Characterisation of the Acute Inflammation During Murine Pneumococcal Pneumonia

A thesis submitted for the degree of Doctor of Philosophy of the University of Glasgow

by

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Declaration

This thesis is the original work of the author except where otherwise stated

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Abstract

We have established a murine model of pneumococcal pneumonia in order to characterise the host inflammatory response.
Intranasal infection with *Streptococcus pneumoniae* resulted in constant bacterial loads within airways of susceptible mouse strains whilst the pathogen burden increased in both the lung tissues and bloodstream. The bacteria induced inflammation that was evident as perivascular cell recruitment in histological sections. Mast cell granule staining indicated that this population degranulated early in the inflammatory response, releasing TNFα into the lung environment. Levels of TNFα and IL-1β increased during mid infection with release of IL-6 and the anti-inflammatory cytokine IL-10 not occurring until late infection. Kinetics of IL-10 were too slow to prevent inflammation causing damage to the host tissues. The total protein levels in bronchoalveolar lavage fluid (a marker of the integrity of the alveolar/capillary barrier) increased significantly during the experiment.
Dose response results indicate that there is likely to be a threshold number of bacteria required to induce this inflammatory response both in the lungs and bloodstream.

Inoculation of bacteria into a mouse strain resistant to the above infection resulted in bacterial clearance from both the pulmonary airways and tissues, with few mice becoming bacteraemic. The location and timing of the inflammatory response in this mouse strain was significantly different. Inflammatory cell influx occurred mainly within the airways, with perivascular areas unaffected. Mast cell degranulation occurred rapidly following infection. TNF activity and IL-1β levels within lung airways were induced earlier and to a greater extent than in susceptible mice. In contrast, the levels of TNF activity and IL-6 within lung tissues were lower in resistant mice. Although damage to lung integrity still occurred, this was only transient and evident during early/mid infection. Higher endogenous IL-10 levels were implicated in the ability of resistant mice to regulate the pulmonary inflammatory response before it could become detrimental. Lymphocytes, previously not thought to play a role in pneumococcal pneumonia, may be the source of regulatory IL-10.

Investigation of core body temperature and activity via a telemetry system revealed that peak levels of inflammation in both resistant or susceptible mouse strains were rapidly followed by mice becoming hypothermic.

Pneumococci were capable of entering the bloodstream of aged mice significantly earlier than aged mice following intranasal infection. In addition, aged mice were found to display an elevated pro-inflammatory response to the infection. TNF activities within the airways and lung tissues were higher in aged mice than in young animals. NO levels were also found to increase significantly during the infection in samples from aged mice but not from young mice.

Modulation of the host response indicated that surfactant, TNFα and NO were all important mediators of the pulmonary host response during pneumococcal pneumonia. Co-administration of the infectious dose in artificial surfactant significantly reduced the pulmonary bacterial load but did not alter overall survival.
Systemic and locally released TNFα were also implicated in the control of pathogen burden within the lungs and in the bloodstream. Treatment of mice with anti-TNFα antibodies significantly increased the bacterial counts recovered in lung and blood samples. Signalling via the TNFα p55 receptor was more important than via the p75 receptor in this respect. Mice deficient in the iNOS enzyme were less able to control pneumococci within the lungs following intranasal infection than wild type animals, indicating that NO was beneficial during *S. pneumoniae* pulmonary infection. Utilisation of the intravenous infection route revealed that NO was detrimental during Gram-positive bacteraemia.

Taken together these results indicate the requirement for a rapid inflammatory response following infection with pneumococci, but also for tight regulation of the response in order to prevent pathology and death.

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ABBREVIATIONS

-/-	Deficient
A	Absorbance
ALEC	Artificial Lung Expanding Compound
BSA	Bovine serum albumin
C	Complement
°C	Degrees Celsius
Cbp	Choline binding protein
Cat.	Catalogue
CFU	Colony forming units
cNOS	Constitutive nitric oxide synthase
CRP	C-reactive protein
D39	*S. pneumoniae* strain D39
eNOS	Endothelial nitric oxide synthase
EGF	Epidermal growth factor
Fc	Crystallisable fragment
FCS	Foetal calf serum
x g	Acceleration in the earth's gravitational field
g	Gram
GCP-2	Granulocyte chemoattractant protein-2
hrs	Hour(s)
HIV	Human immunodeficiency virus
ICAM-1	Intracellular adhesion molecule 1
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
L	Litre
LPS	Lipopolysaccharide
LytA	Pneumococcal autolysin
M	Molar
mAb	Monoclonal antibody
MASP	Mannose binding lectin serine protease
MBL	Mannose binding lectin
mg	Milligrams
min	Minute(s)
MIP	Macrophage inflammatory protein
ml	Millilitre

mM	Millimolar
µg	Microgram
µl	Microlitre
µM	Micromolar
NK	Natural killer
nm	Nanometer
ND	Below detection limit of viable count assay
NO	Nitric oxide
NOS	Nitric oxide synthase
No.	Number
nNOS	neuronal nitric oxide synthase
NS	No survivors at this time point
OD	Optical Density
p55	TNFα p55 receptor
p75	TNFα p75 receptor
PAF	Platelet activating factor
PBS	Phosphate buffered saline
Psa	Pneumococcal surface adhesin
Psp	Pneumococcal surface protein
RANTES	Regulated upon activation normal T expressed and secreted
rpm	Revolutions per minute
sec	Second(s)
spp	Species
TNF	Tumour necrosis factor alpha
Tco	Core body temperature
U	Units
UV	Ultraviolet light
VCAM-1	Vascular Cell Adhesion Molecule
v/v	volume/volume
wks	weeks

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Chapter 1

General Introduction

1.1 *Streptococcus pneumoniae*

Streptococcus pneumoniae (the pneumococcus) was simultaneously identified by Pasteur and by Sternberg in 1881 (Pasteur 1881) & (Sternberg 1881). Pasteur discovered the organism in saliva from a child who had died of rabies, while Sternberg found pneumococci in his own saliva. Each injected saliva into rabbits and both subsequently recovered diplococci from the blood of these animals. Two years later these bacteria were implicated in disease when Frieländer noted the association of the organism with lobar pneumonia (Frieländer 1883).

Formerly called *Diplococcus pneumoniae*, *S. pneumoniae* is a Gram-positive encapsulated bacterium. Pneumococci are lancet shaped cocci, 0.5-1.25μM in diameter (McKinsey and Bisno 1980), generally present in pairs (or short chains). The bacteria form alpha haemolytic colonies when cultured on blood agar base with their sensitivity to the antibiotic optochin (ethyl hydrocuprein) readily distinguishing them from other alpha-haemolytic streptococci.

The pneumococcus is now recognised as a major human pathogen in infancy, childhood and adult life. *S. pneumoniae* is capable of causing both non-invasive disease such otitis media, as well as invasive infections such as pneumonia, meningitis and sepsis.

1.2 Structure

The pneumococcal outer surface is comprised of 3 main parts: the capsule, cell wall and the plasma membrane (Figure 1.1).

1.2.1 Capsule

Although *S. pneumoniae* exists in both encapsulated and unencapsulated forms the great majority of clinical isolates and strains of *S. pneumoniae* recovered from carriage sites possess a polysaccharide capsule (Kalin 1998), of which there are currently 90 known types (Henrichsen 1995). The majority, if not all, of these capsular serotypes are capable of causing serious illness in humans (Kalin 1998). There have been few reports of atypical unencapsulated pneumococci being carried or causing infection in humans

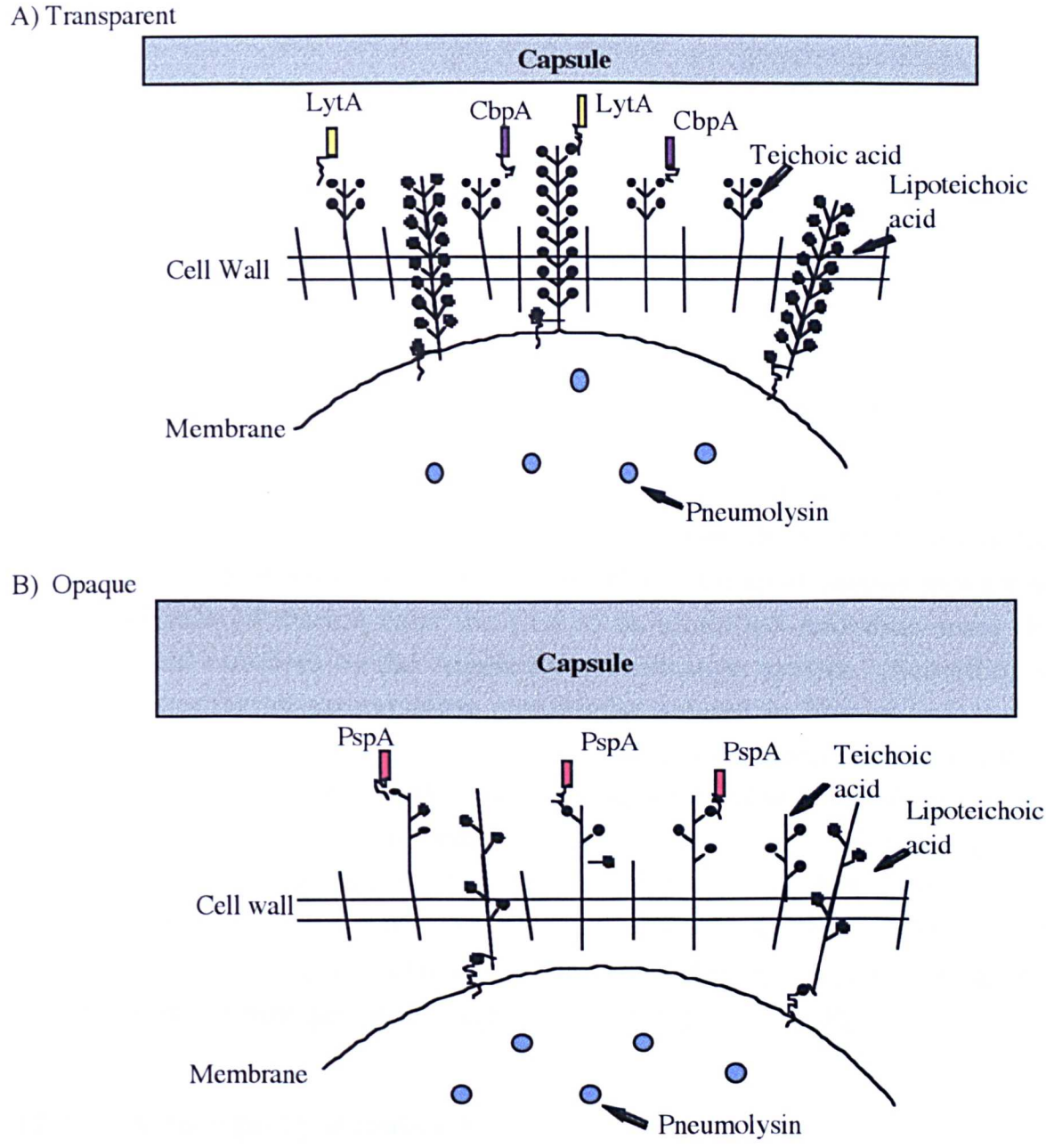

Figure 1.1 Surface of *S. pneumoniae* in different body sites. Pneumococci vary between a transparent phenotype (A) adapted to the nasopharynx and an opaque phenotype (B) adapted to the bloodstream. This phase variation is reversible and involves changes in the amount of choline in the cell wall and the amount of several important surface proteins. These include the protective antigen PspA, the adhesin CbpA, and the autolysin, LytA. Dark circles represent choline. Adapted from (Tuomanen 1999)

(Müller-Graft *et al.* 1999). These strains are mainly isolated from immunocompromised individuals such as HIV-seropositive patients. Thus the majority of unencapsulated pneumococci are only found in the laboratory.

Capsular types were originally differentiated by the use of type specific antiserum, the Quellung reaction. The addition of specific antiserum causes both macroscopic agglutination and microscopically visible swelling of the capsule (Neufeld 1902). However, when the test involves pneumococci with small capsules the reaction can be difficult to interpret and the results dubious. More recent methods employ fluorescent antibodies directed against the capsule (Wicher *et al.* 1982).

Capsular type is classified by one of two classification systems: the American assigns numbers in the chronological order of their discovery whereas the Danish system is based on antigenic similarities. Virulent strains that cause most outbreaks of disease were the first to be identified and thus were the first assigned numbers by the American classification system. Accordingly, the lower numbered serotypes are commonly isolated in human infections. The lowest numbered serotypes such as 1 and 2 are classified identically by both the American and Danish systems. Higher numbered serotypes in the American system that share antigenic similarities with lower numbered types are grouped together by the Danish system. Thus American type 48 becomes Danish 7B (as it is similar to Danish type 7A, American type 7) with serotype 50 becoming 7C (Lee 1987). It is the Danish nomenclature for serotypes that is now generally employed (vanDam *et al.* 1990).

1.2.2 Capsular type 2 pneumococci

At the beginning of this century type 2 pneumococci (e.g. D39) were associated with a high proportion of cases of both pneumonia and bacteraemia (Austrian 1981) & (Findland and Barnes 1977). In subsequent years, recovery of type 2 pneumococci as clinical isolates has declined to the extent that it is almost never detected in industrialised nations (Kalin 1998). These pneumococci do remain a problem in non-industrialised countries, in Nigeria and Senegal they are commonly isolated (Greenwood *et al.* 1980). In Papua New Guinea (an area known to have high rates of pneumococcal

disease) type 2 pneumococci do not frequently colonise, but when they do they are highly likely to cause invasive disease.
Furthermore, type 2 pneumococci are one of the serotypes most likely to cause epidemic pneumonia or bacteraemia (Austrian 1981). Cases of epidemic pneumonia have recently increased in the western world in a range of settings including military camps (Musher *et al.* 1997), nursing homes (Musher 1998), jails (Hoge *et al.* 1994) and child-care centres (Cherian *et al.* 1994). Additionally *S. pneumoniae* has become one of the most common causes of bacteraemia. The pneumococcus was the third most frequently isolated micro-organism (after *Escherichia coli* and *Staphylococcus aureus*) from patients with severe sepsis or septic shock in a recent world-wide study (Cohen and Abraham 1999).
The historical importance of type 2 pneumococci has led to D39 becoming a well characterised experimental strain. Thus a wealth of genetic information is available concerning this isolate. In addition, D39 is virulent in mice and has frequently been employed in animal models (Canvin *et al.* 1995), (Rubins *et al.* 1995) & (Kadioglu *et al.* 2000), making it an ideal candidate for use in the establishment and characterisation of this model of pneumococcal pneumonia.

1.2.3 Cell wall

The typical Gram-positive cell wall is composed mainly of peptidoglycan (PG): glycan chains of alternating N-acetlyglucosamine and N-acetylymuramic acid. Acidic polysaccharides termed teichoic acids are attached to the cell wall (Figure 1.1).
The cell wall polysaccharide, a complex teichoic acid containing phosphorylcholine residues common to all pneumococcal serotypes, is also attached to PG (Jennings *et al.* 1980). The phosphorylcholine residues of cell wall polysaccharide are a recognition site for N-acetylymuramic acid-L-alanine amidase (autolysin), an enzyme that cleaves the bond between the glycan chain and the peptide side chain of the cell wall leading to cellular autolysis (Paton *et al.* 1993), releasing cytoplasmic contents such as pneumolysin (Section 1.3.2).
The cell wall is believed to play a significant role in instigating the inflammatory response that occurs during pneumococcal pneumonia. The

minimum cell wall component capable of initiating an inflammatory response has now been identified as teichoic acid attached to the glycan backbone and some small-stem peptides (Tuomanen *et al.* 1987).
By binding to pulmonary epithelia and causing separation of tight junctions, cell wall components are likely to increase the permeability of alveolar epithelia (Tuomanen *et al.* 1987). This in turn permits influx of inflammatory cells that may be recruited via the ability of cell wall teichoic acids to activate the alternative complement pathway (Section 1.5.3.4) (Winkelstein and Tomasz 1978). Pro-inflammatory cytokine production (such as TNFα and IL-1) is also implicated in this cell recruitment. A separate pro-inflammatory mediator released by incubation with cell wall preparations is nitric oxide (NO) (Orman *et al.* 1998). As mentioned in Section 1.5.3.5, NO is also implicated in upregulation of the inflammatory response and is capable of causing direct damage to the lungs.

1.2.4 Plasma Membrane

The plasma membrane surrounds the cytoplasm and is composed mainly of lipid with lipoteichoic acid and protein also present (Brock and Madigan 1991). This permeable layer is responsible for maintaining the integrity of cytoplasmic contents.
This is also the location of the Forssman antigen (Tomasz 1981), a lipoteichoic acid found in all pneumococci that functions as a highly specific inhibitor of autolysin. Release of the Forssman antigen during the stationary phase of growth leads to cell separation/ cell lysis (AlonsoDeVelasco *et al.* 1995).

1.2.5 Pneumococcal phase variation

Three interchangeable pneumococcal variants have been recorded: opaque, semitransparant, and transparent (Weser *et al.* 1994) (see Figure 1.1 for a diagram of transparent and opaque phenotypes). These phenotypes vary in their suitability to different body compartments, determined by variations in the amount of capsule expressed as well as an array of surface proteins expressed in the cell wall (Figure 1.1). Transparent phenotypes are adapted for survival in the nasopharynx since they are capable of adhering to the

platelet activated factor receptor on epithelial cells that is upregulated during infection (Section 1.4.2). In contrast opaque variants are unable to adhere to this receptor but the upregulation of PspA (Section 1.3.5) and downregulation of choline (reducing the capacity for C-reactive protein to opsonise [Chapter 6] adapts them for survival within the bloodstream.

1.3 Virulence Factors

Pneumococci produce several factors that may be involved in the pathogenesis of disease. These include structural components, surface proteins and toxins (Figure 1.2). Publication of the complete DNA sequence of *S. pneumoniae* by the Institute for Genomic Research in November 1997 (http://www.tigr.org/tdb) will aid the identification of novel virulence factors by allowing comparison of sequences of known virulence factors from other organisms with the *S. pneumoniae* sequence.
Virulence of pneumococci is lost with repeated passage *in vitro* requiring periodic passage of the bacteria through mice for *in vivo* studies (Johnston 1991). This might reflect alterations in some of these virulence factors.

1.3.1 Capsule as a virulence factor

One of the oldest recognised virulence factors is the polysaccharide capsule. Proof of the importance of the capsule to virulence was established in the 1930s. Dubos and Avery found that an enzyme obtained from a soil bacillus could remove the serotype 3 capsular polysaccharide (Avery and Dubos 1931). Infection of mice with bacteria treated in this way resulted in 100% survival whilst challenge with untreated bacteria caused a fatal infection.
The role of the capsule in virulence stems from its anti-phagocytic activity (Jonsson *et al.* 1985). In the nonimmune host, antibody to cell wall constituents (ubiquitous in adults) becomes attached to the surface of the organisms and in turn binds complement. The presence of the capsule prevents complement factor C3b and the Fc of immunoglobulins on the bacterial cell surface from interacting with receptors on phagocytic cells and the organisms remain extracellular (Musher 1992). The role of the capsule in

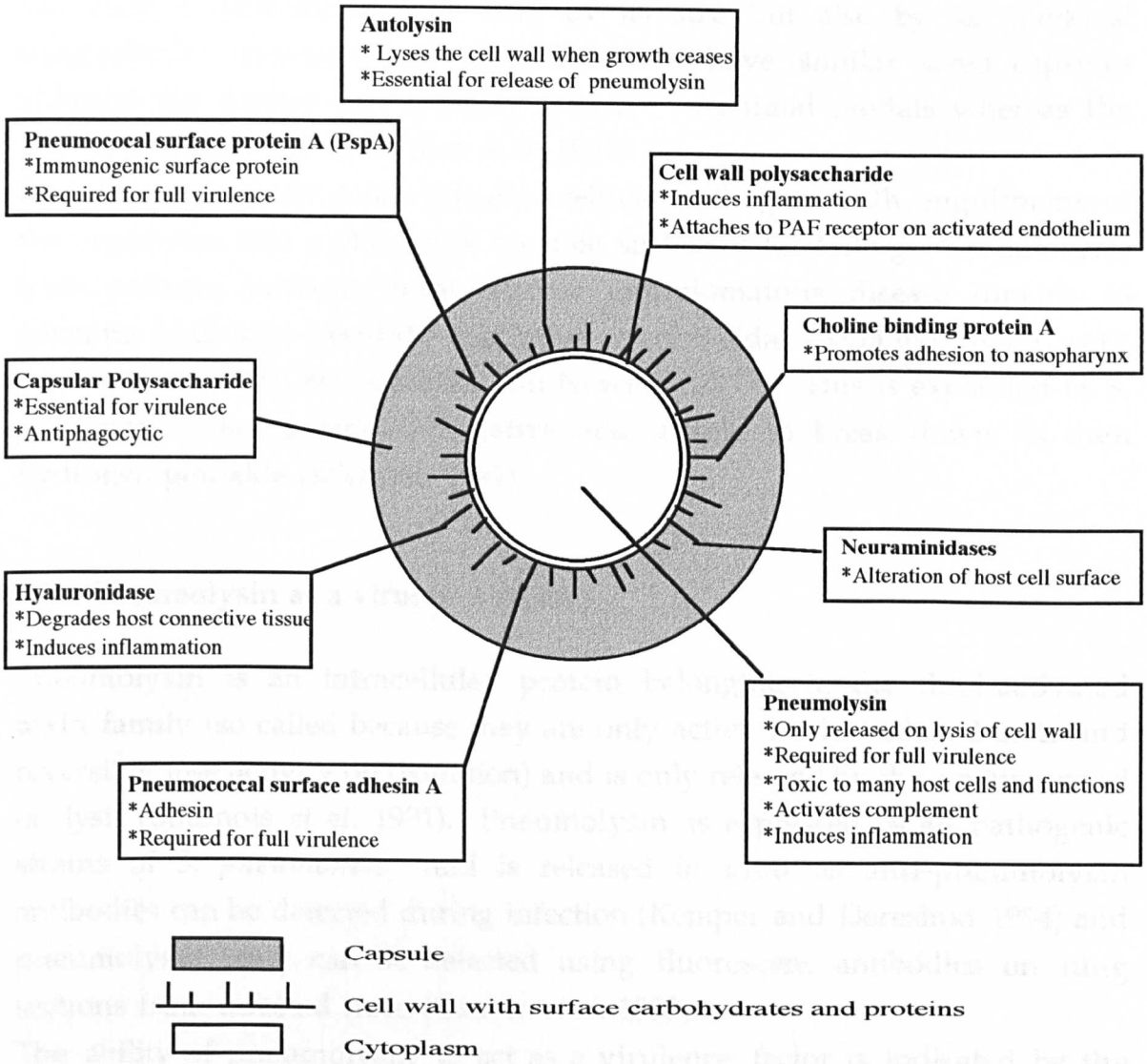

Figure 1.2 Schematic diagram of the main virulence factors of *S. pneumoniae* including their main functions and cellular location. PMN= polymorphonuclear leukocyte. See text for a more detailed description.

virulence is determined not only by its size but also by its chemical composition. Pneumococcal types 3 and 37 have similar sized capsules although the former type is highly invasive in animal models whereas the later is relatively avirulent (Lee *et al.* 1991).

Pneumococci are the prototypic extracellular pathogens with engulfment of the organisms into a phagocytic vacuole sufficient for killing. Granulocytes from patients suffering from chronic granulomatous disease (unable to generate hydrogen peroxide and other microbicidal oxidants) retain anti-pneumococcal activity (Johnston and Newman 1977). This is explained by *S. pneumoniae* being catalase negative and unable to break down its own hydrogen peroxide (Johnston 1991).

1.3.2 Pneumolysin as a virulence factor

Pneumolysin is an intracellular protein belonging to the thiol-activated toxin family (so called because they are only active in the reduced state and reversibly lose activity on oxidation) and is only released by the pneumococci on lysis (Boulnois *et al.* 1991). Pneumolysin is expressed by all pathogenic strains of *S. pneumoniae* and is released *in vivo* as anti-pneumolysin antibodies can be detected during infection (Kemper and Deresinki 1994) and pneumolysin itself can be detected using fluorescent antibodies on lung sections from infected mice (Canvin *et al.* 1995).

The ability of pneumolysin to act as a virulence factor is indicated by the reduced virulence of mutant bacteria unable to produce the toxin (Berry *et al.* 1989). Mice infected with pneumolysin-deficient bacteria survive significantly longer than those infected with pneumolysin-sufficient *S. pneumoniae*. These bacteria are less able to survive and multiply within the lungs and are incapable of multiplying to the same high levels in bloodstreams as wild type pneumococci (Kadioglu *et al.* 2000).

This virulence factor is capable of exerting differing effects on the immune response, depending on its concentration. At lower concentrations (~1ng/ml) pneumolysin is capable of a range of activities indicated in Table 1.1.

At higher concentrations (~10μg/ml) the toxin can lyse cells. The toxin is believed to bind to cholesterol in the target cell membrane, insert into the

Target	Effect	Reference
Monocytes	Inhibits respiratory burst	(Nandoskar *et al.* 1986)
	Decreases anti-pneumococcal activity	(Nandoskar *et al.* 1986)
	Stimulates pro-inflammatory cytokine production	(Houldsworth *et al.* 1994)
Neutrophils	Inhibits respiratory burst & migration	(Paton and Ferrane 1983)
Pulmonary Epithelial cells	Disrupts integrity	(Rubins *et al.* 1993)
	Inhibits cilial beating	(Steinfort *et al.* 1989)
Pulmonary Endothelial cells	Disrupts integrity	(Rubins *et al.* 1992)
B cells	Decreases antibody production	(Boulnois *et al.* 1991)
Peripheral blood Lymphocytes	Inhibits proliferation and antibody production	(Ferrante *et al.* *1984)*

Table 1.1 Effects of low levels of pneumolysin relevant to pneumococcal infection.

membrane, oligomerise and form transmembrane pores, thus disrupting membrane integrity and causing cell lysis (Paton 1996).

These higher concentrations of pneumolysin are also implicated in the inflammatory response that occurs during pneumococcal pneumonia. Injection of recombinant toxin into ligated bronchi of rats induces a similar pattern of inflammation in that area to the host response seen during infection with virulent *S. pneumoniae*. The lobe becomes consolidated with inflammatory cells and there was marked disruption to lung architecture (Feldman *et al.* 1991). Furthermore, *in vitro* pneumolysin is capable of stimulating the classical complement pathway (Section 1.5.3.4.1) in the absence of pneumolysin-specific antibody, thereby instigating an inflammatory response in the fluid layer, diverting the response away from intact pneumococci (Paton *et al.* 1984). In addition, opsonisation with human serum treated with pneumolysin significantly reduced *in vitro* phagocytosis of the bacteria by neutrophils. Thus complement activation correlates with a significant inhibition of serum opsonic activity. The mechanism that pneumolysin utilises to exert this effect is currently unknown but is believed to involve non-specific binding of IgG Fc by the toxin (Mitchell *et al.* 1991).

As mentioned in Table 1.1, pneumolysin is capable of stimulating release of TNFα and IL-1β, from human monocytes. Both these cytokines possess potent pro-inflammatory activities (Section 1.6.1.1). Lower levels of TNFα, IL-6 and IFNγ being released during infection with pneumolysin deficient *S. pneumoniae* mutants than wild type bacteria provides indirect evidence of the *in vivo* inflammatory role played by pneumolysin (Benton *et al.* 1998). Pneumolysin can also stimulate the activity of phospholipase A by pulmonary artery endothelial cells (Rubins *et al.* 1994). Phospholipases release products of the arachidonic acid pathway which have been shown to be involved in neutrophil recruitment and activation of inflammatory cells during pneumococcal pneumonia (Tuomanen *et al.* 1987). In addition, phospholipase A is capable of degrading phospholipids in cell membranes perhaps aiding spread of the pneumococci. Microscopic examination of cells treated *in vitro* with pneumolysin has indicated that the toxin is also capable of mediating direct damage to host cells. Disruption has so far been identified to occur to bronchial and alveolar epithelial cells as well as pulmonary endothelial cells (Steinfort *et al.* 1989), (Rubins *et al.* 1993) & (Rubins *et al.* 1992). Pneumolysin may not act in isolation in these

inflammatory activities, as it may also potentiate the inflammatory effects of cell wall products. Such synergy has been previously documented for the alpha toxin of *S. aureus* (Bhakdi *et al.* 1989).

Finally, infection with pneumococci deficient in the ability to synthesise pneumolysin is known to induce significantly less pulmonary inflammation than infection with wild-type bacteria. This laboratory has shown that although the histopathological changes that occur within the lungs of mice infected with pneumolysin deficient bacteria are similar to those occurring during wild type infection, the kinetics are delayed and severity lessened (Canvin *et al.* 1995). In addition we have recently characterised the pulmonary cellular response during infection with pneumolysin deficient bacteria (Kadioglu *et al.* 2000). Following intranasal infection with 10^6 CFU, these pneumococci were incapable of reaching the same levels of bacterial loads within both the lungs and bloodstreams of MF1 mice. Thus the presence of pneumolysin is essential for bacterial multiplication in the alveoli, especially within the initial 6-8h of infection. It has been previously shown that instillation of recombinant pneumolysin concurrently with pneumolysin deficient bacteria returns the pattern of bacteria growth to that of wild-type bacteria (Rubins *et al.* 1995) proving that it is the loss of this one virulence factor that is responsible for reduced pneumococcal proliferation.

In addition mice infected with pneumolysin deficient bacteria did not display marked symptoms of infection, unlike their counterparts infected with wild-type bacteria (Section 3.2.3.1).

Our investigation of the pulmonary cell influx in response to these infections revealed a delayed and reduced cell recruitment to the lungs of mice inoculated with pneumolysin deficient pneumococci than infection with wild-type bacteria. Analysis of the cell types involved indicated that neutrophil responses were most affected with the redistribution of T and B lymphocytes in and around inflamed bronchioles also delayed and reduced. Whether these changes are due to the reduced bacterial loads within both lungs and circulations of mice infected with pneumolysin deficient bacteria acting as a poorer inflammatory stimulus, or due to pneumolysin directly instigating damage by wild-type bacteria is currently unknown.

Immunisation with inactivated pneumolysin has been shown to offer enhanced survival following subsequent challenge with pneumococci (Paton *et al.* 1983). Survival times of mice subsequently infected with 5×10^6 CFU *S. pneumoniae* were increased from 2.48 days for untreated mice to 5.52 days in

immunised animals. Although this effect is not as marked as the protection elicited by anti-capsule antibodies, protection is not sero-type specific. This laboratory has previously demonstrated protection against subsequent infection with 9 different pneumococcal serotypes for mice immunised with pneumolysin toxoid prepared from type 2 pneumococci (Alexander *et al.* 1994). Pneumolysin has thus been proposed as a promising antigen for use in novel vaccines.

1.3.3 Neuraminidases as virulence factors

All clinical isolates of *S. pneumoniae* tested produce this surface associated enzyme capable of cleaving N-acetylneuraminic acid from glycolipids, lipoproteins and oligosaccharides on cell surfaces or in body fluids (Camara *et al.* 1994). Two separate pneumococcal neuraminidase genes, *nanA* and *nanB*, have now been identified (Camara *et al.* 1994) and (Berry *et al.* 1996). Production of neuraminidases may cause direct damage to the host or may unmask potential binding sites for the organism.
Immunisation of mice with formaldehyde inactivated neuraminidase significantly increases survival times of mice subsequently challenged with *S. pneumoniae* (Lock *et al.* 1988), but not to the extent seen with pneumolysin or capsular polysaccharide.

1.3.4 Hyaluronidase as a virulence factor

Almost all clinical isolates secrete this enzyme which acts on hyaluronic acid, a component of mammalian connective tissue and extracellular matrix. Such degradation may aid bacterial spread and colonisation, as demonstrated for hyaluronidase from other micro-organisms. In the case of *Treponema pallidum*, hyaluronidase induces vascular leakage and dissemination of the organism (Fitzgerald and Repesh 1987).
In addition, hyaluronidase may potentiate pulmonary inflammation during pneumococcal pneumonia via complex interactions with pro-inflammatory cytokines and chemokines. *In vitro*, TNFα and IL-1β are capable of inducing the production of hyaluronic acid by fibroblasts (Irwin *et al.* 1994). Hyaluronic acid can promote further cytokine secretion by binding to CD44 on host cells. The system is further complicated by the ability of IL-1 to act in

combination with high levels of hyaluronic acid to release hyaluronidase, thereby degrading hyaluronic acid (and surrounding connective tissue).
Breakdown products of hyaluronic acid have been shown to stimulate chemokine expression by macrophages *in vitro* (McKee *et al.* 1996). Such expression *in vivo* would induce cell recruitment, potentiating inflammation. Furthermore, these degradation products are also capable of elevating the phagocytosis of IgG opsonised latex beads and chemotaxis (in response to casein) of granulocytes *in vitro* and *in vivo* (Hükansson *et al.* 1980).
Thus the expression of hyaluronidase by *S. pneumoniae* during pneumococcal pneumonia would disrupt normal hyaluronic acid metabolism leading to direct tissue disruption, upregulation of pro-inflammatory cytokines and cell recruitment.

1.3.5 Pneumococcal surface protein A (PspA) as a virulence factor

PspA is a highly variable protein expressed by all medically important pneumococcal serotypes (Crain *et al.* 1990) and found non-covalently attached to the choline of the cell wall.
Mutant *S. pneumoniae* variants unable to produce PspA are less virulent in systemic disease as they are more easily cleared from the bloodstream (McDaniel *et al.* 1987). PspA may act by preventing the deposition of C3b onto the surface of pneumococci. Alternatively, this virulence factor may inhibit the formation of the alternate complement pathway C3 convertase (Tu *et al.* 1999). By either method, PspA reduces the effectiveness of complement receptor-mediated mechanisms of clearance.
More recently PspA has been identified as a lactoferrin binding protein (Hammerschmidt *et al* 1999). By interacting with lactoferrin *S. pneumoniae* may be able to overcome the iron deficiency of mucosal sites and progress to cause disease.

1.3.6 Other surface proteins as virulence factors

An additional range of pneumococcal surface proteins have recently been identified and proposed as virulence factors. One such protein is choline binding protein A (CbpA, Figure 1.1), involved in bacterial adhesion to the

nasopharynx (Section 1.2.5). Immunisation of mice with the same protein but under the name PspC provides mice with protection against subsequent intravenous challenge with either PspC producing or non-producing strains of *S. pneumoniae* (Brooks-Walter *et al.* 1999). This cross-protection was found to be due to the production of antibodies cross-reactive against both PspC and PspA (the two proteins displays high homology).

Although the receptors on human cells that pneumococci adhere to have been identified (Section 1.4.2), pneumococcal adhesins are currently unknown. Cundell *et al* have found that mutations in genes identified as peptide permeases reduced pneumococcal adherence to both of the receptors expressed on resting lung or endothelial cells (Cundell *et al.* 1995). These permeases are believed to bind and transport small peptides across the bacterial membrane, presumably for metabolic utilisation. A homologue of these permeases, PsaA, has been ascribed a role in pneumococcal virulence (Talkington *et al.* 1996), Immunisation of mice with PsaA prior to intravenous infection with 450 CFU type 3 pneumococci resulted in 75% survival whilst there were no survivors in the un-immunised group. PsaA deficient bacteria have also been constructed and shown to display attenuated adhesion to type II pneumocytes *in vitro* (Berry and Paton 1996). These same mutants were less virulent following intranasal or intraperitoneal challenge of mice (assessed by longer survival times and higher survival rates).

More recently PsaA and three homologues, PsaB, PsaC and PsaD have been shown to be manganese transporters (Novak *et al.* 1998). Mutations upstream of the genes for these permeases reduced pneumococcal adherence to lung epithelial cells *in vitro*. Rather than being directly responsible for pneumococcal adhesion, it is now believed that these permeases act in a regulatory capacity to alter adhesion by acting on Cbp expression (manganese may be required for the export of Cbp).

1.4 Pneumococcal Pneumonia

1.4.1 Epidemiology of pneumococcal pneumonia

Pneumonia is the sixth leading cause of death in the United States and the most common cause of death from an infectious disease in the non-industrialised world (Garibaldi 1985) & (Niederman *et al.* 1993). Current

estimates of mortality rates in children under the age of 5 are 2.7 million per year (World Bank 1993).

Pneumonia is also a major cause of death in the adult population. Every year an estimated 100,000 adults between the ages of 45 and 59 die from this infection, with 1.2 million deaths in the over 60 age group (Murray and Lopez 1996). *S. pneumoniae* is believed to be responsible for most of these deaths in the industrialised and non-industrialised worlds. Estimates currently stand at 40-60% of cases being due to this one micro-organism (Fang *et al.* 1990) & (Mulholland 1999).

One reason for the prevalence of pneumococcal pneumonia is the ubiquity of the bacteria, they can be found colonising the nasopharynx of up to 70% of the community (Kemper and Deresinki 1994). Such colonisation occurs early in life with most infants colonised with pneumococci on at least one occasion before they are 2 years old (Gray *et al.* 1980). Carriage of one serotype of pneumococci does not protect against colonisation with other serotypes as up to four serotype have been found to be carried by an individual at any one time (Tuomanen *et al.* 1995).

1.4.2 Pathogenesis of pneumococcal pneumonia

Pneumococcal pneumonia is believed to occur after spread of the bacteria from the nasopharynx to the lungs via aspiration (Musher 1992). Within the alveoli the organisms are thought to preferentially attach to type II pneumocytes (Cundell and Tuomanen 1994). There are two classes of receptors expressed on resting pneumocytes: N-acetyl-D-galactosamine linked by either β1,3 or β1,4 to galactose (GalNAcβ1-3Gal and GalNAcβ1-4Gal) (Cundell *et al.* 1995). Only 0.1% of pneumococci are capable of attaching to these receptors on resting cells (Tuomanen 1997). Activation of pneumocytes leads to the expression of a novel receptor N-acetylglucosamine (GalNAc), the platelet activating factor (PAF) receptor (Cundell *et al.* 1995). The appearance of this receptor results in a rapid enhancement of pneumococcal adhesion (Tuomanen 1997).

Adherence is thought to be followed by bacterial internalisation into host cell vacuoles via receptor-mediated endocytosis (Cundell *et al.* 1995) & (Talbot *et al.* 1996). Pneumococci are then able to pass through these cells and gain

access to the bloodstream. Pneumococci may also enter the circulation via the pulmonary draining lymph ducts.

1.4.3 Predisposing factors for pneumococcal pneumonia

Despite the high carriage rates, comparatively few individuals go on to develop pneumococcal pneumonia. Many factors are responsible for determining the outcome of colonisation and these can generally be separated into physiological, non-immunological and immunological (Table 1.2).

1.4.3.1 Age as a predisposing factor for pneumococcal pneumonia

People at the extremes of age are most likely to suffer from pneumococcal disease. The highest incidences are found in children between 18 and 24 months, infections are rarer in teenagers and young adults but increase with middle age to peak again in individuals in their 70's and 80's (Austrian and Gold 1964) & (Burnman *et al.* 1985).

This age distribution is also associated with variations in the frequency of capsular types identified in clinical cases. Danish types 6B, 19F and 14 are most likely to cause disease in children whereas 1, 2 and 3 are more common in adults (Leach *et al.* 1997).

The immature status of B cells in infants (lacking the CD40 surface antigen required for IgG antibody production during T cell-dependent responses) is responsible for their poor response to pneumococcal polysaccharide (Dullforce *et al.* 1998). Therefore this deficiency also increases their susceptibility to other encapsulated bacteria such as *Haemophilus influenzae.*

At present the increased incidence of pneumococcal pneumonia in the elderly population is not understood. Environmental factors, nutritional status and underlying infections undoubtedly play a role but the immune response itself is also likely to be involved. Immune responses are altered in elderly individuals with the major effects of ageing found in the cell mediated arm of immunity. The most well known deficiency is the loss of thymic tissue which leads to an elevated number of immature T cells within the circulation (Simons and Reynolds 1990). The antibody response is also known to be diminished as investigation of antibody levels 2 weeks post immunisation with pneumococcal capsular polysaccharide detects

Type of Factor	Factor
Physiological	Age of under 2 yr or over 65 yr
	Male gender
Non-immunological	Disruption of bronchial epithelium (influenza, smoking)
	Ethanol, narcotic intoxication
	Decreased vascular perfusion (e.g. sickle cell anaemia or congestive heart failure)
	Cold weather
Immunological	Immunocompromised status for example HIV+
	Phagocytic abnormalities-neutropenia, hyposplenia
	Hypogammaglobulinemia
	Specific antibody deficiency
	Complement deficiency (C2, C3)

Table 1.2 Host factors that predispose individuals to pneumococcal infection (adapted from (Johnston 1981) & (Burman *et al.* 1985)).

significantly lower levels in elderly individuals than in young adults (Ammann *et al.* 1980). Recently the reduced response of the aged population to pneumococcal vaccination was studied in an *in vitro* system. By culturing spleen cells from young and old mice with the vaccine, Garg *et al* discovered that cells from young mice generated significantly more vaccine-specific plaque-forming cells (Garg *et al.* 1996). The authors showed that this was not due to elevated T-cell mediated suppression but rather the result of reduced accessory cell function (an effect overcome by the addition of exogenous IL-1β). It is well known that IL-1 secretion is diminished with age. Both peritoneal macrophages and T cells from 2yr old mice possess less IL-1 activity (as assessed by the ability to induce thymocyte proliferation) than do cells from 4 month old animals (Inamizu *et al.* 1985). IL-1 is capable of stimulating B lymphocytes to proliferate, increasing antibody secretion and inducing IL-2 production (IL-2 in turn causes T lymphocyte proliferation). Thus with reduced expression of IL-1 by accessory cells (such as macrophages) from aged individuals, a poorer response to vaccination is inevitable.

The innate immune defences also diminish with age. Phagocytosis of zymosan opsonised yeast particles is significantly impaired in aged human when compared to young individuals. Such phagocytic impairment is evident in subjects in their 30s when compared to those in their 20s or teens. This effect becomes progressively more evident and significant in subsequent decades (Emanuelli, *et al.* 1986).

In contrast to these immune deficiencies, inflammatory responses are elevated in the elderly. This effect has previously been noted in patients suffering from pneumococcal infection (Bruunsgaard, *et al.* 1999). This effect is likely to represent elevated production of inflammatory cytokines such as TNFα and IL-6 (Foster, *et al.* 1992).

These results indicate that a decrease in neutrophil recruitment and activity or reduced antibody response may be responsible for the elevated susceptibility to pneumococcal pneumonia in aged individuals. Pathology may be worse in this population due to overzealous inflammatory responses, which may result from impaired regulation by lymphocytes.

1.4.3.2 HIV status as a predisposing factor for pneumococcal pneumonia

Pneumococcal infection occurs between 5 and 17 fold more frequently in HIV positive populations, making *S. pneumoniae* the most commonly isolated respiratory pathogen in HIV positive individuals in both industrialised and non-industrialised nations (Moore *et al.* 1998) & (Gilks *et al.* 1996). Furthermore, the risk of bacteraemic pneumococcal pneumonia is increased approximately 100 fold (Redd *et al.* 1990), with a mortality rate in AIDS patients of 57% whereas the rate in other populations is 22-39% (Pesola and Charles 1992).

This problem is compounded by the increased incidence of antibiotic resistant strains of pneumococci in HIV positive patients (Paul *et al.* 1995). As most antibiotic resistant strains of pneumococci are of the serotypes covered by the present vaccine, it would suggest that the problem could be overcome by widespread vaccination of HIV positive individuals. Unfortunately there have been frequent reports of poor vaccine efficacy. Rodriguez-Barradas *et al* found that subjects with CD4 counts below 500 are far less likely to respond to vaccination as reflected in the low post-vaccination total IgG titres to pneumococcal serotypes 3, 4, 6A, 8 and 23F in these individuals (Rodriguez-Barradas *et al.* 1992). Furthermore HIV seropositive individuals who respond well to vaccination may have very low antibody levels again 12 weeks following treatment (Loeliger *et al.* 1995).

1.4.3.3 Gender as a predisposing factor for pneumococcal pneumonia

The incidence of pneumococcal infection in humans is greater in males than in females, with a ratio of 2.1:1 (Burman *et al.* 1985). However, the reason for such a difference is currently unknown. Male subjects do not have a higher rate of carriage of pneumococci than females. Furthermore, male individuals do not appear to be colonised by capsular types with higher virulence than do females, as Scott *et al* found similar capsular types were isolated from both males and females in over 7,000 cases of invasive pneumococcal infection (Scott, *et al.* 1996).

Such data indicates that a defect in the male immune response increases their susceptibility to pneumococcal infection. Indeed it is widely recognised that in most species studied, males have weaker immune responses than females. Gender differences in antibody levels against pneumococcal

capsular polysaccharide have been documented with males responding less significantly than women following vaccination (Roghmann *et al.* 1987).
Most data in this field of research indicates that hormonal control of the immune response is responsible for these gender differences. By using mouse strains that vary in their sensitivity to (but not circulating levels of) androgen, Cohn and her laboratory have revealed that the response to pneumococcal polysaccharides is mediated by this one hormone (Cohn 1979). Male mice with high tissue responses (assessed by seminal vesicle growth) to androgen displayed lower antibody levels following immunisation than did females from the same mouse strain or males from strains with low sensitivity.
At present the reason for androgen sensitivity mediating immune responses is unclear. It may be that androgen mediated responses directly affect the immune response. Alternatively the androgen response may exert its effects via intermediaries such as cytokines. The effects of this sensitivity to androgen on the inflammatory response during pneumococcal pneumonia would therefore be an interesting future direction for our research to take.

1.4.3.4 Other genetic factors predisposing to pneumococcal pneumonia

It has previously been noted that adoptees have a five-fold greater risk of dying from an infectious disease if a biological parent died from the same infection (with pneumonia accounting for 29% of the infections). The risk was not increased if an adoptive parent died from an infection (Sørensen *et al.* 1988). These facts indicate that there is a strong genetic component determining the outcome to infectious diseases with environmental factors playing a minor role.
Recognition of the above has led to investigation of host genetic factors which might be responsible. A locus on mouse chromosome one, termed Bcg, Lsh or Ity, is important in controlling infections due to intracellular organisms such as *Mycobacterium* spp., *Listeria monocytogenes* and *Salmonella typhimurium* (Forget *et al.* 1981), (Skamene *et al.* 1979), and (Plant *et al.* 1982). Ity encodes a natural resistance macrophage protein 1 (Nramp1) which affects the macrophage's ability to destroy ingested intracellular parasites early in the infectious process (Vidal *et al.* 1993).

Nramp1 mRNA is not ubiquitously expressed but found in high levels in phagocytic cells located within the spleen and liver. Immunofluorescence and confocal microscopy studies have shown that Nramp1 is located in the lysosomal compartment of phagocytes (Gruenheid *et al.* 1997). Upon phagocytosis, Nramp1 is recruited to the membrane of the phagosome where it remains during maturation to a phagosome. As of yet the exact mechanism of Nramp1 action has not been identified, although the identification of a separate member of the Nramp family by cross hybridisation cDNA clones (Nramp2) may aid the discovery. Nramp2 is highly similar to Nramp1 (78% homology) but is ubiquitously expressed (Gruenheid *et al.* 1995). A role for Nramp2 as a transporter for divalent cations has been suggested due to a mutation in Nramp2 being discovered in the *mk* mouse mutant (a mouse model of iron deficiency caused by severe defects in intestinal iron absorption) (Fleming *et al.* 1997). This association of Nramp2 has led to the hypothesis that Nramp1 is also involved in divalent cation transport. By depleting the phagosomal environment of Fe^{2+} or other divalent cations such as Mg^{2+}, Zn^{2+} or Ca^{2+}, Nramp1 may remove nutrients required for microbial growth and survival.

The human homologue NRAMP 1 has now been identified and contrary to the murine gene is found expressed in the lungs, liver, and spleen but is most abundant in peripheral blood neutrophils (Cellier *et al.* 1997). Although the function of human *Nramp1* has been characterised far less than the mouse gene, it is also associated with susceptibility to *Mycobacterium* spp. (Bellamy *et al.* 1998).

More recently, mutations in the human IFNγ receptor I have also been identified as mediating susceptibility to infections with *Mycobacteria*, (reviewed in (Abel and Dessein 1997)). IFNγ is a potent macrophage activator and appears to be required for development of optimal macrophage anti-mycobacterial activity.

In addition, currently unidentified host factors influence susceptibility to infections due to *Pseudomonas aeruginosa* (Morissette *et al.* 1995), and *Candida albicans* (Ashman and Papadimitriou 1992).

Notably missing from the above list of pathogens are extracellular bacteria. To this date no candidate genes for resistance to such organisms have been identified. During the 1930s it was first recognised that pathology during pneumococcal pneumonia was dependant on the strain of mouse as well as the capsular serotype of the bacterium, suggesting a host genetic component

to disease susceptibility (Schütze *et al.* 1936). Such findings were also described more recently by Briles *et al* (Briles *et al.* 1986).
At present the nature of any host factor(s) involved in determining the outcome to pneumococcal infection is unknown. For this reason we have established models of pneumococcal pneumonia in inbred strains of mice that are genetically resistant or susceptible to the infection with the long term goal of identifying possible genes (Gingles *et al.* Manuscript in Preparation). By infecting a range of inbred strains of mice BALB/c were found to be resistant and CBA/Ca mice susceptible to intranasal infection with 10^6 CFU *S. pneumoniae*. Investigation of bacterial loads following infection revealed that BALB/c mice were able to control the organisms within their lungs and prevent spread to the bloodstream thereby surviving the infection. In contrast pneumococci proliferated unchecked within the lungs and circulation of CBA/Ca to result in a median survival time of only 27h post challenge.
Analysis of the host cellular response to infection (by whole lung homogenates) revealed that both the total number of leukocytes and the number of neutrophils recruited to BALB/c lungs by 12h and 24h post challenge were significantly higher than the levels seen in CBA/Ca mice. This difference was also evident in histological studies with inflammatory lesions visible earlier and to a greater extent in BALB/c lung sections than in CBA/Ca lungs. Thus the inflammatory response is upregulated earlier and to a greater extent in mice that are genetically resistant to pneumococcal pneumonia. As described below, cell influx is initiated and controlled by a range of host inflammatory mediators secreted by resident lung cells. We have therefore hypothesised that differences in the ability of inbred strains of mice to produce these inflammatory mediators may explain their susceptibility/resistance to pneumococcal pneumonia. This hypothesis was tested as part of this thesis (Chapter 5).

1.4.4 Problems with control of pneumococcal pneumonia

The standard outpatient treatment for pneumococcal pneumonia in the United Kingdom is oral administration of penicillin G for 5-7 days (Musher

1992), with hospitalised patients being treated intravenously. In non-industrialised nations treatment is generally with oral amoxycillin (providing a substantially broader spectrum of coverage than required for pneumococcal infection) with more serious cases given parenterally administered penicillin. The efficacy of all therapeutic agents is currently being threatened by antibiotic resistance (Goldsmith *et al.* 1997) & (Hart and Kariuki 1998).

1.4.4.1 Antibiotic resistant *S. pneumoniae*

The first clinically significant pneumococcal strains with reduced sensitivity to penicillin were identified in Australia and Papua New Guinea during the 1960s (Hansmann and Bullen 1967). By 1974 the incidence of penicillin resistant pneumococci in this area was 12% (Hansmann *et al.* 1974) and by 1980 it had reached 30% (Gratten *et al.* 1980).

Recent studies have identified a real problem in Western nations, some areas of North America are currently reporting that 40% of pneumococcal isolates are penicillin resistant (Butler and Cetron 1999). In non-industrialised countries percentages of antibiotic resistant bacteria are increasing even more dramatically due to unregulated use of antibiotics in humans and animals (Hart and Kariuki 1998).

At present vancomycin is the antibiotic of last resort, with no isolates of vancomycin resistant pneumococci reported. Recently, emergence of vancomycin tolerance (the ability of pneumococci to survive but not grow in the presence of antibiotics) has been detected (Novak *et al.* 1999). Tolerance is generally believed to be a precursor phenotype to resistance. Thus in the short term, strains of *S. pneumoniae* resistant to all appropriate antibiotics may emerge.

1.4.4.2 Ineffective pneumococcal vaccine

Despite successful research into type-specific protection with capsular polysaccharide preparations in the 1930s and 1940s (Lee *et al.* 1991), interest in vaccines against *S. pneumoniae* infection was to wane quickly with the discovery of antibiotics. The scientific community became complacent, believing that a vaccine was now redundant, despite the fact that around 20%

of patients over 65 years of age with pneumococcal bacteraemia die following appropriate antibiotic treatment (Butler and Cetron 1999). Recognition of this fact in the 1970s led to renewed interest in the development of improved pneumococcal vaccines. In 1983 a vaccine containing capsular polysaccharide against 23 serotypes of pneumococci became available. This vaccine is effective against those serotypes responsible for greater than 90% of cases of pneumococcal pneumonia in the United States and Europe (AlonsoDeVelasco *et al.* 1995) but less than 70% of pneumococcal infections in Asia (Lee 1987).

The vaccine is associated with low efficacy rates due to certain groups having poor antibody responses to polysaccharide based vaccines (White 1988). Development of a more effective vaccine for use in infants may result from conjugation of capsular polysaccharide to a protein carrier (e.g. *H. influenzae*) (Ahman *et al.* 1998). These work on the basis that the infant's immune system responds to protein antigens which act as a boost for the polysaccharide antigen (Käyhty and Eskola 1996). Phase three clinical trials are currently being carried out with these preparations. Disadvantages still exist in that fewer capsular serotypes can be covered (at present only 9) and they are likely to be too expensive for use in non-industrialised countries.

1.4.5 Disease progression of pneumococcal pneumonia

In the classic description of pneumococcal pneumonia there are four overlapping stages: engorgement, red hepatisation, grey hepatisation and resolution.

The arrival of bacteria in the alveoli results in congestion of the alveolar capillaries and outpouring of serous fluid. This fluid acts both as a nutrient source and a mode of transport for the bacteria outward via the pores of Kohn to adjacent alveoli (Harford and Hara 1950).

Further engorgement of the capillaries with erythrocytes leads to red hepatisation when neutrophils, a few macrophages and many erythrocytes pass from capillaries via the interstitium to fill the alveolar spaces (Loosli and Baker 1962). Large amounts of fibrin are evident in alveolar and serosal exudates.

Within a few hours, as exudate continues to accumulate in the alveoli, capillaries are compressed, red cell content reduced, and leukocyte content is

increased (McKinsey and Bisno 1980). The intravascular deposition of fibrin causes a decline in perfusion, resulting in an appearance known as grey hepatisation (Kline and Internitz 1915).

Pneumococci that manage to evade the immune response spread into the interstitial lung tissues and then to the bloodstream. In immunocompetent patients a period of several days to weeks can pass before disease progresses to this stage, although most deaths from bacteraemic pneumococcal pneumonia occur within the first 5 days of illness (Sato *et al.* 1998). In addition, splenectomised individuals can succumb in 18-24h with no symptoms (Musher 1992).

The cause of death in pneumococcal pneumonia remains a puzzle. Even after antibiotic treatment has begun, with the pneumonia clearing and the previously infected site sterile, death can still occur (Austrian and Gold 1964). More detailed understanding of the roles played by host factors in disease pathogenesis would be expected to help answer this question and thus aid the search for more effective control measures.

1.4.6 Resolution of pneumococcal pneumonia

A striking finding with patients that recover fully from pneumococcal pneumonia is that their lungs possess no permanent injuries despite the massive inflammatory response they have endured (Catterall 1999).

Resolution is believed to occur after the appearance of anti-capsular antibodies, which enable more efficient engulfment of the bacteria by phagocytes. Following phagocytosis of pneumococci, neutrophils themselves must be removed from the airways and tissues of the lungs. The widespread belief with regard to this process is that the cells undergo necrosis prior to their engulfment by macrophages. More recent results indicate that neutrophils become apoptotic rather than necrotic (Haslett 1992) & (Haslett 1997). With such "programmed cell death" neutrophils retain their granule contents and membrane integrity but no longer have the capability to secrete contents in response to external stimuli (Haslett 1992). In addition, macrophages that have ingested apoptotic PMN do not release pro-inflammatory mediators. These macrophages are then cleared by mucociliary transport (Section 1.5.1).

Deposited fibrin acts as a provisional matrix into which fibroblasts migrate in order to repair damaged lung interstitium (Simon and Paine 1995). Type II epithelial cells also spread over this matrix before proliferating to repair damaged epithelia. The inflammatory cytokines IL-1β and TNFα (Section 1.6) have been shown to act as primers for alveolar epithelial type II cell chemotaxis (as measured by passage through filters) when cells were pre-incubated with these cytokines prior to addition of a type II cell chemokine (epidermal growth factor) (Lesur *et al* . 1996). In contrast IL-6 was shown to regulate the chemotaxis in a negative manner. Following fibroblast and epithelial cell migration the fibrin scaffolding is removed by plasminogen activators (Bertozzi *et al.* 1990).

Clearance of fluid, proteins and debris occurs by active transport mechanisms (Chapter 6), lymphatic drainage and phagocytosis by macrophages.

As the alveolar walls are not destroyed during pneumococcal pneumonia (McKinsey and Bisno 1980), the pulmonary parenchyma returns to normal following resolution of the infection.

1.5 Protective immunity against *S. pneumoniae*

Inhaled air and aspirated aerosols of upper-respiratory secretions contain high levels of viable bacteria, often as high as 10^8 or 10^9 CFU/ml. Despite such a constant threat from both commensal and environmental organisms, in most cases the lungs remain free of infection. It is the responsibility of the defence mechanisms of the respiratory tract (structural, mechanical, cellular, and secretory) to ensure that this is the case (Figure 1.3).

1.5.1 Structural and mechanical defences against *S. pneumoniae*

The first line of defence against the foreign bacteria is the anatomical design of the respiratory tract. Large particles of 10μm or more will be deposited in the nasal passages due to their curved architecture. The frequent branching of bronchi causes particles of 2μm-10μm to impact in the peripheral conducting airways. Coughing and sneezing also act to prevent particles passing beyond the respiratory bronchioles (Pison *et al.* 1994).

Only particles between 0.5μm and 2μm therefore become deposited in the terminal respiratory units and alveoli (Nelson and Summer 1998).

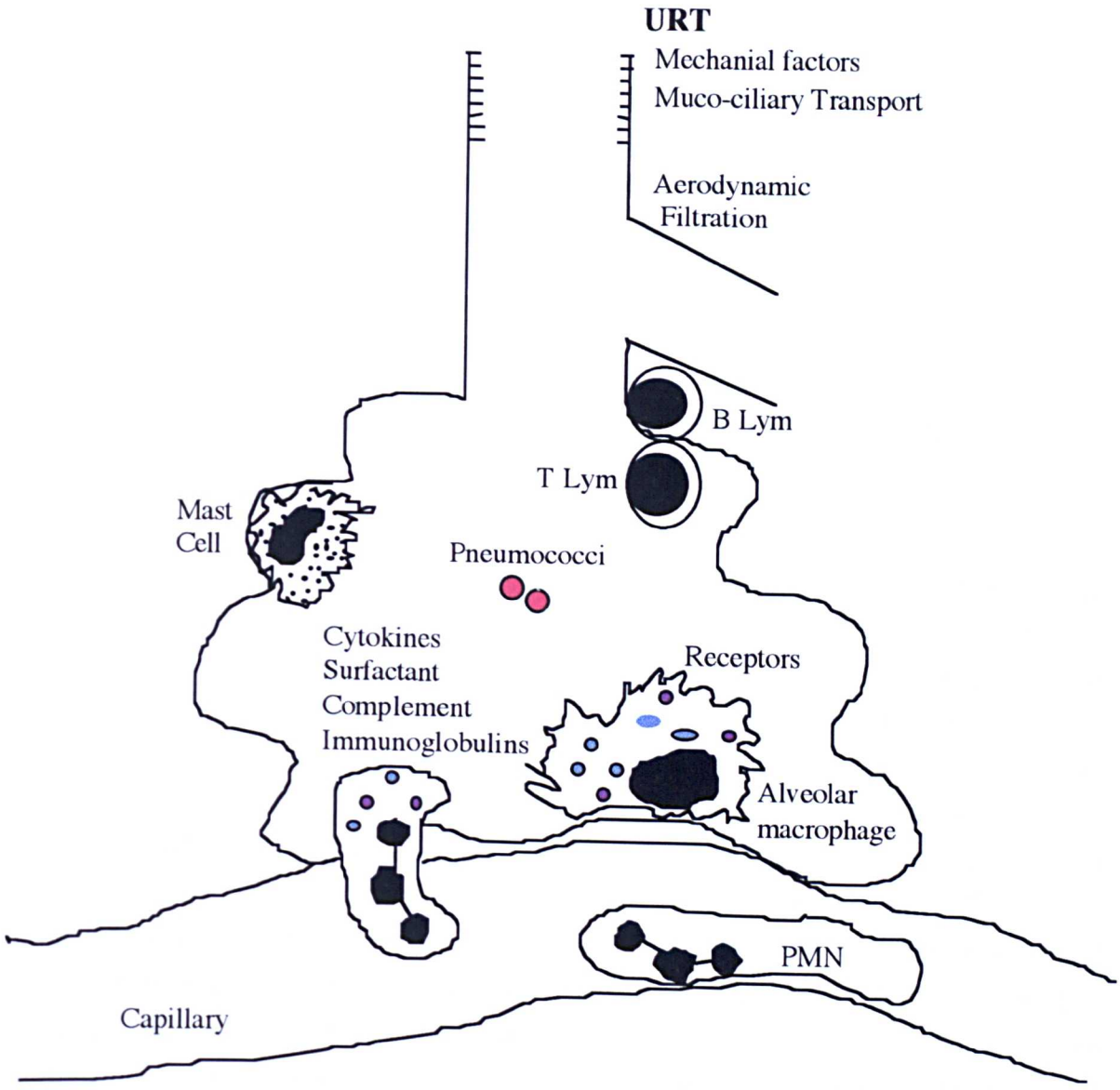

Figure 1.3 Alveolar unit enlarged to illustrate local defences that can combat pneumococci entering it. URT= upper respiratory tract, B Lym= B lymphocyte, T Lym= T lymphocyte, PMN= polymorphonuclear leukocyte (neutrophil).

Following contact with the walls of the respiratory tract, foreign particles become opsonised in respiratory secretions and embedded in the mucous layer overlying the ciliated epithelium. By placing markers such as India Ink or radio-labelled particles in the airways and measuring the distance travelled with time, it has been estimated that the beating movement of the ciliated epithelium continuously moves the mucous layer upwards at a rate of 0.5-1mm/min in the small airways and 5-20mm/min in the larger airways (Wanner 1977).

1.5.2 Cellular defence mechanisms defences against *S. pneumoniae*

1.5.2.1 Macrophages

Alveolar macrophages constitute the first line of cellular anti-bacterial defence and are ideally situated between the lung tissue and air (Lohmann-Matthes *et al.* 1994). In a normal resting animal, these cells constitute greater than 90% of the cells of bronchoalveolar lavage fluid (Reynolds 1987).
Alveolar macrophages are believed to mainly originate from monocytes recruited out of the bloodstream to differentiate within the alveoli. The rapid recruitment of macrophages during acute inflammation suggests that local proliferation of the population may also occur (Toews *et al.* 1979).
Additional populations of macrophages within the lungs include interstitial and intravascular macrophages as well as dendritic cells. Interstitial macrophages are located within the lung connective tissue and function in both an innate immunity and an antigen presenting capacity (Lohmann-Matthes *et al.* 1994). Dendritic cells can be found in low numbers within the lung interstitium. This population have low phagocytic function and are specialised for antigen presentation. Intravascular macrophages are evident attached to the endothelium of pulmonary capillaries (Dehring and Wismar 1989). They are highly phagocytic (unlike monocytes) and can remove bloodborne bacteria entering the lungs via the circulation (Warner *et al.* 1987), although they do not appear to play a major role in mice (Brain *et al.* 1999).
Alveolar macrophages are capable of engulfing bacteria rapidly following infection. In the case of *S. aureus*, 80% of the bacteria are ingested within 1h (Goldstein *et al.* 1974). In order to engulf bacteria macrophages express a

range of receptors on their surface including the Fc receptors, complement receptors and lectin receptors (Lohmann-Matthes *et al.* 1994).
Following phagocytosis pulmonary macrophages possess several mechanisms of killing ingested bacteria. The phagosomes fuse with lysosomes in the cell cytoplasm. These lysosomes contain acid hydrolases that constitute an anaerobic killing pathway. Unlike monocytes and neutrophils, airway macrophages do not possess much myeloperoxidase and therefore do not utilise hypochlorous acid as an anti-microbial. They do, however, exhibit a "respiratory burst" producing reactive oxygen intermediates and reactive nitrogen intermediates (Poulter 1997) (Figure 1.4), They also release free oxygen radicals, proteases and acid hydrolases into their environment and thus may contribute to extracellular killing of pneumococci.
In addition to their role as phagocytes, alveolar macrophages are central to the induction of pulmonary inflammatory responses. These cells are capable of secreting a wide range of inflammatory mediators including cytokines (Section 1.6), nitric oxide (Section 1.5.3.5) and metabolites of the arachidonic acid pathways. These mediators can act in both a paracrine and autocrine manner to mediate macrophage functions such as further cytokine release, antigen presentation and phagocytosis (Cavaillon 1994).

1.5.2.2 Polymorphonuclear leukocytes (neutrophils)

Within the healthy host the majority of neutrophils are found in the circulation, although small numbers are present in the air spaces (less than 0.15% (Vial *et al.* 1984)). Additional neutrophils are not recruited to the lungs until the defences of the alveolar macrophages are overcome (Toews *et al.* 1979). When required, PMN are sequestered from blood vessels (it is estimated that 40% of the body's neutrophils are marginated within the lung capillaries (Nelson *et al.* 1995) or released from the bone marrow.
Normal traffic of neutrophils is along the inner surface of blood vessels, making contact with the endothelium. Initial tethering occurs via the L-selectin constitutively expressed on neutrophils and the counter receptor expressed on endothelial cells (Hogg and Walker 1995). This interaction is not strong and is easily broken resulting in neutrophils "rolling" along the endothelial surface. More secure attachment occurs via the E (endothelial)

and P (initially formed in platelets) selectin molecules on the endothelium and their receptors on neutrophils. These interactions are assisted by integrins on the surface of leukocytes (CD11/CD18) which interact with immunoglobulin-like counter-receptors on the endothelium (intercellular adhesion molecules 1&2 [ICAM-1, ICAM-2] and vascular cell adhesion molecule 1 [VCAM-1]) (Hogg and Walker 1995). Neutrophils then migrate out of post-capillary venules at locations where three endothelial cells meet as the tight junctions between cells are incomplete at these sites (Walker *et al.* 1991). Chemotactic stimuli include complement proteins (Section 1.5.3.4) and chemokines (Section 1.6.1.1.11).

On engulfing bacteria, neutrophils utilise both oxygen dependent and independent anti-microbial mechanisms (Burnett 1997). The secretion of toxic oxygen metabolites (also known as the respiratory burst) occurs after the formation of NADPH oxidase on the surface of activated neutrophils (and macrophages./ monocytes). This enzyme progressively adds single electrons to O_2, forming $O_2^{\bullet}$ which in turn can be reduced to H_2O_2, directly toxic to micro-organisms. H_2O_2 can be reduced further to $HO^{\bullet}$, which when finally reduced, generates H_2O. H_2O_2 can also be metabolised in the presence of myeloperoxidase (MPO, found in neutrophil granules) and chloride ion to hypochlorous acid (HOCl), which is a potent anti-microbial (Nelson and Summer 1998), Figure 1.4.

Oxygen-independent anti-microbial systems include acid hydrolases such as elastases, proteases which can degrade proteins in bacteria and lysozyme with its ability to degrade cell walls (Jacquot *et al.* 1987). Defensins constitute around 5% of the protein content of PMNs and are capable of creating channels in bacterial lipid membranes, destroying permeability barriers (Nelson and Summer 1998).

1.5.2.3 Lymphocytes

Lymphocytes within the lungs are found as aggregates below the mucosal layer of airways (bronchus-associated lymphoid tissue [BALT]), within the air spaces and interstitium and in the intravascular compartment (Pabst 1997).

We and others have shown that the number of lymphocytes within lungs of infected animals increases during pneumococcal pneumonia (Kadioglu *et al.* 2000) & (Bergeron *et al.* 1998). However, the roles of these recruited

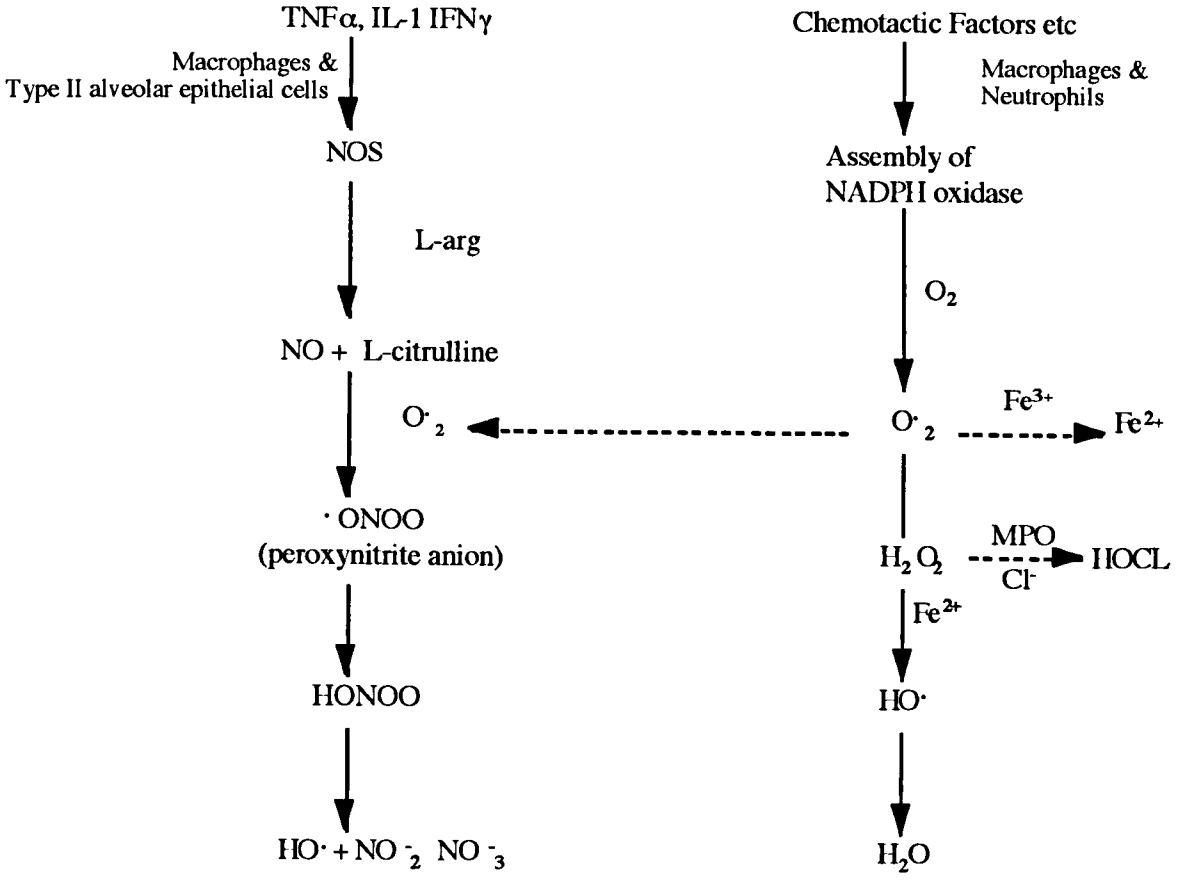

Figure 1.4 Oxidant -generating pathways within the infected lung.

lymphocytes are not fully understood. Nude mice (T cell deficient) are no more susceptible to pneumococcal infection than wild-type animals (Winkelstein and Swift 1975) suggesting that T cells are not required for a protective response against *S. pneumoniae*. Conversely, Sestini *et al* have indicated that lymphocytes are involved in antibody mediated cytotoxicity against *S. pneumoniae in vitro* (Sestini *et al.* 1987). The lymphocytes responsible possess the phenotype of T helper cells and utilised IgA in order to mediate their anti-bacterial activity (Sestini *et al.* 1988).
Lymphocytes are known to be capable of releasing a range of cytokines (Section 1.6) that possess the ability to activate and potentiate the effects of other cell populations. Thus the importance of lymphocytes in pneumococcal pneumonia may be due to their regulatory capacity.
Our recent finding that pulmonary lymphocytes underwent redistribution during pneumococcal pneumonia (Kadioglu *et al.* 2000) led us to investigate the role of lymphocytes in controlling bacterial loads and contributing to pulmonary and systemic inflammation as part of this study.

