Regulation of Cytokines and Chemokines during Lung Infection with Nontypeable *Haemophilus influenzae*

By

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Abbreviations

APC	Antigen presenting cell
BAL	Bronchoalveolar lavage
CBA	Chocolate blood agar
ChoP	Phosphorylcholine
CFU	Colony forming unit
COPD	Chronic obstructive pulmonary disease
DEPC	diethyl pyrocarbonate
ELISA	Enzyme linked immunosorbent assay
GM-CSF	Granulocyte macrophage-colony stimulating factor
Hib	*Haemophilus influenzae* type b
ICAM	Intracellular adhesion molecule
IFN	Interferon
IL	Interleukin
IPP	Intra Peyer's patch
IP	Intra peritoneal
IT	Intra tracheal
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MCP	Monocyte chemoattractant protein
MIP	Macrophage inflammatory protein
MLN	Mesenteric lymph node
NTHi	Nontypeable *Haemophilus influenzae*
OD	Optical density
OMP	Outer membrane protein
PAF	Platelet activating factor

PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMN	Polymorphonuclear leukocyte
RSV	Respiratory syncytial virus
SARS	Severe acute respiratory syndrome
SEM	Standard error of the mean
SIDS	Sudden infant death syndrome
SPF	Specific pathogen free
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
URT	Upper respiratory tract
WBC	White blood cell
WKC	Whole killed cell

Summary

An animal model of respiratory infection was used to determine the effect of various factors, thought to influence the ability of the host to clear bacteria, on the host's innate response to an NTHi lung infection.

Mucosal immunisation with NTHi has previously been shown to enhance the clearance of NTHi from the lung in an animal model of infection through the increased recruitment of phagocytes. Comparisons of cytokine and chemokine kinetic profiles were made in order to determine differences between innate and acquired immune response and the way in which mucosal immunisation controls the innate immune response to NTHi. Increased production of pro-inflammatory cytokines and chemokines in the early stages of NTHi lung infection enhanced the ability to clear bacteria from the rat lung in the immune animals through the increased recruitment of phagocytes to the site. Mucosal immunisation was found to alter the cytokine and chemokine mRNA profiles of CD4+ and CD8+ cells, with increased levels of MCP-1 protein being detected in both types of immune cells.

An antecedent viral infection has been shown to increase the chance of developing a respiratory bacterial infection. The NTHi model of respiratory infection was used to characterise the effect that a viral infection had on the host response to the host's innate response to a bacterial infection and the ability to clear the bacteria. The host's ability to clear NTHi from the rat lung was enhanced by an antecedent viral infection through alterations to the innate immune response and the cytokine and chemokine kinetic profiles.

The use of a mutant strain of NTHi deficient in a component of Lipooligosaccharide (LOS), Phosphorylcholine (ChoP), was utilised as a tool to characterise the innate immune response to LOS. Animals challenged with the LOS mutant strain had a reduced inflammatory response to NTHi through the decreased production of pro-inflammatory cytokines and chemokines and the reduced recruitment of phagocytes to the site of infection.

This thesis has contributed valuable information to enable a better understanding of the host's innate immune response to respiratory infection. This study has identified the role of cytokines and chemokines in the innate response to a respiratory bacterial infection and the enhanced ability of the host to clear NTHi from the lung.

INTRODUCTION

CHAPTER ONE

1.1 RESPIRATORY INFECTION IN THE LUNG

The respiratory system is a component vital to the survival of the host and therefore it is essential that the function of the lungs and the respiratory tract be maintained and protected from foreign particles and infectious agents. In order to facilitate gas exchange the lung surface consists of epithelial cells which, apart from their intended function, also provide an attractive niche for potential pathogens. Whilst the lower respiratory tract of a healthy individual is a normally sterile environment, devoid of microbes, the upper respiratory tract (URT) is continuously colonised by microbes and is constantly exposed to foreign particles. In order to maintain the sterility of the lung, host mechanisms are in place that act to inhibit the introduction of foreign particles and enhance the clearance of particles that are able to infiltrate the area.

1.1.1 Defense systems

The initial stages of defense against either a foreign particle or an infectious agent are very similar through the production of mucous secretions and the presence of cilia in the URT that aid clearance and prevent the progression of the particle or microbe into the lung. Impairment of this mucociliary clearance mechanism is a commonly reported characteristic of many lung disorders including cystic fibrosis (Cowley, Wang et al. 1997), asthma (Del Donno, Bittesnich et al. 2000) and chronic obstructive pulmonary disease (COPD) (Smaldone, Foster et al. 1993). In response to a pathogen in the lung a coordinated response between the innate and acquired immune systems is necessary for not only successful elimination of the pathogen but also a

controlled inflammatory response in order to minimise damage to the lung tissue.

1.1.1.1 The innate immune system

The innate immune response to a pathogen is the initial response that is non-specific and uses pathogen recognition to identify the intruder as 'non self'. There are many components of both bacteria and viruses that can be recognised by the innate response, including DNA, RNA, lipoproteins, lipopolysaccharides (LPS) and Lipooligosaccharide (LOS) to name a few. In 1989 Janeway suggested that the host was able to detect invading pathogens through recognition of specific conserved regions of the pathogen (Janeway 1989). These conserved regions are now commonly referred to as pathogen-associated molecular patterns (PAMPs). The mechanism by which PAMPs are detected by the host was not determined until sometime later when a family of receptors know as toll-like receptors (TLRs) were described (Medzhitov, Preston-Hurlburt et al. 1997). The interaction between PAMP and TLRs initiates host defense mechanisms through a signaling pathway that results in the activation of the transcription factor NF-κB (Kopp and Medzhitov 1999). In terms of the innate response to pathogens in the lung it is the interaction with the respiratory epithelial cells and resident alveolar macrophages that will be considered further.

1.1.1.1.1 Respiratory Epithelial Cells

Epithelial cells have several functions in the innate immune systems. In the initial stages of a lung infection the first interaction is between the microbe and

respiratory epithelial cells. Epithelial cells act not only as a barrier but also to alert the immune system to the presence of disease-inducing microbes. In order for a pathogen to multiply in the respiratory tract it must first attach itself to the host's respiratory epithelial cells. As part of their contribution to the innate immune response, epithelial cells invoke components of their defense system to avoid being colonised by potentially pathogenic bacteria. Mucin that is secreted from the epithelial surface of the respiratory tract aids in mechanical clearance of bacteria (Knowles and Boucher 2002). In response to an invading pathogen the epithelial cell up-regulates the production of receptors on the cells surface (Avadhanula, Rodriguez et al. 2006), produces and releases antimicrobials (Harder, Meyer-Hoffert et al. 2000), cytokines and chemokines that signal other components of the innate immune system (Strieter, Belperio et al. 2002). The production of cytokines and chemokines and the role they play will be discussed later in this chapter.

1.1.1.1.2 Macrophages

Alveolar macrophages protect the lung through surveillance and detection of foreign particles such as bacteria. As a resident of the lung tissue alveolar macrophages are an important part of the early innate immune response and are present in the normal lung (Foxwell, Kyd et al. 1998). Activation of alveolar macrophages (North 1978) can occur directly through contact with the pathogen, through recognition of PAMPs (Zhang, Summer et al. 2000), or indirectly after stimulation by cytokines and chemokines produced by respiratory epithelial cells (Lafleur, Abrahamsen et al. 1998). Degranulation of the macrophage commonly occurs following activation, which allows the macrophage to change shape and

engulf the invading pathogen (Djaldetti, Salman et al. 2002). Activation of the alveolar macrophages also stimulates production of a number of cytokines and chemokines that enhance inflammation through the recruitment of neutrophils and circulating macrophages to the lung (Lentsch, Czermak et al. 1999). Alveolar macrophages are not only recruited to the site of infection but also have the ability to proliferate locally (Sibille and Reynolds 1990).

In addition to the phagocytosis of bacteria, alveolar macrophages also play a role in the clearance of apoptotic neutrophils from the lung (Savill, Wyllie et al. 1989). The clearance of apoptotic cells from the lung is an important part of the resolution of inflammation and the prevention of tissue damage.

1.1.1.1.3 Neutrophils

Neutrophils or polymorphonuclear leukoctyes (PMNs) are not normally found in the healthy lung but are recruited rapidly and in high numbers following infection such as with the respiratory pathogen Nontypeable *Haemophilus influenzae* (NTHi) (Foxwell, Kyd et al. 1998). In order for the neutrophils to travel from the blood into the lung tissue and alveolar space a signal, in the form of a cytokine or chemokine, must be produced. The upregulation of intracellular adhesion molecules (ICAM) 1 on the surface of cells in response to cytokines such as Tumor necrosis factor (TNF)-α facilitates the migration of neutrophils to the lung (Frick, Joseph et al. 2000). Activation of neutrophils in the lung leads to an oxidative burst followed by phagocytosis of bacteria (Kragsbjerg and Fredlund 2001).

1.1.1.2 The acquired immune system

The acquired system is developed through previous exposure to a pathogen through either a prior infection or through vaccination. One of the challenges in developing a successful vaccine is ensuring that protection against the pathogen is not strain-specific, as is often seen with naturally occurring acquired protection, but is able to protect against infection by a wide range of strains.

It was initially believed that the innate and acquired immune systems acted in isolation in response to a pathogen. However the discovery of TLRs has provided a link between the two systems and it appears that it is the ability of the acquired immune system to regulate the innate immune response that is important in providing protection and limiting damage to the surrounding tissue.

Better knowledge and understanding of how effective host immunity protects against infection, specifically how the acquired immune response modulates the innate response to remove infectious agents and control the host's inflammatory response is needed in order to develop effective therapies and vaccinations.

1.1.2 Lung disease

Bacteria and viruses play an important role in many major lung conditions such as; COPD, chronic bronchitis, pneumonia and cystic fibrosis. Of particular interest is COPD, the fourth leading cause of death worldwide (Pauwels, Buist et al. 2001). In a study of acute exacerbations it was determined that a pathogen was the aetiological agent in up to 80% of cases (Sethi 2000). It has been proposed that the mechanisms by which pathogens and more specifically

bacteria contribute to COPD is through the development of a vicious cycle that leads to a continued state of inflammation (Murphy and Sethi 1992). *Streptococcus pneumoniae,* NTHi and *Moraxella catarrhalis* are the three major causes of infection in COPD patients (Sethi and Murphy 2001). The ability of these bacteria to continually infect and re-infect can be attributed, in part, to the strain-specific immune response following naturally acquired infection.

1.1.2.1 Viral lung infection

Viruses in the respiratory system are important not only in their ability to cause disease themselves but also for their ability to predispose the host to an array of bacterial infections. In recent years the role of viruses in respiratory infection has gained significant attention through increased reports of infection caused by avian influenza and Severe acute respiratory syndrome (SARS) to name a few. Although the importance of such viruses should not be undermined there exists a multitude of other respiratory viruses capable of causing serious infection in both susceptible and otherwise healthy individuals. For example, respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract infection in infants and young children (Selwyn 1990) and has even been suggested as a contributing factor in sudden infant death syndrome (SIDS) (Cubie, Duncan et al. 1997).

Respiratory viral infections are known to have an ability to predispose the host to bacterial infections. *H. influenzae* received its name in the initial stages of the influenza pandemic in 1918 as it was commonly isolated from the respiratory tract of patients and thought to be the source of the pandemic until it

was identified that the bacterial infection was secondary to a viral infection (Brown 2003). The mechanisms by which a viral infection is able to increase susceptibility to bacterial infection are not well understood and various theories have been suggested. Many of these theories surround an increased ability of the bacteria to colonise the respiratory surface or changes to the innate immune response.

Viruses commonly replicate in the epithelial cells of the respiratory tract during infection often leading to decreased function of the cell or apoptosis (Mori, Komatsu et al. 1995). This process may enable the bacteria to bind more readily to the epithelial cells, through increasing expression of receptors and thereby increasing colonisation. An *in vitro* study of cultured epithelial cells has shown that *S. pneumoniae* is more able to bind to the cells after the epithelial cells had been damaged by the influenza virus (Avadhanula, Rodriguez et al. 2006). In the same study enhancement of colonisation was also involved in the interaction between NTHi and cultured RSV infected epithelial cells.

Bacterial otitis media infections are often associated with a previous respiratory viral infection in humans. In a study of children with symptoms of the common cold caused by various respiratory viruses it was found that abnormal middle ear pressure often developed (Winther, Hayden et al. 2002). This abnormal negative pressure can lead to bacterial otitis media through aspiration of the nasopharyngeal fluid containing potentially pathogenic bacteria into the middle ear space. A combined study of Influenza A and *S. pneumoniae* documented changes in the Eustachian cell surface carbohydrate structure (Tong, Grants et al. 2002). This altered structure may be a factor in increasing the risk of

bacterial infection through providing a greater number of binding sites for bacteria.

During a viral infection the upregulation of adhesion molecules can be both beneficial and detrimental to the host. Whilst these molecules are necessary for the mechanisms of the innate immune response and recruitment of cells to the area, they are also able to act as bacterial adhesion molecules. An example of this is the Platelet activating factor (PAF) receptor. During viral infection this receptor is upregulated. Phosphorylcholine (ChoP), which is a component of LOS from NTHi and other Gram negative bacteria, is able to bind to the PAF receptor expressed on bronchial epithelial cells (Swords, Buscher et al. 2000). Hence it might be concluded that upregulation of the PAF receptor during a viral infection could lead to increased colonisation with bacteria that express ChoP, such as NTHi.

Differing cytokine and chemokine profiles between cells recruited in response to viral infection or bacterial infection is common. During a viral infection CD4 + and CD8+ T cells at the site of infection play a major role in the ability to resolve the infection. During infection these cells have differing roles. CD4+ T cells act as helper cells, controlling the immune response through the production of cytokines and chemokines whilst CD8+ T cells play a direct role in the killing of the virus (Doherty, Topham et al. 1997). The production of cytokines and chemokines from T cells in the respiratory tissue in response to viral infection may enhance the ability to clear the virus but may affect the mechanisms called into effect during a bacterial infection. For example, Monocyte chemoattractant protein (MCP)-1 has been implicated in innate host defense in animal models of

influenza virus. Not only does MCP-1 have a role in recruitment of phagocytes but also activation and therefore phagocytic activity (Chensue 2001). An increase in phagocytic activity may lead to an increase in apoptotic cells which may, in turn, affect the ability to clear a secondary bacterial infection as phagocytes play an important role in the clearance of bacteria from the lung.

The combination of viral infection plus bacterial infection may cause both to become more pathogenic through increased inflammation leading to tissue damage. Following stimulation with LPS, macrophages previously exposed to influenza A virus had an enhanced ability to produce TNF-α. This did not appear to be due to increased protein storage or upregulation of mRNA prior to LPS exposure but due to the priming of these cells (Nain, Hinder et al. 1990). Increases in TNF-α are associated with tissue damage (Qureshi, Harmsen et al. 2003) and may attribute to the enhanced pathogenesis during a co-infection with virus and bacteria.

Sendai virus, a parainfluenza virus, is often used in animal models of respiratory infection due to it being a natural rodent pathogen. A study conducted by Avadhanula and co-workers involving epithelial cells and a human parainfluenza virus found that NTHi adhesion to epithelial cells was enhanced following exposure of the cells to the virus (Avadhanula, Rodriguez et al. 2006). In response to sendai virus the rodent host elicits a CD4+ and CD8+ T cell response resulting in the recruitment of these cells to the lung (Cole, Katz et al. 1994; Usherwood, Hogan et al. 1999). A greater understanding of the mechanisms by which a viral infection may pre-dispose the host to a bacterial infection will contribute to the development of treatments.

1.1.2.2 Bacteria in the lung

Both Gram-negative and Gram-positive bacteria play a role in infection of the lung, however, the mechanisms by which they induce an inflammatory response often differ greatly. The basis for these differences can be explained in part by the preferential binding to different members of the TLR family. Traditionally it is believed that Gram-negative bacteria preferentially bind to TLR4 through the interaction with LPS (Hoshino, Takeuchi et al. 1999), while components of the Gram-positive bacteria cell wall interact with TLR2 (Yoshimura, Lien et al. 1999). The three most commonly isolated pathogens in lung infections; *S. pneumoniae*, *M. catarrhalis* and NTHi differ greatly in their structures and components of their outer surface.

1.2 NONTYPEABLE *HAEMOPHILUS INFLUENZAE*

NTHi is a Gram-negative bacteria that is a commonly isolated commensal from the nasopharynx of healthy individuals (Murphy and Apicella 1987) but it is also capable of causing infection. NTHi is a human specific microbe that has a carriage rate of up to 80% in healthy children (Harabuchi, H. Faden et al. 1994). Although NTHi rarely causes death it commonly causes an array of respiratory infections including otitis media (Kilpi, E. Herva et al. 2001), sinusitis (Kalcioglu, Durmaz et al. 2003), bronchitis (Bandi, Apicella et al. 2001) and pneumonia (Faden, Duffy et al. 1996) and is an important cause of lower respiratory tract infection in adults with COPD (Murphy and Sethi 1992)}.

The introduction of a vaccine against *Haemophilus influenzae* type b (Hib) in 1987 was successful in reducing the morbidity and mortality rates associated

with infection caused by Hib (Adams, Deaver et al. 1993)}. The Hib vaccine is directed against the b serotype capsule and as such does not provide protection against other capsular types or NTHi which is unencapsulated. The introduction of this vaccine also further highlighted the prevalence of infections caused by NTHi as previously many were overlooked and attributed to Hib. Currently there is no vaccine available that protects against infection with NTHi and in order to develop a successful vaccine a better understanding of the interaction between NTHi and the host is required.

1.2.1 Virulence Factors

Many of the virulence factors of NTHi add to its' pathogenesis, assist in colonisation and enable it to avoid or modify the innate immune response. The ability of specific strains of NTHi to adhere to epithelial cells is important to virulence and can often be mediated by factors such as certain outer membrane proteins (OMPs) that are capable of binding to a component of mucin in the respiratory tract (Reddy, Berstein et al. 1996). Pili (Gilsdorf, McCrea et al. 1997) and fimbria (Jiang, Nagata et al. 1999) also enable NTHi to bind to the epithelial cells of the respiratory tract. Increased colonisation is an important virulence factor as high colonisation loads are often associated with an increased risk of infection due to only a small increase in numbers being required to shift the balance from colonisation to infection.

In addition to surface associated virulence factors NTHi has the ability to secrete proteins that can assist in avoidance of the host's immune response through the interaction with host molecules. A 2002 study by Vitovski and co-

workers revealed the importance of IgA proteases as virulence factors of NTHi (Vitovski, Dunkin et al. 2002). The secretion of IgA at mucosal surfaces in an important component of the immune response to pathogens (Kraehenbuhl and Neutra 1992). The production of IgA proteases by NTHi enables it to avoid this component of the innate system and increases its ability to colonise.

Increasingly it is being recognised that the formation of biofilms is a mechanism utilised by NTHi in order to evade the host's immune system. The presence of biofilms are often associated with the development of chronic infections such as COPD. Studies on biofilm formation by NTHi in the respiratory tract have provided evidence of an increased inflammatory response and decreased susceptibility to antibiotics (Ehrlich, Veeh et al. 2002; Swords, Moore et al. 2004; Starner, Zhang et al. 2006). It has been suggested that one of the ways that biofilms add to the pathogenesis of NTHi is by mimicking a component of mucin which leads to failure of the innate immune response to detect the pathogen (Greiner, Watanabe et al. 2004). Recently, it was reported that NTHi biofilms *in vivo* contain both type IV pilin protein and a significant amount of double-stranded DNA (Jurcisek and Bakaletz 2007). The double-stranded DNA appears to form a mesh-like matrix providing structural stability and may even impose a protective barrier for the bacteria. There are still questions remaining regarding whether all strains are capable of forming biofilms and if they are only formed under certain host conditions.

Vaccines based on virulence factor components can be very successful in conferring immunity as often many of these factors are highly conserved due to the fact that they are important for the pathogenic characteristics of the

bacterium. Although NTHi has many specific components that are able to act as virulence factors it must be noted that antigenic diversity is also one of the mechanisms by which NTHi avoids strain-specific immunity and this diversity can be considered a key feature of NTHi virulence. Infection is often the result of the acquisition of a new strain (Eldika and Sethi 2006). Epidemiological studies of NTHi colonisation in children revealed that whilst only a single dominant strain was present at any one time, over a 2 year period children had been colonised with up to as many as 7 different strains (Faden, Duffy et al. 1996). In adult COPD patients, it is common for an NTHi strain to be isolated from sputum followed by periods where this strain is absent in sputum culture and then the same original strain of NTHi seems to reappear (Murphy, Brauer et al. 2004). It would appear that NTHi has multiple mechanisms of survival that prevent detection of the bacteria through the use of sputum culture to assess the presence or absence of an NTHi strain. Such persistence by NTHi has important implications in understanding the role of bacteria in the course and pathogenesis of COPD, a condition that is underpinned by airway inflammation.

A characteristic of the pathogenicity of NTHi is the release of LOS during bacterial growth. Both cell bound and released LOS have the ability to stimulate production of pro-inflammatory cytokines, such as TNF-α, interleukin (IL)-1β and IL-6 from host cells. LOS has been shown to be capable of stimulating increased production of cytokines by up to ten-fold in human monocyte cultures (Gu, Tsai et al. 1995). It must therefore be considered that the ability of differing strains of NTHi to release large amounts of LOS could contribute to its ability to stimulate the innate immune response and cause increased or persistent inflammation.

1.2.2 Pathogenesis

1.2.2.1 Cell Invasion

The location of NTHi is not just confined to the surface of the epithelial cell. A key factor in the pathogenesis of NTHi is in its ability to invade cells. The ability to gain cellular access through macropinocytosis was demonstrated in a study by Ketterer and co-workers, where scanning electron microscopy demonstrated the presence of NTHi within the vacuoles of the respiratory epithelial cells (Ketterer, Shao et al. 1999). Furthermore NTHi has been shown to invade the epithelial barrier by passing in between epithelial cells using a process known as paracytosis (van Schilfgaarde, van Alphen et al. 1995). By gaining entry into the epithelial cell or through the epithelial layer NTHi is able to avoid killing by bactericidal antibodies or antibiotics, a mechanism that may be important in COPD (Sethi and Murphy 2001). In a study using lung explants from patients that were undergoing lung transplant due to cystic fibrosis, COPD and other similar conditions, *in situ* hybridization and Polymerase chain reaction (PCR) showed that H. *influenzae* was present in 'the epithelium, the submucosa of the bronchi, the bronchioles, the interstitium, and the alveolar epithelium (Moller, Timens et al. 1998). This study also showed an association between the bacteria and macrophages. Subsequently, Craig observed that one of the key differences between virulent and avirulent NTHi strains was their ability to survive in macrophage cells. In the J774 mouse macrophage-like cell line, he demonstrated that the avirulent strains died within 24 hours, however the virulent strains survived for as long as 72 hours (Craig, Cliffe et al. 2001). These observations indicate that when NTHi colonises the human respiratory tract, the bacterium can be present in both the epithelial and subepithelial layers. Persistence in the subepithelia is believed to be significant for maintaining a

chronic low-grade infection resulting in inflammatory responses. This response may act synergistically with other agents that cause inflammation, for example a viral infection, to induce the exacerbations associated with chronic disease or may be a marker of compromised local immunity in itself.

1.2.2.2 Modulation of Immune Response

The ability of bacterial components to stimulate the immune response through interaction with host receptors such as members of the TLR family is a common characteristic of pathogenic bacteria. The outer membrane of NTHi is composed of an array of molecules capable of modulating the host's immune response and thereby contributing to the bacteria's pathogenesis. These molecules include outer membrane proteins such as P2 and P6 to name a few in addition to various components of LOS (Avadhanula, Rodriguez et al. 2006).

1.2.2.2.1 Lipooligosaccharide

NTHi, like other Haemophilus sp. and members of the Neisseria and Branhamella families has an endotoxin component of its outer membrane that contains LOS. Unlike LPS from other Gram-negative bacteria, LOS does not contain the O-antigen (Gibson, Melaugh et al. 1993) but is still able to induce an inflammatory response in the host. Early efforts to characterise LOS from NTHi revealed that it has significant antigenic diversity not only between different species (Campagnari, Spinola et al. 1990) but also between strains (Patrick, Kimura et al. 1987).

LOS is able to stimulate the production of cytokines through the phosophorylation of the stress-activated kinase, p38 (Lee, Laydon et al. 1994). Previous studies have provided information on the effect that LOS from NTHi has on the concentration of various cytokines and chemokines. A study by Tong and coworkers (Tong, Chen et al. 2001)} showed that NTHi mutants that contained a disruption in the LOS gene were less effective at stimulating expression of genes for certain inflammatory cytokines than the complete NTHi. This suggests that the initial inflammatory response to NTHi is at least in part stimulated directly by the LOS. NTHi has developed mechanisms to avoid the host's immune response such as the incorporation of host-derived sialic acid into the LOS structure (Bouchet, Hood et al. 2003). This mechanism enables NTHi to avoid the activation of TLR-4.

LOS is comprised of oligosaccharide chains that contain molecules that are capable of mimicking molecules from the host (Mandrell and Apicella 1993). This mechanism it thought to enhance the ability of NTHi to colonise epithelial cells. One molecule of particular interest from NTHi LOS, ChoP, is capable of binding to the PAF receptor on the host's respiratory epithelial cells (Swords, Buscher et al. 2000). Not only does the interaction between ChoP and the PAF receptor facilitate binding of NTHi to the host cell but it also begins a cascade of events that eventually allow internalisation of the bacterium (Swords, Ketterer et al. 2001), which enhances the ability of the bacterium to avoid the host's immune response.

1.2.2.2.2 Other bacterial factors triggering host responses

A range of other bacterial components have been shown to be capable of interacting with the host in such a way that they induce host responses. P6 is a highly conserved lipoprotein component of the outer membrane of NTHi (Karalus and Murphy 1999) that has been found to bind to TLR2, leading to the activation of NF-κB (Shuto, Xu et al. 2001). Culturing human macrophages with P6 exhibited the ability of the lipoprotein to elicit an immune response. Following stimulation with P6 macrophages responded through the production of cytokines TNF-α and IL-8. This response was far greater than that produced through the stimulation of macrophages with either a complete NTHi OMP preparation or LOS (Berenson, Murphy et al. 2005). The ability of P6 to induce an immune response coupled with its high conservation among NTHi strains makes it an attractive vaccine candidate. Construction of a P6-deficient mutant provided insight into the role of P6 in the pathogenesis of NTHi (Murphy, Kirkham et al. 2006). *In vitro* studies of this mutant revealed decreased growth in culture and an increased susceptibility to a number of antimicrobial agents. *In vivo* studies involving P6-deficient NTHi mutants and their ability to modulate the host's immune response may allow further characterisation of P6 and its role in the pathogenesis of NTHi.

A recent study involving the interaction between NTHi and neutrophils revealed a cascade of events that could contribute to inflammation and possibly tissue damage. Upon stimulation with NTHi neutrophils were able to phagocytise the bacteria but this did not always lead to death of the bacteria and quite often the neutrophil itself became apoptotic and upon death released factors such as IL-8

(Naylor, Bakstad et al. 2007). The release of IL-8 further signals the migration of neutrophils to the site, increasing inflammation and the possibility of tissue damage. Although the specific NTHi factors involved in this occurrence have not been described it provides further evidence of the ability of NTHi to modulate the host's innate immune response. Other factors such as OMP P2, a porin from NTHi are also capable of inducing an inflammatory response (Galdiero, Galdiero et al. 2004). Further studies characterising the host's response to each individual factor may aid in the development of a greater understanding of how NTHi affects the host's inflammatory response.

1.2.3 Effect of NTHi immunisation

Currently there is no licensed vaccine available that protects against NTHi. An important part of developing a vaccine is gaining an understanding of the host response to the pathogen and what sort of response an effective vaccine would need to elicit to provide protection. Animal models of infection and immunisation are useful tools that enable researchers to view the mechanisms involved in the response.

The rat model of NTHi infection has been successfully used to characterise the response to a NTHi lung infection (Wallace, Clancey et al. 1989; Foxwell, Kyd et al. 1998). NTHi is not a natural pathogen in the rat host and is therefore cleared from the lung rapidly, usually within 24h of challenge, even in naïve animals (Foxwell, Kyd et al. 1998). However, it does provide a useful tool for determining the effectiveness of immunisation through the kinetics of clearance and characterisation of the cellular responses.

Mucosal immunisation has been found to be effective in enhancing the clearance of NTHi from the lung of rats (Wallace, Clancey et al. 1989; Kyd, Dunkley et al. 1995; Tan, Ferrante et al. 1995; Foxwell, Kyd et al. 1998). The idea of a common mucosal system (McDermott and Bienenstock 1979) has lead to the development of a mucosal immunisation regime involving the injection of antigen into the gut-associated lymphoid tissue (Wallace, Clancey et al. 1989). A modified model of mucosal immunisation involving injection of the antigen into the Peyer's patches, aggregates of lymphoid tissue in the small intestine, followed by an intra-trachael boost was found to be more effective in enhancing the clearance of NTHi from the rat lung (Kyd, Dunkley et al. 1995).

The enhanced clearance of NTHi from the lungs of mucosally immunised animals has been associated with the migration of cells into the alveolar space. In a study of the effects of mucosal immunisation with OMP26, an outer membrane from NTHi, on clearance of bacteria from the lung, an enhanced ability to clear NTHi from the lung in the immune animals corresponded with an increased number of cells present in the Bronchoalveolar lavage (BAL) in comparison with the naïve animals (Kyd and Cripps 1998). This result was also seen in a previous study involving immunisation with P6, a component of the outer membrane of NTHi (Kyd, Dunkley et al. 1995) and a study of the effect of mucosal immunisation with whole killed cell (WKC) NTHi (Foxwell, Kyd et al. 1998). The differing numbers of phagocytic cells recruited to the alveolar space in response to the mucosal immunisation is an indication that the innate immune response is being controlled.

The mechanism by which mucosal immunisation is able to enhance bacterial clearance is believed to be primarily cell-mediated. This was demonstrated in a study of the protective ability of CD4+ T cells after mucosal immunisation. Enhanced bacterial clearance was seen in naïve animals that received CD4+ T cells isolated from mucosally immunised animals (Wallace, Cripps et al. 1991; Dunkley, Clancy et al. 1994). The effect of antigen specific antibody in enhancing clearance of NTHi from mucosally immunised animals is believed to be minimal, as demonstrated in a study involving bronchial washings from immunised animals by Wallace and co-workers (Wallace, Cripps et al. 1991). The differing CD4+ and CD8+ T cell response seen in immune and non-immune animals following infection with NTHi provides further evidence that mucosal immunisation affects cellular immunity. Immediately following pulmonary challenge with NTHi an influx of CD8+ cells to the lung was seen in immune animals but not in naïve animals (Foxwell, Kyd et al. 2001). This influx of CD8+ cells may provide a mechanism by which the acquired immune response is able to modulate the innate immune response enhancing the protective response. In this study the later CD4+ T cell response seen in mucosally immunised animals indicates that T cell recruitment is under different signals, differentially attracting the CD4+ and CD8+ cells to the lungs. The later response by CD4+ cells may be an important factor in controlling the innate immune response to limit damage to the host tissue.

1.3 CYTOKINES AND CHEMOKINE IN INNATE DEFENSE IN THE LUNG

Uncontrolled tissue inflammatory responses to micro-organisms may lead to irreversible lung injury. Cytokines play a role in the recruitment of neutrophils

and mononuclear cells and the removal of the invading micro-organism. The innate system is able to recognise similar patterns of antigens, for example, LOS on the surface of Gram-negative bacteria. Pattern recognition leads to production of cytokines that subsequently amplifies the innate immune response.

The cytokines that stimulate the innate immune response are not constitutively expressed but their expression is enhanced by specific signals that alert the host to invading micro-organisms (Strieter, Belperio et al. 2002). TLRs are host defense signaling receptors that alert the host to invading micro-organisms. Human TLRs recognize Gram-negative bacteria via LPS or LOS binding to the receptor, triggering signal transduction pathways (Hoshino, Takeuchi et al. 1999; Ozinsky, Underhill et al. 2000), and this leads to increased production of pro-inflammatory cytokines such as IL-1β, IL-6 and IL-8.

The interaction between LOS from NTHi and the respiratory epithelial cells is one of the most important interactions in initiating the cytokine cascade which acts to induce the inflammatory response. NF-κB is a host transcriptional factor that is activated following the interaction between LOS and TLR2 on the surface of the respiratory epithelial cell (Moon, Woo et al. 2007). Following activation of NF-κB respiratory cells release a number of cytokines and chemokines. In a study of cultured epithelial cells incubated with either NTHi or LOS isolated from NTHi the production IL-6, IL-8, TNF-α and MCP-1 increased immediately after incubation (Clemans, Bauer et al. 2000). When the amount of isolated LOS was matched against the LOS from the whole NTHi cell it was determined that the response to NTHi was greater than seen with LOS only. This suggested

that other components of NTHi are capable of stimulating the expression of cytokines and chemokines from epithelial cells. Henderson and co-workers describe the action of bacterial modulins, such as components associated with the cell membrane for example P6 and LOS, that are capable of eliciting a cytokine response from the host (Henderson and Wilson 1996).

The NF-κB activation pathway also occurs through the interaction of NTHi with TLR4. In a study designed to characterise the role of this receptor in the innate immune response to respiratory challenge with NTHi, the absence of TLR4 from mice resulted in delayed clearance of the bacteria (Wang, Moser et al. 2002). The delay in clearance of NTHi was attributed to the lack of neutrophils present in the alveolar space due to decreased production of TNF-α, IL-1β, IL-6 and Macrophage inflammatory protein (MIP)-2. Upregulation of TNF-α mRNA was only seen in animals expressing TLR4. These results lead to the conclusion that TNF-α mRNA expression is upregulated by activation of NF-κB through the interaction of NTHi with TLR4. In the study by Clemans and co-workers detailed in the previous paragraph (Clemans, Bauer et al. 2000), the absence of IL-1β in the culture supernatant provided evidence that it is not produced in epithelial cells in response to NTHi or LOS. However in this study of TLR4, IL-1β was present in the alveolar space. This provides evidence to suggest that the alveolar macrophages are a major source of IL-1β.

IL-1β is an example of a cytokine where the presence of protein production does not always follow the increase in mRNA expression. In a study of human middle ear epithelial cells cultured with NTHi, IL-1β mRNA expression was upregulated but no protein was detected in the culture supernatant (Tong, Chen

et al. 2001). It has been suggested that the lack of correlation between IL-1β mRNA expression and protein production is due to two separate regulatory mechanisms that individually control transcriptional activation and translational efficiency (Schindler, Clark et al. 1990). More recently host factors have been attributed to the controlled production of IL-1β. α-defensins were shown to be capable of blocking the release of IL-1β from macrophages following their stimulation with LPS (Shi, Aono et al. 2007). In order for a cytokine to perform its function transcription, translation and secretion must all occur. Any one of these steps can be controlled through both host and pathogen derived mechanisms.

Alveolar macrophages are an important source of chemokines and cytokines in the initial stages of respiratory infection with NTHi. Alveolar macrophages are able to recognise modulins from bacteria and respond in defense. Outer membrane proteins from NTHi have been shown to stimulate the production of IL-10, IL-8 and TNF-α protein from human macrophages (Berenson, Murphy et al. 2005). Alveolar macrophages are also the major source of MCP-1 and macrophage inflammatory protein 2 (MIP-2). MCP-1 and MIP-2 are chemokines that are produced in the lung in response to bacterial infection (Deiters and Muhlradt 1999; Zhang, Nelson et al. 2002; Wissel, Schulz et al. 2005). MIP-2 is a chemokine produced in rodents that is a functional homologue of IL-8 produced in humans (Bozic, Gerard et al. 1994). Chemokines are chemotactic factors important for the recruitment of phagocytic cells to the site of infection (Tessier, Naccache et al. 1997; Diab, Abdalla et al. 1999). MCP-1 is produced from both respiratory epithelial cells (Olszewska-Pazdrak, Casola et al. 1998) and alveolar macrophages (Yano, Yanagawa et al.

1996) in response to pathogen stimulation. Studies have shown it has an important role in the recruitment of macrophages and T cells (Huffnagle, Strieter et al. 1995) but there is no evidence to suggest that it has any effect on the migration or activation of neutrophils. MIP-2 on the other hand has identical cellular sources to MCP-1 but, unlike MCP-1, is a potent attractant of neutrophils (Schmal, Shanley et al. 1996). Once recruited to the site of infection neutrophils are also an important source of MIP-2 (Naylor, Bakstad et al. 2007). The differing roles of these two chemokines indicates that they may both be necessary for a successful immune response against NTHi.

Many cytokines exhibit similar characteristics and may work together to achieve a common goal. IL-1β, TNF-α and IL-6 are all classed as pro-inflammatory cytokines due to their ability to induce responses either directly or indirectly, through the activation and upregulation of cells, of other factors, such as adhesion molecules, and influencing the migration of cells to the site of stimulation. A study using IL-6 deficient mice revealed a compensatory mechanism controlling the production of pro-inflammatory cytokines (Fattori, Cappelletti et al. 1994). After injection of bacterial LPS into the mice, the IL-6 deficient mice had concentrations of TNF-α three times that found in the wild-type mice.

