

**Differential Alveolar Epithelial Injury and Protein
Expression in Pneumococcal Pneumonia**

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ABSTRACT

Rationale: *Streptococcus pneumoniae* is a major cause of community-acquired pneumonia and its damaging effects on lung tissue can be extensive. The alveolar epithelium consists of alveolar type I (ATI) cells, involved in gas exchange, ATII cells, which are progenitor cells which secrete surfactant. Cell-specific marker proteins can be used to look at targeted epithelial injury by measurement in broncho-alveolar lavage (BAL) fluid, and expression of cell-specific proteins in lung tissue can be used to identify changes in cell population and functionality. The initial purpose of this thesis was to examine differential alveolar epithelial injury in pneumococcal pneumonia.

Results: Non-lethal rat models of acute (24 hours), resolving (72 hours) and recovering (3 weeks) pneumococcal pneumonia were set up, by way of intratracheal inoculation of wildtype D39 *S. pneumoniae*. Models were characterised by bacterial clearance with an acute inflammatory response and damage to the air-blood barrier that was resolving by 72 hours. Lungs were histologically normal after 3 weeks. Assessment of ATI cell-specific protein RTI40 demonstrated possible regulation in protein expression in both BAL fluid and lung tissue, rendering it inappropriate as a marker of injury in this model, and expression differed from that of other ATI cell-specific proteins. A new method of quantifying damaged ATII cells, using cell-specific proteins APN/MMC4, RTII70 and Pro-SP-C, showed targeted injury to these cells by 24 hours, which was shown to be resolving by 72 hours. Differential expression of ATII cell-specific surfactant proteins cytoplasmic Pro-SP-C and secreted SP-D was identified. Subsequent study to elucidate the mechanism of this differential protein expression compared the initial study with other models of lung infection. To determine the effect of pneumococcal toxin pneumolysin, PLN-A pneumolysin-deficient bacteria were used. Results showed that changes in expression of RTI40 and SP-D were not dependent on pneumolysin production. Further investigation to determine the extent that differential protein expression was characteristic of other Gram-positive infections *S. aureus*, wildtype 8325-4 strain was used. Results demonstrated that differential expression of RTI40 was specific to pneumococcal

infection but that expression of SP-D was common to other Gram-positive infections. To further explore the mechanism, *in vitro* bacterial co-culture experiments were carried out using *S. pneumoniae* D39 and the SV40-T2 alveolar epithelial cell line, to establish if the observation was caused by a direct effect by bacteria or bacterial products. These showed that expression of RTI40 and SP-D were not altered by *S. pneumoniae* alone.

Conclusions: Pneumococcal pneumonia induces specific injury to ATII cells and differential protein expression in both ATI and ATII cells. Expression of RTI40 is specific to the pneumococcus but expression of SP-D is common to other Gram-positive bacteria. Expression of neither protein was mediated by pneumolysin or by direct interaction of bacteria or bacterial products. These results provide valuable insight into host response to bacterial pathogens.

DECLARATION

All the work presented in this thesis has been carried out by myself, except where acknowledged otherwise.

This work has not been submitted for any other degree or qualification.

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ABBREVIATIONS

8325-4 = wildtype strain of *S. aureus*
A. fumigatus = *Aspergillus fumigatus*
AM = Alveolar macrophage
APC = Antigen presenting cell
APN = Aminopeptidase N
AQP = Aquaporin
ARDS = Acute respiratory distress syndrome
ATI = Alveolar type I
ATII = Alveolar type II
BAB = Blood agar base
BAL = Broncho-alveolar lavage
BSA = Bovine serum albumin
Can = Collagen-binding adhesin
CAP = Community acquired pneumonia
CbpA = Choline binding protein A
CC10(16) = Clara cell protein 10(16)
Clf = Clumping factor
C. neoformans = *Cryptococcus neoformans*
CNS = Central nervous system
COPD = Chronic Obstructive Pulmonary Disease
C. pneumoniae = *Chlamidia pneumoniae*
D10 media = DMEM + 10% FBS + L-glutamine
D39 = Wildtype strain of *S. pneumoniae*
DMEM = Dulbecco's modified eagle's medium
dNTPs = Deoxynucleotide-triphosphates
DPBS = Dulbecco's phosphate buffered saline
DPBS ^{+/+} = Dulbecco's phosphate buffered saline containing calcium and magnesium
DPBS ^{-/-} = Dulbecco's phosphate buffered saline without calcium and magnesium
DPPC = Dipalmitoylphosphatidylcholine
DTT = Dithiothreitol
ECL = Enzymatic chemiluminescence
ECM = Extracellular matrix
E. coli = *Escherichia coli*
EDTA = Ethylenediaminetetraacetic acid
ELISA = Enzyme-linked immunosorbent assay
FACS = Fluorescence-activated cell-sorting
FBS = Foetal bovine serum
FBI = Fluorescent bead immunoassay
FITC = Fluorescent isothiocyanate
FnBP = Fibronectin binding protein
GM-CSF = Granulocyte macrophage colony-stimulating factor
GMO = Genetically modified organism
GORD = Gastro-oesophageal reflux disease
H₂O₂ = Hydrogen peroxide
H&E = Haemotoxylin and eosin
HAP = Hospital-acquired pneumonia
HCAP = Health-care-associated pneumonia
H. influenzae = *Haemophilus influenzae*
Hla = Alpha toxin
Hlb = Beta toxin

Hld = Delta toxin
Hlg = Gamma toxin
HRP = Horseradish peroxidase
HT156 = Human type I 56
Hyl = Hyaluronate lyase
ICAM-1 = Intercellular adhesion molecule 1
ICU = Intensive care unit
IFN- γ = Interferon gamma
Ig = Immunoglobulin
IIPs = Idiopathic interstitial pneumonides
IL = Interleukin
IPCVD = Interstitial pneumonia with collagen vascular disease
IPD = Invasive pneumococcal disease
IPF = Interstitial pulmonary fibrosis
LBP = Lipopolysaccharide binding protein
L. pneumophila* = *Legionella pneumophila
LPS = lipopolysaccharide
LRTI = Lower respiratory tract infection
LTA = Lipoteichoic acid
LytA = N-acetylmuramic acid L-alanine amidase
KGF = Keratinocyte growth factor
K. pneumoniae* = *Klebsiella pneumoniae
MCP-1 = Monocyte chemotactic protein 1
MIP = Macrophage inflammatory protein
MMC4 = APN
MMC6 = protein on surface of ATII cells
M.O.I = Multiplicity of infection
M. pneumoniae* = *Mycoplasma pneumoniae
MRSA = methicillin-resistant *S. aureus*
NCTC = National collection of type cultures
NK = Natural killer
OCT = Optimum cutting temperature compound
OD = Optical density
P. aeruginosa* = *Pseudomonas aeruginosa
PAMP = Pathogen-associated molecular pattern
PAP = Pulmonary alveolar proteinosis
PBS = Phosphate buffered saline
P. carinii* = *Pneumocystis carinii
PCP = *Pneumocystis carinii* pneumonia
PCR = Polymerase chain reaction
Strep-PE = Streptavidin-phycoerythrin
PG = Peptidoglycan
PLN-A = Pneumolysin-deficient mutant of D39 *S. pneumoniae*
Ply = Pneumolysin
PMNs = Polymorphonuclear cells
PRR = Pattern recognition receptor
PsaA = Pneumococcal surface adhesin A
PspA = Pneumococcal surface protein A
PVDF = Polyvinylidene fluoride
PVL = Panton-valentine leukocidin
RBC = Red blood cell
RDS = Respiratory distress syndrome

RIPA = Radioimmuno precipitation assay
RTI40 = Rat type I 40
RTII70 = Rat type II 70
RT-PCR = Reverse transcriptase polymerase chain reaction
SARS = Severe acute respiratory syndrome
S. aureus* = *Staphylococcus aureus
SDS-PAGE = Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SP = Surfactant protein
SpA = Staphylococcal protein A
SPF = Specific pathogen free
S. pneumoniae* = *Streptococcus pneumoniae
SV40-T2 = Simian virus-transformed ATII cell line
TBS = Tris-HCl buffered saline
TBST = TBS containing 0.05% Tween 20
TGF- β = Transforming growth factor beta
TNF- α = Tumour necrosis factor alpha
TLR = Toll-like receptor
TSST-1 = Toxic shock syndrome toxin 1
VAP = Ventilator-associated pneumonia
VEGF = Vascular endothelial growth factor

CHAPTER 1: INTRODUCTION

1.1 : PNEUMONIA

1.1.1 Clinical Importance of Pneumonia

Pneumonia is an important infectious pathological lung condition, which was recognised as far back as ancient Greece and has been a key disease throughout history (Blasi et al., 2007). Even today pneumonia is still a significant cause of morbidity and mortality worldwide, and in ‘developed’ countries such as the USA it is the sixth most common cause of death, and has a mortality rate of 13.4 per 100,000 (Blasi et al., 2007; Franquet, 2001).

Most cases of pneumonia can be classified into one of two categories. community-acquired pneumonia (CAP), can affect even young healthy people, with an incidence of 6/1000 between the ages of 18-39, which rises to 34/1000 in the over-75s (Blasi et al., 2007). Hospital admission can be required in 20-50% of cases, and 5-10% need intensive care treatment (Blasi et al., 2007; BTS Guidelines, 2001; Franquet, 2001; Hoare and Lim, 2006). Mortality is higher in developing countries, the elderly and children (Blasi et al., 2007; Franquet, 2001; Hoare and Lim, 2006). When managed in the community, mortality is less than 1%; however, this rises to 5-12% of hospital admissions and is over 50% in patients admitted to the Intensive Care Unit (ICU) (Blasi et al., 2007; BTS Guidelines, 2001; Hoare and Lim, 2006). Nosocomial or Hospital-Acquired Pneumonia (HAP) occurs when the patient contracts the disease after admission to hospital and includes Ventilator-Associated Pneumonia (VAP) and Health-Care-Associated Pneumonia (HCAP) (ATS Guidelines, 2005; Franquet, 2001). Among hospital-acquired infections, HAP is a leading cause of death, with a mortality rate of 20-55% in the ICU (Franquet, 2001).

Pneumonia can be caused by a variety of microbiological agents, including fungi, viruses and bacteria. Fungal pneumonias are most significant in immunocompromised AIDS patients, particularly *Pneumocystis carinii* (*Pneumocystis jirovecii*) (Agarwal et al., 2006) pneumonia (PCP) (Franquet, 2001). Viruses, mostly influenza viruses, are responsible for 5-20% of out-patient CAP, especially in children, and are often associated with secondary bacterial superinfection (Blasi et al., 2007). They tend to be uncommon in severe pneumonia, i.e. requiring admission to the ICU; however, there are obvious exceptions to this, such as Severe Acute Respiratory Syndrome (SARS) and the 'avian flu' virus H5N1 (Blasi et al., 2007; Hoey, 1998; Zhang, 2003). Bacteria are the most common cause of pneumonia and treatment is usually based on antibiotics. However the increasing incidence of antibiotic resistance has resulted in a pressing need for additional therapies (Blasi et al., 2007). Vaccines against the most common causes of the disease, such as *Streptococcus pneumoniae* (pneumococcus), can be highly effective (AlonsoDeVelasco et al., 1995; Bricks and Berezin, 2006; Jedrzejcas, 2001), but it is unlikely to be practical to vaccinate against all causative agents, especially given that different patient groups can be susceptible to different organisms.

An estimated 5 million children under the age of 5 still die every year from pneumonia worldwide (Jedrzejcas, 2001). It therefore remains a major human disease that requires urgent further study. A more thorough understanding of how each organism instigates and develops the disease is vital in order to develop appropriate treatments.

1.1.2 Disease and Aetiology

Pneumonia is classified as an acute lower respiratory tract infection (LRTI), characterised by symptoms including shortness of breath and rapid breathing, cough often with production of sputum, and pleuritic chest pain combined with an increase in white blood cell count and fever (BTS Guidelines, 2001; Hoare and Lim, 2006). However identification of consolidation by shadowing in a chest radiograph is vital to accurate diagnosis (Franquet, 2001; Hoare and Lim, 2006). Figure 1.1 shows a chest radiograph from a patient with pneumonia (<http://www.emedicine.com>).

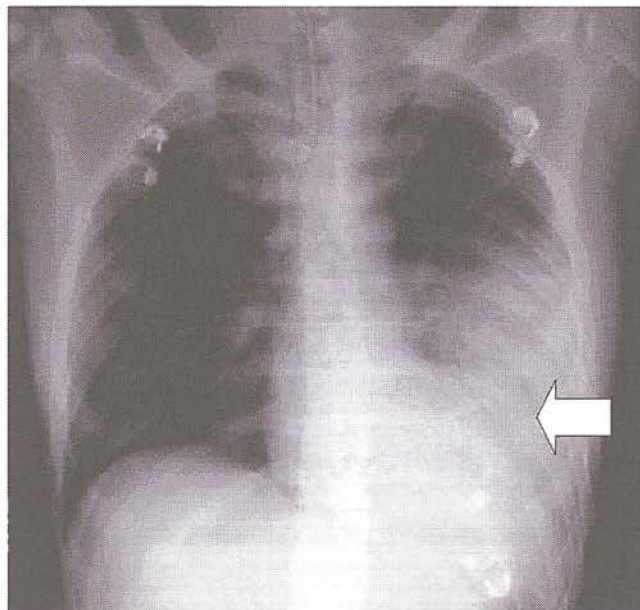


Figure 1.1 – A chest radiograph from a patient with bacterial pneumonia. Arrow shows consolidation in the lower left region.

Aetiological agents of pneumonia can differ considerably according to a variety of patient risk factors, as well as geographical location (Blasi et al., 2007). However, in a high proportion of cases the causative pathogen is never actually identified (Blasi et al., 2007; BTS Guidelines, 2001). By far the most common aetiological agents are bacteria, although this can be in conjunction with or following a viral infection (Blasi et al., 2007). Actual figures, even for Western Europe and North America, can vary

markedly, from 3-76%, but it is well established that the most common cause of CAP is *S. pneumoniae* (Jones et al., 2000; Lim et al., 2001; Logroscino et al., 1999; Sopena et al., 1999). Other common species such as *Haemophilus influenzae* can cause 10-20% of CAP cases treated in the community (BTS Guidelines, 2001; Vilar et al., 2004), but this drops to 2-7% in hospitalised patients (BTS Guidelines, 2001). *Mycoplasma pneumoniae* has been shown to account for up to 37% of CAP cases although numbers are highly variable due to cyclical epidemics, and this species tends to affect younger patients rather than the elderly (Blasi et al., 2007; BTS Guidelines, 2001; Vilar et al., 2004). Another so-called 'atypical' and difficult to diagnose intracellular pathogen, *Chlamydia pneumoniae*, is often coexistent with *M. pneumoniae* (Vilar et al., 2004) and is responsible for up to 20% of CAP cases, particularly in hospitalised patients (Blasi et al., 2007; BTS Guidelines, 2001; Vilar et al., 2004). *Legionella pneumophila* is rare in community-treated patients but is the causative agent for up to 17% of severe pneumonias admitted to the ICU (Blasi et al., 2007; BTS Guidelines, 2001). Aerobic Gram-negative bacilli such as Enterobacteriae, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* are rare in CAP, as is the Gram-positive coccus *Staphylococcus aureus*, however these species are much more prevalent in HAP infection (ATS Guidelines, 2005; Blasi et al., 2007; BTS Guidelines, 2001; Vilar et al., 2004).

Thus, although the pathogen-specific aetiology of pneumonia is by no means clear cut on presentation by the patient, the importance of bacteria as causative agents is well established, particularly that of *S. pneumoniae* in CAP.

1.1.3 Pathogenesis

Broadly speaking, there are two radiologically distinctive types of pneumonia, and although pathology is not necessarily indicative of causal agent, some pathogens can often exhibit radiological characteristics (Franquet, 2001; Vilar et al., 2004). Lobar pneumonia is characterised by continuous expression of disease, usually affecting only one lobe or segment (Vilar et al., 2004). In CAP lobar pneumonia is most frequently caused by *S. pneumoniae* (Franquet, 2001; Vilar et al., 2004), although other causes can be *K. pneumoniae*, *L. pneumophila*, *H. influenzae* and occasionally *M. pneumoniae* (Franquet, 2001). In contrast, bronchopneumonia has a diffuse multifocal distribution involving more lobes and is associated more in HAP with *S. aureus* and *H. influenzae* (Franquet, 2001; Vilar et al., 2004), although it can be also be caused by *S. pneumoniae* (Vilar et al., 2004). Interstitial pneumonia involves the walls of the bronchi and bronchioli and tends to be caused more by viral pathogens or unusual bacterial pathogens such as *M. pneumoniae* and *C. pneumoniae* (Franquet, 2001; Vilar et al., 2004).

Despite the variety of causative agents of pneumonia, and variations in pathological type, the basic pathogenesis of the disease remains essentially the same. Although other routes of infection such as direct inhalation can be important for environmental pathogens like *L. pneumophila* (Vilar et al., 2004), colonisation often begins in the nasopharynx with normally asymptomatic commensal organisms such as *S. pneumoniae* (AlonsoDeVelasco et al., 1995; Catterall, 1999; Tuomanen et al., 1995) and *S. aureus* (Lindsay and Holden, 2004). Adhesion to the mucosal epithelium is an important factor in nasal colonisation (Catterall, 1999; Tuomanen et al., 1995). Dissemination of bacteria from the nasopharynx to the lungs is dependent on a

number of factors, including bacterial virulence and host immune status. Pre-existing viral infection can reduce the host's immune capability (AlonsoDeVelasco et al., 1995; Catterall, 1999; Tuomanen et al., 1995), and microaspiration of bacteria from the nasopharynx is thought to enable translocation to the lower respiratory tract (Ben-David et al., 2005). If local defence systems are unable to deal with the invasion, inflammation becomes established and damage to lung tissue can occur, including that caused by the bacteria themselves and secondarily by factors released by inflammatory cells. This series of events is discussed in detail in Section 1.2.2 (Host Defence of the Lung). Alveoli become flooded with protein-rich exudate and erythrocytes from the circulatory system as the air-blood barrier is damaged and made more permeable and an acute inflammatory response with recruitment of neutrophils is initiated (Catterall, 1999; Tuomanen et al., 1995). Then, as consolidation is established, alveoli continue to fill up with blood products such as erythrocytes due to progressive leakage into the lungs (Tuomanen et al., 1995). As consolidation of the lungs progresses, deposition of fibrin reduces the influx of fluid across the air-blood barrier and allows clearance by recruited neutrophils (Catterall, 1999; Tuomanen et al., 1995). Finally, resolution occurs as leukocytes phagocytose any remaining debris and apoptotic neutrophils are cleared by macrophages (Catterall, 1999; Tuomanen et al., 1995). Diagram 1.2 shows normal (A) and pneumonic (B) human lung at the microscopic level (<http://teaching.path.cam.ac.uk>)

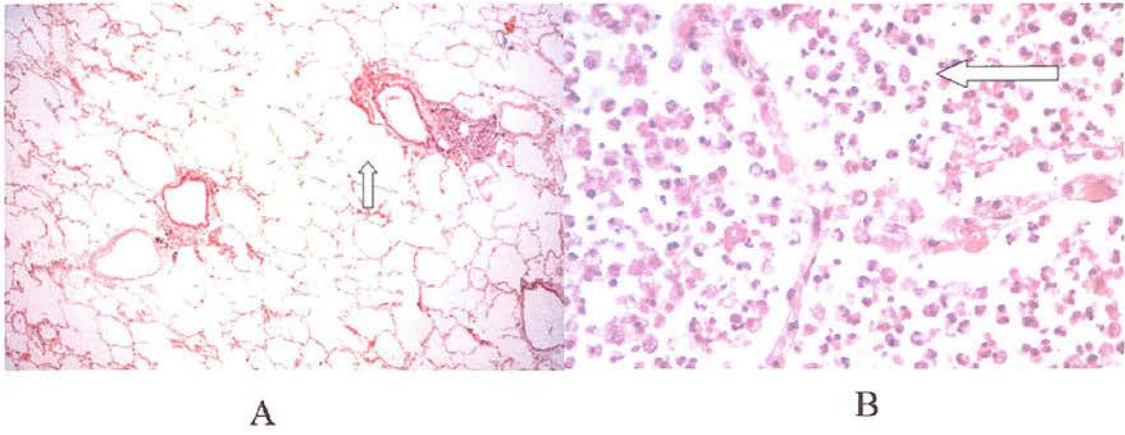


Figure 1.2 showing normal (A) and pneumonic (B) lung at the microscopic.
A. Arrow illustrates clear airspaces and conserved alveolar structure
B. Arrow shows alveoli filled with inflammatory cells and exudate

Although the basic pathogenesis of the disease is roughly similar regardless of pathogen-specific aetiology, there are variations between different causative agents, which can affect recovery. For example, lungs tend to completely return to normal in *S. pneumoniae* infection, whereas pneumonia caused by *S. aureus* and *K. pneumoniae* often leads to abscesses and fibrosis (Catterall, 1999; Tuomanen et al., 1995).

1.1.4 Summary

Pneumonia is a consolidated lower respiratory tract infection, which is a significant source of mortality and morbidity throughout the world. Bacterial infection is the cause of most cases. *S. pneumoniae* is the most important aetiological agent in community-acquired disease, although other species like *S. aureus* and *P. aeruginosa* are more prevalent in pneumonia contracted in hospital. Bacterial species, pre-existing viral infection and the ability of the host to defend itself are important factors in determining risk of development of disease. Colonisation often begins in the nasopharynx and progresses to the alveolar regions, leading to progression of disease then resolution. Pneumonia still kills millions of children throughout the world every year, and understanding the effects and interactions of causative agents on the host systems is vital to successful treatment and prevention.

1.2: THE LUNG

1.2.1 Structure and Function

1.2.1.1 Overview

The fundamental purpose of the lungs is to facilitate gas exchange between the external environment and the circulatory system. It does this by providing a large surface area open to the atmosphere, enabling haemoglobin in blood erythrocytes to exchange excreted CO₂ for fresh O₂ (Weibel, 1983). In 1962 Weibel and Gomez described 3 functional zones in the lung;

1. The Conducting Zone, facilitating the distribution of gases
2. The Respiratory Zone, dedicated to gas exchange
3. The Intermediate or Transitory Zone, linking the conducting and respiratory zones and containing aspects of both (Weibel, 1973).

Figure 1.3 shows the main features of the Conducting, Intermediate and Respiratory Zones of the lung. (Adapted from http://www.anatomie.uni-tuebingen.de/project/proj1/stoffkatalog/Kursbuch.html#Trachea9_1)

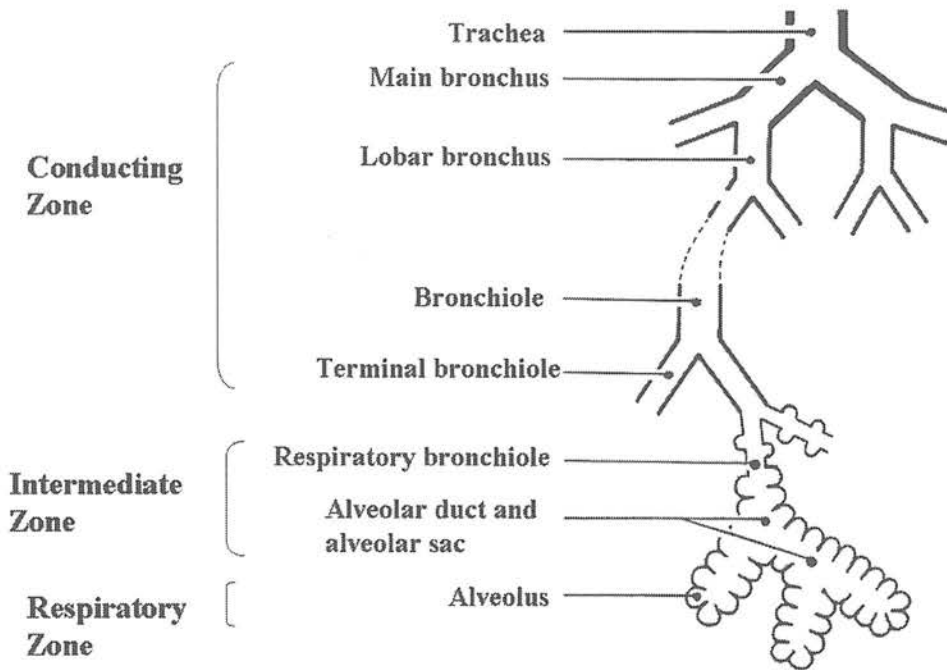


Figure 1.3 – Features of the Conducting, Intermediate and Respiratory Zones of the lung. The Conducting Zone comprises the bronchi and bronchioles as far as the terminal bronchioles. The Intermediate Zone is made up of the respiratory bronchioles, alveolar ducts and alveolar sacs. The Respiratory Zone is the alveoli. Adapted from http://www.anatomie.uni-tuebingen.de/project/projI/stoffkatalog/Kursbuch.html#Trachea9_1.

The basic unit of gas exchange, the acinus, comprises the respiratory bronchioles, the alveolar ducts and the alveolar sacs, which are clusters of alveoli (~0.25mm dia), where gas exchange between the air and bloodstream takes place (Burns et al., 2003; Sinnatamby, 1999; Weibel, 1973).

1.2.1.2 Respiratory Zone (Alveolar Region)

1.2.1.2.1 Overview

Figure 1.4 shows a stylized diagram adapted from Ware and Matthay (2000) showing salient features of a normal alveolus.

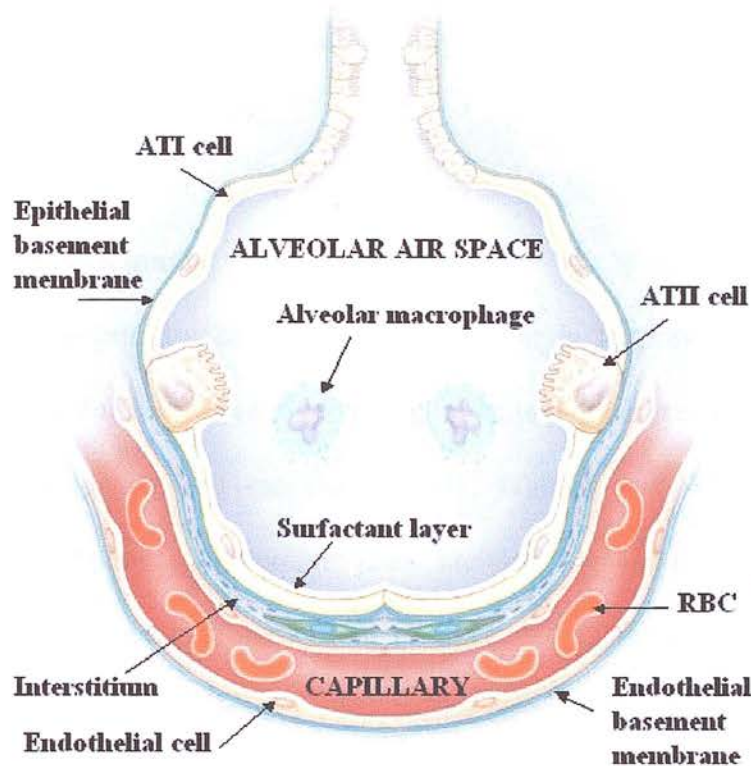


Figure 1.4 – Features of a normal alveolus, showing airspace and capillary. Note the alveolar epithelium covered by surfactant layer, and alveolar macrophages in the airway. The basement membrane and lung interstitium separate these from the blood vessel, which is lined with endothelial cells. Adapted from Ware and Matthay (2000).

The alveolar region, which is where exchange of gases takes place, is made up of fine tissue comprising a multitude of separated airspaces in order to maximise surface area available for gas exchange. The alveolar region is highly vascularised, and close contact (0.2-0.5 μ m) between the capillary endothelia and alveolar epithelia, separated

by a thin Extracellular Matrix (ECM) enables efficient movement of CO₂ and O₂ across the boundary (Burns et al., 2003; Weibel, 1973; Weibel, 1983). Because of the delicate nature of this alveolar airspace, it is highly susceptible to collapse. To prevent this it is lined with a phospholipid-protein layer known as surfactant (Weibel, 1973; Weibel, 1983). Underneath the surfactant layer, the alveolar epithelium is covered by an aqueous lining fluid, which is involved in the dissemination of surfactant components (Wright, 1997).

1.2.1.2.2 Surfactant

The primary purpose of pulmonary surfactant is to prevent alveolar collapse by providing a surface-active layer at the air-liquid interface, thus decreasing surface tension (Haagsman and van Golde, 1991; Whitsett and Weaver, 2002). However, some of the factors in the protein phase are also involved in protection of the lung as part of the innate immune system (Wright, 1997). Surfactant is made up of two components, 90% lipids and 10% surfactant-specific protein (Haagsman and van Golde, 1991), both of which are produced, degraded and recycled by lamellar bodies in alveolar epithelial type II (ATII) cells (Haagsman and van Golde, 1991; Whitsett and Weaver, 2002; Wright, 1997). Surfactant constituents are released into the aqueous fluid lining the epithelium (Haagsman and van Golde, 1991; Wright, 1997), where the surfactant layer, a biofilm, is assembled, along with the lamellar bodies forming tubular myelin, important for maintaining surfactant structure (Haagsman and van Golde, 1991; Whitsett and Weaver, 2002; Wright, 1997). Here, also, lipid and protein constituents are degraded and recycled by ATII cells, although degradation is also carried out by alveolar macrophages (Haagsman and van Golde, 1991; Kuroki and Voelker, 1994; Whitsett and Weaver, 2002; Wright, 1997).

The lipid phase of surfactant is the constituent that is primarily involved in decreasing surface tension, and is made up of 80-90% phospholipids which contains a very high proportion of phosphatidylcholine, particularly dipalmitoylphosphatidylcholine (DPPC), which is the main active ingredient of the biofilm. The additional presence of phosphatidylglycerol is important for the formation of tubular myelin (Haagsman and van Golde, 1991; Wright, 1997).

Despite the surface-tension-lowering capabilities of surfactant phospholipids, on their own as surfactant they perform poorly, being inflexible and slow to form lipid layers. The addition of surfactant-associated proteins enables efficient formation and modulation of surface tension (Whitsett and Weaver, 2002). In total there are 4 currently-identified surfactant proteins (SP); SP-A, SP-B and SP-C can be considered fully surfactant-associated, as they are bound to the phospholipid layer. SP-D, although categorised as a surfactant protein, differs in being mostly free from the lipid layer and is not localised to lamellar bodies (Beers and Fisher, 1992; Kuroki and Voelker, 1994). Surfactant proteins can be further classified into two groups; the hydrophobic proteins (SP-B and SP-C), and the hydrophilic collectins (SP-A and SP-D) (Haagsman and Diemel, 2001).

The hydrophobic surfactant proteins SP-B and SP-C are both small molecules; 8-15kDa and 3.5-6 kDa respectively, produced by post-translational cleavage of larger precursor proteins; Pro-SP-B and Pro-SP-C (Beers and Fisher, 1992; Kuroki and Voelker, 1994). Together they are involved in promoting the formation of phospholipid layers by enhancing adsorption, assimilation and organisation of lipids

into the monolayer and improving stability. They are also involved in the recycling of surfactant components, by enhancing the uptake of phospholipid by ATII cells (Haagsman and Diemel, 2001; Kuroki and Voelker, 1994; Whitsett and Weaver, 2002). SP-B, along with SP-A, is essential for the formation of tubular myelin (Kuroki and Voelker, 1994; Whitsett and Weaver, 2002). SP-B is produced both by ATII cells and bronchiolar Clara cells, and attachment of the protein to the lipid layer via cationic regions of the molecule enables lysis and fusion of vesicles to create layers of phospholipids (Kuroki and Voelker, 1994; Whitsett and Weaver, 2002). By this method it aids the formation of monolayers as well as organising storage within the lamellar bodies (Haagsman and Diemel, 2001; Whitsett and Weaver, 2002) and may also be how it aids the formation of tubular myelin from lamellar bodies (Whitsett and Weaver, 2002). In contrast, SP-C is localised specifically to ATII cells (Kuroki and Voelker, 1994; Whitsett and Weaver, 2002). It is highly hydrophobic and very abundant, making up about 4% of the surfactant components (Whitsett and Weaver, 2002). SP-C is palmitoylated, which enables it to incorporate within and between lipid layers, helping the formation of monolayers and disruption of vesicles allowing lipid dissemination (Whitsett and Weaver, 2002). Thus the hydrophobic surfactant proteins are crucial for adequate construction and homeostasis of the phospholipid surfactant layer.

The hydrophilic surfactant proteins, or collectins, are large, water-soluble oligomers that are highly post-translationally modified and produced by both ATII and bronchiolar Clara cells (Haagsman and Diemel, 2001; Kuroki and Voelker, 1994). Although both SP-A and SP-D are involved in maintenance of surfactant, their most important role is local host defence and they recognise a wide variety of microbial

pathogens and particles as well as interacting with alveolar macrophages to aid clearance (Haagsman and Diemel, 2001; Kuroki and Voelker, 1994). Like the hydrophobic proteins, SP-A is involved in regulation of secretion and reuptake of surfactant phospholipid (Kuroki and Voelker, 1994) and has opsonic activity, binding to alveolar macrophages and stimulating phagocytosis and chemotaxis (Crouch, 1998a; Kuroki and Voelker, 1994; Wright, 1997). SP-D, unlike other surfactant proteins, is not usually bound to the phospholipid layer, but is located in the aqueous lining fluid covering the alveolar epithelium (Kuroki and Voelker, 1994). Despite this, SP-D is thought to have some effect on surfactant homeostasis, although less so than SP-A (Kuroki and Voelker, 1994). Like SP-A, it is also an opsonin and binds to alveolar macrophages, but is more involved in bacterial killing, by enhancing aggregation of micro-organisms to improve phagocytosis and inducing O₂ radicals (Crouch, 1998a; Haagsman and Diemel, 2001; Kuroki and Voelker, 1994). In addition, both SP-A and SP-D have been shown to exhibit antifungal (McCormack et al., 2003) and antibacterial (Wu et al., 2003) capabilities.

The hydrophilic collectins thus provide a wide range of services within the airspace, including surfactant maintenance and host defence.

In summary, surfactant is a complicated amalgamation of lipids and proteins that work together in protecting the alveolus from both physical collapse and attack by external agents.

1.2.1.2.3 Alveolar Epithelium

The alveolar epithelium accounts for >99% of the internal surface area of the lung, and consists of two main cell types, known as alveolar epithelial type I (ATI), and

ATII cells (Dobbs et al., 1998; Weibel, 1973). ATI cells are large, thin, elongated, squamous cells (Weibel, 1973), with a diameter of 50-100 μm and a volume of 2000-3000 μm^3 (Chen et al., 2004a), exhibiting fine cytoplasmic extensions (Weibel, 1973). Although they constitute only 10-15% of the total number of lung cells, they account for about 95% of the alveolar surface, which is about 70 m^2 in humans (Rishi et al., 1995). ATI cells are thought to be important for exchange of gases (Rishi et al., 1995). They are anchored together by tight junctions (Weibel, 1973), and this, combined with the short diffusion pathway between the airspaces and bloodstream, make ATI cells efficient conduits for O_2 and CO_2 exchange. There is also evidence that ATI cells have other functions in addition to simple gas exchange. They have been shown to be highly water-permeable (Dobbs et al., 1998), contain transport proteins and ions (Dahlin et al., 2004) and have the capability to carry out liquid clearance, indicating a regulatory role in lung fluid dynamics (Ridge et al., 2003). It has long been assumed that these highly specialised ATI cells are terminally differentiated; however, there is some evidence suggesting that, in a foetal system at least, they are able to transdifferentiate into ATII cells (McElroy and Kasper, 2004).

In contrast to ATI cells, ATII cells are small, cuboidal cells (Weibel, 1973), with a diameter of 10 μm and a volume of 450-900 μm^3 (Chen et al., 2004a). They are located in the corners of alveoli, between ATI cells, and are identified by characteristic apical microvilli and cytoplasmic lamellar bodies (McElroy and Kasper, 2004; Weibel, 1973). Like ATI cells, they also account for 10-15% of total lung cells, although they make up only 5% of the alveolar surface area (Rishi et al., 1995). ATII cells are responsible for the production, secretion and recycling of surfactant components. The other important role that they are thought to fulfil is during repair, by repopulating the

alveolar epithelium following injury (McElroy and Kasper, 2004). They do this by a process of proliferation and differentiation into ATI cells to cover the denuded basement membrane (McElroy and Kasper, 2004). This differentiation can be observed easily *in vitro*, as isolated ATII cells readily undergo transdifferentiation into ATI-like cells over time, losing their ATII-like characteristics, such as lamellar bodies and microvilli, and adopting a flattened ATI cell phenotype (Borok et al., 1998a; Dobbs et al., 1988). ATII cells seem to regulate this repair process using subpopulations of proliferative daughter cells, which develop into ATI-like cells via intermediate cell types (Borok et al., 1998a; Reddy et al., 2004; Tyrrell et al., 2005). Thus ATII cells can be considered the progenitor cells of the alveolar epithelium. Additionally, ATII cells are involved in lung fluid regulation (McElroy and Kasper, 2004; Wright, 1997) and may also contribute to vascularisation due to their production of Vascular Endothelial Growth Factor (VEGF) (McElroy and Kasper, 2004; Raoul et al., 2004).

As well as the two main alveolar epithelial cell types, an additional type III or alveolar brush cell has also been described. These cells are very unusual and are characterised by large microvilli, which are consistently twice the length of those found on ATII cells, but do not contain lamellar bodies or produce surfactant (Kosłowski et al., 1998; Weibel, 1973).

Alveolar epithelial cells also have a role to play in lung defence. On their cell surface are Pattern Recognition Receptors (PRRs), such as Toll-Like Receptors (TLRs), that recognise Pathogen-Associated Molecular Patterns (PAMPs) expressed by microorganisms (Martin and Frevert, 2005; Paterson and Mitchell, 2006). Epithelial cells

produce inflammatory mediators such as cytokines and acute phase proteins as well as antimicrobial peptides (Delclaux and Azoulay, 2003; Martin and Frevert, 2005). ATII cells are particularly important due to their production of immunomodulatory collectins SP-A and SP-D as well as complement components and cytokines such as Monocyte Chemoattractant Protein 1 (MCP-1), Transforming Growth Factor Beta (TGF- β), Tumour Necrosis Factor Alpha (TNF- α) and a variety of Interleukins (ILs) (Fehrenbach, 2001; Wright, 1997).

In summary, the alveolar epithelium is populated mainly by two highly specialised cell types and is responsible for the primary function of lung tissue, gas exchange. The main purpose of ATI cells is to carry out this process, supported by ATII cells that have a protective role by providing repair capabilities and the production of surfactant. Both cell types contribute to lung defence against pathogens.

1.2.1.2.4 Pulmonary Endothelium and ECM

The pulmonary capillaries are lined with endothelial cells, 0.1-0.2 μm thick and 1000 μm^2 (Burns et al., 2003; Weibel, 1973), keeping the distance between the airway and bloodstream as narrow as possible to facilitate gas exchange. The ECM, consisting of the basement membrane and lung interstitium, lies between the pulmonary endothelia and epithelia and provides scaffolding for the lung ultrastructure (Burns et al., 2003; Weibel, 1983). The thickness of the ECM varies considerably. In places it consists of only a fused basement membrane between the endothelial and epithelial layers, but in other places they are separated by interstitium, made up of fibronectin, elastic fibres and collagen (Burns et al., 2003; Weibel, 1973).

Thus both the capillary endothelium and ECM maintain a thin barrier between the airways and blood system.

1.2.1.2.5 Alveolar Macrophages

Alveolar macrophages (AMs) are the resident defence cells in the gas-exchanging regions of the lung, responsible for protection against inhaled particles and microorganisms. AMs are located in the external environment of the alveolar airspaces (Dorger and Krombach, 2002; Knapp et al., 2003; Weibel, 1973). These cells are mononuclear phagocytes derived from stem cells in the bone marrow, entering the bloodstream as monocytes and migrating to the lung where they differentiate (Gjomarkaj et al., 1999; Takemura and Werb, 1984). These highly motile cells are abundant in the airways, making up the majority of cells recovered from Broncho-Alveolar Lavage (BAL) fluid (Wright, 1997). AMs have housekeeping roles in the normal lung by clearing up dead and dying host cells, and are involved in surfactant degradation (Delclaux and Azoulay, 2003; Wright, 1997). AMs are also involved in clearing the airways of invasive materials by phagocytosis (Delclaux and Azoulay, 2003; Dorger and Krombach, 2002; Weibel, 1973; Wright, 1997). Although they are quite capable of doing this without specific stimuli (Wright, 1997), their phagocytic potential can be enhanced by means of PAMP recognition (Martin and Frevert, 2005) and opsonisation (Delclaux and Azoulay, 2003; Dorger and Krombach, 2002; Martin and Frevert, 2005). Once the cell has engulfed the offending invader, it attacks it with intracellular toxic agents, such as reactive oxygen and nitrogen species and antimicrobial peptides (Delclaux and Azoulay, 2003; Dorger and Krombach, 2002).

As well as phagocytosing invading organisms, AMs also co-ordinate inflammation by releasing inflammatory mediators, including pro-inflammatory molecules like IL-1, 6 and 8, TNF- α and Macrophage Inflammatory Proteins (MIP) 1 and 2 and anti-inflammatory cytokines such as IL-10 (Delclaux and Azoulay, 2003; Dorger and Krombach, 2002). These mediators can influence lymphocytes (Delclaux and Azoulay, 2003) and activate Antigen Presenting Cells (APCs) such as lung dendritic cells (Martin and Frevert, 2005). By releasing these and other immunomodulatory proteins such as complement factors, the AM is able to orchestrate both the inflammatory and adaptive immune responses (Delclaux and Azoulay, 2003; Dorger and Krombach, 2002). Thus the AM has a dual role in protection of the lung; by clearing unwanted materials from the alveoli, and by initiating a more extensive immune response should it be unable to deal with the threat.

1.2.1.3 Summary

There are 3 main functional regions in the lungs. The Conducting Zones transports gases to and from the Respiratory Zone or Alveolar Region, where exchange of O₂ and CO₂ takes place. The Intermediate Zone spans both of these regions. The Alveolar Region comprises a delicate and complex network providing a short diffusion pathway to the bloodstream to enable effective gas exchange. This structure is maintained and protected from physical collapse by surfactant, a composite of lipids and proteins which decrease surface tension in the alveoli. The alveolar epithelium consists of two main cell types; ATI cells are elongated and thin, providing a large surface area through which gas exchange can occur. They are supported by ATII cells, which maintain the population of the alveolar epithelium and produce surfactant. AMs are the resident defence cells of the Alveolar Region, phagocytosing and clearing

inhaled agents, aided by surfactant proteins. In the event that the local airway defence systems become overwhelmed, the alveolar macrophages are able to co-ordinate a systemic inflammatory and adaptive immune response.

1.2.2 Host Defence of the Lung

1.2.2.1 Introduction to Host Defence

There are essentially two different facets to host defence in the lung, which work in close accordance to optimise protection against insult. Innate immunity is a ‘natural’ response involving a variety of host defence systems aimed at clearing infectious agents. The response is rapid and non-specific, covering a wide range of pathogens and is functional regardless of previous exposure (Boyton and Openshaw, 2002; Delclaux and Azoulay, 2003). In contrast, acquired immunity is an adaptive response to a given infectious agent that is swifter and more efficient on subsequent exposure to the same pathogen (Delclaux and Azoulay, 2003) but requires a prolonged activation period (Boyton and Openshaw, 2002; Delclaux and Azoulay, 2003; Rogan et al., 2006).

1.2.2.2 Innate Immunity

The first stage of lung host defence is in the nose, where convolutions in the passages, combined with nasal hairs, encourage adhesion of inhaled particles (Cohen and Gold, 1975). In the conducting airways, physical tracheo-bronchial clearance is co-ordinated by the muco-ciliary ladder (Chilvers and O'Callaghan, 2000). Secreted mucus provides a physical and chemical barrier to invasive agents and its highly adhesive nature enables particles to easily stick to it (Chilvers and O'Callaghan, 2000). Ciliated epithelial cells beat in a co-ordinated caudal to cephalic manner throughout the

epithelium to transport the mucus out of the airways to the oropharynx, where it is swallowed (Chilvers and O'Callaghan, 2000). Reflexes such as sneezing and cough are also important protective mechanisms (Boyton and Openshaw, 2002; Cohen and Gold, 1975; Delclaux and Azoulay, 2003).

If physical defences are evaded and foreign material reaches the Alveolar Region, detection of this material is crucial to efficient protection of the lung. The most important method of achieving this is through the use of PRRs, stimulating inflammatory functions and clearance (Paterson and Mitchell, 2006). These molecules include the TLRs, which are expressed on both innate immune cells and the respiratory epithelium and mediate production of inflammatory cytokines (Kadioglu and Andrew, 2004; Martin and Frevert, 2005; Paterson and Mitchell, 2006). In the case of bacterial infection, TLRs 2 and 4 are particularly important (Carpenter and O'Neill, 2007; Kadioglu and Andrew, 2004; Martin and Frevert, 2005; Paterson and Mitchell, 2006). Soluble proteins such as pulmonary Lipopolysaccharide binding protein (LBP) and soluble CD14 are also involved (Martin and Frevert, 2005; Paterson and Mitchell, 2006). The pulmonary collectins also function as opsonins, as do components of the complement cascade (Boyton and Openshaw, 2002; Paterson and Mitchell, 2006).

Various soluble factors and cell types then work together, to neutralise the infectious agents. Secreted products such as antimicrobial peptides, manufactured by immune and epithelial cells, work in concert with each other to destroy pathogens (Boyton and Openshaw, 2002; Delclaux and Azoulay, 2003; Martin and Frevert, 2005; Rogan et al., 2006). Components of the complement cascade, cytokines and the pulmonary collectins can also be directly bactericidal (Boyton and Openshaw, 2002; Delclaux

and Azoulay, 2003; Rogan et al., 2006). Resident AMs are the first cellular line of defence in alveoli, engulfing pathogens and using intracellular toxins to destroy them. Neutrophils, recruited by inflammatory mediators, are stimulated by opsonised particles, neutralising phagocytosed pathogens using intracellular toxins in a similar manner to the macrophage (Boyton and Openshaw, 2002; Delclaux and Azoulay, 2003). Natural Killer (NK) cells are lymphocytes that detect infected cells and lyse them by forming a pore in the membrane and injecting cytotoxin into the cell (Boyton and Openshaw, 2002; Delclaux and Azoulay, 2003).

Neutrophils are not usually present in high numbers in the airways and in order to deal with pathogen invasion they must be recruited from the bloodstream (Delclaux and Azoulay, 2003). Important inflammatory cytokines produced by activated macrophages and epithelial cells include IL-1, IL-6, neutrophil chemoattractant IL-8 (Boyton and Openshaw, 2002; Delclaux and Azoulay, 2003; Fehrenbach, 2001; Martin and Frevert, 2005) and MCP-1 (Martin and Frevert, 2005). Inflammatory cytokines help to control inflammation in the airways by activation of immune cells (Delclaux and Azoulay, 2003; Martin and Frevert, 2005) and upregulating the release of antimicrobial factors (Boyton and Openshaw, 2002), and soluble CD14 (Delclaux and Azoulay, 2003; Martin and Frevert, 2005). The pulmonary collectins can also activate macrophages. Neutrophils themselves also produce pro-inflammatory mediators and anti-microbial peptides (Boyton and Openshaw, 2002; Delclaux and Azoulay, 2003). Anti-inflammatory cytokines like IL-10, released by macrophages but not neutrophils, are responsible for controlling the influx of inflammatory products and decrease production of pro-inflammatory cytokines (Boyton and Openshaw, 2002; Delclaux and Azoulay, 2003; Martin and Frevert, 2005).

As well as mediating a localised inflammatory response, the innate immune system can also stimulate the adaptive response, particularly by the presentation of foreign antigens to acquired immune cells. This is done by APCs, the most notable of which is the dendritic cell which processes antigen and presents it to T lymphocytes (Boyton and Openshaw, 2002; Delclaux and Azoulay, 2003). Macrophages are less efficient APCs but can transport antigen to dendritic cells in the lymph nodes (Martin and Frevert, 2005). Soluble innate immune mediators such as IL-1, IL-6 and IL-10 can also activate the adaptive response, including stimulation of lymphocyte proliferation and antibody production by B cells, as well promotion of the T helper cell immune response (Delclaux and Azoulay, 2003; Martin and Frevert, 2005). The acquired immune system can also be stimulated by the complement cascade and NK cells (Boyton and Openshaw, 2002; Delclaux and Azoulay, 2003; Martin and Frevert, 2005).

1.2.2.3 Adaptive Immunity

The adaptive immune system relies on the recognition of specific foreign antigen to stimulate production of antibodies and effector T lymphocytes. In response to cues from the innate immune system, such as processing and presentation of foreign antigens and secretion of cytokines, pathogen-specific B and T lymphocytes proliferate (Boyton and Openshaw, 2002; Delclaux and Azoulay, 2003). They then release a variety of effector molecules, including cytokines by T lymphocytes and high affinity immunoglobulin (Ig) antibodies by B cells (Boyton and Openshaw, 2002; Delclaux and Azoulay, 2003). As a consequence, both T and B lymphocyte

memory cells are generated, enabling a swifter and more efficient response on additional exposure to the same pathogen (Boyton and Openshaw, 2002).

1.2.2.4 Summary

The defensive systems in place for defending the lung against microbial attack consist of two main aspects. The innate system includes physical, chemical and cellular elements that reduce the incidence of invasive agents in the delicate alveolar region and clear them if they do appear. In the event that these procedures are unable to deal with infection, chemical mediators released by immune cells and the respiratory epithelium are able to recruit and regulate a more systemic inflammatory response, bringing in other specialised immune cells from the circulation. The innate immune system is also able to regulate, and be regulated by, the adaptive immune system, which has chemical and cellular components to organise a longer-lasting specific response to any particular pathogen.

1.3: INJURY AND REPAIR OF THE LUNG

1.3.1 Lung Injury and Disease

1.3.1.1 Acute Injury of the Lung

Acute pulmonary damage can result from a variety of different causes, however the main response to injury is relatively conserved. Acute injury of the lung is characterised by compromise of the air-blood barrier, including damage of alveolar epithelium and capillary endothelium causing an influx of plasma and blood cells from the capillaries into the airspaces and interstitium (Matthay et al., 2003; Ware and Matthay, 2000). Alveolar macrophages respond to damaging stimuli such as bacteria by secreting cytokines, such as IL-1 and IL-8, which recruit neutrophils from the circulation to the airways to help combat the threat. Movement of materials across the air-blood barrier can go in either direction (Ware and Matthay, 2000). ATI cells are more vulnerable to injury than ATII cells; however, when the progenitor cells are damaged this can potentially affect both surfactant production and epithelial repair. Damage resulting in the death of these cells may strip the alveolar basement membrane (Matthay et al., 2003; Ware and Matthay, 2000). Figure 1.5 shows a stylised diagram adapted from Ware and Matthay (2000) illustrating the main features of alveolar injury and inflammation compared to the normal alveolus.

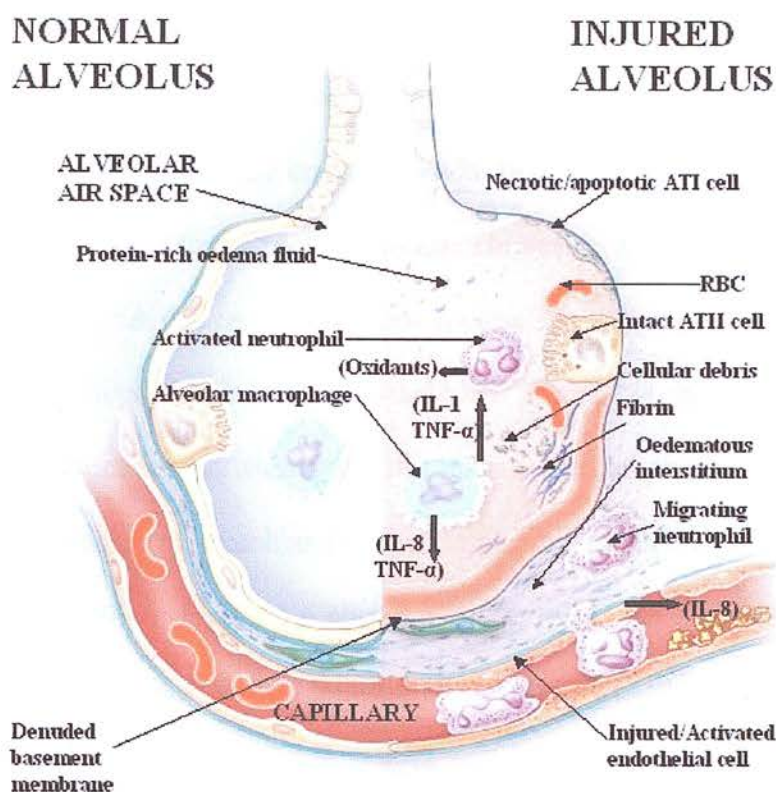


Figure 1.5 – Features of acute alveolar injury (right) and inflammation compared to the normal alveolus (left). In the normal alveolus, note the clear airspace with intact epithelium and absence of neutrophils. In the injured alveolus there is damage to the epithelium and a denuded basement membrane. Alveolar macrophages are releasing inflammatory cytokines IL-1 and TNF- α and neutrophil chemoattractant IL-8. Neutrophils are recruited from the bloodstream and travel to the airways via the interstitium through the endothelium. Airspaces are filled with debris, red blood cells (RBC), fibrin and neutrophils releasing oxidants. Adapted from Ware and Matthay (2000).

Following acute lung injury, there are two main outcomes; repair and resolution of the damage, or prolonged inflammation and remodelling leading to fibrosis (Wallace et al., 2007). If the injurious stimulus is neutralised rapidly, ATII cells can proliferate and differentiate into ATI cells, repopulating the alveolar epithelium and re-

establishing appropriate fluid dynamics (Ware and Matthay, 2000). Extraneous protein and apoptotic neutrophils are phagocytosed by macrophages, with some clearance of protein carried out by epithelial cells, bringing about the restoration of normal lung tissue (Ware and Matthay, 2000). However, a sustained stimulus or remodelling abnormalities can result in fibrosis (permanent scarring resulting from the inability to properly repair the alveolar epithelium). Fibrosis is characterised by the loss of alveolar structure, activation of fibroblasts and continued deposition of extracellular matrix molecules such as fibronectin and collagen (Wallace et al., 2007; Ware and Matthay, 2000). Figure 1.6 shows a stylised diagram adapted from Ware and Matthay (2000), demonstrating resolution versus fibrosis in the alveolus.

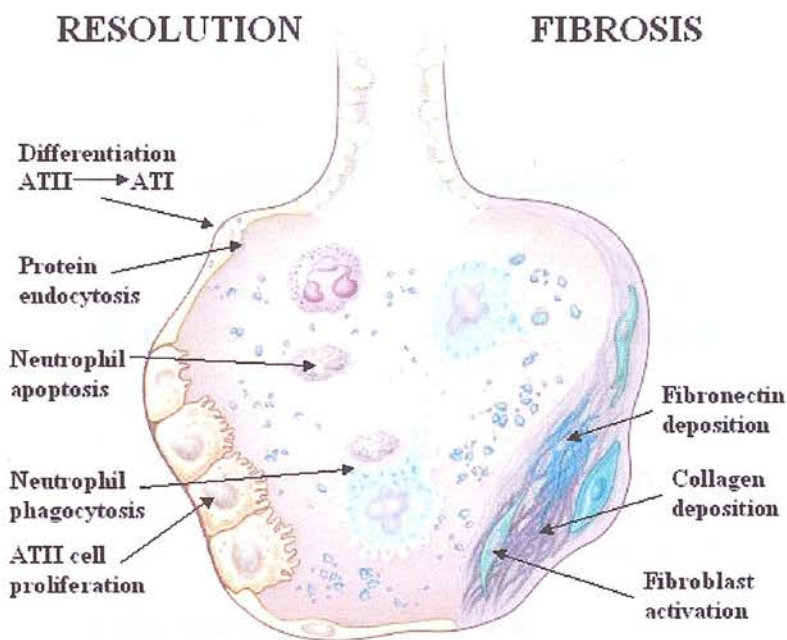


Figure 1.6 – Features of injury resolution (left) compared to fibrosis (right). In resolution, note the repopulation of the basement membrane by proliferation and differentiation of ATII cells and clearance from the airways of protein and apoptotic neutrophils by epithelial cells and macrophages. In fibrosis, note the

activation of fibroblasts and deposition of fibronectin and collagen, leading to a change in alveolar architecture. Adapted from Ware and Matthay (2000).