1.5.2.4 Mast cells

Mast cells are derived from CD34+ progenitor cells within the bone marrow (Kirschenbaum *et al.* 1991). These progenitors migrate to tissues such as the genitourinary, gastrointestinal and respiratory tracts which are primary sites of infection for a wide range of organisms.
The most striking feature of mast cells is the presence of 50-200 densely packed granules within their cytoplasm (Stites *et al.* 1994). These granules contain histamine, serotonin, and TNFα (Section 1.6.1.1). Mast cells are the only cells known to store pre-formed TNFα which they quickly release (along with newly synthesised TNFα) on activation (Gordon and Galli 1990) & (Gordon and Galli 1991). Degranulation also releases histamine and serotonin into the cell's environment. Each of these three substances is capable of causing disruption to the surrounding tissue with the latter two also known to cause vasodilation when present in high concentrations. This effect increases blood flow to the site of infection, permitting a greater influx of inflammatory cells (Szarek *et al.* 1992) & (Cushing and Cohen 1992). Physical contact between the mast cell and bacteria is not required for mast

cell activation as the cells can be activated at a distance by bacterial products such as toxins (Abraham and Malaviya 1997).
In addition to TNFα, mast cells have been found to secrete a range of cytokines such as IL-1, IL-6, IL-8, IL-10 and several chemokines (Lorentz *et al.* 2000) & (Mecheri and David 1997). This implicates mast cells as important first line defences against pathogenic micro-organisms. Indeed mast cells are able to phagocytose and kill both Gram-positive and Gram-negative bacteria. Uptake of Gram-positive bacteria is higher and associated with far higher TNFα than Gram-negative organisms(Arock, Ross *et al.* 1998). However, the phagocytic efficiency of these cells appears to be far less than that of macrophages and neutrophils.
The importance of mast cells during pulmonary and systemic infections has been highlighted by experiments carried out in mast cell deficient mice (Malaviya *et al.* 1996). These animals are less able to clear invading bacteria such as *Klebsiella pneumoniae* due to impaired neutrophil recruitment at sites of infection (both systemic and local). This was shown to reflect a reduction in TNFα levels. In addition, increasing the number of mast cells by treating mice with c-kit ligand (also referred to as mast cell growth factor) results in animals that are more resistant to bacterial peritonitis induced by cecal ligation and puncture (Maurer *et al.* 1998).

1.5.3 Secretory pulmonary defences against *S. pneumoniae*

Proteins within respiratory secretions can either be produced locally within the airway lumen or they can pass through the alveolar/capillary interface (Stockley 1997). The degree of transudation depends on the molecular size of the substance and the integrity of the blood-air barrier.

1.5.3.1 Surfactant

Surfactant (*surf*ace *act*ive *agent*) is comprised of phospholipid-rich lipoproteins and acts to reduce the surface tension in the lungs permitting their expansion. In addition to this physiological role, the ability of surfactant preparations to modulate the immune response has recently been recognised. The majority of these functions are attributed to surfactant proteins, of which five are currently known, SP-A 1 & 2, SP-B, SP-C & SP-D.

SP-A and SP-D are members of the collectin family of proteins which are characterised by the presence of both a collagenous region and a lectin domain, which mediates binding to carbohydrates on the surface of micro-organisms. This family also contains mannose binding lectin (MBL) and two bovine serum lectins, conglutinin and CL-43. MBL is now recognised to activate the alternative complement system and will be discussed in more detail in Section 1.5.3.4.3. Recent data suggest that the lung collectins (SP-A and SP-D) are able to modulate pulmonary inflammatory responses.

SP-A is highly efficient at opsonising bacteria (including *S. pneumoniae*) in order to promote their phagocytosis by alveolar macrophages. Indeed addition of SP-A to FITC (fluorescein isothiocyanate) labelled pneumococci in the presence of rat alveolar macrophages resulted in a higher level of phagocytosis than IgG opsonised bacteria (Schagat *et al.* 1999). When both SP-A and IgG were present the two opsonins acted synergistically.

In vitro both SP-A and SP-D are known to act as chemoattractants for neutrophils and macrophages, with SP-D more effective in this capacity than SP-A as higher levels of SP-A were required to exert the same effect as lower levels of SP-D (Madan *et al.* 1997) & (Wright and Youmans 1993).

Following phagocytosis, both pulmonary collectins can increase the production of reactive oxygen species (assessed by chemiluminescence) by either neutrophils or macrophages (van Iwaarden *et al.* 1990) & (van Iwaarden *et al.* 1992). The effects of SP-A can be attenuated by addition of surfactant lipids but such treatment does not affect SP-D induced production

Finally SP-A has also been shown to be capable of modulating the pulmonary inflammatory response by altering cytokine production. This protein can act to induce release of immunoglobulins and pro-inflammatory cytokines such as TNFα, IL-1 and IL-6 from peripheral blood mononuclear cells, splenocytes and alveolar macrophages (Kremlev and Phelps 1994). Conversely, SP-A has also be ascribed an anti-inflammatory role in that it can reduce TNFα activity released from LPS-stimulated alveolar macrophages *in vitro* (McIntosh *et al.* 1996). Such differences may reflect the local environment the inflammatory stimulus and levels of other inflammatory mediators.

1.5.3.2 Lysozyme

Lysozyme is a muraminidase capable of degrading bacterial cell wall peptidoglycan. This enzyme is the major secretory enzyme of the alveolar macrophage, accounting for approximately 25% of the protein material released (Canto *et al.* 1994). Although its effects have not been widely studied it is known to be active against *S. pneumoniae* (Jacquot *et al.* 1987). Culture of pneumococci in media with 1000µg/ml final concentration of human airway lysozyme resulted in delayed growth of the bacteria. Furthermore, although the addition of lysozyme to a culture in early logarithmic growth phase still permitted the bacteria to multiply, reduced viability counts were recovered when compared to media alone cultures.

1.5.3.3 Immunoglobulins

IgA, IgG, IgD, IgE, and IgM immunoglobulins are all detectable within respiratory secretions (Stockley 1997). Whilst a role in innate lung defence has been identified for the initial two isotypes (upregulation of phagocytosis by alveolar macrophages (Richards and Gauldie 1985)), roles for the latter three isotypes have not been identified. The importance of antibody production for protection against *S. pneumoniae* is highlighted by the increased susceptibility of hypogammaglobulinemic patients to pneumococcal infections (Rosen and Janeway 1966).

1.5.3.4 Complement

Like other Gram-positive bacteria, the pneumococcus is resistant to the bactericidal and lytic activities of complement (Kemper and Deresinki 1994). The role of complement in *S. pneumoniae* infection is therefore to aid phagocytosis of pneumococci by opsonisation.

1.5.3.4.1 The classical complement pathway

Formation of an immune complex occurs when antibodies of either IgG or IgM class combine with an antigen (Figure 1.5) A conformational change then occurs in the Fc portion of the antibody molecule permitting the immune complex to bind and activate the C1 component of complement. Activated C1 possesses the ability to activate C2 and C4 which in turn cleave

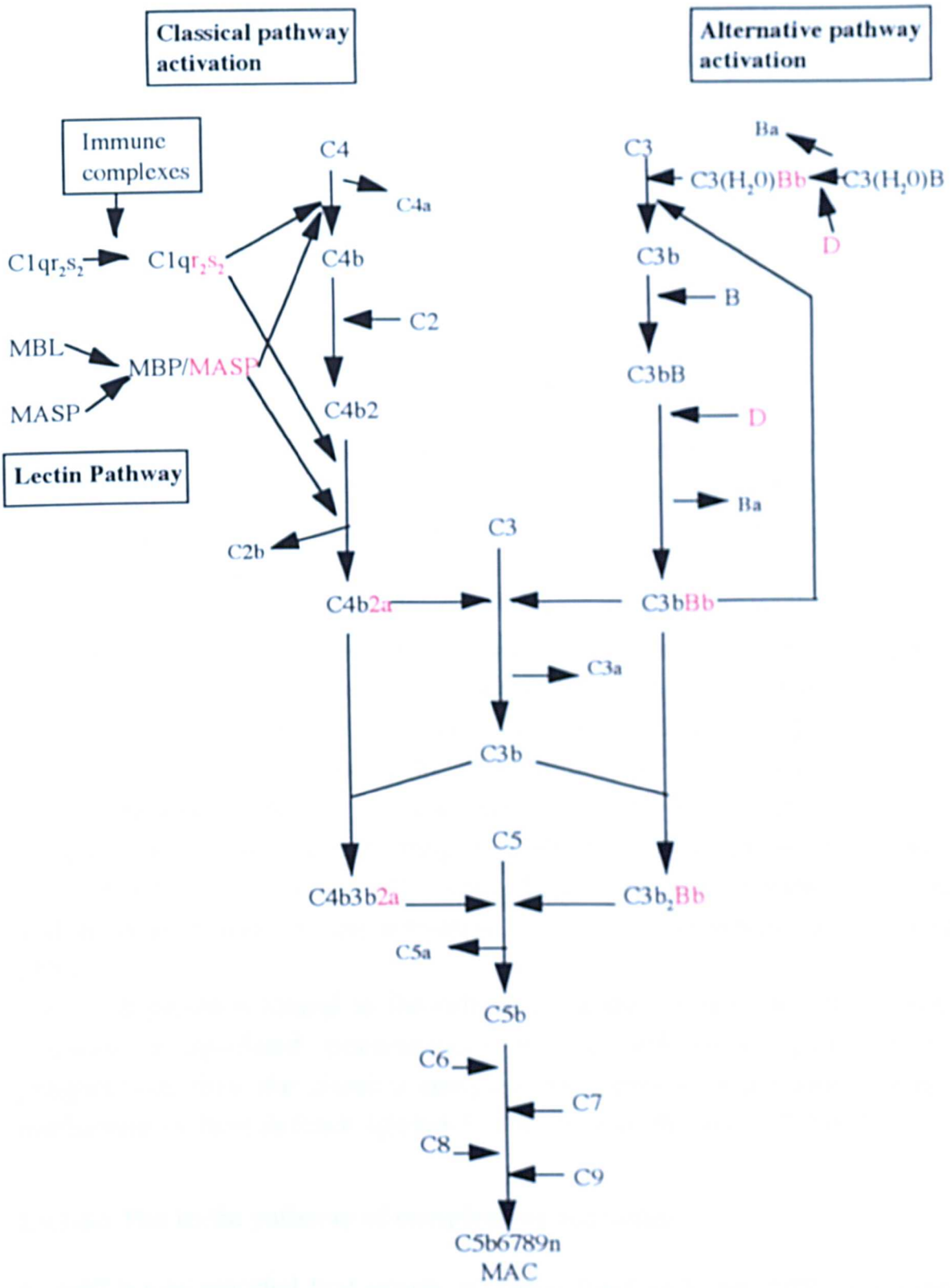

Figure 1.5 The complement cascade. MBL= mannose binding lectin, MASP= mannose binding lectin serine protease, D= factor D, B= factor B and MAC= membrane attack complex. Red text indicates active components, see text for details.

C3 into C3a and C3b. Interaction of C3b with C2 and C4 activates the remaining components of the cascade (Winkelstein 1981).
Activation of the classical complement system by pneumococci coated in anti-capsular antibodies permits deposition of C3b on the outer surface of pneumococci and is believed to play a major role in host defence against *S. pneumoniae* by increasing efficacy of phagocytosis (Brown *et al.* 1983).
Pneumolysin is also capable of initiating the classical complement pathway (Section 1.3.2).

1.5.3.4.2 The alternative complement pathway

The activating complex in this pathway is comprised of C3b and the cleavage product of Factor B (Bb) which is generated by active factor D (Figure 1.5). This C3b,Bb complex is highly efficient and cleaves C3 as for the classical pathway (Winkelstein 1981).
As the alternative pathway does not require the presence of specific antibodies in order to function, this pathway is probably of more importance during early infection before type-specific antibodies are generated.
Pneumococci are capable of direct activation of the alternative pathway. By testing a range of pneumococcal preparations *in vitro*, Winkelstein & Tomasz discovered that the cell wall was responsible for depleting C3 levels in serum and thus was activating the alternative complement pathway (Winkelstein and Tomasz 1977). They then went on to identify cell wall teichoic acid residues as the activating fraction (Winkelstein and Tomasz 1978).
Since C3b becomes bound to the cell wall via the alternative complement pathway, encapsulated pneumococci are not efficiently opsonised for phagocytosis, thus the classical complement pathway is a more effective mechanism of host defence against *S. pneumoniae* (Brown *et al.* 1983).

1.5.3.4.3 The lectin pathway of complement activation

In 1987 it was reported that serum mannose binding lectin (MBL) possessed the ability to activate the classical complement pathway (since sheep erythrocytes coated in mannan were lysed by incubation in MBL depleted

complement) (Ikeda *et al.* 1987). MBL has since been shown to share structural homology with C1q.
In order to activate complement, MBL associates with mannose binding lectin serine protease (MASP), a serine protease with the same function as activated C1s and is found in the same fraction of human serum as MBL when separated by sequential affinity chromatography. This cleaves both C2 and C4, generating C4b2a complexes that possess the ability to convert C3 into C3a and C3b (Matsushita and Fujita 1992) (Figure 1.5).
It is now generally accepted that this pathway does occur in human serum deficient in C1q but whether it occurs physiologically in the presence of C1q remains a contentious issue.

1.5.3.4.4 Sources of complement proteins

Most complement components within the lung are derived from plasma during inflammation (Stockley 1997). However, low levels of complement proteins must be present within the lungs during health as a functioning alternative complement pathway has been detected within the lavage fluid of normal rats (Coonrod and Yoneda 1981). This fact may be explained by the ability of pulmonary macrophages and epithelial cells to synthesise and secrete C2, C4, C3, and C5 (Strunk *et al.* 1988).

1.5.3.4.5 Effects of complement proteins

The most important complement factors in pneumococcal infection are C3 and C5. Both C3a and C5a are capable of stimulating mast cell and neutrophil degranulation and affecting vascular permeability (Lukacs and Ward 1996). C5a appears to be more potent than C3a in these respects and possesses the additional ability of stimulating neutrophil migration and degranulation. In addition to these inflammatory roles played by C3a and C5a, it has recently been recognised that *in vitro* C1q can also mediate inflammatory responses by elevating the production of pro-inflammatory cytokines (IL-6, IL-8 and MCP-1) by human endothelial cells (van den Berg *et al.* 1998).
C3b and C5b are able to opsonise pneumococci, as revealed by depleted C3 and C5 levels in serum following incubation with the bacteria and

agglutination and capsular swelling of opsonised bacteria following the addition of anti-C3 serum opsonins (Shin *et al.* 1969).
Direct evidence of the role of C5 in protection against *S. pneumoniae* is highlighted by the inability of mice deficient in C5 to clear the organisms, unless exogenous C5 is administered, due to impaired neutrophil recruitment (Toews and Vial 1984). In addition, humans with complement factor deficiency are more likely to become infected with *S. pneumoniae* than are immunocompetent individuals (Newman *et al.* 1978).

1.5.3.5 Nitric oxide (NO)

Nitric oxide is a gaseous free radical that exhibits multiple biological properties. The molecule was first shown to be produced by living cells in 1987 and was subsequently confirmed to be identical to the endothelium-derived relaxing factor (Ignarro *et al.* 1987) & (Palmer *et al.* 1987).

1.5.3.5.1 NO production

NO is generated when L-arginine is oxidised to produce one molecule each of L-citrulline and NO. This reaction is catalysed by the enzyme nitric oxide synthase (NOS) (Ward 1997).
There are three isoforms of NOS in mammalian systems, two of which are constitutively expressed and dependant on elevated intracellular Ca^{2+}, eNOS (endothelial NOS or NOS3) and nNOS (neuronal NOS, NOS1). eNOS and nNOS are collectively referred to as cNOS (MacMicking *et al.* 1997). The third isoform, iNOS (NOS2), is inducible and independent of intracellular Ca^{2+}. All three mammalian isoforms of NOS are thought to catalyse the production of NO by the same biochemical pathway represented in Figure 1.4.
Stimulation of macrophages with pro-inflammatory cytokines induces NOS production. NOS converts L-arginine into nitric oxide (NO) and L-citrulline. NO can then react with superoxide anion ($O^{\bullet}_2$) generated from another pathway to form the highly reactive peroxynitrite anion ($^{\bullet}ONOO$), which, when protonated in an acidic environment readily forms an unstable species, HONOO. This cleaves itself into the hydroxyl radical ($HO^{\bullet}$) and ultimately, the formation of nitrite (NO^{-}_2) and nitrate (NO^{-}_3), which are

quantitative indicators of the amount of NO originally formed. For explanation of second pathway see Section 1.5.2.2.

Generally, low-output NO production sustained by the cNOSs regulates physiologic functions of NO in the healthy host, while the high-output due to iNOS is engaged during inflammation and infection (MacMicking *et al.* 1997). cNOS is responsible for producing NO for minutes at a time whilst iNOS releases NO for as long as 5 days post stimulation (MacMicking *et al.* 1997).

Within the lungs, NO can be formed in epithelial cells, macrophages, neutrophils, mast cells, smooth muscle cells, fibroblasts, and endothelial cells (reviewed in Lukacs and Ward 1996). A given monotypic cell population can express one, two, and perhaps all three of the isoforms of NOS. Macrophages produce NO via both eNOS and iNOS (Miles, Bowman *et al.* 1998), and lymphocytes only rarely express any NOS.

1.5.3.5.2 Effects of NO

Table 1.3 highlights how variable the effects of NO can be, depending on its level of expression and the environment it is acting in (Nathan 1997). NO has been shown to diminish lung injury (Kageyama *et al.* 1997) or it can induce it (Karupiah *et al.* 1988). Chemotaxis of both LPS stimulated and unstimulated human neutrophils has been shown to be attenuated *in vitro* by the addition of nitric oxide synthase inhibitor (Belenky *et al.* 1993). This is in direct contrast to the data presented in Table 1.3 by Kubes *et al.*

Despite these anti-inflammatory effects, NO is also capable of up-regulating host defence mechanisms against micro-organisms. Growth of a range of Gram-positive bacteria, including *S. pyogenes* has been shown to be inhibited by addition of nitrite (Castellani and Niven 1955) & (Shank *et al.* 1962) and NO gas in deoxygenated media hindered the growth of *E. coli* and *L. monocytogenes.*

NO mediates its antibacterial activities via several mechanisms. Reactive nitrogen intermediates are capable of directly damaging DNA or oxidising proteins (Fang 1997). Nitrosylation of tyrosine residues can disrupt metabolic pathways involving tyrosine phosphorylation preventing these pathways from occurring, or modifying their output.

Target	Effect	Reference
Mast cells	Inhibit degranulation	(Iikura *et al.* 1998)
Monocytes	Increase pro-inflammatory cytokine production	(Eigler *et al.* 1993)
	Decrease adhesion and chemotaxis	(Bath *et al.* 1991)
Macrophages	Decrease pro-inflammatory cytokine production	(Thomassen *et al.* 1997)
	Inhibits NF-κB activation	(Raychaudhuri *et al.* 1999)
Neutrophils	Down-regulate Adhesion & Emigration	(Kubes *et al.* 1991)
	Decrease microbicidal activity	(Daher *et al.* 1997)
Endothelial cells	Decrease expression of VCAM-1	(De Caterina *et al.* 1995)
	Reduce expression of pro-inflammatory cytokines (IL-6 & IL-8)	(De Caterina *et al.* 1995)
	Disrupt integrity	(Beckman *et al.* 1990)
Airway & vascular smooth muscle	Relaxation	(Lukacs and Ward 1996)

Table 1.3 Effects of nitric oxide on the immune response.

Recently pulmonary NO production has been shown to be elevated using a murine model of pneumococcal pneumonia (Bergeron *et al.* 1998). A brief release of NO occurred within the airways of infected mice 1h post challenge. This was followed by a sustained release in lung tissues from 24h post challenge until the end of the experiment.
Despite such elevated NO and that fact that NO has potent anti-microbial activities, to our knowledge there have been no previous investigations of the role played by NO during pneumococcal pneumonia. As part of this thesis, the production and effects of NO during pneumococcal pneumonia were investigated in our model system via utilisation of mice deficient in iNOS activity.

1.6 The role of cytokines in inflammation and pneumococcal pneumonia

1.6.1 Cytokines

The body requires a wide range of effector molecules to act as intercellular mediators in the regulation of cellular function. Cytokines are one family of such effector molecules that act within the immune response. Members include the interleukins, tumour necrosis factors, interferons, colony stimulating factors and chemokines (Curfs *et al.* 1997).
Cytokines were originally described on the basis of their functions, but are now classified into a range of families, the numerical positioning in the families reflecting their order of discovery.
These molecules are highly potent, often acting at femtomolar concentrations and only requiring occupancy of around 10% of membrane cytokine receptors in order to have an effect (Fernandez-Botran 1999). Locally produced cytokines are believed to act in a paracrine and autocrine manner in order to activate the inflammatory and immune response in a beneficial manner for the host.
The interactions of cytokines with other mediators in instigation of alveolar inflammation during pneumococcal pneumonia is depicted in Figure 1.6.
Due to the important balance between pro- and anti-inflammatory cytokines in determining outcome to infection (Walley *et al.* 1996) the production of several pro-inflammatory and one anti-inflammatory cytokine was

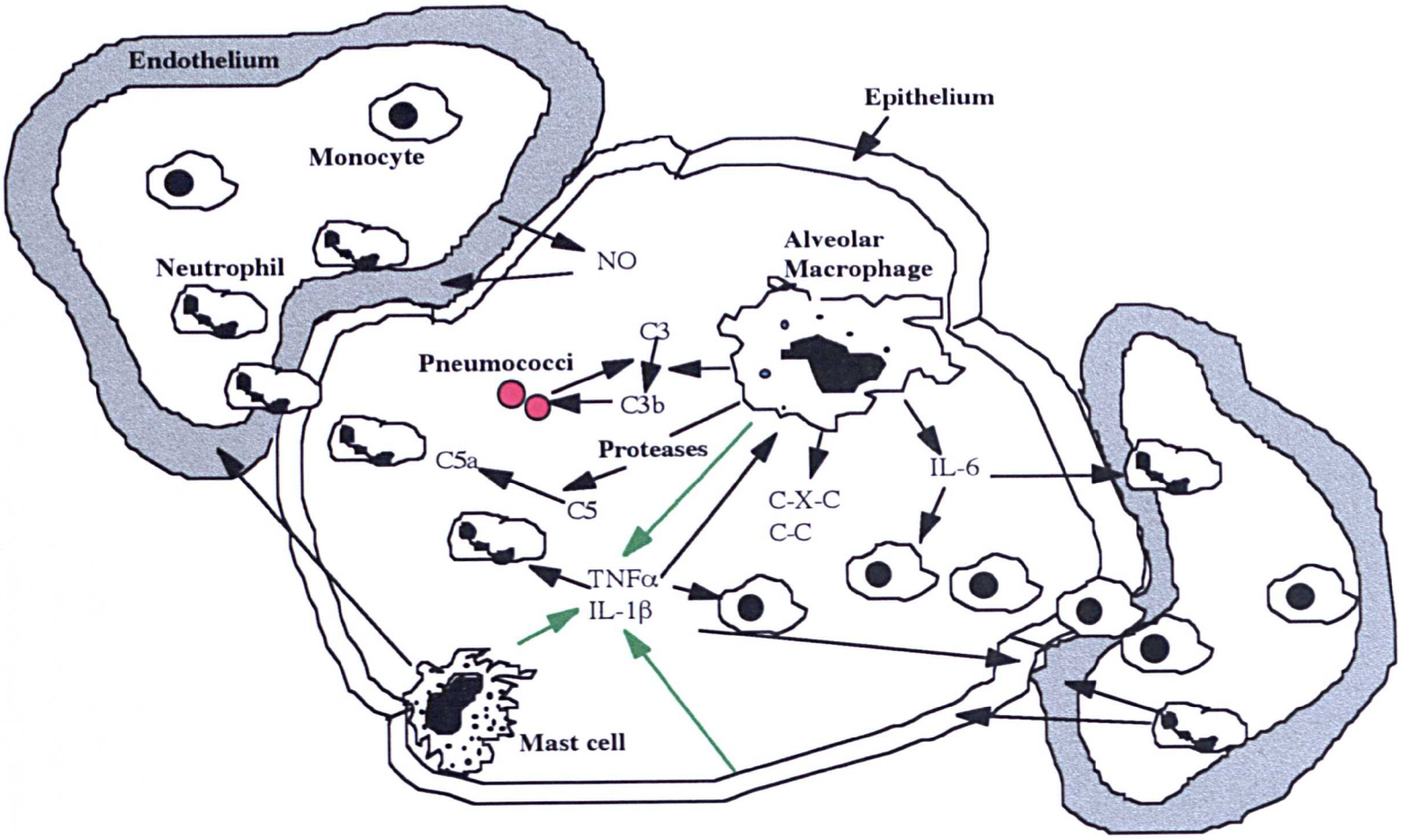
Endothelium
Monocyte
Neutrophil
NO
Epithelium
Alveolar
Macrophage
C3
Pneumococci
C3b
Proteases
C5a
C5
IL-6
C-X-C
C-C
TNFα
IL-1β
Mast cell

Figure 1.6 Initiation of alveolar inflammatory responses during pneumococcal pneumonia. Green arrows represent initial response.

Arrival of pneumococci in the alveolus induces secretion of TNFα and IL-1β from alveolar macrophages, mast cells and epithelial cells. These cytokines induce monocyte and neutrophil recruitment (aided by release of CXC and CC chemokines) as well as up-regulating the activities of these cells. TNFα and IL-1β can also regulate macrophage activities in an autocrine manner. IL-6 released by macrophages is capable of activating monocytes and up-regulating the respiratory burst of neutrophils. Macrophage derived proteases activate the complement pathways, as can pneumococci themselves, increasing the efficacy of phagocytosis and releasing chemotactic complement products. NO released by the endothelium acts in an autocrine capacity to increase the permeability of the alveolar epithelium/ vascular endothelium barrier, along with TNFα, IL-1β, mast cell serotonin and histamine and proteases released by activated neutrophils. See following sections for further explanation.

characterised in this murine model of pneumococcal pneumonia. These cytokines are by no means the only mediators involved in host response to pneumococcal pneumonia but time prevented further investigation of this field. A detailed description of the candidate cytokines follows.

1.6.1.1 Pro-inflammatory Cytokines

1.6.1.1.1 Tumor Necrosis Factor α (TNFα)

TNFα was initially identified as a factor in the serum of endotoxin treated rodents, previously infected with *Mycobacterium bovis* strain Bacillus Calmette-Guérin, that caused haemorrhagic necrosis of tumours in mice (Carswell *et al.* 1975). This was quickly followed by the recognition that the same factor was responsible for the wasting syndrome cachexia (Beutler *et al.* 1985). Since then TNFα has been implicated in a wide range of aspects of the inflammatory and immune responses.

1.6.1.1.2 TNFα producing cell types

The main cellular sources of TNFα are blood monocytes and tissue macrophages (for example alveolar macrophages) (Vassalli 1992) but many other cell types including neutrophils, mast cells, endothelial cells and NK cells are known to synthesis TNFα. Of these cell types only mast cells are known to pre-store TNFα and are thus capable of immediate release of the cytokine upon activation (Gordon and Galli 1990) & (Gordon and Galli 1991) but under certain conditions mRNA for TNFα can exist within macrophages in an untranslated form (for example in thioglycollate-elicited murine peritoneal macrophages) (Beutler *et al.* 1986). Within stimulated lungs, TNFα is predominantly expressed by macrophages (Walley *et al.* 1999) although epithelial cells may release low levels of this cytokine within healthy lungs (Pendino *et al.* 1994).

1.6.1.1.3 TNFα receptors

TNFα mediates its effects on target cells via one of two cell surface receptors named p55 (TNFRI) and p75 (TNFRII) due to their molecular weights (Brockhaus *et al.* 1990).

In order to characterise the roles of these two receptors, TNF mutants that either bind selectively to p55 or p75 have recently been developed. Intravenous administration of recombinant TNFα or the p55 agonist to primates caused the increased heart rate and drop in an arterial blood pressure characteristic of sepsis along with increased levels of IL-6 and IL-8 (Van Zee *et al.* 1994). Separate investigators have also indicated that induction of ICAM-1, E-selectin, VCAM-1, and CD44 expression on endothelial cells are mediated via p55 (Mackay *et al.* 1993). p55 is therefore believed to regulate the pro-inflammatory effects of TNFα, whilst p75 is thought to regulate these pro-inflammatory effects by acting in an antagonistic capacity and is also involved in TNFα's cytotoxic and apoptotic properties (Peschon *et al.* 1998) & (Heller *et al.* 1992).

1.6.1.1.4 Soluble TNFα receptors

Soluble TNFα receptors were originally identified as proteins within human urine that could bind and inactivate TNFα (Olsson *et al.* 1993). These proteins were later shown to represent soluble forms of the extracellular part of TNFα receptors.

Soluble TNFα p55 and p75 receptors are formed by proteolytic cleavage of the extracellular domain (Black *et al.* 1997) & (Moss *et al.* 1997), occurring in response to several stimuli including TNFα itself (Cope *et al.* 1995) and cell activation (Bemelmans *et al.* 1994).

Soluble p55 and p75 are thought to bind TNF competitively with their surface bound counterparts and therefore act as antagonists, dampening down the inflammatory response. These soluble forms may also enhance inflammation, TNF activity has been shown to undergo spontaneous decay (Aderka *et al.* 1992) but in the presence of its receptors this decay does not occur indicating that soluble receptors can stabilise TNFα. TNFα-receptor complexes may also act as reservoirs that slowly release bioactive TNFα and facilitate interactions with surface bound receptors at other sites in the body. An additional possibility is that a soluble receptor may bind to a cell which previously did not express it, allowing it to respond to TNFα (Rose-John and Heinrich 1994) & (Aderka *et al.* 1992).

Target	Effect	Reference
Monocytes	Directly chemotactic	(Ming *et al.* 1987)
	Enhance monocyte cytotoxicity	(Philip and Epstein 1986)
	Upregulate expression of itself	(Philip and Epstein 1986)
Macrophages	Enhance activation and anti-microbial activity	(Suzuki *et al.* 1994)
Neutrophils	Upregulate recruitment	(Lukacs *et al.* 1995)
	Directly chemotactic	(Ming *et al.* 1987)
	Induce NO production	(Gosselin *et al.* 1995)
	Stimulate respiratory burst	(Dusi *et al.* 1996)
	Upregulate degranulation	(Klebanoff *et al.* 1986)
	Enhance phagocytosis	(Shalaby *et al.* 1985)
Endothelial Cells	Upregulate adhesion molecules	(Neumann *et al.* 1996)
	Disrupt integrity	(Geelen *et al.* 1993)
Epithelial Cells	Increases expression of IL-6	(Cromwell *et al.* 1992)

Table 1.4 Effects of TNFα on the immune response.

1.6.1.1.5 Effects of TNFα on the immune response

TNFα is a pleiotropic cytokine acting on many aspects of the immune and inflammatory responses. This cytokine is capable of a wide range of inflammatory effects, some of those mediated by TNFα *in vitro* are described in Table 1.4.

In addition TNFα has been shown to be required for effective *in vivo* host defence against a range of micro-organisms such as *K. pneumoniae*, *Pneumocystis carinii* and *Chlamydia trachomatis* (Laichalk *et al.* 1996), (Chen *et al.* 1992a) & (Williams *et al.* 1990). The ability of TNFα to recruit and up-regulate the anti-bacterial activities of inflammatory cells is the likely explanation. However, these same effects of TNFα are implicated in the pathology occurring during systemic inflammation as TNFα neutralisation promotes survival during Gram-positive and Gram-negative septic shock (Hindshaw *et al.* 1992) & (Fang *et al.* 1989).

1.6.1.1.6 Interleukin 1 (IL-1)

The initial effects attributed to Interleukin-1 were the ability to induce fever, stimulation of the acute phase response and augmentation of lymphocyte responses (Curfs *et al.* 1997). It has since been discovered that there are two forms of IL-1, IL-1α and IL-1β, the former is cell membrane associated whilst the latter is released from cells. IL-1 is produced by a wide range of cells including monocytes/macrophages, neutrophils, T- and B-cells, epithelial cells and endothelial cells (Dinarello *et al.* 1987) & (Warner *et al.* 1987). It has been shown that IL-1β is a major product of human monocytes, constituting 1-2% of the total polyadenylated RNA following LPS stimulation (Auron *et al.* 1984).

Both IL-1α and IL-1β are synthesised are precursor forms. In this form IL-1α is fully active (Mosely *et al.* 1987) but is not secreted, remaining intracellular or membrane bound until death of the cell. IL-1α can also be released from the cells by calcium dependent membrane-associated cysteine proteases called calpains (Kobayashi *et al.* 1990). In contrast, the pro-form of IL-1β is inactive until cleaved by the intracellular IL-1β converting enzyme (ICE), a member of the cysteine protease family called caspases (proteases that cleave after an aspartic acid residue) (Cerretti *et al.* 1992). Once cleaved active IL-1β is secreted from the cell.

1.6.1.1.7 IL-1 receptors & regulation

The effects of IL-1α and β are mediated through 2 IL-1 receptors, the type I receptor IL-1RI transduces a signal whereas the type II receptor IL-1RII binds IL-1 but does not transduce a signal, thereby antagonising IL-1's effects (Sims *et al.* 1993).
IL-1's activities are also regulated by a third member of the IL-1 family, called IL-1 receptor antagonist (IL-1Ra). This is the naturally occurring inhibitor of IL-1 and binds to IL-1 receptors but does not induce any intracellular response.
Soluble forms of IL-1 type I and type II receptors are formed by proteolytic cleavage of the membrane receptors. These are found within the circulation of healthy humans with an increase occurring during disease (Fernandez-Botran 1999). These soluble receptors are capable of binding IL-1 and dampening down IL-1 responses in a dose-dependent fashion (Dinarello 1997).

1.6.1.1.8 Effects of IL-1

Despite TNFα and IL-1 binding to different cellular receptors, both cytokines have multiple overlapping and synergistic activities. Those relevant to inflammation are summarised in Table 1.5.
Effects of IL-1 during pneumococcal infection have not been widely studied. However, IL-1 has been shown to be required for resistance to both systemic and pulmonary infectious disease caused by a range of micro-organisms such as *P. carinii* and *L. monocytogenes* (Havell *et al.* 1992) & (Chen *et al.* 1992b).
TNFα is known to be a potent inducer of IL-1 expression (Dinarello *et al.* 1986) and proof of the synergy between TNFα and IL-1 *in vivo* was found by Waage *et al* (Waage and Espevik 1988). They found that administration of low levels of IL-1 intravenously into mice does not result in mortality whilst TNFα treatment alone can result in 50% mortality by 72h post injection. Co-administration of both cytokines increased mortality to 100% in the same time frame. Similar results have since been gathered in a rabbit system by other investigators (Okusawa *et al.* 1988).

Target	Effect	Reference
Neutrophils	Recruitment to site of infection	(Rogers *et al.* 1994)
	Enhance adhesion to endothelium	(Belvilacqua *et al.* 1985)
Monocytes	Increase expression of itself	(Dinarello *et al.* 1987)
	Enhance adhesion to endothelium	(Belvilacqua *et al.* 1985)
Macrophage	Activation	(Rogers *et al.* 1994)
Epithelial cells	Increase expression of IL-6	(Cromwell *et al.* 1992)
Endothelial cells	Upregulation of ICAM-1 expression	(Dustin *et al.* 1986) & (De Caterina *et al.* 1995)
	Increase expression of itself	(Warner *et al.* 1987)
	Disrupt integrity	(Geelen *et al.* 1993)

Table 1.5 Effects of IL-1 on the inflammatory response.

1.6.1.1.9 Interleukin 6 (IL-6)

IL-6 was originally cloned due to its ability to induce B cell differentiation but has since been recognised as a product of a wide range of other cell types including T cells, monocytes, alveolar macrophages, neutrophils, mast cells, endothelial cells and epithelial cells (Biffl *et al.* 1996). As TNFα and IL-1 are major inducers of IL-6, kinetics of IL-6 release are generally slower than those for TNFα and IL-1 production (Van Damme *et al.* 1987).
IL-6 mediates its effects by binding to a specific receptor called IL-6 R. Only a very short portion of IL-6R is located within the cytoplasm of cells and this does not transduce signals. For signal transduction the associated glycoprotein gp130 is required (Hibi *et al.* 1990). The current belief is that binding of IL-6 to IL-6R is of a low affinity but interaction with gp130 stabilises this complex. IL-6 itself does not have any binding specificity for gp130.

1.6.1.1.10 Effects of IL-6

In addition to the *in vitro* effects in Table 1.6, there is much proof of IL-6's activities *in vivo*. IL-6 deficient mice were unable to mount an inflammatory response to localised tissue damage generated by turpentine injection, indicating the pro-inflammatory capacity of IL-6. In contrast, during systemic inflammation (induced by intravenous administration of LPS) elevated inflammation results in significantly higher TNFα levels. Such data supports an anti-inflammatory role for IL-6 (Fattori *et al.* 1994). In separate models of septic shock or endotoxaemia IL-6 has been ascribed both beneficial and detrimental roles as IL-6 neutralisation can either result in increased or decreased mortality (Barton and Jackson 1996) & (Starnes *et al.* 1990). Furthermore, following systemic infection with *S. pneumoniae* sustained production of IL-6 has been shown to be associated with elevated mortality in a porcine model (Ziegler-heitbrock *et al.* 1992).
IL-6 is required for a protective response in *L. monocytogenes* (Dalrymple *et al.* 1995) and *E. coli* infection (Dalrymple *et al.* 1996) as IL-6 deficient mice are more susceptible to these infections than are wild type animals. In contrast IL-6 is not required for clearance of *P. carinii* during pulmonary infection (Chen *et al.* 1993) although it does control pulmonary inflammation (by reducing cell influx) and attenuate antibody responses during resolution.

Target	Effect	Reference
Neutrophils	Induces release from bone marrow	(Dalrymple *et al.* 1995)
	Synergises with TNFα to increase respiratory burst	(Mullen *et al.* 1995)
Myeloid cells	Differentiation into macrophages	(Shabo *et al.* 1988)
Monocytes	Activation	(Borish *et al.* 1989)
B cells	Promotes maturation & differentiation	(Hirano *et al.* 1986)
T cells	Promotes differentiation into cytotoxic T cells	(Okada *et al.* 1988)
Endothelial cells	Increases permeability	(Maruo *et al.* 1992)
Hepatocytes	Induces expression of acute phase response proteins	(Gauldie *et al.* 1987)

Table 1.6 Effects of IL-6 on the immune response.

It therefore seems likely that the balance between IL-6 and other mediators is crucial in determining whether IL-6 plays an inflammatory or anti-inflammatory role and the effect on disease outcome during pneumococcal pneumonia.

1.6.1.1.11 Chemokines

Chemokines are the class of cytokines capable of direct cellular recruitment. There are at least four chemokine families, grouped on the basis of the position of the first two cysteine residues.

The CXC family members appear to be more closely related to acute inflammatory reactions and are generally characterised by their ability to initiate neutrophil influx into the tissues. This family includes IL-8, macrophage inflammatory protein-2 (MIP-2) and granulocyte chemoattractant protein-2 (GCP-2).

The CC chemokines appear to be primarily chemotactic for monocytes, lymphocytes, basophils, and eosinophils. This family contains several related proteins, including MIP-1α, MIP-1β, and regulated upon activation normal T expressed and secreted (RANTES).

The only known member of the C family is Lymphotactin which is released by lymphocytes and appears to act solely on T lymphocytes (Kelner *et al.* 1994). Fractaline is also the sole member of a "family" of chemokines, the CXXXC and exerts its effects on neutrophils, monocytes and T lymphocytes (Bazan *et al.* 1997). In contrast to the other classes of chemokines, fractaline is mainly produced by somatic cell lines such as endothelial cells, with little or no expression in peripheral blood monocytes, T and B cells and NK cells.

Roles in anti-bacterial defences have been ascribed to certain cytokines. MIP-2 is able to initiate anti-bacterial activity *in vitro* by increasing the ability of neutrophils to phagocytose and kill *E. coli* (Standiford *et al.* 1996). MIP-1α is capable of similar effects on alveolar macrophages. In addition, CC chemokine receptor 2 deficient mice display increased susceptibility to infection with *L. monocytogenes*, displaying 100% mortality by 5 days post intravenous infection whilst all wild type animals survive (Kurihara *et al* 1997). During a pulmonary model of *K. pneumoniae* infection, inhibition of MIP-2 activity results in decreased neutrophil recruitment and bacterial clearance with an increase in mortality (Greenberger *et al.* 1996).

Despite these facts, roles of chemokines in pneumococcal pneumonia have not been investigated. Indeed effects of chemokines *in vivo* during other bacterial pneumonias remain poorly understood but warrant further investigation.

1.6.1.2 Anti-inflammatory Cytokines

1.6.1.2.1 Interleukin 10 (IL-10)

IL-10 was initially described as a factor produced by Th2 cells that was capable of inhibiting cytokine release by Th1 cells (Fiorenino *et al.* 1989). Production of this cytokine has since also been detected in monocytes/macrophages, mast cells, neutrophils, epithelial cells, and B lymphocytes (O'Garra *et al.* 1990) & (Moore *et al.* 1993).

1.6.1.2.2 Effects of IL-10

Interleukin 10 is a potent anti-inflammatory cytokine with a range of activities, summarised in Table 1.7.
IL-10 is constitutively expressed by bronchial epithelial cells (Bonfield *et al.* 1995), placing the cytokine in an ideal location to control inflammation within normal lungs. Indeed the fact that IL-10 knockout mice spontaneously develop enterocolitis is further evidence of IL-10's regulatory capacity (Kühn *et al*. 1993). It is currently unknown whether IL-10 deficient mice also spontaneously develop overwhelming lung inflammation, presumably because the enterocolitis is so severe the animals do not survive long enough.
IL-10's ability to inhibit cytokine inhibition occurs mainly via inhibition of gene transcription (Wang *et al.* 1994) since IL-10 is a potent inhibitor of NF-κB activation in human monocytes (Wang *et al.* 1995). In addition IL-10 can also induce cytokine mRNA degradation (Bogdan *et al.* 1992) & (Kasama *et al.* 1994) and increase levels of cytokine soluble receptors such as p55 and p75 (Bemelmans *et al.* 1993).
IL-10 is also capable of down-regulating non-cytokine inflammatory mediators, such as NO. This effect is indirect via the ability of IL-10 to down regulate TNFα (Oswald *et al.* 1992).

Target	Effect	Reference
Macrophage	Reduce TNFα production	(Zisselet *al.* 1996) & (Bogdan *et al.* 1991)
	Inhibit Microbicidal activity	(Oswald *et al.* 1992) & (Fleming and Campbell 1996)
	Inhibit release of reactive oxygen intermediates	(Bogdan *et al.* 1991)
	Inhibit release of reactive nitrogen intermediates	(Bogdan *et al.* 1991)
	Inhibit apoptosis	(Bingisser *et al.* 1996)
Mast cells	Inhibit TNFα production	(Arock and Pretolani 1996)
Monocyte	Enhance IL-1Ra production	(Jenkins *et al.* 1994)
	Inhibit release of TNFα	(Armstrong *et al.* 1996)
	Down-regulate ICAM-1 expression	(Willems *et al.* 1994)
	Down-regulate MHC II expression	(de Waal malefyt *et al.* 1991)
Neutrophils	Inhibit pro-inflammatory cytokine release	(Cassatella *et al.* 1993)
	Promote apoptosis	(Cox 1996)
	Down-regulate chemokine expression	(Kasama *et al.* 1994)
	Increase IL-1Ra release in response to TNFα	(Marie *et al.* 1996)
Endothelial cells	Suppresses NO release	(Koedel *et al.* 1996)

Table 1.7 Effects of IL-10 on the immune response.

Recognition of IL-10's wide array of anti-inflammatory effects has led to trials of this cytokine as a treatment during septic shock/endotoxaemia, with highly variable results. Marchant *et al* and Gérard *et al* found that treatment with IL-10 reduced levels of pro-inflammatory cytokines during experimental endotoxaemia and was required for host survival (Marchant *et al.* 1994) and (Gérard *et al.* 1993). Conversely Remick *et al* found that IL-10 treatment did not alter mortality during sepsis (Remick *et al.* 1997). It appears that beneficial effects are only observed if IL-10 is given either before or very early after the inflammatory stimulant (Pajkrt *et al.* 1997).

1.6.1.3. Detrimental effects of cytokines

It has recently been shown that outcome of sepsis or pneumococcal pneumonia is determined not only by the magnitude of the inflammatory response. More critical is the balance between inflammatory and anti-inflammatory cytokines (Walley *et al.* 1996) & (Mohler *et al.* 1998). In acute inflammation it is hypothesised that the levels of TNFα and IL-1 produced are higher than can be neutralised by available anti-inflammatory mediators. Inflammation then spreads to the circulation where cytokines act in an endocrine manner inducing endothelial damage, which leads to multiple organ failure (due to tissue hypoperfusion) and eventually death (Molloy *et al.* 1993).

The importance of cytokines to these responses is highlighted by the ability of systemic administration of TNFα or IL-1 to result in many of the symptoms of septic shock (Okusawa *et al.* 1988). In addition, pathology and mortality and can be reduced by neutralisation of these cytokines either prior to shortly after instigation of the septic shock (Remick *et al.* 1990) & (Hindshaw *et al.* 1992).

1.6.1.4 Cytokine regulation

As cytokines are highly potent molecules, capable of both beneficial and detrimental activity, their activity is tightly regulated. Regulation occurs at several levels, including transcriptional regulation, short half lives, soluble cytokine receptors and anti-inflammatory cytokines.

Nuclear factors such as NF-κB act to upregulate transcription of cytokine genes such as those for TNFα and IL-1. NF-κB in turn can be down-regulated by anti-inflammatory cytokines such as IL-10 (Wang *et al.* 1995). As with promoters for pro-inflammatory cytokines, the iNOS promoter has an NF-κB recognition site (Xie *et al.* 1994) and thus NO production can be initiated along with that of inflammatory cytokines. However, NO can also act upstream of NF-κB in a regulatory capacity (Walley *et al.* 1999). This reduces the amount of NF-κB available for binding to the regulatory region of pro-inflammatory cytokine genes and reduces their transcription.

The presence of UA-rich sequences in the 3' untranslated region of mRNA transcripts leads to instability and a short half-life for TNFα transcripts (Caput *et al.* 1986). Such regions are also conserved in mRNA transcripts of other pro-inflammatory cytokines including IL-1 and IFN-γ.

During sepsis, expression of cell surface TNFα receptors on circulating monocytes and granulocytes is down regulated with a corresponding increase in soluble receptor concentrations and an increase in IL-Ra levels (Calvano *et al.* 1993). In contrast to TNFα and IL-1, there does not appear to be any specific IL-6 antagonists *in vivo*. Soluble IL-6R does exist but appears to facilitate interactions with membrane bound gp130 (Rose-John and Heinrich 1994) rather than have an antagonistic effect.

1.6.1.5 Microbial factors responsible for cytokine induction

Most investigations of the roles of cytokines in infectious disease concentrate on the effect of the cytokines in controlling the micro-organisms. Less emphasis has been placed on the possibility that the microbes induce cytokines in order to utilise these factors for their growth/spread or at least have adapted in order to utilise inflammatory mediators for their benefit. For example, IL-1 has been found to increase growth of *E. coli* if the bacteria are grown in sub-optimal media such as fresh serum (Porat *et al.* 1991).

The mechanism by which micro-organisms utilise cytokines for their growth is currently not understood. They may be used as simple nutrients as an effect on proliferation only occurs when the incubation occurs in nutrient starved conditions. This appears to be unlikely, however, as heat inactivation or antibody treatment of the cytokine blocks the enhancement (Denis and Porat 1995).

In addition, interaction of organisms with cytokines may result in the formation of mediator/bacteria complexes, capable of interacting with cytokine receptors on the surface of lung cells. Such complexes have been shown to occur between TNFα and *Shigella flexneri* and may promote uptake of the organism in the intestine (Luo *et al.* 1993). A range of bacteria have been tested for this interaction and although Gram-negative bacteria such as *S. flexneri* bind TNFα to the highest extent, Gram-positive organisms such as *Streptococcus mitis* and *S. aureus* are also capable of binding this cytokine.

Cytokine induction may also prove favourable for bacteria that require cells to be activated before expressing receptors for their attachment. As mentioned in Section 1.4.2, *S. pneumoniae* may attach to 2 different receptors on the surface of resting lung cells, but only a very low percentage of bacteria do so. Activation of lung cells via TNFα and IL-1 causes up-regulation of the PAF receptor and a 200-300 fold increase in pneumococcal adherence (Cundell *et al.* 1995). Thus pneumococci may utilise the inflammatory response to aid their entry to the bloodstream.

A range of pneumococcal factors have been shown to induce production of pro-inflammatory cytokines *in vitro*. Heat killed pneumococci and capsular polysaccharides are capable of stimulating *in vitro* release of TNFα from macrophages (Simpson *et al.* 1994). The same effect has been noted for pneumococcal lipoteichoic acid (Keller *et al.* 1992). *In vitro* data with monocytes have shown the induction of IL-1 and IL-6 following stimulation with individual cell wall constituents of pneumococci (Riesenfeld-Orn, *et al.* 1989) & (Heumann *et al.* 1994). These factors are also capable of inducing TNFα, but require more than 100 times the amount of cell wall to do so than for IL-1 release.

The role of pneumolysin in induction of inflammation has already been discussed in Section 1.3.2.

1.6.1.6 Pulmonary cytokine production during pneumococcal pneumonia

Since beginning these studies several reports have been published on the production of cytokines during murine models of pneumococcal pneumonia. Most of publications have concentrated on single cytokines, with the balance between pro- and anti-inflammatory cytokines poorly

understood and results often conflicting (Takashima *et al.* 1997) & (van der Poll *et al.* 1997a). The investigations in this thesis were therefore carried out in order to characterise this important balance and to expand data on cytokine interactions. The following section represents a review of the current state of art research in this field.

The importance of pro-inflammatory cytokines in host defence against *S. pneumoniae* is provided indirect support by the reduced ability of transgenic mice with impaired NF-κB activity to control type 2 pneumococcal infection (Sha *et al.* 1995). As mentioned in Section 1.6.1.4, NF-κB is a transcription factor capable of regulating the expression of pro-inflammatory cytokines. NF-κB is composed of two subunits, p50 and p65 and mice with targeted disruptions in the p50 subunit of NF-κB have recently been produced. Infection of these mice intraperitoneally with 100 CFU type 2 *S. pneumoniae* resulted in reduced survival times (24h post challenge) when compared to wild-type mice (36-72h post challenge) (Sha *et al.* 1995). Furthermore high numbers of viable pneumococci were recovered in the blood and organs from the transgenic mice. Thus these animals were impaired in their ability to contain and kill the bacteria within their peritoneal cavities.

Direct evidence of the involvement of cytokines in the host response to *S. pneumoniae* is provided in several recent mouse models using various strains of immunocompetent mice. The most extensive of these was published by Bergeron *et al* in 1998 using a model of pneumonia initiated by infecting female CD1 mice with 10^7 CFU type 3 pneumococci (Bergeron *et al.* 1998). This caused acute inflammation with mice dying approximately 72h post challenge. We have since found similar histological disruption and cell recruitment with a rapid influx of neutrophils to the lungs, initially in perivascular areas but then spreading around infected bronchioles (Kadioglu *et al.* 2000). Neutrophils were progressively replaced by monocytes and low numbers of lymphocytes at 72h to 96h post challenge.

In the Bergeron paper, this cell influx was accompanied by rapid cytokine release. The first cytokine to be recovered in bronchoalveolar lavage fluid was TNFα. Significantly elevated levels were detected 1h post challenge and these continued to increase until 12h into the experiment. By 24h post challenge levels were returning to baseline. A similar transient production of TNFα was evident within the lung tissues. Pulmonary IL-1α levels also reached their peak at 12h post challenge but with slower kinetics and in lower concentrations than TNFα. IL-1α levels remained elevated within

lung tissues from mid infection until the end of the experiment. Airway IL-6 production was biphasic, peaking very early at 4h post challenge and again at 48h post challenge. Constant levels of this cytokine were detected within the lung tissues from 4h until the end of the experiment.
In summary Bergeron *et al* found (in addition to the elevated release of NO mentioned in Section 1.5.3.5.2) initial ineffective phagocytosis of pneumococci leading to cytokine release in bronchoalveolar lavage fluid. This cytokine release occurred over 4-24h as pneumococci grew in the alveoli. Following injury to alveolar structure and bacterial access to the bloodstream, cytokine release in the lungs was downregulated.
These kinetics of cytokine production are supported by those found in other models of pneumococcal pneumonia. Intranasal infection with either type 3 or type 19 pneumococci resulted in rapid production of TNFα within the lungs (van der Poll *et al.* 1997a) & (Takashima *et al.* 1997).
Although investigation of both host pro- and anti-inflammatory responses has not previously been carried out, van der Poll *et al* have characterised the production of IL-10 during pneumococcal pneumonia (van der Poll *et al.* 1996). They found elevated levels of IL-10 from 12h post challenge but peak production did not occur until 72h post challenge.

1.6.1.7 Systemic cytokine production during pneumococcal pneumonia

Bergeron *et al* also investigated the systemic production of cytokines in their model of pneumococcal pneumonia (Bergeron *et al.* 1998). It was found that TNFα levels in serum increased towards the end of the experiment as bacteria gained access to the bloodstream. IL-1α levels increased very transiently at 12h post challenge, at the same time point as maximum lung levels were detected. This may indicate that systemic detection was the result of overspill from the lungs. IL-6 levels were found to increase during early infection (4h post challenge) and again with increasing magnitude towards the end of the experiment, correlating well with bacterial loads.
Other investigators in this field have found similar results for systemic TNFα kinetics but have reported highly variable amounts (van der Poll *et al.* 1997a), supported by similar variability in human investigations (Marks *et al.* 1990).

The rapidity and sustained nature of IL-6 production within the circulation has also been reported by van der Poll *et al* (van der Poll *et al.* 1997b).
Serum IFNγ levels have been found to correlate well with the degree of bacteraemia and the severity of illness (Rubins and Pomeroy 1997). However, in these experiments mice surviving until 72h post challenge with either serotype 1, 2, 3 or 4 pneumococci possessed the lowest IFNγ levels despite elevated bacteraemia.

1.6.1.8 Effects of cytokines during pneumococcal pneumonia

By altering levels of individual cytokines during the infection, either by antibody neutralisation or utilisation of transgenic mice, it is possible to discover the roles played by these mediators.
Systemic neutralisation of TNFα has been found to result in elevated bacterial loads and increased mortality following subsequent infection with *S. pneumoniae* (van der Poll *et al.* 1997) & (Takashima *et al.* 1997). Contradictory reasons for these effects of TNFα have, however been reported. van der Poll *et al* found that TNFα neutralisation in combination with infection with type 3 pneumococci resulted in significantly elevated pulmonary bacterial loads but bacteraemia was unaffected. In addition, anti-TNFα treatment reduced IL-1β levels but had no effect on IL-6, IL-10 or IFN-γ levels or neutrophil recruitment to the lungs (van der Poll *et al.* 1997). Thus the authors hypothesised that TNFα was required in order to increase the anti-bacterial activity of resident host cells during pneumococcal pneumonia but did not recruit additional cells to the site of infection.
In contrast Takashima *et al* found that neutralisation of TNFα prior to infection with type 19 pneumococci did not affect pulmonary bacterial loads but did significantly increase levels of bacteraemia (Takashima *et al.* 1997). Furthermore significantly lower numbers of circulating neutrophils were found following TNFα neutralisation. This data indicates that TNFα is responsible for increasing circulating neutrophil numbers during pneumococcal pneumonia and these prevent overwhelming bacteraemia. This conflicting data prompted us to investigate what role, if any TNFα was exerting during our model of pneumococcal pneumonia (Chapter 4).
Although most previous research has been aimed at identifying the role of TNFα during pneumococcal pneumonia, the effects of other cytokine have also been investigated. Intranasal infection of mice deficient in IL-6 results

in a six fold increase in bacterial loads and a higher mortality rate during pneumococcal infection (van der Poll *et al.* 1997b). This was evident despite higher levels of pulmonary TNF, IL-1β and IFNγ at the end of the infection (highlighting the importance of pro-inflammatory cytokines during early infection but not at end-stage disease).

Interferon gamma knockout mice are significantly more susceptible to infection with type 2 pneumococci than their wild-type counterparts with an LD_{50} 20-fold lower (2.50×10^6 and 5.00×10^7 CFU respectively) (Rubins and Pomeroy 1997), likely to be due to the ability of IFNγ to increase neutrophil phagocytosis.

Finally, antibody neutralisation of IL-10 2h prior to intranasal infection with 10^6 CFU type 3 pneumococci has been shown to result in increased pulmonary TNFα and IFNγ levels, with a corresponding six-fold decrease in pulmonary and systemic bacterial loads and increased survival. Pre-treatment with recombinant IL-10 had the opposite effects (van der Poll *et al.* 1996). This indicates that overly high levels of IL-10 during pneumococcal pneumonia reduce the levels of pro-inflammatory cytokines, permitting greater bacterial loads and ultimately reduced host survival.

Thus there are many possible interactions and roles of inflammatory mediators during pneumococcal pneumonia and other inflammatory responses. Recognition of this fact led us to investigate the production of both inflammatory (TNF, IL-1β, IL-6 and NO) and anti-inflammatory (IL-10) mediators following infection with a well characterised and utilised laboratory strain of pneumococci. Both an outbred mouse strain (presumably containing a wide range of genetic backgrounds) as well as in two inbred mouse strains (differing significantly in their susceptibility to pneumococcal pneumonia) were used. It was hypothesised that this would characterise the balance between inflammatory and anti-inflammatory cytokine production kinetics in a typical population. In addition it permitted investigation of the "ideal" inflammatory response that occurs if subjects are to control the infection. We were also able to characterise the host response in mice that were overcome by the infection. By comparing these it was possible to identify differences in the early response to pneumococcal pneumonia that may be responsible for the significantly different susceptibilities.

Chapter 2

Materials and Methods

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich, Dorset, UK unless otherwise stated.

2.1 Mouse strains

The mouse strains used in these studies are listed in Table 1.
BALB/c *scid* mice were bred and housed in isolators within the central research facility (CRF) at the University of Glasgow. On infection these mice were moved into filter top cages. All other strains of mice obtained from the Department of Immunology were bred and housed in filter top cages.
Mouse rooms were maintained at 21°C +/- 1°C and 55-65 % relative humidity with 12h artificial light from 0700 to 1900h. Mice were fed pelleted rat and mouse standard diet (B&K Universal, North Humberside, England). Both water and food were administered on an *ad libitum* basis and in the case of iNOS -/-, MF1/129, BALB/c *scid* and TNFα receptor deficient mice these were irradiated
In order to allow acclimatisation, mice from commercial suppliers were kept in the CRF for a period of at least one week prior to use.

2.2 Microbiological techniques

2.2.1 Bacterial stock preparation

Streptococcus pneumoniae D39, serotype 2, was obtained from the National Collection of Type Cultures (NCTC 7466; Central Public Health Laboratory, London). Bacteria were grown on Blood Agar Base Number 2 (Oxoid, Basingstoke, UK) plus 5% (vol/vol) defibrinated horse blood (E&O Laboratories, Bonnybridge, UK) (BAB). Stock cultures were prepared by inoculating a sweep of 4-5 colonies into 25ml Brain-Heart Infusion broth (BHI, Oxoid). Cultures were grown statically at 37°C overnight before 1ml aliquots containing 15% sterile glycerol were frozen at -70°C until use. Strain validation was carried out by confirming sensitivity to the antibiotic Optochin (Difco, Detroit, USA). Serotype verification was performed by checking for the presence of type specific polysaccharide capsule using anti-type 2 capsule antiserum (Statens Seruminstitut, Copenhagen, Denmark).

Mouse Strain	Genetic Background	Inbred/ outbred	Age (wks)	Weight (g)	Gender
MF1 [a]	MF1	Outbred	young adults 8-16 aged adults 52	25-30 55-75	Female Female
iNOS knockouts [d]	MF1/129	Outbred	8-11	20-25	Female
iNOS controls [d]	MF1/129	Outbred	8-11	20-25	Female
CBA/Ca[a]	CBA/Ca	Inbred	9-12	15-20	Female
BALB/c[a,b,c]	BALB/c	Inbred	9-12	20-25	Female
BALB/c *scid* [e]	BALB/c	Inbred	9-12	20-25	Female & Male
TNFα receptor knockouts (p55 & p75) [f]	C57Bl/6	Inbred	11-17	25-30	Female & Male
p55 & p75 controls[a]	C57Bl/6	Inbred	10-16	20-25	Female & Male

Table 2.1 Mouse strains used in infection studies.

[a]were purchased from Harlan Olac (Bicester, UK), [b]were purchased from Charles River UK Ltd (Kent, UK), [c]were obtained from CRF own stock, [d]were obtained from Professor F.Y. Liew, [e]were obtained from Dr P. Garside, [f]were obtained from Dr A. Mowat, Department of Immunology, University of Glasgow.

2.2.2 Viable counting of *Streptococcus pneumoniae*

As previously described (Kadioglu *et al.* 2000), serial dilutions of samples were prepared by the addition of 20µl to 180µl sterile PBS (Appendix) in sterile round bottomed 96 well microtitre plates (Life Technologies, Paisley, UK). With smaller samples (such as tail bleeds) 10µl of sample were mixed with 90µl PBS then 20µl of this serially diluted as before. Lavage fluid and lung homogenate sample dilutions were continued to 10^{-5} with undiluted sample also plated out. All other samples were plated out in the range of 10^{-1} to 10^{-6}.

Dry BAB plates were marked into 6 sectors and 3 x 20µl of each dilution were plated on one sector. This procedure was done in duplicate and repeated for each dilution. The plates were allowed to dry before being incubated overnight at 37°C. Colonies were counted in the sector that had between 40-200 colonies on each spot for the lowest dilution The following equation was used to calculate the number of colony forming units per ml of sample:

CFU per ml = Mean number of colonies per sector x 50 x Dilution Factor.

These methods resulted in a detection limit of 8.33 pneumococci per ml lung sample and 83.3 pneumococci per ml of other samples.

2.2.3 Growth characteristics

In order to prepare the starting culture, aliquots of stock bacteria were thawed quickly in a water bath at 37°C and 900µl of each centrifuged at 13,000rpm (Eppendorf centrifuge 5417C) for 3min before resuspending in 900µl sterile PBS. An appropriate volume was added to 20ml BHI with 20% v/v heat inactivated FCS (Life Technologies, Paisley, UK) to give a starting Optical Density at 600nm of 0.04. Cultures were grown statically at 37°C and every hour the OD_{600} and a count of colony forming units was determined. From this data the OD_{600} at which the bacteria were in mid-log phase was determined.

2.3 *In vivo* techniques

2.3.1 Telemetry chip implantation

Telemetry chips (Minimitter, Oregon, USA) were sterilised by immersion in Cidex™ (Johnston & Johnston Medical Limited, Skipton, UK) for 10min and then rinsed twice in sterile distilled water.

1-2 weeks prior to bacterial challenge, mice to be implanted were lightly anaesthetised with 2.5% v/v Halothane (Zeneca Pharmaceuticals, Macclesfield, UK) over oxygen (1.5 L/min), administered using a calibrated vaporiser and anaesthesia confirmed by observing no pinch reflex reaction. The abdomen was shaved and the mouse placed on a sterile drape upon a heat mat. The mice were then deeply anaesthetised with 3-5% v/v Halothane over oxygen (1.5 L/min), administered using a calibrated vaporiser.

A sterile scalpel was used to make the initial incision in the abdomen and the peritoneum opened with sterile scissors. The telemetry chip was placed inside the peritoneum and the inner wound fastened using 5-0 Dexon II suture (Cyanamid of Gt. Britain Ltd, Hampshire, UK). The outer wound was fastened using surgical clips (Becton Dickinson). Mice were placed in a cage warmed from below by a heat mat and left to recover for several hours before being returned to normal housing conditions. Implanted mice were weighed every day and their wounds inspected to ensure total recovery from surgery. Any mice that displayed continued weight loss for three days were fed on human baby food (Boots, Nottingham, UK) until their weight returned to normal. One week after surgery the outer clips were removed but the weighing continued until the end of the experiment.

Telemetry readings were gathered using the VitalView® data acquisition system. The implanted chip captures energy from the field of radio waves generated by the coils of an energiser/receiver box placed underneath each individually caged animal. The powered chip emits a signal and the frequency of this signal informs the receiver box of the core body temperature and activity of the animal. The VitalView® software gathers the information and presents the data for each individual animal. Data can then be transferred into appropriate software packages for statistical evaluation.

2.3.2 Preparation of bacterial inoculum

Standard inoculum was prepared as previously described by this laboratory with minor modifications (Canvin *et al.* 1995). 100µl of sterile PBS containing approximately 10^5 CFU were injected into the peritoneal cavity of mice with an insulin syringe (F. Baker Scientific, Runcorn, UK). On development of lethargy, mice were culled by cervical dislocation, the chest cavity carefully opened up and a section of ribcage removed in order to provide access to the heart. A 23 gauge needle was inserted into the right ventricle and blood slowly collected into the attached syringe. 50µl of infected blood was used to grow passaged bacteria in 10ml BHI statically overnight at 37°C. Bacteria were harvested by centrifugation at 3,000 rpm for 10min (Heraeus medifuge), resuspended in fresh BHI containing 20% v/v FCS to an A_{600} of 0.04, and then incubated at 37°C until they reached mid-log phase (as judged by optical density). 1ml aliquots of the suspension were stored at -70°C, until required. After 24h at -70°C viability of the suspension was measured by viable counting and optochin sensitivity was confirmed. Viability was unaffected by storage at -70°C for at least 3 months.

2.3.3 Intranasal infection of mice

Aliquots of standard inoculum were rapidly thawed. A 900µl sample was pelleted by centrifugation at 13,000rpm (Eppendorf centrifuge 5417C) and resuspended in 900µl sterile PBS. Bacteria were diluted in sterile PBS to give 1 x 10^6 CFU/50µl (unless otherwise stated) and this confirmed by viable counting (as described previously).

For experiments with heat-killed pneumococci, a preparation of bacteria was prepared as above and then placed in a 60°C water bath for 10min. The viable count was then re-determined.

In order to inoculate the bacteria, mice were lightly anaesthetised as described prior to abdomen shaving in Section 2.3.1 (Kadioglu *et al.* 2000). Halothane was used due to its rapid recovery time (approximately 5 min) and its ability to depress pulmonary mucociliary clearance mechanisms, thereby aiding infection (Forbes 1976). Once anaesthetised the animals were scruffed, with the nose held upright, and pneumococci introduced intranasally by adding a series of small droplets of the 50µL inoculum in to the nostril for the mice to

involuntarily inhale. After inoculation the mice were laid on their backs until recovery and the viable count of the inoculum was re-determined.
To assess whether this procedure itself induced an inflammatory response, some mice were inoculated intranasally with 50µl of PBS alone.

2.3.4 Intravenous infection of mice

Mice to be infected were placed in a heated, ventilated perspex box for 5min before bleeding. The animal was then placed in a ventilated tube and the infectious dose as a 50µl volume was administered into the lateral tail vein via an insulin syringe (Wolfensohn and Lloyd 1995). A blood sample was then removed from a separate vein (see below) in order to determine the initial level of bacteraemia.

2.3.5 Survival experiments

Experiments designed for survival data involved infecting groups of mice and then monitoring them frequently between 18h (signs of illness were not apparent prior to this time point) and 336h post challenge (or until the mice became moribund (Toth 1997) at which point the experiment was ended). Signs of illness were recorded on a "pain-score" system devised by the this laboratory. This system grades the progression from normality through mild and severe hunched stance, mild and severe piloerection and mild and severe lethargy to the moribund state. In experiments where mice were actively culled the time of reaching moribund was taken as the time of death.

2.3.6 Tail bleeding mice

Mice to be bled were warmed as for intravenous infection. An insulin syringe was used to remove up to 100µl of blood. Collected samples were placed on ice until processing.

2.3.7 Blood sampling for bacterial loads from sacrificed mice

At pre-chosen intervals after infection, randomly determined groups of mice were carefully sacrificed by cervical dislocation as to ensure an intact trachea.

The chest cavity was opened up and blood removed as for preparation of standard inoculum (see above). The samples were kept on ice prior to viable counting as previously described.

2.3.8 Serum sampling for cytokines

Blood samples were removed as for bacterial loads. The samples were then left to clot for 30 min at room temperature (van der Poll *et al.* 1996) before being centrifuged at 6,000rpm (Eppendorf centrifuge 5417C). Serum was stored at -70°C until use.

2.3.9 Sampling lungs for bacterial loads

In order to carry out bronchoalveolar lavage, the skin and muscles above the trachea were separated and a section of the ribcage was removed in order to allow expansion of the lungs. Lavage was carried out in a similar manner to that used by Van der Poll *et al* (van der Poll *et al.* 1996), the trachea was clamped with a pair of Spencer-Wells forceps (Fisher Scientific, Loughborough, UK) in order to prevent fluid flowing up the trachea. A 16 gauge non-pyrogenic angiocath (F. Baker Scientific) was inserted into the trachea and lavage was carried out with 2 x 1ml volumes of PBS. The usual recovery volume was 1.25 - 1.50 ml per mouse. For CBA/ca mice, the smaller lung size meant 2 x 0.75ml volumes of PBS were used. This generated between 0.80 - 1.00 ml lavage fluid per mouse. A small aliquot of this fluid was placed on ice for bacterial counts prior to snap freezing the remainder for cytokine analysis (see below).

The lavaged lungs were then removed and placed in 5ml sterile PBS with homogenisation carried out using a glass handheld tissue homogeniser (Jencons, Leighton Buzzard, UK). The viable count for the recovered lavage fluid and lung homogenates were then determined as previously.

In order to investigate the contribution of bloodborne pneumococci to lung bacterial loads, perfusion of blood from the lung tissues was carried out in one experiment. This involved rupturing the left atrium prior to insertion of a syringe containing 5ml non-pyrogenic saline into the right ventricle. The syringe contents were slowly injected into the heart and the resulting

fluid was allowed to drain from the left ventricle. The perfused lungs were then rapidly removed as previously described.

2.3.10 Alveolar cell recovery

For BALB/c cell recovery experiments lavage was carried out during late afternoon in order to prevent firm adherence by erythrocytes in addition to airway cells. Lavage volume was increased to 5 x 1ml aliquots of non-pyrogenic saline (Baxter Healthcare Ltd Norfolk, UK), resulting in 4.0 - 4.25ml fluid recovered per mouse. For CBA/Ca cell sampling, 5 x 0.75ml volumes were instilled this gave 3.0-3.25ml per animal. In order to prevent cell adherence to the collection tube, the recovered fluid was kept on ice at all times.

2.3.11 Sampling lungs for cytokines

Bronchoalveolar lavage was carried out as above but in this case the fluid was collected into a sterile cryotube (Sarstedt, Leicester, UK) before immersion in liquid nitrogen. Following lavage, the lungs were removed and wrapped in aluminium foil and also snap-frozen in liquid nitrogen. Both bronchoalveolar lavage fluid and whole lavaged lung samples were stored at -70°C until further processing.

2.3.12 Processing of lung samples for cytokines

On thawing, the whole lungs were weighed and then homogenised as previously. Following the protocol by van der Poll *et al* (van der Poll *et al.* 1996), homogenates were centrifuged at 1600g for 30min at 4°C to remove cell debris. The cell-free supernatants were then centrifuged at 5000g (Sigma 4K15 centrifuge) for 30 min at 4°C after which the supernatants were filter sterilised (0.2μM, Gelman Sciences, Northampton, UK) and stored at -70°C. Once thawed, lavage fluid samples were centrifuged at 13,000 rpm (Eppendorf centrifuge 5417C) for 3 min in order to remove any cells present before cytokine determination was carried out

2.3.13 Sampling of lungs for histological analysis

Mice chosen for histological analysis were carefully culled and the ribcage opened, trachea clamped and a catheter inserted as above. The lungs were then inflated with 1ml (CBA/Ca mouse lungs were fixed with 0.75ml) formal saline (Appendix) in order to improve clarity of histologic lesions (Wood Jr 1940). The lungs were removed intact and placed in 5ml formal saline in a plastic bijoux . A piece of tissue paper was then placed at the top of the fluid in order to ensure that the lungs remained submerged at all times. Following 48h in fixative, the heart and trachea were removed.

2.4 Histological techniques

2.4.1 Basic histological analysis

After two weeks in preservative lungs were removed, embedded in paraffin and blocked utilising standard histological protocols by either Ms J. Hope or Miss C. Macleod at the Glasgow Dental Hospital and School or by the Glasgow veterinary pathology department.

Lung blocks were sectioned at 5μm prior to staining with haematoxylin and 1% eosin.

Histopathology was scored blind using Table 2.2 (adapted from (Bergeron *et al.* 1998)):

2.4.2 Mast cell staining

Mast cell staining was performed by Miss Y. Paterson at the University of Glasgow. The lung sections were initially warmed to 56 °C for 1hr followed by 2 x 5min washes in Histoclear (National Diagnostics, Georgia, USA) and 2 x 5min washes in 100% ethanol. The sections were then rinsed in each of the following for 2min, 90% ethanol, 70% ethanol, 50% ethanol and finally tap water. The slides were then left in 0.5 % Toluidine blue (National Diagnostics, Appendix) for 24h before being rinsed in 0.7N hydrochloric acid and stained in 0.5% safranine O (Appendix) for 10min. Background counterstain was removed by rapid submersion in each of 70% ethanol, 90% ethanol, 2 x 100% ethanol and finally Histoclear for 1min. Slides were then mounted with Histomount and left to set.