Subsequently the increased production of other cytokines was attributed to IL-6 having that capability to exhibit both proinflammatory and anti-inflammatory characteristics (Xing, Gauldie et al. 1998). In this study IL-6 deficient mice received an aerosol dose of endotoxin and the production of TNF-α and MIP-2 protein were both produced in higher quantities than in wild-type mice capable

of producing IL-6. Although this increased TNF-α and MIP-2 could be explained by a compensatory mechanism as suggested above, an increase in neutrophil recruitment was also seen in the IL-6 deficient mice. This later result would support the theory that IL-6 can act as an anti-inflammatory cytokine through regulating the production of pro-inflammatory cytokines and chemokines. In the lung production of IL-6 can be stimulated in respiratory epithelial cells (Khair, Devalia et al. 1994) and alveolar macrophages (Krause, Hohberg et al. 1997) in response to an invading pathogen. In response to this pathogen it is possible that IL-6 has the capability to act as both a proinflammatory cytokine, through its ability to recruit neutrophils, and as an anti-inflammatory cytokine by controlling the production of other more traditional proinflammatory cytokines.

One cytokine, IL-10, has a clearly defined role in suppressing the immune response through controlling the production of pro-inflammatory cytokines. IL-10 acts to control the immune response through inhibition of the mRNA encoding inflammatory mediators such as TNF-α (Murray 2005). Stimulation of human monocytes with LPS was demonstrated to induce the production of TNF-α, IL-1β, IL-6 and IL-10 (Donnelly, Freeman et al. 1995). The production of IL-10 lagged behind that of the pro-inflammatory cytokines indicating the possible presence of a feedback mechanism being utilised to control the inflammatory response. IL-10 is found to be constitutively produced by health human respiratory epithelial cells (Bonfield, Konstan et al. 1995) providing evidence of a role in maintaining the normal function of cells through controlling inflammation.

IL-10 production itself is under the influence of other cytokines. Interferon (IFN)-γ has been found to suppress IL-10 production in a dose-dependant

manner (Donnelly, Freeman et al. 1995). This suppression of IL-10 coupled with the ability of IL-10 to inhibit production of pro-inflammatory cytokines indicates that the inflammatory response is under the control of a wide variety of factors working in succession to resolve infection but also minimalising the negative effects of inflammation. IL-10 protein production appears to be under the control of post-transcriptional factors. IL-10 mRNA contains a sequence that leads to destabilization of the mRNA. Factors that stabilise the mRNA on stimulation increase the half-life and may allow for translation of IL-10 protein (Powell, Thompson et al. 2000).

Characterisation of the role of IL-10 may provide an important link between the innate and acquired immune response to infection as the production of IL-10 has been found to be essential in the induction of antigen-specific regulatory T cells (Sonoda, Faunce et al. 2001). Although IL-10 is commonly referred as an anti-inflammatory cytokine, a dual role for IL-10 has been suggested. While the inhibitory effect of IL-10 on the production of pro-inflammatory cytokines was discussed earlier IL-10 also exhibits pro-inflammatory characteristics through its ability to attract CD8+ T cells (Jinquan, Larsen et al. 1993).

Many cytokines and chemokines do not appear to have any post-transcriptional regulation. In these cases the upregulation of mRNA leads to protein production and there is a direct correlation between the two. MCP-1 and MIP-2 protein production was found to be directly associated with mRNA expression in model of viral infection in the lung (Weinberg, Lutzke et al. 2002). There is also evidence that production of IFN-γ is controlled at the transcriptional level (Penix, Weaver et al. 1993).

Cytokines do not act in isolation, but usually act in synergy with or to induce or inhibit other cytokines, and in doing so create a complex network of chemical signals. These cytokine networks are the main controlling element in the inflammatory and the innate immune responses that occur in response to infectious agents.

1.4 AIMS AND OBJECTIVES

Gaps in our understanding of the response to NTHi infection and how mucosal immunisation protects the host relate to the way in which the acquired immune response works with the innate immune system. Characterisation of the innate response to NTHi in terms of cytokine and chemokine response in the lung will further our understanding. This study has tested the hypothesis that factors affecting the innate immune response will cause changes in the production of cytokines and chemokines.

The specific aims of this study were to compare cytokine and chemokine response in order to:

1. Investigate the response to NTHi in animals following mucosal immunisation in comparison to naïve animals and how the acquired immune response may modulate the innate immune response in order to clear the infection;
2. Characterise the production and secretion of inflammatory mediators in both the lung tissue and alveolar space;
3. Investigate the effect that an antecedent respiratory viral infection has on the innate immune response and the ability of the host to respond to a bacterial infection in the lung;
4. Investigate the role of the ChoP component of LOS in the innate response to NTHi in order to determine if components of LOS affect the host's production of cytokines and chemokines;
5. Characterise the specific responses of CD4+ and CD8+ T cells from the lymph nodes to NTHi to determine how these cells may be primed to modulate the immune response.

MATERIALS AND METHODS

CHAPTER TWO

2.1 BACTERIA

2.1.1 Bacterial Strains

2.1.1.1 NTHi 289

Nontypeable *Haemophilus influenzae* 289 was originally isolated from the sputum of an adult suffering from chronic bronchitis. NTHi 289 was chosen due to its use in previous studies by our research group.

2.1.1.2 NTHi 2019

Nontypeable *Haemophilus influenzae* 2019 is a clinical isolate from a patient suffering from chronic obstructive pulmonary disease. This strain was kindly provided by Dr Michael Apicella.

2.1.1.3 NTHi 2019 licD::kan

Nontypeable *Haemophilus influenzae* 2019 *licD::kan* is an isogenic mutant of strain 2019 kindly provided by Dr Michael Apicella (Swords, Buscher et al. 2000).

2.1.2 Bacterial suspensions

NTHi 289 and 2019 were grown overnight at 37ºC in 5% CO_2 atmosphere on chocolate blood agar (CBA) comprising of brain heart infusion agar (Oxoid, Hampshire, UK) supplemented with partially lysed 5% defribrinated horse blood (Amadeus International, Australia). NTHi 2019 *licD::kan* was grown as above but with the addition of 15µg/ml kanamycin (Amresco, Ohio, USA).

2.1.3 Live bacteria

Live bacteria were harvested from plates by scraping with a sterile glass slide, then washed three times in sterile phosphate buffered saline (PBS), pH 7.4 (Dulbecco's formula; Amresco, Ohio, USA) through centrifugation at 10 000 x *g* for 3 min at 4˚C. The concentration of bacteria in PBS was estimated through the measurement of the optical density (OD) at 405nm (BioRad Microplate Reader model 3550, BioRad, Ca, USA) and calculations from a regression curve. The estimated concentration was then confirmed by plating ten fold serial dilutions and colony counts following overnight incubation.

2.1.4 Killed bacteria

In order to prepare the bacteria for immunisation NTHi 289 was killed by suspension in 1% (v/v) formaldehyde in PBS and incubated for 2 h at 37˚C. The bacteria were then washed 3 times in PBS. An overnight culture was prepared to ensure the killing was successful. The concentration of NTHi was estimated by measuring the OD at 405nm and adjusted to a concentration of $2x10^{10}$ colony forming units (CFU) per ml.

2.1.5 Sendai Virus

Sendai virus (Enders strain) was provide by Dr Brett Lidbury. The virus was grown in the allantoic cavity of 10 day-old embryonated chicken eggs and concentrated ($10^{7.5}$ $TCID_{50}$/mL) using a sucrose gradient centrifugation and stored below -70^{0}C. A 1:6 dilution of stock in PBS was used as an infectious dose.

2.2 ANIMALS EXPERIMENTS

2.2.1 Animal strain

Specific pathogen free (SPF) DA male rats aged between 8-10 weeks were obtained from the Animal Resources Centre, Perth, Australia and maintained under SPF conditions with access to sterile food and water ad libitum. During immunisations they were removed and returned back to SPF conditions until bacterial challenge. All animal work was approved by the University of Canberra, Animal Ethics Committee

2.2.2 Animal Model

The animal model used measured the kinetics of bacterial clearance and host responses at timepoints in the first 24 h post induction of an acute pulmonary infection. In Chapters 3, 4 and 7, the animal groups consisted of mucosally immunised and non-immunised rats. In Chapter 5 non-immunised rats were divided into a group that received a viral infection 3 days before bacterial infection and a bacterial infection only group. In Chapter 6 all the animals were non-immune and were challenged with different NTHi strains.

2.2.3 Immunisation and bacterial challenge

The mucosal immunisation regime used in this study was based on a previously established model by Wallace and co-workers (Wallace, Clancey et al. 1989). The immunisation regime consisted of an intra Peyer's patch (IPP) immunisation on day 0, followed by an intra-tracheal (IT) boost on day 14 then pulmonary challenge with live bacteria on day 21.

2.2.3.1 Peyer's patch immunisation

The killed bacterial cell suspension prepared in 2.1.4 was emulsified with Freund's incomplete adjuvant (IFA; Sigma, MO, USA) at a 1:1 ratio to get a final concentration of $1x10^{10}$ CFU/ml. Each rat received approximately $5x10^{10}$ CFU of bacteria. The rats were anaesthetised by an intra-peritoneal (IP) injection of pentobarbitone sodium (Nembutal, Australia) at a dose of 40 mg per kg of body weight. The small intestine was exposed by a mid line incision of the lower abdomen and the killed bacterial suspension was injected subserosal to each Peyer's patch. The non-immune group of animals did not receive an IPP immunisation. It had been previously established that sham immunized animals did not differ from untreated immunised. The peritoneum was sutured and the animals allowed to recover.

2.2.3.2 Intra-tracheal boost immunisation

An IT boost was given 14 days post-IPP immunisation. The rats were sedated by inhalation of halothane (Rhone Merieux, Vic, Australia). Each rat received an IT boost of killed bacteria (section 2.1.4) via a 20 G cannula (Terumo, MD, USA) inserted into the trachea and dispersed with 2 x 5 ml volumes of air. The immunised group received 50µl of killed NTHi 289 at a concentration of $1x10^{10}$ CFU/ml in PBS while the non-immune group received 50µl of PBS. Animals were allowed to recover before being returned to a SPF environment until challenge.

2.2.3.3 Bacterial challenge NTHi 289

Pulmonary challenge with live bacteria as prepared in section 2.1.3 was performed on day 21 (post-IPP immunisation). The rats were sedated through inhalation of halothane. A bolus inoculum of $5x10^8$ CFU of live NTHi 289 in 50µl of PBS was introduced into the lung via an IT cannula and dispersed with two 5 ml volumes of air. Rats were euthanased by an overdose of pentobarbitone sodium administered by IP injection at 1, 2, 4, 8, 12 or 24 h post-challenge or prior to challenge (0 h).

2.2.3.4 Bacterial challenge NTHi 2019/NTHi 2019 *licD::kan*

Pulmonary challenge with live bacteria with either NTHi 2019 (section 2.1.2) or NTHi 2019 *licD::kan* (section 2.1.2) was performed as above (section 2.2.3.3). Rats were euthanased at 2 h post-challenge.

2.2.3.5 Viral infection

Rats were sedated with halothane through inhalation and infected with a 1:6 dilution of concentrated Sendai virus in PBS (section 2.1.5) delivered via intranasal inoculation in 50µl of PBS. Rats were allowed to recover then maintained in a clean environment separately until challenge with NTHi 289 3 days later.

2.2.4 Tissue Collection

2.2.4.1 Bronchoalveolar lavage

Following euthanasia and collection of the blood, the trachea was exposed in the neck and lung lavage was performed with five 2 ml volumes of PBS via the trachea and pooled for individual animals. The BAL was assessed for bacterial clearance by plating ten-fold serial dilutions of the washings on CBA at 37˚C in 5% (v/v) CO_2 in air overnight for CFU determination.

2.2.4.2 Lung Homogenate

Following lavage, the lungs, trachea and heart were removed and the heart and connective tissue were removed and discarded. The lungs were placed in 10 ml of sterile PBS and homogenized using a Heidolph Diax 600 disperser (Kelheim, Germany). The lung homogenate was assessed for bacterial clearance by plating ten-fold serial dilutions of the washings on CBA at 37˚C in 5% (v/v) CO_2 in air overnight for CFU determination. The homogenised lung was then centrifuged at 1,500 rpm for 10 min at 5˚C using a Jouan BR3.11 benchtop centrifuge (St. Nazaire, France). The supernatant was removed and stored at below -20˚C and the pellet was resuspended in 3 ml of RNA extraction buffer [47.26% phenol (Sigma), 47.26% Guanidine thiocyanate (MW 118.16), 4.76% 4M sodium acetate, 0.72% β-mercaptoethanol (MW 78.13)] and stored at -80˚C.

2.2.4.3 Differential counts on cells in BAL

Cytospin slides were prepared to differentiate PMN, macrophage and other cells present in the BAL. 100 µl of BAL was spun for 100 min at 20 x g onto a glass microscope slide with a cytospin (Shandon, Cheshire, UK). Slides were then fixed and stained in Diff Quick (Lab Aids, NSW, Australia) and percentages determined from three differential cell counts on each slide.

2.2.4.4 BAL cell counts

BAL was separated into two 10 ml samples and centrifuged at 200 x *g* for 10 min at 5˚C using a Jouan BR3.11 benchtop centrifuge (St Nazaire Cedex, France). The supernatants were removed and stored at below -20˚C. One pellet was resuspended in RNA extraction buffer and stored at -80˚C. The other pellet was resuspended in a known volume of PBS, and the total number of cells present in the BAL fluid determined by staining with methylene blue and counting with a hemocytometer.

2.2.4.5 Mesenteric Lymph nodes

Mesenteric lymph nodes (MLNs) were removed from the immune and non-immune rats and placed into 2ml of Buffer A (PBS w/ 0.1% bovine serum albumin (BSA)+ 0.6% Na-citrate). The MLNs placed in a Petri dish and the liquid removed. They were then minced with a scalpel blade and gently pushed through a stainless steel to separate and suspend the cells. The sieve was washed with buffer A until the cells were suspended in approximately 7 ml of buffer. The cells were allowed to stand for 30 min before being centrifuged at

1000 rpm for 10 min using a Jouan BR3.11 benchtop centrifuge (St Nazaire Cedex, France). The pellet was resuspended in buffer A. The viability of the cells was determined using trypan blue staining. Cells were then centrifuged as previously and then discarded. The pellet was resuspended in an appropriate amount of buffer A to obtain a concentration of $1x10^7$ cells/ml.

2.2.4.6 Spleen cells

Spleens were removed from non-immune rats and chopped up in 2 ml of buffer A (as above) The spleen was then pushed through a nylon sieve (Falcon, Marathon lab supplies) with the plunger end of a syringe, washing it though with buffer A until there was a 10 ml solution. The cells were allowed to stand for 30 min before centrifugation for 10 min at 1000rpm using a Jouan BR3.11 benchtop centrifuge (St Nazaire Cedex, France). The supernatant was discarded and 0.5ml of 0.17M NH_4Cl (pH 7.65) was added to the pellet for 1 min to lyse the red blood cells. The cells were then diluted to 10ml with buffer A (as above) and centrifuged as before. The supernatant was discarded and the pellet resuspended in buffer A. The total number of cells present in the spleen samples was determined by staining with methylene blue and counting with a hemocytometer . The cells were then diluted with buffer A to obtain a concentration of $2X10^7$ cells/ml. All non-immune spleen samples were then pooled. 1.5 ml of pooled spleen cell suspension was centrifuged as before and resuspended in 1ml of RPMI supplemented with 10% v/v foetal calf serum and 100µg/ml Fungizone™. Mitomycin C was added at a concentration of 25µg/ml and the spleen cells were incubated at 37˚C for 30 min. The cells were then washed with RPMI + supplements and resuspend to obtain a concentration of $2x10^7$ cells/ml.

2.3 CELL CULTURE

2.3.1 CD4+T-cell and CD8+T-cell isolation

CD4+ and CD8+ cells were positively selected using immunomagnetic separation with the DynaBead system following DYNAL CELLection Pan Mouse IgG kit protocol. I ml of MLNs isolated in 2.2.4.5 were incubated with either 5µg CD4 or 3µg CD8-specific primary antibody (Serotec) and incubated at 4˚C for 10 min. The cells were washed using PBS with 0.1% BSA, ph 7.4, centrifuged at 300 x *g* for 8 min, then the supernatant was discarded. The cells were resuspended using PBS with 0.1% BSA, ph 7.4. 25 µl of Dynabeads were added and incubated for 20 min on ice with constant rocking. The tubes were placed in the Dynal magnet and the supernatant removed for flow cytometry analysis. The sample was removed from the magnet and the cells released from the beads using the releasing buffer. The suspension was then placed back in the magnet and the supernatant containing either CD4+ or CD8+ cells was removed. The total number of cells present was determined by staining and counting with a hemocytometer. CD4+ and CD8+ cells were then made up to a concentration of $2x10^6$ cells/ml with RPMI+supplements. The cells were cultured in plates using a 1ml volume of either CD4+ or CD8+ cells plus 50µl of antigen presenting cells (APCs) (mitomycin C treated spleen cells prepared in section 2.2.4.6). The plates were cultures overnight at 37˚C. Live or WKC NTHi 289 (sections 2.1.3 and 2.1.4) were added to cultures. The cultures were then incubated for 30 min or 1 h at 37˚C. Following incubation cells were centrifuged at 12 000 rpm. The supernatant was removed and stored at -20˚C. The pellet was resuspended in RNA extraction buffer and stored at -80˚C.

2.3.2 Flow cytometry

Flow cytometry was used to determine if the target CD4+ or CD8+ cell had been separated. Both the complete MLN cell samples and the remaining depleted subsets from immune and non-immune animals were anaylsed. Analysing the selected cells would have meant there were insufficient cells to perform individual culture analyses. $1x10^6$ cells from each sample were incubated with 30µl of a 1 in 50 dilution of mouse anti-rat CD4 or CD8 primary antibody (Serotec) and incubated at 4˚C for 30 min. The cells were then washed 3 times in wash buffer (0.25ml FCS, 0.0225 M sodium azide, PBS). Cell samples were then incubated with 30µl of 1 in 50 dilution of FITC labeled anti-mouse IgG for 30 min at 4˚C. The cells were then washed a further 3 times in wash buffer and resuspended in 0.5ml of PBS plus 0.5ml 2% formaldehyde and analysed on a Flow Epic ® XL-MCL Flow Cytometer (Beckman Coulter, NSW, Australia).

2.4 CYTOKINE AND CHEMOKINE mRNA EXPRESSION

2.4.1 RNA extraction

150µl of chloroform was added to 1ml of BAL cells, lung homogenate or cell culture sample in RNA extraction buffer and vortexed for 20 sec. The tubes were placed on ice for 20 min before being centrifuged at 14,000 rpm for 15 min at 4˚C. The top aqueous layer was removed into a new tube and an equal amount of isopropanol was added and the tube vortexed. The tube was then stored overnight at -20˚C in order to precipitate the RNA. The next day the tubes were centrifuged at 14,000 rpm for 20 min at 4˚C. The supernatant was removed and the tube inverted on absorbent paper to remove excess liquid.

The pellet was washed with 75% Ethanol (cold at -20˚C) and placed at -20˚C for 20 min. The wash steps were then repeated, the final supernatant was removed and the tubes inverted to air dry. The final pellet was resuspended in 100µl diethyl pyrocarbonate (DEPC) water with 1 mM EDTA. Optical densities (OD) of each sample at 260 nm and 280 nm were measured. Purity was determined by calculating OD260/OD280. If the purity value was <1.7 the RNA extraction was repeated. The concentration of RNA was determined using OD260x2000.

2.4.2 RNase Protection Assays

In the Chapter 3 experiments the expression of different cytokine and chemokine genes was determined using two Multi-Probe Template Sets (PharMingen) with the RiboQuant RNase protection assay system (PharMingen) according to manufacturer's directions. The first template set rck-1 was used in the detection of IL-10, TNF-α and IFN-ɣ. The second template set, a custom-made template set, was used in the detection of the chemokines MCP-1 and MIP-2. The housekeeping gene L32 was present on both template sets and was used as an internal standard for the normalisation of cytokine and chemokine gene expression. The band intensity for each cytokine and chemokine was determined using Quantity One Quantitation Software (BioRad). The actual band intensity was normalised and expressed as a percentage of the L32 band intensity. mRNA expression was measured at various time points up to 12 h for the custom made template and up to 24 h for the rck-1 template.

2.4.3 Real-time reverse transcriptase PCR

2.4.3.1 cDNA synthesis

cDNA synthesis was performed on extracted RNA samples using a Superscript III First-strand synthesis system for RT-PCR kit according to the manufacturer's instructions (Invitrogen).

2.4.3.2 Primers

Quantitect primers for rat Granulocyte macrophage-colony stimulating factor (GM-CSF), IFN-γ, IL-1β, IL-6, IL-10, MCP-1, MIP-2, TNF-α and β-actin (control gene) were purchased and prepared according to manufacturers instructions. (Qiagen, Australia).

2.4.3.3 Sample preparation

The concentration of each cDNA sample was determined based on the concentration of RNA determined above. Samples from treatment groups were then pooled in equal concentrations. PCR master mix consisted of 2.5µl of PCR buffer, 1.5µl of $MgCl_2$, 1.0ul dNTPs, 0.2µl Platinum taq polymerase, and 1.0µl of SYBR green diluted 1 in 1000. 1µg of pooled cDNA and 0.5µl of both forward and reverse primers were added to the master mix and then DEPC-H_20 was added to make a final volume of 25µl. All reagents were purchased through Invitrogen.

2.4.3.4 Real- Time PCR protocol

Real-time PCR was run on the Rotorgene 3000 (Corbett research, Australia) according to the following protocol;

Hold 1 @ 95˚C for 2 min,

Cycling (50 repeats) - Step 1@ 94˚C, hold 15 secs

Step 2@ 55˚C, hold 30 secs

Step 3@ 72˚C, hold 60 secs

Hold 2 @ 72˚C, hold 15 secs

All samples were run in triplicate.

The $2^{-\Delta\Delta C}{}_T$ Method of analysis was used to calculate mRNA expression (Livak and Schmittgen 2001).

2.5 DETECTION OF CYTOKINES AND CHEMOKINES

2.5.1 Levels of IFN-γ, IL-6, IL-10, MCP-1 and MIP-2

Levels of IFN-γ, IL-6, IL-10, MCP-1 and MIP-2, were estimated in BAL, lung homogenate and cell culture supernatants using commercially available immunoassay kits (Biosource International, CA, USA) which estimate cytokine concentration using solid phase sandwich Enzyme linked immunosorbent assay (ELISA). The sensitivities of the assays are as follows;

IFN-γ	<13pg/mL
IL-6	<8pg/mL
IL-10	<5pg/mL
MCP-1	<8pg/mL
MIP-2	<1pg/mL

2.5.2 Levels of GM-CSF, IL-1β and TNF-α

Levels of GM-CSF, IL-1β and TNF-α were estimated in BAL, lung homogenate and cell culture supernatants using commercially available immunoassay (Quantikine, R&D systems Inc, MN, USA) which estimate cytokine concentration using solid phase sandwich ELISA. The sensitivities of the assays are as follows;

GM-CSF	<1.8pg/mL
IL-1β	<5pg/mL
TNF-α	<5pg/mL

2.6 STATISTICAL ANALYSIS

The data has been expressed as the means ± standard error of the mean (SEM). All data was compared for statistical significance using ANOVA with Bonferroni post tests (GraphPad Prism).

2.7 ANIMAL ETHICS

All experiments involving animals were done after approval from the animal ethics committee at the University of Canberra.

THE EFFECT OF MUCOSAL IMMUNISATION ON THE PRODUCTION OF CYTOKINES AND CHEMOKINES

CHAPTER THREE

3.1 ABSTRACT

This study was designed as a pilot study to investigate the production of cytokines and chemokines over time in response to an NTHi lung infection in mucosally immunised animals. Clearance of NTHi from the lung was enhanced in mucosally immunised animals. At 4 h post-challenge more than double the number of macrophages were present in the BAL from the immune animals in comparison to the non-immune animals. mRNA expression and protein production of IFN-γ, IL-10, MCP-1, MIP-2 and TNF-α were measured in the BAL of the immune and non-immune animals. The production of chemokines MCP-1 and MIP-2 was increased at 1 h post-challenge in the immune animals. Resolution of inflammation was enhanced by the decreased production of TNF-α at 4 h and MCP-1 and MIP-2 at 12 h. This study has demonstrated that the enhanced clearance of NTHi corresponded with differences in the production of cytokines and chemokines and that further investigation to more fully quantitate these differences was warranted.

3.2 INTRODUCTION

NTHi is a respiratory pathogen that is a common cause of otitis media in children, sinusitis in adults and lower respiratory infections associated with subjects with COPD (Murphy and Sethi 1992). NTHi is normally a harmless commensal found in the respiratory tract and the mechanisms by which it becomes a pathogen are not well understood.

Lipooligosaccharide (LOS) is a major surface antigen of certain bacteria, including NTHi, and has been found to be an important stimulator of the inflammatory process through production of pro-inflammatory cytokines from host cells (Kopydlowski, Salkowski et al. 1999; Tong, Chen et al. 2001). LOS stimulates the production of proinflammatory cytokines through phosphorylation of the stress-activated kinase, p38, which is subsequently involved in the production of inflammatory cytokines such as IL-1β and TNF-α (Lee, Laydon et al. 1994) and also chemokine IL-8 (Wang, Cleary et al. 2003). Mucosal immunisation with NTHi has been shown to be effective in enhancing clearance of the bacteria from the lung in a, previously well published, rat model of infection (Wallace, Clancey et al. 1989; Foxwell, Kyd et al. 2001). In order to understand how immunisation effectively protects the host against infection and controls the host's inflammatory responses, the roles of cytokines and chemokines in the inflammatory processes must be defined.

Bacterial endotoxins exert the majority of their toxic effects through the unregulated release of certain inflammatory cytokines (Sabirov, Kodama et al. 2001). It can therefore be assumed that the protective effects of immunisation

are due to a controlled regulation of cytokine and chemokine production which leads to a controlled immune response to the pathogen. The initial stimulation of epithelial cells leads to the production and release of these inflammatory cytokines (Khair, Davies et al. 1996; Clemans, Bauer et al. 2000; Ahren, Eriksson et al. 2003; Wang, Cleary et al. 2003). The way in which immunisation regulates cytokine and chemokine production to protect the host against infection could be through either enhancement or inhibition of protein or mRNA production or a combination of both. NTHi has previously been found to stimulate the release of TNF-α and IL-8 at both the transcriptional and post transcriptional level (Shuto, Xu et al. 2001) and studies from this laboratory have shown that immunised animals have a different TNF-α response to infection (Foxwell, Kyd et al. 1998).

TNF-α is a pro-inflammatory cytokine produced in the early stages of lung infection with NTHi. Primarily TNF-α is a mononuclear phagocyte-derived cytokine that activates macrophages and neutrophils and also plays an indirect role in the recruitment of phagocytes to the site of infection (Drost and MacNee 2002). It affects the migration of neutrophils though regulation of the production of the CXC family of chemokines, including MIP-2 and increasing the expression of ICAM by bronchial cells (Myers, Wertheimer et al. 1992). MIP-2, a chemokine produced in rats, is a functional homologue of IL-8 (Bozic, Gerard et al. 1994). In the initial stages of infection, production of MIP-2 by epithelial cells is increased after stimulation with TNF-α (Khair, Devalia et al. 1994). MIP-2 has a direct role in neutrophil recruitment to the site of infection (Castagliuolo, Keates et al. 1998). Although other cells are capable of producing MIP-2 the

major source in the lung is the alveolar macrophage. TNF-α also stimulates production of another chemokine, MCP-1. MCP-1 has previously been found to be upregulated in normal development (Alam, York et al. 1996), but increases have been reported after stimulation with LPS (Zisman, Kunkel et al. 1997). MCP-1 is thought to play a role in the sustained recruitment of monocytes (Chae, Im et al. 2002).

Downregulation of the inflammatory response produced after stimulation with bacteria is important in order to prevent damage to the host which can lead to a situation that could favour the establishment of chronic disease. IL-10 is considered to be a major downregulator of TNF-α. The mechanism by which IL-10 downregulates production of TNF-α has been attributed to various means including, disruption of the translation of TNF-α mRNA into protein, inhibition of transcription and decreasing the half-life of mRNA (Bogdan, Vodovotz et al. 1991). IL-10 also blocks expression of other pro-inflammatory cytokines and chemokines including IFN-γ, MIP-2 and MCP-1 through promoting degradation of mRNA (Opal and V 2000; Strieter, Belperio et al. 2002).

There have been many more studies involving the measurement of cytokine and chemokine concentrations in the BAL than there have been about their concentrations in the lung tissue. Analysis of the BAL and lung tissue will yield results on the total response to infection, including measurement of membrane bound or cell-associated protein, not just the protein that is secreted into the alveolar spaces.

This part of the study was performed as a pilot investigation in order to screen cytokine and chemokine production and gather information to help in the experimental design of the major parts of this thesis. The mucosal immunisation regime used in the following study has been previously used as an effective tool in identifying the mechanisms of immunity to NTHi in the lung (Foxwell, Kyd et al. 1998). In order to further understand the mechanisms by which immunisation protects the host against infection through the regulation of cytokine and chemokine the level of mRNA and protein production were measured for TNF-α, IFN-γ, IL-10, MIP-2 and MCP-1 and associated with the NTHi bacterial clearance and cell recruitment data.

3.3 MATERIALS AND METHODS

3.3.1 Bacteria: NTHi 289 was prepared for immunisation and challenge as detailed in section 2.1.

3.3.2 Animals Strain, immunisation and challenge: SPF DA male rats aged between 8 and 10 weeks were used. They were immunised and challenged with either NTHi or PBS following the protocol detailed in sections 2.2.1, 2.2.2, 2.3.1-2.3.3 and 2.2.4.1-2.2.4.4.

3.3.3 Cytokine and chemokine mRNA expression: The RiboQuant® RNase Protection Assay system was used to determine IFN-γ, IL-10 TNF-α, MCP-1 and MIP-2 mRNA expression in lung homogenate as detailed in sections 2.4.1 and 2.4.2. A representative image of the results in shown in figure 3.1.

3.3.4 Determination of cytokine and chemokine concentration: IFN-γ, IL-10 TNF-α, MCP-1 and MIP-2 protein concentrations were measured in lung homogenate using commercially available ELISA kits as specified in section 2.5.

3.3.5 Statistical analysis: The data has been expressed as the means ± SEM. The data was compared for statistical significance by ANOVA with Bonferroni post tests (GraphPad Prism).

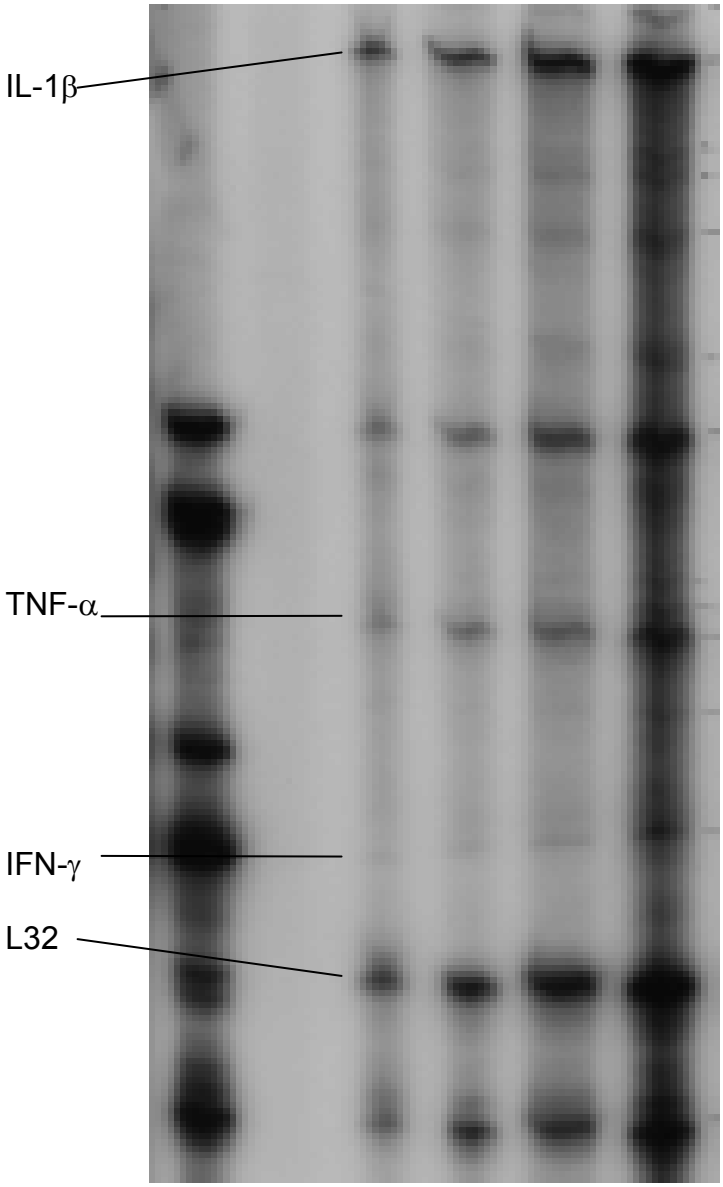

Figure 3.1 Representative image of cytokine gene expression in the lungs of rats challenged with NTHi. Cytokine mRNA levels were determined using the RiboQuant ® RNase Protection Assay System as described in materials and methods. The intensity of the bands was determined and expressed as a percentage of the intensity of the internal control L32.

3.4 RESULTS

3.4.1 Effect of Immunisation on bacterial clearance: The ability to clear NTHi from the rat lung was significantly enhanced following immunisation (figure 3.2). At between 1 and 4 hour post-challenge the difference in enhanced clearance of the bacteria was minimal for immune and non-immune animals. After 4 h post-challenge the rate of bacterial clearance in the immune animals increased rapidly from the lung and by 8 h no NTHi was present in the lungs of the immune animals while the non-immune animals still retained a bacterial load of 10^6 CFU. At 24 h post-challenge, the final time-point measured, the non-immune animals had still not successfully cleared all the bacteria from the lung.

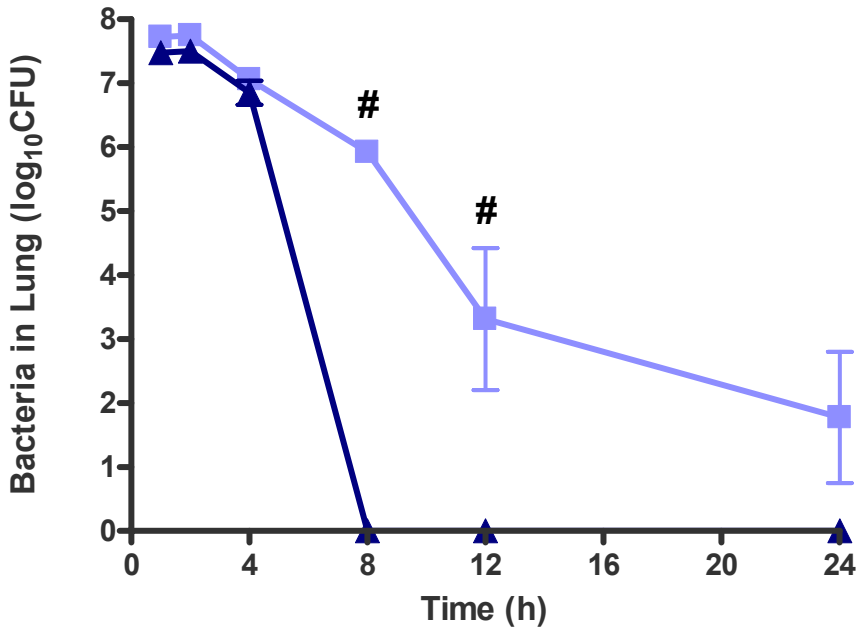

Figure 3.2 Recovery of NTHi from the lung of immunised rats (▲) and non-immunised rats (■) from time of challenge until 24 h post-challenge. After 2 h post-challenge both immune and non-immune animals begin to rapidly clear bacteria from the lung. By 8 h post challenge the immune animals have cleared all of the NTHi whereas non-immune animals still had bacteria present at 24 h. Differences between immune and non-immune animals were significant at 8 and 12 hours post challenge with NTHi (# $p < 0.05$). The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

3.4.2 Effect of immunisation on phagocyte recruitment: A rapid increase in the number of phagocytes recruited to the lung in response to the bacterial challenge was seen in both the immune and the non-immune animals (figure 3.3). The number of phagocytes present in BAL peaked at 8 h post-challenge. In the immune animals the number of phagocytes appeared to decrease to pre-challenge numbers slightly ahead of the non-immune animals.