Acute injury of the lung can be caused by a variety of physical, chemical and biological agents. These injuries to the lung can result from direct insult or occur indirectly as a consequence of sepsis or trauma (Ware and Matthay, 2000). Even techniques devised to physically aid respiration, such as ventilation, can cause damage; for example if high tidal volumes are used (ARDSnet, 2000; Frank et al., 2002). A vast array of chemicals is also able to cause injury to the lungs. These include industrial silica, air pollutants (Chauhan and Johnston, 2003; Hastings et al., 2002a), high levels of oxygen (Hastings et al., 2002b) and drugs such as bleomycin (Wilberding et al., 2001). The injurious effects of hydrochloric acid on lung tissue seen in patients who have aspirated gastric contents is also clinically important (Frank et al., 2002; Ware and Matthay, 2000). Pneumonia induced by respiratory pathogens is one of the most common causes of biologically-induced lung damage (Ware and Matthay, 2000). In addition to external factors, host defence systems themselves can initiate injury to the lungs, particularly in situations of chronic inflammation where tissue remodelling can lead to fibrosis (Wallace et al., 2007).

1.3.1.2 Bacterial Infection of the Lungs

Bacterial infection is a significant and common cause of lung injury. This section will focus on the pathogenesis of pneumonia induced by two clinically important Gram-positive pathogens; *S. pneumoniae*, which is the chief aetiological agent of CAP, and *S. aureus*, an important cause of HAP. These are the two species of bacteria used in this thesis.

In vivo studies have shown that both pneumococcal and staphylococcal pneumonias are characterised by an acute, mainly neutrophilic inflammatory response (Kadioglu et al., 2000; Labandeira-Rey et al., 2007; McElroy et al., 1999; Yoneda and Coonrod, 1980). Alveoli are filled with plasma and erythrocytes following damage to the air-blood barrier (Labandeira-Rey et al., 2007; McElroy et al., 1999; Rubins et al., 1992; Yoneda and Coonrod, 1980), giving rise to the characteristic pneumonic 'hepatisation' (Bergeron et al., 1998). Both bacteria induce considerable injury to lung tissue (Bergeron et al., 1998; Wardenburg et al., 2007). Much of this damage is caused by pore-forming toxins, which are virulence factors produced by the bacteria. *S. pneumoniae* also releases an oxidant, hydrogen peroxide (H₂O₂), which can damage epithelial cells (Duane et al., 1993; Feldman et al., 2002).

1.3.1.3 Summary

Acute injury to the lung can be caused by a variety of environmental factors, not least of which is bacterial pneumonia. It is characterised by compromise of the air-blood barrier, complete with resultant flux of moieties across it, acute inflammation and damage to alveolar epithelial cells in particular. Injury to pulmonary tissue can result in either repair and resolution or fibrosis. Much of the response to clinically important Gram-positive bacteria is mediated by toxins produced by the microorganisms.

1.3.2 Animal Models of Lung Disease

1.3.2.1 Overview

Animal models, particularly those using rodents, can be a useful way to study the mechanisms of lung disease, although they are clearly not able to fully reproduce the clinical situation. In pneumonia, for example, the full natural progression from commensal nasal colonisation to lung consolidation in humans is dependent on a range of factors which would be impossible to reproduce experimentally. However, it is feasible to examine individual aspects of disease provided that conclusions are reached based on the scope of the experiment conducted and not extrapolated to other aspects. Similarly, host responses to stimuli differ between species; for example, rodents do not manufacture the antimicrobial peptide and elastase inhibitor Elafin which is present in humans (Sallenave et al., 1998). Nevertheless, models in whole living mammalian systems enable detailed examination of events from initiation to resolution, in intact respiratory and immune systems, that is not possible with clinical and *in vitro* studies.

1.3.2.2 Techniques

When considering the use of animal models of lung injury, the first thing to bear in mind is the method of injury. In pneumonia models, this entails the delivery of infectious agents into the lungs. Previously, this has involved surgical intubation, i.e. tracheotomy and instillation via a cannula inserted directly into the lungs (Rhodes et al., 1989a; Rhodes et al., 1989b; Yoneda and Coonrod, 1980). More recently, researchers have employed non-surgical intubation, where a cannula is inserted down the trachea via the mouth (McElroy et al., 2002; Rubins et al., 1995; Tyrrell et al., 2005). These techniques enable direct delivery of a set number of bacteria to the

alveolar region and are often able to model a precisely located lobar pneumonia. Other techniques involve inhalation of infectious material. These include intranasal methods (Bergeron et al., 1998; Brown et al., 2002; Dallaire et al., 2001; Garcia-Suarez et al., 2007; Kadioglu et al., 2000) as well as the use of nebulisers (Clement et al., 2008). While both of these techniques may be representative of clinical methods of microaspiration, they are likely to allow for less control over the numbers of bacteria reaching the alveoli. In this respect the use of nebulisers could be more reliable, as this technique would be less likely to result in loss of inoculum by swallowing and attachment of bacteria to the nasopharynx. Having established the lung injury, it is then necessary to assess the extent of the damage and inflammatory response. The most obvious way to do this is to examine lung tissue morphologically using histological techniques, including both light and electron microscopy and immunohistochemical methods (Rhodes et al., 1989a; Rhodes et al., 1989b; Tyrrell et al., 2005; Yoneda and Coonrod, 1980). However, if a more quantitative result is required this is usually done by collection of BAL fluid, which can then be analysed as being representative of events occurring in the airways. The degree of inflammation can be measured in a number of ways, including direct counts of inflammatory cells and assessment of inflammatory cytokines (Bergeron et al., 1998; Dallaire et al., 2001; McElroy et al., 2002). Damage to the air-blood barrier can be assessed by measurement of protein flux across the barrier. A simple method of doing this involves measurement of protein in BAL fluid (McElroy et al., 1999; McElroy et al., 2002; Rubins et al., 1995). Other methods include the use of radio-labelled albumin (Frank et al., 2005; McElroy et al., 1995; McElroy et al., 1997a).

As well as assessing the injury to the air-blood barrier it is also possible to quantify differential damage to specific cell types within the lung, using cell-specific proteins. It is possible to use these cell-associated proteins to measure injury to lung cells by analysing their presence in BAL fluid. Many researchers have used this technique to quantify injury to ATI cells, using the RTI40 protein (Frank et al., 2002; Frank et al., 2005; McElroy et al., 1995; McElroy et al., 1997b; McElroy et al., 1999; McElroy et al., 2002). Analysis of lung tissue itself can also provide information about effects of disease on the host. Assessment of lung tissue using cell-specific proteins can also provide information about changes in cell populations during injury (Tyrrell et al., 2005).

1.3.2.3 Models of Pneumococcal and Staphylococcal Pneumonia

Many researchers have used rodent models of pneumococcal and staphylococcal pneumonia to elucidate mechanisms of injury (Garcia-Suarez et al., 2007; McElroy et al., 1999; McElroy et al., 2002; Rubins et al., 1996) and host response (Bergeron et al., 1998; Brown et al., 2002; Coonrod and Yoneda, 1982; Kadioglu et al., 2000; Rubins et al., 1996), to study bacterial pathogenesis (Benton et al., 1997; Berry et al., 1989a; Berry et al., 1992; Dallaire et al., 2001; Feldman et al., 1991; McElroy et al., 1999; McElroy et al., 2002; Rubins et al., 1995), and to test new treatments (Ellbogen et al., 2003; Hegde et al., 2008; Karzai et al., 1999; Lock et al., 1992). This section will review *in vivo* research concerning lung injury and host response in pneumococcal and staphylococcal pneumonia.

Although there is a large body of work studying staphylococcal pneumonia, particularly using mice, there are relatively few papers that address the question of

mechanisms of injury to lung tissue. McElroy et al's initial *S. aureus*-induced pneumonia model in the rat used a high bacterial dose for 4 hours in ventilated animals, and demonstrated an acute inflammatory response with significant damage to the air-blood barrier and targeted injury to ATI cells (McElroy et al., 1999). This was assessed using the cell-specific protein RTI40 in BAL fluid. Using mutant bacteria, this selective injury was shown to be mediated by staphylococcal α -toxin, although this was not thought to be a direct cytotoxic effect on the epithelial cells (McElroy et al., 1999). A recovering model (which the author was directly involved in) was then established using a lower dose of bacteria in non-ventilated rats. This showed that even though the bacteria were cleared from the lungs after 24 hours, they still induced an acute inflammatory response with compromise of the air-blood barrier. However, the increase in RTI40 protein in BAL fluid was not significantly different from controls (McElroy et al., 2002). Animals continued to recover over time and by 96 hours post-inoculation the inflammatory response was abated and protein movement across the air-blood barrier was decreased. As with the previous model, this study used mutant bacteria to demonstrate that *in vivo*, the presence of fibronectin-binding proteins stimulates clearance of *S. aureus* from the lungs (McElroy et al., 2002). This model was used again at a 72 hour timepoint to demonstrate a decrease in ATI cells and an increase in ATII cells in the lungs during repair, by assessing cell-specific proteins in lung tissue (Tyrrell et al., 2005). In addition an intermediate cell type co-expressing proteins specific to both ATI and ATII cells was demonstrated (Tyrrell et al., 2005).

A morphological study using a rat model of recovering lobar pneumonia induced by *S. pneumoniae* serotype 25 examined the histological features of pathogenesis, but

differential damage to alveolar epithelial cells was not addressed (Yoneda and Coonrod, 1980). Another rat model demonstrated targeted damage to ATI cells; however, this study used the related bacterium *Streptococcus sanguis* as representative of pneumococcal disease (Rhodes et al., 1989b). The same authors, using pneumococcal serotype 25, showed damage to ATII cells, but this model appeared to induce an unusual fibrotic pathology (Rhodes et al., 1989a) and it is questionable how relevant these latter two results are to the typical disease caused by *S. pneumoniae*. There is also an extensive body of work in mice, which establishes the importance of the pneumococcal toxin pneumolysin (Ply) as a virulence factor. In particular, the use of mutant bacteria to induce pneumococcal pneumonia has shown that Ply affects host survival (Berry et al., 1989a; Berry et al., 1992; Rubins et al., 1996), bacterial growth and bacteraemia (Kadioglu et al., 2000; Rubins et al., 1995; Rubins et al., 1996). Animal models have also shown that Ply contributes to air-blood barrier damage (Rubins et al., 1995) and affects the inflammatory response (Kadioglu et al., 2000; Rubins et al., 1996). Studies using wildtype bacteria, usually serotypes 2 or 3, have shown *S. pneumoniae* to induce an increase in BAL leukocytes, particularly neutrophils, up to 24 hours post-instillation (Bergeron et al., 1998; Dallaire et al., 2001; Kadioglu et al., 2000; Rubins et al., 1996), an increase in lung TNF- α and IL-1, including IL-1 β , by 12 hours and MCP-1 by 24 hours (Bergeron et al., 1998; Dallaire et al., 2001). Damage to the air-blood barrier was detected as early as 3 hours post-inoculation (Rubins et al., 1995; Rubins et al., 1996). However, to date, no studies have looked at specific damage to individual cell types and most of this research has involved the use of lethal rather than recovering pneumonia models.

1.3.2.4 Summary

Rodent models of infective lung injury are a good way of studying mechanisms of disease. A variety of techniques can be used to provide information about pulmonary injury and host response. Rat models of staphylococcal pneumonia have shown an acute inflammatory response and damage to the air-blood barrier, particularly ATI cells. *In vivo* models of pneumococcal pneumonia in mice have also demonstrated an inflammatory response and pulmonary injury; however, information about cell-specific injury in a recovering model is lacking.

1.4: BIOMARKERS

1.4.1 Overview

Biomarkers can be defined as quantifiable moieties, measurement of which provides information about either normal or abnormal biological processes, and include the use of imaging techniques, and assessment of DNA and protein (Gundert-Remy et al., 2005). Disease can induce changes in both the expression of genes and function of proteins. Studying the dynamics of these changes not only enables more efficient diagnosis and assessment of prognosis, but also can offer insights into how disease affects the body. Proteins in particular can be obtained by a variety of invasive and non-invasive techniques and used to monitor disease. For example, detection of microalbuminuria is a commonly-used marker of kidney damage and cardiovascular disease (Erdmann, 2006) and podoplanin (RTI40) has been used as a marker of the lymphatic endothelium, both as a prognostic indicator and to establish a lymphatic origin of tumours (Birner et al., 2000; Sinzelle et al., 2000). In the lung, cell-specific proteins have been used to quantify targeted cell injury and identify changes in cell populations during injury and repair. Studying differential changes in cell-specific proteins following infection provides information about bacterial effects on the host as well as regulation of specific proteins.

1.4.2 ATI Cell-Specific Proteins

1.4.2.1 RTI40

1.4.2.1.1 Overview

RTI40 (GENE 54320, OMIM 608863) is a 40kDa protein located on the apical surface of ATI cells (Dobbs et al., 1988). It was first identified in the lung using a monoclonal antibody against rat ATI cells, and was consequently abbreviated from 'Rat Type I cell, 40kDa' (Dobbs et al., 1988). Although at the time it was thought to be unique to the lung, a number of homologues in other species and tissues have been discovered. The protein has also been identified in murine, canine and human systems and detected in kidney, lymphatic and vascular endothelium, bone, testis, Central Nervous System (CNS), thymus, dermal fibroblasts and a variety of benign and malignant neoplasms. Currently identified variants are T1 α protein (Rishi et al., 1995), podoplanin (Breiteneder-Geleff et al., 1997; Breiteneder-Geleff et al., 1999; Kahn and Marks, 2002; Kimura and Kimura, 2005; Parr and Jiang, 2003; Sinzelle et al., 2000), gp36 (Zimmer et al., 1999), gp38p (Boucherot et al., 2002), gp38 (Farr et al., 1992a; Farr et al., 1992b), gp 40 (Zimmer et al., 1995; Zimmer et al., 1997), E11 antigen (Wetterwald et al., 1996), RANDAM-2 (Kotani et al., 2002; Kotani et al., 2003), PA2.26 (Gandarillas et al., 1997; Martin-Villar et al., 2005), Aggrus/gp44 (Kato et al., 2003; Kato et al., 2005; Watanabe et al., 1988), OTS-8 (Nose et al., 1990; Ohizumi et al., 1998; Taniguchi et al., 2000), D2-40 and M2A antigens (Bailey et al., 1986; Marks et al., 1999).

Such extensive expression of a single protein in very different cells in a variety of organs may be explained by the complex structure of RTI40. The size of RTI40 varies between species, from about 36kDa in humans up to 44kDa in the mouse (Dobbs et

al., 1988; Watanabe et al., 1988; Zimmer et al., 1995; Zimmer et al., 1997). The open reading frame (ORF) codes for a predicted protein considerably smaller than this (Farr et al., 1992b; Kotani et al., 2002; Ma et al., 1998; Rishi et al., 1995; Scholl et al., 1999; Taniguchi et al., 2000; Wetterwald et al., 1996; Zimmer et al., 1997; Zimmer et al., 1999). However, there are multiple sites for post-translational modification, particularly glycosylation (Breiteneder-Geleff et al., 1997; Farr et al., 1992b; Martin-Villar et al., 2005; Scholl et al., 1999; Zimmer et al., 1999). These facts, combined with some decrease in molecular weight of the protein when treated with O-glycosidase (Kotani et al., 2003; Scholl et al., 1999; Toyoshima et al., 1995; Zimmer et al., 1995; Zimmer et al., 1997; Zimmer et al., 1999), are consistent with the idea that the RTI40 is indeed highly post-translationally glycosylated. This may explain the presence of different forms of the protein in various cells, tissues and species. Epitopes bound by different antibodies may be located on variable regions of modification, producing conflicting results from different research groups. Indeed, the E11 antibody (raised against a rat osteoblastic cell line) does not bind to the 18kDa core protein expressed from cDNA in transfected cells, whereas the RTI40 antibody (raised against isolated rat ATI cells) does (Williams et al., 1996). In addition, treatment with O-glycanase resulted in loss of reactivity to another antibody against the protein in the mouse (Toyoshima et al., 1995).

1.4.2.1.2 Function

Although RTI40 has been shown to be a platelet aggregation factor (Toyoshima et al., 1995; Watanabe et al., 1988; Watanabe et al., 1990), and is important for cancer metastasis (Sugimoto et al., 1991), as well as being a receptor for the Influenza C virus (Zimmer et al., 1995), its function in the normal adult lung is as yet unknown.

Despite its expression on ATI cells, which demonstrate high water permeability, the protein has been shown not to be involved in water transport or regulation of water channels (Ma et al., 1998), or transport of amino acids or folic acid (Boucherot et al., 2002). However, production of RTI40-deficient mice demonstrated that the protein is crucial for normal lung development (Ramirez et al., 2003). In the normal foetal rodent lung, RTI40 levels progressively increase during gestation (Williams and Dobbs, 1990). RTI40-deficient mice die of respiratory failure shortly after birth, demonstrating poorly-developed lungs with narrow airspaces and a decreased inflation volume, although a few ATI cells do develop (Ramirez et al., 2003). This phenomenon indicates that RTI40 is involved in pattern formation, perhaps through regulation of cell shape or motility. Indeed it has been demonstrated in a number of different cell types that RTI40 is not only frequently localised to a specific surface of the cell (Breiteneder-Geleff et al., 1997; Dobbs et al., 1988; Zimmer et al., 1997) and membrane projections, but that cells transfected to express the protein actively demonstrated a more elongated morphology and increased membrane protrusions, as well as tube-forming behaviour (Gandarillas et al., 1997; Martin-Villar et al., 2005; Schacht et al., 2003; Scholl et al., 1999). There is also evidence of a role for RTI40 in cell migration. Not only has the protein been shown to be involved in regulation and organisation of the actin cytoskeleton (Scholl et al., 1999; Scholl et al., 2000), but a wound-healing assay also demonstrated increased motility in RTI40-transfected keratinocytes (Scholl et al., 1999). *In vivo*, physical wounding of both skin and bone in rodents has resulted in induction or upregulation of the protein, suggesting a role in injury/repair (Gandarillas et al., 1997; Hadjiargyrou et al., 2001).

1.4.2.1.3. ATI Cell Marker

Despite the RTI40 protein's extensive expression by cells in different organs, much research has been carried out using it as a cell-specific marker. In non-respiratory organs, it has been used as a marker for glomerular epithelial cells (podocytes) in the rodent kidney (Kohda et al., 2000; Schiwiek et al., 2004), human lymphatic endothelium (Birner et al., 2000; Cursiefen et al., 2002; Hirakawa et al., 2003; Nisato et al., 2003) and as a marker of differentiation from osteocyte to osteoblast in tooth and bone in rats (Liu et al., 1997; Schwab et al., 1999; Tenorio et al., 1993; Wetterwald et al., 1996).

In the lung, RTI40 is a marker of rodent ATI cells. It has been used as a laboratory tool for improving the purity of isolated alveolar epithelial cells (Chen et al., 2004a; Dobbs et al., 1988; Roper et al., 2003); however, it is used more commonly to specifically identify ATI cells in various situations. This has included developmental ontology studies in both normal (Meneghetti et al., 1996; Williams and Dobbs, 1990; Williams et al., 1996) and transgenic (Cole et al., 2004) animals, and as a marker of differentiation from the ATII to ATI cell phenotype, where it characterises an intermediate transition cell type (Tyrrell et al., 2005). It has been shown both *in vitro* (Borok et al., 1998a; Gutierrez et al., 1998; Gutierrez et al., 1999; Gutierrez et al., 2003) and developmentally *in vivo* (Kitterman et al., 2002; Yoshizawa et al., 2003), that distension causes an increase in RTI40 expression, while contraction decreases it. *In vitro* this corresponds with a decrease in ATII cell-specific proteins and a more ATI cell-like phenotype. Other factors, such as serum, Keratinocyte Growth Factor (KGF) and VEGF result in a decrease in RTI40 and inhibition or delay in the development of the ATI phenotype (Borok et al., 1998a; Raoul et al., 2004).

1.4.2.1.4 Injury Marker

As well as being a well-established cell-specific marker in a variety of organs, RTI40 has also been used as a marker of injury and disease. Its induction in some cancer cells has led to its use as a human diagnostic marker of neoplasms, including mesothelioma (Kimura and Kimura, 2005), lymphangioma (Sinzelle et al., 2000), seminoma, dysgerminoma (Bailey et al., 1986) and Kaposi's sarcoma (Gessain and Duprez, 2005; Schmid et al., 2003). Expression on the lymphatic endothelium has also enabled investigation of lymphangiogenesis and prognostic significance in human tumours (Renyi-Vamos et al., 2005; Schoppmann et al., 2004; Valencak et al., 2004). In the lung it has been extensively used as a marker of ATI cell injury in a variety of rodent models. This was initially established using an acute rat model of pneumonia induced by *P. aeruginosa* (McElroy et al., 1995), which showed an 80-fold increase in RTI40 in BAL fluid, associated morphologically with extensive damage to ATI cells. However, this method has also been found to have a wide sensitivity range, with increases of about 2-fold being associated with less extensive damage to the alveolar epithelium (McElroy et al., 1997b; McElroy et al., 1997a). The technique has been used to assess an array of clinically relevant insults, including mechanical and chemical challenges and bacterial infections. Assessment of RTI40 has demonstrated injury to ATI cells in rat models of hyperoxia, high tidal ventilation volume following acid injury, bleomycin-induced pulmonary fibrosis and NO₂-induced emphysema (Frank et al., 2002; Koslowski et al., 1998; McElroy et al., 1997b; McElroy et al., 1997a). Bacterial lung infections caused by *S. aureus*, and *P. aeruginosa* have also shown damage to ATI cells, and have also been pivotal in identifying virulence factors (McElroy et al., 1995; McElroy et al., 1999; McElroy et

al., 2002). RTI40 has therefore been broadly used as a marker of ATI cell injury *in vivo*.

1.4.2.1.5 Summary

RTI40 is a protein that is widely used as a cell-specific marker in a number of organs. Its highly post-translationally modified structure has led to some controversy in the literature. In the lung, RTI40 is restricted to ATI cells and has been used as a biomarker both to identify ATI cells and quantify injury to them. RTI40 is essential for lung development, but its function in the normal adult lung is still unknown. It may have a role in the regulation of cell shape, migration and pattern formation.

1.4.2.2 Aquaporin 5

There are 11 known mammalian aquaporins (AQPs), which act as water channels, facilitating fluid movement by increasing the water permeability of the plasma membrane. They are therefore mainly found in tissues with high regulation of fluid (Gabazza et al., 2004; King and Agre, 1996). AQP5 is mercury-sensitive (Borok et al., 1998b; Krane et al., 2001), and was first identified in the salivary gland (King and Agre, 1996). In human and rat respiratory systems, it has been identified at the apical surface of sub-mucosal gland secretory cells of the nasopharynx and proximal airways, but is predominantly found on ATI cells (Gabazza et al., 2004; King et al., 1997). However, in mouse lung it is more extensively expressed, including on tracheal and bronchial epithelium and ATII cells (Krane et al., 2001), making rat models perhaps more representative of human lungs. The exact mechanism of AQP5 fluid transport is not well understood. AQP5-deficient mice demonstrated decreased saliva secretion and although there was a decrease in water permeability of the air-

blood barrier, fluid absorption from the lungs was not affected (Ma et al., 2000). In addition, they also showed hypersensitivity to cholinergic-stimulated bronchoconstriction (Krane et al., 2001). AQP5's specific location to ATI cells in rats and humans has led to its use as an ATI cell-specific marker *in vitro*, particularly to identify transdifferentiation of isolated ATII cells (Borok et al., 1998b; Chen et al., 2004b). It has also been used in conjunction with RTI40 *in vivo* to identify changes in cell populations during repair in a model of *S. aureus*-induced pneumonia (Tyrrell et al., 2005). However, decreased expression of AQP5, independent of changes in cell population, has been demonstrated in the lung in a number of models of injury, including adenoviral infection (Towne et al., 2000), bacterial product instillation (Jiao et al., 2002), and bleomycin-induced fibrosis (Gabazza et al., 2004). It has also shown to be downregulated by TNF- α in cultured mouse epithelial cells (Towne et al., 2001). Thus, while AQP5 has important potential uses as a cell-specific marker of ATI cells, expression may be differentially affected by a variety of factors.

1.4.2.3 MMC6

MMC6 is an antigen localised to the apical surface of ATI cells in the rat lung (Franklin, 2006). In the adult rat, MMC6 is unique to the lung, being undetectable in kidney, thymus, liver, brain, stomach, bladder, muscle, placenta and gonads, small and large intestine and macrophages (Franklin, 2006). The antigen has been identified as an epitope on a membrane-associated protein (Franklin, 2006) co-localised on the apical surface of ATI cells along with RTI40, and is not expressed on ATII cells, capillary endothelium, Clara cells or bronchiolar epithelial cells (Franklin, 2006; McKechnie, 2008). Developmentally, MMC6 is not detectable until E20, when it is co-expressed with RTI40 on squamous cells. However, RTI40 was expressed as early

as E17, suggesting that these are not the same protein. Like RTI40, MMC6 is not expressed in isolated ATII cells but was detected in cultured cells by day 3 (McKechnie, 2008). The protein has not been characterised and its function is as yet unknown (Franklin, 2006; McKechnie, 2008). The specific location of MMC6 suggests its use as a possible marker of rat ATI cells. Indeed, it has been used in conjunction with RTI40 to assess levels of epithelial cell damage in a recovering model of *S. aureus*-induced pneumonia in rats, where the increase in BAL fluid of MMC6 was shown to correlate well with that of RTI40, indicating its use as a marker of ATI cell injury (Franklin, 2006). It was also shown to correlate with RTI40 levels in BAL fluid in a haemorrhagic shock model of lung injury in the rat (McKechnie, 2008). In summary, although the identity and function of MMC6 is currently unknown, it has potential use as a cell-specific marker of ATI cells in rat models of lung injury.

1.4.3 ATII Cell-Specific Proteins

1.4.3.1 SP-C

As stated in section 2.1.3.1.2 (Surfactant), SP-C is a small, hydrophobic protein, produced only by ATII cells, which is involved in the formation and homeostasis of surfactant. Like its associated hydrophobic surfactant protein, SP-B, the mature form of SP-C is generated by post-translational cleavage of a larger precursor protein. This precursor protein, known as Pro-SP-C, is about 21kDa in size and is restricted to the cytoplasm, where it is processed in multivesicular bodies. Mature SP-C, about 3.5kDa, is then secreted into the airways (Beers and Fisher, 1992; Whitsett and Weaver, 2002). The study of SP-C has proved challenging in the past due to its highly hydrophobic nature; however, the production of antibodies against both Pro-SP-C and

SP-C have enabled progress to be made (Beers et al., 1992; Schmidt et al., 2002). Defects in the production of SP-C are characteristic of a number of lung diseases, including Acute Respiratory Distress Syndrome (ARDS) (Schmidt et al., 2002), Respiratory Distress Syndrome (RDS) of the newborn (Tong et al., 2006) and idiopathic interstitial pneumonides (IIPs) (Glasser et al., 2003). Indeed, studies on SP-C-deficient mice have demonstrated pulmonary pathology reminiscent of IIPs (Glasser et al., 2003) and SP-C is a crucial component of the synthetic surfactant used to treat RDS of the newborn (Beers and Fisher, 1992). Production of SP-C has been shown to be modulated by a variety of conditions, being increased by corticosteroids and hyperoxia (Beers and Fisher, 1992). It has been used as a biomarker both to investigate surfactant dysfunction (Russo et al., 2002; Savani et al., 2001; Vaporidi et al., 2005) and to determine the effects of mitogens on ATII cells (Raoul et al., 2004; Tong et al., 2006; Yano et al., 2000). SP-C has also been used as a marker of ATII cell phenotype (Gutierrez et al., 1998; Gutierrez et al., 1999; Gutierrez et al., 2003; Meneghetti et al., 1996; van Tuyl et al., 2005).

1.4.3.2 RTII70

RTII70 is an antigen located on the surface of rat ATII cells (Dobbs et al., 1998). Developmentally, it is expressed at E20 on ATII-like cells (McKechnie, 2008). RTII70 has been used as a cell-specific marker of ATII pneumocytes in a number of *in vivo* studies, including investigation of oligohydramnios and tracheal occlusion in rat foetuses (Kitterman et al., 2002; Yoshizawa et al., 2003), and a rat model of *S. aureus*-induced pneumonia (McElroy et al., 2002). The protein has also been used in conjunction with APN/MMC4 to identify intermediately differentiated alveolar epithelial cells during repair in a *S. aureus*-induced rat model of pneumonia, as well

as aiding in the description of a novel ATI cell-specific protein, MMC6 (McKechnie, 2008; Tyrrell et al., 2005). Although RTII70 has not yet been characterised and its function is unknown, it is an established cell-specific marker of rat ATII cells.

1.4.3.3 SP-D

As discussed in section 1.1.3.1.2 (Surfactant), SP-D is a large, hydrophilic, post-translationally modified protein expressed by both ATII and Clara cells. It is only minimally involved in surfactant maintenance and its main function is to interact with foreign particles to enhance their removal by phagocytes. As well as being present in high levels in the lungs, SP-D is also found in the gastrointestinal and genitourinary tracts, as well as a variety of glands (Griese and Starosta, 2005; Reid, 1998). The protein is a 43kDa monomer, but in most cases tends to form dodecamers, made up of four trimeric units in a cruciform structure. The presence of carbohydrate-binding domains, which are able to bind to a variety of microbial moieties in a calcium-dependent manner, are crucial to its role in recognising PAMPs (Crouch, 1998b). SP-D has been shown to be important in the clearance of DNA (Palaniyar et al., 2005) as well as a host of microorganisms. It is able to bind to, agglutinate and enhance neutrophil binding to viruses such as Influenza A (Hartshorn et al., 1998; Ofek et al., 2001; Restrepo et al., 1999), and can bind to and inhibit replication of HIV (Meschi et al., 2005). The protein also binds and aggregates a variety of fungi. These include *Aspergillus fumigatus*, for which it also enhances clearance by neutrophils (Madan et al., 1997), *P. jirovecii*, for which it enhances macrophage association but not phagocytosis (Ofek et al., 2001) and *Cryptococcus neoformans* (Restrepo et al., 1999). SP-D also affects Gram-negative bacteria, particularly through its binding lipopolysaccharide (LPS) leading to agglutination (Ofek et al., 2001). Specifically, it

has been shown to bind to and aggregate *E. coli*, to increase phagocytosis by neutrophils and to enhance association with macrophages, although it does not affect macrophage phagocytosis (Hartshorn et al., 1998; LeVine et al., 2000; Restrepo et al., 1999). *P. aeruginosa* is bound and agglutinated by SP-D, and is more readily phagocytosed by macrophages, although there is some controversy as to whether agglutination is required for this (Bufler et al., 2004; Giannoni et al., 2006; Griese and Starosta, 2005; Restrepo et al., 1999; Wright et al., 1999). *K pneumoniae* is bound, aggregated and phagocytosed more efficiently by neutrophils in the presence of SP-D (Ofek et al., 2001; Restrepo et al., 1999) and internalisation of *Chlamydia* spp by macrophages is also enhanced (Oberley et al., 2004). SP-D has been shown to bind to lipoteichoic acid (LTA) and peptidoglycan (PG) from Gram-positive bacteria (van de Wetering et al., 2001); however, there is some controversy over the actual effects. While there is evidence that it does indeed bind to and agglutinate pneumococcus (Hartshorn et al., 1998; Jounblat et al., 2004), contention remains as to whether it affects phagocytosis by alveolar macrophages (Kuronuma et al., 2004; Sano et al., 2006) and neutrophils (Hartshorn et al., 1998; Jounblat et al., 2004; Restrepo et al., 1999). Nevertheless, it is clear that SP-D influences clearance of *S. pneumoniae* from the lungs (Jounblat et al., 2005). Similarly, there is conflicting evidence about the aggregation properties of SP-D on *S. aureus* (Bufler et al., 2004; Hartshorn et al., 1998), although it does appear to enhance uptake by neutrophils (Hartshorn et al., 1998; Restrepo et al., 1999).

SP-D can be used as a marker of surfactant production. It has been shown to be decreased in BAL fluid in cystic fibrosis (Hartl and Griese, 2006; Postle et al., 1999), ARDS (Hartl and Griese, 2006; Kuroki et al., 1998), interstitial pulmonary fibrosis

(IPF), interstitial pneumonia associated with collagen vascular disease (IPCVD) (Kuroki et al., 1998), gastro-oesophageal reflux disease (GORD) and RSV infection (Hartl and Griese, 2006). SP-D is increased in BAL fluid in pulmonary alveolar proteinosis (PAP) (where it has been used as a diagnostic indicator) (Kuroki et al., 1998), sarcoidosis, asthma, and eosinophilic pneumonia (Hartl and Griese, 2006). Levels in serum are increased in Chronic Obstructive Pulmonary Disease (COPD) (Sin et al., 2007), IPF, PAP, IPCVD (Hartl and Griese, 2006; Kuroki et al., 1998) and sarcoidosis (Hartl and Griese, 2006). There is no effect on SP-D in serum in bacterial pneumonia (Kuroki et al., 1998). Thus, SP-D tends to be used as a marker of surfactant function, although it has been used as a cell-specific marker in differentiation during repair (Ohtsuki et al., 2007).

1.4.3.4 APN/MMC4

In 2001 an antigen was identified on the apical surface of rat ATII and Clara cells, during the development of a monoclonal antibody against isolated ATII cells. As well as being expressed in the lung, it was also found in the kidney and small intestine, but not the liver or on alveolar macrophages. This antigen was thought to be a novel protein, with the potential to be used as a marker of ATII and Clara cell injury in the rat, much in the same way that RTI40 is utilised to quantify ATI cell damage. It was designated MMC4 (Boylan et al., 2001). Purification of MMC4, however, revealed it to be rat aminopeptidase N (APN), a membrane-bound metallopeptidase, which is homologous to CD13 in the mouse (Franklin, 2006). This finding was surprising, as APN had already been eliminated as a candidate for MMC4 due to its presence in the liver and on the surface of macrophages (Boylan et al., 2001). However, further study showed that MMC4 was indeed expressed on a small percentage of alveolar

macrophages (Franklin, 2006). Although levels of the protein were increased in BAL fluid in a rat model of mutant (expressing the normally MRSA-specific surface protein Pls) *S. aureus*-induced pneumonia (but not using the wildtype strain) the APN/MMC4 protein was not considered suitable as a marker of damage to ATII and Clara cells due to its expression on alveolar macrophages (Franklin, 2006). However, the fact that the level of APN/MMC4 did not significantly increase in the wildtype staphylococcal pneumonia model, despite an obvious inflammatory response, suggests that there might still be a role for the protein as a preliminary screen to identify rat models that warrant further investigation into ATII and Clara cell injury. Furthermore, its use as a marker of ATII cells has already been established, as demonstrated by its contribution to the identification and investigation of an intermediate cell phenotype during differentiation, both *in vitro* and during repair *in vivo* (McKechnie, 2008; Tyrrell et al., 2005).

1.4.4 Clara Cell-Specific Proteins

1.4.4.1 CC10

Clara Cell Protein (GENE ID 7356, OMIM 192020), also known as CC10, CC16 and Clara Cell Specific Protein (CCSP), is a 16kDa protein secreted by bronchiolar Clara cells. Clara cell functions include protection of the respiratory tract, production of surfactant proteins and repair of the epithelium following injury by proliferation and differentiation into ciliated cells (Boylan et al., 2001; Broeckert et al., 2000; Magdaleno et al., 1997). CC10 has also been shown to be the same as alpha-microprotein (protein 1) in urine from patients with tubular dysfunction (Bernard et al., 1992; Magdaleno et al., 1997) and uteroglobulin in the uterus (Muller-Schottle et al., 1999). The exact function of CC10 is as yet unknown, but it is thought to play a

role in protection of the epithelium by immunosuppression, as it has been shown to have anti-inflammatory properties, inhibiting both Interferon gamma (IFN- γ) and TNF- α , as well as being upregulated by IFN- γ (Broeckaert et al., 2000; Magdaleno et al., 1997). As expected, CC10 is present at highest levels in the lung, but is detectable in both serum and urine (Halatek et al., 1998). Although CC10 has been used as a direct marker of Clara cells in the rat (Barth and Muller, 1999) and to measure renal injury in both rodents and humans (Bernard et al., 1992; Halatek et al., 1998; Hermans et al., 1998), its most common use as a biomarker is to assess air-blood barrier permeability and Clara cell damage. Its elevation in serum has been used in humans to demonstrate damage of the air-blood barrier in response to smoke, ozone and LPS-induced lung inflammation as well as in sarcoidosis (Bernard et al., 1992; Broeckaert et al., 2000; Hermans et al., 2001). A decrease in both BAL fluid and serum CC10 has been taken as evidence of damage to Clara cells in COPD, lung cancer and in response to tobacco smoke and crystalline silica in humans (Bernard et al., 1992; Broeckaert et al., 2000). In addition, it has also been described as a marker of leakage from the lungs into the pleural space in humans (Hermans et al., 1998). Thus, CC10 is a well-established marker of Clara cells, and has been used in a variety of settings to assess both Clara cell injury and air-blood barrier permeability.

1.4.5 Summary

Biomarkers, particularly proteins, can be a valuable source of information about inflammation, alveolar damage and surfactant production in the lung. Cell-specific proteins in particular can prove useful in investigating targeted cell injury, cell population dynamics and differentiation. In the lung these include RTI40, AQP5 and MMC6 (ATI cells), SP-C and RTII70 (ATII cell only), SP-D and APN/MMC4 (ATII and Clara cells) and CC10 (Clara cells only).

1.5: BACTERIA

1.5.1 Overview

Although there is a wide range of bacteria important in human lung infection, this section will focus on two clinically relevant microorganisms; *S. pneumoniae* and *S. aureus*.

1.5.2 *Streptococcus pneumoniae*

1.5.2.1 Clinical Importance of *Streptococcus pneumoniae*

S. pneumoniae is a major worldwide human pathogen, responsible for a variety of important diseases including pneumonia, bacteraemia, meningitis, otitis media and sinusitis (Bricks and Berezin, 2006; Garcia-Suarez et al., 2006; Lopez, 2006). However it is most important as the commonest aetiological agent of CAP.

Pneumococcal disease is highest in the young, the elderly and the immunocompromised (Bricks and Berezin, 2006; Garcia-Suarez et al., 2006; Lopez, 2006). In the developed world the incidence of invasive pneumococcal disease (IPD) is 10-20 per 100,000, with an overall mortality of 7-36%, although socio-economic factors can also contribute to risk (Parsons and Dockrell, 2002). In the developing world pneumococcal disease kills over 1 million children under the age of 5 (Bricks and Berezin, 2006) and worldwide approximately 5 million die every year due to pneumonia, the most common cause of which is *S. pneumoniae* (Jedrzejak, 2001).

S. pneumoniae is sensitive to a variety of antibiotics, particularly penicillin (Jedrzejak, 2001; Lopez, 2006; Restrepo et al., 2005). However, the emergence of penicillin-resistance was documented as early as the 1970s and is continuing to increase (Fuller

et al., 2005), with a worrying trend toward multi-drug resistance including cephalosporin and macrolides (Fuller et al., 2005; Jedrzejewski, 2001; Lopez, 2006; Pallares et al., 1995). Tolerance to vancomycin, a drug used only as a last resort, has already been shown (Novak et al., 1999), suggesting that development of full resistance is only a matter of time.

Development of a vaccine for pneumococcal disease started as far back as 1914 and a hexa-valent (6 serotype) vaccine was developed after WWII but was withdrawn in favour of antibiotic treatment (Watson et al., 1993). Emergence of antibiotic resistance in the 1970s fuelled interest in alternative treatments, and a 14-valent vaccine, based on the 14 serotypes causing 80% of disease, was finally developed in 1978 (AlonsoDeVelasco et al., 1995; Watson et al., 1993). A 23-valent vaccine, based on the most prevalent serotypes at the time, was licensed in 1983, but protection is low in those most susceptible to pneumococcal disease, such as children under the age of 2 (Bricks and Berezin, 2006; Jedrzejewski, 2001; Kalin, 1998). A heptavalent pneumococcal conjugate vaccine, which included those serotypes responsible for 85% of pneumococcal disease in the USA, was more suitable for young children and was licensed in 2000 (Bricks and Berezin, 2006). This appears to have had a positive effect on pneumococcal disease (Bricks and Berezin, 2006).

1.5.2.2 Bacterial Morphology

S. pneumoniae (known historically as *Diplococcus pneumoniae* and pneumococcus) was simultaneously and independently identified and cultured by both Louis Pasteur and George Sternberg from human saliva in 1881 (Watson et al., 1993). It was

established as an aetiological agent of lobar pneumonia in the late 19th century (Watson et al., 1993).

In taxonomic terms, *S. pneumoniae* is a Gram-positive bacterium, of the genus *Streptococcus* (Facklam, 2002; Hoskins et al., 2001). As a Gram-positive species, the surface of *S. pneumoniae* comprises a thick cell wall containing many layers of PGs, LTAs and teichoic acids (Schleifer and Kandler, 1972). *Streptococcus* spp are distinguished from *S. aureus* by their inability to produce catalase (an enzyme catalysing the breakdown of H₂O₂) (Wong, 1987). Furthermore, *S. pneumoniae* can be distinguished from other streptococci by its demonstration of α (incomplete) rather than β (complete) haemolysis on blood agar, and its sensitivity to optochin (ethyl hydrocupreine) (Bowers and Jeffries, 1955; Garcia-Suarez et al., 2006). Within the species there are 91 known serotypes of *S. pneumoniae* (Henrichsen, 1995; Park et al., 2007), although not all are pathogenic (Kalin, 1998). *S. pneumoniae* exhibits natural transformation competence i.e. exchange of genetic material between strains and uptake of extracellular DNA from the environment (Lorenz and Wackernagel, 1994), which has important implications for the development of novel clinical serotypes as well as antibiotic resistance (Mitchell, 2000; Watson et al., 1993).

S. pneumoniae is roughly 1 μm in diameter (AlonsoDeVelasco et al., 1995). When viewed on smears it tends to appear in pairs and has a tendency to grow in chains in liquid media (Watson et al., 1993). Figure 1.7 shows a Gram stain of *S. pneumoniae* on a sputum smear (<http://www.bact.wisc.edu>)

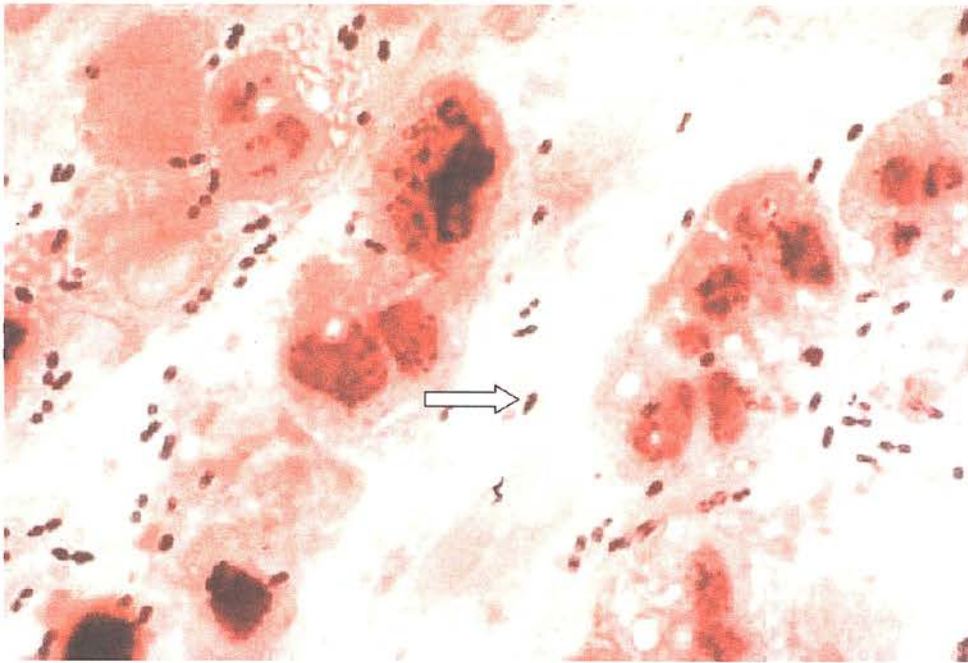


Figure 1.7 – Gram stain of a sputum smear from a lobar pneumonia. Arrow shows *S. pneumoniae*. Note characteristic pairing of bacteria

S. pneumoniae is a facultative anaerobe and a fastidious organism requiring complex media for growth (Hoskins et al., 2001), stemming in part from its inability to produce catalase (Restrepo et al., 2005). Laboratory culture of *S. pneumoniae* requires an external source of catalase, such as blood, to neutralise the H_2O_2 produced by the bacterium (Restrepo et al., 2005). In addition, *S. pneumoniae* tends to be prone to a characteristic autolysis when subjected to nutritional deficits such as that encountered during stationary phase (Mitchell et al., 1997).

1.5.2.3 Virulence Factors

1.5.2.3.1 Overview

Virulence factors are moieties that increase a bacterium's pathogenicity. They can promote enhancement of adhesion, colonisation and invasion, modulate the host

immune system and also include toxins. Some of the most important virulence factors of *S. pneumoniae* will be addressed here.

1.5.2.3.2 Intracellular Factors

1.5.2.3.2.1 Pneumolysin

Pneumolysin (Ply), known historically as pneumococcal haemolysin, was first identified by Libman in 1905 (Lorian et al., 1973). By the 1980s, the toxin had been purified, cloned and sequenced (Paton et al., 1986; Shumway and Klebanoff, 1971; Walker et al., 1987) and was being used diagnostically to identify pneumococcal disease (Kalin et al., 1987).

Purification of the toxin led to an extensive body of work determining its pathological effects. Instillation of Ply into rat lungs induced all the relevant histological features of pneumonia (Feldman et al., 1991), and it has been shown to cause damage to both pulmonary endothelial and epithelial cells (Rubins et al., 1992; Rubins et al., 1993), and to inhibit ciliary beat in respiratory epithelial cells (Feldman et al., 2002; Jedrzejewski, 2001). Ply can influence immune cells, inhibiting respiratory burst and bacterial killing in neutrophils and monocytes (Nandoskar et al., 1986; Paton and Ferrante, 1983), as well as decreasing chemotaxis and migration by neutrophils (Paton and Ferrante, 1983). It also stimulates the production of inflammatory cytokines TNF- α , IL-1 β and IL-8 (AlonsoDeVelasco et al., 1995; Hirst et al., 2004) and binds to TLR-4 (Malley et al., 2003). The toxin also affects lymphocytes by inhibiting proliferation and production of both antibodies and lymphokines (Rubins and Janoff, 1998). Immunisation with purified Ply has also conferred protection against subsequent insult (Lock et al., 1992; Paton et al., 1993). The development of

pneumolysin-deficient bacteria, and demonstration of their decreased virulence in pneumococcal pneumonia (Berry et al., 1989b), has led to a number of studies contributing to the knowledge about the extent of the toxin's importance in lung pathogenesis. Such studies have shown that pneumolysin enhances expansion of *S. pneumoniae* in both pneumonia and sepsis (Benton et al., 1997; Kadioglu et al., 2000; Rubins et al., 1995), damage to the air-blood barrier including pulmonary endothelial cells (Rubins et al., 1992; Rubins et al., 1995), and inflammation, including neutrophil influx and lymphocyte accumulation (Kadioglu et al., 2000). In pneumococcal pneumonia, expression of Ply is histologically associated with lung damage, apoptosis in leukocytes and bronchial epithelial cells, and infiltration of inflammatory cells (Garcia-Suarez et al., 2007).

Further studies on the toxin, found in all clinical isolates of the pneumococcus, have shown it to be a 53kDa polypeptide which is stored in the bacterial cytoplasm and can be released on autolysis and during logarithmic growth (Jedrzejewski, 2001; Rubins and Janoff, 1998; Spreer et al., 2007). The toxin interacts with cholesterol in the plasma membrane of the target cell, leading to insertion of the molecule and the formation of a pore (Jedrzejewski, 2001). There are two main activities of Ply, cytotoxicity and complement activation, both of which are important for virulence (Garcia-Suarez et al., 2006). The cytolytic effect is most critical for bacterial growth earlier on in infection, and contributes to tissue damage and neutrophil recruitment (Rijneveld et al., 2002; Rubins et al., 1995; Rubins et al., 1996). Ply can also activate the classical complement pathway by binding to Fc domain of IgG (Paton et al., 1993). *In vivo* studies have shown this activity to be more important for bacterial growth during the later stages of infection, and that it is not involved in tissue injury or leukocyte

recruitment but affects killing by neutrophils. The mechanism for these effects is not yet known (Rubins et al., 1995; Rubins et al., 1996). Studies using mutant bacteria which are separately deficient in the cytotoxic and complement activating activities have identified an additional, independent property of the pneumolysin toxin, but its purpose is as yet unknown (Berry et al., 1999). In summary, Ply is an important virulence factor for *S. pneumoniae*, affecting bacterial growth, tissue injury and inflammation.

1.5.2.3.2 Neuraminidase

Neuraminidase is a sialidase enzyme produced by a number of human microbial pathogens, including *S. pneumoniae*, *P. aeruginosa* and *influenza viruses*. It acts by cleaving terminal sialic acids from carbohydrate moieties on host cell surfaces or in body fluids, enhancing bacterial adhesion (Davies et al., 1999; Jedrzejewski, 2001; von Itzstein M., 2007). In the pneumococcus it is released from the cytoplasm following autolysis, thus contributing to pneumococcal pathogenesis (Jedrzejewski, 2001). There are at least 2 active forms of the enzyme, NanA and NanB (Jedrzejewski, 2001; Mitchell, 2000). The virulence of NanA has been demonstrated *in vivo*, and immunisation with the enzyme confers partial pneumococcal protection (Mitchell, 2000; Paton et al., 1993). Although the precise contribution of neuraminidase to pathogenesis is not yet known, its action may damage host tissues, expose bacterial adhesion receptors on the surface of epithelial cells and decrease the viscosity of mucus, thus aiding bacterial colonisation (Jedrzejewski, 2001; Mitchell et al., 1997; Mitchell, 2000).

1.5.2.3.2.3 Hydrogen Peroxide

H₂O₂ is a strong oxidant, produced by *S. pneumoniae*, as a by-product of aerobic metabolism (Spellerberg et al., 1996). However, it has also been shown to be an important virulence factor *in vivo* (Spellerberg et al., 1996). *In vitro* it has been demonstrated to be toxic to lung epithelial cells, as well as affecting ciliary function in both lung and neural cells (Duane et al., 1993; Feldman et al., 2002; Hirst et al., 2000), and demonstrates a bactericidal effect on other competing bacteria such as *H. influenzae* and *S. aureus* (Pericone et al., 2000; Regev-Yochay et al., 2006).

1.5.2.3.3 Surface Factors

1.5.2.3.3.1 Capsule and Cell Wall Components

Variations in the pneumococcal capsule are the basis of serotype classification. Although both capsulated and unencapsulated forms of the bacteria are found naturally, the capsule is required for virulence and clinical isolates are always encapsulated (Catterall, 1999). The capsule is itself non-toxic, non-inflammatory, antiphagocytic and inhibits the alternative complement pathway (AlonsoDeVelasco et al., 1995; Catterall, 1999; Garcia-Suarez et al., 2006), all of which contribute to bacterial growth in host tissues. In contrast, cell wall polysaccharide, particularly LTA, is highly inflammatory, binding to TLR-2 (Carpenter and O'Neill, 2007; Kadioglu and Andrew, 2004; Martin and Frevert, 2005; Paterson and Mitchell, 2006), thus potentially contributing to tissue damage. Inflammatory responses induced by pneumococcal cell wall components include activation of the alternative complement pathway, leading to recruitment of inflammatory cells and production of cytokines. The presence of phosphorylcholine in LTA also enables adhesion of the bacteria to

activated cells via inflammatory receptors on the plasma membrane (Catterall, 1999; Garcia-Suarez et al., 2006).

1.5.2.3.3.2 Autolysin

Four bacterial peptidoglycan hydrolases, or autolysins, have so far been identified in the pneumococcal cell wall (Moscoso et al., 2005). These enzymes are responsible for the autolytic phenomenon demonstrated by *S. pneumoniae* and by the breaking of a choline bond, leading to break down of the cell wall (Jedrzejewski, 2001; Mitchell, 2000; Paton et al., 1993). There are a number of potential functions for this process, including roles in the separation of daughter cells and competence in genetic transformation (Paton et al., 1993). However, the most obvious result of autolysis is the release of other bacterial virulence factors, including inflammatory cell wall PGs and LTAs, as well as intracellular toxins such as Ply and neuraminidase (Garcia-Suarez et al., 2006; Jedrzejewski, 2001; Mitchell, 2000). LytA (N-acetylmuramic acid L-alanine amidase) is the main pneumococcal autolysin (Jedrzejewski, 2001) and has been shown *in vivo* to be important for virulence (Jedrzejewski, 2001; Mitchell, 2000) but there have been some conflicting results regarding the mechanism of this (Balachandran et al., 2001; Berry et al., 1989a; Berry et al., 1992; Lock et al., 1992).

1.5.2.3.3.3 Other Surface Proteins

In addition to the capsule, cell wall and autolysin there are a number of other virulence factors found on the bacterial surface. Pneumococcal surface protein A (PspA) is expressed on all clinical pneumococci (Jedrzejewski, 2001; Mitchell, 2000) and inhibits alternative complement activation, thus decreasing complement-mediated clearance and phagocytosis (Garcia-Suarez et al., 2006; Jedrzejewski, 2001).

Hyaluronidase, or more specifically hyaluronate lyase (Hyl), is a surface-secreted enzyme that breaks down hyaluronan (hyaluronic acid), present in connective tissue and the ECM. This activity may contribute to bacterial dissemination and the enzyme may also be involved in altering function of the innate immune system (Jedrzejewski, 2001; Mitchell, 2000). Bacterial adhesion is critical to successful colonisation, thus pneumococcal adhesion-associated proteins are important factors in virulence (Mitchell et al., 1997). These include direct adhesins such as Choline binding protein A (CbpA) (Hammerschmidt et al., 2007; Jedrzejewski, 2001; Mitchell, 2000) and adhesion-mediators like Pneumococcal surface adhesin A (PsaA) (Jedrzejewski, 2001).

1.5.2.4 Summary

S. pneumoniae, an encapsulated, Gram-positive coccus, is an important respiratory pathogen. Virulence factors such as pneumolysin, adhesion molecules and the capsule and cell wall components all contribute to its highly efficient lifecycle and pathogenesis.