Parameter	Score	Interpretation
Inflammatory Cells	0	No neutrophils, lymphocytes, or eosinophils in alveoli and lung tissue; normal amount of alveolar macrophages
	1	Slight increase in the number of any inflammatory cell population
	2	Recruitment of a large number of inflammatory cells
Haemorrhage	0	No erythrocytes in alveoli
	1	Few erythrocytes in alveoli
	2	Numerous erythrocytes
Oedema	0	Absent
	1	Minimal swelling of tissue interstitium
	2	Widespread swelling of tissue interstitium
Tissue injury	0	Absent
	1	Minimal modification in alveolar architecture
	2	Widespread disorganisation of lung tissue, including epithelial hyperplasia

Table 2.2 Scoring grid for histopathologic examination of lung tissue.

2.4.3 Immunohistochemistry

Immunohistochemistry was carried out by Miss C. MacLeod at the Glasgow Dental Hospital and School, utilising the following procedure. Lung sections were deparafinised in xylene and rehydrated and graded through ethanol (99% and 95%) before being washed with distilled water and then treated with 3% hydrogen peroxide in methanol for 15min.

The sections were again washed with distilled water before being microwaved three times in 0.1M EDTA for 5 min. Sections were allowed to cool to room temperature and then washed in PBS for 5 minutes. Blocking serum was prepared by adding three drops of rabbit serum to 10ml PBS, 200µl of blocker were added to each slide followed by an incubation at room temperature for 20min.

Blocking serum was replaced by primary antibody diluted in PBS/blocking serum to a pre-determined optimal titration (see below), this was left overnight at 4°C or for 1-2h at room temperature. The slides were then washed in PBS/ 0.01% Tween (v/v) for 5min and in PBS alone for an additional 5min.

A biotinylated anti-mouse IgG (Vectastain, Vector Laboratories, Peterborough, UK) was prepared by adding 1 drop to 10ml rabbit serum/PBS (prepared as above). 200µl of prepared antibody were placed on each slide and incubated for 30min at room temperature.

An alkaline phosphatase/anti phosphatase HRP complex (Vectastain A/B mix, Vector Laboratories) was prepared according to manufacturers instructions by the addition of 2 drops solution A and 2 drops solution B to 5ml PBS with a 30min incubation at room temperature prior to use. Sections were washed twice in PBS/ Tween for 5min and once in PBS for 5min before addition of 200µl A/B mix and an additional incubation at room temperature for 30min.

A further 2 x 5min washes in PBS/Tween were followed by a single wash in PBS alone for 5 min before the addition of 200µl developing solution. Developer (Fast Red TR Napthol AS Mix) was prepared by dissolving one Tris buffer tablet in 10ml distilled water before dissolving one Fast Red TR Napthol AS tablet. This was left on slides for 15-30min at room temperature, or until a signal was detected.

Sections were then washed twice in distilled water before being counter stained in Haematoxylin for 10min and mounted with Aquamount (Merck Ltd, Leicestershire, UK)

The optimal titrations of primary antibodies were as follows:
Rat anti mouse IL-10 monoclonal (Endogen Cat. No. MM-010-S, TCS biologicals, UK) 1/100 dilution. This antibody is of the JES-2A5 clone specific for mouse IL-10.

Rat anti-mouse TNFα monoclonal (Serotec Ltd, Oxford, UK Cat. No. MCA1487) 1/50 dilution. This antibody is of the MP6-XT3 clone specific for murine TNFα.

Rat anti-mouse PMN (Serotec Cat No. MCA7719) 1/100 dilution. This antibody of the 7/4 clone recognises a polymorphic 40kD antigen expressed by polymorphonuclear cell, but absent on macrophages.

2.5 Immunological techniques

In each assay samples were run either in duplicate or triplicate, as stated in the method. Actual measurements were determined by comparing the mean sample absorbance to the standard curve.

2.5.1 Detection of TNFα by Quantikine™ ELISA kit from R & D Systems

Following the protocol supplied with the kit, the required number of wells were placed in the plate frame and 50µl of assay diluent RD1W added to each well. The standard curve was prepared by reconstitution of the mouse TNF-α standard with calibrator diluent RD5T to give a standards with concentrations of 1500-23.44pg/ml. Unused standard was aliquoted into single use volumes and frozen at -70°C until required. The batch specific control was resuspended as directed in distilled water and excess stored as single use aliquots frozen at -70°C. 50µl of standard, control or undulate sample were added to the wells in duplicate with calibrator diluent serving as the zero standard. Well contents were mixed by gentle tapping and the

plate was covered with an adhesive strip and incubated at room temperature for 2hrs.
Wells were then emptied and washed for a total of 5 washes using diluted wash buffer. The anti-mouse TNF-α conjugate was diluted in conjugate diluent and 100µl added to each well, the plate was covered with a fresh adhesive strip and incubated at room temperature for 2hrs.
The wash step was repeated prior to addition of 100µl of substrate (prepared by mixing equal volumes of colour reagents A and B) to each well and the plate incubated in the dark for 30min to allow the colour change to occur. Colour development was stopped by the addition of 100µl stop solution to each well. The A_{450} nm was read with correction at A_{570} using a Dynatech MRX ELISA reader with Biolinx 2.21™ software.

2.5.2 Detection of IL-1β by Quantikine™ ELISA kit from R & D Systems

Following the protocol supplied with the kit. The required number of wells were placed in the plate frame and 50µl of assay diluent RD1-14 added to each well. IL-1β standard curve was prepared by reconstitution of the mouse IL-1β standard with calibrator diluent RD5T to give a standards with concentrations of 500-7.5pg/ml. The remainder of the standard was aliquoted into single use volumes and frozen at -70°C until required. A batch specific control was resuspended as directed in distilled water and unused control stored as single use aliquots at -70°C. Lavage fluid were diluted 1 in 2 and lung homogenates 1 in 4 in calibrator diluent before the addition of 50µl of standard, control or sample in duplicate to the wells. Calibrator diluent served as the zero standard. The plate was tapped gently to mix the well contents and covered with an adhesive strip and incubated at room temperature for 2 hrs.
The wells were emptied and washed for a total of 5 washes using diluted wash buffer. An anti-mouse IL-1β conjugate was diluted in conjugate diluent and 100µl added to each well before covering the plate with a fresh adhesive strip and incubating it at room temperature for 2 hrs.
The wells were then emptied and washed as above. Substrate was prepared (by mixing equal volumes of colour reagents A and B), and 100µl added to each well and the plate incubated in the dark for 30min to allow the colour change to occur. Colour development was stopped by the addition of 100µl

stop solution to each well. The A_{450} nm was read with correction at A_{570} using an MRX ELISA reader.

2.5.3 Detection of IL-6 by ELISA

A sandwich ELISA was performed to detect IL-6 in samples. Plastic flat-bottomed 96-well maxisorb™ plates (Life Technologies) were coated with 50µl per well of a 2.0µg/ml preparation of the purified anti-mouse mAb to IL-6 (Pharmingen Cat No.18071D, Becton Dickinson) in coating buffer (Appendix), sealed with cling-film to prevent evaporation and incubated overnight at 4°C.
Wells were then emptied and unbound areas blocked by the addition of 200µl blocking buffer (Appendix) at room temperature for 2h.
Blocking buffer was removed from all wells and the plates were washed 3 times with PBS/0.05% polyoxyethylenesorbitan monolaurate (Tween 20). The recombinant murine IL-6 standards (Pharmingen Cat No. 19251V) were prepared in blocking buffer/Tween (Appendix) in the range of 10U/ml - 0.04 U/ml. Lavage fluids were diluted 1 in 2 and lung homogenates 1 in 4 in blocking buffer/Tween prior to the addition of 100µl of standard or sample in duplicate. Plates were then incubated at room temperature for 2h or for 16h at 4°C.
Plates were washed 4 times with wash buffer and 100µl of a 1.0µg/ml solution of biotinylated anti-mouse mAb to IL-6 (Pharmingen Cat No.18082D) was added, followed by a 1h incubation at room temperature.
Following 4 washes with wash buffer, 100µl streptavidin-HRP (1/2000 dil of Genzyme Cat No. 80-3546-03) was added for 30min at room temperature.
After 5 washes with wash buffer, specifically bound IL-6 was visualised with 100µl of ELISA substrate (Appendix). Plates were incubated in the dark at room temperature for 30min and the reaction stopped by addition of 100µl 1M H_2SO_4. A_{450} was determined by reading the plates on an MRX ELISA plate reader with correction set at A_{570}.

2.5.4 Detection of IL-10 by ELISA

Plastic flat-bottomed 96-well maxisorb™ plates (Life Technologies) were coated with 100µl per well of a 4.0µg/ml preparation of the purified anti-

mouse mAb to IL-10 (Genzyme, Kent, UK Cat No. 80-4858-02) in coating buffer (Appendix), sealed with cling-film and incubated overnight at 4°C.
Plates were washed 3 times with PBS/0.05% Tween 20 and unbound areas blocked with 250µl blocking buffer (Appendix) at 37°C for 2h.
The recombinant murine IL-10 standards (1600-0pg/ml, Genzyme, Cat No. 80-4857-02) were prepared in standard diluent (Appendix). Blocking buffer was removed from all wells before addition of 100µl of standard in duplicate. 50µl of standard diluent were added the remaining wells before addition of 50µl sample (resulting in a 1 in 2 dilution) in duplicate and plates incubated at 37°C for 2h. From this point on all solutions were made up in standard diluent.
Plates were washed 4 times with wash buffer and 100µl of a 0.2µg/ml solution of biotinylated anti-mouse mAb to IL-10 (Genzyme Cat No. 80-4862-02) was added, followed by a 1h incubation at 37°C.
Following 4 washes with wash buffer, 100µl streptavidin-HRP (1/2000 dil of Genzyme Cat No. 80-3546-03) was added for 30min at 37°C.
After 5 washes with wash buffer, specifically bound IL-10 was visualised with 100µl of ELISA substrate (Appendix). Plates were incubated in the dark at room temperature for 30min and the reaction stopped by addition of 100µl 1M H_2SO_4. A_{450} was determined by reading the plates on an MRX ELISA plate reader with correction set at A_{570}.

2.5.5 Nitric oxide determination

Nitric Oxide (NO) has a half-life of only milliseconds before being oxidised to nitrite (NO_2^-) and nitrate NO_3^-). Both NO_2^- and NO_3^- are more stable forms and can be measured in serum as an indication of NO production. Nitrate levels in samples were determined using the conversion assay described by Moncada (Moncada *et al.* 1991). The degree of conversion was assessed by the Greiss reaction (Migliorini *et al.* 1991).
Standards of both NO_2^- and NO_3^- were prepared in the range 400µM to 6.25µM by diluting a 0.1M stock solution of $NaNO_2$ and $NaNO_3$ respectively in distilled water. 30µl of standard, sample or blank (dH_20) were added to the wells of a flat bottomed 96 well microtitre plate (Life Technologies) in duplicate and then 30µl of conversion buffer (Appendix) added. Plates were incubated at 37°C for 2h and then 60µl of Greiss solution (Appendix) was

added. After 10min at room temperature the plate was read in an MRX ELISA plate reader at 540nm. NO levels were determined from the nitrite standard curve with the nitrate standard curve utilised to ensure proper conversion had taken place.

2.5.6 Total protein determination

Total protein measurements were determined via the Bradford assay (Bradford 1976). Standard curves were prepared by the dilution of a 2mg/ml stock solution of BSA in PBS giving a range from 1500µg/ml - 62.5µg/ml. 10µl of sample, standard or PBS (blank) were added to the wells of a flat bottomed 96 well plate. 200µl of Bradford's reagent were then added to each well and the plate incubated at room temperature for 10 min. Colour development was determined at 570nm on an MRX ELISA plate reader. Total protein levels within the samples were calculated by comparison to the standard curve.

2.6 Tissue culture methods

2.6.1 Standard passage of L929 cells

The L929 mouse connective tissue cell line was purchased from the European Collection of Animal Cell Cultures (ECACC, No. 85011425). Once flasks reached confluency the flasks were left one more day before removing the growth media (RPMI 1640 plus 10% heat inactivated FCS, 50 IU/ml penicillin and 50µg/ml streptomycin) and washing the cells by the addition of 10ml sterile PBS. On removal of this, 1ml trypsin/EDTA (Life Technologies) was added, the cells coated and excess discarded. The flasks were incubated at 37°C and 5% CO_2 until the monolayer began to lift from the flask (usually 5-10 min) The cells were the resuspended in 10ml fresh media. 2ml of the resulting suspension were used to inoculate 18ml of fresh media. The caps were loosely closed and the flasks incubated at 37°C and 5% CO_2.

2.6.2 Cryopreservation of mammalian cells

Once the cells became confluent the cells were trypsinised (as described above) and resuspended in freeze media (normal medium with 10% v/v DMSO). The cells were aliquoted into 1ml volumes in sterile cryotubes and frozen to -70°C at a rate of 1°C/min by placing them in a box surrounded with isopropanol (Mr Frosty™ Fisher Scientific) in a -70°C freezer. Cells were then transferred to a liquid nitrogen store.

2.6.3 Recovery of cells from liquid nitrogen storage

A vial of cells was removed from liquid nitrogen storage and defrosted quickly at 37°C by immersion in a water bath after loosening off the lid. The tube was the swabbed with 80% industrial methylated spirits to ensure sterility. The cells were resuspended in 10ml growth medium and pelleted by centrifugation at 1000rpm (Sigma 4K15 centrifuge) for 10min. The supernatant was discarded and the pellet resuspended in 5ml growth medium, transferred to a (25 cm^2) flask and incubated at 37°C and 5% CO_2.

2.6.4 TNF bioassay

A TNF bioassay was developed and optimised from a method routinely run at AstraZeneca pharmaceuticals, and published cytotoxicity assays (Levesque *et al.* 1995) and (Coote and Arain 1995). This assay was based upon the ability of TNF to cause cytotoxicity on fibroblast cells. Cell death was assessed by the inability of cell mitochondria to breakdown MTT (3-[4,5-Dimethylthiazol-2y1]-2-5, diphenyltetrazolium bromide) into a formazan product.

During late afternoon (in order prevent target cells from proliferating to an unusable level before the addition of samples), flasks were trypsinised as above and viable cells counted using 500µl trypan blue, 300µl media and 200µl cells. This was left for 5min before counting the cells in an improved Neubauer chamber. The number of cells was adjusted to $3x10^5$/ml in media and 200µl of this suspension was added to each well of a flat bottomed 96 well tissue culture plate.

Following an overnight incubation at 37°C and 5% CO_2,the media was removed from all wells and replaced by 50µl of a 20µg/ml solution of

Actinomycin D in PBS (to prevent L929 multiplication in wells without TNF activity). The TNFα standard was diluted 1/3 in media giving a range of concentrations from 2500 U/ml to 0.014 U/ml. Samples were subjected to 3 x 1/3 dilutions in media as well as being tested undiluted (for serum, the volumes recovered meant that samples were already diluted 1/15 before being tested in this assay). 25µL of TNFα standard or sample was then added in triplicate to the appropriate wells with 25µl of media were added in triplicate to separate wells as a 0% kill control.
The plates were returned to 37°C and 5% CO_2 for an overnight incubation. Cell viability was then indicated by the addition of 100µl of 1mg/ml MTT with a 4h incubation.
After removal of the media, the formazan product of MTT was solubilised by the addition of 100µl of dimethyl-sulphoxide/0.04N hydrochloric acid and gently tapping the plates.
The A_{540} was then read with correction set at A_{630} in an MRX ELISA plate reader. A logarithmic standard curve was used to determine the activity of the samples by the 50% kill method. This involved determining the TNFα standard activity that was capable of killing 50% of the cells per well. The corresponding absorbance was then compared to the diluted samples. This gives a dilution factor that was used to determine the actual TNFα activity of the sample (Thorpe *et al.* 1992).

2.6.5 *In vitro* airway cell stimulation assays

Cells recovered as in Section 2.3.10 were washed by centrifugation at 1000rpm (Sigma 4K15 centrifuge) and 4°C for 5min. The pellet was resuspended in 500µl RPMI 1640 culture medium used in TNFα bioassay. Cell viability was measured as above and the number adjusted to 2.0×10^5 cells/ml. 100µl of this cell suspension were plated per well in a flat bottomed 96 well microtitre plate. Cells were incubated overnight in 37°C and 5% CO_2 before being washed twice with 100µl sterile PBS. Adherent cells were then stimulated with LPS, purified pneumolysin or heat killed pneumococci.
LPS (from *E. coli* 0127:B8) was utilised at a range of concentrations from 50,000ng/ml - 0.0005ng/ml or media alone for 24h. The supernatant fluid was sampled and TNFα total protein and activity measured as above. This incubation period has previously been shown to be sufficient for TNFα production to occur (Houldsworth *et al.* 1994).

Recombinant pneumolysin protein was obtained from Ms J. Search, Division of Infection & Immunity. This protein was known to possess biological activity as it was haemolytic for red blood cells. The concentrations of pneumolysin utilised as stimulants were 100ng/ml, 1ng/ml, 10pg/ml or 3pg/ml which were previously shown to be capable of stimulating TNFα and IL-1β production by human monocytes (Houldsworth *et al.* 1994). Supernatant fluids from pneumolysin stimulated cells were only assayed for TNFα by ELISA due to unpublished observations that pneumolysin is cytotoxic for L929 cells.

For heat killed pneumococci stimulation experiments the number of bacteria used were either 10^6 , 10^5 or 10^4 CFU. These numbers of heat killed pneumococci have been shown to be capable of releasing IL-1β from human monocytes (Riesenfeld-Orn *et al.* 1989). With heat killed bacterial stimulation, supernatant fluid was centrifuged at 13,000 rpm (Eppendorf 5417 centrifuge) for 3min in order to remove bacteria from samples prior to detection of TNF by bioassay or ELISA.

In a preliminary experiment 10^6 CFU heat killed pneumococci were combined with 1ng/ml pneumolysin.

2.7 Immune modulation techniques

2.7.1 Effect of exogenous surfactant on pneumonia

Artificial lung expanding compound (ALEC, Britannia Pharmaceuticals Ltd, Surrey, UK) was re-suspended in non-pyrogenic saline to a concentration 41.67mg/ml. This was shaken gently and left at 4°C for 30min to allow the viscosity to settle before experiments were carried out. The initial *in vitro* experiment involved preparing a suspension of approximately 2.0 x10^7 CFU/ml and investigating viability over time.

This was followed by investigation of bacterial growth *in vitro* in BHI supplemented with 5% (v/v) ALEC (Rubins *et al.* 1996). An equal volume of non-pyrogenic saline was added to BHI as a control. Viable bacteria were counted hourly until control cultures were seen to pass through each stage in a typical growth curve. Absorbance readings could not be obtained for the cultures containing ALEC due to the opacity of a 5% ALEC solution.

In vivo investigations involved intranasal administration of 50µl ALEC either 2h prior to challenge with 10^6 CFU *S. pneumoniae*, at the same time as the bacteria, 2h after pneumococci, or alone with bacterial loads at 24h and overall survival determined.

2.7.2 Anti TNFα antibody modulation

Affinity-purified neutralising anti-TNFα polyclonal antibody (R & D Systems) was re-suspended in sterile non pyrogenic saline to a concentration of 200µg/ml. The amount of antibody required to neutralise the level of TNFα produced *in vivo* during pneumococcal pneumonia was determined using the TNF bioassay. TNFα standard was prepared at the highest activity detected in lavage fluids or per total animal during pneumococcal pneumonia and the level of antibody required to neutralise this activity calculated. Accordingly, 40µg of antibody was given intraperitoneally to groups of 5 mice at 2h prior to infection with 1×10^6 CFU *S. pneumoniae*. The negative control was 40µg Goat IgG (as the anti-TNF-α antibody was a goat anti-mouse IgG antibody) and 200µl anti-pneumococcal type 2 capsule antiserum (diluted 1 in 200 in sterile non-pyrogenic saline) provided the positive control. In the case of survival times an additional control set consisted of an untreated group of mice.
Animals were monitored until 24h after infection when they were sacrificed and bacterial loads determined in lavage fluid, lung homogenates and blood. Aliquots of lavage fluid were also snap frozen for cytokine analysis.
Neutralisation of airway TNFα was achieved by intranasal administration of 10µg anti-TNFα antibody, 10µg Goat IgG, or 50µl anti-type 2 capsule antiserum 2h prior to infection with 1×10^6 CFU *S. pneumoniae*. Samples were taken at 24h post challenge as for the intraperitoneal modulation route.

2.7.3 Effect of SNAP on *S. pneumoniae* growth *in vitro*

The effect of NO on the growth and viability of *S. pneumoniae in vitro* was determined using s-nitroso-acetyl penicillamine (SNAP, Calbiochem, Nottingham, UK), a NO donor (Kaplan *et al.* 1996). SNAP was freshly prepared in BHI and filter sterilised to give a range of concentrations from 200-25µM. These preparations were incubated at 37°C for 24h, with 1ml

samples removed and frozen at -70°C at several time points. Total nitrite was determined in these samples using the Greiss reaction. From this experiment the concentration of SNAP required to induce a rapid and easily detectable production of NO was determined.

S. pneumoniae was obtained from vials of standard inoculum and was therefore in mid log phase of growth. This preparation was added to BHI containing 200μM SNAP to give a bacterial load of around $5x10^4$ cfu/ml. *S. pneumoniae* was also incubated in controls (BHI alone, D-penicillamine [to control for SNAP] and sodium nitrite [to control for nitrite production]), both at the same concentrations as SNAP. The cultures were incubated at 37°C and growth of the bacteria was measured each hour by optical density at 600nm and by viable counting. Culture samples were stored at -70°C prior to nitrite levels being determined by Greiss reaction as previously.

2.8 Statistical analysis

Results are expressed as geometric mean +/- 1 SEM unless otherwise stated.

Comparisons of survival times following infection and core body temperatures were carried out using non-parametric Mann Whitney U analysis with $P<0.05$ or $P<0.01$ considered statistically significant for all analyses (Wardlaw 1997). If a value of greater than 0.05 was obtained, this is not mentioned in the text. Levels of inflammatory mediators were also compared by Mann Whitney U analysis with a Bonferroni correction for multiple analyses.

Comparisons of bacterial load data from time course experiments were compared using one way analysis of variance (ANOVA) with Scheffe's post hoc test. Where samples contained fewer CFU/ml than the lower detection limit for the viable counting assay (Log 1.92) they were ascribed a value just below the detection limit (Log 1.91) in order to prevent artificial reduction of the geometric mean. Comparisons between mouse strains or treatment groups were carried out using multiple unpaired student t tests with a Bonferroni correction for multiple analyses.

Chapter3

Inflammation in MF1 mice

3.1 INTRODUCTION

A murine model of pneumococcal pneumonia has been used to study the pathogenesis of the infection. This model is based on that described by Canvin *et al* (Canvin *et al.* 1995) and involves respiratory challenge of 9 week old adult MF1 mice. An acute infection is initiated, resulting in bacteraemic pneumococcal pneumonia within 48h.
Previous investigations utilising this model have concentrated on roles played by the bacteria during the infection (Kadioglu *et al.* 2000). This chapter characterises the host inflammatory response to the inoculated pneumococci.

The effect of reducing the infectious dose on the inflammatory response was characterised in order to determine whether there was a threshold number of bacteria required to induce the inflammatory response. In addition the possibility that the magnitude of the host response correlates with the infectious dose was investigated.

Epidemiological studies have shown age to be a very important factor in determining outcome after pneumococcal pneumonia. Infants under 2 years of age and elderly individuals older than 65 years have the greatest infection rates and the poorest outcome to infection (Burman *et al.* 1985). Immune alterations responsible for increased susceptibility in infants are better understood than those in the elderly population. The murine model of pneumococcal pneumonia was therefore utilised in order to determine whether there was also an age bias in mice and whether an altered inflammatory response could account for such an effect (Bruunsgaard *et al.* 1999) and Section 1.4.3.1.

3.2 RESULTS

3.2.1 Growth characteristics of D39

In vivo experiments were carried out with pneumococci that were in mid log phase, requiring the growth characteristics of D39 to be investigated. Figure 3.1 shows the growth curve for *S. pneumoniae* in serum broth with lag phase, logarithmic phase, stationary phase and then decline evident. Logarithmic growth is associated with a doubling time of approximately 25 min. Mid-log phase of growth occurs at an OD_{600} of approximately 0.600.

3.2.2 Viability of D39 on ice

As *S. pneumoniae* is known to be a labile bacterium (Jonsson *et al.* 1986) & (Johnston 1991) viability of the challenge dose on ice was investigated. Viability of *S. pneumoniae* prepared in PBS and kept on ice drops with time (Figure 3.2). The greatest decrease occurs within the initial 20min with a more gradual loss of viability occurring in the following 40min, suggesting that the decline in viability is levelling off. All animal inoculations were therefore performed within this 40min window.

3.2.3 Intranasal infection

During the initial 18h post intranasal infection with D39, MF1 mice displayed normal behaviour (Figure 3.3 A) and are highly alert. There then followed progression through a range of clinical indications of pneumococcal infection. The primary observation was a hunched stance (Figure 3.3 B) as animals walked or stood still. The next clinical sign of pneumococcal pneumonia displayed by MF1 mice is piloerection. The hairs covering the back of the mouse in Figure 3.3 C are standing on end. This mouse is no longer interested in its surroundings and is displaying a marked hunched appearance. All further clinical signs of illness are manifested in the movement of the mice and cannot thus be displayed in a figure. Mice become more lethargic in their movement before finally becoming unwilling to move (moribund (Toth 1997)). Animals reaching this stage were sacrificed.

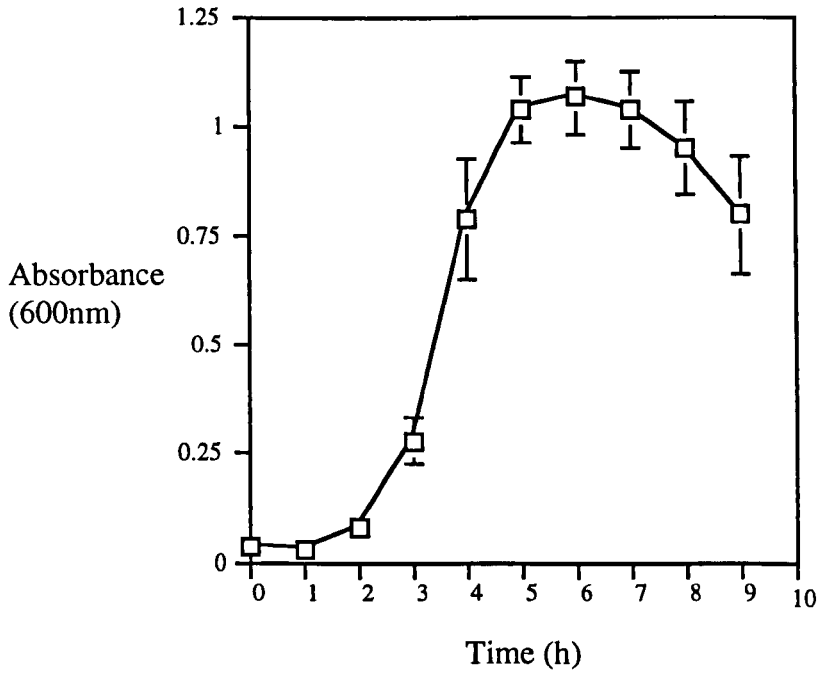

Figure 3.1 Growth characteristics of *S. pneumoniae* in BHI + 20% v/v heat-inactivated fetal calf serum. These results represent four bacterial suspensions grown on two separate occasions.

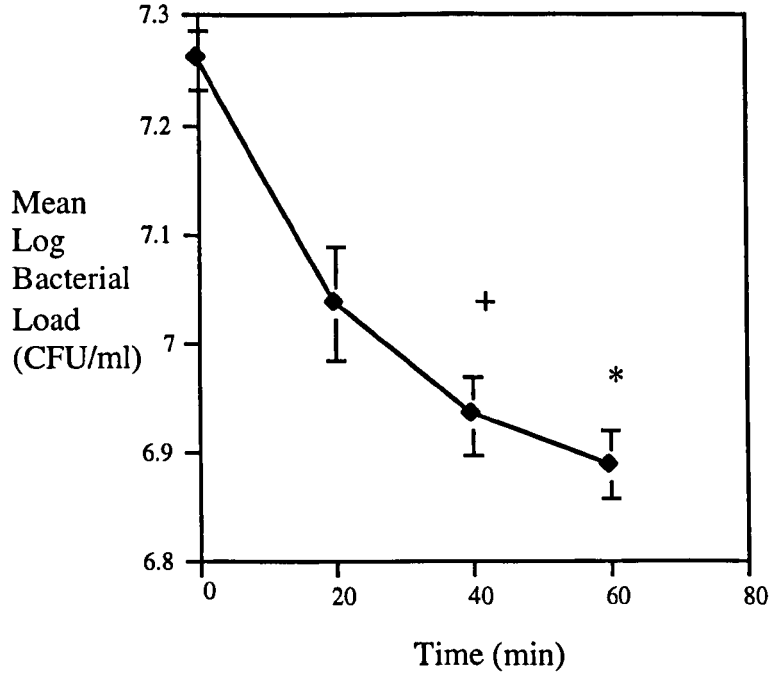

Figure 3.2 Viability of *S. pneumoniae* resuspended in PBS and kept on ice. n=3, *, $P<0.01$ and $^{+}$, $P<0.05$ reduced viability when compared to 0min results.

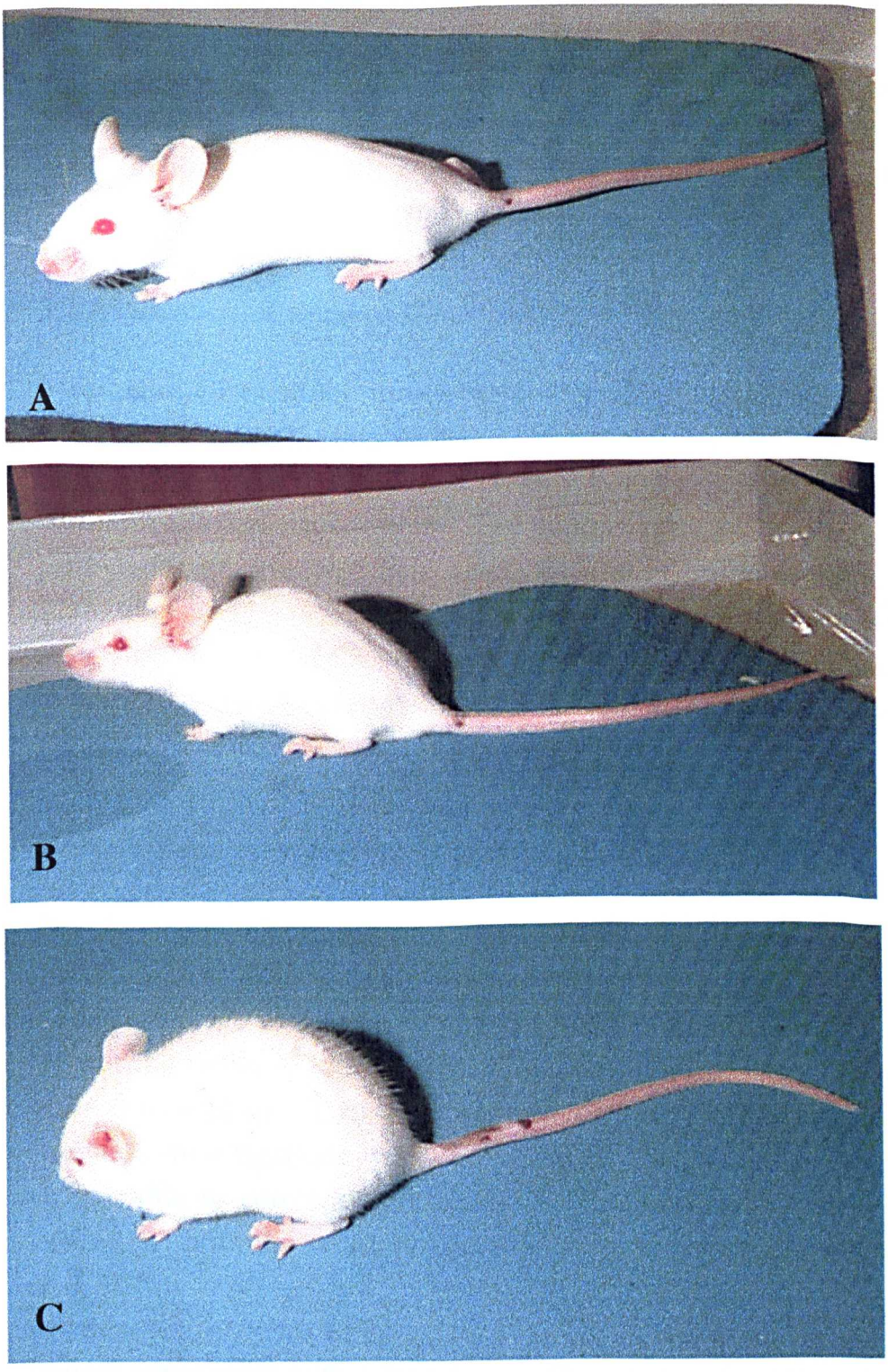

Figure 3.3 Clinical signs of pneumococcal pneumonia displayed by MF1 mice following infection with 10^6 CFU *S. pneumoniae*. A= healthy mouse, B= mouse displaying early signs of infection with a hunched stance, and C= mouse with piloerection during mid/late infection.

Development of these signs of illness was followed by a "pain-score" system developed by this laboratory (Section 2.3.5). This involved frequent recording of the severity of each of the above signs throughout the experiments, in order to monitor disease progression.

3.2.3.1 Survival & signs of illness following intranasal infection

Mice succumbed to the infection rapidly after intranasal challenge with 10^6 CFU *S. pneumoniae* (Figure 3.4). The onset of signs of infection occurred at around 18h into the experiment with a hunched appearance being the most common clinical observation. The earliest time of death recorded was 26h post challenge, followed by a steady decline in the percentage survival until 118h after infection. In seven experiments only two mice (out of 53) survived the infection. Statistical comparison of the survival times gained in the 7 separate experiments revealed no significant differences, showing the reproducibility of the model.

3.2.3.2 Telemetry following intranasal infection

The "pain-score" monitoring system is not ideal as it does not permit continual monitoring, e.g. during the night. In addition, it is difficult to utilise pain-score data in a quantitative method. For these reasons a telemetry system was employed. This permitted constant monitoring of core body temperature (Tco) and activity of mice throughout the infection. Previous investigations have shown telemetry systems to yield reliable data, comparable to conventional methods of disease monitoring such as rectal probes (without the constant distress to the experimental animals) (Dilsaver *et al.* 1992).

In this set of experiments mice were infected at 08:00h on 12/08/98 (month/day/year) with telemetry readings taken for 12h prior to infection. Although a cyclical alteration could be seen in the temperature of sham infected mice, no significant alteration in core body temperature was found (Figure 3.5). In addition, activity counts for sham infected animals remained high during the experiment (Figure 3.6). Periods of no activity occurred infrequently and with short duration.

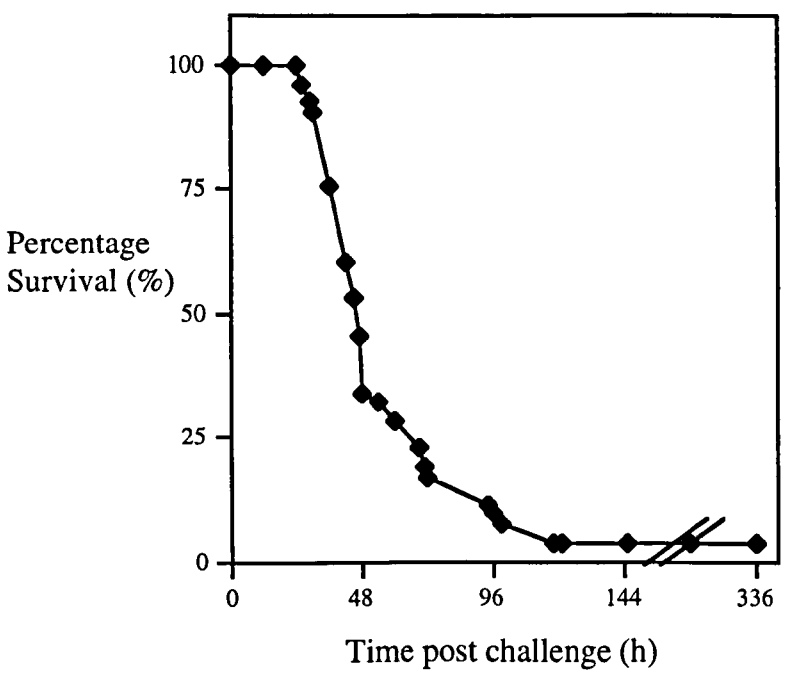

Figure 3.4 Survival of female adult MF1 mice after intranasal challenge with 9.76×10^5 CFU *S. pneumoniae*. n= 53 from 7 experiments.

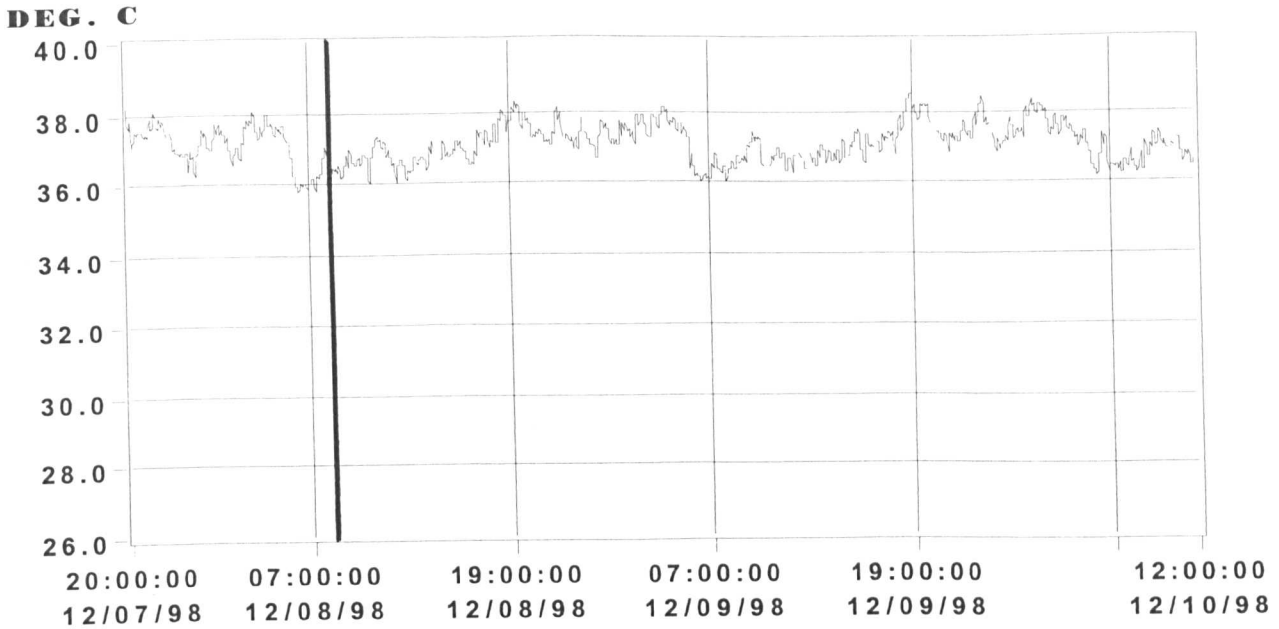

Figure 3.5 Core body temperature (°C) of an MF1 mouse sham infected with PBS. Vertical line represents time of "infection".

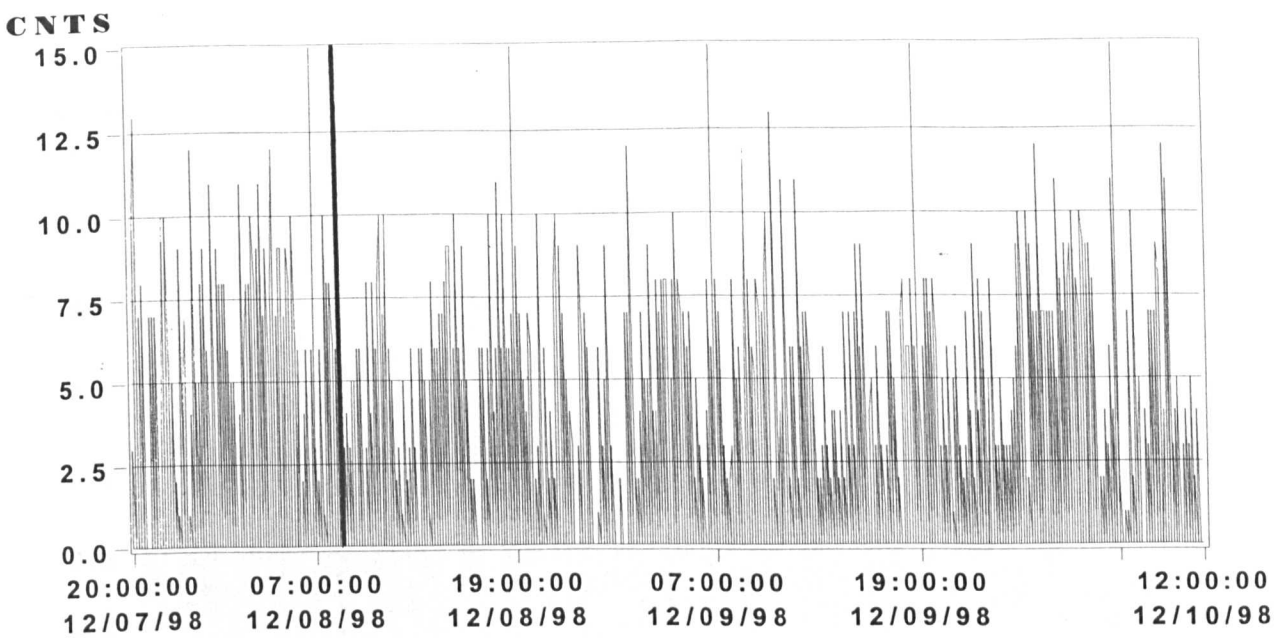

Figure 3.6 Activity score (arbitrary units) of an MF1 mouse sham infected with PBS. Vertical line represents time of "infection".

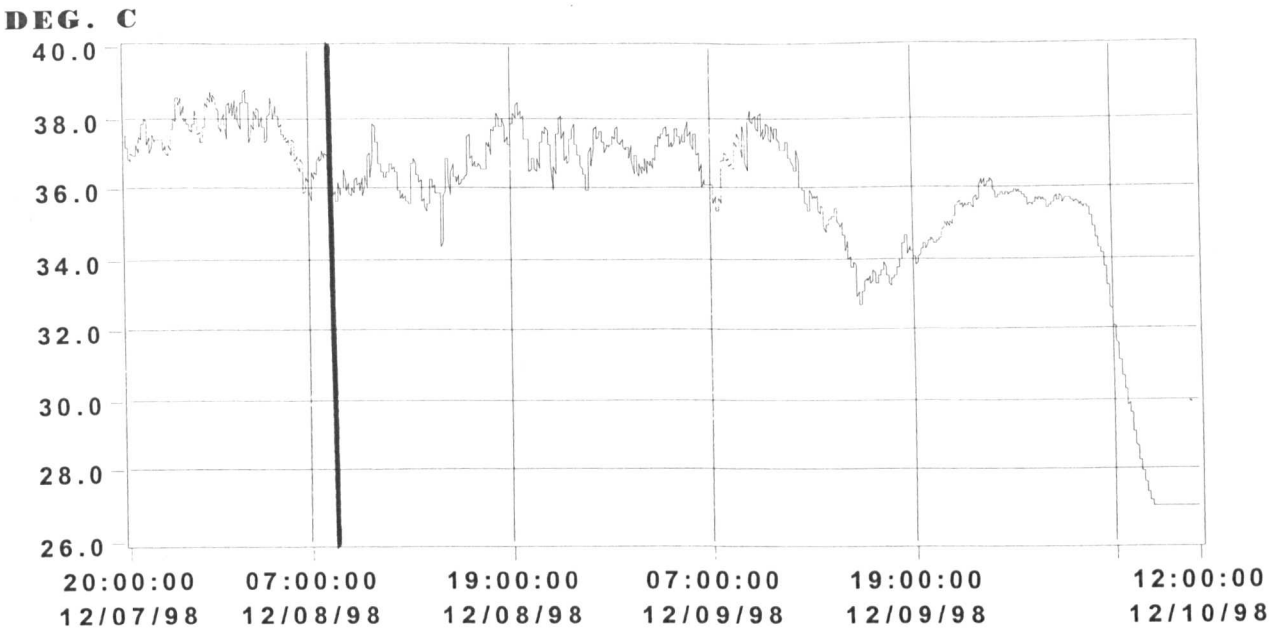

Figure 3.7 Core body temperature (°C) of an MF1 mouse infected with 10^6 CFU *S. pneumoniae*. Vertical line represents time of infection.

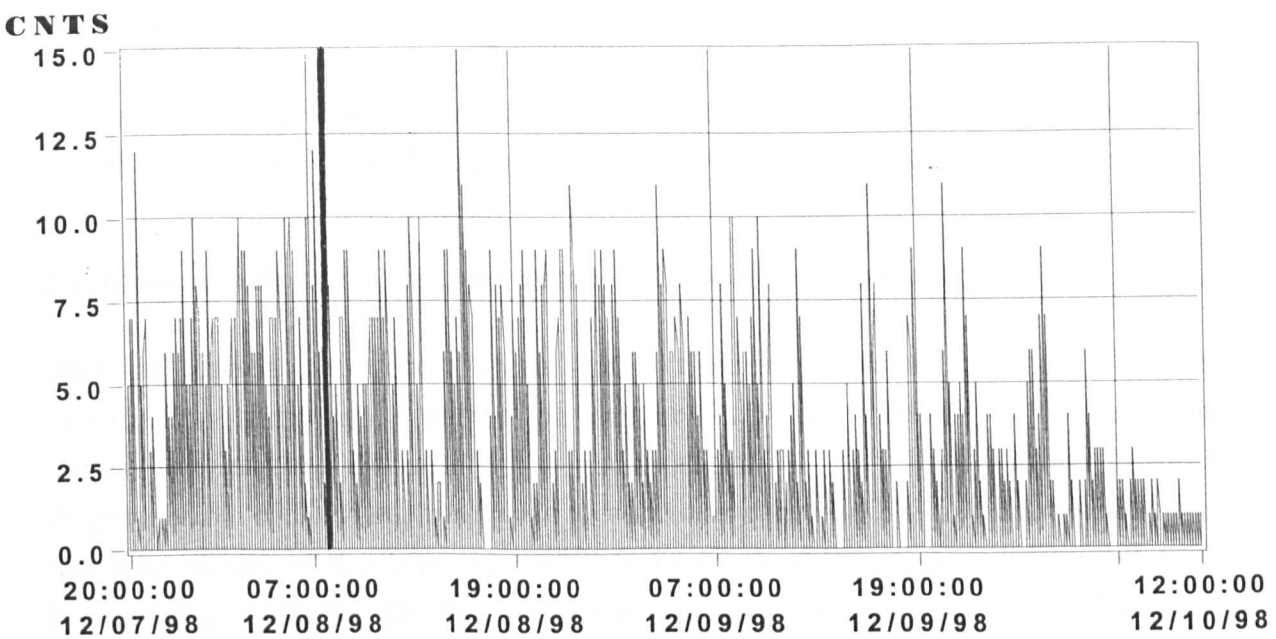

Figure 3.8 Activity score (arbitrary units) of an MF1 mouse infected with 10^6 CFU *S. pneumoniae*. Vertical line represents time of infection.

During the initial 24h of the experiment core body temperature *in S. pneumoniae* infected animals was not seen to alter significantly from controls and a similar cyclical alteration of Tco as seen in PBS treated mice was documented (Figure 3.7). The circadian rhythm of infected animals then became less pronounced and mild, transient hypothermia developed in 8/20 implanted MF1 animals succumbing to the infection, Tco in infected mice then partially returned to baseline before the animals went on to develop marked hypothermia. The other 12 animals displayed only one period of marked hypothermia. Core body temperature dropped to ambient room temperature, over a period of around 6h with time of death around 09:00h on 12/08/98.

At the initiation of hypothermia clinical symptoms of infected mice were found to be moderate with hunched appearance, piloerection and mild lethargy. When ambient room temperature was reached signs of infection had progressed to the stage that mice were deemed moribund.

Activity records show that MF1 mice are highly active during the initial 30h of the experiment (Figure 3.8). Concurrently with the development of mild hypothermia activity scores were reduced. High activity counts were recorded less frequently as the end stages of disease were reached and Tco dropped below 34°C.

3.2.3.3 Bacteriology in the pulmonary air spaces following intranasal infection

Counts of viable pneumococci within pulmonary air spaces did not alter significantly from time 0h values during the infection (Figure 3.9).

3.2.3.4 Bacteriology in the lung tissues following intranasal infection

Bacterial loads recovered from lung tissues increase immediately on inoculation although a period of greater than 12h appears to be required before the bacteria become fully acclimatised to their new environment (Figure 3.10). Following this time the counts increased significantly towards the later stages of the infection with a mean doubling time of 2h whilst in logarithmic growth. By the end of the disease, as many as 10^7 CFU were found associated with each ml of lung homogenate.

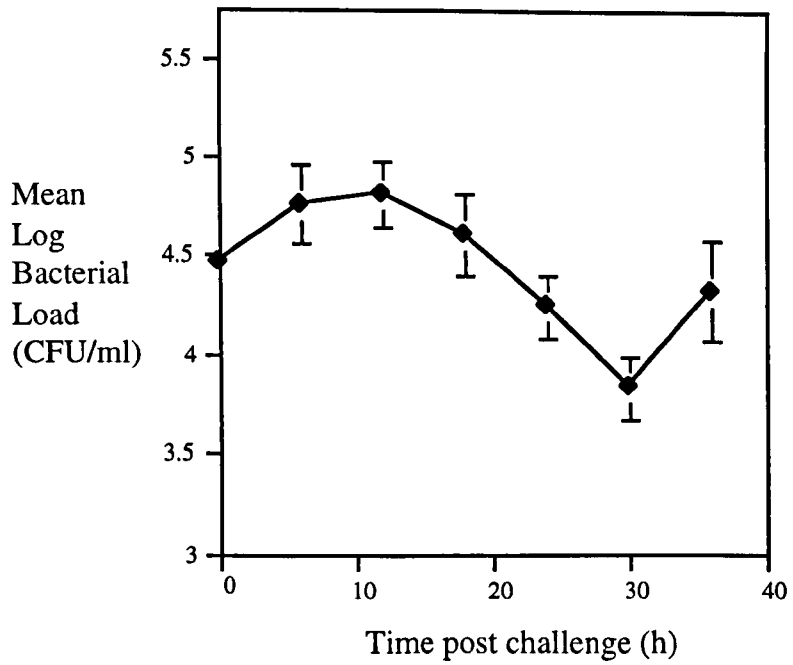

Figure 3.9 Bacterial loads within the airways of MF1 mice following intranasal infection with 9.75 x 10^5 CFU *S. pneumoniae*. n=14-24.

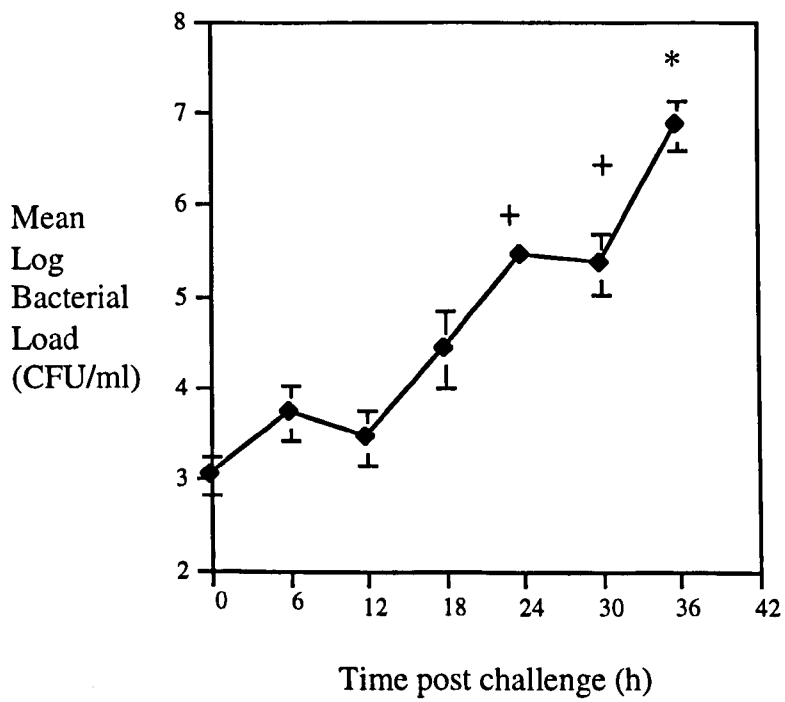

Figure 3.10 Bacterial loads within the lung tissues of MF1 mice following intranasal infection with 9.75 x 10^5 CFU *S. pneumoniae*. n=5-9, *, $P<0.01$, +, $P<0.05$ higher when compared to time 0h values.

3.2.3.5 Systemic bacteriology following intranasal infection

S. pneumoniae was able to enter the bloodstreams of 6 of the 7 infected mice by 12h post challenge (6 of the 17 were bacteraemic at this time) [Table 3.1]. By 18h into the experiment only one mouse did not have detectable pneumococci in the bloodstream. Once in the circulation pneumococcal counts increased until the end of the infection when greater than 10^7 CFU were cultured from each ml of blood. The mean doubling time of bloodborne bacteria was 1.4h (slightly more rapid than the 2h seen within lung tissues).

3.2.3.6 Bacteriology in perfused lung tissues following intranasal infection

As such high bacterial counts are present in the blood of infected mice and the lungs contain a high proportion of this blood it was a possibility that the elevated bacterial counts seen in the lung tissues was due to blood-borne and not tissue associated pneumococci. Blood was therefore perfused from selected lungs prior to removal and homogenisation. Bacterial loads recovered from these lung homogenates were not significantly different from those detected in unperfused lungs (Figure 3.11) at all times investigated. Therefore the increase in bacterial numbers seen in the lung homogenates is not solely due to blood-borne bacteria.

3.2.3.7 Histology following intranasal infection

Histological investigation revealed the development of acute inflammation within the lungs following intranasal infection with 1.02×10^6 CFU *S. pneumoniae* (Figure 3.12). The pattern of inflammation shows that bronchopneumonia has developed. As little as 6h into the infection some areas of lung tissue were already displaying oedema. In addition, increased numbers of neutrophils could be seen within the blood vessels. These cells were found both among erythrocytes in the middle of the blood vessels and in association with the endothelium. By the 12h time point areas of oedema were more common between the normally closely associated blood vessels and bronchioles and a slightly higher number of inflammatory cells could be found in these areas. By 24h these perivascular areas contained appreciable

	Time post challenge (h)						
	0h	**6h**	**12h**	**18h**	**24h**	**30h**	**36h**
Bacterial load (Log CFU/ml)	ND	ND	2.13 +/- 0.10	4.19 +/- 0.39 *	5.22 +/- 0.34 *	5.80 +/- 0.35 *	7.12 +/- 0.46 *

Table 3.1 Mean log bacterial loads within the bloodstream of MF1 mice infected 9.75 x 10^5 CFU *S. pneumoniae*. n=14-24, *, P<0.01 higher when compared to 0h counts. ND= below detection limit of viable count assay (<log 1.92 CFU/ml).

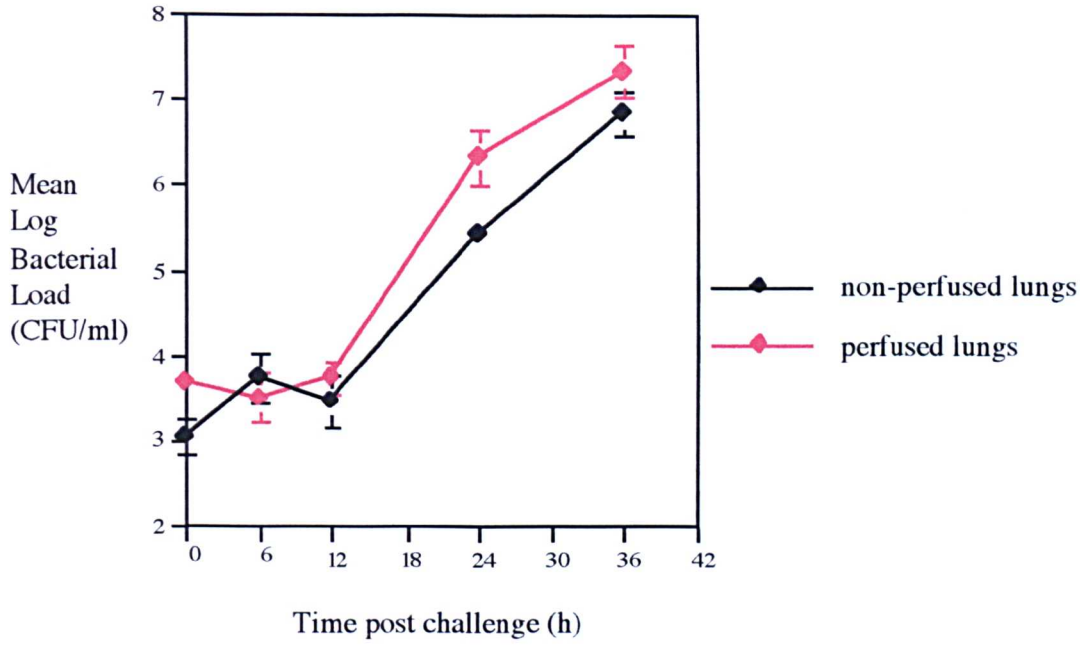

Figure 3.11 Bacterial loads within the perfused lung tissues of MF1 mice following intranasal infection with *S. pneumoniae*. Infectious dose for perfused group was 7.12×10^5 CFU and for non-perfused group 1.01×10^6 CFU, n=5-9.

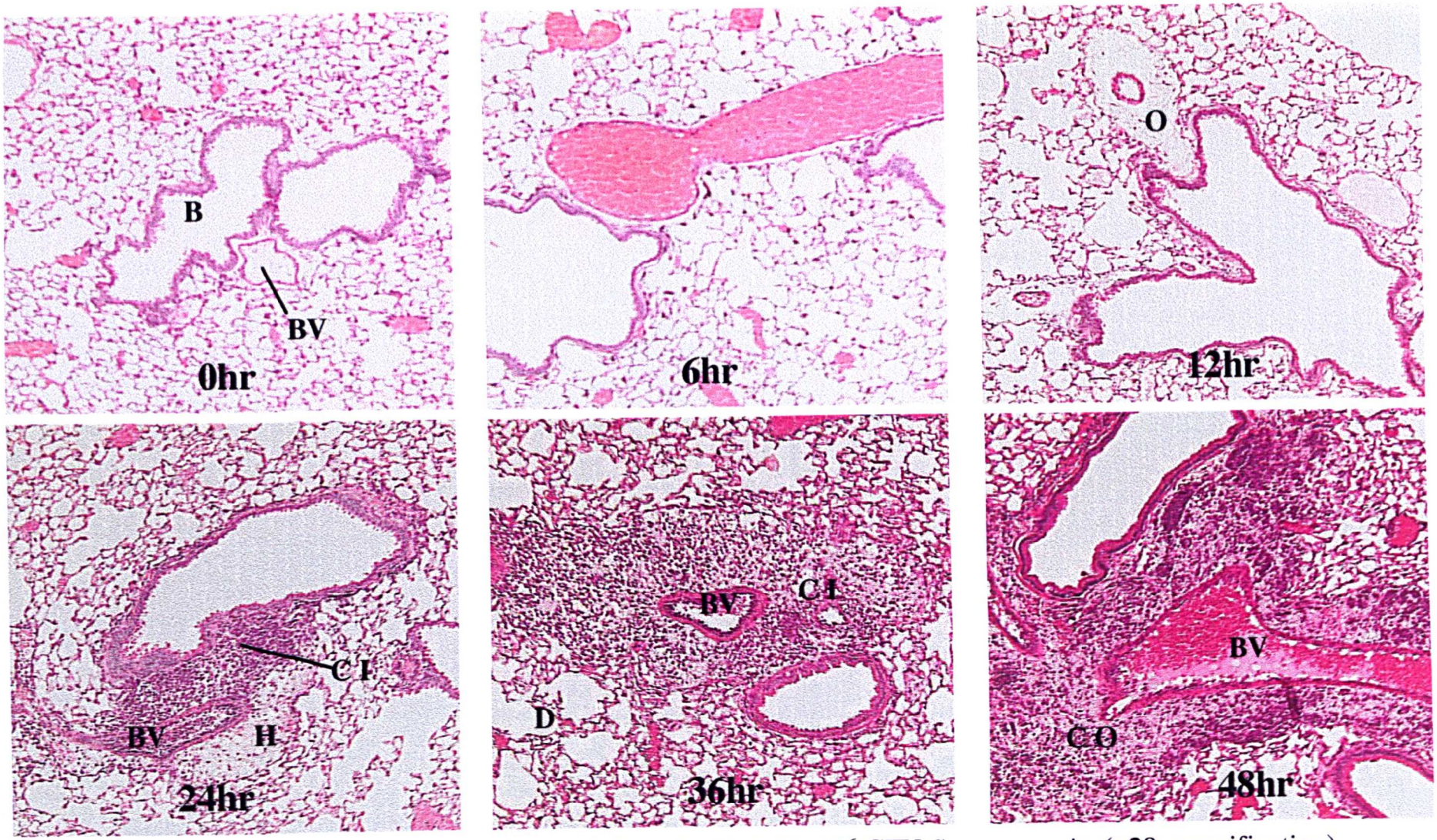

Figure 3.12 Histogical sections of mice infected with 1.01×10^6 CFU *S. pneumoniae*(x20 magnification).

Figure 3.12 Histological sections from the lungs of MF1 mice at various times following intranasal infection with 10^6 CFU *S. pneumoniae* (x200 magnification) stained with haematoxylin and eosin. At time 0h there is a tight interface between the bloodvessel (BV) and the two bronchioles (B). Six hours later there are no major changes to lung architecture but there are more neutrophils evident within bloodvessels. By 12h post challenge oedematous areas (O) are evident between bloodvessels and bronchioles with recruited inflammatory cells entering these areas by 24h post challenge (CI), along with erythrocytes (H). Cell influx continues through 36h with disruption also occurring to the surrounding tissues (D). By 48h post challenge the lungs contain many large areas that are consolidated with recruited cells (CO).

numbers of infiltrating cells. Microscopic examination of the nuclei of these cells identified them as mainly being neutrophils. The lesions grew in size through 36h until 48h post challenge when the areas were masses of inflammatory cells. Only low levels of haemorrhage (as determined by presence of erythrocytes in alveoli) could be detected at any time post challenge.

Utilisation of a scoring grid for histopathologic examination of lung tissue adapted from that used by Bergeron *et al* (Bergeron *et al.* 1998), (Section 2.3.13), permitted a numerical value to be associated with lung histopathology. Results of this analysis are presented in Table 3.2.

Histopathologic scores increased slightly immediately following infection and continued to increase until 12h post challenge when a significantly higher score was obtained than at time 0h ($P<0.01$). The level of inflammation then remained constant for 12h prior to increasing at 36h and 48h post challenge, also significantly higher than time 0h scores.

3.2.3.8 Mast cell detection following intranasal infection

Mast cells were differentiated within lung tissues by virtue of their characteristic granules. This stain is not intense and is not readily reproduced by photography, for this reason data is presented in Table 3.3.

Mast cells were detected in the lungs of untreated MF1 mice, of PBS sham infected mice and of MF1 mice immediately following intranasal infection with 10^6 CFU *S. pneumoniae.* Immediately following infection cells containing granules were present in high numbers and were mainly located around blood vessels. 6h into the experiment, the majority were associated with blood vessels but were also scattered around lung interstitium. By 12h into the experiment most cells had degranulated (or migrated out of the lungs) as fewer positive staining cells were evident, however this was not a statistically significant reduction (indeed the number of granule containing cells did not alter significantly from time 0h values throughout the experiment). At 24h post challenge they were again present in slightly higher numbers, with the number of granule containing cells remaining constant until the end of the experiment. Mast cells were never found within the foci of inflammation but were located in the immediate surrounding areas of lung.

	Time post challenge (h)					
	0	**6**	**12**	**24**	**36**	**48**
Mean score (arbitrary units)	0.00	1.83 +/- 0.70	3.75 +/- 1.03 *	3.50 +/- 0.56 *	5.67 +/- 0.62 *	5.00 +/- 1.03 *

Table 3.2 Histopathologic score for MF1 lung tissue following intranasal infection with 1.01 x 10^6 CFU *S. pneumoniae*. n=4-6 sections per time point, *, P<0.01 higher when compared to 0h scores.

	Time post challenge (h)				
	0	**6**	**12**	**24**	**36**
Mean No. cells/field of vision	63.2 +/- 10.78	79.80 +/- 3.12	31.20 +/- 7.83	75.60 +/- 13.17	78.60 +/- 5.88

Table 3.3 Mean number of mast cells with positive staining granules per field of vision (x40 magnification) in MF1 lung tissue following intranasal infection with 1.01 x 10^6 CFU *S. pneumoniae*. n=5 field of visions per time point.

3.2.3.9 Production of inflammatory mediators following intranasal infection

Recent investigations have highlighted the important balance that exists between pro- and anti-inflammatory markers during infection (Walley *et al.* 1996) & (Mohler *et al.* 1998). Contrary to absolute amounts of such mediators, their levels in relation to each other and the kinetics of their production are more important in determining outcome. In order to investigate whether this balance is altered during pneumococcal pneumonia, the biological mediator NO and the cytokines TNFα, IL-1β, IL-6 and IL-10 were selected for investigation. The former four are believed to act in a pro-inflammatory manner (Section 1.6.1.1) whilst IL-10 is a potent anti-inflammatory mediator (Section 1.6.1.2) (although IL-6 can also act in this manner in certain circumstances). Such characterisation of baseline levels was also required prior to modulation experiments (Section 4.2.2).
Total protein levels within lavage fluid of mice can be used as an indicator of the permeability of the alveolar/capillary barrier (Rubins *et al.* 1995). Disruption to this barrier during the course of infection, either by bacteria moving out of the lungs into the bloodstream or by recruitment of effector cells in the opposite direction, increases the level of total protein as serum proteins enter the airways.
Components of the pneumococcal cell wall have been shown to induce production of inflammatory cytokines both *in vitro* and *in vivo* (Section1.6.1.5). *In vivo* inflammation was therefore investigated following instillation of heat killed pneumococci in order to assess the ability of the cell wall of D39 to promote inflammation in this model of pneumococcal pneumonia. TNF activity and IL-10, NO and total protein levels were selected as markers of the pro-inflammatory/ anti-inflammatory balance.

3.2.3.9.1 Production of inflammatory mediators in the pulmonary air spaces following intranasal infection

TNF activity was undetectable within airways of mice immediately following infection (Figure 3.13 A). Levels became detectable during the mid and late stages of infection. TNF activity rapidly increased between 12h and 18h post challenge and equally rapidly decreased by the 24h time point (significantly reduced in comparison to 18h $P<0.01$). A second, smaller peak in activity

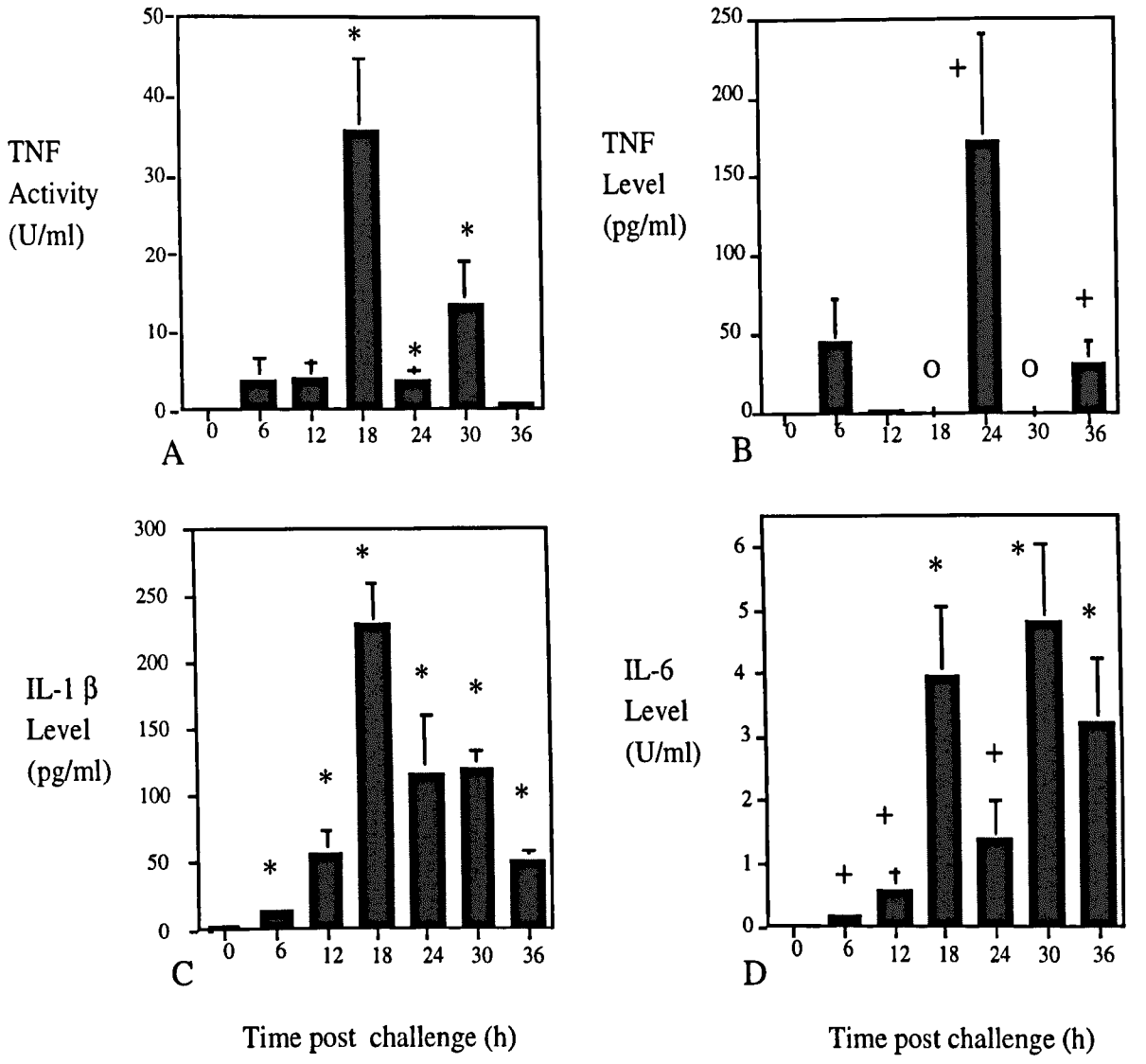

Figure 3.13 Production of pro-inflammatory mediators within the airways of MF1 mice following intranasal infection with 9.75 x 10^5 CFU *S. pneumoniae*. A= TNF activity n=11-21, B= TNFα protein levels n=5-7, C= IL-1β Levels n=5, D= IL-6 levels n=5, *, $P<0.01$ and +, $P<0.05$ higher when compared to time 0h values. O, no samples were tested for TNFα protein at 18h and 30h post challenge.