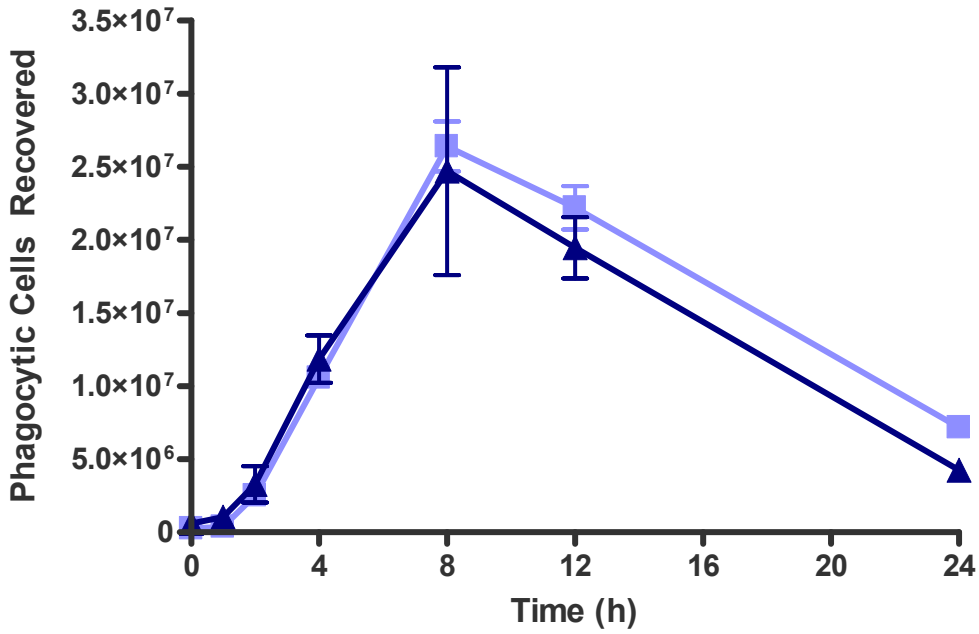

Figure 3.3 Total number of white cells in the BAL of immunised rats (▲) and non-immunised rats (■) from time of challenge until 24 h post-challenge. The profiles of phagocyte recruitment were not significantly different between immune and non-immune animals. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

Although immunisation had very little effect on the total number of phagocytes recovered in the BAL, more obvious differences were observed in the type of phagocyte isolated from BAL. As expected, the number of neutrophils increased rapidly after the NTHi challenge and with the immune animal numbers peaking at a slightly lower number and decreasing sooner than the non-immune animals (figure 3.4).

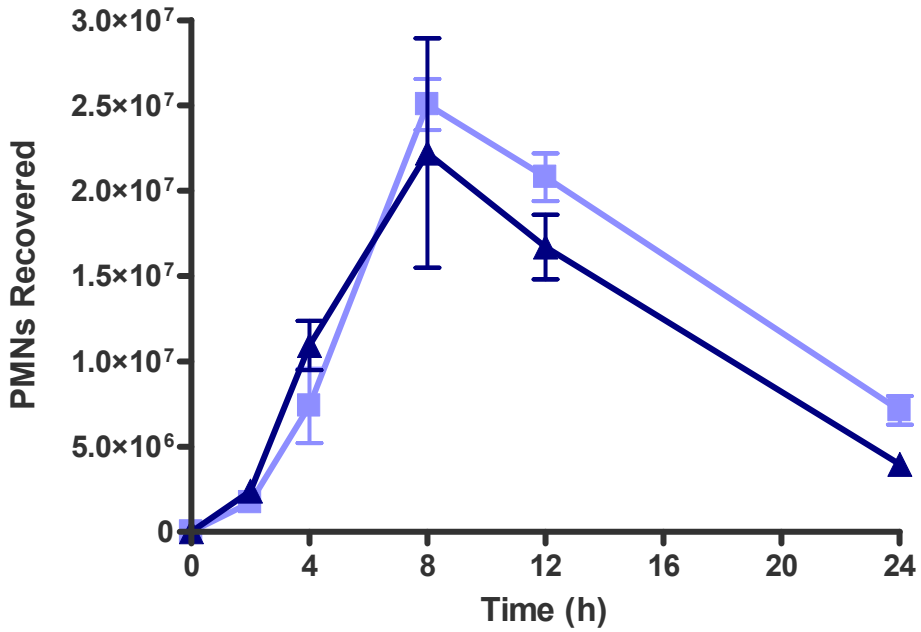

Figure 3.4 Number of PMN cells in the BAL of immunised rats (▲) and non-immunised rats (■) from time of challenge until 24 h post-challenge. The profiles of PMN recruitment were not significantly different between immune and non-immune animals. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

The most significant differences appeared in the number of macrophages recovered from BAL. The non-immune animals showed a slight increase at 2 h, but otherwise maintained a baseline number of macrophages, whereas the immune animals showed a bi-phasic response with an initial small peak at 2 h then a second, larger peak at 8-12 h. The numbers of macrophages in non-immune and immune animals were found to be significantly different at both 8 and 12 h post-challenge with there being more than twice as many macrophages present in immune animals at both of these time points (figure 3.5).

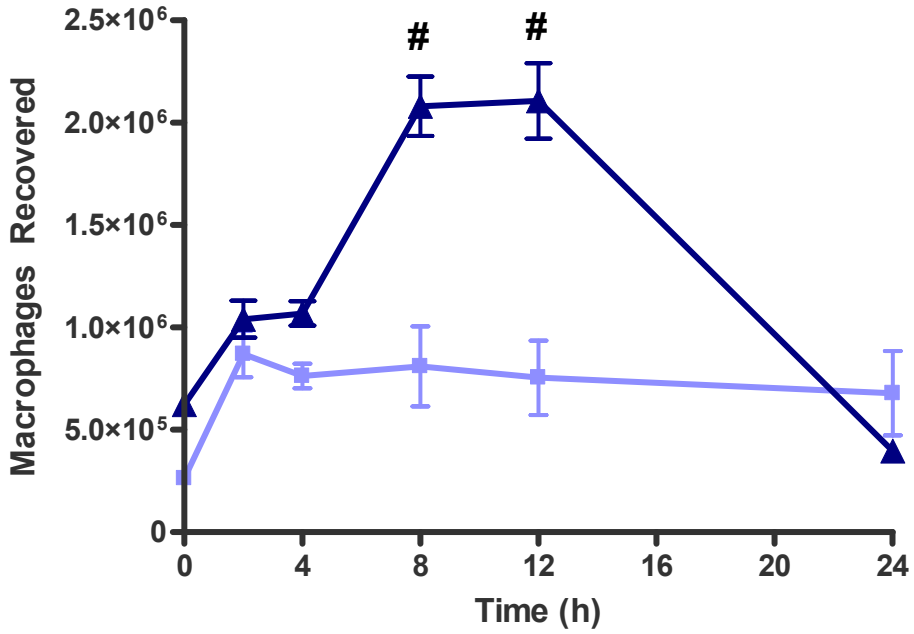

Figure 3.5 Number of macrophages in the BAL of immunised rats (▲) and non-immunised rats (■) from time of challenge until 24 h post-challenge. The profiles of macrophage recruitment were significantly different between immune and non-immune animals. At 8 and 12 h post challenge there were significantly more macrophages found in the BAL of immune animals (# $p < 0.05$). The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

3.4.3 Cytokine and Chemokine gene expression after NTHi exposure: mRNA was detected in the lung homogenate at some point for all the cytokines and chemokines studied (figure 3.6). The mRNA profiles for IL-10, MCP-1 and MIP-2 were found to be the same in immune and non-immune groups, whilst some differences were found for IFN-γ and TNF-α. MCP-1 and MIP-2 mRNA levels were measured at various time-points up to only 12 h due to insufficient sample, unlike the cytokines, which were measured up to 24 h.

Both MCP-1 and MIP-2 mRNA levels remained relatively consistent throughout the time period until 12 hours post-challenge when the levels dropped below pre-challenge levels.

No detectable IL-10 gene expression was found until 12 h post-challenge. TNF-α gene expression steadily increased after challenge until a peak expression was reached at 12 h in both immune and non-immune animals.

Immune animals had a significantly higher level of TNF-α gene expression at both 1 h and 2 h post-challenge. At 24 hours post-challenge no TNF-α mRNA was detected in immune animals whilst a significant amount was still present in non-immune animals.

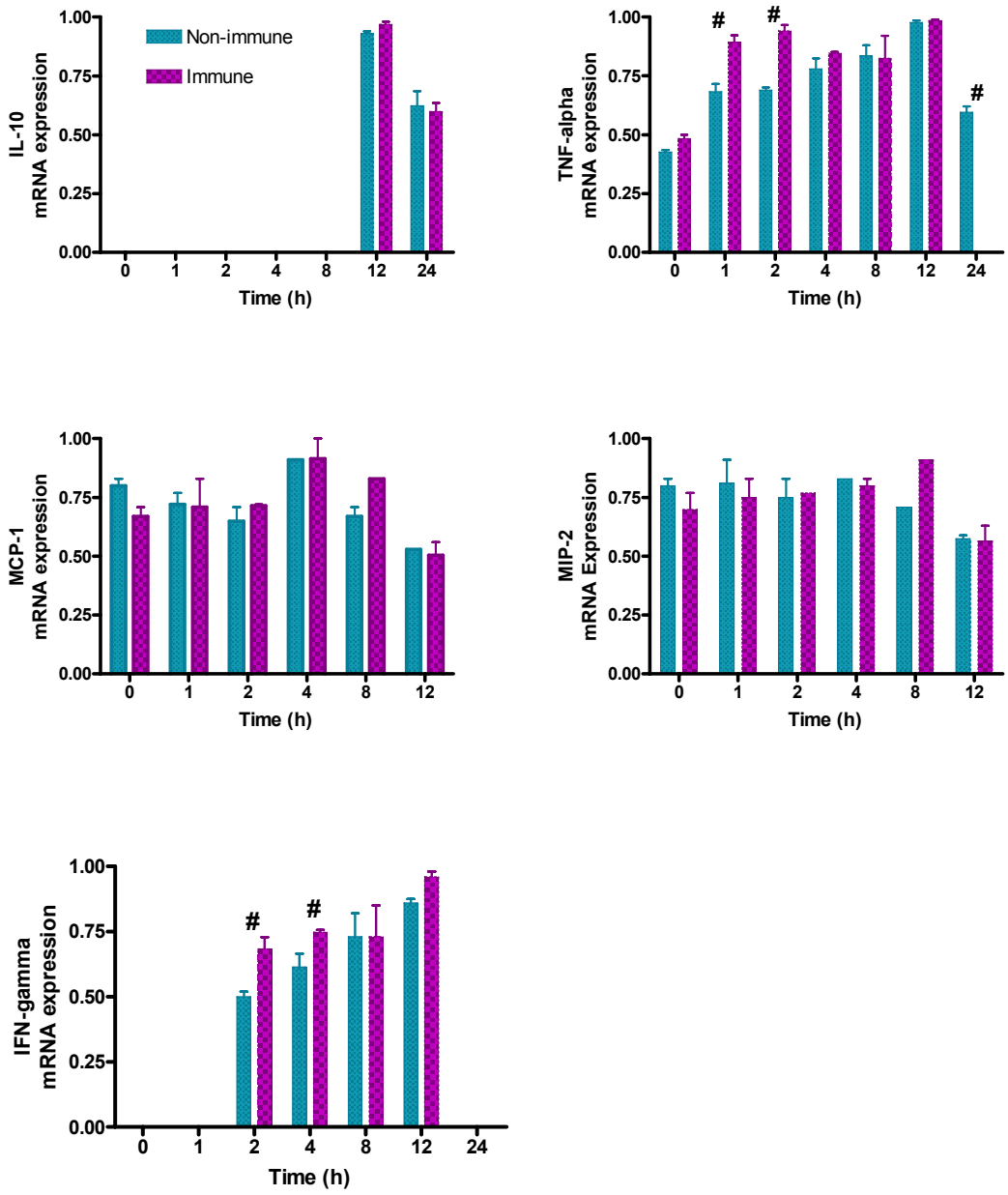

Figure 3.6 IL-10, TNF-α and IFN-γ mRNA expression were measured in the lung homogenate recovered from immunised (■) and non-immunised rats (■) challenged with NTHi from time of challenge until 24 h post-challenge. MCP-1 and MIP-2 mRNA expression was measured until 12 h post-challenge. TNF-α and IFN-γ mRNA expression was significantly higher in immune animals at both 2 and 4 hours post-challenge (# $p < 0.05$). At 24 hours post-challenge no mRNA for TNF-α was detected in lung homogenate from immune animals. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

The IFN-γ gene expression follows a similar profile for both immune and non-immune groups although significant differences are seen at 2 and 4 hours post-challenge when the gene expression is higher in immune animals. Pre-challenge there was no detectable IFN-γ mRNA present. mRNA levels begin to increase from 2 h until they reach a peak at 12 h before dropping back down to undetectable level.

3.4.4 Kinetics of cytokine and chemokine protein production after NTHi exposure: Profiles for the cytokines and chemokines were generated by measuring protein levels in the lung homogenate at various time points post-challenge (figure 3.7). The profiles generated for TNF-α and IL-10 were very similar for both non-immune and immune animals with TNF-α only revealing a significant difference between the two groups at 4 hours, prior to a large decrease in protein concentration in the animals.

IL-10 was the only cytokine or chemokine to decrease following challenge. Before challenge with NTHi a detectable amount of IL-10 was present in all non-immune and immune animals. Immediately after challenge the IL-10 concentration dropped to undetectable levels.

MIP-2 and MCP-1 demonstrated similar trends in the animals tested with significant differences being found between immune and non-immune animals in the production of the two chemokines studied. Unlike MIP-2, there was an initial decrease in the concentration of MCP-1 detected 1 h post challenge in both immune and non-immune animals, at which time there was a significant difference in the two groups. After the initial reduction in concentration the

protein level increased and both MCP-1 and MIP-2 reached a peak at 4 h post challenge when they decreased to reach normal levels. Significant differences in protein concentration between immune and non-immune animals were found at two time-points for both MCP-1 and MIP-2. At 1 h post challenge immune animals had a significantly higher concentration of MCP-1 and MIP-2, whilst at 12 h immune animals had a significantly lower concentration than the non-immune animals.

IFN-γ was not detected in the lungs of any animals (data not shown).

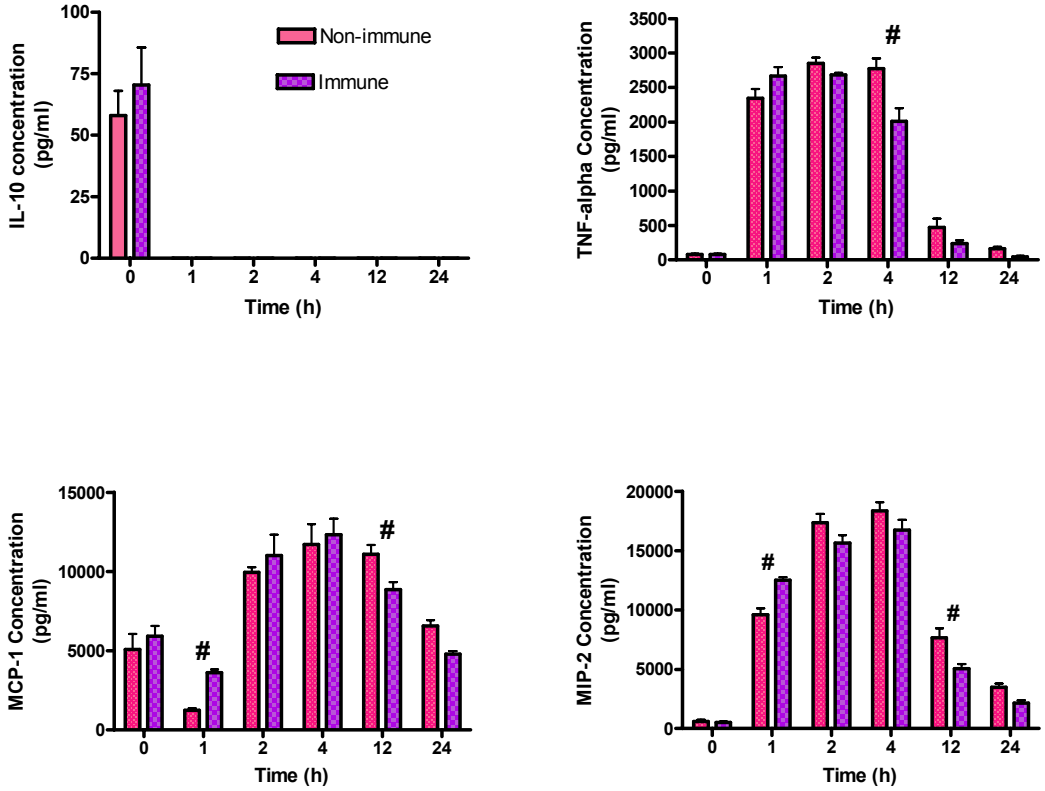

Figure 3.7 Concentration of IL-10, TNF-α MCP-1 and MIP-2 in lung homogenate recovered from immunised (■) and non-immunised rats (■) challenged with NTHi from time of challenge until 24 h post-challenge. Significantly more TNF-α was found in non-immune animals at 4 h post-challenge (# $p < 0.05$). Both MCP-1 and MIP-2 concentrations were significantly higher in non-immune animals at 12 h whilst significantly less MCP-1 was found in non-immune animals at 1 h post challenge. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

3.5 DISCUSSION

This part of the study was performed as a pilot investigation to undertake a preliminary screen in order to determine if there were differences in the immune modulation of cytokines and chemokines in the lungs of rats exposed to infection with NTHi. Both protein production and mRNA expression were measured for several specifically selected cytokines and chemokines in non-immune animals and animals that had received mucosal immunisation prior to exposure to NTHi. The difference in profiles for the various cytokines and chemokines may help to explain how immunisation modulates the innate immune system to enhance the clearance of NTHi from the lungs and regulate the host's inflammatory response to infection.

It was known that NTHi stimulates production of IL-8 (human MIP-2 homologue) and TNF-α from human epithelial cells (Clemans, Bauer et al. 2000; Tong, Long et al. 2003). This study has supported the evidence that production of TNF-α and MIP-2 was increased at the site of infection, in this case the lung, after exposure to NTHi. The increased production cannot be solely attributed to epithelial cells as white blood cells are also an important source of these molecules. The increase in white blood cells at the site of infection is an obvious source of cytokines and chemokines. In some cases it is the cytokines produced by macrophages as the first line of defense that may activate the alveolar epithelial cells (Thorley, Ford et al. 2007).

IL-10 protein was present before exposure to NTHi but then undetectable immediately following stimulation with NTHi. This could suggest that NTHi might be suppressing the production of IL-10 immediately after its introduction or that the intracellular store of IL-10 were released as part of the early host response. IL-10 is believed to block expression of pro-inflammatory cytokines such as TNF-α through promotion of degradation of mRNA (Donnelly, Freeman et al. 1995). The disappearance of IL-10 after the introduction of NTHi corresponds with an upregulation of TNF-α mRNA and protein production at 1 h.

The rat model for investigation of respiratory infection described in this chapter is a previously well published model (Wallace, Clancey et al. 1989; Kyd, Dunkley et al. 1995; Foxwell, Kyd et al. 1998). Experiments in this laboratory have shown that mucosal immunisation with whole-killed NTHi enhances clearance of the bacteria from the lung (Foxwell, Kyd et al. 1998; Foxwell, Kyd et al. 1998). The enhanced clearance of NTHi from the lung in immune animals appears not to be directly determined by the total number of phagocytes recruited to the site of infection but may be due to the enhanced ability of the cells to phagocytise the bacteria. The number of neutrophils recruited to the lung was actually lower in the immune animals. Increased activation of the neutrophils in the immune animals could account for an increase in the ability of these neutrophils to clear the bacteria. The major difference in cell recruitment to the lung between the immune and non-immune groups was in the number of macrophages. The large number of macrophages present in the immune lung at 8 h corresponded with the clearance of all bacteria. The continued presence of the macrophages at 12 h was most likely due to the need to resolve

inflammation through phagocytosis of apoptotic neutrophils as it was during this time that the numbers of neutrophils were reduced (Aderem and Underhill 1999). In regards to the bi-phasic macrophage response, the initial peak at 2 h could be due to the initial response to stimulation of the epithelial cells by LOS located on the surface of NTHi. After this time the influx of neutrophils to the lung would provide another source of stimulation for the recruitment of macrophages.

Initial production of TNF-α by 1 h may further influence the release of other pro-inflammatory cytokines and chemokines (Wilson, Seymour et al. 1998). It is the production of these cytokines and chemokines that leads to the recruitment of cells to the site of infection. This would account for the initial increase of macrophages at 2 h. The subsequent decrease at 4 h in the non-immune animals could be credited to the initial suppression of MCP-1 after NTHi challenge that was more apparent in these animals.

It has previously been suggested that some bacteria have an ability to suppress the production of cytokines and chemokines from cells (Ceponis, McKay et al. 2003; Hauf and Chakraborty 2003; Petrofsky and Bermudez 2005). MCP-1 protein was found to be constitutively expressed in the lungs of both immune and non-immune rats. The initial reduction in MCP-1 production could therefore be due to the bacteria suppressing production of MCP-1 in the lung or degrading MCP-1 protein that was present prior to the challenge. It may also be due to the utilisation of MCP-1 stores and a lag in the host response. The differences in the level of MCP-1 at 1 h in the immune and non-immune animals provides evidence of a controlled immune response as the immune animals

have an increased amount of MCP-1 which would be due to a faster response. This reduction in suppression of MCP-1 in immune animals could play a role in the later increase of macrophages in the lung and more rapid clearance of NTHi.

As important as the clearance of bacteria during infection, is the resolution of the inflammatory response. Prolonged inflammation of the lung can lead to tissue damage through the release of the substances produced by the cells recruited to the lung to clear the bacteria (Kolb, Margetts et al. 2001). In the immune animals, the number of phagocytes present in the lung decreased faster than observed in the non-immune animals. This difference can be attributed to an earlier decrease in the concentration of the chemokines MCP-1 and MIP-2 in the immune animals. Both MCP-1 and MIP-2 play major roles in the sustained recruitment of phagocytes to the site of infection (Castagliuolo, Keates et al. 1998; Wilson, Seymour et al. 1998). Therefore the differences in MCP-1 and MIP-2 concentrations between immune and non-immune groups provides evidence for a controlled inflammatory response in previously immunised animals.

It was found that the levels of the cytokine and chemokine proteins produced did not always correlate with the measure of the gene expression. IL-10 protein was only detected prior to infection however IL-10 mRNA was produced at 12 and 24 h after the infection. This may indicate that the cells were beginning to replenish IL-10 stores that were present prior to infection. TNF-α exhibited similar patterns for protein and mRNA, although there was not an exact relationship. mRNA production increased after the peak in measured protein

levels. A direct relationship between protein production and mRNA production was not apparent for MCP-1, MIP-2 and IFN-γ. The discrepancies between mRNA and protein production could be due to a number of factors including, the release of pre-formed protein, the sensitivity of the assay, small sample size or the timing of samples taken.

In conclusion, the protection against infection with NTHi that was afforded by immunisation appears, in part, to be achieved through increasing the recruitment of macrophages to the site of infection. In immunised animals this recruitment appears to be influenced by the pro-inflammatory chemokine MCP-1. The resolution of inflammation in immunised animals as being under the immune control was supported by the decreased production of the chemokines MCP-1 and MIP-2.

This investigation provided information on the host's response to infection with NTHi and some of the mechanisms by which immunisation is able to influence this response. Some of the differences between the responses in the immune and non-immune groups support broadening the study to include more cytokines and chemokines and not only in the lung tissue but also in the alveolar spaces for comparison of the compartmentalisation of responses.

CONTROL OF CYTOKINE AND CHEMOKINE PRODUCTION IN THE LUNG TISSUE AND ALVEOLAR SPACE FOLLOWING MUCOSAL IMMUNISATION

CHAPTER FOUR

4.1 ABSTRACT

The mRNA expression and protein concentration of a number of cytokines and chemokines were measured in both the BAL and lung tissue of immune and non-immune animals in response to an NTHi lung infection. Mucosal immunisation altered the kinetic profiles of both mRNA expression and protein production of cytokines and chemokines in response to lung infection with NTHi. Protein production of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α was increased immediately following challenge with NTHi in both the lung tissue and BAL of the immune animals. Production of these cytokines also decreased more rapidly in the immune animals following an increase in the protein production of anti-inflammatory cytokine IL-10 in the BAL at 8 h. MCP-1 and MIP-2 protein production peaked earlier and higher in the BAL of the immune animals. The early increase of pro-inflammatory cytokines and chemokines corresponded with enhanced clearance of NTHi from the lungs of the immune animals. This study revealed that enhanced clearance of NTHi from the lung corresponds with the differing kinetic profiles of cytokine and chemokine production generated in mucosally immune animals in comparison with Naïve animals.

4.2 INTRODUCTION

Mucosal immunisation has previously been found to enhance the clearance of NTHi from the rat lung (Wallace, Clancey et al. 1989; Foxwell, Kyd et al. 1998; Foxwell, Kyd et al. 2001)(Chapter 3). In these studies infection with NTHi was characterised by an increase in the number of macrophages and neutrophils in the lung in both the immune and non-immune animals. These cells, however acted differently dependant on immunisation status. In the previous chapter a pilot study was described which examined cytokine and chemokine mRNA expression and protein production in lung homogenate samples in non-immune and immune animals. The results demonstrated differences between the immune and non-immune animals, indicating that a more extensive investigation would be informative.

The majority of cytokines and chemokines are not constitutively expressed in the lung (Strieter, Belperio et al. 2002). Initially, after stimulation with bacteria and in this case NTHi, cytokine and chemokine production in the lung can largely be attributed to the interaction of the endotoxin (LOS) with resident alveolar macrophages (Lafleur, Abrahamsen et al. 1998; Toews 2005) and respiratory epithelial cells (Clemans, Bauer et al. 2000; Avadhanula, Rodriguez et al. 2006). While infection with NTHi stimulates and recruits macrophages to the lung, a large number of neutrophils also migrate to the site of infection and assist in bacterial clearance (Foxwell, Kyd et al. 1998; Foxwell, Kyd et al. 2001; Sabirov, Kodama et al. 2001).

The choice of cytokines measured in this study was extended to include IL-6, IL-1β and GM-CSF. IL-6 and IL-1β are pro-inflammatory cytokines that increase in both mRNA expression and protein production after stimulation with NTHi in epithelial cells and macrophages (Clemans, Bauer et al. 2000; Melhus and Ryan 2000; Tong, Chen et al. 2001). GM-CSF also enhances inflammatory conditions through increasing alveolar macrophage proliferation and activation (Agostini, Trentin et al. 1992). It was therefore postulated that GM-CSF might be a causative determinant of the significant difference in the macrophage numbers present in previously immunised animals that coincide with the enhanced clearance of NTHi (Foxwell, Kyd et al. 1998).

Response to infection with NTHi in the lung relies on extravasation of polymorphs and macrophages from the lung tissue to the alveolar spaces (Foxwell, Kyd et al. 1998). Therefore to gain a further understanding of the role of cytokines and chemokines produced in the lung following challenge with NTHi concentrations of these proteins and mRNA expression in both the lung tissue and alveolar spaces were examined.

While mRNA was measured in the pilot study using the RiboQuant® method, the more widely used tool of Real-time RT-PCR was used for greater sensitivity and reliability in the quantitative results (Dumoulin, Nischalke et al. 2000; Stordeur, Poulin et al. 2002; Ponchel, Toomes et al. 2003).

4.3 MATERIALS AND METHODS

4.3.1 Bacteria: NTHi 289 was prepared for immunisation and challenge as detailed in section 2.1.

4.3.2 Animals Strain, immunisation and challenge*:* SPF DA male rats aged between 8 and 10 weeks were used. They were immunised and challenged with either NTHi or PBS following the protocol detailed in sections 2.2.1, 2.2.2, 2.3.1-2.3.3 and 2.2.4.1-2.2.4.4.

4.3.3 Cytokine and chemokine mRNA expression: Cytokine Real-time RT PCR was used to determine GM-CSF, IFN-γ, IL-1β, IL-6, IL-10 TNF-α, MCP-1 and MIP-2 mRNA expression in lung homogenate and BAL as detailed in sections 2.4.1 and 2.4.3.

4.3.4 Determination of cytokine and chemokine concentration: GM-CSF, IFN-γ, IL-1β, IL-6, IL-10 TNF-α, MCP-1 and MIP-2 protein concentrations were measured in lung homogenate and BAL using commercially available ELISA kits as specified in section 2.5.

4.3.5 Determination of total protein concentration*:* Total protein concentration was measured in the lung homogenate and BAL using the method described in section 2.5.3.

4.3.6 Statistical analysis: The data has been expressed as the means ± SEM. The data was compared for statistical significance by ANOVA with Bonferroni post tests (GraphPad Prism).

4.4 RESULTS

4.4.1 Effect of immunisation on cell recruitment and bacterial clearance from the lung tissue and BAL: The animal challenge performed in Chapter 3 was repeated in order to obtain BAL samples for cytokine and chemokine analysis. The results for bacterial clearance and cell recruitment to the lung did not differ from the results obtained in Chapter 3 (figures 3.1-3.5). Profiles of NTHi clearance from the BAL did not differ from clearance from the lung homogenate (data not shown).

4.4.2 Increased production of pro-inflammatory cytokines after challenge with NTHi: Protein production of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α increased in the BAL and lung homogenate samples after challenge with NTHi in both the immune and non-immune animals (figures 4.1, 4.2 and 4.3). This increase in production was observed immediately after challenge in all except for IL-6 in the BAL which did not begin to increase in production until after 1h. These cytokines increased more rapidly in the immune animals, often peaking higher than in the non-immune animals. Protein levels also began to decrease earlier in the immune animals. This decrease was more apparent in the BAL than in the lung homogenate for IL-1β and IL-6.

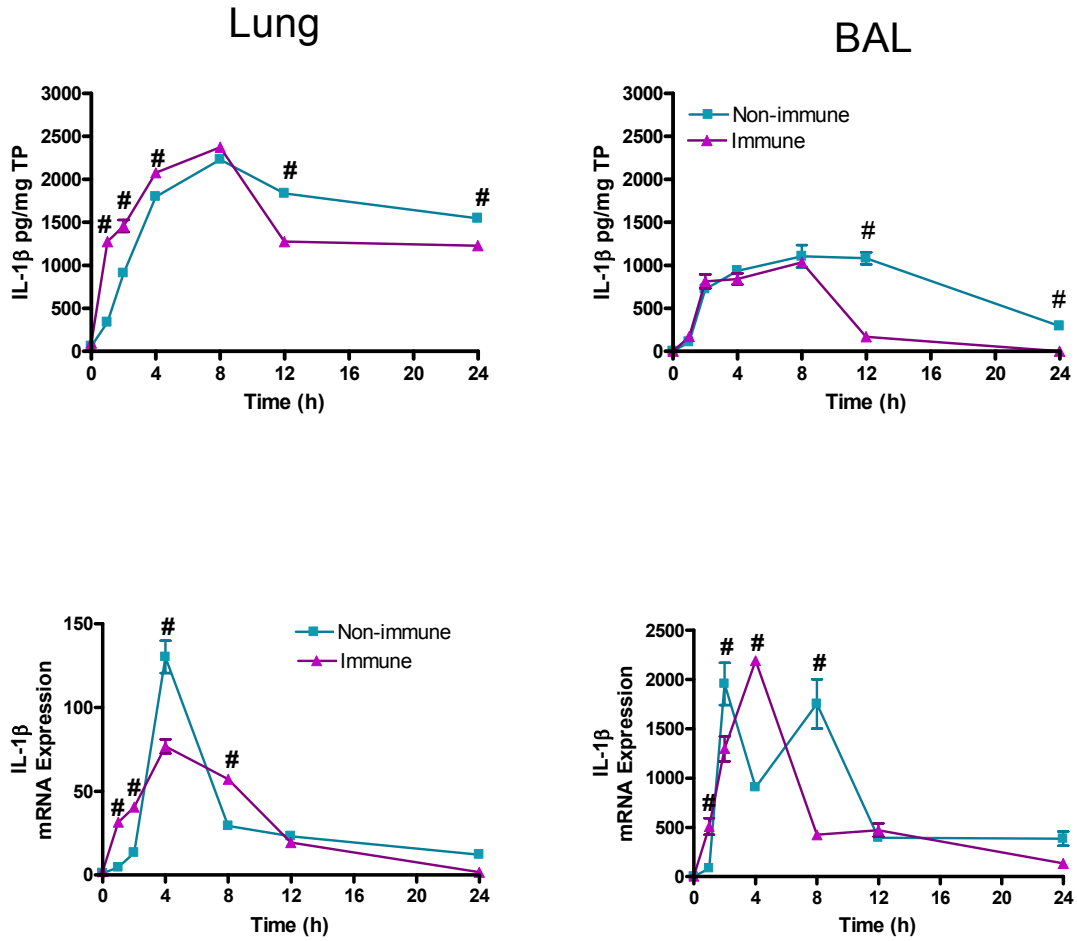

Figure 4.1 IL-1β protein concentration and mRNA expression were measured in the lung homogenate and BAL recovered from immunised and non-immunised rats challenged with NTHi from time of challenge until 24 h post-challenge. Protein concentration has been expressed per mg of total protein. A significant difference in protein concentration between immune and non-immune animals was seen in lung homogenate at 1, 2, 4, 12 and 24 h post-challenge and at 12 and 24 h in BAL (# $p < 0.05$). Y-axis scales differ between individual mRNA expression graphs. mRNA expression was significantly different between immune and non-immune animals at 1, 2, 4 and 8 h in both lung homogenate and BAL. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

IL-1β, IL-6 and TNF-α mRNA expression also peaked in the lung homogenate following the introduction of NTHi. This large increase in mRNA expression was not seen for IL-6 or TNF-α in the BAL of the non-immune animals but was seen in immune animals. Following the primary peak in mRNA, expression dropped for all except IL-1β in the BAL of the non-immune animals and TNF-α in the lung homogenate of the immune animals. In both of these cases a lower secondary peak appeared. The kinetic profiles for pro-inflammatory cytokine protein production closely followed the profiles for mRNA expression.

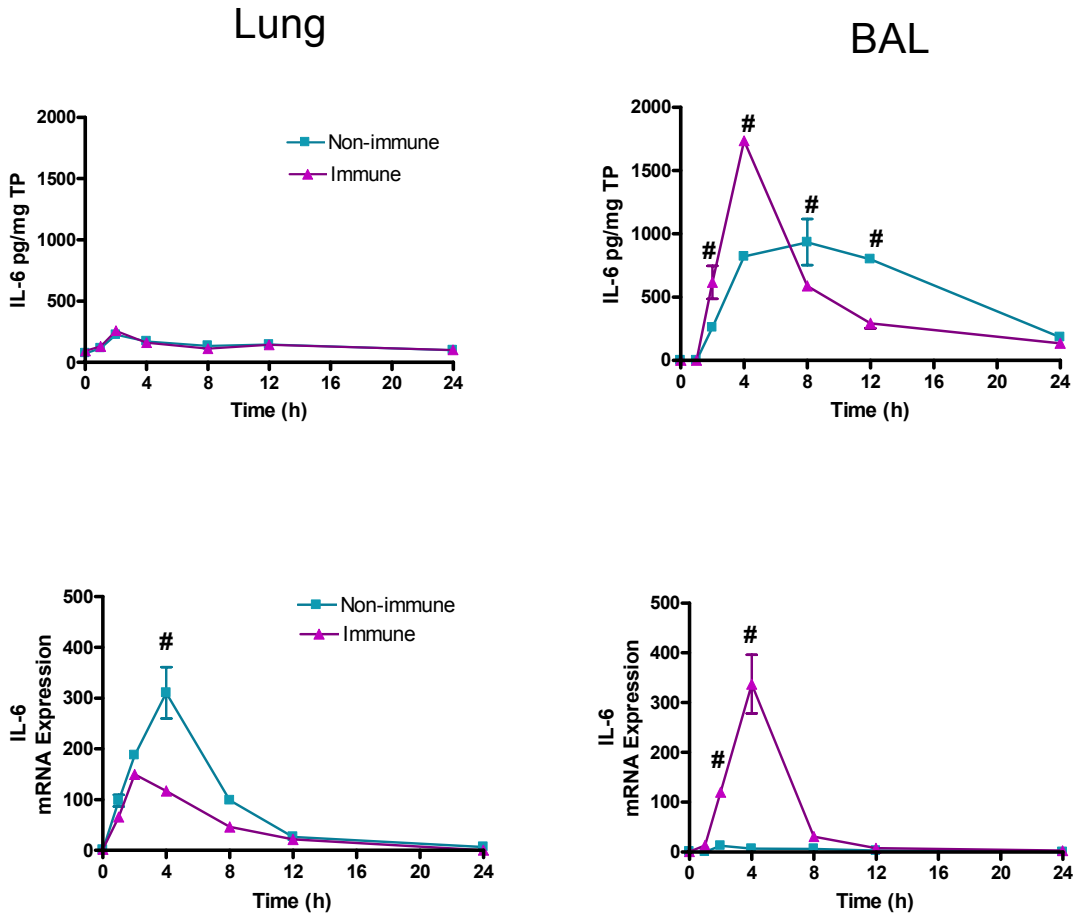

Figure 4.2 IL-6 protein concentration and mRNA expression were measured in the lung homogenate and BAL recovered from immunised and non-immunised rats challenged with NTHi from time of challenge until 24 h post-challenge. Protein concentration has been expressed per mg of total protein. No significant difference between immune and non-immune animals was seen in protein concentration in lung homogenate whilst differences were seen in BAL at 2 and 4 h post-challenge (# $p < 0.05$). mRNA expression was determined to be significantly different between immune and non-immune animals at 4 h in lung homogenate and 2 and 4 h in BAL. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

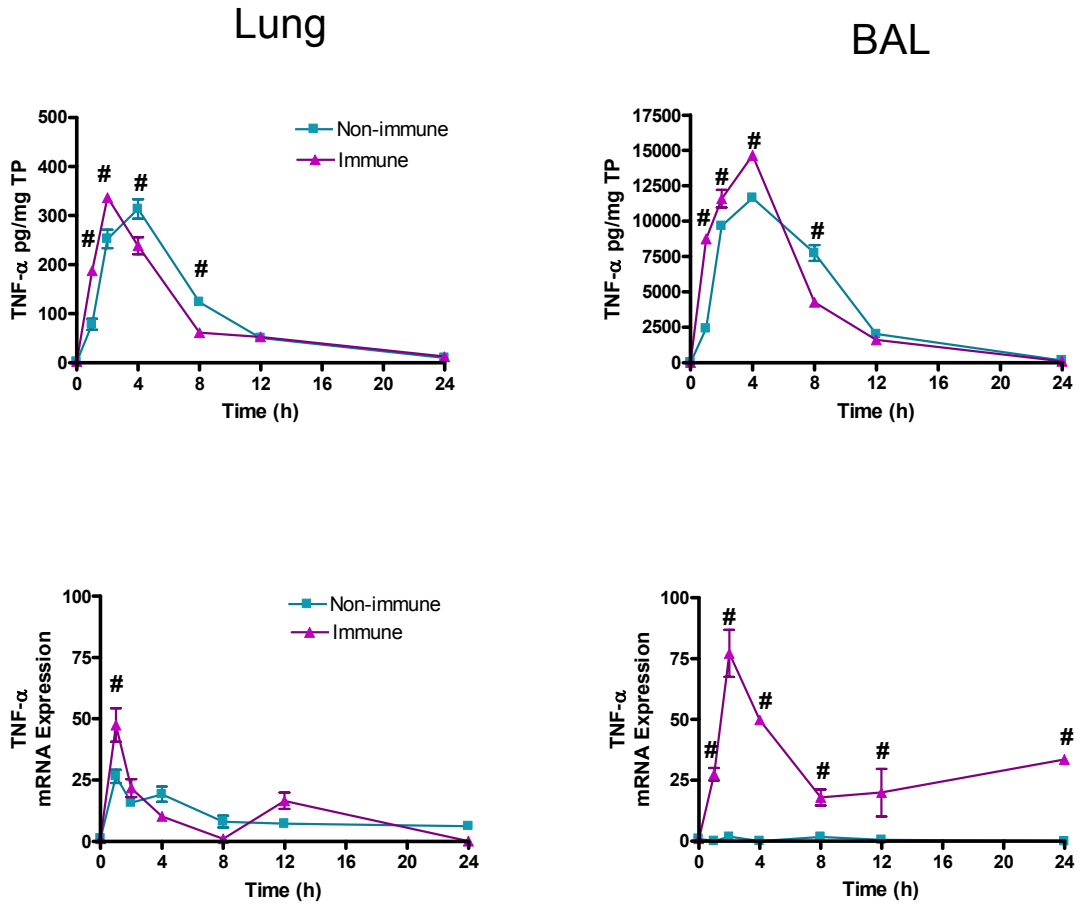

Figure 4.3 TNF-α protein concentration and mRNA expression were measured in the lung homogenate and BAL recovered from immunised and non-immunised rats challenged with NTHi from time of challenge until 24 h post-challenge. Y-axis scales differ between individual protein production graphs. Protein concentration has been expressed per mg of total protein. A significant difference in protein concentration between immune and non-immune animals was seen in lung homogenate at 1, 2, 4, and 8 h post-challenge and at 1, 2, 4 and 8 h in BAL (# $p < 0.05$). mRNA expression was significantly different between immune and non-immune animals at 1 h in lung homogenate and at all time-points post-challenge in BAL. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

4.4.3 Initial suppression of IL-10 production following challenge: Low levels of IL-10 protein were detected prior to challenge with NTHi in all samples and groups (figure 4.4). After challenge, a drop in IL-10 protein production was seen in the BAL and lung homogenate samples in both the immune and non-immune animals. IL-10 protein levels remained suppressed throughout the time-period in the BAL of non-immune animals but increased in the immune animals and in the lung homogenate of both groups. Similar profiles were seen in IL-10 production from the lung homogenate and BAL of the immune animals but differed in non-immune animals.

mRNA expression remained constant throughout the time-period in non-immune animals but increased rapidly after 2 h in immune animals. The mRNA profiles from the immune animals were similar to those of actual protein production.