1.5.3 *Staphylococcus aureus*

1.5.3.1 Clinical Importance of *Staphylococcus aureus*

S. aureus is an important human pathogen (Diekema et al., 2001), particularly relevant as a cause of infections in the bloodstream, soft tissue and the lower respiratory tract (Diekema et al., 2001). *S. aureus* is the most common cause of infections acquired in hospital and is particularly important as an aetiological agent of HAP (ATS Guidelines, 2005; Lindsay and Holden, 2004). The bacterium colonises nares of between 30% and 70% of the population and usually lives commensally, but is able to infect the body in the event of a breach in the skin or mucous membranes

(Lindsay and Holden, 2004). Antibiotics remain the primary treatment for staphylococcal infection (Lindsay and Holden, 2004; Micek, 2007). However, the emergence of methicillin-resistant *S. aureus* (MRSA) leaves relatively limited options for therapy of MRSA infections, which are often treated with vancomycin or linezolid (Lindsay and Holden, 2004; Micek, 2007). Of particular concern are strains inducing severe infection, such as those expressing Panton-valentine leukocidin (PVL) which can cause haemolytic pneumonia (Lindsay and Holden, 2004). In addition, MRSA is becoming a more common cause of severe CAP (Stankovic et al., 2007). Widespread contamination by MRSA throughout hospitals in the developed world, and growing evidence of strains that are now resistant to vancomycin (Lindsay and Holden, 2004; Micek, 2007), make *S. aureus* infection a substantial burden to the health services.

1.5.3.2 Bacterial Morphology

S. aureus is a Gram-positive, spherical (~1µm diameter), catalase-producing facultative anaerobe (Brown et al., 2005). Unlike most other staphylococci, *S. aureus* synthesises coagulase (a trait that can be used in bacterial identification) (Brown et al., 2005), and often demonstrates haemolysis when cultured on blood agar, due to production of α -toxin (McClatchy and Rosenblum, 1966). *S. aureus*, named because of the golden colour of colonies on agar (Kloos, 1980) is also known historically as *Staphylococcus pyogenes* and was first identified in 1880 by Alexander Ogston, who demonstrated its pathogenicity in pustulent abscesses (Smith, 1982). Encapsulation of *S. aureus* varies considerably between serotypes (O'Riordan and Lee, 2004; von Eiff et al., 2007). Those accounting for 80% of clinical isolates, demonstrating a less pronounced capsule, are often described as microencapsulated (von Eiff et al., 2007). There are currently 13 identified serotypes of *S. aureus* (von Eiff et al., 2007).

1.5.3.3 Virulence Factors

S. aureus expresses a number of factors affecting virulence. The polysaccharide capsule can play an important role in bacterial pathogenesis. Highly encapsulated strains (serotypes 1 and 2) are resistant to neutrophil phagocytosis by inhibiting binding by complement and IgG, and are more virulent than strains lacking capsule. However, serotypes 5 and 8, the most clinically relevant strains, express different primary polysaccharides and are less virulent than those in serotypes 1 and 2 (O'Riordan and Lee, 2004), although the capsule still confers virulence (Foster, 2005). This suggests that other factors are more important for clinical pathogenicity. However, expression of capsule is heavily dependent on culture conditions (O'Riordan and Lee, 2004), which may explain this inconsistency.

S. aureus also expresses surface proteins, which contribute to virulence and pathogenesis. Protein A (SpA) has been shown to be a virulence factor *in vivo* (Patel et al., 1987), binding to Fc regions of IgG and making them unavailable for binding to neutrophils, thus inhibiting phagocytosis (Foster, 2005). It also demonstrates pro-inflammatory capabilities (Gomez et al., 2004), stimulates apoptosis of B lymphocytes (Foster, 2005; Goodyear and Silverman, 2004) and contributes to bacterial adhesion (Gomez et al., 2006; Hartleib et al., 2000). There are a number of other surface molecules that promote adhesion of *S. aureus*. Fibronectin-binding proteins (FnBps) expressed on the surface of the bacterium are important for internalisation in host epithelial cells, although *in vivo* they enhance bacterial clearance of the lung (Dziewanowska et al., 1999; McElroy et al., 2002), suggesting a role in immune system avoidance that may be more important for colonisation than during established disease. Clumping factors (Clf) or fibrinogen-binding proteins are

able to stimulate cell aggregation and inhibit bacterial phagocytosis (Foster, 2005; Higgins et al., 2006; Miajlovic et al., 2007). Collagen-binding adhesin (Cna) has also been shown to be a virulence factor in a variety of disease models (Elasri et al., 2002; Patti et al., 1994; Rhem et al., 2000).

S. aureus also secretes a number of virulence factors, including exotoxins. A-toxin or α -haemolysin (Hla), responsible for the haemolytic effect of *S. aureus* on blood agar, damages host cells by forming a pore in the plasma membrane (Bhakdi and Tranum-Jensen, 1991; Jonas et al., 1994). It also stimulates pro-inflammatory cytokine production (Bhakdi et al., 1989; Grimminger et al., 1997) and is an important virulence factor in a number of disease models, including pneumonia (Bramley et al., 1989; McElroy et al., 1999; Patel et al., 1987). It does not cause direct damage to alveolar epithelial cells however (McElroy et al., 1999), suggesting mediation of indirect effects on lung tissue. Inoculation with Hla has also been shown to protect against lethal staphylococcal pneumonia *in vivo* (Deleo and Otto, 2008). Other haemolytic toxins include β -toxin (Hlb), γ -toxin (Hlg) and δ -toxin (Bramley et al., 1989; Dinges et al., 2000; Foster, 2005). PVL, another pore-forming toxin, is expressed by a small proportion of clinical isolates but is important in staphylococcal pneumonia (Foster, 2005; Labandeira-Rey et al., 2007; Wardenburg et al., 2007). Other factors associated with *S. aureus* virulence include 'superantigens', such as staphylococcal enterotoxins and toxic shock syndrome toxin-1 (TSST-1), that stimulate T lymphocyte proliferation regardless of antigenic specificity (Dinges et al., 2000; Foster, 2005; Proft et al., 2003).

1.5.3.4 Summary

S. aureus, like *S. pneumoniae*, is an important Gram-positive respiratory pathogen, particularly in HAP. The emergence of resistant strains of MRSA and its widespread infection of many organs other than the lung make it especially significant as a human pathogen. *S. aureus* produces a variety of surface adhesions and exotoxins, which contribute to pathogenesis and help the bacterium avoid the host immune system.

1.6 THESIS OBJECTIVES

Subsequent to the discussion set forth in this Chapter, the following initial hypothesis was generated.

Hypothesis 1

Pneumococcal pneumonia induces differential damage to ATI and ATII cells

The results of this hypothesis are detailed in Chapter 3. Following on from this, two further hypotheses were developed.

Hypothesis 2.1

Pneumococcal virulence factor pneumolysin mediates differential protein expression

Hypothesis 2.2

Differential protein expression in pneumococcal pneumonia is due to host response to Gram-positive bacteria

Data from experiments investigating these hypotheses are presented in Chapter 4.

To further investigate results generated in Chapter 3 a final hypothesis was devised.

Hypothesis 3

Differential protein expression in pneumococcal infection is due to direct interaction with bacteria or bacterial products

Results examining this theory are detailed in Chapter 5

A discussion of results chapters 3-5 will be presented in Chapter 6

CHAPTER 2 – MATERIALS AND METHODS

2.1: REAGENTS, ANIMALS AND GMO

2.1.1 Reagents

All reagents were supplied by Sigma, Poole, UK unless stated otherwise. All plastics were supplied by Fisher Scientific, Leicester, UK unless stated otherwise. Syringes and needles were supplied by B. D. Biosciences, Oxford, UK. Suppliers are listed in Appendix 1.

2.1.2 Animals

All animal experiments were carried out with approval according to the Home Office Animals (Scientific Procedures) Act, 1986. All animals were supplied by Harlan UK Ltd, Oxton, UK. Rats were male Sprague Dawley Specific Pathogen Free (SPF) weighing 300-350g and were kept on standard chow. All anaesthetics supplied by Genusxpress Ltd, Aberdeen, UK unless stated otherwise.

2.1.3 GMO

All use of Genetically Modified Organisms (GMO) was carried out according to the Genetically Modified Organisms (Contained Use) Regulations (2000).

2.2: BACTERIA

All plates, media and antibiotics for bacterial culture were supplied by B. D. Biosciences, Oxford, UK.

2.2.1 *Streptococcus pneumoniae*

2.2.1.1 Strains

Two strains of *S. pneumoniae* were used in this study, a wildtype laboratory strain (D39) and an isogenic pneumolysin-deficient mutant (PLN-A) which was resistant to erythromycin (Berry et al., 1989). Both wild-type and mutant bacterial strains were a kind gift from Tim Mitchell, University of Glasgow. Bacterial stocks were kept at -80°C in Brain/ Heart Infusion broth plus 30% glycerol Ultra.

2.2.1.2 Bacterial culture

For all experiments, strains were grown overnight at 37°C from the same batch of glycerol stocks on Blood Agar Base (BAB) plates containing 5% sheep blood, one colony was then cultured overnight at 37°C in 3ml of Brain/ Heart Infusion broth, with no agitation. Growth curves carried out on bacterial cultures indicated that pneumococci reached mid log phase after approximately 9 hours and stationary phase after 15 hours. Bacteria were harvested at approximately 12-14 hours, due to practical considerations. This was estimated to be late log phase, where the bacteria were thought to be still viable and producing virulence factors.

The identity of each *S. pneumoniae* strain was checked during each experiment using

antibiotic (erythromycin) disks. To get an initial estimate of bacterial growth within the culture, the optical density of the overnight cultures was assessed using a Biomate 3 spectrophotometer (Thermo Electron, Reading, UK). To prepare instillates, overnight cultures were washed twice with sterile, endotoxin-free Dulbecco's Phosphate Buffered Saline containing calcium and magnesium ions (DPBS ^{+/+}). This was done by centrifuging for 3 minutes at 11,300g then resuspending the pellet in DPBS and repeating. The final pellet was resuspended in 0.5ml DPBS ^{+/+} for all experiments (McElroy et al., 2002). To confirm the concentration of bacteria, instillates were serially diluted in DPBS ^{+/+} at 10⁻³ and 10⁻⁵ or 10⁻⁶, plated on BAB plates and incubated overnight at 37°C. The number of colonies was counted and the number of colony forming units (cfu) instilled was calculated.

2.2.2 *Staphylococcus aureus*

One wildtype laboratory strain of *S. aureus* (8325-4) was used in this study (McElroy et al., 2002). Bacterial stocks were kept at -80°C in Todd Hewitt broth plus 30% glycerol Ultra.

For all experiments, *S. aureus* was grown overnight at 37°C from glycerol stocks on BAB plates, then one colony was cultured overnight at 37°C in 3ml of Todd Hewitt broth with agitation. Preparation of bacteria for experiments was subsequently the same as for *S. pneumoniae*.

2.3: MODELS OF INFECTION

2.3.1 *In vivo* Models of Pneumonia

2.3.1.1 Pneumonia Models

Rats were weighed immediately prior to being anaesthetised with an intraperitoneal injection of 1ml of a mixture of Hypnorm (0.315mg/ml fentanyl citrate and 10 mg/ml fluanisone) and Hypnovel (5ml/ml midazolam hydrochloride) (Wolfensohn and Lloyd, 1998). The mouth was opened with the aid of a laryngoscope and an intubation wedge, in the form of a modified 2ml Eppendorf tube (Jou et al., 2000), was inserted to improve accessibility of the trachea. A catheter (Portex Fine Bore Polythene Tubing) of 0.96mm external diameter, 9cm in length (Portex Ltd, Hythe, UK) was inserted into the distal airways. 0.5ml bacterial suspension (approximately 10^6 , 10^8 or 10^9 cfu/0.5ml) was inserted into the distal airways via the catheter. 0.5ml of DPBS $^{+/-}$ was instilled into the distal airways of control rats (McElroy et al., 2002). Animals were kept for 24 hours, 72 hours or 3 weeks and were weighed and monitored regularly throughout recovery and immediately prior to harvest.

2.3.1.2 Processing of Animal Tissue

2.3.1.2.1 Biochemical analysis

At the end of the experimental period, rats were terminally anaesthetised with Sagatal or Pentoject (45mg/kg/i.p. pentobarbital) containing heparin (500units/kg/i.p) (Leo Pharmaceuticals, Princes Risbourough, UK). Blood was collected from the ascending aorta. Pleural lavage fluid was collected by lavaging the pleural cavity with 2 x 1ml DPBS $^{+/-}$. Lungs were examined for injury. The trachea was then cannulated using a

modified Monoject Aluminium Hub Blunt Needle (Harvard Apparatus, MA, USA) and the cannula secured with Ethicon mersilk sterile black non-absorbent suture (3s Healthcare, Bristol, UK). BAL fluid was collected by lavaging the lungs with 2 x 10 mls of DPBS ⁺/₊. The lungs were then removed and placed into DPBS ⁺/₊ (McElroy et al., 2002).

Blood and pleural fluid were plated out undiluted; BAL fluid was serially diluted in DPBS onto BAB plates, which were incubated at 37°C overnight. Blood was then centrifuged at 1000g for 15 mins; serum was collected then kept at -80°C. Pleural lavage and BAL fluid was centrifuged at 900g for 5 minutes (McElroy et al., 1997; McElroy et al., 2002). Pleural lavage and BAL fluid leukocytes were quantified by haemocytometric counting. Approximately 200,000 cells were added to a disposable double cytofunnel (Thermo Electron, Reading, UK) and cytocentrifuged at 10g for 3 mins in a Cytospin 2 cytocentrifuge (Thermo Electron, Reading, UK). Cytospins were then fixed with methanol for 2 mins and stained with Diff-Quick Red, then Diff-Quick Blue (Gamidor Technical Serviced Ltd, Abingdon, UK), for 1 min each. Differential cell counts on pleural and BAL fluid cells were performed on cytocentrifuged preparations. A total of 300 cells were counted per cytospin. Pleural lavage and BAL fluid was then aliquoted and stored at -80°C (McElroy et al., 2002).

All lung tissue was weighed and homogenised in Tris-HCl (2.42mM)-buffered saline (TBS), pH 8.2, containing 154mM NaCl, with the addition of Complete Protease Inhibitor Cocktail (Roche Diagnostics, Loughborough, UK), to prevent degradation of

protein in the samples. Lungs were homogenised by using an Ultra Turrax T25 homogeniser (IKA-Werke, Staufen, Germany) on ice at approximately 4,000 rpm for 2 x 5 seconds each. Samples were serially diluted in DPBS and plated out on BAB plates, which were incubated at 37°C overnight (McElroy et al., 2002). Lung homogenates were then aliquoted and stored at -80°C.

The number of cfu present in blood, BAL, pleural fluid and lung homogenate was then calculated.

2.3.1.2.2 Morphological analysis

Rats were anaesthetised as described above. Blood was collected and processed as described above, and the trachea was cannulated. Lungs were examined for injury.

For electron microscope analysis, lungs were then inflated with 10mls of 4% paraformaldehyde (made up using DPBS 7-), secured using Ethicon mersilk sterile black non-absorbent suture, then removed into 4% paraformaldehyde and left at 4°C for at least 24 hours. Cubes were cut from the injured region and fixed in glutaraldehyde buffer 3% containing sodium cacodylate (0.1M). Similar regions were processed from control lungs.

Tissues were then postfixed in osmium tetroxide buffer (1%) containing sodium cacodylate (0.1M), then dehydrated in a series of graded alcohols and embedded in polyethylene capsules in fresh Araldite epoxy resin. Lung sections were cut, placed on 200-mesh uncoated grids then stained with uranyl acetate and lead citrate for electron microscopic studies. Sections were viewed with a Philips CM12 electron microscope.

Processing * to * was carried out by Steve Mitchell

For immunofluorescence and histological analysis, the lungs were inflated with 10mls of 4% paraformaldehyde containing 4% Tissue-Tek Optimum Cutting Temperature compound (OCT) (Bayer, Newbury, UK), secured using Ethicon mersilk sterile black non-absorbent suture, then removed to 4% paraformaldehyde/ OCT. Lungs were left at 4°C for at least 72 hours. For immunofluorescence analysis, lung tissue was processed for frozen thin sections. Small cubes were cut from macroscopically inflamed and control lungs and fixed overnight in 30% (876mM) sucrose in non-sterile Phosphate Buffered Saline (PBS), containing 4% OCT. †Frozen sections 3-4µm thick were then cut on a Leica CM1850 cryostat onto Superfrost Plus microscope slides (VWR, Poole, UK)† and were subsequently frozen at -20°C. For histological analysis, pieces of tissue were processed for paraffin section. ‡ Tissue was dehydrated and embedded in paraffin wax in a tissue processor, then set into a tissue mould. Sections 3-4µm thick were cut on a Microtome then stained with Harris haematoxylin and eosin (H&E) before mounting‡. Processing † to † and ‡ to ‡ was carried out by Susan Harvey and Robert Morris.

2.3.2 *In vitro* Models of Bacterial Co-culture

2.3.2.1 Bacterial Co-culture Model

2.3.2.1.1 Reagents and plastics

All tissue culture media and reagents were supplied by PAA Laboratories, Yeovil, UK unless otherwise stated. All plastics were supplied by Fisher Scientific, Leicester, UK unless otherwise stated.

2.3.2.1.2 Cell lines

The rat alveolar epithelial cell line, SV40-T2, was used in the models of bacterial infection (Clement et al., 1991).

2.3.2.1.3 Cell Culture

Cells were grown at 37°C in a 5% CO₂ humidified incubator in D10 media, consisting of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Foetal Bovine Serum (FBS) and additional amino acids (2mM l-glutamine). The D10 media was supplemented with penicillin (100 U/ml) and streptomycin sulfate (100ug/ml) to control unwanted bacterial infection in the cultures. Cells were grown on 75cm² plastic flasks.

2.3.2.1.4 Bacterial Co-culture

For each experiment, cells were grown to approximately 80% confluency and washed with 2 x 10mls DPBS without calcium and magnesium ions (DPBS %). 10mls of fresh D10 media, without penicillin and streptomycin sulphate, was then added along with 0.5ml bacterial suspension or DPBS % control.

2.3.2.2 Cell Processing

2.3.2.2.1 Protein Analysis

At the end of each experimental period, cell culture media was removed, plated serially, aliquoted and frozen at -80°C. The remaining cells were then washed with DPBS % and detached from the flask using 1ml of 0.5mg/ml Trypsin/0.22mg/ml EDTA (ethylenediaminetetraacetic acid). Cells were resuspended in 10 ml of D10 media and

spun at 1000g for 3 mins. The cell pellet was then resuspended in 1ml RIPA (RadioImmuno Precipitation Assay) buffer (1% Igepal + 12mM sodium deoxycholate in PBS) containing Complete Protease Inhibitor Cocktail (Roche Diagnostics) to prevent degradation of protein in the samples. Samples were vortexed and drawn up and down in a 1ml syringe in a 25 gauge needle at least 6 times. Samples were then centrifuged at 11,300g for 15 minutes at 4°C, the supernatant collected and mixed well before being aliquoted and stored at -80°C.

2.3.2.2.2 RNA Analysis

Cells were initially treated as for protein analysis described above. After the media was removed and cells washed, cells were then treated for RNA isolation using TRIZOL[®] reagent (Invitrogen, Paisley, UK). In summary, cells were incubated on ice for 5 minutes in 1 ml TRIZOL[®]. Cells were then scraped from the flask using a cell scraper. 1ml of the TRIZOL[®] solution was incubated on ice for 5 minutes with 200µl of chloroform, and then centrifuged at 11,300g for 15 minutes at 4°C in order to separate the organic and aqueous phases. 600µl of the clear aqueous phase was then mixed with an equal amount of isopropanol and kept at -70°C for at least one hour so as to precipitate out the RNA. After thawing, the samples were then centrifuged at 11,300g for 20 minutes at 4°C and 300µl of ice-cold ethanol added to the pellet to precipitate RNA from DNA. Another 11,300g centrifuge for 10 minutes at 4°C yielded an RNA pellet which was air-dried and resuspended in 43µl of sterile nuclease-free H₂O (Applied Biosystems, Warrington, UK). The RNA solution was then stored at less than 4°C.

Normal rat lung tissue was used as a positive control in RNA analysis of lung-specific proteins. Lung tissue was removed directly into an appropriate volume of TRIZOL[®] reagent, cut into small pieces and homogenised on ice using a PowerGen 125 homogeniser (Fisher Scientific, Leicester, UK). 1ml of tissue homogenate was then processed for RNA isolation as described above.

2.4: ANALYSIS

2.4.1 Protein Assessment

2.4.1.1 Biochemical Analysis

2.4.1.1.1 Protein Assay

Protein concentration of BAL and pleural lavage fluid, lung homogenates and cell lysates were determined by the Bio-Rad Bradford assay (Bio-Rad Laboratories, Hemel Hempstead, UK), using bovine serum albumin (BSA) solution (Pierce Warriner, Chester, UK) as a standard (McElroy et al., 1999; McElroy et al., 2002). Samples were analysed at 630 nm in a 96-well tissue culture plate using a Dynatech Laboratories MRX Microplate Reader, and Revelation software.

2.4.1.1.2 Protein Analysis

Details of antibodies used in specific protein analysis, including suppliers, are detailed in Table 2.1. All primary antibodies were diluted in non-sterile DMEM containing 10% FBS, except loading controls which were diluted in 2.5% bovine casein in TBS. Rockland secondary antibodies supplied by Lorne Laboratories, Twyford, UK were used in all Dot and Western blots. Zymed® secondary antibodies supplied by Invitrogen, Paisley, UK were used for Enzyme-Linked ImmunoSorbent Assay (ELISA). All secondary antibodies were diluted in 2.5% casein.

The concentration of specific proteins present in samples was evaluated using a variety of methods, including ELISA, ELISA-based Dot blot, Western blot and multiplex bead array techniques.

Analysis of BAL fluid for membrane-associated proteins (RTI40, APN/MMC4, and MMC6) was carried out on the insoluble fraction, which was obtained by treating at 500,000g for 10 minutes in a Beckman Optima ultracentrifuge. The resultant pellet was resuspended in TBS. Lung homogenates were centrifuged at 11,300g for 30 seconds to remove any large pieces of tissue and assays were carried out on the resulting supernatant. All other assays were carried out on untreated sample.

2.4.1.1.2.1 Dot Blot

Dot blots were carried out as described previously (McElroy et al., 1995; McElroy et al., 1997). Protein from samples was immobilised on Immobilon-P polyvinylidene fluoride (PVDF) membrane which had been pre-treated with methanol, using a dot blot manifold (Whatman Schleicher & Schuell, Keene, USA) connected to a vacuum pump. The membrane was then blocked for 1-2 hours in 2.5% bovine casein in TBS, to prevent non-specific antibody binding. The membrane was then incubated in primary antibody for 30 minutes at room temperature or at 4°C overnight. Primary antibody was removed by washing thoroughly with 0.05% polyethylene sorbitan monolaurate (Tween 20) in TBS (TBST) for 25-30 minutes before incubation with goat IgG secondary antibody conjugated to horseradish peroxidase (HRP), diluted to 0.15µg/ml, for 30 minutes at room temperature. Secondary antibody was removed by washing in TBST as before and the blots were visualised using Enzymatic Chemiluminescence (ECL) Western Blotting Detection Reagents (Amersham, Little Chalfont, UK). Finally, blots were developed using Kodak BIOMAX XAR film in a Xograph Compact X4 X-ray film processor.

Densitometry values for the dots were measured on a Dynatech Laboratories MRX Microplate Reader, using Revelation software. All relevant samples were assayed on the same blot; therefore data are expressed as a percentage of the mean control value.

2.4.1.1.2.2 Western Blot

Western blots were carried out using the NuPage[®] Bis-Tris Electrophoresis System (Invitrogen, Paisley, UK) as follows. Samples of approximately equal protein, along with protein standard MultiMark[®] Multi-Colored Standard (Invitrogen, Paisley, UK) were loaded and run on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. On reducing gels, samples were treated with reducing agent 0.5M Dithiothreitol (DTT) and antioxidant (Invitrogen, Paisley, UK) according to the NuPage System protocol. Gels were run at 200v and transferred at 30v for 2 hours onto methanol-treated PVDF membrane, which were then blocked for 1 hour in 2.5% casein. Membranes were then treated as for dot blots, except that secondary antibodies were diluted to 0.5 μ g/ml. The blots were analysed using upc grabit and upc gel plate software as average volume of bands. All relevant samples were assayed on the same blot; therefore data is expressed as a percentage of the mean control value.

β -actin or GAPDH antibodies were used as loading controls.

2.4.1.1.2.3 ELISA (Enzyme-Linked ImmunoSorbent Assay)

(E11 Antibody)

A direct ELISA technique was used. As purified protein was not available all relevant samples were assayed on the same blot; therefore data is expressed as a percentage of the mean control value. Protein was coated onto a 96-well, flat-bottomed, high-binding tissue culture plate by incubating 100µl of sample overnight at 4°C. Unbound sample was then removed and wells were washed x 3 with TBST then blocked in 200µl of 2.5% casein for 2 hours at 4°C. Blocking buffer was removed and wells rinsed with TBST then incubated for one hour in 100µl of E11 antibody. Primary antibody was washed off x 3 in TBST then wells were incubated in 100µl of secondary antibody, diluted to 0.5µg/ml, for 30 mins at 4°C. Secondary antibody was washed off as described previously, and then wells were incubated for 5-20 mins in 100µl of SureBlue TMB Microwell Peroxidase Substrate (Insight Biotechnology, Wembley, UK) developing solution. Once the colour had developed, the reaction was stopped using 50µl of 1M H₂SO₄ and the plate was analysed at 560nm with 650nm correction using a Dynatech Laboratories MRX Microplate Reader.

TABLE 2.1: BIOCHEMICAL PROTEIN ANALYSIS: ANTIBODY SUMMARY

Protein	Tissue	Antibody	Antibody Source	Detection Method	Antibody Dilution/ conc	Secondary Antibody
RT140	BAL, lung, cell lysates	Mouse anti-rat monoclonal IgG ₁ Mouse anti-rat monoclonal IgG ₁	Dobbs Wetterwald	Dot/ Western blot, ELISA	1:50 1:20	Goat anti-mouse IgG HRP Goat anti-mouse IgG ₁ HRP
Aquaporin 5	Lung	Rabbit anti-rat polyclonal (affinity purified)	Chemicon, Southampton, UK	Western blot	2.5ug/ml	Goat anti-rabbit IgG HRP
MMC6	BAL and lung	Mouse anti-rat monoclonal IgG _{2b}	McElroy	Dot blot	1:1	Goat anti-mouse IgG HRP
APN/MMC4	BAL and lung	Mouse anti-rat monoclonal IgG _{2a}	McElroy	Dot blot	1:1	Goat anti-mouse IgG HRP
Pro-SP-C	Lung	Rabbit anti-rat polyclonal Antisera	Beers	Western blot	1:300	Goat anti-rabbit IgG HRP
SP-D	BAL and lung	Rabbit anti-human polyclonal Antisera	Beers	Western blot	1:4000	Goat anti-rabbit IgG HRP
β-actin	Lung and lysates	Rabbit anti-human polyclonal (affinity purified)	Abcam, Cambridge, UK	Western blot	333ng/ml	Goat anti-rabbit IgG HRP
GAP-DH	Lung and lysates	Mouse anti-rabbit monoclonal (protein A purified)	Abcam, Cambridge, UK	Western blot	400ng/ml	Goat anti-mouse IgG HRP

2.4.1.1.2.4 Multiplex Fluorescent Bead Immunoassay (FBI)

A Bender Medsystems FlowCytomix rat Cytokine 6plex bead array (Invitrogen, Paisley, UK) assessing IL-1 α , MCP-1, TNF- α , IFN- γ , Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) and IL-4 was carried out (http://www.bendermedsystems.com/bm_products/MAN/825FF.pdf). Lysophilised standards were reconstituted in Assay Buffer (1% BSA in PBS) and serially diluted. 25 μ l of sample or standard was added to a 96-well round-bottomed tissue culture plate and incubated with 25 μ l of antibody-coated fluorescent beads and 50 μ l of biotin-conjugated secondary antibody for 2 hours with agitation at 500rpm. Beads were pelleted at 300g for 5 minutes at 4°C and unbound sample and biotin-conjugated antibody was then washed off with Assay Buffer. The washing procedure was repeated once more then beads were pelleted again and incubated with 50 μ l Streptavidin-Phycoerythrin (PE) for 1 hour with agitation. After an additional wash, beads were resuspended in Assay Buffer and processed on a FACSCalibur flow cytometer (B. D. Biosciences, Oxford, UK). Data was analysed using BenderMedSystems FlowCytomix Pro 2.1 Software (Invitrogen, Paisley, UK). Data was measured as picograms of protein but is expressed as a percentage of the mean control.

2.4.1.1.2.5 FACS (Fluorescence-activated cell-sorting) Analysis

(RTI40/ICAM-1 double staining)

SV40-T2 cells were suspended in FACS wash buffer (30 μ M BSA in PBS containing 15.4mM NaN₃ sodium azide) at approximately 10⁷ cells/ml and 100 μ l (about 1 million cells) per well was added to a round-bottomed 96-well tissue culture plate. Cells were pelleted at 300g for 5 minutes at 4°C and the supernatant removed. Cells were blocked

for 15 minutes at 4°C in 20% goat serum in FACS wash. 100µl of E11 (RTI40) primary antibody only, at 1:20 dilution, was then added and the cells were incubated for a further 30 minutes at 4°C. The cells were then pelleted as described previously and washed twice in FACS wash. 200µl of Alexa Fluor® 647-conjugated goat anti-mouse IgG₁ secondary antibody (Invitrogen, Paisley, UK), diluted in FACS wash to 20µg/ml, was added and cells were incubated for 30 minutes at 4°C. Cells were pelleted and washed as above. Only after staining of RTI40 was complete were the cells incubated for 30 minutes at 4°C in 200µl of 5µg/ml Intercellular Adhesion Molecule 1 (ICAM-1) primary antibody [1A29] conjugated to fluorescent isothiocyanate (FITC) (Abcam, Cambridge, UK). This was done as both primary antibodies were isotype IgG₁, so as to avoid additional binding of the secondary antibody to the FITC-conjugated antibody. Cells were then pelleted and washed, then resuspended in 100µl of FACS wash before fixation in 100µl of 8% formaldehyde. Unstained and secondary antibody only cells were also processed to serve as controls. Cells were analysed on a FACSCalibur flow cytometer. 5000 events were recorded per sample, including unstained, secondary only and primary antibody stained cells.

2.4.1.2 Immunohistochemical Analysis

2.4.1.2.1 Immunofluorescence Staining

Frozen lung sections were rinsed with PBS before immersion for 15 minutes in 100µl of blocking buffer (2.2g fish gelatin in 20% goat serum) containing 1% Triton to permeabilise the cell membrane. Sections were then incubated with primary antibody for 30 minutes at room temperature, or overnight at 4°C, and then rinsed thoroughly with

PBS. Tissue was then incubated in 100µl of 20µg/ml goat Alexa Fluor®-conjugated secondary antibody (Invitrogen, Paisley, UK) for 30 minutes, rinsed thoroughly with PBS and then immersed in a 0.1% To-Pro-3 in PBS solution, before rinsing in PBS and mounting using Mowiol mounting medium (Merck Biosciences, Nottingham, UK). Stained slides were analysed using a Zeiss LSM 510 meta confocal microscope.

2.4.1.2.2 Antibodies

Table 2.2 below shows antibodies used for immunofluorescence staining

<i>Primary Antibody</i>	<i>Dilution</i>	<i>Secondary Antibody</i>	<i>Dilution</i>
RTI40	1:50	Alexa Fluor® Goat anti-mouse IgG1	1:100
MMC4	1:1	Alexa Fluor® Goat anti-mouse IgG2a	1:100
MMC6	1:1	Alexa Fluor® Goat anti-mouse IgG2b	1:100
RTII70	1:1	Alexa Fluor® Goat anti-mouse IgG3	1:100
Pro-SP-C	1:50	Alexa Fluor® Goat anti-rabbit IgG	1:100

2.3.4 RNA Assessment

2.3.4.1 RNA Purity Assessment

Before mRNA analysis was carried out, the purity of isolated RNA was determined. RNA preparations were diluted 1:100 in H₂O and the OD (optical density) of RNA (260nm) and protein (280nm) was assessed using a spectrophotometer. Isolated RNA was considered to be adequately pure if the ratio of RNA:Protein was more than 1.6. From the OD values, it was also possible to determine the concentration of RNA. The quality of the RNA was also assessed, by running 6µl of preparation, in loading buffer on a 2% agarose gel, using SYBR Safe™ DNA Gel Stain (Invitrogen, Paisley, UK). A DNA ladder (Promega, Southampton, UK) was run alongside to assess band sizes. Gels were analysed using a Versadoc™ Model 4000 and Quantity One software (Bio-rad Laboratories,

Hertfordshire, UK). Assessment of the 28S and 18S bands served as a rough guide of the integrity of RNA in isolates.

2.3.4.2 RT-PCR (Reverse Transcription Polymerase Chain Reaction)

Analysis of RNA was carried out by RT-PCR (Reverse Transcription Polymerase Chain Reaction) analysis.

2.3.4.2.1 Reverse Transcription

All reagents for Reverse Transcription and Polymerase Chain Reaction were supplied by Promega, Southampton, UK unless otherwise stated.

cDNA was synthesised from isolated RNA using the ImProm-II™ Reverse Transcription System. In summary, 1µg of RNA was combined with either Oligo (dT)₁₅ or Random Primers at 50ng/µl and denatured for 5 minutes at 70°C, then chilled on ice for 5 minutes. RNA/primer solution was then incubated in 1x ImProm-II™ Reaction Buffer, 6mM MgCl₂, 1mM dNTPs, 0.4U Recombinant RNasin® Ribonuclease Inhibitor and 5% ImProm-II Reverse Transcriptase enzyme. The solution was initially annealed at 25°C for 4 minutes, then first strand synthesis was carried out at 42°C for 60 minutes. Finally the Reverse Transcriptase enzyme was inactivated at 70°C for 15 minutes.

2.3.4.2.2 PCR

The amount of mRNA corresponding to each protein of interest was assessed using the GoTaq™ DNA Polymerase GoTaq™ system and was optimised for each specific set of primers, summarised in Table 2.3.

Gene	Accession No.	Primer Sequences	Product size	BSA	cDNA
IL-1 α	NM_017019	F <i>tcgggaggagacgactctaa</i> R <i>gaaagctgcggatgtgaagt</i>	201bp	No	1 μ l
MCP-1	M57441	F <i>atgcagttaatgccactc</i> R <i>ttccttattggggtcagcac</i>	167bp	No	1 μ l
RTI40	NM_019358	F <i>agtgttgctctgggtttgg</i> R <i>cgccttggcagttgacttat</i>	184 bp	No	1 μ l
SP-D	M81231	F <i>gatggaggctttgaatggaa</i> R <i>cgtgccacatctgtcatalc</i>	271 bp	Yes	2 μ l
GAP-DH	BC087743	F <i>aactttggcattgtggaagg</i> R <i>acacattgggggtaggaaca</i>	223bp	No	1 μ l
B-actin	NM_031144	F <i>agccatgtacgtagccatcc</i> R <i>ctctcagctgtgggtgaa</i>	228 bp	No	1 μ l

Table 2.3: PCR Protocols

Table 2.3 shows mRNA accession numbers, forward and reverse primer sequences, product size in base pairs (bp) and specifics of optimised PCR protocols.

The generic PCR protocol was as follows. cDNA was incubated in 1x GoTaq™ Reaction Buffer, 1.75mM MgCl₂, 250 μ M dNTPs, 1U GoTaq™ DNA Polymerase enzyme and 250nM each of specific forward and reverse primers. PCR was carried out as follows:

1. Denatured at 94°C for 3 mins
2. Denature at 94°C for 1 min
3. Anneal at 60°C for 1 min

4. Extend at 72°C for 1 min
5. Repeat Steps 2 – 4 for 35 cycles
6. Final extension/ blunt ending at 72°C for 5 mins

β -actin and GAPDH were used as loading controls.

Resultant PCR products were then run on a 2% agarose gel in the same way as for RNA purity assessment above. Densities of bands were analysed using Quantity One software on a Versadoc™ Model 4000. As all relevant samples were assayed on the same gel, data are expressed as a percentage of the mean control value.

2.5 STATISTICAL ANALYSIS

Data were tested for normality (Kolmogorov and Smirnov) and subsequently tested using parametric methods (usually 1-way ANOVA). Non-normally distributed data were either transformed (reciprocal or log) and tested as normal or tested non-parametrically (usually Kruskal-Wallis). Where sample sizes were too small to test for normality or variances between groups was significantly different (Bartlett's), non-parametric tests were used. Comparisons were considered significant at $p < 0.05$.

CHAPTER 3

3.1. INTRODUCTION

Pneumococcal pneumonia is an important source of mortality and morbidity throughout the world (Section 1.1 Pneumonia). While much research has been carried out to determine the pathogenesis of this disease, there is a lack of data regarding differential injury to alveolar epithelial cells (1.3.2.3 Models of Pneumococcal and Staphylococcal Pneumonia).

3.2. HYPOTHESIS 1

Rat models of *S. aureus*-induced pneumonia have demonstrated specific injury to ATI cells in the acute phase and proliferation of ATII cells during repair, ascertained using cell-specific biomarker proteins (1.3.2.3 Models of Pneumococcal and Staphylococcal Pneumonia). Using this premise, the following hypothesis was formulated.

Pneumococcal pneumonia induces differential damage to ATI and ATII cells

The aims of this chapter were to set up and characterise a rat model of acute injury, resolution and recovery of *S. pneumoniae*-induced pneumonia and to then use cell-specific proteins to assess differential alveolar epithelial cell damage.

3.3. METHODS

The methodology used to set up the recovering rat model was based on that employed previously in our laboratory (McElroy et al., 2002; Tyrrell et al., 2005). It was considered that while surgical intubation to deliver the bacteria (Rhodes et al., 1989a; Rhodes et al., 1989b; Yoneda and Coonrod, 1980) might be appropriate for ventilated animals under terminal anaesthesia (McElroy et al., 1995; McElroy et al., 1999), this would be inappropriate in a recovering model, both for animal welfare reasons and

since it would require recovery from a procedure in addition to the bacterial inoculation. Intranasal instillation (Bergeron et al., 1998; Brown et al., 2002; Dallaire et al., 2001; Garcia-Suarez et al., 2007; Kadioglu et al., 2000) allows for the possibility of the animals swallowing some of the inoculum and may not guarantee a consistent quantity of bacteria delivered directly into the lungs. Thus a non-surgical intra-tracheal instillation procedure, delivered under light anaesthetic, was selected. The wildtype D39 strain of *S. pneumoniae*, a virulent, encapsulated, serotype 2, laboratory strain, NCTC 7466 (National Collection of Type Cultures, London, UK) (Berry et al., 1989), was used for all experiments. Doses used were 10^6 , 10^7 , 10^8 or 10^9 cfu delivered in 0.5ml PBS vehicle. Control animals were instilled with PBS vehicle.

A preliminary time course indicated that the following time points represented acute injury (24 hours), resolution (72 hours) and recovery (3 weeks) and these were selected for further investigation. 24 hour, 72 hour and 3 week infected animals were treated during different experiments along with controls for each.

In all graphs data presented is as a result of at least 2 separate experiments. Data points shown are means unless stated otherwise. Error bars are 1x standard deviation (SD). Asterisks on graphs represent significance compared to control unless otherwise stated. Other levels of significance between experimental groups are qualified in the figure legend if appropriate. In graphs comparing 24 and 72 hour time points, 24 hour data is the same as (or representative of) data presented in the previous 24 hour time point graph. In this case asterisks are not used to demonstrate significance between 24

hour controls and experimental groups as this data has already been presented. Analysis of specific proteins is presented as a percentage of the mean control value.

3.4. RESULTS

3.4.1 Basic Characterisation of *S. pneumoniae*-induced Pneumonia Model

3.4.1.1 Aim

The aim of this section was to set up and characterise a rat model of acute injury, resolution and recovery of *S. pneumoniae*-induced pneumonia

3.4.1.2. Weight Loss

Loss of body weight was used as a crude measure of animal health during the experiments (Figure 3.1). All animals had a starting weight of 300-350g. Rats instilled with the higher doses of *S. pneumoniae* (10^8 and 10^9 cfu) showed an 8% decrease in body weight after 24 hours. The lower doses (10^6 and 10^7 cfu) were not significantly lower than controls. At 48 hours the rats given the higher doses still weighed significantly less than controls. By 72 hours the rats given the highest dose were still lower in weight, although this was starting to increase. Rats inoculated with 10^8 cfu were of equivalent weight to controls although values were highly variable. From 72 hours to 3 weeks, weights of infected animals continued to increase in a similar manner to controls (Figure 3.1).

3.4.1.3 Histology

Instillation with *S. pneumoniae* in most cases induced a lobar pneumonia that was restricted to the left lobe. By 24 hours the affected areas of lung were macroscopically livid and red, whereas after 72 hours were more of a grey colour. Three weeks after

instillation lungs were barely distinguishable from normal. H&E staining was carried out on control and *S. pneumoniae*-infected lung sections to determine microscopic changes in lung architecture. Control sections show clear airspaces and normal lung architecture (Figure 3.2a) but after 24 hours there is an influx of inflammatory cells and red blood cells (RBCs) (Figure 3.2b). By 72 hours inflammatory cells are still present and the alveolar walls are thickened (Figure 3.2c) but by 21 days the lungs look normal again (Figure 3.2d).

3.4.1.4 Bacterial Recovery

The number of viable bacteria recovered from BAL fluid and lung homogenate in *S. pneumoniae*-infected rats was used as a measure of bacterial growth and clearance.

Ninety nine percent of instilled bacteria were cleared from the lungs by 24 hours (Figure 3.3a). After 72 hours there was an additional 10-fold decrease (Figure 3.3b). Positive blood cultures were observed in some of the infected animals after 24 hours. This ranged from 0% at the 10^6 dose to 55% at 10^9 cfu. Overall, these data suggest that the lung is able to clear most of the *S. pneumoniae* by 24 hours with further but not complete clearance by 72 hours.

3.4.1.5 Airway Inflammation

In order to characterise inflammation in the airways, assessment of inflammatory cells in BAL fluid was carried out, including total and differential leukocyte counts. Instillation of *S. pneumoniae* induced a significant, dose dependent, up to 100-fold increase in the number of BAL fluid leukocytes recovered after 24 hours (Figure 3.4a). This infiltration decreased after 72 hours (Figure 3.4b). Of BAL fluid leukocytes, the most abundant in controls was the macrophage, accounting for more

than 80% of cells. In all *S. pneumoniae* infected rats after 24 hours however, the ratio of macrophages to neutrophils significantly reversed, regardless of dosage (Figure 3.5a) even though the absolute numbers of macrophages increased dose-dependently (Control: $1.16 \times 10^4 \pm 6.07 \times 10^3$; 10^6 cfu: $2.58 \times 10^4 \pm 8.24 \times 10^3$; 10^7 cfu: $6.77 \times 10^4 \pm 3.2 \times 10^4$; 10^8 cfu: $8.91 \times 10^4 \pm 5.62 \times 10^4$; 10^9 cfu: $1.52 \times 10^5 \pm 1.07 \times 10^5$). By 72 hours the neutrophil percentage had dropped to about 50%, which was significantly different from both controls and instilled animals after 24 hours (Figure 3.5b). The number of macrophages in infected animals after 24 and 72 hours was similar (24 hrs: $1.52 \times 10^5 \pm 1.07 \times 10^5$; 72 hrs: $1.45 \times 10^5 \pm 1.05 \times 10^5$).

3.4.1.6 Damage to Air-Blood Barrier

In order to quantify injury to the air-blood barrier, analysis was carried out on protein in BAL fluid as a measure of protein flux across the barrier. *S. pneumoniae* induced a significant, dose-dependent, up to 5 fold increase in BAL fluid protein after 24 hours, the 10^9 cfu inoculation level containing protein concentrations significantly higher than lower inoculation levels (Figure 3.6a). By 72 hours the amount of protein had decreased significantly although it was still significantly higher than controls (Figure 3.6b). These data suggest that *S. pneumoniae* causes damage to the air-blood barrier after 24 hours, but that this is starting to resolve by 72 hours.

At this point it was considered that the two higher doses of bacteria provided good evidence of appropriate injury and inflammation whilst still demonstrating resolution and recovery. Thus further analysis was carried out with inoculations of 10^8 and 10^9 cfu only.

3.4.1.7 Pleural Space

In order to determine if infection had disseminated from the lungs to the pleural cavity, assessment was carried out on pleural lavage fluid. This included recovery of viable bacteria (for bacterial dissemination), total and differential leukocyte counts (for influx of inflammatory cells) and measurement of protein (for leakage from lungs to pleural space). Positive bacterial cultures of pleural lavage fluid were recorded for 20-25% of rats infected with 10^8 and 10^9 cfu. Although there was no significant difference between the total numbers of leukocytes in *S. pneumoniae*-infected pleural lavage fluid and controls either after 24 or 72 hours (Figure 3.7a + b), the bacteria did induce a significant increase in percentage of neutrophils, and a decrease in macrophages at 24 hours (Figure 3.8a). This had returned to normal by 72 hours (Figure 3.8b) and there was no effect on either eosinophils or mast cells at either time point (Figure 3.8). The presence of 10% eosinophils in both control and bacteria-treated animals was unexpected. This result may be explained by some kind of low-grade infection. While animals were initially supplied as SPF, the animal unit in which they were housed for a short time prior to treatment was not, and rats may have acquired pathogens from their environment during this time. Figure 10a shows that protein levels in pleural lavage were very variable, and there was no significant difference between controls and *S. pneumoniae*-infected animals at 24 hours. Variability at 72 hours was high, with bacterially instilled rats showing a significantly higher amount of pleural lavage protein than those at 24 hours. However, control values at the 72-hour time point were also noticeably lower than those at 24 hours.

As there were no significant differences between control groups at 24 hours and 72 hours in any measured parameters the two control groups were subsequently pooled.

3.4.1.8 Inflammatory Mediators

In order to determine changes in inflammatory mediators in the lungs, measurements were made of the cytokine IL-1 α and chemokine MCP-1. Both proteins were only detectable in infected BAL fluid and not in controls. Control lung homogenates contained 450 ± 208 pg/ml of IL-1 α and 100 ± 90 pg/ml of MCP-1. Due to assay limitations of space samples from each treatment group were randomly selected from 2 experiments to assess. *S. pneumoniae* induced a significant (10 fold) increase in IL-1 α after 24 hours, which was not dose-dependent (Figure 3.10a). This increase was maintained at 72 hours (Figure 3.10b). Similarly, levels of MCP-1 also increased, by 25 fold after 24 hours (Figure 3.11a), this increase being sustained until 72 hours (Figure 3.11b). TNF- α , IFN- γ and GM-CSF were all below the detection limit of the assay.

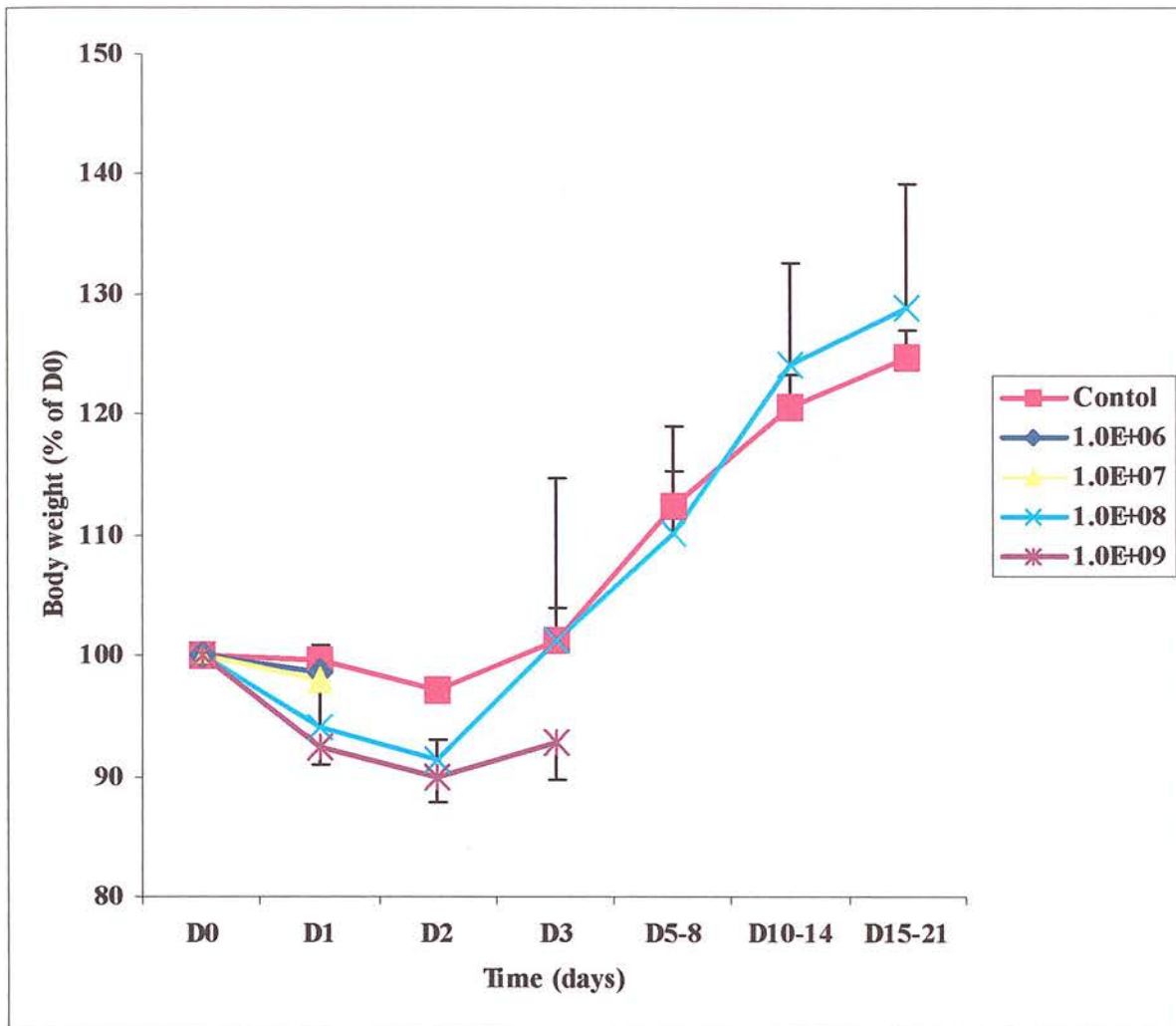


Figure 3.1 - Rat body weight

Data expressed as a percentage of initial weight at Day 0.

D1: Control, D39 10⁶, 10⁷, 10⁸, 10⁹

After 24 hours weight decreased in a dose-dependent manner, significant at doses of 10⁸ and 10⁹ bacteria.

Kruskal-Wallis p < 0.0001

Dunns post-test

Control vs D39 10 ⁸	***	p < 0.001
Control vs D39 10 ⁹	***	p < 0.001
D39 10 ⁶ vs D39 10 ⁸	**	p < 0.01
D39 10 ⁶ vs D39 10 ⁹	***	p < 0.001
D39 10 ⁷ vs D39 10 ⁹	**	p < 0.01

D2: Control, D39 10⁸, 10⁹

After 48 hours differences between groups were still significant (Kruskal-Wallis p = 0.0302)

D3: Control, D39 10⁸, 10⁹

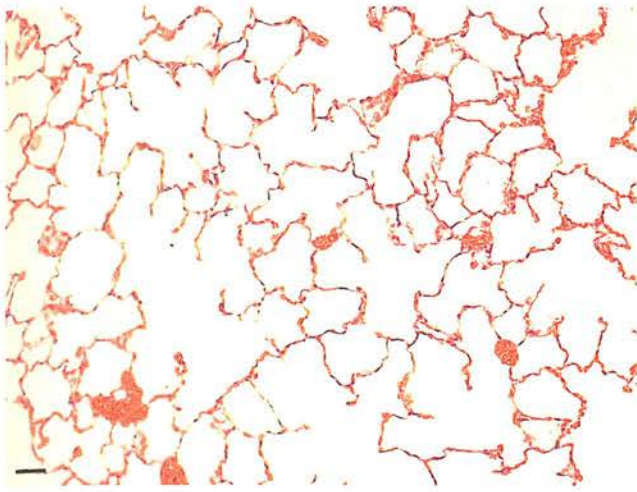
After 72 hours differences between groups were still significant (Kruskal-Wallis p < 0.0047)

D5-21: Control, D39 10⁸

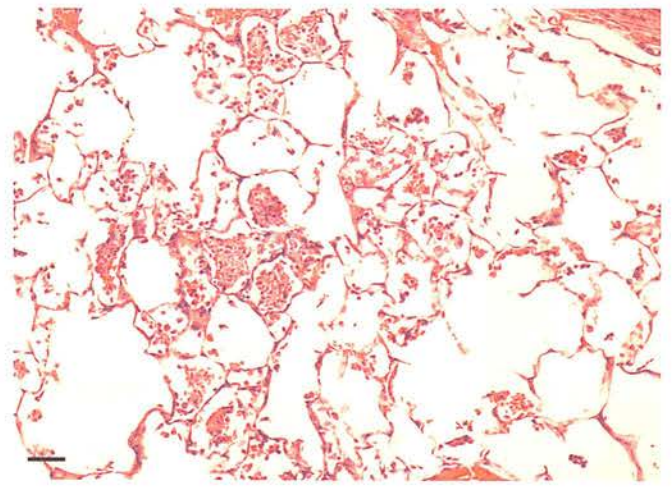
By 5-21 days the differences between groups were not significant

Sample Numbers

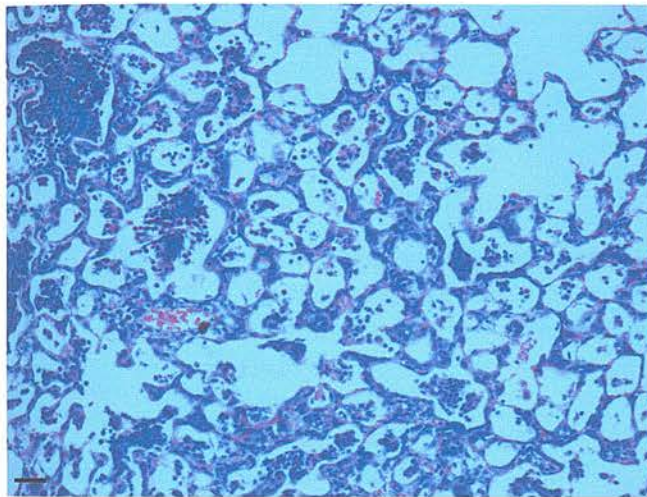
Time point	PBS	D39 10 ⁶	D39 10 ⁷	D39 10 ⁸	D39 10 ⁹
D1	26	5	6	21	18
D2	3	-	-	3	4
D3	8	-	-	6	6
D5-8	3	-	-	7	-
D10-14	3	-	-	8	-
D15-21	5	-	-	12	-



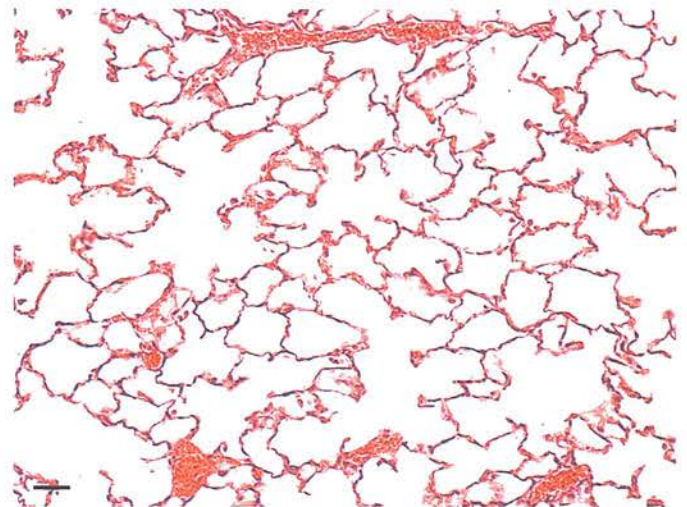
A



B



C

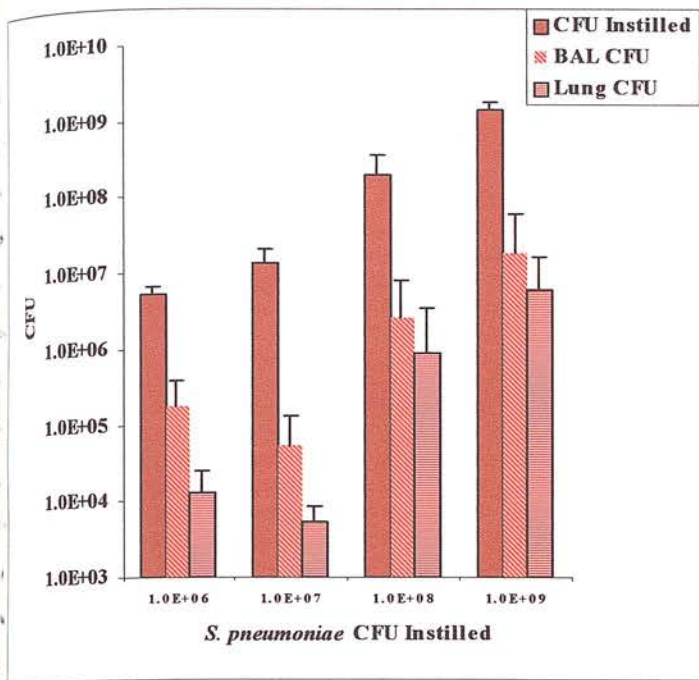


D

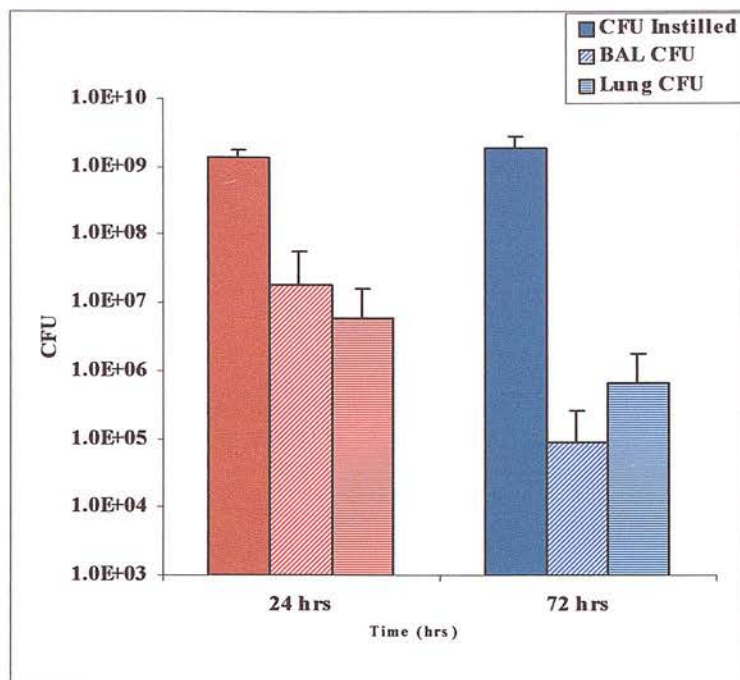
Figure 3.2 – H&E of rat lung

Original magnification x 10. Scale bars are 100µm

- A. Normal airways and lung architecture
- B. *S. pneumoniae* 10⁸ cfu at 24 hours
RBCs and inflammatory cells in airways
- C. *S. pneumoniae* 10⁸ cfu at 72 hours
Inflammatory cells in airways and thickened alveolar walls
- D. *S. pneumoniae* 10⁸ cfu at 21 days
Normal airways and restoration of lung architecture



A



B

Figure 3.3 – CFU recovered from lungs

Data expressed as total cfu shown on a log scale

Bars are Instillation cfu, BAL fluid cfu and Lung tissue cfu

Samples in each treatment group are taken from at least 3 separate experiments

A. 24 hour dose response

There were no significant differences between *S. pneumoniae* dosage groups after 24 hours (red).