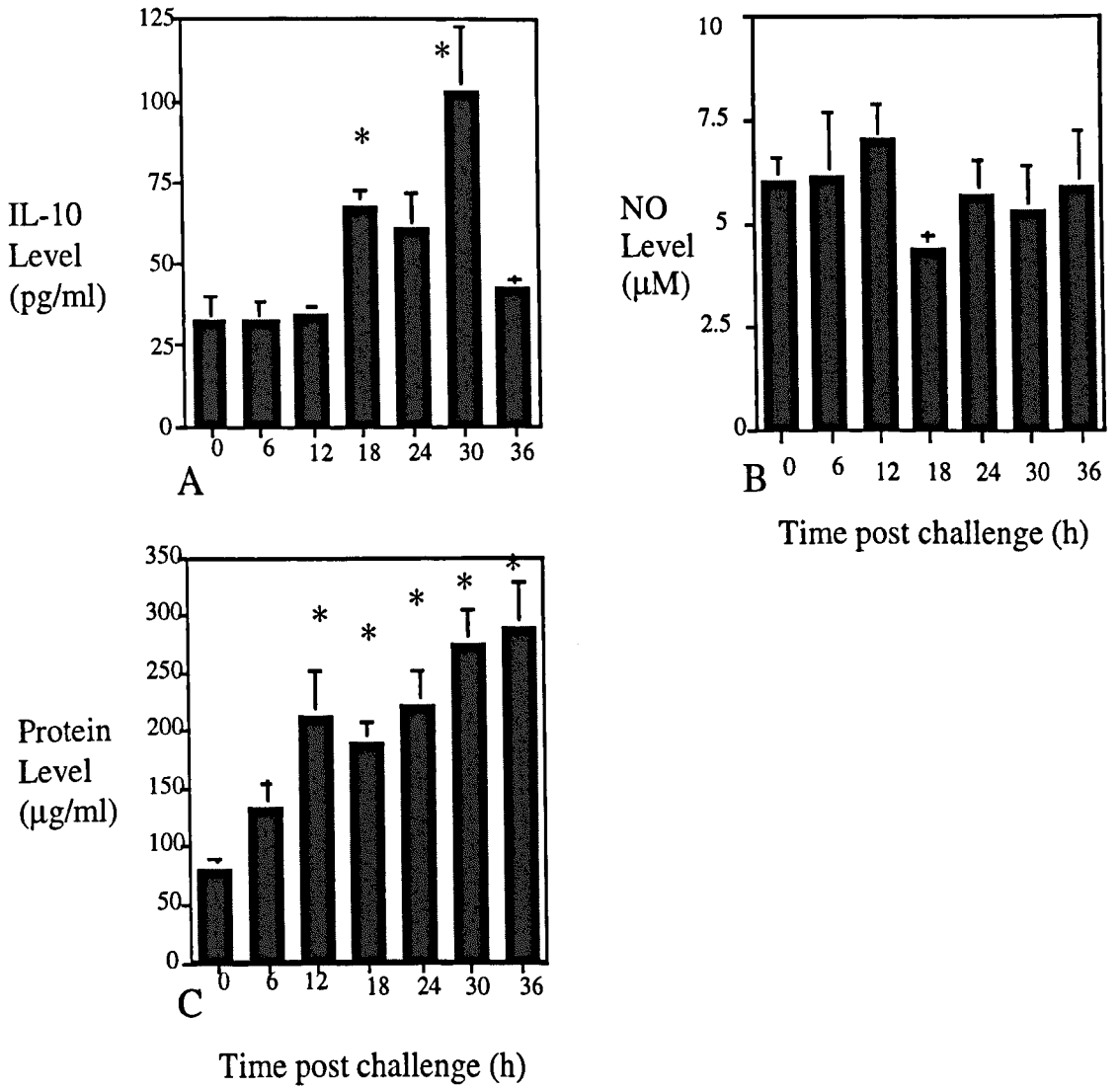

Figure 3.14 Production of inflammatory mediators within the airways of MF1 mice following intranasal infection with 9.75 x 10^5 CFU *S. pneumoniae*. A= IL-10 levels n=6-12, B= NO levels n=11-14, and C= Total protein content n=6-14, *, $P<0.01$ higher when compared to time 0h values.

occurred 30h post challenge, again transient in nature but a significant increase comparison to 24h results ($P<0.05$).

Similar kinetics were evident for TNFα protein levels although in this case 18h samples were not taken (Figure 3.13 B). Peak levels were thus recorded at 24h post challenge with production decreased at 36h.

IL-1β levels had already increased by 6h following infection, with this trend continuing until 18h post challenge (Figure 3.13 C). At this time maximal levels of this cytokine were detected. A gradual decline in IL-1β levels then occurred towards the end of the experiment although a significant decrease from this peak production was not evident until 36h post challenge ($P<0.05$ when 18h and 36h results are compared).

Detection of IL-6 increased from early during the infection with high levels detected during mid infection (Figure 3.13 D). Most IL-6 was detected during the final 18h of the experiment. As with TNF, IL-6 release may be occurring in two waves, perhaps explained by direct IL-6 induction by TNF although the decrease in IL-6 levels at 24h post challenge is not significant when compared to 18h results.

Interleukin 10 was present in appreciable levels within the airways of mice from the time of infection (Figure 3.14 A). Such levels remained constant until after the 12h time point. IL-10 production then increased slowly with peak amounts detected 30h into the experiment. High levels of IL-10 were not sustained as only 6h later they had returned to baseline.

Nitric oxide was investigated for its effects on epithelial and endothelial disruption in other models of disease (Section 1.5.3.5.2). As with the other pro-inflammatory mediators, low levels of NO were found within the airways of mice at the time of infection (Figure 3.14 B). These levels did not increase significantly from baseline values during the experiment.

Disruption to the alveolar/capillary barrier, as measured by protein leakage, occurred rapidly after instillation of the bacteria (Figure 3.14 C). By 6h post challenge protein levels in lavage fluids were already elevated and by a further 6h significantly higher levels of protein were detected than immediately following infection. These levels continued to increase almost unchecked until the end of the infection.

Heat killed pneumococci were incapable of causing the release of detectable TNF activity within the air spaces of mice (data not shown). A similar lack of effect was noted on NO levels.

Inoculation of heat killed bacteria did not significantly increase lavage fluid IL-10 levels during the experiment. These facts indicate that mild inflammation, undetected by the above assays may still have occurred. Further evidence is found with the increase in lavage fluid total protein levels that occurred at the end of the experiment ($P<0.01$).

Methods employed to initiate infections were not shown to initiate inflammation. The airways from PBS sham infected mice did not possess detectable TNF activity, nor did they contain significantly elevated NO, IL-10 levels or total protein (data not shown).

3.2.3.9.2 Production of inflammatory mediators in the lung tissues following intranasal infection

TNF activity was detected in low amounts during the first 12h of infection. This was followed by a rapid and significant increase at 18h post challenge of a similar magnitude to that seen in the lung airways (Figure 3.15 A). TNF activity levels remained significantly elevated for the remainder of the experiment.

Detection of TNFα protein was not possible within the lung tissues of infected mice until after 12h (Figure 3.15 B). Following this time (again a 18h time point was not carried out), there was a significant increase in its detection. Heightened TNFα levels were sustained until after the final time studied.

Significantly elevated IL-1β levels were detected within lung tissues rapidly after infection (Figure 3.15 C). By 18h post challenge they were at their highest detected level. After this time they decreasing slowly towards the end of the infection although they remained elevated in comparison to time 0h values.

IL-6 production did not increase significantly until after 12h into the experiment (Figure 3.15 D). Maximum levels were not detected until 30h post challenge with production not sustained but rather decreasing with time. The capacity of lung tissues to release this cytokine, as well as TNF and IL-1β, is significantly higher than that found within the pulmonary airways (Figures 3.15 & 3.15).

IL-10 levels within the lung tissues increase from after 6h into the experiment until after the 30h time point. Elevated levels of this cytokine within the tissues significant at the level of $P=0.028$ were found 30h post

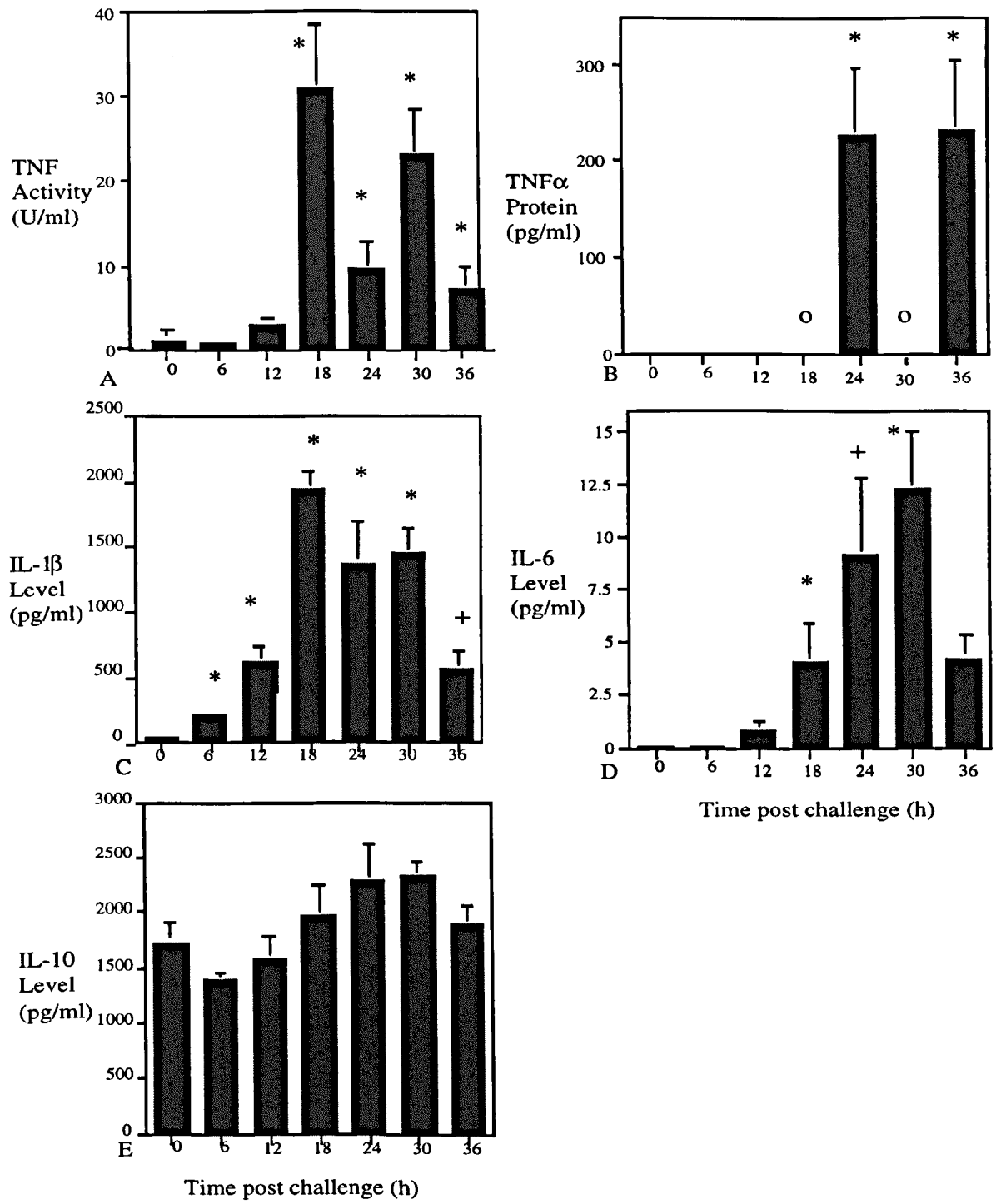

Figure 3.15 Production of inflammatory mediators within the lung tissues of MF1 mice following intranasal infection with 9.75 CFU x 10^5 *S. pneumoniae*. A= TNF activity n=5-17, B= TNFα protein n=5-7, C= IL-1β levels n=5, D= IL-6 level n=5, and E= IL-10 levels n=5-6.*, $P<0.01$, $^{+}$, $P<0.05$ higher when compared to time 0h values. O, no samples were tested for TNFα protein at 18h and 30h post challenge.

challenge. This elevation was of a far smaller magnitude than that seen in the airways although more IL-10 was found in association with the lung tissues than in the airways (Figure 3.15 E and Figure 3.14 A).

TNF activity remained undetectable within the lung tissues of all mice inoculated with heat killed pneumococci (data not shown). However, homogenate IL-10 levels increased significantly at 6h ($P<0.05$) and 24h post challenge ($P<0.01$) when compared to time 0h values.
Lung tissues from PBS sham infected animals did not contain detectable levels of TNF activity although lung tissue IL-10 levels were elevated at 6h and 12h post challenge ($P<0.05$) (data not shown).

Similar kinetics of TNF production were gathered using the TNF bioassay and the ELISA. However, the higher levels of variation evident with the ELISA resulted in all further TNF measurements in this thesis being carried out by bioassay

3.2.3.9.3 Systemic TNF activity following intranasal infection

TNF activity was undetectable within the circulation of mice until 24h post challenge (Figure 3.16). Following this time detection of TNF activity was highly variable within serum from infected mice. High levels of activity were found in serum from mice which were highly bacteraemic at 24h and 30h post challenge. Since the majority of mice did not possess systemic TNF activity a high level of variability was associated with the mean activity levels at 24h and 30h post challenge. This resulted in no statistical significance when these time points were compared to baseline.
This highly variable detection of TNF within serum from infected mice was found to be directly related to their level of bacteraemia. Comparison of all MF1 mice infected with *S. pneumoniae* and investigated for systemic TNF activity led to the discovery that only one mouse with a level of bacteraemia lower than 2.88×10^7 CFU/ml possessed such activity, whilst all 12 mice with higher bacteraemia had systemic TNF levels.
In table 3.4, all young MF1 infected in the experiments described in this chapter and sampled after 24h post challenge are separated into two groups on the basis of the magnitude of their bacteraemia. All mice in the high bacteraemia group (greater than 2.88×10^7 CFU/ml) possessed systemic TNF

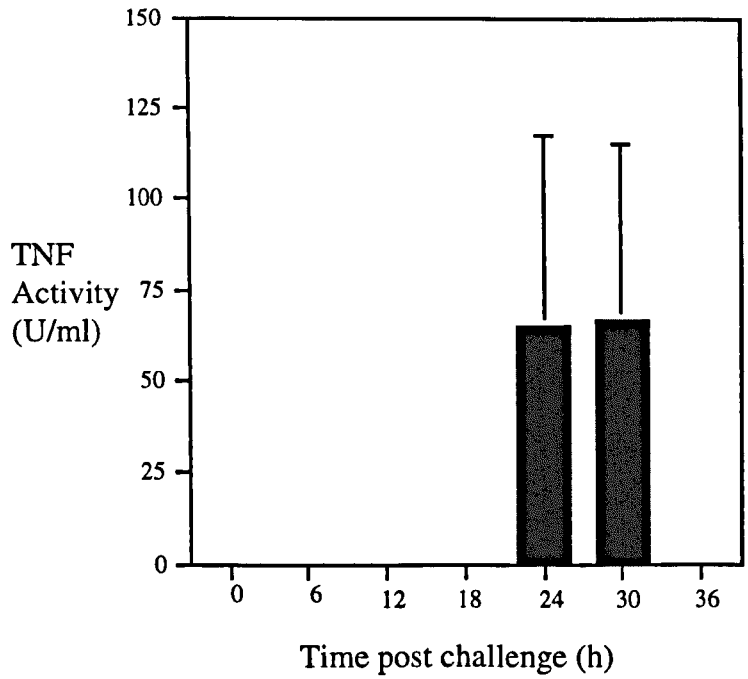

Figure 3.16 TNF activity within serum of MF1 mice following intranasal infection with 9.75 x 10^5 CFU *S. pneumoniae*. n=6-16.

Group description	Mean bacterial load (CFU/ml) +/- SEM	Mean TNF activity (U/ml) +/- SEM
Mice with level of bacteraemia higher than log 7.46 CFU/ml n=12	8.00 +/- 0.20	718.30 +/- 201.41 *
Mice with level of bacteraemia lower than log 7.46 CFU/ml n=75	3.75 +/- 0.22	2.97 +/- 2.97

Table 3.4 TNF activity levels in MF1 mice with levels of bacteraemia higher than or lower than log 7.46 CFU/ml following intranasal infection with 9.75 x 10^5 CFU *S. pneumoniae*. n=12-75, *, P<0.01 higher when compared to low bacteraemia group.

activity whilst only one mouse in the lower group did so. Furthermore, the mean level of TNF activity in the high bacteraemia group is significantly higher than that displayed in the low bacteraemia group ($P<0.0001$). This data indicates that there is a threshold number of pneumococci required in the bloodstream of an outbred population before high levels of systemic TNF activity are released and that this threshold is approximately 2.88×10^7 CFU/ml.

This fact also explains why no TNF was detected in mice prior to 24h post challenge and after 30h post challenge in Figure 3.13, as none of the mice in these groups had more than 2.88×10^7 CFU pneumococci/ml of blood.

TNF activity was not detected in serum from mice either inoculated with PBS or heat killed pneumococci (data not shown).

3.2.3.10 Immunohistochemistry following intranasal infection

Histological analysis was only capable of describing the kinetics and gross characteristics of pulmonary inflammation during pneumococcal pneumonia. Immunohistochemistry was thus employed in order to better define the roles played by certain cell populations.

Immunohistochemistry revealed that TNFα and IL-10 are expressed in low levels within the alveoli of untreated animals and mice immediately after infection, a finding previously reported by other investigators (Pendino *et al.* 1994) & (Bonfield *et al.* 1995). The location and nuclear morphology of the cells responsible implicates alveolar macrophages and bronchial epithelial cells.

Early during infection TNFα positive cells were detected both within the alveoli and bronchial epithelium with granular cells also frequently staining positively. Following inflammatory cell recruitment (24h-36h post challenge) most of the lung associated TNFα, as detected by immunohistochemistry, was located in these areas of cell influx (Figure 3.17 A). Both cell associated and secreted TNFα was detected.

IL-10 expressing cells were present in low numbers within alveoli and bronchial epithelium until 24h post challenge. Following this time, as with TNFα, IL-10 could mainly be detected within areas of inflammation and was present as both a cell bound and secreted form (3.17 B). However, during late infection IL-10 staining was more widespread and more intense than TNFα,

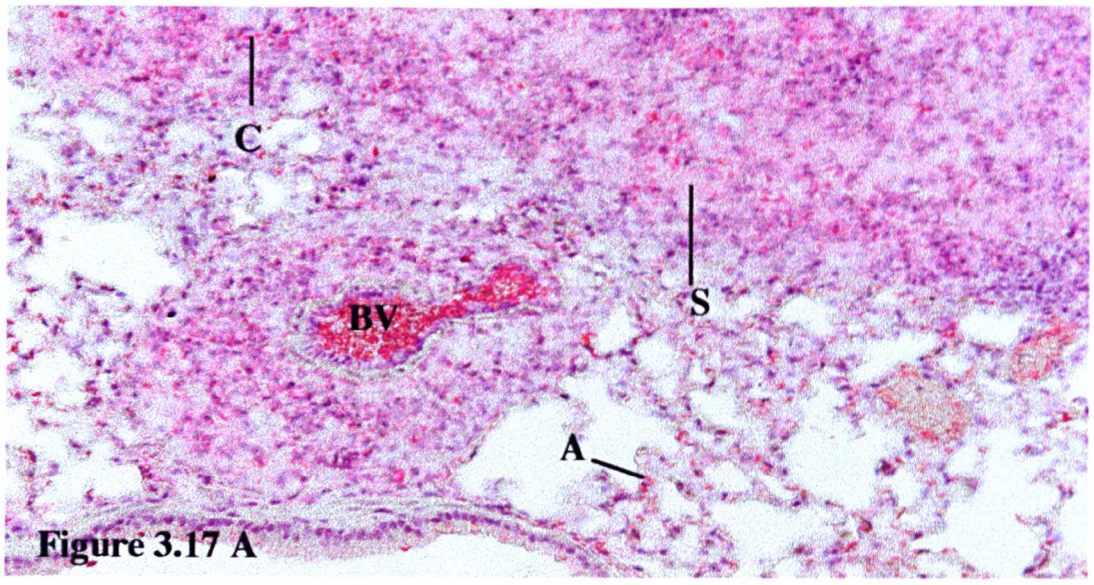

Figure 3.17 A

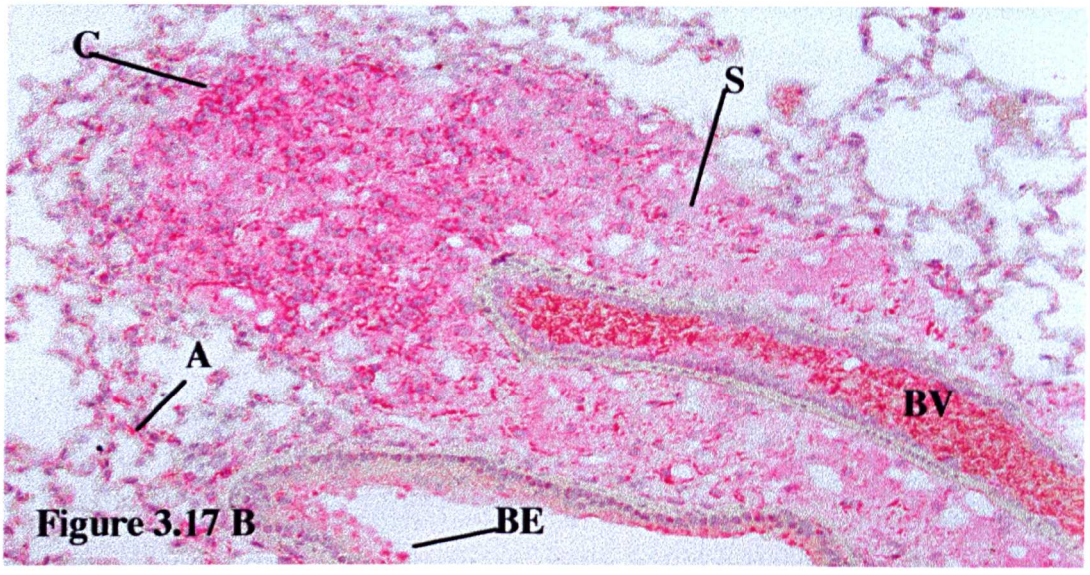

Figure 3.17 B

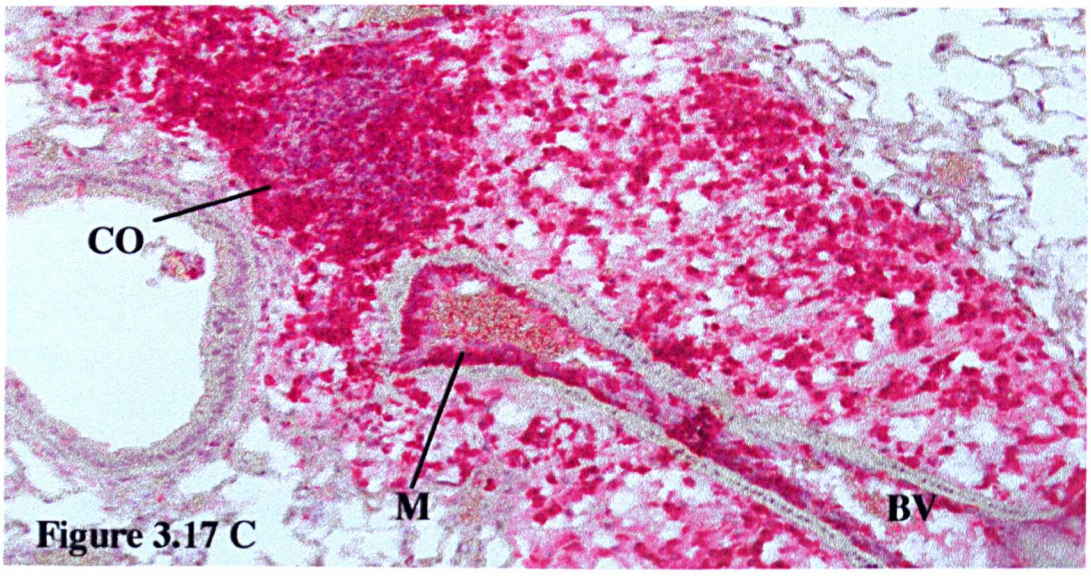

Figure 3.17 C

Figure 3.17 Immunohistochemistry on sections of lung from a mouse 36h post challenge with 1.01 x 10^6 CFU *S. pneumoniae* (x 200 magnification).

Figure 3.17A Immunohistochemical detection of TNFα. A consolidated area around a bloodvessel (BV) is shown. A signal for TNFα is detected both in the alveoli (A) and the foci of inflammation. In the areas of cell influx both cell associated (C) and secreted (S) forms of TNFα can be seen.

Figure 3.17B shows immunohistochemical detection of IL-10 in a serial section of the same lung. A stronger signal can be seen for IL-10 with more cells staining pink than are seen for TNFα in Figure 3.17A. Again both cell associated (C) and secreted (S) IL-10 can be detected. Alveolar cells are also expressing this anti-inflammatory cytokine, as are bronchial epithelial cells (BE).

Identification of the majority of recruited inflammatory cells has been carried out with immunohistochemical staining for a neutrophil marker in the same section (Figure 3.17 C). Most of the cells in the area of consolidation (CO) are staining positive for this marker. In addition to areas of consolidation, neutrophils are also evident marginating through the endothelium of the bloodvessel (M).

with bronchial epithelial and alveolar cells releasing the anti-inflammatory cytokine but not TNFα.
Both TNFα and IL-10 were found within the same areas of inflammatory foci during end infection. This fact may be explained if the initial cells to become stimulated had already expressed TNFα and were now synthesising IL-10 whilst newly recruited cells would still at the producing TNFα stage.
Light microscopy identified most of the recruited inflammatory cells as being neutrophils, an identification supported by immunohistochemical staining with a neutrophil marker (3.17 C).

3.2.4 Dose response

In each of the subsections associated with the effect of lowering the infectious dose, data for 10^6 CFU have been discussed elsewhere in this chapter and will not be reiterated.

3.2.4.1 Signs of infection & survival following intranasal infection with reduced doses of *S. pneumoniae*

Reducing the challenge dose from 10^6 CFU to 10^5 CFU was sufficient to significantly increase survival times ($P<0.01$) [Figure 3.18]. Mice inoculated with 10^5 CFU started to display clinical symptoms 24h into the experiment but did not begin to succumb of infection until 48h post challenge. No further mice succumbed to the infection after 121h into the experiment.
An infectious dose of 10^4 CFU increased survival times even more significantly ($P<0.01$ when compared to 10^6 CFU). Mice displaying signs of illness following infection with 10^4 CFU developed them with similar kinetics as with the other doses and all deaths occurred by 74h.
However, these statistics do not take into account the fact that many of the mice challenged with lower infectious doses survived the infection. In order to account for this fact it is necessary to separate the groups into those that resist the infection and those that succumb.
Table 3.5 shows that reducing the infectious dose from 10^6 CFU to 10^5 CFU significantly increases the survival times of MF1 mice that do succumb to the infection. The variability associated with resistance/susceptibility with this dose prevents its use as a less acute model of infection.

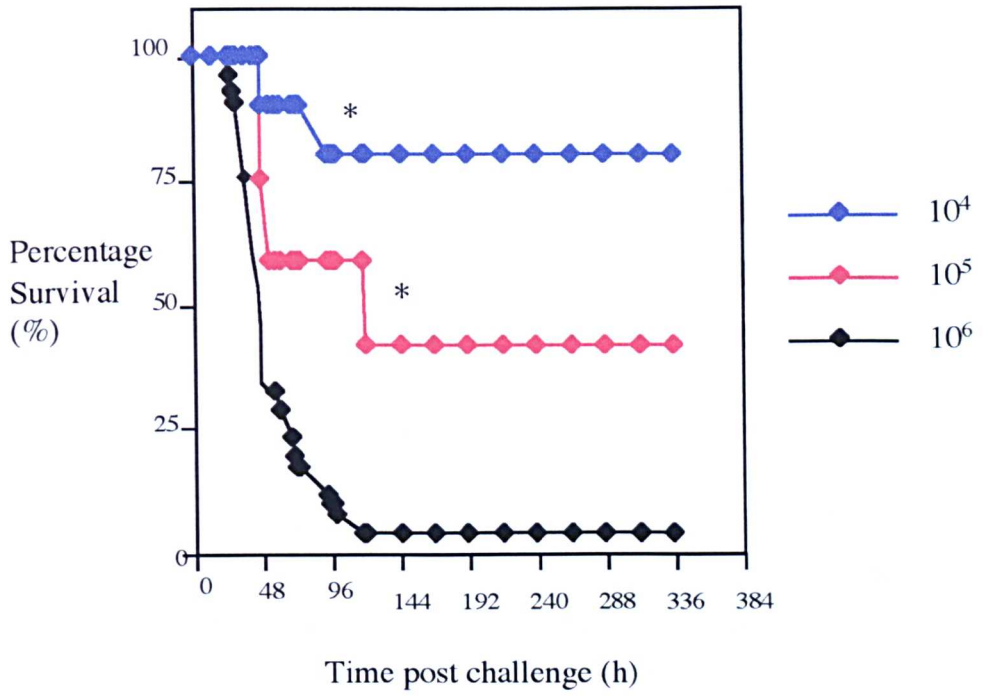

Figure 3.18 Survival of MF1 mice following inoculation of 9.76 x 10^5 CFU, 9.95 x 10^4 CFU, or 1.02 x 10^4 CFU *S. pneumoniae*. n= 10-53, *, P<0.01 longer survival times when compared to 10^6 mice.

Infectious Dose	No. of animals that succumb	Mean survival time (h)
10^6	51/53	53.38
10^5	7/12	70.57 *
10^4	2/10	60.00

Table 3.5 Survival times of MF1 mice following inoculation of 9.76 x 10^5 CFU, 9.95 x 10^4 CFU, or 1.02 x 10^4 CFU *S. pneumoniae* that succumb to pneumococcal pneumonia. *, P<0.05 longer when compared to 10^6 CFU group.

Mice inoculated with 10^4 CFU also had longer mean survival times than those infected with 10^6 CFU. However, the small group size prevents statistical significance.

Analysis of the following data from the dose response experiments revealed a marked intra-group variation in outcome following intranasal challenge with either 10^5 or 10^4 CFU *S. pneumoniae*. As the infectious dose was reduced the proportion of mice that did not succumb to the infection but rather were resistant, increased. This led to the data being analysed by additional procedures. The mean data are presented (as above for 10^6 CFU dose) in order to allow the overall kinetics of inflammation to be compared between infectious doses. The bacteraemia results were then separated into 2 groups on the basis of whether the mice appeared to resist the infection and not develop bacteraemia, or succumbed. Production of inflammatory mediators was also investigated in a similar manner with the infectious dose groups separated on the basis of an elevated response in comparison to baseline data. All mice that did respond in this manner from 6h post inoculation of reduced doses were put in one group whilst those that did not respond were placed in another group. This permitted analysis of whether mice challenged with reduced doses of pneumococci displaying a host response did so to a similar extent as mice infected with 10^6 CFU *S. pneumoniae*. This method of analysis could not be performed elsewhere in this thesis due to small group sizes in either the responding (for example BALB/c bacteraemia, Section 5.2.1.5) or non-responding (MF1/129 non-bacteraemic animals Section 4.2.4.2.3) groups.

3.2.4.2 Bacteriology following intranasal infection with reduced doses of *S. pneumoniae*

Lung tissues samples were utilised for the analysis of production of inflammatory mediators. Bacteriological profiles could therefore only be investigated in lavage fluid and blood samples from mice infected with reduced doses of *S. pneumoniae*.

3.2.4.2.1 Bacterial loads within pulmonary air spaces following intranasal infection with reduced doses of *S. pneumoniae*

The number of viable bacteria found associated with the lung airways on challenge with 10^5 CFU was around ten fold lower than that recovered from lungs of mice challenged with 10^6 CFU (Figure 3.19). This in turn was higher than that discovered in airways of mice given 10^4 CFU.

Bacterial loads within the airways of mice inoculated with 10^5 CFU contained similar numbers of viable pneumococci at all times studied. All of the 57 mice in the 10^5 CFU group were found to have viable pneumococci within their airways during the experiment. A slight increase in bacterial load occurred during late infection and at 36h there were similar numbers of bacteria within the airways of mice given either 10^6 CFU or 10^5 CFU *S. pneumoniae* (prior to this point there were significantly lower bacterial loads within lungs of mice infected with 10^5 CFU than with the higher dose [$P<0.01$]).

Bacterial viability within airways of mice challenged with 10^4 CFU D39 dropped immediately on instillation. This trend continued towards the later time points with fewer than 10^4 CFU/ml found in all mice but one throughout the experiment. However, very few (7/54) mice inoculated with 10^4 CFU *S. pneumoniae* had undetectable bacteria within their lungs during the experiment. At all times bacterial loads within airways of mice infected with 10^4 CFU pneumococci were significantly lower than those in airways of mice infected with 10^6 CFU ($P<0.01$) and only at 24h were the levels in the 10^4 CFU group not significantly lower than in the 10^5 CFU animals, at all other times a P value of $P<0.01$ was found.

3.2.4.2.2 Systemic bacterial loads following intranasal infection with reduced doses of *S. pneumoniae*

Grouping all mice inoculated with the various infectious doses permitted development of bacteraemia in these groups to be used as a marker of disease progression (Table 3.6).

Viable pneumococci were not detected within the bloodstream of any mice until 12h post challenge. At this time similar numbers and levels of positive cultures were found in each of the groups (Table 3.6).

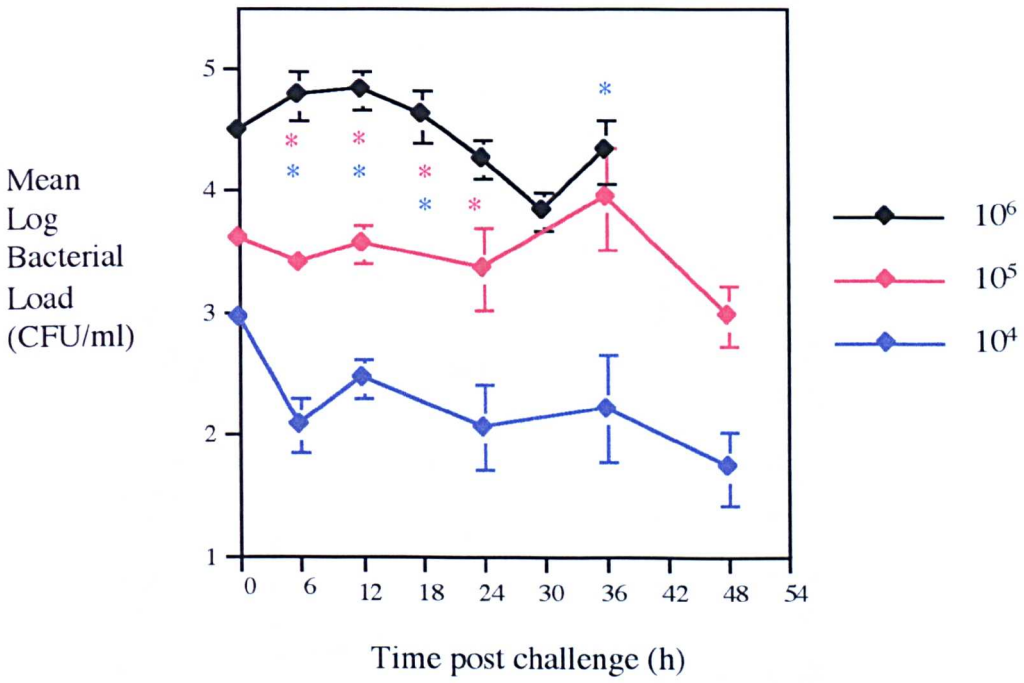

Figure 3.19 Bacterial loads within the airways of MF1 mice following inoculation of either 9.75 x 10^5, 9.56 x 10^4 or 9.55 x 10^3 CFU *S. pneumoniae*. n=8-24, red asterisk denotes P<0.01 lower bacterial load in 10^5 CFU group and blue asterisk denotes P<0.01 lower bacterial load in the 10^4 CFU group, when compared to the 10^6 CFU group.

Infectious Dose	Time post challenge (h)							
	0	6	12	18	24	30	36	48
10^4 CFU	ND	ND	2.03 +/- 0.12	-------	2.30 +/- 0.24 *	-------	2.56 +/- 0.64 *	2.21 +/- 0.25 +
10^5 CFU	ND	ND	2.10 +/- 0.13	-------	2.88 +/- 0.37 †	-------	5.67 +/- 0.93	5.40 +/- 0.75
10^6 CFU	ND	ND	2.13 +/- 0.10	4.19 +/- 0.39	5.22 +/- 0.34	5.80 +/- 0.35	7.12 +/- 0.46	-------

Table 3.6 Mean log bacterial loads within the bloodstreams of all MF1 mice following intranasal infection with either 9.75×10^5, 9.56×10^4 or 9.55×10^3 CFU *S. pneumoniae*. n=8-24, * $P<0.01$ lower for 10^4 CFU group compared to 10^6 CFU group. +, $P<0.01$ lower for 10^4 CFU group compared to 10^5 CFU group. †, $P<0.01$ lower for 10^5 CFU group compared to 10^6 CFU group. ND= below detection limit of viable count assay (< Log 1.92 CFU/ml).

The mean data from the 10^5 group revealed that bacteria were able to continue to increase in number with significantly more colony forming units detected 36h and 48h post challenge when compared to time 0h ($P<0.01$). At 24h post challenge the level in the 10^5 CFU was significantly lower than that in the 10^6 CFU group ($P<0.01$) but by 36h post challenge both groups of mice had a reached levels that were not significantly different.
The rate of increase in pneumococcal numbers within the circulation of mice inoculated with 10^5 CFU was slightly slower than that in the group challenged with 10^6 CFU (mean doubling time of 2.2h versus 1.4h for 10^6 CFU).
In mice inoculated with 10^4 CFU, mean levels of bacteraemia did not increase above the level of approximately 10^2 CFU/ml with no significant alteration from the time 0h levels. Bacteria in the bloodstreams of mice in this group had a slow doubling time of 12.6h.
From 24h post challenge onwards, mice inoculated with 10^4 CFU had significantly fewer viable pneumococci within their bloodstreams than mice infected with 10^6 CFU ($P<0.01$). Only at 36h and 48h post challenge were the levels of bacteraemia in the 10^4 CFU group reduced in comparison to the 10^5 CFU group ($P<0.05$ at 36h and $P<0.01$ at 48h).

As with the survival data, these overall data do not account for many of the mice remaining free of bacteraemia during the experiment. In order to investigate whether mice that succumbed to the disease after infection with reduced doses did so to comparable levels as the mice infected with 10^6 CFU D39 it is necessary to divide the data. In Table 3.7 mice that did not develop bacteraemia are removed, leaving only those that did. All three infectious doses resulted in similar levels of bacteraemia at 12h into the experiment although the highest number of mice with positive blood cultures were infected with 10^6 CFU.
Infection with 10^4 CFU did not result in high levels of bacteraemia in most mice, only one animal in this group had a very high level (7.70, occurring 36h post challenge). Statistically fewer pneumococci were present in blood from these than in mice infected with 10^6 CFU at 24h ($P<0.01$) and 36h post challenge ($P<0.05$). The mean doubling time for D39 within the circulation of this batch of mice was 4h with no significant alteration in viable counts detected on comparison to time 0h.

Infectious Dose	Time post challenge (h)					
	12	18	24	30	36	48
10^4 CFU	3.03 n=1/9	-------	3.09 +/- 0.48 n=3/9 *	-------	3.85 +/- 1.93 n=3/9 ▲	3.10 +/- 0.80 n=2/8 †
10^5 CFU	2.72 +/- 0.32 n=2/9	-------	3.85+/- 0.38 n=5/10 +	-------	6.09 +/- 0.93 n=9/10 +	6.39 +/- 0.46 n=7/9
10^6 CFU	3.10 +/- 0.46 n=7/17	4.36+/- 0.37 n=13/14	5.48 +/- 0.30 n=23/24	5.80 +/- 0.35 n=17/17	7.12 +/- 0.46 n=16/16	-------

Table 3.7 Mean log bacterial loads +/- SEM within the bloodstreams of MF1 mice developing detectable bacteraemia following intranasal infection with either 9.75 x 10^5, 9.56 x 10^4 or 9.55 x 10^3 CFU *S. pneumoniae*. n= number bacteraemic/number tested. *, P<0.01 lower for 10^4 CFU group when compared to 10^6 CFU group. +, P<0.05 lower for 10^5 CFU group when compared to 10^6 CFU group. ▲, P<0.05 lower for 10^4 CFU group when compared to 10^6 CFU group. †, P<0.05 lower for 10^4 CFU group when compared to 10^5 CFU group.

Reducing the infectious dose to 10^5 CFU reduced the level of bacteraemia in comparison to the 10^6 CFU group at 24h post challenge and 36h post challenge ($P<0.05$). Furthermore, the rate of development of bacteraemia was slower with this reduced dose as it took until 36h for the magnitude of bacteraemia to reach that displayed by the 10^6 CFU group at 30h post challenge. Significantly higher bacterial loads were found in bacteraemic mice infected with 10^6 CFU by 18h post challenge ($P<0.05$) and 24h post challenge ($P<0.01$) whilst to took until 36h for the level to change significantly following inoculation with 10^5 CFU ($P<0.01$ at 36h in the 10^5 CFU group).The mean doubling time for pneumococci in the bloodstream of mice inoculated with 10^5 CFU was 1.43h.
Thus spread of pneumococci into the circulation is reduced when a low dose of 10^4 CFU is administered. However, lowering the infectious dose to 10^5 CFU does not reduce the level of bacteraemia associated with pneumococcal pneumonia but does marginally slow down its development (this may be due to a slightly lower number of D39 gaining access to bloodstream at 12h in this group).

3.2.4.3 Histology following intranasal infection with a reduced dose of *S. pneumoniae*

Histological examination of lung tissues compared mice inoculated with 10^5 CFU *S. pneumoniae* to those of mice infected with 10^6 CFU. Lung histology was not determined following infection with 10^4 CFU due to the highly variable nature of infection following this dose. A similar pattern of event occurred within the lungs of mice inoculated with 10^5 CFU as with 10^6 CFU, areas of oedema between blood vessels and airways were the earliest sign, followed by slight haemorrhage and cell influx. More significant cell influx then occurred until large areas of the lungs were consolidated with inflammatory cells (data not shown).

The same histopathology scoring system as in Section 3.2.3.4 was utilised in order to give this inflammation an arbitrary score in order to permit comparisons between the two doses (Table 3.8).
Histopathology was slower to develop within lungs of mice inoculated with 10^5 CFU *S. pneumoniae* despite two sections displaying mild inflammation immediately following infection. In addition, lower inflammatory scores

Infectious dose	Time post challenge (h)					
	0	6	12	24	36	48
10^6 CFU	0.000	1.83 +/-0.70	3.75 +/- 1.03	3.50 +/- 0.56	5.67 +/- 0.62	5.00 +/- 1.03
10^5 CFU	0.67 +/- 0.42	0.50 +/-0.34	1.00 +/- 0.45	1.50 +/- 0.72	2.83+/- 0.79 +	3.83 +/- 0.83

Table 3.8 Histopathologic score for MF1 lung tissue following intranasal infection with either 1.01 x 10^6 or 9.80 x 10^4 CFU *S. pneumoniae*. n=4-6 sections per time point, +, $P<0.05$ lower for 10^5 CFU scores when compared to 10^6 CFU scores.

were detected within lung tissues of mice inoculated with 10^5 CFU when compared to those infected with 10^6 CFU pneumococci. Inflammatory scores for the 10^6 CFU dose were significantly increased by 12h post challenge, but a similar effect was not evident in the 10^5 CFU group until 48h post challenge.

As with the bacteriology data in Section 3.2.4.2, in order to better detect any histopathology in mice inoculated with 10^5 CFU it is necessary to separate the animals into those that did display inflammation and those that did not, Table 3.9.

Inflammatory scores from lungs of mice that did respond with lung pathology following infection with 10^5 CFU were lower than those that responded following infection with 10^6 CFU. Furthermore, infection with the reduced dose required a longer period of time before marked inflammation became evident. These results indicate that although a similar pattern of lung pathology occurs following infection with 10^5 CFU as with 10^6 CFU these events require longer in the reduced dose group.

3.2.4.4 Production of inflammatory mediators within pulmonary air spaces following intranasal infection with a reduced dose of *S. pneumoniae*

Only one lavage fluid sample from the 54 tested in the 10^5 CFU challenged group showed measurable TNF activity (Figure 3.20 A). This occurred at 36h post challenge with 2.89 U/ml detected. This mouse had the highest level of viable pneumococci within its airways with log 5.60 CFU/ml.

Mice challenged intranasally with 10^4 CFU *S. pneumoniae* did not produce detectable TNF within their airways during this experiment. This is in agreement with none of these mice having high numbers of viable bacteria in their airways.

Production of IL-6 within the lavage fluids of mice inoculated with 10^5 CFU was delayed in comparison to that in the 10^6 CFU group (Figure 3.20 B) with significantly lower levels at 12h post challenge ($P<0.05$). Elevated IL-6 levels in the former group did not occur until 24h post challenge and even then levels were low and only present in (2/8) mice, significance was not obtained until 36h post challenge ($P<0.01$) but this was maintained until the end of the experiment.

Reducing the dose to 10^4 CFU greatly reduced the amount of IL-6 produced during the infection with no significant production discovered. Very few

Infectious Dose	Time post challenge (h)				
	6	12	24	36	48
10^6 CFU	2.75 +/- 0.63 n=4/6	3.75 +/- 1.03 n=4/4	3.50 +/- 0.56 n=6/6	5.67 +/- 0.62 n=6/6	5.00 +/- 1.03 n=6/6
10^5 CFU	1.50 +/- 0.50 n=2/6	1.50 +/- 0.50 n=4/6	3.00 +/- 0.58 n=3/6	2.83 +/- 0.79 n=6/6 *	3.83 +/- 0.83 n=6/6

Table 3.9 Histopathologic score for MF1 lung tissue in mice displaying lung pathology following intranasal infection with either 1.01 x 10^6 or 9.80 x 10^4 CFU *S. pneumoniae*. *, P<0.01 lower for 10^5 CFU scores when compared to 10^6 CFU scores.

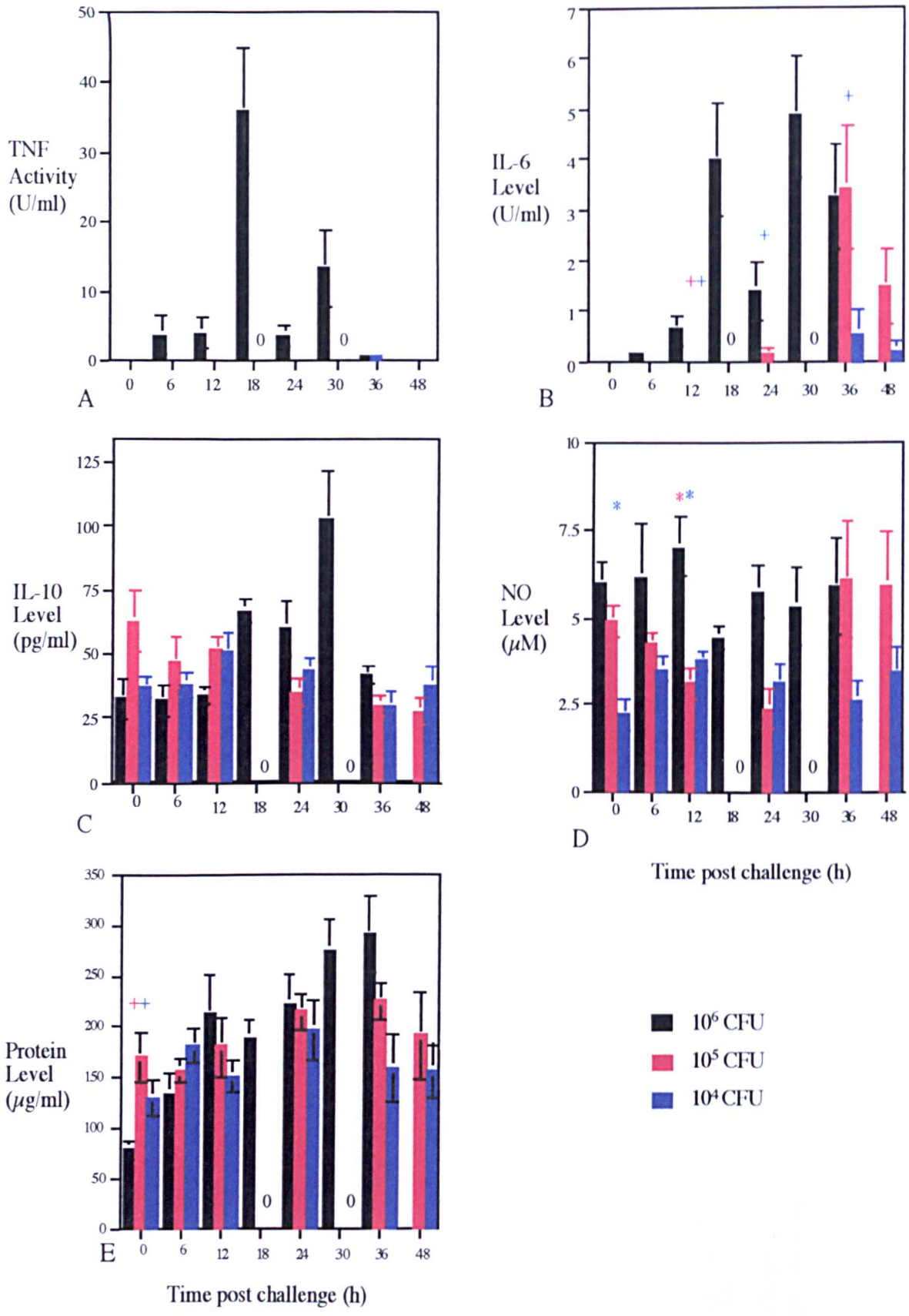
TNF Activity (U/ml)
A
IL-6 Level (U/ml)
B
IL-10 Level (pg/ml)
C
NO Level (μM)
D
Protein Level (μg/ml)
E
Time post challenge (h)
Time post challenge (h)
10⁶ CFU
10⁵ CFU
10⁴ CFU

Figure 3.20 Levels of inflammatory mediators within the airways of MF1 mice following inoculation of 9.75 x 10^5 CFU, 9.58 x 10^4 CFU, or 9.55 x 10^3 CFU *S. pneumoniae*. A= TNF activity, n=9-21, B= IL-6 levels, n=5-11, C= IL-10 levels, n=6-12, D= NO levels, n=8-14, and E= total protein level, n=8-23. Red asterisk denotes $P<0.01$ different in 10^5 CFU group and blue asterisk denotes $P<0.01$ different in the 10^4 CFU group when compared to the 10^6 CFU group. Red cross indicates $P<0.05$ different in 10^5 CFU group and blue cross represents $P<0.05$ different in 10^4 CFU group when compared to 10^6 CFU group. 0, no samples were tested from reduced doses at 18h and 30h post challenge.

(2/56) mice in the lowest dose group had detectable levels of IL-6 during the infection and only one of these had high levels (4.56 U/ml). From 12h onwards the 10^4 CFU group had significantly lower levels of IL-6 associated with their airways than did the 10^6 CFU group ($P<0.05$).

Lavage fluid from mice inoculated with 10^4 CFU contained similar levels of IL-10 at the time of infection as did the 10^6 CFU control group (3.20 C). Detection of this anti-inflammatory cytokine did not alter significantly during the experiment in this group of mice.

The highest amounts of IL-10 associated with the group of mice inoculated with 10^5 CFU D39 were found at the time of infection although these were not significantly higher than levels found in the other two groups. From that time onwards the IL-10 levels decreased, although not significantly so, until the end of the experiment.

Similar levels of NO were found associated with lavage fluid from mice inoculated with 10^5 CFU as with 10^6 CFU at the time of infection (Figure 3.20 D). A significant decrease in detectable NO in the 10^5 CFU group occurred after 12h into the experiment when compared to time 0h results ($P<0.05$) and this was sustained until 24h. At these same time levels in the 10^5 CFU group were significantly reduced in comparison to those in the 10^6 CFU group ($P<0.01$). By the penultimate time studied NO levels in the airways of mice inoculated with 10^5 CFU had returned to those originally detected.

Infection with 10^4 CFU resulted in lower levels of NO being detected throughout the experiment when compared to the other doses. At the time of infection these levels were significantly lower than levels found in the other groups ($P<0.01$). A significant, but transient, elevation did occur in the amount of NO that was recovered during mid infection ($P<0.05$) but even following this increase, levels were lower than those seen at the time of infection with the other doses.

Lavage fluids recovered from mice inoculated with 10^5 or 10^4 CFU *S. pneumoniae* contained significantly higher amounts of protein at the time of infection than did the 10^6 CFU group [$P<0.05$] (Figure 3.20 E). Levels in the 10^5 CFU group remained constant during the initial 6h of the infection after which they increased slightly, but not significantly, towards the end of the experiment.

Protein levels in mice inoculated with 10^4 CFU also increased slightly during mid infection. This increase occurred to a reduced extent than in the higher doses and had disappeared by the end of the experiment.

3.2.4.5 Production of inflammatory mediators within lung tissues following intranasal infection with a reduced dose of *S. pneumoniae*

Elevated TNF activity levels were detected in the lung tissues of mice inoculated with 10^5 CFU *S. pneumoniae* but not until 24h post challenge (Figure 3.21 A) resulting in significantly lower levels at 12h post challenge compared to 10^6 CFU group ($P<0.05$). At this time similar levels of TNF activity were found as detected in the 10^6 CFU group at 12h post challenge. By 36h post challenge TNF activity in the group of animals inoculated with 10^5 CFU was significantly elevated in comparison to time 0h values ($P<0.05$) but by 48h post challenge significance was no longer obtained.

No TNF activity was found in lung tissues of mice inoculated with 10^4 CFU D39 until mid/late infection when very low levels of activity were detected. Most mice (49/53) inoculated with this dose of *S. pneumoniae* did not respond by inducing detectable TNF activity. Thus significantly reduced TNF activity was detected in this group than in the 10^6 CFU group ($P<0.05$ at 12h and $P<0.01$ at 24h).

IL-6 production was induced less rapidly and to a lower extent within the lung tissues of mice inoculated with 10^5 CFU than those infected with 10^6 CFU (Figure 3.21 B). No mice responded with elevated IL-6 levels until 24h post challenge when 6/9 mice did so to significantly elevate IL-6 levels above baseline ($P<0.05$). Similarly significant levels continued to be detected throughout the remainder of the experiment.

Inoculation of 10^4 CFU did not result in significant IL-6 induction with only (3/54) mice inoculated with this dose of *S. pneumoniae* had elevated IL-6 levels in their lung tissues giving a P value of <0.05 when results from the 10^4 CFU group were compared to the 10^6 CFU group at 24h post challenge.

Similar levels of IL-10 were detected within lung tissues from all mice immediately following infection with *S. pneumoniae* (Figure 3.21 C). Production of IL-10 was only increased slightly (but not significantly) following infection with 10^5 CFU and this occurred at 36h post challenge.

Elevated IL-10 levels were not found within the lung tissues of mice inoculated with 10^4 CFU *S. pneumoniae*. Thus these data suggest that the only infectious dose capable of altering IL-10 levels within lung tissues is 10^6 CFU D39.

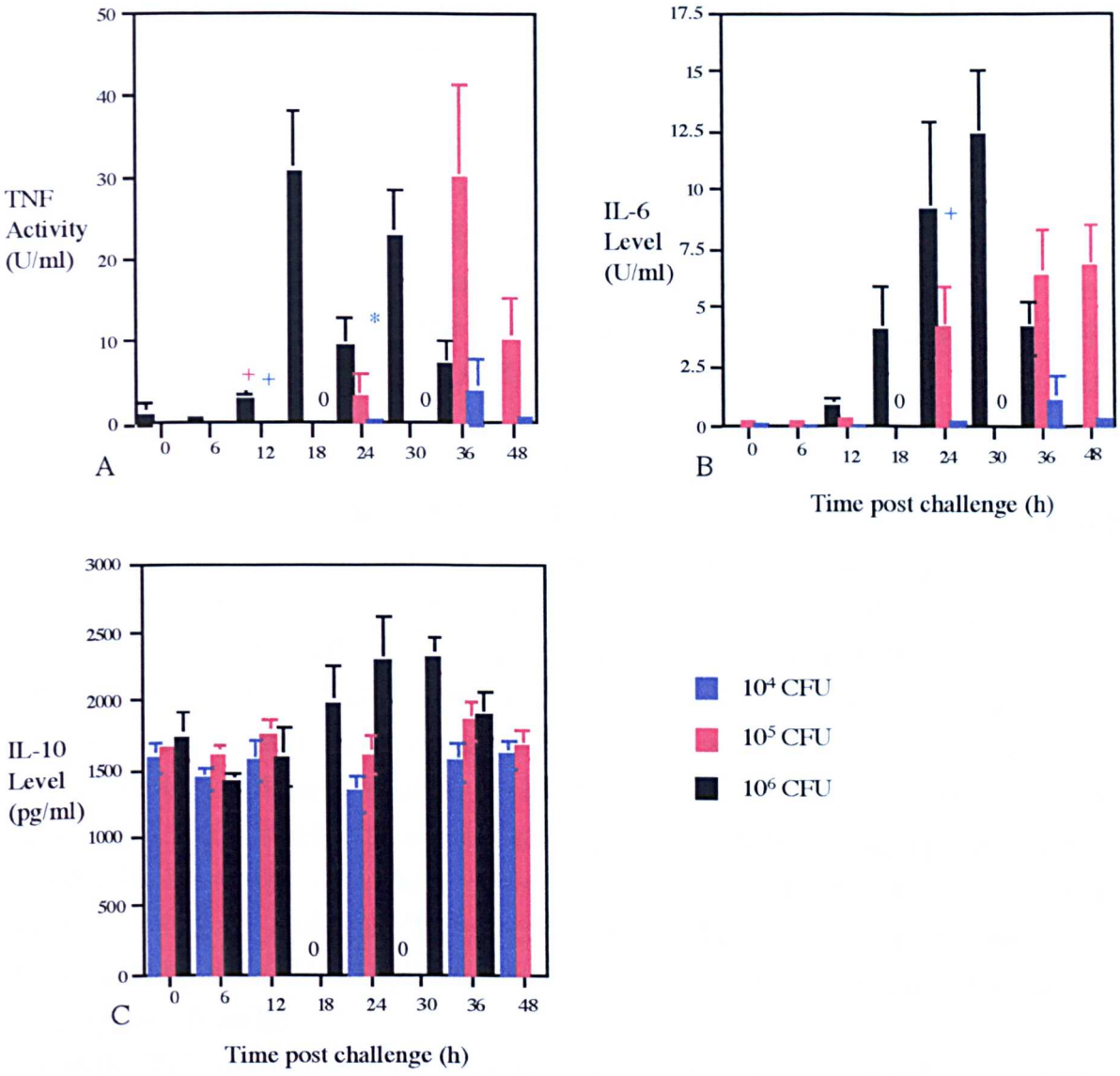

Figure 3.21 Levels of inflammatory mediators within the lung tissue of MF1 mice following inoculation of 9.75 x 10^5 CFU, 9.58 x 10^4 CFU, or 9.55 x 10^3 CFU *S. pneumoniae*. A= TNF activity levels, n=5-9, B= IL-6 levels, n=5-9, C= IL-10 levels,5-10. Blue asterisk denotes P<0.01 different in the 10^4 CFU group when compared to the 10^6 CFU group. Red cross indicates P<0.05 different in 10^5 CFU group and blue cross represents P<0.05 different in 10^4 CFU group when compared to 10^6 CFU group. 0, no samples were tested from reduced doses at 18h and 30h post challenge.

3.2.4.6 Production of inflammatory mediators in mice that display a host response following inoculation of reduced doses of *S. pneumoniae*

In order to determine whether mice inoculated with reduced doses *of S. pneumoniae* were capable of releasing similar levels of inflammatory mediators as the 10^6 CFU group, mice were separated into responding and non-responding phenotypes as described in Section 3.2.4.1.

Reducing the challenge dose significantly reduced the number of mice that responded to infection by increasing TNF activity (Table 3.10). A lower level of TNF was also found in the single mouse that released detectable TNF following infection with 10^5 CFU *S.pneumoniae*. The low group size prevents statistical analysis.

Although the number of mice responding to infection by releasing higher levels of TNF activity in lung airways was reduced in a dose dependent manner, the levels expressed by mice responding to the inoculated bacteria were not significantly different between the 10^4 and 10^6 groups. The possibility that similarly high bacterial loads were present in the lung tissues of mice that increased TNF activity cannot be excluded. Numbers of viable pneumococci within lung homogenates could not be investigated in these experiments. Indeed, elevated bacteraemia levels in many of the 10^5 CFU inoculated mice at later time points suggests that lung tissue bacterial loads would be increased (due to correlation of systemic/lung tissues bacterial loads mentioned earlier). This fact would explain why lung tissues from mice in this group had significantly higher TNF activity levels than the 10^6 CFU group ($P<0.05$).

As with TNF, the number of mice responding to pneumococcal pneumonia by increasing their IL-6 levels within lung airways and tissues increased in a dose related manner (Table 3.11). In this case however mice inoculated with reduced doses of *S. pneumoniae* responding with elevated airway IL-6 levels did so to a similar extent as the 10^6 CFU group. Within the lung tissues, however, only mice inoculated with the two higher doses were capable of releasing high levels of this pro-inflammatory cytokine. Levels of IL-6 released by the 10^4 CFU group were significantly lower than those in the other two groups ($P<0.01$).

A 100 fold reduction in infectious dose significantly reduced the level of NO released by mice responding to the infection [$P<0.01$] (Table 3.12). This might be explained by the reduced bacterial loads within these samples. Mice in the

Infectious Dose	Sample	No. responding	Mean Level +/- SEM (U/ml)
10^4	Lavage fluid	0/46	ND
10^5	Lavage fluid	1/45	2.89
10^6	Lavage fluid	44/68	18.99 +/- 3.59
10^4	Lung homogenate	4/44	11.21 +/- 8.27
10^5	Lung homogenate	11/46	35.34 +/- 7.95 +
10^6	Lung homogenate	31/43	16.48 +/- 2.51

Table 3.10 TNF activities within lung samples from MF1 mice responding with elevated production of this mediator after inoculation of either 9.75 x 10^5, 9.56 x 10^4 or 9.55 x 10^3 CFU *S. pneumoniae*. ND= none detected, +, P<0.05 higher when compared to 10^6 CFU group.

Infectious Dose	Sample	No. responding	Mean Level +/- SEM (U/ml)
10^4	Lavage fluid	2/47	2.37 +/- 0.17
10^5	Lavage fluid	16/47	3.16 +/- 0.86
10^6	Lavage fluid	27/30	2.64 +/- 0.47
10^4	Lung homogenate	9/45	1.34 +/- 0.80 *
10^5	Lung homogenate	26/44	5.84 +/- 1.06
10^6	Lung homogenate	25/30	6.11 +/- 1.25

Table 3.11 IL-6 levels within lung samples from MF1 mice responding with elevated production of this mediator after inoculation of either 9.75 x 10^5 , 9.56 x 10^4 or 9.55 x 10^3 CFU *S. pneumoniae*. *, P<0.01 reduced when compared to other infectious doses.

Infectious Dose	No. responding	Mean Level +/- SEM (μM)
10^4	9/46	3.84 +/- 0.21 *
10^5	12/47	8.47 +/- 1.39
10^6	12/62	9.56 +/- 1.34

Table 3.12 NO levels within lung samples from MF1 mice responding with elevated production of this mediator after inoculation of either 9.75 x 10^5, 9.56 x 10^4 or 9.55 x 10^3 CFU *S. pneumoniae*. *, P<0.01 reduced when compared to 10^6 CFU group.

Infectious Dose	No. responding	Mean Level +/- SEM (μg/ml)
10^4	30/46	203.12 +/- 13.00
10^5	27/47	243.04 +/- 11.18
10^6	56/65	247.44 +/- 16.42

Table 3.13 Total protein levels within MF1 lung samples from mice responding with elevated production of this mediator after inoculation of either 9.75 x 10^5, 9.56 x 10^4 or 9.55 x 10^3 CFU *S. pneumoniae*.

Infectious Dose	Sample	No. responding	Mean Level +/- SEM (pg/ml)
10^4	Lavage fluid	24/44	54.17 +/- 2.77 *
10^5	Lavage fluid	4/45	82.83 +/- 14.23
10^6	Lavage fluid	41/49	69.63 +/- 6.92
10^4	Lung homogenate	12/41	1900.58 +/- 72.52
10^5	Lung homogenate	19/43	2018.26 +/- 49.66
10^6	Lung homogenate	23/30	2098.19 +/- 94.81

Table 3.14 IL-10 levels within MF1 lung samples from mice responding with elevated production of this mediator after infection with either 9.75×10^5, 9.56×10^4 or 9.55×10^3 CFU *S. pneumoniae*. *, $P<0.01$ reduced when compared to 10^6 CFU group.

10^6 and 10^5 CFU groups were equally capable of releasing NO within their airways.

Reducing the infectious doses did not significantly reduce the total protein levels associated with lung airways during pneumococcal pneumonia (Table 3.13). This suggests that similar levels of disruption occurred to the alveolar/capillary barrier in mice succumbing to the infection in each of the groups.

Few mice in the 10^5 CFU group responded to infection by increasing lavage fluid IL-10 levels above that seen immediately after infection (Table 3.14). This may be explained by the unusually high IL-10 levels seen at time 0h. Those mice that did respond in this manner did so to a similar level as the 10^6 CFU group.

Although half of the mice challenged with 10^4 CFU *S. pneumoniae* upregulated their airway IL-10 levels following infection, the magnitude of elevation was significantly lower than that seen in the 10^6 CFU group ($P<0.01$).

This dose dependency was absent from lung homogenate IL-10 levels as mice in all three groups were equally capable of up-regulating tissue levels of this anti-inflammatory cytokine.

Thus although the overall data for IL-10 (Figure 3.17 C) suggested that only a very high infectious dose was capable of altering lung tissue IL-10 levels, Table 3.14 shows that this is not the case. All three infectious doses are capable of releasing similar levels of IL-10.

3.2.4.7 Systemic inflammation following intranasal inoculation of reduced doses of *S. pneumoniae*

Serum TNF activities were also measured in the dose response samples in order to further explore the possibility of a threshold number of pneumococci required for initiation of systemic inflammation.

Mice inoculated with any of the doses of *S. pneumoniae* were capable of releasing systemic levels of TNF activity (Figure 3.22). The highest levels were found in mice inoculated with 10^5 CFU and occurred at 36h post challenge, 6h later than that seen in the 10^6 CFU group. At 36h post challenge of the mice inoculated with 10^5 CFU had detectable TNF activity within their circulations with levels ranging from 68U/ml to 2400U/ml. At this same time point, one mouse inoculated with 10^4 CFU had detectable

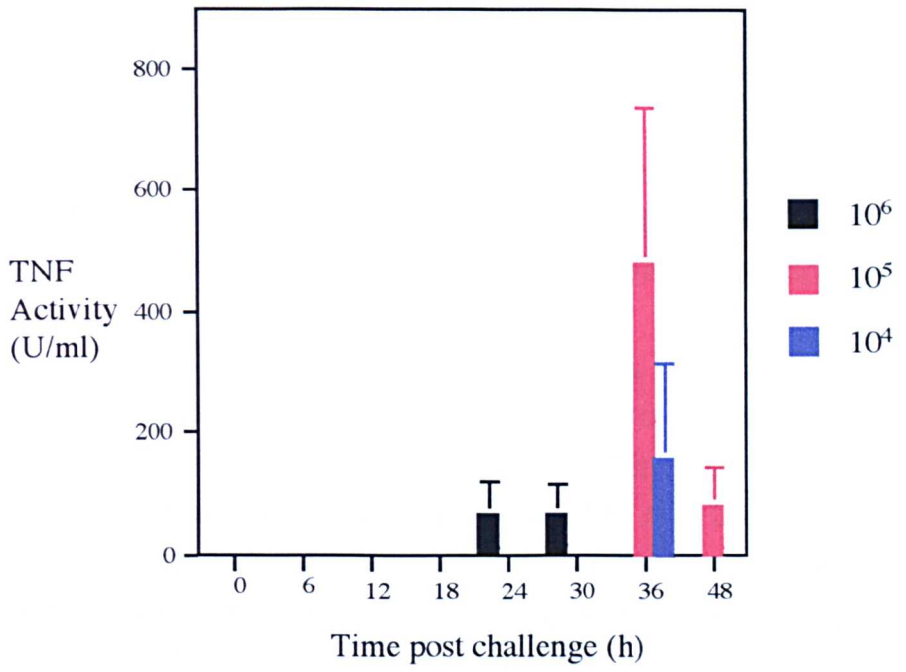

Figure 3.22 TNF activity within serum from MF1 mice following intranasal infection with either 9.75 x 10^5, 9.58 x 10^4, or 9.55 x 10^3 CFU *S. pneumoniae*. n=6-16.

Dose	No. responding	TNF activity (mean +/- SEM)
10^4	1/38	1400
10^5	7/40	782.10 +/- 325.86
10^6	5/59	393.56 +/- 148.95

Table 3.15 TNF activity within serum samples from MF1 mice responding with elevated production of this mediator after infection with either 9.75 x 10^5, 9.56 x 10^4 or 9.55 x 10^3 CFU *S. pneumoniae*.

TNF activity in its circulation, the only mouse to do so in this group throughout the experiment.

By separating these mice into responders and non responders, as with pulmonary TNF activities, it is possible to better understand the effects of dose on systemic inflammation.

Reducing the infectious dose to 10^4 CFU significantly reduced the number of mice with detectable systemic TNF (Table 3.15). Only one mouse in this group possessed such activity, although its serum did contain a very high level.

Mice in the 10^5 CFU group displayed the greatest signs of systemic inflammation, the largest number of positive mice and the highest activity levels. Systemic inflammation also occurred in mice in the 10^6 CFU group, but in fewer animals and to a reduced extent to that in the 10^5 CFU group.

All of these findings directly relate to the level of bacteraemia in these groups, with low levels in the 10^4 CFU group, higher in the 10^6 CFU group and the highest in the 10^5 CFU category. See Table 3.4 for further investigation of TNF activity/level of bacteraemia relationship.

3.2.5 Effect of age on response to intranasal infection

In order to investigate the effects of ageing on host response to *S. pneumoniae*, 52 week old MF1 mice were infected with 10^6 CFU and their survival times, bacterial loads and inflammatory response compared to young mice.

3.2.5.1 Survival and signs of illness following intranasal infection of aged and young mice

By 24h into the experiment 13/15 aged mice displayed symptoms of illness. At this time, a hunched appearance was the most common although one mouse was lethargic. Some mice passed rapidly through the range of signs of illness with 2 mice culled at 28h post challenge whilst others progressed slowly through the infection. One mouse apparently recovered from the illness with symptoms disappearing after 92h and surviving until the end of the experiment at 336h.

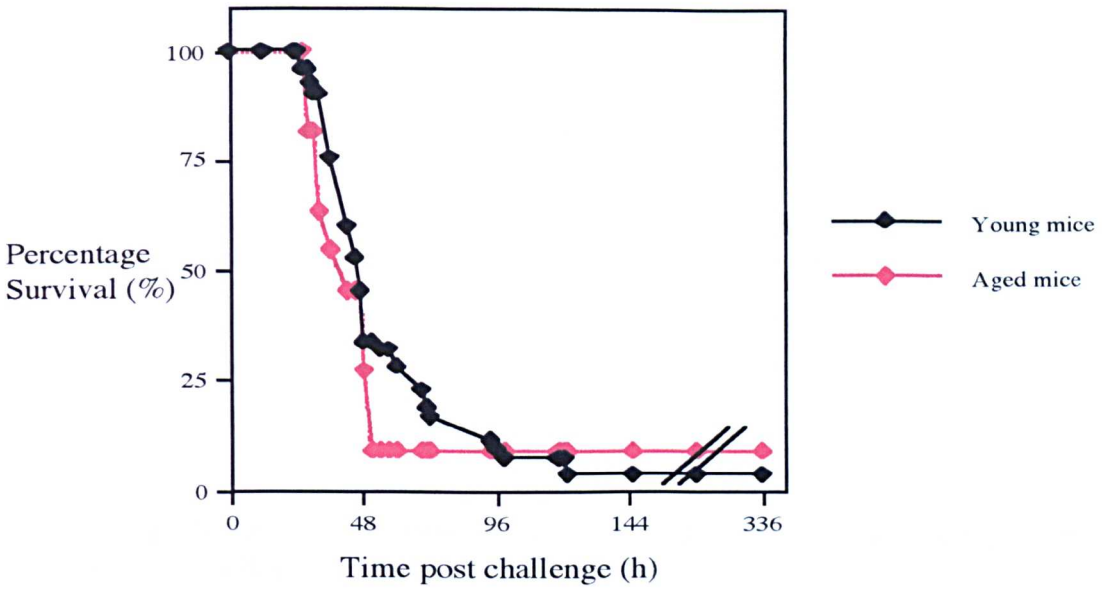

Figure 3.23 Survival of aged and young MF1 mice following intranasal infection with either 1.01 x 10^6 CFU (aged mice) or 9.76 x 10^5 CFU (young controls) *S. pneumoniae*. n=11-53.