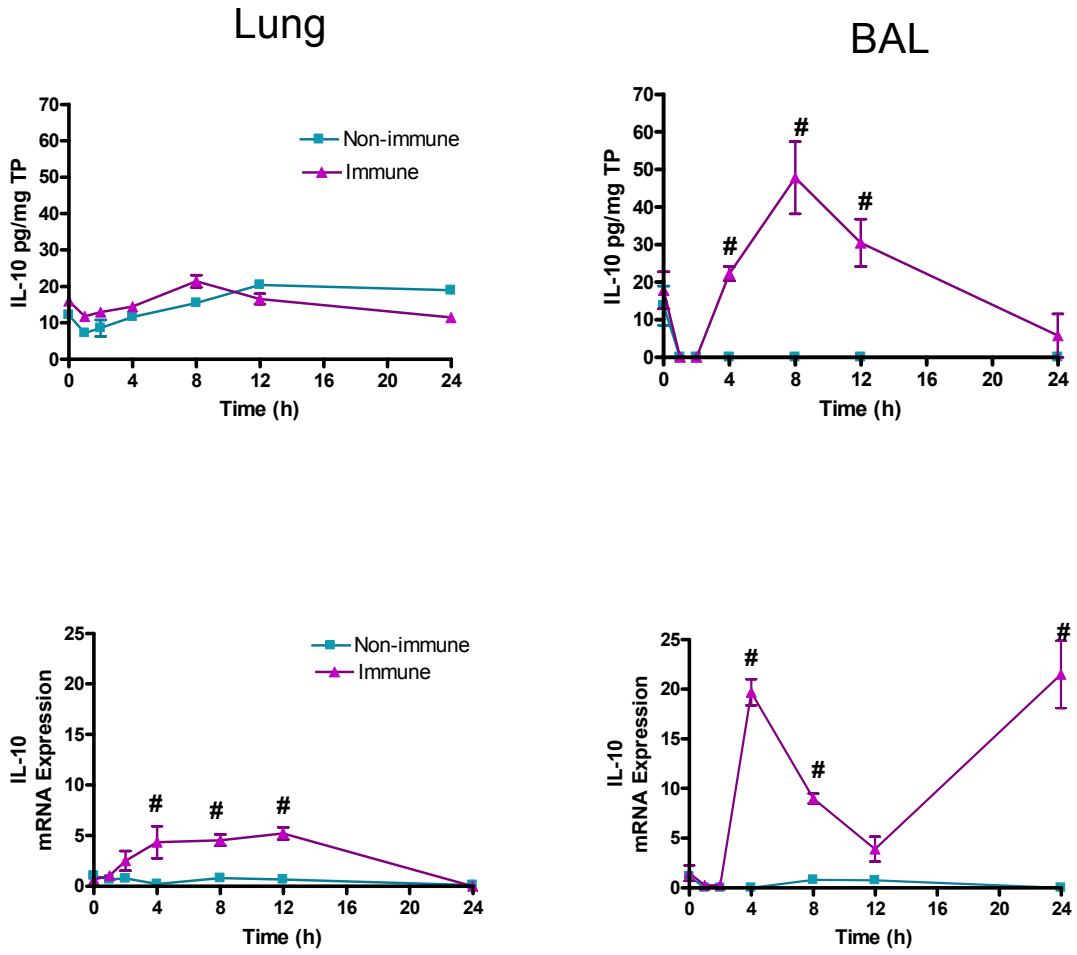

Figure 4.4 IL-10 protein concentration and mRNA expression were measured in the lung homogenate and BAL recovered from immunised and non-immunised rats challenged with NTHi from time of challenge until 24 h post-challenge. Protein concentration has been expressed per mg of total protein. No significant difference between immune and non-immune animals was seen in protein concentration in lung homogenate whilst differences were seen in BAL at 4, 8 and 12 h post-challenge (# $p < 0.05$). mRNA expression was determined to be significantly different between immune and non-immune animals at 4, 8 and 12 h in lung homogenate and 4, 8 and 24 h in BAL. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

4.4.4 Chemokine production increased after challenge with NTHi: An increase in the production of both of the chemokines, MCP-1 and MIP-2, was detected in the BAL and lung homogenate samples post-challenge with NTHi (figures 4.5 and 4.6). The protein production profiles for MCP-1 and MIP-2 were similar with only one major difference of interest, which was an initial reduction of MCP-1 protein in the lung homogenate from the non-immune animals and to a lesser extent in the immune animals. This drop was reversed by 2 h post-challenge. The major differences between the immune and non-immune animals were the earlier peak in the immune animals followed by an earlier decline.

Whilst the protein profiles are very similar for MCP-1 and MIP-2, the mRNA profiles differ greatly. MCP-1 mRNA expression was bi-phasic whilst MIP-2 mRNA expression peaked then fell for all samples except the non-immune BAL, where expression remained elevated throughout. Very few similarities in the profiles for mRNA expression and protein production were seen, however peaks in MCP-1 mRNA expression in the immune animals and MIP-2 in the non-immune corresponded with peaks in the respective protein production. Following the peak at 2 h the MIP-2 mRNA expression in the non-immune animals remained elevated throughout the time-course while protein production decreased.

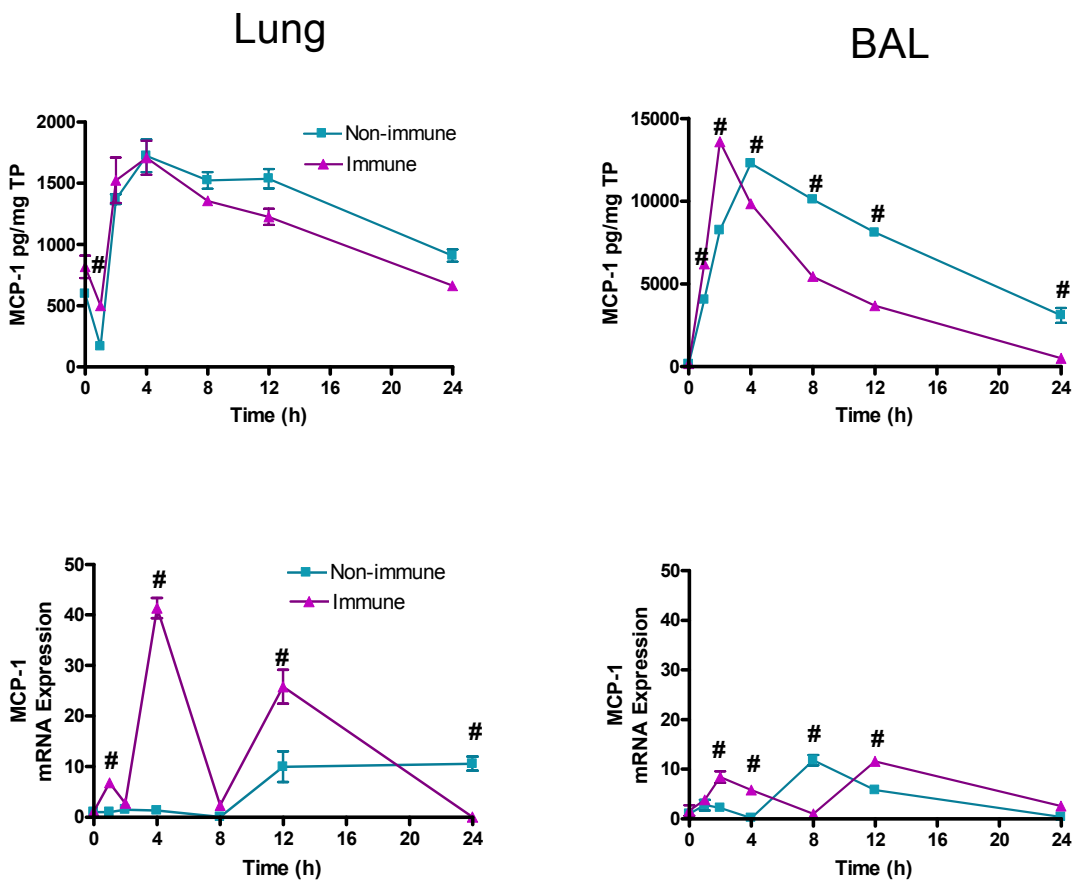

Figure 4.5 MCP-1 protein concentration and mRNA expression were measured in the lung homogenate and BAL recovered from immunised and non-immunised rats challenged with NTHi from time of challenge until 24 h post-challenge. Y-axis scales differ between individual protein production graphs. Protein concentration has been expressed per mg of total protein. A significant difference in protein concentration between immune and non-immune animals was seen in lung homogenate at 1 h post-challenge and in BAL at all time points post-challenge (# $p < 0.05$). mRNA expression was significantly different between immune and non-immune animals at 1, 4, 12 and 24 h in lung homogenate and at 2, 4, 8, 12 and 24 h in BAL. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

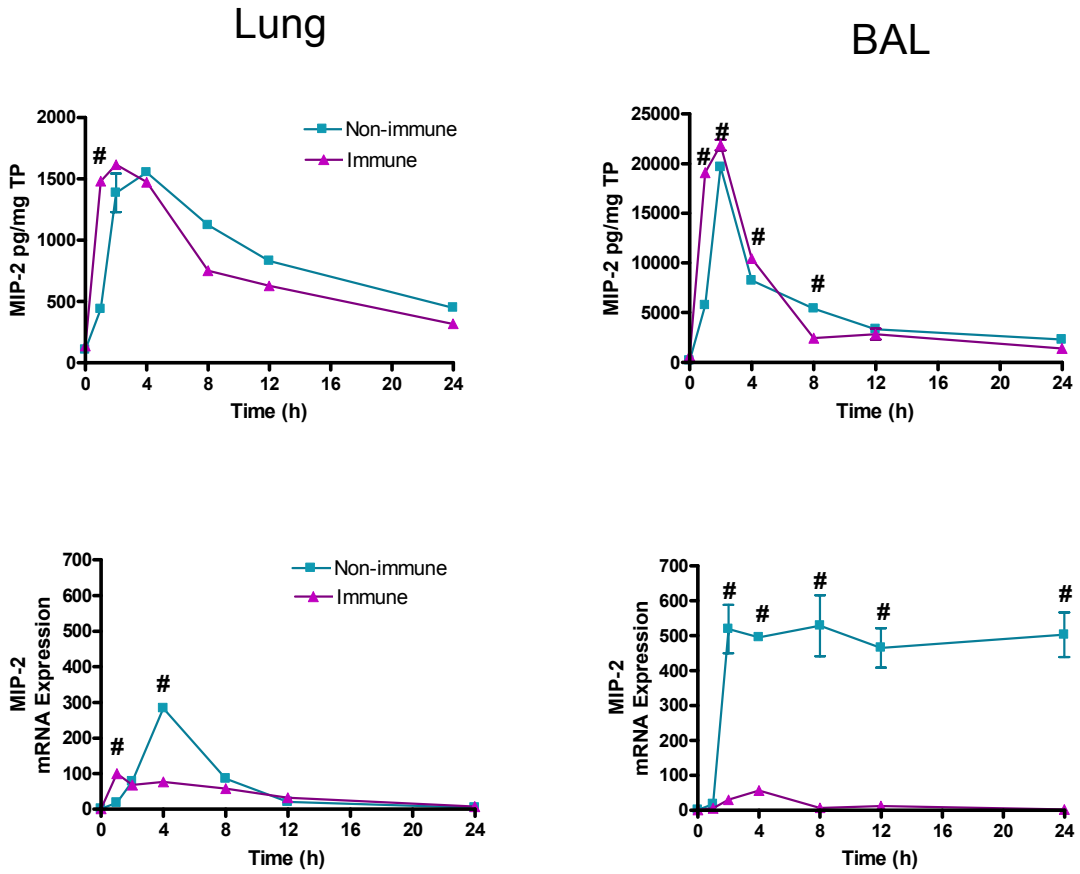

Figure 4.6 MIP-2 protein concentration and mRNA expression were measured in the lung homogenate and BAL recovered from immunised and non-immunised rats challenged with NTHi from time of challenge until 24 h post-challenge. Y-axis scales differ between individual protein production graphs. Protein concentration has been expressed per mg of total protein. A significant difference in protein concentration between immune and non-immune animals was seen in lung homogenate at 1 h post-challenge and in BAL at 1, 2, 4 and 8 h post-challenge (# $p < 0.05$). mRNA expression was significantly different between immune and non-immune animals at 1 and 4 h in lung homogenate and at all time point post-challenge in BAL. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

4.4.5 No IFN-γ detected in immune animals: No IFN-γ protein was detected in either the BAL or lung homogenate of immune animals at any time (figure 4.7). Low levels were detected in the non-immune animals at 8 and 12 h post-challenge but the concentration was just above the detection levels.

The detection of protein was in contrast to the IFN-γ mRNA expression. IFN-γ mRNA expression was up-regulated more quickly in the immune animals in both the BAL and lung homogenate. In the non-immune animals the mRNA expression appeared to be up-regulated after the production of protein suggesting that the levels of protein detected may not be directly linked to the mRNA responses observed.

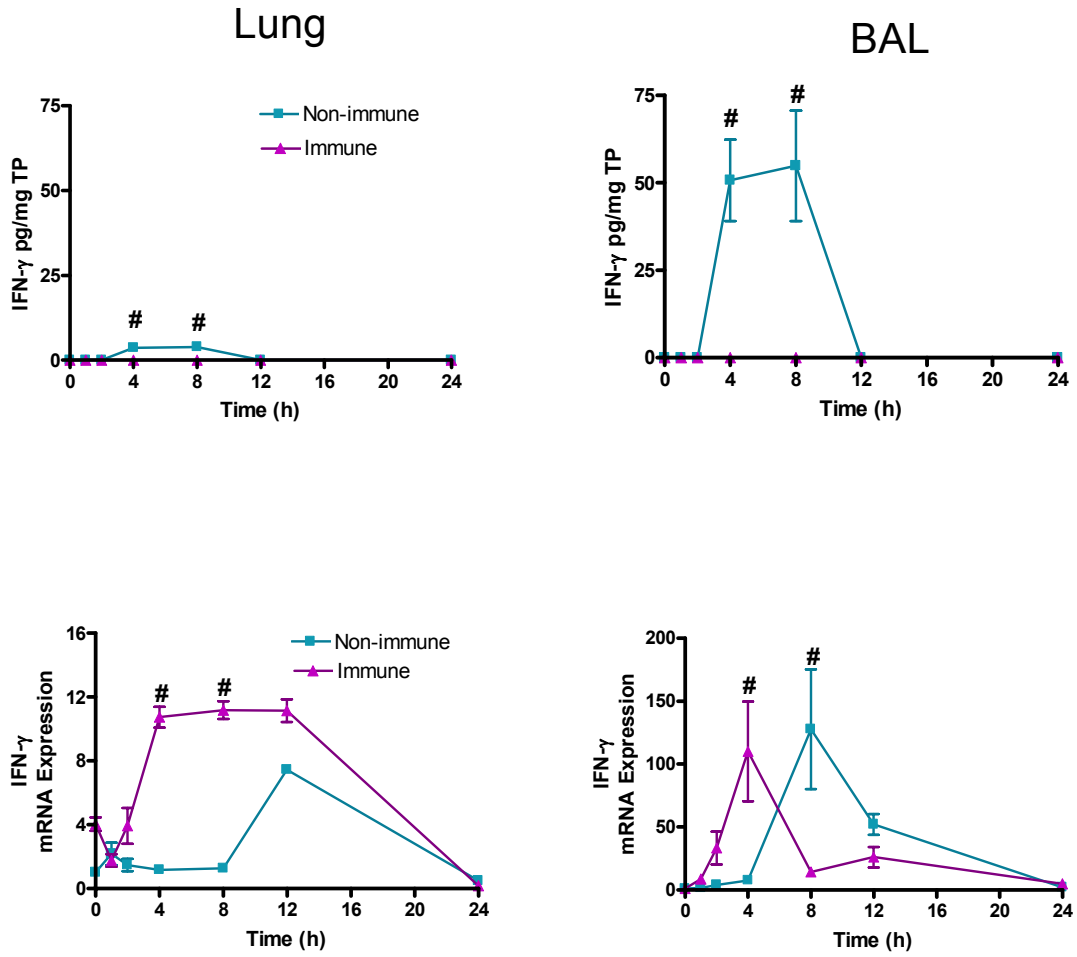

Figure 4.7 IFN-γ protein concentration and mRNA expression were measured in the lung homogenate and BAL recovered from immunised and non-immunised rats challenged with NTHi from time of challenge until 24 h post-challenge. Protein concentration has been expressed per mg of total protein. A significant difference in protein concentration between immune and non-immune animals was seen in both lung homogenate and BAL at 4 and 8 h post-challenge (# $p < 0.05$). Y-axis scales differ between individual mRNA expression graphs. mRNA expression was significantly different between immune and non-immune animals at 4 and 8 h in both lung homogenate and BAL. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

4.4.6 Suppression of GM-CSF protein production: No GM-CSF protein was detected in any lung tissue or BAL sample at any time point even though increases in mRNA expression were seen in the lung tissue of both the immune and non-immune animals and in the BAL of the immune animals (figure 4.8). Significantly different profiles of mRNA expression between the non-immune and immune animals were seen in the BAL with expression remaining elevated throughout the time-period in the immune animals whilst no increase in mRNA expression was seen in the BAL of the non-immune animals.

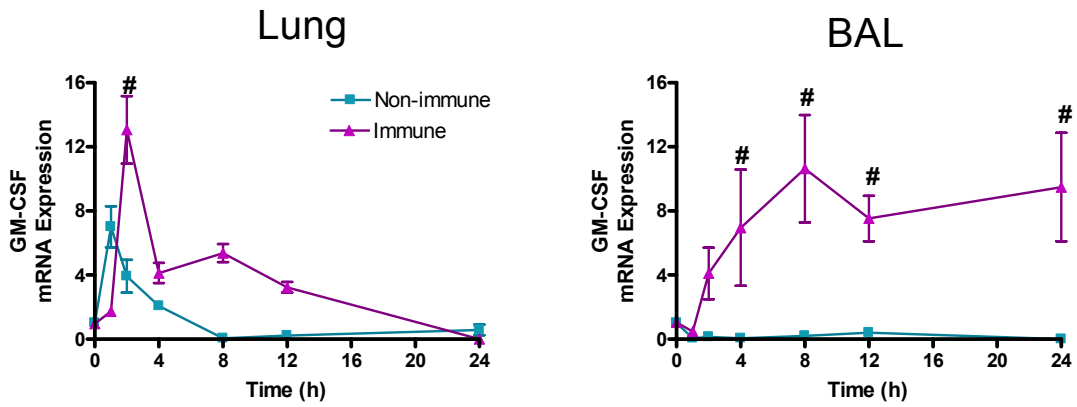

Figure 4.8 GM-CSF protein concentration and mRNA expression were measured in the lung homogenate and BAL recovered from immunised and non-immunised rats challenged with NTHi from time of challenge until 24 h post-challenge. No protein was detected in any of the samples. mRNA expression was significantly different between immune and non-immune animals at 2 h in lung homogenate and at 4, 8, 12 and 24 h post-challenge in BAL (# $p < 0.05$). The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

4.5 DISCUSSION

Mucosal immunisation has been shown to be effective in providing protection from NTHi in a rat model of respiratory infection (Wallace, Clancey et al. 1989; Foxwell, Kyd et al. 1998; Foxwell, Kyd et al. 2001)(Chapter 3). Differences in the recruitment of white blood cells to the lung after challenge with NTHi were seen between the immune and non-immune groups. More specifically, significant differences were seen in the recruitment of macrophages. Resident alveolar macrophages were present in the alveolar spaces of both the immune and non-immune animals prior to challenge (Foxwell, Kyd et al. 1998) and it is these cells that are an important source of pro-inflammatory cytokines in the initial stages of infection.

Rapid increases in the production of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α and the chemokines MCP-1 and MIP-2 were seen in the lung tissue and alveolar spaces after challenge with NTHi. These results are in agreement with prior studies of cytokine and chemokine production after stimulation with NTHi (Khair, Devalia et al. 1994; Sato, Liebeler et al. 1999; Clemans, Bauer et al. 2000; Tong, Chen et al. 2001). The differences in cytokine and chemokine production between the immune and non-immune groups provide evidence of immunisation controlling the inflammatory response. It is these differences in production that lead to differences in phagocyte recruitment and activation and further lead to enhanced bacterial clearance from the immune lung.

As the non-immune animals have not previously been exposed to NTHi the immune response to the challenge was completely innate in nature.

Differences in cytokine and chemokine production between the immune and non-immune groups as early a 1 h post-challenge indicated that the acquired immune system responds almost immediately following infection with NTHi. In the immunised animals, the acquired immune system exerts an influence on innate responses (Strieter, Belperio et al. 2002) to effectively recruit and activate cells and as such co-ordinates a balance that results in a controlled inflammatory response.

Inflammation in the respiratory tract involves a series of interactions aimed to control the area of tissue affected by infections, remove the infecting agent and finally to repair and restore function to the affected tissue. In response to infection it is important that the host recruits and activates the appropriate leukocyte population and then selectively de-activates and resolves these cells. To do this in a naïve state, the host relies on signals and responses associated with the innate immune system. In the immune state, it is hypothesised that effective control of inflammation in response to infection is achieved by immune regulation of the innate response in addition to the antigen-specific responses associated with antibody and antigen-specific lymphocytes.

Chemotactic and pro-inflammatory cytokines such as TNF-α, IL-1 and IL-8 (MIP-2 in rodents) released from macrophages (Standiford, Kunkel et al. 1991) act as both an attractant to neutrophils and an activator for the expression of cell adhesion molecules on endothelial cells (Detmar, Tenorio et al. 1992). The activation of phagocytic cells is also under the influence of pro-inflammatory cytokines (Malazdrewich, Ames et al. 2001). It can therefore be hypothesised that the enhanced ability to clear NTHi from the lung seen in mucosally

immunised animals is due to the increased production of pro-inflammatory cytokines and that the production of these cytokines is controlled by the acquired immune response.

Chemokines MCP-1 and MIP-2 protein were found to be constitutively expressed in the lung tissue and may plan a role in sustaining the presence of resident alveolar macrophages in the normal rat lung. MCP-1 acts mainly on macrophages through the expression of adhesion molecules and the regulation of cytokine production (Jiang, Beller et al. 1992). The initial decrease in MCP-1 after challenge may be as a result of the movement of this chemokine from the lung tissue into the interstitial space in order to promote the migration of macrophages. Early increased production of MIP-2 and MCP-1 in the alveolar spaces of the immune animals was followed by a greater number of macrophages being recruited.

LPS and LOS of Gram-negative bacteria express unique molecular patterns that are distinguished by the host as being foreign (Ozinsky, Underhill et al. 2000). This pattern recognition is part of the innate immune response. Following stimulation with LOS both resident alveolar macrophages and lung epithelial cells produce and release a variety of cytokines and chemokines. Alveolar macrophages release TNF-α, IL-1β, IL-6, MCP-1 and MIP-2, whilst lung epithelial cells have been found to produce IL-6, IL-8 and TNF-α (Lafleur, Abrahamsen et al. 1998; Clemans, Bauer et al. 2000). This production corresponds with the results found in this study.

IL-10 is well known for its anti-inflammatory characteristics and its ability to suppress production of TNF-α and to a lesser extent MIP-2 (Donnelly, Freeman

et al. 1995; Murray 2005). The increase in IL-10 mRNA expression followed by protein production in the BAL of immune animals corresponds with a rapid decrease in TNF-α production. As sustained increased levels of TNF-α have been associated with tissue damage in lung infection (Matuschak, Munoz et al. 1998; Tsao, Hong et al. 1999; Dumitru, Ceci et al. 2000), the down-regulation an important part of the resolution of the inflammatory response and a further indication of a controlled response.

The only cells present in the alveolar space were neutrophils and macrophages. It can therefore be safely concluded that the mRNA profiles generated from the BAL can be attributed solely to these cell. The differing mRNA expression between the immune and non-immune animals provides evidence that one of the ways the acquired response is able to influence the innate response is through regulation of mRNA expression in phagocytic cells recruited to the site of infection.

Neutrophils were recruited in large numbers to the lung following challenge with NTHi in both the non-immune and immune animals. Although there were no notable differences in cells numbers between the immune and non-immune animals, cytokines and chemokines can act to control the activation of these cells and therefore their ability to phagocytise the bacteria. The earlier increase in TNF-α production in both the lung tissue and alveolar spaces of the immune animals may lead to greater activation as TNF-α in known to be a potent activator of neutrophils (Ferrante, Martin et al. 1993). This increase in production of TNF-α appears to be under transcriptional control as there is an obvious relationship between mRNA expression and protein production.

The early presence of IFN-γ has been associated with a predominately innate immune response (Strieter, Belperio et al. 2002) and its presence in only samples from the non-immune animals further substantiates this observation. Although mRNA expression was up-regulated in the immune animals this did not lead to translation of detectable levels of protein at any time. This may indicate that post-transcriptional control was in place in the immune animals.

GM-CSF is required for normal lung function (Christensen, Bailie et al. 2000) and also has a role in proliferation of macrophages during infection (Hawgood, Akiyama et al. 2001). The most likely explanation for the lack of GM-CSF detection in all samples would lie with the sensitivity of the assay. GM-CSF has been detected in mouse lung homogenate using bead assay but at a very low concentration (McDuffie, Obert et al. 2006). If similar concentrations of GM-CSF were present in the samples obtained in this study they would be below the level of detection for the assay.

One of the most notable differences between the two animal groups that may play an important role in the resolution of inflammation in the immune animals was the sustained increase of macrophages from 8-12 h post-challenge. Not only do macrophages act to phagocytise bacterial pathogens but also apoptotic and damaged cells (Savill, Wyllie et al. 1989). This could explain the number of macrophages still present in the BAL even after all the bacteria had been cleared from lung tissue and alveolar spaces.

Although neutrophils and macrophages are not thought to be able to act as memory cells they play an important role in the acquired immune response through the way in which they are controlled. T-cells are indirectly capable of controlling the migration and activation of phagocytes through the production and regulation of cytokines and chemokines (Kambayashi, Assarsson et al. 2003) as is proposed to have happened in mucosally immunised animals.

This study has expanded on the results from the pilot study in Chapter 3 and further demonstrated that mucosal immunisation influences the production of cytokines and chemokines in the lung following infection and the enhanced clearance of NTHi from the lung can be attributed to cytokines and chemokines in both the alveolar space and lung tissue controlling the recruitment, and most likely activation of phagocytic cells to the site of infection.

EFFECT OF AN ANTECEDENT VIRAL INFECTION ON NONTYPEABLE HAEMOPHILUS INFLUENZAE LUNG INFECTION

CHAPTER FIVE

5.1 ABSTRACT

Bacterial clearance and the immune response to NTHi were measured in animals with an antecedent respiratory Sendai viral infection. Clearance of NTHi was enhanced in animals that had been infected with the Sendai virus 3 days prior to bacterial challenge. Enhanced clearance of NTHi was associated with an early influx of macrophages to the lungs of the virally infected animals. This early influx of macrophages corresponded with an early increase in production of chemokines MCP-1 and MIP-2. Prior to challenge with NTHi the mRNA expression of several cytokines and chemokines was up-regulated in animals that had been virally infected. In this model of infection it was shown that an antecedent viral infection enhanced clearance of NTHi from the rat lung most likely through the priming of cells associated with the innate immune response.

5.2 INTRODUCTION

Respiratory viral infections are well known for their ability to predispose the host to bacterial infections but the mechanisms by which this happens are currently not fully understood. It has been suggested that this could be through promotion of colonisation of more pathogenic strains of bacteria or impairment of the host's innate immune response (Fernandez-Sesma, Marukian et al. 2006). In the case of otitis media, it is commonly believed that viral infections impair Eustachian-tube functions (Suzuki and Bakaletz 1994; Tong, Grants et al. 2002). The Eustachian tube is important for maintaining middle ear pressure and abnormal middle ear pressure has been observed in children following a respiratory viral infection (Winther, Hayden et al. 2002) with suggested consequences of aspiration of nasopharyngeal fluids and development of bacterial otitis media. Studies from this laboratory have further shown than an antecedent Sendai virus has a significant effect on the development of otitis media in animal models (Krishnamurthy 2007).

In the respiratory tract, the epithelial cells act as a first line of defense against bacterial invasion. The ability of bacteria to adhere to these cells has been proven to increase after exposure to viral pathogens (Hakansson, Kidd et al. 1994; Jiang, Nagata et al. 1999; Avadhanula, Rodriguez et al. 2006). It is this increase in the bacteria's ability to adhere to respiratory cells that leads to an increase in colonisation (Tong, Weiser et al. 2001) and possible increased chance of developing infection as seen in a colonisation epidemiology study of children (Faden, Duffy et al. 1996).

The interaction between the host's innate immune response and invading viral pathogens can also reduce the effectiveness of the host's ability to prevent bacterial infection (White, Crouch et al. 2005; McNamee and Harmsen 2006). Experiments by Engelich et al have demonstrated a decrease in neutrophil survival following incubation with influenza A virus and *S. pneumoniae in vitro* (Engelich, White et al. 2001). This decreased survival of neutrophils could allow bacteria colonising the respiratory tract to have an advantage in evading the host's innate immune response.

As cytokines and chemokines play an integral role in the innate response to invading pathogens, changes in their concentration and the kinetics of production following a viral infection may further indicate some of the mechanisms by which viral infections predispose the host to bacterial infections.

In the investigation presented in this Chapter, rats were inoculated with Sendai virus, followed 3 days later, by inoculation with NTHi at the time when viral titer was at its peak (Moore, Lidbury et al. 2001). Sendai virus is a rodent respiratory pathogen commonly used in models of viral infections (Moore, Lidbury et al. 2001; Strahle, Garcin et al. 2003). In order to gauge the innate immune response to the co-infection, cytokine and chemokine mRNA expression and protein concentrations were measured and compared to the bacterial clearance and cell recruitment responses following infection. It was predicated that a viral infection would initiate an innate immune response that might modulate the host's response to NTHi.

5.3 MATERIALS AND METHODS

5.3.1 Bacteria: NTHi 289 was prepared for challenge as detailed in section 2.1.

5.3.2 Virus: Sendai virus was prepared for challenge as detailed in section 2.2.3.5.

5.3.3 Animal strain and challenge*:* SPF DA male rats aged between 8 and 10 weeks were used. They were challenged with either Sendai virus or PBS followed 3 days later by NTHi following the protocol detailed in sections 2.2.1, 2.2.2, 2.2.3.3 and 2.2.3.5-2.2.3.10.

5.3.4 Cytokine and Chemokine mRNA expression*:* Real-time RT PCR was used to determine IL10, TNF-α, IFN-γ, MCP-1, MIP-2, IL-1β, IL-6 and GM-CSF mRNA expression in lung homogenate as detailed in sections 2.4.1 and 2.4.3. No samples were collected for assessment of mRNA in the BAL between these groups.

5.3.5 Determination of cytokine and chemokine concentration*:* IL10, TNF-α, IFN-γ, MCP-1, MIP-2, IL-1β, IL-6 and GM-CSF protein concentrations were measured in lung homogenate and BAL using commercially available ELISA kits as specified in section 2.5.

5.3.6 Determination of total protein concentration*:* Total protein concentration was measured in the lung homogenate and BAL using the method described in section 2.5.3.

5.3.7 Statistical analysis*:* The data has been expressed as the means ± SEM. The data was compared for statistical significance by ANOVA with Bonferroni posttests (GraphPad Prism).

5.4 RESULTS

5.4.1 Pre-existing viral infection increases rodent's ability to clear NTHi from lung tissue and BAL: The ability to clear NTHi from the rat lung and BAL was significantly enhanced in animals infected with Sendai virus 3 days prior to bacterial challenge (figures 5.1 and 5.2). The Sendai infected+NTHi challenged animals cleared NTHi by 8 h and 12 h from the lung tissue and BAL respectively while the NTHi-only challenged animals had not cleared all the NTHi from either site until between 12 and 24 h post-bacterial challenge as seen in the previous chapters. Significantly more NTHi was detected at 1 h following challenge in both the lung homogenate and BAL of the Sendai infected+NTHi challenged animals but by 8 h in the lung homogenate and at 12 h in both the lung homogenate and BAL there was significantly more NTHi detected in the NTHi-only challenged animals.