D39 10⁶ n = 5, D39 10⁷ n = 3, D39 10⁸ n = 10, D39 10⁹ n = 5

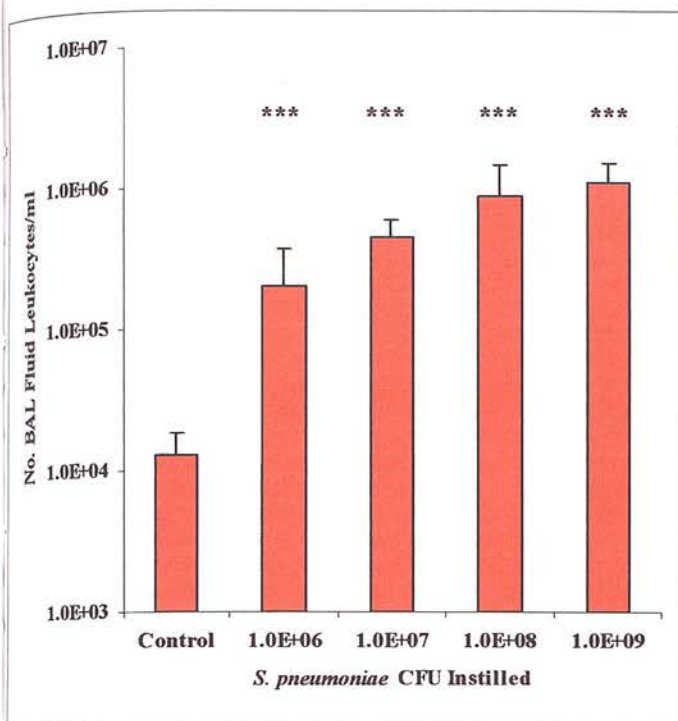
B. 24 hours vs 72 hours (10⁹ cfu)

Data expressed for 24 hours is the same as that presented in Figure A (red).

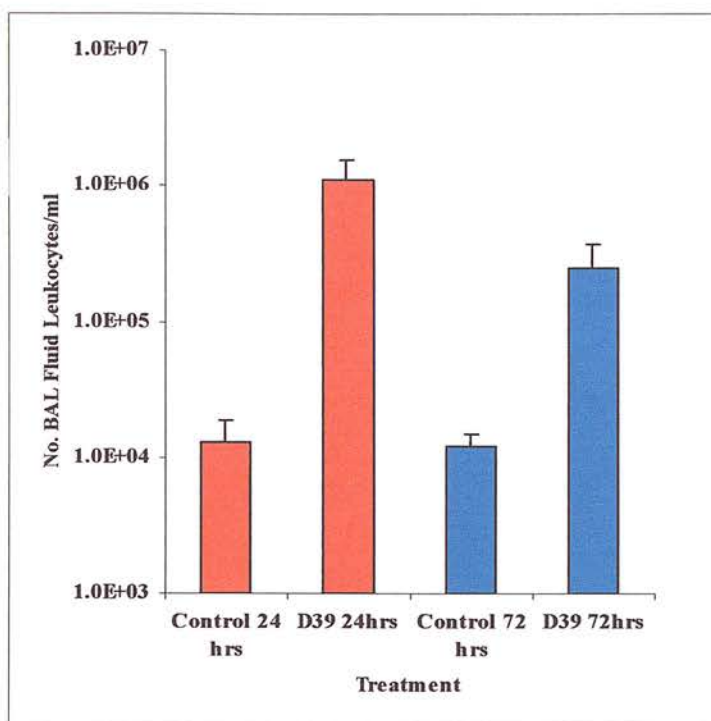
24 and 72 hour infected groups were treated during separate experiments.

Although the trend was for less bacterial recovery at 72 hours (blue), there were no significant differences between 24 hours (red) and 72 hours (blue),

D39 24 hours n = 5, D39 72 hours n = 6



A



B

Figure 3.4 – Number of leukocytes in BAL fluid

Data expressed as number of cells per ml of BAL fluid shown on a log scale
 Samples in each treatment group are taken from at least 3 separate experiments

A. 24 hour dose response

S. pneumoniae induced a dose-dependent increase in BAL leukocytes after 24 hours (red), significant at all inoculation levels.

Transformed (Log) ANOVA $p < 0.0001$

Tukey post-test	Control vs D39	Significance	p-value
Control vs D39 10 ⁶	***	$p < 0.001$	
Control vs D39 10 ⁷	***	$p < 0.001$	
Control vs D39 10 ⁸	***	$p < 0.001$	
Control vs D39 10 ⁹	***	$p < 0.001$	
D39 10 ⁶ vs D39 10 ⁸	***	$p < 0.001$	
D39 10 ⁶ vs D39 10 ⁹	***	$p < 0.001$	

Control $n = 9$, D39 10⁶ $n = 5$, D39 10⁷ $n = 4$, D39 10⁸ $n = 10$, D39 10⁹ $n = 5$

B. 24 hours vs 72 hours (Control + 10⁹ cfu)

Data expressed for 24 hours is the same as that presented in Figure A (red).

24 and 72 hour infected groups were treated during separate experiments.

The number of BAL fluid leukocytes recovered after 72 hours (blue) was lower than at 24 hours (red) but higher than in Controls. Differences between groups were significant (Kruskall-Wallis $p = 0.0002$) but only at 24 hours (see A)

There was no significant difference between 24 and 72 hours controls.

Control 24 hours $n = 9$, Control 72 hours $n = 7$, D39 24 hours $n = 5$, D39 72 hours $n = 6$

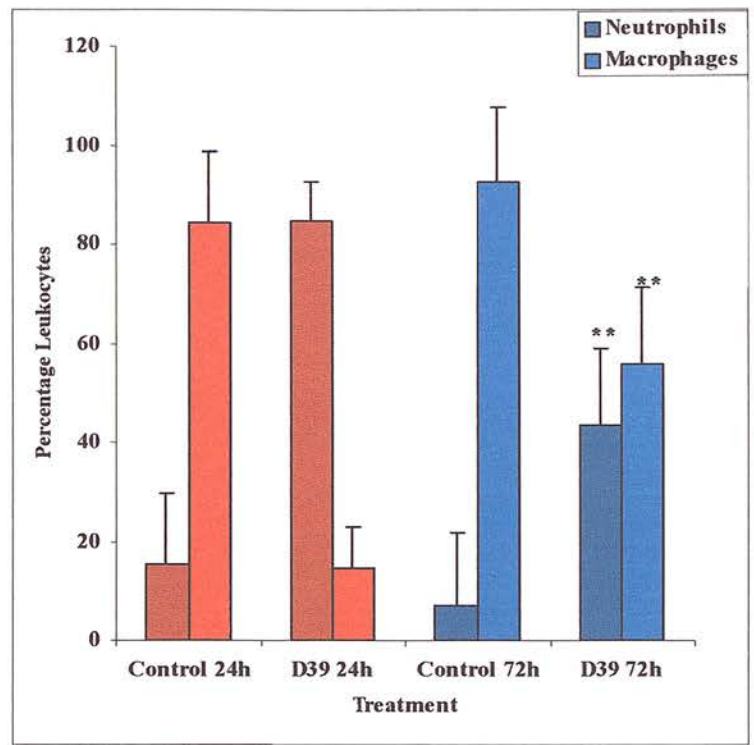
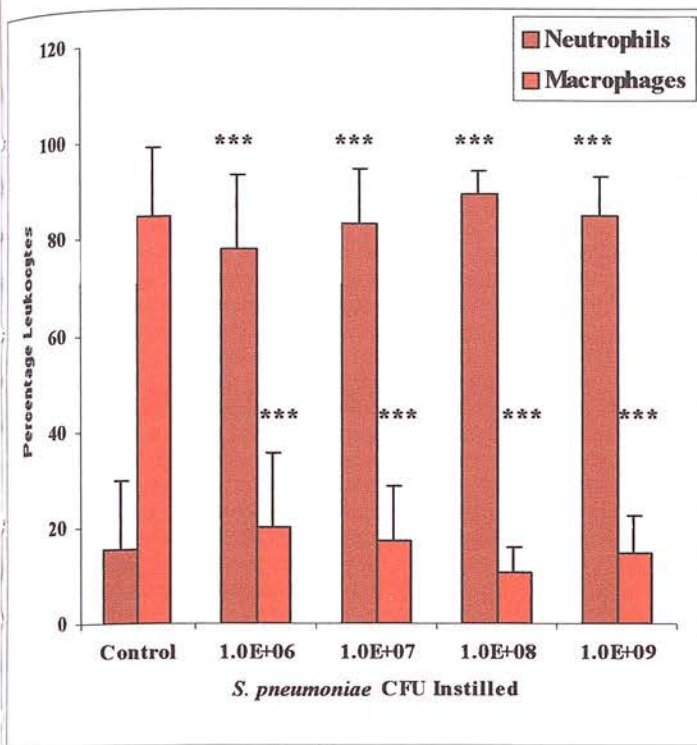


Figure 3.5 – Differential leukocyte count in BAL fluid
 Data expressed as percentage macrophages and neutrophils. Percentage of other leukocytes <1%.

Samples in each treatment group are taken from at least 3 separate experiments

A. 24 hour dose response
 There was a significant difference between percentage macrophages and neutrophils in all groups after 24 hours (red). *S. pneumoniae* induced a significant increase in the ratio of macrophages:neutrophils compared to controls. This was not dose-dependent. There were no differences between *S. pneumoniae*-infected groups.

ANOVA $p < 0.0001$

Tukey post-test All groups:

Neutrophils vs macrophages *** $p < 0.001$

Neutrophils:

Controls vs all D39 groups *** $p < 0.001$

Macrophages:

Control vs all D39 groups *** $p < 0.001$

Control $n = 9$, D39 10^6 $n = 5$, D39 10^7 $n = 4$, D39 10^8 $n = 10$, D39 10^9 $n = 5$

B. 24 hours vs 72 hours (Control + 10^9 cfu)

Data expressed for 24 hours is the same as that presented in Figure A (red). 24 and 72 hour infected groups were treated during separate experiments.

The ratio of macrophages : neutrophils significantly decreased at 72 hours (blue) compared to 24 hours (red), however it was still significantly higher than controls. There was no significant difference between 24 and 72 hours controls.

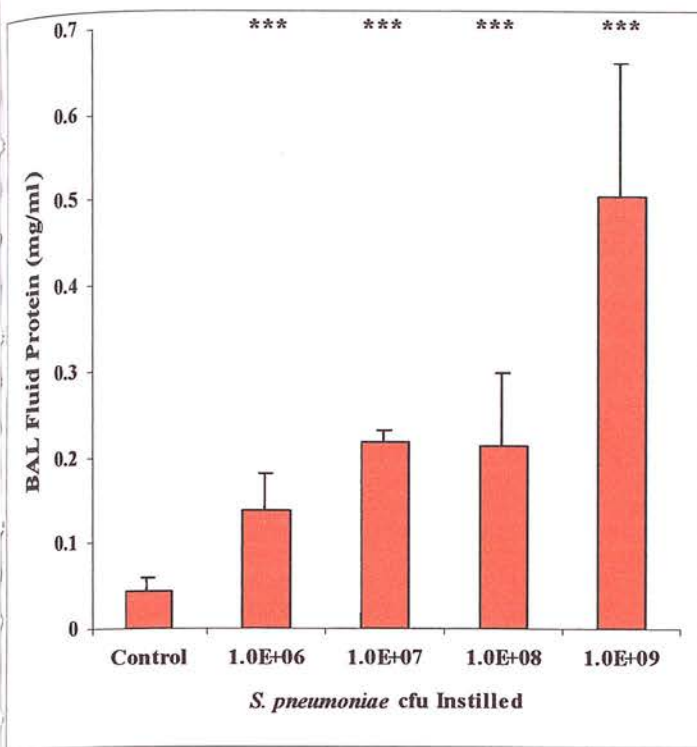
ANOVA $p < 0.0001$

Tukey post-test Macrophages and Neutrophils

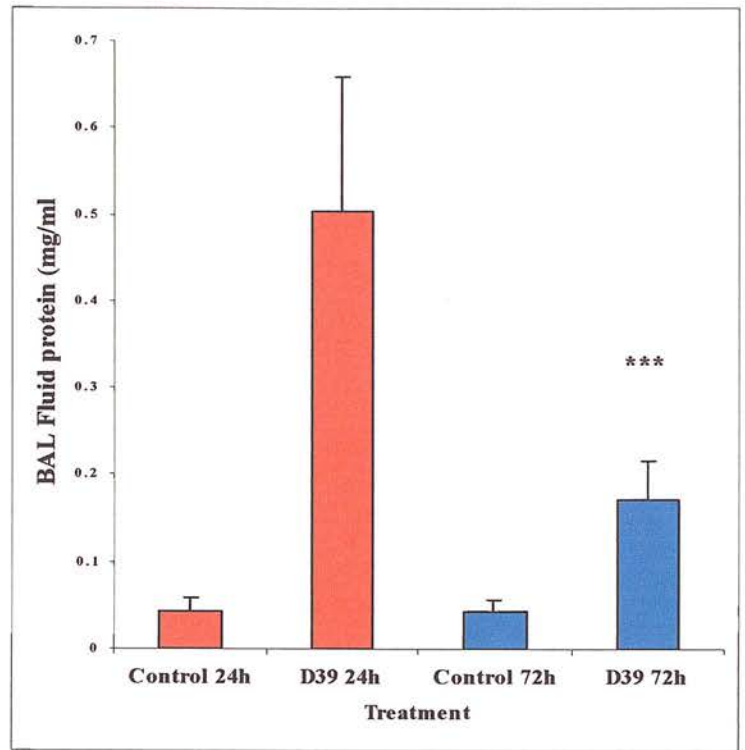
All Controls vs D39 72 hours ** $p < 0.01$

D39 24 hours vs D39 72 hours *** $p < 0.001$

Control 24 hours $n = 9$, Control 72 hours $n = 7$, D39 24 hours $n = 5$, D39 72 hours $n = 6$



A



B

Figure 3.6 – Protein concentration in BAL fluid

Data expressed as protein (mg) per ml BAL fluid

Samples in each treatment group are taken from at least 3 separate experiments

A. 24 hour dose response

S. pneumoniae induced a dose-dependent increase in BAL fluid protein after 24 hours (red) which was significant at all inoculation levels.

Transformed (Log) ANOVA $p < 0.0001$

Tukey post-test Control vs D39 10 ⁶	***	$p < 0.001$
Control vs D39 10 ⁷	***	$p < 0.001$
Control vs D39 10 ⁸	***	$p < 0.001$
Control vs D39 10 ⁹	***	$p < 0.001$
D39 10 ⁶ vs D39 10 ⁹	***	$p < 0.001$
D39 10 ⁷ vs D39 10 ⁹	*	$p < 0.05$
D39 10 ⁸ vs D39 10 ⁹	**	$p < 0.01$

Control n = 11, D39 10⁶ n = 5, D39 10⁷ n = 4, D39 10⁸ n = 10, D39 10⁹ n = 5

B. 24 hour vs 72 hours (Control + 10⁹ cfu)

Data expressed for 24 hours is the same as that presented in Figure A (red). 24 and 72 hour infected groups were treated during separate experiments.

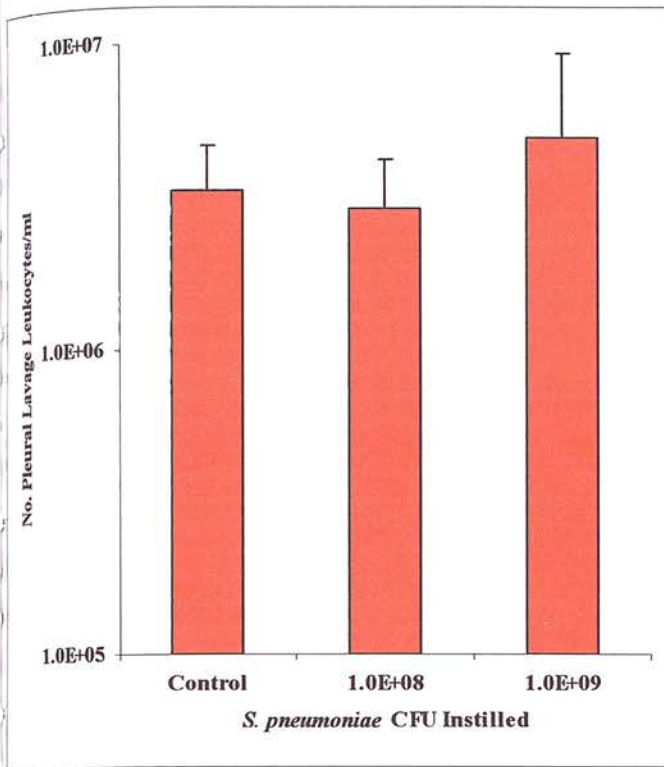
S. pneumoniae still induced a significant increase in BAL fluid protein after 72 hours (blue) compared to controls (black), although values were significantly lower than those at 24 hours (red).

There was no significant difference between 24 and 72 hours controls.

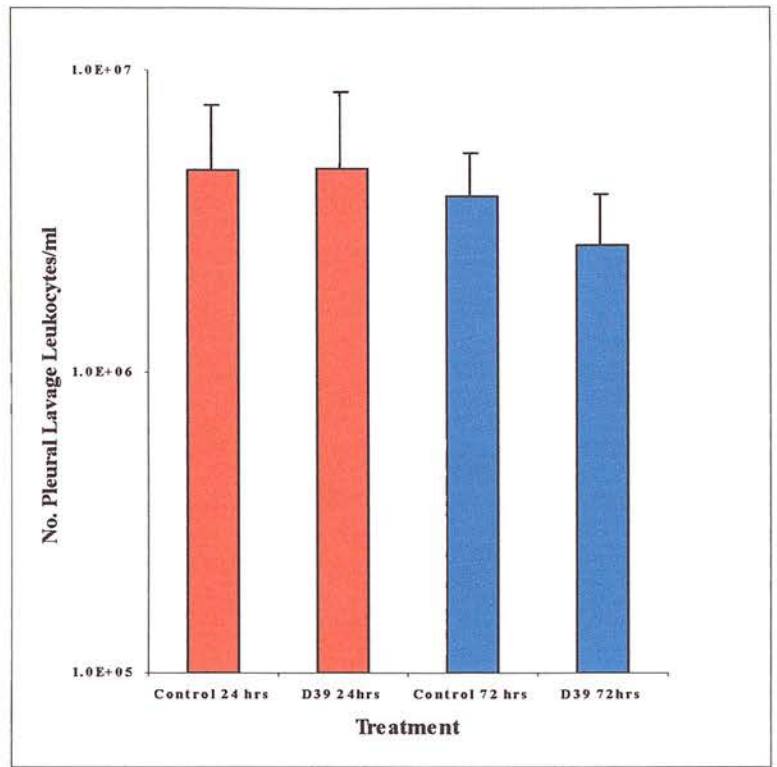
Transformed (Log) ANOVA $p < 0.0001$

Tukey post-test All Controls vs D39 72 hours	***	$p < 0.001$
D39 24 hours vs D39 72 hours	***	$p < 0.001$

Control 24 hours n = 11, Control 72 hours n = 7, D39 24 hours n = 5, D39 72 hours n = 6



A



B

Figure 3.7 – Number of leukocytes in pleural lavage fluid shown on a log scale

Data expressed as number of cells per ml pleural lavage fluid

Samples in each treatment group are taken from at least 3 separate experiments

A. 24 hour dose response

There was no significant difference in the number of pleural lavage fluid leukocytes between control and *S. pneumoniae*-infected samples after 24 hours (red)

Control n = 8, D39 10⁸ n = 6, D39 10⁹ n = 5

B. 24 hours vs 72 hours (Control + 10⁹ cfu)

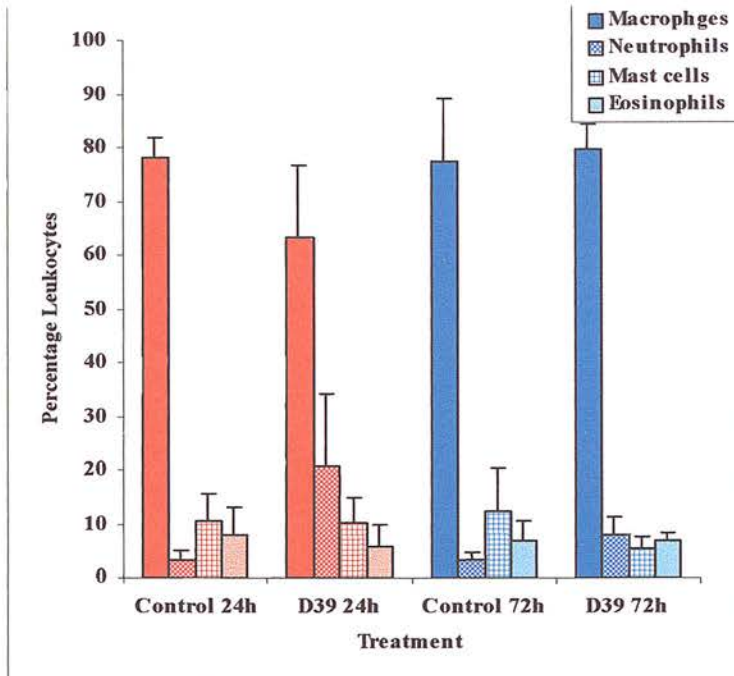
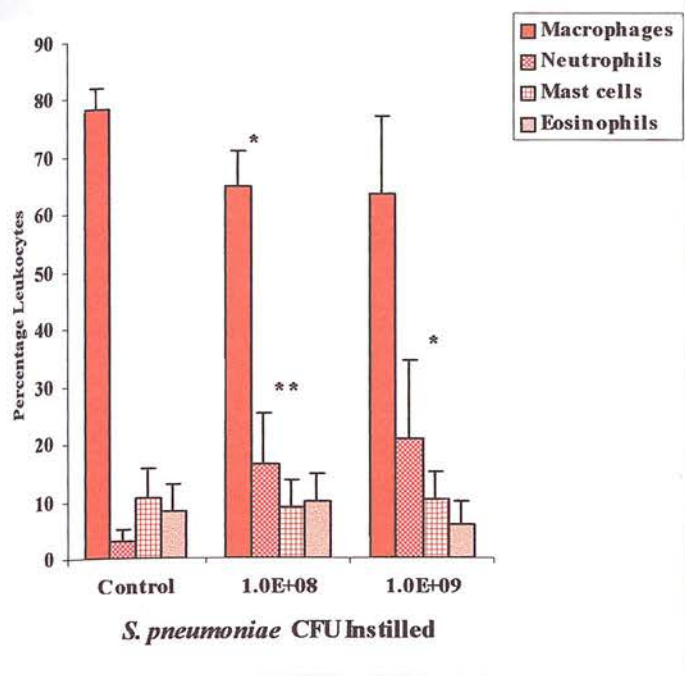
Data expressed for 24 hours is the same as that presented in Figure A (red).

24 and 72 hour infected groups were treated during separate experiments.

There was no significant difference in the number of pleural lavage fluid leukocytes between 24 (red) and 72 (blue) hours.

There was no significant difference between 24 and 72 hours controls.

Control 24 hours n = 8, Control 72 hours n = 7, D39 24 hours n = 5, D39 72 hours n = 6



A

B

Figure 3.8 – Differential leukocyte count in pleural lavage fluid

Data expressed as percentage macrophages, neutrophils, mast cells and eosinophils.

Percentage of other leukocytes <1%.

Samples in each treatment group are taken from at least 3 separate experiments

A. 24 hour dose response

S. pneumoniae induced a significant, dose-dependent increase in percentage neutrophils in pleural lavage fluid after 24 hours (red), as well as a significant decrease in the percentage of macrophages. There were no differences in proportions of eosinophils or mast cells.

Neutrophils:

Transformed (Log) ANOVA p = 0.0034

Tukey post-test Control vs D39 10⁸ = ** p < 0.01
Control vs D39 10⁹ = * p < 0.05

Macrophages:

Kruskall-Wallis p = 0.0068

Dunns post-test Control vs D39 10⁸ = * p < 0.05

Control n = 8, D39 10⁸ n = 6, D39 10⁹ n = 5

B. 24 hours vs 72 hours (Control + 10⁹ cfu)

Data expressed for 24 hours is the same as that presented in Figure A (red).

24 and 72 hour infected groups were treated during separate experiments.

The percentage of neutrophils in pleural lavage decreased between 24 (red) and 72 (blue) hours, although the latter value was still higher than controls there was no significant difference between groups. The percentage of macrophages increased to control levels by 72 hours (blue) but was not significantly different from 24 hours (red). There were no differences between percentages of eosinophils and mast cells.

There was no significant difference between 24 and 72 hours controls.

Control 24 hours n = 9, Control 72 hours n = 7, D39 24 hours n = 5, D39 72 hours n = 6

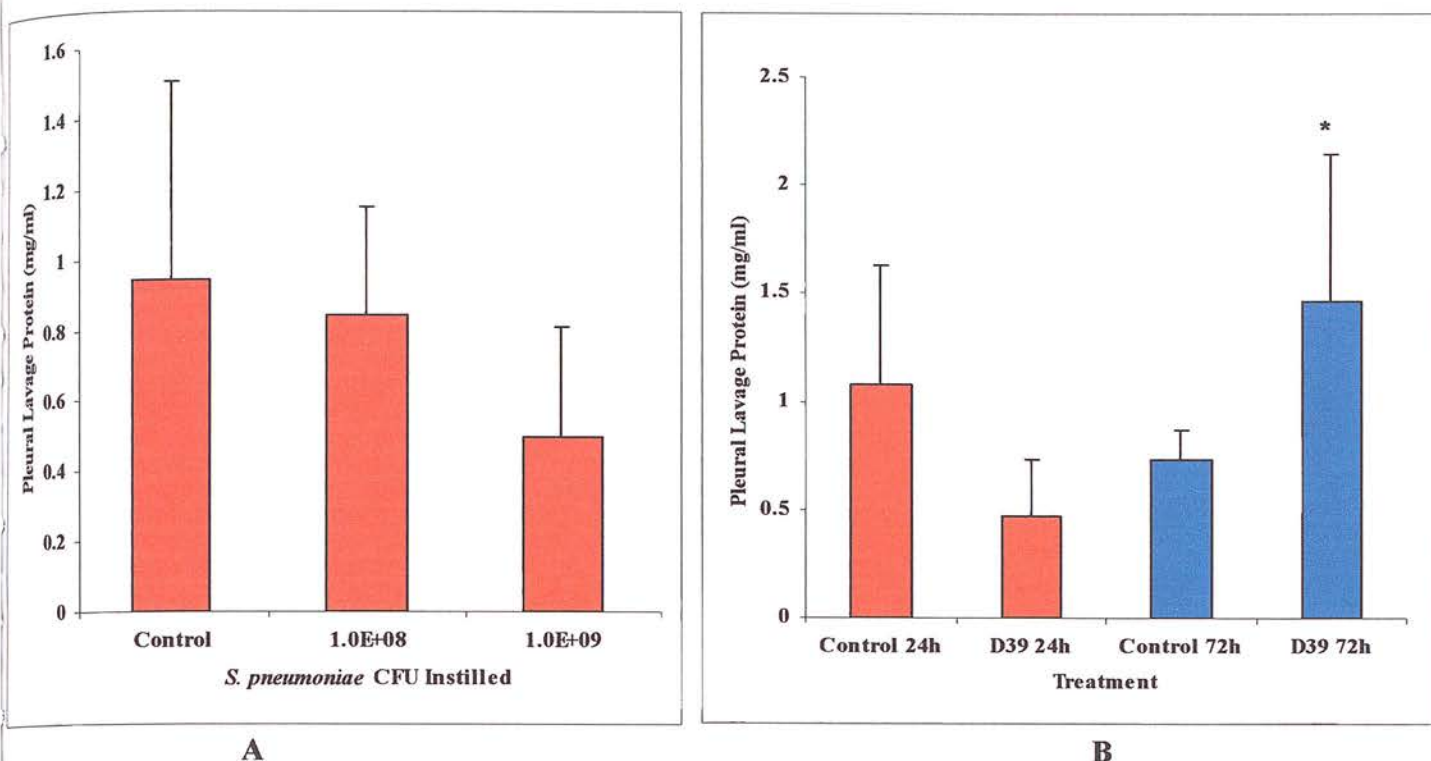


Figure 3.9 – Protein concentration in pleural lavage fluid

Data expressed as protein (mg) per ml pleural lavage fluid

Samples in each treatment group are taken from at least 3 separate experiments

A. 24 hour dose response

Although pleural lavage fluid protein appears to decrease with *S. pneumoniae* infection, there was no significant difference between groups after 24 hours (red).

Control n = 10, D39 10⁸ n = 6, D39 10⁹ n = 5

B. 24 hours vs 72 hours (Control + 10⁹ cfu)

Data expressed for 24 hours is the same as that presented in Figure A (red).

24 and 72 hour infected groups were treated during separate experiments.

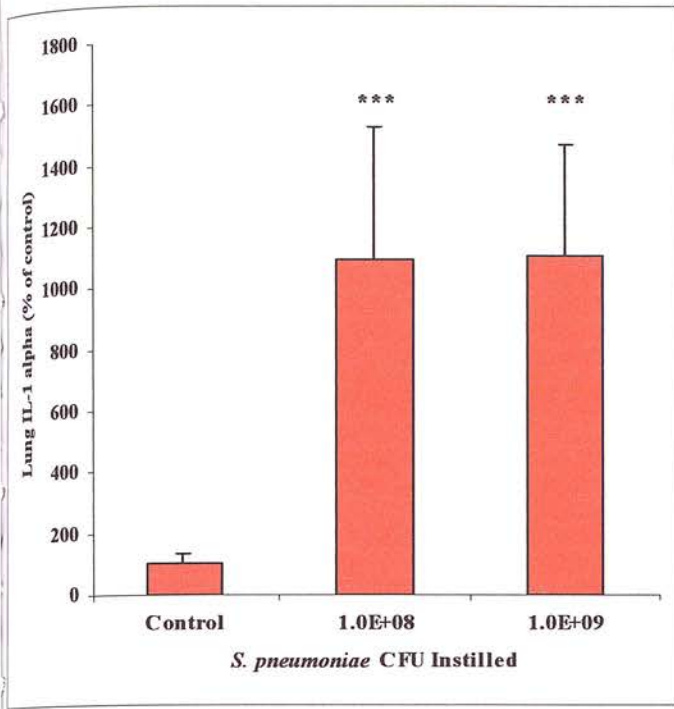
Asterisk shows significance between *S. pneumoniae* groups at 24 and 72 hours

Protein in pleural lavage fluid appears to increase with *S. pneumoniae* infection after 72 hours (blue) and is significantly higher than at 24 hours (red). However, control values at 72 hours (blue) are also lower than those at 24 hours (red).

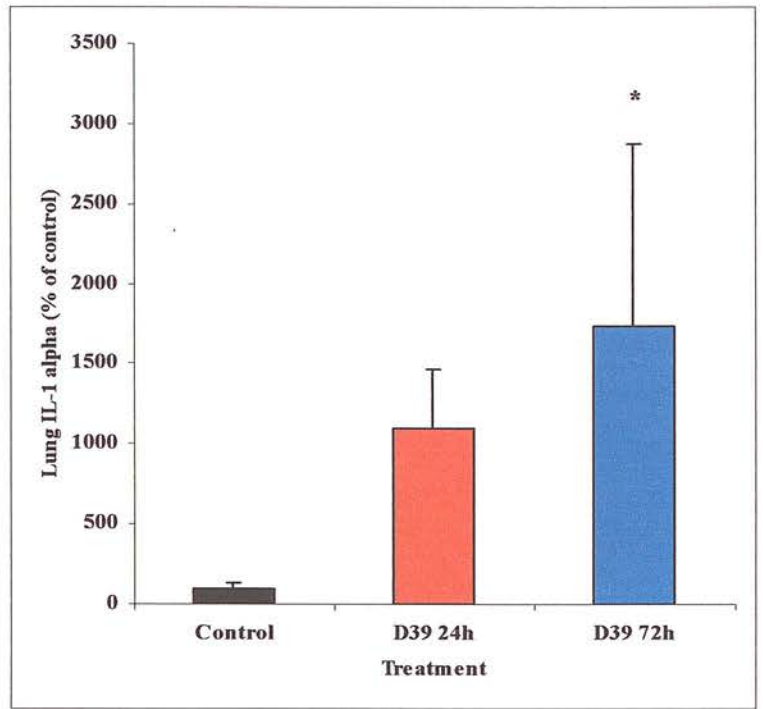
Transformed (Log) ANOVA p = 0.317

Tukey post-test D39 24 hours vs D39 72 hours * p < 0.05

Control 24 hours n = 10, Control 72 hours n = 5, D39 24 hours n = 5, D39 72 hours n = 5



A



B

Figure 3.10 – IL-1 α in lung tissue

Data expressed per mg lung protein and presented as a percentage of control

Samples in each treatment group are taken from 2 separate experiments

A. 24 hour dose response

S. pneumoniae induced a significant increase in IL-1 α in lung tissue after 24 hours (red), which was not dose-dependent.

ANOVA Transformed (Log) $p < 0.0001$

Tukey post-test Control vs D39 10⁸ *** $p < 0.001$

Control vs D39 10⁹ *** $p < 0.001$

Control $n = 4$, D39 10⁸ $n = 4$, D39 10⁹ $n = 4$

B. 24 hours vs 72 hours (Control + 10⁹ cfu)

Data expressed for 24 hours is the same as that presented in Figure A (red).

24 and 72 hour infected groups were treated during separate experiments.

S. pneumoniae continued to induce a significant increase in IL-1 α after 72 hours (blue) compared with controls (black). Although levels did increase, there was no significant difference between 24 hours (red) and 72 hours (blue).

Kruskall-Wallis $p = 0.009$

Dunns post-test Control vs D39 72 hours * $p < 0.05$

Control $n = 4$, D39 24 hours $n = 4$, D39 72 hours = 4

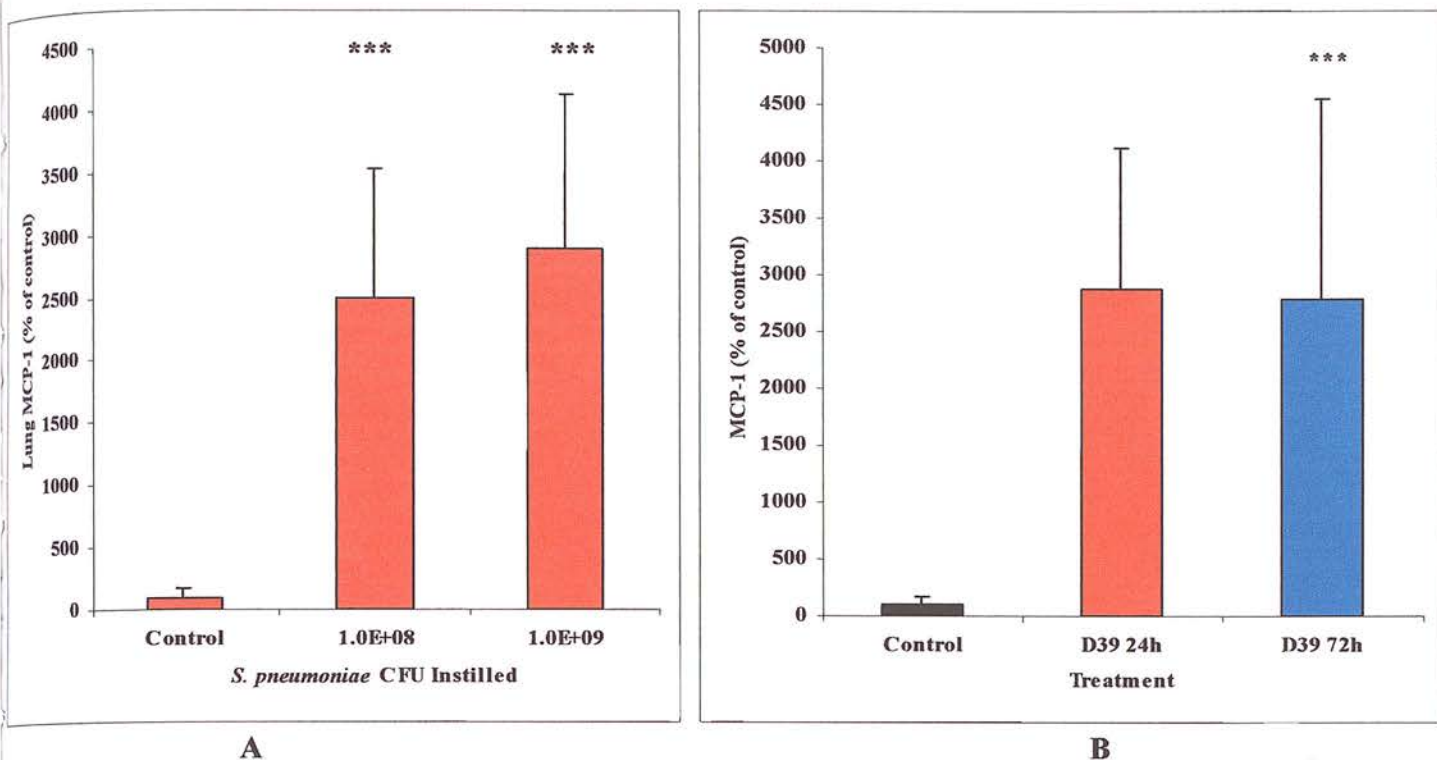


Figure 3.11 – MCP-1 in lung tissue

Data expressed per mg lung protein and presented as a percentage of control

Samples in each treatment group are taken from 2 separate experiments

A. 24 hour dose response

S. pneumoniae induced a significant increase in MCP-1 in lung tissue after 24 hours (red), which was not dose-dependent.

Transformed (Log) ANOVA $p < 0.0001$

Tukey post-test Control vs D39 10^8 *** $p < 0.001$

Control vs D39 10^9 *** $p < 0.001$

Control $n = 4$, D39 10^8 $n = 4$, D39 10^9 $n = 4$

B. 24 hours vs 72 hours (Control + 10^9 cfu)

Data expressed for 24 hours is the same as that presented in Figure A (red).

24 and 72 hour infected groups were treated during separate experiments.

S. pneumoniae continued to induce a significant increase in MCP-1 after 72 hours (blue) compared to controls (black). There was no significant difference between 24 hours (red) and 72 hours (blue).

Transformed (Log) ANOVA $p < 0.0001$

Tukey post-test Control vs D39 72 hours *** $p < 0.001$

Control $n = 4$, D39 24 hours $n = 4$, D39 72 hours $n = 4$

3.4.1.9 Summary and Conclusions

- A recovering model of pneumococcal pneumonia was set up at the acute (24 hours), resolving (72 hours) and recovering (21 days) phases
- Rats showed a decrease in body weight which recovered over time
- The model was characterised by progressive bacterial clearance
- The model demonstrated an acute neutrophilic response in the airways, air-blood barrier damage, and some evidence of inflammation in the pleural space, all of which started to resolve by 72 hours
- The model demonstrated an increase in pro-inflammatory IL-1 α and MCP-1 by 24 hours which was maintained up to 72 hours

The purpose of this section was to establish a recovering model of *S. pneumoniae*-induced pneumonia. This was characterised by histological evidence of consolidation, resolution and recovery from 24 hours, 72 hours and 3 weeks. Experimental animals demonstrated a loss of body weight suggesting a detrimental effect on health that improved over time. Bacteria did not actively grow in the lungs in this model. Even at the acute phase (24 hours) bacterial clearance was apparent, with further clearance at 72 hours, despite bacterial dissemination into the blood and pleural space in a few individuals. The pneumonia model was also characterised by an acute neutrophilic inflammatory response in the airways, with some evidence of neutrophilia in the pleural space, and stimulation of production of pro-inflammatory cytokines. By 72 hours the inflammatory response is attenuated indicating resolution, however the maintenance of high levels of cytokines at this time point suggests further mediation of inflammation at this time point. Damage to the air-blood barrier was also apparent at 24 hours, with decreased protein levels in BAL fluid at 72 hours indicating the initiation of repair.

3.4.2 ATI Cell Injury

3.4.2.1 Aim

To assess damage to ATI cells in the *S. pneumoniae*-induced pneumonia model.

3.4.2.2 Assessment of RTI40

In order to quantify injury to ATI cells, assessment of the ATI cell-specific protein RTI40 was carried out in BAL fluid and lung tissue. This is a technique that has been widely used by the author (McElroy et al., 2002; Tyrrell et al., 2005) and others (McElroy et al., 1995; McElroy et al., 1997b; McElroy et al., 1997a; McElroy et al., 1999) both as a measure of ATI cell-specific injury (analysis of BAL fluid) and as representative of the ATI cell population in the lung (analysis of lung tissue). The principle of the BAL fluid analysis is dependent on the membrane-associated nature of the RTI40 protein. During normal turnover in healthy lungs a basal amount of this protein is present in the airways. However, during lung injury, if ATI cells are damaged they break apart or detach from the basement membrane. As a consequence there is an increase in RTI40 in the airways, which can be sampled by biochemical analysis of BAL fluid in an ELISA-based dot blot. Assessment of the protein in lung tissue provides information about changes in the ATI cell population in the lung during injury. Due to assay limitations of space samples from each treatment group were randomly selected from 2 experiments to assess.

Using the RTI40 antibody, it was demonstrated that *S. pneumoniae* induced a significant decrease in detectable RTI40 in both BAL fluid and lung tissue after 24 hours (Figure 3.12a). This effect was unexpected and dose dependent in lung tissue. Having established that this model allows for resolution and recovery of animals,

implying that ATI cells are not completely destroyed, this result indicates that this method of BAL fluid RTI40 analysis is not an appropriate marker of ATI cell injury in this model. Furthermore these results suggest that regulation of RTI40 levels occurs in this model of infection. By 72 hours, levels of RTI40 in lungs were significantly higher than at 24 hours, although still 60% less than in controls (Figure 3.12b). To further investigate this phenomenon samples from the time point at 24 hours were run on a Western blot, which demonstrated total loss of RTI40 reactivity (Figure 3.12c). To determine whether the decrease in RTI40 was due to alteration of the epitope recognised by the monoclonal antibody, BAL fluid and lung samples from the 24 hour time point were assessed by direct ELISA using the E11 antibody. This antibody is thought to recognise another epitope not on the core protein, unlike the original RTI40 antibody (Williams et al., 1996). This produced similar results in both BAL fluid and lung tissue (Figure 3.12d).

3.4.2.3 Assessment of MMC6

In order to further investigate damage to ATI cells, assessment of the ATI cell-specific MMC6 protein was carried out on BAL fluid and lung tissue. Like RTI40, MMC6 protein is a membrane-associated antigen and has been shown in an ELISA-based dot blot to reflect RTI40 levels in BAL fluid, demonstrating injury to ATI cells in models of staphylococcal pneumonia and haemorrhagic shock (McKechnie, 2008). Due to assay limitations of space samples from each treatment group were randomly selected from 2 experiments to assess

In contrast to the RTI40 data, *S. pneumoniae* induced a significant (2-3 fold) increase in MMC6 in BAL fluid after 24 hours, which was dose dependent. There were no

significant differences in levels in lung tissue (Figure 3.13a). However, after 72 hours the significant increase in BAL fluid was maintained (Figure 3.13b).

In order to further investigate differential expression of RTI40 and MMC6 proteins and injury to ATI cells in this model, frozen sections of tissue were stained for both RTI40 and MMC6. Gross differences in protein expressed by immunofluorescence were assessed by eye but were not quantified. Figure 3.14a shows a control section, with ATI cells co-expressing both RTI40 (green) and MMC6 (red). 24 hours after infection with *S. pneumoniae*, extensive loss of reactivity to RTI40 was observed. Much of the epithelium is still stained with MMC6, although some slight decrease in expression was observed, (Figure 3.14b). By 72 hours RTI40 staining of the epithelium is still reduced (Figure 3.14c). Figure 3.15 shows that after 72 hours, those sections of the epithelium not stained with MMC6 (blue) are stained with ATII cell-specific marker APN/MMC4 (red). It is also worth noting that APN/MMC4 stained cells present in the airways are more likely to be macrophages than sloughed ATII cells at this time point, however additional ATII cell or macrophage markers were not used to confirm this.

3.4.2.4 Assessment of Aquaporin 5

To further investigate differential protein expression and injury in ATI cells, analysis of Aquaporin 5 was carried out. Aquaporin 5 is another membrane-associated protein expressed on the surface of ATI cells and has been used in conjunction with RTI40 as a marker of ATI cells (Tyrrell et al., 2005). The amount of Aquaporin 5 in BAL fluid was below the detection level of the Western blot so only lung tissue was assessed. Due to assay limitations of space samples from each treatment group were randomly

selected from 2 experiments to assess. Although at the highest inoculation level there was a slight decrease in Aquaporin 5, there was no significant difference between *S. pneumoniae*-infected lung tissue and control (Figure 3.16a). At the 10^8 cfu dose Aquaporin 5 appears to increase, although Figure 3.16b demonstrates that this is in fact due to a solitary outlier and should not therefore necessarily be considered different from control. After 72 hours, levels of Aquaporin 5 in rats inoculated with 10^9 cfu were beginning to increase again, although they were not significantly different (Figure 3.16c). Despite the fact that assessment of BAL fluid was not possible, lung data for Aquaporin 5 appear to correlate with those for MMC6.

3.4.2.5 Morphological Assessment

Given that biochemical methods of assessing ATI cell injury were proving inconclusive in this model, a morphological analysis by EM was carried out on lung tissue at 24 hours. There was no evidence of ATI cell damage and the cells looked healthy and intact (Figure 3.17).

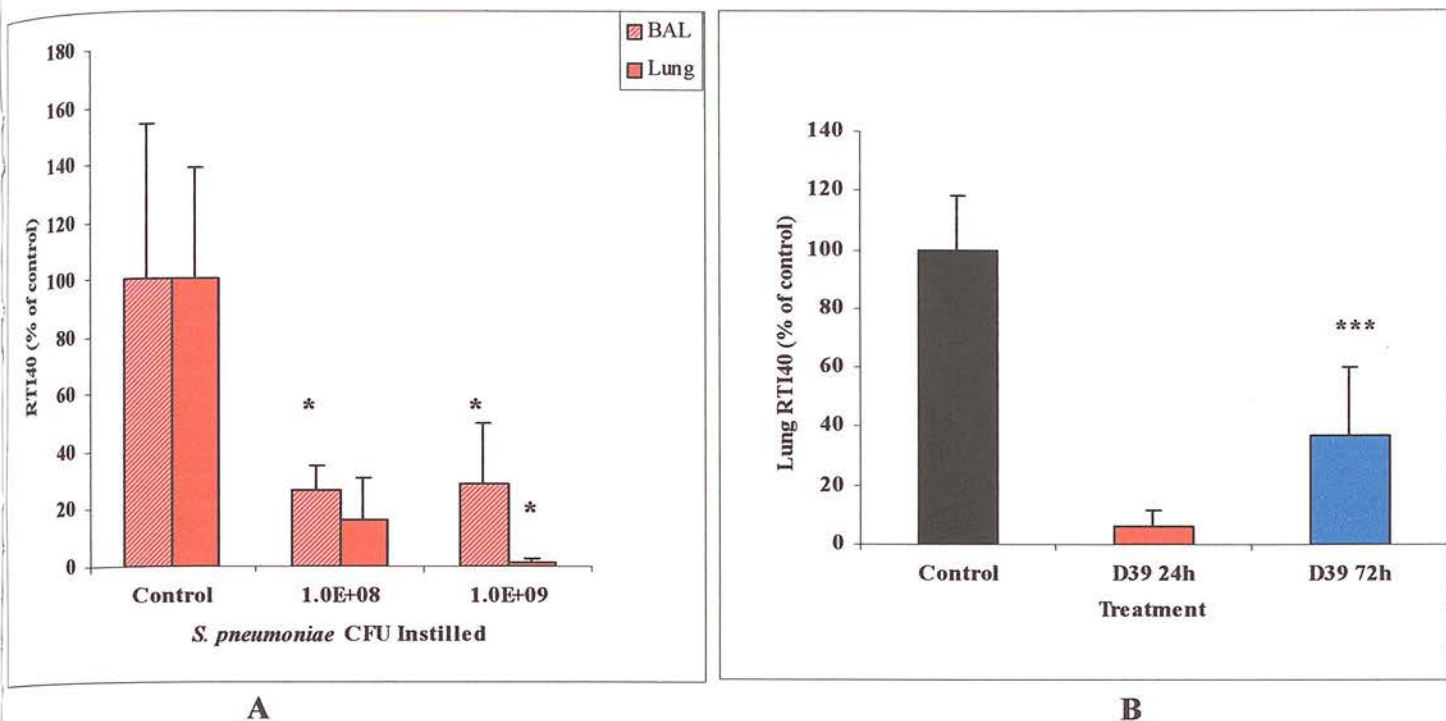


Figure 3.12 – RTI40 in BAL fluid and lung tissue

Samples in each treatment group are taken from 2 separate experiments

A. 24 hour dose response (BAL/ Lung)

Data expressed per ml BAL fluid or per mg lung protein and presented as percentage of control

S. pneumoniae induced a significant decrease in RTI40 in both BAL fluid and lung tissue after 24 hours (red).

BAL fluid: Transformed (Log) ANOVA $p = 0.008$
 Tukey post-test Control vs D39 10^8 * $p < 0.05$
 Control vs D39 10^9 * $p < 0.05$

Control $n = 4$, D39 10^8 $n = 7$, D39 10^9 $n = 5$

Lung tissue: Kruskal-Wallis $p < 0.0001$
 Dunns post-test Control vs D39 10^9 * $p < 0.01$

Control $n = 3$, D39 10^8 $n = 7$, D39 10^9 $n = 5$

B. 24 hours vs 72 hours - Lung only (Control + 10^9 cfu)

Data expressed per mg lung protein as percentage of control

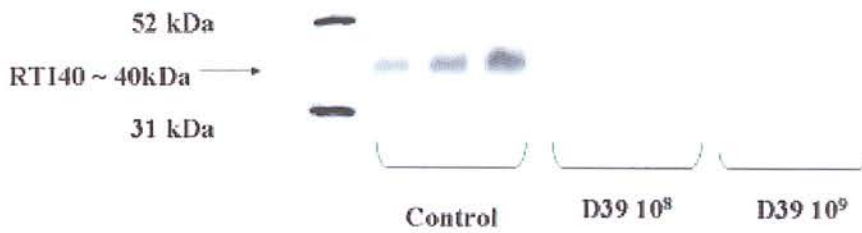
Data expressed for 24 hours is representative of that presented in Figure A (red).

24 and 72 hour infected groups were treated during separate experiments.

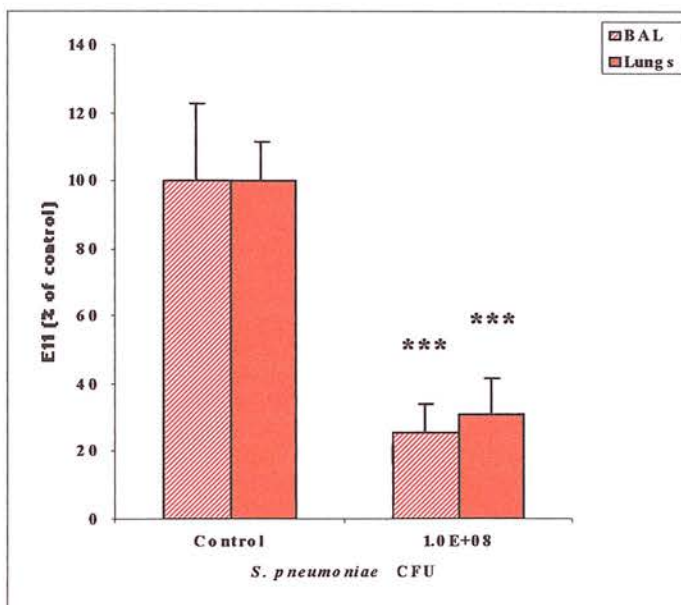
S. pneumoniae still induced a significant decrease in RTI40 in lung tissue at 72 hours (blue) compared to control (black), however levels were also significantly higher than at 24 hours (red).