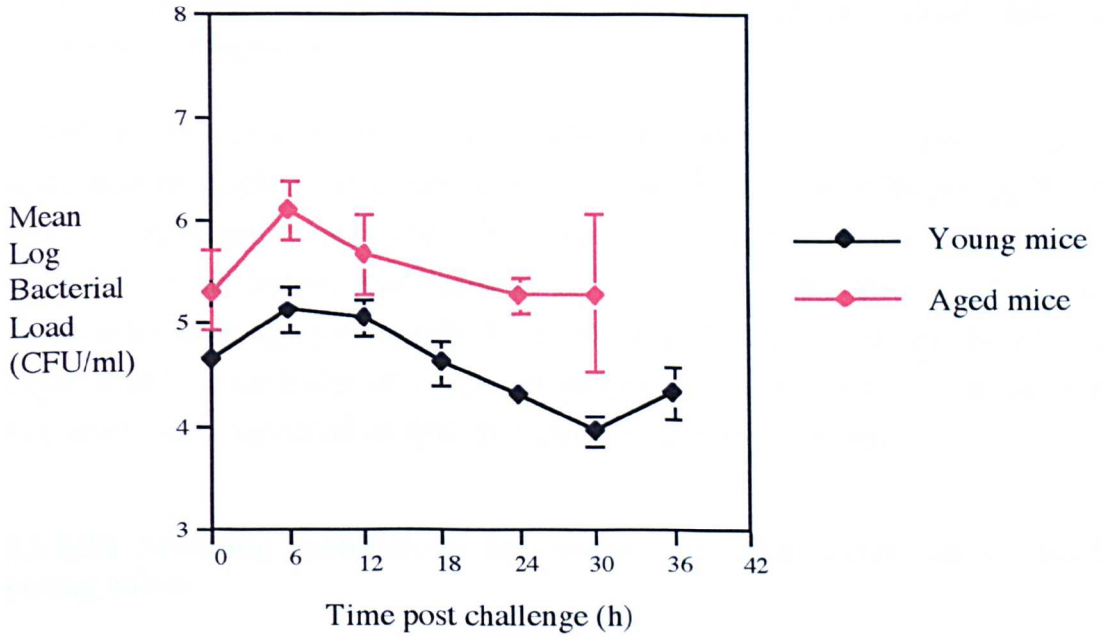

Figure 3.24 Bacterial loads within the airways of aged and young MF1 mice following intranasal infection with either 1.06 x 10^6 (aged mice) or 9.75 x 10^5 CFU (young controls) *S. pneumoniae*. n=4-28.

Despite 5 aged mice dying within 36h, the overall survival times of these mice were not significantly different from those of young mice (Figure 3.23).

3.2.5.2 Bacteriology following intranasal infection of aged and young mice

At the time of conducting these experiments a technical problem had arisen with the bacterial preparations used in infections. This resulted in higher counts recovered within the lungs immediately following intranasal infection than were previously (and since) found. However, comparisons of the trends between groups are still possible.

3.2.5.2.1 Bacteriology in the pulmonary air spaces following intranasal infection of aged and young mice

Bacterial loads within the airways of aged mice were higher at all points than young controls (Figure 3.24). No significant alteration in bacterial loads was detected in either group during the infection.

3.2.5.2.2 Bacteriology in the lung tissues following intranasal infection of aged and young mice

Numbers of viable pneumococci associated with lung tissues of aged mice increased throughout the experiment (Figure 3.25). As with young mice, the initial increase was slow in occurring and required around 12h acclimatisation before the number of viable bacteria increased rapidly to reach a level of approximately 10^6 CFU/ ml of lung tissue by the end of the experiment. The level of variation associated with aged mouse lung tissue bacterial loads resulted in this increase being insignificant.

3.2.5.2.3 Systemic bacteriology following intranasal infection of aged and young mice

Bacteraemia developed earlier in aged mice than in young controls following intranasal infection with *S. pneumoniae* (Table 3.16). By 6h post challenge all aged mice had culturable pneumococci within their circulations a significant increase ($P<0.05$), whilst none of the young controls had detectable bacteraemia. Blood counts continued to increase rapidly in the aged group

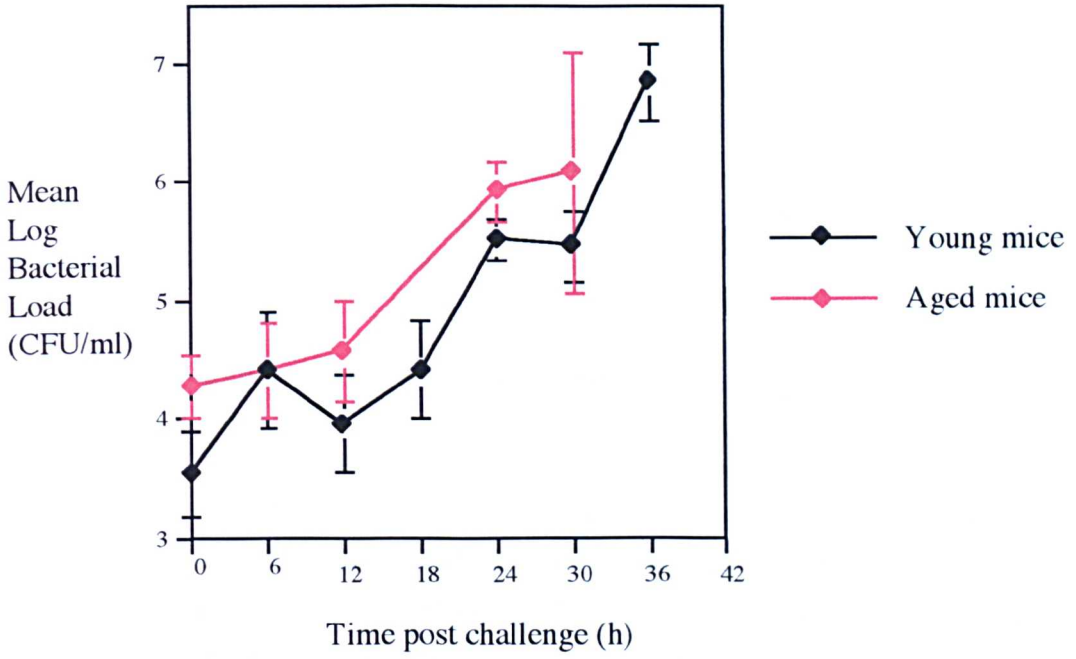

Figure 3.25 Bacterial loads within the lung tissues of aged and young MF1 mice following intranasal infection with either 1.06 x 10^6 (aged mice) or 9.75 x 10^5 CFU (young controls) *S. pneumoniae.* n=4-11.

Time post challenge (h)	Aged mice bacterial load (log CFU/ml)	Young mice bacterial load (log CFU/ml)	P value
0	ND	ND	P>0.999
6	2.460 +/- 0.269	ND	P<0.01
12	3.702 +/- 0.416	2.414 +/- 0.213	P<0.05
24	6.853 +/- 1.116	5.315 +/- 0.316	P>0.05 (NS)
30	7.900 +/- 0.274	5.801 +/- 0.347	P<0.01

Table 3.16 Bacterial loads (mean +/- SEM) within the bloodstreams of aged and young MF1 mice following intranasal infection with either 1.06 x 10^6 (aged mice) or 9.75 x 10^5 CFU (young controls) *S. pneumoniae.*. n=4-26. ND= below detection limit of viable count assay (<log 1.92 CFU/ml), NS= not significantly different.

until the end of the experiment (at 30h post challenge). By this time around 10^8 CFU were present per ml of blood. Similar kinetics of increasing bacterial viability were found in the circulation of young mice, the mean doubling time for D39 in young mice was 1.4h and in aged mice 1.3h. There was a delay in bacterial loads becoming significantly increased (not until 12h post challenge), with counts lagging behind by a factor of 1 log until 30h post challenge when a 2 log difference was evident.

3.2.5.3 Histology following intranasal infection of aged and young mice

Due to the rapid progression through symptoms of illness, and the short survival times of some aged mice, all time courses carried out with aged mice were finished at 30h post challenge instead of 36h.

Lung sections sampled immediately following infection displayed minor signs of inflammation in "healthy" aged mice There was evidence of slight inflammatory cell influx and disruption to alveolar architecture. By 6h post challenge, sections from aged mice displayed marked neutrophil recruitment especially around blood vessels (both marginating through endothelium and already located with surrounding lung tissue [Figure 3.26]). Histopathology then followed a similar pattern in aged mice as that described for young mice in Section 3.2.3.7 with aged animals displaying more rapid kinetics (Table 3.17).

3.2.5.4 Production of inflammatory mediators in the pulmonary airways following intranasal infection of aged and young mice

Upregulation of TNF activity occurred earlier and to a significantly greater extent in the airways of aged mice when compared to young animals (Figure 3.27 A). At 6h, 12h and 24h post challenge significantly higher TNF levels were found in aged mouse samples than in young samples ($P<0.01$). In both sets of mice TNF activity was transient with lower levels found at the later time points.

NO levels were significantly lower in the airways of aged mice immediately following intranasal infection with *S. pneumoniae* ($P<0.01$, Figure 3.27 B). Levels of NO did increase in lavage fluid towards later time points but it was not until peak production that levels reached those normally found in young mice.

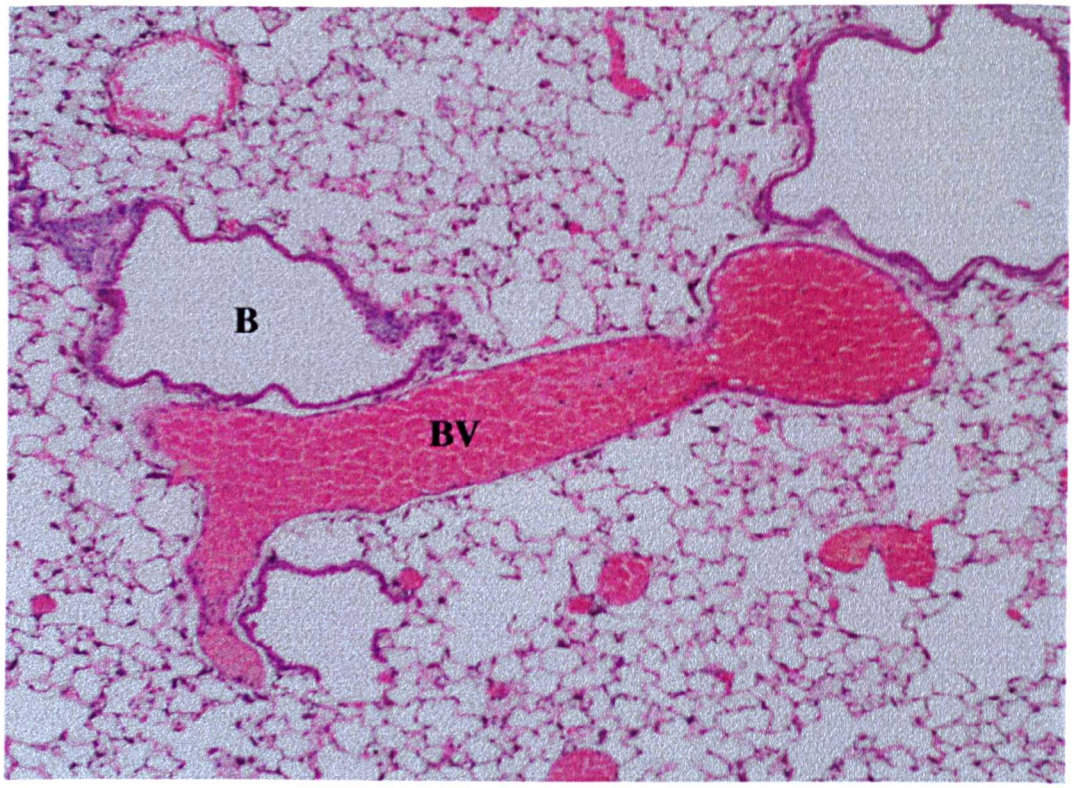

Figure 3.26 A

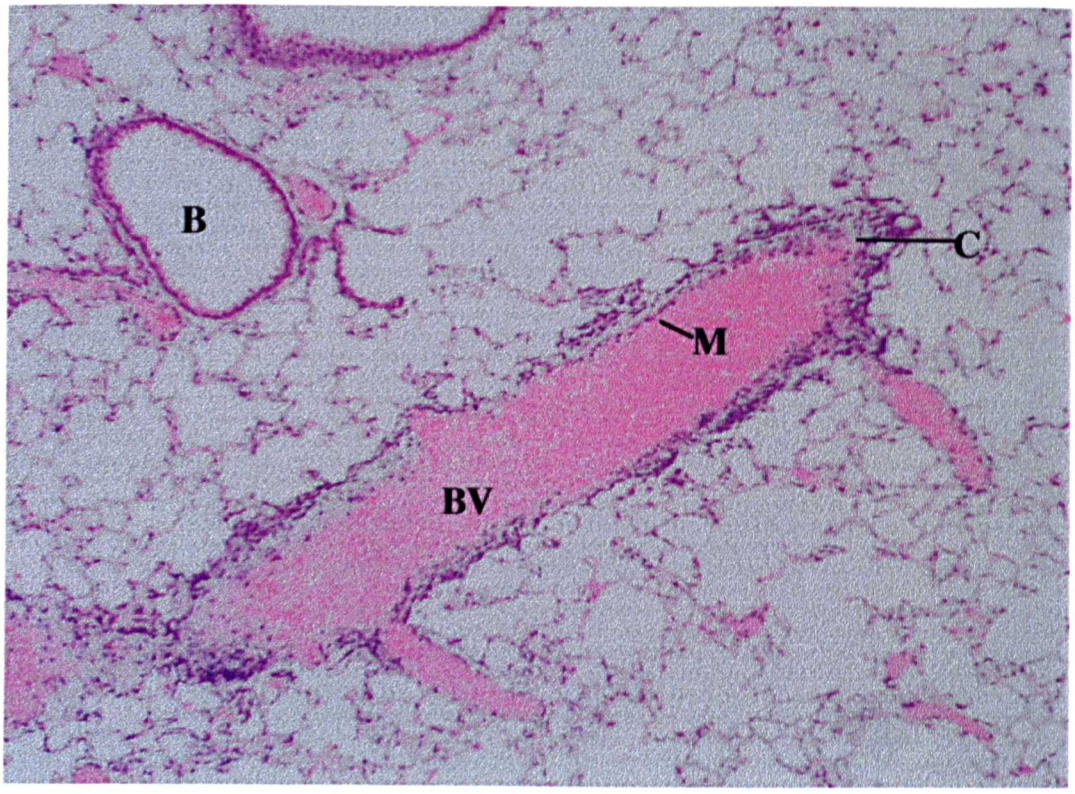

Figure 3.26 B

Figure 3.26 Comparison of cell influx to the lungs of aged and young mice early after intranasal infection with 10^6 CFU *S. pneumoniae.* A major bloodvessel (BV) and bronchiole (B) is evident in each section.

A) Histological section of lung from an 9 week old MF1 mouse 6h post infection with 10^6 CFU *S. pneumoniae* stained with haematoxylin and eosin. Minor inflammatory cell presence is evident both inside a major bloodvessel and in the surrounding tissue (x200 magnification).

B) Histological section of lung from a 52 week old MF1 mouse 6h post infection with 10^6 CFU *S. pneumoniae.* There is marked inflammatory cell influx in the tissues surrounding the major blood vessel (C) and marginating through the endothelium (M) (x20 magnification).

Mouse age	Time post challenge (h)						
	0	6	12	24	30	36	48
9wks	0.00	1.83	3.75	3.50	-------	5.67	5.00
52wks	2.00	4.00	6.00	5.00	8.00	-------	-------

Table 3.17 Histopathologic score for lung tissue from 9 or 52 week old MF1 mice following intranasal infection with 1.01 x 10^6 CFU *S. pneumoniae*. n=1-3 mice per time point. SEM have been omitted from the 9wk group as there are no SEM for aged mice samples. At most times only one aged mouse was sampled and at the others the two mice had the same histopathology score.

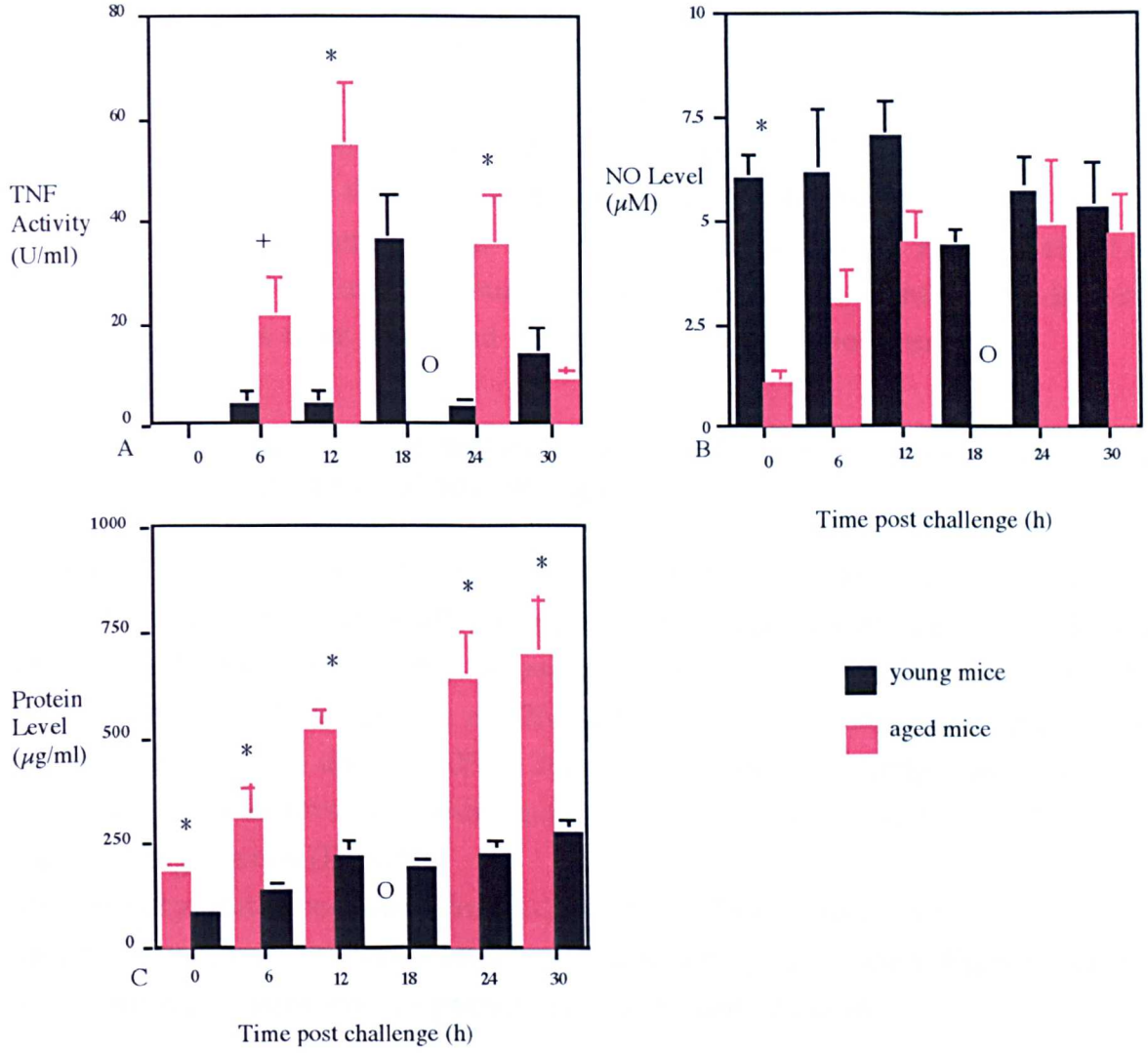

Figure 3.27 Production of inflammatory mediators within the airways of aged and young MF1 mice following intranasal infection with 9.75 x 10^5 (young mice) or 1.06 x 10^6 (aged mice) CFU *S. pneumoniae*. A= TNF activity n=4-21, B= NO levels n=4-12 , and C= Total protein content, n=8-14. 0= no samples for aged mice at 18h post challenge. *, $P<0.01$ higher for aged mice samples when compared to young mouse samples. +, $P<0.05$ higher for young mouse samples when compared to aged mouse samples

A high level of disruption occurred to lung integrity of aged mice during the infection (Figure 3.27 C). Protein levels increased steadily and highly significantly from 6h post challenge indicating that damage occurred to the alveolar/capillary barrier early during the experiment. At each time point investigated, lavage fluid from aged mice contained significantly higher levels of total protein than in lavage fluid from young mice ($P<0.01$).
Preliminary data gathered for IL-10 suggests that age did not have an effect on production of IL-10 during pneumococcal pneumonia. Similar levels were found in samples from aged and young animals (data not shown).

3.2.5.5 Production of inflammatory mediators in the lung tissues following intranasal infection of aged and young mice

Elevated levels of TNF activity were evident within the lungs of aged mice from 6h post with significantly elevated levels present at 12h and 24h post challenge (Figure 3.28). Lung tissues from aged mice contained significantly higher levels of TNF activity at 12h and 24h post challenge than did young mice ($P<0.01$ at 12h and $P<0.05$ at 24h). By 30h post challenge however, the relationship was reversed with more TNF activity associated with young mouse lung tissues ($P<0.05$).
Preliminary data recovered for IL-10 within these same samples indicates that production of this cytokine was no different in aged mice than in young mice during pneumococcal pneumonia (data not shown).

3.2.5.6 Systemic production of inflammatory mediators following intranasal infection of aged and young mice

Only 4 aged mice had detectable levels of TNF levels in their serum during infection. One mouse mice displayed low levels of systemic TNF activity at 24h with two doing so at 30h post challenge. These results are not significantly different from those obtained with young mice (Figure 3.16).

3.2.5.7 Immunohistochemistry following intranasal infection of aged and young mice

As with young mice, TNF and IL-10 could be detected at low levels within lungs immediately following infection, mainly within bronchial epithelial

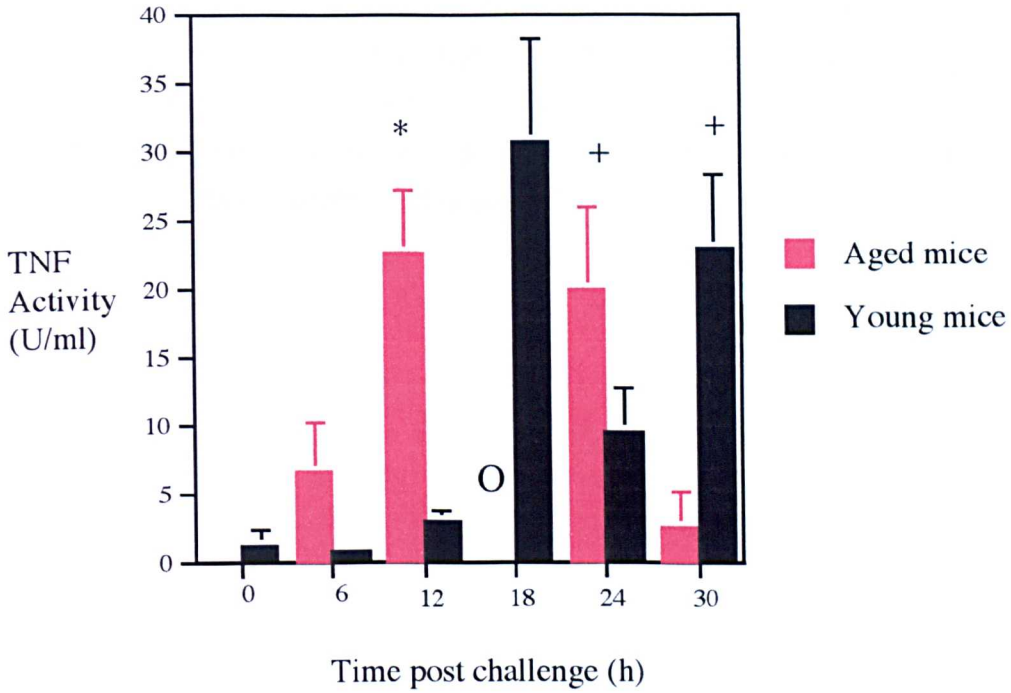

Figure 3.28 TNF activity levels within the lung tissues of aged and young MF1 mice following intranasal infection with either 1.06 x 10^6 CFU (aged mice) or 9.75 x 10^5 CFU (young mice) *S. pneumoniae*. n= 4-17, *, P<0.01 higher for aged mouse samples when compared to those from young mice, +, P<0.05 different for young mouse samples when compared to aged mouse samples. 0= no samples for aged mice at 18h post challenge.

cells. Similar results were obtained 6h post challenge. At 12h post challenge TNFα was mainly located in areas of cell influx with lower expression in the bronchial epithelium. At 24h post challenge there were high levels of TNF present throughout the lungs. At 30h post challenge TNF was only found associated with areas of inflammatory cell influx and even then was present at very low levels. As with young mice, both cell-attached and secreted forms of TNFα were present although secreted constituted the majority (Figure 3.29 B). A histological section is included as Figure 3.29A as a point of reference.

From 12h onwards IL-10 expression was mainly found at sites of cell influx. This anti-inflammatory cytokine was detected in similar areas to TNFα at the end of the infection but a stronger signal was detected suggesting more IL-10 was present that TNFα (Figure 3.29 C).

As with young mice, neutrophils constituted the majority of recruited cells to the lungs of aged animals (Figure 3.29 D).

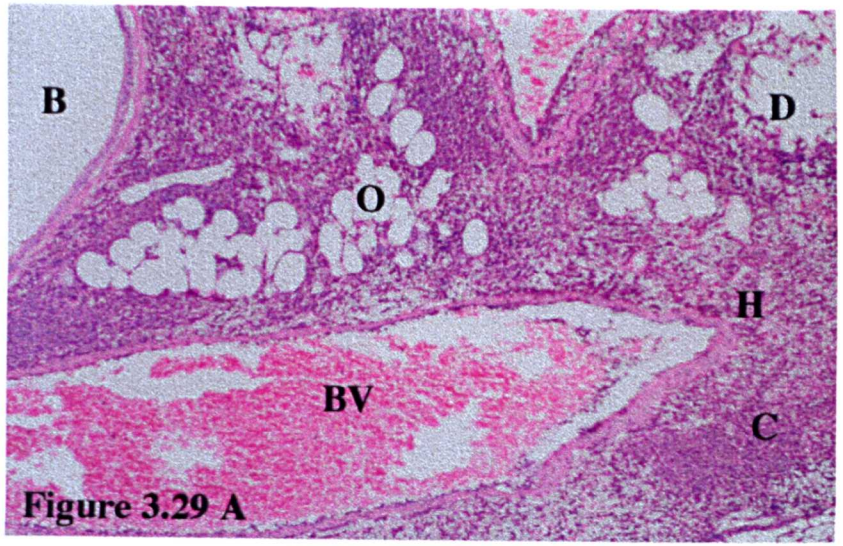

Figure 3.29 A

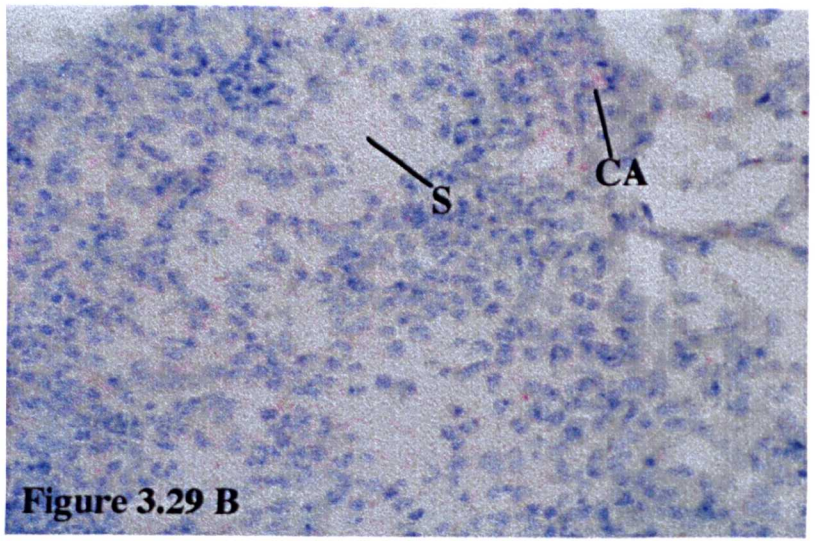

Figure 3.29 B

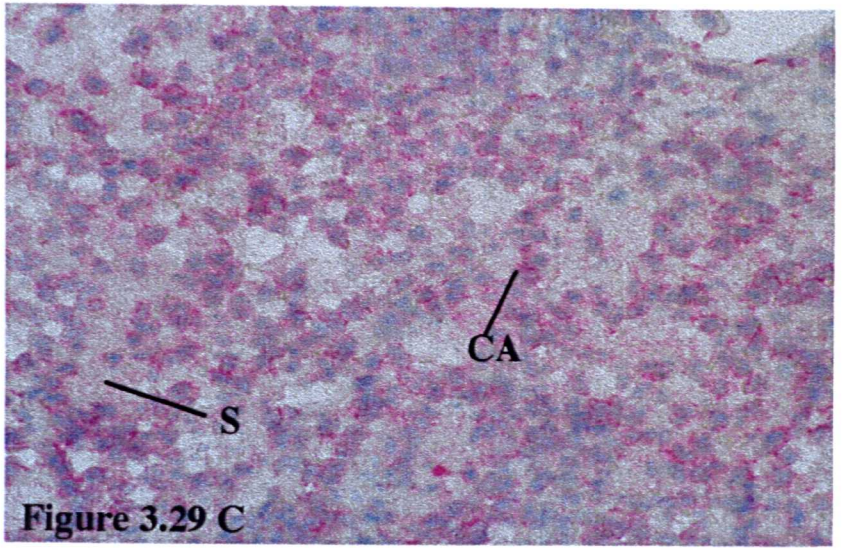

Figure 3.29 C

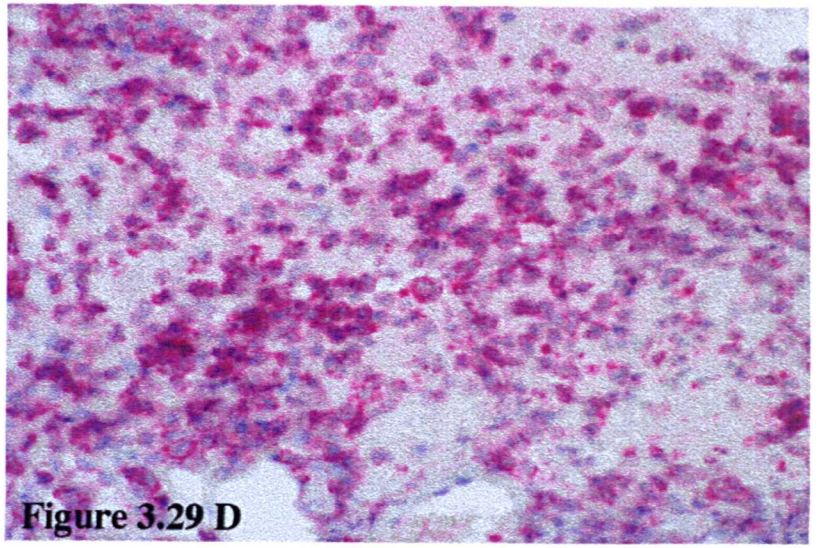
Figure 3.29 D

Figure 3.29 Histological and immunohistochemical sections of lungs from a 52 week old MF1 mouse 30h post infection with 1.06 x 10^6 CFU *S. pneumoniae* (x100 magnification for histology stained with haematoxylin and eosin [3.29 A] and x400 magnification for immunohistochemistry [3.29 B-D]).

In Figure 3.29A a major bloodvessel (BV) is shown at the bottom of the picture with a bronchiole (B) on the left. Widespread consolidation with inflammatory cells is evident (C) as is oedema (O), haemorrhage (H) and disruption to surrounding alveoli (D).

In Figure 3.29B a consolidated area of the lung section is stained for immunohistochemical identification of TNFα. The staining is mainly weak detecting secreted TNFα forms (S), although some cell-associated TNFα still remains (C).

The staining for IL-10 on a serial section (Figure 3.29C) detects a stronger signal with again both cell associated and secreted forms of the cytokine present.

Figure 3.29 D shows the immunohistochemical identification of the majority of cells in this same area of consolidation to be neutrophils.

3.3 SUMMARY

This chapter describes the characterisation of the acute inflammatory response that occurs following intranasal infection of MF1 mice with 10^6 CFU *S. pneumoniae*.

Such mice rapidly succumb to the infection with a median survival time of 47h post challenge.

The number of viable pneumococci found within the pulmonary air spaces did not alter greatly during infection, whilst the number of pneumococci associated with the lung tissues and bloodstream increased significantly. There was a significant correlation between the bacterial loads in lung tissues and bloodstreams ($P<0.05$), i.e. as the number of bacteria associated with lung tissues increased so did the level of bacteraemia.

The bacteria induced inflammation that was evident in histological sections. From 24h post challenge, disruption to the normal lung architecture was evident throughout the lobes. The majority of inflammatory lesions evident within infected lungs were perivascular, a pattern of disease previously reported by this laboratory (Canvin *et al.* 1995) and others (Branch and Stillman 1924). Candidate cell types involved in this inflammation are neutrophils which were rapidly recruited in large numbers and mast cells which appeared to release their granular contents.

One of these granule contents was likely to have been TNF. Pulmonary activity of this pro-inflammatory cytokine did increase during mid infection. Kinetics suggested two waves of production, one at 18h and one at 30h post challenge. The early peak may have been released by resident pulmonary cells with recruited cells responsible for the later peak.

IL-1β levels detected within bronchoalveolar lavage fluid also increased during mid infection. Peak IL-6 and IL-10 production was delayed in comparison, although IL-6 production displayed similar trends to TNF activity. Kinetics of production of all inflammatory mediators were similar in the tissues and air spaces. NO could only be detected within lung airways, with no significant increase in production evident during the infection.

The protein content of these lavage fluids increased from early in the experiment indicating that damage did occur to the integrity of the alveolar/capillary barrier rapidly after instillation of the pneumococci.

Infected mice became severely hypothermic at the end of the infection, with kinetics closely following elevated production of several inflammatory

mediators, suggesting that one of these might have been the stimulus for the decline in T_{CO}.

PBS sham infection did not induce TNF activity in lung airways or tissues, suggesting that the inflammation was due to the bacteria and not due to the procedures involved. Sham infected mice did display a very transient increase in the production of IL-10, which may constitute an attempt to down regulate any detrimental inflammation directed against a harmless liquid. Disruption to lung integrity was also detected in these mice.

TNF activity was not detected in either the pulmonary airways or tissues of mice challenged with heat killed bacteria. This indicates that live pneumococci are required for activating this arm of the inflammatory response. IL-10 levels were elevated in these mice as was the protein content of lavage fluids. These findings suggest that inflammation not mediated by TNFα is occurring within lungs of mice inoculated with heat killed *S. pneumoniae*. IL-1β may be a likely replacement for TNFα in such situations (Riesenfeld-Orn *et al.* 1989).

Reducing the infectious dose decreased overall mortality associated with pneumococcal pneumonia. In addition, mean data indicate that the kinetics of the host response following inoculation of a reduced dose of *S. pneumoniae* are delayed in comparison to those evident following infection with 10^6 CFU.

By separating mice into responding and non-responding phenotypes it was possible to investigate whether similar responses resulted with reduced challenge doses. Results showed that an infectious dose of 10^5 CFU significantly lengthened the survival times of mice during the infection. However, in respect of bacterial loads and inflammatory mediator production, mice that were overcome by 10^5 CFU responded in a similar manner, but more slowly than those infected with the control dose of 10^6 CFU. Reducing the challenge dose even further to 10^4 CFU resulted in a marked difference in not only response kinetics but also the magnitude of several components. Even in mice responding with elevated lung tissue IL-6, airway NO or IL-10 release, levels were significantly reduced in comparison to the other doses. Such a phenomenon indicates that there may be a threshold number of bacteria required to induce pulmonary inflammation. Inoculation of 10^4 CFU does not result in bacterial loads that

reach this threshold and the host response is diminished. With higher doses the threshold is reached and mice succumbing to the infection all respond in a similar manner, albeit with slower kinetics in the 10^5 CFU group (a longer period of time may be required for pneumococci to reach the threshold).
Data from Tables 3.10, 3.12 and 3.14 indicate that the threshold number of pneumococci may be higher within the airways than in the lung tissues. No TNF activity at all was observed in the lavage fluid from animals inoculated with 10^4 CFU. There was also significantly reduced NO levels and IL-10 levels (suggesting that there was a lower general pro-inflammatory stimulus for IL-10 production) whilst levels in lung tissues were generally no different from the other doses. Such a hypothesis supports the idea that the host would not benefit from inflammation caused by low numbers of non-pathogenic organisms encountered in the airways during everyday life. Rather, high levels of inflammation would only be beneficial if the bacteria became associated with the lung tissues.
The pulmonary threshold hypothesis is also supported by the inability to detect systemic TNF activity in mice with a level of bacteraemia lower than 2.88×10^7 CFU/ml. Thus a systemic threshold number of bacteria must be reached before the cytokine cascade is initiated.

Bacteraemia was found to develop earlier in aged mice than in young controls following intranasal infection with *S. pneumoniae*. Cytokine analysis in the aged mice revealed a heightened production of TNF during the infection, reduced NO levels and greatly elevated disruption to lung tissues.
Significant inflammatory cell recruitment around major blood vessels in aged mice lung sections occurred by 6h post challenge. Marked disruption to lung tissues was evident from 12h post challenge until the end of the experiment. These results indicate that an overzealous inflammatory response occurs in aged mice during pneumococcal infection.

Chapter 4

Modulation of Response

4.1 INTRODUCTION

In an attempt to define the mechanism of inflammation in response to pneumococci we decided to investigate the roles played by selected components of the host system. An overview of the factors is given before a detailed description of the results.

One of the first lines of non-specific defence is surfactant (Section 1.5.3.1). Surfactant is a substance capable of lowering the surface tension in the lungs and is produced by type II pneumocytes within the alveoli (Dobbs 1989). Surfactant contains 20% protein and 80-90% lipid by weight and of this, around 80% is phosholipid (Dobbs 1989).

Surfactant replacement therapies were originally designed to aid lung function in pre-term infants (Shapiro 1998). We have employed the synthetic surfactant preparation called artificial lung expanding compound (ALEC) (Bangham *et al.* 1979) in attempts to modulate the disease process in this model of pneumococcal pneumonia. This preparation is comprised of a mixture of 70% phosphatidylcholine (the major phospholipid of natural surfactant) and 30% phosphatidylglycerol and has been shown to function well in a surface tension regulating capacity (Morley 1987), although its effects on the immune system during disease is unknown. As this is used clinically it is of interest to determine whether it confers any protection to infectious disease in neonates.

The majority of host defence functions of surfactant are attributed to surfactant proteins. The possibility that surfactant phospholipids can have effects on host defence during pneumococcal pneumonia has therefore not been extensively studied.
ALEC was thus employed as a convenient method of elucidating the roles of surfactant phospholipids in immunity to infectious disease.

The first transgenic mice were successfully generated in 1980 (Gordon, Scangos *et al.* 1980). Transgenic animals have since become a standard method of investigating the effects of an individual mediator during infection (van der Poll *et al.* 1997a & b), (Decken *et al.* 1998) (Deckert-Schlüter *et al.* 1998) & (McInnes *et al.* 1998) and inflammation (Kühn *et al.* 1993), (Peschon *et al.* 1998).

These animals have advantages over antibody neutralisation of mediators due to assurance of complete removal of the cytokine of interest. Additionally, substances used to manipulate one cytokine are likely to have unknown effects on others.
One disadvantage of cytokine "knockout" mice is that they do not permit investigation of selective depletion of a cytokine within one body compartment, e.g. the lungs. For this reason, antibody neutralisation of cytokines still has a role to play in elucidating the effects of cytokines during infections.

As described elsewhere in this thesis (Chapter 1) TNFα is known to play a major role in host defence during infections caused by pneumococci and other organisms. Antibody neutralisation of TNFα during disease has revealed roles for this cytokine in the recruitment of inflammatory cells to the site of infection thereby reducing bacterial loads, preventing development of bacteraemia and improving survival rates (Britton *et al.* 1998), (Chen *et al.* 1992a), (Gosselin *et al.* 1995), (Laichalk *et al.* 1996), (Takashima *et al.* 1997), (van der Poll *et al.* 1997a), and (Williams *et al.* 1990). For this reason TNF was selected as a target to modulate the immune response during pneumococcal pneumonia. Neutralising antibodies and TNF receptor deficient mice were both used.

TNFα 's activities are mediated via two structurally related receptors, p55 and p75, named due to their molecular masses (Brockhaus *et al.* 1990) (Section 1.6.1.1.4). These receptors can be found as cell surface bound on most cell types, or as free soluble receptor after proteolytic cleavage of the extracellular domain (Black *et al.* 1997) & (Moss *et al.* 1997). TNFα's more classic functions such as up-regulation of adhesion molecules and increased phagocyte anti-microbial activity (i.e. inflammatory effects) are mediated through p55. p75 is thought to be responsible for TNFα's cytotoxic and apoptotic responses and down regulation of TNFα's inflammatory reactions (Peschon *et al.* 1998). In certain situations, however, p75 can act to enhance p55 mediated responses (Weiss *et al.* 1997).
Mice deficient in either or both TNF receptors have now been described. p55-/- mice display heightened susceptibility to a range of infections including intracellular micro-organisms (Everest *et al.* 1998), (Castanos-Velez *et al.*

1998) & (Garcia *et al.* 1995), fungi (Steinshamn *et al.* 1996) and systemic *S. pneumoniae* infection (O'Brien *et al.* 1999).
In general p75-/- mice do not display heightened susceptibility to infectious disease (Deckert-Schlüter *et al.* 1998).

Infections in humans and experimental animals are often associated with significant increases in systemic and local NO production (Bergeron *et al.* 1998). NO production by the inducible NO synthase isoform (iNOS) is stimulated by pro-inflammatory cytokines such as TNFα and IL-1β, as well as by microbial products such as lipoteichoic acid (Fang 1997).
Literature concerning the anti-microbial activities of NO mainly concerns intracellular organisms such as Chlamydia (Igietseme *et al.* 1998) and Mycobacteria (Nozaki *et al.* 1997). Roles for NO in controlling infections due to extracellular bacteria is much less well understood with limited literature available (Kaplan *et al.* 1996) & (Tsai *et al.* 1997), although a recent report does implicate NO in cell recruitment during pneumococcal pneumonia (Bergeron *et al.* 1998).
As with anti-cytokine antibodies, most of the pharmacological inhibitors of iNOS also have an effect on cNOS and all have activities that are unrelated to the ability to down-regulate iNOS (MacMicking *et al.* 1997). Thus, use of genetically engineered mice unable to produce iNOS was a more appropriate method of discovering the roles exclusive to iNOS during infection.

Viable and fertile genetically engineered mice deficient in iNOS (iNOS-/-) were originally produced by Wei *et al* (Wei *et al.* 1995). Peritoneal macrophages from these mice completely lack expression of iNOS but not of cNOS following LPS stimulation.
Survival of iNOS-/- mice is markedly diminished upon infection with bacteria such as *L. monocytogenes*, and the protozoan *Leishmania major*, in comparison to wild-type mice of comparable genetic background (MacMicking *et al.* 1995) and (Wei *et al.* 1995). Where examined, decreased survival correlated with major increases in microbial burden in the affected organs. In contrast these mice are more resistant to endotoxic shock than are wild-type mice.
Experiments were thus carried out in an attempt to discover whether iNOS depletion has any effects in pneumococcal pneumonia and the resulting local inflammation.

4.2 RESULTS

4.2.1 Artificial lung expanding compound (ALEC)

4.2.1.1 Effect of ALEC on infectious dose *in vitro*

Prior to using ALEC in the *in vivo* model, it was necessary to assess any effects of preparation of the infectious dose in ALEC in order to ensure that the intended viable doses of *S. pneumoniae* would be administered.
Viability of *S. pneumoniae* D39 was not altered significantly by incubation in ALEC (Figure 4.1). Bacterial survival drops similarly in both treatment groups from initial preparation until the end of the experiment. Although the rate of decline in viability does appear to be slightly greater for the ALEC preparation than that for PBS alone, at no time are the numbers of viable bacteria significantly different.

4.2.1.2 Effect of ALEC on bacterial loads *in vivo*

An *in vivo* experiment was carried out in order to measure any effects of ALEC on bacterial loads during pneumococcal pneumonia. ALEC was administered in several regimes, either 2h before, 2h after, at the same time as the bacteria, or alone.
Administration of ALEC either 2h prior to or 2h post introduction of the bacteria had no effect on lung associated bacterial loads 24h post challenge (Figure 4.2). Inoculation of pneumococci concurrently with ALEC reduced the number of viable bacteria that could be recovered 24h into the experiment. In this group, approximately 2 log fewer CFU were recoverable per ml of homogenate, a significant reduction in viability when compared to no ALEC group ($P<0.01$).
The possibility that reduced bacterial loads were due to the viscosity of the bacteria/ALEC preparation hampering infection of the mice was investigated. Sacrifice of 5 mice directly after challenge revealed that the mean log bacteria count associated with each lung was 5.61 +/- 0.21. This level is not lower than total lung associated counts recovered from other experiments (not involving ALEC) where bronchoalveolar lavage fluid counts and lung homogenate counts were gathered (4.84 +/- 0.08). This

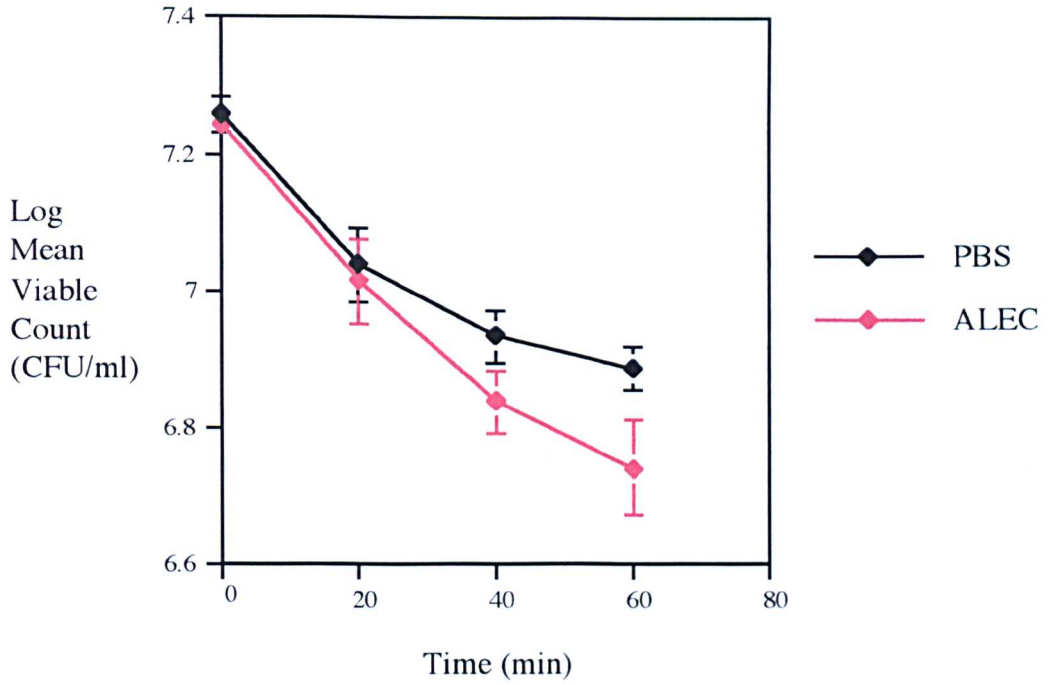

Figure 4.1 Viability of D39 when suspended in ALEC or PBS. n=4 for PBS suspensions and n=3 for ALEC preparations.

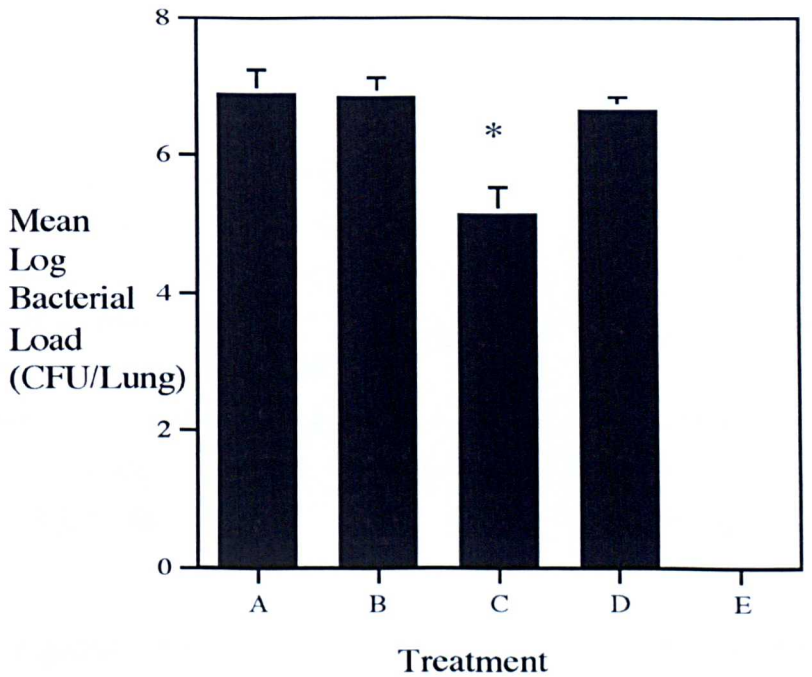

Figure 4.2 Bacterial loads within the lungs of mice 24h after undergoing various ALEC treatment regimes in connection with intranasal administration of either 1.06 x 10^6 (groups A,B, and D) or 9.40 x 10^5 (Group C) CFU *S. pneumoniae*. A= ALEC 2h prior to infection, B= no ALEC, C= pneumococci in ALEC, D= ALEC 2h post challenge, E= no pneumococci. n=8 for each group. *, P<0.01 lower when compared to no ALEC group.

suggests that the lowered bacterial loads 24h following intranasal infection with D39 in ALEC was not a consequence of impaired bacterial inoculation.

4.2.1.3 Effect of ALEC on pneumococcal growth *in vitro*

The ability of ALEC to prevent growth of pneumococci was investigated by culture of *S. pneumoniae* in BHI plus 5% ALEC or 5% non-pyrogenic saline. Growth of D39 in 5% surfactant was not inhibited in comparison to that in BHI plus 5% saline (Figure 4.3). Both cultures had the same mean doubling time (approximately 25min) and reached similar maximum viability levels 8h post inoculation. Bacteria grown in 5% ALEC displayed an earlier decline phase at the end of growth and by the final time point the number of viable bacteria within the 5% ALEC preparations was lower than those in the controls, but not significantly so.

4.2.1.4 Effect of ALEC treatment on survival during pneumococcal pneumonia

As co-administration of ALEC and *S. pneumoniae* reduced bacterial loads 24h post challenge, it was a possibility that this would lead to an alteration in survival times. Development of signs of illness in mice administered *S. pneumoniae* in ALEC was delayed in comparison to those in the D39 alone group. Clinical signs of illness were not apparent in the former group until 36h post challenge with 9/10 mice at this time displaying symptoms. Mice infected with D39 in PBS displayed signs of illness from 18-24h post challenge. Symptoms of mice in the ALEC treated group reached the same severity as mice infected with D39 alone after 36h. All mice then progressed through illness at a similar rate to control animals. Despite the earlier appearance of clinical symptoms, ALEC treated mice did not survive significantly longer than mice given bacteria in PBS (Figure 4.4).

Results thus suggest that co-administration of pneumococci in ALEC initially reduced bacterial viability in comparison to control animals. This reduction was associated with a delayed appearance of signs of illness but no overall effect on survival.

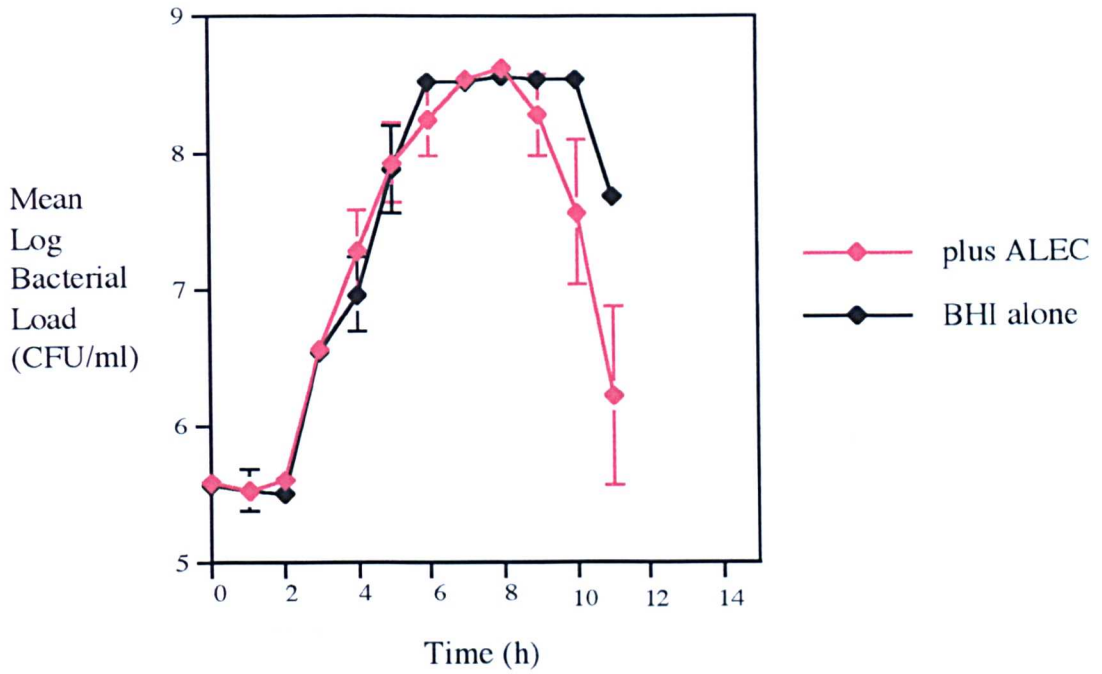

Figure 4.3 Viable counts of D39 grown in BHI alone or in BHI plus 5% ALEC (v/v). n= 3 for both cultures.

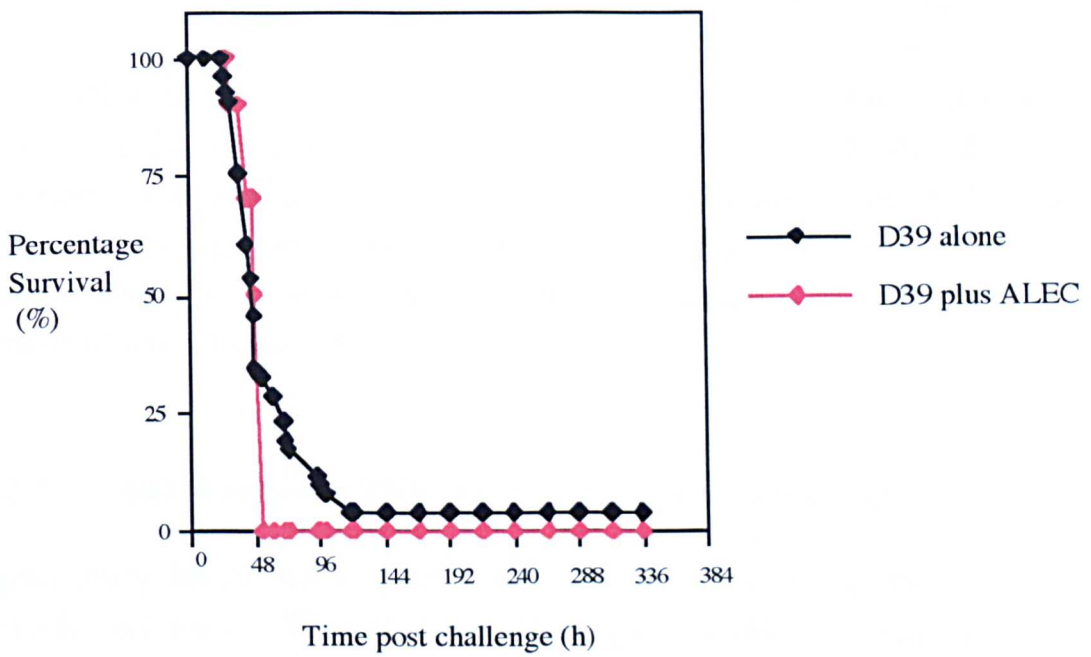

Figure 4.4 Survival of MF1 mice following intranasal infection with 9.76×10^5 (D39 alone) or 9.60×10^5 (D39 plus ALEC) CFU *S. pneumoniae* alone or in combination with ALEC. n=10-53.

4.2.2 Antibody neutralisation of TNFα

Previous investigations of TNFα's role in pneumococcal pneumonia have indicated that this cytokine is required for a protective response (Takashima *et al.* 1997) & (van der Poll *et al.* 1997a). These studies did not identify interactions of TNF with other inflammatory mediators (e.g. IL-10). Furthermore, no previous investigations have been carried out into the ability of local neutralisation within the lungs to alter the response to pneumococcal pneumonia. In the studies reported here mice were treated either systemically or locally with anti-TNFα antibody sampled at 24h post challenge and samples investigated for cytokine levels.

4.2.2.1 Systemic neutralisation

Neutralisation of TNF activity was carried out using a goat polyclonal antibody against mouse TNFα. *In vitro*, 40μg of this antibody were capable of neutralising 80 U TNF activity. This level of TNF activity is equivalent to the total activity (lavage fluid plus lung homogenate and serum) detected at peak levels of TNF activity within the lungs (18h post challenge). 40μg of antibody were thus administered intraperitoneally to mice 2h prior to intranasal infection with 10^6 CFU D39. The effect of this treatment on bacterial loads 24h after bacterial inoculation was then investigated.
In order to determine interactions of TNFα with other inflammatory mediators during pneumococcal pneumonia, levels of IL-10, NO and total protein were also measured within the bronchoalveolar lavage fluid recovered from these mice.

4.2.2.1.1 Effect of systemic TNFα neutralisation on bacteriology

Significantly fewer viable pneumococci were recovered from lung airways ($P<0.05$) and tissues ($P<0.01$) from mice pre-treated with anti-capsule anti serum than with no treatment (Table 4.1). This treatment also prevented development of detectable bacteraemia in all but one mouse ($P<0.01$). Such

	Bacterial Load/ treatment			
	No treatment	Anti-type 2 capsule	Goat IgG	Anti TNFα
Lavage fluid	4.24 +/- 0.16	3.38 +/- 0.12	3.76 +/- 0.21	4.49 +/- 0.35
Lung homogenate	5.42+/-0.18	3.05 +/- 0.11	4.77 +/- 0.20	6.40 +/- 0.35 *
Blood	5.22 +/-0.34	0.48 +/- 0.48	5.51 +/- 0.60	7.47 +/- 0.65

Table 4.1 Effect of systemic administration of neutralising anti-TNFα antibody on bacterial loads within pulmonary airways, lung tissues and circulation 24h post infection with 1.02 x 10^6 CFU *S. pneumoniae*. n= 5-24 mice, *, P<0.01 higher for anti-TNFα treatment when compared to goat IgG treatment.

	Mediator level/ treatment			
	None	Anti-capsule	Goat IgG	Anti-TNFα
TNF activity (U/ml)	9.45 +/- 3.31	6.43 +/- 2.26	6.17 +/- 1.87	5.38 +/- 2.34
IL-10 level (pg/ml)	60.57 +/- 10.88	73.50 +/- 2.42	41.50 +/- 5.97	69.00 +/- 4.11
NO level (μM)	5.69 +/- 0.77	5.22 +/- 0.85	4.17 +/- 1.35	6.33 +/- 1.13
Total protein (μg/ml)	221.72 +/- 28.83	231.14 +/- 11.65	263.60 +/- 47.39	309.69 +/- 54.88

Table 4.2 Levels of inflammatory mediators within bronchoalveolar lavage fluid recovered 24h post challenge with 1.02 x 10^6 CFU *S. pneumoniae* from untreated controls or mice treated intraperitoneally with anti-capsule anti serum, anti-TNFα antibody, or Goat IgG. n=5-24.

findings are in agreement with the type specific protective effects of anti-capsule previously reported (MacLeod *et al.* 1945).
Bacterial loads within lung tissues recovered from Goat IgG treated animals were lower than those normally found with no modulation of the immune response ($P<0.01$). In the setting of this experiment, Goat IgG treatment constitutes a more appropriate control than no treatment at all (in order to control for any non-specific effects of IgG treatment and to account for the treatment procedure itself inducing alterations). Systemic neutralisation of TNF activity results in higher bacterial loads within each site investigated when compared to Goat IgG treated mice and the untreated group. Anti-TNFα treatment resulted in less than 1 log of an increase in viable bacteria associated with pulmonary airways when compared to Goat IgG treated control mice. In lung tissues and bloodstreams TNFα neutralisation lead to an almost 2 log increase in associated bacteria ($P<0.01$ for lung tissues and $P=0.058$ in blood).

4.2.2.1.2 Effect of systemic TNFα neutralisation on the production of inflammatory mediators

Lavage fluid TNF levels in the three treatment groups were not statistically different from those in untreated mice (Table 4.2). Anti-TNFα treatment neutralised 100% of TNF activity within the airways of two mice but high levels of activity were recorded in lavage fluid from the remaining 3 mice.
IL-10 levels were similar in untreated mice or in those mice treated with anti-capsular anti serum or with anti-TNFα. In addition anti-TNFα treatment did not significantly alter NO levels from those in Goat IgG treatment group or untreated animals.
The total protein content of lavage fluid recovered from untreated control animals were similar to those levels detected in anti-capsule anti serum or Goat IgG treated mice. Higher levels were found within the lungs of anti-TNFα treated mice, but this difference was not significant. The highest levels were found in lavage fluids that contained the most TNF activity.
Systemic TNF activity could only be detected in one mouse given anti-TNF antibody and in one mouse given Goat IgG. Both of these Mice had levels of bacteraemia greater than log 7.5 CFU/ml.

4.2.2.2 Effect of local neutralisation of TNFα

After successfully indicating that systemic levels of TNFα do play a role in controlling pneumococcal viability, modulation of pulmonary TNF was attempted.
10μg anti-TNFα antibody were found to neutralise the peak levels of TNFα activity produced normally within the airways following intranasal challenge with 10^6 CFU. Intranasal delivery of this amount of antibody 2h prior to infection with 1 x 10^6 CFU *S. pneumoniae* was thus carried out, with bacterial loads investigated 24h later.
As with systemic modulation, it was of interest to determine levels of other inflammatory mediators during the infection. The aim was to investigate local interactions of TNFα within the lungs.

4.2.2.2.1 Effect of local TNFα neutralisation on bacteriology

In contrast to the ability of systemic anti-capsule antiserum to significantly reduce pneumococcal viability, local administration did not have an effect on bacterial loads in any body compartment when compared to either non-treated or IgG treated animals.
Neutralisation of TNFα within the airways results in significantly higher bacterial loads in both the air spaces and lung tissues when compared to the other three groups (Table 4.3) [$P<0.01$ for air spaces and $P<0.05$ for lung tissues when compared to Goat IgG group and $P<0.01$ for air spaces when compared to no treatment group]. Bacteraemia levels were also higher in anti-TNFα treated mice, although this difference was not significant when compared to goat IgG treated animals.

4.2.2.2.2 Effect of local TNFα neutralisation on the production of inflammatory mediators

TNF activity was undetectable within lavage fluid from all mice treated with anti-capsule anti serum, anti-TNFα antibody or goat IgG (Table 4.4). Comparison of these levels to those associated with the no treatment group did not result in a statistically significant difference due to the variability in TNF activity in the latter group.

	Bacterial Load/ treatment			
	No treatment	Anti-type 2 capsule	Goat IgG	Anti TNFα
Lavage fluid	4.24 +/- 0.16	4.02 +/- 0.24	4.42+/- 0.19	5.74 +/- 0.03 *
Lung homogenate	5.42+/-0.18	5.31 +/- 0.23	5.04 +/- 0.27	6.03 +/- 0.37 +
Blood	5.22 +/-0.34	5.99 +/- 0.60	6.02 +/- 0.37	6.62 +/- 0.45

Table 4.3 Effect of neutralisation of pulmonary airway TNFα activity on bacterial loads within pulmonary airways and tissues and circulation 24h post challenge with 1.01 x 10^6 CFU *S. pneumoniae*. n= 5-24, +, $P<0.05$ and *, $P<0.01$ for anti-TNFα treatment when compared to goat IgG treatment.

	Mediator level/ treatment			
	None	Anti-capsule	Goat IgG	Anti-TNFα
TNF activity (U/ml)	9.45 +/- 3.31	0.00	0.00	0.00
IL-10 level (pg/ml)	60.57 +/- 10.88	61.00+/- 16.27	47.50 +/- 6.96	48.50 +/- 6.59
NO level (μM)	5.69 +/- 0.77	3.89 +/- 0.36	5.28 +/- 1.45	4.67 +/- 1.50
Total protein (μg/ml)	221.72 +/- 28.83	229.57 +/- 15.70	179.08 +/- 20.54	221.86 +/- 44.50

Table 4.4 Levels of inflammatory mediators within bronchoalveolar lavage fluid recovered 24h post challenge with 1.01 x 10^6 CFU *S. pneumoniae* from untreated controls or mice treated locally with anti-capsule anti serum, anti-TNFα antibody, or Goat IgG. n=5-24

IL-10 levels were not significantly different in any of the groups of mice, although lower levels of IL-10 were found in airways of animals treated with either TNFα antibody or goat IgG.
Similar levels of NO and total protein were found in samples recovered from all mice during this experiment with no statistical difference between any treatment groups.
No mice were found to have detectable levels of TNF activity within their circulations.

4.2.3 Pneumococcal pneumonia in TNFα receptor -/- mice

Following demonstration that systemic TNFα neutralisation was capable significantly altering pneumococcal viability following respiratory infection with *S. pneumoniae*, it was decided to investigate TNFα's role further. Utilisation of TNFα receptor deficient mice permitted the effect of an inability to mediate TNFα's functions on overall survival to be determined. Further analysis of TNFα's effects on bacterial loads were possible at multiple time points. These experiments also enabled the roles of TNFα's two receptors to be investigated independently during pneumococcal pneumonia.

4.2.3.1 Signs of illness and survival of TNFα receptor -/- mice

Despite all control mice surviving the infection (Figure 4.5), almost all infected animals displayed clinical signs of illness during the experiment. In the case of the C57Bl/6 controls these were of mild nature, with a slight hunched appearance from around 36h post challenge. By 72h post challenge all mice had returned to normal behaviour.
p75-/- mice also displayed a hunched appearance during the experiment. In this case it occurred at 18-24h into the experiment and the symptoms were gone by 72h post challenge.
In addition to displaying transient signs of infection, all p75-/- and C57Bl/6 mice survived the infection (mean survival time >336h), making them more resistant than MF1 (Figure 3.4) and CBA/Ca mice (Figure 5.1).
p55-/- mice were hunched in appearance from around 24h post challenge. In all but 2 of the p55-/- mice, symptoms were not displayed in the order

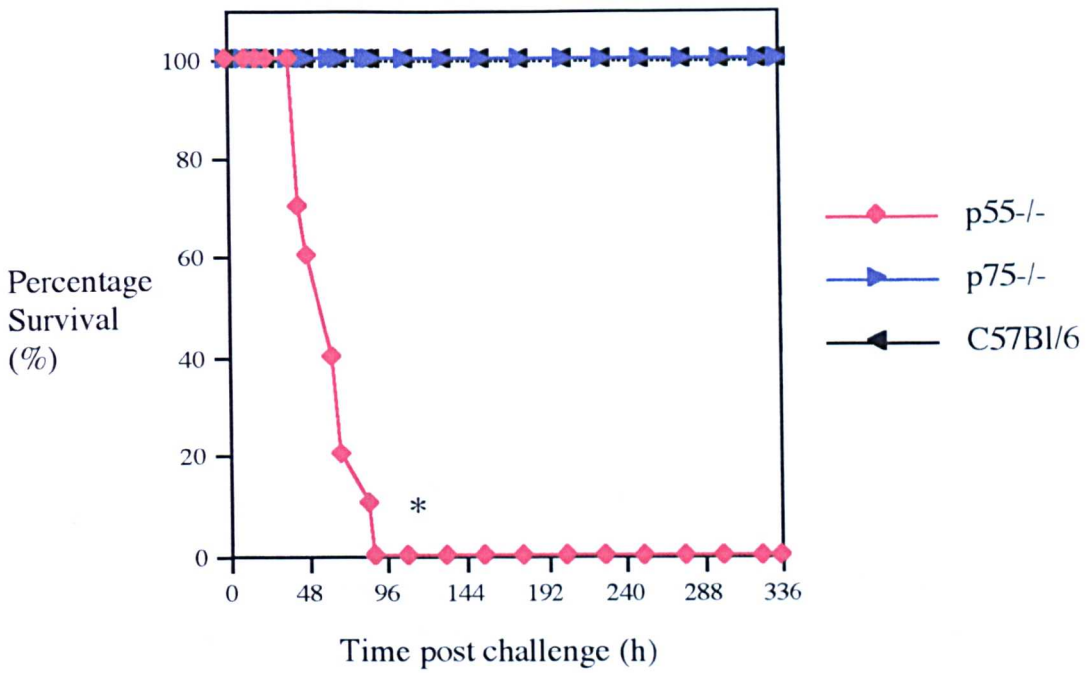

Figure 4.5 Survival of p55-/-, p75-/- or wildtype C57Bl/6 mice following intranasal infection with 1.01 x 10^6 CFU *S. pneumoniae*. n=10, *, P<0.01 shorter survival times for p55-/- when compared to p75-/- and C57Bl/6 mice.

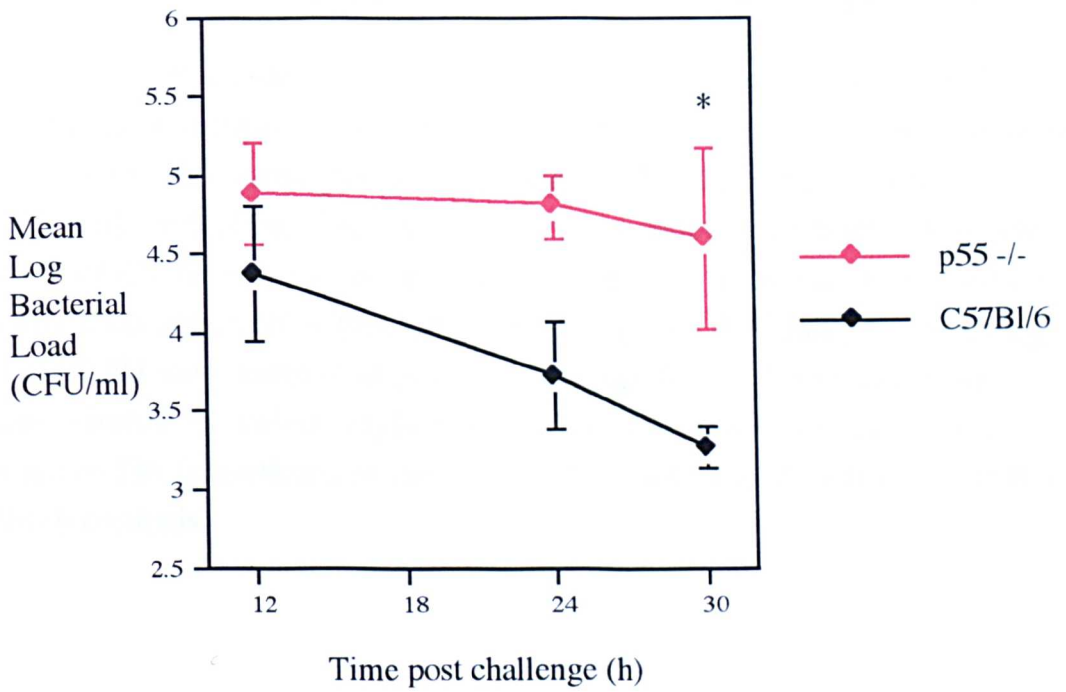

Figure 4.6 Bacterial loads within the lung airways of p55-/- and C57Bl/6 mice following intranasal infection with 1.01 x 10^6 CFU *S. pneumoniae*. n=3-7, *, P=0.01 higher bacterial load in p55-/- airways than in C57Bl/6 airways.

normally seen in susceptible mice, rather mice proceeded from mild signs of illness to being moribund in a matter of approximately 6h. There was no evidence of piloerection or lethargy in these animals.
p55-/- deficient mice were susceptible to the infection, with 100% mortality by 89h into the experiment (median survival time 63h). Statistical comparison of survival times of each group of mice revealed that p55-/- mice survived for significantly shorter periods following intranasal infection with 10^6 CFU *S. pneumoniae* than did both p75-/- and C57Bl/6 mice ($P< 0.01$).

4.2.3.2 Bacteriology in TNFα receptor -/- mice

As p75-/- mice were no more susceptible to pneumococcal pneumonia than C57Bl/6 controls, further experiments were mainly carried out in p55-/- and C57Bl/6 mice.
Bacterial loads were only investigated in p75-/- mice at 24h post challenge. At this time bacterial loads in lavage fluid (4.35+/- 0.22 CFU/ml), lung tissues (3.15 +/- 0.22 CFU/ml) or blood (0.66 +/- 0.43 CFU/ml) from p75-/- mice were no different from those in C57Bl/6 mice 24h into the experiment.