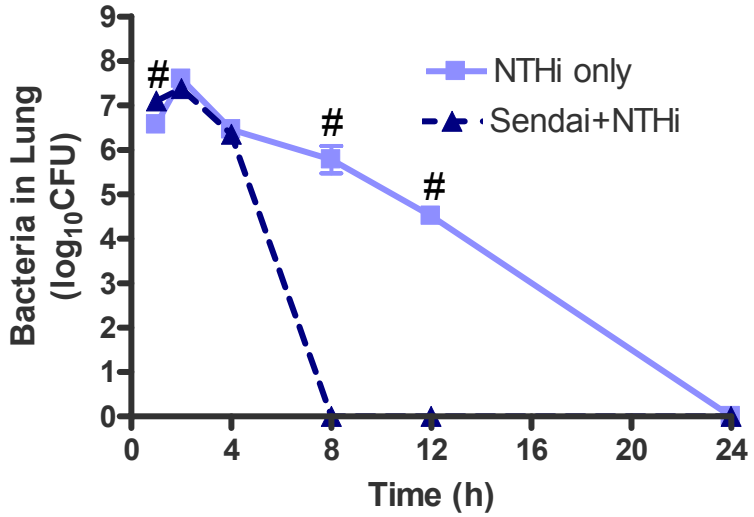

Figure 5.1 Recovery of NTHi from the lung of rats infected with the Sendai virus 3 days previously (▲) and rats that received NTHi only (■) from time of challenge until 24 h post-challenge. Animals received $5x10^8$ CFU at time 0. At 1 h post-challenge there was significantly ($P<0.05$) more bacteria recovered from the lungs of Sendai+NTHi rats. After 2 h post-challenge both the Sendai+NTHi and the NTHi only animals begin to rapidly clear bacteria from the lung. By 8 h post challenge the Sendai+NTHi animals had cleared all of the NTHi whereas NTHi only animals did not clear all the bacteria until 24 h. Differences between Sendai+NTHi and NTHi only animals were significant at 8 h ($P<0.001$) and 12 h ($P<0.001$) post challenge with NTHi. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically. (# indicates significant statistical difference)

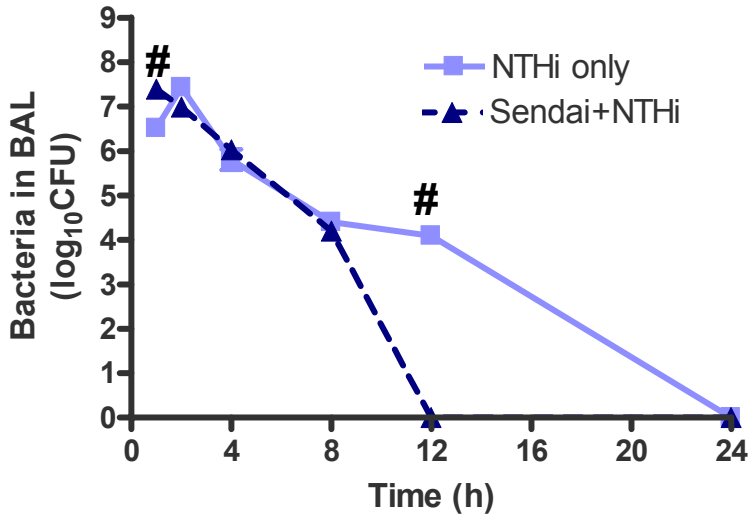

Figure 5.2 Recovery of NTHi from the BAL of rats infected with the Sendai virus 3 days previously (▲) and rats that received NTHi only (■) from time of challenge until 24 h post-challenge. Animals received $5x10^8$ CFU at time 0.At 1 h post-challenge there was significantly ($P<0.001$) more bacteria recovered from the BAL of Sendai+NTHi rats. 1 h was also the time at which the number of bacteria peaked in the BAL of Sendai+NTHi animals, whilst NTHi only animals reached a peak at 2 h. After 2 h post-challenge both Sendai+NTHi and NTHi only animals begin to rapidly clear bacteria from the BAL. By 12 h post challenge the Sendai+NTHi animals cleared all of the NTHi whereas NTHi only animals did not clear all the bacteria until 24 h. Differences between Sendai+NTHi and NTHi only animals were significant at 12 hours post challenge with NTHi (# $p< 0.001$). The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically. (# indicates significant statistical difference)

5.4.2 Effect of previous viral infection on recruitment of phagocytes to the lung: A rapid increase in the number of phagocytes isolated in the BAL after bacterial challenge was seen in both the Sendai infected+NTHi challenged and NTHi-only challenged groups (figure 5.3). The profiles of both groups were identical until after 4 h post–challenge. There was a peak in total phagocytes for both groups at 8 h however there were significantly (P<0.001) less cells in the Sendai infected+NTHi challenged animals at this time and also at 12 and 24 h post bacterial challenge.

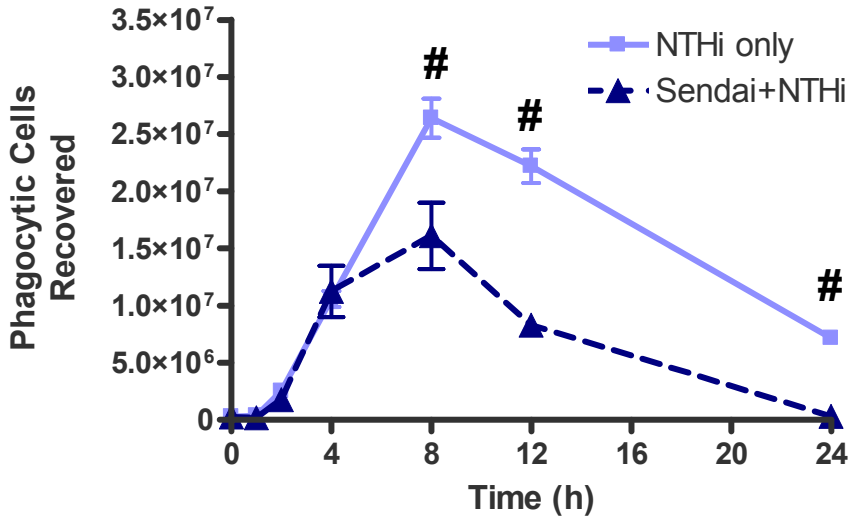

Figure 5.3 Recruitment of phagocytic cells to BAL of rats infected with the Sendai virus 3 days previously (▲) and rats that received NTHi only (■) from time of challenge until 24 h post-challenge. The profiles of phagocyte recruitment were significantly different (# P<0.001) between Sendai+NTHi and NTHi animals at 8, 12 and 24 h when there were more cells isolated in the BAL of NTHi only animals. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

The kinetics of PMN recruitment to the lung followed a pattern identical to that of total phagocytes in both the Sendai infected+NTHi challenged animals and NTHi-only challenged animals (figure 5.4). The number of macrophages isolated in the BAL of the Sendai infected+NTHi challenged animals reached a peak at 2 h. This was significantly higher than seen in the NTHi-only challenged animals, whose macrophage numbers peaked later at 4 h (figure 5.5). After macrophage numbers peaked in virally challenged animals there was a rapid decrease in numbers and by 24 h macrophages had returned to post-challenge levels. After peaking, macrophage numbers remained significantly increased throughout the time-period in animals challenged with NTHi only.

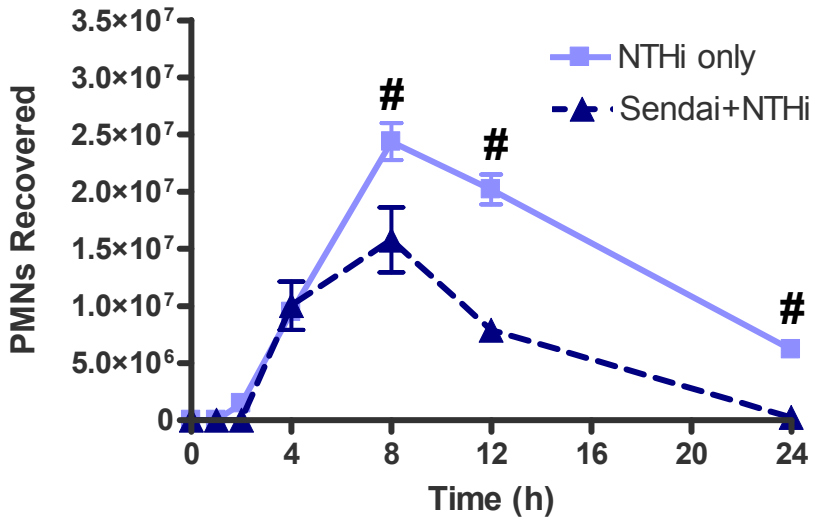

Figure 5.4 Recruitment of PMN cells to the BAL of rats infected with the Sendai virus 3 days previously (▲) and rats that received NTHi only (■) from time of challenge until 24 h post-challenge. The profiles of phagocyte recruitment were significantly different between Sendai+NTHi and NTHi animals at 8h ($P<0.001$), 12h ($P<0.001$) and 24h ($P<0.01$) when there were more cells isolated in the BAL of the NTHi only animals. By 24 h post-challenge no PMNs were found in the BAL of Sendai+NTHi only animals. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically. (# indicated significant statistical difference)

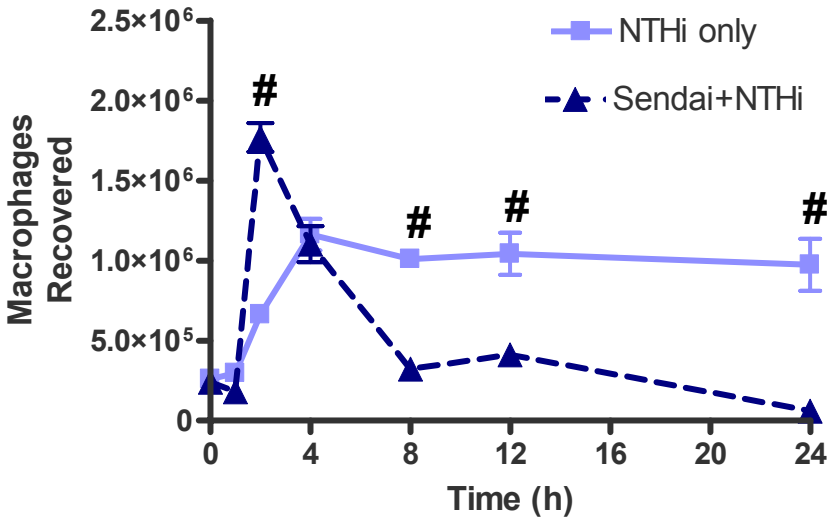

Figure 5.5 Recruitment of macrophages to BAL of rats infected with the Sendai virus 3 days previously (▲) and rats that received NTHi only (■) from time of challenge until 24 h post-challenge. The profiles of macrophage recruitment were significantly different between Sendai+NTHi and NTHi only animals. At 2 h post challenge there were significantly more macrophages isolated from the BAL of Sendai+NTHi animals. At 8, 12 and 24 h post challenge there were significantly more macrophages found in the BAL of NTHi only animals (# $p < 0.001$). The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

5.4.3 The effect of a viral infection on cytokine and chemokine mRNA expression: Prior to bacterial challenge with NTHi there were only a small number of significant differences in cytokine and chemokine mRNA expression or protein production between the Sendai infected+NTHi challenged and NTHi-only challenged animals. This was particularly that case for IL-10, MCP-1 and GM-CSF mRNA where expression was greater in Sendai infected+NTHi challenged animals prior to bacterial challenge (figures 5.6 and 5.7).

IL-10 mRNA expression profiles differed between the two groups (figure 5.6). The NTHi-only challenged animals maintain a low level of expression throughout the time-period, while in the Sendai infected+NTHi challenged animal's IL-10 mRNA expression was elevated and peaked several times following bacterial challenge.

IL-1β and IL-6 mRNA expression peaked at 4 h in both groups with the only differences being a greater peak in IL-1β mRNA expression in animals challenged with NTHi only (figure 5.6). TNF-α mRNA expression also peaked at 4 h in the virally infected animals but the peak was seen earlier, at 2 h, and the magnitude remained lower in the animals challenged with NTHi only (figure 5.6).

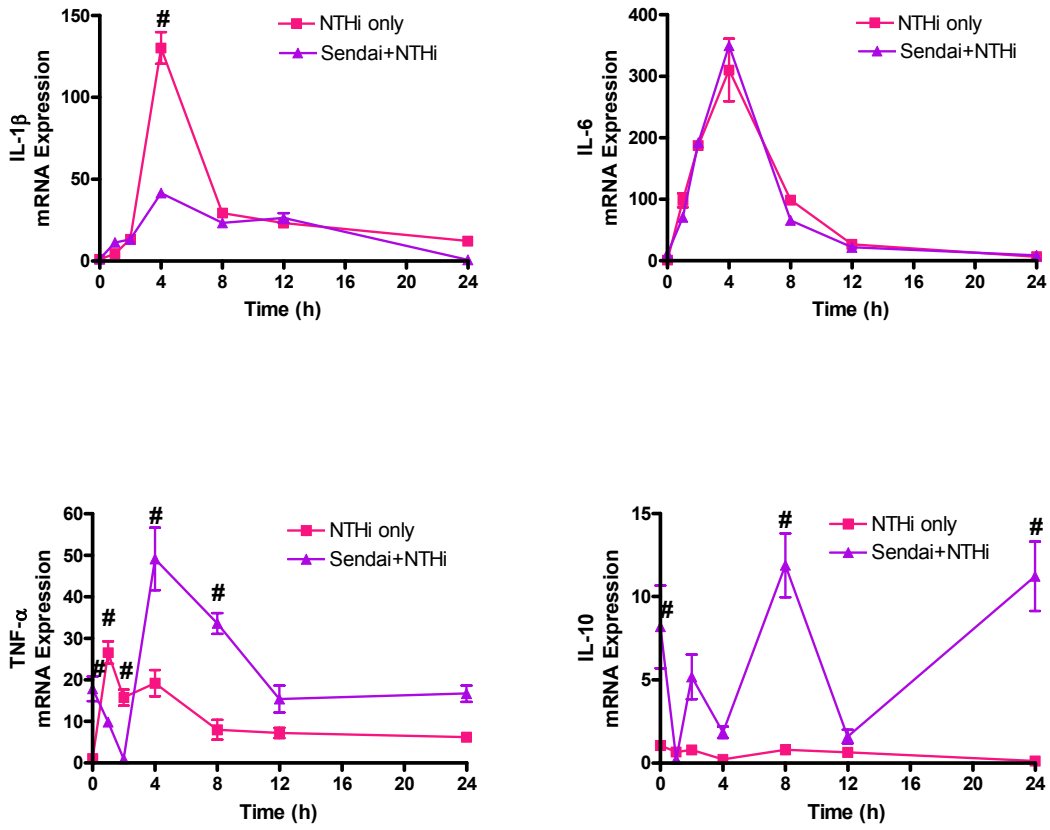

Figure 5.6 IL-1β, IL-6, TNF-α and IL-10 mRNA expression was measured in the lung homogenate. Y-axis scales differ between individual graphs. mRNA expression was significantly different between Sendai+NTHi and NTHi only animals at 4 h for IL-1β, at 0, 1, 2, 4 and 8 h for TNF-α and at 0, 8 and 24 h for IL-10 (# $p < 0.05$). The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

A small increase in MIP-2 mRNA expression was seen immediately following bacterial challenge in the Sendai infected+NTHi challenged animals but returned to pre-bacterial challenge levels soon after (figure 5.7). Expression peaked significantly higher and later in the NTHi-only challenged animals. MCP-1 mRNA expression profiles differed greatly between groups (figure 5.7). Expression remained low in the animals that received NTHi-only challenge until 12 h post–challenge after which time expression remained elevated.

After bacterial challenge a drop in MCP-1 mRNA expression was seen in the Sendai infected+NTHi challenged animals, followed by a significant increase at 8 h. Expression decreased rapidly after this time but remained slightly elevated at 24 h post-bacterial challenge.

A biphasic-type profile was found for IFN-γ mRNA expression in the Sendai infected+NTHi challenged animals with an initial peak at 2 h and a further peak at 12 h (figure 5.7). The later peak coincided with the single peak seen in the NTHi-only animals.

A slight increase in GM-CSF mRNA expression was detected following bacterial challenge in the NTHi-only group (figure 5.7). GM-CSF mRNA expression peaked prior to bacterial challenge in the Sendai infected+NTHi challenged animals. Directly after bacterial challenge the mRNA expression dropped rapidly but immediately began to increase again to a peak at 4 h. At 24 h mRNA expression remained elevated.

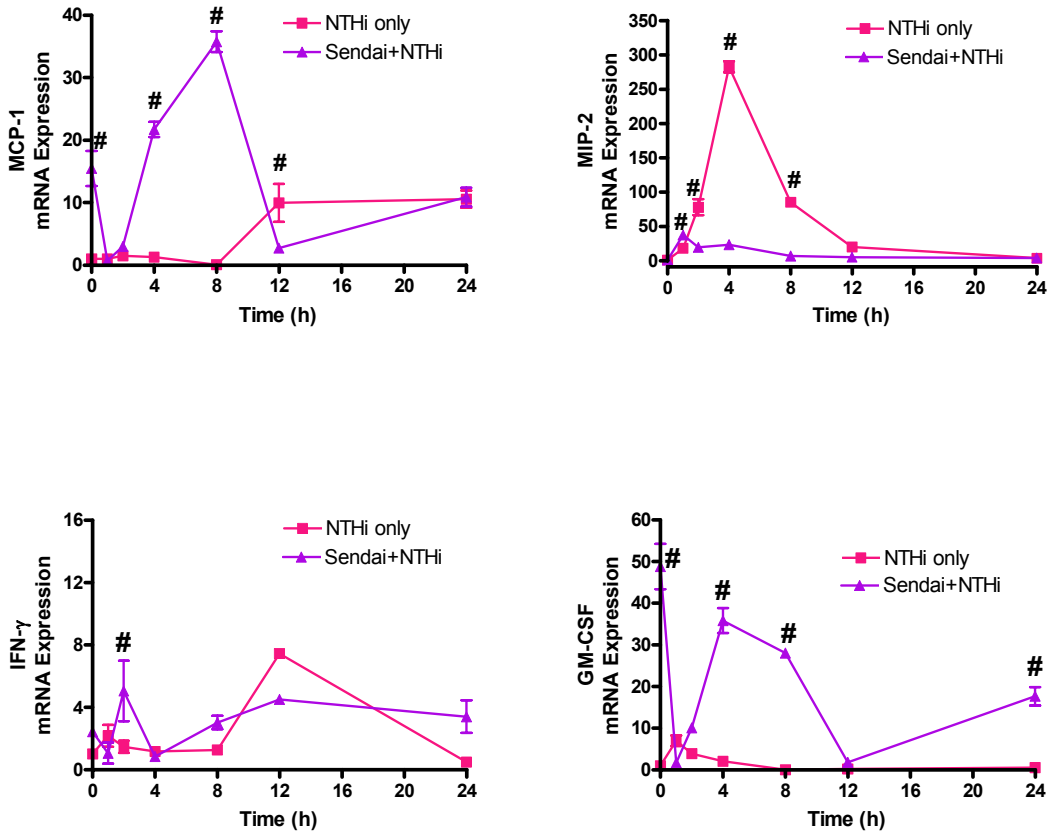

Figure 5.7 MCP-1, MIP-2, IFN-γ and GM-CSF mRNA expression were measured in the lung homogenate. Y-axis scales differ between individual graphs. mRNA expression was significantly different between Sendai+NTHi and NTHi only animals at 0, 4, 8 and 12 h for MCP-1, at 1, 2, 4 and 8 h for MIP-2, at 2 h for IFN-γ and at 0, 4, 8 and 24 h for GM-CSF (# $p < 0.05$). The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

5.4.4 IL-10 protein production decreases following bacterial challenge:

IL-10 was present in the BAL prior to bacterial challenge in the NTHi-only challenged animals whilst none was detected in the Sendai infected+NTHi challenged animals at any stage (figure 5.8). Immediately following challenge there was a decrease in IL-10 protein in the NTHi-only group and at no time were detectable levels measured again throughout the 24 h. In the lung homogenate there was measurable IL-10 just prior to bacterial challenge for both groups which was followed by an immediate decrease (figure 5.8). Following this initial decrease, IL-10 protein production increased to peak at 12 h. This level was still maintained at 24 h post-bacterial challenge in both groups.

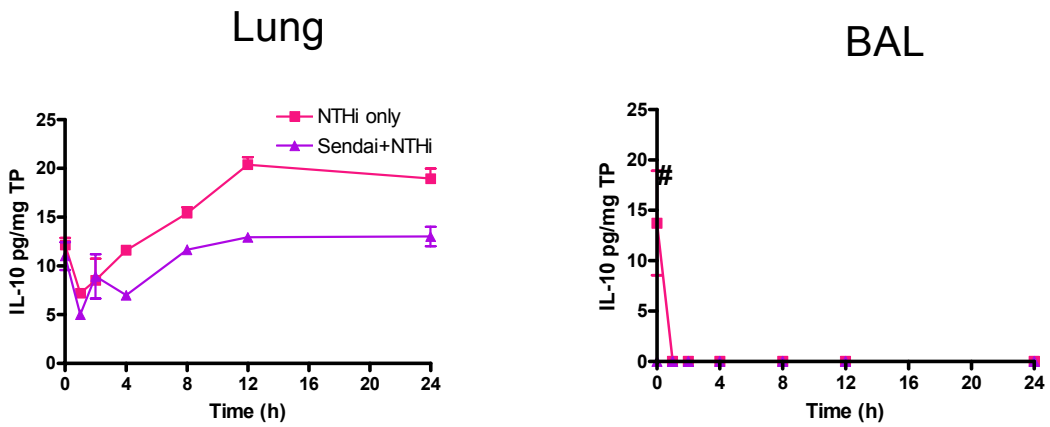

Figure 5.8 IL-10 protein concentration was measured in the lung homogenate and BAL recovered from animals that were challenged with NTHi only and animals that were infected with the Sendai virus from the time of bacterial challenge until 24 h post-challenge. Protein concentration has been expressed per mg of total protein. A significant difference in protein concentration between Sendai+NTHi and NTHi only animals was seen in the BAL prior to challenge (# $p < 0.05$). No significant difference was in seen protein production in the lung homogenate. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

5.4.5 Increase in pro-inflammatory cytokine production following bacterial challenge: Protein production of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α increased following bacterial challenge in both the Sendai infected+NTHi challenged and NTHi-only challenged animals (figures 5.9, 5.10 and 5.11). IL-1β protein production peaked at 8 h post-bacterial challenge in the lung homogenate and BAL in both groups however the kinetics of the profiles differed. In the lung homogenate IL-1β protein production increased more rapidly following bacterial challenge in the Sendai infected+NTHi challenged animals whilst in the BAL it was higher in NTHi-only challenged animals.

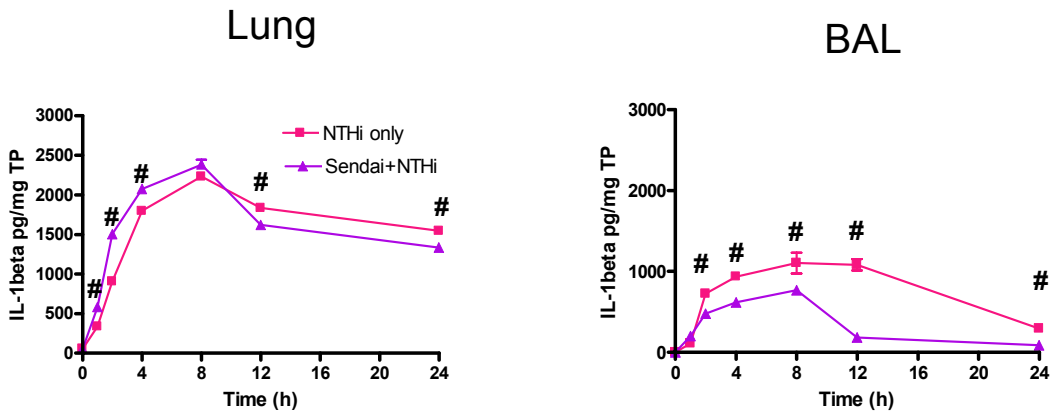

Figure 5.9 IL-1β protein concentration was measured in the lung homogenate and BAL recovered from animals that were challenged with NTHi only and animals that were infected with the Sendai virus from the time of bacterial challenge until 24 h post-challenge. Protein concentration has been expressed per mg of total protein. A significant difference in protein concentration between Sendai+NTHi and NTHi only animals was seen in lung homogenate at 1, 2, 4, 12 and 24 h post-challenge and at 2, 4, 8, 12 and 24 h in BAL (# $p < 0.05$. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

IL-6 and TNF-α protein production in the BAL increased more rapidly in the Sendai infected+NTHi challenged animals and also dropped more quickly following the peak (figures 5.10 and 5.11). The TNF-α peak was significantly lower in both the lung homogenate and BAL of these animals. There was no difference in IL-6 protein levels in the lungs of both groups (figure 5.10).

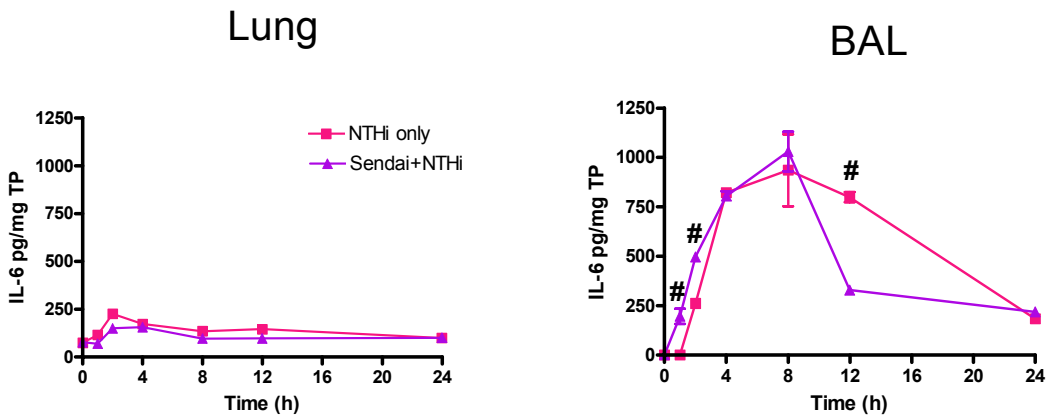

Figure 5.10 IL-6 protein concentration was measured in the lung homogenate and BAL recovered from animals that were challenged with NTHi only and animals that were infected with the Sendai virus from the time of bacterial challenge until 24 h post-challenge. Protein concentration has been expressed per mg of total protein. A significant difference in protein concentration between Sendai+NTHi and NTHi only animals was seen in the BAL at 1, 2, and 12 h post-challenge (# $p < 0.05$). The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

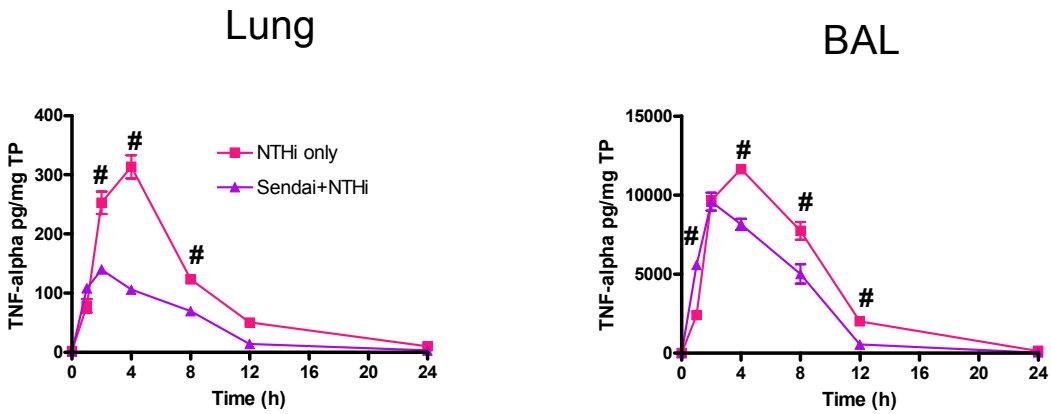

Figure 5.11 TNF-α protein concentration was measured in the lung homogenate and BAL recovered from animals that were challenged with NTHi only and animals that were infected with the Sendai virus from the time of bacterial challenge until 24 h post-challenge. Protein concentration has been expressed per mg of total protein. Y-axis scales differ between individual graphs. A significant difference in protein concentration between Sendai+NTHi and NTHi only animals was seen in the lung homogenate at 2, 4, and 8 h post-challenge and at 1, 4, 8, and 12 h in BAL (# $p < 0.05$). The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

5.4.6 Increased chemokine production in Sendai infected+NTHi challenged animals: Immediately following challenge with NTHi there was a greater increase in MCP-1 and MIP-2 protein concentration seen in the lung homogenate and BAL of the virally challenged animals (figures 5.12 and 5.13). This immediate difference at 1 h was the only difference seen between the groups in the lung homogenate. In both the lung homogenate and BAL, MCP-1 and MIP-2 peaked and returned to pre-bacterial-challenge concentrations more quickly in the Sendai infected+NTHi challenged animals. At 24 h there was still a significant amount of MCP-1 and MIP-2 protein remaining in the BAL of animals that received NTHi-only.

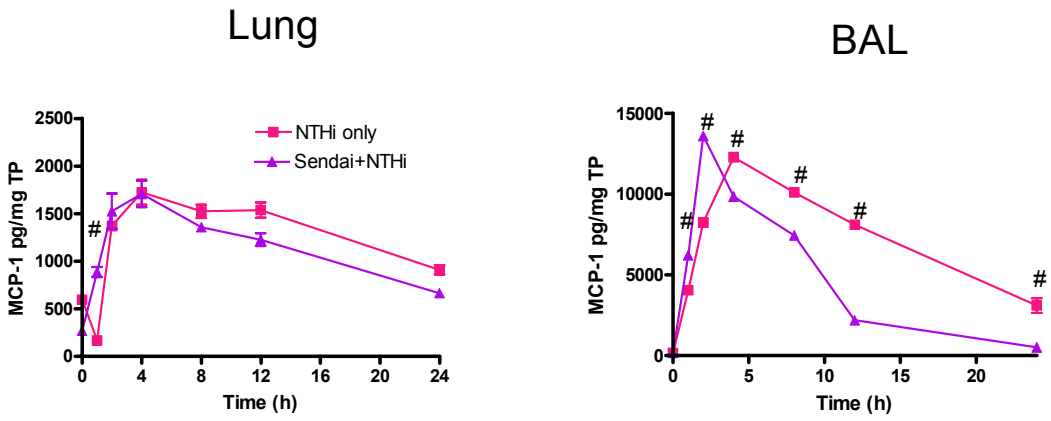

Figure 5.12 MCP-1 protein concentration was measured in the lung homogenate and BAL recovered from animals that were challenged with NTHi only and animals that were infected with the Sendai virus from the time of bacterial challenge until 24 h post-challenge. Protein concentration has been expressed per mg of total protein. Y-axis scales differ between individual graphs. A significant difference in protein concentration between Sendai+NTHi and NTHi only animals was seen in lung homogenate at 1 h post-challenge and at all time-points post-challenge in BAL (# $p < 0.05$). The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

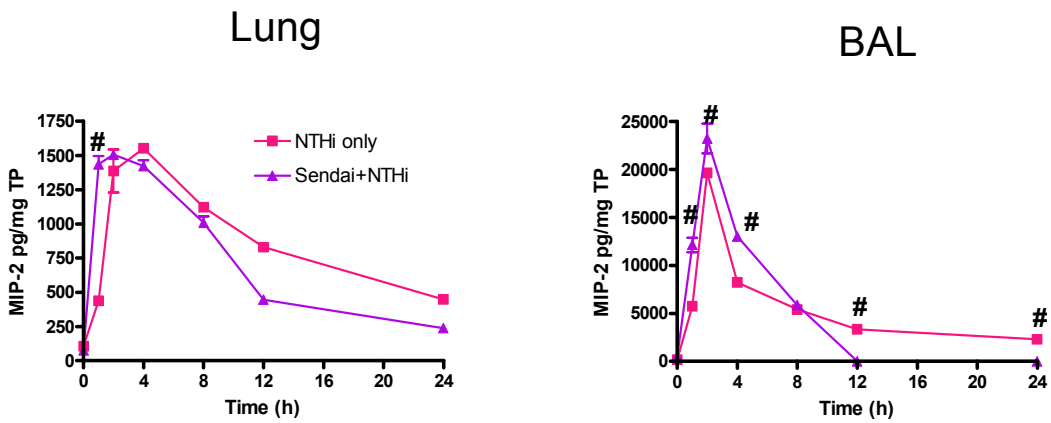

Figure 5.13 MIP-2 protein concentration was measured in the lung homogenate and BAL recovered from animals that were challenged with NTHi only and animals that were infected with the Sendai virus from the time of bacterial challenge until 24 h post-challenge. Protein concentration has been expressed per mg of total protein. Y-axis scales differ between individual graphs. A significant difference in protein concentration between Sendai+NTHi and NTHi only animals was seen in lung homogenate at 1 h post-challenge and at 1, 2, 4, 12 and 24 h in BAL (# $p < 0.05$. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

5.4.7 Lack of protein translation following NTHi challenge: No IFN-γ was detected in the lung homogenate or BAL of the Sendai infected+NTHi challenged animals at any time-point but was detected at low concentrations at 4 and 8 h in both sample types from the NTHi-only challenged animals (figure 5.14).

No GM-CSF protein was detected in either the lung homogenate or BAL at any time in either group of animals (data not shown). This contrasts with the mRNA levels in figure 5.7.

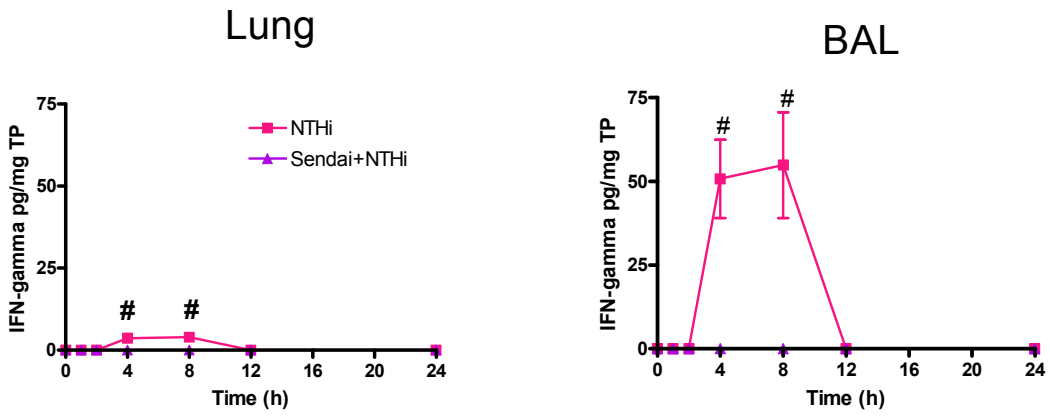

Figure 5.14 IFN-γ protein concentration was measured in the lung homogenate and BAL recovered from animals that were challenged with NTHi only and animals that were infected with the Sendai virus from the time of bacterial challenge until 24 h post-challenge. Protein concentration has been expressed per mg of total protein. A significant difference in protein concentration between Sendai+NTHi and NTHi only animals was seen in both the lung homogenate and BAL at 4 and 8 h post-challenge(# $p < 0.05$). mRNA expression was significantly different between Sendai+NTHi and NTHi only animals at 1 h in lung homogenate. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

5.5 DISCUSSION

Clearance of NTHi from the rat lung and alveolar spaces was found to be enhanced by an antecedent Sendai virus infection. This was not an expected result as viral infection has been shown to predispose the host to bacterial infection. The unexpected result still highlights differences in the host response when a viral infection is present with a bacterial infection. The model used was an acute infection model and not a colonisation model so the dynamics associated with the ability of NTHi to increasingly colonise the respiratory tract and descend into the lungs after a viral infection does not play a role in this model. NTHi is not a natural pathogen of the rat and therefore infection is cleared from the lung tissue by the innate immune response with relative ease following challenge in naïve animals.

Profiles of bacterial clearance from the lung tissue and alveolar spaces of the Sendai infected+NTHi challenged animals were almost identical to those seen in the immunised animals (Chapters 3 and 4). This provides evidence that the innate immune response had been primed by the viral infection and the end clearance results appear to mimic the immune animal. The way by which the viral infection affected the enhanced clearance of NTHi appeared to be through the recruitment and activation of phagocytic cells.

The reduced number of neutrophils recruited to the alveolar space in the Sendai infected+NTHi challenged animals in comparison with the immune animals in Chapter 3 indicated that enhanced clearance of NTHi may be due to the activation of these cells and not the number of cells present. The enhanced

clearance may have also been due to the increased number and activation of macrophages. This increased activation of neutrophils could be explained by the early increase in TNF-α and IL-6 proteins in the alveolar spaces as both of these cytokines act as potent activators of neutrophils.

Increased MCP-1 and MIP-2 protein in the alveolar spaces at 1 and 2 h in the Sendai infected+NTHi challenged animals corresponded with a peak in the number of macrophages recovered. This early increase in macrophages along with the activation of the neutrophils provides a possible explanation for the enhanced clearance in the Sendai infected+NTHi challenged animals. The way in which the viral infection indirectly influenced these phagocytic cells was not further investigated but was likely to be as a result of the normal innate response to the presence of the virus.

Regulation of neutrophil recruitment to the alveolar spaces appears to be under the influence of IL-1β. The kinetic profiles of IL-lβ secretion into the alveolar spaces directly corresponded with the number of neutrophils present in both the Sendai infected+NTHi challenged animals and the NTHi-only challenged animals. Whilst IL-1β is not a chemoattractant for neutrophils it is able to indirectly influence recruitment through up-regulation of adhesion molecules (Myers, Wertheimer et al. 1992). The primary source of IL-1β in the lung is the alveolar macrophage (Frick, Joseph et al. 2000) and increased mRNA expression in the lung tissue of the NTHi-only challenged animals was followed by increased protein being detected in the BAL of these animals. mRNA expression was not determined in the BAL in this chapter and further

investigation may reveal differences in the expression of IL-1β between the two groups.