ANOVA $p < 0.0001$
 Tukey post-test Control vs D39 72 hours *** $p < 0.001$
 D39 24 hours vs D39 72 hours * $p < 0.05$

Control $n = 9$, D39 24 hours $n = 5$, D39 72 hours $n = 4$



C. Western blot showing loss of RTI40 in lung tissue



D. E11 antigen in BAL/ Lung at 24 hours

Data expressed as per ml BAL fluid or per mg lung protein as percentage of control

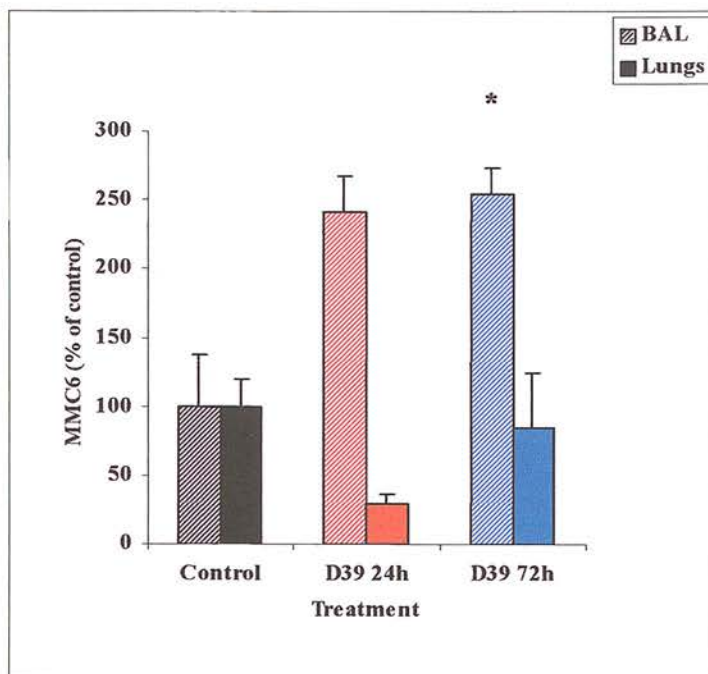
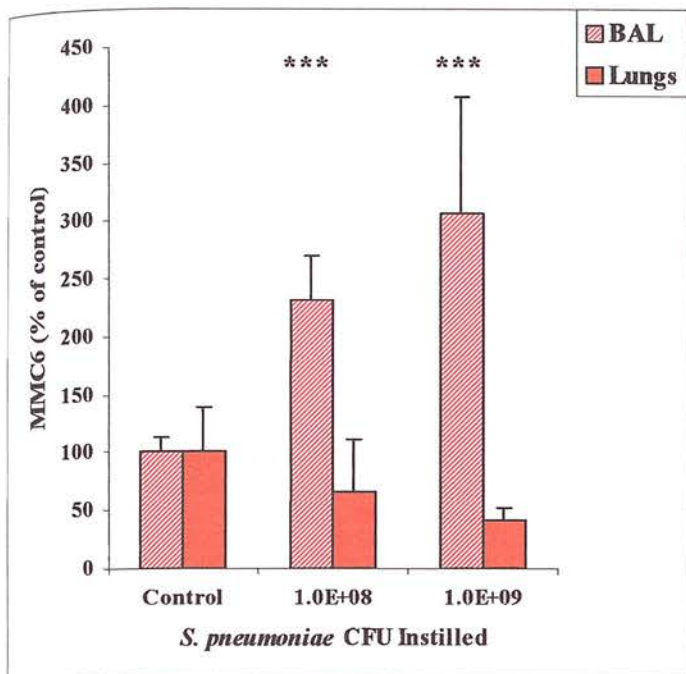
Samples in each treatment group are taken from 2 separate experiments

S. pneumoniae induced a significant decrease in E11 antigen in both BAL fluid and lung tissue after 24 hours (red).

BAL fluid: Unpaired t test Control vs D39 10⁸ *** p = 0.0008

Lung tissue: Unpaired t test Control vs D39 10⁸ ** p = 0.0019

Control n = 4, D39 10⁸ n = 4



A

B

Figure 3.13 – MMC6 in BAL fluid and lung tissue

Data expressed per ml BAL fluid or per mg lung protein and presented as a percentage of control

Samples in each treatment group are taken from 2 separate experiments

A. 24 hour dose response

S. pneumoniae induced a significant increase in MMC6 in BAL fluid after 24 hours which was dose-dependent. There was also a decrease in MMC6 in lung tissue but this was not significant.

BAL fluid: Transformed (Log) ANOVA $p < 0.0001$

Tukey post-test Control vs D39 10^8 *** $p < 0.001$

Control vs D39 10^9 *** $p < 0.001$

Control $n = 4$, D39 10^8 $n = 7$, D39 10^9 $n = 5$

B. 24 hours vs 72 hours (Control + 10^9 cfu)

Data expressed for 24 hours is representative of that presented in Figure A (red).

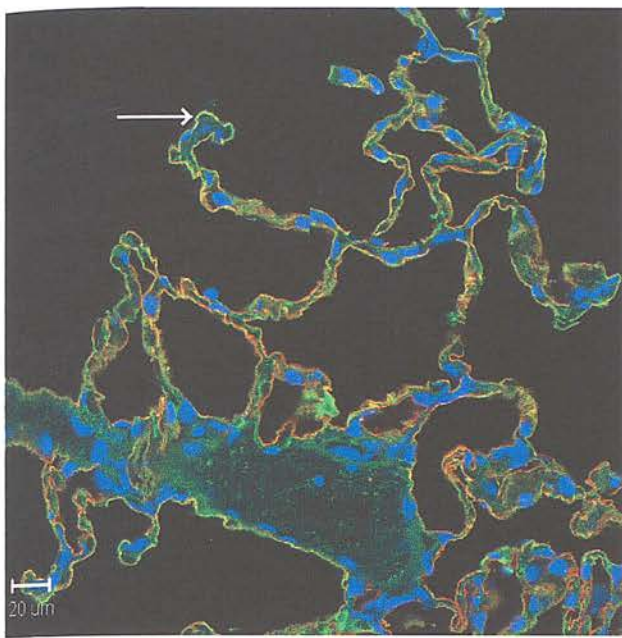
24 and 72 hour infected groups were treated during separate experiments.

S. pneumoniae still induced a significant increase in MMC6 in BAL fluid after 72 hours (blue) compared to controls (black). There was no difference in MMC6 levels between 24 hours (red) and 72 hours (blue). There was no difference between groups in MMC6 in lung tissue.

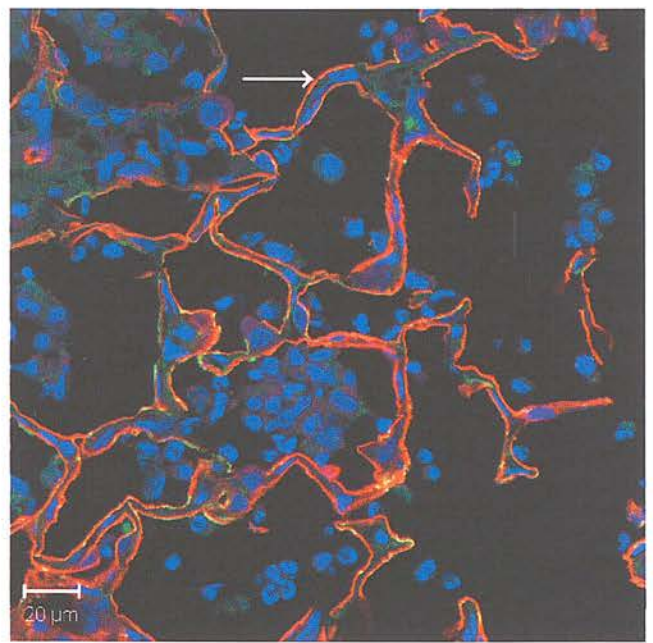
BAL fluid: Kruskal-Wallis $p = 0.0062$

Dunns post-test Control vs D39 72 hours * $p < 0.05$

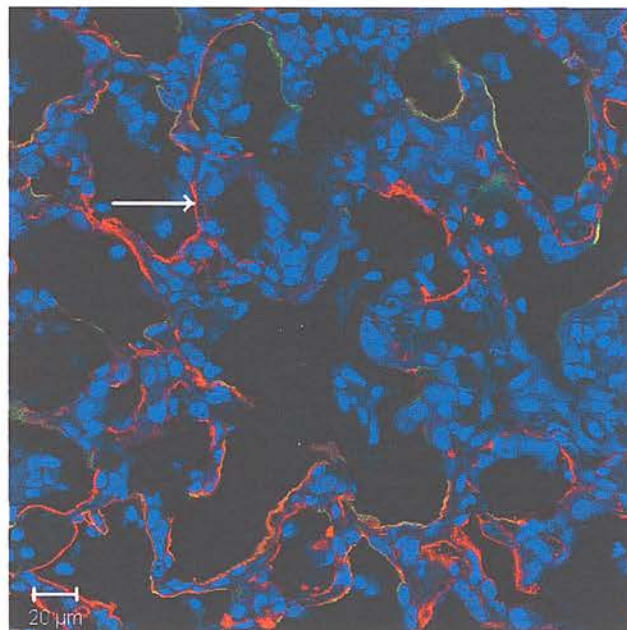
Control $n = 4$, D39 24 hours $n = 3$, D39 72 hours $n = 4$



A



B



C

Figure 3.14 – Immunofluorescence staining of rat lung

Original magnification x63

Sections stained with **RTI40** (green) and **MMC6** (red) with nuclear stain **To-Pro-3** (blue)

A. Control

Arrow shows both RTI40 and MMC6 co-expressed on the surface of ATI cells

B. *S. pneumoniae* (10^9 cfu) at 24 hours

Extensive loss of RTI40 expression. Arrow shows areas of alveolar epithelium stained with MMC6 only. Some small sections are still stained with both RTI40 and MMC6

C. *S. pneumoniae* at (10^9 cfu) 72 hours

24 and 72 hour infected groups were treated during separate experiments.

RTI40 loss maintained but recovering. Arrow shows areas of alveolar epithelium still stained with only MMC6. More staining of RTI40 is apparent than at 24 hours.

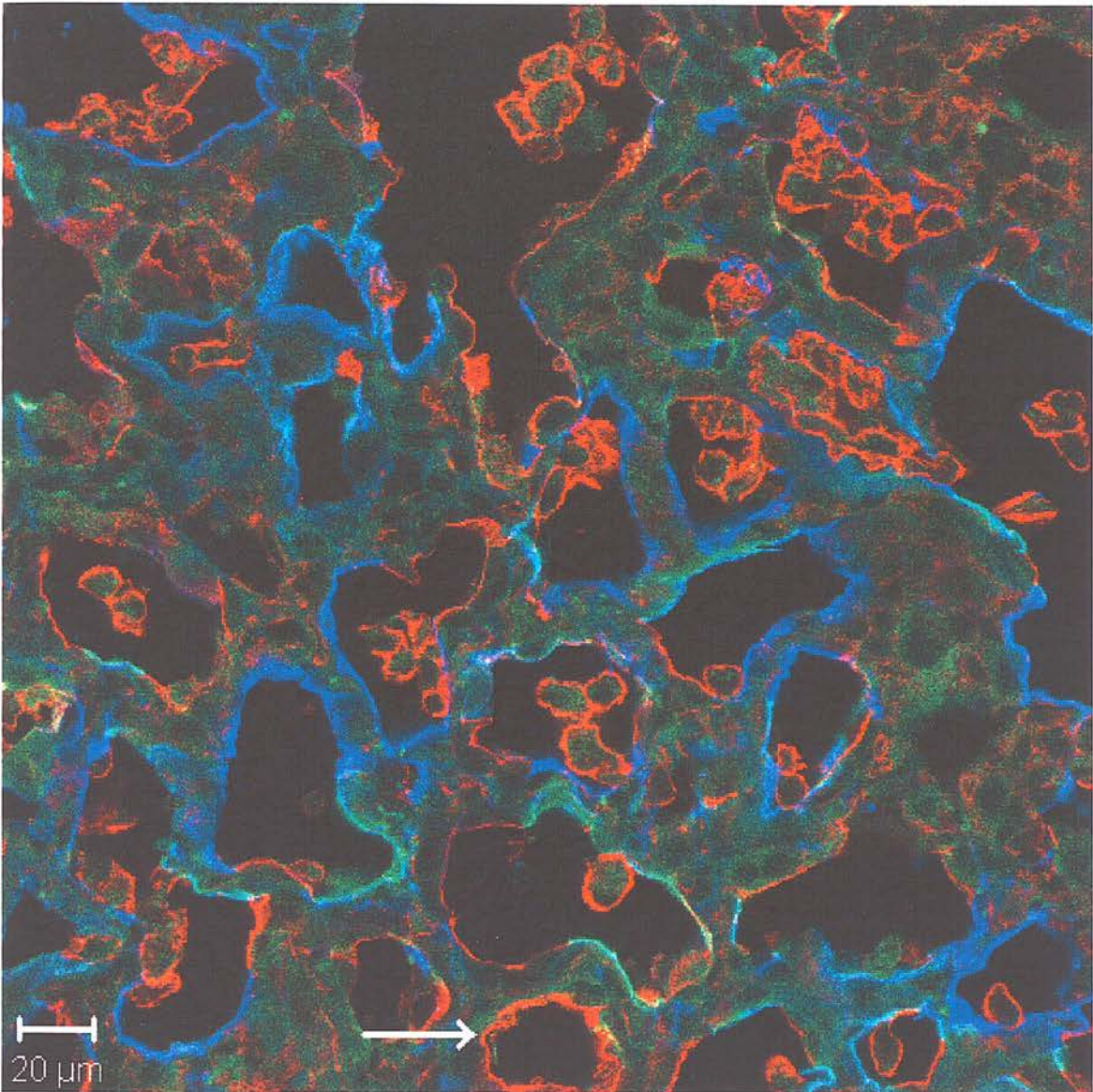
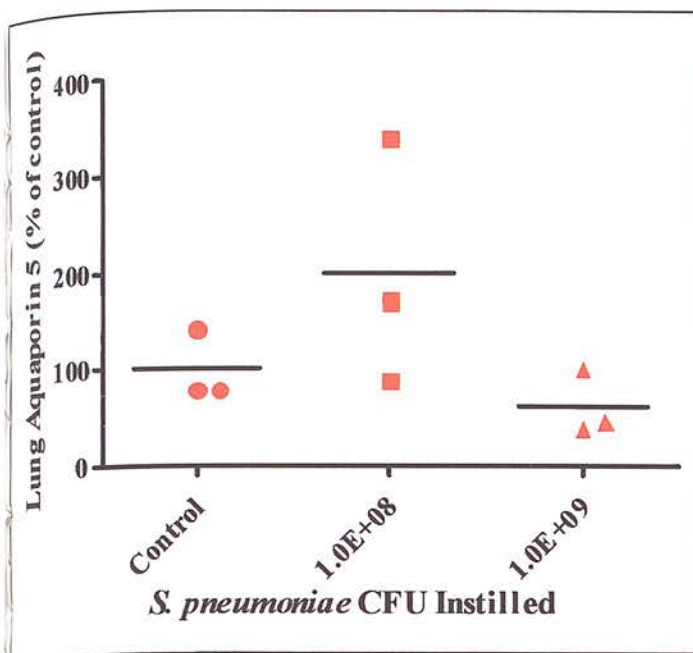


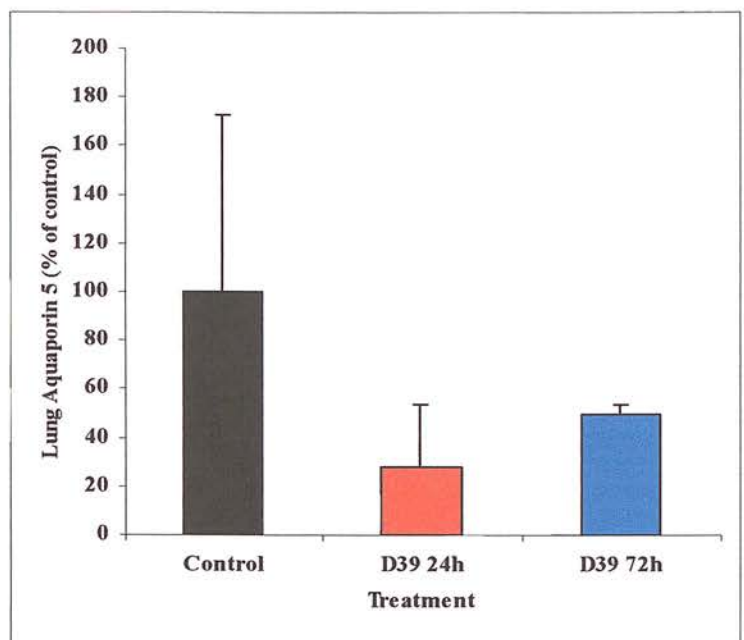
Figure 3.15 – Immunofluorescence staining of *S. pneumoniae*-infected rat lung
Original magnification x 63

Sections stained with **RTI40 (green)**, **MMC6 (blue)** and **APN/MMC4 (red)**

Image shows infected (10^9 cfu) lung after 72 hours. Arrow shows that most of the epithelium not stained with MMC6 is stained with APN/MMC4. Note that cells expressing APN/MMC4 in the airways are likely to be macrophages rather than ATII cells.



A



B

Figure 3.16 – Aquaporin 5 in lung tissue

Data expressed per mg lung protein and presented as a percentage of control

Samples in each treatment group are taken from 2 separate experiments

A. 24 hour dose response (showing individual data points)

S. pneumoniae did not induce a significant change in Aquaporin 5 in lung tissue after 24 hours (red). The increase in Aquaporin 5 at the 10^8 dose was due to one outlier.

Control n = 3, D39 10^8 n = 3, D39 10^9 n = 3.

B. 24 hours vs 72 hours (Control + 10^9 cfu)

Data expressed for 24 hours is representative of that presented in Figure A (red).

24 and 72 hour infected groups were treated during separate experiments.

S. pneumoniae did not induce a significant difference in Aquaporin 5 in lung tissue after 72 hours (blue) compared to controls (black), nor was there any difference between 24 hours (red) and 72 hours (blue).

Control n = 3, D39 24 hours n = 3, D39 72 hours n = 3

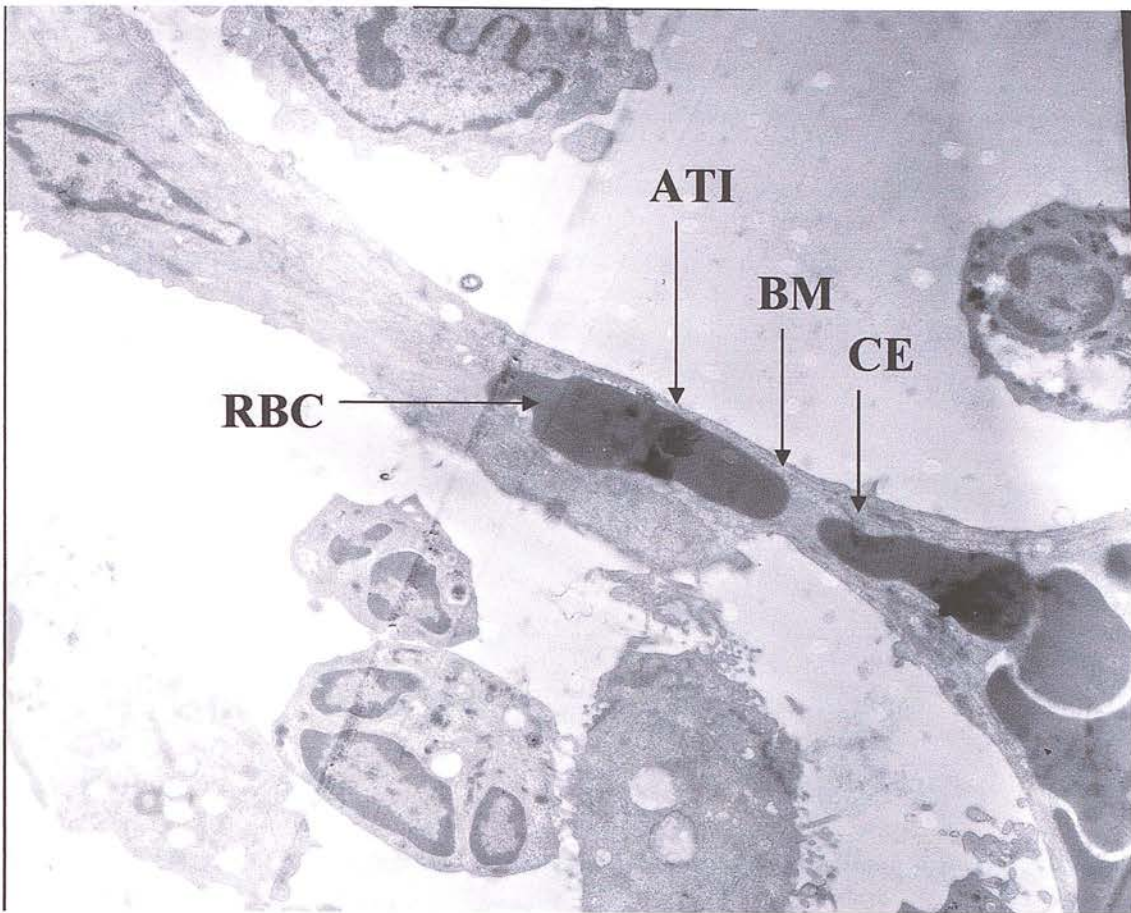


Figure 3.17 – EM of *S. pneumoniae*-infected (10^8 cfu) rat lungs at 24 hours

Original magnification x 3,000

Image shows a healthy ATI cell

Arrows indicate lung ultrastructure such as RBC, ATI basement membrane (BM) and capillary endothelium (CE)

3.4.2.4 Summary and Conclusions

- *S. pneumoniae* induced a pronounced decrease in RTI40 protein in BAL fluid and lung tissue by 24 hours, shown biochemically and by immunofluorescence, which was starting to normalise by 72 hours
- The decrease in RTI40 at 24 hours was shown biochemically with two different antibodies against the protein
- *S. pneumoniae* induced an increase in MMC6 in BAL fluid after both 24 and 72 hours
- There was no significant decrease in MMC6 or Aquaporin 5 in lung tissue
- There was no morphological evidence of injury to ATI cells

In summary, it was established that infection with *S. pneumoniae* induced a significant decrease in RTI40 in both BAL fluid and lung tissue after 24 hours. One possible explanation of this phenomenon is the wide-scale destruction of ATI cells. However, the fact that experimental animals were still alive at 24 hours and went on to recover fully from the infection suggests that this is not the case. Furthermore, levels of protein in BAL fluid were not consistent with a high degree of lung injury. In addition, immunofluorescence staining on infected lung tissue, which showed a loss of RTI40 staining without a corresponding decrease in MMC6, suggests the continued presence of the ATI cell population. The lack of a significant decrease in MMC6 and Aquaporin 5 levels in lung tissue also supports this idea. It was possible that the decrease in RTI40 was due to a loss of antibody reactivity against the protein. However, the effect was detected biochemically using two separate antibodies against the RTI40 protein, thought to bind to different epitopes. These data therefore suggest that *S. pneumoniae* induces a specific decrease in RTI40 protein that differs from that

of other ATI cell-specific proteins. The subsequent increase in RTI40 expression suggests that this effect may be reversible following resolution, and the full recovery of animals after 72 hours implies that the decrease in RTI40 expression does not appear to adversely affect resolution of the disease. These data also indicate that the method of RTI40 assessment in BAL fluid as previously used is not representative of damage to ATI cells in this in this model of lung injury RTI40.

An increase of MMC6 in BAL fluid after 24 hours of infection implied that damage to ATI cells was taking place. However, the maintenance of this increase at 72 hours, despite other indications of resolution at that time point, makes this data difficult to interpret, and gives rise to two possibilities. Firstly, that the increase of MMC6 in BAL fluid at 72 hours reflects continued injury to ATI cells, and in spite of progressive clearance of bacteria, attenuation of inflammation and repair of the air-blood barrier, further damage to ATI cells is occurring, although the lack of morphological evidence of injury at 24 hours does not support this. The alternative is that *S. pneumoniae* induces shedding of MMC6 that is independent of ATI cell injury. Although MMC6 is thought to be an integral membrane protein, the lack of knowledge about its structure and function suggests that this could indeed be a possibility.

3.4.3 ATII/Clara Cell Injury

3.4.3.1 Aim

To assess damage to ATII and Clara cells in the *S. pneumoniae*-induced pneumonia model

3.4.3.2 Rationale

Unlike ATI cells, there is no established method of quantifying damage to ATII cells. This would provide valuable information about disease pathogenesis and if used in conjunction with ATI cell injury markers could enable investigation into targeted injury and differential damage to alveolar epithelial cells, perhaps with a view to treatment design.

3.4.3.3 Evidence of ATII Cell Injury

In order to determine whether there was any morphological evidence of injury to ATII cells in this model of *S. pneumoniae*-induced infection EM was carried out on *S. pneumoniae*-infected (10^8 cfu) lung tissue after 24 hours. Figure 3.18a shows a normal ATII cell, with characteristic microvilli and lamellar bodies, firmly embedded in the basement membrane of the lung. Figure 3.18b however, representative of a number of cells observed in infected tissue, shows an ATII cell that is sloughing away from the basement membrane. This data suggests injury to ATII cells in *S. pneumoniae*-induced pneumonia.

3.4.3.4 Quantification of ATII Cell Injury

3.4.3.4.1 Rationale

This thesis describes biochemical and morphological techniques to quantify injury to ATII cells. In particular it describes a novel morphological immunofluorescence method of ATII cell injury quantification, using monoclonal antibodies against ATII cell-specific proteins on lung sections.

3.4.3.4.2 Immunofluorescence Quantification

Control and *S. pneumoniae*-infected frozen lung sections were stained for 2 proteins; RTII70, a membrane-associated protein restricted to rat ATII cells (Dobbs et al., 1998); and APN/MMC4, a membrane-associated protein on the surface of rat ATII cells, Clara cells and macrophages (Franklin, 2006). Membrane co-localisation of these proteins was used as the principle of ATII cell identification in this technique, with cell type confirmation obtained by triple staining with the addition of a polyclonal antibody against Pro-SP-C, an ATII cell specific cytoplasmic precursor protein to surfactant protein SP-C (Beers et al., 1992). Stained sections were viewed by confocal microscopy under x40 magnification. Four areas of approximately 1mm² (0.85mm²) from each section were randomly selected and scanned using a tiling system. All morphologically damaged cells stained with RTII70 and APN/MMC4 were counted in each scanned tile. Damaged cells were considered to be completely detached from the epithelium into the airspace or cells which appeared to be in the process of sloughing off from the basement membrane. Figure 3.19a shows a normal ATII cell double stained with RTII70 (green) and APN (red). The two proteins are co-localised on the apical membrane of the cell. Figure 3.19b demonstrates morphology that was considered to be 'sloughing', with the ATII cell detaching from the basement

membrane. Confirmation of the identity of these sloughing cells by triple staining with the addition of Pro-SP-C (blue), staining the cytoplasm of cells already labelled with RTII70 and APN is shown in Figure 3.19c.

Figure 3.19d shows the results of this quantification, and demonstrates that at 24 hours there were significantly more sloughing cells than controls. Between 24 and 72 hours the numbers further decreased, but levels were not significantly different from those either in controls or at 24 hours.

3.4.3.4.2 Biochemical Quantification

To further investigate injury to ATII cells and determine if it was possible to detect and quantify damage to ATII cells biochemically, other ATII cell-specific proteins were used. Unfortunately neither blotting nor direct ELISA techniques worked using the RTII70 antibody. Due to assay limitations of space samples from each treatment group were randomly selected from 2 experiments to assess.

3.4.3.4.2.1 Assessment of Pro-SP-C

Levels of Pro-SP-C in BAL fluid were below the detection sensitivity of Western blotting so only lung tissue was analysed. Unfortunately, due to subsequent technical difficulties with the Pro-SP-C antibody on Western blots, it was only possible to assess samples from the 24 hours time point biochemically. *S. pneumoniae* induced a significant (16 fold) decrease in Pro-SP-C in lung tissue at the highest dosage after 24 hours (Figure 3.20a). In order to determine whether this was representative of a reduction in the ATII cell population or decreased protein expression, immunofluorescence staining of Pro-SP-C in conjunction with 2 other ATII cell-

specific proteins, RTII70 and APN/MMC4 was carried out on lung tissue. Control lung (Figure 3.20b) showed relatively low expression of Pro-SP-C in the cytoplasm of RTII70 and APN/MMC4-positive ATII cells, as expected. 24 hours after instillation of *S. pneumoniae* however, staining intensity did not appear to be different to that of controls, nor did there appear to be an obvious decrease in the ATII cell population. Indeed there seemed to be an increase in the number of Pro-SP-C/RTII70/APN/MMC4-positive cells overall at both 24 hours (Figure 20b) and 72 hours (Figure 20c). This is discussed on p129-130.

3.4.3.4.2.2 Assessment of SP-D

SP-D is a surfactant protein secreted by both ATII and Clara cells. To determine whether *S. pneumoniae* infection affected cell function or the production of SP-D indicative of cell-specific damage, biochemical analysis of SP-D on BAL fluid and lung tissue was carried out.

Control levels of SP-D in both BAL fluid and lung tissue were about 250-350ng/ml, as assessed against a standard curve of recombinant rat SP-D, kindly provided by Erica Crouch, Washington University, St Louis. Infection with *S. pneumoniae* induced a significant dose-dependent increase in the protein in BAL fluid, as much as 6-fold more than controls at the highest inoculation level, after 24 hours. SP-D in lung tissue was higher in infected rats than controls, but this was not significant (Figure 3.21a). After 72 hours, levels significantly increased even further than at 24 hours. SP-D in BAL fluid was 8-fold higher than controls, although the difference between 24 and 72 hours was not significant. The increase of SP-D in lung tissue was particularly pronounced, showing up to 43-fold more than controls (Figure 3.21b).

Although the values were highly variable, Figure 3.21c shows that they were consistently higher than at 24 hours.

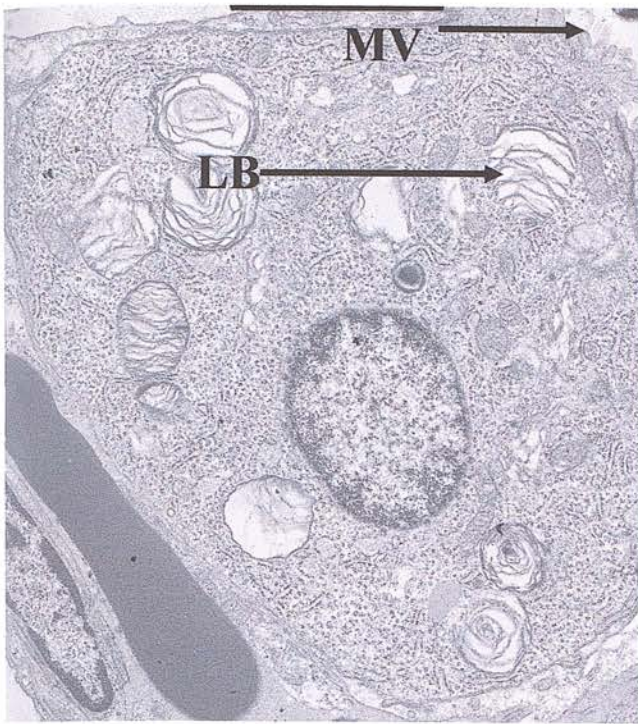
3.4.3.4.2.3 Assessment of APN/MMC4

In order to further investigate biochemical methods of injury assessment in ATII cells, ELISA-based dot blot analysis was carried out on BAL fluid and lung tissue using a monoclonal antibody against rat APN/MMC4 (found on ATII, Clara cells and macrophages). Despite the protein's expression on alveolar macrophages, previous work has shown that levels of APN/MMC4 in BAL fluid vary between models of wildtype and mutant *S. aureus*-induced pneumonia, despite an equivalent inflammatory response (Franklin, 2006). This has suggested that APN/MMC4 might still be used as a preliminary screen for ATII and Clara cell injury to identify rat models warranting further investigation. As the current model of pneumococcal pneumonia has established and quantified injury to ATII cells, it was decided that this system would be a good opportunity to test the APN/MMC4 assessment technique. The most pronounced damage to ATII cells was at 24 hours, so this time point was selected to investigate further. Figure 3.22 shows that *S. pneumoniae* induced a significant, dose-dependent increase in APN/MMC4 in BAL fluid, with no particular effect on lung tissue at 24 hours. This increase was as high as 2.5-fold at the highest inoculation level.

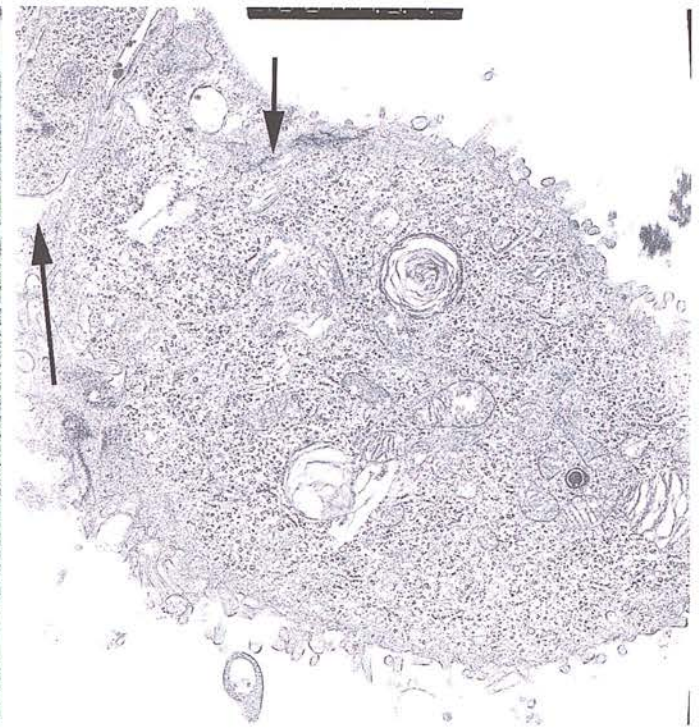
3.4.3.5 Assessment of Clara Cell Injury

3.4.3.5.1 Assessment of CC10

Measurement of Clara cell-specific protein CC10 in BAL fluid and serum has been used as a measure of air-blood barrier permeability and Clara cell damage (Section 1.4.4.1 CC10). Assessment of CC10 in rat BAL fluid and serum was kindly carried out by A. Bernard, Catholic University of Louvain, using an automated latex immunoassay (Halatek et al., 1998). Control levels in BAL fluid were 3785 ± 698 ng/ml and 31 ± 8 ng/ml in serum. Figure 3.23 demonstrates that instillation of *S. pneumoniae* induced a significant 2-fold decrease in CC10 in BAL fluid and a similar increase in serum.



A – Normal ATII cell



B – Sloughing ATII cell

Figure 3.18 – EM of *S. pneumoniae* (10^8 cfu) infected lungs at 24 hours

A. Normal ATII cell

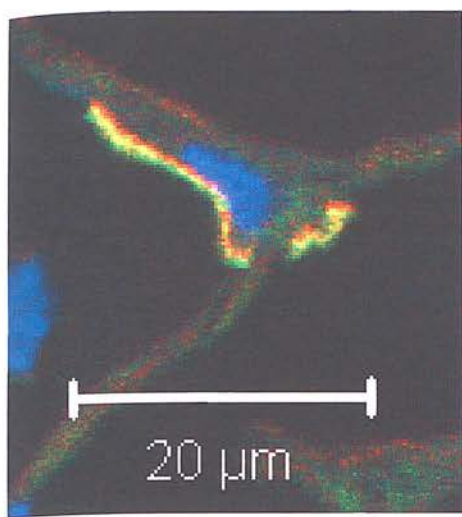
Original magnification x 10,000

Arrows show normal ATII cell with characteristic microvilli (MV) and lamellar bodies (LB), attached to basement membrane

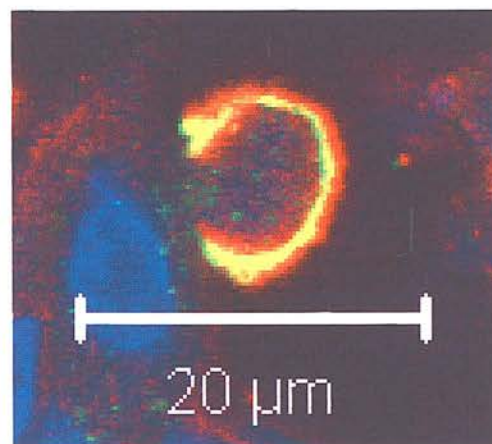
B. Sloughing ATII cell

Original magnification x 8,000

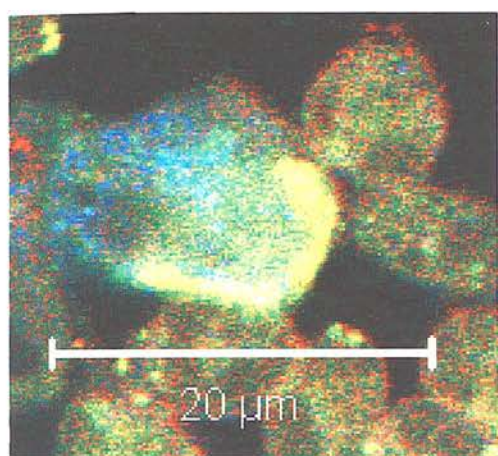
Arrows show ATII cell detaching from basement membrane



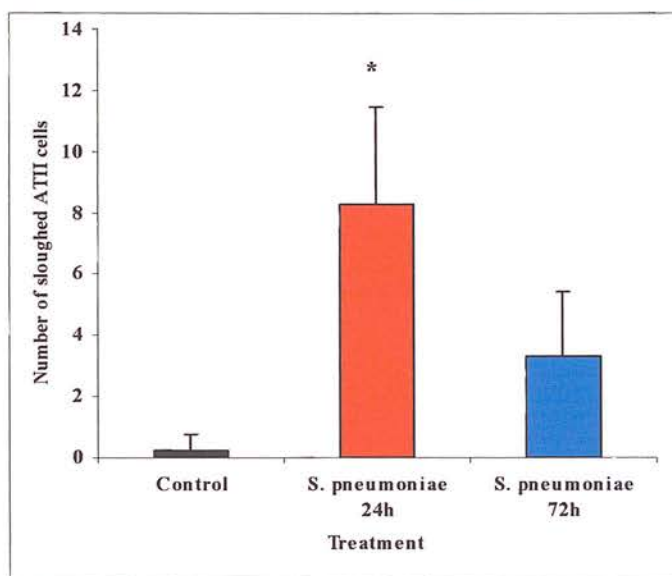
A



B



C



D

Figure 3.19 – Quantification of ATII cell sloughing in *S. pneumoniae*-infected rat lungs (10^9 cfu)

Samples in each treatment group are taken from 2 separate experiments

A. Double immunofluorescence staining of normal ATII cell stained for membrane proteins RTII70 (green) and APN/MMC4 (red) with nuclear stain To-Pro-3 (blue).

Original magnification x 40.

Shows normal ATII cell co-expressing RTII70 and APN/MMC4 (yellow).

B. Double immunofluorescence staining of sloughing ATII cell

Stained for membrane proteins RTII70 (green) and APN (red) with nuclear stain To-Pro-3 (blue).

Original magnification x 40.

Shows cell coexpressing RTII70 and APN/MMC4 sloughing off from basement membrane.

C. Triple immunofluorescence staining of sloughing ATII cell

Stained for membrane proteins RTII70 (green), APN (red) and cytoplasmic protein Pro-SP-C (blue).

Original magnification x63.

Shows RTII70 and APN/MMC4-positive sloughing cell stained with Pro-SP-C.

D. Sloughing ATII cells at 24 and 72 hours (Control + 10^9 cfu)

Data expressed as number of sloughing cells

24 and 72 hour infected groups were treated during separate experiments.

S. pneumoniae induced a significant increase in the number of sloughing ATII cells after 24 hours (red) compared to controls (black). By 72 hours (blue) numbers were still elevated above controls (black) but below levels at 24 hours (red) and were not significantly different from either.

Kruskal-Wallis $p = 0.0139$

Dunns post-test Control vs D39 24 hours

*

$p < 0.05$

Control $n = 3$, D39 24 hours $n = 3$, D39 72 hours = 3

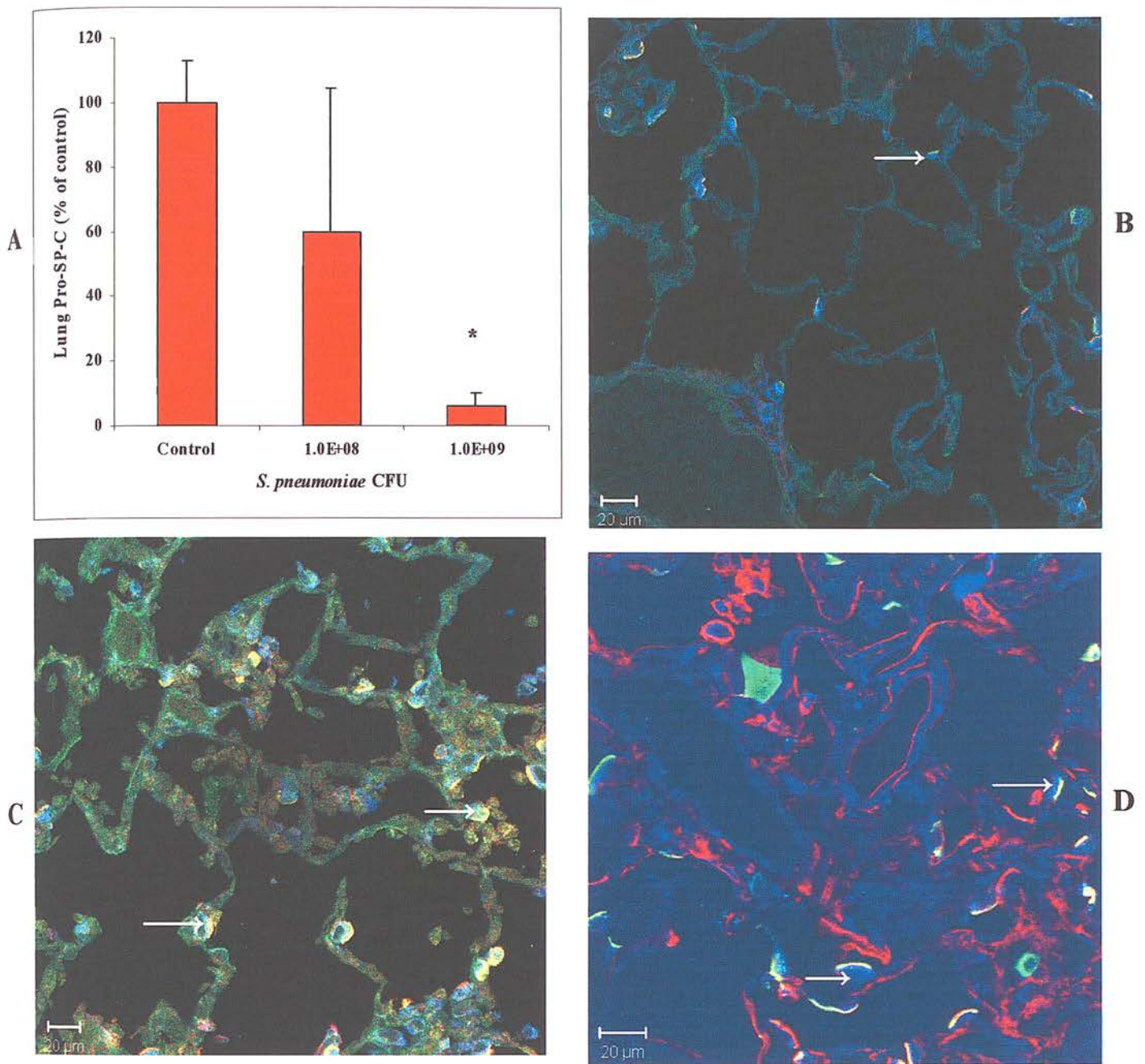


Figure 3.20 – Pro-SP-C in lung tissue

A. 24 hour dose response

S. pneumoniae induced a significant decrease in Pro-SP-C in lung tissue after 24 hours (red), which was roughly dose dependent.

Kruskal-Wallis $p = 0.0248$

Dunn's post-test Control vs D39 10^9

$p < 0.05$

Control $n = 3$, D39 10^8 $n = 4$, D39 10^9 $n = 4$

B. Immunofluorescence of control lung tissue

Stained for membrane proteins RTII70 (green), APN/MMC4 (red) and cytoplasmic protein Pro-SP-C (blue).

Original magnification $\times 40$

Arrow shows staining of Pro-SP-C restricted to cytoplasm of RTII70 and APN/MMC4-positive ATII cells.

C. Immunofluorescence of *S. pneumoniae*-infected tissue at 24 hours (10^9 cfu)

Stained for membrane proteins RTII70 (green), APN/MMC4 (red) and cytoplasmic protein Pro-SP-C (blue).

Original magnification $\times 40$

Image shows Pro-SP-C restricted to RTII70 and APN/MMC4-positive cells. Staining intensity does not appear reduced.

Arrow indicates the presence of an abundance of ATII cells.

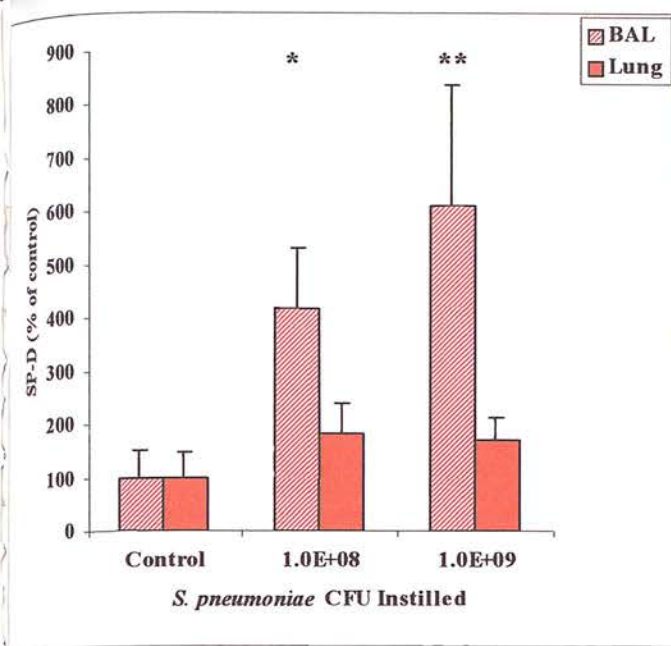
D. Immunofluorescence of *S. pneumoniae*-infected tissue at 72 hours (10^9 cfu)

Stained for membrane proteins RTII70 (green), APN/MMC4 (red) and cytoplasmic protein Pro-SP-C (blue).

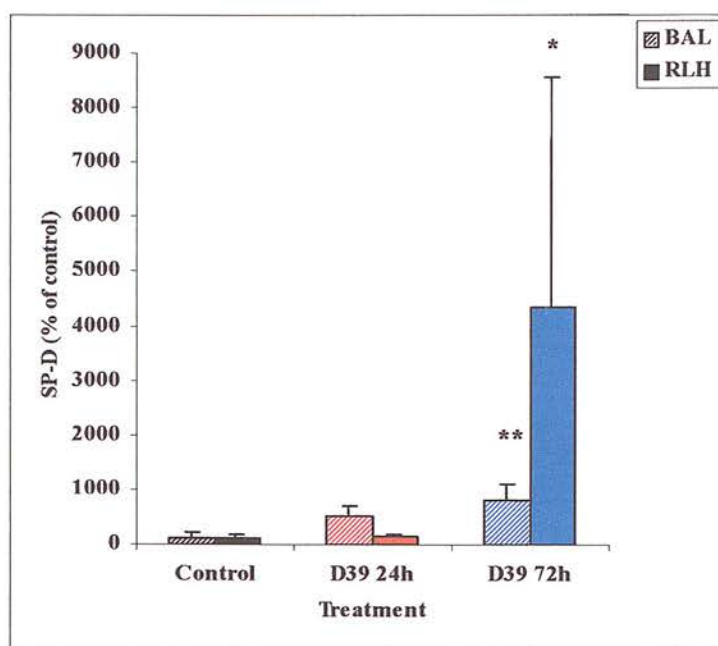
Original magnification $\times 4$

Image shows Pro-SP-C restricted to RTII70 and APN/MMC4-positive cells. Staining intensity does not appear reduced.

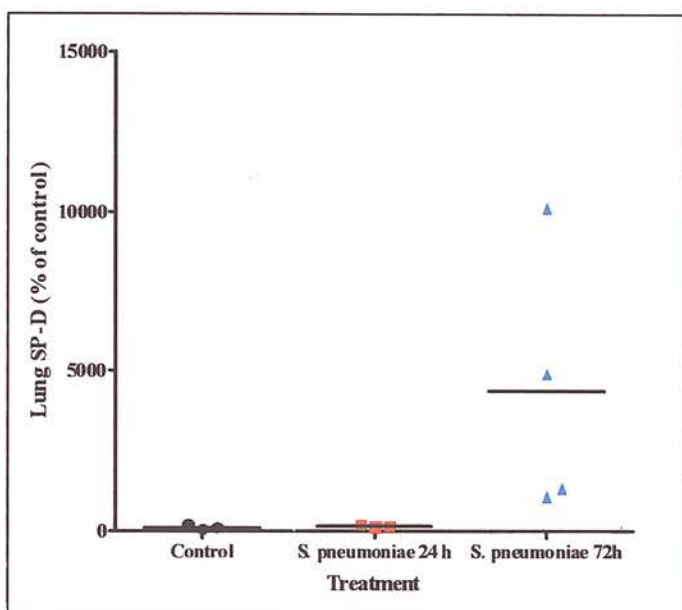
Arrow indicates the presence of an abundance of ATII cells.



A



B



C

Figure 21 – SP-D in BAL fluid and lung tissue

Data expressed per ml BAL fluid or per mg lung protein as a percentage of control
 Samples in each treatment group are taken from 2 separate experiments

A. 24 hour dose response

S. pneumoniae induced a significant increase in SP-D in BAL fluid after 24 hours (red) which was dose-dependent. There was no significant increase in lung tissue.

BAL fluid: ANOVA $p = 0.001$

Tukey post-test

Control vs D3910⁸ * $p < 0.05$

Control vs D39 10⁹ ** $p < 0.01$

Control $n = 4$, D39 10⁸ $n = 5$, D39 10⁹ $n = 5$

B. 24 hours vs 72 hours (Control + 10⁹ cfu)

Data expressed for 24 hours is representative of that presented in Figure A (red).

24 and 72 hour infected groups were treated during separate experiments.

S. pneumoniae still induced a significant increase in SP-D in BAL fluid after 72 hours (blue) compared to controls (black). There was also a particularly large increase in lung tissue after 72 hours (blue), however there was no significant difference between 24 hours (red) and 72 hours (blue) in BAL fluid or lung tissue

BAL fluid: ANOVA $p = 0.0028$

Tukey post-test Control vs D39 72 hours ** $p < 0.01$

Control $n = 4$, D39 24 hours $n = 4$, D39 72 hours = 4

Lung tissue: Kruskal-Wallis $p = 0.0062$

Dunns post-test Control vs D39 72 hours * $p < 0.05$

Control $n = 3$, D39 24 hours $n = 4$, D39 72 hours $n = 4$

C. Individual values 24 vs 72 hours (lung tissue only)

Values at 72 hours (blue) were consistently higher than control (black) or 24 hours (red).

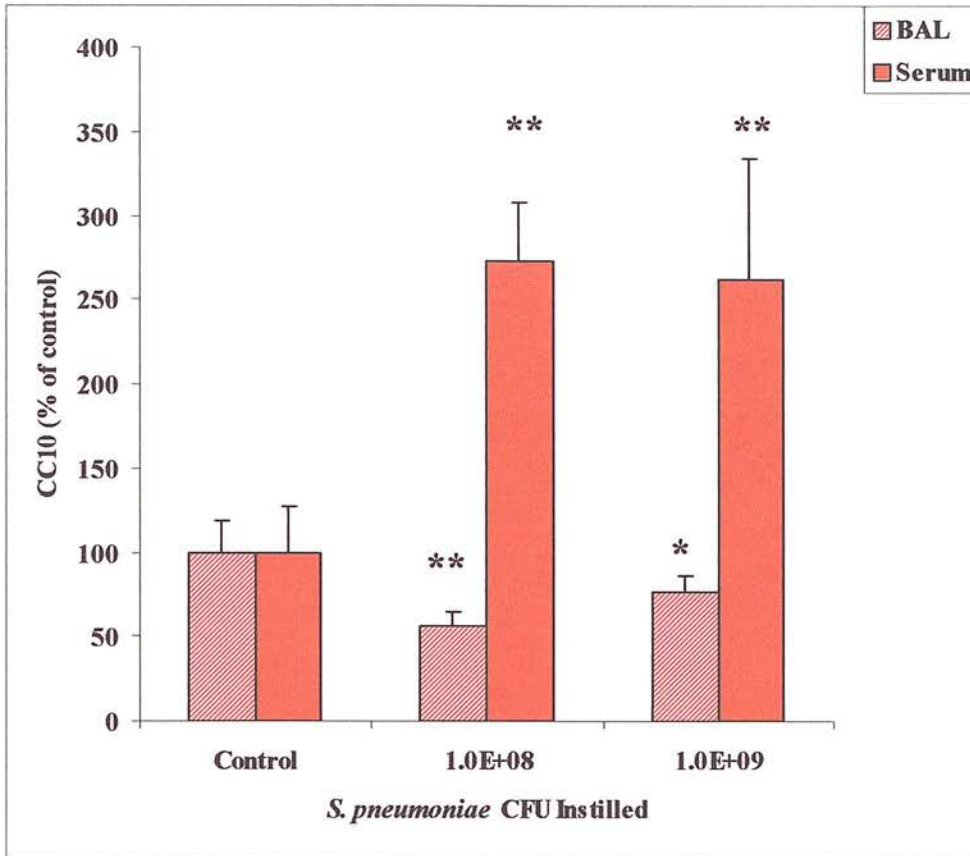


Figure 3.23 – CC10 in BAL fluid and serum at 24 hours

Data expressed per ml BAL fluid or serum and presented as a percentage of control

Samples in each treatment group are taken from 2 separate experiments

S. pneumoniae induced a significant decrease in CC10 in BAL fluid and an increase in serum which was not dose-dependent.

BAL fluid: ANOVA $p = 0.0026$

Tukey post-test	Control vs D39 10^8	**	$p < 0.01$
	Control vs D39 10^9	*	$p < 0.05$

Serum: ANOVA $p = 0.0012$

Tukey post-test	Control vs D39 10^8	**	$p < 0.01$
	Control vs D39 10^9	**	$p < 0.01$

Control $n = 4$, D39 10^8 $n = 4$, D39 10^9 $n = 5$

3.4.3.6 Summary and Conclusions

- *S. pneumoniae* induces injury to ATII cells
- A novel method of immunofluorescence quantification of ATII cell injury was developed that demonstrated damage to ATII cells 24 hours after infection with *S. pneumoniae* which was resolving after 72 hours
- *S. pneumoniae* induced a decrease in Pro-SP-C in lung tissue by Western blot after 24 hours but this was not supported by immunofluorescence data
- *S. pneumoniae* induced an increase in SP-D in BAL fluid after 24 hours which was maintained at 72 hours, and an increase in lung tissue after 72 hours
- *S. pneumoniae* induced an increase in APN/MMC4 in BAL fluid after 24 hours
- *S. pneumoniae* induced an increase in CC10 in serum but a decrease in BAL fluid

The purpose of this section was to assess injury to ATII and Clara cells in the model of pneumococcal pneumonia. Having established the nature of this damage in the case of ATII cells as sloughing of cells away from the basement membrane into the airways, this was quantified using a novel method. Injury was detected at 24 hours and was shown to be resolving by 72 hours, suggesting that ATII cell damage was not adversely affecting the repair process. A biochemically demonstrated decrease in ATII cell-specific protein Pro-SP-C was observed. Although this was initially thought to be representative of a decrease in the ATII cell population following cell-specific injury, immunofluorescence data did not support this, suggesting that there was no evidence of decreased ATII cells or Pro-SP-C staining intensity at 24 or 72 hours. This implies that the biochemical decrease may be due to artefact of the blotting

process, the bacteria perhaps affecting the protein's ability to be degraded by SDS. Overall the data suggests that Pro-SP-C did not appear to be a good biochemical marker of the ATII cell population in this injury model, although the immunofluorescence data indicates that damage to the cells did not affect their proliferative capabilities. Surfactant-associated protein SP-D secretion, as measured in BAL fluid, was stimulated by infection with *S. pneumoniae* after 24 hours, although production, as measured in lung tissue, was more greatly increased after 72 hours. This implies the release of stored SP-D early on during infection, but in order to maintain this during the resolution phase, an increase in protein production is initiated. Furthermore, this data suggests that injury to ATII cells either does not adversely affect collectin production or that this is compensated for by Clara cells. Investigation of APN/MMC4 demonstrated an increase in the protein in BAL fluid 24 hours after *S. pneumoniae* infection. While this result may be due to the increase in macrophages number during infection, it may also correspond to ATII cell injury at this time point. These data suggest that further investigation is required but APN/MMC4 may still be a candidate for preliminary screening of ATII/Clara cell damage in rat models of injury. Infection with *S. pneumoniae* induced a significant increase in levels of Clara cell-specific protein CC10 in serum and a corresponding decrease in BAL fluid. This data is consistent with damage to the air-blood barrier as CC10 from the airways leaks across into the bloodstream. Extensive injury or functional inhibition of Clara cells, would result in a decrease in CC10 in both BAL fluid and serum, due to loss of cells or a failure to produce the protein. This is not consistent with the data observed in this model.

