DESCRIPTION AND CHARACTERISATION OF A
NOVEL MODEL OF PERSISTENT INFECTION
WITH *STREPTOCOCCUS PNEUMONIAE* IN THE
LOWER AIRWAYS OF MICE

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Abstract:
This thesis reports the development of a murine model of low-level, long-term colonisation of the lower respiratory tract with \textit{S. pneumoniae}, which would mimic the bacterial colonisation observed in COPD and severe asthma in humans. For a successful model of persistent infection, viable pneumococci would be recovered from the lower respiratory tract for a minimum of 28 days, mice would be asymptomatic but there would be an inflammatory response. After a single intranasal challenge of \textit{S. pneumoniae} strain LgSt215, CBA/Ca mice had recoverable numbers of viable pneumococci in the lower airways for at least 28 days post-infection, with an accompanying inflammatory response that consisted of a transient neutrophilia but progressed to being predominantly monocytic with foci of fibroplasia at the transitional airways from 14 days post-infection.

The next stage was to combine the new model of persistent pneumococcal infection with models that mimic other pathologies observed in COPD and asthma. Consequently an acute model of lipopolysaccharide-induced lung injury was combined with the model of persistent infection. This resulted in an increase in the number of neutrophils in the lower airways. However when the persistent pneumococcal infection model was combined with a model of ovalbumin-induced eosinophilic inflammation, the number of eosinophils induced was reduced, suggesting that the inflammatory response was no longer one of allergy, but mediated primarily by a Th1-mediated immune response. The observed phenotype mimicked the non-eosinophilic phenotype observed in half of mild-to-moderate asthmatics. A new NADPH oxidase 4 inhibitor was tested in this combined model of inflammation, and it was seen that after administration of this inhibitor an improvement in ciliary function was observed.

This model of persistent infection is an ideal tool to test hypotheses of triggers of exacerbation and for study of the role that bacterial infection play in the progression of COPD and asthma.
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List of Abbreviations:

*S. pneumoniae* – *Streptococcus pneumoniae*

AHR – Airway hyperresponsiveness

BA – Blood agar

BALF – Bronchoalveolar lavage fluid

BHI – Brain heart infusion

BSA – Bovine serum albumin

BTS – British Thoracic Society

CBF – Ciliary beat frequency

CFU – Colony forming unit

cm - Centimetre

CO₂ – Carbon Dioxide

COPD – Chronic obstructive pulmonary disease

CRP – C-polysaccharide reactive protein

DAB - 3,3’-diaminobenzidine tetrahydrochloride

DMSO – Dimethyl Sulfoxide

EDTA - Ethylenediaminetetraacetic acid

ELISA – Enzyme-linked immunosorbent assay

FACS – Fluorescence-activated cell sorting

FBS – Foetal bovine serum

FCS – Foetal calf serum

FEV₁ - Forced expiratory volume in 1 second

Fps – Frames per second

FRC - Forced residual capacity

FVC - Forced vital capacity

g - gram

g – gravity

GOLD – Global initiative for chronic obstructive pulmonary disease

H&E - Haemotoxylin and Eosin

HBSS – Hank’s buffered salt solution

HDM – House dust mite

HRP – Horse radish peroxidase

H₂SO₄ – Sulphuric acid
IFN-γ - Interferon – γ
Ig - Immunoglobulin
IL – Interleukin
IMS – Industrial Methylated Spirits
IP-10 - Interferon gamma induced protein 10
ITAC - Interferon-inducible T-Cell alpha chemoattractant
kPa – kilopascal
L – Litre
LPS - Lipopolysaccharide
Ltd – Limited
mg – Milligram
MIG - Monokine Induced by Interferon-γ
ml – Millilitre
N - Normality
NHS – National Health service
O₂ - Oxygen
OD – Optical density
OVA - Ovalbumin
PBS – Phosphate buffered saline
PEF – Peak expiratory flow
PPE – Porcine pancreatic elastase
psi – Pounds per square inch
RPMI-1640 – Roswell Park Memorial Institute medium formulation 1640
SEM – Standard error of the mean
Th – T Helper cell
THY – Todd Hewitt Broth + 0.5% w/v Yeast extract
TLC – Total lung capacity
TV – Tidal volume
UK – United Kingdom
v/v – volume/volume
w/v – weight/volume
μl – Microlitre
μm – Micrometre
1 Introduction:

Pathogens such as *Streptococcus pneumoniae* have been associated with the pathogenesis of severe respiratory diseases, such as chronic obstructive pulmonary disease (COPD) (Obert and Burgel, 2013). The aim of this project was to develop and characterise a model of long-term bacterial infection in the lower airways with a clinically relevant human pathogen, to better understand how it contributes to the pathology of severe respiratory disease.

1.1 Asthma and Chronic Obstructive Pulmonary Disease

The respiratory diseases, asthma and COPD, are major public health problems in both developed and developing countries. The World Health Organisation (WHO, 2008) reported that COPD was the fourth leading cause of death worldwide in 2004, and it predicted that by 2030 COPD would become the third leading cause of death worldwide.

Respiratory diseases cost the National Health Service (NHS) £6.6 billion each year (BTS Report 2006) and were responsible for one fifth of the deaths in the UK. Of these, COPD is responsible for 23% of all respiratory deaths in the UK and it affects 1.6% of the population in England, at an annual cost of £800 million to the NHS (NICE Clinical Guideline 101, 2011). Asthma is responsible for a smaller proportion of deaths in the UK, (1,272 in 2000 (Office for National Statistics, 2000)), however it costs the NHS £850 million annually (Hoskins et al. 2000).

Both COPD and asthma are diseases that are characterised by airway obstruction (Berge et al. 2011). In asthma this is variable and reversible, but in COPD it is progressive and largely irreversible (Barnes 2008a). In asthmatics, this variable airflow obstruction is often triggered by an abnormal immune response to environmental antigens (Essilfie et al. 2012). In contrast, in COPD the airflow obstruction is progressive and does not change markedly over several months (Burge and Wedzicha, 2003). Both diseases are also distinguished by the cellular inflammation observed in the airways and by a decline in respiratory function (Vasquez and Spina, 2000). In COPD and asthma the inflammation is driven predominantly by different T-cell subsets, T helper 1 (Th1) in
COPD and Th2 in asthma, (Barnes, 2008a). As both diseases are characterised by airway obstruction, it has also been hypothesised that both may represent disease states along a continuum, with varying degrees of each disease present in the same patient (Elias, 2004).

In the 1960’s the ‘Dutch hypothesis’ was proposed, which stated that COPD and asthma were different forms of a common disease (chronic obstructive disease) (Barnes 2008b). At the first Bronchitis Symposium at the University of Groningen in 1961, Orie and Sluiter proposed that various forms of airway obstruction (asthma, chronic bronchitis and emphysema) should be considered as different expressions of the same disease entity – chronic nonspecific lung disease (CNSLD) (Orie et al. 1961). In 1969, at the third Bronchitis Symposium, Fletcher and colleagues termed this hypothesis the ‘Dutch Hypothesis’ (Postma and Boezen, 2004). Fletcher et al. (1959) had proposed a separate hypothesis (the ‘British’ hypothesis) that chronic bronchitis could be separated into three main types of abnormality. The debate over whether the ‘Dutch’ or ‘British’ hypothesis is the most accurate goes on. There are many distinguishing features between COPD and mild asthma; however these distinctions are less apparent between COPD and severe asthma (Barnes 2008b). To make this even more complicated, roughly 10 % of COPD patients are thought to also suffer from asthma and therefore exhibit pathological features of both diseases, which has been labelled ‘wheezy bronchitis’ (Barnes, 2000).

1.1.1 COPD

COPD is an umbrella term that describes diseases that include emphysema and chronic bronchitis (Read, 1999). It is characterised by an airflow obstruction that is not fully reversible (Celli et al. 2004) and is diagnosed, and the degree of severity is assessed, with spirometry (Pauwels et al. 2001). In developed countries it is predominantly caused by cigarette smoking, yet only a small proportion of smokers develop the disease (Barnes and Cosio 2004). Roughly ninety per cent of deaths from COPD can be attributed to smoking (Chen, 1999), but other factors, such as air pollution and prolonged exposure to noxious particles e.g. biomass, can also cause COPD (Ko and Hui, 2012). Smoking cessation early in the disease onset can halt disease progression (Lokke et al. 2006); however lung function will not improve (Fletcher and Peto, 1977).
Pauwels *et al.* (2001) also noted that the prevalence of COPD was highest in countries where cigarette smoking was or still is common e.g. United States of America.

COPD is a disease of the small airways and the lung parenchyma (Barnes 2008a). Parenchymal disease is also termed emphysema, which is defined as permanent abnormal enlargement of the gas-exchanging airspaces, accompanied by destruction of alveolar walls but not by excessive fibrosis (Thurlbeck *et al.*, 1990). This destruction of the alveolar walls and enlargement of airspaces causes a reduction in the elastic recoil of the lung (Cosio Piqueras and Cosio 2001), which in turn causes a reduction in the efficiency of the lung. As well as emphysema, COPD sufferers also suffer from chronic bronchitis, which results in a persistent cough. This is caused by a ‘chronic’ over-production of sputum, where chronic is defined as occurring for more than three months per year for two or more successive years (Thurlbeck, 1990).

As can be seen in Figure 1, inhaled irritants including pollution and cigarette smoke activate epithelial cells and macrophages which in turn release chemotactic factors that attract inflammatory cells to the lungs (Barnes, 2008a). Epithelial cells release growth factors, including transforming growth factor–β (TGF–β) and fibroblast growth factor (FGF), which stimulate fibroblast proliferation (Barnes, 2008b). These stimulated fibroblasts then secrete chemokines, which direct the trafficking of dendritic cells (Kitamura *et al.* 2011). As well as signalling dendritic cells, the stimulated fibroblasts also cause collagen deposition, which contributes to the fibrosis of the small airways in COPD (Bonner, 2007).

Macrophages are one of the most abundant cell types in the respiratory tract (Boorsma *et al.*, 2013) and are the pivotal cells in COPD pathogenesis (Barnes, 2004). Macrophages are phagocytic cells that as part of the immune system, engulf and destroy foreign particles (Gordon, 2007). In the airways, macrophages can be classified into three subtypes; alveolar macrophages, interstitial macrophages and intravascular macrophages (Lohmann-Matthes *et al.* 1994). Each subtype is classified by its location in the lungs e.g. alveolar macrophages are found on the surface of the alveoli (Schneberger *et al.* 2011). Alveolar macrophages are thought to be the key subtype involved with COPD disease progression (Barnes, 2004).
When macrophages are activated, by particulates such as smoke, many chemokines are released, as shown in Figure 1 (Barnes, 2008a). One such chemokine is CCL2 (monocyte chemoattractant protein (MCP)-1) which attracts monocytes from the circulation to the airways (Deshmane et al. 2011). Once in the airways these monocytes differentiate into macrophages (Shi and Pamer, 2011). Other chemokines that are released by activated macrophages are the CXC chemokines; CXCL10 (Interferon gamma induced protein 10 (IP-10)), CXCL11 (Interferon-inducible T-Cell alpha chemoattractant (I-TAC)), and CXCL9 (Monokine Induced by Interferon-γ (MIG)) (Barnes, 2008b). All three chemokines bind to the CXC receptor 3 (CXCR3), which is expressed on activated T lymphocytes and Natural Killer cells (Farber, 1997).

T lymphocytes are another key immune cell for maintaining immune homeostasis and combating pathogens. T lymphocytes can be classified further and a key subtype is the CD4 T helper (Th) cell (Wan, 2010), which can be further distinguished by the cytokines which they secrete (Romagnani, 1991). The immune response in COPD is thought to be predominantly controlled by Th1 cells, unlike asthma where Th2 cells drive the immune response (Ichinose, 2009). Th1 cells secrete cytokines which include interferon – γ (IFN-γ), TNF-α and TNF-β, whereas Th2 cells are defined by their production of the cytokines IL-4, IL-5, IL-9, IL-10 and IL-13 (Wan, 2010). New research suggests that the inflammation seen in COPD could also be driven by Th17 cells which secrete IL-17, a cytokine which induces bronchial epithelial cells and fibroblasts (Curtis et al. 2007).

As well as CXCR3 being activated on Th1 cells, another subtype of T lymphocytes that are activated are type 1 cytotoxic (T_C1) T cells. These T_C1 cells have an increased expression of IFN-γ which contributes to a positive feedback loop inducing the chemokines CXCL9, 10 and 11, which are secreted by activated macrophages (Barnes and Cosio 2004). T_C1 cells have been shown to contribute to the emphysemic alveolar wall destruction (Barnes, 2008a).

Other contributors to the alveolar wall destruction are proteases that are secreted by macrophages and neutrophils (Owen, 2008). Another phenotype that is stimulated is mucus hypersecretion, which is produced by the increased numbers of goblet cells in the airway epithelium of COPD patients as shown in Figure 1.1, (Nadel, 2000).
**Figure 1.1**– Inflammatory and immune cells involved in chronic obstructive pulmonary disease (Barnes, 2008).

### 1.1.1.1 Diagnosis of COPD

Clinicians diagnose COPD, and its severity, from a combination of patient symptoms, history of exposure to certain risk factors and age. This diagnosis is then confirmed with spirometry. The global initiative for chronic obstructive lung disease (GOLD, 2011) recommends that patients who are aged over 40 and have dyspnea, chronic cough or sputum production, as well as a history of exposure to either tobacco smoke, smoke from home cooking and heating oils or occupational dusts and chemicals, should be considered as having COPD (Pauwels et al. 2001). It is then recommended that spirometry is performed to confirm this diagnosis (GOLD, 2013).

Spirometry is a range of techniques that are used to assess the mechanical ability of the lung to function. The two most commonly measured values are the forced expiratory volume in one second (FEV₁) and the forced vital capacity (FVC). FEV₁ is the maximal volume exhaled in the first second of a forced expiration from a position of inspiration.
A reduction in this value from predicted is indicative of an airflow obstruction. The larger the decrease of FEV\textsubscript{1} from predicted values, the greater the airway obstruction. Normal predicted values for FEV\textsubscript{1} are based on general population statistics and are dependent on factors such as age, ethnicity and sex. FVC is the maximal volume of air exhaled from a maximal inspiration (Miller et al. 2005). In other words, it is the largest volume that can be exhaled from the lungs. These values can be used independently, but the ratio between the two values is also informative. An FEV\textsubscript{1}: FVC ratio > 70 are considered normal, whereas patients that have a post-bronchodilator FEV\textsubscript{1}: FVC ratio < 70 are clinically diagnosed as having COPD (GOLD, 2013). A bronchodilator is used to confirm that the airflow limitation is persistent, rather than reversible as seen in asthma (Siafakas et al. 1995).

The severity of COPD can be classified into mild, moderate or severe (GOLD, 2011). This is based on a combination of lung function measurements and patient symptoms. Table 1.1, shows how spirometry is used to classify COPD severity. The classification of COPD disease severity is a pragmatic approach to give a general indication for disease management in the clinic (Pauwels et al. 2001). As the airflow limitation seen in COPD is progressive, disease severity increases over time and with further exposure to noxious stimuli e.g. cigarette smoke (Thurlbeck, 1990).
<table>
<thead>
<tr>
<th>Stage</th>
<th>Characteristics</th>
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| 0: At Risk  | Normal spirometry – no airflow limitation  
Chronic symptoms (cough, sputum production that is not due to specific disease e.g. tuberculosis) |
| 1: Mild COPD| FEV₁ / FVC < 70 %  
Predicted FEV₁ ≥ 80 % - a mild airflow limitation  
With or without chronic symptoms (cough, sputum production), patient may not be aware of symptoms |
| 2: Moderate COPD | FEV₁ / FVC < 70 %  
30 % ≤ FEV₁ < 80 % predicted – worsening airflow limitation  
With or without chronic symptoms (cough, sputum production, dyspnea), worsening quality of life |
| 3: Severe COPD | FEV₁ / FVC < 70 %  
Predicted FEV₁ < 30 % or the presence of respiratory failure or clinical signs of right heart failure – severe airflow limitation, poor quality of life |

Table 1.1 – Classification of disease severity in COPD (adapted from Pauwels et al. 2001).

1.1.1.2 Disease progression in COPD

A characteristic of COPD is the periodic exacerbation of symptoms (Burge and Wedzicha, 2003). As COPD is a heterogeneous condition, the severity and frequency of exacerbations are individual to the sufferer (Sapey and Stockley, 2006). However as the severity of the disease increases, the frequency of these exacerbations also increases (Silverman, 2007). This increased frequency of exacerbations has been shown to have a negative impact on the sufferer’s quality of life (Seemungal et al. 1998).

Due to the heterogeneous nature of COPD disease exacerbations there is no definitive definition of an exacerbation (Sapey and Stockley, 2005). However an exacerbation can be defined as a sustained worsening of the patient’s condition from the stable state and beyond normal day-to-day variations, which is acute in onset and may warrant additional treatment (Burge and Wedzicha, 2003). It has been shown that exacerbations are the driving force in lung function decline in COPD sufferers and profoundly affect the quality of life (Hurst, 2011). During exacerbations, patients complain of breathlessness, wheeze, cough and sputum production (Wilson, 1998).
These exacerbations are a major contribution to the morbidity, mortality and healthcare costs associated with COPD (Sethi et al. 2007). There is much debate over the triggers for exacerbations with some groups suggesting bacterial infections (Patel et al. 2002), viral infections (Wedzicha, 2004), changes in the weather (Donaldson et al. 1999) or exposure to air pollutants (Ko and Hui, 2011) to be triggers. In a study to better understand whether COPD exacerbations that required hospitalisation were associated with inflammation, Papi and colleagues, found that viral and/or bacterial infections were detected in seventy eight per cent of exacerbations. It was also observed by them that the sufferers with exacerbations, and who had a detectable infection, had a greater impairment to lung function and also required a longer period of hospitalisation (Papi et al. 2006). It is important to better understand the triggers of exacerbations as seventy per cent of the annual cost of COPD is attributed to exacerbations that require hospitalisation (Sullivan et al. 2000).

Due to the heterogeneity of the disease it is likely that exacerbations could be triggered by all these factors, through either exposure to one or a combination of the triggers mentioned above. The key to treating each individual exacerbation could be to determine the trigger for that particular episode. Groups have looked into the presence of inflammatory biomarkers that could better predict the development of certain comorbidities (Thomsen et al. 2012) or aid treatment during exacerbations (Bafadhel et al. 2011).

1.1.1.3 COPD and Bacterial Infection

It has already been discussed that viral and bacterial infections are associated with a high proportion of exacerbations that require hospitalisation (Papi et al. 2006). In acute exacerbations of COPD with respiratory infection, the causal agent is more likely to be bacterial rather than viral (Anzueto et al. 2007). As well as being associated with exacerbations, microorganisms, particularly Streptococcus pneumoniae and non-typeable Haemophilus influenzae, often colonise the airways of COPD patients in a stable state (Hirschmann, 2000). Hill and colleagues noted that the bacterial load and species present in COPD patients between exacerbations contributed to the airway inflammation present, but stated that further investigation was needed to see if it had any association with increased exacerbation frequency or mortality (Hill et al. 2000).
Soon after this, it was shown, that there was a significant relationship between lower airway colonisation in the stable state and an increase in exacerbation frequency (Patel, \textit{et al.} 2002). This was described by Sethi \textit{et al.} who showed that there was a significantly increased risk of exacerbation frequency when \textit{S. pneumoniae} and \textit{Moraxella catarrhalis} were recovered from stable state patients (Sethi \textit{et al.} 2002).

It is known that during the progression of COPD, there is an increase in mucus production, depressed ciliary function and epithelial cell injury, which may allow bacteria not normally present in the airway below the larynx, to colonise the diseased epithelium (Read, 1999). The presence of bacteria in the lower airways during the stable state is often labelled as colonisation (Hill \textit{et al.} 2000, Patel, \textit{et al.} 2002). Sethi’s hypothesis (Sethi, 2000) is that this colonisation is actually a low-grade infection that induces chronic airway inflammation. Medical dictionaries define colonisation as, ‘the presence and multiplication of microorganisms without tissue invasion or damage’. Whereas, an infection is defined as ‘the invasion of the body by pathogenic microorganisms that reproduce and multiply, causing disease by local cellular injury, secretion of a toxin, or antigen-antibody reaction in the host’ (Mosby’s medical dictionary, \textit{8\textsuperscript{th} Edition}, 2009). Therefore as Sethi hypothesises (Sethi, 2000) and according to these definitions, the bacterial presence seen in the stable state of COPD should be classified as a low-grade infection, because it has been shown to contribute to airway inflammation (Hill \textit{et al.} 2000).

It is also thought that in these low-grade persistent infections there is a clinically stable state, with an underlying inflammatory condition that can be unbalanced by change in the \textit{in vivo} environment. These changes could be the host response to a new viral or a new bacterial infection or perhaps a strain shift in an existing colonising population (Hurst \textit{et al.} 2006, Mallia and Johnston, 2006, Sethi \textit{et al.} 2004). It has also been suggested that the increased risk of having an exacerbation is actually 4 – 8 weeks after acquisition of a new bacterial strain in the lower airways (Anzueto \textit{et al.} 2007).

There is much debate on the role bacteria play in the pathogenesis of COPD. It is generally accepted that a significant proportion of exacerbations are due to respiratory infections (Sullivan \textit{et al.} 2000). It is also accepted that bacterial colonisation in stable COPD leads to a functional decline in FEV\textsubscript{1} (Wilkinson \textit{et al.} 2003). One other role that
has been proposed is that lower respiratory infections, during childhood, impair lung growth and are reflected by a lower FEV₁ in adulthood (Murphy and Sethi, 1992). Sethi (Sethi, 2000) states that this alone would not cause symptomatic pulmonary disease but may predispose individuals to the effects of agents such as tobacco smoke.

Figure 1.2 shows that after an initial lung injury from an irritant such as tobacco smoke, the lungs become more prone to microbial infection. The acute cycle represents lungs that are more sensitive to infection and acquire a mucosal infection, which causes an exacerbation of symptoms and damages the lung further resulting in it becoming more prone to further mucosal infections. The chronic cycle is representative of the “vicious circle hypothesis” (Sethi 2009). Sethi (2000) first described this hypothesis and stated that once bacterial pathogens have been able to colonise the lower respiratory tract due to impaired mucociliary clearance caused by smoking, they persist by further impairing this clearance mechanism and inducing inflammation.

Figure 1.2 – A flow diagram showing the two distinct infection cycles of chronic obstructive pulmonary disease, (Sethi, 2009).
1.1.2 Asthma

Asthma, like COPD, is a major public health problem (WHO, 2008). Hallmarks of asthma include an intermittent wheeze and airway inflammation (Bel, 2004). Asthma is characterised by episodes of reversible airway obstruction, airway hyperresponsiveness (AHR) and IgE production (Meyts et al. 2006). It is most commonly caused by exposure to allergens; however there are also some non-allergic triggers too such as exposure to cold air or exercise (Stevenson and Birrell, 2011). It affects 300 million individuals worldwide (Hilty et al. 2010). In the UK 21% of children are diagnosed with asthma and there is a history of wheezing in one third of children and adults (BTS report, 2006).

It was generally believed that the airway inflammatory infiltrate in asthmatics was predominantly eosinophilic and that this eosinophilia was a hallmark of asthma (Bousquet et al. 2000). However, as with COPD, there is much debate in the classification of patients and several phenotypes / endotypes have emerged (Lotvall et al. 2011). The most distinct phenotype is still eosinophilic asthma, however not all asthmatics fall into this phenotype. There is a distinct subgroup of patients with mild to moderate asthma, who have predominantly neutrophilic inflammation (Green et al. 2002). This subgroup, which can also be classified as being persistently non-eosinophilic, has recently been shown to make up approximately half of mild to moderate asthmatics (McGrath et al. 2012).

In allergic asthma, it is thought that epithelial and dendritic cells detect an inhaled antigen, which causes them to become active (Hansel et al. 2013). As can be seen in Figure 3, the detection of antigen by dendritic cells initiates the release of the chemokines, CC-chemokine ligand 17 and 22 (CCL17 and CCL22 respectively). These then bind to the CC-chemokine receptor 4 (CCR4) to attract Th2 cells (Barnes, 2008a). In asthmatic airways the majority of CD4+ T cells in the airways are Th2 cells, whereas in healthy human airways the majority are Th1 cells (Meyer, 2008). Once activated, Th2 cells orchestrate a response by releasing the cytokines, interleukin (IL)-5, IL-13 and IL-4 (Bradding et al. 1995). The recruitment and activation of eosinophils in the airways of asthmatics is thought to contribute to the histopathological changes of the disease (Jacobsen et al. 2007). The growth, survival, differentiation and activation of...
eosinophils are regulated by the cytokine IL-5 in conjunction with the cytokine eotaxin, which regulates the distribution of airway eosinophils (Pope et al. 2001). The other key cytokine secreted by Th2 cells is IL-13, which causes a switch from IgM to IgE production (Borish and Steinke 2003). It is believed that IgE is one of the key effector molecules in the propagation and induction of allergic diseases like asthma (Eckel-Dorna and Niederberger, 2013).

Figure 1.3 shows that mast cells are activated by inhaled antigen (Barnes, 2008a). It is hypothesised that the infiltration of mast cells into the airway smooth-muscle is associated with the airway hyper-responsiveness seen in asthmatics (Brightling et al. 2002). This infiltration of mast cells into the bronchial epithelium does not occur in healthy airways, but is seen in the airways of asthmatics (Sanmugalingam et al. 2000). Mast cells also release several bronchoconstrictors including histamine, leukotriene C, D and E, as well as prostaglandin (Barnes 2008). The release of these mediators is thought to contribute to the variable nature of the bronchoconstriction seen in asthmatic airways (Bousquet et al. 2000).

Figure 1.3 – Inflammatory cytokines, mediators and immune cells involved in asthma, (Barnes, 2008a).
Inhaled corticosteroids are the established first-line treatment in adults and children with persistent asthma (Barnes and Adcock, 2003). The establishment of different endotypes of asthma is important, as it has been shown that if asthmatics have predominantly neutrophilic inflammation, then they respond poorly to inhaled corticosteroids (Green et al. 2002, McGrath et al. 2012). Classifying asthmatics by endotype could be beneficial, as physicians could deliver a treatment regime that had been tailored to specific phenotypes seen in asthma (Fahy, 2009). It is generally considered that the airflow limitation observed in asthmatics is largely reversible (Sciurba, 2004). However it has been seen that there is a relationship between neutrophilic inflammation in the airway and a persistent airflow limitation in asthmatics (Woodruff et al. 2001). This was also observed by Shaw et al. (2007), who reported that there was no bronchoconstriction in asthmatics with high levels of airway neutrophilia.

1.1.2.1 Asthma and Bacterial Infection

Unlike COPD, the role of bacteria in the disease progression of asthma is not well understood (Armann and Mutius, 2010). For many years it has been accepted that respiratory pathogens such as *H. influenzae* and *S. pneumoniae* are associated with an increase in airways obstruction in asthmatics (Pauwels et al. 1980). Specific serum IgE antibodies to *H. influenzae* and *S. pneumoniae* surface antigens have been measured in a cohort of 1,380 teenage asthmatics and it was seen that they were inversely associated with asthma risk in healthy atopic teenagers (Hollams et al. 2010). Hollams et al. (2010) suggested that the presence of IgE specific to the surface antigens of these pathogens could be a consequence of a strong exposure to these pathogens, which induces a strong Th1 response that must be counterbalanced by Th2 immunity.

It is known that in COPD, infectious exacerbations are a frequent cause of death and chronic low level infections are thought to contribute to a progressive decline in lung function, yet a systematic study of the organisms in airways disease was yet to be done (Hilty et al. 2010). Hilty et al. (2010) compared the microbiome of healthy subjects with the microbiomes of COPD and asthma sufferers. They saw that the microbiota of patients with asthma was more similar to that of COPD sufferers compared to healthy subjects. They also reported that the pathogenic *Haemophilus* spp. were more frequent.
in the airways of adult asthmatics and COPD sufferers than in the healthy controls. It has been seen in COPD sufferers that the microbiome diversity is reduced compared to healthy controls (Erb-Downward et al. 2011).

The ‘hygiene hypothesis’ proposes another role that pathogens such as *S. pneumoniae* and *H. influenzae* may play in the development of asthma (Okada et al. 2010). The hygiene hypothesis states that a reduced exposure to allergens in early life is solely implicated in the rise of allergic diseases (Platts-Mills et al. 2005). It has been shown that asthma prevalence in children at 5 years of age was significantly increased if they had been asymptomatically colonised in the hypopharynx with *S. pneumonia*, *M. catarrhalis* and *H. influenza* (Bisgaard et al. 2007). However, in Latin American countries with high infection rates, asthma prevalence is also high which would argue against the idea that microbial pressure early in life protects against atopic asthma by suppressing Th2 immune responses (Brooks et al. 2013).

1.2 Modeling respiratory disease *in vivo*

COPD and asthma are heterogeneous conditions, with patients displaying varied symptoms (Wedzicha, 2000). This represents a challenge to scientists to model all aspects of these diseases in one *in vivo* model, for the development of new compounds to treat COPD and asthma. Animal models are useful tools that allow studies to be carried out in the setting of an intact immune and respiratory system (Zosky and Sly, 2007). Current *in vivo* models sometimes offer poor translation to the human condition, novel compounds that have been proven to work in animals do not always show efficacy in human trials (Rennard et al. 2007). This could be due to a limited mechanistic understanding of the progression of COPD and asthma, as both are diagnosed by a series of physiological changes rather than a specific mechanistic change. However, as clinical understanding of both of these diseases improves better preclinical models can be developed (Stevenson and Birrell, 2011).

There are many published models of inflammation that mimic different aspects of these diseases (Brusselle et al. 2006). However no models have been described that have a chronic bacterial presence with in the lower airways (Stevenson and Birrell, 2011). Current *in vivo* models with the three most commonly associated pathogens with COPD
and asthma; *S. pneumoniae, H. influenzae* and *M. Catarrhalis*, are acute or transient and do not mimic the low level persistent bacterial presence seen in COPD sufferers in the stable state (Sethi, 2002).

In currently, published models of *S. pneumoniae*, mice either quickly succumb to infection (within 48 hours) or the infection is cleared within a few days (Chin et al. 2005, Drannik et al. 2004). These models are acute in nature, and depending on the genetic sensitivity of the strain of mouse, after intranasal infection with serotype 2 pneumococci, mice develop a severe pneumonia which progresses to septicaemia, resulting in animals having to be culled (Gingles et al. 2001). These acute models mimic pneumonia and sepsis, however do not provide a tool for understanding why in COPD a low-level persistent bacterial presence is observed in the lower airways and how this may contribute to COPD disease progression and an increase in exacerbation frequency (Patel et al. 2002, Stevenson and Birrell, 2011). Another model of pneumococcal infection has been developed for the assessment of antibiotic efficacy. For this, mice were infected with an aerosolised bolus of *S. pneumoniae* and within 2-4 days pneumonia developed, with mice having to be culled at 5-10 days post-infection (Tateda et al. 1996). The advantage of this was that there was a greater period of time where antibiotic efficacy could be assessed compared to the model used by Gingles et al. (2001). Another model that has been described with *S. pneumoniae* is a model of nasopharyngual colonisation, where pneumococci persist in the nasopharynx of mice for a month asymptomatically but no pneumococci is recovered from the lower airways (Kadioglu et al. 2002). This model does not mimic the colonisation of the lower airways seen in COPD as the pneumococci persist asymptomatically in the upper respiratory tract of mice with no pneumococci present in the lower respiratory tract. The ability of the pneumococcus to colonise the nasopharynx plays an important role in the ability of *S. pneumoniae* to cause an infection, as all pneumococcal diseases are believed to start with colonisation of the nasopharynx (McCool and Weiser, 2004).

There are many small animal models that mimic different aspects of COPD and asthma (Stevenson and Birrell, 2011). In brief, inflammatory models that mimic asthma are typically induced by exposing mice to a sensitising agent such as house dust mite (HDM) and looking for the expression of eosinophils and an increase in airway hyper-responsiveness. Whereas for COPD, animals are exposed to a noxious agent such as
smoke, to induce neutrophils and macrophages which cause an irreversible airflow obstruction and emphysema (Brusselle et al. 2006).

### 1.2.1 Models of inflammation typically associated with COPD

Modelling COPD in mice is complicated because it is a condition that consists of four anatomical lesions; emphysema, small airway remodelling, vascular remodelling with pulmonary hypertension, mucus overproduction and chronic bronchitis (Churg et al. 2008). There are also six factors which have been associated with functional decline in COPD, these are; smoking, exposure to occupational dusts, chemicals and air pollutants, infections and host predisposition (Ramsey and Hobbs, 2006).

For the past 50 years the proteinase-anti-proteinase imbalance hypothesis has been the main explanation for explaining COPD disease progression (Stevenson and Birrell, 2011). It was developed to explain why smokers with a deficiency of α1-antitrypsin were at increased risk of pulmonary emphysema (Laurell, 1963). The hypothesis suggested that smoking caused an increase in the numbers of neutrophils and macrophages in the lungs and these inflammatory cells released proteolytic enzymes (Abboud and Vimalanathan, 2008). The released proteases were not fully inhibited by anti-proteases and this led to degradation of lung connective tissue and in turn emphysema (Gross et al. 1965). The proteinase-anti-proteinase imbalance hypothesis led to two models of lung injury – one induced by lipopolysaccharide (LPS) and one induced by elastase.

The endotoxin, LPS, is a ubiquitous contaminant found in air pollution and cigarette smoke (Vernooy et al. 2002). When LPS is instilled in the airways of rodents there is a large influx of neutrophils. These cells are thought to be the driving force in COPD disease progression due to the release of neutrophil elastase. Models of LPS-induced injury can be classified as either acute or chronic. In the acute lung injury model, mice were exposed to a single intranasal challenge with LPS. This induced an influx of neutrophils, which caused pulmonary damage (Matute-Bello et al. 2008). Acute damage was seen two to four hours after instillation, with maximal damage observed between 24 and 48 hours post-instillation (Szarka et al. 1997). For chronic exposure to high levels of LPS, 5 µg/instillation of LPS was intratracheally given twice weekly for
twelve weeks. The LPS caused a chronic mononuclear inflammatory response and airway wall thickening (Vernooy et al. 2002). This model of lung injury is no longer used as a “disease” model for testing prospective COPD therapies as the inflammation it induces does not mimic the progressive, steroid-insensitive inflammation that is a hallmark of COPD (Fox and Fitzgerald, 2009). It is now used to investigate mechanisms, for example regulation of toll-like receptor (TLR)-mediated neutrophilia (Stevenson and Birrell, 2011).

For the elastase-induced model of emphysema Pancreatic Porcine Elastase (PPE) is dosed intranasally or intratracheally (Mahadeva and Shapiro, 2002). The instillation of elastase into the lung was shown to induce acute inflammation followed by increases in average airspace area that was maintained over a 10-week period (Birrell et al., 2005). Though the mechanisms behind this elastase emphysema are unclear, disease progression can still be observed after elastase activity can no longer be detected (Wright et al. 2008).

Neither of these lung injury models mimic the complex response to cigarette smoke. As cigarette smoking is considered to be the main etiologic factor in COPD, many groups have focussed on using this noxious stimulus to induce lung damage. There are many smoke protocols in use and, similar to LPS induced lung injury, investigations focus on either acute or chronic exposure to smoke. Chronic smoke inhalation models are often long-term (daily exposure for 12 weeks) because mice are resistant to its effects (Gaschler et al. 2009). For example, mice can tolerate the inhalation of two cigarettes per day for one year, with minimal effects seen on body weight (Mahadeva and Shapiro, 2002). Mice exposed to smoke four times daily for a period of 24 weeks had an emphysematous phenotype, which included pulmonary inflammation and airspace enlargement (Gosker et al. 2009). Dhami and colleagues (2000) also achieved an emphysematous phenotype in C57Bl/6 mice, but this time in an acute model of damage and a single exposure to smoke. As with models of elastase injury, this acute model of cigarette exposure had a transitory inflammation with effects being measured from 6 to 48 hours post-smoke inhalation. However, due to the shorter length of these acute models, compared to the chronic models of smoke-exposure, they are sometimes more practical to use. However the main disadvantage they have is that due to the short time frame no airway remodelling is observed (Bracke et al. 2007). Rats and guinea pigs
have also been used for models of smoke exposure, though in rats smoke exposure only causes minimal changes in pathology (Wright et al. 2008).

It is hypothesised that cigarette smoke is the major environmental factor responsible for the development of emphysema but as < 20% of smokers develop this disease, other risk factors, such as genetics, must be important. Guerassimov and colleagues (2004) investigated and characterised the effects of smoke-induced emphysema after an exposure period of six months in five different inbred strains of mouse. They found that the different strains had differing susceptibilities to smoke which resulted in differing Th1 cell inflammatory responses. For example, the AKR/J strain was classified as super-susceptible because after exposure to smoke significant weight loss was observed as well as the most intense pulmonary inflammatory response with significant increase in polymorphonuclear cells, alveolar macrophages and T cells. In contrast the NZW Lac/J strain was considered to be resistant because after exposure to the same smoke protocol, no weight loss was observed and the only change in the inflammatory cell populations observed was an increase in alveolar macrophages.

The two most commonly utilised models of lung injury used in rodents to better understand disease progression of COPD are exposure to smoke or PPE. Both models can be acute or chronic and this is dependent on the number of doses that are administered. Both induce lung injury but both are transient in nature and do not induce airway remodelling. Other groups are using mutant mouse strains and gene-knockout mouse lines to explore the involvement of immune factors. For example over-expression of the inflammatory mediator IL-13, leads to airway space enlargement (Stevenson and Birrell, 2011). However, like the smoke, LPS and elastase models, when run in isolation they only mimic some aspects of COPD and do not successfully mimic the heterogeneity of the condition.

1.2.2 Models of inflammation typically associated with asthma

Allergic asthma is the predominant form of the disease (Corren, 2013), so most in vivo models developed to mimic asthma aim to mimic allergen-induced lung inflammation and lung function changes (Stevenson and Birrell, 2011). The two most commonly used models require animals to be sensitised to either HDM extract or ovalbumin (OVA)
before challenging the airways (Bates et al. 2009). By sensitising and challenging the immune system with such agents, the aim is to provoke a Th2 driven allergic response (Zosky and Sly, 2007).

The standard approach for modeling asthma over the past 100 years has been to use OVA, to induce an allergic response in the lungs of rodents (Stevenson and Birrell, 2011). Research groups have optimised their own protocols; however the general principle remains the same, animals are sensitised with an intraperitoneal injection of OVA absorbed onto an adjuvant, such as aluminium hydroxide (Bates et al. 2009). This happens between one and three times, with each sensitisation being a minimum of a week apart. After sensitisations, mice are most commonly exposed to an aerosolised challenge of OVA, but this can also be administered intranasally or intratracheally (Nials and Uddin, 2008). By administering OVA in these ways it is possible to induce airway hyperresponsiveness (AHR), eosinophilic lung inflammation, mucus hyper-secretion and elevated IgE, which are all hallmarks of the human condition (Epstein, 2006).

There is also a genetic element involved in the development of asthma, which is seen in both animals and humans, (Brewer et al. 1999, Whitehead et al. 2003, Zhang et al. 1997). Whitehead and colleagues (2003) compared AHR and lung inflammation in nine strains of mouse exposed to OVA and found that strains differed in their response. They found that five of the inbred strains tested (129/SvIm, Balb/cJ, C3H/HeJ, DBA/2J and FVB/NJ) showed significantly increased AHR, whereas the other four strains (A/J, BTBR+(T)/tf/tf, CAST/Ei and C57BL/6J) did not. Another group has focused on comparing the OVA sensitisation and challenge model in three mouse strains; Balb/c, C57/BL6 and CBA/Ca mice (Tumes et al. 2007, Tumes et al. 2008, Tumes et al. 2009). In 2008, Tumes et al. (2008) noted that CBA/Ca mice mounted a robust IgE and OVA-specific IgE response, yet showed relatively little pathology in response to OVA (Tumes et al. 2008). They hypothesised that the minimal changes in pathology could be due to a rapid turnover of eosinophils in CBA/Ca mice compared to the other two strains. After further investigation, they reported that the rapid turnover of lung eosinophils was due to a low expression of survivin (Tumes et al. 2009). Survivin is involved in the regulating the maturation and function of cells in the immune system (Vassina et al., 2006 and Altznauer et al. 2004).
Much has been learnt from using the OVA sensitisation and challenge model because it induces a lung inflammation that is associated with asthma. However the induction of the allergic response has two major short comings. Firstly the antigen (OVA) is not naturally associated with lung inflammation in humans and secondly the method of sensitisation is an issue. To sensitise animals with OVA, the antigen has to be absorbed onto an adjuvant and be administered intraperitoneally. For an ideal model that mimics asthma, sensitisation to the antigen would be via direct exposure to the respiratory tract because this is believed to be how the disease develops in humans (Stevenson and Birrell, 2011). Nevertheless, OVA produces a robust model of allergic inflammation, which induces eosinophilic inflammation, increases serum IgE (specific to OVA), AHR and tissue remodelling (Birrell et al. 2010). Both of the disadvantages of the OVA induced allergic inflammation model can overcome by using HDM extract as an allergen (Stevenson and Birrell, 2011).

House dust mite is one of the most common allergens worldwide and has been associated with asthma (Willart et al. 2012). It is hypothesised that early exposure leads to sensitisation, and then repeated exposure leads to airway inflammation and bronchial hyperresponsiveness (Roche et al. 1997). In 2004, Cates et al. (2004) first described this model of allergy, in which HDM extract was administered intranasally to Balb/c mice daily for ten days. This treatment elicited increased total IgE, increased HDM specific IgG1, eosinophils and AHR to methacholine challenge. As sensitisation to allergen occurs via the respiratory mucosa it provides a platform for groups to better understand how sensitisation occurs. However the HDM allergen is complex and the commercially available HDM extracts all have different biochemical properties which affect the induced inflammation (Post et al. 2012).

