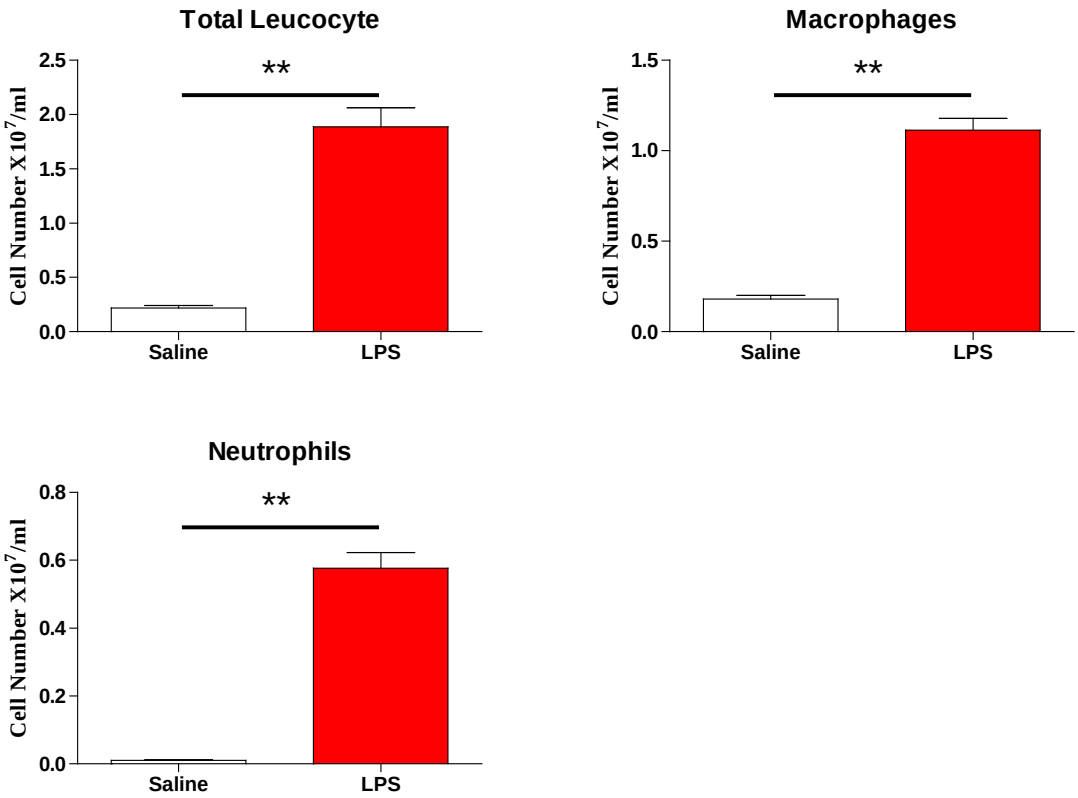


Figure 3.9 The Effect of Nine Consecutive 15µg/ml LPS Exposures on Cell Influx into the Lung. Consecutive LPS exposures showed a significant increase in all cell types compared to the saline control. Mean ±SEM, N=6, **P<0.01, Mann-Whitney test.

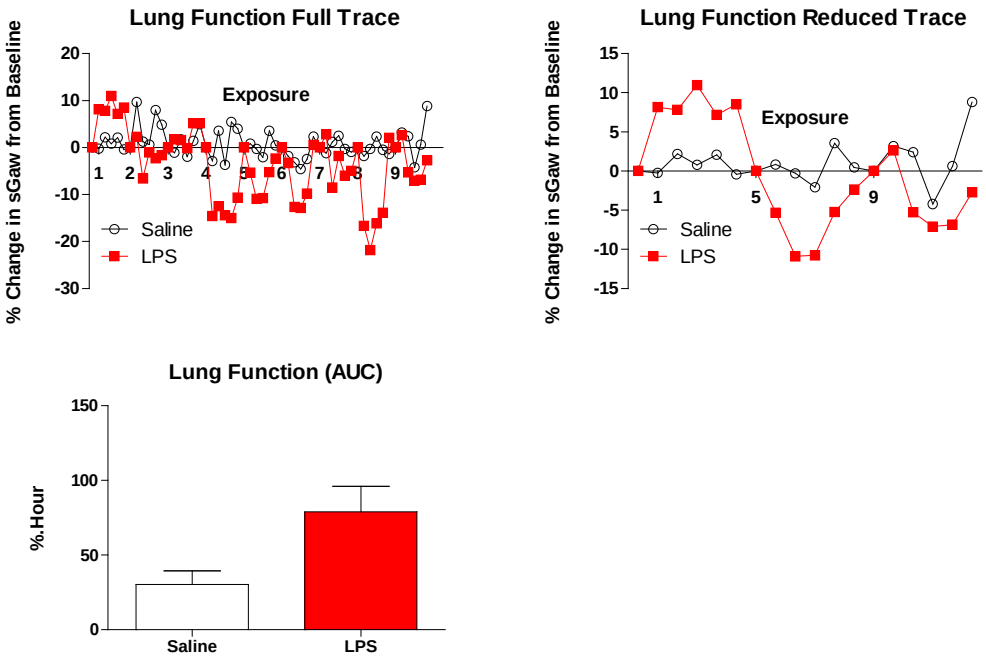


Figure 3.10 The Effect of Nine Consecutive 15µg/ml LPS or Saline Exposures on the Lung Function of Guinea-pigs. The full 9 exposure trace and the first middle and last exposure trace showed very small variations around the baseline for saline challenged animals. LPS challenged animals showed a bronchodilation after the first exposure, with a consistent bronchoconstriction after the fourth exposure up to the 8th exposure. The peak bronchoconstriction was observed at the 8th exposure before a decrease in bronchoconstriction after the 9th exposure. An increase in AUC signifies a decrease in lung function. Consecutive LPS challenge shows no significant difference in lung function compared to consecutive saline challenged animals. Mean ±SEM, N=6, P > 0.05, Mann-Whitney test. Error bars have been removed from sGaw traces for clarity.

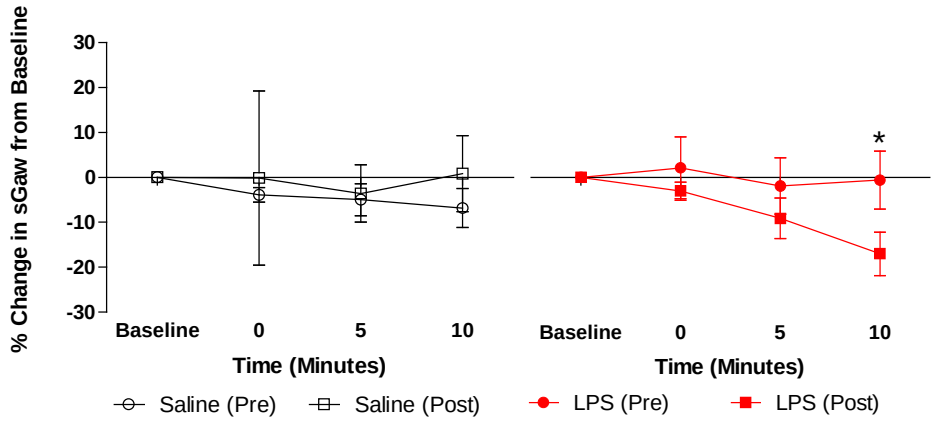


Figure 3.11 The Effect of Histamine (0.3mM) Before and After Nine Consecutive 15µg/ml LPS or Saline Challenge. There was no significant difference in histamine responses before or after saline challenge at 0, 5 and 10 minutes. Similarly LPS 15µg/µl showed no significant change in responses at 0 and 5 minutes, but there was a significant increase in AHR after LPS challenge at 10 minutes. Mean ± SEM, N=6, *P<0.05, Paired t-test.

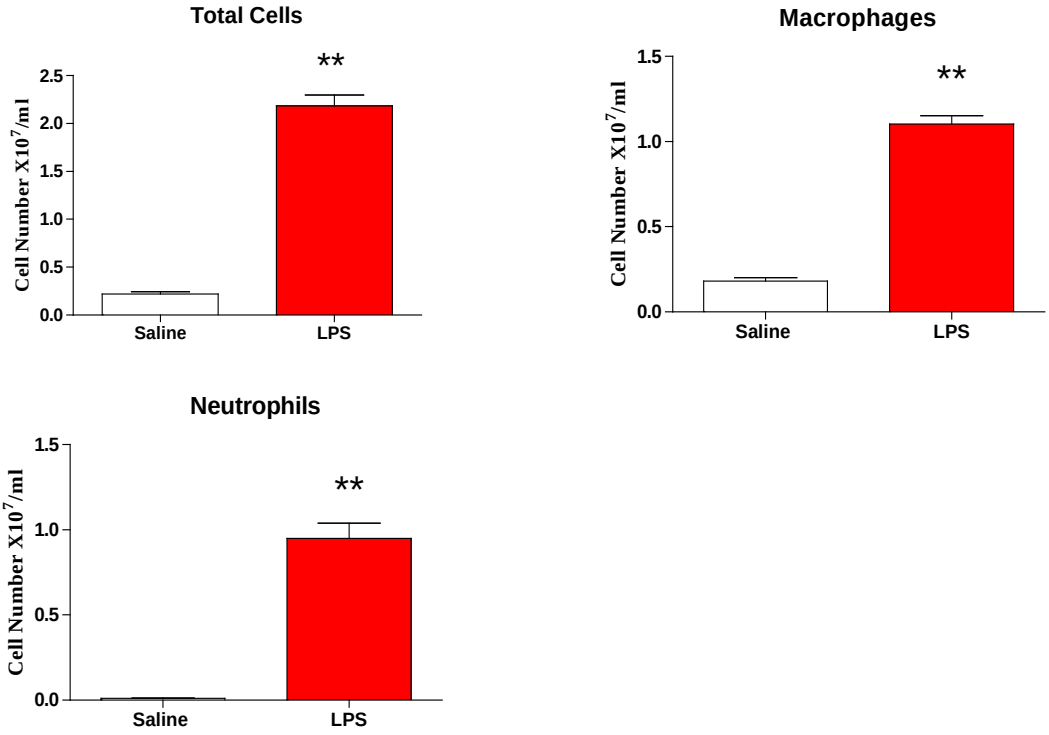


Figure 3.12 The Effect of Chronic Saline or Chronic LPS Challenge on Cell Influx into the Lung of Guinea-pigs. Chronic LPS showed a significant increase in all cell types compared to chronic saline challenged animals. Mean ± SEM, N=6, **P<0.01, Mann-Whitney test.

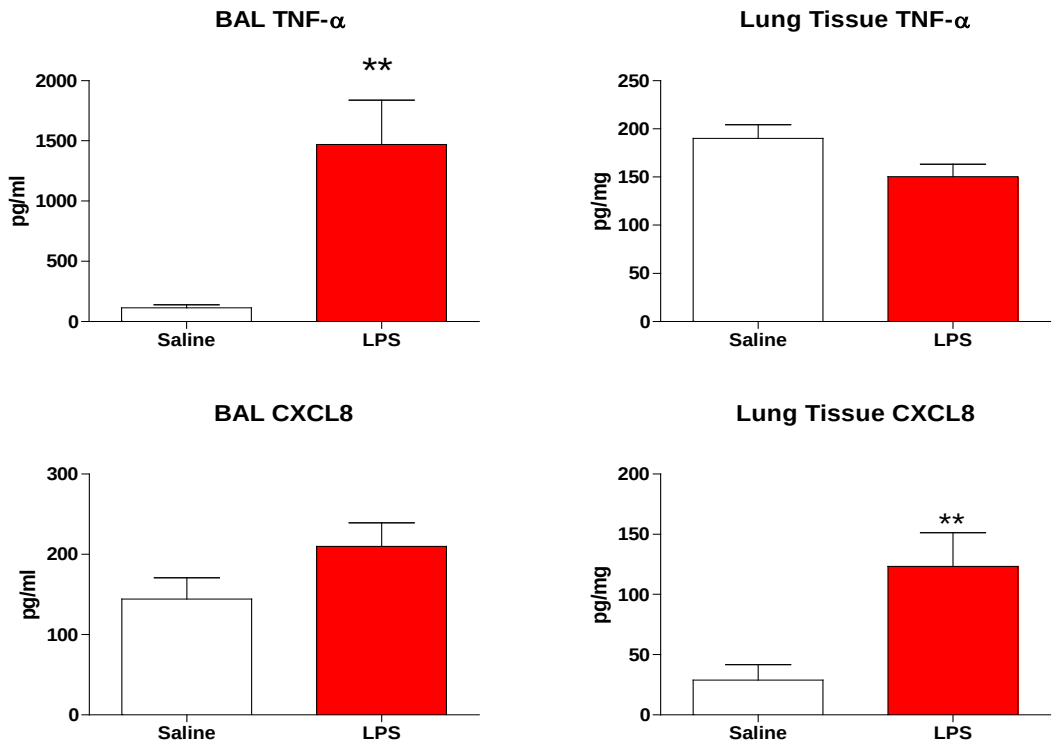


Figure 3.13 The Effect of Chronic Saline or Chronic LPS Challenge on TNF-α and CXCL8 Levels in the BALF and Lung Tissue of Guinea-pigs. TNF-α showed a significant difference in the BALF but no significant change in the lung tissue. While CXCL8 showed the reverse, a significant difference in the lung tissue but no change in the BALF. Mean ± SEM, N=6, **P<0.01, Mann-Whitney test.

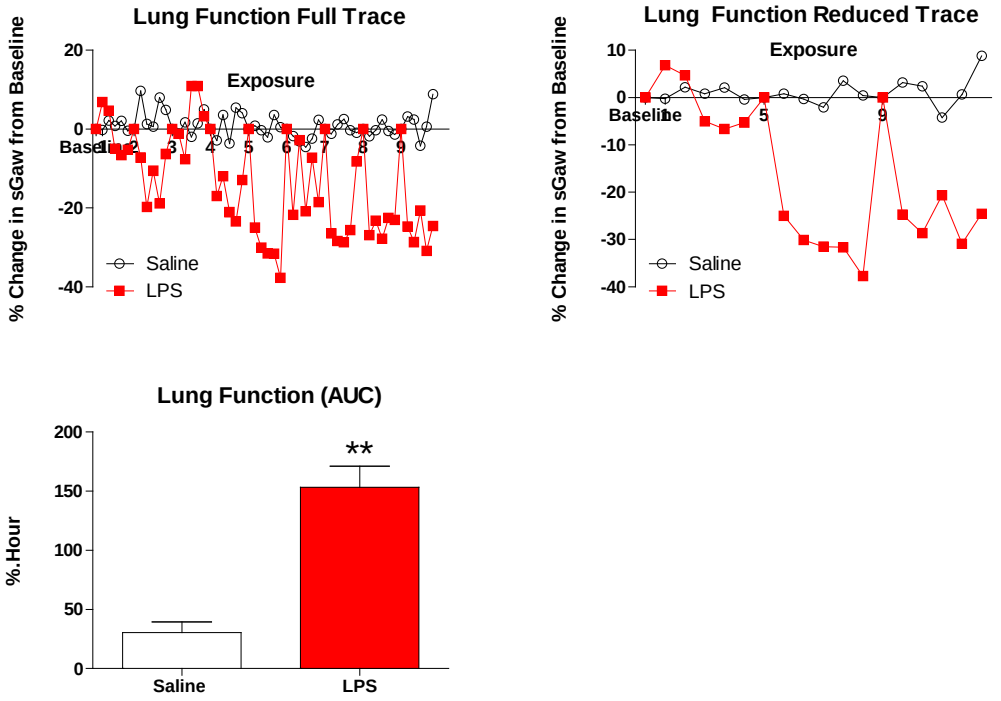


Figure 3.14 The Effect of Chronic Saline or Chronic LPS Challenge on the Lung Function of Guinea-pigs. The full 9 exposure trace and the first middle and last exposure trace showed very small variations around the baseline for saline challenged animals. Chronic LPS challenged animals showed a steady increase in bronchoconstriction after each exposure up to exposure 5, excluding exposure 3. After the peak bronchoconstriction at exposure 5 there is a steady bronchoconstriction in response to LPS up to exposure 9, excluding exposure 6.An increase in AUC signifies a decrease in lung function. Chronic LPS challenge shows a significant difference in lung function compared to chronic saline challenged animals. Mean ±SEM, N=6, **P<0.01, Mann-Whitney test. Error bars have been removed from sGaw traces for clarity.

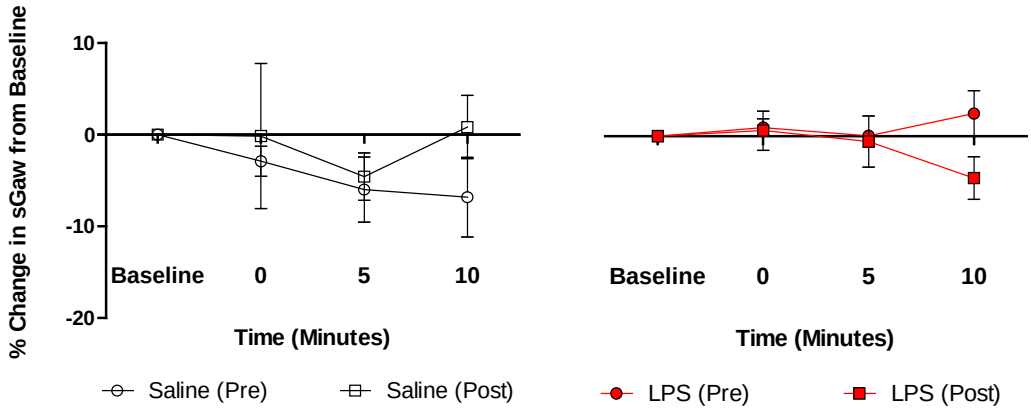


Figure 3.15 The Effect of Histamine (0.3mM) Before and After Chronic Saline or Chronic LPS Challenge. There was no significant difference in histamine induced bronchoconstriction after either chronic saline or chronic LPS challenge compared to pre challenge responses. Mean % change from baseline ± SEM, N=6, P>0.05, Paired T-Test.

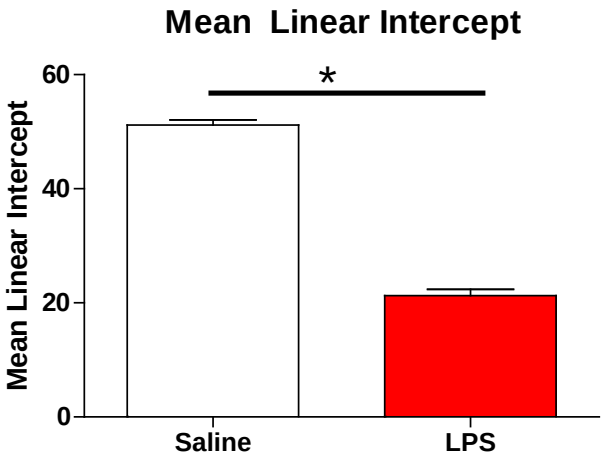


Figure 3.16 The mean linear intercept for chronic saline and chronic LPS challenged Guinea pigs. Chronic LPS exposed animals show a significant decrease in mean linear intercept compared to chronic saline challenged animals, signifying an increase in emphysema. Mean ±SEM, N=6, *P<0.05, Mann-Whitney test.

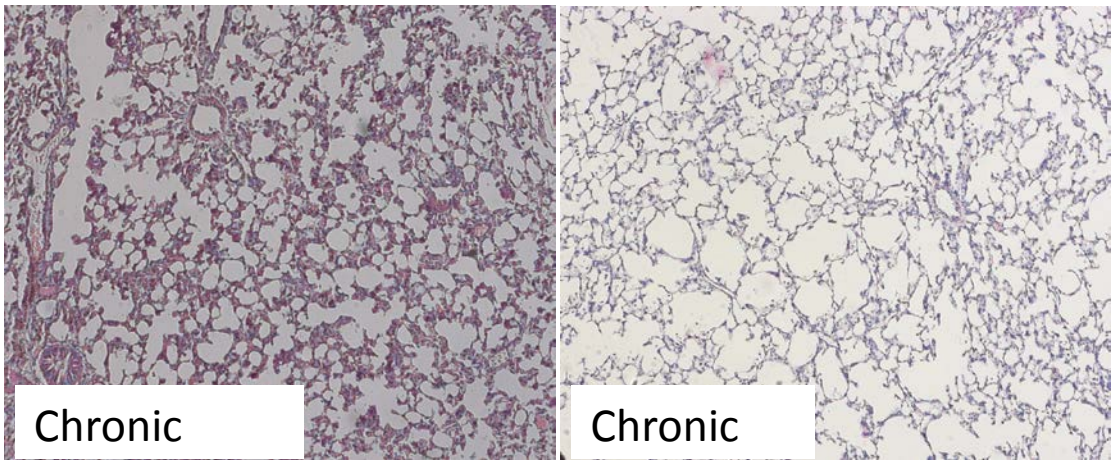


Figure 3.17 An example of the histology of the guinea pig lung after chronic saline or chronic LPS challenge. The chronic LPS challenged lung showed larger emphysematous spaces compared to the chronic saline exposed animals. Magnification x100

3.5 Discussion

Many different animal models have been developed to try and understand the complex mechanisms that take place in COPD. Acute LPS models in many different species, including mice (Hakansson *et al.*, 2012), rats (Spond *et al.*, 2001), guinea pigs (Toward and Broadley, 2000) and non-human primates (Seehase *et al.*, 2012), have shown an increase in inflammation, which is predominantly neutrophil and macrophage driven. They also show an increase in inflammatory mediators, such as CXCL8 and TNF-α.

The inflammation after acute LPS challenge in healthy humans is similar to that observed in COPD patients. Healthy people exhibit a ~69% neutrophil and a ~26% macrophage profile after single LPS challenge (Aul *et al.*, 2012), while COPD patients show a ~58% neutrophil and ~33% macrophage profile (Beeh *et al.*, 2003). Similarly the inflammatory response observed in the guinea pig to acute LPS challenge showed a distribution of ~49% neutrophils and ~48% macrophages. This signifies that, by using inflammatory percentage profiles, LPS is a suitable model to study COPD inflammation. The acute model also demonstrated increases in TNF-α and CXCL8 in the lung tissue only, while there was no difference between the groups in the BALF. This could be due to differential responses between 'resident' inflammatory cells within the lung tissue and 'new' cell influx in the BALF, differences in cytokine degradation between the two compartments or continued release of cytokines in the short time between lavaging the animal and snap freezing the lung tissue.

Chronic LPS exposure, 30µg/ml with alternating days proved to be the maximal, well tolerated dose in the guinea pig and showed a similar inflammatory profile to

COPD. Nine LPS exposures were chosen as previous experience with this model in this lab demonstrated airway hyperresponsivness was present at 24 hours only after the eighth exposure, while bronchoconstriction to LPS was only prolonged after the ninth (Toward and Broadley, 2001). Upon histological examination emphysema was also present after nine exposures meaning further exposures would not be required. This makes this model advantageous over a similar model by Kaneko *et al*., (2007) who demonstrated emphysema after the 15th exposure.

The chronic model also showed elevated TNF-α levels in the BALF compared to control, while the acute model demonstrated little difference. TNF-α levels have been shown to be increased in the BALF and sputum of patients suffering from COPD (Soler 1999; Blidberg *et al*, 2012) and have been correlated with the severity of the disease, with higher levels signifying more severe COPD (Hacievliyagil *et al.*, 2006), while CXCL8 levels have also been shown to be increased in COPD (Yammamoto *et al.*, 1997). This means the chronic model also exhibits a similar inflammatory profile to COPD. The chronic LPS model, where LPS was administered every 48 hours, would, however, result in peaks and troughs in inflammation (Toward and Broadley, 2001). It is for this reason a consecutive model (i.e. LPS exposures every 24 hours) was also investigated. This would mean inflammation would not resolve but would instead be peaking by the time the next exposure was given. Unfortunately the same dose of LPS could not be used in both models as the 30µg/ml of LPS used in the chronic model was not well tolerated in the consecutive model, meaning the dose had to be halved.

To differentiate the acute and chronic models, the most important thing to note in terms of acute LPS models is the lack of structural changes. There are no structural changes in an acute model, while COPD exhibits emphysematous changes, due to

the loss of parenchymal cells (Barnes, 2012), as well as narrowing of the airways and increased fibrosis (Barnes, 2011). Goblet cell hyperplasia is also observed, resulting in increased mucous secretion and mucous plugging (Rogers, 2000). No structural changes have been reported in the literature after acute LPS challenge in any species. Hakansson *et al.* (2012) state that any alteration in lung function that is observed after an acute LPS challenge, is solely down to the influx of inflammatory cells and not due to any structural changes, which are the cause of obstructive airflow in COPD. So unsurprisingly, an acute LPS model of a chronic disease process, which takes many decades to occur, has few clinically relevant manifestations of COPD, so a more chronic model is required.

Chronic LPS as a model of COPD, just like acute models, have been used in many species including mice (Veernoy *et al.*, 2002; Brass *et al.* 2008), rats (Harkema and Hotchkiss, 1993) and guinea pigs (Toward and Broadley, 2002). Toward and Broadley (2002) showed repeated exposure of the guinea pig to LPS resulted in neutrophilia, goblet cell hyperplasia and a decrease in sGaw, representing a bronchoconstriction. While Kaneko *et al* (2007) also describe neutrophilia, as well as emphysematous changes after 15 exposures to LPS.

The chronic LPS guinea pig model does not show any goblet cell hyperplasia, indeed goblet cells could rarely be detected at all. However, this may not be surprising as studies have shown that guinea pigs exhibit goblet cells in the proximal trachea and rarely have any goblet cells beyond this point (Widdicombe *et al.*, 2001; Goco *et al.*, 1963); However, Toward and Broadley (2002) did observe goblet cell hyperplasia in this model. The chronic LPS guinea pig model does, however, exhibit a significant increase in emphysema compared to the chronic saline control. This was evaluated using mean linear intercept (MLI), where a decrease in MLI signifies an increase in

emphysema due to the loss of alveolar walls. MLI was performed on a separate group which had to be rested for two weeks after the final exposure to allow for the inflammation to clear. This was the only group that was rested, with the sole aim of studying the histology of the lung. All other groups were culled 24 hours after the final LPS exposure. Although the MLI reached significance, greater significance might be possible if the guinea pigs did not have to recover for a couple of weeks which may allow time for some regeneration of the lung tissue.

The chronic model exhibited emphysematous changes while the consecutive model did not. This may be due to the length of time proteinases are present and active in the lung. Proteases, such as neutrophil elastase (Janoff *et al.*, 1977; Snider *et al.*, 1984) and MMPs released by inflammatory cells, result in remodelling of the lung (Finlay *et al.*, 1997). Secreted proteases are inactivated by anti-proteases such as TIMPs (Okada *et al.*, 1999), α_1 anti-trypsin and α_2 macroglobulins (Poller *et al.*, 1989). The length of time proteases were present and active in the different models are the likely cause of the differences in histology. The chronic model had active proteases present in the lungs up to twice as long as the consecutive model possibly explaining the differences between the histology.

With any model using LPS, the concept of LPS tolerance must also be considered. LPS tolerance is a reduction in response to LPS after a previous exposure. LPS tolerance has been shown to affect cytokine release, with some pro-inflammatory cytokines, such as TNF-α showing decreased levels (del Fresno *et al.*, 2009) while the anti-inflammatory cytokine IL-10 is increased (Cole *et al.*, 2012). The molecular mechanisms for LPS tolerance are not fully elucidated but several theories have been hypothesized. Repeated exposure to LPS has been demonstrated to decrease TLR4

receptor expression on the cell surface (Fan *et al.*, 2002; Zhong *et al.*, 2008). The signaling pathways themselves can be affected such as increased negative feedback by inhibitors of TLR signaling such as IRAK-M (Escoll *et al.*, 2003). Impairment of IRAK-1 and MAPK activation in the MyD88 dependent pathway has also been described (Fan and Cook, 2004). Differences in Nf-κB dimer state, with less pro-inflammatory P50 homodimers having an increased prevalence during endotoxin tolerance, while the more pro-inflammatory P50/P65 heterodimers show increased prevalence during normal LPS responses (Porta *et al.*, 2009). The consecutive model may be affected more by LPS tolerance than the chronic model due to the challenge regimen. Repeated LPS exposure in a short time frame will result in LPS tolerance, while repeated LPS exposure with 48 hours between exposures may mean LPS tolerance does not build up, with any mechanisms activated after the first exposure resolving prior to the next.

Many studies have been performed to assess the correlation of emphysema with lung function, which show that the two are poorly related (Robbesom *et al.*, 2003), with emphysema also varying widely between patients in the same stage of COPD (Makita *et al.*, 2007). This highlights the heterogeneity of the disease and the difficulties in producing a model for such a varied condition.

Lung function in COPD is usually measured using spirometry. A FEV_1/FVC ratio below 0.7 is the diagnosing criteria for COPD, with the severity depending on the percentage of FEV_1 expected for the individual's age, height etc. (Celli *et al.*, 2004). The measuring of these parameters is not possible in conscious animals, which were chosen for experiments as it allows for repeated measurements over time, as well as preventing interference in recording respiration by an anesthetic (Flecknell and Mitchell, 1984). Experiments have been performed comparing the conventional

spirometry measurements with plethysmography in COPD patients, which found that while FEV_1 was more reproducible, sGaw was better at detecting bronchoconstriction and dilation in the airways, and may therefore be the best way to measure lung function in patients (Borrill *et al.*, 2004; Gimeno *et al.*, 1993). FEV_1 has also been shown to cause changes in the airways due to the effort of deep inspiration and forced expiration itself (Burns and Gibson, 2002). These studies rationalize the use of a parameter such as sGaw, signifying that the measurement has clinical relevance in COPD.

The results in the guinea pig show a steadily progressive airflow limitation, with sGaw decreasing until 5 LPS exposures and then plateauing until the ninth exposure. This results in a significant reduction in airflow compared to the control when analyzed by area under the curve. Airway hyperresponsiveness was not observed after chronic LPS treatment, while Toward and Broadley (2001) showed airway hyper reactivity was present 24 hours after the final LPS exposure in the same LPS model. The method of histamine delivery differed between the two studies, with Toward and Broadley using 1mM histamine for a 20 second nose only exposure, while this study used 0.3mM, (calculated as a sub threshold dose of histamine) for 1 minute at 20% duty, delivered at 2 litres per minute, into the head chamber of the plethysmography machine. It is possible that this dose of histamine is too low; however, Turner *et al.* (2011) demonstrated that this dose, delivered in the exact same way as in this study, was able to cause a significant bronchoconstriction in ovalbumin challenged guinea pigs, but had no effect prior to challenge. Airway hyperresponsiveness used to be used as a way of differentiating between patients with asthma and patients with COPD (Irvin, 2012). However, COPD is now understood to be more complex than originally thought and also exhibits signs of

hyper responsiveness. The symptom is very diverse, with some COPD patients showing signs of hyperresponsivness while others do not. AHR has been linked to a greater increase in disease progression as a result of a decrease in lung function, as well as a diminished response to bronchodilators (Han *et al.*, 2010). Due to this variability in COPD phenotype, a general model of COPD does not necessarily need to exhibit airway hyperresponsiveness.

This data shows that the chronic LPS model, where guinea pigs were exposed to 30µg/ml on alternating days, exhibits similar inflammation and structural changes as observed in COPD and also results in a decrease in lung function. Since the model exhibits several important aspects of COPD this model could be used to investigate steroid sensitivity and exacerbations.

CHAPTER 4

The Steroid Sensitivity and Viral Exacerbation Potential of the Chronic LPS Model

4.1 COPD and Steroid Insensitivity

It has previously been discussed that two important aspects of COPD are steroid insensitivity, and the tendency for COPD patients to develop acute exacerbations, which are poorly treated. A model that can be used to investigate these two processes and help improve treatments is urgently required.

The exact reason for steroid insensitivity in COPD remains unknown and needs to be investigated; however, many theories have been hypothesised. One theory is that it is due to the lack of steroid responses in subtypes of neutrophils during inflammation (Strickland *et al.,* 2001). The most widely reported theory is the effect of oxidative stress on the enzyme histone deacetylease 2 (HDAC-2) reducing its activity. This reduced activity can decrease transcription of proinflammatory cytokines by regulating the acetylation of histone proteins. To *et al* (2004) demonstrated that the severity of COPD is inversely proportional to HDAC activity, with more severe disease exhibiting a larger decrease in HDAC activity. Low dose theophylline has been demonstrated to restore HDAC activity in steroid resistant COPD, with a six fold increase in alveolar macrophage HDAC activity in COPD patients (Cosio *et al,* 2004), but how this effect translates into whole animal models or indeed the disease itself is largely unknown.

4.1.1 Theophylline

Theophylline is a methylxanthine that acts as a non-specific phosphodiesterase inhibitor, allowing cAMP to accumulate, causing bronchodilation. This mechanism of action is described in chapter one. Inhibition of phosphodiesterases can also have an anti-inflammatory effect by increasing cyclic AMP (cAMP). cAMP is known to have an anti-inflammatory effect by decreasing pro-inflammatory cytokines, such as CXCL8 and TNF-α and decreasing ROS, as well as increasing the anti-inflammatory

cytokine Il-10 (Currie *et al,* 2008). The anti-inflammatory effect of low dose theophylline has been demonstrated by Ito *et al* (2002) to work independently of PDE, with other PDE inhibitors not demonstrating the same effects. This is beneficial as the side effects associated with theophylline are due to its PDE inhibiting properties (Barnes *et al,* 2004). This suggests theophylline is also acting on HDAC; however, this mechanism as yet remains unclear.

4.2 Exacerbations of COPD

Exacerbations are also an important aspect of COPD. Exacerbations are described as "*An acute event characterised by a worsening or the patient's respiratory symptoms that is beyond normal day-to-day variations and leads to a change in medication"* (GOLD, 2011). Exacerbations decrease quality of life, increase morbidity and mortality and frequently result in hospital admissions for COPD patients.

Respiratory viral infections play an important role in exacerbations as they have been shown to be responsible for approximately half of all exacerbations, either in conjunction with bacteria or on their own (Wedzicha & Seemungal, 2007). Treatment of exacerbations, like the stable disease, is poor. An animal model of acute exacerbations of COPD (AECOPD) is desperately needed to assess the mechanisms of AECOPD to improve treatments.

4.2.1 Human Parainfluenza 3 Virus

Human parainfluenza viruses (HPIV) is an enveloped, single stranded RNA virus that belongs to the taxonomy *Paramoxyviridae,* which are subdivided into 4 types (Greenwood *et al,* 2007*).* HPIV 3 is the cause of croup in infants, but is also a cause of respiratory infections in the immuno-compromised and is a recognised cause of exacerbations in COPD patients (Dimpoloulos *et al.* 2012).