3.4.4 Overall Conclusions

The initial purpose of this chapter was to set up a series of acute, resolving and recovering models of pneumococcal pneumonia and investigate differential damage to ATI and ATII cells. These models showed acute inflammation and damage to the air-blood barrier that resolved over time leading to full recovery. Assessment of ATI cell injury demonstrated unexpected differential expression of ATI cell-specific proteins RTI40, MMC6 and Aquaporin 5 but no visible morphological evidence of specific cell damage. This included for the first time evidence of regulation of RTI40 protein expression. Injury to ATII cells was identified and quantified using a novel immunofluorescence method. Biochemical techniques of quantification were also evaluated, including the use of APN/MMC4 as a screen for ATII and Clara cell injury. Damage to ATII cell in this model did not appear to functionally inhibit the repair process. Infection with *S. pneumoniae* stimulated differential expression in surfactant-associated proteins SP-D and Pro-SP-C, with the identification of increased secretion and production of SP-D.

CHAPTER 4

4.1 INTRODUCTION

In Chapter 3 it was established that pneumococcal pneumonia induced visible ATII cell injury and differential expression of injury-associated cell-specific proteins in both ATI and ATII cells. In particular, the pronounced decrease in ATI expression of RTI40 compared to Aquaporin 5 and MMC6 (Section 3.4.2 ATI Cell Injury) was intriguing. Furthermore, a significant increase in surfactant protein SP-D in both BAL and lung tissue was observed, which differed markedly from expression of Pro-SP-C (Section 3.4.3 ATII Cell Injury).

Bacteria can interact with host tissue in a huge variety of ways, some of which are mediated by species-specific virulence factors and some involve pathways that are common to other pathogens. Further investigation of the effects of these factors on the observations presented in Chapter 3 might provide valuable information about the mechanism underlying these results. It was therefore postulated that these effects might be mediated by the pneumococcal virulence factor pneumolysin, or be due to an effect common to Gram-positive bacteria. Comparison of the initial model of *S. pneumoniae*-induced lung injury with other models, induced by pneumolysin deficient mutant *S. pneumoniae* and by *S. aureus* (another Gram positive bacterium) was therefore undertaken.

4.2 PNEUMOLYSIN

4.2.1 Introduction

Pneumolysin is an important virulence factor of *S. pneumoniae* and is responsible for a variety of pathological effects in the lung (Section 1.5.2.3.1 Pneumolysin). Thus it may modulate differential protein expression in pneumococcal pneumonia.

4.2.2 Hypothesis 2.1

Pneumococcal virulence factor pneumolysin mediates differential protein expression

The aim of this section was to establish a model of pneumolysin-deficient pneumococcal pneumonia and to compare cell specific protein expression with the wildtype model.

4.2.3 Methods

The pneumolysin-deficient pneumonia model was set up as for the wildtype established in Chapter 3 (see Chapter 2 – Materials and Methods). An isogenic mutant of D39, PLN-A was used, which is deficient in both cytotoxic and complement-inducing functions of pneumolysin and is resistant to erythromycin (Berry et al., 1989). In all experiments the identity of PLN-A was confirmed using erythromycin discs. Production of pneumolysin was tested in both wildtype and mutant bacteria by reduced Western blot using a mouse monoclonal antibody ([9.1/2/3/6]) against the recombinant protein (Abcam, Cambridge, UK), diluted 1:1000 and a Zymed® goat anti-mouse IgG2_b HRP secondary antibody (Invitrogen, Paisley, UK) diluted 1:2000. Bacterial cultures were harvested at late logarithmic/stationary phase, centrifuged to pellet bacteria and supernatant was collected. The bacterial pellet was then lysed

using RIPA buffer. See Chapter 2 (Materials and Methods) for detailed protocols. Samples of *S. aureus* strain 8325-4 were used as negative controls. For the *in vivo* study the 24-hour time point was selected for investigation, as this was when acute injury was detected and significant differences in expression of proteins specific to ATI and ATII cells was apparent in the wildtype infection (Chapter 3). Instillation doses of PLN-A at 10^6 ($5.82 \times 10^6 \pm 2.91 \times 10^6$) and 10^8 ($2.02 \times 10^8 \pm 7.03 \times 10^7$) cfu were comparable to those of the wildtype D39 ($5.27 \times 10^6 \pm 1.34 \times 10^6$ and $1.8 \times 10^8 \pm 1.61 \times 10^8$). Wildtype and pneumolysin-deficient bacteria were instilled during the same experiments. For technical reasons, due to problems with the antibodies, it was not possible to assess levels of Aquaporin 5 and Pro-SP-C in samples. The APN/MMC4 protein was used as a preliminary screen to determine if there was any potential mediation of ATII/Clara cell injury by pneumolysin. As in Chapter 3, all graphs show data generated as a result of at least 2 separate experiments. Graphs show means with SD error bars. Asterisks illustrate significant differences between experimental data and controls unless stated otherwise. Analyses of specific proteins are presented as a percentage of the mean control value. As with Chapter 3, assay limitations of space necessitated the random selection of samples from 2 separate experiments for specific protein analysis.

4.2.4 Results

4.2.4.1 Basic Comparison of *S. pneumoniae* Wildtype and Pneumolysin-Deficient Models

Figure 4.2.1 shows a Western blot confirming that the wildtype D39 produced pneumolysin but the mutant PLN-A does not. Both D39 and PLN-A caused a loss of body weight after 24 hours, and although rats infected with the mutant strain were heavier, there was no significant difference (Figure 4.2.2). Similarly, the number of bacteria recovered from the lungs did not differ between wildtype and pneumolysin-deficient strains (Figure 4.2.3) and there were also no particular variations in terms of dissemination into the bloodstream or pleural space. Both strains stimulated similar increases in BAL fluid leukocytes (Figure 4.2.4), proportion of neutrophils (Figure 4.2.5) and protein (Figure 4.1.6). As with the D39 strain, PLN-A showed no difference in total pleural lavage fluid leukocytes (Figure 4.2.7a) or protein (Figure 4.2.7c) compared with controls. However, the significant increase in the percentage of neutrophils compared to controls observed when using D39 was not seen with the pneumolysin mutant, although there was no significant difference between the strains (Figure 4.2.7b), suggesting attenuation of this effect in the mutant. Both D39 and PLN-A induced significant increases in pro-inflammatory cytokines IL-1 α (Figure 4.2.8) and MCP-1 (Figure 4.2.9) in lung tissue. The pneumolysin-deficient strain generated lower levels of cytokines than the wildtype, although this was only significant with MCP-1.

4.2.4.2 Differential Protein Expression

Both PLN-A and D39 induced significant decreases in expression of RTI40 in both BAL fluid and lung tissue (Figure 4.2.10), as well as similar increases in MMC6 in BAL fluid (Figure 4.2.11). Lower values for the pneumolysin-deficient bacteria for MMC6 were not significant from wildtype. Expression of SP-D in BAL fluid was also increased with both PLN-A and D39, however the pneumolysin-deficient strain was again lower than the wildtype and the increase was not significant (Figure 4.2.12). PLN-A and D39 both induced a significant increase in levels of APN/MMC4 in BAL fluid, and although the pneumolysin-deficient strain was again lower than the wildtype there were no significant differences (Figure 4.2.13).

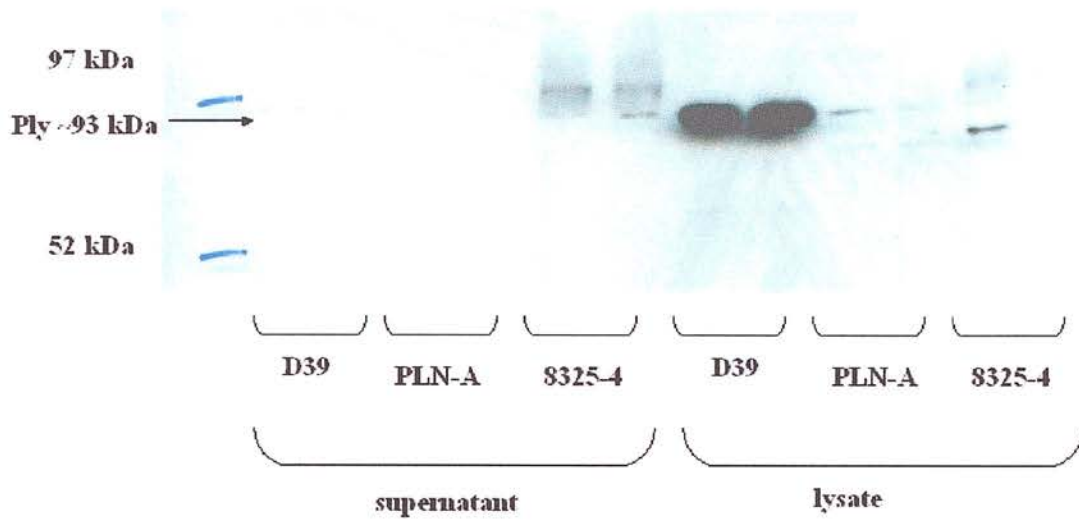


Figure 4.2.1 – Pneumolysin expression in *S. pneumoniae* D39 and PLN-A
Western blot showing expression of pneumolysin in bacterial supernatants and lysates. Negative control is *S. aureus* 8325-4.
 Expression of pneumolysin is limited to D39 bacterial lysates.

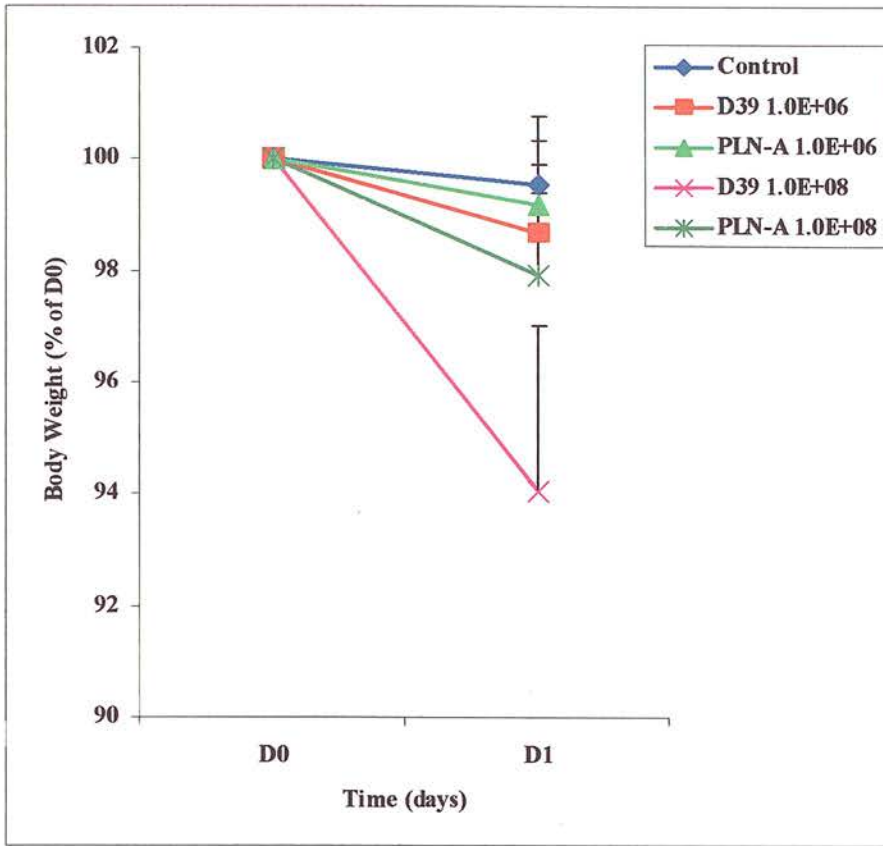


Figure 4.2.2 - Rat body weight after 24 hours

Data expressed as a percentage of initial weight at Day 0 over time

Data expressed for D39 (red) is the same as that presented in Chapter 3 at 10^6 and 10^8 cfu

Although body weight decreased in all *S. pneumoniae*-infected groups, there was no significant difference between PLN-A (green) and controls after 24 hours. There were also no significant differences between equivalent inoculation of D39 (red) and PLN-A (green).

Control n = 26, D39 10^6 n = 5, D39 10^8 n = 21, PLN-A 10^6 n = 6, PLN-A 10^8 n = 6

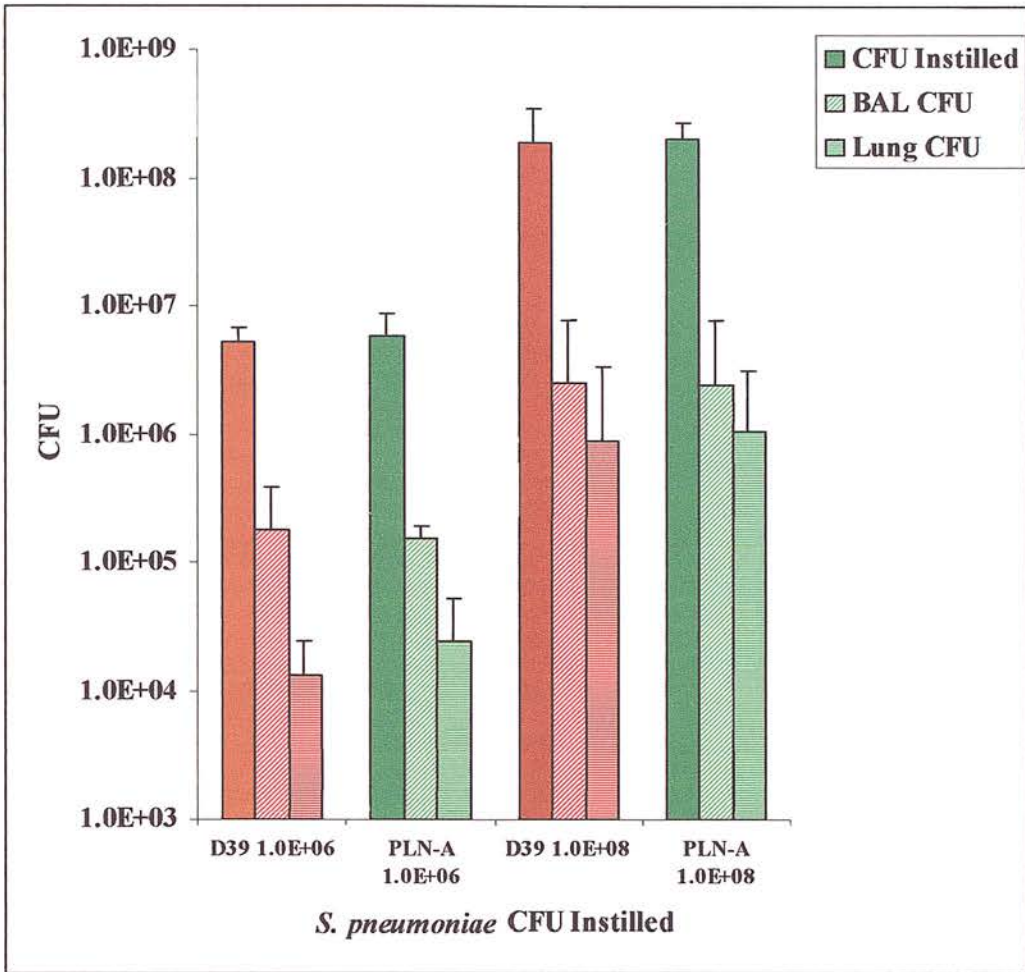


Figure 4.2.3 – CFU recovered from lungs after 24 hours

Data expressed as total cfu shown on a log scale

Bars are Instillation cfu, BAL fluid and Lung tissue

Samples in each treatment group are taken from at least 2 separate experiments

Data expressed for D39 (red) is the same as that presented in Chapter 3 at 10^6 and 10^8 cfu.

There were no differences in cfu recovered from BAL fluid and lungs between PLN-A (green) and D39 (red) at any instillation level.

D39 10^6 n = 5, D39 10^8 n = 10, PLN-A 10^6 n = 4, PLN-A 10^8 n = 6

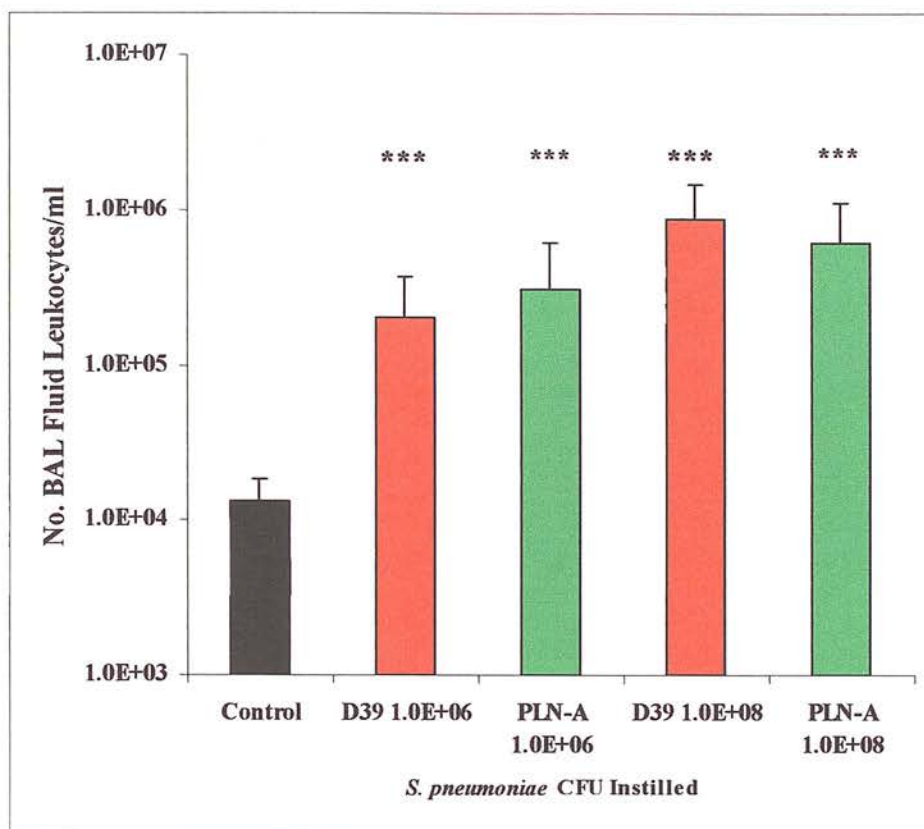


Figure 4.2.4 – Number of leukocytes in BAL fluid after 24 hours shown on a log scale

Data expressed as number of cells per ml of BAL fluid

Samples in each treatment group are taken from at least 2 separate experiments

Data expressed for D39 (red) is the same as that presented in Chapter 3 at 10^6 and 10^8 cfu.

Both D39 (red) and PLN-A (green) induced a significant increase in BAL fluid leukocytes compared to controls, but there were no differences between strains at any instillation level, or between instillation levels for each strain.

Transformed (Log) ANOVA $p < 0.0001$

Tukey post-test	Control vs PLN-A 10^6	***	$p < 0.001$
	Control vs PLN-A 10^8	***	$p < 0.001$
	(Control vs D39 10^6	***	$p < 0.001$)
	(Control vs D39 10^8	***	$p < 0.001$)

Control $n = 9$, D39 10^6 $n = 5$, D39 10^8 $n = 10$, PLN-A 10^6 $n = 4$, PLN-A 10^8 $n = 6$

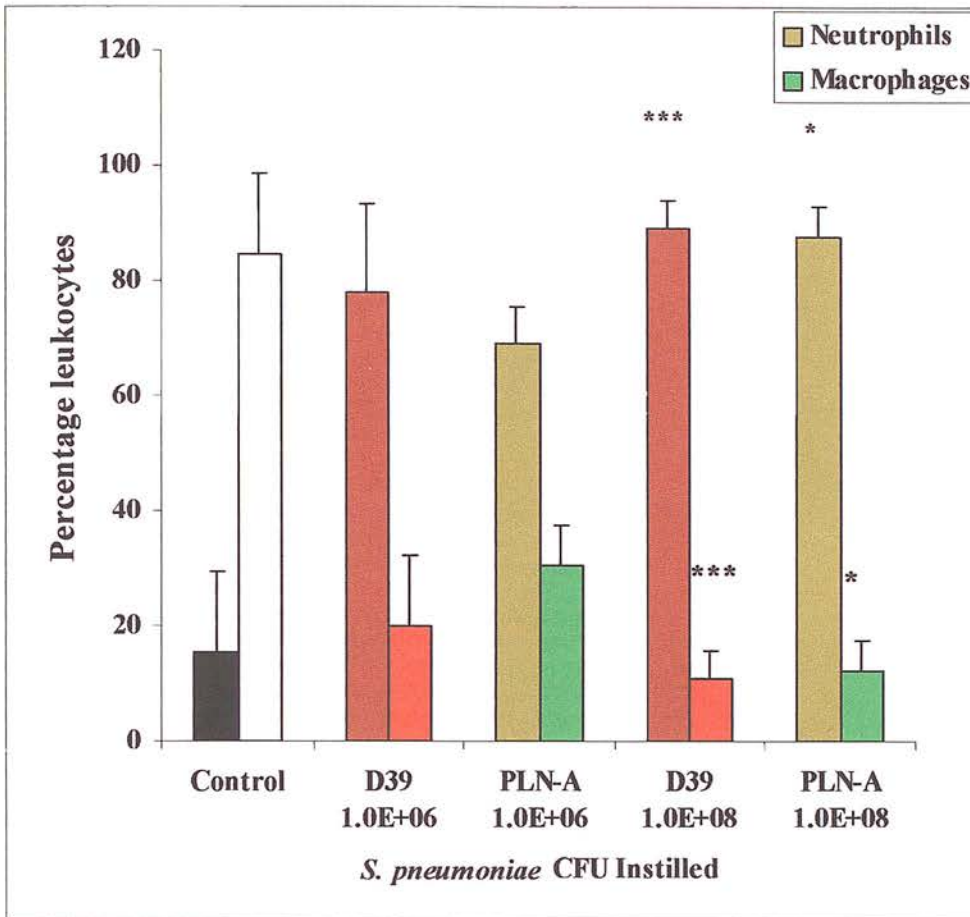


Figure 4.2.5 – Differential leukocyte count in BAL fluid after 24 hours
 Data expressed as percentage macrophages and neutrophils. Percentage of other leukocytes <1%

Samples in each treatment group are taken from at least 2 separate experiments

Data expressed for D39 (red) is the same as that presented in Chapter 3 at 10^6 and 10^8 cfu.

Both PLN-A (green) and D39 (red) at the higher instillation level induced a significant increase in the ratio of neutrophils:macrophages compared to controls, but there was no difference between the two strains.

Kruskal-Wallis $p < 0.0001$

Dunns post-test	Control vs PLN-A 10^8 macrophages	*	$p < 0.05$
	Control vs PLN-A 10^8 neutrophils	*	$p < 0.05$
	(Control vs D39 10^8 macrophages	***	$p < 0.001$)
	(Control vs D39 10^8 neutrophils	***	$p < 0.001$)

Control $n = 9$, D39 10^6 $n = 5$, D39 10^8 $n = 10$, PLN-A 10^6 $n = 4$, PLN-A 10^8 $n = 6$

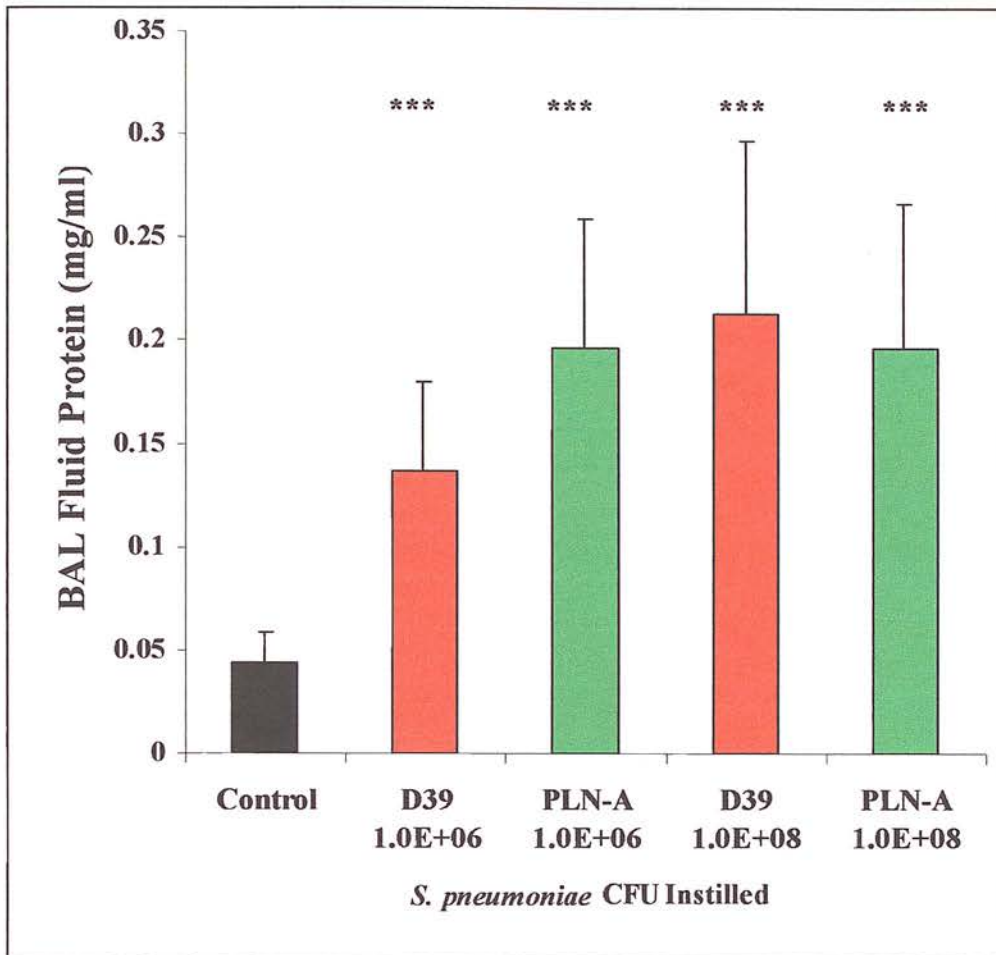


Figure 4.2.6 – Protein concentration in BAL fluid after 24 hours

Data expressed as protein (mg) per ml BAL fluid

Samples in each treatment group are taken from at least 2 separate experiments

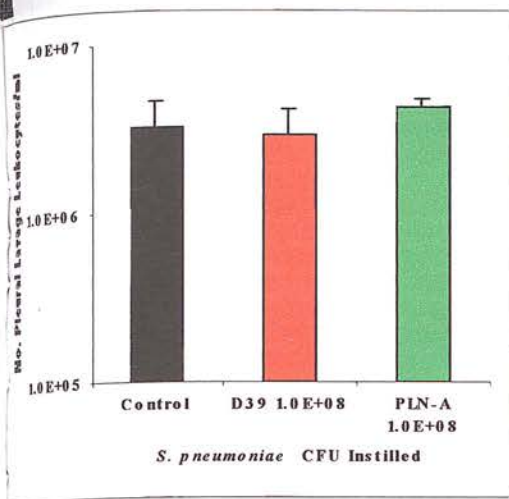
Data expressed for D39 (red) is the same as that presented in Chapter 3 at 10^6 and 10^8 cfu.

Both PLN-A (green) and D39 (red) induced a significant increase in BAL fluid protein after 24 hours at all inoculation levels. There were no differences between the two strains.

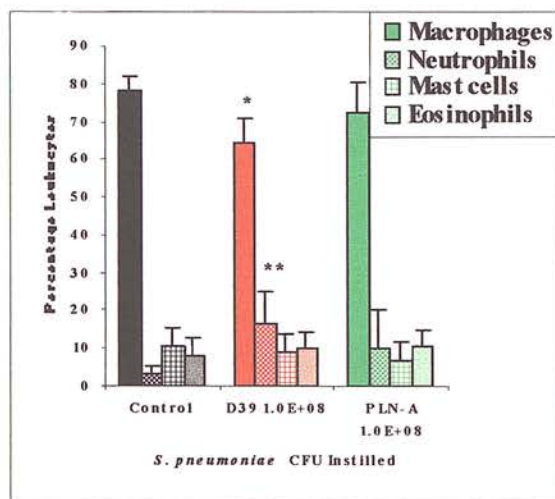
Transformed (Log) ANOVA $p < 0.0001$

Tukey post-test	Control vs PLN-A 10^6	***	$p < 0.001$
	Control vs PLN-A 10^8	***	$p < 0.001$
	(Control vs D39 10^6	***	$p < 0.001$)
	(Control vs D39 10^8	***	$p < 0.001$)

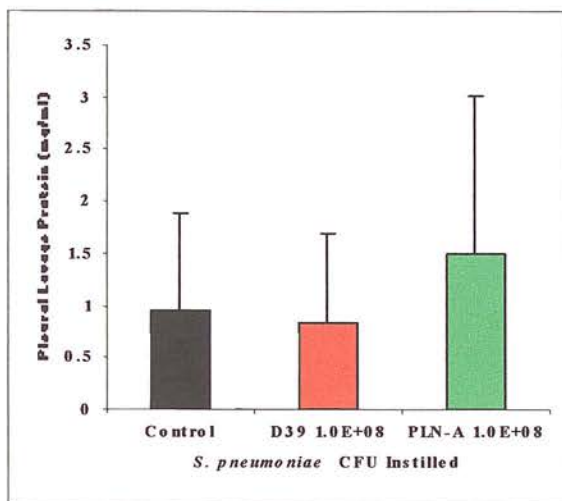
Control $n = 11$, D39 10^6 $n = 5$, D39 10^8 $n = 10$, PLN-A 10^6 $n = 4$, PLN-A 10^8 $n = 6$



A



B



C

Figure 4.2.7 – Pleural Lavage Fluid after 24 hours

Samples in each treatment group are taken from at least 2 separate experiments

A. Number of leukocytes shown on a log scale

Data expressed as log number of cells per ml pleural lavage fluid

Data expressed for D39 (red) is the same as that presented in Chapter 3 at 10^6 and 10^8 cfu. There were no differences in the number of pleural lavage fluid leukocytes between PLN-A (green), D39 (red) and controls.

Control n = 8, D39 10^8 n = 6, PLN-A 10^8 n = 6

B. Differential leukocyte count

Data expressed as percentage macrophages, neutrophils, mast cells and eosinophils. Percentage of other leukocytes <1%.

Data expressed for D39 (red) is the same as that presented in Chapter 3 at 10^6 and 10^8 cfu. Unlike D39 (red), PLN-A (green) did not induce a significant increase in percentage neutrophils or decrease in the percentage of macrophages in pleural lavage fluid.

Neutrophils:

Transformed ANOVA (Log) p = 0.0123

Tukey post test (Control vs D39 10^8 ** p < 0.01)

Macrophages:

Transformed (Log) ANOVA p = 0.003

Tukey post test (Control vs D39 10^8 ** p < 0.01)

There were no significant differences between PLN-A (green) and control (black) or D39 (red).

There were no differences in proportions of eosinophils or mast cells.

C. Protein concentration

Data expressed as protein (mg) per ml pleural lavage fluid

Data expressed for D39 (red) is the same as that presented in Chapter 3 at 10^6 and 10^8 cfu. Although pleural lavage fluid protein appeared to increase with PLN-A (green) infection above control (black) and D39 (red) levels, there were no significant differences between groups.

Control n = 10, D39 10^8 n = 6, PLN-A 10^8 n = 6

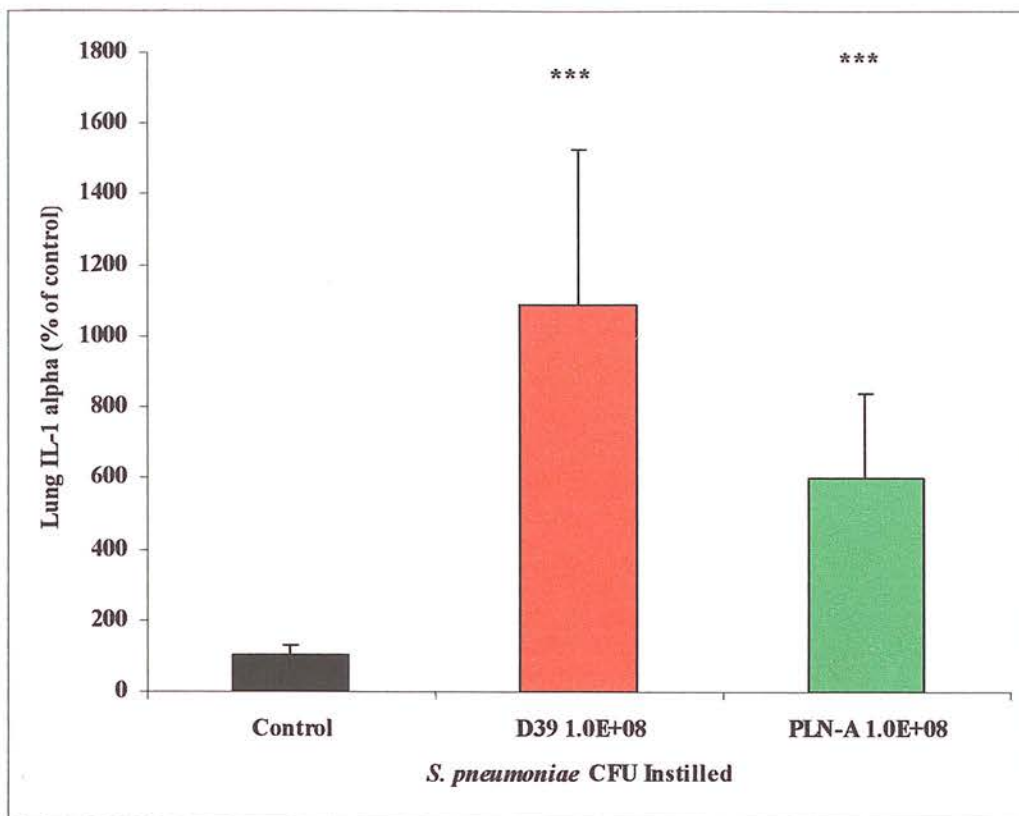


Figure 4.2.8 – IL-1 α in lung tissue after 24 hours

Data expressed per mg lung protein and presented as a percentage of control

Samples in each treatment group are taken from at least 2 separate experiments

Data expressed for D39 (red) is the same as that presented in Chapter 3 at 10^8 cfu.

Both PLN-A (green) and D39 (red) induced a significant increase in IL-1 α in lung tissue. Although values for PLN-A (green) were lower than D39 (red), there was no significant difference between strains.

Transformed (Log) ANOVA $p < 0.0001$

Tukey post-test	Control vs PLN-A 10^8	***	$p < 0.0001$
	(Control vs D39 10^8)	***	$p < 0.0001$

Control $n = 4$, D39 10^8 $n = 4$, PLN-A 10^8 $n = 4$

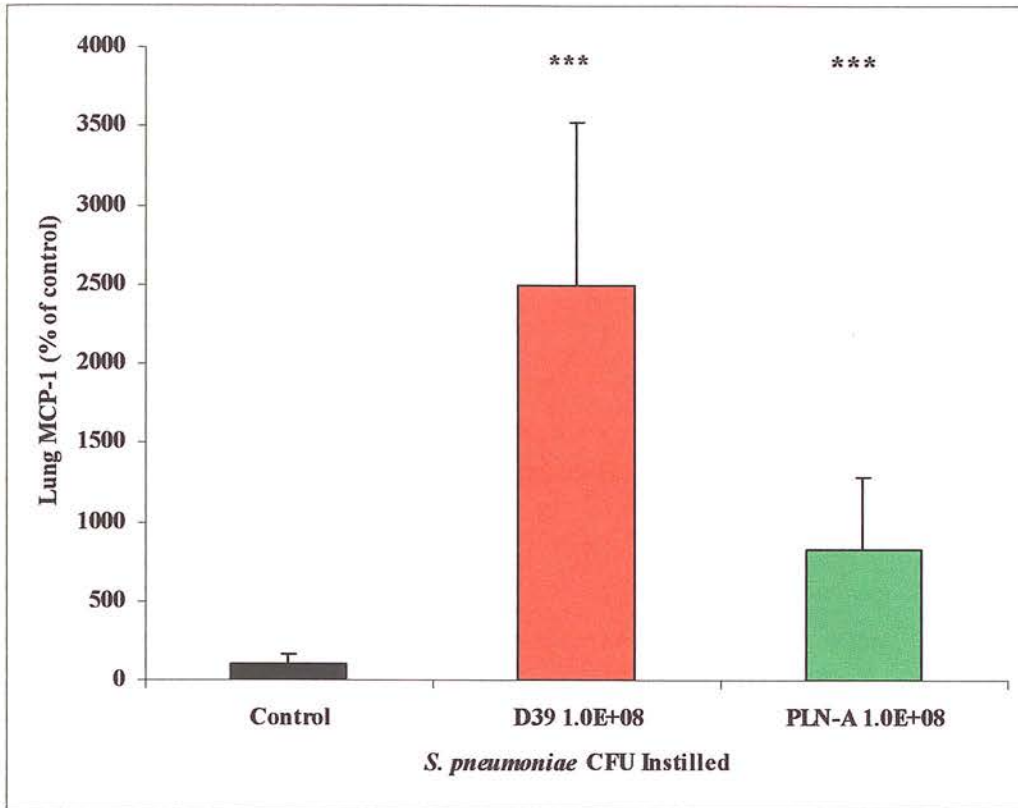


Figure 4.2.9 – MCP-1 in lung tissue after 24 hours

Data expressed per mg lung protein and presented as a percentage of control

Samples in each treatment group are taken from at least 2 separate experiments

Data expressed for D39 (red) is the same as that presented in Chapter 3 at 10^8 cfu.

Both PLN-A (green) and D39 (red) induced a significant increase in MCP-1 in lung tissue. Levels with PLN-A (green) were also significantly lower than those for D39.

Transformed (Log) ANOVA $p < 0.0001$

Tukey post-test	Control vs PLN-A 10^8	***	$p < 0.0001$
	D39 10^8 vs PLN-A 10^8	*	$p < 0.05$
	(Control vs D39 10^8)	***	$p < 0.0001$

Control $n = 4$, D39 10^8 $n = 4$, PLN-A 10^8 $n = 4$

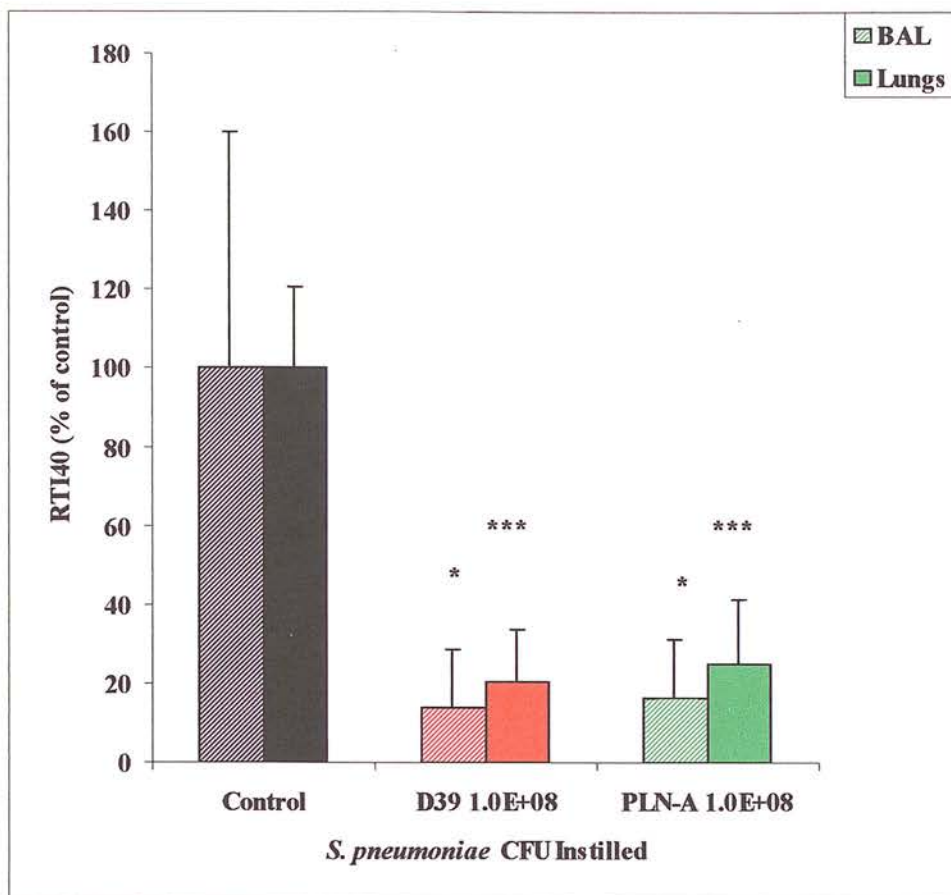


Figure 4.2.10 – RTI40 in BAL and lung tissue after 24 hours

Data expressed per ml BAL fluid or per mg lung protein and presented as a percentage of control

Samples in each treatment group are taken from at least 2 separate experiments

Data expressed for D39 (red) is representative of that presented in Chapter 3 at 10^8 cfu.

Both PLN-A (green) and D39 (red) induced a decrease in RTI40 in both BAL fluid and lung tissue, with no differences between the two strains.

BAL:

ANOVA $p = 0.02$

Tukey post-test	Control vs PLN-A 10^8	*	$p < 0.05$
	(Control vs D39 10^8)	*	$p < 0.05$

Control $n = 4$, D39 10^8 $n = 3$, PLN-A 10^8 $n = 4$

Lung tissue

ANOVA $p < 0.0001$

Tukey post-test	Control vs PLN-A 10^8	***	$p < 0.0001$
	(Control vs D39 10^8)	***	$p < 0.0001$

Control $n = 5$, D39 10^8 $n = 7$, PLN-A 10^8 $n = 6$

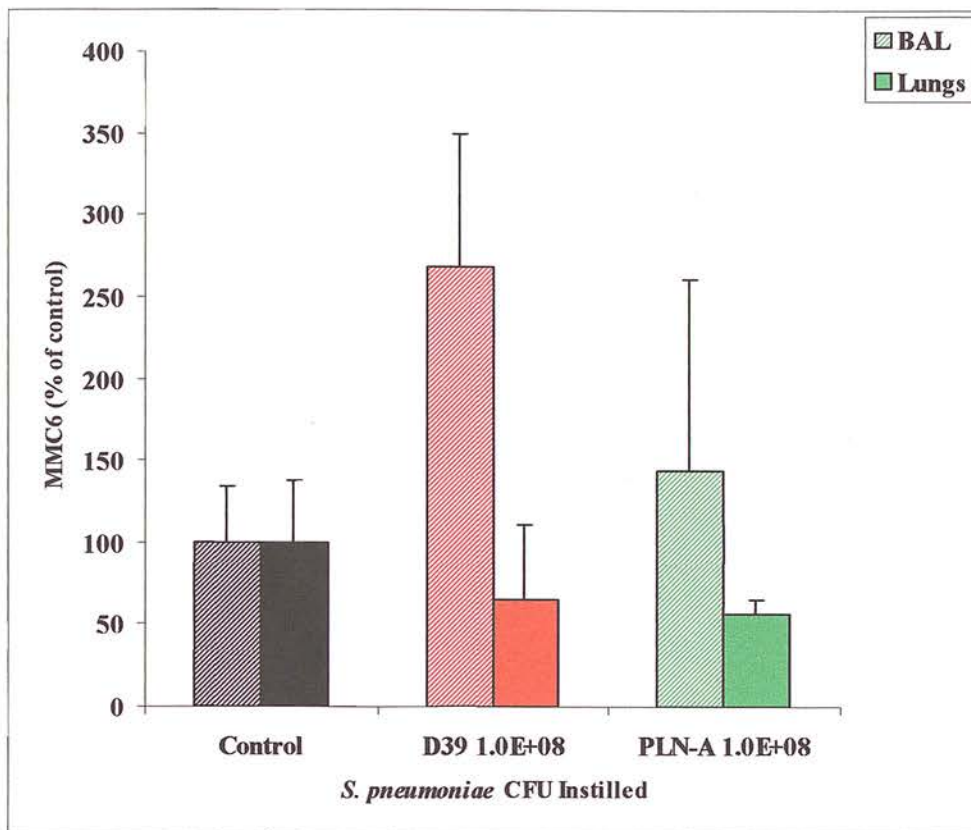


Figure 4.2.11 – MMC6 in BAL and lung tissue after 24 hours

Data expressed per ml BAL fluid or per mg lung protein and presented as a percentage of control

Samples in each treatment group are taken from at least 2 separate experiments

Data expressed for D39 (red) is representative of that presented in Chapter 3 at 10^8 cfu.

Both PLN-A (green) and D39 (red) induced an increase in MMC6 in BAL fluid with a slight decrease in lung tissue. Values for PLN-A (green) in BAL fluid were lower than D39 (red), but this difference was not significant.

Control n = 4, D39 10^8 n = 4, PLN-A 10^8 n = 4

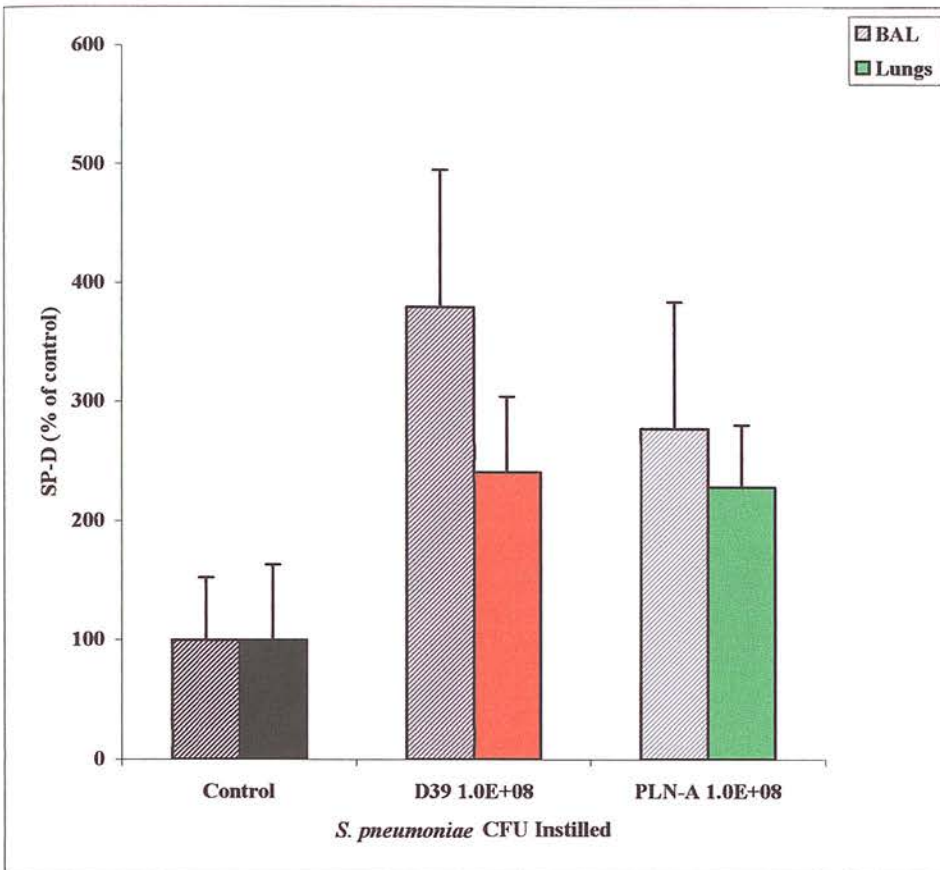


Figure 4.2.12 – SP-D in BAL fluid and lung tissue after 24 hours

Data expressed per ml BAL fluid or per mg lung protein and expressed as a percentage of control

Samples in each treatment group are taken from at least 2 separate experiments

Data expressed for D39 (red) is representative of that presented in Chapter 3 at 10^8 cfu.

Both PLN-A (green) and D39 (red) induced an increase in SP-D in BAL fluid, however unlike D39 (red), this difference was not significantly different from controls (black) for PLN-A (green) (Kruskall-Wallis $p = 0.006$). Both strains induced a similar, non-significant decrease in SP-D in lung tissue.

Control $n = 4$, D39 10^8 $n = 4$, PLN-A 10^8 $n = 4$

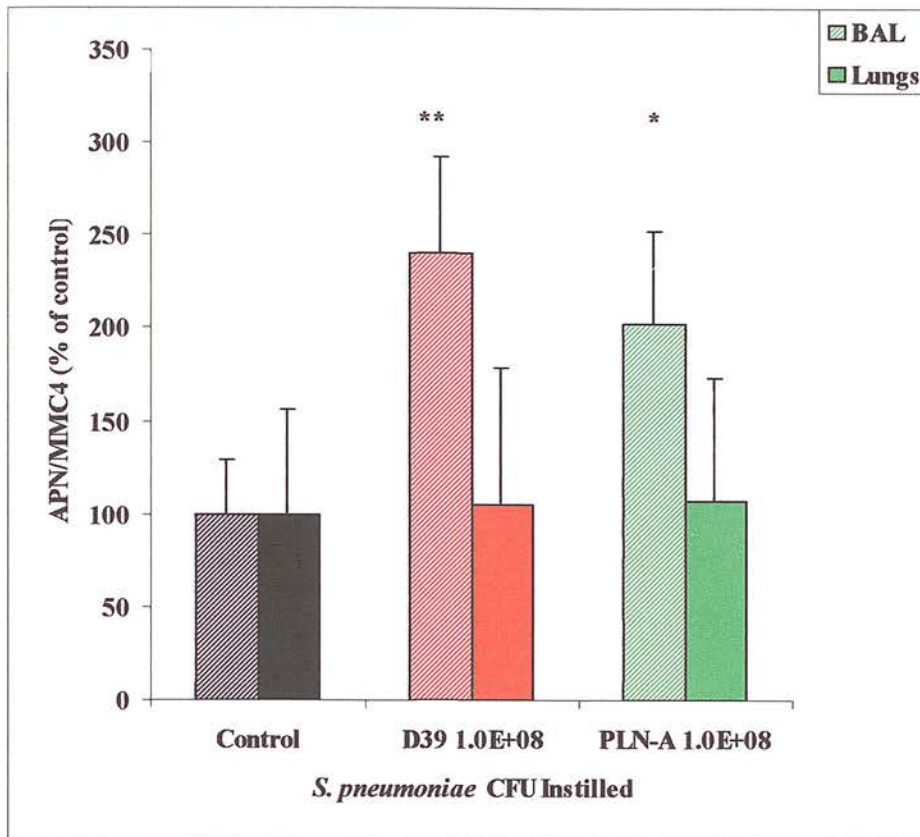


Figure 4.2.13 – APN/MMC4 in BAL fluid and lung tissue after 24 hours

Data expressed per ml BAL fluid or per mg lung protein and presented as a percentage of control

Samples in each treatment group are taken from at least 2 separate experiments

Data expressed for D39 (red) is representative of that presented in Chapter 3 at 10^8 cfu.

Both PLN-A (green) and D39 (red) induced a significant increase in APN/MMC4 in BAL fluid, and although PLN-A (green) was lower than D39 (red), there was no significant difference between them.

BAL:

ANOVA $p = 0.002$

Tukey post-test

Control vs PLN-A 10^8
(Control vs D39 10^8)

* $p < 0.05$

** $p < 0.05$

Control $n = 5$, D39 10^8 $n = 6$, PLN-A 10^8 $n = 4$

4.3 *S. aureus*

4.3.1 Introduction

S. aureus is a Gram positive bacterium and a clinically important lung pathogen. A recovering model of *S. aureus*-induced pneumonia had already been established in the laboratory (McElroy et al., 2002; Tyrrell et al., 2005). The differential cell-specific protein expression identified in pneumococcal pneumonia may have been due to an effect common to Gram-positive bacteria.

4.3.2 Hypothesis 2.2

Differential protein expression in pneumococcal pneumonia is due to host response to Gram-positive bacteria

The aim of this chapter was to compare cell-specific protein expression in the model of pneumococcal pneumonia with that of caused by *S. aureus*.

4.3.3 Methods

The model of staphylococcal pneumonia used in this chapter was equivalent to that already established by the author (McElroy et al., 2002), using the wildtype laboratory strain 8325-4 (derived from NCTC 8325-4) (Novick, 1967) at 10^8 cfu ($1.97 \times 10^8 \pm 3.50 \times 10^6$), enabling comparison with the streptococcal model at the same dose ($1.87 \times 10^8 \pm 1.54 \times 10^8$). As for section 4.2 (Pneumolysin), the 24-hour time point was selected. Infected animals were treated during different experiments along with controls for each. For technical reasons due to problems with the antibodies it was not possible to assess levels of Pro-SP-C in these samples. This set of experiments also provided the opportunity to further investigate the use of APN/MMC4 as a preliminary indicator of ATII/Clara cell injury by comparing two different models of

bacterial infection. As in Chapter 3, all graphs show data generated as a result of at least 2 separate experiments. Graphs show means with SD error bars unless stated otherwise. Asterisks illustrate significant differences between experimental data and controls unless stated otherwise. Analyses of specific proteins are presented as a percentage of the mean control value. As with Chapter 3, assay limitations of space necessitated the random selection of samples from 2 separate experiments for specific protein analysis.