1.2.3 The combination of several models of inflammation to better mimic asthma and COPD

Historically, models of inflammation have been treated as stand-alone models, but groups are starting to run models of inflammation in parallel to increase translatability to the human condition. One group have combined a model of *H. influenzae* infection with a model of OVA-induced allergic airway disease to mimic the neutrophilic airways
inflammation observed in severe asthma (Essilfie et al. 2012). Another group has combined a model of elastase and LPS-induced lung injury, to produce a model that mimicked COPD-like pathology features; goblet cell metaplasia, emphysema and decreased elastic recoil (Ganesan et al. 2010). The same group has taken this combination of elastase and LPS-induced lung injury and combined it with a viral insult with rhinovirus (Sajjan et al. 2009) and also a bacterial insult with nontypeable H. influenzae (Ganesan et al. 2012). Both research groups combined models of inflammation with a bacterial insult with H. influenzae. However in both studies the bacterial infection was acute with mice challenged with bacteria alone clearing the infection from the lower airways within a few days. An interesting point to note was that both groups showed that when the bacterial insult was combined with another insult of lung injury, viable bacteria persisted in the lower airways for a longer period of time compared to when it was given alone.

These are just examples from two groups, but there are other groups combining inflammatory models such as; smoke, cigarette smoke extract, virus, acute models of bacterial infection and allergic airways disease (Hardaker et al. 2010, Thorburn et al. 2012,).

1.3 *Streptococcus pneumoniae*

*Streptococcus pneumoniae* (the pneumococcus) is a Gram positive, bacterial pathogen which can cause pneumonia, septicaemia, otitis media, and meningitis (Kadioglu et al. 2000). This is a reflection in the ability of the pneumococcus to survive in many ecological niches within the human host (Weiser et al. 1994). One such niche is the mucosal surface of the upper respiratory tract (Kadioglu et al. 2008), where an individual strain could be carried asymptomatically for weeks to months before eventual clearance (Gray et al. 1980). It is thought that most humans will be colonised by pneumococci in the upper respiratory tract at some stage during their lifetime (Paton et al. 1993) but usually without symptoms. However the ability of the pneumococcus to colonise the nasopharynx plays an important role in pneumococcal diseased because all pneumococcal diseases are believed to start with colonisation of the nasopharynx (McCool and Weiser, 2004).
1.3.1 Evasion of the Host Response

*S. pneumoniae* exists in both an encapsulated and an un-encapsulated form, however only encapsulated strains have been isolated from clinical samples (Catterall, 1999). The ability for strains to produce capsular polysaccharide has been shown to be essential for virulence (Morona *et al.* 2006). Encapsulated forms have a biochemically distinct polysaccharide capsule that is generally covalently attached to the host cell wall distinguishes the serotype of the pneumococcus, currently there are 91 known serotypes (Weinberger *et al.* 2009). The serotype of pneumococcus is a determining factor in the ability for carriage and the cause of invasive disease (Hogberg *et al.* (2007) Kadioglu *et al.* (2008)). It has been shown that the capsule is not toxic, however the cell wall induces inflammation (Tuomanen *et al.* 1987). Magee and Yother (2001) hypothesize that a reduction in the amount of capsule is required for colonisation, as it would allow an increased exposure of surface molecules necessary for adherence.

The outer cell wall of the pneumococcus is composed of peptidoglycan, teichoic and lipoteichoic acids and phosphorylcholine (Bergman and Hammerschmidt, 2006). Attached to the cell wall, there are the cell surface proteins, which include lipoproteins as well as the choline-binding proteins (CBPs), PspA, PspC and autolysin (LytA), (Kadioglu *et al.* 2008). The choline-binding proteins are anchored to the cell wall by the phosphorylcholine residues (Bergman and Hammerschmidt, 2006). Any surface-exposed phosphorylcholine residues are recognised and bound by the C-polysaccharide reactive protein (CRP), which is part of the hosts innate defence system (Fischer, 2000).

The proteins pneumolysin and autolysin have been shown to play an important role in the virulence of *S. pneumoniae* (Canvin *et al.* 1995). Autolysin is an enzyme, which causes cell lysis, which in turn releases pneumolysin (Kadioglu *et al.* 2008). Pneumolysin is a 53 kDa cytosolic protein that is released upon cell lysis (Kanclerski and Mollby 1987). Pneumolysin has a direct role cytokine-mediated inflammation, as it is able to stimulate TNF α and IL-1β production from human monocytes (Kadioglu *et al.* 2004). Pneumolysin is toxic to most host cells and once released is able to activate complement (Catterall, 1999).
1.4 The Airway Microbiome

It is known that the upper respiratory tract has a diverse microbiome which can include pathogens such as *S. pneumoniae*, *H. Influenzae* and *M. catarrhalis* (Konno et al. 2006). In contrast, for many years it was considered that the lower respiratory tract of healthy individuals was sterile as no bacteria could be cultured; however studies using quantitative-PCR and pyrosequencing are challenging this view (Charlson et al. 2011). Independently of the presence of a lung microbiome in healthy individuals, it has been noted that COPD sufferers often have a low level bacterial presence in the lower airways and the three most common species of bacteria, recovered by culture techniques are *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* (Hirschmann, 2000). This bacterial colonisation of the lower airways has been associated with neutrophilic inflammation in the lower airways and it is thought that it could contribute to the progression of airway disease in COPD (Sethi et al. 2006). The presence of the *Streptococcus* species in the bronchial tree of COPD patients has also been confirmed by quantitative-PCR and pyrosequencing (Erb-Downward et al. 2011, Cabrera-Robio et al. 2012).

1.5 Aim of the Project

The low level bacterial presence has been linked with increased exacerbation frequency and severity in COPD and asthma. The overall aim of this project was to investigate whether the low level bacterial presence in the lower airways resulted in a more severe / exaggerated response to stimuli used to investigate COPD and asthma.

The initial aim of this project is to establish and characterise a murine model of long-term pulmonary infection with *S. pneumoniae*, in which bacteria persist in the lungs for a minimum of 28 days with an accompanying measurable inflammatory response. Once a model of persistent infection in the lower airways was established the next step would be to combine the infection model with other models of inflammation that mimic different aspects of COPD and asthma. This model could also then be used to investigate the triggers of exacerbations and see if those triggers incited a change in the bacterial number or inflammatory condition. The stand-alone model of persistent pneumococcal infection and its combination with other models of inflammation would provide a platform to test pre-clinical compounds.
2 Materials and Methods:

2.1 Materials:

All chemicals were supplied by Sigma-Aldrich, (Dorset, UK) and Oxoid, (Hampshire, UK) supplied bacteriological growth media, unless otherwise stated.

2.2 Culture Media for *S. pneumoniae*

**Blood Agar (BA):** 16 g Blood agar base medium was suspended in 400 ml distilled water, then autoclaved at 15 psi (103 kPa) for 20 minutes. Molten medium was allowed to cool to < 56 °C, and 20 ml (final concentration 5% v/v) defibrinated horse blood (Oxoid, Hampshire, UK) was added. For Blood Agar + Gentamicin (BA + Gentamicin) plates, 84 µl of Gentamicin (Stock concentration 10 mg/ml) (Sigma, UK) was added at the same time as the blood, to give a final concentration of 2 µg/ml.

**Brain Heart Infusion Broth (BHI):** 14.8 g Brain heart infusion medium was dissolved in 400 ml distilled water and then autoclaved at 15 psi (103 kPA) for 20 minutes.

**Todd Hewitt Broth + 0.5 % (w/v) Yeast Extract (THY):** 15 g Todd Hewitt broth (Becton Dickinson, UK) and 2.5 g yeast extract (Becton Dickinson, UK) were dissolved in 500 ml distilled water and then autoclaved at 15 psi (103 kPA) for 20 minutes.

**Phosphate Buffered Saline (PBS):**
Four phosphate buffered saline tablets (Oxoid, Hampshire, UK) were dissolved in 400 ml distilled water and then autoclaved at 15 psi (103 kPA) for 20 minutes.

2.3 Pneumococcal Strains:

All strains used, were obtained from the frozen bead stock collection of Laboratory 227 Department of Infection and Immunity, Leicester, UK. Table 2.1 below describes where each strain was obtained before being placed in the laboratory bead collection.
Table 2.1 – Origin of pneumococcal strains used.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>D39</td>
<td>2</td>
<td>National Collection of Type Culture (NCTC) 7466, London, UK</td>
</tr>
<tr>
<td>TIGR4</td>
<td>4</td>
<td>Clinical Isolate</td>
</tr>
<tr>
<td>BHN100</td>
<td>19F</td>
<td>Brigitte Normack (Karolinska Institute, Sweden)</td>
</tr>
<tr>
<td>BHN191</td>
<td>6B</td>
<td>Brigitte Normack (Karolinska Institute, Sweden)</td>
</tr>
<tr>
<td>ST138</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BHN418</td>
<td>6B</td>
<td>Brigitte Normack (Karolinska Institute, Sweden)</td>
</tr>
<tr>
<td>ST138</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LgSt215</td>
<td>19F</td>
<td>Herminia de Lencastre (Instituto de Tecnologia Química e Biológica (ITQB), Universidade Nova de Lisboa, Oeiras, Portugal</td>
</tr>
</tbody>
</table>

2.3.1 Viable Counting

Viable counting was carried out using the Miles and Misra method (Miles and Misra, 1938). Using a round-bottomed microtitre plate (Nunc, UK), cultures were ten-fold serially diluted in sterile PBS. Three 20 µl drops, were plated onto a BA plate (in duplicate) and incubated at 37 °C in a candle jar overnight. Colonies were counted at the dilution with 30-300 visible colonies.

2.3.2 Stocks of Non-passaged S. pneumoniae

From Laboratory 227 bead stocks, the strain required was streaked to single colonies onto a BA plate and incubated at 37 °C in a candle jar overnight. Optochin (ethylhydrocupreine hydrochloride) sensitivity was confirmed by placing an optochin disc (Oxoid, Hampshire, UK) onto the initial site of streaking. Optochin sensitivity is a simple diagnostic test to distinguish S. pneumoniae from Streptococcus viridans, which has been shown to be consistently optochin resistant (Bowers and Jeffries, 1955). The following day, a sweep of 5 – 10 colonies were inoculated into 10ml BHI broth in a Universal tube and incubated statically for 16 – 18 hours at 37 °C. After incubation, the culture was centrifuged at 1500 g for 15 minutes at room temperature. The supernatant was then discarded and the remaining pellet re-suspended in 1 ml BHI serum broth (80 % (v/v) BHI broth + 20 % (v/v) filtered foetal bovine serum (Sigma, UK). Seven hundred microlitres of the suspension was added to 10 ml BHI serum broth and OD$_{500\text{nm}}$ was adjusted to ~ 0.70 and the culture was incubated statically at 37 °C.
until the OD$_{500nm}$ was ~ 1.60. 0.5 ml aliquots were put into sterile cryotubes and frozen at -80 °C (Canvin et al. 1995). After 48 hours, the viability and optochin sensitivity were confirmed as described in section 2.3.1.

For strain LgSt215 a different protocol was used. The strain was taken from Laboratory 227 bead stocks, streaked to single colonies onto a BA plate and incubated at 37 °C in a candle jar overnight. Optochin sensitivity was confirmed by placing an optochin disc (Oxoid, Hampshire, UK) onto the initial site of streaking. Once optochin sensitivity was confirmed, colonies from overnight growth on BA plates were suspended in 3 ml BHI until OD$_{600nm}$ = 0.2. From this suspension 100 µl was taken and inoculated into 10 ml fresh BHI and incubated statically at 37 °C until OD$_{600nm}$ = 0.6 – 0.8 which correlated with mid – late exponential phase. Glycerol was then added to give a final concentration of 10 % v/v and aliquots of 750 µl were put into sterile cryotubes and frozen at -80 °C. After 48 hours, the viability and optochin sensitivity were confirmed as described in section 2.3.1. This protocol was adapted from Hyams et al. (2010).

2.4 Murine Strains

Female outbred MF1, inbred Balb/cOlaHsd (Balb/c), inbred CBA/CaOlaHsd (CBA/Ca) and inbred C57BL/6 mice were obtained from Harlan Olac (Bicester, UK); female outbred MF1 mice were also obtained from Charles River (Kent, UK). Mice were used when they were at least 9 weeks old but less than 20 weeks. Before use, mice were kept for at least one week, under standard conditions in the University of Leicester’s Division of Biomedical Services, with access to water and food ad libitum.

2.4.1 Disease Sign Scoring

All mice were scored for signs of disease; the three signs monitored were a hunched posture, piloerection and lethargy. As laid out in the Home Office Project Licence (80/2111 and 60/4327) each sign was scored as either; 0 = no sign, 1+ = starting to show sign or 2+ = clearly showing the sign (Morton and Griffiths, 1985). For assessing disease sign progression, disease severity was graded on a scale of 0 – 7, with 0 being normal and 7 having to be culled. Scores 1 - 6 were cumulative depending how many signs were observed, so a score of 1 would be starting to be hunched or showing piloerection on the haunches. In accordance with
the Home Office Licence, all animals that became 2+ lethargic were culled before they became moribund.

2.4.2 Passage of *S. pneumoniae* Through Animals

*Streptococcus pneumoniae* was passaged by a modification of the method described by Canvin *et al.* (1995). The strain required was streaked for single colonies onto a BA plate and incubated at 37 °C in a candle jar overnight. Optochin sensitivity was confirmed by placing an Optochin Disc (Oxoid, Hampshire, UK) onto the initial site of streaking. The following day a sweep of 5-10 colonies were inoculated into 10 ml BHI broth in a Universal tube and incubated statically overnight at 37 °C. After, incubation the culture was centrifuged at 1500 g for 15 minutes at room temperature. The supernatant was then discarded and the remaining pellet re-suspended in 5 ml sterile PBS. Under the Personal Licence 40/9645 two MF1 mice (Harlan Olac, Bicester, UK) were injected intraperitoneally with 100 µl of the suspension. As soon as possible after completion of infections, viable counts were performed (Section 2.3.1).

After 22-28 hours, mice were scored for signs of disease and mice that were 2+ Hunched, 2+ Starry were deeply anaesthetised with 5 % (v/v) fluothane (AstraZeneca, Macclesfield, UK) with 1.6 – 1.8 L O₂/minute using a Fluotec 3 calibrated vaporiser (Cyprane). Blood was collected by cardiac puncture and the animal was exsanguinated while under terminal anaesthesia. For each mouse, 50 µl blood was inoculated into 10 ml BHI broth in a Universal tube and incubated statically for 16 – 18 hours at 37 °C (OD₅₀₀nm >1.4). After incubation, the supernatant was removed from the sedimanted red blood cells, placed into a fresh Universal tube and centrifuged at 1500 g for 15 minutes at room temperature. The supernatant was then discarded and the remaining pellet re-suspended in 1 ml BHI serum broth (80 % (v/v) BHI broth + 20 % (v/v) filtered foetal bovine serum (Sigma, UK)). Seven hundred microlitres of the suspension was added to 10 ml BHI serum broth and OD₅₀₀nm was adjusted to ~ 0.70 and the culture was incubated statically at 37 °C until the OD₅₀₀nm was ~ 1.60. Then 0.5 ml aliquots were made in sterile cryotubes and frozen at -80 °C.

After 48 hours the viability and optochin sensitivity were confirmed as previously stated in section 2.3.1. Aliquots could be kept frozen for a maximum of 3 months, with viability being confirmed periodically.
2.4.3 Preparation of Infectious Dose

As described by Kadioglu et al. (2000), a single aliquot from the – 80 °C freezer was thawed (in the hand), and then centrifuged at 12000 g for 3 minutes in a benchtop microcentrifuge. The supernatant was removed and the pellet re-suspended in 400 µl PBS. As the viability of the aliquot had been determined previously, as described in Section 2.3.1 set volumes were taken to make up the required concentration for infection.

2.4.4 Intranasal Infection

As described by Kadioglu et al. (2000), mice were lightly anaesthetised with 2.5 % (v/v) fluothane (AstraZeneca, Macclesfield, UK) in 1.6 – 1.8 litre O₂/minute, using a Fluotec 3 calibrated vaporiser (Cyprane). Once anaesthetised mice were scuffed and held upright. The required dose of S. pneumoniae was then placed in a series of droplets into the nostrils of the anaesthetised animal. Animals were placed onto their back in a new cage and allowed to recover from the anaesthesia (Mitchell and Paterson, 2007).

2.4.5 Virulence Test of Passaged Stocks of S. pneumoniae

From a frozen aliquot of passaged stock, a suspension of 2 x 10⁷ CFU / ml in PBS was prepared. 50 µl of this suspension was administered intranasally to 5 x MF1 (Harlan Olac, Bicester, UK) mice. Disease signs were monitored for 72 hours. For the stock to be classed as ‘virulent’, the mice had to become 2+ lethargic within 44 – 52 hours. Once mice became 2+ lethargic, they were culled and the time was recorded.

2.5 Collection and Processing of In Vivo Samples

2.5.1 Culling of Animals

At pre-determined time points post-infection, or once mice became 2+ lethargic, mice were culled. If large amounts of blood were required for experiments and / or the trachea did not need to be intact, mice were deeply anaesthetised with 5 % (v/v) fluothane (AstraZeneca, Macclesfield, UK) in 1.6 – 1.8 L O₂/minute, using a Fluotec 3 calibrated vaporiser (Cyprane). Death was confirmed by cervical dislocation. If the trachea needed to remain intact, mice were terminally anaesthetised with an intraperitoneal injection of 250 µl Pentoject (Pentobarbitone Sodium 20 % w/v) (AnimalCare Ltd, York, UK). Death was confirmed by non-responsiveness to noxious stimuli (hind paw pinch) and lack of heartbeat.
2.5.2 Tissue Harvest

2.5.2.1 Collection of Sera

The method of collection was dependent on the method of culling. Blood was either collected by cardiac puncture in which the animal was exsanguinated while under terminal anaesthesia with 5 % (v/v) flouthane (AstraZeneca, Macclesfield, UK) in 1.6 – 1.8 L O₂/minute, using a Fluotec 3 calibrated vaporiser (Cyprane, UK). Or mice were terminally anaesthetised with Pentoject and post-mortem, blood was collected from the vena cava using a 1 ml Insulin Syringe (U-100 insulin (Terumo, UK)). For both techniques, blood was collected into a 1.5 ml eppendorf and if serum was required; blood was allowed to clot at room temperature, then centrifuged at 7000 g for 10 minutes. Serum was collected and stored at -80 °C.

2.5.3 Collection of Nasopharynx and Lungs

After death, lungs and nasopharynx were removed and placed into a pre-weighed volume of sterile PBS and the weight of the tissue was determined. Tissues were homogenized for 15 seconds with a tissue disrupter (IKA-Werke, UK). Viable counts (as previously described in section 2.3.1) were determined in the tissue homogenates (Canvin et al. 1995).

2.5.4 Collection of Bronchoalveolar Lavage Fluid (BALF)

At pre-determined time points post-infection, mice were terminally anaesthetised with an intraperitoneal injection of 250 µl Pentoject. Death was confirmed by non-responsiveness to noxious stimuli (hind paw pinch) and lack of heartbeat. The trachea was exposed and a cannula was placed into the trachea via a small incision and held in place with a suture. Three hundred microlitres of PBS were flushed into the lungs with a 1 ml syringe and collected again with the same syringe and placed into a pre-weighed 1.5 ml eppendorf. This was repeated three times and kept at room temperature. To determine the amount of BALF recovered the eppendorf was weighed before and after sample collection.

2.5.5 Differential Cell Counts

BALF samples were centrifuged at 1850 g for 10 minutes and supernatants were placed at -80 °C for cytokine analysis (as described in section 2.5.4). The pellet was re-suspended in 200 µl PBS and 50 µl of suspension was pipetted into a plastic chamber and placed into a cytopsin slide centrifuge (Shandon Southern Products Ltd, UK). Slides were centrifuged at 160 g for 3
minutes and then were allowed to dry overnight before staining with a REASTAIN Quick-Diff kit (Reagena Ltd, Toivala, Finland) according to the manufacturer’s instructions. Slides were dipped 10 times in Quick-Diff FIX solution (100 % methanol), then dipped 6 times in Quick-Diff RED solution (0.12 % (w/v) Eosin) and finally dipped 10 times in Quick-Diff BLUE solution (0.09 % (w/v) Azur II, 5 % (w/v) Glycerol). Once dry, a coverslip was mounted onto the slides with DPX (Fisher, Loughborough, UK).

2.5.6 Histopathology

2.5.6.1 Sample collection

At pre-determined time points post-infection, mice were terminally anaesthetised with an intraperitoneal injection of 250 µl Pentoject (20 % (w/v) sodium pentobarbitone (AnimalCare, UK)). Death was confirmed by non-responsiveness to noxious stimuli (hind paw pinch) and lack of heartbeat. The lungs were distended with 10 % (v/v) neutral buffered formalin (Sigma, UK) for histopathological analysis of inflammation. A cannula was placed into a pre-exposed trachea and the lungs were inflated with approximately 400 µl of 10 % (v/v) neutral buffered formalin via a 5 ml syringe, before tying the lungs off. The lungs were carefully extracted from the thorax and placed into 5 ml of 10 % (v/v) neutral buffered formalin. The spleen was also removed and placed into 2 ml of 10 % (v/v) neutral buffered formalin.

2.5.6.2 Sectioning

Samples were kept in 10 % v/v neutral buffered formalin (Sigma) for a minimum of 24 hours. Samples were then transferred to the Pathology Department, AstraZeneca R & D, Charnwood, UK and processed using their standard protocols. The tissue was then dissected into slices approximately 3mm thick. Tissues were embedded overnight with a Leica Tissue Processor (LEICA TP 1050 fully enclosed vacuum tissue processor). Tissues were dehydrated through graded alcohols and into paraffin wax, then embedded on a Leica Histoembedder, positioned and set into a paraffin wax block. Blocks were stored at room temperature. 4 µm sections were taken with a Leica Jung RM2155. Cut sections were floated on water (37°C), and transferred to charged slides. The slides were dried overnight at 45 °C in an oven.

2.5.6.3 Haemotoxylin and Eosin Staining

Slides were de-waxed by placing in xylene for a minimum 20 minutes and then rehydrated. To rehydrate, slides were taken through graded alcohols (100 % ethanol, 100 % and 70 % industrial methylated spirits (IMS)). Slides spent a minimum of 5 minutes in each alcohol and
were then placed into water. The slides were stained with Gills Haematoxylin for 1 minute (Pioneer Research Chemicals Ltd, UK). Slides were then washed in running water for around 10 minutes to “blue” the haematoxylin. The slides were submerged in eosin Y (high purity, Acros Organics, New Jersey, USA) for approximately 2 minutes, washed briefly in running water and cleared through graded alcohols to xylene. To rehydrate slides were placed for 5 minutes at room temperature in each of the following alcohols; 70 %, 100 % IMS and 100 % Ethanol. A coverslip was then mounted with Hystomount (Hughes and Hughes, Somerset, UK) onto each section.

### 2.5.6.4 Gram Staining

As described in Section 2.5.5.3 slides were de-waxed in xylene and rehydrated through graded alcohols. Slides were stained with crystal violet (Sigma, UK) for 3 minutes, washed with running water then in the mordant, iodine (Sigma, UK) for 5 minutes. The iodine was washed off with running water and the slides were blotted. Slides were rapidly decolourised with acetone (Sigma), dipped in water and then counterstained for 10 seconds with safranin O (Sigma). Slides were washed briefly in water and rapidly dehydrated through graded alcohols (70 %, 100 % IMS and 100 % Ethanol) to xylene and a coverslip was mounted with DPX (Fisher Scientific, Loughborough, UK) onto each section.

### 2.5.6.5 Immunohistochemistry, Staining Against the Antigen CD3

Slides were de-waxed in xylene and rehydrated through graded alcohols to water (as described in 2.5.5.3). After comparing different solutions, optimum antigen retrieval was found to be microwaving for 30 seconds in high pH antigen retrieval solution (1 mM EDTA, pH 9.0 (Sigma, UK)), slides were then left in the solution for 10 minutes. Slides were then rinsed in distilled water and a PAP pen (Dako, Ely, UK) was used to form a hydrophobic ring around the tissue. Slides were quenched for 5 minutes at room temperature for in 1 % v/v Hydrogen Peroxide (Fisher, Loughborough, UK) in methanol (Fisher, Loughborough, UK). Slides were washed three times with wash buffer for 2 minutes at a time (PBS + 1 % w/v BSA + 0.05 % v/v Tween). Slides were blocked with 20 % v/v goat serum (Dako, Ely, UK) in Antibody diluent (1 % w/v BSA (Sigma, UK) in PBS (Oxoid, UK)) for 20 minutes at room temperature. Excess protein block was removed by tipping and 2 µg / ml anti-CD3 antibody (AbCam, Cambridge, UK) in antibody diluent was added for 1 hour and slides were placed in a humidity chamber at room temperature. Isotype control was normal rabbit IgG (Vector Laboratories, Peterborough, UK), at the same dilution as the primary antibody. After 1 hour slides were washed three times for 2 minutes in wash buffer. Slides were incubated for 30
minutes with 0.1 % v/v biotinylated anti rabbit (Vector Laboratories, Peterborough, UK) in antibody diluent. Slides were washed as before and Vectastain ABC kit (Vector Laboratories, Peterborough, UK) made up as per manufacturer’s instructions was added to each section for 20 minutes. Slides were washed again in wash buffer as before and 3, 3’ Diaminobenzidine (DAB) chromogen (Vector Laboratories, Peterborough, UK) was added. DAB was made fresh as per manufacturer’s instructions, slides were incubated for 6 minutes and the reaction was stopped with wash buffer. Slides were counterstained with Haemotoxylin (Pioneer Research Chemicals Ltd) for 8 seconds, washed in running water for 10 minutes to “blue” and then dehydrated through alcohols to xylene. Once slides were in xylene they were mounted with DPX mountant (Merck) and a coverslip was placed on top.

2.5.7 FACS analysis of In Vivo Samples

2.5.7.1 Preparation of BALF samples

BALF samples were collected as described in section 4.5.3. BALF samples were centrifuged at 1850 g for 10 minutes; pellets were re-suspended in 500 µl FACS running buffer (PBS containing 2 mM EDTA (Sigma, UK) and 0.5 % w/v BSA (Sigma, UK)).

2.5.7.2 Preparation of Whole Lung samples

At necropsy, lungs were placed in 2 ml Hank’s buffered salt solution (HBSS) (Sigma, UK) containing 1 mg / ml Collagenase D (Roche) and chopped into approximately 2 mm² cubes. Tissue was then incubated at room temperature for approximately 90 minutes. Lungs were passed through a 70 µm nylon cell strainer (BD Falcon) with 10 ml complete rodent mix (RPMI 1640 (Gibco) containing 5 % v/v FCS (Sigma, UK)). Cells were centrifuged at 1500 g for 5 minutes. The supernatant was then discarded and the pellet was re-suspended in 4 ml BD PharmLyse (BD Bioscience) and incubated in the dark at room temperature for 5 minutes to lyse erythrocytes. Reaction was quenched with 11 ml PBS and cells were then centrifuged at 1500 g for 5 minutes. The supernatant was discarded and pellets were re-suspended in 750 µl FACS running buffer (PBS containing 2 mM EDTA (Sigma, UK) and 0.5% w/v BSA (Sigma, UK)) if stained the same day. Samples being analysed at a future point were re-suspended in 1ml RPMI (Gibco) containing 15 % v/v FCS (Sigma, UK) and 10 % v/v DMSO (Sigma, UK) and stored at -80 °C.
2.5.7.3 Staining of Samples

One hundred microlitres of each sample was incubated with an equal volume of staining mix. Staining mix was a 1 to 100 dilution of Fc Block (anti-CD16/32 antibody to reduce non-specific binding, BD Bioscience, UK) and a range of conjugates from BD Biosciences, eBioscience and R & D systems, in FACS running buffer (PBS containing 2 mM EDTA (Sigma) and 0.5 % w/v BSA (Sigma)). The conjugates used were dependent on which cells were being stained. Cells, conjugates and Fc Block were incubated at room temperature, in the dark, for 20 minutes. After incubation, 1 ml of FACS running buffer was added and cells were centrifuged at 1500 g for 5 minutes. Supernatants were discarded and samples were re-suspended in FACS running buffer. Samples were analysed with a Cytomics FC500 FACS machine (Beckman Coulter).

2.5.8 Cytokine Analysis of In Vivo Samples by Multiplex Analysis

The MILLIPLEX Mouse Cytokine / Chemokine panel (Millipore) was used to analyse BALF and serum samples. The panel was used according to the manufacturer’s instructions. The microtiter filter plate was pre-wetted with 200 µl of wash buffer (provided in the kit at a 10 x concentration and diluted to a 1 x working concentration with deionised water). Plate was incubated for 10 minutes at room temperature on a plate shaker. Wash buffer was removed by vacuum, 25 µl of assay buffer was added to all wells followed by 25 µl of standard or sample, 25 µl of premixed antibody-immobilized beads were added to all wells. Plate was then incubated under agitation at room temperature for 2 hours. Liquid was removed by vacuum and each well was washed twice with 200 µl wash buffer. Detection antibodies (25 µl) were added to each well and allowed to incubate for 1 hour at room temperature with agitation. After incubation, 25 µl Streptavidin-Phycoerythrin was added and incubated under agitation at room temperature for 30 minutes. Well contents were removed by vacuum and washed twice with 200 µl wash buffer. Sheath fluid (150 µl) was added to all wells to re-suspend the beads, the plate was then analysed with a Luminex 100 IS.

2.5.9 Cytokine Analysis of In Vivo Samples by ELISA

Duoset Mouse ELISA kits (R & D systems) were used to measure the levels of cytokines present within samples. Kits were used according to the manufacturer’s instructions. Ninety-six well Maxisorp ELISA plates (Nunc, UK) were coated with 100 µl capture antibody
diluted in PBS (concentration specific to kit used) and left to incubate at room temperature overnight. Wells were washed 3 times with 300 µl wash buffer (PBS containing 0.05 % v/v Tween 20 (Sigma). Plates were then blocked for 1 hour with 300 µl reagent diluent (PBS containing 1 % w/v BSA (Sigma)). Wells were washed 3 times with 300 µl wash buffer, 100 µl of standards and unknown samples were added to wells. BALF samples were used neat and serum samples were diluted 1 in 40 in reagents diluent. For each plate, standard curves were prepared in duplicate, according to the manufacturer’s instructions. Samples and standards were incubated for 2 hours at room temperature. Wells were washed as previously described and 100 µl detection antibody diluted in PBS (concentration specific to kit used) was added and left to incubate at room temperature for 2 hours. After incubation with detection antibody wells were washed and 100 µl Streptavidin – HRP (diluted 1 to 200 in reagent diluent) was added and left to incubate in the dark. After 20 minutes wells were washed and 100 µl of BD OptEIA TMB substrate solution (BD Bioscience) was added and after 20 minutes the reaction was stopped using 2 N H₂SO₄. Absorbance was then read at 450 nm using a Microplate reader (BioRad Model 680) and raw data were initially analysed using the software Microplate Manager 5.2.1.

2.5.10 Analysis of Serum Samples by Direct ELISA for IgG levels

Direct ELISA was performed to analyse the titre of non-specific IgG antibody. Ninety-six well Maxisorp ELISA plates (Nunc, UK) were coated with serum samples starting with a dilution of 1:50 serially diluted by two eleven times and incubated overnight at 4˚C. The following day plates were washed twice with PBS and 0.05% v/v tween, and plates were then blocked for 1 hour with 300 µl reagent diluent (PBS containing 1 % w/v BSA (Sigma)). After blocking plates were washed twice with PBS and 0.05% v/v tween and 100 µl of the goat anti- mouse IgG biotinylated (eBioscience) secondary antibody was added at 1:5000 dilution in reagent diluent to each well. The plates were then incubated for 1 hour at room temperature and then washed twice with PBS and 0.05% v/v Tween. Then 100 µl Streptavidin – HRP (R & D systems, UK), (diluted 1 to 200 in reagent diluent) was added and left to incubate in the dark. After 20 minutes wells were washed and 100 µl of BD Opt EIA TMB substrate solution (BD Bioscience) was added and after 5 minutes the reaction was stopped using 2 N H₂SO₄. Absorbance was then read at 450 nm using a Microplate reader (BioRad Model 680) and raw data was initially analysed using the software Microplate Manager 5.2.1.
2.5.11 Post mortem Gravimetric Analysis of In Vivo Samples to assess Pulmonary Oedema

At pre-determined time points post infection, or once mice became 2+ Lethargic, mice were deeply anaesthetised with 5 % (v/v) fluothane (AstraZeneca, Macclesfield, UK) with 1.6 – 1.8 L O₂/minute using a Fluotec 3 calibrated vaporiser (Cyprane). Blood was collected by cardiac puncture and the animal was exsanguinated while still under anaesthesia. Post death, lungs were excised, cleared of all extra pulmonary tissue and placed into a pre-weighed container and the total wet weight of the tissue was determined. Lungs were then placed at 60 °C and dried, after 24 and 48 hours weights of the lungs were determined to find the total dry weight. Pulmonary oedema was expressed as the ratio of total wet weight to total dry weight.

2.5.12 Tracheal Ciliary Function

At pre-determined time points post-infection, mice were terminally anaesthetised with an intraperitoneal injection of 250 µl Pentoject (20 % (w/v) sodium pentobarbitone (AnimalCare, UK)). Death was confirmed by non-responsiveness to noxious stimuli (hind paw pinch) and lack of heartbeat. Trachea’s were exposed and embedded in 4 % UltraPure low melting point agarose (Invitrogen, Paisley, UK) in situ. Embedded trachea’s were excised and placed into a petri dish filled with low melting point agarose and placed on ice. The trachea’s were then placed onto a temperature controlled vibrotome (Campden Instruments, Leicester, UK) and cut into 300 µm sections. Sectioned rings were placed into Gibco Medium 199, pH 7.3 (Invitrogen, Paisley, UK) containing antibiotic solution (streptomycin 50 µg / ml, penicillin 50 µg / ml, Gibco, UK) until they were ready to be analysed.

To view cilia the method described by Chilvers and O’Callaghan (2000) was used. In this method tracheal sections were suspended in a chamber formed by the separation of a cover slip and glass microscope slide by two adjacent cover slips. The microscope slide was then placed onto a heated stage (37 °C) of a Leica DMLB Motion Pro X4 microscope mounted on an anti-vibration table (Wentworth Laboratories Ltd, Sandy, UK).

For each ring tracheal ring the morphology was viewed using an X10 interference contrast lens and the proportion of ciliated cells and intact epithelium were estimated. Ciliated edges were then examined using an X100 interference contrast lens and edges were recorded using a digital high-speed camera at a rate of 500 frames per second. To assess the number of beating
cilia (motility index) the number of motile ciliated cells, number of ciliated cells and total intact cells were counted in a 512 x 200 pixel field. To determine the average cilia beat frequency (CBF) the cilia beat frequency of cells in a field of view was assessed individually. To do this the number of frames required for five full sweeps of a clearly observed ciliary tip were counted. This was converted to CBF by a simple calculation (CBF = 250 / (number of frames for 5 beats) x 5), (Smith et al, 2013).

2.5.13 Lung Function

Lung function was assessed using the eSpira Forced Manoeuvres systems (EMMS, Borden, UK). Mice were anesthetized with an intraperitoneal injection of anaesthetic solution containing ketamine (100 mg/kg; Fort Dodge Animal Health, Southampton, UK) and medetomidine (0.25 mg/kg; Dechra Veterinary Products Ltd., Shrewsbury, UK) and the trachea cannulated. Mice were allowed to breathe spontaneously and were monitored in a whole body plethysmograph with a pneumotachograph connected to a transducer (EMMS). Transpulmonary pressure was assessed via an oesophageal catheter (EMMS). Mice were pre-treated with a volume history manoeuvre whereby the lungs were inflated to 20 cmH2O for 1000 ms to improve airway patency prior to the start of the manoeuvres. Baseline lung function was measured for 1 minute; baseline airway resistance was calculated using the eDaq software. Three semi-automatic manoeuvres were performed in triplicate per mouse: forced expiratory volume (FEV), functional residual capacity (FRC) and quasi-static pressure volume curves. The FEV manoeuvre recorded FEV at 25 ms (FEV25), FEV40, FEV50, FEV60 and FEV75, forced peak expiratory flow (PEF) and forced vital capacity. FRC was determined with Boyle’s Law FRC. Total lung capacity (TLC), residual volume (RV), tidal volume (TV) and compliance were calculated from quasi-static pressure volume curves.

2.6 Statistical Analyses

GraphPad Prism software version 6 was used to analyse all data. Analysis of survival graphs was carried out using the Mantel-Cox test. The non-parametric Mann-Whitney test was used to compare differences between two sets of data, when more than two sets of data were being analysed the non-parametric Kruskal-Wallis with a Dunn’s post-test was used. Results were considered significant when p was < 0.05 and error bars in all Figures show the standard of the mean (SEM).
3 Establishment of model of persistent pneumococcal infection:

The aim of the project was to establish a model of long-term *S. pneumoniae* colonisation of the lower airways of mice. The successful model would consistently have viable *S. pneumoniae* present in the lower airways for a minimum of four weeks post-infection, with few or no outward signs of disease, but there would be an accompanying cellular inflammatory response. This would occur in over 70% of mice after a single intranasal infection. It was thought that if the infection persisted for four weeks, a stable phenotype would be observed that would mimic what Sethi (2000) defined as colonisation. To develop this model, four factors were investigated; dose volume, number of viable pneumococci in the dose, serotype of *S. pneumoniae* and strain of mouse.

3.1 Investigation of the effects of dose volume on pneumococcal virulence

Initially outbred MF1 mice and the pneumococcal serotype 2, strain D39 were chosen, because this was the combination that was proven to work in the previously established acute model of pneumococcal infection (Gingles *et al.* 2001). In this acute model, a dose of $1 \times 10^6$ CFU strain D39 suspended in 50 µl of PBS was administered intranasally to outbred MF1 mice. Subsequently there was progressive increase in the level of disease signs observed, until animals had to be culled between 48 – 72 hours post infection (Canvin *et al.*, 1995).

3.1.1 Severity of pneumococcal infection can be altered by changing the volume in which the dose is administered

The first question asked was whether the lethality of the challenge could be changed by decreasing the volume in which the dose was administered. Outbred MF1 mice were intranasally challenged with $1 \times 10^6$ viable pneumococci (strain D39) suspended in 20, 30, 40 or 50 µl of PBS. Percentage survival was assessed and the number of pneumococci present in the blood, lungs and nasopharynx were enumerated at the predetermined time point of 7 days post-infection.

As can be seen in Figure 3.1A, there was a direct correlation between the dose volume and the dose lethality. By lowering the dose volume, the number of animals that
survived increased, even though the number of infecting bacteria was unchanged. It was also interesting to note that this was also reflected in the time of first appearance of signs of disease. As seen in Figure 3.1B, when the dose was administered in 20 µl it took 66.5 hours for mice to show signs of disease, whereas when the dose was administered in 40 µl mice showed signs of disease after 26 hours.

![Figure 3.1](image)

**Figure 3.1** – Survival of MF1 mice intranasally infected with $1 \times 10^6$ CFU *S. pneumoniae* strain D39 in 20, 30, 40 or 50 µl PBS (n = 5). A – percentage survival, B – average disease sign rating for the first 91 hours post-infection.

To better understand why there was a delay in disease progression, when the same number of pneumococci were intranasally infected in a smaller dose volume, the experiment described in Figure 1 was repeated. Mice were culled immediately after infection and the pneumococcal numbers were determined in the lungs and nasopharyngeal tissue. Figure 3.2 shows the recovered numbers of viable pneumococci, expressed as percentage of the dose given. It can be seen that as the dose volume increases, the percentage of dose reaching the lungs increases up to 40 µl. There was a significant effect ($p < 0.001$) of dose volume on recovery of bacteria from the lungs.
Figure 3.2 suggests that as the dose volume increases the percentage of dose that remains in the nasopharynx increases, however the effect was not significant (p < 0.05).

**Figure 3.2** – Percentage of dose recovered from the lungs and nasopharynx immediately after intranasal challenge of MF1 mice intranasally infected with $1 \times 10^6$ CFU *S. pneumoniae* strain D39 in 20, 30, 40 or 50 µl PBS (n = 5).

From Figure 3.1 it can be seen that altering the dose volume altered the lethality of the dose. The next question asked in the development of the model was whether the survivors from the experiment of Figure 3.1 had detectable numbers of pneumococci in the lungs or whether the survival was due to animals clearing the infection. At seven days post-infection all surviving animals were culled and the numbers of viable pneumococci were enumerated in the nasopharynx, blood and lungs. In all mice culled there were no detectable viable pneumococci in the lungs and blood, but viable pneumococci were recovered from the nasopharynx. Figure 3.3 shows the number of viable pneumococci recovered from the nasopharynx of mice that survived to 7 days post-infection. Outbred MF1 mice were intranasally challenged with $1 \times 10^6$ viable pneumococci (strain D39) suspended in 20, 30, 40 or 50 µl of PBS. There appeared to be no effect of dose volume on the number of viable pneumococci recovered from the nasopharynx 7 days post-infection, however due to their only being one survivor in the two highest dose volume groups this could not be tested for statistical significance.
Figure 3.3 – Number of viable pneumococci recovered from the nasopharynx of mice that survived to 7 days post-intranasal challenge of MF1 mice intranasally infected with $1 \times 10^6$ CFU $S.\ pneumoniae$ strain D39 in 20, 30, 40 or 50 µl PBS ($n = 5$).

For the desired model of long term colonisation, viable pneumococci needed to be recovered from the lower airways at 7 days post-infection. This experiment showed that for the desired model the combination of outbred MF1 mice with the pneumococcal strain D39 was not the right combination, but the experiments did prove that the dose volume would be a key variable in the establishment of the desired model.