A viral infection triggers both the innate and the adaptive immune response. The innate immune response primarily induces apoptosis of virus infected cells by the activation of CD8+ cytotoxic lympohcytes as well as activation of Toll like receptors by viral components, with TLR-7 recognising single stranded viral RNA. As described in chapter 3, activation of TLRs activates the MyD88 dependent and independent pathways, resulting in the expression of NF-κB and interferons. Interferons are important in viral infections as they are used as messengers between cells that a viral infection is taking place. Interferon causes neighbouring cells to decrease protein synthesis and increase RNA destruction within the cell, therefore decreasing viral replication in possible virus infected cells (Fensterl & Sen, 2009).

Like HPIV3, the common viruses associated with AECOPD, rhinovirus and influenza virus, are single stranded RNA viruses, but they have been shown to activate TLR3 during replication due to the synthesis of intermediary double stranded RNA (Wang *et al.*, 2009; Guillot *et al.*, 2005). Polyinosinic: polycytidylic acid (Poly I:C) is a synthetically manufactured compound closely resembling double stranded RNA and has been demonstrated to activate TLR3 (Alexopoulou *et al.*, 2001). It can be used to mimic viral infection and has advantages over viruses as it is not contagious and the amount of poly I:C given is able to be carefully controlled.

4.3 Aim

The aim of this chapter iss to investigate the steroid sensitivity, the effect of low dose theophylline on the steroid sensitivity and exacerbation potential of the chronic LPS model.

4.3.1 Objectives

- Investigate the response to the inhaled corticosteroid budesonide and the systemic corticosteroid dexamethasone, measuring inflammatory cell influx, TNF-α and CXCL8 release
- Investigate the effect of low dose theophylline on improving steroid sensitivity, measuring the same parameters as steroid alone.
- Investigate human parainfluenza 3 virus and Poly I:C as possible exacerbation agents in the guinea pig, measuring inflammatory cell influx and airway hyperresponsivenss,.

4.4 Methods

4.4.1 Animal Husbandry

Animals were housed as described in 2.1

4.4.2 Challenges

4.4.2.1 Lipopolysaccharide Exposures

Guinea pigs received 30µg/ml of lipopolysaccharide (LPS) (E.Coli 026:B6, Sigma), or a control solution of saline, nebulised using a Wright nebuliser at 0.3mls/min at a constant pressure of 20 psi in a sealed Perspex chamber (15x15x32cm) for 1 hour.

4.4.2.2 Acute Lipopolysaccharide Exposures

A single 30µg/ml LPS or saline exposure was performed before the guinea pigs were culled 24 hours after the exposure. A bronchoalveolar lavage was performed (as described in 2.6) to determine total and differential leucocyte numbers, as well as cytokine levels. Drug treated animals received drugs as described in 2.5 for 6 consecutive days prior to the lavage.

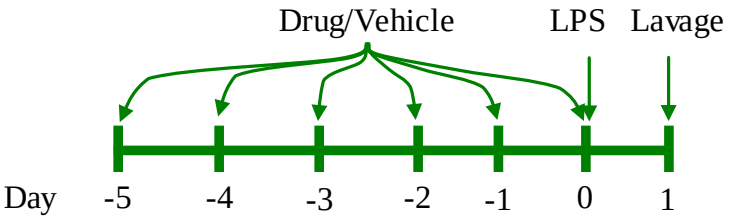


Figure 4.1 A schematic representation of the acute LPS protocol with six day drug treatment

4.4.2.3 Chronic Lipopolysaccharide Exposures

Exposures were performed using the method described by Toward and Broadley (2001). Guinea pigs were exposed to 30µg/ml of LPS or saline every other day until a total of nine exposures were reached. Airway function was recorded using whole body plethysmography (Buxco) after the first, fifth and ninth exposure using

standard airway conductance (sG_{aw}) as the measured parameter. sG_{aw} was measured at 0 minutes, 1, 2, 3 and 4 hours after exposure and compared to measurements performed immediately prior to the corresponding LPS or Saline exposure. This allowed for percentage change in sG_{aw} to be calculated. Animals were killed 24 hours after the ninth exposure with a sodium pentobarbital overdose (400mg/kg), and a bronchoalveolar lavage was performed to determine total and differential cell counts and cytokine levels.

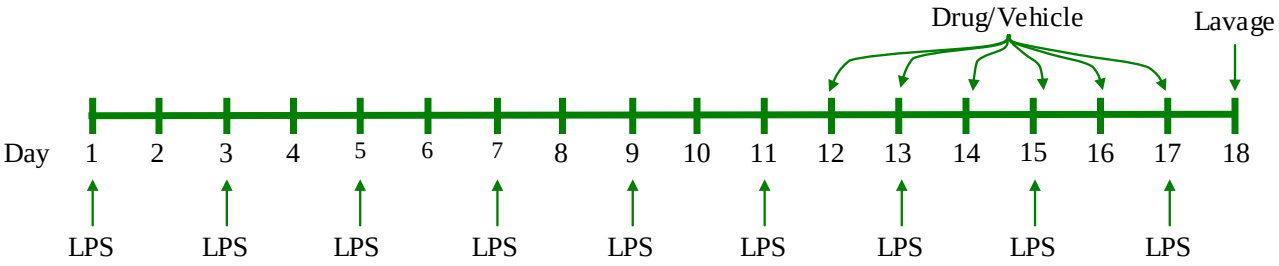


Figure 4.2 A diagram representing the chronic protocol for saline/LPS challenged guinea pigs.

4.4.2.4 Bronchoconstrictive Dose of Histamine

A bronchoconstrictive dose of histamine was also determined in order to evaluate the effectiveness of bronchodilator drugs, such as theophylline. 0.7mM of histamine delivered for 2 minutes, at 20 duty per chamber and a constant flow rate of 2 litres per minute per chamber, exhibited significant bronchoconstriction in naive animals (figure 4.3).

4.4.3 *In Vivo* Drug administration

4.4.3.1 Corticosteroids

The effects of the corticosteroids budesonide (Sigma Aldrich, UK) and dexamethasone (Sigma Aldrich, UK) were evaluated against the LPS model. All drugs were given once a day for 6 consecutive days before the end of an experiment

and were received thirty minutes before a challenge. Details relating to doses, vehicles and administration can be found in table 2.1.

4.4.3.2 Theophylline

The effect of theophylline was evaluated in the chronic LPS model. Theophylline was administered orally via a gavage needle, twice a day for 6 consecutive days before the end of an experiment. Animals receiving theophylline alone did so 30 minutes before any challenge. Animals receiving both theophylline and a corticosteroid, firstly received theophylline 15 minutes before the steroid was administered. Details relating to doses, vehicles and administration can be found in table 2.1.

To evaluate the bronchodilator effects of theophylline against a bronchoconstrictive dose of histamine, theophylline was administered 30 minutes before a histamine challenge to allow for adequate absorption. Details relating to doses, vehicles and administration can be found in table 2.2.

4.4.4 Exacerbating Agents

4.4.4.1 Human Parainfluenza 3 Inoculations

Human parainfluenza 3 virus was cultured in a BSC-1 cell line to achieve a minimum viral titre of at least 10^8 virus particles per ml. The virus particles were quantified by RT-PCR following RNA extraction as described in 2.6.

Animals received 0.3mM histamine on day 1 to determine airway responsiveness prior to any viral inoculations taking place. Guinea pigs received either 125µl of virus or a control of virus free media, twice in each nostril on days 2 and 3. All viral inoculations were performed in a laminar flow cupboard to prevent viral spread. As well as this, after the first inoculation with virus, animals were kept in an isolator

with an independent air supply to prevent viral spread. They were housed in twos or threes, dependant on size, in plastic cages with steel cage tops. Each cage contained hay, wooden blocks and cardboard tubes for environmental enrichment. Animals inoculated with medium were placed back in the main animal house facility. Animals received their second dose of 0.3mM histamine on day 7 to test for AHR, and were then killed by a sodium pentobarbital overdose and a bronchoalveolar lavage was performed.

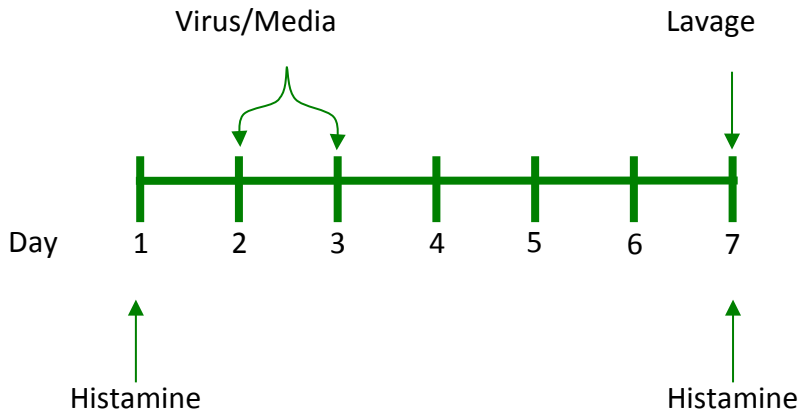


Figure 4.3: A schematic diagram of the 7 day virus/media inoculation protocol.

4.4.4.2 Poly I:C Inoculations

Animals received 0.3mM histamine on day 1 to determine airway responsiveness prior to any inoculations taking place. Guinea pigs received 125ul of poly I:C (2mg/ml) or a saline control per nostril per day, on days 2, 3 and 4. Airway responsiveness to 0.3mM histamine was measured on day 5, 24 hours after the final poly I:C. The animals were then killed by an overdose of sodium pentobarbital and a bronchoalveolar lavage was performed.

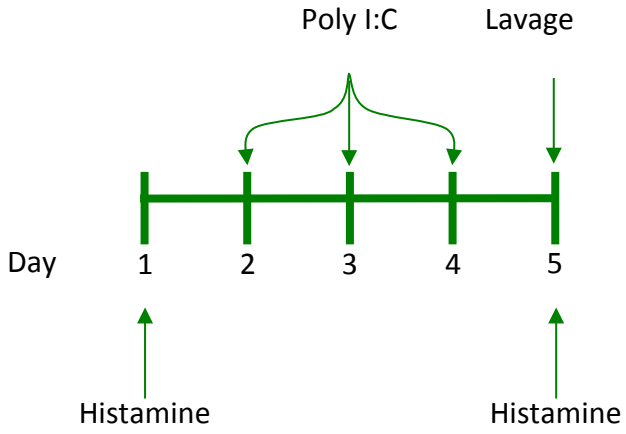


Fig 4.4 A schematic diagram of the 5 day poly I:C model.

4.5 Results

The Effect of Inhaled Budesonide on Cell Influx into the Lung in Acute LPS Challenged Guinea-pigs.

Budesonide showed no significant difference in total leucocyte numbers at either 0.6 or 1.2 mg/ml compared to the vehicle control group. Vehicle, 0.6 and 1.2mg/ml budesonide treated animals also showed no significant difference in macrophage or neutrophil numbers (figure 4.5).

The Effect of Systemic Dexamethasone on Cell Influx, TNF-α and CXCL8 Levels after Acute LPS Challenged Guinea-pigs.

Dexamethasone significantly reduced total leucocyte numbers compared to the vehicle control group (1.6 ± 0.25 and 3.0 ± 0.17 $x10^7$cells/ml respectively). Dexamethasone also significantly reduced macrophage ($P<0.05$) and neutrophil ($P<0.01$) numbers compared to the vehicle (Fig 4.6). Dexamethasone showed no significant difference in TNF-α and CXCL8 levels compared to the vehicle control group in both the BALF and lung tissue.

The Effect of Systemic Dexamethasone on Cell Influx, TNF-α and CXCL8 Levels After Chronic LPS Challenge

Dexamethasone was able to significantly reduce neutrophil numbers compared to vehicle treated animals (0.46 ± 0.17 and 1.2 ± 0.04 $x10^7$cells/ml respectively) (fig 4.8). However, there was no significant difference in total leucocyte or macrophage numbers when vehicle and dexamethasone groups were compared.

Dexamethasone significantly reduced TNF-α levels in the BALF compared to vehicle treated animals (147 ± 22 and 251 ± 20pg/ml respectively). However, there was no significant difference in TNF-α levels in the lung tissue with vehicle and

dexamethasone treated animals having very similar values. There was no significant difference between CXCL8 levels in the BALF or lung tissue between vehicle and dexamethasone treated animals (fig 4.9).

The Effect of High or Low Dose Theophylline Treatment on Histamine Induced Bronchoconstriction in the Guinea-pig.

High dose theophylline treatment significantly reduced the bronchoconstriction to histamine compared to pre-treatment responses at 0 and 5 minutes after histamine exposure ($P<0.01$) which returned to similar levels by 10 minutes This contrasted with the low dose theophylline treatment, which exhibited no significant difference in histamine responses before or after theophylline treatment at 0 or 5 minutes after exposure, but showed a significant difference in responses at 10 minutes (-26.0 ± 5.7 and -9.9 ± 1.4 % change from baseline respectively) (fig 4.10).

The Effect of Low-Dose Theophylline and Dexamethasone Co-treatment on Cell Influx, TNF-α and CXCL8 levels in Chronic LPS Challenge.

There was a significant reduction in macrophage number when dexamethasone and low dose theophylline co-treated animals were compared with animals receiving dexamethasone only ($P<0.05$)(fig 4.11). There was no significant difference in total cell or neutrophil numbers between the groups

There was a significant increase in TNF-α levels in the BALF of co-treated animals compared to dexamethasone only treatment ($P<0.01$) (Fig 4.12). This was the only parameter to show any significant change, with CXCL8 in both the BALF and lung tissue and TNF-α in the lung tissue remaining unchanged.

The Effect of Human Parainfluenza 3 Virus on Cell Influx and Airway Hyperresponsiveness

There was no significant difference in total leucocyte, macrophage or neutrophil numbers when naïve, and virus challenged animals were compared (fig 4.13). There was also no significant difference in airway responses between pre and post media and pre and post virus challenge at 0, 5 and 10 minutes after histamine challenge (fig 4.14). There was also no significant difference in viral titre between media and virus challenged animals in both lung tissue and the BALF (fig 4.15).

The Effect of Poly I:C on Cell Influx and Airway Hyperresponsiveness

Poly I:C significantly increased total leucocyte numbers and macrophages in the lung compared to saline challenged animals ($P<0.05$). However, there was no significant difference in neutrophil numbers between saline and poly I:C challenged animals (2.9 ± 0.62 and 4.6 ± 0.95 $x10^7$cells/ml respectively) (fig 4.16) and there was no significant difference in airway responses between pre and post saline and pre and post poly I:C challenge at 0, 5 and 10 after histamine challenge (fig 4.17).

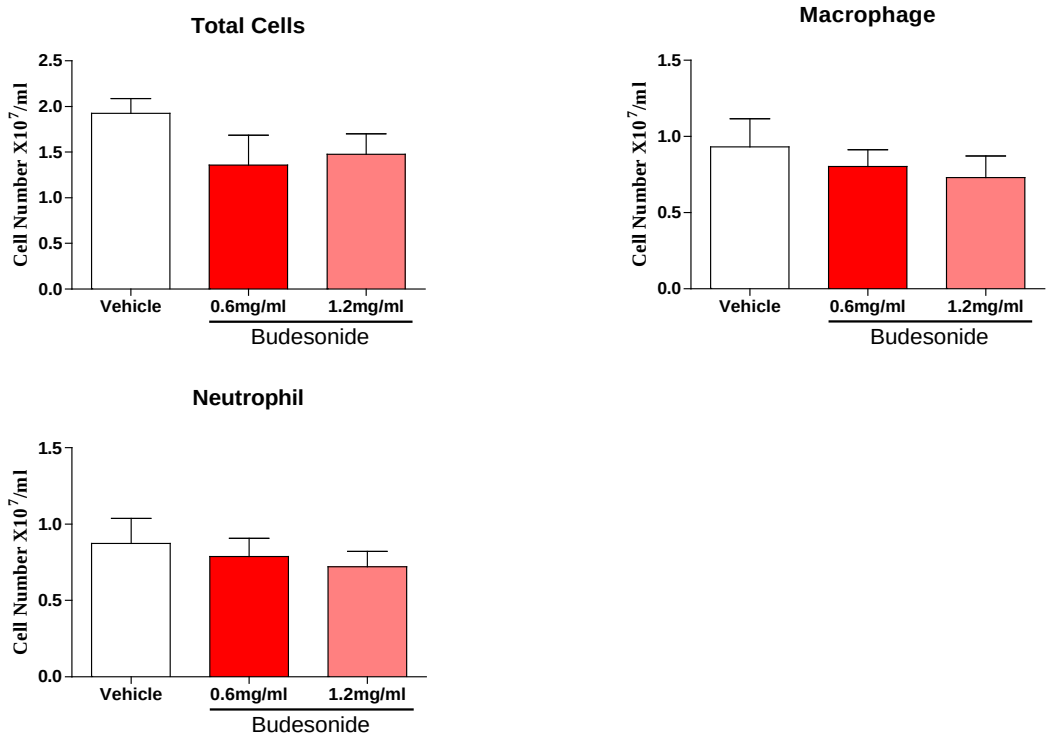


Figure 4.5 The Effect of Inhaled Budesonide on Cell Influx into the Lung in Acute LPS Challenged Guinea-pigs. Both doses of budesonide, 0.6 and 1.2mg/ml (inhaled, *s.i.d)*, failed to show a significant reduction in cell numbers, with the maximum concentration of budesonide being reached due to insolubility at higher concentrations. Mean ± SEM, N=6, *P<0.05, Kruskal-Wallis test *post hoc* Dunns.

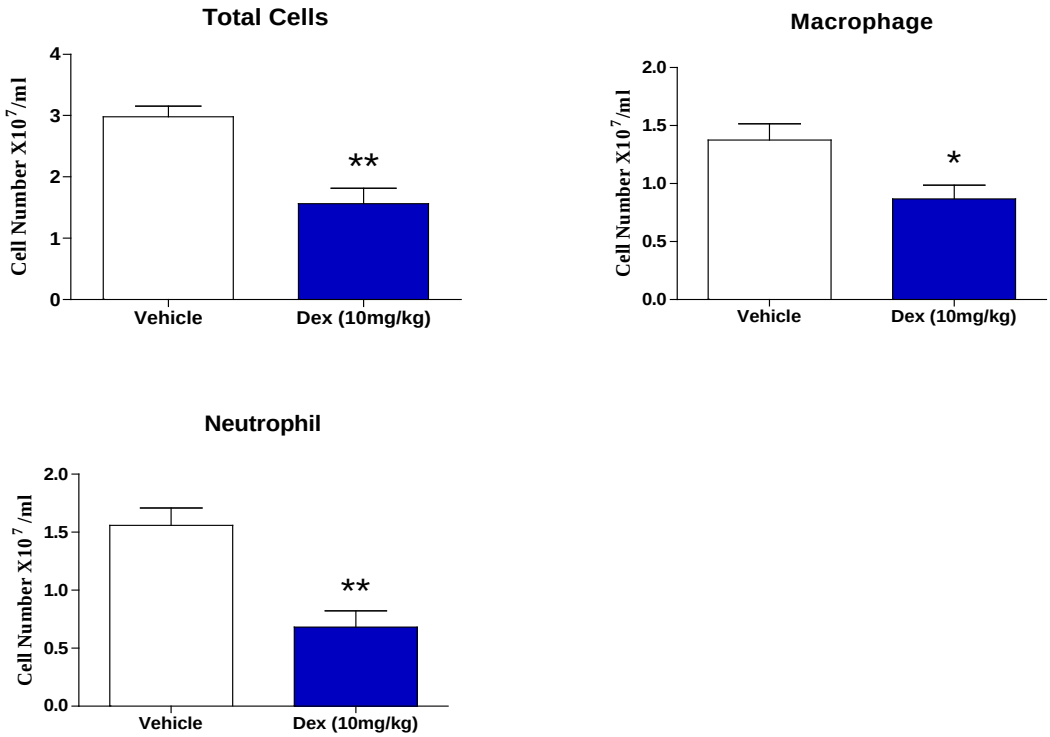


Figure 4.6 The Effect of Systemic Dexamethasone on Cell Influx into the Lung in Acute LPS Challenged Guinea-pigs. 10mg/kg dexamethasone (*s.i.d, i.p*) significantly reduced total cell numbers, including macrophages and neutrophils. Mean ± SEM, N=6, *P<0.05, **P< 0.01, Mann-Whitney test.

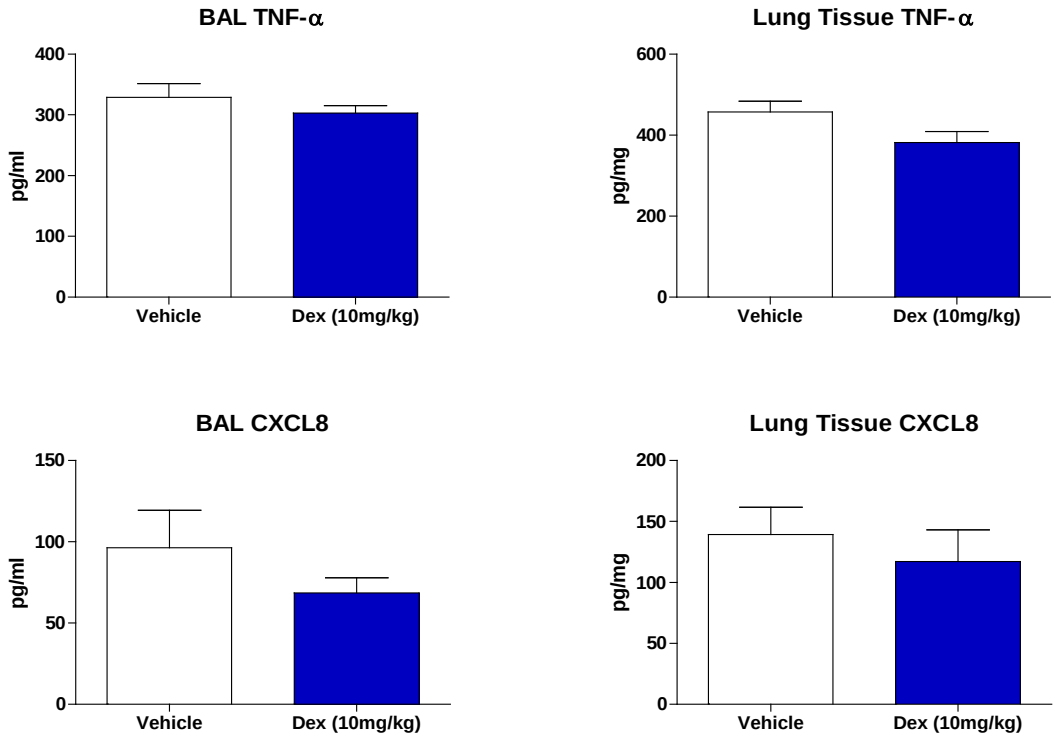


Figure 4.7 The Effect of Systemic Dexamethasone on TNF-α and CXCL8 Levels in the BALF and Lung Tissue of Acute LPS Challenged Guinea-pigs. Dexamethasone (10mg/kg *s.i.d, i.p*) showed no significant difference in both TNF-α and CXCL8 levels in either the BALF or the lung tissue. Mean ± SEM, N=6, P>0.05, Mann-Whitney test *post*.

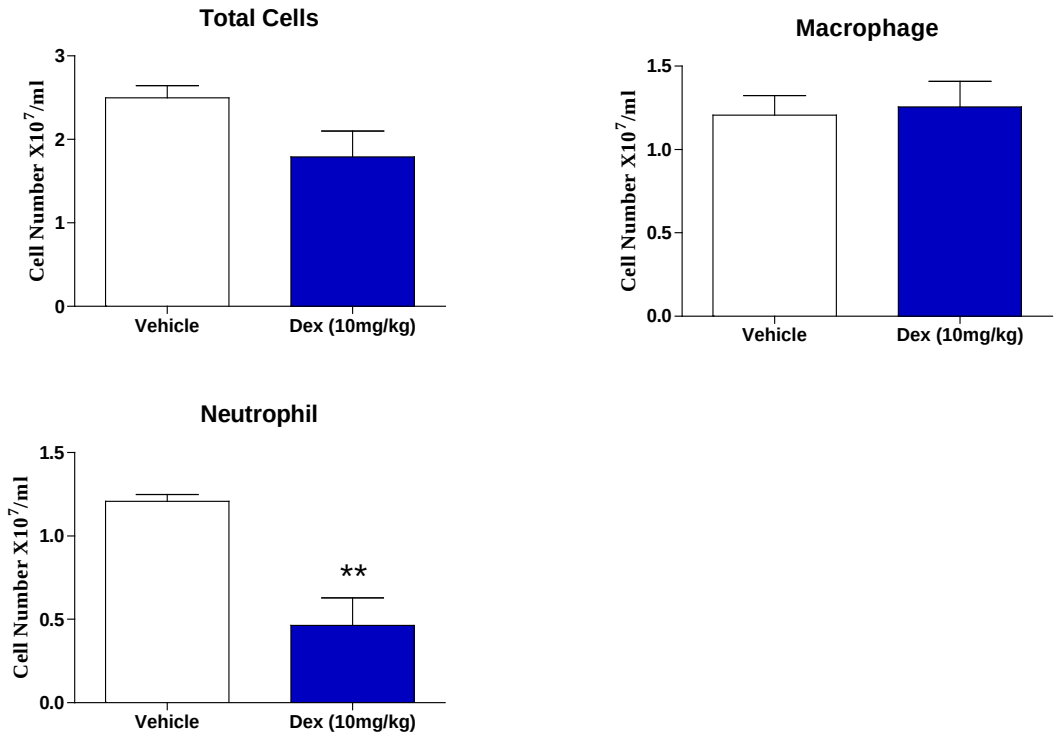


Figure 4.8 The Effect of Systemic Dexamethasone on Cell Influx into the Lung After Chronic LPS Challenge. Dexamethasone (10mg/kg *s.i.d, i.p)* significantly reduced neutrophils without having a significant impact on total or macrophage numbers compared to the vehicle control group. Mean ±SEM, N=5, **P<0.01, Mann-Whitney test

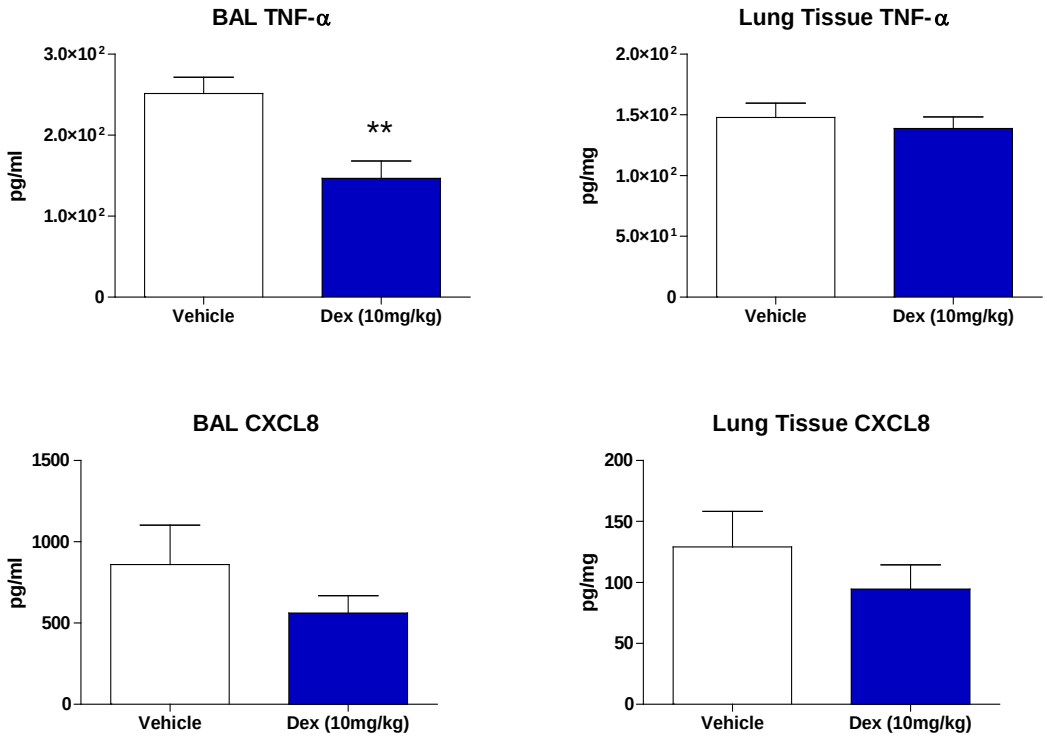


Figure 4.9 The Effect of Systemic Dexamethasone on TNF-α and CXCL8 Levels After Chronic LPS Challenge. Dexamethasone (10mg/kg *s.i.d, i.p*) significantly reduced TNF-α in the BALF but had no significant impact in the lung tissue. Dexamethasone also failed to have a significant difference on CXCL8 levels in both the BALF and lung tissue. Mean ±SEM, N=5, **P<0.01, Mann-Whitney test

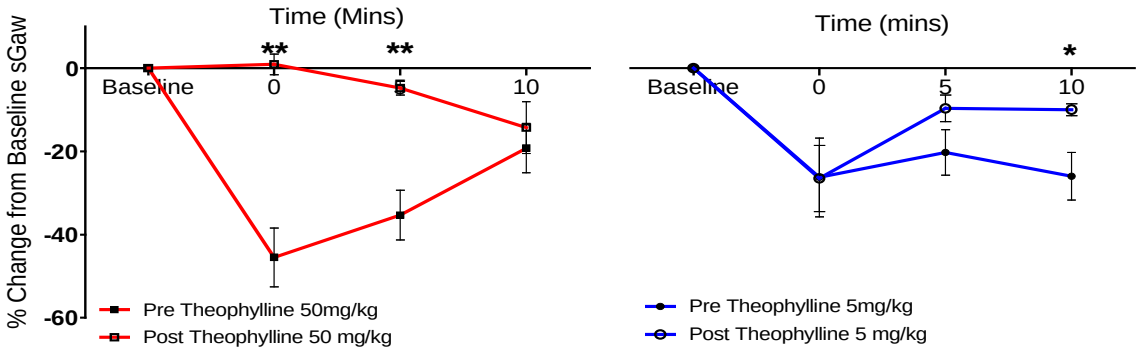


Figure 4.10 The Effect of High or Low Dose Theophylline Treatment on Histamine Induced Bronchoconstriction in the Guinea-pig. High dose theophylline (50mg/kg) treatment significantly impaired bronchoconstriction compared to pre treatment responses, while low dose theophylline (5mg/kg) treatment showed no significant change in bronchoconstriction to histamine (0.7mM, inhaled). Mean % change from baseline ±SEM, N=6,*P<0.05, **P<0.01, Paired T-Test.

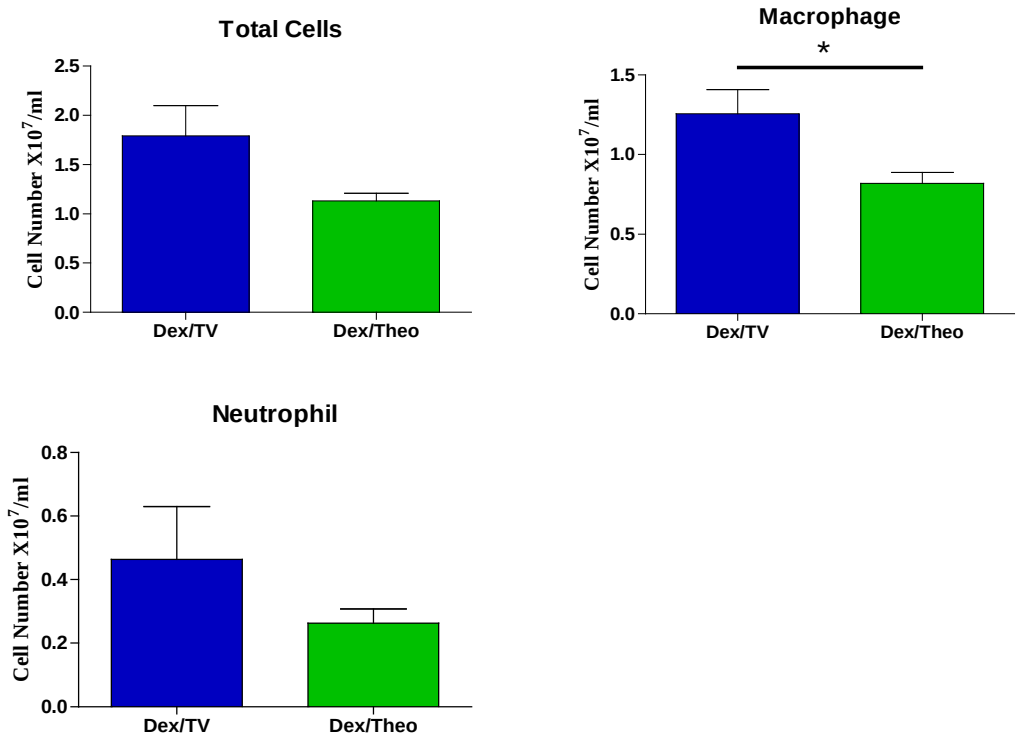


Figure 4.11 The Effect of Low-Dose Theophylline and Dexamethasone Co-treatment on Cell Influx into the Lung of Chronic LPS Challenged Guinea-pigs. Combined dexamethasone (10mg/kg *s.i.d, i.p*) and low-dose theophylline (5mg/kg *b.i.d, p.o*) treatment showed a significant reduction in macrophage numbers compared to the dexamethasone only treated group. Mean ±SEM, N=5, *P<0.05, Mann-whitney test.