4.2.3.2.1 Bacteriology in pulmonary air spaces of TNFα receptor -/- mice

The numbers of viable pneumococci within the lung airways of p55-/- mice did not alter significantly during the infection (Figure 4.6). Approximately 10^5 CFU were recovered per ml of lavage fluid at each time point.
During early infection there were similar numbers of bacteria within the airways of C57Bl/6 mice as in p55-/- mice. A decrease in pneumococcal viability then occurred within C57Bl/6 airways so that 30h post challenge just over 10^3 CFU were recovered per ml of lavage fluid. Comparison of the two mouse strains revealed higher numbers of pneumococci within p55-/- airways at 24h (significant at the level of P=0.026) and at 30h (P=0.01) than in C57Bl/6 controls.

4.2.3.2.2 Bacteriology in lung tissues of TNFα receptor -/- mice

At 12h post challenge both strains of mice have similar numbers of pneumococci associated with their lung tissues (Figure 4.7). The numbers then increased slowly within p55-/- lungs during the next 12h, with a rapid increase in recoverable pneumococci occurring during the final 6h studied. By 30h post challenge a statistically significant increase in pneumococcal viability over that at 12h was found ($P<0.01$).
Pneumococcal viability in C57Bl/6 lungs did not alter between the 12h and 24h time points. A rapid increase in their number did occur during the final 6h with a statistically significant increase compared to levels at 12h by 30h in the experiment ($P<0.01$). Significantly lower numbers of viable pneumococci were still found associated with these lung tissues than in p55-/- mice ($P<0.05$ at 24h and $P<0.01$ at 30h).

4.2.3.2.3 Systemic bacteriology in TNFα receptor -/- mice

At 12h post challenge the two strains of mice had similarly low levels of pneumococci in their bloodstreams (Figure 4.8). Numbers of viable bacteria then increased rapidly in p55-/- mice until the end of the experiment when a significantly higher number of pneumococci were cultured in the samples ($P<0.01$). In C57Bl/6 mice, pneumococci were controlled during the next 12h with recoverable numbers not increasing until after the 24h time point ($P<0.01$ at 30h).
From 18h onwards significantly higher numbers of pneumococci were cultured from p55- blood than from C57Bl/6 samples ($P<0.01$)

4.2.3.3 Production of inflammatory mediators within airways of TNFα receptor -/- mice

Production of inflammatory markers was investigated at 24h post challenge. This single time point was selected as the low numbers of samples currently recovered at the other times would result in ineffective statistical comparisons.
At this time TNF activity could not be detected within lavage fluid from C57Bl/6 or p55-/- mice (Table 4.5). At 24h post challenge lavage fluids from 3

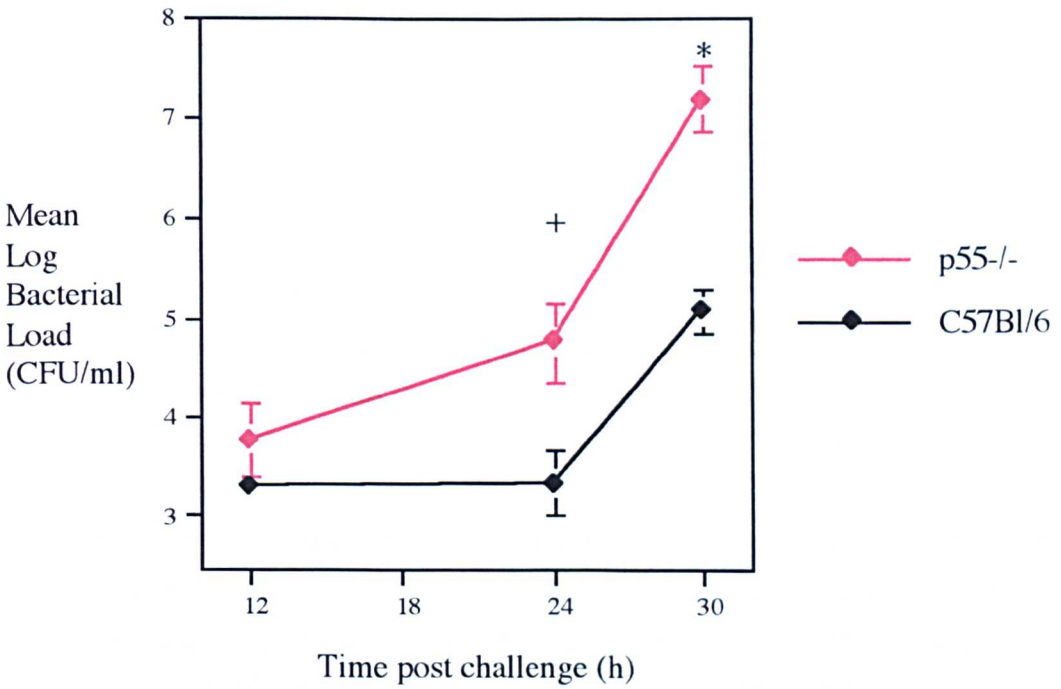

Figure 4.7 Bacterial loads within the lung tissues of p55-/- and C57Bl/6 mice following intranasal infection with 1.01 x 10^6 CFU *S. pneumoniae*. n=3-6, +, $P<0.05$ and *, $P<0.01$ higher for p55-/- lungs than for C57Bl/6 samples.

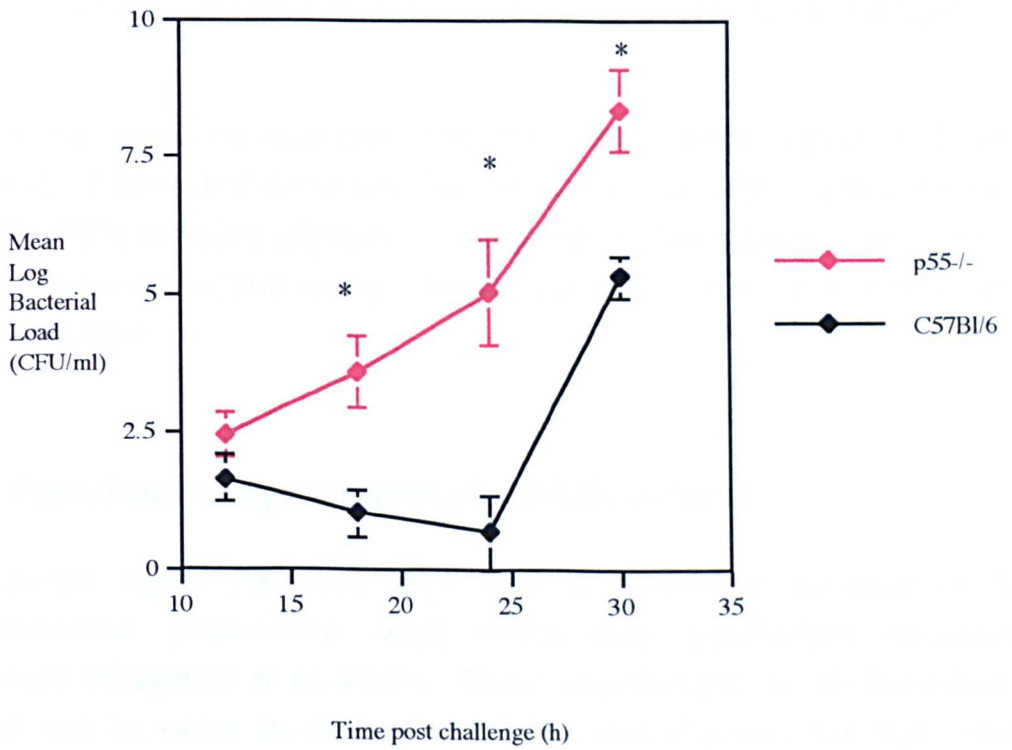

Figure 4.8 Bacterial loads within the blood of p55-/- and C57Bl/6 mice following intranasal infection with 1.01 x 10^6 CFU *S. pneumoniae*. n=4-18, *, $P<0.01$ higher for p55-/- samples than for C57Bl/6.

out of the 5 p75-/- mice possessed detectable TNF activity but these results were not significantly different from the other groups although they do agree with the p75 receptor acting as a TNF scavenger (Peschon *et al.* 1998) during pneumococcal pneumonia.

Similar levels of IL-10 were found in p55-/- and p75-/- mice. Control C57Bl/6 samples only contained around a third of this amount, but this difference was not significant.

NO production was significantly lower in both TNF receptor deficient mouse strains during mid infection when compared to C57Bl/6 controls ($P<0.05$). The lowest levels were found in p55-/- mice with slightly higher levels in p75-/-.

Similar levels of disruption to the alveolar/ capillary barrier occurred to in both p75-/- and C57Bl/6 mice. These mice had around double the amount of protein associated with their lavage fluids than did p55-/- mice. The difference between p55-/- and p75-/- mice was not significant but comparison of protein levels in p55-/- lavage fluids with those from C57Bl/6 mice revealed that mice with the ability to signal through the p55 TNF receptor had significantly more pulmonary disruption during the infection ($P<0.05$).

Inflammation appeared to be contained within the lungs as no TNF activity could be detected within serum from any mice during the experiment.

During pneumococcal pneumonia in p55-/- mice there appears to be slightly higher IL-10 levels but decreased NO and lung integrity. Signalling through the p55 TNFα receptor appears to upregulate inflammation within the lungs and mediates disruption to the alveolar/capillary barrier permitting influx of serum proteins.

4.2.4 Pneumococcal pneumonia in iNOS -/- mice

In a recent report NO was implicated in monocyte recruitment during pneumococcal pneumonia with overzealous production involved in pathology (Bergeron *et al.* 1998). These experiments to be described were carried out in order to determine if this was the case for this model of pneumococcal pneumonia, and to also investigate interactions of NO with other inflammatory mediators. Mice unable to produce NO via the

Lavage fluid marker	Mouse Strain		
	p55-/-	p75-/-	C57Bl/6
TNF activity (U/ml)	0.00 U/ml	4.98 U/ml +/- 3.64	0.00 U/ml
NO level (μM)	2.72 μM +/- 0.56 +	4.00 μM +/- 0.53 +	5.39 μM+/- 0.20
IL-10 level (pg/ml)	36.00 pg/ml +/- 9.35	34.80 pg/ml +/- 16.42	12.60 pg/ml +/- 6.63
Total protein content (μg/ml)	128.28 μg/ml +/- 32.83 +	278.71 μg/ml +/- 56.80	247.57 μg/ml +/- 19.31

Table 4.5 Levels of inflammatory markers within the lavage fluid from TNFα receptor deficient or control mice at 24h post intranasal challenge with 1.01 x 10^6 CFU *S. pneumoniae*. n=5, +, P<0.05 lower for p55-/- mice than for p75-/- and C57Bl/6 mice.

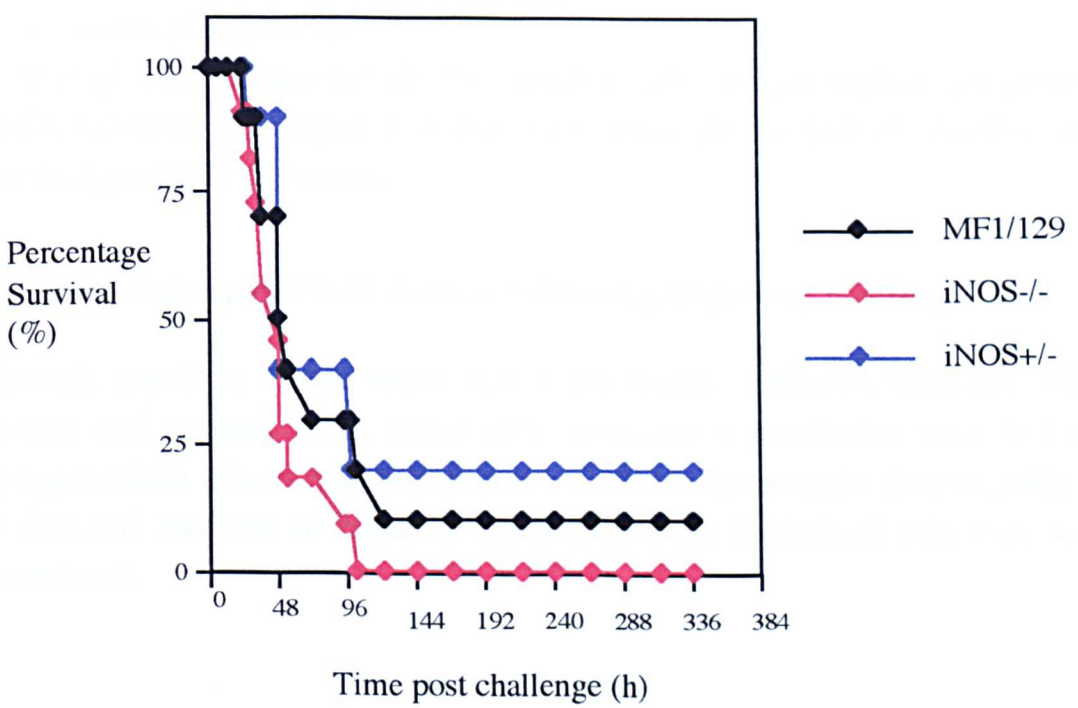

Figure 4.9 Survival of iNOS-/-, iNOS +/- and wildtype MF1/129 mice following intranasal challenge with 1.05 x 10^6 CFU *S. pneumoniae*. n=9-10.

inducible nitric oxide synthase pathway were infected and their response characterised. The role of NO in Gram-positive septic shock was also investigated.

4.2.4.1 Signs of illness and survival of iNOS -/- mice following intranasal challenge

Development of symptoms in control MF1/129 mice was highly variable. At the same time as 1 mouse was moribund (24h post challenge) 2 other mice that did succumb to the infection later were still completely normal. Additionally, the speed that MF1/129 mice passed through the range of clinical indications was also not uniform.

Symptoms in the heterozygous iNOS+/- group tended to occur by 24h post challenge with a hunched stance being the most common. In general these mice passed slowly through the range of symptoms.

iNOS-/- mice displayed piloerection from 24h into the experiment, although at this time one mouse had already succumbed to the illness. iNOS-/- mice developed the most severe signs of illness of the three mouse strains. This occurred shortly after intranasal infection with all iNOS-/- mice succumbing to the illness by 101h.

Although there appeared to be a shift to lower survival times for iNOS-/- mice, statistical analysis revealed no significant difference between any of the mouse strains (Figure 4.9).

As iNOS+/- mice displayed similar survival times and symptom progression to MF1/129 mice, subsequent studies were only performed on iNOS-/- and the wild type MF1/129 strain

4.2.4.2 Bacteriology in iNOS -/- mice following intranasal challenge

Although survival times were not significantly different between iNOS deficient and normal mice, there still remained a possibility that NO was playing a subtle role in host defence. In order to investigate that hypothesis, the size and location of bacterial loads following intranasal infection were determined.

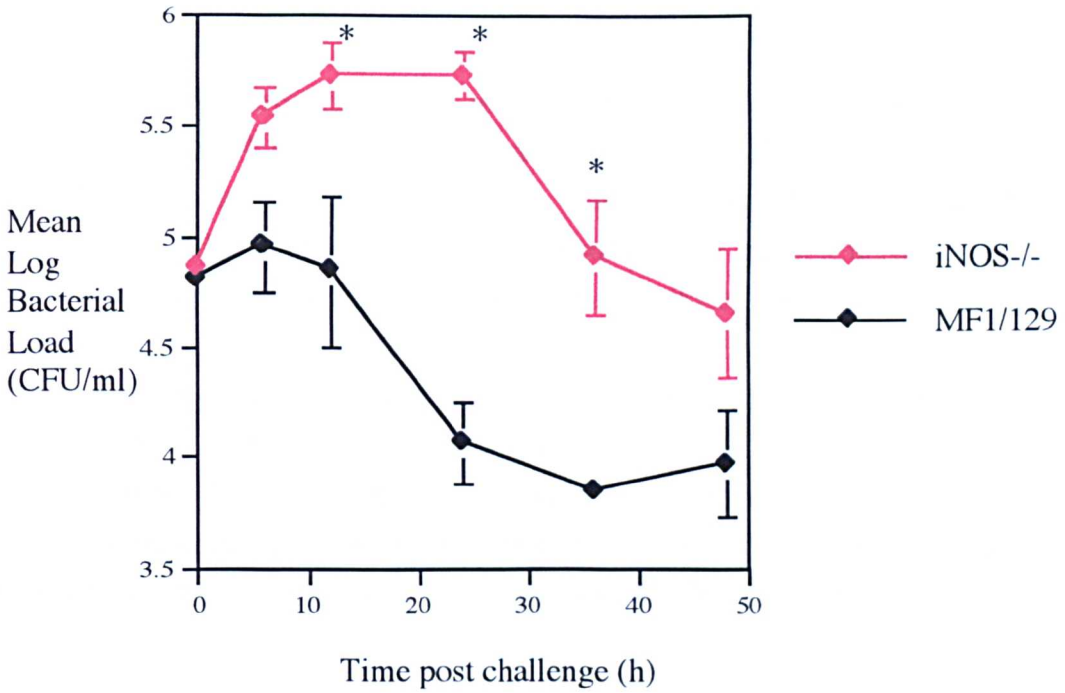

Figure 4.10 Bacterial loads within the airways of iNOS-/- and control MF1/129 mice following intranasal infection with 1.03 x 10^6 CFU *S. pneumoniae.* n= 5-10, *, P<0.01 higher for iNOS-/- than for MF1/129 samples.

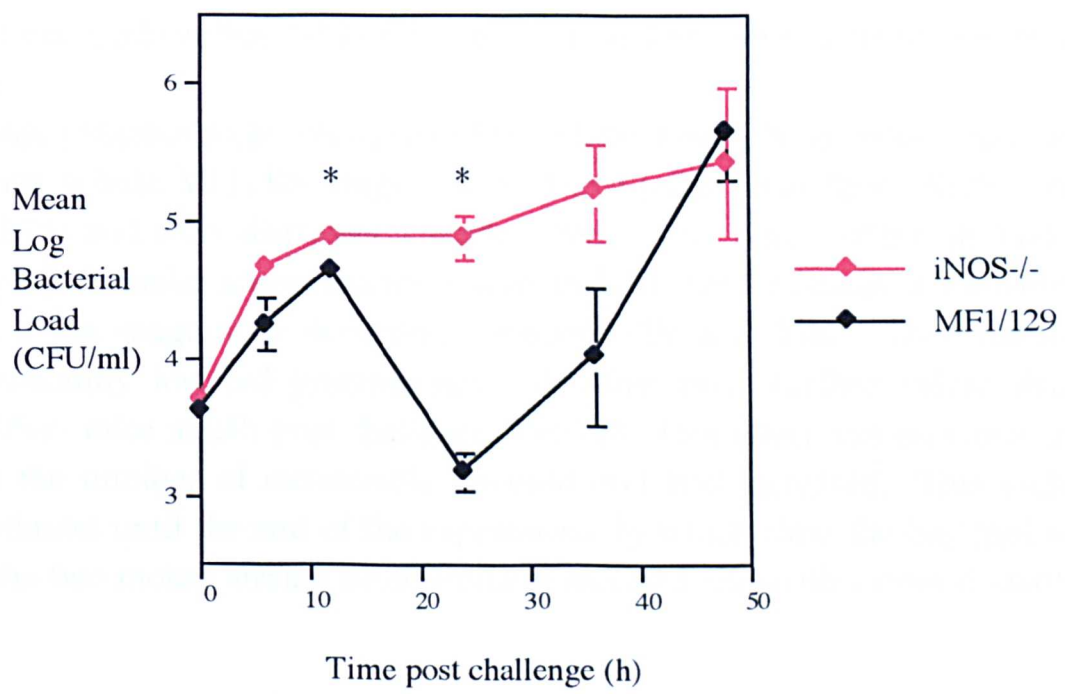

Figure 4.11 Bacterial loads within the lung tissues of iNOS-/- and control MF1/129 mice following intranasal infection with 1.03 x 10^6 CFU *S. pneumoniae.* n= 5-10, *, P<0.01 higher for iNOS-/- than for MF1/129 samples.

4.2.4.2.1 Bacteriology in the pulmonary air spaces of iNOS -/- mice

Viability of pneumococci within the airways of iNOS-/- mice increased immediately following infection with around a 1 log increase by 12h post challenge (Figure 4.10). This trend was not evident in samples from MF1/129 mice. Indeed, following 12h into the experiment MF1/129 mice rapidly reduced the bacterial counts by around 1 log per ml lavage fluid, with levels remaining constant until the end of the experiment.
A similar, but delayed reduction in bacterial viability was observed within airways of iNOS-/- mice, reducing viability to a level comparable with that detected immediately following infection.
From 12h into the experiment, the enhanced ability of normal mice to control pneumococcal viability resulted in significantly fewer pneumococci cultured from their lungs than from lungs of iNOS-/- mice ($P<0.01$).

4.2.4.2.2 Bacteriology in lung tissues of iNOS -/- mice

Bacterial viability within the lung tissues of both iNOS-/- and MF1/129 mice increased initially during the experiment (Figure 4.11). Similar levels were found in the two mouse strains at 6h and 12h post challenge although iNOS -/- lung tissues contained slightly higher counts. Following 12h, viable bacterial loads within iNOS-/- lungs continued to increase, albeit at a reduced rate.
Whilst pneumococcal counts in iNOS-/- lung tissues were increasing, viable counts within MF1/129 lungs increased to a lower level than iNOS-/- mice ($P<0.01$) and then displayed a marked reduction. The decline in viability displayed similar kinetics to those seen in MF1/129 air spaces, a reduction of 1 1/2 log magnitude occurring between 12h and 24h. This reduction significantly lowered pneumococcal viability even further below that in iNOS-/- mice at 24h post challenge ($P<0.01$). This effect was transient as by 24h the number of recoverable pneumococci had increased. This increase continued until the end of the experiment, by which time the bacterial loads in the two mouse strains were similarly elevated above 0h values ($P<0.01$).

Time post challenge (h)	Mean Log Bacterial Load (CFU/ml)	
	MF1/129	iNOS-/-
0	ND	ND
6	ND	ND
12	2.27 +/- 0.21	2.42 +/- 0.27
24	3.85 +/ 0.55	3.91 +/- 0.50
36	4.77 +/- 0.95	5.64 +/- 0.60
48	4.82 +/- 0.81	6.79 +/- 0.64

Table 4.6 Bacterial loads within the bloodstreams of iNOS-/- and control MF1/129 mice following intranasal infection with 1.03 x 10^6 CFU *S. pneumoniae*. n= 5-10. ND= below detection limit of viable count assay (<log 1.92 CFU/ml).

4.2.4.2.3 Systemic bacteriology in iNOS -/- mice

No blood-borne bacteria were found until 12h post challenge (Table 4.6). At this time 7 iNOS-/- mice and 3 MF1/129 had developed bacteraemia. Kinetics of increasing bacterial viability within the bloodstream's of iNOS-/- mice are similar to those in MF1/129 mice. Blood culture counts continued to increase almost unchecked until the end of the infection in iNOS-/- mice so that they were almost a 2 log higher than in MF1/129 mice.

4.2.4.3 Production of inflammatory mediators by iNOS -/- mice during pneumococcal pneumonia

The ability of NO to interact with several other inflammatory mediators has been widely studied (Chapter 1). In order to characterise such interactions during this infection, TNF activity and IL-10 levels were measured in iNOS-/- mice.

Despite the inability to release high levels of NO via the Ca_2^+ independent pathway, iNOS-/- mice retain the ability to release lower levels via the cNOS pathway. The possibility that this occurred during pneumococcal pneumonia was investigated by measuring levels of NO released in the airways of both iNOS deficient and sufficient strains of mice.

NO is known to effect the integrity of endothelial barriers (Beckman *et al.* 1990). In order to determine whether this occurred to the alveolar/capillary barrier during pneumococcal pneumonia, total protein levels within the airways of mice unable to produce high levels of this mediator were recorded and compared to NO sufficient controls.

4.2.4.3.1 Production of inflammatory mediators in lungs of iNOS -/- mice during pneumococcal pneumonia

TNF activity was undetectable within lavage fluid from all mice during early infection (Figure 4.12 A). 12h post challenge similar levels of activity were found in iNOS-/- and MF1/129 mice. Throughout the remainder of the experiment, no TNF activity was detected within MF1/129 samples. iNOS-/- samples contained high levels of TNF activity at both 24h and 36h post challenge which were significantly higher than those in MF1/129 samples

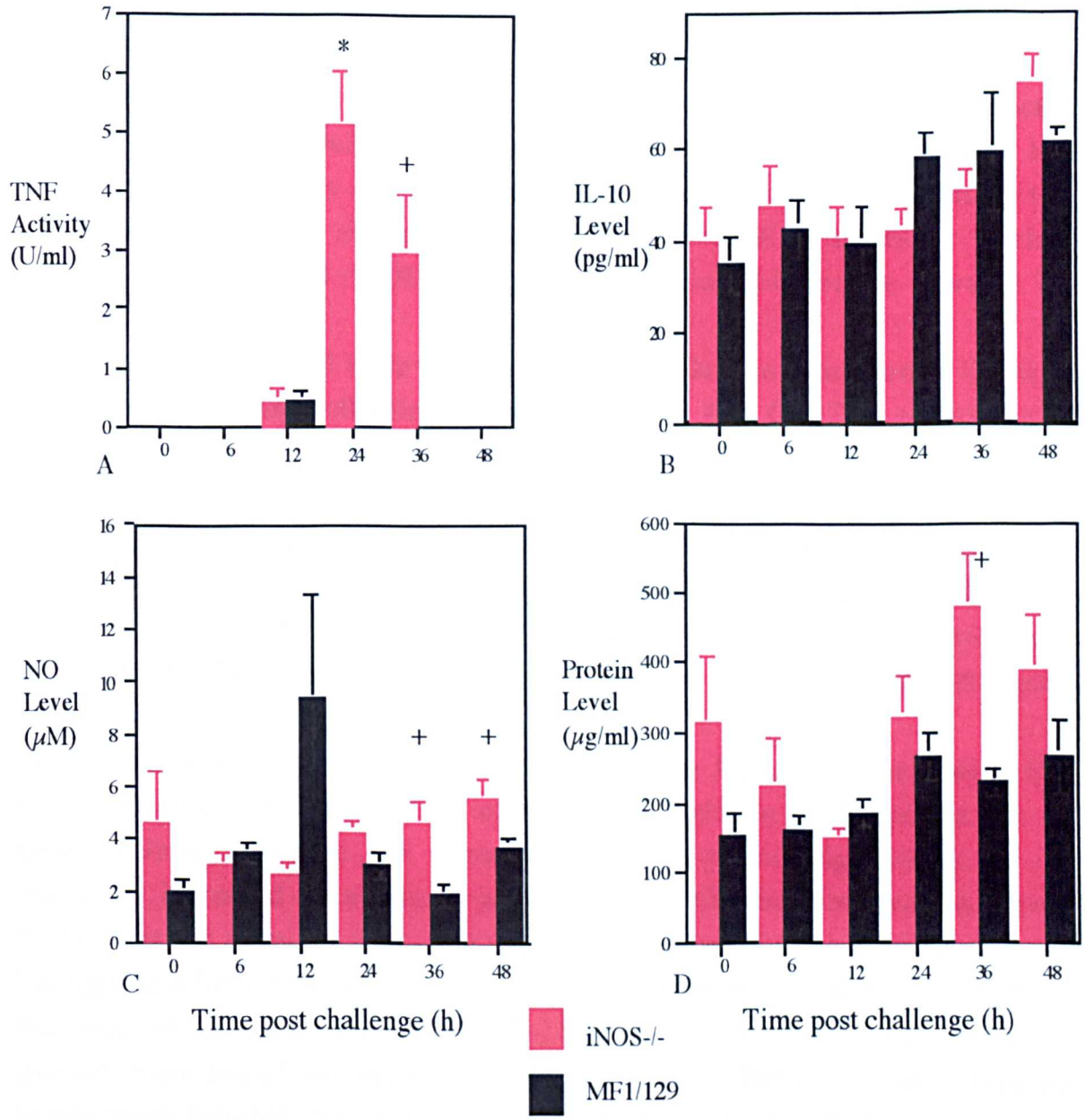

Figure 4.12 Levels of inflammatory mediators within bronchoalveolar lavage fluid from iNOS-/- and MF1/129 mice following intranasal infection with 1.03 x 10^6 CFU *S. pneumoniae*. A= TNF activity, B= IL-10 levels, C= NO levels, and D= total protein content. n=5-10, *, $P<0.01$, $^+$ $P<0.05$ higher for iNOS-/- when compared to MF1/129 results.

($P<0.01$ at 24h and $P<0.05$ at 36h). By 48h such activity had returned to undetectable levels.

The inability to synthesise NO via the iNOS pathway did not reduce the capacity to release IL-10 (Figure 4.12 B). Similar levels of IL-10 were detected within the lavage fluid from iNOS-/- and MF1/129 mice during the infection. Both strains displayed a significant increase in IL-10 levels by the end of the experiment ($P<0.01$ for MF1/129 and $P<0.05$ for iNOS-/-). Within iNOS-/- mice, elevated cNOS activity may be responsible for the elevated IL-10 production.

Very high levels of NO were detected within the lavage fluid from 1 iNOS-/- mouse immediately following infection (Figure 4.12 C). The remaining 4 mice had similar levels to MF1/129 controls. NO production within the airways of iNOS-/- mice increased slowly during the infection to peak at 5.5 μM 48h post challenge. Levels of NO within iNOS-/- mice were significantly higher than those in MF1/129 airways at 36h post challenge ($P<0.05$). The high variation in MF1/129 NO levels at 12h post challenge resulted in a insignificant difference between the two mouse strains at this time post challenge.

Low levels of NO were found in lavage fluid from MF1/129 mice during the first 6h of infection. Production increased markedly in 4/10 mice at 12h to peak at 9μM, although the variation did not permit significance to be reached. Increased production was transient with baseline values again being detected from 12h post challenge until the end of the experiment.

Lavage fluid from 2/5 iNOS-/- mice contained high levels of total protein at the time of infection (Figure 4.12 D). During the next 12h, low levels of protein were found associated with the airways of iNOS-/- mice. Higher levels were detected during the final 24h of the experiment indicating that disruption to the alveolar/capillary barrier occurred around 24h into the experiment despite the absence of NO.

Samples from MF1/129 mice contained similar levels of protein during the initial 12h of the infection. From 24h post challenge, protein contents did increase in MF1/129 mice, but not to the same extent as they did in iNOS-/- mice. At 36h post challenge lavage fluid from iNOS-/- mice contained significantly more protein than samples from MF1/129 mice ($P<0.05$).

These results show that airways of iNOS-/- mice have a greater inflammatory stimulus due to elevated bacterial loads. This leads to significantly more TNF, NO and total protein within pulmonary air spaces

and perhaps the more severe signs of illness displayed by these animals during infection.

Investigation of TNF activity within the lung tissues of mice sampled at the time point of 24h post challenge revealed that mice unable to produce NO via the iNOS pathway had lower levels in comparison to MF1/129 mice (Table 4.7). Due to the small group size (n=5) this difference is not significant.
At 24h post challenge iNOS-/- mice released slightly higher (but not significantly so) levels of this anti-inflammatory cytokine than did tissues from MF1/129 mice.
Systemic TNF activity was found in only 3 mice in these studies. One iNOS-/- and one MF1/129 serum sample at 24h and one MF1/129 sample at 36h.

4.2.4.4 Systemic pneumococcal infection in iNOS-/- mice

There was no difference in levels of bacteraemia within iNOS-/- and MF1/129 mice following intranasal infection with *S. pneumoniae* (Table 4.6). This suggests that any difference in bacterial survival within NO sufficient and NO deficient infected animals is solely mediated by the lungs. In order to investigate this hypothesis, groups of both strains of mice were infected intravenously. If the hypothesis is correct, pneumococci would be equally able to proliferate in the bloodstreams of iNOS-/- and MF1/129 mice.

4.2.4.4.1 Signs of illness and survival following systemic pneumococcal infection in iNOS-/- mice

All of the mice displayed symptoms of illness during the experiment. Development of symptoms in MF1/129 mice was variable with 2 mice still displaying normal appearance at 30h post challenge whilst another had already succumbed to the infection. Once signs of illness became apparent, mice rapidly developed full blown disease with 100% mortality by 72h post challenge.
All iNOS-/- mice displayed clinical signs of illness by 30h post challenge. At this time these were mild in nature with only a slightly hunched stance evident. 2 mice progressed to succumb to the bacterial challenge whilst the

Mouse strain	Cytokine Level	
	TNF (U/ml)	IL-10 (pg/ml)
MF1/129	53.84 +/-16.56	471.00 +/- 23.18
iNOS-/-	17.87 +/- 5.61	588.50 +/- 45.54

Table 4.7 TNF and IL-10 levels within the lung tissues of MF1/129 and iNOS-/- mice 24h post intranasal infection with 1.03×10^6 CFU *S. pneumoniae*. n=5.

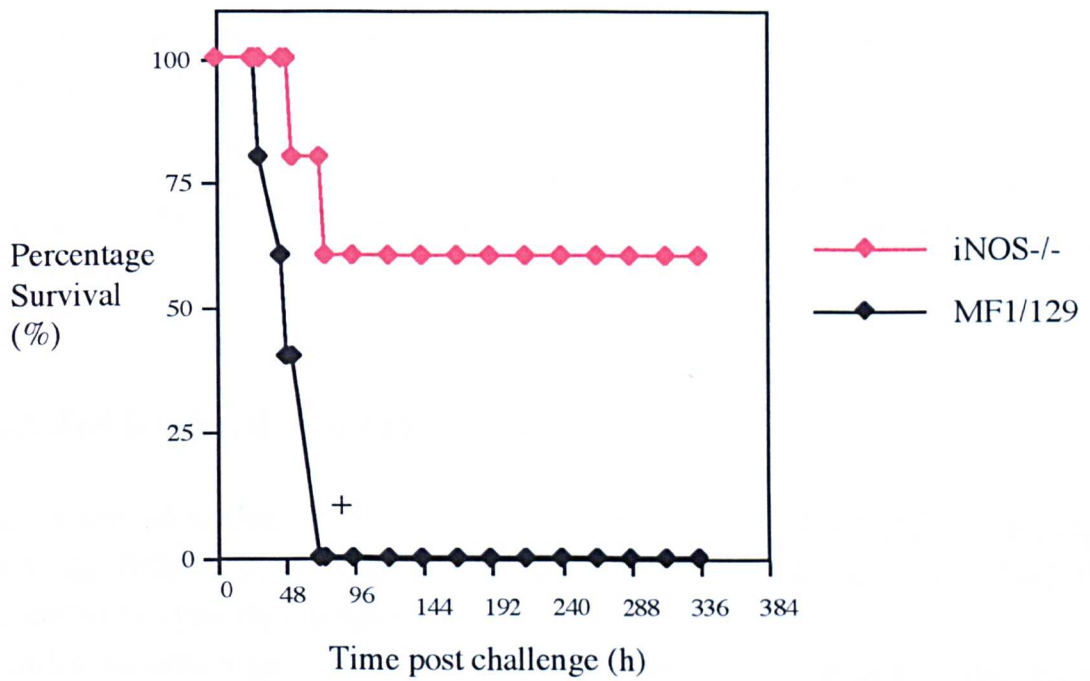

Figure 4.13 Survival of iNOS-/- and MF1/129 mice following intravenous infection with 7×10^4 CFU *S. pneumoniae*. n=5 group, +, $P<0.05$ shorter survival times for MF1/129 mice than for iNOS-/- mice.

remaining 3 were again normal by 72h post challenge and remained so until the end of the experiment.
Statistical analysis revealed that the survival times of MF1/129 mice were significantly lower than those of iNOS-/- mice ($P<0.05$ [Figure 4.13]).

4.2.4.4.2 Bacteriology following systemic pneumococcal infection in iNOS-/- mice

Similar numbers of pneumococci were recovered from the circulation immediately following intravenous infection (Figure 4.14). During the following 24h this number increased slowly in MF1/129 mice with greater than 10^5 CFU found in each ml of blood by 24h post challenge. iNOS-/- mice were better able to control the inoculated bacteria, with numbers of viable pneumococci actually decreasing slightly in the 12h following challenge to a level significantly lower than in MF1/129 mice ($P<0.01$). No further significant alteration in pneumococcal viability was detected in the final 12h of the experiment.

The survival data and bacteriology indicate that the ability to produce high output NO via the iNOS pathway is detrimental during Gram-positive septic shock.

4.2.5 Antibacterial effects of NO *in vitro*

The enhanced ability of NO sufficient mice to control bacterial loads within the lungs following intranasal infection with *S. pneumoniae* indicated that NO could be directly bactericidal for pneumococci.
In order to investigate this, a NO donor (SNAP) was added to the growth media of *S. pneumoniae* cultures. SNAP has previously been employed to prove the direct effects of NO on other micro-organisms, for example (Kaplan *et al.* 1996). The ability of NO to prevent or delay pneumococcal proliferation was therefore investigated.

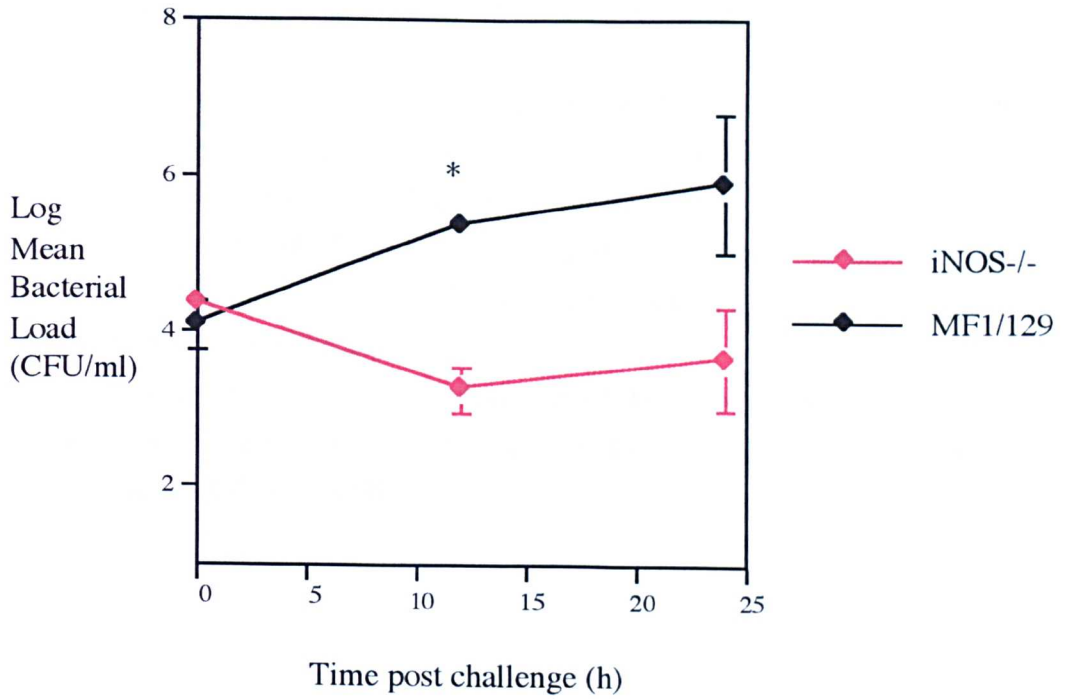

Figure 4.14 Bacteraemia in iNOS-/- and MF1/129 following intravenous infection with 7.00×10^4 CFU *S. pneumoniae*. n=5, *,P<0.01 higher for MF1/129 when compared to iNOS-/-.

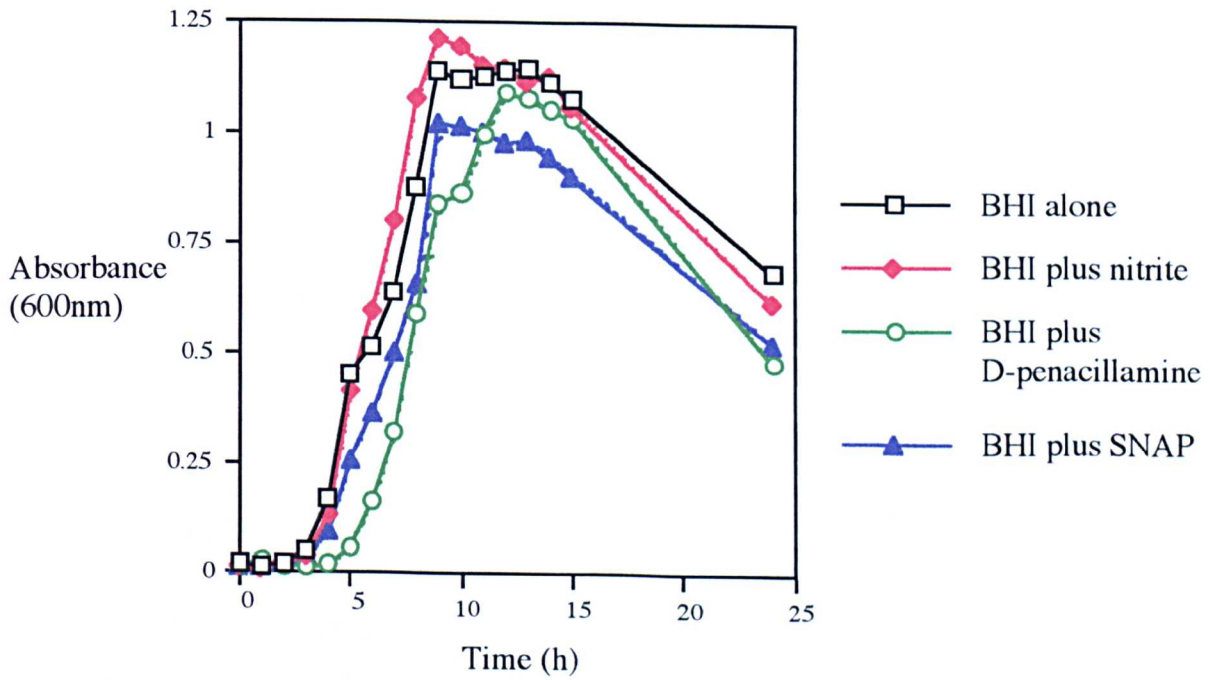

Figure 4.15 Effect of SNAP on growth of *S. pneumoniae* in BHI n= 3 cultures/ group. Each point represents the mean, for simplicity SEM are left off.

4.2.5.1 Effect of SNAP on growth of *S. pneumoniae*

Incubation of *S. pneumoniae* in BHI plus 200μM of the NO donor SNAP has no effect on the ability of bacteria to proliferate (as revealed by viable counts) [Figure 4.15]. The peak absorbance in this group was slightly lower but this difference was not significant. The negative control, D-penicillamine, did delay the initiation of growth slightly but the overall growth of *S. pneumoniae* was unaffected.

When statistical analysis was carried out there were no significant differences in pneumococcal growth between any of the preparations either by optical density or by viability counts.

4.3 SUMMARY

The effects of surfactant, the ablation of TNF receptors, or of the enzyme, iNOS, all on the induction of pneumococcal pneumonia in mice are reported in this chapter.

Surfactant phospholipids were capable of reducing pneumococcal viability *in vivo* if administered at the same time as the bacteria. This did not result in an alteration in survival times.

The effect of ALEC on pneumococci was not direct cytotoxicity as viability of the organism was unaffected by incubation in the surfactant. In addition, growth of D39 was not altered in BHI and 5% ALEC, although viability at the end of growth was.

TNFα was shown to be an important mediator of host defence in the setting of pneumococcal pneumonia. Neutralisation of TNFα, either systemically or locally, significantly altered bacterial loads in the lung airways and tissues. Bacteraemia was also elevated in mice depleted in systemic TNFα.

Signalling through the p55 TNFα receptor was found to be important for controlling bacterial levels and promoting survival during pneumococcal pneumonia.

In addition to the beneficial effects of TNFα, signalling through the p55 receptor was involved in the disruption to lung integrity and development of clinical symptoms during the infection.

Nitric oxide was not found to be directly bactericidal for pneumococci *in vitro*, nor absolutely required for survival during pneumococcal pneumonia. It was found to control bacterial viability within the lungs. Mice unable to produce NO via the iNOS pathway were found to have higher numbers of viable pneumococci within their pulmonary airways and lung tissues.

Production of NO by the iNOS pathway was associated with reduced airway TNFα levels and lowered disruption to the alveolar/capillary barrier although IL-10 production was unaffected.

During systemic infection the relationship was reversed. Mice unable to utilise the iNOS pathway were significantly less susceptible to pneumococcal infection. This indicates that production of high levels of NO during Gram-positive sepsis is detrimental.

Chapter 5

Host Response in Susceptible/resistant Mouse Strains

5.1 INTRODUCTION

As mentioned in Chapter 1 (Section 1.4.3), host genetic factors are known to affect the outcome during several infectious diseases and are believed to play a role during pneumococcal pneumonia. At present the nature and effects of these factors is not understood.

In order to better understand the host response to pneumococcal infection, a detailed comparison of mouse strains was undertaken in this laboratory. Initial studies (Gingles *et al.* manuscript in preparation) identified BALB/c and CBA/Ca mouse strains as being resistant and susceptible, respectively, to intranasal infection with *S. pneumoniae.*

Further experiments were undertaken to compare the nature of the inflammatory response in these two mouse strains. The aim was to identify differences in the inflammatory response mounted by CBA/Ca and BALB/c mice that could account for the outcomes of infection.

Such link between the production of cytokines and outcome of disease has been found in several other infection models. In a pulmonary model of *Cryptococcus neoformans* infection, resistance correlates with elevated IFNγ and IL-2 secretion (Hoag *et al.* 1995). During experimental leishmaniasis or *Listeria monocytogenes* infection, insufficient production of TNFα is associated with death. High levels of TNFα have been correlated with severe complications and death during malaria (Garcia *et al.* 1995). TNFα also plays a role in determining resistance of mouse strains to pulmonary *Pseudomonas aeruginosa* infection (Gosselin *et al.* 1995). Finally, individuals with a low TNFα or high IL-10 response (as determined by *ex-vivo* endotoxin stimulation of whole-blood) are more likely to die from an episode of meningococcal meningitis than are patients with a lower anti-inflammatory response (Westendorp *et al.* 1997).

5.2 RESULTS

5.2.1 Intranasal Infection

5.2.1.1 Signs of illness following intranasal infection of BALB/c or CBA/Ca mice

Following intranasal infection with 10^6 CFU *S. pneumoniae,* significantly different signs of illness and survival times were displayed by CBA/Ca and BALB/c mice (Figure 5.1). These results confirm the initial observations from this laboratory (Gingles *et al.* Manuscript in Preparation). 4h into the experiment all but one CBA/Ca mouse (out of 96 studied) displayed signs of illness. The most common clinical symptoms at this time were a hunched stance and a degree of piloerection, although 10 of the mice had already passed through these signs of illness to become lethargic. Over the next 12h all mice became so ill they were deemed moribund. The median survival time for infected CBA/Ca mice was 36h post challenge. During time course experiments it was necessary to finish experiments at 36h post challenge in order to gather a full data set.

In comparison, although 24 of the 166 BALB/c mice used in these studies displayed some symptoms of infection, these tended to be of a milder nature with hunched appearance and slight piloerection. Such signs of illness were mainly recorded between 24h and 72h post challenge. Due to the nature of these experiments, with each mouse sacrificed prior to sampling, it could not be determined whether symptoms were transient in nature. Only 4 of the 166 BALB/c mice passed through lethargy to become moribund by 144h. The remaining BALB/c mice were deemed to have survived the experiment and were sacrificed at 336h post challenge. The median survival time for the resistant mice was greater than 336h.

Comparison of the survival times for both strains of mice revealed that those for non-implanted CBA/Ca were significantly lower than those displayed by non-implanted BALB/c mice during pneumococcal pneumonia ($P<0.01$).

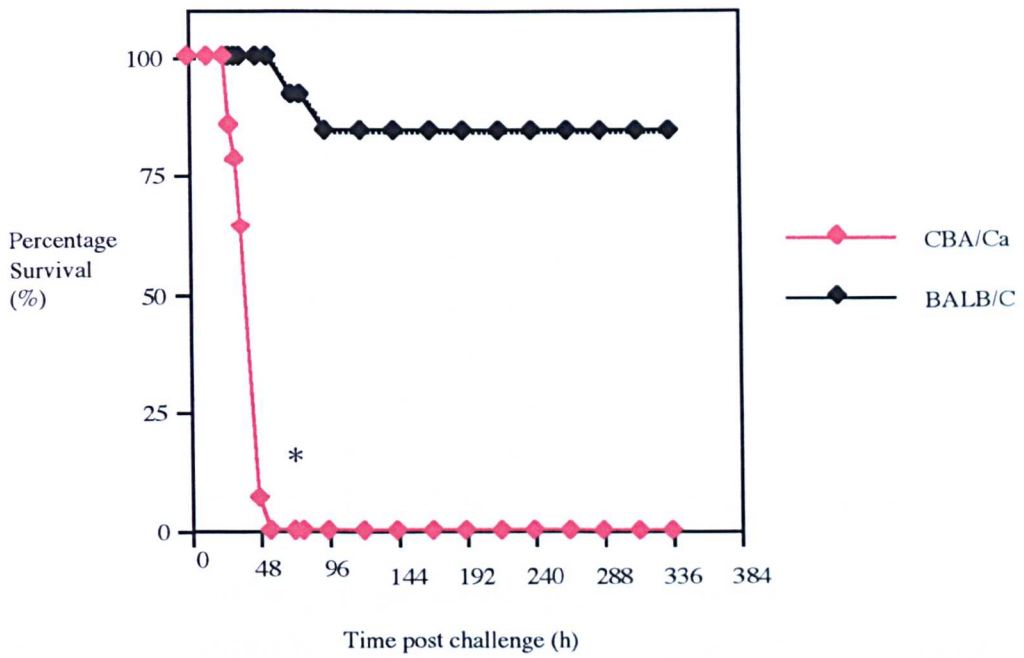

Figure 5.1 Survival of CBA/C and BALB/c mice following intranasal infection with either 9.75 x 10^5 (BALB/c mice) or 9.82 x 10^5 (CBA/Ca mice) CFU *S. pneumoniae*. n= 14 for CBA/Ca and n= 11 for BALB/c, *, P< 0.01 shorter for CBA/Ca survival times compared to BALB/c.

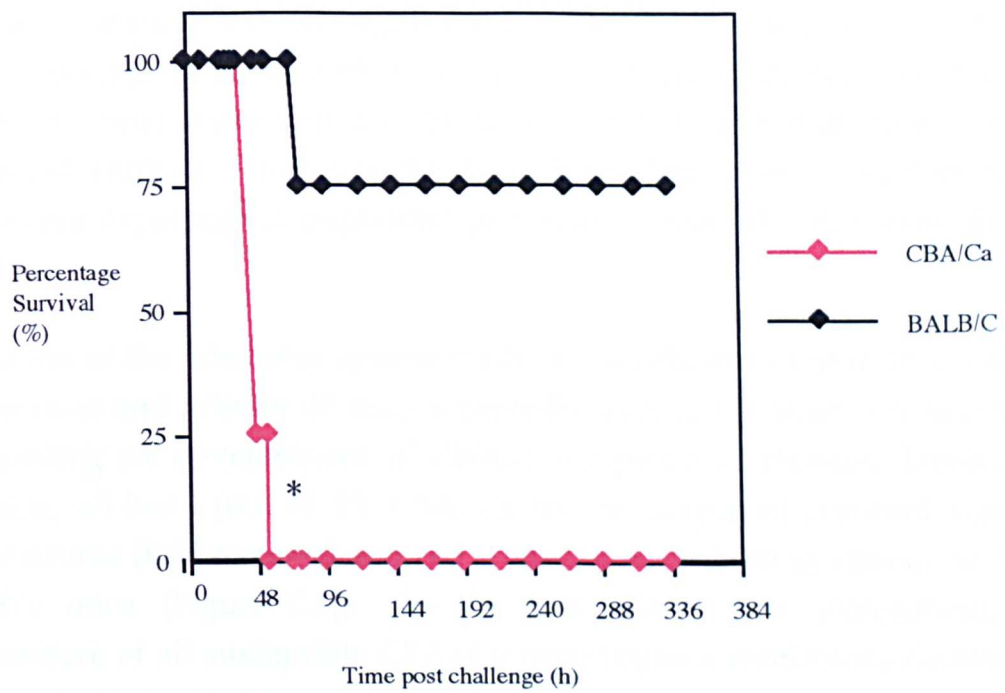

Figure 5.2 Survival of implanted CBA/C and BALB/c mice following intranasal infection with 1.01 x 10^6 (BALB/c mice) or 9.95 x 10^5 (CBA/Ca mice) CFU *S.pneumoniae*. n=8, *, P<0.01 shorter survival times for CBA/Ca mice compared to BALB/c.

5.2.1.2 Telemetry following intranasal infection of BALB/c or CBA/Ca mice

In order to better define the nature of symptoms in the two strains we used a system of telemetry implants. The effect of chip implantation on the response of the mice was first determined.
As with non implanted animals, implanted CBA/Ca mice displayed clinical symptoms earlier than did BALB/c mice following intranasal infection with *S. pneumoniae*. By 24h post challenge all CBA/Ca mice were hunched with 4/8 displaying piloerection. Signs of illness did not progress from this stage until after 32h into the experiment. The mice then rapidly passed through the range of symptoms so that there was only one survivor 48h post challenge.
Two implanted BALB/c mice succumbed to the infection. These mice displayed mild symptoms from mid infection (32h & 52h post challenge) and survived until 79h post challenge. The remaining BALB/c animals showed no signs of illness and survived the experiment.
Survival times displayed by implanted CBA/Ca mice were again significantly shorter than BALB/c counterparts ($P<0.01$).

Chip implantation did not significantly alter the survival time of BALB/c mice following infection with *S. pneumoniae* (Figure 5.2), but survival times of CBA/Ca mice were found to be significantly longer than those of non-implanted animals. However the overall trend was the same, therefore in subsequent experiments implanted and non-implanted mice were grouped together.

Utilisation of the telemetry system made it possible to monitor the core body temperature and activity of mice repeatedly during the infection in addition to recording the development of clinical symptoms of disease. During early infection, all but 1 (out of 15) CBA/Ca mouse displayed constant core body temperatures (this mouse displayed transient hypothermia similar to that in BALB/c mice [Figure 5.4]). In the final 7h of the experiment, body temperature of all susceptible CBA/Ca mice began a continuous decline until ambient room temperature was reached (Figure 5.3).
36/43 resistant BALB/c mice monitored for longer than 24h developed transient hypothermia during early infection [around 20h post challenge] (Figure 5.4). In 18 of the mice hypothermia was seen as a single episode but

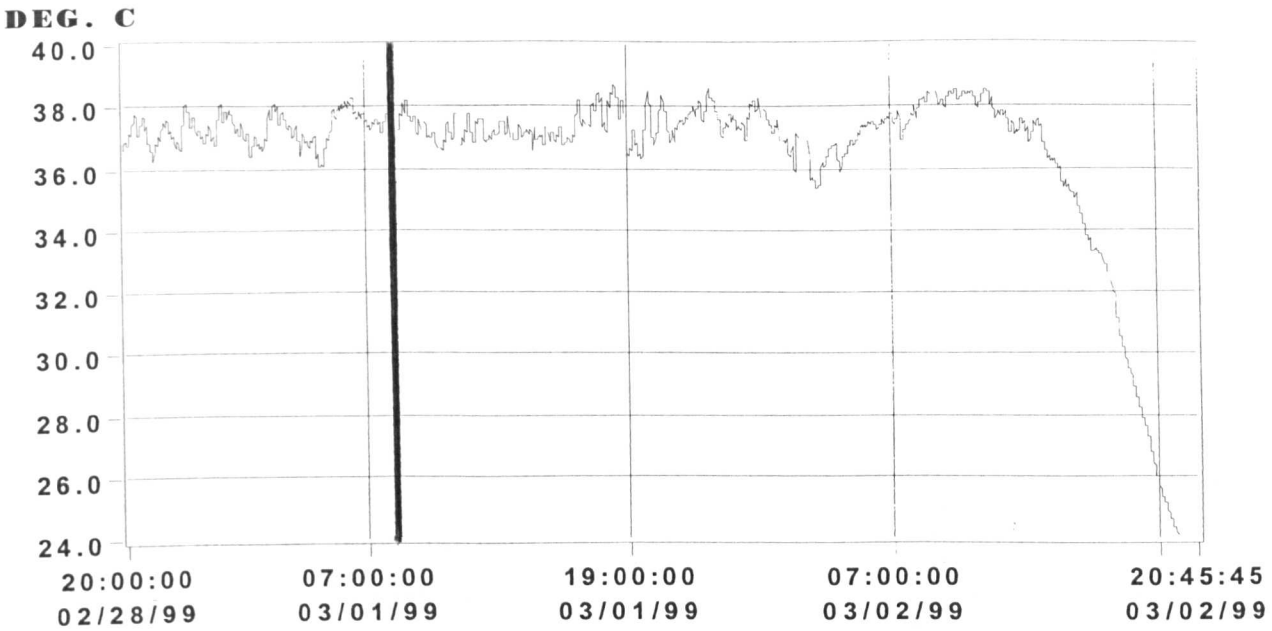

Figure 5.3 Core body temperature (°C) of a CBA/Ca mouse infected with 10^6 CFU *S. pneumoniae*. Vertical line represents time of infection.

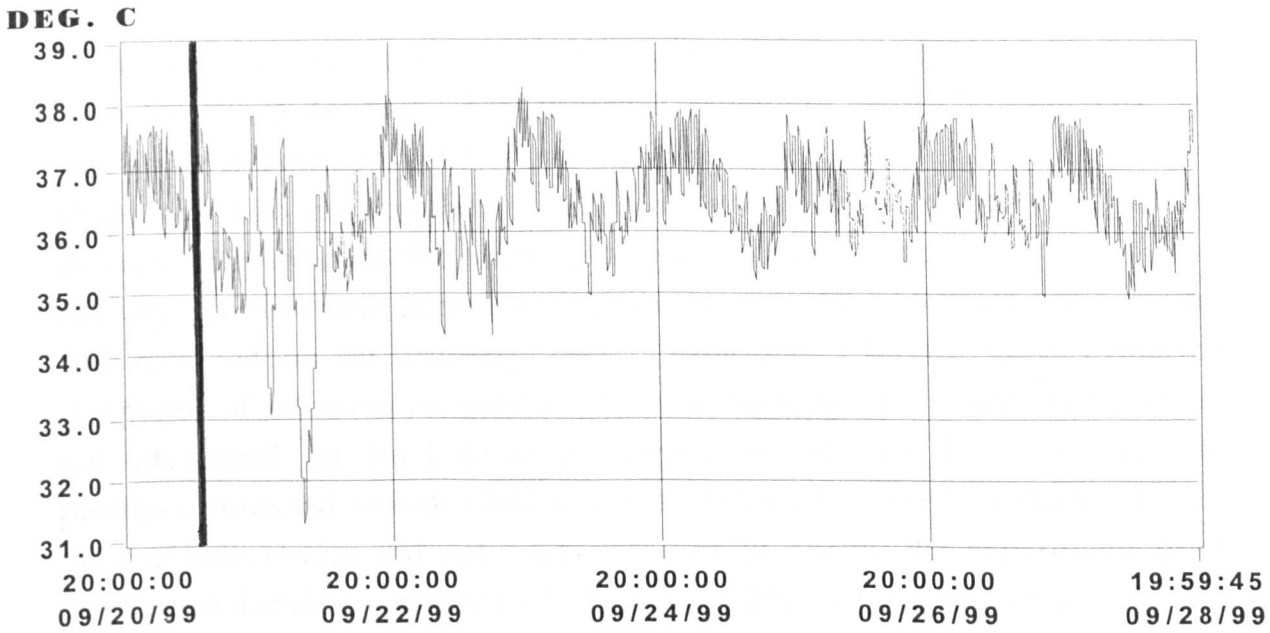

Figure 5.4 Core body temperature (°C) of a BALB/C mouse infected with 10^6 CFU *S. pneumoniae*. Vertical line represents time of infection.

in the other 18 there was one period, recovery and constant Tco for 1-2h and then a second transient period of hypothermia.

During these periods of hypothermia (second drop if double episode), core body temperature dropped to a mean of 31.5 °C for approximately 5h, a significant drop in Tco ($P<0.01$). A further 8-12h were required before T_{co} finally returned to baseline, where it remained until the end of the experiment. The earlier period of hypothermia in double episode was less marked (approximately 4°C) and lasted around 1h.

Periods of hypothermia appeared to occur as the normal circadian rhythm of the mice was on a downwards trend. The subsequent increase in temperature also corresponded in time frame to a shift in circadian rhythm towards elevated temperature.

Two implanted BALB/c mice succumbed to pneumococcal pneumonia. These mice still displayed the transient hypothermia during early infection but also displayed the constant decline in Tco seen in CBA/Ca mice at the end of the experiment

Core body temperatures of PBS sham infected mice of both backgrounds did not display hypothermia although a cyclical alteration in core body temperature was evident (data not shown) as seen with MF1 mice (Figure 3.5).

There was no correlation of hypothermia to signs of illness as only four animals with a decreased Tco displayed symptoms whilst 2/7 animals not displaying hypothermia still showed clinical symptoms.

There was also no correlation between hypothermia and survival as the animals with constant Tco went on to survive the experiment.

Activity profiles were determined concurrently with core body temperature and recorded on an arbitrary scale. Although CBA/Ca mice displayed symptoms of infection as early as 24h post challenge, their activity score did not alter until the final 2h of the experiment (Figure 5.5). The activity profiles of infected resistant BALB/c mice (Figure 5.6) and PBS sham infected mice of both strains did not alter significantly during the experiment (data not shown for sham infected BALB/c and CBA/Ca mice but similar to that for sham infected MF1 mice Figure 3.6). In addition the activity counts displayed for the representative mice show that the BALB/c animal was generally more active than its CBA/Ca counterpart throughout the experiment.

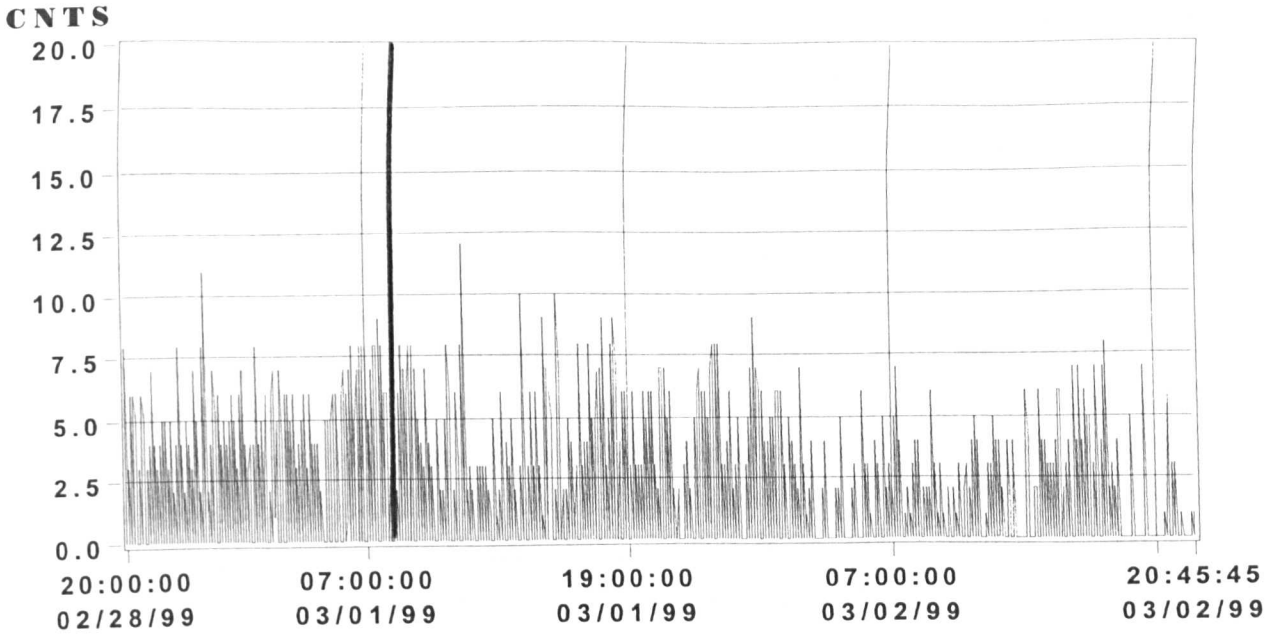

Figure 5.5 Activity score (arbitrary units) of a CBA/Ca mouse infected with 10^6 CFU *S. pneumoniae*. Vertical line represents time of infection.

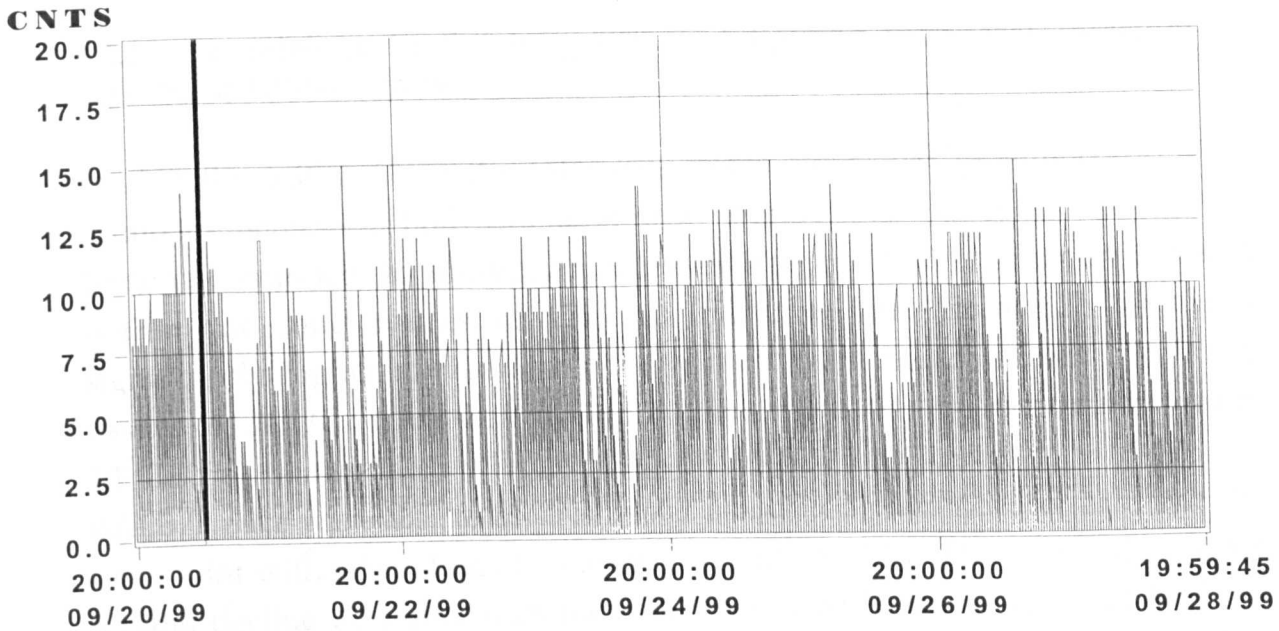

Figure 5.6 Activity score (arbitrary units) of a BALB/C mouse infected with 10^6 CFU *S. pneumoniae*. Vertical line represents time of infection.

5.2.1.3 Bacteriology in the pulmonary air spaces following intranasal infection of BALB/c or CBA/Ca mice

Inoculation of 10^6 CFU of pneumococci into the nose resulted in approximately 10^5 CFU being recovered immediately in the airways of each animal (Figure 5.7). Although higher numbers of viable pneumococci were initially recovered in CBA/Ca mice than in the BALB/c mice ($P<0.01$), by 6h into the experiment this relationship had reversed with significantly more bacteria being found within airways of BALB/c mice ($P<0.01$). Following the 12h time point, the number of viable bacteria in both strains began a decline that was sustained until the end of the experiment with BALB/c air spaces containing significantly fewer pneumococci from 24h onwards ($P<0.01$). In CBA/Ca mice this reduction was constant but in BALB/c mice there were two stages. Between 12h and 48h there is a sharp decline in the viability of pneumococci with the number of viable bacteria recovered reduced from approximately 10^6 CFU/lung to around 10^1 CFU/lung. This was followed by a more gradual reduction in bacterial viability so that by 336h post challenge no viable bacteria could be detected within the airways.

5.2.1.4 Bacteriology in the lung tissues following intranasal infection of BALB/c or CBA/Ca mice

During the initial 6h of the infection, tissue associated pneumococci were found to increase in both strains of mice (Figure 5.8). After this point, the bacteria continued to multiply unchecked (mean doubling time of 3.00h) within the lung tissues of CBA/Ca mice so that from 12h onwards significantly higher numbers of pneumococci were associated with the lung tissues of CBA/Ca mice than in BALB/c mice ($P<0.01$) By the time of death 10^7 CFU were associated with each ml of CBA/Ca lung tissue.
BALB/c mice were able to control tissue associated pneumococci after the 6h time point with a levelling off of bacterial loads by 12h. This was followed by a rapid decline in viable bacteria which occurred between 12h and 36h. A slower, but steady reduction in bacterial viability resulted in around 10^2 CFU being recovered at 2wks into the experiment.

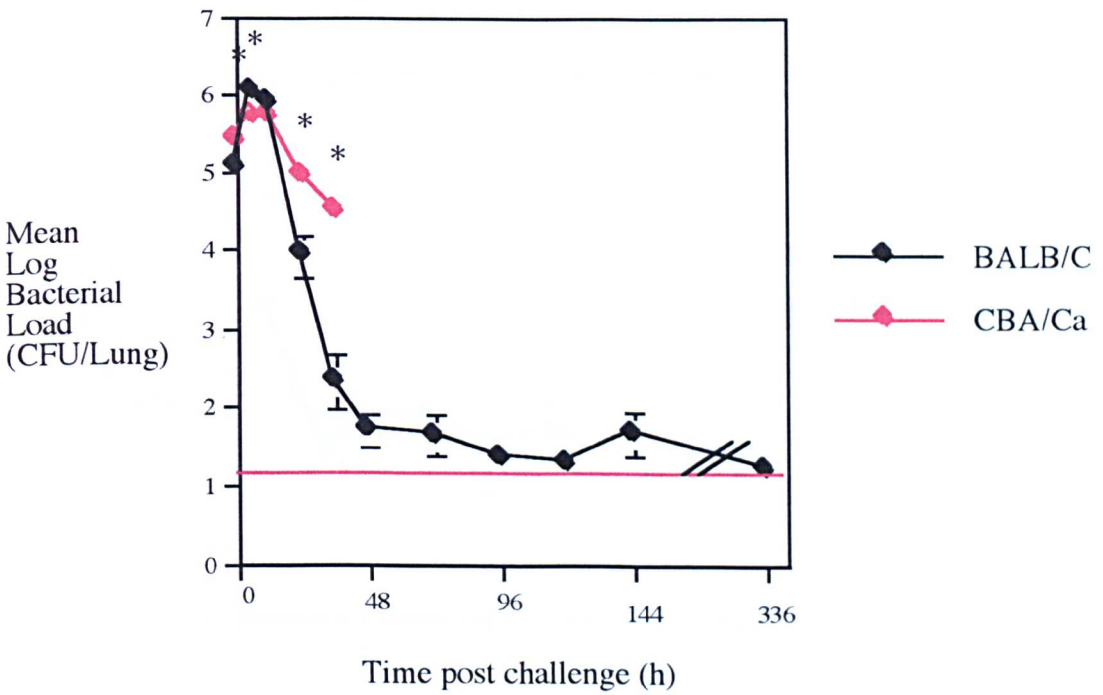

Figure 5.7 Bacterial loads within lavage fluids from BALB/c and CBA/Ca mice during infection with 9.91×10^5 (BALB/c mice) or 9.02×10^5 (CBA/Ca mice) CFU *S. pneumoniae*. n= 9-11, *, $P<0.01$ different for CBA/Ca and BALB/c samples. Red horizontal line represents detection limit of BALB/c viable count assay. No results for CBA/Ca mice after 36h post challenge as there were no survivors in this group past this time.