### 3.2 Investigation of the effects of pneumococcal serotype on pneumococcal virulence

As shown in Figure 3.1, disease severity and onset could be altered by changing the volume of the dose, but the serotype of pneumococcus can also be a determining factor in carriage or invasive disease (Hogberg et al. 2007). From the two experiments shown in Figure 3.1, Figure 3.2 and Figure 3.3, it was observed that in MF1 mice, strain D39 (serotype 2) was either too invasive or could be cleared within a few days. To see if disease severity could be decreased, but without the bacterium being cleared from the lower airways, the next variable investigated was pneumococcal serotype.
3.2.1 Investigation of the effect of animal passage on the virulence of serotype 6B, strain BHN418

All experiments with strain D39 (serotype 2) were performed with pneumococci that had been passed through animals, as this was the method of bacterial preparation that Canvin et al (1995) described. This process, also termed as animal passage, is used to standardise strain virulence, however not all research groups that study the virulence of *S. pneumoniae* use this method. Strain BHN418 was kindly given to the University of Leicester by Herminia de Lencastre (ITQB, Universidade Nova de Lisboa, Portugal). This group does not routinely use the method of animal passage (Sandgren et al. 2005), so the first experiment performed was to determine if there was an effect of animal passage on this strain. Outbred MF1 mice were intranasally infected with $1 \times 10^6$ viable pneumococci, strain BHN418 suspended in 50 µl of PBS. Percentage survival was assessed (Figure 3.4) and the number of pneumococci present in the blood, lungs and nasopharynx were enumerated at time of culling or at 7 days post-infection. All mice that were culled before 7 days post-infection had high numbers of viable pneumococci in the blood, lungs and nasopharynx at time of death. This suggested that the disease signs mice displayed were due to a pneumococcal infection. Figure 3.4 showed that there was no significant difference ($p > 0.05$) between the percentage survival of mice dosed with pneumococci that had been passaged compared to mice infected with non-passaged pneumococci.

![Figure 3.4](image-url)  
**Figure 3.4** – Comparison of survival between outbred MF1 mice that had been intranasally infected with $1 \times 10^6$ CFU / 50 µl of PBS of passaged or non-passaged *S. pneumoniae* strain BHN418 ($n = 5$).
The only difference observed between the two methods was that viable pneumococci (1 CFU / mg tissue) were recovered from the lungs of the surviving mouse dosed with non-passaged pneumococci. The surviving mouse from the group dosed with passaged pneumococci did not have any recoverable pneumococci in the lower airways.

The one animal that survived to 7 days post-infection with non-passaged strain BHN418, dosed in a volume of 50 µl, had 1 CFU / mg lung tissue at time of culling. This was one step closer to the desired model than had been seen using strain D39 in MF1 mice. Because different pneumococcal serotypes have differing abilities to cause invasive disease, the next step was to confirm whether the disease severity could be reduced by lowering the volume of the dose, as was seen with strain D39 in Figure 1. For this, outbred MF1 mice were intranasally challenged with $1 \times 10^6$ viable non-passaged strain BHN418 suspended in 20, 30, 40 or 50 µl of PBS. Percentage survival was assessed and the number of pneumococci present in the blood, lungs and nasopharynx were enumerated at the pre-determined time point of 7 days post-infection. As can be seen in Figure 3.5, lowering the dose volume did not reduce the disease severity in the same way as seen with strain D39 in Figure 3.1A. The group dosed with a dose volume of 30 µl had the highest percentage survival (40 %) but this was lower than that seen with strain D39 (60 %). Unlike strain D39, strain BHN418 pneumococci were recovered from the lungs of three of the four surviving mice at 7 days post-infection, as shown in Table 3.1. This confirmed that using a lower dose volume than the 50 µl dose volume used in the published acute model of pneumococcal infection (Canvin et al. 1995), would be the most suitable for the future development of the desired model.
**Figure 3.5** – Percentage survival of MF1 mice intranasally infected with $1 \times 10^6$ CFU *S. pneumoniae* strain BHN418 in 20, 30, 40 or 50 µl PBS (n = 5).

<table>
<thead>
<tr>
<th>Volume of Dose</th>
<th>Number of Mice at 7 days post-infection</th>
<th>Number of Mice with Viable Pneumococci in the Lungs</th>
<th>CFU / mg lung tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 µl</td>
<td>1</td>
<td>1</td>
<td>27</td>
</tr>
<tr>
<td>30 µl</td>
<td>2</td>
<td>1</td>
<td>73</td>
</tr>
<tr>
<td>40 µl</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>50 µl</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 3.1** – Survival at 7 days post-infection and the number of MF1 mice with viable pneumococci in the lungs after intranasal infection with $1 \times 10^6$ CFU *S. pneumoniae* strain BHN418 in 20, 30, 40 or 50 µl PBS.
3.3 A study using Factorial Experimental Design (FED)

3.3.1 Investigating the virulence of serotype 6B in different mouse strains

It had already been demonstrated that by decreasing the dose volume, the lethality of the dose could be reduced and that by switching from strain D39 to strain BHN418 it was possible to recover pneumococci from the lower airways of mice 7 days post-infection. Another variable that could have contributed to the development of the model of persistent infection in the lower airways of mice was the strain of mouse used.

It had already been highlighted that there were many variables that could contribute to the development of the desired model. Figures 3.1 and 3.2 show that dose volume was a contributing factor to the severity of disease and the data in Figure 3.4 demonstrated that the pneumococcal strain could be a factor. Currently there are 91 known serotypes (Weinberger et al. 2009) and many different pneumococcal strains that are classified as each serotype. When mouse strain was then considered as another variable, the number of combinations to be investigated increased significantly.

The initial approach that had been used to plan experiments was to investigate one variable factor at a time; by taking this approach the design space was limited and required many individual experiments. In conjunction with Mike Dymond, a statistician at AstraZeneca (Charnwood, Loughborough, UK (Address when study was designed and run)), an FED study was designed. This study investigated the three factors that had been focussed on before; mouse strain, pneumococcal strain and dose volume. Whereas in the previously described studies parameters were investigated singly, in an FED approach several combinations could be investigated in one experiment and any interactions between those combinations could be observed. Because 3 out of 4 animals (as shown in Table 3.1) had recoverable pneumococci in the lungs 7 days post-infection with a serotype 6B pneumococcus two strains of this serotype (BHN418 and BHN 191) were investigated, with $1 \times 10^6$ CFU administered in 20 or 50 µl to either MF1, C57Bl/6 or Balb/c mice. To run all these combinations in a FED study it required twelve combinations, as shown in Table 3.1. For each combination, four animals were tested.
<table>
<thead>
<tr>
<th>Combination</th>
<th>Mouse Strain</th>
<th>S. pneumoniae strain</th>
<th>Dose Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C57/Bl6</td>
<td>BHN418</td>
<td>20 µl</td>
</tr>
<tr>
<td>2</td>
<td>C57/Bl6</td>
<td>BHN418</td>
<td>50 µl</td>
</tr>
<tr>
<td>3</td>
<td>C57/Bl6</td>
<td>BHN191</td>
<td>20 µl</td>
</tr>
<tr>
<td>4</td>
<td>C57/Bl6</td>
<td>BHN191</td>
<td>50 µl</td>
</tr>
<tr>
<td>5</td>
<td>MF1</td>
<td>BHN418</td>
<td>20 µl</td>
</tr>
<tr>
<td>6</td>
<td>MF1</td>
<td>BHN418</td>
<td>50 µl</td>
</tr>
<tr>
<td>7</td>
<td>MF1</td>
<td>BHN191</td>
<td>20 µl</td>
</tr>
<tr>
<td>8</td>
<td>MF1</td>
<td>BHN191</td>
<td>50 µl</td>
</tr>
<tr>
<td>9</td>
<td>Balb/c</td>
<td>BHN418</td>
<td>20 µl</td>
</tr>
<tr>
<td>10</td>
<td>Balb/c</td>
<td>BHN418</td>
<td>50 µl</td>
</tr>
<tr>
<td>11</td>
<td>Balb/c</td>
<td>BHN191</td>
<td>20 µl</td>
</tr>
<tr>
<td>12</td>
<td>Balb/c</td>
<td>BHN191</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

**Table 3.2** – Combinations tested in Factorial Experimental Design Study

All animals were culled when they became severely lethargic or at 7 days post-infection. At time of culling the number of pneumococci present in the blood, lungs and nasopharynx was enumerated and time of death was recorded.

Figure 3.6 below shows the percentage survival for each of the combinations tested. Figure 3.6A shows that 50 % of outbred MF1 mice were susceptible to both BHN418 and BHN191 when 1 x 10^6 CFU was administered intranasally in 50 µl PBS. However when BHN418 was dosed in the smaller volume (20 µl) all MF1 mice survived to 7 days post-infection. Figure 6B showed that the susceptibility of C57Bl/6 mice could be dependent on the pneumococcal strain rather than the volume that the dose was administered in. Figure 3.6B it can be seen that C57Bl/6 mice had a 75 % survival rate with strain BHN418 compared to a 100 % survival rate seen with strain BHN191. Balb/c mice appeared to be the least susceptible to pneumococcal infection as Figure 3.6C shows that only 75 % mice survived when dosed with strain BHN418 in the larger (50 µl) dose volume, but showed 100 % survival for the other conditions tested.
Figure 3.6 – Percentage survival of mice intranasally infected with 1 x 10^6 CFU S. pneumoniae of strain BHN418 or BHN191 in either 20 or 50 µl PBS. A –MF1, B – C57Bl6, C – Balb/c (n = 4).

The next step was to investigate whether any of the surviving mice had pneumococci present in the lower airways at 7 days post-infection. In total 83% of mice survived to 7 days post-infection. Out of these none of the 14 surviving C57Bl/6 mice had any detectable pneumococci in the lower airways at time of culling. However out of the other two mouse strains; four MF1 mice and two Balb/c mice had recoverable pneumococci in the lungs as shown in Table 3.3. The number of pneumococci was expressed as the number of viable pneumococci recovered from the entire lung.
<table>
<thead>
<tr>
<th>Strain of Mouse</th>
<th>Strain of S. pneumoniae</th>
<th>Dose Volume</th>
<th>Percentage Survival</th>
<th>Number of Mice with Pneumococci in the Lungs</th>
<th>Total Lung CFU / mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF1</td>
<td>BHN418</td>
<td>20 µl</td>
<td>100 %</td>
<td>2</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 µl</td>
<td>50 %</td>
<td>1</td>
<td>305</td>
</tr>
<tr>
<td></td>
<td>BHN191</td>
<td>20 µl</td>
<td>75 %</td>
<td>1</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 µl</td>
<td>50 %</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C57 BL/6</td>
<td>BHN418</td>
<td>20 µl</td>
<td>75 %</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 µl</td>
<td>75 %</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>BHN191</td>
<td>20 µl</td>
<td>100 %</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 µl</td>
<td>100 %</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Balb/c</td>
<td>BHN418</td>
<td>20 µl</td>
<td>100 %</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 µl</td>
<td>75 %</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>BHN191</td>
<td>20 µl</td>
<td>100 %</td>
<td>1</td>
<td>833</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 µl</td>
<td>100 %</td>
<td>1</td>
<td>667</td>
</tr>
</tbody>
</table>

Table 3.3 – Shows the percentage survival of mice to 7 days post-infection after 3 strains of mice (MF1, C57Bl/6 or Balb/c) were intranasally infected with 1 x 10^6 CFU S. pneumoniae of strain BHN418 or BHN191 in either 20 or 50 µl PBS. The table also shows the proportion of survivors that had recoverable pneumococci in the lungs at 7 days post-infection and the number of pneumococci recovered expressed as CFU / whole lung harvested of each mouse (n = 4).

As can be seen in Table 3.3, two Balb/c mice dosed with strain BHN191 had recoverable numbers of viable pneumococci in the lungs. The mouse challenged with the dose in 20 µl had 833 CFU / lung (5 CFU / mg lung tissue) whereas the mouse challenged with the dose in 50 µl had 667 CFU / lung (3 CFU / mg lung tissue). As there were recoverable numbers of pneumococci in the lungs when 1 x 10^6 CFU of BHN191 was dosed intranasally to inbred Balb/c mice, it suggested that this could be a viable combination for the desired model.

To investigate whether a higher proportion of mice would have recoverable numbers of pneumococci in the lungs 7 days post-infection Balb/c mice were dosed with BHN191. From the previous experiments with the pneumococcal strain D39, it was observed that the number of pneumococci that were instilled in the lungs at the time of infection could be altered by changing the dose volume (Figure 3.1). Figure 3.1 shows that by altering the dose volume the progression of disease could be delayed. With the aim of increasing
the proportion of mice with viable pneumococci at 7 days post-infection five Balb/c mice were dosed intranasally with $1 \times 10^6$ CFU of strain BHN191 in 30 µl. Pneumococcal numbers were enumerated in the lungs, nasopharynx and blood at 7 days post-infection. All mice survived to 7 days post-infection, at this point one mouse had 5 CFU / mg lung tissue (1208 CFU / total lung).

This fulfilled the criterion for the desired model that mice should have persistent pneumococcal infection in the lower airways, with no detectable pneumococci in the blood. However, only 20 % of Balb/c mice were ever observed to have recoverable numbers of pneumococci at 7 days post-infection when intranasally dosed with strain BHN191, which was independent of dose volume.

3.3.2 Strain LgSt215 persists in the lungs of 40 % MF1 mice for 7 days post-infection

The experiments with the serotype 6B strain showed some promising results however the combination of serotype 6B with the Balb/c strain of mouse did not result in the desired model. After performing a literature review for clinically relevant pneumococcal serotypes in COPD it was seen that serotype 19F was often recovered from the lungs of COPD patients in the stable state (Bogaert et al. 2004b, Malley et al. 2007). Several groups have also described that certain strains of serotype 19F can cause focal pneumonia in the absence of bacteraemia (Briles et al. 2003, Sandgren et al. 2005). A serotype 19F strain was obtained from Herminia de Lencastre (ITQB), Universidade Nova de Lisboa, Oeiras, Portugal (Strain LgSt215).

A dose volume titration experiment was carried out with strain LgSt215 in outbred MF1 mice. As can be seen in Figure 3.7, all mice survived to the pre-determined time point of 7 days post-infection. At this point all animals were culled and the numbers of pneumococci were enumerated in the lungs, blood and nasopharynx. As can be seen in Table 3.4, 40 % of mice that had received strain LgSt215 in a dose volume of 30 µl had recoverable numbers of S. pneumoniae in the lower airways. It was also observed that for dose volumes 20 and 40 µl, 20 % of animals had recoverable numbers of pneumococci. From these observations 30 µl was chosen as the dose volume for further development of the model.
Figure 3.7 - Percentage survival of MF1 mice intranasally infected with $1 \times 10^6$ CFU *S. pneumoniae* strain LgSt215 in 20, 30, 40 or 50 µl PBS (n = 5).

<table>
<thead>
<tr>
<th>Dose Volume</th>
<th>Percentage of Group that had recoverable numbers of pneumococci in the lungs</th>
<th>Viable Pneumococci CFU / mg Lung Tissue, each individual mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 µl</td>
<td>20 %</td>
<td>131</td>
</tr>
<tr>
<td>30 µl</td>
<td>40 %</td>
<td>54</td>
</tr>
<tr>
<td>40 µl</td>
<td>20 %</td>
<td>123</td>
</tr>
<tr>
<td>50 µl</td>
<td>0 %</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3.4 – Proportion of mice with viable pneumococci in the lungs at 7 days post-infection and the number of pneumococci recovered.

The next objective was to increase the proportion of mice with viable pneumococci in the lower airways at 7 days post-infection. Initially 7 days post-infection was used as it was a more practical time point than 28 days post-infection. Once the model was shown to be reproducible at 7 days post-infection then later time points would be assessed.

In an attempt to increase the proportion of mice with viable pneumococcus in the lower airways, doses of $5 \times 10^5 – 7 \times 10^6$ CFU in 30 µl PBS were tested with strain LgSt215. As can be seen in Table 3.5, in outbred MF1 mice persistent pneumococcal presence in the lower airways was not seen in more than 50 % of the animals initially challenged with pneumococci. From Table 3.5 it can be seen that dosing with a higher CFU (4.42 x
$10^6 / 30 \mu l$ gave a higher proportion of mice with pneumococci in the lungs at 7 days post-infection compared to a dose approximately 4 times lower ($9.75 \times 10^5 / 30 \mu l$).

<table>
<thead>
<tr>
<th>Viable CFU / Dose</th>
<th>Dose Volume</th>
<th>Proportion of mice with Recoverable number of Pneumococci at 7 days post-infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>$4.98 \times 10^5$</td>
<td>$30 \mu l$</td>
<td>2 out of 5 (40 %)</td>
</tr>
<tr>
<td>$9.75 \times 10^5$</td>
<td>$30 \mu l$</td>
<td>1 out of 5 (20 %)</td>
</tr>
<tr>
<td>$1.16 \times 10^6$</td>
<td>$30 \mu l$</td>
<td>2 out of 10 (20 %)</td>
</tr>
<tr>
<td>$2.05 \times 10^6$</td>
<td>$30 \mu l$</td>
<td>2 out of 5 (40 %)</td>
</tr>
<tr>
<td>$2.14 \times 10^6$</td>
<td>$30 \mu l$</td>
<td>2 out of 5 (40 %)</td>
</tr>
<tr>
<td>$4.42 \times 10^6$</td>
<td>$30 \mu l$</td>
<td>5 out of 10 (50 %)</td>
</tr>
<tr>
<td>$6.80 \times 10^6$</td>
<td>$30 \mu l$</td>
<td>2 out of 5 (40 %)</td>
</tr>
</tbody>
</table>

**Table 3.5** – Proportion of outbred MF1 mice that had recoverable numbers of pneumococci (strain LgSt215) in the lower airways 7 days post-infection with a range of infectious doses in a dose volume of 30 µl.

### 3.4 Investigation of the effects of pneumococcal virulence in a different strain of mouse

#### 3.4.1 Strain LgSt215 persists in the lungs of CBA/Ca mice for 7 days post-infection

To increase the percentage of the population that had a persistent pneumococcal infection in the lower airways, the CBA/Ca strain was chosen because this was a mouse strain has been shown to be more susceptible to pneumococcal infection with strain D39 (Gingles *et al.* 2001). It has also been shown that 90 % of CBA/N mice were able to have recoverable numbers of pneumococci in the lungs 5 days post intranasal challenge with 3 strains of serotype 19F (Briles *et al.* 2003). The CBA/Ca strain of mouse was chosen over the CBA/N strain for practical reasons.
As can be seen in Figure 3.7, pneumococcal strain LgSt215 was not lethal in MF1 mice and was able to persist in the lungs of < 50% of MF1 mice (Table 3.5). To increase the proportion of mice with a persistent pulmonary infection, inbred CBA/Ca were dosed with either $3 \times 10^6$ or $5 \times 10^6$ CFU LgSt215 intranasally in 30 µl, and culled at either 7 or 14 days post-infection. Figure 3.8A shows that with the higher dose a small proportion of animals had to be culled due to the severity of the disease signs observed. This demonstrated that even though the strain could persist in the lungs it could also become invasive. Figure 3.8B demonstrated that the model had been achieved, with both doses tested recoverable numbers of viable pneumococci in the lungs at 14 days post-infection. There was no significant difference in pneumococcal numbers at day 7 or day 14 post-infection. Another condition for the model to be successful was that mice should not display signs of disease. It could be seen in Figure 3.8C that although mice did display disease signs for the first 48 hours, these were mild – mice (other than the one that had to be culled) did not show disease signs above 2 + hunched, 1 + starchy. By fourteen days post-infection no disease signs were observed, which fitted with the criterion for the desired model of persistent pulmonary infection.
The desired model of persistent infection had been achieved to 14 days post-infection in over 70% of inbred CBA/Ca mice intranasally dosed with pneumococcal strain LgSt215. The next step would be to see if this pneumococcal infection in the lower airways of mice persisted for 28 days post-infection and whether there was any associated cellular inflammation.
3.5 Discussion

There were many factors that could have been influential when establishing the model of persistent *S. pneumoniae* colonisation in the lower airways of mice. The four main factors investigated were; dose volume, pneumococcal strain, the effect of passage on the virulence of different serotypes and mouse strain. Other factors that could have been investigated were the route of administration of the dose (Chiang *et al.* 2009) or the additional dosing of compounds such as sialic acid to alter the progression of the pneumococcal infection (Trapetti *et al.* 2009).

As there were so many factors that could have been investigated, initial experiments were carried out using outbred MF1 mice and the pneumococcal serotype 2 strain D39. This combination was chosen as it was the combination that had been proven to work in the previously established acute model of pneumococcal infection (Gingles *et al.* 2001). In this acute model of infection, a dose of 1 x 10⁶ CFU strain D39 suspended in 50 µl of PBS was administered intranasally to outbred MF1 mice, a progressive increase in disease signs were subsequently observed before animals had to be culled between 48 – 72 hours post-infection (Canvin *et al.*, 1995). As the majority of models with *S. pneumoniae* are acute with animals succumbing to or clearing the infection within the first 72 hours post-infection, initial experiments were run to 7 days post-infection.

3.5.1 Dose Volume

In the previously established acute model of pneumococcal infection (Gingles *et al.* 2001), a dose volume of 50 µl was utilised. Yet it has also been published that when the same pneumococcal strain was administered in a smaller dose volume (10 µl) nasopharyngeal carriage was established with no pneumococci recovered from the lower airways (Ogunniyi *et al.* 2007). To investigate this further the same numbers of viable pneumococci (strain D39) were administered in a range of dose volumes between 20 – 50 µl. It was seen that as the dose volume decreased the percentage survival within the group increased.

As well as the increase in percentage survival, the length of time before the onset of disease signs was also increased suggesting that the volume in which the dose was administered altered the progression of disease. It is widely thought that *S. pneumoniae*
infection requires the colonisation of the nasopharynx before it can progress to the lower airways (Kadioglu, 2002). However, the change in the progression of the disease could be due to the size of the nasopharynx, with a larger dose volume a higher proportion of the dose would be washed directly into the lungs at the time of infection due to the nasopharynx only being able to hold a proportion of the dose.

To investigate this, MF1 mice were dosed with the same number of pneumococci (strain D39) suspended in a range of dose volumes (20 – 50 µl). However, instead of following the progression of disease signs and culling at 7 days post-infection, mice were culled immediately after infection and the nasopharynx, lungs and blood were harvested. In the blood, no viable bacteria were present at the level of detection, but the most interesting result was the percentage of the dose that was recovered from the lungs. When the dose was administered in a volume of 20 µl, less than 10 % of the dose was recovered from the lungs, however when the same number of viable bacteria was intranasally dosed in 40 µl roughly 70 % of the dose was recovered from the lungs. This suggested that the nasopharynx of outbred MF1 mice could only hold a volume smaller than 20 µl, which could confirm why Ogunniyi et al. (2007) only saw nasopharyngeal colonisation with a dose volume of 10 µl.

With the larger dose volumes, the majority of the dose was flushed into the lungs at the time of infection. This directly correlated with the onset of disease signs; the larger the proportion of the dose flushed into the lungs at time of infection, the sooner the onset of disease signs. Miller et al. (2012) have also investigated the effect of intranasal administration different dose volumes on the onset of disease with the Gram negative bacterium Francisella tularensis. They tested four dose volumes, 10, 20, 50 and 100 µl and found that the larger the dose volume the higher the proportion of dose that reached the lungs at the time of infection, except for the two highest dose volumes where the bacterial burden measured in the lower airways was the same. This was similar to what was observed with the higher dose volumes in this experiment, with MF1 outbred mice when the dose was administered in 50 µl only 40 % of the dose was recovered from the lungs, but when it was administered in 40 µl roughly 70 % of the dose was recovered from the lower airways. This could be due to the volume being greater than the respiratory tract can deal with, one explanation could be that some of the dose enters the digestive system. This hypothesis has not been tested with S. pneumoniæ however it
has been shown with a radioactive labelled nonabsorbable tracer that there was an optimum dose volume (35 µl in inbred Balb/c mice) for the highest proportion of the dose to reach the lower airways, it was also shown that radioactivity was detected in the gastrointestinal tract (Southam et al. 2002).

In conclusion, this data shows that with *S. pneumoniae* serotype 2 (D39) one way to control the progression of disease was to utilise different dose volumes. By reducing the dose volume the onset of disease symptoms could be delayed. However there was a point where the volume was too small (10 µl) and only carriage in the nasopharynx was observed (Mitchell and Paterson 2007) when tested with pneumococcal serotype 2 (D39).

These initial experiments showed that by lowering the dose volume the level of survival could be increased. However all mice culled at 7 days post-infection with serotype 2 (D39) had no viable pneumococci recovered from the lungs. From this it could be deduced that MF1 outbred mice intranasally infected with serotype 2 (D39) were able to clear the infection from the lower respiratory tract. For a successful asymptomatic model of persistent infection this combination was not successful.

### 3.5.2 Effect on pneumococcal serotype on pneumococcal virulence

Even though disease severity could be altered by changing the dose volume, no outbred MF1 mice intranasally dosed with serotype 2 (D39) had recoverable numbers of viable pneumococcus in the lungs at 7 days post-infection. The next option chosen to investigate for establishing a model of long term pulmonary infection was the serotype of the pneumococcus. It is widely accepted that different serotypes of pneumococcus vary greatly in their ability to cause invasive disease (Brueggemann et al. 2003). It was decided that the next serotype to be tested was serotype 6B, strain BHN418. This serotype is one of the most prevalent globally and has been associated with respiratory disease (Hausdorff et al. 2005).
3.5.3 The effect of animal passage and dose volume on the virulence of serotype 6B strain BHN418

All experiments with strain D39 (serotype 2) were performed with pneumococci that had been passed through animals, as this was the method of bacterial preparation that Canvin et al (1995) described. This process, also termed as animal passage, is used to standardise strain virulence, however not all research groups that study the virulence of *S. pneumoniae* use this method. It has also been published that as well as standardising the virulence, it also reduces phase variability (Mitchell and Paterson, 2007). However not all research groups that study *S. pneumoniae* routinely use the method of animal passage (Sandgren et al. 2005). To determine whether there was an effect of animal passage on pneumococcal serotype 6B, strain BHN418, an experiment was performed to compare the virulence of passaged strain BHN418 and non-passaged strain BHN418. No difference was observed in the virulence; however the one surviving animal that had been dosed with non-passaged strain BHN418 had recoverable numbers of pneumococci in the lower airways. This suggested that for the establishment of a model of long-term pneumococcal infection in the lower airways using pneumococci that had not been passaged could be more successful. The process of passaging bacteria through animals selects for pneumococcal colonies that are able to cause septicaemia. *S. pneumoniae* has high genome plasticity (Claverys et al. 2000) and has a distinct gene expression profile depending on whether it was recovered from the blood or lungs of mice (Oggioni et al. 2006). For the development of the persistent infection model the ability to cause septicaemia is not a desirable phenotype as the aim of the model was to have a pneumococcal infection in the lower airways that did not progress to the bloodstream. All experiments from this point used non-passaged pneumococci.

The same number of viable non-passaged pneumococcal strain BHN418, serotype 6B was dosed to outbred MF1 mice in a range of dose volumes (20 – 50 µl). By lowering the dose volume with strain D39, the proportion of mice that survived increased, however this was not the case with strain BHN418. Lowering the dose volume did not significantly increase survival, this could suggest that strain BHN418 is more virulent than strain D39. It has been published that serotype 6B has been responsible for 3.9 – 10.8 % of invasive pneumococcal disease in children in Germany (Linden et al. 2013), suggesting that this serotype is virulent. An increased virulence could also explain why
it was possible to detect viable pneumococci in the lower airways at 7 days post-infection, whereas it was not possible with the pneumococcal strain D39.

For a successful model of persistent pneumococcal infection in the lower airways the pneumococcal strain and the dose it was administered in, would need to be invasive enough that it remained in the lower airways but not so invasive that the infection of the lower airways progressed to septicaemia. Conversely the ideal mouse strain would be susceptible enough to pneumococcal infection than an infection could establish and remain in the lower airways without the infection being cleared.

It was seen that in MF1 mice infected with strain D39 that mice either succumbed to infection or cleared the pneumococci from the lower airways before 7 days post-infection. Yet with strain BHN418 pneumococci did persist in MF1 mice at 7 days post-infection, however the majority of MF1 mice infected with strain BHN418 succumbed to disease within a few days of infection. In an attempt to find the right combination of mouse strain, dose volume and pneumococcal strain that persisted in the lower airways of mice for a minimum of 28 days a study was designed using factorial experimental design.

3.5.4 A study using Factorial Experimental Design (FED)

As part of working under a Home Office license for animal research, all researchers should continually strive to reduce the number of animals, refine the techniques that are used and if possible replace the experiment with a non-animal method (The 3R’s). If all possible combinations had been tested for the development of this model then this would not have been followed. In an attempt to reduce and refine the next set of experiments, a statistical method called factorial experimental design (FED) was used. An FED study uses statistics to plan and increase the statistical power of experiments while aiming to minimise the number of animals used in experiments (Festing, 1994). By using an FED study approach the design space was increased and the interactions between three or more variables could be investigated in a single experiment.

The experiments that have already been discussed looked at one variable at a time. With this method a single variable at a time is changed, once one variable is optimised then the
next variable is investigated. However this means that if two variables interact and are not independent of each other, then the optimum combination may be missed (Sheridan et al. 2004). As previously mentioned, there were many variables that could be investigated, so by using a one variable at a time approach a large number of combinations would have to be tested to make sure that the design space was adequately covered. To get a better coverage of the design space, FED was used to design an experiment where three variables could be compared simultaneously; strain of mouse, pneumococcal strain and dose volume. This meant that in a single experiment, three variables could be investigated and any synergy between the variables could also be identified.

With the FED study, two strains of serotype 6B (strain BHN418 and BHN191), were administered in two dose volumes (20 and 50 µl) to three different strains of mouse (MF1, Balb/c and C57Bl/6). It had already been observed that it was possible to recover viable pneumococci from the lower airways of MF1 mice 7 days post-infection with strain BHN418, by including this within the experiment it gave a point of reference. The inbred strains C57Bl/6 and Balb/c were chosen as they have been shown to be less susceptible to pneumococcal strain D39 compared to MF1 mice (Gingles et al. 2001). In MF1 mice the disease severity could be reduced with pneumococcal strain D39 by reducing the volume it was dosed in, however with pneumococcal strain BHN418 the virulence was unaltered by changing the dose volume. This could be because MF1 mice were more susceptible to pneumococcal strain BHN418 than to pneumococcal strain D39. By including mouse strains in the FED study that have been shown to be less susceptible to pneumococcal strain D39 it could be inferred that they might also be less susceptible to pneumococcal strain BHN418.

It was observed with the FED study that both Balb/c and C57Bl/6 mice were less susceptible to both pneumococcal serotype 6B strains investigated compared to MF1 mice. This was one step closer to finding the ideal model of persistent infection as the percentage survival was increased. However none of the C57Bl/6 mice had detectable numbers of pneumococci in the lower airways at 7 days post-infection. This suggested that the combination of either of the two strains of pneumococcal serotype 6B and the mouse strain C57 BL/6 were not right for the persistent model of pneumococcal infection in the lower airways. At 7 days post-infection there were recoverable numbers
of pneumococci in the lower airways of MF1 mice dosed with strains BHN418 and strain BHN191 and the lower airways of Balb/c mice dosed with BHN191. As 100 % of Balb/c mice survived to 7 days post-infection when dosed with strain BHN191 and two mice had recoverable numbers of pneumcocci in the lower airways, the next step was to try and increase the proportion of Balb/c mice with recoverable numbers of pneumococci in the lower airways at 7 days post-infection.

In the FED study all Balb/c mice that were dosed with pneumococcal strain BHN191 survived to 7 days post-infection and two animals had recoverable numbers of pneumococci in the lower airways, this was the combination chosen to investigate further. By administering a higher number of viable pneumococci (strain BHN191) to an inbred strain of mouse shown to be more resistant to type 2 pneumococcus, it was hypothesised that a higher number of Balb/c mice at 7 days post-infection would have recoverable pneumococci in the lower airways. It was seen though that only 20 % of mice had recoverable numbers of pneumococci in the lungs. This fulfilled the criterion that was set out for a model of persistent infection as it was possible to recover pneumococci from the lower airways with no detectable pneumococci in the blood. However this was only possible in 20 % of mice that were initially infected. As the model was asymptomatic it was not possible to distinguish between the 80 % of mice that had cleared the infection from the 20 % of mice that did. This model would not fulfil the 3 R’s set out in the Home Office License as for every mouse that had the infection and fulfilled the model; four would have to be culled that had cleared the infection.

As there are over 90 pneumococcal serotypes (Weinberger et al. 2009), each with multiple strains, then testing them one by one was not feasible. As it is known that certain pneumococcal clones and serotypes are more likely to cause invasive disease (Sandgren et al. 2004), a literature review was performed. The aim of the literature review was to find a pneumococcal serotype that was associated with severe respiratory disease and has been shown to persist in the lower airways of mice for longer than the acute model of pneumococcal infection (72 hours).
3.5.5 Investigations with a serotype 19F pneumococcus

For the next set of experiments *S. pneumoniae* strain LgSt215 (serotype 19F) was chosen, as it is one of the most prevalent pneumococcal serotypes recovered from the lungs of COPD patients (Bogaert, 2004b and Domenech, 2011). Initial studies with strain LgSt215 were carried out in outbred mice as this is the strain that the acute model of pneumococcal infection has been published in (Canvin *et al*., 1995).

The first experiment with pneumococcal strain LgSt215 was a repeat of the dose volume experiment to see if the ability to control survival with dose volume was unique to serotype 2 (D39). Outbred MF1 mice were infected intranasally with $1 \times 10^6$ CFU re-suspended in 20, 30, 40 and 50 µl PBS, progression of disease signs were followed for 7 days post-infection. All mice survived to 7 days and had recoverable numbers of viable pneumococcus from the nasopharynx. However unlike D39, 20 % of mice intranasally dosed with LgSt215 had recoverable levels of viable pneumococcus in the lungs, 7 days post-infection. This was spread across three different dose volumes – 20, 30 and 40 µl.

As more mice in the 30 µl dose volume group carried the infection further work was performed using this dose volume. The group size was increased and a range of CFU doses were tested. To have a viable model of persistent infection it was decided that a minimum of 75 % of infected animals must carry the infection 28 days post-infection. However no combination could be found in MF1 outbred mice where more than 40 % of the group had a persistent pneumococcal infection 7 days post-infection in the lungs. Another literature review was performed and it was found that a model of latent pneumococcal infection has also been published, where CBA/J mice have a low level of recoverable pneumococci in the lungs for two to four days post-infection with mice succumbing to pneumococcal infection within ten days of an aerosolised challenge, with a relatively low number of organisms (in comparison to the acute intranasal model of infection) (Tateda *et al*. 1996, Nuermberger *et al*. 2005). It has also been shown that 90 % of CBA/N mice had recoverable numbers of pneumococci in the lungs five days post infection with 3 strains of serotype 19F pneumococcus (Briles *et al*. 2003). This suggested that the CBA background could be suitable for the model of persistent infection.
To test whether the model of persistent infection could be established in the CBA background with CBA background with serotype 19F, strain LgSt215, CBA/Ca inbred mice were intranasally infected with either $3 \times 10^6$ or $5 \times 10^6$ viable pneumococci suspended in 30 µl. Disease signs were monitored for 14 days post-infection with animals being culled at 7 and 14 days post-infection to assess whether pneumococci were present in the lower airways and also the number of viable pneumococci that were present in the blood, lung and nasopharyngeal tissue.

It was seen that with both doses over 70 % of mice had recoverable levels of pneumococcus in the lungs 14 days post-infection. Some mice displayed disease signs at 24 hours however by 48 hours post-infection this had dissipated. As the majority of animals did not display disease signs at 7 or 14 days post-infection, and those that did display minimal disease signs did not do so consistently, this could not be used as a measure of whether there was a persistent pneumococcal presence in the lower airways.

It has been shown that with two different doses of viable pneumococci, strain LgSt215 suspended in 30 µl, dosed to inbred CBA/Ca mice resulted in recoverable numbers of viable pneumococci in the lower airways at 7 and 14 days post-infection. This suggested that the correct combination of pneumococcal strain, dose volume and mouse strain had been found for the persistent model of pneumococcal infection. The next step was to investigate whether the pneumococcal presence in the lower airways could persist for longer than 14 days post-infection and also characterise what was happening at a cellular level.
4 Characterisation of a model of persistent pneumococcal infection in the lower airways of CBA/Ca mice:

As previously stated, the aim of the project was to establish a model of long-term \textit{S. pneumoniae} colonisation in the lower airways of mice. As described in section 3.4.1, in over 70 \% CBA/Ca mice the pneumococcal strain LgSt215 persisted for 14 days post-infection, with no outward signs of disease from 7 days post-infection.

4.1 Investigation into whether \textit{S. pneumoniae} persists in the lower airways for 28 days post – infection

A novel, reliable and reproducible model of long-term pulmonary infection has been established. One of the criteria for the model to be successful was that \textit{S. pneumoniae} should persist for a minimum of 4 weeks post-infection, with an accompanying inflammatory response. To test this possibility inbred CBA/Ca mice were intranasally dosed with $4 \times 10^6$ CFU (strain LgSt2150) suspended in 30 µl. Percentage survival was assessed and the number of pneumococci present in the blood and BALF were enumerated at the pre-determined time points of 1, 7, 14, 21 and 28 days post-infection. These days were chosen arbitrarily, to better understand how the recoverable numbers of viable pneumococci changed over the 4 weeks post-infection that was being investigated.

Figure 4.1A shows that the number of viable pneumococci recovered in the BALF peaked at 24 hours post-infection. The pneumococcal numbers then plateaued at approximately $10^4$ CFU / ml BALF from 14 to 28 days post-infection. Figure 4.1B demonstrated the proportion of mice initially infected with \textit{S. pneumoniae} that had detectable numbers of viable pneumococci in the lungs at the time of culling; these data were collected from a minimum of five experiments that have been performed over the course of the project. In Figure 4.1B it can be seen that 100 \% of mice had recoverable numbers of pneumococci in the lungs at 24 hours post-infection. There were a small proportion of mice that did not have detectable numbers of pneumococci in the BALF at the later time points. On average, at 7 days post-infection 98 \% of mice had detectable numbers of pneumococci in the BALF, whereas at 14 days post-infection this dropped
to 79.5%. At 21 days and 28 days post-infection the proportion of mice with a detectable persistent pneumococcal infection were 90% and 89% respectively. However the proportion of mice that had detectable numbers of pneumococci was not significantly different between all-time points assessed (p > 0.05). As there were few or no signs of disease this phenotype could not be used to determine if the pneumococci were still present in the lower airways.

Figure 4.1 - CBA/Ca mice were intranasally infected with strain LgSt215. Mice were culled at 24 hours, 7, 14, 21 and 28 days post-infection. Figure 1A shows the number of viable pneumococcus present in the bronchoalveolar lavage fluid collected from the lower airways, samples were taken across 3 experiments (n = 21 – 25). Figure 1B shows the proportion of mice with recoverable numbers of viable pneumococci in the lungs at each time point post-infection, data taken from a minimum of 3 experiments. Error bars show SEM.

Over 80% of inbred CBA/Ca mice had a persistent pneumococcal infection in the lungs 28 days post-infection, after a single intranasal instillation of pneumococci, as shown in Figure 4.1. To assess whether the presence of *S. pneumoniae* was being recognised by the host, levels of pulmonary oedema were assessed by the ratio of total wet weight to the total dry weight of excised lung tissue. Figure 4.2 demonstrates that the persistent presence of viable *S. pneumoniae* in the lower airways of mice caused a significant increase in the levels of pulmonary oedema. At 28 days post-infection the percentage of dry to wet weight was significantly different to that of naïve mice (p < 0.05), suggesting that pulmonary oedema is present. At 7 days post-infection levels of pulmonary oedema were significantly raised compared to naïve mice (p < 0.05) but not at 14 days post-infection (p > 0.05). This could suggest that there was a change in the inflammatory phenotype between 7 and 14 days post-infection.
Figure 4.2 - CBA/Ca mice were intranasally infected with strain LgSt215. Mice were culled at 7, 14, 21 and 28 days post-infection. Lungs were harvested and the levels of pulmonary oedema were assessed gravimetrically. The wet and dry lung weights are expressed as a percentage (n = 5). Kruskal-Wallis non-parametric test with a Dunns post-test were used to compare differences between time post-infection and naive, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.

4.2 Characterisation of the cellular responses to a persistent pneumococcal infection

To determine whether the inflammatory response was due to the persistence of viable pneumococci an experiment was devised. CBA/Ca mice were intranasally dosed with 4 x 10^6 CFU (strain LgSt2150) suspended in 30 µl. One group of animals was dosed with viable pneumococcus (n = 8), whereas before infection, the dose for the second group (n = 4) was heat killed. To heat-kill, the dose was placed in a 60 °C water bath for 10 minutes, viable counting was used to confirm that all pneumococci in this dose was no longer viable. At 24 hours, 7 days or 14 days post-infection both groups were culled and BALF was harvested and stained within 3 hours for the inflammatory markers myeloid differentiation antigen (Gr-1) or F4/80. Gr-1 is a 21-25 kDa protein which is expressed on peripheral neutrophils and the marker F4/80 is a 160 kDa glycoprotein expressed by murine macrophages. Figure 4.3A shows the proportion of Gr-1 positive and/or F4/80 positive cells in the BALF of mice after intranasal administration of pneumococcus. As can be seen, at 24 hours post infection there was a large increase in Gr-1 positive cells.
in the BALF of mice that had been challenged with live pneumococcus but not in the BALF of mice that had been challenged with heat-killed pneumococcus. Figure 4.3A also shows that at 7 and 14 days post-infection only animals that had been dosed with live pneumococci had Gr-1 positive cells in the BALF. This can be seen in Figure 4.3B where only the proportion of Gr-1 positive cells in the BALF are graphed. In this Figure it can be seen that at each of the time points post-infection investigated there were significantly higher numbers of neutrophils in the BALF of mice challenged with live pneumococcus compared to the BALF of animals dosed with heat-killed pneumococcus (p < 0.05). Figure 4.3C shows the proportion of F4/80 positive cells in the BALF. At 24 hours and 14 days post-infection there were no significant differences (p > 0.05) seen in the proportion of F4/80 positive cells in the BALF of mice dosed with either heat-killed or live pneumococcus. At 7 days post-infection the proportion of F4/80 positive cells was significantly higher (p < 0.01) in the BALF of animals dosed with heat-killed S. pneumoniae compared to the BALF of animals challenged with live bacteria.
Figure 4.3 - CBA/Ca mice were intranasally infected with $3 \times 10^6$ viable or heat killed strain LgSt215. Mice were culled at 24 hours, 7 and 14 days post-infection, BALF samples were harvested and stained against the inflammatory markers Gr-1 or F4/80, numbers of cells were enumerated by FACS analysis. A – Proportion of Gr-1 positive, F4/80 positive, or cells positive for both cellular markers, B – Percentage of gated Gr-1 positive cells, C – Percentage of gated F4/80 positive cells. Labels for graphs are how long post-infection the sample was taken and whether the animal had had live or dead (heat-killed) pneumococcus, for example; 24 hours live, were animals that were dosed with viable pneumococcus and were culled 24 hours post-infection ($n = 8$). The non-parametric Mann Whitney T test was used to compare differences in cell numbers between mice dosed with live or dead pneumococcus at each time-point evaluated, * $p < 0.05 > 0.01$, ** $p < 0.01$. 
4.2.1 Further characterisation of the cellular responses to a persistent pneumococcal infection

Figure 4.3B demonstrated that the increased numbers of Gr-1 positive cells was dependent on the presence of viable *S. pneumoniae* in the lower airways. To determine the effect of the persistent infection on other immune cell populations, differential cell counts were performed on the BALF. Lungs were lavaged as described in section 2.5.3 and samples were cytocentrifuged before staining with the Diff-quick staining kit to distinguish different immune cell populations. Figure 4.4 shows differential cell counts of the immune cells in the BALF. Figure 4.4A shows that there was a transient increase in neutrophil numbers at 24 hours post infection (p < 0.05) but numbers declined to control levels by 14 days post-infection. In contrast at 21 and 28 days post-infection lymphocytes, macrophages and monocytes were increased compared to naïve mice. Figure 4.4B shows that the numbers of lymphocytes were significantly higher at 7 (p < 0.05), 21 (p < 0.05) and 28 (p < 0.001) days post-infection in comparison to that seen in the BALF of naïve animals. It can be seen in Figure 4.4C that macrophage numbers increased progressively and were significantly higher than naïve mice (p < 0.05) at 21 and 28 days post-infection. Figure 4.4D shows that monocyte numbers were also significantly increased at 7 (p < 0.01), 14 (p < 0.01), 21 (p < 0.001) and 28 (p < 0.05) days post-infection compared to naïve mice. The number of eosinophils was not enumerated but was not graphed as less than five were seen across the whole experiment which is as expected as eosinophils are usually associated with allergy not bacterial infection.
Figure 4.4 - CBA/Ca mice were intranasally infected with *S. pneumoniae* strain LgSt215. At pre-determined time points post-infection, bronchoalveolar lavage fluid (3 x 300 µl PBS) was harvested (n = 10). A – Number of neutrophils, B – Number of lymphocytes, C – Number of macrophages and D – Number of monocytes counted in 10 fields of view at X 400 magnification. Kruskal-Wallis non-parametric test with a Dunns post-test were used to compare differences between time post-infection and naive, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.