4.3.4 Results

4.3.4.1 Basic Comparison of *S. aureus* and *S. pneumoniae* Models

Both *S. aureus* and *S. pneumoniae* induced a similar loss of body weight (Figure 4.3.1). The number of bacteria recovered from the lungs was comparable in both models, although it is worthy of note that with *S. pneumoniae* there were more cfu in BAL fluid than lung tissue but more in lung tissue than BAL fluid in the staphylococcal model (Figure 4.3.2), perhaps suggesting different mechanisms of virulence between the two species. It is also of interest that in contrast to pneumococcal infection, no evidence of dissemination into the bloodstream was observed in any *S. aureus*-infected animals. Both strains induced similar significant increases in BAL fluid leukocytes (Figure 4.3.3) and proportions of neutrophils (Figure 4.3.4). The increase in BAL fluid protein induced by *S. aureus* was significantly higher than that induced by *S. pneumoniae* (Figure 4.3.5). *S. aureus* infection also induced higher levels of pro-inflammatory cytokines, significant for IL-1 α (Figure 4.3.6) but not MCP-1 (Figure 4.3.7) compared to the pneumococcal model. Also of note is the fact that *S. aureus*-infected lung tissue contained a large amount

(887±224pg/ml) of IFN- γ , a pro-inflammatory cytokine that was barely detectable in either control or *S. pneumoniae*-infected tissue.

4.3.4.2 Differential Protein Expression

Expression of the ATI cell-specific protein RTI40 was significantly different between *S. aureus* and *S. pneumoniae*-induced lung infections. The decrease in both BAL fluid and lung tissue observed in the pneumococcal model was not seen in the staphylococcal one (Figure 4.3.8). Other ATI cell-specific proteins MMC6 (Figure 4.3.9) and Aquaporin 5 (Figure 4.3.10) showed similar decreases in lung tissue and increases in BAL fluid (MMC6 only). As with the streptococcal model, *S. aureus* induced a similar increase in ATII/Clara cell-specific protein SP-D in both BAL fluid and to a lesser extent lung tissue (Figure 4.3.11). Finally, the increase in APN/MMC4 in BAL fluid from *S. pneumoniae*-infected lungs was not observed in the staphylococcal model (Figure 4.3.12). Both infections demonstrate similar percentages (4.3.4.1 Basic Comparison of *S. aureus* model with *S. pneumoniae*) and numbers (*S. pneumoniae* $8.9 \times 10^4 \pm 4.8 \times 10^3$ vs *S. aureus* $9.4 \times 10^4 \pm 3.2 \times 10^3$) of macrophages in BAL fluid, and while it is possible that this result could be due to differential effects of the pathogens on protein expression on the macrophages, it could also be that these data suggest that in the model of pneumococcal pneumonia levels of APN/MMC4 are representative of injury to ATII/Clara cells.

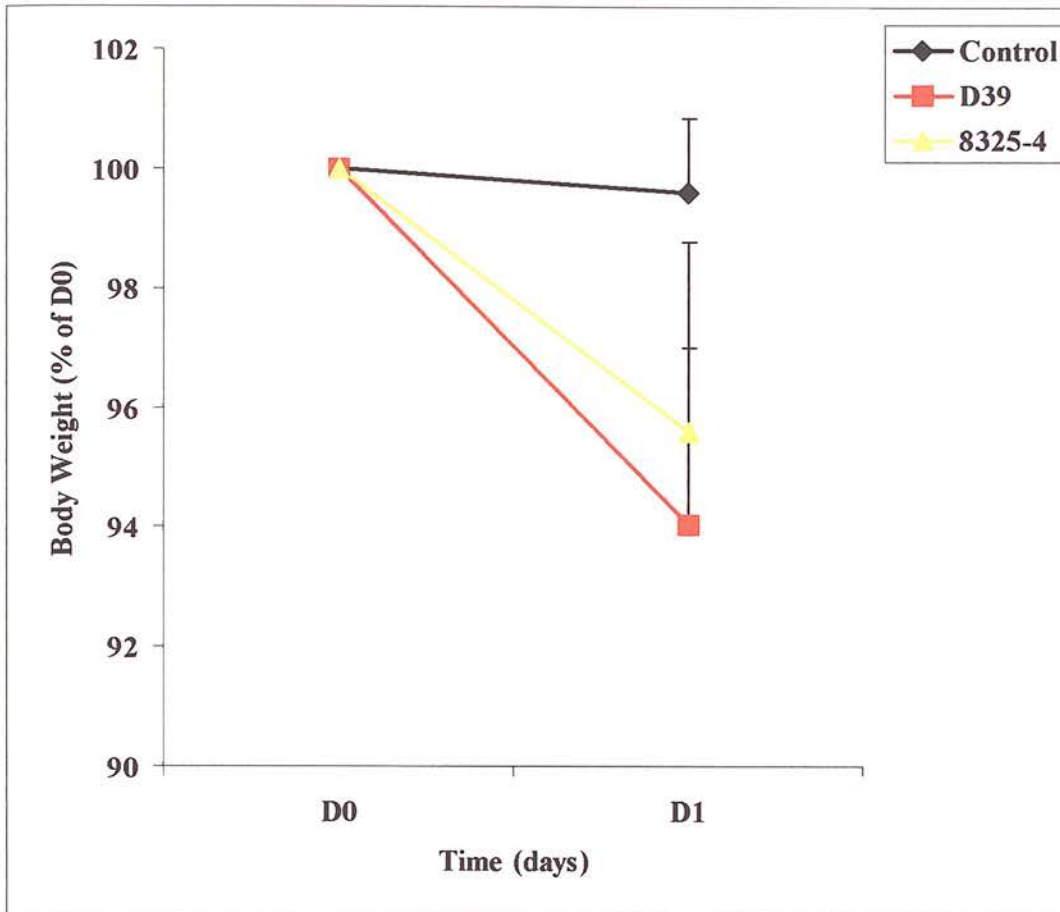


Figure 4.3.1 - Rat body weight after 24 hours

Data expressed as a percentage of initial weight at Day 0

Data expressed for D39 *S. pneumoniae* (red) is the same as that presented in Chapter 3 at 10^8 cfu.

S. pneumoniae and *S. aureus* infected animals were treated during different experiments.

After 24 hours weight significantly decreased in both 8325-4 *S. aureus* (yellow) and D39 *S. pneumoniae*-infected rats. More weight was lost in the D39 *S. pneumoniae* (red) model but the difference was not significant.

ANOVA $p < 0.0001$

Tukey post-test	Control vs 8325-4 10^8 cfu	***	$p < 0.001$
	(Control vs D39 10^8 cfu	***	$p < 0.001$)

Control $n = 29$, D39 10^8 $n = 21$, 8325-4 10^8 $n = 15$

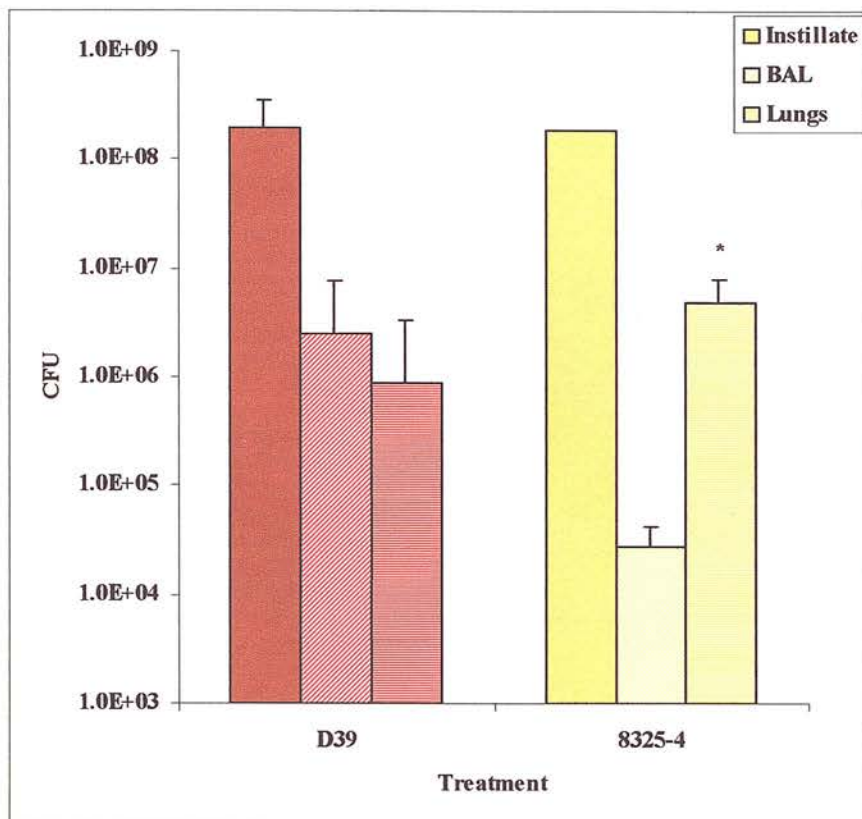


Figure 4.3.2 – CFU recovered from lungs after 24 hours

Data expressed as total cfu shown on a log scale

Bars are Instillation cfu, BAL fluid and Lung tissue

Samples in each treatment group are taken from at least 2 separate experiments

Asterisk represents significant difference between D39 and 8325-4

Data expressed for D39 *S. pneumoniae* (red) is the same as that presented in Chapter 3 at 10^8 cfu.

S. pneumoniae and *S. aureus* infected animals were treated during different experiments.

There was no difference in total numbers of bacteria recovered from lung tissue between the 8325-4 *S. aureus* (yellow) and D39 *S. pneumoniae* (red) models. CfU in BAL fluid was noticeably lower with *S. aureus* (yellow), but this was not significant. Similarly, cfu in lung tissue was higher in *S. aureus* (yellow), which was significant.

Mann-Whitney D39 vs 8325-4 * p = 0.0336
 D39 10^8 n = 10, 8325-4 10^8 n = 4

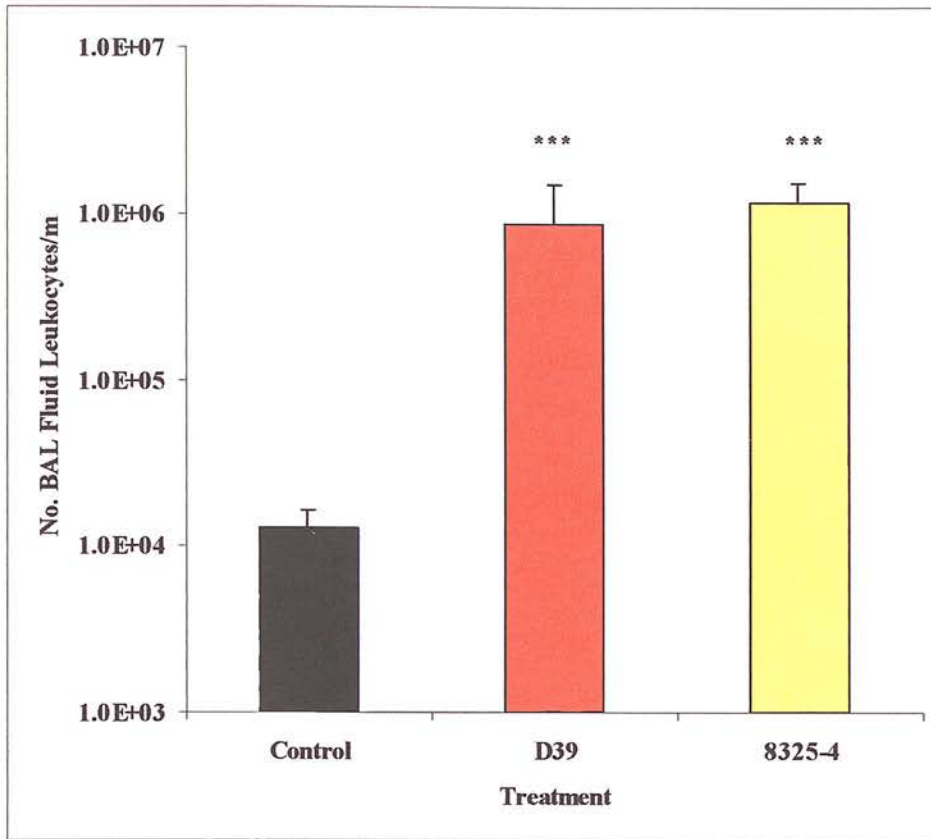


Figure 4.3.3 – Number of leukocytes in BAL fluid after 24 hours

**Data expressed as number of cells per ml of BAL fluid shown on a log scale
 Samples in each treatment group are taken from at least 2 separate experiments**

Data expressed for D39 *S. pneumoniae* (red) is the same as that presented in Chapter 3 at 10^8 cfu.

S. pneumoniae and *S. aureus* infected animals were treated during different experiments.

Both 8325-4 *S. aureus* (yellow) and D39 *S. pneumoniae* (red) induced a significant increase in BAL leukocytes after 24 hours, but there was no difference between the two strains.

Transformed (Log) ANOVA $p < 0.0001$

Tukey post test	Control vs 8325-4 10^8	***	$p < 0.001$
	(Control vs D39 10^8)	***	$p < 0.001$

Control $n = 17$, D39 10^8 $n = 10$, 8325-4 10^8 $n = 4$

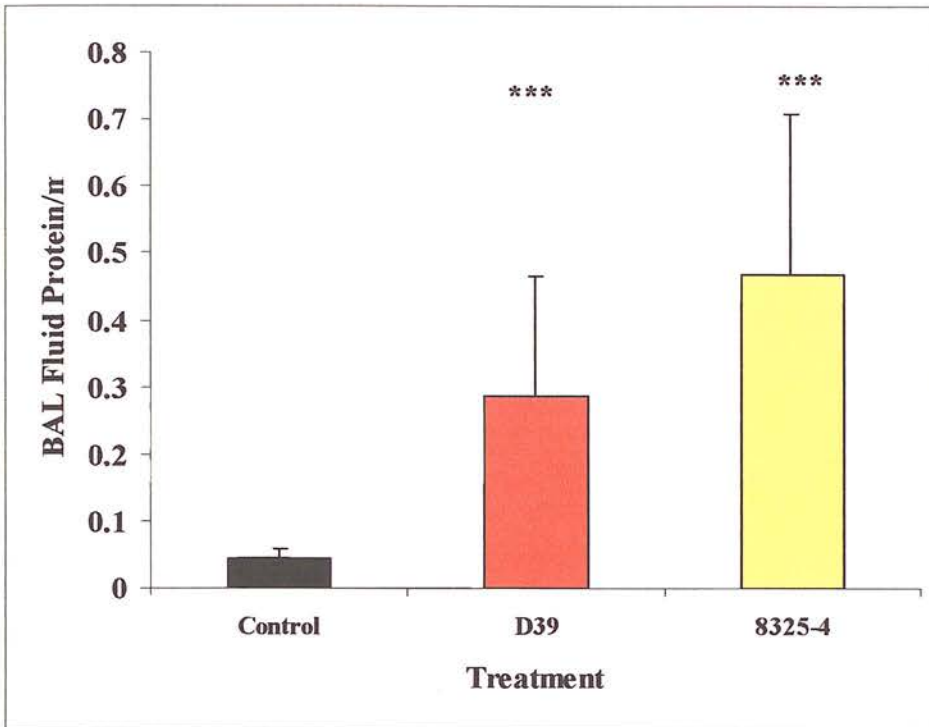


Figure 4.3.5 – Protein concentration in BAL fluid after 24 hours

Data expressed as protein (mg) per ml BAL fluid

Samples in each treatment group are taken from at least 2 separate experiments

Data expressed for D39 *S. pneumoniae* (red) is the same as that presented in Chapter 3 at 10^8 cfu (red).

S. pneumoniae and *S. aureus* infected animals were treated during different experiments.

Both 8325-4 *S. aureus* (yellow) and D39 *S. pneumoniae* (red) induced a significant increase in BAL fluid protein after 24 hours. Values for 8325-4 *S. aureus* (yellow) were significantly higher than D39 *S. pneumoniae* (red).

Transformed (Log) ANOVA $p < 0.001$

Tukey post-test Control vs 8325-4 10^8 *** $p < 0.001$

D39 10^8 vs 8325-4 10^8 ** $p < 0.01$

(Control vs D39 10^8 *** $p < 0.001$)

Control $n = 18$, D39 10^8 $n = 10$, 8325-4 10^8 $n = 4$

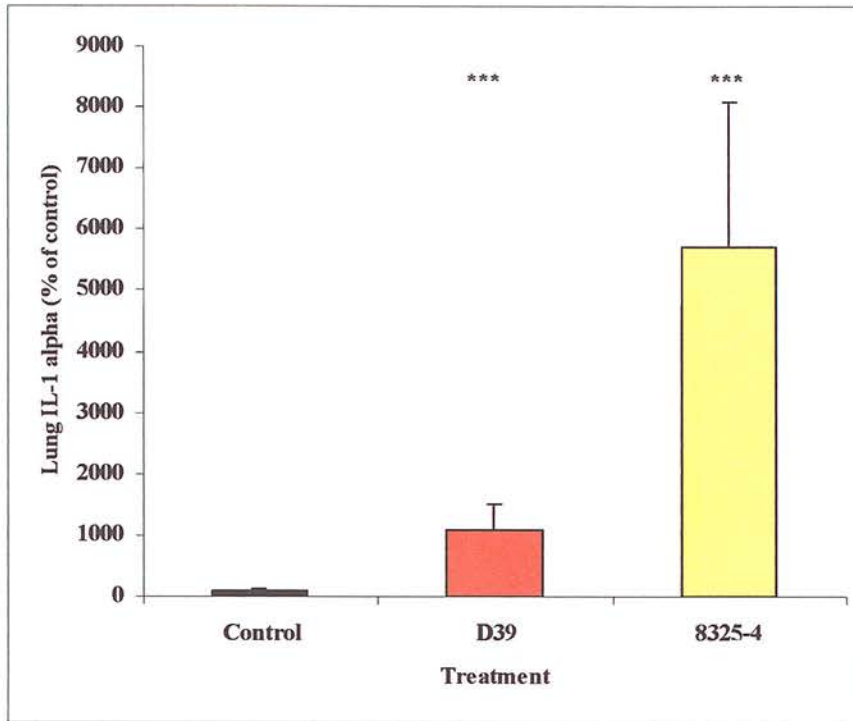


Figure 4.3.6 – IL-1 α in lung tissue after 24 hours

Data expressed per mg lung protein and presented as a percentage of control

Samples in each treatment group are taken from at least 2 separate experiments

Data expressed for D39 *S. pneumoniae* (red) is the same as that presented in Chapter 3 at 10⁸ cfu (red).

S. pneumoniae and *S. aureus* infected animals were treated during different experiments.

Both 8325-4 *S. aureus* (yellow) and D39 *S. pneumoniae* (red) induced a significant increase in IL-1 α in lung tissue after 24 hours (red). Levels IL-1 α in 8325-4 *S. aureus*-infected tissue (yellow) were also significantly higher than those using D39 *S. pneumoniae* (red).

Transformed (Log) ANOVA $p < 0.0001$

Tukey post-test	Control vs 8325-4 10 ⁸	***	$p < 0.0001$
	D39 10 ⁸ vs 8325-4 10 ⁸	***	$p < 0.0001$
	(Control vs D39 10 ⁸)	***	$p < 0.0001$

Control $n = 4$, D39 10⁸ $n = 4$, 8325-4 10⁸ $n = 4$

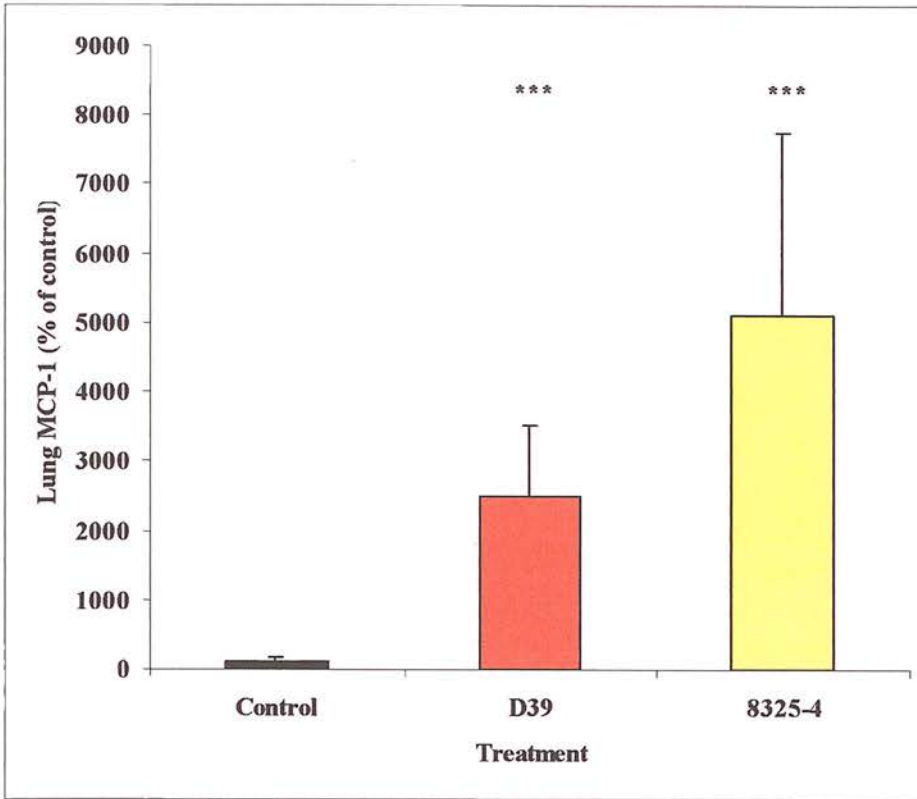


Figure 4.3.7 – MCP-1 in lung tissue after 24 hours

Data expressed per mg lung protein and expressed as a percentage of control

Samples in each treatment group are taken from at least 2 separate experiments

Data expressed D39 for *S. pneumoniae* (red) is the same as that presented in Chapter 3 at 10^8 cfu (red).

S. pneumoniae and *S. aureus* infected animals were treated during different experiments.

Both 8325-4 *S. aureus* (yellow) and D39 *S. pneumoniae* (red) induced a significant increase of MCP-1 in lung tissue after 24 hours. Levels with 8325-4 *S. aureus* (yellow) were higher than D39 *S. pneumoniae* (red) but this difference was not significant.

Transformed (Log) ANOVA $p < 0.0001$

Tukey post-test	Control vs 8325-4 10^8	***	$p < 0.0001$
	(Control vs D39 10^8)	***	$p < 0.0001$

Control $n = 4$, D39 10^8 $n = 4$, 8325-4 10^8 $n = 4$

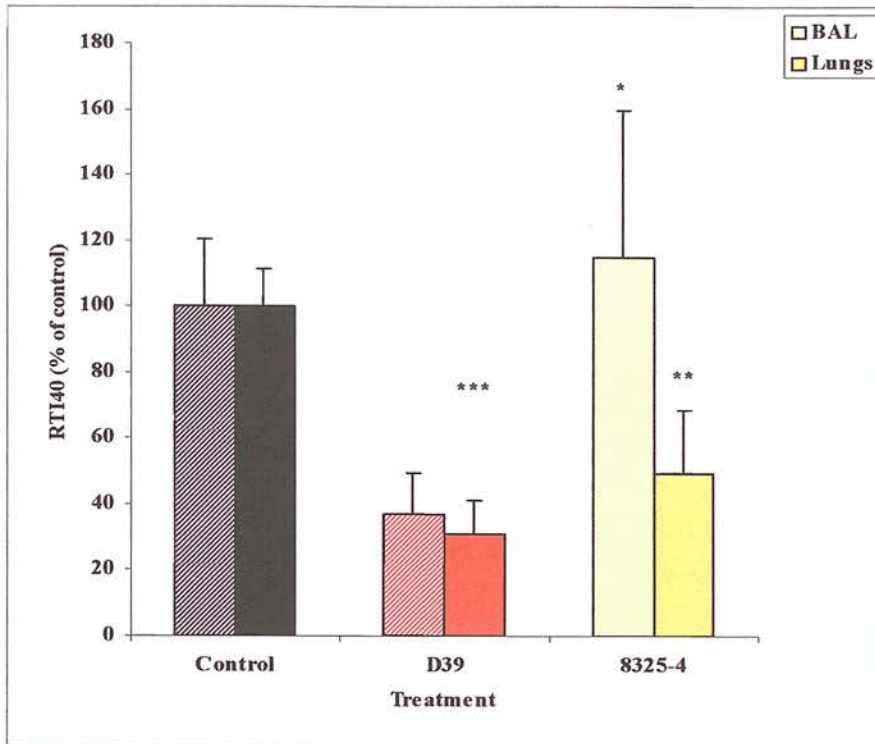


Figure 4.3.8 – RTI40 in BAL and lung tissue after 24 hours

Data expressed per ml BAL fluid or per mg lung protein and expressed as percentage of control

Samples in each treatment group are taken from at least 2 separate experiments
 Asterisks above BAL fluid bars represent a significant difference between D39 *S. pneumoniae* and 8325-4 *S. aureus*

Asterisks above lung tissue bars represent a significant difference between bacterial infection and control

Data expressed for D39 *S. pneumoniae* (red) is representative of that presented in Chapter 3 at 10^8 cfu (red).

S. pneumoniae and *S. aureus* infected animals were treated during different experiments.

There was no difference in RTI40 levels in BAL fluid between 8325-4 *S. aureus*-infected (yellow) lungs and controls, compared to the lower levels in D39

S. pneumoniae infection (red). There was also a significant difference between D39 *S. pneumoniae* and 8325-4 *S. aureus*. Levels of RTI40 in lung tissue were similarly significantly reduced in both models (ANOVA $p < 0.0002$)

BAL Fluid:

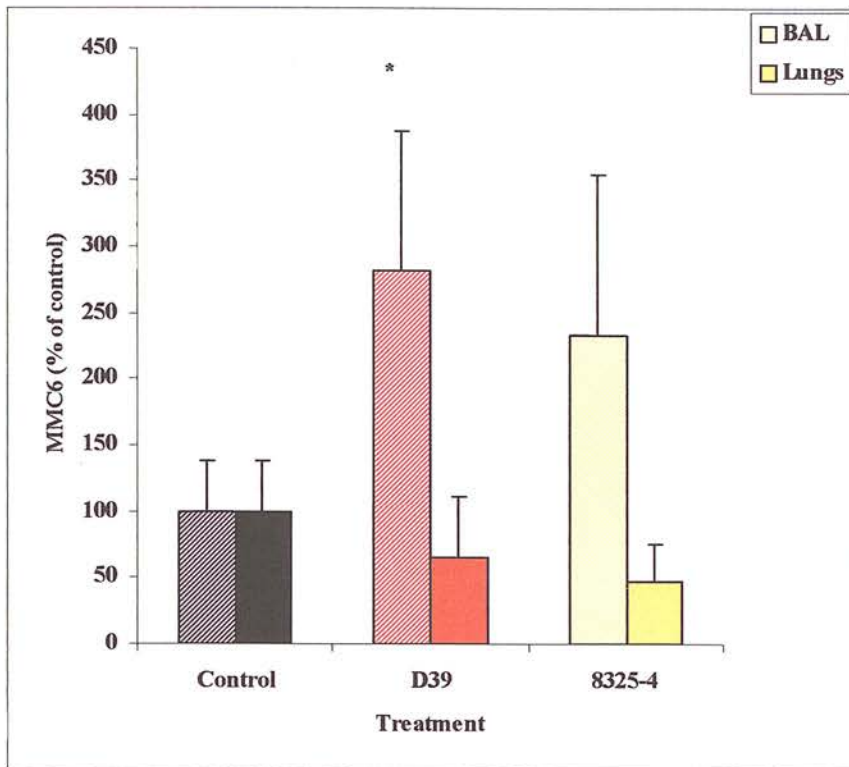
Kruskal-Wallis $p = 0.009$

Dunns post-test D39 10^8 vs 8325-4 10^8 * $p < 0.05$

Lung tissue:

ANOVA $p = 0.0002$

Tukey post-test Control vs 8325-4 10^8 ** $p < 0.001$
 (Control vs D39 10^8) ** $p < 0.001$



Control n = 4, D39 10⁸ n = 4, 8325-4 10⁸ n = 4

Figure 4.3.9 – MMC6 in BAL and lung tissue after 42 hours

Data expressed per ml BAL fluid or per mg lung protein and expressed as a percentage of control

Samples in each treatment group are taken from at least 2 separate experiments

Data expressed for D39 *S. pneumoniae* (red) is representative of that presented in Chapter 3 at 10⁸ cfu (red).

S. pneumoniae and *S. aureus* infected animals were treated during different experiments.

Both 8325-4 *S. aureus* (yellow) and D39 *S. pneumoniae* (red) induced a similar increase in MMC6 in BAL fluid, although this was only significant in the streptococcal model (Kruskal-Wallis p = 0.0261; Dunns - Control vs D39 10⁸ * p < 0.05). Both bacteria also induced a similar non-significant decrease in MMC6 in lung tissue.

Control n = 4, D39 10⁸ n = 4, 8325-4 10⁸ n = 4

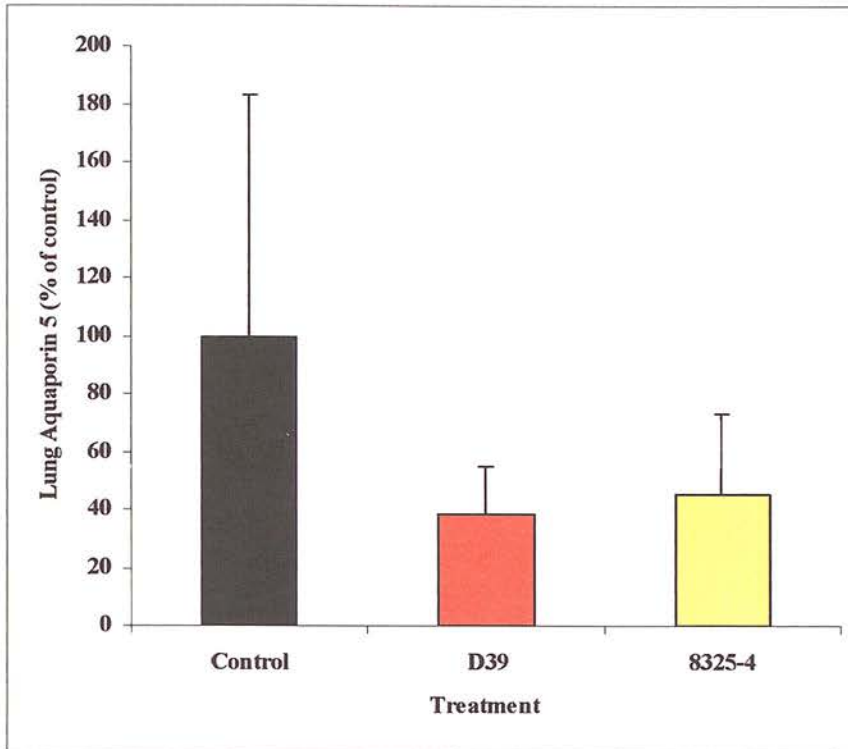


Figure 4.3.10 – Aquaporin 5 in lung tissue after 24 hours

Data expressed per mg lung protein and expressed as a percentage of control

Samples in each treatment group are taken from at least 2 separate experiments

Data expressed for D39 *S. pneumoniae* (red) is representative of that presented in Chapter 3 at 10^8 cfu (red).

S. pneumoniae and *S. aureus* infected animals were treated during different experiments.

Both 8325-4 *S. aureus* (yellow) and D39 *S. pneumoniae* (red) induced a similar non-significant decrease in Aquaporin 5 in lung tissue.

Control n = 3, D39 10^8 n = 3, 8325-4 10^8 n = 4

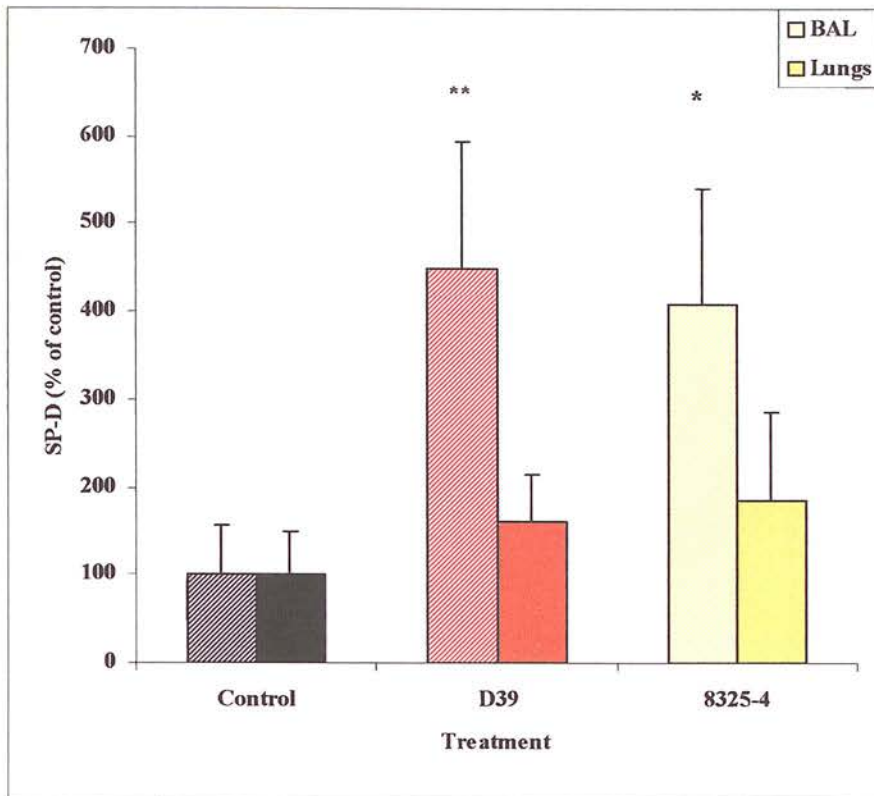


Figure 4.3.11– SP-D in BAL fluid and lung tissue after 24 hours

Data expressed per ml BAL fluid or per mg lung protein and presented as percentage of control

Samples in each treatment group are taken from at least 2 separate experiments

Data expressed for D39 *S. pneumoniae* (red) is representative of that presented in Chapter 3 at 10^8 cfu (red).

S. pneumoniae and *S. aureus* infected animals were treated during different experiments.

Both 8325-4 *S. aureus* (yellow) and D39 *S. pneumoniae* (red) induced a similar significant increase in SP-D in BAL fluid, and a non-significant increase in lung tissue. There were no differences between the two bacteria.

BAL:

ANOVA $p = 0.0043$

Tukey post-test	Control vs 8325-4 10^8 *	$p < 0.05$
	Control vs D39 10^8 **	$p < 0.01$

Control $n = 4$, D39 10^8 $n = 4$, 8325-4 10^8 $n = 4$

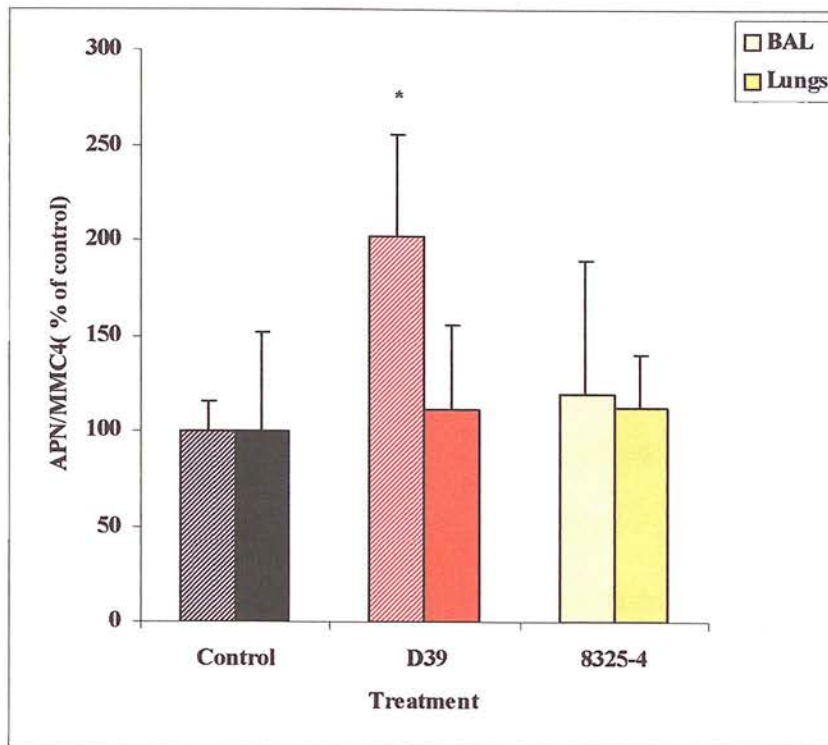


Figure 4.3.12– APN/MMC4 in BAL fluid and lung tissue after 24 hours

Data expressed per ml BAL fluid or per mg lung protein and presented as percentage of control

Samples in each treatment group are taken from at least 2 separate experiments

Data expressed for D39 *S. pneumoniae* (red) is representative of that presented in Chapter 3 at 10^8 cfu (red).

S. pneumoniae and *S. aureus* infected animals were treated during different experiments.

8325-4 *S. aureus* (yellow) did not induce an increase in APN/MMC4 in BAL fluid, unlike *S. pneumoniae* (ANOVA $p = 0.0162$; Tukey – Control vs D39 10^8 * $p < 0.05$).

There were no differences between bacteria or controls in lung tissue.

Control $n = 4$, D39 10^8 $n = 7$, 8325-4 10^8 $n = 4$

4.4 Summary and Conclusions

- Pneumolysin-deficient bacteria induced an equivalent lung infection to wildtype
- Pneumolysin-deficient bacteria induced an attenuated neutrophilia in the pleural space compared to wildtype
- Pneumolysin-deficient bacteria stimulated an attenuated response of pro-inflammatory cytokines compared to wildtype
- Pneumolysin-deficient bacteria induced an equivalent decrease in RTI40 and MMC6 expression compared to wildtype
- Pneumolysin-deficient bacteria demonstrated attenuated SP-D secretion compared to wildtype
- Pneumolysin-deficient bacteria induced an equivalent increase in APN/MMC4 in BAL fluid compared to wildtype
- *S. aureus* induced a similar inflammatory response to *S. pneumoniae* but demonstrated a more pronounced increase in inflammatory cytokines and air-blood barrier damage
- *S. aureus* did not induce a decrease in RTI40, but expression of MMC6, Aquaporin 5 and SP-D was equivalent to *S. pneumoniae*
- *S. aureus* did not induce an increase in APN/MMC4 in BAL fluid

The purpose of this series of experiments was to attempt to investigate the mechanism(s) by which pneumococcal pneumonia induces differential expression of alveolar epithelial cell-specific proteins. This was done by comparing the model of *S. pneumoniae*-induced pneumonia with rat models of lung infection induced by a pneumolysin-deficient mutant pneumococcus and *S. aureus*, another Gram-positive

bacterium. Section 4.2 (Pneumolysin) illustrated that in these rat models pneumolysin was not required for infection, inflammation or damage to the air-blood barrier, although attenuation of some aspects of inflammation, particularly production of pro-inflammatory cytokines, was observed. *S. aureus* stimulated a comparable lung infection to that induced by *S. pneumoniae* however there was greater damage to the air-blood barrier and stimulation of pro-inflammatory cytokines. Changes in expression of the RTI40 protein were not mediated by pneumolysin, but decreased expression of the protein was not observed in the staphylococcal infection, despite the increased levels of air-blood barrier damage with *S. aureus* and variations in pro-inflammatory cytokine production in both pneumolysin-deficient and staphylococcal models. This not only suggests that the decrease in RTI40 may be unique to the pneumococcus, but also that the effect is not likely to be mediated by the pro-inflammatory cytokines assessed here. An attenuated increase in SP-D secretion was observed in pneumolysin-deficient bacteria, whereas levels were similar in infection with both wildtype Gram-positive bacteria, again suggesting that this is not related to the degree of air-blood barrier damage or levels of the pro-inflammatory molecules studied. The difference in expression of APN/MMC4 protein in BAL fluid between the two Gram-positive models, despite an equivalent macrophage presence in the airways, suggests that this biochemical technique may be of some use as a preliminary screen of ATII and Clara cell injury in the streptococcal model, although it is acknowledged that this may also be due to differential effects on macrophages. This injury was not mediated by pneumolysin.

CHAPTER 5

5.1 INTRODUCTION

Chapters 3 and 4 established that pneumococcal pneumonia induces differential expression of ATI cell-specific proteins that is not due to a generic response to Gram-positive bacteria or mediated by pneumolysin. Increased production of ATII and Clara cell protein SP-D occurs in both Streptococcal and Staphylococcal pneumonia and is attenuated by pneumolysin. However, the mechanism behind these changes in protein expression is as yet unknown, and this suggested the idea that *S. pneumoniae* might be directly influencing expression of the proteins.

5.2 HYPOTHESIS 3

Differential protein expression in pneumococcal infection is due to direct interaction with bacteria or bacterial products

The main aim of this chapter was to set up an *in vitro* model of bacterial co-culture with cells expressing RTI40 and SP-D and to determine whether or not infection with *S. pneumoniae* induces protein regulation.

5.3 METHODS

In order to establish an *in vitro* model to test protein responses to *S. pneumoniae*, it was necessary to acquire rat cells that were able to express both RTI40 and SP-D. While isolated ATII cells do progressively demonstrate ATI characteristics such as expression of RTI40, (Chapter 1.4.2.1 RTI40), the logistics and validity of processing primary ATII cells at different stages of culture, combined with the technically challenging nature of the isolation procedure were prohibitive to their use in these studies. The rat alveolar epithelial cell line SV40-T2, was initially created to study

proliferation in ATII cells and indeed they maintain their characteristic lamellar bodies (Clement et al., 1991). However, SV40-T2 cells have also been shown to express RTI40 (McElroy et al., 2002; Rishi et al., 1995). This suggested that these cells might be of use in investigating bacterial effects on both RTI40 and SP-D. IL-1 α and MCP-1 were used in this *in vitro* model as a measure of the inflammatory response stimulated by the bacteria. For the co-culture experiments only the wildtype D39 *S. pneumoniae* was used. Experiments were carried out in T75 flasks containing approximately 10⁶ cells that were infected with 10⁷ (2.59x10⁷ \pm 3.37x10⁶) cfu of bacteria (M.O.I. 10). Preliminary time course experiments indicated that by 8 hours post-infection the plasma membrane of cells was compromised, so a 4 hour time point when cells were still viable was selected for study. As with Western blots, mRNA analysis was carried out with all samples run on the same gel, which were also assessed using a loading control, and analysed by densitometry (Chapter 2 Materials and Methods). As in Chapters 3 and 4, all graphs show data generated as a result of at least 2 separate experiments. Graphs show means with SD error bars. Asterisks illustrate differences between experimental data and controls unless stated otherwise. Analyses of specific proteins are presented as a percentage of the mean control value. As with Chapters 3 and 4, assay limitations of space necessitated the random selection of samples from 2 separate experiments for specific protein analysis.

5.4 RESULTS

5.4.1 Characterisation of SV40-T2 Cells

Pro-inflammatory IL-1 α and MCP-1 proteins (Figure 5.1a) were reproducibly expressed in SV40-T2 cell lysates. However in cell culture medium protein levels of MCP-1 were variable and IL-1 α was not detectable. Expression of IL-1 α (Figure 5.1b)

and MCP-1 (Figure 5.1c) mRNA was also detected in cells. The presence of RTI40 protein was demonstrated by Western blot (Figure 5.2a) and flow cytometry (Figure 5.2b), and mRNA was demonstrated by RT-PCR (Figure 5.2b). Similarly, SP-D protein was detected in cell lysates (Figure 5.3a), but was not found in culture medium at any point. SP-D mRNA was also detectable in cells (Figure 5.3b). These data show that SV40-T2 cells express all the molecules of interest.

5.4.2 Characterisation of *In Vitro* Bacterial Co-Culture System

The number of viable bacteria recovered from cell culture media had significantly decreased by 4 hours (Figure 5.4). As IL-1 α was not detected in control media, in order to quantify in some way the changes in expression following bacterial infection, the control mean was allocated 100 and data was presented as a percentage of this as was done previously (Figure 5.5b). The levels of pro-inflammatory cytokines IL-1 α mRNA (Figure 5.5c) and protein in both cells (Figure 5.5a) and cell culture medium (Figure 5.5b) increased following infection with D39. While levels of MCP-1 protein in cells increased significantly (Figure 5.6a), protein in culture media showed a pronounced decrease (Figure 5.6b), with no difference shown in mRNA (Figure 5.6c).

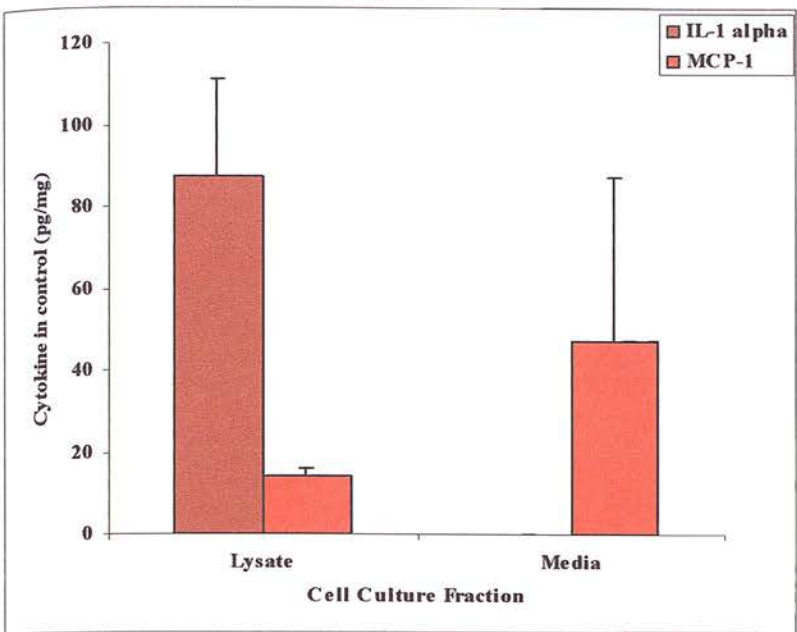
5.4.3 Differential Protein Expression

Assessment of RTI40 by FACS analysis (Figure 5.7a) showed a shift of 99% of events in the lower left quadrant (secondary antibody alone), to 89.67% in the upper right (primary antibodies) in control samples. *S. pneumoniae*-infected cells differed slightly in showing more auto-fluorescence (probably due to damaged cells) with 95.7% in the lower left quadrant (secondary antibody alone), shifting to 96.12% in the upper right (primary antibodies). While this small change alone is unlikely to be

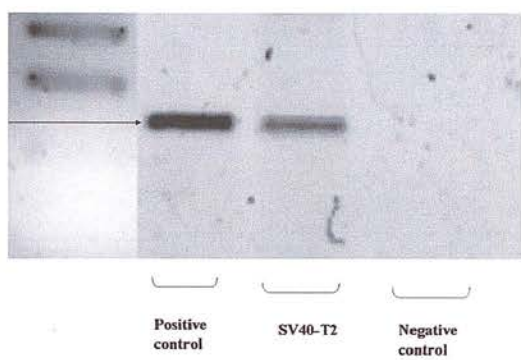
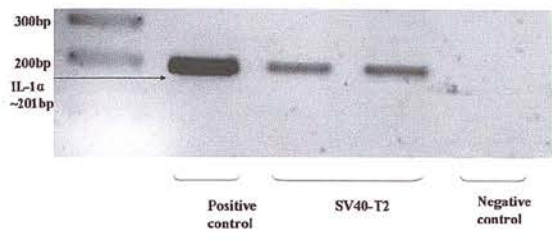
indicative of a reduction in RTI40 expression, the proportion of mean fluorescence intensity of ICAM:RTI40 was higher in controls (1:5) than infected samples (1:3). This may be indicative of a reduction in RTI40 expression, however it may also be due to differences in cell population due to cell damage as biochemical analysis of lysed cells showed no difference (Figure 5.7b). Expression of mRNA showed a significant increase after 4 hours (Figure 5.7c). In contrast, neither SP-D protein (Figure 5.8a) nor mRNA (Figure 5.8b) changed after incubation with D39. The *in vitro* results are summarised in Figure 5.9.

5.4.4 Distribution of RTI40 Expression *In Vivo*

As well as using *in vitro* bacterial co-culture to determine direct bacterial effects on expression of RTI40, this question could also be further investigated by going back to the *in vivo* situation where it was first observed. This was done by examining the distribution of RTI40 staining on different areas of infected lung tissue. Instillation of *S. pneumoniae* usually instigated a lobar pneumonia, so inflamed lung tissue was separated from unaffected tissue and immunofluorescence staining of RTI40 was compared between the two. Figure 5.10 shows that after 72 hours non-inflamed areas of infected lungs (C) demonstrated a similar loss of RTI40 protein expression to that of inflamed areas (B), despite lung architecture being microscopically comparable to control lungs (A).



A



B

C

Figure 5.1– Expression of IL-1 α and MCP-1 in control SV40-T2 cells

A. IL-1 α and MCP-1 protein in cell lysates and media

Data expressed as pg/mg of protein

Samples taken from at least 2 separate experiments

Both IL-1 α and MCP-1 were expressed in cell lysates, although

IL-1 α was undetectable in media and MCP-1 very variable

n = 4

B. RT-PCR data showing IL-1 α mRNA in cells

Images representative of at least 2 separate experiments

Image shows a band at 201bp in SV40-T2 cells

Positive control is rat lung tissue

C. RT-PCR data showing MCP-1 mRNA in cells

Images representative of at least 2 separate experimant

Image shows a band at 167bp in SV40-T2 cells

Positive control is rat lung tissue

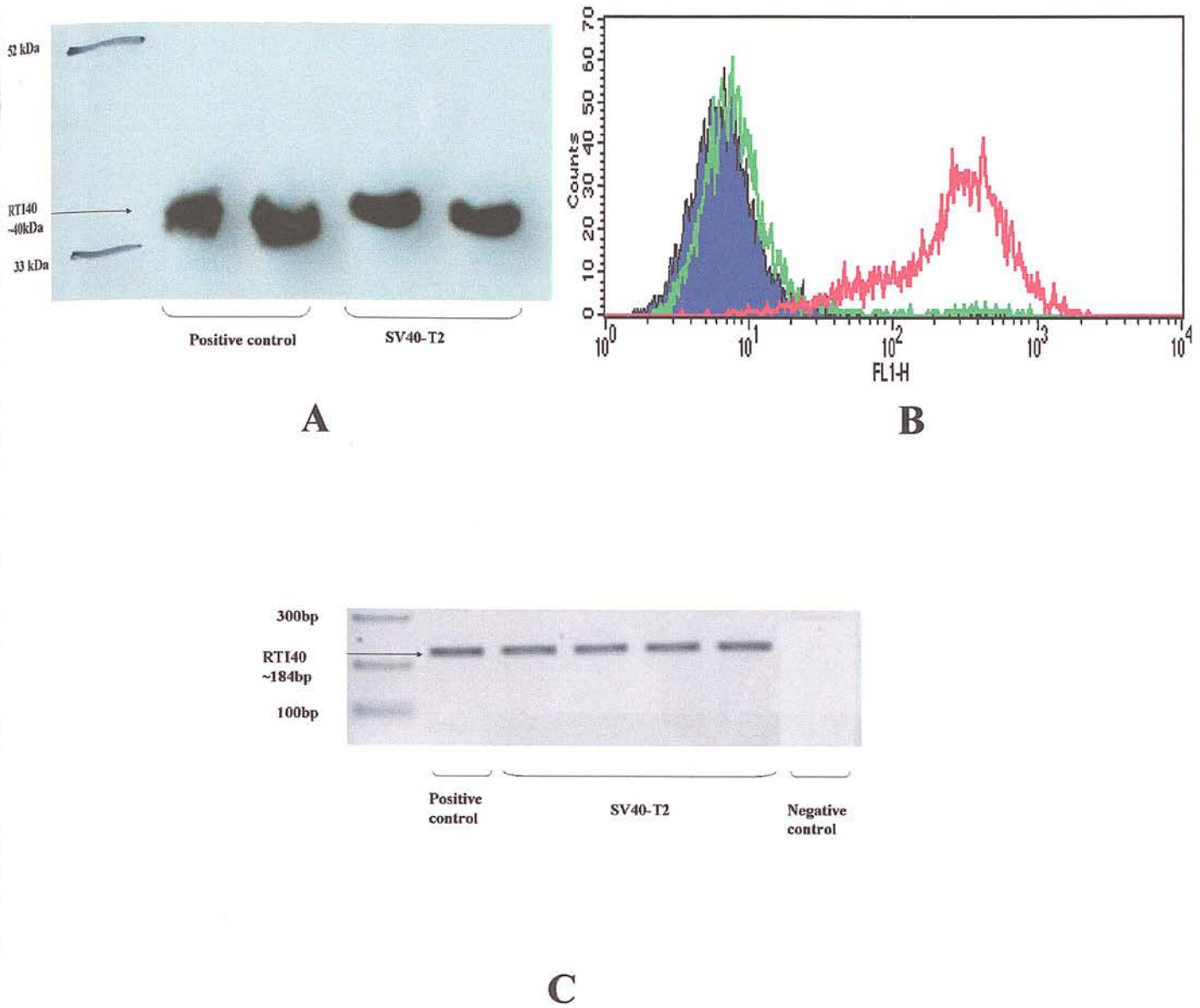


Figure 5.2 – Expression of RTI40 in control SV40-T2 cells

Images representative of at least 2 separate experimentants

A. Western blot showing RTI40 protein in cell lysates

Blot shows a band at 40kDa in SV40-T2 cell lysates.

Positive control was rat lung tissue.

B. FACS data showing RTI40 protein on cell surface

Graph shows unstained cells (purple), overlaid with

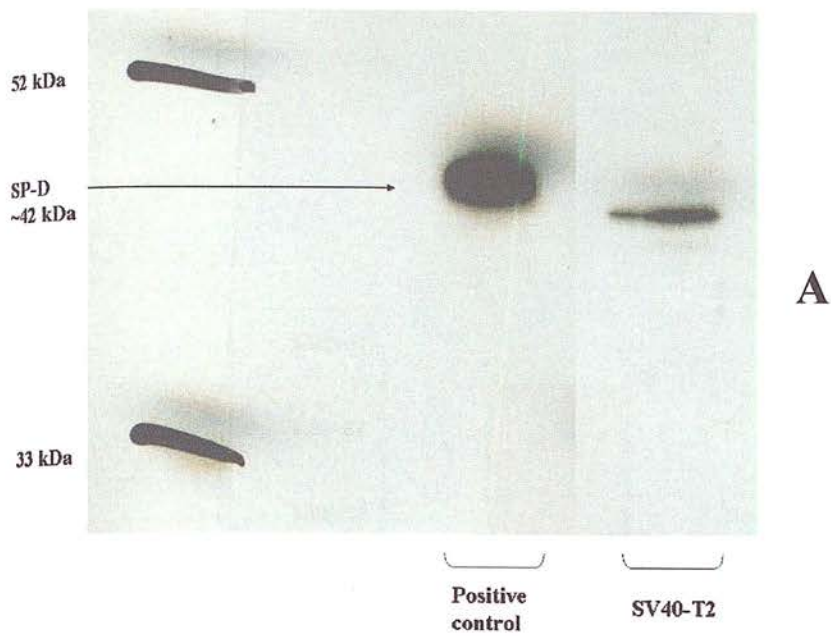
secondary antibody only (green) and RTI40 antibody (pink)

Graph shows a fluorescence shift with RTI40 stained cells only

C. RT-PCR data showing RTI40 mRNA in cells

Image shows a band at ~184bp in SV40-T2 cell lysates.