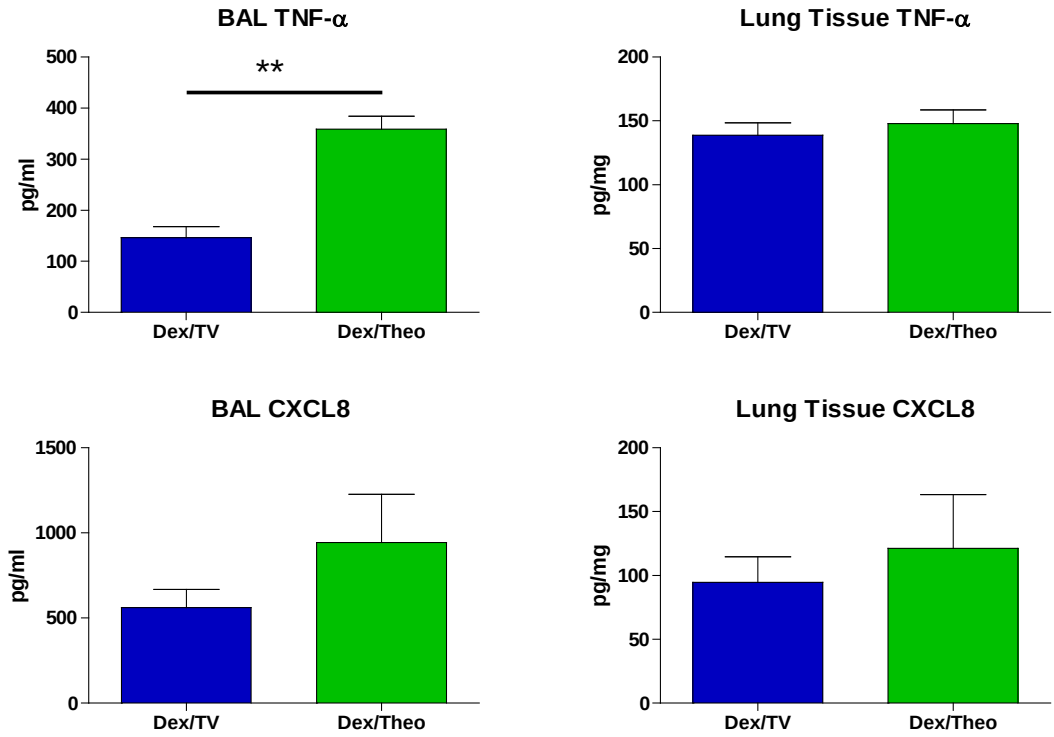


Figure 4.12 The Effect of Low-Dose Theophylline and Dexamethasone Co-treatment on TNF-α and CXCL8 levels in Chronic LPS Challenged Guinea-pigs. TNF-α in the BALF was the only measured parameter that showed a significant difference, with levels increased in the combined dexamethasone (10mg/kg *s.i.d, i.p*) and low-dose theophylline (5mg/kg *b.i.d, p.o*) treatment groups compared to dexamethasone alone. Mean ±SEM, N=5, **P<0.01, Mann-Whitney test.

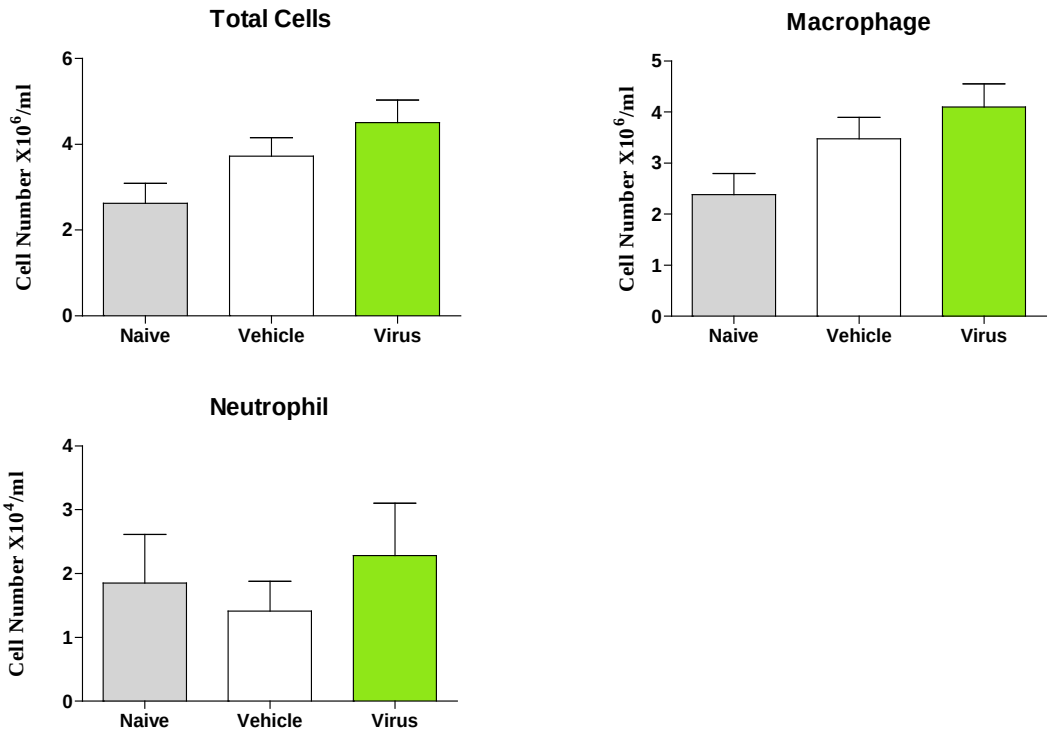


Figure 4.13 The Effect of Human Parainfluenza 3 Virus on Cell Influx into the Lung of Guinea-pigs. Virus (~1.25^7 virions, *b.i.d, i.n)* showed no significant change in any cell type compared to both the vehicle control and to naïve animals. Mean ±SEM, N=6, 1 P>0.05, Kruskal-wallis test *post hoc* Dunn's.

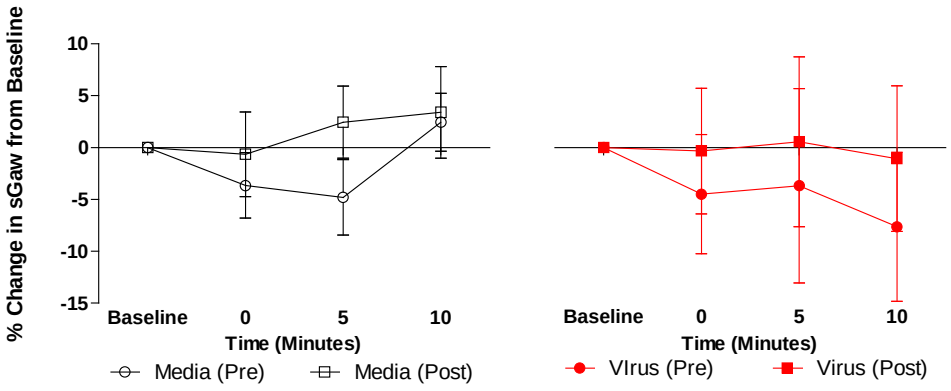


Figure 4.14 The Effect of Media and Parainfluenza 3 Virus Challenge on Airway Responses to 0.3mM Histamine. There was no significant difference in histamine responses before or after media or before or after virus 0, 5 and 10 minutes after histamine challenge. Mean ± SEM, N=6, P>0.05, Paired t-test.

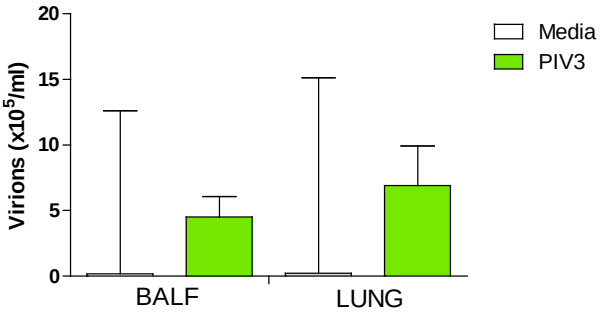


Figure 4.15 The Viral Titre After Media or Parainfluenza 3 Virus Challenge. There was no significant increase in virus titre in PIV3 challenged animals compared to media challenged animals, in either the BALF or lung tissue. Mean ± SEM, N=6, P>0.05, Mann-Whitney test.

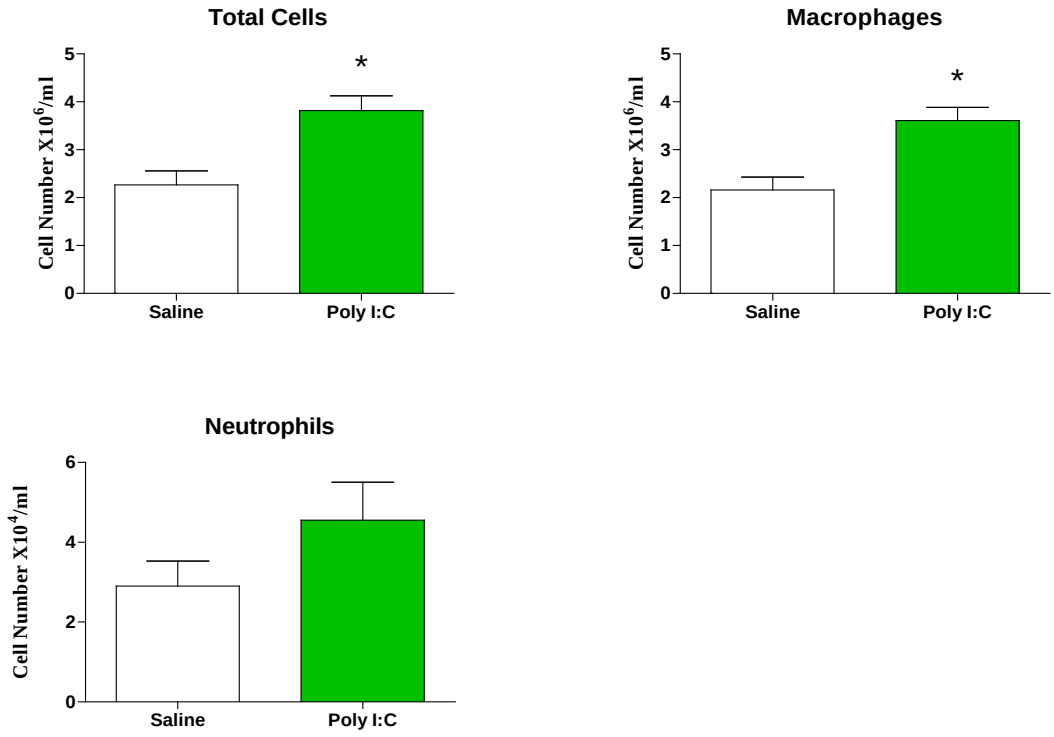


Figure 4.16 The Effect of Poly I:C on Cell Influx into the Lung of Guinea-pigs. Poly I:C (0.25mg *s.i.d, i.n*) showed a significant increase in total cell and macrophage numbers compared to the vehicle challenged animals. There was no significant change in neutrophil numbers. Mean ±SEM, N=6, *P<0.05, Mann-Whitney test

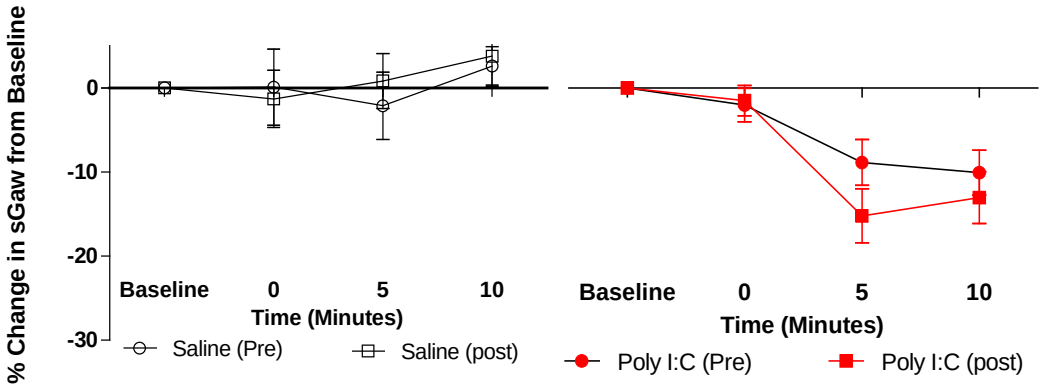


Figure 4.17 The Effect of Saline and Poly I:C Challenge on Airway Responses to 0.3mM Histamine. There was no significant difference in histamine responses before or after saline or before or after Poly I:C 0, 5 and 10 minutes after histamine challenge. Mean ± SEM, N=6, P>0.05, Paired t-test.

4.6 Discussion

Inflammation in COPD patients is largely unaffected by corticosteroids (Barnes, 2009), however acute LPS challenge was steroid sensitive. Guinea pigs have been shown to be less responsive to corticosteroids than other rodents (Keightley and Fuller, 1994), which may make them a better choice of species. This may not only be true in COPD models, but in many disease models, as humans are also less steroid sensitive than many rodents (Claman, 1972).

From the results shown here, acute LPS-induced inflammation was reduced by the systemic corticosteroid dexamethasone, with significant reductions observed for total leucocytes, neutrophils and macrophages, with no change in CXCL8 or TNF-α levels. Whelan *et al.* (1995) also showed steroid sensitivity in an acute LPS guinea pig model with reductions in neutrophils. Although the acute model showed no effect on inflammation after inhaled budesonide treatment, the reason for this may not be steroid insensitivity, but either the failure to reach a high enough dose due to solubility problems or the inability of inhaled steroids to exhibit an effect, while systemic steroids are effective. Nevin & Broadley (2004) showed that 0.3mg/ml of budesonide was able to suppress eosinophils 24 hours after a single exposure to LPS but was unable to lower total cell numbers or any other cell type, including neutrophils. However, the authors hypothesised this effect was due to the anti-inflammatory properties of the DMSO vehicle which exhibited similar results. This could mean that inhaled corticosteroids are ineffective at altering the neutrophillic inflammation in the lung, with inhaled budesonide having been demonstrated to have limited effects on patients with COPD. The treatment fails to decrease the inflammation, so also fails to decrease the rate of disease progression (Pauwels *et al*, 1999; Vestbo *et al*, 1999).

Many studies have implicated the macrophage in COPD as one of the causes of steroid insensitivity (Culpitt *et al.*, 2003; Cosio *et al.*, 2004). The chronic LPS model supports this hypothesis, with dexamethasone significantly reducing neutrophils, while the macrophage numbers remain completely unaffected. Dexamethasone reduced CXCL8 levels in the BALF and lung tissue by 35% and 27% respectively but this failed to reach significance, TNF-α showed a reduction in the BALF but remained unchanged in the lung tissue. Toward and Broadley (2001) demonstrated dexamethasone, 20mg/kg, drastically reduced neutrophil numbers in the chronic LPS model, while macrophage numbers were relatively unchanged. Experiments have been performed on alveolar macrophages, isolated from the sputum of COPD patients, which show macrophages do not decrease CXCL8 release in response to dexamethasone (Culpitt *et al.*, 2003).

As previously discussed, it has been hypothesized that the reason for this reduction in macrophage sensitivity is due to a reduction in HDAC activity. HDAC is important in the regulation of inflammation due to its recruitment by steroids, resulting in the removal of acetyl groups from histone proteins, decreasing pro-inflammatory transcription. Barnes *et al* (2004) propose that this reduction in HDAC is due to the increased oxidative stress burden in COPD. Cosio *et al.* (2004) showed macrophages isolated from COPD patients had a 51% reduction in HDAC activity compared to normal control subjects. This was shown to be reversed using a low dose of theophylline, increasing the activity of HDAC tenfold, which could be blocked by a HDAC inhibitor. This increase in activity resulted in increased sensitivity to dexamethasone and decreases in CXCL8. Low dose theophylline alone has also been shown to decrease neutrophil activity and CXCL8 levels in COPD (Kobayashi *et al.*, 2004). The same effect was observed by To *et al.* (2010) when

either low dose theophylline or a PI3K inhibitor was administered, that led to the hypothesis that theophylline is exerting its action by inhibiting PI3K. PI3Ks are enzymes responsible for phosphorylating phosphoinositides, lipid second messengers, which play a role in cell signalling by binding to Akt (protein kinase B) (To *et al.* 2010). Akt is able to control key intracellular processes by acting on NF-κB and IKK, which has previously been described to be involved in the Toll-like receptor pathway and COPD. IKK is bound to NF-κB resulting in an inactive form of NF-κB. Subsequent activation of Akt by PI3K results in the phosphorylation and resultant degradation of IKK, releasing active NF-κB and increasing transcription of inflammatory cytokines and oxidative stress. It has previously been shown that oxidative stress alone is able to decrease the activity of HDAC and increase the acetylation of histones (Tomita *et al*, 2003), so inhibition of PI3K ultimately leads to a reduction in active NF-κB, a decrease in transcription and a decrease in oxidative stress.

These *in vitro/ex vitro* effects of low-dose theophylline and dexamethasone treatment reported for alveolar macrophages isolated from COPD patients were tested in the chronic LPS guinea pig model. The dose of theophylline required to exert an anti-inflammatory effect has been shown to be significantly below the therapeutic bronchodilator dose (Ito *et al.*, 2002a). In this study, 50mg/kg theophylline was shown to have a significant effect on histamine induced bronchoconstriction, while the 10% dose had no effect. While there are large variations in initial histamine response between the two groups due to fluctuations in the response of guinea pigs to histamine (fig 4.10), the fact that the same guinea pigs are compared before and after treatment, reduces the error of this reading.

Low dose theophylline alone had no effect on cells or cytokines, but combined with dexamethasone significantly improved the responses to dexamethasone, reducing total leucocytes and neutrophils compared to the double vehicle control, but not significantly compared to dexamethasone alone. Macrophage responses were also improved, with a reduction in numbers but again this did not reach significance compared to dexamethasone only. CXCL8 levels remained unchanged, while TNF-α levels in the BALF were significantly elevated with co-treatment compared to dexamethasone only.

Clinical trials with theophylline and the inhaled corticosteroid fluticasone in COPD patients showed similar results to the *in vitro* experiments in terms of HDAC activity, which increased nine-fold, but there was no change in neutrophil numbers (Ford *et al.*, 2010). On the other hand, Kanehera *et al.* (2008) showed low dose theophylline reduced neutrophil numbers, but did not affect CXCL8 levels. There is very little clinical data showing the effects of low-dose theophylline alone or in combination with a corticosteroid. This means that the effects observed in *in vitro/ex vitro* alveolar macrophages are as yet unknown in the complete disease process. The decrease in macrophages observed in the chronic LPS model did not reach significance, however, the repeated exposure of a strong agonist such as LPS, may overpower any effect low-dose theophylline could exert.

With the chronic LPS model exhibiting aspects of steroid resistance, it is also important for a model of COPD to have the ability to be exacerbated, increasing the inflammation above what is observed in the stable disease. COPD exacerbations account for 15.9% of all hospital admissions in the UK, and are a major cause of morbidity and mortality (Wedzicha and Seemungal, 2007). Studies have shown that

viruses are detected in roughly half of acute exacerbations (47-64%), while detection limits are low during stable disease (Seemungal *et al.*, 2001; Rohde *et al.*, 2003). The most common viruses isolated are rhinovirus, influenza, coronavirus and parainfluenza (Wedzicha and Seemungal, 2007). A study looking into the aetiology of exacerbations found parainfluenza 3 to be the third most common cause of hospitalizations (Ko *et al.*, 2007a). Rhinovirus, influenza and coronavirus are viruses that are contagious and require high levels of containment, as well as some requiring a vaccination program. Parainfluenza, however, is a childhood associated infection with most children acquiring a parainfluenza infection before the age of 10 (Gardner, 1969). This gives most people immunity; however, this immunity is not complete and can result in further infections down the line (Henrickson, 2003) and has been shown to be a cause of exacerbations in COPD. This partial immunity makes parainfluenza a relatively safe, clinically relevant virus.

Parainfluenza 3 has been shown to infect guinea pigs (Freyer and Jacoby, 2012; Buckner *et al.*, 1981). Broadley *et al.* (2010) and Toward *et al.* (2005) showed intranasal administration of parainfluenza 3 virus in the guinea-pig significantly increased cell influx into the lung, which is predominantly macrophage driven, with slight increases in eosinophils and neutrophils. They also showed an increase in airway hyper responsiveness to histamine after virus compared to before. However, the studies performed here, did not show an increase in total, macrophage or neutrophil numbers, an increase in AHR or any difference in PCR titre between media and virus challenged animals. The PCR titre retrieved was also lower and not higher than the $1.25x10^7$ virions/ml inoculated, indicating a lack of infection and replication. This could be due to mutations in the virus after repeated culture, or due to mutations in the guinea pig. Bailley *et al* (2000) demonstrated that a change in the

nucleocapsid protein of PIV3 virus decreases its virulence in humans. PIV3 initially binds to neuraminidase acid receptors on the cell surface via haemagglutanin (Henrickson, 2003). A slight mutation on either of these proteins could result in decreased ability of the virus to attach and therefore infect the cell. As the virus is cultured more and more in the BSC-1 cell line, the virus is likely to mutate into a more virulent strain for this specific cell line, to the possible detriment of virulence in other species and cell types.

The TLR3 agonist poly I:C was also used in this study as an alternative for possible use as an exacerbating agent. It also failed to produce an adequate response, with no significant increase in neutrophil numbers. Poly I:C is a double stranded RNA mimetic which acts via activation of the MYD88 dependent and independent pathways as a result of TLR3 activation. A study performed by Stowell *et al,* (2009) showed intranasal inoculation of poly I:C in anesthetised mice caused a reduction in lung function and an increase in cellular influx. Field *et al* (1972) showed that the failure to initiate an exacerbation with poly I:C is possibly due to the route of administration and not due to the chemical failing to trigger a response. The study showed poly I:C injected intravenously in guinea pigs developed antibodies to poly I:C while intranasal administration failed to do so.

There were a number of possible reasons for the failure to achieve adequate infection to exhibit inflammation. Intranasal inoculation in conscious animals is not very accurate, with animals attempting to clear their noses of virus infected media or poly I:C by sneezing. Nardelli-Haefliger *et al* (2001) demonstrated that intranasal bacterial inoculation in conscious mice significantly reduces antibody responses compared to anaesthetised animals. Conscious mice inhaled only 0.1% of the

inoculated dose compared to 30% in anaesthetised mice, which would result in the minimum infective dose of PIV3 not being achieved if the same results transferred to the guinea pig. This shows that intranasal inoculation in conscious animals is a very unreliable method of initiating an exacerbation and may be the reason the model failed to show an adequate exacerbation.

In summary, the chronic LPS model exhibits aspects of steroid resistance, with macrophages remaining unchanged with systemic dexamethasone treatment. Low-dose theophylline improves the action of dexamethasone by decreasing macrophage numbers, but not significantly, and has no effect on cytokines. This means the model is exhibiting aspects of theophylline responses observed in *ex vitro* studies in alveolar macrophages, but the effect of theophylline in COPD as a whole still remains unclear. The model also failed to exhibit an adequate inflammatory response to parainfluenza 3 virus or poly I:C, with no increase in neutrophil numbers for either inoculations. Therefore a new model that can be used to investigate exacerbations needs to be developed.

CHAPTER 5

Development of a Chronic Cigarette Smoke Induced Inflammatory Model in the Mouse

5.1 Cigarette Smoke Exposure as a Model of COPD

5.1.1 Cigarette smoke

Cigarette smoke is the most common risk factor for COPD, accounting for 73% of COPD related deaths in developed countries and 40% in less-developed countries (WHO, 2010). It is not just direct smoking that is detrimental; passive smoking has also been implicated in the development of COPD (Yin *et al.*, 2007), with 55% of smoke produced by a cigarette ending up as passive smoke (Yoshida and Tuder, 2007). This makes cigarette smoke the most clinically relevant stimulus in models of COPD.

Cigarette smoke is a complex cocktail, containing over 4700 different chemicals or oxidative species (Pryor and Stone, 1993). Studies are being performed to evaluate the toxicity of individual components, including aldehydes (Facchinetti *et al.*, 2007), nicotine (Smith and Hansch, 2000) acrolien (Pfeifer *et al.*, 2002) and reactive oxygen species (Rahman *et al.*, 2002)

5.1.2 Pathophysiology of Smoke Exposure

The pathological response to cigarette smoke has previously been discussed in chapter 1. Briefly, there is an increase in proinflammatory cytokines, such as CXCL8 and TNF-α. This results in inflammatory cell influx into the lungs, which are predominantly the neutrophil and macrophage, as well as cytotoxic T-lymphocytes. This results in three key processes occurring;

- A protease/anti-protease imbalance
- An oxidant/antioxidant imbalance
- A failure to repair and maintain the lung

This is summarised in figure 5.1.

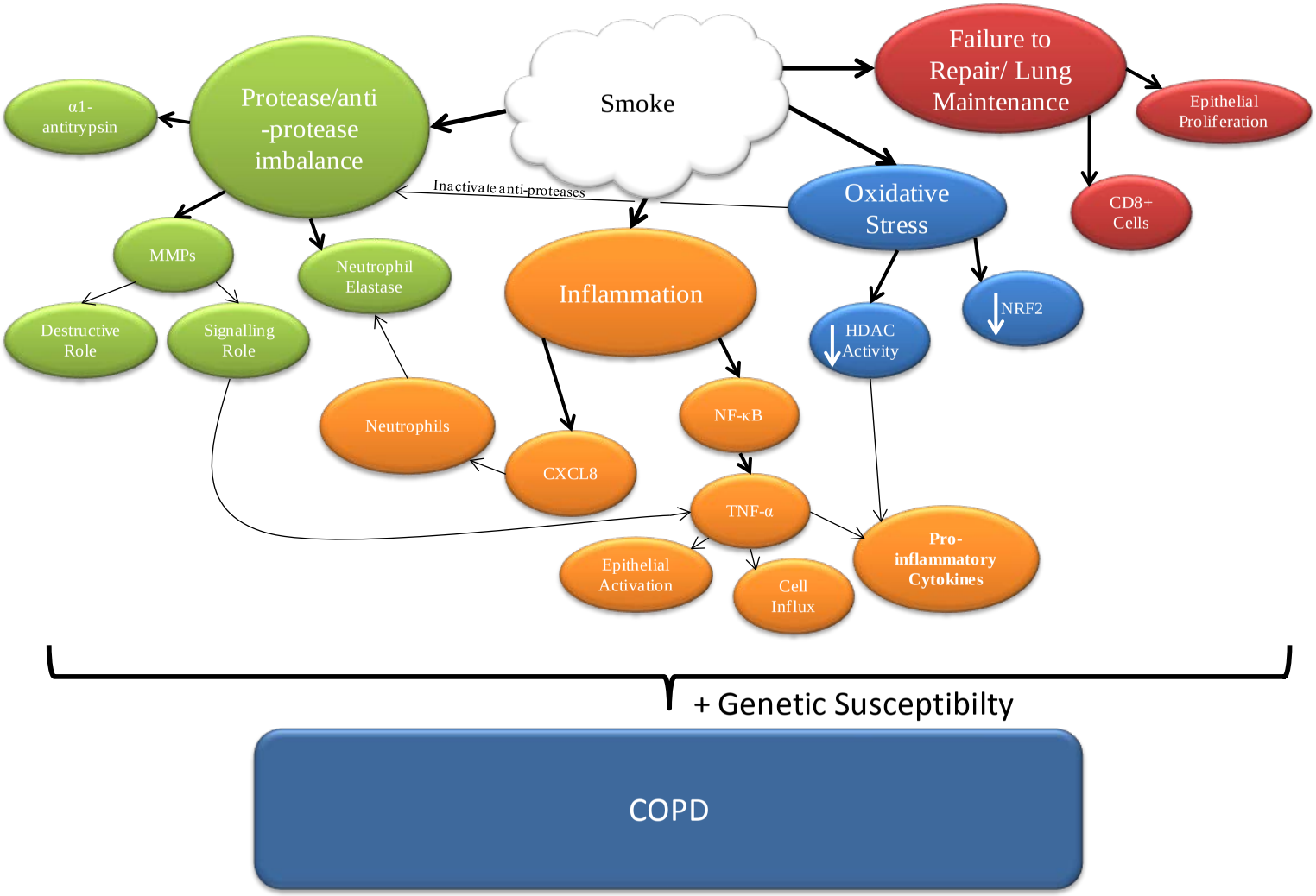


Figure 5.1 The effects of cigarette smoke in the lung. Cigarette smoke induces four main pathways in the lung: A protease/anti-protease imbalance, inflammation, oxidative stress and failure to repair the lung, which all results in COPD if the patient is genetically susceptible to it.

Recent studies have been conducted to elucidate the mechanisms by which cigarette smoke increases inflammation, which have implicated the innate immune receptors TLR4 (Doz *et al.,* 2008) and TLR9 (Mortaz *et al.,* 2009) in increasing CXCL8 levels in response to cigarette smoke. All these mechanisms result in the conditions associated with COPD; chronic bronchiolitis, emphysema and increased mucous production; However, the exact mechanism by which cigarette smoke exerts this large range of effects still remains unclear.

5.1.3 Cigarette Smoke Models

A large number of smoke models have been used in many different animal species in order to better understand COPD. Acute smoke models in mice, rats and guinea pigs have shown an increase in inflammation which is primarily neutrophil driven (Thatcher *et al.,* 2005; Hardaker *et al.,* 2010; Nishakawa *et al.,* 1999) and fails to show changes in histology. Chronic cigarette smoke challenge in rodents have demonstrated increases in inflammation that was more similar to COPD with a larger macrophage involvement as well as neutrophils (Churg *et al.,* 2004; Yang *et al.,* 2007; Wright *et al.,* 2002), and importantly have been demonstrated to exhibit histological changes, such as emphysema (Bartalesi *et al.,* 2005).

5.2 Aim

The aim of this chapter is to develop cigarette smoke induced inflammation in the mouse exhibiting a similar phenotype to COPD.

5.2.1 Objectives

- To investigate the differences in acute and chronic exposure to cigarette smoke in the mouse by measuring inflammatory cell influx, TNF-α and CXCL1 levels.

- Investigate the effect of chronic cigarette smoke exposure on lung function, measuring static compliance and total lung capacity, as well as lung histology.

5.3 Methods

5.3.1 Animal Husbandry

Animals were housed as described in 2.1

5.3.2 Cigarette Smoke Challenge

Balb/C Mice received cigarette smoke (1R3F Kentucky research cigarettes, table 5.1) or air (sham) into a sealed perspex chamber (7000cm^3) at a flow rate of 0.6 l/min for 30 minutes. Smoke was produced by peristaltic suction with 4 seconds of smoke per minute. The average particulate matter was recorded and adjustments to the amount of smoke entering the chamber altered accordingly to maintain a consistant reading across all groups. The average total particulate matter was 444.1 ± 1.9 tpm/M^3

Ingredient	Percentage (W/W)
Flue-Cured	32.54
Burley	20.04
Turkish	11.09
Maryland	1.06
Reconstituted Sheet	27.17
Invert Sugar	5.3
Glycerine	2.8

Table 5.1 The blend of 1R3F research cigarettes acquired from the University of Kentucky

5.3.2.1 Acute Cigarette Smoke Challenge

Mice were exposed to cigarette smoke or air for 30 minutes, twice a day, for 3 consecutive days. A minimum of 5 hours was allowed between exposures (fig 5.2). 24 hours after the final exposure the mice were culled and a bronchalveolar lavage

was performed to determine total and differential leucocyte numbers and cytokines as described in 2.7.

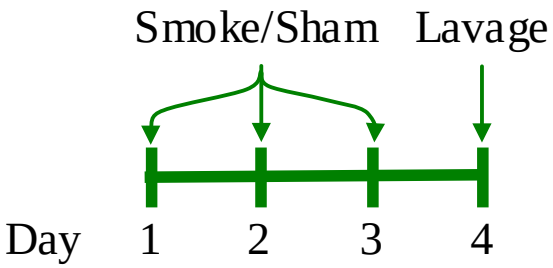


Figure 5.2 A diagramatic representation of the acute cigarette smoke model

5.3.2.2 Chronic Cigarette Smoke Challenge

Mice were exposed to cigarette smoke or air for 30 minutes, twice a day, 5 days a week, for 5 weeks. On the sixth week, mice recived twice daily smoke or air for 3 days (fig 5.3) Animals were killed 24 hours after the final smoke or air challenge and a bronnchoalveolar lavage was performed to determine total and differntial leucocyte numbers as described in 2.7. Animals were also killed 96 hours later to determine lung function changes after inflammation had subsided.

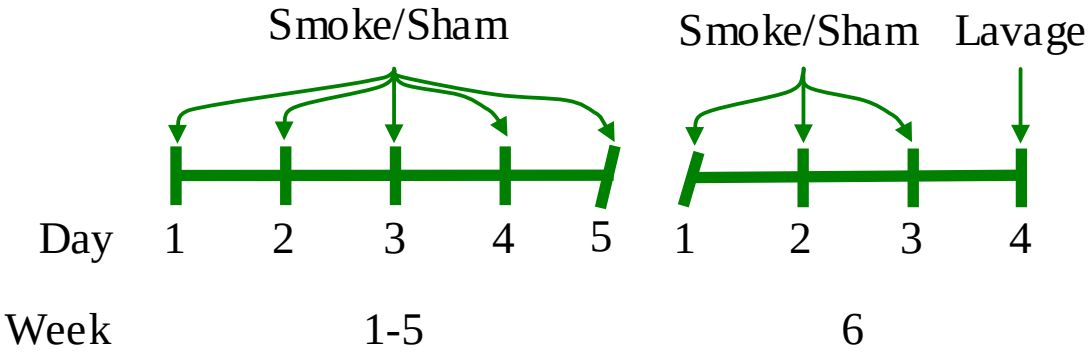


Figure 5.3 A diagramatic representation of the chronic cigarette smoke model

5.4 Results

The Effect of Acute Cigarette Smoke or Acute Air Challenge on Cell Influx into the Lung of Mice

Acute cigarette smoke challenge significantly increased total leucocyte numbers in the lung compared to sham challenged animals (489.7 ± 69.3 and 110.8 ± 15.2 $x10^3$cells/ml respectively) (fig 5.4). This was predominantly neutrophillic, with acute cigarette smoke challenged mice expressing significantly higher neutrophil numbers compared to sham challenged animals ($P<0.001$), but no significant difference in macrophage numbers between the groups.

The Effect of Acute Cigarette Smoke or Acute Air Challenge on CXCL1 and TNF-α Levels in the BALF and Lung Tissue of Mice

There was no significant difference between BALF CXCL1 levels or TNF-α levels in acute sham and acute cigarette smoke challenged animals (fig 5.5). However, there was a significant increase in CXCL1 levels ($P<0.001$) and TNF-α levels (18.3 ± 4.8 and 0.8 ± 0.8pg/mg respectively) in the lung tissue when acute cigarette smoke challenged groups were compared with their control group.

The Effect of Chronic Cigarette Smoke or Chronic Air Challenge on Cell Influx into the Lung of Mice.

Chronic cigarette smoke challenge significantly increased total leucocytes, neutrophils and macrophages compared to chronic sham challenged animals ($P<0.001$) (fig 5.6).

The Effect of Chronic Cigarette Smoke or Chronic Air Challenge on CXCL1 and TNF-α Levels in the BALF and Lung Tissue of Mice

Chronic cigarette smoke challenge significantly increased CXCL1 levels in the BALF and lung tissue compared to chronic sham challenged animals ($P<0.05$) (fig

5.7). In contrast, TNF-α levels in both the BALF and lung tissue showed no significant difference in levels when chronic sham and chronic cigarette smoke challenged animals are compared.

The Effect of Chronic Cigarette Smoke or Chronic Air Challenge on Total Lung Capacity and Static Compliance in Mice

Chronic cigarette smoke significantly increased total lung capacity and static compliance ($P<0.01$) in the mice compared to chronic sham challenged animals (fig 5.8).

The Effect of Chronic Cigarette Smoke or Chronic Air Challenge on Emphysema in the Mouse Lung.