4.2.2 Characterisation of the cytokine and chemokine responses to a persistent pneumococcal infection

Figure 4.4 demonstrated how the cellular responses in the BALF shifted from being predominantly neutrophilic at 24 hours post-infection to being predominantly monocytic and lymphocytic from 7 to 28 days post-infection. To better understand the drivers of this inflammatory change, cytokine and chemokine levels were assessed in the BALF and serum at pre-determined times post-infection. Inbred CBA/Ca mice were
intranasally dosed with $4 \times 10^6$ CFU (strain LgSt2150) suspended in 30 µl. At the pre-
determined time points 24 hours, 7 days and 14 days post-infection, bronchoalveolar
lavage fluid (3 x 300 µl PBS) and serum was harvested.

A combination of twenty cytokines and chemokines were analysed in the BALF and
serum with a MILLIPLEX Mouse Cytokine / Chemokine panel (Millipore, Billerica,
USA) kit. The results of this have been included in the appendices in Table 10.2. The
MILLIPLEX panel allowed twenty cytokines and chemokines to be screened in one
assay with a relatively small volume of sample. After this screening, individual cytokine
levels were analysed by sandwich ELISA. For all cytokine analysis by ELISA, inbred
CBA/Ca were intranasally dosed with $4 \times 10^6$ CFU (strain LgSt2150) suspended in 30
µl. At pre-determined time points, mice were culled and bronchoalveolar lavage fluid (3
x 300 µl PBS), whole lung homogenate and serum were harvested and samples were
frozen. For analysis samples were defrosted and R & D Duoset kits were used as per
manufacturer’s instructions to measure individual cytokines and chemokines.

From the data summarised in Table 8.2 it was decided to focus initially on the
inflammatory CXC chemokines; CXCL10 (Interferon gamma induced protein 10 (IP-
10)) and CXCL9 (Monokine Induced by Interferon gamma (MIG)). IP-10 and MIG are
both ligands for the CXCR3 receptor, which is primarily expressed on activated T
lymphocytes and natural killer cells (Farber, 1997). It can be seen in Table 8.2 that IP-
10 was significantly raised ($p = 0.03$) in the serum at 14 days post-infection compared
to naïve mice and was significantly raised ($p < 0.05$) in the BALF at 24 hours and 7
days post-infection, but was not significantly raised ($p > 0.05$) at 14 days post-infection.
Figure 4.5A confirms this result. In the BALF, IP-10 was significantly raised ($p < 0.05$)
at 24 hours and 7 days post-infection compared to naïve mice. In the lung homogenate,
as can be seen in Figure 4.5B, levels of IP-10 were significantly lower ($p < 0.05$) than
naïve mice at 14 days post-infection but no difference was observed at the earlier time
points. However, Figure 4.5C shows that, in the serum, levels of IP-10 were
significantly raised ($p < 0.05$) compared to naïve mice. Table 8.2 shows that levels of
MIG were significantly raised ($p < 0.05$) in the BALF at 24 hours post-infection
compared to naïve mice, but was the same level as was found in infected and in naïve
mice at all other time-points tested, in the BALF and serum. This result was confirmed
by ELISA as seen in Figure 4.5D and F. Figure 4.5E shows that at 7 days post-infection
levels of MIG in the lung homogenate were significantly raised ($p < 0.05$) compared to naïve mice.

**Figure 4.5** – CBA/Ca mice were intranasally infected with *S. pneumoniae* strain LgSt215 ($n = 16 - 25$). Whole Lung homogenate, BAL and serum samples were collected at 24 hours, 7 and 14 days post infection. IP-10 Cytokine levels are shown in sections A, B and C, A shows data for the BALF, B shows data for the whole lung homogenate and C shows data for the sera. MIG cytokine levels are shown in sections D, E and F, D shows data for the BALF, E shows data for the whole lung homogenate and F shows data for the sera. Kruskal-Wallis non-parametric test with a Dunn’s post-test were used to compare differences between individual time points against that of naïve mice, * $p < 0.05 > 0.01$, ** $p < 0.01 > 0.001$, *** $p < 0.001$. 
Figure 4.5 confirmed that the results seen with the MILLIPLEX assay could be repeated with individual ELISA kits. The next step was to look at the levels of cytokines in the BALF at up to 28 days post-infection. Levels of the chemokines IP-10 and MIG were analysed in the BALF at 21 and 28 days post-infection but there was no significant difference (p > 0.05) observed in comparison to the level of these two chemokines in the BALF of naïve mice.

Other cytokines were also looked at in the BALF of mice with a persistent pneumococcal infection. Two cytokines that are associated with an innate immune response are tumour necrosis factor alpha (TNF α) and interferon gamma (IFN γ) (Borish and Steinke, 2003). Table 8.2 showed that in the BALF and serum level of TNF α were not significantly different (p > 0.05) from naïve mice at any of the three time-points measured post-infection. Figure 4.6A shows that when this cytokine was measured in the BALF by ELISA it was significantly higher (p < 0.001) than when measured in naïve mice at 24 hours, 7 and 14 days post-infection. No significant difference (p > 0.05) in levels of TNF α in the BALF of infected animals was observed at 21 and 28 days post-infection in comparison to naïve mice. Table 8.2 showed that IFN γ was significantly raised (p < 0.05) in the BALF of mice 7 days post-infection but not at 24 hours or 14 days post-infection (p > 0.05) compared to naïve mice. Figure 4.6B shows that in the BALF levels of TNF α were significantly higher (p < 0.05) at 7 and 14 days post-infection compared to that measured in the BALF of naïve mice. No significant differences (p > 0.05) in the levels of TNF α in the BALF were observed at 24 hours, 21 or 28 days post-infection compared to naïve mice.
Figure 4.6 – CBA/Ca mice were intranasally infected with *S. pneumoniae* strain LgSt215 (n = 10). BALF samples were collected at 24 hours, 7, 14, 21 and 28 days post infection. Cytokine levels were measured by sandwich ELISA, A – levels of TNF α measured, B – levels of IFN γ measured in the BALF. Kruskal-Wallis non-parametric test with a Dunn’s post-test were used to compare differences between individual time points against that of naïve mice, * p < 0.05 > 0.01, ** p < 0.01.

The next two cytokines which were analysed by sandwich ELISA were Keratinocyte induced-chemokine (KC) and Interleukin 6 (IL-6). Both are secreted by activated macrophages; KC is the murine homologue of human IL-8, which is a neutrophil chemoattractant (Lee *et al.* 1995) and IL-6 induces acute-phase protein production (Heinrich *et al.* 1990). As can be seen in Figure 4.7A, levels of KC were significantly raised (p < 0.0001) in the BALF at 24 hours post-infection compared to the level seen in the BALF of naïve mice. Figure 4.7A also showed that KC levels in the BALF were significantly raised (p < 0.05) at 7 days post-infection compared to naïve mice. From 14 days post-infection the level of KC in the BALF was not significantly different (p > 0.05) from that seen in naïve mice. Figure 4.7B shows that the same profile is observed in the whole lung homogenate. The amount of KC present in the whole lung
homogenate was significantly higher (p < 0.05) at 24 hours and 7 days post-infection compared to naïve mice. From 14 days post-infection, no significant differences (p > 0.05) in the levels of KC in the whole lung homogenate were observed, compared to naïve mice. In Figure 4.7C the cytokine IL-6 was significantly raised (p < 0.01) in the BALF at 24 hours post-infection compared to the level measured in the BALF of naïve mice. Figure 4.7C also shows that at the later time points assessed post-infection, no significant changes (p > 0.05) to the level of IL-6 in the BALF were observed when compared to the level measured in naïve mice. Figure 4.7D shows that at 24 hours post-infection there was a significant increase (p < 0.001) in the level of IL-6 in the whole lung homogenate compared to naïve mice. At 7 days post-infection the level of IL-6 appears to be raised but this was not statistically significant (p > 0.05) compared to naïve mice. Figure 4.7D also show that at 14, 21 and 28 days post-infection there was no statistical difference (p > 0.05) observed between naïve mice or mice with a persistent pneumococcal infection in the lower airways.

**Figure 4.7** – CBA/Ca mice were intranasally infected with *S. pneumoniae* strain LgSt215 (n = 10). BALF and whole lung homogenate samples were harvested at 24 hours, 7, 14, 21 and 28 days post infection. KC cytokine levels are shown in sections A and B, A shows data for the BALF, B shows data for the whole lung homogenate. IL-6 cytokine levels are shown in sections C and D, C shows data for the BALF and D shows data for the whole lung homogenate. Kruskal-Wallis non-parametric test with a Dunn’s post-test were used to compare differences between individual time points against that of naïve mice, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001 > 0.0001, **** p <0.0001.
The final cytokine that was measured by sandwich ELISA was IL-12 p40. This cytokine is secreted by activated macrophages and dendritic cells and activates natural killer cells and induces CD4 T cell differentiation into Th1 like cells (Trinchieri, 2003). Figure 4.8A shows that in the BALF, IL-12p40 was significantly raised (p < 0.001) compared to naïve mice at 24 hours, 7 and 14 days post-infection, but not at 21 or 28 days post-infection. In Figure 4.8B it can be seen that in the whole lung homogenate, levels of IL-12p40 were significantly raised (p < 0.05) at 24 hours, 7 and 14 days post-infection, but not at the later time points measured compared to naïve mice. In the serum levels, of IL-12p40 were significantly raised (p < 0.001) at 7, 14, 21 and 28 days post-infection, but not 24 hours post-infection compared to naïve mice as shown in Figure 4.8C.
Figure 4.8 - CBA/Ca mice were intranasally infected with *S. pneumoniae* strain LgSt215 (n = 10). BALF whole lung homogenate and serum samples were harvested at 24 hours, 7, 14, 21 and 28 days post infection. Levels of IL-12p40 were measured by sandwich ELISA. A shows data for the BALF, B shows data for the whole lung homogenate and C shows data for the serum. Kruskal-Wallis non-parametric test with a Dunn’s post-test were used to compare differences between individual time points against that of naïve mice. * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001 > 0.0001, **** p <0.0001.

This project aimed to develop a model of persistent pneumococcal infection in the lower airways of mice for a minimum of 28 days post-infection, with an underlying inflammatory response. There were large changes in the levels of IL-12p40 observed at 24 hours and 7 days post-infection in the BALF and whole lung homogenate which
could cause the smaller changes observed at later time points post-infection to appear less significant. Figure 4.8C showed that at 21 and 28 days post-infection levels of IL-12p40 were significantly raised in the serum compared to naïve mice, but in Figure 4.8B levels of IL-12p40 in the whole lung homogenate appeared to be raised, but this was not statistically significant (p > 0.05). Figure 4.9 shows the levels of IL-12p40 in the BALF and whole lung homogenate at 21 and 28 days post-infection in infected mice compared to that seen in naïve mice. It can be seen in Figure 4.9A that at 21 and 28 days post-infection, there was no significant difference (p > 0.05) in the levels of IL-12p40 in the BALF when compared to naïve mice but Figure 4.9B shows that there was a difference in the whole lung homogenate. Figure 4.9B shows that at 21 and 28 days post-infection levels of IL-12p40 were significantly raised (p < 0.05 and p < 0.01 respectively) in the whole lung homogenate compared to naïve mice.

Figure 4.9 - CBA/Ca mice were intranasally infected with S. pneumoniae strain LgSt215 (n = 10). BALF and whole lung homogenate samples were harvested at 21 and 28 days post infection. Levels of IL-12p40 were measured by sandwich ELISA, A shows data for the BALF and B shows data for the whole lung homogenate. Kruskal-Wallis non-parametric test with a Dunn’s post-test were used to compare differences between individual time points against that of naïve mice, * p < 0.05 > 0.01, ** p < 0.01.

4.2.3 Characterisation of the histopathological responses to a persistent pneumococcal infection

As discussed earlier in this thesis, it has been observed that there was an associated inflammation in response to a persistent pneumococcal infection in the lower airways of mice. To better understand this inflammation, lungs were harvested for histological
analysis. CBA/Ca mice were intranasally dosed with $4 \times 10^6$ CFU (strain LgSt2150) suspended in 30 µl. Mice were culled at the pre-determined time points of 24 hours, 7, 14, 21 or 28 days post-infection and lungs were distended and fixed with formalin, before being paraffin embedded, sectioned and stained. For each time point a minimum of 8 lungs were harvested. The Figures in this section show the typical response that was observed at each time point.

### 4.2.4 Study of the overall inflammation

Sections were stained with a standard haematoxylin and eosin (H&E) stain as described in section 2.5.5.3, to assess any changes in the histopathology of the lung. To confirm that the pathology was as expected; lungs from naïve mice were harvested. Figure 4.10 shows the situation of the naïve lung. In Figure 4.10A no significant pathology was observed, alveolar spaces were clear and bronchioles were free of debris. At the higher magnification (X30) shown in Figure 4.10B, clear alveolar spaces and bronchioles can be seen.

![Image](image.png)

**Figure 4.10** – Formalin fixed, paraffin embedded, H&E stained lung section from a naïve CBA/Ca mouse. A – Low magnification image of the left lobe, B – higher magnification to show airways and alveolar spaces, b = bronchiole, v = blood vessel.

After confirmation that the naïve mice in the studies had no findings of pathological significance, the next step was to view whether the persistent presence of the pneumococci in the lower airways caused any changes to pathology.
At 24 hours post-infection all lobes showed a diffuse, severe neutrophilic infiltration and extensive microvascular leakage in the alveolar bed as well as occasional foci of inflammation that were more severe. This diffuse neutrophilic infiltration can be seen in Figure 4.11A as neutrophils and inflammatory debris were present in the alveolar airspaces. The arrows in Figure 4.11A and B indicate that neutrophils were also localised around blood vessels (v) and bronchioles (b). This localisation of immune cells can be referred to as perivascular and peribronchiolar cuffs. In Figure 4.11A it can be seen that the majority of immune cells were focused in small areas; one of the more severe foci of inflammation is shown in Figure 4.11B. Figure 4.11C shows the diffuse presence of neutrophils that were present in the alveolar bed, as well as the extensive microvascular leakage.

![Figure 4.11](image.png)

**Figure 4.11** – H&E stained CBA/Ca mice, 24 hours post-infection with pneumococcal strain LgSt215. A – diffuse inflammation across the alveolar bed with small focal areas of inflammation, B – higher magnification of focal area of inflammation shown in A, C – Inflammation in the alveolar bed. Arrow indicates inflammation, b = bronchiole, v = blood vessel.

Sections at 7 days post-infection showed a distinct change in pathology, from a widespread diffuse neutrophilia with occasional areas of more severe foci to a more
focal severe pathology that was not always present in all lobes. Figure 4.12A shows pneumonic consolidation of the alveolar bed with inflammatory cells and debris. The consolidated tissue is shown at X 100 magnification in Figure 4.12B. It consisted primarily of inflammatory cells (neutrophils, alveolar macrophages and foam cells), cellular debris and hypertrophic type II pneumocytes.

As well as the large areas of consolidated inflammation, smaller foci of inflammation were observed in all lobes sectioned, however the severity of the observed pathology varied between lobes. Two such foci of inflammation are shown in Figure 4.12 C and D. This inflammatory infiltrate is within the alveolar bed but is also localised to blood vessels and airways. It can also be seen in Figure 4.12D that within the airways, the epithelial cells appeared to be hypertrophic and hyperplastic and the lumen were occasionally filled with cellular debris.

Figure 4.12 - H&E stained CBA/Ca mice, 7 days post-infection with pneumococcal strain LgSt215. A – consolidation of inflammation, B – higher magnification of inflammation in consolidated areas shown in A, C – perivascular and peribronchiolar cuffing, D - perivascular and peribronchiolar cuffing and inflammation in the alveolar spaces. Arrow indicates inflammation, b = bronchiole, v = blood vessel.
At 7 days post-infection mice showed no outward signs of disease even though there were large areas of consolidated inflammation. At the time of culling these dense areas of inflammation could be seen macroscopically, as shown in Figure 4.13. The large areas of inflammation appeared as smooth glossy areas of lung indicated by the letter I in Figure 4.13.

![Image](image.png)

**Figure 4.13** – Gross pathology of CBA/Ca mouse lung 7 days post-infection with pneumococcal strain LgSt215. I – inflamed area of lobe, N – area of lobe that appears ‘healthy’.

At 14, 21 and 28 days post-infection similar pathology was observed. Similar to 7 days post-infection there was consolidation of inflammation, however this was in small foci similar to that observed in Figure 4.12C, rather than the lobular consolidation seen in Figure 4.12A. These smaller foci can be seen in Figure 4.14A, unlike at 7 days post-infection not all lobes had these foci of inflammatory cells. The inflammation at a cellular level was also different. Whereas at 7 days post-infection the inflammation was predominantly neutrophilic, from 14 days post-infection onwards the inflammation was more mononuclear with larger numbers of macrophages and lymphocytes being present. As well as this change in inflammation, areas of fibroplasia were seen. Figure 4.14C shows one of these areas of fibroplasia at 21 days post-infection, it was noted that these areas of fibroplasia were focal to transitional zones (where the cellular profile of the airway changes). Foci of fibroplasia were observed at 14, 21 and 28 days post-infection, but appeared to be more prevalent at 14 days post-infection. However at no point did these foci of fibroplasia progress to fibrosis. Figure 4.14D shows that as well as peribronchiolar cuffing, inflammation also infiltrated the airways. As indicated by the ‘M’, cellular infiltrate was also present in the airways. This inflammatory infiltrate consisted predominantly of mononuclear cells.
Figure 4.14 - H&E stained CBA/Ca mice, 14, 21 and 28 days post-infection with pneumococcal strain LgSt215. A – foci of consolidation of inflammation at 14 days post-infection, B – higher magnification of inflammatory foci at 14 days post-infection, C – fibroplasia (Fb) observed at 21 days post-infection, D – inflammatory infiltrate in the bronchioles (M). Arrow indicates inflammation, b = bronchiole, v = blood vessel.

Figure 4.14C showed fibroplasia at 21 days post-infection. This fibroplasia could suggest that the presence of a persistent pneumococcal infection in the lower airways could lead to airway remodelling and collagen deposition. To investigate this further paraffin sections were stained with a Masson Trichrome Stain. This is a differential stain, which stains connective tissue and collagen blue, fibrin pink, nuclei dark purple and muscle fibres and erythrocytes red (Goldner, 1938).

Figure 4.15A shows a section of lung from a naïve mouse, collagen fibres (blue and indicated by arrowheads) are present in the adventitia around the peribronchioles and blood vessels. At 21 days post-infection the amount of collagen fibres was increased (collagen deposition), as shown in Figure 4.15B. Figure 4.14C depicts an area of fibroplasia at 21 days post-infection. These apparent areas of fibroplasia at the transitional zones of airways were observed at 14, 21 and 28 days post-infection. Figure 4.15C and D confirm that this fibroplasia contained fibrin. The higher magnification
image in Figure 4.15D indicates that the fibroplasia was very cellular, which indicates that this could still be a very early stage of collagen deposition (as indicated by the arrowheads). Some collagen staining was observed but this was relatively weak compared to the collagen staining around the peribronchiole (Figure 4.15B).

Figure 4.15 - Paraffin sections of lung tissue stained with a Masson Trichrome Stain, sections from CBA/Ca mice at various time points post-infection with pneumococcal strain LgSt215. A – section from a naïve mouse to show normal level of connective tissue (stained blue and indicated by an arrowhead) around airways and blood vessels (V), B – collagen deposition at 28 days post-infection, C – low power magnification (x 100) image of fibroplasia at 21 days post-infection, D - higher power magnification (x 200) image of fibroplasia at 21 days post-infection. Arrowheads indicate stained collagen / connective tissue, Fb = fibroplasia and V = blood vessel.

4.2.5 Distribution of the inflammation across the whole lung

Taking a single section of paraffin embedded formalin fixed tissue provides a lot of information on the general changes in pathology. However as only one section was taken per lung, this only provided a snapshot of what was happening. To determine how the pathology was distributed throughout the whole lung, CBA/Ca mice were
intranasally dosed with 4 x 10^6 CFU (strain LgSt2150) suspended in 30 µl. Mice were culled at the pre-determined time points of 7 or 28 days post-infection (n = 3) and lungs were distended and fixed with formalin, before being paraffin embedded. Step sections were harvested every 300 µm to a total of 11 sections per block and stained with a standard H & E stain.

At 7 days post-infection, inflammation was observed in all lobes and was similar to that shown in Figure 4.12. Where lesions were observed they appeared to span across much of the lobe. At 28 days post-infection there were fewer lesions observed compared to 7 days post-infection. Similar to that seen in the previous studies, foci of inflammation were observed, but these were not present in all lobes. However where foci of inflammation were present, the lesion was continuous throughout the entire lobe. At both 7 and 28 days post-infection no bias was observed to a particular lobe, although the two larger lobes were most often affected. However the inflammation did appear to be focussed upon the main tracheobronchial tree.

4.2.6 Study to better understand the cellular inflammation

It has already been described that the inflammation was predominantly neutrophilic at 24 hours and 7 days post-infection, progressing to be more monocytic and lymphocytic from 14 days post-infection. To confirm that at the later time points this inflammation was lymphocytic, sections were stained for the cellular marker CD3, which is a marker that is present on all types of T cells (Chetty and Gatter, 1994).

At 21 days post-infection positive CD3 staining was observed on lymphocytes present in the perivascular cuffs, especially in the cuffs that were present along the main axial airway and those adjacent to areas of epithelial hyperplasia or damage. The most intense lymphocyte staining was observed in the more severe areas of inflammation. Figure 4.16 shows paraffin sections of lungs at 21 days post-infection with pneumococcal strain LgSt215; CD3-positive cells appear brown. For all sections an appropriate isotype control was also stained and these isotype controls showed no positive staining. In Figure 4.16A CD3-positive cells (indicated by the arrowheads) are scattered throughout the alveolar bed, with several focussed next to the blood vessel at the top left of the Figure. Figure 4.16B shows a peribronchiolar cuff with a mix of CD3-
positive cells (indicated by the arrowheads) and other mononuclear cells. The epithelium of the bronchiole (E) is also hyperplastic (Figure 4.16B).

**Figure 4.16** – Paraffin section of lung tissue stained for CD3, sections from CBA/Ca mice at 21 days post-infection with pneumococcal strain LgSt215. A – CD3 positive cells scattered through alveolar bed, B – Peribronchiolar cuff with mixed cellular infiltrate. Arrowheads indicate CD3 positive cells, L = lumen, A = alveolar space and E = epithelium.

### 4.2.7 Gram stain and staining with anti 19F capsular serum

To confirm whether the inflammation and pathology observed was due to the presence of pneumococci, sections were cut and Gram stains were carried out. At 7 days post-infection Gram-positive diploid bacteria were observed as can be seen in Figure 4.17B. The box in Figure 4.17A shows the localisation of Figure 4.17B, the Gram-positive bacteria were located within the interstitial membrane around the peribronchioles.
Figure 4.17 - CBA/Ca mice were intranasally infected with pneumococcal strain LgSt215. Sections taken at 7 days post-infection. A – Low magnification (x 100) H&E stained section, square shows localisation of Gram-positive bacteria, B – Gram stained section at high magnification (x 630) under oil, arrows point to short chains of Gram positive bacteria.

To confirm whether the Gram-positive bacteria observed in Figure 4.17B were *S. pneumoniae*, immunohistochemistry was used. Slides were stained as described in section 2.5.5.5.1 against the serotype 19F pneumococcal capsule. Positive staining was observed in the interstitial membrane around the peribronchioles, however no images were captured.

4.3 Persistent *S. pneumoniae* colonisation caused damage to the tracheal epithelium

There are physical defence mechanisms to prevent bacterial infection of the lower airways; one is the presence of beating cilia in the trachea. Healthy respiratory cilia beat in a co-ordinated manner with a specific frequency and pattern to clear debris or mucus from the airways (Chilvers et al. 2003). It has been published that *S. pneumoniae* can damage ciliated epithelium in the brain of rats (Hirst et al. 2003) and also reduce the ciliary beat frequency and cause disruption of the epithelium of human epithelial strips and cells *ex vivo* (Steinfort et al. 1989).

A technique was developed for this project at the University of Leicester to view the beating of the respiratory cilia *ex vivo* from mice. It was developed with help from Dr R. Hirst, a senior scientist in the National Diagnostic Service for Primary Ciliary Dyskinesia in Leicester. To analyse and evaluate human respiratory cilia this service harvests respiratory cilia by taking nasal brushings. These brushings remove strips of
ciliated epithelium from the nasopharynx, which are then placed into Medium 199, containing antibiotic solution, and viewed with a Leitz Diaplan microscope. Ciliary motion can then be recorded with a high speed camera (Chilvers et al. 2003).

Initially to keep the technique comparable to that used in the clinic, brushings of murine trachea were taken and analysed in the same way. Intact strips of ciliated epithelium were able to be recovered, as seen in Figure 4.18, but the majority of cilia viewed were static. The only beating cilia observed were on the largest strips of intact epithelium, and approximately only one in ten ciliated cells were beating, as seen in the video ‘Cilia beat work up’ on the supplementary DVD.

Delmotte and Sanderson (2006) harvested and analysed viable respiratory cilia from the airways of mice. Beating cilia were viewed and analysed on slices of lung and trachea that had been insufflated with 3 % w/v agarose, harvested from mice and then sliced with a vibrotome. From this report a method was developed to harvest tracheal rings, as described in section 2.5.11. Initially tracheas were filled and embedded with 3 % w/v low melting point agarose; however the process of filling the trachea with agarose caused some damage to the cilia. It was found that embedding the outside of the trachea with low melting point agarose provided enough stability for the trachea to be sectioned with a vibrotome. From these tracheal rings, ciliated cilia could be viewed in healthy naïve mice as shown in the videos naïve A and B, as detailed in Table 4.1 When analysing the cilia, four outcomes were measured; a description of the epithelium, the

![Figure 4.18](image)  
**Figure 4.18** – Strip of ciliated epithelium from tracheal brushing. Intact cilia are indicated by the arrows. ‘E’ indicates the columnar epithelial cells that the cilia are attached to. All cilia on this strip were static.
proportion of intact epithelium on each tracheal ring, the proportion of cilia that were beating and the frequency at which the cilia were beating.

Figure 4.19 is a still photograph from the video on the supplementary DVD labelled Naïve B. It shows intact beating cilia on intact columnar epithelial cells. Due to the angle of the video, only the top of the columnar epithelial cells (labelled E) can be seen. The top of all of the epithelial cells was covered in cilia (shown by the arrow) but only some cilia were in the correct plane of view to be in focus. The average proportion of intact epithelium per naïve tracheal ring was 79%. The average motility rate of intact naïve cilia were 93.5%, which was beating at a frequency of 12.49 Hz.

**Figure 4.19** – Functional, beating cilia on an intact healthy epithelium, tracheal ring harvested from a naïve CBA/Ca mouse. Arrow points to intact, functional cilia, CE – columnal epithelium cell. All columnar epithelium cells in picture have attached beating cilia, but not all are in focus.

As the technique of using tracheal rings has been shown to work in naïve CBA/Ca mice, a group of CBA/Ca mice was intranasally dosed with $4 \times 10^6$ CFU (strain LgSt2150) suspended in 30 µl. At the pre-determined time points of 1, 7, 14, 21 and 28 days post-infection, three mice were culled and tracheas were harvested, and sliced with a vibrotome into tracheal rings. All data presented below are from tracheal rings, from animals that did have a persistent pneumococcal infection in the lower airways. A minimum of three slices per mouse were first evaluated for phenotypic changes associated with the presence of *S. pneumoniae*. Lungs from all animals were also harvested to confirm the presence of *S. pneumoniae*. Sections were viewed at two magnifications, at low power (X 100) to assess the tracheal ring topology and at high power (X 1000) to assess ciliary function and presence. Stills in Figures 4.20 – 4.23 have been taken from video sequences that had been recorded at 30 bps. The phenotype
of each point post-infection has been described and video sequences were recorded for the analysis of the motility rate and ciliary beat frequency. Table 4.1 summarises the video sequences that have been included on the supplementary DVD to illustrate the points that have been described in the text.

At 24 hours post-infection two different phenotypes of damage could be observed, as shown in Figure 4.20. Figure 4.20A demonstrates that the columnar epithelial cells (E) were uniform in size and shape. Ciliated epithelial cells remained attached to the basement membrane, cilia (as indicated by the arrow) were intact, but they were static, as shown in video ‘24 hours A’. As well as the ciliated columnar epithelial cells, there were large cytoplasmic extrusions (CE), these appeared to be filled with bacteria, which can be seen in the supplementary video. Figure 4.20C shows that sections of the tracheal ring were also denuded, as indicated by the D, leaving basement membrane. The columnar epithelium cells (E) that remain were irregular in shape and very few cilia remained attached.

Figure 4.20 – Sections of tracheal rings of CBA/Ca mice, 24 hours post-infection with pneumococcal strain LgSt215. A and B – uniform columnar epithelial cells with intact, static cilia (arrow) and cytoplasmic extrusions (CE). C – columnar epithelial cells have detached (D) from the basement membrane (BM), the majority of epithelial cells (E) that remain, are irregular in shape and the cilia has also detached.
At 7 days post-infection the phenotype observed was different, as shown in Figure 4.21. Again more than one type of damage was observed. Figure 4.21A shows the columnar epithelium (E) was disrupted and irregular in shape but remained attached. Most of the epithelial cells were devoid of cilia; however some cilia remained intact, as indicated by the arrow. As well as disrupted epithelium, there was also the presence of white blood cells (marked by the star in Figure 4.21A) suggesting that the damage observed was due to the bacteria. Other damage seen at 7 days post-infection was large sections of denuded epithelium as shown in Figure 4.21(B and C). As well as large areas of denuded basement membrane, red blood cells (RBC) were also observed. Figure 4.21B shows a low power magnification (X100) of a tracheal ring with focal patches of red blood cells, Figure 4.21C shows one of these areas at a higher magnification. Figure 4.21C also shows that the basement membrane is exposed because all the ciliated columnar epithelial cells are no longer present.

![Figure 4.21 - Sections of tracheal rings of CBA/Ca mice, 7 days post-infection with pneumococcal strain LgSt215. A – disrupted and damaged epithelium (E), some cilia were still attached (arrow) and leukocytes were also present (star), B – low power magnification (X100) to show foci of red blood cells (RBC), C – high power magnification of red blood cell present on denuded epithelium.](image)

At 14 days post-infection, red blood cells were not observed in the lumen, however there were still large areas of damage. Figure 4.22A shows damage similar to that in Figure 4.20B. In Figure 4.22A it can be seen that columnar epithelial cells (E) were
partially sheared away from the basement membrane (BM) but still attached to each other. As seen in the left hand side of Figure 4.22A patches of the basement membrane were denuded of epithelial cells. Columnar epithelial cells had some cilia still attached but the majority were missing. Figure 4.22(B and C) show damaged and disrupted columnar epithelium cells, the cilia that was still attached, indicated by the arrow, in Figure 4.22 cilia were either static or appearing to beat at a lower frequency (10.70 Hz) than that seem on the epithelium of naïve mice (12.49 Hz). Cytoplasmic extrusions can be observed in Figure 4.22C (CE).

Figure 4.22 - Sections of tracheal rings of CBA/Ca mice, 14 days post-infection with pneumococcal strain LgSt215. A – disrupted and damaged columnar epithelium cells (E), that have started to detach from the basement membrane (BM), some cilia were still attached (arrow) to these cells but the majority had been lost. B and C – disrupted and damaged epithelium, columnar epithelium cells were still attached to the basement membrane (BM) and cilia were still attached (arrow). CE indicates the cytoplasmic extrusions that were also observed at the earlier time points.

Figure 4.23 shows the damage that was observed at 28 days post-infection. In Figure 4.23A it can be seen that the columnar epithelial cells (E) are disrupted and some are protruding. There was beating cilia attached to the epithelial cells, which were beating as shown in video ‘28 days A’. Above the beating cilia there was debris and mucus, indicated with an M in Figure 4.23A. As with the focal nature of inflammation seen at 28 days in the histology, there were sections of the trachea that were comparable to naïve mice. Figure 4.23B shows a section of intact, uniform columnar epithelial cells (E) with attached functioning cilia (indicated by the arrow). On the left of Figure 4.23A
a detached columnar epithelium cell (*E) can be seen that was being moved by the beating cilia as shown in video ‘28 days B’.

Figure 4.23 - Sections of tracheal rings of CBA/Ca mice, 28 days post-infection with pneumococcal strain LgSt215. A – disrupted and damaged epithelium (E) with beating cilia (arrow) with a piece of mucus (M), B – healthy beating cilia (arrow) on uniform columnar epithelial cells (E), with a detached ciliated epithelial cell (*E).
<table>
<thead>
<tr>
<th>Time sample harvested post-infection</th>
<th>Video Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve</td>
<td>Naïve A</td>
<td>Beating cilia on a healthy epithelium. Epithelial cells were columnar in appearance and cilia were intact.</td>
</tr>
<tr>
<td></td>
<td>Naïve B</td>
<td>Closer view of non-damaged beating cilia, can see the full range of motion of the beating epithelium.</td>
</tr>
<tr>
<td>24 hours</td>
<td>24 hours A</td>
<td>Cilia were present but static, epithelial cells were relatively intact but there were large cytoplasmic extrusions which were full of bacteria.</td>
</tr>
<tr>
<td></td>
<td>24 hours B</td>
<td>Area of damaged epithelium, left hand side of epithelium is denuded, cells on right side of video are disrupted and the cilia have been sheared off. The cilia that remain intact were static.</td>
</tr>
<tr>
<td>7 days</td>
<td>7 days A</td>
<td>Right half of video shows apparently healthy epithelium with beating cilia. The ciliated epithelium on the left has been damaged, cells remain ciliated but the cilia are static.</td>
</tr>
<tr>
<td></td>
<td>7 days B</td>
<td>Very damaged epithelium, some cilia are still attached but the majority are static. Those that are beating are beating slowly and do not have the full range of motion of healthy cilia. Cytoplasmic extrusions are still present.</td>
</tr>
<tr>
<td>14 days</td>
<td>14 days A</td>
<td>Cells are ciliated, the cilia is beating, at a slower frequency than that of naïve mice. A large amount of mucus was being moved by the cilia but not being cleared.</td>
</tr>
<tr>
<td></td>
<td>14 days B</td>
<td>Some slow moving cilia, epithelium was disrupted.</td>
</tr>
<tr>
<td>28 days</td>
<td>28 days A</td>
<td>Disrupted and damaged epithelium,</td>
</tr>
<tr>
<td></td>
<td>28 days B</td>
<td>Carpet of beating cilia, epithelial cells were columnar and uniform in shape. The beating cilia were moving a ciliated epithelial cell that was no longer attached to the basement membrane.</td>
</tr>
</tbody>
</table>

**Table 4.1** – Brief descriptions of the video sequences on the supplementary DVD. CBA/Ca mice were intranasally infected with strain LgSt215. Mice were culled at 24 hours, 7, 14, 21 and 28 days post-infection, trachea’s were harvested and the respiratory cilia were viewed with a Leitz Diaplan microscope, cilia motion was then recorded with a high speed camera and evaluated with the ‘motion plus’ software.
As well as looking at the damage, the proportion of intact epithelium can be measured, as well as the ciliary motility rate and the ciliary functioning. Firstly the proportion of intact epithelium present on each tracheal ring was estimated. On a healthy tracheal ring, three layers can be seen; the basement membrane, columnar epithelial cells and the cilia attached to them. Figure 4.24 shows the proportion of columnar epithelium cells present at each point post-infection. As can be seen in Figure 4.24A, the percentage of intact epithelium was significantly less (p < 0.005) at 24 hours and 7 day post-infection compared to that of naïve mice. At 14 and 28 days post-infection the percentage of intact epithelium appeared to be lower than that of the naïve mice but this was not statistically significant (p > 0.05) with the Kruskal-Wallis analysis, but it was significantly (p < 0.05) reduced when compared directly with naïve mice with a Mann-Whitney analysis.

Figure 4.24B shows the motility index at set times post-infection. The motility index was assessed by counting the number of beating ciliated epithelial cells and expressing this as a percentage of the total intact ciliated cells. As can be seen in Figure 4.24B, at 24 hours post-infection the average motility index was 0 %, because all cilia were static. However as can be seen in Figure 4.20A, ciliated cells were present, at 7 and 14 days post-infection, the assessed the motility index, showed that the ciliary beat frequency was significantly slower (p < 0.05). At 28 days post-infection, however, the ciliary beat frequency appears slower than naïve mice, but this was not statistically significant (p > 0.05) with the Kruskal-Wallis analysis, but it was significantly (p < 0.05) reduced when compared directly with naïve mice with a Mann-Whitney analysis.
Figure 4.24 - CBA/Ca mice were intranasally infected with *S. pneumoniae* strain LgSt215 and at the pre-determined time points 24 hours, 7, 14 and 28 days post-infection mice were culled. A - for each tracheal ring viewed percentage of intact epithelium was estimated, B – Percentage of ciliated epithelium that was beating. Kruskal-Wallis non-parametric test with a Dunn’s post-test were used to compare differences between individual time points against that of naïve mice, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.

Figure 4.25 shows the ciliary beat frequency. This was assessed on video sequences that were recorded at 500 frames per second, as described in section 2.5.11. At 24 hours post-infection all cilia were static. At 7 and 14 days post-infection the beat frequency was not significantly different (p > 0.05) from that of naïve mice, even though sections observed had a significantly lower (p < 0.05) motility rate (Figure 4.23B). At 28 days
post-infection the beat frequency was significantly lower \((p < 0.05)\) than that of naïve mice.

**Figure 4.25** - CBA/Ca mice were intranasally infected with *S. pneumoniae* strain LgSt215 and at the pre-determined time points 24 hours, 7, 14 and 28 days post-infection mice were culled. For each ciliated cell, beat frequency was assessed using the Motion Plus software. Kruskal-Wallis non-parametric test with a Dunn’s post-test were used to compare differences between individual time points against that of naïve mice, * \(p < 0.05 \leq 0.01\), ** \(p < 0.01 \leq 0.001\), *** \(p < 0.001\).

### 4.4 *S. pneumoniae* in the lower airways causes a reduction in lung functioning

As well as looking at the level of inflammation at a cellular level, during the time-course of infection lung function was assessed with a forced manoeuvres system (eSpira, EMMS). This system measures the end points; forced expiratory volume (FEV), total lung capacity and peak expiratory flow, as well as other parameters such as respiratory rate, tidal volume and breathing patterns.

In humans, spirometry is a range of techniques that are used to assess the ability of the lung to function. FEV\(_1\) (volume of air forcefully exhaled in 1 second) is part of the GOLD standard for assessing airway obstruction and is one of the measures used to classify if a patient has COPD, and its severity. The larger the decrease of FEV\(_1\) from predicted values the greater the airway obstruction (Jakeways *et al.* 2003). To measure this and other parameters, such as tidal volume and respiratory rate, mice were
anaesthetised and cannulated. Cannulated mice were able to breathe spontaneously, except during one of the three forced manoeuvres where the breathing was controlled. The FEV was measured at set points over a time course (25 – 75 milliseconds). Figure 4.24 shows the FEV curves measured at set points from 25 – 75 milliseconds. The Figure has been split so that changes can be seen more distinctly. In Figure 4.24A it can be seen that at 24 hours post-infection there was no statistically significant reduction in FEV compared to naïve mice but at 7 days post infection there was a significant reduction (p < 0.001). Figure 4.24B shows that at 14 and 21 days post-infection the FEV curves were significantly reduced (p < 0.001) compared to naïve mice, but at 28 days post-infection a significant statistical difference to naïve mice was not seen (p > 0.05).
Figure 4.26 - CBA/Ca mice were intranasally infected with strain LgSt215. At predefined time points post-infection lung function parameter FEV$_1$ was assessed using a forced manoeuvres system (EMMS) (n = 8). A – 1 and 7 days post-infection, B – 14, 21 and 28 days post-infection.