The differing profiles between macrophage numbers for the immune animals in Chapter 3 and the Sendai infected+NTHi challenged animals in this chapter may be due to the immune systems regulation of these cells. In addition, there was, almost twice as many neutrophils present in the BAL of the immune animals compared with the Sendai infected+NTHi challenged animals. Macrophages not only clear bacteria but they are also able phagocytise apoptotic neutrophils (Savill, Wyllie et al. 1989). It can therefore be suggested that the difference in macrophage numbers between the Sendai infected+NTHi animals and the immune animals of Chapter 3 may be associated with the way the acquired immune response initiates effective resolution of apoptotic neutrophils. NTHi specific antibody present in the immune animals may also play a role in attracted different populations of phagocytes to the site of infection.

The decrease in MCP-1 protein detected following challenged with NTHi in the lungs of the NTHi-only challenged animals and the immune animals from Chapter 4 was not seen in the Sendai infected+NTHi challenged animals. In these animals there was an up-regulation of MCP-1 mRNA expression detected following viral infection prior to challenge. The production of MCP-1 protein is stimulated in respiratory epithelial cells (Clemans, Bauer et al. 2000) and alveolar macrophages (Huffnagle, Strieter et al. 1995) following exposure to bacteria. It was suggested in the previous chapter that the perceived decrease in MCP-1 production in the lung could be due to secretion of the protein into the alveolar space and while it is likely that the Sendai infected+NTHi challenged

animals also secreted the protein following NTHi challenge, the increased mRNA expression may mean that these animals were more capable of increasing protein production immediately.

An explanation for the differences in cytokine and chemokine production in the Sendai infected+NTHi challenged animals may lie with cells that were recruited to the lung tissue prior to bacterial infection. Following a respiratory viral infection CD8+ T-cells are recruited to the lung tissue (Ely, Cauley et al. 2003; Ely, Cookenham et al. 2006). The presence of these cells during the bacterial challenge may explain why the innate response seen in the Sendai infected+NTHi challenged animals was similar to that seen in the immune animals in the previous chapters as other studies in this laboratory have provided evidence that increased CD8+ T-cell recruitment to the lungs of mucosally immunised animals following challenge with NTHi was significant for the acquired immune response (Foxwell, Kyd et al. 2001).

In this animal model the antecedent viral infection did not predispose the animals challenged with a bacterial infection to enhanced disease, however the study has provided an indication of how an antecedent viral infection may change the host's normal response to infection. The increased number of bacteria present in both the lung tissue and alveolar space in the Sendai-infected+NTHi challenged animals immediately following challenge may indicate an increased ability to adhere to virally infected epithelial cells as has been previously suggested (Tong, Grants et al. 2002; Avadhanula, Rodriguez et al. 2006). Priming of the cells, through upregulation of mRNA, in the Sendai infected+NTHi challenged animals was the likely mechanism by which these

animals were able to overcome any increased ability to colonise. The timing of the bacterial challenge may also have affected the host's response. 3 days post viral infection was chosen as the time of challenge as this has previously been shown to be the time at which the viral load was the greatest (Moore, Lidbury et al. 2001). Differing host results may be achieved by allowing the host to recover from the viral infection before the NTHi challenge.

This study has shown that an antecedent viral infection does not decrease the ability to clear NTHi from the rat lung and that there was no apparent suppression of the innate immune response by the virus. It is more likely that the mechanisms by which an antecedent viral infection may predisposes the host to bacterial infection is through increased colonisation or compromising the susceptible host through the observed differences in the innate immune responses at another stage of the viral infection cycle. So while the viral infection in this model resulted in effective clearance of the bacterial infection, different models of colonisation and infection may result in different outcomes for the likelihood of developing a secondary bacterial infection.

CYTOKINE AND CHEMOKINE PRODUCTION IN RESPONSE TO NONTYPEABLE *HAEMOPHILUS INFLUENZAE* LOS ASSOCIATED ChoP

CHAPTER SIX

6.1 ABSTRACT

The immune responses to different strains of NTHi and LOS associated ChoP from NTHi were determined in relation to bacterial clearance, cellular recruitment and the production of cytokines and chemokines in the lung. Enhanced clearance of bacteria was seen at 2 h in animals challenged with a ChoP deficient mutant strain of NTHi in comparison with the wild-type strain, NTHi 2019. Less neutrophils and macrophages were present on the BAL of animals challenged with the ChoP$^-$ mutant strain. An increase in the production of pro-inflammatory cytokines and chemokines in the BAL was associated with the presence of ChoP. MCP-1 protein was undetectable in animals that had been challenged with the ChoP$^-$ mutant strain, indicating a role for ChoP in the pathway that leads to production of MCP-1. The production of TNF-α was significantly increased in the BAL of animals challenge with wild-type NTHi 289 in comparison with wild-type NTHi 2019 but this increased production did not lead to increased recruitment of phagocytes to the lung. These results have highlighted variations in the inflammatory response to different strains of NTHi and the potential role of ChoP in the pathogenesis of NTHi.

6.2 INTRODUCTION

The pathogenesis of NTHi can be attributed, in part, to the interaction of LOS, a component of the outer membrane, with the host. The response to LOS is similar to the response seen to the LPS of other Gram-negative bacteria. In the initial stages of infection with NTHi the LOS is involved in stimulating the production of cytokines and chemokines from cells in the respiratory tract, including epithelial cells (Clemans, Bauer et al. 2000) and alveolar macrophages (Berenson, Murphy et al. 2005). LPS has been found to be the major stimulator of TNF-α release from macrophages (Cui, Morrison et al. 2000) and correlations between the presence of LPS and TNF-α and IL-1β levels have been found in the middle ear fluid from children with otitis media (Willet, Rezaee et al. 1998).

NTHi mutants with LOS gene disruption have been used to show that the way in which LOS contributes to pathogenesis is not necessarily just through increasing colonisation but more due to influencing the ability of the bacteria to induce inflammation (DeMaria, Apicella et al. 1997). The LOS structure is a highly variable assortment of short oligosaccharide chains branching from the lipid A core region. Within the branch structure, NTHi LOS expresses host carbohydrate structures such as ChoP (Rahman, Gu et al. 1999; Risberg, Alvelius et al. 1999). ChoP expression is determined in the *lic1* operon (Weiser, Love et al. 1989) and it is expressed as a terminal structure on the oligosaccharide (Lysenko, Richards et al. 2000). Using LOS gene disruption tools a decrease in the colonisation of human epithelial cells was observed after disruption of LOS biosynthesis (Swords, Buscher et al. 2000).

Tong and coworkers demonstrated that incubation of human middle ear epithelial cells with an NTHi strain containing a disruption to the LOS gene induced reduced production of IL-6, IL-8 and MCP-1 protein in comparison to the wild-type strain (Tong, Chen et al. 2001). When all these studies are taken into account it appears that the pathogenic characteristics of LOS are due to its ability to not only influence colonisation but to also stimulate the production of pro-inflammatory cytokines and chemokines.

ChoP is a component of LOS that is capable of mimicking host structures in order to increase the bacteria's ability to colonise (Mandrell and Apicella 1993; Weiser, Shchepetov et al. 1997; Tong, Blue et al. 2000). This was evident in a study which showed that $ChoP^-$ mutant strains of NTHi were cleared from the rat nasopharynx more quickly than $ChoP^+$ strains (Weiser, Pan et al. 1998). The way in which ChoP is thought to aid colonisation is through binding of the PAF receptor on epithelial cells (Swords, Buscher et al. 2000). PAF receptors can couple with G-protein complexes and therefore activation of PAF receptors initiate host cell signals (Shukla 1992). This pathway is not the only mechanism by which NTHi affects the activity of cells. Host TLR-2 plays an important role in activating host inflammatory and immune responses and NTHi has been shown to activate NFκB via the TAK-1-dependant NIK-IKKα/β-IκBα and MKK3/6-p38 MAP kinase signaling pathways (Shuto, Xu et al. 2001). This activation has recently been shown to be involved in MCP-1 regulation (Moon, Woo et al. 2007). NTHi also activates TLR-4 on cells such as macrophages through its LOS moieties, initiating host inflammatory responses (Ahrén, Bjartell et al. 2001). In general though, it is believed that NTHi avoids TLR-4 activation

through the incorporation of host-derived sialic acid into the LOS structure (Bouchet, Hood et al. 2003). Other structures on the surface of NTHi have also been shown to selectively induce host inflammatory responses, for example, P6 was shown to be a key effector for IL-8 and TNF-α induction in human macrophages (Berenson, Murphy et al. 2005).

This investigation aimed to examine what effect the presence/absence of ChoP had on the production of cytokines and chemokines in the lung in an animal model of infection with NTHi. Based on the results obtained from previous chapters and the documented effects of LOS, it was determined that two hours post-challenge with NTHi would be the most suitable time-point to assess differences in the host response as this was when many of the most significant increases in cytokine and chemokine production were observed and, in part, hypothesised to be in response to the effects of LOS.

6.3 MATERIALS AND METHODS

6.3.1 Bacteria: NTHi 289, NTHi 2019 and NTHi 2019 *licD::kan* were prepared for challenge as detailed in section 2.1. NTHi 289 and NTHi 2019 are both wild-type strains of NTHi. NTHi 2019 *licD::kan* is a mutant strain that expresses a negative ChoP glycoform. All challenges were performed using $5x10^8$ CFU of live bacteria.

6.3.2 Animal strain challenge: SPF DA male rats aged between 8 and 10 weeks were used. They were immunised and challenged with either NTHi or

PBS following the protocol detailed in sections 2.2.1, 2.2.2, 2.3.1-2.3.3 and 2.2.4.1-2.2.4.4. Samples were taken for analysis at 2 h post-challenge.

6.3.3 Cytokine and Chemokine mRNA expression: Real-time RT-PCR was used to determine IL10, TNF-α, IFN-γ, MCP-1, MIP-2, IL-1β, IL-6 and GM-CSF mRNA expression in lung homogenate and BAL as detailed in sections 2.4.1 and 2.4.3.

6.3.4 Determination of cytokine and chemokine concentration: IL10, TNF-α, IFN-γ, MCP-1, MIP-2, IL-1β, IL-6 and GM-CSF protein concentrations were measured in lung homogenate and BAL using commercially available ELISA kits as specified in section 2.5.

6.3.5 Determination of total protein concentration: Total protein concentration was measured in the lung homogenate and BAL using the method described in section 2.5.3.

6.3.6 Statistical analysis: The data has been expressed as the means ± SEM. The data was compared for statistical significance by ANOVA with Bonferroni posttests (GraphPad Prism).

6.4 RESULTS

6.4.1 Bacteria in the lung homogenate and BAL: Although all three groups were initially challenged with equal numbers of the corresponding NTHi strain, different numbers of bacteria were recovered in both the lung homogenate and BAL at 2 h post-challenge (figure 6.1). There was significantly less NTHi isolated from both the lung homogenate and BAL of animals challenged with NTHi 2019 *licD::kan* when compared with the wild-type NTHi 2019. Significantly more NTHi 289 was isolated in both the lung and BAL in comparison to NTHi 2019.

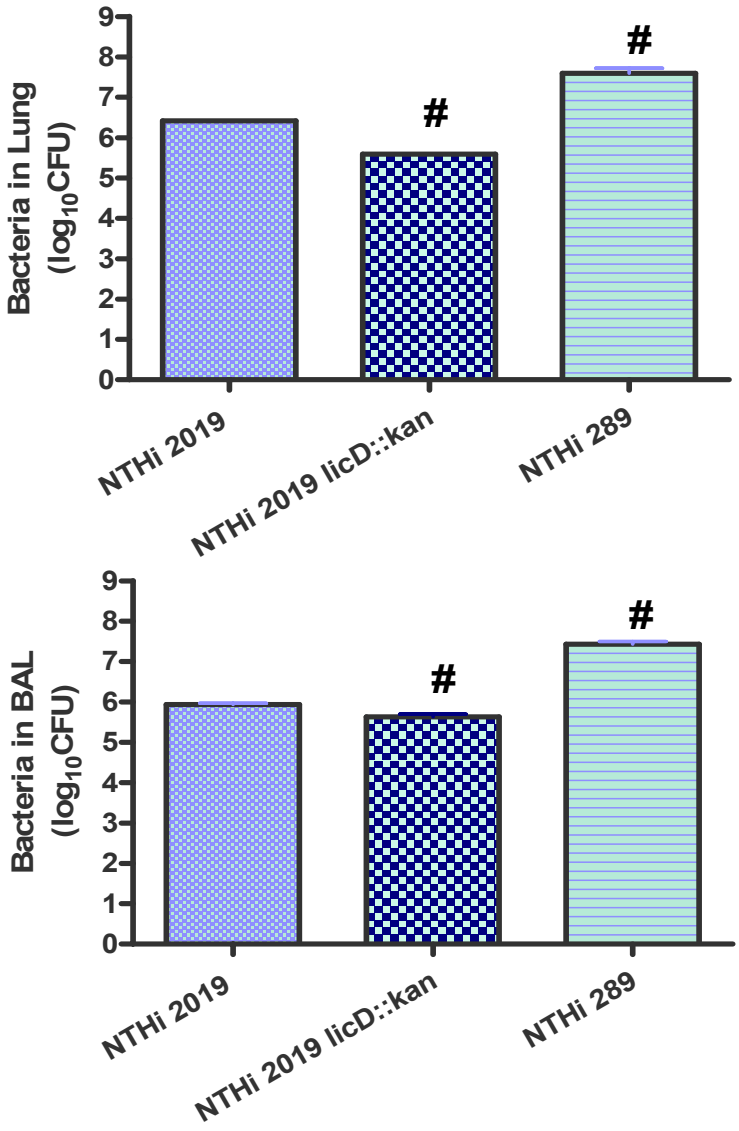

Figure 6.1 Recovery of NTHi from the lung homogenate and BAL of rats challenged with either NTHi 2019, NTHi 2019 *licD::kan* or NTHi 289 2 h post-challenge. Each animal was challenged with $5x10^8$ CFU of live organism. Significant difference from NTHi 2019 is indicated by # for $p< 0.05$. NTHi 2019 *licD::kan* and NTHi 289 bacteria numbers were significantly different from NTHi 2019 in the lung homogenate and BAL. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

6.4.2 Phagocytic cells isolated from the BAL: At 2 h post-challenge significantly fewer phagocytes were isolated from the BAL of animals challenged with the mutant strain of NTHi 2019 when compared with the wild-type strain (figure 6.2). When the types of phagocytes were differentiated it was also found that there were significantly less macrophages and PMNs isolated from animals challenged with the mutant strain. Whilst the difference in total phagocytic cell numbers between NTHi 2019 and NTHi 289 did not reach statistical significance differences were noted when the cells were differentiated. There were significantly less macrophages and PMNs in animals challenged with NTHi 289.

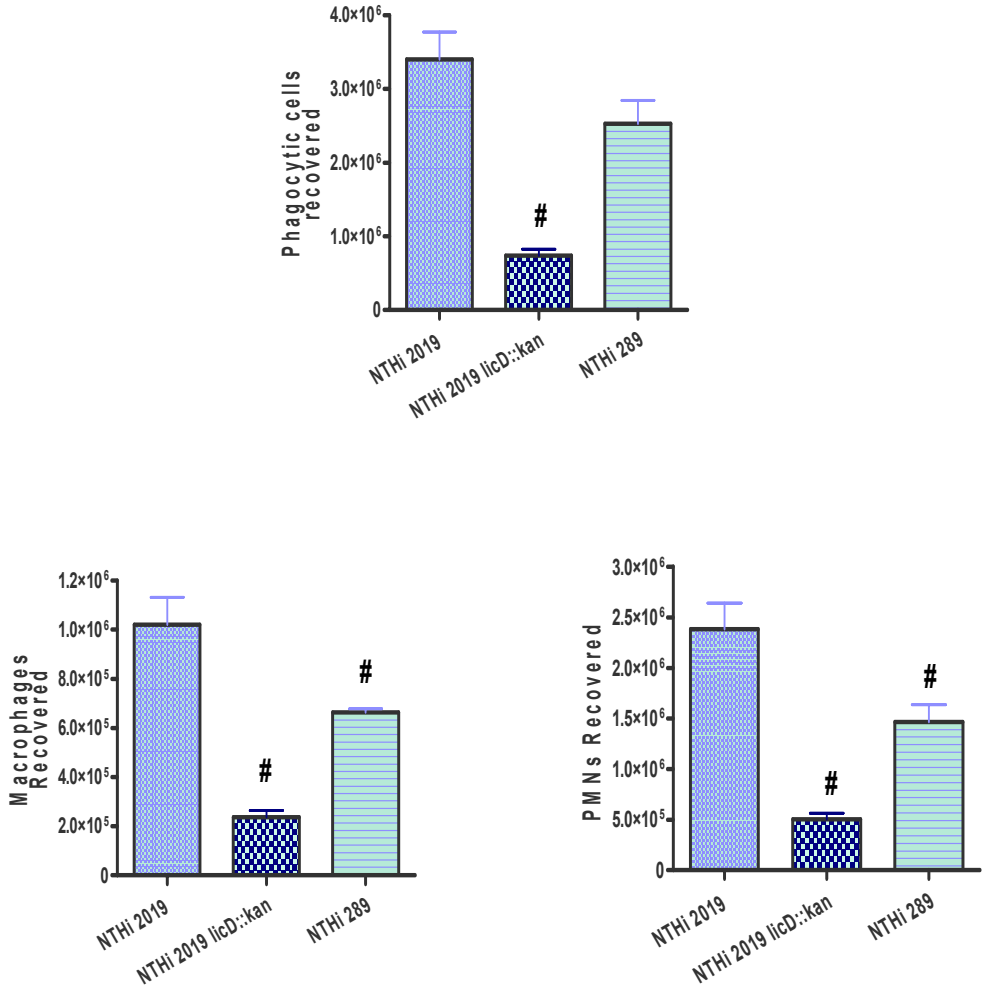

Figure 6.2 Total WBCs, PMNs and macrophages isolated from the BAL of rats challenged with either NTHi 2019, NTHi 2019 *licD::kan* or NTHi 289 2 h post-challenge. Each animal was challenged with $5x10^8$ CFU of live organism. Significant difference from NTHi 2019 is indicated by # for $p< 0.05$. Significantly less PMNs and macrophages were isolated from the BAL of animals challenged with both NTHi 2019 *licD::kan* and NTHi 289. Only NTHi 2019 *licD::kan* total WBC numbers differed significantly from NTHi 2019. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

6.4.3 Pro-inflammatory cytokine production after NTHi challenge: IL-1β, IL-6 and TNF-α protein production in the BAL was significantly decreased in the animals challenged with NTHi 2019 *licD::kan* 2 h post-challenge in comparison the wild-type NTHi 2019 whereas only IL-1β was decreased only in the lung homogenate (figures 6.3, 6.4 and 6.5). TNF-α mRNA expression was found to be lower in the BAL of animals challenged with the mutant strain of NTHi but not in the lung homogenate whilst IL-6 mRNA expression was up-regulated in the lung tissue of these animals.

Differences were also seen in proinflammatory cytokine production between the NTHi wild-type groups (figures 6.3, 6.4 and 6.5). Increases in IL-1β and IL-6 protein in the BAL were seen in the animals challenged with NTHi 289 and increased TNF-α was detected in the animals challenged with NTHi 2019. In the lung homogenate only IL-1β differed between the groups with a higher concentration being detected in the NTHi 2019 group. No differences in mRNA expression were detected in the lung tissue but in the alveolar spaces mRNA expression was increased for IL-1β in the NTHi 289 animals and for TNF-α in the NTHi 2019 animals.

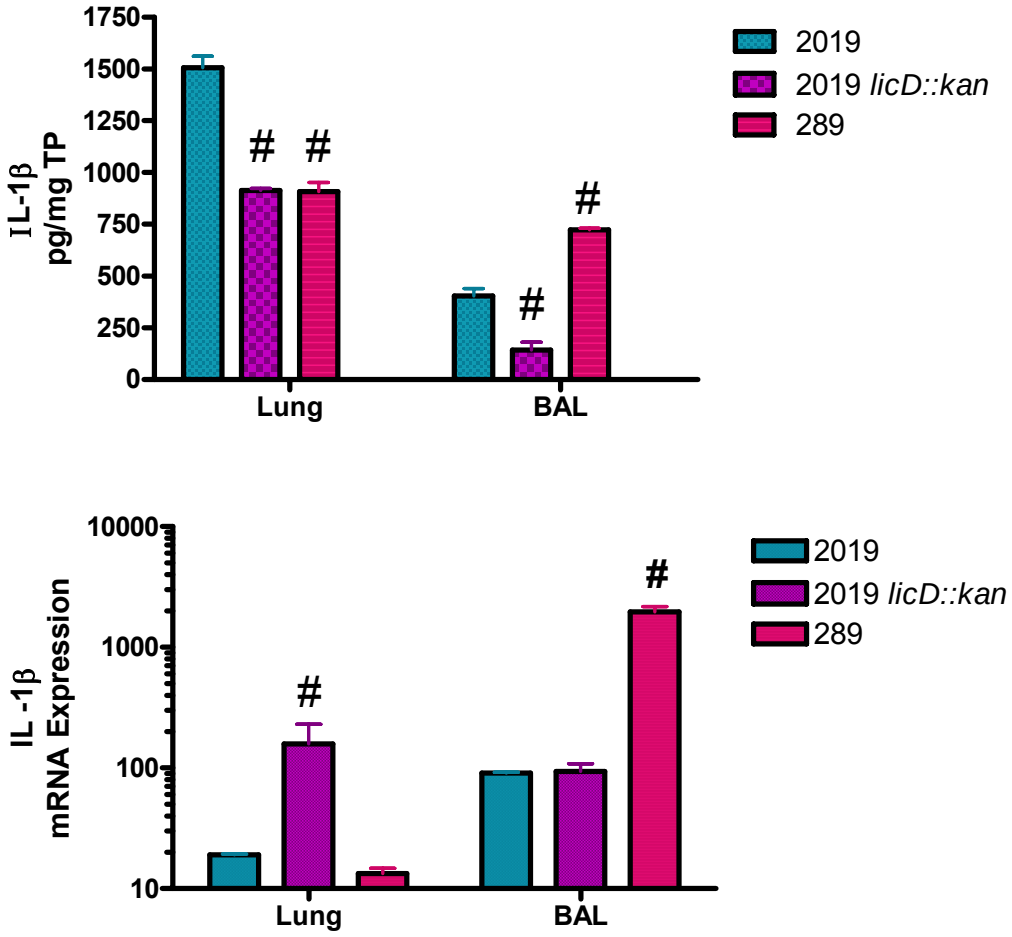

Figure 6.3 IL-1β protein concentration and mRNA expression in the lung homogenate and BAL recovered from rats challenged with NTHi 2019, NTHi 2019 *licD::kan* or NTHi 289 2 h post-challenge. Each animal was challenged with $5x10^8$ CFU of live organism. Protein concentration has been expressed per mg of total protein. Significant difference from NTHi 2019 is indicated by # for $p< 0.05$. Significantly less IL-1β protein was detected in the lung homogenate of both the 2019 *licD::kan* and NTHi 289 groups and in the BAL of 2019 *licD::kan* animals. Significantly more protein was detected in the BAL of animals challenged with NTHi 289. mRNA expression was significantly higher in the lung of 2019 *licD::kan* animals and in the BAL from NTHi 289 animals. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

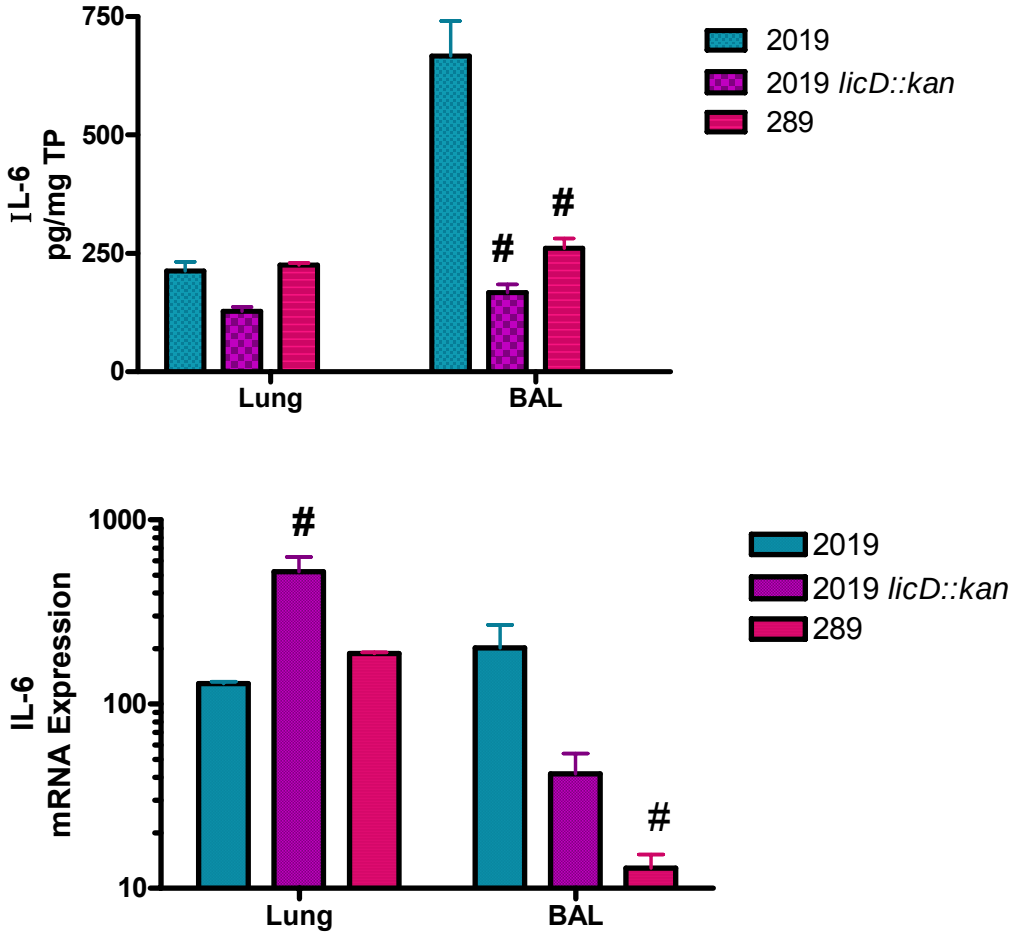

Figure 6.4 IL-6 protein concentration and mRNA expression in the lung homogenate and BAL recovered from rats challenged with NTHi 2019, NTHi 2019 *licD::kan* or NTHi 289 2 h post-challenge. Each animal was challenged with $5x10^8$ CFU of live organism. Protein concentration has been expressed per mg of total protein. Significant difference from NTHi 2019 is indicated by # for $p< 0.05$. Significantly less IL-6 protein was detected in the BAL of both the 2019 *licD::kan* and NTHi 289 groups. No significant difference was seen in protein production in the lung homogenate. Animals challenged with 2019 *licD::kan* had significantly higher IL-6 mRNA expression. mRNA expression was significantly lower in the BAL from NTHi 289 animals. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

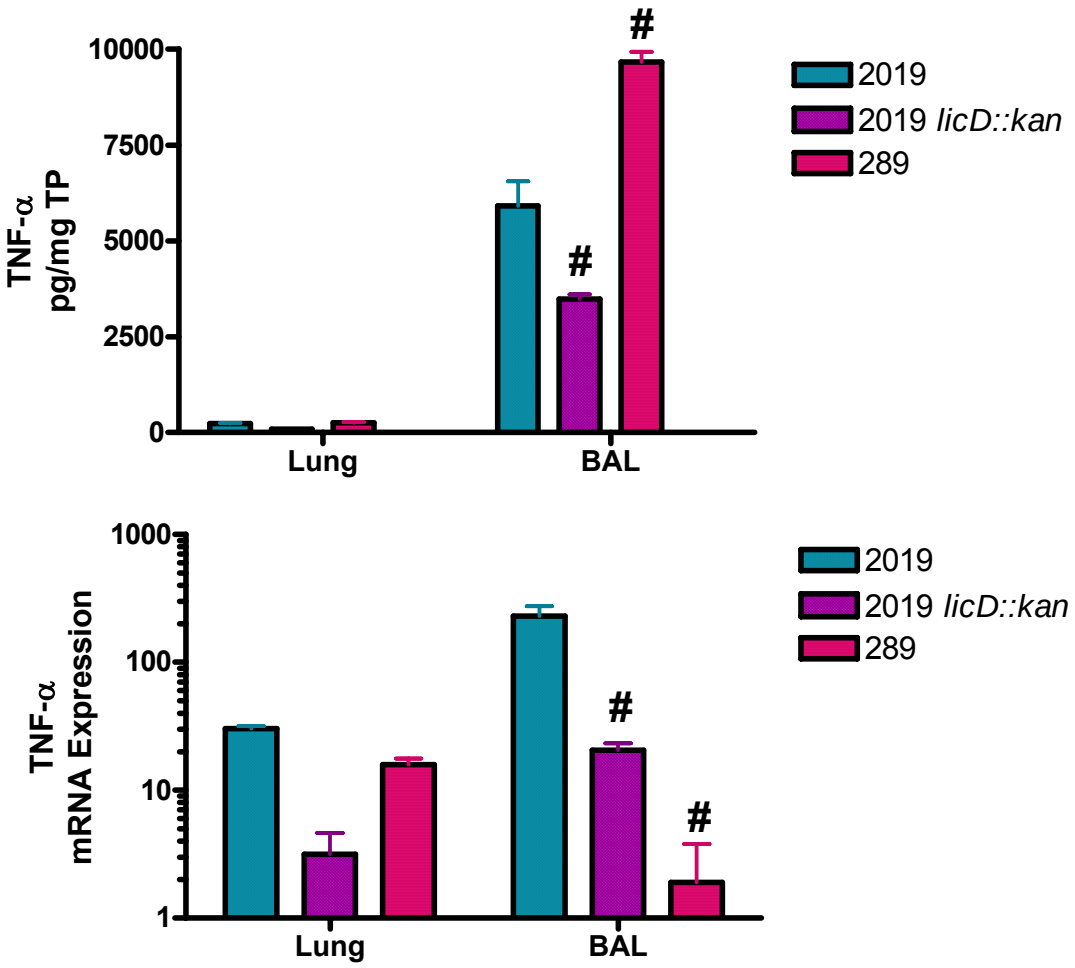

Figure 6.5 TNF-α protein concentration and mRNA expression in the lung homogenate and BAL recovered from rats challenged with NTHi 2019, NTHi 2019 *licD::kan* or NTHi 289 2 h post-challenge. Each animal was challenged with $5x10^8$ CFU of live organism. Protein concentration has been expressed per mg of total protein. Significant difference from NTHi 2019 is indicated by # for $p< 0.05$. Significant differences were seen in the production of TNF-α protein in the BAL of both groups. No significant differences were seen in protein in the lung homogenate. mRNA expression was significantly lower in the BAL of animals challenged with NTHi 289 and NTHi 2019 *licD::kan* compared with NTHi 2019. No significant differences were detected in mRNA expression in the lung homogenate. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

6.4.4 IFN-γ and GM-CSF protein was not detected: 2 h after challenge with NTHi, neither IFN-γ nor GM-CSF protein was detected in the lung homogenate or BAL in any of the groups that were challenge with either of the wild-type strains or the mutant strain (data not shown). GM-CSF mRNA expression differed between the groups in the lung homogenate with significant upregulation seen in the animals challenged with the wild-type 2019 strain when compared with the mutant strain (figure 6.6). IFN-γ mRNA expression was significantly higher in the wild-type NTHi 2019 group than both the other groups (figure 6.7). The NTHi 289 levels of expression were similar to or lower than those observed for the mutant strain.

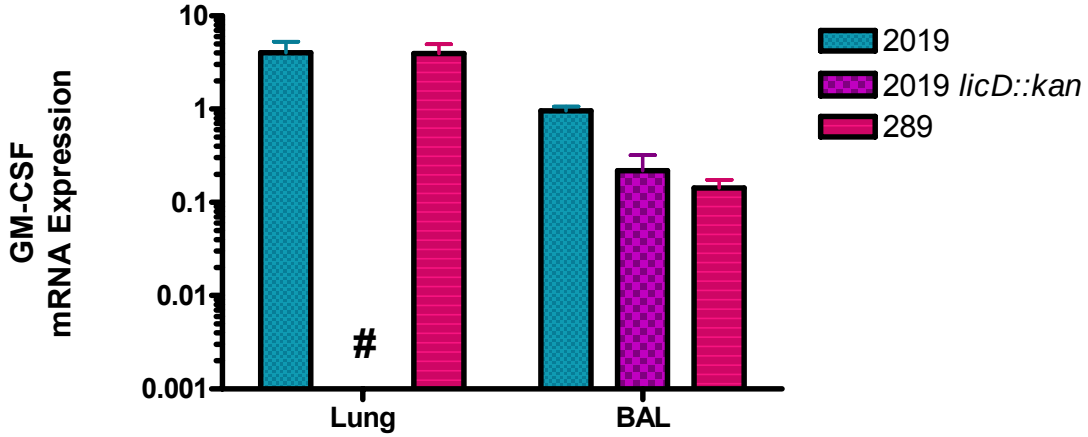

Figure 6.6 GM-CSF protein concentration and mRNA expression in the lung homogenate and BAL recovered from rats challenged with NTHi 2019, NTHi 2019 *licD::kan* or NTHi 289 2 h post-challenge. Each animal was challenged with $5x10^8$ CFU of live organism. No GM-CSF protein was detected in any sample. Significant difference from NTHi 2019 is indicated by # for $p< 0.05$. No significant differences were detected in mRNA expression in the BAL. Significantly lower mRNA expression was seen in the lung homogenate of the group challenged with NTHi 2019 *licD::kan*. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

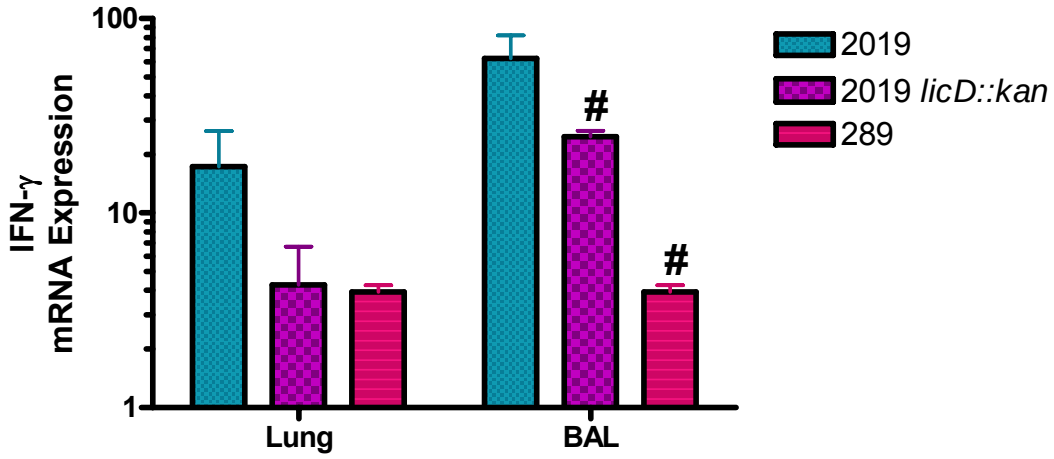

Figure 6.7 IFN-γ protein concentration and mRNA expression in the lung homogenate and BAL recovered from rats challenged with NTHi 2019, NTHi 2019 *licD::kan* or NTHi 289 2 h post-challenge. Each animal was challenged with $5x10^8$ CFU of live organism. No IFN-γ protein was detected in any sample. Significant difference from NTHi 2019 is indicated by # for $p< 0.05$. No significant differences were detected in mRNA expression in the lung homogenate. Significantly lower mRNA expression was seen in the BAL of both NTHi 2019 *licD::kan* and NTHi 289. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

6.4.5 Effect of ChoP mutation on chemokine production:

Concentrations of MIP-2 and MCP-1 protein in the BAL were lower in the animals that had been challenged with NTHi 2019 *licD::kan* in comparison with the animals challenged with the wild-type NTHi 2019 (figures 6.8 and 6.9). MCP-1 mRNA expression was also lower in the BAL from the animals challenged with the mutant NTHi strain but this was not seen in MIP-2 mRNA expression. In the lung tissue no differences in chemokine mRNA expression were determined between the wild-type and mutants strains of NTHi 2019 however MIP-2 was significantly higher for NTHi 289. IN comparison with animals challenged with NTHi 2019, MCP-1 protein production in the lung tissue was significantly higher in animals challenged with NTHi 289 but lower in those challenged with NTHi 2019 *licD::kan.*