Chronic cigarette smoke challenge significantly decreases the mean linear intercept compared to chronic air challenged mice (27.9 ± 0.92 and 38.3 ± 1.1 respectively) (fig 5.9). This signifies an increase in emphysema in cigarette smoke challenged mice compared to sham challenged animals. Figure 5.10 shows chronic cigarette smoke challenged mice exhibit larger emphysematous space than chronic air challenged mice.

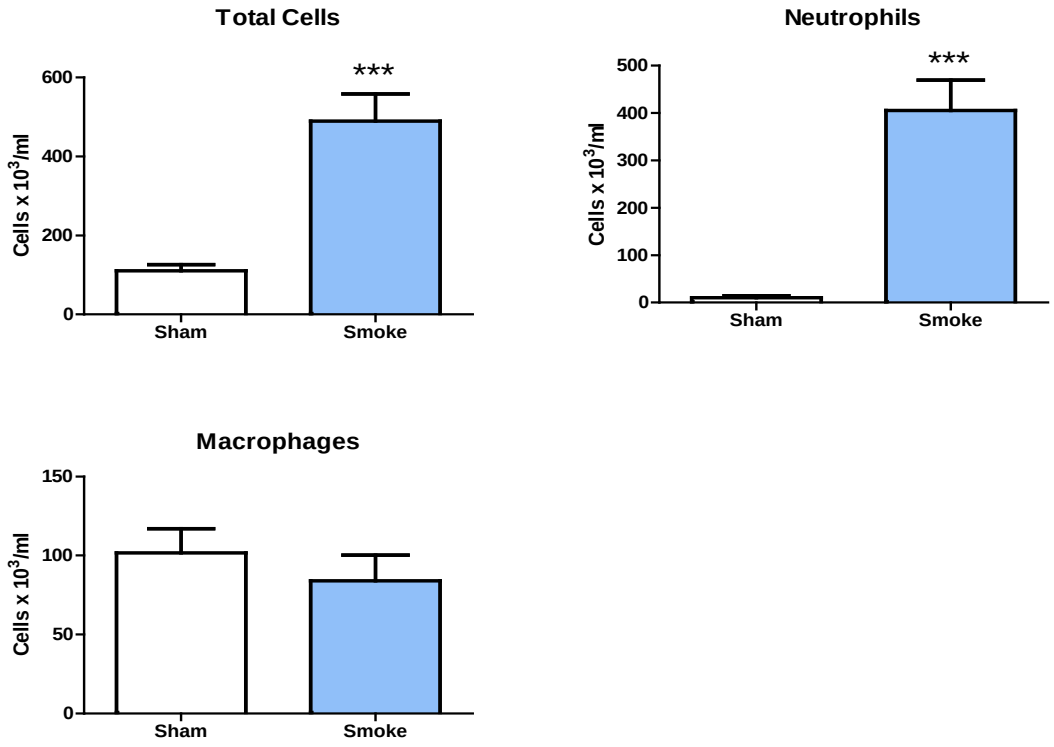


Figure 5.4 The effect of acute cigarette smoke challenge on total cell, macrophage and neutrophil numbers in the mouse. Acute cigarette smoke challenge significantly increases total cell and neutrophil numbers compared to sham challenged animals, with macrophages showing no significant difference. Mean ± SEM, N=10, ***P<0.001, Mann-Whitney test.

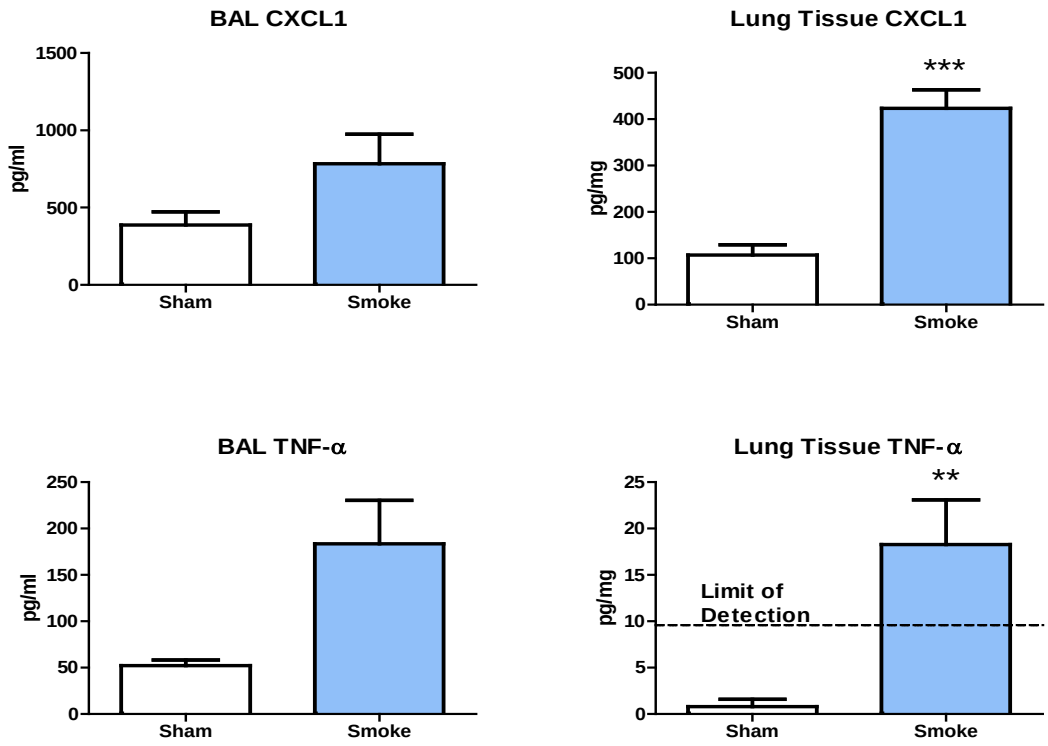


Figure 5.5 The effect of acute cigarette smoke challenge on the cytokines CXCL1 and TNF-α in the mouse. Acute cigarette smoke challenge significantly increases CXCL1 levels in the lung tissue compared to sham challenged animals, while there is no significant difference in the BALF. TNF-α shows no significant change in either BALF or lung tissue. Mean ± SEM, N=10, **P<0.01, ***P<0.001, Mann-Whitney test, ----- limit of detection.

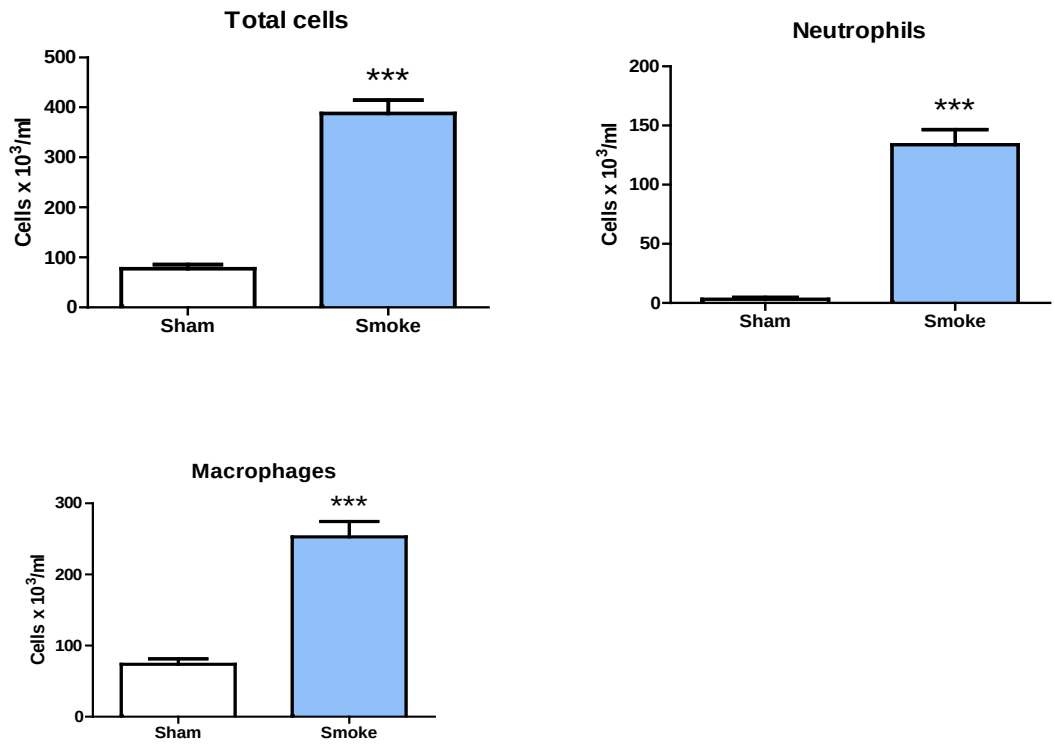


Figure 5.6 The effect of chronic cigarette smoke challenge on total cell, neutrophil and macrophage numbers in the mouse. Chronic cigarette smoke challenge significantly increases all parameters compared to sham challenged animals. Mean ± SEM, N=10, ***P<0.001, Mann-Whitney test.

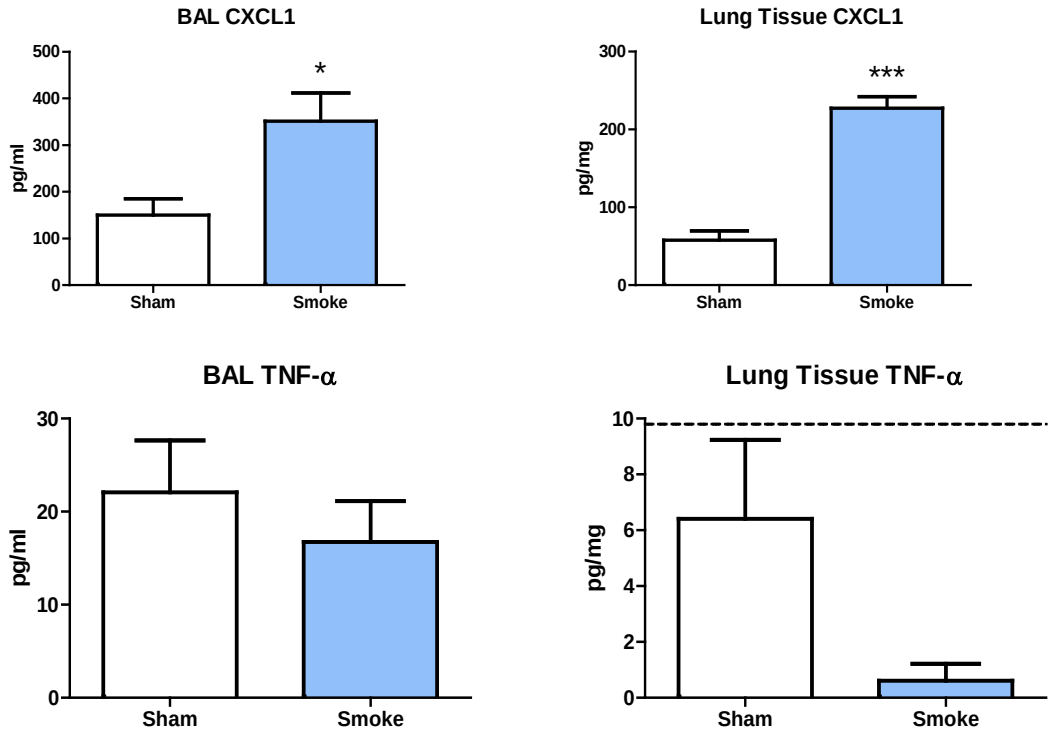


Figure 5.7 The effect of chronic cigarette smoke challenge on the cytokines CXCL1 and TNF-α in the BALF and lung tissue of the mouse. Chronic cigarette smoke challenge significantly increases CXCL1 in both the BALF and lung tissue compared to sham challenged animals. TNF-α shows no significant difference in both the lung tissue and the BALF compared to sham challenged animals. Mean ± SEM, N=10, *P<0.05, ***P<0.001, Mann-Whitney test ---- limit of detection.

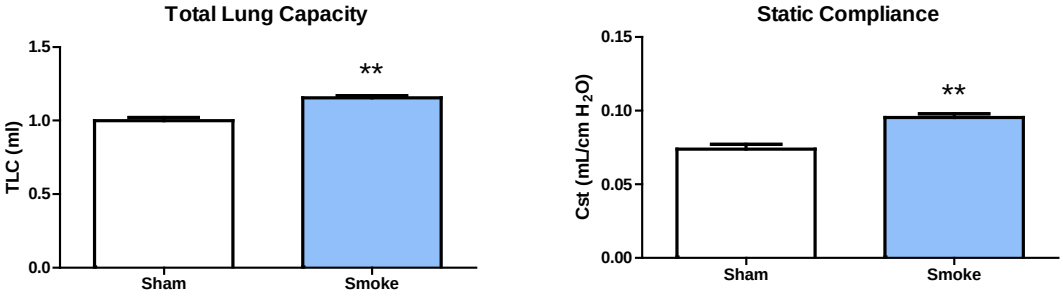


Figure 5.8 The effect of chronic cigarette smoke challenge on the lung function of mice. Chronic cigarette smoke challenge results in a significantly different total lung capacity and static compliance when compared to sham challenged animals. Mean ± SEM, N=10, **P<0.01, Mann-Whitney test.

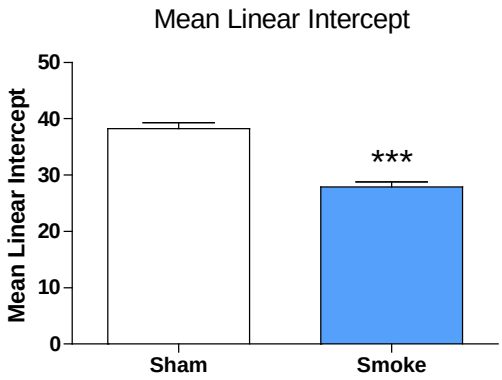


Figure 5.9 The mean linear intercept for chronic sham and chronic smoke challenged mice. Chronic smoke challenged animals show a significant decrease in mean linear intercept compared to chronic sham challenged animals, signifying an increase in emphysema. Mean ±SEM, N=10, ***P<0.001, Mann-Whitney test.

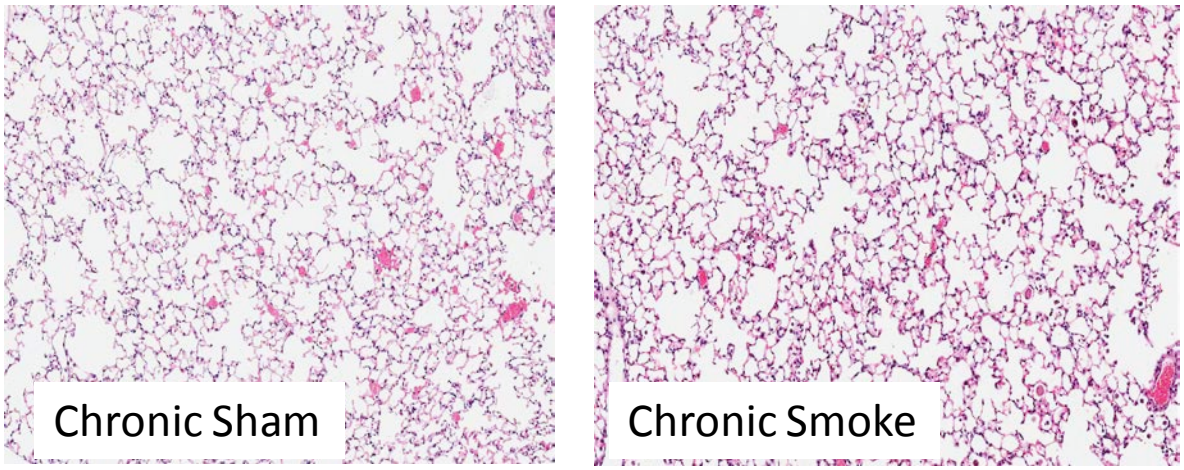


Figure 5.10 An Example of the Histology of the Mouse Lung After Chronic Saline or Chronic Cigarette Smoke Challenge. Chronic cigarette smoke increases the emphysematous air spaces in the lung compared to chronic air challenged mice.

5.5 Discussion

Many varied smoke models have been used in many different animal species, in an attempt to examine the complex pathways involved in the development of COPD. Acute smoke models are routinely employed using mice (Thatcher *et al.*, 2005), rats (Hardaker *et al.*, 2010) and guinea pigs (Nishikawa *et al.*, 1999). Smoke is delivered in varying different ways; nose only inhalation, nose and mouth inhalation, whole body smoke exposure, anesthetized inhalation and intratrachael inhalation (van der Vaart *et al.*, 2004), with varying acute smoking schedules, making it particularly difficult to compare these non-standardised protocols. However, they all show an increase in inflammation which is neutrophil and macrophage driven.

The acute smoke model shows a purely bronchoalveolar inflammation, with no inflammatory infiltration into the lung (from previous studies at Novartis) and an inflammatory profile of 83% neutrophils and 17% macrophages, while COPD shows a 58% neutrophil and 33% macrophage profile (Beeh *et al.*, 2003). This means that acute cigarette smoke exposure is a predominantly neutrophillic response, with little macrophage involvement. Wan *et al.* (2010) also demonstrated that acute, 3 day cigarette smoke exposure in mice was almost entirely neutrophillic, with no macrophage involvement. Macrophages play a very important role in the pathophysiology of COPD (Barnes, 2004a), meaning the acute cigarette smoke model is not expressing the correct pathophysiology for the disease.

The most important thing to note in terms of acute cigarette smoke models is the lack of structural changes and lung function impairment. There are no structural changes in an acute model, while COPD exhibits emphysematous changes, due to the loss of parenchymal cells (Barnes, 2012). No structural changes have been reported in the literature after acute cigarette smoke challenge in any species. Churg *et al.* (2002)

did show acute smoke exposure resulted in measurable markers of elastin and collagen breakdown after 24 hours, but did not lead to structural changes due to insufficient time for these changes to occur. Previous studies at Novartis have also shown that this model does not exhibit any change in lung function after acute cigarette smoke exposure, as well as being sensitive to corticosteroids. Despite these flaws, the acute smoke model is favoured in drug discovery over the more chronic models due to time constraints and the expense of keeping and dosing animals for a prolonged period of time. However, with the wrong distribution of inflammation and no change in lung structure or lung function, the relevancy of any results obtained in this model is questionable. A more chronic model with more clinically relevant manifestations of COPD is required.

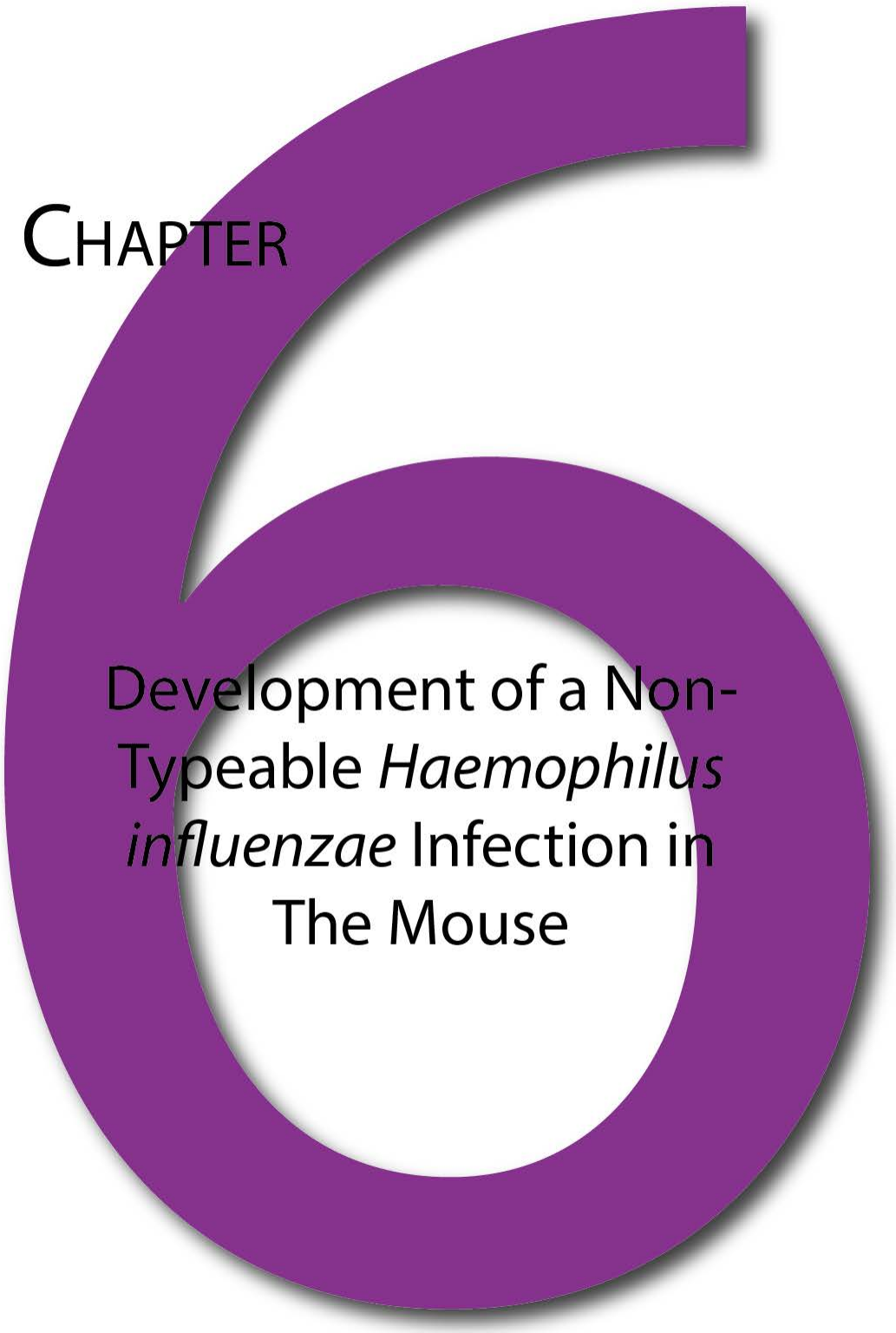
Chronic cigarette smoke as a model of COPD, like the acute model, has been used in many different species, including mice (Churg *et al.*, 2004), rats (Yang *et al.*, 2007), and guinea pigs (Wright *et al.*, 2002). Chronic cigarette smoke significantly increased total, neutrophil and macrophage numbers, with an inflammatory profile of 35% neutrophils and 65% macrophages meaning this model displays a larger, and more representative, macrophage response than the acute model. The chronic model displayed increases in CXCL1 in both the BALF and lung tissue just like in COPD (Yammamto *et al.*, 1997; de Boer *et al.*, 2000), unlike in the acute smoke model, which only showed elevations in CXCL1 in the lung tissue. However, the model exhibited no increase in TNF-α in either the lung tissue or the BALF, with very similar levels as observed in the sham exposed animals. These cytokines were chosen for the preliminary investigation to make the results comparable with the LPS model. Since cytokines were measured using an MSD 10-plex assay, other cytokines did show differences, but significance was only observed in IL-10 in the BALF and

IL-1β in the lung tissue which were significantly impaired and increased by smoke respectively ($P<0.01$). Many studies have shown that TNF-alpha is increased in COPD (Profita *et al.,* 2003; Cosio *et al.,* 2004), while there is increasing evidence that TNF-α release from macrophages is decreased after exposure to cigarette smoke as a result of oxidative stress (Droeman *et al.,* 2005; Berenson *et al.,* 2006a). The exact effect cigarette smoke has on the inflammatory pathways in macrophages as yet remains poorly understood, but will be discussed in more detail in chapters 7 and 8. Another possible reason for a decrease in TNF-α levels is due to chronic exposure to a stimulus desensitizing receptors of the innate and adaptive immune system, decreasing the response to the stimulus over time (Parker *et al.,* 2004).

Unlike in the acute cigarette smoke model, the chronic model displayed decreases in lung function. In COPD forced expiratory volume (FEV_1) is decreased, while total lung capacity (TLC) (O'Donnel *et al.,* 2004) and static compliance (C_{stat}) (Zanotti *et al.,* 1995) are measurably increased. This is due to loss of elasticity in the lungs increasing compliance, resulting in hyperinflation and increases in total lung capacity (Vlahos *et al.,* 2006). This makes patients feel short of breath as the lungs are unable to efficiently exhale all the air in the lungs, decreasing the amount of oxygen that can then be inhaled. As in COPD, the chronic smoke model shows significant increases in total lung capacity and static compliance and also demonstrates emphysematous changes, with a significant decrease in mean linear intercept in smoke challenged animals compared to the sham controls. A decrease in mean linear intercept signifies a reduction in the number of alveolar walls, due to the loss of parenchyma (Barnes, 2011). As previously mentioned, many studies have been performed to assess the correlation of emphysema with lung function, which

show that the two are poorly related (Robbesom *et al.*, 2003), with emphysema also varying widely between patients in the same stage of COPD (Makita *et al.*, 2007).

In summary, this data has shown that the chronic cigarette smoke model exhibits similar inflammation and structural changes as COPD and also results in a decrease in lung function. This makes the chronic model a better model over acute cigarette smoke exposure in exhibiting several important aspects of COPD. Therefore, the chronic model is the model that should be used in further studies investigating exacerbations and pathways.



Development of a Non-Typeable *Haemophilus influenzae* Infection in The Mouse

6.1 Bacteria and COPD

Bacteria's role in COPD is poorly understood due to the difficulties in sampling and identification; however, studies are beginning to suggest that bacteria also play a vital role in the pathophysiology of COPD. Bacterial colonization of the lungs is believed to not only cause increased numbers of exacerbations, but to also increase stable COPD inflammation, independent of cigarette smoke, increasing the severity and progression of the disease (Sethi *et al.,* 2006). The most commonly isolated bacterium in COPD is non-typeable *Haemophilus influenza* (NTHi), followed by *Streptococcus pneumoniae* and *Morexella catharrlis* (Wedzicha & Seemungal, 2007).

6.2 NTHi and COPD

Haemophilus influenza was first discovered in 1892 by Pfeiffer (Murphy, 2001) and was the first organism to have its entire genome sequenced in 1995 (Todar, 2008). *Haemophilus influenza* and non-typeable *Haemophilus influenza* differ in the presence or absence of a polysaccharide capsule. *Haemophilus influenza* has six serotypes, *a-f,* dependent on the type of capsule present. NTHi lacks a capsule, so is therefore termed 'non-typeable'. Differing strains of NTHi can be distinguished using electrophoresis or enzymatic analysis of external proteins (Foxwell *et al.,* 1998).

NTHi is a Gram negative, pleomorphic coccobaccillus that can grow aerobically (in the presence of hemin and NAD) or anaerobically. NTHi is a human specific pathogen and colonizes 50% of children by the age of six, and 75% of healthy adults are colonized by this bacteria, but it is also an opportunistic pathogen (King, 2012). Non-typeable *Haemophilus influenza* is the most frequently isolated bacterium in COPD, accounting for between 30% and 60% of all stable COPD samples (Sethi *et*

al., 2006) and up to 87% of samples during acute exacerbations (Bandi *et al.,* 2001). Rossell *et al.* (2005) also showed NTHi was the most frequently sampled bacteria in healthy smokers. Studies have shown that impaired immune states and decreases in the ability of macrophages to phagocytose NTHi in COPD could be responsible for the persistent colonization as well as the increased rate of infection of NTHi in these patients (Berenson *et al.,* 2006).

NTHi have many virulence factors to evade detection and increase infection. The *Haemophilus inflenzae* capsule is a virulence factor that allows it to be more invasive than NTHi; however NTHi has several other virulence factors that increase its virulence. NTHi is a diverse species with very large genetic variability. This allows the bacteria to increase virulence, evade the host's immune system and better acquire the necessary nutrients (Garmendia *et al.,* 2012). NTHi has been shown, for example, to decrease the rate of ciliary clearance from the lungs due to the modulation of protein kinase C (Bailey *et al.,* 2012). It has also been demonstrated to evade complement by altering its lipooligosaccharide composition, preventing recognition and subsequent binding of IgM. (Langeris *et al.,* 2012). NTHi has also been shown to secrete IgA proteases to prevent detection (Erwin and Smith, 2007; Murphy *et al.,* 2011), as well as entering epithelial cells to evade phagocytosis (Morey *et al.,* 2011).

6.3 Aim

The aim of this chapter iss to determine the dose of NTHi required to cause an infection and inflammation in Balb/C mice and whether there is a difference in response to live or heat-inactivated NTHi.

6.3.1 Objectives

- Correlate the optical density of NTHi in broth to the CFU/ml for accurate determination of dose
- Identify the dose of NTHi that gives maximal response in inflammatory cell influx
- Investigate the inflammatory cell and cytokine responses as well as the bacterial load at 6, 24, 96 and 168 hours after NTHi inoculation.
- Identify the time taken to heat-inactivate NTHi at 70°C
- Quantify the LPS levels in live and heat-inactivated NTHi
- Investigate the response to live and heat-inactivated NTHi in the mouse measuring neutrophil influx and activity (Myeloperoxidase levels), as well as CXCL1 and TNF-α levels.

6.4 Methods

6.4.1 Animal Husbandry

Animals were housed as described in 2.1

6.4.2 Bacterial Growth Curve

6.4.2.1 Optical Density Measurements

NTHi was grown on chocolate agar plates (Becton Dickinson, UK) for 16 hours at 37°C with 5% CO_2 to achieve a lawn growth. Colonies were then added to 1.5mls of brain-heart infusion broth, supplemented with 1% hemin and 0.1% NAD to achieve a value greater than 1.1 when read in a spectrophotometer at a wavelength of 600nm (OD_{600}). 30mls of the supplemented brain heart infusion broth was placed in a culture flask and inoculated with the NTHi infected broth to give a starting optical density of 0.05. The flask was then placed in a shaking incubator at 37°C and 225rpm. Every 30 minutes, ranging from 0-480 minutes after inoculation, 1ml was taken and placed in a cuvette and the OD_{600} value was recorded.

6.4.2.2 Colony Forming Unit Analysis

A flask of supplemented brain heart infusion broth was prepared and incubated at 37°C with constant shaking at 300rpm. Every 30 minutes, ranging from 0-480 minutes after inoculation, 1ml was taken and serial dilutions were made in broth ranging from 1 in 10 to 1 in 1000000. 100µl of the dilutions ranging were streaked onto separate chocolate agar plates and incubated at 37°C with 5% CO_2 for 24 hours. The colony forming units per ml of the streaked plates were then determined on a protoCOL 2 plate reader (Symbiosis, UK)

6.4.3 Dose Response to Non-typeable *Haemophilus influenzae*

NTHi was grown on chocolate agar plates (Becton Dickinson, UK) before being inoculated into supplemented brain heart infusion broth (BHi) (Becton Dickinson,

UK) at an OD600 reading of greater than 1.1. A 1 in 10 dilution of the infusion was adjusted accordingly to give an OD600 reading of 0.4, which corresponded to $3x10^9$ CFU/ml.

Mice were anaesthetised with an intraperitoneal injection of hypnorm/hypnovel before receiving 50µl of supplemented brain-heart infusion broth or NTHi at concentrations ranging from $6x10^7$ to of $3x10^9$ CFU/ml *intra-nasally.* Animals were killed 24 hours after *intranasal* administration (unless otherwise stated). All bacterial inoculations took place in a laminar flow cupboard to prevent bacterial spread. After bacterial inoculation animals were housed in groups of 5 in an OptiMice bio-containment cage system (Animal Care Systems, USA) with an independent air supply.

6.4.4 Time-course of Non-typeable *Haemophilus influenzae* infection

Mice were inoculated with $1x10^8$cfu/mouse NTHi intranasally as described in 2.4.3. Mice were culled 6, 24, 96 and 168 hours after inoculation and a bronchoalveolar lavage performed, as described in 2.7, to determine inflammatory cells and cytokines.

6.4.5 Quantification of Colony Forming Units

100µl of lung homogenised in brain heart infusion broth (as described in 2.7.10) was streaked on chocolate agar plates (Becton Dickinson, UK) with a large dilution range using 1 in 10 serial dilutions in BHi broth. The plates were incubated at 37°C in 5% CO_2 for 16 hours. Plates were read on a protoCOL 2 colony counter (Synbiosis, UK) with the limit of detection ranging from a minimum of 30 to a maximum of 300 colonies per plate. Plates falling below 30 were considered to have no growth while plates exceeding 300 were considered too confluent for accurate counting. The

relevant dilution factors were input into the program to give colony forming units (CFU)/ml

6.4.6 Heat-inactivated NTHi

NTHi was grown on chocolate agar plates (Becton Dickinson, UK) for 16 hours at 37°C with 5% CO_2 to achieve a lawn growth. Colonies were then added to 1.5mls of brain-heart infusion broth, supplemented with 1% hemin and 0.1% NAD to achieve on OD_{600} value greater than 1.1. 30mls of the supplemented brain heart infusion broth was placed in a culture flask and inoculated with the NTHi infected broth to give a starting optical density of 0.05. The flask was then placed in a shaking incubator at 37°C and 225rpm for 380 minutes to allow the bacteria to be in the log phase of growth. A sample was then taken and the optical density of the broth measured. The flask was adjusted accordingly to achieve an optical density of 0.4 ($3x10^9$cfu/ml). 1 ml was then placed in boiling tubes and placed in a water bath set at 70°C. A tube was removed every ten minutes ranging from, 0-90 minutes after being placed in the bath. Serial dilutions were made in broth ranging from 1 in 10 to 1 in 1000000. 100µl of each dilution was streaked onto separate chocolate agar plates and incubated at 37°C with 5% CO_2 for 24 hours. The colony forming units per ml of the streaked plates were then determined on a protoCOL 2 plate reader (Symbiosis, UK).

6.4.7 Quantification of LPS Levels

LPS levels were determined using a limulus amebocyte lysate (LAL) assay kit (Thermo Scientific, UK) according to the instructions. Levels were determined for live and heat inactivated NTHi at $3x10^9$cfu/ml. Briefly, standards were made by dilution of the supplied standard in endotoxin-free water to give a range from 1EU/ml to 0.1EU/ml. An endotoxin-free 96 well plate was pre-incubated at 37°C for 10 minutes. 50µl of each standard, blank and sample (diluted 1:5000) was added to

the appropriate wells, covered and incubated for 5 minutes at 37°C. 50µl of the supplied LAL (dissolved in endotoxin free water) was added to each well before the plate was gently shook on a plate shaker for 10 seconds and incubated at 37°C for 10 minutes. 100µl of the supplied chromogenic substrate (dissolved in endotoxin- free water to yield a concentration of 2mM) was added to each well and then gently shaken on a plate shaker for 10 seconds and incubated at 37°C for 6 minutes. 50µl of the stop reagent (25%acetic acid) was added and the plate gently shaken for the final time for 10 seconds. The absorbance was then read at 410nm in a spectramax plate reader.

6.5 Results

The Time-Course of Growth of *Haemophilus influenza* Determined by Optical Density and Colony Forming Unit.