Another flow derived measure is the Peak Expiratory flow (PEF), it is the maximal flow (or speed) achieved during the maximally forced expiration initiated at full inspiration, measured in liters per minute. A reduction in PEF would suggest that the lung was being constricted. It can be measured when the mouse is breathing unaided (spontaneous (Figure 4.27A)), or while the animal’s breathing is being controlled by the lung function apparatus (forced (Figure 4.27B)). In Figure 4.27A it can be seen that the volume of the PEF, while the mice were breathing spontaneously, was significantly reduced (p < 0.05) compared to naïve mice at 24 hours, 7, 14 and 21 days post-
infection. At 28 days post-infection the decrease in spontaneous PEF was not statistically significant (p > 0.05). Figure 4.27B demonstrates that the forced PEF was decreased when the persistent pneumococcal infection in the lower airways was present. It can be seen in Figure 4.27B that at 24 hours and 14 days post-infection this reduction was not statistically significant (p > 0.05). However this reduction in forced PEF in comparison to naïve mice was significantly (p < 0.05) decreased at 7, 21 and 28 days post-infection.

Figure 4.27 - CBA/Ca mice were intranasally infected with strain LgSt215. At predetermined time points post-infection, flow derived lung function parameters were assessed with an eSpira forced manoeuvres system (EMMS). A – Spontaneous peak expiratory flow (PEF), B – Forced PEF (n = 8). Kruskal-Wallis non-parametric test with a Dunn’s post-test were used to compare differences between individual time points against that of naïve mice. * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001 > 0.0001, **** p < 0.0001.

As well as the flow-derived parameters FEV and PEF, the forced manoeuvres system (EMMS) was able to measure parameters such as breathing frequency and airway resistance. Figure 4.28A shows that the breathing frequency of mice at 7 days post-infection was significantly lower (p <0.05) compared to the breathing frequency of naïve mice. At 24 hours, 14, 21 and 28 days post-infection the breathing frequency was not statistically different (p > 0.05) from that of naïve mice (Figure 4.28A). It can be seen in Figure 4.28B that the airway resistance was not affected by the presence of pneumococci in the lower airways. At the five time points assessed post-infection, airway resistance was not statistically different (p > 0.05) from the airway resistance of naïve mice (Figure 4.28).
Figure 4.28 - CBA/Ca mice were intranasally infected with strain LgSt215. At predetermined time points post-infection, lung function parameters were assessed with a forced manoeuvres system (EMMS). A – Breathing frequency expressed as breaths per minute, B – Airway resistance (n = 8). Kruskal-Wallis non-parametric test with a Dunn’s post-test were used to compare differences between individual time points against that of naïve mice, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.

The final set of parameters assessed by the forced manoeuvres system (EMMS) were based on the volume or capacity of the lung at different points of the respiratory cycle. During spontaneous respiration only a small proportion of the lung is utilised, this is known as the resting tidal volume (Al-Ashkar et al. 2003). The resting tidal volume is shown in Figure 4.29. It can be seen that at 24 hours, 7, 14 and 21 days the tidal volume was significantly reduced (p < 0.01) compared to the tidal volume of naïve mice. At 28 days post-infection, tidal volume appears to be reduced in comparison to naïve mice but this is not statistically significant (p > 0.05).
Figure 4.29 - CBA/Ca mice were intranasally infected with strain LgSt215. At predetermined time points post-infection, changes in tidal volume at rest were assessed with a forced manoeuvres system (EMMS) \((n = 8)\). Kruskal-Wallis non-parametric test with a Dunn’s post-test were used to compare differences between individual time points against that of naïve mice, * \(p < 0.05 > 0.01\), ** \(p < 0.01 > 0.001\), *** \(p < 0.001\).

As well as the tidal volume at rest, four other measures of lung capacity were assessed with the forced manoeuvres system (EMMS). These were; the inspiratory capacity, the forced vital capacity (FVC), the functional residual capacity (FRC) and the residual volume.

Figure 4.30A shows the inspiratory capacity, this is the total volume of air that can be taken in at the time of inspiration, which includes the volume of air that is inspired at rest as well as the inspiratory reserve volume. It can be seen in Figure 4.30A that at 7 and 14 days post-infection the inspiratory capacity is significantly reduced \((p < 0.05)\) in comparison to naïve mice. But at 24 hours, 21 and 28 days post-infection there is no statistically significant differences in inspiratory capacity compared to naïve mice (Figure 4.30A).

Figure 4.30B shows the FVC, this is the maximal volume of air that can be expelled from the lungs during a forced manoeuvre. In Figure 4.30B it can be seen that this was significantly reduced \((p < 0.01)\) at 7 days post-infection compared to naïve mice but not at any other point measured post-infection.
FRC is the volume of air that remains in the lungs after passive respiration. Figure 4.30C shows that the FRC is not statistically different from naïve mice at 24 hours, 21 and 28 days post-infection. However it can be seen that at 7 and 14 days post-infection the FRC was significantly reduced ($p < 0.01$) in comparison to naïve mice (Figure 4.30C). Even after forced expiration there was still a residual volume of air that remained in the lung. The residual volume is shown in Figure 4.30D, it can be seen that at 24 hours, 7 and 14 days post-infection the residual volume in the lung was significantly reduced ($p < 0.05$) in comparison to naïve mice. But at 21 and 28 days post-infection the residual volume was not statistically different ($p > 0.05$) when compared to that of naïve mice (Figure 4.30D).

**Figure 4.30** - CBA/Ca mice were intranasally infected with strain LgSt215. At predetermined time points post-infection, lung function parameters to assess changes in lung volume/capacity were assessed with a forced manoeuvres system (EMMS). A – Inspiratory capacity, B – Forced Vital Capacity (FVC), C – Functional residual capacity (FRC), D – Residual Volume ($n = 8$). Kruskal-Wallis non-parametric test with a Dunn’s post-test were used to compare differences between individual time points against that of naïve mice, * $p < 0.05$ > 0.01, ** $p < 0.01$ > 0.001, *** $p < 0.001$ > 0.0001, **** $p < 0.0001$. 
4.5 Investigation into whether the model of persistent infection could be established in a different inbred strain of mouse

A model of persistent pneumococcal infection of the lower airways has been established, where inbred CBA/Ca mice, had a persistent pneumococcal presence in the lower airways for a minimum of 28 days, after a single intranasal challenge with pneumococcal strain LgSt215. The next aim was to combine the newly established model of persistent pneumococcal infection with other models of inflammation to understand triggers for exacerbations in severe respiratory diseases such as asthma and COPD. Many published studies of respiratory inflammation that are used to better understand the mechanisms involved in the pathogenesis of asthma and COPD have been established in inbred Balb/c mice, but none are reported in CBA/Ca mice (Bartlett et al. 2008, Natarajan et al. 2010, Nials and Uddin, 2008). Therefore the next step was to assess whether the model of persistent infection could be established in inbred Balb/c mice.

Inbred Balb/c mice were intranasally dosed with $4 \times 10^6$ CFU (strain LgSt2150) suspended in 30 µl. Percentage survival was assessed and the number of pneumococci present in the BALF was enumerated at the pre-determined time points of 1, 7 and 14 days post-infection. As can be seen in Figure 4.31A, the number of detectable pneumococci in the BALF was significantly lower ($p < 0.0001$) in Balb/c mice at 24 hours, 7 and 14 days post-infection compared to CBA/Ca mice at the same time points. In Figure 4.31B it can be seen that at 24 hours only 90% of Balb/c mice had detectable numbers of pneumococci in the BALF. By 7 days post-infection this decreased to 50% of Balb/c mice and at 14 days post-infection no Balb/c mice had detectable numbers of pneumococci in the BALF. At all-time points evaluated, the proportion of Balb/c mice with detectable numbers of pneumococci in the BALF was lower than the proportion of CBA/Ca mice with detectable numbers of pneumococci in the BALF at the same point post-infection.
Figure 4.31 - CBA/Ca or Balb/c mice were intranasally infected with strain LgSt215. Mice were culled at 24 hours, 7 and 14 days post-infection. Figure 1A shows the number of viable pneumococcus present in the bronchoalveolar lavage fluid collected from the lower airways (n = 10). Figure 1B shows the percentage of mice with recoverable numbers of viable pneumococci in the lungs at each time point post-infection. The non-parametric Mann Whitney T test was used to compare differences in the numbers of detectable pneumococci in the BALF between inbred CBA/Ca or Balb/c mice at each time-point evaluated, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001 > 0.0001, **** p < 0.0001.

As can be seen in Figure 4.31, Balb/c mice no longer had a recoverable numbers of viable pneumococci 14 days post-infection with strain LgSt215, in contrast to CBA/Ca mice. To better understand this observation, the cellular profile was assessed in the BALF. As can be seen in Figure 4.32A and B, the number of neutrophils and macrophages in the BALF of CBA/Ca mice were significantly higher (p < 0.0001) compared to the BALF of Balb/c mice. The levels of the neutrophil chemoattractant KC paralleled the profile of neutrophil numbers. It can be seen in both CBA/Ca mice and Balb/c mice there was an increase in the number of neutrophils and the level of KC which was significant (p < 0.01) compared to the corresponding naïve control. At later
time points this increase was no longer seen in either strain of mouse. Figure 4.32D shows that there was no statistical difference ($p > 0.05$) in the level of the chemokine IP-10 in the BALF between the two inbred mouse strains at any time point evaluated.

**Figure 4.32** - CBA/Ca or Balb/c mice were intranasally infected with strain LgSt215, mice were culled at 24 hours, 7 and 14 days post-infection (n=10). At time of culling BALF was harvested and the number of immune cells were enumerated and the levels of the cytokine KC and the chemokine IP-10 were evaluated by sandwich ELISA. A – number of neutrophils, B – number of macrophages, C – level of KC and D – level of IP-10 in the BALF. The non-parametric Mann Whitney T test was used to compare immune cell number or cytokine concentration in the BALF between inbred CBA/Ca or Balb/c mice at each time-point evaluated, * $p < 0.05 > 0.01$, ** $p < 0.01 > 0.001$, *** $p < 0.001 > 0.0001$, **** $p < 0.0001$.

### 4.6 Discussion

The first and main aim of this project was to develop and characterise a model of long-term pulmonary infection with *S. pneumoniae*. The ideal model would have a persistent pneumococcal ‘infection’ in a minimum of 75 % of the group, 28 days post-infection with few or no signs of disease and a low-level inflammation that could be quantified.
This was achieved; it was consistently observed that at 28 days post-infection, 89% of inbred CBA/Ca mice remain infected with a serotype 19F pneumococcus. This model is novel, because currently animal models of lung infection with *S. pneumoniae* are either acute in nature with animals developing bacteraemia and having to be culled within a few days of infection (Chiavolini *et al.* 2008), or only the upper respiratory tract is colonised for a period of time, with no outward signs of disease (McCool and Weiser, 2004). This is the first description of a robust, murine model with a persistent *S. pneumoniae* infection of the lower airways that lasts for a minimum of a month.

The number of viable pneumococci recovered from the lower airways changed as time progressed. At 24 hours post-infection the majority of mice (> 75%) and 7 days post-infection some mice (< 20%), but not all mice had greater numbers of viable pneumococci recovered from the lungs than the number given in the initial dose. This suggested that the host response was not successful in clearing the infection and the pneumococcus was able to proliferate in the lungs of the host. However, by fourteen days post-infection this was no longer the case, as the number of pneumococci recovered from the lungs of all mice was lower than the initial dose given and also that enumerated at 24 hours post-infection. As at 28 days post-infection viable pneumococci were recovered from the lower airways, it suggested that the host was unable to sterilise the infection from the lower airways, but was able to prevent the pneumococci from invading the blood.

It was also observed that the proportion of mice with detectable numbers of pneumococci in the lower airways changed over the 28 days post-infection at the different time points evaluated. It was seen that at 24 hours post-infection 100% of CBA/Ca mice had detectable numbers of pneumococci in the lungs. It was seen that the average proportion of CBA/Ca mice with detectable numbers of pneumococci in the lower airways dropped from 100% at 24 hours post-infection, to 98% at 7 days post-infection then down to 79.5% at 14 days post-infection. Yet at 21 and 28 days post-infection the proportion of mice with detectable numbers of pneumococci in the lungs increased from 79.5% at 14 days post-infection to 90 and 89% at 21 and 28 days post-infection respectively. This drop in the proportion of mice at 14 days with a pneumococcal presence in the lower airways was consistently seen across all experiments. It could suggest that mice were either clearing the infection and being re-
infected or that pneumococci was present in a higher proportion of mice at 14 days post-infection but the number of pneumococci was below the level of detection. For all experiments the lowest limit of detection was 17 colony forming units per millilitre of BALF harvested or 83 colony forming units per lung harvested. It was most likely that viable pneumococci were present, but the number present was below the limit of detection. It has been shown that when mice infected with *S. pneumoniae* were co-housed with naïve mice, transmission of *S. pneumoniae* to the naïve mice could only occur if all mice were also infected with influenza A virus (Diavatopoulos *et al.* 2010).

As *S. pneumoniae* could be recovered from the lungs of mice 28 days post-infection, the next thing to be understood was whether the presence of the pneumococcus was being recognised by the host. To do this several parameters were evaluated, including the number of inflammatory cells present and any changes in levels of cytokines.

The first parameter that was analysed was whether the presence of pneumococci in the lower airways was inducing oedema. An increase was seen in the ratio of the wet weight of the lung to the dry weight, this suggested that there pulmonary oedema was present. This change was significant at 7 and 28 days post-infection but not at 14 or 21 days post-infection, suggesting that the levels of oedema, just like the number of pneumococci, changed over the time course of the infection. However it is also known that this method of assessing pulmonary is quite crude and can be quite variable so a high number of repeats are required to get results of statistical significance (Parker and Townsley, 2004).

The next question asked was whether the oedema measured was due to a persistent present of viable pneumococci or just the presence of dead pneumococci. To do this, CBA/Ca mice were infected with either viable pneumococci or pneumococci that were no longer viable due to being heat killed. This was an important question as it has been shown that heat-killed *S. pneumoniae* can elicit an immune response similar to that of viable pneumococcus (Dominis-Kramaric *et al.* 2011). This could be due to the acute inflammatory response to *S. pneumoniae* being mediated by pattern recognition receptors which non-specifically recognise pathogen-associated molecular patterns (Santos-Sierra *et al.* 2006).
FACS was used to analyse the cellular influx into the BALF, the two cellular markers chosen were Gr-1 and F4/80 as both have been reported to be expressed on cells involved in the innate immune response to *S. pneumoniae* (Hatta *et al.* 2010). Gr-1 is expressed on peripheral neutrophils and F4/80 is expressed by murine macrophages. It was observed that at 24 hours post-infection, heat-killed pneumococci elicited an influx of Gr-1 positive cells into the lung, however this influx was significantly lower than the influx of Gr-1 positive cells in the lower airways elicited by the presence of live *S. pneumoniae*. At 7 and 14 days post-infection with the heat-killed pneumococci the cellular profile observed was the same as that seen with naïve mice suggesting that the mouse’s immune system was able to clear the heat-killed pneumococci between 24 hours and 7 days post-instillation. However at 7 and 14 days post-infection with viable *S. pneumoniae* there was an influx of Gr-1 positive cells, suggesting that an immune response was still occurring in response to the viable pneumococcus. No differences were observed in the number of gated F4/80 positive cells at the time points assessed.

As it was confirmed that the presence of live pneumococci was required to elicit an immune response past 24 hours post-infection then the next step was to better understand what the immune response was. Firstly the immune cell population in the BALF was evaluated at set time point’s up to 28 days post-infection.

Two distinct phases of inflammatory response was seen, during the first 7 days there was a transient neutrophilia. By 14 days post-infection this transient neutrophilia was no longer present in the BALF, but the number of monocytes, macrophages and lymphocytes were significantly increased. It is generally considered that neutrophils are one of the key effector cells in the host defense against the pneumococcus (Standish and Weiser, 2009). This model shows that the neutrophilic response in CBA/Ca was limited and was unable to sterilise the lower airways from the pneumococcal strain LgSt215. However the initial influx of neutrophils at 24 hours post-infection could be the reason why the initial bacterial numbers decreased. From fourteen days post-infection the numbers of neutrophils present in the BALF were the same as that observed in naïve mice; however there were an increased number of macrophages. Taylor *et al.* (2010) suggest that in COPD, macrophage innate responses are suppressed which could lead to bacterial colonisation. Our model also shows that in CBA/Ca mice, the numbers of macrophages were increased compared to naïve, but they were also unable to sterilise
the lower airways. This suggests that there may be underlying reasons that are independent of the impairment seen in COPD patients, why macrophages were unable to clear a persistent low-level bacterial colonisation. It is known that macrophages can be subdivided into M1/classically activated macrophages or M2/alternatively activated macrophages (Boorsma et al. 2013). It has been reported that in response to cigarette smoke there is a down regulation of M1 macrophages and an increase in M2 macrophages which could explain the change in the innate response as M1 macrophages are induced by Th1 signals like IFN-γ (Shaykhiev et al. 2009). This could suggest that a similar response is occurring to the persistent pneumococcal infection, further classification of the macrophage populations could be done to better understand this.

To better understand the underlying drivers of the cellular response the cytokine profile was also determined in the BALF, lung and sera. As an exploratory exercise a panel of cytokines were analysed using a MILLIPLEX kit from Millipore. This enabled a large number of cytokines to be tested with a relatively small amount of sample. When this assay was run there were only four animals in the control group and eight animals tested at the three pre-determined time points post-infection, so further work would always need to be done to increase the significance of the data. The second technical problem with this assay was due to the kits age, not all of the standard curves for the cytokines worked as the standard samples had degraded, so some of the data was worked out on only the fluorescence measured rather than the concentrations of protein. As this was data was only ever used as an indication of which cytokines and chemokines could be of interest this did not matter as all data was confirmed by sandwich ELISA.

The key advantage of using the MILLIPLEX assay was that twenty cytokines could be analysed on the same assay using very small amounts of sample. The disadvantage to this method is that it is not as sensitive as testing each individual cytokine or chemokine by sandwich ELISA, which meant that although it could identify cytokines and chemokines that may be of interest, further work needed to be done to elucidate actual cytokine changes.

After assessing a range of cytokines and chemokines by sandwich ELISA it was seen that at 24 hours post-infection the levels of cytokines Keratinocyte induced-chemokine (KC) and IL-6 were increased. Both are secreted by activated macrophages; KC is the
murine homologue of human IL-8, which is a neutrophil chemoattractant (Lee et al. 1995) and IL-6 induces acute-phase protein production (Heinrich et al. 1990). It has been shown that during pneumococcal pneumonia, IL-6 down-regulates the activation of the cytokine network in the lung and contributes to host defence (van der Poll and Opal, 2007). At 14 days post-infection neither KC nor IL-6 were raised, this could be due to downstream events of the host response are clearing the pneumococcal infection. Or the pneumococcal infection could no longer be causing a host response, but was still proliferating.

Levels of IP-10 and MIG were also raised at these earlier time points, however by 14 days post-infection, the levels of IP-10 and MIG were no longer significantly raised. Both IP-10 and MIG are substrates for the receptor CXCR3 which is expressed on T cells and has been shown to be important in the recruitment of effector T cells (Hu et al. 2010). This suggests that the early innate response that was occurring was shifting to a more adaptive immune response. To confirm whether an adaptive immune response was occurring, the expression of T cells needs to be analysed. However as will be discussed, due to the low level of inflammation observed challenges in analysing the inflammation occurred.

Levels of IP-10 were significantly raised in the sera 14 days post infection, which could suggest a role as a potential biomarker to signify that the infection is still persisting in the lungs. It has been suggested that the levels of this chemokine in serum could be a potential biomarker of human rhinovirus infection at exacerbation (Quint et al. 2010). By finding a successful biomarker for the persistent asymptomatic pneumococcal infection, it would reduce the number of mice being sacrificed, as organs would not need to be harvested for viability counts. Unfortunately this chemokine was not robust enough to be a potential biomarker; further work still needs to be carried out for a potential biomarker to be found.

Other cytokines that were measured were TNFα and IFNγ, which are both associated with an innate immune response (Borish and Steinke, 2003). In the BALF levels of both cytokines were significantly increased at 7 and 14 days post-infection. Only levels of TNFα were significantly increased at 24 hours post-infection, suggesting that it was involved in the early immune response, whereas levels of IFNγ were not significantly
increased until at least 7 days post-infection. At 21 and 28 days post-infection there were no significant differences observed in the levels of either cytokine in the BALF.

The only cytokine that was significantly increased at all the time points assessed was IL-12p40. The subunit IL-12p40 and the IL-12p70 heterodimer bind together to form the biologically active cytokine IL-12, which is produced by macrophages and B lymphocytes to stimulate the production of IFN-γ (Scott, 1993). However the IL-12p40 subunit has also been shown to bind with the subunit IL-23p19 to form the biologically active cytokine IL-23. IL-23 is expressed by activated dendritic and phagocytic cells and is thought to be important for the recruitment of inflammatory cells required for chronic inflammation (Langrish et al. 2004). The ELISA assay used in this study was specific for the sub-unit IL-12p40, therefore it was not possible to distinguish between the biologically active forms of the cytokines IL-12 and IL-23.

When bound the IL-12p40 subunit is known to activate and induce proliferation of natural killer cells, T-helper cells and cytotoxic lymphocytes (Borish and Steinke, 2003). The increased levels of this cytokine at all the time points assessed, could suggest that there was a persistent inflammation associated with the pneumococcal infection. To understand this further the inflammation in the lungs was assessed by histopathology.

From the lung sections taken for histology it could be seen that the inflammation in response to the infection changes over time. At twenty-four hours post-infection there was a diffuse inflammatory response that was present in all lobes of the lungs assessed. This consisted of a diffuse alveolitis with moderate perivascular and peribronchiolar cuffing of neutrophils and mononuclear cells. There was also a ‘pneumonic’ reaction observed, where a diffuse severe neutrophilic infiltration and extensive microvascular leakage in the alveolar bed was seen.

Though by 7 days post-infection the inflammation observed had changed, consolidation of airspaces with neutrophils, macrophages, foam cells, necrotic debris and type II pneumocytes were seen. In approximately half of the animals this consolidation was focal, however in the other half this consolidation was seen across the entire lobe. As well as these lobular and focal areas of consolidation a diffuse alveolitis with moderate
perivascular and peribronchiolar cuffing of neutrophils and mononuclear cells was still seen. It is important to remember that even though there were large areas of consolidated inflammation, the mice appeared asymptomatic.

By 14 days post-infection the inflammatory response had changed again however the same phenotype was also observed at both 21 and 28 days post-infection, though at 21 and 28 days post-infection the inflammatory response was milder. Consolidation of the airspaces was still observed however this was predominantly focal rather than lobular as seen at 7 days post-infection, by 28 days post-infection the incidence of these foci of consolidation was also reduced. Cuffing of the tracheobronchial tree with inflammatory cells was also observed, however the predominant cell type had shifted from being neutrophilic at 24 hours and 7 days post-infection to becoming predominantly monocytic. As well part of this change in inflammatory response, foci of fibroplasia were observed at the transitional airways (where the airway meets the alveolar bed); these were most common at 14 and 21 days post-infection but were still observed at 28 days post-infection. After seeing these foci of fibroplasia sections were stained with Masson Trichrome and at 14–28 days post-infection deposition of matrix was observed. Fibroplasia is a precursor of fibrosis, which could be associated with airway remodelling, which is a phenotype that has been observed in asthma and COPD (Redington, 2000). There are many recognised animal models of pulmonary fibrosis, which are induced by agents such as bleomycin, but it is generally recognised that S. pneumoniae is not one of these. Fibrosis is defined by the overgrowth, hardening or scarring of various tissues and is attributed to excess deposition of extracellular matrix components including collagen (Wynn, 2007). It is associated with the presence of a more chronic inflammation and damage that is more permanent, it is thought that an acute inflammatory response is generally cellular and rapidly cleared leaving no lasting evidence of damage (Wynn, 2008). This presence of fibroplasia suggests that the pneumococcal infection induces a more chronic inflammatory response. One hypothesis of how this fibroplasia arises is based on the presence of bronchoalveolar duct junctions (BADJ) at the transitional airways. These BADJ are known niches of progenitor cells, which could be activated by the persistent inflammation which results in these foci of fibroplasia (Snyder et al. 2009).
As well as observing inflammation that could be associated with a more chronic inflammatory response, inflammation was observed that could be associated with an adaptive immune response. Using immunohistochemistry it was possible to determine that at 21 and 28 days post-infection CD3-positive cells were present in perivascular cuffs of inflammation and also to inflammatory cuffs that were adjacent to areas of epithelial hyperplasia and damage. CD3 has been used as a generic marker for lymphocytes (D’Acquisto and Crompton, 2011). The presence of CD3-positive cells suggest that an adaptive response is occurring, however to better understand this response further work needs to be carried out to further define the subsets of the T cells present.

As the inflammatory response was focal in nature at the later time points a distribution study was conducted to understand whether there was any bias to which lobes the inflammatory response was observed in and also to understand how widespread the inflammatory response. It has been reported that in models of inflammation the method of instillation can be important as the distribution of the inflammatory compound can be altered by the route of administration (Donnelley et al. 2012). At 24 hours post-infection a diffuse inflammatory response was viewed in all lobes, suggesting that there was a uniform distribution of viable pneumococci after intranasal administration. However environmental factors such as the oxygen concentration in different parts of the lung may influence the localisation of the foci of inflammation as it is known that *S. pneumoniae* is a facultative anaerobe (Gingles et al. 2001). It has also been reported that *S. pneumoniae* was able to alter gene expression depending on whether it was growing planktonically or in a biofilm-like state (Oggoni et al. 2006). It would be interesting to investigate the gene expression of the pneumococci at different time points during the persistent infection to see if changes in gene expression could be linked to the inflammatory response observed in the host.

At 21 and 28 days post-infection it could be seen that foci of inflammation were present, but for all cytokines except IL-12p40 no difference was seen compared to naïve animals. This histopathology suggests an inflammatory response was occurring, but the majority of the lung was comparable to that of a naïve animal. To analyse any changes in the inflammatory response it would be best to look at the foci of inflammation rather than any changes across the whole lung. This was not within the scope of this project
but it could be done by using techniques such as micro-dissection or by using in situ hybridisation.

It was known that viable pneumococci could be recovered from the lungs of mice up to 28 days post-infection and an inflammatory response was observed in the host. To confirm that the inflammation observed was due to the pneumococcal infection sequential paraffin embedded lung sections were Gram stained. Lanceolate shaped Gram-positive diplococcal bacteria could be seen at seven days post-infection around the peribronchioles and in the perivascular adventitia. With the Gram stained sections, bacteria were not seen at any other time point, but this could be due to the low numbers of pneumococci in the lung. However sections stained with Periodic Acid Schiff Reagent (PAS) showed that lanceolate diplococci were present at other times post-infection. The PAS reaction stains complex carbohydrates such as glycogen and is known to stain bacteria (Moats, 1959), due to the polysaccharides in the cell wall. Although this did not confirm that the bacteria present is Gram positive, it did confirm the localisation of the bacteria and in conjunction with the viability counts determined from the BAL fluid suggested that the bacteria visualised with the PAS stain was *S. pneumoniae*.

As well as looking at the inflammation at a cellular level, during the time-course of infection lung function was assessed with an eSpira forced manoeuvres system. In humans spirometry, is a range of techniques that are used to assess the ability of the lung to function. FEV₁ is part of the GOLD standard for assessing airway obstruction and is one of the measures used to classify if a patient has COPD, and its severity (GOLD, 2013).

The eSpira forced manoeuvres system provided the ability to measure end points to assess lung functioning routinely measured in humans, in anaesthetised mice. The manoeuvres performed included; FEV, TLC, resistance and PEF. In humans, to assess the severity of airway obstruction, the FEV₁ is measured and compared to predicted values, the larger the decrease of FEV₁ from predicted values the greater the airway obstruction. Predicted values are based on the general population statistics and are dependent on factors such as age, ethnicity and sex. Unlike humans, mice cannot be instructed to breathe on command and also have a higher respiratory rate (anaesthetised
average number of breaths per minute was 170 for CBA/Ca mice). To counteract this, mice were anaesthetised, the trachea was then cannulated and the cannula was attached to a machine that was able to manipulate breathing in a controlled repeatable manner. Cannulated mice were able to breathe spontaneously, except during one of the three forced manoeuvres where the breathing was controlled. The FEV was measured at set points over a time course (25 – 75 milliseconds) as it was unknown how the FEV\textsubscript{1} in humans translates to FEV in mice. The GOLD report states that COPD sufferers with a predicted FEV\textsubscript{1} of between 50 and 80% are defined as having moderate COPD (GOLD, 2011). A reduction of this magnitude was seen at 7 days post-infection suggesting that the model that had been set up induced changes in lung mechanics that were clinically relevant to COPD. However a significant decrease in FEV was also observed at 14 and 21 days post-infection but not at 24 hours or 28 days post-infection.

Another flow based parameter that was analysed was PEF, which is the maximal flow (or speed) achieved during the maximally forced expiration initiated at full inspiration, measured in liters per minute. The reduction in PEF suggests that the lung was being constricted, which could be due to the inflammatory cells in the airways. All the changes observed in the lung function were associated with an increase in inflammation rather than any structural changes in the lungs such as changes seen with small airways disease or emphysema. These structural changes would be associated with an increase in residual volume and total lung capacity, whereas a decrease in these parameters was observed.

As well as looking at the rate of flow, different end points that measured the capacity volume of the lungs were assessed at different times post-infection. It was observed that significant reductions in the capacity and volume of the lungs was observed at 7 and 14 days post-infection which is when the inflammation observed was highest. This suggested that the reduction in capacity was directly related to the presence of inflammation to the inflammatory response.

In summary, the model achieved consistently had viable S. pneumoniae recovered from the lower airways four weeks post-infection in mice that showed no outward signs of disease. Low levels of inflammation were present; initially this was predominantly neutrophilic however by 14 days post-infection the main inflammatory population was
monocytic and lymphocytic with foci of fibroplasia. The presence of the pneumococcus also caused physiological changes to the lung such as a decreased PEF which could be due to the increased inflammation.

In the majority of experiments for the characterisation of this model naïve mice were used as the control group. What would have been better would have been to have a group of animals that had received a mock intranasal dose of saline at each time-point assessed post-infection. If the lung function experiment is taken as an example; five time points post-infection were assessed plus one control group of naïve mice, for each group ten mice were sacrificed which meant that there were sixty animals in the experiment. If naïve mice had been substituted with a group of ten mice mock infected with saline at each time point post-infection then an extra forty mice would have been used. It was decided to use naïve mice as if mice were mock infected with saline then they would have to be time matched with mice infected with pneumococcus. When planning in vivo experiments the number of mice being sacrificed has to be considered as one of the 3R’s is to reduce the number of animals used in research. Would it have been ethical to cull forty extra mice for use as negative controls? As well as considering whether it would have been ethical the practicality and cost of running an experiment with one hundred mice compared to sixty needed to also be considered. By increasing the size of the experiment by this number it would have meant that the quality of the data gathered from the experiment would also have been compromised as it could not be run competently by one person which would have invalidated the whole of the experiment.

4.6.1 Investigation into whether the model of persistent infection can be established in a different inbred strain of mouse

The next step was to assess whether the model of persistent infection could be established in inbred Balb/c mice rather than inbred CBA/Ca mice. During the establishment of this model, it was seen over 50% of MF1 mice did not have detectable pneumococci in the lungs 7 days post-infection with pneumococcal strain LgSt215. It has been reported that different mouse strains have differing susceptibilities to pneumococcal infection with a serotype 2 pneumococcus, with Balb/c mice able to clear
the infection, whereas CBA/Ca mice succumbed within 36 hours of infection (Gingles et al. 2001).

This suggested that the Balb/c strain of mouse would be able to clear the persistent pneumococcal infection however many models of inflammation have been optimised in the Balb/c strain of mouse (Bartlett et al. 2008, Natarajan et al. 2010, Nials and Uddin, 2008). As the next aim of the project was to combine the newly established model of persistent pneumococcal infection with other models of inflammation to better understand triggers for exacerbations in severe respiratory diseases such as asthma and COPD it would be beneficial to be able to demonstrate that the model of persistent infection could be established in the Balb/c strain of mouse.

It was observed that by 7 days post-infection 50 % of Balb/c mice had cleared the persistent pneumococcal infection and by 14 days post-infection there were no detectable viable pneumococci from the lungs of Balb/c mice. At all times assessed post-infection there were significantly lower numbers of pneumococci, neutrophils and macrophages in the BALF of Balb/c mice compared to in the BALF of CBA/Ca mice. This suggested that Balb/c mice were able to clear the pneumococci and that the level of inflammation could be directly associated with pneumococcal number. It has been reported that in response to a serotype 2 acute infection the cytokine Balb/c mice express a higher level of the cytokine TGF-β in the lungs compared to CBA/Ca mice which led to a rapid rise in T regulatory cells (Neill et al. 2012). This would suggest that it is the initial immune response of the host that is important in the establishment or clearance of a pneumococcal infection.

Even though Balb/c mice were able to clear the pneumococcal infection with strain LgSt215 by 14 days post-infection at 7 days post-infection 50 % of mice did have detectable numbers of viable pneumococci. This could potentially could be used to test hypotheses of whether other models of inflammation impair the clearance of pneumococci. It has been suggested that insults such as cigarette smoke impair bacterial clearance (Phipps et al. 2010), however in this study an acute model of pneumococcal infection. By combining a model of cigarette smoke-induced lung injury, with a model of low-level pneumococcal infection where half of mice were able to clear the infection it would provide the ability to follow inflammatory effects. Something similar has been
done by Essilfie et al. (2012), who showed that by combining a model of OVA-induced inflammation with a *H. influenzae* infection that the infection persisted for longer when the OVA-induced inflammation was present.
Combination of the persistent pneumococcal infection with a model of inflammation associated with COPD:

Development of a model of airway exacerbation

One of the hallmarks in the disease progression of COPD is the periodic, acute worsening of symptoms, which is termed an exacerbation (Seemungal et al. 1998b). After a model of persistent pneumococcal infection in the lower airways had been established, then the next aim was to combine this with another model of inflammation that was associated with severe respiratory disease, such as asthma and COPD. This would be done to better understand the role that bacterial colonisation may play in the pathology of these diseases.

Establishment of a murine model of neutrophilia

One model that has historically been used to study COPD is a model of LPS-induced lung injury in the mouse (Wright et al. 2008). After intranasal or intratracheal instillation of LPS, a large influx of neutrophils into the lungs was observed (Matute-Bello et al. 2008). It is accepted that neutrophils are one of the key cell types that contribute to the disease progression of COPD, due to their release of neutrophil elastase (Abboud and Vimalanathan, 2008), thus the LPS-induced model is viewed as appropriate.

As there was no established model of LPS induced neutrophilia at the University of Leicester a model of LPS inflammation needed to be established, before it could be used in combination with the model of persistent pneumococcal infection. For the establishment of this lung injury model, two intranasal concentrations of LPS (Sigma, UK) were tested (1.75 and 3.5 µg /50 µl) and animals were culled at 24 and 48 hours post intranasal challenge to determine when the highest number of neutrophils were observed. At time of culling, BALF was harvested as described in section 2.5.3 and differential cell counts were performed.

Figure 5.1A shows the differential cell counts, at both concentrations of LPS tested at 24 and 48 hours post instillation. The number of immune cells was increased compared to mice that had been administered an equivalent volume of saline. The main cell population of interest was the neutrophils and as can be seen in Figure 5.1B, the number of neutrophils in the BALF at 24 hours with both concentrations tested was significantly raised (p < 0.05) compared to animals dosed with saline. The number of neutrophils was raised at 48 hours post challenge with 1.75 µg /50 µl of LPS but this was not significant (p > 0.05). In Figure 5.1C it can be
seen that the number of macrophages was unaffected at both time points and at both doses, compared to animals dosed with saline. The number of immune cells in the group that was dosed with 3.5 µg /50 µl of LPS at 48 hours was too high to count (> 350 cells / field of view) so has been excluded from the results.

**Figure 5.1** – Differential cell counts of the BALF of CBA/Ca mice after intranasal challenge with LPS. A – total cell counts, B – number of neutrophils and C – number of macrophages in the BALF of CBA/Ca mice. A Kruskal-Wallis non-parametric test with a Dunns post-test was used to compare differences, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.

### 5.1.2 Does an underlying pneumococcal infection in the lower airways increase the inflammatory response to a model of neutrophilia?

As a model of acute LPS neutrophilia had now been established in CBA/Ca mice it was decided to combine it with the model of persistent pneumococcal infection. CBA/Ca mice were intranasally dosed with 4 x 10⁶ CFU (strain LgSt2150) suspended in 30 µl, fourteen days later mice were challenged with 3.5 µg LPS suspended in 50 µl and culled 6 hours later. At time of culling BALF was harvested and lungs were distended with 10 % formalin for histology.

The first question investigated was whether the introduction of an acute neutrophilia caused a change in the number of pneumococci present in the lower airways. As can be seen in Figure
there was no statistical difference (p > 0.05) in the number of pneumococci in the BALF of CBA/Ca mice that had been challenged with both LPS and strain LgSt215, compared to mice that had just been dosed with pneumococcal strain LgSt215.

Figure 5.2 – Viable pneumococci in the BALF of CBA/Ca mice were enumerated at the time of culling. A persistent pneumococcal infection with LgSt215 was established in CBA/Ca mice, 14 days after the induction of infection an intranasal challenge of LPS was administered and mice were culled on day 15, each point is one mouse.

As an LPS challenge on day 14 of the persistent pneumococcal infection with strain LgSt215 in CBA/Ca mice did not alter the number of pneumococci in the BALF, the next question asked was whether the inflammatory cellular profile was altered. As shown in Figure 5.3A, there was a significant increase (p < 0.001) in the number of neutrophils in the BALF in CBA/Ca mice that had an LPS challenge on day 14 of persistent pneumococcal infection compared to the number of neutrophils in the BALF of naïve mice. Figure 5.3A suggests that CBA/Ca mice challenged with either LPS alone or pneumococcal infection alone had a small increase in neutrophils compared to mice dosed with saline but this was not significant (p > 0.05) when analysed with a Kruskal-Wallis non-parametric test with a Dunns post-test. However, when the level of neutrophils in either LPS alone or infection alone groups were compared to mice dosed with saline and analysed with a Mann-Whitney statistical test then the number of neutrophils were significantly raised (p < 0.05) compared to mice dosed with saline for both conditions. Figure 5.3B shows that no statistical differences (p > 0.05) were observed in the number of macrophages in the BALF of any of the conditions investigated with either a Mann-Whitney or a Kruskal-Wallis statistical test.
Differential cell counts of the immune cells in the BALF of CBA/Ca mice were performed at the time of culling. A persistent pneumococcal infection with LgSt215 was established in CBA/Ca mice, 14 days after the induction of infection an intranasal challenge of LPS was administered and mice were culled 6 hours post LPS challenge. A – number of neutrophils and B – number of macrophages in the BALF, a Kruskal-Wallis non-parametric test with a Dunns post-test was used to compare differences, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.

To better understand the inflammatory cell profile, the levels of four cytokines; KC, TNFα, IL-12 p40 and IL-6 were assessed in the BALF by sandwich ELISA. As can be seen in Figure 5.4A the cytokine KC was significantly raised (p < 0.05) in the BALF of CBA/Ca mice that had been challenged with LPS alone and LPS at day 14 of a persistent pneumococcal infection compared to the level measured in the BALF of mice mock challenged with saline. Figure 5.4A also shows that the level of KC was significantly raised (p < 0.05) in the BALF of CBA/Ca mice with a persistent pneumococcal infection and which had been challenged with LPS compared to CBA/Ca mice that only had a persistent pneumococcal infection. It can be seen in Figure 5.4B that the cytokine TNFα was significantly raised (p < 0.05) in the BALF of CBA/Ca mice challenged with both LPS and pneumococcal strain LgSt215 compared to the level of TNFα in the BALF of mice dosed with saline or CBA/Ca mice that had only been challenged with pneumococcal strain LgSt215. The level of TNFα was higher in the BALF of mice that had been challenged with LPS alone, was not significantly higher (p > 0.05) compared to mice challenged with saline alone (Figure 5.4B). Figure 5.4C shows that the level of IL-12 p40 was significantly (p < 0.05) higher in mice that had been challenged with LPS and pneumococcal strain LgSt215 compared to the level measured in the BALF of mice dosed with either saline or only pneumococcal strain LgSt215. The last cytokine to be
measured was IL-6. As can be seen in Figure 5.4D, this cytokine was not significantly different (p > 0.05) in the BALF of any of the conditions investigated.

**Figure 5.4** – Cytokine levels in the BALF of CBA/Ca mice at time of culling were measured by sandwich ELISA. A persistent pneumococcal infection with LgSt215 was established in CBA/Ca mice, 14 days after the induction of infection an intranasal challenge of LPS was administered and mice were culled 6 hours post challenge. A – level of KC, B – level of TNFα, C – level of the cytokine subunit IL-12 p40 and D – level of IL-6, a Kruskal-Wallis non-parametric test with a Dunns post-test was used to compare differences. * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.

From the differential cell counts it could be seen that there was a significant increase (p < 0.05) in the number of neutrophils in the BALF when an intranasal LPS challenge was performed on day 14 and mice were culled 6 hours later compared to mice dosed with saline. Histopathology was assessed for localisation of inflammatory cells and whether there were
any changes in pathology. As can be seen in Figure 5.5A, 6 hours post-LPS challenge there was an influx of neutrophils which localised to mild peribronchiolar and perivascular cuffs. Figure 5.5B shows the typical foci of inflammation observed on day 14 post-infection with strain LgSt215 in CBA/Ca mice, severe perivascular cuffs can be seen with a diffuse pneumonitis and there were also an increased number of granulocytes and alveolar macrophages in the alveolar bed. In Figure 5.5C it can be seen that the LPS challenge on day 14 of a pneumococcal infection still induced perivascular cuffing, however there were also type II pneumocytes proliferating in the alveolar bed, causing consolidation of the airspaces.

**Figure 5.5** – Formalin fixed, paraffin embedded, H&E stained lung sections of CBA/Ca mice challenged with LPS of pneumococcal strain LgSt215, A – LPS challenge only, B – Pneumococcal infection only, C – LPS challenge on day 14 of a pneumococcal infection with strain LgSt215. Arrows indicate peribronchiolar and perivascular cuffing, v = blood vessel, b = bronchiole.
As well as the pathology observed in Figure 5.5A, Figure 5.6 shows more pathology associated with the LPS challenge on day 14 of a pneumococcal infection. Figure 5.6A is a low power magnification of Figure 5.6B which shows the extent of the observed pneumonitis. There is a mix of leukocytes (Figure 5.6B, arrows) and proliferating type II pneumocytes (Figure 5.6B, arrow heads), which are beginning to fill up alveolar spaces.