Positive control was rat lung tissue



B

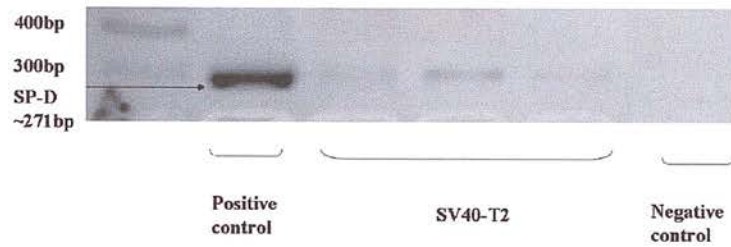


Figure 5.3 – Expression of SP-D in control SV40-T2 cells

Images representative of at least 2 separate experiment

A. Western blot showing SP-D protein in cell lysates

Blot shows a band at 42 kDa in SV40-T2 cell lysates

Positive control is rat lung tissue

B. RT-PCR data showing SP-D mRNA in cells

Image shows a band at 271bp in SV40-T2 cells

Positive control is rat lung tissue

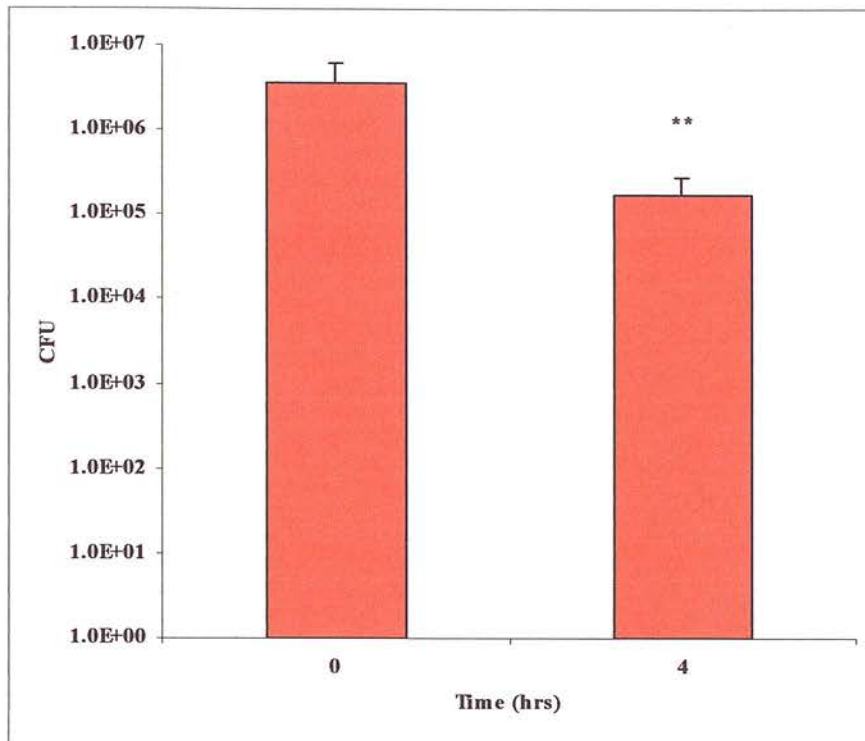
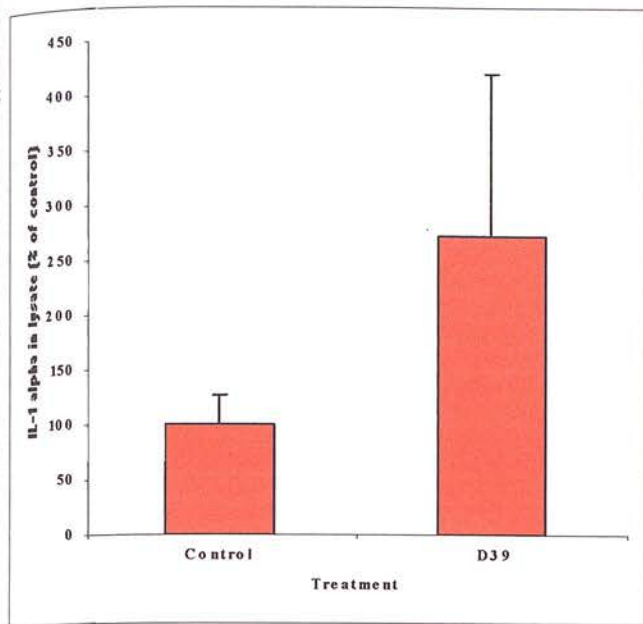


Figure 5.4 – CFU recovered from *S. pneumoniae*-infected cell culture media
Data expressed as cfu/ml of cell culture media shown on a log scale
Samples in each treatment group are taken from at least 2 separate
experiments

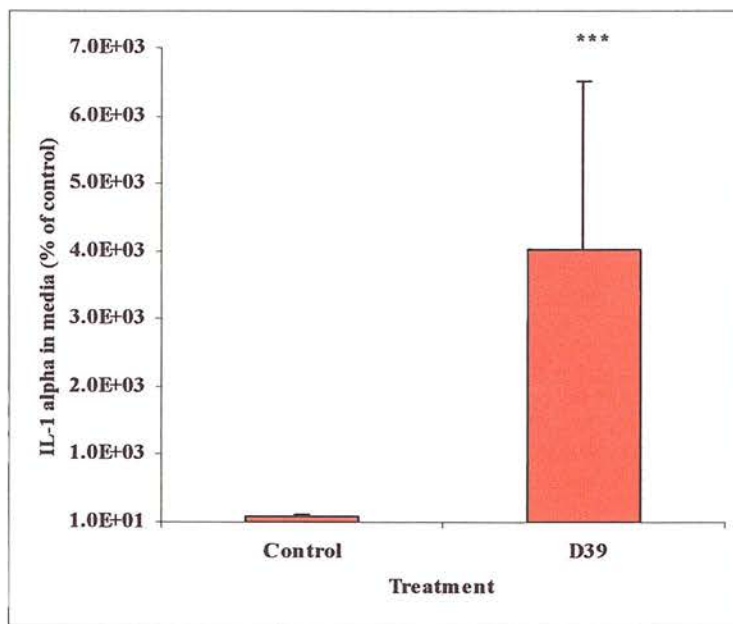
Numbers of *S. pneumoniae* show a significant decrease in cell culture media between 0 and 4 hours

Transformed (Log) Unpaired t-test $p = 0.0023$

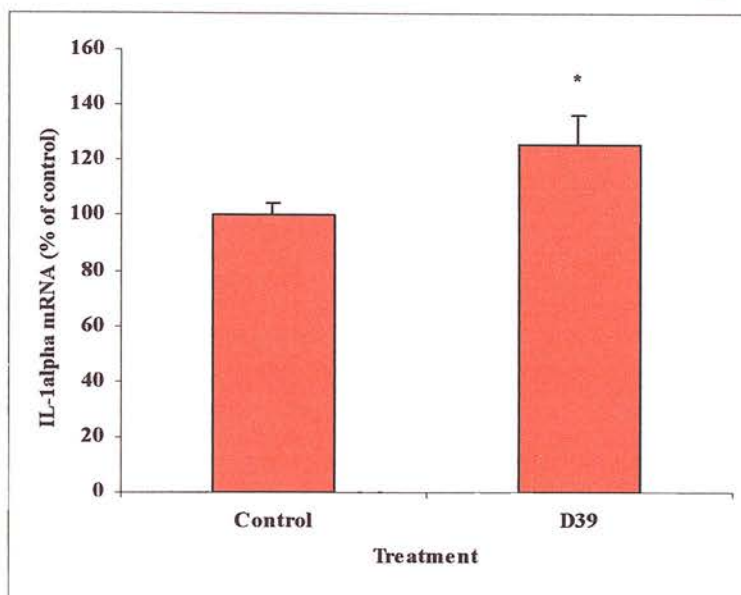
0 hrs $n = 4$, 4 hrs $n = 4$



A



B



C

Figure 5.5 – IL-1 α expression after 4 hours

Samples in each treatment group are taken from at least 2 separate experiments

Data presented as a percentage of control

A. Expression of IL-1 α protein in cell lysates

Data expressed per mg of protein

S. pneumoniae induced an increase in IL-1 α protein after 4 hours which was not quite significant

(Transformed (Log) Unpaired t-test $p = 0.06$)

Control $n = 4$, D39 4 hrs $n = 4$

B. Expression of IL-1 α protein in cell culture media

Data expressed per mg of protein

S. pneumoniae induced a significant increase in IL-1 α protein in cell culture media after 4 hours

Transformed (Log) Unpaired t-test *** $p = 0.0002$

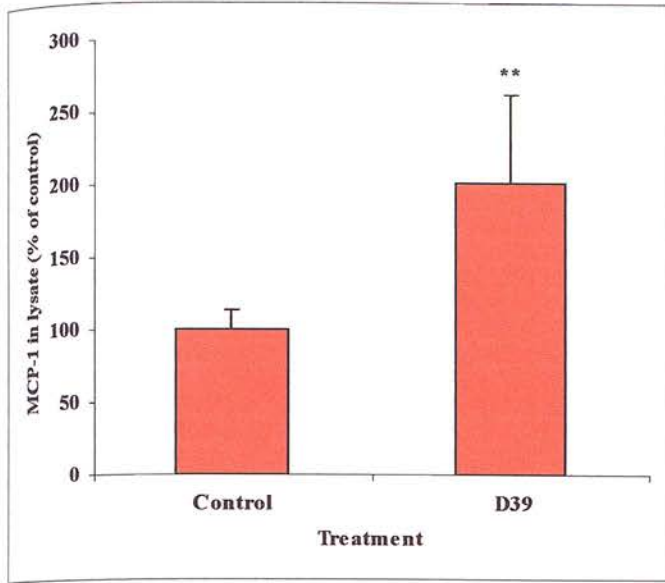
Control $n = 3$, D39 4 hours $n = 5$

C. Expression of IL-1 α mRNA in cell layer

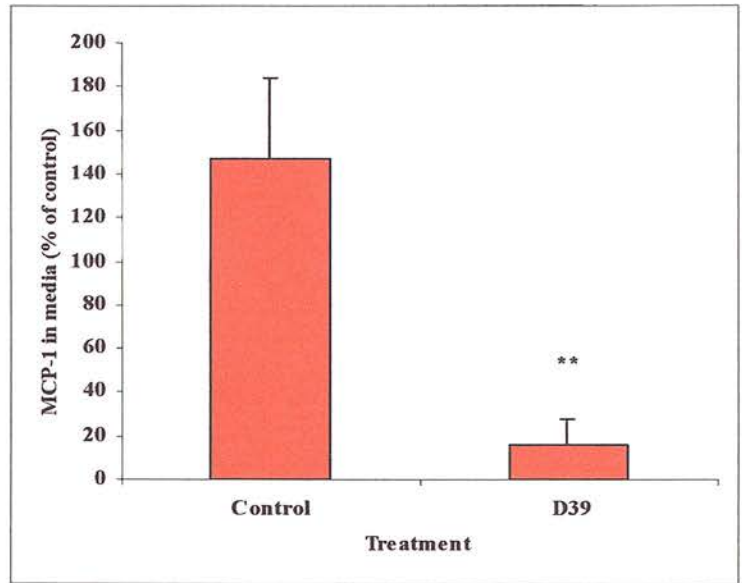
S. pneumoniae induced an increase in IL-1 α mRNA in the cell layer after 4 hours

Unpaired t-test * $p = 0.0174$

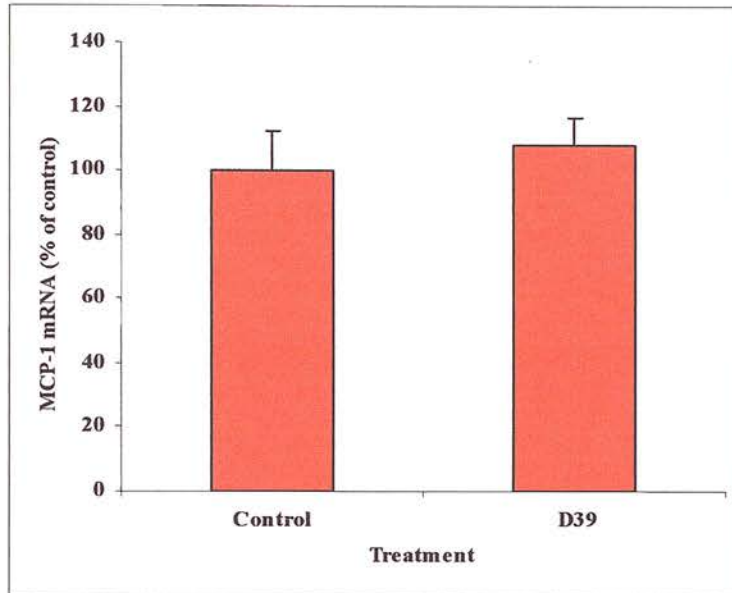
Control $n = 3$, D39 4 hours $n = 3$, D39 8 hours $n = 3$



A



B



C

Figure 5.6 – MCP-1 expression after 4 hours

Data expressed as percentage of control

Samples in each treatment group are taken from at least 2 separate experiments

A. Expression of MCP-1 protein in cell lysates

Data expressed per mg of protein

S. pneumoniae induced a significant increase in MCP-1 protein in cell lysates after 4 hours

Transformed (Log) Unpaired t-test ** p = 0.003

Control n = 4, D39 n = 4

B. Expression of MCP-1 protein in cell culture media

Data expressed per mg of protein

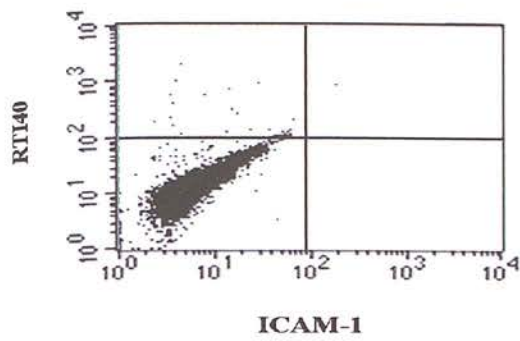
S. pneumoniae induced a significant decrease in MCP-1 protein in cell culture media after 4 hours

Unpaired t-test ** p = 0.002

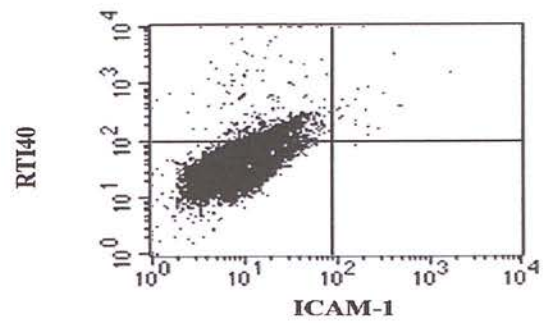
A. Expression of MCP-1 mRNA in cell layer

S. pneumoniae induced no change in MCP-1 mRNA in the cell layer

Control n = 3, D39 4 hours n = 3, D39 8 hours n = 3

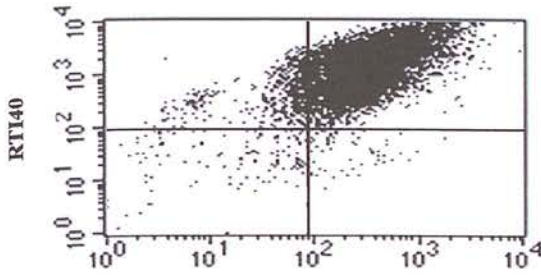


Secondary
antibody
only

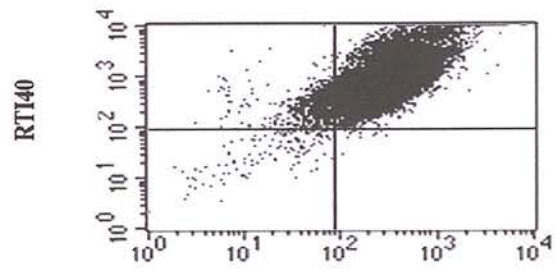


A

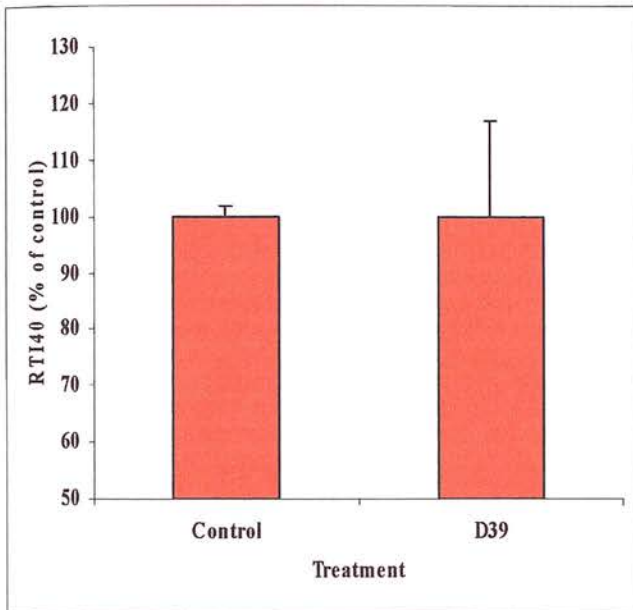
RTI40/
ICAM



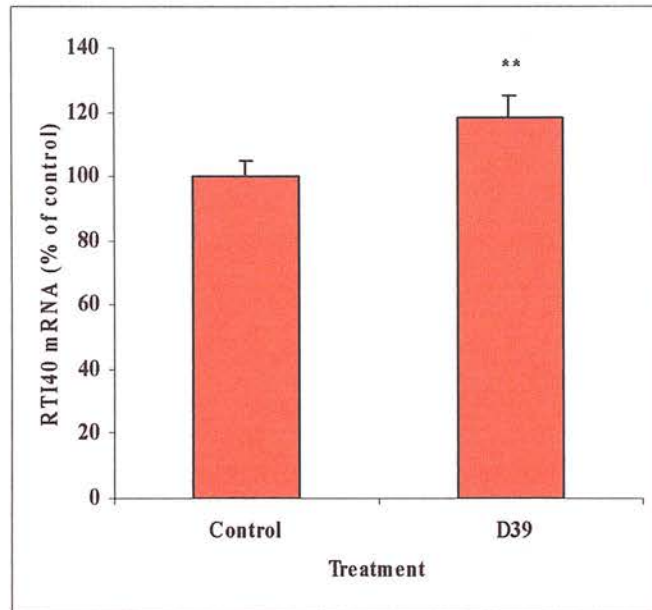
Control



D39



B



C

Figure 5.7 – RTI40 expression after 4 hours

A. FACS data showing RTI40 and ICAM-1 protein on cell surface

Figure representative of 2 separate experiments shows cells stained with secondary antibody only (upper graph) and RTI40/ICAM-1 (lower graph).

Graphs show consistent expression of both RTI40 and ICAM-1 in both control and D39-infected cells
Control n = 3, *S. pneumoniae* 4 hours n = 3. *S. pneumoniae* 8 hours n = 3

B. Expression of RTI40 protein in cell lysates

Data expressed per mg of protein and presented as a percentage of control

S. pneumoniae did not induce any change in expression of RTI40 protein in cell lysates after 4 hours

Control n = 5, D39 n = 3

C. Expression of RTI40 mRNA in cell layer

Data expressed as percentage of control

S. pneumoniae induced a significant increase in RTI40 mRNA in the cell layer after 4 hours

Unpaired t-test ** p = 0.003

Control n = 4, D39 n = 4

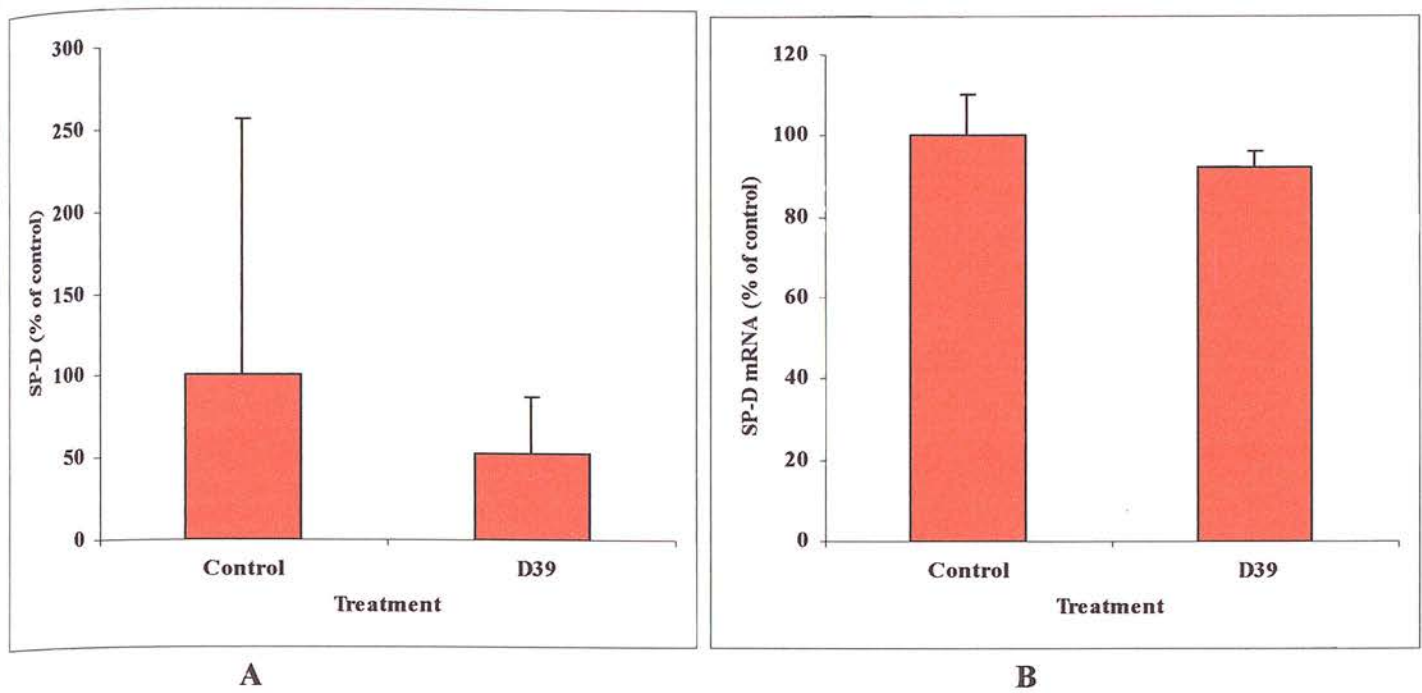


Figure 5.8 – SP-D expression after 4 hours

Data presented as a percentage of control

Samples in each treatment group are taken from at least 2 separate experiments

A. Expression of SP-D protein in cell lysates

Data expressed per mg of protein

S. pneumoniae induced no change in SP-D protein in cell lysates after 4 hours

Control n = 4, D39 n = 3

B. Expression of SP-D mRNA in cell layer

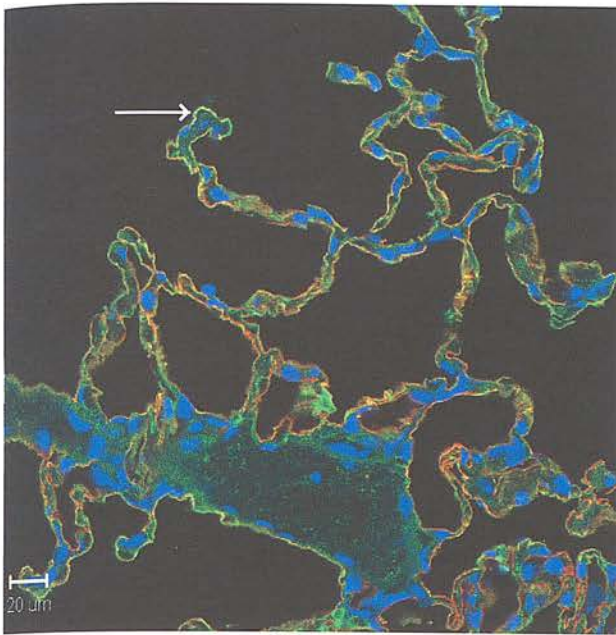
S. pneumoniae induced no change in SP-D mRNA in the cell layer after 4 hours

Control n = 3, D39 4 hours n = 4

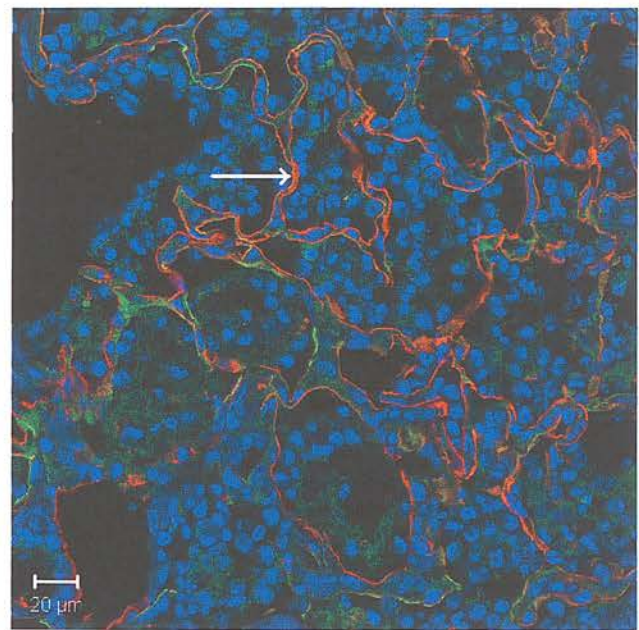
Figure 5.9 – *In vitro* summary table

Table summarises expression of protein and mRNA with and without *S. pneumoniae*

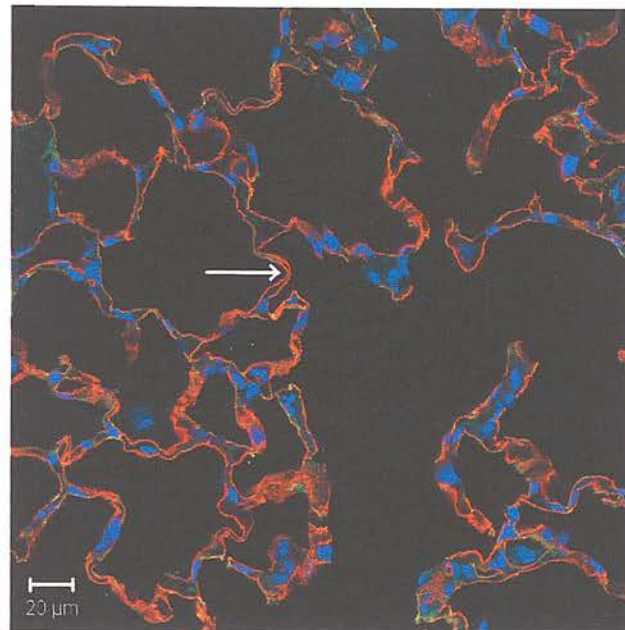
Gene	Control		<i>S. pneumoniae</i>	
	Protein	mRNA	Protein	mRNA
IL-1α	+ve (cell layer + media) (biochem)	+ve (cell layer) (RT-PCR)	Increase (cell layer + media) (biochem)	Increase (cell layer) (RT-PCR)
MCP-1	+ve (cell layer and media) (biochem)	+ve (cell layer) (RT-PCR)	Increase (cell layer) Decrease (media) (biochem)	Increase (cell layer) (RT-PCR)
RTI40 (ATI)	+ve (cell layer) (biochem, flow, immuno)	+ve (cell layer) (RT-PCR)	No change (cell layer) (flow, biochem)	Increase (cell layer) (RT-PCR)
SP-D (ATII/Clara)	+ve (cell layer) (biochem)	+ve (cell layer) (RT-PCR)	No change (cell layer) (biochem)	No change (cell layer) (RT-PCR)



A



B



C

Figure 5.10 – Immunofluorescence staining of rat lung at 72 hours
Sections stained with RTI40 (green) and MMC6 (red) with nuclear stain
To-Pro-3 (blue)

A. Control

Arrow shows both RTI40 and MMC6 co-expressed on the surface of ATI cells

B. *S. pneumoniae* – Inflamed area of lungs

Arrow shows areas of inflamed alveolar epithelium stained with MMC6 only.

C. *S. pneumoniae* – Non-inflamed area of lungs

Arrow shows areas of non-inflamed alveolar epithelium stained with MMC6 only

5.5 Summary and Conclusions

- SV40-T2 cells express IL-1 α , MCP-1, RTI40 and SP-D protein and mRNA *in vitro*
- Co-cultured *S. pneumoniae* did not actively grow in cell culture medium *in vitro*
- *S. pneumoniae* induced an overall increase in production of IL-1 α and MCP-1 *in vitro*
- *S. pneumoniae* did not change RTI40 protein expression but mRNA was increased *in vitro*
- *S. pneumoniae* did not affect expression of SP-D protein or mRNA *in vitro*
- *In vivo* the decrease in RTI40 protein in *S. pneumoniae* infection was not limited to areas of inflammation

The purpose of this chapter was to determine if the changes in cell-specific proteins in pneumococcal pneumonia was due to a direct effect of bacteria or bacterial products. An *in vitro* system of bacterial co-culture was set up using a rat alveolar epithelial cell line SV40-T2 that was shown to express RTI40 and SP-D as well as pro-inflammatory molecules IL-1 α and MCP-1. *S. pneumoniae* (which did not actively grow in media, representing the situation *in vivo*) induced an overall increase in cytokine production, but initiated active secretion of IL-1 α , while either inhibiting secretion of, or cleaving, MCP-1. However, these data do suggest that incubation of SV40-T2 cells with *S. pneumoniae* results in an inflammatory response by the cells. RTI40 showed no change in protein expression biochemically, although there is some evidence of a reduction by FACS analysis, although levels of mRNA were shown to increase, suggesting that while the decrease in protein observed *in vivo* was not

reproduced, the bacteria did demonstrate a direct effect *in vitro*. Expression of SP-D did not change at all after infection with *S. pneumoniae*. These data suggest that changes in the protein expression observed in pneumococcal pneumonia require additional stimuli than just bacteria and protein. However this should be considered within the confines of the *in vitro* system. Limitations of both co-culture incubation time due to monolayer damage, and peculiarities of cell line protein expression such as the lack of SP-D secretion will likely have affected the results obtained. Returning to the *in vivo* observation, immunofluorescence analysis of infected lungs showed that the decrease in RTI40 protein expression was not restricted to areas showing the most response to bacterial infection, supporting the *in vitro* data in concluding that RTI40 protein modulation is not a result of direct interaction between protein and bacteria.

CHAPTER 6: DISCUSSION

6.1 SUMMARY OF RESULTS

Experiments presented in Chapters 3-5 of this thesis have demonstrated the following novel findings.

Pneumococcal pneumonia: A recovering rat model of pneumococcal pneumonia showed differential expression of ATI and ATII cell-specific proteins. A pronounced decrease in RTI40 expression on ATI cells was observed compared to MMC6 and Aquaporin 5, in the absence of visible damage to ATI cells. Synthesis and secretion of SP-D by ATII cells and/or Clara cells was increased in contrast to expression of Pro-SP-C.

Pneumolysin-deficient pneumococcal pneumonia: Pneumolysin-deficient pneumococcal pneumonia induced a similar lung infection to the wildtype organism although inflammation was attenuated in some aspects. Pneumolysin did not mediate the decrease in RTI40 or increase in SP-D.

Staphylococcal pneumonia: *S. aureus*-induced lung infection showed similar levels of lung inflammation to *S. pneumoniae*, but demonstrated an increase in pro-inflammatory cytokine response and injury to the air-blood barrier. SP-D was increased, but in contrast to *S. pneumoniae* there was no decrease in RTI40.

Injury to ATII cells: Targeted injury of ATII cells was identified in the pneumococcal model and novel methods of quantification using cell-specific proteins were investigated. An immunofluorescence technique was shown to be effective, but biochemical Pro-SP-C expression was not found to be a reliable marker of ATII cells. Measurement of APN/MMC4 in BAL fluid was shown to be representative of ATII cell injury in the pneumococcal model. This injury was not apparent in staphylococcal infection and was not mediated by pneumolysin.

Mechanism of protein regulation: An *in vitro* model of *S. pneumoniae* infection of alveolar epithelial cells showed that expression of RTI40 and SP-D protein was not directly mediated by bacteria, or bacterial products. This was further supported *in vivo* for RTI40 by the absence of expression in non-inflamed areas of infected lung.

6.2 DISCUSSION

The purpose of this thesis was to test the following hypotheses:

1. Pneumococcal pneumonia induces differential damage to ATI and ATII cells

2.1 Pneumococcal virulence factor pneumolysin mediates differential protein expression

2.2 Differential protein expression in pneumococcal pneumonia is due to host response to Gram-positive bacteria

3. Differential protein expression in pneumococcal infection is due to direct interaction with bacteria or bacterial products

Detection of an increase in RTI40 protein in BAL fluid is a well-established technique for identifying targeted damage to rat ATI cells (Franklin, 2006; McElroy et al., 1995). In this thesis a pronounced decrease in RTI40 in both BAL fluid and lung tissue was detected in a model of pneumococcal pneumonia. This is the first time that such regulation of RTI40 expression has been identified independent of morphological ATI cell injury. Even in a highly injurious, ventilated model of *P. aeruginosa*-induced lung infection, levels of RTI40 showed a huge (80-fold) increase in BAL fluid as opposed to a decrease (McElroy et al., 1995). The only previous studies that demonstrated a decrease in RTI40 were in lung tissue. In a model of staphylococcal pneumonia this was interpreted as a measure of shift to a more ATII cell phenotype during lung repair (Tyrrell et al., 2005). A rat model of bleomycin-induced lung fibrosis also showed a decrease in RTI40 expression in lung tissue, associated with an increase in BAL fluid indicating ATI cell injury (Koslowski et al., 1998). Although morphological evidence of ATI cell injury was not identified in the pneumococcal model, the contrast in expression between RTI40 and other ATI

cell-specific proteins, MMC6 and Aquaporin 5, suggests that *S. pneumoniae* is affecting ATI cells in some way. Nevertheless, these results do indicate that the established interpretation of RTI40 in BAL fluid as a marker of ATI cell damage does not apply in this model. This result highlights the importance of using an array of ATI-cell specific proteins in this type of analysis, particularly when the function and regulatory mediators of the protein are unknown. The fact that the decrease is not observed in another model of Gram-positive bacterial lung infection, mediated by pneumolysin or occurring as a result of direct bacterial effects, while inconclusive, is consistent with the idea that it is induced by a specific host response to pneumococcal infection. However, the increased expression of pro-inflammatory cytokines stimulated by *S. aureus* compared to *S. pneumoniae*, and a similar induction of SP-D secretion combined with an absence of effect on RTI40, indicates a process that is likely to be complex. A host of inflammatory and anti-microbial molecules produced and regulated in response to the pneumococcus, or interactions between them, could be responsible for these results. Similarly, the mechanism by which the protein is decreased has yet to be established. Despite the fact that the function of RTI40 in the adult lung is unknown, its extensive expression on normal lung epithelium suggests an important role. Indeed, expression of the protein is vital to normal lung development (Ramirez et al., 2003). The indication that the decrease in RTI40 expression is reversible, as demonstrated by an increase in protein after 72 hours, combined with the full resolution of disease over time suggests that decrease in protein expression does not appear to adversely affect disease progression, and may imply a lack of protein detection rather than active down regulation. The demonstration of decreased RTI40 using two different antibodies that are thought to bind to different epitopes (Williams et al., 1996) suggests that if this is the case, the

conformational change induced in RTI40 must be considerable. There is a possibility that the post-translational modification of RTI40 could be involved in this, however given that the same effect was observed using antibodies that are thought to bind to both the core protein and post-translational modification, this is likely to be a complex procedure.

Like RTI40, expression of MMC6 in the pneumococcal model has been difficult to interpret. Maintenance of an increase in MMC6 in BAL fluid by 72 hours may be explained by three possible theories: continued damage to ATI cells in the resolution phase; protein shedding into the airway stimulated by bacterial infection; or some combination of the two. As mentioned earlier, an absence of visible damage to epithelial cells does not necessarily preclude injurious effect. In a severe model of *P. aeruginosa*-induced pneumonia, it was noted that an increase in ATI cell-specific protein in BAL fluid was detected 2 hours post-infection, before evidence of morphological damage (McElroy et al., 1995). While this might explain the MMC6 data at 24 hours, it does not rationalise further injury during resolution. To this end, it is worthy of note that of all parameters measured, the only other proteins that demonstrated an up regulation of expression at both 24 and 72 hours are those involved in innate immunity, namely pro-inflammatory IL-1 α and MCP-1, and the surfactant collectin SP-D. So little is known about the function of MMC6 that it is tempting to speculate the protein might therefore have some role to play in the innate immune system. CD14, an important PRR for the pneumococcus (Paterson and Mitchell, 2006) and other bacterial pathogens (Martin and Frevert, 2005), exists in two forms, soluble and membrane-bound, the soluble form being shed from the cell membrane (Martin and Frevert, 2005). Although MMC6 is restricted to ATI cells and

there is no evidence of protein shedding in the normal lung, it is possible that under certain pathological conditions this is activated, perhaps as some form of specific defence for the alveolar epithelium's most vulnerable cells. The increase in MMC6 in BAL fluid in both *S. pneumoniae* and *S. aureus*-induced lung infections suggests that this could be stimulated by factors in common to Gram-positive bacteria such as PG, LTA and teichoic acids.

The identification of differential protein expression in this model of pneumonia has demonstrated the flaws of relying on one assessment parameter when using cell-specific proteins to quantify injury, and suggests that an array of markers be used to provide more reliable results.

SP-D is an important molecule in the innate immune system of the lung, and aids lung clearance by binding to a variety of different microbial pathogens (Giannoni et al., 2006; Griese and Starosta, 2005; Hartshorn et al., 1998; Restrepo et al., 1999), including the pneumococcus (Hartshorn et al., 1998; Jounblat et al., 2004; Jounblat et al., 2005). Although it has been used as a marker of surfactant production in a number of human diseases (Hartl and Griese, 2006; Kuroki et al., 1998; Postle et al., 1999), and the effects of specific lung pathogens in SP-D-deficient mice has been investigated (Giannoni et al., 2006; Jounblat et al., 2005; LeVine et al., 2000) few studies have been carried out on protein expression responses *in vivo*. A study of lung infection in pigs showed an increase in SP-D immunoreactivity in bronchopneumonia induced by *S. aureus* and Gram-negative *Actinobacillus pleuropneumoniae* (Soerensen et al., 2005). This thesis has presented for the first time a quantified increase in secretion and production of SP-D in response to two Gram-positive lung

pathogens, *S. aureus* and *S. pneumoniae*. This supports the pig data, and suggests stimulation of SP-D in response to a wide range of bacterial pathogens. Modulation of SP-D was not shown to be as a result of direct stimulation by *S. pneumoniae* or associated products, as demonstrated by *in vitro* data. This implies that while direct interaction between SP-D and pathogen does occur, regulation of protein expression is modulated in an indirect manner, perhaps by some interaction with host defence mechanisms. Unlike RTI40 however, the similar increase of SP-D expression induced by both Gram-positive species suggests that this could be initiated by pro-inflammatory molecules, such as IL-1 α and MCP-1, which are both up regulated in the lung in response to *S. aureus* and *S. pneumoniae*.

This thesis has presented, for the first time, evidence of targeted injury to ATII cells in a clinically relevant model of pneumococcal pneumonia. There is evidence of *S. pneumoniae* adhesion to alveolar epithelial cells, particularly ATII cells (Cundell and Tuomanen, 1994). Adhesions and adhesion-mediators are important virulence factors for the pneumococcus (Jedrzejewski, 2001; Mitchell et al., 1997) and adhesion has been shown to facilitate internalisation of other Gram-positive bacteria into host cells (Dziewanowska et al., 1999; McElroy et al., 2002). Thus preferential attraction of *S. pneumoniae* to ATII cells may facilitate cell damage, perhaps by stimulation of internalisation. This thesis has also demonstrated a variety of methods to quantify this damage, of which there were previously no well-established techniques. The use of cell-specific proteins has been fundamental to this. Difficulties in biochemical analysis of membrane-associated molecules, such as RTII70, suggested the value of employing an immunofluorescence-based quantification technique, which was able to quantify ATII cell-specific injury in this model. Biochemical investigation of ATII

cytoplasmic Pro-SP-C proved inconsistent with immunofluorescence data while an increase in SP-D expression indicated that injury to ATII cells did not adversely affect global production of surfactant protein. Assessment of APN/MMC4 demonstrated that, despite the protein's expression on the surface of macrophages, increased levels in BAL fluid during the acute phase of disease was likely to be representative of damage to ATII cells. Clearly, this expression by macrophages would mean that this would not be a definitive technique by which ATII and Clara cell injury could be quantified; however it might prove useful as a quick and easy preliminary screen to filter out models of lung infection worthy of further investigation using the immunofluorescence method, which is considerably more time consuming.

In the presented model of pneumococcal infection bacteria did not actively grow either *in vivo* or *in vitro*. Despite this there is evidence of injury to both lungs and cultured cells. This is representative of recovering pneumonia where bacteria are cleared from the lungs rather than being disseminated and resulting in a more systemic infection. Other groups have used rat models of pneumococcal infection. After 18 hours Boe *et al* showed recovery of approximately 1×10^5 cfu of bacteria from lung tissue, in contrast to the 1×10^4 recovered here after 24 hours (Boe et al., 2001). This may have been due to the difference in time point assessed, as their study showed a progressive decrease in viable bacteria between 6 and 18 hours, however over the course of 8 days *S. pneumoniae*-infected animals showed low mortality as did the model presented here, and it is possible that assessment of an earlier time point might have shown an increase in bacterial numbers followed by clearing by 24 hours. An additional control to confirm instillation dose by harvest of freshly infected lungs could also have been done, as could additional tests to confirm the identity of the

bacteria such as Gram-stain and optochin sensitivity. This could also apply to stocks of *S. aureus* by carrying out catalase tests. A lack of bacterial growth *in vitro* however, was surprising. This may have been due in part to the previous culture of epithelial cells in media supplemented with antibiotics at a bacteria-static level. This was carried out due to persistent infections of cultures. Despite the fact that cells were washing thoroughly and cultured in antibiotic-free media during bacterial infection, it is possible that this may have affected pneumococcal growth *in vitro*. However, preliminary studies comparing bacterial growth with cells cultured in antibiotic versus antibiotic-free media suggested that this did not have an effect. Other alternative explanations may be that bacteria were harvested at an earlier growth phase might have been better able to grow in media, although the presence of viable bacteria in culture and injury to epithelial cells suggests that pneumococci were still able to function.

Pneumolysin is an important virulence factor of *S. pneumoniae* and pneumonia induced by pneumolysin-deficient bacteria has been shown to demonstrate decreased bacterial growth, damage to the air-blood barrier and inflammation (Berry et al., 1989; Kadioglu et al., 2000; Rubins et al., 1995). The pneumonia model presented in this thesis, however, showed no difference in bacterial recovery, inflammation or damage to the air-blood barrier compared to the wildtype, although some attenuation in expression of innate immune mediating proteins was observed. While it was demonstrated that the wildtype bacteria did indeed express the protein and the deficient mutant did not, this was established under laboratory conditions and was not tested in lung tissue *in vivo*. This, combined with the unexpectedly similar results from the two pneumonia models suggests two possible explanations. It is possible that

the mutant strain reverted to wildtype *in vivo*; however, this is unlikely since these strains have been used in a number of other *in vivo* studies demonstrating phenotypic differences (Benton et al., 1997; Berry et al., 1989; Kadioglu et al., 2000; Rubins et al., 1995). A more plausible explanation might be that the wildtype strain was not producing significant amounts of pneumolysin *in vivo*. Previous studies illustrating the importance of pneumolysin as a virulence factor have been in non-recovering mouse models (Benton et al., 1997; Berry et al., 1989; Kadioglu et al., 2000; Rubins et al., 1995). This suggests that the recovering nature of the pneumonia studied in this thesis is fundamental to the differences observed. Pneumolysin is an intracellular toxin, released only on autolysis during stationary phase (Jedrzejewski, 2001; Rubins and Janoff, 1998); however, in this model most of the bacteria were cleared from the lungs by 24 hours, suggesting a lack of growth in the respiratory tract. If bacteria were unable to reach stationary phase they would be unable to release the toxin, although clearly some basal levels of autolysis will have occurred. The complicated cascade by which inflammatory molecules such as IL-1 α , MCP-1 and SP-D are stimulated may have been slightly influenced by this small amount of pneumolysin release in a way that other measures of inflammation and tissue injury were not, perhaps by the interaction of pneumolysin with TLR-4 (Kadioglu and Andrew, 2004; Paterson and Mitchell, 2006). These results suggest that pneumolysin may be less important as a virulence factor in recovering pneumonia, perhaps due to the inability of the bacteria to release pneumolysin in any significant quantities.

Figure 6.1 shows a schematic of mechanisms by which pneumococcal pneumonia might mediate alveolar epithelial cell injury and protein expression.

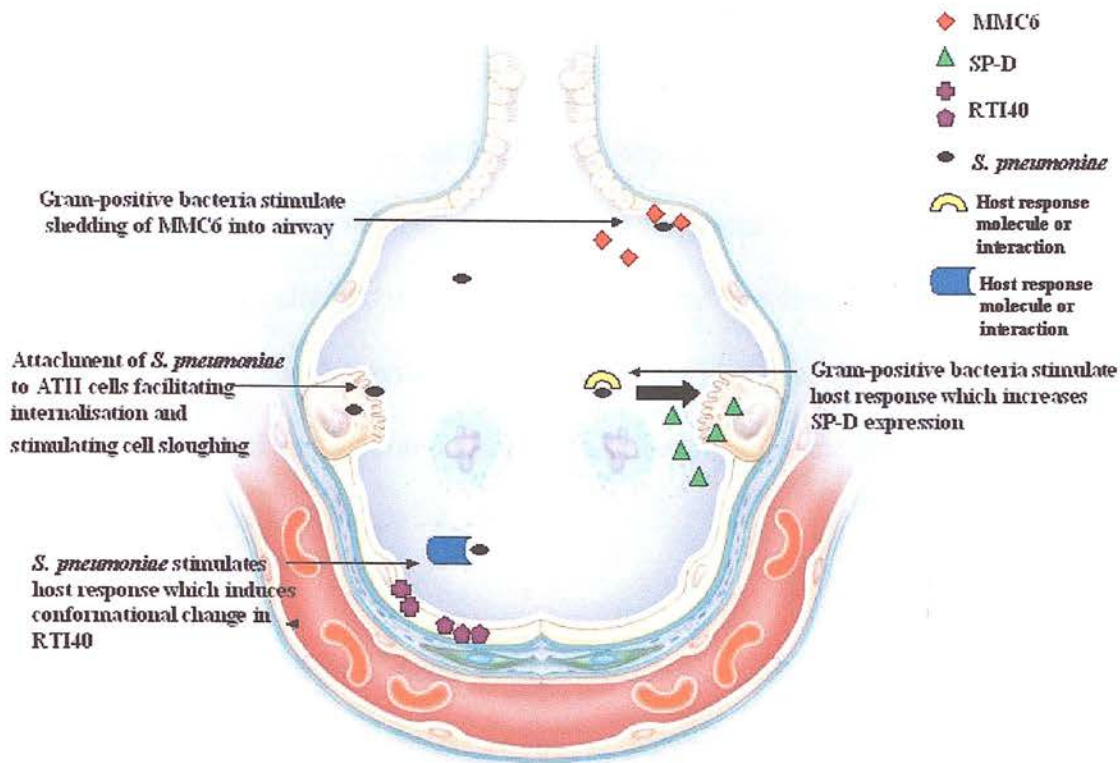


Figure 6.1 Possible mechanisms of *S. pneumoniae* mediation of alveolar epithelial cell injury and protein expression. *S. pneumoniae* stimulates sloughing of ATI cells by preferential attachment and internalisation. A combination of host response factors specific to the pneumococcus induces conformational change in RTI40 protein on the surface of ATI cells. Generic responses initiated by Gram-positive bacteria stimulate shedding of MMC6 protein from the surface of ATI cells, contributing to host defence. Similar host response factors induce an increase in production and secretion of SP-D by ATI and/or Clara cells. Basic alveolar structure adapted from Ware and Matthay (2000).

This thesis has included comparisons of animals receiving different treatments on different days (*S. pneumoniae* 24hrs vs 72 hrs, *S. pneumoniae* vs *S. aureus*). This was done due to practical reasons outwith the author's control. Although this method may not have been as scientific as setting up experiments with all treatment groups represented, there are a number of experimental systems whereby this method is used by necessity e.g. clinical studies with patients or experiments using treatment of genetically modified animals. While this is not the case in this study, and the limitations of this kind of comparison are acknowledged, there is no statistical reason why it should invalidate the evaluation.

In summary, this thesis presents novel research demonstrating differential alveolar epithelial injury and protein expression in pneumococcal pneumonia, along with novel techniques of assessment. Potential mechanisms were investigated and provided insight into disease pathogenesis, as well as highlighting the limitations of using cell-specific proteins as markers of lung epithelial cell injury.

6.3 FUTURE WORK

The mechanisms by which *S. pneumoniae* induces the effects presented in this thesis warrant further investigation. Determination of whether targeted attachment of bacteria to ATII cells is the means by which cell sloughing occurs would provide more information about this phenomenon, and the novel methods of ATII cell damage quantification could be applied to other models of lung injury. In addition, study of what exactly is happening to the cells during sloughing i.e. are they undergoing necrosis or apoptosis, would provide information about bacterial effects in pneumococcal pneumonia.

Assessment of mRNA levels *in vivo* could help to establish whether the decrease in RTI40 is in fact due to a change in protein configuration. Analysis of RTI40 protein structure could also provide insight. An investigation of host response factors found to be specific to pneumococcal infection could offer potential targets for further study to determine the regulatory stimulus for RTI40 decrease. Anti-microbial peptides, anti-inflammatory cytokines and complement components may warrant particular attention, as they are important factors in bacterial infection. Once identified, these factors could then be tested in the *in vitro* model presented here. Further examination to elicit the function of RTI40 in the normal adult lung would provide insights into mechanisms behind regulation, as well as potentially affording a means of testing the functionality of the protein in the event of extensive conformational change.

The identification and sequencing of MMC6 is crucial to interpreting the function of the protein in normal and infected lung. Studies using purified protein might establish if it has a role to play as a PRR or other innate immune function. Investigation of the potential shedding phenomenon could be further examined by looking at other Gram-positive models of lung infection at the resolving phase.

Despite the flaws in using cell-specific proteins as markers of injury in the lung, when properly supported these still provide valuable insights into disease pathogenesis. An important progression from this is the use of human marker proteins to assess clinical conditions. Although there is one currently identified human ATI-cell specific marker, HTI56 (Newman et al., 1999), techniques to analyse other proteins such as RTI40 and MMC6 are currently unavailable. Human antibodies against RTI40 do exist (Breiteneder-Geleff et al., 1999; Marks et al., 1999; Martin-Villar et al., 2005; Roy et al., 2005; Zimmer et al., 1999); however, variability in detection in different tissues, most likely due to difficulties of protein post-translational modification, mean that there is as yet no reliable marker of RTI40 in human lung alveolar epithelium. An antibody recognising such a protein would therefore be useful in human disease, although a previous attempt by the author to generate this proved unsuccessful. If accomplished this could contribute to purification of human epithelial cell isolation. In addition it could enable assessment of BAL fluid from CAP patients with a view to assessing ATI cell injury in a clinical situation or perhaps as an aid to aetiological diagnosis.

Further investigation of Gram-positive-stimulated host factors could also prove useful to find out more about regulation of SP-D production. Furthermore, the questions raised in this thesis regarding the role of pneumolysin in recovering pneumonia could be easily addressed by determining whether the toxin is actually produced *in vivo*, and by assessing the importance of TLR-4 in the stimulation of inflammation in this model of pneumococcal pneumonia.

APPENDIX 1: SUPPLIERS

REAGENTS/ EQUIPMENT

Reagents/equipment	Supplier
Alexa Fluor® conjugated goat anti-mouse/rabbit IgG secondary antibodies	Invitrogen
Antioxidant	Invitrogen
Biomate 3 spectrophotometer	Thermo Electron
Bio-Rad	Bio-Rad Laboratories
BSA	Pierce Warriner
Complete Protease Inhibitor Cocktail	Roche Diagnostics
Cytofunnel (double)	Thermo Electron
Cytospin 2 cytocentrifuge	Thermo Electron
Diff-Quick	Gamidor Technical Services Ltd
DNA ladder	Promega
Dot blot manifold	Whatman Schleicher and Schuell
DPBS	PAA Laboratories
DTT	Invitrogen
ECL Western Blotting Detection Reagents	Amersham
Eppendorf	Fisher Scientific
Ethicon mersilk black non-absorbent suture	3s Healthcare
FACSCalibur flow cytometer	B. D. Biosciences
FlowCytomix rat Cytokine 6plex bead array and analysis software (Bender Medsystems)	Invitrogen
Goat anti-mouse IgG HRP secondary antibody (Rockland)	Lorne Laboratories Ltd
Goat anti-mouse IgG HRP secondary antibody (Zymed®)	Invitrogen
Goat anti-rabbit IgG HRP secondary antibody (Rockland)	Lorne Laboratories Ltd
Goat anti-rabbit IgG HRP secondary antibody (Zymed®)	Invitrogen
H ₂ O (sterile, nuclease-free)	Applied Biosystems
Heparin	Leo Pharmaceuticals
ICAM1 antibody [1A29] (FITC)	Abcam
Mouse anti-rabbit GAP-DH monoclonal antibody [6C5]	Abcam
Mouse anti-rat ICAM-1 monoclonal antibody [1A29] conjugated to FITC	Abcam
Mowiol	Merck Biosciences
MultiMark® Multi-Colored Standard	Invitrogen
Monoject Aluminium Hub Blunt Needle	Harvard
Nuclease-free water	Applied Biosystems
NuPage Bis-Tris Electrophoresis System	Invitrogen
OCT	Bayer
PCR primers	MWG Biotech
Portex Fine Bore Tubing	Portex
PowerGen 125 homogeniser	Fisher Scientific

Rabbit anti-rat Aquaporin 5 polyclonal antibody	Chemicon
Rabbit anti-human β -actin polyclonal antibody	Abcam
Superfrost Plus slides	VWR
SYBR Safe™ DNA Gel Stain	Invitrogen
TMB Microwell Peroxidase Substrate	
TRIZOL®	Invitrogen
Ultra Turrax	IKA-Werke
Versadoc™ Model 4000 and Quantity One software	Bio-rad

LIST OF SUPPLIER ADDRESSES

3s Healthcare, Bristol, UK
 Abcam, Cambridge, UK
 Amersham, Little Chalfont, UK
 Applied Biosystems, Warrington, UK
 Bayer, Berkshire, UK
 B. D. Biosciences, Oxford, UK
 Bio-Rad Laboratories, Hertfordshire, UK
 Chemicon, Southampton, UK
 Fisher Scientific, Leicester, UK
 Gamidor Technical Services Ltd, Abingdon, UK
 Genusxpress Ltd, Aberdeen, UK
 Insight Biotechnology, Wembley, UK
 Harlen UK Ltd, Oxton, UK
 Harvard Apparatus, MA, USA
 Hycult Biotechnology Uden, Netherlands
 IKA-Werke, Germany
 Invitrogen, Paisley, UK
 Leo Pharmaceuticals, Princes Risborough, UK
 Lorne Laboratories Ltd, Twyford, UK.
 Merck Biosciences, Nottingham, UK
 MWG Biotech, London, UK
 PAA Laboratories Ltd, Yeovil, UK
 Pierce Warriner, Chester, UK
 Portex Fine Bore Polythene Tubing, Portex Ltd, Kent, UK
 Promega, Southampton, UK
 R&D Systems Abingdon, UK
 Roche Diagnostics, Loughborough, UK
 Sigma, Dorset, UK
 Thermo Electron, Reading, UK
 VWR, Dorset, UK
 Whatman Schleicher and Schuell, Keene, NH, USA

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PhD References Chart