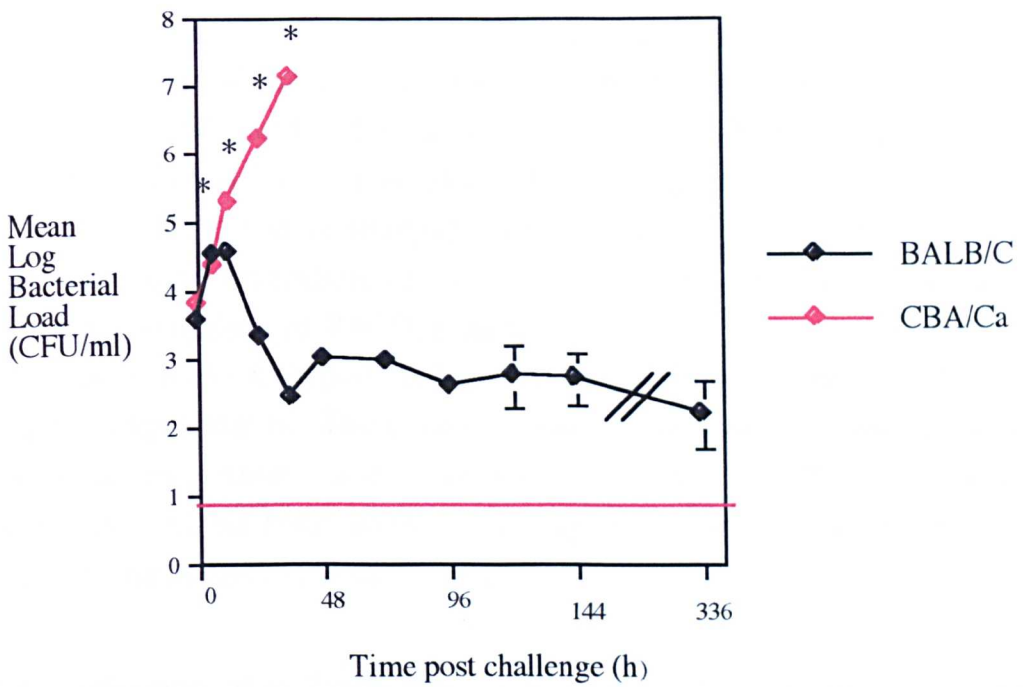

Figure 5.8 Bacterial loads within lung homogenates from BALB/c and CBA/Ca mice during infection with 9.91 x 10^5 (BALB/c mice) or 9.02 x 10^5 (CBA/Ca mice) CFU *S. pneumoniae*. n=5, *, P<0.01 higher for CBA/Ca when compared to CBA/Ca. Red horizontal line represents detection limit of viable count assay. No results for CBA/Ca mice after 36h post challenge as there were no survivors in this group past this time.

5.2.1.5 Systemic bacteriology following intranasal infection of BALB/c or CBA/Ca mice

By culturing blood samples recovered from the same mice during the experiment it was possible to investigate the development of bacteraemia in both resistant and susceptible mice during pneumococcal pneumonia (Table 5.1).
5 out of 12 CBA/Ca mice developed detectable bacteraemia by the first time point investigated (6h after intranasal challenge), and all had by 12h ($P<0.01$). Once in the bloodstream the bacteria multiplied exponentially (mean doubling time 0.7h) until the end of the experiment when greater than 10^7 CFU were recovered per ml of blood ($P<0.01$ higher than time 0h counts at both 24h and 36h post challenge). From 12h onwards CBA/Ca mice had significantly higher numbers of viable pneumococci within their circulation than in the circulation of BALB/c mice ($P<0.01$).
Pneumococci were recorded within the circulation of only 5 BALB/c mice during the experiments. The earliest cases of bacteraemia were discovered at 12h into the experiment and after 96h no further BALB/c mice developed bacteraemia. At no time were there significant numbers of pneumococci within the circulation of BALB/c mice.

5.2.1.6 Production of inflammatory mediators following intranasal infection of BALB/c or CBA/Ca mice

The same range of pro- and anti- inflammatory mediators was investigated within the lungs and circulation of BALB/c and CBA/Ca mice as were studied in MF1 mice (Section 3.2.3.9). An imbalance in the pro-inflammatory/ anti-inflammatory cytokine relationship was hypothesised to explain the different outcomes to infection evident in this chapter.

5.2.1.6.1 Production of inflammatory mediators in pulmonary air spaces following intranasal infection of BALB/c or CBA/Ca mice

TNF activities increased within the airways of both BALB/c and CBA/Ca mice during pneumococcal pneumonia (Figure 5.9 A). The peak of activity in both mouse strains occurred at 6h post challenge with 75U of TNF activity detected per lung in BALB/c mice but a significantly lower 5U/lung in CBA/Ca mice ($P<0.01$). The activity level declined in both strains post 6h

Time post challenge (h)	Mean Log Bacterial Load (CFU/ml)	
	BALB/c mice	CBA/Ca mice
0	ND	ND
6	ND	2.00 +/- 0.06
12	2.06 +/- 0.11	5.07 +/- 0.18 *
24	ND	6.14 +/- 0.42 *
36	ND	7.79 +/- 0.49 *
48	2.11 +/- 0.20	NS
72	2.16 +/- 0.20	NS
96	2.12 +/- 0.21	NS
120	ND	NS
144	ND	NS
336	ND	NS

Table 5.1 Bacterial loads within bloodstreams of BALB/c and CBA/Ca mice during infection with 9.91 x 10^5 (BALB/c mice) or 9.02 x 10^5 (CBA/Ca mice) CFU *S. pneumoniae*. n= 10-13 mice/group, *, P<0.01 higher for CBA/Ca samples than for BALB/C samples. ND= below detection limit of viable count assay (< log 1.92), NS=No survivors in this group at these times.

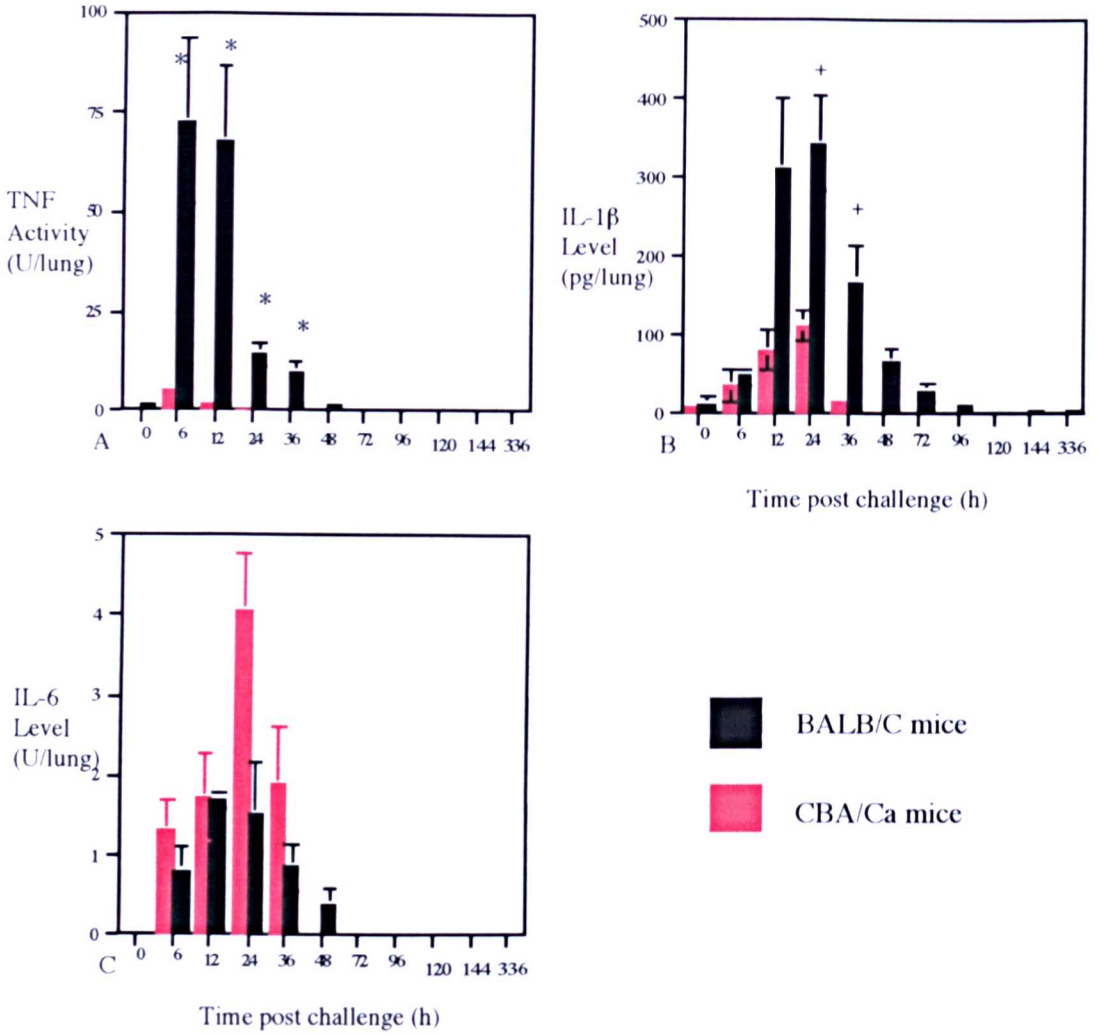

Figure 5.9 Production of pro-inflammatory mediators within the pulmonary airways of BALB/c and CBA/Ca mice following intranasal infection with 9.91 x 10^5 (BALB/c mice) or 9.02 x 10^5 (CBA/Ca mice) CFU *S. pneumoniae*. A= TNF activity n=10, B= IL-1β level n=4-6, and C= IL-6 levels n=5. Values are corrected for volume differences and expressed per lung. *, $P<0.01$, $^+$, $P<0.05$ higher for BALB/c values when compared to CBA/Ca values. No results for CBA/Ca mice after 36h post challenge as there were no survivors in this group past this time.

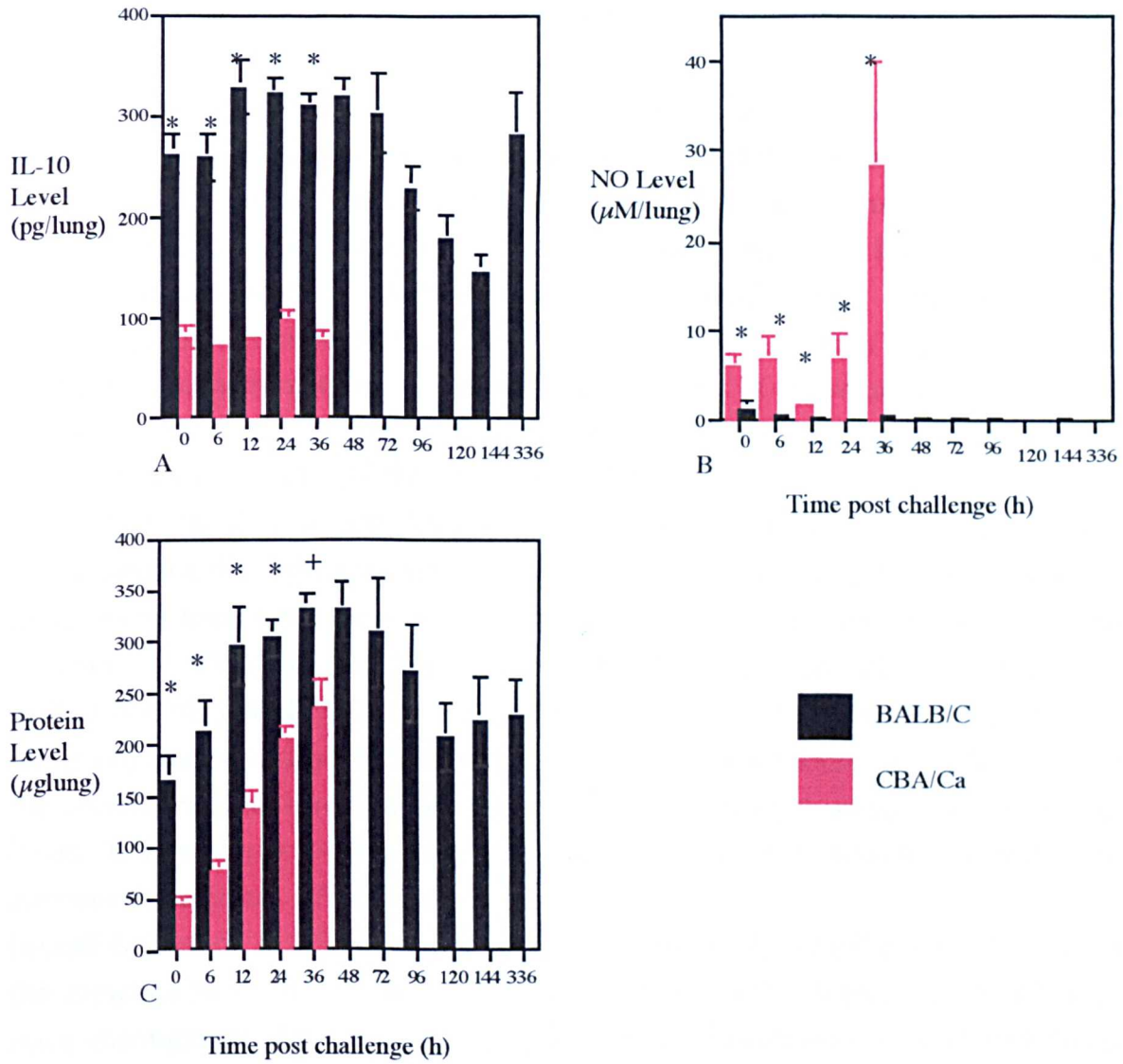

Figure 5.10 Production of inflammatory mediators within the pulmonary airways of BALB/c and CBA/Ca mice following intranasal with 9.91 x 10^5 (BALB/c mice) or 9.02 x 10^5 (CBA/Ca mice) CFU *S. pneumoniae*. A= IL-10 levels n=9-10, B= NO levels n=8-11, and C= total protein levels n=10. Values are corrected for volume differences and expressed per lung. *, $P<0.01$, +, $P<0.05$ for BALB/c values when compared to CBA/Ca values. No results for CBA/Ca mice after 36h post challenge as there were no survivors in this group past this time.

and by 36h TNF activity was no longer detectable in the airways of CBA/Ca. Significantly elevated TNF activity could not be detected in BALB/c mice after 24h post challenge.

IL-1β protein production within the airways was investigated by ELISA (Figure 5.9 B). IL-1β is up-regulated with similar kinetics in both strains of mice with the highest levels also detected at the same time post challenge (24h). This increased production occurred later than the increase in TNF activity (Figure 5.9A). The levels of IL-1β detected at this time in BALB/c mice are more than three times that seen in CBA/Ca mice. Following this peak, IL-1β concentrations declined until the end of the experiment in both groups of mice and by 36h they had returned to baseline in CBA/Ca mice. This did not occur in BALB/c mice until 72h.

As with IL-1β, IL-6 protein levels were determined by ELISA (Figure 5.9 C). IL-6 levels of this cytokine within the airways of both BALB/c and CBA/Ca mice were found to increase significantly above baseline by 6h into the experiment ($P<0.01$). Maximum production was seen at 12h post challenge in BALB/c mice and at 24h in CBA/Ca. The levels of IL-6 detectable in both strains of mice decreased towards the end of the experiment, in CBA/Ca mice the concentration found at 36h was still significantly higher than baseline. From 48h onwards significant levels of IL-6 were not detected within the airways of resistant BALB/c mice.

Investigation of the levels of the anti-inflammatory cytokine IL-10 within the airways by ELISA revealed that high levels were detectable in BALB/c mice throughout the infection (Figure 5.10 A). Initial levels were increased by 12h into the experiment and they remained elevated until the 96h post challenge time point. By 120h, the level of IL-10 detected was significantly lower than that found at the beginning of this experiment ($P<0.05$). This reduction continued for the next 24h with levels only returning to baseline by 336h. The decline in IL-10 levels during late infection are likely to be due to IL-10 exerting a negative feedback mechanism on its own production (Brown *et al.* 1996). Peak production of IL-10 occurred at the same time points as TNFα, IL-1β and IL-6 levels were declining, suggesting a role for IL-10 in regulation of these other cytokines.

Lavage fluid from infected CBA/Ca mice also contained detectable levels of IL-10 throughout the experiment. At all times investigated, these levels were significantly lower than those in BALB/c samples ($P<0.01$). Although there was a slight increase in IL-10 production within CBA/Ca airways at 24h

post challenge, this was not significant. Indeed, the level of IL-10 detected in these samples did not alter significantly at any time during the experiment.
Nitrate was converted in to nitrite prior to carrying out a Greiss reaction. This permitted the total levels of NO within the airways during pneumococcal pneumonia in both strains of mice to be determined (Figure 5.10 B).
Lavage fluids from CBA/Ca mice displayed higher concentrations of NO than BALB/c samples from the initial time point until the end stages of infection ($P<0.01$). There is a high level of variation within CBA/Ca lavage fluids at the earlier time points resulting in no obvious trend. By 36h the levels of NO detectable are higher than baseline, although this result is not significant.
NO within the airways of BALB/c mice was only present at very low levels during the infection. The highest level detected was actually at the time of infection when 1.26μM nitrite were found per ml of lavage fluid. Following this, detection did not follow any discernible trend with no significant alterations found.
Total protein analysis of bronchoalveolar lavage fluid by Bradford reagent was carried out in order to monitor the integrity of the alveolar/capillary barrier during the infection using serum protein as a marker (Figure 5.10 C). Higher endogenous protein levels were present within the airways of BALB/c mice than CBA/Ca mice ($P<0.01$). These protein levels increased from very early post challenge indicating disruption of the barrier occurred rapidly in both strains of mice. The disruption to BALB/c lungs appears to be reversible, with protein levels only increasing during the mid stages of infection (from 6h to 48h post challenge). By 120h into the experiment the level of total protein associated with the lavage fluids has returned to baseline as the lung parenchyma returns to normal and excess fluid is removed by epithelial active transport (Chapter 6).
In the susceptible CBA/Ca mice the increase in total protein levels occurs steadily throughout the experiment. Disruption occurred early during the experiment and by the final time point the level had increased significantly to 235μg/ml total protein ($P<0.01$). The relative disruption to lung integrity was greater in susceptible CBA/Ca mice than in BALB/c mice (correlating with higher NO levels).

5.2.1.6.2 Production of inflammatory mediators in lung tissues following intranasal infection of BALB/c or CBA/Ca mice

With BALB/c mice, the lung tissue TNF activity profile again peaked at 6h into the experiment with around 10U TNF activity/ml lung homogenate (Figure 5.11 A) [$P<0.05$]. Levels then decreased so that by 36h post challenge no TNF activity could be detected in any mice.

In CBA/Ca lung homogenates there was a delayed but greater TNFa activity peak at 24h into the experiment ($P<0.01$) with an activity level of almost 40 U/ml reached per ml of lung tissue. This level had decreased slightly by 36h when the experiment was ended.

The increased production of IL-1β within lung tissues during the infection, is similar in both strains (Figure 5.11 B). At the height of production (around 12h into the experiment) equal levels of IL-1β were expressed by lung tissues of both strains of mice. Following this peak, IL-1β production declined until the end of the experiment in both strains of mice. Levels were no longer significantly increased in BALB/c samples 120h post challenge although there was again significant release by 336h post challenge ($P<0.05$).

IL-6 is detectable at similar levels within the lung homogenates of both strains of mice at the time of infection (Figure 5.11 C). This level increased significantly by 24h into the infection in BALB/c mice ($P<0.05$), although this change was not sustained After 24h there was a significant reduction in levels at 120h & 144h post challenge ($P<0.01$ compared to time 0h) with these levels returning to baseline by 336h into the experiment.

In susceptible CBA/Ca mice, greatly elevated IL-6 concentrations were detected within the lung tissues. From 12h post challenge until the end of the experiment these were significantly higher than baseline ($P<0.01$) and higher than those in BALB/c samples from the same times.

As with lung airways, BALB/c mice produced higher amounts of IL-10 in their lung tissues than CBA/Ca mice at all times investigated (Figure 5.11 D). By 12h post challenge this level was significantly elevated when compared to that originally found and remained significantly higher ($P<0.05$) until 144h. By 336h, however the level had returned to being higher than baseline ($P<0.01$).

IL-10 concentrations within CBA/Ca lung homogenates were increased significantly from 12h into the experiment ($P<0.05$) until the final time

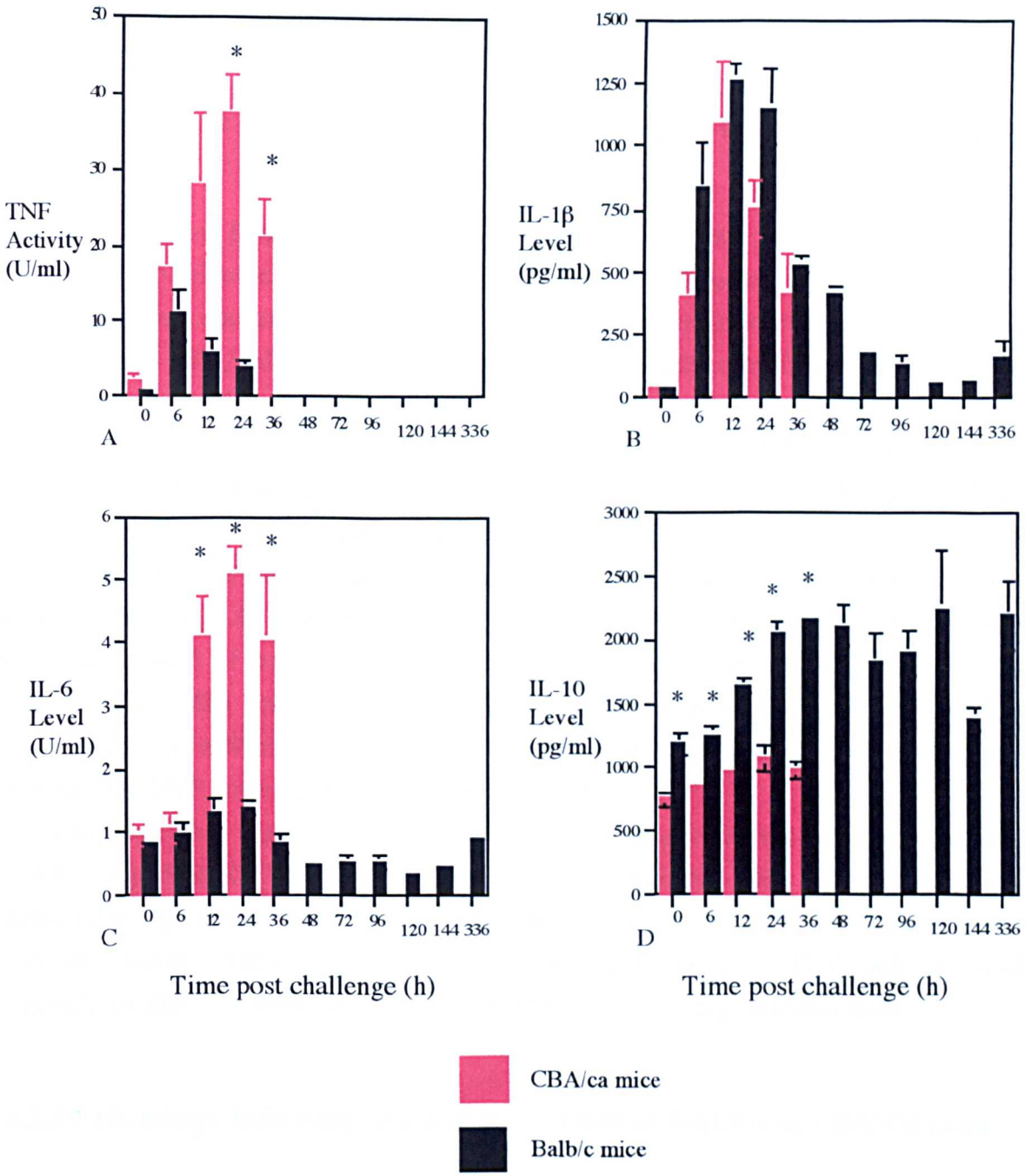

Figure 5.11 Production of inflammatory mediators within the lung tissues of BALB/c and CBA/Ca mice following intranasal infection with 9.91 x 10^5 (BALB/c mice) or 9.02 x 10^5 (CBA/Ca mice) CFU *S. pneumoniae.* A= TNF activity n=5-7, B= IL-1β levels n=4-5, C= IL-6 levels n=5, and D= IL-10 levels n=5-7. *, $P<0.01$ different for BALB/c values when compared to CBA/Ca values. No results for CBA/Ca mice after 36h post challenge as there were no survivors in this group past this time.

point. This increase was not as marked as that in BALB/c lung tissues and was followed by a slight decline by 36h after infection.

5.2.1.6.3 Systemic TNFα activity following intranasal infection of BALB/c or CBA/Ca mice

Serum samples from the same mice were also tested for TNF bioactivity (Figure 5.12). Systemic TNF activity could not be detected within the serum of BALB/c mice at any point during the experiment (detection limit of assay was around 38U/ml for serum samples). This results correlates with the low number of viable pneumococci present in these samples. Detection was possible in serum from the susceptible CBA/Ca mice at 24h and 36h post challenge. At these times serum from only 50% of mice displayed TNF activity, but positive samples did contain very high levels (up to 440 U/ml). All mice that had detectable systemic TNF activity had levels of bacteraemia higher than log 6.46 (2.88×10^6) CFU/ml, indicating that there might also be a threshold level of bacteraemia to initiate systemic inflammation in CBA/Ca mice (as in MF1 mice Section 3.2.3.9.3). This threshold was not readily identifiable as although 11 mice with bacteraemia greater than log 6.46 had detectable TNF activity in their serum, 3 didn't.
In addition, the three CBA/Ca mice with highest levels of bacteraemia, (above log 8.68 CFU/ml, all at 36h post challenge) did not possess any detectable systemic TNF activity. The short half life of TNF in the circulation (around 6min (Aderka *et al.* 1992)) might indicate that TNF activity had already peaked in these mice and had returned to being undetectable.

5.2.1.7 Histology following intranasal infection of BALB/c or CBA/Ca mice

Lung sections from BALB/c mice sampled immediately after challenge did not show marked signs of inflammation. This was altered by 6h into the experiment when lungs had marked haemorrhage and oedema and a slight cell influx in these same areas. Lungs sampled at 12h post challenge had a similar histopathologic score as those at 6h but with most recruited cells present within the airways. By 24h post challenge large numbers of inflammatory cells were present within the airways causing significant disruption to alveolar integrity (Figure 5.13 A) whilst perivascular areas

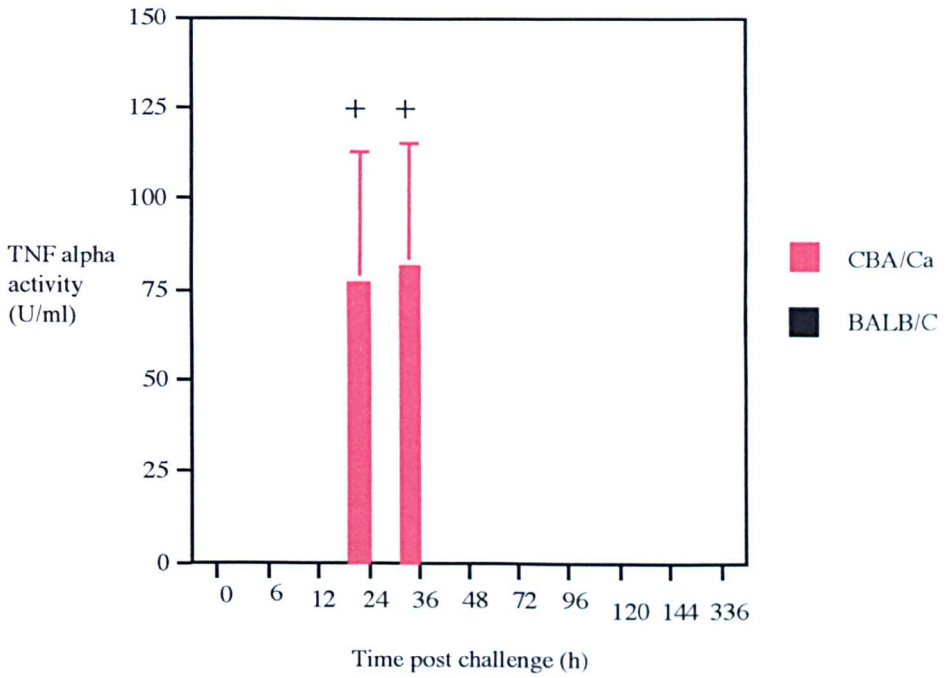

Figure 5.12 TNF activity levels within serum from BALB/c and CBA/Ca mice during infection with 9.91 x 10^5 (BALB/c mice) or 9.02 x 10^5 (CBA/Ca mice) CFU *S. pneumoniae*. n= 11-14, +, $P<0.05$ higher for CBA/Ca values when compared to BALB/c values. No results for CBA/Ca mice after 36h post challenge as there were no survivors in this group past this time.

Mouse strain	Time post challenge (h)									
	0	**6**	**12**	**24**	**36**	**48**	**72**	**96**	**120**	**336**
BALB/c	1.50	4.00	4.50	7.50	7.00	5.50	5.50	5.00	5.50	4.00
CBA/Ca	1.50	2.50	4.50	6.50	6.00	NS	NS	NS	NS	NS

Table 5.2 Mean histopathologic score for lung sections from BALB/c and CBA/Ca mice following intranasal infection with either 9.75 x 10^5 (BALB/c mice) or 9.82 x 10^5 (CBA/Ca mice) CFU *S. pneumoniae*. n= 2 mice per time point. NS= no surviving CBA/Ca animals after this time.

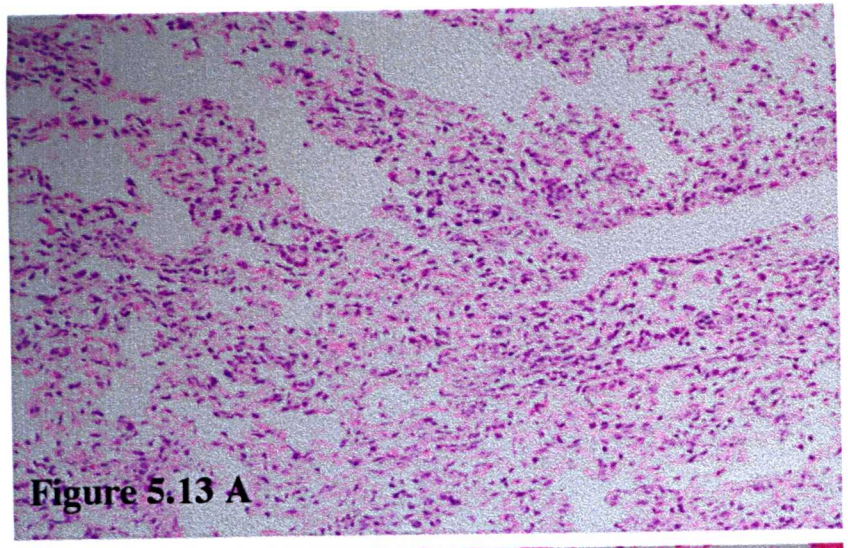

Figure 5.13 A

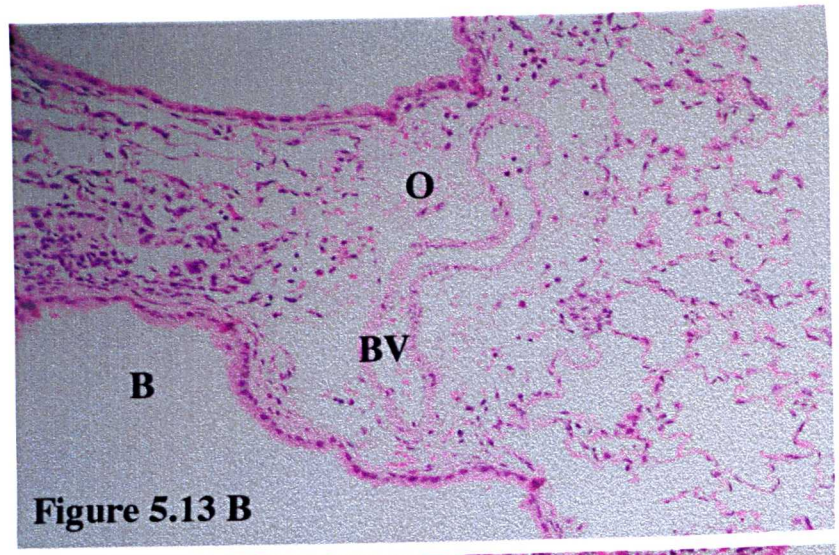

Figure 5.13 B

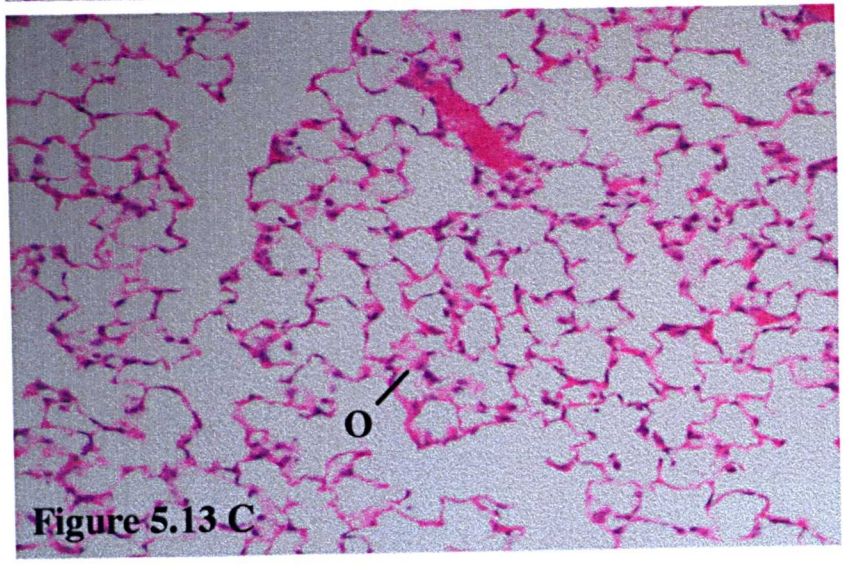

Figure 5.13 C

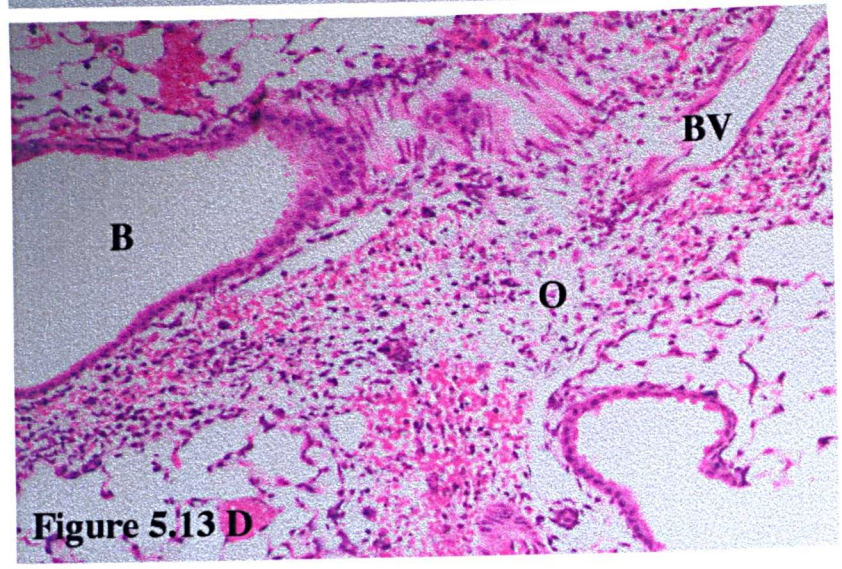

Figure 5.13 D

Figure 5.13 Histological sections from resistant BALB/c mice (A & B) and susceptible CBA/Ca mice (C & D) 24h post infection with either 9.75 x 10^5 CFU (BALB/c) or 9.82 x 10^5 CFU (CBA/Ca) *S. pneumoniae* , (X200 magnification) stained with haematoxylin and eosin.

In Figure 5.13 A the BALB/c alveoli are congested with high numbers inflammatory cells making differentiation of alveolar walls difficult. In contrast BALB/c perivascular areas display only minor oedema with few inflammatory cells or erythrocytes present (Figure 5.13B). Thus the inflammatory response appears to occur mainly within the airways of resistant mice.

Figure 5.13 C reveals that CBA/Ca alveoli appear un-consolidated although there is some evidence of oedema (O) as certain alveolar walls are enlarged. The majority of inflammation in these lungs is perivascular (Figure 5.13 D). The interface between bronchioles and bloodvessels is swollen and oedematous (O) with high numbers of inflammatory cells and erythrocytes present. This indicates that the inflammatory response in susceptible CBA/Ca mice occurs mainly within the lung tissues.

remained less consolidated (Figure 5.13 B). Neutrophils were seen to represent the majority of these cells with a few macrophages also present. From 36h until the end of the experiment at 336h post challenge a similar level of cell influx was noted although the ratio of neutrophils/macrophages decreased with time, as did disruption to alveolar architecture and haemorrhage.

Lungs from healthy, uninfected CBA/Ca mice and animals sacrificed immediately following challenge contained marked areas of oedema around airways. This histopathology did not alter greatly during the initial 12h. From this time point onwards, haemorrhage was evident within alveoli and a higher number of inflammatory cells were present. These cells were mainly situated at the interface between bloodvessels and airways (Figure 5.13 D) with few present within the airways (Figure 5.13 C). These cells displayed multi-lobed nuclei leading to their identification as neutrophils.

Table 5.2 displays the above results as histopathologic scores. It can be seen that pathology occurs earlier in BALB/c mice than in CBA/Ca animals (due to more rapid cell influx and haemorrhage in the former). Both strains have similar peak levels of histopathology at 24h and 36h post challenge although they differ in the location of this pathology. As mentioned above, BALB/c pathology is situated within airways and in CBA/Ca lungs it is found in perivascular areas of tissue.

5.2.1.8 Mast cell detection following intranasal infection of BALB/c or CBA/Ca mice

By counting the number of cells containing positive staining granules it was possible to quantify the number of mast cells within the lungs of BALB/c and CBA/Ca mice during pneumococcal pneumonia. Table 5.3 shows that immediately following infection with 10^6 CFU *S. pneumoniae* resistant BALB/c mice have significantly more granular mast cells within their tissues than do susceptible CBA/Ca animals ($P<0.01$). At the time mast cells were generally found at the outer edges of the lung lobes or surrounding blood vessels.

Following infection the number of granular mast cells decreased in BALB/c lung sections until they were significantly lower by 12h post challenge ($P<0.01$). This reduction was maintained until after 36h post challenge, with

Mouse strain	Time post challenge (h)									
	0	6	12	24	36	48	72	96	120	336
BALB/c	13.6 +/- 2.86 *	9.4 +/- 3.17	2.00 +/- 0.71	2.60 +/- 0.40	5.00 +/- 1.52	8.00 +/- 2.07	2.80 +/- 0.74	9.60 +/- 1.47	6.80 +/- 1.07	4.60 +/- 1.47
CBA/Ca	0.80 +/- 0.37	5.80 +/- 1.24	4.40 +/- 1.50	4.40 +/- 2.02	4.60 +/- 0.68	NS	NS	NS	NS	NS

Table 5.3 Mean number of mast cells with positive staining granules per field of vision (x40 magnification) in lung sections from BALB/c and CBA/Ca mice following intranasal infection with either 9.75×10^5 (BALB/c mice) or 9.82×10^5 (CBA/Ca mice) CFU *S. pneumoniae*. n= 5 field of visions/ time point, *, $P<0.01$ higher number of granular mast cells in BALB/c lung sections than in CBA/Ca at the same time. NS= no surviving CBA/Ca animals after this time.

levels at 48h, 96h and 120h not significantly different from the numbers detected at time 0h.

In contrast, the number of detectable mast cells within CBA/Ca lungs increased during the infection. By 6h post challenge a significant increase of the level ($P<0.01$) was obtained with $P<0.05$ found for the remaining time points.

This data indicates that mast cells within the lungs of resistant BALB/c lungs are normally primed with granular contents, an inflammatory stimulus (such as *S. pneumoniae*) causes de-granulation of these cells. As they release their granular contents they are no longer detectable by the stain, explaining the reduction in numbers during infection. Mast cells within CBA/Ca lungs do not endogenously contain high levels of granular contents and require time in order to synthesise TNFα with granular cells increasing during the experiment, but still to a significantly reduced level in comparison to BALB/c lungs at the time of infection ($P<0.05$ lower for CBA/Ca lungs at 6h, 12h, 24h and 36h when compared to BALB/c sections at 0h).

5.2.2 *In vitro* stimulation of airway cells recovered from BALB/c or CBA/Ca mice

Following the discovery that 15 fold higher TNFα activity was associated with BALB/c airways after infection with 10^6 CFU *S. pneumoniae*, the possibility that BALB/c airway cells were better able to produce this cytokine upon stimulation thus explaining the results gained *in vivo*. This was investigated by *in vitro* stimulation of freshly recovered airway cells from both mouse strains with various concentrations of LPS, heat killed *S. pneumoniae* or recombinant pneumolysin (Figure 5.14).

Suspensions of 10^4 and 10^5 CFU heat killed *S. pneumoniae* were incapable of stimulating the production of TNFα protein by airway cells from either mouse strain (Figure 5.14 A). Increasing the stimulus to 10^6 CFU did result in the production of TNFα protein by cells from both BALB/c and CBA/Ca mice. BALB/c cells released 126pg/ml whilst CBA/Ca supernatants contained only 12pg/ml.

Although such levels of TNFα protein could be measured, these same supernatant fluids did not possess and any TNF activity, as assessed by the bioassay. The fact that heat killed pneumococci released lower amounts of

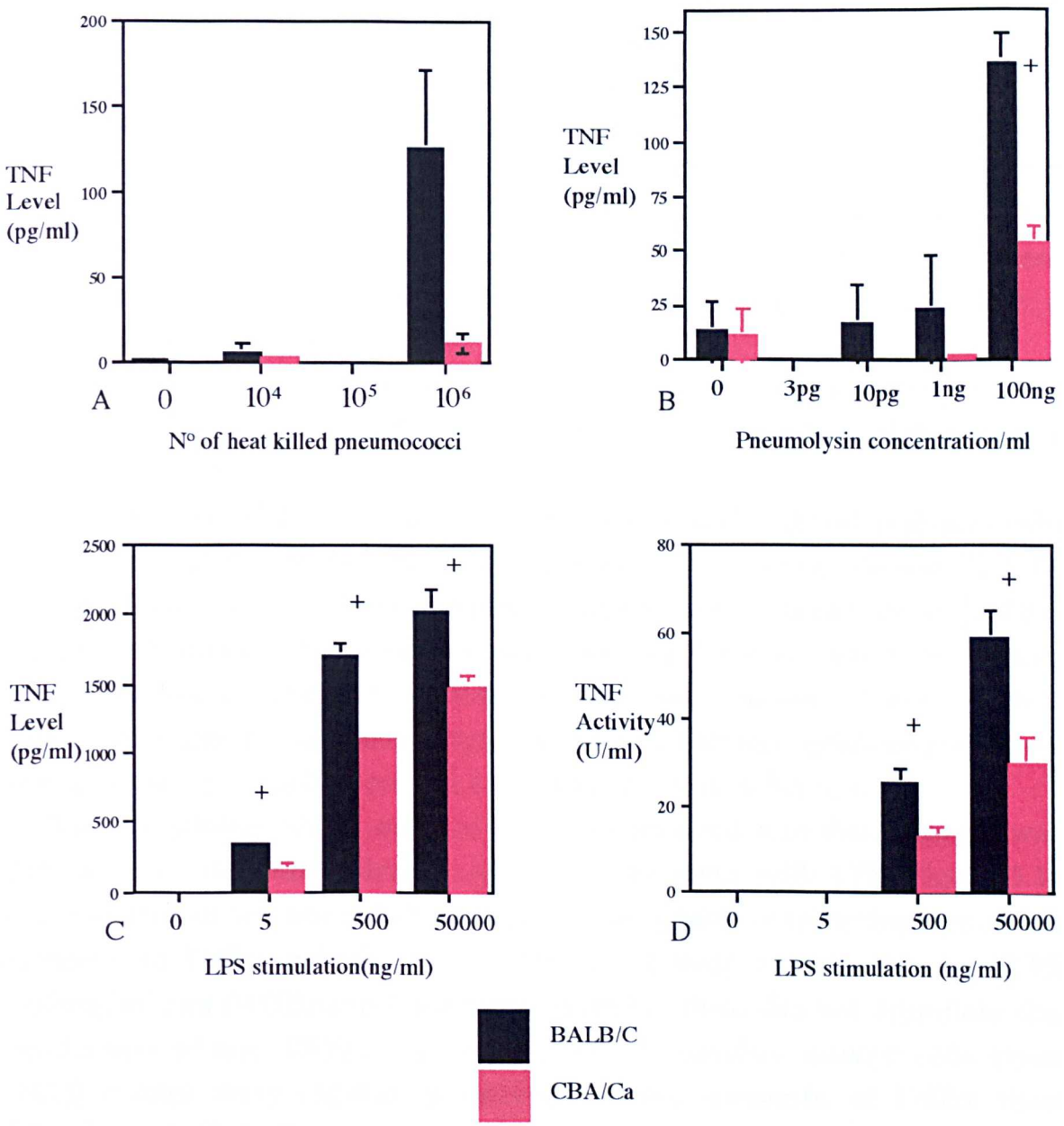

Figure 5.14 TNFα protein and activity levels produced by airway cells stimulated *in vitro* with various stimuli. A= heat killed D39, B= recombinant pneumolysin, C= LPS for TNFα protein, & D= LPS for TNF activity. n=3-5 wells per stimulus, $^{+}$, $P<0.05$ higher for BALB/c results when compared to CBA/Ca.

TNF protein than did LPS (Figure 5.14 C & D) may have resulted in TNF activity levels too low to be detected on the bioassay. Such a discrepancy between ELISA and bioassay data has also been found for *in vitro* TNF release from human monocytes stimulated with staphylococcal peptidoglycans (Timmerman *et al.* 1993).

Airway cells incubated with 100ng/ml pneumolysin contained significant amounts of TNFα protein (Figure 5.14 B). BALB/c cells released the highest level with 135pg/ml detected, CBA/Ca cells produced 54pg/ml. Thus there was again a marked difference between mouse strains, in this case a significant one ($P<0.05$).

TNF activity was not determined in these samples due to preliminary results which indicate that pneumolysin is directly cytotoxic for the L929 indicator cell line in the bioassay.

A combination of 10^6 CFU heat killed bacteria and 1ng/ml pneumolysin resulted in significantly higher TNFα protein levels being released by cells from BALB/c mice than from CBA/Ca cells ($P<0.05$) [data not shown]. This stimulus resulted in the most marked difference between cells from mouse strains (80 fold compared to 10 fold for heat killed bacteria alone). Such a difference suggests that there might be synergy between pneumolysin and pneumococci when acting on BALB/c cells but not on CBA/Ca.

TNFα total protein levels detected by ELISA revealed that this cytokine was produced *in vitro* when airway cells were stimulated with LPS (Figure 5.14 C). As small an amount of LPS as 5ng/ml was capable of inducing significant amounts of TNFα in both cell populations. Lower concentrations of LPS (0.05ng/ml and 0.0005ng/ml) were also tried but these did not stimulate the production of any TNFα. At each level of stimulus airway cells from BALB/c mice were capable of releasing higher amounts of TNFα than CBA/Ca cells ($P<0.05$).

Analysis of the same LPS stimulated cell supernatants by TNF bioassay revealed that not only was TNFα protein released, but this protein was active (Figure 5.14 D). An LPS concentration of at least 500ng/ml was required to induce significant TNF activity. This level of stimulation resulted in significantly more TNF activity from BALB/c cells than from CBA/Ca cells ($P<0.05$). Increasing the LPS stimulus to 50,000ng/ml resulted in more TNF activity detected in supernatant from cells of both mouse strains but did not alter the relationship between strains. As with TNFα total protein, lower

levels of LPS were used as stimuli but these were found ineffective at stimulating any TNF activity.
Thus, although higher levels of TNFα protein were detected following stimulation with LPS than with pneumococcal factors, the most striking difference between the two mouse strains resulted from stimulation with the latter.

5.2.3 Role of lymphocytes in resistance to pneumococcal pneumonia and cytokine production

In order to attempt to discover whether lymphocytes are required for the resistance of BALB/c mice to pneumococcal pneumonia, BALB/c *scid* mice were infected intranasally with 10^6 CFU *S. pneumoniae*. The B and T lymphocytes of these mice are incapable of expressing functional immunoglobulin or T cell receptors on their surfaces, hence cell-mediated and humoral defense mechanisms are impaired.
In these experiments *scid* mice were either culled at 24h and bacterial loads and cytokine levels determined, or monitored for survival time.

5.2.3.1 Signs of illness and survival times following intranasal infection of BALB/c or BALB/c *scid* mice

The control BALB/c data have already been described and showed the increased resistance of BALB/c mice to pneumococcal infection. BALB/c *scid* mice developed symptoms of illness by 20h into the experiment. At this time a hunched stance was the predominant clinical manifestation. Mice sampled for the 24h time point described below were all displaying mild signs of illness, mainly a hunched stance with some mice exhibiting piloerection. Female mice passed through the range of symptoms at a faster rate than male mice, with most female mice had developing full symptoms by 52h post challenge. Male mice did not do so until around 75h into the experiment. By the end of the experiment all male mice had succumbed to the infection and only one female mouse had survived (Figure 5.15).
Despite the differences in kinetics of symptom appearance, survival times were not statistically different for female and male BALB/c *scid* mice. Comparison of survival times for BALB/c and BALB/c *scid* mice revealed

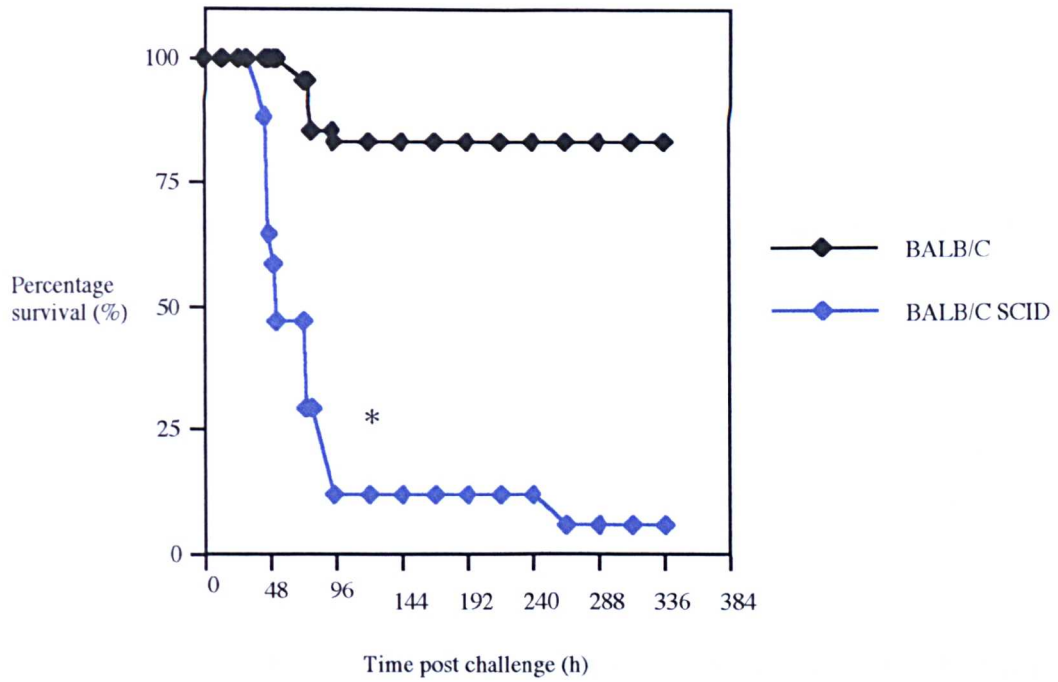

Figure 5.15 Survival of BALB/c and BALB/c *scid* mice following challenge with either 9.75 x 10^5 (BALB/c mice) or 1.01 x 10^6 (BALB/c *scid* mice) CFU *S. pneumoniae*. n= 21 for BALB/c and n=17 for BALB/c *scid*. *, P<0.01 shorter survival times of BALB/c *scid* mice when compared to BALB/c.

Mouse Strain	Bacterial Load CFU/ml	
	Lavage Fluid	Blood
BALB/c	3.63 +/- 0.26	0.00
BALB/c *scid*	2.95 +/- 0.39	6.11 +/- 0.24 *

Table 5.4 Bacterial loads within BALB/c *scid* mice lavage fluids and blood 24h after intranasal infection with either 9.91 x 10^5 (BALB/c mice) or 9.75 x 10^5 (BALB/c *scid* mice) CFU *S. pneumoniae*. n= 5/10, *, P<0.01 higher for BALB/c bacteraemia than for BALB/c bacteraemia.

that the immunodeficient mice (total of males and females) were significantly more susceptible to intranasal infection with 10^6 CFU D39 (P<0.01).

5.2.3.2 Bacteriology following intranasal infection of BALB/c or BALB/c *scid* mice

There was no significant difference between the bacterial loads in BALB/c and BALB/c *scid* airways at 24h post challenge. However there was a highly significant difference between bacteraemia levels in the two strains at this time (P<0.01). At this time BALB/c *scid* mice possessed comparable numbers of viable pneumococci within their circulation as CBA/CA mice (Table 5.4)

5.2.3.3 Production of Inflammatory mediators by BALB/c or BALB/c *scid* mice following intranasal infection

As the function of lymphocytes in BALB/c *scid* mice is impaired it was a possibility that the production of inflammatory mediators might be disregulated in these mice, explaining their increased susceptibility.
BALB/c *scid* mice were able to produce higher levels of TNF, IL-1β and IL-6 within the lung airways (Table 5.5) and tissues (Table 5.6) during pneumococcal pneumonia although none of these results were significant. Production of NO was significantly higher in the airways of BALB/c *scid* mice (P<0.01), as was the amount of total protein associated with the airways (P<0.01).
Levels of the anti-inflammatory cytokine IL-10 were significantly reduced in the airways (to the level of P=0.028) and lung tissues (P<0.01) of BALB/c *scid* mice with non functional lymphocytes.
Additionally, the BALB/c *scid* mice had detectable levels of TNFα activity with their circulations (77.91 U/ml), although levels were highly variable. The two BALB/c *scid* mice that possessed systemic TNF activity were those that had the highest levels of bacteraemia log 6.53 and 6.55).

Overall these results indicate that BALB/c *scid* mice have heightened inflammatory reactions within their lungs and circulation due to decreased levels of IL-10.

Mouse strain	TNFα	IL-1β	IL-6	NO	Protein	IL-10
BALB/c	7.01 +/- 1.71	170.67 +/- 30.31	0.76 +/- 0.32	0.00	151.12 +/- 8.86	160.80 +/- 8.04
BALB/c *scid*	5.56 +/- 1.68	154.67 +/- 18.33	1.43 +/- 0.42	3.61+/- 0.62*	270.28 +/- 30.26 *	128.00 +/- 19.90 *

Table 5.5 Levels of inflammatory mediators within lavage fluids of BALB/c and BALB/c *scid* mice 24h post challenge with either 9.91 x 10^5 (BALB/c mice) or 9.75 x 10^5 (BALB/c *scid*) CFU *S. pneumoniae*. n=4-10, *, P<0.01 different for BALB/c *scid* samples when compared to BALB/c.

Mouse strain	TNFα	IL-1β	IL-6	IL-10
BALB/c	3.60 +/- 1.05	1151.55 +/- 148.81	1.40 +/- 0.14	2052.00 +/- 85.36
BALB/c *scid*	6.88 +/- 3.36	984.38 +/- 124.60	2.81 +/- 1.00	477.85 +/- 56.66 *

Table 5.6 Cytokine levels within lung tissues of BALB/c and BALB/c *scid* mice 24h post challenge with either 9.91 x 10^5 (BALB/c mice) or 9.75 x 10^5 (BALB/c *scid* mice) CFU *S. pneumoniae*. n=4-5, *, P<0.01 lower for BALB/c *scid* samples when compared to BALB/c.

5.2.4 Systemic susceptibility of BALB/c and CBA/Ca mice to *S. pneumoniae* bacteraemia

5.2.4.1 Survival and signs of illness following intravenous infection of BALB/c or CBA/Ca mice

In order to determine whether the resistance of BALB/c mice in comparison to CBA/Ca mice was due to pulmonary defences alone, intravenous infections were initiated with 2.40 $x10^3$ CFU or with 5.00 x 10^4 CFU *S. pneumoniae*. The development of symptoms, bacteraemia and survival times were recorded. These doses were chosen as 5.00 x 10^4 was similar to the highest recorded level of bacteraemia in a BALB/c mouse following intranasal infection (Table 5.1). The lower dose was selected in order to determine whether CBA/Ca were able to resist pneumococcal infection if very few bacteria gained access to the circulation from the lungs.

BALB/c mice display a heightened resistance to systemic challenge with *S. pneumoniae*. CBA/Ca mice displayed symptoms from 24h into the experiment at both doses used. At 24h post challenge they were hunched and had a degree of piloerection. Rapidly after this point they passed through a similar set of symptoms as with pulmonary challenge until all mice had succumbed to the infection by 33h post challenge (Figure 5.16). There was no difference between symptoms displayed on infection with 2.4 x 10^3 CFU than with 5.0 x 10^4 CFU *S. pneumoniae*.

BALB/c mice displayed mild symptoms in comparison, with a slight hunched appearance being the worst symptom displayed during mid infection (33h post challenge). By 49h all mice had returned to normal behaviour and appearance. Furthermore, no BALB/c mice succumbed to systemic infection with D39 and when the experiment was actively finished pneumococci could not be cultured from blood samples from any of the mice.

Thus BALB/c mice displayed significantly longer survival times than CBA/Ca mice following systemic challenge ($P<0.01$).

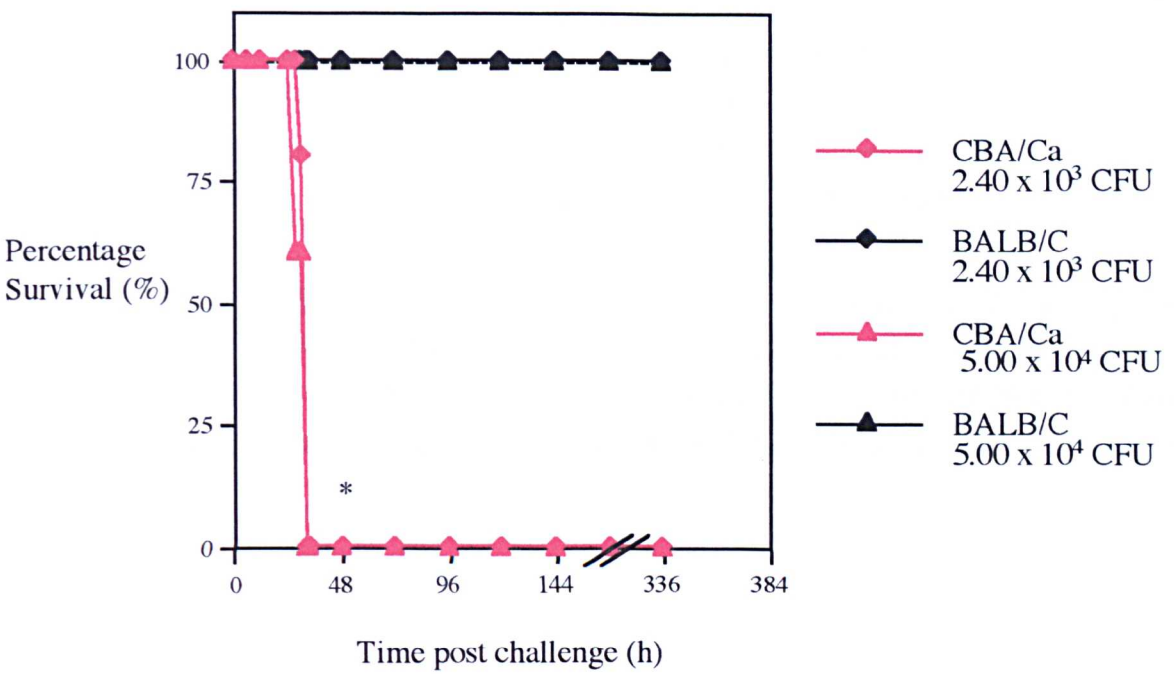

Figure 5.16 Survival of BALB/c and CBA/Ca mice after intravenous infection with 2.40 x 10^3 or 5.00 x 10^4 CFU*S. pneumoniae*. n= 5 mice, *, P<0.01 for CBA/Ca when compared to BALB/c (both experiments).

5.2.4.2 Systemic bacteriology following intravenous infection of BALB/c or CBA/Ca mice

Serial tail-bleeds permitted the level of bacteraemia to be monitored for the initial 24h following intravenous infection with two doses of *S. pneumoniae*.

Similar levels of bacteraemia were found in all CBA/Ca and BALB/c mice immediately following infection (Figure 5.17). Despite this fact, by 12h into each experiment, the two mouse strains had significantly different numbers of systemic bacteria ($P<0.01$). By 12h BALB/c mice showed a slight reduction in bacterial numbers. This decrease was more marked by 24h into the experiment when 4/5 BALB/c mice no longer had detectable levels of bacteraemia.

In CBA/Ca mice, pneumococci had multiplied significantly by 12h post challenge. This proliferation occurred unchecked until the end of the experiment when around 10^8 CFU were present in each ml of blood. The mean doubling time following infection with 5.00×10^4 was 2.08h and with 2.40×10^3 1.88h.

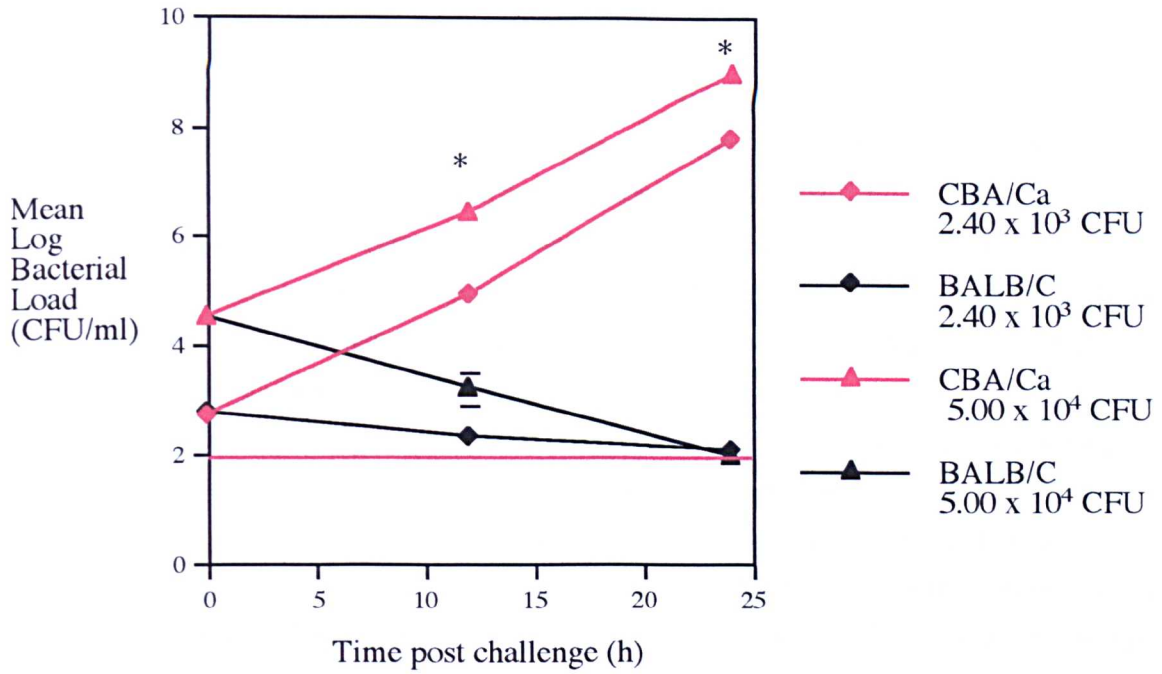

Figure 5.17 Mean +/- SEM level of bacteraemia in BALB/c and CBA/Ca mice after intravenous infection with 2.40 x 10^3 or 5.00 x 10^4 CFU *S. pneumoniae*. n=5, *, P<0.01 higher for CBA/Ca bacterial loads than for BALB/c (both experiments). Red horizontal line represents detection limit of viable count assay.

5.3 SUMMARY

It has long been recognised that there is a genetic component to susceptibility to pneumococcal infections. Results in this chapter consolidate this hypothesis with survival times and the inflammatory response produced by two inbred mouse strains varying significantly following infection with 10^6 CFU type 2 *S. pneumoniae.*

CBA/Ca mice are highly susceptible and are unable to control the organisms within the lungs and bloodstream and succumb around 36h post challenge. These mice had developed bacteraemia by 6h post challenge (the first time point studied, thus further experiments are required to establish how early pneumococci can access the bloodstream in these mice following intranasal infection). As low a number of pneumococci as 2000 CFU was capable of instigating a fatal infection in these mice.

In contrast BALB/c mice are able to reduce the viability of pneumococci within their lungs and prevent access to the bloodstream. Even if pneumococci entered the circulation, protective systemic immunity was capable of removing 5.00×10^4 CFU, indicating that a constant shedding of larger numbers of these organisms from the lungs would be required for fatal infection in this mouse strain.

Infection of the two strains results in alteration in Tco. Resistant BALB/c animals display transient hypothermia during early infection lasting approximately 6h. Susceptible CBA/Ca mice become severely hypothermic during end stage disease with temperature dropping to ambient room temperature.

Inflammation is rapidly instigated within the lungs of both strains of mice. In BALB/c mice this is predominantly contained within the airways (as are the pneumococci) and is manifested mainly by TNF. In CBA/Ca mice inflammation spills over into the lung tissues where TNF and IL-6 are released in very high levels and into the bloodstream where TNF activity could be detected as a marker of inflammation.

Airway cells of BALB/c mice are capable of releasing significantly higher levels of TNF than those from CBA/Ca. This appears to be especially

relevant to Gram-positive infections and explains the ability of BALB/c mice to mount a significant inflammatory response rapidly following infection.

The location of cytokine production is directly related to the site of inflammatory cell influx. In infected BALB/c animals neutrophils arrive in the air spaces approximately 12h post challenge and it is within the airways that most inflammatory cytokines (especially TNF) are released in these animals. Maximum pro-inflammatory cytokine release in infected CBA/Ca mice is within the tissues themselves, supported by the location of recruited cells. Inflammatory cell influx into these lungs predominantly occurs in perivascular areas, with minimal recruitment to the airways. The rapidity with which these animals develop bacteraemia is likely to cause cells surrounding blood vessels to secrete cytokines and chemokines early during infection, explaining this pattern of cell influx.

Lymphocytes, previously not thought to play a role in pneumococcal pneumonia, are important in this model. Mice with incompletely functioning T and B lymphocytes display elevated susceptibility to *S. pneumoniae* infection. This may be due to a disregulation of inflammation on the behalf of IL-10 permitting an overzealous response.

Chapter 6

General Discussion

The results presented in this thesis demonstrate the characterisation of the inflammatory response occurring during pneumococcal pneumonia caused by a frequently studied isolate of *S. pneumoniae*. Effects on bacterial loads, lung histology, inflammatory mediators and core body temperature are reported.

Although the viability of pneumococci is reduced immediately following resuspension in PBS (Figure 3.2), no such drop in viability was seen following inoculation into pulmonary airways. Constant numbers of *S. pneumoniae* were cultured from the lavage fluid of MF1 mice from immediately after intranasal inoculation of 10^6 CFU D39 until the end of the experiment (Figure 3.9). Thus the pulmonary air spaces are more suitable for pneumococcal survival than is PBS.
Viability of the inoculum does drop immediately following intranasal challenge when bacterial counts are performed on the lungs as a whole (un-lavaged lung homogenates) (Kadioglu *et al.* 2000). Therefore the different environments that exist within the lungs vary in their suitability for survival of *S. pneumoniae*, possibly connected to the immune cell types and numbers that the organisms encounter in each.

The ability of pneumococci to survive within the airways also varied with mouse strains. An elevation in viability occurred immediately after intranasal challenge of either BALB/c or CBA/Ca mice (Figure 5.7). This indicates that *S. pneumoniae* readily adapts to airways in these mouse strains. It is possible that BALB/c and CBA/Ca mice express higher levels of the pulmonary receptors utilised by pneumococci (Section 1.4.2) than do MF1 mice. From 12h onwards viability declined in both mouse strains. Separate causes may be responsible for these reductions in the two mouse strains. In the resistant mice it is probable that the reduction in bacterial viability is a real reflection of pneumococcal kill. In CBA/Ca mice lavage fluid counts may decrease as the bacteria become more tightly associated with the lung tissues from mid infection onwards. They would therefore be recovered and counted within the lung homogenates. Indeed bacterial counts within CBA/Ca lung tissues at the same time points do increase. If it were a simple matter of a reduction in bacterial viability a corresponding reduction within the CBA/Ca lung tissues would also be expected.

The ability of BALB/c airway defences to control pneumococci occurs in two stages (Figure 5.7). The earlier reduction in viability is more effective with a 6 fold difference resulting over a matter of 24h. The second stage is a more gradual decline with complete removal of viable organisms not detected until a further 312h.

In addition to there being several mechanisms of pneumococcal kill acting within the airways of resistant BALB/c mice, there may also be separate methods of bacterial kill occurring within the environment of the lungs as a whole. At the same time as the airway counts are reduced 6 fold, only a 2 fold reduction is evident within the tissues (Figure 5.8). It is possible that the mechanism responsible within BALB/c airways for the initial rapid kill is not present within the lung tissues whilst the second mechanism active within the airways is also found in the tissues.

Although there was complete clearance of bacteria from the airways of BALB/c mice, there were still viable pneumococci associated with the lung tissues at 336h into the experiment. A more chronic infection was therefore established in these mice. Indeed the slight increase in certain cytokine levels (IL-6, IL-1 & IL-10- see below) in the lung homogenates (but not lavage fluid) by the end of the infection indicates that an inflammatory stimulus remains within these tissues.

In contrast to the ability of resistant BALB/c mice to control pulmonary *S. pneumoniae* viability, lung homogenate counts from susceptible mouse strains increased throughout the experiment (Figures 3.10 & 5.8). Infections in MF1 mice revealed a significant correlation between lung homogenate and blood pneumococcal viable counts. The possibility that bloodborne bacteria were responsible for the increase in lung homogenate bacterial loads was therefore tested by lung perfusion. Removal of blood from the pulmonary vasculature did not significantly reduce lung homogenate bacterial loads (Figure 3.11), a result that has since been repeated by collaborators. This experiment does not exclude the possibility that the bacteria are adherent within the bloodvessels and not readily removed by perfusion. However, Harrow *et al* have previously shown that the number of bacteria present within the blood of the lung does not meaningfully contribute to the total number of lung associated bacteria (Harrow *et al.* 1975).

This model therefore stands as one of bacteraemia following pneumococcal pneumonia and not pneumococcal bacteraemia alone.

The bacteria are readily recognised by the immune system of the host and an inflammatory response occurs rapidly following their inoculation. This response is primed for action in healthy individuals with low levels of TNF, IL-1 and IL-6 mRNA constitutively expressed in the spleen, liver, kidney and peripheral blood of healthy individuals (the lungs were not examined) (Tovey *et al.* 1988).

The pulmonary inflammatory response within both resistant and susceptible mice was initially characterised by an increase in TNFα protein and activity. Such elevated TNFα levels are found within the lungs of patients with bacterial pneumonia (Dehoux *et al.* 1994). Similar results been found in a range of animal models of pulmonary infection including those caused by extracellular bacteria; *S. pneumoniae* (Takashima *et al.* 1997), (Bergeron *et al.* 1998) & (van der Poll *et al.* 1997a), *Klebsiella pneumoniae* (Laichalk *et al.* 1996) and *Pseudomonas aeruginosa* (Gosselin *et al.* 1995); the intracellular bacterium *Legionella pneumophila* (Brieland *et al.* 1995) and the fungus *Pneumocystis carinii* (Chen *et al.* 1992a).

TNF activity within MF1 airways and tissues increased in 2 waves (Figure 3.13). The number of levels of variation in the MF1 experiment prevent statistical analysis to determine whether the dual peaks of TNF activity were real or experimental artefacts. If repeat experiments support the dual peak hypothesis, separate cell populations may be responsible for these kinetics. Resident cells such as mast cells (secreting both pre-formed and newly synthesised TNF) and alveolar macrophages could have caused the 18h post challenge peak in TNF activity. The 30h post challenge activity peak may have been released by newly recruited neutrophils and additional macrophages. Histological examination revealed no significant cell influx until 24h post challenge (Figure 3.12), supporting this separate population hypothesis. Mast cell staining revealed their degranulation (or efflux of the cells themselves) by 12h post challenge, the time point immediately preceding the earlier peak in TNF activity. Mast cell release of TNF has previously been implicated in host resistance to infectious disease (Malaviya

et al. 1996) & (Abraham and Malaviya 1997). It is therefore probable that these cells are responsible for early TNF production, initiating the host inflammatory response during pneumococcal pneumonia. Further work is planned to investigate this hypothesis both by investigation of mast cell protease levels (Wastling *et al.* 1997) and through the use of mast cell deficient mice (Malaviya *et al.* 1996).