**Figure 5.6** – Formalin fixed, paraffin embedded, H&E stained lung sections of CBA/Ca mice challenged with LPS on day 14 of a pneumococcal infection with strain LgSt215. A – Low magnification (x 100), B – higher magnification (x 200). Arrows indicate leukocytes, arrow heads indicate proliferating type II pneumocytes, v = blood vessel, b = bronchiole.

Overall the pathology observed showed that although the addition of LPS on day 14 of a pneumococcal infection increased the neutrophil infiltrate, it did not significantly alter any other observed pathology seen at 14 days post-infection with pneumococci.

### 5.2 Treatment of a COPD exacerbation

As part of the management of COPD, inhaled and oral corticosteroids are used, which can lead to small improvements in postbronchodilator FEV\(_1\) (Celli et al. 2004), even though a large proportion of COPD sufferers are resistant to even high doses of corticosteroids (Barnes, 2013). However, during an exacerbation one of the recommended therapies is a daily dose of 30 – 40 mg / kg prednisolone per day for 10-14 days (GOLD, 2013). Though there is much debate on whether dosing for a shorter period time (5 days) could be as effective, and also have the advantage of fewer side effects from prolonged use of corticosteroids (Leuppi et al. 2013).
5.2.1 Effect of corticosteroid treatment on a persistent pneumococcal infection of the lower airways

In the first instance, the effect of administration of corticosteroid affected the established model of persistent pneumococcal infection of the lower airways was investigated. In the clinic, the use of inhaled corticosteroids has been associated with an excess risk of pneumonia hospitalisation (Ernst et al. 2007). This could be due to the suppressive effect that corticosteroids have on the inflammatory profile, which could lead to increased susceptibility to pulmonary bacterial infections. Patterson et al. (2012) showed that exposure of inbred mice to inhaled corticosteroid, followed by a pulmonary challenge of Klebsiella pneumoniae led to an increased bacterial burden as well as decreased survival.

When considering the effects of steroid on the persistent bacterial infection model it was important to remember that different pathologies were observed during the time-course of the persistent pneumococcal infection of the lower airways. Two different questions were asked, the first was whether the administration of the corticosteroid prednisolone affected the establishment of pneumococcal infection (Figure 5.7A) and the second question asked was whether the same corticosteroid affected an established low level pneumococcal infection of the lower airways (Figure 5.7B). Both questions were important because both pathologies can be observed in the clinic (Rodrigues et al. 1992).

![Figure 5.7](image)

**Figure 5.7** – Dose regime protocols that were tested in inbred CBA/Ca mice. On the days indicated with a star, CBA/Ca mice were dosed orally with 10 mg / kg prednisolone. On the days indicated with an orange hour CBA/Ca mice were intranasally infected with pneumococcal strain LgSt215. A – prophylactic protocol, mice were dosed with prednisolone from day -1 to day 5 post-infection, mice were culled on day 4 to assess lung function and day 6 to assess changes in inflammation, B – therapeutic protocol, mice were dosed with prednisolone day 14 to day 17 post-infection and culled 24 hours later on day 18.
5.2.2 Investigation into how prednisolone affected the establishment of a persistent pneumococcal infection of the lower airways

To assess the progression of disease and how the corticosteroid was affecting this, CBA/Ca mice were weighed daily. Figure 5.8A shows how the weights changed. It can be seen that CBA/Ca mice lose weight after being challenged with pneumococcal strain LgSt215; however this was increased when prednisolone was also administered daily. As shown in Figure 5.8B, at time of culling the percentage loss in weight was significantly higher ($p < 0.05$) in CBA/Ca mice that were dosed with prednisolone and challenged with pneumococcal strain LgSt215.

**Figure 5.8** – CBA/Ca mice were dosed daily with 10 mg / kg prednisolone from the day before intranasal infection with pneumococcal strain LgSt215 until day 6. A – Mice were weighed daily, B – percentage change in weight at time of culling. A Kruskal-Wallis non-parametric test with a Dunns post-test was used to compare differences, * $p < 0.05 > 0.01$, ** $p < 0.01 > 0.001$, *** $p < 0.001$.

As has been shown in Figure 5.8, there was a significant reduction in the weights of CBA/Ca at time of culling when they received corticosteroid and pneumococcal infection. As well as this weight loss, the administration of prednisolone caused a significant increase ($p < 0.01$) in the number of viable pneumococci in the BALF compared to mice that were only challenged with pneumococci (Figure 5.9).
Figure 5.9 – CBA/Ca mice were dosed daily with 10 mg / kg prednisolone from the day before intranasal infection with pneumococcal strain LgSt215 until day 6. At time of culling CFU in the BALF was enumerated. A Mann-Whitney test was used to compare differences, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.

Differential cell counts were performed on the BALF harvested at 4 and 6 days post-infection. Figure 5.10A shows that there were less inflammatory cells in the BALF at 4 days post-infection compared to 6 days post-infection. It can also be seen in Figure 5.10A that the mice that were challenged with both steroid and pneumococcal infection had the highest number of inflammatory cells in the BALF compared with the other groups. However at 6 days post-infection (Figure 5.10B) the group that only had a persistent pneumococcal infection had the highest number of inflammatory cells in the BALF, with the infected group dosed with prednisolone having a reduced number of inflammatory cells. Never the less this was higher than the number of inflammatory cells in the BALF of mice that had only been dosed with saline or steroid.
Figure 5.10 - Differential cell counts of the immune cells in the BALF of CBA/Ca mice were performed at the time of culling. CBA/Ca mice were dosed daily with 10 mg / kg prednisolone from the day before intranasal infection with pneumococcal strain LgSt215 until culling. A – total cell counts in the BALF at 4 days post-infection and B – total cell counts in the BALF at 6 days post-infection.

Individual cell populations are shown in Figure 5.11A and B show the neutrophil numbers in the BALF at time of culling, whereas Figure 5.11C and D show the macrophage number. It can be seen in Figure 5.11A that the number of neutrophils in the BALF was significantly higher (p < 0.001) in inbred CBA/Ca mice that had been dosed with prednisolone and pneumococcal infection compared to mice that had only been dosed with saline or pneumococcal infection alone. However by 6 days post-infection there was no significant effect of prednisolone on the number of neutrophils in the BALF in the model of persistent infection. Figure 5.11B shows that the number of neutrophils in the BALF of mice that had a persistent pneumococcal infection and mice that had a persistent pneumococcal infection and had also been dosed daily with 10 mg / kg prednisolone was significantly higher (p < 0.05) compared to mice that had only been dosed with saline. As can be seen in Figure 5.11C, at 4 days post-infection there was no difference observed in the number of macrophages in the BALF of any of the conditions tested. At 6 days post-infection the number of macrophages in
the BALF of mice that had been dosed with the infection model alone was not different to the saline control group (p > 0.05).

**Figure 5.11** – Differential cell counts of the immune cells in the BALF of CBA/Ca mice were performed at the time of culling. CBA/Ca mice were dosed daily with 10 mg / kg prednisolone from the day before intranasal infection with pneumococcal strain LgSt215 until culling. A – neutrophils at 4 days post-infection, B – neutrophils at 6 days post-infection, C – macrophages at 4 days post-infection and D – macrophages at 6 days post-infection, a Kruskal-Wallis non-parametric test with a Dunns post-test was used to compare differences, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.

Physiological parameters were only assessed on mice at 4 days post-infection because mice at day 6 post-infection had lost over 25 % of their body weight, they were too thin to be anaesthetised and attached to the eSpira forced manoeuvres system (EMMS). As can be seen
in Figure 5.12, no changes to the physiological functioning of the lung were observed. Figure 5.12A – C show that the flow-based parameters FEV, tidal volume and peak expiratory flow (forced) respectively were unaffected by the presence of infection or corticosteroid. Figure 5.12D shows that there were no significant changes in airway resistance (p > 0.05).

**Figure 5.12** – CBA/Ca mice were dosed daily with 10 mg / kg prednisolone from the day before intranasal infection with pneumococcal strain LgSt215 until 4 days post-infection when lung function was assessed with an eSpira forced manoeuvres system (EMMS). A – FEV was assessed at set time points over a time course (25 – 60 milliseconds), B – Total lung capacity, C – peak expiratory flow (forced) and D – airway resistance, a Kruskal-Wallis non-parametric test with a Dunns post-test was used to compare differences, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.

No significant changes in physiology were observed in saline control groups and animals treated with steroid alone. When steroid was given to CBA/Ca mice that also received a pneumococcal infection with strain LgSt215, there was an increase in the areas of consolidation and necrotic debris, as well as an increase in incidence of inflammatory foci compared to mice that had only been challenged with pneumococci. There was also an
apparent reduction in the number of alveolar macrophages and foam cells in the alveolar bed in areas of inflammation in animals that had been dosed with prednisolone and pneumococcal infection compared to pneumococcal infection alone. Figure 5.13A shows that the pathology observed with the pneumococcal infection alone at day 6 post-infection was similar to that seen previously at 7 days post-infection. Severe perivascular cuffs were observed as well as pneumonitis and debris in the bronchioles. Figure 5.13B shows areas of consolidation with airspaces entirely filled with type II pneumocytes, granulocytes and alveolar macrophages. When prednisolone was given in conjunction with the pneumococcal challenge, a similar pathology was observed compared to that seen with infection alone, however there were some differences. As can be seen in Figure 5.13C, there was debris in the transitional airways (shown by a star), as well as many inflammatory cells in the alveolar bed (arrow) and hypertrophic type II pneumocytes (arrowhead). In Figure 5.13D foci of neutrophilia were observed, which were similar to those seen with the pneumococcal infection alone.

Figure 5.13 – Formalin fixed, paraffin embedded, H&E stained lung sections of CBA/Ca mice challenged with pneumococcal strain LgSt215 and dosed daily with 10 mg / kg prednisolone, A and B – pneumococcal infection only, C and D – pneumococcal infection and dosed daily with prednisolone. Arrows indicate inflammatory cells in the alveolar bed, star indicates debris in transitional airways, arrowheads indicate proliferating type II pneumocytes, v = blood vessel and B = bronchiole.
5.2.3 Investigation into how prednisolone affected an established persistent pneumococcal infection of the lower airways

As shown in the previous section, when the corticosteroid prednisolone was given prophylactically from the day before pneumococcal infection to day 6 post-infection, there was a significant (p < 0.05) increase in disease severity. This showed how prednisolone affected the establishment of a pneumococcal infection, but did not test what effect corticosteroid had on an established low level persistent pneumococcal infection in the lower airways. To do this, CBA/Ca mice were infected with the pneumococcal strain LgSt215, 14 days post-infection, prednisolone was given daily at 10 mg/kg for 4 days, as shown in Figure 5.14A. As can be seen in Figure 5.14B, at the time of culling there was no significant difference (p > 0.05) in the number of viable pneumococci in the BALF of CBA/Ca mice that had been dosed with prednisolone in addition to a pneumococcal infection, compared to CBA/Ca mice that had only been dosed with pneumococci. It was observed, that when prednisolone was given prophylactically in combination with the pneumococcal infection, mice lost a significant proportion of their body weight. With the dosing regimen shown in Figure 5.14A a significant drop in weight was noted (Figure 5.14C) compared to animals dosed with saline. However as can be seen in Table 5.1, the overall average weight loss when prednisolone was given therapeutically was 9.1 %, but when given prophylactically, the average weight loss observed was 26.3 %.
Figure 5.14 - CBA/Ca mice were dosed daily with 10 mg / kg prednisolone from the day 14 to day 18 post-infection with pneumococcal strain LgSt215. A – protocol used, B – the number of viable pneumococci in the BALF was enumerated at the time of culling and C – percentage change in weight at time of culling. A Kruskal-Wallis non-parametric test with a Dunns post-test was used to compare differences, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.

Table 5.1 – Average (mean) weight loss observed on day of culling compared to the weight of CBA/Ca mice at the start of the protocol described in Figure 5.7.

<table>
<thead>
<tr>
<th>Group</th>
<th>Prophylactic Dosing Regime Change in weight (%)</th>
<th>Therapeutic Dosing Regime Change in weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>- 5.4</td>
<td>+ 4.0</td>
</tr>
<tr>
<td>Prednisolone alone</td>
<td>- 5.8</td>
<td>0.0</td>
</tr>
<tr>
<td>Infection Alone</td>
<td>- 14.0</td>
<td>- 3.6</td>
</tr>
<tr>
<td>Infection + Prednisolone</td>
<td>- 26.3</td>
<td>- 9.1</td>
</tr>
</tbody>
</table>

When prednisolone was given prophylactically there was a significant (p < 0.01) increase in the number of neutrophils in the BALF compared to CBA/Ca mice that only had a pneumococcal infection in the lower airways. However, as can be seen in Figure 5.15B although the number of neutrophils in the BALF of CBA/Ca mice that received both
prednisolone and pneumococci were significantly higher (p < 0.05) compared to CBA/Ca mice dosed only with saline, there was no significant difference (p > 0.05) with CBA/Ca mice that had a persistent pneumococcal infection. Figure 5.15B shows that there were no statistical differences (p > 0.05) in the number of macrophages in the BALF of any of the conditions evaluated.

**Figure 5.15** - Differential cell counts of the immune cells in the BALF of CBA/Ca mice were performed at the time of culling. CBA/Ca mice were dosed daily with 10 mg / kg prednisolone from day 14 to day 18 post-infection with pneumococcal strain LgSt215. A – neutrophils, B – macrophages, a Kruskal-Wallis non-parametric test with a Dunns post-test was used to compare differences, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.

As well as investigating changes at a cellular level, physiological parameters were determined with an eSpira forced manoeuvres system (EMMS). Figure 5.16A shows that although there was an apparent decrease in FEV with infection, and infection plus prednisolone, this reduction was not significant (p > 0.05). Figure 5.16B and C show that the other flow based parameters measured (tidal volume and peak expiratory flow (forced)) were also unaffected by the presence of infection or corticosteroid. Though Figure 5.16C does show that the forced peak expiratory was significantly lower (p < 0.05) in CBA/Ca mice that had been dosed therapeutically with prednisolone in addition to a pneumococcal infection, compared to mice that had only been dosed with prednisolone. Figure 5.16D shows that no significant changes in airway resistance were observed in any of the groups studied.
Figure 5.16 - CBA/Ca mice were dosed daily with 10 mg / kg prednisolone from day 14 to day 18 post-infection with pneumococcal strain LgSt215, on day 18 lung function was assessed with an eSpira forced manoeuvres system (EMMS). A – FEV was assessed at set time points over a time course (25 – 60 milliseconds), B – Total lung capacity, C – peak expiratory flow (forced) and D – airway resistance, a Kruskal-Wallis non-parametric test with a Dunns post-test was used to compare differences, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.

5.3 Effect of corticosteroid treatment on a combined model of LPS-induced lung injury and a persistent pneumococcal infection of the lower airways

As shown in section 5.1.2, there was a significant (p < 0.05) increase in neutrophilia after CBA/Ca mice with a persistent pneumococcal presence in the lower airways were challenged intranasally with 3.5 µg / 50 µl LPS. This increase could represent a model of COPD exacerbation, with an intranasal challenge of LPS acting as a trigger of a worsening of disease severity. As previously discussed (section 5.2) corticosteroids are used in the clinic to treat exacerbations. It was decided to combine the ‘model of COPD exacerbation’ with administration of prednisolone to see whether it would change the inflammatory profile.

CBA/Ca mice were intranasally dosed with 4 x 10⁶ CFU (strain LgSt2150) suspended in 30 µl, fourteen days later 10 mg/kg prednisolone was administered orally, one hour later mice
were challenged with 3.5 µg LPS suspended in 50 µl and then culled 6 hours after the intranasal challenge of LPS. At time of culling BALF was harvested and lungs were distended with 10 % v/v formalin for histology.

Differential cell counts were performed, Figure 5.1 shows the number of neutrophils in the BALF and it can be seen that the number of neutrophils was significantly increased (p < 0.05) in the groups that received LPS and prednisolone, infection and LPS and infection, LPS and prednisolone compared to the number of neutrophils in the BALF of CBA/Ca mice dosed with saline. There was also a significant difference (p < 0.05) in neutrophil number in infected CBA/Ca mice given LPS compared to CBA/Ca mice that were challenged with pneumococci and prednisolone. Figure 5.17B shows that although there were significant changes in the neutrophil number in the BALF, there were no significant differences (p > 0.05) in the numbers of macrophages in any group in the BALF.

Figure 5.17 - CBA/Ca mice challenged with prednisolone and LPS on day 14 of a pneumococcal infection with strain LgSt215, BALF was harvested at time of culling and differential cell counts were performed, A – neutrophils and B – macrophages. Kruskal-Wallis non-parametric test with a Dunns post-test was used to compare differences, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.

As well as differential cell counts, the levels of four cytokines; KC, TNF α, IL-12 p40 and IP-10 were determined in the BALF by sandwich ELISA. Figure 5.18A shows the level of KC in
the BALF of CBA/Ca mice. It can be seen in Figure 5.18A that KC levels were significantly (p < 0.05) raised in the BALF of animals dosed with LPS compared to CBA/Ca mice dosed with saline, LPS and prednisolone, infection plus prednisolone and pneumococcal infection alone. Figure 5.18B shows the levels of TNFα in the BALF, levels of TNFα were significantly raised (p < 0.01) compared to animals dosed with saline alone and infection with prednisolone, however they were significantly lower than TNFα levels in the BALF of CBA/Ca mice challenged with LPS and pneumococcal infection. Levels of TNFα were significantly decreased (p < 0.01) when prednisolone was also given in addition to the pneumococcal infection with an LPS challenge.

**Figure 5.18** - CBA/Ca mice challenged with prednisolone and LPS on day 14 of a pneumococcal infection with strain LgSt215, BALF was harvested at time of culling and the levels of cytokines were determined by sandwich ELISA. A – level of KC and B – level of TNFα. Kruskal-Wallis non-parametric test with a Dunns post-test was used to compare differences, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.

The other two cytokine/chemokine levels that were measured were for the subunit IL-12 p40 and IP-10. Figure 5.19A shows that an oral dose of prednisolone did not statistically (p > 0.05) affect the level of IL-12 p40 in the BALF. Levels of IL-12 p40 was significantly (p < 0.05) raised in the BALF of CBA/Ca mice that had a persistent pneumococcal infection and had an intranasal insult of LPS, regardless of whether prednisolone had also been given. Levels of IL-12 p40 were significantly raised (p < 0.05) in the BALF of CBA/Ca mice that had a persistent pneumococcal infection and had also been challenged with LPS and
prednisolone, compared to CBA/Ca mice with a persistent pneumococcal infection that had only been challenged with LPS (Figure 5.19A). It can be seen in Figure 5.19B that levels of IP-10 were not significantly raised (p > 0.05) in the BALF of CBA/Ca mice under any of the conditions tested.

![Graph showing cytokine levels](image)

**Figure 5.19** - CBA/Ca mice challenged with prednisolone and LPS on day 14 of a pneumococcal infection with strain LgSt215, BALF was harvested at time of culling and the levels of cytokines were determined by sandwich ELISA, A – level of IL-12 p40 and B – level of IP-10. Kruskal-Wallis non-parametric test with a Dunns post-test was used to compare differences, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.

As well as the differential cell counts and sandwich ELISA data, histopathology was used to better understand the effect prednisolone had on the inflammation induced by the LPS challenge on a persistent pneumococcal infection. As can be seen in Figure 5.20A, 6 hours after the intranasal LPS challenge, peribronchiolar and perivascular cuffing was observed (Figure 5.20A arrows), which was typical of an LPS response. Figure 5.20B shows that the oral administration of prednisolone one hour before the LPS challenge caused a reduction in the severity of this peribronchiolar and perivascular cuffing (Figure 5.20B arrows).

It can be seen in Figure 5.20C that, 14 days into the persistent pneumococcal infection, pneumonitis was observed, as well as perivascular leukocytes (predominantly macrophages and granulocytes) although there were also granulocytes and alveolar macrophages in the
alveolar bed. This was similar to the response that has been seen previously (section 4.2.4.1), in response to a persistent pneumococcal infection of the lower airways at 14 days post-infection. However after a single dose of prednisolone, there appeared to be reduction in the incidence of the pneumonitis, but this inflammation was replaced with a severe alveolitis which consisted predominantly of granulocytes (Figure 5.20D). Large perivascular and peribronchiolar cuffs were observed regardless of whether prednisolone had been dosed or not.

As shown in section 5.1.2, an intranasal challenge of LPS on day 14 of a pneumococcal infection led to an increase in neutrophilic infiltrate within the alveolar bed (Figure 5.20E). Treatment with prednisolone an hour before the LPS challenge on day 14 of the persistent pneumococcal infection caused a diffuse severe alveolitis consisting of neutrophils, macrophages and debris. Dosing with prednisolone seemed to reduce the incidence of consolidation. In Figure 5.20F multifocal accumulations of granulocytes (Figure 5.20F, stars) in the alveolar bed were seen in response to prednisolone being given before an LPS challenge on day 14 of a persistent pneumococcal infection. However treatment with prednisolone was unable to resolve the inflammation entirely because severe perivascular and peribronchiolar cuffs were still observed (Figure 5.20F).
Figure 5.20 - Formalin fixed, paraffin embedded, H&E stained lung sections of CBA/Ca mice challenged with prednisolone and LPS on day 14 of a pneumococcal infection with strain LgSt215. A – LPS alone, B – LPS and prednisolone, C – infection alone, D – infection and prednisolone, E – infection and LPS and F – infection, LPS and prednisolone. Arrows indicate peribronchiolar and perivascular cuffs, stars indicate granulocytes in alveolar bed, v = blood vessel, b = bronchiole.
5.4 Discussion:

There are many published models of inflammation, which mimic different aspects of the respiratory diseases; asthma and COPD. However current in vivo models sometimes offer poor translation to the human condition. It has been seen that novel compounds that have been proven to work in animals don’t always show efficacy in human trials (Rennard et al. 2007). One model of inflammation that has been missing is a model of long-term bacterial colonisation with human pathogens such as *S. pneumoniae* and nontypeable *H. Influenzae* (Stevenson and Birrell, 2011). This project has addressed this by developing a novel model of long-term persistent infection with the human respiratory pathogen *S. pneumoniae*. As a standalone model of infection it is unique, however to increase the translational value of the model it was combined with other models of inflammation, which mimic different phenotypes observed in respiratory disease.

This is not the first time that standalone models of inflammation have been combined together to model the largely heterogeneous phenotypes of inflammation and varied symptoms displayed in patients with the respiratory diseases; asthma and COPD, (Stevenson and Birrell, 2011, Wedzicha, 2000). Due to the heterogeneous nature of these diseases, it is a challenge to scientists to model this disease in one in vivo model. For many years models were developed that involved a single insult or a repeated insult with a single compound for example LPS (Savov et al. 2002), smoke (Churg et al. 2004) or elastase (Hamawaka et al. 2011). These successfully mimic some aspects of respiratory disease however the development of models of inflammation is an iterative process and it is a challenge to scientists developing these models to make them as clinically relevant as possible (Stevenson and Birrell, 2011).

One approach research groups have started to use is to combine two or more models of inflammation that mimic different aspects of respiratory disease with the aim of having a more clinically relevant model. One group have combined a model of *H. influenzae* infection with a model of OVA-induced allergic airway disease to mimic the neutrophilic airways inflammation observed in severe asthma (Essilfie et al. 2012). Whereas another group have gone one step further and combined a model of elastase and LPS-induced lung injury, to produce a model that mimicked COPD-like pathology features; goblet cell metaplasia, emphysema and decreased elastic recoil (Ganesan et al. 2010). The same group have taken this model of combined elastase and LPS-induced lung injury and combined it with a viral
insult with rhinovirus (Sajjan et al. 2009) and also a bacterial insult with nontypeable *H. influenzae* (Ganesan et al. 2012). Both groups combine models of inflammation with a bacterial insult with *H. influenzae*, however both models of infection were acute with mice challenged with bacteria alone clearing the infection from the lower airways within a few days. But, an interesting point to note was that both groups showed that when the bacterial insult was combined with another lung injury challenge, viable bacteria persisted in the lower airways for a longer period of time compared to when it was dosed alone. As it is well recognised that respiratory infections are important in the progression and exacerbations of these respiratory diseases (Starkey et al. 2013) it is important to be able to mimic the asymptomatic presence of pathogens in the lower airways (Stevenson and Birrell, 2011).

As it has been shown that the low level presence of pathogens, such as *S. pneumoniae* contribute to the disease progression in COPD (Sethi et al. 2006). It was decided that the first model of inflammation that the model of persistent infection was to be combined with was a model of LPS-induced lung injury as this has historically been used to model COPD (Wright et al. 2008). As there was no established model of LPS induced neutrophilia at the University of Leicester a model of LPS inflammation needed to be established, before it could be used in combination with the model of persistent pneumococcal infection.

Models of inflammation induced by LPS can either be classed as acute or chronic (Wright et al. 2008). In a chronic model of LPS-induced lung injury, LPS is repeatedly administered over a period of weeks, leading to chronic airway inflammation, which included perivascular lymphocytic aggregations, irreversible airway enlargement and airflow obstruction (Vernooy et al. 2002). Whereas for an acute model of LPS induced lung injury, mice were exposed to a single intranasal challenge of LPS, which induced an influx of neutrophils, which caused pulmonary damage (Matute-Bello et al. 2008). It was decided that an acute rather than chronic model of LPS induced injury would be most appropriate.

It has been observed that after a single administration of LPS to the lungs to Balb/c mice; damage was seen two to four hours after instillation, with maximal damage being observed between 24 and 48 hours post-instillation (Szarka et al. 1997). So for a successful acute model of LPS-induced lung injury, an influx of neutrophils into the lungs of CBA/Ca mice needed to be observed after a single intranasal instillation of LPS suspended in PBS.
With both doses of LPS tested an influx of neutrophils into the lungs, was seen at both 24 and 48 hours post-instillation in CBA/Ca mice. This fulfilled the criteria for success, as both doses induced neutrophilia at both time points tested, either dose could be utilised for future work.

The next step was to combine this newly established model of LPS-induced lung injury with the model of persistent pneumococcal infection. In order to do this it had to be decided when the induction of LPS should happen during the course of infection. As previously discussed in this thesis it was seen that different phases of inflammation were observed at different time points post-infection. At 24 hours post-infection a diffuse inflammation is observed across all the lobes of the lung and at 7 days post-infection consolidation of inflammation is observed that is typical of pneumonia in humans. As both these early time points post-infection are acute in the inflammatory profile observed, with high numbers of neutrophils in the BALF it was decided to choose a later time point post-infection in which to induce the acute model of LPS-induced lung injury, when a more chronic inflammation is observed in response to the persistent pneumococcal infection. From 14 days post-infection with the pneumococcal model alone, a more chronic inflammatory profile was observed which remains stable for a minimum of 2 weeks. It was decided that this should be the point when the acute model of LPS-induced should be induced, as there was a measurable neutrophilia present but this was at a lower level than observed at 24 hours and 7 days post-infection. This time point post-infection could also be thought to most closely mimic the low level progressive inflammation observed in stable state COPD sufferers with a low level persistent bacterial infection of the lower airways (Sethi et al. 2006).

At 14 days post-infection with the persistent model of pneumococcal infection mice were challenged intranasally with a single dose of LPS and culled 6 hours later. It was decided that mice should be culled at this earlier time point so that any additive changes in neutrophilia could be observed, as at 24 hours and 48 hours a strong neutrophilic response was observed yet it has been published that an increase in neutrophils occurs as early as 6 hours post-LPS challenge which is lower than that observed at 24 hours post-instillation (Ferretti et al. 2003).

At 6 hours post-instillation with LPS neutrophilia was observed in CBA/Ca mice, when LPS was instilled on day 14 post-infection with pneumococci however there was a significant increase in the neutrophilia observed. As an additive effect was observed in the number of neutrophils present it suggested that the neutrophils induced by the instillation of LPS was by
a different mechanism compared to the neutrophils present at 14 days post-infection with pneumococci.

To better understand the drivers of this neutrophilic inflammation, cytokine levels were measured in the BALF by sandwich ELISA. It was seen that at 6 hours post-instillation with LPS alone that KC was significantly raised compared to naive mice, although TNFα and IL-6 both appeared raised this change was not significant when compared to naive mice. At 14 days post-infection with pneumococci alone IL-12p40 and IL-6 also appeared to be raised against naive mice but neither was shown, to be statistically different from naive with a Kruskal-Wallis statistical test with a Dunn’s post-test. This statistical test was chosen as it takes into account all other differences observed. When the LPS was instilled on top of the pneumococcal infection it was observed that KC, TNFα and IL-12p40 were all significantly raised compared to naive animals and animals that had only received a pneumococcal infection. Again, there was a suggestion that there was an increase in IL-6 but this was not significant. It has been published that in mice the Toll-like receptor 4 (TLR4) is important for the inflammatory response to inhaled LPS in mice (Lorenz et al. 2001) and humans (Arbour et al. 2000). TLR4 mediates this response via the adaptor molecule MyD88 as this molecule has been shown to be essential for an inflammatory response to inhaled LPS. Mice deficient in the adaptor molecule MyD88 did not produce the cytokines TNF, IL-12p40 and KC in response to an inhaled LPS challenge (Noulin et al. 2005). It has also been seen that LPS and TNFα synergistically induce KC production by Clara cells (Elizur et al. 2007). TNFα is secreted mainly by monocytes and macrophages immediately after pathogen recognition, yet IL-12p40 is also produced by macrophages as well as other immune cells as a late response to TLR activation (Avni et al. 2009).

With the persistent pneumococcal infection model, at 14 days post-infection an adaptive immune response is already in progress as discussed previously in this thesis. As cells that are able to produce IL12p40 are already activated, this could be why there is a significant increase in the levels of IL-12p40 in the BALF at 6 hours after an inhaled challenge of LPS on day 14 of the pneumococcal infection that is not seen when the LPS challenge was dosed to a naïve mouse.

The enhanced immune response to an inhaled LPS challenge, when given on day 14 of a pneumococcal infection could mimic what is classified as an exacerbation in COPD. It has
been hypothesised that exacerbations in COPD occur when a new bacterial strain is acquired (Veeramachaneni and Sethi, 2006). As the LPS used in these experiments was derived from *Pseudomonas aeruginosa*, which is a respiratory pathogen, this could mimic the acquisition of a new bacterial strain. Other studies that have typically used a model of LPS-induced lung injury utilised LPS derived from *E. coli* which is not as clinically relevant (Vernooy *et al.* 2002).

5.4.1 **Effect of Prednisolone on a Persistent Pneumococcal Infection**

The corticosteroid prednisolone is one of the recommended drugs for the treatment of an exacerbation in COPD (GOLD, 2013). However in the clinic, the use of inhaled corticosteroids has been associated with an excess risk of pneumonia hospitalisation (Ernst *et al.* 2007).

When considering the effects of steroid on the persistent pneumococcal infection model it was important to remember that different pathologies were observed during the time-course of the persistent pneumococcal infection of the lower airways. Two different questions were asked, the first was whether the administration of the corticosteroid prednisolone affected the establishment of pneumococcal infection and the second question asked was whether the same corticosteroid affected an established low level pneumococcal infection of the lower airways. Both questions are important as, both pathologies are seen in the clinic (Rodriques *et al.* 1992).

The first question that was asked was whether administration of prednisolone effected the establishment of pneumococcal disease. To do this a prophylactic treatment regime with prednisolone was established where prednisolone was dosed daily from the day before the pneumococcal infection was given until day 6 post-infection. On day 6 animals that had received both prednisolone and pneumococci had to be culled as they had lost on average 26.3% of their body mass from the start of the protocol. This weight loss directly correlated with the number of viable pneumococci recovered in the BALF, the greater the weight loss observed, the higher the bacterial load. The increase in bacterial load was also associated with a significant increase in neutrophils in the BALF at both 4 and 6 days post-infection. At 4 days post-infection the number of neutrophils in the BALF of mice that received both prednisolone and pneumococci was significantly compared to naive mice and mice that received both only the infection. However at 6 days post-infection the numbers of
neutrophils in the BALF were significantly higher in both groups that had received pneumococci and the administration of prednisolone made no significant difference to the number of neutrophils in the BALF. This suggested that when animals were dosed with both prednisolone and pneumococci neutrophils were induced into the lung at an earlier time-point compared to animals dosed with only pneumococci. The increase in bacterial load with inhaled corticosteroids has been observed previously. It has been shown that fluticasone propionate (another corticosteroid) impairs clearance of *K. pneumoniae* and that after 8 days post-infection bacterial burden was significantly increased, but no affect was seen in alveolar neutrophil recruitment (Patterson *et al.* 2012).

Inhaled corticosteroids are given for treatment of asthma and COPD as they lower the number of inflammatory cells in the induced sputum and decrease bronchial hyperactivity (Mastalerz and Kasperkiewicz, 2011). The impaired clearance of bacteria after inhaled corticosteroids could be due to the reduction in inflammatory cells, especially macrophages in the sputum, which would impair the host’s ability to clear the infection. This study shows that inhaled corticosteroids linked to an increased bacterial burden if they are given before an infection is induced.

The second question was asked was whether administration of corticosteroid affected an established low level pneumococcal infection of the lower airways. To do this, the persistent pneumococcal infection was run to 14 days post-infection so that a stable ‘chronic’ inflammatory phenotype was observed. On day 14 post-infection with pneumococci animals were given prednisolone daily for four days before being culled. Prednisolone had no effect on the bacterial burden observed on day 18 post-infection. However there was a significant decrease in body mass when prednisolone was dosed in combination with the pneumococcal infection. This was the same observation as when prednisolone was administered during the establishment of infection, but the weight loss observed on day 18 post-infection was much lower compared to that seen at day 6 post-infection. However no differences were observed in macrophage number in the BALF, but there was an increase in the number of neutrophils in the BALF of animals that received both pneumococci and prednisolone compared to naive mice, however no difference was observed between mice that received both pneumococci and prednisolone compared to mice that only received pneumococci. With regards to lung function no improvements in lung function was observed between animals dosed with pneumococci compared to animals that were dosed with pneumococci and prednisolone. This
would suggest that the inflammation observed at 14 to 18 days post-infection was insensitive to corticosteroids. One of the pathologies of COPD is that a large proportion of sufferers are resistant to even high doses of corticosteroids (Barnes, 2013). This would suggest that from 14 days post-infection the model of persistent pneumococcal infection exhibits pathologies similar to that observed in humans with COPD.

The final question asked was whether this apparent steroid insensitivity would still occur on a model of COPD ‘exacerbation’. To do this animals received the model of persistent pneumococcal challenge and on day 14 post-infection, the corticosteroid prednisolone was dosed orally, one hour later a challenge of LPS was given intranasally and mice were culled 6 hours after the LPS challenge.

One of the controls for this experiment was ‘LPS + Prednisolone’ with no infection. It has been published that prednisolone (10 ml / kg) reduces LPS induced pulmonary neutrophilia in the BALF of rats (Kubo et al. 2012). However this reduction in neutrophilia was not observed, but there was a significant reduction in the concentrations of KC in the BALF of mice that had been dosed with both prednisolone and LPS compared to mice that had only been dosed with LPS. As KC is a neutrophil chemoattractant this reduction would suggest that the future recruitment of neutrophils could be being reduced. Further work needs to be done to elucidate why there was no reduction in neutrophilia, one possibility could be that the concentration of LPS used was too high.

It was seen that the administration of prednisolone onto a model of COPD exacerbation (LPS and infection) caused a significant reduction in the levels of TNFα, but this was the only endpoint that was significantly affected. There was a suggestion that prednisolone led to an apparent reduction in neutrophils but this change was not significant. The administration of prednisolone onto a model of COPD exacerbation (LPS and infection) had no significant effects on the number of neutrophils or macrophages observed in the BALF and also no effect on the cytokines KC or IL12p40. From the histopathology there was a suggestion that prednisolone may have reduced the inflammation present and increased the amount of inflammatory debris in the alveolar bed but this was not quantified. An increase in inflammatory debris in the alveolar bed could be an indication of impaired clearance.
It could be suggested that prednisolone could reduce the overall inflammation of a COPD ‘exacerbation’ but would not reduce the inflammation to that observed in naive animals. All changes that were observed after the administration of prednisolone reduced the inflammation observed with LPS and infection to a level of inflammation observed with the pneumococcal infection alone. This suggests that the inflammation induced by the addition of the inhaled LPS challenge could be reduced by the administration of prednisolone but the established inflammation from the chronic infection could not.

There is much debate over whether inhaled corticosteroids increase the risk of pneumonia in the clinic (Singanayagam et al. 2010). From these experiments in vivo it would suggest that if a bacterial infection is acquired during inhaled corticosteroid treatment then the severity of disease will be greater. However if there is already an underlying pneumococcal infection then the addition of a treatment of corticosteroid would not affect bacterial burden or inflammation. As COPD is a largely heterogeneous condition then both pathologies may be observed in the clinic which could explain why there is such debate over whether corticosteroids do increase the risk of pneumonia.
6 Combination of the persistent pneumococcal infection with a model of inflammation associated with allergy:

6.1 Development of a model of non-eosinophilic asthma with a bacterial presence

6.1.1 Establishment of a murine model of ovalbumin-induced allergy

A model of bacterial infection has been established, it was decided to combine this model, with a model of allergy to mimic the pathology seen in a subset of asthmatics, which have a low level bacterial presence (Hilty et al. 2010). One well-described model of allergy is the ovalbumin (OVA) sensitisation and challenge model. As the University of Leicester had no established model of allergy a protocol had to be established and validated. It was decided that the criterion for the success of this model was that after exposure to OVA, animals had increased eosinophilia in the airways. The OVA sensitisation and challenge model has been most commonly used in Balb/c mice, because as this strain has been shown to have a strong, reliable response (Whitehead et al. 2003).

There are many published protocols of OVA sensitisation and challenge (Stevenson and Birrell, 2011). Because AstraZeneca in Charnwood had an established model of OVA sensitisation and challenge it was decided to use their dosing regimen. Figure 6.1A shows the protocol that was adapted from the one established at AstraZeneca in Balb/c mice. The only difference between the protocol shown in Figure 6.1A and that established by AstraZeneca was the method of OVA challenge. AstraZeneca used an aerosolised OVA challenge, whereas for practical reasons at the University of Leicester this challenge had to be administered intranasally. The OVA sensitisation and challenge model has been published with dosing intranasally, as well as by aerosol (Amano et al. 2007, Tunes et al. 2008, Qui et al. 2011) which suggested that this would be a viable alternative.

Another issue with the regimen established by AstraZeneca was that it had been optimised in inbred Balb/c mice. As shown in section 4.5 Balb/c mice were able to clear the pneumococcal infection with strain LgSt215, which meant in this strain of mouse there was no pulmonary persistence with pneumococci. As the model of persistent infection has been established in CBA/Ca mice, the OVA sensitisation and challenge model to be established at the University of Leicester also had to be optimised in
CBA/Ca mice. In the first experiment Balb/c and CBA/Ca mice were given the protocol shown in Figure 6.1A to judge any differences in eosinophilia.

Figure 6.1A shows how CBA/Ca and Balb/c mice were intraperitoneally challenged with 100 µg Ovalbumin adsorbed onto 1 mg aluminium hydroxide (Alum) in saline in a volume of 300 µl on days 0, 7 and 14, then on day 21 mice were intranasally challenged with; 0.5, 1 or 1.5 mg in 50 µl saline. Mice were culled on days 22 or 23 and lung inflammation was assessed. It can be seen in Figure 6.1 (B & C) that eosinophilia was induced in both CBA/Ca and Balb/c mice. Eosinophilia was only induced when animals received both sensitisation and challenge with ovalbumin. There was no significant difference (p > 0.05) between the level of eosinophilia induced at 48 hours post-challenge with the three concentrations of OVA given intranasally or between the two mouse strains tested. Figure 6.1B shows that eosinophils were induced at both 24 hours and 48 hours post-challenge (days 22 and 23 respectively), whereas Figure 6.1C shows that in CBA/Ca mice eosinophilia was only induced 48 hours post-challenge.

Figure 6.1 – Comparison of an OVA-induced model of allergic inflammation in Balb/c and CBA/Ca mice (n = 9). A - protocol of ovalbumin sensitisation and challenge, B – differential cell counts in BALF of Balb/c mice and C – differential cell counts in BALF of CBA/Ca mice.

Figure 6.1 demonstrated that eosinophilia could be induced in CBA/Ca mice, albeit at a slightly (but not significant (p > 0.05) lower level than Balb/c mice and at 48 hours
post-challenge. In Balb/c mice, eosinophilia was present at 24 hours post-challenge, which was not seen in CBA/Ca mice.

One disadvantage of this dose regimen was that twenty per cent mortality was observed, with both mouse strains, after the third sensitisation with ovalbumin. To refine the model and reduce this level of mortality three different sensitisation regimes were then compared and tested in CBA/Ca mice. For all three regimens of OVA sensitisation and challenge, mice were intranasally challenged on day 21 with 100 µg/50 µl OVA, only the number and timings of sensitisations were changed. As can be seen in Table 6.1, for all three dose regimes tested eosinophilia could be induced at 48 hours post-infection, which was significantly higher than that seen in naïve mice (p < 0.05). This meant that all three dose regimes would fit with the original criterion, however as seen in Table 6.1 the only dose regime that had no mortality was when mice were sensitised on days 0 and 14.

<table>
<thead>
<tr>
<th>Sensitisation Regime</th>
<th>Challenge Dose on day 21</th>
<th>Level of Mortality</th>
<th>Eosinophilia Increased Compared to Naïve Mice at 48 hours post-challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0 and 7</td>
<td>100 µg/50 µl</td>
<td>10 %</td>
<td>Yes (p &lt; 0.05)</td>
</tr>
<tr>
<td>Day 0 and 14</td>
<td>100 µg/50 µl</td>
<td>0 %</td>
<td>Yes (p &lt; 0.05)</td>
</tr>
<tr>
<td>Day 0, 7 and 14</td>
<td>100 µg/50 µl</td>
<td>20 %</td>
<td>Yes (p &lt; 0.05)</td>
</tr>
</tbody>
</table>

Table 6.1 – Comparison of the three OVA sensitisation and challenge regimes tested in CBA/Ca mice to induce eosinophilia (n=5).