The optical density time-course for *Haemophilus influenza* showed three distinct phases. The lag phase starts at 0 minutes and ends around 180 minutes, with optical densities ranging from 0.053 ± 0.003 to 0.223 ± 0.043 respectively. The next phase was the log phase ranging from 210 minutes to 390 minutes with optical densities of 0.377 ± 0.072 and 1.730 ± 0.056 correspondingly; and the final phase was the stationary phase, where growth plateaus, ranging from 420 to 480 minutes with optical densities of 1.853 ± 0.054 and 1.907 ± 0.041 respectively (fig 6.1). The same pattern was observed for the colony forming unit time-course, although it was less distinct with a lot more variation. Here, the lag phase spanned 0 to 120 minutes with colony forming unit measurements of 4.1± 2.0 and 5.8 ± 3.7 $x10^8$CFU/ml correspondingly. The log phase ranged from 150 to 390 minutes with colony forming units of 25.2 ± 8.3 $x10^8$CFU/ml and 15.9 ± 2.7 $x10^9$CFU/ml respectively. The stationary phase spanned the same length of time as the optical density measurements, 420 to 480 minutes (17.1 ± 1.3 and 11.5 ± 2.1 $x10^9$CFU/ml respectively) (fig 6.1).

There was a good correlation between optical density and colony forming unit, with an R^2 value of 0.87. The equation of the graph was: $Y = 7.0x10^9x + 1.975\ x10^8$. From this equation it was inferred that a colony forming unit value of $3x10^9$CFU/ml requires an optical density of 0.4 (fig 6.2).

The Effect of Varying Doses of Haemophilus influenza on Cell Influx and Bacterial Retrieval from the Lung of Mice.

There was no significant difference in total leucocyte numbers between media challenged animals and mice challenged with $3x10^6$CFU/mouse of *Haemophilus influenza* (fig 6.3). However, there was a significant increase in leucocytes, when 1, 3 ($x10^7$CFU/mouse) ($P<0.01$) and $1x10^8$CFU/mouse were compared to media only challenged animals ($P<0.001$). Similarly, the exact same pattern was observed in neutrophil responses, with media and mice challenged with $3x10^6$CFU/mouse of *Haemophilus influenza* showing no significant difference, but the mice challenged with $1x10^7$CFU/mouse, $3x10^7$CFU/mouse and $1x10^8$CFU/mouse of *Haemophilus influenza* showed a significant increase when compared to the media only group. Macrophages, on the other hand, showed no significant difference for mice challenged with *Haemophilus influenza* at any dose compared to the media control group (152.0 ± 19.1 $x10^3$cells/ml).

There was no significant difference in bacterial retrieval in mice challenged with $3x10^6$CFU/mouse and $1x10^7$CFU/mouse *Haemophilus influenza* compared to media only animals, but there was a significant increase in retrieval when mice challenged with *Haemophilus influenza* at $3x10^7$CFU/mouse and $1x10^8$CFU/mouse were compared to media only animals ($P<0.01$) (fig 6.3).

The Bacterial Retrieval Time-Course Response to *Haemophilus influenza* Challenge in the Mouse.

There was a significant difference in viable bacterial clearance from the lungs between mice challenged with BHi media only and mice challenged with $1x10^8$ CFU/mouse of *Haemophilus influenza* at 6 hours and 24 hours ($P<0.001$) after *intra-*

nasal inoculation (fig 6.4). By 96 hours there was no significant difference between media only and *Haemophilus influenza* challenged animals, and by 168 hours both media and *Haemophilus influenza* challenged mice had a retrieval of 0.0 ± 0.0CFU/ml.

The Time-Course of Cell Influx into the Lung after *Haemophilus influenza* challenge in the Mouse

Total leucocyte and neutrophil influx into the lung was significantly increased in NTHi challenged mice compared to the control group at 6 hours ($P<0.001$), peaks at 24 hours ($P<0.01$ and $P<0.001$ respectively) and total leucocytes were still significantly increased at 96 hours ($P<0.05$), while neutrophils returned to similar levels as the control. There was no significant difference in total or neutrophil cell numbers by 168 hours after inoculation. Macrophages however, remained unchanged at 6 and 24 hours after NTHi challenge compared to their respective controls, but were significantly increased in NTHi groups compared to media only groups at 96 hours ($P<0.001$)). Macrophage numbers returned to similar numbers between NTHi groups and media only groups by 168 hours. Lymphocytes, like macrophages, remained unchanged in NTHi groups compared to their respective controls at 6 and 24 hours after challenge, however, at 96 and 168 hours lymphocyte numbers were significantly increased in NTHi animals compared to media only mice ($P<0.001$) (fig 6.5).

The Time-Course of CXCL1 and TNF-α Levels in the BALF and Lung Tissue after *Haemophilus influenza* challenge in the Mouse

CXCL1 levels in NTHi challenged animals were increased at six hours compared to media only challenged mice (1856.0 ± 529.5 and 145.4 ± 55.8pg/ml respectively) but

failed to reach significance (figure 6.6). However, at 24 hours the groups were significantly different <P<0.01), but returned to very similar levels at 96 and 168 hours after challenge. Similarly, CXCL1 levels in the lung tissue were increased in NTHi challenged mice compared to media only groups at 6 and 24 hours, but these differences were significantly different (P<0.001). As in the BALF, CXCL1 returned to very similar levels between the groups at 96 and 168 hours. TNF-α in the BALF was also raised at 6 hours in NTHi challenged animals compared to media only but again this did not reach significance. 24 hours after challenge however, NTHi groups had significantly elevated levels compared to the control (522.6 ± 223.6 and 12.8 ± 3.0pg.ml respectively), while at 96 and 168 hours the groups had very similar levels. TNF-α levels in the lung tissue were undetectable at any time point.

The Time Required to Heat-Inactivate NTHi at 70C

At the 0 minute time point the CFU/ml was 1.4 ± 0.6 $x10^{10}$. There was a rapid decrease in viable bacteria numbers up until 20 minutes, with a CFU/ml of 9238 ± 2531, which plateaued until 40 minutes with a CFU/ml count of 4368 ± 1634. There was then a sharp decline at 50 minutes (138 ± 137 CFU/ml), until all bacteria were killed at 60 minutes (0 ± 0 CFU/ml). Bacteria remained at 0 ± 0CFU/ml at 70, 80 and 90 minutes (fig 6.7).

Determination of LPS levels and Bacterial Viability in Media, Live and Heat-inactivated NTHi

The brain-heart infusion media had small levels of LPS, with an optical density value of 0.4. However, both the live and heat-inactivated NTHi had much higher values compared to the broth but had the exact same value as each other with an optical density value of 2.92 each (fig 6.8). Media and heat-inactivated NTHi showed no

evidence of viable bacteria with a CFU/ml value of 0 ± 0 each. Live NTHi showed a significantly increased bacterial viability (10466 ± 4781 CFU/ml) compared to both the media and heat-inactivated NTHi challenged groups ($P<0.01$) (fig 6.9).

The Effect of Live and Heat-Inactivated NTHi on Neutrophil Influx and Myeloperoxidase Levels in the BALF of Mice.

Live and heat-inactivated NTHi showed a significantly increased neutrophil response compared to media only challenged animals ($P<0.01$), however, there was no significant difference between live and heat-inactivated NTHi. The exact same pattern was observed for myeloperoxidase levels in the BALF, with media only challenged animals expressing significantly lower levels of MPO compared to both live and heat-inactivated NTHi ($P<0.05$). Again, there was no significant difference in MPO levels between live and heat-inactivated NTHi (figure 6.10).

The Effect of Live and Heat-Inactivated NTHi on CXCL1 and TNF-α levels in the BALF and Lung Tissue of Challenged Mice.

Live NTHi challenged animals showed a significantly higher expression of CXCL1 in the BALF compared to media only challenged animals ($P<0.01$). However, heat-inactivated NTHi showed no significant difference between either media only of live NTHi challenged mice. Similarly, the lung tissue CXCL1 levels were significantly elevated in live NTHI challenged mice compared to media only challenge ($P<0.001$), but the heat-inactivated NTHi group ($P<0.01$) also showed a significant increase in levels compared to media only animals. Again, there was no significant difference between live and heat-inactivated NTHi challenged groups.

TNF-α levels in the BALF increased significantly when both live ($P<0.05$) and heat-inactivated ($P<0.001$) NTHi challenged mice were compared to media only challenged animals. Again, there was no significant difference in levels between the live and heat-inactivated NTHI challenged groups. TNF-α levels in the lung tissue, although live and heat inactivated NTHi increased the expression of the cytokine, this was not significantly different from the media only challenged animals, nor each other (figure 6.11).

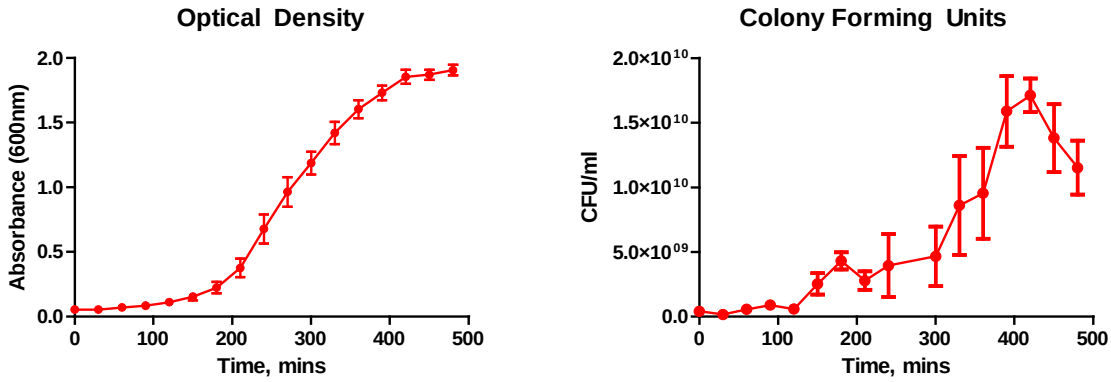


Figure 6.1 The Time Course of Growth for NTHi Measured by Optical Density and Colony Forming Units. Optical density showed a smooth growth curve with distinct lag, log and stationary phases, reaching optimum rate at ~200minutes and reaching peak at ~400minutes. The colony forming unit time course also showed rough lag, log and stationary phase around the same time as the optical density time course. Mean ± SEM, N=3.

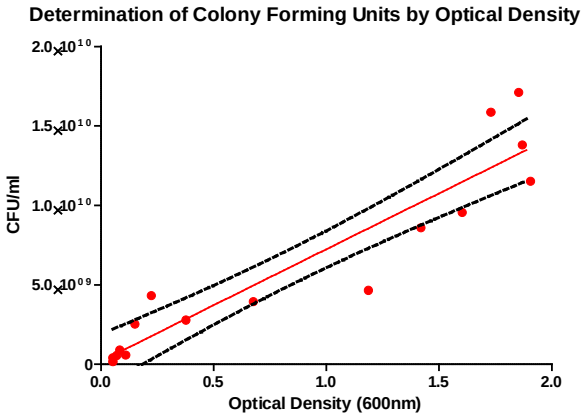


Figure 6.2 The Comparison of Optical Density and Colony Forming Units of NTHi. The graph shows the optical density required to obtain a set cfu/ml of bacteria. To achieve a cfu/ml of $3x10^9$, an optical density of 0.4 is required Mean ± SEM, N=3.

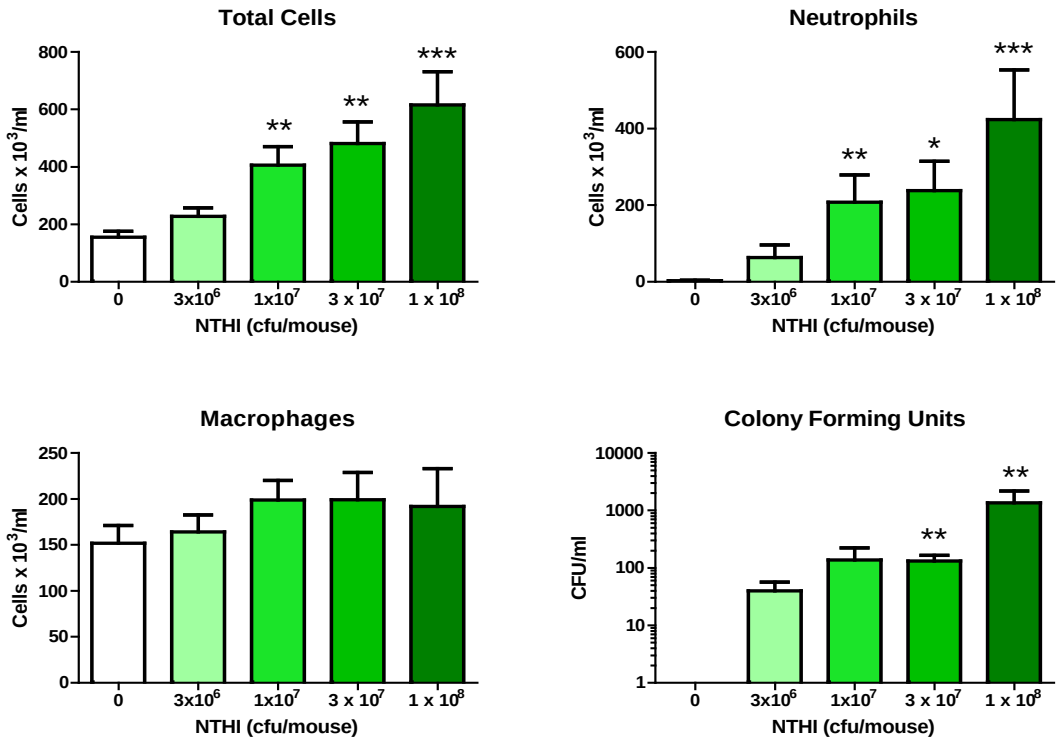


Figure 6.3 The Effect of Varying Concentrations of NTHi (0, $3x10^6$, $1x10^7$, $3x10^7$, and $1x10^8$ cfu/mouse) on Total Cell, Neutrophil and Macrophage Numbers in the BALF and Viable Bacterial Return from the Lung Tissue. Concentrations ranging from $1x10^8$ to $1x10^7$ showed significant increases in total cell and neutrophil numbers compared to the media control group. No group showed a significant difference in macrophages. Only $1x10^8$ and $3x10^7$ showed a significant difference in bacterial return compared to the control. $3x10^9$cfu/ml had the most statistically significant difference from control of all the concentrations. Mean ± SEM, N=10, *P<0.05, **P< 0.01, ***P<0.001 Kruskal-Wallis test *post hoc* Dunns

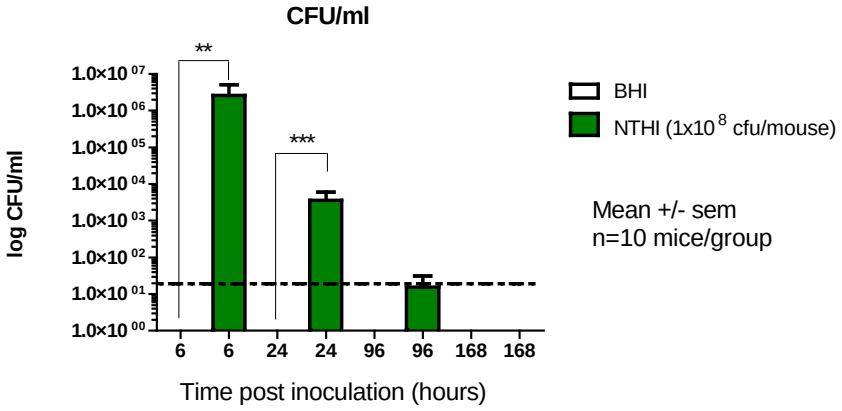


Figure 6.4 Viable Colony Forming Units/ml Retrieved from Lung Tissue at 6, 24, 96 and 168 Hours After NTHi Inoculation (1x10^8 cfu/mouse *i.n*). There was a significant difference between NTHi infected animals and their corresponding control group at 6 and 24 hours. NTHi infected animals did not show any significant difference to the controls at 96 and 168 hours with cfus falling below the limit of detection. BHi = Brain heart infusion broth, Mean ± SEM, N=10, **P< 0.01, ***0.001, Mann-Whitney test.

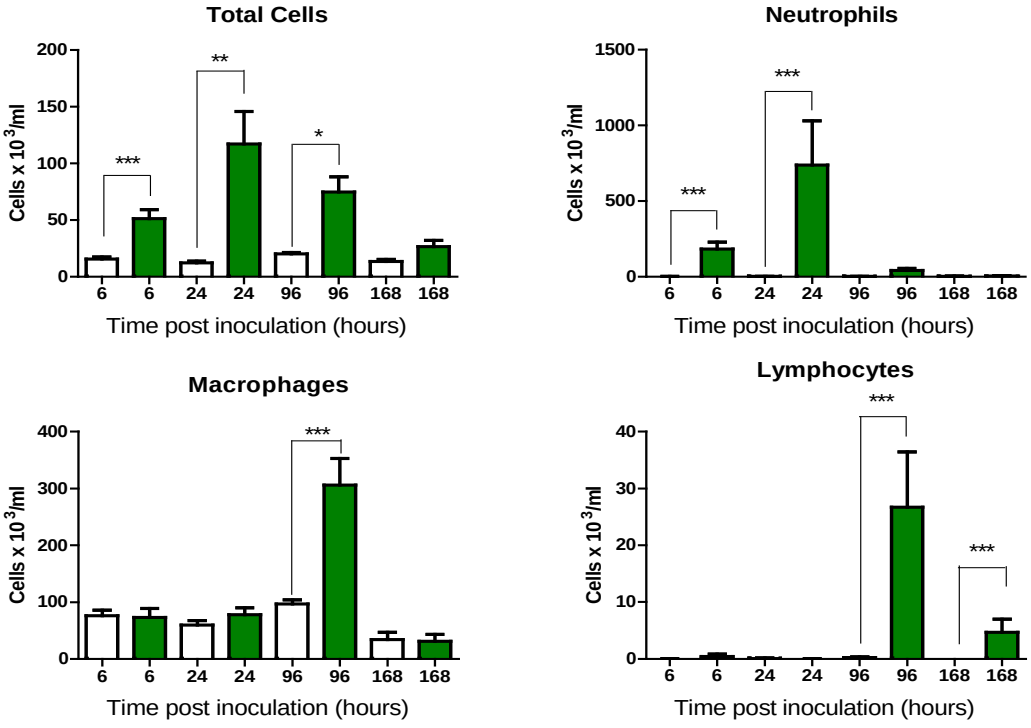


Figure 6.5 The Effect of NTHi Inoculation (1x10^8 cfu/mouse *i.n*) on Total Cell, Neutrophil, Macrophage and Lymphocyte Numbers at 6, 24, 96 and 168 Hours after Inoculation. Total cell and neutrophil numbers were significantly increased at 6 and 24 hours for NTHi infected animals compared to the respective control groups. Total cells remained significantly different at 96 hours after inoculation as did macrophages and lymphocytes. Lymphocytes also remained significantly elevated at 168 hours after inoculation. Mean ± SEM, N=10, *P<0.05, **P< 0.01, ***0.001, Mann-Whitney test.

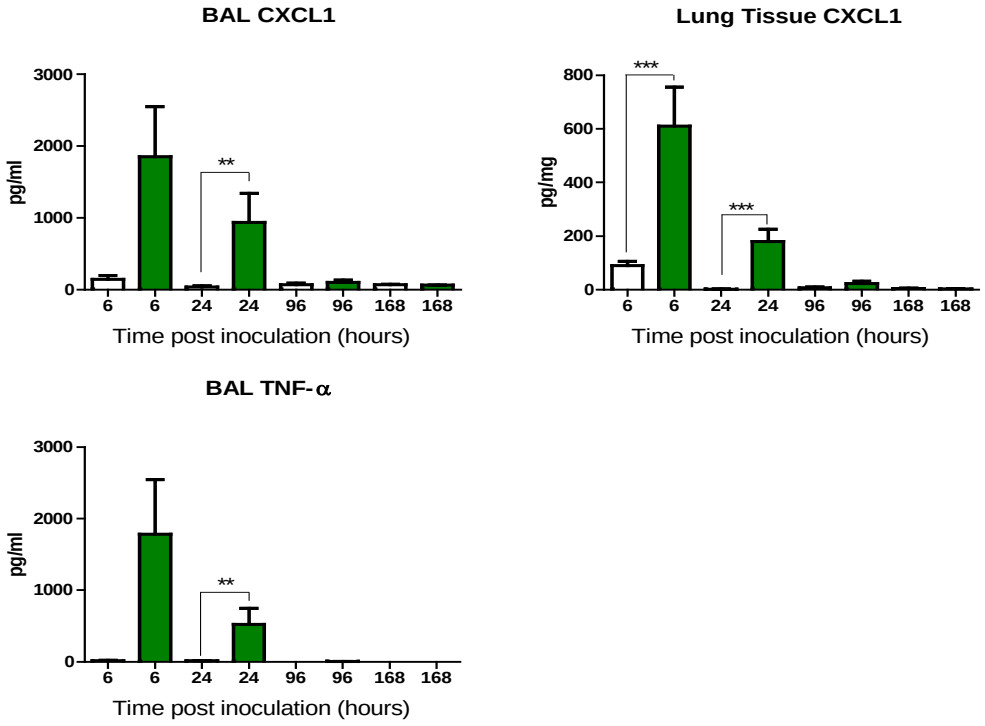


Figure 6.6 The Effect of NTHi Inoculation (1x10^8 cfu/mouse *i.n*) on CXCL1 and TNF-α Levels in the BALF and Lung Tissue at 6, 24, 96 and 168 Hours After Inoculation. CXCL1 was significantly increased in the BALF 24 hours after inoculation. It was also increased at 6 and 24 hours in the lung tissue, while TNF-α was only significantly increased in the BALF 24 hours later. TNF-α was undetectable in the lung tissue at any time point. Mean ± SEM, N=10, **P< 0.01, ***P<0.001, Mann-Whitney test.

6 Hours	24 Hours	96 Hours
TNF-α (BAL only)	Serum Amyloid A (Blood)	
CXCL1 (+ Blood)	Il-1β (BAL)	
IL-10 (BAL Only)		
Il-1β (Lung Tissue)		
Il-12p70		
Il-6		

Table 6.1 Table of Other Recorded Cytokines Measured at 6, 24 and 96 hours After NTHi inoculation (1X10^9 cfu/mouse *i.n*). Table represents the peak response of each cytokine, with peaks occurring in BALF and lung tissue unless otherwise stated. Cytokines failed to reach significance (raw data in appendix 2). Mean ± SEM, N=10, P> 0.05, Mann-Whitney test.

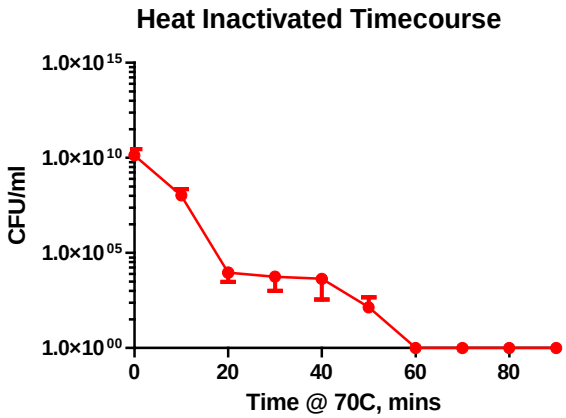


Figure 6.7 The Time Required to Heat- Inactivate NTHi at 70°C. NTHi was heat inactivated after 60 minutes at 70°C, with no growth occurring at or after this time point. Mean ± SEM, N=3

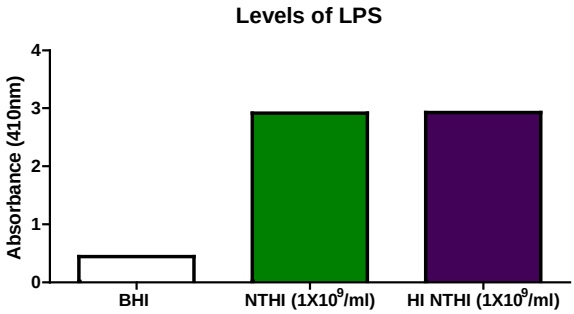


Figure 6.8 Quantification of LPS Levels of Live and Heat-Inactivated NTHi. There was no difference in LPS levels between live and heat inactivated NTHi. Mean, N=3,

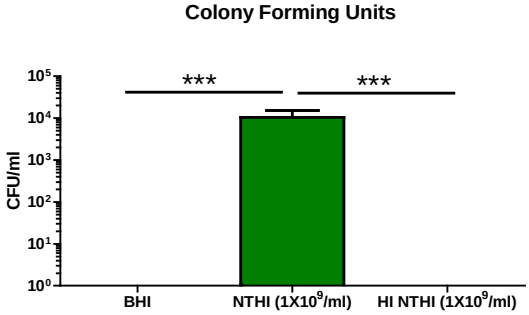


Figure 6.9 Viable NTHi Recovery from the Lungs of Live and Heat-Inactivated Inoculated Mice. There was a significant difference in CFUs when live infection was compared to both the control group and the heat-inactivated NTHi challenged animals. Heat-inactivated NTHi showed no significant difference compared to the control group. Mean ± SEM, N=10, ***P<0.001, Kruskal-wallis *post hoc* Dunn's test.

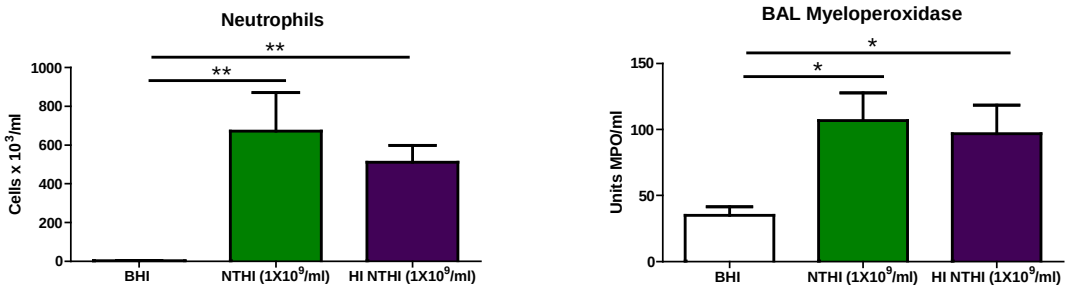


Figure 6.10 The Effect of Live and Heat-Inactivated NTHi on Neutrophil Numbers and Neutrophil Secreted Myeloperoxidase Levels in the BALF. Both live and heat-inactivated NTHi showed a significant difference in neutrophil numbers and myeloperoxidase levels compared to the control group. Live and heat-inactivated NTHi showed no significant difference from each other in cell number or activity. Mean ± SEM, N=10, *P<0.05,**P<0.01, Kruskal-wallis *post hoc* Dunn's test.

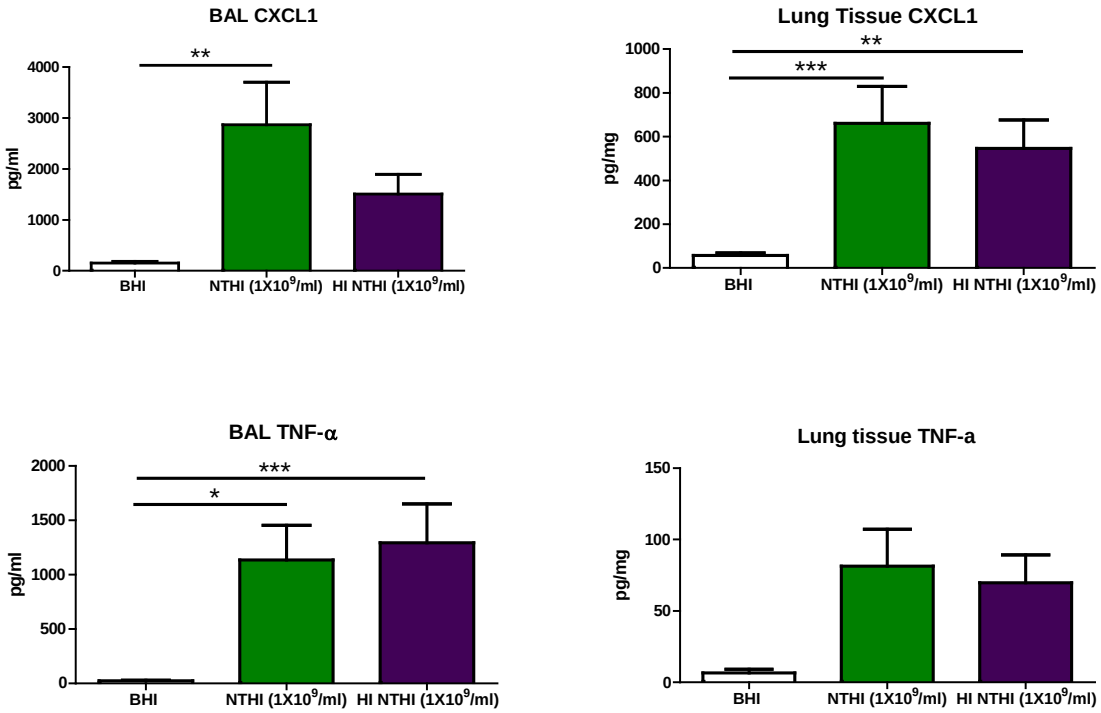


Figure 6.11 The Effect of Live and Heat-Inactivated NTHi on CXCL1 and TNF-α Levels in the BALF and Lung Tissue. CXCL1 showed a significant difference between live NTHI and the control in the BALF and a significant change in levels in both live and heat-inactivated NTHi compared to the control group in the lung tissue. Live and heat-inactivated showed no significant difference from each other in either the BALF or the lung tissue. TNF-α showed a significant increase in levels for both live and heat-inactivated NTHi in the BALF compared to the control but no significant difference from each other. TNF-α levels in the lung tissue showed no significant difference between any of the groups. Mean ± SEM, N=10, *P<0.05, **P<0.01, ***P<0.001, Kruskal-wallis *post hoc* Dunn's test.

6.6 Discussion

Non-typeable *Haemophilus influenza* is the most commonly isolated bacteria in stable and exacerbated COPD (Eldika and Sethi, 2006). It is therefore a clinically relevant bacterium in exacerbation experiments. The growth of this strain of NTHi was investigated in order to correlate an optical density reading with the number of colony forming units/ml. This would allow for accurate determination of the number of bacteria being administered using optical density readings.

The optical density growth curve (Fig 6.1) clearly shows three out of the four distinct phases of bacterial growth; the lag, log and stationary phases (figure 6.12)

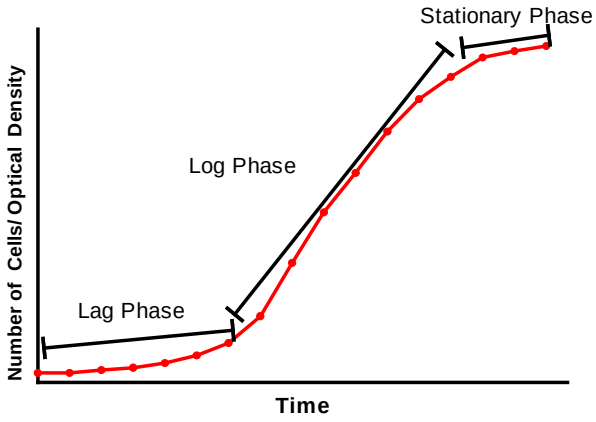


Figure 6.12 A Diagrammatic Representation of the Three Phases of Bacterial Growth. The graph shows three of the four distinct phases of bacterial growth, the Lag, Log and Stationary Phase

The lag phase is where bacteria acclimatize to the new surroundings and prepare for growth. The log phase is a period of exponential growth, where the bacteria double in number after each generation, which is followed by the stationary phase, where the nutrients are depleting and the growth rate and death rate are in equilibrium. The final, fourth phase is the death phase, where nutrients become so depleted that the equilibrium shifts, with more cells dying than are being generated (Pommerville and

Alcamo, 2012). Both the optical density and CFU/ml graphs show these phases, with both reaching the peak of log phase at approximately 400 minutes. Correlating the two measurements gives a cfu/ml value of $3x10^9$cfu/ml at an OD_{600} of 0.4.

NTHi has been investigated in numerous mouse models in the drive to better understand the pathways involved in exacerbations of COPD. NTHi concentration ranges of $1x10^6$ (Gaschler *et al.,* 2010) to $2x10^8$ CFU/mouse (Koyama *et al.,* 2007) have been described in the literature. Half log increases in CFU/mouse from $3x10^6$ to $1x10^8$ were investigated for peak inflammatory cell responses. Figure 6.3 shows a dose dependent increase in total leucocytes and neutrophils. Macrophages remain fairly stable across all concentrations, while the highest concentration gives the highest CFU/ml return. Gaschler *et al.* (2010) also show similar responses to NTHi in Balb/C mice, an increase in total leucocytes that are predominantly neutrophilic. From these results, the peak inflammatory response occurs at $1x10^8$ CFU/mouse, so was chosen for future experiments.

NTHi is a specialist human colonizer and pathogen, failing to naturally infect other species (Garmendia *et al.,* 2012). This can be seen in the bacterial load responses from the mouse. The bacterial load peaks at 6 hours but is less than what was administered. $1x10^8$ CFU/mouse was given intranasally but only $2x10^5$ CFU/mouse was recovered 6 hours later. Similarly, the number of viable bacteria recovered from the lungs 24 hours after inoculation was $2x10^2$ CFU/mouse. This shows that NTHi fails to colonize or replicate in the mouse lung and is cleared quite quickly. Infection with NTHi in animal models have been described where replication occurs, but they are usually obtained by firstly damaging the mucosal lining (Slater, 1990). Despite this drawback, NTHi was still chosen as the exacerbating agent in the mouse, as

cigarette smoke may cause enough damage to the epithelial lining to allow infection to occur and be investigated.

The fast clearance of NTHi can be linked to the fast response of the immune system. The neutrophil chemoattractant CXCL1, and the pro-inflammatory cytokine TNF-α show peak responses at 6 hours. This means neutrophils are significantly increased by 6 hours and peak at 24 hours. CXCL1 and neutrophil influx are a key response to an NTHi infection (Look *et al.*, 2006). This has been shown to be partly due to TLR4 recognition, with neutrophils in TLR4 deficient mice taking longer to arrive at the site of infection, as well as increasing the time taken to clear the bacteria (Wang *et al.*, 2002). Essilfie *et al.* (2011) also showed similar responses to NTHi in a time course study in the mouse. The viable bacterial load was almost halved by 24 hours, while there were peak responses in neutrophils after 24 hours which returned to normal by day 5. Lymphocytes were then significantly increased by day 5.