A reduced capacity of recruited cells to produce TNF (in comparison to resident cells) would explain the reduced magnitude of the second peak in TNF activity at 30h. The recruited cells may be of a different phenotype (neutrophils are less capable of releasing cytokines than are cells of the monocyte lineage (Cassatella 1995). Alternatively, higher levels of anti-inflammatory mediators (IL-10 and soluble TNF receptors) at 30h may be antagonising TNF activity. Further proof of action of an antagonist is found in the ability to detect sustained levels of TNFα by ELISA and immunohistochemistry at 36h post challenge whilst activity is reduced (Figures 3.13 & 3.17).
Only single waves of TNF activity were evident within the lungs of all other strains sampled in this study; perhaps a reflection of the longer sampling interval.

A marked difference was detected in the levels of TNF activity associated with lung airways from susceptible CBA/Ca and resistant BALB/c mice (Figure 5.9). At peak production airways of BALB/c mice contained 15 fold higher levels of this inflammatory cytokine than CBA/Ca airways although kinetics of TNF production are similar in both strains of mice. TNF is therefore a prime candidate for a factor that might be responsible for mediating the susceptibility to pneumococcal pneumonia. Significantly elevated production of TNF within the airways of BALB/c mice during *P. aeruginosa* infection when compared to susceptible C57Bl/6 mice, has also been suggested to play a role in resistance (Sapru *et al.* 1999) & (Gosselin *et al.* 1995). Elevated TNF levels in BALB/c airways may play a role in chemotaxis of phagocytic cells which arrive earlier and in greater numbers than in CBA/Ca. TNF can cause up regulation of V-CAM and E-selectin in lung tissue *in vivo* as little as 4 h after stimulation (Neumann *et al.* 1996). A correlation between location of TNFα and neutrophil recruitment has also

been discovered (Nelson *et al.* 1989). In addition to recruiting neutrophils into the air spaces, TNF will activate these cells so that they are primed for anti-microbial activity on arrival at the site of infection (Dusi *et al.* 1996), (Klebanoff *et al.* 1986) & (Shalaby *et al.* 1985).
It is not clear whether the amount of TNF released during infection in the two mouse strains is regulated by different levels of transcription of the gene or by other post transcription/translation events. For example it could be that the secretion of active TNF is reduced in susceptible CBA/Ca mice. TNFα has two active forms, one is a surface bound 26kDa protein and the other is a 17kDa secreted protein released from cell surfaces by cleavage of the 26kDa form. This cleavage is regulated by TNFα converting enzyme (Black *et al.* 1997) & (Moss *et al.* 1997). A disruption to the activities of this enzyme in CBA/Ca mice would result in lower levels of secreted TNFα, leading to impaired host defences within the airways whilst lung tissue levels would be elevated (Figures 5.9 & 5.11).

Variations in the cell type and number at lung airways in BALB/c and CBA/Ca mice would also explain the difference in TNF activities. A candidate cell type would be the mast cell. Significantly elevated numbers of mast cells were found in the lungs of BALB/c mice immediately following infection with *S. pneumoniae* than were found in the lungs of CBA/Ca mice. These BALB/c mast cells were primed for release of granule contents within hours of infection, perhaps explaining the rapid nature of TNF response. In contrast, the lower number of granule containing cells within lungs of CBA/Ca animals required longer to synthesise and secrete TNF which they continued to do throughout the experiment (as granule containing cells did not decrease in number once elevated). This continual release of TNFα is more likely to be involved in pathology than protection.
A striking finding of these studies was the significantly reduced numbers of granule containing cells in BALB/c and CBA/Ca mice when compared to MF1 animals (Table 3.3 and 5.3). As BALB/c mice displayed the most rapid TNF activity kinetics these mice might have been expected to have more mast cells than MF1 mice. Although mast cells from MF1 mice have more granule contents, the TNFα content might be reduced in comparison to the histamine or serotonin content. In comparison, BALB/c granules may

contain a significantly greater proportion of pre-synthesised TNFα, primed for release on inoculation of the pneumococci.
Epithelial cells may also contribute to the cytokine profile by release of cytokines on interaction with bacteria (Khair and Davies 1996). Previously epithelial cells were thought of as bystander cells with no real role to play in inflammatory responses. More recent research indicates that interaction of pneumococci with BALB/c airway epithelial cells could be responsible for the high TNF activity levels seen during pneumococcal pneumonia. *Staphylococcus aureus* has been shown to induce cytokine expression by epithelial cells (IL-8 release from canine tracheal epithelial cells *in vitro*) (Massion *et al.* 1995). This cytokine release was also shown to mediate neutrophil recruitment Although this field of research is in its infancy, it is possible that the ability of BALB/c airway epithelial cells but not CBA/Ca cells to respond in this manner explains the marked difference in TNF levels.
Ongoing studies are being carried out to investigate the cell populations within BALB/c and CBA/Ca mice both before and during pneumococcal pneumonia.

Thus a deficiency in TNF activity in CBA/Ca mice is likely to be involved in the heightened susceptibility of these mice to intranasal infection with *S. pneumoniae*. In order to investigate whether the reduced lavage fluid TNF activities from CBA/Ca mice was the result of airway cells from CBA/Ca being less able to respond to stimuli by the production of TNF than BALB/c mice, *in vitro* stimulation of airway cells recovered from BALB/c and CBA/Ca mice was carried out (Figure 5.14).
When low concentrations of pneumolysin were utilised as a stimulus, a complete lack of TNFα protein production was evident. This is in contrast to findings previously published by this laboratory (Houldsworth *et al.* 1994). The disagreement is likely to be explained by the different cell types used, that is rodent airway cells in place of human monocytes. Increasing the stimulus to 100pg/ml resulted in significant TNFα release from BALB/c cells but not from CBA/Ca cells, giving a statistically significant difference between the cell types.
Similar results were found with another pneumococcal stimulus. Incubating airway cells with numbers of heat killed pneumococci utilised *in*

vivo resulted in BALB/c cells releasing high levels of the cytokine. In contrast, CBA/Ca cells still only released minimal levels. This result is supported by the ability of other investigators to induce the production of TNFα by macrophages in vitro with heat killed *S. pneumoniae* (Simpson *et al.* 1994).

The most significant difference in TNF levels between cells from the two strains was achieved by stimulating with a combination of pneumolysin and heat killed *S. pneumoniae.* Such a result suggests that pneumolysin may act in synergy with cell wall products to induce inflammation in BALB/c cells but not in CBA/Ca cells. This synergy has previously been shown to occur following stimulation of human monocytes with the alpha toxin of *Staphylococcus aureus* (Bhakdi *et al.* 1989).

The least significant difference in TNFα levels between cell from the two mouse strains was evident following stimulation with the Gram-negative factor, LPS. Although cells from BALB/c mice stimulated with LPS were capable of producing higher levels of TNF protein and activity, the difference in the two strains was lower than the 15 fold difference seen *in vitro.* This result indicates that the difference between cell responses is most significant during Gram-positive infections.

The recognition of a pathogen involves receptors that bind molecular structures (pathogen-associated molecular patterns (PAMPS) which are shared by large groups of micro-organisms (Medzhitov and Janeway Jr 1997) . These receptors are referred to as pattern-recognition receptors (PRRS). One such group of receptors are the recently identified Toll-like receptors which are believed to mediate innate responses against foreign bacteria via interaction with the transcription factor NF-kB (Kopp and Medzhitov 1999). Toll-like receptor 4 (TLR4) is believed to be the major receptor involved in recognition of Gram-negative bacteria whilst TLR2 is implicated in responses to Gram-positive organisms (Yoshimura *et al.* 1999).

If CBA/Ca airway cells express a high percentage of TLR4 they would be expected to respond best to Gram-negative bacteria. Alternatively, if BALB/c cells expressed more TLR 2 they would be better able to respond to *S. pneumoniae* cell wall (Yoshimura *et al.* 1999).

The relationship between BALB/c and CBA/Ca TNF activities within the airways is reversed in the rest of the lungs, with CBA/Ca mice displaying the highest TNF activities (Figure 5.11). This result has also been documented in the *Toxoplasma gondii* encephalitis model which utilises BALB/c animals as resistant mice whilst CBA/Ca mice are susceptible. In that model, equal peak levels of TNFα and IFNγ were found within mesenteric lymph nodes but elevated TNF levels persisted for longer in CBA/Ca mice (Hunter *et al.* 1994). A difference in location of the inflammatory stimuli might explain this finding in our model of pneumococcal pneumonia. Compartmentalised TNFα production does occur following either intravenous or intratracheal administration of LPS (Nelson *et al.* 1989), suggesting that TNF production only occurs where there is a direct stimulus. Pneumococci do not leave the airways in BALB/c mice but they readily gain access to the lung tissues of CBA/Ca mice. However, bacterial loads within the airways and lung tissues of the two mouse strains are similar at 6 and 12h post challenge indicating that a difference in bacterial loads is not responsible for the difference in cytokine production. Therefore the elevated BALB/c airway TNF levels are involved in reducing bacterial loads within the tissues and not vice versa.
TNF within the lungs of BALB/c mice appears to be present at levels that are sufficient to carry out its functions and are contained within the airways but are not great enough to cause overwhelming pathology. In CBA/Ca mice the TNF production is not contained to the airways but is mainly found within the lung tissues. This could play a role in pathology due to overzealous production of this mediator too late to have a beneficial effect but rather leading to further disruption to lung integrity.
If the total level of TNF activity per lung is calculated for both mouse strains the values obtained are approximately the same. This indicates that the role played by TNF in determining the outcome of pneumococcal pneumonia is mediated by the location and timing not the absolute level.

Further investigation of the role played by TNFα during pneumococcal pneumonia in the MF1 model was carried out via utilisation of anti-TNFα neutralising antibodies (Section 4.2.2).
An effect of systemic anti-TNFα treatment on bacterial loads within the lungs has previously been controversial. We have shown that with type 2 pneumococci systemic neutralisation of TNF activity is associated with

impaired bacterial clearance both within the lungs and bloodstream (Table 4.1). van der Poll *et al* found a similar result (van der Poll *et al.* 1997a), whilst Takashima *et al* could only detect a difference in pneumococcal viability within the bloodstream (Takashima *et al.* 1997). In all published results this treatment was associated with reduced survival. Therefore, although the effect of anti-TNFα treatment on survival time was not assessed in this model, data from other models (and that for TNFα p55 receptor deficient mice (Figure 4.5) indicate that survival times would have been shortened following anti-TNFα treatment and therefore that systemic TNFα is required for an effective host response during pneumococcal pneumonia.

There have been no previous reports testing the effects of local TNFα within the lungs during pneumococcal pneumonia. We therefore neutralised pulmonary TNF activity via intranasal administration of the anti-TNFα antibody.
Elevated pulmonary bacterial loads following local neutralisation of TNFα revealed that pulmonary TNFα activity does play a protective role during the infection. This role is only sufficient to alter *S. pneumoniae* viability within the lungs and does not prevent the bacteria gaining access to the circulation (Table 4.3). Thus during bacteraemic pneumococcal pneumonia systemic TNFα production plays a more critical role in controlling *S. pneumoniae* than does locally produced TNFα.

In an attempt to identify which TNF receptor mediated the protective responses of TNFα during pneumococcal pneumonia receptor deficient mice were infected. As described in chapter 3, mice infected with *S. pneumoniae* pass through a range of symptoms. The first to appear is a hunched stance, they then develop piloerection before becoming lethargic. The final symptom is the unwillingness to move (moribund). On infecting TNFα p55 knockout mice 8/10 did not display the normal range of signs of illness, rather they rapidly progressed from being slightly hunched to becoming moribund. This group of mice were susceptible to the infection, with very high bacterial loads and 100% mortality by 89h into the experiment. TNFα therefore mediates the development and progression of symptoms during pneumococcal infection. Indeed animals infused with TNFα display biochemical and physiological changes similar to those seen in animals with

Gram-positive or Gram-negative bacteraemia, that is hypotension, oedema of lung interstitium and intravascular thrombosis that is fatal within minutes to hours (Tracey *et al.* 1987). Furthermore, these alterations can be inhibited by administration of anti-TNFα antibodies (Tracey *et al.* 1986).

p55-/- mice were unable to control pneumococci either within the lungs or within the bloodstream. Whether TNFα is directly responsible for reducing pneumococcal viability within lungs (e.g. by activating phagocytes) or whether p55 is involved in the up-regulation of a separate mediator is currently unknown.
Despite high levels of bacteraemia at 12h post challenge pneumococcal viability did not increase as rapidly in these mice as it did in MF1, MF1/129, iNOS-/- or CBA/Ca mice. This indicates that the heightened susceptibility of p55 mice is due to the lack of TNFα in the lungs not bloodstream (this could be proven by infection via the intravenous route). Indeed mice unable to signal through the p55 TNF receptor are more resistant to septic shock than are animals capable of signalling via p55 (see below & (Garcia *et al.* 1995)).

This increased susceptibility of p55-/- mice to pneumococcal pneumonia is supported by a previous report on these mice in *S. pneumoniae* infection (O'Brien *et al.* 1999). p55-/- mice infected intraperitoneally with pneumococci have a LD50 10,000 fold lower than that of controls with no detectable defect in neutrophil migration or acute phase response. The p55 receptor may thus act to up-regulate the anti-pneumococcal activity of those phagocytic cells already present without altering their recruitment.
Only preliminary histological examination has been carried out in our model of pneumococcal pneumonia in TNFα receptor deficient mice. Results support those of O'Brien in that there was no difference in cell recruitment at 24h post challenge. However this time point may be too early for any difference to be apparent.
Therefore it is likely that TNFα's protective role in pneumococcal pneumonia is to upregulate phagocyte antibacterial activity, signalling via the p55 receptor.

Infections of TNFα receptor deficient mice also provided evidence that TNF interacts with NO during pneumococcal pneumonia. Airways of p55-/- mice

contained significantly reduced NO levels than did p75-/- and C57B/6 mice (Table 4.5). Such a result agrees with reduced NO levels within lungs from p55-/- and p55 and p75 double deficient mice during *Toxoplasma gondii* infection (Deckert-Schlüter *et al.* 1998). These mice were unable to elevate NO levels despite elevated IFNγ, TNFα and IL-1β levels. Decreased NO levels were ultimately responsible for death in these experiments. Such an NO deficiency is unlikely to be the cause of death during pneumococcal pneumonia as iNOS-/- mice are not significantly more susceptible to *S. pneumoniae* infection than are iNOS sufficient animals (see below).

NO is not the only inflammatory mediator that TNFα interacts with. The production of TNF during pneumococcal pneumonia initiates a cascade of further mediators which act together in an attempt to control the foreign bacteria (Bergeron *et al.* 1998). In our MF1 model, increased pulmonary production of IL-1β was initiated with similar kinetics to that of TNF (Figure 3.13). By 18h post challenge production appeared to be at its peak with levels decreasing towards the end of the experiment.
Higher IL-1β levels expressed *in vivo* during pneumococcal pneumonia were detected within the airways of BALB/c mice than in CBA/Ca mice, although the difference was not of the magnitude seen with TNF activity (Figure 5.9).

TNFα and IL-β are likely to be involved in the induction of the other inflammatory mediators studied in these experiments. As with TNF activity, there appeared to be two peaks in IL-6 levels within MF1 lavage fluid, suggesting rapid induction of IL-6 by TNF (Figure 3.13).
Comparative levels of IL-6 were recovered within the airways of both BALB/c and CBA/Ca mouse strains (Figure 5.9). Thus BALB/c mice do not simply possess a greatly elevated intrinsic ability to produce pro-inflammatory cytokines within their airways. Within lung tissues, however, CBA/Ca mice were capable of releasing far higher levels of IL-6 than BALB/c mice (Figure 5.11).
It is currently unknown whether IL-6 was playing an inflammatory or anti-inflammatory role within the lungs during these infections. A previous investigation of the role of IL-6 in pneumococcal pneumonia using IL-6 deficient mice indicated that within the lungs the latter was true. These mice

displayed higher levels of TNFα and IL-10 levels within lung tissues than did control mice. However it was impossible to determine whether the significantly elevated bacterial loads were directly responsible for inducing higher levels of cytokines (van der Poll *et al.* 1997b).
Thus the two mouse strains display different pro-inflammatory cytokine profiles during pneumococcal pneumonia. To summarise, airways from resistant BALB/c animals contain higher TNF activities and similar IL-1 and IL-6 when compared to CBA/Ca animals. Samples from BALB/c lung tissues possess less TNF activity, similar IL-1 and less IL-6 than samples from CBA/Ca animals.

Results of the MF1 and BALB/c mice studies indicate that NO release is not up-regulated in murine lungs as part of the normal host response to clear an infectious challenge with *S. pneumoniae* (Figures 3.14 & 5.10). A similar lack of effect for NO was found in a mouse model of pulmonary *P. carinii* infection (Shellito *et al.* 1996).
The possibility remains that these mice do not induce high levels of NO during pneumococcal pneumonia as the lower levels are optimal for bacterial control without playing a role in pathology. Indeed, those effects of NO that occur prior to high levels of detectable NO may be more important than later effects. That is, cNOS may be more important than iNOS. For example, moderate levels of NO are more effective inducers of TNF than are very high levels (Eigler *et al.* 1993). Alternatively the higher levels of other inflammatory mediators may make NO redundant, perhaps the case with TNF in BALB/c airways.

These facts do not prove that NO is redundant within the setting of this model of pneumococcal pneumonia. NO may be mediating more subtle actions during the infection that were being masked by other factors in the previous experiments. Indeed results from infections of iNOS-/- mice reveal that NO is capable of controlling pneumococcal viability within the lungs during the initial stages of infection.
iNOS sufficient MF1/129 mice are able to prevent the initial increase in bacterial viability evident within airways of iNOS-/- animals (Figure 4.10). This is followed by MF1/129 significantly reducing bacterial viability

associated with the lung airways following the 12h time point. A similar reduction does occur in iNOS-/- air spaces but not until 12h later.
Such kinetics indicate that it is unlikely to be a direct antibacterial activity of NO and its breakdown products that is responsible for pneumococcal control. More probable is that NO up-regulates the effects of other mediators such as pro-inflammatory cytokines (Eigler *et al.* 1993) or relaxes vascular smooth muscle (Palmer *et al.* 1987), increasing blood flow to the site of infection, increasing cell recruitment.

Although iNOS -/- mice are deficient in RNI they retain the ability to release ROI. Interaction between RNI and ROI has been shown to augment cytotoxicity of the mediators (Beckman *et al.* 1990). Perhaps this interaction to increase cytotoxicity is required to control pneumococci and occurs later in iNOS-/- mice as they depend upon cNOS whereas MF1/129 mice will be able to initiate this method of kill earlier via iNOS.
A similar reduction in pneumococcal viability within MF1/129 lung tissues occurs at the same time post challenge (between 12h And 24h) [Figure 4.11]. This indicates that the same mechanisms is likely to be responsible, i.e. a factor capable of acting both within the lung tissues and airways.

Pneumococci associated with MF1/129 lung tissues are not permanently controlled despite the availability of NO. The mechanism that they utilise in order to overcome this control is not clear. The protective levels of NO may be reduced by some factor following the 24h time point e.g. IL-10 (Cunha *et al.* 1992) or NO itself (Assreuy *et al.* 1993). The balance between NO and other inflammatory mediators may alter NO's role from pro- to anti-inflammatory. In addition, higher levels of NO may inhibit production of certain cytokines (MacMicking *et al.* 1997), whilst low levels actually promote their release. It is possible that during early infection the low levels of NO induce high TNF thereby reducing bacterial viability (van der Poll *et al.* 1996), whilst later on higher NO leads to low TNF and in turn higher bacterial loads.

Alternatively, the pneumococci may produce a factor that enables the bacteria to resist NO's anti-bacterial effects. *S. pneumoniae* is known to release an inhibitor of the oxidative response (Perry *et al.* 1993). As this

inhibitor is believed to be released in high levels by autolysis, its presence would increase at later time points (perhaps also released by neutrophil-mediated killing of pneumococci) thus explaining the delay in action until late infection (Perry *et al.* 1994). As mentioned in Section 1.3.2, pneumolysin is also capable interfering with the neutrophil respiratory burst. In addition, pneumococcal extracts prepared by sonication or lysis with deoxycholate have been found to inhibit elastase activity (Vered *et al.* 1984). Elastase in a enzyme contained in neutrophil granules that is capable of degrading proteins in bacterial cell walls. Thus an elastase inhibitor may protect the bacteria against recruited neutrophils.

An inability to produce high output NO via the iNOS pathway resulted in an upregulated inflammatory response within the lungs (Figure 4.12). Significantly elevated TNF activity was evident in bronchoalveolar lavage fluid from iNOS-/- mice at 24h and 36h post challenge when compared to MF1/129 samples. As these elevated TNF activities were associated with elevated levels of total protein within lungs of iNOS-/-, TNF is implicated in mediating disruption to alveolar/capillary barrier in iNOS-/- mice. If NO was directly responsible for this disruption, NO depletion would be expected to prevent disruption. Instead removal of NO caused higher levels of TNFα and this in turn affected lung integrity.

There are several possible explanations for this elevated inflammatory response. NO may routinely act in an anti-inflammatory capacity within lungs during pneumococcal pneumonia (Kageyama *et al.* 1997). The low levels of NO produced by cNOS may be more capable of elevating pro-inflammatory cytokines than are higher levels induced by iNOS (Eigler *et al.* 1993). Finally, the higher pneumococcal viability within airways of iNOS mice is likely to act as a more potent stimulus of inflammation.

Thus during a localised infection NO production is beneficial in that it reduces bacterial loads and controls inflammatory response. This is in contrast to the role of the same mediator during systemic infection (see below).

In vitro culture of *S. pneumoniae* in the presence of the NO donor SNAP did not alter growth of the organism thus indicating that NO does not exert a direct anti-bacterial activity on *S. pneumoniae* (Figure 4.15). However, at

pH5 but not pH7 (these experiments were carried out at the latter) the NO_2^- anion is converted into nitrous acid, a compound with bactericidal activity that can generate addition RNI species such as ·NO (Kaplan *et al.* 1996). Thus at a lower pH a significant antibacterial activity may have been recorded. Such an effect has previously been documented for against *M. bovis* at pH5 but not pH7 (O'Brien *et al.* 1994). However, SNAP has been shown to exert an anti-bacterial effect against *S. aureus* (Kaplan *et al.* 1996) at pH7 . The possibility that SNAP (and thus NO) is anti-bacterial for *S. pneumoniae* at lower pH could easily be investigated in the future.

Production kinetics of the anti-inflammatory cytokine IL-10 within the lungs were delayed in comparison to those of the pro-inflammatory mediators (Figures 3.14 & 5.10). Therefore an elevated inflammatory stimulus is required before IL-10 production is increased. Within lung airways and tissues maximum levels of this IL-10 were detected concurrently with the decline in pro-inflammatory cytokine levels. Such kinetics implicate IL-10 in the control of TNFα and IL-1 (and likely a range of other inflammatory mediators) during pneumococcal pneumonia (van der Poll *et al.* 1996).
Down-regulation of inflammatory cytokines during late infection in all strains of mice might also be explained by the fact that alveolar macrophages become hyporesponsive during pneumococcal pneumonia (Dehoux *et al.* 1994). Such cells release lower levels of pro-inflammatory cytokines than either circulating monocytes from the same individual or alveolar macrophages from healthy controls. In addition, pulmonary defence against *P. aeruginosa* is significantly reduced during sepsis, an effect recently attributed to over production of IL-10 (Steinhauser *et al.* 1999). Thus pulmonary defences in CBA/Ca mice would be impaired further on the development of bacteraemia, serving to perpetuate pneumococcal survival.

BALB/c lavage fluid IL-10 levels increased during mid infection but then dropped dramatically during late infection, with a significant reduction by 120h post challenge. This decrease in IL-10 is likely to be due to the ability of IL-10 to negatively feedback upon its own production (Brown *et al.* 1996) via the destabilisation of mRNA over a period of hours. However, it is also possible that a pneumococcal product is playing a role in the down-

regulation of IL-10 levels. Cytokines have previously been shown to be susceptible to degradation by bacterial proteases. For example, the bioactivities of recombinant TNFα and IFNγ are significantly reduced by incubation with proteases from *Pseudomonas aeruginosa* (Parmely *et al.* 1990). If this is the case during pneumococcal pneumonia, it suggests that pneumococci are gaining an advantage by perpetuating the inflammation, perhaps aiding their spread out of the lungs.
IL-10 levels were higher within the lung tissues and airways of BALB/c than in the susceptible mouse strains from the initiation until the end of the infection. Elevated production of "Th2" cytokines by BALB/c mice has been recognised in other infections such as pulmonary infection with *Pseudomonas aeruginosa* (Moser *et al.* 1999). The higher tissue IL-10 levels may explain lowered TNF activities in these mice i.e. due to tighter anti-inflammatory regulation. This is the reciprocal finding from that in susceptible BALB/c *scid* mice.

This inflammatory response was accompanied by a rapid increase in the level of total protein within pulmonary airways (Figures 3.14 & 5.10). Such an increase is believed to be an indication that the integrity of the alveolar/capillary barrier has been affected permitting serum proteins to cross the barrier (the majority of which is albumin) (Rubins *et al.* 1995). The bacteria may be able to utilise some of these proteins as energy sources at the site of infection. The rapid kinetics of lung disruption have previously been demonstrated via electron microscopy in another model of pneumococcal pneumonia (Yoneda and Coonrod 1980).
This disruption was evident until the end of the experiment in susceptible mice. The relative lung disruption in these animals was greater than that in resistant BALB/c mice. Furthermore, disruption in resistant BALB/c mice was transient, only occurring until mid infection, protein levels then reached a plateau before declining again toward the end of the experiment as resolution occurred.

Cells of the immune system may be responsible for this disruption to the alveolar/capillary barrier. Increased demand for neutrophils during pneumococcal pneumonia results in release of immature cells from the bone marrow. Such cells have reduced deformability in comparison to mature

neutrophils and are less able to migrate into the alveoli (Lawrence *et al.* 1996). However the immature neutrophils do have an elevated capacity to produce oxygen radicals and proteolytic enzymes than mature cells. Partial activation of neutrophils before they migrate out of the pulmonary capillaries can result in damage occurring to healthy tissues (Hogg 1994), causing disruption to the alveolar capillary membrane and protein and fluid leakage from the vascular space into the lung interstitium and air spaces (Flick *et al.* 1981).
However, the presence of early epithelial and vascular changes in the absence of significant numbers of inflammatory cells suggests that these cells are not required for the initial disruption. A bacterial factor or early response cytokines (e.g. TNF and IL-1)are more likely candidates.

Cell wall constituents have been found to be responsible for accumulation of protein with lavage fluid. They are capable of binding to epithelia and endothelia *in vitro*, leading to separation of cells from each other, removing the endothelium's barrier (Tuomanen *et al.* 1987). The direct pore forming action of pneumolysin, may alter the integrity of the alveolar/capillary barrier permitting influx of protein. Formation of pores leads to ionic disequilibrium which in turn creates gaps in the endothelium between cells (Suttrop *et al.* 1988). In addition, pneumolysin is capable of direct interaction with the inflammatory response (for example complement, Section 1.5.3.4), perhaps initiating damage to lung integrity. *In vitro* pneumolysin is also capable of inducing the production of TNFα and IL-1 by monocyte cell lines. These cytokines have been implicated in disruption to the alveolar/capillary barrier as their *in vivo* administration results in damage to pulmonary vascular endothelium (Okusawa *et al.* 1988) & (Stephens *et al.* 1987). Furthermore, release of TNFα and IL-1 from endothelial cells occurs in response to pneumococcal adherence. These cytokines were shown to cause separation of tight junctions and eventual disruption of monolayers. This effect was not mediated by NO as the NO inhibitor N-monomethylarginine did not inhibit the cytopathic effects (Geelen *et al.* 1993).
Further evidence for TNFα's involvement in this disruption is found in the lower levels of protein associated with lavage fluids from p55-/- mice than half those in C57Bl/6 or p75-/- mice. This fact implicates the p55 TNFα receptor in disruption to the alveolar/capillary barrier during pneumococcal

pneumonia, either directly or via induction of NO (as NO levels were significantly reduced in p55-/- mice). TNFα and IL-1 may also play a role in the instigation of tissue damage via production of iNOS. This enzyme then leads to release of free radicals and oxidants via one of the two pathways described in Figure 1.4. Such products of iNOS are known to mediate epithelial/endothelial damage. The elevated NO levels within airways of CBA/Ca animals (Figure 5.10) may therefore be involved in disruption to pulmonary integrity during end-stage disease.
Alveolar epithelial cells are capable of active transport of salt and water from the airspace to the interstitial compartment (Cott *et al.* 1986). Indeed removal of excess fluid from the alveoli occurs in part by active transport mechanisms (Sakuma *et al.* 1994). Resolution of inflammation within BALB/c lungs is likely to be aided by these transport mechanisms.

Initiation of this disruption to lung integrity was quickly followed by recruitment of phagocytic cells, a characteristic of inflammatory responses initiated by cells resident at the site of infection. The cell influx to infected MF1 lungs was evident from 12h post challenge as oedematous areas with few associated cells (Figure 3.12). This was followed by a rapid increase in cell numbers through 24h and 36h until by 48h post challenge large areas of the lungs were consolidated with recruited cells. Utilisation of immunohistochemistry identified the majority of cells associated with this bronchopneumonia as neutrophils, a finding confirmed by previous work of this laboratory (Kadioglu *et al.* 2000) & (Canvin *et al.* 1995) and others (Bergeron *et al.* 1998). A similar cell influx was evident within infected CBA/Ca lungs (Figure 5.13).
Rapid neutrophil recruitment was also evident within the lungs of BALB/c mice. In these mice cell influx mainly occurred within the airways and occurred by 12h post challenge (Figure 5.13). Additional macrophages begin to arrive inside inflamed bronchioles and in perivascular tissue areas surrounding such bronchioles 24-48h post challenge (Kadioglu *et al.* 2000) & (Bergeron *et al.* 1998). These cells are responsible for a longer lived but slower anti-microbial effect and resolution of the disease.

Resident macrophages within the alveoli and interstitium of all strains of mice are ideally situated to act as primary defences against the inoculated

bacteria (Jonsson *et al.* 1985) and are capable of recruiting additional cells as required. These cells constitute the vast majority of cells recovered within bronchoalveolar lavage fluid from healthy mice whilst neutrophils constitute less than 0.15% of total cells (Vial *et al.* 1984). Activation of resident macrophages may be responsible for recruitment of neutrophils via production of pro-inflammatory cytokines or chemokines or via activation of the complement pathway (Section 1.5.3.4). Neutrophils are highly efficient phagocytes capable of arriving in significant numbers within the airways and tissues by as little as 4h after instillation of pneumococci (Vial *et al.* 1984), (Doerschuk *et al.* 1994) & (Pierce *et al.* 1977) with migration finished by around 24h post challenge (Haslett 1992), fitting with the kinetics of reduced pneumococcal viability evident in Figures 4.10 & 4.11.
A defect in the ability of CBA/Ca neutrophils to reach the lung airways as early as those in BALB/c mice would play a role in determining the outcome to infection. Potential reasons for different neutrophil recruitment in the two mouse strains include the original chemotactic signal or a different intrinsic response on behalf of the neutrophils themselves.

Observations in this laboratory (Gingles *et al.* manuscript in preparation) have found that BALB/c mice have statistically higher numbers of neutrophils within their lungs (whole lungs only, airway cell influx was not measured) than CBA/Ca mice at both 12h and 24h post challenge with 10^6 CFU D39. Maximal neutrophil recruitment occurred between 6h and 12h post infection. BALB/c mice also develop a higher level of pathology during *Toxoplasma gondii* encephalitis but go on to survive whilst CBA/Ca mice develop less of an inflammation and are susceptible (Hunter *et al.* 1994). Additionally, Morissette *et al* (Morissette *et al.* 1995) have shown that BALB/c mice, resistant to *Pseudomonas aeruginosa* infection, have a heightened neutrophil influx during early infection. Cellular recruitment correlated to the ability of BALB/c mice to control the growth of bacteria within the lungs.
Neutrophils could conceivably be responsible for the initial, rapid pneumococcal kill evident within BALB/c airways and tissues whilst other cell types e.g. additional macrophages are responsible for the more gradual reduction in pneumococcal viability.

Pulmonary lymphocyte numbers are also known to change during pneumococcal pneumonia. By 24h post challenge significantly elevated numbers of these cells surround inflamed bronchiole walls. During end stage disease lymphocytes migrate further into the tissue surrounding these bronchioles and into the perivascular tissues (Kadioglu *et al.* 2000).
Lymphocytes have previously not been implicated in the host response during pneumococcal pneumonia (Winkelstein and Swift 1975). This influx to and migration around the lungs led us to investigate their role via infections of BALB/c *scid* mice.

Systemic bacterial loads are significantly higher in the immunocompromised mice than in control BALB/c animals (Table 5.3) indicating that lymphocytes are required for protective immunity against *S. pneumoniae.*
Pro-inflammatory cytokine production was not impaired (Tables 5.4 & 5.5), so lymphocytes are not responsible for the production of TNFα, IL-1 and IL-6 during pneumococcal pneumonia. Engwerda *et al* have shown that *scid* mice infected with *Leishmania donovani* have a much lower pro-inflammatory response than BALB/c mice (Engwerda *et al.* 1996). This suggests that the elevated bacterial loads with *S. pneumoniae* infected mice do act as a stronger stimulus of cytokines in this model. Lymphocytes are likely to be major producers of the anti-inflammatory cytokine IL-10 during pneumococcal pneumonia since levels of this cytokine were significantly reduced in BALB/c *scid* mice.

In order to validate these findings it would have been useful to adoptively transfer some B and T cells from BALB/c mice into BALB/c *scid* mice and then infect them as previously. Such treatment has previously been shown to prevent movement of *E. coli* out of the gastrointestinal tract in mice depleted of T cells by thymectomy and administration of anti-T cell antibody (Gautreaux *et al.* 1995). A resistant phenotype would indicate that B and T cells are required for protection in pneumococcal pneumonia whilst a susceptible phenotype would indicate that the difference documented in these experiments are due to other factors for example defects in other myeloid cells such as γδ T cells.
The classic use of *scid* mice in infection studies is to investigate innate defence mechanisms as specific responses are depleted. The classic

hypothermia increasing survival times (Eiseman *et al.* 1956). Thus the early induction of hypothermia in BALB/c mice may be beneficial. Pneumococci cannot multiply as quickly at hypothermic temperatures as at 37°C (both in vivo and in vitro (Eiseman *et al.* 1956) & (Eiseman *et al.* 1964), indicating that reducing core body temperature may be the hosts attempt to halt bacterial proliferation.
Additionally the immune response may be affected. Low temperatures (~30°C) down-regulate cytokines levels and activities released by helper T cells (Hanson 1997), phagocytic index is reduced during hypothermia and leukopenia is present (Eiseman, *et al.* 1956) & (Eiseman *et al.* 1964). Taken together these results suggest that hypothermia is associated with a down-regulation of the inflammatory response. Timing of this effect in BALB/c mice suggests that lowered T_{co} regulates inflammation before it can have a detrimental effect. The delayed onset of this response in CBA/Ca and MF1 mice occurs too late to have a beneficial effect as overwhelming inflammation has already been initiated.

The initiation of hypothermia in these mouse strains occurred rapidly after peak pulmonary inflammation. Such kinetics suggest that one or more of the inflammatory mediators may be playing a role in the development of hypothermia displayed by infected mice.
Each of the mediators studied have been implicated in Tco alteration. Injection of TNFα alone in to mice induces a period of hypothermia (Kozak *et al.* 1995) whilst the same treatment in rabbits induces a febrile response (Dinarello *et al.* 1986). Neutralisation of TNFα activity *in vivo* results in elevated febrile responses following LPS injection whilst injection of recombinant TNFα prolongs the fall in Tco evident following LPS treatment. It has recently been found that systemic TNF is more likely to be detected and at higher levels within the circulation of hypothermic patients than in hyperthermic patients (>80% vs.<20%) (Arons *et al.* 1999). It has also become clear that lower levels of TNFα induce febrile responses whilst higher levels induce hypothermia (Kettlehut *et al.* 1987).

As IL-6 is known to induce febrile responses (Leon *et al.* 1998) & (Helle *et al.* 1989), it may also be able to prevent the development of hypothermia. Therefore the elevated IL-6 levels within the lungs of CBA/Ca and MF1 mice

in comparison to BALB/c mice may prevent the decline in T_{CO} that is evident in BALB/c mice during early infection.
The ability of these mediators to induce hypothermia during pneumococcal pneumonia could be tested by the neutralisation of TNFα or IL-6 with antibodies (as in Section 4.2.2) prior to infection of implanted animals. If these mediators are responsible, their neutralisation would be expected to block the development of hypothermia. Conversely, the administration of recombinant TNFα or IL-6 would be expected to cause a decline in Tco, also readily examinable in this model.

The classic model of temperature alteration states that production of pro-inflammatory cytokines such as IL-1β, TNFα and IL-6 in the circulation acts as an inducer of Tco change (Netea *et al.* 1999). On reaching the brain these cytokines induce the release of prostaglandins which are responsible for the actual response. More recently, the possibility that cytokines can alter core body temperature without being detectable in the circulation has been suggested (Netea *et al.* 1999). The highly labile nature of pro-inflammatory cytokines within the bloodstream makes this a likely possibility.

Alternative methods that pulmonary cytokines may employ to alter core body temperature include the induction of mediators such as phospholipase 2 (Rintala and Nevalainen 1993). This in turn stimulates prostaglandin release and temperature change. Alternatively the cytokines may remain membrane-bound and therefore undetectable in serum. The cell association would not prevent them activating endothelial cells within the brain and stimulating temperature change. Indeed Munoz *et al* have shown that cell-associated TNFα, IL-1α and IL-1β are more frequently identified in the circulations of febrile individuals than are the soluble forms (Munoz *et al.* 1991).

It has previously been shown that instillation of lower numbers of bacteria into the lungs causes a reduced inflammation to that seen with higher doses. This indicates that resident pulmonary cells are capable of containing low levels of inflammation, only recruiting additional cells once they are overcome (Toews *et al.* 1979). In addition, the number of inflammatory cells recruited to the lung depends on the size of the bacterial inoculum (Vial *et*

al. 1984). Taken together these data indicates that there is a threshold number of bacteria required to initiate the pulmonary inflammatory response during pneumococcal pneumonia. Infections were thus carried out with lower doses of *S. pneumoniae* in order to investigate whether the inflammatory response would be reduced following intranasal infection with reduced doses of *S. pneumoniae.*

Decreasing the number of bacteria inoculated permitted the innate defences of most mice to successfully counter the bacteria. However, reducing the infectious dose 100 fold still resulted in some of the MF1 population succumbing to the infection (Figure 3.18). Those mice challenged with the 10^5 CFU dose of *S. pneumoniae* that succumbed to the infection mainly responded in a similar manner as those infected with 10^6 CFU but with slightly delayed kinetics. However, reducing the dose 10 fold further did significantly reduce the inflammatory response.

The majority of mice in the 10^4 or 10^5 CFU groups did not have TNF activity within their airways, nor did these mice have high numbers of viable bacteria in their airways. 11/54 in the 10^4 CFU group had less than log 3.00 CFU/ml with only 4/56 animals in the 10^5 CFU group possessing higher than log 4.00 CFU/ml throughout these experiments. Such low levels of bacteria were only found in association with lavage fluid TNF activity in the 10^6 CFU dose when a long "incubation time" had occurred e.g. 24h or 30h post challenge. Therefore, pneumococci instilled into the lungs of mice at reduced doses require a lengthened incubation period on order to induce detectable TNF activity. This hypothesis would explain the slightly slower kinetics of inflammation and death seen following infection with 10^5 and 10^4 CFU *S. pneumoniae.* This possibility could be investigated by varying incubation periods of pneumococci during the *in vitro* stimulation assay reported in Chapter 5.

As MF1 mice are an outbred population, a group of these mice would contain a range of genotypes. Some mice will be more similar to CBA/Ca mice with others similar to BALB/c. This explains the ability of some mice to resist infection with lower numbers of pneumococci whilst others remain susceptible.

Previous reports indicate that inflammatory responses are altered in elderly populations (Bruunsgaard *et al.* 1999). It is therefore possible that an altered inflammatory response during pneumococcal pneumonia is responsible for the elevated susceptibility of elderly humans to pneumococcal pneumonia (Burman *et al.* 1985). This possibility was investigated in the MF1 mouse model of the disease.

1yr old MF1 mice displayed slightly lower survival times than young MF1 mice but there was no significant alteration suggesting that 1yr old MF1 mice were not elderly enough (Figure 3.23). A previous report found that this age of mice were no different from 2 month old mice in respect of cytokine and NO production during endotoxic shock (Chorinchath *et al.* 1996). However, studies with these MF1 mice could not have been postponed for a further year as they were already displaying mortality due to natural causes.

Time course experiments for bacterial loads and cytokine levels in aged mice were ended at 30h post challenge in contrast to the normal 36h in young mice due to several of the aged mice displaying high levels of bacteraemia (which also developed earlier) and appearing moribund. These mice were found to possess elevated pro-inflammatory cytokine production within their lungs. Aged animals are known to produce heightened levels of TNF within their serum during endotoxic shock (Chorinchath *et al.* 1996). Significantly earlier and elevated TNF responses within airways of aged mice in this model of pneumococcal pneumonia support this hypothesis (Figure 3.27). TNF is not the only pro-inflammatory cytokine that is upregulated with age. Following LPS injection, elevated TNF and IL-6 levels are displayed in the serum of aged Fischer rats when compared to young animals (Foster *et al.* 1992).

Aged mice constitutively released significantly lower levels of NO than younger mice. However, aged animals were capable of rapidly and significantly increasing this NO production whilst young mice did not. This indicates that iNOS activity is elevated during ageing whilst cNOS activity may decline. Previous research has also indicated that NO production following endotoxin treatment of mice increases with age (Chorinchath *et al.* 1996).

The literature in this field and results presented here show that elderly mice and humans have an upregulated and prolonged inflammatory response during infection. Rather than providing protection, this response is likely to be involved in lung pathology. These facts explain the earlier progression to systemic infection during pneumococcal pneumonia evident in aged mice. Upregulated inflammation may cause more pulmonary disruption earlier following infection, permitting *S. pneumoniae* earlier access to the circulation.

In addition to aged MF1 mice, BALB/c mice have an upregulated early inflammatory response within their lungs, but in this case it is controlled and protective. The elevated TNF activities within BALB/c airways may not be the only factor involved in the resistant phenotype of BALB/c mice during pneumococcal pneumonia.

The presence of increased endogenous protein levels in the lungs of BALB/c mice may result in increased availability of proteins related to innate immunity. These could include antibodies, complement or surfactant. Alternatively a protein that possess direct bactericidal properties (such as lysozyme) may account for the elevated protein levels.

The role played by surfactant in this model of pneumococcal infection was further investigated in these studies. Surfactant phospholipids did not alter viability of pneumococci in vitro indicating a lack of direct bactericidal action (Figure 4.1). A similar lack of effect has been gathered for incubation of *S. pneumoniae* in human alveolar lining material (Jonsson *et al.* 1986). However, Coonrod *et al* have previously found that rat pulmonary surfactant will accelerate the decline in viability of *S. pneumoniae* over several hours, an effect attributed to a detergent-like action of long-chain free fatty acids (Coonrod *et al.* 1984). ALEC might have exerted a similar effect in these experiments if the incubation time had been extended. It is a possibility that this is the effect that ALEC exerted when it was co-administered with *S. pneumoniae* into the lungs of mice (Figure 4.2). The viability of the bacteria may not have been affected immediately following inoculation into the lungs but with time it could have declined until the levels detected at 24h were reached.

In addition to a possible direct bactericidal action of ALEC, pneumococcal viability may have been reduced by virtue of the ability of surfactant material

to enhance alveolar macrophage recruitment *in vitro* (Schwartz and Christman 1979) or to increase phagocytosis of Gram-positive bacteria (*S. aureus*) by rat alveolar macrophages *in vitro* (O'Neill *et al.* 1984).
However, bacterial loads within lungs 24h post challenge with co-administration of ALEC are similar to those normally recovered immediately following infection. This suggests that ALEC does not exert its effect by decreasing pneumococcal viability within the lungs but rather by preventing the increase. ALEC may be in some way capable of preventing *S. pneumoniae* from gaining access to nutrients. Indeed a modified natural surfactant preparation containing 99% lipids and 1% protein (no SP-A or SP-D) instilled into the lungs of pre-term rabbits was shown to reduce proliferation of B streptococci in comparison to streptococci within untreated lungs (Herting *et al.* 1997). As with the *in vivo* ALEC experiments this treatment did not significantly alter survival times.
Whatever the action, some pneumococci were obviously resistant since administering the infectious dose in ALEC did not significantly alter survival times. Perhaps 10^6 CFU was too high a dose to be affected completely by surfactant (Figure 4.4). These pneumococci were capable of proceeding to cause the infection and subsequent death of the animals.

ALEC was unable to alter *in vitro* growth of *S. pneumoniae* in optimal growth conditions (BHI growth media plus 5% ALEC, Figure 4.3). In similar investigation by Rubins *et al* (Rubins *et al.* 1996), incubation in 20% pulmonary surfactant was found to inhibit growth and then cause a rapid decline in bacterial viability. As little as 2.5% pulmonary surfactant was found to have a bactericidal effect on pneumococci, with 5% causing a significant reduction in bacterial survival in vitro. Such a discrepancy indicates that some other constituent of surfactant rather than phospholipid is responsible for this effects, for example the protein constituent (Schagat *et al.* 1999).

Although surfactant can enhance immunity as stated above, it can also suppress some aspects of it. Surfactant has been shown to prevent the activation and proliferation of lymphocytes exposed to various stimuli. Surfactant lipids (from humans, pigs or rabbits) are capable of significant suppression of the proliferative response of a mixed lymphocyte population

from human blood following phytohaemagglutinin treatment (Wilsher *et al.* 1988). By fractionating human amniotic fluid surfactant Catanzaro *et al* have shown that the surfactant associated lipids phosphatidylcholine, phosphatidylglycerol and phosphatidylinositol (i.e. constituents of ALEC) are responsible for the inhibition of lymphocyte effector functions such as NK cell mediated lysis of human myeloid target cells (Catanzaro *et al.* 1988). This result indicates that surfactant does not inhibit lymphocyte activity by simply preventing proliferation. Artificial surfactants are also known to act in an anti-inflammatory manner, capable of reducing TNF, IL-1 and IL-6 production by human alveolar macrophages stimulated with endotoxin (Thomassen *et al.* 1992). Neutrophil and macrophage functions can also be inhibited by surfactant preparations. A reduced capacity to adhere *in vitro*, to respond to chemotaxis and to produce superoxide have all been recorded in the past (Suzuki *et al.* 1992) as has a reduction in pulmonary macrophage bactericidal activity (Sherman *et al.* 1988).

It is possible that ALEC may initially confer resistance to the host by preventing pneumococcal proliferation. The anti-inflammatory activities of surfactant phospholipids would then have the opposite effect, phagocytes would not be recruited due to the lack of cytokine and chemokine expression. This would permit pneumococci to proliferate, reaching comparable levels to those in mice not treated with ALEC and causing the subsequent death of the animals.

A separate mechanism of resistance against *S. pneumoniae* that may be employed by BALB/c mice is the endogenous possession of type-specific antibodies. The ability of BALB/c mice to clear approximately 10^6 CFU *S. pneumoniae* from the lungs has previously been described (Vial *et al.* 1984), in this case capsular type 3 pneumococci were used. Resistance therefore does not appear to be dependent upon capsular type of the pneumococcus and it is unlikely to be due to endogenous anti-capsule antibodies (unless the mice possess antibodies against a range of capsular types). Indeed the possibility that BALB/c mice possess antibodies against type 2 pneumococci has previously been investigated by this laboratory. No such antibodies could be detected in unchallenged mice (Gingles *et al.* manuscript in preparation).

The protective effects of type specific anti-capsule antibody in the circulation during pneumococcal pneumonia are well known (MacLeod *et al.* 1945) & (Wood Jr 1940). These effects have not previously been investigated within the local environment of the lungs. Results presented in this thesis show that anti-capsule anti serum mainly exerts its effects in the bloodstream. Systemic administration resulted in reduced pneumococcal viability within each body site investigated but intranasal administration did not exert significant effect. The larger population of phagocytes located within the spleen and liver than in the lungs is likely to be involved in this observation. Indeed studies in both animals and humans have revealed the spleen and liver as the major organs involved in clearing bloodborne bacteria (Bruyn *et al.* 1992). As in the lungs, effective clearance of pneumococci by the liver is dependent on opsonisation by type-specific antibodies or complement (Friedman and Moon 1980). The spleen does not require this process to have taken place (Brown *et al.* 1981) due to its anatomical arrangement which allows prolonged contact between the phagocyte and the blood borne pathogen (Wara 1981) although opsonisation will still improve phagocytosis.

Although possession of anti-capsule antibodies is unlikely to be involved in BALB/c resistance to the infection it may be that these mice possess higher levels of some other protective antibodies, e.g. anti-phosphocholine antibodies. Such antibodies have been detected in the serum from normal mice (Briles *et al.* 1981) and were shown to be protective against intravenous challenge with type 3 pneumococci.
McDaniel *et al* have shown that these antibodies do increase blood clearance of the organisms (McDaniel *et al.* 1984). The most likely explanation for the mechanism of protection is that these antibodies act by mediating phagocytosis.
Another opsonin that may be responsible for increasing pneumococcal clearance from the circulation of BALB/c mice is C-reactive protein (CRP). A major role of CRP in infection is to opsonise bacteria by initiating the early complement pathway which leads to deposition of C3b on the bacterial surface (Kaplan and Volanakis 1974). This promotes phagocytosis of the bacteria and therefore clearance from the host . Additional relevant effects of CRP on the immune response include its ability to induce monocytes to

release monocyte chemottractant protein-1 (MCP-1) thereby recruiting addition cells to a site of infection (Zhou *et al.* 1995) and up-regulation of microbicidal activities of monocytes and neutrophils by increasing O_2^{-}. and H_2O_2 levels (Zeller and Sullivan 1992).

Complement has also previously been shown to play an important role in the clearance of pneumococci from the bloodstream (Hosea and Frank 1978). Treatment of guinea pigs with cobra venom factor significantly impaired their ability to clear pneumococci administered intravenously. In contrast C4 deficient animals were able to control the bacteraemia, indicating that the alternative complement pathway was most important for host defense in this setting.

The presence of higher levels of anti-phosphocholine antibodies, CRP or complement within the circulation of BALB/c mice could also account for some of the non-specific immunity to *S. pneumoniae* and may act to control the infection until protective levels of anti-capsular antibodies can be induced around 5 to 6 days following challenge (Briles *et al.* 1981).

One cytokine that has also been implicated in systemic host response during pneumococcal pneumonia is IFNγ, known to be protective against intracellular organisms. Rubins and Pomeroy have shown that IFNγ deficient mice have a 20-fold lower LD_{50} dose following intranasal infection with virulent *S. pneumoniae* than do wild type animals (Rubins and Pomeroy 1997). Investigation of IFNγ production in wild type mice revealed that systemic levels increased significantly during the infection whilst pulmonary levels were unchanged. These results thus indicate that IFNγ is an important mediator of the host defense against *S. pneumoniae* in the bloodstream but may be less effective in the lungs.

An additional range of cytokines have been shown to be involved in host defence against pneumococcal bacteraemia caused by less virulent strains of *S. pneumoniae*. In this setting intravenous inoculation of D39 leads to exponential growth of the bacteria until death of the mice 24-36h post challenge when the level of bacteraemia reaches approximately 10^9 CFU/ml (Benton *et al.* 1998). In contrast, infection with pneumolysin deficient bacteria results in bacteraemia that does not pass the $10^6/10^7$ CFU/ml level with mice not succumbing to the infection for several days. A subsequent inoculation of pneumolysin sufficient D39 into these same mice is no longer as rapidly fatal, indicating that immunity is conferred to the host by infection

with pneumolysin-deficient bacteria. Cytokines have now been implicated in this protective response. Mice infected with pneumococci unable to produce pneumolysin release lower levels of TNFα within their circulation than do animals infected with D39. These low levels of TNFα are in some way protective as their neutralisation significantly reduces survival during bacteraemia caused by pneumolysin deficient bacteria. A similar effect on survival was observed following IL-1β neutralisation (Benton *et al.* 1998). Taken together these data indicate that low systemic levels of TNFα and IL-1β are implicated in the protective host response and immunity evident following infection with less virulent *S. pneumoniae* strains. These cytokines may exert their effects locally within the spleen (by increasing phagocytosis of pneumococci) and thus would not be found in high levels within the circulation. Therefore low levels of pro-inflammatory cytokines within the circulation result in protection without pathology.

Utilisation of the intravenous route of infection permitted investigation of the hypothesis that the resistant phenotype of BALB/c was mediated solely by the lungs. Systemic infection resulted in the same clinical signs of infection as in pulmonary model within a similar time frame. CBA/Ca mice rapidly progressed through piloerection and lethargy to become moribund by around 30h post challenge. Similar numbers of pneumococci were detected within the circulation of intravenously challenged CBA/Ca as at the end of pulmonary infection (Table 5.1 & Figure 5.17). Thus there is a threshold number of bacteria responsible for initiating the lethal event within this model of pneumococcal infection (see below).

The nature of the intranasal infection experiments precluded us from determining whether any BALB/c mice that displayed bacteraemia would progress to develop the disease or survive. However, the ability of BALB/c mice to resist intravenous infection with 7.00 $x10^4$ CFU *S. pneumoniae* shows that a level of bacteraemia lower than this would not prove fatal for BALB/c mice. In a situation where pneumococci were constantly being shed from the lungs into the bloodstream, they may no longer be able to contain the infection, explaining why four mice did die during these experiments. In addition, Briles *et al* have previously shown that BALB/c mice are susceptible to intravenous infection with D39 but the LD_{50} was greater than

10^6 CFU (Briles *et al.* 1986). Therefore, with low numbers of pneumococci, BALB/c mice can control the infection and survive, but with larger inoculations of the micro-organisms, the defence mechanisms are overcome and the infection becomes fatal.

In contrast to pulmonary inflammation, systemic inflammation is likely to be detrimental during infection, contributing to septic shock (Sriskandan and Cohen 1995).
Systemic TNF activity was only detected during the end stages of infection in all of these experiments. This fact can be explained by the low levels of viable bacteria within bloodstreams until this time. During all experiments with young MF1 mice, detectable TNF was only found in serum from one mouse with fewer than 2.88×10^7 CFU/ml, whilst all mice with more severe bacteraemia had detectable TNF activity. In addition, all CBA/Ca mice with detectable systemic TNF activity possessed levels of bacteraemia higher than log 6.46 (2.88×10^6 CFU/ml) whilst BALB/c mice did not have high levels of bacteraemia nor circulating TNF activity.
This threshold level is in agreement with data presented by Benton *et al* (Benton *et al.* 1998). In this study, mice infected intravenously with 5×10^4 CFU of *S. pneumoniae* deficient in pneumolysin did not produce TNFα in significant levels whereas D39 infected mice did. It was not until levels of bacteraemia in D39 infected mice reached approximately $5x10^7$/ml that detectable levels of TNF protein were induced. Such high numbers of bloodborne bacteria were not seen in mice infected with pneumolysin deficient bacteria, perhaps explaining the lack of systemic TNF in these mice.
The threshold hypothesis also fits with the data from the *in vitro* stimulation experiments reported earlier. Stimulation of airway cells with heat killed pneumococci only resulting in TNF activity when the highest dose of bacteria (10^6 CFU) was employed (Figure 5.14).

The exact mechanism of death during bacteraemic pneumococcal pneumonia remains unknown. Even days after initiation of antibiotic treatment, when the site of infection has returned to being sterile, patients can still die (Austrian and Gold 1964). The general belief is that an overzealous host response to infection is the main culprit with antibiotics

releasing increased levels of pneumolysin and cell wall products to increase the inflammation.

During severe sepsis overwhelming inflammation is believed to lead to death. In less severe sepsis higher levels of anti-inflammatory cytokines are able to control the inflammation and promote survival (Walley *et al.* 1996). Similar results have also been reported in a model of pneumococcal pneumonia (Mohler *et al.* 1998) where infection with virulent *S. pneumoniae* resulted in delayed and increased expression of pro-inflammatory cytokines compared to an avirulent strain. Thus, the fine balance between survival and death in infectious disease depends on the balance between pro-inflammatory and anti-inflammatory cytokines.

During the studies reported in this thesis, production of a range of inflammatory mediators has been characterised. All of these have been implicated in the pathology and even lethality following infection or endotoxin shock. Although TNF levels were the only marker of systemic inflammation investigated, comparison to other models of Gram-positive bacteraemia can help identify production kinetics of other cytokines in this model. Yao *et al* discovered that IL-1 levels increased concurrently with TNF, peaking early after bacterial introduction with IL-6 production not maximal until 72h post stimulus (Yoa *et al.* 1997). Thus TNFα is implicated in the initiation of inflammation.

Levels of detectable TNF activity were highly variable in all of the mouse strains used in these studies. Such variability is also associated with human disease, Marks *et al* were able to measure TNF in only 12 of 32 patients suffering from Gram-positive sepsis (Marks *et al.* 1990). It is thus a possibility that the variability seen in human models is also dependant on the level of bacteraemia.

Despite these highly variable reports of systemic TNFα detection during Gram-positive bacteraemia, one factor that is reproducible in human models of bacteraemia is the positive correlation between circulating TNF and mortality (Hindshaw *et al.* 1992) & (Marks *et al.* 1990). TNF is implicated in pathogenesis of septic shock as administration of antibodies directed against TNFa has been shown to be protective in mice (Tracey *et al.* 1986). However, investigations of the role of TNF during pneumococcal pneumonia have attributed this cytokine a protective role (Section 4.2.2, (Takashima *et al.* 1997) & (van der Poll *et al.* 1997a)), up-regulating recruitment of phagocytes and

preventing bacteraemia. Such a dual effect of TNFα has been demonstrated in other models of infectious disease. For example, in *Listeria* infection excessive production of TNFα may be associated with dramatic shock and organ failure, while neutralisation of TNFα activity resulted in increased bacterial loads and decreased circulating granulocytes (van Furth *et al.* 1994). Therefore the role of TNF is likely to be dependent on the location, timing and magnitude of the response.

The slower disease progression in p55-/- mice than in MF1 (or CBA/Ca) animals indicates that TNFα does initiate the lethal event during Gram-positive bacteraemia. However, as mice unable to signal via p55 are resistant to septic shock (Garcia *et al.* 1995) it is unlikely to be TNFα that is directly responsible, however. A similar prolonged infection occurred in BALB/c *scid* mice. Despite these animals having very high bacteraemia levels and detectable TNF activity at 30h post challenge the median survival time was 63h. Furthermore, the CBA/Ca mice with highest levels of bacteraemia did not have detectable systemic TNF activity at the time of their death (Section 5.2.1.6.3).

IL-1 production is a likely candidate for mediator of lethal event in the absence of high levels of TNFα. *In vivo* administration of IL-1β results in hypotension, elevated heart rate and leukopenia (as does TNFα administration), all symptoms of a shock-like state which might be fatal (Okusawa *et al.* 1988). Plasma levels of both TNFα and IL-1β correlate well with severity of shock and mortality (Grüdlund, *et al.* 1995).
IL-1β may act independently or it can induce production of IL-6, also capable of mediating pathological events. High levels of IL-6 have been observed in patients and animals with Gram-positive and Gram-negative sepsis (Calandra *et al.* 1991). In comparison to other inflammatory cytokines, levels of IL-6 detected in serum from patients during sepsis correlate well with outcome. Excessive production of this cytokine is considered a good marker of an elevated inflammatory response and poor prognosis both in humans and in rodents (Damas *et al.* 1992). In a porcine model of pneumococcal septicaemia over production of IL-6 was associated with mortality (Ziegler-heitbrock *et al.* 1992). Furthermore, treatment of sepsis with anti-TNF antibody reduces IL-6 levels and leads to 100% survival (from 0%)

(Hindshaw *et al.* 1992). A similar improvement in survival in a mouse model where lethal intraperitoneal doses of *E. coli* were inhibited with anti-IL-6 antibodies, has been reported (Starnes *et al.* 1990). Conversely a protective role has been ascribed to IL-6 in a model of septic shock initiated by LPS-galactosamine. Neutralisation of IL-6 in this setting enhance mortality (Barton and Jackson 1996). Thus the balance of IL-6 and other mediators is likely to determine the role played by this cytokine during disease.

TNFα, IL-1 and IFNγ can act synergistically to induce production of NO in vascular smooth muscle cells (Sriskandan and Cohen 1995). Indeed, serum levels of this mediator are elevated during sepsis (Evans *et al.* 1993). Furthermore, inhibition of iNOS in endotoxin treated animals prevents the development of shock (Evans *et al.* 1994). Despite this fact, inhibition of nitric oxide synthase has not been conclusively demonstrated to promote survival in models of live bacterial sepsis. We therefore investigated the role played by iNOS in pneumococcal bacteraemia via intravenously infecting iNOS-/- mice.

MF1/129 mice succumbed significantly earlier (and in higher numbers) following intravenous infection than did iNOS-/- mice, with significantly elevated pneumococcal viability associated with their circulations (Figures 4.13 & 4.14). Results suggest that the low dose of pneumococci used in this experiment was around the threshold number of bacteria required to initial a fatal bacteraemia in mice on the MF1/129 background (as 3/5 iNOS-/- cleared the bacteraemia and survived). However, this dose of *S. pneumoniae* was sufficient to initiate a fatal Gram-positive septic shock via an NO mediated pathway. NO is known to cause relaxation of vascular smooth muscle (Palmer *et al.* 1987), contributing to hypotension. NO is also implicated in the reduced myocardial contractility evident during sepsis. It was shown that TNFα, and IL-6 could prevent contractions of hamster heart muscles *in vitro* and that these contractions were blocked by NO inhibition (Finkel *et al.* 1992). The development of hypotension and hypoxaemia due to vasodilation and cardiac dysfunction leads to acidosis and hypoperfusion of tissues and eventually leads to death (Sriskandan and Cohen 1995). Further investigation of the interactions of NO with other inflammatory mediators during Gram-positive septic shock may be possible with this model. Results may prove particularly relevant as the lung represents the source of infection

in the majority of cases of septic shock in humans (Sriskandan and Cohen 1995).

IL-10 is known to regulate the levels of all of these inflammatory mediators implicated in systemic inflammation. Indeed repeated injection of mice from birth with anti-IL-10 antibody renders them highly susceptible to endotoxic shock (Ishida *et al.* 1993). Thus during overwhelming sepsis the levels of IL-10 released into the bloodstream, along with other anti-inflammatory mediators (such as soluble TNF receptors and IL-1Ra) are insufficient to control the inflammation and shock results.

The overall body of evidence presented in this thesis and in other literature suggests that the "inflammatory balancing act" that occurs during bacterial infection depends on whether the infection is localised or systemic. During localised lung infections, a pro-inflammatory response is required to rapidly and effectively clear the bacteria before dissemination can occur. In the setting of systemic infection, an overwhelming pro-inflammatory response will result in shock and death.

In the case of this model of pneumococcal pneumonia the balance must tip in favour of an inflammatory response during the early stages of disease thus elevated TNF activity (mediated by the p55 receptor) and the ability to express iNOS is beneficial. If and when systemic infection occurs the balance must tip in the opposite direction with tight regulation of inflammatory mediators by IL-10. Data from this model and others implicates TNFα as the initial mediator in the shock-like response during bacteraemic pneumococcal pneumonia. TNF induces other factors such as NO which is implicated in the shock like end-stages of disease. However, the inflammatory mediators studied in this thesis are by no means the only repertoire that the host produces to combat *S. pneumoniae*. It is highly likely that a range of other host factors such as other interleukins, chemokines and metabolites of the arachidonic acid pathways, which were beyond the scope of this study, are involved in both the protection and pathology of bacteraemic pneumococcal pneumonia.

A detailed description of the initiation and regulation of the alveolar inflammatory response by mediators investigated in this thesis is given in Figure 6.1.

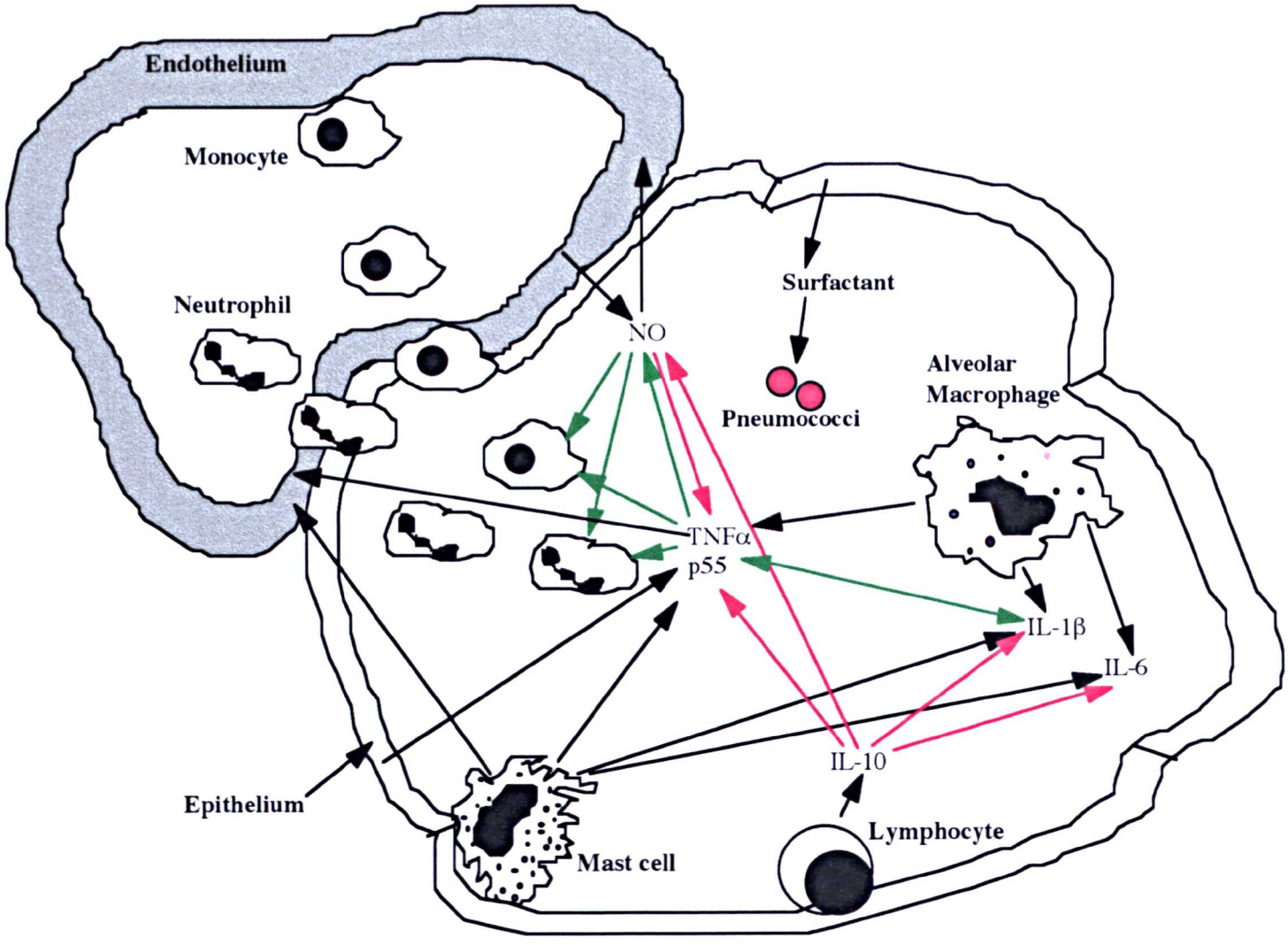
Endothelium
Monocyte
Neutrophil
NO
Surfactant
Pneumococci
Alveolar
Macrophage
TNFα
p55
IL-1β
IL-6
IL-10
Epithelium
Mast cell
Lymphocyte

Figure 6.1 Initiation and regulation of the alveolar inflammatory response by mediators investigated in this thesis. Green arrows indicate a potentiation of effects whilst red arrows indicate an inhibitory effect.
Arrival of pneumococci within the airways initiates an inflammatory response orchestrated by alveolar macrophages and mast cells. These populations release TNFα and IL-1 β. Signalling via the p55 TNFα receptor is responsible for upregulation of either inflammatory cell recruitment or activity as anti-TNFα neutralisation or infection of p55-/- mice resulted in significantly elevated local and systemic bacterial loads. TNF signalling via p55 also initiates production of NO, a mediator capable of controlling bacterial loads within the lungs. NO can feedback upon TNFα (as iNOS-/- mice display heightened levels of TNF activity) but can also act directly upon inflammatory cells to modulate their recruitment or activation. Surfactant phospholipids also control pulmonary bacterial loads but this effect is transient with inoculation of pneumococci in artificial surfactant unable to significantly alter the outcome of pneumococcal pneumonia. In addition to TNF and IL-1 β, alveolar macrophages and mast cells are also capable of releasing IL-6 during pneumococcal pneumonia but the role of this cytokine is currently uncertain. Lymphocytes, previously not thought to play a role in host defence against *S. pneumoniae* appear to control the inflammatory response and prevent it from becoming overzealous by the release of IL-10. This anti-inflammatory cytokine is capable of down-regulating the levels of TNF, IL-1 β, IL-6 and NO.
If the bacteria evade this range of host defences they are able to gain access to the circulation. Such pneumococcal spread is due to disruption to the alveolar/capillary barrier either by pneumococcal or host factors. Possible host factors include contents of mast cell granules such as serotonin and histamine, TNFα signalling via p55, IL-1β or NO release. Indeed NO produced by the endothelium in response to the airway inflammation can feedback to cause endothelial damage

The ability of BALB/c mice to release high levels of IL-10 is likely to be involved in controlling the pro-inflammatory cytokines within the airways thereby preventing lung tissue or bloodstream inflammation. In contrast, susceptible mice cannot control the inflammation which may aid the dissemination of the bacteria. Pneumococci then pass into the bloodstream where a large host response is detrimental with bacterial loads increasing until death of the animals, via a mechanism similar to NO mediated septic shock.

Appendix

PHOSPHATE BUFFERED SALINE

80.0g NaCl
11.6g Na_2HPO_4
2.0g KH_2PO_4
2.0g KCL
Make up to 10L; pH to 7.0

In all experiments directly involving mice, PBS was made from tablets (Oxoid) by addition of 1 tablet to 100ml dH_2O

FORMAL SALINE

3.06g	NaH_2PO_4 Anhydrous
6.70g	Na_2HPO_4 Anhydrous
100ml	Formaldehyde
9g	NaCl (Sigma)

Made up to 1 litre with distilled water.

TOLUIDINE BLUE STAIN FOR MAST CELLS

0.5% Toluidine blue in 0.5M HCl; pH to 0.3

MAST CELL COUNTER STAIN

0.5% Safranine O in 0.125M HCl

IL-6 ELISA COATING BUFFER

0.1M Na_2HPO_4 adjust pH to 9.0 with 0.1 M NaH_2PO_4

IL-6 BLOCKING BUFFER

PBS with 10% FCS (v/v) and 0.25% Tween 20.

IL-6 AND IL-10 ELISA SUBSTRATE

Sufficient substrate for 1 plate is obtained by mixing 10ml solution A with 100µl solution B and adding 4µl H_2O_2

(A) 1.36g sodium acetate in 100ml dH_20, pH adjusted to 5.5 with citric acid

(B) 6mg Tetramethylbenzidine (TMB) in 1ml DMSO

IL-10 ELISA COATING BUFFER

Citrate coating buffer, is obtained by mixing 33ml of solution A with 17ml solution B, making up the volume to 50ml and adjusting the pH to 4.0.

(A) 0.1M citric acid

(B) 0.1M sodium citrate

IL-10 ELISA BLOCKING BUFFER

PBS with 1.0% BSA and 0.25% Tween 20.

IL-10 ELISA STANDARD DILUENT

PBS with 1% BSA, 0.25% Tween 20 and 25% heat inactivated FCS

GREISS REAGENT

The Greiss reagent is obtained by mixing in equal volumes, the two following solutions:

(A) 0.1% alpha-naphtyl-amine in distilled water.

(B) 1% sulphanilamide in phosphoric acid.

Both solutions were stored in the dark at 4°C. Fresh Greiss reagent was prepared every time.

NITRATE CONVERSION BUFFER

Stock Reagents

NADPH	5mg/ml in distilled water. Aliquots of 500µl stored at -20°C.
FAD	41.5 mg/ml in distilled water. Aliquots of 500µl stored at -20°C.
Nitrate reductase	34mg/ml in distilled water. Aliquots of 50µl stored at -70°C
KH_2PO_4 (anhydrous)	0.5M, pH 7.5

Buffer

For 50 samples and standards in duplicate:

500µl NADPH
500µl FAD
500µl KH_2PO_4
500µl distilled water
50µl nitrate reductase freshly diluted in 450µl distilled water.

TISSUE CULTURE GROWTH MEDIA

RPMI 1640 media with 300mg/L L-Glutamine (Life Technologies,) plus 10% heat inactivated FCS (Life Technologies) and 100IU/ml penicillin and 100µg/ml streptomycin (Life Technologies).

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