After optimisation the only regime that had no mortality but still induced eosinophilia 48 hours post-challenge was when two sensitisations were given 14 days apart, as shown in Figure 6.2. To confirm how long mice remained sensitised, mice were sensitised with ovalbumin adsorbed onto alum (prepared as described in section 6.1.1) on day 0 and day 14 (Figure 6.2A). Sensitised animals received a single intranasal challenge with 1 mg ovalbumin in 50 µl physiological saline on either day 21, 28 or 35, samples were collected 48 hours post-challenge. Figure 6.2B shows, that the length of time between the second sensitisation and the intranasal challenge had no effect on the individual immune cell populations in the BALF. Neutrophils, macrophages and
Eosinophils were significantly raised (p < 0.05) whatever the time of challenge compared to the numbers observed in the BALF of naïve animals. Figure 6.2C shows the eosinophil numbers counted in the BALF. The length of time between the second sensitisation and the intranasal challenge had no effect on the number of eosinophils induced in the BALF 48 hours after the ovalbumin challenge.

Figure 6.2 – CBA/Ca mice were sensitised to OVA on days 0 and 14. CBA/Ca mice were then challenged intranasally with OVA on day 21, 28 or 35 of the regime (n = 5 – 8). A - protocol of ovalbumin sensitisation and challenge, B – differential cell counts in BALF of CBA/Ca mice and C – Log eosinophil cell counts in BALF of CBA/Ca mice. Kruskal-Wallis non-parametric test with a Dunns post-test were used to compare differences between time post-challenge and naive, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.

Figure 6.3 shows the effects of the OVA sensitisation and challenge model on the physiological functioning of the lung after administering the dose regime shown in Figure 6.2A. Figure 6.3A shows that the OVA sensitisation and challenge model significantly reduced (p < 0.05) the functional residual capacity of the lung 48 hours post-challenge. This was regardless of whether the challenge was administered on day 21, 28 or 35. As can be seen in Figure 6.3B, the airway resistance was significantly
increased (p < 0.05), 48 hours post-challenge when the challenge was administered on day 21 or day 28. However 48 hours after the challenge being given on day 35, airway resistance was not significantly increased (p > 0.05). The total lung capacity was significantly decreased (p < 0.05) in comparison to naïve mice 48 hours post-OVA challenge, at all three time points tested. Figure 6.3D shows that the tidal volume was also was significantly decreased (p < 0.05) 48 hours post-challenge, when the challenge was administered on day 21 or day 35, but not on day 28 (p > 0.05).

Figure 6.3 – Physiological airway responses Ovalbumin sensitisation and challenge model in CBA/Ca mice, investigation to see how long mice remain sensitised. At predetermined time points post-infection lung function was assessed using a forced manoeuvres system (EMMS) (n = 8). A – Functional Residual Capacity, B – Airway Resistance, C – Total Lung Capacity and D – Tidal Volume (n = 7 - 15). Kruskal-Wallis non-parametric test with a Dunns post-test were used to compare differences between time post-challenge and naive, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.
A model of OVA sensitisation and challenge model has been established. In this model eosinophils were significantly increased 48 hours post-intranasal challenge with ovalbumin.

6.2 Combination of the persistent infectious model with a model of allergic response

As can be seen in Figure 6.1 to 6.3, a model of sensitisation and challenge was established in inbred CBA/Ca mice at the University of Leicester. The next step was to combine this model with the model of persistent pneumococcal infection. As both models are complex, there were many possibilities of how the persistent pneumococcal infection model and the OVA sensitisation and challenge model could be combined.

Three different combinations of the model were tested as shown in Figure 6.4. For all three regimes tested, two parameters were kept constant; the two IP OVA sensitisations were always done on days 0 and 14 and all animals were culled 48 hours after the intranasal OVA challenge. The first two dose regimes shown in Figure 6.4A and B asked the question; does the presence of a low level pneumococcal infection in the lower airways increase the response to an intranasal challenge with OVA. Whereas the dose regime shown in Figure 6.4C asked whether the presence of a persistent low level pneumococcal infection in the lower airways affected the establishment and outcome of a Th2 response to the OVA sensitisation and challenge.

Figure 6.4A shows the first regime that was tested. In this regime the pneumococcal infection was instilled on day 7, halfway between the two OVA sensitisations. This meant that the induction of the eosinophilic inflammation with the intranasal OVA challenge occurred 14 days into the pneumococcal infection. The second regime tested was an adaption of this question and is shown in Figure 6.4B. In this regime the infection was instilled 7 days after the second OVA sensitisation and the OVA intranasal challenge was done 7 days into the pneumococcal infection of the lower airways. The third regime shown in Figure 6.4C was different. In this regime the pneumococcal infection was allowed to establish for 14 days before the OVA sensitisation and challenge was introduced. For the OVA sensitisation and challenge
model sensitisations with OVA and alum were done on days 0 and 14, with the intranasal OVA challenge dosed on day 21 of the protocol.

Figure 6.4 – Three dose regimes for the combination of infection with the OVA and sensitisation and challenge model. Dose regimes A and B asked whether pneumococcal infection of the lower airways changed the host response to an intranasal challenge of OVA. The third dose regime (C) asked whether the presence of a low level pneumococcal infection in the lower airways altered the establishment and outcome of a model of OVA sensitisation and challenge.

6.2.1 Does the presence of a persistent pneumococcal infection of the lower airways alter the response to an intranasal challenge of OVA

With the protocol shown in Figure 6.4, on day 14, within a few hours of the second OVA sensitisation a high number of animals reached the 2+ lethargic endpoint and had to be culled. This was observed in groups that had a persistent pneumococcal infection of the lower airways and had received 2 sensitisations with OVA. When compared to animals that had received only two sensitisations with OVA but no pneumococci, the level of mortality observed in the two groups that had received two OVA sensitisations and the pneumococcal infection was significantly higher (p < 0.05).
Figure 6.5 – Percentage survival of CBA/Ca mice after administration of a combination of OVA challenge and sensitisation and the persistent pneumococcal infection (n = 5) (dose regime shown in Figure 6.4A).

Even though a high rate of mortality was observed, it was possible to measure other endpoints. At 48 hours post-infection, inflammatory cells were enumerated in the BALF. Figure 6.6A shows the dose regime used and Figure 6.6B shows the total inflammatory cell counts in the BALF. It can be seen in Figure 6.6B that the highest numbers of inflammatory cells were observed in the group that had received the OVA sensitisation and challenge combined with the persistent pneumococcal infection. Figure 6.6C shows the number of eosinophils present in the BALF. From this it can be seen that the OVA sensitisation and challenge model alone induced eosinophilia, however when the persistent pneumococcal infection was present there was an apparent reduction in the number of eosinophils but this was not significant (p > 0.05). The persistent infection model alone did not cause an influx of eosinophils into the BALF. In Figure 6.6D it can be seen that neutrophils were present in the BALF of animals that had received either the OVA sensitisation and challenge model alone or a combination of the two models as shown in Figure 6.6A. However these were only significantly raised (p < 0.05) in the BALF of animals that had received both OVA sensitisation and challenge and pneumococci compared to the number of neutrophils in the BALF of naïve mice.
Figure 6.6 – Combination of OVA sensitisation and challenge administration with pneumococcal infection in CBA/Ca mice, protocol 1 (n = 5). A – protocol 1 of ovalbumin sensitisation and challenge, B – differential cell counts in BALF of CBA/Ca mice, C – eosinophil cell counts in BALF of CBA/Ca mice and D – neutrophil cell counts in BALF of CBA/Ca mice. Kruskal-Wallis non-parametric test with a Dunns post-test were used to compare differences, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.

As well as performing differential cell counts, the levels of four cytokines / chemokines; KC, IP-10, IL-12p40 and eotaxin were measured in the BALF supernatant by sandwich ELISA. As can be seen in Figure 6.7A, the level of the cytokine KC was not significantly different (p < 0.05) from that of naïve mice, in any of the combinations tested. Figure 6.7B shows that the chemokine IP-10 was significantly raised (p < 0.05) in the BALF of mice that had only been exposed to OVA sensitisation and challenge compared to the BALF of naïve mice. Levels of IP-10 appear to be raised in the BALF of mice that were exposed to both models; however this difference was not significant (p > 0.05). As shown in Figure 6.7C, the levels of the cytokine subunit IL-12 p40
appear to be raised in the BALF compared to naïve mice when both models were combined, but this was not significant (p > 0.05). Figure 6.7D shows the level of eotaxin in the BALF. It appeared that the level of eotaxin in the BALF could be raised in the BALF of mice that were administered OVA alone but this change was not significant (p > 0.05). It can be seen in Figure 6.7D that the level of eotaxin was not significantly different (p < 0.05) in any of the model combinations tested.

**Figure 6.7** – Cytokines levels in the BALF of CBA/Ca mice 48 hours post intranasal challenge with OVA. CBA/Ca mice were either naïve or were dosed with either the OVA sensitisation model or the persistent pneumococcal infection model or both models as described by protocol 1. All cytokines were measured by sandwich ELISA. A – Levels of the cytokine KC in the BALF, B – Levels of the chemokine IP-10 in the BALF, C – Levels of the cytokine subunit IL12 p40 in the BALF and D – Levels of the cytokine Eotaxin in the BALF. Kruskal-Wallis non-parametric test with a Dunns post-test were used to compare differences, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.
6.2.2 Regime adapted to reduce the mortality observed in the first dose regime

As there was a high level of mortality within a few hours of the second sensitisation in the first dose regime, it was decided that the infection should be instilled 7 days after the second sensitisation, as shown in Figure 6.8A.

Figure 6.8B shows the combined immune cell counts observed in the BALF. It can be seen that in all groups that received either one or both of the inflammatory models the number of immune cells are raised compared to in the BALF of naïve mice. Figure 6.8C and D show the individual results for eosinophils and neutrophils respectively. As shown in Figure 6.8C, the number of eosinophils in the BALF of mice that received the full protocol shown in Figure 6.8A was significantly reduced (p < 0.001) compared to the number of eosinophils in the BALF of mice that had received only the OVA sensitisation and challenge model alone. Figure 6.8D shows that the number of neutrophils was significantly raised (p < 0.05) in all animals that had received one or both of the inflammatory regimes compared to the number of neutrophils in the BALF of naïve mice.
Figure 6.8 - Combination of the administration of OVA sensitisation and challenge with a persistent pneumococcal infection in CBA/Ca mice, protocol 2. A - protocol 2 of ovalbumin sensitisation and challenge, B – differential cell counts in BALF of CBA/Ca mice, C – eosinophil cell counts in BALF of CBA/Ca mice and D – neutrophil cell counts in BALF of CBA/Ca mice. Kruskal-Wallis non-parametric test with a Dunns post-test were used to compare differences, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.

The levels of cytokines were then analysed in the BALF supernatant by sandwich ELISA. As shown in Figure 6.9A, the level of the cytokine KC was significantly raised (p < 0.05) in the BALF of mice that had received both models of inflammation compared to the level of KC in the BALF of naïve mice, but was unchanged in mice that had only received the OVA sensitisation and challenge model alone. Figure 6.9B shows that levels of the chemokine IP-10 in the BALF of mice that had been exposed to the OVA sensitisation and challenge model were significantly raised (p < 0.05) compared to in the BALF of naïve mice. In Figure 6.9B it appears that IP-10 was also
raised in the BALF of mice that had been exposed to both models of inflammation but this was not significant ($p > 0.05$). It can be seen in Figure 6.9C that IL-12 p40 was significantly raised ($p < 0.05$) in the BALF of mice that had only been given the OVA sensitisation and challenge compared to the BALF of naïve mice and mice that were exposed to both OVA and pneumococci. Figure 6.9D shows that the level of eotaxin in the BALF was unchanged in either of the inflammation models tested.

**Figure 6.9** – Cytokines levels in the BALF of CBA/Ca mice 48 hours post intranasal challenge with OVA. CBA/Ca mice were either naïve, dosed with the OVA sensitisation and challenge model or a combination of the OVA sensitisation and challenge model with the persistent pneumococcal infection model as described in protocol 2. All cytokines were measured by sandwich ELISA. A – Levels of the cytokine KC in the BALF, B – Levels of the chemokine IP-10 in the BALF, C – Levels of the cytokine subunit IL12 p40 in the BALF and D – Levels of the cytokine Eotaxin in the BALF. Kruskal-Wallis non-parametric test with a Dunns post-test were used to compare differences, * $p < 0.05$ > 0.01, ** $p < 0.01$ > 0.001, *** $p < 0.001$.

Physiological parameters were also assessed. As can be seen in Figure 6.10A, no differences were observed in the FEV in any of the groups tested. This is mirrored in Figure 6.10B, which shows that no changes were observed in the tidal volume, and also
Figure 6.10C, which shows that there no changes were also observed in the forced peak expiratory volume, suggesting that there was no airway obstruction. Figure 6.10D shows that significant differences were observed in the airway resistance. In Figure 6.10D it can be seen that the airway resistance in the lungs of mice that were exposed to OVA sensitisation and challenge, regardless of whether mice had also been exposed to pneumococcal infection was significantly increased (p < 0.01) compared to the airway resistance of naïve mice and mice that were only exposed to the pneumococcal infection.

**Figure 6.10** – Assessment of the lung functioning with an eSpira forced manoeuvres system (EMMS) of inbred CBA/Ca mice that were either naïve, dosed with the OVA sensitisation and challenge model, dosed with the persistent pneumococcal infection model or a combination of the two models of inflammation as described in protocol 2. A – FEV was assessed at set time points over a time course (25 – 60 milliseconds), B – Tidal volume, C – Peak expiratory flow (forced) and D – airway resistance. Kruskal-Wallis non-parametric test with a Dunns post-test were used to compare differences. * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.
6.3 Investigation into whether the low level presence of a pneumococcal infection in the lower airways affects the establishment and outcome of a model of OVA sensitisation and challenge

To investigate whether the presence of a persistent low level pneumococcal infection affected the OVA sensitisation and challenge model the protocol shown in Figure 6.11A was tested. Figure 6.11A shows the combined differential cell counts in the BALF, it can be seen that the presence of infection was responsible for an increase in the number of inflammatory cells in the BALF. As can be seen in Figure 6.11C, the number of eosinophils in the BALF of mice that had received only OVA sensitisation and challenge was significantly higher (p < 0.01) compared to the number of eosinophils in the BALF of any other group tested. It is of interest to note in Figure 6.11C that the presence of infection with OVA sensitisation and challenge caused a significant decrease (p < 0.01) in the number of eosinophils present in the BALF, compared to mice that received only the OVA sensitisation and challenge model alone. Figure 6.11D shows that the presence of the pneumococcal infection in the lower airways caused a significant increase in the number of neutrophils in the BALF compared to naïve mice.
Figure 6.11 - Combination of the OVA sensitisation and challenge model with the model of pneumococcal persistence in CBA/Ca mice, protocol 3. A - protocol 3 of ovalbumin sensitisation and challenge, B – differential cell counts in BALF of CBA/Ca mice, C – eosinophil cell counts in BALF of CBA/Ca mice and D – neutrophil cell counts in BALF of CBA/Ca mice. Kruskal-Wallis non-parametric test with a Dunns post-test were used to compare differences, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.

As well as enumerating the number of immune cells in the BALF the levels of four cytokines were also measured by sandwich ELISA. As shown in Figure 6.12A, in neither model of inflammation was there a significant (p > 0.05) change in the level of KC in the BALF of mice compared to the level measured in the BALF of naïve mice. Figure 6.12B shows that the cytokine TNF α was significantly (p < 0.05) decreased in the BALF of mice that had received either the pneumococcal infection alone or the pneumococcal infection and OVA sensitisation and challenge, compared to the BALF of naïve mice. It can also be seen in Figure 6.12B that although the level of TNF α appeared to be lower in the BALF of mice that had only received the OVA sensitisation and challenge when compared to the BALF of naïve mice, this change was not
significant (p > 0.05). Figure 6.12C shows that the cytokine sub unit IL-12 p40 was significantly raised (p < 0.05) in the BALF of mice that had received both pneumococci and OVA sensitisation and challenge compared to in the BALF of naïve mice. There was no significant difference (p > 0.05) in the levels of IL-12 p40 compared to naïve mice when the inflammation models were run in isolation. The levels of the cytokine eotaxin in the BALF were not significantly different (p >0.05) from the levels seen in the BALF of naïve mice when the models of inflammation were combined or run separately.

Figure 6.12 – Cytokines levels in the BALF of CBA/Ca mice 48 hours post intranasal challenge with OVA. CBA/Ca mice were either naïve or were dosed with either the OVA sensitisation and challenge model or the persistent pneumococcal infection model or both models as described in protocol 3. All cytokines were measured by sandwich ELISA. A – Levels of the cytokine KC in the BALF, B – Levels of the cytokine TNFα in the BALF, C – Levels of the cytokine subunit IL12 p40 in the BALF and D – Levels of the cytokine Eotaxin in the BALF. Kruskal-Wallis non-parametric test with a Dunns post-test were used to compare differences, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.
To determine whether any of the changes in the inflammatory cell profile translated to a physiological change, the functioning of the lung was assessed with the eSpira forced manoeuvres system. As shown in Figure 6.13A the models of inflammation had no effect on the FEV across the whole time course assessed (25 – 60 ms) compared to the FEV measured in naïve mice. Figure 6.13B and C showed that with the flow derived parameters tidal volume and forced peak expiratory flow respectively no significant differences (p > 0.05) were observed in the models of inflammation tested compared to naïve mice. Figure 6.13D also showed that the models of inflammation did not have a significant effect (p > 0.05) on airway resistance compared to naïve mice. In Figure 6.13D it appears that lung resistance was marginally increased in the OVA sensitisation and challenge model alone compared to the airways resistance of naïve mice but this change was not significant (p >0.05).
Figure 6.13 – Assessment of the lung functioning at time of culling with an eSpira forced manoeuvres system (EMMS) of inbred CBA/Ca mice that were either naïve, dosed with the OVA sensitisation and challenge model, dosed with the persistent pneumococcal infection model or a combination of the two models of inflammation as described in protocol 3. A – FEV was assessed at set time points over a time course (25 – 60 milliseconds), B – Tidal volume, C – Peak expiratory flow (forced) and D – airway resistance. Kruskal-Wallis non-parametric test with a Dunns post-test were used to compare differences, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.

As well as assessing lung function the pathology was also observed. It was observed that there were eosinophils in the peribronchiolar cuffs (Figure 6.14A and B) in animals that were sensitised and challenged with OVA. Figure 6.14C and D show the inflammation observed on day 37 of a pneumococcal infection which is similar to that observed at days 14, 21 and 28, with the inflammation being predominantly monocytic. The presence of a pneumococcal infection during sensitisation and challenge with OVA led to an inflammation that was predominantly monocytic with a few neutrophils in the peribronchiolar cuffs (Figure 6.14E and F).
Figure 6.14 - Formalin fixed, paraffin embedded, H&E stained lung sections of CBA/Ca mice that had been sensitised and challenged with OVA and 14 days into a persistent pneumococcal infection. A and B – OVA sensitisation and challenge, C and D – day 37 of a pneumococcal infection, E and F – OVA sensitisation and challenge started on day 14 of a pneumococcal infection. Triangles indicate eosinophils in the tissue.

6.4 A model of non-eosinophilic asthma

Moderate, mild and severe asthmatics can be classified into distinct phenotypes (Green et al. 2002). Although eosinophilic inflammation is typically thought of as a hallmark of asthma, some studies are challenging this convention. It has been published that eosinophilic airway inflammation may only be present in up to 50% of asthmatics (Douwes et al. 2002, McGrath et al. 2012). It is important to be able to model non-eosinophilic asthma because it is a sub group that has been shown to be insensitive to standard asthma steroid therapies (Green et al. 2002).
By combining an *in vivo* model that was known to trigger a Th2 driven inflammatory response (OVA sensitisation and challenge model) with a model that has been shown to trigger a Th1 driven inflammatory response (persistent pneumococcal infection model) a model of non-eosinophilic asthma has been developed. As shown in Figure 6.6 this combined model of allergy and persistent pneumococcal infection resulted in an inflammatory response which consisted of neutrophilic inflammation but with a significant reduction in induced eosinophils compared to the OVA sensitisation and challenge model alone.

### 6.4.1 Can the inhibition of NADPH oxidase 4 (NOX4) improve lung function in a model of non-eosinophilic asthma?

NADPH oxidases (NOX) are enzymes which mediate physiological functions, including host defense and cell signalling, through the generation of reactive oxygen species (Lambeth *et al.* 2008). It has been suggested that over-expression of NOX4 in asthma is critical in the promotion of oxidative stress and, in turn, airway smooth muscle hypercontractility (Sutcliffe *et al.* 2012). The hypothesis is that in a model of non-eosinophilic asthma the inhibition of NOX4 would lead to the prevention of reactive oxygen production, which in turn would lead to an improvement in disease progression.

To test this hypothesis, the model described in Figure 6.4B was utilised because as shown in section 6.2.2 it could be interpreted to be a model of non-eosinophilic asthma. An inhibitor of NADPH oxidase 4 (NOX4) (donated from the biotechnology company GenKyoTex, Geneva, Switzerland) was administered to CBA/Ca mice as shown in Figure 6.15. This was based on the regimen shown in Figure 6.4B.

As the effect of the NOX4 inhibitor on an *in vivo* pneumococcal infection was unknown, it was decided that compound should be given after the infection had established in the lower airways of mice. This is because the hypothesis being tested was not what effect a NOX4 inhibitor had on the establishment of a persistent pneumococcal infection but what effect it would have in a model of non-eosinophilic asthma. To allow the establishment of infection and the compound to be given for 7 days, the OVA challenge was moved to day 28 from day 21 (Figure 6.15). As shown
previously, during the establishment of the OVA sensitisation and challenge model a similar level of eosinophilic inflammation (Figure 6.2) and change in lung function (Figure 6.3) could be achieved whether the OVA challenge was performed on day 28 or day 21.

As can be seen in Figure 6.15, CBA/Ca mice were intraperitoneally sensitised with OVA on days 0 and 14. After sensitisation, CBA/Ca mice were dosed intranasally with $4 \times 10^6$ CFU / 30 µl with pneumococcal strain LgSt215 on day 17 of the protocol. From day 24 of the protocol which was 7 days post-infection with S. pneumonia. The NOX 4 inhibitor (compound) was given once daily for 7 days at a dose of 40 mg / kg (concentration of compound was based on internal data from GenKyoTex). Compound was suspended in 1.2 % w/v Methylcellulose (Sigma) and 0.1 % w/v Polysorbate 80 (Sigma) in distilled water and administered in a volume of 10 ml / kg (mice were weighed before each administration of compound). On day 28 mice were intranasally challenged with 1 mg / 50 µl OVA one hour post-oral administration of the compound. Forty eight hours post-intranasal challenge with OVA, mice were assessed for the following outcomes; number of viable pneumococci in the BALF, cellular inflammation in the BALF, tracheal ciliary beat frequency, lung functioning and the overall pathology. For all endpoints (except tracheal ciliary beat frequency) a minimum of 8 mice were either dosed with the full regimen (model + compound), the regimen but with no pharmacological intervention (model alone), with NOX4 inhibitor but no OVA or infection (compound alone) or saline instead of OVA or infection (saline alone).

Figure 6.15 – The dosing regimen established to investigate the effect of dosing an inhibitor of NOX4 on a model of non-eosinophilic asthma in CBA/Ca mice.
<table>
<thead>
<tr>
<th>Group Name</th>
<th>Regime Given</th>
<th>No. of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>Saline - days 0, 14, 17 and 28 Vehicle - days 24 - 30</td>
<td>9</td>
</tr>
<tr>
<td>Compound alone</td>
<td>Saline - days 0, 14, 17 and 28 NOX4 Inhibitor - days 24 - 30</td>
<td>9</td>
</tr>
<tr>
<td>Model</td>
<td>OVA - days 0, 14, and 28 Pneumococcal Infection day 17 Vehicle - days 24 - 30</td>
<td>9</td>
</tr>
<tr>
<td>Model + Compound</td>
<td>OVA - days 0, 14, and 28 Pneumococcal Infection day 17 NOX4 Inhibitor - days 24 - 30</td>
<td>9</td>
</tr>
</tbody>
</table>

**Table 6.2** – Summary of the conditions investigated when a NOX4 inhibitor was given to CBA/Ca mice who had also received a regimen of OVA sensitisation and challenge combined with a persistent pneumococcal infection.

As can be seen in Figure 6.16, as expected, only the two groups of mice that had been dosed with pneumococci (model and model + compound) had recoverable numbers of *S. pneumoniae* in the BALF at time of culling. There was no statistical difference (p < 0.05) in the number of viable pneumococci in the BALF of animals that had or had not been dosed with the NOX4 inhibitor.

![Graph](image.png)

**Figure 6.16** – A novel inhibitor of NOX4 (compound) was administered to CBA/Ca mice who had received a combination of OVA sensitisation and challenge and a pneumococcal infection (model) daily for 7 days before culling. The number of pneumococci in the BALF was assessed at the time of culling. Each point represents an individual mouse.
To determine the effect of the NOX4 inhibitor (compound) on the murine model of non-eosinophilic asthma on the immune cell populations, differential cell counts were performed on the BALF. Lungs were lavaged, as described in section 2.5.3, and samples were cytocentrifuged before staining with Diff-quick to distinguish different blood cell populations. Figure 6.17 shows the differential cell counts of the immune cells in the BALF: Figure 6.17A shows the combined cell counts and Figure 6.17B-D show the individual results for neutrophils, eosinophils and macrophages respectively. Mice that were given compound alone had similar differential cell counts in the BALF to mice that had just received saline. As can be seen in Figure 6.17A, mice subjected to the combined pneumococcal/OVA challenge to generate the non-eosinophilic asthma model, showed an increase in the BALF the inflammatory cells; neutrophils, macrophages, lymphocytes and monocytes. Figure 6.17B shows that the numbers of neutrophils were significantly increased (p < 0.05) in the model of non-eosinophilic asthma compared to the compound and saline alone controls. The NOX4 inhibitor had no significant effect (p > 0.05) on the number of neutrophils present in the BALF in the model of non-eosinophilic asthma. In Figure 6.17C it can be seen that when compared to the saline control group, the number of eosinophils in the BALF was not statistically different (p > 0.05) in any of the groups of CBA/Ca mice. The single outlier in the non-eosinophilic asthma group given NOX4 inhibitor had no recoverable pneumococci in the BALF (Figure 6.17C), confirming that the presence of pneumococci was required for the suppression of Th2 responses such as eosinophil induction. In Figure 6.17D the number of macrophages in the BALF, was significantly increased (p < 0.05) in the non-eosinophilic asthma model compared to the group that only received the NOX4 inhibitor. It can also be seen in Figure 6.17D that the number of macrophages in the BALF was not significantly different (p > 0.05) between any of the other combinations tested.
**Figure 6.17** – In combination with a regimen to induce a model of non-eosinophilic asthma (model) CBA/Ca mice were dosed daily for 7 days before culling with a NOX4 inhibitor (compound). Differential cell counts of immune cells in the BALF of CBA/Ca mice were assessed at the time of culling, counts are based on the number of immune cells counted in 10 fields of view at X 400 magnification. A – Differential cell counts in BALF of CBA/Ca mice, B – Number of neutrophils, C – Number of eosinophils and D – Number of macrophages. Kruskal-Wallis non-parametric test with a Dunns post-test were used to compare differences, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.

Dosing with either saline (Figure 6.18A and B) or compound alone (Figure 6.18C and D) did not cause any significant pathology in the alveolar bed (Figure 6.18A and C) or the transitional airways (Figure 6.18B and D). Alveolar spaces were clear with no signs of inflammation and the appearance of bronchioles, including the transitional zones were normal.
Figure 6.18 – Formalin fixed, paraffin embedded, H&E stained lung sections of CBA/Ca saline and compound control mice. A – Alveolar bed from an animal dosed with saline, B – transitional airway of a mouse dosed with saline, C – alveolar bed from an animal dosed with NOX4 inhibitor alone and D – transitional airway of a mouse dosed with NOX4 inhibitor alone. All images were taken at an x 50 magnification.

As the negative controls were free from inflammation, the next question to ask was whether the NOX4 inhibitor compound had an effect on the inflammation observed with the model of non-eosinophilic asthma. Figure 6.19A shows the typical inflammation observed in the lungs of CBA/Ca mice with non-eosinophilic asthma. As can be seen in Figure 6.19A, there was a marked inflammatory infiltration of perivascular and peribronchiolar adventitia (Figure 6.19A, arrows) and marked inflammatory infiltration into the alveolar bed (Figure 6.19A, star). It can be seen in Figure 6.19B that after treatment with NOX4 inhibitor the inflammation was less severe, but a diffuse pneumonitis and inflammatory infiltration into the alveolar spaces could still be observed. Similar to that shown in Figure 6.19A, Figure 6.19C shows that without NOX4 treatment the inflammation in the model of non-eosinophilic asthma had pneumonic consolidation of the alveolar bed across entire lobes of CBA/Ca mice. Figure 6.19D shows that after administration of the NOX4 inhibitor, diffuse
pneumonitis with pneumonic consolidation of the alveolar bed was still seen. However, even though the severity of inflammation remained comparable, the incidence of areas of more severe inflammation was reduced after administration of compound.

**Figure 6.19** - Formalin fixed, paraffin embedded, H&E stained lung sections of CBA/Ca mice that had been given the NOX4 inhibitor compound on top of the protocol to induce non-eosinophilic asthma. A and C – marked inflammatory infiltrate (arrow) and pneumonic consolidation (star) seen with the model of non-eosinophilic asthma, B and D – model of non-eosinophilic asthma treated with NOX4 inhibitor orally for 7 days.

As well as changes to the inflammation across the alveolar bed, after administration of the NOX4 inhibitor there were also changes to the epithelial damage observed. As can be seen in Figure 6.20A, in the model of non-eosinophilic asthma fibroplastic foci (*) were observed which had a spindle-like appearance with cells aligned within the foci. As well as fibroplasia, diffuse pneumonitis and inflammation can be observed within the alveolar bed (Figure 6.20A). Figure 6.20B shows that after administration of the NOX4 inhibitor, the same pathology was still observed. As well as the presence of fibroplasia in the transitional airways, damage to the epithelium within the bronchioles was also observed. With the model of non-eosinophilic asthma, it can be seen in Figure
6.20C that the epithelial cells lining the bronchiole are hypertrophic, and the surrounding alveolar bed is consolidated with inflammation. After administration of the NOX4 inhibitor compound damage to the epithelial cells lining the bronchioles could still be seen, but there was a higher proportion of intact epithelium and the inflammation present in the alveolar spaces was reduced (Figure 6.20D).

**Figure 6.20** – Formalin fixed, paraffin embedded, H&E stained lung sections of CBA/Ca mice that had been given the NOX4 inhibitor compound on top of the protocol to induce non-eosinophilic asthma in CBA/Ca mice. A - fibroplasia (*) seen with the model of non-eosinophilic asthma, B - fibroplasia (*) seen with the model of non-eosinophilic asthma after treatment with NOX4 inhibitor, C – epithelial damage (triangle) seen with the model of non-eosinophilic asthma, D - epithelial damage (triangle) seen with the model of non-eosinophilic asthma after treatment with NOX4 inhibitor.

A similar severity in pathology was observed in the model of non-eosinophilic asthma after NOX4 inhibitor compound administration compared to the model alone when inflammation was present. However the incidence of inflammation appeared to be reduced after compound NOX4 inhibitor administration. To assess whether this was the
case a semi-quantitative analysis was performed, to evaluate whether there were any
differences in the inflammation observed. Each lobe of the mouse was scored
individually, and then assigned a numerical score between 1 – 5, for either the level of
alveolar inflammation or the level of epithelial injury, as shown in Table 6.3. To assess
the overall pathology, the two scores given for the level of alveolar inflammation and
the level of epithelial injury were combined. If no inflammation was observed lobes
were scored as zero.

<table>
<thead>
<tr>
<th>Alveolar Inflammation / Injury</th>
<th>Epithelial Injury</th>
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<tr>
<td><strong>Type of Inflammation</strong></td>
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<tr>
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</tr>
<tr>
<td>Focal pneumonitis</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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<td>5</td>
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<tr>
<td><strong>Type of Inflammation</strong></td>
<td><strong>Score</strong></td>
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</tr>
<tr>
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</tr>
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<td>3</td>
</tr>
<tr>
<td>Epithelial injury and repair</td>
<td>4</td>
</tr>
<tr>
<td>Epithelial injury and fibroplasia</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 6.3 – Semi-quantitative scoring system used to compare changes in inflammation of the formalin fixed, paraffin embedded, H&E stained lung sections of CBA/Ca mice that had been given the NOX4 inhibitor compound on top of the protocol to induce non-eosinophilic asthma. A total of four lungs were scored for both types of injury, each lobe was scored separately.

The pathology was assessed to determine whether the NOX4 inhibitor had an effect on
the model of non-eosinophilic asthma. No changes in pathology were observed in the
lungs of mice dosed with either saline or NOX4 inhibitor alone. As seen in Figure
6.21A, the NOX4 inhibitor significantly (p < 0.01) reduced the level of overall
inflammation observed in the non-eosinophilic model of inflammation. To better
understand how the inflammation was reduced each of the two types of lung injury
(alveolar inflammation or epithelial injury) were also analysed. Figure 6.21B shows
that the NOX4 inhibitor significantly (p < 0.01) reduced the level of alveolar
inflammation observed and Figure 6.21C also shows that the NOX4 inhibitor
significantly reduced (p < 0.05) the level of epithelial injury and fibroplasia observed.
Figure 6.21 – Semi-Quantitative analysis of the pathology of formalin fixed, paraffin embedded, H&E stained lung sections of CBA/Ca mice. In combination with a regimen to induce a model of non-eosinophilic asthma (model) CBA/Ca mice were dosed daily for 7 days before culling with a NOX4 inhibitor (compound). A – overall pathology observed, B – Level of alveolar inflammation and C – level of epithelial injury. The non-parametric Mann Whitney T test was used to compare differences in pathology, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.

As well as looking at the level of inflammation at a cellular level, during the time-course of, infection lung function was assessed with a forced manoeuvres system (eSpira). The first parameter that was assessed was FEV, which was measured at set points over a time course (25 – 75 milliseconds). As can be seen in Figure 6.22, in the model of non-eosinophilic asthma there was a significant decrease in FEV across the time course that was assessed.
FEV was measured at time of culling in CBA/Ca mice that were exposed to a regimen of OVA sensitisation and challenge and pneumococcal infection to induce a model of non-eosinophilic asthma (model). Saline control CBA/Ca mice and CBA/Ca mice that had non-eosinophilic asthma were dosed daily for 7 days before culling with a NOX4 inhibitor (compound).

Figure 6.22 – FEV was measured at time of culling in CBA/Ca mice that were exposed to a regimen of OVA sensitisation and challenge and pneumococcal infection to induce a model of non-eosinophilic asthma (model). Saline control CBA/Ca mice and CBA/Ca mice that had non-eosinophilic asthma were dosed daily for 7 days before culling with a NOX4 inhibitor (compound).

Figure 6.23A shows that for the flow derived measure, PEF, during forced exhalation the volume that could be exhaled in the model of non-eosinophilic asthma with or without treatment with the NOX4 inhibitor compound was significantly reduced (p < 0.05) compared to the saline and compound alone control groups. Other parameters to assess the ability of the lung to function were also measured, however no significant (p > 0.05) changes were observed. Figure 6.23B shows that lung compliance was not affected by the model of non-eosinophilic inflammation or the administration of the NOX4 inhibitor compound. In all parameters of lung function assessed the NOX4 inhibitor did not have a therapeutic effect when given in combination with the regimen to induce non-eosinophilic asthma, however it also did not worsen the ability of the lung to function.
In combination with a regimen to induce a model of non-eosinophilic asthma (model) CBA/Ca mice were dosed daily for 7 days before culling with a NOX4 inhibitor (compound). At time of culling, flow derived lung function parameters were assessed with an eSpira forced manoeuvres system (EMMS). A – peak expiratory flow (forced) B – compliance of airways was assessed. Kruskal-Wallis non-parametric test with a Dunns post-test were used to compare differences, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.

As well as investigating the effect of the NOX4 inhibitor on the cellular inflammation and the overall ability of the lung to function, the cilia in the trachea were also observed. After the regimen described in Figure 6.15 a small group of mice (n = 1 for saline and compound alone, n = 2 for model alone and model + compound) were used to test ciliary function. The tracheas were embedded in low melting point agarose and excised. After culling, the excised tracheas were sectioned on a vibrotome, as described in section 2.5.11. A minimum of three tracheal rings were harvested per mouse. For each tracheal ring the proportion of intact epithelium still attached to the basement membrane was evaluated. As can be seen in Figure 6.24A, the proportion of intact epithelium was significantly (p < 0.05) reduced in the model of non-eosinophilic asthma compared to the saline and compound alone control. In Figure 6.24A, it also can be seen that after administration of NOX4 inhibitor in non-eosinophilic asthma there no longer was a reduction in intact epithelium (p > 0.05) compared to the saline or compound only control groups. However, Figure 6.24B shows that after treatment with the NOX4 inhibitor compound, the ciliary beat frequency was significantly increased (p < 0.05) compared to the group that were given non-eosinophilic asthma.
Figure 6.24 – Assessment of the amount of intact epithelium and the ciliary beat frequency observed on tracheal rings of CBA/Ca mice that had been given a regimen to induce a model of non-eosinophilic asthma (model) CBA/Ca mice were dosed daily for 7 days before culling with a NOX4 inhibitor (compound). A - for each tracheal ring viewed percentage of intact epithelium was estimated, B - for each ciliated cell with beating cilia, beat frequency was assessed using the Motion Plus software. The Kruskal-Wallis non-parametric test with a Dunn’s post-test was used to compare differences, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.

6.5 Discussion

As the initial aim of the project had been achieved the next aim was to better understand the role that a persistent pneumococcal infection of the lower airways may have in asthma and COPD. In the previous section, the role that pneumococcal infection might have in COPD disease and exacerbation was investigated. This was done by using an acute challenge of LPS to induce neutrophilic inflammation on day fourteen of the persistent pneumococcal infection. To look at the role pneumococcal infection may have in asthma and COPD it was decided to use a model of OVA-induced allergic inflammation to induce tissue eosinophilia. One of the phenotypes that have been observed in a proportion of COPD patients is tissue eosinophilia (Elias, 2004). The presence of eosinophilia in both COPD and asthma has been associated with the effectiveness of inhaled corticosteroid treatment (Badfahel et al, 2011, McGrath et al. 2012).
The model of OVA-induced inflammation is one of two in vivo models that have been classically used to investigate asthma (Bates et al. 2009). There is no ‘classic’ model of OVA sensitisation and challenge as groups use a wide range of protocols to induce the required inflammatory response (Kumar et al. 2008). For the model of OVA-induced inflammation mice have to first be ‘sensitised’, to do this mice are given a series of intraperitoneal injections of OVA in combination with an adjuvant such as alum (Nials and Uddin 2008). This acts to ‘prime’ the immune system, after this sensitisation period, mice are then ‘challenged’ with either one or a series of intranasal or nebulised administrations of OVA (Zosky and Sly, 2007). After this challenge an induction of Th2 immune cells, eosinophils are observed (Fernandez-Rodriguez et al. 2008). It has also been published that in response to OVA sensitisation and challenge the airways of mice become hyper-responsive to a challenge of methacholine, serum levels of IgE are increased compared to naïve animals, as well as an increase in the cytokines; IL-4, IL-5, IL-13 and eotaxin (Nials and Uddin 2008, Smith and Broadley, 2007). The model of OVA sensitisation and challenge has most commonly been established in the Balb/c strain of mouse as this mouse has been proven to show a strong immune response to OVA (Whitehead et al. 2003).

As there was no established model of OVA-induced allergy in mice at the University of Leicester, a protocol of OVA sensitisation and challenge first needed to be established. There are many established protocols of OVA sensitisation that have been published (Kumar et al. 2008), and each protocol is optimised by a research group to display the asthma phenotype that they are most interested in (Stevenson and Birrell, 2011). The protocols vary in the number of injections (generally between one and three), the length of sensitisation, the number of OVA challenges and also the concentration of OVA used throughout the protocol (Stevenson and Birrell, 2011). The published models can either induce a chronic or an acute inflammation and this is based on the regimen of sensitisation and challenge utilised (Zosky and Sly, 2007).

AstraZeneca (Charnwood) had an established model of acute OVA sensitisation and challenge, so it was initially decided that this should be the regimen that would be used. Unfortunately this regimen had been established in the Balb/c strain of mouse. As the persistent pneumococcal infection has been established in the CBA/Ca strain of mouse and in section 6.2.2 it was shown that the Balb/c strain of mouse was able to clear the
persistent pneumococcal infection, then the OVA sensitisation and challenge regime at
the University of Leicester needed to be established in CBA/Ca mice. Tumes et al.
(2007), compared the susceptibility of three mouse strains; Balb/c, C57/Bl6 and
CBA/Ca, to the OVA-induced inflammation. It was seen that there the propensity to
develop an ‘asthma’ phenotype was strain dependent and that CBA/Ca mice were
though able to develop this phenotype, it was at a much lower level than compared to
Balb/c and C57/Bl6 mice (Tumes et al. 2007). It has been hypothesised that the lower
level of lung eosinophilia observed was due to the regulation of and survival of
eosinophils in the lung (Tumes et al. 2008).

To find out whether CBA/Ca mice had a different response compared to Balb/c mice
when sensitised and challenged with OVA, both Balb/c and CBA/Ca mice were
subjected to three OVA sensitisations a week apart followed by a single intranasal
challenge with OVA. One change however that was made was how the challenge of
OVA was given; AstraZeneca used an aerosolised OVA challenge, but for practical
reasons it was decided to challenge the OVA intranasally. Intranasal administration of
the OVA challenge has been published (Qui et al. 2011) and shown to be more effective
than an aerosolised OVA challenge in Balb/c mice (Swedin et al. 2010). As there were
several published OVA protocols that all used different concentrations of OVA for the
intranasal challenge it was decided that three different concentrations of OVA should be
tested for the intranasal challenge (Essilfie et al. 2012, Qui et al. 2011, Swedin et al.
2010). The Balb/c strain of mouse was tested alongside CBA/Ca mice to act as a
positive control for the protocol. AstraZeneca had already proven that this protocol
worked in the Balb/c strain of mice when the OVA challenge was given as an aerosol so
if eosinophils were not induced it could suggest that the intranasal administration of the
OVA challenge could have affected the results.