Neutrophils are able to respond quickly to pulmonary infections as even when healthy they are more concentrated in the pulmonary circulation than anywhere else in the body (Doerschuk *et al.*, 1999). When an infection is detected, CXCL1 is secreted by alveolar macrophages and epithelial cells and neutrophils migrate into the alveoli (Downey *et al.*, 1993). Once in the airspaces neutrophils have three primary defences against bacteria. The first is phagocytosis, where neutrophils ingest opsonised bacteria into phagosomes where they are destroyed (Mancuso *et al.*, 2001). The second is degranulation, where neutrophils secrete antimicrobial compounds and proteins such as bactericidal/permeability-increasing protein (BPI) and myeloperoxidase (MPO) (Standish and Weiser, 2009). The final method is neutrophil extracellular traps (NETs) which are extracellular protrusions, consisting

primarily of DNA and contain proteases and oxidant species to decrease the virulence of bacteria trapped within the NETs (Brinkman *et al.*, 2004).

Similar to Essilfie *et al.* (2011) who show lymphocytes peak at 5 days, this study shows lymphocytes and macrophages appear and peak 4 days after inoculation of NTHi. Macrophages appear at the end of an infection to phagocytose inflammation fragments and apoptotic neutrophils. Phagocytosis is increased by the release of interferon-ɣ and by T helper lymphocytes (Kedzierka *et al.*, 2004). T helper cells and B lymphocytes are also involved in the production of antibodies to impart host immunity if the same bacteria are encountered in the future (Alberts *et al.*, 2008).

The time taken to heat-inactivate NTHi was also investigated in order to assess whether there was a difference in immune responses between live and heat-inactivated bacteria. NTHi was heat-inactivated by 60 minutes at 70°C, with no viable cell growth being detected. Bailey *et al.* (2012) showed NTHi was heat inactivated at 95°C for 5 minutes. They also showed inactivation by freeze thawing, with other methods such as formalin inactivated NTHi being employed in other studies (Zhao *et al.*, 2011). The lower temperature and longer time frame was used in order to inactivate the bacteria as gently as possible, keeping the bacteria as structurally intact as feasible, preventing the release of LPS. This meant that when LPS levels were tested there was no difference between the levels in live or heat-inactivated NTHi. Therefore, any differences that might be observed between the live and heat-inactivated bacteria was not due to variations in LPS levels. The viable bacterial return of heat inactivated NTHi in the mouse confirmed that there was no viable bacteria in the lungs after 24 hours, while the live bacteria showed a significant bacterial return.

There was no difference between the number or activity of neutrophils (MPO), while CXCL1 and TNF-α levels were also the same between live and heat-inactivated NTHi groups. With no difference between live and heat inactivated bacteria there is a suggestion that the response is solely down to LPS and other external proteins, with no active infection component. Bosnar *et al.,* (2009) showed that pure LPS in the mouse significantly increased total leucocytes which were predominantly neutrophilic, with increases in CXCL1 and TNF-α being observed.

However, live NTHi is advantageous over heat-killed in investigating exacerbations of a chronic cigarette smoke model as epithelial damage may allow for infections to occur, as well as allowing the effect cigarette smoke has on bacterial clearance to be studied. Alternatively, heat inactivated NTHi can be used *in vitro*, where live NTHi would kill the cells, having been shown to have the same effect *in vivo.*

In conclusion, the peak inflammatory response to NTHi was $1x10^8$ CFU/mouse, which is achieved by an OD_{600} reading of 0.4. The peak inflammatory response is after 24 hours with a predominantly neutrophil response, which switches to a macrophage and lymphocyte resolutory profile at 4 days. All bacteria were cleared from the lungs 96 hours after inoculation, while live and heat-inactivated NTHi showed no difference in LPS levels or inflammatory response. This means that live NTHi can be used *in vivo* to study inflammation and bacterial clearance, while heat inactivated NTHi can be used *in vitro* to study the inflammation.

CHAPTER 7

Interactions Between Chronic Cigarette Smoke Exposure and Bacterial Infection by NTHI on Lung Inflammation.

7.1 Exacerbations of COPD

Exacerbations are an important aspect of COPD. Exacerbations occur 1-3 times a year (Sethi *et al.,* 2002), and decrease the quality of life, increase morbidity and mortality and frequently result in hospital admissions for COPD patients. Bacterial infections of the lungs play an important role in exacerbations as they have been shown to be responsible for approximately half of all exacerbations, either in conjunction with viruses or on their own (Veeramachaneni and Sethi, 2006; van der Valk *et al.,* 2004).

7.2 Bacterial Exacerbations of COPD

The role of bacteria in acute exacerbations of COPD (AECOPD) is controversial and is still poorly understood. Bacteria, as previously discussed, are known to colonize the airways of COPD patients. Species of bacteria isolated during an exacerbation were the same as those isolated during stable disease, leading to the conclusion that bacteria played no role in AECOPD (Fagon and Chastre, 1996). However, it is now understood that strain variations in bacteria that are already established colonizers can lead to an infection and an exacerbation (Sethi *et al.*, 2002). During a bacterial exacerbation, inflammation and pro-inflammatory cytokines are elevated, sputum purulence is increased, lung function declines and a change in medication and/or hospitalization is required (Gompertz *et al.,* 2001). The most frequently isolated bacteria implicated in AECOPD are *Haemophilus influenza*, *Moraxella catarhallis* and *Streptococcus pneumonia* (Biswal *et al.,* 2012; Sethi, 2011). Of these, nontypeable *Haemophilus influenza* (NTHi) is the most commonly isolated bacteria during AECOPD (Miravitalles *et al.,* 2012), accounting for up to 87% of all exacerbations (Bandi *et al.,* 2001).

Treatment of exacerbations, like the stable disease, is poor. An animal model of AECOPD is desperately needed to investigate the mechanisms and improve treatments.

7.3 Aims

The aim of this chapter is to develop an exacerbated cigarette smoke model using non-typeable *Haemophilus influenza* and the effect cigarette smoke has on the inflammatory response to NTHi.

7.3.1 Objectives

- Investigate the response to NTHi on top of the chronic smoke model measuring-

  - Inflammatory cell influx at 24 and 96 hours.

  - cytokine release, including TNF-α and CXCL8, in the BALF and lung tissue at 24 and 96 hours

  - Bacterial load of NTHi to determine bacterial clearance

- Investigate the effect cigarette smoke has on the inflammatory response to NTHi measuring the same parameters as above.

7.4 Methods

7.4.1 Animal Husbandry

Animals were housed as described in 2.1

7.4.2 Chronic Cigarette Smoke Challenge

Balb/C Mice received cigarette smoke (1R3F Kentucky research cigarettes,) or air (sham) into a sealed perspex chamber (7000cm^3) at a flow rate of 0.6 l/min for 30 minutes. Smoke was produced by peristaltic suction with 4 seconds of smoke per minute. The average particulate matter was recorded and adjustments to the amount of smoke entering the chamber altered accordingly to maintain a consistant reading across all groups. The average total particulate matter was 444.1 ± 1.9 tpm/M^3

Mice were exposed to cigarette smoke or air for 30 minutes, twice a day, 5 days a week, for 5 weeks. On the sixth week, mice received twice daily smoke or air for 3 days. Animals were killed 24 hours after the final smoke or air challenge and a bronnchoalveolar lavage was performed to determine total and differential leucocyte numbers.

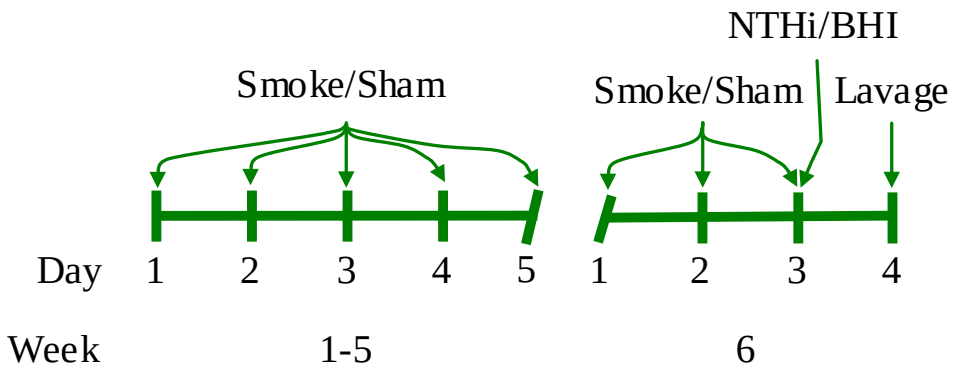


Figure 7.1 A diagrammatic representation of the chronic cigarette smoke model with NTHi inoculation. NTHi = Nontypeable *Haemophilus inflienzae*, BHi = Brain heart infusion broth

In NTHi infected groups of mice and their controls, animals were killed 24 hours after the final smoke exposure, as described above, as well as groups af animals being terminally anaesthetised 96 hours after inoculation.

7.4.3 Non-typeable *Haemophilus influenzae* Inoculations

NTHi was grown on chocolate agar plates (Becton Dickinson, UK) before being inoculated into supplemented brain heart infusion broth (BHi) (Becton Dickinson, UK) at an OD_{600} reading of greater than 1.1. A 1 in 10 dilution of the infusion was adjusted accordingly to give an OD_{600} reading of 0.4, which corresponded to $6x10^9$ CFU/ml.

Mice were anaesthetized with an intraperitoneal injection of hypnorm/hypnovel before they received 50µl of supplemented BHi or NTHi at a concentration of $6x10^9$ CFU/ml intra nasally. All bacterial inoculations took place in a laminar flow cupboard to prevent bacterial spread. After bacterial inoculation animals were housed in groups of 5 in an OptiMice bio-containment cage system (Animal Care Systems, USA) with an independent air supply. Animals were killed 24 or 96 hours after intranasal administration and a bronchoalveolar lavage performed as described in 2.7. Inflammatory cells and cytokines were analysed as described in 2.7.2-2.7.10

7.5 Results

The Effect of NTHi on Total Cell Influx into the Lung in Chronic Smoke or Chronic Sham Challenged Mice 24 and 96 Hours after Challenge.

At 24 hours, there was no significant difference in the total cell number between any of the groups. At 96 hours, Smoke NTHi was significantly increased compared to both Smoke/BHi and Sham/NTHi ($P<0.05$) (fig 7.2).

The Effect of NTHi on Neutrophil Influx and Myeloperoxidase Levels in the BALF in Chronic Smoke or Chronic Sham Challenged Mice, 24 and 96 Hours after Challenge.

Twenty Four hours after challenge, Smoke/NTHi challenged mice showed a significantly elevated neutrophil number compared to Smoke/BHi challenged animals ($P<0.01$) (fig 7.3). The myeloperoxidase levels measured in the BALF in the however were no different between the two groups, nut was significantly increased in Sham/NTHi compared to Smoke/NTHi ($P<0.05$).

At 96 hours, Smoke/NTHi and Smoke/BHi challenged animals showed no significant difference in neutrophil numbers. Smoke/NTHi neutrophils were significantly elevated compared to the Sham/NTHi group ($P<0.001$). BAL MPO was significantly increased in the Sham/NTHi challenged animals compared to the Smoke/NTHi group at both time points ($P<0.05$), while there was no significant difference in MPO levels between Smoke/NTHi and Smoke/BHi at either time point.

The Effect of NTHi on CXCL1 Levels in the BALF and Lung Tissue in Chronic Smoke or Chronic Sham Challenged Mice, 24 and 96 Hours after Challenge.

Twenty-four hours after challenge, there was a significant increase in CXCL1 levels in Smoke/NTHi challenged animals compared to Smoke/BHi challenged mice in the BALF ($P<0.001$) and lung tissue ($P<0.05$)(fig 7.4). There was no significant difference between Smoke/NTHi and Sham/NTHi in both the BALF and lung tissue at either time point.

At 96 hours, there was no significant difference in CXCL1 levels in the BALF and lung tissue between Smoke/BHi and Smoke/NTHi, however, there was a significant increase in levels when Smoke/NTHi is compared to sham/NTHi in both the BALF ($P<0.05$) and lung tissue ($P<0.001$).

The Effect of NTHi on TNF-α Levels in the BALF and Lung Tissue in Chronic Smoke or Chronic Sham Challenged Mice 24 Hours after Challenge.

Smoke/BHi and Smoke/NTHi TNF-α levels in the BALF and the lung tissue were not significantly different. The Smoke/NTHi challenged group was significantly different from the sham/NTHi in both the BALF ($P<0.05$) and the lung tissue ($P<0.01$). TNF-α was undetectable in both BALF and lung tissue 96 hours after NTHi/BHi challenge (fig 7.5).

The Effect of NTHi on Interferon -ɣ and Il-10 Levels in the BALF in Chronic Smoke or Chronic Sham Challenged Mice 24 Hours after Challenge.

As observed for TNF-α, interferon-ɣ and IL-10 levels were increased in sham/NTHi challenged mice compared to the Smoke/NTHi challenged in the BALF ($P<0.05$ and

P<0.001 respectively). Smoke/NTHi however had no significant difference in both interferon-ɣ and IL-10 levels compared to Smoke/BHi challenged animals (fig 7.6).

The Effect of NTHi on Lymphocyte numbers in the BALF and Lung Tissue in Chronic Smoke or Chronic Sham Challenged Mice 96 Hours after Challenge.

There was no significant difference between any of the groups (fig 7.7). Sham/NTHi had the highest average score of 2.1 ± 0.3, smoke/NTHi and smoke/BHi challenged animals had a very similar score of 1.5 ± 0.2 and 1.1 ± 0.1 respectively, while sham/BHi challenged mice had the lowest score of 0.1 ± 0.1.

The Effect of NTHi on Macrophage numbers in the BALF in Chronic Smoke or Chronic Sham Challenged Mice, 24 and 96 Hours after Challenge.

Smoke/BHi and Smoke/NTHi challenged animals showed no significant difference in macrophage number at 24 hours. Smoke/NTHi challenged animals however had significantly elevated numbers compared to Sham/NTHi challenged mice (P<0.001)(Fig 7.8).

By 96 hours, this was reversed. There was a significant increase in macrophage numbers when Smoke/NTHi and Smoke/BHi challenged groups were compared (P<0.01). There was no significant difference between Smoke/NTHi and Sham/NTHi.

The Effect of Chronic Smoke or Chronic Sham Challenge on Bacterial Load of NTHi from the Lungs of Mice 24 Hours after Challenge.

Smoke/NTHi challenged mice showed a significantly reduced bacterial load compared to sham/NTHi challenged animals (P<0.05) (fig 7.9)

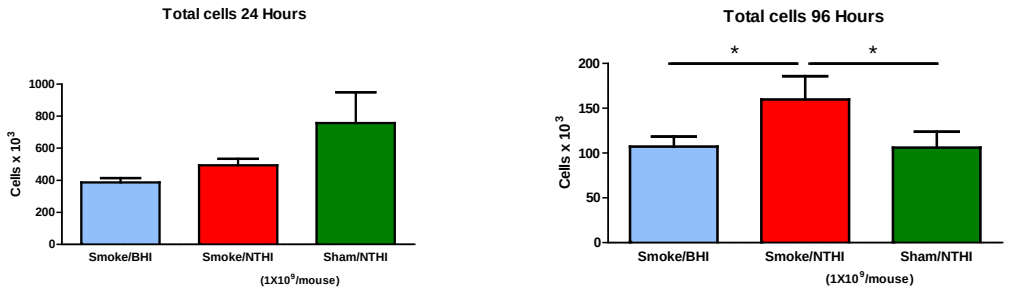


Figure 7.2 The Effect of NTHi on Total Cell Influx into the BALF in Chronic Smoke or Chronic Sham Challenged Mice 24 and 96 Hours after Challenge. There was no significant difference in total cell number between any of the groups at 24 hours. Smoke/NTHi total cells were significantly increased compared to both smoke/BHi and sham/NTHi at 96 hours. Mean ± SEM, N=10, *P<0.05 Mann-Whitney test.

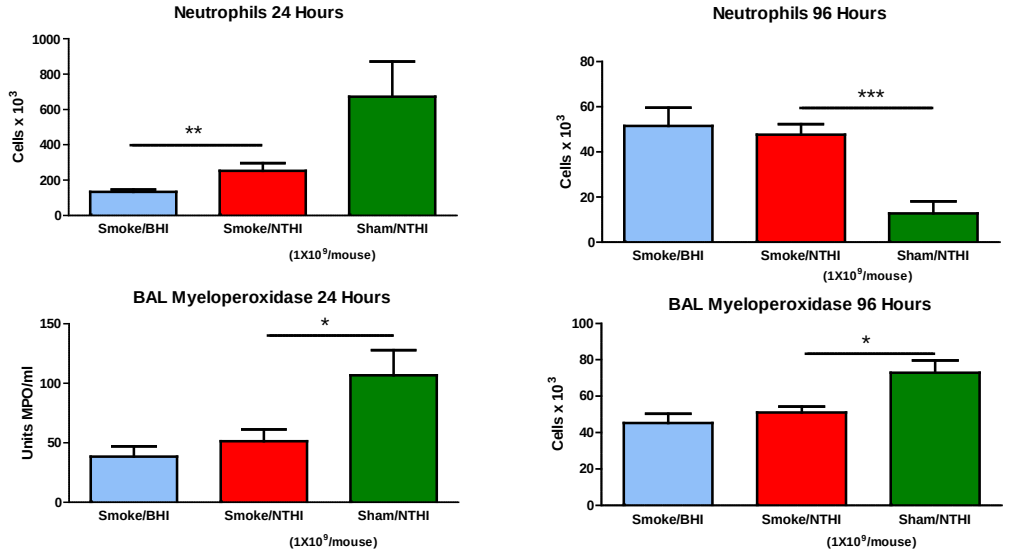


Figure 7.3 The Effect of NTHi on Neutrophil Influx and Myeloperoxidase Levels in the BALF in Chronic Smoke or Chronic Sham Challenged Mice, 24 and 96 Hours after Challenge. Smoke/NTHi challenged animals showed a significant increase in Neutrophil numbers at 24 hours compared to smoke/BHi challenged mice. Sham/NTHi and Smoke/NTHi challenged mice showed no significant difference, however they did show a significant difference in MMPO levels at 24 hours. Smoke/BHi and smoke/NTHi showed no difference. Smoke/NTHi had significantly elevated neutrophil numbers at 96 hours compared to Sham/NTHi but no significant change compared to Smoke/BHi. The same pattern was observed for MPO activity at 96 hours. Mean ± SEM, N=10, *P<0.05, **P< 0.01, ***P<0.001 Mann-Whitney test

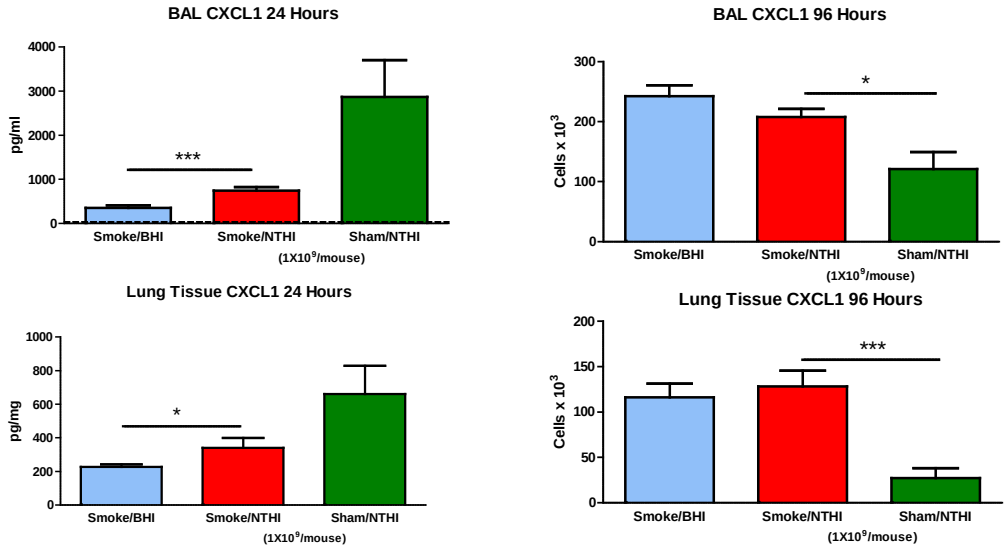


Figure 7.4 The Effect of NTHi on CXCL1 Levels in the BALF and Lung Tissue in Chronic Smoke or Chronic Sham Challenged Mice, 24 and 96 Hours after Challenge. There was a significant increase in CXCL1 levels in both the BALF and lung tissue 24 hours after inoculation in Smoke/NTHi compared to Smoke/BHi challenged animals. Sham/NTHi challenged animals showed no significant difference compared to Smoke/NTHi challenge. At 96 hours, Smoke/NTHi showed a significant increase in CXCL1 levels in both the BALF and lung tissue compared to Sham/NTHi challenged mice, while there was no significant difference in levels between Smoke/NTHi and Smoke/BHi in the BALF or lung tissue. Mean ± SEM, N=10, *P<0.05, ***P<0.001 Mann-Whitney Test.

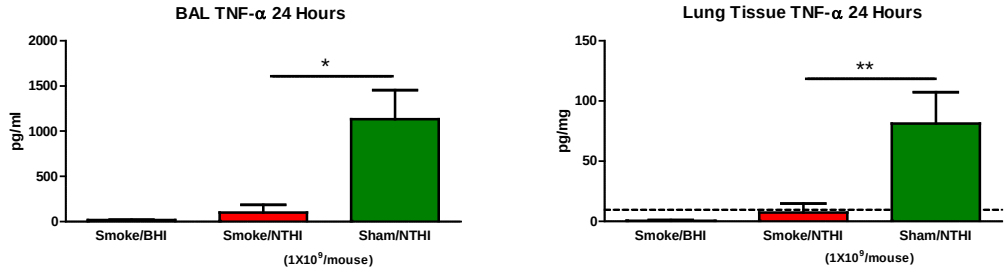


Figure 7.5 The Effect of NTHi on TNF-α Levels in the BALF and Lung Tissue in Chronic Smoke or Chronic Sham Challenged Mice 24 Hours after Challenge. TNF-α levels were significantly elevated in the Sham/NTHi challenged animals compared to Smoke/NTHi challenged mice in both the BALF and lung tissue. There was no significant difference in TNF-α levels between Smoke/NTHi and Smoke/BHi challenged animals at 24 hours in both the BALF and lung tissue. Mean ± SEM, N=10, *P<0.05, **P< 0.01, Mann-Whitney Test.

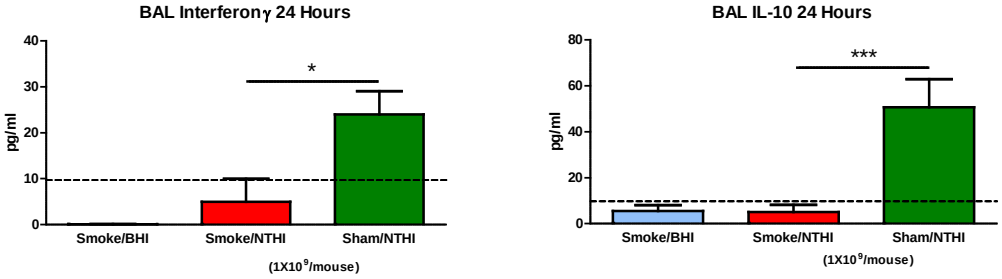


Figure 7.6 The Effect of NTHi on Interferon -ɣ and Il-10 Levels in the BALF in Chronic Smoke or Chronic Sham Challenged Mice 24 Hours after Challenge. There was a significant reduction in both interferon-ɣ and IL-10 in Smoke/NTHi challenged animals compared the NTHi only challenged group. There was no significant difference in levels in Smoke only and Smoke/NTHI challenged animals. Mean ± SEM, N=10, *P< 0.05, ***P<0.001 Mann-Whitney Test.

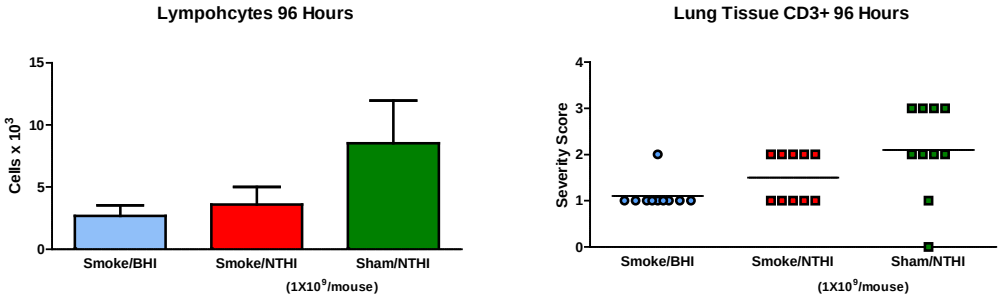


Figure 7.7 The Effect of NTHi on Lymphocyte numbers in the BALF and lung tissue in Chronic Smoke or Chronic Sham Challenged Mice 96 Hours after Challenge. There was no significant difference between any of the groups. Mean ± SEM, N=10, Mann-Whitney Test.

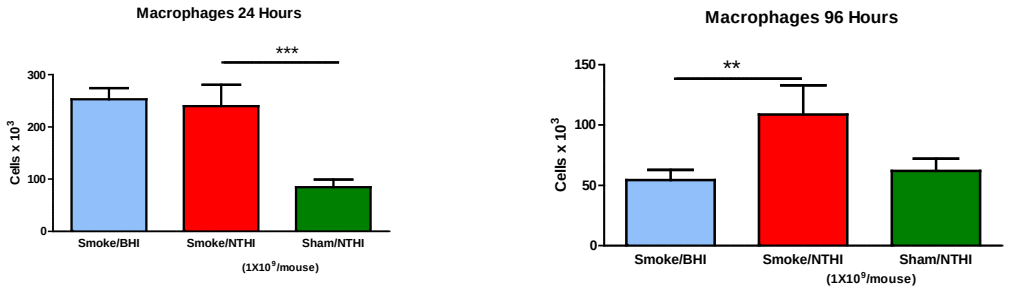


Figure 7.8 The Effect of NTHi on Macrophage numbers in the BALF in Chronic Smoke or Chronic Sham Challenged Mice, 24 and 96 Hours after Challenge. 24 hours after NTHi inoculation there was a significant increase in macrophages in Smoke/NTHi challenged animals compared to Sham/NTHi challenged mice, but no significant difference was observed between Smoke only and NTHi only challenged mice. 96 hours after challenge Smoke/NTHi showed a significant increase in Smoke/BHi challenged mice while no difference was observed between Smoke/NTHi and Sham/NTHi challenged animals. Mean ± SEM, N=10, **P< 0.01, ***P<0.001 Mann-Whitney Test.

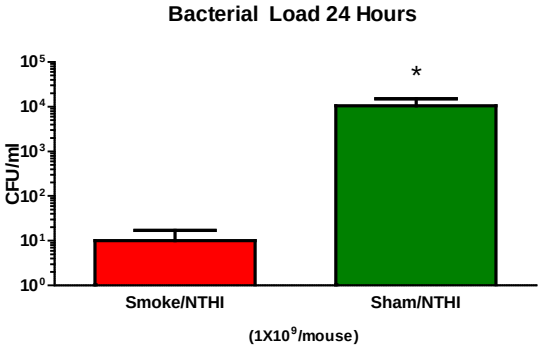


Figure 7.9 The Effect of Chronic Smoke or Chronic Sham Challenge on Bacterial Load of NTHi from the Lungs of Mice 24 Hours after Challenge. Twenty-four hours after NTHi inoculation there was a significant increase in bacterial clearance/decrease in bacterial load, in smoke/NTHI challenged mice compared to Sham/NTHi challenged animals. Mean ± SEM, N=10, *P<0.05 Mann-Whitney Test

7.6 Discussion

Exacerbations of COPD are an important aspect of the disease. They cause a worsening of symptoms, decrease a patient's quality of life and remain poorly treated. Nontypeable *Haemophilus influenza* (NTHi) is the most frequently sampled bacteria implicated in AECOPD (Miravitalles *et al.,* 2012), so is a very important and clinically relevant pathogen. An NTHi exacerbation of COPD results in an increase in pro-inflammatory cytokines and an influx of pro-inflammatory cells, including macrophages and neutrophils (Clemans *et al.,* 2000; Berenson *et al.,* 2005). This study shows that an NTHi exacerbation in a murine chronic cigarette smoke model mimics this, with significantly increased neutrophils and the neutrophil chemoattractant CXCL1, compared to smoke alone (figs 7.2 and 7.3). TNF-α is also elevated in NTHi challenged mice, however this fails to reach significance. This could be due to TNF-α being an acute cytokine that peaks between 2 and 5 hours after challenge (Tsutsui *et al.,* 1997), so may be significant at an earlier time point. Total cells and macrophages were also significantly elevated in NTHi challenged animals four days (96 hours) after challenge. Similarly, Gaschler *et al.* (2010) demonstrated that an NTHi infection on top of chronic cigarette smoke in the mouse increases inflammation compared to smoke alone.

The data from this study demonstrate that an exacerbation model has been achieved with significant increases in cells and cytokines similar to those observed during an AECOPD (Aaron *et al.,* 2001).

Interestingly, the level of inflammation seen after an NTHi exacerbation in the cigarette smoke model does not reach the same magnitude as observed in sham/NTHi challenged animals. This signifies that chronic cigarette smoke in fact

impairs the immune response to pathogens. The possible reason for this will be discussed later. Little clinical data is available comparing the inflammatory responses in humans of healthy individuals and COPD patients to an infection. To my knowledge Mallia *et al.* (2010) is the only study and have shown that interferon responses to a rhinovirus infection are impaired in COPD patients compared to healthy controls.

Here the impairment of the initial immune response by cigarette smoke observed 24 hours after NTHi inoculation will be discussed as well as the possible theories from the literature as to why cigarette smoke impairs these responses. The effect cigarette smoke then has on the resolution of inflammation 4 days after inoculation will also be reviewed.

7.6.1 The Initial Twenty-Four Hour Response to NTHi

Firstly focusing on the initial 24 hour response to the pathogen, this study shows that smoke impairs the inflammatory response to NTHi, decreasing cellular influx and cytokine release. Total inflammatory cells in the smoke/NTHi challenged group were decreased compared to sham/NTHi challenged animals as well as neutrophil numbers and activity, CXCL1, TNF-α, interferon-ɣ and Il-10 levels. The only exception to this pattern was macrophages, which were comparatively increased, but this was solely due to cigarette smoke, with no difference between smoke only and smoke/NTHi challenged groups.

This impairment in inflammatory response is counterintuitive with little clinical evidence available highlighting this impairment, with most clinical studies examining the effects of exacerbations compared to stable disease and/or ‘healthy smokers’. This study highlights the problems in using control groups such as these

(e.g infected non-smokers) which do not occur in clinical trials. For example, while there is an increase in neutrophilia in the exacerbated (Smoke/NTHi) group compared to the stable (Smoke/BHi) group in this study (fig 7.3), this would not reveal the attenuation of the neutrophil response to NTHi by smoke compared to an infected control (Sham/NTHi).

The impaired neutrophil response may be linked to chemotaxis, which has been been shown to be decreased in COPD in response to an infection compared to healthy nonsmokers (Venge *et al.,* 1991; Yoshikawa *et al.,* 2007). This is despite COPD patients exhibiting increased neutrophilia in the airways. The levels of CXCL1 in this murine model, although increased in response to Smoke/NTHI compared to smoke alone, were impaired when compared to NTHi alone. The activity of neutrophils present in the airways of COPD patients have also been shown to be decreased compared to the healthy controls, including decreased phagocytic potential (Fietta *et al.,* 1988; Yoshikawa *et al.,* 2007). Again, this is observed in the mouse model, with decreased levels of MPO compared to NTHi alone.

The impairment of neutrophils is not only a COPD phenomenon; asthma patients with frequent exacerbations have also been shown to have decreased neutrophil responses in the lung (Baines *et al.,* 2009), signifying that impaired neutrophil responses may impact on the severity and frequency of exacerbations.

This model also highlights several cytokines that are impaired by cigarette smoke. TNF-α levels from COPD alveolar macrophages have been shown to be decreased when responding to NTHi antigens, while monocytes from COPD patients have the same response as healthy individuals (Berenson *et al.,* 2006a). TNF-α levels have also been shown to be decreased in epithelial cells when challenged with bacterial

products after chronic cigarette smoke extract challenge (Laan *et al.,* 2004). Other chronic mouse models have shown attenuation of TNF-α levels to NTHi after smoke challenge (Gaschleer *et al.,* 2009). Epithelial cells also secrete TNF-α and are damaged in COPD (Lapperre *et al.,* 2007) as well as undergoing increased apoptosis (Hodge *et al.,* 2005). This decreases the number of epithelial cells present in the airways, possibly decreasing the amount of TNF-α released. Birrell *et al.,* (2007) have shown that cigarette smoke depletes the antioxidant enzyme glutathione, increasing oxidative stress which impairs NF-kB and leads to a reduction in TNF-α.

Levels of the anti-inflammatory cytokine Il-10 were also impaired in this model by cigarette smoke, with similar responses having been demonstrated by Takanashi *et al.* (1999) in the sputum of COPD patients and in smokers in general, compared to healthy controls. IL-10 is released from lymphocytes and macrophages and has been shown to down regulate a large number of pro-inflammatory cytokines. Impaired release of IL-10 impairs the ability of the body to decrease inflammation causing further damage to the lungs (Takanashi *et al.,* 1999).This reduction in Il-10 shows that the reduction of pro-inflammatory cytokine release is not being mediated by this anti-inflammatory cytokine.

Many studies implicate the macrophage in this impaired response. Twenty-four hours after NTHi challenge the smoke/NTHi challenged animals have a significantly larger number of macrophages compared to the NTHi alone animals. However, this is solely down to the smoke, as the same levels were observed in the smoke only challenged animals. It may be partly for this reason, along with increased neutrophils, why the bacterial load was reduced by cigarette smoke (fig 7.9). One possible reason for this reduced bacterial load is an increased clearance by phagocytosis by neutrophils and macrophages. Alveolar macrophages from COPD

patients as well as macrophages exposed to cigarette smoke have however demonstrated a diminished phagocytic ability (Taylor *et al.,* 2010; Marti-Lliteras *et al.* 2009). The increase in bacterial clearance by chronic cigarette smoke observed in the present study may be partially down to the sheer numbers of inflammatory cells present in the lungs prior to inoculation, so despite a decrease in activity they are still able to be quickly phagocytosed. This may be different if a pathogenic strain of bacteria was used. *Streptococcus pneumonia* for example is able to infect mice (Ludewick *et al.,* 2011). A *Streptococcus* infection on top of cigarette smoke may mean the impaired activity of macrophages and neutrophils allow the bacteria to take hold more readily than in healthy mice and multiply (Phipps *et al.*, 2010). Gaschler *et al.* (2010) however, hypothesize that the increased clearance is not due to increased existing neutrophilia due to the same rate of clearance being observed in a less severe smoke model with much lower numbers of neutrophils. They propose that the increased clearance is due to increased IgA in the lungs, opsonising the bacteria and increasing phagocytosis by macrophages.