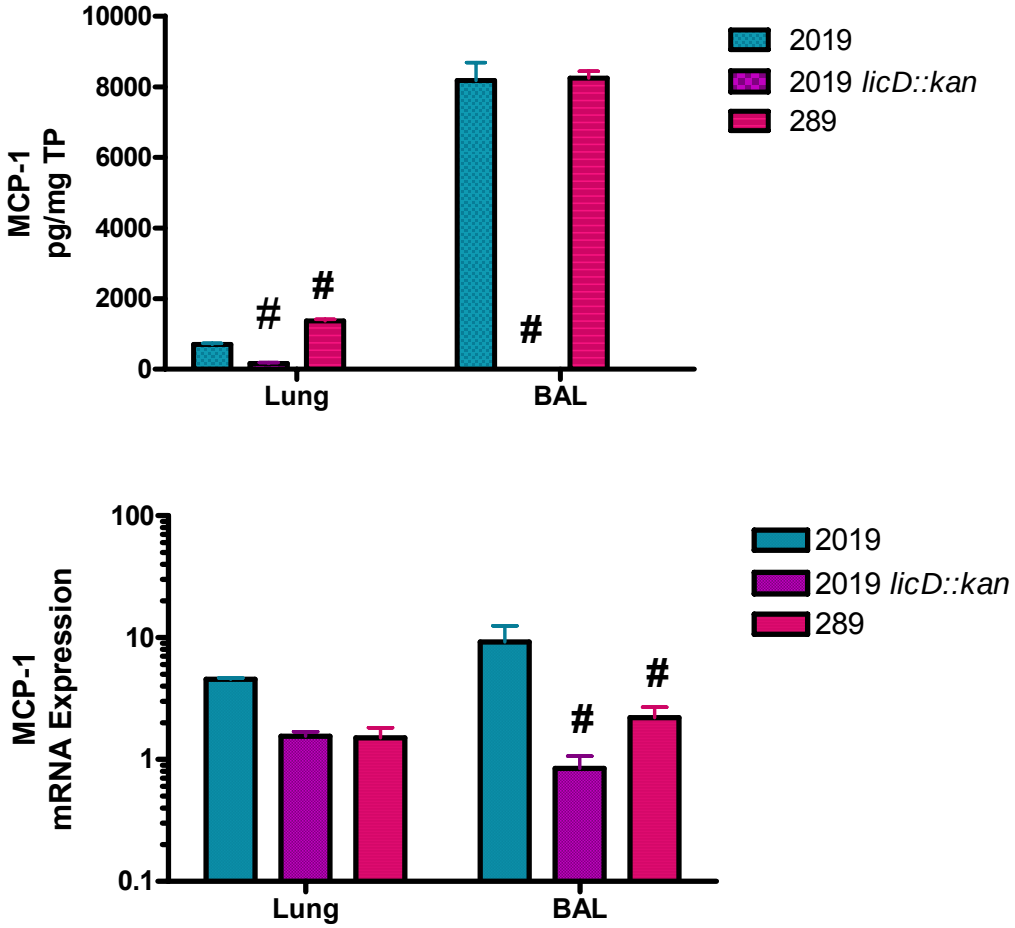

Figure 6.8 MCP-1 protein concentration and mRNA expression in the lung homogenate and BAL recovered from rats challenged with NTHi 2019, NTHi 2019 *licD::kan* or NTHi 289 2 h post-challenge. Each animal was challenged with $5x10^8$ CFU of live organism. Protein concentration has been expressed per mg of total protein. Significant difference from NTHi 2019 is indicated by # for $p< 0.05$. Significantly more MCP-1 protein was detected in the lung homogenate of the group challenged with NTHi 289. Significantly less MCP-1 protein was detected in the lung homogenate from the NTHi 2019 *licD::kan* group. No MCP-1 protein was detected in the BAL of the 2019 *licD::kan* group, this was statistically significant. Animals challenged with 2019 *licD::kan* and NTHi 289 had significantly lower MCP-1 mRNA expression. No significant differences were detected in mRNA expression in the lung homogenate. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

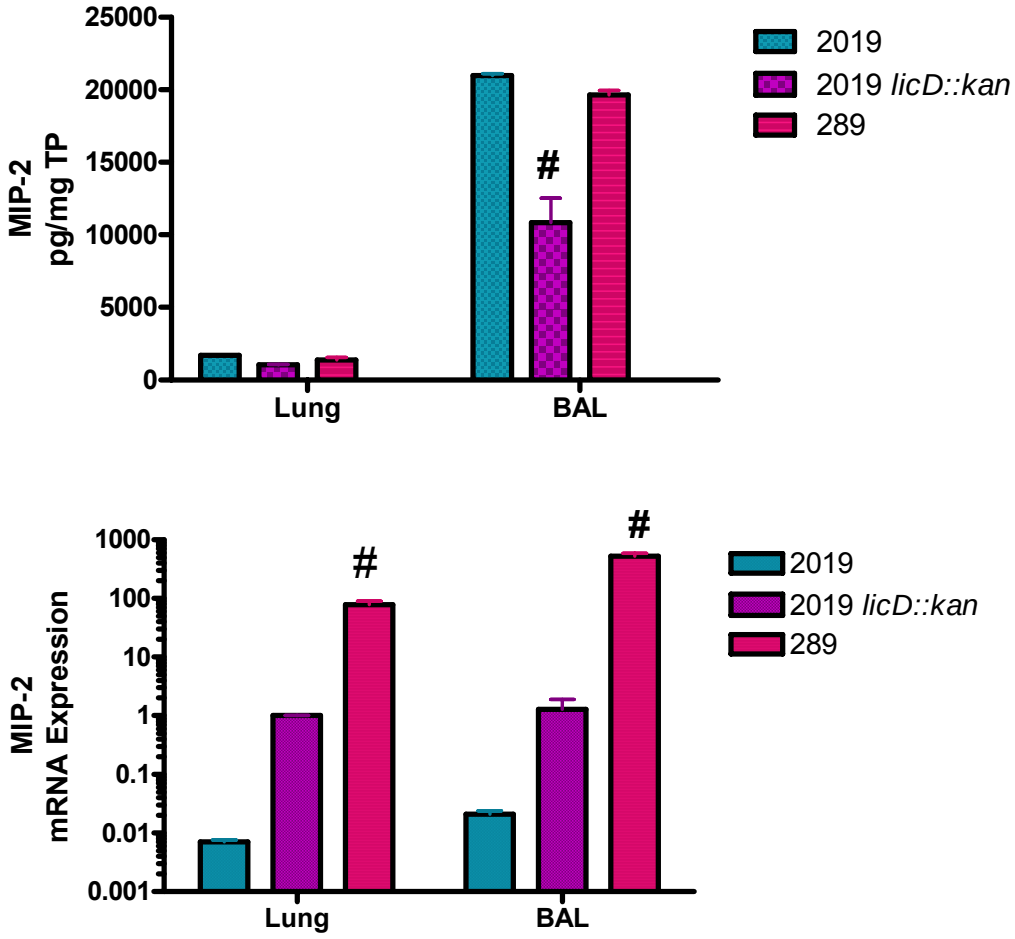

Figure 6.9 MIP-2 protein concentration and mRNA expression in the lung homogenate and BAL recovered from rats challenged with NTHi 2019, NTHi 2019 *licD::kan* or NTHi 289 2 h post-challenge. Each animal was challenged with $5x10^8$ CFU of live organism. Protein concentration has been expressed per mg of total protein. Significant difference from NTHi 2019 is indicated by # for $p< 0.05$. Significantly less MIP-2 protein was detected in the BAL of the group challenged with NTHi 2019 *licD::kan*. No significant differences were seen in protein production in the lung homogenate. mRNA expression was higher in the BAL and lung homogenate from animals challenged with NTHi 289. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

Comparisons between the two different wild-type strains, NTHi 289 and NTHi 2019 (figures 6.8 and 6.9) showed that MCP-1 mRNA expression in the BAL was lower in the animals challenged with NTHi 289 but the amount of protein produced did not differ between the two groups. MCP-1 protein production in the lung tissue was higher in animals challenged with NTHI 289. In these animals there was an increase in MCP-1 protein production in the lung tissue. The only difference for MIP-2 between the two wild-type challenged groups was in mRNA expression in the BAL, where expression was higher in the animals challenged with NTHi 289. The level of protein in the BAL was equivalent between the two wild-type groups and contrasts with the difference observed in the number of phagocytes recruited to the lungs at this time-point (figure 6.2).

6.4.6 IL-10 production differs in lung tissue and BAL: IL-10 protein was not detected in any of the BAL samples 2 h post-challenge with either NTHi 289, NTHi 2019 or NTHi 2019 *licD::kan* but was detected in all of the lung homogenate samples (figure 6.10). Protein production was significantly higher in animals challenged with the mutant strain of NTHi 2019 compared with the wild-type and also appeared to be higher in the NTHi 289 challenged group. mRNA expression was also up-regulated in the lung homogenate samples taken from these animals. The only difference detected between the groups challenged with either of the wild-type strains of NTHi was that IL-10 mRNA expression was lower in the BAL from the animals challenged with NTHi 289.

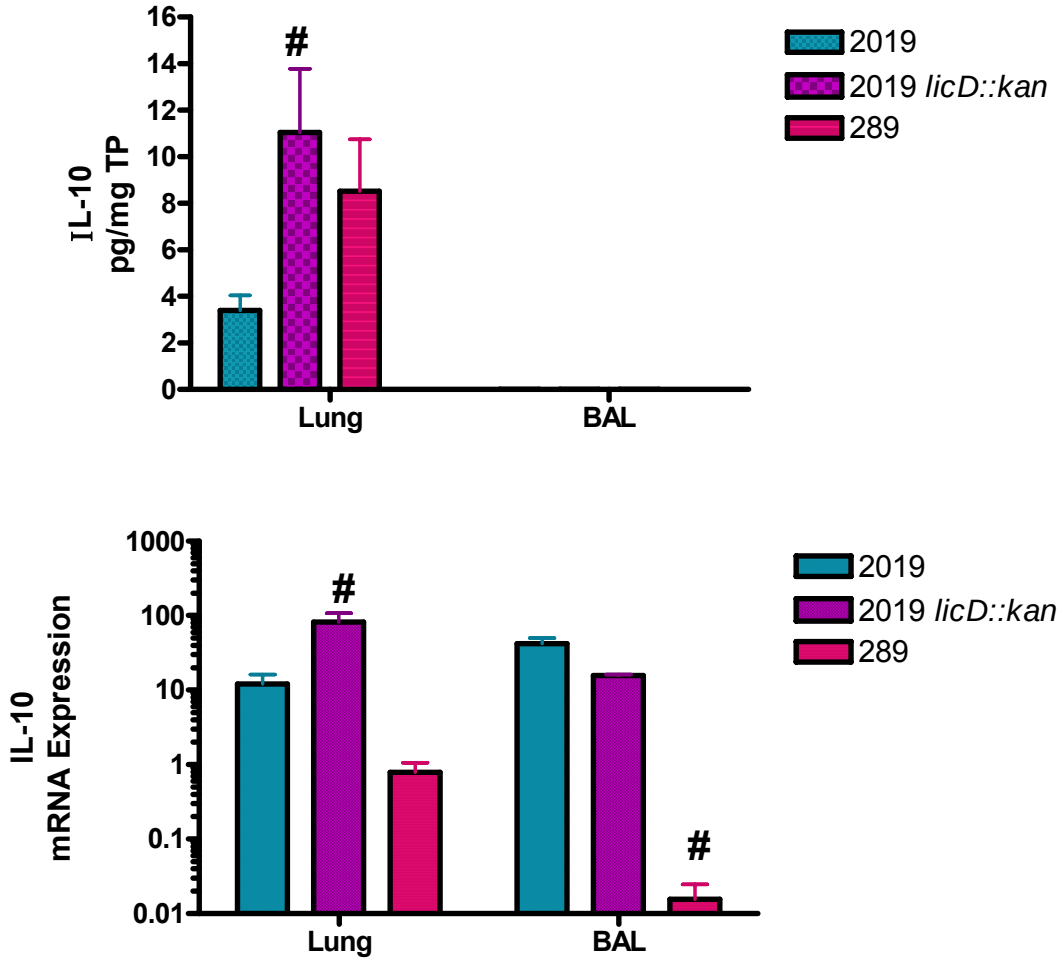

Figure 6.10 IL-10 protein concentration and mRNA expression in the lung homogenate and BAL recovered from rats challenged with NTHi 2019, NTHi 2019 *licD::kan* or NTHi 289 2 h post-challenge. Each animal was challenged with $5x10^8$ CFU of live organism. Protein concentration has been expressed per mg of total protein. No IL-10 protein was detected in the BAL of any group. Significant difference from NTHi 2019 is indicated by # for $p< 0.05$. Significantly more IL-10 was detected in the lung homogenate of animals challenged with 2019 *licD::kan*. Significantly more protein was detected in the BAL of animals challenged with NTHi 289. mRNA expression was significantly higher in the lung homogenate of animals challenged with NTHi 2019 *licD::kan* and significantly lower in the BAL of NTHi 289 animals. The values represent the mean ± SEM for n=4 rats. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

6.5 DISCUSSION

In order to determine the contribution of the NTHi LOS to the production of cytokine and chemokine responses in the lung observed in the previous chapters, a ChoP$^-$ mutant strain was studied. ChoP is a component of LOS that mimics the structure of host molecules in order to colonise and invade cells and to avoid the host's innate immune response (Mandrell and Apicella 1993; Weiser, Shchepetov et al. 1997; Weiser, Pan et al. 1998). Its presence on LOS structures is phase-variable and so strains can be considered differently according to their category of ChoP variance (Weiser, Shchepetov et al. 1997). One of the mechanisms by which ChoP interacts with host cells is through interaction with the PAF receptor. The natural ligand of PAF contains ChoP and this mimicry enables the bacteria to adhere to and invade host cells (Swords, Buscher et al. 2000). The animal host was more able to reduce the bacterial load of the ChoP$^-$ mutant strain of NTHi in comparison to the wild-type strain in both the lung tissue and alveolar space. This may well be linked to the decreased ability of the ChoP$^-$ mutant strain to adhere to the epithelial cells.

For comparison with the previous results, NTHi 289, a strain that has been used in many studies (Kyd, Dunkley et al. 1995; Foxwell, Kyd et al. 1998; Foxwell, Kyd et al. 1998; Foxwell, Kyd et al. 2001), was included to identify any differences that may have been wild-type strain specific. The differing virulence of various strains of NTHi can be attributed to both the composition of the LOS and the amount present in the outer membrane (Melhus, Hermansson et al. 1998). Of significant interest is that although a higher number of bacteria were present in the lung tissue and alveolar spaces of the animals challenged with NTHi 289 more neutrophils and macrophages were present in the alveolar

spaces of animals challenged with the other wild-type strain, NTHi 2019. LOS is a variable component between strains of NTHi. The differences in cell recruitment between the wild-type strains may be a result of strain differences, including LOS expression. This decreased number of phagocytic cells recruited to the alveolar spaces corresponded with the concentration in the BAL of the pro-inflammatory cytokines IL-1β and IL-6 but not with any differences in MIP-2 or MCP-1. These cytokines may have been indirectly affecting the migration of phagocytes through their ability to influence the up-regulation of adhesion molecules such as ICAM-1 (Frick, Joseph et al. 2000).

The protein concentration of all of the cytokines and chemokines detected in the BAL corresponded directly with phagocytes recruitment and the presence of bacteria. As was seen in the previous chapters, mRNA expression was not always matched with protein production. As detected in the BAL, only TNF-α and MCP-1 appeared to have a direct relationship between mRNA expression and protein production. This lack of relationship seen with other cytokines and chemokines is due to mechanisms such as post-transcriptional modification, cellular production in the lung tissue or BAL and then release to the site of action, such as the alveolar spaces (Le, Lazorick et al. 1991; Powell, Thompson et al. 2000).

A direct relationship between the number of NTHi present and the concentration of TNF-α protein in the BAL was seen after challenge with each of the 3 strains of NTHi included in this study. This may indicate that the presence of TNF-α in the alveolar space was in direct response to the number of bacteria present and was not significantly affected by the change to the LOS structure of individual

bacteria. Other components of the outer membrane of NTHi have been shown to directly influence the innate immune response. Berenson and co-workers, in a study of components of the outer membrane of NTHi showed evidence that P6, an outer membrane protein, stimulated the production of TNF-α and IL-8 from human macrophages (Berenson, Murphy et al. 2005).

There was a lack of an apparent relationship between the migration of phagocytes and TNF-α. Although TNF-α is not directly chemotactic for neutrophils it is able to influence migration through activation of adhesion molecules (Gamble, Harlan et al. 1985; Myers, Wertheimer et al. 1992). The concentration of IL-6 and IL-1β protein in the BAL appeared to have more of an effect on the migration of phagocytic cells into the alveolar space than did TNF-α.

An important difference between the wild-type and mutant strain of NTHi 2019 was in IL-10 protein production in the lung. IL-10 was the only cytokine or chemokine where the concentration was higher in the animals challenged with the ChoP$^-$ mutant strain. IL-10 is known as an anti-inflammatory cytokine for its ability to suppress production of the pro-inflammatory cytokines (Murray 2005). An inverse relationship between IL-10 protein production in the lung tissue and the presence of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in the BAL was apparent. This relationship was also apparent in the BAL from the immune animals in Chapter 4.

MIP-2 and its human homologue, IL-8, are potent neutrophil chemoattractants (Diab, Abdalla et al. 1999). The presence of less MCP-1 and MIP-2 protein in

the BAL from animals challenged with the ChoP$^-$ mutant strain of NTHi 2019 in comparison with the wild-type corresponded with a reduction in the number of both neutrophils and macrophages present. This can be supported by the observation that chemokines are responsible for the initial influx of neutrophils to the alveolar space (Lafleur, Abrahamsen et al. 1998). The effect of LOS on cytokine and chemokine production appears to be due to regulation of mRNA expression as it has previously demonstrated that MCP-1 and MIP-2 mRNA expression is increased in macrophages following exposure to LPS (Kopydlowski, Salkowski et al. 1999). A very recent study investigated the pathways involved in up-regulation of MCP-1. It was demonstrated that MCP-1 production may be up-regulated through TLR2-dependant activation of NF-κB (Moon, Woo et al. 2007). These results coupled with the decreased production of MCP-1 in animals challenged with the ChoP$^-$ mutant strain indicates that ChoP may play an important role in the NF-κB pathway through activation of TLR2.

This study has shown that ChoP, a component of LOS, has an effect on the up-regulation of the host's innate immune response to infection with NTHi. The decreased production of pro-inflammatory cytokines and chemokines and the resulting decreased recruitment of cells to the site of infection provide evidence that ChoP is an important virulence factor of NTHi. Future studies into the kinetics of clearance and *in vitro* studies could provide further insight into the mechanism by which LOS and ChoP influence cytokine and chemokine production and more specifically the production of MCP-1 and its activation of the NF- κB pathway.

THE EFFECT OF IMMUNE CD4+ AND CD8+ CELLS ON THE PRODUCTION OF CYTOKINES AND CHEMOKINES

CHAPTER SEVEN

7.1 ABSTRACT

The effect of mucosal immunisation on CD4+ and CD8+ cell regulation of production of cytokines and chemokines in response to NTHi was determined by culturing cells isolated from mesenteric lymph nodes of immune and non-immune animals. Prior to incubation with NTHi the cytokine and chemokine mRNA expression profiles from both CD4+ and CD8+ cells had been altered by mucosal immunisation. Increases in GM-CSF, IL-10 and MCP-1 mRNA expression in CD4+ cells and increases in IL-6 mRNA expression in CD8+ cells were detected. Following incubation of the cell cultures with either live or killed NTHi, differences in the production of MCP-1 protein were detected with production being increased in cell cultures containing immune CD4+ or CD8+ cells. This study revealed that mucosal immunisation affected the cytokine and chemokine mRNA expression profiles of CD4+ and CD8+ cells.

7.2 INTRODUCTION

Mucosal immunisation has been shown to be effective in enhancing NTHi clearance in an animal model of infection (Buret, Dunkley et al. 1994; Cripps, Dunkley et al. 1994; Kyd, Dunkley et al. 1995; Foxwell, Kyd et al. 2001). Previous chapters have focused on the role of cytokines and chemokines in the lung in relation to both bacterial clearance and phagocytic cell recruitment. It has been determined that enhanced clearance is associated with the recruitment of both macrophages and neutrophils (Foxwell, Kyd et al. 1998)(Chapter 3) and that cytokine and chemokine production in the lung acts to influence the recruitment of these cells (Foxwell, Kyd et al. 1998)(Chapters 3 and 4). In order to further understand the mechanisms by which mucosal immunisation protects the host it is necessary to look at the cells that are directly affected by immunisation.

Previous studies in this laboratory have demonstrated that CD8+ T cells play an important role in enhancing the clearance of NTHi from the lungs of immune animals (Foxwell, Kyd et al. 2001). The role of CD4+ T cells in protection afforded by immunisation has been demonstrated through the transfer of CD4+ T cells from immune animals into naïve animals and the resulting enhanced clearance of bacteria from the lung (Wallace, Cripps et al. 1991; Dunkley, Clancy et al. 1994). It is suggested that memory T-cells are polarized to produce a particular cytokine profile following initial antigen exposure, which is then reproduced upon re-exposure to the same antigen (Richter, Lohning et al. 1999). In a study by Graham and co-workers, differing cytokine mRNA

expression profiles were induced in lymphocytes isolated from immune and non-immune animals after challenge (Graham, Henderson et al. 1993).

In order to determine the effect of immunisation on cytokine and chemokine profiles from T cells upon re-stimulation, CD4+ and CD8+ T cells were isolated from the mesenteric lymph nodes of immune and non-immune animals and cultured with either live or WKC NTHi. Certain stimulators of cytokine and chemokine production may only be up-regulated by the live bacterial cell. The cytokine and chemokine mRNA expression and protein production profiles generated from these cells should aid in developing a further understanding of the mechanisms involved in providing protection from NTHi infection through mucosal immunisation.

7.3 MATERIALS AND METHODS

7.3.1 Bacteria: NTHi 289 was prepared for immunisation and cell culture as detailed in section 2.1.

7.3.2 Animal strain and immunisation: SPF DA male rats aged between 8 and 10 weeks were used. They were immunised with either NTHi or PBS following the protocol detailed in sections 2.2.1, 2.2.2, 2.3.1-2.3.3 and 2.2.4.1-2.2.4.4. Briefly, animals received a mucosal immunisation consisting of injection of WKC NTHi 289 into the Peyer's patches located in the small intestine followed 14 days later by an IT boost. 21 days after the initial immunisation the animals were sedated briefly with halothane before being euthanased by an overdose of pentobarbitone sodium.

7.3.3 Tissue Collection: Cells were collected from animals following protocol detailed in sections 2.2.4.5 and 2.2.4.6.

7.3.4 Cell Culture: Isolation and culture of CD4+ and CD8+ T-cells was performed using the protocol detailed in section 2.3. Briefly, either CD4+ or CD8+ T cells were positively selected using immunomagnetic separation beads with the depleted sub-set being used for flow cytometry anaylsis. The CD4+ or CD8+ T cells were then cultured at a concentration of $2x10^6$ cells per ml in RPMI with the addition of Mitomycin C treated spleen cells at a concentration of $2x10^7$ as a source of APCs. The following day cell cultures were incubated with either live or WKC NTHi for 30 min or 1 h. Following incubation cells were centrifuged with the supernatant being used for the measurement of protein and the cell pellet for mRNA expression.

7.3.5 Cytokine and Chemokine mRNA expression: Real-time RT-PCR was used to determine IL10, TNF-α, IFN-γ, MCP-1, MIP-2, IL-1β, IL-6 and GM-CSF mRNA expression in culture cells as detailed in sections 2.4.1 and 2.4.3.

7.3.6 Determination of cytokine and chemokine concentration: IL10, TNF-α, IFN-γ, MCP-1, MIP-2, IL-1β, IL-6 and GM-CSF protein concentrations were measured in cell culture supernatant using commercially available ELISA kits as specified in section 2.5.

7.3.7 Statistical analysis: The data has been expressed as the means ± SEM. The data was compared for statistical significance by ANOVA with Bonferroni posttests (GraphPad Prism).

7.4 RESULTS

7.4.1 CD4+ and CD8+ lymphocyte isolation: CD4+ or CD8+ lymphocytes were isolated from the mesenteric lymph nodes using immunomagnetic separation. The target cell subset presence and subsequent removal from the lymph node cells was confirmed by flow cytometry (figures 7.1-7.2). The selected cells were found to retain their viability and were set up as a monoculture of CD4+ or CD8+ cells plus mitomycin C treated spleen cells.

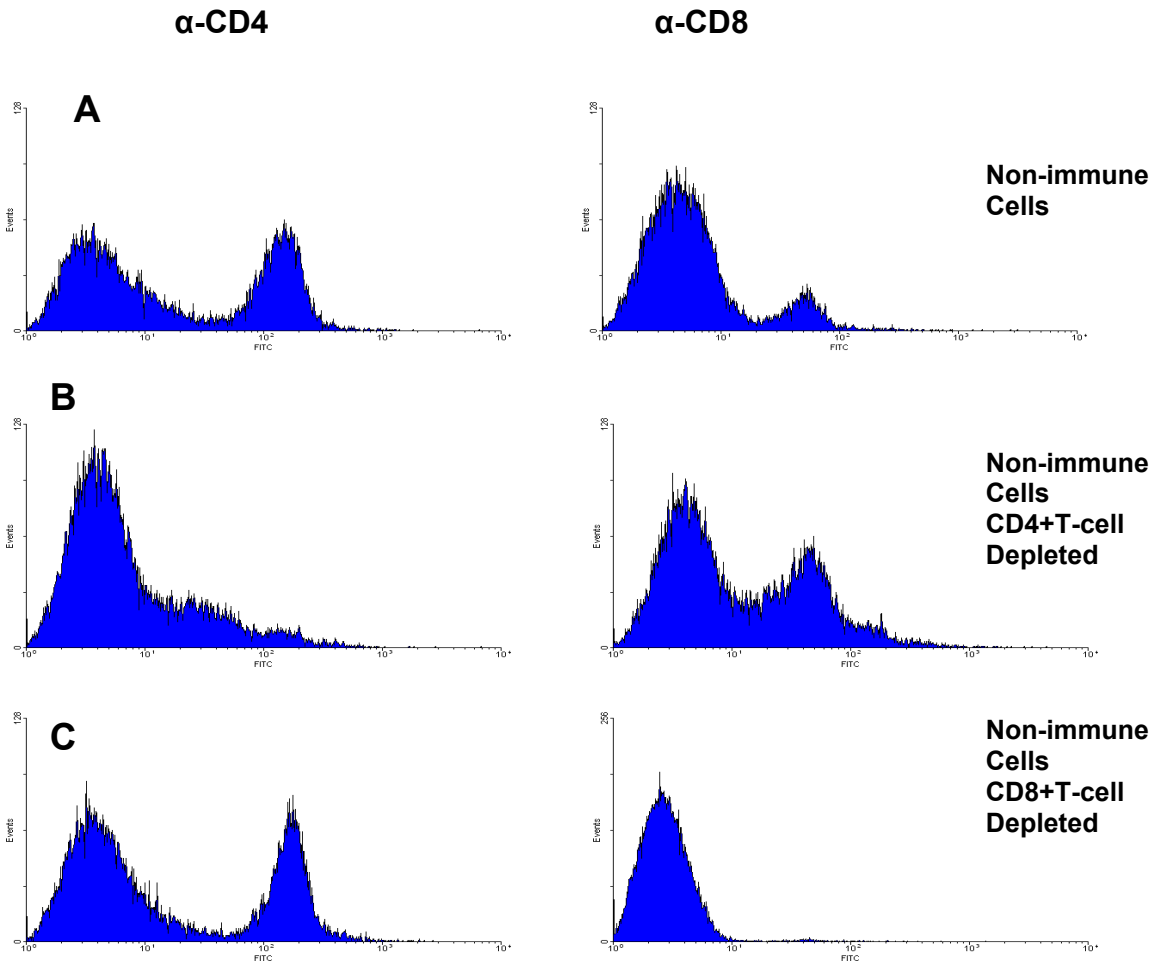

Figure 7.1 Histograms from flow cytometric analysis of cells isolated from the MLNs of non-immune animals before (A) and after $CD4^+$T-cell (B) and $CD8^+$T-cell (C) depletion. Cells were incubated with either CD4 or CD8 primary antibody (α-CD4; α-CD8) followed by incubation with secondary FITC labeled antibody.

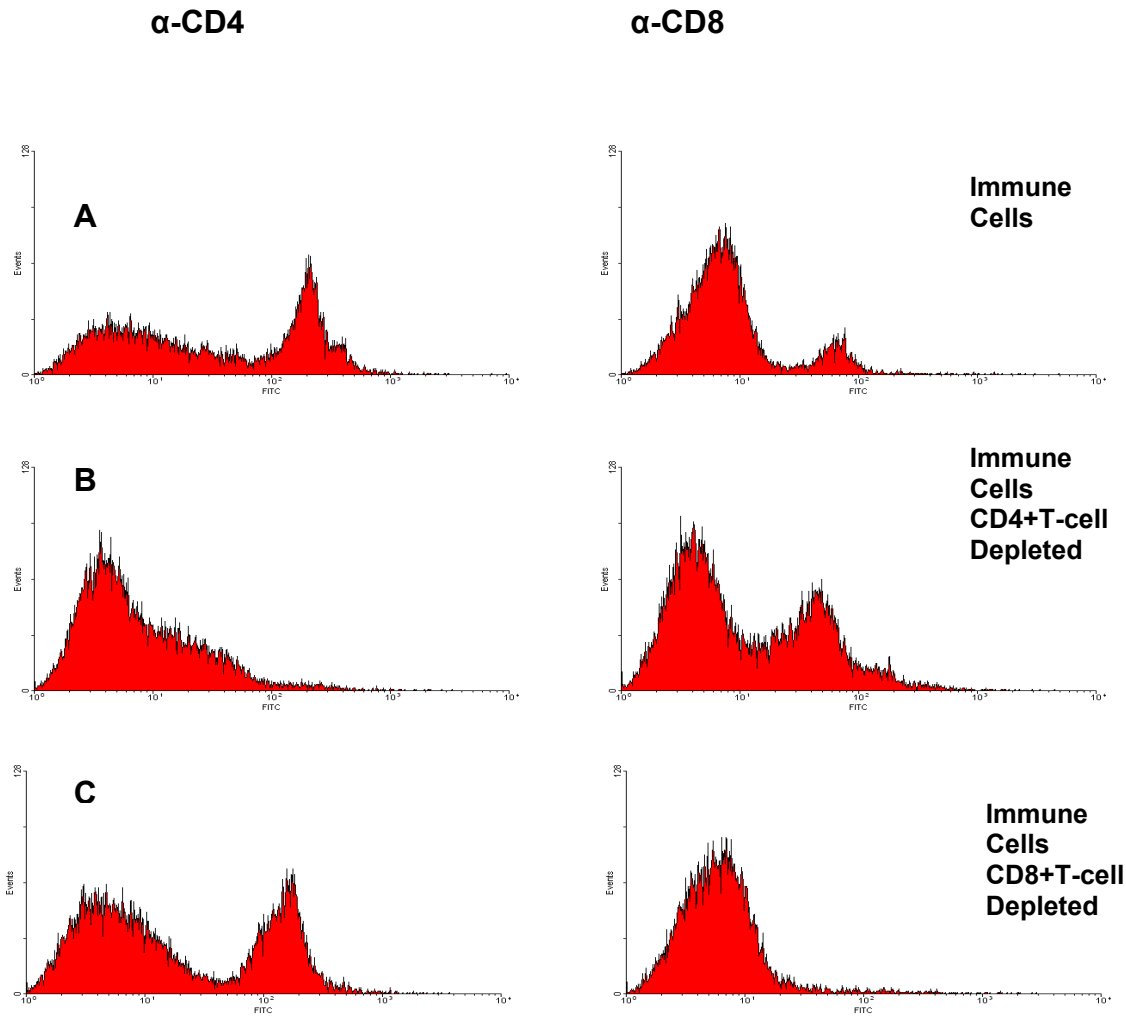

Figure 7.2 Histograms from flow cytometric analysis of cells isolated from the MLNs of immune animals before (A) and after $CD4^+$T-cell (B) and $CD8^+$T-cell (C) depletion. Cells were incubated with either CD4 or CD8 primary antibody followed by incubation with secondary FITC labeled antibody.

7.4.2 TNF-α, MCP-1 and MIP-2 mRNA expression and protein production in lymphocytes: Only TNF-α, MCP-1 and MIP-2 protein were detected in the supernatant from cultures containing CD4+ or CD8+ T-cells isolated from NTHi immune and non-immune animals (figure 7.3, 7.4 and 7.5). All other cytokines where either absent or below the level of detection. Prior to incubation with either live or WKC NTHi, differences in MCP-1 protein production between immune and non-immune cells were seen in both CD4+ and CD8+ T-cell cultures, with significantly more MCP-1 protein being produced from cells isolated from the mucosally immunised animals. This increase in MCP-1 protein production in CD4+ T-cells from the immune animals corresponded with an up-regulation of MCP-1 mRNA expression (figure 7.4). Following incubation with NTHi MCP-1 protein production remained significantly higher in cells from the mucosally immunised animals.

Prior to incubation with NTHi, TNF-α was secreted from CD4+ and CD8+ T-cell cultures isolated from both the immune and non-immune animals but at no time following incubation was any TNF-α protein detected in any of the cell cultures. This did not directly correspond with TNF-α mRNA expression as the increases in mRNA expression were detected following NTHi incubation (figure 7.3). In both CD4+ and CD8+ T-cell cultures, mRNA expression was higher in the cells from the non-immune animals. Incubation with either live or WKC NTHi also had an effect on TNF-α mRNA expression with expression generally being higher in cells incubated with live NTHi.