It was observed that in both CBA/Ca and Balb/c mice eosinophils could be induced at
48 hours post-infection with all three concentrations of OVA tested. However Balb/c
mice did have eosinophils present in the BALF at 24 hours post-OVA challenge,
whereas CBA/Ca mice did not. This could be due to the rate CBA/Ca mice recruit
eosinophils to the lungs after the OVA challenge being different to that of Balb/c mice
(Tumes et al. 2008). It has also been shown that CBA/Ca mice approximately 50 % less
circulating eosinophils in the blood compared to Balb/c mice so there are less
eosinophils to recruit to the lungs initially, which could explain why it takes longer for eosinophils to appear in the BALF (Grubb et al. 2004).

There are many phenotypes that have been published that are induced by sensitisation and challenge with OVA, which include; airway hyperresponsiveness to methacholine, eosinophilia and an increase in serum IgE (Smith and Broadley, 2007). It was decided that the criterion for a successful model of OVA sensitisation and challenge, that there should be a measurable increase in eosinophils in the BALF. This was because tissue eosinophilia in both COPD and asthma has been observed, and in these groups there has been an association with the effectiveness of inhaled corticosteroid (Badfahel et al, 2011, McGrath et al. 2012). The model that was set up in Leicester had increased numbers of eosinophils.

As it could be seen that eosinophils were present in the BALF of CBA/Ca mice 48 hours post-challenge with OVA it suggested that a successful model of OVA sensitisation and challenge had been established in CBA/Ca mice. Unfortunately, after the third sensitisation with OVA, twenty per cent mortality was observed. In an attempt to eliminate the mortality observed, it was decided to try two different protocols, where the number of sensitisations was reduced from three to two. It was observed that the level of mortality could be decreased by reducing the number of sensitisations; however the level of eosinophils in the BALF 48 hour’s post-OVA challenge was still significantly increased when compared to that of naïve mice.

It was decided to use the sensitisation protocol where mice were sensitised with OVA on days 0 and 14 of the protocol and then challenged on day 21 as no mortality was observed and this was similar to protocols already published (Qui et al. 2011). It is known that Balb/c mice do not stay sensitised indefinitely (unpublished data AZ), and the induced inflammation tends to resolve quickly after the cessation of exposures to antigen (Kumar et al. 2004). The next question asked, was how long do CBA/Ca mice stay sensitised and have a response to an inhaled OVA challenge after the second intraperitoneal injection of OVA in combination with alum. To test this CBA/Ca mice were sensitised with OVA on days 0 and 14 then mice were challenged with OVA on either day 21, 28 or 35. Forty-eight hours after OVA challenge, the number of immune
cells in the BALF were assessed as well as cytokine levels and lung function was assessed.

At all three time points evaluated, a significant increase in eosinophils was observed compared to naive mice. However no differences in the number of eosinophils induced was observed between each time-point assessed, suggesting that mice did remain sensitised.

One of the phenotypes that have been associated with allergic airways disease and the OVA-induced model of inflammation is airway hyperresponsiveness (Kumar and Foster 2012). To assess airway hyperresponsiveness a dose response curve to a bronchoconstrictor such as methacholine is performed (Fernandez-Rodriguez et al. 2008). If there is airway hyperresponsiveness then a change in the lung function measures of compliance or resistance would be observed which would be greater than that measured in a naïve or saline control mouse (Preston et al. 2011). Protocols were worked up for this; however the eSpira forced manoeuvres system used in this project was not sensitive enough to detect these changes. Another difficulty that was faced was that the methacholine had to be given intravenously. Groups commonly give this challenge with a nebuliser (Stevenson and Birrell, 2011), however the system at the University at the University of Leicester did not have the necessary adaptor to do this. It has been shown though that the route of administration for the methacholine challenge is important (Cojacaru et al. 2008).

However even though the response to methacholine could not be measured at the University of Leicester, a significant increase in airway resistance was observed. Airway resistance is observed and also thought to contribute to the pathology of obstructive lung disease (Kaminsky, 2012). Decreases in functional residual capacity, tidal volume and total lung capacity were also observed suggesting that there was a reduction in flow and volume which could be due to increased airway inflammation and airway resistance.
6.6 Combination with the Ovalbumin Model

The next question asked was what effect a persistent low level infection with *S. pneumoniae* would have on OVA-induced lung inflammation. It has been shown by Preston *et al.* (2011), that when ethanol killed *S. pneumoniae* were administered intratracheally ten days after a single OVA sensitisation, a suppression of the number of eosinophils was significantly reduced. This study was carried out in Balb/c mice and was attributed to the induction of regulatory T cells (Preston *et al.* 2011). However it has been shown that in response to an acute infection with *S. pneumoniae* Balb/c mice induce regulatory T cells but CBA/Ca mice do not (Neill *et al.* 2012).

Two different protocols were assessed; the first asked the question; does the presence of a low level pneumococcal infection in the lower airways increase the response to an intranasal challenge with OVA. Whereas, the second protocol, asked the question; does the presence of a persistent low level pneumococcal infection in the lower airways, affected the establishment and outcome of OVA-induced inflammation.

To answer whether the presence of a low level pneumococcal infection in the lower airways increase the response to an intranasal challenge with OVA a dose regimen was designed where the pneumococcal infection was administered between the two OVA sensitisations. Unfortunately when mice, which had a persistent pneumococcal infection in the lungs, were administered the second sensitisation that contained OVA and alum, 50 % of mice had to be culled within two hours of the procedure. Mice were culled as they became severely lethargic, which is the stated severity limit of the Home Office License for this project. As animals had to be culled within a few hours and did not show the usually progression in disease signs observed with pneumococcal infection (Mitchell and Paterson, 2007) this suggested the lethargy was not due to a sudden increase in pneumococcal numbers. It was observed that after OVA sensitisation mice would become cold to the touch and “quiet”, however the change in body temperature was never quantified. It was seen though, that animals that were placed on a heat mat after sensitisation were less likely to become severely lethargic (results not shown). This could be due to contamination of the OVA with endotoxin such as LPS (Tsuchiya *et al.* 2012) as administration of LPS to mice has been shown to cause hypothermia (Liu
et al. 2012). This induction of hypothermia could have been more fatal due to the presence of a persistent pneumococcal infection in the lower airways.

To reduce the number of animals that did not reach the end of the protocol it was decided to introduce the pneumococcal infection after the sensitisations had been administered but still before the OVA challenge. It had been previously shown that similar changes in lung function and cellular influx into the BALF was observed when the OVA challenge was given either seven or fourteen days after the second sensitisation with OVA. Using this protocol the mortality seen with the second sensitisation was reduced.

It was seen with both protocols that when the pneumococcal infection was present there was a significant decrease in the number of eosinophils induced after OVA sensitisation and challenge compared to animals that received only OVA. This was the same result as was seen by Preston et al. (2011). To better understand the drivers for the reduction in eosinophils cytokines were analysed.

When mice were sensitised and challenged with OVA alone there was no significant increases in the levels of cytokines associated with Th2 inflammation, such as IL-4, IL-5 and eotaxin (not all data shown), however there was a significant increase in the chemokine IP-10. It has been shown that IP-10 is able to down regulate the expression of the cytokines IL-4, IL-5 and IL-13 but up regulate the expression of IFN-γ (Romagnani et al. 2005). The chemokine IP-10 has been shown to exert chemotactic activity on Th1 cells as its receptor CXCR3 has been shown to be preferentially expressed on these cells (Bonecchi et al. 1998). The level of IP-10 in the BALF was still significantly induced when the persistent pneumococcal infection was present in combination with the OVA sensitisation and challenge suggesting that it was independent of the change in eosinophil number.

One cytokine that was increased in the OVA sensitisation challenge model alone but was not raised in the model when the pneumococcal infection was present in the adapted protocol was IL-12p40. The cytokine that this subunit is part of is IL-12, which has been associated in abrogating the phenotype of experimental asthma (Schwarze et al. 1998). The ELISA kit for the detection of the subunit IL-12p40 is non-specific and
can also detect the subunit IL-23p40 (R & D systems product information). It has been shown in mice that IL-23 is also able to differentially regulate Th1 and Th2 responses and that overexpression of IL-23 led to an increase in eosinophil number (Peng et al. 2010). This suggests that the eosinophilic inflammation observed in CBA/Ca mice was induced by IL-23 rather than IL-12. This is of clinical relevance as levels of IL-23 have been shown to be statistically higher in asthmatic children compared to healthy children (Ciprandi et al. 2012a). To confirm that it was changes in IL-23 rather than IL-12 that was being measured Th17 cells could be measured as it has been demonstrated that the cytokine IL-23 induces Th17 cells (Ciprandi et al. 2012b).

The ability of the lung to function was also assessed with the adapted protocol. No changes in the flow based parameters; FEV, tidal volume or forced peak expiratory flow were observed in any of the conditions tested. However it was seen that there was a significant increase in airway resistance in mice that had received OVA sensitisation and challenge regardless of whether the persistent pneumococcal infection of the lowers airways was present. This suggested that although the presence of pneumococcal infection was able to reduce the eosinophilic inflammation induced by the OVA sensitisation and challenge it did not alter the physiological effect on the airways.

The other protocol tested asked whether the presence of a persistent low level pneumococcal infection in the lower airways, affected the establishment and outcome of OVA-induced inflammation. The results with this protocol reflected what was seen with the first protocol tested. The population of inflammatory cells in the BALF was the same as observed with the previous protocols, there was a significant decrease in eosinophils after OVA challenge when the pneumococcal infection was also present. The cytokine protocol was similar too, with levels of IL-12p40 being raised in the BALF of animals that had both the pneumococcal infection and the sensitisation and challenge with OVA compared to naïve mice.

With all three protocols tested, there was a significant reduction in the number of eosinophils in the BALF when mice had a pneumococcal infection and had been challenged with OVA, compared to mice that were only exposed to OVA sensitisation and challenge. As previously discussed the cytokine IL-23 may be involved in this reduction in eosinophilia. It has also been shown that components of S. pneumoniae
(pneumolysin and type-3 polysaccharide) have suppressive properties in eosinophilic asthma by inducing regulatory T cells (Thorburn et al., 2012). It was shown that when both of these components were administered during an established model of ovalbumin the induction of eosinophils was reduced. This reduction in eosinophilia was seen when the OVA challenge was administered to mice with a persistent *S. pneumoniae* infection in the lungs. To determine whether the suppression of eosinophils in is due to the presence of pneumolysin, mice could be challenged with a strain of *S. pneumoniae* serotype 19F that had been genetically engineered to have the gene for pneumolysin knocked out. If it was due to the presence of pneumolysin then this strain would not reduce the eosinophilia induced by the OVA challenge. Pneumolysin could be suppressing the same pathway that IL-23 is part of.

The reduction in eosinophil numbers in the BALF suggests a switch from a predominantly Th2 response induced by the OVA, to a Th1 response. This could be due to the cytokine IL-23 inducing Th17/Th2 regulatory cells in mice that receive only the OVA sensitisation and challenge (Ciprandi et al. 2012b). It is now believed that some regulatory T cells express both Th17/Th2 and that these cells could play a role in the pathogenesis of asthma (Cosmi et al. 2011). To investigate this further the populations of T regulatory cells would need to be defined in animals that received both a pneumococcal infection and the OVA sensitisation and challenge.

Whether the OVA induced a Th2 response in CBA/Ca mice should be further investigated. One way to do this is to assess changes in cytokines associated with a Th2 response such as IL-4, IL-5 and eotaxin. These had been measured in the BALF for all experiments where OVA was administered, however no differences were observed in animals sensitised and challenged with OVA compared to the levels measured in the BALF of control mice. A second way to assess whether the sensitisation had been successful would be to assess any increases in serum IgE (Beck and Spiegelberg, 1989). Serum was collected for all experiments and a direct ELISA for the measurement of ovalbumin specific IgE was developed, however no OVA-specific IgE could be detected when assessing changes in individual mice. Further optimisation of this protocol would be needed for the future assessment of changes in serum IgE to confirm that the sensitisation had been successful. However eosinophils were only observed in the BALF of mice that had been both challenged and sensitised with OVA. Control
groups that received OVA sensitisation or OVA challenge did not have eosinophils present in the BALF. This demonstrated that mice had to receive both the OVA sensitisation and challenge for eosinophils to be induced which suggests that the sensitisation was required.

6.7 Can the inhibition of NADPH oxidase 4 (NOX4) improve lung function in a model of non-eosinophilic asthma?

As previously mentioned there are a significant proportion of mild-to-moderate asthmatics that have predominantly neutrophilic inflammation in the airways which are resistant to inhaled corticosteroid treatment (McGrath et al. 2012, Green et al. 2002). Other treatments need to be found that can reduce the inflammation and the lung damage observed in these mild-to-moderate asthmatics. One potential therapeutic target are the NADPH oxidases (NOX) enzymes (Lambeth et al. 2008). NOX enzymes mediate physiological functions including host defense and cell signalling, through the generation of reactive oxygen species (Lambeth, 2004). It has been suggested that overexpression of NOX4 in asthma is critical in the promotion of oxidative stress and in turn airway smooth muscle hypercontractility (Sutcliffe et al. 2012). The hypothesis is that in a model of non-eosinophilic asthma the inhibition of NOX4 would lead to the prevention of reactive oxygen overexpression which in turn would lead to an improvement in disease progression.

By combining an in vivo model that was known to trigger a Th2 driven inflammatory response (OVA sensitisation and challenge model) with a model that has been shown to trigger a Th1 driven inflammatory response (persistent pneumococcal infection model) a model of non-eosinophilic asthma has been developed. This combined model of allergy and persistent pneumococcal infection resulted in an inflammatory response which consisted of neutrophilic inflammation but with a significant reduction in induced eosinophils compared to the OVA sensitisation and challenge model alone. Yet there was still an increase in airway resistance in all animals that had received OVA sensitisation and challenge, regardless of if pneumococcus was present in the lower airways.

To test whether the inhibition of NOX enzymes did lead to an increase in lung function in this novel model of ‘non-eosinophilic asthma’ an inhibitor of NADPH oxidase 4
(NOX4) (donated from the biotechnology company Genkyotex) was administered. It was recommended by Genkyotex that the compound should be dosed orally for a minimum of 7 days. To accommodate this the day the pneumococcal infection was administered was moved from day 21 to day 17 of the protocol.

As previously discussed when the corticosteroid was dosed prophylactically and therapeutically to CBA/Ca mice that had been dosed intranasally with pneumococcal strain LgSt215 there were two different outcomes. When dosed prophylactically there was a significant worsening in disease signs and mice had to be culled 6 days post-infection yet when dosed therapeutically from 14 days post-infection there was no apparent effect of steroid. As the effect of the NOX4 inhibitor on an in vivo pneumococcal infection was unknown it was decided that compound should be dosed therapeutically once the infection had established in the lower airways of mice as the question being asked was not what effect a NOX4 inhibitor had on the establishment of a persistent pneumococcal infection but what effect it would have on a model of non-eosinophilic asthma. To allow the establishment of infection and the compound to be dosed for 7 days the OVA challenge dose was moved to day 28 from day 21. As seen during the establishment of the OVA sensitisation and challenge at the University of Leicester, a similar level of eosinophilic inflammation and change in lung functioning could be achieved when the OVA challenge was dosed on day 28 as it was if it was administered on day 21 after OVA sensitisations on days 0 and 14.

It was observed that the NOX4 inhibitor had minimal effects on the cellular inflammation in the BALF and on the functioning of the lungs when compared to the inflammation observed in the model of non-eosinophilic asthma. However the key thing to note was that although therapeutic effects were minimal there was no worsening of symptoms. It was observed that there was no change in the number of pneumococci recovered in the BALF, if anything there was a slight suggestion that the number of pneumococci were reduced after treatment with the NOX4 inhibitor. This could be due to the improvement in ciliary beat frequency and also the increase in the proportion of intact epithelium. Ciliary function is important as respiratory cilia beat in a co-ordinated manner with a specific frequency and pattern to clear mucus and debris from the airways (Chilvers et al. 2003). It has been suggested that bacteria such as S. pneumoniae are able to disrupt the ciliary epithelium and detrimentally affect the cilia
In the chapter of this thesis that describes the characterisation of the persistent pneumococcal infection it was shown that a persistent presence of *S. pneumoniae* in the lower airways was able to disrupt the epithelium and reduce ciliary beat frequency. This reduction in intact epithelium and ciliary beat frequency was also observed in the model of non-eosinophilic asthma. However administration of a NOX4 inhibitor increased the proportion of intact epithelium and cilia beat frequency.

As well as this improvement in ciliary function the administration of the NOX4 inhibitor resulted in a reduction of the incidence of inflammation observed in the histopathology. It was observed that in the model of non-eosinophilic asthma foci of inflammation were observed which consisted of a neutrophilic infiltration, with some consolidation of the alveolar bed. Epithelial damage was also observed as well as foci of fibroplasia at the transitional airways, which was also observed from 14 days post infection with the pneumococcal strain LgSt215. Animals that had received both the model of non-eosinophilic asthma and the NOX4 inhibitor showed the same range of inflammatory responses that were observed in the non-eosinophilic model alone, however there was an apparent reduction in the incidence of the foci of inflammation. To assess whether this was the case a semi-quantitative scoring system was applied to the histopathology sections to grade the inflammation observed. The semi-quantitative scoring system was then split into two phenotypes; the first graded the inflammation present in the alveolar bed and the second was used to grade the level of injury observed to the epithelium. It was observed that there was a significant reduction in the level of inflammation in the alveolar bed after administration of the NOX4 inhibitor, suggesting the level of inflammatory cell consolidation was reduced. The administration of the NOX4 inhibitor also significantly reduced the incidence of epithelial injury and also focal fibroplasia.

Overall this suggested that the NOX4 inhibitor did have a small but significant effect on the incidence of inflammation and the ciliary function in the model of non-eosinophilic asthma. To increase the potency of the NOX4 inhibitor it could be administered for a longer duration or it could be dosed more frequently as it was only dosed once daily in this study. In previous studies performed by Genkyotex with this compound, it was administered for longer and more frequently so this could be an option.
7 Discussion:

The aim of this project was to develop and characterise a model of persistent *S. pneumoniae* colonisation in the lower airways of mice. It is known that some microorganisms for example *S. pneumoniae* and *H. influenzae*, often colonise the airways of COPD patients in a stable state (Hirschmann, 2000). Sethi (2000) hypothesises that this colonisation is a low-grade infection that induces chronic airway inflammation. However, it is hypothesised that in these low-grade persistent infections there is a clinically stable state, with an underlying inflammatory condition that can be unbalanced by change in the *in vivo* environment. During the progression of COPD, there is an increase in mucus production, depressed ciliary function and epithelial cell injury, which may allow bacteria not normally present in the airway below the larynx, to colonize the diseased epithelium (Read, 1999). A study by Patel *et al.* (2002) has shown that the presence of bacterial colonisation in the stable state was associated with an increased frequency of exacerbations.

Before this project, the majority of pneumococcal infection models in the lower airways of mice were acute in nature with animals succumbing to disease within 48 hours of infection (Chiavolini *et al.* 2008). Whereas the aim of this project was to develop a model that mimicked a chronic infection with animals having a persistent pneumococcal presence in the lower airways for a minimum of four weeks, with no outward signs of disease. It was hypothesised that if the infection persisted for four weeks a stable phenotype would be observed that would mimic what (Sethi, 2000) defines as colonisation in the clinic. It would also model the phenotype in the severe respiratory diseases asthma and COPD in humans where a low level bacterial presence is observed. This is thought to contribute to the pathogenesis of both diseases (Stevenson and Birrell, 2011).

The initial criteria that defined whether the model was successful were; that the model would consistently have a persistent pneumococcal presence in the lower airways with few or no outward signs of disease, but there would be an accompanying inflammatory response at a cellular level. This would occur in over 70 % of mice after a single intranasal infection. As one criterion of the model of persistent infection was that it was asymptomatic, it meant that disease signs could not be used to determine whether an
animal had a persistent pneumococcal infection in the lower airways or whether the pneumococci was no longer present in the lower airways. The only way to confirm the presence of pneumococci in the lower airways was to cull the mice and harvest BALF or lungs so that the number of viable pneumococci present could be enumerated by viable counting. Future work could be to investigate whether a biomarker of the persistent infection could be measured non-invasively. Cytokines in the serum of mice have been used to monitor disease progression in animal models of bacterial-induced inflammatory bowel disease (Torrence et al. 2008). A promising chemokine appeared to be IP-10, however IP-10 and other cytokines analysed at the later time points in the serum (from 14 days post-infection) of the model of persistent infection, no cytokines measured in the serum were significantly raised compared to the level measured in the serum of naïve mice. Further work could be done to evaluate a wider range of cytokines. As well as using cytokines in the serum, analysis of the breath could be used to search for biomarkers. One biomarker in the human breath that has been associated with atopic asthma is exhaled nitric oxide (Kharitonov and Barnes, 2002). Exhaled nitric oxide has been measured in a spontaneously breathing mouse (Weicker et al. 2001). Work was carried out to optimise this technique in mice, however it was not possible to consistently and reliably measure exhaled nitric oxide from spontaneously breathing mice, with the equipment at the University of Leicester.

Further work could be done to understand why the pneumococcal infection with strain LgSt215 persisted in the lower airways of CBA/Ca mice but was cleared from the lower airways of Balb/c and MF1 mice. One explanation could be the induction of T regulatory cells, as it has been demonstrated that Balb/c mice were able to induce higher numbers of these inflammatory cells after an infection with a serotype 2 pneumococcus compared to CBA/Ca mice (Neill et al. 2012). It has been shown that CD3-positive cells are induced in response to the persistent pneumococcal infection but further work needs to be done to elucidate the role that they play during the pneumococcal infection.

Respiratory diseases such as severe asthma and COPD are complex, as they are made up of a combination of different endotypes / phenotypes (Lotvall et al. 2011). They are largely heterogeneous conditions, (Wedzicha, 2000), with patients displaying varied symptoms. This represents a challenge to scientists when trying to model this disease. Current in vivo models sometimes offer poor translation to the human condition, novel
compounds that have been proven to work in animals don’t always show efficacy in human trials (Rennard et al. 2007). This could be due to a limited mechanistic understanding of the progression of COPD, as COPD like asthma is diagnosed by a series of physiological changes rather than a specific mechanistic change. To address this, a combination of approaches is being used. One approach research groups have started to use is to combine models of inflammation that mimic on aspect of respiratory disease with others e.g. rhinovirus and OVA, Haemophilus and OVA, PolyIC and LPS, LPS and smoke. This is in an attempt to develop more clinically relevant in vivo models. One model that has been missing is a model of long term bacterial colonisation with human pathogens such as S. pneumoniae and nontypeable H. Influenzae (Stevenson and Birrell, 2011). This project has developed a model with S. pneumoniae and combined it with two other models of inflammation that have been used historically to investigate asthma and COPD.

After characterisation the first model of inflammation that the model of persistent pneumococcal infection was combined with was a model of LPS-induced lung injury. An acute model of LPS-induced lung injury induced an influx of neutrophils, which caused pulmonary damage after a single intranasal challenge of LPS (Matute-Bello et al. 2008). It was seen that six hours after an acute challenge of LPS was dosed on day fourteen of a persistent pneumococcal infection with strain LgSt215 an increase in the number of neutrophils in the BALF was observed. It is known that both models of inflammation induce a low level of neutrophils at this point, however when given in combination with each other the influx of neutrophils was additive. This could suggest that this combination of inflammatory models could mimic a COPD exacerbation which is defined as a sustained worsening of the patient’s condition from the stable state and beyond normal day-to-day variations, which is acute in onset and may warrant additional treatment (Burge and Wedzicha, 2003).

During an exacerbation one of the recommended therapies is a daily dose of 30 – 40 mg / kg prednisolone per day for 10-14 days (GOLD, 2013). The effects of the inhaled corticosteroid prednisolone were investigated on the persistent pneumococcal infection model as well as on the influx of neutrophils that was induced by the combination of the LPS-induced lung injury with the pneumococcal infection. It was observed that when prednisolone was dosed from the day before infection with strain LgSt215, it had a
detrimental effect with an increased bacterial load and inflammatory response being observed. This could be because corticosteroids are potent inhibitors of inflammatory processes (van der Velden, 1998). It is generally thought that T cells and eosinophils are more susceptible to corticosteroids compared to neutrophils (Belvisi, 2004). This could suggest that T cells as well as neutrophils could be a key immune cell population in host response to a pneumococcal infection (Neill et al. 2012), as the suppression of both inflammatory cell populations leads to a proliferation in pneumococci number. However to confirm this more work would need to be done. It was also seen that when prednisolone was given orally for four days from 14 days post-infection no significant differences were observed in bacterial load or inflammatory cells. This suggests that the infection at this point could be steroid ‘insensitive’ which is a phenotype that has been seen in COPD and neutrophilic asthma (Barnes, 2013).

When corticosteroid was given on day 14 of the pneumococcal infection one hour before an intranasal challenge with LPS, some reduction in inflammation was observed. However there was still a measureable inflammatory response present which was comparable to that seen with day 14 of the pneumococcal infection alone without an LPS challenge. This would suggest that the corticosteroid could reduce the inflammatory response to the new stimulus (the LPS challenge) but did not affect the existing inflammation. From this it could be thought that the mode of action of prednisolone is to block the induction of inflammatory cells, rather than to induce the apoptosis of the inflammatory cells that are already present (van der Velden, 1998).

As well as investigating the response of LPS-induced injury in combination with a persistent pneumococcal infection it was decided to combine the persistent pneumococcal infection with a model of allergy. The model of allergy chosen induces an inflammatory response by sensitising and challenging mice with the protein OVA. Sensitisation and challenge with OVA induces an influx of eosinophils into the BALF of animals (Stevenson and Birrell, 2011).

It was seen that when the persistent pneumococcal infection was combined with sensitisation and challenge with OVA the eosinophilic inflammation was significantly reduced. This has been seen previously in response to ethanol-killed S. pneumoniae (Thorburn et al. 2012), but not to a low level persistent presence of inflammation. This
suggests that the eosinophilic inflammatory response could be skewed by the innate response. This could possibly be regulated by Th17 cells, due to the cytokine sub units that have been detected in response to OVA induced inflammation and the pneumococcal infection (Peng et al. 2010). Further work needs to be done to elucidate the mechanisms by which this occurs, however as discussed previously due to the inflammatory response to the persistent pneumococcal infection being focal from 14 days post-infection understanding changes in the inflammatory response could be a challenge.

It could be thought that the combination of the persistent pneumococcal infection with induced inflammation from OVA sensitisation and challenge could mimic the non-eosinophilic endotype observed in approximately 50% of mild-to-moderate asthmatics (McGrath et al. 2012). This is an important endotype to be able to mimic in vivo as these asthmatics are known to be steroid insensitive (Green et al. 2002). Corticosteroids are one of the key treatments used in the management of asthma (Belvisi, 2004), so alternative treatments need to be researched.

One compound that is being investigated for the treatment of asthma is a NOX4 inhibitor developed by Genkyotex. NOX enzymes mediate physiological functions including host defense and cell signalling, through the generation of reactive oxygen species (Lambeth, 2004). It has been suggested that over expression of NOX4 in asthma is critical in the promotion of oxidative stress and in turn airway smooth muscle hypercontractility (Sutcliffe et al. 2012). It was seen that when the NOX4 inhibitor was given in the possible model of non-eosinophilic asthma then ciliary function was improved and there was a reduction in the incidence of foci of inflammation. This suggested that it could be having a beneficial effect in the model. However further work needs to be done to investigate this further. Other work that could be done is to see whether this model of non-eosinophilic asthma is sensitive to steroids.

The key aims of this project have been fulfilled. A model of persistent pneumococcal infection in the lower airways of mice has been established and characterised that persists for a minimum of a month. This model has also been combined with two inflammatory insults (LPS and OVA) that have historically been used to study the mechanisms involved in COPD and asthma. As well as this the effects of an established
corticosteroid (prednisolone) and also a pre-clinical compound (NOX4 inhibitor) have been investigated on the pneumococcal infection. Further work needs to be done to elucidate the mechanisms involved in the inflammatory response but this project has developed a clinically relevant, robust and novel model of inflammation with a clinically relevant serotype of the human pathogen *S. pneumoniae*. 
8 Appendix

8.1 Statistical Analyses

Throughout the thesis non-parametric statistical tests were used to analyse the data and to confirm whether changes observed were statistically significant. Non-parametric tests should be used when the data does not fit a normal (Gaussian) distribution; whereas when the data does fit a normal distribution then a parametric test should be used. To test whether the data was normally distributed a D’Agostino-Pearson omnibus test was used.

8.1.1 Worked Example

8.1.1.1 Characterisation of the cellular responses to a persistent pneumococcal infection

To determine the effect of the persistent infection on immune cell populations, differential cell counts were performed on the BALF. Lungs were lavaged as described in section 2.5.3 and samples were cytocentrifuged before staining with the Diff-quick staining kit to distinguish different immune cell populations. The number of immune cells per sample was determined by the number of immune cells counted in 10 fields of view at X 400 magnification.

In Table 8.1 shows that the majority of data was normally distributed (p < 0.05) when tested with the D’Agostino-Pearson omnibus test, but this was not the case for all data sets. All of the data collected from the control group (naïve mice) was shown to not be normally distributed when using the D’Agostino-Pearson omnibus test (p < 0.05).
Was the data normally distributed?

<table>
<thead>
<tr>
<th>Cell Type Counted</th>
<th>Time post-infection</th>
<th>24 hours</th>
<th>7 days</th>
<th>14 days</th>
<th>21 days</th>
<th>28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cells</td>
<td>Naive</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.05</td>
<td>p &gt; 0.05</td>
<td>p &lt; 0.05</td>
<td>p &gt; 0.05</td>
<td>p &gt; 0.05</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.05</td>
<td>p &gt; 0.05</td>
<td>p &lt; 0.05</td>
<td>p &gt; 0.05</td>
<td>p &gt; 0.05</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Macrophages</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.05</td>
<td>p &gt; 0.05</td>
<td>p &gt; 0.05</td>
<td>p &gt; 0.05</td>
<td>p &gt; 0.05</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>Monocytes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.05</td>
<td>p &gt; 0.05</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.05</td>
<td>p &gt; 0.05</td>
<td>p &gt; 0.05</td>
<td>p &gt; 0.05</td>
<td>p &gt; 0.05</td>
<td>p &gt; 0.05</td>
</tr>
</tbody>
</table>

Table 8.1 – The D’Agostino-Pearson omnibus test was used to determine whether the data was normally distributed.

As the data was a combination of both normally and not normally distributed values, then the same data was compared with both the non-parametric Kruskal-Wallis test with a Dunn’s post-test (Figure 4.4) and the parametric one-way ANOVA test (Figure 8.2). Figure 4.4 and Figure 8.2 both show the differential cell counts of the immune cells in the BALF. Figure 4.4A shows that there was a transient increase in neutrophil numbers at 24 hours post infection (p < 0.05) but numbers declined to control levels by 14 days post-infection, this was confirmed by the one-way ANOVA analysis (Figure 8.2A). Both statistical tests showed that the numbers of neutrophils in the BALF were raised at 24 hours and 7 days post-infection (p < 0.05), but only the non-parametric test showed the increase to be significant (p < 0.05) at 14 days post-infection.

Similar results were observed with both the non-parametric and the parametric analysis of the data. Though the parametric analysis showed that less time-points were significantly different from the naïve data set (p > 0.05). However this was not the case for the number of macrophages observed, where using a parametric analysis that the number of macrophages in the BALF were significantly higher at 7, 21 and 28 days post infection compared to the number of macrophages in the BALF of naïve mice.
compared to the non-parametric test which showed this to be the case at only 21 and 28 days post-infection.

**Figure 8.1** - CBA/Ca mice were intranasally infected with *S. pneumoniae* strain LgSt215. At pre-determined time points post-infection, bronchoalveolar lavage fluid (3 x 300 µl PBS) was harvested (n = 10). A – Number of neutrophils, B – Number of lymphocytes, C – Number of macrophages and D – Number of monocytes counted in 10 fields of view at X 400 magnification. Kruskal-Wallis non-parametric test with a Dunns post-test were used to compare differences between time post-infection and naive. * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.
Figure 8.2 - CBA/Ca mice were intranasally infected with *S. pneumoniae* strain LgSt215. At pre-determined time points post-infection, bronchoalveolar lavage fluid (3 x 300 µl PBS) was harvested (n = 10). A – Number of neutrophils, B – Number of lymphocytes, C – Number of macrophages and D – Number of monocytes counted in 10 fields of view at X 400 magnification. One-way ANOVA parametric test with a Dunnett’s multiple comparison post-test were used to compare differences between time post-infection and naive, * p < 0.05 > 0.01, ** p < 0.01 > 0.001, *** p < 0.001.

As the D’Agostino-Pearson omnibus test for normality showed that the data collected was a mixture of normally and not normally distributed data, both a parametric and a non-parametric statistical analysis could be used, though all experiments should be analysed with either a non-parametric or a parametric analysis. In this thesis, non-parametric statistical tests were used as it could not be assumed that all data was normally distributed.
8.2 Characterisation of the cytokine and chemokine responses to a persistent pneumococcal infection

Differential counts demonstrated how the cellular responses in the BALF shifted from being predominantly neutrophilic at 24 hours post-infection to being predominantly monocytic and lymphocytic from 7 to 28 days post-infection. To better understand the drivers of this inflammatory change, cytokine and chemokine levels were assessed in the BALF and serum at pre-determined times post-infection. Inbred CBA/Ca mice were intranasally dosed with $4 \times 10^6$ CFU (strain LgSt2150) suspended in 30 µl. At the pre-determined time points 24 hours, 7 days and 14 days post-infection, bronchoalveolar lavage fluid (3 x 300 µl PBS) and serum was harvested.

A combination of twenty cytokines and chemokines were analysed in the BALF and serum with a MILLIPLEX Mouse Cytokine / Chemokine panel (Millipore, Billerica, USA) kit. Table 8.2 shows whether the levels of cytokine and chemokines measured in the BALF or serum were significantly raised compared to naïve at each time point tested (24 hours, 7 days or 14 days post-infection). In the BALF and serum, the cytokines and chemokines eotaxin, GM-CSF, IL-2, IL-10, IL12-p40, MIP2 and TNFα were not significantly raised ($p > 0.05$) compared to that of a naïve animal at any of the three time points tested. As can be seen in Table 8.2, most changes in chemokine and cytokine levels were observed at 24 hours post-infection. At 24 hours post-infection ten of the cytokines and chemokines tested were significantly raised ($p < 0.05$) in the BALF and five were significantly raised ($p < 0.05$) in the serum compared to that of naïve animals. In the serum, no cytokines or chemokines were significantly raised ($p > 0.05$) at 7 days post-infection compared to naïve animals. However the chemokines Interferon gamma induced protein 10 (IP-10) and monocyte chemotactic protein 1 (MCP 1), were significantly raised ($p < 0.05$) against naïve at 14 days post-infection. In the BALF Interferon γ (IFN γ), IP-10 and LPS Induced CXC chemokine (LIX) were significantly raised ($p < 0.05$) at 7 days post-infection compared to naïve mice. LIX was also significantly raised ($p < 0.05$) in the BALF at 14 days post-infection compared to naïve mice.
<table>
<thead>
<tr>
<th>Cytokine / Chemokine</th>
<th>Was the chemokine / cytokine significantly raised against naïve mice in the BALF?</th>
<th>Was the chemokine / cytokine significantly raised against naïve mice in the sera?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hours</td>
<td>7 days</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>p &gt; 0.05</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>GM - CSF</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>p &gt; 0.05</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>IFN – γ</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>p &gt; 0.05</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>IL – 1β</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.05</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>IL – 2</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>p &gt; 0.05</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>IL – 6</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.05</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>IL – 10</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>p &gt; 0.05</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>IL – 12 p40</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>p &gt; 0.05</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>IL – 12 p70</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.05</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>IP – 10</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>KC</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.01</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>LIX</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>MCP 1</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>p &gt; 0.05</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>MIG</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.05</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>MIP 1 α</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.05</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>MIP 1 β</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>p &gt; 0.05</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>MIP 2</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>p &gt; 0.05</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>Rantes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.05</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>TNF α</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>p &gt; 0.05</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>VEGF</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.05</td>
<td>p &gt; 0.05</td>
</tr>
</tbody>
</table>

Table 8.2 – Summary of the changes seen in 20 cytokine and chemokine levels in the BALF and Serum from CBA/Ca mice were intranasally infected with *S. pneumoniae* strain LgSt215, measured by a MILLIPLEX assay (naïve n = 4, experimental groups n = 8). Each column represents a different time point post-infection.
The MILLIPLEX panel allowed twenty cytokines and chemokines to be screened in one assay with a relatively small volume of sample. After this screening, individual cytokine levels were analysed by sandwich ELISA. For all cytokine analysis by ELISA, inbred CBA/Ca were intranasally dosed with $4 \times 10^6$ CFU (strain LgSt2150) suspended in 30 µl. At pre-determined time points, mice were culled and bronchoalveolar lavage fluid (3 x 300 µl PBS), whole lung homogenate and serum were harvested and samples were frozen. For analysis samples were defrosted and R & D Duoset kits were used as per manufacturer’s instructions to measure individual cytokines and chemokines.

8.2.1 Discussion

As *S. pneumoniae* could be recovered from the lungs of mice 28 days post-infection, the next thing to be understood was whether the presence of the pneumococcus was being recognised by the host. To do this several parameters were evaluated, including the number of inflammatory cells present and any changes in levels of cytokines.

To better understand the underlying drivers of the cellular response the cytokine profile was also determined in the BALF, lung and sera. As an exploratory exercise a panel of cytokines were analysed using a MILLIPLEX kit from Millipore. This enabled a large number of cytokines to be tested with a relatively small amount of sample.
<table>
<thead>
<tr>
<th>Cytokine / Chemokine</th>
<th>Role in Inflammation</th>
<th>BALF</th>
<th>Sera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eotaxin</td>
<td>Eosinophils modulation</td>
<td>No Change</td>
<td>No Change</td>
</tr>
<tr>
<td>GM - CSF</td>
<td>Stimulates growth of cells of monocytic lineage</td>
<td>No Change</td>
<td>No Change</td>
</tr>
<tr>
<td>IFN – γ</td>
<td>Macrophage activation</td>
<td>Raised at 7 days post-infection</td>
<td>Raised at 24 hours post-infection</td>
</tr>
<tr>
<td>IL – 1β</td>
<td>Fever, T cell and Macrophage Activation</td>
<td>Raised at 24 hours post-infection</td>
<td>No Change</td>
</tr>
<tr>
<td>IL – 2</td>
<td>T cell growth factor</td>
<td>No Change</td>
<td>No Change</td>
</tr>
<tr>
<td>IL – 6</td>
<td>Differentiation of mature and immature T cells</td>
<td>Raised at 24 hours post-infection</td>
<td>Raised at 24 hours post-infection</td>
</tr>
<tr>
<td>IL – 10</td>
<td>Potent suppressor of macrophages</td>
<td>No Change</td>
<td>No Change</td>
</tr>
<tr>
<td>IL – 12 p40</td>
<td>Activates Natural Killer Cells and induces CD4 T cell differentiation into Th1 like cells</td>
<td>No Change</td>
<td>No Change</td>
</tr>
<tr>
<td>IL – 12 p70</td>
<td></td>
<td>Raised at 24 hours post-infection</td>
<td>No Change</td>
</tr>
<tr>
<td>IP – 10</td>
<td>Recruitment of activated T cells</td>
<td>Raised at 24 hours and 7 days post-infection</td>
<td>Raised at 14 days post-infection</td>
</tr>
<tr>
<td>KC</td>
<td>Neutrophil chemoattractant</td>
<td>Raised at 24 hours post-infection</td>
<td>Raised at 24 hours post-infection</td>
</tr>
<tr>
<td>LIX</td>
<td>Induced by bacterial lipopolysaccharides</td>
<td>Raised at 24 hours, 7 and 14 days post-infection</td>
<td>No Change</td>
</tr>
<tr>
<td>MCP 1</td>
<td>Monocyte chemoattractant</td>
<td>No Change</td>
<td>Raised at 14 days post-infection</td>
</tr>
<tr>
<td>MIG</td>
<td>Chemoattractant for stimulated T cells</td>
<td>Raised at 24 hours post-infection</td>
<td>No Change</td>
</tr>
<tr>
<td>MIP 1 α</td>
<td>Causes local inflammatory responses</td>
<td>Raised at 24 hours post-infection</td>
<td>Raised at 24 hours post-infection</td>
</tr>
<tr>
<td>MIP 1 β</td>
<td>Enhances activities of GM - CSF</td>
<td>No Change</td>
<td>No Change</td>
</tr>
<tr>
<td>MIP 2</td>
<td>Chemoattractant for segmented neutrophils</td>
<td>No Change</td>
<td>No Change</td>
</tr>
<tr>
<td>Rantes</td>
<td>Chemoattractant for T cells, basophils and increases the adherence of monocytes to epithelial cells</td>
<td>Raised at 24 hours post-infection</td>
<td>No Change</td>
</tr>
<tr>
<td>TNF α</td>
<td>Promotes inflammation, endothelial activation</td>
<td>No Change</td>
<td>No Change</td>
</tr>
<tr>
<td>VEGF</td>
<td>Monocyte chemoattractant</td>
<td>Raised at 24 hours post-infection</td>
<td>No Change</td>
</tr>
</tbody>
</table>

Table 8.3 – Summary of the changes seen in 20 cytokine and chemokine levels in the BALF and Serum from CBA/Ca mice were intranasally infected with *S. pneumoniae* strain LgSt215, measured by a MILLIPLEX assay.
The advantage of using the MILLIPLEX assay was that twenty cytokines could be analysed on the same assay using very small amounts of sample. The disadvantage to this method is that it is not as sensitive as testing each individual cytokine or chemokine by sandwich ELISA, which meant that although it could identify cytokines and chemokines that may be of interest, further work needed to be done to elucidate actual cytokine changes. There were also technical problems with the assay which have been discussed in Chapter 4, which is why the data collected from the MILLIPLEX assay was only used to identify possible cytokines and chemokines of interest. These cytokines were then tested further by sandwich ELISA to elucidate whether the changes observed were statistically significant.
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