Another possible explanation for the decreased bacterial load observed in the chronic smoke infected mice is due to the inhospitable environment present in the lungs. Cigarette smoke is full of reactive oxidant species, with the inflammatory process also releasing oxidant species (MacNee, 2001). Bacteria are damaged by reactive oxygen species, with *Haemophilus* having been shown to cope less well with oxidative stress than other bacteria, despite extensive mechanisms to combat it (Harrison *et al.,* 2012). This may result in decreased viable bacterial load. Also, as previously discussed, NTHi has been described evading host defences by crossing into epithelial cells (Morey *et al.,* 2011). Here they can avoid detection and subsequent phagocytosis by macrophages. New pattern recognition receptors have

been discovered intracellularly to detect bacteria called nucleotide-binding oligomerization domain (NOD)-like receptors and can complement the innate immune response via Toll-like receptors (TLRs) to subsequently increase pro-inflammatory cytokines (Kanneganti *et al.*, 2007; Shaw *et al.*, 2008). However, it has been demonstrated that these receptors are down regulated as a result of cigarette smoke exposure, so may play a part in impaired recognition and therefore reduced epithelial entry (Gaschler *et al.*, 2007). Unfortunately, attempts in the present study to identify whether NTHi did in fact enter epithelial cells using both Gram stain and immunohistochemistry failed to detect any NTHi in the airways, even in samples known to have bacteria present, so this process of evasion could not be examined. NTHi has also been shown to cause necrosis of neutrophils in humans. This may be a host specific response, which does not occur in mice, resulting in increased clearance of NTHi by neutrophils in a murine model compared to those in a human model (Gaschler *et al.*, 2009). The bacterial load at 96 hours in this smoke model may be of interest, possibly showing differential responses compared to NTHi only.

7.6.2 The Response to NTHi After Four Days

The initial impairment of the innate immune response subsequently affects the inflammatory profile of the lungs 4 days after NTHi inoculation. Neutrophils and the neutrophil chemoattractant CXCL1 were significantly elevated in cigarette smoke challenged animals compared to the sham challenged group after NTHi inoculation, but this was due to the cigarette smoke. Cigarette smoke still impaired neutrophil activity, while macrophages were still elevated. The lymphocyte response was also impaired in smoke challenged animals indicating that the adaptive immune response may also be impaired along with the innate immune response.

COPD patients have more severe and longer infections compared to healthy individuals with increased inflammation for longer after an infection and a marked decrease in lung function. COPD patients also suffer from bacterial colonization, which may, in part, be due to impaired innate responses in the clearance of bacteria (Marti-Lliteras *et al.,* 2009). However, with reduced lymphocyte numbers 4 days after an infection, cigarette smoke may also be impairing the adaptive immune response.

Lymphocyte development may be impaired in COPD due to a decrease in mature dendritic cells in the airways (Tsoumakidou *et al.,* 2009). These cells, along with macrophages are responsible for presenting antigens to T cells and causing them to become appropriate effector T cells depending on the stimulus. A decrease in mature cells as well as a decrease in alveolar macrophage activity could decrease the lymphocyte responses.

Knobloch *et al.* (2010) describe impaired-helper cell type 1 immune responses in COPD and in smokers in general, in response to Gram–negative bacteria. They surmise that this is a result of decreased TLR4 expression and altered signaling pathways by cigarette smoke exposure of lymphocytes. This decreases the release of interferon-ɣ, which improves the phagocytic activity of macrophages in response to an infection. Takabatake *et al., (*2004) also showed impaired interferon-ɣ secretion in COPD patients. King *et al.* (2002) also describe an impairment of the Th1 response in COPD patients, who tend to shift to a more Th2 response than healthy individuals in response to NTHi. However, other studies have shown LPS impairs Th2 responses, pushing for a more Th1 response (Koch *et al.*, 2007a). Counteracting this, IL-5, a cytokine responsible for B cell proliferation, has been shown to be decreased by Th2 cells in response to LPS (Schild *et al.,* 2011). CD8+ lymphocytes have also

been shown to be affected by cigarette smoke, with increases in numbers and activity in smokers and in COPD patients (Koch *et al.*, 2007), which has been implicated in the structural remodeling and destruction of parenchymal cells in COPD. As observed in some studies investigating the immune response of COPD to infections, this chronic cigarette smoke model also expresses decreased interferon-ɣ release in response to NTHi, possibly contributing to the impaired responses of the alveolar macrophages. Natural killer cells have also been shown to have reduced cytotoxicity in COPD patients, which can be linked to viral exacerbations and lung cancer (Prieto *et al.*, 2001).

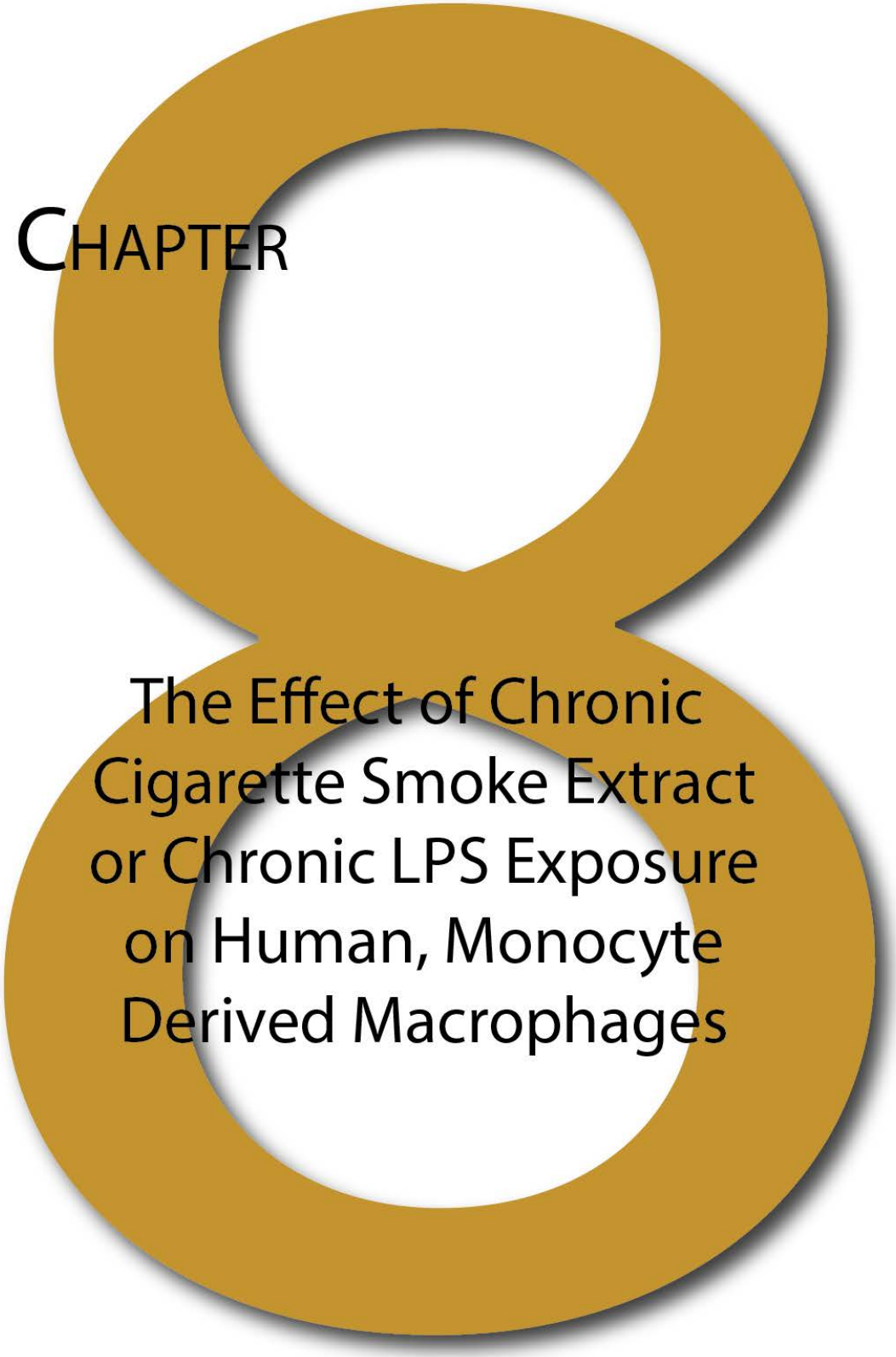
There are many theories as to why cigarette smoke diminishes the immune response to pathogens, but there has been very little research to elucidate specific mechanisms. Firstly, an oxidant/anti-oxidant imbalance can cause a significant amount of damaging reactive oxygen species. As previously noted, Birrell *et al.* (2007) showed cigarette smoke depleted the anti-oxidant enzyme glutathione, increasing oxidative stress and resulting in lower NF-κB production but higher AP-1. Drost *et al.* (1992; 1993) have implicated oxidative stress in the plasticity of neutrophils, with more oxidative stress decreasing the degree with which neutrophils can deform. This decreases the progression of neutrophils from the blood into the airspaces, and is reversed by the antioxidant glutathione.

The pattern recognition receptors themselves have also been implicated in the decreased inflammatory response to pathogens in COPD. Droeman *et al.* (2005) have shown that the gram-positive pathogen receptor TLR2 is down regulated in cigarette smokers and in COPD patients. Gaschler et al., (2007) show that the Toll-like receptors TLR3 and TLR4 are not down regulated in response to smoke but the inflammation after stimulation is attenuated due to decreased activation of NF-kB

and AP-1. As previously discussed a large proportion of cigarette smoke is LPS, with studies showing chronic LPS exposure desensitizes the TLR4 receptor (Liew *et al.*, 2005) as well as scavenger receptors (Ganesan *et al.*, 2012), decreasing pro-inflammatory cytokine release.

Cigarette smoke itself has also been shown to contain immune-regulatory compounds, such as NKK and acrolien, which have been demonstrated to suppress inflammation (Therriault *et al.*, 2003; Li *et al.*, 1998). Carbon monoxide inhalation, at levels appropriate to levels in cigarette smoke can decrease inflammation (Bathoorn *et al.*, 2007) and nicotine, as well as having antimicrobial activity, has also been shown to have anti-inflammatory activity (Geng *et al.*, 1996).

This model has shown an NTHi exacerbation on top of chronic cigarette smoke exposure significantly increases inflammation. This model also highlights cigarette smoke has a significant impact on the immune response to NTHI, impairing both innate and adaptive responses. This model could therefore be used to elucidate mechanisms and possible therapeutic treatments for 1) AECOPD and 2) impaired immunity. This could slow disease progression, reduce exacerbation frequency and improve exacerbation treatments.


CHAPTER
8
The Effect of Chronic Cigarette Smoke Extract or Chronic LPS Exposure on Human, Monocyte Derived Macrophages

8.1 Macrophages in COPD

Alveolar macrophages (AM) are an important cell type in COPD, with increased numbers in the lung and increased levels of associated cytokines compared to healthy individuals (Tetley, 2002). Important cytokines released by AMs in COPD are the neutrophil chemo-attractant CXCL8, the pro-inflammatory cytokines TNF-α and IL-1β and the regulatory cytokine IL-6 (Kent *et al.*, 2008). Macrophages also play a key role in tissue remodeling due to the release of proteolytic enzymes such as matrix metalloproteinases, which break down the extra cellular matrix of the lung and lead to changes in lung histology (Demedts *et al.*, 2006).

The release of these cytokines by AMs *in situ* means the cells may be important in 'orchestrating' and mediating the inflammation in the lung during COPD (Barnes, 2004a). Alveolar macrophages play a key role in the response to inhaled particles by migrating into the lungs to phagocytose them, preventing infections and/or irritations. In COPD irritants are present in cigarette smoke, with 'healthy smokers' exhibiting elevated macrophage numbers in the lungs, which have been shown to have reduced ability to regulate inflammation (Mikuniya *et al.*, 1999). While in the lungs, AMs phagocytose these irritants and secrete the aforementioned cytokines, increasing the influx of neutrophils and other inflammatory cell types (Barnes, 2012).

There are two distinct subpopulations of macrophages, the M_1 subtype, primarily thought to be pro-inflammatory, and the M_2 subpopulation, generally thought of as anti-inflammatory or immune-regulatory and pro-remodeling. They vary by expression of receptors on their cell surface and secretion of cytokines (Benoit *et al.*, 2008), and can be driven to an M_1 or M_2 phenotype *in vitro* by GM-CSF or M-CSF

respectively (Verreck *et al.,* 2006). It has been demonstrated that macrophages are able to quickly change from one subtype into another, with pro-inflammatory macrophages switching to an M_2 phenotype and aiding the resolution of inflammation (Porcheray *et al.,* 2005). The proportion of subtypes in COPD however is as yet unclear (Hodge *et al.,* 2011) but studies have demonstrated that macrophages from COPD patients have exhibited down-regulated M_1 genes and up regulated M_2 genes leading to increased tissue remodeling (Shaykhiev *et al*., 2009).

During an infection, macrophage responses to a lung pathogen are pro-inflammatory, recruiting other inflammatory cells to the source of the infection as well as phagocytosing the pathogen and presenting it to the adaptive immune system. This is primarily through activation of Toll-like receptor pathways by agonists such as lipopolysaccharide (LPS) (Nau *et al.,* 2002). In healthy individuals this is a predominantly M_1 response; however in COPD this response is more muddled, with a heterogeneous mix of M_1 and M_2 macrophages (Gutierrez *et al.,* 2010). Droeman *et al.* (2005) showed that certain Toll-like receptors on AMs are down regulated in COPD and in healthy smokers decreasing their ability to recognize and respond to pathogens. This may partly explain the results by Taylor *et al.* (2010) and Berenson *et al.* (2006) demonstrating the phagocytic ability of macrophages were diminished in AM and monocyte derived macrophages (MDMs) from COPD patients. This may play a part in the increased bacterial colonization and exacerbations observed in COPD patients (Patel *et al.,* 2002).

However, cigarette smoke is not the only agent shown to affect macrophage responses. LPS is a significant component of cigarette smoke and is also, as previously discussed, an integral constituent of Gram-negative bacteria. Studies have

shown that chronic colonization of the airways by bacteria causes a chronic inflammation in the lungs, increasing inflammatory cytokines (Sethi and Murphy, 2001; Hill *et al.,* 2000). The increased colonization of the airways in COPD increases the amount of LPS in the lung which has been shown to affect MDM responses *in vitro* (Doyle *et al.*, 2010).

8.2 Aim

The aim of this chapter is to investigate the effect of chronic, low level cigarette smoke and LPS on monocyte derived macrophage (MDM) cytokine release in response to NTHi and their phagocytic ability.

8.3 Methods

8.3.1 Peripheral Blood Mononuclear Cell Isolation from Human Whole Blood

Blood was acquired from Novartis's on site donor panel with all relevant consent acquired and donors remaining anonymous. Mononuclear cells were separated from whole blood and differentiated in GM-CSF as described in 2.11. Cells were seeded into wells at a concentration of 100,000 cells per well in the 96 well format and 30,000 cells per well in the 384 well format. Cells were allowed to adhere for 4 days before the media was changed

8.3.2 Cigarette Smoke Extract/LPS Challenge of Monocyte Derived Macrophages

Cigarette smoke extract was produced by passing five cigarettes (1R3F Kentucky research cigarettes) through 100mls of RPMI 1640 media at a flow rate of 0.6l/min. This solution was considered 100% cigarette smoke extract (CSE). One in ten serial dilutions were performed ranging from 3% to 0.03% CSE, 1 in 10 serial dilutions of LPS (E. Coli O26:B6) were also performed ranging from 1ng/ml to 0.01ng/ml in GM-CSF enriched RPMI 1640. MDMs were treated with the dilutions of CSE, LPS or GM-CSF RPMI control on the fifth day of the experiment. Media was changed on days 8 and 10 of the experiment and replaced with GM-CSF enriched media containing CSE, LPS or GM-CSF alone. Cells were challenged continuously for a total of 1 week before parameters were analyzed.

8.3.4 Heat Inactivated NTHi

NTHi was heat inactivated as described in 6.4.6 and was shown to have the same effect as live NTHi in chapter 6. Heat inactivated NTHi was used as live bacteria

would kill the cells and contaminate the tissue incubators. One hundred µl of brain heart infusion

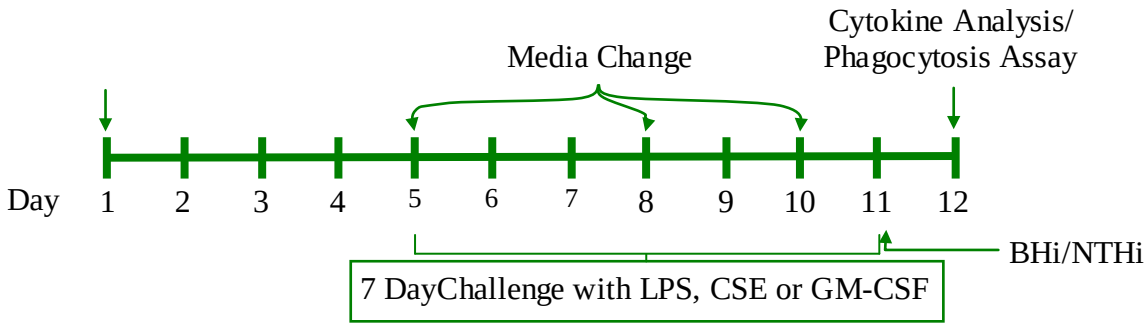


Figure 8.1 A schematic of the monocyte-derived macrophage protocol. LPS-Lipopolysaccharide, CSE-cigarette smoke extract, GM-CSF-Granulocyte macrophage colony stimulating factor, BHi-Brain heart infusion broth, NTHi-Nontypeable *Haemophilus influenza*.

broth (BHi) or heat inactivated NTHi at concentrations of $1x10^8$, $3x10^8$, $1x10^9$ and $3x10^9$cfu/ml were added to the relevant wells on day 11 of the experiment. Cells were then incubated for 24 hours before the supernatant was removed for future cytokine analysis.

8.3.5 Cytokine Analysis

On the twelfth day of the experiment the supernatant was removed and stored at -80°C for further cytokine analysis. Cytokines were analyzed using the MesoScale Discovery system previously described in 2.9.9. The Human 10 Plex base kit was used, which contained plates pre-coated in antibodies to detect GM-CSF, IL-1β, IL-10, IL-12p70, IL-2, IL-4, IL-5, IL-6, CXCL-8 and TNF-α. Cytokine analysis was performed on cells seeded in the 96 well format.

8.3.6 Phagocytosis Assay

On the twelfth day of the experiment a phagocytosis assay was performed on cells seeded in the 384 well format as described in 2.11.3. Briefly, after the addition of

fluorescently labelled *E.coli* and the cytoskeleton fixative cytochalasin D, which inhibits phagocytosis, to the relevant wells the plate was read immediately at an excitation wavelength of 544nm and read at an emission wavelength of 612nm. The plates were then incubated at 37°C and read every 30 minutes up to 4 hours with incubation between readings.

8.4 Results

The Dose-Dependent Effect of Heat-Inactivated NTHi on CXCL8, TNF-α, IL-1β and IL-6 Levels in Monocyte-Derived Macrophages.

All heat-inactivated NTHi challenged groups exhibited a significant increase in CXCL8, TNF-α, IL-1β and IL-6 levels compared to the media control group ($P<0.001$) (fig 8.2). CXCL8, TNF-α and IL-1β showed maximal responses at a concentration of $1x10^9$CFU/ml of heat-inactivated NTHi, while IL-6 showed peak responses at $3x10^9$CFU/ml of heat-inactivated NTHi; Therefore, $1x10^9$CFU/ml of heat-inactivated NTHi was used in future experiments.

The Dose-Dependent Effect of Cigarette Smoke Extract Challenge on CXCL8 Levels in Monocyte-Derived Macrophages

CXCL8 levels peaked at the highest concentration of 3% cigarette smoke extract. 0.3 and 0.03% CSE had very similar CXCL8 levels, with all CSE challenged groups exhibiting increased levels of CXCL 8 compared to the media challenged group. 0.3% CSE was the submaximal response, so was the dose used in further experiments (fig 8.3).

The Effect of Heat-Inactivated NTHi on CXCL8, TNF-α, IL-1β and IL-6 Levels in GM-CSF or Cigarette Smoke Extract Challenged Monocyte-Derived Macrophages

There was no significant change in CXCL8, TNF-α and IL-6 levels when heat-inactivated NTHi challenged groups for both the CSE and control groups were compared (fig 8.4); however, they were all increased compared to their respective vehicle control groups. When Il-1β levels for heat-inactivated NTHi challenged cells for both 0.3% CSE and GM-CSF groups were compared there was a significant

reduction in levels in CSE challenged groups ($P<0.05$), which were elevated compared to the respective control groups.

The Effect of Cigarette Smoke Extract Challenge on Phagocytosis by Monocyte-Derived Macrophage.

Cigarette smoke extract challenged groups showed a 22% decrease in the initial two hour rate of phagocytosis compared to the GM-CSF treated group, but this failed to reach significance. Both groups showed an increased rate compared to their cytochalasin D control groups. There was a very similar pattern in the peak response, with CSE challenged groups expressing a 21% decrease in peak phagocytosis compared to the GM-CSF treated, but again this failed to reach significance, but both groups showed an increase rate compared to their respective cytochalasin D treated groups (fig 8.5).

The Dose-Dependent Effect of LPS Challenge on CXCL8 Levels in Monocyte-Derived Macrophages

CXCL8 levels peaked at the highest (1ng/ml) concentration of LPS, with a dose dependent increase in levels as the dose increased from 0.01 to 0.1ng/ml, with all groups being elevated compared to the control. 0.1ng/ml of LPS being the submaximal response and was therefore chosen for future experiments (fig 8.6).

The Effect of Heat-Inactivated NTHi on CXCL8, TNF-α, IL-1β and IL-6 Levels in GM-CSF or LPS Challenged Monocyte-Derived Macrophages

There was no significant change in CXCL8 levels when heat-inactivated NTHi challenged groups, for both the LPS and control groups, were compared (fig 8.7), however, CXCL8 levels were increased compared to their respective controls. TNF-α levels between heat-inactivated NTHi challenged groups for LPS and GM-CSF

also showed no significant change in levels, despite having an almost 50% reduction. There was a significant difference in IL-1β levels between heat-inactivated NTHi groups challenged with either GM-CSF or LPS, with the LPS group exhibiting a significant reduction ($P<0.05$). LPS and vehicle challenged cells exhibited similar IL-1β levels compared to the LPS NTHi group but showed increased levels compared to the GM-CSF and vehicle challenged cells. Similar to the IL-1β response, IL-6 levels also exhibited a significant change in the heat-inactivated NTHi and GM-CSF or LPS challenged groups ($P<0.01$) and again LPS and vehicle challenged cells had similar levels of IL-6 compared to the LPS and NTHi challenged group but were increased compared to the GM-CSF and vehicle challenged group.

The Effect of LPS Challenge on Phagocytosis by Monocyte-Derived Macrophage.

LPS challenged cells showed a significant 54% impairment in the initial two hour rate of phagocytosis, compared to GM-CSF treated cells ($P<0.001$), while both groups showed an increased rate compared to their respective cytochalasin D treated group. There was a similar 55% significant reduction in the peak response in LPS challenged cells compared to the GM-CSF treated group ($P<0.001$), which was again increased compared to their respective cytochalasin D treated group which remained unchanged (fig 8.8).

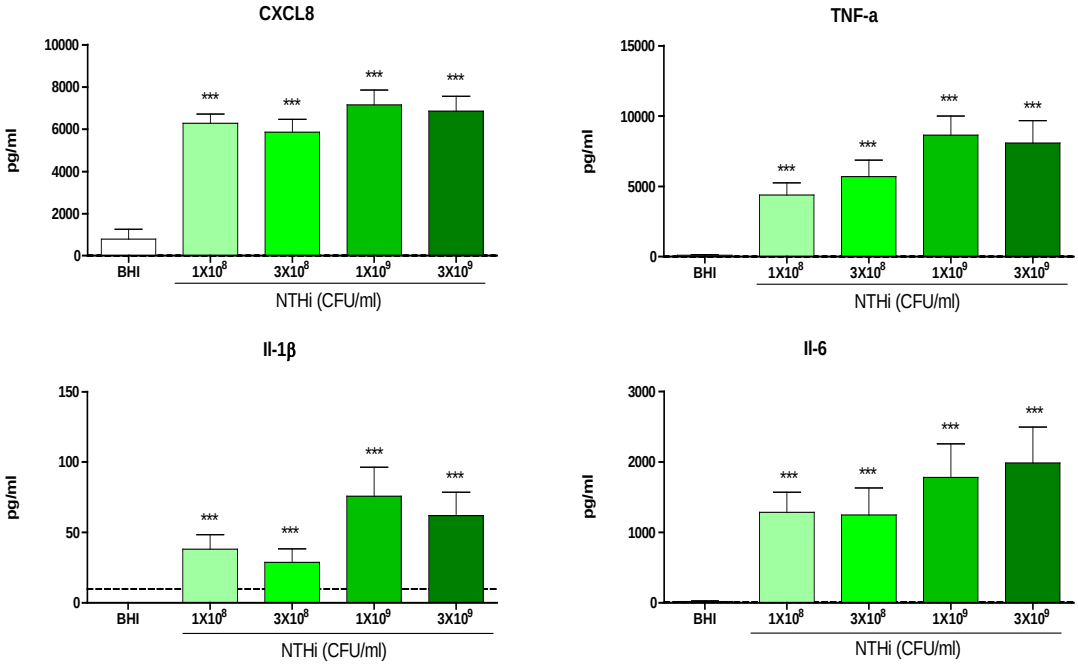


Figure 8.2 The dose dependent effect of heat-inactivated NTHi ($1x10^8$, $3x10^8$, $1x10^9$ and $3x10^9$cfu/ml) on CXCL8, TNF-α, IL-1β and IL-6 levels in MDMs. All cytokines are significantly elevated at each NTHi concentration compared to the control, with CXCL8, TNF-α and IL-1β reaching maximal response at $1x10^9$ cfu/ml and IL-6 achieving maximal response at $3x10^9$cfu/ml. ***P<0.001, Mean ± SEM, N=6.

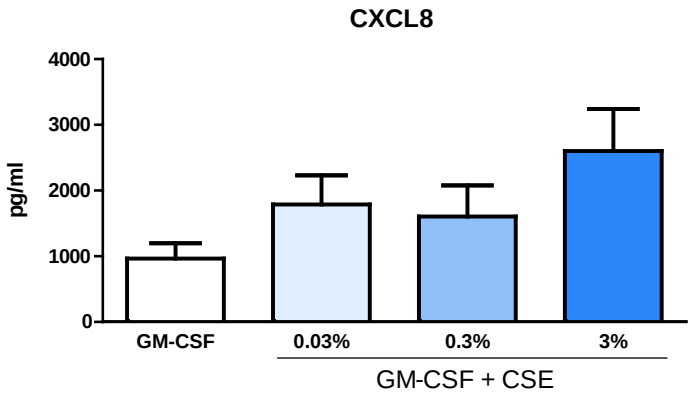


Figure 8.3 The dose dependent effect of cigarette smoke extract on CXCL8 levels in monocyte derived macrophages. Monocyte derived macrophages were challenged for one week with 1 in 10 serial dilutions ranging from 3 to 0.03% cigarette smoke extract. All concentrations of cigarette smoke extract raised CXCL8 levels, with 0.3% being the sub-maximal response. Mean ± SEM, N=6.

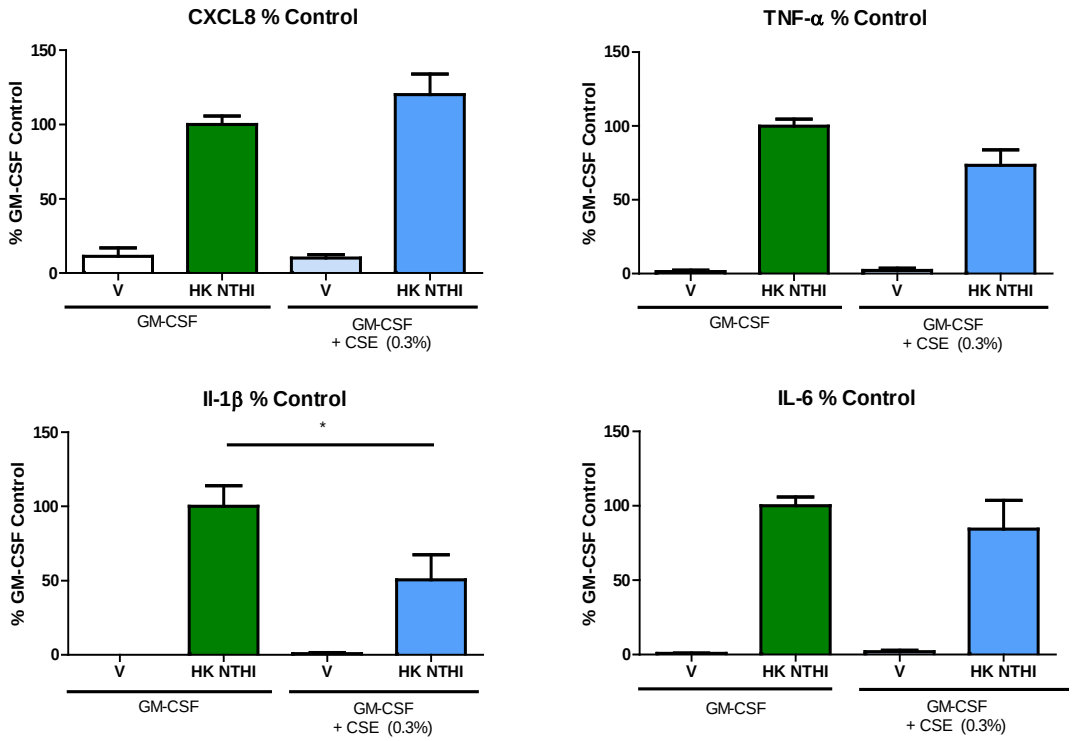


Figure 8.4 The effect of NTHi (1x10^9 cfu/ml) on monocytes differentiated in GM-CSF or GM-CSF and 0.3% cigarette smoke extract for 7 days. There is no significant difference in CXCL8, TNF-α and IL-6 levels between GM-CSF or cigarette smoke extract differentiated monocytes after challenge with NTHi. There was a significant difference between these groups for IL-1β. Mean ± SEM, N=6, *P<0.05, Kruskal-Wallis *post hoc* Dunn's test.

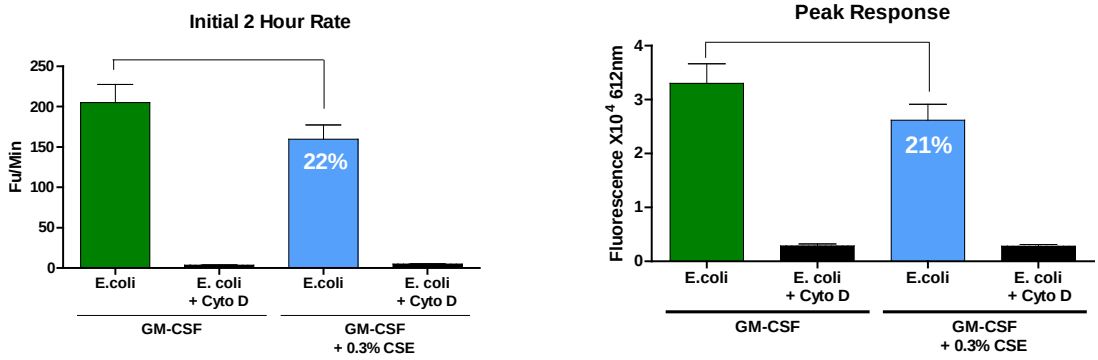


Figure 8.5 The effect of cigarette smoke extract (0.3%) on the rate and peak phagocytosis response in monocyte-derived macrophages. Cigarette smoke extract showed no significant change in rate or peak phagocytosis response compared to the control group. Mean ± SEM, N=3, P>0.05, Kruskal-Wallis *post hoc* Dunn's test.

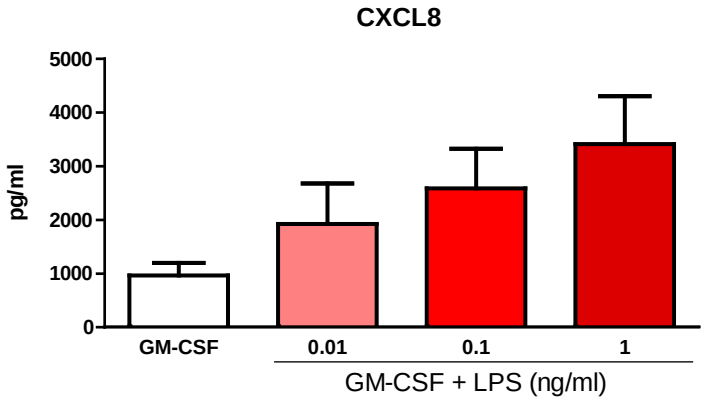


Figure 8.6 The dose dependent effect of LPS on CXCL8 levels in monocyte derived macrophages. Monocyte derived macrophages were challenged for one week with 1 in 10 serial dilutions ranging from 1 to 0.01ng/ml of LPS. All concentrations of LPS raised CXCL8 levels, with 0.1ng/ml being the sub-maximal response. Mean ± SEM, N=6.