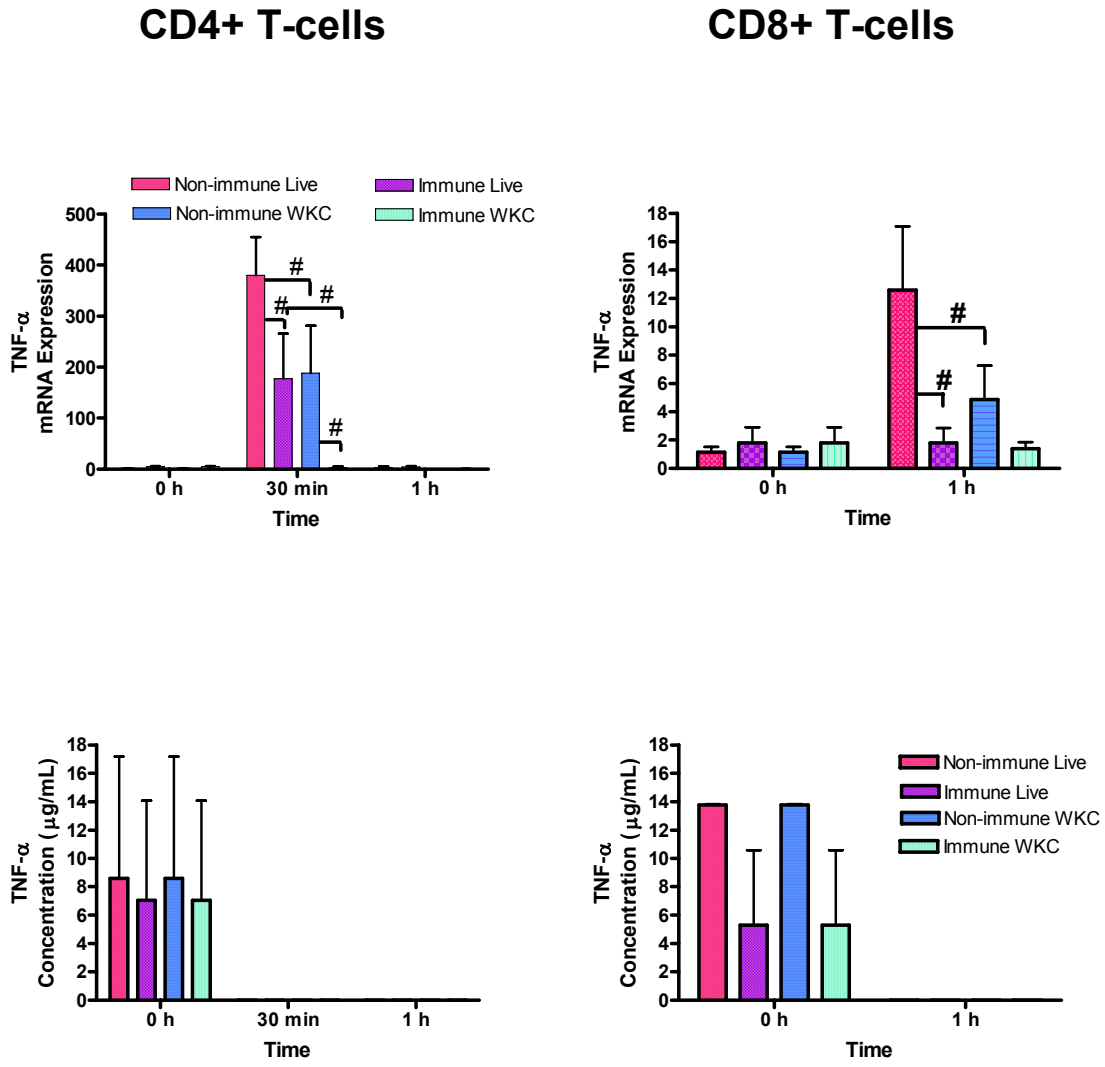

Figure 7.3 TNF-α mRNA expression and protein production in $CD4^+$ and $CD8^+$ T-cell cultures isolated from the MLN of immune and non-immune animals. Cells were cultured overnight with mitomycin C treated spleen cells prior to incubation for 30 min or 1 h with either live or WKC NTHi 289. Significant differences between cells isolated from non-immune or immune animals and cells incubated with either live or WKC NTHi were represented by # (# $p < 0.05$). Y-axis scales differ between individual mRNA expression graphs. The values represent the mean ± SEM for n=3. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

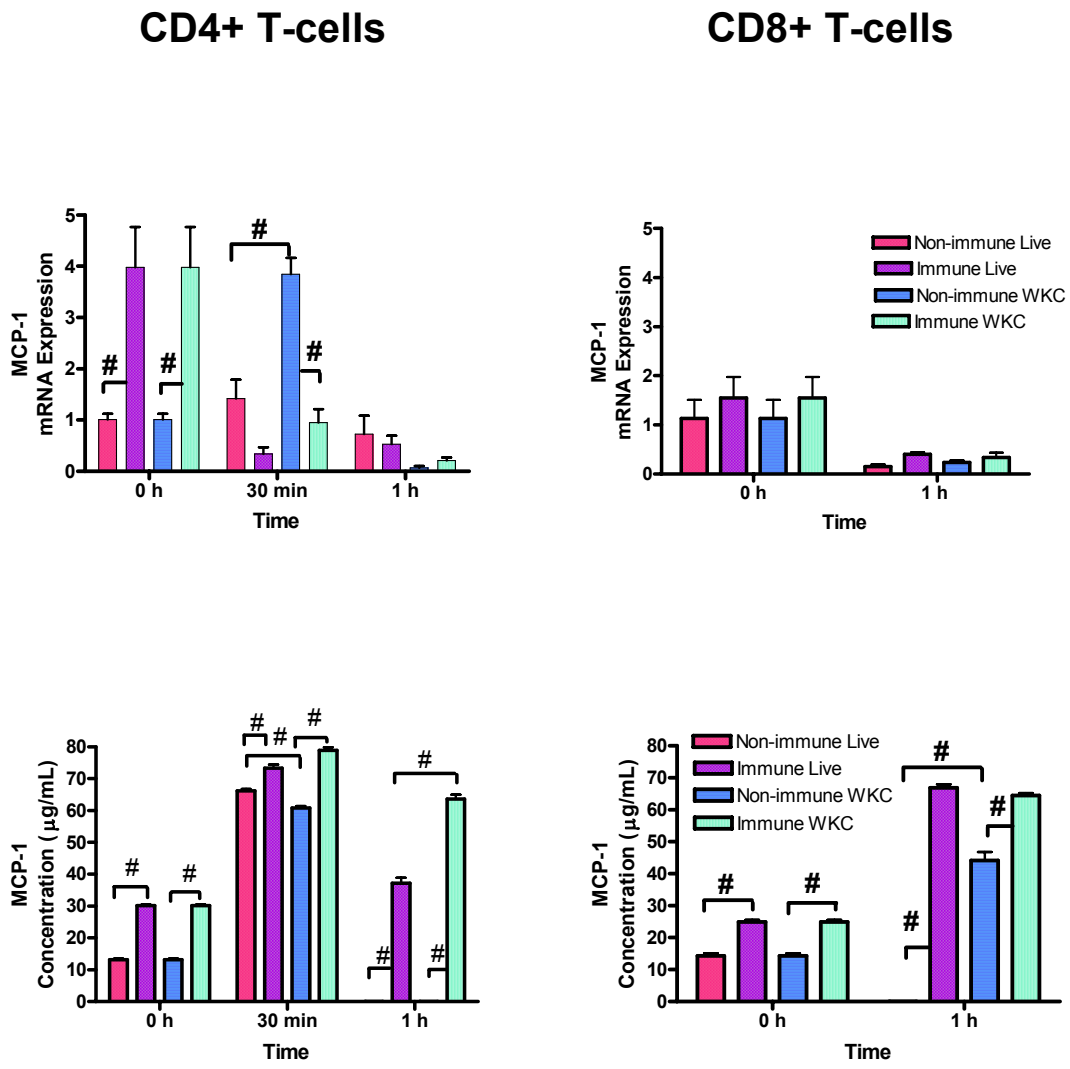

Figure 7.4 MCP-1 mRNA expression and protein production in $CD4^+$ and $CD8^+$ T-cell cultures isolated from the MLN of immune and non-immune animals. Cells were cultured overnight with mitomycin C treated spleen cells prior to incubation for 30 min or 1 h with either live or WKC NTHi 289. Significant differences between cells isolated from non-immune or immune animals and cells incubated with either live or WKC NTHi were represented by # (# $p < 0.05$). The values represent the mean ± SEM for n=3. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

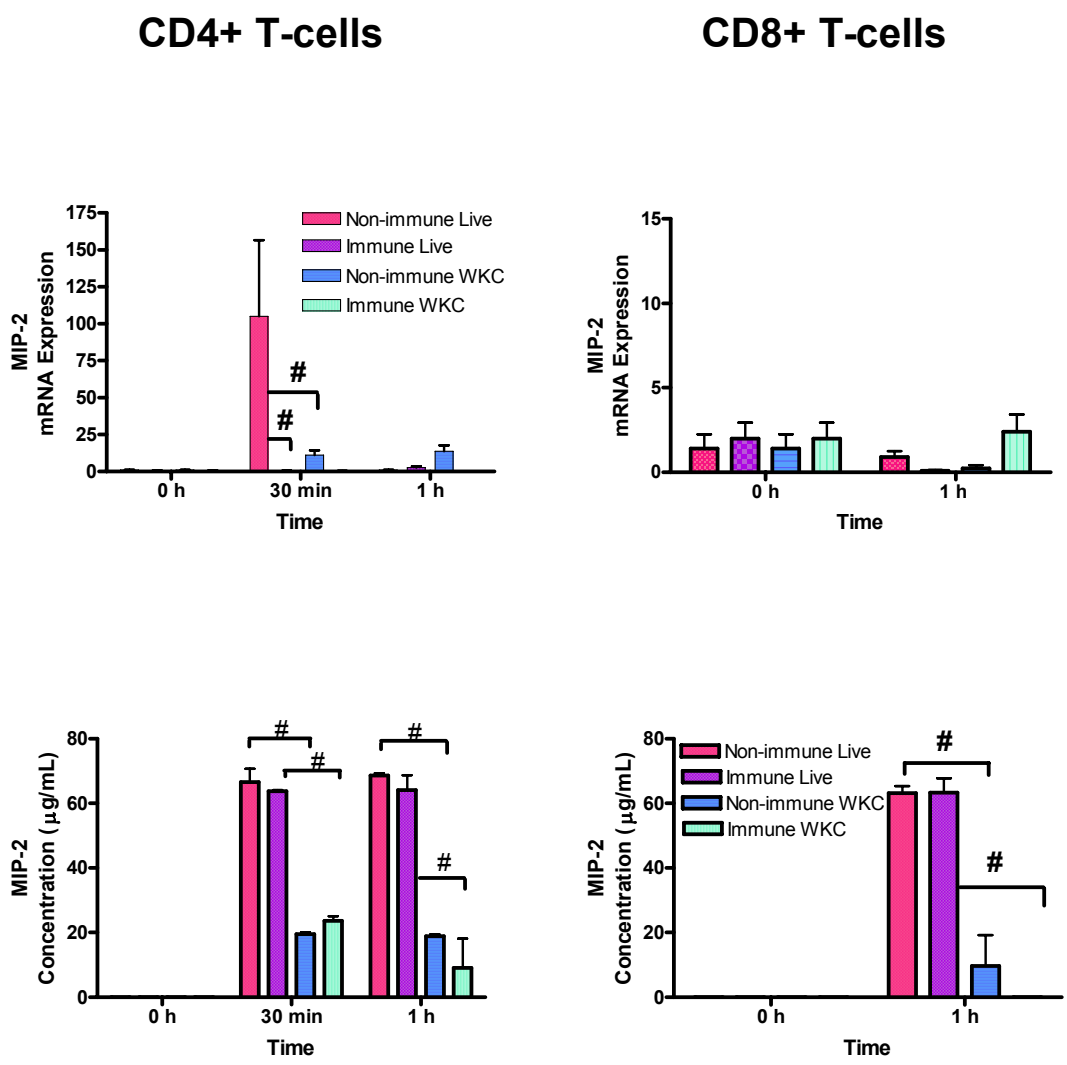

Figure 7.5 MIP-2 mRNA expression and protein production in $CD4^+$ and $CD8^+$ T-cell cultures isolated from the MLN of immune and non-immune animals. Cells were cultured overnight with mitomycin C treated spleen cells prior to incubation for 30 min or 1 h with either live or WKC NTHi 289. Significant differences between cells isolated from non-immune or immune animals and cells incubated with either live or WKC NTHi were represented by # (# $p < 0.05$). Y-axis scales differ between individual mRNA expression graphs. The values represent the mean ± SEM for n=3. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

Following incubation with NTHi MIP-2 protein production was detected in both CD4+ and CD8+ T-cell cultures but no differences were seen between the immune and non-immune cells. However differences in production were seen between cells incubated with either live or WKC NTHi. Stimulation of cells with live NTHi produced more MIP-2 protein than stimulation with WKC NTHi.

7.4.3 Cytokine mRNA expression in CD4+ T-cells: Prior to incubation with either live or WKC NTHi, differences were seen in GM-CSF and IL-10 mRNA expression between the cultures of CD4+ T-cell isolated from either the immune or non-immune animals (figure 7.6). In both cases expression was higher in cells isolated from the mucosally immunised animals. After 1 h of incubation with NTHi differences in IL-10 mRNA expression were seen. Expression in the non-immune CD4+ T-cell cultures incubated with WKC NTHi was higher than both the immune cells incubated with the WKC NTHi and the non-immune cells incubated with live NTHi.

No differences were seen in either IFN-γ or IL-1β mRNA expression between any of the cell culture groups. Expression of both of these cytokines was down-regulated in the CD4+ T-cell cultures following incubation with NTHi, live and WKC. IL-6 mRNA expression only differed between the immune and non-immune cells at 1 h after incubation with live NTHi, with expression being higher in the non-immune cells. At this time incubation with live NTHi, as opposed to WKC, also resulted in an up-regulation of mRNA expression in the non-immune cells only.

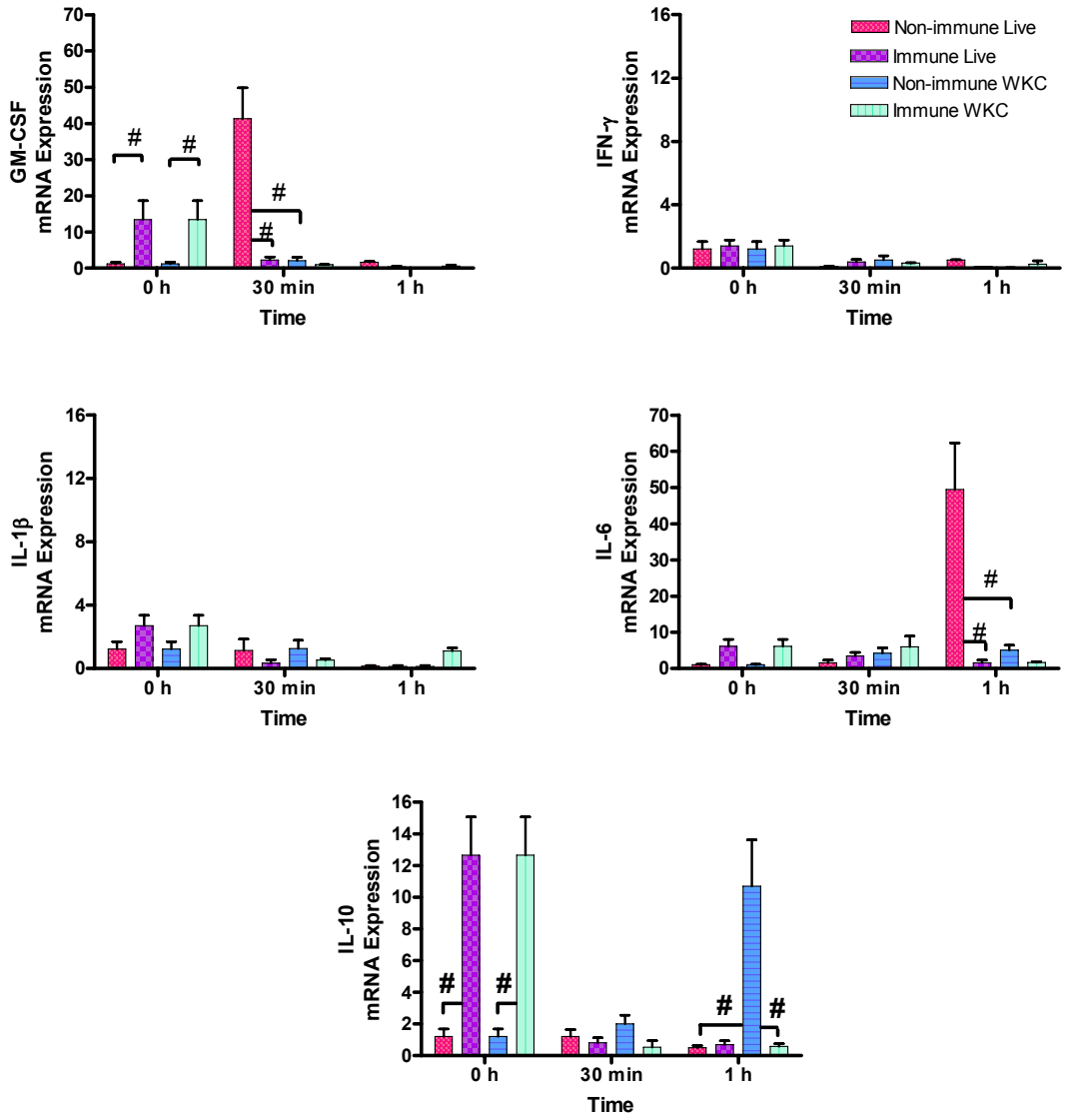

Figure 7.6 GM-CSF, IFN-γ, IL-1β, IL-6 and IL-10 mRNA expression in $CD4^+$ T-cell cultures isolated from the MLN of immune and non-immune animals. Cells were cultured overnight with mitomycin C treated spleen cells prior to incubation for 30 min or 1 h with either live or WKC NTHi 289. Significant differences between cells isolated from non-immune or immune animals and cells incubated with either live or WKC NTHi were represented by # (# $p < 0.05$). Y-axis scales differ between individual mRNA expression graphs. The values represent the mean ± SEM for n=3. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

7.4.4 GM-CSF, IFN-γ and IL-10 mRNA expression in CD8+ T-cells:

IL-6 mRNA expression was effected by immunisation, with expression being upregulated in the CD8+ T-cell cultures from the immune animals prior to incubation with NTHi (figure 7.7). Similar trends were seen for IL-10 and IFN-γ but these were not significant. Immunisation had no effect on GM-CSF mRNA expression in CD8+ cell cultures. There was no difference in expression between cells incubated with live or WKC NTHi.

Following incubation with NTHi the only difference in cytokine mRNA expression between the immune and non-immune CD8+ T-cell cultures was in IL-1β mRNA expression (figure 7.7). Expression was upregulated in the non-immune cells incubated with live NTHi. This expression was significantly higher than in cells incubated with WKC NTHi.

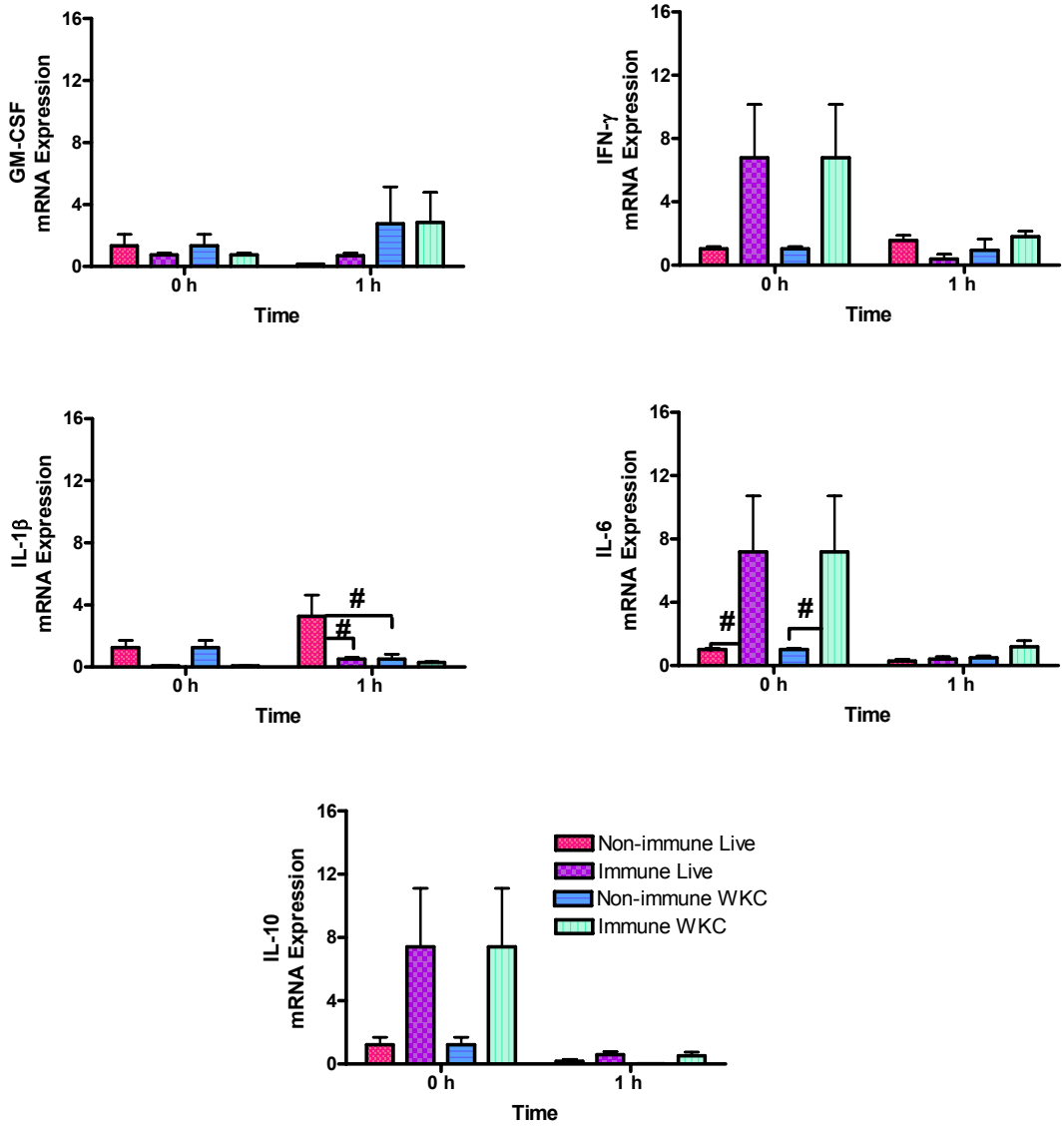

Figure 7.7 GM-CSF, IFN-γ, IL-1β, IL-6 and IL-10 mRNA expression in $CD8^+$ T-cell cultures isolated from the MLN of immune and non-immune animals. Cells were cultured overnight with mitomycin C treated spleen cells prior to incubation for 30 min or 1 h with either live or WKC NTHi 289. Significant differences between cells isolated from non-immune or immune animals and cells incubated with either live or WKC NTHi were represented by # (# $p < 0.05$). The values represent the mean ± SEM for n=3. Where no SEM bars are indicated, the SEM was too small to be shown graphically.

7.5 DISCUSSION

The way in which mucosal immunisation affords protection against infection with NTHi cannot be explained through the increased production and activation of phagocytic cells alone. Neutrophils and macrophages are part of the innate immune system and whilst they can act as effector cells in an adaptive T cell mediated immune response they do not have the capability of a memory response to recognise pathogens after re-exposure.

In Chapters 3 and 4 and in other investigations in this laboratory it has been shown that the enhanced clearance of NTHi from the lung in the immune animals was related to the ability to recruit macrophages to the alveolar spaces (Foxwell, Kyd et al. 1998). It was further demonstrated in previous chapters that the profiles of cytokine and chemokine protein production differed between the immune and non-immune animals and that this was the likely cause of the altered macrophage response in the immune animals. Following these discoveries the question still remained, in what way does the acquired immune response control the production of cytokines and chemokines in response to NTHi. It is well established that the mucosal immunisation regimen used in this study induces antigen-specific T cell responses that can be measured in the mesenteric lymph nodes (Kyd, Dunkley et al. 1995).(Kyd and Cripps 1998). These nodes are part of the common mucosal immune system and cells from these nodes home to other mucosal sites, including the lung (Mestecky, Michalek et al. 1997). Foxwell *et al* showed that both CD4+ and CD8+ cells were recruited to the lungs of the immune animals following NTHi infection, although surprisingly CD8+ cells were recruited in the earlier stages of the infection and CD4+ when the infection was resolving (Foxwell, Kyd et al. 2001).

The current study aimed to identify differences in the cytokine and chemokine profiles of the cells from the mesenteric lymph nodes that are part of the population that can migrate to the lungs following mucosal immunisation.

Of the cytokines and chemokines studied only TNF-α, MCP-1 and MIP-2 protein were detected in the supernatant from CD4+ and CD8+ T cell cultures. This corresponded with the high concentrations of these proteins detected in the lung in Chapter 3 and the BAL in Chapter 4. Other cytokine protein levels were below the detection level of the kits used in this investigation. The process of cell separation results in a small number of T cells available from the rat mesenteric lymph nodes which limits the cell concentration of the culture and limitations on scaling up the cultures may have attributed to the low levels of cytokines and chemokines being produced in the cell cultures. In addition, this study looked at the response associated with the T cells and did not distinguish which cells in the culture produced the response. It is well established that the CD4+ cells from the mesenteric lymph nodes proliferate in response to antigen presented by antigen presenting cells (Kyd, Dunkley et al. 1995). The mitomycin C is a potent DNA cross-linker and prevents cells from replicating. This does not prevent the cells treated with this agent from functioning, in this case, the spleen antigen presenting cells are capable of processing antigen for presentation to and response by the non-treated T cells.

It appears likely that GM-CSF would play a role in the increased macrophage numbers in the lung of immunised animals as it has a role in increased proliferation of alveolar macrophages (Chen, Mueller et al. 1988; Lemaire, Yang et al. 1996). The upregulation of GM-CSF mRNA expression in immune CD4+

T cells prior to re-exposure with NTHi is evidence that immunisation has primed the immune system by altering the cytokine mRNA expression profile.

The cause of the decline in GM-CSF mRNA expression in the immune CD4+ T cells at 1 h following incubation with NTHi may be due to the translation of this mRNA into protein. The ability of CD4+ T cells to increase levels of GM-CSF may be a mechanism responsible for the increased number of macrophages detected in the lungs of the mucosally immunised animals seen in Chapters 3 and 4. Although no GM-CSF protein was detected in either the cell cultures in this chapter or the lung homogenate and BAL samples from previous chapters, the likely scenario is that GM-CSF was not absent but that the concentrations required to affect the macrophage levels were lower than detected by the method used.

Although studies from this laboratory have shown that the number of CD4+ T cells in the lung tissue do not change in the early stages of infection with NTHi (Foxwell, Kyd et al. 2001) this does not mean that they do not play a role in the acquired immune response seen in the immune animals. The lung is not devoid of these cells *in situ* and some of these cells would have been primed to recognize the NTHi infection. It is well established that effector B and T cells home to mucosal sites following immunisation (Mestecky, Michalek et al. 1997). Although alveolar macrophages suppress proliferation of the CD4+ T cells, activation and production of cytokines and chemokines still occurs (Upham, Strickland et al. 1995). The differing abilities of the immune and non-immune cells to produce cytokines and chemokines that control phagocytic cell recruitment and activation were evident in this chapter.

The differing mRNA expression profiles seen in CD4+ T cells may play a role in the recruitment of CD8+ T cells to the site of infection. Following challenge with NTHi increased numbers of CD8+ T cells have been detected in the lung tissue of mucosally immune animals (Foxwell, Kyd et al. 2001). It has not been established whether the CD8+ T cells that are recruited as part of the protective immune response against NTHi were antigen-specific cells. Their early increase in the lung may not be due to priming of the CD8+ T cells through immunisation but as a result of other antigen-specific cells signaling to increase their presence at the site of infection.

Further evidence for the role of CD4+ T cell in protection after immunisation lies with the production of MCP-1 protein. MCP-1 production was increased in the immune cells. MCP-1 is a potent chemoattractant of T cells and its production *in vivo* could lead to the proliferation of CD8+ T cells (Loetscher, Seitz et al. 1994). CD8+ T cells from the immune animals also produced more MCP-1 than the non-immune cells and their increased presence in the lung tissue would result in increased macrophage recruitment (Zisman, Kunkel et al. 1997) and ultimately the enhanced ability to clear NTHi (Foxwell, Kyd et al. 2001)(Chapters 3 and 4).

An important characteristic of the response to NTHi in the immune animals was the ability to resolve inflammation (Chapter 3). IL-10 was found to be constitutively expressed in the normal rat lung but absent following challenge with NTHi. Following clearance of all NTHi, IL-10 protein production was once again upregulated in the immune animals (Chapter 4). This increased production of IL-10 corresponded with the increase in CD4+ T cells in the

immune lung seen in a previous investigation by Foxwell *et al* (Foxwell, Kyd et al. 2001). In this investigation a further link between CD4+ T cells, the production of IL-10 and resolution of inflammation was revealed through the upregulation of IL-10 mRNA expression in the CD4+ T cell cultures isolated from the immune animals.

The differing cytokine and chemokine mRNA expression and protein production profiles seen between CD4+ and CD8+ T cell cultures isolated from either immune or non-immune animals has provided insight into the way in which mucosal immunisation may be priming cells to enable enhanced clearance of NTHi from the lung. These primed cells are able to control cytokine and chemokine production, which leads to regulation of cellular recruitment and activation at the site of infection and appropriate resolution of the innate and inflammatory responses.

DISCUSSION AND CONCLUSION

CHAPTER EIGHT

This study was undertaken to better understand the host response to NTHi infection and how mucosal immunisation protects the host during infection. Specifically the study determined the way in which the acquired immune response controls the innate immune system during NTHi infection in the lung by investigating the production and regulation of cytokines and chemokines in the lung, the alveolar spaces and in *ex vivo* T cells. Investigation of the regulation of the cytokines and chemokines assessed in this thesis by factors that can affect the clearance of NTHi from the lung has contributed to a better understanding of how the acquired immune response controls the innate immune response during an infection.

It has been well established that protective antigens delivered by mucosal immunisation effectively enhances clearance of NTHi from the lung in the rat model of infection (Wallace, Clancey et al. 1989; Kyd, Dunkley et al. 1995; Kyd and Cripps 1998). Characterisation of the mechanisms involved in this enhanced clearance of NTHi over a 24 h time-period by Foxwell (Foxwell, Kyd et al. 1998) set down a framework for the design of an investigation into the role of cytokines and chemokines in this animal model of infection. A cell-mediated response to immunisation was identified as a major contributing factor of enhanced clearance of NTHi from the lung (Wallace, Witt et al. 1995; Foxwell, Kyd et al. 1998).

The mucosal immunisation regime that was a combination of a primary immunization delivery of killed whole cell NTHi to the Peyer's patches in the small intestine followed by a boost delivered to the lungs had been established as inducing the most effective immune response in this model. Animals that

had been mucosally immunised cleared the bacteria from the lung more rapidly that the non-immune animals and showed control and earlier resolution of inflammation. The kinetics of infection clearance accompanied by the white cell responses in the lung reported in Chapters 3 and 4 were similar to that previously been reported (Foxwell, Kyd et al. 1998) but are from a different strain of rat.

The pilot study (Chapter 3) was able to provide an indication of the way in which mucosal immunisation enhanced clearance of NTHi from the lung is through the regulation of mRNA expression and production of cytokines and chemokines. Based on these results, reports on cytokine and chemokine responses to NTHi in the literature (Khair, Devalia et al. 1994; Clemans, Bauer et al. 2000; Melhus and Ryan 2000; Tong, Chen et al. 2001) and previous results from this laboratory (Foxwell, Kyd et al. 1998) the scope of the study was expanded. This has enabled investigation of a range of cytokines and chemokines that clearly demonstrate a role in the regulation of the immune response NTHi in both naïve and immune states. In particular, the results in Chapter 3 highlighted the importance of assessing the mRNA and signaling molecule protein levels in both the lung tissue and BAL.

Differences in the recruitment of neutrophils and macrophages to the lung following NTHi infection were seen between this study and a previous study from this laboratory (Foxwell, Kyd et al. 1998). Both the Foxwell study and this thesis showed that the enhanced clearance of bacteria in the immune animals was linked to a distinct difference in the migration of macrophages to the lung. What was important was that the two studies used different rat strains. In this

thesis inbred DA rats were used while the Foxwell study used the outbred Wistar strain. Even though there were some differences between the way the different rat strains responded, importantly in both strains the increased number of macrophages was significant. The trend in the neutrophil recruitment following infection (Chapter 3) suggests that the period between 4 and 8 hours may be an important period for numbers of these cells in the BAL in the immune animals. There were some differences in the relative numbers of phagocytes at the different times in the two animal strains highlighting the fact that some observations to NTHi in animals may be dependent on each individual rodent host. Likewise, it is apparent from various studies of human infection that there are differences in individual susceptibility to the range of infections caused by NTHi and particularly studies in children have shown that factors such as ethnicity can affect the colonisation load and susceptibility to otitis media.

The recruitment of phagocytes to the lung in response to NTHi was accompanied by an increase in the production of pro-inflammatory cytokines and chemokines. Rapid increases in the production of IL-1β, IL-6, TNF-α, MCP-1 and MIP-2 in both the lung tissue and alveolar spaces were seen immediately following challenge with NTHi in both naïve animals and mucosally immunised animals. Significant regulation of both mRNA and translated protein for these cytokines and chemokines by mucosal immunisation was identified (Chapter 3 and 4). These differences support the hypothesis that one of the ways immunization is effective is by regulating or controlling the inflammatory response to the infectious agent.

Viral infections have been shown to predispose the host to bacterial infection. In contrast to the expected result, an antecedent viral infection actually enhanced clearance of NTHi from the rodent lung (Chapter 5). This result does not necessarily dispute the role of viral infections in predisposing the host to a subsequent bacterial infection but may help to define the mechanism by which this occurs. In this study there did not appear to be an impairment of the innate immune response following viral infection. The theory that the predisposition to bacterial infection may be due to the increased ability of NTHi bind to virally infected cells (Avadhanula, Rodriguez et al. 2006). As such, this study was not a colonisation model and so any impact that the viral infection may have had on upper respiratory tract colonization would not be detected. Additionally, these rats did not have any particular underlying conditions that are usually present in the human host that is susceptible to bronchitis, pneumonia or COPD exacerbations. The design of an animal colonisation model that could assess the ability of a viral infection to predispose the host to NTHi infection in terms of the ability to increase colonisation could provide the evidence required to support this theory.

Although the antecedent viral infection enhanced clearance of NTHi (Chapter 5) to a degree similar to that observed in the mucosally immunised rats (Chapter 3), the mechanisms by which clearance was enhanced were very different. The decreased number of neutrophils recruited to the lungs of virally infected animals following NTHi challenge in comparison to the immune animals indicated that priming of the innate response by a viral infection most likely increased the phagocytic ability of neutrophils. The differing profiles of cellular recruitment and cytokine and chemokine responses highlighted distinct

differences between those induced as a result of an antigen-specific immune response and that of a non-specific innate response upregulated by some other trigger. The key characteristics of an antigen specific immune response included increased secretion of the pro-inflammatory cytokines TNF-α and IL-6 in the alveolar space and an increase in the anti-inflammatory cytokine IL-10 following clearance of bacteria (Chapter 4). In the virally infected animals no IL-10 was detected in the BAL at any time-point (Chapter 5). This finding may be quite significant since IL-10 is important in the resolution of inflammation through decreasing the production of pro-inflammatory cytokines and chemokines (Bogdan, Vodovotz et al. 1991; Opal and V 2000; Strieter, Belperio et al. 2002) and so in the susceptible host may contribute to a situation of sustained inflammation increasing the likelihood of tissue damage and a secondary bacterial infection.

In the early stages of a lung infection with NTHi the initial response can be, to a large extent, attributed to the interaction of bacterial surface antigens such as LOS with the respiratory epithelial cells and alveolar macrophages. NTHi has been found to stimulate the production of pro-inflammatory cytokines from host cells through the activation of TLRs. In Chapter 6 the production of these pro-inflammatory cytokines was influenced by the strain of NTHi. ChoP, a component of LOS was shown to be a potent stimulator of MCP-1 protein production. This observation was significant and coincided with a recent publication by Moon et al that revealed that the regulation of MCP-1 is through the TLR2-dependant activation of the NF-κB pathway (Moon, Woo et al. 2007). It can therefore be hypothesised that NTHi activates the NF-κB pathway through the binding of ChoP to TLR2. Further studies are required to test this

theory and determine the significance of these observations for the host's response to acute and chronic disease.

The chemokines, MCP-1 and MIP-2, are very important for recruiting phagocytic cells to the infection site. Production of these chemokines was increased in both the immune animals (Chapter 4) and the virally infected animals (Chapter 5), indicating their importance in the host's ability to respond to NTHi in the lung through the recruitment of phagocytes to the site of infection (Huffnagle, Strieter et al. 1995; Schmal, Shanley et al. 1996). The increased presence of MCP-1 and MIP-2 appeared to be directly related to the increased ability to clear bacteria from the lung.

In had previously been reported that infiltrates of CD8+ T cells were important in the acquired immune response in the lung (Foxwell, Kyd et al. 2001) and that transferring $CD4^+$ T cells conferred protection to naïve rats (Wallace, Cripps et al. 1991; Dunkley, Clancy et al. 1994). In order to understand if these T cells are capable of specific cytokine profiles from primary exposure to a pathogen, their profiles were assessed following *in vitro* restimulation with both live and killed NTHi. An initial increase in pro-inflammatory cytokine protein production in the lung tissue and alveolar space (Chapters 3 and 4) is likely to be associated with immune priming of CD4+ T cells and possibly CD8+ T cells (Chapter 7). In addition, the role of CD8+ T cells in the host response to viral infection is well established (Kedzierska, La Gruta et al. 2006). The results of Chapter 7 along with the previous results from this laboratory (Foxwell, Kyd et al. 2001) suggest that the CD8+ T cells play an important role in enhancing the clearance of NTHi through possibly both the proliferation of cells at the site of infection and the

priming of the cells both recruited to and resident in the lung. The altered cytokine and chemokine profile seen in CD4+ T cells seen *in vitro* (Chapter 7) may influence the increased recruitment of CD8+ T cells to the lung tissue that was found by Foxwell et al (Foxwell, Kyd et al. 2001).

Protein production of only three out of the eight cytokines and chemokines measured in the CD4+ and CD8+ T cell cultures were detected (Chapter 7). MCP-1, MIP-2 and TNF-α had the highest concentrations in the lung tissue in Chapter 3 and 4 and the BAL in Chapter 4. The other cytokine proteins may have been present in the cell culture supernatant, but at levels below the detection limits of the assays. The difference observed in Chapter 7 indicate that further investigation is warranted to better understand how immune antigen priming of these cells contributes to the host response to infection.

The differences in GM-CSF mRNA expression profiles between the various groups indicated that there may be significant differences in the production of GM-CSF protein but that the concentration of GM-CSF was so low that it was below the sensitivity of the assay. It may also be that once produced, it is utilized rapidly as part of the infection response.

This study was undertaken to test the hypothesis that mucosal immunization induces an acquired immune response that is able to regulate the host's innate immune system in response to an NTHi infection. Specifically, this study has found that the chemokine and cytokine signals associated with the recruitment, activation and inflammatory responses differ significantly from those induced in the naïve state. Importantly, the study has also shown that resolution of these

responses as the infectious organism is removed was also under immune control. Some of the important new findings and contributions to a better understanding of host responses to infection include: (1) The fact that the chemokine MCP-1 is present in the normal lung (Chapters 3 and 4) and plays a role in the sustained recruitment of mononuclear phagocytes, in this case alveolar macrophages (Chae, Im et al. 2002). (2) IL-10 was also present as it was constitutively produced by respiratory epithelial cells in order to maintain normal cellular function and control inflammation (Chapters 3 and 4)(Bonfield, Konstan et al. 1995). IL-10 decreased in the lung tissue following infection with NTHi and as TNF-α and MIP-2 increased production (Chapter 3 and 4)(Donnelly, Freeman et al. 1995; Murray 2005). In the immune animals, IL-10 mRNA and protein levels increased at a time critical to controlling resolution of the inflammatory response and confirmed that IL-10 is associated with this control in the immune system. (3) The study found distinct differences in the TNF-α, IL-1β and IL-6 responses, with different kinetics and concentrations in the BAL and lung as well as between immune and non-immune animals. These finding help to better understand the partitioning of host responses in the lung and importantly how immunisation regulates the inflammatory response to infection as part of host protection. (4) Although the parameter of bacterial clearance during a viral infection appeared to mimic the kinetics of immune clearance, the assessment of the cytokine and chemokine responses had several differences that contribute to helping understand how these responses may contribute to more significant secondary bacterial infection in the compromised host. (5) Components on the bacteria were important in the host's innate response and this study has shown that LOS moities such as the ChoP play an important role. (6) The study has also shown that T cells produce

different cytokine profiles upon restimulation with bacteria in vitro, supporting the hypothesis that these cells are important contributors of the acquired immune response to infection through their ability to affect the host's innate immune system.

In summary, this thesis has contributed to our understanding of host responses to NTHi infection through characterising the innate immune response to NTHi infection and the regulation of the production of cytokines and chemokines in the lung. The keys factors identified were the mechanism by which mucosal immunisation enhances clearance of NTHi from the lung through the controlled regulation of cytokines and chemokines. Control of the innate immune response appears to be important for responding to and resolving the bacterial infection and inflammatory response in the lung. A controlled innate response is important in regulating factors that may lead to an enhanced disease state.

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