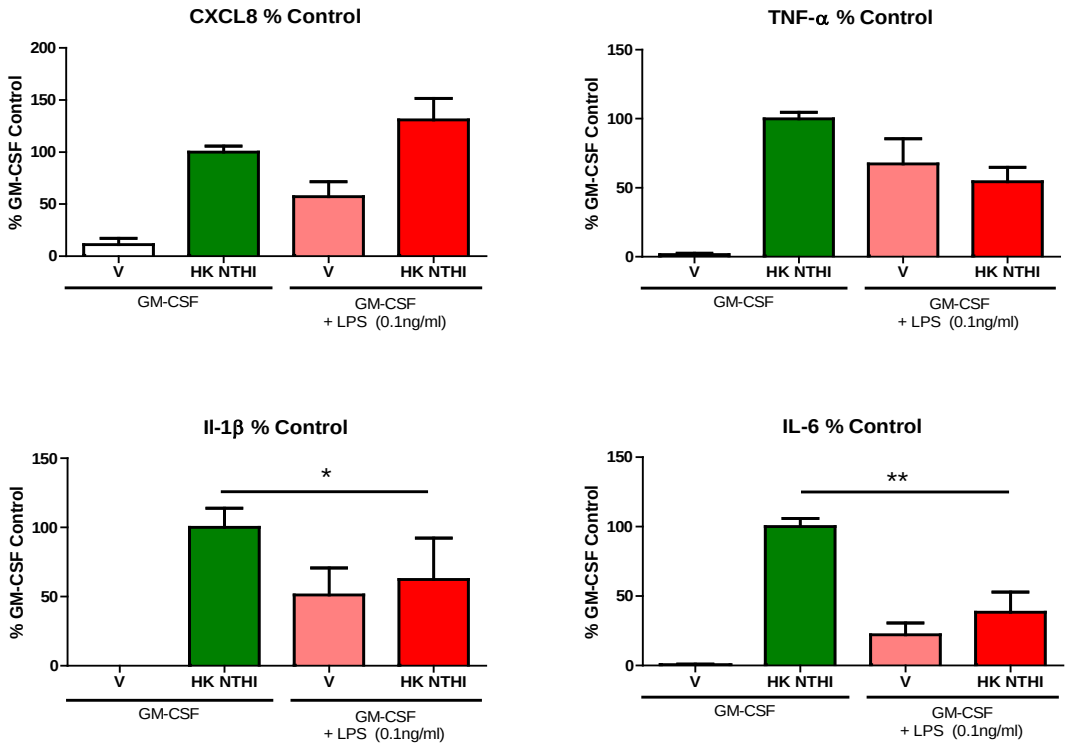


Figure 8.7 The effect of NTHi (1x10^9 cfu/ml) on monocytes differentiated in GM-CSF or GM-CSF and LPS (0.1ng/ml) for 7 days. There is no significant difference in CXCL8 or TNF-α levels between GM-CSF or LPS differentiated monocytes after challenge with NTHi. There was a significant difference between these groups for IL-1β and IL-6. Mean ± SEM, N=6, *P<0.05, **P<0.01, Kruskal-Wallis *post hoc* Dunn's test.

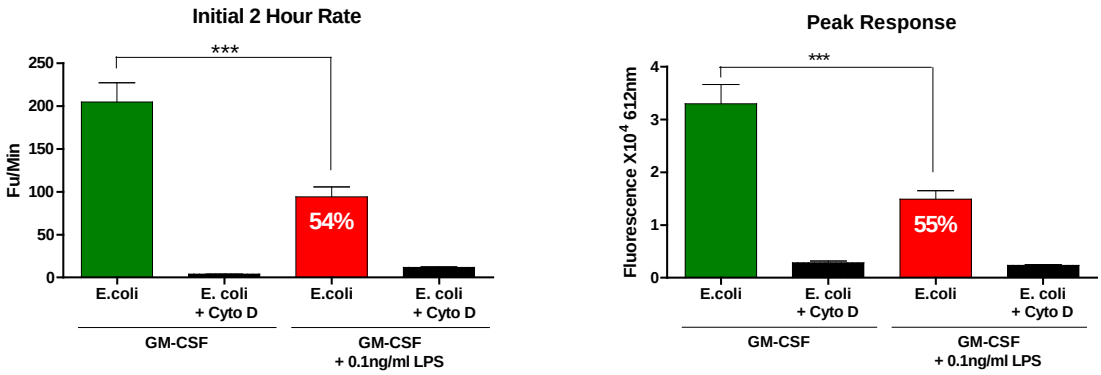


Figure 8.8 The effect of LPS (0.1ng/ml) on the rate and peak phagocytosis response in monocyte-derived macrophages. LPS showed a significant difference in both initial rate and peak phagocytosis response compared to the control group. Mean ± SEM, N=3, ***P<0.001, Kruskal-Wallis *post hoc* Dunn's test.

8.5 Discussion

To my knowledge this study is novel in investigating the effect of low level chronic CSE or LPS exposure on monocyte derived macrophages (MDMs). This study has shown that stimulation with CSE or LPS impairs the response of MDMs to NTHi. Many studies have been performed on alveolar macrophages and monocyte derived macrophages from COPD patients, healthy individuals and animal models to examine the differences in their gene and cytokine profiles. The results from this study showed that mild but prolonged stimulation of MDMs with CSE significantly reduced the release of IL-1β in response to heat-inactivated NTHi while LPS significantly reduced Il-1β and Il-6.

Varying concentrations of CSE have been described in many different macrophage models to investigate its effect on their responses; however the very nature of producing CSE means that batches vary considerably, meaning results are not perfectly comparable and must be assessed with caution. The effect of high concentrations on cell viability must also be taken into account (Yang, 2006). The effect of low concentrations of CSE on cell viability was not assessed in this study, but flow cytometry could be employed to determine whether this was a factor.

A low concentration of CSE was desired to more accurately reflect the type of exposure that might be expected in smokers. 0.3% CSE was a relatively low concentration and was shown to be submaximal in this study. Kent *et al.* (2008) used 1, 10 and 25% CSE which suppressed 24, 340 and 627 genes respectively after an acute 6 hour exposure in MDMs from COPD patients, however CXCL8 expression was increased. Ouyang *et al.* (2000) demonstrated a concentration of CSE as low as ~0.1% (after correction to current study protocols) for 27 hours significantly

suppressed Il-1β, Il-2 and IFN-ɣ release by MDMs in response to an anti-CD3 antibody. Similarly, Doyle *et al.* (2010) demonstrated alveolar macrophages taken directly from the lungs of COPD patients as well as healthy smokers exhibited a significantly down regulated IFN-ɣ pathway compared to non-smokers.

The results seen in this study with LPS challenged MDMs further suppressed cytokines after NTHi compared to CSE, significantly impairing Il-1β and IL-6. TNF-α was also decreased compared to non challenged cells; however this failed to reach significance. To my knowledge there are no studies in the literature examining the effect of chronic low-level LPS challenge on MDMs. Many studies have been performed evaluating the effect of LPS challenge acutely on AMs from COPD patients which showed that they had an impaired innate immune response compared to healthy AMs (Berenson e*t al.,* 2006). Studies have also shown challenge with LPS down regulates key inflammatory receptors and pathways (Maris *et al.*, 2006; Ganesan *et al.*, 2012; Armstrong *et al.*, 2009), possibly explaining why the response to NTHi is diminished.

This down regulation of macrophage responses by cigarette smoke and LPS may appear to be beneficial, reducing inflammatory cytokines; however on the other hand the impaired response can also have a detrimental effect on the host's response to pathogens, leading to increased colonization and exacerbations. Further suppression of the response by dexamethasone has also been observed, which may increase the detriment in this process, questioning the rationale of steroid therapy in COPD (Kent *et al.*, 2010). It has previously been noted that bacteria and increased colonization can affect the progression of COPD and increase the frequency of exacerbations (Sethi and Murphy 2001).

As formerly mentioned NTHi is the most frequently isolated bacteria during stable and exacerbated disease, so is a very clinically relevant pathogen (Sethi *et al.*, 2006). This study has shown that the response of the macrophage to NTHi is significantly impaired by CSE and LPS. Berenson *et al.* (2006a) have shown that AM responses to NTHi antigens were significantly suppressed by smoking and in COPD compared to non-smokers, impairing the response to pathogens. Unfortunately, standard fluorescently labeled *E. coli* was used in the phagocytosis experiments due to the time constraints not allowing for NTHi to be labeled. This study showed a 21% inhibition in phagocytosis by CSE, which although is not significant, many studies have shown cigarette smoke can significantly suppress the phagocytic potential of macrophages. Significant impairment of phagocytosis may be possible in this study if the concentration or length of challenge of CSE was increased. LPS challenged cells on the other hand exhibited a significant decrease in phagocytic ability of 55% compared to unchallenged cells.

Martí-Lliteras *et al.* (2009) demonstrated significant impairment in phagocytosis of NTHi by alveolar macrophages, from smokers and COPD patients, and macrophage cell lines challenged with CSE. Beresson *et al.*, (2006) also showed AM from COPD patients were impaired in phagocytosing NTHi. While Taylor *et al.* (2010) showed this is not only applicable to NTHi, as phagocytosis of *Streptococcus pneumonia* was also impaired. Hodge *et al.* (2003) also demonstrated alveolar macrophages from COPD patients had impaired efferocytosis of apoptotic epithelial cells.

There are many possible explanations as to why cigarette smoke and LPS down regulates macrophage innate immunity and phagocytosis. Firstly chronic exposure to an antigen such as CSE or LPS has been shown to result in down regulation of toll-

like receptors and their associated co-receptors (Droeman *et al.*, 2005; Wang *et al.*, 2002a; Lin *et al.*, 2004; Oshikawa and Sugiyama, 2003). Ganesan *et al.* (2012) demonstrated in mice exposed to LPS a link in an impaired phagocytic ability of macrophages with a decrease in scavenger receptors. This decreases the inflammatory response of the macrophage in response to the antigen over time, as well as impairing its ability to recognize specific pathogen antigens in the future. As previously discussed in chapter 3, activation of Toll-like receptor pathways causes the activation of NF-κB and AP-1 via the MyD88 pathways. Kent *et al.* (2008) showed CSE affected NF-κB and AP-1 decreasing the expression of inflammatory genes with the exception of the neutrophil chemoattractant CXCL8. This study showed very little variation in CXCL8 levels for CSE with similar increases in response to NTHi challenge; however Il-1β was impaired. LPS alone increased CXCL8 levels as would be expected, but again responses to CXCL8 were not impaired after further challenge with NTHi but Il-1β and Il-6 were.

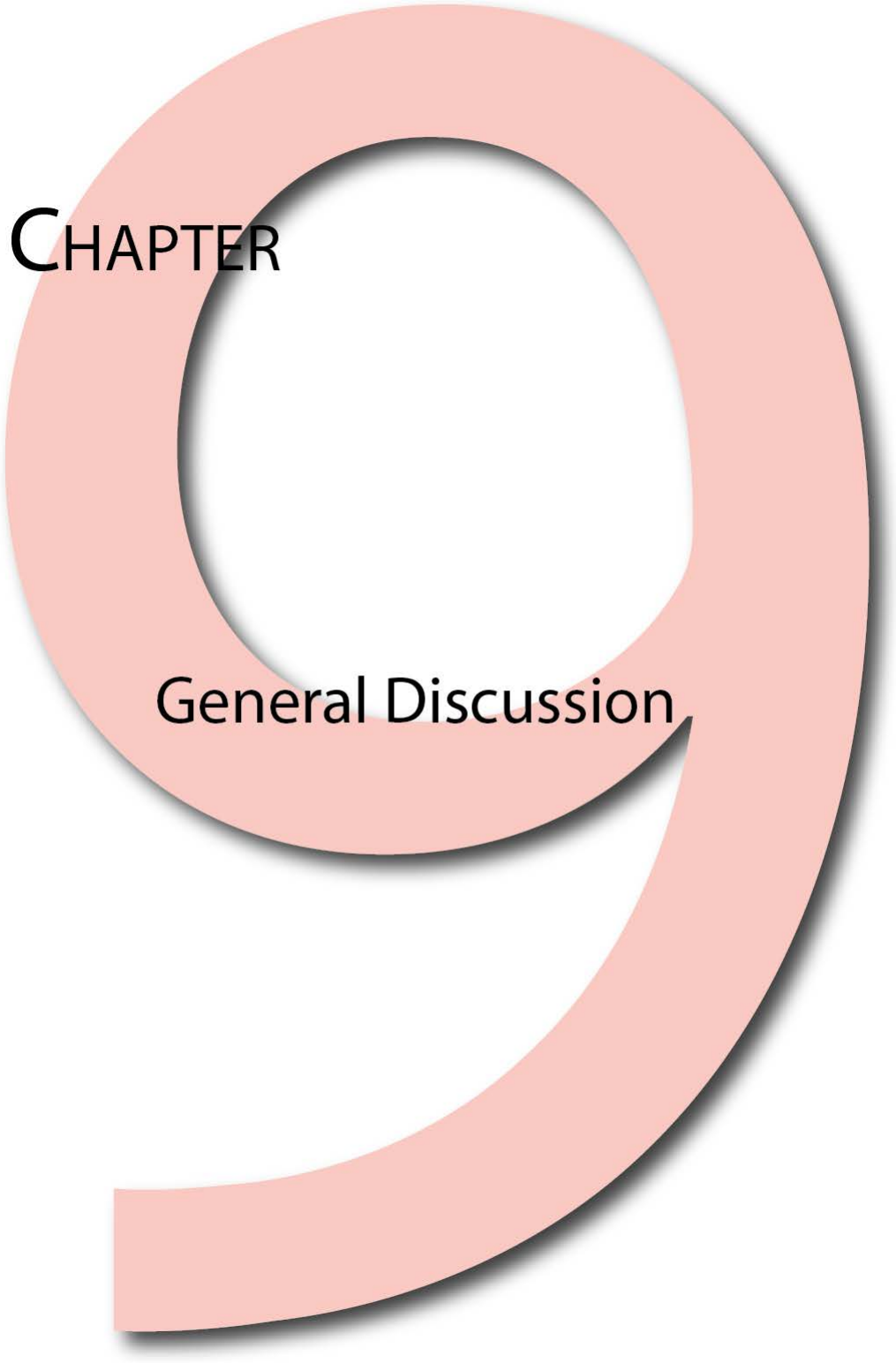
Birrell *et al*'s. (2007) study in AM exposed to CSE proposes this affect may be due to oxidative stress and glutathione depletion. While increased oxidative stress attenuated the NF-κB pathway resulting in a decrease in some cytokines, the AP-1 pathway was up regulated, resulting in increases in other cytokines, most notably CXCL8. This response was blocked by glutathione. CSE has also been shown to affect the regulation of genes and inflammation due to its effect on histone deacetylase 2 (HDAC2). This has been discussed in chapter 4, but briefly HDAC2 is responsible for removing acetyl groups from histone proteins, allowing DNA to bind more tightly to the protein, preventing the transcription of genes by obscuring the promoter regions. Adenuga *et al.* (2009) demonstrated that CSE impaired HDAC2 in

a macrophage cell line as well as in bronchial epithelial cells, resulting in abnormal inflammation.

The difference in M_1 and M_2 responses may also be important. Cells in this study were stimulated with GM-CSF which drives them to an M_1 phenotype, while cells differentiated in M-CSF would exhibit a more M_2 phenotype (Verreck *et al.*, 2006). LPS has also been shown to drive cells to an M1 phenotype (Mantovani *et al.*, 2004), while cigarette smoke shows a more heterogeneous mix of M1 and M2 AMs in COPD (Hodge *et al.*, 2011). It has previously been mentioned that studies have observed reductions in M1 type cytokines and up regulation of M_2 cytokines in COPD AMs (Shaykhiev *et al.*, 2006), so with GM-CSF pushing an M_1 phenotype, the impaired response observed here is similar to the disease, however a possible homogenous population of M1 macrophages may react differently than a more heterogeneous M_1/M_2 population. Further studies should be performed to establish the phenotype present, as well as examining the effect CSE and LPS challenge has on their gene profiles.

It is important to note that while CSE and LPS were treated as separate entities, their interplay in COPD cannot be ignored. As previously noted, CSE has been shown to down regulate phagocytosis in AMs of COPD patients, which may lead to increased colonization and exacerbations in COPD. This in turn could lead to more LPS present in the airways, decreasing the response to pathogens further and possibly leading to a vicious cycle of increased colonization with decreased innate immunity and more frequent exacerbations. The effect of CSE and LPS co-challenge on MDMs may be of interest, to investigate whether the decrease in innate immune response to NTHi is compounded.

In summary, this study has demonstrated that chronic low level CSE was able to significantly decrease Il-1β release from MDMs in response to NTHi challenge. LPS had a greater effect, decreasing IL-1β and IL-6 as well as impairing phagocytosis. This study has suggested that LPS from bacterial colonization may also play a key role in impairing the innate immune response to pathogens, increasing the frequency of exacerbations.


CHAPTER
9
General Discussion

The aim of this thesis was to create an animal model of COPD that could be exacerbated with a pathogen. This model could then be used to investigate acute exacerbations of COPD (AECOPD), identifying pathways as possible therapeutic targets.

Firstly, the chronic LPS guinea pig model demonstrated a similar phenotype to COPD, with increased inflammatory cell influx, decreased lung function and changes in lung histology, notably emphysema. Guinea pigs were chosen as they have a more similar lung structure and pharmacology to humans compared to mice. Furthermore, Broadley *et al.* (2010) and Toward *et al.* (2004) demonstrated an infection in guinea pigs after intranasal administration of PIV3. However, initial experiments in this thesis with human parainfluenza 3 virus (PIV3) and poly I:C failed to show infection or adequate increases in inflammation respectively, meaning the model could not be exacerbated with these agents. This may have arisen through mutations in the virus making it less virulent. Another possible explanation is conscious guinea pigs expelled a large amount of the virus from their noses, meaning the infective dose may not have been met, possibly explaining why poly I:C also failed to adequately increase inflammation. Nardelli-Haefliger *et al.* (2001) demonstrated mice that were anaesthetised, inhaled 300 times more of the inoculation than conscious animals, so anaesthetising the animals may have overcome this problem. This would still leave investigations into pathways and cytokine responses difficult to investigate due to lack of specific reagents for guinea pigs.

The guinea pig model demonstrated similar responses as COPD to the drug theophylline as described in the literature, with theophylline improving the response to the glucocorticoid dexamethasone by decreasing leucocyte numbers, neutrophils

in particular. The use of steroids in COPD is controversial (Barnes 2000b; Calverly 2000), with the disease (Alasaeedi *et al.,* 2002; Culpitt *et al.,* 1999; Keatings *et al.,* 1997) and the chronic LPS guinea pig model demonstrating a lack of response to inhaled steroids. Many studies have been performed to investigate the mechanisms of COPD steroid insensitivity and whether this could be reversed. Low dose theophylline has been shown to increase the effectiveness of glucocorticoids (Ford *et al.,* 2010), which is believed to be due to the effect of PI3Kδ inhibition on HDAC2. This results in increased HDAC2 activity, decreasing NF-κB and oxidative stress resulting in decreased inflammation and increased steroid sensitivity (To *et al.,* 2010). However, research into improving steroid responses in COPD may do more harm than good. This is because reducing the response of the immune system with glucocorticoids may make the disease worse by impairing the clearance of aetiological and exacerbating agents, such as viruses and bacteria.

Chronic cigarette smoke model in the mouse highlights the negative effect impairing the immune response could have. The chronic cigarette smoke model exhibited a similar phenotype to COPD, with increased inflammation, structural changes and decreased lung function. Chronic cigarette smoke induced inflammation has historically been shown to be resistant to steroids. However, this mouse model does have its drawbacks, in that it is expensive, time consuming, has relatively mild inflammation and takes a long time to develop structural changes, but it is generated with a disease-relevant substance, which is a more representative heterogeneous mix of chemicals than a singular toxin such as LPS.

The chronic cigarette smoke model was exacerbated with the bacteria, non-typeable *Haemophilus influenza* (NTHi) with significant increases in neutrophils. NTHi is the most frequently isolated bacteria in acute exacerbations of COPD, so was a clinically

relevant bacterium to use (Mitravelles *et al.,* 2012). The addition of NTHi in the chronic smoke model resulted in an increase in inflammation on top of the inflammation caused by cigarette smoke as observed during an AECOPD; however, the results in the chronic smoke model demonstrated a significant impairment in many cytokines and cells of the innate and adaptive immune response to NTHi compared to non-smoke exposed animals.

This impairment in immune response has rarely been reported previously. Many models of AECOPD focus on an increase in inflammation with a pathological agent on top of a stimulus, such as cigarette smoke or LPS, compared to the stable disease model. However, there is increasing evidence that cigarette smoke impairs the response of the immune system, rather than enhancing it as would be expected in an exacerbation. This would result in a decreased ability to fight infections. Kent *et al.* (2008) demonstrated down regulation of numerous inflammatory genes in monocyte-derived macrophages (MDMs) from COPD patients challenged with CSE, while many other studies have demonstrated down regulation of cytokines in the disease (Takanashi *et al.,* 1999; Doyle *et al.,* 2010), cell models (Ouyang *et al.,*2000) and in animal models (Gascheler *et al.,* 2009) challenged with CSE.

With relatively little research into the blunting of the immune response, the exact reasons for this impairment is not fully understood. Theories include the down regulation of pattern recognition receptors such as toll like receptors (Droeman *et al.,* 2005; Liew *et al.,* 2005; Wang *et al.,* 2002a; Lin *et al.,* 2004; Oshikawa and Sugiyama, 2003) and scavenger receptors (Ganesan *et al.,* 2012), impairment of the NF-kB pathway (Gaschler *et al.,* 2007, Birrell *et al.,* 2007; Kent *et al.,* 2008), differential responses between macrophage subtypes (Shaykhiev *et al.,* 2006), oxidative stress (Drost *et al.,* 1992, 1993; Birell *et al.,* 2007), down regulation of

HDAC2 (Adenuga *et al.,* 2009) and anti-inflammatory compounds in cigarette smoke (Therriault *et al.,* 2003; Li *et al.,* 1998; Bathoom *et al.,* 2007; Geng *et al.,* 1996), but much more research is needed in this area to elucidate mechanisms and pathways involved.

The mouse model, with down regulated immunity would be expected to show decreased bacterial clearance, however, the reverse was true, with a lower bacterial load in smoke/NTHi mice compared to sham/NTHi animals. This could be due to several reasons. Firstly NTHi is not a bacteria that can infect mice, so does not actively replicate making it easier to clear. This was highlighted by the similar responses observed between live and heat-inactivated NTHi. An infective strain of bacteria in the mouse, such as *Streptococcus pneumoniae* (Ludewick *et al.,* 2011), which is present in 15 % of COPD exacerbations (Sethi, 2011), making it a clinically relevant pathogen, may show decreased colonisation in this impaired immunity model. Secondly, while macrophages may experience decreased phagocytic ability, the sheer numbers present in the lungs prior to inoculation may overcome this and result in increased bacterial clearance. Thirdly, the inhospitable environment in the lungs of cigarette smoke exposed mice, with increases in oxidative species, could decrease the viability of the bacteria. Lastly, NTHi has been demonstrated to cross into epithelial cells to evade the host immune response (Morey *et al.,* 2011). If this happened in this model, more bacteria could cross into epithelial cells of the chronic cigarette smoke mouse lung than non-smoked due to increased epithelial cell damage in the model. Unfortunately attempts with Gram stain and immunohistochemistry to elucidate whether this did occur failed. The phagocytic ability of macrophages in this model should be investigated to determine whether it was affected by chronic cigarette smoke exposure as observed in the disease (Taylor *et al.,* 2010). The model

should also be validated by using pharmacological agents that have been proven beneficial in treating AECOPD, such as phosphodiesterase 4 inhibitors (Reannard *et al.*, 2011).

This impairment of the immune response was also observed in the study with MDMs differentiated in GM-CSF and challenged with CSE or LPS. COPD alveolar macrophages have demonstrated an impaired immune response (Doyle *et al.*, 2010; Berenson *et al.*, 2006) and phagocytic ability (Marti-Lliteras *et al.*, 2009). Monocyte derived macrophages in this study also showed that chronic, low level stimulus with cigarette smoke or LPS reduces cytokine release and impairs phagocytosis. This may be partly responsible for the increased bacterial colonisation associated with the disease and lead to a vicious cycle of impaired immunity, increased bacterial colonisation/exacerbation and lead to faster disease progression. This is summarised in figure 9.1. It would also be preferable to fluorescently label NTHi and *Streptococcus pneumoniae* to investigate the phagocytosis in both the chronic cigarette smoke model and the MDM model rather than using pre-prepared E.coli. Analysing and comparing the pathways involved in phagocytosis in both models may help pinpoint specific therapeutic targets to up- regulate phagocytosis, with the aim of decreasing colonisation and exacerbations and breaking the vicious cycle. These pathways must then be analysed in the disease to determine whether the pathways are similar in both the model and in COPD.

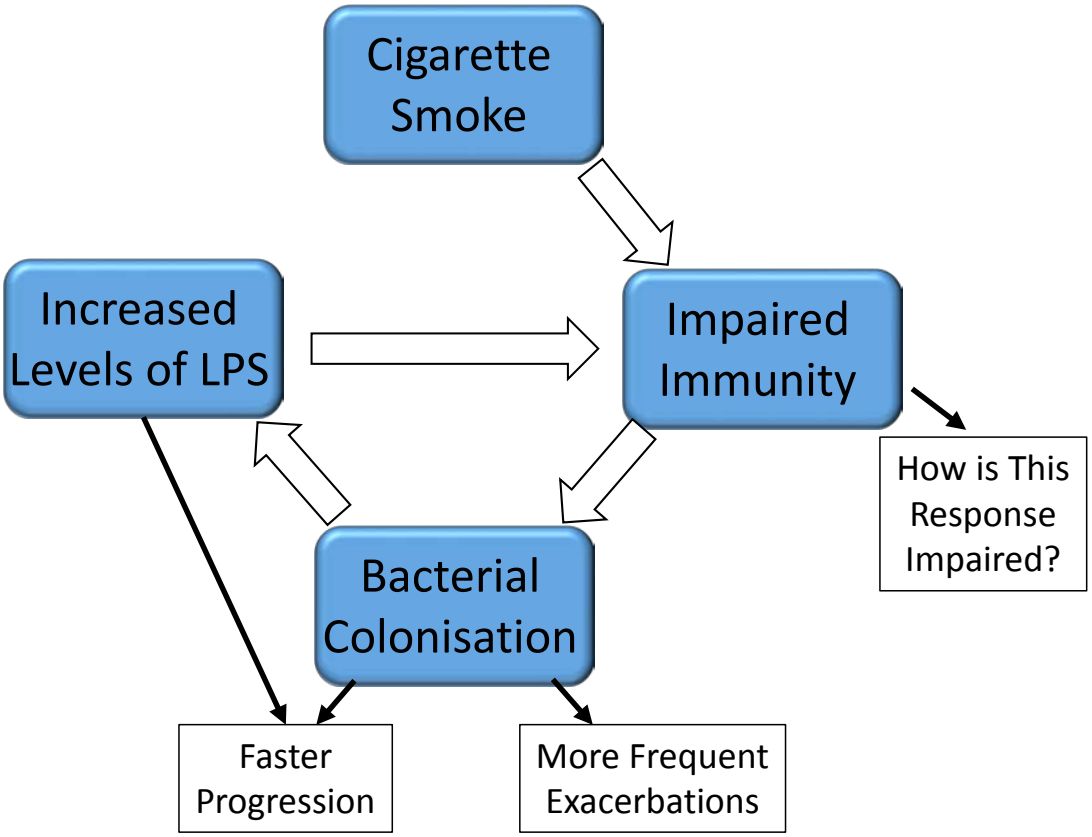


Figure 9.1: The Vicious Cycle of Impaired Immunity and Increased Bacterial Colonisation and Exacerbation in COPD.

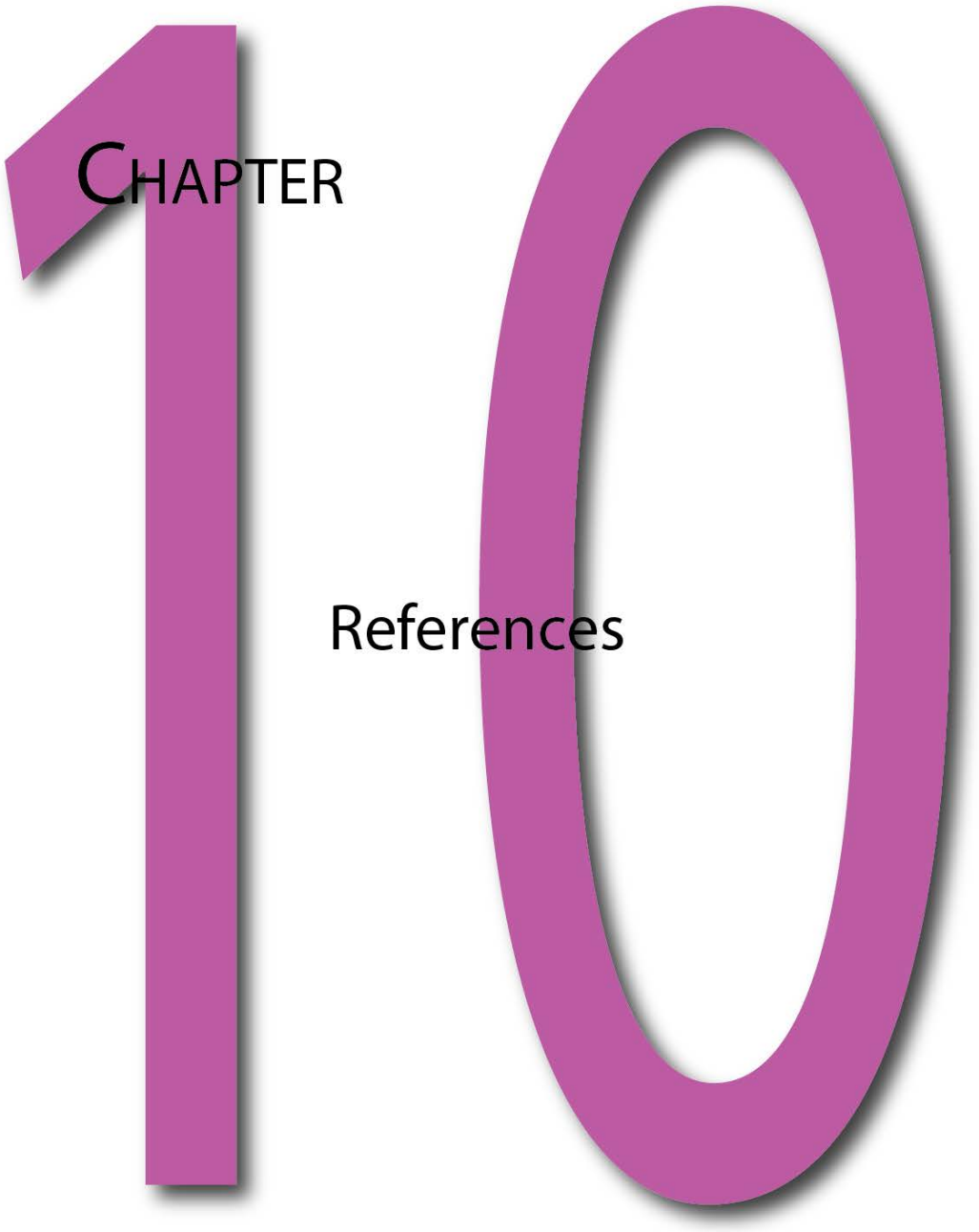
Another important aspect that must be investigated in the chronic cigarette smoke model is the macrophage subpopulations that are present. As previously discussed studies have shown distinct differences in macrophage subpopulation responses in COPD, with the pro-inflammatory M_1 macrophages being typically impaired, while the anti-inflammatory M_2 macrophages are generally up-regulated (Shaykhiev *et al.*, 2006). The subpopulation of macrophages in COPD comprise of both M_1 and M_2 macrophages (Verreck *et al.*, 2006). The subpopulation of macrophages in the chronic smoke model should be investigated, with gene analysis determining which pathways are up-regulated and down-regulated within the different populations and whether this correlates with the disease.

Differentiating MDMs in M-CSF as opposed to GM-CSF would cause the macrophages to display a more M_2 phenotype. The response of this separate population to chronic cigarette smoke extract or LPS challenge could also be investigated to examine differences between the two subpopulations in response to the same stimulus. The addition of CSE and LPS co-challenge may also be interesting to investigate to see whether there is a compounding of the impaired response.

It is important, whatever model is used, that pathways investigated and identified in the models or in the disease itself are cross checked with each other to determine whether the models and the disease are responding similarly and via the same pathways. A model that heavily involves pathways which the disease does not have may be of little use in understanding and drug discovery. However, it may also be important to note large phenotypes within the disease, which may exhibit different pathways but have the same outcome, should be investigated with many different models focussing on particular phenotypes instead of attempting to generalise an entire disease within a small number of models.

In conclusion this study has shown that a chronic LPS model in the guinea pig developed similar inflammation, lung function and structural changes observed in COPD, however it was unable to be exacerbated by PIV3 or Poly I:C. Low dose theophylline did increase steroid sensitivity in the model but this failed to reach significance. It was also shown that a model of COPD exacerbation in the mouse was achieved, with increased inflammation after NTHi and smoke compared to smoke alone. The model also demonstrated impaired responses to NTHi after smoke challenge, with decreased inflammatory cell and cytokine respsonses. This was replicated in monocyte derived macrophages challenged with cigarette smoke and

LPS, with down regulated cytokine release and phagocytosis in response to bacteria. This impairment may lead to a vicious cycle of impaired immunity, increased colonisation/exacerbation and faster disease progression. Studies must be performed to elucidate specific mechanisms by which this impaired response occurs with the aim of specifically up regulating inflammation to healthy levels, which may slow down the vicious cycle of colonisation and impairment and lead to resolved inflammation.



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Appendix

Your name:

Today's date:

COPD Assessment Test

How is your COPD? Take the COPD Assessment Test™ (CAT)

This questionnaire will help you and your healthcare professional measure the impact COPD (Chronic Obstructive Pulmonary Disease) is having on your wellbeing and daily life. Your answers, and test score, can be used by you and your healthcare professional to help improve the management of your COPD and get the greatest benefit from treatment.

For each item below, place a mark (X) in the box that best describes you currently. Be sure to only select one response for each question.

Example: I am very happy 0 X 2 3 4 5 I am very sad

			SCORE
I never cough	0 1 2 3 4 5	**I cough all the time**	
I have no phlegm (mucus) in my chest at all	0 1 2 3 4 5	**My chest is completely full of phlegm (mucus)**	
My chest does not feel tight at all	0 1 2 3 4 5	**My chest feels very tight**	
When I walk up a hill or one flight of stairs I am not breathless	0 1 2 3 4 5	**When I walk up a hill or one flight of stairs I am very breathless**	
I am not limited doing any activities at home	0 1 2 3 4 5	**I am very limited doing activities at home**	
I am confident leaving my home despite my lung condition	0 1 2 3 4 5	**I am not at all confident leaving my home because of my lung condition**	
I sleep soundly	0 1 2 3 4 5	**I don't sleep soundly because of my lung condition**	
I have lots of energy	0 1 2 3 4 5	**I have no energy at all**	

TOTAL SCORE

RES/QST/09/43163/1 Date of preparation: September 2009.

MODIFIED MEDICAL RESEARCH COUNCIL DYSPNEA SCALE

ID NUMBER:									FORM CODE: MRC VERSION: 1.0 10/26/10	Visit Number			SEQ #			

0a) Form Date ..... ☐☐/☐☐/☐☐☐☐ 0b) Initials..... ☐☐☐

Instructions: *This form should be completed during the participant's visit. Choose the one best response.*

Please choose the one best response to describe your shortness of breath.

Grade

0 "I only get breathless with strenuous exercise"

1 "I get short of breath when hurrying on the level or walking up a slight hill"

2 "I walk slower than people of the same age on the level because of breathlessness or have to stop for breath when walking at my own pace on the level"

3 "I stop for breath after walking about 100 yards or after a few minutes on the level"

4 "I am too breathless to leave the house" or "I am breathless when dressing"

1. Grade ..... ☐